Results and Problems in Cell Differentiation 54

Robert B. Denman *Editor*

Modeling Fragile X Syndrome

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For his seminal discoveries in neuroscience and especially those relating to understanding fragile X syndrome, we the authors dedicate this book to Dr. William T. Greenough.

Preface

In the beginning of 2005, after finishing the galley proofs for "The Molecular Basis of Fragile X Syndrome, Research Signpost" earlier that fall, I was invited to participate in a conference on fragile X syndrome. This was one of the famed Banbury conferences which were held on the picturesque campus of Cold Spring Harbor Laboratory. I had attended the inaugural one in 2000, where I met a childhood idol, Dr. James Watson. As with all conferences there are highlights, the things that leave an indelible impression on your memory, and there is the rest, which you, in short order, forget. For this particular Banbury conference, there was one talk which bears on the creation of this book that I will never forget.

The talk was given by Dr. Richard Paylor of Baylor University and it concerned the recent new behavioral tests that were being used in his laboratory to assess the several different fragile X mouse model strains that currently existed. His group's work definitively showed that specific behaviors and particular phenotypes produced by the loss of the fragile X mental retardation protein were significantly affected by the mouse strain under investigation. He summarized his findings by constructing the behavior equivalent of a gene expression heat map and put forth the provocative thesis that in order to understand fragile X syndrome one must assess phenotypes in a variety of model strains. I remember afterwards thinking, in true Darwinian fashion, that if strains could produce such profound effects, how much more so the species. So to tease out the true fragile X phenotype, we may need to examine behaviors in several species and would not that make an interesting book project to edit.

Except perhaps for the closing fragment in that last sentence such an idea was not novel because the *Drosophila* dFmr1$^{-/-}$ model of fragile X syndrome was already well established in the literature and work characterizing the fragile X gene family member expression in frogs and zebra fish had just been published. Nevertheless, it took a few more years before an opportunity arose to gestate this project. That opportunity came by way of an inquiry from Dr. Henri Tiedge, co-editor of "*Results and Problems in Cell Differentiation*", as to whether I would be interested in editing a volume on fragile X syndrome for the series. I jumped at the chance and

could not have been more pleased with the outcome. I hope that you, the reader and especially those who are my colleagues in the fragile X field, agree with this assessment.

It should be self-evident that like a symphony conductor an editor's role in the book-making process is mainly one of preparation and coordination; although often the focus of the audience's attention, a conductor should merely serve as a bridge, accepting the audience's applause on behalf of the orchestra. The real kudos belong to the individual members for their performances. This differentiates the roles of editors and conductors, as editors are often unheralded, anonymous fellows and that is how it should be. In contrast, authors are utterly like their orchestral counterparts in deserving praise. Therefore, I humbly and gratefully acknowledge my immense debt to each of the chapter authors: first for doing the majority of the primary research that enabled this project to be initiated and second for their willingness to cogently distill and disseminate their results here in these next pages. They have truly turned my dream into reality and collaborating with them has been one of the highlights of my short editing career.

Staten Island, NY, USA Robert B. Denman
2011

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Chapter 1
Introduction: Reminiscing on Models and Modeling

Robert B. Denman

"From man or angel the great Architect did wisely to conceal, and not divulge his secrets to be scanned by them who ought rather admire; or if they list conjecture, he his fabric of the heavens left to their disputes, perhaps to move his laughter at their quaint opinions wide hereafter, when they come to model heaven calculate the stars how will they wield the mighty frame, how build, unbuild, contrive to save appearances, how gird the sphere with centric and eccentric scribbled o'er, and epicycle, orb in orb."

John Milton – Paradise Lost

"Models are to be used, not believed."

H. Theil – Principles of Econometrics

Abstract This chapter answers three basic questions, which are: (1) Why build models, (2) why build models of fragile X syndrome, and (3) what has been learned from the models of fragile X syndrome that have been made? The first question is used to frame the other two questions, providing the appropriate context by which the rest of the book should be examined. Of necessity the last two questions are only addressed briefly, and from one man's point of view, as they contain the subject matter of the entirety of the book. Thus, the reader is introduced to the various topics under review and urged to read for him/herself their contents, drawing such conclusions as he/she thinks are warranted.

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1.1 Origins and Necessity of Modeling

"Be fruitful and multiply, fill the earth and subdue it and have dominion over it." The human imperative found in the biblical account of creation, and so to fulfill their destiny men, became natural scientists, observing the world and its inhabitants, cataloging their observations, and distilling the data into useful theories that ultimately transformed their environs and so too, themselves. But despite the fact that this process has gone on since the beginning of historical time, humanity has yet to obtain the complete dominion it quests for. All you have to do is look around or watch a "year in review" on the news to know that this is true. Ravages due to earthquakes, hurricanes, tornados and floods, not to mention global warming, species extinction, famine, pestilence, disease, and strife all testify to the fact that we as a species have not mastered the world, its inhabitants, or ourselves.

Why is this so? Quite simply, because the world and its inhabitants are complex and men are limited. Our poets have rightly asked, "What is man that thou art mindful of him"? Our songwriters have declared us to be, "dust in the wind," and our philosophers often despair of humans knowing much of anything at all (Russell 1912).

If we do not wish to acquiesce to our poets, our songwriters and our philosophers how then are we to proceed to total global dominion in the face of our limitations? One of the tools in the scientist's arsenal is the model, a set of simplifying features that allow them to clarify underlying problems and extract potentially useful conclusions. Rendered in this sense a model is a theoretical construct that uniquely impinges or corresponds to the natural world via its assumptions. But do not take my word for it. The mathematician, John von Neumann, eerily echoes this when he states, "the sciences do not try to explain, they hardly even try to interpret; they mainly make models. By a model is meant a mathematical construct, which, with the addition of certain verbal interpretations describes observed phenomena." I can still remember sitting in my undergraduate physical chemistry class and being mesmerized by Professor Rolf Steinmann's description of the particle in the box, that is, a particle, which is set in a well of length L whose sides have infinite potential. As you will recall, the particle can move in any of the three Cartesian directions, but for simplicity is constrained to move only along the X axis. The solution to this problem is found in solving the Schrödinger wave equation:

$$\frac{\mathrm{d}\psi}{\mathrm{d}x^2} + \frac{4\pi^2}{\lambda^2}(\psi) = 0$$

where ψ is the wave function, and λ is the wavelength.

In solving this equation, one remarkably finds that the kinetic energy of the particle: h^2/mL^2 (where h is Planck's constant and m is the mass) has solutions, if and only if, $L = n\ \lambda/2$; that is, the energy of the system can only take on certain discrete values ($n = 1, 2, 3\ldots$) or is "quantized." It is just a short, albeit mathematically intensive jaunt into polar coordinates to describe flesh and blood atoms in terms of their three quantum numbers n, l, and m and begin to understand their

intrinsic properties. *Thus, by first simplifying and then by adding complexity models help us understand our universe and have dominion over it.*

1.2 Utilitarian Features of Modeling

The above description of a model differs significantly from what you will find in literature and the social sciences. There models are often rather artificial constructs of dubious worth that are added to a work as an organizing principle. In his recent bestseller entitled "Genius," the noted Yale literary critic Harold Bloom discourses on the work of 100 literary geniuses (Bloom 2002). Although he explicitly acknowledges the randomness of both genius and organizational principles, he nevertheless endeavors to group the geniuses into ten sets of ten and assigns each set a specific "Divine attribute" from the Kabbalah, which supposedly exemplifies the basic characteristics of those writers. Within each set, the group of ten is broken down further into two groups of five and each group of five is ordered from the genius best exemplifying the Divine attribute to the one that least represents it. How these assignments are actually made is never explicitly stated, and so one perceives at the outset a sense of arbitrariness in this framework. Moreover, neither comparisons within each set of geniuses nor contrasts among the sets are ever made, so as an analytical tool this model of how to examine literary genius is more bluster than it is science. *What is most disconcerting to me about this type of modeling is that it fails the test of utilitarianism, i.e., for a model to be worthwhile, it must have some predictive power.* Channeling von Neumann again, "the justification of a mathematical construct (model) is solely and precisely that it is expected to work." However, if you are looking for a harangue about how Shakespeare is a superior writer to the Bible's authors or why Iago is the most authentically human character ever written it is good read.

Of course, physical scientists and social scientists are not the only ones that construct and use models. In the realm of medicine and disease we who seek cures often model human maladies with other mammals, particularly primates (Fiandaca and Bankiewicz 2010) and rodents (Cryan and Holmes 2005). For example, there are currently mice that model Alzheimer's disease, Parkinsonism, prion diseases, hypercholesterolemia, Crohn's disease, Down syndrome, and Prader–Willi syndrome to name a few. Here the model is less the theoretical framework described above than an actual physical entity, which has been designed and engineered to mimic a particular disease, something a logical positivist would love. *The utility of these models is that they phenocopy one or more aspects of a disease and in doing so can be used as tools to understand the molecular bases of a disease as well as a screening agent for particular remedies.*

Along with these complex, "big brain" models (primates and rodents), there has also been the concomitant development of "small brain" models like those found in small vertebrates [zebrafish (Steenbergen et al. 2011; van Tijn et al. 2011; Peal et al. 2010) and frogs (Pienaar et al. 2010)] as well as other eukaryotes such as worms, flies, and honeybees (Burne et al. 2011). In fact, this trend to smallness can be seen in the recent

development of induced pluripotent stem cells (iPSs) disease models for spinal muscular atrophy and other diseases (Vogel 2010). These less complex models allow us to more easily or more precisely define anatomical pathways, and or social behaviors, turn genes on and off, dissect molecular pathways, screen and test compounds, and evaluate theories of a disease's etiology in the hope that this will lead to an understanding that, in due course, yields a cure. *In short, by modeling human disease in animals we seek and sometimes acquire dominion of a small slice of the world.*

1.3 Modeling Fragile X Syndrome

To those inquisitive minds that have picked up this volume, you will find the collected efforts of a small community to model fragile X syndrome (FXS). FXS is, as all of our authors routinely say in their published work, "the most common inherited cause of mental impairment and the most common known cause of autism." In the United States, FXS is as common as muscular dystrophy and cystic fibrosis. The cause of this syndrome is a triplet repeat expansion in the promoter of the *FMR1* gene, which ultimately results in the loss of an essential RNA-binding protein known as the fragile X mental retardation protein (FMRP).

In actuality, the situation is more complex than the simple loss of a protein. Normal individuals have between 30 and 55 copies of a CGG triplet repeat in the 5′ untranslated region of their *FMR1* gene. Individuals with more than 55 copies of the repeat are classified as having the premutation (Bat et al. 1997; Cunningham et al. 2011). As the number of repeats increases from 55, there is an increased transcription of FMR1 mRNA but a corresponding lag in protein production (Kenneson et al. 2001). When the number of repeats reaches 200, the gene becomes hypermethylated and subject to transcriptional silencing (Verkerk et al. 1991). Individuals with 200 or more repeats are classified as having the full mutation and exhibit all of the hallmarks of FXS. However, premutation males have been shown to suffer from a neurodegenerative disorder termed Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) (Hagerman and Hagerman 2004), while premutation females exhibit premature ovarian failure (POF) (Oostra and Willemsen 2003).

But while FXS is that sterile clinically white-washed diagnosis, it is also much more. It is families struggling for answers and coping with learning and behavioral problems that seem baffling and at times insurmountable. More than anything however, FXS represents the loss of potential of our collective human soul, and the sadness that is usually reserved for those great ones among us that die too soon. That is why we model FXS; that is why we will not rest until we find a cure and claim dominion over a small niche in this wide world.

So, how far have we come on our journey? While the reader ultimately will render the final judgment on this matter, allow me as the first reader of this book to provide an initial assessment, affixing some guideposts along the way. At the time of this writing, there is currently no cure for FXS; thus, most boys and many girls

afflicted with the disease remain significantly affected throughout their lives, although appropriate education and medications can help minimize the effects and maximize the potential of each child. Nevertheless, the cost to society for treatment, special education, and lost income is staggering (Clapp and Tranfaglia 2011). While this is quite sobering I believe there is reason to hope. Many of these reasons lie in the research accomplishments that you will find in perusing this tome.

First of all, it should be self-evident from simply the *Table of Contents* that this book tends to concentrate on animal models of FXS. Seven of the chapters detail an impressive total of 22 distinct animal models ranging from the "large brain" mouse knockouts (Chaps. 4, 11, 12–14) and double knockouts (Chaps. 12, 19) to the well-known small brain models of *Drosophila* (Chaps. 6–8), *zebrafish* (Chap. 19) and the more exotic *Xenopus* (Chap. 9), *zebra finch* (Chap. 10), *Cnidarian* (Chap. 19), *Ciona* (Chap. 19), *Aplysia* (Chap. 19), and *Gryllus* (Chap. 19) models. From this diverse group, certain general and species-independent functions of Fmrp are extracted namely that it is a dendritic- and axonal-localized RNA-binding protein complexed in large neuronal granules that are involved in translational regulation at the synapse and coupled to several receptor-mediated signaling pathways. Correspondingly, its loss results in both subtle and profound changes in neuronal architecture, neuronal networking, circadian rhythm, and synaptic plasticity. A second feature emanating from the multiple models is presented here, and this is especially true of the double knockout models described is a focus on remediating FXS through both engineering and the development of small molecule therapeutics, which is explored in Chaps. 6, 12, and 19. These efforts have paved the way for an initial round of drug trials which are described by Hagerman et al. in Chap. 17.

The molecular alterations in gene expression, translational regulation, and signaling that derive from the loss of the fragile X mental retardation protein give rise to anatomical and neuroanatomical defects that result in networking or wiring abnormalities, which in turn produce the distinct behavioral phenotypes that we classify as FXS. The allure of stem cell biology is that by introducing "corrected" self-renewing neurons in a particular location at a precise moment in the developing brain, all of these defects, molecular anatomical, networking, and behavioral can be corrected. Here, Castren et al. (Chap. 3) and Qurashi et al. (Chap. 8) explore what has been learned about the role Fmrp plays in the developing nervous system using mouse, human, and *Drosophila* stem cell models. The results show points of convergence and points of divergence between the big brain and small brain models, much like the functional similarities and dissimilarities observed between the molecular aspects of each system. Moreover, the recent findings that Fmrp is expressed in astrocytes (Pacey and Doering 2007) have allowed Jacobs et al. (Chap. 2) to begin to outline the important role these cells play in shaping synaptogenesis.

Declaring that the fly, *Drosophila melanogaster,* has been a workhorse of fragile X research is either an oxymoron or a *speciespomorphism*, but nonetheless it is also a truism. As a foil for the mouse, allowing us to understand with greater clarity the essential features produced by the loss of Fmrp, as a unique behavioral model, and as a drug screening tool, the humble fly has driven and illuminated research on FXS.

These and other qualities of the *Drosophila* model of FXS are mapped out by Bell et al. (Chap. 6), Tessier et al. (Chap. 7), and Qurashi et al. (Chap. 8).

While the quintessential "small brain" model, *Drosophila*, has garnered the lion's share of attention, fragile X researchers are nothing if not extremely innovative and having not been content with a single counterpoint to the mouse have gone on to model in a variety of other species, which are summarized in (Chap. 19). However, two of the more recent and unique animal models deserve special attention. They are the *Xenopus* model (Chap. 9) and the zebra finch model (Chap. 10).

As an experimental model of early development, the frog clearly rivals the mouse (Kay and Peng 1991). Moreover, the ease by which frog eggs can be extracted and manipulated have fostered work that ranges from examining the effect cancer genes have on development (Sung et al. 1996), to determining the role translational regulation plays in said process (Luo et al. 2011). Previous studies by Huot et al. demonstrated the use of this system in studying the fragile X homolog, FXR1P (Huot et al. 2005), [reviewed in Denman (2008)]. Here (Chap. 9) Huot et al. expand their analyses to include work by Gessert et al., which tantalizingly shows a potential link between FXR1P, FMRP, and specific micro-RNAs (miRNAs) in regulating developmental programs involving the eye and connective tissues (Gessert et al. 2010).

A constellation of fragile X pathologies little spoken of outside the clinic involve speech. Fragile X patients exhibit several speech-related deficits, both in speech production (articulation, perseveration) and in language competence (syntax, pragmatics) reviewed in Hagerman and Cronister (Bennetto and Pennington 1996). But how to model these deficits? Flies and zebrafish do not speak and mice, high-pitched squeaks notwithstanding, only talk in cartoons. The song bird, *Taeniopygia guttata,* however, sings and as Winograd et al. persuasively argue (Chap. 10) both the way zebra finch learn to sing and the anatomical makeup of their song nuclei are extremely similar to that of humans. Therefore, they hypothesize that zebra finch will be an extremely valuable model for understanding fragile X speech defects. The only caveat is that it still must be made, which could be accomplished using viral vectors used by Zeier et al. to locally ablate Fmr1 gene expression (Zeier et al. 2009).

The role environment and epigenetics play in producing our phenomes and how they combine to produce a disease state is an area that is just beginning to be explored (Houle et al. 2010). Here Zuppan and Toth (Chap. 13) recount a novel breeding strategy that allows them to tease out the genotypic effects of a particular phenotype from nongenetic, maternal-genotype-dependent ones. They demonstrate that heterozygous wild-type offspring, i.e., Fmr1 heterozygous females bred to wild-type males are more active than wild type–wild type offspring. Moreover, male Fmr1 knockout mice born to heterozygous or Fmr1 knockout mothers are even more active than the heterozygous wild-type offspring. Thus, hyperactivity in the Fmr1 knockouts is the result of the combination of the maternal and the offspring genotype effects. On the other hand, hyperreactivity, audiogenic seizure susceptibility, and macroorchidism do not have a maternal-genotype component.

As mentioned above, instability in CGG-repeat sizes above 55 in the *FMR1* promoter lead to its expansion and for repeats above 200 chromatin hypermethylation ensues followed by transcriptional silencing. Intermediate repeats, i.e., between 55 and 200 however, are associated with an RNA gain-of-function toxicity syndrome, FXTAS, whose etiology is outlined by Tassone and Hagerman (Chap. 18). Efforts to model FXTAS in mice are described by Hunsaker et al. (Chap. 14). Interestingly, while many of the features of the human disease are faithfully recapitulated in mice expressing CGG-repeats in the intermediate range, the hallmark symptom, gait ataxia, is either absent, or occurs only in advanced age depending on the model assessed. This along with the difficulties of modeling some of the more complex cognitive and behavioral phenotypes in mice lead these authors to call for the development of novel tasks that can be used to properly test for subtler FXTAS phenotypes.

1.4 Modeling Within the Model

As mentioned above, an attribute of the animal models described here is their ability to specifically test whether a putative molecular mechanism operates to cause all or some of the phenotypic features of the disease. The synaptic plasticity model is perhaps the most all-encompassing of the mechanistic models of FXS as it subsumes the most telling neuroanatomical anomaly of the disease, namely altered dendritic spines, with the host of molecular mechanisms by which this defect might arise (Waung and Huber 2009). From the careful quantitative work of Comery et al. (Comery et al. 1997) showing that the mouse model of FXS recapitulates the spine defects observed in human fragile X patients (Hinton et al. 1991) to the more recent transcranial two-photon imaging techniques demonstrating that the long immature dendritic spines found in fragile X result from increased turnover, leading to an increase in the number short-lived spines that are insensitive to sensory experience, providing a mechanistic basis for these observations has been a consuming interest of fragile X researchers. Here, Kindler and Kreienkamp (Chap. 5) explore the role the postsynaptic density plays in FXS. Their analyses link the activity-dependent protein synthesis-dependent pathway, which utilizes a variety of kinases and phosphatases to control the synthesis of Fmrp target mRNAs directly to expression and function of particular postsynaptic density proteins. Similarly, Goebel-Goody and Lombroso (Chap. 12) add a new and important player in this process, striatal-enriched protein tyrosine phosphatase (STEP), an enzyme that regulates AMPA receptor recycling and whose synthesis is under the direct control of Fmrp.

Transcranial two-photon imaging requires a fluorescently labeled population of neurons to examine, and the development of transgenic mouse lines that selectively express autofluorescent proteins (AFPs) has been instrumental in the success of this technology (Evanko 2007; Sigler and Murphy 2010). It turns out that cultured neurons expressing yellow fluorescent protein (YFP) provide an appropriate backdrop to perform a variety of imaging analyses, for example, allowing one to

determine when and where a particular protein is locally synthesized. Importantly, Kao et al. have shown that the Fmrp target mRNA, CamKIIα, is selectively translated in mGluR5-containing dendritic spines following stimulation with S-3, 5-dihdroxyphenylglycine (DHPG) (Kao et al. 2010). These data confirm the critical role Fmrp plays in the activity-dependent regulation of target message expression (discussed in Chap. 19).

Most researchers, psychiatrists, and clinicians tend to view FXS through the window of its cognitive impairments. It is after all an example of an X-linked mental retardation isn't it (Raymond 2006; Koukoui and Chaudhuri 2007)? However, the more visionary among us would point out that FXS is much more than just the cognitive impairments we tend to focus on. El Idrissi et al. (Chap. 11) have expanded their groundbreaking work on the GABAergic dysfunction in the Fmr1 knockout mouse by showing that organs such as the pancreas that utilize GABA are as substantially misregulated as the brain. This results not only from deficits in the GABA system, but also decreases in voltage sensitive calcium channels (VSCCs) and in somatostatin production, which, in turn, give rise to their own set of anatomical defects. They put forward the startling hypothesis that FXS may best be viewed as a "channelopathy" disease. Similarly alterations in the spinal sensory system in FXS are just beginning to be considered. Based on detailed behavioral analyses of Fmr1 knockout mice, Price and Melemdjian (Chap. 4) argue that the self-injurious behavior (SIB) characteristic of fragile X patients may be related to a decreased ability to sense painful stimuli. Their results not only highlight that the loss of FMRP leads to profound changes in the peripheral nervous system, they also provide additional behavioral tests to conduct when we examine strategies aimed at correcting the fragile X phenotype (discussed in Chap. 19).

1.5 The Human Model

The development of the various fragile X animal models described here is predicated upon their ability to recapitulate the phenotype(s) associated with the disease. That is why we must always go back to the one true model, humans. There, along with the patients and their families, we encounter the other cast members of our mortality play, the psychiatrists and clinicians. Regarding this book, the distinguished actors playing these roles are Dr. Michael Tranfaglia, the chief scientific officer of the FRAXA Research Foundation (FRAXA) and Dr. W. T. Brown, the director of the New York State Institute for Basic Research in Developmental Disabilities (IBR). In his chapter, Dr. Tranfaglia details the cognitive and behavioral features that uniquely identify fragile X patients and provides an extensive list of all of the medications that are used to treat the symptoms of the disease (Chap. 16). Importantly, he predicts a future in which the symptomatic approach to treatment that is currently used will be supplanted by a disease-specific approach. Whereas psychiatrists deal with patient profiling and treatment clinicians deal with disease diagnoses. Here Dr. Brown describes the methods used to diagnose FXS (Chap. 14).

As the Southern blotting approach has given way to the information-rich precision of RT-PCR for determining CGG-repeat size, Brown looks forward to the development of a rapid quantitative sandwich ELISA assay for "low-cost newborn screening to identify affected males."

1.6 Modeling FXS: Future Promises, Future Challenges

As the reader will discover in the ensuing chapters, FMRP is a broad specificity RNA-binding protein and as such regulates a host of neuronal and nonneuronal mRNAs. In turn, FMRP is also regulated by a variety of stimulus-induced receptor pathways. These two features have combined to yield a plethora of druggable targets some of which are beginning to be evaluated in various sorts of drug trials. From antagonists to the mGluR5 receptor such as fenobam, AFQ056 and STX107, or arbaclofen, which targets the $GABA_B$ receptor to the AMPA modulator, CX516, or the matrix metalloproteinase inhibitor, minocycline, a spate of small-scale trials have been undertaken and some candidates like donapezil have graduated to larger trials. As Hagerman et al. point out in their chapter (Chap. 17), and this is echoed by Tranfaglia (Chap. 16), it is likely that combined treatments with multiple drugs will probably be a necessary feature of future targeted FXS therapy.

It would be very tempting to close this Introduction on modeling FXS here on the fruits of our current efforts, these drug trials. But to do so would be a grave disservice to the reader as it would leave her/him with the false impression that our labors were both complete and successful. In many respects however, we, the fragile X community of researchers, are really near the beginning of our quest. On a molecular level, we have barely scratched the surface of what we need to know in order to develop targeted and effective medications. Most of the FMRP-regulated mRNAs obtained via high throughput assays (Sung et al. 2000; Brown et al. 2001; Darnell et al. 2001; Chen et al. 2003; Miyashiro et al. 2003) have yet to be validated by alternative means much less understood within a framework of FMRP interaction. Similarly, though we have identified three unique FMRP–RNA interaction motifs, i.e., G-quartets, the kissing complex, and U-rich stretches (Schaeffer et al. 2001; Chen et al. 2003; Darnell et al. 2005), we know very little about how FMRP accesses them in vivo in an intact full-length mRNA, whether multiple RNA–protein interactions can occur and if they can what the spacing and other structural requirements for the interactions are, and how such interactions may be modified by other members of the FXRP family or other FMRP-interacting proteins. Finally, FMRP–messenger ribonucleoprotein particles (mRNPs) are not independent entities, rather they form heterogeneous higher order complexes with a variety of auxiliary and RNA-binding proteins that are collectively termed neuronal granules. These granules are translationally inactive and are used for transport and stimulus-induced translocation into dendritic spines where they play an intimate role in shaping the postsynaptic density via local translation. However, while neuroscientists have worked out this scenario over the last 30 years (Rao and

Steward 1991; Krichevsky and Kosik 2001; Kanai et al. 2004; Elvira et al. 2006), the remodeling, which forms the molecular basis of this mechanism is still a black box, although it is likely governed, at least in part, by posttranslational modifications (Dolzhanskaya et al. 2006; Xie and Denman 2011). Thus, we are currently years behind the story of the NOVA RNA network model (Zhang et al. 2010), and fragile X researchers have the added burden of dealing with a variety of mutually expressed isoforms that likely modulate both RNA binding and protein–protein interactions. Nevertheless, the success of our modeling efforts to date has brought us closer to our goal of understanding and treating FXS and provides hope for our future endeavors. If I were a reader, I would be anxiously awaiting the publication of the sequel to this volume.

Robert B. Denman, editor
Staten Island, NY, 2011

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Chapter 2
Probing Astrocyte Function in Fragile X Syndrome

Shelley Jacobs, Connie Cheng, and Laurie C. Doering

Abstract Astrocytes have been recognized as a class of cells that fill the space between neurons for more than a century. From their humble beginnings in the literature as merely space filling cells, an ever expanding list of functions in the CNS now exceeds the list of functions performed by neurons. In virtually all developmental and pathological conditions in the brain, astrocytes are involved in some capacity that directly affects neuronal function. Today we recognize that astrocytes are involved in the development and function of synaptic communication. Increasing evidence suggests that abnormal synaptic function may be a prominent contributing factor to the learning disability phenotype. With the discovery of FMRP in astrocytes, coupled with a role of astrocytes in synaptic function, research directed to glial neurobiology has never been more important. This chapter highlights the current knowledge of astrocyte function with a focus on their involvement in Fragile X syndrome.

2.1 Historical Synopsis of Astrocytes

The term astrocyte is first mentioned in 1891 by Michael von Lenhossek in the German journal "Verhandlungen der Anatomischen Gesellschaft" (Transactions of the Anatomical Society). He indicated in his writings that glial cells should be considered to consist of more than one cell type. Lenhossek wrote "I would suggest that all supporting cells be named spongiocytes. And the most common form in vertebrates be named spider cells or astrocytes, and use the term neuroglia *only cum grano salis* (with a grain of salt), at least until we have a clearer view" (von Lenhossek 1891).

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Today, we use the term neuroglia as a broad inclusive term for all cells in the nervous system that are not neurons. Most articles cite Rudolph Virchow (1821–1902) as introducing the term neuroglia (often translated as nerve glue, cement or putty) to the scientific community. In 1856, Virchow used the term neuroglia or "nervenkitt" to describe the connective or interstitial tissue substance in the brain, recognizing that it differed in appearance and consistency from other organs. Kettenman and Ransom (2005) point out that (although not highlighted in most historical accounts) Virchow only used the term neuroglia to refer to the interstitial substance and not to the cellular elements contained within the substance. The brain tissue that Virchow was likely referring to was the neuropil or the astrocyte processes that we readily identify today. As an interesting side note, it was a student of Rudolph Virchow who stenographed a lecture series given by Virchow at Berlin University. It was this stenographing that placed the neuroglia concept into print for the first time with subsequent dissemination around the world (Kettenmann and Verkhratsky 2008). Somjen (1988) provides further insight into the historical evolution of the broad separation of glial cells from neurons by scientists including Santiago Ramon y Cajal, Camillo Golgi, and Otto Deiters.

2.2 Developmental Origin of the Astrocyte

Research on the glial lineage has expanded dramatically in recent years. With advances in microscopic technology and improved methods of cellular identification, the complexity of astrocyte differentiation and function becomes increasingly apparent each year.

Astrocytes develop from precursor cells in at least three different regions of the brain (Goldman 2003). In the developing cerebrum, these include precursor cells in the subventricular zone (SVZ), the radial glia of the ventricular zone (VZ), and from glial precursors not necessarily confined to the SVZ or the VZ layer.

The SVZ, located just beneath the ventricular walls, is a principal source of neural stem cells in the adult mammalian brain. Stem cells in this zone generate thousands of progenitors that form neurons and glial cells each day during development (Alvarez-Buylla et al. 2001; Doetsch et al. 1999). In the presence of exogenous mitogens like fibroblast growth factor, SVZ-derived neural stem cells grown in vitro self-renew and differentiate into all three lineages including astrocytes (Reynolds and Weiss 1992; Chiasson et al. 1999; Gritti et al. 2002).

In addition to the stem cells of the SVZ, another distinct group of cells referred to as radial glia give rise to astrocytes. Radial glia were repeatedly identified at the end of the nineteenth century (Magini 1888; Retzius 1893; von Lenhossek 1895; Ramon y Cajal 1899). Consistent with what is known today, the illustrations in these early papers accurately showed these cells as having their cells bodies in the VZ with processes spanning the complete thickness of the developing cerebral cortex.

Differentiated astrocytes that reenter the cell cycle can also serve as precursors to astrocytes and other cell types (Ganat et al. 2006). These astrocytes located throughout all regions of the brain represent a form of plasticity in the brain and they respond in cases of injury or dysfunction.

Freeman (2010) reviews various intrinsic epigenetic mechanisms that converge with extrinsic signals to generate astrocyte differentiation from neural precursor cells. Signaling through the Wnt and JAK-STAT pathways are prominent in driving precursor cells to an astrocyte fate.

Our initial concept of just one or two different types of astrocytes is gradually being redefined. For example, many subtypes of astrocytes are now being identified according to their location in the brain and on the spatial domain or microniche of the astrocyte environment. Three subpopulations of astrocytes in the white matter of the spinal cord have been identified on the basis of a combinatorial expression of the guidance molecules Reelin and Slit1. The positional identities of these astrocyte subtypes are specified by the homeodomain transcription factors Pax6 and Nkx6.1 (Hochstim et al. 2008). With a transcriptome database now available (Cahoy et al. 2008), insight into new astrocyte subtypes from developmental and functional perspectives will continue to emerge.

2.3 Astrocytes Link Developmental Form with Function

Studies from recent years have revealed that astrocytes perform a significantly wider range of functions than previously appreciated. Technological advances in molecular approaches continue to reveal an ever expanding list of functions for the astrocyte in all ages of the nervous system. Beyond the more commonly described functions of modulating neurovascular blood flow (Attwell et al. 2010) and the regulation of the extracellular ionic milieu (homeostasis) (Walz 1989), research has shown that astrocytes shape the synaptic environment (Ullian et al. 2004) and generate signaling mechanisms within neural networks via calcium waves (Volterra and Meldolesi 2005). Table 2.1 outlines several astrocyte functions and corresponding review papers. The most recent comprehensive reviews of astrocyte function include Wang and Bordey (2008) and Kimelberg (2010).

2.3.1 Astrocyte Cytoarchitecture

The classical Golgi impregnation techniques and immunocytochemical methods used to identify glial fibrillary acidic protein (GFAP) reveal protoplasmic astrocytes with a relatively simple stellate appearance. In contrast to the classical descriptions of astrocyte shape, the work of Bushong et al. (2002), Halassa et al. (2007), Ogata and Kosaka (2002), and Oberheim et al. (2006) reveal astrocytes with exceedingly dense arrays of processes that radiate in a symmetrical fashion from the cell body.

Table 2.1 Listing of various astrocyte functions in the CNS

Astrocyte function	Sub-function	Description	Suggested papers/ reviews
Homeostatic regulation of the neural microenvironment	Extracellular ion buffering	Clearance by uptake of excess extracellular K^+ ions; distribution of the ions through the astrocytic syncytium	Walz (2000)
	Neurotransmitter reuptake and release	High-affinity uptake of glutamate and GABA mediated by plasma membrane transporters	Kimelberg (2007)
		Release of glutamate or ATP in a vesicular, Ca^{2+}-dependent manner	Schousboe and Waagepetersen (2004)
	Metabolic support	Uptake and metabolism of glutamate into glutamine for re-distribution to neurons	Schousboe and Waagepetersen (2004)
		Uptake of glucose via GLUT1 transporter found in astrocyte endfeet surrounding capillaries	Porras et al. (2008)
		Regulate neuronal metabolic responses to activity via: (1) astrocytic glycogen (short term repository for glucose in the brain) and (2) lactate (released to neurons as energy substrate)	Pellerin et al. (2007)
	Blood brain barrier (BBB)	Regulate induction, maintenance and permeability of BBB (tight junction formation, expression of various transport systems and secretion of molecules)	Abbott (2000), Abbott et al. (2006), Haseloff et al. (2005)
Neural development	Neurogenesis	GFAP-expressing cells in the SVZ or SGZ can contribute to cell genesis both as stem cells and as neural components of the neurogenic niche	Barkho et al. (2006), Lie et al. (2005)
Synaptic regulation	Modulate synaptic transmission and neural activity	Astrocytic glutamate release modulates synaptic transmission by activating presynaptic and postsynaptic glutamate receptors	Paixao and Klein (2010)
		Generate signaling mechanisms within neural networks via calcium waves	Volterra and Meldolesi (2005)

(continued)

Table 2.1 (continued)

Astrocyte function	Sub-function	Description	Suggested papers/ reviews
	Synaptogenesis	Promote synaptogenesis between CNS neurons by release of diffusible molecules	Christopherson et al. (2005), Ethell and Pasquale (2005), Ullian et al. (2004)
	Synaptic plasticity	Modulate synaptic function through their role in glutamate re-uptake at the synapse by action of excitatory amino acid transporters (EAATs)	Paixao and Klein (2010)
		Modulate intensity and duration of postsynaptic activation (eg. release of glutamate prompting LTP, preservation of synaptic strength by release of TNF-α, etc)	Barker and Ullian (2010), Beattie et al. (2002), Bergami et al. (2008)
Other	Vasomodulation	Coordinate blood flow to the brain (functional hyperaemia)	Iadecola (2004)
		Control of blood glucose and O_2 by neurotransmitter mediated signaling (predominantly by glutamate)	Attwell et al. (2010), Iadecola and Nedergaard (2007)
		Secrete vasoactive agents to induce vasoconstriction or vasodilation (correlated with increased intracellular Ca^{2+})	Zonta et al. (2003)
	Detoxification	Prevent excitotoxic neuronal death by capturing excess ammonia and glutamate (converting them to glutamine)	Chung et al. (2008), Struzynska et al. (2001)
		Uptake of toxic or heavy metals	
	Immune activation	Bridges CNS and immune system:	
		(1) Express MHC II and costimulatory molecules important for T-cell activation and antigen presentation	Dong and Benveniste (2001)
		(2) Express receptors involved innate immunity	Farina et al. (2007)
		(3) Secrete a wide variety of chemokines and cytokines that act as immune mediators	

The complexity of astrocyte morphology is further highlighted by the facts that a single astrocyte (in the rodent) can contact 300–600 dendrites (Halassa et al. 2007), and each astrocyte oversees in excess of 100,000 synapses (Bushong et al. 2002). In the human, these values are further increased due to the larger size of the protoplasmic astrocytes. Oberheim et al. (2006) estimate that a single astrocyte in the human brain contacts on the order of two million synapses. Clearly, while difficult to conceptualize the numerical value of astrocyte contacts with neurons, the morphological arrangement of astrocyte processes at the synaptic level is critical to proper function.

Specialized anatomical tracing techniques have revealed that mature astrocytes occupy distinct, nonoverlapping domains in the brain (Bushong et al. 2002; Halassa et al. 2007). During development, the astrocyte processes appear quite ragged and display overlapping zones with adjacent astrocytes. By the 3rd to 4th week after birth, neighboring astrocytes occupy very distinct spatial domains with no overlapping processes. Remarkably, there is a striking similarity of these modern images to a figure published by von Lenhossek in 1893 (see Fig. 2.1). It is believed that astrocytes "tile" with one another through a mechanism that is similar to dendritic tiling (reviewed by Freeman 2010). This anatomical arrangement has been taken one step further in theory, with the suggestion that the defined domains of astrocytes function as synaptic islands (Halassa et al. 2007). This concept proposes that all the synapses confined within the boundaries of an individual astrocyte are modulated by the gliotransmitter environment of the same astrocyte.

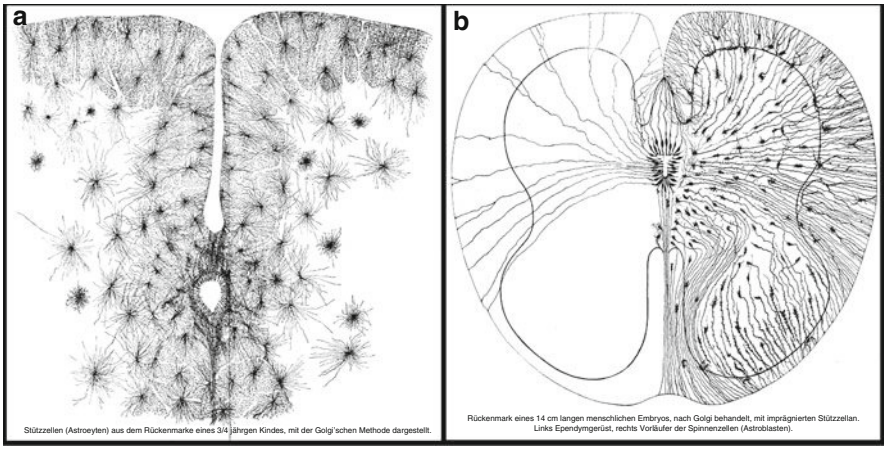


Fig. 2.1 Reproduction of the diagram from the 1893 article by Michael von Lenhossek. (**a**) Golgi impregnation of astrocytes in the spinal cord of a 9 month old child. Note the exquisite pattern of astrocyte "tiling" that is observed by modern day methods of cell filling. (**b**) Spinal cord preparation of a 14 cm human embryo reveals patterns of radial glial spanning the entire thickness of the cord. Golgi impregnation

2.3.2 Gliotransmitters in Astrocytes

Astrocytes are now recognized as "excitable" cells. When astrocytes are activated by internal or external signals, they communicate with neighboring cells in the form of gliotransmission. Astrocytes release various transmitters and factors such as glutamate, GABA, acetylcholine, noradrenaline, D-serine, ATP, nitric oxide, and brain-derived neurotrophic factor (BDNF) (reviewed by Volterra and Meldolesi 2005). In concert with the release of transmitters, many different receptors for neurotransmitters are expressed on the astrocyte cell membrane. These receptors respond with a particular form of excitability involving Ca^{2+} oscillations (Porter and McCarthy 1997).

Modulation of neuronal excitability and synaptic transmission by astrocytes was first shown to be mediated by glutamate release (Haydon 2001). With astrocytes providing local neuronal excitation via glutamate, they provide a source of neuronal activation that may be critical in controlling the synchronous depolarization of neurons (Fellin et al. 2006). At the same time, astrocytes can also suppress synaptic transmission by releasing purines. Through these coordinated actions, the astrocyte is thought to provide balanced excitation and inhibition mediated by two distinct transmitter systems.

Astrocyte excitation, which is chemically encoded, can be detected experimentally by assays of Ca^{2+} transients and oscillations. Two main forms of astrocyte excitation are well-documented: one that is generated by chemical signals in neuronal circuits (neuron-dependent excitation) and one that occurs independently of neuronal input (spontaneous excitation). Numerous studies highlight the release of glutamate from astrocytes in response to neuronal activity. In the case of glutamate, synaptic-like glutamatergic microvesicles have been identified in astrocytes and these vesicles are released via Ca^{2+}-dependent exocytosis (Bezzi et al. 2004).

2.3.3 Astrocytes Modulate Synapse Development and Function

Various insect and vertebrate animal models indicate that glial cells and neurons function together to guide axons during development (Chotard and Salecker 2004). When axons reach their target, glial cells contribute to the specification of the appropriate synaptic connections. The importance of the neuron–astrocyte interaction in synaptic development and function has been highlighted in several papers (Haydon 2001; Ullian et al. 2001; Slezak and Pfrieger 2003; Schipke and Kettenmann 2004). Astrocytes secrete diffusible factors, such as cholesterol (Mauch et al. 2001), tumor necrosis factor-α (Beattie et al. 2002), activity-dependent neurotrophic factor (Blondel et al. 2000), and thrombospondins–extracellular matrix glycoproteins (Ullian et al. 2004; Christopherson et al. 2005) to promote synapse formation. Other classes of cell adhesion molecules such as the γ-protocadherins, a family of neuronal adhesion molecules that are critical for

synaptogenesis, are expressed by astrocytes (Garrett and Weiner 2009). Direct astrocytic contacts also upregulate synapse formation in a protein kinase C-dependent manner (Hama et al. 2004).

Astrocyte contacts may induce local structural and functional modifications of dendritic segments or individual synapses. Membrane-bound ligands on astrocytes, such as ephrin-A3, have been shown to regulate spine morphology in the hippocampus (Murai et al. 2003), suggesting local activation of EphA receptors on spines by astrocytic ephrin-A3. Using organotypic hippocampal slice preparations, Haber et al. (2006) showed that astrocytes can rapidly extend and retract fine processes to engage and disengage from postsynaptic dendritic spines. These dynamic structural changes in astrocytes possibly control the degree of neuron–glia communication at the synapse. With two-photon time-lapse imaging methodology (Nishida and Okabe 2007), they revealed that astrocyte motility in the form of protrusive activity acts as a key local regulator for stabilization of individual dendritic protrusions and subsequent maturation into spines.

2.3.4 The Neurovascular Unit

Considering the contacts made between astrocytes and blood vessels, it has been estimated that in excess of 99% of the brain vasculature is ensheathed by astrocytic processes (Takano et al. 2006). This active interaction between the neuron, astrocyte, and blood vessel has been termed the neurovascular unit and is essential for the regulation of blood flow (Takano et al. 2006; Koehler et al. 2009). The importance of regulating blood volume in the brain is highlighted by the fact that the brain consumes approximately 20% of the energy produced by the body at rest. The control of blood glucose and O_2 are tightly controlled by neurotransmitter mediated signaling (predominantly by glutamate) and this control is modulated by astrocytes (see review by Attwell et al. 2010). The increase in glia research and evolution of the importance of astrocytes to normal neuronal and vasculature function is also highlighted by numerous reviews (Attwell et al. 2010; Freeman 2010; Pfeiffer and Huber 2010; Eroglu and Barres 2010; Barker and Ullian 2010).

2.4 Astrocytes in Neurological Disorders

With an evident role of astrocytes in normal neural function at all cellular and molecular levels, it is not surprising that astrocytes have been implicated in virtually all pathological conditions in the nervous system. Dysregulated astrocyte function has been linked with the progressive pathology of stroke and to a number of neurodegenerative diseases including Alzheimer's disease, Huntington's disease, and Parkinson's disease (Maragakis and Rothstein 2006). While a comprehensive review of astrocytes in the various pathologies is beyond the scope of this chapter, the involvement of astrocytes in the development of Rett syndrome (RTT) is very

applicable. Recently, Ballas and colleagues (2009) demonstrated that astrocytes and astrocyte-conditioned media from the RTT mouse model failed to support normal dendritic morphology. Taken together with our findings in Fragile X (discussed in the next section), and the consistent synaptic alterations seen in Fragile X, learning impairments and autism spectrum disorders, the possibility of an astrocyte involvement in multiple childhood neurodevelopmental disorders certainly becomes evident.

2.5 The Fragile X Astrocyte

With overall synaptic function standing as a prominent link to the expression of the disease phenotype in a number of neurodevelopmental disorders, and knowing that astrocytes influence synapse development and function, our lab initiated experiments to evaluate the role of astrocytes in Fragile X neurobiology. These experiments were preceded by the observation that astrocytes, in addition to neurons, also express the Fragile X Mental retardation Protein (FMRP) (Pacey and Doering 2007). At the time of this finding, FMRP expression in the brain was considered to be primarily neuronal. FMRP had been reported in oligodendrocyte precursor cells, but not mature oligodendrocytes by Wang et al. (2004). When studying stem and progenitor cells from the brains of wildtype and knockout Fragile X mice, approximately 50% of the cells in culture coexpressed FMRP and GFAP. Parallel immunocytochemical studies in vivo also showed the coexpression of FMRP and GFAP in the embryonic and adult developing hippocampus.

With the identification of FMRP in astrocytes and knowledge of their role in synaptogenesis, we initiated experiments to explore neuronal development and synapse formation in the Fragile X mouse. A coculture design was used to selectively combine cells from the *Fmr1* KO mouse and its wild-type (WT) counterpart (Jacobs and Doering 2009). With this tissue culture approach, neurons and astrocytes were independently isolated to explore four different combinations of neuronal-astrocyte cultures (WT neurons + WT astrocytes, WT neurons + *Fmr1* KO astrocytes, *Fmr1* KO neurons + WT astrocytes and *Fmr1* KO neurons + *Fmr1* KO astrocytes). The cells were grown for 7, 14, or 21 days and then processed for immunocytochemistry to analyze the morphological and synaptic profiles.

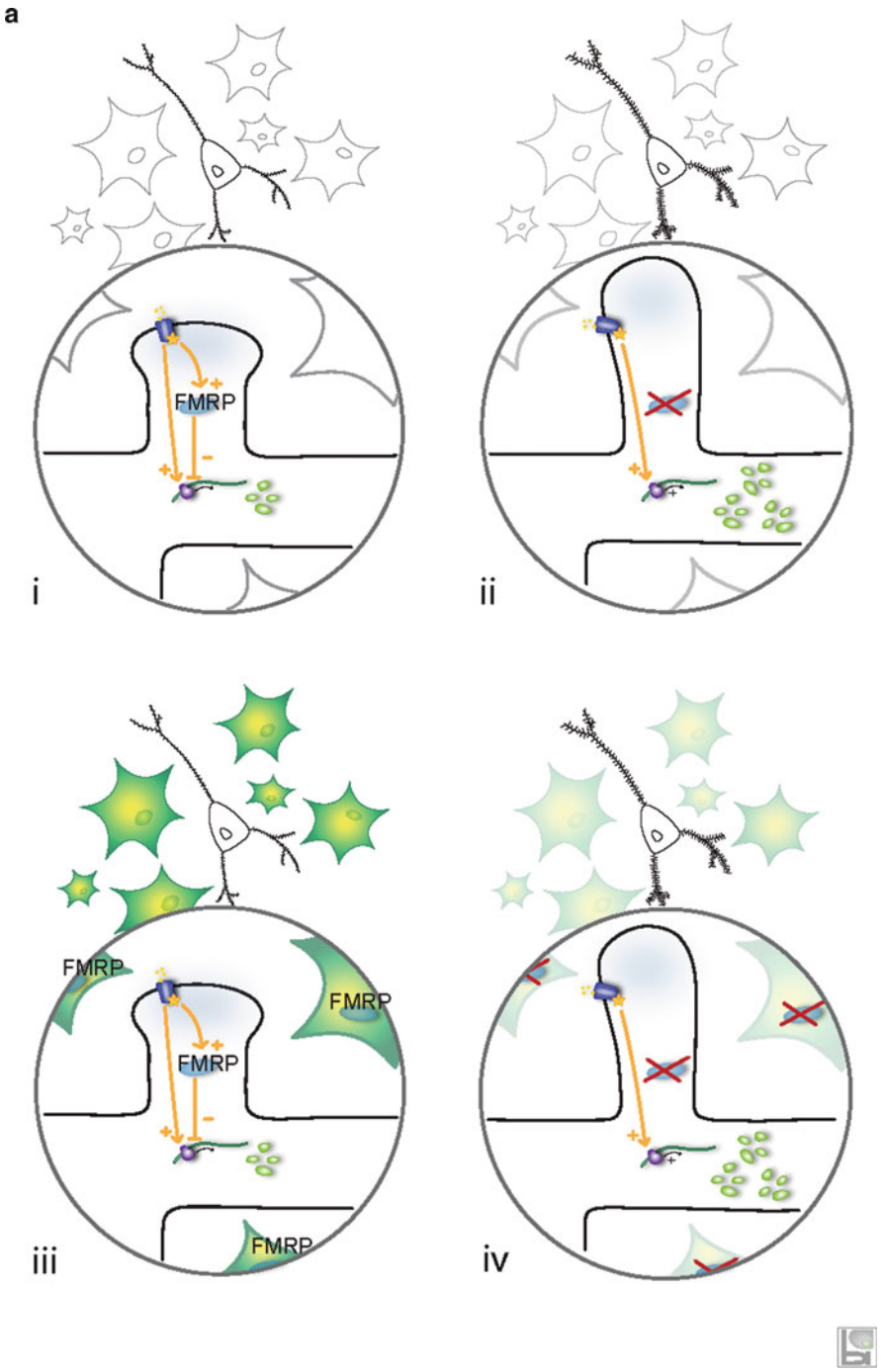
The first set of experiments focused on neurons in each of the four combinations, cultured for 7 days (Jacobs and Doering 2010). The neurons were studied with an antibody directed against microtubule-associated protein-2, (MAP2; a dendritic marker) and the pre- and postsynaptic proteins, synaptophysin and postsynaptic density protein-95 (PSD-95), respectively. The WT neurons grown on the *Fmr1* KO astrocytes had significantly altered dendritic arbor morphologies, with a shift toward a more compact and highly branched dendritic tree. These neurons also displayed a significant reduction in the number of pre- and postsynaptic protein aggregates. However, when the *Fmr1* KO neurons were cultured with the WT astrocytes, the alterations in dendritic morphology and synaptic protein expression were prevented. In fact, their morphological characteristics and synaptic protein

expression approached the appearance of the normal neurons grown with WT astrocytes. These experiments were the first to suggest that astrocytes contribute to the abnormal dendritic morphology and the dysregulated synapse development seen in Fragile X syndrome.

In the next phase of this research, we wanted to determine if these altered characteristics represented a developmental delay imparted by the *Fmr1* KO astrocytes (Jacobs et al. 2010). Focusing on WT neurons grown in the presence of WT or *Fmr1* KO astrocytes, we evaluated the dendritic arbor morphology and synaptic protein expression at 7, 14, and 21 days in culture. If we considered the developmental pattern of the WT neurons on the WT astrocytes to reflect a normal pattern of dendrite and synaptic protein development, we found a significant alteration to these patterns when WT neurons were grown with *Fmr1* KO astrocytes. Our results revealed that the WT neurons grown with *Fmr1* KO astrocytes displayed significantly altered morphological and synaptic protein profiles at 7 days (when compared to the WT condition); however, by 21 days in culture these differences were no longer significantly different from normal. On the basis of this research at this time, it appears that the astrocytes in the Fragile X mouse may contribute to the altered characteristics of neurons seen in Fragile X syndrome, in a developmentally regulated manner.

In preliminary studies to examine if neuronal subsets are preferentially affected, we performed Sholl analyses on the morphology of the neurons in the experiments described above. Our findings suggest that there is a bias in the extent of the morphological alterations imparted by the astrocytes to a subset of neurons with a stellate dendritic arbor morphology (unpublished results). However, it should be noted that in these experiments, the astrocyte involvement was assessed independently of the alterations that would be observed due to a lack of *Fmr1* (and therefore, FMRP) in the Fragile X neurons themselves. Therefore, the situation in vivo, having both neurons and astrocytes affected by a lack of FMRP may not truly reflect the experimental results in vitro. Additional experiments with a rigorous method of identifying subtypes of neurons (e.g., excitatory versus inhibitory neuronal markers) should be performed to specifically address this possibility.

These studies create numerous new avenues to identify and detail the role of astrocytes in the morphological alterations of neurons seen in Fragile X. Establishing key aspects to altered molecular relationships between astrocytes and neurons in Fragile X will lead to new therapeutic possibilities (Fig. 2.2). Are the alterations due to a lack of FMRP in the astrocytes or are the astrocytes abnormal because they develop and function in a diseased microenvironment? If the absence of FMRP in the astrocytes is the primary source of dysfunction, how are these effects translated to the neurons? For example, is the astrocyte–neuron signaling disrupted due to a lack of astrocyte-FMRP? How, where, and when do these signals act? Is the abnormal astrocyte–neuron communication mitigated by a membrane associated or a soluble factor? Finally, can these abnormalities observed in vitro be studied in vivo? These, and many other questions about the Fragile X astrocyte are now important targets for Fragile X research – the answers important in gaining a full understanding of the underlying neurobiology that contributes to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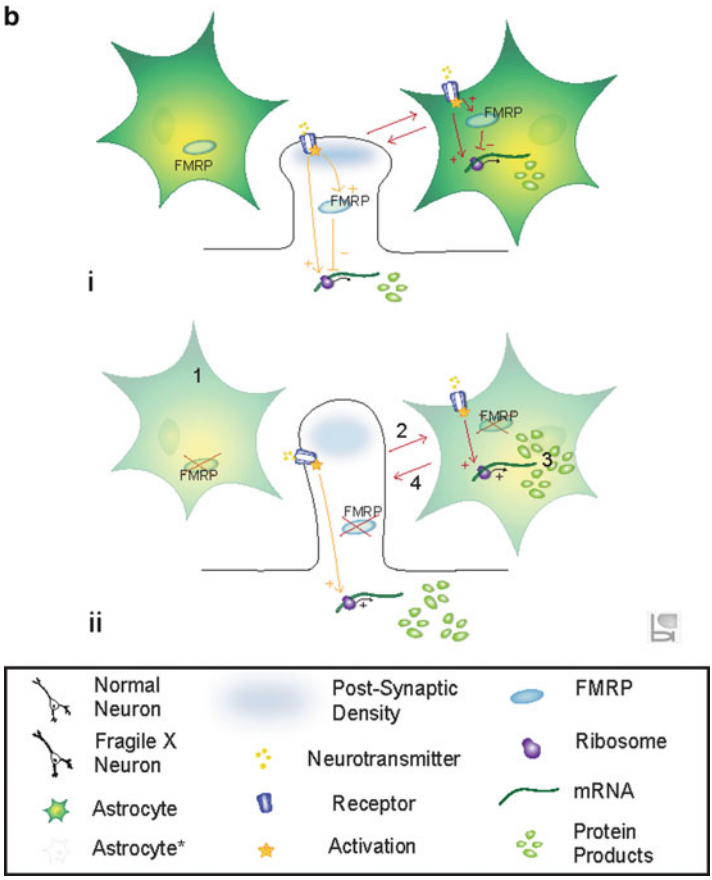

Fig. 2.2 The role of FMRP in astrocytes in Fragile X syndrome. (**a**) Historically, FMRP has only been associated with neurons. (i) With FMRP present there is regulated protein synthesis, normal dendritic spine morphology and no abnormalities associated with Fragile X syndrome (FXS). (ii) In Fragile X (in humans and in mouse models) there is a lack of FMRP in neurons leading to the dysregulation of synaptic protein synthesis, abnormal dendritic spine morphologies and features associated with FXS. (iii) Recent studies indicate that FMRP is present in both neurons AND astrocytes. (iv) In the *Fmr1* KO mouse (and presumably in FXS) FMRP is absent from both neurons AND astrocytes, and the astrocyte FMRP plays an important role in shaping the neuron morphology and synaptic protein profiles. (**b**) It is now important to investigate the role of astrocytes in Fragile X. (i) FMRP may play a similar role in astrocytes as in neurons, functioning as a regulator of protein translation. (ii) There are a number of possibilities for how the lack of FMRP in astrocytes may contribute to the abnormal neurobiology of FXS. (1) The astrocytes may be abnormal as a consequence of developing in an abnormal environment (and therefore not due to a direct effect of astrocyte-FMRP). (2) The neuron–glia signaling may be altered as a result of dysregulated FMRP-dependent protein synthesis, which in turn could alter astrocyte function (again, not due directly to astrocyte FMRP). (3) The translation of a subset of glial proteins may be dysregulated in the absence of astrocyte-FMRP. (4) The glia–neuron signaling may be disrupted due to an abnormal glial signaling protein profile (membrane bound or secreted) as a result of a lack of astrocyte-FMRP. *Presence of astrocyte but not a key player. Figure © Biomedical Illuminations, 2011

the morphological phenotype seen in Fragile X, and in the potential of a future treatment for individuals with Fragile X syndrome.

2.6 Astrocyte Research in the Future

With each year passing, neuroscience research continues to unfold aspects of astrocyte involvement in health and disease. Each new molecular and cellular finding builds into the extensive functioning of how glial cells control and modify neuronal structure and communication.

Subtle changes in the connectivity patterns within subsets of neurons may significantly alter the output of the neuronal circuitry. Interestingly, mutations in the synaptic proteins neurexin 1 and neuroligins 3 and 4 are associated with autism spectrum disorders and mental impairment (Sudhof 2008). The postsynaptic scaffolding molecule and interacting protein of neuroligin SHANK3 (ProSAP2) is also associated with autism (Durand et al. 2007). Accumulating evidence illustrates roles for FMRP in synapse development and corresponding alterations in synaptic molecules in Fragile X (Pfeiffer and Huber 2009). In fact, synaptic function and structure may be the converging point of malfunction in many neurodevelopmental disorders such as Fragile X, RTT, and autism (Walsh et al. 2008; Geshwind 2008).

Together, the last three decades have created a more complete image of synaptic development and function both in health and in diseases of neurological dysfunction – one that is highly dependent on the glial cells of the CNS. Keystone papers by Pfrieger and Barres (1997), Ullian et al. (2001), Christopherson et al. (2005), and others revealed that astrocytes play a major role in the modulation of the development and functioning of synapses. Given the recent findings of astrocyte involvement in neurodevelopmental disorders such as RTT and FXS, it is realistic to now consider astrocytes as holding the key to avenues of intervention for learning disabilities that we previously did not appreciate.

Since many aspects of CNS development involve a neuron–glial interaction, solving neurological dysfunction will require solutions that include glial cells as part of the picture. To maintain a healthy microenvironment for neurons, it will be important to continue research efforts that target our understanding of how astrocytes interface with neuronal circuitry at the cellular and molecular levels. Modes of pharmacological therapy should indeed concentrate on the health of the astrocyte. With astrocytes as "gatekeepers" of neuronal health and function, if we can target astrocytes, then they may in turn take care of the neurons.

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Chapter 3
Neural Stem Cells

Maija Castrén

Abstract Neural stem/progenitor cell (NPC) cultures are a tool to study the differentiation of neuronal cells and can be used to model disease conditions in studies investigating the pathological mechanisms affecting the development and cellular plasticity of the central nervous system. There is evidence that abnormalities of NPCs and their differentiation contribute to the pathophysiology of fragile X syndrome. The results obtained with NPC cultures derived from human and mouse brain tissue with the fragile X mutation are in line with the abnormalities of *Fmr1*-knockout mouse brain *in vivo* indicating that NPC cultures can be useful as a model for fragile X syndrome.

Abbreviations

aNPCs	Adult neural precursor cells
bFGF	Basic fibroblastic growth factor
CNS	Central nervous system
EGF	Epidermal growth factor
ES	Embryonic cells
FXS	Fragile X syndrome
IPC	Intermediate progenitor cell
iPS cells	Induced pluripotent stem cells
NPC	Neural precursor cell
RG	Radial glia
SVZ	Subventricular zone

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3.1 Neural Stem Cells

Neural stem cells are undifferentiated cells that have the capacity for self-renewal and the potential to give rise to all the main cell types of the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes (McKay 1997; Gage 2000). Multipotent neural precursor cells (NPCs) include neural progenitor cells that are immature cells, which may have less broad differentiation potential than primary neural stem cells or limited renewal capacity (McKay 1997; Gage 2000). The novel techniques developed during the 1990s to culture neural stem cells enabled researchers to verify the existence of neural stem cells in the brain (Reynolds and Weiss 1992; Richards et al. 1992). NPCs were found in both the developing and the adult mammalian nervous system (Lindvall and Kokaia 2010). In agreement with the fact that embryonic cells exhibit active neurogenesis and regenerative capacity, cells isolated from all regions of the embryonic brain and grown in culture are multipotent. The cultured NPCs sequentially generate first neuronal and thereafter glial restricted progenitors correlating with the *in vivo* developmental sequence (Qian 2000). In the adult mammalian brain, NPCs are known to be associated with specialized germinal centers (Gage 2002; Alvarez-Buylla and Lim 2004; Ming and Song 2005). Neurogenesis persists in the subventricular zone (SVZ) in the walls of the lateral ventricles from where the new neurons migrate to the olfactory bulb to replace local interneurons (Alvarez-Buylla and Lim 2004). New neurons are also generated in the subgranular layer of the dentate gyrus (Gould and Cameron 1996; Kempermann et al. 1997). Cells with *in vitro* NPC properties may be obtained from other brain regions of the adult brain, but the physiological function of these cells is unknown and it is unclear whether the cells give rise to neurons *in vivo*.

Neurons and macroglia are ultimately derived from a pseudostratified neuroepithelium that lines the embryonic cerebral ventricles early in the development (Kriegstein and Alvarez-Buylla 2009). Neuroepithelial cells begin to exhibit features of glial cells during the second embryonic week and the conversion of tight junctional complexes of neuroepithelial cells to adherens junctions is associated with the transition of neuroepithelial cells to radial glia (RG) cells that are neural precursors throughout the CNS. Both neuroepithelial and RG cells undergo interkinetic nuclear migration that regulates the exposure of progenitor cell nuclei to neurogenic or proliferative signals (Conti et al. 2005; Glaser and Brustle 2005). Initially, all dividing cells are attached to the embryonic ventricle. The asymmetric cell divisions of RG cells generate a daughter cell that is either a neuron or an intermediate progenitor cell (IPC) (Noctor et al. 2004). IPCs are transit amplifying cells that migrate a short distance to form the SVZ where they may proliferate one to two mitotic cycles. The number of IPC divisions may vary in different brain regions and in different species. It has been suggested that IPCs contribute to the large population of mitotic cells in the outer SVZ of the fetal primate brain and the extensive cortical expansion within the primate cortex (Kriegstein et al. 2006). The tightly controlled differentiation and migration of newborn cells in a spatial and temporal order is essential for the establishment of functional cellular networks that form the basis for capacity for sensation and behavior.

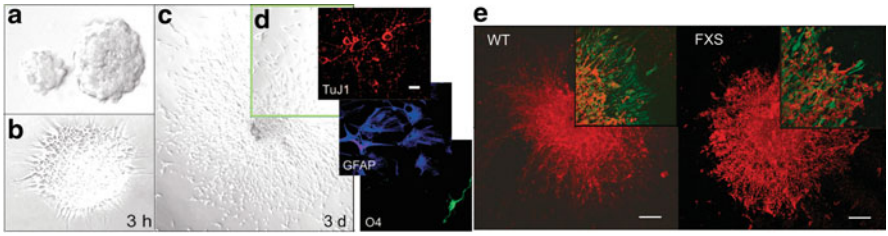

Fig. 3.1 Multipotent NPCs grow and divide in cell aggregates called neurospheres in the presence of EGF and bFGF (**a**). NPCs start to differentiate upon mitogen removal (**b**) and give rise to neurons (red, β-III-tubulin; clone TuJ1), astrocytes (blue, glial fibrillary acidic protein; GFAP), and oligodendrocytes (green, oligondendrocyte marker O4) upon mitogen removal (**c**, **d**). The neuronal differentiation (*red*,TuJ1) of human FXS neurospheres differ from the differentiation of control neurospheres (**e**). (*Insets*) The difference in neuronal (*red*) and glial (*green*) differentiation of FXS NPCs can be particularly seen at the edge of the neurosphere during the first day of differentiation.

3.2 *In Vitro* Culturing of Neural Stem/Progenitor Cells

Epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF) have proliferative effects on NPCs (Gage et al. 1995). In the presence of these mitogens, cultured neural progenitors give rise to new clones of immature cells that proliferate in cell aggregates called neurospheres (Fig. 3.1a) (Reynolds and Weiss 1992). The cells within these neurospheres differentiate to neurons, astrocytes, and oligodendrocytes upon mitogen removal (Fig. 3.1b–d). The cultured progenitors propagated from the mouse embryonic cortex show high neurogenic potential that decreases over time (Qian et al. 2000). The changes in the capacity to generate neurons and glia follow the in vivo developmental sequence. Although responses to growth factors change with time, the factors that influence precursors in early development appear to remain important in adulthood. In neurosphere cultures, the differentiation of NPCs is a dynamic process that is connected with the migration of cells from the original cell cluster (Kärkkäinen et al. 2009). Multiple cell-intrinsic and cell-extrinsic factors regulate NPC proliferation, migration, and neuronal differentiation. NPC cultures provide an excellent model to study the production of diverse CNS cells during distinct phases of development and alterations of NPCs in neurodevelopmental diseases, including FXS.

3.3 Neural Stem Cells as a Model for FXS

NPCs propagated from human fetal brains carrying the mutation causing FXS syndrome and from immature and mature brains of *Fmr1*-knockout mice, the mouse model for FXS, show unique properties compared to their wild-type NPC counterparts (Castrén et al. 2005; Bhattacharyya et al. 2008; Luo et al. 2010). A few

studies have been performed on pluripotent stem cells lacking fragile X mental retardation protein (FMRP) encoded by the *FMR1* gene (Eiges et al. 2007; Urbach et al. 2010). Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells reprogrammed from somatic cells are pluripotent stem cells that have the capacity to differentiate to cells of all germ layers, including the neuroectodermal layer. It is possible to obtain pluripotent cells of human origin and they are potentially important tools for modeling human genetic disorders, including FXS (Urbach et al. 2010). The *FMR1* gene is expressed in undifferentiated ES cell lines generated from human embryos with the fragile X mutation but transcriptionally silenced after ES cell differentiation (Eiges et al. 2007), so ES cells with FXS could be particularly valuable in studies elucidating the regulation of histone modifications and gene methylation that control stem cell differentiation. Recently, iPS cells were successfully reprogrammed from fibroblasts with the fragile X mutation in the *FMR1* gene (Urbach et al. 2010). Since the *FMR1* gene is not activated by the reprogramming process and remains transcriptionally silenced, the iPS cells derived from FXS individuals may open new avenues for patient-specific studies in FXS research. The effects of modifier genes on the disturbed signaling cascades associated with the single gene disorders such as FXS can be studied in patient-derived neuronal cultures and thereby, improved understanding of the molecular mechanisms that cause FXS facilitates also research on the neurobiology of other neurodevelopmental diseases.

3.3.1 NPCs Derived from Mammalian Brains with FXS

Studies of NPCs lacking FMRP, propagated from the human cerebral cortex of fragile X full-mutation male fetuses, have revealed unique intrinsic characteristics (Castrén et al. 2005; Bhattacharyya et al. 2008). For example, NPCs derived from an 18-week-old fragile X fetus generate an increased number of cells responsive to metabotropic glutamate receptor activation and give rise to more neurons with a short neurite phenotype than the age-matched control NPCs (Fig. 3.1e) (Castrén et al. 2005). Cortical NPCs derived from a fragile X full-mutation male fetus at 14-week of gestation display expression changes in genes, which encode components of intracellular signal transduction cascades, including receptors, second messengers, and transduction factors (Fig. 3.2) (Bhattacharyya et al. 2008). At that developmental stage, however, no abnormalities are found in the neuronal differentiation suggesting that the absence of FMRP causes alterations of neurogenesis in specific neuronal populations and/or during distinct phases of development (Bhattacharyya et al. 2008). In NPCs derived from the embryonic brain of the *Fmr1*-knockout mouse, an increase in expression of TrkB receptors in undifferentiated NPCs is associated with alterations in the differentiation of subpopulations of neurons with TrkB expression (Louhivuori et al. 2011). Differentiating neurons in cultures of human NPCs derived from the fetal brain during early neurogenesis express high levels of FMRP (Castrén et al. 2005). FMRP is also detectable in the

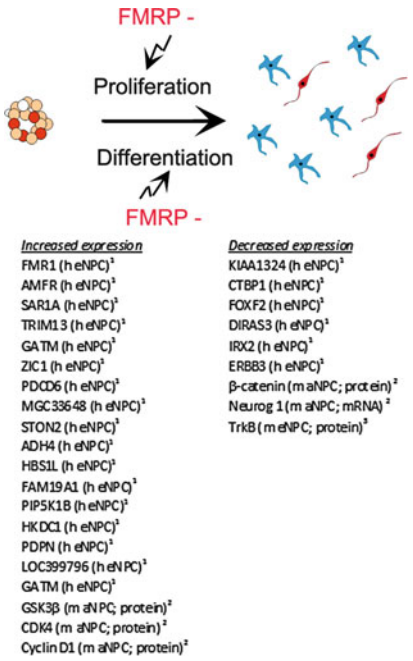

Fig. 3.2 Alterations in gene/protein expression in mammalian FXS NPCs.[1] Bhattacharyya et al. (2008), [2]Luo et al. (2010) and [3]Louhivuori et al. (2011), *h* human, *m* mouse, *eNPC* embryonic neural stem cells, *aNPC* adult neural stem cells

lineages of astrocytes in differentiated mouse NPC cultures during the gliogenic period (Pacey and Doering 2007). Transient expression of FMRP during early oligodendrocyte differentiation in oligodendroglia precursors and immature oligodendrocytes but not in mature oligodendrocytes supports the role of FMRP in the regulation of the differentiation of neuronal cell populations during distinct phases of CNS development (Wang et al. 2004; Pacey and Doering 2007).

The absence of FMRP in adult NPCs (aNPCs) derived from the dentate gyrus of the hippocampus and the forebrain of adult *Fmr1*-knockout mice has been shown to increase proliferation and alter fate determination of aNPCs (Luo et al. 2010). Changes in the Wnt/β-catenin signaling pathway and subsequently the downregulation of a downstream effector, neurogenin I, have been identified as putative mechanisms for the differentiation of an increased number of glial cells and reduced number of neurons in the transgenic aNPCs (Fig. 3.2).

3.3.2 Neurogenesis in the Brain of Fmr1-Knockout Mice

Alterations in neuronal differentiation in FXS NPC cultures has been verified in the brain of *Fmr1*-knockout mouse *in vivo*. Enhanced neuronal differentiation in cultures of FMRP-deficient NPCs of embryonic origin *in vitro* is consistent with a significant increase in the number of IPCs in the developing brain of *Fmr1*-knockout mouse (Castrén et al. 2005; Tervonen et al. 2009). An accumulation of

newborn neurons expressing a mutated FMRP with gain of function properties in the SVZ of developing mouse brain also confirms impaired migration and differentiation of newborn neurons lacking functional FMRP (Tervonen et al. 2009). In addition, the gene expression changes and altered differentiation of subsets of neurons in NPCs lacking FMRP have been shown to be in line with the alterations of gene expression and neuronal cell differentiation in the *Fmr1*-knockout mouse brain (Louhivuori et al. 2011). Furthermore, the abnormalities of cultured aNPCs are in agreement with the defects shown in hippocampal neurogenesis in the adult brain of *Fmr1*-knockout mouse (Eadie et al. 2009).

3.4 Summary

There is a body of evidence that FMRP is involved in the regulation of neuronal differentiation (Castrén et al. 2005; Castren 2006; Eadie et al. 2009; Tervonen et al. 2009; Callan et al. 2010; Luo et al. 2010). Neuronal differentiation has been shown to be affected in NPC cultures derived from both immature and mature mammalian brains with FXS. Alterations of the neuronal production may modulate neuronal network formation and defects of adult neurogenesis likely contribute to the impaired learning and memory in FXS (van Praag et al. 1999). Neurogenic potential of cultured NPCs is known to be influenced by the developmental stage of the NPCs as well as cell culturing conditions and the diverse data obtained from studies of FMRP-deficient NPCs could be explained by differential effects of FMRP on multiple subtypes of cells during distinct phases of development. Furthermore, aberrances of neuronal differentiation are associated with alterations of the glial differentiation and defects in astrocytes may contribute to the abnormalities of the neuron phenotype and synapse formation in FXS (Jacobs and Doering 2010).

Cultured NPCs provide an excellent tool to study the differentiation of neuronal cells and can be used to model disease conditions in studies investigating the pathological mechanisms affecting CNS development and cellular plasticity in the mature brain. Cultured cortical NPCs represent pathways necessary for normal mammalian cortical maturation. Neuronal cell lineage development of NPCs is a gradual developmental process that involves the sequential induction of specific receptors, acquisition of factor responsiveness and complex lineage interdependence. Further histochemical, behavioral, and functional studies of NPCs will improve our understanding of the NPC biology and delineate the limitations of NPCs with genetic mutations to model human diseases. From a clinical perspective, the finding that NPC differentiation is altered in FXS is highly interesting. Most NPC studies are performed with rodents but the introduction of novel stem cell techniques may allow broader use of human cells and patient-specific studies in neurodevelopmental diseases, including FXS.

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Chapter 4
Fragile X Mental Retardation Protein (FMRP) and the Spinal Sensory System

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Abstract The purpose of this chapter is to discuss the role of the fragile X mental retardation protein (FMRP) in the spinal sensory system and the potential for use of the mouse model of fragile X syndrome to better understand some aspects of the human syndrome as well as advance knowledge in other areas of investigation, such as pain amplification, an important aspect of clinical pain disorders. We describe how the Fmr1 knockout mouse can be used to better understand the role of Fmrp in axons using cultures of sensory neurons and using manipulations to these neurons in vivo. We also discuss the established evidence for a role of Fmrp in nociceptive sensitization and how this evidence relates to an emerging role of translation control as a key process in pain amplification. Finally, we explore opportunities centered on the Fmr1 KO mouse for gaining further insight into the role of translation control in pain amplification and how this model may be used to identify novel therapeutic targets. We conclude that the study of the spinal sensory system in the Fmr1 KO mouse presents several unique prospects for gaining better insight into the human disorder and other clinical issues, such as chronic pain disorders, that affect millions of people worldwide.

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4.1 Why Study Fmrp in the Spinal Sensory System?

4.1.1 Links to Fragile X Syndrome in Humans

Silencing of the fragile X mental retardation gene (FMR1) causes fragile X syndrome. This gene encodes a protein, fragile X mental retardation protein (FMRP), which plays a multifunctional role in protein synthesis and neuronal development (Bagni and Greenough 2005). FMRP binds to mRNAs and is involved in transporting them to distal sites in cells while repressing their translation. In neurons, upon intense synaptic stimulation, Fmrp is thought to dissociate from its target mRNA, leading to a derepression of translation (Bassell and Warren 2008). Synaptic synthesis of new proteins plays a key role in synaptic plasticity initiation and maintenance and all evidence indicate that Fmrp plays a crucial role in this process (Bassell and Warren 2008). Two forms of synaptic plasticity are altered in several brain regions in a mouse model of fragile X syndrome (Fmr1 knockout mouse): long-term depression (LTD) is enhanced (Bear et al. 2004) and long-term potentiation (LTP) is absent in some, but not all, brain regions (Li et al. 2002; Larson et al. 2005; Wilson and Cox 2007; Hu et al. 2008). A mouse model of fragile X syndrome was created in 1994 (Consorthium 1994) and the long-standing existence of this mouse, coupled with interest in the role of translation regulation in synaptic plasticity (Kelleher et al. 2004) and the high prevalence of fragile X syndrome (Turner et al. 1996) has led to an extraordinarily in-depth understanding of the role Fmrp plays in synaptic plasticity that continues to develop into new areas of discovery and possible therapeutic intervention.

While the primary focus of research into the role of Fmrp in neuronal plasticity is aimed at understanding this from the perspective of developing therapeutics around the developmental intellectual disability (Bear et al. 2004), there is good evidence from humans that the disorder includes pathology of the sensory spinal system. This is implied by the prominence of self-injurious behavior (SIB), especially among males affected by fragile X syndrome (Symons et al. 2010). Despite the prevalence of SIB in many genetic developmental disorders associated with severe intellectual impairment, very little is known about the neurobiological underpinnings of this comorbidity. SIB occurs in different sectors of the normal population, but its frequency is much higher among individuals with developmental disorders, including fragile X syndrome (Symons et al. 2003, 2010), that negatively influence brain function. The reasons for this are unclear; however, several recent advances in preclinical models of such disorders (including fragile X syndrome and Rett syndrome) have led to a greater understanding of how mutations in genes that cause these diseases lead to changes in the structure and function of the central nervous system (CNS). At the same time, a greater appreciation of plasticity in the CNS as it pertains to chronic pain conditions has led to the recognition that molecular mechanisms of learning and memory and pain amplification are remarkably similar (Ji et al. 2003). We undertook a study using the preclinical model of fragile X syndrome in an effort to ascertain whether loss of Fmrp led to deficits in

sensitization of pain pathways (Price et al. 2007). This study, which will be discussed at length below, concluded that Fmr1 knockout (KO) mice have profound and specific deficits in nociceptive sensitization. Based on this evidence, we speculated that the persistence of SIB in humans with fragile X syndrome may be linked to a failure of the nociceptive system to amplify incoming pain signals, leading to the absence of a neurobiological stop signal for SIB. This hypothesis requires further testing and is unlikely to explain the emergence of SIB but does provide a testable neurological basis for the persistence of SIB in fragile X syndrome and other developmental intellectual disorders.

Further evidence of a pathology in the spinal sensory system in fragile X syndrome comes from emerging evidence of deficits in pain sensation in humans with the disorder (Symons et al. 2010). This study suggests, based on parental reports, that children with fragile X syndrome have higher pain thresholds than other children unaffected by the disorder. Unfortunately, to date, no studies have assessed this directly with quantitative sensory testing [as has been done in Rett syndrome (Downs et al. 2010)]. Based on studies in the preclinical model (discussed below), we would not anticipate changes in acute pain thresholds in fragile X syndrome; however, pain amplification would be expected to be impaired. This is a fundamentally more difficult problem to address in human studies because it requires some intervention to induce sensitization, a manipulation that may be viewed as unethical in humans with fragile X syndrome. However, the fragile X premutation tremor/ataxia syndrome does provide some interesting potential insight into the hypothesis that pain amplification should be decreased in humans with fragile X syndrome. The fragile X premutation tremor/ataxia syndrome, unlike the full mutation that leads to fragile X syndrome (Hagerman and Hagerman 2002), does not repress FMRP expression but, rather, leads to an increase in FMRP mRNA expression (Hessl et al. 2005). Based on the hypothesis stated above, an increase in pain amplification might be expected in the premutation based on the increase, as opposed to loss, in FMRP expression. Interestingly, humans with the fragile X premutation tremor/ataxia syndrome frequently develop peripheral neuropathies, which have a high frequency of associated pain (Berry-Kravis et al. 2007; Brega et al. 2009). Moreover, the incidence of the functional pain disorder, fibromyalgia, is significantly increased in patients with fragile X premutation tremor/ataxia syndrome (Coffey et al. 2008). Hence, taken together, the preclinical and clinical evidence strongly suggest a major role for the Fmr1 gene in pain amplification. We believe that this clinical evidence provides a strong rationale to further understand the role of Fmrp in the spinal sensory system using the Fmr1 KO mouse.

4.1.2 Role of Fmrp in Axons

While the majority of work in CNS structures has focused on the role of Fmrp in dendrites (Bassell and Warren 2008), there is emerging evidence that Fmrp is found in axons as well and may play a functional role during development or even in

synaptic plasticity (Akins et al. 2009; Antar et al. 2006; Centonze et al. 2008). Sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglion (TG) are the initial gateway of the pain, proprioceptive, and tactile sensation pathways. These neurons, unlike CNS neurons, are pseudo-unipolar and are made up of a single axon that emerges from the soma and bifurcates at close distance from the cell body, sending an axonal extension both to the periphery and into the spinal cord. These axons are longer than most CNS axons, with the possible exception of corticospinal and spinal motor neurons. Moreover, removing these neurons from adult rodents and developing primary cultures of these cells are a relatively straightforward process (see Malin et al. 2007 for detailed methods). Extensions from these neurons in vitro maintain axonal properties even after many days or weeks in vitro and these neurons maintain a phenotype consistent with their in vivo properties as well (Price et al. 2005; Malin et al. 2007). For manipulations in vivo, axons of these neurons are relatively accessible. Manipulations can be made to the sciatic nerve with a straightforward surgery that is selective for DRG and motor neurons (Decosterd and Woolf 2000) and the spinal process of the DRG (which contains no motor component) is also accessible for manipulation (Kim and Chung 1992). Lesions to these nerves are often used to assess neuropathic pain, which is caused by injury to peripheral sensory neurons, but such manipulations can also be made to assess a response to axonal injury (with caveats to include difference in regrowth capacity of CNS vs. peripheral neurons). We have utilized a model of injury to the sciatic nerve, to assess the role of Fmrp in neuropathic pain using the mouse model of fragile X syndrome.

As mentioned above, DRG and TG neurons can be used to generate primary cultures for in vitro studies. These neurons, if cultured from adult animals, can be grown in the presence or absence of growth factors (such as nerve growth factor) and they can survive for days to weeks in vitro. These neurons do not extend neurites with dendritic properties but, rather, extend neurites with axonal properties, affording the ability to study axons in isolation. The proliferation of new techniques to study selectively different compartments of these neurons at distance from the soma [e.g., microfluidic devices (Taylor et al. 2005; Park et al. 2006)] also provides a unique opportunity to study the role of Fmrp in axons when using these neurons from Fmr1 KO mice. Hence, these neurons may provide a readily accessible model for studying the role of Fmrp in the axonal compartment that may be relevant to gaining better insight into pathologies related to the human disorder.

4.1.3 Translation Control of Nociceptive Plasticity

A growing body of evidence indicates that translation control plays an important role in sensitization of the pain pathway (Price and Geranton 2009), both in sensory neurons of the DRG and in second-order neurons of the spinal dorsal horn. In fact, some of the first evidence for a direct role of translation control in pain

amplification came from studies done in Fmr1 KO mice (Price et al. 2007). Because Fmrp is involved in transporting mRNAs to distal sites in neurons and in releasing these mRNAs for translation upon neuronal stimulation (Bassell and Warren 2008), as this area of research continues to blossom, the Fmr1 KO mouse may play an important role in identifying novel therapeutic targets. These opportunities and the existing evidence for a role of Fmrp in the spinal sensory pathway will be discussed at length below.

4.2 Evidence for a Role of Fmrp in Pain Pathology

4.2.1 Sensory Neurons and Their Axons

In the CNS, Fmrp localizes primarily to the soma and the dendritic compartment, leading to the view that Fmrp was segregated from the axon, at least in the adult. While several studies have now indicated that Fmrp localizes to the axonal compartment in the adult CNS (Akins et al. 2009; Christie et al. 2009), DRG and TG neurons lack dendritic arbors but robustly express Fmrp in the soma, and Fmrp immunoreactivity is also observed in the axons of these neurons (Price et al. 2006). Most, if not all, DRG and TG neurons express Fmrp and, in the peripheral branch of these pseudo-unipolar neurons, Fmrp immunoreactivity localizes to most axons. Interestingly, we noted that in the centrally projecting branch of DRG neurons, there appears to be less Fmrp expression and many axons do not contain Fmrp immunoreactivity (Price et al. 2006). Finally, at the central terminal of these neurons, located throughout the dorsal horn of the spinal cord, we did not observe any Fmrp immunoreactivity. Somewhat remarkably, Fmrp is not the only protein involved in translation control that shows this distribution as mammalian target of rapamycin (mTOR) immunoreactivity also is excluded from the central terminals of DRG neurons in the spinal dorsal horn (Geranton et al. 2009). This is in stark contrast to sensory neuropeptides and channels (e.g., TRPV1) that are robustly expressed at central terminals of sensory neurons. Hence, it is possible that translation machinery is excluded from the projection of DRG neurons as they traverse the dorsal root entry zone. The reasons and mechanisms of this apparent exclusion are not currently known.

What is the purpose of Fmrp in the axonal extensions of DRG and TG neurons? The traditional view of the axon is that translation does not occur in this compartment, but studies over the past decade have made it clear that the axons of these neurons contain mRNAs (Mohr and Richter 2000; Aronov et al. 2001; Tohda et al. 2001; Willis et al. 2005), a variety of RNA transport proteins (e.g., Fmrp and staufen) (Bassell et al. 1998; Hirokawa and Takemura 2005; Antar et al. 2006; Price et al. 2006; Li et al. 2009), ribosomal proteins (Koenig 1979; Twiss et al. 2000), golgi components (Merianda et al. 2009), and functional RNA interference (Murashov et al. 2007) and that protein synthesis does, indeed, occur in this compartment (Brittis et al. 2002; Martin 2004; Willis and Twiss 2006; Lin and

Holt 2008; Melemedjian et al. 2010). Most of the work in this area has focused on axonal regeneration and growth cone guidance and collapse (Brittis et al. 2002; Martin 2004; Willis and Twiss 2006; Lin and Holt 2008); however, we have recently demonstrated that growth factors (such as NGF) and cytokines (such as interleukin-6, IL-6) are capable of stimulating protein synthesis in the axon of DRG and TG neurons and this process is directly linked to the development of nociceptive sensitization by these endogenous pain mediators (Melemedjian et al. 2010). We presume that Fmrp may play an important role in NGF- and IL-6-mediated translation and, in support of this, we have recently shown that IL-6-induced sensitization is strongly blunted in FMRP KO mice (Asiedu et al. 2011).

4.2.2 Fmrp in the Spinal Dorsal Horn

In addition to Fmrp expression in DRG and TG neurons, FMRP is richly expressed in the spinal cord of mice (Price et al. 2006). Like many other CNS regions, FMRP expression in the spinal cord is isolated to neurons and our previous findings suggest that all spinal cord neurons express Fmrp. Fmrp localization to the dendrites of spinal cord neurons has not been assessed and, in the Fmr1 KO, it is not known if dendritic spines of spinal neurons show abnormal morphology as they do in many other CNS regions (Comery et al. 1997).

4.2.3 Behavioral Pain Phenotype of Fmr1 Knockout Mice

4.2.3.1 Deficits in Nociceptive Sensitization Linked to Peripheral Stimulation

Sensitization of peripheral nociceptors is a primary mechanism of pain amplification. This process involves local signaling within the peripheral terminal of the nociceptor. One of the best-studied forms of peripheral sensitization is thermal hyperalgesia. Research on thermal hyperalgesia was greatly enhanced by the discovery of the noxious heat and capsaicin receptor TRPV1 in the late 1990s (Caterina et al. 1997). TRPV1 is expressed on peripheral terminals of nociceptors and its activity is enhanced by a number of kinase signaling cascades, such as protein kinases A and C, through direct phosphorylation of the receptor (Caterina et al. 1997, 2000; Tominaga et al. 1998; Caterina and Julius 2001). TRPV1 phosphorylation leads to a leftward shift in the temperature response curve of the channel such that it becomes more sensitive to temperature, leading to a drop in threshold for activation of the receptor. Interestingly, recent evidence suggests that this process is mediated by an endogenous agonist and not temperature itself (Patwardhan et al. 2009, 2010). Hence, in many cases, thermal hyperalgesia can be explained simply as a signaling cascade that occurs locally to change the activation threshold of nociceptive sensory neurons (Tominaga et al. 1998).

