Metabotropic Glutamate Receptor 5: a Therapeutic Target in Fragile X and a Regulator of Plasticity in Visual Cortex

by

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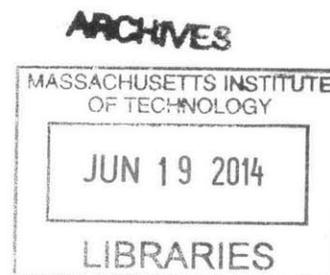PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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Abstract

The synaptic proteins Fragile X mental retardation protein (FMRP) and metabotropic glutamate receptor 5 (mGluR5) act in functional opposition to regulate local translation of synaptic mRNAs. Fragile X is the most common form of inherited intellectual disability and autism, and is expressed by a loss of FMRP. Previous studies have implicated mGluR5 in the pathogenesis of the disease, but a crucial unanswered question is if pharmacological mGluR5 inhibition is able to reverse an already established FX phenotype. Here we use the novel, potent and selective mGluR5 inhibitor CTEP to address this issue. Chronic treatment beginning in young adulthood results in comprehensive phenotype correction, suggesting that FX is in part a disease of acutely altered synapses. These results hold promise for clinical trials currently underway using similar approaches. Identifying mouse phenotypes to model cognitive impairment is especially important in FX because intellectual disability is at the core of the disorder. Here, we describe instrumental extinction as an assay for testing cognitive function, and report that this process is altered in FX model mice. In parallel, we characterize the role of mGluR5 in regulating synaptic and experience-dependent plasticity in wild-type mouse visual cortex. We report that NMDA receptor-dependent long-term depression (LTD) is reduced specifically in layer IV of visual cortex in mGluR5 knockout mice, as well as in wild-type mice treated chronically with CTEP. However, LTD induction is normal in the presence of acute mGluR5 antagonism in wild-type mice, suggesting an important difference between acute and chronic mGluR5 function. In vivo, monocular deprivation results in experience-driven weakening of synaptic strength, which occurs through similar mechanisms as LTD in vitro. We report that this ocular dominance plasticity is impaired following chronic mGluR5 inhibition. This study shows that specifically in layer IV, chronic but not acute downregulation of mGluR5 signaling has important consequences for forms of NMDA receptor-dependent plasticity in vitro and in vivo. Taken together, this work addresses both the basic function of mGluR5 as a regulator of plasticity and its potential as a therapeutic target in Fragile X.

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Michalon A*, **Sidorov MS***, Ballard TA, Ozmen L, Spooren W, Wettstein J, Jaeschke G, Bear MF, and Lindemann L (2012). Chronic pharmacological mGlu5 inhibition corrects Fragile X in adult mice. *Neuron* 74(1): 49-56.

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PRESENTATIONS

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Sidorov MS*, Michalon A*, Ballard TA, Ozmen L, Spooren W, Wettstein J, Jaeschke G, Bear MF, and Lindemann L. Chronic pharmacological mGlu5 inhibition corrects Fragile X in young adult mice.

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Chapter 1

Roles for mGluR5 in regulating Fragile X and wild-type synaptophysiology

1.1: Introduction

Synaptic plasticity is a well-studied neuronal process by which long-term memories may be formed and maintained. Two proteins, Fragile X mental retardation protein (FMRP), and metabotropic glutamate receptor 5 (mGluR5), interact with postsynaptic translation machinery to regulate the induction of certain forms of synaptic plasticity. In mouse models of Fragile X syndrome, disruption of synaptic mRNA translation has important consequences for plasticity as well as behavior. This chapter will introduce Fragile X syndrome, mGluR5, and the molecular mechanisms underlying synaptic plasticity in the hippocampus and visual cortex of wild-type and Fragile X model mice.

1.2: Fragile X syndrome

1.2.1: Historical roots and clinical manifestations

Martin-Bell syndrome was first described in 1943 (Martin & Bell, 1943) as a "pedigree of mental defect showing sex-linkage." At the time, such a pattern was remarkable and extremely rare - the original 1943 report cites only one prior published report of a hereditary history of intellectual disability (ID). The affected family had eleven affected males in the course of two generations (Figure 1.1A). At the time, inheritance of Martin-Bell syndrome was presumed to occur through normal Mendelian recessive X-linked mechanisms. In 1969, a karyotyping study identified for the first time chromosomal abnormalities associated with X-linked intellectual disability (Lubs, 1969). But it was not until 1982 when a fragile site was identified on the X chromosome (Figure 1.1B) (Moore *et al.*, 1982), that "Fragile X syndrome" was coined. In 1991, researchers discovered that Fragile X is caused by lengthened CGG repeats at the fragile chromosomal site Xq27.3, now known to be the FMR1 gene (Figure 1.1C, and see section 1.2.3) (Verkerk *et al.*, 1991).

Martin and Bell's initial report emphasized intellectual disability, and noticed no

categorical mental or physical features from which to distinguish Martin-Bell syndrome from other cases of intellectual disability. Intellectual disability (ID), historically called mental retardation, is indeed highly co-morbid with FX, with 90% of male and 50% of female FX patients reaching criteria for ID (Schneider *et al.*, 2009). Fragile X is also associated with with autism (30-60%), epilepsy (15-20%), hyperactivity, anxiety, attentional problems, macroorchidism (enlarged testicles), and hyperreactivity to sensory stimuli (Berry-Kravis, 2002, Miller *et al.*, 1999, Musumeci *et al.*, 1999, Nielsen *et al.*, 1982, Schneider *et al.*, 2009).

We will discuss in greater depth the importance of Fragile X syndrome as a model of autism (see section 1.2.7). Like Martin-Bell syndrome, autism was first described in 1943 (Kanner, 1968). The initial report of Martin and Bell did notice at least one patient with autistic characteristics, despite the lack of a real framework for describing "autism" at the time. They wrote that this subject displayed "almost complete mental detachment from his surroundings, with mutism and unceasing stereotyped movements" (Martin & Bell, 1943) (Figure 1.1A). However, these symptoms were not noted categorically or as primary features of the disorder. Even the 1982 report of Fragile X-linked intellectual disability, which additionally discussed macroorchidism, did not mention autistic symptoms in parallel (Moore *et al.*, 1982). Thus it is key to remember that while FX is associated with autism and considered a single-gene model of autism, it is intellectual disability and not autistic features, which is the single most prominent clinical and historical condition associated with Fragile X.

1.2.2: Genetics

Fragile X is typically caused by excessive CGG repeats in the 5' untranslated region of the FMR1 gene, located at Xq27.3. This results in hypermethylation and silencing the gene, and an absence of its protein product, Fragile X mental retardation protein (FMRP) (Pieretti *et al.*, 1991, Sutcliffe *et al.*, 1992, Verkerk *et al.*, 1991). Unaffected individuals have ~5-50 CGG repeats in this region; Fragile X patients have

>200 (Figure 1.2). In FX, hypermethylation is restricted to the FMR1 locus (Alisch *et al.*, 2013). There also have been reports of individuals with rare point mutations in the *FMR1* gene, including at the I304N site, which also result in Fragile X (De Boulle *et al.*, 1993, Lugenbeel *et al.*, 1995), but 98% or more of all cases of FX are caused by expanded CGG repeat length, hypermethylation, and gene silencing. Individuals with ~50-200 repeats are carriers of FX premutation – the *FMR1* gene is not silenced and they do not show most symptoms of FX; however, some psychological symptoms have been reported (Hagerman *et al.*, 2011). Individuals with premutation are also at risk for Fragile X-associated tremor/ataxia syndrome and Fragile X-associated primary ovarian insufficiency (Hagerman & Hagerman, 2004, Hagerman & Hagerman, 2002). The premutation is much more common than the full mutation (~1 in 250 vs. ~1 in 4000) (Hagerman *et al.*, 2011). While full mutation leads to loss of *FMR1* transcription and FMRP, premutation leads to increased transcription of *FMR1* resulting in increased *FMR1* mRNA (Tassone *et al.*, 2000). Despite the increased levels of mRNA, there are lower levels of FMRP protein associated with premutation (Qin *et al.*, 2011, Tassone *et al.*, 2007) (Figure 1.2). Premutation phenotypes are likely caused by a combination of decreased FMRP and by *FMR1* RNA toxicity. The process of RNA toxicity in premutation is not yet well understood, but it is thought that expansion of the CGG repeat sequesters more RNA binding proteins, preventing their normal function elsewhere (Hagerman & Hagerman, 2013).

