Genetic interaction between *Fmr1* and *Grm5*:

A role for mGluR5 in the pathogenesis of

Fragile X Syndrome

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"The road to excess leads to the palace of wisdom... for we never know what is enough until we know what is more than enough."

--William Blake

Preface

Fragile X syndrome (FXS) is the leading inherited cause of mental retardation and an identified cause of autism. Despite impressive progress understanding the etiology of the disease, it is still unknown how disruption of brain function by a mutation in *FMR1* leads to the devastating syndrome that includes altered neural development, cognitive impairment, and childhood epilepsy. Currently, there is no effective treatment for FXS and the prospects for therapy by gene replacement are not promising. Future therapeutic approaches must therefore be based on a more complete understanding of the basic pathogenesis of the disease.

Fragile X mental retardation protein (FMRP) and mRNA are enriched postsynaptically in the brain, particularly at synapses that use the major excitatory neurotransmitter glutamate, so much attention has been focused on synaptic dysfunction in FXS. Recently a "metabotropic glutamate receptor (mGluR) theory" of fragile X pathogenesis has been proposed (appendix 1), based on the following four observations: (i) FMRP can function as a repressor of mRNA translation at synapses; (ii) synaptic protein synthesis is stimulated potently by activation of group 1 (Gp1) mGluRs, comprising mGluR1 and mGluR5; (iii) many of the lasting consequences of activating Gp1 mGluRs depend on synaptic mRNA translation; and (iv) in the absence of FMRP, several protein synthesis-dependent consequences of activating mGluRs are exaggerated. Together, these findings have led to the idea that FMRP and Gp1 mGluRs normally work in functional opposition, and that in the absence of FMRP, unchecked mGluR-dependent protein synthesis leads to the pathogenesis of FXS.

The goal of these studies was to test a key prediction of the mGluR theory—that aspects of fragile X syndrome can be corrected by down-regulating signaling through group 1 mGluRs. Each analysis was designed to examine a different dimension of the disorder in mice with relevance to the human syndrome, ranging from the cognitive to the somatic. The experiments assayed dysfunction in very different neural circuits; and for each, three outcomes were possible: amelioration, exacerbation, or persistence of *Fmr1* mutant phenotypes in mice with reduced expression of mGluR5. Thus, it is remarkable that by reducing mGluR5 gene dosage by 50%, we were able to bring 6 of 7 diverse fragile X phenotypes significantly closer to normal.

Although a range of phenotypes were studied, a simple way to conceptualize the constellation of findings is that fragile X is a disorder of excess—excessive sensitivity to environmental change, synaptic connectivity, memory extinction, protein synthesis, body growth, and excitability—and these excesses can be corrected by reducing mGluR5 signaling. Although the precise molecular basis of the interaction remains to be determined, the data show unambiguously that mGluR5 and FMRP act as an opponent pair in several functional contexts, and support the theory that many CNS symptoms in fragile X are accounted for by unbalanced activation of Gp1 mGluRs. These findings have major therapeutic implications for fragile X syndrome and autism.

Chapter 1: *Genetic analysis*. Identification of the FMR1 gene will be described, followed by an overview of strategies for genetic analysis of genetic interaction between Fmr1 and Grm5. Results section will include generating cross mice, genotyping, and confirmation of mGluR5 knockdown.

Chapter 2: *FMRP and mGluR5*. This chapter will begin with a description of the function, developmental profile, and putative interaction between, the proteins implicated in the pathogenesis of FXS. Results of metabolic labeling experiments to determine the genetic interaction between *Fmr*1 and *Grm*5 at the level of protein synthesis will be provided and discussed.

Chapter 3: *Cognitive function*. An overview of the cognitive disruption in FXS will be provided, followed by a discussion of the phenotypes relevant to the cognitive features of the disease. Evidence for genetic interaction between Fmr1 and Grm5 in the regulation of ocular dominance plasticity, dendritic spine density, and learning and memory behaviors will be provided and discussed.

Chapter 4: *Syndromic features.* Syndromic features including neurologic, endocrine and dysmorphic, of FXS will be discussed. Evidence for genetic interaction in the regulation of seizure and growth, but not macroorchidism, will be provided and discussed.

Chapter 5: *Implications, future directions and conclusions*

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Chapter 1: Genetic analysis

Introduction

Fragile X syndrome (FXS) is a genetic disease. Therefore we will begin our discussion with brief description of how the gene that is mutated in FXS, *FMR1*, was discovered. Next we will consider the methodology used in genetic analysis, with a focus on genetic interaction, followed by a description of some of the key tools used in genetic engineering, particularly the generation of mutant animal models. Following this general introduction to genetic engineering, we will consider animal models of FXS as well the metabotropic glutamate receptor 5 (mGluR5) knockout animal. In the results section, we will see how mutant cross animals were generated. Finally, we will end with a short discussion of how genetic interaction between *Fmr1* and *Grm5* (the gene that encodes mGluR5) can be interpreted in the context of the pathogenesis of FXS, as well as implications for therapy.

Identifying the *FMR1* gene

The *FMR1* gene is located at Xq27.3; this designation indicates that it is found on the long arm (q) of the X chromosome at position 27.3. As shown in **Figure 1A**, staining chromosomes with the Giemsa stain produces a reliable pattern of bands; because the pattern of bands on each chromosome is unique, it can be used to identify and number genes. The ideogram (**Figure 1A**, middle) is the map of the chromosome, and is divided into two parts the "p" (for petit) and "q" (alphabetically after p); position 27 is indicated. Before the *FMR1* gene could be identified and implicated in the disease, a number of key observations had to be made. First, evidence for a genetic etiology (based on pedigree patterns of families with affected individuals) had to be established (*1*). Second, the locus of the disease was hypothesized (*2, 3*) and later confirmed (*4*) to be a Fragile site on the X chromosome. This cytogenic marker, originally designated FRAXA (for fragile site, FRA, on the X chromosome, A since it was the first identified) (shown in **Figure 1A**, right), allowed the identification of many more patients whose DNA could be studied. This required the ability to reliably induce the fragile site using the appropriate culture conditions (*5*). In order to appreciate the breakthroughs that followed, first we must have a basic understanding of DNA recombination, cloning and mapping, described below:

1. A sample of human DNA, obtained from tissue, is cut up, by using enzymes called restriction endonucleases (e.g. *Eco*R1). These enzymes are a class of DNA-binding proteins that bind to the DNA and cut it at specific sites. They can cut long chromosome sized DNA into thousands of smaller fragments.

2. Next, each fragment is fused with a vector chromosome (e.g. the yeast artificial chromosome, YAC, which can accept very large pieces of DNA, shown in **Figure 1B** (*6*)). Typically, the vector itself has been cut with the same restriction enzyme used to cut the genomic DNA, and the "sticky" ends (staggered cuts with single-stranded ends that can base pair to a complementary sequence) are used to *hybridize* (stick together) the cut ends of the genomic DNA with the cut ends of the vector DNA. This fused molecule is called *recombinant DNA*.
3. The recombinant DNAs are inserted into bacterial cells, (one recombinant molecule per cell), and replicated along with bacterial DNA during normal growth and division, resulting in a *clone* of identical cells each caring the recombinant DNA molecule (this method is therefore called *DNA cloning*).
4. This process can be used to make *genomic libraries* which are large collections of cloned genomic material that represent the cut up pieces of the genome's worth of DNA. Because they are generated by semi-randomly cutting up the DNA, they can represent DNA segments of varying size, sometimes containing overlapping sequences that are redundantly represented in the library.
5. Once the library has been produced, the specific clone that represents the genomic region of interest must be identified. This can be done in a variety of ways, but for the purposes of this discussion we will limit ourselves to two:
    a. Southern Blotting
    b. Positional Cloning (gene mapping) (e.g. by breakpoint cluster, CpG island, restriction site mapping)
6. Once the clone has been matched to the region of interest, the nucleotide sequence

of the DNA contained in the clone can be determined.

7. This sequence can then be scanned (by a computer) in search of possible protein coding regions that begin with an ATG initiation codon and end with a stop codon. These regions are called open reading frames (ORFs), and represent sequences that are candidate genes.

In order to identify the *FMR1* gene, Verkerk and colleagues (*7*) began with an 80 kB YAC (named RS46) derived from the distal portion of the long arm of the human X chromosome (*8*), **Figure 1B**. In order to determine whether this segment of the DNA contained the Fragile site, it was compared to segments of DNA obtained from patients with Fragile X. These "translocation hybrids" (8 pairs of them) were known to contain the fragile site, because they had been "broken" as described previously (*9*), so they provided a reliable way to screen potential clones for the Fragile site, **Figure 1C**. As mentioned above, matching the clone to the gene of interest can be done in a number of ways; this approach represents a form of breakpoint mapping, since the breakpoint provides a reliable landmark of where the gene might be. These hybrids come in two forms, because the DNA is broken into two pieces; these pieces were called proximal and distal (relative to the centromere) translocation hybrids. The YAC clone RS46 hybridized to the proximal hybrids, but not the distal hybrids, providing evidence that the RS46 contains some, but not all of the genomic sequence involved in fragile X.

Thus, the authors wanted to find another YAC that was longer and might contain both sides of the break. Using RS46-specific oligonucleotide primers (*8*), a YAC library was screened to find such a clone. This screen identified a much larger YAC (475 kB) named

209G4, which completely overlaps YAC RS46, and includes sequences both proximal and distal to the breakpoint, **Figure 1B**.

In order to further confirm the match between these YACs and the Fragile X site, the authors performed a second kind of mapping, called restriction endonuclease linkage analysis, with a polymorphism called DXS548 that provided a second landmark for the fragile X site (*10*), **Figure 1C**. Verkerk and colleagues showed that this landmark was also contained within the two YAC sequences, providing further evidence that these clones contained the fragile X site.

A third landmark found within the YAC sequence was the CpG island, **Figure 1C**. CpG islands are called islands because CG dinucleotide pairs are sparsely and unevenly distributed in the human genome, but when they do occur they are 1-2 thousand nucleotide pairs long, and often surround the promoters (5' end) of genes; when they are methylated they can regulate gene expression by turning off genes. Thus, this third landmark for confirming the YAC sequence, served the additional purpose of suggesting the location of the gene, and a mechanism by which it might be misregulated (i.e. by hypermethylation) (*11*).

In order to focus in on this important landmark, the authors generated sequence contig (contiguous sequence) cosmids (smaller vectors than YAC, that can carry 35-45 kb inserts) surrounding the CpG island; these cosmids were subclones from the larger YAC sequence. These subclones were then reconfirmed and sorted by hybridization to the breakpoint hybrids and Southern blotting.

The *FMR1* gene was now ready to be sequenced, **Figure 1D**. Using the subclones, the authors screened a cDNA library derived from normal human fetal brain RNA;

cosmid 4.1 matched a cDNA clone called BC22. This cDNA was sequenced and the open reading frame for the gene identified. In order to test whether the gene could be found in other organisms, BC22 was tested for hybridization against DNA from several species; interestingly it showed good hybridization for all species examined except for *drosophila*.

The BC22 cDNA contains the gene, but is only a small part (70 kB). Therefore, next the authors wanted to determine the location of BC22 relative to the CpG island and the breakpoint cluster. Using the other cosmids derived from the YAC sequences, the authors were able to show that the *FMR1* gene is adjacent to the CpG island and the region where the majority of fragile X breakpoints occur. The fragment that they identified is 5.1 kb in normal X-chromosomes, but undergoes a large expansion (7.4 kb) in the fragile X chromosome, **Figure 1E**. This expansion was later determined to be the triplet repeat expansion that, not only explained the the inheritance pattern of the disease, but also provided a mechanism for transcriptional silencing of the *FMR1* gene (*12, 13*).

Genetic analysis

It is often useful to think of genes as the basic chromosomal unit that *determines* a given phenotype. While this notion is useful shorthand, it is somewhat oversimplified, since it is clear that no gene exerts its effect on a given phenotype in isolation. In order for a gene to have any influence on phenotype it must act in coordination with many other genes, and with the external and internal environment. One method for understanding what role a gene has in the expression of a phenotype is genetic analysis. In its most basic formulation, this approach involves creating a mutant animal and examining its phenotype. Further iterations of this approach generate crosses between

mutants (e.g. double mutants) to determine the contribution of multiple genes to a single phenotype, and how and if the genes interact. Perhaps the simplest way to appreciate genetic interaction is in terms of *direct physical interaction* between genes and gene products (proteins, DNA, or RNA). To illustrate, several types of interactions are exemplified below:

1. Transcription of one gene might be regulated by the protein product of another: one gene encodes a regulatory protein that binds to a region in front of the second gene that turns on or off transcription (e.g. transcription factors)
2. Enzyme proteins encoded by one gene may be necessary for the production of the substrate for the enzyme proteins encoded by a second gene (e.g. synthesis of catecholamine neurotransmitters)
3. Proteins encoded by one gene may bind to proteins from other genes to form a protein complex, which acts as a functional unit (e.g. subunits of a receptor protein)
4. Proteins encoded by one gene may modify proteins encoded by a second gene in order to activate or deactivate protein function (e.g. activation of a receptor protein leads to phosphorylation of another receptor protein)

This simple picture is complicated by the fact that cellular signaling usually involves multiple gene products, which can be modified by environmental signals like hormones (that act as transcription factors) or neurotransmitters (that activate effector second messenger cascades). Additionally the discovery of alternative splicing, translational

regulation of RNA, and dominant-negative mutations, adds further complexity to these genetic interactions. Therefore, while it is easiest to conceptualize genetic interaction in terms of direct physical contact between gene products, often the physical interaction is unknown. Interpretation is further complicated by the fact that genes are temporally and regionally regulated, such that interaction between two genes can vary depending on the developmental stage and cell type required for the expression of the phenotype.

Over a century of genetic interaction studies in bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*), flies (*Drosophila melanogaster*), fish (*Danio rerio*) and worms (*Caenorhabditis elegans*) have provided a framework for understanding the contribution of multiple genes to a phenotype, even when the direct physical interaction is unknown. For example, when two genes interact in an *epistatic* fashion, the interaction provides evidence that the two genes are in the same cellular pathway. Epistasis means, "to stand upon" reflecting the fact that one mutation prevents the expression of another in the double mutant (the overriding mutation is *epistatic*, the overridden is *hypostatic*) (*14*). In the example given above (2) a mutation that prevents the expression of dopamine decarboxylase (mutant 1) would lead to the absence of Dopamine (DA) and norepinephrine (NE), whereas a mutation in the gene that encodes DA ß hydroxylase (DBH) (mutant 2) would lead to the absence of NE alone. A double mutant would have the phenotype of mutant 1, since DA is further upstream in the biosynthetic pathway (i.e. mutant 1 is epistatic, mutant 2 is hypostatic) (**Figure 2A**). The logic behind the interpretation of epistatic interaction is deceptively simple, and can be rapidly

complicated when pathways involve multiple points of positive and negative regulation (**Figure 2B**)(*15*).

Another form of genetic interaction is *suppression*. There are two types of genetic suppression, *intragenic* and *extragenic,* depending on whether the suppressors are in the same gene or different genes as the starting mutation. Only extragenic suppression is relevant to the current discussion, and will be referred to forthwith simply as genetic suppression (for further discussion of intragenic suppression see, (*16*)). A genetic suppressor is a mutant allele of one gene that reverses the effect of a mutation of another gene, resulting in a wild-type phenotype. The suppressor mutation sometimes has no effect in the absence of the other mutation, in which case it has a wild-type phenotype. In other cases, the suppressor mutation produces its own abnormal phenotype. Analysis of suppressor mutations can also aid in the dissection of contributions of different genes to a common phenotypic endpoint. In addition to identifying points of regulation, these studies can also be used to suggest therapeutic strategies, especially when the mutation being suppressed is implicated in a human disorder (see below for further discussion). At the molecular level, suppressors can work in a variety of ways, each time restoring the wild-type phenotype in the double mutant:

1. Bypass suppression (**Figure 3**). This form of genetic suppression occurs when convergent pathways intersect in the regulation of a common phenotypic endpoint. The mutation is suppressed by a second mutation that bypasses the defective component of the first mutation to produce a wild-type phenotype. Bypass may occur by up or down-regulating the expression of a related protein

that can compensate for the lack of a particular protein. It can also occur by up or down-regulating the activity of a parallel protein pathway (*16*). Bypass suppression represents the least direct kind of genetic interaction, because it suggests that the two gene products of interest are not in the same linear pathway, but instead in two pathways that converge at some downstream regulatory step.

2. Component suppression (**Figure 4**). This form of genetic suppression represents the most direct form of interaction, and occurs when the function of a mutant protein is restored by changes in another protein in the same complex (*16*). The simplest way to conceptualize this interaction is in terms of receptor subunits that when brought together act as a receptor protein complex (obviously other more complicated protein complexes exist in cells, but the principle of interaction is the same). In this case a mutation in one subunit prevents the formation of the receptor complex, and only when the other subunit is correspondingly mutated is the receptor complex formed, and function restored. Note that in this case, the two mutants have the same phenotype, but in the double mutant the wild-type phenotype is restored.

3. Suppression by removal of a toxic protein or protein complex (**Figure 5**). The appearance of an abnormal phenotype is sometimes the result of the production of a toxic protein. Any alteration that removes the toxic protein, or prevents it from acting, will act as a suppressor (*16*). Although there are a number of potential mutations that could lead to the production of a toxic protein (e.g. metabolic

pathways, mutagens etc), for the purposes of the current discussion we will focus on dominant negative mutations. A dominant negative mutation occurs when the gene product adversely affects the normal, wild type gene product within the same cell. This usually occurs if the product can still interact with the same elements as the wild-type product, but block some aspect of its function. For example, for a receptor protein that requires two dimerized subunits for its proper function, a mutation that removes the functional domain, but retains the dimerization domain would cause a dominate negative phenotype, because some fraction of protein dimers would be missing one of the functional domains. A suppressor mutation could reverse these dominant negative effects by out-competing the mutant subunit, and restore wild-type function.

4. Suppression by modulators of activity (**Figure 6**) (*16*). The efficiency of a signal transduction pathway is usually modulated by interacting components that can increase or decrease the strength of a signal. This is especially true in the mammalian nervous system, where the same pathway may be used for multiple purposes in different tissues, and can be fine-tuned by modulators to allow optimal function in each context. As an example, imagine the case where a mutation interferes with the phosphorylation site on a receptor; a suppressor mutation that enhances the activity of the kinase that phosphorylates the site, could in principle restore wild-type function.

5. Suppression by epistasis (**Figure 7**). Recall from the discussion above that

epistatic interactions occur when gene products are in the same cellular pathway. In some cases, epistatic interactions can lead to suppression of a mutant phenotype (*16*) by antagonistic mutations in the pathway. In neuroscience the most familiar cellular pathways are those that mediate signal transduction events in response to neurotransmitter receptor activation. These signal transduction cascades are often very complicated, involving both pre- and post-synaptic partners, receptor proteins of various types, second messengers, translation machinery, and transcription factors. At each step the interactions may be either positive or negative. In many cases the epistatic relationships of mutations in the different genes can be used to deduce the order and nature of the steps involved (*15*). An example of epistatic suppression by antagonistic mutation acting at a downstream step is diagramed in **Figure 7A**. In this example, the suppressor mutant has no phenotype of its own, and acts as an epistatic suppressor of the hypostatic mutant. Often signal transduction involves pathways are differentially recruited depending on the stimulus condition. In the example shown in **Figure 7B**, the same pathway is activated in a different way, (e.g. a stimulus is applied), and the epistatic mutation's phenotype is revealed. Under these conditions, epistatic suppression would no longer occur. This situation will be further discussed below in the context of the specific genetic interactions studied herein.

A final form of genetic interaction is *enhancement*. In a sense, this is just the opposite of suppression, since here the mutant enhancer gene *exacerbates* the effect of a mutation of another gene, resulting in a more severe phenotype. In some cases, this

exacerbation leads to death, and these double mutants are therefore called synthetic lethals. Sometimes exacerbation is due to the loss of a redundant pathway, such that in the absence of duplicate pathways the more severe phenotype is revealed (analogous to bypass suppression). In other cases, the interacting genes each have partial function when mutated that is compensated in the presence of the normal allele, but when both alleles are only partially functional, the phenotype is revealed (analogous to component suppression). As with suppression, enhancement by epistasis is also possible, and once again involves negative and positive regulatory steps.

The discussion thus far has focused on the molecular interactions that underlie the contribution of multiple genes in the expression of a phenotype. From a molecular genetics perspective, interaction may be implied even if single mutant phenotypes are in opposite directions (since regulatory steps are often bi-directionally regulated) (*16*). However, another way to estimate genetic interaction, used by population geneticists, is to calculate inheritance ratios. This sort of analysis is useful when individual genotypes within a population are not known (but is not necessary when genotypes are known) (*14*). Although a detailed description of this quantification will not be given here, suffice it to say that, if the inheritance pattern from a cross is *additive across loci*, there is no evidence for genetic interaction (in other words, if the number of individuals with the wild type phenotype is equal to the number of individuals expected to have a wild type genotype, then there is no genetic interaction). This sort of addition should not be confused with additive molecular effects of mutations that regulate the same process (sometimes in the same direction, sometimes in opposite directions).

Genetic engineering: mouse models

Among model genetic organisms, the genome of the house mouse, *Mus musculus* most closely resembles the human genome. Although its diploid chromosome number is 40 (compared to 46 in human) and its genome is slightly smaller than that of humans, it contains approximately the same number of genes (about 25-30 thousand), and all mouse genes have a counterpart in humans (*14*). There are two transgenic technologies used for creating mouse models: *ectopic insertions* and *gene targeting*. Ectopic insertions involve random insertion of the transgene into the genome, whereas targeted mutations replace designated sequences in the genome with homologous transgenes.

Ectopic insertions

The first successful production of transgenic mice was reported in 1980 (*17*). These animals were generated by injecting DNA directly into the nucleus of fertilized mouse oocytes at the one-cell stage (to avoid mosaicism) (**Figure 8A**). In these studies the injected DNA was a modified bacterial plasmid that contained both an origin of DNA replication and a gene that encodes thymidine kinase (tk), which is not found endogenously in the mouse and therefore could be used to screen for incorporation. Injected embryos were then implanted into the oviducts of surrogate recipient mothers, and allowed to develop. The incorporation of this transgenic DNA into the genome occurs randomly, relatively infrequently (in this case only 2 out of 78 mice), and often undergoes mutation. If the injected DNA is incorporated into the germline, then the ectopic mutation can be passed on to future generations. More recently, essentially the same strategy has been used to generate an wide number of different transgenic mice, including those that express various fluorescent proteins (*18*) (**Figure 8B**). Because

genes are incorporated randomly, the expression patterns of ectopic genes are determined by the regulatory environment of the host genome (*14*). This feature of the technique has been exploited to generate several mouse lines with ubiquitous or restricted neuronal expression of fluorescence (*18*) (**Figure 8C**). The YFP-h mouse, which has restricted cortical expression of neuronal YFP, will be discussed in later chapters.

Gene targeting

The gene targeting approach was first used successfully to generate transgenic animals in 1988 (*19*). This approach can be used to make a wide variety of transgenic animals, but for the purposes of the current discussion, generation of so called *knockout* mice by gene disruption will be described (**Figure 9** adapted from (*19*)). As shown in **Figure 9A**, gene targeting begins by cloning the gene of interest and insertion of the neomycin resistance gene into an exon (expressed codon) region, which disrupts the expression of gene. This so called neo-cassette will also later serve to select cells that have incorporated the targeting vector into their genome (see below). A gene encoding the exogenous thymidine kinase (tk) is also added to one end of the vector; this marker will later serve to deselect cells have incorporated the marker into their genome (see below). Next (**Figure 9B**) the targeting vector is added to cultured mouse embryonic stem cells (ES cells). Three outcomes are possible at this stage. If the desired event occurs, the vector replaces the endogenous gene in the ES cells, through a process called homologous recombination (although this a somewhat rare event, it occurs spontaneously) (*14*). Importantly, when insertion happens through this mechanism, the tk gene is *not* incorporated. In addition, many vectors become inserted into the host genome by the mechanism described earlier, ectopic insertion, which is a random event (no

targeting). When insertion happens through this mechanism, the tk gene *is* incorporated. The majority of vectors are not incorporated, and the host genome remains unchanged.

In order to select for ES cells that have the appropriate insertion, all the cells are placed in medium containing neomycin (or its equivalent) and ganciclovir (**Figure 9C**). Cells that have incorporated the neomycin resistance gene are protected against neomycin, whereas cells that have incorporated the tk gene are susceptible to ganciclovir. Thus only cells that contain the neo-cassette, but not the tk gene, survive. These cells are then inserted into a developing embryo (in the blastocyst stage) (**Figure 9D**). The host embryo comes from an animal whose coat color is different from the coat color of the ES cell donor mouse, so animals that have successfully incorporated ES cells are born with a chimeric coat color, making them easy to identify (**Figure 9E**). After several rounds of breeding, knockout mice are generated, as shown in **Figure 9F**. This strategy for generating knockout mice has been widely used, including for the generation of the *Fmr1* KO (*20*), the *Fxr1* KO (*21*), *Fxr2* KO (*22*) and the *Grm5* KO (*23*) discussed below.

Animal models of Fragile X syndrome

The molecular basis for Fragile X syndrome in humans is a triplet repeat (CGG) expansion in the 5' untranslated region of the *FMR1* gene, located on the long arm of the X chromosome at position 27.3 (Xq27.3) (*7*) (see above for a more detailed discussion of how this gene was identified). As a consequence of this repeat expansion, the *FMR1* promoter and the repeat itself become methylated, leading to silencing of transcription and translation of the *FMR1* gene (*12, 24*). Recently the *FMR4* gene has been identified, and shares its promoter with *FMR1* (but is transcribed in the reverse direction), raising

the possibility that features of the disease are caused by additional silencing of this gene (unpublished results presented by Khalil, V and Wahlestedt, C., at the Conquer Fragile X Conference 2007, West Palm Beach Florida). Because the *FMR4* gene is only found in primates, these results raise the possibility that a deletion of the murine homolog *Fmr1* (which shows 97% sequence homology with human *FMR1* (*25*)) may be an incomplete model of the disease. However, studies have shown that rare mutations (e.g. at the I304N site) that disrupt the *FMR1* gene in isolation are sufficient to cause disease (*26, 27*), suggesting that the contribution of disrupted *FMR4* is negligible. The expression pattern of FMRP (mRNA and protein) is similar in humans and mice across tissues (*28*) and many of the disease features are recapitulated in the mouse model (*20, 29*), see later chapters for a more detailed discussion.

Using the gene targeting approach described above, the *Fmr1* KO mouse was generated by the insertion of a targeting vector into the mouse genome by homologous recombination in ES cells (*20*). The targeting vector, pMG5, consisted of mouse genomic DNA containing exons 4-9 of the *Fmr1* gene, which was interrupted with a neo-cassette insertion at exon 5, as well as the tk-cassette inserted 3' of exon 9 (the original fragment was obtained by probing with a human FMRi cDNA sequence) (*20*). Because the *Fmr1* gene is disrupted at exon 5, these mice transcribe exons 1-4, leaving open the possibility that phenotypes in these mice are due to dominant negative effects of this non-functional mRNA fragment. More recently, the generation of a second *Fmr1* KO mouse (*Fmr1* KO2) has overcome this confound by disrupting the *Fmr1* gene at exon 1; these animals make no transcript (*30*) and share many of the same behavioral features as the original *Fmr1* KO, with two notable exceptions: absence of the conditioned fear

phenotype described in the original KO (*31*) and an additional impairment in motor coordination not observed in the original KO (unpublished results, presented by Paylor, R. at the Banbury conference 2007, Cold Spring Harbor, NY).

Interestingly, in the process of generating the *Fmr1* KO2, these investigators also developed a conditional *Fmr1* KO (*Fmr1* CKO), by the addition of flanking lox P sites that allow for tissue specificity and temporal regulation of expression of the null allele (after crossing CKO mice with tissue specific or inducible cre-recombinase expressing mice) (*30*). These mice will be useful tools for future examinations of the tissue specific and developmental effects of loss of *Fmr1*. Most recently, a fourth mouse model of the disease has been developed (J. Darnell, unpublished); these mice have a mutation in the I304N site (I304N knock-in) and largely recapitulate the phenotypes described for the original *Fmr1* KO, with the possible addition of an *adult* audiogenic seizure phenotype (unpublished results, presented by Paylor, R. at the Banbury conference 2007, Cold Spring Harbor, NY). While these new models represent potentially useful alternatives to the original *Fmr1* KO, they have only recently become available, and were not used in the current study.

Non-mammalian animal models of FXS have also been developed and used for the study of the disease (*32-35*). While these models have some advantages (mainly they are relatively easy to manipulate, have short breeding cycles, and are inexpensive) they have several limitations for the study of human disease (*36*). Human *FMR1* belongs to a family of three Fragile X related (*FXR)* genes (FMR1, FXR1, and FXR2), of which only one (*FMR1*) is disrupted in FXS (*37*). In contrast, *Drosophila* have only one FXR orthologue (which has been variably termed dfxr and dfmr) (*34, 37, 38*), thus null

mutants in this model organism necessarily represent disruption of the entire gene family, making correlations to the specific functions of *Fmr1* difficult. Recent experiments in double KO (*Fxr2* and *Fmr1*) mice report a more severe phenotype, suggesting that in mammals, these genes have redundant functions that can partially compensate each other (this interaction represents a form of genetic enhancement, discussed above) (*39*). Phenotypes reported in the fly model may therefore represent more severe disruptions than those seen in the human disorder.

This potential confound is avoided in the zebrafish, since this organism has all three FXR genes; the published FXS model in zebrafish uses a morpholino knockdown strategy (binding of an antisense oligonucleotide to the start codon of the target gene blocks transcription) (*35*). These animals were reported to have severe craniofacial abnormalities as well as disruptions in axon guidance and neurite outgrowth (*35*); however these phenotypes are not recapitulated in recently developed knockout fish model, calling into question the reliability of the morpholino knockdown for studying the disease (unpublished results, presented by Oostra, B. at the Banbury conference 2007, Cold Spring Harbor, NY).

More importantly, for the purposes of the current study, neither fly nor fish have the mammalian complement of metabotropic glutamate receptors (mGluRs) (*40, 41*). Mammals have 8 mGluRs; these are categorized into three groups: Group I, II and III. Group I mGluRs (GpI mGluRs) are coupled to phospholipase C (PLC) and phosphoinositide (PI) hydrolysis, while Group II and III mGluRs couple to adenylate cyclase (*42*). This diversity is not recapitulated in flies and fish, where mGluR orthologs couple to adenylate cyclase rather than PLC (*40, 41*). Moreover, because the purpose of

these studies was to examine the role of Group I mGluRs in the pathogenesis of FXS, fly and fish models were deemed inappropriate (*36*), though they have been used for this purpose (*35, 43*).

One of the advantages of simpler organisms for genetic studies is their relatively short life cycle, making examination of multiple generations of offspring feasible within hours to days, depending on the organism (e.g. the average life cycle in a fly is about 50 days). While this short life span has its advantages, it presents a problem when studying developmental processes. For example, one early study in the fly model of FXS showed an important role for the MAP1B homologue Futsch in the pathogenesis of the disease (*38*). However later studies in the mammalian model showed that this phenotype (overexpression of MAP1B in the KO) is restricted to very early developmental time points (*44*) (but see (*45*)), calling into question the role it plays in the pathogenesis of the disease later. This is relevant since *Fmr1* is developmentally regulated, and may have different roles during early and late development (*46*). One strategy to overcome this problem in flies is to heat treat animals, which extends their life span (*47*), but it is unclear how this manipulation affects normal developmental programs. An equivalent problem exists for mouse models of neurodegenerative disease, since both accumulation of mutant protein and neurodegeneration seem to depend, in part, on the amount of time that a neuron is exposed to the mutant protein. Thus these diseases are often modeled by overexpression to achieve the disease phenotype during the normal lifespan of a mouse (*48*). This limitation of the mouse is less of a concern for FXS which does not have a reported neurodegenerative component (*49*) (but see, (*50*)), but may become important when modeling related diseases (i.e. FXTAS) (*51*).

In addition, the nervous system of non-mammalian species differs from mammals in a number of important ways. In the mammalian central nervous system, an estimated 80% of synaptic transmission occurs through glutamatergic signaling (*52*), while in the periphery, acetylcholine (ACh) is the dominant neurotransmitter. This situation is reversed in the fly, where glutamate is released at the neuromuscular junction and ACh is used centrally. Studies in the fly extrapolate disruptions in glutamatergic signaling and synapse formation from studies of the neuromuscular junction (*53*), but these inferences can be misleading since they only relate to the presynaptic neuron. Furthermore, while some structures such as the hippocampus have functional orthologs in the fly (i.e. the mushroom body), others, such as the neocortex, are notably absent and have no equivalent structure. While studies in the fly have attempted to overcome this problem by studying simple behaviors (*54*), this becomes more difficult when modeling complex behaviors that require the cortex (*55*) and may be involved in human disorders such as FXS and autism (*56*).

Grm5 knockout mouse

In the same year that the fragile X mutation was identified, cloning of the mGluR molecule (*57, 58*) brought further insight into the structure and function of these receptors. As mentioned above, the 8 human subtypes are all found in the mouse genome, of which mGluR5 was of particular interest. In mouse, the *Grm5* gene codes for mGluR5, and is found on chromosome 7 (in humans it is located on chromosome 11). Using the same targeting approach described above, the *Grm5* KO mouse was created by the insertion of a targeting vector into the mouse genome by homologous recombination

in ES cells (*23*). The mouse *Grm5* gene clone was isolated from a DNA library prepared by screening with rat mGluR5 cDNA; the exon 1 region was then sequenced and showed 98% homology with rat (data not shown) (*23*). The targeting vector, pPNT, was made by deleting a 0.4-kb fragment containing part of exon 1 and a part of intron 1 by replacement with a 1.8-kb fragment containing the PGK–*neo* cassette. The PGK-tk cassette was inserted downstream to serve as a negative selection marker. Because the *Grm5* gene was disrupted at exon 1, these mice are unlikely to produce any transcript (although this control was not shown, instead absence of mGluR5 *protein* in KO mice was demonstrated by western blotting with an mGluR5 specific antibody) (*23*). Since their generation, these mice have been used in a number of studies (*23, 59-66*), which will be discussed elsewhere as they become relevant.

Results

Generation of mutant crosses

As shown in **Figure 10A**, *Fmr1* heterozygous females (*20*) were crossed with *Grm5* heterozygous males (*23*) to produce *Fmr1* knockout animals with a selective reduction in mGluR5 expression. Littermates with 4 different genotypes were created from this cross: Wild type [*Fmr1* (+/Y)/*Grm5* (+/+)], *Fmr1* knockout [*Fmr1* (-/Y)/*Grm5* (+/+)], *Grm5* heterozygote [*Fmr1* (+/Y)/*Grm5* (+/-)], and the knockout/heterozygote cross [*Fmr1* (-/Y)/*Grm5* (+/-)]; these animals are termed WT, KO, HT, and CR, respectively. In addition, for a subset of experiments, Fmr1+/-/Grm5-/- (HK) females were crossed with Grm5-/- (mKO) males to generate double KO animals (dKO) and mGluR5 KO controls, but as had been reported previously (*23*), these animals did not breed well. Breeding was

somewhat improved when the animals were placed on a high fat diet, suggesting that low maternal body weight accounts for the poor breeding in these animals (unpublished observations).

Genotyping (**Figure 10B**)

As had been published previously (*20*), screening for the presence or absence of the wild-type allele on the *Fmr1* locus was performed using primer S1(5' GTG GTT AGC TAA AGT GAG GAT GAT 3') and S2 (5' CAG GTT TGT TGG GAT TAA CAG ATC 3') and the knockout allele using primer M2 (5' ATC TAG TCA TGC TAT GGA TAT CAG C 3') and N2 (5' GTG GGC TCT ATG GCT TCT GAG G 3'). For the *Fmr1* locus the following PCR program was used: 95 °C for 5 min; 34 PCR cycles were performed composed of 30 sec at 95 °C, 30 sec at 61 °C, and 1min at 72°C. KO and WT PCR reactions were run separately; the reaction products were then combined and electrophoresed on a 1.5% agarose gel [WT: 465 BP (S1/S2); KO: 800 BP (M2/N2)].

Previously published genotyping strategies for the *Grm5* knockout allele used primers that were both directed a the neo-cassette (*23*). Using these primers in our cross led to the detection of false positives, since both the *Fmr1*KO and the *Grm5* KO mouse were generated with a neo-cassette insertion to disrupt the gene (i.e. animals that were *Fmr1* KO but WT for *Grm5*, would consistently come up positive for the *Grm5* KO allele, since these primers were recognizing the neo-cassette in the *Fmr1*KO allele). In order to avert this problem, primers were redesigned so that one of the primers (F) would recognize sequences within the undisrupted coding region on chromosome 7 but sufficiently close to the primer within the neo-cassette (primer P) so that amplification

could occur. Thus the new genotyping protocol is as follows: screening for the presence or absence of the knockout allele on the *Grm5* locus was performed using primer P(5' AGG GGA GGA GTA GAA GGT GGC GCG A 3') and F(5'GCT CAC ATG CCA GGT GAC ATT ATT ATT GGA 3') and the wild-type allele using F (see above) and R (5' CCA TGC TAG TTG CAG AGT AAG CAA TCT GAG GT 3'). For the *Grm5* locus the following PCR program was used: 95 °C for 5 min; 34 PCR cycles were performed composed of 30 sec at 95 °C, 55 sec at 61 °C, and 1min at 72 °C. KO and WT PCR reactions were run separately; the reaction products were then combined and electrophoresed on a 1.5% agarose gel [WT: 445 BP (F/R); KO: 550 BP (F/P)]. All primers were generated by Invitrogen.

Confirmation of reduced mGluR5 signaling

To increase the therapeutic relevance, we concentrated on animals with a 50% reduction in mGluR5 rather than a complete knockout (which impairs brain function (*23, 64*)).

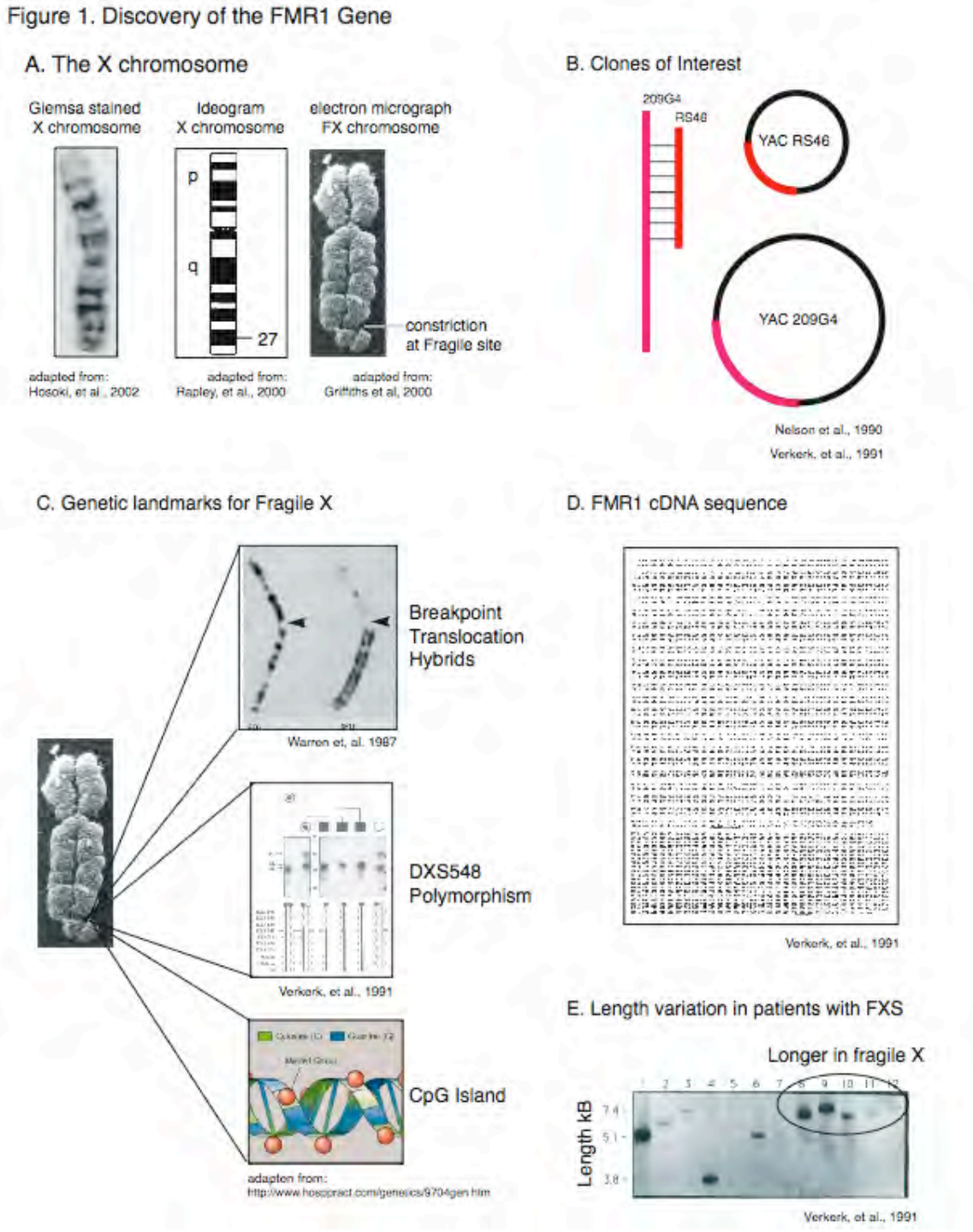
As shown in **Figure 10C** the 50% reduction in *Grm5* gene dosage was confirmed by probing for mGluR5 protein levels in animals from the four genotypes. Western blot analysis of total mGluR5 expression in visual cortical homogenates from P30 normalized to WT (+/+, +/Y) mice, KO (+/+, -/Y), HT (+/-, +/Y), and CR (+/-, -/Y) (n = 11 per genotype, ANOVA $P < 0.0001$). *Grm5* KO (-/-, +/Y) control sample was run 4 times (n = 1 animal) and normalized to age matched (postnatal day 45) WT. Representative blot is shown above. The reduction in mGluR5 protein expression in *Grm5* heterozygotes (shown above) is known to be functionally significant. As shown in **Figure 11,** Long-term synaptic depression triggered by mGluR activation (mGluR-LTD), a phenomenon

that depends on mRNA translation at the synapse, is significantly impaired in the mGluR5 heterozygotes

Interpretation of genetic interaction

Genetic interaction studies are a valuable tool for deciphering the contribution of various genes in the production of a phenotype. In 6 out of 7 of the phenotypes we have examined, we show that a 50% reduction in *Grm5* suppresses *Fmr1* KO phenotypes. However, these studies were unusual because genetic interaction studies are ordinarily carried out in full KO's rather than heterozygous mutants; nevertheless we see evidence for dominant suppression (since a mutation in a single allele can suppress the effects of a full mutation in the *Fmr1*KO). While many of the suppressive interactions described above are unlikely, by themselves, these experiments do not allow us to distinguish between bypass suppression and suppression by epistasis (**Figures 12 and 13**). Assuming the epistatic suppression model (**Figure 12**), currently it seems that under basal conditions, the remaining 50% of mGluR5 activity is sufficient to maintain normal activity (no phenotype in the HT). A phenotype in the *Grm5* HT is only apparent when the pathway is activated by monocular deprivation; but the remaining receptors are sufficient to drive the system normally in the absence of inhibition by FMRP (as in the in the cross). However, the bypass suppression model, shown in **Figure 13**, might just as easily describe this situation. Future experiments in the double KO could help to parse out which of these models describes the interaction between *Fmr1* and *Grm5*. For example, if suppression of the ocular dominance phenotype by 50% reduction in *Grm5*

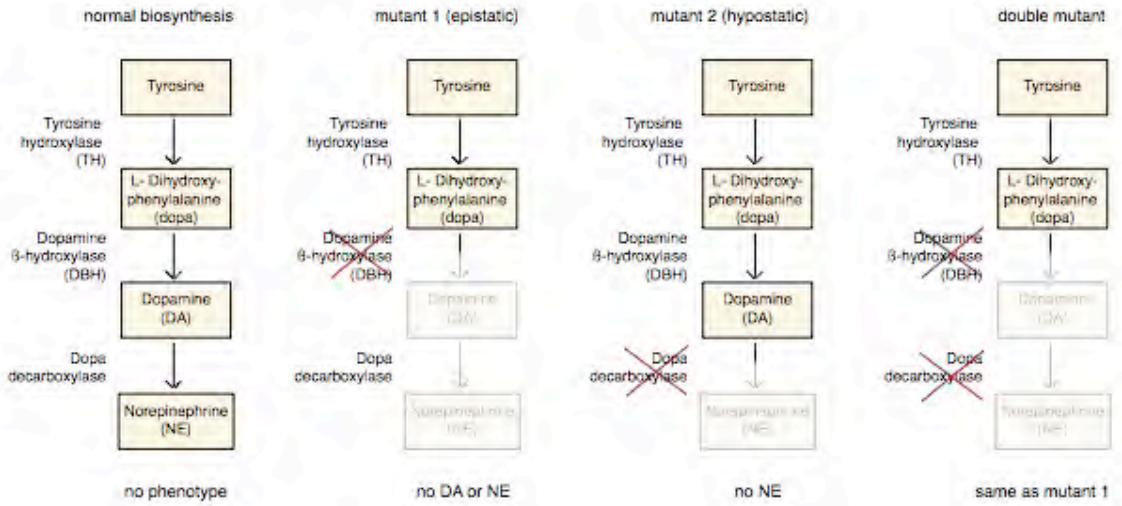
gene dosage occurs through epistasis, then we would predict that in the double KO, the complete loss of *Grm5* would prevent the rescue, and the phenotype in the double KO would be indistinguishable from the *Grm5* KO phenotype (*Grm5* epistatic, *Fmr1* hypostatic). If on the other hand, the complete loss of *Grm5* in the *Fmr1* KO leads to a wild type phenotype, then it would be more likely that *Fmr1* and *Grm5* converge on a common intermediate, and rescue in the double KO occurs via bypass suppression.



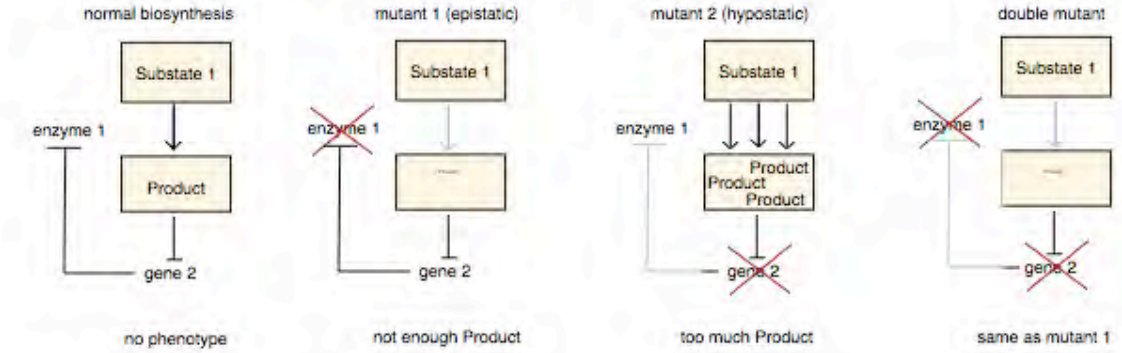
Discovery of the FMR1 gene (A) The X chromosome can be visualized in a number of ways: giemasa stain, ideogram, electron micrograph. (B) Yeast artificial chromosomes containing overlapping clones (RS46 and 209G4) of putative sites of FX mutation. (C) Three landmarks at the FRAXA site used to validate clones. (D) FMR1 gene sequence. (E) BC222 clone is longer in FXS patients.

Figure 2. Epistasis

A. Catecholemine biosynthesis epistasis

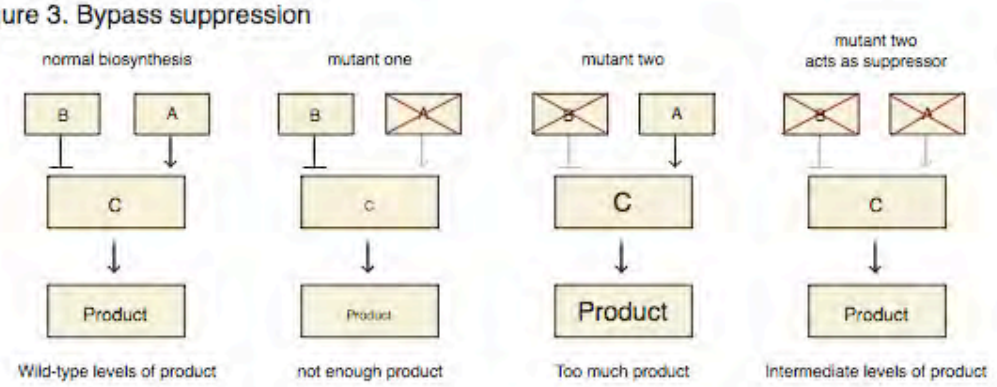


B. Negative feedback epistasis



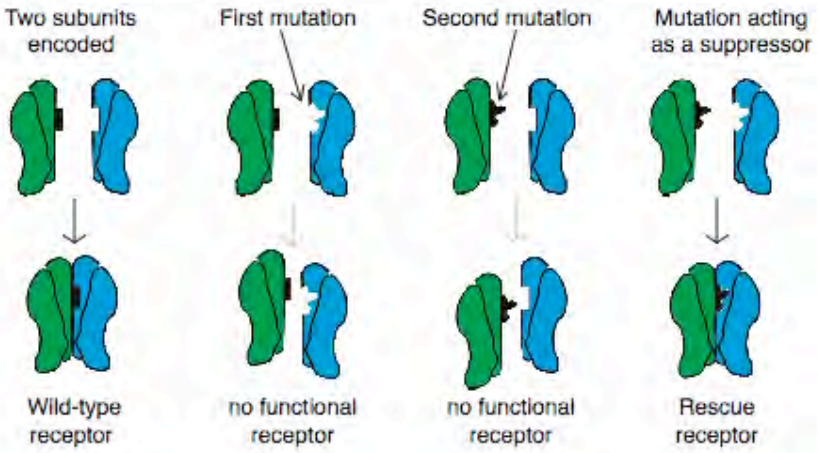
Genetic interaction: Epistasis. (A) Double mutant phenotype is the same as mutant 1, which is epistatic to mutant 2. (B) Epistasis can also occur when negative feedback loops are involved.

Figure 3. Bypass suppression



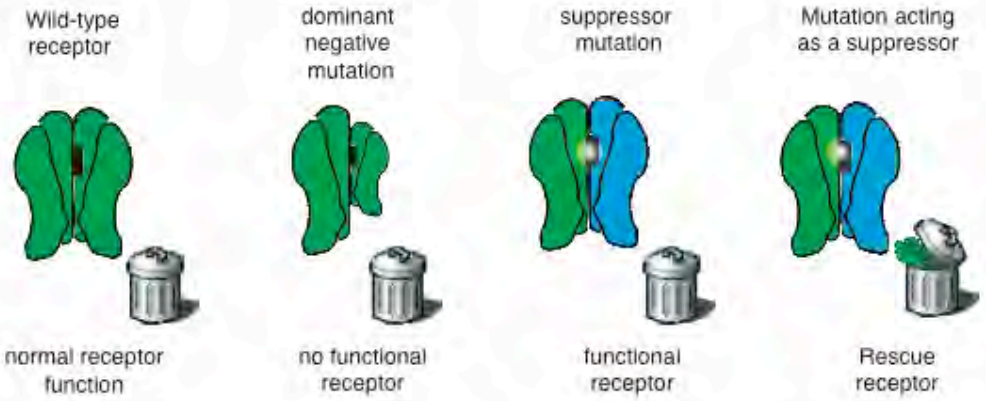
Bypass suppression. The mutation (one) is suppressed by a second mutation that bypasses the defective component of the first mutation to produce a wild-type phenotype.

Figure 4. Suppression by interacting components



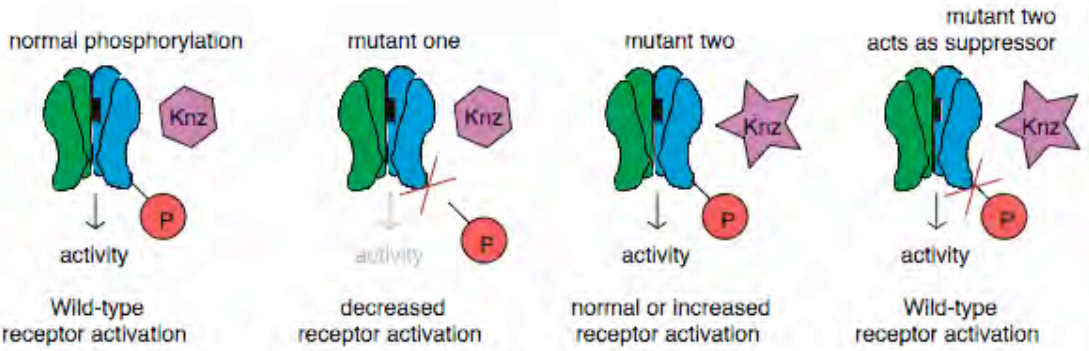
Component Supression. The function of one mutant protein is restored by changes in another protein in the same complex. Double mutant has wild-type phenotype.

Figure 5. Suppression by removal of dominant negative



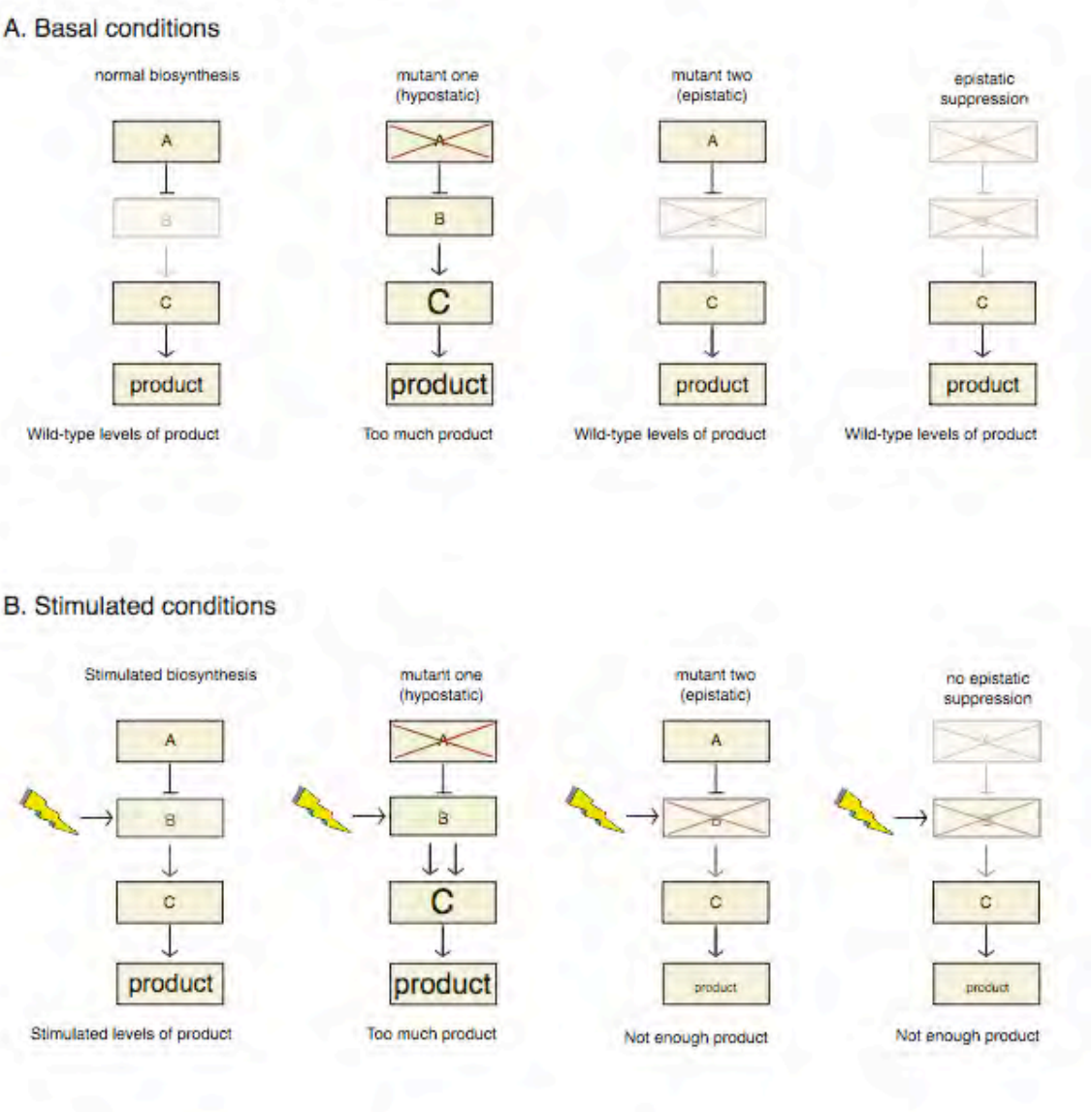
Suppression by removal of a toxic protein or protein complex. Dominant negative mutation of a receptor protein is suppressed by a mutation that allows functional subunits to out compete mutant subunit and restore WT function.

Figure 6. Suppression by modulators of activity



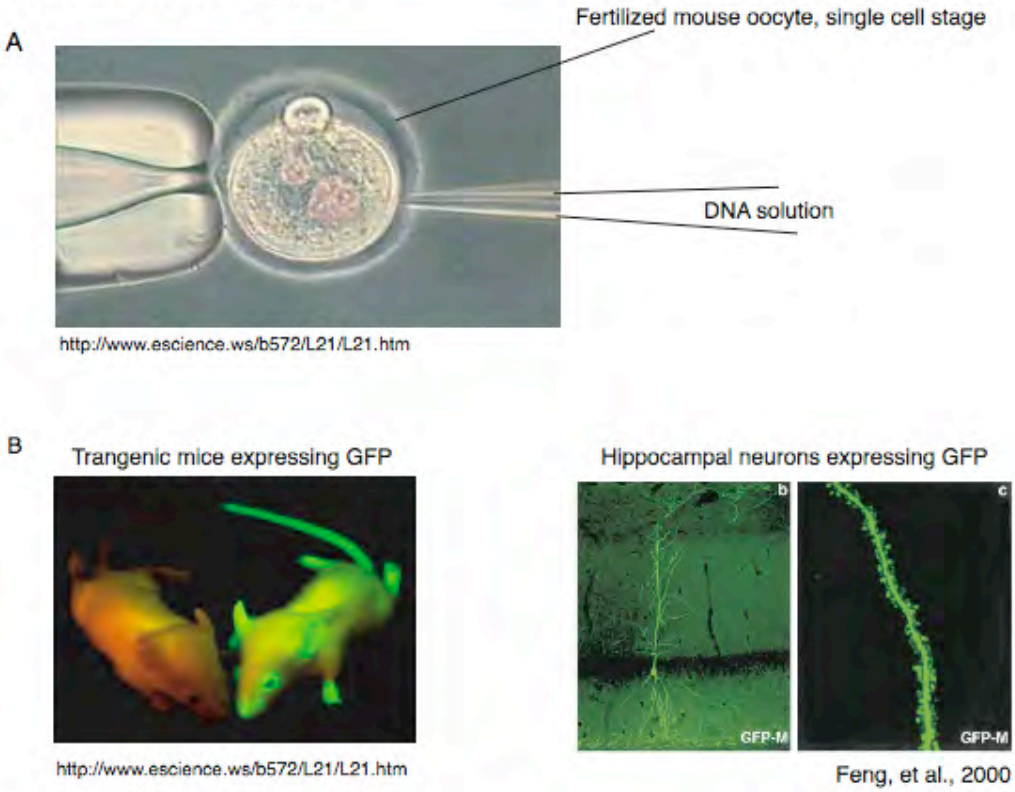
Supression by modulators of activity. A mutation that enhances the activity of a kinase can restore wild type function to a mutation in a phosphorylation site.