As mentioned above, we did not observe any deficits in normal mechanical or thermal thresholds in Fmr1 KO mice. On the contrary, several deficits in nociceptive sensitization were found, some of which were linked to peripheral sensitization, while others gave clear indications of CNS deficits (Price et al. 2007). We were able to separate the contributions of peripheral and central sensitization based largely on the administration of a group I metabotropic glutamate receptor (mGluR1/5) agonist. These experiments were facilitated by two lines of evidence: experiments leading to the development of the mGluR theory of fragile X syndrome (Bear et al. 2004) and experiments demonstrating a clear role of mGluR1/5 in nociceptive sensitization (Karim et al. 2001; Adwanikar et al. 2004; Hu et al. 2007). In terms of peripheral sensitization, previous experiments had demonstrated that mGluR1/5 are expressed by DRG neurons and that these receptors localize to the peripheral terminals of these neurons (Bhave et al. 2001). Stimulation of mGluR1/5 with the specific agonist DHPG leads to the development of thermal hyperalgesia in normal animals (Bhave et al. 2001), an event which has subsequently been linked, on the molecular level, to sensitization of the noxious heat and capsaicin receptor TRPV1 (Kim et al. 2009). We found that while thermal hyperalgesia was present in wild-type mice in response to intradermal DHPG administration, it failed to develop in Fmr1 KO mice (Price et al. 2007). Because thermal hyperalgesia in response to local injection of DHPG likely occurs through local sensitization of TRPV1, effectively dropping the thermal threshold of this subset of nociceptors, this finding provides strong evidence of a lack of peripheral sensitization in response to mGluR1/5 stimulation in the periphery in Fmr1 KO mice (Fig. 4.1).

4.2.3.2 Deficits in Nociceptive Sensitization Linked to Spinal Processing

Windup

When a noxious stimulus, such as biting one's hand, is applied, it causes an initial stinging or sharp pain with a short latency (called "first pain") and is followed by a more persistent burning-type pain, which commonly possesses a burning quality. This so-called second pain has a longer latency and is thought to be associated with windup of dorsal horn neurons (Price et al. 1977; Price 1972). Windup involves a progressive increase in action potential generation in spinal dorsal horn neurons in response to repetitive firing of peripheral afferents synapsing in the dorsal horn. This windup takes less than 1 sec to begin and can be observed in most dorsal horn neurons that receive a nociceptive input. While the pharmacology of windup is complex, its basic mechanisms involve glutamatergic neurotransmission and postsynaptic glutamate receptors of the NMDA type. Existing evidence points to progressive depolarization through NMDA channels as a primary means through which frequency-dependent amplification of dorsal horn neuron firing is augmented (Dickenson and Sullivan 1987). In addition to the increase in the output firing of dorsal horn neurons relative to the afferent input, windup can also lead to after-discharge in these neurons (continued firing despite the absence of continued input). Because windup takes place over such a

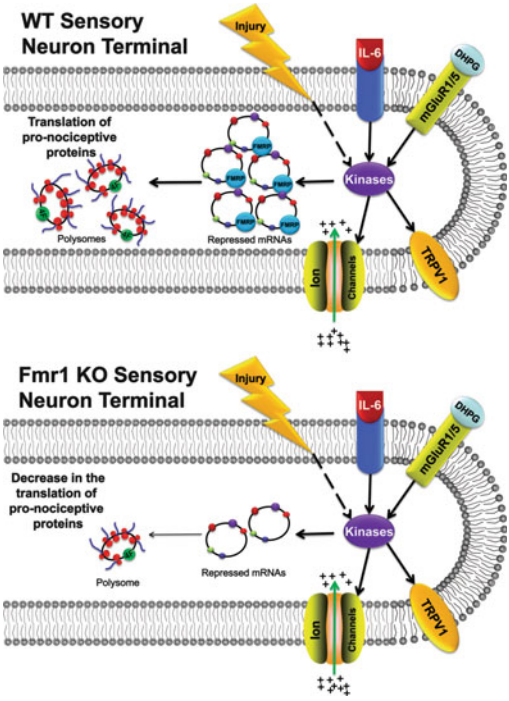

Fig. 4.1 In the peripheral termini of WT sensory neurons, Fmrp facilitates the transport and translational repression of mRNA destined for the axon. Injury, cytokines such as IL-6, and the mGluR1/5 agonist DHPG activate various kinases that increase the excitability of sensory neurons by modulating the activity of TRPV1 and other ion channels. Moreover, activated kinases can induce the initiation of translation [via increased eIF4F complex formation (4 F)], leading to the local synthesis of pronociceptive proteins that enhance and maintain nociceptive sensitization of the primary afferents. In contrast, absence of Fmrp results in the dysregulation of mRNA trafficking and translational repression. Hence, nociceptive inputs that induce prolonged sensitization of the primary afferents may not efficiently induce the local translation of pronociceptive proteins. This results in abrogated responses to injury, IL-6, and DHPG in Fmr1 KO mice

short time course, it is commonly viewed as a primary mechanism for short-term plasticity in the nociceptive system (Herrero et al. 2000).

We recorded responses in ascending fibers of second-order dorsal horn neurons from Fmr1 KO mice after afferent volleys that are sufficient to induce windup in most wild-type neurons. Strikingly, windup was absent in the vast majority of fibers in FMRP KO mice and some of these fibers even demonstrated a decrease in their input–output function, which we termed winddown (Fig. 4.2a) (Price et al. 2007). The molecular mechanisms of this effect are not known, but this provides compelling evidence for a specific deficit in this form of short-term sensitization in the spinal dorsal horn of Fmr1 KO mice. This effect may be explained by abnormal synaptic connections and/or changes in NMDA receptor expression in the spinal dorsal horn, but these possibilities have not been tested. However, these hypotheses

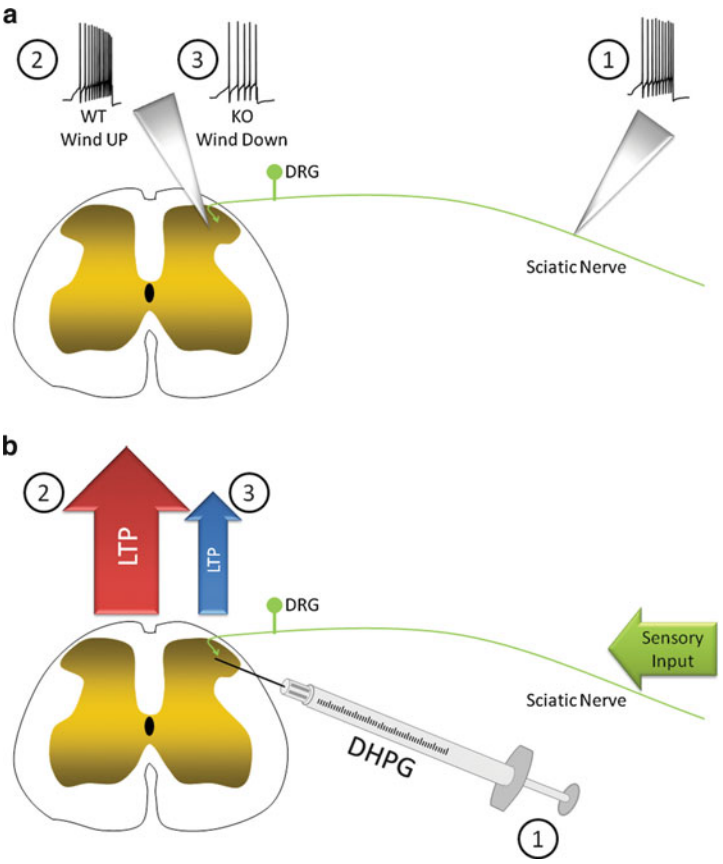

Fig. 4.2 Spinal plasticity in Fmr1 KO mice. (**a**) Repetitive stimulation of peripheral nociceptors (1) induces windup (an increase in the number of spikes relative to the peripheral input) in the ascending second-order neurons of the spinal cord in WT mice (2). However, in Fmr1 KO mice, a lack of windup, and even winddown (3), was observed in response to the stimulation of peripheral nociceptors (1). (**b**) Intrathecal injection of mGluR1/5 agonist DHPG (1) may induce LTP in second-order neurons of the spinal cord in WT mice (2), resulting in robust nociceptive behavior. However, intrathecal injection of DHPG fails to induce nociceptive behavior in Fmr1 KO mice (3) compared to that in WT. This lack of nociceptive behavior may reflect a reduction in, or absence of, spinal LTP in Fmr1 KO mice

are supported by alterations in dendritic morphology in the absence of Fmrp and Fmrp-mediated control, via microRNA association, of NMDA receptor expression (Edbauer et al. 2010).

Long-term Potentiation

Unlike windup, LTP involves an increase in synaptic efficacy that has a longer latency to onset and can persist for days to weeks and may even be permanent.

As such, most work on LTP has focused on establishing its mechanistic role in learning and memory. In fact, several lines of evidence suggest that LTP occurs during learning and memory (Whitlock et al. 2006) and inhibition of molecular maintenance mechanisms of LTP reverses established memories (Pastalkova et al. 2006; Shema et al. 2007). Moreover, LTP is impaired in preclinical models of Rett syndrome (Moretti et al. 2006) and fragile X syndrome (Zhao et al. 2005; Wilson and Cox 2007). In terms of pain signaling, LTP has recently been recognized as an important synaptic amplifier mechanism in the dorsal horn (Ikeda et al. 2006; Sandkuhler 2007). While LTP can be induced in dorsal horn neurons by artificial high-frequency stimulation of nociceptors, it can also be observed after natural stimulation that mimics persistent inflammation and/or injury to the peripheral nervous system (Ikeda et al. 2006), pointing to the physiological importance of this type of plasticity in chronic pain states. While the ability of LTP to explain the full sequelae of chronic pain symptoms is still controversial (Sandkuhler 2010; Latremoliere and Woolf 2010), it is, nevertheless, a critical amplification mechanism for pain pathways that leads to enhanced pain perception in human subjects (Lang et al. 2007; Klein et al. 2004).

We tested the effect of intrathecal (direct spinal injection) DHPG in Fmr1 KO mice. Previous studies indicated that DHPG elicits a nociceptive response when injected intrathecally through postsynaptic stimulation of mGluR1/5 receptors, leading to extracellular signaling-regulated kinase (ERK) activation (Karim et al. 2001; Adwanikar et al. 2004; Hu et al. 2007). While DHPG stimulated a robust nociceptive response in wild-type animals, this response was virtually absent in Fmr1 KO mice (Price et al. 2007). Our findings on links between mGluR1/5 and Fmrp and deficits in nociceptive sensitization in the mouse model of fragile X syndrome may seem to contradict findings in the hippocampus where mGluR1/5-dependent LTD is enhanced in the absence of Fmrp, suggesting hyperactive mGluR1/5 signaling in neurons lacking FMRP (Bear et al. 2004). However, mGluR1/5-dependent LTP is absent in the visual neocortex of Fmr1 KO mice, indicating that differences in mGluR1/5-mediated plasticity in the absence of Fmrp can differentially influence LTP and LTD (Wilson and Cox 2007). In the hippocampus, DHPG can induce LTD, however, in the spinal cord, and in the visual cortex, mGluR1/5 recruitment is required for the establishment of LTP, similarly to the visual neocortex (Wilson and Cox 2007). Although mGluR1/5 activation does induce LTD in some spinal cord neurons (Heinke and Sandkühler 2005), the clear role of mGluR1/5 in spinal LTP (Azkue et al. 2003) leads us to speculate that there may be deficits in spinal LTP in Fmr1 KO mice (Fig. 4.2b). While we speculate, based on this evidence, that spinal LTP may be absent in Fmr1 KO mice, this hypothesis has not been tested to date.

4.2.3.3 Neuropathic Pain

Neuropathic pain presents one of the greatest clinical challenges facing pain neuroscientists and clinicians today (Campbell and Meyer 2006; Woolf 2010). Neuropathic pain is largely intractable to common analgesics and the prolonged

duration of the disease state makes the use of such medicines challenging due to adverse side effects (Baron 2006; Campbell and Meyer 2006). Hence, gaining a better understanding of neuropathic pain mechanisms and identifying new targets for neuropathic treatment are of great importance. Neuropathic pain is generally caused by injury to the peripheral nervous system, although it is not always the case that an injury can be directly identified. In such cases, neuropathic pain is often assigned as a diagnosis based on symptoms and effective analgesics (e.g., serotonin and norepinephrine reuptake inhibitors). Injury to the peripheral nervous system causes the generation of ectopic activity in sensory neurons, leading to consistent afferent input into the spinal dorsal horn causing continuous activation of pain pathways (Baron 2006; Campbell and Meyer 2006; Devor 2006). It is also thought that this afferent discharge induces changes in spinal circuitry (Latremoliere and Woolf 2009; Woolf 2010) and even abnormal neuroimmune interactions (Romero-Sandoval et al. 2008) in the spinal dorsal horn that drive amplification of pain pathways. These pathologies manifest as continuous ongoing pain, mechanical allodynia (in most cases), and the presence of thermal (generally cold) hypersensitivities (Baron 2006).

We hypothesized that translation control may play a key role in neuropathic pain (Price and Géranton 2009). Previous studies had indicated that injury to the peripheral nervous system in the form of a preconditioning nerve crush lesion induces alterations in mRNA localization to DRG neuron axons (Zheng et al. 2001; Willis et al. 2005). As an initial test of this hypothesis, we assessed whether Fmr1 KO mice develop neuropathic pain after injury to branches of the sciatic nerve. Strikingly, Fmr1 KO mice failed to develop neuropathic allodynia (the major measure of neuropathic pain in preclinical models) for several weeks after injury to the sciatic nerve. Moreover, even when these mice did develop a drop in mechanical thresholds, these mice failed to develop full neuropathic allodynia compared to wild-type mice (Price et al. 2007). Subsequent studies from our group have indicated that injury to the peripheral nervous system in mice and rats induces a robust increase in Fmrp localization in peripheral sensory neuron axons (Melemedjian and Price, unpublished observations). This finding, taken together with data from Fmr1 KO mice, strongly suggests a role of Fmrp in neuropathic pain.

More recent investigations have substantiated the case for translation control as a key aspect of neuropathic pain. Local inhibition of mTOR acutely reduces hyperalgesia to mechanical stimulation after injury of the peripheral nervous system (Jiménez-Díaz et al. 2008; Geranton et al. 2009). Another study has indicated that the mRNA for the voltage-gated sodium channel, NaV1.8, increases in DRG axons after injury to the peripheral nervous system (Thakor et al. 2009). This finding parallels other studies that have demonstrated that NaV1.8 protein increases within the sciatic nerve in the setting of neuropathic pain (Gold et al. 2003). Pharmacological blockade of NaV1.8 reduces neuropathic pain in preclinical models, as does knockout or knockdown of NaV1.8 with antisense technology (Lai et al. 2002; Roza et al. 2003; Jarvis et al. 2007). Hence, local synthesis of this voltage-gated sodium channel may contribute to sensory neuron hyperexcitability and ectopic activity (Devor 2006), providing a direct link between translation

control and sensory neuron pathology in neuropathic pain. How Fmr1-based mouse models and Fmrp association with mRNAs may be utilized to advance our understanding of neuropathic pain will be discussed below.

4.3 Open Questions and How to Address Them

4.3.1 Dissecting the Role of Fmrp in DRG Versus Spinal Neurons (Conditional Knockouts)

Although certain pain phenotypes in the Fmr1 KO mouse model are indicative of altered peripheral (mGluR1/5 thermal hyperalgesia) or central (windup) sensitization, others are harder to categorize and will require further experimentation. One example is decreases in the nociceptive responses of the Fmr1 KO mouse in the formalin test (Price et al. 2007), a common test for assessing analgesic efficacy of novel therapeutics (Mogil 2009). The formalin test consists of two phases: the first, which lasts for 10 min, is associated with the initial nociceptor discharge in response to formalin and the second, which lasts from 20 to 45 min postformalin injection, is classically considered a test of central sensitization (Mogil 2009). Despite this commonly held view, there is clear evidence of a peripheral component to the second phase of the formalin test (Taylor et al. 1995; Puig and Sorkin 1996). It is currently not clear if decreased sensitization in the mouse model of fragile X syndrome is due to peripheral or central effects, or both, but there are several tools available for the potential solution to this and other problems.

The generation of mice harboring LoxP sites to excise the Fmr1 gene in a conditional fashion has the potential to advance fragile X syndrome research greatly (Mientjes et al. 2006). Likewise, the generation of CRE-expressing mice for conditional knockout of floxed alleles in certain populations of sensory neurons has led to major advances in our basic understanding of pain mechanisms. One such Cre recombinase-harboring mouse is the NaV1.8-Cre mouse (Nassar et al. 2004; Stirling et al. 2005). Because this voltage-gated sodium channel is only expressed in a population of nociceptors, this mouse can be used to generate mice with conditional knockout of genes only in this subset of cells. A decrease in formalin-induced pain in NaV1.8-Cre mice crossed with Fmr1-floxed mice would strongly suggest a predominate role of peripheral Fmrp in formalin-induced sensitization. Likewise, peripherin-Cre mice have been created and these mice can be used for conditional knockout of floxed alleles selectively in unmeylinated sensory neurons (Zhou et al. 2002). Similar technologies can be used to delete genes in the spinal cord conditionally but, thus far, no Cre-harboring mice have been created that generate a dorsal horn-specific knockout of floxed alleles.

While we have used the formalin pain phenotype of the Fmr1 KO mouse as an example above, these types of experiments have broader implications than simply parceling out peripheral vs. central components of the formalin test. These model systems have been particularly important for neuropathic pain research and

a sensory neuron-specific deletion of Fmr1 would be useful for advancing our understanding of the contribution of sensory neuron Fmrp expression for neuropathic pain.

4.3.2 Viral Vectors to Assess Changes in Adult Animals

A common criticism of knockout mouse studies, including conditional knockouts, is the potential for developmental compensatory changes and their contribution to the presence or absence of phenotypes in such mice. While this is likely less of a concern for Fmr1 KO mice due to their link to the human disorder, one way around this problem is to allow for development to proceed normally in the presence of floxed alleles and then delete these genes in a conditional fashion in adult animals through transduction of cells with a viral vector expressing Cre (van der Neut 1997). In this regard, the spinal cord is accessible through a relatively simple procedure [intrathecal injection (Hylden and Wilcox 1980)] and several studies have shown that this is an effective route for viral transduction in adult mice (Milligan et al. 2005; Chou et al. 2005). Because selective Cre mice for dorsal horn expression have not been created to date, this may be a more effective approach for the investigator interested in deleting Fmr1 in the adult spinal cord. Somewhat surprisingly, the DRG and TG systems are also relatively accessible for viral transduction in adult animals. Certain viruses are selectively taken up by DRG and TG neurons and transduction of these ganglia can be induced by simple intradermal injection of virus at the appropriate anatomical location in adult animals (Tzabazis et al. 2007; Gu et al. 2005; Jackson et al. 2005). Hence, generating conditional deletion of Fmr1 in adult animals may prove to be particularly simple in the spinal sensory system with the use of floxed Fmr1 mice. These experiments may be important for better understanding how Fmrp contributes to pain plasticity.

4.3.3 Opportunities to Better Understand the Role of Fmrp in Axons: New Mechanisms and Targets for Pain Control

Our view is that understanding the role of Fmrp in sensory neuron axonal plasticity may play an important role in unlocking new therapeutic targets for neuropathic pain. The question is how to harness Fmrp to discover these new targets. Because Fmrp is a well-known RNA-binding protein, much research has been dedicated to identify its mRNA-binding targets (Darnell et al. 2005). Extremely stringent experimental protocols have been elucidated to identify these targets in CNS neurons (Brown et al. 2001; Darnell et al. 2001, 2009) but, thus far, these techniques have not been applied to sensory neurons and their axons. They have also not been applied to in vivo conditions that represent important preclinical models of neuropathic pain.

As mentioned above, cultured sensory neurons maintain their in vivo phenotype [although this phenotype may best represent a "neuropathic" one (Dussor et al. 2003)] in culture for days and even weeks. Certain phenotypes can also be enriched by altering the growth factors present in the culture media (Price et al. 2005; Malin et al. 2007). Hence, this model system affords the opportunity to enrich axons from these neurons (using microfluidics or other techniques) in an effort to identify Fmrp-bound mRNAs that localize to the axonal compartment. Identifying these mRNAs could provide insight into proteins that might be translated locally within the axonal compartment in an Fmrp-dependent fashion (a hypothesis that can be assessed using the Fmr1 KO mouse). The repertoire of mRNAs bound to Fmrp in the axons of sensory neurons in culture could then be compared to in vivo conditions, with or without injury to the peripheral nervous system. Because we have observed a striking neuropathic phenotype in Fmr1 KO mice (Price et al. 2007), and an increase in Fmrp in the axons of DRG neurons after peripheral nerve injury (Melemedjian and Price, unpublished observations), these studies have the potential to identify targets linked to Fmrp that may lead to the development of novel therapeutics for the treatment of neuropathic pain.

4.4 Conclusions

The sensory spinal system has received less attention than other, higher CNS structures in Fmrp research. From a fragile X syndrome therapeutic standpoint, there are good reasons for this disparity; however, we have tried to argue that studying Fmrp in the sensory spinal system is highly relevant both to a better understanding of certain aspects of the disorder and to gaining insight into other human diseases such as neuropathic pain. While work into translation control and nociceptive sensitization is just beginning, studying Fmrp is a natural gateway to understand how translation control contributes to nociceptive plasticity that may lead to the development of novel mechanism-based therapeutics for human pain disorders.

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Chapter 5
The Role of the Postsynaptic Density in the Pathology of the Fragile X Syndrome

Stefan Kindler and Hans-Jürgen Kreienkamp

Abstract The protein repertoire of excitatory synapses controls dendritic spine morphology, synaptic plasticity and higher brain functions. In brain neurons, the RNA-associated fragile X mental retardation protein (FMRP) binds in vivo to various transcripts encoding key postsynaptic components and may thereby substantially regulate the molecular composition of dendritic spines. In agreement with this notion functional loss of FMRP in patients affected by the fragile X syndrome (FXS) causes cognitive impairment. Here we address our current understanding of the functional role of individual postsynaptic proteins. We discuss how FMRP controls the abundance of select proteins at postsynaptic sites, which signaling pathways regulate the local activity of FMRP at synapses, and how altered levels of postsynaptic proteins may contribute to FXS pathology.

5.1 Introduction

In the mammalian central nervous system, excitatory synapses are localized on tiny protrusions emanating from the shafts of dendrites, named dendritic spines. In electron microscopic images, the postsynaptic side of these synapses is characterized by a dark (i.e., electron dense) structure, which is attached to the postsynaptic plasma membrane and extends into the cytosol of the dendritic spine. As this structure is both electron dense and easily isolated due to its high buoyant density, it has been named postsynaptic density (PSD). According to current knowledge (reviewed in Chua et al. 2010; Kim and Sheng 2009; Sheng and Hoogenraad 2007), the PSD performs several functions:

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1. It contains the postsynaptic neurotransmitter receptors, and formation of the PSD is required to position these receptors at the correct site opposite the presynaptic nerve terminal.
2. The PSD provides a physical linkage of receptors to the actin-based cytoskeleton of the dendritic spine, which is thought to serve an anchoring function.
3. Formation of the PSD during development is inextricably linked with the establishment of a dendritic spine, such that the size of the PSD and the abundance of some of its components affect spine morphology.
4. By incorporating numerous signaling proteins, the PSD provides a platform for postsynaptic signal transduction.
5. In the PSD, signaling events allow for the incorporation or removal of postsynaptic receptors, phenomena that are crucial elements of synaptic plasticity.

To perform these functions, a large and variable set of proteins assembles in the PSD (Fig. 5.1). Ever more rigorous proteomic analysis methods have identified somewhere between 100 and 400 different proteins, which constitute the average PSD isolated from the rodent forebrain (Husi et al. 2000; Jordan et al. 2004; Peng et al. 2004; Walikonis et al. 2000). The copy number of individual PSD components is variable. Estimates are available for certain scaffold proteins, which are present in 60–400 copies per PSD, whereas some of the glutamate receptor proteins are present in 10–30 copies. Taking into account these numbers, in combination with biophysical

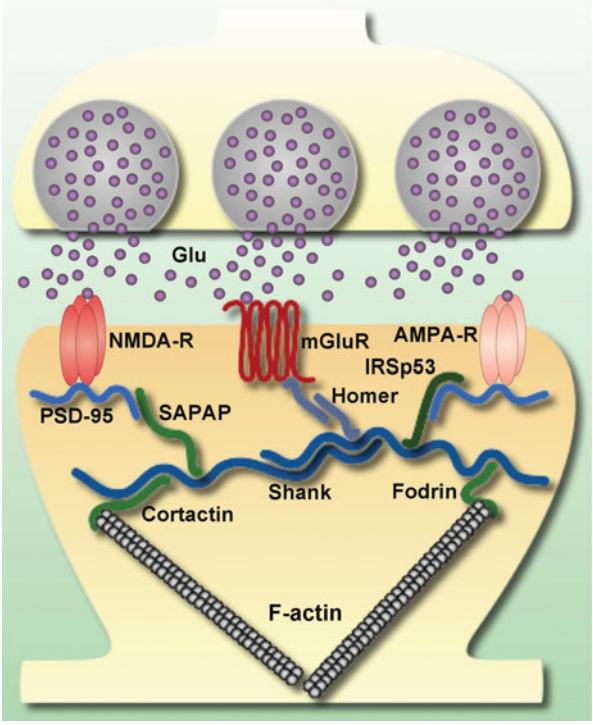

Fig. 5.1 Schematic illustration of an excitatory mammalian brain synapse. At the presynaptic bouton (*upper yellow structure*), synaptic vesicles (*gray spheres*) fuse with the presynaptic membrane to release the neurotransmitter glutamate (*purple spheres*) into the synaptic cleft. Glutamate binds to different type of receptors, which are anchored in the postsynaptic membrane via a dense network of scaffold proteins and filaments of the actin cytoskeleton. See text for further details

parameters, the average molecular weight of the PSD complex was determined to be around 1.1 GDa (Chen et al. 2005; Cheng et al. 2006; Sugiyama et al. 2005).

PSD components may be roughly categorized into four different groups:

1. Neurotransmitter receptors and other cell surface proteins
2. Scaffold proteins, which through multiple protein interaction motifs link different PSD components together
3. Signaling proteins, such as protein kinases and elements of GTPase signaling pathways
4. Cytoskeletal proteins, mostly of the actin-based cytoskeleton

Several mRNAs coding for PSD proteins are associated with the RNA-binding fragile X mental retardation protein (FMRP). Particularly, transcripts encoding members of the SAPAP/GKAP family of scaffold proteins were among the most strongly enriched mRNAs in FMRP immunoprecipitates in the microarray analysis presented by Brown et al. (2001). Further studies have repeatedly identified mRNAs coding for additional scaffold and receptor proteins such as Shank1, PSD-95, and *glutamate receptor* subunits to be associated with FMRP (Schütt et al. 2009; Zalfa et al. 2007). These data suggest that the composition of the PSD may be substantially regulated by FMRP. Given the high relevance of the PSD for synaptic signaling, plasticity, and higher brain functions, we can assume that changes in PSD composition resulting from the loss of FMRP strongly contribute to the cognitive impairments observed in patients suffering from the fragile X syndrome (FXS).

Here, we will first describe the functional relevance of selected components of the PSD. We will then discuss how FMRP may influence postsynaptic levels of individual PSD components, which signaling pathways control the synaptic function of FMRP, and how an altered abundance of PSD proteins may contribute to FXS pathology.

5.2 Membrane Proteins: Glutamate Receptors

Both ligand-gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors convert the appearance of glutamate in the synaptic cleft into a postsynaptic signal (Chua et al. 2010; Kim and Sheng 2009; Sheng and Hoogenraad 2007). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPA-Rs) perform the main business of synaptic transmission as glutamate-gated ion channels for monovalent cations. Tetrameric AMPA-Rs are assembled from four different subunits, with GluR1 and GluR2 being most abundant. In addition, transmembrane AMPA-R associated proteins (TARPs) such as stargazin need to be attached to the receptors for efficient cell surface expression and targeting to postsynaptic sites (Chen et al. 2000; Schnell et al. 2002). As the strength of a synapse is largely defined by the number of functional AMPA-Rs, this is the major parameter that may be regulated during periods of synaptic plasticity,

such as long-term potentiation (LTP) or long-term depression (LTD). The number of available cell surface receptors is tightly regulated by exo- and endocytosis (e.g., Scholz et al. 2010). Once on the cell surface, AMPA-R/TARP complexes diffuse into or out of the PSD. Here, TARPs provide a C-terminal PDZ domain-binding motif (consensus: Xxx-Ser/Thr-Xxx-Val-COO^-) that allows for anchoring of the complex by distinct PDZ domain-containing scaffold proteins of the PSD (Bats et al. 2007; Schnell et al. 2002).

N-methyl-D-aspartate receptors (NMDA-Rs), built mostly from NR1, NR2A, and/or NR2B subunits, constitute another class of ionotropic glutamate receptors, which have a high permeability for Ca^{2+}. Activation of NMDA-Rs under depolarizing conditions triggers Ca^{2+}-dependent alterations in AMPA-R number. Thus, NMDA-Rs are the major inducers of synaptic plasticity. In contrast to AMPA-Rs, the number of NMDA-Rs is more constant and not as highly regulated as the number of AMPA-Rs. NMDA-Rs undergo endocytosis early in development (Lavezzari et al. 2004), but are rather stable at the PSD in later stages. Similar to AMPA-Rs, NMDA-R subunits are anchored at the PSD via C-terminal PDZ domain-binding motifs located on NR2 subunits (Kornau et al. 1995).

Finally, metabotropic glutamate receptors (mGluRs) may modulate postsynaptic signaling by activation of heterotrimeric G-proteins. Postsynaptic mGluR1/mGluR5 subtypes are physically linked to their downstream signaling components phospholipase C and the inositol trisphosphate (IP3) receptor via Homer and Shank proteins, allowing for efficient production of IP3 and release of Ca^{2+} from intracellular stores via IP3 receptors (Tu et al. 1999). Treatment of hippocampal slices with the mGluR agonist (RS)-3,5-dihydroxy-phenyl-glycine elicits one form of LTD characterized by enhanced endocytosis of AMPA-Rs (Huber et al. 2002; Waung et al. 2008), demonstrating that the different glutamate receptors in the PSD are linked to form a large regulatory network.

5.3 PSD-95/SAP90 and Other Membrane-Associated Guanylate Kinases

PSD-95/SAP90 and the other members of the membrane-associated guanylate kinase (MAGUK) family, PSD-93, SAP102, and SAP97, may be considered as prototypic scaffolds as they contain at least five protein interaction domains, which enable interactions with other postsynaptic proteins (Chua et al. 2010; Kim and Sheng 2009). Through the three PDZ domains, PSD-95 associates not only with membrane proteins via their C-terminal PDZ ligand motifs (Kim et al. 1995; Kornau et al. 1995), but also with other scaffold and signaling proteins of the PSD such as SynGAP and IRSp53 (Chen et al. 1998; Soltau et al. 2004). In addition, multimerization of PSD-95 via its N-terminal domain has been observed, which depends on palmitoylation at two conserved N-terminal cystein residues. As these fatty acid modifications provide anchorage in the postsynaptic plasma membrane,

the majority of postsynaptic PSD-95 molecules should be in close apposition to the membrane (El-Husseini Ael et al. 2002). This has been confirmed by immunoelectron microscopy, where PSD-95 was indeed found closer to the membrane than other postsynaptic scaffolds such as Shank or SAPAP/GKAP isoforms (Valtschanoff and Weinberg 2001; see below). We currently assume that PSD-95 (and other MAGUKs) assemble into a two-dimensional network of open binding sites, which allow for docking of membrane receptors carrying appropriate PDZ ligand motifs (i.e., NMDA-Rs carrying NR2A or NR2B subunits, TARP-associated AMPA-Rs, and cell adhesion molecules such as neuroligins). On average, 300–400 MAGUKs per synapse are available for this purpose (Chen et al. 2005; Cheng et al. 2006; Sugiyama et al. 2005). The abundance of PSD-95 is, therefore, highly relevant for synaptic strength, as it determines the amount of additional TARP/AMPA-R complexes, which can be accommodated at the postsynapse during periods of synaptic plasticity (as observed during LTP; Stein et al. 2003). In this context, it is important that the postsynaptic PSD-95 content can be dynamically regulated. Using two-photon microscopy in vivo, Gray et al. (2006) demonstrated that PSD-95 cycles between synaptic and nonsynaptic localizations. Several signaling pathways involving posttranslational modification of the protein control PSD levels of PSD-95. The aforementioned palmitoylation that is essential for incorporation into the PSD is highly dynamic, such that rapid palmitoylation/depalmitoylation cycles are associated with a high turnover of the protein at synapses (reviewed in Keith and El-Husseini 2008). Furthermore, phosphorylation of PSD-95 by cyclin-dependent kinase 5 reduces multimerization and postsynaptic clustering of PSD-95, whereas phosphorylation at another serine residue by Jun N-terminal kinase promotes synaptic accumulation (Kim et al. 2007; Morabito et al. 2004).

Mice deficient in PSD-95 exhibit dramatically altered synaptic plasticity, accompanied by learning deficits (Migaud et al. 1998). However, as these mice are viable and do not exhibit severe insufficiencies in synapse formation, it must be assumed that other MAGUK family members (in particular, PSD-93) compensate for the loss of PSD-95. Nevertheless, it becomes clear that control of postsynaptic PSD-95 levels is a major pathway for the regulation of synaptic efficiency.

5.4 SAP90/PSD-95-Associated Proteins (SAPAPs)/Guanylate Kinase-Associated Proteins (GKAPs)

The guanylate kinase domain of MAGUKs appears to be catalytically inactive, and instead acts as a protein interaction module, which mediates an association with members of the SAPAP/GKAP family (SAPAP1–4) (Chua et al. 2010; Kim and Sheng 2009). In contrast to PSD-95, these proteins are not directly associated with the postsynaptic membrane but are positioned at an intermediate layer of the PSD. Copy numbers of SAPAP family members have been determined in the range of

150–170 molecules per PSD (Chen et al. 2005; Cheng et al. 2006; Sugiyama et al. 2005). Though the expression patterns of individual SAPAP genes strongly overlap, the striatum is one tissue where only one isoform is highly expressed, namely SAPAP3 (Kindler et al. 2004; Welch et al. 2004, 2007). Deletion of the SAPAP3 gene in mice is associated with an obsessive–compulsive disorder-like behavior, and a strongly reduced transmission via cortico-striatal synapses. In addition, SAPAP3-deficient synapses fail to switch from a juvenile type (characterized by NMDA-Rs containing the NR2B subunit) to the adult type (containing NR2A). These data suggest that SAPAPs perform essential functions in regulating synaptic circuitry.

The number of known direct interaction partners for SAPAP isoforms is rather limited. An interaction with a light chain shared by dynein and myosin motor proteins (Haraguchi et al. 2000) suggests that they may be involved in attaching postsynaptic protein complexes to motor proteins involved in dendritic transport (as observed in Gerrow et al. 2006 for SAPAP/PSD-95/Shank complexes). In addition, C-termini of SAPAP proteins contain a PDZ ligand motif, which interacts tightly with postsynaptic scaffolds of another family:

5.5 Proline-Rich Synapse-Associated Proteins (ProSAPs) or SH3- and Ankyrin-Containing Proteins (Shanks)

Shanks/ProSAPs associate with SAPAPs via a central PDZ domain (Naisbitt et al. 1999) that is highly conserved in all three known Shank variants (Shank1/SSTRIP/synamon, Shank2/ProSAP1/CortBP1, and Shank3/ProSAP2) (Kreienkamp 2008). A distinguishing feature of Shanks is, however, their large proline-rich region of about 800–1,000 amino acid residues, which contains several individual proline-rich interaction motifs. These allow for binding to numerous postsynaptic proteins such as Cortactin, Homer, IRSp53, and Abi1 (Bockmann et al. 2002; Du et al. 1998; Proepper et al. 2007; Soltau et al. 2002; Tu et al. 1999). The N-terminal portion of Shanks contains an ankyrin repeat region and an Src homology three domain, which provide further interactions with the cytoskeletal protein α-Fodrin (Böckers et al. 2001), Sharpin, and Densin-180 (Lim et al. 2001; Quitsch et al. 2005). Finally, the C-terminal sterile alpha motif (SAM) domains of Shank2 and Shank3 (but not Shank1) may multimerize in a zinc-dependent manner (Baron et al. 2006; Grabrucker et al. 2011).

Shanks are thought to connect the postsynaptic complex with the actin cytoskeleton of the dendritic spine. Consequently, compared to MAGUK/SAPAP members, they are positioned more remote from the plasma membrane (Valtschanoff & Weinberg 2001) and interact with only a few membrane proteins directly (e.g., Kreienkamp et al. 2000). Sala et al. (2001) were the first to show that Shanks may have a significant effect on the formation of the PSD as well as spine morphology. Overexpression of Shank1 causes enhanced maturation of spines. This depends on

the Shank1-binding sites for SAPAPs and Homer. Homer in particular aids the recruitment of smooth ER membranes to the spine through its interaction with the IP3 receptor. Roussignol et al. (2005) extended these studies by showing that Shank3 overexpression induces spine formation in otherwise spineless neurons. Recent work by Grabrucker et al. (2011) indicates that at least one Shank isoform needs to be present at the PSD for maintenance of synapses.

Despite high similarity in their protein interaction motifs, and a highly overlapping expression pattern (Böckers et al. 2004), Shank isoforms do not appear to be functionally redundant. Shank1 is incorporated into PSDs at a later time point than Shank2 and Shank3, and is not able to increase spine density upon overexpression in cultured neurons in early stages of differentiation (Grabrucker et al. 2011). This may be related to the inability of the Shank1 SAM domain to undergo zinc-dependent polymerization. So far, genetic deficits in *SHANK3* and to a lesser extent *SHANK2* have been associated with mental disease in humans, whereas no disease-associated alterations in the human *SHANK1* gene have been observed (Berkel et al. 2010; Bonaglia et al. 2001; Durand et al. 2007). Both autism and mental retardation have been linked to deletions and point mutations in *SHANK3*. Mouse models suggest that Shank3 is most relevant to the function of hippocampal synapses, as mice lacking one copy of the gene exhibit reductions in basal synaptic transmission, LTP, and the number of synaptic AMPA-Rs. These changes coincide with deficits in social interactions, suggesting that haploinsufficiency of *SHANK3* causes autism-like disorders in mice as well as in man (Bozdagi et al. 2010). After preparation of this manuscript, several publications analyzed the phenotype of additional lines of Shank3 deficient mice (Wang et al., 2011; Peca et al., 2011). Here phenotypic changes such as deficits in synaptic transmission or plasticity were observed only in homozygous mice. Interestingly, mice heterozygous for a truncated Shank3 gene exhibited almost complete loss of the wild type Shank3 protein due to proteasomal degradation, coincident with an autistic behavioural phenotype (Bangash et al., 2011). Importantly, all manuscripts published so far support the view that the Shank3 protein is required for normal synaptic function as well as normal behaviour. On the contrary, homozygous deletion of the Shank1 gene in mice also leads to reduced basal synaptic transmission but does not affect social interactions (Hung et al. 2008). Interestingly, mRNAs coding for Shank isoforms as well as SAPAP3 are prominently localized in neuronal dendrites (Böckers et al. 2004; Kindler et al. 2005; Schütt et al. 2009; Welch et al. 2004), indicating that the proteins are synthesized locally at synapses.

5.6 arg3.1/Arc: A Regulator of AMPA Receptor Trafficking

This protein is a rather temporary component of postsynaptic sites, as its levels are rather low under resting conditions but strongly increase upon excitatory synaptic activity. Newly formed arg3.1/Arc mRNA is rapidly transported into dendrites,

where it is locally translated to provide activated synapses with the arg3.1/Arc protein (Link et al. 1995; Lyford et al. 1995; Steward and Worley 2001). There arg3.1/Arc interacts with proteins of the endocytosis machinery, in particular with endophilin 2/3 and dynamin-2, and thus facilitates endocytosis of AMPA-Rs (Chowdhury et al. 2006). This is particularly relevant for mGluR-dependent LTD. Here, basal levels of arg3.1/Arc are required for the reduction of synaptic AMPA-Rs by rapid endocytosis during the early phase of LTD. For the maintenance of LTD, however, new arg3.1/Arc needs to be synthesized locally to enable a higher rate of AMPA-R endocytosis (Waung et al. 2008). The physiological significance of this mechanism was illustrated by Plath et al. (2006) who showed that arg3.1/Arc-deficient mice exhibit impaired memory formation.

5.7 Calcium/Calmodulin-Dependent Protein Kinase II (CaMKII): A Major Signaling Molecule and Scaffold of the PSD

The α-subunit of the CaMKII is probably the most abundant signaling molecule in PSDs. Similar to arg3.1/Arc, its mRNA is present in dendrites and it is supplied to the PSD by local synthesis (Miller et al. 2002). αCaMKII forms heterooligomers with βCaMKII, which fulfill an essential role in NMDA-R-dependent LTP. The protein is activated upon Ca^{2+} influx through NMDA-Rs, followed by autophosphorylation at threonine 286, which converts the kinase into a permanently active enzyme. Active CaMKII affects synaptic incorporation of AMPA-Rs and at the same time phosphorylates regulators of the actin-based cytoskeleton, thereby leading to enlargement of dendritic spines. Interestingly, CaMKII subunits may also be considered as structural or scaffold proteins, as βCaMKII binds and eventually also bundles actin filaments (see review by Okamoto et al. (2009) and references therein). On the other hand autophosphorylated αCaMKII binds to proteasomes and promotes their recruitment into dendritic spines (Bingol et al. 2010). This process leads to the degradation of polyubiquitinated proteins in spines, thereby enhancing structural reorganization of synapses in response to synaptic stimulation.

5.8 Control of Neuronal mRNA Metabolism by FMRP

The finding that FMRP contains several typical RNA-binding motives and in vitro associates with distinct RNAs, strongly suggested a role as a regulator of RNA metabolism of eukaryotic cells (Ashley et al. 1993; Brown et al. 1998). In agreement with this notion, FMRP was found to associate selectively with about 4% of mRNAs present in the mammalian brain, including transcripts that encode key

components of the PSD, such as SAPAP3 and SAPAP4 (Brown et al. 2001). Currently available data suggest that in mammalian neurons, FMRP controls mRNA metabolism in at least three distinct ways, namely by regulating dendritic mRNA targeting, transcript stability, and mRNA translation (Bassell and Warren 2008; Levenga et al. 2010).

5.9 FMRP and Dendritic Targeting of mRNAs Encoding PSD Components

Utilizing in situ hybridization techniques to determine the subcellular localization of different dendritic transcripts in the mouse hippocampus, Steward et al. (Steward et al. 1998) showed that loss of FMRP does not alter dendritic levels of mRNAs encoding the PSD components αCaMKII and arg3.1/Arc, as well as the dendritic proteins dendrin and microtubule-associated protein 2. These findings suggested that FMRP is not necessary for dendritic mRNA targeting. In agreement with this notion, dendritic levels of transcripts encoding αCaMKII and PSD-95 are not obviously altered in the neocortex, hippocampus, and dentate gyrus of FMRP-deficient mice (Muddashetty et al. 2007). Nevertheless, Dictenberg et al. (2008) reported that in cultured primary neurons from wild-type, but not from FMRP-deficient mice, activation of mGluRs induces dendritic targeting of several mRNAs. These include the messages coding for αCaMKII, SAPAP4, MAP1b, and RGS5. In addition, Kao et al. (2010) observed that mGluR activation induces a significant and FMRP-dependent enrichment of αCaMKII transcripts in dendritic spines compared to shafts of dendrites. Currently, the physiological significance of these cell culture observations for the intact mammalian brain remains unclear, as in vivo αCaMKII but not SAPAP4 transcripts were identified as prominent dendritic mRNAs (Burgin et al. 1990; Kindler et al. 2004; Welch et al. 2004, see also The Allen Brain Atlas at http://www.brain-map.org/). Taken together, these data suggest that FMRP does not play an obligatory role in extrasomatic trafficking of most dendritic mRNAs. However, it may still to some extent modulate activity-dependent targeting of particular transcripts into dendrites.

5.10 Role of FMRP in Controlling Stability of Transcripts for Postsynaptic Proteins

Recently, FMRP has also been implicated in regulating the stability of dendritic transcripts. In particular, total levels of PSD-95 mRNA were reported to be drastically reduced in the hippocampus but not neocortex of FMRP-deficient mice compared to wild-type animals, indicating that binding to FMRP enhances the stability of PSD-95 mRNAs in a cell type-specific manner (Zalfa et al. 2007). In contrast,

using semiquantitative real-time polymerase chain reaction, Schütt et al. (2009) found both overall and synaptic levels of PSD-95 mRNAs in the hippocampus and the neocortex to be unaltered upon loss of FMRP. At the same time, this study also showed that distinct from other dendritic mRNAs such as Shank1 and SAPAP3 transcripts, relative PSD-95 mRNA levels at synapses versus whole neurons are rather minor. This finding is consistent with earlier in situ hybridization data on brain slices (Cho et al. 1992; Kistner et al. 1993, see also The Allen Brain Atlas), suggesting that PSD-95 messages are not prominent dendritic mRNAs. Thus, the questions whether FMRP may stabilize some of its target mRNAs, including PSD-95 transcripts, and whether it may perform this regulatory function locally at synapses await further analysis.

5.11 FMRP-Mediated Translational Control of mRNA Targets Encoding PSD Components

One hallmark of the FXS in humans and its Fmr1$^{-/-}$ mouse model is the hyper-abundance of rather immature dendritic spines along dendrites (Grossman et al. 2006; Irwin et al. 2001, 2002). FMRP associates with actively translating polyribosomes in vivo (Corbin et al. 1997; Feng et al. 1997; Khandjian et al. 2004; Stefani et al. 2004) and represses mRNA translation in vitro (Laggerbauer et al. 2001; Li et al. 2001). The presence of FMRP along dendrites has, therefore, led to the hypothesis that FMRP regulates local protein synthesis at synapses and thereby contributes to dynamic changes in synaptic structure and function that are critical for learning and memory (Bassell and Warren 2008; Levenga et al. 2010). Synaptic phenotypes of the FXS are, therefore, thought to at least in part result from an impaired translational control of transcripts encoding PSD components. Analyzing PSD fractions isolated from mouse brain, we recently showed that a select group of postsynaptic proteins, including Shank1, Shank3, SAPAP1–3, NMDA receptor subunits NR1 and NR2B, and AMPA receptor subunit GluR1, is enriched in PSDs of FMRP-deficient mice (Schütt et al. 2009). mRNAs encoding these PSD components are in vivo associated with FMRP; however, loss of FMRP does not affect the concentration of these transcripts, neither whole cell nor synaptic levels. These data indicate that FMRP regulates the translation, but not the stability of the respective mRNAs. Functional assays in primary neurons further suggest that FMRP represses translation of Shank1 transcripts via an interaction with their 3′UTR, an inhibition that is abolished upon activation of mGluRs. As Shank1 stabilizes dendritic spines, its deregulated postsynaptic synthesis may significantly contribute to the aberrant dendritic spine morphology observed in the absence of FMRP.

Interestingly, the exact mode by which FMRP regulates mRNA translation is not yet clear. Current experimental evidence suggests that four regulatory mechanisms may contribute to FMRP-mediated translational control (Fig. 5.2). In the first

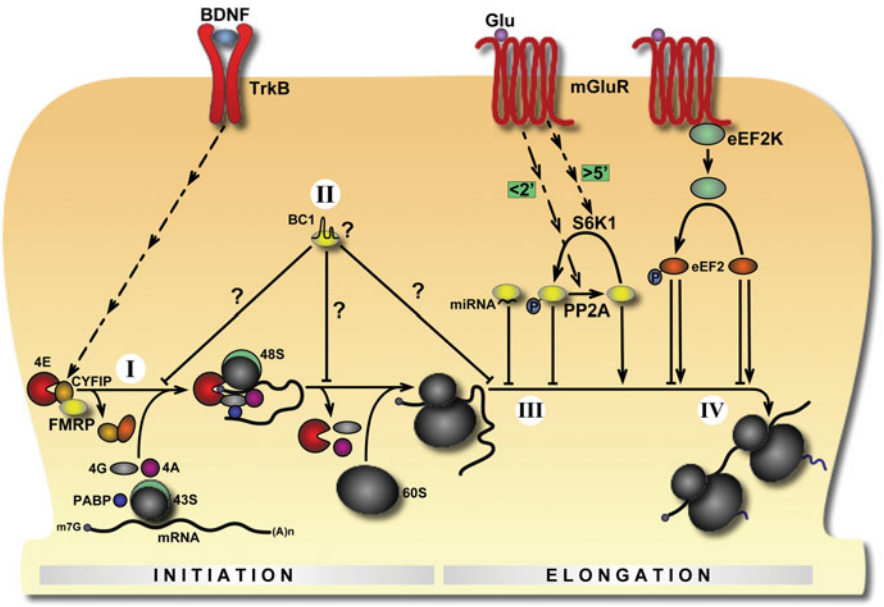

Fig. 5.2 Schematic representation of FMRP controlled pathways regulating protein synthesis at synapses. FMRP has been implicated in the control of both 5′cap-dependent initiation (*left*) and elongation (*right*) of translation. Factors are not drawn to scale. Abbreviations: 4A, eIF4A; 4E, eIF4E; 4G, eIF4G; 48S, pre-initiation complex bound to initiator codon of mRNA, 7-methylguanosine cap structure, m7G; BDNF receptor, TrkB; glutamate, Glu; large ribosomal subunit, 60S; poly(A)-binding protein, PABP; poly(A) tail of mRNA, (A)n. See text for detailed explanations and further abbreviations

scenario, FMRP is thought to target initiation of mRNA translation. During initiation, the eukaryotic initiation factor eIF4E binds to the 5′cap of the mRNA and helps to recruit additional factors, including eIF4G, eIF4A, and the so-called 43S pre-initiation complex. Thus, association of eIF4E with the 5′cap is a critical step in the initiation process (Jackson et al. 2010; Sonenberg and Hinnebusch 2009). eIF4E activity is regulated by different binding proteins (4E-BPs) that can sequester eIF4E, occupy the eIF4G-binding site on eIF4E, and therefore block translation initiation. The cytoplasmic FMRP interacting protein 1 (CYFIP1) has been suggested to act as a 4E-BP that inhibits eIF4E function and translation initiation of FMRP-associated mRNAs, including αCaMKII transcripts, at synapses (Napoli et al. 2008). Upon activation of BDNF receptors or mGluRs, eIF4E and CYFIP1 dissociate and thus eIF4E may be free to promote initiation.

As a second mode of action, FMRP has been proposed to associate with the small untranslated RNA BC1 to repress translation of particular dendritic target mRNAs such as αCaMKII and arg3.1/Arc messages, which base pair with BC1 (Zalfa et al. 2003). However, several other groups have subsequently been unable to verify specific binding of BC1 to FMRP (Iacoangeli et al. 2008a). Also, while BC1

has been shown to repress initiation of translation (Wang et al. 2002, 2005), the preferential association of FMRP with actively translating polysomes instead implies a role during the elongation phase (Corbin et al. 1997; Feng et al. 1997; Khandjian et al. 2004; Stefani et al. 2004). Thus, it remains a matter of debate whether the proposed mutual regulatory interaction between FMRP and BC1 is indeed implemented at mammalian synapses (Bagni 2008; Iacoangeli et al. 2008b).

A third proposed cellular mechanism involves micro RNAs (miRNAs) (Cheever and Ceman 2009; Jin et al. 2004), small RNAs of 21–24 nucleotides in length, which act as repressors of translation through partial base pairing with particular target mRNAs (Slezak-Prochazka et al. 2010). In the mammalian brain, FMRP associates with different miRNAs, including miR-125b and miR-132 (Edbauer et al. 2010). miR-125b hybridizes with mRNAs encoding postsynaptic NMDA receptor subunits and thereby suppresses their translation. Currently, it is unknown if a concerted action of FMRP with various miRNA also regulates local synthesis of other PSD components at excitatory synapses.

As a fourth possibility, FMRP targets the elongation phase of mRNA translation (Waung and Huber 2009). In particular, it appears to be involved in inhibiting arg3.1/Arc mRNA translation at synapses at the basal state (Park et al. 2008). Upon mGluR activation, the receptor-associated eukaryotic elongation factor 2 kinase (eEF2K) dissociates from mGluRs and phosphorylates eukaryotic elongation factor 2 (eEF2). Phospho-eEF2 inhibits general protein synthesis by slowing the elongation step of translation while simultaneously abolishing FMRP-mediated translational block on arg3.1/Arc mRNAs, thereby triggering rapid arg3.1/Arc synthesis. Newly synthesized arg3.1/Arc associates with endophilin 2/3 and dynamin to induce enhanced internalization of AMPA-Rs, leading to LTD of synapses. Thus, loss of this regulation in FMRP-deficient mice leads to excessive LTD, which is independent of new protein synthesis (Waung et al. 2008).

In FMRP, Ser499 can be phosphorylated by S6 kinase 1 (S6K1). Whereas non-phosphorylated FMRP preferentially associates with actively translating polysomes, its phosphorylated form tends to associate with apparently stalled polysomes (Ceman et al. 2003) and thus appears to inhibit translation (Ceman et al. 2003; Narayanan et al. 2008). At synapses, activation of mGluRs induces a short-lived dephosphorylation of phospho-FMRP by protein phosphatase 2A (PP2A), which removes the FMRP-mediated translational block of dendritic transcripts, such as SAPAP3 mRNAs. Soon thereafter, the same receptor activity leads to rephosphorylation of FMRP by S6K1. Loss of S6K1 activity, therefore, results in the absence of phospho-FMRP and increased levels of postsynaptic SAPAP3 (Narayanan et al. 2008). Thus, activation of the mGluR-FMRP signaling pathway allows for only a brief phase of synaptic protein synthesis followed by repression of translation after an appropriate period of time (Levenga et al. 2010). Alternatively, synaptic translation of SAPAP3 mRNAs can also be triggered by stimulation of dopamine D1 receptors (Wang et al. 2010).

5.12 Consequences of the Loss of FMRP-Mediated Translation Control for Synaptic Contacts

As described above, increasing recent evidence suggests that in FMRP-deficient mice, levels of several key components of postsynaptic signaling complexes are dysregulated, including major PSD scaffolds such as Shank and SAPAP isoforms. It is likely that some of the observed molecular changes contribute to the hyperabundance of immature dendritic spines in the FMRP-deficient mammalian brain (Grossman et al. 2006; Irwin et al. 2001, 2002), a morphological alteration that is generally believed to underlie learning deficits and behavioral abnormalities in both FXS patients and FMRP-deficient mice (Bassell and Warren 2008; Penagarikano et al. 2007). In particular, dendritically localized Shank1 and SAPAP3

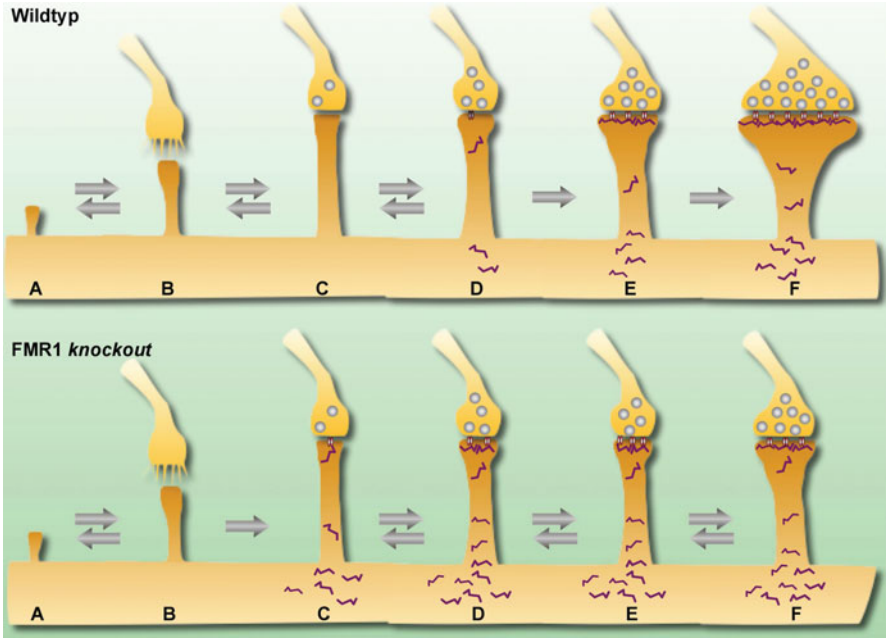

Fig. 5.3 Schematic representation of dendritic spine development. *Upper panel* a filopodial protrusion extends from the dendritic shaft of a wild-type neuron. (A). Upon contact with an axonal growth cone (B), the intercellular contact site is stabilized (C) and may develop into an "immature" synapse (D). Depending on distinct factors, such as the synaptic signaling intensity, the newly formed contact may either disappear (D > A) or develop to a "mature" synapse containing a mushroom-type dendritic spine (E and F). Final spine maturation critically depends on the spatially and timely regulated recruitment of postsynaptic scaffold proteins, such as proteins of the Shank and SAPAP family (*purple lines*). In FMRP-deficient neurons (*lower panel*), loss of FMRP results in an excess synthesis of distinct postsynaptic components, including Shank1. This may lead to an enhanced accumulation of Shank1 in immature spines (C, D, and E), which may thus be unable to either develop into mature synapses (F) or entirely disappear (C > A). Thus, FMRP-deficient neurons possess significantly more immature spines as their wild-type counterparts

mRNAs have been identified as in vivo FMRP targets (Brown et al. 2001; Narayanan et al. 2008; Schütt et al. 2009). Overproduction of Shank1 and increased postsynaptic protein levels, as observed in the neocortex and hippocampus of Fmr1$^{-/-}$ mice, may be a major cause for the synaptic FXS phenotype. As described above, Shank isoforms are considered as master scaffolds of the PSD (Gundelfinger et al. 2006) and are dynamically involved in the generation and stabilization of dendritic spines. Overexpression of Shank1 or Shank3 induces spine formation and maturation (Roussignol et al. 2005), whereas Shank1-deficient mice exhibit a reduced number and size of dendritic spines (Hung et al. 2008). Thus, excess production of Shank1 at synapses may lead to an erroneous stabilization of nascent dendritic spines and may, therefore, cause aberrant dendritic spine morphology, density, and function (Fig. 5.3). Further genetic studies show that tight control of neuronal Shank levels is highly relevant for mental health in humans, as loss of only one copy of the human *SHANK3* gene is associated with mental retardation (Bonaglia et al. 2001) and mutations in one *SHANK3* allele have been identified in patients suffering from autism spectrum disorders (ASD; Durand et al. 2007). Mutations in *SHANK2* have also been associated with both mental retardation and ASD (Berkel et al. 2010). Similarly, loss of SAPAP3 in mice results in abnormal behavior and synaptic function (Welch et al. 2007). In particular, these mice fail to progress from a "juvenile" to an "adult" complement of NMDA-R subunits at synapses, indicating that SAPAP3 plays a crucial role in synapse maturation. In the future, to better understand the significance of individual dysregulated PSD proteins for the synaptic FXS phenotype, it will be helpful to establish appropriate in vivo models, which allow for the fine-tuning of the levels of individual PSD components. Thus, it will be possible to test whether modulation of postsynaptic concentrations of single proteins may compensate for morphological and function abnormalities observed in the FMRP-deficient brain.

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Chapter 6
Behavior in a Drosophila Model of Fragile X

Sean M. McBride, Aaron J. Bell, and Thomas A. Jongens

Abstract This chapter will briefly tie together a captivating string of scientific discoveries that began in the 1800s and catapulted us into the current state of the field where trials are under way in humans that have arisen directly from the discoveries made in model organisms such as *Drosophila* (fruit flies) and mice. The hope is that research efforts in the field of fragile X currently represent a roadmap that demonstrates the utility of identifying a mutant gene responsible for human disease, tracking down the molecular underpinnings of pathogenic phenotypes, and utilizing model organisms to identify and validate potential pharmacologic targets for testing in afflicted humans. Indeed, in fragile X this roadmap has already yielded successful trials in humans (J. Med. Genetic 46(4) 266–271; Jacquemont et al. Sci Transl Med 3(64):64ra61), although the work in studying these interventions in humans is just getting underway as the work in model organisms continues to generate new potential therapeutic targets.

6.1 The History

In 1850, 9 years before Charles Darwin published "On the Origin of Species" and 15 years before the first public presentation of Gregor Mendel's revolutionary experiments laying out the foundation of genetics presented in the lecture "Experiments in Plant-Hybridisation," Felix Dujardin first proposed that the mushroom bodies (MBs) in the insect brain were analogous to the human hippocampus (Darwin 1859; Mendel 1866; DuJardin 1850). DuJardin postulated that the MBs

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must, therefore, be responsible for memory in insects due to the extensive folding and resemblance to the human hippocampal structure (DuJardin 1850). These initial studies were followed up by extensive anatomical studies of the insect brain by Kenyon published in 1896 stimulating further speculation that this may represent a structure involved in memory formation in insects (Kenyon 1896). The high proportion of mentally retarded males relative to mentally retarded females was first recognized from US census data in 1897, although no rationale was provided (Johnson 1897). Thomas Hunt Morgan at the turn of the century began working on *Drosophila* (fruitfly) genetics and expanding on the Mendelian theory of inheritance bringing to prominence the power of *Drosophila* as an organism in which to examine genetics due to its low cost, fast generation time, and the ability to use a forward genetic approach to study naturally occurring mutations (Morgan 1919). Indeed, the power of the model organism, *Drosophila,* has become particularly clear since the completion of the human and *Drosophila* genomic sequencing projects. Results from these projects have reinforced the notion that a great deal has been conserved genetically over the course of evolutionary history (Yandell et al. 2006; Celniker and Rubin 2003; Rubin et al. 2000; Rubin and Lewis 2000). Today, *Drosophila* represents a powerful and cost-effective genetic model and the vast majority of the basic cell biological pathways have shown strong conservation between *Drosophila* and mammals (Dauwalder and Davis 1995; Muqit and Feany 2002).

6.1.1 Conservation of Cellular signaling

The conservation of pathways between Drosophila, other invertebrates and mammals amazingly extends beyond cell biology and into pathways important for learning and memory. This is highlighted by the fact that the proteins that have been identified to be important for learning and memory in Drosophila, Aplysia (sea slug), and mammals are highly conserved (Kandel 2001). Indeed many of these proteins are also critical for the establishment of long-term potentiation (LTP) and long-term depression (LTD) in mammals, suggesting that the phenomena of activity-dependent modification of synaptic efficacy represent the cellular mechanisms that underlie plastic cognitive behavioral process conserved across many species, and thought to be critical for learning and memory (Cajal 1894; Konorski 1948; Hebb 1949; Bailey et al. 2004; Bear and Abraham 1996; Hawkins et al. 1993; Sherman and Atwood 1971; Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973; Skoulakis and Grammenoudi 2006). Although it has yet to be definitively demonstrated that LTD and LTP are required for associative memory formation, LTP and LTD have been demonstrated to correlate with learning and memory in mammals (Whitlock et al. 2006; Manahan-Vaughan and Braunewell 1999). Indeed the earliest studies on the molecular substrates of memory were performed in invertebrates (for review see Kandel 2001). Furthermore, circadian behavior also demonstrates a similar high degree of conservation across species from Drosophila to mammals. The molecular

underpinnings of circadian rhythms were also first elucidated in the model organism Drosophila (Reddy et al. 1984; Zehring et al. 1984; Bargiello and Young 1984; Jackson and Newby 1993; Young 1996).

Although a high degree of conservation exists in the molecular pathways regulating cognition as well as other behaviors including social interaction and circadian rhythmicity, this still leaves open the question of whether or not Drosophila can be useful to model human diseases that afflict such processes. An ideal disease model not only replicates important aspects of the human disease of interest, but also provides clues toward effective treatment routes. This not only depends on how closely the model recapitulates the genetic condition leading to the disease state, but also on how well the genetic alteration recapitulates the defects leading to the human disease state.

6.1.2 Why Drosophila?

Almost all of the cases of fragile X syndrome are due to a trinucleotide expansion in the 5′ untranslated region of the FMR1 gene, leading to hypermethylation of the FMR1 gene and resulting in no or very little functional expression of the protein product FMRP (Hagerman and Hagerman 2002). Drosophila possesses a highly conserved gene called *dfmr1*, whose protein products contain several highly conserved biochemical domains that are also found in human FMRP (Wan et al. 2000). Given this high degree of homology, several groups set out to develop a model based on loss of *dfmr1* expression, by isolating Drosophila mutants that contained deletions of all or most of the *dfmr1* gene (Zhang et al. 2001; Morales et al. 2002; Dockendorff et al. 2002; Inoue et al. 2002). In addition to these models, mutants that contained point mutations in the *dfmr1* coding region were isolated that also appear to eliminate all *dfmr1* activity (Xu et al. 2004). Thus several Drosophila mutants exist that should recapitulate the same genetic situation of having no functional FMR1 protein as found in most patients with fragile X syndrome.

From a signaling network perspective the loss of *dfmr1* expression theoretically allows for an approximation of the signaling dysregulation in human patients, allowing the possible modeling of several clinically relevant aspects (phenotypes) of fragile X in Drosophila. The hope is that this will expedite the elucidation of molecules and pathways involved in pathologic behaviors associated with fragile X because Drosophila is amenable to pharmacologic and genetic screens for suppression or enhancement of phenotype, a hope that has already begun to be realized.

6.1.3 How to Construct a Model in Drosophila

There are three basic approaches that are currently used to develop models in Drosophila. The first approach is that potential models are identified from screens using forward-genetics, in which a large number of mutations are created, screened

for the appropriate phenotype, and then are seen as candidate genes in human disease. This forward genetic screen is a strategy that revolutionized the Drosophila field of genetics with the development of large-scale ethyl methanesulfonate EMS screens (Jenkins 1967a, b; Epler 1966). The second approach is reverse-genetics in which a gene that represents a potential homologue of a human disease gene is manipulated to alter its expression and then the phenotype is analyzed (Jenkins 1972). The candidate gene expression can be increased or reduced. This approach was quickly adapted for the analysis of behavioral phenotypes in Drosophila by a seminal work from the pioneering lab of Seymour Benzer (Benzer 1973). The third approach utilizes the GAL4/UAS system to induce site-directed expression of human proteins in Drosophila. In this system, the gene of interest is placed downstream of a UAS (upstream activating sequence) in a transgenic construct. The transcriptional activator GAL4 binds the UAS sequence. However, without the influence of GAL4, the gene remains inactive. Several thousand fly stocks containing transgenic constructs that express GAL4 in temporal, or cell-type specific patterns are currently available. When such transgenic constructs are genetically introduced into flies that contain the UAS transgene, the gene of interest is expressed in a pattern that mirrors the pattern of the GAL4 "driver transgene" construct (Brand and Perrimon 1993). Furthermore, utilizing recently developed optogenetic approaches, the precise temporal as well as spatial expression of the gene of interest can be manipulated in active Drosophila (Miesenbock 2009). Additionally, Drosophila has nearly complete genome coverage of deletions, insertional mutants, and RNAi libraries, making this model system unmatched in the alacrity with which neurogenetic dissection of behavior can be accomplished (for reviews see (Sokolowski 2010; Muqit and Feany 2002; Bell et al. 2009).

6.1.4 The Phenotype or Endophenotype of Interest

The ability to make genetically relevant models raises the question of what are the most important phenotypes displayed by the model to study once it is obtained? For fragile X syndrome, the most debilitating clinical feature, according to the majority of parents and physicians, is cognitive impairment with the average IQ score in males with fragile X syndrome being 50 (Hagerman and Hagerman 2002). One proposed explanation of the learning and memory deficits is altered shape and number of dendritic spines. This phenotype was found in the mouse model of fragile X, the FMR1 knockout mice (Comery et al. 1997), identified in affected humans at autopsy (O'Donnell and Warren 2002), and is consistent with the theory that dendritic spine "dysgenesis" may be involved in mental retardation in humans (Purpura 1974). Cognitive impairment is a social and medical obstacle, keeping many afflicted patients from living independently and being able to sustain needed medical treatments independently.

Other devastating aspects of the disease include autistic behaviors (social interaction such as communication and repetitive behaviors), which have been shown to

increase in severity with age and circadian dysregulation, resulting in impaired function and impaired sleep. Autism afflicts 25–67% of male patients with fragile X syndrome, which increases in severity with age (Zafeiriou et al. 2007; Moldin 2005; Moldin et al. 2006; Jacquemont et al. 2007; Hatton et al. 2006). Female fragile X patients are affected at a lower percentage (Hatton et al. 2006; Jacquemont et al. 2007; Zafeiriou et al. 2007). Fragile X patients afflicted with autism tend to have worse cognitive function compared to non-autistic fragile X patients (Hatton et al. 2006). For many years it has been thought that upwards of 75% of autistic patients suffered from cognitive impairment (as defined by an IQ < 70). However, it is currently thought that approximately 50% of patients diagnosed with autism spectrum disorder (ASD) also suffer from cognitive impairment (Gernsbacher et al. 2005). Therefore, the most obvious phenotypes displayed by a fragile X model must include the histology of the relevant tissue or organ, in this case the Drosophila brain. Additionally, social interaction, cognitive ability, and circadian behavior would seem to be intimately related to the human disease. Therefore, studies of the fragile X fly model have included examination of brain morphology, social interaction, cognitive ability, circadian behavior, and sleep.

6.1.5 Learning and Memory in Drosophila

Learning and memory (cognitive abilities) can be examined in *Drosophila* by utilizing available learning and memory paradigms. The two most popular are an olfactory classical conditioning paradigm (also known as the odor-shock paradigm) and conditioned courtship paradigm (also known as the courtship conditioning paradigm). The first to be established was the odor-shock paradigm, wherein flies learn to associate electric shocks with olfactory cues (Quinn et al. 1974; Dudai et al. 1976; Jellies 1981; Tully and Quinn 1985; Davis 1993; Tully et al. 1994; Skoulakis and Grammenoudi 2006). An initial odor is given concurrently with electric foot shocks, while a subsequent odor is given without the electric shock. The first odor given is the conditioned stimulus and the foot shock is the unconditioned stimulus. The flies are then given a choice between the two odors in a T-maze, and the flies that have learned to associate the conditioned and unconditioned stimuli then avoid the odor that was given with the shock.

In the conditioned courtship paradigm, a male fly learns to modify his courtship behavior after experience with an unreceptive female (Siegel and Hall 1979; Hall 1994; Skoulakis and Grammenoudi 2006). The conditioned courtship paradigm is an ethologically relevant and more complex associative memory paradigm, which involves the visual, olfactory, gustatory, tactile, and acoustic sensory systems (Tompkins et al. 1982, 1983; Tompkins 1984; Ackerman and Siegel 1986; Siwicki et al. 2005; Ejima et al. 2005, 2007).

In courtship, male flies perform a characteristic sequence of behaviors: orienting toward and following the female, tapping her with his forelegs, vibrating one or both wings, licking her genitalia, and attempting copulation (Sturtevant 1915;

Bastock 1955, 1956; Hall 1994; Dickson 2008). These behaviors are repeated with some variation until successful copulation occurs. Virgin females will generally respond by mating; however, recently mated females will be unreceptive and will not allow copulation to occur (Spieth 1974). They display different behaviors (Bastock 1955, 1956; Burnet and Connolly 1973; Connolly 1973) and have an altered, although somewhat overlapping, pheromonal profile (Cobb 1996; Ejima et al. 2007).

A naïve male paired with a mated female will initially court her, but his courtship activity soon decreases displaying a form of learning. This "learning during training" (LDT) is quantified by comparing the percentage of time the male spends courting the mated female (courtship index, CI) during the first 10 min to the CI of the last 10-min period of a 1-h pairing. Wild type flies typically show a 40% or more decrease in courtship activity during the training session (Joiner Ml and Griffith 1997; Kane et al. 1997). Hence LDT is a form of behavioral plasticity; however, it is distinct and separate from courtship suppression assayed post-training, which is a form of associative memory (Ackerman and Siegel 1986; Tompkins et al. 1983). When a male is paired with a virgin female, after the 1 h of training experience with a mated female, his courtship remains depressed for 2–3 h (Siegel and Hall 1979). This effect is not a general suppression of all courtship activity, since trained males do not modify their courtship of other pheromonally distinct targets (Gailey et al. 1984; Ejima et al. 2005; Siwicki et al. 2005). This indicates that the depression in courtship activity is not the result of fatigue. After training with a mated female, memory is measured as a decrease in CI toward virgin females in trained males relative to naïve controls.

6.1.6 The Phases of Memory in Drosophila

Genetic and pharmacologic dissection has resulted in the elucidation of 5 phases of memory in *Drosophila* (Greenspan 1995). Depending on when the fly is assayed, there is an immediate recall memory 0–2 min post-training (also referred to as immediate memory); short-term memory (STM) out to just over 1 h; medium-term memory (MTM) out to 6 h (also referred to as middle term memory); anesthesia resistant memory (ARM) out to 2 days; and long-term memory (LTM) which lasts up to 9 days post training (Greenspan 1995; Margulies et al. 2005; McBride et al. 1999; Skoulakis and Grammenoudi 2006; Tully et al. 1990, 1994; Yin et al. 1994, 1995; McBride 1995). In addition, LDT can also be assayed in the conditioned courtship paradigm (Joiner Ml and Griffith 1997; Kane et al. 1997).

A major advance for the field was the invention of LTM paradigms which established phases of memory in *Drosophila* that fully parallel the phases of memory found in other invertebrate and vertebrate models of memory. This was achieved in the odor-shock paradigm, with memory lasting out to 7 days (Tully et al. 1994; Yin et al. 1994, 1995), as well as in the conditioned courtship paradigm, with memory lasting out to 9 days (McBride et al. 1999; McBride 1995). Immediate-recall and STM are independent of translation and transcription. MTM requires protein

synthesis from pre-existing mRNA messages. Anesthesia-resistant memory is independent of de novo protein synthesis. In contrast, LTM requires de novo translation and transcription. This dissection of the phases of memory has also led to further understanding of the cellular pathways involved in memory by isolating memory mutants with deficits in specific phases of memory.

6.1.7 The Anatomical Structures Involved in Memory Formation

Beyond knowing the phases and signaling pathways involved in learning and memory, the next important question becomes what are the areas of the brain that affect these behaviors in *Drosophila* so that a focused search for pathological alterations in the brain can be instigated. Dujarin first proposed the involvement of the mushroom bodies as an anatomical location of memory formation. In *Drosophila*, the MBs arise from bilateral clusters of about 2,500 Kenyon cells located in the dorsal and posterior cortex (Davis 1993; Strausfeld et al. 1995; Yang et al. 1995). Information from various sensory systems including olfactory, gustatory, visual, and thoracic sensory systems feed into the MBs, making them an ideal candidate to form associations from various environmental stimuli (Power 1943; Strausfeld et al. 1995; de Belle and Heisenberg 1994; Heisenberg et al. 1995; Barth and Heisenberg 1997; McBride et al. 1999).

To examine the role of the MB in memory, Erber et al. (1980) used cooling experiments, which disrupted mainly the MBs but involved other brain structures as well, to link the MBs to olfactory memory in *Apis mellifera* (honeybees) (Erber 1980). In 1980, Heisenberg utilized the mushroom body *deranged* mutation to demonstrate that there was an impairment of immediate-recall memory; however, again, the mutation affected many areas of the brain including the antennal lobes (Heisenberg 1980). Later, the mushroom body *miniature* mutation was used to demonstrate that the MBs are involved in memory in the odor-shock paradigm, with the problem again being that other structures in the brain were also abnormal as a consequence of this mutation (Heisenberg et al. 1985). In 1994, deBelle and Heisenberg utilized a chemical ablation procedure to conclude that the MBs are involved in immediate memory in the odor-shock paradigm (de Belle and Heisenberg 1994). This procedure took advantage of the fact that in the developing nervous system only five sets of neuroblasts are actively dividing from 0 to 8 h post-larval hatching. Of the five pairs of CNS neuroblasts dividing in this time period, four of these generate the Kenyon cells of the MBs, and the fifth pair generates a portion of the antennal lobes (Truman and Bate 1988; Prokop and Technau 1991; Ito and Hotta 1992). In this paper, the effects of possible antennal lobe damage were not delineated from that of mushroom body ablation with regard to memory. However, in spite of this fact, the conclusion was drawn that the MBs are necessary for immediate memory in the odor-shock paradigm (de Belle and Heisenberg 1994). In 1995, memory was isolated to a specific anatomical structure in the insect brain, without decrement to memory immediately post-training, for the first time in

insects (McBride 1995). STM was isolated to the MBs in an ablation experiment utilizing the conditioned courtship paradigm and was found to be independent of any antennal lobe damage effects (McBride et al. 1999; McBride 1995). This result was later confirmed by elegant experiments in the olfactory association paradigm (odor shock) where again, the experimenters were able to isolate effects on the MBs from those on the antennal lobes (Zars et al. 2000; McGuire et al. 2001; Dubnau et al. 2001). This was a critical advance because it meant that the MBs should be thoroughly examined in cases of STM impairments.

Another important question was which of the structures in the insect brain are required for the establishment of LTM in *Drosophila*. A paradigm for the establishment of LTM in *Drosophila* was first developed in the odor-shock paradigm, where it was found that a series of spaced training sessions could induce LTM (Tully et al. 1994). This was quickly followed by the development of two paradigms for the establishment of LTM-utilizing courtship behavior (McBride et al. 1999; McBride 1995). The MBs were shown to be required for LTM in the conditioned courtship paradigm (McBride et al. 1999), as well as in the odor-shock paradigm (Pascual and Preat 2001). Additionally, the antennal lobes were shown to be involved in memory formation lasting out to 30 min post training in honeybees and fruit flies (Faber et al. 1999; McBride et al. 1999). This was later confirmed in the odor-shock paradigm as well (Yu et al. 2004).

6.2 The Drosophila Model of Fragile X

All of the initial publications of a *Drosophila* model of fragile X used homozygous *dfmr1* null mutant lines. The findings from these first papers included the identification of altered circadian rhythms, altered synaptic arborization, altered activity at the neuromuscular junction (which was partially rescued by altering levels of the MAP1B homologue, *futsch*), and altered courtship levels (social interaction) (Dockendorff et al. 2002; Morales et al. 2002; Zhang et al. 2001). Additionally, it was determined that vision, olfaction, and overall locomotor activity were grossly intact (Dockendorff et al. 2002). Fragile X males also appeared phenotypically normal, were able to walk, fly, and copulate with females.

6.2.1 Social Interactions in the Fragile X Model

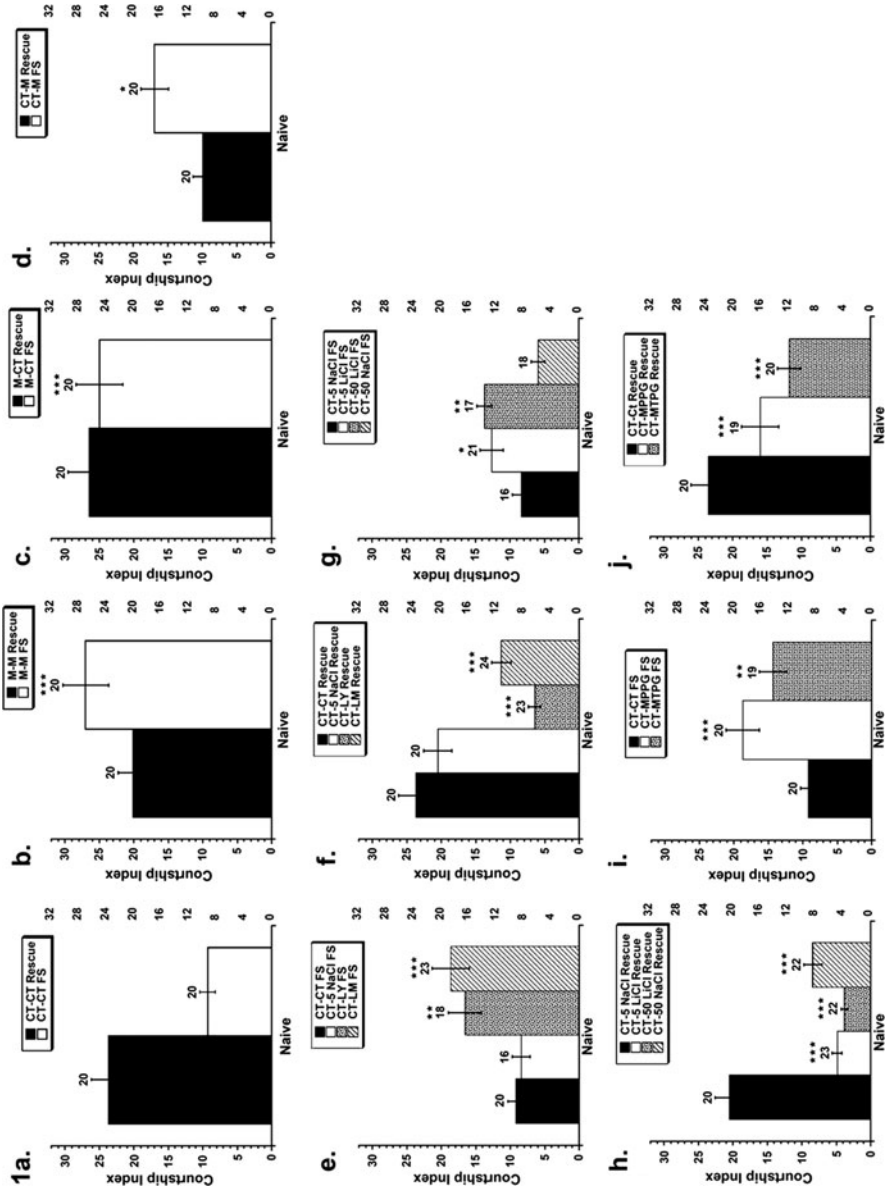
Social interactions in the fragile X model were first studied utilizing courtship behavior in the *Drosophila* model. Indeed social impairments are a core symptom of ASDs. Fragile X, along with several other known monogenetic disorders such as tuberous sclerosis type 1, tuberous sclerosis type II, neurofibromatosis type 1, Retts and Rubinstein–Taybi syndrome make up 15–20% of the autistic population (Zafeiriou et al. 2007). Fragile X is a monogenetic disorder with a high incidence

of autism and autistic behaviors in fragile X patients. Therefore, fragile X represents an ideal genetic model to study autism, a disease that has remained elusive in animal modeling, due to the fact that in the majority of cases of autism, the genetic or environmental causes remain unknown.

In initial work, social interactions were demonstrated to be decreased in naïve courtship for *dfmr1* males. The courtship activity of naïve *dfmr1* males paired with virgin female targets, was significantly less than naive control males paired with virgin females (Dockendorff et al. 2002). This deficit was also observed in a slightly altered social paradigm. *Drosophila* naïve adult males will generally court immature males, which is speculated to be a way of facilitating courtship success in the immature male when it is ready to court a female. In testing, fragile X model flies also displayed decreased courtship of immature males, indicating that the decrease in social interaction was not specific for the virgin female targets. Also on average, fragile X flies exhibited the same number of courtship attempts as control flies, but they failed to sustain the courtship ritual. A lower percentage of fragile X flies progressed to the later steps of courtship (genital licking and copulation attempt) compared to control flies. This was speculated to be analogous to ADHD, which is common in humans with fragile X (Dockendorff et al. 2002). Overall these studies demonstrated for the first time that a social deficit existed in an animal model of fragile X, which also could be considered a disease model for autism (Dockendorff et al. 2002).

6.2.2 Restoring Social Interactions in dfmr1 Mutants

The naïve courtship results validated the utility of studying models of autism with a focus on social behavior in *Drosophila*, a system which has already been extensively studied and has begun to be genetically dissected in this regard (Sokolowski 2001, 2010). In 2005, McBride et al. again examined the social interactions of the fragile X fly model, but this time with a focus on pharmacologic treatments as a way to test for rescue of social impairments. Currently, pharmacologic treatment of social impairments in ASD remains an enormous unmet medical need. For reasons described below, drugs that antagonize metabotropic glutamate receptor (mGluR) signaling were used in an attempt to pharmacologically rescue phenotypes displayed by *dfmr1* mutant flies. Indeed, it was demonstrated that mGluR antagonist treatment of the fragile X flies could be used to rescue social behavior when given in development alone, i.e., during the larval stages, or when given in both development and adulthood. No increase in social behavior was observed in control flies indicating that the effectiveness of the drug treatments was specific for the disease state (McBride et al. 2005) (Fig. 6.1). Another interesting observation from these studies and in contrast to the prevailing dogma of neurodevelopment diseases at the time, treatment with mGluR antagonists during adulthood alone was also able to rescue social impairments in the fragile X model. Furthermore, lithium treatment in adulthood only was also able to rescue social


1a.
CT-CT Rescue
CT-CT FS
b.
M-M Rescue
M-M FS
c.
M-CT Rescue
M-CT FS
d.
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CT-M FS
e.
CT-CT FS
CT-5 NaCl FS
CT-LY FS
CT-LM FS
f.
CT-CT Rescue
CT-5 NaCl Rescue
CT-LY Rescue
CT-LM Rescue
g.
CT-5 NaCl FS
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h.
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i.
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CT-MPPG FS
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CT-MPPG Rescue
CT-MTPG Rescue
Courtship Index
Naive

impairments in the fragile X model (Fig. 6.1). Treatment of control flies had the opposite effect. Treatment during adulthood only with mGluR antagonists or with lithium greatly decreased the social interactions with virgin females in both cases (Fig. 6.1) (McBride et al. 2005).

This work represented the first rescue of social behavior in a disease model with a high association of autism by pharmacologic treatment and raised the potential efficacy of mGluR antagonist or lithium treatment in alleviating social impairments in fragile X as well as extending the results to some subset of other autism patients. These studies also demonstrated that social impairments were not set in stone by immutable developmental circuitry, but that adulthood signaling was important in social behavior and that modulating adulthood signaling could ameliorate social impairments. This represented a paradigm shift with regard to thinking of ASD and gives hope that even afflicted adults could reap benefits from new treatments on the horizon (Moldin 2005; Moldin et al. 2006; Walsh et al. 2008; Volkmar et al. 2009).