Historically, a puzzling observation was noted in family pedigrees: the incidence of FX increases with subsequent generations (Sherman *et al.*, 1985, Sherman *et al.*, 1984). This suggests an inheritance pattern at odds with a standard X-linked recessive disorder. We now know that inheritance of CGG repeat length is unstable, and that the risk of expansion from premutation to FX greatly increases as a function of the number of CGG repeats in the premutation carrier (Jin & Warren, 2000). Thus with each successive generation, there is an increased likelihood of CGG expansion to from premutation-length to FX-length. The study of mechanisms underlying CGG expansion and how expanded CGG regions result in gene silencing are areas of active research. There is evidence suggesting that SIRT1, a class 3 histone deacetylase, may be

involved in *FMR1* gene silencing (Biacsi *et al.*, 2008), and some histone modification sites have been identified within the FMR1 gene (Kumari & Usdin, 2010).

1.2.3: Fragile X animal models: phenotypes

The *Fmr1/FMR1* gene is highly conserved from rodent to human, and the expression pattern of FMRP is similar between mouse and human brain (Ashley *et al.*, 1993b, Eichler *et al.*, 1993, Hinds *et al.*, 1993). Several animal models have been developed to study Fragile X, most notably the *Fmr1* knockout mouse (Consortium, 1994). In these mice, *Fmr1* is knocked out rather than functionally inactivated via hypermethylation and gene silencing. The results, however, are the same: complete loss of its protein product, FMRP. The *Fmr1* KO mouse model shows a wide range of phenotypes that closely mirror symptoms of Fragile X in humans (Wijetunge *et al.*, 2013). These phenotypes range from synaptic and cellular-level changes to broad impairments in cognition. Noteworthy phenotypes in *Fmr1* KO mice include:

Learning deficits and cognitive impairments: Inhibitory avoidance extinction, a hippocampally-encoded behavior, is deficient in *Fmr1* KO mice (Dolen *et al.*, 2007). Other standard learning assessments in mice such as the Morris water maze and radial arm maze show subtle and inconsistent differences between *Fmr1* KO and WT (D'hooge *et al.*, 1997, Mineur *et al.*, 2002, Yan *et al.*, 2004). Distinct from these learning tasks, higher-order cognitive deficits have also been shown in *Fmr1* KO mice on a visuospatial discrimination assay linked to prefrontal cortex (PFC) function (Krueger *et al.*, 2011). Overall, it is often difficult to map mouse behavior onto human behavior, but of any reported phenotypes, these cognitive, PFC-encoded deficits may prove most useful as a model for intellectual disability and impaired executive function (see Chapter 3).

Social deficits: Social deficits are one of the core symptoms of autism in humans but are much subtler to define in mice (Patterson, 2011). *Fmr1* KO mice show increased social anxiety and abnormal social interactions with other mice (Mcnaughton *et al.*,

2008, Spencer *et al.*, 2005), as well as impaired ultrasonic vocalizations (Lai *et al.*, 2013, Rotschafer *et al.*, 2012, Roy *et al.*, 2012), thought to be a form of communication in mice.

Epilepsy/hyperexcitability: In both C57BL/6 and FVB backgrounds, *Fmr1* KO mice show induced audiogenic seizure rates of ~60-90%, compared to near zero in WT littermates (Yan *et al.*, 2004). Additionally, *Fmr1* KO mice show a hypersensitivity to auditory stimuli, measured by looking at the startle response to tones with increasing but sub-seizure amplitude (72-90 dB) (Chen & Toth, 2001, Nielsen *et al.*, 2002). Physiological manifestations of hyperexcitability have also been reported at the cellular level in *Fmr1* KO mice (Chuang *et al.*, 2005, Gibson *et al.*, 2008, Osterweil *et al.*, 2013).

Hyperactivity has been reported in *Fmr1* KO mice by measuring movement in an open field as well as in other contexts (Consortium, 1994, Kramvis *et al.*, 2013, Mineur *et al.*, 2002).

Enhanced protein synthesis and mGluR-LTD: As discussed thoroughly in section 1.2.4, FMRP is a repressor of translation. *Fmr1* KO mice show increased protein synthesis in the absence of FMRP, as well as increased magnitude of metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD), a form of synaptic weakening which is protein synthesis-dependent (Huber *et al.*, 2002, Qin *et al.*, 2005). FMRP has a wide range of target mRNAs (Darnell *et al.*, 2011), and it is likely that dysregulated translation of numerous synaptic proteins accounts for much of the pathophysiology in Fragile X.

Abnormal/immature dendritic spine morphology: Humans with FX and *Fmr1* KO mice show altered dendritic spine morphology. In man and mouse, FX spines generally have characteristics of developmental immaturity: they are longer, thinner, and there is also a higher spine density (Comery *et al.*, 1997, Cruz-Martin *et al.*, 2010, Irwin *et al.*, 2001). It is not clear whether altered spine morphology occurs directly upstream of other

phenotypes or if they occur in parallel (Portera-Cailliau, 2011). Better understanding this relationship could be important to make the link between disrupted protein synthesis and behavior.

Macroorchidism has been reported in mice as well as humans (Bernardet & Crusio, 2006).

Beyond the widely studied *Fmr1* knockout mouse, other mouse models have been developed to study aspects of Fragile X. Conditional *Fmr1* KO (cKO) mice (Mientjes *et al.*, 2006) allow for regional and temporal specificity in knocking down FMRP using Cre recombinase. Region-specific knockouts have been used, for example, to characterize the effect of FMRP deletion in Purkinje cells (Koekkoek *et al.*, 2005). Additionally, a model of FX premutation has recently been characterized (Qin *et al.*, 2011). This model has 120-140 CGG repeats, increased levels of *Fmr1* mRNA, and decreased levels of FMRP. These mice display dendritic spine abnormalities, subtle behavioral deficits, and impairments in induction of synaptic plasticity (Hunsaker *et al.*, 2012, Qin *et al.*, 2011). Additionally, an I304N *Fmr1* knock-in mouse has been developed to model the severe point mutation seen in at least one FX patient (section 1.2.3) (De Boulle *et al.*, 1993, Zang *et al.*, 2009). This mouse displays impaired FMRP function (specifically, impaired mRNA binding) and phenocopies many behavioral and electrophysiological impairments seen in *Fmr1* KO mice. As described in section 1.2.4, these results suggest that FMRP's role as a translational inhibitor is critical to how it regulates synaptic function as well as behavior.

Beyond the mouse, models of FX have been developed and characterized in Drosophila (Dockendorff *et al.*, 2002, Mcbride *et al.*, 2005) and zebrafish (Ng *et al.*, 2013), and currently are being developed and tested in rat (unpublished data). Each of these has species-specific advantages as a model system. Drosophila can be used for rapid, large-scale screens of potential compounds, zebrafish provide another non-rodent vertebrate model in which to generalize knowledge, and *Fmr1* KO rats will provide an avenue to study cognitive behavioral impairments in much greater depth.

1.2.4: FMRP regulates synaptic protein synthesis and synaptic plasticity

A hallmark of FX in both humans and the *Fmr1* KO mouse is enhanced synaptic protein synthesis (Aschrafi *et al.*, 2005, Bolduc *et al.*, 2008, Dolen *et al.*, 2007, Huber *et al.*, 2002, Osterweil *et al.*, 2010, Qin *et al.*, 2005). Normally, FMRP acts as a repressor of translation at the synapse (Laggerbauer *et al.*, 2001, Li *et al.*, 2001). In the absence of FMRP, local protein synthesis is increased and this is thought to account for numerous downstream effects in the disease. This effect can be measured both directly by metabolic labeling and indirectly by using hippocampal mGluR-LTD as a proxy. This form of LTD requires protein synthesis (Huber *et al.*, 2000) and is enhanced in *Fmr1* KO mice (Huber *et al.*, 2002).