Figure 7. Suppression by epistasis



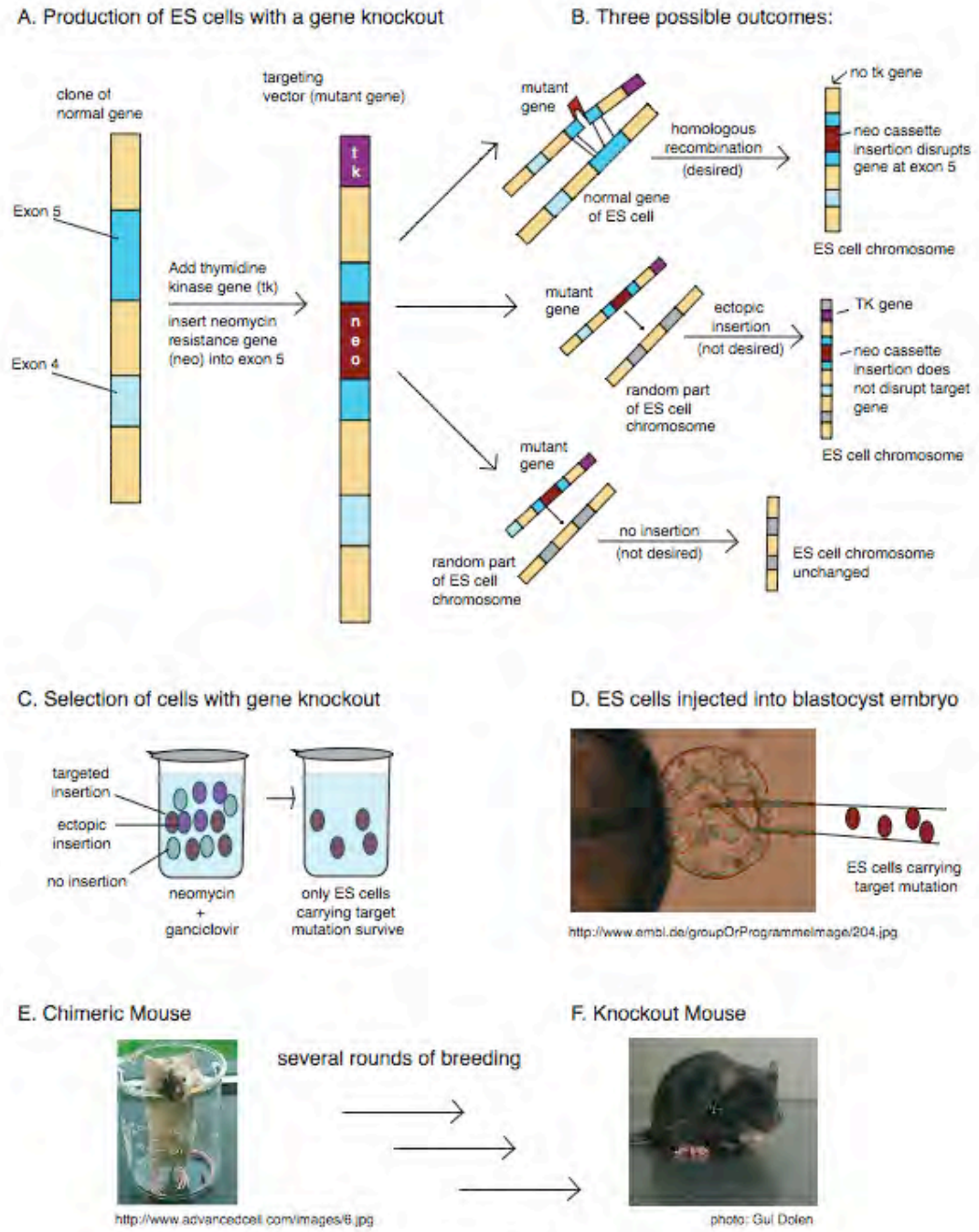
Suppression by epistasis. (A) Wild-type pattern is restored in double mutant, even though mutant proteins are in the same linear pathway. (B) Suppression may be apparent under somte stimulus conditions, but not others

Figure 8. Ectopic insertions



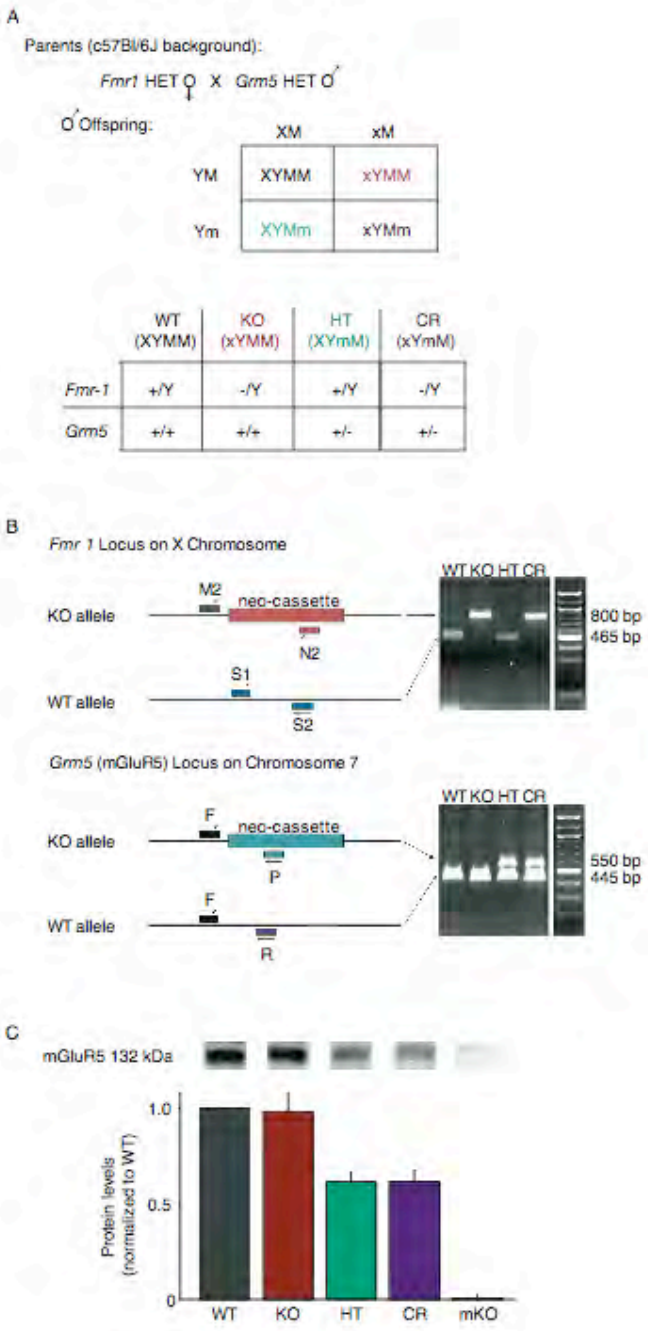
Ectopic insertion. (A) DNA is injected directly into the nucleus of a fertilized mouse oocuyte. Incorporation of transgenic DNA into genome occurs randomly. (B) Transgenic mice expressing GFP. (C) Random insertion allows for generation of different lines of mice with expression patterns determined by the regulatory environment of host genome at point of insertion. YFP H line expresses in layer 5 pyramidal neurons of the cortex

Figure 9. Gene Targeting



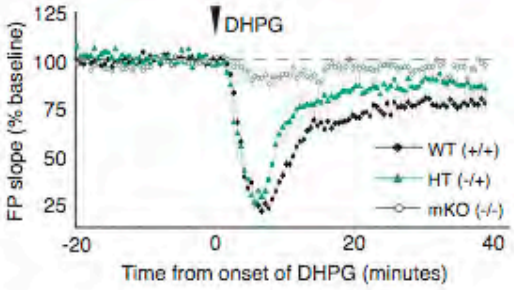
Gene targeting. (A) Gene of interest is cloned, neo and tk cassettes are inserted. Neo-cassette insertion disrupts the gene. (B) Targeting vector is added to cultured embryonic stem cells, incorporation into genome occurs in one of three ways. If homologous recombination occurs the neo cassette but not the tk cassette is inserted. (C) neomycin and gancyclovir are added to ES cell culture media, only cells with a neo cassette, but not a tk cassette insertion survive. (D) ES cells are injected into developing embryo. (E) Chimeric mice provide rapid detection of successful ES incorporation. (F) KO mouse is bred from chimeras.

Figure 10. Generating the Cross



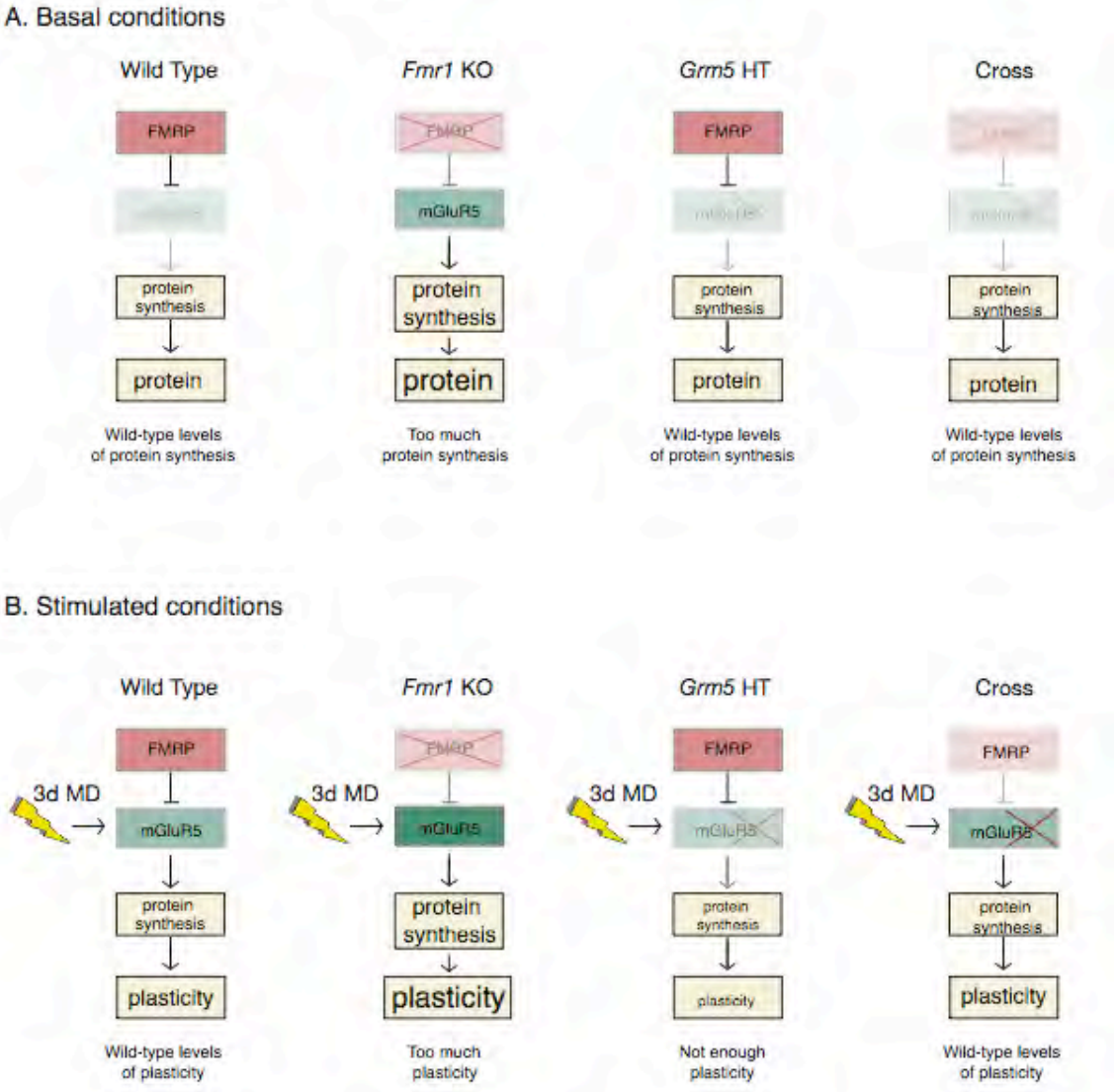
Generation *Fmr1* KO mice with a selective reduction of *Grm5* gene dosage. (**A**) *Fmr1* heterozygous females were crossed with *Grm5* heterozygous males to generate littermate male offspring of four genotypes: wild type, WT (black); *Fmr1* KO, KO (red); *Grm5* HET, HT (green); and *Fmr1* KO/*Grm5* HET, CR (purple). (**B**) Genotyping strategy. Screening for the presence or absence of the wild-type allele on the *Fmr1* locus on the X chromosome was performed using primer S1 (forward) and S2 (reverse) and the knockout allele using primer M2 (forward) and N2 (reverse). [*Fmr1* WT: 465 BP (S1/S2); *Fmr1* KO: 800 BP (M2/N2)]. Screening for the presence or absence of the knockout allele on the *Grm5* locus of chromosome 7 was performed using primer P (reverse) and F (forward) and the wild-type allele using F (forward) and R (reverse). [*Grm5* WT: 445 BP (F/R); *Grm5* KO: 550 BP (F/P)]. Representative gel for mice of four genotypes is shown; WT mice show a single band (465 bp) on the *Fmr1* gel and a single band (445 bp) on the *Grm5* gel; KO mice show a single band (800 bp) on the *Fmr1* gel and a single band (445 bp) on the *Grm5* gel; HT mice show a single band (465 bp) on the *Fmr1* gel and a double band (445 bp and 550 bp) on the *Grm5* gel; CR mice show a single band (800 bp) on the *Fmr1* gel and a double band (445 bp and 550 bp) on the *Grm5* gel. **(C)** Western blot analysis of total mGluR5 expression in visual cortical homogenates from P30 normalized to WT (+/+, +/Y) mice, KO (+/+, -/Y), HT (+/-, +/Y), and CR (+/-, -/Y) (n = 11 per genotype, ANOVA $P < 0.0001$). *Grm5* KO (-/-, +/Y) control sample was run 4 times (n = 1 animal) and normalized to age matched (postnatal day 45) WT. Representative blot is shown above.

Figure 11. LTD in WT, *Grm5* HT and *Grm5* KO (mKO) mice



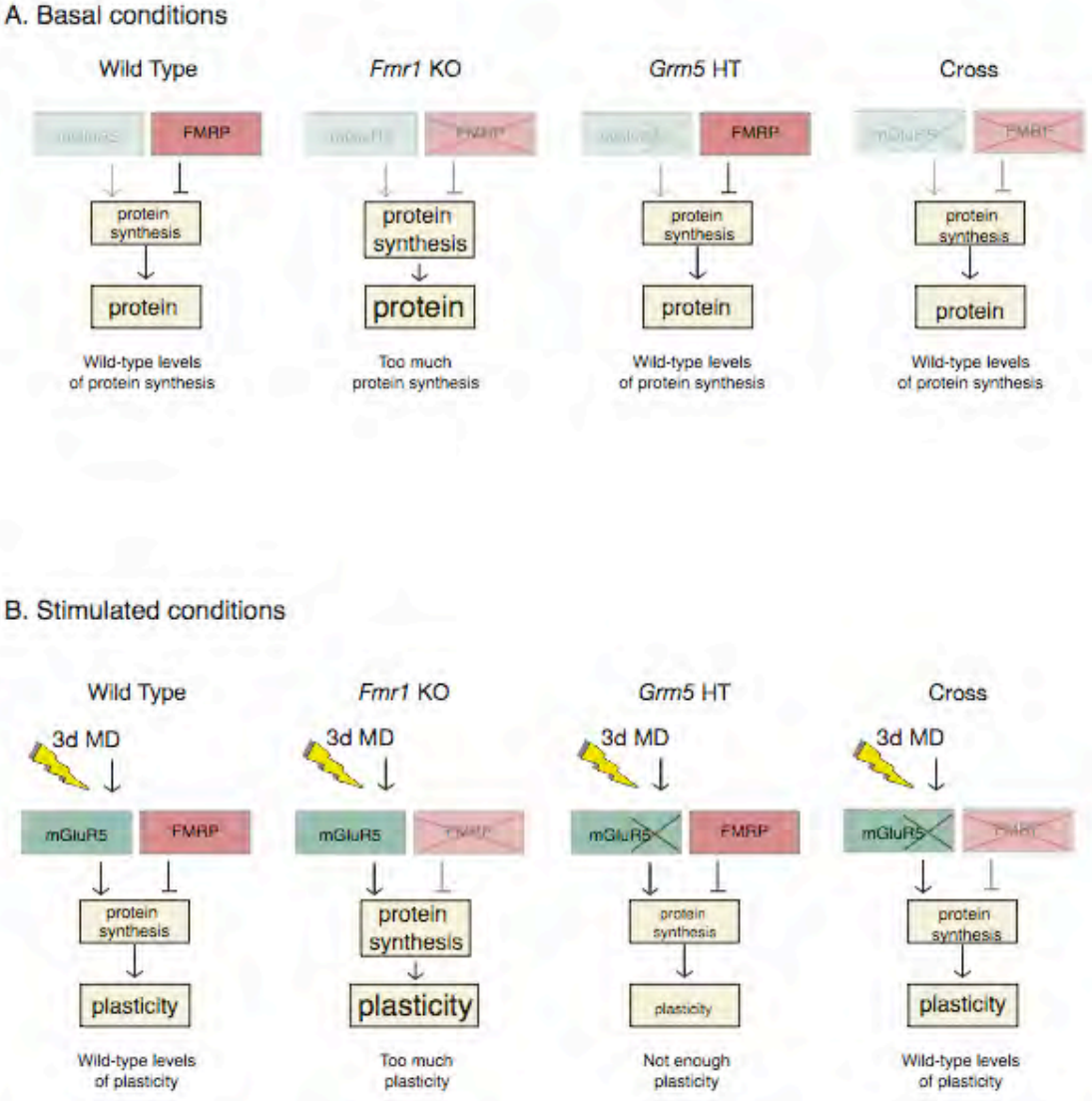
Effect of mGluR5 gene dosage on protein synthesis-dependent mGluR-LTD. These data re-plotted from (*61*). LTD induced by 50 μM, DHPG ((RS)-3,5-dihydroxyphenylglycine; 5 min) is absent in *Grm5* KO and reduced by 50% in *Grm5* HT (-/+) compared to WT (+/+) (n = 9 WT, n = 8 KO, n = 6 HT, slices from 5 animals per genotype). Repeated measures ANOVA, compacted variable: time, 40 minutes after DHPG application; global genotype $P < 0.0001$, genotype*time $P < 0.0001$; WT:HT genotype $P = 0.0400$, genotype*time $P < 0.0001$; WT:KO genotype $P < 0.0001$, genotype*time $P < 0.0001$; HT:KO genotype $P = 0.0017$, genotype*time $P < 0.0001$).

Figure 12. Suppression by epistasis



Interpretation of genetic interaction between Fmr1 and Grm5: epistasis. (A) Under basal conditions the cross mice exhibit WT phenotypes since a 50% reduction in mGluR5 exactly counterbalances the absence of negative regulation by FMRP. (B) 3 days of MD; this manipulation stimulates mGluR5, and the hypoplasticity phenotype in the HT mice is revealed under these conditions. WT phenotype is restored to the CR since the remaining mGluRs are sufficient to drive plasticity when they are not inhibited by FMRP

Figure 13. Bypass Suppression



Interpretation of genetic interaction between Fmr1 and Grm5: Bypass. (A) Under basal conditions wild-type phenotype is restored since mGluR5 and FMRP converge on a final common pathway; under these conditions mGluRs are minimally activated so Grm5 HT does not express a phenotype. (B) the Grm5 HT phenotype is revealed by stimulating mGluRs with 3d MD, but once again rescued in the cross.

Chapter 1 References

1. J. Martin, B. J, *J Neurol Neurosurg Psychiatry* **6**, 154 (1943).
2. R. Lehrke, *Am J Ment Defic* **76**, 611 (May, 1972).
3. H. A. Lubs, *Am J Hum Genet* **21**, 231 (May, 1969).
4. B. W. Richards, P. E. Sylvester, C. Brooker, *J Ment Defic Res* **25 Pt 4**, 253 (Dec, 1981).
5. G. R. Sutherland, *Science* **197**, 265 (Jul 15, 1977).
6. D. T. Burke, G. F. Carle, M. V. Olson, *Science* **236**, 806 (May 15, 1987).
7. A. J. Verkerk *et al.*, *Cell* **65**, 905 (May 31, 1991).
8. D. L. Nelson *et al.*, *Proc Natl Acad Sci U S A* **88**, 6157 (Jul 15, 1991).
9. S. T. Warren, F. Zhang, G. R. Licameli, J. F. Peters, *Science* **237**, 420 (Jul 24, 1987).
10. B. A. Oostra *et al.*, *Genomics* **6**, 129 (Jan, 1990).
11. D. Heitz *et al.*, *Science* **251**, 1236 (Mar 8, 1991).
12. M. Pieretti *et al.*, *Cell* **66**, 817 (Aug 23, 1991).
13. Y. H. Fu *et al.*, *Cell* **67**, 1047 (Dec 20, 1991).
14. W. Griffiths, Lewontin, Gelbart, Suzuki, Miller, *Introduction to genetic analysis* (W. H. Freeman and Company, New York, ed. 8th, 2005), pp.
15. L. Avery, S. Wasserman, *Trends Genet* **8**, 312 (Sep, 1992).
16. J. Hodgkin, in *WormBook* P. Anderson, Ed. (the C. elegans research community, http://www.wormbook.org, 2005).
17. J. W. Gordon, G. A. Scangos, D. J. Plotkin, J. A. Barbosa, F. H. Ruddle, *Proc Natl Acad Sci U S A* **77**, 7380 (Dec, 1980).
18. G. Feng *et al.*, *Neuron* **28**, 41 (Oct, 2000).
19. S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (Nov 24, 1988).
20. T. D.-B. F. X. Consortium, *Cell* **78**, 23 (Jul 15, 1994).
21. E. J. Mientjes *et al.*, *Hum Mol Genet* **13**, 1291 (Jul 1, 2004).
22. C. J. Bontekoe *et al.*, *Hum Mol Genet* **11**, 487 (Mar 1, 2002).
23. Y. M. Lu *et al.*, *J Neurosci* **17**, 5196 (Jul 1, 1997).
24. C. Verheij *et al.*, *Nature* **363**, 722 (Jun 24, 1993).
25. C. T. Ashley, Jr., K. D. Wilkinson, D. Reines, S. T. Warren, *Science* **262**, 563 (Oct 22, 1993).
26. Y. Feng *et al.*, *Mol Cell* **1**, 109 (Dec, 1997).
27. K. A. Lugenbeel, A. M. Peier, N. L. Carson, A. E. Chudley, D. L. Nelson, *Nat Genet* **10**, 483 (Aug, 1995).
28. H. L. Hinds *et al.*, *Nat Genet* **3**, 36 (Jan, 1993).
29. R. F. Kooy *et al.*, *Am J Med Genet* **64**, 241 (Aug 9, 1996).
30. E. J. Mientjes *et al.*, *Neurobiol Dis* **21**, 549 (Mar, 2006).
31. D. Van Dam *et al.*, *Behav Brain Res* **117**, 127 (Dec 20, 2000).
32. Y. Q. Zhang *et al.*, *Mol Cell Proteomics* **4**, 278 (Mar, 2005).
33. T. C. Dockendorff *et al.*, *Neuron* **34**, 973 (Jun 13, 2002).
34. L. Wan, T. C. Dockendorff, T. A. Jongens, G. Dreyfuss, *Mol Cell Biol* **20**, 8536 (Nov, 2000).
35. B. Tucker, R. I. Richards, M. Lardelli, *Hum Mol Genet* **15**, 3446 (Dec 1, 2006).
36. G. Dolen, M. F. Bear, *Neuron* **45**, 642 (Mar 3, 2005).

37. L. L. Kirkpatrick, K. A. McIlwain, D. L. Nelson, *Genomics* **78**, 169 (Dec, 2001).
38. Y. Q. Zhang *et al.*, *Cell* **107**, 591 (Nov 30, 2001).
39. C. M. Spencer *et al.*, *Hum Mol Genet* **15**, 1984 (Jun 15, 2006).
40. T. K. Bjarnadottir, R. Fredriksson, H. B. Schioth, *Gene* **362**, 70 (Dec 5, 2005).
41. T. K. Bjarnadottir, H. B. Schioth, R. Fredriksson, *Ann N Y Acad Sci* **1040**, 230 (Apr, 2005).
42. P. J. Conn, J. P. Pin, *Annu Rev Pharmacol Toxicol* **37**, 205 (1997).
43. S. M. McBride *et al.*, *Neuron* **45**, 753 (Mar 3, 2005).
44. R. Lu *et al.*, *Proc Natl Acad Sci U S A* **101**, 15201 (Oct 19, 2004).
45. L. Hou *et al.*, *Neuron* **51**, 441 (Aug 17, 2006).
46. J. H. Lim, A. B. Booker, J. R. Fallon, *J Cell Physiol* **205**, 170 (Nov, 2005).
47. A. A. Khazaeli, M. Tatar, S. D. Pletcher, J. W. Curtsinger, *J Gerontol A Biol Sci Med Sci* **52**, B48 (Jan, 1997).
48. K. Watase, H. Y. Zoghbi, *Nat Rev Genet* **4**, 296 (Apr, 2003).
49. R. J. Hagerman, P. J. Hagerman, *Fragile X Syndrome: Diagnosis, Treatment, and Research* (The Johns Hopkins University Press, ed. 3 edition, 2002), pp. 552.
50. J. Larson, R. E. Jessen, D. Kim, A. K. Fine, J. du Hoffmann, *J Neurosci* **25**, 9460 (Oct 12, 2005).
51. R. J. Hagerman, M. Y. Ono, P. J. Hagerman, *Curr Opin Psychiatry* **18**, 490 (Sep, 2005).
52. J. Spacek, M. Hartmann, *Anat Embryol (Berl)* **167**, 289 (1983).
53. L. Pan, Y. Q. Zhang, E. Woodruff, K. Broadie, *Curr Biol* **14**, 1863 (Oct 26, 2004).
54. C. Margulies, T. Tully, J. Dubnau, *Curr Biol* **15**, R700 (Sep 6, 2005).
55. B. J. Wiltgen, R. A. Brown, L. E. Talton, A. J. Silva, *Neuron* **44**, 101 (Sep 30, 2004).
56. M. Bernardet, W. E. Crusio, *ScientificWorldJournal* **6**, 1164 (2006).
57. M. Masu, Y. Tanabe, K. Tsuchida, R. Shigemoto, S. Nakanishi, *Nature* **349**, 760 (Feb 28, 1991).
58. K. M. Houamed *et al.*, *Science* **252**, 1318 (May 31, 1991).
59. M. J. Bradbury *et al.*, *J Pharmacol Exp Ther* **313**, 395 (Apr, 2005).
60. C. Chiamulera *et al.*, *Nat Neurosci* **4**, 873 (Sep, 2001).
61. K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **86**, 321 (Jul, 2001).
62. A. J. Hannan *et al.*, *Nat Neurosci* **4**, 282 (Mar, 2001).
63. L. J. Miller *et al.*, *Am J Med Genet* **83**, 268 (Apr 2, 1999).
64. Z. Jia *et al.*, *Learn Mem* **5**, 331 (Sep-Oct, 1998).
65. J. Brodkin *et al.*, *Eur J Neurosci* **16**, 2241 (Dec, 2002).
66. S. A. Brody, M. A. Geyer, *Neurotox Res* **6**, 79 (2004).

Chapter 2: FMRP and mGluR5

Introduction

Transcriptional silencing of the FMR1 gene leads to the absence of the fragile X mental retardation protein (FMRP) in the majority of patients with the disease. Insight into the function of this protein is therefore a major goal for understanding the disease. Increasing evidence suggests that this protein is involved in the regulation of protein synthesis. Metabotropic glutamate receptors have also been implicated in protein synthesis regulation. As we will see below, there is now substantial evidence that these two proteins regulate translation in a reciprocal fashion, whereby FMRP serves as the "brake" and group I mGluRs serves as the "gas." Furthermore, here we provide evidence that genetic interaction between these proteins can rescue the exaggerated protein synthesis phenotype seen in the *Fmr1* KO model of the disease.

The Fragile X mental retardation protein (FMRP)

Transcriptional regulation

The *FMR1* gene, with its 17 exons, spans 38kB at Xq27.3 (see **Figure 1A**)(*1*). Because of alternative splicing of the precursor mRNA, this gene encodes as many as 12 partially different proteins, that share the same N- and C- terminus, but differ in various internal segments (*2-4*); in the mouse 10 splice variants have been detected (*5*). Collectively these various isoforms are called FMRP (fragile X mental retardation protein), and often appear on Western Blots as multiple bands with molecular weights ranging from 67 to 80 kDA (*2, 6*). The FMRP isoforms do not seem to show differential tissue specificity, and the functional significance of the splice variants is not known (*3*).

The expression of *FMR1* is both temporally and spatially regulated. As shown in Figure 1B, FMR1 mRNAs are expressed in many types of proliferating and migrating cells, the retina, and non-nervous tissues of the developing human embryo (shown at 9 weeks, **Figure 1B** left)(*7, 8*), but by adulthood, mRNA and protein expression is largely restricted to cortical, cerebellar, and testicular regions, with lower expression in kidney and lung, and absent in heart, liver and skeletal muscles (*6, 9*) (see **Figure 1B**, right). A similar developmentally defined expression pattern is found in the mouse (*9*), where mRNA is detectable by embryonic day 10 (E10), peaks at E13, and progressively declines thereafter (**Figure 1C**, top). Later in development, expression is highest in the brain, testes, ovaries, thymus, esophagus and spleen, with only moderate expression in kidney, liver, colon, uterus, thyroid and lung, and none in muscle heart or aorta (see **Figure 1C**, bottom) (*9*). Expression of FMRP continues to decline over development (see **Figure 1C**, right) (*10*).

This temporally and regionally restricted expression is likely to be coordinated by regulation of *FMR1* transcription. As shown in **Figure 2A,** the promoter region of the *FMR1* gene contains a transcription initiation site (TIS) and binding sites for at least 4 transcription factors (a-Pal/Nrf1, SP1, AP-2, and USF1/2) (*11*). One of these transcription factors, AP-2, has been studied extensively *in vivo*, and has been shown to regulate *Fmr1* expression during early development in both mice an *Xenopus* (*12, 13*).

The AP-2 family of transcription factors consists of 5 isoforms which show overlapping but distinct expression patterns; of these AP-2a is of special interest since it has been shown to play a role in neural development (*14-16*). As shown in **Figure 2B**, *Fmr1* is also expressed during *Xenopus* development (*13*); furthermore AP-2a and *Fmr1* transcripts are co-localized during early development in *Xenopus* (as shown in **Figure 2B**), and dominant-negative AP-2a represses *Fmr1* transcription in embryonic tissue in this species (*12*). Because AP-2a KO mice die at birth, these authors generated conditional AP-2a KO's to study this regulatory role in mice; when crossed with *Six3* Cre recombinase lines, the mutation is restricted to the eyes of embryonic and adult mice. When conditional AP-2a KO animals were examined for *Fmr1* transcript across two developmental time points, an interesting pattern emerged. Specifically, in these mice *Fmr1* transcripts were reduced in embryonic but not in adult retina (as expected, normal levels of transcript were seen in WT and leg tissue where AP-2a was not deleted) (See **Figure 2C**) (*12*). These results suggest that *Fmr1* expression is regulated by the AP-2a transcription factor during development, but not adulthood, which could at least in part explain the expression patterns of *Fmr1* described above. Furthermore, because *Fmr1* is differentially regulated during embryogenesis than later in life, these results suggest the

possibility that the function of the protein also changes across development (see below for more).

While regulation gene transcription by proteins that associate with the promoter is the canonical method of gene regulation, covalent modification of the DNA itself is also possible. Of special interest for FXS is transcriptional inactivation by methylation. Because methylation is found primarily on transcriptionally silent regions of the genome (inactive chromosomes or genes that are inactivated in certain tissues), it is thought that this modification plays a role in gene silencing. A detailed description of this process will not be attempted here, but briefly it involves the addition of a methyl group (-CH3) to cytosine nucleotides (particularly when found in a CG sequence). A family of proteins that bind methylated DNA interacts with chromatin remodeling complexes and histone deacetylases (HDAC) that condense chromatin so it becomes transcriptionally inactive.

As noted earlier, in the majority of patients with FXS, FMRP is absent because DNA methylation of the CGG repeats in the 5' untranslated region of *FMR1* turns off the gene (*17, 18*). Normal individuals have CGG sequence repeats that are limited to within 5-50 repeats with no functional change in FMRP expression. Pre-mutation carriers have an expanded repeat size of between 50-200, but these are unmethylated and transcript levels are not decreased (indeed they may by elevated); permutation carriers are largely unaffected (although there is growing evidence for premature ovarian failure and ataxia, which is thought to be related to dominant negative effects of the transcript) (*19*). Affected individuals have CGG repeats in excess of 200 and feature methylation-dependent repression of the FMR1 gene (*18*). At the transcriptional level, *FMR1* gene activity is associated with the acetylation of histones H3 and H4 in cells from normal

individuals, but is significantly reduced in FXS, suggesting the assembly of a repressive chromatin structure on the *FMR1* gene. Moreover, in cells derived from FXS patients, treatment with inhibitors of DNA methylation and HDAC can remodel chromatin and increase the transcriptional competence of the gene (*17, 20*). These findings suggest exciting new therapeutic strategies for the treatment of FXS.

FMRP as a repressor translation

Although, as mentioned above, most patients with the disease are missing FMRP because the *FMR1* gene is transcriptionally silenced, in one particularly severe case, the disease is caused by a *de novo* mutation in the I304N site of the gene (*21*). This site codes for the RNA-binding domain of the protein (KH2, see below); the severity of the phenotype in the patient carrying this mutation suggests that RNA-binding is critical to the biological function of FMRP (*22*). As shown in Figure 1A, FMRP contains three RNA binding domains: an RGG box and two K- Homology (KH) domains (*4*). In addition, these studies, as well as others (*23*), suggest that FMRP regulates nearly 4% of human fetal brain mRNA, including its own transcript. With the discovery of these binding domains, several labs have attempted characterize mRNA targets of FMRP, in the hope of determining which mRNAs are misregulated in FXS (*23-25*). However the value of this approach depends on the functional significance of the binding interaction (*26*). For example, recent studies show that the association between FMRP and translating polyribosomes (see below) depends on the KH domain, but not the RGG box (*22, 26, 27*). Thus targets identified by RGG box binding (*24*) must be validated by other methods (*26*).

FMRP is cytoplasmic (*2, 6*) and predominantly expressed in neurons, but not glia in

postnatal tissue ((*28*) but see, (*29*)). In order to understand its role in translational regulation, several studies have examined its association with the sub-cellular translation machinery (ribosomes, polyribosomes, granules) using sucrose density gradients (*5, 22-24, 28, 30-34*). Translating polyribosomes have been known to be localized to the base of dendritic spines for over 20 years (see **Figure 3A**) suggesting the possibility that local protein synthesis can regulate synaptic function (*35*). Localization of FMRP to polyribosomes therefore suggests a putative mechanism for the misregulation of synapses in FXS.

Interpretation of sucrose gradient fractionation results has been controversial (*31*), in part because fractionation profiles are sensitive to extract preparation (*34*) and in part because the functional significance of the granule peak is not limited to regulation of translation (*36*). For example, several studies have shown that FMRP co-sediments with polyribosomes and that this co-sedimentation is lost with I304N mutation, consistent with the idea that FMRP regulates protein synthesis by binding to actively translating mRNAs (*5, 22, 23, 28, 30, 34, 37-39*) (see **Figure 3B**). In contrast, other studies have suggested an alternate mechanism whereby FMRP interacts with BC1 (a non-translatable RNA) to repress translation indirectly at the *initiation* step; this BC1-FMRP complex associates instead with monomeric 80s ribosomes and with mRNPs in the lighter fractions (and not polyribosomes) ((*40*), see also (*41*)). However later studies have shown that the reported lack of polyribosome co-sedimentation is due to detergent artifacts, calling into question this alternate mechanism for translational regulation (*27, 34*).

FMRP has also been co-localized with high-density granules (*30*), which may represent large ribosomal aggregates whose translation has been stalled (**Figure 3C**) (*36*).

While this study additionally reports a smaller granule peak in the *Fmr1* KO, it must be noted that no other study, under any treatment condition, has reported either the presence of this peak or its association with FMRP (*5, 22-24, 28, 31-34, 39*) (See **Figure 3B**). The polyribosome profile reported by Aschrafi, et al., lacks the normal peaks and valleys associated with a clean fractionation, and the FMRP signal (by Western blotting) is weak. The interpretation of co-fractionation is further confounded by disagreement between the fractionation profile and protein levels (for example, the S20 protein is not expressed in the 40S peak, suggesting misalignment and calling into question the association between FMRP and the gradient fraction). Even setting aside methodological concerns, this sort of analysis is at best an indirect measure of translational regulation, since conclusions are based on association between FMRP and the granules, and inferences about the functional consequences of such association.

A number of studies now suggest that FMRP acts as a negative regulator of translation (*23, 30, 40-51*). As mentioned above, some of this evidence comes from mRNA binding studies (*23, 40, 50*) as well as fractionation profiles (*30*) in *Fmr1* KO mice. In addition, a number of studies have shown increased expression of specific proteins in *Fmr1*/*dFmr1* KO animals (*42, 44, 48, 50, 51*). However, it should be noted that other studies have reported either no change in protein expression (*52, 53*) or decreases in protein expression (*25, 54-56*). Further support for the idea that FMRP acts to suppress protein synthesis comes from studies that have examined mGluR-LTD which have shown that in *Fmr1* KO mice this translation dependent process is exaggerated and persists in the presence of protein synthesis inhibitors (*43, 46-48*). While many of these studies provide indirect support for the role of FMRP as a repressor of translation, they

cannot distinguish between direct changes due to loss of FMRP dependent protein synthesis regulation and compensatory homeostatic changes independent of translational regulation.

Three studies have examined the role of FMRP in regulating protein synthesis directly, two *in vitro* (*41, 45*) and one *in vivo* (*49*); in all three cases the results suggest that FMRP acts to inhibit translation (**Figure 4**). Each of these studies will be described briefly:

Li, et. al., examined the effect of recombinant FMRP on translation in rabbit reticulocyte lysate (RRL). This *in vitro* system for measuring translation involves the addition of Tmethionine to mRNA, and exposing the mixture to varying amounts of FMRP (3 minutes) after which translation is initiated by addition of RRL; the amount of translation is then assayed by TCA precipitation of protein products and measurement (by scintillation counting) of radioactivity (see **Figure 4B**, right). Using this system, these authors show FMRP dose-dependent suppression of translation of brain RNA (from cerebral cortex of P 60-90 rat), but not reticulocyte RNA (from rabbit blood) (see **Figure 4B**, left), suggesting that the regulation of protein synthesis is specific to FMRP binding partners, rather than a general interference with the translation machinery (*45*). Furthermore, this suppressive effect was abolished by competitive inhibition of FMRP, and when FMRP-binding sequences were removed from mRNA transcripts, providing further evidence for the specificity of the interaction between FMRP and the template mRNA. Finally, this effect was shown to be due specifically to translation suppression, rather than increased mRNA degradation, since exposure of mRNA's to FMRP in the absence of the translation machinery (from RRL), produced no decrease in template

levels (assayed by Northern Blot), nor was there a decrease in mRNA after translation in FMRP treated versus non-treated controls.

Using the same RRL assay as well as microinjected *Xenopus* oocytes, Laggerbauer, et al., have shown a similar negative regulatory role for FMRP (*41*). In addition, these authors have shown that repression of translation is lost in FMRP with the I304N mutation in both systems, consistent with the idea that this domain confers translation regulatory function to the protein. Using the sucrose fractionation method described above, these authors show that FMRP blocks the formation of the 80s ribosomal RNA, and the I304N mutation prevents this block on translation initiation; although these authors did not collect polyribosome fractions, this finding is nevertheless difficult to reconcile with earlier studies that show FMRP/polyribosome co-fractionation (*5, 22, 23, 28, 30, 34, 37-39*), but consistent with the idea that FMRP is involved in regulating translation initiation (*40*). Oddly, this mutation did not alter the mRNA binding ability of FMRP, which is surprising given its location within the KH2 mRNA binding domain (*22*), but consistent with earlier reports that mRNA binding of I304N mutant FMRP is only altered in high salt concentrations (*52, 57*). Furthermore, both normal FMRP and I304N mutant FMRP shifted mRNA to a higher molecular mass in a gel mobility shift assay (another measure of mRNA binding -- protein bound mRNAs move more slowly through the gel). Instead these authors report that I304N mutant FMRP is unable to oligomerize to itself (binding to normal FMRP, FXR1, and FXR2 is unaltered). It is still unclear how this homo-oligomerization defect relates to loss of regulatory function.

Finally, Qin, et al., have examined a protein synthesis regulatory role for FMRP *in vivo*. WT and *Fmr1*KO mice were catheterized and injected with an intravenous pulse of

radioactive leucine (100 Ci/kg L-[1- 14C]leucine). In order to control for plasma clearing of the label, several arterial blood samples were collected over the course of an hour, following which, mice were sacrificed, brains removed, and sectioned. Autoradiograms of radioactively labeled sections were quantified by brain region and compared across two developmental ages and genotypes. In both genotypes, the authors report an age dependent decrease in the amount of protein synthesis across brain regions. In addition, in a number of brain regions, but especially the hippocampus (See **Figure 4C**), the authors report an increase in protein synthesis in the *Fmr1* KO versus WT brain.

Taken together, these findings are largely in agreement that FMRP functions as a negative regulator of translation. The mechanism of this translational regulation has been more controversial. Several possibilities, described above, are under current investigation, including direct binding to messenger RNA, indirect binding and regulation of translation initiation (via BC1), or association with the granule fraction. In addition, recent studies in *drosophila* have implicated the microRNA and siRNA pathways in *dfmr* mediated regulation of mRNA degradation, translation inhibition, and/or chromatin silencing (*58-64*); however, a detailed description of these putative mechanisms will not be attempted here. As we will see below, the Gp 1 mGluR signaling pathway has also been implicated in protein synthesis.

The cargo-hypothesis

Some have suggested that rather than acting as a regulator of protein synthesis, FMRP instead acts as a shuttle protein, transporting its cargo from one part of the cell to the other (*65*). This, so called "cargo-hypothesis," proposes that the regulatory function of FMRP is to select target mRNA from a large pool of mRNAs in the nucleus and

transport them down dendrites to the spines where they are translated. It is largely based on the following observations: that the I304N mutant FMRP shuttles more rapidly between the nucleus and the cytoplasm (*38*); that FMRP and its mRNA are found both in the soma and the dendrite and that this localization is protein synthesis independent (*66*); that in the absence of FMRP, mGluR5 activation does not lead to protein synthesis (*67*); and finally that FMRP, through its interaction with the messenger ribonucleoprotein (mRNP) complex (*40*), associates with proteins involved in transport (*25, 65*). Consistent with this idea, these authors have recently shown that the FMRP-RNP particle contains several cargo proteins (i.e. alphaCaMKII and BC1 RNAs as well as Staufen and CPEB) and that following mGluR activation, the FMRP-mRNP complex moves into spines (*68*). Because this complex has been proposed to be represent stalled translation, these results suggest that mGluR5 activation leads to repression of translation rather than its stimulation (see below for discussion) and that previously reported stimulation of protein synthesis by DHPG (*69*), is due to de novo protein synthesis at the synapse, independent of regulation by FMRP. This interpretation relies heavily on the functional significance of co-sedimentation of FMRP with the mRNP, which as mentioned above, has been controversial. Somewhat consistent with the shuttling function of FMRP are the findings of overexpression of cytoskelatal proteins in KO models, including MAP1b (and its orthologue Futsch), actin, tubulin, tropomyosin and profilin (an actin binding protein) (*42, 50, 51*). However, it must be re-emphasized that these findings cannot distinguish between proximal and distal consequences of loss of FMRP, and may instead reflect compensatory changes due to, for example, increased synapse turnover.

Metabotropic glutamate receptors (mGluRs)

Signal transduction

Glutamate (or glutamic acid) is the most abundant excitatory neurotransmitter in the mammalian nervous system. Glutamate that is released by the presynaptic cell is detected postsynaptically by binding of the transmitter to one of two types of receptors: ionotropic and metabotropic receptors. The ionotropic glutamate receptors (iGluRs) include alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and N-Methyl-D-Aspartate (NMDA) receptors (see **Figure 5**). By definition, ionotropic receptors are transmitter-gated ion channels that couple receptor binding to direct (and consequently rapid) changes membrane potential. In contrast, metabotropic glutamate receptors (mGluR's) (also called heterotrimeric GTP-binding protein linked glutamate receptors) do not have an integral ion channel, and mediate their effects by biochemical cascades. The discovery of this type of modulatory transmission (*70*) at glutamatergic synapses was remarkable because it implied that glutamate could *modulate* activity at the same synapses at which it elicits fast excitatory transmission (through iGluRs). This modulatory role had previously been thought to be the exclusive domain of the monoamines (dopamine, acetylcholine, serotonin, and norepinephrine) and other neuromodulators, released from non-glutamatergic afferents. Because of their near ubiquitous localization at CNS synapses, it is perhaps not surprising that activation of mGluRs has an extremely diverse range of electrophysiological effects, including: inhibition of potassium and calcium currents; activation of potassium, calcium, and non-specific cation currents; mediation of slow excitatory postsynaptic potentials; presynaptic inhibition of transmitter release; potentiation of AMPAR and NMDAR synaptic

responses; and generation of oscillatory and epileptiform activity. In addition to being activated by synaptic glutamate, they have also been found to be responsive to increases in extracellular calcium. This diversity of electrophysiological effects cannot be covered here (see (*71-74*) for full review), but specific aspects will be discussed as they are relevant.

Cloning of the mGluR molecule (*75, 76*) brought further insight into the structure and function of these receptors. These and subsequent studies have revealed 8 variants of the mGluR in mammals, categorized into three groups: Group I, II and III (based on their sequence homology, agonist and antagonist pharmacology, and coupling to signal transduction pathways) (See **Figure 5**) (*73*); for the purposes of the current discussion, special attention will be given to Group I mGluRs, especially mGluR5. The human homologues of mGluRs, including mGluR5 (*77*), have been cloned and show 93-96% sequence homology with rodents (*73*). Note that the *drosophila* orthologue, DmGluRA, is most closely related to group II mGluRs (showing 43% sequence homology with mGluR3) (*78*). Two splice variants of mGluR5 have been identified (mGluR5a and mGluR5b, **Figure 5**); although some have noted differential developmental expression patterns (*79, 80*), the functional significance of the variants is yet to be elucidated since they appear, at least in heterologous expression systems, to couple to the same signal transduction cascades (*81*).

Group I mGluRs (GpI mGluRs), which are further subdivided into mGlur1 and mGluR5 subtypes, couple to Gq-like G-proteins and signal through activation of phospholipase C (PLC) (see below). In contrast, both Group II and III mGluRs couple to Gi-like G-proteins and signal through adenylate cyclase (*73*). Groups II and III are mostly

presynaptically localized, and primarily modulate neurotransmitter release. In contrast, Gp1 mGluRs are primarily postsynaptic (see Figure 5A) (*82, 83*). However, there is at least one example of presynaptically mediated GpI mGluR activity worthy of note. In the adult hippocampal area CA1, activation of GpI mGluRs can reduce both GABA and glutamate release at these synapses (*82, 84-86*). These presynaptic effects have now been shown to occur through retrograde endogenous endocannabinoid mediated signaling (coupled to mGluRs via diacylglycerol (DAG) production (see below) which is a direct precursor of 2-arachidonylglycerol (2-AG), the endogenous endocannabinoid operating in the hippocampus) (*87-89*).

The canonical Gq signaling cascade (shown in **Figure 6A**) involves activation of phospholipase C (PLC). Activated PLC converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into two second messengers : DAG and inositol-1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC); IP_3 binds to calcium channel receptors on the smooth ER and other membrane-enclosed organelles in the cell, causing the organelles release their stores of Ca^{2+}. It is important to remember that many of these mechanisms were derived from heterologous receptor expression studies, and that in neurons the coupling to signaling cascades may be more complicated (*74*). For example, PKC and PKA inhibitors as well as postsynaptic calcium chelators fail to block DHPG-LTD (*90-92*). Because DHPG is a selective mGluR5 agonist (and DHPG-LTD is absent in *Grm5* KO mice) (*93*), these results suggest that mGluR5 must also couple to other transduction cascades. For example, the GpI mGluRs have been implicated in the activation of a variety of intracellular molecules including those belonging to the tyrosine kinase (*94-97*) and to the mitogen-activated protein kinase (MAPK) signal transduction cascades (*98-*

112). Indeed, DHPG-LTD at the CA1 synapse has now been shown to be dependent on ERK, a member of the MAPK signaling pathway, and both DHPG-LTD and the MAPK pathway have been linked to protein synthesis (see below for more) (shown in **Figure 6B**) (*98, 106, 113, 114*).

As shown in **Figure 5**, postsynaptic mGluRs do not exist in isolation at the synapse. They are tethered to the post-synaptic density (PSD) by their interaction with the Homer and Shank family of scaffolding proteins, which crosslink them to PSD-95 and NMDA's (*115*). At the synapse, mGluR5 is concentrated in an annulus around the postsynaptic density, but it also exists extrasynaptically (*83*). Activation of mGluR5 with DHPG has been shown increase mGluR5 diffusion into this extrasynaptic pool, whereas the addition of Homer 1b decreases receptor mobility, and implicates these scaffolding proteins in the dynamic regulation of the composition of the synapse (*116*). The Homer 1b/c proteins have also been shown to link mGluRs to the ERK signaling pathway (*117*). Studies from many labs now provide compelling evidence that the trafficking and recycling of glutamate receptors at the synapse mediates synaptic plasticity (*118*). For example, one study from our own lab has shown that the expression mechanism of mGluR LTD is the internalization of AMPA and NMDA receptors (*119*). Finally, as mentioned above, the *Grm5* KO mouse shows no DHPG-LTD, as expected (*93*), but in addition shows deficits in NMDA receptor dependent forms of LTP, despite normal expression of AMPA and NMDA receptors (*120, 121*), consistent with the idea that mGluRs can also modulate NMDA receptor function (*122*).

Developmental regulation of mGluRs

Metabotropic glutamate receptor signaling, like FMRP, is regionally and developmentally regulated (*80, 82, 123-130*). As shown in **Figure 7A** (left), mGluR5 is expressed widely in the cortex, hippocampus and striatum, but noticeably absent from the cerebellum (mGluR1 shows complementary expression, with high levels in the cerebellum but almost none in the cortex, not shown)(*131*). In addition, both mGluR1 and mGluR5 are expressed in the testicles (mGluR5 expression is shown in **Figure 7A** right) (*127*). The expression of mGluR5 protein is developmentally down-regulated (younger animals express more receptor protein than adults) (Western blots from rat shown in Figure 7B) (*123*) see also (*126*). Although earlier studies had found no evidence for receptor downregulation, they had shown developmental decreases in phosphoinositide (PI) turnover, consistent with decreased receptor function (*128, 129*). In addition, to this general decrease in receptor expression/function, a number studies have shown evidence for laminar redistribution of mGluR5 expression in the visual cortex across development, with the highest density of layer 4 expression corresponding to the height of the critical period (shown in **Figure 7C**) (*125, 130*), consistent with a role for this receptor in modulating cortical plasticity (*129*).

Interestingly, as mentioned above, the two mGluR5 splice variants (mGluR5a and mGluR5b) show differential developmental regulation (*79, 80*). Specifically, while mGluR5b is dramatically upregulated across development, the relative proportion of mGluR5a is dramatically downregulated; however, as the authors themselves point out, the mGluR5a interpretation should be viewed with caution, because in the absence of a specific antibody for this variant, these values are only estimates based on measured

values for mGluR5b and total mGluR5 protein expression (*80, 123*).

While studies in heterologous expression systems have shown identical pharmacologic and signal transduction profiles for these splice variants (*81*), the more recently identified ERK signaling pathways were not examined in these studies (incidentally, neither mGluR5 a nor b showed any response to MCPG, which is expected since this drug has been shown previously to have no effect on PI turnover (*132*)). Therefore future studies examining differential coupling to ERK mediated protein synthesis between these splice variants will be of significant interest. This is a particularly enticing possibility since recently it has been shown that the protein synthesis dependence of DHPG-LTD is developmentally regulated (neonatal LTD is protein synthesis independent, in contrast with DHPG-LTD in older animals) (*124*). These studies also indicate that DHPG-LTD in neonatal animals is presynaptically mediated (but not through the aforementioned endocannabinoid mechanism), so it will also be of interest to determine whether mGluR5 splice variants show differential pre or post-synaptic localization. Moreover, in the case that the splice variants are differentially coupled to protein synthesis, their relative distributions may be different in the *Fmr1* KO mouse compared to WT (despite normal levels of total mGluR5 protein, see Chapter 2).

Protein synthesis dependent function of Gp1 mGluRs

It is now clear that activation of Gp I mGluR's stimulates protein synthesis at the synapse (*133-135*). As noted above, the translational machinery required for protein synthesis is localized to the base of dendritic spines (the postsynaptic compartment of excitatory synapses). This arrangement allows independent modification of individual

synapses through the *local* synthesis of proteins and thereby confers a vast amplification in the computational capacity of the neuronal network. Moreover, it is thought that protein synthesis is a requirement for stable, enduring (on the order of days) modification of the synapse (*35, 136*). It is perhaps not surprising then, that activation of Gp I mGluR's can have lasting functional and even structural consequences; and that these consequences, when examined, have be found to be dependent on the translation of new proteins (*98, 106, 119, 124, 137-147*).

Specifically, mGluR5 dependent forms of long-term depression, LTD (*98, 106, 124, 137, 140, 144*), long-term potentiation, LTP (*143, 145*), late-phase LTP (L-LTP) (*139*) and synaptic depotentiation (*147*) are all protein synthesis dependent. In addition, application of DHPG to organotypic slice cultured neurons induces a protein synthesis dependent increase in the density of long thin spines (*146*). Because DHPG application in cell culture also induces rapid protein synthesis dependent internalization of AMPA and NMDA receptors (*119*), receptor internalization may be the preamble to morphologic remodeling and synapse elimination/recycling. Consistent with the involvement of these plasticity processes in learning and memory, behavioral tests indicate that some forms of memory consolidation are both mGluR5 and protein synthesis dependent (*138*). Finally, increases in epileptiform activity in response to mGluR5 stimulation are also protein synthesis dependent (*142, 148*), suggesting that in addition to synapse specific changes, circuit level modulation of excitability is sensitive to the state of mGluR5 dependent protein synthesis (*145*). This list represents a small subset of diverse range of known electrophysiological effects of mGluR5 receptor activation, and may be expanded as the protein synthesis dependence of these effects is examined. That being said, it is also

likely that not all effects of mGluR5 activation will be directly coupled to protein synthesis (*88, 149, 150*).

FMRP and mGluR5

The first evidence to suggest a link between FMRP and Gp I mGluRs came from the discovery that FMRP is translated near synapses in response to Gp I mGluR activation (*69*). In the same year, the first evidence emerged to suggest a role for FMRP in translational regulation of protein synthesis at the synapse (*22, 28*). Taken together, these data suggested the possibility that FMRP might be important for effecting the protein synthesis dependent downstream consequences of Gp I mGluR activation, and that in the absence of FMRP these consequences would be deficient or absent. One such protein synthesis dependent consequence of mGluR-5 activation is hippocampal long-term depression (LTD) (*99, 137, 151*). The finding from our lab that this form of LTD was in fact *enhanced*, rather than deficient, in the *Fmr1* KO mouse model of FXS was therefore quite surprising (*43*). Nevertheless, this was an exciting discovery because it suggested that FMRP and mGluR5 work as an opponent pair, where mGluR5 activates protein synthesis and FMRP suppresses it. In addition, as mentioned above, mGluR5 activation in hippocampal culture leads to changes in dendritic spine morphology that exactly parallels the neuropathology seen in Fragile X syndrome (*146, 152*). Like LTD and glutamate receptor internalization, this effect of mGluR5 activation is blocked by protein synthesis inhibitors (*146*). Because synaptic plasticity is the foundation of most theories of learning and memory in the brain, these results implicate GpI mGluRs in the pathogenesis of the mental retardation phenotype seen in FXS. As we will see later

(Chapter 4 and 5) we now have significant evidence to support this interaction between FMRP and mGluR5 in regulating synaptic plasticity, spine morphology and learning and memory.

At this point, although there was no direct evidence for it, it seemed possible that FMRP also functions as a negative regulator of epileptiform activity through mGluR-mediated mechanisms. Interestingly group I mGluR mediated induction of epileptiform activity has a protein synthesis dependent mechanism (*142*) which suggested the possibility that in fragile X syndrome, the absence of FMRP may account for increased epileptiform activity via disrupted mGluR regulation. As we will see later (Chapter 5), we now have evidence to support this interaction between FMRP and mGluR5 in regulating seizure phenotypes in FXS.

Underlying each of these ideas is the mechanistic assumption that mGluR5 and FMRP interact in opposite directions at the level of protein synthesis. This interaction is supported by the observation that FMRP acts as a negative regulator of protein synthesis (*41, 45, 49*) while mGluR5 activation acts as a stimulator of protein synthesis (*133*). In addition, because mGluR5 and FMRP show largely overlapping spatial and temporal expression patterns, these proteins seem optimally positioned to co-regulate translation at the synapse. To date, the best evidence to suggest an interaction between FMRP and mGLuR5 comes from a study that showed that FMRP co-fractionates with mRNA granules, which are decreased in *Fmr1*KO mice, and increased in animals treated with MPEP (an mGluR5 antagonist)(*30*). However, as mentioned above, this sort of *in vitro* co-fractionation study provides only indirect evidence for protein synthesis, and relies on the assumption that granules are involved in translational regulation. Furthermore,

because MPEP has known off-target activity (*153, 154*), these results must be viewed with caution.

Results

Our genetic manipulations provided an opportunity to test more directly the interaction of mGluR5 and FMRP in the regulation of protein synthesis. Based on the work of Carolyn Smith and colleagues (*49*), we chose to focus on the hippocampus of adult animals. As shown in **Figure 8**, we discovered that the increased rate of cerebral protein synthesis in *Fmr1* KO mice could also be detected in acutely prepared slices of ventral hippocampus, even though "basal" neural activity in slices is far different from that *in vivo*. Consistent with the conclusions of Aschrafi *et al*. (2005), the increased protein synthesis in KO mice was corrected by a 50% reduction in mGluR5 expression. However, unlike Aschrafi *et al*. (2005), we saw no effect on protein synthesis of reducing mGluR5 in the WT background. This discrepancy may be related to differences in the basal levels of mGluR5 activity *in vivo* and *in vitro*, or to differences in the genetic background of the animals.

FMRP is estimated to regulate nearly 4% of all cellular proteins (*23*). Electrophoretic separation of radiolabeled translation products (**Figure 8**, right) shows that increased protein synthesis in the *Fmr1*KO is not limited to one or few predominant protein species, but rather extended across a broad range of resolved molecular weights. These data imply that the exaggerated translation phenotype in the *Fmr1* KO reflects subtle increases in many individual proteins, whose compound effect is reflected in the ~20% total increase quantified in **Figure 8** (left); identification of these individual proteins will therefore necessitate further in depth investigation in the future.

As mentioned above, recently it has been suggested that mGluR5 activation leads to increased localization of stalled proteins to synapses, consistent with the cargo-hypothesis of FMRP (*31, 68*). In addition this same group has very recently shown that FMRP binds to mRNA encoding PSD-95, that this binding serves to increase stability of the message, and that mGluR activation serves to further stabilize the message (*155*). Together, these results suggest that rather than acting in opposition, mGluR5 and FMRP work in the same direction in the regulation of proteins. However, it is difficult to reconcile these results with our findings here. First since increased protein synthesis, measured directly in the *Fmr1*KO mouse is normal in the CR, our results suggests that if FMRP does in fact act as a molecular shuttle (*68*), this activity is unrelated to the increased protein synthesis phenotype reported in FXS, both *in vivo* and *in vitro* ((*41, 45, 49*) and here). Zalfa, et al., further suggest that dysregulation of mRNA stability may contribute to the cognitive impairments in individuals with FXS. Nevertheless here (see chapter 4 and 5) we show that synaptic plasticity, spine morphology, and learning and memory are normal in *Fmr1*KO mice with a 50% reduction in *Grm*5. Given these results, the functional significance of the mRNA stabilizing function of FMRP, and the role of mGluR5 activation, is something of a mystery, and awaits further investigation to determine its relevance to the pathogenesis of cognitive phenotypes in FXS.