Work on social behavior in the fragile X model has continued to expand. In the first paper to dissect out specific domains of the protein involved in social behavior, Banerjee et al. demonstrated that critical isoleucines in the two KH domains of dFMR1 were required for proper courtship behavior. When either of these

Fig. 6.1 The effect of mGluR inhibitors and lithium on naive courtship in flies lacking dfmr1 activity. (**a**–**d**) Naive courtship of Rescue and FS flies exposed to 86 μM MPEP. *Filled bars* indicate Rescue males (dfmr13 + wild-type rescue fragment); *open bars* indicate FS males (dfmr13 + frame-shifted rescue fragment). Mean CIs (±SEM) are plotted; Ns are indicated above each bar for all groups. For levels of significance, $*p < 0.005$; $**p < 0.0005$; $***p < 0.0001$. Flies were raised on either control food (CT) or food supplemented with 86 μM MPEP (M). . All flies were placed on CT food 24 h before measurement of naive courtship levels. The first abbreviation indicates the food type that the larvae grew up on, and the second indicates the food type the adult males were placed on within 4 h of eclosion. (**a**) FS and rescue flies without drug treatment (CT–CT). Comparisons in panels (**b**)–(**d**) are made relative to the CT–CT mean of the same genotype in (**a**). (**b**) MPEP containing food administered to FS and rescue flies during both development and adulthood (M–M). (**c**) Rescue and FS flies treated with MPEP as larvae and then placed on CT food as adults (M-CT). (**d**) FS and rescue flies treated with MPEP only as adults (CT-M). (**e**–**j**) The Naive Courtship Levels of Flies Lacking dfmr1 Activity and Treated with Low Doses of MPEP, LY341495, Lithium, MPPG,or MTPG. The naive courtship levels of FS flies(**e**, **g** and **i**) and Rescue flies (**f**, **h** and **j**) were tested after a diet of CT food during development and then food containing either NaCl or a test drug for 4 days during adulthood. Levels of significance are indicated as follows: $*p < 0.05$; $**p < 0.005$; $***p < 0.001$. (**e**–**f**) Adult male flies were fed food containing 8.6 μM MPEP (CT-LM), 400 nM LY341495 (CT-LY), or 5 mM NaCl (CT-5 NaCl). (**g**–**h**) Adult male flies were fed food containing 5 mM NaCl (CT-5 NaCl), 50 mM NaCl (CT-50 NaCl), 5 mM LiCl (CT-5 LiCl), or 50 mM LiCl (CT-50 LiCl). The naive courtship levels shown for CT–CT FS flies and CT–CT Rescue flies in figures (**e**–**f**) and (**i**–**j**) are replicated from (**a**) as a reference point to compare with the CT-5 NaCl, CT-MPPG, and CT-MPTG groups. In (**e**–**h**), comparisons are made relative to the 5 mM NaCl control treatment group of the same genotype. In previous experiments we determined that this treatment did not affect naive courtship levels for both genotypes. (**i**–**j**) Comparisons are made relative to the CT–CT group. The levels of significance are indicated as follows: $**p < 0.005$; $***p < 0.0001$. FS males (**i**) and Rescue males (**j**) raised on control food and then fed either control food (CT–CT) or food containing 573 μM MPPG (CT-MPPG) or 348 μM MTPG (CT-MTPG)

isoleucines was mutated, courtship behavior was suppressed (Banerjee et al. 2007). Later a large screen of small molecules identified potential therapeutic agents in fragile X as stimulating GABAergic signaling. This was validated by demonstrating that several of the pro-GABAergic signaling molecules increased naïve courtship behavior in fragile X flies (Chang et al. 2008). This finding enhanced and supported the findings in the mouse model that the GABAergic system may represent a novel target for therapeutic intervention.

6.2.3 A Novel Social Interaction Assay

The fragile X model has also been examined in a newly developed assay for social interaction in *Drosophila*, which examined the interactions of individual flies and scored them to delineate either receptive or expressive behaviors. In this new assay it was found that fragile X flies may have intact receptive behaviors but impaired expressive behaviors toward other flies, which fits nicely with the results from the naïve courtship studies where the male that is actively courting the target female fly displays decreased courtship activity (Bolduc et al. 2010).

6.2.4 Social Interactions in Aging

Social behavior over the lifetime of fragile X flies has also been examined. In fragile X flies that are aged and tested at 20 days of age post-eclosion (late middle age for a fly), it was determined that social interactions were similar to males that were 5 days post-eclosion, indicating that there was no further decline with age. The ability to examine courtship activity over a significant portion of the fly's lifespan was utilized to examine the more long-term effects, as well as the perdurance of rescue obtained with drug treatment. In these studies, it was demonstrated that treatments with either mGluR antagonists or lithium during development alone failed to rescue social behavior in 20-day-old fragile X flies, which is in contrast to the full rescue observed when tested at 5 days of age. However, if the drug treatments were given in development and adulthood, or solely in adulthood for the entire aging process, rescue of the social impairments was observed (Choi et al. 2010). This demonstrates the efficacy of such treatments throughout the course of life in the fragile X flies.

6.2.5 Common Signaling Dysregulation Between Fragile X and Alzheimer's Disease Genes

Genetic studies aimed at identifying pathways affected by loss of *dfmr1* activity, which led to the behavioral phenotypes displayed by the *Drosophila* fragile X model, identified an interesting interaction with a gene associated with an

aggressive form of Alzheimer's disease. *Presenilin* is a gene that when mutated to loss of function (as biochemically assayed in model organisms) is causally implicated in early onset Alzheimer's disease. It was demonstrated in a *Drosophila* model of this disease that flies containing one normal and one copy of a mutant allele of *presenilin* display normal social behavior, i.e., naïve courtship. It is known that having one mutant *dfmr1* and one normal *dfmr1* gene also results in no courtship impairments. However, when trans-heterozygous flies are made having one normal and one loss of function allele of each gene, the social interaction drastically decreased (McBride et al. 2010). This finding strongly links signaling dysregulation in fragile X to signaling dysregulation in Alzheimer's disease, with the prospect that some pharmacologic therapeutic agents may have efficacy in the treatment of both disorders in humans. Indeed, it has now been demonstrated that treatment with lithium or mGluR antagonists is able to rescue memory impairments in *Drosophila* with decreased *presenilin* activity (McBride et al. 2010).

6.2.6 Cognition in the Fragile X Model

Consistent impairments in cognitive processes such as learning and memory have remained elusive in the mouse models of fragile X. Therefore the fly model was characterized with regard to cognition. This raised the possibility of increasing the speed with which the underlying signaling dysregulation leading to defects in cognition could occur and to identify and test novel therapeutic targets. In 2005, cognition was first examined in the fragile X model utilizing the conditioned courtship paradigm. In this study, LDT was examined as well as purely associative memory in the phases of immediate recall (0 min after training) and short term memory (60 min after training). In young fragile X flies, it was found that LDT was intact. Therefore, the fragile X flies were capable of plasticity in the form of decreasing courtship behavior during the training session. In contrast, the young mutant males had impairments in the immediate recall and STM phases of memory (McBride et al. 2005) (Fig. 6.2). Additionally, defects in the morphology of the mushroom bodies, known to be required for short- and long-term memory formation, were also identified. Specifically, there is an increased propensity of midline crossing of the beta lobes of the mushroom bodies in fragile X flies compared to control flies (McBride et al. 2005; Restifo 2005).

6.2.7 Dysregulated Signaling in Cognition

The identification of cognitive defects in the fragile X model led to the initiation of studies aimed at dissecting the molecular signaling cascade alterations causing these phenotypes. Initially it was hypothesized by McBride et al. (2005), that in fragile X there would be overactive inositol trisphosphate activity and underactive

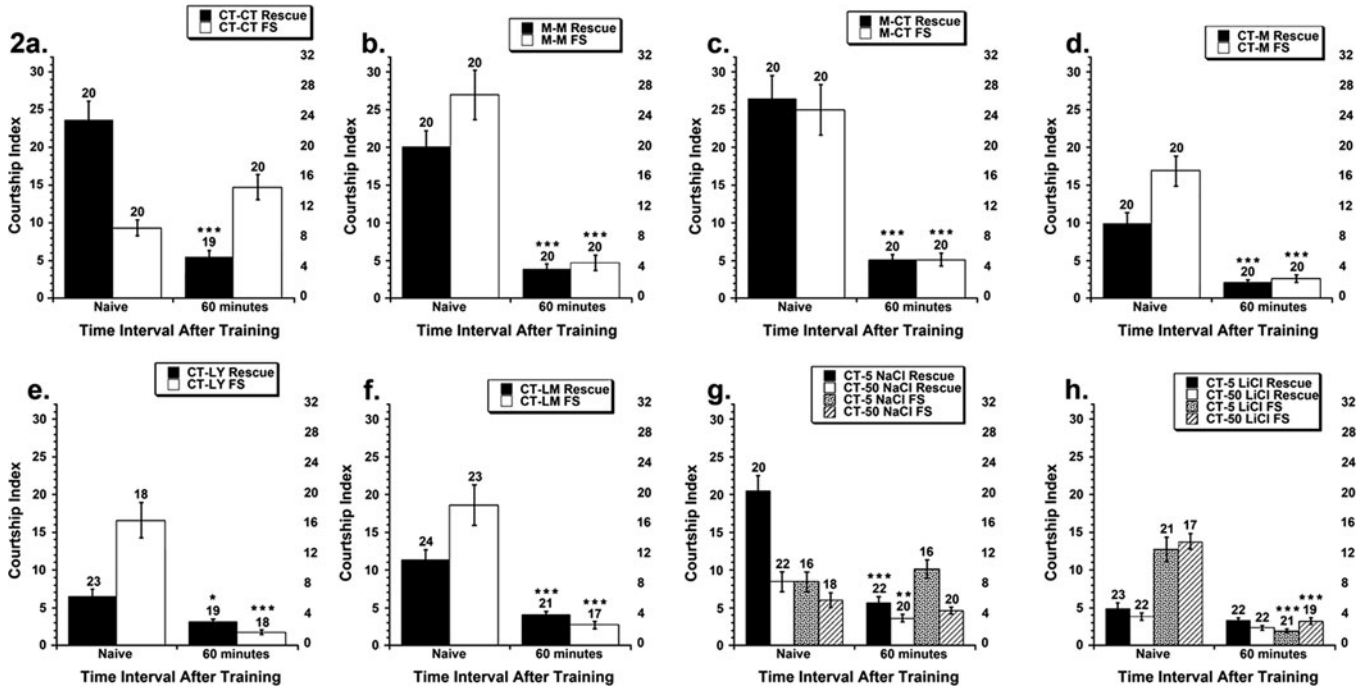

Fig. 6.2 Effects of drug treatment on Short-Term Memory (STM) in flies lacking *dfmr1* Activity. (**a–h**) Short-term (60 min) memory was measured in Rescue and FS flies that were either given control food or administered various drug treatments as described below. For (**a**)–(**d**), the mean CIs (±SEM) and levels of significance are plotted as described in the legend of Fig. 6.1. (**a–f**) *Black bars*, CIs of Rescue flies; *open bars*, CIs of FS flies. (**a**) STM of Rescue and FS flies are compared to the naïve courtship levels of both groups. (**b–d**) Rescue and FS flies fed 86 μM MPEP during development and adulthood (M–M Rescue and M–M FS) (**b**); during development alone (M-CT Rescue and M-CT FS) (**c**); or during adulthood alone (CT-M Rescue and CT-M FS) (**d**) are compared to the naive courtship levels obtained for similarly treated flies. For (**e–h**), mean CIs (±SEMs) and the levels of significance are plotted as described in the legend of Fig. 6.1 (**e–h**). (**e–f**) Examination of short-term memory in Rescue and FS flies that were either fed food containing 400 nM LY341495 (CT-LY Rescue or CT-LY FS) (**e**) or 8.6 μM MPEP as adults (CT-LM Rescue or CT-LM FS) (**f**). (**g–h**) Examination of short-term memory in Rescue and FS flies that were either fed food containing 5 mM NaCl (CT-5 NaCl Rescue and CT-5 NaCl FS) or 50 mM NaCl (CT-50 NaCl Rescue and CT-50 NaCl FS) as adults (**g**) or were treated with 5 mM LiCl (CT-5 LiCl Rescue and CT-5 LiCl FS) or 50 mM LiCl (CT-50 LiCl Rescue and CT-50 LiCl FS) as adults (**h**)

cAMP signaling with resulting overactive GSK-3beta signaling. This hypothesis culminated from an extensive review of the literature encompassing several diverse experimental techniques (Berry-Kravis and Ciurlionis 1998; Berry-Kravis et al. 1995; Berry-Kravis and Huttenlocher 1992; Darnell et al. 2001; Brown et al. 2001; Lakin-Thomas 1993; Tanji et al. 2002; Hamada et al. 1999; Dal Santo et al. 1999; Dockendorff et al. 2002; Berridge 1993; Berridge et al. 1989; Mak et al. 1998; Khodakhah and Armstrong 1997; Fujii et al. 2000, 2003; Inoue et al. 1998; Nagase et al. 2003; Yin et al. 1994, 1995; Nishiyama et al. 2000; Roman and Davis 2001; Vitolo et al. 2002; Bozon et al. 2003; Chen et al. 2003; Tully et al. 2003; Takei et al. 1998; Bullock and Habener 1998; Grimes and Jope 2001; Mai et al. 2002) (For further explanation please see McBride et al. 2005 and Choi et al. 2010). Therefore, a search was initiated to find a way to manipulate these pathways to lower inositol trisphosphate mediated calcium signaling and to increase cAMP signaling after synaptic stimulation. The *Drosophila* mGluR receptor was identified as a target that could be treated with antagonists and lithium was identified as a potential way to effect similar changes in intracellular signaling. This work was immensely aided by the timely and independent finding of enhanced mGluR dependent LTD in the CA1 region of the mouse hippocampus and the subsequent proposal of the mGluR theory of fragile X by Mark Bear and colleagues in 2004, a proposal that altered the landscape of research into neurodevelopmental disorders (Huber et al. 2002; Bear et al. 2004). Research on cognition in the *Drosophila* model of fragile X provided the first test of this theory.

6.2.8 The Drosophila DmGluRA

Although mammalian genomes contain eight different mGluR, subdivided into three groups (I, II, and III), the *Drosophila* genome contains a single mGluR, called DmGluRA. Characterization of DmGluRA indicates that it activates signaling pathways downstream of both Group I and Group II mGluRs (Choi et al. 2011; McBride et al. 2005; Pan and Broadie 2007; Pan et al. 2008). Although some work had been done regarding the characterization of the *Drosophila DmGluRA,* when the initial study of cognition in the fragile X *Drosophila* model was commencing, it was limited to one paper (Parmentier et al. 1996). This study demonstrated that DmGluRA, like group II mGluRs, possessed the ability to signal through trimeric G-protein complexes containing the Gi-alpha subunit, but whether it possessed Gq signaling activity remained open. McBride et al. 2005, proposed that DmGluRA possessed the ability to signal through both Gi and Gq trimeric G-proteins, effectively performing both group I and II signaling, based on the precedence from the mammalian literature (see McBride et al. 2005 or Choi et al. 2011 for additional discussion). This hypothesis has since been validated (Pan and Broadie 2007; Pan et al. 2008).

Since DmGluRA was not well characterized, a question of how best to manipulate its activity was raised. The authors decided on pharmacologically manipulating

the receptor because this would allow titration of dosing and increased temporal control over genetic manipulation of the receptor. This, however, left open the question of what pharmacologic agents to utilize in *Drosophila*. Given the limited work on DmGluRA, and no existing research on any pharmacology regarding the receptor in vivo, a strategy of utilizing several distinct pharmacologic agents to antagonize DmGluRA was chosen. Since this was going to be an *in vivo* treatment, it was hypothesized that the best evidence for any of the compounds antagonizing the *Drosophila* mGluR by feeding would be to see if the chosen compounds had similar effects on the phenotypes of interest. The two mGluR antagonists MPPG and MTPG, which had been demonstrated to antagonize DmGluRA in an exogenous expression system, were included in the study (Parmentier et al. 1996). Also an extremely elegant set of experiments, to characterize the binding pocket of the mammalian mGluR5 antagonist MPEP, had been performed (Malherbe et al. 2003; Pagano et al. 2000). The genetically defined MPEP binding pocket is highly conserved between mammalian mGluR5 and the *Drosophila* mGluRA (see supplemental section of McBride et al. 2005 for further discussion), so MPEP was therefore included in the study. Later, in a meticulous series of assays covering a diverse set of phenotypes, MPEP treatment was demonstrated to phenocopy genetic loss of function of DmGluRA, thereby conclusively demonstrating that in vivo, MPEP antagonizes DmGluRA (Pan and Broadie 2007; Pan et al. 2008). Finally a fourth mGluR antagonist, LY341495, was chosen to be examined based on its high affinity for mammalian mGluRs, specifically group II, at low concentrations but all of the mammalian mGluRs at higher concentrations. LY341495 was later demonstrated to antagonize DmGluRA in an exogenous expression system (Bogdanik et al. 2004). The use of these four compounds was designed to ensure that the efficacy of any potential therapeutic effect on *dfmr1* phenotypes was indeed due to a reduction in DmGluRA activity and not due to any off-target effects.

6.2.9 Rescuing Memory with mGluR Antagonist Treatment

When fragile X flies were treated with mGluR antagonists in development alone, both immediate recall and short-term memory were rescued (Fig. 6.2). Additionally, mGluR antagonist treatment also partially rescued the midline-crossing defect in the mushroom bodies, the associative memory center in the *Drosophila* brain. When mGluR antagonist treatment was performed during development and adulthood, memory was also rescued in fragile X flies (Fig. 6.2). An equally extraordinary finding was that memory could be rescued by treating with mGluR antagonists beginning in adulthood (Fig. 6.2). This was the first demonstration that mGluR antagonist treatment could ameliorate phenotypes in fragile X models. This was quickly followed by the demonstration that pharmacological treatment of MPEP prevented audiogenic seizures in the fragile X mouse model (Yan et al. 2005). Later, Dolen et al. demonstrated that genetically decreasing mGluR5 expression in the fragile X mouse rescued several disease-related phenotypes including optic

density, dendritic spine morphology, and memory (Dolen et al. 2007). In addition, it was recently found that chronic treatment with the group II mGluR antagonist LY341495 can rescue synaptic plasticity defects in the fragile X model mouse (Choi et al. 2011). Indeed, several mGluR antagonist drug trials have already begun in humans (Berry-Kravis et al. 2009; Jacquemont et al. 2011) and reviewed in Chap. 17.

6.2.10 Rescuing Memory with Lithium Treatment

In the above-described studies, it was also demonstrated that lithium, which has effects similar to decreasing the downstream signaling of both the group I and group II mGluRs, (see McBride et al. 2005 and Choi et al. 2011 for further explanation) could also rescue the memory impairment in fragile X flies when the treatment was initiated in adulthood (Fig. 6.2; McBride et al. 2005). Lithium has been demonstrated to inhibit inositol trisphosphate synthesis and recycling via inhibition of IPPase and IMPase (Acharya et al. 1998; Baraban et al. 1989; Berridge et al. 1989; Hallcher and Sherman 1980; Williams et al. 2002), as well as to inhibit GSK-3β activity (Klein and Melton 1996). Since lithium was already FDA approved for the treatment of other disorders, these results were quickly translated to human patients where lithium was demonstrated to improve cognition and other behaviors in human subjects with fragile X, as reported in a recent open label clinical trial (Berry-Kravis et al. 2008).

These results, especially those demonstrating the efficacy of adult only treatment, provided an impetus for examining the effect of lithium treatment in the mouse fragile X model. In fact, lithium treatment, or in some cases treatment with GSK-3beta inhibitors, has been found to rescue enhanced audiogenic seizures and ameliorate aberrant behaviors in *Fmr1* KO mice, as assessed by open-field activity, elevated plus-maze, and passive avoidance assays (Min et al. 2009; Yuskaitis et al. 2010). Lithium treatment in adulthood or beginning in development has also been demonstrated to rescue synaptic plasticity defects in the fragile X mouse model (Choi et al. 2011).

6.2.11 Renewed Hope for Treatment

The initial findings of rescuing the memory impairments in the *Drosophila* model of fragile X (McBride et al. 2005), a disease highly associated with cognitive impairment, provide hope that such diseases may be effectively treated one day, as exemplified by the following quote:

> "The first therapeutic treatments of animal models of mental retardation have been reported, a *Drosophila* model of fragile X syndrome has been treated with lithium or metabotropic glutamate receptor (mGluR) antagonists and a mouse model of NF1 has been treated with the HMG-CoA reductase inhibitor lavastatin, which improves the learning and memory skills in these models" (Raymond and Tarpey 2006).

Here, NF1 stands for neurofibromatosis type 1, a monogenetic disease, where approximately 1–4% of afflicted patients have mental retardation, but a larger percentage have some degree of learning disability or autistic behaviors. The quote refers to NF1 in the context of work from the Silva lab where they initially demonstrated that cognitive impairments in mice could be rescued by pharmacologic treatment (Li et al. 2005).

These two publications have another common connection; they were the first publications demonstrating that treatments started in adulthood could rescue memory deficits in neurodevelopmental disorders, where the dogma was that developmental circuitry impairments would remain immutable. Indeed, it has now been demonstrated that interventions in adulthood can rescue cognitive impairments in tuberous sclerosis complex type 2 and Retts disease models as well (Ehninger et al. 2008; Guy et al. 2007). The idea that aspects of these disorders, particularly cognition and social behavior could be treated in adulthood has been referred to as a remarkable finding (Walsh et al. 2008) and the ensuing intellectual medical fervor regarding the opportunity to treat these diseases in adulthood is captured by the following quote from Matthew State (State 2010):

> What is indisputable is that the conceptual transitions reflected in these efforts is remarkable: the notion that intellectual disability and ASD associated with FMRP or TSC-1 [2] mutations may not [be] set in stone early development represents a seismic shift in thinking regarding the opportunities to treat these conditions and underscores the transformative potential of the interplay of human genetic findings and basic neurobiology.

Cleary, the field of fragile X research has been at the forefront in transforming how we in the health care community view the treatment or potential treatment of neurodevelopmental disorders and ASDs. This has been accomplished through the synergy of research efforts on model organisms and afflicted human patients with each finding propelling forward the other to then expound upon.

6.2.12 Long-Term Memory in the Fragile X model

Demonstrating that fragile X flies had impairments in immediate recall memory and short-term memory left open the question of the role of *dfmr1* in LTM. Utilizing the odor-shock paradigm, it was demonstrated that fragile X model flies have impairments in LTM as well. Additionally, it was demonstrated that there were genetic interactions between *dfmr1* and *staufen* as well as *dfmr1* and *argonaute 1* in LTM formation (Bolduc et al. 2008).

A genetic interaction was also demonstrated between *dfmr1* and *cheerio*, the *Drosophila* ortholog of *filamin A* (Bolduc et al. 2010). Mutations in the filamin A cause periventricular nodular heterotopia, which is associated with cerebral malformation, epilepsy, and cognitive impairments. It was found that heterozygous loss of function of either *dfmr1* or *cheerio* did not impair LTM, but that heterozygous loss of function of both led to impairments in LTM (Bolduc et al. 2010).

6.2.13 A Prion-Like Domain and Isoform Regulation Play Critical Parts in the Orchestration of Memory Involving dfmr1

Using the *Drosophila* model of fragile X, a dissection of specific regions of the protein product of *dfmr1* required for memory has begun, and thus far a critical role for multiple isoforms of the protein has been demonstrated (Banerjee et al. 2010). Two isoforms of the *Drosophila* protein product of *dfmr1* that differ with regard to the presence or absence of a prion-like protein domain, which is potentially utilized for protein–protein interactions, were characterized with regard to memory.

In 1984, Francis Crick proposed that stable cooperative protein interactions could act as a stable tag for synapse activity (Crick 1984). The proposal was that specific events at a synapse would lead to a set of proteins being directed to select synapses. The set of proteins would provide a code for the synaptic event and through cooperative interaction lead to a stable protein complex that could continue to properly regulate signaling at the synapse in a self-propagating manner in spite of continual protein influx, efflux, and turnover. Since the initial postulation, some additional ideas regarding potential synaptic tags have been put forth. Currently, a synaptic tag is defined as a way to temporally and spatially mark a synapse in response to previous activity in order to locally determine to activate specific translation or to capture specific translational and transcriptional products in order to alter transmission efficacy (Martin and Kosik 2002; Frey and Frey 2008). Indeed, it may be thought of that there is no one molecular synaptic tag, but a set of tags that influence the production and capture of plasticity related proteins in order to impact the transmission efficiency of a synapse. Every synaptic tag should exhibit the following characteristics: have the ability to be spatially restricted, be reversible (have an on and an off state), control the translation of and influence the capture of plasticity related proteins, be capable of interacting at the cell wide level after strong synaptic stimulation, help produce long lasting alterations in synaptic strength (weakening or strengthening), and be able to self aggregate in order to either perpetuate the signal or allow for it to be turned on or off (Frey and Frey 2008; Martin and Kosik 2002).

The potential utility of prion and prion-like domains in proteins influencing synaptic plasticity was first postulated in two papers proposing that the prion domains in CPEB3 (*orb2* in *Drosophila*) could be involved in memory, specifically LTM formation (Si et al. 2003a, b). However, a missing aspect of the proposed mechanism with regard to being a synaptic tag, is that there is no clear way to achieve different conformations in a regulated manner; the alterations in conformation appear to be rare and random. In other words, as of yet, there is no way to revert back to the original state.

Since FMRP is involved in local translation, having one or more stable conformations could also affect this process locally at the synapse. The prion-like domain that is found in the *Drosophila* gene is highly conserved in both mouse and human genes. In addition, the splice sites that allow for the production of prion containing

and prion lacking forms of the protein are also highly conserved between the genes of these three organisms. In *dfmr1*, the prion-like domain in the primary sequence is a C-terminal amino acid sequence of which 49 of 112 residues are either Q or N, or 44%. In mammalian *FMR1,* there is an amino acid stretch in which 34 of 106 residues are Q, N, or G, or 34%. Therefore, in the *Drosophila* and mammalian proteins this could potentially indicate a prion-like domain. However, it should be noted that in this first work examining the prion-like domain of *dfmr1* in *Drosophila*, the protein was not demonstrated to fulfill all of the criteria to be considered a classical prion protein.

However, since FMRP is involved in local translation and the prion-like domain is conserved, the idea that FMR1 might play a role in synaptic tagging is intriguing. This is particularly appealing as the alternative splicing allows for a mechanism to shift between the prion-like domain containing and lacking isoforms, so that a particular synapse or microdomain within a synapse could change in accordance with previous synaptic activity in order to effect the future activity at the synapse in a temporally and spatially restricted manner. This would allow the fragile X protein to be an ideal synaptic tag candidate, where the prion-like domain-containing or missing proteins can be made and delivered to synapses to encode new memories and guide future signaling at the synapse in a highly regulated manner dependent on previous synaptic activity (Banerjee et al. 2010).

The question of whether or not this domain has a role in memory was therefore explored. LTM was found to be impaired in fragile X model flies using the conditioned courtship paradigm, just as had been demonstrated in the odor-shock paradigm. In the fragile X model fly background, *dfmr1* transgenes that encode the prion-like domain-containing (long isoform) or the prion-like domain-missing (short isoform) were expressed. In flies expressing the short isoform, immediate recall memory was intact, but both short-term and LTM were impaired. This suggests that the prion-like domain containing isoform is required for both short-term and LTM. In flies expressing the long isoform, both immediate recall memory and STM were intact, but LTM was impaired. This suggests that the prion-like domain lacking isoform is also required for LTM. Since LTM was rescued with a wild type genomic transgenic rescue construct, one intriguing idea is that the expression of both the long and short forms is required for proper LTM. Clearly, further experiments are required to delineate the role that different FMR protein isoforms play in memory formation. Nonetheless, these experiments add to an ever-increasing body of literature indicating a role for protein isoform diversity in the fine-tuning of synaptic plasticity.

6.2.14 Age Dependent Cognitive Decline

The role of the fragile X protein in aging in the brain has not been thoroughly explored in animal models. There appears to be progressive cognitive decline and increased autistic features with age in fragile X patients, although some have argued

that the finding may be related to the types of testing that were performed (Wright-Talamante et al. 1996; Hay 1994; Hatton et al. 2006; Hagerman et al. 1989). Nonetheless, extended longitudinal studies with adult patients have not been done, leaving a critical potential gap in our information regarding performance of fragile X patients with aging (Wright-Talamante et al. 1996; Jacquemont et al. 2007; Hay 1994; Hagerman et al. 1989). In animal models, this type of longitudinal study can be performed quickly and cost-effectively. Furthermore, characterizing the effect that the loss of the protein product of FMR1 (FMRP) in aging exhibits may provide clues as to the pathophysiology of fragile X-associated tremor/ataxia syndrome (FXTAS), an age onset disease afflicting some FMR1 premutation carriers.

Studies with regard to aging have begun in the *Drosophila* model of fragile X. The initial studies answer two critical questions. First, are there additional cognitive impairments with aging in the fragile X model, which could indicate a role for the fragile X protein in normal aging, and if additional impairments are found, would they be amenable to the same treatments? Second, can treatments that are effective in early adulthood remain effective throughout the lifetime of the flies?

An age-dependent cognitive decline in LDT was found in the *Drosophila* fragile X model (Choi et al. 2010). Treatment with mGluR antagonists or lithium can prevent this age-dependent cognitive impairment. Surprisingly, treatment with mGluR antagonists or lithium during development alone were able to rescue this age-dependent impairment, possibly indicating protective epigenetic effects of treatment in this regard. Furthermore, continuous treatment with either lithium or mGluR antagonists during aging effectively rescues all of the cognitive impairments (Choi et al. 2010). This work indicates a role for the fragile X protein in healthy aging and places fragile X in a category with several other diseases that result in age-dependent cognitive decline.

6.2.15 Circadian Rhythms in the Fragile X Model

Organisms ranging from the fungus *Neurospora* to humans display circadian behavior, i.e., behavior that is set by the light:dark cycle of the day. As shown for learning and memory, research on circadian regulation has uncovered an evolutionarily conserved pathway, referred to as the "clock," which drives the cyclical nature of circadian behavior. The molecular components of the clock are expressed in a subset of "clock neurons" in the brain and operate as an autoregulatory transcriptional feedback loop that cycles with a period of approximately 23 h (Gerstner and Yin 2010; Crocker and Sehgal 2010). Hundreds of genes, whose expression is regulated in a circadian pattern, are transcribed in response to the transcriptional activators Clock and Cycle. This large set of genes includes *timeless* and *period* that encode proteins that work in concert to repress the activity of Clock and Cycle. As *timeless* and *period* are transcribed and translated, their concentration in the cytoplasm increases where they associate with one another and form a complex that enters the nucleus and represses activity of Clock and Cycle, hence reducing the transcription of the downstream genes including

timeless and *period* themselves. This repression is slowly released as the nuclear concentration of Period and Timeless proteins diminishes with time through protein degradation, allowing Clock and Cycle to reactivate transcription of their target genes.

The precision of the molecular clock is such that normal flies, entrained to a 12 h light:12 h dark cycle, are capable of maintaining normal circadian behavior even when maintained in total darkness for a few weeks. This capability, referred to as "free running rest:activity rhythms" results in normal levels of activity during the corresponding daylight hours and relative inactivity during the corresponding night time.

Behavioral characterization of the *dfmr1* mutants revealed a clear deficit in free running rest:activity rhythms (Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002). These three reports found that in general, *dfmr1* mutants displayed near normal entrainment to a light:dark conditions, but failed to displayed any rhythmic locomotor activity in free running conditions. This is contrast to control flies that display normal free running rest:activity rhythms (Fig. 6.3).

Examination of the molecular clock and clock neurons has not yet identified a cause for the circadian defect displayed by the *dfmr1* mutants. The cycling of Period and Timeless proteins appears to be normal at times when the *dfmr1* mutants display arrhythmic behavior in free running conditions. These data along with the fact that the *dfmr1* mutants are capable of entraining their activity patterns to a light: dark cycle indicated that the core molecular clock functions normally (Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002).

A more likely explanation for the arrhythmic behavior of the *dfmr1* mutants is that a defect exists in the circadian output pathway, e.g., genes and pathways regulated downstream of the clock. Consistent with this hypothesis, two defects in circadian output have been reported. First, the timing of eclosion has been found to be affected in the *dfmr1* mutants (Dockendorff et al. 2002; Morales et al. 2002). Normally *Drosophila* adults hatch out of their pupal case during the early morning hours. The *dfmr1* mutants, however, were found to hatch much later in the day on average. Although two groups reported this phenotype using different alleles, another study has reported that this phenotype depends on the genetic background of the fly as this phenotype can be genetically unlinked from the *dfmr1* mutation (Sekine et al. 2008). The second output defect was in CREB (cAMP responsive element binding protein) regulation. The activity of CREB protein, itself being required for proper circadian activity, cycles throughout the day and this cyclical pattern is under circadian control (Gerstner and Yin 2010). Although robust cycling is observed in control flies, *dfmr1* mutants display a dramatically reduced level in cycling amplitude under free running conditions (Dockendorff et al. 2002; Morales et al. 2002).

Although the cause of the defect in circadian behavior is not yet known, a subtle defect in a key neuron involved in circadian regulation has been identified (Gatto and Broadie 2008; Dockendorff et al. 2002; Morales et al. 2002; Reeve et al. 2005). The small ventral lateral neurons (sLN_v) were found to display subtle morphological defects in axonal branching and elaboration of their axonal termini.

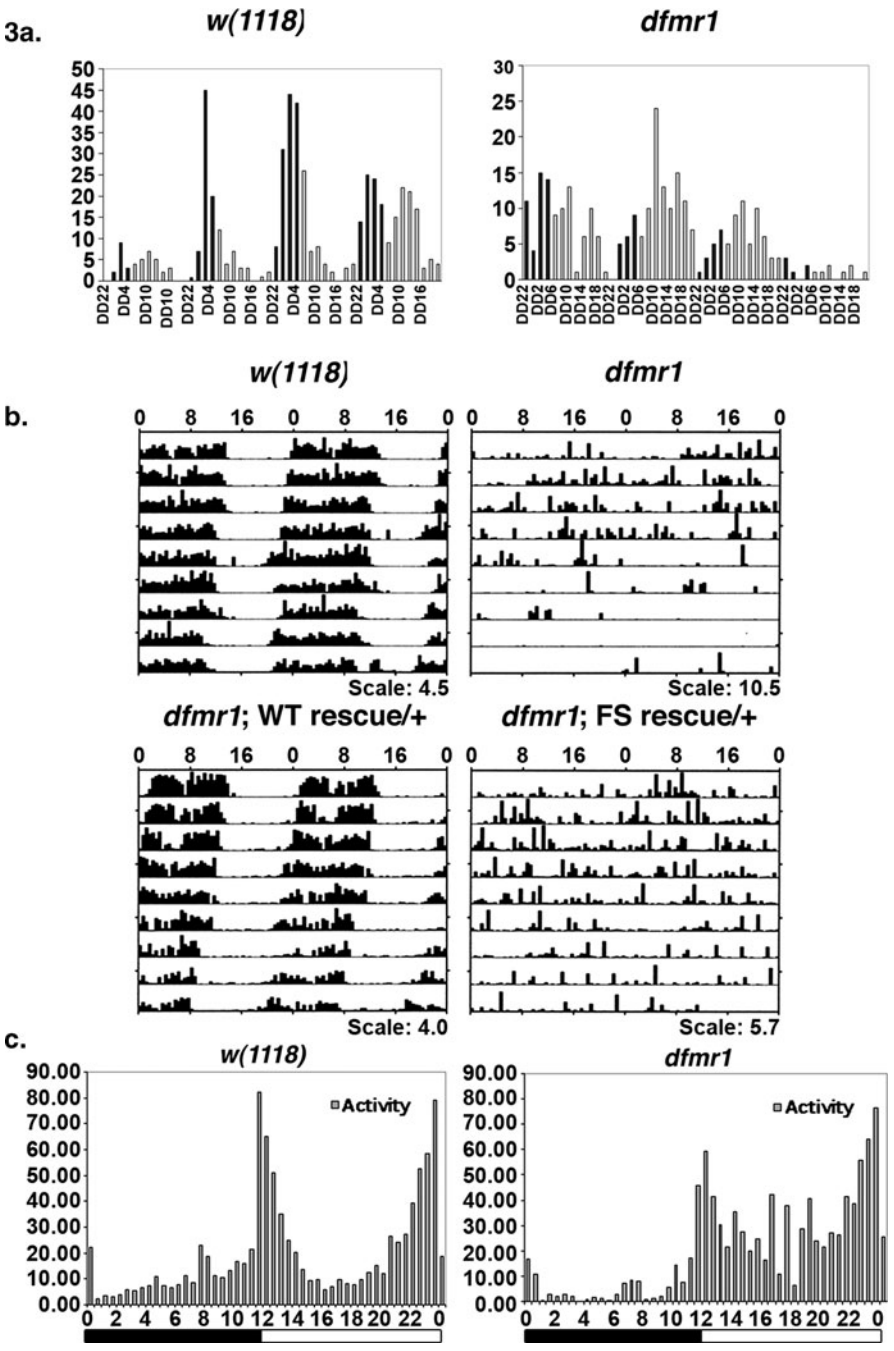

Fig. 6.3 Rest activity of *dfmr1* mutant and control flies. (**a**) Eclosion timing of *w1118* ($n = 501$) and *dfmr1* ($n = 298$) flies in Dark/Dark (DD) cycle. Pupae from larvae that had been entrained in Light/Dark (LD) for 5 days were placed in eclosion monitors in constant darkness for several days. The number of flies eclosing relative to the time of day were plotted. The *dark bars* indicate the

These neurons are known to be a key component of the circadian pathway as they produce a peptide hormone called (PDF) that is released from their axonal termini. The release of PDF occurs in a circadian pattern that is regulated by the clock and is thought to signal to other neurons in the brain, the "time of day." Although the morphological defect in these neurons has been consistently observed by several groups, its contribution to the observed arrhythmic behavior of *dfmr1* flies has yet to be demonstrated (Helfrich-Forster 1997; Helfrich-Forster and Homberg 1993; Wu et al. 2008). Reeve et al. (2005) determined that the phenotype of the sLN_v is likely due to the overexpression of profilin, an actin binding protein. They found that profilin mRNA is bound by and translationally repressed by dfmr1 protein. They also demonstrated that a similar sLN_v phenotype to that observed in *dfmr1* mutants can be obtained by overexpressing profilin protein, but that this does not recapitulate the arrhythmic behavior observed in *dfmr1* mutants, indicating that the morphological aspect of the phenotype alone is not the cause of the arrhythmic behavior (Reeve et al. 2005). Another study has determined that *dfmr1* activity is required during late brain development in order to rescue the morphological defects observed in the sLN_v (Gatto and Broadie 2008). How this correlates to the temporal requirements of *dfmr1* for normal circadian behavior has yet to be determined.

Another connection to the circadian output pathway has been made by the identification of an interaction between *dfmr1* and another gene that encodes an RNA binding protein and is also required for proper circadian output. Sofola et al. found that dFMR1 protein co-purifies with another RNA binding protein called Lark (Sofola et al. 2008). With respect to circadian behavior, the results of genetic studies suggest that Lark acts to antagonize dFMR1 activity and that dFMR1 promotes Lark function. The biochemical basis for this genetic relationship has yet to be resolved.

6.2.16 Examining Sleep in the Fragile X model

In one other behavioral study, the sleep of the *dfmr1* mutants was examined (Bushey et al. 2009). Recent studies in flies, mice, zebra fish, humans as well as other organisms have revealed that sleep too, is regulated by conserved pathways

Fig. 6.3 (continued) "circadian gate," the time period each day when most wild-type flies eclose. Although the majority of *w1118* flies eclosed during the gate, the *dfmr1* mutant flies eclosed over an extended period of time. (**b**) Representative actograms from flies of the genotypes indicated above each actogram. Flies that had been entrained to a light: dark cycle were placed in the activity monitors in constant darkness and their activity was recorded for 9 days. Flies expressing *dfmr1* have rhythmic patterns of rest and activity, while flies lacking wild-type *dfmr1* have erratically timed short bouts of relatively high activity. **(c)** Locomotor activity of *w1118* ($n = 19$) and *dfmr1* ($n = 14$) flies averaged over 9 days in LD. Mean activity levels are reported across flies for each time point (zeitgeber time). The *black bars* across the bottom of each plot indicate the 12 h of darkness and the *open bars* indicate the hours of light. The activity profiles of both *w1118* and *dfmr1* were similar

(Cirelli 2009; Crocker and Sehgal 2010). Although aspects of sleep are certainly affected by the circadian pathway and mutants exist which affect both behaviors, several aspects of sleep regulation are physiologically and genetically separable (Crocker and Sehgal 2010; Cirelli 2009). Thus, despite the circadian defects previously identified in *dfmr1* mutants, sleep-specific defects could be examined. In fact, one study found that *dfmr1* mutants have prolonged sleep due to an increase in the number of sleep episodes and conversely that over-expression of *dfmr1* leads to reduced sleep. This trend was observed in both light/dark conditions (12 h-Light; 12 h-Dark) and in constant darkness (Bushey et al. 2009). Sleep homeostasis was also affected, in that *dfmr1* mutants did not sleep longer as normal flies do, when recovering from sleep deprivation (Bushey et al. 2009). Mapping the requirements of *dfmr1* in the brain revealed that expression in just the mushroom body restores normal sleep (Bushey et al. 2009). The mushroom body has been identified in other studies as a region of sleep control in the fly brain (Joiner et al. 2006). Thus it appears that *dfmr1* is specifically required in this region of the brain for sleep regulation.

6.3 Conclusion

Hopefully the preceding discussion has given the reader a glimpse of where the *Drosophila* portion of the fragile X field has recently been and is currently going. We will conclude with a quote that highlights the potential of the field of fragile X research as a whole by S.O. Moldin (Moldin 2005):

> Symptomatic commonalities among FXS and other pervasive developmental disorders like autism and Rett syndrome may reflect an overlap in underlying neural circuits and pathways and hence shared pathophysiologic mechanisms. This raises the intriguing possibility that new therapeutics developed to treat FXS also may have efficacy in treating aspects of autism and Rett syndrome. And herein lies the promise of a truly successful roadmap for translational research, in which converging basic research in molecular, cellular, and genomic neuroscience across multiple model systems leads us in the direction of new therapeutics for complex human diseases.

It is this roadmap constructed from the scientific discoveries from cell lines, *Drosophila* and mouse models as well as humans afflicted with the disease that the fragile X community of patients, parents, families, researchers, and physicians are attempting to follow to a cure.

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Chapter 7
Molecular and Genetic Analysis of the *Drosophila* Model of Fragile X Syndrome

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Abstract The *Drosophila* genome contains most genes known to be involved in heritable disease. The extraordinary genetic malleability of *Drosophila*, coupled to sophisticated imaging, electrophysiology, and behavioral paradigms, has paved the way for insightful mechanistic studies on the causes of developmental and neurological disease as well as many possible interventions. Here, we focus on one of the most advanced examples of *Drosophila* genetic disease modeling, the *Drosophila* model of Fragile X Syndrome, which for the past decade has provided key advances into the molecular, cellular, and behavioral defects underlying this devastating disorder. We discuss the multitude of RNAs and proteins that interact with the disease-causing *FMR1* gene product, whose function is conserved from *Drosophila* to human. In turn, we consider *FMR1* mechanistic relationships in non-neuronal tissues (germ cells and embryos), peripheral motor and sensory circuits, and central brain circuits involved in circadian clock activity and learning/memory.

7.1 Introduction

The genomic locus responsible for Fragile X Mental Retardation Syndrome (FXS) was mapped in 1991 to an unstable CGG trinucleotide repeat region at Xq27.3, which can rapidly expand and lead to DNA hypermethylation and transcriptional silencing of the *fragile X mental retardation-1* (*FMR1*) gene (Kremer et al. 1991; Pieretti et al. 1991; Poustka et al. 1991; Yu et al. 1991). An *Fmr1* knockout mouse

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model of the disease was quickly generated to investigate cellular and behavioral phenotypes (Consortium 1994; Kooy et al. 1996). This model has proven absolutely vital to advancing our understanding of FXS, but nevertheless has some limitations. First, the long generation time and high colony maintenance costs of mice significantly slow the rate of experimental analyses. Second, the identification of genes that interact with *FMR1* via forward genetic interaction screens is impractical in mice. Third, even with a candidate gene approach, creating multiply mutant mice to probe FMR1 interactions is time-consuming and laborious. Finally, many *Fmr1* knockout mouse phenotypes are subtle and highly dependent on genetic background, and thus relatively difficult to analyze. Owing to these limitations, there was a clear need for an additional genetic model to enhance our understanding of the FXS disease state. *Drosophila* was the obvious candidate model to fill in many of these gaps; with inexpensive maintenance and rapid generation time, excellent suitability for forward genetic screening and the ability to readily interrogate multiple genetic elements in single animals. Importantly, *Drosophila* has well-defined neural circuits driving a range of complex behaviors defective in FXS patients, including circadian patterned activity and learning formation coupled to robust memory consolidation (Dykens et al. 1988; Elia et al. 2000; Miano et al. 2008). The *Drosophila* genome contains a highly-conserved *FMR1* gene, *dfmr1*, which was targeted for mutation by mobilization of transposable P-elements located in the 5′ portion of the locus (Wan et al. 2000; Zhang et al. 2001). The *Drosophila* FXS model was established in 2001 by imprecise P-element excision to produce *dfmr1* null mutants with robust phenotypes (Zhang et al. 2001). Subsequently, a wide array of *dfmr1* alleles and transgenes have been created to facilitate FXS research in this classic genetic model (Dockendorff et al. 2002; Lee et al. 2003; Morales et al. 2002).

When considering this model, it is critical to note that the *Drosophila* genome contains only a single *FMR1* gene, whereas mammalian genomes contain three highly related genes: *FMR1* and two paralogs (*FXR1* and *FXR2*). The three gene products have been suggested to have overlapping functions, although loss of *FMR1* function solely results in FXS, and FXR1/2 mutations are not linked with any disease state (Cavallaro et al. 2008; Coffee et al. 2010; Darnell et al. 2009; Mientjes et al. 2004; Siomi et al. 1995; Zhang et al. 1995). The *dfmr1* gene product, *Drosophila* Fragile X Mental Retardation Protein (dFMRP), is approximately equally identical to all three mammalian gene products (~35% identity, ~60% similarity), and shows particularly high sequence conservation (~70% identity) in critical protein–protein and RNA-binding domains (Kirkpatrick et al. 2001). Consistently, murine and *Drosophila* FMRP have been shown to be RNA-binding proteins with conserved functions in multiple mRNA regulative processes, including transcript stability, localization, and repression of mRNA translation (De Diego Otero et al. 2002; Feng et al. 1997; Laggerbauer et al. 2001; Weiler et al. 2004; Xu et al. 2008; Zalfa et al. 2003, 2007; Zhang et al. 2007). Importantly, transgenic expression of all three human gene family members in the *Drosophila* FXS model shows that only the disease-linked gene, *FMR1*, is able to rescue molecular and cellular defects (Coffee et al. 2010). Human *FMR1* completely rescues all

requirements in the *Drosophila* disease model, just as effectively as the native *Drosophila* gene, whereas FXR1/2 completely fail to provide any detectable rescue of *dfmr1* loss of function phenotypes in the nervous system. Interestingly, however, both human *FXR* paralogs are able to rescue non-neuronal defects in spermatid development and male fecundity in the *Drosophila* FXS model (Coffee et al. 2010). These findings confirm the evolutionary conservation of *FMR1* function in the nervous system, show that *FXR1/2* are not required for this conserved *FMR1* neuronal function, and suggest *FXR1/2* have diverged to assume non-neuronal roles that are not essential to the behavioral and cognitive defects of the disease state. These results validate the single gene *Drosophila* knockout as a model of the FXS human disease caused by exclusive loss of *FMR1* function.

Drosophila has been used for more than a century to dissect the molecular genetic bases of developmental processes, making this model system a particularly appealing venue to study FXS developmental defects. A tremendous amount of information on genes involved in *Drosophila* development is readily available, and the availability of mutations in these developmental genes allows for experimentation with combinatorial mutations to identify *dfmr1* interactions. In addition, *Drosophila* has been used for many decades to systematically study the nervous system. The availability of mutations in neurological genes, coupled to excellent in vivo imaging, electrophysiological, and behavioral analyses, makes *Drosophila* a powerful system to specifically investigate FXS neurological defects. This chapter will discuss how *Drosophila* genetic interaction tests and forward genetic screens have been harnessed to identify dFMRP developmental and neurological roles. Multiple stages of *Drosophila* development (embryo, larva, pupa and adult) have been used to investigate dFMRP neuronal and non-neuronal requirements. Our discussion will begin by considering non-neuronal roles of dFMRP, and then move on to focus the bulk of our attention on dFMRP roles within the nervous system that are the heart of FXS neurological dysfunction.

7.2 dFMRP Roles in Non-neuronal Development

Although dFMRP is most highly enriched in neurons, it is also widely expressed in other tissues during development where it has important functions (Schenck et al. 2002; Wan et al. 2000). In particular, the role of dFMRP in the formation of *Drosophila* germ cells has been investigated at several levels. Following fertilization, syncitial blastoderm nuclei rapidly divide, and nuclei fated to form germ cells migrate to the posterior pole of the embryo. Maternal mRNAs and proteins localized at the pole trigger cellularization of these pole cells, which then function as progenitors to the germ line. This process of pole cell cellularization is disrupted in *dfmr1* null embryos lacking maternal dFMRP (Deshpande et al. 2006). Mutant embryos show a significant reduction in the overall number of embryonic pole cells and a consistent reduction in the number of germ cells present in the embryonic gonads. Interestingly, as with many *dfmr1* phenotypes, the penetrance of this defect

is highly variable, with some embryos containing near wildtype numbers of pole cells and others containing virtually zero pole cells (Deshpande et al. 2006). One proposed mechanism for this *dfmr1* defect is misregulation of the cytoskeleton. Cytoskeletal contractile rings around nuclei are required for proper pole cell cellularization, and myosin-binding Annilin and actin-binding Chickadee/Profilin are both grossly mislocalized in *dfmr1* null pole cells (Deshpande et al. 2006). Interestingly, the *chickadee/profilin* transcript has been characterized as a direct mRNA binding target of dFMRP (Reeve et al. 2005). A manifestation of this binding interaction could be mRNA mislocalization or inappropriate translation, which may account for the unstable cellularization process in *dfmr1* mutant embryos. However, other processes also appear misregulated. Maternally contributed gene products normally suppress transcriptional activity in pole cells during the blastoderm stage, with transcription subsequently activated later during gonad development (Leatherman and Jongens 2003). This early quiescent state is absent in some *dfmr1* embryos, as activated RNA polymerase II can be detected in the blastoderm pole cells (Deshpande et al. 2006). Moreover, the disruption of pole cell formation may also hinge on the regulation of microRNA pathways (Megosh et al. 2006). dFMRP associates with maternally contributed PIWI (P-element induced wimpy testis) and the RNA helicase Dicer-1 proteins, both involved in RNA silencing mechanisms. Their loss of function similarly reduces the number of pole cells and, in some cases, results in a complete loss of pole plasm (Megosh et al. 2006). It is possible that loss of dFMRP may disrupt PIWI/Dicer-1 association, which would be predicted to misregulate miRNAs involved in pole cell formation. This hypothesis needs to be tested experimentally, but could provide a mechanistic basis for dFMRP involvement during pole cell cellularization.

Following cellularization, a mid-blastula transition (MBT) occurs when maternally contributed gene products cease to be utilized and zygotic transcription is activated, requiring new regulation of mRNA synthesis and degradation (Tadros and Lipshitz 2005). At this stage, maternally-contributed dFMRP appears necessary to regulate specific zygotic mRNAs (Monzo et al. 2006). In particular, dFMRP binds *trailerhitch* mRNA and activates its translation, and Trailerhitch protein levels are reduced and mislocalized in *dfmr1* null embryos (Monzo et al. 2006). This result is surprising given that dFMRP characteristically acts as a translational repressor, and thus direct targets are usually increased in *dfmr1* loss of function mutants. Also surprising is that this reduction of protein coincides with an increase in *trailerhitch* mRNA levels in *dfmr1* nulls. Nevertheless, loss of function *trailerhitch* mutants exhibit cleavage furrow defects similar to *dfmr1* nulls (Monzo et al. 2006). Trailerhitch and dFMRP proteins co-sediment in ribonucleoparticles, although they are not thought to interact directly to control MBT cellularization. In contrast, the RNA-binding translational regulator Caprin, which also associates with dFMRP at the MBT, does coordinately control common mRNA targets (Papoulas et al. 2010). Although *caprin/dfmr1* double mutants exhibit a normal spindle apparatus, embryos show premature entry into mitosis due to misregulation of mutual downstream targets, including Cyclin B and Frustart (FRS) (Papoulas et al. 2010). The transcripts of these cell cycle regulators are

bound by both Caprin and dFMRP, but the proteins are inversely regulated during early MBT: Cyclin B is upregulated and FRS is downregulated in the absence of Caprin/dFMRP. Interestingly, expression changes are transient, with normal levels of each protein returning by late MBT (Papoulas et al. 2010). Consistently, elevated Cyclin B causes premature entry into mitosis at an early stage, within minutes of cellularization, but has no effect at later stages (Royou et al. 2008). Thus, the role of dFMRP in the developing embryo is also likely transient. The mammalian homolog of Caprin has been implicated in regulating specific subsets of mRNAs involved in synaptic plasticity (Shiina et al. 2005; Solomon et al. 2007). Thus, while the dFMRP/Caprin interaction was identified in embryos, a similar interaction could exist in the nervous system. The association with Caprin highlights the idea that dFMRP participates in different ribonucleocomplexes, which may possess specific functions dictated by the precise composition of each particle.

Additional dFMRP mRNA targets may also contribute to defects during embryogenesis. In particular, 3 subunits of the 8-subunit CCT (Chaperonin containing TCP) complex are direct dFMRP mRNA targets in MBT embryos (Monzo et al. 2010). The CCT complex is an ATP-dependent chaperone which functions in protein folding of a large range of substrates, most notably actin and tubulin (Dekker et al. 2008; Yam et al. 2008). The 3 targeted subunits are misexpressed in *dfmr1* mutants in varying ways; CCT7 is upregulated, CCT4 is downregulated, and CCT3 is inappropriately posttranslationally modified (Monzo et al. 2010). Interestingly, all subunits not identified as dFMRP mRNA targets also appear unchanged at the protein level. It is not clear how these changes to specific complex subunits affect the overall CCT function, but the complex does not appear to properly form in *dfmr1* nulls, suggesting that chaperone activity is likely compromised. Consistently, disruption of the CCT complex with loss of function subunit mutants leads to cellularization defects (Monzo et al. 2010). Combining these mutations with the *dfmr1* null further exacerbates the phenotype, suggesting direct interaction between dFMRP and the complex. While the complete substrate identities of CCT are not known, the septin Peanut, which is known to be required for furrow formation, is one target that is mislocalized in both CCT mutants and even more so in *dfmr1* mutants (Adam et al. 2000; Deshpande et al. 2006; Neufeld and Rubin 1994). Thus, another mechanism by which dFMRP affects cellular processes may be indirectly through protein folding programs essential for proper protein expression. Certainly more analysis into the functions of these proteins in embryonic development and elsewhere is warranted to explore the full extent of this intriguing mechanism.

Additional layers to the complexity of dFMRP mechanistic functions have been revealed in germline cells. In particular, dFMRP binds to and represses the translation of the *Drosophila* cytoplasmic polyadenylation element binding protein (dCPEB) Orb (Costa et al. 2005). Orb functions in an autoregulatory feedback loop required for the translation of localized mRNAs in egg chambers and, as such, regulates its own expression to ensure efficient translational control (Tan et al. 2001). As predicted from their opposing molecular functions, mutations in *dfmr1* and *orb* antagonize each other: *dfmr1* null ovaries exhibit an increase in egg

chambers, and this defect can be rescued by *orb* mutations. Likewise *orb* mutants show a defect in dorsal–ventral polarity, favoring ventralized eggs, which can be partially or completely rescued by reducing dFMRP expression (Costa et al. 2005). Thus, dFMRP antagonizes the Orb translational pathway in a dose-dependent manner. Parallels for this mechanism exist in the nervous system, where *Drosophila* Orb2 genetically interacts with *dfmr1*, although the consequence of this interaction on FXS-related phenotypes is unknown (Cziko et al. 2009). However, mammalian CPEBs function in synaptic plasticity and memory formation, suggesting an intriguing possibility that Orb2 provides a similar role in *Drosophila* (Keleman et al. 2007). In parallel ovarian studies, microarray analysis identified the transcript of E3 ubiquitin ligase, cbl, as another potential binding target of dFMRP (Epstein et al. 2009). Indeed, *cbl* mRNA levels are elevated in *dfmr1* mutant ovaries, although Cbl protein levels are unaffected, suggesting that dFMRP may regulate the stability or trafficking of *cbl* mRNA as opposed to its translation. Just as with *dfmr1* and *orb* double mutants, loss of function *cbl* alleles either completely or partially rescue the aberrant egg chamber counts in combination with *dfmr1* null alleles (Epstein et al. 2009). Interestingly, loss of *dfmr1* seems to cause defects by altering germ cell proliferation, and rates of cell cycle progression, possible via increased expression of cyclin E. Null *dfmr1* germ cells overexpress cyclin E, are hyper-proliferative, and progress through the cell cycle at a slower rate than controls (Epstein et al. 2009). Taken together, these studies reveal a surprising complexity of dFMRP-mediated mRNA regulation controlling the proliferative capacity of germline cells.

7.3 dFMRP Roles in Larval Neuronal Development

Clues about dFMRP function in oogenesis are providing direct insights into similar functions during early neuronal development. *Drosophila* neuronal stem cells (neuroblasts) populate the nervous system through a series of asymmetric divisions occurring in two periods; in the embryo, to produce larval neurons, and in the larva through pupa, to produce adult neurons. The *dfmr1* null larval central nervous system manifests hyperproliferation from these stem cells (Callan et al. 2010). Null *dfmr1* neuroblast lineages exhibit excessive 5-bromo-2-deoxyuridine (BrdU) incorporation and produce a greater number of differentiated adult neurons per stem cell compared with controls. Although the length of the cell cycle in *dfmr1* null neuroblasts is not altered, a greater number of neuroblasts escape early quiescence in the absence of dFMRP, which leads to the increased production of differentiated neurons (Callan et al. 2010). Somewhat similarly, in the mouse FXS model, loss of FMRP leads to an increase in glial cell differentiation, although the penetrance of the phenotype is low (Hessl et al. 2004; Luo et al. 2010). There are also mild increases in cell numbers, in both *Drosophila* and mouse FXS models, but this defect may nevertheless have important ramifications for the human disease state. In some FXS patients, some brain regions appear larger than controls, which could

be the result of increased cellular proliferation (Hoeft et al. 2008, 2010a, b). Moreover, if FMRP differentially regulates the differentiation of specific subsets of progenitor cells, this could have consequences on neuronal connectivity and brain function. A more detailed description of the role of FMRP in stem cell maintenance and differentiation is described in Chaps. 3 and 8.

Apart from the above recent work, most effort has been focused on the function of dFMRP in late nervous system development, especially during synaptogenesis and synaptic refinement. In the *Drosophila* system, the larval neuromuscular junction (NMJ) is a particularly well-characterized and easily accessible glutamatergic synapse, which is an excellent model for glutamatergic synapse structural and functional development (Ball et al. 2010; Keshishian et al. 1996; Koh et al. 2000; Korkut et al. 2009; Rohrbough et al. 1999). Null *dfmr1* mutants exhibit a number of NMJ phenotypes. The neuronal branches innervating muscles are over-elaborated in *dfmr1* null mutants, indicating a role for dFMRP in repressing growth morphology (Zhang et al. 2001). Consistently, the number of synaptic boutons, sites of glutamate neurotransmitter release, is increased in mutant animals. These findings resemble synaptic defects in the mouse model and FXS patients. Indeed, the classical cellular hallmark of FXS patients is cortex neurons with supernumerary dendritic postsynaptic spines (Comery et al. 1997; Irwin et al. 2000, 2001). Spines in patients and mice have been described as "long", "thin," and "torturous", and suggested to improperly mature in the FXS disease state. Consistently, *dfmr1* null NMJs exhibit an increased number of immature synaptic boutons referred to as "mini" or "satellite" boutons (Coffee et al. 2010; Gatto and Broadie 2008). These satellite boutons are developmentally-arrested at an early stage when normal synapses have morphologically and functionally matured (Beumer et al. 1999; Dickman et al. 2006). Structural changes are accompanied by alterations in synaptic function (Gatto and Broadie 2008; Repicky and Broadie 2009; Zhang et al. 2001). Both spontaneous and evoked neurotransmission currents are increased in *dfmr1* null NMJs, and FM1-43 dye imaging confirms an elevated level of synaptic vesicle turnover (Gatto and Broadie 2008; Zhang et al. 2001). Based on these core phenotypes, the *Drosophila* NMJ has been used as a tool to probe dFMRP function and dissect dFMRP interactions with other neuronal gene products.

One explanation for the synaptic function changes in *dfmr1* null NMJs is a change in neurotransmitter receptor composition. Glutamate receptors are the primary excitatory ionotropic channels located in the postsynaptic muscle juxtaposed to presynaptic sites of glutamate release (DiAntonio 2006; Schuster et al. 1993). At the larval NMJ, GluRII AMPA-like receptors are expressed as tetrameric complexes containing three common subunits (GluRIIC, D and E) combined with either GluRIIA or GluRIIB subunits, to make two distinct classes of receptor (Featherstone et al. 2005; Qin et al. 2005). These receptor classes differ in their regulation, subcellular localization, and functional conductance properties. Interestingly, the distribution of each receptor class is differentially altered in the absence of dFMRP (Pan and Broadie 2007). Null *dfmr1* NMJs exhibit an increase in GluRIIA receptors and a concomitant decrease in GluRIIB receptors, though notably the overall number of total receptors does not change. Thus, dFMRP

regulates the ratio of different GluR subclasses at a single synapse. In contrast, overexpression of dFMRP exclusively in the muscle using the targeted UAS-GAL4 expression system reduces expression of all GluRIIs at the NMJ (Pan and Broadie 2007). Similar findings have been seen in the mouse model of FXS where loss of FMRP results in changes in AMPA receptor surface expression which may be dependent on both the precise brain region examined and particular upstream signaling events (Nakamoto et al. 2007; Soden and Chen 2010; Suvrathan et al. 2010; Wang et al. 2010). Overexpression of dFMRP in the presynaptic neuron does not affect GluRII expression, but increases spontaneous glutamate release amplitude and frequency (Pan and Broadie 2007; Zhang et al. 2001). Consistently, ultrastructural analysis reveals increased synaptic vesicle density around presynaptic active zones in *dfmr1* null terminals. These findings indicate both presynaptic and postsynaptic requirements for dFMRP function. Importantly, while most analysis in the mouse is focused on the postsynaptic requirement of FMRP, presynaptic roles are also beginning to be defined which share many similarities to those identified in *Drosophila* (Akins et al. 2009; Antar et al. 2006; Christie et al. 2009; Hanson and Madison 2007). These changes in excitatory transmission properties highlight an important element in both murine and *Drosophila* systems: FMRP-dependent phenotypes are often revealed under states of heightened neuronal activation and are likely due to the functioning of FMRP downstream of synaptic activity (Antar et al. 2006; Aschrafi et al. 2005; Bear et al. 2004; Khandjian et al. 2004; Muddashetty et al. 2007; Nosyreva and Huber 2006; Park et al. 2008; Repicky and Broadie 2009; Stefani et al. 2004; Tessier and Broadie 2008; Todd et al. 2003). Identifying the specific activation conditions that drive FMRP function will likely be critical for understanding the molecular nature of the disease.

FMRP functions downstream of both broad neuronal activation and specific neuronal signaling pathways including Gq-coupled receptors, which has been best characterized for the group 1 metabotropic glutamate receptor (mGluR) (Bear et al. 2004; Volk et al. 2007). mGluR-dependent LTD/LTP synaptic plasticity mechanisms depend on FMRP activity, and mGluR antagonists either partially or completely alleviate structural, functional, and behavioral FXS phenotypes in both murine and *Drosophila* disease models (Choi et al. 2010, 2011; de Vrij et al. 2008; Dolen et al. 2007; McBride et al. 2005; Osterweil et al. 2010; Pan et al. 2008; Yan et al. 2005). Together, these studies have led to the "mGluR theory of FXS", which hypothesizes that FMRP limits the translation of specific proteins under the influence of mGluR stimulation (Bear et al. 2004). The *Drosophila* genome encodes only a single mGluR (DmGluRA), compared to the eight separate receptors in mammals (Bogdanik et al. 2004). The simplicity of the *Drosophila* system, coupled with the evolutionary conservation of the activation pathways, has provided an excellent basis to test the mGluR hypothesis in the *Drosophila* FXS model. Molecular analyses reveal an inverse regulative relationship between dFMRP and DmGluRA: DmgluRA is overexpressed in *dfmr1* null animals and dFMRP is overexpressed in *DmGluRA* nulls (Pan et al. 2008). These results are consistent with dFMRP regulating DmGluRA downstream of the receptor signaling, though whether this is directly through mRNA binding or indirectly through an

intermediate pathway is unknown. However, compared to the *dfmr1* null, the *DmGluRA* null shows only minor structural defects in NMJ structuring and synaptic bouton number/size (Bogdanik et al. 2004). Thus, at most, DmGluRA-mediated glutamatergic signaling is only one factor influencing the role of dFMRP in shaping NMJ architecture, and there must be additional signaling factors at play. In contrast to mild structural defects, the *DmGluRA* null shows more striking defects in activity-dependent synaptic function, including elevated transmission amplitudes during high frequency stimulation and abnormally strong hyperpotentiation following high frequency stimulation (Bogdanik et al. 2004; Pan et al. 2008; Repicky and Broadie 2009). The functional defects are more severe in *DmGluRA* mutants compared to the *dfmr1* null, suggesting again at least some differential requirement. The fact that this functional overlap is only partial may be due to a diverse set of pathways that are initiated downstream of DmGluRA signaling, which appear to only partially overlap with mechanisms regulated by dFMRP. Finally, at a molecular level, loss of DmGluRA leads to an overall increase of both GluRII receptor classes, and therefore elevated total ionotropic GluR abundance at the NMJ synapse. This is in contrast to the loss of *dfmr1* which differentially alters the ratio of GluRIIA and GluRIIB receptor classes (Pan and Broadie 2007). Once again, this relationship indicates a convergence between DmGluRA signaling and dFMRP dependent pathways, but only a partial overlap of function.

The critical analysis of the link between DmGluRA and dFMRP signaling has come from a combinatorial genetic approach in doubly null mutant animals. The double null mutant partially restores the *dfmr1* null synaptic overgrowth defects at the NMJ (Pan et al. 2008). However, while the *dfmr1* null branching over-elaboration is reduced by removal of DmGluRA, the double mutants actually produce an even greater number of synaptic boutons per terminal. Thus, the overlap in regulation of synaptic architecture is partial. At the ultrastructural level, the density of synaptic vesicles clustered around active zone sites is restored to normal in double mutants, rescuing the increase in density in *dfmr1* null terminals (Pan et al. 2008). However, functional readouts of DmGluRA–dFMRP interaction are more complex. Double mutants exhibit enhanced short-term facilitation and long-term augmentation during high frequency stimulation, which are both equally or more severe than defects in the *DmGluRA* null alone (Bogdanik et al. 2004; Repicky and Broadie 2009). Conversely, after a high frequency stimulus train, the enhanced potentiation characterizing *DmGluRA* null animals is completely rescued in the double mutant. In addition, *dfmr1* null NMJs show a characteristic cycling of transmission amplitudes after high frequency stimulation, and this is only partially restored by removing DmGluRA (Repicky and Broadie 2009). The structural and functional results together illustrate that dFMRP is required for only some of the DmGluRA-dependent signaling pathways. At a molecular level, the increase in the GluRIIA receptor class observed in both *dfmr1* and *DmGluRA* nulls alone is additively increased in double mutants (Pan and Broadie 2007). Likewise, the decrease in the GluRIIB receptor class in *dfmr1* null animals is lessened in double mutants, presumably due to the additive effect of the increase in GluRIIB numbers in the *DmGluRA* mutant. These data indicate that pathways induced by DmGluRA

activation converge with pathways regulated by dFMRP, but do not indicate a linear pathway from DmGluRA to dFMRP. Taking these data together, a picture emerges of the translational regulator, dFMRP, able to compensate partially for loss of the signaling receptor DmGluRA, and the receptor also able to partially compensate for loss of dFMRP. Clearly more work is required to dissect these two converging pathways in order to determine precisely the mechanism by which these genes modulate synaptic transmission and thereby affect the developmental defects in FXS.

In addition to identifying signaling activities that control dFMRP function, a critical question is to determine when dFMRP functions, and therefore whether targeted interventions may need to be performed during specific windows of development as opposed to maturity. The *Drosophila* model is well suited to this dissection as transgenic methods allow gene expression to be temporally controlled (Fischer et al. 1988; McGuire et al. 2004). Using the inducible GeneSwitch system to express dFMRP during a precise window of development, constitutively throughout development or only at maturity, the ability to rescue *dfmr1* null larval NMJ phenotypes has been thoroughly assessed (Gatto and Broadie 2008, 2009; Osterwalder et al. 2001). Using constitutive expression, targeted neuronal presynaptic reintroduction of dFMRP fully rescues all NMJ structural defects, though there is no rescue of defects in synaptic vesicle cycling. Reintroduction of dFMRP only during a short window (12 h) in the early larval stages is equally effective in rescuing NMJ structural defects (Gatto and Broadie 2008). One important caveat to this finding is that the in vivo half-life for dFMRP is approximately 25 h (at 25°C), which extends the window of targeted reintroduction. Nevertheless, dFMRP clearly has a primary function during early stages of synaptogenesis to enable subsequent synaptic maturation. In contrast to the early temporal rescue, reintroduction of dFMRP in late third instar stages only marginally rescues *dfmr1* null NMJ structural defects (Gatto and Broadie 2008). These results indicate that structural plasticity is required to remove excess synaptic branches and supernumerary boutons present in the mutant condition. It is clear that the *Drosophila* NMJ is capable of such plasticity, but the time period available in the GeneSwitch analysis may have limited the ability for structural corrections to manifest (Eaton et al. 2002; Heckscher et al. 2007; Rohrbough et al. 2000). This consideration aside, these results indicate an early requirement for dFMRP in synaptogenesis, which can only be weakly compensated for by later reintroduction of dFMRP. Whether early intervention is similarly necessary to alleviate functional defects at the NMJ, or behavioral readouts of the affected circuit, remains to be tested. It will be critical to understand how early versus late intervention strategies affect downstream dFMRP-dependent molecular pathways that directly control synaptic development.

To test the in vivo significance of interactions between dFMRP and its mRNA targets, the great power of the *Drosophila* system is the ability to make combinatorial mutations in a single animal (Table 7.1). This methodology has been critical in identifying genes that genetically interact with dFMRP at the larval NMJ (Zhang et al. 2001). In particular, *Drosophila* Futsch, homolog to mammalian microtubule associated binding protein 1B (MAP1B), was the first protein shown to genetically

Table 7.1 Genes identified to genetically interact with dFMRP

Genes	Function	Tissue interaction identified	mRNA bound by dFMRP	mRNA levels in *dfmr1* nulls	Protein levels in *dfmr1* nulls	References
Trailerhitch	mRNA processing	Embryo	Yes	Upregulated	Downregulated	Monzo et al. (2006)
Caprin	mRNA translation	Embryo	ND	ND	ND	Papoulas et al. (2010)
CCT3	Chaperone complex	Embryo	Yes	ND	Posttranslationally modified	Monzo et al. (2006)
CCT4	Chaperone complex	Embryo	Yes	ND	Downregulated	Monzo et al. (2006)
CCT7	Chaperone complex	Embryo	Yes	ND	Upregulated	Monzo et al. (2006)
Orb1	mRNA translation	Ovary	ND	ND	Upregulated	Costa et al. (2005)
Cbl	Ubiquitin ligase activity	Ovary	ND	Upregulated	Unaffected	Epstein et al. (2009)
Bantam miRNA	miRNA	Ovary	Yes	Unaffected	–	Yang et al. (2009)
Sticky	Citron kinase	Eye	ND	ND	ND	Bauer et al. (2008)
Orb2	mRNA binding	Eye	ND	ND	ND	Cziko et al. (2009)
Futsch/MAP1B	Microtubule binding	Larval NMJ	Yes	ND	Upregulated	Zhang et al. (2001)
dmGluRA	G-protein coupled receptor	Larval NMJ	ND	ND	Upregulated	Pan and Broadie (2007) and Repicky and Broadie (2009)
Lgl	Cytoskeletal binding	Larval NMJ/Eye	ND	ND	ND	Zarnescu et al. (2005)
aPKC	Protein kinase	Larval NMJ	ND	ND	ND	Zarnescu et al. (2005)
Argonaut-1	miRNA processing	Larval NMJ/Eye	ND	ND	ND	Jin et al. (2004)
Argonaut-2	miRNA processing	Larval NMJ	ND	ND	ND	Pepper et al. (2009)
Spastin	Microtubule severing	Larval NMJ/Eye	ND	ND	Unaffected	Yao et al. (2010)
Rac1	GTPase activity, actin organization	DA neurons	Yes	ND	ND	Lee et al. (2003)
Pickpocket	Sodium ion channel	Sensory neurons	Yes	Upregulated	ND	Xu et al. (2004)
Chickadee/profilin	Actin binding	Lateral clock circuit neurons	Yes	Upregulated	Upregulated	Reeve et al. (2005) and Tessier and Broadie (2008)
Lark	mRNA binding, actin organization	Clock circuit/Eye	ND	ND	Unaffected	Sofola et al. (2008)
Staufen	mRNA translation	Adult brain	ND	ND	ND	Bolduc et al. (2008)
Cheerio/filiminA	Actin binding	Adult brain	ND	ND	Down after spaced training	Bolduc et al. (2010)

ND not detected

interact with FMRP in vivo (Zhang et al. 2001). Mutations in *futsch* alone produce NMJ structural and functional phenotypes that are the inverse of those produced by mutations in *dfmr1*. Moreover, *futsch* hypomorphic alleles, which reduce Futsch expression by approximately 50%, completely restore the *dfmr1* null NMJ axonal branching, supernumerary synaptic bouton and neurotransmission defects of the *Drosophila* FXS model (Zhang et al. 2001). dFMRP binds *futsch* mRNA and represses Futsch translation. Null *dfmr1* animals express approximately twofold more Futsch than controls, and *dfmr1* overexpression reduces Futsch expression, showing that dFMRP acts as a negative regulator of *futsch* translation in vivo (Zhang et al. 2001). Importantly, this FMRP–MAP1B interaction was subsequently confirmed in mammalian systems, confirming the evolutionary conservation of the molecular mechanism (Lu et al. 2004; Wei et al. 2007). Consistent with defects in neuronal microtubule regulation, *dfmr1* null testes spermatid axonemes fail to properly form the microtubule array needed for sperm mobility and male fecundity (Coffee et al. 2010; Zhang et al. 2004). In the mutant condition, microtubule stability is impaired and the characteristic "9 + 2" microtubule arrangement is lost, often resulting in an aberrant "9 + 0" array lacking the central pair of microtubules. Together, studies in the NMJ and testes both suggest that FMRP plays a prominant role in the regulation of the microtubule cytoskeleton.

Using a similar approach to that used to identify a Futsch–dFMRP interaction, other genes have been demonstrated to function with dFMRP at the larval NMJ. For example, loss of function mutations of the cytoskeletal-binding protein Lethal Giant Larvae (Lgl), originally identified as a tumor suppressor gene in cellular proliferation and cell polarity development (Bilder et al. 2000; Strand et al. 1995), dominantly suppress *dfmr1* overexpression phenotypes (Zarnescu et al. 2005). At the NMJ, testing for genetic interaction in the double heterozygote *lgl*/+; *dfmr1*/+ condition revealed structural defects similar to the *dfmr1* null; specifically, a greater than twofold increase in synaptic bouton number. Neither single heterozygotic mutation alone causes a phenotype, but rather the double mutants act convergently to produce synaptic hyperplasia. dFMRP and Lgl co-localize in cells, can be co-fractionated on density gradients and co-immunopreciptate from similar complexes (Zarnescu et al. 2005). Although the two proteins likely do not interact directly, the complexes they inhabit share an overlapping set of mRNAs constituents, which each protein presumably regulates in common during transcript transport and/or translation regulative control. It is possible that Lgl interacts with the cytoskeleton to localize or stabilize a subset of mRNAs regulated by dFMRP, although the mechanism of this putative cooperation is uncertain. This cooperative interaction may involve the Par protein complex and atypical protein kinase C (aPKC), which are necessary for the distribution of proteins defining cell polarity (Ohno 2001). Interestingly, all of these proteins are necessary for proper NMJ development. Loss of aPKC results in a reduction in the number of synaptic boutons caused by improper organization of the microtubule cytoskeleton in both pre- and postsynaptic compartments (Ruiz-Canada et al. 2004). *aPKC* nulls exhibit increased GluRIIA and concomitantly increased transmission amplitudes in NMJ synaptic terminals. The Par complex colocalizes with aPKC at NMJs, and mutations in Par complex

components mimic aPKC mutants in reduced bouton numbers, increased GluRIIA expression and increased evoked synaptic transmission amplitudes (Ramachandran et al. 2009; Ruiz-Canada et al. 2004). Loss of the complex component Baz/Par3 disrupts postsynaptic actin organization similarly to loss of aPKC. aPKC phosphorylates Lgl, thereby releasing it from the actin cytoskeleton and altering the subcellular distribution of Lgl bound targets (Betschinger et al. 2003; Tian and Deng 2008) Heterozygotic removal of aPKC should hyperactivate Lgl and, interestingly, restores *dfmr1* null NMJ synaptic bouton numbers back to wildtype levels (Zarnescu et al. 2005). Therefore, as with the Futsch interaction, it appears that modulation of cytoskeletal properties is a central aspect of dFMRP function.

More recent evidence for the importance of the regulation of the microtubule cytoskeleton in *dfmr1* null animals has come from the identification of a genetic interaction of dFMRP with *Drosophila* Spastin (Yao et al. 2011). Spastin is a microtubule severing protein whose mutation is one prominent cause of Hereditary Spastic Paraplegia (HSP) (Salinas et al. 2007). RNAi knockdown of Spastin suppresses the rough eye phenotype caused by overexpression of dFMRP, and importantly, double mutant combinations of *dfmr1* and *spastin* display a combinatorial increase in NMJ bouton number over either individual null allele alone (Yao et al. 2011). In direct imaging of the microtubule networks around nuclei in muscle cells, *dfmr1* null cells show a dramatic increase in the ratio of microtubules around the perinuclear region compared to the middle nuclear region. The phenotype caused by dFMRP overexpression in the muscle is the inverse; less microtubule complexity in the perinuclear region. Interestingly, though no differences in Spastin expression are seen in *dfmr1* nulls, muscle overexpression of dFMRP leads to a nearly three-fold increase in Spastin expression, suggesting that dFMRP positively regulates Spastin. However, it is not known whether this change in protein level is due to direct regulation of *spastin* mRNA by dFMRP. The functional relevance of the alteration in microtubule complexity may be to impact mitochondria transport processes (Chen et al. 2009). Null *dfmr1* axons contain more mitochondria, and these mitochondria appear more motile than in controls. The interpretation is that dFMRP-dependent misregulation of Spastin leads to an inability to properly regulate the microtubule network, thereby altering the dynamic localization of mitochrondria. Such a defect may have important consequences for activity-regulated mechanisms of dFMRP function.

In addition to regulation of microtubules, dFMRP also regulates the actin cytoskeleton. This function has been best characterized in dendritic arborization (DA) mechanosensory neurons that extend sensory dendritic processes underlying the larval epidermis (Lee et al. 2003; Schenck et al. 2003). As at the NMJ, loss of dFMRP results in an increase in neuronal branch arborization in the sensory dendrites. Moreover, dFMRP overexpression results in a more simplified dendritic architecture with fewer branches. The small RhoGTPase Rac1 plays a critical role in mediating branching by modulating actin cytoskeleton dynamics to control this growth (Luo et al. 1994; Ng et al. 2002). In multiple neural circuits, loss of Rac1 leads to a reduced structural complexity similar to the dFMRP overexpression phenotype. Indeed, simultaneous overexpression of both dFMRP and Rac1 in DA

neurons leads to a modest restoration of dendritic branch structure toward the wildtype architecture (Lee et al. 2003). dFMRP directly binds to *rac1* mRNA, although the effect of this interaction on Rac1 protein levels has not been assessed. However, it is attractive to speculate that dFMRP represses Rac1 translation, perhaps locally in dendritic processes, to control actin-mediated branching. An additional component of this mechanism is the Cytoplasmic Fragile X Interacting Protein (CyFIP), which binds both Rac1 and dFMRP, although no complexes containing all three proteins have been identified (Schenck et al. 2003). CyFIP is an eukaryotic initiation factor 4E-binding protein (eIF4E-BP) that is capable of regulating the initiation of translation at synapses, and specifically in response to neuronal activity (Napoli et al. 2008). Thus, it is possible that CyFIP acts as a bridge between the translational repressor activity of dFMRP and the Rac1-dependent induction of actin remodeling. Since dFMRP regulates *rac1* mRNA directly, it is likely that a feedback mechanism ensures that the actin cytoskeleton is precisely modulated, particularly during times of neuronal activity (Lee et al. 2003). Yet another component of this pathway in *Drosophila* is the actin-binding protein Profilin/Chickadee (Reeve et al. 2005). Profilin/Chickadee mediates the dynamic turnover of actin by destabilizing F-actin. The role of Profilin/Chickadee in the Rac1-FMRP pathway has been best characterized in the adult circadian activity circuit (Reeve et al. 2005), and will therefore be discussed below. The discovery of elements of this cytoskeletal regulatory mechanism in multiple *Drosophila* neural circuits once again highlights the importance of misregulation of cytoskeletal dynamics in the FXS disease state.

7.4 dFMRP Roles in Adult Brain Neural Circuit Development

To correlate dFMRP-dependent pathways to higher order behaviors, there is an obvious need to push the *Drosophila* FXS model assays into adult brain neural circuits. Two particularly attractive circuits are (1) the mushroom body (MB) circuit required for olfactory learning and memory consolidation, and (2) the circadian clock circuit involving small and large pigment dispersing factor (PDF) lateral neurons that coordinate daily activity cycles (Dahdal et al. 2010; Dubnau and Tully 2001; Helfrich-Forster et al. 2007; Sheeba et al. 2008; Zars 2000). Both circuits are excellent locations for *Drosophila* FXS model study for several reasons. First, both are very well characterized with respect to the neuronal subtypes comprising the circuit. In the MB circuit, a great deal is known about connectivity in the upstream olfactory lobe (OL), the projection neurons (PNs) innervating the MB, and the three classes of MB Kenyon Cells (KCs) required for memory storage (Dubnau et al. 2001; Krashes et al. 2007; Tully and Quinn 1985). In the circadian clock circuit, the cellular components are similarly well defined at the level of individually-identifiable neurons (Fernandez et al. 2007; Nitabach and Taghert 2008). Second, in both circuits, a great deal is known about how each neuron class contributes to the associated behavior. Third, a broad array of genetic tools

is available for studying both MB and clock circuitry at the level of small groups of neurons, or even the single cell level. This includes an array of specific GAL4 driver lines for controlled gene expression and the targeted delivery of genetic manipulations and reporter constructs, as well as GAL4-based clonal techniques for similar manipulation of single neurons. Single cell analysis has been particularly critical for understanding activity-dependent mechanisms of architectural and functional refinement associated with the cell-autonomous loss of dFMRP (Tessier and Broadie 2008, 2011).

The first CNS circuit investigated in the *Drosophila* FXS model was the circadian circuit controlled by the dorsal cluster (DC) photoreception responsive cells and the lateral PDF clock neurons (Morales et al. 2002). Interestingly, dFMRP may function in opposing roles in these two components of the same circuit. In DC neurons, the number of axons that project from the lobula to the medulla is reduced in *dfmr1* nulls compared to controls. Concomitantly, however, the connecting large lateral PDF neurons, which normally project via a fasiculated axon bundle toward the dorsal medial lateral horn where synaptic connections are formed, are overgrown in *dfmr1* null brains (Morales et al. 2002). Axonal pathfinding in this part of the circuit appears somewhat inaccurate, with extra projections prematurely branching off to the medial part of the brain. However, the most robust phenotype is that the synaptic puncta in the lateral horn are more numerous and more broadly dispersed in the *dfmr1* null mutant (Coffee et al. 2010; Gatto and Broadie 2009; Morales et al. 2002). Overexpression of dFMRP results in the opposite consequence, with synaptic arbors collapsed to occupy a much smaller area containing fewer definable synaptic boutons. The combination of these gain and loss of function *dfmr1* phenotypes in the lateral PDF clock neurons shows that dFMRP normally functions to limit neuronal growth and limit synaptic development, comparable to the role at the NMJ synapse. Importantly, the timing of this dFMRP requirement appears limited to a precise stage of circuit development (Gatto and Broadie 2009). Restoring dFMRP activity to this circuit using the GeneSwitch conditional paradigm is only effective at rescuing *dfmr1* null phenotypes when dFMRP is reintroduced during a late pupal stage of brain development, a period of synaptogenesis, remodeling, and synaptic refinement (Gatto and Broadie 2009). Reintroduction of dFMRP either during the earlier stages of neurogenesis and axonal outgrowth, or during mature stages in adult animals, completely fails to rescue the *dfmr1* null defects in clock circuit architecture. Thus, as in the larval motor circuit, dFMRP functions in a precise developmental window to sculpt synaptic connectivity. Identifying the molecular players interacting with dFMRP at this stage of brain maturation will be critical to understanding the molecular mechanism of dFMRP function within the developing clock circuit.

From embryonic and larval dFMRP analyses, there are many strong indications that dFMRP plays a prominent role in regulating the dynamic properties of the cytoskeleton. Similarly in the adult clock circuit, genetic interaction between dFMRP and Rac1 has been identified in the regulation of the downstream actin-destabilizing protein Profilin/Chickadee (Reeve et al. 2005). Double loss of function mutations in *rac1* and *dfmr1* significantly exacerbate the branching defects of

the lateral PDF neurons compared to the *dfmr1* null alone. Similarly, *chickadee* mutants genetically interact with *dfmr1* to control clock circuit architecture. Overexpression of *chickadee* alone results in a phenocopy of the *dfmr1* null synaptic overgrowth, and co-overexpression of *chickadee* and *dfmr1* rescues the synaptic arborization of the lateral PDF neurons (Reeve et al. 2005). The mechanism of this interaction is likely direct, as dFMRP binds to and represses the translation of *chickadee* mRNA. This regulation may also involve either an effect on the transcription or stability of *chickadee* mRNA, as there is an elevated level of *chickadee* mRNA in *dfmr1* null brains compared to controls (Tessier and Broadie 2008). Interestingly, the mRNA level increase is transient, peaking during late stages of pupal brain maturation, immediately prior to eclosion. This window is the same developmental window identified for the transient rescue requirement of dFMRP, and reinforces the conclusion that dFMRP has a primary function during neural circuit refinement (Gatto and Broadie 2009). These findings, combined with the evidence of Rac1 and CyFIP genetic interactions in the larval peripheral circuits, strongly indicate that dFMRP mediates regulation of the actin cytoskeleton via translational control of Chickadee/Profilin, in concert with a feedback mechanism to upstream Rac1 signaling molecules.

A particular benefit to studying dFMRP function in the circadian clock circuit is the ability to easily correlate molecular and cellular changes with circadian behavior. Consistent with gross changes in circuit architecture, the *Drosophila* FXS model exhibits profound circadian activity defects (Banerjee et al. 2007; Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Sekine et al. 2008). In locomotor analysis during alternating light and dark cycles, a large portion of *dfmr1* null animals are totally arrhythmic; they do not cycle between active and inactive periods, but rather exhibit heightened activity without a clear sleep period. Confoundingly, when sleep periods are measured directly, *dfmr1* null animals show prolonged sleep, while animals over-expressing dFMRP show reduced bouts of sleep (Bushey et al. 2009). This seeming contradiction certainly requires further investigation. Under conditions of constant darkness, *dfmr1* nulls exhibit an even more pronounced arrhythmicity, indicating a defect in circadian clock output (Dockendorff et al. 2002; Inoue et al. 2002; Sekine et al. 2008; Sofola et al. 2008). Importantly, the severity of the arrhythmicity of each individual *dfmr1* null animal positively correlates with the degree of overexpansion of the lateral PDF neuron synaptic contacts in the dorsal horn (Sekine et al. 2008). The striking behavioral defect helped to identify a genetic interaction between dFMRP and Lark (Sofola et al. 2008), an RNA-binding protein which globally regulates genes involved in circadian behavior (Newby and Jackson 1996; Zhang et al. 2000). Lark and dFMRP can be coimmunoprecipiated, suggesting that some mRNA targets may overlap between the two proteins (Sofola et al. 2008). Interestingly, loss of function *lark* mutants exhibit reduced levels of dFMRP, although *dfmr1* null animals express wildtype levels of Lark, thus perhaps indicating that dFMRP is directly regulated by Lark but not vice versa (Sofola et al. 2008). Moreover, overexpressing Lark in the lateral clock neurons leads to a modest behavioral arrhythmicity, which can at least be partially rescued by introduction of the

heterozygous *dfmr1* null mutation. In addition, genetic knockdown of *lark* and concomitant overexpression of dFMRP leads to a dramatic loss of rhythmicity, reminiscent of *dfmr1* null animals (Dockendorff et al. 2002; Inoue et al. 2002). These strong behavioral readouts certainly demand further investigation of the downstream targets of these two interacting RNA-binding proteins to elucidate the precise mechanisms of circadian control.