The long-term maintenance of many forms of synaptic plasticity requires the synthesis of new proteins. While the role of experience-dependent somatic gene transcription in long-term memory has been well studied (Kandel, 2001), many mRNAs are trafficked to dendrites, suggesting an additional role for local synaptic control of protein synthesis (Steward & Levy, 1982). Indeed, activity-dependent translation of pre-existing dendritic mRNA at the synapse is necessary for the expression of multiple forms of synaptic plasticity (Huang & Kandel, 2005, Huber *et al.*, 2000, Kang & Schuman, 1996). FMRP influences this synaptic plasticity by functioning as a key regulator of mRNA translation (Darnell *et al.*, 2011, Laggerbauer *et al.*, 2001, Li *et al.*, 2001, Qin *et al.*, 2005).

In this section we briefly review the evidence, mostly from the *Fmr1* KO mouse, suggesting a role for FMRP in synaptic plasticity. Broadly, long-term changes in synaptic strength induced by experience are considered a likely mechanism of memory formation and storage. We will further discuss of the importance of and mechanisms underlying forms of synaptic plasticity in section 1.3. Although the distinction is not always clear-cut, it is conceptually important to separate disruptions of synaptic plasticity that are *consequences* of altered brain development from those disruptions of synaptic plasticity that *cause* altered brain function in the *Fmr1* KO. While both are important for understanding disease pathophysiology, only the latter is relevant to the question of how FMRP contributes to synaptic plasticity in the wild-type brain.

FMRP regulates translation

FMRP associates with mRNAs through one of three RNA-binding domains (Ashley *et al.*, 1993a, Siomi *et al.*, 1993), in some cases in conjunction with adaptor proteins (El Fatimy *et al.*, 2012, Napoli *et al.*, 2008). There is evidence that FMRP can repress translation both by blocking initiation and elongation (Bhakar *et al.*, 2012, Santoro *et al.*, 2012). A point mutation in one FMRP/mRNA binding domain is sufficient to recapitulate plasticity phenotypes seen in the *Fmr1* KO mouse (Zang *et al.*, 2009) and in at least one case FX in a human patient (De Boulle *et al.*, 1993). Thus it is likely that FMRP regulates plasticity mainly in its role as a repressor of translation.

FMRP is regulated by posttranslational modifications. Phosphorylated FMRP stalls ribosomal translocation and inhibits translation, whereas dephosphorylation of FMRP upregulates translation (Ceman *et al.*, 2003, Coffee *et al.*, 2012, Muddashetty *et al.*, 2011). Bidirectional regulation of FMRP phosphorylation by the S6 kinase and protein phosphatase 2A (PP2A) in response to activity provide a potential link between synaptic stimulation and local translation (Santoro *et al.*, 2012).

FMRP is well-positioned to regulate synaptic plasticity

FMRP is well-positioned to be a key regulator of synaptic plasticity for three main reasons. First, the protein is found in dendritic spines (Antar *et al.*, 2004, Feng *et al.*, 1997, Ferrari *et al.*, 2007, Weiler *et al.*, 1997), important postsynaptic sites of plasticity induction and maintenance. Secondly, FMRP regulates dendritic mRNA translation (Bassell & Warren, 2008, Garber *et al.*, 2006), which is required for multiple forms of plasticity (Sutton & Schuman, 2006). Finally, FMRP itself is dynamically regulated by activity: experience and synaptic activation can trigger its local translation and rapid degradation, in addition to the posttranslational regulation mentioned above. Multiple experimental manipulations associated with synaptic plasticity have been shown to increase FMRP levels, including exposure to an enriched environment, a complex learning task, and pharmacological activation of group 1 mGluRs (Gabel *et al.*, 2004, Irwin *et al.*, 2000, Todd *et al.*, 2003b, Weiler *et al.*, 1997). Importantly, FMRP is synthesized rapidly, on the same time scale (10-30 minutes) as induction of stable

synaptic plasticity (Gabel *et al.*, 2004). In hippocampal cultures, activity- and mGluR-dependent increases in dendritic FMRP may result from increased trafficking of existing FMRP, rather than *de novo* FMRP synthesis (Antar *et al.*, 2004, Antar *et al.*, 2005, Dictenberg *et al.*, 2008). Either way, FMRP is an ideal candidate to be involved in regulating synaptic plasticity because of its rapid, transient rise in dendrites following well-characterized plasticity induction paradigms, as well as its role as an inhibitor of translation.

FMRP regulates mGluR-LTD via protein synthesis

Long-term potentiation (LTP) and long-term depression (LTD) are well-characterized forms of synaptic plasticity associated with learning and memory. These persistent changes in synaptic strength can be induced by a variety of manipulations and their expression mechanisms are diverse (see section 1.3). Different induction protocols rely on different mechanisms for maintenance, including the requirement for protein synthesis. A particularly compelling example of a form of plasticity requiring local translation is mGluR-LTD in the CA1 region of the hippocampus. Activation of group 1 mGluRs (mGluR1 and 5), either with paired-pulse low-frequency synaptic stimulation (PP-LFS) (Huber *et al.*, 2000) or with the selective agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) (Huber & Bear, 1998, Huber *et al.*, 2001, Palmer *et al.*, 1997), results in a persistent decrease in synaptic strength that is mechanistically distinct from classical NMDA receptor (NMDAR)-dependent LTD (Oliet *et al.*, 1997, Palmer *et al.*, 1997). It is important to note that there are several mechanisms downstream of mGluR activation that can depress synaptic transmission, and these can be differentially expressed depending on the induction protocol, age, rearing history, and species (Auerbach *et al.*, 2011, Moult *et al.*, 2008, Nosyreva & Huber, 2005, Oliet *et al.*, 1997, Zakharenko *et al.*, 2002). However, under appropriate experimental conditions the maintenance of mGluR-LTD requires rapid protein synthesis within minutes of induction (Hou *et al.*, 2006, Huber *et al.*, 2002, Huber *et al.*, 2000). This protein synthesis is likely to be synaptic, as mGluR-LTD can still be induced if the dendritic layer is physically severed from the cell body layer (Huber *et al.*, 2000).

mGluR-LTD is expressed, in part, by the removal of AMPA receptors from synapses, which also requires rapid *de novo* translation (Snyder *et al.*, 2001). The new protein synthesis may be instructive rather than merely permissive for synaptic plasticity since activation of group 1 mGluRs rapidly stimulates protein synthesis in hippocampal slices (Osterweil *et al.*, 2010), dendrites and synaptoneurosomes (Job & Eberwine, 2001, Weiler & Greenough, 1993).

Fmr1 knockout mice show enhanced hippocampal mGluR-LTD (Bhattacharya *et al.*, 2012, Hou *et al.*, 2006, Huber *et al.*, 2002, Michalon *et al.*, 2012) (Figure 1.3). A subsequent study found a similar enhancement in cerebellar mGluR-LTD, which shares many of the same expression mechanisms (Koekkoek *et al.*, 2005). Consistent with the electophysiological data, loss of FMRP leads to excessive mGluR-mediated AMPAR internalization (Nakamoto *et al.*, 2007). In addition, mGluR-LTD no longer requires new protein synthesis in the *Fmr1* KO mice (Hou *et al.*, 2006, Nosyreva & Huber, 2006). These results, combined with what is known about FMRP function, suggest that FMRP acts to inhibit the synthesis of proteins required for mGluR-LTD. In the absence of FMRP, these "LTD proteins" are already available or over-expressed in dendrites resulting in enhanced magnitude and protein synthesis-independent persistence of this form of plasticity (Figure 1.4A) (Bear *et al.*, 2004). Conversely, postnatal overexpression of FMRP reduces the magnitude of mGluR-LTD in both wildtype and *Fmr1* KO neurons (Hou *et al.*, 2006) and restores its protein synthesis dependence (Zeier *et al.*, 2009). Moreover, reducing mGluR5 signaling in *Fmr1* KO mice restores both protein synthesis rates and LTD magnitude in the hippocampus to wildtype levels (Dolen *et al.*, 2007, Michalon *et al.*, 2012), suggesting that mGluR5 and FMRP act in functional opposition to maintain an optimal level of synaptic protein synthesis throughout development and into adulthood (Figure 1.4A, and see section 1.2.5).