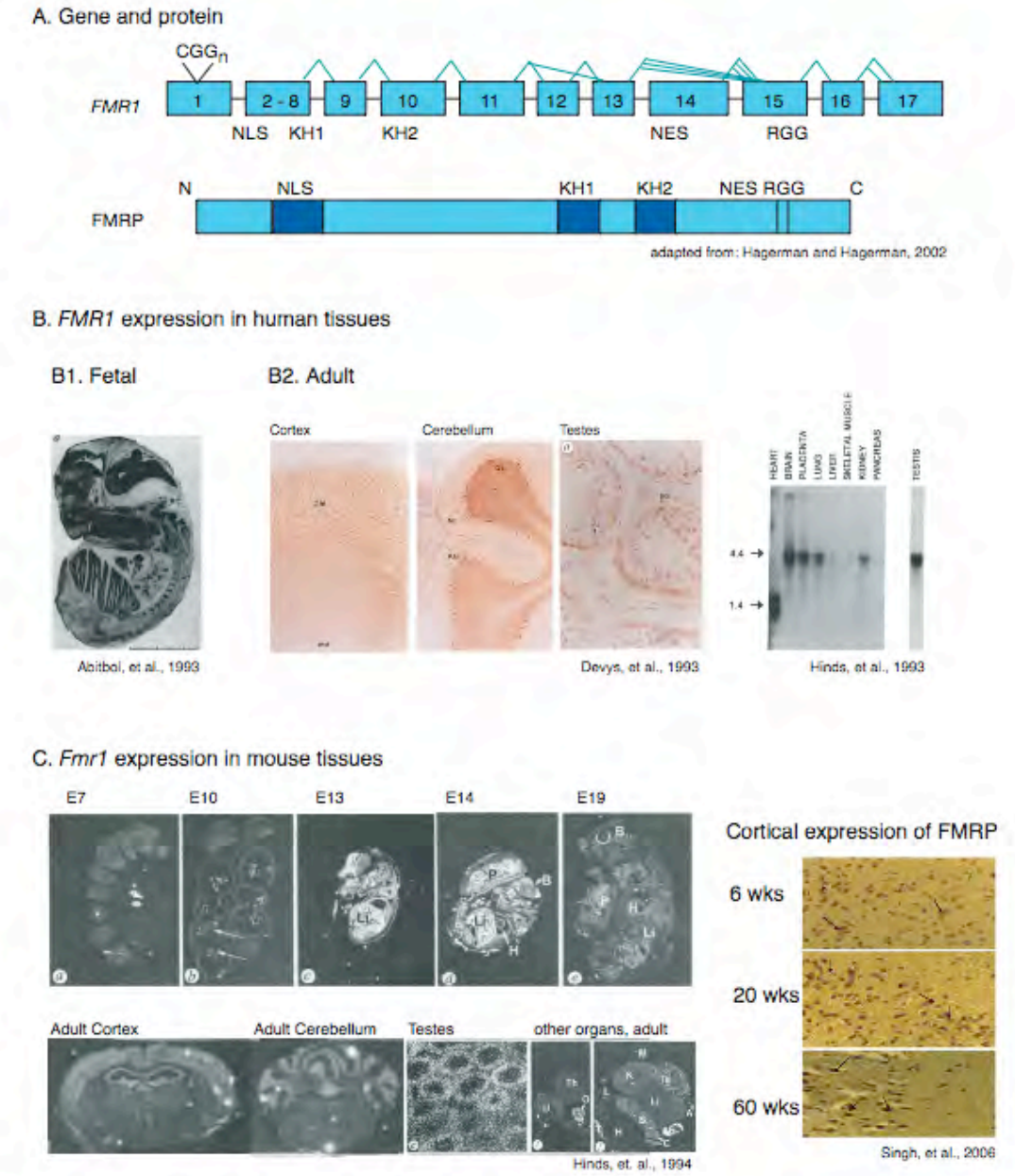
Methods

Experiments performed were similar to those described in Raymond et al. (*143*). Pilot studies indicated that the difference between WT and KO protein synthesis was most pronounced in the ventral hippocampus so all subsequent experiments were restricted to

slices from this region (see appendix 1). Briefly, 500 μm sections were prepared from one hippocampus per animal (average age P84) using a tissue slicer (Stoelting Co.), and transferred to a custom-made incubation manifold with multiple separate inserts (15 mm diameter netwells, Electron Microscopy Sciences) suspended in carbogenated, 32.5°C artificial cerebral spinal fluid (ACSF) (124 mM NaCl; 5 mM KCl; 1.25 mM NaH_2PO_4; 26 mM $NaHCO_3$; 0.8 mM $MgCl_2$; 1.8 mM $CaCl_2$; and 10 mM dextrose saturated with 95% O_2, 5% CO_2). Following a 4 hour recovery, netwells containing slices were transferred to a second manifold chamber containing 100 ml carbogenated ACSF with 11 μCi/ml ^{35}S-Met/Cys (EasyTag Express protein labeling mix, Perkin Elmer) and incubated for 1 hour. Netwells were then transferred to 12-well dish containing ice-cold dissection buffer to stop protein synthesis and remove excess ^{35}S-Met/Cys, and slices were subsequently removed and homogenized in ice-cold homogenization buffer. Samples were then incubated in Trichloroacetic acid (TCA; 10% final) for 10 minutes on ice, spun at 21,000xg, 10 minutes, 4°C, and the pellet washed with ice-cold ddH_2O. Pellets were then resolubilized in 37°C 1N NaOH, and pH adjusted with 1N HCl. For each sample, triplicate aliquots were then read with a scintillation counter (Beckman Instruments) using HiSafe 2 scintillation cocktail (Perkin Elmer), and triplicate aliquots were subjected to a protein concentration assay (Bio-Rad). These triplicate readings were averaged, and the counts per minute (CPM) obtained were divided by the CPM obtained (in triplicate) from the ^{35}S-Met/Cys ASCF used for incubation. Final data were thus expressed as incorporated CPM per μg protein. Equal amounts of protein from each sample were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Autoradiography was performed on the dried membranes. Staining with MemCode blot stain (Pierce)

confirmed equal loading. One dimensional line scans were performed using the gel analyzer tool in Image J to visualize the breadth of radiolabeled species across genotypes.

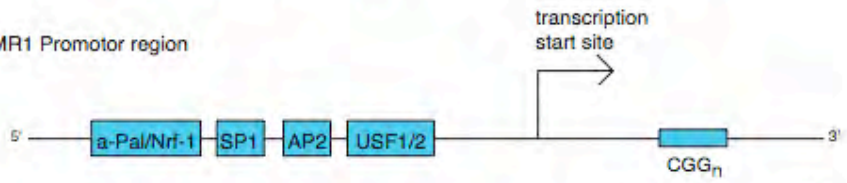
Figure 1: *FMR1* expression



Fmr1 expression. (A) Fmr1 gene encodes 12 splice variants. The protein product contains a nuclear localization signal (NLS) two KH mRNA binding domains, a nuclear export signal (NES) and an RGG box. (B) Fmr1 is widely expressed in human fetal tissues (b1) but expression is restricted in the adult (b2). (C) similar developmental and regional expression pattern is seen in the mouse.

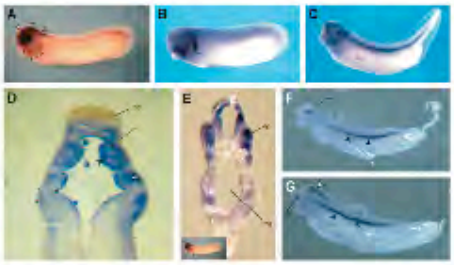
Figure 2. Transcriptional regulation of *Fmr1*

A. FMR1 Promotor region



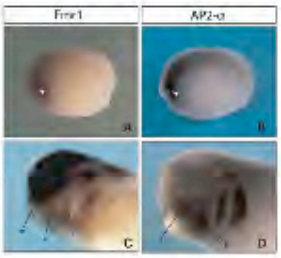
adapted from: Lim, et. al., 2005

B. Fmr1 expression in Xenopus development



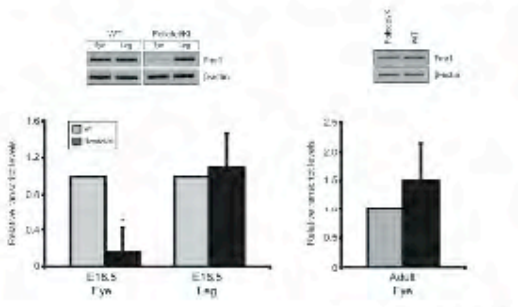
Lim, Luo, et al. 2005

B. Co-expression of Fmr1 and AP-2A transcripts in Xenopus larvae



Lim, Booker, et al. 2005

C. AP-2a regulates Fmr1 transcription during early development in mouse

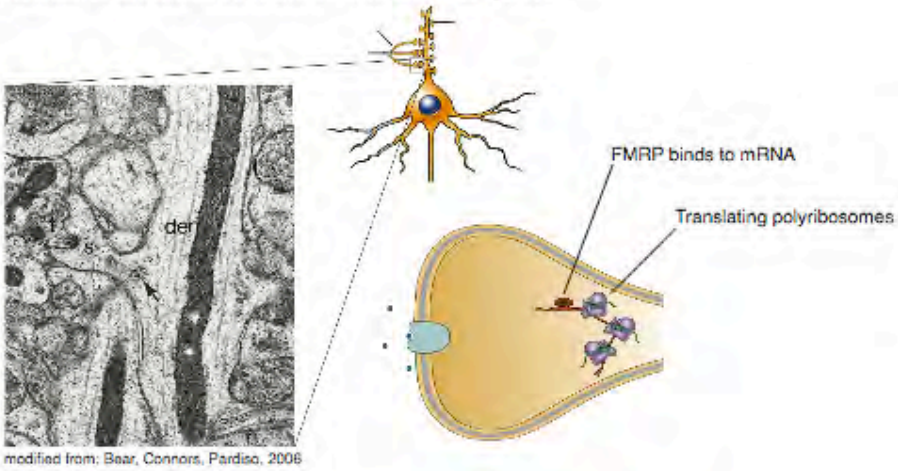


Lim, Booker, et al. 2005

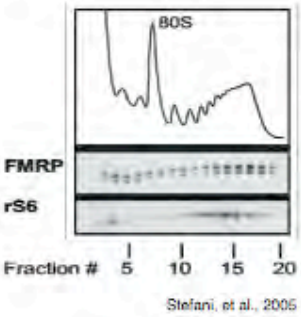
Regulation of transcription. (A) Transcription factor binding sites are found within the promotor region the Fmr1 gene.(B) Fmr1 expression is regulated in xenopus embryo (C) Fmr1 expression (left) and Ap2 a (right) in the xenopus embryo (D) Fmr1 expression is significantly decreased in the ap2 KO tissues during development by not in adulthood.

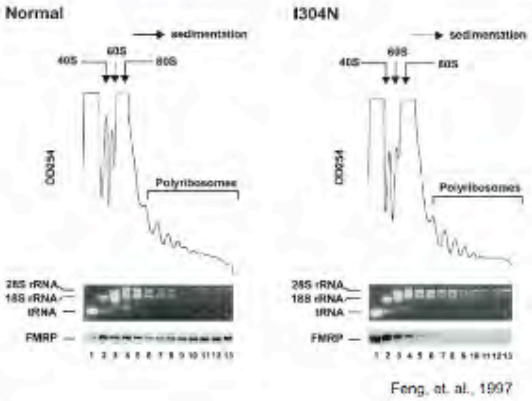
Figure 3

A. Polyribosomes are localized to the base of dendritic spines

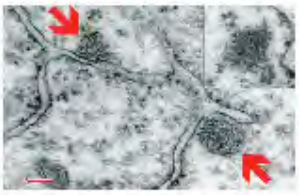


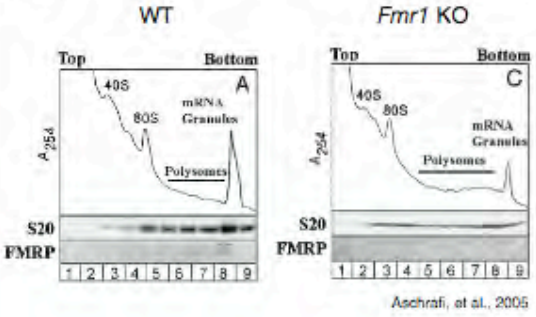
B. FMRP localizes to polyribosomes





C. FMRP localizes to granules on sucrose gradients

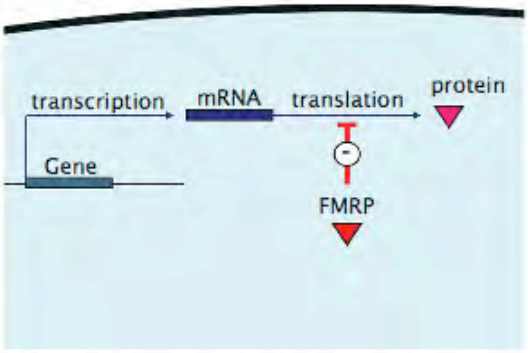




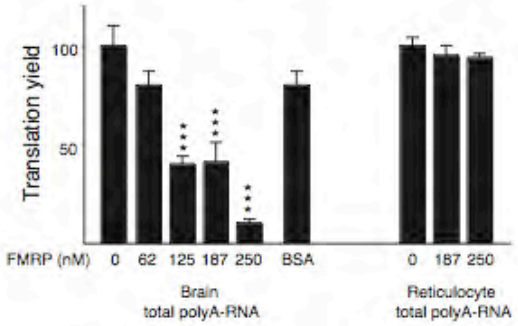
FMRP (A) FMRP is localized to polyribosomes which are localized to the base of dendritic spines. (B) sucrose gradients reveal colocalization of FMRP to polyribosome fraction. This colocalization of FMRP is absent in I304N mutants. (C) RNA granules (left) colocalized with FMRP (right). The granule peak is reduced in Fmr1 KO.

Figure 4. FMRP regulates protein synthesis

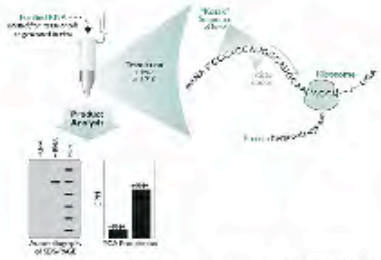
A. FMRP acts as a repressor of translation



B. Mamalian FMRP inhibits mRNA translation *in vitro*

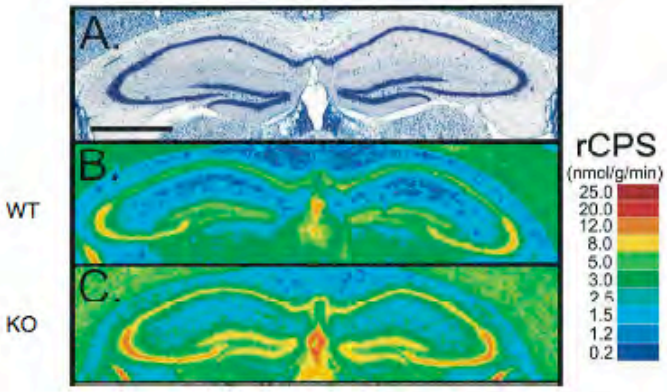


redrawn from Li, et al, 2001



http://www.ambion.com/figs/f00100.gif

C. Increased protein synthesis in the *Fmr1* KO *in vivo*

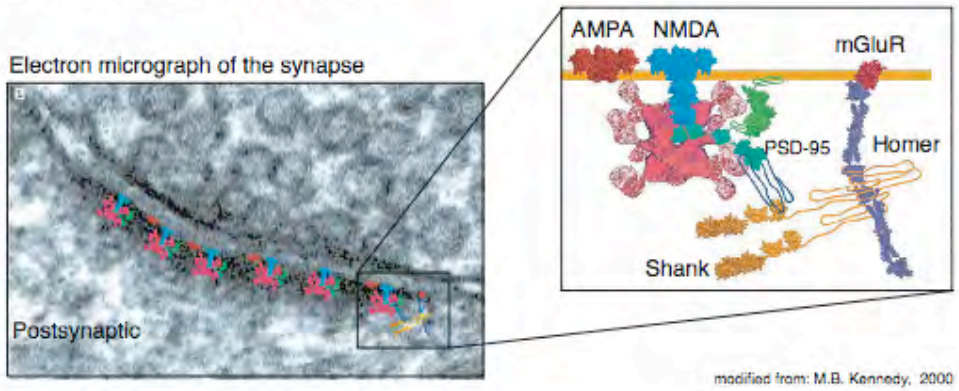


Qin, et al., 2005

FMRP function. (A) Negative regulator of translation. (B) In vitro evidence. Increasing concentrations of FMRP reduces translation yield in RRL of brain RNA but not reticulocyte RNA. (C) In vivo evidence. Increased protein synthesis in Fmr1 KO mouse hippocampus.

Figure 5. metabotropic glutamate receptors

A. Postsynaptic glutamate receptors



modified from: M.B. Kennedy, 2000

B. mGluR familly tree

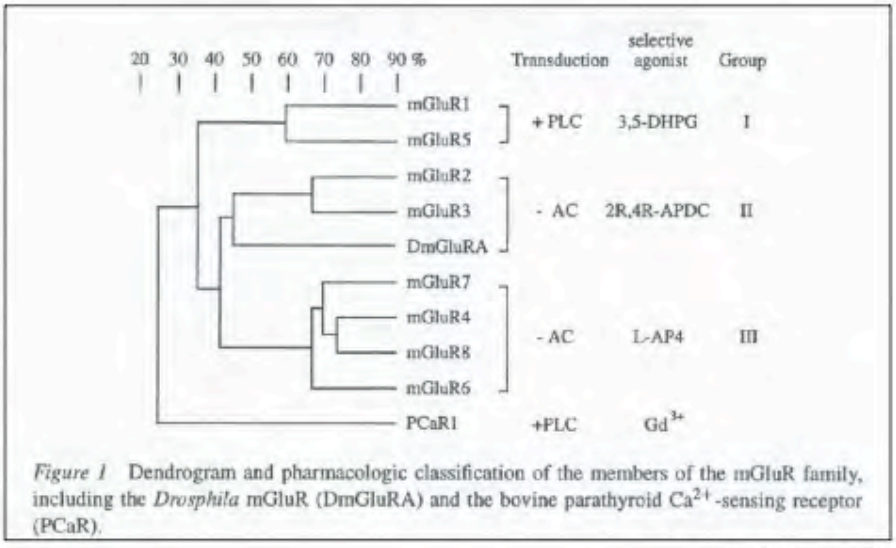
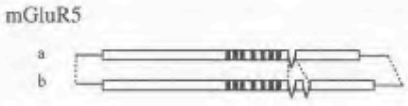


Figure 1 Dendrogram and pharmacologic classification of the members of the mGluR family, including the *Drosphila* mGluR (DmGluRA) and the bovine parathyroid Ca^{2+}-sensing receptor (PCaR).

from: Conn and Pin, 1997

C. mGluR5 splice variants



from: Conn and Pin, 1997

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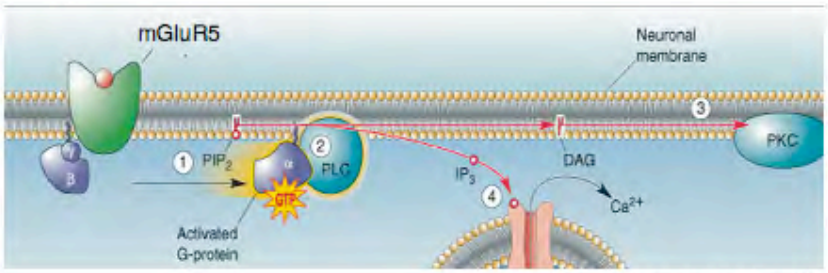
mGluR5 (A) Electron micrograph showing localization of mGluR5 at postsynaptic density. Crosslinking of mGluR5 to PSD via interaction with Homer and Shank. (B) mGluR5 dendrogram (C) Two mGluR5 splice variants (a and b) differ by one exon in the internal region (transmembrane domains are shown in black).

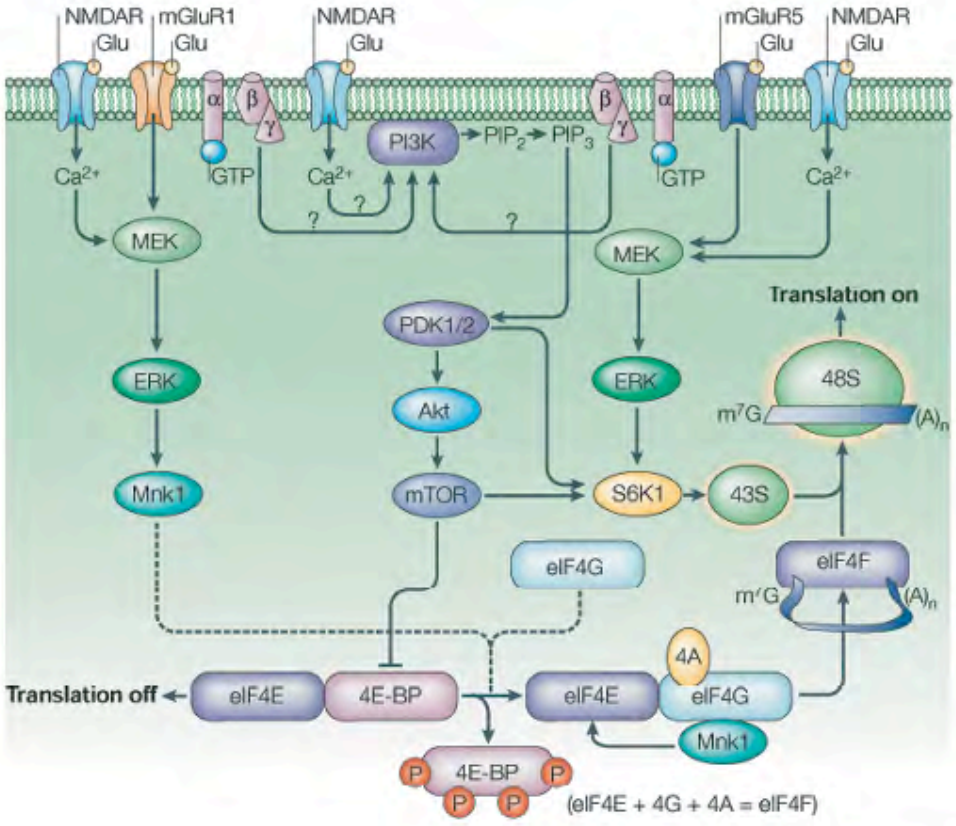
Figure 6. mGluR5 signaling cascades

A. Canonical pathway



adapted from: Bear, Connors, Paradiso, 2006

B. MAPK pathway



From: Klann and Dever, 2004

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mGluR5 signaling. (A) In the canonical pathway stimulation of mGluR5 leads to activation of PLC and the subsequent formation of IP3 and DAG. IP3 binds to Ca++ channels and leads to release of internal stores; DAG activates PKC. (B) MAP K pathway. Stimulation of mGluR5 activates ERK and turns on translation.

Figure 7. mGlur5 expression

A. Regional distribution of mGluR5 expression

B. Developmental downregulation of mGluR5 expression

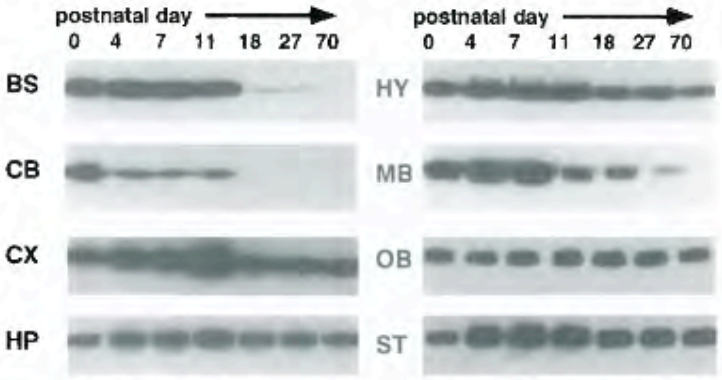
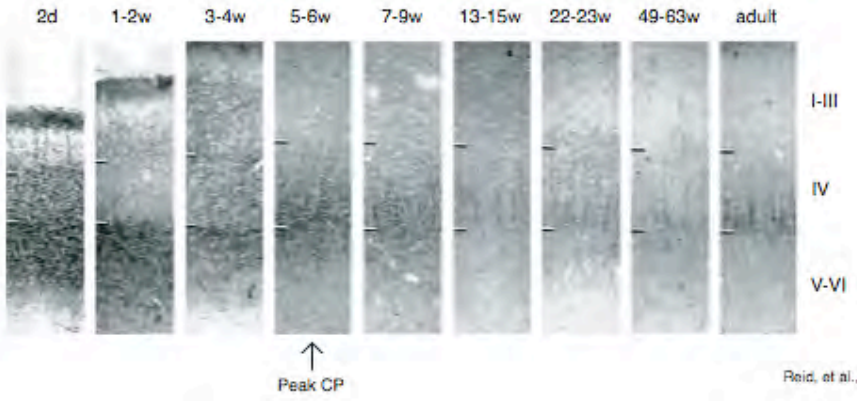


Fig. 1. Western blot analysis of metabotropic glutamate receptor 5 (mGluR5) in developing brain regions. Membrane proteins were prepared from rat brain regions as described in Material and Methods using brains from rats at the specified age and run (25 μg protein/lane) on 7.5% gels before blotting and probing with antibody selective for mGluR5. BS, brainstem; CB, cerebellum; CX, cerebral cortex; HP, hippocampus; HY, hypothalamus; MB, midbrain/thalamus; OB, olfactory bulb; ST, striatum.

Romano, et al., 1996

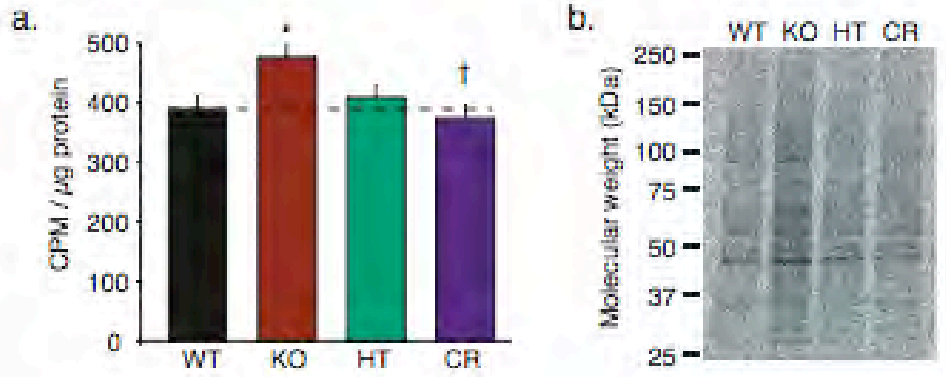
C. Laminar redistribution of mGluR5 expression (visual cortex, cat)



Reid, et al., 1997

mGluR5 expression (A) Widely expressed in the brain and testes. (B) several brain regions show dramatic down regulation of receptor expression across development. (C) MGluR5 expression in layer 4 peaks at the height of the critical period (5-6 weeks, cat).

Figure 8. Protein synthesis phenotype is rescued



Genetic rescue of protein synthesis phenotype in FXS. (**A**) Significant differences in the levels of protein synthesis exist across genotypes in the ventral hippocampus (n = 10 samples, 5 animals per genotype). KO mice showed increased protein synthesis (mean ± SEM: WT 389 ± 33.77 cpm/μg; KO 476 ± 29.98 cpm/μg; post-hoc paired t-test WT:KO P = 0.004). Protein synthesis levels in the HT mice were no different than WT (HT 409 ± 42.99 cpm/μg). Increased protein synthesis seen in the KO were rescued in the CR mice (CR 374 ± 50.81 cpm/μg). Post-hoc paired t-tests: * indicates significantly different from WT, † indicates significantly different from KO. (**B**) Representative autoradiogram shows that synthesis of many protein species is elevated in the KO compared to all other genotypes.

Chapter 2 References

1. E. E. Eichler, S. Richards, R. A. Gibbs, D. L. Nelson, *Hum Mol Genet* **2**, 1147 (Aug, 1993).
2. C. Verheij *et al.*, *Nature* **363**, 722 (Jun 24, 1993).
3. A. J. Verkerk *et al.*, *Hum Mol Genet* **2**, 1348 (Aug, 1993).
4. C. T. Ashley, Jr., K. D. Wilkinson, D. Reines, S. T. Warren, *Science* **262**, 563 (Oct 22, 1993).
5. E. W. Khandjian *et al.*, *Hum Mol Genet* **4**, 783 (May, 1995).
6. D. Devys, Y. Lutz, N. Rouyer, J. P. Bellocq, J. L. Mandel, *Nat Genet* **4**, 335 (Aug, 1993).
7. M. Abitbol *et al.*, *Nat Genet* **4**, 147 (Jun, 1993).
8. C. Agulhon *et al.*, *J Neuropathol Exp Neurol* **58**, 867 (Aug, 1999).
9. H. L. Hinds *et al.*, *Nat Genet* **3**, 36 (Jan, 1993).
10. K. Singh, P. Gaur, S. Prasad, *Mol Biol Rep* (Nov 29, 2006).
11. J. H. Lim, A. B. Booker, J. R. Fallon, *J Cell Physiol* **205**, 170 (Nov, 2005).
12. J. H. Lim *et al.*, *Hum Mol Genet* **14**, 2027 (Jul 15, 2005).
13. J. H. Lim, T. Luo, T. D. Sargent, J. R. Fallon, *Int J Dev Biol* **49**, 981 (2005).
14. S. Brewer, W. Feng, J. Huang, S. Sullivan, T. Williams, *Dev Biol* **267**, 135 (Mar 1, 2004).
15. J. Zhang *et al.*, *Nature* **381**, 238 (May 16, 1996).
16. J. A. West-Mays *et al.*, *Dev Biol* **206**, 46 (Feb 1, 1999).
17. S. P. Chandler, P. Kansagra, M. C. Hirst, *BMC Mol Biol* **4**, 3 (Mar 21, 2003).
18. Y. H. Fu *et al.*, *Cell* **67**, 1047 (Dec 20, 1991).
19. R. J. Hagerman, M. Y. Ono, P. J. Hagerman, *Curr Opin Psychiatry* **18**, 490 (Sep, 2005).
20. K. N. Harikrishnan *et al.*, *Nat Genet* **37**, 254 (Mar, 2005).
21. K. De Boulle *et al.*, *Nat Genet* **3**, 31 (Jan, 1993).
22. Y. Feng *et al.*, *Mol Cell* **1**, 109 (Dec, 1997).
23. V. Brown *et al.*, *Cell* **107**, 477 (Nov 16, 2001).
24. J. C. Darnell *et al.*, *Cell* **107**, 489 (Nov 16, 2001).
25. K. Y. Miyashiro *et al.*, *Neuron* **37**, 417 (Feb 6, 2003).
26. J. C. Darnell, O. Mostovetsky, R. B. Darnell, *Genes Brain Behav* **4**, 341 (Aug, 2005).
27. J. C. Darnell *et al.*, *Genes Dev* **19**, 903 (Apr 15, 2005).
28. Y. Feng *et al.*, *J Neurosci* **17**, 1539 (Mar 1, 1997).
29. H. Wang *et al.*, *Hum Mol Genet* **13**, 79 (Jan 1, 2004).
30. A. Aschrafi, B. A. Cunningham, G. M. Edelman, P. W. Vanderklish, *Proc Natl Acad Sci U S A* **102**, 2180 (Feb 8, 2005).
31. F. Zalfa, T. Achsel, C. Bagni, *Curr Opin Neurobiol* **16**, 265 (Jun, 2006).
32. M. C. Siomi, K. Higashijima, A. Ishizuka, H. Siomi, *Mol Cell Biol* **22**, 8438 (Dec, 2002).
33. A. Schenck, B. Bardoni, A. Moro, C. Bagni, J. L. Mandel, *Proc Natl Acad Sci U S A* **98**, 8844 (Jul 17, 2001).
34. G. Stefani, C. E. Fraser, J. C. Darnell, R. B. Darnell, *J Neurosci* **24**, 7272 (Aug 18, 2004).

35. O. Steward, E. M. Schuman, *Annu Rev Neurosci* **24**, 299 (2001).
36. P. Anderson, N. Kedersha, *J Cell Biol* **172**, 803 (Mar 13, 2006).
37. D. E. Eberhart, H. E. Malter, Y. Feng, S. T. Warren, *Hum Mol Genet* **5**, 1083 (Aug, 1996).
38. F. Tamanini *et al.*, *Hum Mol Genet* **5**, 809 (Jun, 1996).
39. F. Corbin *et al.*, *Hum Mol Genet* **6**, 1465 (Sep, 1997).
40. F. Zalfa *et al.*, *Cell* **112**, 317 (Feb 7, 2003).
41. B. Laggerbauer, D. Ostareck, E. M. Keidel, A. Ostareck-Lederer, U. Fischer, *Hum Mol Genet* **10**, 329 (Feb 15, 2001).
42. Y. Q. Zhang *et al.*, *Cell* **107**, 591 (Nov 30, 2001).
43. K. M. Huber, S. M. Gallagher, S. T. Warren, M. F. Bear, *Proc Natl Acad Sci U S A* **99**, 7746 (May 28, 2002).
44. R. Lu *et al.*, *Proc Natl Acad Sci U S A* **101**, 15201 (Oct 19, 2004).
45. Z. Li *et al.*, *Nucleic Acids Res* **29**, 2276 (Jun 1, 2001).
46. B. E. Pfeiffer, K. M. Huber, *J Neurosci* **27**, 3120 (Mar 21, 2007).
47. E. D. Nosyreva, K. M. Huber, *J Neurophysiol* **95**, 3291 (May, 2006).
48. L. Hou *et al.*, *Neuron* **51**, 441 (Aug 17, 2006).
49. M. Qin, J. Kang, T. V. Burlin, C. Jiang, C. B. Smith, *J Neurosci* **25**, 5087 (May 18, 2005).
50. Y. Q. Zhang *et al.*, *Mol Cell Proteomics* **4**, 278 (Mar, 2005).
51. S. P. Reeve *et al.*, *Curr Biol* **15**, 1156 (Jun 21, 2005).
52. C. Verheij *et al.*, *Hum Mol Genet* **4**, 895 (May, 1995).
53. R. Giuffrida *et al.*, *J Neurosci* **25**, 8908 (Sep 28, 2005).
54. P. K. Todd, K. J. Mack, J. S. Malter, *Proc Natl Acad Sci U S A* **100**, 14374 (Nov 25, 2003).
55. J. Li, M. R. Pelletier, J. L. Perez Velazquez, P. L. Carlen, *Mol Cell Neurosci* **19**, 138 (Feb, 2002).
56. A. El Idrissi *et al.*, *Neurosci Lett* **377**, 141 (Apr 4, 2005).
57. H. Siomi, M. Choi, M. C. Siomi, R. L. Nussbaum, G. Dreyfuss, *Cell* **77**, 33 (Apr 8, 1994).
58. P. Jin, R. S. Alisch, S. T. Warren, *Nat Cell Biol* **6**, 1048 (Nov, 2004).
59. A. A. Caudy, M. Myers, G. J. Hannon, S. M. Hammond, *Genes Dev* **16**, 2491 (Oct 1, 2002).
60. A. Ishizuka, M. C. Siomi, H. Siomi, *Genes Dev* **16**, 2497 (Oct 1, 2002).
61. A. A. Caudy, G. J. Hannon, *Methods Mol Biol* **265**, 59 (2004).
62. H. Siomi, A. Ishizuka, M. C. Siomi, *Ment Retard Dev Disabil Res Rev* **10**, 68 (2004).
63. M. Nakamoto, P. Jin, W. T. O'Donnell, S. T. Warren, *Hum Mol Genet* **14**, 3813 (Dec 15, 2005).
64. P. Jin *et al.*, *Nat Neurosci* **7**, 113 (Feb, 2004).
65. C. Bagni, W. T. Greenough, *Nat Rev Neurosci* **6**, 376 (May, 2005).
66. L. N. Antar, J. B. Dictenberg, M. Plociniak, R. Afroz, G. J. Bassell, *Genes Brain Behav* **4**, 350 (Aug, 2005).
67. I. J. Weiler *et al.*, *Proc Natl Acad Sci U S A* **101**, 17504 (Dec 14, 2004).
68. F. Ferrari *et al.*, *Mol Cell Neurosci* **34**, 343 (Mar, 2007).
69. I. J. Weiler *et al.*, *Proc Natl Acad Sci U S A* **94**, 5395 (May 13, 1997).

70. F. Sladeczek, J. P. Pin, M. Recasens, J. Bockaert, S. Weiss, *Nature* **317**, 717 (Oct 24-30, 1985).
71. R. Anwyl, *Brain Res Brain Res Rev* **29**, 83 (Jan, 1999).
72. P. J. Conn, *Ann N Y Acad Sci* **1003**, 12 (Nov, 2003).
73. P. J. Conn, J. P. Pin, *Annu Rev Pharmacol Toxicol* **37**, 205 (1997).
74. O. Valenti, P. J. Conn, M. J. Marino, *J Cell Physiol* **191**, 125 (May, 2002).
75. M. Masu, Y. Tanabe, K. Tsuchida, R. Shigemoto, S. Nakanishi, *Nature* **349**, 760 (Feb 28, 1991).
76. K. M. Houamed *et al.*, *Science* **252**, 1318 (May 31, 1991).
77. R. Minakami, F. Katsuki, T. Yamamoto, K. Nakamura, H. Sugiyama, *Biochem Biophys Res Commun* **199**, 1136 (Mar 30, 1994).
78. A. Ramaekers, M. L. Parmentier, C. Lasnier, J. Bockaert, Y. Grau, *J Comp Neurol* **438**, 213 (Sep 17, 2001).
79. R. Minakami, K. Iida, N. Hirakawa, H. Sugiyama, *J Neurochem* **65**, 1536 (Oct, 1995).
80. C. Romano, S. Smout, J. K. Miller, K. L. O'Malley, *Neuroscience* **111**, 693 (2002).
81. C. Joly *et al.*, *J Neurosci* **15**, 3970 (May, 1995).
82. C. Romano *et al.*, *J Comp Neurol* **355**, 455 (May 8, 1995).
83. M. B. Kennedy, *Science* **290**, 750 (Oct 27, 2000).
84. O. Manzoni, J. Bockaert, *Eur J Neurosci* **7**, 2518 (Dec 1, 1995).
85. R. W. t. Gereau, P. J. Conn, *J Neurosci* **15**, 6879 (Oct, 1995).
86. R. W. t. Gereau, P. J. Conn, *J Neurophysiol* **74**, 122 (Jul, 1995).
87. N. Varma, G. C. Carlson, C. Ledent, B. E. Alger, *J Neurosci* **21**, RC188 (Dec 15, 2001).
88. M. Uchigashima *et al.*, *J Neurosci* **27**, 3663 (Apr 4, 2007).
89. R. I. Wilson, R. A. Nicoll, *Science* **296**, 678 (Apr 26, 2002).
90. R. Schnabel, I. C. Kilpatrick, G. L. Collingridge, *Br J Pharmacol* **132**, 1095 (Mar, 2001).
91. S. M. Fitzjohn *et al.*, *J Physiol* **537**, 421 (Dec 1, 2001).
92. R. Schnabel, I. C. Kilpatrick, G. L. Collingridge, *Neuropharmacology* **38**, 1585 (Oct, 1999).
93. K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **86**, 321 (Jul, 2001).
94. J. C. Siciliano, M. Gelman, J. A. Girault, *J Neurochem* **62**, 950 (Mar, 1994).
95. C. Heuss, M. Scanziani, B. H. Gahwiler, U. Gerber, *Nat Neurosci* **2**, 1070 (Dec, 1999).
96. A. R. Boxall, *J Physiol* **524 Pt 3**, 677 (May 1, 2000).
97. M. A. Abdul-Ghani, T. A. Valiante, P. L. Carlen, P. S. Pennefather, *J Physiol* **496 (Pt 1)**, 139 (Oct 1, 1996).
98. S. M. Gallagher, C. A. Daly, M. F. Bear, K. M. Huber, *J Neurosci* **24**, 4859 (May 19, 2004).
99. V. Y. Bolshakov, S. A. Siegelbaum, *Science* **264**, 1148 (May 20, 1994).
100. E. D. Roberson *et al.*, *J Neurosci* **19**, 4337 (Jun 1, 1999).
101. R. D. Peavy, S. D. Sorensen, P. J. Conn, *J Neurochem* **83**, 110 (Oct, 2002).
102. R. D. Peavy, M. S. Chang, E. Sanders-Bush, P. J. Conn, *J Neurosci* **21**, 9619 (Dec 15, 2001).

103. R. D. Peavy, P. J. Conn, *J Neurochem* **71**, 603 (Aug, 1998).
104. P. Calabresi *et al.*, *Mol Pharmacol* **60**, 808 (Oct, 2001).
105. F. Karim, C. C. Wang, R. W. t. Gereau, *J Neurosci* **21**, 3771 (Jun 1, 2001).
106. J. L. Banko, L. Hou, F. Poulin, N. Sonenberg, E. Klann, *J Neurosci* **26**, 2167 (Feb 22, 2006).
107. Q. Wang, D. M. Walsh, M. J. Rowan, D. J. Selkoe, R. Anwyl, *J Neurosci* **24**, 3370 (Mar 31, 2004).
108. C. C. Huang, J. L. You, M. Y. Wu, K. S. Hsu, *J Biol Chem* **279**, 12286 (Mar 26, 2004).
109. L. Mao *et al.*, *J Biol Chem* **280**, 12602 (Apr 1, 2005).
110. D. Cumiskey, M. P. Butler, P. N. Moynagh, J. O'Connor J, *Brain Res* **1136**, 13 (Mar 9, 2007).
111. Y. Edling, M. Ingelman-Sundberg, A. Simi, *Glia* **55**, 328 (Feb, 2007).
112. Q. Wang, L. Chang, M. J. Rowan, R. Anwyl, *Neuroscience* **144**, 110 (Jan 5, 2007).
113. E. Klann, T. E. Dever, *Nat Rev Neurosci* **5**, 931 (Dec, 2004).
114. L. J. Volk, C. A. Daly, K. M. Huber, *J Neurophysiol* **95**, 2427 (Apr, 2006).
115. J. C. Tu *et al.*, *Neuron* **23**, 583 (Jul, 1999).
116. A. Serge, L. Fourgeaud, A. Hemar, D. Choquet, *J Neurosci* **22**, 3910 (May 15, 2002).
117. L. Mao *et al.*, *J Neurosci* **25**, 2741 (Mar 9, 2005).
118. G. L. Collingridge, J. T. Isaac, Y. T. Wang, *Nat Rev Neurosci* **5**, 952 (Dec, 2004).
119. E. M. Snyder *et al.*, *Nat Neurosci* **4**, 1079 (Nov, 2001).
120. Y. M. Lu *et al.*, *J Neurosci* **17**, 5196 (Jul 1, 1997).
121. Z. Jia *et al.*, *Learn Mem* **5**, 331 (Sep-Oct, 1998).
122. S. Alagarsamy, S. D. Sorensen, P. J. Conn, *Curr Opin Neurobiol* **11**, 357 (Jun, 2001).
123. C. Romano, A. N. van den Pol, K. L. O'Malley, *J Comp Neurol* **367**, 403 (Apr 8, 1996).
124. E. D. Nosyreva, K. M. Huber, *J Neurosci* **25**, 2992 (Mar 16, 2005).
125. S. N. Reid, C. Romano, T. Hughes, N. W. Daw, *J Comp Neurol* **389**, 577 (Dec 29, 1997).
126. G. Casabona *et al.*, *Eur J Neurosci* **9**, 12 (Jan, 1997).
127. M. Storto *et al.*, *J Endocrinol* **170**, 71 (Jul, 2001).
128. S. M. Dudek, W. D. Bowen, M. F. Bear, *Brain Res Dev Brain Res* **47**, 123 (May 1, 1989).
129. S. M. Dudek, M. F. Bear, *Science* **246**, 673 (Nov 3, 1989).
130. S. N. Reid, C. Romano, T. Hughes, N. W. Daw, *J Comp Neurol* **355**, 470 (May 8, 1995).
131. R. M. Shigemoto, N., *Metabotropic glutamate receptors– immunocytochemical and in situ hybridization analysis*. O. P. S.-M. Ottersen, J., Ed., Handbook of Chemical Neuroanatomy. (Elsevier, New York, 2000), pp.
132. K. M. Huber, N. B. Sawtell, M. F. Bear, *J Neurosci* **18**, 1 (Jan 1, 1998).
133. I. J. Weiler, W. T. Greenough, *Proc Natl Acad Sci U S A* **90**, 7168 (Aug 1, 1993).
134. C. Job, J. Eberwine, *Proc Natl Acad Sci U S A* **98**, 13037 (Nov 6, 2001).
135. C. Y. Shin, M. Kundel, D. G. Wells, *J Neurosci* **24**, 9425 (Oct 20, 2004).

136. O. Steward, E. M. Schuman, *Neuron* **40**, 347 (Oct 9, 2003).
137. K. M. Huber, M. S. Kayser, M. F. Bear, *Science* **288**, 1254 (May 19, 2000).
138. A. Tiunova, K. Anokhin, S. P. Rose, R. Mileusnic, *Neurobiol Learn Mem* **65**, 233 (May, 1996).
139. W. Francesconi, M. Cammalleri, P. P. Sanna, *Brain Res* **1022**, 12 (Oct 1, 2004).
140. L. Karachot, Y. Shirai, R. Vigot, T. Yamamori, M. Ito, *J Neurophysiol* **86**, 280 (Jul, 2001).
141. A. C. Lee, R. K. Wong, S. C. Chuang, H. S. Shin, R. Bianchi, *J Neurophysiol* **88**, 1625 (Oct, 2002).
142. L. R. Merlin, P. J. Bergold, R. K. Wong, *J Neurophysiol* **80**, 989 (Aug, 1998).
143. C. R. Raymond, V. L. Thompson, W. P. Tate, W. C. Abraham, *J Neurosci* **20**, 969 (Feb 1, 2000).
144. K. Naie, D. Manahan-Vaughan, *Neuropharmacology* **49 Suppl 1**, 35 (2005).
145. R. Stoop, F. Conquet, B. Zuber, L. L. Voronin, E. Pralong, *J Neurosci* **23**, 5634 (Jul 2, 2003).
146. P. W. Vanderklish, G. M. Edelman, *Proc Natl Acad Sci U S A* **99**, 1639 (Feb 5, 2002).
147. W. M. Zho, J. L. You, C. C. Huang, K. S. Hsu, *J Neurosci* **22**, 8838 (Oct 15, 2002).
148. R. K. Wong, R. Bianchi, G. W. Taylor, L. R. Merlin, *Adv Neurol* **79**, 685 (1999).
149. R. Vigot, R. T. Kado, C. Batini, *Arch Ital Biol* **142**, 69 (Feb, 2004).
150. H. H. Yin, M. I. Davis, J. A. Ronesi, D. M. Lovinger, *J Neurosci* **26**, 11811 (Nov 15, 2006).
151. S. H. Oliet, R. C. Malenka, R. A. Nicoll, *Neuron* **18**, 969 (Jun, 1997).
152. S. A. Irwin *et al.*, *Am J Med Genet* **98**, 161 (Jan 15, 2001).
153. P. M. t. Lea, A. I. Faden, *CNS Drug Rev* **12**, 149 (Summer, 2006).
154. C. A. Heidbreder *et al.*, *Synapse* **50**, 269 (Dec 15, 2003).
155. F. Zalfa *et al.*, *Nat Neurosci* **10**, 578 (May, 2007).

Chapter 3: Cognitive function

Introduction

Over a hundred years ago, William James coined the term *plasticity* to describe structural changes in the nervous system: "*Plasticity*…means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once…nervous tissue seems endowed with a very extraordinary degree of plasticity of this sort" (*1*). Although the synapse had not yet been discovered, James argued that "the change of [brain] structure here spoken of need not involve the outward shape; it may be invisible and molecular …"(*1*) While he primarily wrote of plasticity as a mechanism of habit learning, including addiction, he also implicates it in the development of the nervous system: "Man is born with a tendency to do more things than he has ready-made arrangements for in his nerve-centres"(*1*). Over the last few decades this notion of plasticity has been expanded to include any long-lasting form of synaptic modification (strengthening or weakening) that is synapse specific and depends on correlations between pre- and postsynaptic firing (*2-4*). Consistent with James' original intuition, synaptic plasticity manifests as both morphological and molecular changes and provides the basis for most models of learning and memory, as well as the development of response selectivity and cortical maps.

The brain contains nearly a hundred billion neurons and a hundred trillion synapses. During development, the interconnections between cells are organized in several waves of maturation. In the first period, which occurs mostly before birth, neurons are born, migrate, sprout neurites (axons and dendrites) and form synapses with target neurons. This process occurs largely via molecular cues and genetic programs. For example, in the visual system, ganglion cells in the retina (RGCs) are born and send their

axons to appropriate target neurons in the lateral geniculate nucleus (LGN). Before they can reach their targets, they must decide whether they will decussate at the optic chiasm, and become contralaterally projecting, or not, and become ipsilaterally projecting. It has recently been shown that ipsilaterally projecting RGCs, but not contralaterally projecting RGCs, express a transcription factor, *Zic2* (*5*). Interestingly the expression of this transcription factor is temporally and spatially coincident with the expression of EphB1 receptors at the axon terminal of RGCs; when these receptors encounter Ephrin-B2, secreted at the optic chiasm, they cause the axon terminals to turn away from the midline and proceed to the LGN on the same (ipsilateral) side of the brain (*6, 7*). In this way, it is thought much of the basic circuitry of the brain is established by molecular cues during early development.

After birth, through late adolescence, these interconnections are largely remodeled by experience. Experience dependent remodeling of the brain represents a second wave of maturation, the so-called "critical period" of plasticity. Some of the first evidence for this sort of plasticity was provided by experiments in the visual system, which showed that if visual experience of an animal was modified by monocular deprivation, the primary visual cortex could be reorganized to a remarkable extent (*8*). When these manipulations were carried out in adulthood, no such remodeling occurred (*9*), suggesting that this plasticity is temporally constrained across development. Although these dramatic cortical rearrangements do not occur after the critical period, some plasticity is still possible, and is thought to be necessary for learning and memory during adulthood (*10-12*). Finally, during senescence, neurons begin to die and synaptic

connections are lost; this period of development is characterized by a decline in learning and memory.

While the end of the critical period is typically lamented -- quoting again from James, "Could the young but realize how soon they will become mere walking bundles of habits, they would give more heed to their conduct during the plastic state" -- there is accumulating evidence that plasticity processes are constricted in a programmed way, suggesting that the stabilization of brain circuitry is an important part of normal maturation. For example, in the visual cortex, it has been suggested that plasticity diminishes as synaptic transmission matures (i.e. by changes in expression of functional mGluRs (*13-15*) or NMDA receptor subunit composition (*16-19*)). Other possibilities that have been proposed include: maturation of inhibition, decreased effectiveness of neuromodulator-enabled transmission, increased myelination of axons, or increased amounts of extracellular matrix proteins and growth factors (*20-27*).

A number of diseases of the brain are thought to reflect derailments of developmental programs, and can be broadly categorized by the epoch that is disrupted. For example, the transcription factor *Zic2* mentioned above, has been implicated in holoprosencephaly, the most common congenital brain malformation in humans (*28-30*). This disease represents a disruption of early brain development, and is characterized by a failure to separate the forebrain into two hemispheres, resulting in a single forebrain 'holosphere' and a continuous cerebral cortex (with no decussations) across the midline (*31*). In contrast, Alzheimer's disease (AD) is thought to be a disorder of senescence. AD primarily affects the elderly, and is characterized by abnormal accumulation of mutated forms of amyloid precursor protein (APP) that leads to neurodegeneration and a

parallel decline in cognitive function. Another class of brain disorders, the "synaptopathies," can be defined as diseases that affect synaptic plasticity; here we provide evidence that FXS is a developmental synaptopathy, since its pathophysiology involves changes in dendritic spine morphology, plasticity and learning, which can be corrected by reducing mGluR5, a receptor protein implicated in the programmed maturation of synaptic transmission.

Human studies: Cognitive profile in FXS

Mental retardation is more common in males than females; this gender asymmetry has been attributed to X-linked mental retardation (XLMR), since X-linked inheritance dictates that mutations on the X chromosome will preferentially affect males, who have only a single copy. Several XLMR disorders have been identified, but of these FXS is the single most common, accounting for more cases of mental retardation than all other identified XLMR causes combined (*32*). By definition, mental retardation is a developmental disorder (*33*). At birth, children with FXS appear largely normal. However within the first and second year, they begin to miss developmental milestones, and IQ testing reveals moderate to severe mental retardation (IQ's in the 30-50 range)(*34*). In addition, many studies have reported a developmental decline of IQ, beginning as early as 2 years and continuing into adolescence (*35-40*). Nevertheless, some adaptive skills continue to improve with age, even in adulthood (*41*). Based on these studies it is unclear whether developmental decline reflects a perpetual state of immaturity, (that only appears as a decline since IQ values are compared to population norms matched for age) or instead a true decline reflecting the accumulation of neural

insults. Brain imaging studies are somewhat difficult to interpret, since not all studies have used the same control parameters, sample sizes are low, and variability between subjects large. In general, there seems to be no consistent abnormality in *gross* brain anatomy; however, some studies have reported increased brain volume (*42-44*). Because reports of hippocampal, caudate, amygdalar, thalamic, and ventricular enlargements were not consistently normalized to total volume, it is unclear whether there is a relative or proportional enlargement in these structures (*43*). Decreases in volume have also been reported, including in the superior temporal gyrus (*42*) and posterior cerebellar vermis (Shown in Figure 1)(*42, 43, 45, 46*). While to date these gross anatomical findings have not been replicated in the mouse model (*47*), dendritic spine abnormalities have been reported in both human patients (*48, 49*) and the mouse model of the disease (*50-54*). This phenotype will be described in more detail below.

In vivo **cortical plasticity in the mouse**

Landmark demonstrations of experience dependent plasticity and its underlying synaptic mechanisms were historically carried out by manipulations of visual experience (e.g. monocular deprivation, binocular deprivation, monocular inactivation) and *in vivo* recordings in the visual cortex (*8, 9, 20, 55-69*). For a number of methodological reasons (for a more detailed discussion, see appendix 1), experiments using the reduced slice preparation *in vitro*, have greatly augmented our mechanistic understanding of these processes (*12, 16, 18, 19, 70-81*). More recently, the advent of molecular genetic manipulations has made it possible to return to the *in vivo* preparation for mechanistic studies. The value of *in vivo* recording stems from the fact that brain circuits are intact,

and in the case of monocular deprivation, experience dependent changes are induced using the naturally occurring patterns of neuronal activity. These activity patterns are mimicked *in vitro* by various induction protocols, however it must be remembered that these activation patterns are artificial estimates, derived largely by trial and error. Furthermore, because *in vitro* recordings experiments are temporally limited (maximum recording times approximately 10 hours), they cannot report naturally occurring plasticity time scales. Genetic manipulations are currently most feasible in the mouse. Most of the earlier landmark studies were performed in monkeys, cats, and ferrets; consequently a number of labs have devoted considerable energy into developing the mouse model for the study of experience dependent plasticity *in vivo (12, 22, 82-91).*

Almost in anticipation of the molecular genetic revolution, the first studies of visual cortical plasticity in mouse were carried out nearly 30 years ago by Ursula Dräger (*82, 90, 91*). These pioneering studies established the basic circuitry of the mouse visual system, as well as the receptive field properties of neurons in the primary visual cortex. A few salient results will be mentioned here. First, unlike the visual system of human and non-human primates, the ipsilateral and contralateral projections from the retina to the binocular region of primary visual cortex in mouse are not equal; this difference likely reflects the degree of binocularity in the mouse which is directly proportional to the lateral position of the eyes within the head. As shown in the autoradiograph in **Figure 2A,** injection of radioactive label (tritiated proline) into the retina is able to pass transynaptically to thalamocortically projecting neurons in the LGN. The projections to the cortex are unevenly distributed between the ipsilateral and contralateral hemisphere, and show a two-fold disparity in contralateral relative to ipsilateral projections (*90*); this

2 to 1 ratio was later confirmed electrophysiologically (*82, 91*). As in other species, these projections make their first synapse in layer 4 of the binocular region of visual cortex (V1b, also called area 17) (shown in **Figure 2B**).

Subsequent electrophysiological studies by Dräger revealed that unlike in other species, cells at this first synapse in layer 4 are binocularly responsive (*91*). This finding is consistent with the absence of ocular dominance columns, which are a feature of some, but not all higher species and reflect the segregation of eye-specific inputs into columns in layer 4 (binocular cells in are found in the second synapse, in layer 2/3). Extensive mapping of the eye-specific receptive field properties in layer 4, revealed the topography of the binocular zone of visual cortex in mouse (*91*) (shown in **Figure 3**, binocular units are highlighted in blue for clarity).

Dräger also pioneered monocular deprivation studies in the mouse visual cortex as an assay of cortical plasticity (*82*). Here, animals were monocularly lid sutured at postnatal day 12, and remained deprived of vision until adulthood (6 months – 1 year). As expected from previous work in other species, this manipulation produced a dramatic shift in ocular dominance recorded electrophysiologically, whereby neurons in the contralateral hemisphere were now driven primarily by the ipsilateral eye (although contralateral eye responses were still measured) (*82*) (shown in **Figure 4A**). As shown in **Figure 4B**, injection of radiolabel into the contralateral eye produced the same labeling pattern (2:1 contra:ipsi) in both deprived and non-deprived animals. These results suggest that the shift in ocular dominance profiles occurs at the synaptic level, and unlike the dramatic OD column rearrangements seen in other species, are not reflected as large-scale rearrangements in thalamocortical projections in the mouse (*82*).

Subsequent work by Michael Stryker's group has largely confirmed these results in mice using shorter deprivation periods (4 days), and have defined a "critical period" for the induction of this form of plasticity between P19 and P32, with maximal shifts occurring at P28 (*83*). However, as will be discussed below, this plastic period might be more appropriately called a "sensitive period" since subsequent work from our own lab and others has shown that plasticity can be induced in the adult with longer deprivation protocols (*12, 86*). In addition, work from the Stryker lab has suggested that subtler anatomical rearrangements of thalamocortical axonal branching (particularly in the ipsilateral eye inputs) can occur after longer periods of monocular deprivation (P20-P60) (*26*).

One of the limitations of single unit recordings is that they are susceptible to sampling bias, since ocular dominance histograms are generated by sampling a population of cells and determining the strength of the response (assayed by firing rates) to each eye. A protocol developed by Lamberto Maffei and colleagues averts this problem by recording instead field potentials evoked by pattern visual stimuli (VEPs) in the mouse visual cortex (*87*). As shown in **Figure 5**, these VEPs vary with laminar position within the visual cortex, having the largest amplitude at a depth of 400μm from the surface. This depth corresponds to deep layer 4, consistent with thalamocortical inputs to this layer. These studies also characterized the spatial and temporal aspects of VEPs, and confirm that acuity, contrast threshold, response latency, and motion sensitivity measured by VEPs are comparable to single unit data. Most importantly, VEPs showed contribution from both eyes, although biased towards the eye contralateral to the recorded cortex (this VEP bias is reported as 3:1 in anesthetized animals, but subsequent

studies in our lab have shown that in awake behaving animals the contra:ipsi ratio is 2:1, comparable to ratios reported previously). These results have also been confirmed using behavioral measures of acuity **(Figure 6)**, which show that changes in visual response properties recorded by VEPs are isometric with changes in visual acuity measured behaviorally (*84*).

Results

Visually evoked potentials (VEPs) were recorded in visual cortex of awake mice (**Figure 7A**), as described previously (*12, 92*). We initially assessed absolute levels of visual responsiveness across genotypes on postnatal day (P) 28 and found no difference (**Figure 7B**). Additional mice were studied before and after MD begun on P28. Previous studies using the chronic VEP method have shown how visual responses evolve during the course of MD (**Figure 8**). Closure of the contralateral eyelid initially causes depression of responses to the deprived- (contralateral-) eye (apparent at 3 d. MD), followed by potentiation of nondeprived- (ipsilateral-) eye responses (apparent by 7 d. MD) (*92*). Because they are recorded chronically, changes in VEPs for each animal can be conveniently described by two values: the fractional change from baseline in contralateral-eye response, and the fractional change from baseline in the ipsilateral-eye response. For reference, average effects (± SEM) of 3 and 7 days of MD in WT mice from a previous study (*92*) appear in **Figure 8**.

In the current study we also found that the response to 3 d MD in WT mice was dominated by deprived-eye depression, as expected. In KO littermates, however, the challenge of 3 d MD uncovered an interesting and unexpected phenotype. The response

to brief MD in the KO mice was characterized by substantial open-eye potentiation, reminiscent of what happens in WT mice after longer periods of MD. On the other hand, the HT mice showed a "hypoplastic" response to MD, as they lacked significant deprived-eye depression. However, crossing the two mutant mice resulted in a phenotype very similar to WT that was again dominated by deprived-eye depression (**Figure 7C**).

Plots of the average (± SEM) fractional changes after 3 d MD in the 4 genotypes are shown in **Figure 7D**. The KO mice displayed increased plasticity compared to the WT (MANOVA WT:KO, P = 0.011); HT mice displayed diminished plasticity compared to WT (MANOVA WT:HT, P = 0.013); CR mice showed a rescue of the KO phenotype and were not significantly different from WT (MANOVA WT:CR, P = 0.8268, KO:CR P = 0.037, HT:CRS P = 0.161).

Since the KO and HT mutations affected ocular dominance plasticity in opposite directions, one could question whether the CR phenotype reflects rescue or the simple addition of two independent effects. However, a compound phenotype would be the absence of deprived-eye depression (the effect of reducing mGluR5) and an exaggeration of open-eye potentiation (the effect of reducing FMRP). Instead, we observe a phenotype in the CR mice that is significantly different from KO mice, and not significantly different from WT. Thus, reducing mGluR5 by 50% corrects the defect in plasticity caused by the absence of FMRP.

We next examined the kinetics of synaptic plasticity with a second deprivation duration (1day MD) **(Figure 9)**. Although global differences across genotypes after 1 day of monocular deprivation are not statistically significant (MANOVA: p = 0.9203); subtler trends nevertheless exist. For example, while WT, KO, and CRS mice all exhibit a trend

towards response depression, the magnitude of the KO depression is greater (17% depression KO, 12% depression WT, 14% depression CRS) and only in the *Fmr1 KO* is this response depression statistically significant (paired t-test WT pre:post p = 0.088, KO pre:post p = 0.027, CRS pre:post 0.054). These results suggest that, rather than limiting cortical LTP, FMRP regulates cortical plasticity in general. Taken together, the 1day and 3day MD results suggest that in the absence of FMRP, *Fmr1 KO* mice display accelerated plasticity dynamics, and that reducing the mGluR5 gene dosage in *FMR1* KO mice attenuates this hyper-plasticity.

Discussion

Early studies examining a possible deficit in synaptic plasticity in the *Fmr1*KO were discouraging. For example, Godfraind and colleagues reported normal long-term potentiation (LTP) at the CA1/Schaffer collateral synapses induced by high-frequency stimulation in the *Fmr1* KO; paired-pulse facilitation was not different, suggesting that presynaptic transmission was also normal (animal age was not reported) (*93*). Paradee and colleagues examined late-phase LTP (L-LTP) in these mice, with the prediction that, since this form of LTP requires protein synthesis (*94*), differences in synaptic plasticity would be observed in the *Fmr1*KO. However, using a theta burst induction protocol in adult animals (also at the CA1/Schaffer collateral synapse), once again reveled no deficit in either short or long phase LTP (*95*). These authors also performed input/output curves at the beginning and end of each experiment, and again found no difference, suggesting that *Fmr1*KO mice have normal basal synaptic transmission.