The second CNS focus of the *Drosophila* FXS model has been the MB learning and memory center. MB neurons come in 3 Kenyon Cell (KC) classes (alpha, beta, and gamma neurons), which are required for different aspects of learning, short- and amnesia-resistant memory formation, and long-term memory consolidation (Akalal et al. 2010; Blum et al. 2009; Isabel et al. 2004; Lee et al. 1999; Wang et al. 2008b; Yu et al. 2006). This circuitry has particular relevance due to the cognitive impairments of FXS patients. The characteristic MB lobe structure can be analyzed either for gross anatomical alteration or for fine architectural changes at the single neuron level using the genetic clonal technique of Mosaic Analysis with Repressible Cell Marker (MARCM) (Lee and Luo 2001). The powerful MARCM technique allows GFP labeling of a single *dfmr1* mutant neuron in an otherwise wildtype brain, permitting characterization of cell autonomous effects of *dfmr1* loss or gain of function. At a gross anatomical level, the *dfmr1* null MB exhibits defects in axonal lobe formation, of variable penetrance and dependent on genetic background (Chang et al. 2008; McBride et al. 2005; Michel et al. 2004; Pan et al. 2004). Phenotypes range from dramatic loss or rearrangement of alpha/beta lobes, to improper beta lobe axonal projections across the brain midline. Single cell MARCM analysis consistently reveals over-branched and over-extended axons with supernumerary synaptic varicosities, similar to excessive neuronal growth and bouton formation in the clock and larval motor circuits (Pan et al. 2004; Tessier and Broadie 2008). Overgrowth is also apparent in dendritic arbors, where PNs connect to their postsynaptic KC inputs of the MB circuit, indicating that dFMRP plays cell-autonomous roles in the structural maturation of both sides of the synapse. Overexpression of dFMRP in single MB neurons again caused the opposite consequence of *dfmr1* loss of function: reduced and simplified synaptic connections in both dendritic inputs and axonal outputs (Pan et al. 2004). Importantly, *dfmr1* synaptic overgrowth phenotypes are both developmentally regulated and activity-dependent (Tessier and Broadie 2008). During the late stages of pupal brain development, *dfmr1* null axonal branches are overgrown, with excessive synaptic contacts. Subsequently, at late stages of development and during early adult use, there is a failure to properly eliminate synaptic contacts via an activity-dependent pruning mechanism. Using light to hyperexcite gamma neurons expressing a light-activated cation channel, wildtype neurons prematurely undergo axonal pruning and synapse elimination, but no refinement was induced in *dfmr1* null neurons (Tessier and Broadie 2008). Thus, pruning is dependent on both dFMRP and activity. This dependence is at least somewhat bidirectional, as hyperexcitation leads to reduced dFMRP expression and loss of activity leads to an upregulation of dFMRP (Tessier and Broadie 2008). Consistently, mammalian FMRP associates with polyribosomes in an activity-dependent manner to regulate

the translation of its own mRNA (Khandjian et al. 2004; Laggerbauer et al. 2001; Stefani et al. 2004). Thus, activity-induced translation is tightly controlled at many levels to ensure that the timing of production of growth factors is well coordinated with both intrinsic and extrinsic signals.

One critical consequence of neuronal activity is the mobilization of calcium in response to membrane depolarization. Many types of calcium-signaling pathways function in neurons, and these depend on the source of calcium, the magnitude and duration of the calcium changes, the frequency of calcium influx, and the precise developmental stage of the neuron (Berridge et al. 2000; Lnenicka et al. 2006; Lohmann 2009). Calcium signaling is known to play key roles in axonal outgrowth, synapse formation, and the refinement of synaptic connections. *dfmr1* null MB neurons exhibit a developmentally regulated defect in processing both internal and external calcium signals after depolarizing stimulation (Tessier and Broadie 2011). MB calcium dynamics were investigated in *dfmr1* null animals using the genetically-encoded calcium sensor gCAMP, which changes fluorescence depending on calcium concentration (Akerboom et al. 2009; Nakai et al. 2001). In *dfmr1* null neurons in intact brain MB circuits, there is a defect in (1) calcium release from internal organelles as well as (2) calcium influx across the plasma membrane (Tessier and Broadie 2011). These defects are cell intrinsic as evidenced by consistent findings at the single cell level in a dissociated MB cell culture system. One possible joint mechanism for both phenotypes is misregulation of calcium-buffering proteins. Both Calmodulin and Calbindin sequester cytosolic calcium and expression of their mRNAs are severely reduced in *dfmr1* null brains (Tessier and Broadie 2011). The reduced expression of calcium buffers could have profound impacts on neuronal development and physiology. In addition to developmental roles, the regulation of dFMRP on calcium dynamics could play key functions in the activity-dependent mechanisms described above. Interestingly, the role in regulating activity could involve cooperative interactions between dFMRP and the CPEB Orb protein. During oogenesis, translation is positively regulated by Orb, but this regulation is maintained within reasonable limits by the repressor dFMRP, just as dFMRP is thought to mitigate local translation after neuronal firing. Indeed, a nervous system-specific CPEB, Orb2, is present in *Drosophila* and genetically interacts with *dfmr1* in the eye (Cziko et al. 2009). CPEBs play critical roles in synaptic plasticity and may act as "memory markers" to help identify synapses during memory formation (Keleman et al. 2007). It will therefore be critical to determine if dFMRP functions in a similar feedback control mechanism for nervous system translation via mitigation of neuronal CPEB/Orb2 expression.

A great advantage of MB circuit analysis is the ability to assay well-characterized MB-dependent learning/memory behaviors. Consistent with MB circuit defects, *dfmr1* null animals exhibit a significant defect in learning and striking loss of long-term memory formation, a protein synthesis-dependent process (Bolduc et al. 2008; Tully et al. 1994). A memory defect also occurs when dFMRP is overexpressed, indicating that precise levels of translational control are necessary for memory storage. This behavioral readout has been exploited to identify multiple dFMRP interactors. For example, the RNA-binding translational repressor Staufen

colocalizes in similar cytoplasmic granules as dFMRP and functions in long-term memory formation, suggesting an overlapping function with dFMRP (Barbee et al. 2006; Brendel et al. 2004; Dubnau et al. 2003). Consistently, double heterozygotic mutation of *staufen* and *dfmr1* causes a dramatic reduction in the ability to form long-term memory, although each single heterozygous mutation shows no memory defects (Bolduc et al. 2008). Long-term memory is also dependent on dFMRP interaction with the microRNA pathway. As with *staufen*, double heterozygous mutations of *dfmr1* and the RNA Induced Silencing Complex (RISC) core component *argonaut-1* produce a dramatic reduction in long-term memory storage. Blocking protein synthesis with chemical inhibitors rescues this genetic interaction, further confirming that deregulated protein synthesis destroys memory capacity (Bolduc et al. 2008). The specific mRNAs translated in response to memory-inducing cues, however, remain to be identified. One possible convergent mRNA target of Staufen and dFMRP is *cheerio/filamin A*, which is required for reorganizing the actin cytoskeleton (Flanagan et al. 2001; Li et al. 1999). The *cheerio* and *dfmr1* mutations interact to disrupt protein synthesis-dependent memory (Bolduc et al. 2010). dFMRP positively regulates Cheerio expression in response to memory-inducing stimuli, although it is not clear if this is a direct or indirect mechanism. Also, whether the dFMRP positive regulation of Cheerio integrates with the microRNA-mediated protein synthesis mechanism of memory storage also remains to be determined. Nonetheless, as with the larval circuits and the adult clock circuit, these results further suggest that dFMRP-dependent regulation of the cytoskeleton is a critical pathway underlying FXS disease-relevant behavioral defects.

7.5 dFMRP and the microRNA Pathway

Despite a great deal of effort, the molecular mechanism by which FMRP regulates mRNAs remains quite uncertain. A number of possibilities have been proposed including control of mRNA stability and/or trafficking, repression of translation mediated by direct mRNA binding via specific secondary structure (G-quartet), tertiary structure and other modes of recognition, and translation regulation through microRNA pathways (Ashley et al. 1993; Caudy et al. 2002; Chen et al. 2003; Darnell et al. 2005a; De Diego Otero et al. 2002; Feng et al. 1997; Laggerbauer et al. 2001; Schaeffer et al. 2001; Sung et al. 2003; Weiler et al. 2004; Xu et al. 2004; Zalfa et al. 2003, 2007; Zhang et al. 2007). In *Drosophila*, dFMRP was initially discovered to associate with multiple components of the cellular machinery responsible for the maturation and function of microRNAs (Caudy et al. 2002; Ishizuka et al. 2002; Jin et al. 2004). dFMRP can cofractionate and coimmunoprecipitate with multiple ribosomal proteins, the double stranded RNA nuclease Dicer and Argonaut-1/2 components of the RNA induced silencing complex (RISC), which functions to degrade mRNAs or repress translation (Caudy et al. 2002; Ishizuka et al. 2002; Megosh et al. 2006).

The dFMRP-Argonaute association is critical for regulating the expression of a specific dFMRP target mRNA encoding the degenerin/epithelial sodium channel Pickpocket (Xu et al. 2004). dFMRP binds directly to *pickpocket* mRNA, and *dfmr1* null animals exhibit an increase in *pickpocket* mRNA levels (Xu et al. 2004). Conversely, overexpression of dFMRP results in a decrease in *pickpocket* mRNA levels, indicating a bidirectional mode of regulation. Loss of function *argonaut-2* mutants similarly exhibit an elevation of *pickpocket* mRNA, but this effect is not dependent on dFMRP. However, the reduced *pickpocket* mRNA levels caused by dFMRP overexpression can be rescued by *argonaut-2* mutation, suggesting a cooperative effect by which dFMRP may act as a target locator for RISC components (Xu et al. 2004).

In addition to these molecular interactions, *dfmr1* mutants genetically interact with both *argonaut-1* and *argonaut-2* mutants (Jin et al. 2004; Pepper et al. 2009). In the *Drosophila* eye, a loss of function *argonaut-1* mutant rescues the rough eye phenotype caused by dFMRP overexpression, which confirms the overlapping pathways of regulation between these two proteins (Jin et al. 2004). At the larval NMJ, double trans-heterozygous mutations in *dfmr1* and *argonaut-1*, or *dfmr1* and *argonaut-2*, cause synergistic over-production of synaptic boutons; in the case of the *dfmr1*/+; *argonaut-1*/+ combination, more numerous boutons than in the homozygous *dfmr1* mutant alone (Jin et al. 2004; Pepper et al. 2009). However, it is not clear yet whether specific mRNAs may be differentially regulated by Argonaut-1/dFMRP and Argonaut-2/dFMRP complexes, or how those complexes may regulate synapse development. In the absence of dFMRP there is a reduced association of Argonaut-1 with Dicer, which may be responsible for the reduced expression of miRNA-124 in *dfmr1* null animals (Xu et al. 2008). Interestingly, miRNA-124 overexpression limits dendritic development in DA neurons and thus, the dendritic overgrowth defects associated with FXS may be due to a failure of RISC to silence targets of miRNA-124 (Xu et al. 2008). Indeed, excessive protein synthesis of miRNA targets may be at least partially responsible for FXS memory defects (Bolduc et al. 2008). Future efforts to identify these targets and determine their role in synapse development will certainly be required to test this hypothesis. In support of these efforts, target identification is increasingly feasible as more refined miRNA target parameters become defined. Multiple databases such as mirBase (http://www.mirbase.org) and microCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) are valuable tools in performing candidate gene targeted approaches to determine the full extent by which this pathway is affected in the *Drosophila* FXS model.

The reduced Dicer/RISC complex association in the absence of dFMRP likely has global effects on miRNAs. In the *Drosophila* ovary, dFMRP associates with at least 30 miRNAs, including the well-characterized *bantam* miRNA, suggesting that downstream targets of many miRNAs may contribute, at least in part, to *dfmr1* null phenotypes (Yang et al. 2009). Bantam miRNA functions in germ cell development, where it genetically interacts with dFMRP to control the maintenance of germ line stem cells. Interestingly, *bantam* miRNA is also required in epithelial cells, but not the neurons themselves, to regulate the arborization of adjoining

sensory DA neuron dendrites in *Drosophila* larvae (Parrish et al. 2009). It will be important to determine if this role is specific to *bantam* miRNA, or if other developmentally regulated miRNAs, such as miRNA-124, are also able to regulate synapse development in a similar trans-tissue mechanism. This work suggests a potential new mechanism by which dFMRP regulates neuronal morphogenesis from neighboring cell types, rather than neurons themselves. This hypothesis can be easily tested in the *Drosophila* FXS model using the powerful array of tissue-specific gene expression systems combined with genetic mutations in each gene. In the future, it will be important to expand dFMRP/miRNA interaction analyses from larval peripheral circuits into the central brain circuits to determine whether this mechanism allows dFMRP to regulate the development of central synapses responsible for FXS behavioral defects.

7.6 Developmental Molecular Analysis of dFMRP Function

FMRP has been proposed to bind hundreds of transcripts and may act as a general mRNA regulator (Ashley et al. 1993; Brown et al. 2001; Darnell et al. 2005b; Miyashiro et al. 2003; Sung et al. 2000). However, attempts to validate these mRNAs as *bone fide* targets of FMRP regulation in vivo have largely been unsuccessful. Therefore, the scope of FMRP function still remains a mystery. The majority of validated FMRP/dFMRP mRNA targets have been identified based on a candidate gene approach, rather than systematic analyses. The candidate approach is subject to serendipity, proceeds in a piecemeal fashion, and does not provide a broad overall picture of cellular functions controlled by FMRP. To circumvent these imitations, several attempts have been made to perform global analysis of FMRP mRNA-binding activity, and mRNA expression in null mutant animals, using microarray technologies (Bauer et al. 2008; Brown et al. 2001; D'Agata et al. 2002; Gantois et al. 2006; Zarnescu et al. 2005). For example, to identify dFMRP targets, immunoprecipitation was used to purify directly bound mRNAs that were then probed on microarrays (Zarnescu et al. 2005). As in mammals, the list of putative targets is long and, confoundingly, there is little overlap between mRNA sets identified by different approaches or different laboratories. Nevertheless, the use of combined microarray analysis on genes that genetically interact with dFMRP has proven valuable in identifying key dFMRP regulatory networks. For example, microtubule regulation was again highlighted in microarray analyses of dFMRP and its genetic interactor, Sticky, a citron kinase that regulates microtubule organization (Bauer et al. 2008). Heterozygous loss of function *sticky* mutants can rescue rough eye phenotypes caused by dFMRP overexpression, and similarly loss of dFMRP exacerbates *sticky* mutant eye phenotypes. Indeed, there is considerable overlap between the identities of mRNAs which show varied expression in *dfmr1* and *sticky* loss of function mutants, as assessed by microarray and quantitative reverse transcription PCR (Bauer et al. 2008). The high degree of mRNA regulation overlap strongly indicates that these

diverse proteins regulate convergent pathways that alter the cytoskeleton, once again implicating the cytoskeleton as a key target of dFMRP regulation.

In addition to the still unknown range of FMRP mRNA targets, the nature of FMRP regulatory functions on those targets is similarly unclear. Both mammalian and *Drosophila* FMRP has been implicated in regulating mRNA transport, mRNA stability and mRNA translation (Ashley et al. 1993; Bechara et al. 2009; De Diego Otero et al. 2002; Fahling et al. 2009; Feng et al. 1997; Laggerbauer et al. 2001; Sung et al. 2003; Weiler et al. 2004; Xu et al. 2004; Zalfa et al. 2003, 2007; Zhang et al. 2007). For example, total brain mRNA levels are elevated in a development stage-dependent manner in *dfmr1* null animals (Tessier and Broadie 2008). In addition, many single candidate target transcripts have altered levels in *dfmr1* loss of function mutants (Table 7.1) (Epstein et al. 2009; Monzo et al. 2006, 2010; Tessier and Broadie 2008, 2011; Xu et al. 2004; Zalfa et al. 2007; Zhang et al. 2007). Clearly, from the developmental work in the *Drosophila* FXS model, RNA-binding studies will need to be performed on controlled developmental time points. Indeed, many targets of dFMRP are likely transient in nature, identifiable only during specific windows of dFMRP function (Gatto and Broadie 2008; Papoulas et al. 2010; Tessier and Broadie 2008, 2011). Moreover, the sensitivity of *dfmr1* phenotypes to neuronal activity levels indicates that coupled activity-dependent assays are also needed. The *Drosophila* FXS model is particularly attractive for these studies because the entire tiled array of the *Drosophila* genome can be spotted on a single chip, compared to the multichip platform needed for mammalian genomes (Bertone et al. 2004; Oliver 2006; Stolc et al. 2004). Tiled arrays would identify a wealth of additional information over classical arrays, including specific mRNA isoforms, noncoding mRNA segments and other classes of RNAs such as microRNAs (Matsumoto et al. 2007). Also promising is the rapidly advancing technology of RNA-Seq which can provide direct sequence analysis of dFMRP-bound transcripts as has been done previously with the splicing factor NOVA (Licatalosi et al. 2008; Marioni et al. 2008; Wang et al. 2009). Indeed, this powerful approach will likely soon overtake standard array technologies.

Of course, the *Drosophila* model is most valuable as a truly unique vehicle for genetic screens. Comparable screens cannot practically be done in the mouse FXS model. Unfortunately, this vital research avenue has yet to be fully explored, and the few limited *Drosophila* screens to date have focused on phenotypes arising from dFMRP overexpression (Reeve et al. 2008; Yao et al. 2011; Zarnescu et al. 2005). In the eye, excess dFMRP produces a degenerative rough eye phenotype, and an enhancer/suppressor screen identified both classes of genetic interactors. The majority of these genes have yet to be characterized, although multiple *lgl* mutations were defined as *dfmr1* suppressors, as discussed above (Zarnescu et al. 2005). Additionally, ubiquitous overexpression of dFMRP causes lethality, allowing a simple search for viable suppressors. Perhaps not surprisingly, the majority of suppressors were mapped to point mutations in *dfmr1* itself, and this has provided the basis for a detailed structure–function analysis of the dFMRP N-terminus, whose function was previously unknown (Reeve et al. 2008). Also, a non-genetic, small chemical screen has been used to identify pharmacological

suppressors of *dfmr1* (Chang et al. 2008). For unknown reasons, null *dfmr1* animals die when raised on high levels of dietary glutamate, and drugs that interfere with either GABA synthesis or transport are able to prevent this glutamate-mediated lethality. This work strongly suggests that GABAergic pathways are a critical component of dFMRP regulation (Chang et al. 2008). Consistently, an independent study showed that GABA receptor subunit expression is significantly decreased in *dfmr1* null brains (D'Hulst et al. 2006). Thus, drugs targeting GABAergic pathways may be a new starting point for therapeutic intervention in the human disease state. These results also inform about a possible FXS mechanism, specifically that the FXS disease state may represent an imbalance between excitatory and inhibitory synaptic connections (Gatto and Broadie 2010). Further investigations of this hypothesis will be necessary to identify dFMRP mRNA targets involved in each type of circuit, and to determine how misregulation of those targets may be causally linked to the FXS disease state.

7.7 Proteomic Analysis of dFMRP Function

FMRP is best characterized as a translational regulator. Therefore, it is of the highest importance to understand changes in the proteome that occur in the absence of FMRP. A number of different proteomic approaches have been used in different tissues to assess which proteins may be aberrantly expressed in FXS disease models (Liao et al. 2008; Monzo et al. 2010; Papoulas et al. 2010; Zhang et al. 2004, 2005). Surprisingly, while FMRP is reported to bind to hundreds of different mRNAs, the total number of proteins that detectably change by proteomic analysis is astoundingly small. Only a relative handful of proteins have been identified to either be up- or downregulated in null mutants. The primary technology employed for these analyses has been 2-dimensional differential gel electrophoresis (2-DIGE) combined with mass spectrometry analysis (Monzo et al. 2010; Papoulas et al. 2010; Zhang et al. 2004, 2005). This technology has been steadily advancing over the years and is well equipped to characterize either large changes in the complete proteome, or changes to single proteins. Nevertheless, as a standard electrophoretic gel is integral to this technique, there are many limitations, including the limited size of proteins that can be resolved on the gel and the requirement for the protein to be relatively abundant, i.e., low abundance proteins may not be identifiable. In the *Drosophila* FXS model, 2-DIGE has been performed on null *dfmr1* whole heads and isolated brains (Zhang et al. 2003, 2005). Among over 1,400 proteins queried, only 32 proteins were identified to have a significant change in level in the absence of dFMRP; that is, only ~2% of the proteins detectably changed (Zhang et al. 2005). Most of the abundance changes were exceedingly small ($<10\%$), and many consisted of shifts in the ratio of post-translationally modified protein variants. Nevertheless, an important discovery from this initial proteomic work was the finding that brain monamine biosynthesis pathways as regulated by dFMRP. Both dopamine and serotonin levels are significantly elevated in the *dfmr1* null brain,

owing to misregulation of rate-limiting biosynthetic enzymes (Zhang et al. 2005). These results are consistent with recent findings suggesting that different neurotransmitters and enzymes involved in neurotransmitter biosynthesis are misregulated in the FXS disease state (Fulks et al. 2010; Wang et al. 2008a, 2010). Thus, despite limitations, 2-DIGE has proven effective at identifying physiologically relevant changes in protein expression.

2-DIGE proteomics has also been performed on *dfmr1* null adult testes and embryos (Monzo et al. 2010; Zhang et al. 2004). In mutants, testes are enlarged and fail to properly form microtubules needed for spermatid axoneme development, and cleavage furrows fail to form properly in embryos. Once again, an exceedingly small number of protein changes were identified in these locations: only 23 and 45 proteins, respectively, were significantly altered in *dfmr1* null animals (Monzo et al. 2010; Zhang et al. 2004). Similar to results obtained from microarray analysis, the identity of proteins characterized in different 2-DIGE studies seldom overlaps. However, a few classes of proteins do seem to be constant. In particular, metabolic genes and chaperone/heat shock proteins are consistently identified in all analyses. In embryos, this later group was found to be directly targeted by dFMRP, as the chaperone complex CCT was misregulated in *dfmr1* mutants and the mRNA for specific subunits can be bound by dFMRP (Monzo et al. 2010). Nonetheless, each of these 2-DIGE analyses carries the same limitations. In no case has it been possible to effectively monitor the complete proteome; only proteins within a restricted size and PI range can be assayed. Moreover, only a single time point has been analyzed and no developmental time lines have been examined. This latter point is a particular concern giving that many mammalian and *Drosophila* phenotypes are only relevant during short periods of neuronal maturation, as discussed above. Thus, it is extremely likely that such limited proteomic analyses have missed significant protein changes that are critical to our understanding of the FXS disease state.

New proteomic approaches are being developed to address these concerns (Friedman et al. 2009). We have recently undertaken multiple methodologies to characterize protein changes in brain and synapse development. First, we have revisited advanced 2-DIGE proteomics with mass spec analysis of protein spots to perform the first studies controlled for developmental time. We have focused our work on four developmental time points, which show significant changes in candidate protein expression during late stages of brain maturation and activity-dependent neural circuit refinement (Fig. 7.1). Using a multiplexed gel system comprising four replicates for each time point, highly significant results have been obtained comparing not just how proteins change between *dfmr1* null to wildtype brains, but also how proteins change in each genotype across developmental time points. Principal component analysis (PCA) of the complete proteomics data set validates this time-point-based methodology. The largest component of variation across all samples is developmental stage, accounting for 35% of the variation, while the second largest component is genotype, accounting for 30% of the variation (Fig. 7.2). Consistent with earlier studies, the number of identified protein changes remains relatively small, albeit several times larger than in adult tissues and with more striking

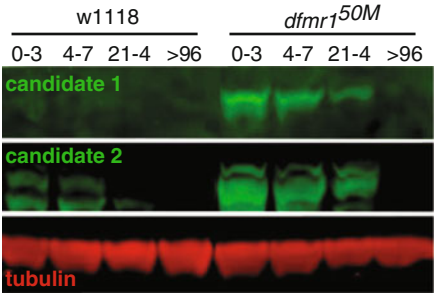

Fig. 7.1 dFMRP transiently suppresses protein expression during development. Protein was extracted from control (w^{1118}) and *dfmr1* null ($dfmr1^{50M}$) heads at the indicated hours post-eclosion at 25°C. Two candidate proteins probed by Western Blot analysis indicate transient upregulation during the first 24 h in the absence of dFMRP. α-Tubulin is a loading control

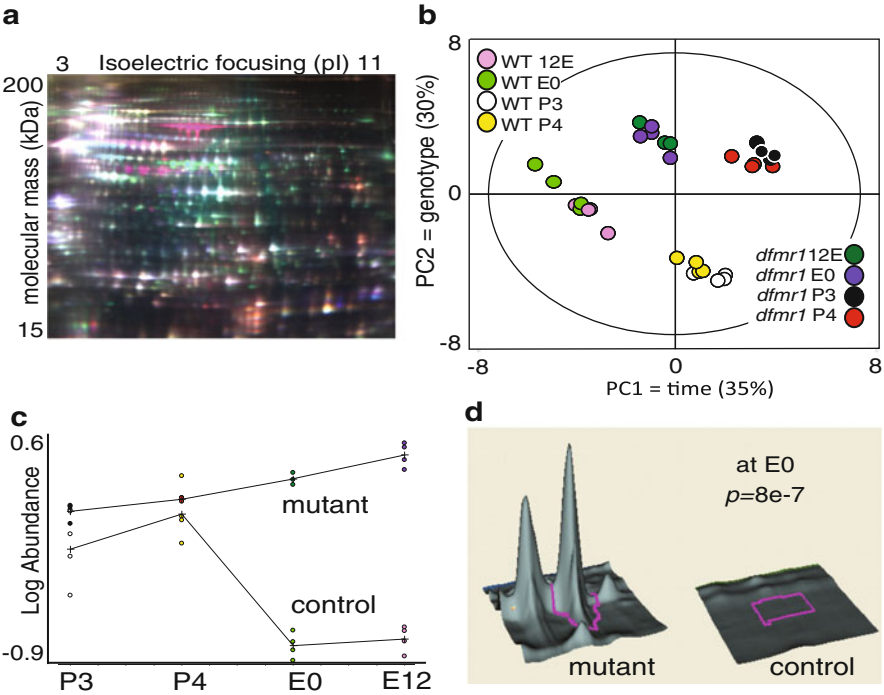

Fig. 7.2 2-DIGE proteomic analysis of *dfmr1* nulls over developmental time. Protein was extracted from wildtype (WT) and *dfmr1* null heads at four developmental times points: 72 h (P3) and 96 h (P4) after puparium formation, and 0–3 h (E0) and 12 h (12E) after eclosion. (**a**) Extracts from four biological replicates at each time point were labeled and subjected to 2-DIGE. A representative gel is shown. (**b**) Principal component analysis reveals strong statistical correlations for each of four replicates for the two genotypes. This analysis identifies the largest source of variance among the data as developmental time (PC1), and the second largest source as genotype (PC2). (**c**) Individual proteins show developmental stage-specific changes in expression. An example shown for the four time points shows aberrant persistence of high expression in the mutant at E0 and E12. (**d**) A single time point from panel C at eclosion, showing the relative abundance of a protein in the control and null mutant

changes in protein levels (Monzo et al. 2010; Zhang et al. 2004, 2005). As before, metabolic proteins are the primary class found to be affected in the *dfmr1* null condition. However, this analysis also reveals significant effects on microtubule and actin cytoskeletal proteins, consistent with the genetic interaction studies described above. This latter finding further suggests that cytoskeletal dynamics may be regulated differently in *dfmr1* mutants at different developmental time points. Thus, temporal analysis will add tremendous depth to our knowledge of the dynamic nature of dFMRP function.

Another recent advancement in proteomics is the use of isobaric tag labeling for quantitative mass spectrometry (Li et al. 2007; Unwin 2010). The lack of gel separation in this technique removes any limitations based on protein size or PI range, which are unavoidable with gel-based technologies. The multiplexing of different mass tags allows for a significant number of biological replicates to be analyzed in unison, thus permitting acquisition of statistically significant quantitative data. We have performed this proteomic approach using proteins from *dfmr1* null and control brain synaptosomal preparations to enrich for proteins at the synapse (Fig. 7.3). This fractionation dramatically eliminated the large number of metabolic and chaperone/heat shock proteins that have been isolated by other techniques. Instead, the majority of proteins found to change in abundance in *dfmr1* null synaptosomes are involved in neurotransmission and cytoskeletal dynamics, as parallel studies have predicted. Thus, selecting not just developmental time points, but specific subcellular locations for proteomic analysis is providing more pinpointed information about dFMRP function than classical global analyses have been able to reveal. Unfortunately, there is currently no one silver bullet methodology to achieve a complete picture of the function of dFMRP. Proteomic analysis, while critical for understanding functional changes, will not provide information as to whether affected proteins are directly or indirectly regulated by dFMRP. Likewise, classical global analysis of mRNA levels will also miss this

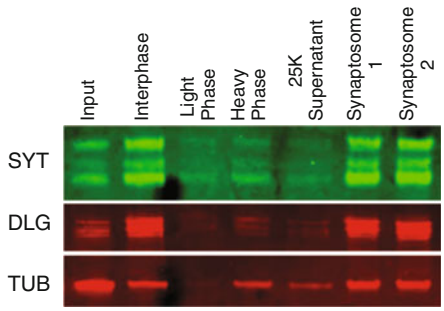

Fig. 7.3 Purification of synaptoneurosomes for proteomic analyses. Head protein extracts were subjected to multiple rounds of high speed centrifugation over sucrose gradients to purify the synaptosomal compartment. Western analysis of each step shows strong enrichment for presynaptic protein synaptotagmin (SYT) and postsynaptic proteins discs large (DLG), but not α-Tubulin. Synaptosomal preparations can be used to increase targeting for synaptic protein changes in proteomic analyses

important distinction. Probing microarrays, or using advanced RNA-Seq techniques with mRNAs that have been purified from dFMRP bound complexes will advance our understanding of direct versus indirect regulations though neither will inform us about the molecular significance of the protein-mRNA association. We will still have to ask, are these mRNAs regulated at the level of translation, stability, or localization? Clearly, combinatorial techniques will need to be applied, and harnessing the power of *Drosophila* genetics to include multiple mutations into these global analyses will greatly enrich our understanding of dFMRP spatial and temporal regulation of mRNA targets.

7.8 Moving Forward

The *Drosophila* FXS disease model has proven to be an extremely powerful system to analyze the molecular players and cellular mechanisms involved in this debilitating disease. While the focus is rightly still on the core player, FMRP itself, genetic analysis is making it increasingly clear that understanding how other proteins cooperate to regulate pathways in common with dFMRP is equally important for our ability to design successful intervention strategies. To this end, exploiting the genetic strengths of the *Drosophila* system will be absolutely critical for advancing future research. Primarily, this means designing and undertaking new forward genetic screens to take advantage of the robust phenotypes in the *Drosophila* FXS model. In addition, we must incorporate new techniques and embrace new understandings of *Drosophila* biology. For example, both the adult brain mushroom body circuit and embryonic motor circuit have been shown to remodel in a developmentally controlled manner in response to neuronal activity (Tessier and Broadie 2008; Tripodi et al. 2008). This is contrary to the dogma that invertebrate circuits are hardwired. Rather, the plasticity exhibited by *Drosophila* circuits closely resembles the plasticity in mammalian systems, and the *Drosophila* FXS model work highlights the need to understand dFMRP function in activity-dependent pathways in critical developmental refinement processes. Furthermore, although the *Drosophila* system is rightly valued for genetic prowess, new advances in electrophysiology and in vivo imaging are providing critical new tools for cellular and physiological analyses in this disease model. It is now possible to record single neuron synaptic responses within intact brains, and to image dynamic physiological responses to stimuli such as light or odor (Gu and O'Dowd 2007; Jayaraman and Laurent 2007; Yu et al. 2006; Zhang et al. 2010). New generation genetically encoded tools such as advanced calcium reporters (e.g. gCAMP3.0), light-activated ion channels (e.g. channelrhodopsin2) and the newly-described potassium selective glutamate channels (HyLighter) can be specifically targeted to neuron subsets and used to directly monitor or drive synaptic function (Janovjak et al. 2010; Lin 2011; Schoenenberger et al. 2011; Tian et al. 2009). In addition, new fluorescent probes can be genetically encoded and targeted to virtually any neural circuit. For example, the DenMARK probe specifically localizes to dendritic regions and allows for

previously unattainable resolution of these densely packed arborizations (Nicolai et al. 2010). All of these tools are available for *Drosophila* FXS model researchers and can be used to probe the intersections between the developmental programs and the activity-dependent mechanisms in which dFMRP is intertwined. Lastly, it is imperative to correlate molecular and genetic analyses with behavioral outputs of targeted circuits. The *Drosophila* system has a long and distinguished history in the study of learning and memory, circadian activity, aggression, courtship or other behavioral paradigms, and applying these assays to the *Drosophila* FXS model will continue to enhance our understanding of the evolutionarily conserved underpinnings of Fragile X Syndrome.

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Chapter 8
Fragile X Mental Retardation Protein and Stem Cells

Abrar Qurashi, Xuekun Li, and Peng Jin

Abstract Stem cells, which can self-renew and produce different cell types, are regulated by both extrinsic signals and intrinsic factors. Fragile X syndrome, one of the most common forms of inherited mental retardation, is caused by the functional loss of fragile X mental retardation protein (FMRP). FMRP is a selective RNA-binding protein that forms a messenger ribonucleoprotein (mRNP) complex that associates with polyribosomes. Recently, the role of Fmrp in stem cell biology has been explored in both *Drosophila* and the mouse. In this chapter, we discuss the role of FMRP in regulating the proliferation and differentiation of stem cells.

8.1 Introduction

Fragile X syndrome, one of the most common forms of inherited mental retardation, is caused by the functional loss of fragile X mental retardation protein (FMRP/Fmrp) (1994). FMRP is an RNA-binding protein that associates with polyribosomes and silences gene expression at synaptic sites (Bassell and Warren 2008). Fmr1 knockout mice exhibit abnormal synaptogenesis, synaptic structures, and function, which were previously observed in fragile X syndrome patients. Fmrp-deficient mice also display learning and memory deficits; however, how the functional deficiency of Fmrp results in learning and memory deficits and the mechanisms of Fmrp-mediated regulation of gene expression are still intensively studied (Edbauer et al. 2010; Li and Jin 2009). Drosophila melanogaster contains a single dFMR1 protein, which shares a high level of homology with FMRP, FXR1P, and FXR2P, and displays similar properties as well as the role with respect to RNA and protein binding (Gao 2002). In addition, as shown for FMRP, as well as its autosomal homologs in mammals, FXR1P and FXR2P, the dFMR1 has been

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found associated with the RNAi silencing machinery (Jin et al. 2004). Flies lacking the dFMR1 protein have been produced in several laboratories that display multiple neuronal defects that are associated with memory formation, eclosion, and circadian rhythms (Zarnescu et al. 2005).

Stem cells, which can self-renew and produce different cell types, are regulated by both extrinsic signals and intrinsic factors (Lin 2002). Neural stem cells (NSCs) have the capability to differentiate into multiple lineages of cells in the central nervous system (Ming and Song 2005). The process of the proliferation of NSCs, their maturation, and the integration of newly generated neurons into existed neural circuitry is termed as neurogenesis. In mammals, neurogenesis has been linked to learning and memory (Zhao et al. 2008). Considering the phenotype of Fragile X syndrome, it would be fascinating to explore whether neurogenesis is altered in the context of FMRP deficiency, thus affecting learning and memory and studies doing just that have been initiated. So far, the results from NSCs from *Drosophila*, mice, and human patients provide a unique window to study the function of FMRP in regulating NSCs and neurogenesis both in vitro and in vivo (Callan et al. 2010; Castren et al. 2005; Luo et al. 2010; Tervonen et al. 2009).

8.2 FMRP in Drosophila Germline Stem Cells

In the *Drosophila* ovary, a very small population of germline stem cells (GSCs) is maintained in a well-defined microenvironment, which provides an attractive system for investigating the regulatory mechanisms that determine the fate of stem cells (Lin 2003; Spradling et al. 2001). A typical *Drosophila* ovary is composed of 16–20 ovarioles. Each ovariole consists of an anterior functional unit, called a germarium, and a linear string of differentiated egg chamber posterior to the germarium. At the tip of the germarium, GSCs normally divide asymmetrically to ensure that one daughter cell remains attached to the niche cells for self-renewal, while the other is displaced from the niche, becoming a cystoblast (CB) that initiates differentiation and sustains oogenesis (Spradling et al. 1997) (Fig. 8.1). Studies from multiple laboratories have identified the genes that are essential for GSC fate determination (Lin 2008; Wong et al. 2005). Recently, the microRNA (miRNA) pathway was also found to be required for controlling GSC self-renewal, since mutations in *Dicer-1*, *Ago1*, and *loquacious*, which are involved in miRNA production and function in *Drosophila*, lead to rapid loss of GSCs (Hatfield et al. 2005; Jin and Xie 2007; Park et al. 2007; Yang et al. 2007a). MiRNAs regulate gene expression through translational repression and mRNA degradation by binding to the 3′ untranslated region (UTR) of their target mRNAs (Plasterk 2006). However, the specific miRNAs required for the regulation of GSC self-renewal and fate specification are yet to be determined.

Recently, we have shown that *dFmr1* is required for both GSC maintenance and repressing differentiation (Yang et al. 2007b). We demonstrated that in *Drosophila* ovary, *dFmr1* protein (dFmrp) interacts with Argonaute protein 1 (AGO1), a key

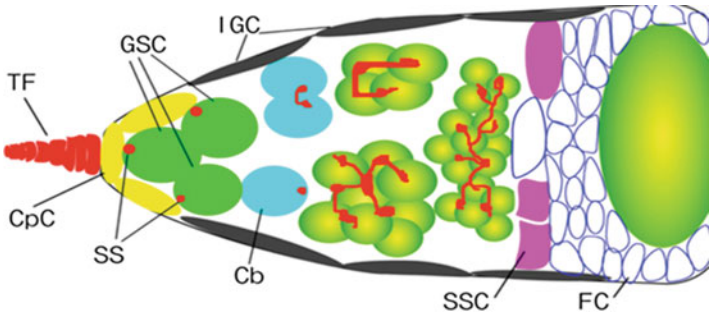

Fig. 8.1 A schematic diagram of a *Drosophila* germarium with different cell types labeled by different colors. *GSCs* germline stem cells, *CB* cystoblast and cysts, *SS* spectrosomes, *TF* terminal filaments , *CPC* cap cells, *IGC* inner germarium sheath cells, *FC* follicle cells, *SSC* somatic stem cells and fusomes

component of the miRNA pathway. Hence, *dFmr1* could modulate the fate of GSCs, likely via the miRNA pathway. Furthermore, we have found that dFmrp is associated with specific miRNAs, such as the bantam miRNA, in *Drosophila* ovary. Like *dFmr1*, the bantam miRNA is not only required for repressing primordial germ cells (PGCs), but also functions as an extrinsic factor for GSC maintenance. We have shown that *bantam* genetically interacts with *dFmr1* to regulate the fate of GSCs. These results support the notion that FMRP-mediated translational control functions through specific miRNA(s) to control stem cell behavior. So it will be important to further explore how *dFmrp* could utilize specific miRNAs to regulate the translation of specific mRNAs and modulate the fate of GSCs.

8.3 FMRP in Drosophila NSCs

Given the role of Fmrp in self-renewal and differentiation of stem cells in the *Drosophila* germline (Costa et al. 2005; Epstein et al. 2009; Yang et al. 2007b, 2009), the role of Fmrp in *Drosophila* larval neuroblasts has also been studied recently. In *Drosophila,* larval brain neuroblasts (NB) undergo asymmetric divisions to produce two daughter cells of distinct size and fate. The larger daughter retains its neuroblast identity and can further divide asymmetrically and self-renew, whereas the smaller daughter, namely the ganglion mother cell (GMC), is committed to the differentiation pathway and divides terminally to produce two neurons or glial cells. Thus, like other stem or progenitor cells, *Drosophila* neuroblasts through repeated self-renewing asymmetric divisions can generate a large number of differentiated cells of the central nervous system (Boone and Doe 2008; Chia et al. 2008; Doe 1992). Depending upon self-renewal/differentiation properties, larval neuroblasts are of two types: type I NB and type II NB. For type I NB that comprises the majority of NB in the larval brain, each GMC divides symmetrically a single time to produce two Ganglion Cells (GC) that each differentiate only into neurons. However, the GMCs in

type II NB that reside mostly on the dorsal–medial side of the brain divide asymmetrically to generate small basal daughter cells called intermediate progenitors (INPs), with each INP generating 6–12 neurons.

Although the role of dFmr1 has been much studied in differentiated neurons, the analysis of this gene in neuroblast has made it possible to uncover its novel aspect in early neurogenesis (Callan et al. 2010). Examination of dfmr1 mutant whole larval brain with variety of cell cycle and neuroblast specific markers have identified significantly more number of mitotic NB in the S and G2/M stages of the cell cycle. The significant increase in the number of mitotic NB latter at the onset of the third larval stages with no obvious defects in asymmetric cell division, suggests an aberrant mitotic control occurring in dFmr1 mutant NB. Developmental studies coupled with live imaging experiments indicate that dFmrp is necessary for correct cell cycle progression in NSCs. These cell cycle defects correspond to NB exiting the quiescence phase prematurely and beginning their proliferative activities sooner than their wild-type counterparts.

The analysis of dFmr1 mutant supranumerary NB has further benefited from using conditional mutations *via* MARCM (Mosaic Analysis with a Repressible Cell Marker) clonal analysis, allowing for the generation of single mutant cells (Lee and Luo 1999). The advantage of knocking out a gene in specific NBs and or/stages is that widespread requirements of dFmr1 throughout brain can be overcome. This also allows the concomitant GFP marking the mutant NB, which enables all the daughter cells produced by a single neuroblast developing larval brain to be traced. Using this approach, it has been shown that *dFmr1* mutant NB have an increased proliferative capacity and generate more number of cells within neuroblast lineages. Although this study was short of analyzing type II NB that generate complex lineages containing both neurons and glia, analysis on dFmr1 mutant type I NB lineages suggest that the supranumerary cells produced differentiate in a neuronal fate in the developing brain, in which they persist through adulthood (Callan et al. 2010). However, these studies demonstrated that the dFmr1 mutation had no effect on the proliferation potential of individual GMCs, which is different from what has been observed in mammalian NSCs (see below). Given the persistence of these supranumerary neurons into adulthood, after the larval brain undergoes a significant amount of neuronal remodeling during morphogenesis, it could be speculated that the well-established synaptic and wiring defects found in adult fragile X fly brains could be the consequence of the dFmr1 deficiency in NB early in development.

8.4 FMRP in Mammalian NSCs

NSCs are multipotent cells that are characterized by their abilities to self-renew and to generate differentiated cells specifically in the central nervous system. Neurogenesis is defined as the process of generating new neurons from NSCs, which consists of the proliferation and fate determination of NSCs, migration and

survival of young neurons, and maturation and integration of newly matured neurons (Ming and Song 2005). Since the discovery of adult neurogenesis, neuroscientists and developmental biologists have been exploring the regulatory mechanisms and functions of this fascinating process. Our current knowledge supports a model whereby adult neurogenesis is regulated by both intrinsic programs and extrinsic modulators. Intrinsic programs include genes, genetic background, and epigenetic modifications that are essential for controlling NSC self-renewal and multipotency (Hsieh and Gage 2004; Li and Jin 2010; Li and Zhao 2008). Extrinsic factors include both the microenvironment where NSCs physically reside and the stimuli that NSCs receive due to endocrinal, physiological, and pathological changes (Zhao et al. 2004).

Previous studies had demonstrated that Fmrp is expressed at the highest level during early murine development stage (Jin and Warren 2000), the peak time point of neurogenesis, suggesting a possible association between Fmrp and neurogenesis. Castren et al. studied the effects of Fmrp loss in embryonic and early postnatal NSCs (eNSCs) in mice (Castren et al. 2005). They found that compared with wild-type littermates, Fmrp-deficient eNSCs displayed increased neuronal differentiation, and the neurons generated from Fmr1 knockout eNSCs had fewer and shorter neurites and smaller cell body size (Castren et al. 2005). Consistent with a previous finding that Fmrp is also expressed in glial cells, Fmrp-deficient eNSCs led to the decreased glial differentiation, which was induced by the increased apoptosis. An electrophysiological study found that Fmrp loss also significantly increased the Ca^{2+} oscillation frequency, which indicated a functional alteration of Ca^{2+} signaling in newly generated cells. However, there was no difference observed in eNSC proliferation (Castren et al. 2005). In line with the findings in mouse, the same effects were also found in eNSCs isolated from human fragile X syndrome fetuses (Castren et al. 2005). These results indicated that FMRP modulates the differentiation of eNSCs and the functional maturation of newly generated cells.

During embryonic and early postnatal development of mice brain, acutely mutated FMRP or Fmr1 knockout resulted in the abnormal development of cortex and more cells clustered in the subventricular zone (Tervonen et al. 2009). An increased density of Tbr2 expressing cells, a marker of glutamatergic neurons was also found, suggesting Fmrp deficiency led to the alteration of cell-type specification (Tervonen et al. 2009). It is worth noting that none of the results indicated the abnormalities of brain anatomic structure in adult Fmr1 knockout mice. These studies indicated that Fmrp regulates brain development during the embryonic period and this function could be development-specific.

Considering the vital difference between embryonic and adult neurogenesis, Luo et al. investigated adult neurogenesis in an Fmrp-deficient context (Luo et al. 2010). They found that consistent with previous studies, Fmrp is highly expressed in adult neural stem cells (aNSCs). BrdU pulse labeling showed that the loss of Fmrp increased the proliferation of aNSCs. Fmrp deficiency also lead to decreased neuronal differentiation, but increased glial differentiation both in vitro and in vivo. Acute knockdown of Fmr1 with siRNAs also produced the same effects. Considering the character of FMRP as an RNA-binding protein, arrays of mRNAs were

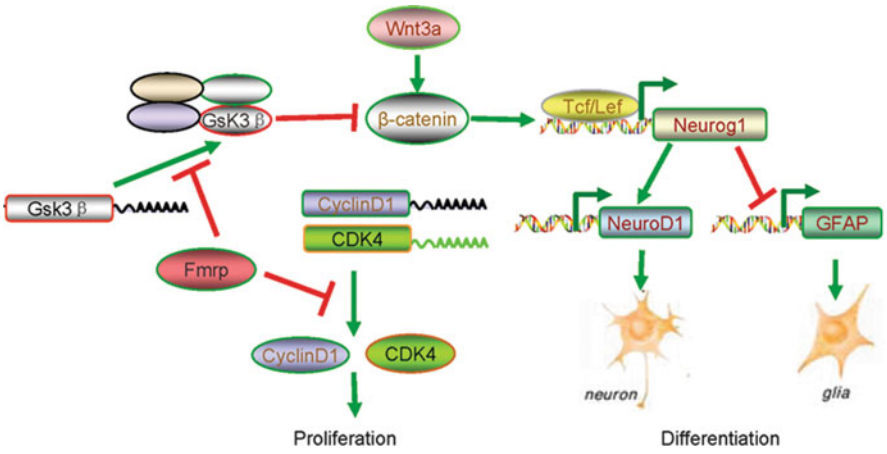

Fig. 8.2 Model of Fmrp functions in adult neurogenesis. By regulating the translation of cyclin D1 and CDK4, Fmrp controls the proliferation of aNPCs. By controlling the translation of GSK3β, Fmrp maintains the proper intracellular levels of β-catenin and Wnt signaling. Upon differentiation, β-catenin positively regulates the expression of Neurog1, which promotes neuronal differentiation and represses glial differentiation

uncovered as the targets of FMRP: CDK4, cyclin D1, and GSK3β, and β-catenin. Furthermore, increased GSK3β and decreased β-catenin in protein levels were also observed. Luciferase reporter assay results showed significantly decreased luciferase activity in Fmr1 knockout aNSCs, which could be partially rescued by inhibiting GSK3β, indicating the involvement of the Wnt signaling pathway. The defective Wnt signaling pathway also led to the decrease of transcriptional factor Neurog1, which is an early initiator of neuronal differentiation and an inhibitor of glial differentiation (Luo et al. 2010). These results demonstrated that in the context of Fmrp loss, the deficits of Wnt pathway underlie the altered differentiation of aNSCs (Fig. 8.2).

Finally, these studies indicated that during neurogenesis, Fmrp not only controls the proliferation of NSCs, but also regulates neuronal and glial differentiation. The alteration of the effects of Fmrp on neurogenesis from embryo to adult suggests Fmrp interacts with different pathways to regulate the proliferation and differentiation of NSCs.

8.5 Summary

Recent studies from both Drosophila and mouse models have suggested a role of Fmrp in stem cell biology, particularly in neurogenesis. Given the neurological phenotypes associated with fragile X syndrome, it will be important to further understand the contribution of altered neurogenesis in the absence of Fmrp to the molecular pathogenesis of fragile X syndrome.

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Chapter 9
Manipulating the Fragile X Mental Retardation Proteins in the Frog

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Abstract The frog is a model of choice to study gene function during early development, since a large number of eggs are easily obtained and rapidly develop external to the mother. This makes it a highly flexible model system in which direct tests of gene function can be investigated by microinjecting RNA antisense reagents. Two members of the Fragile X Related (*FXR*) gene family, namely *xFmr1* and *xFxr1* have been identified in *Xenopus*. While the tissue distribution of their products was found to be identical to that in mammals, the pattern of isoform expression is less complex. Translational silencing of the *xFmr1* and *xFxr1* mRNAs by microinjection of antisense morpholino oligonucleotides (MO) induced dramatic morphological alterations, revealing tissue-specific requirements for each protein during development and in maintaining the steady state levels of a range of transcripts in these tissues.

The power and versatility of the frog model is that the MO-induced phenotypes can be rescued by microinjection of the corresponding MO-insensitive mRNAs. Most importantly, this animal model allows one rapidly to determine whether any member of the *FXR* family can compensate for the absence of another, an approach that cannot be performed in other animal models.

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9.1 Introduction

The Fragile X Related (FXR) gene family has been remarkably conserved during evolution (Fig. 9.1a). This high level of conservation has allowed the development of several animal models and has revealed different facets of the Fragile Mental Retardation Protein (FMRP) and its homologs, Fragile X Related 1 and 2 proteins (FXR1P and FXR2P). Each animal model has its strengths and weaknesses, its supporters and its detractors. Higher organisms have a higher level of complexity of FMRP homologs and isofoms. While mammals express three members of the FXRP family (Zhang et al. 1995), the frog possesses only two, xFmrp and xFxr1p (Blonden et al. 2005; Huot et al. 2005), and the fly has only one, dFmrp (Wan et al. 2000).

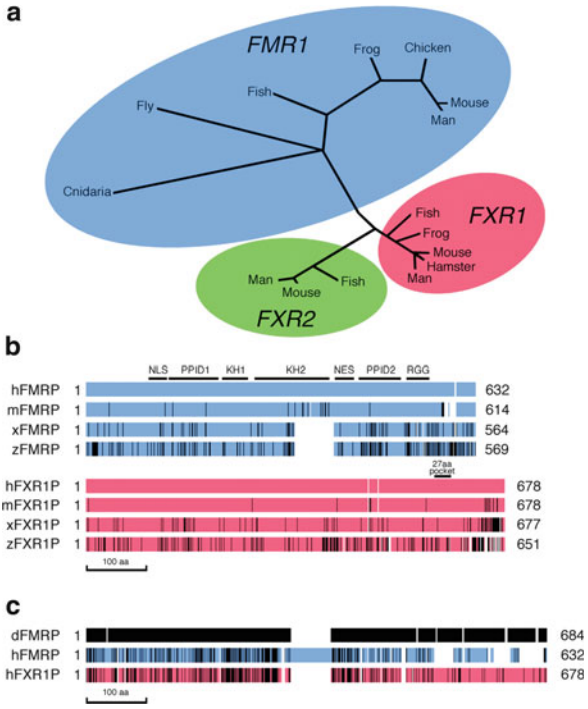

Fig. 9.1 FXR proteins are conserved throughout evolution. (**a**) Phylogenic tree representing the distribution of the known FXR gene family in nature. (**b**) Architectural structure and amino acid alignment between human, mouse, frog and fish in FMRP and FXR1P. Comparisons are based on human FMRP (*blue*) and FXR1P (*red*). Vertical bars in *black* indicate divergent amino acids as compared to human sequences. Note the increased divergence in amino acid back in evolution. The 27 aa pocket found in muscle hFXR1P is conserved from fish to man. (**c**) Amino acids (*in black*) from the fruit fly dFxrp retained in human FMRP and FXRP. Functional domains and their localization in hFMRP are indicated: Nuclear Localization Signal (NLS), Nuclear Export Signal (NES), the RNA-binding domains (RGG box, KH1 and KH2) and the protein–protein interaction domains (PPID1 and PPID2). Note that while these domains have been extensively studied in hFMRP, their presence in the other proteins is inferred by homology

The presence of a single ortholog in the phylum Cnidaria (Guduric-Fuchs et al. 2004), the most primitive living metazoan possessing a nervous system, dating back 600 million years (Greenspan 2007), suggests that these proteins have been conserved to perform a central function specific to the nervous system. This evolutionary convergence of the FXR proteins can be observed by comparing FXR1P and FMRP amino acid sequences from human, mouse, frog and fish (Fig. 9.1b). On the other hand, the fly dFmrp seems to be a hybrid of FMRP and FXR1P and thus appears to be a prototype for both genes (Fig. 9.1c). Overall this range of animal models allows the functional study of FMRP both in the presence and absence of other members of the FXR family. Although none of the existing models alone can explain the Fragile X mental retardation syndrome (FXS), the complementary information they have provided has allowed us to define several causative aspects.

It has been known since the early 1990s that the Fragile X mental retardation syndrome is caused by an irregular expansion of the CGG trinucleotide repeat within the 5′-UTR of the *FMR1* gene. This expansion, and the concomitant hypermethylation of the associated CpG islands, causes the silencing of the gene and results in the abrogation of the expression of the FMRP (O'Donnell and Warren 2002; Bardoni et al. 2006). As no animal models for trinucleotide repeat diseases have been found in nature, a knockout mouse model was developed by selectively inactivating the *Fmr1* gene, generating the first mammal model for FXS (Bakker et al. 1994). This mouse strain exhibits learning and behavioural abnormalities, macroorchidism as well as abnormal neuronal dendritic spines (Irwin et al. 2000; Kooy 2003). These traits essentially recapitulate the phenotype observed in fragile X patients, making it an essential tool to study FMRP's functions in vivo. However, since absence of FMRP impacts embryogenesis and prenatal brain development (Cunningham et al. 2011), mammalian animal models are difficult to handle and do not allow us to easily follow all the steps leading to alterations in brain development. In addition, the high sequence similarity between FMRP and its homologs FXR1P and FXR2P and the formation of heteromers between these proteins (Zhang et al. 1995), raises the problem of functional overlap. This level of complexity increases exponentially if the number of isoforms of each homolog is considered. Indeed in mammals, 6–8 FMRP isoforms, 7 FXR1P isoforms and 2 FXR2P isoforms are observed.

Mouse knockout models have also been developed for *FXR1* (Mientjes et al. 2004) and for *FXR2* (Bontekoe et al. 2002) the two other members of the *FXR* family. The absence of FXR1P has a dramatic impact on muscle development, since the *FXR1* knockout mice die shortly after birth, most likely of heart and respiratory failures. On the other hand, *FXR2* knockout mice show no clear alteration in tissues examined, but subtle behavioral and cognitive deficits were observed, some similar to the *FMR1* knockout model, suggesting that the activity of FXR2P partially overlaps with that of FMRP.

In an effort to better understand the role(s) and function(s) of FMRP, a simpler animal model such as the fruitfly. *Drosophila melanogaster* has attracted the attention of several groups of investigators. Although an extremely elegant and

powerful tool for genetic and developmental studies, this model system suffers from the fact that the three genes *FMR1* and *FXR1* and *FXR2* are essentially all "fused" in one single *dfmr1* gene (Wan et al. 2000). A rapid glance at the sequence alignments of dFmrp with that of hFXR1P and hFMRP (Fig. 9.1c) clearly shows how it becomes impossible to determine which is the true ortholog of dFrmp. Differential tissue expression does not help, since high levels of dFrmp are observed simultaneously in neurons, muscles and gonades in addition to its low level ubiquitous expression in all organs. It is worth noting that although it has been suggested that dFmrp is implicated in translation regulation (Papoulas et al. 2010) no proof has been presented that it is associated with the translation machinery as observed for the mammalian orthologs (Khandjian et al. 2004; Stefani et al. 2004).

9.2 The FXRPs in *Xenopus*

As an alternative to the mouse model, in which the pattern of expression of the FXR proteins is extremely complex, the frog was found to be an attractive vertebrate model. Studies on Fragile-X gene expression in *Xenopus* were simultaneously reported by three groups in 2005. Due to the amino acid conservation between human and frog, several antibodies directed against hFMRP and hFXR1P were successfully used to detect and study the respective frog orthologs. Blonden et al. (2005) reported that xFmrp is expressed ubiquitously in *Xenopus tropicalis* throughout embryogenesis, while a more tissue-specific expression particularly during later development was observed. In adult frogs, xFmrp and xFxr1p proteins are both expressed in most neurons of the central nervous system and in all spermatogenic cells in testis, whereas only xFxr1p is highly expressed in striated muscle tissue.

In parallel, we reported a detailed biochemical study of xFmrp and xFxr1p distribution in *Xenopus laevis* (Huot et al. 2005). Using the mAb1C3 directed against mammalian FMRP, we observed that the *Xenopus* counterpart is less complex than in mammals, as only one single xFmrp band is detected in contrast to the series of 6–8 isoforms present in mammals xFxr1p is also less complex in *Xenopus* since only two isoforms of 82 and 88 kDa are present as opposed to the six mammalian isoforms of 70, 74, 78, 80, 82, and 84 kDa (Fig. 9.2a). This was confirmed at the transcript level using RT-PCR analyses revealing only two distinct mRNAs, as opposed to the seven variant transcripts detected in mouse (Kirkpatrick et al. 1999). The 82 kDa protein is absent in all striated muscles (heart and skeletal muscles) and is replaced by a single 88 kDa protein, that contains a small peptide pocket of 27 aa previously detected in mammalian FXR1P (Khandjian et al. 1998; Dube et al. 2000). It is fascinating that this additional 27 aa pocket has been maintained from fish to man (Khandjian et al. 1998; Engels et al. 2004; Tucker et al. 2004; Huot et al. 2005) with only a very little divergence in fish (Fig. 9.2b), suggesting that it plays a key role in muscle formation and function. Using a specific antibody directed against the 27 aa pocket, we observed, by immunostaining, the

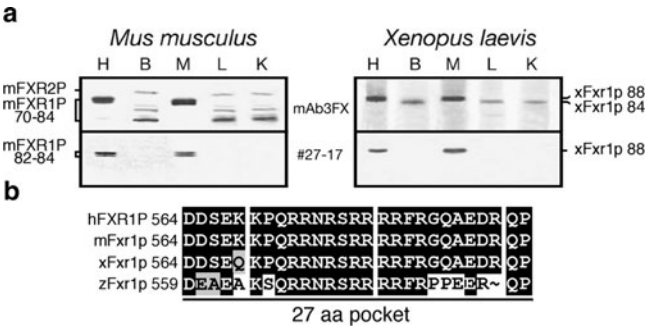

Fig. 9.2 FXR1P display a similar tissue pattern in mouse and frog. (**a**) Distribution of FXR1P in *H* heart, *B* brain, *M* muscle, *L* liver, *K* kidney in *Mus musculus* and *Xenopus laevis* using an antibody directed against an epitope present in all protein isoforms and #27–17 antibody directed against a pocket of 27 amino acid present in the muscle isoform of FXR1P. Six FXR1P isoforms are present in mouse whereas only two bands are detected in frog. (**b**) Sequence homology of the 27 amino acid pocket conserved in the muscle isoform of human, mouse, frog and fish. Adapted from Huot et al. (2005). With permission

exclusive patterns of the longer xFr1p isoform in the frog muscles as is the case for mouse muscles (Fig. 9.3). Finally, Lim et al. (2005) observed that zygotic expression of *xFmr1* began prior to gastrulation and gradually increased during subsequent embryonic stages. Using in situ hybridization, they observed that *xFmr1* transcripts are detected by early tailbud stage in the central nervous system, the eye, and the pharyngeal arches. By late tailbud stage, *xFmr1* expression was shown to be stronger in the CNS and craniofacial regions including the ear vesicle and the eye.

Attempts by two independent groups to detect *xFxr2* in *X. tropicalis* and *X. laevis* have not been successful (Blonden et al. 2005; Huot et al. 2005). It remains puzzling that the frog possesses only the two *xFmr1* and *xFxr1* genes, while all three members were identified in the fish (Tucker et al. 2004).

9.3 *Xenopus* Embryo Manipulation

The frog is a model of choice to study gene function during early development, since large number of eggs are easily obtained and rapidly develop external to the mother. It is a highly flexible model since gene expression can be perturbed by microinjecting antisense morpholino oligonucleotides (MO) in order to directly test the roles and/or functions of the protein of interest (Eisen and Smith 2008; Bill et al. 2009). Most importantly, the strategy to inject one blastomere of the 2-cell stage embryos offers the possibility to directly compare the effects induced on the injected side with the control un-injected side. In addition, different amounts of MO permit one to study the dose-dependent inactivation of the target sequences, allowing a wide range of induced phenotypes to be observed.

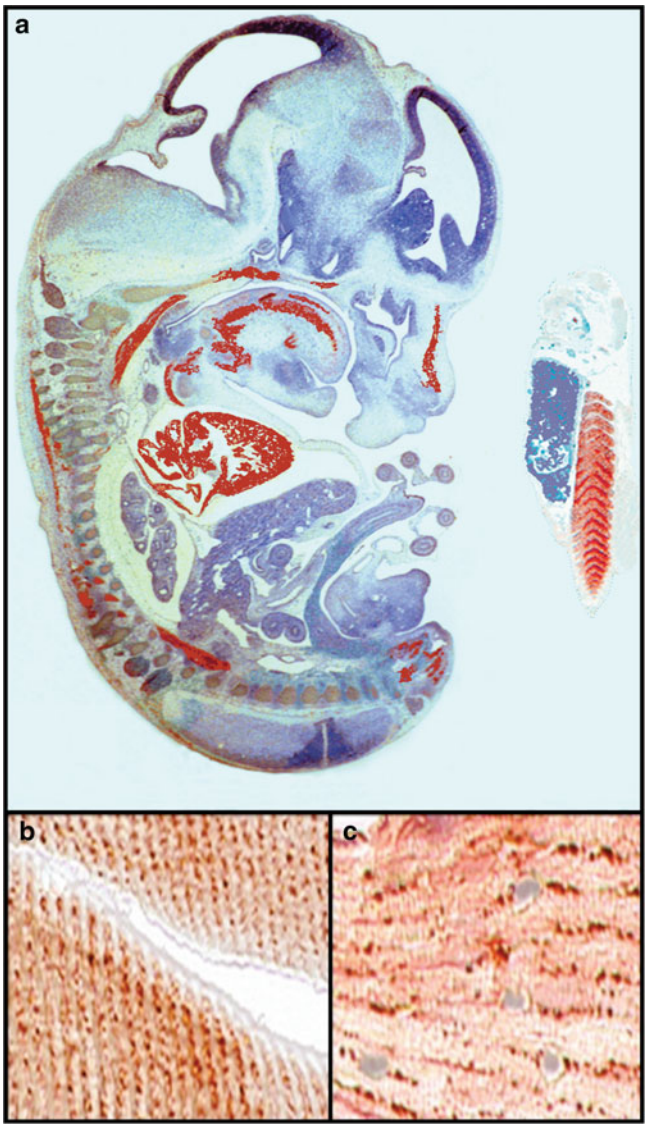

Fig. 9.3 A face to face through evolution. Mouse (*left*) and Xenopus (*right*) embryo sections were immunostained for the muscle specific isoforms. Note the painting of every single muscle in the mouse specimen as well as the chevron-like structure of the muscle in frog typical of swimming animals. Counterstaining with hematoxylin (**a**). *Lower panel*: Higher magnification of the striated muscle showing *dot*-like structures reminiscent of costameres associated with Z bands (**b** mouse and **c** frog). These highly condensed and concentrated foci seem to be structurally conserved throughout evolution. Adapted from Huot et al. (2005)

9.3.1 *Knocking Down* xFxr1 *and* xFmr1 *mRNAs in* Xenopus

In an attempt to circumvent the problems posed by the hypothetical functional redundancy of the FMRP-family and the difficulties, both genetic and biochemical, presented by the mouse system, we identified and characterized the expression of xFxr1p and its requirement in *Xenopus* muscle formation and maintenance (Huot et al. 2005). The data defined xFxr1p as an essential protein in muscle formation and validated *Xenopus* as an ideal model system to determine the biological and molecular functions of xFxr1p.

We used the MO approach to inactivate *xFxr1* mRNA, since these molecules are highly specific, stable and are known for their low level of toxicity when compared to other methods of inactivation (Summerton and Weller 1997). The *xFxr1*-MO was designed to cover the AUG start codon. The strategy was to inject one blastomere of the 2-cell stage embryos, knowing that the first cleavage furrows follows the dorso-ventral axis and determines the left and right sides of the embryo (Klein 1987). Interestingly, phenotypic effects of xFxr1p knockdown were observed only after stage 22, corresponding with the first stages in somitogenesis (Nieuwkoop and Faber 1956).

The observed curled phenotype corresponded with a dose-dependent inhibition or delay in the formation of the somites, proportional to the degree of xFxr1p silencing. Embryos injected with low doses (0.3 pmole) of the *xFxr1*-MO did not show a significant loss of presomitic muscle tissue, but did display an abnormal formation of the somites, implying that a certain minimal level of xFxr1p is required to complete myogenesis. At higher MO doses (1.0 pmole), a dramatic and clear phenotype was induced since all embryos had a tightly curled morphology (Fig. 9.4). Direct proof of the decrease in xFxr1 protein levels in the somites of the injected side is illustrated after immunostaining of a longitudinal section with specific antibodies showing reduced levels of the protein (Fig. 9.5). In addition to the staining of muscle in the unaffected side of the embryo, clear signals were also detected at the level of the head particularly in the eye, heavily decorated in red. In contrast, staining in the injected side was strongly reduced and no signals were observed at the level of the left eye.

The power and versatility of the frog model is that the MO-induced phenotype can be rescued by microinjecting an MO-insensitive muscle specific form of *xFxr1* mRNA synthesized in vitro. Coinjection of this mRNA together with the *xFxr1*-MO resulted in the rescue of ~80% of the injected embryos as their morphology could be restored (Fig. 9.4).

The importance of the presence of the FXRP in eye development was recently confirmed by manipulation studies on *X. laevis*. By silencing either FMR1 or FXR1, after microinjection of the corresponding MO, Gessert et al. (2010) observed abnormal eye development as well as defects in cartilage derived from cranial neural crest cells (Fig. 9.6). These phenotypes could be rescued by microinjection of the wild-type mRNAs. They also observed that the same phenotypes could be provoked by depletion of six defined miRNAs, suggesting a not yet defined role of xFmrp–xFxr1p through an interaction with the miRNA pathway.

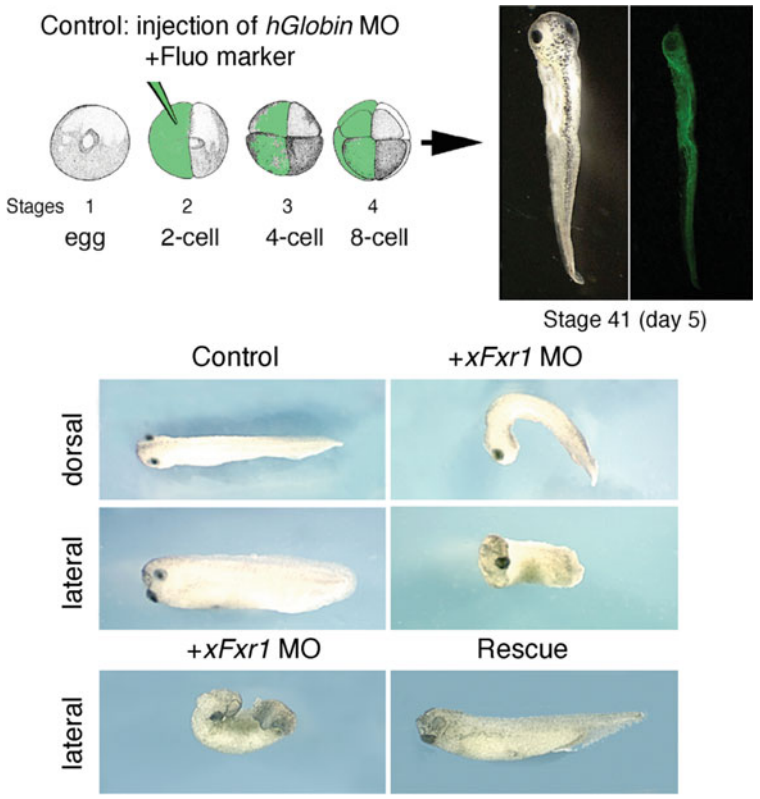

Fig. 9.4 Morphological alterations induced in *Xenopus* embryos after injection of *xFxr1* Morpholino (*xFxr1* MO). Strategy of microinjection of MO in a blastomere of the 2-cell stage embryos. Dorsal views of control embryos showing the diffusion of the fluorescein tagged morpholino directed against the human globin (*hGlob*-MO) injected in the half side of the embryos. Injected embryos with a mismatched *xFxr1* morpholino (Control) shows no apparent phenotype (*left panels*), while injected embryo with *xFxr1* MO shows a curled phenotype (*right panels*). Injection of the wild-type *xFxr1* mRNA was able to rescue the loss of endogenous *xFxr1* expression. For details see Huot et al. (2005)

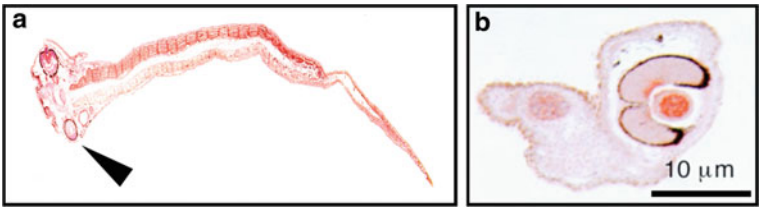

Fig. 9.5 Partial inactivation of *xFxr1* mRNA correlates with decreased Fxr1p levels in the eye. Immunostaining view of a longitudinal section of stage 36 *Xenopus* embryos reacted with antibodies to FXR1P (*left panel*). Note the reduced levels of the staining in the somites and eye (*arrow head*) on the injected side of the embryo (**a**). Immunostaining with antibody to FXR1P showing strong expression in the forming eye (**b**)

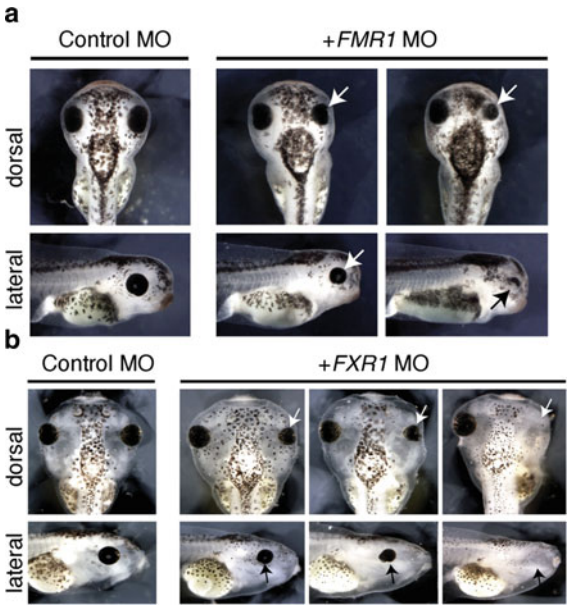

Fig. 9.6 Down regulation of *FMR1* (**a**), and *FXR1* (**b**) result in defects during eye development. For details see Gessert et al. (2010). With permission

9.3.2 *Inactivation of* xFxr1 *mRNA and Alteration in Gene Expression*

To determine whether the absence of xFrx1p has significant impact on the expression of other genes directly or indirectly implicated in muscle formation, we performed microarray analyses and showed that xFxr1p depletion affected the expression of 129 known genes (Huot et al. 2005). Specific transcripts encoding cardiac and striated skeletal muscle proteins such as myosin light chain, tubulin 6, myozenin 1, calcium channel subunit, troponin T, among others, were found to be strongly reduced in the *xFxr1*-MO knockdown embryos. Of the 129 identified mRNAs displaying altered levels, 12% were found to be implicated in muscle formation. This could suggest one of two things, either xFXR1P targets a few key mRNAs essential for a major part of the muscle genetic program, or that most of these mRNAs are directly stabilized by it. Interestingly, while it has been known for some time that FMRP specifically binds to the G-quartet RNA structures (Darnell et al. 2001; Schaeffer et al. 2001), it is only recently that the longest FXR1P isoform, present in muscle, was found also to bind specifically this RNA structure, albeit with a lower affinity as compared to FMRP (Bechara et al. 2007).

Neurospecific mRNAs were also strongly affected by silencing of *xFxr1* mRNA, 71.4% of these showing a drastic downregulation, in particularly mRNAs encoding proteins controlling or implicated in neurite elongation and direction (Huot et al.

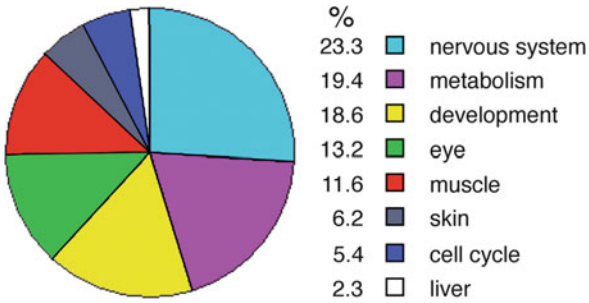

Fig. 9.7 Tissue distribution of the 129 known transcripts affected by the loss of expression of xFxr1p following injection of the *xFxr1* MO. Note that due to overlapping of these transcripts in different tissues the distribution is only relative and cannot be considered as absolute

2005). These results underline the important role that xFxr1p also plays in the control of nervous system development, a neglected aspect, since the 82 kDa form of xFxr1p is strongly expressed in the brain, similar to the 74–80 kDa isoforms in mouse brain. The presence of FXR1P in both the frog and mammal brains suggests a direct role in neuronal function (Fig. 9.7). Interestingly, 13% of the modulated mRNAs were also eye-specific and all showed a downregulation in the knockdown embryos (Huot et al. 2005).

9.4 FMRP in Neurone Granules and FXR1P in Muscle Granules: Same Functions?

In vertebrate muscle, mFXR1P and xFxr1p expression are preferentially detected in granular structures reminiscent of costameres (Khandjian et al. 1998; Dube et al. 2000; Huot et al. 2005) and this distribution is conserved even in zebrafish (Engels et al. 2004). These costamere-like structures have been reported to contain specific mRNAs as well as proteins implicated in muscle contraction and maintenance and are thought to act as a reservoirs of mRNA required for local de novo protein synthesis (Cripe et al. 1993; Morris and Fulton 1994; Fulton and Alftine 1997). It is tempting to draw a parallel between FMRP and FXR1P functions in two very different but large and highly polarized cells such as neurons and muscle cells. In neurons, FMRP is thought to maintain certain classes of mRNAs in a repressed state during their transport in motile granules through neurites to their destination, the synapse (De Diego et al. 2002; Mazroui et al. 2002; Antar et al. 2004; Khandjian et al. 2009). These cargoes are then targeted and anchored to dentritic spines or filopodia, which are the sites where FMRP may regulate synthesis of proteins essential for spine development and maintenance (Miyashiro et al. 2003; Antar et al. 2004; Weiler et al. 2004). Similar to FMRP in neurons, FXR1P may play an equivalent role in muscle to maintain silent mRNAs in costamere structures until

protein synthesis is required. We proposed that in neurons, FMRP is not essential for their maintenance, since the homologs FXR1P and FXR2P, which colocalize in the same repressed granules (De Diego et al. 2002; Davidovic et al. 2005), can partially compensate for the absence of FMRP in fragile X syndrome. In contrast, as FXR1P is the sole member of the FXR protein family to be found in muscle (Khandjian et al. 1998; Bakker et al. 2000), its presence, as seen in the *Xenopus* experimental system, is essential for muscle development and maintenance. Given that xFxr1p silencing leads to downregulation of many muscle-specific mRNAs, our data strongly suggest that as an RNA-binding protein, it might control specific mRNAs that are translated into proteins essential for muscle differentiation. Alternatively, xFxr1p might control a restricted number of mRNAs upstream of a complex cascade leading to the alteration of the steady state levels of a large number of downstream transcripts.

9.5 Interrelational Functions of the FXRPs?

In vertebrates, the high sequence similarity between FMRP and the FXR1 and FXR2 proteins raises the problem of functional overlap between the three proteins. Indeed, it has been proposed that the homologs FXR1P and FXR2P can compensate, though incompletely, for the absence of FMRP in the molecular mechanisms altered in Fragile X Syndrome (Khandjian et al. 1996; Darnell et al. 2009). Indeed, Coffee et al. (2010) recently reported that in the *dfmr1* drosophila null mutant, only *hFMR1* can fully rescue the molecular and cellular defects in neurons. On the other hand, *hFMR1*, *hFXR1*, or *hFXR2* all can rescue mutant fecundity and spermatogenesis defects.

One of the most striking manipulations in the frog model is the possibility to test whether knocking down the expression of a given protein that induces a phenotype can be compensated or restored by the expression of another protein. In the precise situation of knocking down one of the members of the FXRP family, will the other members restore the missing function(s)? We have asked the simple question of whether FMRP or FXR2P could rescue the curled phenotype of *Xenopus* embryos induced by knocking down *xFxr1p*. In other words, would FMRP or FXR2P compensate for the absence of xFxr1p? We first found that human FXR1P, like xFxr1p (Fig. 9.4), rescues xFxr1p knockdown, demonstrating the functional conservation between human and frog (Fig. 9.8a). Rescue of wild-type anatomy in *xFxr1* knockdown embryos was then also scored after coinjecting mRNA encoding the full-length HA-tagged *hFXR2* and *hFMR1* mRNAs. Consistent with a strong degree of functional redundancy between all the FXRP family members, hFXR2P and even hFMRP were able to rescue *xFxr1* knockdown to a significant degree (Fig. 9.8a). These results show: (1) that hFXR1P is as efficient as xFxr1p in rescuing almost 80% of the embryos; (2) hFMRP results in 45% rescue; (3) hFXR2P induces nearly 60% rescue. Such astonishing results were also independently obtained recently by Gessert et al. (2010) who were able to reverse the eye

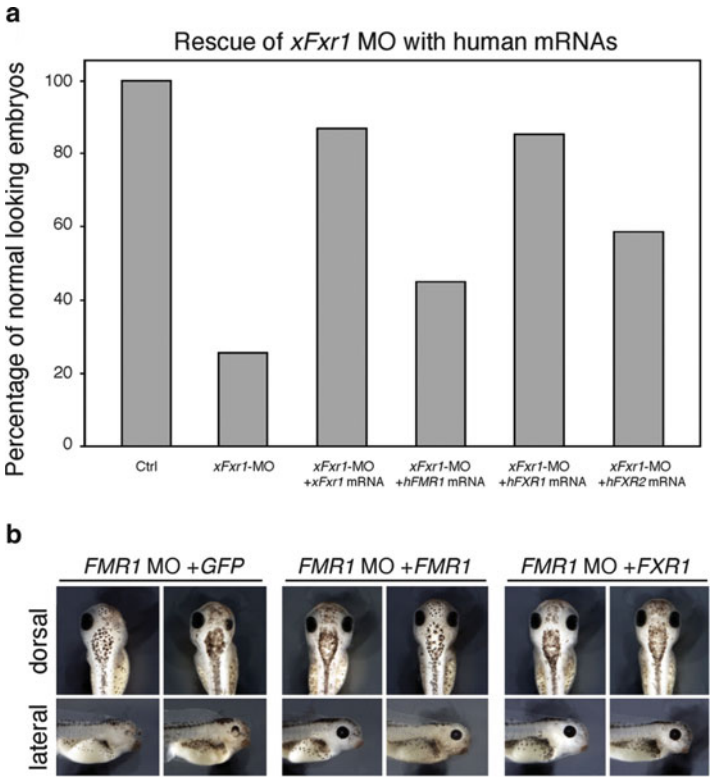

Fig. 9.8 *xFXR1* inactivation can be rescued by other members of the FXR protein family, showing the functional redundancy of all the members of this protein family. (**a**) The *FXR* family share very similar activities. The "curled" phenotype depicted in Fig. 9.4 after the injection of *xFxr1* MO can be efficiently rescued by exogenic mRNA coding for human FXR1P, but also to a significant degree by hFXR2P and even hFMRP. (**b**) Coinjection of an *FMR1* MO together with *FMR1* or *FXR1* mRNAs led to a rescue of the *FMR1* MO-induced eye phenotype. For details see Gessert et al. (2010). With permission

phenotype induced by knocking down *xFxr1* by reintroducing MO-resistant mRNA of either *xFmr1* or *xFxr1* (Fig. 9.8b).

9.6 Concluding Remarks

Using the frog as a model to manipulate the steady state levels of xFmrp and xFxr1p, it is possible to observe specific effects resulting from the modulation of these proteins. While reducing the levels of xFxr1p had effects on muscle development and formation, unexpected phenotypes in other tissues were also provoked, the most striking and visible being at the level of the head. Malformation, if not

absence, of the eye was directly linked to the levels of both xFxr1p and xFmrp. It is worth noting that altered scores in visuospatial tasks are observed in the *Fmr1* knockout mouse in a FVB/N-129 hybrid background was previously attributed to the genetic background of the FVB/N strain which carries retinal degeneration and/or albinism (Dobkin et al. 2000). In view of the results obtained by Gessert et al. (2010), one might ask whether problems of vision in the original *Fmr1* KO C57BL/6J mouse are not also affected as a direct result of FMR1P loss.

One of the most remarkable results obtained with the frog model is that it was possible for the first time to answer the question whether other members of the FXRP family can compensate for the absence of FMRP as hypothesized earlier (Khandjian et al. 1996). As we show, the muscle phenotype induced by knocking down *xFxr1p* can be rescued by complementation with *hFMR1* or *hFXR2* mRNAs. Similarly, the eye phenotype induced by knockdown of *xFmr1p* can be rescued by *xFxr1p* and vice versa. Such cases clearly demonstrate a shared functionality between the FXR proteins. None of these striking results could have been obtained without the availability of the frog model. Studies on the FXR proteins in frogs may yet hold further answers to the perplexing questions surrounding the function(s) of this protein family.

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Chapter 10
Exploring the Zebra Finch *Taeniopygia guttata* as a Novel Animal Model for the Speech–Language Deficit of Fragile X Syndrome

Claudia Winograd and Stephanie Ceman

Abstract Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and presents with markedly atypical speech–language, likely due to impaired vocal learning. Although current models have been useful for studies of some aspects of FXS, zebra finch is the only tractable lab model for vocal learning. The neural circuits for vocal learning in the zebra finch have clear relationships to the pathways in the human brain that may be affected in FXS. Further, finch vocal learning may be quantified using software designed specifically for this purpose. Knockdown of the zebra finch FMR1 gene may ultimately enable novel tests of therapies that are modality-specific, using drugs or even social strategies, to ameliorate deficits in vocal development and function. In this chapter, we describe the utility of the zebra finch model and present a hypothesis for the role of FMRP in the developing neural circuitry for vocalization.

10.1 Introduction to FXS and the Vocal Phenotype

Fragile X syndrome (FXS) is a genetic disease that results in a constellation of features, the most salient of which include intellectual disability and impaired speech and language. FXS is the most common cause of inherited intellectual disability, affecting 1:4,000 males and 1:8,000 females panethnically [reviewed

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in Hagerman et al. (2009)], and 50–90% of children with FXS have speech and language abnormalities such as perseveration and echolalia (Hagerman and Lampe 1999; Kau et al. 2002; Roberts et al. 2007). FXS results from absent expression of the normal *f*ragile X *m*ental *r*etardation *p*rotein (Pieretti et al. 1991; De Boulle et al. 1993) (FMRP; Fmrp in mice and rats; Fmrp in other species; for simplicity it will be written as FMRP hereafter), encoded by the gene FMR1. The FMR1 gene is expressed ubiquitously in the body, excluding the muscles, and it is especially prominent in the testes and brain (Bachner et al. 1993; Devys et al. 1993; Hergersberg et al. 1995); in the brain the protein is primarily neuronal, expressed in glia only during development (Devys et al. 1993; Wang et al. 2004; Pacey and Doering 2007). Within neurons it has been observed in both dendrites (Feng et al. 1997; Weiler et al. 1997; Greenough et al. 2001; Antar et al. 2004; Ling et al. 2004; Antar et al. 2005) and axons (Antar et al. 2006; Price et al. 2006; Tessier and Broadie 2008).

Both speech and language are affected in FXS; our hypothesis is that these features are due to impaired speech and language learning. Before describing the speech and language impairments of FXS, collectively termed for our purposes as vocalization deficits, it is important to define speech and language. Speech is the learned sensorimotor control of vocal movements and sounds while language utilizes speech or signals to communicate a complex meaning, encompassing the cognitive processes required for this communication [reviewed in Brainard and Doupe (2002)]. Verbal language includes vocabulary and grammar, while speech refers to the verbal production of language in terms of pronunciation and rhythm [reviewed in Newbury and Monaco (2010)].

One feature of FXS is delayed onset of vocalization. Before children use words to communicate, they use "prelinguistic" communication such as gestures and coordinated gaze, which are typically supplemented by spoken words by 12 months of age [reviewed in Finestack et al. (2009)]. Children with FXS have delayed onset of this supplementation, with prolonged use of prelinguistic tools (Brady et al. 2006). In fact, vocal delays are found in 69% of children with FXS, based on parental reports (Ferrando-Lucas et al. 2003).

With regard to FXS speech, it has been shown to be perseverative (perseveration is the repetition of one's own words or actions) (Ferrier et al. 1991) and less intelligible than typically developing (TD) peers (Barnes et al. 2009); intelligibility was even worse when FXS boys presented with comorbid autism (Kover and Abbeduto 2010). Interestingly, boys with FXS were shown to have phonological skills similar to TD boys who were of a younger age (Barnes et al. 2009). Additionally, children with FXS are perceived to speak more quickly than their TD peers; however, they actually do not (Zajac et al. 2006). This study was expanded recently to show that the articulation rate, as measured in syllables per second, is the same in FXS as in age-matched controls, but due to speech tone and rhythmicity characteristics (prosody) in children with FXS, their speech is perceived as faster (Zajac et al. 2009).

With regard to language, children with FXS have impaired receptive language capability, as measured by language comprehension (Price et al. 2007); comorbid autism presents with a further decreased receptive language capability (Lewis et al. 2006). Children with FXS also have impaired expressive language

(Ke et al. 2005). This expressive language capability, as judged by expressive vocabulary, is not worsened by comorbid autism (Kover and Abbeduto 2010). Syntax, which is the grammatical arrangement of words in a sentence, is a further measure of expressive language capability. Children with FXS have impaired syntactic skills, as measured by the complexity of their phrases, compared to TD peers and to age-matched children with non-specific intellectual disability. In contrast, children with FXS have stronger syntactic skills than children with Down syndrome (Price et al. 2008). Finally, syntactic ability does not depend on comorbidity with autism (Kover and Abbeduto 2010).