L-LTP appears normal in Fmr1 KO mice

While the effects of protein synthesis inhibition on mGluR-LTD can be seen within minutes, most forms of synaptic plasticity do not require *de novo* synthesis until several hours after induction. This is best characterized by late phase LTP (L-LTP), a

persistent form of potentiation lasting at least 4 hours. The late maintenance phase of L-LTP requires protein synthesis but initial induction does not (Frey *et al.*, 1988, Stanton & Sarvey, 1984). Due to FMRP's conjectured role in translation regulation, L-LTP was one of the first forms of plasticity studied in the *Fmr1* KO mouse (Paradee *et al.*, 1999). Interestingly, no difference has been found in the magnitude of L-LTP in the *Fmr1* KO (Paradee *et al.*, 1999, Zhang *et al.*, 2009). The fact that removal of FMRP affects protein synthesis-dependent LTD but not LTP suggests that FMRP may specifically regulate the translation of proteins required for the expression of LTD (Figure 1.4B). However, while the magnitude of L-LTP is unchanged, it is possible that L-LTP is qualitatively different in its requirement for new protein synthesis when FMRP is absent, as is the case for mGluR-LTD (and LTP priming, see below). Therefore, it will be important to test the protein synthesis-dependency of L-LTP in *Fmr1* KO mice to show that FMRP truly does not play a role in regulating the persistence of LTP.

Alternatively, FMRP may be required for the regulation of local but not somatic translation in the context of L-LTP (1.4C). L-LTP is traditionally induced by multiple trains of high frequency tetanus or theta burst stimulation, protocols that rely on cell-wide transcription and translation (Abraham & Williams, 2003, Krug *et al.*, 1984, Nguyen *et al.*, 1994). L-LTP was characterized in the *Fmr1* KO mouse using these classical paradigms (Paradee *et al.*, 1999, Zhang *et al.*, 2009). However, using a less intense induction protocol results in L-LTP that is maintained specifically by local dendritic translation (Huang & Kandel, 2005, Kelleher *et al.*, 2004). This form of L-LTP, similar to mGluR-LTD, is sensitive to inhibitors of translation but not transcription, and can be maintained in isolated dendrites. It will be interesting to determine if this locally expressed form of L-LTP is regulated by FMRP.

FMRP regulates LTP priming

While the role of FMRP in L-LTP is unclear, FMRP is known to be involved in LTP in other contexts. In particular, FMRP is involved in regulation of an mGluR-dependent form of metaplasticity that sets the threshold for LTP. Originally described in rats (Cohen & Abraham, 1996), weak activation of group 1 mGluRs, in itself insufficient

for LTD induction, facilitates the subsequent induction of LTP ("LTP priming"). As with mGluR-LTD, this facilitation requires translation but not transcription (Raymond *et al.*, 2000). This prompted the examination of the role of FMRP in LTP priming (Auerbach & Bear, 2010). mGluR-dependent priming of LTP is of comparable magnitude in WT and *Fmr1* KO mice; however, while LTP priming requires acute stimulation of protein synthesis in WT mice, it is no longer protein synthesis-dependent in the *Fmr1* KO. Thus, while mGluR-LTD and LTP priming are qualitatively different functional consequences of Gp1 mGluR-stimulated protein synthesis in the hippocampus, both processes are altered by the removal of FMRP (Figure 1.4D). These results suggest that the mRNA under translational control of FMRP may code for proteins required for bidirectional changes in synaptic strength. Thus, the proteins regulated by FMRP should be conceptualized as plasticity gatekeepers rather than solely "LTD proteins."

The induction threshold for LTP and STD-LTP is raised in Fmr1 KO mice

In *Fmr1* KO hippocampal slices, LTP induction is deficient with a weak 5 theta burst protocol but is normal with a strong 10 theta burst protocol (Figure 1.5A) (Lauterborn *et al.*, 2007). In addition, FMRP modulates the induction threshold for spike-timing dependent long-term potentiation (STD-LTP). This form of Hebbian plasticity is induced by temporally staggered presynaptic and postsynaptic activity within a very short window (Bi & Poo, 1998, Markram *et al.*, 1997). In somatosensory and prefrontal cortices, STD-LTP is deficient in *Fmr1* KO neurons (Desai *et al.*, 2006, Meredith *et al.*, 2007). However, if the postsynaptic stimulus strength is increased from a single spike to a burst of five spikes, STD-LTP does occur in KO neurons (Figure 1.5A) (Meredith *et al.*, 2007). Therefore FMRP is not required for expression of STD-LTP, but the threshold is raised in its absence. A possible mechanism for ongoing regulation of LTP thresholds by FMRP is discussed later in this section.

FMRP and other translation-dependent forms of plasticity

In addition to its role in translation-dependent forms of Hebbian plasticity, FMRP can also modulate some forms of homeostatic plasticity. Synaptic scaling is a form of

homeostatic plasticity that acts to keep the strength of synapses within a functional range in response to extreme changes in activity. Broadly, a decrease in activity leads to a subsequent cell-wide increase in synaptic strength ("scaling up") and an increase in activity leads to a decrement in synaptic strength ("scaling down") (Turrigiano, 2008). Two types of scaling up have been described in hippocampal slice culture: one that requires transcription (Ibata *et al.*, 2008) and one that requires local translation (Sutton *et al.*, 2006). Interestingly, only the translation-dependent form of synaptic scaling is deficient in neurons lacking FMRP. Postsynaptic viral expression of FMRP corrects deficient translation-dependent scaling up in *Fmr1* KO neurons (Soden & Chen, 2010). Scaling down of synapses in response to high levels of activity (following prolonged blockade of inhibition) has also been observed (Turrigiano *et al.*, 1998) and requires mGluR5 activation (Hu *et al.*, 2010, Zhong *et al.*, 2012). However, the role of FMRP and local protein synthesis in scaling down has not been directly examined.

While the role of FMRP has been best characterized in mGluR-dependent forms of plasticity, it is not specific to these receptors. Removal of FMRP occludes TrkB-mediated increases in protein synthesis (Osterweil *et al.*, 2010) and alters other forms of G protein-coupled receptor (GPCR)-dependent LTD and LTP (Connor *et al.*, 2011, Volk *et al.*, 2007). The common thread between these processes is their reliance on local dendritic translation. Indeed, evidence suggests that FMRP may specifically be important for the regulation of local rather than somatic translation (Figure 1.4C), as removal of FMRP affects translation but not transcription-dependent forms of Hebbian and homeostatic plasticity.

FMRP and translation-independent plasticity

While many forms of translation-dependent synaptic plasticity are abnormal in *Fmr1* KO mice, other forms of hippocampal plasticity, including NMDAR-dependent LTD and early-phase LTP, are normal (Auerbach & Bear, 2010, Godfraind *et al.*, 1996, Huber *et al.*, 2002, Li *et al.*, 2002, Paradee *et al.*, 1999). These observations suggest that FMRP regulates plasticity mainly in its role as a regulator of translation. However, removal of FMRP has also been shown to affect some forms of synaptic plasticity that

do not require *de novo* translation, such as early-phase LTP in other brain areas, including the cortex and amygdala (Hayashi *et al.*, 2007, Li *et al.*, 2002, Paradee *et al.*, 1999, Suvrathan *et al.*, 2010, Wilson & Cox, 2007, Zhao *et al.*, 2005). Some of these effects could be explained by FMRP modulation of protein synthesis-dependent plasticity thresholds; however it seems likely that many represent end-stage consequences of altered synaptic development in the *Fmr1* KO.

A case in point is altered LTP in the amygdala. A substantial deficit in basal transmission was reported at the same synapses that showed impaired LTP (Suvrathan *et al.*, 2010). Reduced synaptic connectivity might have caused the defective LTP, and might have arisen as a consequence of increased FMRP-dependent protein synthesis during the development of amygdala circuitry.

Candidate plasticity gating proteins regulated by FMRP

In order to determine how FMRP regulates synaptic plasticity, we must identify the synaptic proteins whose translation is regulated by FMRP. FMRP has a wide variety of targets - it has been shown to selectively bind approximately 4% of the mRNA in the mammalian brain (Brown *et al.*, 2001). Recently, over 800 mRNA binding targets of FMRP were identified using a novel high throughput cross-linking immunoprecipitation (HITS-CLIP) assay (Darnell *et al.*, 2011). These targets include genes coding for pre- and post-synaptically expressed proteins: 27% of pre-synaptic protein mRNAs (90 genes) and 23% of postsynaptic protein mRNAs (257 genes) are FMRP targets (Darnell *et al.*, 2011). More specifically, the HITS-CLIP study found that 31% of mRNAs coding for proteins in the NMDAR complex (58 genes), 62% in the mGluR5 complex (32 genes), and 33% in the AMPAR complex (3 genes) are FMRP targets. These three receptor complexes are important for the induction and maintenance of synaptic plasticity, suggesting that FMRP likely acts broadly as a translational regulator rather than solely regulating one or two "plasticity proteins."