A turning point for the field came in 2001, when work from Kim Huber, then a post-doc in our lab, showed that a novel form of long-term depression in the hippocampus (*96, 97*) was *exaggerated* in the *Fmr1* KO (*98*). Unlike the NMDA receptor dependent forms of plasticity examined previously (*93, 95*), this plasticity is induced by activation of metabotropic glutamate receptors (mGluRs); there are two protocols that can be used to induce mGluR-LTD in the hippocampus: paired pulse low frequency stimulation (PP-LFS), which activates mGluRs synaptically, and direct application of DHPG (5 minutes, 100μM) (chemical-LTD). As is shown in **Figure 10**, both of these are exaggerated in *Fmr1*KO mice. Interestingly, this phenotype does not represent a general increase in depression, since low frequency stimulation (LFS), which is an NMDA receptor dependent form of LTD, is normal in KO mice. While the animals used in this study were significantly younger (postnatal day, P, 21-30) than animals used in the earlier reports, subsequent studies have shown that this plasticity phenotype persists in older (P30-60) animals as well (*99*). As discussed previously, mGluR-LTD requires protein synthesis for its stable expression (*96*). Interestingly in the *Fmr1*KO, this dependence on protein synthesis is lost (*99, 100*), raising the possibility that exaggerated signaling through mGluRs stimulates an over-production of "LTD proteins" in the *Fmr1*KO mice, which are available to sustain mGluR-LTD in the absence of *de novo* protein synthesis **(Figure 10)** (*51*).

Subsequent studies have also identified plasticity deficits at cortical (*101-104*), amygdalar (*105*) and cerebellar (*106*) synapses in the *Fmr1* KO. Many of these studies report an absence of LTP (*101-105*), rather than the exaggerated LTD seen in the hippocampus and cerebellum (*98-100, 106*). Because a variety of induction protocols

were used to illicit plasticity in these experiments, it is unclear whether they either have an mGluR dependent component (*101, 104, 105*), and whether GpI mGluR antagonists can correct these phenotypes (*101-106*). Interestingly, Larson and colleagues have noted that deficits in LTP are not present at earlier ages (*104*); suggesting that in addition to the exaggerated plasticity seen during development and adulthood, there is a senescent, perhaps neurodegenerative, decline in plasticity associated with FXS.

Wilson and Cox have recently reported that in brain slices from very young animals (P13-25), an "mGluR mediated form of LTP" is reduced in the *Fmr1*KO, which is not rescued by application of MPEP (*102*). Curiously, in WT animals, this plasticity requires NMDA receptors (partially blocked by CPP), mGlu1 receptors (partially blocked by MCPG (*107*)) and mGlu5 receptors (blocked by MPEP). However, synaptic field potentials in these studies were recorded in layer 5 by stimulating thalamocortical afferents in white matter. Since there is no direct thalamocortical input to layer 5 (*26*) (monosynaptic plasticity is usually studied in this layer by stimulating layer 2/3, from which layer 5 receives its primary feedforward input (*108*)), it is extremely likely that the plasticity reported in these studies reflects activation of a *polysynaptic* circuit (which is also suggested by the broad and shallow waveform). Neither input output curves, nor paired pulse recordings, were reported, so it is unclear whether this "LTP" reflects a change in basal transmission, release probability, or the compound effect of an altered circuit. In the *Fmr1* KO, the absence of plasticity is claimed to be due to a decrease in mGluR5 signaling (*102*), consistent with earlier reports that mGluR5 dependent protein synthesis is absent in the *Fmr1*KO (*109*). Given the contribution of both NMDA and mGlu1 receptors to this form of plasticity however, it is unclear whether the phenotype is

principally due to disrupted mGluR5 signaling (the effect of mGluR1 and NMDA receptor agonists and antagonists on this phenotype in *Fmr1* KO mice were not reported). Moreover, because these slices were prepared from very young animals, and DHPG-LTD has been shown to be protein synthesis independent at this age (*110*), the absence of a rescue by MPEP is likely to be orthogonal to the proposed interaction between Gp1 mGluRs and FMRP at the level of protein synthesis. Without further characterization of the synapse, at best what can be gleaned from these studies is that whatever this phenotype is, it is unaffected by MPEP, suggesting that mGluR5 antagonists should be safe for use as therapeutic agents in FXS.

One novel aspect of the current study was the use of ocular dominance plasticity as an *in vivo* assay of how experience-dependent synaptic modification is altered by the loss of FMRP. Wiesel and Hubel originally discovered that maintenance and refinement of binocular connections require appropriate visual experience in early postnatal life (*111*). Chronic recording experiments later showed that MD sets in motion a sequence of synaptic changes in visual cortex, characterized by a rapid and persistent loss of responsiveness to the deprived eye and a slower compensatory increase in responsiveness to the non-deprived eye (*56, 92*). Because MD triggers mechanisms of synaptic depression and potentiation, as well as homeostatic adaptations to an altered environment, ocular dominance plasticity is a particularly rich paradigm for understanding the interactions of genes and experience. The intersecting trends of using mice to study ocular dominance plasticity mechanisms and to model human diseases provided the opportunity to use this paradigm to get a more precise understanding of how development goes awry in a genetic disorder. The advantages of studying ocular

dominance plasticity *in vivo* over assays of synaptic modification *in vitro* are that brain circuits and activity patterns are intact, and it reflects processes involved in naturally occurring experience-dependent cortical development over a functionally relevant time course.

Previous work suggested that mGluR signaling is highest in visual cortex during the period of maximal synaptic plasticity (*14*), but experiments investigating the role of mGluRs in the response to MD using drug treatments were inconclusive (*cf.* (*58, 107*). The current findings strongly suggest an important role for mGluR5 in ocular dominance plasticity. Although more experiments will be required to pinpoint this role, an obvious clue comes from the genetic interaction with *Fmr1*. FMRP can act as a translational suppressor (*112, 113*), and ocular dominance plasticity, like many forms of persistent synaptic modification, requires protein synthesis (*114*). Thus, our findings suggest the intriguing hypothesis that the rate of plasticity in visual cortex is determined by level of activity-dependent protein synthesis, which is stimulated by mGluR5 and inhibited by FMRP. Consistent with this model, the phenotype in *Fmr1* KO mice appears to reflect "hyperplasticity", since 3 days of MD yielded effects on VEPs that normally require 7 days. This exaggerated plasticity was corrected by reducing mGluR5 expression by 50%.

Reconciling in vivo *and* in vitro *findings:*

Although it seems somewhat contradictory to propose that the hyperplasticity phenotype described here in the *Fmr1* KO may reflect exaggerated LTD, since manifest changes are consistent with increased potentiation, this is nevertheless at least conceptually possible in the context of metaplasticity. The Bienenstock, Cooper, Munroe (BCM) theory is a computational model of the synaptic modification threshold as a

function of the average activity of cortical neurons (*2*). Consistent with this theory, numerous studies have shown that long-term potentiation (LTP) and long-term depression (LTD) require different stimulation frequencies for their induction. These induction requirements can be dramatically altered depending on the recent history of cellular or synaptic activation, a phenomenon known as "metaplasticity" – the plasticity of synaptic plasticity (*115*).

As described above, previous experiments *in vivo* have shown that MD induces a series of changes that evolve according to a defined kinetic time course. Specifically, rapid deprivation (3 days) induced changes are characterized by depression of deprived eye responses. Extended deprivation (7 days) induces a delayed non-deprived eye potentiation (*92*) and an attenuated deprived eye depression. As shown in **Figure 8C**, these results can be understood in terms of the BCM theory. Deprivation of the contralateral eye initially drives synaptic depression in visual cortex. This initial change is a direct consequence of the integrated postsynaptic response function, Φ, and only requires uncorrelated input activity in the absence of pattered visual stimuli. These uncorrelated activity patterns are modeled *in vitro* by various LTD induction protocols such as low frequency stimulation (LFS); evidence that this mechanism is shared with deprivation induced depression comes from studies that have shown that such LTD is occluded in animals that have undergone 3 days of monocular deprivation (*71*). Longer deprivation paradigms reveal a second feature of the modification threshold, namely that it slides (metaplasticity). In the binocular zone of the mouse primary visual cortex, the contralateral eye is the dominant input (*90, 91*); when this input is deprived, cortical activity is sharply reduced, and the BCM theory predicts that the modification threshold

slides to the left, enabling deprivation induced potentiation driven by activity in the non-deprived eye.

In the *Fmr1* KO, exaggerated LTD could potentially lead to a more rapid shift in the modification threshold by excessively driving down cortical activity. Thus in these animals, 3 days of monocular deprivation might resemble 7 days of monocular deprivation in the WT not because LTP is exaggerated, but instead because the modification threshold is more rapidly shifted. This interpretation is suggested by the 1D MD results, since here only *Fmr1* KO depression is significant. According to this conceptual framework, reducing mGluR5 signaling restores appropriate levels of LTD in the *Fmr1* KO, which restores modification threshold sliding to normal rates. However, it must be remembered that "the visual cortex is not just the hippocampus with eyes" (quoting from Rob Crozier), and that future *in vitro* studies in the visual cortex must confirm reports of exaggerated LTD in the hippocampus of *Fmr1* KO mice for this conceptual framework to be viable.

For now, what is clear from these and several other previous studies (*98-100, 106*), is that many forms of plasticity are exaggerated in the *Fmr1*KO, and as has been shown here, this exaggerated plasticity can be rescued by selective reduction in mGluR5 signaling.

Dendritic Spine morphology in FXS

Invented in 1897, and named after its inventor Camillo Golgi, the Golgi stain is still used today to visualize "synapses." Of course, the synapse, which is only about 20 nm in size, is too small to be directly visualized by light microscopy. However, dendritic spines, first

identified by Golgi himself, can be visualized, and are used to estimate the number of excitatory synapses in the brain since each spine receives at least one excitatory input and are the postsynaptic half of the synapse. Dendritic spines are highly modifiable structures, and changes in spine density and morphology have been associated with a wide variety of environmental and experimental factors including hibernation, hormones that regulate the estrus cycle, stress, environmental enrichment, and learning and memory (see Hering and Sheng 2001, for review).

Spine abnormalities have long been associated with human mental retardation of unknown etiology (*116*), as well as with XLMR (x-linked mental retardation)(*117, 118*), Down (*119*), Patau (*120*), Rett (*48, 49*) and Fragile X syndromes (*121*). Among these, FXS is unique, since it is the only mental retardation syndrome associated with *increased* spine density (*50-54*). The increased spine density phenotype has been variably recapitulated in the *Fmr1* KO mouse (*122-125*), but see (*126*). Interestingly, one consequence of activating Gp1 mGluRs *in vitro,* is an increase in the density of long, thin spines *(52-54, 125).* Like LTD and glutamate receptor internalization, protein synthesis inhibitors block this effect of mGluR5 activation. Therefore it seemed plausible that FMRP and mGluR5 opponently regulate dendritic spine density. Here we re-examined the increased spine density phenotype in the *Fmr1*KO and asked whether this anatomical correlate of the disease could be reversed by selective reduction of mGluR5 gene dosage in the CR mouse.

Results

In these experiments, the Golgi-Cox silver staining method was used to visualize neuronal morphology. This fixation method, although over a hundred years old, is a

reliable technique for visualizing dendritic spine morphology *in situ*. Previous studies have focused on layer V pyramidal neurons in visual cortex from adult animals (*12, 83, 92*); however because of the distinct connectivity and plasticity profile of layer II/III pyramidal neurons (*127*), we chose to examine dendritic spine density in these neurons at P30, an age when they are thought to undergo the highest degree of experience dependent remodeling (*128*). It has been suggested that apical and basal compartments are differentially regulated according to laminar position and presynaptic input (*129*). Since apical and basal branches are thought to receive distinct afferent inputs (*130*), we examined dendritic spine density in apical and basal branches separately across the four genotypes (**Figure 11A**). In both apical and basal compartments we see a highly significant 10-15% increase in total dendritic spine density in the *Fmr1* KO, which is rescued by selective reduction in mGluR5 gene dosage in the CR mice (*Grm5* HT mice show no difference from WT) (representative images are shown in **Figure 11A**). This increase in the *Fmr1* KO spine density can be most readily appreciated as a right shift in the cumulative probability histogram, which is rescued in the CR, shown in **Figure 11B** (Apical, Kruskal-Wallis test $P < 0.0001$; Kolmogorov-Smirnov test WT:KO $P < 0.0001$; WT:HT $P = 0.3920$; CR:WT $P = 0.4407$; CR:KO $P < 0.0001$; Basal, Kruskal-Wallis test $P < 0.0001$; Kolmogorov-Smirnov test WT:KO $P < 0.0001$; WT:HT $P > 0.9999$; CR:WT $P > 0.9999$; CR:KO $P < 0.0001$).

We also observe significant differences in spine density between apical and basal branches, however this difference exists uniformly across genotypes (ANOVA Apical:Basal $P < 0.0001$; unpaired t-tests Apical:Basal WT $P = 0.0012$; Apical:Basal KO $P < 0.0001$; Apical:Basal HT $P < 0.0001$; Apical:Basal CR $P < 0.0001$). These results

suggest that while apical and basal compartments are differentially regulated, the loss of FMRP in the *Fmr1* KO causes increases in spine density irrespective of the type of input it receives, and furthermore, that this increase is opponently regulated by mGluR5.

Larkman has noted that spines are unevenly distributed across terminal segments, which is thought to reflect the differential ability of synapses to affect changes in membrane potential depending on proximity to the cell body and the thickness of the dendrite (*48, 49*). Therefore, we also performed a segmental analysis of spine density across the four genotypes. Consistent with Larkman's observations, we see an inverted U shaped distribution of synapses in both apical and basal terminal branches across all genotypes; however, as shown in **Figure 11C**, the density of spines is uniformly increased in the *Fmr1* KO and rescued in the CR (*Grm5* HT mice show no difference from WT) (Repeated measures ANOVA: Apical distance $P < 0.0001$, Apical distance* genotype $P < 0.0001$, Apical genotype $P < 0.0001$, Basal distance $P < 0.0001$, Basal distance*genotype $P = 0.0181$, Basal genotype $P < 0.0001$; ANOVA genotype: Apical, Basal, 10-100, in 10 μm segments $P < 0.0001$; unpaired t-tests Apical, Basal, 10-100, in 10 μm segments WT:KO $P < 0.05$, WT:HT $P > 0.05$, WT:CR $P > 0.05$, KO:CR $P < 0.05$). These results suggest that neither the *Fmr1* KO phenotype, nor the rescue by selective reduction in gene dosage in the CR, reflects a redistribution of synapses within the segment.

Discussion

Our finding here that dendritic spine density is increased in the *Fmr1* KO mouse is consistent with human studies (*51, 52, 54*), as well as with several previous studies in the mouse (*122, 124*). Some reports have suggested that *Fmr1* KO mice show *decreases* in

spine density (*51*); however, it is not clear that differences in tissue preparation or brain region can account for this incongruity, since (consistent with what we find here) more recent experiments (*50, 53*) have shown robust increases in spine density in hippocampal culture. In the somatosensory cortex, there is some evidence to suggest that the spine density phenotype might be developmentally restricted (*122, 124*), however this age restriction, if it exists in the visual cortex (see below) does not share the same time course, since we were able to detect robust increases at P30 (although we cannot exclude differences in cell layer and genetic background). In addition, the possibility remains that reports of decreased spine density (*50*) or developmental restriction of the phenotype (*131*) reflect underreporting of small thin spines, which are the morphometric counterpart of increased spine density in Fragile X (*54*), particularly since fluorescence intensity, and therefore detectability using fluorescence measures, is proportional to the volume of the labeled structure, and is necessarily decreased in the smallest thinnest spines. Early findings in the adult visual cortex (P60-80) suggested that spine density is increased (*125*), but later it was shown that this effect was due to the genetic background of the mice, which had a known mutation that causes retinal degeneration (*52*). Studies from the same group have shown that on the C57Bl6/J clonal background, *Fmr1* KO mice show robust increases in spine density in the adult (*51*). Taken together with our finding here, it seems unlikely that the visual cortex shows a developmentally restricted emergence of this phenotype.

Recent studies have shown a cell-autonomous role for FMRP in the regulation of dendritic spine density (*132-134*). Consistent with this interpretation, we show here that spine density is uniformly increased in the *Fmr1* KO, irrespective of laminar or lateral

distribution of synapses. Furthermore, our results here suggest that the rescue of this *Fmr1* KO phenotype by selective reduction in *Grm5* gene dosage is also cell-autonomous, since CR mice show no difference from WT in either spine density, or in the laminar and lateral distribution of synapses.

Changes in spine number, shape, and size reflect alterations in the strength of excitatory synaptic transmission. For example, the surface area of the postsynaptic density (PSD) is linearly correlated with the volume of the spine that includes it (*135*) and PSD surface area is linearly correlated with AMPA receptor content of the synapse (*136*) (and possibly with AMPA/NMDA receptor ratio (*137*), but see (*133, 138-140*)). The surface of the presynaptic active zone is correlated with PSD surface, and with the number of docked synaptic vesicles, and with physiological measures of presynaptic neurotransmitter release probability (*141, 142*). From such results, the morphology and density of spines at the light microscope level is thought to be correlated with the strength, or state of potentiation, of its synapse. In fact, recent live-imaging work has shown that the estimated volume of a spine is linearly correlated with the amplitude of post-synaptic currents at its synapse, and that long-term potentiation (LTP) of a synapse on an individual spine is associated with enlargement of the spine head (*93, 95, 98*).

Based on these observations, one interpretation of the increased spine density phenotype is that basal synaptic transmission should also be altered in the *Fmr1* KO. However, previous work has shown that *Fmr1* KO have normal basal transmission and excitability (*98-102, 104-106*), which is consistent with our finding here that baseline visual acuity measure *in vivo* is not altered in these mice. However, spine changes may reflect a more sensitive measure of synaptic strength, since unlike electrophysiologic

measures, which necessarily report whole cell or field potentials, dendritic spine measurements, capture changes at individual synapses. Consistent with this view, experimental manipulations of synaptic strength both *in vitro* (*126*) and *in vivo* (here), reveal significant plasticity deficits in the *Fmr1* KO mouse. Significantly, like spine morphology changes (*98-100, 106*), in many cases these plasticity deficits have been related to mGluR activation and protein synthesis (*126*) and (see chapter 2). Here we show direct evidence for the role of mGluR5 and FMRP in regulating synapses, since the increased spine density phenotype seen in *Fmr1* KO mice is completely rescued by reducing mGluR5 gene dosage in CR mice. Although 50% reduction in mGluR5 in the *Grm5* HT has no effect on dendritic spine density on its own, this almost certainly reflects the effect of gene dosage, since as mentioned above, mGluR5 has been shown to regulate dendritic spine morphology in WT animals *in vitro* (*15*) and the *Grm5* KO has been reported to have defects in synaptogenesis (*24, 127*).

Behavioral measures of learning and memory in *Fmr1* KO

Although humans with FXS show mental retardation in the moderate to severe range, prior studies of cognitive performance in *Fmr1* KO mice have revealed only subtle deficits (*47, 95, 105, 106, 143-151*) These studies have recently been summarized in a thorough review (*152*); a summary table from this review is provided in Figure 12 (references for the table are shown in Figure 13). Consistent with earlier observations (*143*), we found that acquisition of one-trial inhibitory avoidance (IA), a hippocampus-dependent memory, did not differ from normal in the *Fmr1* KO mice. However, we were inspired to additionally investigate IA *extinction* (IAE) by a recent report that this process

requires protein synthesis in the hippocampus (*153*). We discovered that IAE is exaggerated in the *Fmr1* KO mouse, and that this phenotype is corrected by reducing expression of mGluR5.

Results

Adult mice of all four genotypes were given IA training, followed at 6 and 24 hours by IAE training (**Figure 14A**). For each animal, we measured the latency to enter the dark side of the box on the first trial (baseline), the latency 6 hours later (post-acquisition) to assess IA memory, and again at 24 and 48 hours (post-extinction 1 and 2, respectively) to assess IAE. As shown in **Figure 14B**, animals of all four genotypes showed both significant IA acquisition at 6 h and extinction by 48 h. A global statistical test suggested that the pattern of learning across time varied across genotypes (repeated measures ANOVA genotype *time P = 0.0239). As shown in **Figure 14C-E**, these differences are likely due to extinction rather than acquisition of inhibitory avoidance. At 6 h, there was no difference across genotypes in latency to enter (6 h ANOVA P = 0.1525); however, at 24 h KO mice showed significantly shorter latencies, suggesting exaggerated extinction in the absence of FMRP. This phenotype was rescued by selective reduction in mGluR5 gene dosage in the CR mice (24 h ANOVA P = 0.0013; t-tests: WT:KO P < 0.0001,WT:HT P = 0.8251, WT:CR P = 0.1156, KO:CR P = 0.0132).

Although the difference was not statistically significant, there was a trend towards diminished latency in the KO mice at the 6 h time point, raising the possibility that the phenotype observed at 24 h might be due to less robust acquisition in addition to exaggerated extinction. Because the primary aim of this study is to examine genetic

interaction between *Fmr1* and *Grm5*, we performed a multivariate analysis which takes into consideration both acquisition and extinction as they vary across genotypes. As shown in **Figure 14F**, KO animals showed significant difference in 24 h latency (memory retention) as it varied with 6 h latency (memory acquisition), and this difference was rescued by the selective reduction in mGluR5 gene dosage in the CR mice (MANOVA for genotype 6:24 P = 0.0054, MANOVA WT:KO P = 0.0005, WT:HT 0.0785, KO:CR P = 0.0490, WT:CR P = 0.1863). The difference in retention across all genotypes was not significant by 48 h. Regardless of whether this KO phenotype reflects exaggerated extinction or diminished stability of the formed memory, there clearly is a significant genetic interaction between *Fmr1* and *Grm5*: The *Fmr1* KO phenotype is rescued by the selective reduction in mGluR5 expression.

Discussion

Animal studies of learning and memory found their champion in Ivan Petrovich Pavlov, the 19th century Russian psychologist who first described classical conditioning in dogs. The name Pavlov is omnipresent in my own memories of childhood, since my mother referred to his behavioral studies on dogs with some frequency. Usually these references were in the context of driving, when upon having taken the exit to her work (which daily conditioning had deeply entrained in her) instead of the exit to the place we were presently trying to get to, she would admonishingly slap her forehead and exclaim: "Just like Pavlov's dog!" Pavlovian conditioning is a form of associative learning where an unconditioned stimulus (US) is paired with a conditioned stimulus (CS) until the two stimuli are associated with one another. Inhibitory avoidance (IA) learning (also sometimes called passive avoidance learning) is a special case of Pavlovian conditioning,

such that a foot shock (which serves as the US, since it is intrinsically unpleasant and does not require conditioning) is paired with a spatial context, the dark side of a training box, (which serves as the CS since it is intrinsically pleasant, and is not feared without conditioning) until the animal learns to avoid the dark side of the box, even in the absence of foot shock. Both the hippocampus and the amygdala have been implicated in this form of learning.

In his classic 1927 book that formalized the study of conditioning, Pavlov also described a second form of learning: extinction. In the example of my mother's driving given above, extinction learning would involve her taking the exit to her work, but upon arrival realizing that her work was no longer there. After a while, this extinction training would presumably teach her not to get off at that exit anymore. This type of learning is distinct from reversal learning (see discussion below for more examples), since here training involves learning a new location to replace the old one (e.g. if my mother's work place had been moved, and she trained herself to drive to a new location). In extinction of inhibitory avoidance (IAE), repeated presentation of the CS (the dark side of the box) in the absence of the US (foot shock) causes conditioned responses to diminish. Pavlov described extinction as a form of inhibitory learning in its own right, ("the CS is not followed by the US") that leads to extinction memory via a protein synthesis-dependent consolidation process (*153-160*).

A second theory has been dubbed "reconsolidation." Here, the consolidated memory, although stable without further intervention, becomes labile again upon further exposure to the context, and is again susceptible to amnestic treatments (*161-166*). Since the retrieval trial activates the already-consolidated memory, this process has been called

reconsolidation, assuming that the labile memory trace corresponds to the original, already-consolidated memory (*162, 164*). Under the appropriate circumstances, reconsolidation is thought to strengthen the original memory. Advocates of this idea point to studies which have shown that the same molecular and cellular machinery becomes activated during the training and retrieval phases, suggesting that the same process is being reactivated during memory reconsolidation (*167*). Recently this idea has been challenged, since differences in the molecular processes have also been reported (*168-174*). Furthermore, Power and colleagues have shown that post-retrieval administration of protein synthesis inhibitors (infused into the hippocampus) only interferes with the memory if *extinction* is blocked, calling into question whether a re-exposure to the context can lead to memory lability, a phenomenon that is at the crux of the reconsolidation hypothesis (*153*).

Others have tried to reconcile extinction and reconsolidation by suggesting that they are processes in competition for the same cellular machinery. This so-called "trace dominance hypothesis" posits that if a retrieval trial leads to extinction of the memory, then reconsolidation does not happen and the memory is weakened. If on the other hand, extinction doesn't happen, then the memory is reconsolidated, and the memory is strengthened (*167, 175*).

Still others have opposed this view since spontaneous recovery of the original memory (due to "reconsolidation") is incomplete after extinction, suggesting that reconsolidation and extinction must be subserved by distinct cellular processes (*176*). These authors put forth an "internal-reinforcement hypothesis", which unlike the reconsolidation, posits that once a memory is consolidated it can not be made labile again

(consistent with (*153, 157, 177*)). Instead, the observed memory strengthening (which some have attributed to reconsolidation), is due to a new memory that has been created by comparing the retrieval context to the internal state of the animal at the time of retrieval. This process might alternately be called reminder learning, and behaviorally opposes extinction learning if the internal state of the animal reminds the animal that the context is unpleasant. In this formulation, the cellular processes underlying memory are informed by reward and punishment signals (e.g. from other brain regions like the amygdala and nucleus accumbens).

While the current experiments were not designed to distinguish between these alternative hypotheses, some attempt will be made here to interpret the findings in the *Fmr1* KO in the context of each. First, if extinction exists but reconsolidation does not (*153*), then these results suggest that *Fmr1* KO has exaggerated extinction. Consistent with this interpretation, mGluR5-LTD is exaggerated in the *Fmr1* KO (*98*), and like extinction is protein synthesis dependent (*96, 153*). A recent study showed that IA induces long-term potentiatiion (LTP) of Schaffer collateral synapses in area CA1 of the hippocampus (*10*); interestingly, neither NMDA dependent LTP in the CA1 area (*93, 95*) nor IA learning (shown in results above and (*143*)) is altered in the *Fmr1* KO. Thus it is tempting to speculate that IA extinction is exaggerated in *Fmr1* KO mice due, at least in part, to excessive mGluR-dependent synaptic weakening.

Second, if extinction and reconsolidation co-exist as opponent processes, it may be that the phenotype we observe in the *Fmr1* KO reflects a deficit in reconsolidation, such that the previously consolidated memories are more labile at the time of retrieval. This possibility is supported by the fact that protein synthesis-dependent reversal

(depotentiation) of CA1 LTP can be elicited by activation of mGluR5 (*178*), although this form of synaptic plasticity has not been examined in the *Fmr1* KO.

Finally, it may be that the internal-reinforcement regime is different for *Fmr1* KO, such that the reward-reinforcing properties of the CS are stronger than the aversive-reinforcing properties of the US. Although this idea is more difficult to conceptualize mechanistically, it is supported by the fact that the *Fmr1* KO phenotype is rescued by reduction in mGluR5 signaling, which has been implicated in reward based-learning. Moreover, studies of Go/No-Go reward learning paradigms in female patients with Fragile X, suggest abnormal patterns of brain activation during this task, consistent with abnormal reward based learning in these patients (*179*).

Strain specific variation has confounded previous attempts to identify a behavioral learning and memory phenotype in the *Fmr1* KO using the Morris water maze task, which tests a hippocampus-dependent form of spatial learning and memory (*152*). Recently, a robust deficit in hippocampus-dependent IA memory was described for *Fmr1* KO mice on the FVB/N clonal background (*148*). However, consistent with earlier findings using the water maze, we were unable to detect a significant IA deficit in the *Fmr1* KO mice on the C57BL/6 background (see also (*143*)). On the other hand, we were able to detect a difference in the rate of IA extinction, using the protocol introduced by Power *et al*. (*153*). Accelerated extinction in the KO mice was corrected by reducing mGluR5 expression.

METHODS

Electrode implantation, monocular deprivation and VEP recording.

Experiments were performed as previously described (*92*). Briefly, P26 mice from all four genotypes were anesthetized with 50 mg/kg (i.p.) ketamine. A head fixation post was attached to the skull just anterior to bregma and reference electrodes were placed bilaterally in prefrontal cortex. Two small (<0.5 mm) burr holes were made in the skull overlying the binocular visual cortex (3.05 mm lateral of lambda), and tungsten microelectrodes (FHC, Bowdoinham, ME) were inserted 450 μm below the cortical surface. Fixation posts and electrodes were secured in place using cyanoacrylate, and the entire exposure was covered with dental cement. Animals were monitored postoperatively for signs of infection or discomfort and were allowed at least 24 hours recovery before habituation to the restraint apparatus. 24 hours after habituation, on postnatal day 28, visually evoked potentials were recorded from awake mice. A total of 100 to 200 stimuli were presented per each condition. VEP amplitude was quantified by measuring peak-peak response amplitude. Stimuli consisted of full-field sine-wave gratings of 0% , 1.5%, 3%, 6%, 12%, 25%, 50% and 100% contrast, square reversing at 1 Hz, and presented at 0.05 cycles/degree. Stimuli were generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on a computer monitor suitably linearized by g correction. VEPs were elicited by either horizontal or vertical bars. The display was positioned 20 cm in front of the mouse and centered on the midline, thereby occupying 92° X 66° of the visual field. Mean luminance, determined by a photodiode placed in front of the computer screen, was 27 cd/m^2. Following

baseline recording, mice were anesthetized by inhalation of isoflurane (IsoFlo 2%–3%); eyelid margins were trimmed and three mattress stitches were placed using 6-0 vicryl, opposing the full extent of the trimmed lids. Mice were recovered by breathing room air and were monitored daily to be sure that the sutured eye remained shut and uninfected. After 3 days of deprivation mice were re-anesthetized, stitches were removed, and lid margins were separated. Eyes were then flushed with sterile saline and checked for clarity under a microscope. Once animals were fully alert, VEPs were recorded again, using the orthogonal stimulus orientation.

Golgi-cox

At postnatal day 30, animals from all four genotypes (n = 8 WT, 8 KO, 6 HT, 8 CR) were sacrificed by barbiturate overdose, and brains from each genotype were dissected and placed in 50 mL of freshly prepared Golgi-cox fixative (5% potassium dichromate, 5% Mercuric chloride, 4% potassium chromate in ddH20) and stored in amber colored glass vials at room temperature (RT) for 6-10 weeks. Brains were then removed from fixative and dehydrated in a series of ethanol washes (50%, 1hour, 70% 1hour, 95% 2 hours, 100% 1 hour), followed by an 1:1 ether:ethanol wash (100% Ether: 100% EtOH). Brains were then placed in 15% Nitrocellulose (15g cellulose nitrate, 50mls 100% Ether, 50mls 100% Ether) overnight; followed by 30% nitrocellulose in a mold. Nitrocellulose was solidified over choloroform overnight, followed by submersion in chloroform for 2-4 weeks. 120 μm thick sections were made using a rotary microtome (Jung RM 2044; Leica, Rueil-Malmaison, France). Sections were then be dehydrated in ethanol, developed in 5% sodium carbonate solution, cleared in cedar wood oil followed by xylene, mounted on slides and coverslipped with DPX mountant. Using the NeuroLucida

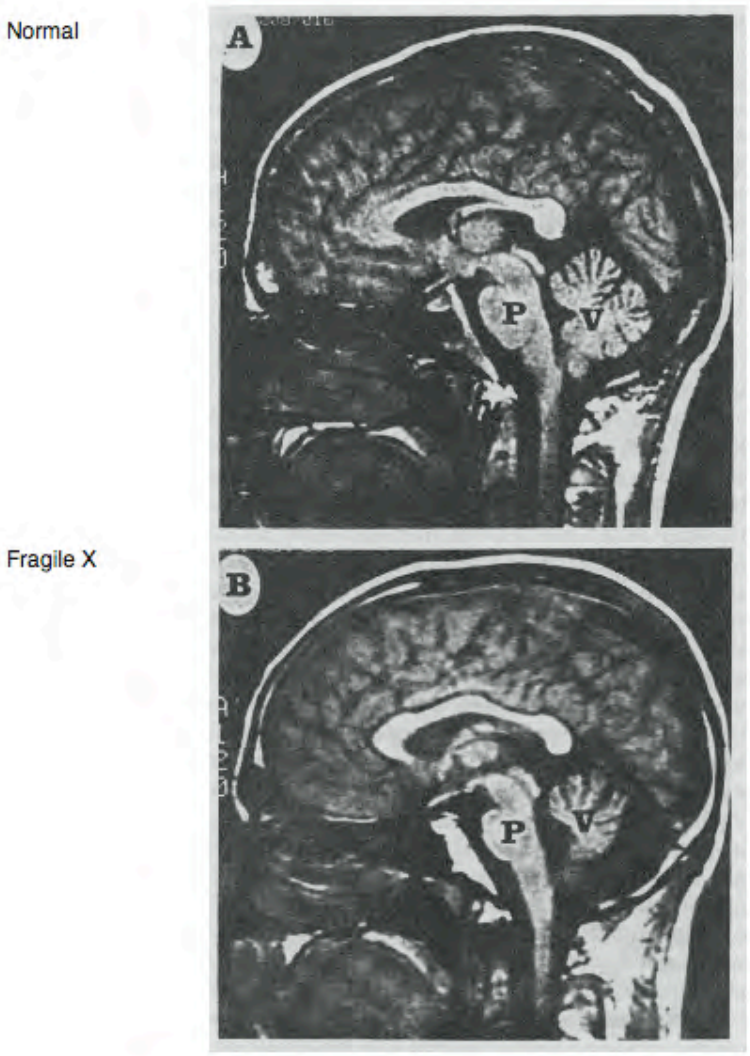
image analysis system (Microbrightfield, Wiliston, VT) attached to an Olympus (Melville, NY) microscope (100, 1.3 numerical aperture, Olympus BX61) spine density was analyzed in layer II/III pyramidal neurons in the binocular zone of visual cortex; apical and basal terminal branches were analyzed separately (n = 80 WT, 80 KO, 60 HT, 80 CR apical branches, and n = 80 WT, 80 KO, 60 HT, 80 CR basal branches). For purposes of segmental analysis, spine densities within each 100μm segment were tallied in 10μm sub-segments. All protrusions, irrespective of their morphological characteristics, were counted as spines if they are in direct continuity with the dendritic shaft.

Inhibitory Avoidance Extinction

Experiments were performed as previously described (*153*). Briefly, on the day before training adult mice of all four genotypes (P60-P110) were moved into the behavioral testing room. On the day of testing, animals were placed into the dark compartment of an IA training box (a two-chambered Perspex box consisting of a lighted safe side and a dark shock side separated by a trap door) for 30 seconds followed by 90 seconds in the light compartment for habituation. Following the habituation period, the door separating the two compartments was opened and animals were allowed to enter the dark compartment. Latency to enter following door opening was recorded ("baseline", time 0, 8a.m.-12p.m.); animals with baseline entrance latencies of greater than120 seconds were excluded. After each animal stepped completely into the dark compartment with all four paws, the sliding door was closed and the animal received a single scrambled foot-shock (0.5mA, 2.0 sec) via electrified steel rods in the floor of the box. This intensity and duration of shock consistently caused animals to vocalize and jump. Animals remained

in the dark compartment for 15 sec following the shock and were then returned to their home cages. Six to seven hours following IA training, mice received a retention test ("post-acquisition", time 6 hours, 2p.m.-6p.m.). During post-acquisition retention testing each animal was placed in the lit compartment as in training; after a 90 second delay, the door opened, and the latency to enter the dark compartment was recorded (cutoff time 540 sec). For inhibitory avoidance extinction (IAE) training, animals were allowed to explore the dark compartment of the box for 200 seconds in the absence of foot-shock (animals remaining in the lit compartment after the cutoff were gently guided, using an index card, into the dark compartment); following IAE training animals were returned to their home cages. Twenty-four hours following initial IA training, mice received a second retention test ("post-extinction 1", time 24 hours, 8a.m-12p.m.). Animals were tested in the same way as at the six hour time point, followed by a second 200 second extinction trial in the dark side of the box; following training animals were again returned to their home cages. Forty-eight hours following avoidance training, mice received a third and final retention test ("post-extinction 2", time 48 hours, 8a.m.-12p.m.).

Figure 1. Imaging (MRI) studies of FXS

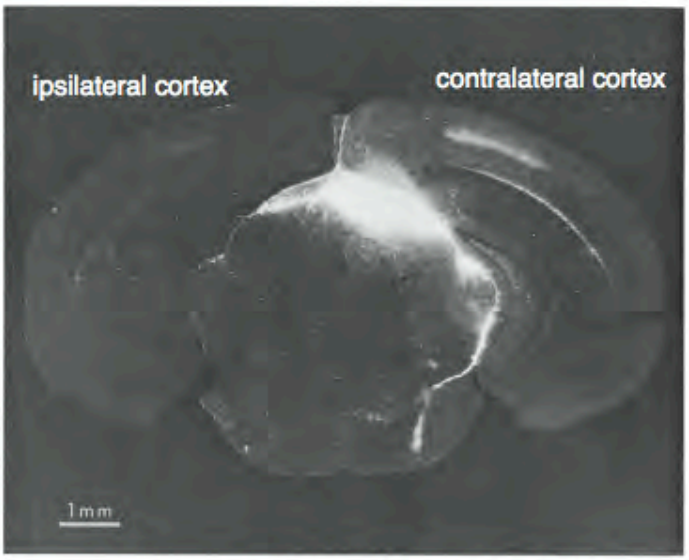


Mostofsky et al., 1988

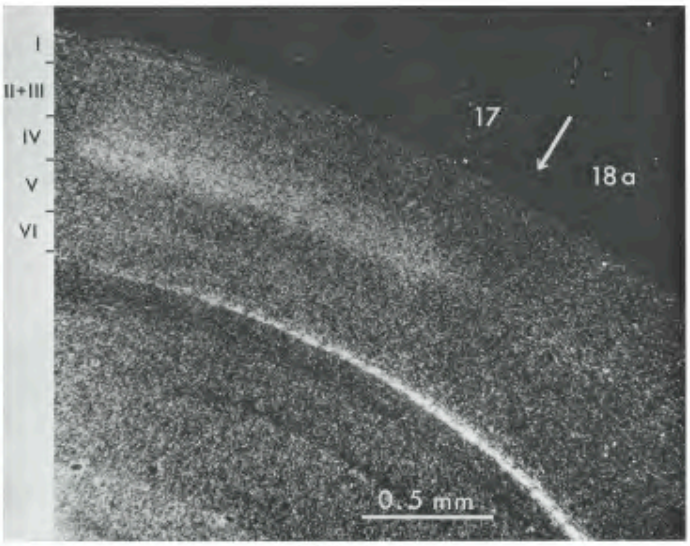
Brain imaging studies in human patients with FXS. Notice the decreased size of the cerebellum.

Figure 2. Pioneering anatomical studies by Ursula Dräger, 1974.

A.



B.



Organization of the mouse visual system.(A) Intraocular injection of radioactive label, which passes transynaptically to the cortex, reveals 2 fold greater projection to the contralateral versus ipsilateral cortex. (B) Cortical label is strongest in layer 4, where thalamocortical axons synapse.

Figure 3. Electrophysiological delineation of binocular zone: Ursula Dräger, 1975.

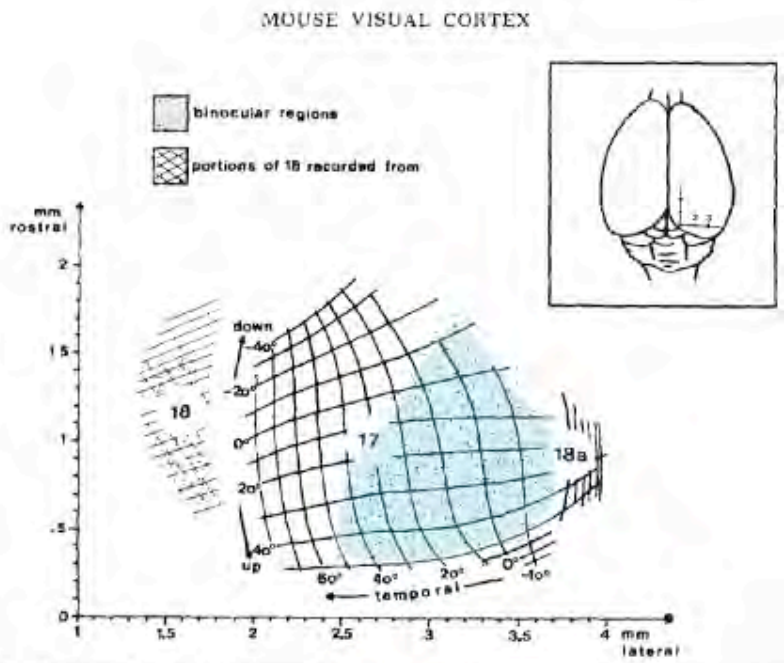


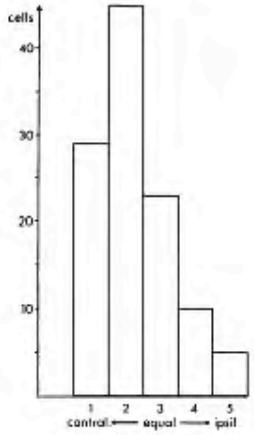
Fig. 9 Topographic map of visual field representation in area 17. Position of the axes, relative to the brain, are indicated in the inset. The positions of the adjoining areas 18 and 18a are merely indicated.

Topographic map of binocular mouse visual cortex. Area highlighted in blue represents region where binocularly responsive cells can be found.

Figure 4. Monocular deprivation: Ursula Dräger

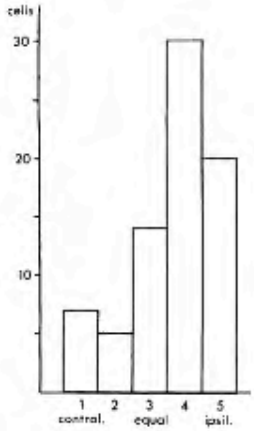
A. Electrophysiological effects of MD

Non-MD (units recorded in contralteral hemisphere)



Adapted from: Drager 1978

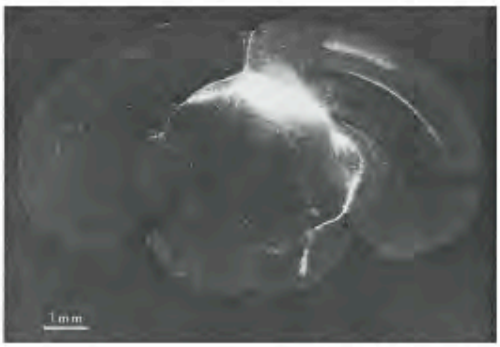
Post MD (units recorded in contralteral hemisphere)



Adapted from: Drager 1978

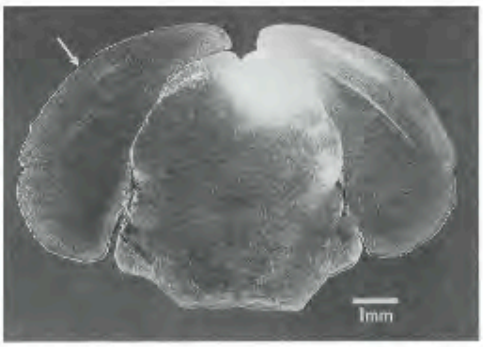
B. Anatomical effects of MD

Non-MD (label injected into contralteral eye)



Adapted from: Drager 1974

Post MD (label injected into contralteral eye)



Adapted from: Drager 1978

Monocular deprivation in mouse. (A) Extended deprivation (before eye opening until adult) leads to dramatic shift in ocular dominance recorded electrically. The percentage of contralaterally responsive cells is dramatically reduced, while the percentage of ipsilaterally responsive cells is increased. (B) The anatomical projection pattern is not dramatically changed in response to this same manipulation.

Figure 5. Visually evoked potentials (VEPs) recorded in mouse visual cortex: Porciatti, 1999

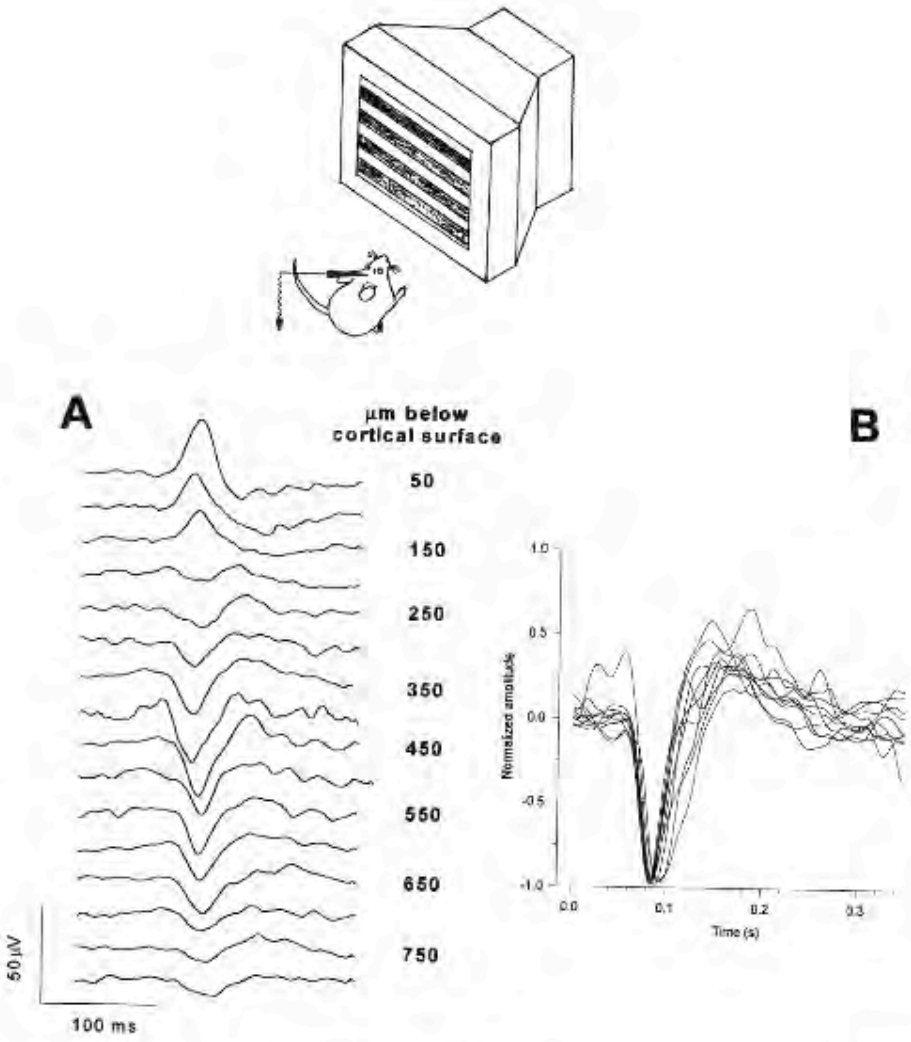


Fig. 1. Laminar analysis of the mouse VEPs. (A) Examples of VEPs recorded at different depths of the binocular visual cortex contralateral to the stimulated eye, in response to a visual pattern constituted of sinusoidal gratings (0.06 c/deg, 90% contrast, 25 cd/m² mean luminance, 81 × 86° field size, 1 Hz contrast reversal). The electrode was inserted 3.0 mm lateral to lambda. Note that the waveform reverses its polarity at about 200 µm cortical depth, suggesting a major dipole generator in layer II–III. (B) VEPs with maximal amplitude are recorded at 400 µm cortical depth, and display a major negative wave with a peak latency of 90–100 ms. Waveforms of different animals (n = 10) are superimposed to show consistency of cortical VEPs in mice.

Visually evoked potentials (VEPs). (A) Contralateral eye stimulation with patterned stimuli, reveals VEPs that have a maximum negativity (peak latency 90-100 ms) at a cortical depth of 400 μm below the surface of the brain. (B) superimposed VEP waveforms from multiple animals (n = 10) show consistency of VEP technique.

Figure 6. Visual acuity can be measured behaviorally in mice. Prusky and Douglas, 2003

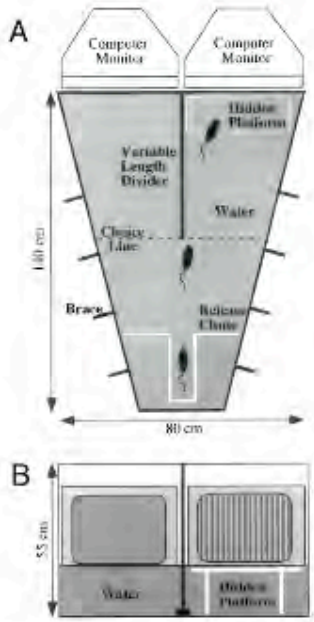


Fig. 1. Schematic diagram and components of the visual water box. (A) View from above showing the major components including pool, midline divider, platform, starting chute and two monitors. The pool is filled with clean water (gray), and the braces are needed to resist its weight. Following release from the chute, animals choose to swim on the side of the pool displaying the grating in order to find the hidden platform and escape from the water. (B) Front view showing monitor screens, submerged platform and midline divider. See text for details.

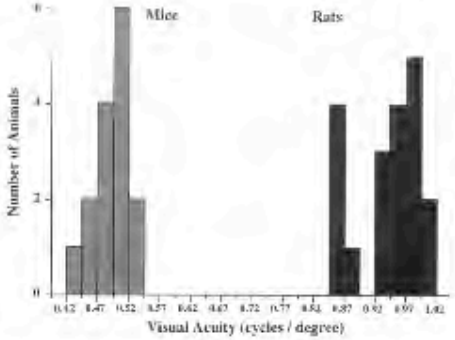
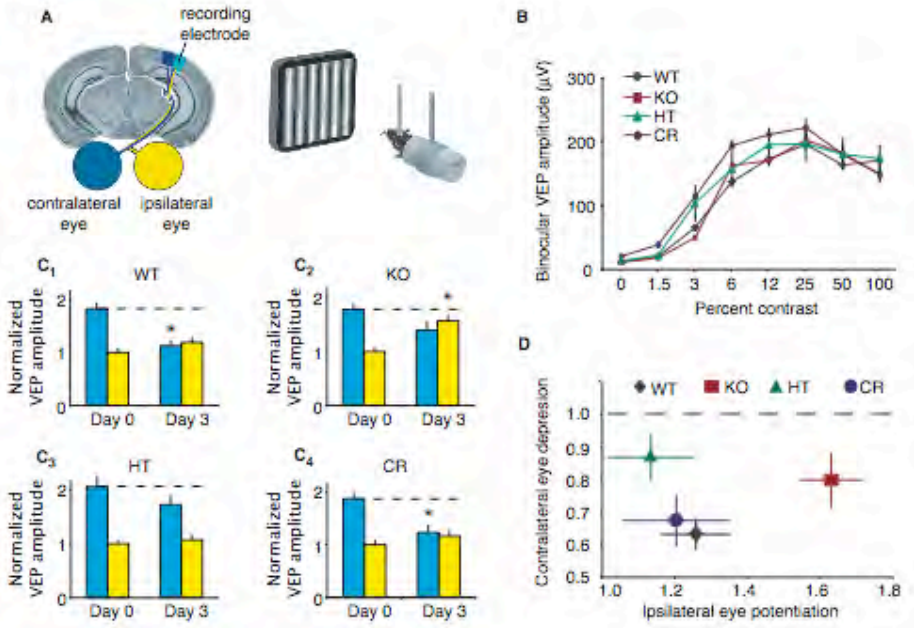


Fig. 5. Distribution of acuities for mice (left) and rats (right). Acuities were clustered within a narrow range for each species, and the average acuity of rats was approximately twice that of mice.

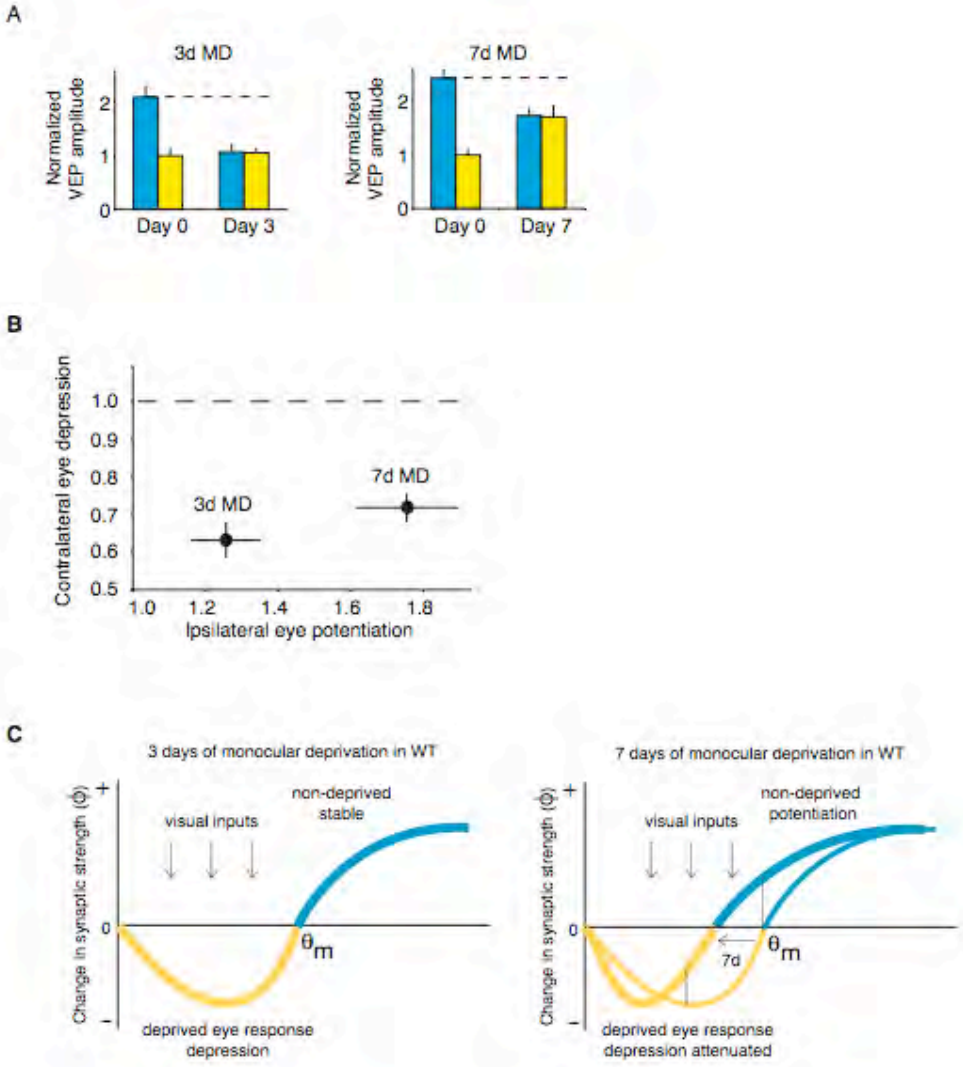
Visual Acuity measured behaviorally. (A) Schematic of visual water box. (B) Mouse acuity peaks at 0.5 cycles/degree.

Figure 7. VEP recording



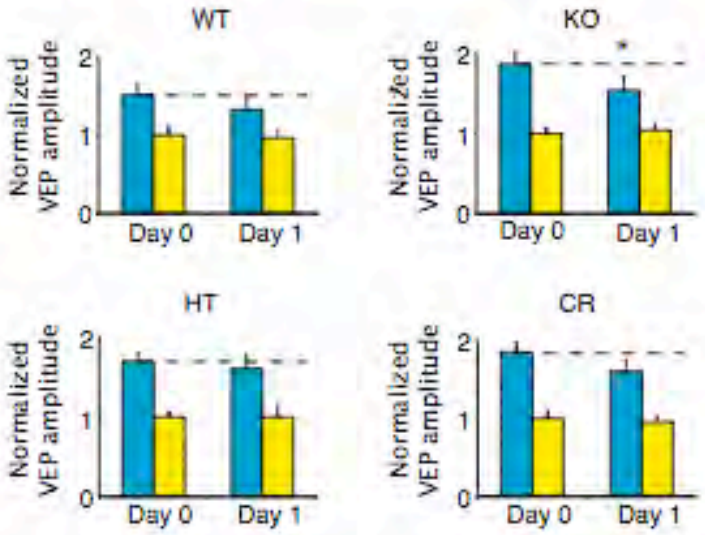
Genetic rescue of ocular dominance plasticity phenotype in FXS. (**A**) Schematic of the mouse visual pathway and position of the recording electrode in primary visual cortex. (**B**) Absolute VEP amplitudes recorded during binocular viewing across contrasts (0 –100%, square reversing at 1 Hz, 0.05 cycles/degree). No significant differences across genotypes. (n = 46 WT, n = 33 KO, n = 8 HT, n = 20 CR hemispheres, MANOVA P = 0.0868). (**C**) Effect of 3 day MD on VEP amplitude (data expressed as mean ± SEM, normalized to day 0 ipsilateral eye value. (**C_1**) WT mice (n = 19). Note significant deprived eye depression. (**C_2**) KO mice (n = 18). Note significant open eye potentiation. (**C_3**) HT (n = 16). Note absence of deprived eye depression. (**E_4**) CR mice (n = 13). Note rescue of KO phenotype. Post-hoc t-tests: * indicates significantly different from baseline (day 0). (**D**) Plots (mean ± SEM) of the fractional change in open and deprived eye responses after 3 day MD show rescue of the KO phenotype in CR mice.

Figure 8. Ocular dominance plasticity varies with duration of deprivation



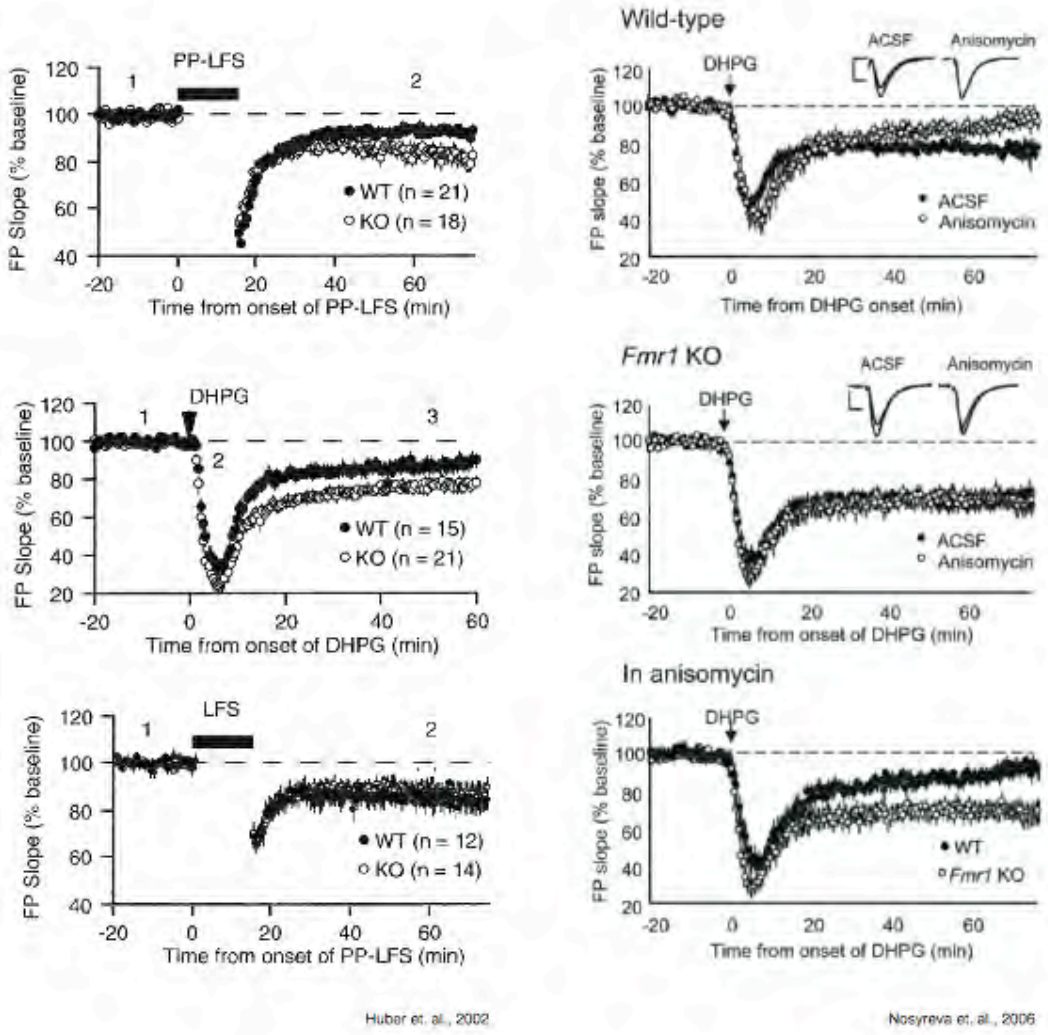
Changes in VEP amplitude as a function of deprivation duration in WT mice. These data are re-plotted from Frenkel and Bear (2004). (**A**) MD of the contralateral eye in WT mice for 3 days induces statistically significant changes in contralateral-eye VEP responses (blue bars representing mean ± SEM, n = 15). (**B**) 7 days of MD in WT mice induces statistically significant changes in contralateral and ipsilateral (yellow bars) VEP responses (n = 17) Post-hoc t-tests: * equals significantly different from baseline (day 0). **(C)** Data from animals in A and B are replotted using a plasticity index that indicates the fractional changes in contralateral and ipsilateral eye responses. Significant differences exist in the plasticity profile between 3 and 7 days MD (MANOVA 3d:7d P = 0.020). (**C**) BCM theory model of metaplastic changes after 3 (left) and 7 (right) days of MD.