An important additional feature of vocalization is pragmatics, which includes the "social norms" of communication, both nonverbal and verbal, such as eye contact and turn-taking [reviewed in Noveck and Reboul (2008)]. Pragmatic function in FXS is impaired in ways related to, but not identical to, autism (Abbeduto et al. 2008; Dalton et al. 2008).

The reduced intelligibility of speech in FXS individuals has been attributed to an impaired oral-motor system (Barnes et al. 2006). The generalized hypotonia, joint laxity, and orofacial hypotonicity observed in FXS individuals may play a role in their unintelligible speech (Hodge 1991; Hagerman et al. 1996; Hagerman and Lampe 1999). In fact, FXS boys scored lower than typically developing boys on oral structure – particularly with regard to lip structure – and some oral function tasks (using lips, tongue, and velopharynx), as patients performed less well on speech function tasks like repeating single and multiple syllable words (Barnes et al. 2006). In addition, other studies suggest that the FXS speech problems indicate higher-level motor encoding problems of linguistic information rather than peripheral articulatory deficits (Vilkman et al. 1988; Hodge 1991; Hagerman et al. 1996). In addition, sensorimotor delays have been observed in FXS children as young as 9–12 months of age (Bailey et al. 2003; Grace et al. 2005), as well as both fine and gross motor skills (Ke et al. 2005; Zingerevich et al. 2009).

Importantly, the vocal deficits are not due to cognitive impairment in general, but rather are unique to FXS, since they have been described in comparison to children with Down syndrome or idiopathic intellectual disability (Sudhalter et al. 1990; Ferrier et al. 1991; Belser and Sudhalter 2001). While the speech–language deficits of FXS have been extensively established and characterized, as outlined above, in the realm of molecular biology, this phenotypic vocal quality of FXS is often overlooked. This paucity of studies is likely because to date there has not been a tractable model organism for the study of the atypical vocalizations.

Humans, songbirds, and certain cetaceans are vocal learners – so-called because they have a sensitive period during postnatal (or posthatch) development in which they must hear the adult vocalizations as well as their own, in order to learn this vocalization (Doupe and Kuhl 1999; Brainard and Doupe 2002; Wilbrecht and Nottebohm 2003). The atypical vocalization in FXS provides a unique opportunity to study the role of FMRP in a novel venue – vocal learning. Because FMRP is involved in learning and memory (Mercaldo et al. 2009), and humans are vocal learners, our hypothesis is that individuals with FXS have impaired vocal learning, leading to their impaired vocalization.

10.2 The Songbird as a Model for Vocal Learning

The fragile X gene ortholog has been identified in a number of species and its knockdown has been induced in the mouse, fruit fly *Drosophila melanogaster*, zebra fish *Danio rerio*, and the cricket *Gryllus bimaculatus* (Consortium 1994; Zhang et al. 2001; Lin et al. 2006; Hamada et al. 2009); however, none of these species are vocal learners. Adult male mice and altricial mouse pups do emit ultrasonic vocalization, and a study has already been performed on murine FoxP2, an important speech gene mutated in a human familial speech disorder (Lai et al. 2001), showing a deleterious effect of a FoxP2 mutation on ultrasonic vocalization of mouse pups (Shu et al. 2005). Nonetheless, it is unclear if these murine vocalizations are learned (Sales 1972; Branchi et al. 2001; Holy and Guo 2005). Further, the mouse does not lend itself to our study because the particular brain regions involved in ultrasonic song have not been mapped. Additionally, the cricket model does not lend itself to our study because the deficit is likely purely motor and not due to a learning deficit in the realm of communication (Hamada et al. 2009). Currently, a model organism with a well-characterized neural circuit for vocalization is the songbird, specifically the zebra finch *Taeniopygia guttata*. In addition to their well-studied anatomy, songbirds have been proposed as invaluable animal models for studying the acquisition of a skilled motor sequence (Vu et al. 1994), as well as a behavioral sequence (Fee et al. 2004). For these reasons, and for its easy management in a laboratory setting, the zebra finch is an excellent animal model for studying the role of FMRP in vocal learning, thereby elucidating the biology of the vocal impairments observed in FXS.

Songbirds and humans are vocal learners. This type of vocalization is different from that of other avian species such as the chicken, for example, which when raised in isolation can still make the proper vocalizations as adults [reviewed in Doupe and Kuhl (1999)]. Researchers of birdsong have identified three stages in its learning and production – sensory, sensorimotor, and the final, crystallized song [reviewed in Brainard and Doupe (2002)].

In the sensory period, a "song template" is formed as the young bird listens to his tutor and learns the tutor's song. This sensory period of the zebra finch spans the first 60 days after hatching. During the sensorimotor period, from about posthatch day 23 (P23) through sexual maturity (approximately P90), the male bird begins to vocalize and to correct his song using auditory feedback, in order to match the tutor. At adulthood, the bird has an established, "crystallized" song, which he will continue to sing for the remainder of his life. A feature of vocal learning is practice. The first vocalizations of a songbird (at about P35) are called subsong, akin to the babbling of a human infant. Human babbling begins at about month seven, followed by the first true word spoken at about 1 year of age, with continued vocal learning that diminishes markedly after sexual maturity (Doupe and Kuhl 1999).

In addition to singing, the bird must be able to hear itself in order to crystallize its song properly, as must a human child hear him or herself in order to learn to speak properly [reviewed in Doupe and Kuhl (1999)]. A deaf songbird cannot learn proper

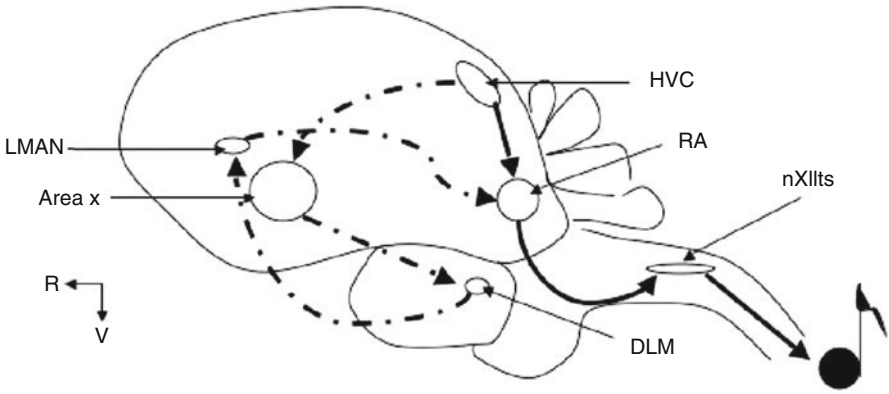

Fig. 10.1 Map of the zebra finch "song circuit". Schematic of sagital view of an adult male zebra finch. Anterior Forebrain Pathway for song learning shown in dashed lines; Posterior Pathway for song production shown in solid lines [Rostral (R) left; Ventral (V) down. Not drawn to scale]. *Song nuclei-Area X* letter-based name, *DLM* DorsoLateral Medial nucleus of the thalamus, *HVC* letter-based name, *LMAN* Lateral Magnocellular nucleus of the Anterior Nidopallium, *nXIIts* Nucleus TrachioSyringealis of cranial nerve XII, *RA* Robust nucleus of the Arcopallium

song (Marler and Waser 1977); similarly, hearing-impaired children cannot, without interventional training, learn proper adult vocalization (Ching et al. 2010).

Song learning and production utilizes many avian brain regions (called "song nuclei"); the five principle song nuclei comprise two merging pathways (Fig. 10.1). The Anterior Forebrain Pathway (primarily for song learning) begins with nucleus HVC (letter-based name; formerly High Vocal Center), projecting to Area X then DLM, LMAN, and finally the RA. The Posterior Pathway (primarily for song production) also begins in the HVC, projecting directly to the RA. The RA then projects to the nucleus of the tracheosyringial region of the 12th cranial nerve (nXIIts) (songbirds have a dual-barreled voicebox, called a syrinx, comparable to the single-barreled human larynx). Each of these nuclei plays a role in song learning and/or production. For a summary of the zebra finch nuclei and the analogous mammalian brain regions, please see Table 10.1 and Reiner et al. (2004a, b).

The Anterior Forebrain Pathway and the Posterior Pathway both begin with the HVC. Behavioral learning (i.e., vocal learning) in the HVC requires rapid synaptic plasticity at this sensorimotor nucleus in response to an instructive experience (Roberts et al. 2010). It is important to note that here, the term sensorimotor is used to denote the integration of sensory (such as visual, auditory, and proprioception) input and motor (such as song) output, not that the HVC is strictly involved in the sensorimotor (as opposed to sensory) phase of song learning.

Considering the Anterior Forebrain Pathway first, HVC projects to Area X, a nucleus considered to be the avian analog of the "direct" striatopallidothalamic pathway of the mammalian basal ganglia (Medina and Reiner 1995; Farries and Perkel 2002). Area X contains spiny neurons that respond to dopamine, thereby influencing song learning and maintenance (Ding et al. 2003). Furthermore, these

Table 10.1 Analogous brain regions in zebra finch and mammals

Zebra finch nuclei		Mammalian brain region
Abbreviation	Full name	
HVC (letter-based name)	Formerly High Vocal Center	Dorsal telencephalon[a]
Area X	Area X	Basal ganglia
DLM	DorsoLateral Medial nucleus of the thalamus	Thalamus
LMAN	Lateral Magnocellular nucleus of Anterior Nidopallium	Dorsal telencephalon[a]
RA	Robust nucleus of the Arcopallium	Unique to avians but best described as vocal premotor cortex, similar to (but distinct from) the somatic telencephalon

[a]Derived from the telencephalic pallial sector of the developing brain (mammalian pallial derivatives are the neocortex, amygdala, and claustrum) (Reiner et al. 2004a, b)

spiny neurons express FOXP2 during song learning, and new neuron recruitment is increased during this period (Rochefort et al. 2007). Of note, lentivirally-driven knockdown of FoxP2 in Area X results in incomplete, inaccurate song learning (Haesler et al. 2007). Neurons in Area X project to the song nucleus DLM (DorsoLateral Medial nucleus of the thalamus) via the neurotransmitter GABA (Luo and Perkel 1999).

DLM neurons are the avian equivalent of mammalian thalamocortical neurons. This alignment is due to both the strong inhibitory GABAergic input from Area X as well as their intrinsic properties (Luo and Perkel 2002). The Anterior Forebrain Pathway continues through DLM to LMAN (Lateral Magnocellular nucleus of the Anterior Nidopallium) via glutamatergic projections onto NMDA receptors as well as AMPA receptors on inhibitory interneurons that then use GABA to inhibit LMAN (Livingston and Mooney 1997); plasticity related to these receptors has been shown to be involved in song learning (Bottjer 2005). Interestingly, the NMDA receptors in LMAN decrease at the synapse between P32 and 40 (corresponding to the young finch's maturation from fledgling to juvenile), suggesting that these synapses are important for sensory, not sensorimotor learning (Livingston and Mooney 1997). LMAN itself remains important in residual plasticity and song maintenance during adulthood (Brainard and Doupe 2001). Finally, neurons in LMAN project to the RA (Robust nucleus of the Arcopallium), a connection that has been shown to be critical for motor learning (Ölveczky et al. 2005).

The RA is the nucleus, which intersects the Anterior Forebrain Pathway and the Posterior Pathway (Fig. 10.1). It is critical for song production and is analogous to mammalian premotor cortex (Nottebohm et al. 1976). The RA receives input from HVC and LMAN by approximately P30 (just preceding the onset of sensorimotor learning) (Konishi and Akutagawa 1985; Mooney 1992; Mooney and Rao 1994), consolidates these inputs, and projects to the brainstem motor nucleus controlling song, nXIIts (tracheo-syringial nucleus of cranial nerve XII). Plasticity at RA has been proposed to play different roles temporally during song learning (Stark and

Perkel 1999) [For a model for the overall long term plasticity at the HVC-RA connections (via both pathways), see: Fiete et al. (2007)]. It is also interesting to note that feedback from the RA to the HVC has been observed, via intermediate song nuclei likely to be involved in the auditory pathway [reviewed in Margoliash (1997)]. In addition, "bottom-up" feedback from the respiratory brainstem to RA and HVC during adult singing has also been observed (Ashmore et al. 2005). The complexities of the song circuit are fodder for elucidating studies into the role of FMRP in vocal learning.

10.3 FMRP Expression in the Song Circuit

Synaptic plasticity, known to be abnormal in FXS [reviewed in Bear et al. (2004)], plays a role at each connection between the nuclei in the song circuit; therefore, we hypothesized that FMRP plays a role in modulation of synaptic plasticity at one or more song nuclei, as part of a greater role in song learning. To test this hypothesis, we set out to examine the zebra finch song circuit for FMRP expression during development and thereby song learning. We found FMRP expressed in HVC, LMAN, Area X, and RA; expression was neuronal and primarily cytoplasmic just as in other species (Winograd et al. 2008) (Fig. 10.2). The DLM is a heavily myelinated nucleus and we were unable to achieve adequate immunofluorescence in this region; therefore, we cannot make conclusions about the expression of FMRP in this thalamic brain region; FMRP expression in nXIIts was not examined. Intriguingly, out of these four song nuclei, we observed elevated FMRP expression in the RA, as compared to surrounding neuropil, an effect that was consistent at P30 (Winograd et al. 2008) (Fig. 10.3).

RA receives inputs from both the nuclei HVC and LMAN as well as from intrinsic interneurons and therefore is not merely a relay nucleus from the HVC to the motor nucleus nXIIts (Spiro et al. 1999 and see Fig. 10.1). Synaptic plasticity in the RA has been proposed as a mechanism for song learning and production (Mooney 1992; Stark and Perkel 1999; Fee et al. 2004), and FMRP is necessary for normal synaptic plasticity [reviewed in Bear et al. (2004)]. It is an exciting prospect that FMRP might be involved in the synaptic maturation of the RA that is associated with song learning.

A likely setting for the facilitation of this maturation is in the postsynaptic compartment of dendritic spines, where FMRP has been shown to play a role in synaptic plasticity in other species (Weiler and Greenough 1999; Greenough et al. 2001; Michel et al. 2004). Specifically, it is possible that this role is via the NMDA receptors. Alterations in RA neurochemistry, such as NMDA receptor subunit expression, occur during song learning (Wang and Hessler 2006). In fact, FMRP has recently been shown to modulate expression of NMDA receptors (Eadie et al. 2010; Edbauer et al. 2010).

To definitively test the role of FMRP in vocalization would entail knocking down FMRP in the zebra finch brain and observing any effects on song learning.

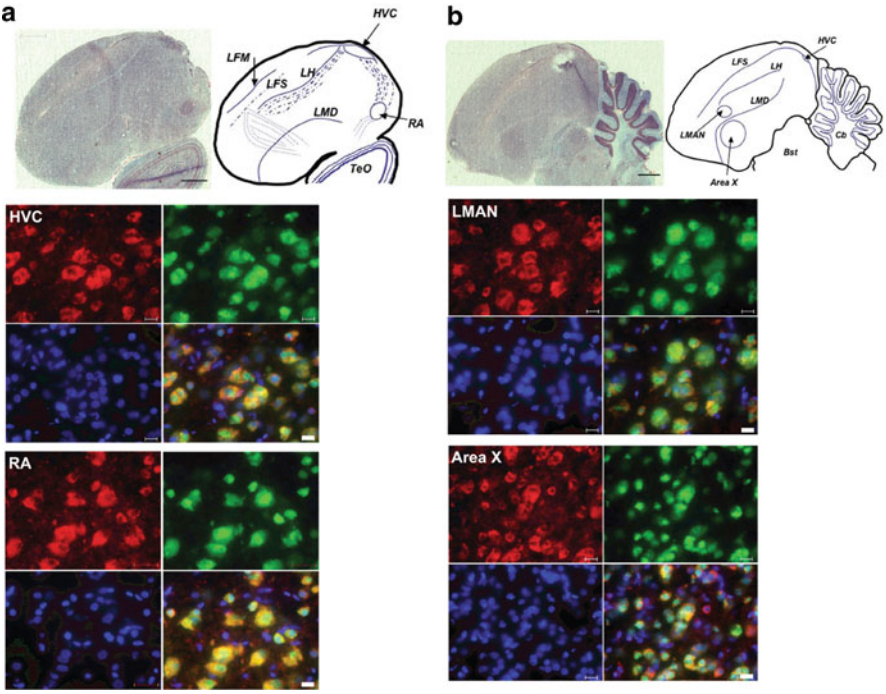

Fig. 10.2 FMRP is expressed in neurons in the male zebra finch brain. Sagital brain sections containing (**a**) HVC (letter-based name) and RA and (**b**) LMAN (Lateral Magnocellular nucleus of the Anterior Nidopallium) and Area X were stained for anatomy with cresyl violet (*bar* = 1,000 μm) (*upper left*). Upper right is an accompanying sketch with the significant anatomical features indicated. Adjacent sections were co-stained with the zebra finch-specific Fmrp antibody 24 (*red*), the neuronal marker NeuN (*green*), and DAPI (*blue*). In the overlay, a *yellow* signal indicates co-fluorescence for *red* and *green*. *Bar* = 20 μm. *Bst* Brainstem, *Cb* Cerebellum, *LFM* Lamina frontalis suprema, *LFS* Lamina frontalis superior, *LH* Lamina hyperstriatica, *LMD* Lamina medullaris dorsalis, *TeO* Optic Tectum. Shown are images from a posthatch day (P) P30 brain; P60 and Adult males showed similar results (data not shown) [Reprinted with permission from Winograd et al. (2008)]

In the zebra finch, gene knockout at the germline level, as accomplished with mouse and fly, is currently not feasible; therefore, epigenetic means such as silencing RNA must be employed. A future experiment would therefore be to use a viral vector to silence the gene encoding zebra finch FMRP in a young finch prior to the onset of song learning, and analyze the resultant song.

10.4 Feasibility of Knockdown Strategy

Researchers have used the zebra finch and its well-characterized song system to study the transcription factor FoxP2 (Haesler et al. 2004; Scharff and Haesler 2005; Schulz et al. 2010). In a human family with a FOXP2 mutation, affected family

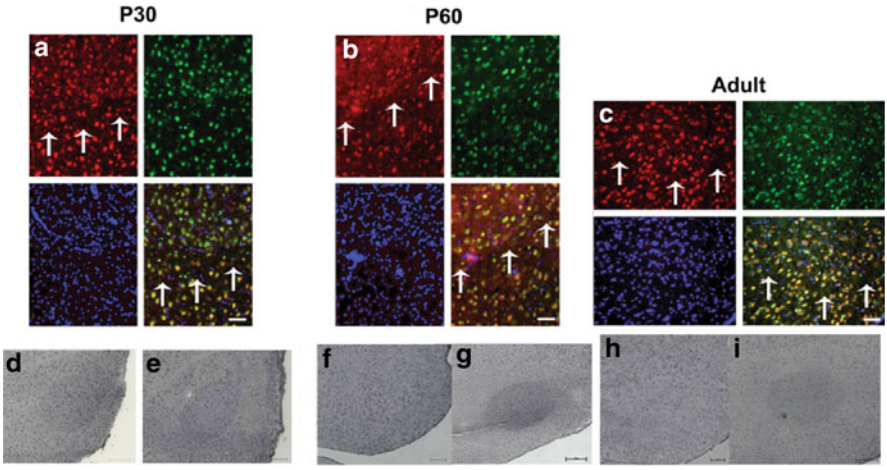

Fig. 10.3 FMRP is consistently elevated in the RA nucleus of a P30 male zebra finch and variably expressed in P60 and Adult males. (**a**–**c**) Representative fluorescent-immunohistochemistry using an antibody specific to zebra finch FMRP on a male P30 (**a**) P60 (**b**) and Adult (**c**) zebra finch RA. Shown are FMRP immunoreactivity (*red*), NeuN stain (*green*), and DAPI-labeled nuclei (*blue*), along with the overlay. *Arrows* denote ventral border of RA. *Bar* = 100 μm. (**d**–**i**) Representative DAB-IHC using anti-zebra finch FMRP antibody on a male P30 (**d**, **e**) P60 (**f**, **g**) and Adult (**h**, **i**) zebra finch RA. *Bar* = 200 μm [Reprinted with permission from Winograd et al. (2008)]

members present with developmental verbal dyspraxia – difficulty with coordinated motor tasks, specifically in the lower face and jaw such that speech is impaired (Lai et al. 2001). There are also language processing impairments and significantly-below-average grammar skills (Vargha-Khadem et al. 1995). In the finch, FoxP2 is expressed ubiquitously in the brain, and principally in the striatum, and its knock-down in the zebra finch Area X via lentivirus-delivered short-hairpin silencing RNA results in imperfect imitation by a male zebra finch of his tutor's song (Haesler et al. 2007). It is important to consider the contrast between the murine study where knockdown of FoxP2 did not affect ultrasonic vocalization, and this avian study, where it did. It is conceivable that certain proteins such as FoxP2 are required for vocal learning but not necessarily the production of innate vocalizations such as murine ultrasonic or nonlearned avian calls. This specified role is also likely with FMRP, as people with FXS are capable of vocalization.

10.5 Strength of Songbirds: Ability to Measure Song Learning

As described above, the charge of the young male zebra finch is to learn the song of the adult male tutor. Zebra finch song consists of a set of notes or syllables – the frequency of which can be measured in kilohertz over time (milliseconds). Thus, a song syllable is defined as a continuous, morphologically discrete trace on a song

spectrogram (Sossinka and Bhoner 1980). The adult song of the zebra finch male begins with several variations of the same introductory syllable, followed by a set of dissimilar syllables. The latter syllables are rendered in a stereotyped sequential order and constitute the "motif". A motif lasts approximately 700 ms (Sossinka and Bhoner 1980), with frequencies ranging from 0.5 to 0.8 kHz (Scharff and Nottebohm 1991). Each adult male song is unique based on the nature of the syllables and their specific assembly into a motif. Thus, each zebra finch song is readily quantifiable. In order to do that, software (SA+) was designed to analyze how well a young male finch learns his tutor's song (Tchernichovski et al. 2000). SA+ software is able to capture and analyze song at all stages of learning and compare it to the tutor; the program identifies individual syllables (notes) and analyzes their temporal structure, characterizing duration, mean pitch, and mean frequency modulation. The software automatically generates and updates a syllable-table for each bird that summarizes every song syllable produced during vocal development. Thus, the song of the adult tutor can be specifically compared to the emerging song of the juvenile male.

10.6 What We Could Hope to Learn from a Knockdown Zebra Finch

Like FoxP2, FMRP is expressed throughout the brain; however, the role of this latter protein in a functional CNS circuit has not been investigated. The zebra finch provides a well-characterized functional CNS circuit for study in its song system.

As described in 10.2, individuals with FXS have developmental delays with sensorimotor deficits (Grace et al. 2005). In addition, a good animal model for the characterization of vocalization deficits is lacking (10.3). Numerous studies in rodent models expressing short hairpin RNAs from viruses have been successful in creating knockdown models (Xia et al. 2004; Bohn 2005; Harper et al. 2005; Ralph et al. 2005; Raoul et al. 2005; Rodriguez-Lebron et al. 2005; Singer et al. 2005; Sapru et al. 2006). We expect that inducing loss of FMRP by viral-driven expression of short hairpin RNAs in the RA would have an effect on the ability to accurately learn the tutor song, since FMRP is elevated in the neuropil of RA (Fig. 10.3). We can imagine at least three possible outcomes of knocking down FMRP expression in RA: (1) the knockdown bird has the same phenotype as the RA-ablated bird where the song is lost (Nottebohm et al. 1976); (2) the knockdown bird is unable to produce normal song due to problems with motor production; (3) the knockdown bird is able to produce song but is unable to *learn* the tutor song.

If the first scenario is true and the knockdown bird is unable to sing, then we would suspect that FMRP is required for vocalization. This result would be surprising because although FXS patients have significant speech delays and deficits, vocalization is not absent. Thus, in zebra finch, FMRP would appear to have a critical role in song production. This is a distinct possibility because in humans and rodents, loss of

FMRP expression is sometimes compensated for by the other autosomal paralogs FXR1 and FXR2 (Siomi et al. 1995; Spencer et al. 2006). In fact, elimination of both FMRP and FXR2 was required to see a circadian defect in mice (Spencer et al. 2006). Although zebra finches express FXR1, they do not express FXR2 (unpublished results), thus, elimination of FMRP in the zebra finch may reveal defects not observed in other species.

The second possible outcome that we can envision is that knockdown of FMRP leads to problems with motor production. We would suspect that this is the case if the features of the song syllables are abnormal in the FMRP knockdown birds compared to those in the control birds. If we find that there are problems with motor production (versus motor learning), then adults should also be affected. We could test this prediction by introducing FMRP silencing viruses and control viruses into adult RA and then analyzing song production before and after virus introduction. If FMRP is required for producing song at any age, then we would expect altered song in adults injected with the silencing virus compared to control virus.

The third possible outcome, which is our hypothesized one, is that knockdown of FMRP expression affects motor learning and that in the absence of FMRP, the manipulated birds are unable to learn their tutor's song. If FMRP is required for the introduction of variability by LMAN into the HVC-driven template song (Kao et al. 2005; Ölveczky et al. 2005), then we would expect early stereotypy, that is, the song would crystallize before attaining similarity to the adult song. Thus, the mean similarity scores and accuracy scores of the analyzed bird song would be significantly different between the FMRP silenced birds and the control birds. This phenotype would be similar to that observed when LMAN was ablated (Scharff and Nottebohm 1991). If the song stereotypes too soon, we would also expect to observe no change in song in the FMRP knockdown birds between P65 and adulthood. Further, the P65 song should not be normal – that is, it should not be similar to the tutor song but should resemble immature song (Scharff and Nottebohm 1991).

Alternatively, if the inability to learn song is due to a defect in motor learning because FMRP is required for reinforcing motor actions, then we would expect no normal stereotypy, that is, the song would continue to change during adulthood. In this case, we would expect the song spectrograms of the knockdown pupils to change continually, where either syllables would be omitted or the duration of the syllable would be imprecisely copied between renditions. This would be reflected in differences in the mean similarity and accuracy scores between knockdown and control groups. Further, an individual's own song would significantly vary from rendition to rendition, which would be reflected in the coefficient of variation.

10.7 Conclusion

In summary, FMRP is a protein involved in learning and memory, specifically in the realm of synaptic plasticity. People with FXS have impaired vocalization, which we propose is due to impaired vocal learning. The songbird zebra finch

provides an exciting animal model for the study of the role of FMRP in vocal learning. We suggest a role for FMRP in the reorganization of the postsynaptic dendritic compartment in the song nucleus RA, possibly through effects on NMDA receptors. It is our hope that knowledge gained on the role of FMRP in song learning can be translated into therapeutics for FXS patients with impaired vocalization due to the absence of expression of FMRP. A knockdown zebra finch would be a useful model for the study of novel interventive approaches because song learning and production can be precisely quantified.

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Chapter 11
Neuroendocrine Alterations in the Fragile X Mouse

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Abstract The expression of $GABA_A$ receptors in the fragile X mouse brain is significantly downregulated. We additionally found that the expression of somatostatin and voltage-sensitive calcium channels (VSCCs) is also reduced. $GABA_A$ and the VSCCs, through a synergistic interaction, perform a critical role in mediating activity-dependent developmental processes. In the developing brain, GABA is excitatory and its actions are mediated through $GABA_A$ receptors.

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Subsequent to GABA-mediated depolarization, the VSCCs are activated and intracellular calcium is increased, which mediates gene transcription and other cellular events. GABAergic excitation mediated through $GABA_A$ receptors and the subsequent activation of the VSCCs are critically important for the establishment of neuronal connectivity within immature neuronal networks. Data from our laboratories suggest that there is a dysregulation of axonal pathfinding during development in the fragile X mouse brain and that this is likely due to a dysregulation of the synergistic interactions of GABA and VSCC. Thus, we hypothesize that the altered expression of these critical channels in the early stages of brain development leads to altered activity-dependent gene expression that may potentially lead to the developmental delay characteristic of the fragile X syndrome.

11.1 Introduction

Histogenesis of brain structures is an articulated sequence of events, involving neurogenesis, migration, axonal growth, dendritic growth, synapse formation, and myelination. This complex process is directed by the genetic program that stems from cellular positional identity and is influenced by neurotransmitters, neuropeptides, hormones, and other modulators. Neuropeptide involvement in the ontogeny of neuronal structures is implied by their early presence within the embryonic brain, which suggests a different role from the classical neurotransmitter or neuromodulator function usually attributed to these molecules. These molecules could act as guidance cues or trophic factors. The transient expression of neuropeptides during early development is seen in various areas of the mammalian brain, for example, in the cerebral cortex (Naus et al. 1988), as well as in the visual (Bodenant et al. 1991), somatosensory (Katz et al. 1992), and auditory systems (Takatsuki et al. 1981, 1982; Kungel and Friauf 1995). Similarly, hormones of either maternal or embryonic origin could influence brain development through their genomic or non-genomic effects. Thus, alterations in the expression of any of these molecules or their receptors could have long-lasting effects on brain development that could subsequently be manifested by developmental delay. In this chapter, we discuss how the alteration of VSCC expression and the GABAergic system in the fragile X mouse brain could lead to developmental delay and alterations in brain function. The fragile X syndrome is a developmental disorder characterized by hyperarousal, hypersensitivity to sensory stimuli, and an increased prevalence of seizures (Wisniewski et al. 1991; Hagerman 2002). The mouse model for this disorder (Bakker et al. 1994) also has increased seizure susceptibility (Musumeci et al. 2000; Chen and Toth 2001; Yan et al. 2004) and may directly parallel elements of the syndrome that predict reduced inhibition/increased excitability. Our investigations of the molecular basis of increased seizure susceptibility in the fragile X mouse demonstrated a reduction in $GABA_A$ receptor expression (El Idrissi et al. 2005). Since these receptors play a major role in cellular inhibition,

their reduction may explain the increased seizure susceptibility of this mouse model for fragile X and suggests that the GABAergic system may be affected in the fragile X syndrome. Furthermore, we showed that the expression of somatostatin (SST) is significantly decreased in the brain of fragile X and this reduced expression of SST is consistent with both central and peripheral features of the fragile X syndrome. Additionally, we found that the VSCCs, involved in exocytosis of hormones, neurotransmitters, and neuropeptides, are drastically reduced in the fragile X mouse brain (El Idrissi et al. 2010). These molecules are ubiquitously expressed in the brain and peripheral organs, and yet their expression is selective and enriched within various brain regions and in the periphery. Thus, their altered expression would have brain region- and organ-specific functional significance. For example, the altered startle response of fragile X mice could be a result of reduced SST expression since SST is transiently expressed in many brain regions during development and influences the maturation of sensorimotor information processing (Kung et al. 1996). Similarly, in the periphery, reduced SST expression in the intestine would result in increased intestinal motility, as this peptide is responsible for inhibiting intestinal motility (Hansen et al. 2000).

In addition to the GABAergic and SST alterations observed in the fragile X mouse, we found a global decrease in the expression of the VSCCs (El Idrissi et al. 2010). Calcium acts as both a charge carrier and a second messenger, and plays a pervasive role within neurons directly or indirectly regulating nearly all the reactions within cells (Augustine et al. 2003). Many physiological processes such as vesicular exocytosis, neuronal excitability, plasticity, and gene transcriptional regulation (Cao 2006) are related to Ca^{2+} signaling events occurring at various sites within cells (Miller 2001). In neurons, Ca^{2+} influx through glutamate receptors and VSCCs provides the major source of Ca^{2+} used for signaling events (Bloodgood and Sabatini 2007). Thus, reduced expression of the VSCCs in the fragile X mouse brain and periphery would obstruct calcium-dependent processes, including those activated during early brain development, which may lead to the developmental delay observed in the fragile X brain. In the following sections, we will discuss the current states of knowledge of GABAergic and somatostatinergic systems, as well as calcium channel changes, with relationship to fragile X syndrome.

11.2 Alterations in the GABAergic System

One of the features of both the human syndrome and the fragile X mouse model is a reduced seizure threshold. To investigate the brain alterations responsible for the increased seizure susceptibility of the fragile X mouse, we examined the expression of the $GABA_A$ receptor, the major inhibitory receptor in the brain. Analysis of the expression of the $GABA_A$ receptor by Western blot and immunohistochemistry showed that $GABA_A$ levels were reduced in the cortex, hippocampus, diencephalon, and brainstem in adult male fragile X mice (El Idrissi et al. 2005; D'Hulst et al. 2009). A reduction in $GABA_A$ receptors would reduce GABAergic inhibition and

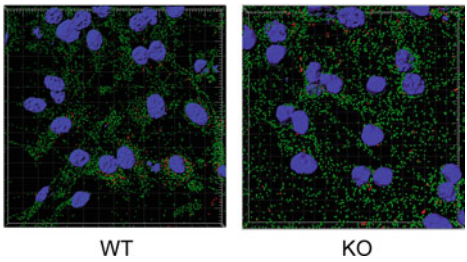

Fig. 11.1 Representative images of $GABA_A$ and VSCCs immunoreactivity in cerebellar granule cells (CGC) in vitro. Dissociated cells were obtained from P7 pups and cultured for 5 days in vitro. Images were obtained using a confocal microscope and z'ed stacks were reconstructed using Imaris software. CGC from controls show significantly higher number of puncta, indicating an increased level of expression of both $GABA_A$ (*green*) and VSCCs (*red*) compared to CGC obtained from *Fmr1* KO mice. All gains and offset were kept constant during image acquisition, and threshold values for each were identical during post-acquisition processing

thus increase excitability. This probably contributes to the increased seizure susceptibility in these animals. We also found reduced $GABA_A$ receptor expression in cerebellar granule cells in vitro (Fig. 11.1), indicating the early onset of GABAergic deficiency in the fragile X mouse brain. This suggests that the alterations in the GABAergic system are cell autonomous and are the result of absent FMRP. We also found increased glutamic acid decarboxylase (GAD) expression in the same brain regions that showed reduced $GABA_A$ receptor expression. GAD is responsible for GABA synthesis in GABAergic neurons. GAD has two isoforms, 65 and 67 KDa (GAD65 and GAD67), encoded by different genes (Erlander et al. 1991). The expression of both isoforms is activity dependent (Babb et al. 1989; Szabo et al. 2000; Nishimura et al. 2001) and influenced by the effectiveness of GABAergic inhibition (Ribak et al. 1988, 1993). We hypothesize that the observed increase in GAD expression is a compensatory mechanism that is activated in response to the reduced expression of the $GABA_A$ receptor in the fragile X mouse brain.

In addition to its role as an inhibitory neurotransmitter in the mammalian brain, GABA plays an important role in the developing nervous system. During early brain development, GABA is expressed transiently in various non-GABAergic cells, in addition to GABAergic neurons (Van Eden et al. 1989; Ma et al. 1992). In vitro investigations of dissociated or cultured cells have shown that GABA affects cell proliferation (LoTurco et al. 1995), cell migration (Behar et al. 1996, 1998), cell survival (Ikeda et al. 1997; Obata 1997), neurite extension (Barbin et al. 1993), and synapse formation (Spoerri 1988).

The ontogeny of membrane properties of excitable cells revealed a unique interaction between GABA and voltage-sensitive sodium and calcium channels. At very early developmental stages, functional $GABA_A$ receptors are expressed in neurons. Activation of $GABA_A$ receptors depolarizes neuroblasts and immature neurons in all brain regions examined, and this transient excitatory action of GABA represents a general feature of the developing brain. This excitatory action of GABA is due to

the elevated intracellular chloride concentration. Activation of $GABA_A$ receptors results in membrane depolarization and activation of voltage-sensitive sodium and calcium channels. Since the GABAergic synaptic connections precede the onset of glutamatergic synapses (Ben-Ari et al. 1989; Hosokawa et al. 1994; Durand et al. 1996), establishment of immature neuronal networks is critically dependent on GABAergic excitation.

Another fundamental feature of developing brain structures is the synchronized neuronal activity, known as giant depolarizing potentials (GDPs) that are triggered by intracellular calcium oscillations. These GDPs have been reported in a wide range of neuronal circuits in both the central and peripheral nervous systems (Ben-Ari et al. 1989; Christie et al. 1989; Wong et al. 1993; Yuste et al. 1995; Feller et al. 1996; Mooney et al. 1996). GDPs are mediated by $GABA_A$ receptors and blocked by bicuculline (Ben-Ari et al. 1989; Xie and Smart 1991; Strata et al. 1995, 1997). Therefore, $GABA_A$ receptors and VSCCs play a critical synergistic role in the ontogeny of functional connectivity within neuronal circuits. The deficiency in the GABAergic system observed in the fragile X mouse brain would have long-term effects on neuronal function spanning brain development and extending into adulthood. We hypothesize that the reduced expression of the $GABA_A$ receptors in early brain development leads to delayed maturation of neuronal networks, leading to an overall developmental delay. Interestingly, we found that the VSCCs are also affected in the fragile X mouse brain (see below). These channels are critically involved in the initiation of $GABA_A$ receptor-mediated GDP and calcium oscillations early in development.

11.3 Alteration in the Somatonergic System

The somatostatin (also known as somatotroph release inhibiting factor) neuropeptide family comprises peptides arising from different posttranslational processing of the 116 amino acid prepro-somatostatin precursor. Only two biologically active SST isoforms have been identified so far: the tetradecapeptide (SST-14) and the amino-terminally extended octacosapeptide (SST-28) (the entire SST-14 sequence is present in the C-terminus of SST-28). Both SST-14 and SST-28 are found in the peripheral and central nervous systems, with SST-14 being the predominant isoform. The relative proportions of the two isoforms differ among various SST-producing tissues; however, SST-14 and SST-28 display overlapping physiological functions (Krantic et al. 2004). We show here that the expression of SST is significantly decreased in the brain of the fragile X mouse (Fig. 11.2). The reduced expression of SST in the fragile X mouse is not limited to the brain but is also observed in the Leydig cells in the mucosa of the intestine, in the testes, and in the islets of Langerhans within the pancreas (Fig. 11.2). One of the anatomical features of fragile X syndrome is enlarged testicular volume. Interestingly, we found that fragile X testes show a decrease in the number of Leydig and sustentacular cells (Fig. 11.2). Since these cells have an endocrine and paracrine function through their

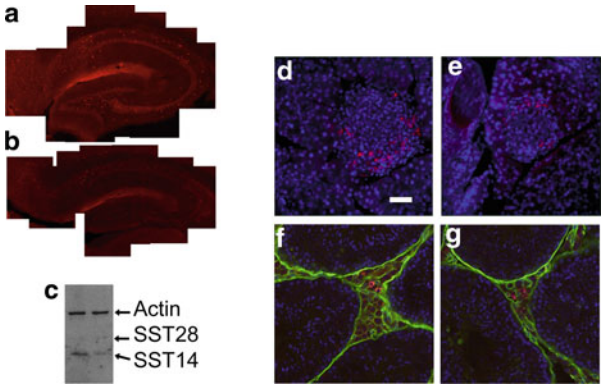

Fig. 11.2 SST expression is reduced in the *Fmr1* KO mouse brain and periphery: Montage images obtained with an epifluorescence microscope equipped with digital camera (×20 objective) from a 30-μm-thick sagittal brain cryosection of the hippocampal formation from WT (**a**) and KO (**b**) showing SST IR. SST-positive neurons were found throughout the hippocampal formation and intense immunoreactivity was present in the hilar and subicular regions of WT hippocampus. KO mouse brain had reduced immunoreactivity for SST and almost lack it in the hilus. (**c**) Western blot of SST and β-actin expression in the cortex of 2-month-old male mice (*Left lane*: WT, *right lane*: KO). The Western blot was probed simultaneously with a rabbit polyclonal antibody that recognized SST (SST28 and SST14) and a monoclonal antibody that recognized β-actin. SST 14 expression level was significantly reduced in the *Fmr1* KO mice (*right lane*). (**d** and **e**) SST IR in the islets of Langerhans. Representative confocal images of 30-μm-thick pancreatic sections. Intense IR and increase in the number of SST-positive δ-cells are seen in the WT islets (**d**) compared to that in KO (**e**). Also, note the reduced islet size typical of KO islets. (**f**) and (**g**) are representative images of SST IR in the testes of WT and KO, respectively. KO testes have reduced SST IR, and reduced number of Leydig and sustentacular cells between the seminiferous tubules. All mice were two months old. *Bar*: 50 μm

steroidogenic capacity, their reduction would add to the endocrine deficiencies observed in the fragile X mouse.

11.4 Reduced Number of SST-Positive Interneurons in the Brain Fragile X Mouse

To facilitate the study of GABAergic inhibitory interneurons, we used a transgenic mouse line that selectively expresses the enhanced green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons. In these mice, an upstream regulatory region from the murine *Gad1* gene (Oliva et al. 2000), which codes for the 67-kDa form of the GABA synthesizing enzyme, GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which served to validate the biochemical observations and allowed us to determine the type of neuro-architectural changes (such as changes

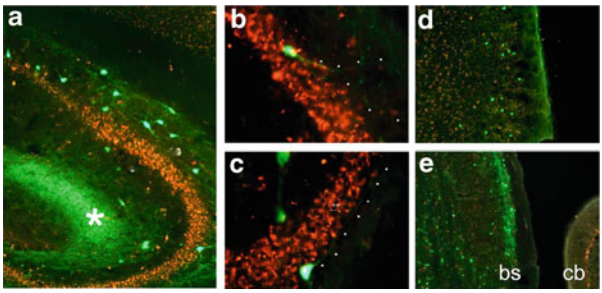

Fig. 11.3 Pattern of EGFP expression found in the brain of 2 months old EGFP transgenic mice. (**a**) Low magnification image of the hippocampus, asterisk denotes the plexus of EGFP-expressing (white) axonal terminals in stratum lacunosum-moleculare (SLM) of area CA3. Cells in stratum oriens (SO) of CA1 (**b**), and area CA3 (**c**), show processes that can be followed for a long distance from the cell body. (*Dots* delineate processes from single cells.) (**d**) Photomicrograph illustrating the pattern of EGFP expression in the somatosensory cortex, which is typical of all cortical areas. The laminar specificity of EGFP expression in the primary cortex is illustrated in this 30-μm-thick section: EGFP-expressing somata are restricted mainly to layers II–IV and upper layer V. (e) Pattern of EGFP expression in the brainstem (BS) and cerebellum (CB). At two months old, the cerebellum does not show any EGFP-positive neurons. Strata abbreviations: *SO* stratum oriens, *SP* stratum pyramidale, *SR* stratum radiatum, *SLM* stratum lacunosum-moleculare, *SM* stratum moleculare, *SG* stratum granulosum, *H* hilus of the dentate gyrus, *SL* stratum lucidum, *GL* internal granule cell layer, *P* Purkinje cell layer, *ML* external molecular layer. Magnifications: A, D, and E ×10; B and C ×40 dry objective. These are images of a 30-μm-thick sagittal brain section from a homozygous mouse. It should be noted that the EGFP fluorescence shown in these images is intrinsic fluorescence and not the product of a fluorophore-labeled antibody immunoreaction. All sections were stained with a red fluorescent Nissl stain (Molecular Probes) to facilitate visualization of neuronal structures. Magnifications: A, D, and E ×10; B and C ×40 dry objective

in the number of inhibitory interneurons or changes in their dendritic morphology) that occur in the fragile X mouse brain. In brain sections prepared from these mice, EGFP appeared to diffuse freely throughout the cytoplasm of expressing neurons (Fig. 11.3). In fixed preparations, the overwhelming majority of EGFP-expressing neurons were very intensely fluorescent, making their processes readily visible and traceable to their terminals. In most instances, neuronal processes could be visualized and followed for hundreds of micrometers from their parent soma. In these mice, induction of hippocampal and cortical EGFP expression was found to begin at approximately postnatal day 5 (Oliva et al. 2000).

In their initial characterization of these transgenic mice, Oliva et al. (2000) have shown that the expression of EGFP in these mice is developmentally regulated and temporally coincides with the terminal differentiation of GABAergic interneurons and the onset of expression of many of the macromolecules that delineate subpopulations of mature GABAergic neurons (Naus et al. 1988; Bergmann et al. 1991; Jiang and Swann 1997). We used fluorescence immunohistochemistry for numerous markers of GABAergic interneurons and found that somatostatin expression was significantly reduced in fragile X mouse brains compared to control mice (Fig. 11.4). Thus, in the fragile X mouse brain, there is a decrease not only in SST expression but also in the number of SST-positive interneurons. While the reduced

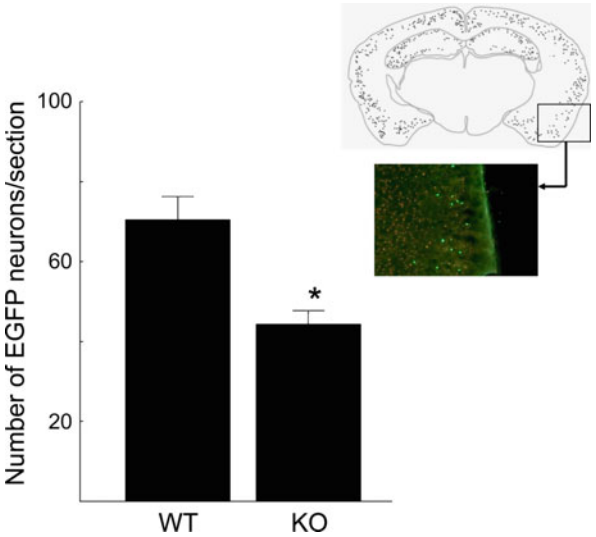

Fig. 11.4 Laminar specificity of EGFP expression shown in primary cortex from a 30-μm-thick section. This expression pattern typifies the one seen in all cortical areas. EGFP-expressing somata are restricted mainly to layers II–IV and upper layer V. Magnifications ×10 objective. Histogram represents a comparison of EGFP expression in WT controls and *Fmr1* KO mice. *Bars* represent the mean ± SD of EGFP-positive neurons obtained from four WT controls and four *Fmr1* KO mice ($n \approx 400$, 100 sections per brain). The decrease in the number of EGFP-positive neurons in the *Fmr1* KO mice was significant ($^*p < 0.05$)

expression of SST could be due to transcriptional or translational events that would suppress or reduce SST gene expression or translation, the reduced number of SST-positive neurons in the fragile X brain suggests an alternative mechanism whereby misregulation of neurogenesis in this population of neurons leads to an overall reduced number of SST-positive inhibitory interneurons.

11.5 Functional Significance for Decreased Somatostatin Expression in Fragile X Mouse

11.5.1 SST Is Important for Pancreatic Remodeling

SST was originally discovered in the 1970s during the search for hypothalamic growth hormone (GH)-releasing factor as a hypothalamic hormone that inhibits growth hormone secretion. SST localization was described in the central and peripheral nervous systems, gastrointestinal tract, pancreas, and immune and cardiovascular systems. Besides its GH-inhibiting activity, SST also inhibits the secretion of other hormones such as thyroid-stimulating hormone (TSH) and most

gastro-enteropancreatic hormones (i.e., gastrin/cholecystokinin, insulin, glucagon, and vasoactive intestinal polypeptide), as well as exocrine gastrointestinal and pancreatic secretions. SST activates five distinct G-protein coupled receptors, which are widely expressed by mammalian cells and show region- and tissue-specific expression patterns. Thus, reduced SST expression should produce tissue-specific effects. To investigate this hypothesis, we examined the functional significance of reduced SST in the pancreas of fragile X mice. In the pancreas, SST tightly controls the secretion of glucagon and insulin, two major hormones regulating glucose homeostasis. SST is also implicated in the ontogeny, remodeling, and trophic support of various cell types within the islets of Langerhans (Strowski and Blake 2008). We have previously shown that depletion of SST through cysteamine treatment completely, selectively, and irreversibly eliminates the insulin-secreting β cells of the islets, rendering the mice diabetic (El Idrissi et al. 2010). Therefore, SST secreted by δ cells acts through a paracrine mechanism to maintain the functional integrity of the islets of Langerhans. In addition to its classical role of regulating insulin and glucagon secretion by the β- and α-cells, respectively, SST provides a trophic support for the other cell types found in the islets. In line with this, we found that the fragile X pancreas had a significant reduction in the number of islets of Langerhans compared to controls (Fig. 11.5). Furthermore, the overall size of these islets was reduced. Since the islets of Langerhans occupy less than 1% of the volume of the pancreas, we could not detect any histological abnormalities in the endocrine or exocrine parts of the pancreas (Fig. 11.5).

Petrik and colleagues have shown that a peak of islet cell apoptosis is maximal in the rat pancreas 14 days after birth and is temporally associated with a fall in the islet cell expression of IGF-II (Petrik et al. 1998). IGF-II functions as an islet survival factor in vitro. The induction of islet cell apoptosis in vivo may involve an increased expression of inducible nitric oxide synthase (iNOS) within β cells. Interestingly, SST inhibits iNOS in various cell types (Kang et al. 2001; Vasilaki et al. 2004; Thermos 2008). Similarly, Scaglia et al. (1997) have shown increased replication and decreased incidence of apoptosis in the β cells in the presence of IGF-II. IGF-II may also induce β-cell proliferation (Rabinovitch et al. 1982;

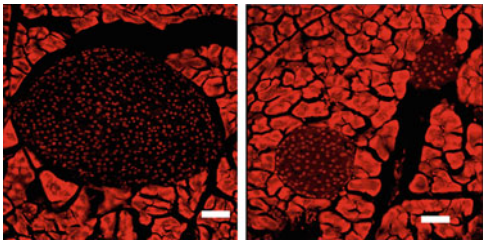

Fig. 11.5 Reduced size of pancreatic islets in the *Fmr1* KO mice. Pancreata were cryosectioned (30 μm) in the longitudinal plane. Each pancreas yielded approximately 150 sections. WT mice had consistently larger islets than did the *Fmr1* KO mice. A representative islet from WT control is shown on the left and a representative *Fmr1* KO islet is on the right. Sections were stained with propidium iodide

Rafaeloff et al. 1993; Hill et al. 1998). These studies indicate that IGF-II exerts a growth-regulating effect on the fetal pancreas, that the endocrine pancreas undergoes significant modification during neonatal life, and that apoptosis and growth are important mechanisms in this remodeling.

11.5.2 Histological Alterations of the Islets and Their Consequences on Glucose Metabolism

Qin et al. (2002) have shown a widespread effect on brain functional activity in fragile X mice as measured by glucose metabolism. Such an increase in brain activity is consistent with hyperactivity observed in fragile X syndrome, and the increase in glucose metabolism could be an adaptive mechanism to the increase in energy demand of neuronal circuits in the fragile X mouse brain. However, increased glucose metabolism in fragile X mouse could also be related to the effects of SST on the pancreas. SST is a potent paracrine modulator that inhibits the secretion of insulin and glucagon from the islets of Langerhans (Kanno et al. 2002). SST secretion is upregulated by high blood glucose via cytosolic Ca^{2+} and cAMP (Arimura and Fishback 1981). The biochemical interaction among the three cell types within the islets of Langerhans is similar to those of inhibitory synapses within the brain in terms of neurotransmitter used, receptor expressed, and enzymes (El Idrissi et al. 2009). In the pancreas, however, there is an added level of complexity associated with the paracrine secretion of various pancreatic hormones. In the brain of fragile X mice, the functional alterations in inhibitory synapses that we previously reported have also functional consequences in the physiology of the pancreas. $GABA_A$ receptor expression is reduced and GAD is increased in the fragile X brain when compared to that in controls. These alterations in $GABA_A$ receptors would reduce GABAergic inhibition, thus increasing excitability. In the pancreas, GABA is co-released with insulin when plasma glucose is elevated. While insulin diffuses into the blood stream and increases glucose transport into hepatic and skeletal muscle cells, GABA binds to $GABA_A$ receptors expressed on the α-cells. Activation of $GABA_A$ receptors leads to hyperpolarization of α-cells and inhibition of the constitutive glucagon release. The functional significance of reduced GABA inhibition of α-cells in the fragile X mouse is diminished inhibition of glucagon release. This may potentially lead to an elevated circulating glucagon and mobilization of glucose through glycogenolysis. This perturbed paracrine hormonal control in the pancreas of fragile X mice would be expected to result in altered plasma glucose homeostasis.

To determine the functional significance of decreased islet size and number in the pancreas of fragile X mice, we tested their tolerance to glucose injection as an indicator of the pancreas' ability to regulate plasma glucose homeostasis. As expected, control mice showed a drastic increase in plasma glucose concentration 30 min after challenge, with a gradual decrease over through 120 min. By the end of

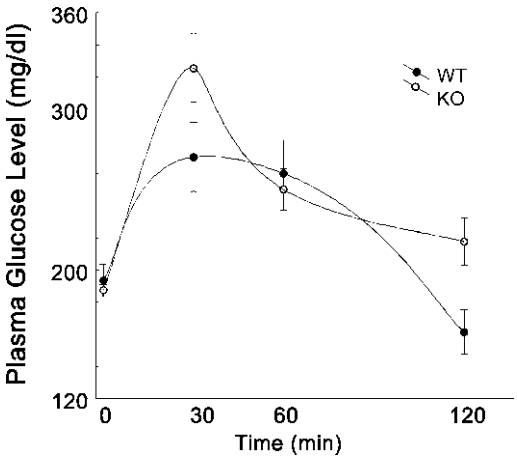

Fig. 11.6 Intraperitoneal glucose tolerance test on overnight-fasted mice. Mice from both groups were fasted overnight (12 h) and then injected intraperitoneally with 0.02 ml/g of body weight D-glucose (7.5 % stock solution in saline). Blood samples were taken by tail venesection at 0 min (just before glucose injection) and at 30-, 60-, and 120-min intervals after the glucose load. Glucose was measured with Ascensia Breeze portable glucose meter (Bayer, Germany). Mice were given only water during the test. Values are expressed as means ± S.E.M obtained from three experiments

the experiment, mice were slightly hypoglycemic relative to baseline (Fig. 11.6). Fragile X mice had similar plasma glucose levels compared to controls. However, 30 min post-glucose injection, plasma glucose level was higher than those of controls. At 60 min, plasma glucose levels were the same as controls, but two hours post-injection, plasma glucose in control mice continued to decline, whereas the fragile X plasma glucose levels remained significantly higher. The results of the glucose tolerance test are consistent with the histological observations of reduced islet size in the fragile X mouse.

11.6 Reduced SST Expression as a Correlate for Fragile X Features

11.6.1 Hyperexcitability and Seizures

Electrophysiological studies in the hippocampus have shown that SST has an inhibitory effect on the spontaneous activity of pyramidal cells and that bath application of the peptide induces dendritic hyperpolarization (Schwarzer et al. 1995). SST-positive neurons in the hippocampus play an important role in hippocampal excitability and epilepsy. SST suppressed chronic susceptibility to kainic acid seizures in rats (Perez et al. 1995). Hippocampal SST also retarded the

acquisition of generalized seizures in electrically kindled rats (Monno et al. 1993). SST is preferentially released from neurons during seizures (Bartfai et al. 1988; Hokfelt 1991; Vezzani et al. 1993), and marked changes in the expression of SST mRNA and the levels of the peptide and its receptors occur after experimentally induced seizures and in human epileptic tissue (Laming et al. 1989). Intracerebral injections of SST, its analogs, or SST-specific antibodies affect seizures and epileptogenesis in rats (Vezzani et al. 2000). Furthermore, there is an inverse relationship between SST content in the entorhinal cortex and interictal paroxysmal activity in the hippocampus of human epileptics (Deutch et al. 1991), suggesting that this neuropeptide has inhibitory actions on seizures, through the modulation of recurrent excitation, similar to that described in the rat hippocampus (Manfridi et al. 1991; Monno et al. 1993; Perez et al. 1995). This is consistent with the data presented here, showing that SST expression and the number of SST-immunoreactive cells are downregulated in the fragile X mouse brain, which exhibits hyperexcitability and has higher seizure susceptibility.

SST mediates its inhibitory modulatory effects on synaptic transmission by inhibiting ionic currents, in that it decreases the probability of a neuron firing an action potential. In rat CA1 hippocampal pyramidal neurons, SST has an inhibitory action, including hyperpolarization of the membrane at rest through an increase in the voltage-insensitive K^+ leak current. SST also augments the voltage-sensitive K^+ M current (Moore et al. 1988). In these cells, SST was shown to inhibit N-type Ca^{2+} currents (Ishibashi and Akaike 1995), thus reducing the probability of neurotransmitter release. SST reversibly depressed evoked excitatory postsynaptic currents (EPSCs) and reduced the frequency of miniature EPSCs (Sun et al., 2002). However, unlike classical neurotransmitters, release of neuropeptides, including SST, is generally thought to depend on high-frequency neuronal discharge. Thus, SST may be preferentially released from neurons during certain forms of rhythmic oscillations or elevated neuronal activity (Vezzani and Hoyer 1999).

11.6.2 Elongated Face and Enlarged Testicular Volume

We hypothesize that these two features of the fragile X syndrome may be related to the regulation of GH secretion. GH release is primarily regulated by two hypothalamic peptides, growth hormone-releasing hormone (GHRH) and SST. GHRH stimulates and SST inhibits the release of GH. GHRH neurons are located in the arcuate nucleus (ARC) and SST neurons are located in the periventricular nucleus (PeN) of the hypothalamus. Although ARC GHRH neurons possess estrogen receptors (Kamegai et al. 2001) and no androgen receptors (Fodor et al. 2001), estradiol and testosterone both increase the release of GHRH from the hypothalamus (Zeitler et al. 1990; Hassan et al. 2001). These results suggest that estradiol may act directly and testosterone may act indirectly on GHRH neurons. On the contrary, PeN-SST neurons possess androgen receptors, but do not contain estrogen receptors (Herbison and Theodosis 1993; Herbison 1994, 1995; Simonian et al. 1998). However, both sex steroids are involved in the regulation of SST genes in the

hypothalamus (Werner et al. 1988). The enlarged testicular volume and the resulting increase in testosterone secretion in the fragile X syndrome would exaggerate the feedback mechanisms for GH release. Elevated testosterone levels would increase GHRH secretion and ultimately GH secretion. This may result in the craniofacial alterations observed in the mouse model and in the human syndrome. Interestingly, the number of pituitary GH and hypothalamic GHRH immunoreactive neurons at two months in male mice is twice that in females (Kuwahara et al. 2004a, b). At 2 years of age, there was no sex difference (Kuwahara et al. 2004a, b). Furthermore, changes in the number of SST-ir neurons differ between mice of both sexes during aging (male: no difference with age; female: a decrease with age (Kuwahara et al. 2004a, b). Therefore, a sexual dimorphism in the ratio of SST-ir to GHGH- and GH-ir could make the regulation of GH secretion more sensitive to changes in the levels of SST in males than females. It should be noted that SST is the accepted treatment for macromegaly.

11.6.3 Hypersensitivity to Sensory Stimuli, Sleep Disorders, and Hyperarousal

The thalamus is a crucial relay station for messages going to the cerebral cortex. Except for olfactory inputs, nothing can get to the cortex from the sensory periphery or from lower brain centers without going through the thalamus. Following a reduction in activating input from the brainstem reticular formation, the thalamus generates powerful synchronized bursts of action potentials during slow-wave sleep, in contrast to the activity in alert states, which is characterized by a single spike firing (e.g., tonic). Repetitive spike bursts, which characteristically appear during early stages of sleep, arise from GABAergic thalamic reticular neurons that generate inhibitory postsynaptic potentials (IPSPs) in glutamatergic thalamocortical neurons (Steriade 1999).

SST decreases thalamic network excitability through multiple actions at different sites, including activation of postsynaptic K^+ channels of reticular neurons, inhibition of glutamate release onto reticular neurons, and consequent dampening of both spindle-like and epileptiform thalamic network oscillations (Sun et al. 2002). These actions of SST, along with the frequency-dependent release and long-term effects characteristic of peptides, suggest that SST may act as an important endogenous regulator of physiological and pathological thalamocortical network activities. Thus, reduction in SST inhibition may contribute to the hypersensitivity, hyperarousal, and sleep disturbances in the fragile X syndrome.

11.7 Reduced Expression of the VSCCs Exacerbates the Neuroendocrine Alterations in the Fragile X Mouse

The islets of Langerhans contain three cell types: beta, alpha, and delta. The β-cells secrete insulin and GABA and express ionotropic glutamate receptors. The α-cells secrete glucagon and glutamate and express $GABA_A$, SST, and metabotropic

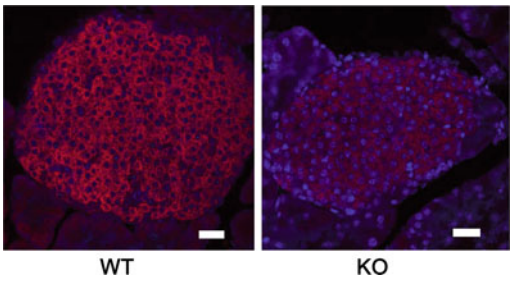

Fig. 11.7 Representative confocal images showing VSCCs IR in pancreatic islets from WT and *Fmr*1 KO mice, respectively. Sections were counterstained with the nuclear stain DAPI. WT islets showed intense IR for the VSCCs in all cells of the islets compared to *Fmr1* KO islets. *Bars*: 30 μm

glutamate receptors. The δ-cells secrete SST and express ionotropic glutamate receptors. The specific antigens they express can identify these cells. We found that the expression of the VSCC is drastically reduced in the islets of the pancreas (Fig. 11.7). Such a reduction in the expression of these channels would result in reduced calcium influx through them and reduced intracellular calcium necessary for vesicular release of pancreatic hormones. Thus, the reduced expression of the VSCC may be partially responsible for the altered glucose homeostasis observed in the fragile X mice.

We further examined the expression of these channels in the brain and found that, similar to the pancreas, the expression of the VSCC in the fragile X brain is drastically reduced. This reduction in the expression of the VSCC was observed at 7 days postnatal and in the adult mouse brain (Fig. 11.8). The reduced expression of these channels in the brain of fragile X mice would lead to reduced intracellular calcium concentrations following neuronal stimulation and reduced neurotransmitter and neuropeptide release: A feature that perhaps leads to altered synaptic plasticity manifested by enhanced LTD (Bear et al. 2004) and reduced LTP (Meredith et al. 2007) in the fragile X mouse brain. More importantly, the reduced expression of the VSCCs in the fragile X mouse early in development may lead to reduced activity-dependent changes in gene transcription that are usually triggered by activity-induced elevation of cytoplasmic Ca^{2+}.

Numerous physiological effects are triggered by Ca^{2+} signaling through VSCCs. Knockout and natural mutant studies suggest that many of the VSCCs act as a neuronal substrate for absence seizures, cerebellar ataxia, and naturopathic pain treatments (Benarroch 2010). VSCCs have been shown to be associated with Parkinson's disease, Alzheimer's disease, and other neurological disorders (Willis et al. 2010). VSCCs are critical in mediating rhythmic burst firing and pacemaker activity (Benarroch 2010); regulating neurotransmitter release, cell differentiation, and synaptic plasticity; and modulating excitatory activity-dependent gene expression and other nervous system functions.

The activity-dependent changes in gene transcription are usually triggered by activity-induced elevation of Ca^{2+} in the cytoplasm. High-throughput gene chip

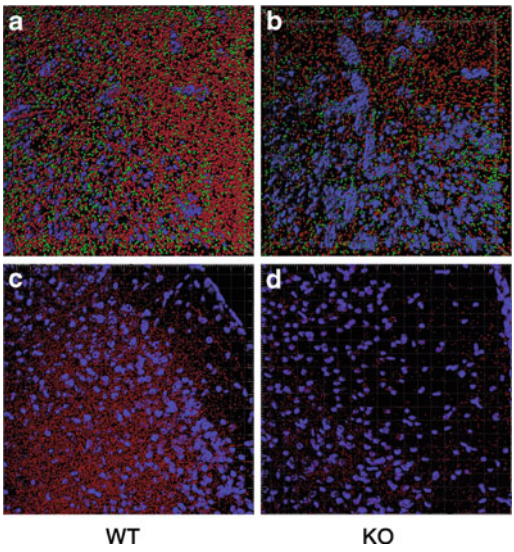

Fig. 11.8 Representative images of $GABA_A$ and VSCCs immunoreactivity from hippocampus of a P7 pup (A and B) and a cortex of a 2 months old mouse (C and D). Images were obtained with a confocal microscope, and Z stacks of a 30-μm cryosections were reconstructed using Imaris software. WT hippocampus (**a**) showed intense IR for both $GABA_A$ (*green*) and VSCCs (*red*) in the CA1 region of the hippocampus compared to *Fmr1* KO IR (**b**). This reduction in $GABA_A$ and VSCC IR was seen throughout the KO brain (except the cerebellum where there was no apparent difference between WT and *Fmr1* KO $GABA_A$ and VSCCs IR). c and d are representative Imaris reconstructions of a Z stack obtained from an adult mouse brain, which illustrate the persistence of this drastic reduction of VSCC punctuate staining in the adult *Fmr1* KO mouse cortex

analysis has revealed a list of 248 genes that are potentially affected by Ca^{2+} signaling, in which the expression of 117 genes was blocked in response to nifedipine, a VSCC blocker (Xiang et al. 2007). This indicates the critical role of Ca^{2+} influx through VSCCs in gene transcription. Thus, the reduced expression of the VSCCs in the fragile X mouse brain and peripheral organs not only affects vesicular exocytosis and calcium-dependent reactions but rather, if the defect in the expression of these channels is prenatal, will also drastically affect the calcium-dependent gene expression. This, in turn, will alter or delay the time course of genomic and cellular events that are set in motion sequentially during development, leading therefore to a developmental delay characteristic of the fragile X syndrome.

11.8 Summary and Conclusion

The findings reported in this study are as follows: $GABA_A$ receptors and VSCCs are drastically downregulated in the fragile X mouse brain and periphery; and SST and SST-positive neurons are reduced in the periphery and the brain, respectively.

Reduced expression of each these proteins in the adult brain or periphery has a particular functional significance that collectively contributes to various aspects of the phenotype characteristic of this syndrome: Reduced $GABA_A$ receptors lead to lower seizure threshold, elevated anxiety, and hyper-excitability, while reduced expression of the VSCCs results in diminished calcium-dependent vesicular exocytosis. Reduced expression of SST leads to misregulation of pancreatic remodeling and exaggerated intestinal motility. Although various traits of the fragile X syndrome could be explained by, or linked to, a functional deficiency in one of these proteins, we propose a new paradigm of channelopathy in the etiology of fragile X syndrome –abnormality in the expression and function of channel molecules. Our working hypothesis is that the fragile X syndrome is a disorder of channel imbalance resulting from channel dysfunction. Of particular importance is the finding that the expression of $GABA_A$ receptors and the VSCCs is reduced in the brain and peripheral organs of the fragile X mouse. We hypothesize that the reduced expression of these two particular channels in early development would result in the developmental delay that is characteristic of the fragile X syndrome.

The ability of GABA to alter neuronal excitability in a direction that is depolarizing rather than hyperpolarizing has profound functional consequences for neurons. $GABA_A$ receptors are ionotropic channels freely permeable to chloride ions. Consequently, the electrochemical gradient for chloride predominantly determines the nature of GABAergic transmission, excitatory versus inhibitory. As part of a self-regulating feedback loop, GABA controls its own fate during development. The rise in intracellular Ca^{2+} levels subsequent to repeated depolarization and $GABA_A$ receptor activation leads to increased expression of potassium-chloride co-transporter (KCC2), which extrudes chloride from neurons and thereby mediates the conversion of GABA-induced depolarizations to hyperpolarizations (Cherubini et al. 1990; Ganguly et al. 2001). When GABA is excitatory, activation of $GABA_A$ receptors depolarize neurons and activate the VSCCs. This in turn initiates calcium oscillations that lead to calcium-dependent gene expression that direct sequential cellular events characteristic of normal development. During this early time of brain development, GABA-initiated, VSCCs-mediated, calcium-dependent gene expression would be critically important for neurogenesis, migration, axonal growth and pathfinding, dendritic growth, synapse formation and myelination, establishment, and strengthening of synaptic connections. This is not to imply that calcium ions or calcium-dependent gene expression is the sole mediator of these processes. Rather, other factors such as guidance cues, trophic factors, hormones, and neuropeptides as well as other neuromodulators are also important in the process of brain development. Nonetheless, calcium is critically important in these processes of brain development. Because of the critical partnership between $GABA_A$ receptors and the VSCCs, their synergistic actions are critically important for normal brain development by activating signal transduction pathways and ultimately resulting in gene expression. Their reduced expression in the fragile X mouse would be expected to result in altered gene expression. The SST gene could be one of the many genes whose downregulation is a consequence of the mismatch between the activity of VSCCs and $GABA_A$ receptors. This remains to be investigated, however.

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Chapter 12
Taking STEPs Forward to Understand Fragile X Syndrome

Susan M. Goebel-Goody and Paul J. Lombroso

Abstract A priority of fragile X syndrome (FXS) research is to determine the molecular mechanisms underlying the functional, behavioral, and structural deficits in humans and in the FXS mouse model. Given that metabotropic glutamate receptor (mGluR) long-term depression (LTD) is exaggerated in FXS mice, considerable effort has focused on proteins that regulate this form of synaptic plasticity. *ST*riatal-*E*nriched protein tyrosine *P*hosphatase (STEP) is a brain-specific phosphatase implicated as an "LTD protein" because it mediates AMPA receptor internalization during mGluR LTD. STEP also promotes NMDA receptor endocytosis and inactivates ERK1/2 and Fyn, thereby opposing synaptic strengthening. We hypothesized that dysregulation of STEP may contribute to the pathophysiology of FXS. We review how STEP's expression and activity are regulated by dendritic protein synthesis, ubiquitination, proteolysis, and phosphorylation. We also discuss implications for STEP in FXS and other disorders, including Alzheimer's disease. As highlighted here, pharmacological interventions targeting STEP may prove successful for FXS.

12.1 Introduction

A priority of fragile X syndrome (FXS) research is to identify potential therapeutic targets by focusing on mRNAs regulated by fragile X mental retardation protein (FMRP) and whose translated proteins regulate the expression of synaptic plasticity. *ST*riatal-*E*nriched protein tyrosine *P*hosphatase (STEP) is one such candidate protein. STEP is a brain-specific tyrosine phosphatase that regulates dendritic proteins involved in synaptic plasticity, including ERK1/2, Fyn, NMDA receptors

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(NMDARs), and AMPA receptors (AMPARs) (Nguyen et al. 2002; Paul et al. 2003; Pelkey et al. 2002; Snyder et al. 2005; Zhang et al. 2008). Dysregulation of these proteins is proposed to contribute to the pathophysiology of FXS (Eadie et al. 2010; Kim et al. 2008; Nakamoto et al. 2007). STEP reduces ERK1/2 activity by dephosphorylating one of its regulatory tyrosine residues, Tyr^{204} (Paul et al. 2003), and inactivates the Src family tyrosine kinase (SFK) Fyn by dephosphorylating its regulatory site (Nguyen et al. 2002). Dephosphorylation of NMDARs and AMPARs promotes internalization of these receptors (Snyder et al. 2005; Zhang et al. 2008), and STEP is thought to mediate group 1 metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) (Gladding et al. 2009; Moult et al. 2006; Zhang et al. 2008). Consistent with the mGluR theory of FXS (Bear et al. 2004), STEP is translated in response to mGluR stimulation (Zhang et al. 2008). Over-activation of mGluRs in the mouse model for FXS [*Fmr1* knock-out (KO)] is associated with a tyrosine phosphatase-dependent reduction in ERK1/2 phosphorylation (Kim et al. 2008), suggesting upregulation of an unknown tyrosine phosphatase in FXS. STEP is an excellent candidate for being this unknown tyrosine phosphatase. In this chapter, we review the current understanding of STEP and its potential role in the physiological and behavioral deficits associated with FXS.

12.2 STEP Basics

12.2.1 Isoforms, Domain Function, and STEP Regulation

STEP was cloned and identified as a brain-specific tyrosine phosphatase 20 years ago (Lombroso et al. 1991, 1993). As its name implies, STEP is enriched in the striatum but is also found in other CNS structures including the hippocampus, cortex, amygdala, optic nerve, and spinal cord (Boulanger et al. 1995; Lorber et al. 2004). STEP is not expressed in the cerebellum (Lombroso et al. 1991). To date, four alternatively spliced variants of STEP have been identified: $STEP_{61}$, $STEP_{46}$, $STEP_{38}$, and $STEP_{20}$ (Fig. 12.1) (Bult et al. 1996, 1997; Sharma et al. 1995). The two major isoforms, $STEP_{61}$ and $STEP_{46}$, contain a signature consensus tyrosine phosphatase sequence, [I/V]HCxAGxxR[S/T]G, that is necessary for its catalytic activity, and a kinase-interacting motif (KIM) essential for substrate binding (Bult et al. 1996). $STEP_{38}$ and $STEP_{20}$ do not contain the consensus tyrosine phosphatase sequence and are, therefore, inactive variants of STEP with unknown function (Bult et al. 1997; Sharma et al. 1995).

Unlike $STEP_{46}$, $STEP_{61}$ contains an additional 172 amino acids at its *N*-terminus targeting it to membranous organelles including the endoplasmic reticulum and the postsynaptic density (Fig. 12.1) (Boulanger et al. 1995; Bult et al. 1996; Oyama et al. 1995). Without this targeting sequence, $STEP_{46}$ is restricted to the cytosol (Bult et al. 1996). The *N*-terminal portion of $STEP_{61}$ also has two polyproline-rich

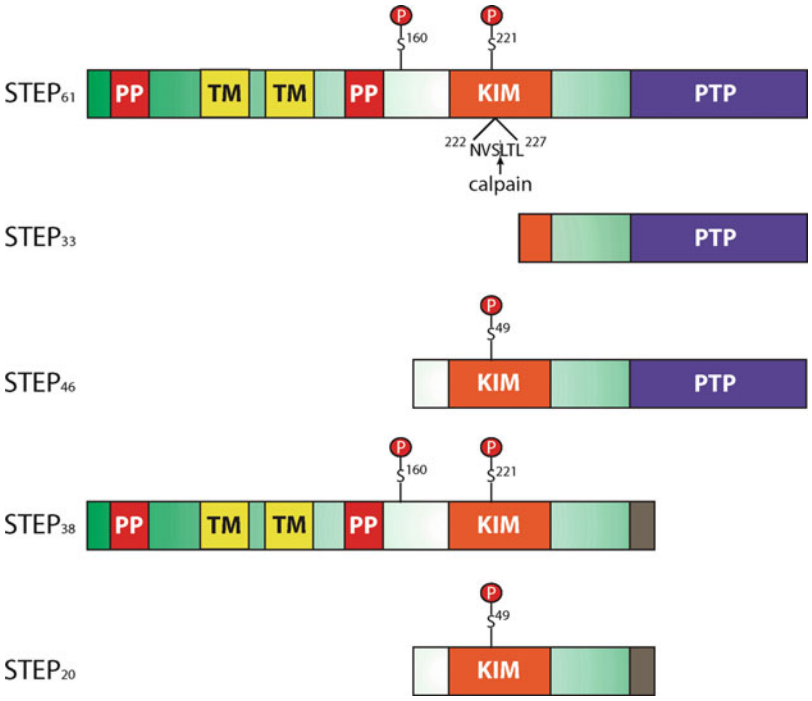

Fig. 12.1 Schematic of STEP. To date, four alternatively spliced variants of STEP ($STEP_{61}$, $STEP_{46}$, $STEP_{38}$, and $STEP_{20}$) and one calpain cleavage product ($STEP_{33}$) have been identified. The kinase-interacting motif (KIM) domain is essential for substrate binding, and the consensus protein tyrosine phosphatase (PTP) sequence, [I/V]HCxAGxxR[S/T]G, is required for phosphatase activity. Since $STEP_{61}$ and $STEP_{46}$ are the only two that contain both the KIM and PTP sequences, they are the only active forms of STEP. $STEP_{38}$ and $STEP_{20}$ do not contain the PTP sequence and are inactive variants of STEP with unknown function. It is possible that these two inactive isoforms function as dominant-negative variants that compete with active STEP variants for substrate binding, or they possess other functions yet to be discovered. A unique ten-amino acid sequence at the C-terminus of $STEP_{38}$ and $STEP_{20}$ is introduced during splicing. A calpain cleavage site resides within the KIM domain between Ser^{224} and Leu^{225} which is utilized to generate $STEP_{33}$. Cleavage at this site disrupts the ability of $STEP_{33}$ to interact with its substrates. $STEP_{61}$ also has an additional 172 amino acids in its *N*-terminus which contains two transmembrane (TM) domains, two polyproline-rich (PP) regions, and an adjacent PEST sequence (not labeled). The TM regions target $STEP_{61}$ to the endoplasmic reticulum, as well as the postsynaptic density. Without these TM regions, $STEP_{46}$ is restricted to the cytosol. The PP regions impart substrate binding specificity. PKA phosphorylates STEP within the KIM domain (Ser^{221} and Ser^{49} on $STEP_{61}$ and $STEP_{46}$, respectively), as well as in the region adjacent to the PP regions (Ser^{160} on $STEP_{61}$). Although the function of the additional phosphorylation site on $STEP_{61}$ remains unclear, current investigations are aimed at determining if phosphorylation at this or other sites is a signal for calpain-mediated cleavage and/or ubiquitination

regions that impart substrate specificity. For example, the first polyproline region is required for the interaction of $STEP_{61}$ with Fyn (in addition to the KIM domain), and $STEP_{61}$ has a tenfold greater affinity for Fyn than $STEP_{46}$ (Nguyen et al. 2002).

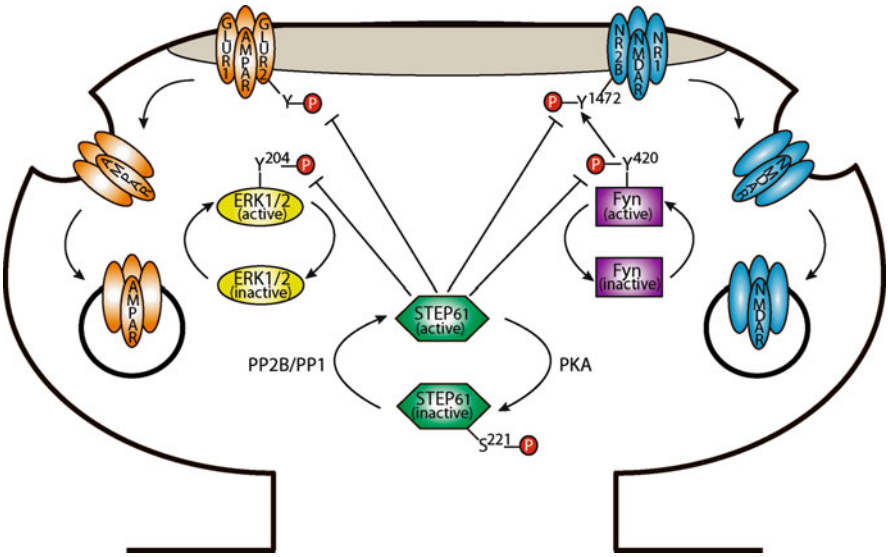

Fig. 12.2 Regulation of STEP and its substrates by phosphorylation. In response to dopamine D1 receptor activation, PKA phosphorylation of $STEP_{61}$ at Ser^{221} sterically prevents binding of $STEP_{61}$ to its substrates. In contrast, stimulation of NMDARs initiates calcium influx and activation of PP2B (calcineurin) and PP1 to dephosphorylate and activate $STEP_{61}$. When active, STEP dephosphorylates ERK1/2 and Fyn at their regulatory tyrosine residues, Tyr^{204} and Tyr^{420} (respectively), and inactivates them. $STEP_{61}$ regulates the phosphorylation of NR2B-containing NMDARs by two parallel mechanisms. First, when Fyn is inactivated by $STEP_{61}$, Fyn is unable to phosphorylate NR2B Tyr^{1472}. Second, $STEP_{61}$ dephosphorylates NR2B Tyr^{1472} directly. Dephosphorylation of Tyr^{1472} promotes the interaction of NR2B with clathrin adaptor proteins and leads to endocytosis of these receptors. $STEP_{61}$ is also required for the internalization of GluR1/GluR2-containing AMPARs following mGluR stimulation. While the molecular mechanisms are still incompletely understood, $STEP_{61}$ appears to promote the endocytosis of AMPARs in a similar manner to NMDARs

As mentioned, the KIM domain is required for binding STEP to its substrates, and this interaction is tightly regulated (Fig. 12.2). In all variants, dopamine-induced PKA phosphorylation of STEP in the KIM domain (Ser^{221} in $STEP_{61}$ and Ser^{49} in $STEP_{46}$) decreases the ability of STEP to bind to substrates due to steric interference (Paul et al. 2003). Conversely, activation of PP2B/calcineurin and PP1 (via NMDAR or α7nAChR stimulation) dephosphorylates STEP at this serine residue and increases its substrate affinity. STEP integrates dopaminergic, glutamatergic, and nicotinic signaling, suggesting a potential role in psychostimulant addiction (Tashev et al. 2009; Valjent et al. 2005) and Alzheimer's disease (Kurup et al. 2010; Snyder et al. 2005; Zhang et al. 2010). Additional evidence for its role in Alzheimer's disease is discussed below.

A truncated STEP product, $STEP_{33}$, is a calpain-mediated cleavage product generated during extrasynaptic NMDAR stimulation (Fig. 12.1) (Gurd et al. 1999; Nguyen et al. 1999; Xu et al. 2009). The calpain cleavage site resides in

the KIM domain between residues Ser^{224} and Leu^{225} (Xu et al. 2009). Cleavage at this site disrupts the ability of STEP to associate with and dephosphorylate its substrates. Consequently, proteolytic cleavage of STEP after extrasynaptic NMDAR stimulation results in the activation of one of STEP's substrates, p38, and initiates the cell death signaling cascade. Preventing STEP cleavage through the use of a competitive peptide significantly attenuates cell death after either glutamate excitotoxicity or oxygen-glucose deprivation models (Xu et al. 2009). The inactivation of STEP by calpain cleavage is a possible mechanism for the observed increase in NMDAR tyrosine phosphorylation following cerebral hypoxia–ischemia (Besshoh et al. 2005; Gurd et al. 1999).

12.2.2 Developmental Profile, Brain Region Specificity, and Subcellular Localization

$STEP_{61}$ and $STEP_{46}$ are differentially expressed during rodent development (Raghunathan et al. 1996). $STEP_{61}$ is abundant at birth and remains relatively unchanged into adulthood, whereas $STEP_{46}$ is virtually undetectable at birth. $STEP_{46}$ appears around postnatal day 6 and is enriched by 14 days (Okamura et al. 1997; Raghunathan et al. 1996). $STEP_{46}$ plateaus at postnatal day 30 and remains constant throughout adulthood. Given that the first few weeks of life are associated with extensive cell migration and synaptogenesis (reviewed in Cayre et al. 2009), the onset of $STEP_{46}$ expression during this time suggests a role in synaptogenesis. In middle aged (12-month old) mice, $STEP_{61}$ is elevated relative to 3–6 months old (Kurup et al. 2010), suggesting that expression of $STEP_{61}$ also changes during aging.

The expression of $STEP_{61}$ and $STEP_{46}$ is brain region specific (Boulanger et al. 1995; Lorber et al. 2004). The striatum, central nucleus of the amygdala, and optic nerve express both STEP isoforms. In contrast, the hippocampus, neocortex, spinal cord, and lateral amygdala express only $STEP_{61}$ (Boulanger et al. 1995). Initial subcellular characterizations showed that $STEP_{46}$ is enriched in cytosolic fractions, while $STEP_{61}$ is enriched in light membrane fractions (which include endoplasmic reticulum, golgi, and endosomes) (Bult et al. 1996; Lombroso et al. 1993). Further investigation with electron microscopy showed that STEP is also targeted to the postsynaptic density (Oyama et al. 1995), and biochemical purification of these densities showed an enrichment of the $STEP_{61}$ isoform (Gurd and Lombroso, unpublished data). Recent work demonstrates that the concentration of $STEP_{61}$ is higher in extrasynaptic membranes than synaptic membranes (Goebel-Goody et al. 2009). Given that clathrin-mediated internalization of membrane proteins is thought to occur extrasynaptically (Blanpied et al. 2002; Racz et al. 2004), enrichment of STEP in extrasynaptic compartments might play a role in facilitating endocytosis of glutamate receptors in this part of the dendritic spine.

12.3 STEP Substrates Implicated in FXS

12.3.1 Mitogen-Activated Kinase ERK1/2

Activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) is critical for the induction and maintenance of synaptic plasticity. These kinases have been implicated in the regulation of membrane electrical properties (via the Kv4 family channels and subsequent NMDAR activation), local dendritic protein synthesis, nuclear transcriptional regulation, and the formation and stabilization of dendritic spines (reviewed in Sweatt 2004). ERK1/2 inactivation disrupts these processes. STEP inactivates ERK1/2 by dephosphorylating the regulatory tyrosine residue (Tyr^{204}) in the activation loop (Fig. 12.2) (Paul et al. 2003). In wild-type synaptoneurosomal preparations, ERK1/2 phosphorylation, as detected by a dual-specificity Thr^{202}/Tyr^{204} phospho-specific antibody, is rapidly increased upon mGluR stimulation via the selective group 1 mGluR agonist (RS)-3,3-dihydroxyphenylglycine (DHPG) (Kim et al. 2008). In contrast, in *Fmr1* KO synaptoneurosomes, ERK1/2 phosphorylation is quickly and abnormally reduced following mGluR stimulation (Kim et al. 2008). A broad-spectrum tyrosine phosphatase inhibitor (orthovanadate) prevents the DHPG-mediated decrease in ERK1/2 phosphorylation, suggesting over-activation of a tyrosine phosphatase in FXS (Kim et al. 2008). Because STEP dephosphorylates and inactivates ERK1/2 (Paul et al. 2003), STEP is a likely candidate for the tyrosine phosphatase that is upregulated in FXS. The early-phase kinetics of ERK activation is delayed in some individuals with FXS, so ERK activation may be an useful biomarker of metabolic status in FXS (Weng et al. 2008).

Further support for the regulation of ERK1/2 by STEP stems from studies of STEP KO mice (Venkitaramani et al. 2009). ERK1/2 phosphorylation is significantly enhanced in the striatum, CA2 region of the hippocampus, and central/lateral amygdala in STEP KOs. Moreover, activation of ERK1/2 phosphorylation following DHPG treatment is more pronounced in STEP KOs relative to that in wild type (Venkitaramani et al. 2009), corroborating earlier work implicating a tyrosine phosphatase in the regulation of ERK1/2 activity following mGluR stimulation (Kim et al. 2008).

ERK1/2 translocation to the nucleus and subsequent initiation of gene transcription are required for the formation of fear memories and the expression of synaptic plasticity in the lateral amygdala (Schafe et al. 2000). Infusion of a substrate trapping membrane-permeable fusion protein of $STEP_{46}$ (TAT-$STEP_{C\text{-}S}$) in the lateral amygdala of rats inhibits Pavlovian fear conditioning (Paul et al. 2007). This mutant STEP protein binds to its substrates, but cannot dephosphorylate them and does not release them, thereby disrupting their downstream signaling. Bath application of this construct also blocks the induction of LTP in the lateral amygdala and prevents ERK1/2 translocation to the nucleus during memory consolidation and synaptic plasticity (Paul et al. 2007). A recent report shows decreased LTP in the lateral amygdala of *Fmr1* KO mice that is not rescued by the mGluR5-specific inverse agonist,

MPEP (Suvrathan et al. 2010). Inhibitors targeted at other proteins upregulated in FXS, such as STEP, should be tested for their capacity to rescue these deficits.