The finding that many FMRP targets encode presynaptic proteins is interesting and illuminating. In the mature nervous system the evidence for local protein synthesis in axons or axon terminals is still sparse; however during early axon development and

synapse formation local protein synthesis is believed to play an important role in pathway and target selection (Akins *et al.*, 2009, Jung *et al.*, 2012). Thus, the absence of FMRP regulation of protein synthesis during early development very likely alters synaptic connectivity well before the onset of experience-dependent postnatal plasticity. In addition, outside the CNS, local control of translation in sensory afferent terminals plays a role in nociceptive sensitization and neuropathic pain (Price & Melemedjian, 2012). FMRP is localized to these terminals and *Fmr1* KO mice show altered nociceptive sensitization (Price *et al.*, 2007). These results suggest that in the spinal cord, presynaptic FMRP may inhibit local translation and can regulate pain plasticity even into adulthood.

We have discussed two major categories of plasticity defects in *Fmr1* KO mice: (1) forms of plasticity requiring FMRP/local translation for their maintenance (mGluR-LTD) and (2) forms of plasticity where FMRP regulates their induction threshold (STD-LTP). We will discuss a few proteins in both categories that are likely involved given their regulation by FMRP and their known roles in plasticity maintenance and threshold-setting in wild-type synapses. These "candidate proteins" are meant to serve as exemplars of how FMRP might regulate synaptic plasticity.

Plasticity maintenance proteins: MAP1B, Arc, and STEP

Recent work has identified proteins whose translation is regulated by FMRP and are involved in mGluR-LTD, including microtubule-associated protein 1B (MAP1B) and activity-regulated cytoskeleton-associated protein (Arc) (Bassell & Warren, 2008, Pfeiffer & Huber, 2009). MAP1B is required for mGluR-depdendent AMPA receptor endocytosis (Davidkova & Carroll, 2007), the mechanism by which mGluR-LTD is expressed. FMRP associates with MAP1B mRNA and represses its translation (Brown *et al.*, 2001, Darnell *et al.*, 2001, Lu *et al.*, 2004, Zalfa *et al.*, 2003), and *Fmr1* KO mice show increased hippocampal MAP1B expression (Hou *et al.*, 2006). However, there may be mouse strain and region-specific variations in how FMRP regulates MAP1B translation. For example, in the cerebellum and hippocampus of FVB mice, FMRP may positively regulate MAP1B expression (Wei *et al.*, 2007).

Arc is involved in AMPAR endocytosis (Chowdhury *et al.*, 2006) and is upregulated in dendrites following mGluR activation (Park *et al.*, 2008, Waung *et al.*, 2008) and behavior (Shepherd & Bear, 2011). Arc is required for hippocampal mGluR-LTD and L-LTP, which are both protein synthesis-dependent, and Arc$^{-/-}$ mice have multiple learning deficits (Park *et al.*, 2008, Plath *et al.*, 2006, Waung *et al.*, 2008). FMRP binds Arc mRNA and suppresses its translation. As a result, Arc expression is increased in *Fmr1* KO dendrites (Iacoangeli *et al.*, 2008, Niere *et al.*, 2012, Zalfa *et al.*, 2003). Since (a) mGluR-LTD is increased in *Fmr1* KO mice, (b) Arc is increased in *Fmr1* KO dendrites, and (c) Arc is required for mGluR-LTD, it seems likely that FMRP regulates mGluR-LTD via Arc. This hypothesis was tested directly using Fmr1/Arc double knockout mice which show deficient (rather than exaggerated) mGluR-LTD (Huber *et al.*, 2002, Park *et al.*, 2008). This finding suggests that increased Arc expression may partially account for the enhanced mGluR-LTD seen in *Fmr1* KO mice.

Mechanistically, dephosphorylation of FMRP by the phosphatase PP2A is required for rapid mGluR-mediated increases in Arc protein (Niere *et al.*, 2012). However in *Fmr1* KO neurons, Arc levels are basally increased, occluding a further effect of DHPG treatment. Acute viral reintroduction of FMRP into *Fmr1* KO neurons normalizes dendritic Arc levels and restores rapid mGluR-mediated Arc synthesis. This provides further evidence that the acute loss of FMRP, rather than developmental abnormality, underlies synaptic plasticity phenotypes in the *Fmr1* knockout mouse.

In addition to MAP1B and Arc, numerous other candidate LTD proteins have been identified in the *Fmr1* KO mouse. One interesting example is striatal-enriched protein tyrosine phosphatase (STEP). Translation of STEP is increased during mGluR-LTD (Goebel-Goody *et al.*, 2012a, Zhang *et al.*, 2008), and STEP mRNA binds to FMRP (Darnell *et al.*, 2011). Genetic reduction of STEP corrects behavioral phenotypes in the *Fmr1* KO mouse; but it is not known whether corresponding LTD phenotypes are affected (Goebel-Goody *et al.*, 2012b). Additional candidate proteins include APP (Westmark & Malter, 2007, Westmark *et al.*, 2011), OPHN1 (Nadif Kasri *et al.*, 2011), CaMKIIα (Hou *et al.*, 2006, Muddashetty *et al.*, 2007, Zalfa *et al.*, 2003), PSD-95

(Muddashetty *et al.*, 2007, Todd *et al.*, 2003a, Zalfa *et al.*, 2007), and PI3K (Gross *et al.*, 2010).

Plasticity threshold-regulating proteins: Kv4.2

A recent review discussing the role of potassium channels in Fragile X provides insight into how FMRP may regulate excitability (Lee & Jan, 2012). FMRP directly regulates the translation of at least three potassium channels: Kv4.2, Kv3.1b, and Slack (Brown *et al.*, 2010, Gross *et al.*, 2011, Lee *et al.*, 2011, Strumbos *et al.*, 2010, Zhang *et al.*, 2012). FMRP's control of Kv4.2 translation may have indirect consequences on regulating the threshold for LTP and STD-LTP induction.

Kv4.2 is an A-type potassium channel that regulates dendritic excitability and the extent of action potential backpropagation (Chen *et al.*, 2006, Hoffman *et al.*, 1997). A-type currents act to dampen dendritic excitability and AP backpropagation. By modulating the strength of backpropagation, Kv4.2 also has been shown to regulate the threshold for LTP and STD-LTP (Chen *et al.*, 2006, Watanabe *et al.*, 2002). In the absence of Kv4.2, dendrites are more excitable and there is a decreased threshold for LTP induction (Chen *et al.*, 2006, Jung *et al.*, 2008).

Fmr1 KO mice have an increased threshold for LTP and STD-LTP induction, as discussed earlier (Desai *et al.*, 2006, Meredith *et al.*, 2007). One potential hypothesis for this phenomenon is that FMRP inhibits the translation of Kv4.2, and *Fmr1* KO mice have excessive Kv4.2 protein synthesized in dendrites. Indeed, FMRP does directly associate with and negatively regulate the translation of Kv4.2 mRNA (Lee *et al.*, 2011). But does this account for the altered LTP/STD-LTP threshold in *Fmr1* KO mice? Pharmacological inhibition of Kv4.2 in *Fmr1* KO mice does correct deficient weak-stimulus hippocampal LTP while strong-stimulus LTP remains unchanged (Lee *et al.*, 2011). This finding suggests that the increased threshold for LTP in the *Fmr1* KO mouse may be accounted for by increased translation of the potassium channel Kv4.2.

Interestingly, another group has shown that under their conditions, FMRP positively regulates the translation of Kv4.2 (Gross *et al.*, 2011). This study did not address the potential consequences of decreased Kv4.2 in the *Fmr1* KO on synaptic

plasticity. One would expect increased dendritic excitability, which has been previously reported in other contexts (Chuang *et al.*, 2005), and a decreased LTP threshold. A very recent study supports these predictions, showing that A-type currents are decreased, backpropagating AP amplitudes are greater, and LTP magnitude is increased in the dendrites of *Fmr1* KO hippocampus (Routh *et al.*, 2013). It will be important to determine the precise experimental and *in vivo* conditions under which each of these opposing patterns of regulation can occur, but it is clear that FMRP's regulation of Kv4.2 in either direction would have important consequences for plasticity.