Figure 9. 1 day of Monocular deprivation



Changes in VEP amplitude across genotypes after 1 day of MD. Although global differences across genotypes after 1 day of monocular deprivation are not statistically significant (MANOVA: p = 0.9203); subtler trends nevertheless exist. For example, while WT, KO, and CRS mice all exhibit a trend towards response depression, the magnitude of the KO depression is greater (17% depression KO, 12% depression WT, 14% depression CRS) and only in the *Fmr1 KO* is this response depression statistically significant (paired t-test WT pre:post p = 0.088, KO pre:post p = 0.027, CRS pre:post 0.054).

Figure 10. mGluR LTD is exaggerated and protein synthesis independent in Fmr1 KO



mGluR5 dependent LTD in the hippocampal CA1. PP-LFS and DHPG LTD are exaggerated in the Fmr1 KO, but LFS is not. In wild-type mice DHPG induced LTD is blocked by anisomycin, a protein synthesis inhibitor. Anisomycin has no effect on this form of LTD, suggesting that in Fmr1 KO mice, de novo protein synthesis is not required for the stable expression of LTD.

Figure 11. Dendritic spine abnormalities in mental retardation syndromes: Marin-Padilla, 1972

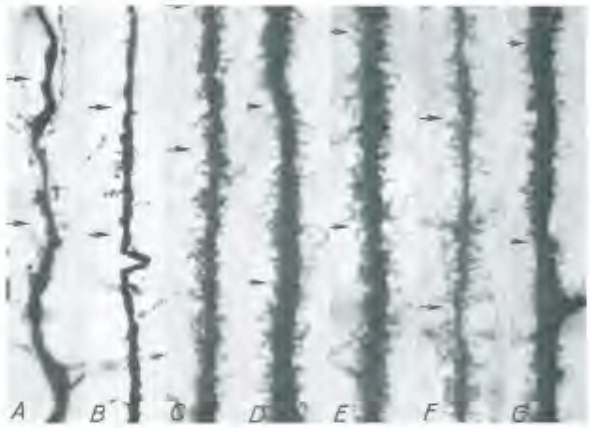


Fig. 1. Detail of the apical dendrites of the giant pyramidal neurons (layer V) of the human cerebral cortex in a variety of normal and abnormal cases. They are all reproduced at the same magnification to facilitate their comparative analysis. Each one represents a section of the apical dendrite of layer V pyramidal neuron crossing the territory of layer III of the cerebral cortex. The apical dendritic segments illustrated here belong to the following cases: (A) a 5-month fetus; (B) a 7-month fetus; (C) a newborn; (D) a 2-month-old infant; (E) an 8-month-old infant; (F) a newborn girl with D1 (13–15) trisomy (Patau syndrome); and (G) an 18-month-old mentally retarded girl with 21 trisomy (Down syndrome, mongolism). They illustrate the morphological characteristics of the human dendritic spine of the pyramidal neurons of the motor cortex during the course of prenatal and early postnatal cortical development, and those of two infants with proven chromosomal trisomies. Rapid Golgi method.

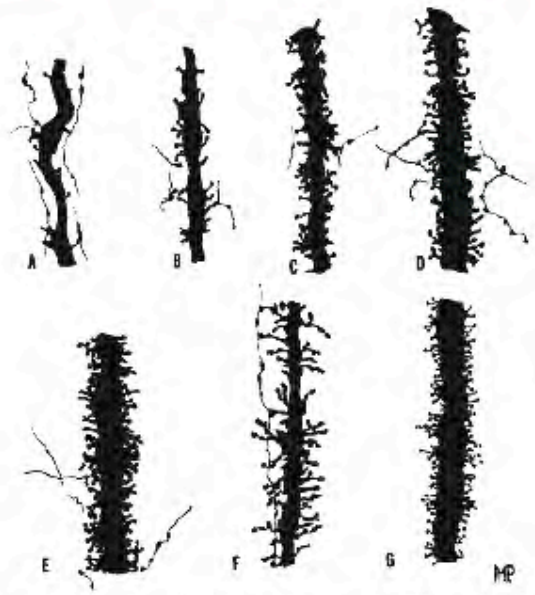
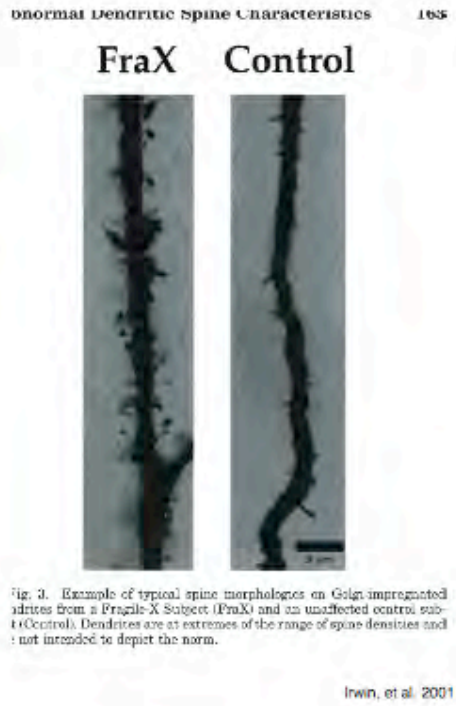


Fig. 2. Camera lucida drawings made from the Golgi preparations of each one of the apical dendrites shown in Fig. 1 to illustrate perhaps clearly and with more detail the morphological characteristics of the apical dendritic spines of normal and abnormal human cerebral cortices. The dendritic segments reproduced in these drawings are those marked by arrows in the apical dendrites shown in Fig. 1. The drawing of each dendritic segment is identified by the same capital letter used in the identification of the different apical dendrites of Fig. 1

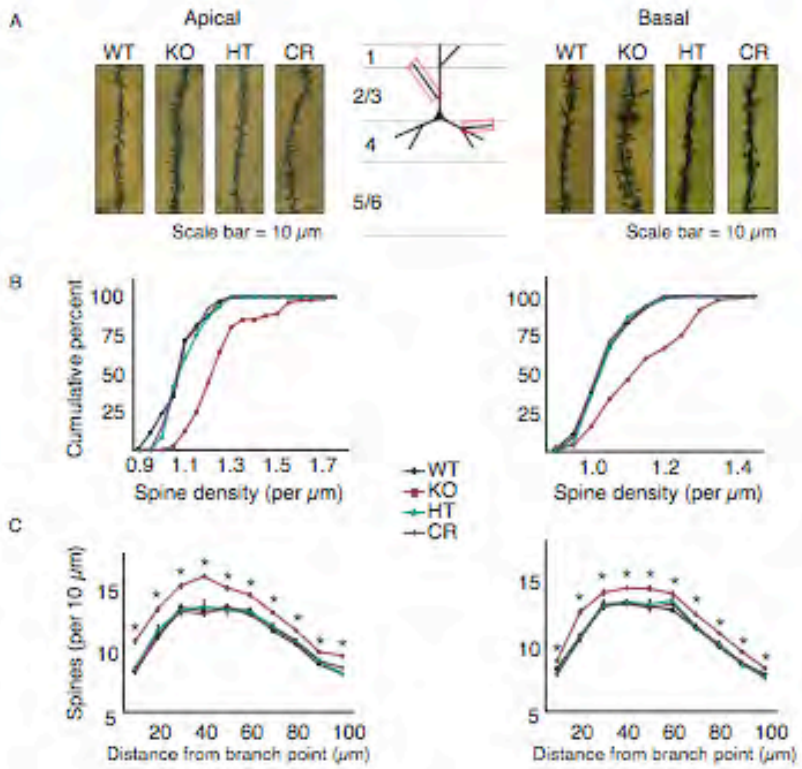
Dendritic spine abnormalities are apparent in a variety of mental retardation syndromes. (A) Golgi-cox labeled segments in normal development (a-e), downs syndrome (g) and Patau syndrome (f). (B)Camera lucida drawings of spine abnormalities.

Figure 12. Increased dendritic spine density in Human patients with fragile X



Dendritic spine phenotype. Increased dendritic spine density in human patients with FXS, compared to unaffected control. Golgi-impregnated dendrites, extreme not representative, examples are shown.

Figure 13. FXS Dendritic spine phenotype rescued by reduction in Grm5 gene dosage



Genetic rescue of dendritic spine phenotype in FXS. (**A**) Representative images from apical (left) and basal (right) dendritic segments of layer 3 pyramidal neurons in the binocular region of primary visual cortex of all four genotypes collected at P30. (**B**) Cumulative percent spines per μm in each dendritic segment; apical branches, left; basal branches right (n = 80 WT, 80 KO, 60 HT, 80 CR apical branches, and n = 80 WT, 80 KO, 60 HT, 80 CR basal branches). (**C**) Segmental analysis of spine density; number of spines per 10 μm bin, given as distance from the origin of the branch, for apical (left) and basal (right) segments across four genotypes.

Figure 14. Summary of learning and memory deficits in the Fmr1 KO mouse

Test	Background	Result	Ref.
Learning and memory			
Cross-shaped water maze	FVB	Correct trials: KO < WT	[102]
	B6	Escape latencies: KO = WT	[98]
		Correct trials:	
		KO < WT	[98]
		KO = WT	[102]
Changing position of platform in water maze	B6	KO = WT	[97,98]
E-shaped water maze	B6	KO = WT	[89]
Morris water maze training:			
Hidden-platform condition	B6	Escape latencies:	
		KO = WT	[96,97,101]
		KO > WT	[89]
		KO > WT the first four trials	[82]
	FVBxB6	Escape latencies: KO > WT	[83]
	B6	Rate of learning: KO = WT	[82,89,102]
	FVB	Rate of learning: KO < WT	[102]
Visible-platform condition	B6	Escape latencies: KO = WT	[82,96]
Radial maze	B6	Working memory: KO = WT	[91]
	FVBxB6	Working memory: KO < WT the first 6 days; reference memory: KO < WT; strong choice design: KO = WT	[83]
Barnes maze	FVBxB6	KO = WT; during probe test: KO < WT	[83]
Fear conditioning: context and conditioned cue	FVB	KO = WT	[102]
	B6	KO = WT	[98,101,102]
	B6	KO < WT	[97]
Trace fear conditioning	B6	KO < WT	[100]
Conditioned eyelid blink reflex	B6	KO < WT	[109]
Passive avoidance (latency to enter dark compartment)	B6	KO = WT	[82]
	FVB	KO = WT	[108]
Lever press escape/avoidance task	B6	KO < WT	[113]
Instrumental conditioning	B6	Conditioning learning : KO = WT	[73]
		Devaluation of reward and omission of lever press : KO > WT	
Olfactory learning and memory tasks	FVBxB6	KO = WT	[83]
Novel object task	FVBxB6	KO = WT	[83]
	FVB	KO < WT	[114]

From: Bernardet and Crusio, 2006

Summary table of studies aimed at determining whether learning and memory deficits are recapitulated in Fmr1 KO mouse.

Figure 15. Summary of learning and memory deficits in the Fmr1 KO mouse (references)

73. Frankland, P.W., Wang, Y., Rosner, B., Shimizu, T., Balleine, B.W., Dykens, E.M., Ornitz, E.M., and Silva, A.J. (2004) Sensorimotor gating abnormalities in young males with fragile X syndrome and *Fmr1*-knockout mice. *Mol. Psychiatry* **9**, 417–425.

82. The Dutch-Belgian Fragile X Consortium (1994) *Fmr1* knockout mice: a model to study fragile X mental retardation. *Cell* **78**, 23–33.

83. Yan, Q.J., Asafo-Adjei, P.K., Arnold, H.M., Brown, R.E., and Bauchwitz, R.P. (2004) A phenotypic and molecular characterization of the *fmr1-tm1Cgr* fragile X mouse. *Genes Brain Behav.* **3**, 337–359.

89. Kooy, R.F., D'Hooge, R., Reyniers, E., Bakker, C.E., Nagels, G., De Boulle, K., Storm, K., Clincke, G., De Deyn, P.P., Oostra, B.A., and Willems, P.J. (1996) Transgenic mouse model for the fragile X syndrome. *Am. J. Med. Genet.* **64**, 241–245.

91. Mineur, Y.S., Sluyter, F., de Wit, S., Oostra, B.A., and Crusio, W.E. (2002) Behavioral and neuroanatomical characterization of the *Fmr1* knockout mouse. *Hippocampus* **12**, 39–46.

96. D'Hooge, R., Nagels, G., Franck, F., Bakker, C.E., Reyniers, E., Storm, K., Kooy, R.F., Oostra, B.A., Willems, P.J., and De Deyn, P.P. (1997) Mildly impaired water maze performance in male *Fmr1* knockout mice. *Neuroscience* **76**, 367–376.

97. Paradee, W., Melikian, H.E., Rasmussen, D.L., Kenneson, A., Conn, P.J., and Warren, S.T. (1999) Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience* **94**, 185–192.

98. Van Dam, D., D'Hooge, R., Hauben, E., Reyniers, E., Gantois, I., Bakker, C.E., Oostra, B.A., Kooy, R.F., and De Deyn, P.P. (2000) Spatial learning, contextual fear conditioning and conditioned emotional response in *Fmr1* knockout mice. *Behav. Brain Res.* **117**, 127–136.

100. Zhao, M.G., Toyoda, H., Ko, S.W., Ding, H.K., Wu, L.J., and Zhuo, M. (2005) Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J. Neurosci.* **25**, 7385–7392.

101. Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., and Nelson, D.L. (2000) (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. *Hum. Mol. Genet.* **9**, 1145–1159.

102. Dobkin, C., Rabe, A., Dumas, R., El Idrissi, A., Haubenstock, H., and Brown, W.T. (2000) *Fmr1* knockout mouse has a distinctive strain-specific learning impairment. *Neuroscience* **100**, 423–429.

108. Qin, M., Kang, J., and Smith, C.B. (2005) A null mutation for *Fmr1* in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior. *Neuroscience* **135**, 999–1009.

109. Koekkoek, S.K., Yamaguchi, K., Milojkovic, B.A., Dortland, B.R., Ruigrok, T.J., Maex, R., De Graaf, W., Smit, A.E., VanderWerf, F., Bakker, C.E., Willemsen, R., Ikeda, T., Kakizawa, S., Onodera, K., Nelson, D.L., Mientjes, E., Joosten, M., De Schutter, E., Oostra, B.A., Ito, M., and De Zeeuw, C.I. (2005) Deletion of FMR1 in Purkinje cells enhances parallel fiber LTD, enlarges spines, and attenuates cerebellar eyelid conditioning in Fragile X syndrome. *Neuron* **47**, 339–352.

113. Brennan, F.X., Albeck, D.S., and Paylor, R. (2006) Fmr1 knockout mice are impaired in a leverpress escape/avoidance task. *Genes Brain Behav.* **5**, 467–471.

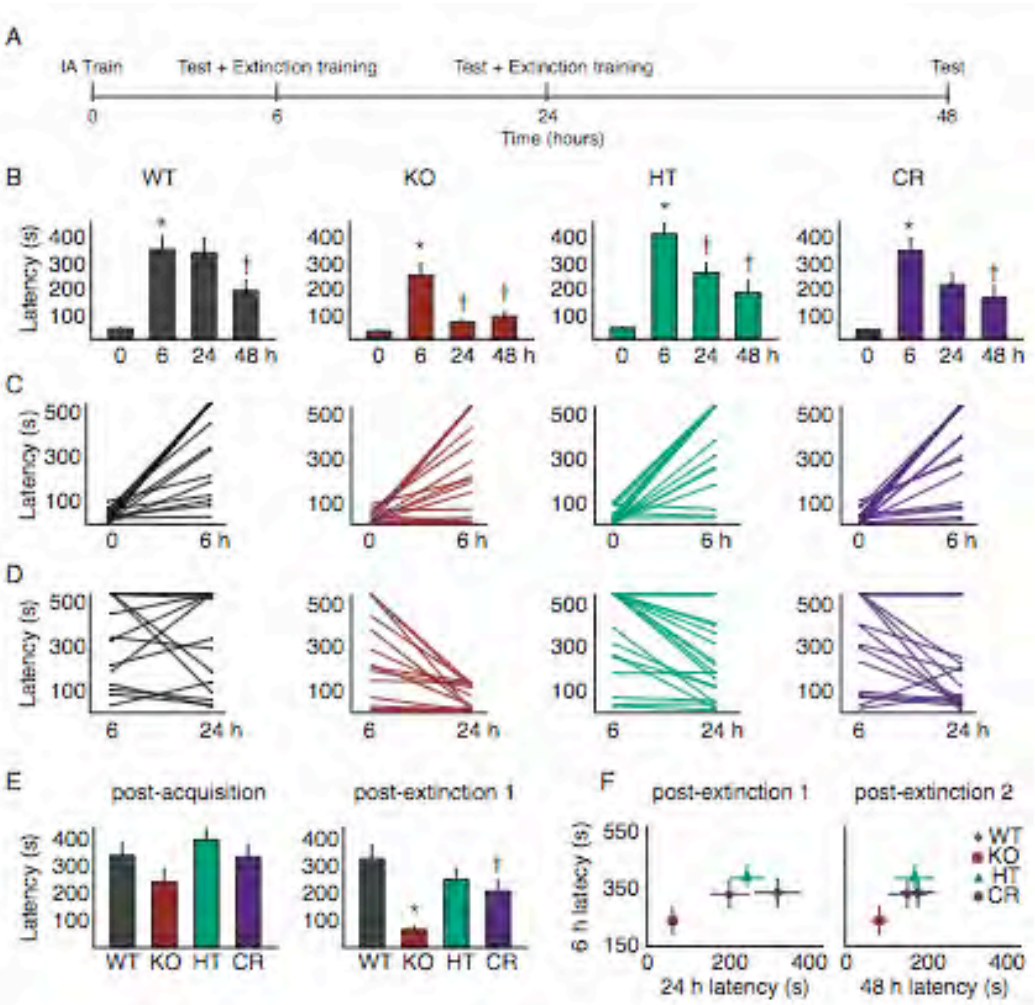
114. Ventura, R., Pascucci, T., Puglisi-Allegra, S., Catania, M.V., and Musumeci, S.A. (2004) Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: Involvement of dopamine in the medial prefrontal cortex. *Behav. Pharmacol.* **15**, 433–442.

115. Musumeci, S.A., Bosco, P., Calabrese, G., Bakker, C., De Sarro, G.B., Elia, M., Ferri, R., and Oostra, B.A. (2000) Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. *Epilepsia* **41**, 19–23.

From: Bernardet and Crusio, 2006

References for summary table shown in Figure 14.

Figure 16



Genetic rescue of behavioral learning and memory phenotype in FXS. (**A**) Experimental design. Animals of all 4 genotypes (n = 15 WT, n = 15 KO, n = 20 HT, n = 17 CR) were given IA training at time 0 and the latency to enter the dark side was measured at 6 h. They were then given IAE training, and latency was again measured at 24 h. Testing was followed by another round of IAE training, and latency was measured again at 48 h. (**B**) Animals in all four genotypes showed significant acquisition and extinction. Post-hoc t-tests: * indicates significantly different from time 0h, † indicates significantly different from time 6h. (**C**) Raw data for acquisition of IA in the four genotypes. Each line represents the change in latency to enter the dark side for one mouse (data from some mice superimpose). (**D**) Raw data for extinction-1 in the four genotypes. (**E**) Comparison of latency (mean ± SEM) across genotypes for 6 hour time point (post acquisition) and 24 hour time point (post extinction 1). Post-hoc t-tests: * indicates significantly different from WT, † indicates significantly different from KO. (**F**) Multivariate analysis of extinction as a function of acquisition at 24 h time point (post extinction-1) and 48 h time point. Plotted are mean latencies ± SEM.

Chapter 3 References

1. W. James, *The Principles of Psychology* (Dover Publications. Inc, New York, ed. Dover edition, 1890), pp.
2. E. L. Bienenstock, L. N. Cooper, P. W. Munro, *J Neurosci* **2**, 32 (Jan, 1982).
3. D. O. Hebb, *The organization of behavior; a neuropsychological theory* (Wiley, New York, 1949), pp.
4. M. F. Bear, L. N. Cooper, F. F. Ebner, *Science* **237**, 42 (Jul 3, 1987).
5. E. Herrera *et al.*, *Cell* **114**, 545 (Sep 5, 2003).
6. S. E. Williams, C. A. Mason, E. Herrera, *Curr Opin Neurobiol* **14**, 51 (Feb, 2004).
7. S. E. Williams *et al.*, *Neuron* **39**, 919 (Sep 11, 2003).
8. T. N. Wiesel, D. H. Hubel, *J Neurophysiol* **28**, 1029 (Nov, 1965).
9. D. H. Hubel, T. N. Wiesel, *J Physiol* **206**, 419 (Feb, 1970).
10. J. R. Whitlock, A. J. Heynen, M. G. Shuler, M. F. Bear, *Science* **313**, 1093 (Aug 25, 2006).
11. H. W. Mahncke *et al.*, *Proc Natl Acad Sci U S A* **103**, 12523 (Aug 15, 2006).
12. N. B. Sawtell *et al.*, *Neuron* **38**, 977 (Jun 19, 2003).
13. S. M. Dudek, W. D. Bowen, M. F. Bear, *Brain Res Dev Brain Res* **47**, 123 (May 1, 1989).
14. S. M. Dudek, M. F. Bear, *Science* **246**, 673 (Nov 3, 1989).
15. A. J. Hannan *et al.*, *Nat Neurosci* **4**, 282 (Mar, 2001).
16. B. D. Philpot, K. K. Cho, M. F. Bear, *Neuron* **53**, 495 (Feb 15, 2007).
17. B. D. Philpot *et al.*, *Neuropharmacology* **41**, 762 (Nov, 2001).
18. B. D. Philpot, A. K. Sekhar, H. Z. Shouval, M. F. Bear, *Neuron* **29**, 157 (Jan, 2001).
19. B. D. Philpot, J. S. Espinosa, M. F. Bear, *J Neurosci* **23**, 5583 (Jul 2, 2003).
20. M. F. Bear, W. Singer, *Nature* **320**, 172 (Mar 13-19, 1986).
21. M. P. Kilgard, M. M. Merzenich, *Science* **279**, 1714 (Mar 13, 1998).
22. Z. J. Huang *et al.*, *Cell* **98**, 739 (Sep 17, 1999).
23. S. Oray, A. Majewska, M. Sur, *Neuron* **44**, 1021 (Dec 16, 2004).
24. N. Mataga, Y. Mizuguchi, T. K. Hensch, *Neuron* **44**, 1031 (Dec 16, 2004).
25. M. Fagiolini *et al.*, *Science* **303**, 1681 (Mar 12, 2004).
26. A. Antonini, M. Fagiolini, M. P. Stryker, *J Neurosci* **19**, 4388 (Jun 1, 1999).
27. J. L. Hanover, Z. J. Huang, S. Tonegawa, M. P. Stryker, *J Neurosci* **19**, RC40 (Nov 15, 1999).
28. L. Brown, M. Paraso, R. Arkell, S. Brown, *Hum Mol Genet* **14**, 411 (Feb 1, 2005).
29. L. Y. Brown *et al.*, *Hum Mol Genet* **10**, 791 (Apr 1, 2001).
30. S. A. Brown *et al.*, *Nat Genet* **20**, 180 (Oct, 1998).
31. J. A. Golden, *J Neuropathol Exp Neurol* **57**, 991 (Nov, 1998).
32. F. L. Raymond, *J Med Genet* **43**, 193 (Mar, 2006).
33. A. P. Association, *Diagnostic and Statistical Manual of Mental Disorders IV-TR* (American Psychiatric Association Press, Washington, DC, ed. 4th, 2000), pp.
34. R. J. Hagerman, P. J. Hagerman, *Fragile X Syndrome: Diagnosis, Treatment, and Research* (The Johns Hopkins University Press, ed. 3 edition, 2002), pp. 552.
35. D. B. Bailey, Jr., D. D. Hatton, M. Skinner, *Am J Ment Retard* **103**, 29 (Jul, 1998).

36. R. J. Hagerman *et al.*, *Am J Med Genet* **33**, 513 (Aug, 1989).
37. R. M. Hodapp *et al.*, *J Am Acad Child Adolesc Psychiatry* **29**, 214 (Mar, 1990).
38. C. Wright-Talamante *et al.*, *Am J Med Genet* **64**, 350 (Aug 9, 1996).
39. G. S. Fisch *et al.*, *Am J Med Genet* **64**, 356 (Aug 9, 1996).
40. A. M. Wiegers, L. M. Curfs, J. P. Fryns, *Birth Defects Orig Artic Ser* **28**, 93 (1992).
41. A. M. Wiegers, L. M. Curfs, E. L. Vermeer, J. P. Fryns, *Am J Med Genet* **47**, 216 (Aug 15, 1993).
42. A. L. Reiss, J. Lee, L. Freund, *Neurology* **44**, 1317 (Jul, 1994).
43. W. R. Kates, M. T. Abrams, W. E. Kaufmann, S. N. Breiter, A. L. Reiss, *Psychiatry Res* **75**, 31 (Aug 8, 1997).
44. P. Jakala *et al.*, *J Clin Invest* **100**, 331 (Jul 15, 1997).
45. S. H. Mostofsky *et al.*, *Neurology* **50**, 121 (Jan, 1998).
46. M. M. Mazzocco, W. R. Kates, T. L. Baumgardner, L. S. Freund, A. L. Reiss, *J Autism Dev Disord* **27**, 415 (Aug, 1997).
47. R. F. Kooy *et al.*, *Am J Med Genet* **64**, 241 (Aug 9, 1996).
48. S. A. Irwin *et al.*, *Am J Med Genet* **98**, 161 (Jan 15, 2001).
49. V. J. Hinton, W. T. Brown, K. Wisniewski, R. D. Rudelli, *Am J Med Genet* **41**, 289 (Dec 1, 1991).
50. E. A. Nimchinsky, A. M. Oberlander, K. Svoboda, *J Neurosci* **21**, 5139 (Jul 15, 2001).
51. B. E. Pfeiffer, K. M. Huber, *J Neurosci* **27**, 3120 (Mar 21, 2007).
52. B. C. McKinney, A. W. Grossman, N. M. Elisseou, W. T. Greenough, *Am J Med Genet B Neuropsychiatr Genet* **136**, 98 (Jul 5, 2005).
53. R. Galvez, W. T. Greenough, *Am J Med Genet A* **135**, 155 (Jun 1, 2005).
54. T. A. Comery *et al.*, *Proc Natl Acad Sci U S A* **94**, 5401 (May 13, 1997).
55. M. F. Bear *et al.*, *Nature* **302**, 245 (Mar 17-23, 1983).
56. L. Mioche, W. Singer, *J Neurophysiol* **62**, 185 (Jul, 1989).
57. C. D. Rittenhouse, H. Z. Shouval, M. A. Paradiso, M. F. Bear, *Nature* **397**, 347 (Jan 28, 1999).
58. T. K. Hensch, M. P. Stryker, *Science* **272**, 554 (Apr 26, 1996).
59. M. F. Bear, J. D. Daniels, *J Neurosci* **3**, 407 (Feb, 1983).
60. A. Kleinschmidt, M. F. Bear, W. Singer, *Science* **238**, 355 (Oct 16, 1987).
61. Q. A. Gu, M. F. Bear, W. Singer, *Brain Res Dev Brain Res* **47**, 281 (Jun 1, 1989).
62. M. F. Bear, A. Kleinschmidt, Q. A. Gu, W. Singer, *J Neurosci* **10**, 909 (Mar, 1990).
63. M. F. Bear, H. Colman, *Proc Natl Acad Sci U S A* **87**, 9246 (Dec, 1990).
64. I. J. Reynolds, M. F. Bear, *Exp Brain Res* **85**, 611 (1991).
65. D. H. Hubel, T. N. Wiesel, *Nature* **221**, 747 (Feb 22, 1969).
66. D. H. Hubel, T. N. Wiesel, *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* **248**, 492 (Aug 19, 1964).
67. D. H. Hubel, T. N. Wiesel, S. LeVay, *Philos Trans R Soc Lond B Biol Sci* **278**, 377 (Apr 26, 1977).
68. T. N. Wiesel, D. H. Hubel, *J Neurophysiol* **28**, 1060 (Nov, 1965).
69. T. N. Wiesel, D. H. Hubel, *J Neurophysiol* **26**, 1003 (Nov, 1963).
70. D. Feldman, J. E. Sherin, W. A. Press, M. F. Bear, *Exp Brain Res* **80**, 252 (1990).

71. R. A. Crozier, Y. Wang, C. H. Liu, M. F. Bear, *Proc Natl Acad Sci U S A* **104**, 1383 (Jan 23, 2007).
72. M. F. Bear, W. A. Press, B. W. Connors, *J Neurophysiol* **67**, 841 (Apr, 1992).
73. A. Kirkwood, M. F. Bear, *J Neurosci* **14**, 3404 (May, 1994).
74. A. Kirkwood, M. F. Bear, *J Neurosci* **14**, 1634 (Mar, 1994).
75. A. Kirkwood, H. K. Lee, M. F. Bear, *Nature* **375**, 328 (May 25, 1995).
76. A. Kirkwood, M. F. Bear, *Biol Res* **28**, 73 (1995).
77. C. D. Aizenman, A. Kirkwood, M. F. Bear, *Cereb Cortex* **6**, 751 (Nov-Dec, 1996).
78. K. M. Huber, N. B. Sawtell, M. F. Bear, *Neuropharmacology* **37**, 571 (Apr-May, 1998).
79. N. B. Sawtell, K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **82**, 3594 (Dec, 1999).
80. A. Kirkwood, C. Rozas, J. Kirkwood, F. Perez, M. F. Bear, *J Neurosci* **19**, 1599 (Mar 1, 1999).
81. C. Rozas *et al.*, *J Neurosci* **21**, 6791 (Sep 1, 2001).
82. U. C. Drager, *J Neurophysiol* **41**, 28 (Jan, 1978).
83. J. A. Gordon, M. P. Stryker, *J Neurosci* **16**, 3274 (May 15, 1996).
84. G. T. Prusky, R. M. Douglas, *Eur J Neurosci* **17**, 167 (Jan, 2003).
85. J. Syken, T. Grandpre, P. O. Kanold, C. J. Shatz, *Science* (Aug 17, 2006).
86. Y. Tagawa, P. O. Kanold, M. Majdan, C. J. Shatz, *Nat Neurosci* **8**, 380 (Mar, 2005).
87. V. Porciatti, T. Pizzorusso, L. Maffei, *Vision Res* **39**, 3071 (Sep, 1999).
88. G. Di Cristo *et al.*, *Science* **292**, 2337 (Jun 22, 2001).
89. M. Y. Frenkel *et al.*, *Neuron* **51**, 339 (Aug 3, 2006).
90. U. C. Drager, *Brain Res* **82**, 284 (Dec 27, 1974).
91. U. C. Drager, *J Comp Neurol* **160**, 269 (Apr 1, 1975).
92. M. Y. Frenkel, M. F. Bear, *Neuron* **44**, 917 (Dec 16, 2004).
93. J. M. Godfraind *et al.*, *Am J Med Genet* **64**, 246 (Aug 9, 1996).
94. S. Sajikumar, S. Navakkode, J. U. Frey, *Curr Opin Neurobiol* **15**, 607 (Oct, 2005).
95. W. Paradee *et al.*, *Neuroscience* **94**, 185 (1999).
96. K. M. Huber, M. S. Kayser, M. F. Bear, *Science* **288**, 1254 (May 19, 2000).
97. K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **86**, 321 (Jul, 2001).
98. K. M. Huber, S. M. Gallagher, S. T. Warren, M. F. Bear, *Proc Natl Acad Sci U S A* **99**, 7746 (May 28, 2002).
99. E. D. Nosyreva, K. M. Huber, *J Neurophysiol* **95**, 3291 (May, 2006).
100. L. Hou *et al.*, *Neuron* **51**, 441 (Aug 17, 2006).
101. J. Li, M. R. Pelletier, J. L. Perez Velazquez, P. L. Carlen, *Mol Cell Neurosci* **19**, 138 (Feb, 2002).
102. B. M. Wilson, C. L. Cox, *Proc Natl Acad Sci U S A* **104**, 2454 (Feb 13, 2007).
103. N. S. Desai, T. M. Casimiro, S. M. Gruber, P. W. Vanderklish, *J Neurophysiol* **96**, 1734 (Oct, 2006).
104. J. Larson, R. E. Jessen, D. Kim, A. K. Fine, J. du Hoffmann, *J Neurosci* **25**, 9460 (Oct 12, 2005).
105. M. G. Zhao *et al.*, *J Neurosci* **25**, 7385 (Aug 10, 2005).

106. S. K. Koekkoek *et al.*, *Neuron* **47**, 339 (Aug 4, 2005).
107. K. M. Huber, N. B. Sawtell, M. F. Bear, *J Neurosci* **18**, 1 (Jan 1, 1998).
108. N. Daw, Y. Rao, X. F. Wang, Q. Fischer, Y. Yang, *Vision Res* **44**, 3377 (Dec, 2004).
109. I. J. Weiler *et al.*, *Proc Natl Acad Sci U S A* **101**, 17504 (Dec 14, 2004).
110. E. D. Nosyreva, K. M. Huber, *J Neurosci* **25**, 2992 (Mar 16, 2005).
111. T. N. Wiesel, *Nature* **299**, 583 (Oct 14, 1982).
112. P. Jin, R. S. Alisch, S. T. Warren, *Nat Cell Biol* **6**, 1048 (Nov, 2004).
113. P. W. Vanderklish, G. M. Edelman, *Genes Brain Behav* **4**, 360 (Aug, 2005).
114. S. Taha, M. P. Stryker, *Neuron* **34**, 425 (Apr 25, 2002).
115. W. C. Abraham, M. F. Bear, *Trends Neurosci* **19**, 126 (Apr, 1996).
116. M. L. Hayashi *et al.*, *Neuron* **42**, 773 (Jun 10, 2004).
117. P. V. Belichenko *et al.*, *J Comp Neurol* **480**, 281 (Dec 13, 2004).
118. M. Marin-Padilla, *J Comp Neurol* **167**, 63 (May 1, 1976).
119. M. Marin-Padilla, *Brain Res* **44**, 625 (Sep 29, 1972).
120. P. V. Belichenko, A. Oldfors, B. Hagberg, A. Dahlstrom, *Neuroreport* **5**, 1509 (Jul 21, 1994).
121. W. E. Kaufmann, H. W. Moser, *Cereb Cortex* **10**, 981 (Oct, 2000).
122. M. Segal, U. Kreher, V. Greenberger, K. Braun, *Brain Res* **972**, 9 (May 16, 2003).
123. A. W. Grossman, N. M. Elisseou, B. C. McKinney, W. T. Greenough, *Brain Res* **1084**, 158 (Apr 21, 2006).
124. K. Braun, M. Segal, *Cereb Cortex* **10**, 1045 (Oct, 2000).
125. S. A. Irwin *et al.*, *Am J Med Genet* **111**, 140 (Aug 1, 2002).
126. P. W. Vanderklish, G. M. Edelman, *Proc Natl Acad Sci U S A* **99**, 1639 (Feb 5, 2002).
127. W. Wallace, M. F. Bear, *J Neurosci* **24**, 6928 (Aug 4, 2004).
128. A. Globus, A. B. Scheibel, *Nature* **212**, 463 (Oct 29, 1966).
129. M. Herkenham, *Science* **207**, 532 (Feb 1, 1980).
130. A. U. Larkman, *J Comp Neurol* **306**, 332 (Apr 8, 1991).
131. S. A. Irwin, R. Galvez, W. T. Greenough, *Cereb Cortex* **10**, 1038 (Oct, 2000).
132. M. Freire, *J Anat* **126**, 193 (May, 1978).
133. K. M. Harris, J. K. Stevens, *J Neurosci* **9**, 2982 (Aug, 1989).
134. J. Spacek, M. Hartmann, *Anat Embryol (Berl)* **167**, 289 (1983).
135. Z. Nusser *et al.*, *Neuron* **21**, 545 (Sep, 1998).
136. Y. Takumi, A. Matsubara, E. Rinvik, O. P. Ottersen, *Ann N Y Acad Sci* **868**, 474 (Apr 30, 1999).
137. C. Racca, F. A. Stephenson, P. Streit, J. D. Roberts, P. Somogyi, *J Neurosci* **20**, 2512 (Apr 1, 2000).
138. V. N. Murthy, T. Schikorski, C. F. Stevens, Y. Zhu, *Neuron* **32**, 673 (Nov 20, 2001).
139. T. Schikorski, C. F. Stevens, *Nat Neurosci* **4**, 391 (Apr, 2001).
140. T. Schikorski, C. F. Stevens, *J Neurosci* **17**, 5858 (Aug 1, 1997).
141. H. Kasai, M. Matsuzaki, J. Noguchi, N. Yasumatsu, H. Nakahara, *Trends Neurosci* **26**, 360 (Jul, 2003).
142. M. Matsuzaki *et al.*, *Nat Neurosci* **4**, 1086 (Nov, 2001).
143. T. D.-B. F. X. Consortium, *Cell* **78**, 23 (Jul 15, 1994).

144. R. D'Hooge *et al.*, *Neuroscience* **76**, 367 (Jan, 1997).
145. D. Van Dam *et al.*, *Behav Brain Res* **117**, 127 (Dec 20, 2000).
146. A. M. Peier *et al.*, *Hum Mol Genet* **9**, 1145 (May 1, 2000).
147. C. Dobkin *et al.*, *Neuroscience* **100**, 423 (2000).
148. M. Qin, J. Kang, C. B. Smith, *Neuroscience* **135**, 999 (2005).
149. F. X. Brennan, D. S. Albeck, R. Paylor, *Genes Brain Behav* **5**, 467 (Aug, 2006).
150. S. A. Musumeci *et al.*, *Epilepsia* **41**, 19 (Jan, 2000).
151. Q. J. Yan, P. K. Asafo-Adjei, H. M. Arnold, R. E. Brown, R. P. Bauchwitz, *Genes Brain Behav* **3**, 337 (Dec, 2004).
152. M. Bernardet, W. E. Crusio, *ScientificWorldJournal* **6**, 1164 (2006).
153. A. E. Power, D. J. Berlau, J. L. McGaugh, O. Steward, *Learn Mem* **13**, 27 (Jan-Feb, 2006).
154. D. E. Berman, Y. Dudai, *Science* **291**, 2417 (Mar 23, 2001).
155. M. R. Vianna, A. S. Coitinho, I. Izquierdo, *Curr Neurovasc Res* **1**, 55 (Jan, 2004).
156. M. R. Vianna, L. M. Igaz, A. S. Coitinho, J. H. Medina, I. Izquierdo, *Neurobiol Learn Mem* **79**, 199 (May, 2003).
157. M. R. Vianna, G. Szapiro, J. L. McGaugh, J. H. Medina, I. Izquierdo, *Proc Natl Acad Sci U S A* **98**, 12251 (Oct 9, 2001).
158. E. Santini, H. Ge, K. Ren, S. Pena de Ortiz, G. J. Quirk, *J Neurosci* **24**, 5704 (Jun 23, 2004).
159. M. E. Pedreira, H. Maldonado, *Neuron* **38**, 863 (Jun 19, 2003).
160. M. E. Bouton, E. W. Moody, *Neurosci Biobehav Rev* **28**, 663 (Nov, 2004).
161. M. Eisenberg, Y. Dudai, *Eur J Neurosci* **20**, 3397 (Dec, 2004).
162. J. Przybyslawski, S. J. Sara, *Behav Brain Res* **84**, 241 (Mar, 1997).
163. M. E. Pedreira, L. M. Perez-Cuesta, H. Maldonado, *J Neurosci* **22**, 8305 (Sep 15, 2002).
164. K. Nader, G. E. Schafe, J. E. LeDoux, *Nat Rev Neurosci* **1**, 216 (Dec, 2000).
165. O. O. Litvin, K. V. Anokhin, *Neurosci Behav Physiol* **30**, 671 (Nov-Dec, 2000).
166. M. Eisenberg, T. Kobilo, D. E. Berman, Y. Dudai, *Science* **301**, 1102 (Aug 22, 2003).
167. K. Nader, *Nature* **425**, 571 (Oct 9, 2003).
168. S. M. Taubenfeld, M. H. Milekic, B. Monti, C. M. Alberini, *Nat Neurosci* **4**, 813 (Aug, 2001).
169. E. Salinska, R. C. Bourne, S. P. Rose, *Eur J Neurosci* **19**, 3042 (Jun, 2004).
170. K. M. Lattal, S. Honarvar, T. Abel, *Behav Brain Res* **153**, 327 (Aug 31, 2004).
171. K. M. Lattal, T. Abel, *Proc Natl Acad Sci U S A* **101**, 4667 (Mar 30, 2004).
172. A. Bahar, N. Dorfman, Y. Dudai, *Eur J Neurosci* **19**, 1115 (Feb, 2004).
173. J. L. Lee, B. J. Everitt, K. L. Thomas, *Science* **304**, 839 (May 7, 2004).
174. C. M. Alberini, *Trends Neurosci* **28**, 51 (Jan, 2005).
175. Y. Dudai, M. Eisenberg, *Neuron* **44**, 93 (Sep 30, 2004).
176. D. Eisenhardt, R. Menzel, *Neurobiol Learn Mem* **87**, 167 (Feb, 2007).
177. J. L. McGaugh, *Science* **153**, 1351 (Sep 16, 1966).
178. W. M. Zho, J. L. You, C. C. Huang, K. S. Hsu, *J Neurosci* **22**, 8838 (Oct 15, 2002).
179. V. Menon, J. Leroux, C. D. White, A. L. Reiss, *Proc Natl Acad Sci U S A* **101**, 3615 (Mar 9, 2004).

Chapter 4: Syndromic features

Introduction

In addition to the cognitive deficits described in the previous chapter, the clinical picture in FXS includes a number of additional features; for this reason, Fragile X is classified as a *syndromic* disorder (*1*). Syndromic features of the disease include characteristic dysmorphic features, neurologic features, and a number of behavioral features that qualify FXS as an autistic spectrum disorder (ASD) (See chapter 6 for further discussion of autistic features). The most common neurological abnormality in FXS is epilepsy, occurring in approximately 20% of children with the disease, and presenting as seizure and EEG abnormalities (*2-6*). Growth abnormalities also suggest an endocrine disturbance in the disease **(Figure 2)** (*7*). Finally, a constellation of dysmorphic features in FXS have prompted the idea that FXS may be a connective tissue dysplasia; these include: macroorchidism **(Figure 3)**, velvet-like skin, hyperextensible joints **(Figure 1)**, hypotonia, flat feet, high palate, mitral valve prolapse, strabismus, prognathism **(Figure 1)** and long protruding ears **(Figure 1)** (*8, 9*).

Epilepsy

As mentioned above, the most common neurological abnormality in FXS is epilepsy; although incidence rates vary among clinical studies, it is most often reported at 15-20% (*2-6*). Seizures present as both partial seizure (beginning at a specific focus, i.e. frontal or temporal lobe) and generalized seizure (begin by involving the whole brain diffusely, less common) (*6*). Furthermore, patients lose consciousness during seizure (complex seizure), and when generalized in form, the seizure episode is characterized by convulsions (e.g. tonic-clonic) (*5*). If uncontrolled, status epilepticus (SE) is a rare but fatal clinical outcome. In addition, another 20% of patients who do not have seizures, present with epileptiform activity, most commonly in the form of centrotemporal spikes (*6*). Finally, the age of onset of these seizures is 2 years, and in most cases seizures and epileptiform activity resolve by puberty(*6*). The pathogenesis of this phenotype in FXS is unknown, but it is interesting to note that patients show hypersensitivity to acoustic stimuli (*10*), and elevated evoked responses to these stimuli in cortex (*11*).

This epilepsy phenotype is recapitulated in the *Fmr1* KO mouse model of the disease. Susceptibility to audiogenic seizures (AGS) is consistently and significantly higher in *Fmr1*-KO mice compared to wild-type littermates (*12-14*). These seizures are generalized, convulsive (tonic-clonic), complex seizures evoked by a loud (125 dbSPL at 0.25m) auditory stimulus. Similar to the human they are present early in postnatal development, and are absent in post-adolescence (*14*). Although the details of the locus of AGS is still being worked out, the inferior colliculus, amygdala and perirhinal cortex have all been implicated in studies of the genetically epilepsy prone rat (*15-18*). In addition, hypersensitivity to auditory stimuli leads to higher seizure susceptibility in

Fmr1-KO mice (*19*) and FMRP expression itself is modulated in response to sensory stimulation and seizure induction *in vivo* (*20, 21*).

Previous studies have not been able to account for increased epileptiform activity in *Fmr1*-KO mice by any of the anticipated mechanisms. For example, no differences have been observed between wild-type and *Fmr1*-KO mice in basal synaptic transmission, excitability, paired pulse facilitation, and long-term potentiation in the CA1 region of the hippocampus (*22, 23*). One recent study suggests that decreased GABAergic signaling in the *Fmr1 KO* might account for the seizure phenotype, since activation of the GABA(a) receptor decreases neuronal excitability (*24*). Unfortunately, these decreases in GABA(a) receptor expression were observed in the hippocampus, cortex, diencephalon, and brainstem of *adult* animals; there relevance to the seizure phenotype (that is restricted to juveniles) is unclear. Furthermore, these results are controversial, since they have not been replicated in a recent study, which shows no difference in GABA(a) receptor expression in the *Fmr1*KO (consistent with our own preliminary observations, data not shown) (D'Hulst, C. unpublished results presented at the Conquer Fragile X foundation conference, April 2007, West Palm Florida)

Interestingly, it has been shown that agonists of group I mGluRs act as convulsants in rodents (*25, 26*). Other studies have shown that selective Gp I mGluR antagonists block seizures in a range of rodent models of epilepsy (*14, 27, 28*). As mentioned above, kainic acid-induced seizures decrease levels of FMRP expression (*20*). Decreases in FMRP in response to seizure may reflect a causal relationship between protein expression and seizure induction (*29*). Specifically, FMRP may normally serve to negatively regulate mGluR5-mediated induction of epileptiform activity.

Growth

Fragile X boys and girls show an increased rate of growth, as measured by height, during the preadolescent period **(Figure 2)** (*7*). Consistent with this precocious growth, total pubertal height gain is diminished in these patients, suggesting the possibility that premature activation of the hypothalamic-pituitary-adrenal (HPA) axis is involved in the pathogenesis of this phenotype (*7*). Furthermore, approximately 20% of patients with Fragile X syndrome show marked clinical obesity **(Figure 2)**. This group of children are a subset, sometimes referred to in the clinical literature as the Prader-Willi-like subset of FXS (*30*). Prader-willi syndrome (PWS) is a genetic imprinting disorder; boys with this disease inherent a mutation on an imprinting region of chromosome 15 from their fathers. Although there are many genes in this region, one candidate gene is CYFIP; this gene is known to interact with FMRP (*31-33*). How this interaction leads to an obesity phenotype in FX and PWS is unknown; however, the HPA axis is also likely to be involved.

The hypothalamus is an integral part of the HPA axis that controls endocrine function. The lateral hypothalamus and the ventromedial hypothalamus are thought to be important for feeding behaviors: lesions of these regions lead to anorexia and obesity (respectively) in mice (*34*). Moreover, both the ventromedial hypothalamus and the lateral hypothalamus have high levels of mGluR5 expression (*35*). In addition, mGluR5 is a regulator of feeding behavior, and mGlur5 antagonists are known to be appetite suppressants (*36*).

Macroorchidism

The macroorchidism phenotype (large testicles) has been recognized for over 20

years, and occurs in over 80% of adult males with FXS (*8*). Nevertheless, this phenotype is not specific to FXS -- it is estimated to occur in nearly 30% of the mentally retarded (male) population, and FXS accounts for only a small subset of these cases (*37, 38*). Macroorchidism has no adverse effect on reproduction, since males with FXS have been documented to be fertile, and capable of reproduction (*39, 40*). Although the pathogenesis of this phenotype is unknown, it may be related to either the connective tissue dysplasia or the endocrine dysfunction seen in the disease. Evidence for an endocrine disruption comes from the observation that this phenotype does not develop until after puberty **(Figure 3)**. The largest increases are seen between ages 8 and 10, coincident with a pubertal spurt in gonadotropin hormone (*41*). By adulthood these increases are fully manifest, and average testicular size in FX patients is about double that of population averages (although dramatic increases up to 4 times the average have also been reported) (*42*).

Evidence for a pathogenesis relating to connective tissue dysplasia comes largely from the expression profile of FMRP. FMRP expression studies in the testicles of mice, indicate that this protein is differentially expressed across development (See chapter 3, Figure 1) (*43*). Early in development FMRP is found in the cytoplasm of germ cells, Sertoli cells, and interstitial cells; however by adulthood, the expression of FMRP is restricted to the spermatogonia (differentiated germ cells) and completely absent in Sertoli and interstitial cells (*44*). In Fmr1 KO mice, there have been reports of increased Sertoli cell proliferation and it has been suggested that this increase could lead to the macroorchidism phenotype (*45*).

Both the phenotype itself and the developmental profile are recapitulated in the *Fmr1*

KO mouse model (*46-48*). Interestingly, GpI mGluR RNA's are abundantly expressed in the testicles, with high levels of both mGluR5 and mGluR1 expression in the seminiferous tubuli and germ cells as well as pronounced mGluR1 expression in the Sertoli cells (see chapter 3, Figure 7) (*49*). These expression studies raise the intriguing possibility that the pathogenesis of the macroorchidism phenotype in FXS is related to exaggerated Gp1 mGluR signaling.

Results

Audiogenic seizures in *Fmr1* KO mice are rescued by reducing mGluR5 expression.

Consistent with neurological findings in fragile X patients, previous studies have shown increased seizure susceptibility in the *Fmr1* KO mouse, using both *in vitro* and *in vivo* epilepsy models (*50*). Here, the audiogenic seizure (AGS) paradigm was used, which shows a robust phenotype in *Fmr1* KO mice and exhibits developmental changes consistent with epilepsy in human FXS (*14*). Because C57Bl/6 WT mice are normally seizure resistant (*51*), seizures in the KO mice are a specific consequence of the absence of FMRP. Experiments were performed as described previously (*14*). Briefly, animals at P19-21 (immediately following weaning) were habituated to the behavioral chamber (28x17.5x12 cm transparent plastic box) for 2 minutes prior to stimulus onset. AGS stimulus was a 125 dbSPL at 0.25 m siren (modified personal alarm, Radioshack model 49-1010, powered from a DC converter). Seizures were scored for incidence during a 5 minute stimulus presentation or until animal reached AGS endpoint (status epilepticus/ respiratory arrest/ death).

As shown in **Figure 4**, significant differences in AGS susceptibility were observed

across the four genotypes examined. WT and HT mice showed zero incidences of AGS, as expected, whereas 72% of the KO mice had a seizure in response to the tone (Mann-Whitney U test WT:KO $P < 0.0001$). This mutant phenotype was significantly attenuated in the CR mice (Mann-Whitney U test CR:KO $P = 0.028$). Thus chronic reduction of mGluR5 gene dosage in KO mice produced a substantial rescue of the seizure phenotype that is caused specifically by the lack of FMRP.

This finding that the AGS phenotype in the *Fmr1* KO mice is attenuated by reducing expression of mGluR5 is consistent with the observations that MPEP can acutely suppress seizures in fragile X mice (*14, 52*). Our results suggest that the drug effects are due to inhibition of mGluR5, rather than off-target activity, and importantly show that compensatory mechanisms cannot overcome the protective effect of chronically reducing mGluR expression. While the mechanism of this reduced seizure threshold is not known , Robert Wong and colleagues have shown that the generation of epileptiform bursts is both mGluR and protein synthesis dependent, consistent with the mechanistic model presented here. Alternately, if the seizure phenotype in FXS is secondary to hypersensitivity to sensory stimuli (*19*) reduction of mGluR5 signaling could decrease this sensitivity(*53*). Another possibility is that mGluR5 regulates circuit level inhibitory balance, perhaps by upregulating GABAergic signaling (*54*). Finally, it has recently been suggested that KCNMA1alpha potassium channels may be upregulated in *Fmr1* KO mice, which results in spike broadening and increases in burst frequency (Vanderklish, P. unpublished results presented at the Banbury conference, 2007 Cold Spring Harbor, New York). Thus it may be that reduction of seizure threshold in CR mice is mediated by regulation of these channels, since it is known that mGluRs can modulate potassium

channel conductance (*55*).

Accelerated growth is recapitulated in mice and rescued by reducing mGluR5 expression. Children with FXS show accelerated prepubescent growth (*7*). Here we provide evidence that this phenotype is recapitulated in the KO mouse, and is rescued by reducing mGluR5 gene dosage (**Figure 5**). At weaning (P20-21), animals from all four genotypes had similar body weights, but by P26 KO mice showed a slight (10%) but significant increase in body weight as compared to WT animals at the same age. This difference was not observed in either the HT or CR mice (ANOVA $P = 0.048$, post-hoc t-tests WT:KO $P = 0.017$, KO:CR $P = 0.004$, CR:WT $P = 0.818$). The WT:KO body weight difference was maximal at P30 (~15%) when it was again rescued by a reduction in mGluR5 gene dosage in the CR mice (ANOVA $P = 0.005$, post hoc t-tests WT:KO $P = 0.020$, KO:CR $P = 0.001$, CR:WT $P = 0.555$). As in humans, the KO growth increase in mice was no longer apparent after adolescence (P45).

Although, as mentioned above, one explanation for this rescue is opponent regulation of HPA axis by FMRP and mGluR5, several other possibilities also exist. For example, studies have shown that insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3, are elevated in patients with FXS (while growth hormone (GH), lutenizing hormone (LH), follical stimulating hormone (FSH), and plasma testosterone levels are normal) (de Vries 1995). At the same time mGluRs are expressed in the pancreas and are thought to regulate insulin secretion (*56*). Thus it may be that the rescue of the growth phenotype reflects opponent regulation of metabolism. Other possibilities include opponent regulation of feeding behaviors, appetitive drive, or dominance

hierarchies. Thus while it is not clear how the reduction in mGluR5 gene dosage leads to a rescue of the *Fmr1*KO growth phenotype, it is clear that at the cellular level, wherever it may manifest, FMRP and mGluR5 work in an opponent fashion to regulate growth.

Macroorchidism in *Fmr1* KO mice is not rescued by reducing mGluR5 expression.

Children with FXS (and KO mice) also have dysmorphic features, including postadolescent macroorchidism. Testes express Gp1 mGluRs (*49*), so we wondered if this phenotype might also be rescued in our CR mice. Consistent with earlier studies, animals from all four genotypes had similar testicular weights prior to sexual maturity (P30) **(Figure 7a)**. However, by adulthood (average age P80), testicular weight differences across genotypes were clearly evident (**Figure 7b**). Testicular weight was increased by 24% in KO mice compared to WT ($P < 0.0004$; t-test); however there was no rescue of this phenotype in the CR mice. To investigate if the absence of rescue was a matter of gene dosage, we generated KO mice that had a complete absence of mGluR5 (*Fmr1*KO/*Grm5*KO, dKO). Again, however, there was no rescue of the testicular phenotype. Of course, the possibility remains that the other Gp1 mGluR, mGluR1, could be responsible for the pathogenesis of this phenotype, since it too is expressed in the testicles. Future studies examining the role of mGluR1 in the pathogenesis of the machroorchidism phenotype will therefore be informative.

It has suggested that depletion in melatonin may be associated with macroorchidism, which has been linked to destruction of the pineal gland (which produces melatonin) (*57, 58*). These authors have found a significant *decrease* in melatonin production in FX patients. Subsequent studies of saliva melatonin have found instead *elevated* levels of

melatonin in FXS patients (*59*), which would lead to decreased secretion of gonadotropins, and argues against a role for melatonin in the pathogenesis of the macroorchidism phenotype. Despite this controversy, recently it has been reported that administration of melatonin rescues the macroorchidism phenotype (as well as several others relating to anxiety) in the *Fmr1* KO, consistent with the deficit in melatonin production (DeDiego Otero, Y.; unpublished results presented at the Conquer Fragile X foundation conference, April 2007).

Conclusions

Fragile X is a syndromic disorder. Here we show compelling evidence to suggest that in addition to the cognitive features of the disease (see chapter 4), reduction in *Grm5* gene dosage can rescue some, but not all syndromic features of the disease. Although the exact mechanism of the interaction between FMRP and mGluR5 in the production of these phenotypes is unclear, several testable hypotheses are evident. Understanding the cellular and circuit level interactions underlying each of these syndromic features will require much future study, but the results presented here are nevertheless encouraging because they suggest that mGluR5 and FMRP act as an opponent pair in several functional contexts, and support the theory that many syndromic symptoms in fragile X are accounted for by unbalanced activation of Gp1 mGluRs. These findings have major therapeutic implications for fragile X syndrome.

Figure 1. Dysmorphic features

Hyperextensible joints

Hagerman and Hagerman, 2002

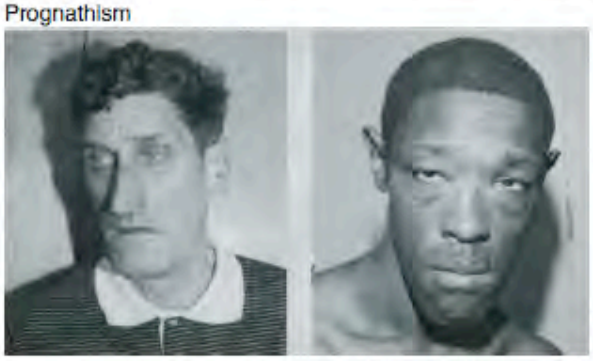
Photo by: Gül Dölen, with permission

Large protruding ears

Hagerman and Hagerman, 2002

Photo by: Gül Dölen, with permission

Prognathism



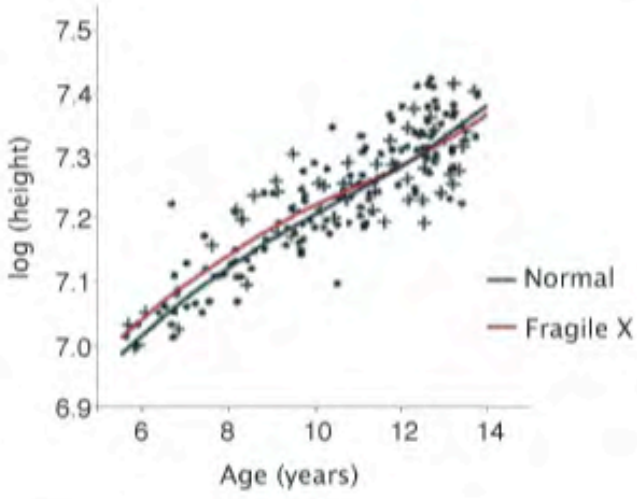
Hagerman and Hagerman, 2002

Photo by: Gül Dölen, with permission

Dysmorphic features of FXS. Includes hyperextensible joints, large protruding ears, prognatihism (elongated protruding jaw).

Figure 2. Growth disturbances in FXS

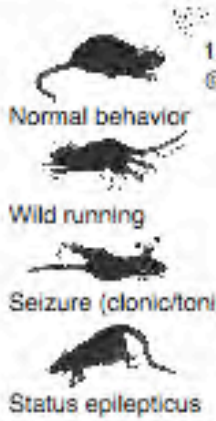
Hagerman and Hagerman, 2002



adapted from: Loesch, 2002

Growth disturbances in FXS. TOP boy with Prader-Willi subtype of FXS. BOTTOM Growth chart during through pubescence for height. Fragile X patients are bigger earlier.

gure 4. *Fmr1* KO recapitulates seizure phenotype which is rescued by 50% reduction in mGluR5

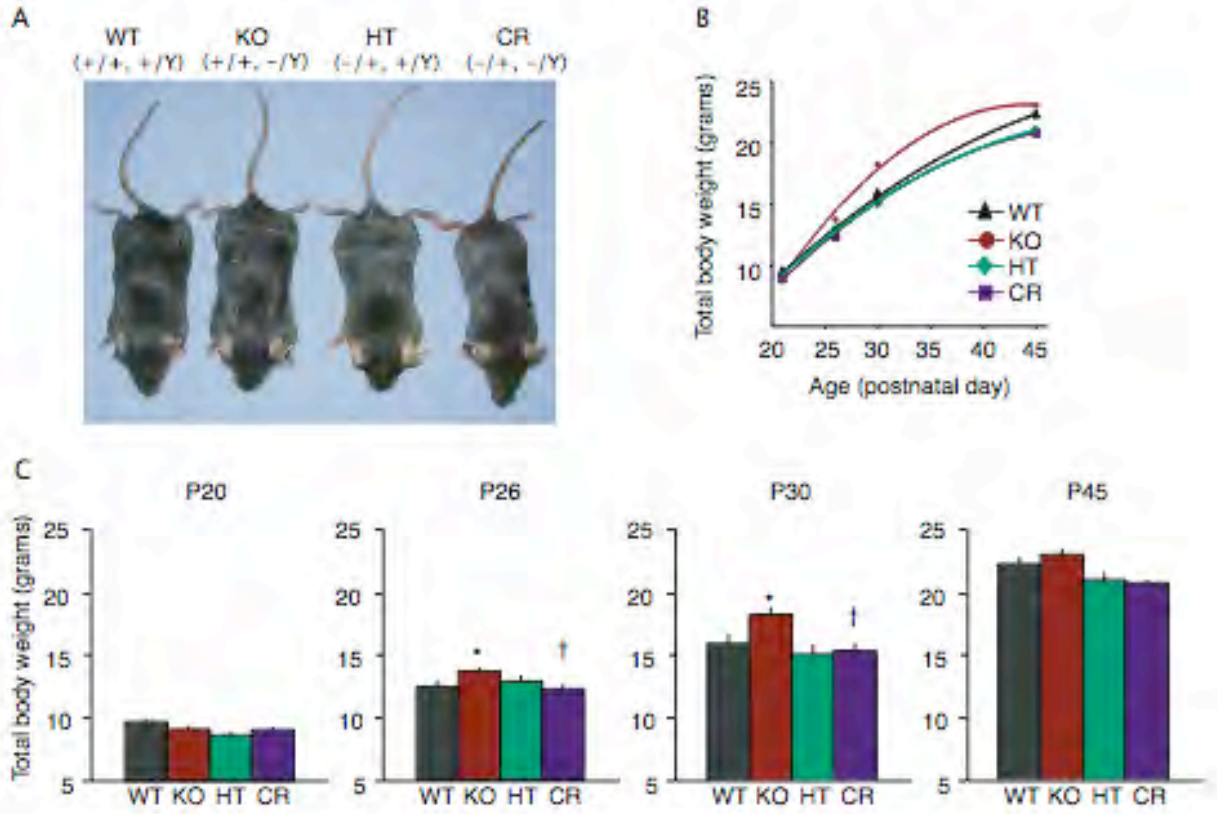


	wild running	seizure (clonic/tonic)	status epilepticus	% incidence
WT	0/15	0/15	0/15	0.0%
KO	13/18	13/18	7/18	72.2% *
HT	0/19	0/19	0/19	0.0%
CR	5/15	5/15	2/15	33.3% * †

Modified from Raisinghani & Faingold, 2005

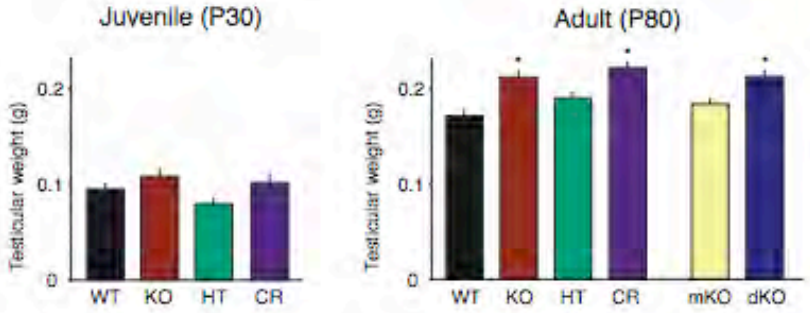
Rescue of audiogenic seizure phenotype in FXS. Significant differences in AGS susceptibility exist across the four genotypes (WT n = 15, n = 18 KO, HT n = 19, CR n = 15). Mann-Whitney U: *indicates significantly different from WT, † indicates significantly different from KO. Cartoon modified from Raisinghani and Faingold, 2005.