12.3.2 Fyn and NMDA Receptors

STEP regulates the phosphorylation and surface expression of NMDARs by two parallel pathways (Fig. 12.2): indirectly via dephosphorylation and inactivation of Fyn, one of the SFKs that phosphorylates NMDARs (Nguyen et al. 2002), and directly by dephosphorylation of the NMDAR subunit NR2B (Snyder et al. 2005; Kurup et al. 2010). When activated, Fyn phosphorylates the NMDAR NR2B subunit at Tyr^{1472} (Nakazawa et al. 2001). Full activation of Fyn is achieved by phosphorylation of Tyr^{420} in its catalytic domain (Smart et al. 1981). STEP dephosphorylates Fyn at this site (Nguyen et al. 2002), thereby inactivating Fyn and reducing Fyn-mediated phosphorylation of NR2B at Tyr^{1472} (Fig. 12.2). Additionally, STEP interacts with NMDARs (Pelkey et al. 2002) and dephosphorylates Tyr^{1472} directly (Kurup et al. 2010; Snyder et al. 2005). Tyr^{1472} resides within a conserved tyrosine-dependent endocytic motif (YXXϕ: X = any amino acid, ϕ = bulky hydrophobic amino acid) (Roche et al. 2001). When not phosphorylated, the tyrosine residue in this motif binds to clathrin adapter proteins via strong hydrophobic interactions (reviewed in Marsh and McMahon 1999). In this way, STEP mediates endocytosis of NR2B-containing NMDARs by promoting the interaction between NMDARs and clathrin adapter proteins (Nakazawa et al. 2006).

Consistent with STEP's role in mediating NMDAR endocytosis, the surface expression of NR1/NR2B receptor complexes is elevated in STEP KO mice and reduced in the presence of increased STEP levels (Kurup et al. 2010; Zhang et al. 2010). STEP's modulation of surface NMDARs also affects their function. For example, application of recombinant STEP to the cytoplasmic face of neurons decreases NMDAR excitatory postsynaptic currents (EPSCs) and prevents the induction of long-term potentiation (LTP), whereas inhibition of endogenous STEP with an anti-STEP antibody enhances NMDAR EPSCs and occludes LTP (Pelkey et al. 2002). Moreover, theta-burst LTP is significantly increased in STEP KO mice relative to that in wild type (Zhang et al. 2010). Taken together, STEP dephosphorylates Tyr^{1472}, promotes internalization of surface NR1/NR2B receptors, and subsequently acts as a "brake" on the induction of NMDAR-dependent LTP (Braithwaite et al. 2006; Kurup et al. 2010; Pelkey et al. 2002; Snyder et al. 2005; Zhang et al. 2010).

Some forms of NMDAR-dependent synaptic plasticity and learning are impaired in *Fmr1* KO mice, lending support to the hypothesis that over-activation of STEP may contribute to hypofunction of NMDARs in FXS. NMDAR-dependent LTP and LTD are significantly attenuated in the dendate gyrus of *Fmr1* KO mice, which is associated with a decrease in NMDAR EPSC amplitude (Eadie et al. 2010). Learning impairments are also observed in the ability of *Fmr1* KOs to discriminate between two similar contexts and during contextual fear extinction, both of which

require functional NMDARs (Eadie et al. 2010). Moreover, Desai et al. (2006) demonstrated that NMDAR-dependent spike timing-dependent plasticity potentiation is attenuated in *Fmr1* KOs relative to WT. While hippocampal CA1 NMDAR-dependent LTD using a 1-Hz low-frequency stimulation protocol is unaffected in *Fmr1* KOs (Huber et al. 2002), it is clear that hypofunction of NMDARs in some brain regions, perhaps via abnormal dephosphorylation by STEP, may contribute to NMDAR-dependent physiological and behavioral deficits in *Fmr1* KO mice.

12.3.3 AMPA Receptors

Pioneering work in the FXS field demonstrated that *Fmr1* KO mice have exaggerated mGluR-dependent LTD (Huber et al. 2002). Both NMDARs and AMPARs are internalized during mGluR stimulation with the pharmacological mGluR agonist DHPG (Snyder et al. 2001), so identification of a common mechanism regulating endocytosis of these receptors would enhance our understanding of synaptic deficits in FXS. Given that STEP also regulates the internalization of GluR1/GluR2-containing AMPARs (Zhang et al. 2008), it is an excellent candidate for regulating this common mechanism.

Mounting evidence supports the hypothesis that a tyrosine phosphatase regulates the expression of mGluR-dependent LTD. Moult et al. (2006) reported that blocking tyrosine phosphatases with a broad-spectrum tyrosine phosphatase inhibitor (phenylarsine oxide) prevents the expression of DHPG-mediated LTD. Pretreatment of slices with the SFK inhibitor PP2 prevents the tyrosine phosphatase-dependent block of mGluR-LTD (Moult et al. 2006), suggesting that SFKs counteract tyrosine phosphatases during this form of synaptic plasticity. Tyrosine phosphorylation of GluR2 is reduced during DHPG-mediated LTD, and this dephosphorylation is associated with internalization of AMPARs (Gladding et al. 2009; Moult et al. 2006). These findings point to a model in which a tyrosine phosphatase is activated during mGluR stimulation to dephosphorylate GluR2-containing receptors and mediate their endocytosis.

STEP appears to be the tyrosine phosphatase that mediates AMPAR internalization during mGluR stimulation (Fig. 12.2) (Zhang et al. 2008). Internalization of surface GluR1/GluR2 receptors is associated with an increase in STEP protein levels following DHPG treatment, and this occurs in conjunction with dephosphorylation of tyrosine residues on GluR2. DHPG-induced internalization of AMPARs is abolished in STEP KO hippocampal slices and cultures and restored with the addition of a wild-type TAT-STEP fusion protein to STEP KO neurons (Zhang et al. 2008). In this way, STEP completes the model proposed by Moult et al. (2006) and Gladding et al. (2009); it is activated by mGluR stimulation and leads to the dephosphorylation and subsequent internalization of AMPARs (Zhang et al. 2008).

While the molecular mechanisms governing AMPAR endocytosis following mGluR activation are still uncertain, tyrosine phosphorylation seems to play an important role. There are four tyrosine residues in the C-terminal tail of GluR2

(Tyr^{837}, Tyr^{869}, Tyr^{873}, and Tyr^{876}) (Hayashi and Huganir 2004); yet none appears to reside within a conserved tyrosine-dependent endocytic motif (YXXϕ). A new model proposed by Scholz et al. (2010) sheds light on this paradox. These authors report that the GluR2 subunit directly interacts with the synaptic protein BRAG2, which is a synaptically localized protein that functions as a guanine exchange factor (GEF) for the GTPase Arf6. This interaction requires dephosphorylation of Tyr^{876} by an unknown tyrosine phosphatase (Scholz et al. 2010). When Arf6 is activated by BRAG2, it recruits the adaptor protein AP2 and clathrin to synaptic membranes (Krauss et al. 2003), thereby promoting endocytosis of GluR2 (Scholz et al. 2010). It is compelling to speculate that STEP is the tyrosine phosphatase that dephosphorylates GluR2 and mediates its internalization. Consistent with this hypothesis, the surface expression of GluR1/GluR2-containing AMPARs is significantly elevated in the absence of STEP (Zhang et al. 2008). Ongoing efforts are directed at confirming the site that STEP dephosphorylates on GluR2.

Given that *Fmr1* KO mice have exaggerated mGluR-dependent LTD (Huber et al. 2002), one prediction is that the steady-state endocytosis of AMPARs would be upregulated in the absence of FMRP. Nakamoto et al. (2007) used siRNA of FMRP in cultured hippocampal neurons to demonstrate that DHPG induces excessive internalization of GluR1/GluR2 in FMRP-deficient neurons compared to controls. More recently, aberrant constitutive AMPAR internalization was reported in hippocampal *Fmr1* KO neurons relative to that in wild-type neurons (Gross et al. 2010), and surface levels of GluR1 are reduced in the amygdala of *Fmr1* KO slices compared to that in wild type (Suvrathan et al. 2010). Linking dysregulation of STEP in *Fmr1* KOs to aberrant endocytosis of AMPARs is the subject of current investigation.

Because of its role in regulating AMPAR endocytosis following mGluR stimulation (Zhang et al. 2008), STEP was coined an "LTD protein" by Luscher and Huber (2010). There are likely several "LTD proteins" that are dysregulated in FXS. For example, microtubule-associated protein 1B (MAP1B) elevated early in development in *Fmr1* KO mice (Lu et al. 2004) and regulates AMPAR surface expression by binding to the AMPAR scaffolding protein GRIP1 (Davidkova and Carroll 2007; Seog 2004). When bound to GRIP1, MAP1B sequesters GRIP–AMPAR bound complexes away from the synaptic surface, thereby negatively regulating AMPAR surface expression (Davidkova and Carroll 2007). Increased levels of MAP1B in *Fmr1* KOs could, therefore, result in greater GRIP–AMPAR bound complexes and may explain increased AMPAR internalization. Even so, there is no evidence to date suggesting that strategies which decrease the expression and/or function of MAP1B improve behavioral performance in *Fmr1* KOs. Another example of an "LTD protein" that is dysregulated in FXS is activity-regulated cytoskeletal associated protein (Arc). Increased polysome-associated Arc mRNA has also been reported in *Fmr1* KOs (Zalfa et al. 2003); however, Arc protein levels do not appear to be significantly upregulated at baseline in *Fmr1* KOs (Park et al. 2008). Arc directly associates with dynamin 2 and endophilin, which are proteins required for AMPAR endocytosis. As a consequence, Arc promotes internalization of AMPARs via interaction with these proteins (Chowdhury et al. 2006;

Rial Verde et al. 2006; Shepherd et al. 2006). Arc is rapidly translated in response to DHPG stimulation in wild-type neurons, but this increase in Arc protein is absent in *Fmr1* KOs (Park et al. 2008), supporting work by Zalfa et al. (2003) which demonstrated elevated constitutive translation of Arc in *Fmr1* KOs. Nonetheless, deletion of Arc in *Fmr1* KO mice does not entirely prevent the exaggerated mGluR-LTD (Park et al. 2008), suggesting that additional proteins contribute to this electrophysiological deficit.

12.4 Regulation of STEP Expression

12.4.1 Synaptic Activity: mGluR Stimulation and FMRP

The mGluR hypothesis of FXS proposes that stimulation of mGluRs leads to local translation of synaptic proteins that are responsible for mediating mGluR-dependent LTD (Bear et al. 2004). Moreover, this theory posits that many FXS-related phenotypes originate in exaggerated signaling through mGluRs. FMRP normally suppresses translation of several mRNAs downstream of mGluR stimulation (Li et al. 2001). In FXS, FMRP is functionally absent, so many of these synaptically localized proteins are upregulated (Gross et al. 2010 but see also Park et al. 2008). Here, we review the evidence that STEP is downstream of mGluR activation (Zhang et al. 2008), interacts with FMRP, and is upregulated in *Fmr1* KO mice (Goebel-Goody et al. 2010). Given that STEP also regulates internalization of both NMDARs and AMPARs (Zhang et al. 2008; Zhang et al. 2010) and that mGluR-dependent LTD is enhanced in *Fmr1* KOs (Huber et al. 2002), a revised model of the FXS mGluR theory emerges where exaggerated signaling through mGluRs causes dysregulation of STEP translation and a subsequent increase in the endocytosis rate of glutamate receptors (Fig. 12.3). These events likely lead to enhanced mGluR-LTD in *Fmr1* KOs.

Stimulation of mGluRs with DHPG leads to a rapid, dose-dependent increase in the translation of STEP (Fig. 12.3a) (Zhang et al. 2008). This increase is also time dependent and occurs within synaptoneurosomes, suggesting that STEP is dendritically translated by mGluR stimulation. Translation inhibitors block the DHPG-induced increase in STEP, whereas transcription inhibitors have no effect. In line with the requirement for the MAPK and phosphoinositide-3 kinase (PI3K) pathways in translation-dependent mGluR-LTD, preincubation of MEK (MAPK kinase) and PI3K inhibitors abolishes the DHPG-induced increase in STEP protein levels (Zhang et al. 2008). The use of specific group I mGluR1 and mGluR5 inhibitors verifies that the DHPG-mediated increase in STEP translation occurs primarily through mGluR5 activation. As discussed previously, this DHPG-induced increase in STEP levels is required for the internalization of AMPARs following mGluR stimulation (Zhang et al. 2008).

We are beginning to uncover the molecular mechanisms underlying the DHPG-induced translation of STEP (Zhang et al. 2008). In agreement with the mGluR

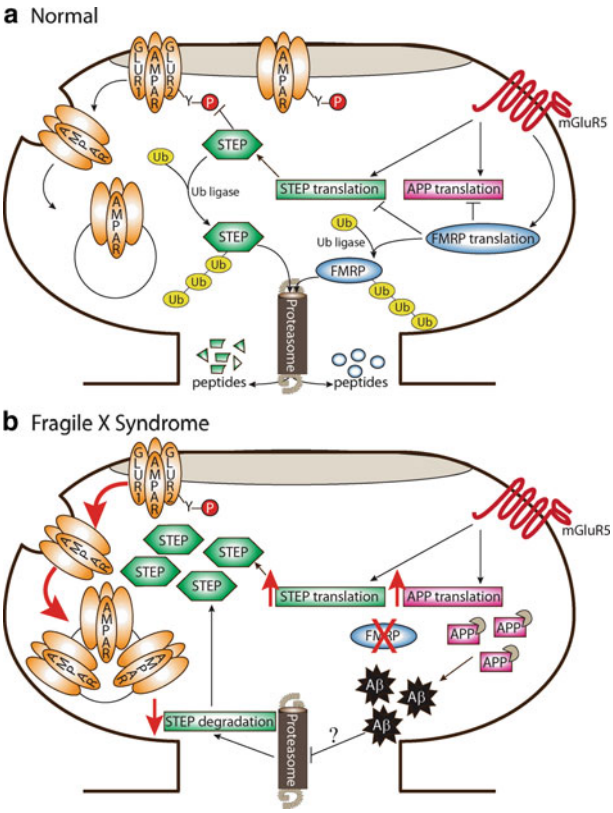

Fig. 12.3 Mechanisms governing STEP protein expression and implications for fragile X syndrome. (**a**) In normal (or wild-type) neurons, brief stimulation of mGluR5 receptors with DHPG triggers translation of STEP mRNA, as well as translation of other mRNAs including APP and FMRP. STEP dephosphorylates a tyrosine residue on GluR2 and initiates endocytosis of AMPARs following DHPG treatment. FMRP associates with both STEP and APP mRNA and likely acts as a translation suppressor to prevent excessive translation of these mRNAs. Ubiquitination and degradation by the proteasome regulate STEP and FMRP levels. Upon stimulation of mGluRs with DHPG, FMRP is rapidly degraded by the proteasome, presumably to permit translation of FMRP targets and allow the expression of LTD. (**b**) In the absence of FMRP, STEP protein expression might be inappropriately elevated by two parallel pathways. First, without the suppression of STEP mRNA translation by FMRP, the steady-state translation rate of STEP would be upregulated. Similarly, translation of APP is increased in *Fmr1* KO mice. Elevated levels of APP provide more targets for β- and γ-secretase-mediated cleavage and result in exacerbated Aβ production in aged *Fmr1* KO mice. Given that Aβ inhibits the ubiquitin proteasome system (UPS) in Alzheimer's disease, it is possible that the UPS is blocked in FXS later in life. Consequently, inhibition of UPS by Aβ could lead to reduced degradation of STEP. Elevated STEP levels in FXS could, therefore, maintain the persistent internalization of AMPARs and exaggerated mGluR-dependent LTD. Of note, for simplicity, NR2B-containing NMDARs, ERK1/2, and Fyn were removed from this figure; however, it is possible that these proteins would also be more dephosphorylated and inactivated in the presence of elevated STEP levels

theory, STEP appears to associate with FMRP (Goebel-Goody et al. 2010; Darnell, unpublished results). Moreover, STEP protein levels are elevated in the brains of adult *Fmr1* KO mice (Goebel-Goody et al. 2010), suggesting aberrant translation of STEP in the absence of FMRP (Fig. 12.3b). These findings are consistent with FMRP suppressing STEP translation under normal conditions and STEP translation being abnormally upregulated in the absence of FMRP.

Accordingly, genetically reducing STEP in *Fmr1* KO mice ameliorates some FXS-associated behavioral deficits. For example, *Fmr1* KO mice are well characterized for their susceptibility to audiogenic seizures (Musumeci et al. 2000; Yan et al. 2005; Dolen et al. 2007). Given that STEP KO mice are more resistant to pilocarpine-induced seizures (Briggs et al. 2011), mice were generated that are null for both STEP and *Fmr1* (Goebel-Goody et al. 2010). STEP/*Fmr1* double KOs have fewer audiogenic seizures and less seizure-induced c-Fos-positive neurons in the periaqueductal gray relative to *Fmr1* KOs. Similarly, genetically reducing STEP in *Fmr1* KOs decreases hyperactivity and spatial anxiety in an open field behavioral task (Goebel-Goody et al. 2010). Thus, inhibitors of STEP may be promising therapeutic strategies in FXS.

12.4.2 Ubiquitination via Beta Amyloid

In addition to regulation of STEP by mGluRs and FMRP, STEP expression is regulated by ubiquitination (Fig. 12.3a) (Kurup et al. 2010). The covalent attachment of ubiquitin targets proteins to the 26S proteasome for degradation (reviewed in Hegde 2010). In some neurological disorders, the ubiquitin proteasome system (UPS) is impaired. One pertinent example is Alzheimer's disease (AD). AD is a debilitating neurodegenerative disorder associated with memory impairments. Accumulation of beta amyloid (Aβ) and the formation of amyloid plaques are characteristic features of AD, both of which are implicated in synaptic loss and cognitive decline (Hardy and Selkoe 2002; Lacor et al. 2004). In both human AD brains and mouse models of AD, the buildup of Aβ inhibits the proteasome and consequently leads to reduced degradation of proteins normally regulated by the UPS (Keller et al. 2000; Lam et al. 2000; Oh et al. 2005; Almeida et al. 2006). Recent studies demonstrate that STEP protein levels are increased in three mouse models of AD (Tg-2576, J20, and 3xTg-AD) (Chin et al. 2005; Kurup et al. 2010; Zhang et al. 2010) and in the prefrontal cortex of human AD patients (Kurup et al. 2010). Elevated STEP levels in AD mouse models results in increased STEP activity, decreased phosphorylation and surface expression of NR2B-containing NMDARs, and decreased cognitive ability (Kurup et al. 2010; Zhang et al. 2010). STEP protein abundance is increased in AD due to Aβ-induced inhibition of the UPS (Kurup et al. 2010). Specifically, $STEP_{61}$–ubiquitin conjugates are increased in the cortex of Tg-2576AD mice and in wild-type cortical slices following Aβ treatment (Kurup et al. 2010). Genetically eliminating STEP in the 3xTg-AD mouse model improves cognitive performance, restores the NR1/NR2B surface

expression deficit, and enhances synaptic plasticity (Zhang et al. 2010), validating STEP as a target for drug discovery for the treatment of AD.

Elucidating the molecular mechanisms underlying AD is relevant to FXS research because amyloid precursor protein (APP), the precursor protein that is cleaved by β- and γ-secretases to generate Aβ, is regulated by mGluRs and FMRP (Fig. 12.3a) (Westmark and Malter 2007). In particular, translation of APP is increased following mGluR stimulation, and APP mRNA associates with FMRP (Westmark and Malter 2007). As predicted by the mGluR hypothesis (Bear et al. 2004), APP translation is elevated in *Fmr1* KO mice (Fig. 12.3b) (Westmark and Malter 2007). Greater levels of APP are also associated with increased soluble Aβ in middle-aged (11–13 months old) *Fmr1* KOs (Westmark and Malter 2007). To study the role of APP and FMRP further, Westmark et al. (2008) created a novel mouse model (FRAXAD) where Tg-2576AD mice were crossed with *Fmr1* KO mice. FRAXAD mice have even greater levels of APP and Aβ than Tg-2576 mice alone (Westmark et al. 2008), suggesting an additive effect of the two mutations. Moreover, FRAXAD mice are more susceptible to seizures than either *Fmr1* KO or Tg-2576 mice (Westmark et al. 2008, 2009), and this deficit is abrogated when mice are pretreated with the mGluR5 antagonist MPEP (Westmark et al. 2009). Taken together, these findings reveal that some FXS-associated behaviors may be due to the accumulation of APP and Aβ in middle-aged *Fmr1* KOs.

Given that Aβ inhibits the UPS, it is possible that the UPS is blocked in FXS later in life (Fig. 12.3b). Aβ-induced inhibition of the UPS could lead to reduced degradation of proteins normally regulated by the UPS. As a result, two possible explanations may exist for the accumulation of proteins in FXS (Fig. 12.3b): (1) lack of translation suppression by FMRP and (2) increased inhibition of the UPS by Aβ. Since STEP is regulated by both FMRP and the UPS, it is a likely candidate for a protein being upregulated by these two mechanisms in FXS. Current investigations are aimed at addressing these intriguing unanswered questions.

The UPS has already been implicated in FXS and fragile X-associated tremor/ataxia syndrome (FXTAS) by a number of studies in recent years (Hou et al. 2006; Greco et al. 2002; Greco et al. 2006; Iwahashi et al. 2006). Inhibitors of the UPS block the expression of mGluR-LTD in wild-type mice (Fig. 12.3a). Specifically, FMRP is rapidly degraded by the UPS after mGluR stimulation, presumably to permit FMRP-bound mRNAs to be translated. In contrast, the enhanced mGluR-LTD observed in *Fmr1* KOs is insensitive to UPS inhibitors. Taken together, these results demonstrate that degradation of FMRP is required for regulating the expression of mGluR-LTD (Hou et al. 2006).

Ubiquitin-positive intranuclear neuronal and astroglial inclusion bodies are a feature of FXTAS, a late-onset neurodegenerative disorder affecting premutation carriers of expanded CGG repeats (50–200) in the 5′ untranslated region of the *Fmr1* gene (Greco et al. 2002; Iwahashi et al. 2006). In particular, a striking correlation exists between CGG length and the number of ubiquitin-positive inclusions (Greco et al. 2006). Given that FXTAS patients present with cognitive decline and dementia, among other symptoms (Garcia-Arocena and Hagerman 2010), it is likely that the presence of inclusion bodies in the hippocampus contribute to these cognitive

deficits. While ubiquitinated proteins are only a minor component of inclusion bodies (Iwahashi et al. 2006), future studies are required which rigorously address whether the UPS is inhibited and what possible ubiquitin-conjugated proteins are upregulated in FXTAS.

12.5 Summary and Conclusions

Over the last two decades, considerable evidence has mounted which supports the role of STEP in synaptic plasticity. Specifically, STEP dephosphorylates both NMDARs and AMPARs and negatively regulates their surface expression by promoting their interaction with clathrin-associated proteins. Additionally, STEP dephosphorylates and inactivates both ERK1/2 and Fyn. For these reasons, STEP acts as a "tonic brake" on LTP and is one of a handful of "LTD proteins" that promote the expression of LTD. STEP's expression and activity are regulated by several mechanisms, including mGluR-stimulated translation, ubiquitination, proteolysis, and phosphorylation. Dysregulation of any of these mechanisms can flip the balance of STEP function such that it is more or less active. Either of these results could contribute to a disease state. In the case of FXS, enhanced activity of mGluRs in the absence of FMRP appears to increase the translation of STEP. Given that APP and Aβ levels are elevated in *Fmr1* KOs and that Aβ inhibits the UPS in AD, it is also an intriguing possibility that Aβ blocks the UPS in FXS and leads to less degradation of STEP. Genetically reducing STEP reduces some FXS-associated behaviors in *Fmr1* KOs. As highlighted here, pharmacological treatments that target STEP may be successful strategies not only for FXS, but also for other neurological disorders such as AD in which increased levels of STEP contribute to cognitive decline. While a number of unanswered questions remain, we continue to take one *step* at a time to advance our understanding of how STEP contributes to the pathophysiology of FXS.

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Chapter 13
Fmr-1 as an Offspring Genetic and a Maternal Environmental Factor in Neurodevelopmental Disease

Bojana Zupan and Miklos Toth

Abstract Since fragile X syndrome (FXS) is a typical X-linked mendelian disorder, the protein product associated with the disease (FMRP) is absent or reduced not only in the affected individuals but, in case of full mutation, also in their mothers. Here, by using the mouse model of the disease, we provide evidence that hyperactivity, a typical symptom of FXS, is not wholly induced by the lack of Fmrp in mice but also occurs as a result of its reduced expression in their mother. Genetically wild-type offspring of mutant mothers also had hyperactivity, albeit less pronounced than the mutant offspring. However, other features of FXS reproduced in the mouse model, such as sensory hyperreactivity and seizure susceptibility, were exclusively associated with the absence of Fmrp in the offspring. These data indicate that *fmr-1*, the gene encoding Fmrp, can be both an offspring genetic and a maternal environmental factor in producing a neurodevelopmental condition.

13.1 Introduction

Fragile X syndrome (FXS) is caused by the absence of a functional fragile X mental retardation protein (FMRP), the product of the *FMR-1* gene. FMRP is an RNA-binding protein involved in the transport, delivery, and local translation of a specific set of mRNAs (Kao et al. 2010). Various approaches identified a large number of mRNAs regulated by FMRP (Brown et al. 2001; Chen et al. 2003; Miyashiro et al. 2003). In addition, FMRP is present in polyribosomal translational complexes, suggesting that it may also regulate global protein synthesis (Zalfa et al. 2006). Indeed, genetic inactivation of *fmr-1* in mice results in an overall increase in protein synthesis (Dolen et al. 2007). Finally, FMRP is known to be involved in

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microRNA-mediated translational regulation (Li et al. 2008), broadening the spectrum of mRNAs and associated proteins whose expression and function are altered in FXS.

Although FMRP is typically discussed and studied as a brain disorder in the context of FXS, it is also expressed outside the CNS and fragile X patients exhibit symptoms related to dysfunctions of the gastrointestinal and immune systems, among others (Ashwood et al. 2010; Hagerman et al. 2008). Considering the widespread expression of FMRP and its fundamental function in protein synthesis, physiological alterations in FXS mothers during pregnancy could affect the in utero environment and consecutively the development of the fetus. Therefore, we propose to expand the role of FMRP further to include functions in the mother that are essential for fetal and postnatal development. This concept is not without precedent. Over 30 alleles, also called teratogenic alleles, that act in the mother to alter fetal development have been identified (Johnson 2003). These include genes linked to maternal metabolic diseases such as maternal phenylketonuria caused by the lack of phenylalanine hydroxylase (characterized by high maternal phenylalanine levels) and spina bifida that is related to maternal polymorphisms in the methionine synthase and methionine synthase reductase genes (Doolin et al. 2002; Rouse and Azen 2004). The list of maternal genes that affect offspring development could be expanded to include those that have an effect on maternal behavior postpartum and during early postnatal life, because maternal care also has a significant effect on offspring development and adult behavior (Meaney 2001).

Here, we propose that *FMR-1* can act not only as an offspring but also as a maternal gene in programming development. According to this dual, *fmr-1*-dependent mode of regulation of offspring development and behavior, illustrated in Fig. 13.1, the maternal *fmr-1* mutation modifies her physiology and behavior, and these changes become the abnormal environmental stimuli that then modify the developmental pattern of the offspring, ultimately contributing to the offspring's abnormal behavior.

In the following sections, we describe data that have, in part, led us to propose the above model. By using the fragile X mouse model, we show that maternal

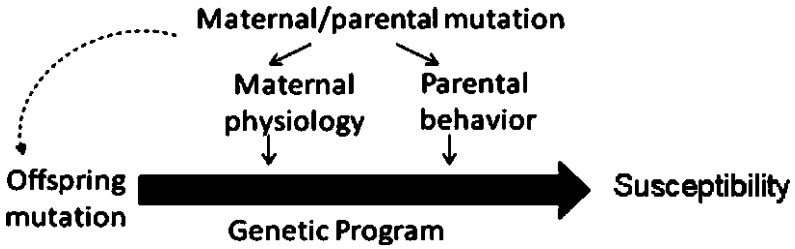

Fig. 13.1 Dual impact model: lack or a deficit in maternal FMRP can modulate (*arrow*) the developmental program establishing the offspring's adolescent/adult behavior, independently of whether the mutation was inherited (*dotted arrow*). However, the lack of FMRP in the mother can interact with the absence of FMRP in the offspring, resulting in a more serious phenotype

genotype-dependent modifications of the environment contribute to lifelong activity changes in the offspring. In addition, we demonstrate that maternal genotype-dependent environmental effects can persist even in offspring that are unaffected by the genetic abnormality which can expand the population that is potentially at risk for neurodevelopmental abnormalities.

13.2 Evidence for a Maternal *fmr-1* Genotype Effect on Offspring Behavior

Since the genetic revolution of the 1990s, the use of mouse models to study human disorders has become quite widespread and common. The fragile X mouse model is based on a "knockout" (KO) mouse line in which the insertion of a neomycin sequence into the *fmr-1* gene interrupts its translation and effectively silences gene expression (Consortium 1994). An early strategy for obtaining KO mice and their appropriate wild-type (WT) controls involved the maintenance of KO and WT animals in separate breeding lines. A more recent breeding strategy, however, is to derive KO and WT littermates by breeding heterozygous (H) parents, or in the case of the X-linked *fmr-1*, by breeding H (technically mosaic) females with WT males. The latter strategy was adopted because the phenotype of KO animals bred separately from their WT controls could be confounded by genetic drift. On the contrary, the littermate breeding strategy is also not without problems. WT mice derived from H parents may have phenotypic abnormalities due to parental effects and, therefore, may not represent "normal" behavior. The only genuinely WT animals free of genetic or parental environmental effect are WT mice derived from non-littermate WT parents. Therefore, to separate the effects of genetic and nongenetic factors on fragile X-related behavioral and physiological traits, we applied a combination of the littermate and non-littermate breeding strategies and compared three groups of animals in parallel. Figure 13.2 illustrates this breeding design. We bred, in a non-littermate fashion, WT offspring from WT parents to obtain "normal" controls identified as $WT_{\text{offspring genotype}}(WT_{\text{maternal genotype}})$. In

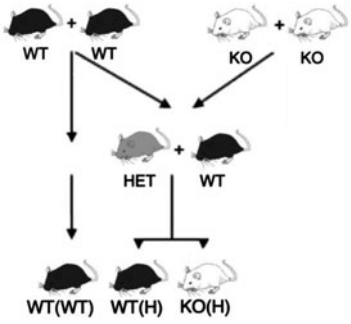

Fig. 13.2 Breeding strategy to generate WT(WT), WT(H), and *fmr-1* KO(H) mice by litter- and non-littermate mating. Breeding involved WT (*black*), H (*gray*), and KO (*white*) mothers

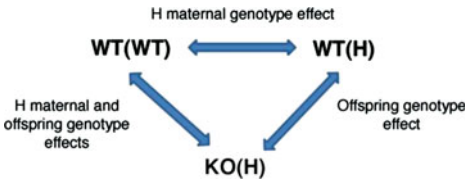

Fig. 13.3 Comparison of genetic and nongenetic maternal effects on behavior

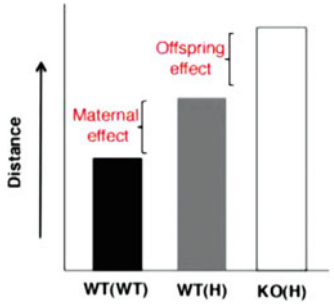

Fig. 13.4 Maternal *fmr-1* genotype-dependent effect on locomotor activity. Activity is measured as distance moved over a 2-h period in WT(WT), WT(H), and KO(H) mice

addition, we obtained H females and bred them with WT males to provide littermate WT and KO male offspring, identified as WT(H) and KO(H) offspring.

By testing the three groups of animals in parallel, we were able to discern between the genetic and nongenetic effects on offspring phenotype. As illustrated in Fig. 13.3, comparing littermate WT(H) and KO(H) offspring allowed us to identify the phenotypes associated with the offspring's own genotype. Since their developmental environment was the same, phenotypic differences can in general be attributed to differences in their own genotype alone. Comparing WT(WT) from non-littermate breeding with WT(H) from littermate breeding allowed us to determine if the WT(H) mouse phenotype had been modified by a nongenetic, maternal genotype-dependent mechanism. Finally, we obtained the combination of genetic and nongenetic effects on offspring behavior by comparing WT(WT) to KO(H) mice.

Using the aforementioned breeding strategy, we assessed some fragile X-associated traits, including hyperactivity, sensory hyperreactivity, seizure susceptibility, and macroorchidism. As reported (Zupan and Toth 2008a), we found that during a 2-h locomotor activity test, the two groups of genetically identical WT animals exhibited different patterns of activity. Specifically, WT(H) animals exhibited higher levels of total locomotor activity than WT(WT) mice (Fig. 13.4). These data indicated that the partial maternal *fmr-1* deficiency was sufficient to induce a maternal genotype-dependent effect on offspring behavior. However, lack of *fmr-1* expression in the offspring also resulted in hyperactivity demonstrated by the higher locomotor activity of the KO(H) than the WT(H) offspring despite the fact that the WT(H) offspring, due to the H maternal environment, already showed increased activity. Therefore, the higher locomotor activity of the KO(H) mice is probably the result of the combination of the maternal and the offspring genotype effects. Upon more detailed examination of locomotor activity, we found that the

hyperactivity of WT(H) mice was apparent only after 20 min of the beginning the test and, therefore, was not a novelty induced but rather a constitutive hyperactivity, typically found in FXS.

In addition to hyperactivity, we also assessed sensory hyperreactivity in our three groups of mice using the startle and pre-pulse inhibition paradigms. Here, we failed to observe any maternal genotype-dependent effects, and as we have reported, animals lacking Fmrp showed decreased startle and enhanced pre-pulse inhibition compared to genetically normal mice (Yun et al. 2006; Zupan and Toth 2008a). Maternal genotype also had no effect on audiogenic seizure susceptibility, or on macroorchidism, both traits distinctly associated with the lack of Fmrp in the offspring (Zupan and Toth 2008a). In all, these findings showed that at least one of the behavioral traits associated with the lack of Fmrp in the mouse model of fragile X is determined not only by the subject's own genetic makeup, but also by the maternal Fmrp, or lack thereof. Some of our findings with respect to the neurobiology of the maternal effect are discussed in the following section, but the actual mechanism(s) of nongenetic transmission of hyperactivity remain unknown.

13.3 Evidence for a Maternal Genotype Effect on Dopamine Neurotransmission

Locomotor activity is regulated in part by the activity of mesolimbic dopamine neurons originating in the midbrain, specifically in the ventral tegmental area, which project to the striatum (Koob and Swerdlow 1988; Szczypka et al. 2001). Hyperactivity has been linked to low tonic dopamine activity promoted by dopamine (DA) D2 autoreceptors and high phasic dopamine neurotransmission (Grace 2001). Activation of D2 autoreceptors, by reducing the amount of DA released into the synapse (presynaptically) and reducing the excitability of the DA neurons (in the somatodendritic compartment), inhibits locomotor activity in rodents (Starke et al. 1989; Cory-Slechta et al. 1996; Usiello et al. 2000) (Fig. 13.5). In addition to the D2 autoreceptors, the activity of mesolimbic DA neurons is regulated by inhibitory $GABA_B$ receptors, which are also located at both the presynaptic and somatodendritic regions of the DA neurons. Presynaptic $GABA_B$ receptors inhibit DA release, while receptors located somatodendritically reduce the firing rate of DA neurons (Engberg et al. 1993; Smolders et al. 1995; Madden and Johnson 1998; Labouebe et al. 2007) (Fig. 13.5). Like D2 auto receptor activation, agonist-induced activation of $GABA_B$ receptors has been shown to reduce locomotor activity in rodents (Cott et al. 1976). In all, both the D2 autoreceptor and the $GABA_B$ receptor regulate locomotor activity via modulation of mesolimbic DA neuron activity.

We probed the function of both these receptors using pharmacological agents to determine if the maternal genotype-based locomotor effects observed in WT(H) mice were in part mediated by altered dopamine and/or gabaergic neurotransmission. By administering quinpirole, a D2/D3 receptor agonist, at low, D2

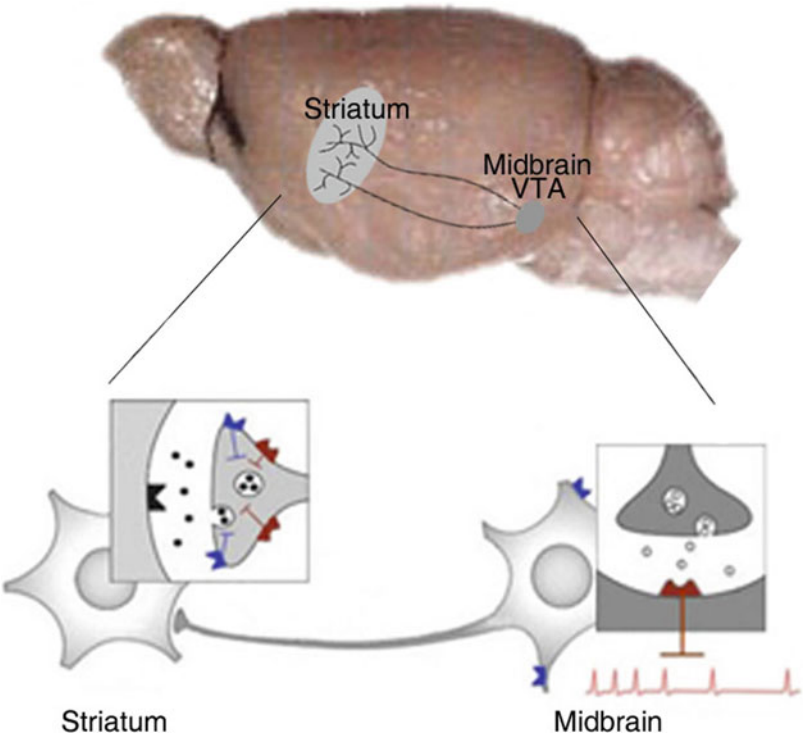

Fig. 13.5 Mesolimbic dopamine neurons. In *blue* are DA D2 autoreceptors located both presynaptically and somatodendritically on the dopamine neurons. In *red* are $GABA_B$ receptors. *VTA* ventral tegmental area

autoreceptor preferring doses, we found that both WT(H) and KO(H) hyperactive mice had reduced D2 autoreceptor activity observed as the attenuated locomotor-suppressing effect of the drug (Zupan and Toth 2008a) (Fig. 13.6). This and additional experiments reported earlier (2008b) lead us to speculate that the reduced quinpirole effect may be due to increased D2 autoreceptor desensitization in the hyperactive mice. Interestingly, unlike the behavioral phenotype of increased locomotor activity in which the WT(H) mice exhibited an intermediate phenotype, here the WT(H) and KO(H) mice were not different (see Fig. 13.6). These findings suggested that the reduced D2 autoreceptor function in hyperactive mice is related to the absence/reduction in Fmrp in the mother rather than in the offspring.

As with the D2 autoreceptor, we assessed $GABA_B$ receptor function by measuring locomotor activity following the administration of the $GABA_B$ agonist baclofen, at doses that had no sedative effects (Zupan and Toth 2008b). In contrast to the reduced D2 autoreceptor function in the hyperactive mice, we found that the $GABA_B$ receptor activity was enhanced in both WT(H) and KO(H) mice relative to that in WT(WT) controls (Fig. 13.6). Specifically, doses that had no locomotor-suppressing effects in the WT(WT) mice produced significant reduction in

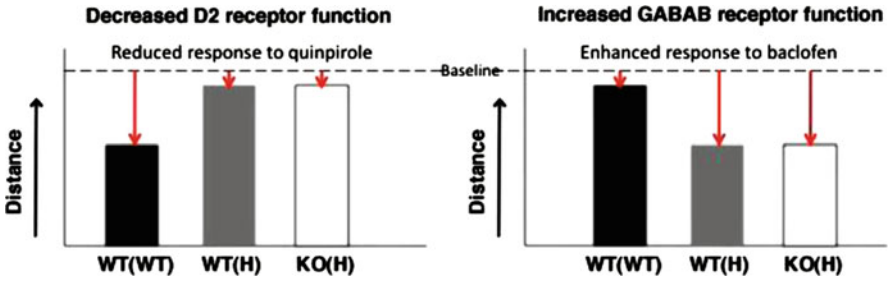

Fig. 13.6 Reduced D2 auto receptor and enhanced $GABA_B$ receptor functions are dependent on the maternal *fmr-1* genotype. Reduced D2 function determined by an attenuated locomotor-suppressing response following quinpirole administration in both WT(H) and KO(H) mice. Enhanced $GABA_B$ receptor function observed as a sensitization to the locomotor-suppressing effects of baclofen

locomotor activity in the offspring of H mothers (Zupan and Toth 2008b). Since a difference was seen between H-derived and WT-derived mice but not between H-derived WT and KO offspring, the increased $GABA_B$ receptor activity, as with the reduced D2 autoreceptor function, was dependent on the maternal rather than the offspring's own *fmr-1* genotype (Figs. 13.3 and 13.6). Taken together, findings from the quinpirole and baclofen experiments lead us to hypothesize that the maternal genotype effect responsible for the hyperactivity in both WT and KO mice born from H females also modifies the function of the D2 autoreceptors and $GABA_B$ receptors. Based on our data, however, we cannot determine whether one of these functional changes is primary and the other a compensatory effect, or if they are both independently associated with the maternal *fmr-1* genotype.

13.4 Maternal Genotype Effects in Disease

Although the fragile X mutation has not been linked to maternal genotype-dependent disease manifestations in humans, genetic polymorphisms in maternal genes have previously been proposed to cause and/or contribute to several neurodevelopmental disorders. Lack of phenylalanine hydroxylase in mothers results in high maternal phenylalanine levels that cause various phenotypes in their children including seizures, microcephaly, and growth retardation (maternal phenylketonuria) and which are different from the symptoms of phenylketonuria in the mother (Rouse and Azen 2004). Also, maternal polymorphisms in the methionine synthase and methionine synthase reductase genes increase the risk for spina bifida (Doolin et al. 2002). There are additional similar examples, but they are all based on statistical data from relatively small populations that will need to be replicated to identify additional genes with maternal genotype effect (Johnson 2003). It is likely that many more maternal alleles exist but they have not been found because most association studies do not include the maternal genotype. Indeed, an interesting

recent study reported maternal genetic mutations in the tryptophan hydroxylase 1 (*TPH1*) gene, presumably because lower than normal maternal 5-HT levels increase the risk of attention deficit and hyperactivity disorder (ADHD) (Halmoy et al. 2010). Importantly, this report also concluded that paternal and offspring *TPH1* mutations had no effect on ADHD risk.

Maternal genetic effects can also influence postnatal development. Variability in maternal behavior, which probably has a genetic basis, has been found to regulate maternal care during early postnatal life (Meaney 2001; Weaver et al. 2004, 2005, 2006). Behaviors in rodents most closely associated with these effects involve patterns of maternal care, such as licking/grooming, arched-back nursing, nest quality, time spent with offspring, etc. Postnatal behavior of the dam can influence the adolescent and adult behavior of the offspring by modifying, at critical developmental periods, the development of neural circuits involved in emotional processing (Francis et al. 1999; Weaver et al. 2004).

13.5 Possible Modes of Transmission of the Maternal Genotype Effect

The behavioral and pharmacological experiments described above showed that the hyperactivity associated with the lack of *fmr-1* in the mouse model of fragile X disorder is not wholly induced only by the lack of the gene in the subject, but also by the decreased expression of the gene in their mother. The question, of course, is the nature of the signal related to the maternal Fmrp deficit, which reaches the fetus, altering its development and eventually the behavior of the adolescent and adult offspring. A prominent mechanism providing communication between mother and fetus is the immune system. The immune system utilizes cytokines and antibodies as signals that can be actively propagated across the placenta and the blood–brain barrier and could program offspring development (Maier 2003). Immunological mechanisms have long been proposed in schizophrenia and mood disorders, and more recently in autism. For example, it has been reported that expectant mothers suffering from asthma, allergies, and psoriasis (an autoimmune condition), all associated with abnormal antibody profiles, have a higher risk of giving birth to an autistic child, especially if diagnosed in the second trimester (Croen et al. 2005). A recent report confirmed the association between maternal autoimmune disease and autism (Atladottir et al. 2009). Furthermore, several reports described increased cytokine levels including those of TNF-α, interleukin 6, interleukin 12, and interleukin 1β in peripheral leukocytes and brain of individuals with ASD (Jyonouchi et al. 2001; Tonelli and Postolache 2005). Animal experiments reproduced these effects by showing that activating the immune system of pregnant mice produces autism-like behaviors in their offspring and that an antibody against interleukin 6 in the offspring mitigates this phenotype (Smith et al. 2007). Considering the overlap between autism and FXS, an immunological mechanism may also be involved in the maternal *fmr-1* genotype effect.

13.6 Implications

In genetic disorders with mendelian inheritance such as fragile X, the disease phenotype is believed to be exclusively related to the genetic defect in the offspring. In the mouse model, however, we still identified a disease-associated behavior that was not or was only partially dependent on the absence of Fmrp in the subject. Since FMRP levels were found to be reduced in conditions unrelated to FXS, maternal effects related to FMRP deficit may not only be relevant to FXS mothers with the full mutation. Specifically, significant reductions in FMRP levels were described in individuals with autism, schizophrenia, bipolar disorder, and major depressive disorder (Fatemi and Folsom 2010; Fatemi et al. 2010).

Importantly, the maternal genotype-dependent behavior was also observed in genetically normal offspring, indicating that disease-associated genetic mutation/variability can produce some degree of disease-associated behavior in genetically unaffected individuals. This finding expands the population that is potentially at risk for psychiatric disease. Moreover, this mechanism may explain, at least partly, the phenomenon of "missing heritability." Missing heritability refers to the discrepancy between high heritability (the proportion of variability in a population attributable to genetic variation among individuals) and the low contribution of genetically identifiable alleles seen in ADHD, autism, and many common disorders. Although missing heritability can be due to a large number of still unidentified alleles [such as copy number variants (CNVs)], some researchers argue that it is an aspect of biology that is not yet known. The maternal genotype effect is such a novel mechanism because it can increase heritability in the population or pedigree but is not detectable at the genetic level in the offspring. Because the disease manifestations are dependent on the mother's rather than the offspring's genotype, the identification of these conditions is difficult in conventional association studies. However, including the maternal genotype in genetic or environmental studies would help to identify maternal genetic variability and mutations that increase the disease risk. Once these maternal genetic factors are identified, the risk for the offspring can be assessed and preventive measures developed. Since the offspring are genetically unaffected, if maternal genotype effects are prevented from occurring, offspring brain and behavioral development would proceed unperturbed. In the case of FXS, a disease predominantly the result of an offspring genotype effect with a possible contribution by the maternal genotype, preventing the maternal effect could mitigate some, presumably psychiatric, symptoms.

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Chapter 14
Mouse Models of the Fragile X Premutation and the Fragile X Associated Tremor/Ataxia Syndrome

Michael R. Hunsaker, Gloria Arque, Robert F. Berman, Rob Willemsen, and Renate K. Hukema

Abstract The use of mutant mouse models of neurodevelopmental and neurodegenerative disease is essential in order to understand the pathogenesis of many genetic diseases such as fragile X syndrome and fragile X-associated tremor/ataxia syndrome (FXTAS). The choice of which animal model is most suitable to mimic a particular disease depends on a range of factors, including anatomical, physiological, and pathological similarities; presence of orthologs of genes of interest; and conservation of basic cell biological and metabolic processes. In this chapter, we will discuss two mouse models of the fragile X premutation which have been generated to study the pathogenesis of FXTAS and the effects of potential therapeutic interventions. Behavioral, molecular, neuropathological, and endocrine features of the mouse models and their relation to human FXTAS are discussed.

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14.1 Introduction

The *FMR1* gene is polymorphic for the length of a tandem CGG trinucleotide repeat in the 5′ untranslated region (UTR). In the general population there are fewer than 55 CGG repeats [mean 30 – Hagerman (2008)]. In some individuals there is a repeat expansion wherein the number of CGG repeats expands beyond 200 repeats in length (i.e., full mutation – FM), and this is associated with *FMR1* promoter and CpG island hyper-methylation and subsequent gene silencing – leading to no measurable *FMR1* transcription and no FMRP translation and Fragile X Syndrome (FXS; Hagerman and Hagerman 2004). This FM occurs in roughly 1:4,000 males and 1:6,000 females, and virtually all FM males will develop FXS and 60% of FM women will develop FXS. CGG repeat lengths between those found in the general population and the FM are called the Fragile X premutation (55–200 CGGs; PM) zone and occurs in ~1:130–200 females and 1:800 males (Hagerman 2008). CGG trinucleotide repeat lengths in the PM were historically considered to lack a clinical phenotype, so the PM was used as a descriptor to emphasize the high probability for the PM to maternally expand into the FM across subsequent generations (Hagerman 2008; Hagerman and Hagerman 2004; Jacquemont et al. 2004; Kraff et al. 2007; Leehey et al. 2007, 2008; Senturk et al. 2009).

In 2001, a late onset neurodegenerative disorder called Fragile-X associated tremor/ataxia (FXTAS) was described in a subset of elderly carriers of PM alleles (Hagerman et al. 2001). FXTAS patients exhibit gait ataxia, intention tremor, and Parkinsonism, as well as presence of eosinophillic, ubiquitin-positive intranuclear inclusions in neurons and astrocytes throughout the brain (Greco et al. 2002, 2006, 2007, 2008; Tassone et al. 2004a). This finding, along with the findings that elevated *FMR1* mRNA levels and concomitant mild reductions in FMRP levels are associated with the PM (Tassone et al. 2000a,b,c, 2004b, 2007; Tassone and Hagerman 2003), has led to the proposal that FXTAS is the result of an RNA gain of function resulting in cellular toxicity, similar to myotonic dystrophy (Garcia and Hagerman 2010; Raske and Hagerman 2009; Sellier et al. 2010; Tassone et al. 2000a). What remains unclear in FXTAS is the cause of incomplete penetrance of FXTAS within PM carriers: in PM carriers from known fragile X probands, only 30% of the males and 10–15% of the females may develop FXTAS, a number that may be lower if samples were ascertained through non-fragile X probands (Jacquemont et al. 2003, 2004).

14.2 Mouse Models of the Fragile X Premutation and FXTAS

The first mouse models were initially developed to model repeat instability and potential expansion to FM across generations. However, these transgenic mouse models, both within and outside the context of the *FMR1* gene, did not show instability in the trinucleotide repeat length (Bontekoe et al. 1997; Lavedan et al. 1997, 1998).

The first model to be reported as a putative model for the PM and potentially FXTAS was the CGG Knock-In mouse model (CGG KI), which was generated by a homologous recombination whereby the endogenous mouse CGG repeat (CGG8) was replaced with a PM length CGG repeat of human origin (CGG98) on the endogenous mouse *Fmr1* promoter (Bontekoe et al. 2001; Willemsen et al. 2003). These CGG KI mice, with minimal changes to the endogenous mouse *Fmr1* promoter, showed moderate instability upon paternal and maternal transmission, and both expansions and contractions have been observed (Brouwer et al. 2007). Later, another CGG-CCG knock-in mouse (CGG-CCG mouse) was developed wherein CGG-CCG repeats (CGG-CCG124) were serially ligated and expressed in the endogenous mouse CGG repeat on the endogenous promoter (Entezam et al. 2007). This model also shows a trend toward gradual increases in CGG (or CGG-CCG) repeat lengths. Furthermore, the CGG-CCG mice show the same general pattern of repeat instability as that reported in the PM, namely that the paternal mutation shows small repeat expansions, and this expansion occurs preferentially in mice lacking ATM, with a bias toward greater expansions in males (Entezam and Usdin 2008, 2009).

Maternally transmitted mutations show larger repeat expansions that occur preferentially in mice lacking ATR. These results support models proposed in the human PM research concerning the differential expansion of male–female PM alleles into FM alleles across generations.

It has recently been reported that there may be environmental contributions to the CGG repeat instability in humans, or at least a contribution of environmental factors in the time course of neurodegeneration (Paul et al. 2010). The CGG-CCG mouse has been used to determine the role of oxidizing agents on CGG-CCG repeat expansion. When a DNA oxidizing agent is introduced to CGG-CCG mice, there appears to be a higher frequency and size of repeat expansions (Entezam et al. 2010). The authors suggest that such oxidizing agents may play a role in CGG repeat expansion seen in the PM and FXTAS.

Recently, another model of FXTAS has been developed in mice (Hashem et al. 2009). These mice used constructs and promoters either independent of the *Fmr1* gene or used non-*Fmr1* promoters. These mice specifically express CGG90 RNA in Purkinje cells with either *Fmr1* or eGFP. Therefore these models target the implications of CGG90 mRNA overexpression for FXTAS. These models expressing an expanded CGG RNA without the context of the *Fmr1* gene are very promising for the study of the RNA gain of function hypothesis.

There is another transgenic mouse model, into which a 1,057 bp fragment of genomic DNA from *FMR1* including the translation initiation site and a repeat of 26 CGG repeats was cloned (Baskaran et al. 2002). These mice show intergenerational instability during both male and female transmission. Baskaran et al. (2002) find methylation in lines lacking repeat expansion and absence of methylation in lines that do show expansion, indicating that methylation and expansion are potentially independent events. This mouse model will not be covered in this chapter, as this mouse serves as a better model for *Fmr1* CGG repeat expansion and gene methylation and thus is a better model for FXS than for FXTAS.

14.3 Utility of CGG KI and CGG-CCG Mice for the Study of FXTAS

As FXTAS is a late onset neurodegenerative disorder, it is difficult to determine precisely the factors that may contribute to the cellular dysfunctions thought to underlie the disease progression across the lifespan of any individual. In FXTAS patients we can only study the end-stage of the disease progression in brain tissue. The benefit of evaluating mouse models of neurodegenerative disorders is the relative shortness of the mouse lifespan. If a researcher wished to determine the natural history of the disease process in FXTAS, both the CGG KI and CGG-CCG mouse models will serve to provide invaluable insight (see Table 14.1).

The CGG KI mouse has been used to evaluate the hypothesis that FXTAS, a late onset neurodegenerative disorder, may be the end stage of earlier, perhaps even neurodevelopmental, effects accumulated across the lifespan (Hagerman and Hagerman 2004; Bourgeois et al. 2011; Cornish et al. 2008a, 2009; Garcia-Arocena and Hagerman 2010). Recently, it has been shown that the CGG KI mouse shows abnormal cortical neuron differentiation and migration patterns in utero (Cunningham et al. 2011). Furthermore, it has been demonstrated in vitro, using primary neuronal cultures from the CGG KI mice, that immature neuronal morphologies predominate (thinner, filapodial dendrites), and reduce cellular viability (Chen et al. 2010). It has also been shown in vivo that CGG KI mice as young as 12 weeks of age show ubiquitin-positive intranuclear inclusions in neurons and astrocytes in the hippocampus and only later similar pathological features appear to develop in the parietal neocortex (Hunsaker et al. 2009). Similarly, intranuclear inclusions are present in the internal granule cell layer in the cerebellum at 12 weeks of age (MR Hunsaker, unpublished observations). These data suggest that there are developmental influences that may contribute to later neurodegenerative processes, or at least that the progressive neuropathology begins to form relatively earlier in life than previously thought.

Table 14.1 Comparison of FXTAS with CGG KI and CGG-CCG FXTAS mouse models

	FXTAS	CGG KI mouse	CGG-CCG mouse
Molecular measures			
CGG Repeat	55–200 repeats	70–350 repeats	120 to >200 repeats
FMR1 mRNA	2–8-fold increase (blood)	3–5-fold increase (brain)	2–6-fold increase (brain)
FMRP Level	Slightly reduced	Slightly reduced	Markedly reduced
Neuropathology			
Inclusions	Neurons and astrocytes	Neurons and astrocytes	In cells of brain
Gross Pathology	Purkinje cell dropout	No gross pathology	Purkinje cell dropout
Motor Function	Tremor and ataxia	Motor deficit with age	Normal motor function
Cognition			
Social	Social anxiety	–	Reduced sociability
Anxiety	Anxiety disorders	Elevated anxiety	Reduced anxiety
Memory	Poor memory	Memory impairments	Memory impairments

14.3.1 Modeling Molecular Correlates of FXTAS in CGG KI and CGG-CCG Mice

Both CGG KI and CGG-CCG mice have been used to evaluate the molecular cascades associated with the PM that potentially underlie FXTAS pathophysiology. The brains of the CGG KI mouse show elevated *Fmr1* mRNA levels and reduced Fmrp levels, similar to those observed in the PM and FXTAS (Tassone et al. 2000a, b, 2004a, 2007; Tassone and Hagerman 2003; Brouwer et al. 2007, 2008a,b, 2009a, b; Entezam et al. 2007). An average of twofold elevation in *Fmr1* mRNA levels was detected as early as 1 week of age in CGG KI mice that persisted throughout development (Willemsen et al. 2003). In contrast to what was reported for the linear correlation between *FMR1* mRNA levels and the repeat size in human FXTAS patients (Kenneson et al. 2001), the increase in *Fmr1* mRNA levels was not correlated with the length of the repeat (Brouwer et al. 2008a). However, the data from the human patients were not from brain samples, but from blood samples or lymphoblasts. Entezam et al. (2007) were able to show a direct relationship between CGG-CCG repeat size and *Fmr1* mRNA levels in the brains of the CGG KI mice, although the number of mice studied for the different repeat sizes was limited. Despite the increase in mRNA levels, both the CGG KI and the CGG-CCG mouse strain show an inverse correlation between CGG repeat length and Fmrp expression in the brain (Entezam et al. 2007; Brouwer et al. 2008c). One explanation is that the CGG repeat hampers the initiation of translation at the ribosome, possibly due to secondary structures formed.

14.3.2 Modeling Cellular Dysfunction Associated with FXTAS in CGG KI and CGG-CCG Mice

The CGG KI mouse has been used (in concert with engineered human cell lines) to demonstrate potential interacting partners of the CGG-expanded Fmr1 mRNA to directly test a model that suggest the CGG repeat itself acts to sequester proteins from the cell and by that mechanism causes cellular dysfunction (Raske and Hagerman 2009; Garcia-Arocena and Hagerman 2010). For example, it was demonstrated that Sam68, a splicing factor, is sequestered by the CGG repeat expansion and thus subsequently titrated out from the rest of the cell. This results in reduced Sam68-dependent splicing events, which may be involved in the events leading up to inclusion formation as increasing Sam68 expression can prevent aggregate formation in mouse and cell lines (Sellier et al. 2010).

The CGG KI mouse has also been used to evaluate more systems level disruptions that may be present in the PM and FXTAS. In addition, the CGG KI mouse has been used to demonstrate altered expression of GABA-B receptors in the cerebellum but not neocortex (D'Hulst et al. 2009), as well as to demonstrate abnormalities along the HPA axis and amygdala similar to those proposed in PM

and FXTAS that might explain the molecular mechanisms underlying the psychopathology in PM carriers and FXTAS patients (Brouwer et al. 2008b).

14.3.3 Modeling Pathological Features of FXTAS in CGG KI and CGG-CCG Mice

Pathologic neuroanatomical features have been demonstrated in the CGG KI mice that appear to phenocopy human FXTAS. Greco et al. (2006) evaluated gray and white matter of brain in a number of cases of FXTAS and found a relatively large percentage (1–5%) of neurons and astrocytes in the brain contained eosinophillic intranuclear inclusions. White matter pallor and apparent thinning of the gray matter were also reported, as well as Purkinje cell dropout and axonal pathology such as torpedo axons in the cerebellum. Both the CGG KI and the CGG-CCG mouse have intranuclear inclusions in neurons throughout the brain (Willemsen et al. 2003; Entezam et al. 2007; Hunsaker et al. 2009; Brouwer et al. 2008a,b; Wenzel et al. 2010) and the CGG KI mouse has further been shown to have intranuclear inclusions in astrocytes, as well as neurons (Wenzel et al. 2010; Fig. 14.1). In addition to the presence of intranuclear inclusions in neurons inclusion presence or absence in astrocytes has not been reported, the CGG-CCG mouse shows reduced numbers of Purkinje cells and evidence for torpedo axonal morphology similar to that reported in FXTAS (Entezam et al. 2007).

In the CGG KI mouse, the distribution of intranuclear inclusions has been carried out in mice ranging from 20 to 72 weeks of age (Willemsen et al. 2003). The analysis

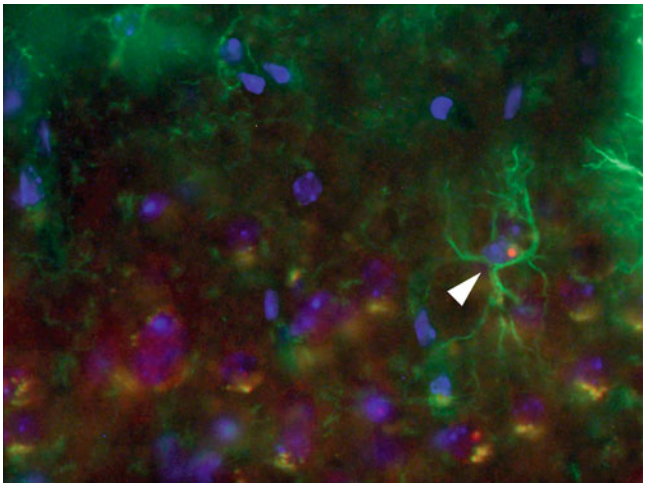

Fig. 14.1 Astroglial cell containing an ubiquitin positive intranuclear inclusion (*white arrow head*) in the motor cortex of a 70 week old female CGG KI mouse with 9, 128 CGG repeats. Green = GFAP, red = ubiquitin, blue = DAPI

suggested that CGG KI mouse displays progressive neuropathological features (i.e., inclusions) that are most prominent in the rostral cortices, hypothalamus, olfactory nucleus, parafasicular nucleus of the thalamus, the inferior colliculus, pontine nuclei, vestibular nucleus, superficial dorsal horn of the spinal cord, and 10th cerebellar lobule. A later study further quantified intranuclear inclusion presence in the pituitary gland and amygdala (Brouwer et al. 2008b). Further analysis of CGG KI mice replicated these findings in a limited sample, but saw a much greater quantity of intranuclear inclusions in the hippocampus, particularly in the dentate gyrus (Brouwer et al. 2008c; Wenzel et al. 2010). The CGG-CCG mouse showed similar inclusions, but no regional quantifications were presented (Entezam et al. 2007).

An intriguing pattern can be seen in the distribution of the relatively early presence of intranuclear inclusions in the more primitive cortical structures, and later presence in more evolutionarily recent cortices (cf., Willemsen et al. 2003). A follow-up analysis of the distribution of intranuclear inclusions undertaken by Wenzel et al. (2010) and to a lesser extent Hunsaker et al. (2009) demonstrated that granular cells within the olfactory bulb, cerebellum, and dentate gyrus show the highest quantity of intranuclear inclusions (roughly 50% of neurons), followed by subcortical structures including the hypothalamus, thalamus, inferior colliculus, septal nuclei, various brainstem nuclei, and the cerebellum. In the cortex, the paleocortex associated with the amygdala and hippocampus and the entorhinal cortex (transitional cortex) show the greatest quantity of inclusions, followed by the limbic cortex and finally the rostral (i.e., sensory and motor cortices) and caudal (i.e. parietal and visual cortices) neocortex. This pattern suggests the potential for a primarily subcortical and limbic involvement in the neuropathology that spreads to the neocortex later in life.

Although the CGG KI and CGG-CCG mouse models appear to provide very good models for the primary neuropathological features present in FXTAS, there are a number of very important differences between the species that needs to be discussed. In FXTAS, a higher percentage of astrocytes in both the grey and white matter contain intranuclear inclusions compared to the local neuron populations (Greco et al. 2002, 2006; Wenzel et al. 2010). Furthermore, in FXTAS the intranuclear inclusions stain easily for eosin in a hematoxylin and eosin (H&E) stain, whereas the inclusions in mice are more difficult to stain – requiring the use of immunocytochemical techniques to identify the presence of intranuclear inclusions, or at least a careful optimization of H&E staining protocols (cf., Willemsen et al. 2003, Fig. 14.2). The reason for these differences is unclear and most likely does not affect the interpretation of the findings in the mouse models; the fundamental differences between species needs to be considered in all studies of comparative neuropathology resultant from the PM. On the other hand, this may be caused by the fact that we study the end stage of the disease in FXTAS patients and the mice we studied might not have reached this stage. These findings highlight the need to study the development of disease progression in the mice instead of focusing solely on the final stage in patients.

What remains unknown about the role of these neuropathological features in the PM and FXTAS is the developmental time course of inclusion formation as well

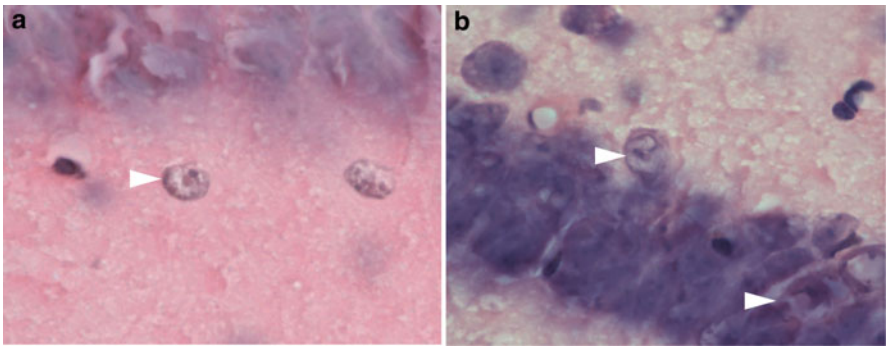

Fig. 14.2 **(a)** H&E stained hippocampus demonstrating an interneuron in the stratum radiatum of CA1 with an intranuclear inclusions (*arrow head*). **(b)** H&E stained hippocampus demonstrating CA1 pyramidal cells with intranuclear inclusions (*arrow heads*). Both images are from a 52 week old female CGG KI mouse with 8, 152 CGG repeats

as the role of these inclusions in cellular processing/toxicity. The first of these questions has been preliminarily addressed, for example, using cellular models (Sellier et al. 2010), but no work to date has evaluated CGG KI or CGG-CCG tissue at ages <12 weeks of age. Such work is necessary to determine a potential age where the brain is free from pathological features to evaluate preventative treatment strategies. However, the Purkinje cell specific transgenic mice (Hashem et al. 2009) very nicely show that the formation of inclusions also occurs when expressing expanded CGG RNA independent of *Fmr1* context, suggesting a strong role for tandem CGG repeat containing RNA toxicity in intranuclear inclusion formation.

14.3.4 Modeling Behavioral Sequelae of FXTAS in CGG KI and CGG-CCG Mice

Until recently, the PM was thought to be free of behavioral and molecular sequelae (Hagerman and Hagerman 2004; Cornish et al. 2005, 2008b, 2009). Once it was determined that there were potential aberrant behavioral and psychiatric phenotypes in the PM prior to FXTAS, the study of the mouse models were expanded to model these phenotypes. Unfortunately, neither the CGG KI nor the CGG-CCG mouse shows classic tremor or ataxia on basic behavioral assays (Van Dam et al. 2005; Qin et al. 2011). This lack of a clear motor phenotype suggests that either the mouse models are lacking, or there are differences between species that prevent potential motor phenotypes from being observed (i.e., methodological differences in tests between species, bipedal gait in humans vs. quadrapedal in mice, etc).

The CGG-CCG mouse has only been preliminarily evaluated for a behavioral phenotype. The CGG-CCG mouse has been shown to be slightly hyperactive and

shows reduced anxiety in the open field and elevated zero mazes. Furthermore, the CGG-CCG mouse shows impaired passive avoidance learning and a slight reduction in social interaction (Qin et al. 2011). They interpret these results to indicate a subtle deficit similar to those reported in the *Fmr1* KO model of FXS.

The CGG KI mouse has been evaluated for the cognitive deficits present in the PM and FXTAS. Van Dam et al. (2005) demonstrated a clear age-related worsening of motor performance on the accelerating rotarod and memory impairments on the water maze. To further characterize these deficits, Hunsaker et al. (2009) evaluated spatial processing in CGG KI mice using tasks designed to more specifically evaluate spatial processing than the water maze. They found that CGG KI mice showed significant deficits in spatial processing compared to littermate control animals as early as 12 weeks of age. On a similar task involving learning the relationship between objects and their location in space, the same mice showed deficits only at 48 weeks of age. Intriguingly, in a separate group of animals, Hunsaker et al. (2009) evaluated the presence of intranuclear inclusions in the dentate gyrus in the hippocampus (which subserves performance in the first task) and the parietal cortex (which subserves performance in the second task) (cf., Goodrich-Hunsaker et al. 2005, 2008). They found that there were inclusions (albeit low in number) in the dentate gyrus of the CGG KI mice as early as 12 weeks of age and progressively more with increasing age. Intranuclear inclusions were only detectable in the parietal cortex at 48 weeks of age. These findings suggest that the development of neuropathology follows a similar time course as the emergence of behavioral dysfunction in the CGG KI mouse, implying a potential neuropathological correlate to the spatial processing deficits.

In a subsequent experiment, female CGG KI mice were tested for their ability to learn and remember short sequences of stimuli. In this task, the mice were presented with three pairs of visual objects for 5 min each separated by 5 min intervals. Afterward, the mice were presented with two tests, one for temporal order, wherein the first object and the last object encountered were presented and the mouse was allowed to preferentially explore. The second test was for novelty, and the first object encountered and a novel, never before seen object was presented. Female CGG KI mice showed a CGG-repeat length-dependent deficit for learning and remembering sequences. Mice with 80–100 CCG trinucleotide repeats performed worse than wild type littermate mice, but performed better than mice with 140–190 CGG repeats. All animals performed the novelty task equally well (Hunsaker et al. 2010). These data suggest that temporal processing is deficient in CGG KI mice. What makes this finding all the more intriguing is that these data were from female mice, who should be 50% as affected as male mice, and thus should show a more subtle phenotype. As such, male mice should show much more profound deficits on the same task; however, this has yet to be assessed.

To better evaluate the cognitive and behavioral phenotypes in CGG KI mice, there is a need to develop a number of novel tasks to more precisely evaluate specific behaviors proposed to be affected by the PM and FXTAS. As it has been suggested previously that the traditional tasks evaluating motor function often miss subtle pathology, task development is needed in this arena.

In order to identify and potentially quantify more subtle motor deficits, Hashem et al. (2009) evaluated mice with expanded CGG repeats expressed from the L7/pcp2 promoter in cerebellar Purkinje cells on the rotarod measure of motor function. They found that these mice showed age-related deficits in the rotarod (i.e., the mice fell from the rod at slower speeds and were unable to stay on the rotating drum as long as controls even at slow speeds).

These findings suggest motor deficits in the mouse models of FXTAS, but to date such robust findings using the rotarod have not been found in the other FXTAS mouse models. However, Van Dam et al. (2005) did find a mild rotarod phenotype in old CGG KI mice. The Purkinje-specific transgenic mice demonstrate that overexpression of the expanded CGG RNA in Purkinje cells is sufficient to cause motor dysfunction.

As the primary tremor present in FXTAS is an intention tremor, it may be worthwhile to evaluate CGG KI and CGG-CCG mice on a skilled forelimb reaching tasks that allow precise quantification of limb use. Such tasks may uncover subtle tremor missed on tests of more gross motor function (Alaverdashvili and Whishaw 2008; Blume et al. 2009; Farr et al. 2006; Farr and Whishaw 2002; Metz and Whishaw 2002, 2009; Ward 1997; Whishaw and Metz 2002; Whishaw et al. 2010). To better model the gait ataxia, skilled walking tasks similar to those used in grid walking paradigms could be applied as they are in models of alcohol intoxication that allow for similarly specific quantification of walking behavior.

Another common cognitive disruption in FXTAS is a sort of dysexecutive syndrome (Brega et al. 2008) involving cognitive control and attentional processing. Although difficult to model in mice, tasks such as the five choice serial reaction time task or biconditional discrimination tasks can be used to model these processes (George et al. 2010; Haddon et al. 2008; Marquis et al. 2007). Similarly, there are attentional tasks in rats that can be modified for mice that can get at specific attentional processes affected in FXTAS (Ward 1997; Ward and Brown 1996).

Furthermore, as the parietal lobe appears to be atrophied in FXTAS, tasks specifically evaluating parietal functions need to be performed in mice [similar to the second task mentioned above from Hunsaker et al. (2009)]. As the time course for the development of neuropathological features has been described in the CGG KI mouse, this mouse provides a unique opportunity to thoroughly evaluate the specific hypotheses concerning the role of molecular factors that may be underlying the neurocognitive deficits present in the PM and FXTAS.

14.4 Utility of CGG KI and CGG-CCG Mice for Interventional Studies

To date, no therapeutic studies have been performed on any of the FXTAS mouse models, primarily because there were no clearly defined behavioral outcome measures and no real biomarkers to speak of. The primary difficulty present in evaluating therapies in the FXTAS mouse models is the fact that FXTAS is defined

as a late onset neurodegenerative disorder characterized by a motor phenotype. This means that, in theory, animals have to be set aside for the better part of a year prior to treatment and then the outcome measures (i.e., latency to fall on the accelerating rotarod) are not all that clear cut. One potential solution to this problem is to use the mouse models reported by Hashem et al. (2009) for evaluating treatments of the motor phenotype. In these mice the motor phenotype is specifically exaggerated in those mice at an age earlier than either the CGG KI or CGG-CCG mice; however, these mice are transgenic and express the CGG repeat in Purkinje cells, not all cells, so this model is incomplete from a clinical perspective.

To better dissect the respective roles of different molecular factors for FXTAS disease progression, further/new transgenic mouse models need to be generated to identify the respective roles of different cell types for FXTAS. The development of transgenic mouse models expressing an expanded CGG RNA in different cell populations at higher levels will facilitate the design of experiments evaluating sufficiency, necessity, and timing of disease progression. The generation of inducible mice will facilitate research into treatment options and outcomes, as well as answer questions concerning the potential reversibility of neuropathology and aid in developing pharmaco- and gene-targeted therapies.

The CGG KI mouse develops subtle behavioral phenotypes that appear to be present from ages as early as 12 weeks or earlier [though the animals have not been tested earlier than 12 weeks of age (Hunsaker et al. 2009, 2010)]. This mouse model, however, does not show motor deficits in the rotarod until advanced ages (Van Dam et al. 2005). A combined strategy of using the CGG KI and the transgenic mice expressing CGG repeats in Purkinje cells to model different aspects of the FXTAS disease process may provide valuable insights into the nature of behavioral and motor problems in FXTAS.

Finally, an additional outcome measure may be to evaluate effect or stress responses in the CGG KI mouse. As Brouwer et al. (2008b) showed CGG KI mice exhibit abnormal HPA activity, which correlated with an abnormal stress response in the amygdala. If these findings extend earlier in life similar to the behavioral measures, then reversing a dysfunctional HPA axis/stress response may provide benefit to FXTAS.

14.5 Conclusion

The CGG KI and CGG-CCG mouse models for the fragile X PM and FXTAS provide an invaluable resource for the translational scientist to generate and evaluate hypotheses into the molecular correlates of FXTAS disease onset and progression. These mouse models further provide outcome measures and putative biomarkers that may aid in the development and evaluation of therapeutic interventions.

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Chapter 15
Clinical Aspects of the Fragile X Syndrome

W. Ted Brown

Abstract Fragile X syndrome patients express a wide array of cognitive and other gender-specific phenotypic features. These manifestations result not only from molecular mechanisms that are altered as a result of the expansion of a CGG-repeat region in the *FMR1* promoter, but also genetic factors such as founder effects and mosaicism. In this chapter, I will summarize the many and varied features of fragile X syndrome as they present themselves in a clinical setting and describe the procedures that are used to diagnose patients. Finally, I will briefly touch on recent developments that will affect patient screening in the future.

15.1 Introduction

The fragile X syndrome is the most common Mendelian inherited form of intellectual deficiency or mental retardation. The name fragile X syndrome comes from the presence of a gap or break near the end of the X chromosome at band q27.3, which appears when cells from an affected individual are cultured in a special media. The fragile X syndrome is usually caused by expansion of an unstable CGG repeat region located within the *FMR1* (fragile X mental retardation type 1) gene which inactivates gene expression. This X-linked form of mental retardation was first recognized as a common and distinct entity in the late 1970s. Current estimates are that approximately 4,000 males and 1 in 8,000 females in the general population have an IQ below 70 as a result of the syndrome, and that 1 in 300 females is a carrier, although various studies have given somewhat differing estimates and there are likely to be founder effects in some ethnic populations (Crawford et al. 2001; Hagerman 2008; Coffee et al. 2009; Hantash et al. 2011). Molecular tests have been

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developed that allow for direct genomic Southern analysis as well as PCR diagnosis (Brown et al. 1993; Brown 2002).

15.2 Features of Affected Males

Compared to individuals diagnosed with many genetic or chromosomal syndromes, affected fragile X males usually are fairly normal in physical appearance. This helps to explain why they are frequently undiagnosed and why the syndrome was only recently recognized as a distinct entity. As adults, they usually have enlarged testicular volume, known as macro-orchidism. This is typically in the range of 30–60 ml, as compared to the normal adult male mean volume of 17 ml. Other recognizable physical features that are variably present include large or prominent ears, highly arched palate, narrow midfacial diameter, narrow intereye distance, long face, large head circumference, prominent forehead, facial asymmetry, prominent thumbs, hyperextensible joints, and mitral-valve prolapse.

Approximately 95% of adult fragile X males have an IQ below 70, with an overall mean of approximately 35. Approximately 70% have an IQ in the moderate to severe range, between 50 and 20, while about 10% have an IQ below 20, and 25% have an IQ above 50. There appears to be a decline in measured IQs among young males as they grow from prepubertal to pubertal ages (Bennetto and Pennington 2002). This appears to be consistent with the findings that a number of young boys with fragile X syndrome often have been considered to be only mildly impaired or learning disabled, while adults with the syndrome are usually moderately to severely impaired. The reason for this decline in measured IQ may be due to a relative inability of young affected males to continue to acquire more complex cognitive abilities with maturity.

Common neurologic features in fragile X males include a static central nervous system encephalopathy without focal lateralizing signs and impairment of fine motor coordination. Approximately 15% of fragile X males have a history of seizures (Berry-Kravis et al. 2010). These may be transient, but occasional subjects have persistent seizures that are usually well-controlled with anticonvulsion medication. Neuropathological studies have shown immaturity and dysgenesis of dendritic spines (Irwin et al. 2001). Volumetric MRI studies have indicated an enlarged caudate nucleus, a decreased amygdala size, a decreased ratio of posterior to anterior cerebellar vermis, and increased volume of fourth and posterior ventricles, similar to findings reported for a subgroup of autistic males (Lightbody and Reiss 2009).

There is a significant association of fragile X syndrome with infantile autism. Autistic individuals are usually males; a 4:1 male to female ratio is commonly observed. Approximately 2–4% of autistic males will be found to have the fragile X mutation on testing. Approximately 25–30% of males with fragile X meet full criteria for autism and another 25–30% have a milder autism spectrum disorder or pervasive developmental disability not otherwise specified (PDD-NOS)

(Harris et al. 2008). Fragile X is widely considered to be the single most common biomedical condition specifically associated with autism.

Hyperactivity with a short attention span is often quite pronounced in young affected males. They frequently have stereotypic movements and unusual hand mannerisms such as hand-flapping and hand-biting which often leads to callus formation at the site of biting. They also frequently show speech delay and a relative lack of expressive language ability. Repetitive speech patterns are quite common and often include stereotypical vocalizations, jargon, dysrhythmia, perseveration, echolalia, conditioned statements, inappropriate tangential comments, and talking to self. Many fragile X males are quite social and have an outgoing personality, but they generally have poor eye contact, are hypersensitive to sensory stimuli, and are tactilely defensive, which may interfere with social interactions and development (Hagerman 2002; Tsiouris and Brown 2004).

Some males with the fragile X syndrome have been found to have a Prader–Willi phenotype (PWP), with obesity and hyperphagia. An analysis of 13 such subjects revealed they had a high incidence of delayed puberty, a small penis or testicles, infant hypotonia, and autism spectrum disorder. Further, analysis of the levels of an FMR1 interacting protein (CYFIP1) showed that mRNA levels for CYFIP1 were significantly reduced in FXS patients with the PWP as compared to those without the PWP (Nowicki et al. 2007).

15.3 Carrier Females

Female carriers of a premutation (see definitions below) are usually mentally normal and unaffected. However, remitting recurrent depressive episodes and anxiety disorder have been described among some female carriers (Roberts et al. 2009). Approximately 16% of carrier females experience premature ovarian failure before age 40 compared to about 1% in the general population (Allingham-Hawkins et al. 1999).

Among females who have a full mutation, approximately 50% have a full-scale IQ score below 70. A characteristic profile of cognitive defects may be present with relatively lower Wechsler IQ performance scores, decreased subtest scores on arithmetic, digit span, block design, and object assembly. They may have increased verbal performance scores and do very well academically. There may exist subtle defects on emotional development based on mild neurocognitive functioning deficits. Shy and socially withdrawn behavior is common. Other variable deficits include socially inappropriate comments, inappropriate affect, poor modulation of verbal tone, tangential speech, and odd communication patterns (Hagerman 2002).

15.4 Features of Transmitting Males

About 1 in 700 males in the general population inherit the fragile X mutation in a nonexpressed or premutation form and are considered to be normal carriers. They are nonpenetrant for the mutation and do not express the fragile site.

They transmit the premutation only to all their daughters who are also generally nonexpressing and carriers of the premutation but who then can have affected sons.

It has been found that some 20–35% of males with the premutation over the age of 50 develop a multisystem, progressive neurologic disorder featuring intention tremor and cerebellar ataxia. This new fragile X tremor/ataxia syndrome (FXTAS) is progressive with a variety of developing symptoms that include short-term memory loss, executive function deficits, cognitive decline, Parkinsonism, peripheral neuropathy, lower limb proximal muscle weakness, and autonomic dysfunction. Symmetrical regions of increased T2 signal intensity on MRI in the middle cerebellar peduncles is thought to be a highly sensitive indicator of this syndrome (Jacquemont et al. 2003, see Chaps. 14 and 18 for a more complete description).

15.5 The Molecular Nature of the Fragile X Mutation

The molecular mutation underlying the fragile X syndrome is usually an expanded string of CGG triplet repeats near the 5′ end of the *FMR1* gene, within a transcribed but untranslated promoter region. The CGG repeat region of the gene is variable in length and undergoes a tremendous length amplification in affected individuals. Expansion of the CGG repeat in the 5′ untranslated region of the *FMR1* gene results in methylation of the upstream CpG island, lack of gene expression, and the fragile X phenotype. The length of the repeat region is polymorphic in normal individuals, with lengths ranging from approximately 5–54 CGGs. The most frequent Caucasian repeat number is 30 followed by 29, 20, 23, and 31. Approximately 5% of normal alleles are 40 or greater (Brown et al. 1996). Interspersed AGGs occur within the normal CGG repeat region that may stabilize the sequence and prevent slippage during DNA replication. Carrier females and transmitting males have an enlargement of the region to a range of approximately 56–200 repeats which is designated as a premutation. Offspring of carrier females, but not of transmitting males, can have enlargements of the repeat to values ranging from 200 to over 2,000 repeats, referred to as the full mutation. Affected individuals have such a full mutation (Nolin et al. 2003).

A minority of affected individuals have a mosaic pattern with some proportion of cells showing sizes of less than 200 repeats and partially active gene expression. Affected full mutation males have fathered normal daughters who carry a premutation-sized allele. Analysis of their sperm samples reveals only premutation-sized alleles, suggesting the amplification process occurs postzygotically (Reyniers et al. 1991). The risk of amplification to the full mutation is greater with increasing size of the premutation in the carrier mother (Nolin et al. 2003). As the premutation is transmitted through subsequent generations within families, it demonstrates "anticipation," that is, greater numbers of affected individuals are observed in later generations than in earlier ones.

15.6 Molecular Diagnosis of Fragile X

Molecular diagnostic testing including prenatal diagnosis is conducted using two methods. The first method is direct genomic Southern blot analysis, which uses a probe that flanks the CGG repeat region. One such probe, StB12.3, uses a double digestion with two restriction enzymes. Digestion with the first enzyme (EcoRI) produces a 5.2 kb band on a Southern blot. The second methylation sensitive enzyme (EagI) cuts unmethylated DNA at the CpG island, producing a 2.8 kb band, but leaves methylated DNA uncut. Thus, a normal female DNA sample will have both a 2.8 kb band, reflecting the active unmethylated X chromosome, and a 5.2 kb band, reflecting the inactive, methylated X chromosome. Premutation alleles generally produce bands in the range of 2.9–3.2 kb for the active X, such as present in transmitting males, and 5.3–5.7 kb for the inactive X. Normal carrier females generally have two doublets on such DNA analysis. Affected males generally have bands or smears in the 5.8–9 kb range.

The second molecular method for detecting the fragile X mutation employs the use of the polymerase chain reaction (PCR). PCR analysis for fragile X mutations is rapid and uses small amounts of starting DNA for analysis. However, because the region is high in CG content, special methods are needed for successful amplification of full mutations (Brown et al. 1993). Some of the larger alleles from both males and affected females may fail to amplify successfully. Hence, there is a need to have both methods available for routine diagnostic and prenatal testing purposes. Immunocytochemistry with monoclonal antibodies has been shown to be potentially effective for the detection of the full mutation in males both pre- and postnatally in whole blood lymphocyte, amniocentesis, and chorionic villus samples (de Vries et al. 1998).

A single type of mutation, the repeat amplification, is far and away the most common cause of the syndrome. A small number of fragile X syndrome patients have been identified that have either a deletion of the gene region or a point mutation within the gene. This points out that it is the absence of *FMR1* gene expression which determines the syndrome. If one of these rare deletion-type patients is suspected, more detailed molecular investigations are needed for diagnosis (Collins et al. 2010).

15.7 Glimpsing the Future

Because fragile X accounts for approximately 1–2% of mental retardation overall, children with mental retardation of unexplained etiology should be evaluated for the syndrome. Since reliable prenatal diagnosis by molecular testing is now available, screening of pregnant women for their carrier status is appropriate, particularly in the setting of a family history of mental retardation (Hill et al. 2010). Newer DNA methods are in development to both increase the information provided and reduce the costs of testing (Chen et al. 2010, Chen et al. 2011, Filipovic-Sadic et al. 2010;

Godler et al. 2010; Lyon et al. 2010). The use of antibody-based assays for quantitation of FMRP are being developed in our lab (LaFauci et al. 2010) and that of P. Hagerman (Iwahashi et al. 2009). Direct quantitation of the protein should soon allow for low-cost newborn screening to identify affected males.

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Chapter 16
Fragile X Syndrome: A Psychiatric Perspective

Michael R. Tranfaglia

Abstract Fragile X syndrome (FXS) is associated with a complex but relatively consistent psychiatric phenotype. Recent research has suggested neural substrates for the behavioral abnormalities typically seen in FXS, and enhanced treatment strategies for managing disabling psychiatric comorbidity. While disease-specific, and possibly disease-modifying, therapeutics are being developed for FXS, currently available psychiatric medications can provide significant symptomatic relief of the hyperactivity, anxiety disorders, and affective disturbances often seen in the course of FXS. However, patients with fragile X may be especially susceptible to the psychiatric side effects of these medications, requiring particular care in prescribing. Recent findings concerning disease mechanisms and treatment strategies are reviewed from the perspective of a clinical psychiatrist, in an effort to enhance conventional pharmacotherapy of FXS.

16.1 Introduction

Fragile X syndrome (FXS or simply fragile X) has commanded the attention of a great many neuroscientists in recent years. No doubt there are many reasons for this, but perhaps the most compelling is that FXS offers the enticing possibility of understanding a complex neuropsychiatric disorder at the synaptic, molecular, and genetic levels. While there is certainly much more to be learned about the pathophysiology of fragile X, research efforts in the past decade have been enormously productive. Enough has already been discovered to lead to clinical trials of potentially disease-modifying, novel, investigational drugs in FXS subjects. Compare this to the state of affairs in the study of more common psychiatric disorders, where

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the search for susceptibility genes is constantly frustrated, animal models are speculative and imprecise, and basic mechanisms of disease are poorly understood.