FMRP, synaptic plasticity, and learning

Long-lasting synaptic potentiation and depression have long been considered potential neural correlates of learning and memory. In conjunction with FMRP's role in synaptic plasticity in multiple brain areas, FMRP is also important for a wide range of behavioral learning tasks in mice. *Fmr1* KO mice show deficient amygdalar trace fear memory (Zhao *et al.*, 2005), cerebellar learning (Koekkoek *et al.*, 2005), inhibitory avoidance learning (Dolen *et al.*, 2007), and have difficulties with a prefrontal cognitive learning task (Krueger *et al.*, 2011). Drosophila mutants lacking FMRP also have impaired long-term memory (Bolduc *et al.*, 2008). Overall, learning and memory deficits in the *Fmr1* KO mouse are a likely behavioral consequence of abnormal synaptic plasticity.

1.2.5: The mGluR theory of Fragile X: correction of Fragile X phenotypes

As discussed above, in FX, loss of FMRP results in increased synaptic protein synthesis, which is likely pathogenic (Figure 1.5A). While FMRP normally inhibits local protein synthesis, protein synthesis is driven by activation of group 1 (Gp1) mGluRs, including mGluR1 and mGluR5 (Figure 1.5B). The mGluR theory of Fragile X (Bear *et al.*, 2004) proposed that reduction of signaling through Gp 1 mGluRs might be able to correct symptoms of the disease (Figure 1.5C).

In 2007, a group from our laboratory showed that crossing the *Fmr1* knockout mouse with a 50% reduced mGluR5 mouse (*Grm5$^{+/-}$*) resulted in a rescue of nearly

every measured Fragile X phenotype, as predicted by the mGluR theory (Dolen *et al.*, 2007). This genetic mGluR5 reduction offers proof of principle for treatment, but has two major limitations. First, a genetic treatment is not feasible in humans. Second, any prenatal approach is also severely limited by not knowing that a child will have Fragile X syndrome before birth, in the absence of genetic testing. Therefore it is crucially important to test whether targeted mGluR5 inhibition can rescue Fragile X symptoms in mice both postnatally and with drug administration.

The most commonly used mGluR5 antagonist to date is 2-methyl-6-(phenylethynyl)pyridine (MPEP). MPEP administration has been shown to correct a number of FX phenotypes in mice including enhanced protein synthesis and seizure rate (Mcbride *et al.*, 2005, Osterweil *et al.*, 2010, Suvrathan *et al.*, 2010, Yan *et al.*, 2005). These results show promise for pharmacological treatment of FX; however, the drug and its analog MTEP are not suitable for chronic treatment in either mice or humans. The half-life of MPEP is only approximately 2 hours in rats and 1 hour in mice (Anderson, 2003). In addition, MPEP and MTEP have off-target effects on NMDA receptors, mGluR1, and monoamine oxidase A (Lea *et al.*, 2005, O'leary *et al.*, 2000). Despite these limitations, there have been reports that chronic once-daily MPEP administration is sufficient to correct spine density and spine length phenotypes in the *Fmr1* KO mouse (Su *et al.*, 2011). This is a surprising finding, considering the short half-life of MPEP. Recently the novel mGluR5 antagonist AFQ056 was shown to correct certain phenotypes in the *Fmr1* KO mouse: it has the advantage of not having off-target effects on NMDARs, however, its half-life is still less than 1 hour (Levenga *et al.*, 2011). We tested a novel mGluR5 antagonist developed by our collaborators at Roche, which is highly selective and has a half-life of over 48 hours (Chapter 2 and see Figure 2.2) (Lindemann *et al.*, 2011). This allowed us to better answer the crucial questions surrounding chronic treatment of FX in mice.

Treatment strategies pioneered in mouse models to directly target mGluR5 are currently being evaluated in clinical trials (Delorme *et al.*, 2013). The only published double-blind study to date using mGluR5 antagonists in FX tested AFQ056 in a population of 30 males using a crossover design (Jacquemont *et al.*, 2011, Wijetunge *et*

al., 2013). This report did not find significantly improved behavior, as measured by their defined endpoint, the Aberrant Behavior Checklist score. However, a subgroup of patients with full *FMR1* methylation and no detectable mRNA did improve significantly while on AFQ056. These results, while not indicating improvement on the defined endpoint, were encouraging and suggest that methylation status of *FMR1* may be an important variable in treatment. This knowledge may help personalize treatments and inform future endpoints. Additionally, other clinical trials are underway testing mGluR antagonists in patient populations (Hoffmann-La Roche).

1.2.6: Beyond the mGluR theory: other mechanisms informing treatment

The mGluR theory and subsequent correction of FX phenotypes in mice has provided a base from which other treatment strategies have been developed. The most direct approach to treating FX would be re-introduction and re-expression of FMRP. Though currently not a realistic strategy in human populations, there is evidence that certain compounds can epigentically unsilence genes in mouse models of disease (Huang *et al.*, 2013), providing hope that such a strategy may eventually be possible to re-express FMRP. For now, direct re-introduction of FMRP in *Fmr1* KO mice was accomplished using a viral vector, and FMRP re-expression corrected enhanced mGluR-LTD (Zeier *et al.*, 2009) and dendritic levels of Arc protein (Niere *et al.*, 2012). This work provides proof of principle that direct re-expression of FMRP can correct altered protein synthesis, but the behavioral consequences of this approach have not yet been characterized. An advantage of using local viral infusion of FMRP is that it provides the potential to test the spatial requirements for FMRP in correcting specific behaviors (e.g. re-expression in prefrontal cortex and cognitive function).

The mGluR theory of Fragile X opened two key mechanistic questions which are active avenues of current research: (1) What signaling pathways link mGluR signaling to protein synthesis? (2) Dysregulation of which specific synaptic proteins is pathogenic in FX? We have partially addressed question (2) in section 1.2.4, mainly in the context of potential "LTD proteins," and it is highly likely that behavioral phenotypes in FX are a

consequence of altered synthesis of hundreds of synaptic proteins (Darnell *et al.*, 2011), rather than one "master regulator." Therefore approaches aimed at normalizing levels of one specific synaptic protein are unlikely to correct the full range of FX phenotypes (unless that protein is FMRP). However, study of the role of individual proteins upregulated in FX remains essential. For example, a full understanding of Kv4.2 function in FX may inform methods to correct hyperexcitability and perhaps seizures.

Understanding how mGluR5 is coupled to synaptic protein synthesis (question 1) has provided additional targets for treatments designed to normalize protein synthesis in FX. Group 1 metabotropic glutamate receptors are G_q-coupled receptors, and mGluR5 activation drives the canonical G_q – PLC – IP_3 pathway, resulting in the release of calcium from intracellular stores and activation of PKC (see section 1.3) (Conn & Pin, 1997, Kim *et al.*, 2008). Other G_q-linked receptors, including M_1 muscarinic acetylchloline receptors (mAChRs), can also regulate local protein synthesis in a similar manner to group 1 mGluRs. For example, mAChR-dependent LTD, like mGluR-LTD, is also protein-synthesis dependent and enhanced in the *Fmr1* KO mouse (Volk *et al.*, 2007). This suggests that in addition to mGluR5 inhibition, targeting other G_q-linked receptors may also have the potential for treatment of FX.

Downstream of mGluR5 and G_q, two major signaling pathways have been implicated in FX: the mammalian target of rapamycin (mTOR) and extracellular regulated kinase (ERK) cascades. These pathways link mGluR5 activation to local translation (see section 1.3.2). Inhibition of ERK brings basal protein synthesis levels back to normal in the *Fmr1* KO mouse, similar to the effect of an mGluR5 inhibitor, and also rescues increased seizure rate (Osterweil *et al.*, 2010). Phosphorylation of mTOR and mTOR activity may also be increased in *Fmr1* KO mice, depending on the experimental preparation (Sharma *et al.*, 2010). Recent work pioneered by Emily Osterweil has used statins to indirectly downregulate the ERK pathway to treat FX in mice (Osterweil *et al.*, 2013) (Chapter 2 and see Figure 2.15A). The advantage to this approach is that statins are already approved to treat high cholesterol and have been used successfully in children. This approach has corrected seizures, enhanced protein synthesis, and physiological measures of enhanced excitability. The ERK pathway is

downstream of mGluR5, but exactly how they are linked is not known. A Canadian clinical trial using lovastatin is currently underway.