Figure 5. *Fmr1* KO recapitulates growth phenotype which is rescued by 50% reduction in mGluR5



Genetic rescue of growth phenotype in FXS. (**A**) Photograph of littermate animals of four genotypes at P29. (**B**) Body weight data across four genotypes fitted to polynomial curves **(C)** Normal body weight (mean ± SEM) across genotypes at weaning (P20) (WT n = 18, KO n = 23; HT n = 24; CR n = 16). Increased body weight in KO mouse at P26, rescued in CR, not different in HT (WT n = 36, KO n = 26, HT n = 20, CR n = 40). Maximal body weight increase in KO mouse at P30, rescued in CR, not different in HT (WT n = 17, KO n = 17, HT n = 10, CR n = 15). Body weight phenotype in KO is absent by P45 (WT n = 17, KO n = 17, HT n = 10, CR n = 15). Post-hoc t-tests: *indicates significantly different from WT, † indicates significantly different from KO.

Figure 6. *Fmr1* KO macroorchidism is NOT rescued by reduction in mGluR5



Macroorchidism phenotype is not rescued by reduction in mGluR5 gene dosage. (**A**) Testicular weights (mean ± SEM) were not significantly different in juvenile mice across genotypes (P30). (**B**) Testicular weight differences across genotypes become evident in adulthood (average age P80). KO phenotype is not rescued in the CR mice or in dKO mice (WT n = 20, KO n = 14, HT n = 10, CR n = 10, mKO n = 7, dKO n = 6). Post-hoc t-tests: * indicates significantly different from WT, † indicates significantly different from KO.

Chapter 4 References

1. H. H. Ropers, B. C. Hamel, *Nat Rev Genet* **6**, 46 (Jan, 2005).
2. S. A. Musumeci *et al.*, *Epilepsia* **29**, 41 (Jan-Feb, 1988).
3. F. Hecht, *Am J Med Genet* **38**, 509 (Feb-Mar, 1991).
4. G. Kluger, I. Bohm, M. C. Laub, C. Waldenmaier, *Pediatr Neurol* **15**, 358 (Nov, 1996).
5. S. A. Musumeci *et al.*, *Epilepsia* **40**, 1092 (Aug, 1999).
6. E. Berry-Kravis, *Dev Med Child Neurol* **44**, 724 (Nov, 2002).
7. D. Z. Loesch, R. M. Huggins, N. H. Hoang, *Am J Med Genet* **58**, 249 (Sep 11, 1995).
8. K. B. Nielsen, N. Tommerup, H. V. Dyggve, C. Schou, *Hum Genet* **61**, 113 (1982).
9. J. M. Opitz, J. M. Westphal, A. Daniel, *Am J Med Genet* **17**, 101 (Jan, 1984).
10. L. J. Miller *et al.*, *Am J Med Genet* **83**, 268 (Apr 2, 1999).
11. M. Castren, A. Paakkonen, I. M. Tarkka, M. Ryynanen, J. Partanen, *Brain Topogr* **15**, 165 (Spring, 2003).
12. S. A. Musumeci *et al.*, *Epilepsia* **41**, 19 (Jan, 2000).
13. Q. J. Yan, P. K. Asafo-Adjei, H. M. Arnold, R. E. Brown, R. P. Bauchwitz, *Genes Brain Behav* **3**, 337 (Dec, 2004).
14. Q. J. Yan, M. Rammal, M. Tranfaglia, R. P. Bauchwitz, *Neuropharmacology* **49**, 1053 (Dec, 2005).
15. D. N. Chakravarty, C. L. Faingold, *Exp Neurol* **157**, 135 (May, 1999).
16. M. Raisinghani, C. L. Faingold, *Brain Res* **967**, 113 (Mar 28, 2003).
17. M. Raisinghani, C. L. Faingold, *Brain Res* **1032**, 131 (Jan 25, 2005).
18. M. Raisinghani, C. L. Faingold, *Brain Res* **1048**, 193 (Jun 28, 2005).
19. L. Chen, M. Toth, *Neuroscience* **103**, 1043 (2001).
20. P. K. Todd, K. J. Mack, *Brain Res Mol Brain Res* **80**, 17 (Aug 14, 2000).
21. S. A. Irwin *et al.*, *Neurobiol Learn Mem* **74**, 87 (Jul, 2000).
22. J. M. Godfraind *et al.*, *Am J Med Genet* **64**, 246 (Aug 9, 1996).
23. W. Paradee *et al.*, *Neuroscience* **94**, 185 (1999).
24. A. El Idrissi *et al.*, *Neurosci Lett* **377**, 141 (Apr 4, 2005).
25. J. P. Tizzano, K. I. Griffey, D. D. Schoepp, *Neuropharmacology* **34**, 1063 (Aug, 1995).
26. P. J. Conn, J. P. Pin, *Annu Rev Pharmacol Toxicol* **37**, 205 (1997).
27. C. Thomsen *et al.*, *J Neurochem* **62**, 2492 (Jun, 1994).
28. A. G. Chapman, K. Nanan, M. Williams, B. S. Meldrum, *Neuropharmacology* **39**, 1567 (Jul 10, 2000).
29. L. R. Merlin, P. J. Bergold, R. K. Wong, *J Neurophysiol* **80**, 989 (Aug, 1998).
30. B. B. de Vries *et al.*, *J Med Genet* **30**, 761 (Sep, 1993).
31. A. Schenck, B. Bardoni, A. Moro, C. Bagni, J. L. Mandel, *Proc Natl Acad Sci U S A* **98**, 8844 (Jul 17, 2001).
32. A. Schenck *et al.*, *Neuron* **38**, 887 (Jun 19, 2003).
33. P. Billuart, J. Chelly, *Neuron* **38**, 843 (Jun 19, 2003).
34. L. L. Bellinger, L. L. Bernardis, *Physiol Behav* **76**, 431 (Jul, 2002).
35. A. N. van den Pol, C. Romano, P. Ghosh, *J Comp Neurol* **362**, 134 (Nov 6, 1995).

36. M. J. Bradbury *et al.*, *J Pharmacol Exp Ther* **313**, 395 (Apr, 2005).
37. D. A. Primrose, R. el-Matmati, E. Boyd, C. Gosden, M. Newton, *Br J Psychiatry* **148**, 655 (Jun, 1986).
38. R. Hagerman *et al.*, *Am J Dis Child* **142**, 1216 (Nov, 1988).
39. P. J. Willems *et al.*, *Hum Mol Genet* **1**, 511 (Oct, 1992).
40. F. Rousseau, L. J. Robb, P. Rouillard, V. M. Der Kaloustian, *Hum Mol Genet* **3**, 927 (Jun, 1994).
41. A. M. Lachiewicz, D. V. Dawson, *Pediatrics* **93**, 992 (Jun, 1994).
42. M. G. Butler, A. Brunschwig, L. K. Miller, R. J. Hagerman, *Pediatrics* **89**, 1059 (Jun, 1992).
43. D. Devys, Y. Lutz, N. Rouyer, J. P. Bellocq, J. L. Mandel, *Nat Genet* **4**, 335 (Aug, 1993).
44. C. E. Bakker *et al.*, *Exp Cell Res* **258**, 162 (Jul 10, 2000).
45. K. E. Slegtenhorst-Eegdeman *et al.*, *Endocrinology* **139**, 156 (Jan, 1998).
46. *Cell* **78**, 23 (Jul 15, 1994).
47. R. F. Kooy *et al.*, *Am J Med Genet* **64**, 241 (Aug 9, 1996).
48. A. M. Peier *et al.*, *Hum Mol Genet* **9**, 1145 (May 1, 2000).
49. M. Storto *et al.*, *J Endocrinol* **170**, 71 (Jul, 2001).
50. M. Bernardet, W. E. Crusio, *ScientificWorldJournal* **6**, 1164 (2006).
51. H. A. Robertson, *Eur J Pharmacol* **66**, 249 (Aug 29, 1980).
52. S. C. Chuang *et al.*, *J Neurosci* **25**, 8048 (Aug 31, 2005).
53. G. Bhave, F. Karim, S. M. Carlton, R. W. t. Gereau, *Nat Neurosci* **4**, 417 (Apr, 2001).
54. R. W. t. Gereau, P. J. Conn, *J Neurosci* **15**, 6879 (Oct, 1995).
55. R. Anwyl, *Brain Res Brain Res Rev* **29**, 83 (Jan, 1999).
56. N. L. Brice, A. Varadi, S. J. Ashcroft, E. Molnar, *Diabetologia* **45**, 242 (Feb, 2002).
57. J. P. Fryns, A. Dereymaeker, M. Hoefnagels, P. Volcke, H. Van den Berghe, *Am J Med Genet* **23**, 213 (Jan-Feb, 1986).
58. R. J. Hagerman, P. J. Hagerman, *Fragile X Syndrome: Diagnosis, Treatment, and Research* (The Johns Hopkins University Press, ed. 3 edition, 2002), pp. 552.
59. E. L. Gould *et al.*, *Am J Med Genet* **95**, 307 (Dec 11, 2000).

Chapter 5: Conclusions and future studies

Implications

The goal of these studies was to test a key prediction of the mGluR theory—that aspects of fragile X syndrome can be corrected by down-regulating signaling through group 1 mGluRs. Each analysis was designed to examine a different dimension of the disorder in mice with relevance to the human syndrome, ranging from the cognitive to the somatic. The experiments assayed dysfunction in very different neural circuits; and for each, three outcomes were possible: amelioration, exacerbation, or persistence of *Fmr1* mutant phenotypes in mice with reduced expression of mGluR5. Thus, it is remarkable that by reducing mGluR5 gene dosage by 50%, we were able to bring 6 of 7 diverse fragile X phenotypes significantly closer to normal.

Although a range of phenotypes were studied, a simple way to conceptualize the constellation of findings is that fragile X is a disorder of excess—excessive sensitivity to environmental change, synaptic connectivity, memory extinction, protein synthesis, body growth, and excitability—and these excesses can be corrected by reducing mGluR5 signaling. Although the precise molecular basis of the interaction remains to be determined, the data show unambiguously that mGluR5 and FMRP act as an opponent pair in several functional contexts, and support the theory that many CNS symptoms in fragile X are accounted for by unbalanced activation of Gp1 mGluRs. These findings have major therapeutic implications for fragile X syndrome and autism.

Future studies

Imaging studies. As described in chapter 4, dendritic spine density phenotype in the *Fmr1* KO is dramatically rescued by reduction of mGluR5 gene dosage. However it remains to be seen whether, in addition to the spine *density* phenotype there exists a spine *motility* phenotype in *Fmr1* KO, and whether this phenotype can similarly be rescued. Spine motility is a developmentally regulated process, such that as maturation progresses dendritic spines are increasingly less motile (*1, 2*). Although the reasons for this decreased motility are not known, one likely possibility is that as plasticity matures, the same mechanisms that constrict synaptic plasticity also impinge on dendritic spine motility. As mentioned previously, these include changes in expression of functional mGluRs, NMDA receptor subunit composition, maturation of inhibition, decreased effectiveness of neuromodulator-enabled transmission, increased myelination of axons, or increased amounts of extracellular matrix proteins and growth factors (*3-15*). For example, recently it has been shown that degradation of the extracellular matrix can enable motility of spines during adulthood (*16*) consistent with the idea that motility, like plasticity is restricted in adult brain by the maturation of the extracellular matrix (*12*). Because two-photon microscopy, that enables visualization of dendritic spine motility *in vivo*, is a relatively new technology, many of the other possibilities have not been tested. Therefore future experiments using a triple mutant cross, which I have recently generated (Fmr1/Grm5/YFPh), will examine the role of FMRP and mGluR5 in this maturation process. As described in chapter 2, these YFP lines express fluorescent protein in a subset of cortical pyramidal neurons (*17*). Like the golgi-cox silver staining method, sparse labeling of neurons (that is nevertheless complete within labeled neurons), enables

the fluorescent visualization of small processes, including dendritic spines. Based on the plasticity and spine density profiles described here, we predict that *Fmr1* KO spines will show exaggerated motility, and that this hypermotility will be corrected by selective reduction in *Grm5* gene dosage.

Recent optical imaging studies from Tropea and colleagues, have suggested that insulin like growth factor 1 (IGF1) applied exogenously can block ocular dominance plasticity evoked by 7 days of monocular deprivation (*18*). This peptide is thought to exert its effects through phosphatidylinositol 3-kinase (PI3K) signaling. Therefore, in a second set of studies (using double mutant animals (*Fmr1*/YFPh)), the effects of IGF1 application on ODP in the *Fmr1*KO will be addressed.

Ocular dominance plasticity. As described in chapter 4, the exaggerated ocular dominace plasticity kinetics phenotype in the *Fmr1* KO is dramatically rescued by reduction of mGluR5 gene dosage. While there is a clear role for NMDA receptor dependence of both *in vitro* and *in vivo* plasticity in the visual cortex (*3, 4, 19-24*), until the current study, evidence for a role for mGlur5 dependent plasticity in the visual cortex has been circumstantial. This evidence had largely been based on the developmentally regulated expression profile of the receptor (*25*) and functionally correlated glutamate stimulated PI turnover (*7, 8*) (reviewed in chapter 3). Hensch and Stryker have argued against a role for mGluRs in ocular dominance plasticity, with evidence that while the mGluR antagonist, MCPG, blocks LTD in the cortex, it has no effect on ocular dominance plasticity *in vivo* (*26*). Subsequent studies have shown that MCPG does not block mGluR dependent PI turnover, nor LTD (*27*), calling into question the interpretation put forth by Hensch and Stryker. Moreover the results presented here

argue against these conclusions directly, since 50% reduction in mGluR5 expression leads to a dramatic absence of ODP *in vivo*. Still, mechanistic evidence, in the form of *in vitro* LTP and LTD mediated by mGluR5 is lacking. Previous studies aimed at identifying such mechanisms in layer IV of the visual cortex have been disappointing (*28*), although they have been implicated in other layers (*24*) as well as the hippocampus (*29-31*). This may reflect that fact that multiple mGluRs act synergistically on downstream targets, which also share downstream signaling pathways with other receptors including NMDA receptors, TrkB, and mAChs to name a few. Blocking a specific mGluR subtype, may not be sufficient to reveal a requirement for mGluR activation. Furthermore, it remains to be seen whether the *in vivo* blockade of ODP by mGluR5 knockdown, is evident *in vitro*, and whether this blockade is mediated principally by signaling cascades coupled to mGluRs or instead by compensatory modulation of other receptors, such as NMDAs. Circumstantial evidence again points to a role for mGluR5 since both ERK and protein synthesis have been implicated *in vivo* (*32, 33*), however the coupling of these signaling mechanisms to mGluR5 will need to be demonstrated *in vitro*.

In addition, *in vitro* studies can also be used to delineate the role of these receptors in regulating metaplasticity (see chapter 4 for discussion of metaplasticity). There is growing evidence that metabotropic glutamate receptors are involved in various forms of metaplasticity (the plasticity of plasticity) (*34*). In some cases mGluRs have been implicated in regulating NMDA receptor dependent LTP (*35, 36*) while others have reported mGluR metaplasticity of mGluR dependent forms of LTD (*37, 38*). A third form of mGluR5 dependent metaplasticity is the so-called molecular switch, whereby previous

mGluR5 activation enables LTP by releasing the requirement of co-activation of mGluRs with tetatanic stimulation for its induction (*39*). While all of these studies were carried in the hippocampus, it seems possible in light of the findings here, that mGluR-mediated metaplasticity also exists in the visual cortex.

Maturation of the somatosensory cortex. Previous studies have implicated mGluR5 in the maturation of the so-called whisker barrels in the somatosensory cortex (also called barrel cortex) (*9*). Barrel cortex maturation, as mentioned previously, has a different time course than visual cortex; here synaptogenesis as well as the critical period for plasticity, occurs nearly 10 days in advance of visual cortex. Therefore, it is of interest to know whether the dramatic rescue of *Fmr1* KO phenotypes also occurs during this early developmental time point, particularly in light of recent findings that suggest that mGluR5 is differentially coupled to proteins synthesis at earlier time points (*40*). Using the same genetic manipulation as the current studies, postnatal day 7-8 animals will be perfused and stained. In the barrel cortex, Layer IV cortical cells segregate to form a cell dense barrel wall around the incoming thalamocortical axons, thus creating a cell sparse barrel hollow. Barrel formation will then be assessed by counting the number cells within the hollow relative to the wall, and comparing across genotypes. Preliminary data (not shown) suggests that *Fmr1* KO have significantly disrupted barrel formation (decreased ratio of wall to hollow cell number), and a trend towards epistatic suppression in CR mice (the *Grm5* HT shows a deficit in the same direction as the *Fmr1* KO and the cross reproduces the HT phenotype). These results suggest that despite regional and developmental differences, reduction of mGluR5 signaling might rescue *Fmr1* KO phenotypes. Future studies to examine the regulatory switch in mGluR5 signaling in the

barrel cortex might clarify the mechanism of the rescue.

Presynaptic phenotypes. Recent evidence suggests that in addition to the postsynaptic spine density and plasticity phenotypes, *Fmr1* KO mice show presynaptically mediated disruptions as well (*41*). Previously, changes in axonal outgrowth have been observed in the Fmr1 KO using the Timms sulfide-silver stain, however these results were contradictatory (*42, 43*). Future studies will therefore reexamine this axonal phenotype, and determine whether, if it exists, it can be rescued by selective reduction in Grm5 signaling.

The Timms stain selectively labels ionic zinc, which is localized within synaptic vesicles of glutamatergic neurons. Staining for synaptic zinc is especially intense in hippocampal mossy fibers, which project from granule cells in the dentate gyrus to the CA3 region of the hippocampus, and stimulation of these fibers causes the release of zinc from synaptic boutons . Although the physiological significance of this released zinc is not known, studies have suggested that it might act as a NMDAR antagonist, and protect cortical neurons from excitotoxicity. Interestingly, synaptic zinc is most abundant in brain areas particularly prone to seizures, and zinc distribution in the brain significantly decreases a result of prolonged seizure activity.

Autism. FXS is a syndromic disease: in addition to mental retardation, clinical features include a behavioral profile that places all patients with the disease on the autistic spectrum. In fact, until the locus of the disease was identified, this disease was included under the umbrella diagnosis of autism; more recently, the disease is categorized as an autistic spectrum disorder (ASD), and labeled as the Fragile X subtype of autism (*44*). While all cases of FXS fall on the autistic spectrum, an estimated 20-30% meet the

full diagnostic criteria for autism, and the degree to which patients display autistic features is correlated to FMRP expression (*45-47*).

Increasing evidence suggests that the etiology of autism is genetic (*47*), however thus far attempts to identify a *single* genetic cause have been unsuccessful, suggesting that it is a multigenic or oligogenic disorder. Genome-wide linkage scans for autism susceptibility loci have identified several chromosomal regions that are likely to be disrupted (*48*). Among the most frequent are the fragile X locus, fra(X)(q27) and anomalies involving proximal 15q, specifically the Prader-Willi and Angelman region (*49, 50*). Linkage, association, and/or chromosome rearrangement studies have identified several ASD candidates, including genes encoding neuroligins and their binding partners, as well as SHANK3 as having disease-associated mutations (*51-54*). Most recently, genetic linkage analysis has implicated the 15q25.2 locus, which contains the HOMER2 gene(*48*). Interestingly, both Shank and Homer proteins (as discussed in Chapter 3) are involved in the crosslinking of mGluR5 to the postsynaptic density (*55*), and misregulated Homer1b and PSD-95 have been implicated in the pathogenesis of FXS (*56, 57*). Thus, not only is FXS the most common identified cause of autism, understanding the signaling pathways relevant to the pathogenesis of FXS may lead to therapeutic strategies relevant to many, if not all, of the causes of autism. The studies described here have identified a link between mGluR5 and FMRP in the pathogenesis of FXS. Future studies should therefore concentrate on delineating the signaling cascade that links mGluR5 to FMRP, including Homer1b, Shank, PSD-95, ERK, and mTOR (reviewed in Chapter 3) to determine how these pathways are misregulated in the *Fmr1* KO, as well as other candidate autism models including the UBE3a KO (for Angelman syndrome) and

MeCP2 KO (for Rhett syndrome).

While the essential behavioral features of autism relate to abnormal social interactions and stereotyped behaviors (*44*), other behavioral features include hyperactivity, attention deficit (*58*), impulsivity, self-injurious behavior, disturbances in mood or affect and disturbed sleep patterns (*59*). Autistic patients can lack fear in some dangerous situations, but show excessive fear in others (*60*). Hypersensitivity to sensory stimuli has also been reported (e.g. a high threshold to pain, and hypersensitivity to noise and physical contact, overreaction to lights or odors, or fascination for certain stimuli) (*61*). Epilepsy also occurs in 5–38% of cases, particularly before 5 years of age (*62*). In addition, most autistic patients show cognitive impairments, despite the well-known but comparatively rare cases of savantism (*63, 64*). These features are largely overlapping with FXS (*65*) (cognitive impairments and epilepsy have been discussed in chapters 4 and 5 respectively, and will not be further addressed here). Because many of the behavioral features relevant to autism are recapitulated in the *Fmr1*KO mouse, the *Fmr1* KO is currently the best available animal model for studying autism (*46, 60*). The behavioral profile has of FXS includes hyperactivity and attention deficit, social anxiety **(Figures 1 and 2)**, hypersensitivity to sensory stimuli, obsessions and compulsions, and self-injurious behaviors (Figure 2)(*65, 66*). As reviewed by Bernardet and Crusio (*60*), these features have largely been recapitulated in the *Fmr1* KO mouse model (see **Figures 3-5** for summary table and list of references), with some notable exceptions. A sampling of these will be reviewed briefly here.

Social anxiety and eye aversion. In the mirrored chamber test, *Fmr1* KO mice spend less time in the mirrored chamber (*67*), consistent with the interpretation that *Fmr1*

KO mice have social anxiety comparable to human patients with the disease. However, as can be seen in the photograph in **Figure 2**, social anxiety in human patients is often paradoxically manifest, a feature dubbed the "Fragile X handshake." Patients with the disease are both interested in social interaction (wanting to shake hands, or play at the pool) but have exaggerated anxiety about this interaction (eye aversion). Interestingly this paradox is also apparent in the *Fmr1* KO, since studies in the tube test for social interaction have shown that KO mice show increased active and receptive social behaviors (*68*). Other tests of social interaction in the *Fmr1* KO show either decreases (*69*) or no change (*68*) in social interaction compared to WT. Recently studies in human patients with autism have shown reduced activity in frontoparietal regions (i.e. mirror neurons) thought to subserve social interaction, imitation, and self-recognition (*70, 71*) . It will be interesting to know whether the mirror neuron system is similarly affected in FXS, and whether these mirror neurons have a homologous structure in the mouse brain that subserves social interaction.

Perseveration. As mentioned above, one of the core behaviors in autism is stereotypy. These behaviors include repetitions, ecolalia, perseverative speech as well as a restricted repertoire of activities. Perseverative behaviors have been modeled in the mouse using reversal trials in morris water maze. As you will recall from the discussion in chapter 4, reversal is distinct from extinction in that here, the animal is asked to learn a new location of the hidden platform, rather than simply extinguishing the old behavior. Several studies have examined reversal trials in the *Fmr1* KO; three of these suggested that these animals perseverate on the previously learned platform position (*72-74*), while two others were not able to replicate these findings (*75, 76*). However it must be noted

that unlike rats, mice do not live in aquatic habitats, and so water mazes are especially aversive, making it difficult to disentangle anxiety responses from perseveration using this task. This confound could potentially explain the inconsistencies across studies, since an exaggerated aversion to water might overcome perseverative behavioral responses. These confounds are avoided in studies of reversal learning using the radial arm maze, however, studies of this kind have yet to be reported.

Attention deficit and hyperactivity (ADHD). FXS shows greater than 70% comorbidity with ADHD (*77*). In mice this behavior is modeled by tests of motor activity, and several reports have suggested that at least on the C57bl6 background, *Fmr1* KO mice show significant locomotor hyperactivity (*67, 69, 72, 78*) but see (*79*). This phenotype is strain specific however since it is absent in the FVB background (which is dominantly suppressive since the phenotype is also absent in the FVB X B6 hybrid background) (*79, 80*) but see (*81*). Interestingly this phenotype is thought to be related to increased striatal activation, and mGluR5 is widely expressed in this brain structure (see chapter 3). Although Grm5 KO mice do not show decreased locomotor activity, studies have shown that increased locomotor activity in response to psychostimulants, like cocaine, are absent in these mice (*82*). While studies using the mGluR5 antagonist MPEP have shown rescue of the locomotor phenotype in *Fmr1* KO mice, these results have not been replicated in studies using the *Fmr1*KO/*Grm5* HT cross (Paylor, R unpublished results presented at the Banbury conference, April 2007, Cold Spring Harbor, New York), suggesting the possibility of off target effects of MPEP. Furthermore, given the variable reproducibility of this phenotype, future studies examining the effect of psychostimulants on this phenotype will be informative.

Paradoxically, these drugs are used to treat ADHD in human patients, but the mechanism of this reversal is not understood. It is unclear whether this treatment would be effective in reversing the increased locomotor activity seen in *Fmr1*KO, and whether reduced mGluR5 signaling would occlude or potentiate this response.

Prepulse inhibition of acoustic startle (PPIS). Several studies in both the FVB and C57Bl6 clonal background have indicated that prepulse inhibition of acoustic startle (PPIS) is increased in the *Fmr1* KO (*72, 79, 83*). Interestingly in *Grm5* KO animals as well as animals with intracerebrally administered mGluR5 antagonists show decreased PPIS (*84-86*). Together these results suggest that FMRP and mGluR5 might opponently regulate this endophenotype of Fragile X. In fact, recent work has suggested that this PPIS endophenotype is partially rescued by reduction in mGluR5 gene dosage (Paylor, R unpublished results presented at the Banbury conference, April 2007, Cold Spring Harbor, New York). However because this rescue is incomplete, it suggests that either the gene dosage is not sufficiently reduced, or that additionally other mechanisms contribute. MGluR1 KO mice show a similar deficit in PPIS, so it is possible that this other Gp1 mGluR is also involved (*87*). The PPIS endophenotype has been used previously to model schizophrenia in animals (*88*); interestingly, studies of females with the full-mutation (who are otherwise asymptomatic for FXS) have shown higher incidence of schizotypal and schizoid personality disorder (*89-91*). Thus this sensorimotor gating endophenotype may relate to avoidant social behaviors as well as hypersensitivity to sensory stimuli reported in fragile X patients (*92*).

Anxiety. A number of studies have suggested that the increased anxiety phenotype seen in FXS and autism may be related to increased activity of the amygdala (*76, 93*).

However, studies in the *Fmr1* KO have suggested that anxiety related behaviors attributed to the amygdala are decreased (*67, 78, 94*) or not changed (*69, 79, 80*). Other studies have suggested that trace fear learning as well as amygdalar LTP are absent in the *Fmr1*KO mice (*80*). Thus at this time it is unclear how amygdalar function relates to the disease. Nevertheless, we have preliminary data to suggest that the increased spine density phenotype observed in the visual cortex of *Fmr*1 KO mice (see chapter 4) is recapitulated in the amygdala, and once again rescued by reducing *Grm5* gene dosage (preliminary data, not shown). Other studies have suggested that glucocorticoid signaling may be altered in FXS since *Fmr1* KO mice show elevated glucocorticoid levels in response to acute immobilization stress and decreased glucocorticoid receptor expression (*95-98*). However, we and others (C.B. Smith, personal communication), have not been able to replicate the increased plasma glucocorticoid phenotype (data not shown), which calls into question the reliability of this model.

A brain region that is intimately interconnected with the amygdala is the ventral hippocampus (review by (*99*)). As has been shown here (chapter 3), this brain region shows marked increase in protein synthesis in the *Fmr1* KO. Lesion studies of the ventral hippocampus have shown that this brain region is also involved in the regulation of startle reflexes and anxiety (*100*). It is tempting to speculate that increased protein synthesis in this hippocampal subregion may account for the increased anxiety phenotype seen in FXS. Behavioral studies aimed specifically at testing this possibility have not been reported, but would be informative. In addition, it has long been known that the ventral hippocampus is especially epileptogenic (*101*), suggesting the possibility that increased protein synthesis in ventral hippocampus might account for the epilepsy

phenotype seen in FXS, which like the increased protein synthesis phenotype is rescued by reduction in *Grm5* gene dosage. Furthermore the ventral subregion of the hippocampus has been differentially implicated in the modulation of endocrine function. Anatomically, the ventral hippocampus is intimately interconnected with the hypothalamus, thus it may be that increased protein synthesis in ventral hippocampus can account for the various endocrine phenotypes seen in FXS, including altered growth which we have also rescued by reduction in *Grm5* gene dosage. While at this point these observations are merely speculative, future studies examining the role of the ventral hippocampus in the pathogenesis of the disease will be informative.

Alternatives to the mGluR theory

The studies reported here largely confirm the tenets of the mGluR theory of Fragile X, and implicate mGlur5 antagonists as valid therapeutic targets for the treatment of the disease. However, other transmitter systems have also been proposed (i.e. BDNF, Dopamine, Serotonin, Acetylcholine), and will briefly be reviewed here (GABA, glucocorticoids, and melatonin, have also been implicated but these have been addressed elsewhere and will not be discussed further).

Brain derived neurotrophic factor (BDNF). Studies of hippocampal culture have shown that BDNF application leads to dramatically decreased expression Fmr1 mRNA levels (*102*). A parallel decrease in Fmr1 mRNA was observed *in vivo* in animals overexpressing the TrkB receptor for BDNF, as well as a parallel but more modest decrease in FMR protein levels (*102*). Although these changes were not reciprocally manifest in *Fmr1* KO animals (no change in BDNF and TrkB mRNAs), these data

implicate BDNF mediated signaling in the pathogenesis of FXS (*102*). Interestingly, it is known that hippocampal dendritic spine changes in response to TrkB activation by BDNF utilize the ERK signaling pathway (*103*), the same signaling pathway implicated in protein synthesis dependent mGluR-LTD (*40, 104-107*). Furthermore, in visual cortical cultures, BDNF mediated rises in intracellular calcium levels require concomitant mGluR5 activation by DHPG (*108*). Aberrant BDNF signaling has been implicated in Rett syndrome, which as mentioned above, is another disease model for autism (*109*). Other interesting parallels to metabotropic glutamate signaling include BDNF mediated maturation of inhibition in the visual cortex, which has been implicated in the closure of the critical period, and changes in the synaptic modification threshold (*11, 15, 110*). Interestingly, BDNF overexpressor mice have increased body weight, consistent with the decreased expression of *Fmr1* mRNA and the precocious growth seen in *Fmr1* KO mice. Taken together, these results suggest the possibility that BDNF and mGluR5 converge on similar signaling cascades, and have overlapping roles in the brain. Moreover, if the rescue effects we report here in the *Fmr1* KO mouse result from modification of that shared signaling cascade, they suggest the possibility that similar rescue effects might be achieved by downregulation of BDNF or the TrkB receptor.

Gq theory. As discussed in chapter 4, mGluR5 mediated synaptic depression can be induced by either DHPG application or synaptic activation (PP-LFS)(50-ms interstimulus interval, pulses at 1 Hz for 20 min) (*30, 31*), and both forms of plasticity are protein synthesis dependent and exaggerated in the *Fmr1* KO. However, while DHPG-LTD is absent in the *Grm5* KO mouse, PP-LFS persists (despite the fact that it is blocked by the broad range mGluR antagonist LY341495), suggesting that this stimulation protocol

additionally activates other transduction cascades (*111*). Recent studies by McMahon and colleagues (*112*) have identified a novel form of hippocampal LTD that is mediated by muscarinic acetylcholine receptors (mACh). Using a protocol introduced by Kirkwood and colleagues (*113*), these authors have shown that hippocampal mLTD is activated by carbachol (CCh) application (an mACh agonist) and is occluded by saturation of LFS-LTD (LFS-LTD induced by 15 minutes of 1 Hz stimulation, single pulses); interestingly saturation of mLTD does not occlude LFS-LTD. These results raise the possibility that PP-LFS that persists in the *Grm5* KO is mediated by muscarinic receptors. Moreover, because PP-LFS LTD is also exaggerated in the *Fmr1* KO, it seems possible the mLTD would also be exaggerated in the *Fmr1* KO. In fact, recent data from Kim Huber's lab suggests that indeed this is so (Huber, K. submitted 2007). In addition like DHPG LTD, mLTD is protein synthesis independent in the *Fmr1* KO, and CCh application, like DHPG, induces expression of FMRP and eIF1alpha. Because mAChs, like Gp1 mGluRs, are also coupled to Gq, these results have prompted a "Gq theory of FXS" whereby exaggerated signaling through all Gq coupled metabotropic receptors account for the pathogenesis of FXS (Huber, K. submitted 2007). It remains to be seen whether selective reduction in mAChR can rescue *Fmr1* KO phenotypes. Although this seems likely, because differences also exist between the coupling of these receptors to signaling cascades upstream of ERK (e.g. PKC inhibitors block mLTD but not DHPG-LTD, mGluRs couple to Homer, mAChRs do not), this hypothesis must be tested.

Along these same lines, neuromodulatory transmitter systems have also been implicated in FXS. Using a protein profiling approach (2d gel proteomics), recent studies have identified two enzymes, Henna and Punch, that are elevated in the dfmr1 null

drosophila model of FXS (*114*). These enzymes are involved in the biosynthesis of dopamine and serotonin, and levels of both of these neuromodulators are elevated in the dfmr1 null (*114*). It must be stressed that these findings cannot distinguish between compensatory and primary pathogenic mechanisms. Furthermore these findings await confirmation in the mammalian model, where pre and postsynaptic regulatory mechanism are more clearly relatable. Many subtypes of the receptor for both dopamine and serotonin also couple to Gq signaling, these findings may represent a further iteration of the Gq theory mentioned above, however this proposition awaits further investigation.

Conclusions

One of the promises of synaptic plasticity research has always been translational: understanding the biological mechanisms of synaptic plasticity will lead to new therapeutic strategies in psychiatric medicine. Thus far, all of the therapies for neuro-psychiatric disorders (including schizophrenia, major depression, and Parkinson's disease) have been discovered more or less accidentally. Here we show the first evidence for a therapeutic strategy in psychiatric medicine based on an understanding of the basic mechanisms of synaptic plasticity.

For comparison, the prevalence of Schizophrenia is about 3 per 1000 (*115*) while the prevalence of autism in children is about 3.4 per 1000 (*116*). In terms of clinical significance, therefore, autism represents a major psychiatric disorder on par with Schizophrenia. While FXS represents only a subset of all patients with autism, it is nevertheless the most common inherited cause of mental retardation, and second only to Down's syndrome in genetic causes overall. Prevalence estimates vary but have been estimated at 1:3500 males in the overall population (but as high as 1:2500 in some subpopulations) (*117*); as identification methods are improved, and related mutations in the *FMR1* gene identified, these estimates are likely to increase. Moreover, it may be that treatments designed for FXS will have therapeutic value for other causes of autism as well, particularly if interconnected signaling cascades are involved in producing a common clinical phenotype.

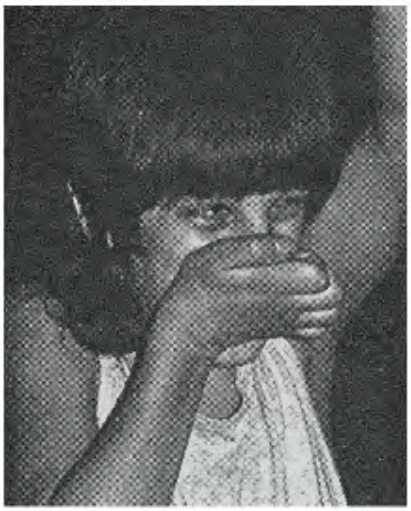
Current medications used for FXS are aimed at treating symptoms of the disease. This treatment regimen includes the usual battery of neuropsychiatric pharmocopia, including mood stabilizers, antipsychotics, anticonvulsants, antidepressants, anxiolytics

and psychostimulatants (for a complete list, refer to the Medication Guide for Fragile X, available at http://www.fraxa.org/aboutFraxa_resources.aspx). While this symptomatic approach can ameliorate certain features, often medication used for one symptom can exacerbate others (e.g. psychostimulants are moderately effective in controlling ADHD symptoms, but can exacerbate sleep disturbances). Most importantly, none of these treatments is effective in correcting cognitive impairment, arguably the most debilitating feature of the disease.

The data presented here provide the first real hope for global therapy for FXS, aimed at treating the whole disease rather than the sum of its parts. Of course this model awaits validation in forthcoming clinical trials in humans. As the signaling cascade between mGluR5 and FMRP is delineated, and the theory broadened to include other relevant neurotransmitter systems, additional targets may be identified. For now, it is important to note that metabotropic glutamate receptors are particularly amenable to pharmacologic manipulation (*118*), and these studies provide compelling evidence that these receptors if targeted appropriately, will have significant therapeutic value for the treatment of FXS and related disorders.

Figure 6. Behavioral features

Self-injurious hand biting



Hagerman and Hagerman, 2002

Hagerman and Hagerman, 2002

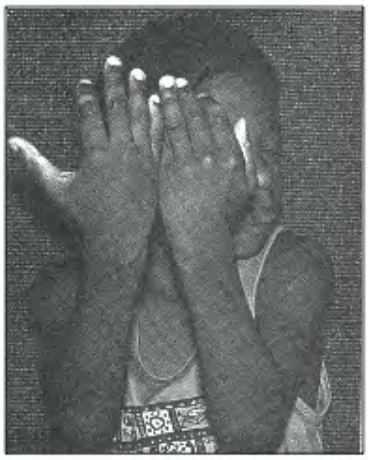
Photograph provided by: FRAXA parent

Thompson and Thompson Medical Genetics

Behavioral Features of FXS. Includes hand biting, hand flapping.

Figure 6. Social anxiety

Photograph provided by: FRAXA parent



Hagerman and Hagerman, 2002

Photograph provided by: FRAXA parent

Social anxiety in FXS. Paradoxical interest in and anxiety about social interaction. Eye aversion is shown in all pictures.

Figure 3. Autistic features of FXS

Test	Background	Result	Ref.
Inappropriate social interactions			
Mirrored chamber test	B6	KO < WT for % time in the mirrored chamber	[94]
Tube test of social dominance	B6	KO < WT vs. unfamiliar WT the first time KO = WT vs. unfamiliar WT the third day KO = WT vs. familiar WT	[94]
Social interaction test	B6	KO vs. WT: Active social behavior: KO > WT Passive social behavior: KO < WT KO vs. KO, WT vs. WT: Sniffing and receptive behavior: KO > WT KO vs. C3H, WT vs. C3H: KO < WT	[94] [95]
Crawley test	B6	KO = WT	[94]
Influence of cage familiarity on response to unfamiliar social partners	B6	In an unfamiliar cage: KO = WT; in a familiar cage: KO < WT during the first 5 min, KO > WT after 20 min	[94]
Perseverance			
Water maze reversal learning:			
Hidden-platform condition	B6	KO = WT	[97,98]
	B6	Escape latencies: KO > WT	[82,89,96]
	B6	Path length: KO > WT	[96]
	B6	Number of trials: KO > WT	[98]
	B6	Rate of learning: KO = WT, KO>WT	 [96] [89]
Visible-platform condition	B6	Escape latencies: KO > WT KO = WT	 [96] [82]
E-shaped water maze reversal learning	B6	KO = WT	[89]
Plus-shaped water maze reversal learning	B6	Escape latencies: KO = WT, but rate of learning: KO < WT	[98]
Anxiety			
Elevated plus maze	FVB	KO = WT	[100]
	B6	KO = WT	[91,107]
	FVBxB6	KO = WT	[107]
	FVBxB6	KO less anxious than WT	[83]
Thigmotaxis in open-field	B6	KO < WT	[94,101]
	FVBxB6	KO < WT	[83]
Boli in open-field	B6	KO < WT	[94]
Light-dark exploration	B6	Transitions between compartments: KO > WT Time spent in both compartments: KO = WT	[82,101]
Corticosterone response to acute stress	B6	Males: Sham and 15 min: KO = WT 0 min: KO < WT 60 min: KO > WT Females: Sham, 0 and 60 min: KO = WT 15 min: KO < WT	[104]
	B6	Males: No stress, 30 min stress: KO = WT 2 h stress: KO > WT	[103]
Conditioned emotional response	B6	KO = WT	[98]

From: Bernardet and Crusio, 2006

Figure 4. Autistic features of FXS, continued

Test	Background	Result	Ref.
Motor abilities			
Rotarod motor coordination and balance	B6	KO = WT	[101]
Aggression			
Neutral cage aggression test	B6	KO = WT	[91]
Hyperactivity			
Open field activity	B6	KO > WT	[91,94,101]
	B6	KO = WT	
	FVBxB6	KO = WT	[107]
	FVB	KO = WT	[107]
	FVB	KO = WT before 18 min	[100]
		KO > WT after 18 min	[108]
Activity cage	FVB	KO > WT	[114]
Motor activity test	B6	KO > WT	[82]
Idiosyncratic responses to sensory stimuli			
Auditory startle response	B6	KO = WT, but increased response with Fmr1gene containing YAC	[101]
	B6	KO > WT at 70 and 80 dB; KO < WT at 120 dB	[107]
	B6	KO < WT at higher intensities, interaction between genotype and intensity	[73]
	FVB	KO < WT	[110]
	FVB	KO = WT under 110 dB; KO< WT from 110 dB and above	[108]
	FVBxB6	KO > WT at 80 dB; KO < WT at 100, 110, and 120 dB	[83]
	FVBxB6	KO = WT	[83]
Prepulse inhibition	B6	KO > WT	[73]
	B6	KO > WT at 67 dB (2 dB above background noise)	[107]
	FVB	KO > WT	[110]
Audiogenic seizures (AS)	FVB	KO after long loud sound and after age 10 weeks	[110]
		KO >> WT (143 ± 5 days)	[115]
		KO >> WT (45 days and under)	[108]
	B6 and FVBxB6	KO display AS, WT do not (21 days)	[83]
	FVB	KO >> WT (30 days)	[83]
Hot plate and tail-flick test	FVB	KO = WT	[100]

From: Bernardet and Crusio, 2006

Figure 3 Syndromic features of FXS, table references

73. Frankland, P.W., Wang, Y., Rosner, B., Shimizu, T., Balleine, B.W., Dykens, E.M., Ornitz, E.M., and Silva, A.J. (2004) Sensorimotor gating abnormalities in young males with fragile X syndrome and *Fmr1*-knockout mice. *Mol. Psychiatry* **9**, 417–425.

82. The Dutch-Belgian Fragile X Consortium (1994) *Fmr1* knockout mice: a model to study fragile X mental retardation. *Cell* **78**, 23–33.

83. Yan, Q.J., Asafo-Adjei, P.K., Arnold, H.M., Brown, R.E., and Bauchwitz, R.P. (2004) A phenotypic and molecular characterization of the *fmr1-tm1Cgr* fragile X mouse. *Genes Brain Behav.* **3**, 337–359.

89. Kooy, R.F., D'Hooge, R., Reyniers, E., Bakker, C.E., Nagels, G., De Boulle, K., Storm, K., Clincke, G., De Deyn, P.P., Oostra, B.A., and Willems, P.J. (1996) Transgenic mouse model for the fragile X syndrome. *Am. J. Med. Genet.* **64**, 241–245.

91. Mineur, Y.S., Sluyter, F., de Wit, S., Oostra, B.A., and Crusio, W.E. (2002) Behavioral and neuroanatomical characterization of the *Fmr1* knockout mouse. *Hippocampus* **12**, 39–46.

94. Spencer, C.M., Alekseyenko, O., Serysheva, E., Yuva-Paylor, L.A., and Paylor, R. (2005) Altered anxiety-related and social behaviors in the *Fmr1* knockout mouse model of fragile X syndrome. *Genes Brain Behav.* **4**, 420–430.

95. Mineur, Y.S., Huynh, L.X., and Crusio, W.E. (2006) Social behavior deficits in the *Fmr1* mutant mouse. *Behav. Brain Res.* **168**, 172–175.

96. D'Hooge, R., Nagels, G., Franck, F., Bakker, C.E., Reyniers, E., Storm, K., Kooy, R.F., Oostra, B.A., Willems, P.J., and De Deyn, P.P. (1997) Mildly impaired water maze performance in male *Fmr1* knockout mice. *Neuroscience* **76**, 367–376.

97. Paradee, W., Melikian, H.E., Rasmussen, D.L., Kenneson, A., Conn, P.J., and Warren, S.T. (1999) Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience* **94**, 185–192.

98. Van Dam, D., D'Hooge, R., Hauben, E., Reyniers, E., Gantois, I., Bakker, C.E., Oostra, B.A., Kooy, R.F., and De Deyn, P.P. (2000) Spatial learning, contextual fear conditioning and conditioned emotional response in *Fmr1* knockout mice. *Behav. Brain Res.* **117**, 127–136.

100. Zhao, M.G., Toyoda, H., Ko, S.W., Ding, H.K., Wu, L.J., and Zhuo, M. (2005) Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. *J. Neurosci.* **25**, 7385–7392.

101. Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., and Nelson, D.L. (2000) (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. *Hum. Mol. Genet.* **9**, 1145–1159.

103. Lauterborn, J.C. (2004) Stress induced changes in cortical and hypothalamic c-fos expression are altered in fragile X mutant mice. *Mol. Brain Res.* **131**, 101–109.

104. Markham, J.A., Beckel-Mitchener, A.C., Estrada, C.M., and Greenough, W.T. (2006) Corticosterone response to acute stress in a mouse model of Fragile X syndrome. *Psychoneuroendocrinology* **31**, 781–785.

107. Nielsen, D.M., Derber, W.J., McClellan, D.A., and Crnic, L.S. (2002) Alterations in the auditory startle response in *Fmr1* targeted mutant mouse models of fragile X syndrome. *Brain Res.* **927**, 8–17.

108. Qin, M., Kang, J., and Smith, C.B. (2005) A null mutation for *Fmr1* in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior. *Neuroscience* **135**, 999–1009.

110. Chen, L. and Toth, M. (2001) Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience* **103**, 1043–1050.

114. Ventura, R., Pascucci, T., Puglisi-Allegra, S., Catania, M.V., and Musumeci, S.A. (2004) Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: involvement of dopamine in the medial prefrontal cortex. *Behav. Pharmacol.* **15**, 433–442.

115. Musumeci, S.A., Bosco, P., Calabrese, G., Bakker, C., De Sarro, G.B., Elia, M., Ferri, R., and Oostra, B.A. (2000) Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. *Epilepsia* **41**, 19–23.

From: Bernardet and Crusio, 2006

Chapter 5 References

1. J. Deng, A. Dunaevsky, *Dev Biol* **277**, 366 (Jan 15, 2005).
2. A. Dunaevsky, R. Blazeski, R. Yuste, C. Mason, *Nat Neurosci* **4**, 685 (Jul, 2001).
3. B. D. Philpot, K. K. Cho, M. F. Bear, *Neuron* **53**, 495 (Feb 15, 2007).
4. B. D. Philpot *et al.*, *Neuropharmacology* **41**, 762 (Nov, 2001).
5. B. D. Philpot, A. K. Sekhar, H. Z. Shouval, M. F. Bear, *Neuron* **29**, 157 (Jan, 2001).
6. B. D. Philpot, J. S. Espinosa, M. F. Bear, *J Neurosci* **23**, 5583 (Jul 2, 2003).
7. S. M. Dudek, W. D. Bowen, M. F. Bear, *Brain Res Dev Brain Res* **47**, 123 (May 1, 1989).
8. S. M. Dudek, M. F. Bear, *Science* **246**, 673 (Nov 3, 1989).
9. A. J. Hannan *et al.*, *Nat Neurosci* **4**, 282 (Mar, 2001).
10. M. F. Bear, W. Singer, *Nature* **320**, 172 (Mar 13-19, 1986).
11. Z. J. Huang *et al.*, *Cell* **98**, 739 (Sep 17, 1999).
12. N. Mataga, Y. Mizuguchi, T. K. Hensch, *Neuron* **44**, 1031 (Dec 16, 2004).
13. M. Fagiolini *et al.*, *Science* **303**, 1681 (Mar 12, 2004).
14. A. Antonini, M. Fagiolini, M. P. Stryker, *J Neurosci* **19**, 4388 (Jun 1, 1999).
15. J. L. Hanover, Z. J. Huang, S. Tonegawa, M. P. Stryker, *J Neurosci* **19**, RC40 (Nov 15, 1999).
16. S. Oray, A. Majewska, M. Sur, *Neuron* **44**, 1021 (Dec 16, 2004).
17. G. Feng *et al.*, *Neuron* **28**, 41 (Oct, 2000).
18. D. Tropea *et al.*, *Nat Neurosci* **9**, 660 (May, 2006).
19. N. B. Sawtell *et al.*, *Neuron* **38**, 977 (Jun 19, 2003).
20. R. A. Crozier, Y. Wang, C. H. Liu, M. F. Bear, *Proc Natl Acad Sci U S A* **104**, 1383 (Jan 23, 2007).
21. W. S. Chen, M. F. Bear, *Neuropharmacology* **52**, 200 (Jan, 2007).
22. A. Kirkwood, M. C. Rioult, M. F. Bear, *Nature* **381**, 526 (Jun 6, 1996).
23. M. F. Bear, A. Kleinschmidt, Q. A. Gu, W. Singer, *J Neurosci* **10**, 909 (Mar, 1990).
24. N. Daw, Y. Rao, X. F. Wang, Q. Fischer, Y. Yang, *Vision Res* **44**, 3377 (Dec, 2004).
25. S. N. Reid, C. Romano, T. Hughes, N. W. Daw, *J Comp Neurol* **389**, 577 (Dec 29, 1997).
26. T. K. Hensch, M. P. Stryker, *Science* **272**, 554 (Apr 26, 1996).
27. K. M. Huber, N. B. Sawtell, M. F. Bear, *J Neurosci* **18**, 1 (Jan 1, 1998).
28. N. B. Sawtell, K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **82**, 3594 (Dec, 1999).
29. Y. M. Lu *et al.*, *J Neurosci* **17**, 5196 (Jul 1, 1997).
30. K. M. Huber, J. C. Roder, M. F. Bear, *J Neurophysiol* **86**, 321 (Jul, 2001).
31. K. M. Huber, M. S. Kayser, M. F. Bear, *Science* **288**, 1254 (May 19, 2000).
32. S. Taha, M. P. Stryker, *Neuron* **34**, 425 (Apr 25, 2002).
33. G. Di Cristo *et al.*, *Science* **292**, 2337 (Jun 22, 2001).
34. W. C. Abraham, M. F. Bear, *Trends Neurosci* **19**, 126 (Apr, 1996).
35. A. S. Cohen, C. R. Raymond, W. C. Abraham, *Hippocampus* **8**, 160 (1998).
36. A. S. Cohen, W. C. Abraham, *J Neurophysiol* **76**, 953 (Aug, 1996).

37. A. M. Rush, J. Wu, M. J. Rowan, R. Anwyl, *J Neurosci* **22**, 6121 (Jul 15, 2002).
38. J. Wu, M. J. Rowan, R. Anwyl, *Neuroscience* **123**, 507 (2004).
39. Z. A. Bortolotto *et al.*, *Neuropharmacology* **49 Suppl 1**, 13 (2005).
40. E. D. Nosyreva, K. M. Huber, *J Neurosci* **25**, 2992 (Mar 16, 2005).
41. J. E. Hanson, D. V. Madison, *J Neurosci* **27**, 4014 (Apr 11, 2007).
42. Y. S. Mineur, F. Sluyter, S. de Wit, B. A. Oostra, W. E. Crusio, *Hippocampus* **12**, 39 (2002).
43. T. L. Ivanco, W. T. Greenough, *Hippocampus* **12**, 47 (2002).
44. A. P. Association, *Diagnostic and Statistical Manual of Mental Disorders IV-TR* (American Psychiatric Association Press, Washington, DC, ed. 4th, 2000), pp.
45. D. B. Bailey, Jr. *et al.*, *J Autism Dev Disord* **28**, 499 (Dec, 1998).
46. R. J. Hagerman, M. Y. Ono, P. J. Hagerman, *Curr Opin Psychiatry* **18**, 490 (Sep, 2005).
47. G. Trottier, L. Srivastava, C. D. Walker, *J Psychiatry Neurosci* **24**, 103 (Mar, 1999).
48. P. Szatmari *et al.*, *Nat Genet* **39**, 319 (Mar, 2007).
49. J. Veenstra-Vanderweele, S. L. Christian, E. H. Cook, Jr., *Annu Rev Genomics Hum Genet* **5**, 379 (2004).
50. J. Veenstra-VanderWeele, E. H. Cook, Jr., *Mol Psychiatry* **9**, 819 (Sep, 2004).
51. C. M. Durand *et al.*, *Nat Genet* **39**, 25 (Jan, 2007).
52. F. Laumonnier *et al.*, *Am J Hum Genet* **74**, 552 (Mar, 2004).
53. S. Jamain *et al.*, *Nat Genet* **34**, 27 (May, 2003).
54. A. A. Chubykin *et al.*, *J Biol Chem* **280**, 22365 (Jun 10, 2005).
55. J. C. Tu *et al.*, *Neuron* **23**, 583 (Jul, 1999).
56. R. Giuffrida *et al.*, *J Neurosci* **25**, 8908 (Sep 28, 2005).
57. F. Zalfa *et al.*, *Nat Neurosci* **10**, 578 (May, 2007).
58. O. Stahlberg, H. Soderstrom, M. Rastam, C. Gillberg, *J Neural Transm* **111**, 891 (Jul, 2004).
59. M. A. Polimeni, A. L. Richdale, A. J. Francis, *J Intellect Disabil Res* **49**, 260 (Apr, 2005).
60. M. Bernardet, W. E. Crusio, *ScientificWorldJournal* **6**, 1164 (2006).
61. W. Perry, A. Minassian, B. Lopez, L. Maron, A. Lincoln, *Biol Psychiatry* **61**, 482 (Feb 15, 2007).
62. R. Tuchman, I. Rapin, *Lancet Neurol* **1**, 352 (Oct, 2002).
63. M. D. Spencer *et al.*, *Neuroimage* **33**, 1136 (Dec, 2006).
64. N. O'Connor, B. Hermelin, *J Child Psychol Psychiatry* **29**, 391 (Jul, 1988).
65. R. J. Hagerman, P. J. Hagerman, *Fragile X Syndrome: Diagnosis, Treatment, and Research* (The Johns Hopkins University Press, ed. 3 edition, 2002), pp. 552.
66. L. S. Freund, A. L. Reiss, *Am J Med Genet* **38**, 542 (Mar 15, 1991).
67. C. M. Spencer, O. Alekseyenko, E. Serysheva, L. A. Yuva-Paylor, R. Paylor, *Genes Brain Behav* **4**, 420 (Oct, 2005).
68. C. M. Spencer *et al.*, *Hum Mol Genet* **15**, 1984 (Jun 15, 2006).
69. Y. S. Mineur, L. X. Huynh, W. E. Crusio, *Behav Brain Res* **168**, 172 (Mar 15, 2006).
70. M. Iacoboni, M. Dapretto, *Nat Rev Neurosci* **7**, 942 (Dec, 2006).
71. M. Dapretto *et al.*, *Nat Neurosci* **9**, 28 (Jan, 2006).

72. T. D.-B. F. X. Consortium, *Cell* **78**, 23 (Jul 15, 1994).
73. R. F. Kooy *et al.*, *Am J Med Genet* **64**, 241 (Aug 9, 1996).
74. R. D'Hooge *et al.*, *Neuroscience* **76**, 367 (Jan, 1997).
75. D. Van Dam *et al.*, *Behav Brain Res* **117**, 127 (Dec 20, 2000).
76. W. Paradee *et al.*, *Neuroscience* **94**, 185 (1999).
77. M. Backes *et al.*, *Am J Med Genet* **95**, 150 (Nov 13, 2000).
78. A. M. Peier *et al.*, *Hum Mol Genet* **9**, 1145 (May 1, 2000).
79. D. M. Nielsen, W. J. Derber, D. A. McClellan, L. S. Crnic, *Brain Res* **927**, 8 (Feb 8, 2002).
80. M. G. Zhao *et al.*, *J Neurosci* **25**, 7385 (Aug 10, 2005).
81. M. Qin, J. Kang, C. B. Smith, *Neuroscience* **135**, 999 (2005).
82. C. Chiamulera *et al.*, *Nat Neurosci* **4**, 873 (Sep, 2001).
83. L. Chen, M. Toth, *Neuroscience* **103**, 1043 (2001).
84. S. A. Brody, S. C. Dulawa, F. Conquet, M. A. Geyer, *Mol Psychiatry* **9**, 35 (Jan, 2004).
85. S. M. Grauer, K. L. Marquis, *Psychopharmacology (Berl)* **141**, 405 (Feb, 1999).
86. S. A. Brody, M. A. Geyer, *Neurotox Res* **6**, 79 (2004).
87. S. A. Brody, F. Conquet, M. A. Geyer, *Eur J Neurosci* **18**, 3361 (Dec, 2003).
88. S. A. Brody, F. Conquet, M. A. Geyer, *Psychopharmacology (Berl)* **172**, 187 (Mar, 2004).
89. P. Franke *et al.*, *Psychiatry Res* **87**, 223 (Oct 11, 1999).
90. P. Franke *et al.*, *Psychiatry Res* **80**, 113 (Aug 17, 1998).
91. P. Franke *et al.*, *Am J Med Genet* **64**, 334 (Aug 9, 1996).
92. L. J. Miller *et al.*, *Am J Med Genet* **83**, 268 (Apr 2, 1999).
93. D. G. Amaral, B. A. Corbett, *Novartis Found Symp* **251**, 177 (2003).
94. Q. J. Yan, M. Rammal, M. Tranfaglia, R. P. Bauchwitz, *Neuropharmacology* **49**, 1053 (Dec, 2005).
95. K. Y. Miyashiro *et al.*, *Neuron* **37**, 417 (Feb 6, 2003).
96. H. T. Sun, S. Cohen, W. E. Kaufmann, *Am J Med Genet* **103**, 81 (Sep 15, 2001).
97. J. C. Lauterborn, *Brain Res Mol Brain Res* **131**, 101 (Nov 24, 2004).
98. J. A. Markham, A. C. Beckel-Mitchener, C. M. Estrada, W. T. Greenough, *Psychoneuroendocrinology* (Apr 16, 2006).
99. M. B. Moser, E. I. Moser, *Hippocampus* **8**, 608 (1998).
100. K. G. Kjelstrup *et al.*, *Proc Natl Acad Sci U S A* **99**, 10825 (Aug 6, 2002).
101. M. Gilbert, R. J. Racine, G. K. Smith, *Brain Res* **361**, 389 (Dec 30, 1985).
102. M. Castren *et al.*, *Neurobiol Dis* **11**, 221 (Oct, 2002).
103. M. Alonso, J. H. Medina, L. Pozzo-Miller, *Learn Mem* **11**, 172 (Mar-Apr, 2004).
104. J. L. Banko, L. Hou, F. Poulin, N. Sonenberg, E. Klann, *J Neurosci* **26**, 2167 (Feb 22, 2006).
105. L. Hou *et al.*, *Neuron* **51**, 441 (Aug 17, 2006).
106. S. M. Gallagher, C. A. Daly, M. F. Bear, K. M. Huber, *J Neurosci* **24**, 4859 (May 19, 2004).
107. B. E. Pfeiffer, K. M. Huber, *J Neurosci* **27**, 3120 (Mar 21, 2007).
108. L. Yang *et al.*, *J Neurosci* **24**, 10846 (Dec 1, 2004).
109. Z. Zhou *et al.*, *Neuron* **52**, 255 (Oct 19, 2006).