FXS is appealing because a mutation in a single gene (FMR1) results in the absence of a single protein (fragile X mental retardation protein, FMRP). This, in turn, results in a characteristic behavioral phenotype which is complex but relatively consistent. This can be understood as a constellation of psychiatric symptoms, in addition to global developmental delays, neurological effects, and a distinct physical phenotype.

Understanding the psychiatric phenotype of fragile X is essential for a number of reasons. Of most immediate concern is the treatment of a patient population burdened by a high symptom load. Fragile X patients suffer enormous functional impairment, above and beyond their intellectual impairment, as a result of the maladaptive behaviors and emotional disturbance we collectively term "the behavioral phenotype of FXS." These symptoms are a frequent reason for families to seek treatment and can lead to institutionalization in more severe cases. While there is excitement in the field for the potentially disease-modifying treatments in development, more conventional psychopharmacological treatments must be used in the interim. Even when novel agents such as mGluR5 antagonists are available for general use in treating FXS, it is likely that psychiatric comorbidity will still require administration of psychiatric medications in many cases. The development process for newer, more specific treatments for FXS also requires an enhanced understanding of the psychiatric features of the disorder. Outcome measures for clinical trials in FXS are heavily weighted toward psychiatric and behavioral symptoms, and these instruments require refinement; while there is great interest in the development of biomarkers or cognitive measures that can detect drug effects during relatively brief clinical trials, none of these are practical for use in the near term. For the time being, clinical researchers must rely almost entirely on their ability to describe and quantify psychiatric aspects of FXS.

As more is discovered about the basic pathophysiology of fragile X, the ability to correlate molecular and synaptic abnormalities with behavioral and emotional symptoms can potentially inform our understanding of all forms of psychiatric illness.

Here, we will focus on the psychiatric diagnosis and treatment of males with full mutation FXS; many of these symptoms can be seen in females with the full mutation, though with great variability, and the basic principles of treatment are similar.

16.2 Behavioral Phenotyping of Fragile X Syndrome

Many of the earliest reports of the psychiatric sequellae of the fragile X mutation emphasized the autism-like presentation of many males with FXS (Brown et al. 1982a, b). Early reports found very high rates of undiagnosed FXS in autism populations (Blomquist et al. 1985; Brown et al. 1986; Fisch et al. 1986;

Wahlström et al. 1986), but as testing for FXS has become more commonplace, the reported rates of undiagnosed fragile X among autistic populations have declined. Still, FXS continues to be associated with high rates of stringently defined autistic disorder and pervasive developmental disorder (Bailey et al. 2001; Hall et al. 2010).

One of the earliest attempts to describe the FXS phenotype comprehensively, including the behavioral phenotype, examined 21 males with fragile X from 2 to 21 years of age. The authors noted that while the well-known physical phenotype often did not manifest until later in life, "the psychological profile of these boys, on the other hand, was remarkably uniform" (Fryns et al. 1984). They observed that intellectual impairment ranged from mild to severe, but with a mean IQ of 43.67 and with most subjects (16/21) in the moderately retarded range. All were described as having significant fine and gross motor coordination problems, and all were significantly delayed in their language development. Of the 21 subjects, 20 showed "hyperkinetism" and 19 were described as having "concentration difficulties."

Fryns went on to study a larger population of fragile X males (Fryns 1984) in which he confirmed the previously described behavioral phenotype of hyperactivity and impaired attention, marked anxiety with poor eye contact, affective lability, aggression, self-injurious behavior (especially the characteristic hand biting), and autistic features described as repetitive, perseverative, and stereotypic behaviors. This basic formulation of the fragile X behavioral phenotype has remained intact to the present day, with substantial confirmation of these basic findings in subsequent studies (Largo and Schinzel 1985; Gillberg et al. 1986; Veenema et al. 1987; Bregman et al. 1988). Also confirmed was the observation that the marked hyperactivity seen in young males with fragile X appeared to follow a distinct developmental course: boys with FXS actually appeared hypoactive early in life, but became markedly hyperactive in the preschool years. This "hyperkinesis" and impulsiveness then clearly decreased with age, even as IQ was observed to decline (Borghgraef et al. 1987). Many of the disruptive behaviors seen in fragile X also appear to decline over time when examined longitudinally, while shy behavior and poor eye contact remain relatively constant and significantly different from control subjects with developmental disorders (Einfeld et al. 1999).

Fragile X males differ from IQ-matched controls with nonfragile X developmental disorders in that they have more abnormal language, tactile defensiveness, poor self-control, poor eye contact/shyness, and hand flapping (Lachiewicz et al. 1994; Einfeld et al. 1994). Using the Aberrant Behavior Checklist, fragile X males were found to show significantly higher levels of hyperactivity, stereotypic movements, and unusual speech compared to matched control subjects (Baumgardner et al. 1995). Counter to the notion that developmental delay per se might explain many FXS symptoms, a distinct behavioral phenotype is seen in FXS subjects compared to groups with fetal alcohol syndrome, tuberous sclerosis, and Prader–Willi syndrome (Steinhausen et al. 2002). As a group, FXS subjects are more anxious than the other disease groups, more autistic, but also paradoxically more empathetic. Fragile X subjects score highly on autism rating scales, yet clearly seek social interaction, suggesting a qualitative difference between FXS and autism, which is not easily captured on many rating instruments.

Few fragile X studies have employed a structured psychiatric interview, but when this methodology is used, the range of psychiatric diagnoses is relatively small. Male children and teens with FXS have high rates of attention deficit hyperactivity disorder (ADHD, 74%) and oppositional defiant disorder (29%), as well as functional enuresis and encopresis (Backes et al. 2000). Separation anxiety disorder and obsessive–compulsive disorder (OCD) are seen in a smaller number of subjects (10% and 2%, respectively). As in autism and other developmental disorders, studies in subjects with fragile X have typically found high levels of behavioral and psychiatric symptoms, but relatively few formally diagnosable psychiatric diagnoses according to current (DSM-III/IV) nomenclature. Cross-sectional studies using parent and teacher ratings have shown that ADHD symptoms occur in 59% of FXS children (Sullivan et al. 2006), compulsive behaviors occur in 72% of boys, and self-injurious behavior (primarily hand biting) occurs in 58%, but the behaviors are not necessarily associated in individuals (Hall et al. 2008).

As is the case with ADHD in FXS, many of the other psychiatric manifestations of FXS appear to follow a developmental course, changing significantly over the lifespan. This is reminiscent of the clinical course of seizures in fragile X, with onset early in life and then decreasing after the end of adolescence, both in fragile X knockout mice (Yan et al. 2004) and in humans with FXS (Musumeci et al. 1999; Sabaratnam et al. 2001). Aggression is often observed to worsen dramatically during early adolescence, before dissipating in young adulthood. This appears to correspond roughly to puberty and the hormonal transition to sexual maturity (Tsiouris and Brown 2004). No systematic studies of older males with fragile X have been published, even though lifespan appears to be normal in FXS. For practical, ethical, and regulatory reasons, most clinical trials of investigational new drugs are conducted in adults, so this represents a major gap in our knowledge base.

16.3 Attention Deficit and Hyperactivity

While ADHD is the most common diagnosable condition in FXS patients, with most males meeting formal criteria at some point in their lives, this condition is typically not stable over time in any given individual (Fryns 1984). Very young children with fragile X are often noted to be physically hypoactive, with somewhat impaired attention. Preschool children can display dramatic increases in activity levels, leading to markedly disruptive behavior. As children grow, hyperactivity declines with increasing body mass, while problems with attention continue throughout life. This can be seen as similar to the course of ADHD in the normal population, though the degree of hyperactivity in FXS is impressive. There is also evidence that the attention deficit seen in males with fragile X has a specific profile (Munir et al. 2000) which is distinct from other causes of developmental disorders, suggesting that the attention problems seen in the course of FXS may represent

more than nonspecific immaturity. Animal models of FXS show a corresponding phenotype: Fmr1 knockout mice display increased locomotor activity (Yan et al. 2004) and impaired attention (Moon et al. 2006); Drosophila dfmr1 mutants have disturbed circadian rhythm, a fly correlate of human hyperactivity (Dockendorff et al. 2002).

Dysfunction in dopamine pathways regulating attention, impulse control, and motivation (Volkow et al. 2010) is hypothesized to underlie the symptoms of ADHD. Dopaminergic dysfunction in fragile X has been suggested by abnormal eye blink rate in boys with fragile X (Roberts et al. 2005). More recent studies in the FXS mouse model suggest specific abnormalities in forebrain dopamine (D1) signaling (Wang et al. 2008), including excessive G protein-coupled receptor kinase 2 (GRK2) function, in agreement with previous evidence that amphetamine can rescue impaired object recognition in Fmr1 knockout mice (Ventura et al. 2004). Additionally, dopamine release may be specifically impaired in Fmr1 knockout mice (Fulks et al. 2010).

A small controlled trial of stimulant medication for ADHD symptoms in fragile X children showed modest benefit over a 1-week treatment period (Hagerman et al. 1988). However, even with brief exposure to active drug, significant psychiatric side effects were seen in some subjects. Anxiety, irritability, and mood lability are commonly noted side effects of stimulant medications in FXS patients (Berry-Kravis and Potanos 2004). This parallels the experience in the treatment of developmental disorders generally, with stimulant medication often poorly tolerated in more intellectually disabled populations (Posey and McDougle 2000).

Dysregulation of autonomic nervous system function has also been noted in children and adolescents with fragile X (Hall et al. 2009), and this may contribute to attention deficit and hyperactivity, as well as the classically described sensitivity to environmental stimuli and stress. In another brief study of mixed stimulant medications, a group of fragile X children with ADHD showed specific improvement in electrodermal responses compared to IQ-matched, nonfragile X subjects with ADHD, suggesting enhancement of inhibitory neurotransmission (Hagerman et al. 2002). Larger controlled stimulant trials in FXS subjects of longer treatment duration are warranted, given the frequent long-term use of these medications in FXS.

The relatively high rate of adverse psychiatric effects seen with the use of stimulant medications in FXS suggests that nonstimulant medications may have a particularly important role in the treatment of ADHD symptoms. One of the largest controlled trials to date in FXS subjects found modest benefit from L-acetyl carnitine (LAC) in the treatment of ADHD symptoms (Torrioli et al. 2008). Small but statistically significant improvements in socialization and adaptive behavior were also noted with LAC treatment, though these effects may not be clinically meaningful, and LAC has not gained widespread acceptance as a treatment for FXS. The mechanism of action of LAC remains unclear. The authors had originally proposed that LAC might partially restore FMRP expression, but this was not found to be the case in the course of these studies. On a similar note, a much smaller, open trial of valproic acid (Torrioli et al. 2010) also sought to restore

FMRP expression via inhibition of histone deacetylase (HDAC). This study found improvement in ADHD symptoms in FXS subjects, but expression of FMRP was not detected. Other nonstimulant medications, especially the sympatholytic agents, clonidine and guanfacine, are frequently utilized to treat hyperactivity and hyperarousal in FXS (Hagerman et al. 2009); however, no systematic studies of these agents in FXS subjects have been performed to date.

16.4 Anxiety in FXS

Males with fragile X display a broad range of anxiety symptoms, but these symptoms often do not fit into the established categories of major anxiety disorders employed by the Diagnostic and Statistical Manual of Mental Disorders. Within the classically described behavioral phenotype of fragile X, elements such as poor eye contact, gaze aversion, and excessive shyness are obviously primarily anxiety based, and strongly reminiscent of social phobia. However, the subjective experience of anxiety may contribute significantly to other aspects of the phenotype, such as hand flapping, hand biting, aggression, and autistic symptoms (Boyle and Kaufmann 2010). Thus, anxiety appears to contribute significantly to the morbidity related to FXS.

A minority of males with FXS meet formal criteria for a diagnosis of OCD, while "compulsive symptoms" have been noted in several studies in a large majority of subjects with FXS. In most cases of FXS, individuals exhibit symptoms strongly reminiscent of obsessions and compulsions, but which do not meet the precise psychiatric definitions for these symptoms. Often, pleasure is derived from repetitive and "compulsive" behaviors, in contrast to the ego-dystonic nature of true obsessions and compulsions. A FXS child may, for example, watch the same 2-min section of a video, over and over, for hours. These symptoms are perhaps more precisely termed repetitive, perseverative, or stereotypic behaviors. Hoarding, counting, and need for symmetry are all typical symptoms of OCD frequently seen in FXS. Similarly, younger children with FXS will meet the criteria for separation anxiety disorder in a small minority of cases (Backes et al. 2000), while symptoms of separation anxiety, social phobia, panic, and agoraphobia are seen clinically at a much higher rate.

Proper function of inhibitory GABAergic circuits appears to be critical for the regulation of anxiety. Indeed, one frequently cited, unifying explanation for many causes of autism spectrum disorders is the disruption of excitatory/inhibitory (E/I) circuit balance (Rubenstein and Merzenich 2003). Deficits in GABAergic function have been proposed as the basis for a number of fragile X symptoms, including anxiety and epilepsy, and decreased expression of GABA receptors in the fragile X mouse model has been demonstrated (El Idrissi et al. 2005; D'Hulst et al. 2006; reviewed in Chap. 11). However, the finding of general downregulation of multiple elements of the GABA neurotransmission apparatus suggests that this may be a compensatory change, perhaps secondary to

abnormalities in glutamate systems. Results appear to vary significantly by brain region, but excitatory (glutamatergic) drive onto inhibitory interneurons in the somatosensory cortex of Fmr1 knockout mice is deficient (Gibson et al. 2008), resulting in decreased inhibitory circuit function. This can be rescued in animal models by a number of $GABA_A$ agonists, including novel agents such as gaboxadol (Olmos-Serrano et al. 2010).

There have been no systematic trials of traditional $GABA_A$ agonists in FXS subjects, perhaps because the clinical experience with these agents has not been encouraging, with generally poor response and high rates of adverse behavioral effects. "Paradoxical" reactions to sedatives are of concern in developmentally disabled populations (Marrosu et al. 1987), and FXS patients can display behavioral disinhibition, irritability, and even aggression when treated with benzodiazepines. However, some patients have been prescribed these treatments for occasional use, such as dental procedures, with good effect. Variability in inhibitory dysfunction across different brain regions may explain the inconsistent response of humans with FXS to $GABA_A$ agonists.

Specific abnormalities in $GABA_B$ function have been demonstrated in the Fmr1 knockout mouse model, and these can be rescued with $GABA_B$ agonists (Yun et al. 2006; Pacey et al. 2009). Curiously, there is also evidence that these abnormalities may have their origins in maternal environment, or other epigenetic factors (Zupan and Toth 2008). Results of a large, multicenter, controlled trial of arbaclofen, a proprietary $GABA_B$ agonist, in 54 subjects with FXS have been reported as positive (http://www.seasidetherapeutics.com/about_us/news_media-release_2010-07-26.pdf), but not yet reviewed or published. However, the study compound apparently failed to separate from placebo on primary outcome measures in the overall study population, showing a statistically significant effect only in a more socially withdrawn subpopulation.

Serotonergic deficits have long been hypothesized in FXS, in light of the broad spectrum of anxiety disorders seen in FXS patients at all ages. Absence of dFMRP leads to dysregulation of monoamine synthesis in Drosophila (Zhang et al. 2005). Subjects with FXS who have the so-called long-form polymorphism of the serotonin transporter (and thus, higher rates of synaptic serotonin reuptake) have higher rates of aggression, self-injurious behavior, and stereotypy (Hessl et al. 2008). While there have been no systematic studies of selective serotonin reuptake inhibitors (SSRIs) or other serotonergic anxiolytic therapies in FXS, SSRIs have been widely used in the clinical treatment of FXS (Berry-Kravis and Potanos 2004), as is the case in autism spectrum disorders generally. However, controlled studies of their effectiveness in ASDs have yielded mixed results (Kolevzon et al 2006; King et al. 2009; Williams et al. 2010), perhaps because of the low doses utilized in some of the studies. Successful trials, especially in adults with ASDs, have utilized higher doses typically associated with the treatment of OCD (McDougle et al. 1996). Parent surveys, while lacking in methodological rigor, indicate a general perception among caregivers of SSRI efficacy in individuals with FXS (Boyle and Kaufmann 2010). Adverse effects of SSRIs include significant activation, especially in children, which can aggravate preexisting symptoms of hyperactivity and

can be a major dose-limiting side effect. GI disturbance associated with SSRIs can also aggravate encopresis, another common symptom of FXS.

16.5 Affective Symptoms

As described by Backes et al., males with FXS rarely meet formal criteria for a diagnosis of a major mood disorder as defined in DSM-III or IV. Diagnoses such as major depression or bipolar disorder require periods of abnormal mood that are sustained, whereas individuals with fragile X will typically exhibit labile mood, irritability, self-injurious behavior, and aggressive outbursts of a more fleeting and episodic nature, not meeting the conventional duration criteria. These episodes are typically provoked by environmental stressors and are less frequent in familiar or more structured settings. However, affective symptoms can be severe and disruptive, and are a common target for psychopharmacologic intervention. While insomnia or disturbed sleep patterns are a frequent problem in children with FXS, these symptoms are rarely associated with abnormal mood states. These are more often long-term behavioral patterns, and can be quite disruptive to family life and resistant to pharmacotherapy.

SSRIs are a commonly employed treatment strategy for affective symptoms, along with other antidepressants, anticonvulsants, and atypical antipsychotics in more severe cases (Tsiouris and Brown 2004). There have been no clinical trials of any size, open or controlled, of antidepressants or anticonvulsants for the treatment of affective symptoms of FXS (curiously, the 2010 study of valproic acid by Torrioli et al. focused exclusively on ADHD symptoms, while valproic acid is used in psychiatry primarily as a mood stabilizer).

While formal thought disorders are generally considered rare in FXS, as in autism spectrum disorders (Solomon et al 2008), antipsychotic medications are commonly used to treat affective lability, agitation, and aggression (McDougle et al. 2008). Indeed, risperidone was the first treatment specifically approved by the FDA for treatment of autism symptoms (specifically, irritability). A small pilot study of open-label aripiprazole showed promising results in the treatment of irritability in fragile X (Erickson et al. 2010). No controlled studies of antipsychotics, either typical or atypical, have been conducted in FXS subjects, despite their high rates of drug utilization.

Dysregulation of cortisol secretion in children with FXS has been shown via salivary sampling (Hessl et al. 2002; Hall et al. 2006), and this has been proposed as a potential mechanism for exaggerated stress responsiveness and affective symptoms in FXS. Rather than showing exaggerated cortisol response to stress, as originally hypothesized, subjects with FXS had high baseline levels of cortisol secretion, which correlated with behavioral disturbances. A small pilot study in FXS subjects of the antiglucocorticoid agent mefepristone (Reiss, unpublished

data) showed no therapeutic benefit, and possible serious adverse effects. Studies in the Fmr1 knockout mouse have yielded conflicting results, with an initial study showing subtle differences in corticosterone (the mouse equivalent of human cortisol) secretion following exposure to stress (Markham et al. 2006), and a subsequent study showing no statistically significant abnormalities at baseline or in response to stress (Qin and Smith 2008).

Abnormal activation of the ubiquitous kinase GSK3β has been demonstrated in the FXS mouse model, and rescue of prominent phenotypes has been accomplished with GSK3β inhibitors and lithium, also a known inhibitor of GSK3β (Mines et al. 2010; Liu et al. 2010: Choi et al. 2011). Since GSK3β inhibition is thought to be the primary mechanism of action of lithium in the treatment of bipolar disorder, numerous GSK3β inhibitors are under investigation as lithium alternatives, although none are currently available for use in psychiatry. The activity of brain GSK3β may, therefore, be seen as an important regulator of mood states, and this abnormality may represent an important area of overlap between FXS and bipolar disorder (Li and Jope 2010). Based partly on this work, an open trial of lithium was conducted in 15 FXS subjects aged 6–23 years; subjects showed significant improvement in total Aberrant Behavior Checklist scores, as well as RBANS List Learning (Berry-Kravis et al. 2008). Lithium treatment also resulted in normalization of ERK phosphorylation rates, a putative biomarker of fragile X. These findings await replication in a larger, controlled trial.

16.6 Discussion

Fragile X syndrome is a single-gene disease in which the deficiency of a single protein causes wide-ranging but consistent physical, neurological, and psychiatric symptoms. Remarkably, even though the absence of FMRP causes significant alterations in CNS function and dendritic proteomics, humans with fragile X are generally physically robust and of normal longevity. This suggests that the normal function of FMRP is limited to fine-tuning of synaptic connectivity, rather than any significant role in basic cellular function. Given the phenotypic overlap between fragile X and autism, this strongly suggests that many autism spectrum disorders may essentially be disorders of synaptic plasticity.

The monoamine hypothesis has dominated thinking in psychiatry for decades, holding that dysregulation of dopamine, serotonin, and norepinephrine transmission underlies most major psychiatric disorders. Indeed, FMRP may be involved in the regulation of major neuromodulatory systems, at least in Drosophila (Zhang et al. 2005). However, fragile X presents as a global neuropsychiatric disorder, with multiple abnormalities in multiple neurotransmitter systems – all the result of a single genetic mutation. Clearly, there are regulatory mechanisms in the brain which transcend individual neurotransmitters, and the FMRP appears to be

involved in these mechanisms. This could be explained by the derangement of glutamate signaling, since glutamate constitutes the vast majority of excitatory neurotransmission; however, accumulating evidence suggests that signaling pathways coupled to many different neurotransmitter receptors are affected in FXS (Volk et al. 2007; Wang et al. 2008). Many of these different signaling pathways share common elements (enzymes and scaffold proteins), and some of these elements appear to be directly regulated by FMRP. The importance of this regulatory role is highlighted by a recent report that suggests that low levels of FMRP may be associated with major psychiatric symptoms in many nonfragile X patients (Fatemi et al. 2010).

These advances in our understanding of the basic mechanisms of disease in FXS are leading to improvements in treatment for this disorder, and perhaps even to disease-modifying therapeutics, as described in Chap. 17. They may also significantly enhance our understanding of other, less homogeneous neuropsychiatric disorders, such as autism. While specific therapeutics are being developed, the mainstays of treatment are the conventional legacy drugs developed during the era of the monoamine hypothesis, and these can still prove effective in treating the symptoms of fragile X.

Unfortunately, clinicians looking to the literature for rigorous clinical trials to guide their current treatment of fragile X patients will find none. There have been no adequate studies of any of the psychopharmacological treatments commonly used in FXS. Anecdotal evidence and pilot studies support the use of general psychiatric treatments in FXS in a symptom-specific approach; for example, agitation in FXS can be treated with atypical antipsychotics, just as agitation in the general psychiatric population can be treated with atypical antipsychotics. Clinical reports suggest that a broad range of affective and anxiety symptoms in FXS patients respond to treatment with SSRIs, as well as other serotonergic antidepressants (clomipramine, nefazodone, etc.). Optimal responses typically require doses at the higher end of the therapeutic range. However, this treatment comes at a cost: younger patients with FXS often experience significant activation and worsening of hyperactivity with SSRIs. Older FXS patients, apparently having "grown out" of their susceptibility to hyperactivity, appear to tolerate these treatments better. ADHD symptoms in FXS appear to respond to conventional psychostimulant medications, but the doses may need to be reduced below those used in the general population to enhance tolerability. As a rule, higher functioning FXS patients are able to tolerate higher doses of stimulants, while lower functioning individuals are more likely to develop psychiatric side effects such as irritability, increased anxiety, or aggression.

The major problem with the symptomatic approach to treatment is that FXS patients typically present with a wide array of symptoms, and treatment for one type of symptom (i.e., SSRI for anxiety) may aggravate another (such as hyperactivity), as summarized in Table 16.1. This can greatly complicate treatment, especially when this iatrogenic factor goes unrecognized because of a different time course. For example, treatment with stimulants can result in immediate improvements in

Table 16.1 Psychiatric medications commonly used in FXS

Drug class	Target symptoms	Side effects (behavioral)	Evidence of efficacy
		↑Anxiety ↑OCB	
	Attention deficit	↑Aggression	
Psychostimulants (methylphenidate, amphetamines, etc.)	Hyperactivity Impulsivity Hyperactivity	↑Irritability ↑Seizures	+++
	Attention deficit	↑Irritability	
Sympatholytics (esp. clonidine and guanfacine)	Hyperarousal	Sedation	++
	Anxiety	↑Attention deficit	
	OCB	↑Hyperactivity	
	Irritability	↑Impulsivity	
SSRIs (fluoxetine, sertraline, citalopram, etc.)	Aggression	↑Encopresis	+++
	Seizures	Cognitive impairment	
Anticonvulsants (valproate, carbamazepine, oxcarbazepine, etc.)	Mood lability Aggression	↑Impulsivity ↑Irritability	++
	Mood lability	↑Enuresis	
Lithium	Aggression	↑Seizures	+++
	Aggression		
Atypical antipsychotics (risperidone, aripiprazole, olanzapine, etc.)	Mood lability Irritability	↑Enuresis ↑Seizures	+++

Abbreviations: OCB = Obsessive–Compulsive Behaviors; a.k.a. repetitive, perseverative, or stereotypic behaviors

++++ specific indication; large prospective clinical trials in FXS support use

+++ established indication; small trials in FXS or ASDs support use

++ off-label indication; small trials in ASDs support use

+ off-label indication; clinical experience supports use

attention and hyperactivity, followed much later by an insidious onset of irritability or aggressive behavior. If the early response was strongly positive, the later adverse effects may not be associated with the drug. This can result in layering of medications, one upon another, to treat these iatrogenic symptoms, leading to an unwieldy and occasionally an unsafe combination of medications. Fragile X is not unique in this regard, as this problem is seen in many cases of developmental disorders. A high level of expertise in psychopharmacology is often required on the part of the treating clinician to provide any benefit to the patient.

The solution, of course, is disease-specific treatment. Fragile X is unique in that it is amenable to the development of therapeutics based upon relatively precise understanding of the mechanism of disease. This is the great hope for the coming years. In all likelihood, this will be an ongoing and iterative process: therapeutic strategies will meet with partial success, but the results will guide future development and further enhance our understanding of the disorder. Throughout this process, appreciation and quantification of the psychiatric phenotype of fragile X will play a central role in these efforts.

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Chapter 17
Fragile X Syndrome and Targeted Treatment Trials

Randi Hagerman, Julie Lauterborn, Jacky Au, and Elizabeth Berry-Kravis

Abstract Work in recent years has revealed an abundance of possible new treatment targets for fragile X syndrome (FXS). The use of animal models, including the fragile X knockout mouse which manifests a phenotype very similar to FXS in humans, has resulted in great strides in this direction of research. The lack of Fragile X Mental Retardation Protein (FMRP) in FXS causes dysregulation and usually overexpression of a number of its target genes, which can cause imbalances of neurotransmission and deficits in synaptic plasticity. The use of metabotropic glutamate receptor (mGluR) blockers and gamma amino-butyric acid (GABA) agonists have been shown to be efficacious in reversing cellular and behavioral phenotypes, and restoring proper brain connectivity in the mouse and fly models. Proposed new pharmacological treatments and educational interventions are discussed in this chapter. In combination, these various targeted treatments show promising preliminary results in mitigating or even reversing the neurobiological abnormalities caused by loss of FMRP, with possible translational applications to other neurodevelopmental disorders including autism.

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17.1 Introduction

We are in an age of targeted treatments for neurodevelopmental disorders that began with advances in neurobiology and the development of appropriate animal models for many neurodevelopmental disorders. Although the focus of this book and this chapter is on fragile X syndrome (FXS) and animal models for this disorder leading to targeted treatments, this is a phenomenon that has occurred for many other neurodevelopmental disorders including tuberous sclerosis (de Vries 2010), neurofibromatosis and other disorders of the RAS MEK pathways (Rauen et al. 2010), Down syndrome (Rueda et al. 2008), Rett syndrome (Maezawa and Jin 2010), and others with a known gene deletion or mutation. Targeted treatment strategies are only just beginning in autism, because it is a heterogeneous disorder with no single gene mutation causing the majority of cases (Bent and Hendren 2010). Most cases of autism involve abnormalities occurring in genes involved with synaptic plasticity, brain connectivity, and/or gamma amino butyric acid (GABA) and glutamate imbalances so that brain function is impaired (Belmonte and Bourgeron 2006; Kelleher and Bear 2008; Pinto et al. 2010). Thus, the autism field is benefitting from advances in targeted treatment for other disorders that are associated with autism, such as FXS, which is the most common single gene disorder associated with autism (Hagerman et al. 2010). One reason that FXS is a good model for autism is because FMRP is an RNA binding protein that transports, stabilizes, and regulates the translation of hundreds of mRNAs at the synapse (Darnell et al. 2005; Zalfa et al. 2007; Bassell and Warren 2008; Darnell et al. 2011; Luo et al. 2010). Not only does FMRP regulate many genes that when mutated lead to autism such as neuroligins, neurorexins, and SHANK proteins (Darnell et al. 2011; Hagerman et al. 2010), but the levels of FMRP in the brains of adult autistic patients have been documented to be low compared to controls even in individuals that do not have a fragile X mutation (Fatemi and Folsom 2010). Not only autism but other neuropsychiatric disorders have been reported to have low levels of FMRP in the CNS, including schizophrenia, severe depression, and bipolar disorder (Fatemi et al. 2010). Although FMRP controls the translation of many mRNAs it is likely there are many cellular mechanisms that control the levels of FMRP, particularly mechanisms associated with neuropsychiatric disorders. The commonalities across disorders is an exciting new finding among neurodevelopmental disorders because it means that therapies developed for one disorder are likely to be helpful for many other disorders (Wang et al. 2010b). Preliminary evidence described later suggests that the new targeted treatments for FXS will also be helpful for autism and perhaps other neuropsychiatric disorders.

In this chapter, we will review animal studies leading to targeted treatments and then review the studies in patients with FXS. Although a number of medications are currently available that are frequently utilized for treatment for FXS they are not considered targeted treatments for FXS because they do not reverse the neurobiological abnormalities of FXS but they are generally helpful for the common symptoms in a variety of neurodevelopmental disorders (for a more complete

review, see Tranfaglia, Chap. 15). Stimulants are effective for treatment of ADHD in FXS (Hagerman et al. 1988; Berry-Kravis and Potanos 2004; Hagerman et al. 2009), selective serotonin reuptake inhibitors (SSRIs) are helpful for the pervasive anxiety in FXS (Hagerman et al. 1994; Berry-Kravis and Potanos 2004; Hagerman et al. 2009), and atypical antipsychotics are helpful for mood stabilization and treatment of aggression and irritability (Erickson et al. 2010b). These commonly used treatments have been reviewed elsewhere (Hagerman et al. 2009). The remaining sections of this chapter focus on targeted treatments for FXS; these are summarized in Table 17.1.

17.1.1 Developing Treatment Strategies for FXS Based on Fmr1-Knockout Mouse Studies

Mutations in the *FMR1* gene that lead to transcriptional silencing and loss of FMRP expression result in FXS. The Fmr1-Knockout (KO) mouse does not express FMRP, exhibits many of the phenotypic characteristics of the human FXS condition, and has been an extremely useful tool to investigate the nervous system abnormalities arising from the loss of FMRP, as well as for the development of potential therapeutics for the treatment of this syndrome. Recent discoveries made from investigations using the Fmr1-KO have given rise to potential therapeutics for FXS, and in particular for the treatment of intellectual disability. As will be discussed, a number of approaches have found some success in the animal model with subsequent human trials. The animal models, to varying degrees, have led to positive outcomes for anatomical, electrophysiological, and behavioral measures across the different strategies.

17.1.1.1 Group I Metabotropic Glutamate Receptor Strategy

One of the first insights into the neurochemical underpinnings of FXS came from work by Bear, Huber, and colleagues on group I metabotropic glutamate receptors (mGluRs). In the hippocampal field CA1, activation of mGluR5 leads to long-term depression (LTD), which is seen as a reduction in synaptic responses. Importantly, LTD triggered by mGluR activation (mGluR-LTD) requires the rapid translation of preexisting mRNA in the postsynaptic dendrites (Huber et al. 2000). Huber et al. found in the Fmr1-KO that hippocampal LTD was more pronounced (greater depression) than in wild types (Huber et al. 2002). This work gave rise to the mGluR theory of Fragile X which argues that the psychiatric and neurological aspects of the syndrome are a consequence of an exaggerated response to group I mGluR1/5 activation (Bear et al. 2004); for more details as to role of FMRP in negatively regulating local protein synthesis, and how the lack of synthesis inhibition leads to exaggerated LTD, the reader is referred to several comprehensive

Table 17.1 FXS targeted treatments in models and man

	Phenotypes reversed		Translational progress
Agent/Target	*dfmr* mutant Fly	*Fmr1* k/o Mouse	Humans with FXS
(1a) Block translational signaling pathway-external			
mGluR5 inhibition (MPEP, fenobam, STX107, AFQ056, RO49917523); MPEP used in models see footnote (a)	Courtship behavior – immediate recall and short-term memory; Mushroom body formation; Odor-shock memory; Survival on glutamate-containing food	Audiogenic seizures[a]; Epileptiform bursts; Open field hyperactivity; Dendritic spine morphology[a]; Amygdala mEPSP frequency; Prepulse inhibition[a]; Marble burying[a]	Fenobam – phase IIa single-dose, open-label trial – PPI improved, anxiety reduced; AFQ056 – phase II[b] trial completed, phase III trial being initiated; RO4917523 – phase II trial in progress STX107 – phase I completed
mGluR5 inhibition by genetic reduction of mGluR5 receptors		Audiogenic seizures; Dendritic spine density; Excessive protein synthesis; Abnormal growth pattern; Ocular dominance plasticity; Inhibitory avoidance extinction	
(1b) Block translational signaling pathway – internal			
Lithium (inhibition of GSK3β and PI turnover)	Courtship behavior – immediate recall and short-term memory; Mushroom body formation	Audiogenic seizures; Open field hyperactivity; Dendritic spine morphology; Learning and anxiety deficits in the elevated-plus maze, elevated zero maze, passive avoidance; Social interaction deficit with new mice and anxiety-related behaviors during social interaction	Open-label trial – behavioral improvement, some adaptive skills and verbal memory improved; ERK biomarker normalized
GSK3β inhibition (AR-A014418 or SB-216763)		Audiogenic seizures	

PAK inhibition by genetic reduction of PAK		Dendirtic spine morphology; Cortical LTP deficits; Open field hyperactivity, repetitive behaviors, center field anxiety deficit; Fear conditioning	
PI3K inhibition (LY294002)		Dendritic spine morphology; mTOR overactivity	
ERK / MEK inhibition (SL327)		Audiogenic seizures; Protein synthesis	
(2) Inhibit activity of individual FMRP-regulated proteins			
Inhibit MMP9 (minocycline)		Dendritic spine morphology; Anxiety in elevated plus maze; Exploratory behavior in Y maze	Improvement in behavior in small open-label trial
Inhibit APP/Aβ with antibody or by genetic reduction of APP		Audiogenic seizures; Dendritic spine morphology; Marble burying	
Inhibit STEP by genetic reduction of STEP		AMPA receptor internalization; Audiogenic seizures; Open field hyperactivity	
(3) Activate surface AMPA receptors			
Ampakines (CX516, CX614)		CX614 increases BDNF which reverses impairments in hippocampal TBS-LTP	CX516 – phase II trial – no cognitive or behavioral effects overall – dose too low but may have helped subjects co-treated with antipsychotics
(4) Other synaptic receptors/proteins			
GABA-B agonists (baclofen, R-baclofen)	Survival on glutamate-containing food; Memory deficits	Audiogenic seizures; Open field hyperactivity; Marble burying	R-baclofen phase II trial – improvement in overall function, social and language function in more socially impaired subject group

(continued)

Table 17.1 (continued)

	Phenotypes reversed	Translational progress
GABA-A agonists (ganaxolone)	Audiogenic seizures	
Anticholinesterase (donazepil)		Open-label trial – behavioral and social improvement
NMDA antagonists (memantine, acamprosate)		Memantine – small open-label trial – no overall improvement Acamprosate – open-label trial in three patients with improved language and socialization
Glutamate uptake inhibition (riluzole)		Riluzole – small open-label trial – no overall improvement, ERK biomarker normalized

[a]Audiogenic seizures – MPEP, fenobam and STX107; Spine shape – MPEP, fenobam, AFQ056; PPI – MPEP, fenobam, AFQ056; Motor learning – MPEP, fenobam, AFQ056; Marble burying – MPEP, fenobam, STX107

[b]All phase II or III trials listed in the table are placebo-controlled double-blind trials unless otherwise noted. Adapted from Berry-Kravis and Knox (2011)

reviews (Waung and Huber 2009; Berry-Kravis et al. 2011; Chap. 18). This work has compelled numerous investigations into the outcomes of blocking group I mGluRs, most particularly the mGluR5 subtype, in the Fmr1-KO on different aspects of the phenotype that align with the human condition.

Studies aimed at blocking mGluR5 have principally used the selective noncompetitive antagonist 6-methyl-2-(phenylethynyl)-pyridine (MPEP) (Gasparini et al. 1999). Reducing mGluR5 function either with MPEP treatment or by lowering mGluR5 levels (~50%) in the Fmr1-KO has been shown to ameliorate a number of abnormal features in the mouse model that, to a great degree, reflect the human FXS phenotype. These features in the mouse mutant and the effects of mGluR5 antagonism/reduction on them are described later.

Dendritic Spine Morphology

Dendritic spine abnormalities have been described in both FXS (Rudelli et al. 1985; Hinton et al. 1991; Wisniewski et al. 1991; Irwin et al. 2001) and the Fmr1-KO (Comery et al. 1997; Irwin et al. 2002; Grossman et al. 2006). By and large, the fragile X mutation results in greater spine density on adult cortical neurons and greater numbers of spines that have an "immature" profile: There is a lower density of mushroom shaped spines with large heads, a greater number of longer spines, and excessive filopodia. In both developing and adult hippocampal neurons, spine abnormalities are present (Braun and Segal 2000; Antar et al. 2006; Grossman et al. 2006; de Vrij et al. 2008; Bilousova et al. 2009). Correcting these defects in spine morphology and numbers has become a standard "litmus test" in the field for evaluating the efficacy of drug treatments. Thus far, spine abnormalities seen in hippocampal neurons in vitro have been rescued with two independent mGluR5 antagonists, MPEP and fenobam (de Vrij et al. 2008). In addition, reducing mGluR5 expression in brain normalized spine density on visual cortical neurons in the adult animal (Dölen et al. 2007). While more work is needed to examine effects on spine morphology in the adult brain, these data suggest that mGluR5 antagonism in FXS could be very beneficial.

Protein Synthesis

FMRP binds to mRNAs (including its own) and regulates their translation within dendrites and spines in response to neural activation (Weiler et al. 1997, 2004) and, in particular, occurs in response to activation by either group I mGluR, (Huber et al. 2002; Antar et al. 2004; Aschrafi et al. 2005), muscarinic (M1) acetylcholine receptors (Volk et al. 2007), and possibly other synaptic Gq-linked receptors including dopamine D1 receptors (Wang et al. 2010a). In the Fmr1-KO, levels of synaptic proteins for a number of FMRP target mRNAs are elevated including MAP1B, PSD95, CaMKII, APP, Arc, and PP2A, amongst others [reviewed in (Berry-Kravis et al. (2011)]. In vivo treatment of Fmr1-KOs with MPEP has been

shown to increase levels of mRNA granules (levels are reduced in the mutant as a consequence of heightened translation) indicating that mGluR5 antagonism can normalize protein synthesis in the KO (Aschrafi et al. 2005). Overall protein synthesis in Fmr1-KO brain also is reduced by lowering mGluR5 levels by half (Dölen et al. 2007), further supporting the idea that blockade of this receptor subtype will normalize protein content in FXS.

Long-term Depression

As described earlier, hippocampal mGluR-dependent LTD is more pronounced in the Fmr1-KO as compared to wild-type mice (Huber et al. 2002; Hou et al. 2006; Nosyreva and Huber 2006; Sharma et al. 2010); as induction of this type of synaptic plasticity is dependent on group I mGluR activation, tests of antagonism of this receptor group have not been conducted. Interestingly though, a recent study by Choi et al. (2010) showed that chronic treatment (8 weeks) with the group II mGluR antagonist LY341495 in Fmr1-KOs reduced their level of hippocampal mGluR-LTD to near wild-type levels suggesting that targeting other mGluRs may also be beneficial. Finally, enhanced LTD has been reported in Fmr1-KO cerebellum as well (Koekkoek et al. 2005), although group I mGluR antagonists have not been tested in this system.

Long-term Potentiation

Long-term potentiation (LTP) reflects greater synaptic strength, and is considered the main cellular substrate thought to underlie learning and memory. In the Fmr1-KO, deficits in LTP have been reported for a number of brain regions including the neocortex (Li et al. 2002; Meredith et al. 2007; Wilson and Cox 2007), the piriform cortex (Larson et al. 2005), the hippocampus (Lauterborn et al. 2007; Shang et al. 2009; Chen et al. 2010), and the amygdala (Zhao et al. 2005; Suvrathan et al. 2010). In the few brain areas surveyed thus far mGluR5 antagonism by MPEP does not correct this aspect of the phenotype (Wilson and Cox 2007; Suvrathan et al. 2010), although this drug has been reported to rescue spontaneous excitatory postsynaptic currents in the Fmr1-KO (Suvrathan et al. 2010; Meredith et al. 2011). These data suggest that some, but not all, synaptic defects may be amenable to group I mGluR-targeted intervention.

Prepulse Inhibition

One of the most common clinical features of FXS is heightened sensitivity to sensory stimulation (Cohen 1995; Miller et al. 1999; Frankland et al. 2004). Prepulse inhibition (PPI) of an acoustic startle response, a widely used model to study basic sensorimotor processing, has been shown to be related to mGluR

signaling (Grauer and Marquis 1999). While PPI is reportedly reduced in humans with the fragile X full mutation (Hessl et al. 2008), studies in the Fmr1-KO are mixed with one group reporting enhanced (Frankland et al. 2004) and another group reporting reduced (de Vrij et al. 2008) PPI, albeit with PPI measurement conducted differently in these two studies. Interestingly, the defect in the Fmr1-KO's PPI response reported by de Vrij et al. (2008) was measured via a similar protocol to that used in humans with FXS and was rescued by MPEP treatment (de Vrij et al. 2008).

Seizures

A substantial number (~15%) of patients with FXS suffer from epilepsy during development (Musumeci et al. 1999; Sabaratnam et al. 2001; Berry-Kravis et al. 2010). While the factors responsible for this hyperexcitability in FXS are poorly understood, enhanced Gp1 mGluR activation has been shown to induce epileptiform activity (Ure et al. 2006; Karr et al. 2010). As in the human condition, loss of FMRP in the mouse model results in a greater tendency towards seizures and, in particular, Fmr1-KOs have a more excitable audiogenic seizure pathway (Chen and Toth 2001; Yan et al. 2005; Musumeci et al. 2007), and more protracted hippocampal seizures following kindling (Qiu et al. 2009), than do wild types. Treatment with MPEP has been shown to suppress both audiogenic and limbic seizures in the KO (Yan et al. 2005; Qiu et al. 2009), and reducing mGluR5 levels by 50% also significantly attenuated audiogenic seizures (Dölen et al. 2007). Similarly, studies of the hippocampus have shown that endogenous glutamatergic transmission induces prolonged synchronized discharges in KOs but not in wild types, suggesting a greater degree of excitability in the mutant (Chuang et al. 2005). This effect in Fmr1-KOs was mediated by Group I mGluRs as it was blocked by both mGluR5 (MPEP) and mGluR1 (LY367385) antagonists. As to the downstream mechanism involved in the induction of prolonged synchronized discharges, previous work has implicated the extracellular signal-regulated kinase 1/2 (ERK1/2; a.k.a. MAPK) (Zhao et al. 2004). Inhibition of ERK signaling in the Fmr1-KO hippocampus with a mitogen-activated protein kinase kinase (MEK) inhibitor also blocked the prolonged synchronized discharges (Chuang et al. 2005).

Learning

While learning deficits have been difficult to reliably assess in the Fmr1-KO perhaps due to strain differences, a number of studies have described learning problems for the mutant in different tasks including Morris water maze (Bakker and Consortium 1994; D'Hooge et al. 1997; Dobkin et al. 2000), radial maze (Mineur et al. 2002), fear conditioning (Paradee et al. 1999; Qin et al. 2005), object discrimination (Ventura et al. 2004), odor discrimination (Larson et al. 2008), and eye blink conditioning (Koekkoek et al. 2005). Surprisingly, little work has been done to test the effect of group I mGluR antagonism on learning in the Fmr1-KO. To date,

Dölen et al. (2007) have shown that genetic reduction of brain mGluR5 levels rescues inhibitory avoidance (extinction) learning in the Fmr1-KO. While further studies are needed to assess mGluR5 antagonists on this aspect of the phenotype in mammals, studies in the *Drosophila* (*dfmr*) model of FXS have shown positive effects of MPEP treatment on learning (McBride et al. 2005; Bolduc et al. 2008).

Motor Behavior

Fmr1-KOs have been reported to have abnormal motor behavior including displaying hyperactivity (Bakker and Consortium 1994; Mineur et al. 2002; Qin et al. 2005; Restivo et al. 2005), increased exploratory behavior (Bakker and Consortium 1994), and spending more time in the center of an open field (Yan et al. 2004; Qin et al. 2005). Treatment with MPEP has been shown to reduce center field behavior in the KO to one indistinguishable from wild type (Yan et al. 2005), but effects of mGluR5 antagonism on other motor behaviors have not been assessed.

Macroorchidism

As in the human FXS condition, postadolescent male Fmr1-KOs have enlarged testes (macroorchidism) (Bakker and Consortium 1994; Kooy et al. 1996; Yan et al. 2004). Neither partial reduction nor full loss of mGluR5 expression in the Fmr1-KO rescues this aspect of the phenotype (Dölen et al. 2007).

Autism-Like Behaviors

The above studies indicate that Group I mGluR antagonism in patients with FXS could have substantial effects across a wide range of clinical features in this syndrome. Importantly though, one aspect of the syndrome that has yet to be addressed in the mouse model, or with mGluR5 antagonism in particular, is autism. About 30% of individuals with FXS are diagnosed with autism, a disorder characterized by abnormal reciprocal social interactions, communications deficits, and repetitive behaviors. While autistic-like behaviors have yet to be fully investigated in the Fmr1-KO they do display some social behavioral abnormalities (Mineur et al. 2006; McNaughton et al. 2008). By comparison, the inbred mouse strain BTBR T + tf/J (BTBR) has been investigated to a greater extent and reported to have a number of features associated with autism (McFarlane et al. 2008). Treatment of BTBR mice with MPEP improves some aspects of their behavior such as reducing repetitive grooming, but does not improve their sociability (Silverman et al. 2010). Further work is needed in both mouse models and human testing to evaluate whether mGluR5 antagonism is effective in the treatment of autism-related behaviors.

17.1.2 mGluR5 Antagonists in Human Trials

The first trial of an mGluR5 antagonist in patients with FXS, sponsored by Neuropharm LTD, involved the use of fenobam in 12 adults with FXS given a single dose (Berry-Kravis et al. 2009). Although the purpose of this single-dose trial was to assess pharmacokinetics and side effects, there was a positive behavioral response with improved communication and eye contact in addition to improvement in the PPI deficit which has been documented in patients with FXS (Hessl et al. 2008). Although this first trial of fenobam was very promising, further development of fenobam was not pursued by Neuropharm due to financial challenges.

The next study of an mGluR5 antagonist in adults with FXS was a European trial of AFQ056 that took place at three centers (Jacquemont et al. 2011). This study was double blind and included 30 patients with FXS, ages 18–35, who were randomized to AFQ056 or placebo, underwent dose up titration, 14 days of full dose treatment, and then down titration (total treatment period 28 days) and then crossed over after a 1-week washout period between treatment sessions. Although the overall patient cohort did not demonstrate efficacy of AFQ056 compared to placebo in the primary measures, there was a positive response to AFQ056 on the Repetitive Behaviors Scale (RBS-R). In an exploratory analysis it was found that those patients who were fully methylated ($n = 7$) demonstrated a significant response to AFQ056 in the primary outcome measures, the Aberrant Behavior Checklist (ABC), and the Clinical Global Impression Scale (CGI) in addition to most of the secondary outcome measures compared to placebo (Jacquemont et al. 2011). This demonstrates a methylation biomarker for drug response, most likely reflective of clinical response after short-term treatment in those that are more affected by FXS.

Other mGluR5 antagonists are being assessed currently in multicenter clinical trials including R04917523 (Roche Pharmaceuticals) and also STX107 (Seaside Therapeutics). Further study will be needed to know if these agents are efficacious in FXS compared to placebo.

17.1.2.1 Targeting GABA Receptors

Work on both the mouse and fly models of FXS demonstrate that they have lower levels of GABA receptors, with the Fmr1-KO exhibiting clear reductions in the GABA-A subtype in brain (El Idrissi et al. 2005; D'Hulst et al. 2006; Gantois et al. 2006). In addition, the Fmr1-KO exhibits reduced inhibitory postsynaptic currents in the amygdala (Olmos-Serrano et al. 2010) and abnormal GABA-A currents in subicular neurons (Curia et al. 2008), but levels of glutamic acid decarboxylase, the rate limiting enzyme for GABA synthesis, in brain are mixed (El Idrissi et al. 2009; Olmos-Serrano et al. 2010). As GABA is the principal inhibitory neurotransmitter of the CNS, the collective findings indicate that the balance between neuronal inhibition and excitation in FXS would favor more overall excitation; this

conclusion is consistent with the observation that seizures are more prevalent in FXS than in the general population. Two approaches for restoring appropriate levels of GABA-mediated inhibition entail use of agonists to either the GABA-A or GABA-B receptor subtypes. GABA-A agonists act to directly compensate for the GABA-A subunit deficiencies, whereas GABA-B agonists act presynaptically to block glutamate release thus decreasing glutamatergic drive in general, but also would be expected to reduce group I mGluR activation and downstream signaling events. In the Fmr1-KO, the GABA-A agonists ganaxolone and taurine have been reported to reduce audiogenic seizures (Kooy et al. 2010) and improve learning in a passive avoidance test (El Idrissi et al. 2009), respectively. The GABA-B agonist R-baclofen (Arbaclofen; the right-sided enantiomer of baclofen) also rescues the audiogenic phenotype in the mouse model (Pacey et al. 2009), and normalizes several behaviors including marble burying and open field locomotor activity (Paylor 2008). Similarly, studies in the *dfmr* mutant fly show that a variety of GABA agonists ameloriate the lethality phenotype from glutamate-containing food, neuropathology, excessive protein translation, and abnormal courtship behavior (Chang et al. 2008).

17.1.2.2 Arbaclofen Trials in Individuals with FXS

In addition to animal data discussed earlier, anecdotal clinical experience suggesting behavioral benefits from racemic baclofen administered to patients with autism and fragile X in a clinical setting, and data from TMS studies demonstrating enhancement of cortical inhibition by racemic baclofen (McDonnell et al. 2007), supported the concept of baclofen as a possible treatment for humans with FXS. Arbaclofen (R-baclofen) has more potent GABA-B agonist activity, leading to development of this molecule for the treatment of FXS. An initial pilot double-blind placebo-controlled crossover trial of arbaclofen for children and adults with FXS, age 6–40 years, conducted by Seaside Therapeutics, involved 4-week periods of placebo and active drug treatment for each subject, with drug washout in between treatment periods. This trial showed benefit for arbaclofen over a placebo in global preference for the treatment period and clinician global impression, and was particularly evident in the subgroups of FXS patients with autism, more severe irritable behavior, or more severe social deficits. In the group with more impairment in social behaviors (ABC Social Withdrawal Score >8), significant improvement on the ABC Social Withdrawal scale, Vineland Play and Leisure Scale, and Visual Analog Scale rating for behavior were also seen (Wang et al. 2011), as well as a significantly increased number of responders ("much" or "very much" improved on the CGI and a $> 25\%$ improvement on the ABC Social Withdrawal subscale) during arbaclofen as opposed to placebo treatment. There were no significant safety issues and a very mild side effect profile. Many subjects are continuing treatment though an extension study, to evaluate the long-term benefits and the toxicity. Anecdotally, many of these subjects continue to show benefits of treatment and further development of arbaclofen is in progress with additional clinical trials pending.

17.1.2.3 Ampakines and Targeting Brain-Derived Neurotrophic Factor

The neurotrophin brain derived neurotrophic factor (BDNF) has been shown in numerous studies to be a positive modulator of synaptic plasticity. In particular, application of BDNF or increasing endogenous levels of BDNF production facilitates hippocampal LTP, as well as memory (Kramár et al. 2004; Minichiello 2009). Recent work has shown that BDNF corrects hippocampal LTP deficits in several rodent models of diseases or conditions that are characterized by memory impairment, including those for Huntington's disease (Lynch et al. 2007; Simmons et al. 2009), middle aging (Rex et al. 2006), and menopause (Kramar et al. 2010). Similarly, BDNF was also tested in the Fmr1-KO to determine if the neurotrophin could restore hippocampal LTP in the mutant: Using theta burst stimulation (TBS) to elicit LTP in the hippocampal CA1 region, the Fmr1-KOs were found to have a higher threshold of induction such that five theta bursts only induced LTP in WT hippocampal slices and not in Fmr1-KOs. However, in the presence of BDNF (nM) five theta bursts elicited LTP in Fmr1-KO slices to the same degree as seen in WT slices (Lauterborn et al. 2007). The fact that BDNF corrected the deficit does not, in and of itself, indicate that BDNF levels are perturbed in the Fmr1-KO. In fact, protein measures for both BDNF and its high affinity receptor TrkB in hippocampus were comparable between KOs and wildtypes. However, recent work by Louhivuori et al. (2011) in the Fmr1-KO has shown that BDNF mRNA is mis-localized in neocortical and hippocampal neurons suggesting that the site(s) of neurotrophin release and signaling may be abnormal. Furthermore, Selby et al. (Selby et al. 2007) have reported that TrkB levels are higher in a subgroup of neocortical GABAergic interneurons suggesting that cell-type specific alterations in the receptor may be present in FXS. Further work is needed to determine if disturbances in BDNF release, and thus availability at the synapse, are present in the Fmr1-KO and if the responsivity of the TrkB receptor is abnormal.

One would predict that drugs that augment BDNF content in brain likely facilitate learning and memory. A class of drugs that does both (increases BDNF expression and enhances learning) is the positive AMPA receptor modulators, also known as "ampakines." Ampakines enhance fast, excitatory transmission at central synapses (Staubli et al. 1994a, b), and produce a variety of acute effects including lowered thresholds for LTP and accelerated learning in animals ((Lynch and Gall 2006) for review); effects on memory encoding in humans also have been reported (Ingvar et al. 1997). Ampakines also increase the expression of BDNF in hippocampal and neocortical neurons, both in vitro and in vivo, with elevated levels of BDNF lasting for days following a single injection/treatment (Lauterborn et al. 2000; Legutko et al. 2001; Lauterborn et al. 2003, 2009). Importantly, ampakine-induced increases in BDNF are neuroprotective in models of insult (Destot-Wong et al. 2009; Jourdi et al. 2009a), and can facilitate both LTP (Rex et al. 2006; Simmons et al. 2009; Kramar et al. 2010) and behavior (Simmons et al. 2009) in different animal models of cognitive impairment. Thus, the overall ampakine strategy for the treatment of cognitive impairment in FXS should be viewed as

having two facets: an immediate effect of the ampakine on AMPAR function and a more protracted effect on synaptic plasticity through longer term effects on BDNF content. As expression of AMPA receptors is reduced in many brain regions of the Fmr1-KO (Li et al. 2002; Muddashetty et al. 2007; Suvrathan et al. 2010), direct positive modulation of residual receptors could be very beneficial for enhancing glutamatergic-mediated synaptic plasticity. While studies are ongoing to assess the ampakines for effects on LTP, spine morphology, and cognitive behavior in the Fmr1-KO, it is important to note that the ampakines effectively increase BDNF expression in this animal model (Lauterborn and Gall 2004) making it possible to test the long-term effects of enhanced neurotrophism on its phenotype.

Finally, a significant finding in the Fmr1-KO is the enhanced internalization of AMPA receptors in this mutant by mechanisms engaged by at least two different receptors, mGluR5 and dopamine D1 (Nakamoto et al. 2007; Wang et al. 2010a). Importantly, mGluR5 antagonism has been shown to block the internalization of the AMPARs (Nakamoto et al. 2007). Thus, it seems reasonable to conclude that combinational therapy with both an mGluR5 antagonist, which increases AMPAR levels at the synapse and reduces exaggerated protein synthesis, and an ampakine, which facilitates synaptic plasticity and enhances neurotrophism, could be particularly efficacious as a treatment strategy for the cognitive and behavioral problems in FXS.

17.1.2.4 Use of Ampakines in FXS

A single human trial has been completed with CX516 (Cortex Phamaceuticals), a direct AMPA receptor positive modulator known to increase LTP and raise BDNF levels (Jourdi et al. 2009b). This was a double-blind placebo-controlled trial of effects of CX516 on the safety and the cognitive and behavioral efficacy measures carried out in a cohort of 49 individuals with FXS (Berry-Kravis et al. 2006). The primary outcome measure was a *z*-score for memory across several verbal and nonverbal memory tasks. Conceptually, it was thought the CX516 would help compensate or correct the AMPA receptor deficit resulting from mGluR pathway overactivity. Realistically, CX516 is a very weak ampakine and provides only weak BDNF induction, and thus no improvement was seen in the primary outcome measure of memory, nor were any other behavioral or cognitive improvements observed across the full subject group. Improvement in global functioning was seen in the subgroup of five patients co-treated with an antipsychotic (known to potentiate ampakine activity), relative to the four patients on placebo and an antipsychotic. This suggests that a more potent ampakine molecule might be successful in treating FXS; however, such molecules have not yet come to clinical trials.

Although this trial did not produce the desired improvement in functioning, there were no major safety issues, providing encouragement for future use of more potent AMPA activators in the FXS population. Further, this trial was the first to demonstrate that large fairly intensive phase II clinical trial could be successfully performed in groups of subjects with FXS, with high completion rates for study procedures.

17.1.2.5 The Dopaminergic System and Stimulants

Individuals with FXS often display hyperactivity, attention deficit, and lack of impulse control (Hagerman and Silverman 1991). Dysfunction in frontal-subcortical circuits (i.e., reduced dopaminergic drive) is thought to give rise to these types of behavior (Hjalgrim et al. 1999), and stimulants that modulate forebrain dopaminergic tone correct them (Solanto 2002). Consistent with this, recent work by Fulks et al. (2010) demonstrated that the Fmr1-KOs have reduced extracellular dopamine levels in striatum. Increased dopamine turnover in the cortical regions, the striatum, and the hippocampus also has been reported for the KO (Gruss and Braun 2004). In addition, dopamine receptor 1 (D1) signaling is impaired in both the striatum and the prefrontal cortex of the mutant, and treatment of Fmr1-KO mice with the D1 receptor agonist SKF81297 partially reversed their hyperactive locomotor activity and enhanced their motor function on the rotarod apparatus (Wang et al. 2008a). The psychostimulant amphetamine has also been shown to elicit a greater increase in dopamine release in the prefrontal cortex of Fmr1-KOs as compared to wild-type mice, and improved their ability to discriminate objects (Ventura et al. 2004), suggesting that stimulants may be useful for restoring some balance in dopaminergic tone in forebrain and improving behavior in FXS.

17.1.2.6 Human Studies of Stimulants and Aripiprazole in FXS

There has only been one controlled trial of stimulants in children with FXS and it demonstrated that two-thirds of the patients responded well to the stimulant compared to the placebo (Hagerman et al. 1988). Stimulants are widely used now in children with FXS who are 5 years or older and the effect is generally positive with improvement in hyperactivity and attention (Amaria et al. 2001; Berry-Kravis and Potanos 2004; Hagerman et al. 2009). Occasionally on a higher dose greater activation or a lower number of verbalizations can be problematic but stimulants are usually well tolerated. A negative response to stimulants in a patient under 5 years of age should not deter a trial after 5 years since this drug class is more likely to be tolerated and effective after age five.

Although aripiprazole is a treatment directed primarily at behavior rather than specific molecular mechanisms, it could be theoretically targeted to dopamine deficits described in the *fmr1* knockout mouse (Wang et al. 2008a, b), given its dopamine agonist activity at lower doses. Apipirazole has shown good success when used empirically in FXS clinic populations (Berry-Kravis and Potanos 2004; Hagerman et al. 2009) and resulted in improvement in the ABC Irritability score, other ABC subscores, and additional behavioral rating scales in 15 participants with FXS treated in a very recently completed open-label trial (Erickson et al. 2010b). Initiation of a double-blind placebo-controlled trial of aripiprazole is planned.

17.1.2.7 Targeting Proteins that Regulate the Spine Actin Cytoskeleton

The spine actin cytoskeleton is a dynamic network that supports the shape, and ultimately the function, of the postsynaptic structure. There are numerous proteins and signaling pathways that act to regulate the actin cytoskeleton including the Rho GTPases, and mutations in a number of these proteins have been associated with different forms of mental retardation [reviewed in (van Galen and Ramakers 2005)]. Recent work in *Drosophila* has shown that dfmr (the fly homologue of FMRP) binds to the mRNA encoding the small Rho GTPase dRac (Lee et al. 2003), suggesting that FMRP regulates proteins critical to actin remodeling. Rac signals through its downstream effector p21-activated kinase (PAK), a family of serine–threonine kinases comprised of at least three members, PAK1, PAK2, and PAK3. The Rac–PAK pathway recently has been shown to be important for the stabilization of newly formed actin filaments that occur following TBS (Rex et al. 2009). Although loss-of-function mutations in the *PAK3* gene are associated with non-syndromic X-linked mental retardation (Allen et al. 1998; Bienvenu et al. 2000), recent work from Hayashi and colleagues (2007) has suggested that excessive PAK activity in Fmr1-KOs may be an underlying cause of the dendritic spine abnormalities. In particular, these authors demonstrated that spine abnormalities in neocortex were partially ameliorated in Fmr1-KOs that expressed a dominant negative *PAK* transgene in the forebrain (Hayashi et al. 2007). Likewise, cortical LTP was fully restored in the Fmr1-KO by reduced PAK expression. Finally, several behavioral abnormalities, including locomotor activity, stereotypy, anxiety, and trace fear conditioning, in the KOs also were ameliorated to some degree by the dominant negative *PAK* transgene. These data suggest that inhibition of PAK activity could be a potentially interesting therapeutic target for aspects of the FXS phenotype. To this end, PAK inhibitors are being developed and in initial testing have shown that they correct spine defects and restore LTP in the neocortex (Vollrath et al. 2010).

With regard to the PAK inhibitors, it is important to note that systemic use of these compounds may still only result in regionally selective effects in brain: Although Hayashi et al. were able to attain reduced PAK levels in both the neocortex and the hippocampus the effect on dendritic spine features was only observed in neocortex (Hayashi et al. 2004). Moreover, recent work by Chen and colleagues (2010) in the Fmr1-KO hippocampus demonstrated that the physiological activation of both Rac and PAK in spines is deficient and, consistent with this, the newly polymerized spine actin that occurs following LTP-producing stimulation fails to properly stabilize. These data suggest that the consequence of FMRP loss on RAC–PAK pathway signaling may be different between the cortex and the hippocampus, and that the use of PAK inhibitors may be regionally effective for certain aspects of the FXS phenotype. Evaluation of the impact of these compounds on the different forms of memory (i.e., those ascribed to hippocampus versus other structures) will be particularly interesting.

17.1.2.8 Targeting Other Intracellular Signaling Pathways: Phosphatase and Kinase Inhibitors

Several signaling pathways that regulate protein translation are perturbed in the Fmr1-KO. In particular, mGluR-dependent translation occurs through two major signaling pathways, the ERK–MAPK and PI3 Kinase-mTOR pathways, with convergence on the translation initiation (eIF4F) complex [reviewed in(Waung and Huber 2009)]; inhibition of either PI3 kinase, mTOR, ERK, or translation initiation itself prevents mGluR-LTD (Huber et al. 2000; Gallagher et al. 2004; Hou and Klann 2004). Studies in the Fmr1-KO have shown that the activation of both ERK and mTOR is misregulated (Kim et al. 2008; Weng et al. 2008; Sharma et al. 2010), consistent with the observation that protein synthesis in the mutant is aberrant. Moreover, other proteins that control gene expression and other cellular processes are also misregulated in the KO including glycogen synthase kinase-3β (GSK3β) (Min et al. 2009; Yuskaitis et al. 2010a). As such, a number of studies have targeted these systems (amongst others) and the results for specific drugs and/or targets are encouraging.

Phosphatase Inhibitors

Weng et al. showed that the phosphorylation of ERK in both neurons and thymocytes of Fmr-1 KOs, and in lymphocytes from peripheral blood of individuals with FXS, is delayed (Weng et al. 2008). Kim et al. (2008) also demonstrated that Group I mGluR-dependent activation of the ERK pathway in the Fmr1-KO is abnormal. Specifically, following mGluR1/5 stimulation ERK is phosphorylated in wild-type cortical synaptoneurosomes but *dephosphorylated* in KO cortical synaptoneurosomes (Kim et al. 2008). These results suggest that in response to synaptic stimulation there is aberrant activation of phosphatases in Fmr1-KO synapses. In agreement with this, both protein phosphatase 2A (PP2A) and tyrosine phosphatase were found to be overactivated after mGluR1 and mGluR5 stimulation, respectively, resulting in the rapid deactivation of ERK in Fmr1-KO samples. Pretreatment with a PP2A blocker, however, fully restored ERK activation in Fmr1-KO synaptoneurosomes. The consequence of overactive phosphatases and a misregulated ERK pathway in FXS could be multifold as the MAPK/ERK pathway is involved in many cellular processes. However, it is important to note that not all aspects of the FXS phenotype may be ameliorated by facilitating ERK activation as Chuang et al. (2005) showed that *inhibition* of ERK signaling in the Fmr1-KO was beneficial for controlling seizure-like activity.

PI3K Inhibitors

Recent work by Gross et al. (2010) has shown that PI3K activity, and downstream signaling to Akt, is markedly increased in Fmr1-KO synapses (Gross et al. 2010).

Interestingly, this elevation in PI3K activity is dependent upon the absence of FMRP but not on the presence of group I mGluRs, although mGluR5 antagonism corrected it (Gross et al. 2010). Antagonism of PI3K signaling with two different drugs, LY294002 and wortmannin, rescued excessive synaptic translation in the KO (Gross et al. 2010); treatment with rapamycin, which inhibits the PI3K downstream signaling molecule mTOR, also reduced translation in the KO and is in line with the observation of increased phosphorylation and activity of mTOR in the absence of FMRP (Sharma et al. 2010). Finally, the same group showed that treatment of *Fmr1* knockout neurons in culture with the PI3K inhibitor LY294002 in vitro reduced AMPAR endocytosis and normalized protrusion (including spines and filopodia) density in Fmr1-KO neurons to WT levels (Gross et al. 2010). These data are intriguing in that they further support the idea of selectively targeting the PI3K-mTOR pathway for the treatment of FXS. Currently, inhibitors of this pathway are being investigated in preclinical models of cancer with some success (McMillin et al. 2009), suggesting the possibility that selective compounds could be available for testing in other disorders such as FXS in the future.

GSKβ Inhibition and Lithium

Lithium is principally used to treat mood disorders and, although the exact mechanism is not understood, likely improves behavior through modulatory effects on various brain chemical systems including serotonin, dopamine, and the neurotrophin BDNF [reviewed in (Bschor et al. 2003; Beaulieu and Caron 2008; Gold et al. 2010)]. In recent years, work has more directly linked the effect of lithium to inhibition of GSK3β, a serine/threonine protein kinase, which in turn promotes β-catenin-dependent gene expression (Wada 2009). Work by Jope and colleagues has shown that the inhibitory form of GSK3β is reduced in the Fmr1-KO brain, liver, and testes, suggesting that this kinase is constitutively overactive in the mutant, and that lithium treatment normalizes these measures (Min et al. 2009; Yuskaitis et al. 2010a, b). Furthermore, lithium treatment recently has been shown to normalize levels of activated ERK (Venkitaramani et al. 2010), indicating that this drug is having effects across several protein synthesis-dependent pathways. In addition, recent work by Choi and colleagues demonstrate that lithium can restore normal mGluR-dependent LTD (Choi et al. 2010). Finally, lithium has been shown to reverse a number of behavioral abnormalities in the Fmr1-KO including open field hyperactivity (Min et al. 2009; Liu et al. 2010; Yuskaitis et al. 2010b), deficits on a social interaction task [(Liu et al. 2010; Mines et al. 2010), learning deficits (Liu et al. 2010; Yuskaitis et al. 2010b), anxiety (Liu et al. 2010; Yuskaitis et al. 2010b), novel object recognition (Venkitaramani et al. 2010), audiogenic seizures (Min et al. 2009), as well as dendritic spine shape (Liu et al. 2010), and macroorchidism (Yuskaitis et al. 2010a). Other GSK3 β inhibitors such as SB-216763 have been shown to reverse a number of these phenotypes as well (Min et al. 2009). Importantly though, the effects of GSK3β inhibitors and mGluR5

blockers are not additive, providing strong evidence that excess GSK3β activity is a direct consequence of excessive mGluR activity (Min et al. 2009).

17.1.2.9 Human Trials of Lithium in FXS

Although a number of intracellular treatment targets have been proposed, including lithium, PI3K inhibitors, GSK3β inhibitors, and PAK inhibitors, in most cases safe and available agents acting on these targets are not yet developed for use in humans. One exception is lithium, for which the preclinical findings in the *dfmr* mutant fly and *fmr1* knockout mouse, as described earlier, suggested promise of therapeutic benefit. Lithium may attenuate activation of the phospholipase C (PL-C) signaling pathway by inhibiting phosphatidyl inositol (PI) turnover (Berridge 1993), and clearly inhibits GSK3β activity (Min et al. 2009; Yuskaitis et al. 2010b) which would decrease phosphorylation of ERK and multiple signaling molecules that regulate translation; all of these effects would theoretically lead to reduction of translational activation. Given that lithium treatment does in fact normalize levels of activated ERK and GSK3β in the *fmr1* knockout (Venkitaramani et al. 2010), it appears that the main effect of lithium in the *fmr1* knockout is to reduce excessive GSK3β activity with resultant reduction in excessive ERK-mediated translation; however, lithium may also directly increase surface expression of AMPA receptors (Du et al. 2010) and reduce excess MAP1B activity (Owen and Gordon-Weeks 2003).

Although lithium has been used for some time to treat mood instability and aggression in FXS (Berry-Kravis and Potanos 2004; Hagerman et al. 2009; Wang et al. 2010b), only anecdotal information on effectiveness existed, prior to a pilot proof-of-concept study initiated by Berry-Kravis et al. (2008a, b) to evaluate the strategy of inhibition of mGluR-activated translational signaling pathways as a treatment for FXS, by systematically exploring the effects of short-term (2 month) treatment with lithium on a broad range of phenotypes including behavior, cognition, and biophysical measures in a small cohort of subjects with FXS. In addition, since ERK (extracellular-signal regulated kinase) was shown to have a reduced rate of activation in the *fmr1* knockout and in lymphocytes from humans with FXS (Weng et al. 2008), ERK activation was explored as a potential biomarker for effects of lithium on cellular signaling and more generally as a model for measuring changes in signaling during treatment with agents that may impact receptor-activated translational regulatory pathways. In this pilot open-label trial in 15 patients with FXS (Berry-Kravis et al. 2008a), lithium treatment resulted in a significant improvement in behavior as was seen in on the Total Aberrant Behavior Checklist-Community Edition (ABC-C) Score, and the Hyperactivity, Inappropriate Speech, and Lethargy (Social Withdrawal) subscales of the ABC, the Maladaptive Behavior subscore from the Vineland Adaptive Behavior Scale (VABS), a parent visual analog scale for target behaviors, and the Clinical Global Impression (CGI) Scale. Improvement in verbal memory on the RBANS List Learning task was also demonstrated in addition to normalization of abnormal ERK phosphorylation rates in lymphocytes (Berry-Kravis et al. 2008a, b). There were no major side

effects but polydipsia and polyuria were seen relatively frequently as expected, and there were a few subjects with abnormal thyroid measurements on lithium. A subgroup of 11 subjects continued on lithium for a year with persistent improvements in behavior on the ABC-C and VABS, and ongoing normalization of the ERK activation biomarker (Berry-Kravis, unpublished data), suggesting the behavioral improvements were less likely to be placebo effects. These data indicated that further studies with a placebo-controlled trial would be indicated, however such studies have not yet been carried out, partly due to concerns about the chronic toxicity of lithium, but also related to hope that less toxic mechanism-based treatments will be available soon.

17.1.2.10 Minocycline and Metalloproteinases

Minocycline is a broad-spectrum tetracycline analogue commonly used to treat acne and other skin diseases. Interest in this drug as a potential therapeutic for CNS disorders began with select studies showing that minocycline was neuroprotective in several mouse models of neurodegenerative disorders including Huntington's disease (Chen et al. 2000) and Alzheimer's disease (Choi et al. 2007). Recently, minocycline was tested in the Fmr1-KO for effects on hippocampal dendritic spine development and behavior (Bilousova et al. 2009). Bilousova et al. (2009) found that minocycline promotes the maturation of hippocampal dendritic spines in young neurons. Specifically, minocycline treatment of cultured Fmr1-KO hippocampal cells resulted in a greater proportion of mushroom shaped spines, thought to reflect more mature spines. While there was no effect of treatment on spine length or density, minocycline did reduce the number of filopodia-like protrusions. Further, minocycline treatment of nursing dams beginning at time of birth for 1 week increased the proportion of hippocampal spines with larger heads and reduced the number of filopdia in the Fmr1-KO pups. While these findings are very encouraging and indicate that treatments with this drug could begin very early in development, tests of minocycline on other aspects of the phenotype including examination of the neocortex where the spine abnormalities are greater and persist through adulthood will be very important.

The mechanism(s) by which minocycline "normalizes" the maturation of hippocampal spines is not known but evidence suggests that it could be through regulation of matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases that degrade extracellular matrix proteins. Treatment of wild-type hippocampal neurons with either MMP-7 or MMP-9 results in a more immature dendritic spine phenotype (more filopodia and fewer mushroom-like spines) (Bilousova et al. 2006, 2009), indicating that aberrant MMP levels could give rise to abnormal spine morphologies. Consistent with this observation, active MMP-9 levels were found to be greater in hippocampal lysates of 1-week-old Fmr1-KO mice versus wild types (Bilousova et al. 2009). These data suggest that reducing MMP activity could help to normalize spine morphology. As minocycline and other tetracyclines are well known to inhibit the expression of MMPs [reviewed in

(Griffin et al. 2010)], and mincycline treatment of Fmr1-KO pups reduced MMP-9 activity (Bilousova et al. 2009), it is likely that the spine effects described by Bilousova and colleagues could be due in part to the inhibition of an overactive enzymatic process.

Finally, Bilousova et al. (2009) also tested the effects of minocycline on several behavioral measures for the Fmr1-KOs; drug was given to nursing dams beginning at birth for 21 days and the pups were tested at 3 weeks of age. Using the elevated plus maze, the time spent in the open arm was used as an indicator of anxiety (less time = more anxious). Minocycline-treated Fmr1-KO mice spent more time in the maze's open arms as compared to nontreated mutants, indicating that the drug reduced their anxiety. Using the Y maze to examine hippocampal dependent memory, the investigators also found that minocycline treatment facilitated the Fmr1-KO's strategic exploratory behavior in the task. Overall, these findings indicate that minocycline could be a potential therapeutic for the treatment of cognitive impairment in FXS.

17.1.2.11 Human Trials of Minocycline

After publication of the Bilousova et al. (2009) report, numerous families began using minocycline in their children with FXS as a targeted treatment. Utari et al. (2010) surveyed 50 families who utilized minocycline in their children or adults with FXS for at least 2 weeks to 20 months (mean duration 3.5 mo). Seventy percent of individuals with FXS (43 males and 7 females mean age 13.3 years; SD 6.2 years) had a positive response to minocycline with improvements in language, behavior, and/or cognition as judged by the parents. Although this was not a controlled trial it suggested that further studies of minocycline are warranted for treatment of FXS. Paribello et al. (2010) reported positive effects in an open trial of minocycline in boys with FXS 13 years and older. Currently, a controlled trial of minocycline is taking place at the MIND Institute for children 3.5 years to 16 years with FXS. So far the trial has not demonstrated significant side effects and the analysis of efficacy will be carried out in 2011.

17.1.2.12 Antioxidants: Melatonin and Vitamin E

Results of studies using the Fmr1-KOs have suggested that antioxidants such as melatonin and vitamin E could be beneficial for aspects of the FXS phenotype. Melatonin is a hormone secreted by the pineal gland that acts to regulate the body's circadiam rhythm, in addition to other hormones. Melatonin is also a strong antioxidant and has been shown to be neuroprotective in animal studies (Singhal et al. 2010). Evidence for oxidative stress in the Fmr1-KO has been reported (el Bekay et al. 2007; Romero-Zerbo et al. 2009); for example, the mutants display reduced glutathione levels (in brain) and elevated lipid peroxidation (in brain and testes). Treatment with melatonin for 1 month normalized these biochemical

measures in the mutant (Romero-Zerbo et al. 2009). In addition, measures of aberrant motor and learning behaviors as well as anxiety in the Fmr1-KOs also were normalized by melatonin treatment (Romero-Zerbo et al. 2009). Similarly, treatment of Fmr1-KOs with the strong antioxidant/free radical scavenger vitamin E (alpha-tocopherol) with or without *N*-acetyl L cysteine (NAC) has been shown to normalize oxidative stress markers, testicular size, and behavior (learning, anxiety) (de Diego-Otero et al. 2008). While these data suggest that melatonin and vitamin E could be useful for FXS, particularly as part of a combinational treatment strategy with another drug approach, for it is important to note that melatonin has a very short half-life. Consequently, synthetic melatonergic agonists may be more effective (Hardeland 2010).

17.1.2.13 Human Trials of Melatonin and Other Antioxidants

A controlled study of melatonin's effect on sleep has been carried out in six children with FXS, one with the premutation and in five children with ASD between the ages of 2 and 15.25 years (SD 3.6) (Wirojanan et al. 2009). This study lasted 4 weeks with a crossover design between melatonin and placebo. Children treated with melatonin demonstrated a significant increase in mean night sleep duration, a decrease in mean sleep onset latency, and an earlier sleep onset time compared to placebo. Dysregulated sleep occurs in 32–77% of patients with FXS (Richdale 2003; Kronk et al. 2010). However, these sleep problems are universal in many neurodevelopmental disorders including autism (Richdale 1999). Other controlled trials of melatonin have also been helpful for ASD (Garstang and Wallis 2006). It is also possible that the therapeutic effects of melatonin are related to its antioxidant effects and ability to normalize synaptic connections in the KO mouse (Romero-Zerbo et al. 2009). Although other antioxidants such as omega 3 fatty acid, vitamin C, vitamin E, and NAC have been utilized by many families often routinely there have been no controlled studies of their use. An exception to this is the controlled study of L acetylcarnitine (LAC) carried out by Torelli and colleagues (2008) in 63 males with FXS and ADHD treated for 1 year in a controlled parallel study of LAC (20–50 mg/kg/day up to 1,000 mg/day) versus placebo. Fifty six patients completed the study and there was a significant improvement in ADHD symptoms on the Conners Global Index Parents scale and also in behavior and socialization on the Vineland Composite and Socalization Scale with LAC compared to a placebo.

17.1.2.14 Human Trials of Donazepil and Other Agents

Other agents acting at an array of receptors have undergone exploratory study in groups with FXS [reviewed in (Berry-Kravis et al. 2011)]. These include donazepil, an anticholinesterase which raises acetyl choline in brain and is extensively utilized for maintenance of cognitive function in Alzheimer's disease. Donazepil showed promise for treatment of behavior and social function in an open-label trial in

participants with FXS and now is being studied in a larger placebo-controlled trial (clinicaltrials.gov). A small open-label study of memantine, an NMDA antagonist, in six individuals with FXS demonstrated modest clinical benefit on a CGI in 4/6 patients, but lack of improvement on behavioral rating scales, and several patients developed substantial irritability that limited treatment (Erickson et al. 2009). An open-label study of riluzole, a sodium channel blocker and glutamate uptake activator that indirectly decreases glutamate receptor activity, showed overall behavioral improvement in only one subject of five patients with FXS treated, although ERK activation rates normalized and there was a suggestion of improvement specifically in hyperactivity symptoms (Erickson et al. 2010c).

Anecdotal treatment experience with three adults with FXS treated with acamprosate, demonstrated improvement in language and behavior in all patients (Erickson et al. 2010a). Acamprosate is a drug approved for assisting with alcohol withdrawal that most likely interacts with multiple receptors but primarily may exert effects by acting as a mixed agonist/antagonist at NMDA receptors and activating GABA-A receptors with possibly inhibitory effects at group I mGluRs (Erickson et al. 2010a). One patient had significant gastrointestinal side effects that are often seen with acamprosate.

Cells from the *fmr1* knockout mouse and from individuals with fragile X show reduced cAMP production (Berry-Kravis et al. 1995; Kelley et al. 2007; Chen et al. 2010) which is dependent on FMRP levels (Berry-Kravis and Ciurlionis 1998). Likewise adenylate cyclase activity modulates mGluR-mediated regulation of FMRP activity (Wang et al. 2008a, b). Although the mechanism through which FMRP regulates cAMP production is not known, FMRP is known to bind adenylate cyclase subunit mRNAs (Darnell et al. 2011). Because of the reduction in cAMP production in FXS tissue, treatment with phosphodiesterase inhibitors such as rolipram has been proposed, however no preclinical work has yet been done in this area.

17.1.2.15 Genetic Manipulations

A few studies have attempted to correct the phenotype in the Fmr1-KO by genetic manipulation. The approach used with some success was either through the reduction of mGluR5 levels (see above) or by expressing FMRP de novo in the Fmr1-KO (Peier et al. 2000; Gantois et al. 2001; Musumeci et al. 2007; Zeier et al. 2009). Expression of FMRP in the Fmr1-KO rescued a number of the phenotypes including normalizing hippocampal LTD (Zeier et al. 2009), macroorchidism (Peier et al. 2000), reducing anxiety (Peier et al. 2000), and reducing audiogenic seizure susceptibility (Musumeci et al. 2007). While gene and/or stem cell therapy for neurological disorders is still in its early phase and has a number of issues, these studies are encouraging and suggest that this approach could correct the FXS phenotype (for comprehensive reviews see Chaps. 2 and 6).

17.1.2.16 Clinical Trial Design and Associated Hurdles in FXS

Although an ever-increasing number of neuronal targets for treating the underlying disorder in FXS are emerging, and have prompted early translational work, there are still many issues regarding optimal trial design and how to best demonstrate treatment effects in a clinical trial setting, in the absence of good models for cognitive treatment trials for any neurodevelopmental disorders [reviewed in (Berry-Kravis et al. 2011)]. FXS, in fact, serves as a good model to develop such designs, particularly because FXS is a single genetic disorder in which all affected individuals have the same cellular defect as the primary cause of their brain disorder, a mouse model is available, information on the synaptic function of FMRP in brain is known, and aspects of FXS model more common disorders likely to have mechanistic overlap, including autistic spectrum disorders and learning disabilities.

Trial design issues that need to be resolved for each targeted treatment trial in FXS include: (1) length of placebo treatment and whether to use crossover designs or open-label extensions to ensure everyone gets a chance at active treatment and increased recruitment; (2) lack of information on optimal dosing and whether to determine this through dose escalation or flexible dosing within or between subjects or multiple fixed-dose arms; (3) how to best detect side effects in cognitively impaired individuals who may not be able to discuss their symptoms; (4) the most appropriate age range to study treatment effects, balancing concerns about safety that dictate studies in adult trials initially, with the possibility that studies at younger ages may be indicated even if there are minimal effects in older individuals, because much more significant results may be seen by treating the underlying disorder in young children who are not as advanced in the process of brain development, and are still in school; (5) understanding of the length of treatment needed to impact brain wiring and demonstrate measurable cognitive improvement; (6) drug formulation and how to best deliver drugs to younger children and individuals with difficulty swallowing pills due to coordination issues; (7) inclusion of females and mosaic individuals, and whether to analyze their responses separately, as individuals with FMRP present in a fraction of cells may have different dosing ranges, responses, and toxicities; (8) whether to allow baseline medications and the balance between the need to analyze treatment effects in the absence of medication interactions, problems with recruitment and patient deterioration if baseline medications have to be weaned, and the importance of demonstrating that new targeted treatments can actually improve symptoms even when the best available symptomatic regimen is already in place; (9) the numbers of study visits and travel issues for a relatively rare disorder in which subject numbers are limited and participants may travel a distance to get to a trial site; and (10) the problem of the lack of validated, sensitive behavioral outcome measures, the lack of well-defined cognitive outcome measures, and the lack of biomarkers known to correlate with functional status in FXS.

The design and validation of outcome measures for clinical trials in FXS and other neurodevelopmental disorders represents the most significant hurdle in trial design for targeted treatments. The choice of optimal outcome measures has been

difficult because of the need to test a broad range of abilities so that there is not too much floor or ceiling effects with high- or low-functioning individuals, issues with co-operation and variable performance, the general lack of information on reproducibility of measures for the population being studied, and because for measures that would appear to quantify core defects, there is insufficient data available on whether they correlate with quality of life or true functional improvement. Only a subset of outcome measures utilized in recent trials have shown good feasibility and validity (Berry-Kravis et al. 2006, 2008a, b, 2009). Thus, recently investigators have begun to develop templates for pretrial feasibility, reproducibility, and validity assessment (Berry-Kravis et al. 2008a, b; Hessl et al. 2008; Knox and Berry-Kravis 2009; Scaggs et al. 2011). The choice of outcome measures must also balance the use of standard accepted behavioral measures with precedent for use in drug registration/FDA approval, which are generally caregiver rating scales (such as the ABC), versus use of novel measures (Hessl et al. 2008; Knox and Berry-Kravis 2009; Scaggs et al. 2011) that are more quantitative and may objectively measure core phenotypes (such as eye tracking or PPI), thus advancing treatment science, but have no precedent for registration and are not yet known to predict a specific functional outcome.

A recent series of NIH-sponsored meetings (2009) aimed at developing a consensus about optimal outcome measures for clinical trials in FXS has resulted in some recommendations about best choice of currently existing measures, validation needs for existing measures, optimal additional measures that need to be developed, and the work needed to develop these. No one behavioral rating scale was felt to capture the range and character of problem behaviors typically observed in individuals with FXS, and the development and validation of a fragile X-specific behavioral scale has been suggested. As initial work in preparation for development of such a scale, ABC ratings have been collected from multiple sites and subjected to factor analysis for FXS relative to age and gender. This early work has indicated that the factor structure of ABC subscales and the number of items incorporated into the scale (which was developed for individuals with general cognitive impairment and has been used extensively in autism) should be modified for good validity in FXS (Hessl et al. 2010).

Several years ago the Fragile X Clinical and Research Consortium (FXCRC) was created to help ensure state-of-the-art care delivery to meet the needs of individuals with FXS in North America and facilitate large-scale research efforts and clinical trials. This organization is developing structure for collaboration of FXS clinics worldwide, in preparation for large multisite clinical trials that will be necessary for regulatory approval of targeted treatments.

17.1.2.17 Combining Targeted Treatments with Educational Interventions

Medications alone will not reverse the phenotype of those with FXS because the learning that has occurred throughout life has to be recovered also. The targeted treatments will improve synaptic connections but cognitive remediation needs to

take place to strengthen these connections and remediate the lost learning unless treatment is started right after birth. This is pertinent to adults who are initiated into targeted treatments because they are out of school and not necessarily in a learning environment. Basic abilities such as reading or writing must be addressed in learning paradigms and often a tutor is an expense that cannot be afforded by many families. Therefore, computer learning programs can be utilized and targeted treatment studies should begin to address the efficacy of learning both with and without targeted treatments.