Rearing *Fmr1* KO mice in an enriched environment also improves behavioral symptoms and dendritic spine differences (Restivo *et al.*, 2005). Though an "enriched" environment for experimental mice is still typically less enriched than what nature would provide, the concept of early intervention is applicable to FX patients. Though not specific to FX or based on the underlying mechanism of FX, early intervention/applied behavioral analysis historically has been the most effective method of improving outcomes for individuals with autism and intellectual disability (Howlin *et al.*, 2009).

1.2.7: Fragile X as a model for autism

Approximately 1 in 4,000 boys and a smaller percentage of girls are born with FX worldwide, making a potential treatment in mice directly relevant for a significant population. But the impact could be much larger considering FX as a model for autism. Autism is a developmental disorder defined by a set of common symptoms, including social communication deficits and repetitive behaviors. Though FX accounts for only 3% of all cases of autism, this makes it the single largest known cause of the disorder, where over 90% of cases have an unknown cause. Despite the genetic heterogeneity underlying autism and developmental syndromes with autism comorbidity, there is phenotypic convergence among these disorders, suggesting a common pathological convergence in brain circuitry. Over 200 genes have been linked with autism, including common variants, rare variants, and de novo mutations - and this number is likely to increase (Berg & Geschwind, 2012). Clinical presentation of autism is quite variable in both the presentation and severity of symptoms, which is not surprising considering its underlying genetic variability. A long-term goal of the National Institute of Mental Health is to create a mental health diagnostic system based on underlying biology rather than symptomology (Cuthbert & Insel, 2013). In autism, extreme genetic complexity and incomplete penetrance of any individual gene makes it unlikely that there will ever be a one-to-one mapping of genes to specific "autisms." Rather, it is likely that many autism risk genes converge upon common molecular pathways and brain circuits. One such

common pathway may be altered synaptic protein synthesis. In addition to FX, other single-gene disorders associated with autism, including tuberous sclerosis, are cause by mutations affecting proteins which regulate protein synthesis (Figure 1.6A) (Kelleher & Bear, 2008). Interestingly, tuberous sclerosis model mice display decreased synaptic protein synthesis and decreased mGluR-LTD, yet similar phenotypes to FX model mice, and these phenotypes are corrected either by positive allosteric modulation of mGluR5 or genetic cross with *Fmr1* KO mice (Figure 1.6B) (Auerbach *et al.*, 2011). Taken together, work on single-gene disorders suggests that altered synaptic protein synthesis may be a common feature in cases of autism and that treatments for FX may also be effective for treating autism with unknown etiology. Indeed, a recent genomic study implicated FMRP's translational regulation of numerous risk genes for nonsyndromic autism (Parikshak *et al.*, 2013). Thus it is quite likely that mechanisms underlying FX and other single-gene causes of autism are also relevant for the vast majority of cases of nonsyndromic autism.

1.2.8: Modeling impaired cognition and intellectual disability

Studies over the past 20+ years characterizing the basic biology underlying the synaptic function of FMRP are beginning to inform our understanding of forms of nonsyndromic autism. However, intellectual disability has been historically and clinically the most common and pervasive impairment associated with Fragile X. Comparatively, ID has been understudied in Fragile X, especially in animal models. In chapter 3, we will discuss our progress and the field's progress in developing cognitive assays to model executive function deficits, which are at the core of Fragile X.

1.2.9: Summary

The past twenty years have been a time of great progress and promise in the Fragile X field. Since the discovery of the genetic cause of FX (Verkerk *et al.*, 1991) and development of the *Fmr1* knockout mouse (Consortium, 1994), great progress has been made in understanding FMRP's basic role as a translational inhibitor (Laggerbauer *et al.*, 2001, Li *et al.*, 2001). Electrophysiological and biochemical measurements have

confirmed that increased synaptic protein synthesis is a hallmark of FX (Huber *et al.*, 2002, Qin *et al.*, 2005), and led to development and testing of the mGluR theory of Fragile X (Bear *et al.*, 2004). Genetic downregulation of mGluR5 protein corrected numerous FX phenotypes (Dolen *et al.*, 2007), but key questions remained: What are the temporal and pharmacological requirements for mGluR-targeted treatments? In chapter 2, we used CTEP, a novel, long-lasting mGluR5 antagonist, to test whether chronic treatment beginning in young adulthood is sufficient to correct FX mouse phenotypes. We found that this approach does correct numerous phenotypes, suggesting that FX is a disease of acutely altered synaptophysiology and that future treatments may not be limited to a critical developmental window. In chapter 3, we address the key core cognitive phenotypes in FX using a novel behavioral extinction assay which likely involves prefrontal cortex, and test whether mGluR5-targeted treatments are sufficient to correct these cognitive phenotypes. In chapter 4, we tested how mGluR5 and chronic mGluR5 antagonism regulate synaptic plasticity in wild-type visual cortex (see section 1.3 for introduction). These studies are relevant both for understanding the basic mechanisms underlying synaptic plasticity in this region, and also in characterizing potential side effects of chronic CTEP treatment.

1.3: Group 1 metabotropic glutamate receptors

1.3.1: Structure, taxonomy, localization, and signaling

Glutamate is the main excitatory neurotransmitter in the mammalian brain. Presynaptically released glutamate can bind to and activate two main categories of postsynaptic receptors: "fast" ionotropic glutamate receptors (iGluRs) and "slow" metabotropic glutatmate receptors (mGluRs). Ionotropic glutamate receptors function by coupling glutamate binding to channel opening, ion flux, and rapid membrane depolarization. These receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors. Until the 1980s, it was thought that glutamate acted exclusively through these ionotropic receptors. A series of studies showed that glutamate could also stimulate phosphoinositide turnover consistent with canonical G-protein coupled receptor (GPCR) signaling (Nicoletti *et al.*, 1986, Sladeczek *et al.*, 1985, Sugiyama *et al.*, 1987). The first mGluR was cloned in 1991 by two independent groups (Houamed *et al.*, 1991, Masu *et al.*, 1991), and this turned out to be what is now known as mGluR1a, a group I mGluR isoform. Subsequent studies cloned other types of mGluRs, which have been broadly characterized by their signaling mechanisms (Figure 1.7A). Broadly, mGluR structure can be broken down into four major structural domains (Figure 1.7B): (1) a glutamate binding "venus flytrap" domain (VFD), (2) a cysteine-rich domain linking the binding sites to (3) seven transmembrane-spanning loops, and (4) an intracellular C-terminal domain (Conn & Pin, 1997, Niswender & Conn, 2010). Typically, two VFDs dimerize and as a result of glutamate binding, undergo conformational changes. This signal is transmitted through the cysteine-rich domain, as mutations in this region impair downstream signaling (Rondard *et al.*, 2006). The second intracellular loop linking transmembrane domains is thought to mediate association with G proteins (Gomeza *et al.*, 1996, Rondard *et al.*, 2006). The C-terminal domain likely modulates G protein coupling, and is a major site for alternative splicing and association with other postsynaptic proteins, such as Homer (Tu *et al.*, 1998). Homer is a key synaptic scaffold, and interactions between Homer, Shank, and PSD95 provide a structural link between mGluR5 and

NMDA receptors (see Chapter 4). For more detailed reviews regarding the structure and basic function of metabotropic glutamate receptors, see (Conn & Pin, 1997, Hermans & Challiss, 2001, Niswender & Conn, 2010, Willard & Koochekpour, 2013).

In mammals, eight variants of mGluRs have been characterized (mGluR1-8), with many of these receptors having multiple isoforms due to alternative splicing of their C-termini. Human and rodent versions of mGluRs have high sequence homology (~95%) (Conn & Pin, 1997). These eight variants, plus their multiple isoforms, have generally been grouped into three categories (Group I, Group II, Group III) based on their signaling mechanisms and function (Figure 1.7A). Group I mGluRs are coupled to G_q, where signaling activates phospholipase C, generates inositol 1,4,5-triphosphate (IP3), and results in (1) activation of PKC and (2) release of calcium from intracellular stores (Conn & Pin, 1997). Group II and group III mGluRs couple to the canonical $G_{i/o}$ pathway, which results in inhibition of adenylate cyclase. Typically, group II and group III mGluRs are localized presynaptically and modulate neurotransmitter release, while group I mGluRs are mostly located postsynaptically (Hermans & Challiss, 2001). We will limit our discussion mainly to group I mGluRs, mGluR1 and mGluR5.