110. K. M. Huber, N. B. Sawtell, M. F. Bear, *Neuropharmacology* **37**, 571 (Apr-May, 1998).
111. L. J. Volk, C. A. Daly, K. M. Huber, *J Neurophysiol* **95**, 2427 (Apr, 2006).
112. E. McCutchen, C. L. Scheiderer, L. E. Dobrunz, L. L. McMahon, *J Neurophysiol* **96**, 3114 (Dec, 2006).
113. A. Kirkwood, C. Rozas, J. Kirkwood, F. Perez, M. F. Bear, *J Neurosci* **19**, 1599 (Mar 1, 1999).
114. Y. Q. Zhang *et al.*, *Mol Cell Proteomics* **4**, 278 (Mar, 2005).
115. E. M. Goldner, L. Hsu, P. Waraich, J. M. Somers, *Can J Psychiatry* **47**, 833 (Nov, 2002).
116. M. Yeargin-Allsopp *et al.*, *Jama* **289**, 49 (Jan 1, 2003).
117. D. C. Crawford *et al.*, *Am J Med Genet* **110**, 226 (Jul 1, 2002).
118. M. J. Marino, P. J. Conn, *Curr Opin Pharmacol* **6**, 98 (Feb, 2006).

Appendix 1:

The mGluR theory of Fragile X

Therapeutic implications of the mGluR theory.

Courting a cure

Recording Synaptic plasticity

Opinion TRENDS in Neurosciences Vol.27 No.7 July 2004

The mGluR theory of fragile X mental retardation

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Many of the diverse functional consequences of activating group 1 metabotropic glutamate receptors require translation of pre-existing mRNA near synapses. One of these consequences is long-term depression (LTD) of transmission at hippocampal synapses. Loss of fragile X mental retardation protein (FMRP), the defect responsible for fragile X syndrome in humans, increases LTD in mouse hippocampus. This finding is consistent with the growing evidence that FMRP normally functions as a repressor of translation of specific mRNAs. Here we present a theory that can account for diverse neurological and psychiatric aspects of fragile X syndrome, based on the assumption that many of the protein-synthesis-dependent functions of metabotropic receptors are exaggerated in fragile X syndrome. The theory suggests new directions for basic research as well as novel therapeutic approaches for the treatment of humans with fragile X, the most frequent inherited cause of mental retardation and an identified cause of autism.

Fragile X is the most common inherited form of human mental retardation. It is typically caused by a trinucleotide repeat expansion in the X-linked *FMR1* gene that prevents expression of the encoded protein, called fragile X mental retardation protein (FMRP) [1]. Brain development in the absence of FMRP gives rise to the major symptoms of fragile X syndrome in humans [2,3]. These include mental retardation in the moderate to severe range, developmental delay, attention deficit and hyperactivity, anxiety with mood lability, and obsessive–compulsive and autistic behaviors. People with fragile X also have poor motor coordination, and an increased incidence of epilepsy. Common peripheral symptoms are heightened sensitivity to tactile irritation and loose bowel movements. Non-neurological symptoms can include a long face, large ears, hyperextensible joints, and enlarged testes in post-pubescent males. Autopsy studies indicate that although the brain is grossly normal, dendritic spines are longer and immature in appearance [4–6]. Spine abnormalities have long been associated with human mental retardation of unknown etiology [7], as well as with Down's and Rett syndromes [8]. Spines, of course, are where excitatory synaptic transmission and several important forms of synaptic plasticity occur.

A key advance for understanding fragile X was the isolation of the *FMR1* gene and subsequent generation of the *Fmr1* knockout mouse [9]. The phenotype of the *Fmr1* knockout mouse is multifaceted, and generally consistent with the human [3]. The most robust and reproducible behavioral phenotypes are increased locomotor activity and reduced habituation in an open field, and increased susceptibility to audiogenic seizure. Additionally, mild learning deficits have been noted [10]. Importantly, the *Fmr1* knockout has dendritic abnormalities analogous to those in humans – more long, thin spines [11,12]. Thus, there is reason to suspect that many aspects of fragile X can be attributed to altered synaptic development and plasticity.

A study of synaptic plasticity in the hippocampus of the *Fmr1* knockout mouse suggested a novel connection between metabotropic glutamate receptor (mGluR) signaling and the fragile X phenotype [13]. The resulting theory has generated some excitement in the fragile X field because it points to a possible therapeutic approach to the disorder. Here we articulate the origins, assumptions, and potential consequences of the 'mGluR theory'. This is a case study in how basic research can lead in unexpected directions.

From long-term synaptic depression to fragile X

Synaptic activity in the brain can trigger long-lasting changes in synaptic strength called long-term potentiation (LTP) and long-term depression (LTD). In neonates, the mechanisms of LTP seem to be important for retaining nascent synapses, whereas LTD mechanisms seem to be important for activity-guided synapse elimination. These same mechanisms, working in concert, contribute to learning and memory storage throughout postnatal life [14].

Understanding the mechanisms and functional significance of LTP and LTD first required the establishment of paradigms in which they can be reliably elicited. In the case of LTD, the first useful model was developed by Ito in the cerebellar cortex [15,16]. For many years it was believed that homosynaptic LTD might be the exclusive province of the cerebellum, where it was specialized for motor learning, coordination, and balance. However, a reliable method for inducing LTD using low-frequency

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synaptic stimulation was eventually established in hippocampus [17], and the study of LTD at synapses throughout the brain has subsequently flourished.

LTD of the parallel fiber to Purkinje cell synapse in the cerebellum is elicited by coincident activation of the parallel fibers and the climbing fibers. Climbing fiber synapses are very powerful, and their activation leads to a large rise in intracellular calcium that is permissive for LTD. However, a key signal that distinguishes active from inactive parallel fiber synapses, and which is required to trigger LTD, is activation of postsynaptic group 1 (Gp1) mGluRs. Gp1 mGluRs, by definition, stimulate phosphoinositide hydrolysis and comprise mGluR1 and mGluR5, which have different tissue and subcellular localization. Induction of cerebellar LTD requires activation of mGluR1 [18,19].

Although the most thoroughly characterized form of LTD in the hippocampus is triggered by activation of postsynaptic NMDA receptors, there is evidence for a second type of LTD that, like in cerebellum, requires activation of postsynaptic Gp1 mGluRs [20]. Interestingly, although both forms of hippocampal LTD can be induced by identical patterns of synaptic stimulation [21,22] and can be expressed as a decrease in the number of postsynaptic AMPA receptors [23,24], they are mechanistically distinct. One of the important distinctions is that LTD triggered by mGluR activation (mGluR-LTD) requires the rapid translation of preexisting mRNA in the postsynaptic dendrites [25]. Although NMDA-receptor-dependent LTD, like LTP, also requires protein synthesis to persist longer than a few hours [26,27], the early expression is protein-synthesis-independent [25,27]. Another distinction is that whereas NMDA-receptor-dependent LTD is readily reversible, mGluR-dependent LTD is not [20]. An irreversible loss of glutamate receptors during mGluR-LTD could be a prelude to synapse elimination [24].

The LTD literature can be confusing because different routes of induction can engage different mechanisms, and these can vary with age and synapse type; so it is important for us to be explicit. Although many details remain to be worked out, particularly the precise role for protein synthesis, the mGluR-LTD in area CA1 that we describe here requires activation of mGluR5 (the major postsynaptic Gp1 receptor in the forebrain) [28], the G_q family of G-proteins [29], and extracellular signal-regulated kinase (ERK), one of the mitogen-activated protein kinases (MAPK) [30]. Although the depression of synaptic transmission and the loss of surface-expressed glutamate receptors occur immediately after mGluR5 activation without new protein synthesis, these changes rapidly revert (within 30 min) if postsynaptic mRNA translation is inhibited (Figure 1a,b). However, new protein synthesis is only required for a finite critical period (<60 min) immediately after activation of mGluRs. A model that captures these features of mGluR-LTD is presented in Figure 1(c).

There were several reasons why it was of interest to investigate the role of FMRP in protein-synthesis-dependent mGluR-LTD. FMRP mRNA is found in dendrites and FMRP protein binds mRNA [31], as we will discuss further. However, the strongest rationale for studying FMRP in

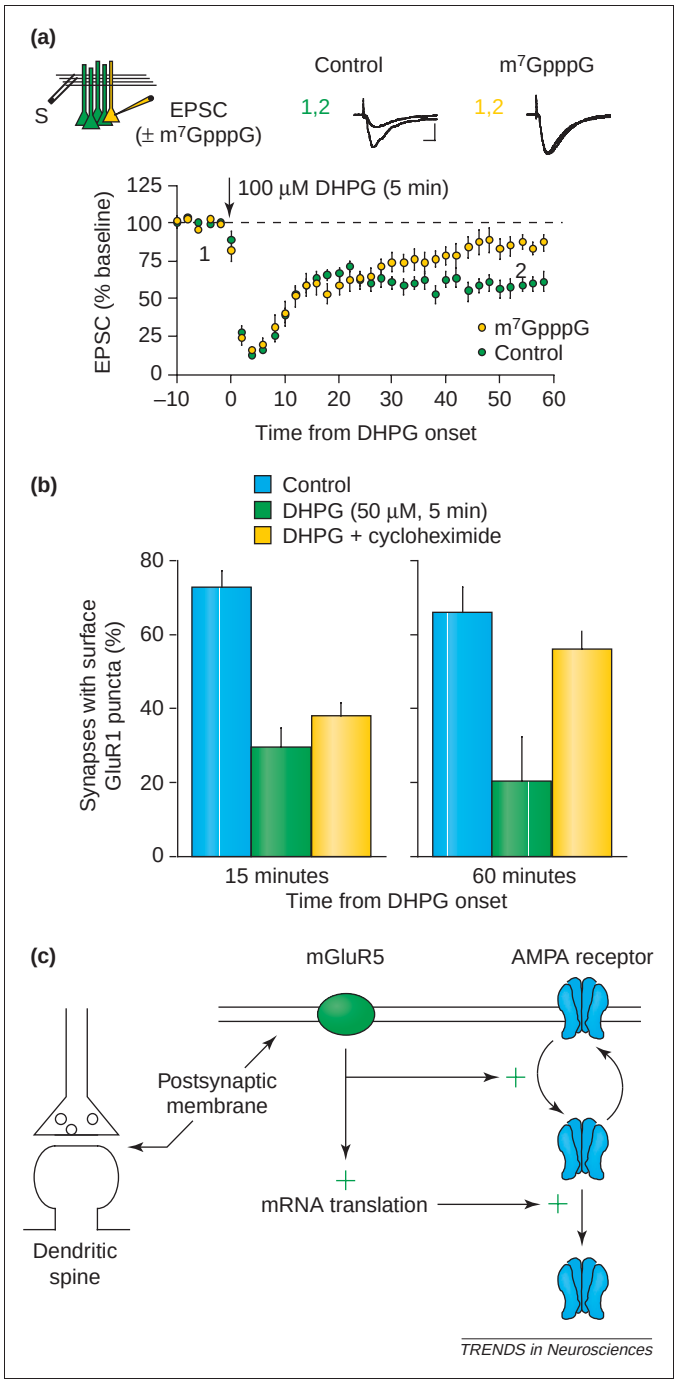

Figure 1. A role for postsynaptic protein synthesis in the stabilization of hippocampal long-term depression (LTD) induced by group 1 (Gp1) metabotropic glutamate receptor (mGluR) activation. **(a)** Summary of experiments in which the effect of activating Gp1 mGluRs in hippocampal slices is compared in the presence or absence of postsynaptic mRNA translation. To activate Gp1 mGluRs, the selective agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) was briefly applied. To block translation, the mRNA cap analogue (m^7GpppG) was introduced into CA1 pyramidal neurons through a patch recording pipette. DHPG rapidly depresses synaptic transmission, but this effect persists only if protein synthesis is allowed to occur. Data replotted from Ref. [25]. Inset: schematic of experimental design showing placement of stimulating electrode (S) and the intracellular recording and injection electrode (EPSC). Representative excitatory postsynaptic potentials (EPSCs) are from a control cell and cell containing m^7GpppG at the times indicated by the numbers on the graph. **(b)** Summary of experiments in which surface expression of synaptic AMPA receptor subunit GluR1 on cultured hippocampal neurons was monitored following DHPG treatment ± the protein synthesis inhibitor cycloheximide. AMPA receptors are rapidly lost from synapses, but are re-expressed on the surface if protein synthesis is inhibited. Data replotted from Ref. [24]. **(c)** Model to account for LTD findings. The loss of surface AMPA receptors is proposed to be at least partially responsible for the depression of synaptic transmission, and this change is stabilized by a process that requires mRNA translation near the synapse. DHPG-induced LTD fails to occur in mGluR5 knockout mice, suggesting this is the key Gp1 mGluR for this response [28].

LTD was that activation of Gp1 mGluRs was reported to stimulate the synthesis of this protein rapidly in synaptoneurosomes [32]. We therefore investigated mGluR-LTD in the *Fmr1* knockout mouse. The anticipated phenotype was defective LTD, so it came as a surprise that mGluR-LTD was actually significantly enhanced in the mutants as compared to wild-type littermates [13]. By contrast, there were no differences in NMDA-receptor-dependent LTD (at least not in the early, protein-synthesis-independent phase), consistent with earlier studies that failed to find any deficits in NMDA-receptor-dependent LTP [33,34]. Thus, the phenotype was specific to the mGluR-dependent form of synaptic plasticity.

Our data showed that one functional consequence of Gp1 mGluR activation – protein-synthesis-dependent LTD – was exaggerated in the absence of FMRP. Based on the evidence that FMRP is normally synthesized following stimulation of Gp1 mGluRs, we proposed a simple model to account for our findings (Figure 2a). According to this model, mGluR activation normally stimulates synthesis of proteins involved in stabilization of LTD and, in addition, FMRP. The FMRP functions to inhibit further synthesis (an example of end-product inhibition), and puts a brake on LTD. Recent research suggests that this 'black box' model is actually consistent with the biology of FMRP.

Emerging functions of FMRP

FMRP has been the subject of several recent reviews [3,35–37]. The excitement stems in part from the fact that fragile X syndrome is caused by a defect in a single gene, so understanding the function of the missing protein promises to provide insight into the pathophysiology of mental retardation, as well as cognition in general. However, the other reason for sudden interest is that FMRP has proven to be a fascinating molecule; it has captured the attention of neurobiologists interested in the synaptic control of protein synthesis, and the role of protein synthesis in changing synaptic structure and function.

Of particular importance for our thesis is the role of FMRP in mRNA translation regulation. FMRP is associated with actively translating polysomes in an RNA-dependent manner via messenger ribonucleoprotein (mRNP) particles [38]. A missense mutation (I304N) in the RNA-binding domain of the protein prevents the polysome association and results in severe mental retardation, suggesting that this interaction is key to the function of the protein [39]. Several innovative approaches have been taken to identify the mRNA targets of FMRP, in the hope of identifying which proteins are misregulated in fragile X [40–43]. This story has taken a very interesting twist recently with the discovery that FMRP specificity can be conferred by binding RNAs that are untranslatable. In one recent study, FMRP was shown to bind *BC1* [44], an untranslated message abundant in dendrites that functions as a translation repressor [45]. It was reported that *BC1* can specifically repress translation of the mRNAs for the synaptic proteins Arc and α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), and the dendritic microtubule associated protein 1b (MAP1b) [44]. Furthermore, FMRP has been shown to be a part of the machinery

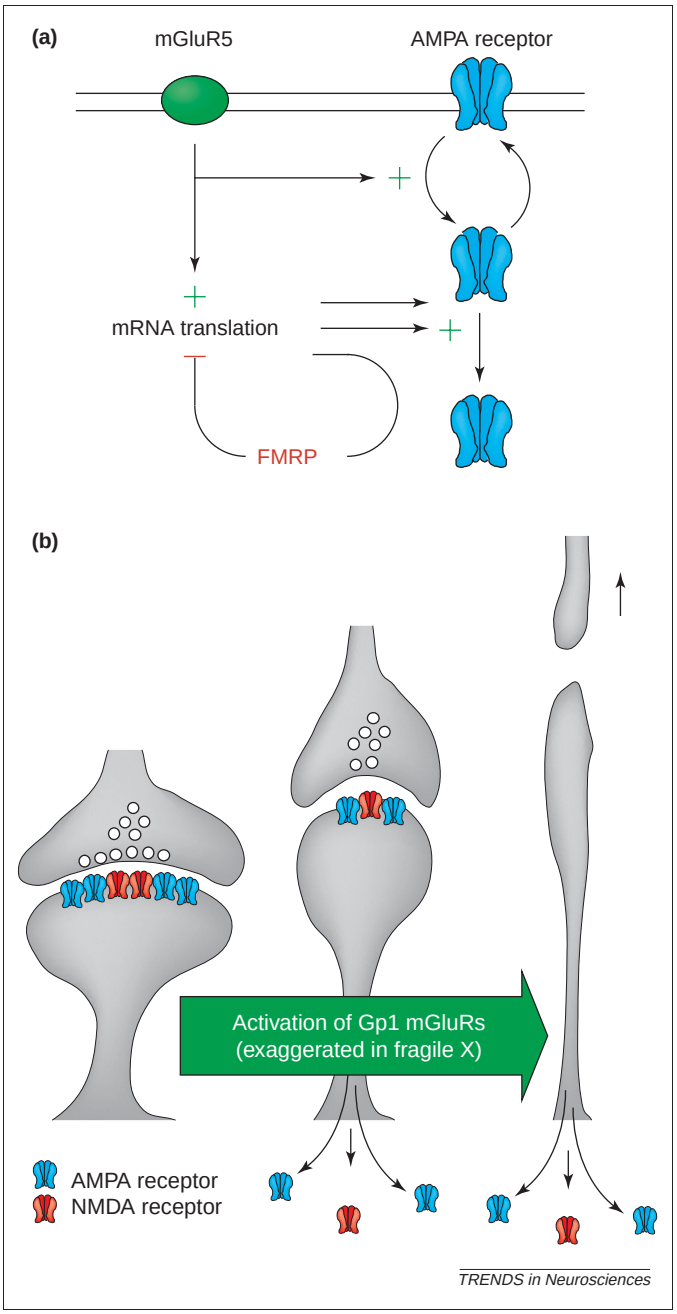

Figure 2. Models of protein synthesis-dependent, functional and structural consequences of group 1 (Gp1) metabotropic glutamate receptor (mGluR) activation at hippocampal synapses, and the role of FMRP. **(a)** Model to account for exaggerated mGluR-long-term depression (mGluR-LTD) the *Fmr1* knockout mouse, based on the assumption that the fragile X mental retardation protein (FMRP) is synthesized in response to mGluR activation and functions as a translational repressor (modified from Ref. [13]). **(b)** Model relating the net loss of synaptic AMPA and NMDA receptors [24] and elongation of dendritic spines [67] observed following Gp1 mGluR activation in cultured hippocampal neurons. We propose that these responses are indicative of increased synapse loss and/or turnover following Gp1 mGluR activation. Both responses require mRNA translation and, if exaggerated in the absence of FMRP, could account for the delay in synaptic maturation and elongated spines in fragile X. According to this view, elongated spines in fragile X are weakened synapses en route to elimination, and/or filopodial extensions of dendrites seeking to replace lost synapses.

for translation regulation by RNA interference (RNAi) [46]. Specifically, FMRP is part of a RISC nuclease complex that represses translation by directing small interfering RNAs (siRNA) to their mRNA targets [47,48].

The case for FMRP as a translational repressor seems particularly strong for MAP1b. First, genetic evidence in

flies *in vivo* shows that the *Drosophila* FMRP homolog (DFXR) represses translation of Futsch, ortholog of the mammalian MAP1b [49]. Second, although anatomical variation has been noted [43], the protein is significantly increased in total brain lysates from *Fmr1* knockout mice [44]. Third, MAP1b mRNA is increased on polysomes in cells derived from fragile X patients, consistent with FMRP negatively regulating translation of this transcript [40]. Finally, the absence of FMRP has recently been shown to directly interfere with the developmentally programmed MAP1b decline in the mammalian brain, with the increased MAP1b leading to increased microtubule stability (Y. Feng *et al.*, unpublished).

Experiments *in vitro* initially suggested the possibility that FMRP is a general repressor of translation [50,51]. However, there are now several studies suggesting this role might be restricted to specific messages. Indeed, synthesis and/or subcellular localization of several proteins appears to be disrupted in the absence of FMRP [42,43,52]. We will return to this point later in the review.

Emerging functions of Gp1 mGluR-stimulated protein synthesis

It has been recognized for many years that the machinery for protein synthesis is present in the dendrites of cortical neurons near synapses [53,54]. Translation of pre-existing mRNAs can be activated in different ways by different signals (e.g. TrkB and NMDA receptor activation), but it is now very clear that activation of Gp1 mGluRs is a potent stimulus for local protein synthesis [52,55,56]. Moreover, in cases where it has been specifically examined, many functional consequences of Gp1 mGluR activation are – like LTD – protein-synthesis-dependent (Table 1).

The first study to show that a lasting effect of Gp1 mGluR activation requires protein synthesis was performed by Merlin *et al.* using hippocampal slices. The phenomenon under investigation was the gradual and persistent prolongation of epileptiform bursts in area CA3 following activation of Gp1 mGluRs with the selective agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG). This action of DHPG on network excitability was blocked by inhibitors of mRNA translation, but not transcription [57–59].

In hippocampal area CA1, brief activation of Gp1 mGluRs can facilitate the induction of LTP without altering baseline responses [60]. However, stronger activation of Gp1 mGluRs can reverse previously induced LTP [61] and, as reviewed above, induce LTD *de novo* [20,62]. All these effects are blocked by protein synthesis inhibitors [25,63,64].

Transient activation of Gp1 mGluRs in hippocampal slices and cultures stimulates the loss of surface-expressed synaptic AMPA and NMDA receptors [24,65] and reduces presynaptic release of glutamate [66]. Prolonged treatment of hippocampal neurons with DHPG also increases the proportion of long, thin dendritic spines [67]. These changes are likely to be related, because synapses on thin spines have a smaller postsynaptic density, fewer AMPA receptors, and a reduced number of synaptic vesicles docked at the presynaptic active zone [68–70]. Again, all these effects require mRNA translation, but not transcription.

Finally, it is noteworthy that the findings on mGluR-LTD in hippocampus inspired a re-examination of cerebellar LTD, which was also found to require rapid protein synthesis [71]. This result suggests that linkage between Gp1 mGluRs and protein synthesis is not restricted to mGluR5, or to hippocampal synapses.

The mGluR theory of fragile X mental retardation

Our studies in the *Fmr1* knockout mouse led us to suggest that exaggerated LTD could slow net synaptic maturation (by tipping the balance away from synapse gain towards synapse loss during the critical period of synaptogenesis), and therefore contribute to the developmental delay and cognitive impairment associated with fragile X (Figure 2b). However, FMRP is widely expressed in the brain, including most, if not all, neurons that express Gp1 mGluRs. We therefore considered the possibility that all functional consequences of Gp1 mGluR-dependent protein synthesis might be exaggerated in the absence of FMRP. An intriguing picture began to emerge. From the literature already reviewed here, overactive or inappropriate Gp1 mGluR signaling might lead to epilepsy, cognitive impairment, developmental delay, an increased density of long, thin dendritic spines, and loss of motor coordination – key features of fragile X syndrome (Table 1).

The picture becomes even more complete when we consider other functions of Gp1 mGluRs not yet tied to protein synthesis. Suspicious coincidences include the following:

- Fear memory formation and LTP in amygdala are mGluR5-dependent [72], and mGluR5 antagonists are anxiolytic [73]. Anxiety and autistic behavior are common in fragile X, and the *Fmr1* knockout mice display abnormal contextual and conditional fear responses [34].

Table 1. The functional consequences of Gp1 mGluR activation that have been shown to require mRNA translation, listed in the order in which they were discovered, and their possible relevance to fragile X syndrome[a]

Effect of Gp1 mGluR-stimulated protein synthesis	Related fragile X phenotype in mouse or human	Refs
Prolongation of epileptiform bursts in hippocampal area CA3	Childhood epilepsy (human) Audiogenic seizure (mouse)	[57,59]
Priming of LTP in hippocampal area CA1	Cognitive impairment, developmental delay	[63]
LTD in hippocampal area CA1	Cognitive impairment, developmental delay	[25]
Internalization of postsynaptic glutamate receptors on cultured hippocampal neurons	Cognitive impairment, developmental delay	[24]
LTD in cerebellar cortex	Loss of motor coordination	[71]
Elongation of dendritic spines on cultured hippocampal neurons	Elongated, immature dendritic spines	[67]
Reversal of LTP (depotentiation) in hippocampal area CA1	Cognitive impairment, developmental delay	[64]

[a]Abbreviations: Gp1 mGluR, group 1 metabotropic glutamate receptor; LTD, long-term depression; LTP, long-term potentiation.

• LTP of the corticostriatal synapse, believed to be important for habit formation [74], requires activation of mGluR1 and mGluR5 [75]. Fragile X syndrome is characterized by obsessive–compulsive behaviors.

• The mGluR5-specific antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP) is anticonvulsant, and raises the threshold for audiogenic seizure in sensitive strains of mice [76]. Enhanced sensitivity to audiogenic seizures is a robust phenotype in *Fmr1* knockout mice in several genetic backgrounds [77].

• mGluR5 activation induces a long-term increase in the excitability of neocortical layer 5 neurons [78]. Fragile X is characterized by heightened behavioral responses to sensory stimuli, and larger sensory evoked potentials [79].

• mGluR1 and mGluR5 knockout mice show impaired pre-pulse inhibition of auditory startle [80]. Pre-pulse inhibition is enhanced in *Fmr1* knockouts [77,81].

• mGluR5 is expressed in C-fiber innervation of the skin [82] where it has been implicated in the mechanisms of hyperalgesia [83,84]. Individuals with fragile X exhibit heightened sensitivity to tactile irritation.

• mGluR5 is expressed in the enteric innervation of the ileum [85,86]. Agonists promote, and antagonists slow, intestinal mobility. Loose bowels are a common complaint in fragile X.

• Translation of the circadian rhythmicity of the molecular clock in the mouse suprachiasmatic nucleus into neural firing requires activation of Gp1 mGluRs [87]. Disrupted circadian rhythm is a striking phenotype in *Drosophila* lacking *dFMR* [88–90].

Putting these pieces together, it appeared that overactive signaling by group 1 mGluRs could contribute to many of the symptoms of fragile X, not just exaggerated LTD and slowed synaptic development. This synthesis suggested a theory – the psychiatric and neurological aspects of fragile X syndrome are a consequence of exaggerated responses to mGluR1/5 activation – that was based on the following assumptions:

(i) Proteins are synthesized in response to activation of mGluR1/5 near synapses in many brain regions, where they contribute to diverse neuronal functions.

(ii) FMRP negatively regulates responses triggered by mGluR-stimulated protein synthesis.

This theory, portrayed in Figure 3(a), was first presented to a collection of experts at a Cold Spring Harbor Laboratory Banbury Meeting in April 2002.

Predictions and progress

A theory can be tested in two ways: (i) assessing the validity of the underlying assumptions, and (ii) spinning out their consequences [91]. The follow-up Banbury meeting in 2003 revealed that such tests are underway in several laboratories, in addition to our own. It is premature to report on these studies, but we can make some explicit predictions.

The first assumption suggests that many of the long-lasting responses to Gp1 mGluR activation will prove to be protein synthesis dependent. In the case of cerebellar LTD, this assumption was tested and validated [71]. We predict that Gp1 mGluR-dependent corticostriatal and amygdala

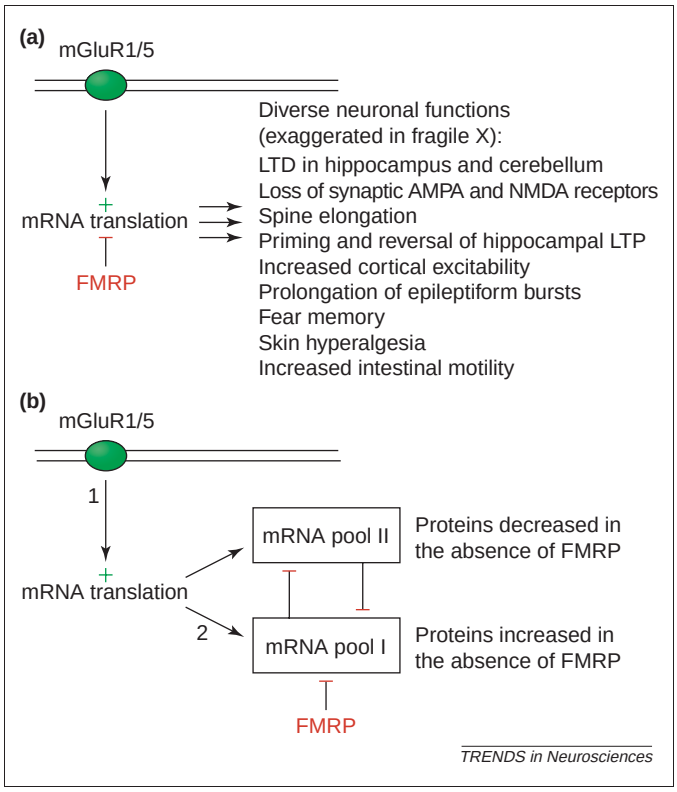

Figure 3. Group 1 (Gp1) metabotropic glutamate receptors (mGluRs) and fragile X: beyond LTD **(a)** The mGluR theory of fragile X. **(b)** Model to explain heterogeneity of translational responses in *Fmr1* knockouts. Activation of Gp1 mGluRs stimulates translation of two pools of mRNA that are in competition for the translational machinery. Repression of pool I by the fragile X mental retardation protein (FMRP) allows efficient translation of pool II. Although step 1 is the therapeutic target suggested by the mGluR theory, this model suggests that step 2 could be a better alternative. Abbreviations: LTD, long-term depression; LTP, long-term potentiation

LTP share this requirement for rapid mRNA translation (without new transcription). We also speculate that experience-dependent priming of audiogenic seizures will prove to be an mGluR5- and protein-synthesis-dependent form of synaptic or cellular plasticity.

A prediction that derives from the second assumption is that other known consequences of Gp1 mGluR-dependent protein synthesis will be exaggerated in the *Fmr1* knockout mouse. These should include increased cerebellar LTD, prolonged epileptiform bursts in hippocampal area CA3, and greater LTP priming and depotentiation in area CA1; and we expect that new behavioral phenotypes could emerge based on these findings. We also predict a greater response to DHPG in cultured hippocampal neurons – longer, thinner spines and an exaggerated loss of glutamate receptors. It is noteworthy that a paper recently appeared showing that one biochemical consequence of Gp1 mGluR activation, *de novo* synthesis of the synaptic protein PSD95, fails to occur in cultured cortical neurons from the *Fmr1* knockout mouse [52]. Thus, in future iterations of the theory, more precision will be required in specifying the protein-synthesis-dependent responses negatively regulated by FMRP (see following discussion).

The most important consequence of the theory, obviously, is that aspects of the fragile X phenotype should be rescued by reducing signaling through Gp1 mGluRs. Partial rescue might be accomplished genetically, for example, by crossing the *Fmr1* knockout mice with mice

lacking one or both genes for mGluR5 and mGluR1. Although less definitive, an even more exciting possibility is pharmacological rescue, for example, with Gp1 mGluR antagonists. First indications are positive: very recent data from Bauchwitz and colleagues indicates that the robust audiogenic seizure phenotype in *Fmr1* knockout mice is prevented by systemic administration of the mGluR5 antagonist MPEP [92].

Prospects for treatment of fragile X syndrome with Gp1 mGluR antagonists

The theory portrayed in Figure 3(a) suggests that it might be possible to overcome the loss of FMRP by dampening the protein synthesis triggered by activation of Gp1 mGluRs – this is the conceptual basis for the use of mGluR antagonists to reverse the fragile X phenotype. However, two caveats must be considered.

First, as mentioned previously, recent research suggests that although some proteins are overexpressed in the absence of FMRP (e.g. Arc and MAP1b) [44], others appear to be under-expressed or misexpressed [42,43,52]. A revision of our model to account for these recent findings is shown in Figure 3(b). According to this scheme, mGluR activation stimulates the translation of two pools of mRNA, those that are negatively regulated by FMRP (pool I) and those that are not (pool II). Competition between the pools for the translation machinery leads to a yin–yang, or push–pull, type of regulation. By inhibiting translation of messages in pool I, FMRP promotes translation of messages in pool II. Conversely, in the absence of FMRP increased translation of pool I inhibits translation of pool II. Such a model might be a better fit to available data, but it does raise concern about the quality of mGluRs as a target for treatment of fragile X. If aspects of the fragile X phenotype are attributable to decreased translation of mGluR-stimulated synthesis of proteins in pool II, it is difficult to see how an mGluR antagonist would be useful (selective blockers of pool I translation would be an alternative). The second (possibly related) caveat is that animals lacking mGluR5 [93] show cognitive deficits. Thus, blocking mGluR5 could potentially exacerbate the cognitive impairments in fragile X.

Despite these potential concerns, the known actions of Gp1 mGluR antagonists clearly suggest considerable therapeutic potential in fragile X. Most attention has been directed to mGluR5 antagonists, because mGluR1 blockers cause ataxia by disrupting cerebellar function. The prototypical mGluR5-selective antagonist is MPEP [94]. In animal models, systemically administered MPEP has been shown to have broad and potent anticonvulsant and anxiolytic actions without causing overt effects on locomotor activity. MPEP can reverse inflammation-induced mechanical hyperalgesia by inhibiting mGluR5 receptors in the C-fibers of the skin. And, by inhibiting mGluR5 receptors in the gut, MPEP can reduce bowel motility. Even the most skeptical would agree it is astonishing that a single compound could target such disparate symptoms of human fragile X syndrome as epilepsy, anxiety, hyperalgesia, and loose bowels.

As for the first caveat raised above, it is possible that we are correct about the utility of mGluR5 antagonists in fragile X for the wrong reasons, or this concern might simply be unwarranted. Regarding the second caveat, proper cognitive function appears to require synaptic plasticity within a finite dynamic range. Mutations that cause this range to be exceeded in either direction (e.g. by too much or too little LTP) impair learning and memory [95]. Antagonists of mGluR5 might correct the mild cognitive deficits seen in the *Fmr1* knockout by bringing synaptic plasticity back into its proper range. Thus, two wrongs (cognitive impairment in *Fmr1* and *mGluR5* knockout mice) could make a right.

We believe mGluR5 antagonists have great promise as a potential treatment for the neurological and psychiatric symptoms of fragile X expressed in adults. However, if the syndrome is a lasting consequence of brain development with exaggerated Gp1 mGluR signaling, it is possible that early intervention with receptor antagonists could prevent some symptoms from occurring altogether.

Beyond fragile X

There is a great deal left to be learned about how protein synthesis is regulated by, and in turn influences, synaptic transmission in the brain. However, two things are certain: (i) FMRP is only one of many proteins and signaling pathways involved in the synaptic regulation of protein synthesis, and (ii) where there is biology, there is pathology. If we are correct that key aspects of fragile X are due to unregulated synaptic protein synthesis, it seems reasonable to anticipate that other disorders with similar symptoms might be traced to defects elsewhere in the same molecular pathways. It is interesting to note that other types of human developmental disorder, including autism, have many of the same core characteristics as fragile X. These include developmental delay and cognitive impairment, increased incidence of childhood epilepsy, a higher proportion of long, thin dendritic spines, reduced motor coordination, heightened anxiety, and altered gastrointestinal function. Thus, the mGluR theory could have broader applicability than just to fragile X.

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References

1 O'Donnell, W.T. and Warren, S.T. (2002) A decade of molecular studies of fragile X syndrome. *Annu. Rev. Neurosci.* 25, 315–338
2 Hagerman, R.J. (2002) The physical and behavioral phenotype. In *Fragile X Syndrome: Diagnosis, Treatment, and Research* (Hagerman, R.J. and Hagerman, P., eds), pp. 3–109, The Johns Hopkins University Press
3 Bakker, C.E. and Oostra, B.A. (2003) Understanding fragile X syndrome: insights from animal models. *Cytogenet. Genome Res.* 100, 111–123
4 Rudelli, R.D. *et al.* (1985) Adult fragile X syndrome. Clinico-neuropathologic findings. *Acta Neuropathol. (Berl.)* 67, 289–295
5 Hinton, V.J. *et al.* (1991) Analysis of neocortex in three males with the fragile X syndrome. *Am. J. Med. Genet.* 41, 289–294
6 Irwin, S.A. *et al.* (2001) Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am. J. Med. Genet.* 98, 161–167
7 Purpura, D.P. (1974) Dendritic spine 'dysgenesis' and mental retardation. *Science* 186, 1126–1128

8 Kaufmann, W.E. and Moser, H.W. (2000) Dendritic anomalies in disorders associated with mental retardation. *Cereb. Cortex* 10, 981–991
9 Bakker, C.E. *et al.* (1994) Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch–Belgian fragile X consortium. *Cell* 78, 23–33
10 Kooy, R.F. (2003) Of mice and the fragile X syndrome. *Trends Genet.* 19, 148–154
11 Nimchinsky, E.A. *et al.* (2001) Abnormal development of dendritic spines in FMR1 knock-out mice. *J. Neurosci.* 21, 5139–5146
12 Irwin, S.A. *et al.* (2002) Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice. *Am. J. Med. Genet.* 111, 140–146
13 Huber, K.M. *et al.* (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc. Natl. Acad. Sci. U. S. A.* 99, 7746–7750
14 Bear, M.F. (1998) The role of LTD and LTP in development and learning. In *Mechanistic Relationships between Development and Learning* (Carew, T.J. *et al.*, eds), pp. 205–225, Wiley
15 Ito, M. *et al.* (1982) Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *J. Physiol.* 324, 113–134
16 Ito, M. (1989) Long term depression. In *Annual Review of Neuroscience* (Vol. 12) (Cowan, M.W. *et al.*, eds), pp. 85–102, Annual Reviews
17 Dudek, S.M. and Bear, M.F. (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of *N*-methyl-D-aspartate receptor blockade. *Proc. Natl. Acad. Sci. U. S. A.* 89, 4363–4367
18 Aiba, A. *et al.* (1994) Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* 79, 377–388
19 Shigemoto, R. *et al.* (1994) Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron* 12, 1245–1255
20 Oliet, S.H. *et al.* (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neuron* 18, 969–982
21 Lee, H.K. *et al.* (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* 112, 631–643
22 Kemp, N. and Bashir, Z.I. (1999) Induction of LTD in the adult hippocampus by the synaptic activation of AMPA/kainate and metabotropic glutamate receptors. *Neuropharmacology* 38, 495–504
23 Carroll, R.C. *et al.* (1999) Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat. Neurosci.* 2, 454–460
24 Snyder, E.M. *et al.* (2001) Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nat. Neurosci.* 4, 1079–1085
25 Huber, K.M. *et al.* (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* 288, 1254–1257
26 Manahan-Vaughan, D. *et al.* (2000) Requirement of translation but not transcription for the maintenance of long-term depression in the CA1 region of freely moving rats. *J. Neurosci.* 20, 8572–8576
27 Sajikumar, S. and Frey, J.U. (2003) Anisomycin inhibits the late maintenance of long-term depression in rat hippocampal slices *in vitro*. *Neurosci. Lett.* 338, 147–150
28 Huber, K.M. *et al.* (2001) Chemical induction of mGluR5- and protein synthesis-dependent long-term depression in hippocampal area CA1. *J. Neurophysiol.* 86, 321–325
29 Kleppisch, T. *et al.* (2001) G(alpha)q-deficient mice lack metabotropic glutamate receptor-dependent long-term depression but show normal long-term potentiation in the hippocampal CA1 region. *J. Neurosci.* 21, 4943–4948
30 Huber, K.M. *et al.* (2001) Evidence for a novel signal transduction pathway in hippocampal mGluR-dependent LTD. Program number 388.7. In *Abstract Viewer/Itinerary Planner*, Society for Neuroscience Online
31 Antar, L.N. *et al.* (2004) Metabotropic glutamate receptor activation regulates fragile X mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. *J. Neurosci.* 24, 2648–2655
32 Weiler, I.J. *et al.* (1997) Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5395–5400
33 Godfraind, J.M. *et al.* (1996) Long-term potentiation in the hippocampus of fragile X knockout mice. *Am. J. Med. Genet.* 64, 246–251
34 Paradee, W. *et al.* (1999) Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. *Neuroscience* 94, 185–192
35 Chiurazzi, P. *et al.* (2003) Understanding the biological underpinnings of fragile X syndrome. *Curr. Opin. Pediatr.* 15, 559–566
36 Jin, P. and Warren, S.T. (2003) New insights into fragile X syndrome: from molecules to neurobehaviors. *Trends Biochem. Sci.* 28, 152–158
37 Antar, L.N. and Bassell, G.J. (2003) Sunrise at the synapse: the FMRP mRNP shaping the synaptic interface. *Neuron* 37, 555–558
38 Feng, Y. *et al.* (1997) Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. *J. Neurosci.* 17, 1539–1547
39 Feng, Y. *et al.* (1997) FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. *Mol. Cell* 1, 109–118
40 Brown, V. *et al.* (2001) Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* 107, 477–487
41 Darnell, J.C. *et al.* (2001) Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 107, 489–499
42 Miyashiro, K.Y. *et al.* (2003) RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. *Neuron* 37, 417–431
43 Chen, L. *et al.* (2003) The fragile X mental retardation protein binds and regulates a novel class of mRNAs containing U rich target sequences. *Neuroscience* 120, 1005–1017
44 Zalfa, F. *et al.* (2003) The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell* 112, 317–327
45 Wang, H. *et al.* (2002) Dendritic BC1 RNA: functional role in regulation of translation initiation. *J. Neurosci.* 22, 10232–10241
46 Jin, P. *et al.* (2004) Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat. Neurosci.* 7, 113–117
47 Caudy, A.A. *et al.* (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* 16, 2491–2496
48 Ishizuka, A. *et al.* (2002) A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev.* 16, 2497–2508
49 Zhang, Y.Q. *et al.* (2001) *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell* 107, 591–603
50 Laggerbauer, B. *et al.* (2001) Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* 10, 329–338
51 Li, Z. *et al.* (2001) The fragile X mental retardation protein inhibits translation via interacting with mRNA. *Nucleic Acids Res.* 29, 2276–2283
52 Todd, P.K. *et al.* (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14374–14378
53 Steward, O. and Schuman, E.M. (2001) Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* 24, 299–325
54 Steward, O. and Schuman, E.M. (2003) Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* 40, 347–359
55 Weiler, I.J. and Greenough, W.T. (1993) Metabotropic glutamate receptors trigger postsynaptic protein synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 90, 7168–7171
56 Job, C. and Eberwine, J. (2001) Identification of sites for exponential translation in living dendrites. *Proc. Natl. Acad. Sci. U. S. A.* 98, 13037–13042
57 Merlin, L.R. *et al.* (1998) Requirement of protein synthesis for group 1 mGluR-mediated induction of epilileptiform discharges. *J. Neurophysiol.* 80, 989–993
58 Lee, A.C. *et al.* (2002) Role of synaptic metabotropic glutamate receptors in epileptiform discharges in hippocampal slices. *J. Neurophysiol.* 88, 1625–1633
59 Stoop, R. *et al.* (2003) Activation of metabotropic glutamate 5 and

NMDA receptors underlies the induction of persistent bursting and associated long-lasting changes in CA3 recurrent connections. *J. Neurosci.* 23, 5634–5644
60 Cohen, A.S. and Abraham, W.C. (1996) Facilitation of long-term potentiation by prior activation of metabotropic glutamate receptors. *J. Neurophysiol.* 76, 953–962
61 Bashir, Z.I. and Collingridge, G.L. (1994) An investigation of depotentiation of long-term potentiation in the CA1 region of the hippocampus. *Exp. Brain Res.* 100, 437–443
62 Palmer, M.J. *et al.* (1997) The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. *Neuropharmacology* 36, 1517–1532
63 Raymond, C.R. *et al.* (2000) Metabotropic glutamate receptors trigger homosynaptic protein synthesis to prolong LTP. *J. Neurosci.* 20, 969–976
64 Zho, W.M. *et al.* (2002) The group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine induces a novel form of depotentiation in the CA1 region of the hippocampus. *J. Neurosci.* 22, 8838–8849
65 Xiao, M.Y. *et al.* (2001) Metabotropic glutamate receptor activation causes a rapid redistribution of AMPA receptors. *Neuropharmacology* 41, 664–671
66 Zakharenko, S.S. *et al.* (2002) Altered presynaptic vesicle release and cycling during mGluR-dependent LTD. *Neuron* 35, 1099–1110
67 Vanderklish, P.W. and Edelman, G.M. (2002) Dendritic spines elongate after stimulation of group 1 metabotropic glutamate receptors in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1639–1644
68 Harris, K.M. and Stevens, J.K. (1989) Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. *J. Neurosci.* 9, 2982–2997
69 Nusser, Z. *et al.* (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* 21, 545–559
70 Schikorski, T. and Stevens, C.F. (1997) Quantitative ultrastructural analysis of hippocampal excitatory synapses. *J. Neurosci.* 17, 5858–5867
71 Karachot, L. *et al.* (2001) Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. *J. Neurophysiol.* 86, 280–289
72 Rodrigues, S.M. *et al.* (2002) The group I metabotropic glutamate receptor mGluR5 is required for fear memory formation and long-term potentiation in the lateral amygdala. *J. Neurosci.* 22, 5219–5229
73 Tatarczynska, E. *et al.* (2001) Potential anxiolytic- and antidepressant-like effects of MPEP, a potent, selective and systemically active mGlu5 receptor antagonist. *Br. J. Pharmacol.* 132, 1423–1430
74 Graybiel, A.M. (1998) The basal ganglia and chunking of action repertoires. *Neurobiol. Learn. Mem.* 70, 119–136
75 Gubellini, P. *et al.* (2003) Corticostriatal LTP requires combined mGluR1 and mGluR5 activation. *Neuropharmacology* 44, 8–16
76 Chapman, A.G. *et al.* (2000) Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). *Neuropharmacology* 39, 1567–1574
77 Chen, L. and Toth, M. (2001) Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience* 103, 1043–1050
78 Sourdet, V. *et al.* (2003) Long-term enhancement of neuronal excitability and temporal fidelity mediated by metabotropic glutamate receptor subtype 5. *J. Neurosci.* 23, 10238–10248
79 Castren, M. *et al.* (2003) Augmentation of auditory N1 in children with fragile X syndrome. *Brain Topogr.* 15, 165–171
80 Brody, S.A. *et al.* (2003) Disruption of prepulse inhibition in mice lacking mGluR1. *Eur. J. Neurosci.* 18, 3361–3366
81 Nielsen, D.M. *et al.* (2002) Alterations in the auditory startle response in Fmr1 targeted mutant mouse models of fragile X syndrome. *Brain Res.* 927, 8–17
82 Tachibana, T. *et al.* (2003) Immunohistochemical expressions of mGluR5, P2Y2 receptor, PLC-beta1, and IP3R-I and -II in Merkel cells in rat sinus hair follicles. *Histochem. Cell Biol.* 120, 13–21
83 Walker, K. *et al.* (2001) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology* 40, 10–19
84 Neugebauer, V. *et al.* (1999) Role of metabotropic glutamate receptor subtype mGluR1 in brief nociception and central sensitization of primate STT cells. *J. Neurophysiol.* 82, 272–282
85 Hu, H.Z. *et al.* (1999) Functional group I metabotropic glutamate receptors in submucous plexus of guinea-pig ileum. *Br. J. Pharmacol.* 128, 1631–1635
86 Liu, M. and Kirchgessner, A.L. (2000) Agonist- and reflex-evoked internalization of metabotropic glutamate receptor 5 in enteric neurons. *J. Neurosci.* 20, 3200–3205
87 Park, D. *et al.* (2003) Translation of clock rhythmicity into neural firing in suprachiasmatic nucleus requires mGluR-PLCbeta4 signaling. *Nat. Neurosci.* 6, 337–338
88 Morales, J. *et al.* (2002) *Drosophila* fragile X protein, DFXR, regulates neuronal morphology and function in the brain. *Neuron* 34, 961–972
89 Dockendorff, T.C. *et al.* (2002) *Drosophila* lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. *Neuron* 34, 973–984
90 Inoue, S. *et al.* (2002) A role for the *Drosophila* fragile X-related gene in circadian output. *Curr. Biol.* 12, 1331–1335
91 Bear, M.F. and Cooper, L.N. (1998) From molecules to mental states. *Daedalus* 127, 131–144
92 Yan, Q. *et al.* (2003) Audiogenic seizures and effects of mGluR5 antagonist MPEP in the fMR1-tm1cgr Fragile X mouse. Program number 634.5. In *Abstract Viewer/Itinerary Planner*, Society for Neuroscience Online
93 Lu, Y.M. *et al.* (1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* 17, 5196–5205
94 Spooren, W.P. *et al.* (2001) Novel allosteric antagonists shed light on mglu(5) receptors and CNS disorders. *Trends Pharmacol. Sci.* 22, 331–337
95 Migaud, M. *et al.* (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396, 433–439

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Review

Therapeutic implications of the mGluR theory of fragile X mental retardation

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Evidence is reviewed that the consequences of group 1 metabotropic glutamate receptor (Gp1 mGluR) activation are exaggerated in the absence of the fragile X mental retardation protein, likely reflecting altered dendritic protein synthesis. Abnormal mGluR signaling could be responsible for remarkably diverse psychiatric and neurological symptoms in fragile X syndrome, including delayed cognitive development, seizures, anxiety, movement disorders and obesity.

Keywords: Anxiety disorder, autism, cognitive development, dendritic protein synthesis, long-term depression, metabotropic glutamate receptors, seizure disorder

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Neuroscientists have long been fascinated by the observation that polyribosomes and the molecular machinery of protein synthesis are localized near dendritic spines, major sites of excitatory synaptic transmission and plasticity (Steward & Schuman 2001). Glutamate is the neurotransmitter at most excitatory synapses in the brain, and there is now considerable evidence that activation of group 1 metabotropic glutamate receptors (Gp1 mGluRs, comprised of mGluR1 and mGluR5) is a potent stimulus for protein synthesis (Job & Eberwine 2001; Shin *et al*. 2004; Todd *et al*. 2003; Weiler & Greenough 1993). Moreover, in cases where it has been specifically examined, many of the lasting functional consequences of Gp1 mGluR activation have been found to be dependent on mRNA translation, but not transcription (Huber *et al*. 2000; Karachot *et al*. 2001; Lee *et al*. 2002; Merlin *et al*. 1998; Raymond *et al*. 2000; Snyder *et al*. 2001; Stoop *et al*. 2003; Vanderklish & Edelman 2002; Zho *et al*. 2002). Other than the common requirement for protein synthesis, however, the precise consequence of activating Gp1 mGluRs varies widely, depending on the neuron and the circuit in which it resides (Fig. 1). Systemic activation or inhibition of Gp1 mGluR-mediated protein synthesis by genetic or pharmacological means therefore would be expected to have diverse effects.

The fragile X mental retardation protein (FMRP) has attracted considerable interest as a potential regulator of dendritic protein synthesis. However, the picture emerging from recent biochemical and cell biological studies is confusing and, at times, contradictory. The fragile X mental retardation protein is contained within ribonucleoprotein granules that traffic specific mRNAs (including *Fmr1*) to sites of synaptic transmission. Activation of Gp1 mGluRs on cultured hippocampal neurons with the selective agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) triggers the delivery of FMRP to dendrites (Antar *et al*. 2004). In cultured cortical neurons, expression of both the FMRP and the synaptic protein PSD-95 is increased after activating Gp1 mGluRs. However, DHPG treatment fails to increase PSD-95 levels in cultures prepared from *Fmr1* knockout (KO) mice lacking FMRP (Todd *et al*. 2003). Similarly, DHPG fails to stimulate polyribosome assembly in synaptosomes prepared from the cortex of *Fmr1* KO mice (Weiler *et al*. 2004). Together, these data are consistent with the proposal that FMRP is a requirement for dendritic protein synthesis. On the other hand, there is also compelling evidence that synthesis of some proteins (e.g. MAP1b) is repressed by FMRP and that this repression is relieved in the *Fmr1* KO and in humans with fragile X (reviewed by Bear *et al*. 2004; see also Warren article, this issue). The disparate findings may be related to differences in the tissue, the preparation, the mRNA and the subcellular compartment under investigation (Miyashiro & Eberwine 2004).

Several years ago, we asked the simple question of how a functional consequence of Gp1 mGluR activation differs in the *Fmr1* KO mouse. The physiological response under investigation was a form of synaptic plasticity, long-term depression (LTD), that is triggered in the CA1 region of hippocampus by appropriate stimulation of mGluR5. In previous studies, we had shown that this type of LTD is protein-synthesis dependent (Huber *et al*. 2000) and expressed, in part, by internalization of glutamate receptors (Snyder *et al*.

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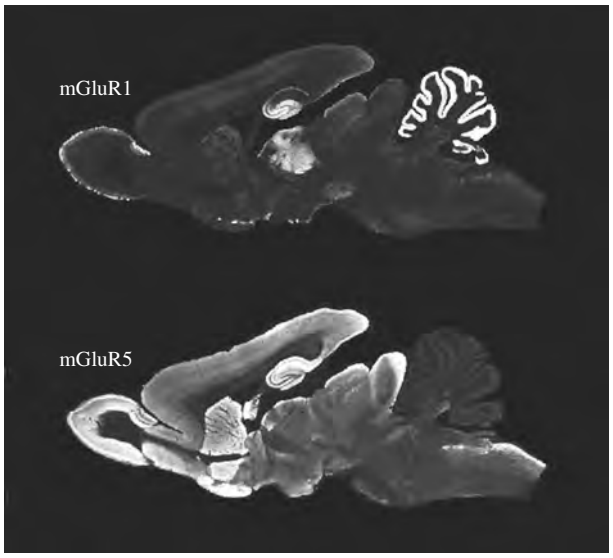

Figure 1: Distribution of group 1 metabotropic glutamate receptor (Gp1 mGluR) protein in the rat brain (modified from Shigemoto & Mizuno 2000).

2001). In the *Fmr1* KO mice, we discovered that mGluR-induced LTD was increased in the hippocampus (Huber *et al*. 2002). These findings are consistent with the hypothesis that FMRP normally represses the protein synthesis required for stable expression of mGluR-dependent LTD – in the absence of repression, we see more LTD in the KO mice. Regardless of the specific mechanism involved, however, the data clearly showed that one functional consequence of mGluR activation is exaggerated in the absence of the fragile X protein.

We next wondered whether all functional consequences of mGluR-dependent protein synthesis might be exaggerated and, if so, whether this could account for aspects of the phenotype in fragile X. The picture that emerged was remarkable and led us to propose the 'mGluR theory' of fragile X (Bear *et al*. 2004). The theory is based on two assumptions that: (1) many lasting consequences of Gp1 mGluR activation require protein synthesis and (2) these are exaggerated in the absence of FMRP. Such theories have value if they stimulate research, and it is encouraging to note that the assumptions have been tested and validated in several functional contexts. More importantly, however, the mGluR theory suggests a sound scientific rationale for the treatment of fragile X syndrome. If symptoms of fragile X arise from excessive signaling through Gp1 mGluRs, then it should be possible, in principle, to treat them with drugs that inhibit the receptors and/or the downstream intracellular signals they initiate.

In this brief review, the author illustrates how the mGluR theory offers a clear molecular logic behind a diverse constellation of symptoms associated with fragile X syndrome. The author will also discuss the prospect of treating these symptoms with drugs that inhibit mGluRs.

Cognitive development

Fragile X is characterized by moderate to severe mental retardation (Bakker & Oostra 2003; Hagerman 2002; Hagerman & Hagerman 2002). Cognition is an emergent property of the cerebral cortex, and the trajectory of cognitive development depends on experience-dependent modifications of synaptic connections among cortical neurons. Synaptic excitation in the cortex is mediated by α-amino-3-hydroxy-5-Methyl-4-isoxazolepropionate (AMPA) and N-Methyl-D-asparate (NMDA) receptors (the major classes of glutamate-gated ion channel). It is therefore of interest that a biochemical phenotype in the *Fmr1* KO mouse is the reduced expression of the AMPA receptor subunit protein *Glu*RI in synaptic plasma membranes prepared from frontal cortex (Li *et al*. 2002).

The experience-dependent delivery and removal of AMPA receptors from cortical synapses are essential for normal cortical development as well as for adult learning and memory. A great deal has been learned about the mechanisms responsible for this synaptic plasticity by the study of the experimental phenomena of long-term potentiation (LTP) and LTD (Malenka & Bear 2004). As mentioned in the introduction, one form of LTD is induced in the CA1 region of hippocampus by activation of mGluR5 (Huber *et al*. 2001), the major Gp1 mGluR in the cerebral cortex (Fig. 1). In cultured hippocampal neurons, activation of mGluR5 with DHPG stimulates the loss of AMPA receptors from synapses, and this is believed to model a mechanism used during cortical development to refine synaptic connections (Snyder *et al*. 2001). Both the LTD and the internalization of AMPA receptors are protein-synthesis dependent, and both the responses are increased in neurons from the *Fmr1* KO mouse (Huber *et al*. 2002) (N. Nagarajan and M.F.B, unpublished observations). Excessive mGluR-dependent LTD during the development could explain the loss of AMPA receptor protein in synaptic plasma membranes from the KO mice.

Neurons in the cerebral cortex of mice and humans lacking FMRP also have a greater proportion of long, thin dendritic spines (Hinton *et al*. 1991; Irwin *et al*. 2001; Rudelli *et al*. 1985). Spine abnormalities have long been associated with human mental retardation of unknown etiology (Purpura 1974) as well as with Down's and Rett syndromes (Kaufmann & Moser 2000). This phenotype may also be related to excessive Gp1 mGluR signaling. Vanderklish and Edelman (this issue) found that prolonged treatment of hippocampal neurons with DHPG also increases the proportion of long, thin dendritic spines (Vanderklish & Edelman 2002). These structural changes and LTD are likely to be related, because synapses on thin spines have a smaller postsynaptic density, fewer AMPA receptors and a reduced number of synaptic vesicles docked at the presynaptic active zone (Harris & Stevens 1989; Nusser *et al*. 1998; Schikorski & Stevens 1997).

Taken together, these findings suggest that exaggerated mGluR5 signaling could contribute to the altered trajectory of

cortical development in fragile X. A number of genetic tests of this hypothesis are feasible in mice; for example, by crossing *Fmr1* KO mice with animals deficient in mGluR5. Another approach would be treatment with a drug that blocks mGluR5, such as 2-methyl-6-phenylethynyl-pyridine (MPEP, with the caveat that at high concentrations, it blocks NMDA receptors). An intriguing possibility is that chronic treatment with an mGluR5 antagonist during a critical period of postnatal development could be 'disease modifying' in animals and humans lacking FMRP.

This possibility has received some striking support very recently in studies of the *Drosophila* model for fragile X syndrome. Flies lacking *dfmr1*, the homologue of *FMR1* in humans, display altered courtship behavior, decreased memory in a conditioned courtship assay and alterations in the structure of the brain (the mushroom bodies) (McBride *et al*. in press). Remarkably, McBride, Jongens and colleagues have found that all of these phenotypes in mutant flies are rescued if they are raised with food containing MPEP or several other drugs that are predicted to affect signaling by the *Drosophila* mGluR, *DmGluRA*. The mushroom body defect could only be rescued when drug treatment was begun at the larval stage of development, but significant behavioral rescue occurred even when treatment began in adult flies (McBride *et al*. in press). These remarkable findings have exciting implications for the treatment of human fragile X syndrome. It is interesting to note that one of the effective agents in flies was lithium, which is currently in widespread use in humans for the treatment of mood disorders.

Seizure disorder

A large proportion of humans with fragile X suffer seizures during childhood (Hagerman 1987; Hagerman 2002; Hagerman & Hagerman 2002), and a robust phenotype in the *Fmr1* KO mice is audiogenic seizures. There are compelling connections between excessive Gp1 mGluR activation and epilepsy (Wong *et al*. 2002).

Electroencephalographic measurements reveal two types of synchronized discharge in epilepsy: brief interictal sharp waves with no perceptible behavioral correlate and prolonged ictal bursts, lasting from seconds to minutes, that produce seizures (Zifkin & Cracco 1990). Hippocampal area CA3 has been used to model the mechanisms involved. Bathing a hippocampal slice in drugs that block inhibition leads to the generation of regularly spaced bursts of synchronous activity in CA3 pyramidal cells that resemble interictal sharp waves. These brief bursts will continue for hours *in vitro* without evolving to ictal-like activity. However, ictal-like activity rapidly appears and persists following transient activation of Gp1 mGluRs (Merlin *et al*. 1998). The requirements for this lasting consequence of mGluR activation are strikingly similar to those for LTD. Induction of ictal-like activity requires activation of extracellular signal-regulated kinase (ERK), a subclass of the mitogen-activated protein kinases (Zhao *et al*. 2004), and mRNA translation but not transcription (Merlin *et al*. 1998).

A prediction of the mGluR theory of fragile X is that this protein-synthesis-dependent response to mGluR activation, like LTD, should be exaggerated in the absence of FMRP. Recently, Wong and colleagues reported a test of this prediction (S. Chuang, Q. Yan, R.P. Bauchwitz, R.K.S. Wong. Program no. 228.5. *2004 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience 2004. Online). They found that ictal-like activity emerged spontaneously in slices from the *Fmr1* KO mouse and that this could be reversed by administering the mGluR5 antagonist MPEP. Thus, in slices from the mutant (but not wild-type), the endogenous activation of mGluR5 by synaptically released glutamate was sufficient to trigger the protein synthesis required for the establishment of ictal-like epileptiform activity. This finding supports a key assumption of the mGluR theory.