An example of a learning computer module is the Cogmed program developed by Torkel Klingberg (Cogmed Cognitive Medical Systems AB, Stockholm, Sweden) for the training of working memory. Working memory (WM), defined as the ability to temporarily store and manipulate information for some purpose (Baddeley 2000), is instrumental for a plethora of daily activities such as keeping track of goals and instructions and planning the next course of action based on current conditions. From a psychometric standpoint, WM can be easily and objectively measured (e.g., through simple span tasks incorporating a simultaneous processing element, such as mentally reordering a series of digits/letters or pattern of blocks) with tasks that most people are capable of performing with varying degrees of success. It is therefore a natural target for cognitive intervention, despite the fact that WM was once thought to be a fixed and highly heritable construct independent of environmental influences (Miller 1956; Niaz and Logie 1993; Kremen et al. 2007; Engel et al. 2008). However, a wealth of recent research measuring the pre- and postintervention changes in response to a working memory training protocol support the plasticity of WM (Gunther et al. 2003; van't Hooft et al. 2003; Holmes et al. 2009; Klingberg 2009; Dahlin 2010; Holmes et al. 2010; Klingberg 2010). Furthermore, changes have also been measured on a neuroimaging level that show increased brain activity in parietal and frontal regions specific to WM after training (Olesen et al. 2004; Westerberg and Klingberg 2007) and on a chemical level through studies showing corresponding decreases in dopamine receptor D1 density as WM performance increases in brain areas known to be critical to WM performance (McNab et al. 2009). It has been shown that dopamine is an important neurotransmitter during WM tasks, a precise balance of which is necessary for optimal WM functioning such that too much or too little would be detrimental (Luciana et al. 1992; Müller et al. 1998; McNab et al. 2009).

Much has been learned from studies of populations with Attention Deficit/Hyperactivity Disorder (ADHD) that can be applied to those with FXS with ADHD. WM is tied very closely to attentional processes, and neuroimaging studies even indicate activation of similar regions in the parietal and prefrontal lobes as controlled attention (Castellanos and Tannock 2002; Kane and Engle 2002; Olesen et al. 2004; Martinussen et al. 2005; Klingberg 2009; Beck et al. 2010). Moreover, individuals with ADHD typically present with deficits in WM function (Barkley 1996; Martinussen et al. 2005). Training in WM could therefore register improvements in cognitive domains, such as attention and focus, and the use of Cogmed in populations with ADHD has been well documented, with parents and teachers reporting reductions of symptoms postintervention (Klingberg et al. 2002, 2005; Beck et al. 2010).

A large percentage, ranging from 41 to 93% based on previous studies, of children with FXS also meet criteria from the Diagnostic and Statistical Manual for Mental Disorders (DSM IV) for ADHD subtypes (Bregman et al. 1988; Freund et al. 1993; Mazzocco et al. 1998; Backes et al. 2000). Furthermore, when compared to three other groups of intellectual disability, the FXS group exhibits a distinct attention impairment above and beyond that found in the other groups (Turk 1998; Munir et al. 2000; Cornish et al. 2004a), consistent with assertions that the fundamental neurocognitive deficit in FXS is in controlling the flow of input and (Cornish et al. 2004b; Mastergeorge et al. 2010), a function which is heavily reliant on WM. In addition, it has been hypothesized that intracellular levels of FMRP may rise in response to a WM load in typically developing individuals (Kwon et al. 2001), which would explain the abundant WM difficulties seen in individuals with FXS (Kwon et al. 2001; Lanfranchi et al. 2009; Baker et al. 2011). This corroborates studies on the Wechsler IQ scales which indicate that performance on the Digit Span subtest (a measure of WM) correlates with FMRP levels in males with FXS (Mastergeorge et al. 2010). This WM deficit is present even after controlling for overall IQ (Kwon et al. 2001), suggesting that there are disproportionately large WM impairments in FXS even in relation to global cognitive impairment. All this is in line with previously described evidence of dopaminergic dysfunction in FXS (Hjalgrim et al. 1999) and the critical role dopaminergic transmission plays in WM function (Luciana et al. 1992; Müller et al. 1998; McNab et al. 2009; Klingberg 2010). Taken together, the fragile X population, with so many core deficits relating to WM and attentional dysfunction, is a prime candidate for Cogmed's working memory training.

Aside from the direct benefits that Cogmed can offer in the form of improvements in WM and the related domains of attention and focus, recent studies on WM also implicate a significant role in higher order cognitive functions such as learning, language/reading comprehension, and reasoning ability (Kyllonen and Christal 1990; Daneman and Merikle 1996; Fry and Hale 2000; Kane et al. 2005; Dahlin 2010), all of which require holding and consistently reevaluating information in WM in the midst of new incoming information. Additionally, WM capacity has also been shown to be an effective longitudinal predictor of academic achievement (Gathercole et al. 2003; Biederman et al. 2004; Alloway and Alloway 2010), correlating most strongly with abilities in reading (Gathercole and Pickering 2000; Swanson and Sachse-Lee 2001b) and arithmetic (Swanson and Sachse-Lee 2001a; Geary et al. 2004). Furthermore, other studies indicate a positive correlation between performance on tasks measuring WM and tasks measuring fluid intelligence, or the ability to reason out novel tasks independent of previously acquired knowledge (Gray et al. 2003; Jaeggi et al. 2008). More broadly, WM tasks have also been shown to correlate with Charles Spearman's *g* (Suß et al. 2002; Conway et al. 2003), a statistical variable describing the theoretical general reasoning ability that underlies the shared variance between all cognitive tests (Spearman 1927). In other words, people who perform well on one cognitive test generally perform well on others, and the common denominator that promotes this correlation is termed "*g*," of which WM is thought to be the most important deriving factor (Suß et al. 2002).

Therefore, training in working memory can have far-reaching effects beyond merely the realm of ADHD; it can also positively influence general intellectual functioning in many different domains of life. Since FXS is the most common known cause of inherited intellectual disability (ID) (IQ < 70; Chonchaiya et al. 2009), WM cognitive training is a natural intervention strategy to explore. Although to date no compelling evidence exists of which the authors are aware that overall IQ scores are affected by WM training, studies of this nature tend to involve IQ testing within a short time frame after training (i.e., Holmes et al. 2010), which may not allow enough time for the full remediating effects of WM training on learning and reasoning abilities to take effect. It's possible that given a longer time frame in the course of years after training, WM training may create a positive feedback loop in which these individuals are more mentally engaged in all aspects of their daily lives and are therefore more prone to further beneficial environmental stimulation (thereby continuously maintaining their WM training), such that performance on IQ tests may improve significantly. Additionally, it's possible that although the effects of WM training on IQ may not be readily apparent in populations such as ADHD, it may be more so in a population such as FXS, where the initial deficits are more pronounced and the lifetime intellectual trajectory has a downward trend relative to the general population, thereby creating more room to detect improvement. This could be especially evident when WM training is used in conjunction with partial correction of synaptic dysfunction by a targeted treatment regimen, such that new cognitive development, not possible with training alone, can now be achieved.

In summary, the current and future aspects of intervention for FXS are promising for improving not only the behavioral aspects but also the cognitive aspects of this syndrome. Future FXS treatment may be a portal for understanding targeted treatments in a variety of neurodevelopmental disorders including autism.

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Chapter 18
The Fragile X-Associated Tremor Ataxia Syndrome

Flora Tassone and Randi Hagerman

Abstract Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder clinically characterized by intention tremor and gait ataxia, in addition to other conditions including hypothyroidism, autonomic dysfunction, hypertension, peripheral neuropathy, and cognitive decline. FXTAS affects some males (approximately 40%) and in less degree female premutation carriers (8–16%) older than 50 years with an age-dependent symptomatology and penetrance. The CGG repeat number appears to influence the severity and the age of onset of the disorder. The neuropathological hallmark of FXTAS is the presence of eosinophillic, ubiquitin-positive intranuclear inclusions in both neurons and astroglia throughout brain. FXTAS is due to RNA toxicity caused by elevated levels of CGG-expanded mRNA containing 55–200 CGG repeats, which is found in the intranuclear inclusions that sequester various proteins including ubiquitin, αB-crystallin, lamin A/C, hnRNP A2, myelin basic protein, and Sam68. The expression of the expanded CGG repeat *FMR1* mRNA also induces a cellular stress response and leads to a disruption of the nuclear lamin A/C architecture. These alterations are observable even in early development, suggesting that the expanded-repeat mRNA triggers pathogenic mechanisms that can provide a molecular basis for the neurodevelopmental abnormalities observed in some children who are carriers of an *FMR1* premutation allele.

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Finally, the presence of cellular dysregulation in older adults who do not present clinical features of FXTAS may suggest that additional genetic or environmental protective factors may play a role in the pathogenesis of FXTAS.

18.1 Introduction

Fragile X-associated tremor ataxia syndrome (FXTAS) is a late adult onset neurodegenerative disorder affecting approximately 40% of male carriers of the fragile X mental retardation gene (*FMR1*) premutation allele (55–200 CGG repeats), generally grandfathers of children with fragile X syndrome. Alleles with 45–54 CGG repeats are named gray zone or intermediate alleles due to their instability in transmission to the next generation (Nolin et al. 2003). Female carriers are affected less frequently (8–16%) and less severely with FXTAS due to the presence of the second X chromosome (Adams et al. 2007; Berry-Kravis et al. 2005; Coffey et al. 2008; Jacquemont 2005; Rodriguez-Revenga et al. 2009). FXTAS was identified in 2001 (Hagerman et al. 2001) following the discovery of elevated *FMR1* mRNA levels in premutation carriers by Tassone et al. (2000). Prior to the discovery of FXTAS, there was evidence of clinical involvement in young boys with the premutation who presented with ADHD, learning disabilities, intellectual disability (ID), or autism (Hagerman et al. 1996; Tassone et al. 2000). In females, there was also documentation of primary ovarian insufficiency (POI) in about 20% of women with the premutation (Allingham-Hawkins et al. 1996; Cronister et al. 1991) and emotional difficulties or subtle physical features (Franke et al. 1998; Riddle et al. 1998; Sobesky et al. 1996). However, much of this involvement was believed not to be related to the premutation, except for the POI. The finding of hard neurological signs of neurodegeneration was impossible to ignore and FXTAS opened up a clinical field of premutation involvement, now recognized as *FMR1*- associated disorders, that continues to grow with a spectrum of neurodevelopmental, emotional, endocrine, autoimmune, and neurological problems associated with the premutation.

The symptoms of FXTAS usually develop in older ages (average age of onset is 60 year) and they appear to be more severe with increased CGG repeat number. The type and severity of FXTAS symptoms can vary among individuals: in some they may progress rapidly, while in others they can remain mild over many years. Symptoms generally include progressive intention tremor, gait ataxia, neuropathy, psychiatric symptoms, cognitive/intellectual decline, parkinsonism, and autonomic dysfunction. Mood changes, increased irritability, outbursts of anger, and inappropriate or disinhibited behavior can also be present.

This neurodegenerative disorder is due to an RNA gain-of-function mechanism, more specifically to RNA toxicity due to the elevated *FMR1* mRNA levels observed in premutation carriers. The neuropathological hallmark of FXTAS observed on autopsy is represented by the presence of ubiquitin-positive intranuclear inclusions throughout the brain. Radiologically, the most distinctive sign in FXTAS is the

presence of bilateral regions of increased T2 signal intensity in the middle cerebellar peduncle (MCP sign); white matter disease in the pons, insula, and periventricular regions; and cerebral atrophy.

Although in this chapter we will focus on FXTAS, it is important to remember that the RNA toxicity associated with the premutation can cause a variety of medical and developmental problems that are not part of the clinical definition of FXTAS. These problems include autism spectrum disorders (ASD) and ADHD in children, particularly boys (Aziz et al. 2003; Farzin et al. 2006); emotional difficulties including depression and anxiety (Bourgeois et al. 2009, 2011; Roberts et al. 2008; Rodriguez-Revenga et al. 2008); neurological problems including neuropathy, autonomic dysfunction, executive function deficits, cognitive decline, and dementia (Brega et al. 2008; Grigsby et al. 2006a, 2008; Hagerman et al. 2007; Seritan et al. 2008; Sevin et al. 2009; Tassone and Berry-Kravis et al. 2010); impotence and testosterone deficiency (Greco et al. 2007); hypertension (Coffey et al. 2008); and autoimmune disease including fibromyalgia and hypothyroidism, particularly in women (Coffey et al. 2008; Greco et al. 2008; Rodriguez-Revenga et al. 2009; Zhang et al. 2009). All of these conditions in addition to POI are likely related to the effects of elevated *FMR1* mRNA, leading to dysregulation and sequestration of a variety of proteins with a consequent cellular dysfunction or even cell death (Garcia-Arocena et al. 2010; Hagerman et al. 2010).

18.2 Clinical Features of FXTAS

Clinical involvement in FXTAS includes two cardinal features, tremor which usually begins first around 62 years followed by ataxia, on average 2 years later (Leehey et al. 2007; Tassone et al. 2007). The tremor typically begins in the dominant hand as an intention or action tremor and within a year or so, the nondominant hand is also involved. It begins to interfere with handwriting and eventually other activities of daily living including pouring liquids, eating, and dressing. The tremor may also eventually involve the head, jaw, tongue, and even the legs. Eventually, the tremor may include a resting tremor but this is typically later in the course, particularly if other parkinsonian features are present (Hall et al. 2009).

The onset of ataxia may be more subtle and a history of falling is seen along with unsteadiness and listing to one side when walking. Often the patients may also say that their legs simply give out or buckle, although this weakness is more prominent later in the course of disease. The patient with FXTAS typically loses stamina for exercise and is able to walk less and less. As ataxia worsens, falls became more frequent and typically occur 6 years after onset of tremor. The need to use a cane and eventually a walker can be necessary by 15 years after onset (Leehey et al. 2007). The increasing weaknesses are likely associated with the mitochondrial deficits in FXTAS that progressively worsen with time (Ross-Inta et al. 2010). There is wide variability in the course of FXTAS and this is related to the sex of the patient, with women having a milder course and less cognitive decline

(Coffey et al. 2008; Hagerman et al. 2004; Seritan et al. 2008) (see case 1) compared to men with FXTAS. The age of onset of FXTAS and the age of death from FXTAS correlate inversely with the CGG repeat number; that is, the higher the CGG repeat, the earlier the onset and the earlier the death of the patient (Greco et al. 2006; Leehey et al. 2008; Tassone et al. 2007). The age of onset of the major motor signs of FXTAS (tremor and ataxia) is 61 ± 7.9 years (mean ± SD) (Tassone et al. 2007) and the penetrance is age related such that 75% of men aged >80 years will develop FXTAS (Jacquemont et al. 2004). Although life expectancy ranges from 5 to 25 years after diagnosis or onset of symptoms (Leehey et al. 2007), we and others have seen that those who have an additional condition, such as Alzheimer's disease, or Lewy body dementia seen in postmortem studies have the shortest lifespan (Greco et al. 2006; Kamm et al. 2005; Louis et al. 2006).

Neuropsychological and neuropsychiatric deficits are observed in FXTAS. Executive cognitive function impairments, including impulsivity, inappropriate behavior, perseveration and apathy, and defective working memory have been well described in FXTAS (Grigsby et al. 2006a, b; Tassone and Elizabeth 2010). Psychiatric problems are often present in premutation carriers whether they have symptoms of FXTAS or not. However, clinically significant symptoms, including obsessive–compulsive behavior, depression, anxiety, psychoticism, and irritability, have a high prevalence in subjects with FXTAS (Bacalman et al. 2006; Bourgeois et al. 2011; Brega et al. 2008; Hessl et al. 2005). Importantly, from the reported studies, it appears that many individuals with FXTAS do not show significant cognitive decline until the later stages of disease, with 50% of males developing significant dementia (Seritan et al. 2008).

Additional features of FXTAS were elaborated by Jacquemont et al. (2003) including neuropathy symptoms, autonomic dysfunction (orthostatic hypotension, hypertension, and urinary and bowel incontinence), radiological features of white matter hyperintensities and brain atrophy, and cognitive decline beginning with executive function deficits. Jacquemont et al. (2003) outlined the diagnostic features for definite FXTAS, probable FXTAS, and possible FXTAS, which are presented in Table 18.1 and grouped by the presence of major and minor clinical features. The additional features associated with FXTAS are now known to sometimes occur before the onset of tremor or ataxia such as neuropathy, involving numbness and tingling in the lower extremities (Hagerman et al. 2007). Interestingly, motor and sensory nerve conduction abnormalities have been observed in male premutation carriers with and without FXTAS, indicating that they may be prodromal to the main neurological FXTAS signs, tremor and gait ataxia. The degree of such abnormalities positively correlates with CGG repeat number and *FMR1* mRNA levels (Soontarapornchai et al. 2008). In addition, lamin A/C and other neurofilaments were detected within the intranuclear inclusions, and a disruption of the lamin A/C architecture within the nucleus was observed in cultured neural cells (Arocena et al. 2005), in cultured skin fibroblasts, and in brain tissue from patients with FXTAS (Garcia-Arocena et al. 2010) as well as in the CGG-expanded knockin (KI) mice (Brouwer et al. 2008). All together, these findings indicate that neuropathy, which is observed in approximately 60% of the subjects,

Table 18.1 Diagnostic features of FXTAS

Molecular	CGG repeat 55–200	
Clinical		
	Intention tremor	
Major	Cerebellar gait ataxia	
	Parkinsonism	
	Moderate to severe short-term memory deficit	
Minor	Executive function deficit	
Radiological		
Major	MRI white matter lesions involving middle cerebellar peduncles	
	MRI lesions involving cerebral white matter disease	
Minor	Moderate to severe generalized brain atrophy	
Diagnostic categories		
Definite	Probable	Possible
One major clinical, and	Two major clinical, or	One major clinical, and
One major radiological, or	One minor clinical, and	One minor radiological
Presence of FXTAS inclusions	One major radiological	

Adapted from Jacquemont et al. (2003) and Hagerman and Hagerman (2004)

represents another clinical presentation in FXTAS. Impotence, which is thought to relate to autonomic dysfunction, is also observed typically a few years before the onset of tremor. Intranuclear inclusions are found in Leydig cells, which produce testosterone, and those with FXTAS typically have a low level of testosterone (Greco et al. 2007).

Although FXTAS by definition involves tremor and ataxia and neurodegeneration, it sometimes blends with problems that started much earlier in development. These problems can start in childhood but are more commonly observed in boys and include ADHD, motor incoordination, anxiety, or even ASD (Bailey et al. 2008; Farzin et al. 2006). More often, psychiatric problems such as depression or anxiety develop in adulthood and these problems are also related to the RNA toxicity in the limbic system with later inclusion formation throughout the amygdala, hippocampus, cingulate cortex, and insula (Brouwer et al. 2008; Greco et al. 2011; Hessl et al. 2005; Hunsaker et al. 2010; Wegiel et al. 2010). Amygdala activation deficits to fearful faces can be seen in young and mid-adult men with the premutation who do not have neurological problems compared to controls (Hessl et al. 2007). Motor coordination problems develop early in the premutation mouse (Van Dam et al. 2005) and sometimes in the patients with the premutation. Before the onset of FXTAS, changes in gray matter (Hashimoto et al. 2011a), diffusion tensor imaging (Hashimoto et al. 2011b), and fMRI deficits can be seen involving the hippocampus and frontal regions of the brain in carriers compared to that in controls (Hashimoto et al. 2011a; Koldewyn et al. 2008). Therefore, subclinical changes in the brain are seen well before the onset of FXTAS, although for some individuals these problems may stay subclinical. The above described brain changes may be associated with additional premutation-associated problems such as migraine headaches, which can occur at higher rate in approximately 50% of female carriers and 40% of male carriers (Akins et al. 2008).

The emergence of autoimmune problems in the form of fibromyalgia (Coffey et al. 2008; Leehey et al. Submitted) and thyroid disease occurs in 43% and 50%, respectively, of women who have FXTAS, which was significantly different from age-matched controls (Coffey et al. 2008). To illustrate the features of FXTAS described above, a typical case of FXTAS in a female is described below.

18.3 Case History

Case 1 is of a 75-year-old woman with 90 CGG repeats and *FMR1* mRNA that is 3.25 times normal, with a history of early onset of tremor at age 32. This was an intention tremor, which gradually worsened until it interfered with the patient's ability to drink liquids at age 35. It was diagnosed as an essential tremor and at age 40, she also developed an intermittent no-no head tremor. Her ataxia began at age 59 and has gradually worsened over the years. At age 67, she was diagnosed with FXTAS, with an MRI demonstrating the MCP sign, although she still manifested normal cognitive function. She subsequently developed neuropathy at about age 70, which included numbness and tingling and intermittent pain in her feet bilaterally. She also developed increasing weakness in her hands, particularly with grasping things, and has severe cramps and weakness in her legs such that they give out on her intermittently, causing her to fall. Over the last 8 years, she has fallen and sustained significant injuries including a compression fracture in her lumbar spine. Falling has also caused her to break her wrist and clavicle. Over the last year, she has developed postprandial lightheadedness, feeling as if she is going to faint, combined with pain in her shoulders. She usually has to sit down because it is almost impossible to walk with the lightheadedness. She has had symptoms of orthostatic hypotension in the past. She has a significant bradycardia and her heart rate is rarely above 50. She is taking nadolol 40 mg, a beta blocker for treatment of her tremor, but it is only mildly helpful and is likely exacerbating her bradycardia. She also has significant osteoporosis and has taken Fosamax over the last 6 years. If she gets a good night's sleep, she is far less likely to have the postprandial lightheadedness, and acetaminophen taken at bedtime helps her sleep because it reduces the pain that she experiences in her feet. She is also on calcium and vitamin D.

Over the last 6 months to 1 year, she feels that her speech is less articulate, sometimes slurred, and this is likely because of an oral motor dyspraxia. She gave up cooking last year because her tremor causes the ingredients to fly across the room and she tires very easily from the pain in her shoulders. She does not sleep excessively during the day. She is unable to use a cane because of the interference of her tremor with cane use.

On examination, she has a continual no-no head tremor that also involves the jaw and the tongue fairly dramatically. Her blood pressure is 149/82, her heart rate is 49, head circumference is 56.5 cm, height is 170 cm, and weight 72.3 kg. She has a very coarse intention tremor that has intermittent myoclonic jerking. The tremor is

present with holding a position and it is worse in the left nondominant hand, compared to the right. She has significant ataxia with heel-to-shin movement, she is unable to tandem walk, and she is very unstable in walking and turning. She has absent deep tendon reflexes in all four extremities and at all levels. Her Babinski reflex is negative but her Hoffman reflex is positive. She has Gegenhalten movements with increased rigidity in her arms. She has absent vibration sense in both ankles and toes bilaterally. Vibration sense is present in her right knee but decreased in the left knee.

Her cognitive function demonstrates gifted abilities in the verbal and full scale IQ. She received a PhD and worked as a college administrator but she is now retired. Her brother, who earned a PhD in engineering, also suffered from FXTAS and had gifted abilities. However, he had a rapid decline to dementia over 5 years after his diagnosis and at the time of death, about 7 years after diagnosis, he had Alzheimer's disease and FXTAS on neuropathological examination (Greco et al. 2006; Mothersead et al. 2005).

Case 1 has had her disease for 43 years with an early onset at 32 years and a very slow decline. She has never experienced autoimmune problems or migraine headaches. She has also never been exposed to toxins, which can exacerbate FXTAS from anecdotal reports (O'Dwyer et al. 2005; Paul et al. 2010). We have also commonly seen the onset of FXTAS symptoms after prolonged general anesthesia (Jacquemont et al. 2004), suggesting that there is neuronal toxicity with the use of isofluorane or other anesthetic agents. Case 1 did not have surgery before her early onset at age 32 years and it is a puzzle why she developed her symptoms so early, but she has been remarkably slow in her progression, so early onset does not necessarily mean rapid decline or more severe disease.

18.4 Cellular Studies

Since the neurons with the premutation can die more easily in culture by 21 days (Chen et al. 2010) and because of mitochondrial abnormalities even in cells from premutation carriers without FXTAS, it is likely that there may be toxins or adverse environmental events that can lead to cell death in the vulnerable premutation neurons. A stressful environment can also more easily lead to cortisol elevations in those with the premutation, as seen in the KI mice (Brouwer et al. 2008). Elevations in cortisol can also lead to cell death and Adams et al. (2007) have demonstrated that elevations in anxiety on the Symptom Checklist-90 (SCL-90) are associated with smaller volumes of the hippocampus in women with the premutation. Stressful situations such as divorce or raising difficult children with FXS may also predispose to premutation-associated disorders including psychiatric problems or neurological problems. Many female carriers complain of memory problems or foggy thinking or chronic pain, but it is not known if these symptoms represent a prodrome to FXTAS or not. Further longitudinal studies are needed.

18.5 The Prevalence of FXTAS

Although population-based studies on the prevalence of FXTAS have not been conducted to date, based on the prevalence of the premutation in the general population and on the penetrance of FXTAS among premutation carriers, it is possible to obtain an estimate of the prevalence of FXTAS in the general population. Reported prevalences of the premutation indicate that they are frequent in the general population, with an estimate of 1 in 259 females and 1 in 813 males in a Canadian study (Dombrowski et al. 2002; Rousseau et al. 1995). However, in certain ethnic populations, this rate is higher; for instance, the prevalence of the premutation in females in Israel is closer to 1 in 110–130 (Hagerman and Hagerman 2002; Toledano-Alhadef et al. 2001) or 1:180 from a pilot study on newborn screening for FXS (Bailey et al. 2010). Also in males, the prevalence of premutation alleles has been reported to be higher and varies between 1:250 in a newborn screening study in Spain (Fernandez-Carvajal et al. 2009) and 1:600 in a newborn screening study in the USA (Bailey et al. 2010). Thus, because of the variance in the prevalence of premutation alleles, large-scale screening studies are necessary to better define the prevalence of the premutation allele in numerous racial and ethnic groups from the general population. In addition, studies of the prevalence of FXTAS in small premutation and gray zone alleles are needed in order to better define the penetrance of FXTAS in male and female carriers.

18.6 Radiological Findings

The brains of individuals affected by FXTAS present with global brain atrophy and white matter diseases in the pons, MCPs, insula, and periventricular regions (Adams et al. 2007; Brunberg et al. 2002). The MCP sign, which is thought to be due to a spongiosis of the deep cerebellar white matter, is present in approximately 60% of the males and 13% of the females with FXTAS (Adams et al. 2007). Although the MPC sign is not specific for FXTAS, as it has been observed in a number of other disorders (Ngai et al. 2006; Storey and Billimoria 2005), it is considered a primary diagnostic criteria for FXTAS (Table 18.1). The majority of individuals with FXTAS also exhibit cerebellar and cerebral atrophy (Brunberg et al. 2002; Greco et al. 2002, 2006). Volumetric changes have also been documented in FXTAS and include increased ventricle size secondary to cerebral, cerebellum, amygdala, and hippocampus volume loss (Adams et al. 2010; Cohen et al. 2006; Loesch et al. 2005). Many of the radiological findings correlate with the CGG repeat number in males with FXTAS and with the severity of FXTAS as defined by the clinical staging scale (see Table 18.2) (Adams et al. 2007; Cohen et al. 2006).

Table 18.2 FXTAS stages

Stage	Clinical description
0	Normal function
1	Subtle or questionable signs such as subtle tremor or mild balance problems, with no interference in activities of daily living (ADLs)
2	Minor, but clear, tremor and/or balance problems with minor interference with ADLs
3	Moderate tremor and/or balance problems and occasional falls with significant interference in ADLs
4	Severe tremor and/or balance problems; uses cane or walker
5	Uses wheelchair on a daily basis
6	Bedridden

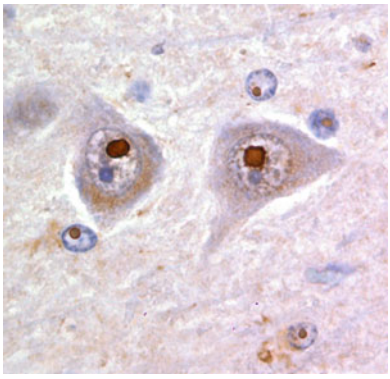

Fig. 18.1 Typical intranuclear inclusions observed in neurons and astroctyes from a FXTAS patient, stained with antiubiquitin staining. Image courtesy of Claudia Greco, MD. Resolution ×400

18.7 Pathological Findings

The presence of intranuclear eosinophilic inclusions that are ubiquitin positive is the most striking pathological finding in FXTAS and was first reported by Greco and colleagues in 2002 (see Fig. 18.1). Although first described in the central nervous system (CNS), it has became clear in the past few years that inclusion formation is not limited to the CNS but involves the peripheral nervous system as well as the neuroendocrine and the reproductive systems also (Gokden et al. 2009; Greco et al. 2007; Louis et al. 2006). Recently, intranuclear inclusions have also been identified in multiple tissues of both FXTAS cases and CGG KI mice, including thyroid, pancreas, intestine, heart, and pituitary glands, among others (Hunsaker et al., Submitted). In the CNS, they are localized within the nuclei of both neural cells and astrocytes and are broadly distributed throughout the cerebrum and the cerebellum, although some brain regions such as the hippocampus (~40%) exhibit a greater percentage of neurons bearing intranuclear inclusions

(Gokden et al. 2009; Greco et al. 2002, 2006; Iwahashi et al. 2006; Tassone et al. 2004). Their presence in the Leydig cells and in the smooth cells of the testicles may be related to the low testosterone levels observed in a number of males with FXTAS and to the presence of impotence (Gokden et al. 2009; Greco et al. 2007). Immunocytochemical studies showed the absence not only of both tau and synuclein but also of polyglutamine peptides, which distinguishes FXTAS from other neurodegenerative disorders including Pick disease, Parkinson's disease, and other trinucleotide repeat disorders. More than 20 proteins have been identified within the inclusions using a flow-based isolation and purification of inclusions from a postmortem FXTAS brain, followed by mass spectrometric analysis. Proteins identified include Lamin A/C, heat shock proteins, hnRPN A2, and several neurofilaments (Iwahashi et al. 2006). Intranuclear inclusions have also been observed in a cell model (Arocena et al. 2005) and in both the mouse (Wenzel et al. 2010) and the Drosophila premutation animal models (Jin et al. 2003). Significant positive correlations have been determined between percent of intranuclear inclusion in neural cells and astrocytes, and the CGG repeat size and age of death (Greco et al. 2006).

18.8 Molecular Findings

Individual carriers of a premutation allele show two to eight times higher *FMR1* mRNA levels, which is CGG number dependent, compared to normal controls (Allen et al. 2004; Kenneson et al. 2001; Peprah et al. 2010; Tassone et al. 2000). A mild deficit of the fragile X mental retardation protein (FMRP) has been demonstrated in premutations and is likely due to a deficit in translational efficiency, especially in the upper premutation range (Kenneson et al. 2001; Peprah et al. 2010; Primerano et al. 2002). FXTAS observed in individuals with the fragile X full mutation who carry an FMR1 allele with >200 CGG repeats followed by transcriptional silencing and absence of FMRP, suggesting that this neurodegenerative disorder is not the consequence of the lack of FMRP, but rather the result of the elevated levels of *FMR1* mRNA observed in premutation carriers. This observation, in addition to the presence of *FMR1* mRNA within the inclusions (Tassone et al. 2004) and to the presence of neurodegeneration and inclusion formation in *Drosophila melanogaster*, following the expression of a reporter containing a CGG expansion (Jin et al. 2003), has led to the hypothesis of a toxic gain-of-function model for FXTAS. Recently, the FXTAS phenotype has been described in a male carrier of an unmethylated full mutation (Loesch et al. 2011) presenting with high FMR1 mRNA levels. This finding further supports notion that elevated levels of FMR1 mRNA can lead to toxicity regardless of the size of the CGG allele. In addition, the presence of rare inclusions, considered restriced to the premutation range, have been detected in 3 males with a full mutation with no clinical neurodegeneration process characteristic of FXTAS (Hunsaker el at., 2011). The RNA toxicity model for FXTAS is

also based on the paradigm established by the myotonic dystrophies DM1 and DM2 where excessive protein binding to the expanded mRNA occur, leading to sequestration and depletion of proteins from the cell pool (sequestration model), impairing several cellular processes (Hagerman and Hagerman 2004). A similar mechanism is possibly operating in FXTAS. In support of the RNA toxicity/sequestration model of FXTAS is the recent findings of colocalization of proteins, including Sam68 (Src-associated substrate during mitosis of 68 KDa), MBNL1 (muscle blind-like), and hnRPN G proteins within CGG aggregates (Sellier et al. 2010). Sam68 is an RNA-binding protein involved in the regulation of alternative splicing, which for some genes is altered in FXTAS (Sellier et al. 2010). More recently, the same group reported that the complex DROSHA/DGCR8, which processes the pri-microRNA into miRNA, is sequestrated within the CGG aggregates in human and mouse FXTAS cell models and also in brain tissue derived from FXTAS subjects (Charlet-Berguerand and Sellier 2010). Thus, it is possible that a global downregulation of the microRNA, and therefore of the microRNA processing machinery, caused by sequestration of the CGG repeat of the DROSHA/DGCR8 complex, is responsible for the pathogenesis of FXTAS.

Recently, as for other trinucleotide repeats, an antisense transcript (*ASFMR1*) has been observed for the *FMR1* gene (Khalil et al. 2008; Ladd et al. 2007). The *ASFMR1* contains a CCG repeat in an open reading frame, coding therefore for a polyproline peptide. The *ASFMR1* transcript is widely expressed in human tissues with relatively high expression in brain tissue. Similarly to the *FMR1* gene, the ASFMR1 is overexpressed in premutation carriers and silenced in individuals with a full mutation (Khalil et al. 2008; Ladd et al. 2007). Several isoforms have been detected and a specific one is detected only in premutations but not in typical developing controls. Thus, the expression of the *ASFMR1*, or the bidirectional transcription of the expanded repeats could play a role in the broad clinical phenotype associated with the premutation by either an RNA or a protein-mediated mechanism (Ladd et al. 2007).

18.9 Animal Models

The mouse premutation model, based on a mouse transgenic originally developed by Bontekoe et al. (2001), is a "knockin" mouse in which the endogenous *Fmr1* gene promoter was replaced by a human repeat element of 98 CGG units in size (Willemsen et al. 2003). Similar to individual carriers of a premutation allele, the KI mice with the expanded CGG repeat number had increased levels of *Fmr1* transcripts by 2–3.5-fold and reduced levels of Fmrp relative to levels from the same brain regions in wild-type, age-matched controls. The expanded CGG repeat element present in a mouse *Fmr1* transgene leads to the formation of ubiquitin-positive, intranuclear inclusions that are morphologically identical to those found in the brains of patients with FXTAS, and they are detected as early as 20 weeks of age

(Brouwer et al. 2008; Willemsen et al. 2003). They are distributed throughout the brain in both neurons and glial cells, and increase in number and size with age (Hashem et al. 2009; Wenzel et al. 2010; Willemsen et al. 2003). Also as in human FXTAS, KI mice display Purkinjie cell degeneration, axonal swellings, and neurotoxicity (Hashem et al. 2009). As in humans, the inclusions observed in CGG-expanded mice stain negatively for tau, presenilin, a synuclein, MAP1B, nucleolin, Fmrp, and Hsp70.

Consistent with the mRNA toxicity model and the neurodegeneration associated with FXTAS, murine hippocampal neurons from CGG-expanded mice show reduced viability and display deficits in dendritic complexity, including shorter dendritic length and fewer branches between 7 and 21 days compared to wild-type, age-matched controls (Chen et al. 2010). The development of a mouse model for FXTAS is important because it enables researchers to study the cellular events that occur with the onset and the progression of the disorder. It also provides the tools to study the mechanism behind the role of RNA toxicity in the pathophysiology of FXTAS and will be useful for developing future target treatments for FXTAS.

Similar observations concerning inclusion formation and eye neurodegeneration have been made in the *Drosophila melanogaster* model, with an expanded 90-CGG transcript (Jin et al. 2003). However, the Drosophila inclusions are substantially cytoplasmic rather than intranuclear, which makes these findings quite different from the ones found in both FXTAS patients and mice containing expanded CGG elements. Thus, the composition of the inclusions and the pathways involved in the neurodegenerative process observed in the fly model could be quite distinct, due to species-specific differences, reflecting the different proteins involved. Nevertheless, the Drosophila model demonstrates that an rCGG in the premutation range, per se, can cause neurodegeneration and importantly enables rapid genetic screens for the identification of potential modifiers of the FXTAS phenotype to be performed. Finally, the findings in both the mice and the Drosophila models establish and support the notion that the expanded CGG repeat plays a direct role in the inclusion formation, leading to a progressive neurodegeneration process.

For a more complete description of the premutation animal models, see Chap. 14.

18.10 The Treatment of FXTAS and Premutation-Associated Disorders

The treatment of premutation-associated disorders is currently symptomatic, although the discovery of mitochondrial abnormalities in those with FXTAS suggests that a specific intervention that is a targeted treatment for the dysregulation of proteins that occurs with RNA toxicity may be available soon. Currently, a controlled trial of memantine is taking place at the MIND Institute at UC Davis, but the results are not available since the trial is ongoing. There are some anecdotal reports that memantine is beneficial for early symptoms of FXTAS, including one

woman who demonstrated improvements in her tremor, ataxia, depression, and neuropathy with memantine and venlafaxine (Hall et al. 2006; Ortigas et al. 2010). Memantine is an NMDA antagonist and it can block glutamate toxicity, which is thought to occur in FXTAS from cellular data (unpublished data) (Hagerman et al. 2008; Tassone and Elizabeth 2010).

The most problematic symptom in early FXTAS is the intention tremor and a variety of medications including beta-blockers, primidone, topiramate, and levetiracetam has been found to be helpful, although typically in less than 50% (Hall et al. 2006). Deep brain stimulation (DBS) has been shown to be helpful in a handful of patients, but sometimes the ataxia or cognitive symptoms may worsen with this intervention (Leehey et al. 2003; Peters et al. 2006). Those without cognitive problems and with only mild ataxia are the best candidates for DBS. We have also seen improvement of tremor symptoms with botulinum toxin (Botox) injections in one patient with repeated injections every 3 months into the flexor muscles under EMG guidance (Hagerman et al. 2008; Tassone and Elizabeth 2010). Treatment of parkinsonian symptoms including the tremor with carbidopa/levodopa was helpful in four of ten patients in a survey study (Hall et al. 2006).

Treatment of ataxia is more difficult than treatment of tremor because there are few medications that have been efficacious. The ataxia in FXTAS may be related to more than one pathological process. The most dominant problem is the cerebellar dysfunction, although the neuropathy symptoms and also episodes of dizziness or vertigo are also common, perhaps related to eighth nerve toxicity or autonomic dysfunction, and these can all be additive to the balance problems. An occasional patient with FXTAS has demonstrated improved ataxia on amantadine (Jacquemont et al. 2004) and one patient who was a smoker improved on varenicline (Chantrix) (Zesiewicz et al. 2009), although the latter was not helpful in subsequent patients who were not smokers. A recent controlled trial of riluzole in patients with ataxia for a variety of reasons demonstrated significant improvement, and one of these patients who improved had FXTAS (Ristori et al. 2010). We have also seen improvement of ataxia in FXTAS with DBS in one patient. The use of physical therapy may also help strength and balance in those with FXTAS.

Psychiatric problems including depression and anxiety are usually responsive to a selective serotonin reuptake inhibitor (SSRI) in those with or without FXTAS (Hagerman et al. 2008). SSRIs are also known to stimulate neurogenesis in the aging brain (Jacobs et al. 2000) and the making of more neurons and astrocytes is certainly of benefit for those with FXTAS (Hagerman et al. 2008). Also, treatment of depression is likely to help in the avoidance of cognitive decline. Duloxetine and venlafaxine, which are serotonin and norepinephrine reuptake inhibitors (SNRIs), may help with pain control and attention and concentration problems that are common in FXTAS. Exercise also stimulates neurogenesis, so prescribing exercise that can be guided by a physical therapist is generally recommended (Hagerman et al. 2008). Because of mitochondrial abnormalities in FXTAS, the use of antioxidants is generally recommended and the combined use of folate and vitamine B12 to lower homocysteine and slow down brain shrinkage with aging has been documented in the general population and also in those with mild

cognitive impairment (Smith et al. 2010). Since brain atrophy is a problem in FXTAS, this regimen will likely be beneficial for these patients. Avoidance of vitamin deficiencies and also hypothyroidism is also important, so blood work is necessary to rule out these problems.

The future of FXTAS treatment will depend on uncovering the molecular triggers of FXTAS and blocking the molecular dysregulation that leads to the problems discussed here. The combination of dietary changes with increasing antioxidants; avoidance of stressors perhaps through therapy or relaxation techniques; avoidance of toxins such as smoking; early treatment of anxiety, depression, hypertension, and hypothyroidism; and regular exercise may be helpful in postponing the onset of FXTAS and perhaps slowing the disease progression.

18.11 Genetic Counseling

A recent collaborative study was developed with the purpose of disseminating protocols for genetic counseling and cascade testing for the multiple disorders associated with the fragile X mental retardation 1 (*FMR1*) mutation including FXTAS (McConkie-Rosell et al. 2007). The collaborative group indicated that families diagnosed with FXTAS should be referred for genetic counseling, which can also address the needs of the patient, the spouse, and other members of the family. The families should be encouraged to include relevant family members in genetic counseling sessions or to talk to other family members about potential risks as the gene mutation leading to FXTAS is inherited, and therefore, additional family members are at risk to develop FXTAS or other *FMR1*-associated disorders. Genetic counseling should, therefore, include a pedigree and risk assessment for carrier status in extended family members. Genetic counseling is also useful in helping patients with FXTAS who have emotional problems, long-term care issues, and end-of-life issues in addition to medical management and target treatment needs. Since FXTAS shares many symptoms with other neurodegenerative conditions including Parkinson's disease, Alzheimer's disease, psychiatric disorders, dementia, stroke, other ataxias, and peripheral neuropathy, patients with FXTAS are often misdiagnosed (Zuhlke et al. 2004; Hall et al. 2005). Thus, testing recommendations have been developed and are based on clinical information including neurological symptoms and neuroimaging findings, and family and personal history (McConkie-Rosell et al. 2007).

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Chapter 19
Vignettes: Models in Absentia

Robert B. Denman

Abstract In this chapter, I will concisely summarize the salient features of all of the fragile X models (ex vivo, non-mouse, mouse, novel mouse, and human) that were not able to be described by their creators in separate chapters. By doing so, it is hoped that this book will become more of an encyclopedic compendium.

19.1 Introduction

When this project was conceived, now more than 2 years ago, it's impetus was to gather together, in one place, all of the models (ex vivo, non-mouse, mouse, novel mouse, and human) that have been used to drive fragile X research. In doing so, it was hoped not only to produce a handy reference book for researchers, but also to assist in crystallizing our understanding of fragile X syndrome (FXS). With that goal in mind, I patiently waited until several models that had been described in conferences took their place in the peer-reviewed literature. Once this occurred, the wheels were set in motion, a syllabus was outlined, and invitations were sent to the nether reaches of the globe. It became readily apparent, almost immediately, that the idealized "Platonic Form" of a comprehensive anthology written by those intimately involved in the creation and use of each model would not be realized. Such is life in the real world and it was probably naïve to even think that it could have turned out any other way. Nevertheless, as I still believe in the merit of this initial goal, I have taken it upon myself to supply what might be described as a roll-call of the missing models. As all of these models are extant in the scientific literature, I will briefly outline the salient points of each, summarize their

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contribution to our understanding of FXS as I see it, and supply the reader with the appropriate literature citations for further evaluation. This will include a hypothetical model, which may be of value if created in the future. Finally, I leave the reader with an outline of the controversial models within the fragile X community that must ultimately be resolved. In doing so, I expect to bring this volume closer to its Platonic ideal.

19.2 Missing Models: Ex Vivo

It is inconceivable to me that a book about FXS models would not have a chapter devoted to synaptoneurosomes. Hollinsworth et al. first introduced the method of gently homogenizing brain tissue, permitting cells to fragment and their membranes reseal; this is followed by size selection through a series of filters of decreasing pore size. The result is an enriched population of resealed, metabolically active synaptic ensembles, including both pre- and postsynaptic elements, i.e., synaptoneurosomes (Hollingsworth et al. 1985). I was literally brought into the field as a pre-reviewer of the classic paper from the Greenough laboratory showing that the messenger RNA for the fragile X mental retardation protein is found in and translated in synaptoneurosomes in response to metabotropic glutamate receptor (mGluR) stimulation (Weiler et al. 1997). Over the years, fragile X laboratories around the world have used synaptoneurosome preparations to demonstrate the following:

1. NUFIP1, a nucleocytoplasmic shuttling protein that interacts with FMRP (Bardoni et al. 1999), is present in active synaptoneurosomes and associates with polyribosomes near synapses (Bardoni et al. 2003).
2. Tip60a mRNA, which binds to the fragile X mental retardation protein (Sung et al. 2000), is selectively enriched in synaptoneurosome preparations (Sung et al. 2004).
3. MicroSpherule protein 58, a novel RNA-binding protein that interacts with the fragile X mental retardation protein, cosediments with FMRP in polyribosomes prepared from synaptoneurosomes (Davidovic et al. 2006).
4. Postsynaptic density 95 (PSD-95) mRNA and calcium/calmodulin-dependent kinase IIα (CaMKIIα) mRNA, which are FMRP target messages (Todd et al. 2003; Zalfa et al. 2003), are reduced in FMRP immunoprecipitates from Fmr1 knockout mice synaptoneurosomes. Correspondingly, newly synthesized PSD-95 and CaMKIIα from *Fmr1* KO mice synaptoneurosomes exhibited dysregulated mGluR5-dependent protein synthesis (Muddashetty et al. 2007).
5. Extracellular signal-regulated kinase (ERK) in Fmr1 knockout mice synaptoneurosomes is rapidly dephosphorylated upon mGluR1/5 stimulation, whereas it is phosphorylated in WT mice. This suggests that aberrant activation of phosphatases occurs in knockout synapses in response to synaptic stimulation (Kim et al. 2008).
6. Fmr1 knockout mice exhibit impaired neuropeptide release. Furthermore, Rab3A, a FMRP target mRNA (Brown et al. 2001; Miyashiro et al. 2003)

involved in vesicle recruitment, is decreased by ~50% in Fmr1 knockout mice synaptoneurosomes; however, the total number of dense-core vesicles does not differ between WT and Fmr1 knockout mice. These data indicate that there may be a fundamental deficit in Rab3A-mediated vesicle release in FXS, which may lead to defective maturation and maintenance of synaptic connections (Annangudi et al. 2010).

Thus, synaptoneurosomes are, and continue to be, an important tool for fragile X researchers.

Similarly, another of the primary tools used by fragile X researchers, cultured cell models, is not separately outlined here, yet their importance to the field is unquestioned. A variety of transformed and primary cell lines has been used to address fundamental questions concerning FMRP biology. From the earliest studies showing that FMRP shuttles between the nucleus and cytoplasm (Eberhart et al. 1996; Sittler et al. 1996), heterodimerizes with fragile X family members FXR1P and FXR2P (Tamanini et al. 1999), associates with polyribosomes (Corbin et al. 1997; Feng et al. 1997), binds RNAs (Ashley et al. 1993; Siomi et al. 1994), and regulates a unique set of mRNAs (Brown et al. 2001; Sung et al. 2003), to the more recent and eye-popping imaging studies demonstrating that FMRP traffics in dendrites in large messenger ribonucleoprotein complexes (Kanai et al. 2004) in response to various types of stimulation (Antar et al. 2004; Dictenberg et al. 2008; Pan et al. 2010) with its loss resulting in aberrant synapses (Pfeiffer and Huber 2007; Gatto and Broadie 2008), cultured cells have been among the reliable tools of FXS research. Coupled with complementary studies of gene expression (Verheij et al. 1995; Dolzhanskaya et al. 2003; Sung et al. 2003; Nishimura et al. 2007), FMR1 gene methylation and reactivation (Chiurazzi et al. 1998; Laird et al. 2004; Tabolacci et al. 2008) in transformed fragile X lymphoblastoid cell lines ex vivo modeling provides both breadth and depth to fragile X research studies.

19.3 Missing Models: Non-mouse

The *zebrafish* model of FXS (Lin et al. 2006; den Broeder et al. 2009), a "small brain" model also could not be reviewed by its principal architects. *Zebrafish* offer the simplicity of a small brain, 78,000 neurons, 1 week postfertilization (Burne et al. 2011), combined with transparency at early developmental stages, which allows intact imaging of live brains. In addition, like mice, *zebrafish* express the three orthologs of the fragile X gene family (van't Padje et al. 2005). *Zebrafish* Fmr1 knockouts are viable and appear to exhibit no gross defects in craniofacial development like Fmr1 knockout mice, although see *Controversial models* below. Interestingly, there may (Lin et al. 2006) or may not (den Broeder et al. 2009) be differences in neuronal architecture as seen in the *Drosophila* dFmr1 knockout (Morales et al. 2002; Gatto and Broadie 2008), although a full and exact comparison of this feature has yet to be made.

Comparative analyses regarding the conservation and evolution of a particular gene or gene family often provide invaluable data regarding their function. Thus, it

becomes important to determine whether a lower organism harbors and expresses an ortholog of a specific disease-causing gene. While the origin and expansion of the FXR gene family have not been exhaustively examined, molecular and bioinformatic analyses have demonstrated that lower eukaryotes such as yeast (Currie and Brown 1999) as well as the worm, *C. elegans* (Shtang et al. 1999), do not contain an FMR1 ortholog. In contrast, invertebrates appear to have a single FXR-protein gene (Wan et al. 2000; Guduric-Fuchs et al. 2004), while frogs have two (Blonden et al. 2005).

The Cnidarian, *Hydractinia echinata*, commonly referred to as snail fur for its propensity to grow on hermit crab shells, is an example of an invertebrate harboring a single FXR-like gene (Guduric-Fuchs et al. 2004). Based on sequence analyses, HyFmr1 corresponds more closely to FMR1 than it does to FXR1 or FXR2. Like *Drosophila* Fmrp (dFmrp), HyFmrp's N-terminal end contains the most highly conserved sequence similarities; however, unlike dFmrp, HyFmrp does not contain a recognizable RGG box. Another invertebrate, the sea squirt, *Ciona intestinalis*, is the smallest of any experimentally manipulable chordate (Guduric-Fuchs et al. 2004; Blonden et al. 2005). It also has a single FXR-like gene, but it appears that in this case the resulting protein, GenBank XP_002127083, is more similar to FXR1P (47% identity; 52% ungapped) than to FMRP (42% identity; 50% ungapped).

In the latter part of 2003, fragile X researchers were tantalized by the description of the discovery of an ortholog of FMR1 in the sea slug, *Aplysia californica* (Kohn et al. 2003). Because of FMR1's association with learning and memory deficits, and with the use of *Aplysia* to understand learning, memory, and behavior (Glanzman 2006; Castellucci 2008), it was hoped that rapid progress in understanding the fundamental defects in FXS would be made. As this chapter was being written at the beginning of 2011, this hope had not yet been fulfilled. But, lo and behold, one last PubMed search identified a new offering describing a novel model system for examining FXS; *Aplysia* had arrived (Till et al. 2011). Analysis of the *Aplysia* FMRP (ApFmrp) sequence shows a strong conservation with the human FXR gene family (40% overall identity, with 60% in the KH domains). ApFmrp does not appear to have the extended KH2 domain of FMRP, but it does alternatively contain spliced exons that bear similarity to those in FMR1 and which are not found in FXR1 and FXR2. Similarly, like *Drosophila* dFmrp, but unlike HyFmrp, ApFmrp contains a significant RG-rich region near its C-terminal end. Furthermore, depending on the alignment, ApFmrp may contain the equivalent of the conserved S_{500} residue that plays an important role in FMRP-mediated translational regulation (Ceman et al. 2003; Narayanan et al. 2008). Consistent with studies of the expression of the FXR gene family in other species, ApFmr1 is found in heart, muscle, neurons, and ovotestis; it is also found in both sensory and motor neurons. Furthermore, overexpression studies using an ApFmrp–ECFP fusion demonstrated that the protein could be observed in punctuate granules in the neurites of sensory-motor neuron cocultures, as is has been in other species. Finally, using antisense oligonucleotides, Till et al. demonstrated that knocking down ApFmr1 resulted in enhanced long-term depression (LTD), but did not affect either short-term or long-term facilitation.

Another recent addition to fragile X models is the cricket, *Gryllus bimaculatus* (Hamada et al. 2009), whose protein sequence is most similar to that of Fmrp from the honey bee, *Apis mellifera* (65.6% identity, 75.6% ungapped in 621 aa overlap) and *Drosophila* (49.9% identity, 53.3% ungapped in 595 aa overlap). As with Fmrp from other species, the main homology is in the N-terminal end of the protein, although unlike HyFmrp, GbFmrp contains an RG-rich region. GbFmr1 mRNA is, as Hamada et al. show, expressed in mushroom bodies, the antennal lobes, and the optic lobes as it is in *Drosophila*; however, we do not yet know whether it is subject to alternative splicing. Using siRNA to reduce GbFmr1 mRNA significantly, the authors found a defect that mimicked the effects of Fmr1 gene ablation in *Drosophila* and mice, namely altered circadian rhythm; they also found that underexpression of GbFmr1 leads to wing defects, which resulted in alterations in the cricket's ability to produce their calling song. These defects are likely due to the proper formation of muscle and/or the development of wing movements and thus, are not likely to be germane to the deficits in speech observed in fragile X patients (for a review of the subject, see Chap. 10).

One of the clear molecular defects in FXS results from altered receptor-mediated signaling. Of the signaling pathways that are known to be misregulated, the metabotropic glutamate receptor pathway (see below) has clearly garnered the most attention. However, it is becoming clear that the BDNF-TrkB pathway is different in Fmr1 knockout mice (Lauterborn et al. 2007) and in neural progenitor cells derived from these mice (Louhivuori et al. 2011). Importantly, it has also been found that within the $GABA_A$ receptor pathway, there are regional changes in $GABA_A$ subunit expression (El Idrissi et al. 2005; D'Hulst et al. 2006; Gantois et al. 2006). In addition, GABA pathway enzymes such as GABA transaminase and succinic semialdehyde dehydrogenase were downregulated in Fmr1 knockout mice during postnatal development (Adusei et al. 2010) and there were compensatory increases in glutamate decarboxylase (GAD) in Fmr1 knockout mice (El Idrissi et al. 2005). In that regard, Chang et al. recently demonstrated a unique lethal phenotype in the *Drosophila* Fmr1 knockout, which occurs when the flies are fed excess glutamate (Chang et al. 2008). This phenotype was then used to screen a chemical library of 2,000 compounds, which identified nine molecules that rescued the lethality, including three that implicate the GABAergic inhibitory pathway. Further tests indicated that these chemicals were also able to rescue mushroom body defects, excess Futsch translation, and abnormal male courtship behavior that occur in these mutant flies. These data show the convergence of the mouse and fly models and suggest that the GABAergic system may be an effective target for therapeutic treatment of FXS.

19.4 Missing Models: Mouse

The model to which all other Fmr1 knockout models look is the original Dutch-Belgian consortium model of FXS (Bakker et al. 1994). These mice were created by inserting a neomycin gene cassette into exon 5 of the *Fmr1* gene into embryonic

stem cell by homologous recombination. The resulting mice produce a variety of mutant alternatively spliced RNAs (Yan et al. 2004), but no detectable protein (Bakker et al. 1994). Importantly, the mice recapitulate several molecular, physical, and behavioral phenotypes that are found in fragile X patients. These include protein synthesis-dependent mGluR long-term depression (mGluR-LTD) (Huber et al. 2002), abnormal dendritic spine length and density (Comery et al. 1997; Nimchinsky et al. 2001), macroorchidism (Bakker et al. 1994), audiogenic seizures (Musumeci et al. 2000; Chen and Toth 2001), and strain-specific learning impairments (Dobkin et al. 2000).

A recent development in the Fmr1 knockout model involved the use of the Cre/loxP system (Brault et al. 2007) to generate both a true null mouse and Fmr1 conditional knockout mice (Mientjes et al. 2006). The use of conditional knockouts allow researchers to limit Fmrp expression to certain tissues and/or certain developmental times during embryogenesis or postnatally. Using this approach, Koekkoek et al. developed a Purkinje cell-specific Fmr1 knockout; they showed that, like Fmr1 null mice, Purkinje cell-specific knockout mice display enhanced cerebral LTD and alterations in dendritic spine morphology and in eyeblink conditioning (Koekkoek et al. 2005).

Another important mouse model of FXS not recounted here is the Fmrp-I304N mutant mouse (Zang et al. 2009). The Fmr1-I304N missense mutation was first observed in a single patient with a severe form of FXS more than 18 years ago (De Boulle et al. 1993); the Fmrp-I304N protein produced by this patient displays defective RNA binding (Siomi et al. 1994; Darnell et al. 2005a), an inability to form proper messenger ribonucleoprotein complexes (Feng et al. 1997; Pfeiffer and Huber 2007) and a reduced capacity to bind to polyribosomes (Darnell et al. 2005b). The Fmrp-I304N mutant mice were constructed to examine the effects of this mutation in a live model. Fmrp-I304N mutant mice have normal levels of Fmr1-I304N mRNA, but reduced levels of the mutant protein compared to wild-type mice. Consistent with the functional defects of the mutant protein Fmrp-I304N, mice also phenocopy the macroorchidism, audiogenic seizures, and specific learning impairments observed in fragile X patients much like Fmr1 knockout mice. These data for the first time directly demonstrated the central role FMRP plays in the pathogenesis of FXS.

The fragile X gene family consists of three homologs, FMRP, FXR1P, and FXR2P, which exhibit both unique and overlapping expression in the brain (Bakker et al. 2000). Consistent with this fact, Fxr2 knockout mice display some similar phenotypic traits as their Fmr1 counterparts (Bontekoe et al. 2002); however, FXR2P cannot compensate for the loss of FMRP, as recently and elegantly demonstrated by Coffee et al. (2010). To further probe the relationship between FMRP and FXR2P, Spencer et al. generated Fmr1/Fxr2 double knockout mice (Spencer et al. 2006). They showed that these mice exhibit exaggerated behavioral phenotypes compared with Fmr1 knockout mice, Fxr2 knockout mice, or wild-type littermates, suggesting that Fmr1 and Fxr2 cooperate in controlling locomotor activity, sensorimotor gating, and cognitive processes.

In addition to modeling a disease phenotype, animals can also be used to test theories that are concerned with correcting one or more aspects of the disease. Invariably, this consists of adding or subtracting particular genes from the model organism. Four such models have been made for FXS. YAC transgenic mice, in which the human FMR1 gene was added back to Fmr1 knockout mice via a yeast artificial chromosome (YAC), were created to determine whether inducing FMRP expression could correct for the loss of the mouse Fmr1 gene (Peier et al. 2000). The authors found that overexpressing the human transgene corrected the macroorchidism caused by the loss of the Fmr1 gene and overcorrected deficiencies corresponding to social anxiety and exploratory behavior.

Recently, Zeier et al. performed a similar but complementary set of experiments that tested whether viral expression of the major central nervous system (CNS) isoform of Fmrp could correct a fragile X phenotype (Zeier et al. 2009). They showed that stereotaxic injection of an Fmrp-expressing adeno-associated virus into the CA1 region of the hippocampus of 5-week-old Fmr1 knockout mice resulted in robust overexpression of Fmrp throughout the pyramidal cell layer of the CA1 3 weeks later. Importantly, Fmrp expression was able to rescue the enhanced LTD exhibited by Fmr1 knockout mice. These data are a proof -of concept showing that at least one important phenotypic feature of FXS can be corrected postnatally by the expression of Fmrp.

Molecular analyses of the function of FMRP have shown that it plays an important role in translational regulation via group I metabotropic glutamate receptor (mGluR) signaling (Antar et al. 2004; Bear et al. 2004; Hou et al. 2006; Muddashetty et al. 2007; Bassell and Warren 2008; de Vrij et al. 2008; Dolen and Bear 2008). While both FMRP translational regulation and the signaling pathways that impinge upon it are quite complex, two of the potential mRNAs that FMRP regulate are mGluR5 and p21-activated kinase (PAK) (Denman 2005; Weiler 2005). Thus, it would be expected that some of the features FXS induced by excessive protein synthesis could be corrected by decreasing or inhibiting the expression of either mGluR5 or PAK. In fact, Hayashi et al. found that the expression of a dominant negative PAK gene was able to rescue specific molecular and behavioral features of the Fmr1 knockout (Hayashi et al. 2007). Similarly, $Fmr1^{-/-}$-$GRM5^{+/-}$ heterozygous mice, in which the metabotropic glutamate receptor-5 was reduced 50%, corrected Fmr1-induced defects in mGluR-LTD, dendritic spine density, basal protein synthesis, and audiogenic seizures (Dölen et al. 2007).

The susceptibility to audiogenic seizures is among the most robust phenotypes of Fmr1 knockout mice and correlates well with seizures experienced by fragile X patients. Pacey et al. recently investigated whether the G-protein inhibitory activity of the regulator of G-protein signaling protein, RGS4, which is decreased in the hippocampus and cerebral cortex of Fmr1 knockout mice (Tervonen et al. 2005), altered the susceptibility to audiogenic seizures (Pacey et al. 2009). Specifically, they demonstrated that $Fmr1^{-/-}$-$RGS4^{-/-}$ double knockout mice exhibited reduced susceptibility to audiogenic seizures compared to age-matched Fmr1 knockout mice. RGS4 is highly expressed in the developing and adult brain,

where it inhibits signaling of group I mGluRs. However, RGS4 has also been shown to associate with $GABA_B$ receptors and inward rectifying K^+ channels (KIR), suggesting that it may also regulate $GABA_B$-mediated signaling. Indeed in further studies, they found that administering the $GABA_B$ receptor agonist baclofen to Fmr1 knockout mice inhibited seizures, while the $GABA_B$ receptor antagonist CGP 46381 increased seizure incidence in double knockout mice but not in wild-type mice. These data show that $GABA_B$ receptor-mediated signaling antagonizes the seizure-promoting effects of the group I mGluRs in Fmr1 knockout mice, and like the studies of Chang et al. in *Drosophila* (Chang et al. 2008), point to the GABAergic system as a point of therapeutic intervention for the treatment of FXS.

19.5 A Hypothetical Model?

Activation of group I metabotropic glutamate receptors by glutamate or agonists such as (*S*)-3, 5-dihydroxyphenylglycine (DHPG) stimulates at least three distinct hippocampal signaling pathways, which ultimately result in LTD, Fig. 19.1a. The first of these pathways, the protein synthesis-dependent pathway, is well-known to fragile X researchers; it was first outlined by Huber et al. (Huber et al. 2002), defined as an important component of the mGluR theory of FXS by Bear et al. (Bear et al. 2004), and subsequently confirmed and refined (Hou et al. 2006; Kim et al. 2008; Ronesi and Huber 2008; Osterweil et al. 2010; Zhao et al. 2011). In this pathway, mGluR5 activation leads to the selective translation of FMRP target mRNAs such as eEF-1A (Sung et al. 2003), Fmr1 (Schaeffer et al. 2001), and those involved in shaping the postsynaptic density βAPP (Westmark and Malter 2004), CamKIIα (Zalfa et al. 2003), MAP1b (Lu et al. 2004), Shank1/3, and SAPAP1-3 (Schuett et al. 2009). Concomitantly, there is a selective repression of other mRNAs via phosphorylation of eukaryotic elongation factor 2 (eEF2); this analysis is based on the segregation of all mRNA into two translational pools [see Fig. 3 in Bear et al. (2004)]. Fmrp produced from the Fmrp-regulated message pool is initially degraded by the 26S proteasome, resulting in a decrease in FMRP allowing translation of other pool I mRNAs and an overall increase in proteins synthesis (Muddashetty et al. 2007); however, 30 min following mGluR5 stimulation, Fmrp levels increase (Zhao et al. 2011) and the system resets itself. The selective synthesis of these proteins, coupled with decreased post-synaptic density protein 95 (PSD-95), results in decreased surface alpha-amino-3-hydroxy-5-methyl-isoxazolepropionic acid receptor (AMPAR) expression and LTD (Ronesi and Huber 2008).

The second pathway stimulated by mGluR5 activation is the endocannaboid synthesis pathway (reviewed in (Di Marzo 2011), which produces anandamide (AEA), a lipid ligand, and is a potent activator of the transient potential receptor 1, or *vanilloid* receptor (TRPV1) (Chavez et al. 2010). TRPV1-stimulated intracellular calcium increases promote a long-lasting, clathrin- and dynamin-dependent endocytosis of AMPA receptors called TRPV1-LTD (Gibson et al. 2008). However,

while this pathway is important in the dentate gyrus (Chavez et al. 2010) and the nucleus accumbens (Grueter et al. 2010), its significance in mediating LTD in the CA1 region of the hippocampus is less certain as Edwards et al. have shown that a stable AEA analog, R-methanandamide, did not produce LTD in hippocampal CA1 pyramidal neurons, and CA1 interneurons, which exhibit R-methyl-anandamide-dependent LTD, do so via a TRPV1-independent mechanism (Edwards et al. 2010).

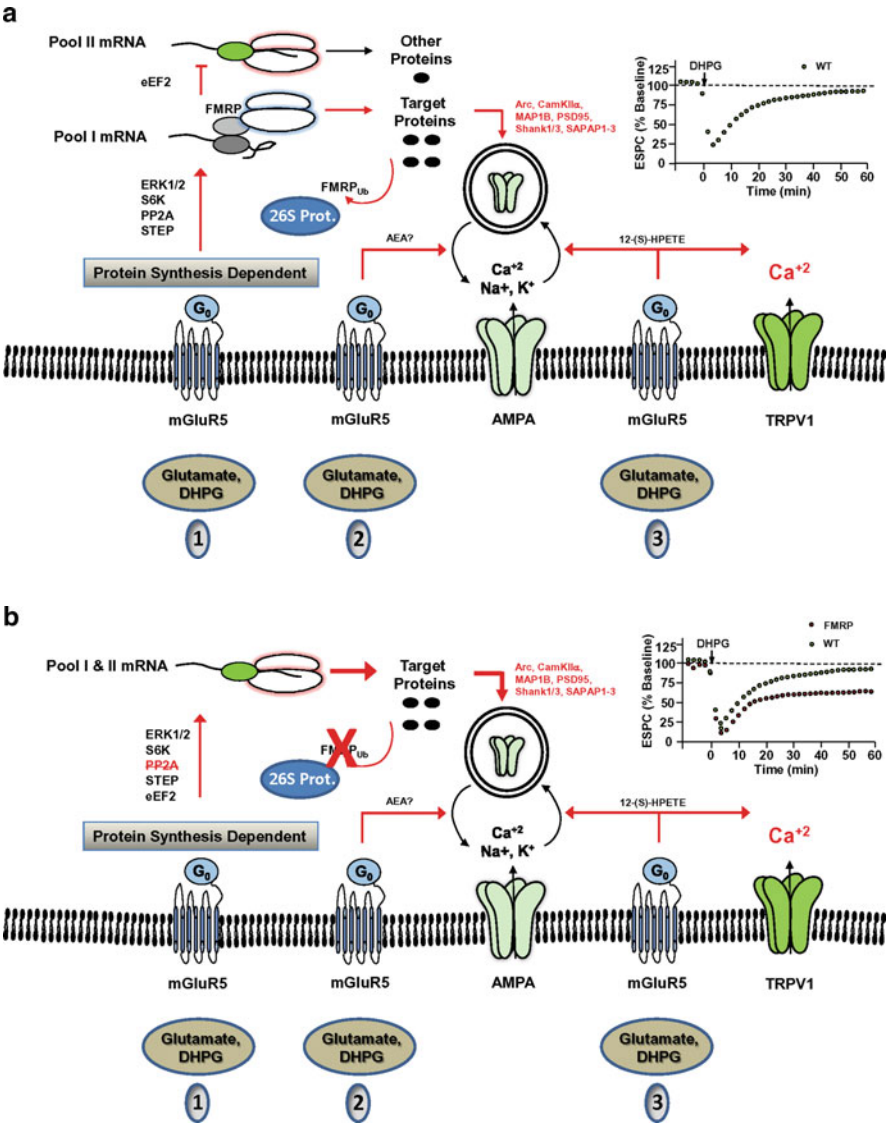

Fig. 19.1 (continued)

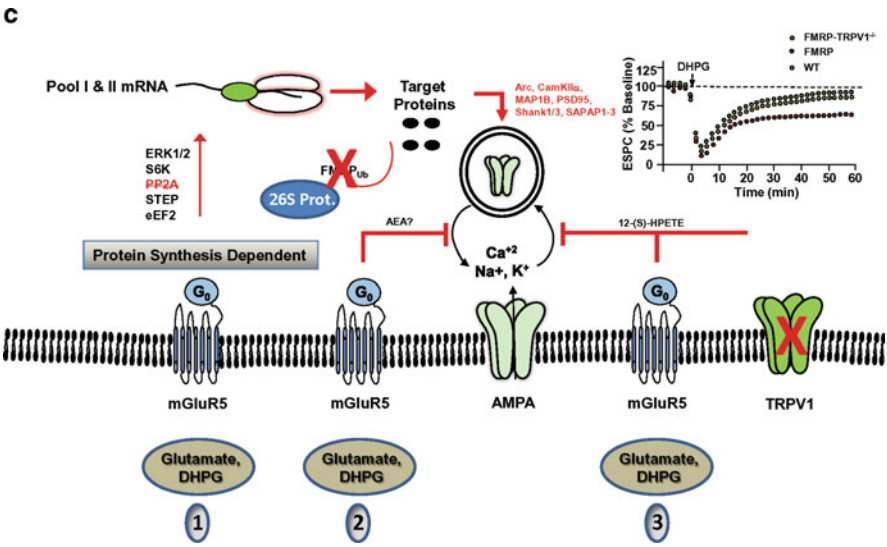

Fig. 19.1 Attenuating TRPV1 activity may correct hippocampal mGluR5- mediated long-term depression (LTD) and LTP. (**a**) Stimulation of wild-type hippocampal cells with glutamate or the glutamate agonist DHPG activates mGluR5 receptors and stimulates three signaling pathways (numbered gray ovals) that combinatorially or synergistically produce long-term synaptic depression (see inset graph). Note: the protein synthesis-dependent pathway 1 consists of two main branches, a kinase-dependent branch orchestrated by the phosphorylation of ERK1/2, or eukaryotic elongation factor (eEF2), or 4E binding protein (4E-BP) via S6 kinase (S6K), and a protein phosphatase branch mediated by PP2A dephosphorylation of FMRP and STEP dephosphorylation of AMPARs and NMDARs. Activation of the endocannaboid pathway is mediated by the lipid ligand anandamide (AEA); here, it is marked with a question mark as it is currently unclear whether it contributes to mGluR-mediated LTD in the hippocampus. (**b**) In Fmr1 knockout mice, the loss of Fmrp results in exaggerated signaling through the protein synthesis-dependent pathway and produces enhanced LTD (see inset graph). Note: the loss of Fmrp in the Fmr1 knockout precludes the ability of PP2A to phosphorylate it. (**c**) In Fmr1-TRPV1$^{-/-}$ double knockout mice, the loss of TRPV1 reduces calcium influx, thereby reducing signaling through pathways 2 and 3 and ultimately correcting the mGluR5-mediated LTD (see inset graph) and possibly LTP. Although it is impossible to determine the extent to which the TRPV1 knockout will rescue mGluR5-mediated LTD a priori, it is represented here as a substantial correction. This is based on the fact that in wild-type mice, treatment of the TRPV1 antagonist capsazepine completely ablates the LTD and in TRPV1 knockout mice do not exhibit LTD (Gibson et al. 2008)

A third pathway that mGluR stimulation activates is one in which 12-lipoxygenase (12-LO) produces 12-hydroperoxyeicosatetraenoic acid (12-(S)-HPETE), an arachidonic acid metabolite and potent TRPV1 activator (Gibson et al. 2008). Again, the activation of TRPV1 increases intracellular calcium concentrations, which lead to the endocytosis of AMPA receptors and LTD. Notably, Gibson et al. demonstrated that in hippocampal pyramidal cells, the TRPV1 agonist capsaicin elicited LTD, while the antagonist capsazepine effectively blocked it. Each of these pathways contributes, possibly in a synergistic or combinatorial or region- or circuit-specific manner, to the establishment of mGluR-LTD.

In FXS, the loss of FMRP leads to a general increase in dendritic protein synthesis, as demonstrated ex vivo by Muddashetty et al. (Muddashetty et al. 2007) and, as shown by Qin et al, in the hippocampus of Fmr1 knockout mice (Qin et al. 2005), which results in the overproduction of FMRP-regulated target proteins that, in turn, produce enhanced hippocampal LTD (Huber et al. 2002), Fig. 19.1b. Osterweil et al. have recently shown that this is not due to the fact that ERK1/2 is constitutively overactive, but rather it is because it is hypersensitive in the absence of FMRP (Osterweil et al. 2010). In fact, using TrkB-mediated ERK1/2 activation, Osterweil et al. went on to demonstrate that all signaling through ERK1/2 is saturated in Fmr1 knockout mice.

As mentioned above, Dölen et al. have shown that crossing Fmr1knockout mice with $GRM5^{+/-}$ mice substantially corrects several of the phenotypic features of FXS (Dölen et al. 2007). However, based on the signaling pathways that are activated when group I metabotropic glutamate receptors are stimulated, there are likely other means of correcting the fragile X phenotype. Here, I propose that crossing Fmr1 knockout mice with readily available TRPV1 knockout mice will rescue some of the phenotypic features of the Fmr1 knockout (Fig. 19.1c). In the $Fmr1^{-/-}$-$TRPV1^{-/-}$ double knockout, the loss of TRPV1 should lower intracellular calcium levels and ameliorate (to an unknown extent) AMPA receptor endocytosis resulting from the activation of either the endocannaboid or 12-lipoxygenase-mediated pathways, thus correcting the enhanced hippocampal LTD observed in the Fmr1 knockout mice. In support of this notion, it has been shown that both pharmacological and genetic ablation of TRPV1 effectively eliminate hippocampal LTD (Gibson et al. 2008).

In addition to enhanced LTD, Fmr1 knockout mice also exhibit decreased hippocampal long-term potentiation (LTP) at threshold levels of stimulation (Lauterborn et al. 2007). Interestingly, LTP was found to be enhanced in the dentate gyrus of TRPV1 knockout mice (Chavez et al. 2010), but whether this is so in the hippocampus is unknown. Nevertheless, this is a testable prediction of the new model. On the contrary, because the central protein synthesis-dependent deficit remains in the $Fmr1^{-/-}$-$TRPV1^{-/-}$ double knockout mice, Fig. 19.1c, it is clear that phenotypic features that are mediated strictly via these mechanisms will not be corrected via this strategy. One of these features may be the susceptibility of Fmr1 knockout mice to audiogenic seizures (Chuang et al. 2005; Osterweil et al. 2010; Zhao et al. 2011); however, Pacey et al. have shown that this phenotype has a $GABA_B$ receptor-mediated component (Pacey et al. 2009).

What will be the effect of the TRPV1 knockout on other phenotypic features of FXS? Dölen et al. examined several different phenotypes in their $Fmr1^{-/-}$-$GRM5^{+/-}$ mice that were altered in Fmr1 knockout mice; these included an examination of increased body weight, ocular dominance plasticity, increased dendritic spine density, increased basal protein synthesis in hippocampus, and the exaggerated inhibitory avoidance extinction (Dölen et al. 2007). In each case, the defects were substantially corrected by the decrease in mGluR5 expression. However, while the results demonstrate that decreasing mGluR5 expression rescues these specific fragile x phenotypes, it does not define the essential mGluR-dependent pathway

(s) that produce this. Thus, it would be interesting to examine whether these phenotypes persist in Fmr1$^{-/-}$-TRPV1$^{-/-}$ mice and thereby define whether the protein synthesis-dependent pathway or the 12-lipoxygenase-mediated pathway was the main determinant in mediating the phenotype.

TRPV1 is a homotetrameric, nonselective ligand-gated cation channel that is activated by a wide range of stimuli; it is also a well-known mediator (Palazzo et al. 2010) of nociceptive pain in the peripheral nervous system (PNS), where it is expressed in C-type neurons in adult rats (Sung et al. 2006) and colocalizes with FMRP (Price et al. 2006). Nociception is a complex phenomenon, consisting of a variety of responses to differing stimuli, and TRPV1's relationship to nociception typifies this complexity. Recent studies have shown that TRPV1 conveys heat pain, but not cold pain or mechanical sensitivity (McKemy 2011); it is also triggered during inflammatory pain responses (Davis et al. 2000). In keeping with its widespread expression during development, often overlapping with the expression of other pain-sensing channels, TRPV1 has also been found to be involved in thermoregulation (maintaining core body temperature) and pruriception (the itch response) (Davis et al. 2000). In Fmr1 knockout mice, various nociceptive behaviors are altered; these include decreased responses to ongoing nociception, the absence of thermal hyperalgesia, delayed development of peripheral nerve injury-induced allodynia, and the absence of wind-up responses in ascending sensory fibers after repetitive C-fiber stimulation (Price et al. 2007; Price and Géranton 2009). A key feature of the altered nociception is that the mechanism appears to be mediated, in part, by mGluR5 activity. These data lead to the view that the loss of FMRP results in decreased nociceptive sensitization, and it has been proposed that this may be related to the repetitive injury behavior of fragile X patients (for a comprehensive review, see Chap. 4).

The connection between mGluR5 activation and TRPV1 appears to extend from the CNS to the PNS where its effects on nociception can be mediated through a dual mechanism. On the one hand, Kim et al. have demonstrated that DHPG injection resulted in a robust increase in spontaneous pain responses in wild-type mice and these responses were significantly reduced in TRPV1$^{-/-}$ mice. Similarly, TRPV1$^{-/-}$ mice display reduced mechanical sensitivity to von Frey hairs (Kim et al. 2009). Along these same lines, Patwardhan et al. showed that the oxidized linoleic acid metabolites 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE), which are formed in mouse and rat skin biopsies by exposure to noxious heat-activated TRPV1. More importantly, however, blocking these substances substantially decreased the heat sensitivity of TRPV1 in rats and mice and *reduced* nociception (Patwardhan et al. 2010). These data suggest that mGluR5 at least partially mediates thermal sensitivity via the 12-lipoxygenase-mediated pathway. Thus, one might expect that the Fmr1-TRPV1$^{-/-}$ mouse might exacerbate the nociceptive deficiencies of Fmr1 knockout mice. However, Karim et al. determined that intrathecal injection of the mGluR5 inhibitor, MPEP, or 7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), a noncompetitive mGluR1-selective antagonist, attenuates formalin-induced ERK activation in the spinal cord dorsal horn (Karim et al. 2001). Furthermore, Hu et al. showed that

activation of mGlu5 leads to ERK-mediated phosphorylation and modulation of Kv4.2-containing potassium channels in dorsal horn neurons. This modulation may contribute to nociceptive plasticity and central sensitization associated with chronic inflammatory pain conditions (Hu et al. 2007). Thus, these data imply that some nociceptive behaviors may be due to the activation of an mGluR-mediated ERK1/2 pathway. If this is the case, $Fmr1^{-/-}$-$TRPV1^{-/-}$ mice would be expected to behave much like the Fmr1 knockout in tests of nociceptive behavior.

It is clear from the above discussion that the creation of an $Fmr1^{-/-}$-$TRPV1^{-/-}$ double knockout will not result in the complete rescue of all of the phenotypic features of FXS. In this regard, these mice are much like the Fmr1-$Grm5^{+/-}$ mice and the Fmr1-RGS4 double knockout mice. Both these mice also do not completely correct the fragile X phenotype (Dölen et al. 2007; Pacey et al. 2011) and several phenotypic features of fragile X (such as reduced nociception) have yet to be tested in either of these mice strains. Nevertheless, the $Fmr1^{-/-}$-$TRPV1^{-/-}$ double knockout should provide a unique window into the molecular basis of some of the features of FXS and for that reason alone, it would be a valuable addition to the fragile x community. Additionally, should mGluR5-mediated LTD and LTP be corrected in these mice, it would provide two additional targets for the development of therapeutics, i.e., TRPV1 antagonists and 12-lipoxygenase-mediated pathway inhibitors.

19.6 Controversial Models

Finally, I would be remiss if I did not say a word about three currently controversial models in the fragile X literature. These involve the role that the small noncoding RNA BC1 plays along with FMRP in translational regulation (Zalfa et al. 2003; Iacoangeli et al. 2008), whether the mammalian target of rapamycin (mTor) is misregulated in FXS (Osterweil et al. 2010; Sharma et al. 2010), and whether the absence of Fmrp in *zebrafish* results in craniofacial and neuronal architecture alterations (Tucker et al. 2006).

The *Wang* and *Zalfa models* of BC1 RNA function, as I will herein refer to them, could not be more structurally or mechanistically different. In the *Wang model*, translational repression is mediated by the 3′-end of BC1 RNA (Wang et al. 2005). The 5′-end, a 74 b hairpin containing a bulge and two internal loops, specifies two dendritic targeting codes (DTE1 and DTE2). The latter is required for long-range dendritic delivery and features a GA kink-turn (KT) motif that specifically interacts with heterogeneous nuclear ribonucleoprotein A2 (Muslimov et al. 2006). Furthermore, the class of messages that are translationally repressed by BC1/BC200 RNA in this model is relatively broad, i.e., those that require eIF4A helicase activity for efficient translation. Notably, Wang et al. specifically investigated whether BC1 RNA interacted with FMRP (Wang et al. 2005). They concluded that FMRP neither bound BC1 RNA in physiological salt nor was it necessary to suppress the translation of mRNA (Wang et al. 2005; Iacoangeli et al. 2008). Moreover, in the *Wang model*, FMRP functions independently of BC1 RNA, orchestrating its translational

effects on polyribosomes, rather than affecting 48S initiation complex formation. Thus, the *Wang model* views the multiple repressors that are found to be associated with neuronal RNA granules (FMRP, Pum, RNG105, UPF1, and BC1/BC200 RNA) as a means of independently modulating activity-dependent translation (Kindler et al. 2005). On the other hand, in the *Zalfa model* of BC1-mediated translational repression, BC1 RNA's 5′-end contains both the binding site for FMRP and the FMRP-target mRNA interaction motif (Zalfa et al. 2003, 2005). Binding of FMRP to BC1 RNA is required to recruit FMRP-target mRNAs to the repressor complex, which interferes with the production of eIF4F complexes (Napoli et al. 2008). Thus, in the *Zalfa model*, the messages repressed by BC1/BC200 RNA are a subset of those undergoing cap-dependent translation. The features of both models are summarized in Table 19.1.

Cellular and molecular signaling cascades, especially those in neurons, are complex, multibranching, often interacting, and terribly plastic (Ihekwaba et al. 2009; Peregrín-Alvarez et al. 2009; Zhang et al. 2010) and because of the maddening numbers of variables that can affect them, I try my best not to think about them in aggregate too much. Hardier intellects than mine, however, have forayed into this area to determine the molecular mechanisms that underlie FXS. Recently, this has led Sharma et al. to conclude that the mTor pathway is dysregulated in FXS and Osterweil et al. to conclude that it is not (Osterweil et al. 2010; Sharma et al. 2010). Regarding the mTor controversy, differences between the experimental preparations used in each of these studies are likely causes of the differing outputs. Hippocampal slice preparations, it must be remembered, are physically excised from an intact mouse brain and it is little appreciated that the resulting trauma due to nerve injury can in some instances mimic learning and memory paradigms from which they may have evolved (Sung and Ambron 2004). Therefore, if we look too early at a slice, we may be investigating a nerve injury response; conversely, if we look too late, we may be seeing the effects of a "dying" or suboptimal hippocampus. Getting it just right and knowing that it is just right will require more work before we can determine whether "Fmrp is a master regulator of global translation" (Cook et al. 2010).

Table 19.1 Differentiating features of the two models of BC1-mediated translational repression

Feature	Wang model	Zalfa model
Class of mRNA regulated	eIF4A-dependent mRNAs	Cap-dependent, FMRP-target mRNAs
Step targeted	48S initiation complex formation	eIF4F complex formation
Required Auxiliary factors	No	CYFIP1
FMRP localizes	Polyribosomes	Light mRNPs
BC1 RNA localizes	Light mRNPs	Light mRNPs
BC1/FMRP function	Independently	Dependently
5′-end of BC1 RNA	Dendritic targeting elements	FMRP binding/mRNA hybridization
3′-end of BC1 RNA	Binds eIF4A/PABP	No function

In 2006, Tucker et al. published their findings concerning the effect loss of Fmrp had in *zebrafish* (Tucker et al. 2006). Using a carefully controlled, paired Fmr1 morpholino oligonucleotide strategy to knock down Fmr1 gene expression, they observed a number of morphological defects that were not present when a control morpholino oligonucleotide was used. Among these defects, although not explicitly mentioned was a "small eye" phenotype (see Tucker et al., Figs. 5 and 6). Moreover, these defects could be unilaterally rescued by the coinjection of the Fmr1 morpholino oligonucleotides and a modified Fmr1 RNA transcript that lacked the morpholino oligonucleotide binding sites. Tucker et al. went on to demonstrate that the injection of the Fmr1 morpholino oligonucleotides also resulted in abnormal axonal branching of Rohon-Beard and trigeminal ganglion neurons, and guidance and defasciculation defects in the lateral longitudinal fasciculus compared to a control morpholino oligonucleotide. Importantly, these defects could be rescued by the mGluR5 antagonist, MPEP, a result consistent with an interaction between mGluR5 signaling and Fmrp function in neurite morphogenesis. However, as mentioned above, genetic ablation of the *zebrafish* Fmr1 gene did not result in any of the defects observed by Tucker et al., implying that though the study had substantial controls, the results were due to an artifact of the particular morpholino oligonucleotides used (den Broeder et al. 2009). Interestingly, Gessert et al. recently observed a "small eye" phenotype in *Xenopus* following treatment with a similarly constructed Fmr1 morpholino oligonucleotide as well as ones targeted to FXR1P, Dicer, microRNAs 23b, 96b, 130a, 196a, 200b, and 219. Although the authors argue that this effect is likely due to the disruption of RISC activity, it is not clear whether this is a general off-target effect, especially as (1) a control morpholino oligonucleotide targeting a microRNA that is not associated with eye development was never presented and (2) the effect of the Fmr1 morpholino oligonucleotide could be blocked by Fxr1p mRNA, which has limited homology to the target. Clearly, additional experiments will be necessary to sort this out.

Like the recent finding that depending on electrophysiological conditions, LTP in the hippocampus is either not altered (Paradee et al. 1999; Li et al. 2002; Zhang et al. 2009), or decreased (Lauterborn et al. 2007; Hu et al. 2008; Yuze et al. 2009) in Fmr1 knockout mice, each of these sets of studies highlight the fact that what we model and how we detect affect and thereby lessen our understanding of the scientific readout, a concomitant of the universality of Heisenberg's uncertainty principle. Although I have definite views concerning each of these models, it would be unfair of me to use these pages as a bully pulpit to lobby for or against any one of them. It would also run directly counter to one of the central goals of this project, which is to gather together, in one place, all of the data concerning all of these models to assist in crystallizing our understanding of FXS. I will, however, continue to do what I have done both publically and privately and that is call on the fragile X community to devise clear proof-of-concept experiments that would sort out the real truth behind these models. In this regard, I find it gratifying that the development and characterization of the BC1 knockout mouse (Centonze et al. 2008; Zhong et al. 2009) and its subsequent crossing to produce an Fmr1-BC1 double knockout (Maccarrone et al. 2010; Zhong et al. 2010) have shed new light

on the action of Fmrp and BC1 RNA and hint at a final resolution to this conflict. Armed with this evidence, we then must abandon the retrograde motion of Ptolemy's epicycles and stride into the new light of the Copernican sun. This may take time, Einstein proposed Special Relativity in 1905 and General Relativity in 1915, yet relativity was not truly confirmed until the late 1950s. Nevertheless, I am confident that this can and will occur regarding our tempests-in-a teapot; an understanding of the molecular basis of FXS and its cure is at stake.

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