Antagonists of mGluR5 have previously been shown to have broad anticonvulsant actions (Spooren *et al*. 2001). These include the prevention of audiogenic seizures in sensitive strains of mice. Recent data from Bauchwitz and colleagues indicate that the robust audiogenic seizure phenotype in *Fmr1* KO mice is also prevented by systemic administration of the mGluR5 antagonist MPEP (R.P. Bauchwitz, Q. Yan, M. Rammal. Program no. 583.20. *2004 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience 2004. Online). Together, the data suggest that mGluR5 antagonists might selectively target the cause of seizures in fragile X syndrome.

Anxiety disorder

Sensory hyperarousal and anxiety are the *sine qua non* of fragile X syndrome in humans (Hagerman & Hagerman 2002). The biological bases of anxiety disorders are poorly understood, but much attention is focused on the control of the hypothalamic-pituitary-adrenal axis by the amygdala. The amygdala is critical for the expression of learned fear. For example, repeated pairing of an auditory stimulus (a tone) with a footshock causes the animal to exhibit fear in response to the tone alone. There is evidence that the tone–shock pairing induces LTP of the synapses bringing the auditory information to the lateral amygdala (Maren & Quirk 2004). Long-term potentiation in the lateral amygdala requires activation of mGluR5 (Rodrigues *et al*. 2002; Rodrigues *et al*. 2004). A clear prediction deriving from the mGluR theory is that this mechanism of LTP will be dependent on translation of pre-existing mRNA and will be enhanced in the *Fmr1* KO mouse. These predictions remain to be tested.

However, there is already extensive evidence that mGluR5 antagonists are highly effective anxiolytics. According to a

recent review of the evidence by Spooren and Gasparini 2004, mGluR5 antagonists exhibit the widest and the most robust anxiolytic activity in preclinical models seen to date. The effects are comparable to those of benzodiazepines with less sedative activity. Thus, although the site(s) and mechanism(s) of action remain to be determined, there is good reason to believe that mGluR5 antagonists will have therapeutic potential for anxiety in fragile X.

Disorders of movement

Two disorders of movement are common in fragile X syndrome: unco-ordinated voluntary movements and repetitive, obsessive-compulsive-like behaviors (Hagerman & Hagerman 2002). Group 1 metabotropic glutamate receptors are highly expressed in two motor-control structures that might contribute to these symptoms: the cerebellum and the striatum (Fig. 1).

Theories of cerebellar function suggest that motor learning occurs by adjustments of the strength of parallel fiber synapses onto Purkinje neurons, based on the relative timing of the parallel fiber activity and 'error signals' conveyed by the climbing fibers arising from the inferior olive. It is now well established that coincident activation of parallel and climbing fibers induces LTD at the parallel fiber–Purkinje cell synapse (Bear & Linden 2001; Ito 1989). Climbing fiber activation is permissive for LTD by elevating intracellular calcium ion concentration; however, the signal that marks the parallel fiber synapse for depression is local activation of Gp1 mGluRs. In the case of cerebellar LTD, the critical receptor is mGluR1 rather than mGluR5. However, similar to the situation in the hippocampus, mGluR-dependent LTD in the cerebellum requires activation of ERK (Endo & Launey 2003) and the translation of pre-existing mRNA (Karachot *et al*. 2001) and is expressed as a loss of AMPA receptors (Steinberg *et al*. 2004). Very recently, cerebellar LTD was examined in the *Fmr1* KO mouse and found to be increased, consistent with the predictions of the mGluR theory (B. Oostra, personal communication). This change in cerebellar synaptic plasticity correlated with impairments in motor learning as assessed by associative eyeblink conditioning. Moreover, comparable defects in eyeblink conditioning were observed in humans with fragile X syndrome. These results suggest that dampening signaling through mGluR1 also could be beneficial in treating fragile X syndrome. However, the therapeutic window for mGluR1 antagonists may prove to be too narrow, because mGluR1 is essential for normal cerebellar function.

Group 1 metabotropic glutamate receptors also play a central role in synaptic plasticity in the striatum believed to be important for the development of habitual motor routines (Gerdeman *et al*. 2003; Gubellini *et al*. 2004). High-frequency stimulation of the cortical afferents to striatal medium spiny neurons can elicit either LTP or LTD, depending on a number of variables such as age and position within the striatum. Both the forms of synaptic plasticity require activation of mGluR1 and/or mGluR5; LTP requires, in addition, activation of NMDA receptors. At present, the picture is most clear for LTD in the dorsal-lateral striatum. Similar to the parallel fiber–Purkinje cell synapse, LTD is induced at corticostriatal synapses by the simultaneous activation of Gp1 mGluRs and a rise in postsynaptic calcium entering through voltage-gated channels. However, unlike the cerebellum, induction of striatal LTD also requires dopamine signaling, and LTD is expressed presynaptically as a reduced probability of glutamate release. The retrograde messenger, signalling from postsynaptic mGluRs to the presynaptic axon terminal, is an endocannabinoid acting on presynaptic CB1 receptors (Gerdeman *et al*. 2002). A role for translation of pre-existing mRNA following mGluR activation has not yet been examined, although this is a clear prediction of the mGluR theory.

The theory also predicts excessive Gp1 mGluR-dependent LTD will be observed in the striatum of the *Fmr1* KO mice. It has been suggested that the development of stereotypies is a consequence of LTD-like changes in the dorsolateral striatum (Graybiel *et al*. 2000). Indeed, striatal activation is deficient in humans with obsessive-compulsive disorder (Graybiel & Rauch 2000; Rauch *et al*. 1997), consistent with the possibility of excessive LTD. Antagonists of Gp1 mGluRs (mGluR5, in particular) could be beneficial for the treatment of compulsive motor routines in fragile X.

Other symptoms and suspicious coincidences

Other symptoms associated with fragile X include obesity, irritable bowel and hyperalgesia. The neurobiological basis for these symptoms remains to be determined in fragile X. Remarkably, however, there is evidence that all of them could be potentially treated with mGluR5 antagonists.

Obesity is likely to arise from altered signaling in the hypothalamus. The ventromedial hypothalamus responds to hormones that signal energy demand and incites feeding behavior via connections with the lateral hypothalamus (Saper *et al*. 2002). Both the ventromedial and the lateral hypothalamus have high levels of mGluR5 expression (van den Pol *et al*. 1995). Very recently, it was reported that mGluR5 KO mice have diminished appetite and weigh less than wild-type littermates. Moreover, treatment of rats with an mGluR5-selective antagonist decreased food intake and caused weight loss (Bradbury *et al*. 2004). These findings suggest that exaggerated mGluR5 signaling could also be responsible for obesity in fragile X.

Gut motility is controlled by a complex interaction of the enteric and central nervous systems (Hunt & Tougas 2002). A population of secretomotor neurons in the ileum contain mGluR5 (Liu & Kirchgessner 2000). Local application of mGluR5 agonists and antagonists increase and decrease, respectively, gut motility (Hu *et al*. 1999).

It is interesting that a majority of patients with irritable bowel syndrome also have altered pain perception (Hunt & Tougas 2002), and hyperalgesia is a common complaint in fragile X. Metabotropic glutamate receptor 5 is expressed by nociceptive C fibers, where it has been implicated in the mechanisms of hyperalgesia.

Concluding remarks

Obviously, there is far more to fragile X than mGluRs. However, evidence continues to accrue that one consistent consequence of the loss of FMRP in neurons is exaggerated signaling via Gp1 mGluRs. This single defect could account for highly diverse neurological and psychiatric symptoms in fragile X syndrome. An exciting prospect is that some or all of these symptoms could be improved by drug therapies that specifically target signaling by Gp1 mGluRs.

References

Antar, L.N., Afroz, R., Dictenberg, J.B., Carroll, R.C. & Bassell, G.J. (2004) Metabotropic glutamate receptor activation regulates fragile x mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. *J Neurosci* **24**, 2648–2655.

Bakker, C.E. & Oostra, B.A. (2003) Understanding fragile X syndrome: insights from animal models. *Cytogenet Genome Res* **100**, 111–123.

Bear, M.F. & Linden, D.J. (2001) The mechanisms and meaning of long-term synaptic depression in the mammalian brain. In Cowan, W.M., Sudhoff, T.C. & Stevens, C.F. (eds), *Synapses.* The Johns Hopkins University Press, Baltimore, pp. 455–517.

Bear, M.F., Huber, K.M. & Warren, S.T. (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* **27**, 370–377.

Bradbury, M.J., Campbell, U., Giracello, D., Chapman, D., King, C.D., Tehrani, L., Cosford, N.D., Anderson, J., Varney, M.A. & Strack, A.M. (2004) Metabotropic glutamate receptor, mGlu5, is a mediator of appetite and energy balance in rats and mice. *J Pharmacol Exp Ther* **313**, 395–402.

Endo, S. & Launey, T. (2003) ERKs regulate PKC-dependent synaptic depression and declustering of glutamate receptors in cerebellar Purkinje cells. *Neuropharmacology* **45**, 863–872.

Gerdeman, G.L., Ronesi, J. & Lovinger, D.M. (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci* **5**, 446–451.

Gerdeman, G.L., Partridge, J.G., Lupica, C.R. & Lovinger, D.M. (2003) It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci* **26**, 184–192.

Graybiel, A.M. & Rauch, S.L. (2000) Toward a neurobiology of obsessive-compulsive disorder. *Neuron* **28**, 343–347.

Graybiel, A.M., Canales, J.J. & Capper-Loup, C. (2000) Levodopa-induced dyskinesias and dopamine-dependent stereotypies: a new hypothesis. *Trends Neurosci* **23**, S71–S77.

Gubellini, P., Pisani, A., Centonze, D., Bernardi, G. & Calabresi, P. (2004) Metabotropic glutamate receptors and striatal synaptic plasticity: implications for neurological diseases. *Prog Neurobiol* **74**, 271–300.

Hagerman, R.J. (1987) Fragile X syndrome. *Curr Probl Pediatr* **17**, 621–674.

Hagerman, R.J. (2002) The physical and behavioral phenotype. In Hagerman, R.J. & Hagerman, P. (eds) *Fragile X Syndrome: Diagnosis, Treatment, and Research*. The Johns Hopkins University Press, Baltimore, pp. 3–109.

Hagerman, R.J. & Hagerman, P.J. (eds) (2002) *Fragile X Syndrome.* The Johns Hopkins University Press, Baltimore, p 540.

Harris, K.M. & Stevens, J.K. (1989) Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. *J Neurosci* **9**, 2982–2997.

Hinton, V.J., Brown, W.T., Wisniewski, K. & Rudelli, R.D. (1991) Analysis of neocortex in three males with the fragile X syndrome. *Am J Med Genet* **41**, 289–294.

Hu, H.Z., Ren, J., Liu, S., Gao, C., Xia, Y. & Wood, J.D. (1999) Functional group I metabotropic glutamate receptors in submucous plexus of guinea-pig ileum. *Br J Pharmacol* **128**, 1631–1635.

Huber, K.M., Kayser, M.S. & Bear, M.F. (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* **288**, 1254–1257.

Huber, K.M., Roder, J.C. & Bear, M.F. (2001) Chemical induction of mGluR5- and protein synthesis-dependent long-term depression in hippocampal area CA1. *J Neurophysiol* **86**, 321–325.

Huber, K.M., Gallagher, S.M., Warren, S.T. & Bear, M.F. (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci USA* **99**, 7746–7750.

Hunt, R.H. & Tougas, G. (2002) Evolving concepts in functional gastrointestinal disorders: promising directions for novel pharmaceutical treatments. *Best Pract Res Clin Gastroenterol* **16**, 869–883.

Irwin, S.A., Patel, B., Idupulapati, M., Harris, J.B., Crisostomo, R.A., Larsen, B.P., Kooy, F., Willems, P.J., Cras, P., Kozlowski, P.B., Swain, R.A., Weiler, I.J. & Greenough, W.T. (2001) Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet* **98**, 161–167.

Ito, M. (1989) Long Term Depression. In Cowan, M.W., Shooter, E.M., Stevens, C.F. & Thompson, R.F. (eds) *Annual Review of Neuroscience*. Annual Reviews, Inc., Palo Alto, California, pp. 85–102.

Job, C. & Eberwine, J. (2001) Identification of sites for exponential translation in living dendrites. *Proc Natl Acad Sci USA* **98**, 13037–13042.

Karachot, L., Shirai, Y., Vigot, R., Yamamori, T. & Ito, M. (2001) Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. *J Neurophysiol* **86**, 280–289.

Kaufmann, W.E. & Moser, H.W. (2000) Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex* **10**, 981–991.

Lee, A.C., Wong, R.K., Chuang, S.C., Shin, H.S. & Bianchi, R. (2002) Role of synaptic metabotropic glutamate receptors in epileptiform discharges in hippocampal slices. *J Neurophysiol* **88**, 1625–1633.

Li, J., Pelletier, M.R., Perez Velazquez, J.L. & Carlen, P.L. (2002) Reduced cortical synaptic plasticity and GluR1 expression associated with fragile X mental retardation protein deficiency. *Mol Cell Neurosci* **19**, 138–151.

Liu, M. & Kirchgessner, A.L. (2000) Agonist- and reflex-evoked internalization of metabotropic glutamate receptor 5 in enteric neurons. *J Neurosci* **20**, 3200–3205.

Malenka, R.C. & Bear, M.F. (2004) LTP and LTD: an embarrassment of riches. *Neuron* **44**, 5–21.

Maren, S. & Quirk, G.J. (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* **5**, 844–852.

McBride, S.M.J., Choi, C.H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., Sehgal, A., Siwicki, K.K., Dockendorff, T.C., Nguyen, H.T., McDonald, T.V. & Jongens, T.A. (2005) Pharmacological rescue of synaptic plasticity, courtship behavior and mushroom body defects in a *Drosophila* model of Fragile X Syndrome. *Neuron* **45**, 642–644.

Merlin, L.R., Bergold, P.J. & Wong, R.K. (1998) Requirement of protein synthesis for group I mGluR-mediated induction of epileptiform discharges. *J Neurophysiol* **80**, 989–993.

Miyashiro, K. & Eberwine, J. (2004) Fragile X syndrome: (What's) lost in translation? *Proc Natl Acad Sci USA* **101**, 17329–17330.

Nusser, Z., Lujan, R., Laube, G., Roberts, J.D., Molnar, E. & Somogyi, P. (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* **21**, 545–559.

van den Pol, A.N., Romano, C. & Ghosh, P. (1995) Metabotropic glutamate receptor mGluR5 subcellular distribution and developmental expression in hypothalamus. *J Comp Neurol* **362**, 134–150.

Purpura, D.P. (1974) Dendritic spine 'dysgenesis' and mental retardation. *Science* **186**, 1126–1128.

Rauch, S.L., Savage, C.R., Alpert, N.M., Dougherty, D., Kendrick, A., Curran, T., Brown, H.D., Manzo, P., Fischman, A.J. & Jenike, M.A. (1997) Probing striatal function in obsessive-compulsive disorder: a PET study of implicit sequence learning. *J Neuropsychiatry Clin Neurosci* **9**, 568–573.

Raymond, C.R., Thompson, V.L., Tate, W.P. & Abraham, W.C. (2000) Metabotropic glutamate receptors trigger homosynaptic protein synthesis to prolong LTP. *J Neurosci* **20**, 969–976.

Rodrigues, S.M., Bauer, E.P., Farb, C.R., Schafe, G.E. & LeDoux, J.E. (2002) The group I metabotropic glutamate receptor mGluR5 is required for fear memory formation and long-term potentiation in the lateral amygdala. *J Neurosci* **22**, 5219–5229.

Rodrigues, S.M., Schafe, G.E. & LeDoux, J.E. (2004) Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. *Neuron* **44**, 75–91.

Rudelli, R.D., Brown, W.T., Wisniewski, K., Jenkins, E.C., Laure-Kamionowska, M., Connell, F. & Wisniewski, H.M. (1985) Adult fragile X syndrome. Clinico-neuropathologic findings. *Acta Neuropathol (Berl)* **67**, 289–295.

Saper, C.B., Chou, T.C. & Elmquist, J.K. (2002) The need to feed: homeostatic and hedonic control of eating. *Neuron* **36**, 199–211.

Schikorski, T. & Stevens, C.F. (1997) Quantitative ultrastructural analysis of hippocampal excitatory synapses. *J Neurosci* **17**, 5858–5867.

Shigemoto, R. & Mizuno, N. (2000) Metabotropic glutamate receptors – immunocytochemical and in situ hybridization analysis. In Ottersen, O.P. & Storm-Mathisen, J. (eds) *Handbook of Chemical Neuroanatomy.* Elsevier, New York, pp. 63–98.

Shin, C.Y., Kundel, M. & Wells, D.G. (2004) Rapid, activity-induced increase in tissue plasminogen activator is mediated by metabotropic glutamate receptor-dependent mRNA translation. *J Neurosci* **24**, 9425–9433.

Snyder, E.M., Philpot, B.D., Huber, K.M., Dong, X., Fallon, J.R. & Bear, M.F. (2001) Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nat Neurosci* **4**, 1079–1085.

Spooren, W. & Gasparini, F. (2004) mGlu5 receptor antagonists: a novel class of anxiolytics? *Drug News Perspect* **17**, 251–257.

Spooren, W.P., Gasparini, F., Salt, T.E. & Kuhn, R. (2001) Novel allosteric antagonists shed light on mglu(5) receptors and CNS disorders. *Trends Pharmacol Sci* **22**, 331–337.

Steinberg, J.P., Huganir, R.L. & Linden, D.J. (2004) N-ethylmaleimide-sensitive factor is required for the synaptic incorporation and removal of AMPA receptors during cerebellar long-term depression. *Proc Natl Acad Sci USA* **101**, 18212–18216.

Steward, O. & Schuman, E.M. (2001) Protein synthesis at synaptic sites on dendrites. *Annu Rev Neurosci* **24**, 299–325.

Stoop, R., Conquet, F., Zuber, B., Voronin, L.L. & Pralong, E. (2003) Activation of metabotropic glutamate 5 and NMDA receptors underlies the induction of persistent bursting and associated long-lasting changes in CA3 recurrent connections. *J Neurosci* **23**, 5634–5644.

Todd, P.K., Mack, K.J. & Malter, J.S. (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc Natl Acad Sci USA* **100**, 14374–14378.

Vanderklish, P.W. & Edelman, G.M. (2002) Dendritic spines elongate after stimulation of group 1 metabotropic glutamate receptors in cultured hippocampal neurons. *Proc Natl Acad Sci USA* **99**, 1639–1644.

Weiler, I.J. & Greenough, W.T. (1993) Metabotropic glutamate receptors trigger postsynaptic protein synthesis. *Proc Natl Acad Sci USA* **90**, 7168–7171.

Weiler, I.J., Spangler, C.C., Klintsova, A.Y., Grossman, A.W., Kim, S.H., Bertaina-Anglade, V., Khaliq, H., de Vries, F.E., Lambers, F.A., Hatia, F., Base, C.K. & Greenough, W.T. (2004) From the cover: fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses. *Proc Natl Acad Sci USA* **101**, 17504–17509.

Wong, R.K., Chuang, S.C. & Bianchi, R. (2002) Metabotropic glutamate receptors and epileptogenesis. *Epilepsy Curr* **2**, 81–85.

Zhao, W., Bianchi, R., Wang, M. & Wong, R.K. (2004) Extracellular signal-regulated kinase 1/2 is required for the induction of group I metabotropic glutamate receptor-mediated epileptiform discharges. *J Neurosci* **24**, 76–84.

Zho, W.M., You, J.L., Huang, C.C. & Hsu, K.S. (2002) The group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine induces a novel form of depotentiation in the CA1 region of the hippocampus. *J Neurosci* **22**, 8838–8849.

Zifkin, B.G. & Cracco, R.Q. (1990) An orderly approach to the abnormal EEG. In Daly, D.D. & Pedley, T.A. (eds) *Current Practices in Clinical Electroencephalography.* Raven, New York, pp. 253–267.

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Small, G.W., Mazziotta, J.C., Collins, M.T., Baxter, L.R., Phelps, M.E., Mandelkern, M.A., Kaplan, A., La Rue, A., Adamson, C.F., Chang, L., et al. (1995). JAMA *273*, 942–947.

Takahashi, R.H., Milner, T.A., Li, F., Nam, E.E., Edgar, M.A., Yamaguchi, H., Beal, M.F., Xu, H., Greengard, P., and Gouras, G.K. (2002). Am. J. Pathol. *161*, 1869–1879.

Yang, A.J., Knauer, M., Burdick, D.A., and Glabe, C. (1995). J. Biol. Chem. *270*, 14786–14792.

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Courting a Cure for Fragile X

Fragile X syndrome is the most common heritable cause of mental retardation. Previous work has suggested that overactive signaling by group I metabotropic glutamate receptors (mGluRs) may be a mechanism underlying many of the disease symptoms. As a test of this theory, McBride et al. show that in a *Drosophila* model for Fragile X syndrome, treatment with mGluR antagonists can rescue short-term memory, courtship, and mushroom body defects.

Fragile X mental retardation syndrome (FXS) is an inherited single-gene disorder. In the afflicted population, the *FMR1* gene is transcriptionally silenced, and the Fragile X mental retardation protein (FMRP) is not made. The consequence in humans is a diverse constellation of psychiatric and neurological symptoms ranging from cognitive impairment to autistic behavior (Hagerman and Hagerman, 2002). Based on research in the *Fmr1* knockout (KO) mouse, the suggestion was made that many of these symptoms could be accounted for by overactive signaling by group I metabotropic glutamate receptors (GpI mGluRs; (Bear et al., 2004; Huber et al., 2002). The implication of this "mGluR theory" is that many symptoms in FXS might respond to treatment with drugs that inhibit GpI mGluRs (Bear, 2005). In this issue of *Neuron*, Tom Jongens, Sean McBride, and colleagues (McBride et al., 2005) describe an audacious test of this notion in fruit flies lacking *dfmr1*, the *Drosophila* homolog of human *FMR1*. Mutant flies exhibit altered courtship behavior, decreased memory in a conditioned courtship assay, and alterations in the structure of the brain (the mushroom bodies). Remarkably, feeding flies drugs that target mammalian mGluR signaling could rescue all three defects. These amazing results fuel a growing sense of optimism that appropriate pharmacological intervention could ameliorate, and possibly even cure, aspects of Fragile X syndrome in humans.

In mammals, mGluRs comprise a family of eight subtypes that are commonly divided into three groups based on their shared signal transduction pathways (Conn and Pin, 1997). Group I mGluRs consist of mGluR1 and mGluR5 and couple to phospholipase C (PLC), which stimulates the turnover of membrane phosphoinositides. Several lines of research led to the mGluR theory of Fragile X (reviewed by Bear et al., 2004). Among them are the findings that (1) activation of GpI mGluRs with a selective agonist stimulates synaptic protein synthesis and trafficking of FMRP, (2) many of the lasting functional consequences of GpI mGluR activation require mRNA translation but not transcription, and (3) when it has been examined, protein synthesis-dependent responses to GpI mGluR activation are exaggerated in the *Fmr1* KO mouse, consistent with a role for FMRP as a translational repressor of selected mRNA transcripts. Considering these findings together with the known consequences of GpI mGluR activation in the brain suggested that many of the symptoms of FXS could be simply accounted for by overactive mGluR signaling (Figure 1A). Because mGluR1 is necessary for proper cerebellar function, mGluR5 has been viewed as the better therapeutic target. There are several drugs that selectively inhibit mGluR5; the most widely used is the noncompetitive antagonist MPEP (2-methyl-6-phenylethynyl-pyridine), with the caveat that at high concentrations it blocks NMDA receptors (Spooren et al., 2001). MPEP was the first food additive used by McBride et al. to treat the behavioral and structural deficits in Fragile X flies.

Courtship behavior in *Drosophila* is innate and involves a complex set of behaviors that ends in copulation (Figure 2A). Conditioned courtship suppression is an associative learning assay that modifies this set of innate behaviors. Briefly, the conditioning paradigm is as follows. During the training phase, the male is placed with an unreceptive trainer (a previously mated female). Initially, he courts the female vigorously, but over time his courtship activity declines (Figure 2B). In the next phase, his memory is tested with a receptive virgin female. After training, wild-type males will not court this female, even though she is receptive. This suppression of courtship lasts 2–3 hr and constitutes the memory phase (Figure 2C).

As had been previously reported (Dockendorff et al., 2002) *dfmr1* KO flies show diminished innate courtship activity (Figure 2A): KO flies court receptive virgin females less vigorously than wild-types. In the current study, McBride et al. extend the analysis to conditioned courtship suppression. They find that during the training phase (Figure 2B) KO flies show normal courtship suppression, indicating that *learning* is intact in these animals. However, courtship suppression *memory* (Figure 2C) is disrupted: KO flies continue to court tester females at naive levels after exposure to unreceptive trainer females. These behavioral changes correlate with an anatomical defect in the mushroom body, the part of the fly brain believed to be responsible for conditioned courtship learning and memory (Michel et al., 2004).

Pharmacological rescue of all three phenotypes was achieved by feeding KO animals MPEP. Maximal rescue was obtained when treatment began in larvae and continued in adults, suggesting a role for mGluR signaling in both the development and adult expression of the phenotypes. The courtship and conditioned courtship phenotypes could still be rescued when treatment was withheld until adulthood. The mushroom body defect, on the other hand, could only be rescued when treatment began early, suggesting that this structural change is a consequence of an altered developmental

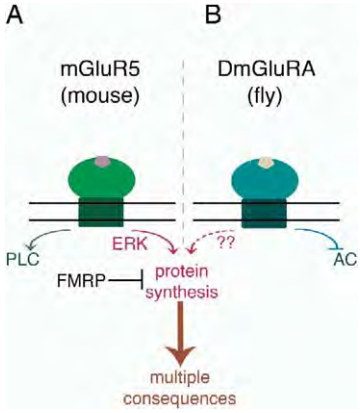

Figure 1. The mGluR Theory of Fragile X

(A) In mammals, many lasting consequences of activating group I mGluRs (mGluRs 1 and 5) require local mRNA translation and appear to be exaggerated in the absence of FMRP. Rather than the "classical" signaling via phospholipase C (PLC), these protein synthesis-dependent actions of mGluR stimulation appear to require activation of extracellular signal-regulated kinase (ERK). (B) In the fly, McBride et al. find that drugs blocking the effect of glutamate at the *Drosophila* mGluR (DmGluRA) can rescue deficits caused by the absence of FMRP. Although DmGluRA resembles vertebrate group II mGluRs that signal via inhibition of adenylyl cyclase (AC), these findings are consistent with the mGluR theory if pathways exist that couple DmGluRA activation to FMRP-regulated protein synthesis.

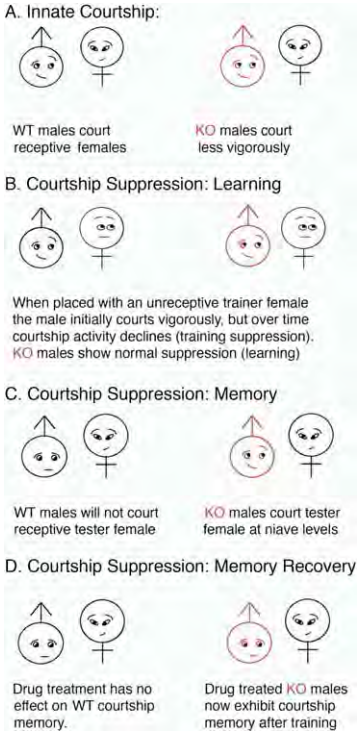

Figure 2. Courtship and Courtship Suppression in Flies

McBride et al. find that courtship suppression memory is impaired in Fragile X flies, but can be completely rescued if the mutants are fed mGluR antagonists during development.

trajectory that cannot be reversed in adults. However, these findings taken together indicate that normal mushroom body anatomy is not required for rescue of the behavioral phenotype. The exciting implication is that pharmacotherapy in early development (which might correspond to a human developmental epoch earlier than the disease is identified) may not be necessary for recovery of a cognitive disruption.

The findings summarized above appear to provide stunning support for the mGluR theory. However, the audacity of Jongens' study stems from the fact that other than the MPEP binding region, the lone functional mGluR in the *Drosophila* genome (DmGluRA) bears little resemblance to mammalian mGluR5. In fact, DmGluRA is an ortholog of vertebrate group II mGluRs that are negatively coupled to adenyl cyclase (AC). Indeed, McBride et al. were able to show that three different group II-selective, competitive mGluR antagonists and lithium chloride also rescue the Fragile X phenotype. Because all four antagonists showed the same effects, the authors argue that DmGluRA is the relevant target. However, future studies to unequivocally establish the role of mGluR activity in pathogenesis must show that reduction in *DmGluRA* gene dosage (by mutation or RNAi) rescues the *dfmr1*-KO phenotypes and occludes drug effects.

It is possible that DmGluRA in *Drosophila* assumes the full burden of metabotropic glutamate signaling that is divided among the eight different mGluRs in vertebrates. Indeed, the *Drosophila* data are compatible with the mGluR theory as it is currently articulated if there exist pathways that couple DmGluRA activation to FMRP-regulated protein synthesis (Figure 1B). Current data in mammals suggest that Gpl mGluRs stimulate protein synthesis via activation of extracellular signal-regulated kinase (ERK; Gallagher et al., 2004; Zhao et al., 2004), rather than the "classical" pathway involving phosphoinositide turnover (and a known target of lithium). It is obviously of great interest to know if DmGluRA activation stimulates mRNA translation in flies and how this is regulated by mGluR antagonists, lithium, and FMRP. This knowledge will be important in making predictions about how the findings in flies might generalize to humans with Fragile X. In the meantime, however, the current study provides a compelling demonstration that pharmacotherapy has the potential to cure aspects of Fragile X.

Mark Bear has a financial interest in Sention, a company developing mGluR antagonists for treatment of Fragile X.

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Selected Reading

Bear, M.F. (2005). Genes Brain Behav. *24*, in press.

Bear, M.F., Huber, K.M., and Warren, S.T. (2004). Trends Neurosci. *27*, 370–377.

Conn, P.J., and Pin, J.P. (1997). Annu. Rev. Pharmacol. Toxicol. *37*, 205–237.

Dockendorff, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., Siwicki, K.K., Sehgal, A., and Jongens, T.A. (2002). Neuron *34*, 973–984.

Gallagher, S.M., Daly, C.A., Bear, M.F., and Huber, K.M. (2004). J. Neurosci. *24*, 4859–4864.

Hagerman, R.J., and Hagerman, P.J. eds. (2002). Fragile X Syndrome, Third Edition (Baltimore, MD: The Johns Hopkins University Press).

Huber, K.M., Gallagher, S.M., Warren, S.T., and Bear, M.F. (2002). Proc. Natl. Acad. Sci. USA *99*, 7746–7750.

McBride, S.M.J., Choi, C.H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., Sehgal, A., Siwicki, K.K., Dockendorff, T.C., Nguyen, H.T., et al. (2005). Neuron *45*, 753–764.

Michel, C.I., Kraft, R., and Restifo, L.L. (2004). J. Neurosci. *24*, 5798–5809.

Spooren, W.P., Gasparini, F., Salt, T.E., and Kuhn, R. (2001). Trends Pharmacol. Sci. *22*, 331–337.

Zhao, W., Bianchi, R., Wang, M., and Wong, R.K. (2004). J. Neurosci. *24*, 76–84.

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METHODS IN ELECTROPHYSIOLOGICAL RECORDING

Before discussing the methods used to record synaptic responses and action potentials, the tissue preparation must be considered. In general, there are two animal preparations used for studies of synaptic plasticity: *in vivo* and *in vitro*. The *in vivo* preparation is in some sense the penultimate "*natural"* environment for making recordings of electrical activity in the brain because neurons, which exist in complex interconnected networks, are recorded intact and virtually undisturbed. Moreover, it is possible, using chronically implanted electrodes, to make recordings over many days, and thereby capture synaptic plasticity events that take place over a longer time course; these recordings can be made in awake behaving animals, eliminating the confound of anesthesia. It is possible to record extracellularly (fields, multiunits, and single units) as well as intracellularly using the *in vivo* preparation; however, most labs studying synaptic plasticity have nevertheless carried out intracellular recordings *in vitro* because of the technical difficulties involved in maintaining an intracellular recording electrode in an intact animal. For example, because of pulsatile blood flow, the brain in a living animal is always moving. This movement can injure the cell being recorded and makes intracellular recording difficult, and in the case of chronic recording, impossible. A second disadvantage of *in vivo* recording is that it is impossible to precisely control the extracellular environment in an *in vivo* preparation. This is particularly true of experiments requiring pharmacologic manipulation. Experimenters have tried to resolve this problem with local application of drugs; however, in some cases it may nevertheless be difficult to control for regulatory, metabolic, and lethal effects of these drugs in an intact animal. Recently, the advent of targeted (region specific) and inducible, molecular manipulations of genes in transgenic and knockout animals has made it possible to study some of the mechanisms of synaptic plasticity in the intact animal without the use of pharmacologic manipulation. However, these studies are currently limited by the time it takes to generate such animals, the degree and specificity of targeting, and by the species (mostly murine models) that are used.

The *in vitro* preparation represents a more reductionist approach and has several varieties: organotypic slice, slice culture and dissociated cell culture. There are several cases where the reductionist approach might be preferred: for example, studies that require the ionic environment to be controlled, studies of the intrinsic properties of the neuron (where the neuron must be isolated from the extrinsic connections), or as mentioned above, intracellular recordings. Furthermore, imaging studies of deep brain structures are limited by the depth that can be optically resolved, even with laser (confocal and two-photon) microscopy; in these cases, *in vitro* preparations expose deeper structures by dissection and/or slicing the tissue. Organotypic slices allow the experimenter to maintain brain tissue for several hours while retaining normal anatomical relationships (for example, in the hippocampus, although input and output axons are usually severed, several synaptic links of the circuit are maintained). Cultured slices and dissociated cell cultures represent a further iteration of the reductionist approach, but are useful when the process being studied, such as developmental synaptic plasticity, takes several days to develop, or when transfection of a gene or application of a drug requires several days of incubation. Some studies require identification of individual neural protrusions and processes, which can be difficult to resolve in highly packed slices; by allowing absolute control over the density of synaptic connections in a dish, dissociated cell culture makes this level of resolution possible.

Neurons are unique among cells for there ability to convert a membrane potential (which exist in almost all cell types) into an electrical signal that can be used to transmit information across cells. This information transfer occurs across the synapse, a specialized junction between nerve cells. As mentioned above, these synapses are subject to modification; such modifications of synaptic strength are generally detected by measuring the electrical activity of the neuron. There are basically two ways to record electrical activity from neurons: extracellular and intracellular. For *extracellular* recording, the electrodes are placed outside the cell and measure changes in the environment next to the cell. For *intracellular* recording one electrode is placed inside the cell and the other electrode is placed outside the cell: activity is recorded between the inside and outside of a *single* cell. In either case, recording the bioelectric signal requires the use of at least two electrodes: one electrode is the active electrode and is placed inside (for intracellular) or near (for extracellular) the signal source; the other electrode serves as the ground.

There are two main electrical signals that the central nervous system uses for information transfer relevant to our discussion of synaptic plasticity. The first is the *action potential* (abbreviated AP, sometimes also referred to as "spike" or "firing"), which is propagated down the shaft of the axon by the sequential opening and closing of Na^+ channels along the axonal membrane. This electrical signal is a large, all or none depolarization of the neuron that can be detected by the extracellular electrode. Extracellular unit recordings, then, refer to recordings made as individual neurons (or "units") fire action potentials. The second electrical signal is the *post-synaptic potential* (abbreviated PSP). When an action potential travels down its axon terminal, the depolarization of the membrane and subsequent Ca^{++} entry causes the release of neurotransmitter from the pre-synaptic terminal into the synaptic cleft. When the neurotransmitter binds to the post-synaptic membrane, ion channels start to open, causing a small change in membrane potential of the post-synaptic neuron, known as the post-synaptic potential. It can be excitatory (ePSP) or inhibitory (iPSP) depending on whether the opening of ion channels depolarizes or hyperpolarizes the cell. This electrical signal is a small, sub-threshold change in membrane voltage, and must be detected by intracellular recording methods. Intracellular recording can also be used to measure current changes, referred to as post-synaptic *currents* (PSC).

Field potentials represent a hybrid signal that is recorded extracellularly: intracellular depolarizations appear extracellularly as small hyperpolarizations relative to a distant ground. These potential changes reflect several hundred synapses that can influence the extracellular potential at a distance. Field potential recordings are particularly useful in the hippocampus, where the separation of cell bodies from their associated synapses, allows recordings of either field postsynaptic potentials (fPSP's) or cell firings (population spikes) by positioning the electrode in either the synaptic or cell body layer. In other brain regions, the interpretation of the field potential is more difficult because changes in potential reflect the combined effect of both synaptic potentials and action potential firing's of nearby cell bodies (that are not sequestered into a strict layers). In these regions current source density (CSD) analysis can be helpful in the interpretation of field potential recordings (a detailed discussion of CSD analysis is beyond the scope of this paper, and will not be attempted here).

Recently the *tetrode* recording method has been developed to circumvent the problem of isolating a signal source when recording units extracellularly. Ordinarily unit recordings are made with a single electrode that detects voltage changes at its tip; but imagine a situation where the electrode tip is exactly equidistant to two neurons. In this case the single electrode would be

incapable of discerning the two signal sources from each other if the two signals had very similar or very complicated spike shapes. By adding three more electrodes, that are sufficiently close to detect the same source, but also sufficiently spread apart to have varying distances from the signal source, tetrodes can generate a electrical fingerprint for a single neuron: two signal sources will each generate a unique set of characteristic waveforms across four electrodes. (Because tetrode recordings represent a variation on the extracellular unit recording method, and because their analysis requires sophisticated statistical methods, they will not be discussed further in this paper).

Finally, electroencephalogram (EEG) recording is yet another extracellular recording method utilized in synaptic plasticity studies. In EEG recordings what is important is not the individual waveforms being recorded, but rather, the frequency of the waves. This is because the potentials recorded using EEG represent the summation of neuronal activity from such a large number of sources, that it would be impossible to differentiate the contribution from any one source from all the others. Nevertheless, several *frequencies* have been correlated with specific behaviors in humans; and are thought to represent the synchronized activity of large groups of neurons. Most relevant to the current discussion is the so-called *theta rhythm* that has a frequency of 4-12Hz. This theta rhythm has been implicated in synaptic plasticity because studies have shown this oscillation to be phase locked with "place" cells, which are hippocampal units that fire when an animal remembers a particular *place* in its environment. Furthermore, as we shall see later, the theta burst protocol for inducing long-term potentiation, is thought to mimic an endogenous theta rhythm that might synchronize behaviorally salient associations and prime them for learning. EEG recordings can be made with scalp electrodes in humans, but in animal studies they are more often electrodes with a very large tip exposure implanted for chronic *in vivo* recording of the awake behaving animal. Interpretation of EEG rhythms is complicated, and can either be done with power spectrum analysis using Fast Fourier Transformation (FFT) or inter-spike interval (ISI) measurements. In either case, the idea is to determine whether a specific oscillatory rhythm, like theta, consistently coincides with a particular behavior.

Extracellular recordings can be made with either insulated metallic electrodes or fluid filled glass micropipette electrodes. Intracellular recordings are made with either *sharp* micropipette electrodes or *patch* micropipette electrodes. Whichever the type of electrode being used, electrodes are chosen according to the desired impedance characteristics. For metallic electrodes, the amount of exposed metal at the tip determines the impedance of the electrode: the larger the exposed tip area, the lower the impedance of the electrode, but the lager the sample area. A larger exposed tip area makes it easier to find sites of electrical activity, but makes it difficult to isolate the electrical activity of single cells. Electrodes with smaller exposed tip areas isolate cells better, but they only sample a small area and have higher-impedance and higher noise. Similarly, the impedance of the glass microelectrode is a function of the tip size. The glass microelectrode is usually filled with a concentrated salt solution that produces a low resistance (or impedance) to recording: the smaller the tip of the microelectrode, the higher the impedance, because only a small cross-sectional area of electrolyte solution is available to carry current at the electrode tip. As mentioned above, extracellular recordings are best used for large strong signals, such as action potentials and field potentials. For smaller electrical events such as postsynaptic potentials and currents, neuron input resistance, and reversal potentials intracellular recording is better. Furthermore, because it is possible to inject drugs directly into the cell, the intracellular technique is well suited to studying the contribution of cytoplasmic components to

synaptic plasticity. The disadvantage of intracellular recording is that it is difficult, and as mentioned above, not well suited to *in vivo*, especially chronic, recordings.

Optical recording represents a third, *indirect*, method of recording electrical activity from neurons. In this case, fluorescing calcium indicators, rather than voltage itself, are used as the signal of electrical activity in the neuron. This is possible because it is known that calcium entry in to the presynaptic cleft immediately precedes and is required for release of neurotransmitter vesicles into the synaptic cleft. Furthermore, because calcium is rapidly sequestered by the endoplasmic reticulum of the presynaptic cell, its presence at the presynaptic terminal is short-lived enough be a sensitive measure of electrical activity (if it were not, after a first action potential induced influx of calcium, several subsequent action potentials could be fired by the cell without detectable changes in calcium levels).

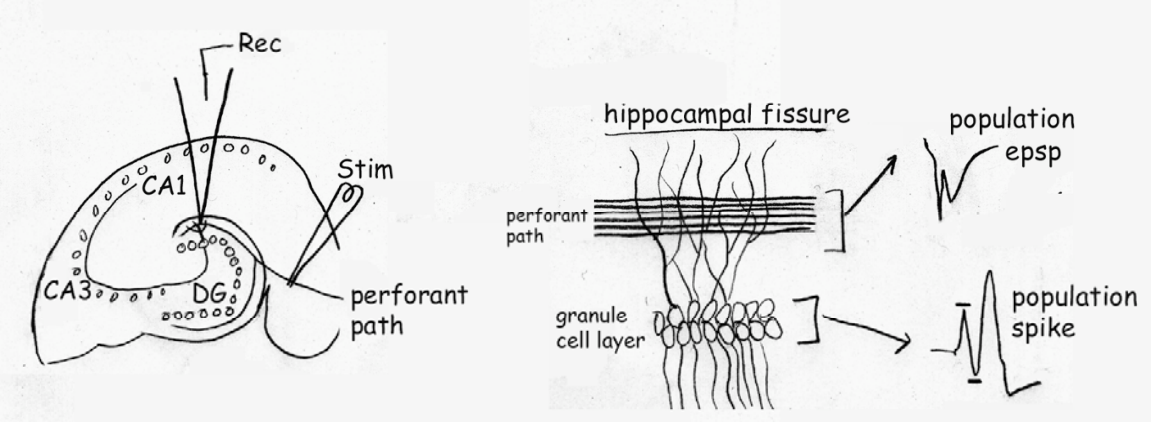
In the next section, the specifics of different recording techniques will be elaborated with four examples from the synaptic plasticity literature. Although this literature is just thirty years old, it is vast, and not every seminal discovery will be covered: preference will be given to papers that represent a wide breadth of techniques and spectrum of topics in synaptic plasticity, including various brain regions and developmental time points.

1. Field Potential's:

Bliss TV, Lomo T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol. 1973 Jul;232(2):331-56.

For a number of compelling reasons the hippocampus is thought to be an important structure for memory processing; it wasn't until the early 1970's however, that it was shown that activity-dependent changes in synaptic efficacy might underlie such processing. In this seminal paper Bliss and Lømo not only report the phenomenon of Long-term Potentiation (LTP), they delineate some of its cardinal features. Specifically, that LTP is long lasting, and input-specific. Input-specificity refers to the fact that when LTP is induced at one set of synapses, the increase in synaptic strength is limited to those synapses; it does not occur at other synapses on the same cell. Theoretically, this specificity increases the storage capacity of the brain because the sites of synaptic plasticity, the dendritic spines, are able to store information independently of each other, and of the soma. The major input to the hippocampus is the entorhinal cortex, which sends axonal projections to the hippocampus via the perforant path. Perforant path axons from the entorhinal cortex synapse onto granule cells of the dentate gyrus, which is located in the dorsal hippocampus. The perforant path fibers course through the molecular layer of the dentate, making *en passant* synapses with dendritic spines from many granule cells. As mentioned earlier, this architecture lends itself well to field potential recordings (redrawn from Bliss and

L ø m o , 1 9 7 3) :



When the recording electrode is placed in the middle third of molecular layer, synaptic potentials are recorded and the trace is a population excitatory postsynaptic potential (*population epsp*). Activation of the synapses here causes a local depolarization of dendritic membrane, causing extracellular current to flow from the cell body layer towards the dendrites, resulting in a negative potential in the synaptic region, and a positive potential in the cell body region relative to the distant electrode (this is reflected in the above trace as a initial negative deflection, with a superimposed positive going spike). When the recording electrode is placed deeper in the granule cell layer, the trace flips such that the initial components of the potential are positive going; this so-called *population spike* reflects the number, amplitude and synchrony of granule cell firing (in essence these are the "units" recorded extracellularly). The population spike is maximally negative in the cell body layer, where the current sink of the spike-generating neuronal hilus is greatest.

For these *in vivo* recordings, under urethane anesthesia, the hippocampus of 18 adult rabbits was exposed by dissecting out the overlying cortex. Stimulating electrodes were placed on the perforant path, and recording electrodes were placed in the appropriate layer of the dentate. A small silver plate was sown into the neck muscles to act as the ground electrode. NaCl-filled glass microelectrodes (resistance of 1-3MΩ) were used for recording. Stimulating electrodes were tungsten wire used to give constant voltage pulses with amplitudes of up to 100V. The circuit was completed with a silver ball electrode placed on a neighboring cut muscle surface. Baseline recordings were made by delivering single shocks at fixed stimulus strength, repeated at 2-3 second intervals. Then conditioning trains of 30 minutes or more were delivered to one side (the experimental pathway); in the other hemisphere, no conditioning stimulus was given (the control pathway). The response evoked by this stimulation paradigm was recorded and analyzed for:

a. Amplitude of the population epsp (negative potential in the synaptic layer, or positive potential in the cell body layer).
b. Amplitude of the population spike (only the cell body layer, from the initial peak positivity to the peak negativity).
c. Latency of the population spike (the time from the stimulus artifact to the initial peak positivity).

The results of these experiments were that after conditioning stimulus is applied, often (but not always) synaptic responses in this pathway were potentiated; the population epsp and spike were increased in amplitude and the latency of the population spike was decreased, despite the fact that the stimulus strength remained fixed before and after the conditioning train. The

significance of this finding is that the conditioning stimulus modified the conditioned synapses so that they were more effective. Moreover, the control pathway, that did not receive conditioning stimulus, did not show this potentiation; LTP is therefore said to be input specific. Interestingly, the authors also use a second conditioning paradigm, which has now become a standard LTP induction protocol, namely, conditioning with high frequency trains. The justification for the use of these100Hz trains of conditioning stimuli was that the authors wanted to localize the changes to the perforant path/granule cell synapse, excluding the influence of granule cell firing. By giving 100Hz trains, they argue, they can prevent firing of the postsynaptic cell during conditioning. Subsequent studies from other labs have shown that varying the frequency of the train can actually reverse the direction of the changes in synaptic efficacy. The frequency dependence however, is not absolute, but rather reflects the requirement of postsynaptic depolarization that is coincident with presynaptic activation (see next paper for discussion of mechanism of this cooperativity rule). Finally, in these experiments, synaptic potentiation lasted for many hours; and subsequent experiments have shown that it can last many weeks. This time course lends support to the idea that LTP like changes in synaptic efficacy could potentially account for the longevity of memories (which can last a lifetime).

2. Unit Recording:

Bear MF, Kleinschmidt A, Gu QA, Singer W. Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. J Neurosci. 1990 Mar;10(3):909-25.

After Bliss and Lomo's seminal discovery of Long-term potentiation in the hippocampus, a flurry of experimental activity over the next ten years identified the NMDA receptor as the molecule that could subserve the requirements of *Hebb's* rule; i.e. it could act as a coincidence detector since NMDA receptors are only unblocked and able to conduct Ca+ ions when presynaptic activation is coincident with postsynaptic depolarization. Using the NMDA antagonist, APV, it was subsequently shown that the NMDA receptor is indeed required for the induction of LTP in hippocampal and visual cortical slices. At this point, the *in vivo* test of an NMDA receptor dependent mechanism of synaptic plasticity required an experimental manipulation that could be correlated with experience dependent changes and test *Hebbian* competitive mechanisms. The well-established ocular dominance shift paradigm of Hubel and Weisel was exactly such an experimental model. To summarize: the two eyes send convergent inputs, via the thalamus, to the binocular visual cortex. There these inputs must compete with one another for synapses on cortical target neurons. By manipulating visual experience, e.g. by monocular deprivation, experimenters can shift ocular dominance towards the dominant (open eye) input. According to *Hebb's* rule, such modification of binocular connections would occur because the dominant input is more effective in activating the target neuron, while the non-dominant input is less effective in activating the target neuron; accordingly, the dominant input is retained, while the nondominant input is lost. Furthermore, these competitive processes occur during postnatal development, and are thought to underlie visual cortical organization by sensory experience.

To answer the question of whether the NMDA receptor mechanism might underlie these ocular dominance shifts, these authors made extracellular single unit recordings from binocular visual cortex, after infusing APV by miniature osmotic pump while manipulating visual experience. In these experiments, single units were recorded in the binocular visual cortex of anesthetized

kittens, using glass micropipettes filled with 1.5 M potassium citrate (impedence 15MΩ). The electrodes were positioned so that they moved along the cortical thickness obliquely (in other words, they wanted to sample many adjacent orientation columns, instead of only one had they penetrated perpendicular to the cortical surface). Because it had been shown that some degree of cell death occurs near the infusion cannula, the authors penetrated the cortex 3-6 mm anterior to the position of the cannula. As each unit along an electrode track was encountered it was classified according to the visual stimulus that could evoke a response (AP firing) from that cell. For example, for ocular dominance a cell was classified as "3" if it responded equally well to a light presented to the right eye as to the left eye, as a "1" or "5" if it responded only to light presented to one eye, and as "2" or "4" if they were binocular but had a preference for one eye or the other. One question, then, is how they were able to find graded responses (more or less responsive to input from an eye) if they are recording AP's, which are all-or-none events? The answer lies in the fact that intensity is coded in the brain as bursts of action potentials, firing in rapid succession; e.g., a "2" neuron would fire a modest number of AP's for one eye, and a larger number of AP's for the other eye. After one week of monocular deprivation in the presence of APV, these authors showed a decrease in the amount of ocular dominance shift (as measured by the comparison of the number of cells responding only to the open eye to those showing a binocular response). These results suggested that the NMDA receptor is critically important for the competitive process involved in organizing the visual cortex with sensory experience.

Important to the conclusions that the authors wanted to draw from these experiments, is the assumption that APV has a specific synaptic effect *in vivo*. One concern was that APV infusion non-specifically blocks light-evoked responses. Therefore, the authors used multiunit recordings with low-impedence platinum iridium electrodes to sample activity in the visual cortex *during* APV infusion. Remember that exposing a larger tip area leads a lager sample area. Because their objective was to map the *percentage* of visually responsive cells (as a function of distance away from the infusion cannula), they used the more broad multiunit approach, which allowed them to sample a larger number of units in a specified area quickly. Using this sort of mapping experiment, the authors were able to show that although APV infusion did lead to a reduction of light-evoked responses, the area that they typically sampled from showed that most (86%) of the multiunit activity was responsive to light.

3. <u>Intracellular Recording and Calcium imaging</u>:

Owens DF, Boyce LH, Davis MB, Kriegstein AR. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci. 1996 Oct 15;16(20):6414-23.

The parameters of synaptic plasticity may be essentially different during early development of the nervous system. These parameters may be determined by intrinsic properties of the neuron fundamental to their excitability, their resting membrane potential, and even equilibrium potentials for the various ions that determine these intrinsic properties. To study the intrinsic properties of developing cortical neurons, which have important implications for plasticity during development, these authors used intracellular recording and calcium imaging. Their results indicate that GABA acts as an excitatory neurotransmitter in early postnatal cells;

moreover, GABA dependent calcium influx in these cells may lead to the establishment or strengthening of inhibitory synapses during development analogous to, and using similar mechanisms as, those involved in the plasticity of excitatory contacts in more mature cells.

In these experiments, the authors use the perforated patch recording technique with the *in vitro* slice preparation. The reason for this is that they were trying to record chloride conductance in developing neurons. Sharp electrode recording is ideal in circumstances where the internal concentration of ions must be maintained; whereas whole cell patch recording has a tendency to dialyze the cell, making it difficult to determine the effect of internal ion concentration on conductance. Unfortunately, impaling small, fragile cells, like developing neurons, with a sharp electrode is difficult without damaging the cell. Perforated patch recording with gramicidin resolves this problem because gramicidin forms membrane pores that are only permeable to cations and small, uncharged molecules, thus leaving internal Cl^- concentration undisturbed while the cell is patched. Thus the patch-clamp method, which is ideal for recording from small cells (no space clamp artifact because cells don't have lots of dendritic arbors that are harder to clamp the further away they are from the site of current injection), can be used without disturbing the internal Cl- concentration. The importance of the difference between the whole cell and perforated patch configuration is shown in the first set of experiments. Here the reversal potential of $GABA_a$ mediated Cl^- (the voltage at which the current due to Cl^- flow is zero) in young neurons is shown to be 40 millivolts lower in the perforated patch configuration. In both recording configurations, reversal potentials are obtained by clamping the cells at different voltages and measuring the current flow. Voltage clamping is achieved by injecting current that exactly counterbalances the current flowing through the voltage-gated membrane channels; the amount of current that must be generated by the voltage clamp to keep the membrane potential constant provides a direct measure of the current flowing across the membrane. The significance of this finding to plasticity is that in young neurons a lower reversal potential means that $GABA_A$ mediated Cl^- entry into the cell will cause a depolarization of these neurons rather than a hyperpolarization as is the case in adult neurons (this depolarizing effect is also dependent on the resting membrane potential of the cell, see below). Furthermore, this result would have been completely obscured in the whole cell patch configuration, emphasizing the importance of choosing the right recording method.

The authors go on to investigate the reason for the lower $GABA_A$ reversal potential, and suggest that a developmentally regulated Cl^- channel might raise the internal Cl^- concentration in young animals. For these experiments, the authors used the dialyzing effect of the whole cell configuration to their advantage. By comparing dialyzed cell conductance to the perforated patch conductance in response to a $GABA_A$ receptor agonist, they were able to show that Cl^- is the principle ion mediating the $GABA_A$ receptor effect across development. Because the effect of $GABA_A$ activation (either depolarizing or hyperpolarizing) depends on both the reversal potential of Cl- and on the resting membrane potential, the authors needed to characterize the resting membrane potential in these developing neurons. To do this experiment, they had to switch to a KCl internal solution for their recording electrodes (in all other experiments they used Cs^+ , which blocks voltage dependent K^+ currents, and helps to isolate GABA currents, but may artificially bias resting membrane potentials to more positive values). Using the KCl internal solution, the authors were able to confirm that the resting membrane potential in these young neurons is indeed lower than the $GABA_A$ equilibrium potential, and that therefore, Cl- entry in response to $GABA_A$ activation would lead to depolarization of these neurons. Next, the experimenters bath apply the chloride transporter blocker, furosemide, and show a reduction in

$GABA_A$ equilibrium potential. This result lends further support to their claim that transport of Cl^- into the cell establishes a high internal Cl^- concentration mediating the reduction of $GABA_A$ equilibrium potential seen in developing neurons.

After showing the mechanism for the lowered $GABA_A$ reversal potential, the authors go on to show the consequences of the depolarizing effect of $GABA_A$ activation in developing neurons and show that $GABA_A$ activation causes reversible calcium entry through voltage-gated Ca^{++} channels (VGCC's). For these experiments the authors took advantage of optical imaging techniques and the Ca^{++} indicator dye, fluo-3. Although the authors don't use this calcium entry for extrapolating electrical changes (they measure these directly using intracellular recording), the method is essentially the same. Cells from sliced tissue are loaded with Fluo-3 by bath immersion, followed by an ACSF wash. Changes in Ca^{++} entry are measured by quantifying optical images taken by confocal microscope before, during, and after application of the $GABA_A$ agonist muscimol (expressed as $\%\Delta F/F$ change in fluorescence over background fluorescence). Furthermore the influx of Ca^{++} in response to $GABA_A$ receptor activation occurs via activation of VGCC's as the effect is blocked by 500uM Cd^{++}. Here the significance of calcium entry into the cell is that the depolarizing effect of GABA activation has similar activation profile, with respect to second messenger signaling, as depolarization via other ion channels, like the NMDA receptor (although here calcium entry is less direct as it occurs through VGCC's, rather than through the $GABA_A$ channel itself).

Next the experimenters switch to the current clamp recording mode to measure spontaneous synaptic currents. In current clamp mode, changes in current are not compensated by voltage injection and spontaneous synaptic events are seen (presumably in response to endogenous presynaptic neurotransmitter release). Application of various drugs into the bath solution helps to isolate the postsynaptic elements responsible for these spontaneous currents. For example, they show that these currents are likely to be $GABA_A$ mediated because they are blocked by bicuculline, a $GABA_A$ receptor antagonist; furthermore they are not glutamatergic because CNQX and APV do not block them. Finally the authors go on to show that spontaneous activity of GABAa dependent depolarization leads to spontaneous Ca^{++} influx. Using the same optical imaging technique as before, they show that bicuculline blocks spontaneous increases in intracellular calcium and are thus mediated by $GABA_A$ receptor activation.

4. <u>EEG recordings:</u>

Seidenbecher T, Laxmi TR, Stork O, Pape HC. Amygdalar and hippocampal theta rhythm synchronization during fear memory retrieval. Science. 2003 Aug 8;301(5634):846-50.

The amygdala is a limbic structure thought to be important for processing the emotional content of incoming stimuli and for storing emotionally salient memories. The importance of the amygdala in learning and memory is apparent from lesion studies. One interesting example comes from patients whose hippocampi have been lesioned; even in the absence of declarative memories about a painful stimuli, these patients will nevertheless avoid such stimuli, indicating that the emotionally salient memory persists in the intact amygdala, even in the absence of frank knowledge of the stimuli that produced them. Furthermore, the amygdala is well connected with

the hippocampus and activation of the amygdala with either stress or direct electrical stimulation, interferes with synaptic plasticity in the hippocampus. The authors of this paper use EEG recordings to investigate the interplay between hippocampal and amygdalar electrical activity in the processing of emotionally salient memories.

Animals were first implanted with stainless steel EEG recording electrodes in the pyramidal cell layer of CA1 and lateral amygdala (electrode placement was confirmed histologically at the end of the experiments). Reference and ground electrodes were also implanted, close to the midline over the nasal and cerebellar regions, respectively. Animals were then allowed to recover from surgery for two to three days before behavioral training. They were then trained using an established model of emotional learning: fear conditioning. Fear conditioned animals were exposed to 3 auditory stimuli (10kHz, 85dB) for ten seconds followed by foot shock (0.4 mA) for one second. Control mice received 3 foot shocks followed by 3 auditory stimuli after a two minute delay. The unconditioned stimulus was a second novel auditory cue; the tones used as conditioned and unconditioned cues did not exhibit any frequency-dependent bias. Following behavioral training, simultaneous EEG recordings from the lateral amygdala (LA) and hippocampal CA1 were made from freely behaving fear-conditioned and control mice.

Before fear conditioning, autocorrelation analysis of these recordings indicated short periods of theta rhythmicity in the CA1 region and no such rhythmicity in the LA. Furthermore, cross-correlation analysis of these same recordings indicated very little synchronization of activity between the two structures. In contrast, fear conditioned animals showed rhythmicity in both regions during presentation of the conditioned stimulus (but not during the unconditioned stimulus) as well as synchronization of this theta activity between CA1 and LA during presentation of conditioned, but not unconditioned stimuli. Exactly how such synchronized theta oscillations contribute to synaptic plasticity is as yet unknown, however; as hinted at earlier, it is known that theta waves and theta frequency stimulation facilitates synaptic plasticity in the hippocampus and amygdala. Based on this evidence, these authors argue that the increased synchronization of the theta rhythm between hippocampus and amygdala might serve to facilitate or stabilize synaptic plasticity in the establishment or retention of fear memory. The evidence presented in this paper underscores the importance of communication between brain regions in the establishment of memories, which are often multifaceted, distributed, and drawing on both sensory and emotional cues for their establishment.

Other Reference materials:

- James, William, 1842-1910. Principles of psychology. Dover Publications; Reprint edition (June 1, 1955).
- Discovering neurons : the experimental basis of neuroscience. Carol Ann Paul, Barbara Beltz, Joanne Berger-Sweeney. Plainview, N.Y. Cold Spring Harbor Laboratory Press, c1997.
- Modern techniques in neuroscience research / Uwe Windhorst, Håkan Johansson (eds.). Berlin ; New York: Springer, c1999.

- Malenka RC, Nicoll RA. Long-term potentiation--a decade of progress? Science. 1999 Sep 17;285(5435):1870-4.

- Bear MF. Bidirectional synaptic plasticity: from theory to reality. Philos Trans R Soc Lond B Biol Sci. 2003 Apr 29;358(1432):649-55.

- Lomo T. The discovery of long-term potentiation. Philos Trans R Soc Lond B Biol Sci. 2003 Apr 29;358(1432):617-20.

- Neuroscience : exploring the brain / Mark F. Bear, Barry W. Connors, Michael A. Paradiso. Baltimore, Md. : Lippincott Williams & Wilkins, c2001.

- Essentials of neural science and behavior. Eric R. Kandel, James H. Schwartz, Thomas M. Jessell. New York : McGraw-Hill, c1995.