Metabotropic glutamate receptors 1 and 5 generally signal through similar mechanisms, but can be distinguished by their localization within the brain and can be isolated pharmacologically. In mouse, both mGluR1 and mGluR5 are enriched in the hippocampus, and mGluR5 is highly enriched in cortex (including visual cortex), whereas mGluR1 is highly enriched in cerebellum (Figures 1.7C-D). The agonist DHPG, used to induce mGluR-LTD (section 1.2.3), is specific to group 1 mGluRs and activates both mGluR1 and mGluR5. The antagonists MPEP and LY367385 are specific for mGluR5 and mGluR1, respectively (Gasparini et al., 1999). The recently developed antagonist CTEP is also highly specific for mGluR5 (see chapter 2). In addition, positive and negative allosteric modulators of mGluR5 and mGluR1 have been developed, which either potentiate or antagonize the receptor at an allosteric site without directly altering glutamate binding (Carroll et al., 2001, Kinney et al., 2005, Knoflach et al., 2001, Varney et al., 1999). Both mGluR1 and mGluR5 have multiple isoforms (mGluR1a-d and mGluR5a-b), which vary structurally in the nature of their C-

terminal tail (Hermans & Challiss, 2001).

Postsynaptic group 1 mGluRs are mainly located perisynaptically and extrasynaptically, i.e. away from the center of the postsynaptic density, unlike AMPA and NMDA receptors, which generally localize to the center of the PSD (Lujan *et al.*, 1996). Activation of mGluR5 with DHPG increases receptor diffusion within the membrane, and association with Homer confines this diffusion (Serge *et al.*, 2002). There is also evidence that some mGluR5 is located intracellularly, on the nuclear and endoplasmic reticular membranes (Jong *et al.*, 2009). It is thought that activation of intracellular mGluR5 also results in release of calcium from intracellular stores upon activation by intracellular transport of glutamate, but generally the role of these receptors is less well understood than that of cell-surface mGluR5.

1.3.2: mGluR5 is coupled to protein synthesis

In addition to canonical G_q-coupled signaling, binding of glutamate to mGluR5 results in the activation of other downstream signaling cascades. These pathways positively couple mGluR5 to synaptic protein synthesis (Figure 1.8) (Bhakar *et al.*, 2012) (Osterweil *et al.*, 2010, Sharma *et al.*, 2010) and represent molecular pathways underlying the mGluR theory of Fragile X (section 1.2.5). The two main pathways linking mGluR5 and regulation of translation are the MEK/ERK cascade and the Akt/mTOR pathway. Coupling of mGluR5 and Akt/mTOR signaling occurs through Homer, which recruits the GTPase PIKE-L, in turn activating PI3 kinase, which is upstream of Akt and mTOR activation (Ahn & Ye, 2005, Bhakar *et al.*, 2012). In addition, mGluR5 is linked to MEK1/2 signaling, which is one step upstream of ERK1/2 activation. It is not known exactly how mGluR5 couples to MEK, but it may be through the upstream activation of the GTPase Ras and/or through association with β–arrestin scaffolding proteins (Dewire *et al.*, 2008). Importantly, both the mTOR and ERK signaling pathways regulate mRNA translation. This coupling is critical for the majority of mGluR-dependent forms of synaptic plasticity.

1.3.3: mGluRs regulate synaptic plasticity

Historically, the experiments of Hubel and Wiesel were the first to show that visual experience can induce long-lasting changes in the brain (Constantine-Paton, 2008, Hubel & Wiesel, 1965, Wiesel & Hubel, 1963a, Wiesel & Hubel, 1963b, Wiesel & Hubel, 1965). In parallel, patterns of electrical stimulation were shown to induce long-lasting changes in synaptic strength (Bliss & Lomo, 1973). Long-term potentiation and long-term depression have long been considered a potential mechanism underlying memory acquisition and storage (i.e. learning). Over the past thirty-plus years, immense progress has been made in linking LTP, LTD, and other forms of synaptic plasticity with learning in various contexts. Entire books have been written on the mechanisms of synaptic plasticity and the relationship between synaptic plasticity and learning; a full description of these processes is beyond the scope of this section. Many forms of synaptic plasticity in vitro and in vivo require NMDA receptors for their induction. We will focus on forms of synaptic plasticity which require mGluR5, as our work addresses mechanisms of mGluR5-mediated synaptic plasticity in hippocampus (Chapter 2) and visual cortex (Chapter 4).

Cerebellar mGluR-LTD and learning

The first reports of long-term depression of synaptic strength came from parallel fiber to Purkinje cell synapses in the cerebellum (Ito et al., 1982). In this preparation, dual activation of parallel fiber and climbing fiber inputs in the rabbit resulted in synaptic depression lasting over one hour. This preparation is especially interesting because it has been associated with eyeblink conditioning in rabbits, a form of associative learning (Mauk et al., 1986). In this paradigm, rabbits learn to associate a tone (the conditioned stimulus) with a puff of air delivered to the eye (the unconditioned stimulus). Classical conditioning occurs as rabbits learn to blink in response to the tone. Neural correlates of the CS and the US have been identified: electrical stimulation of the inferior olive (source of climbing fiber inputs to cerebellum) can serve as the unconditioned stimulus, and electrical stimulation of mossy fiber input can serve as the conditioned stimulus (Lavond et al., 1987, Mauk et al., 1986, Steinmetz et al., 1986). Genetic knockout of

mGluR1, the predominant group 1 mGluR isoform in cerebellum (Figure 1.8D), blocks both LTD and eyeblink conditioning in mouse (Aiba *et al.*, 1994, Kishimoto *et al.*, 2002). Thus cerebellar LTD requires group 1 mGluRs and is likely a mechanism for memory storage.

Hippocampal mGluR-LTD

In rodent hippocampus, the earliest and best-characterized form of LTD is induced by low-frequency stimulation of Schaffer collateral inputs and requires NMDA receptor activation for its induction (Dudek & Bear, 1992). Generally, low-frequency stimulation (1 Hz) induces LTD and high-frequency stimulation (40-100 Hz) induces LTP at hippocampal synapses. The threshold for LTD and LTP induction is not absolute, and the empirically supported Bienenstock, Cooper, and Munro (BCM) model of synaptic modification states that there is a "sliding threshold" for LTP/LTD induction (Bienenstock *et al.*, 1982, Cooper & Bear, 2012). In addition, this threshold, θ_m, depends on the prior history of synaptic activation. Many of the predictions of the BCM theory were verified in visual cortex where dark-rearing and similar manipulations have been shown to shift θ_m. But its lessons are also valid in hippocampus: the induction of NMDAR-dependent LTD and LTP depend on prior history of the system.

In 1998, a novel form of mGluR-dependent synaptic depression was discovered in hippocampus (Huber & Bear, 1998). This mGluR-LTD can be induced by DHPG administration and there is a wealth of evidence suggesting that mGluR-LTD and NMDAR-LTD induction are distinct non-occluding processes which both result in AMPA receptor internalization (see Chapter 4 for more detail). A key aspect of mGluR-LTD is that its induction requires local protein synthesis, whereas induction of NMDAR-LTD does not (Huber *et al.*, 2000). Thus, just as FMRP regulates synaptic plasticity mainly through its role as a translational repressor (Section 1.2.4), mGluR5 induces synaptic plasticity through its positive coupling to translation (Section 1.3.2). It is possible that ERK1/2 and mTOR, both downstream of mGluR5, may regulate different pools of mRNA (Bhakar *et al.*, 2012), and that these pools may represent the "LTD proteins" and "LTP proteins" noted in Figure 1.4D. This hypothesis is based on reports that ERK1/2 is

required for induction of mGluR-LTD (Gallagher *et al.*, 2004) and mTOR may requlate late-phase LTP (Ehninger *et al.*, 2008). The expression mechanisms of mGluR-LTD are developmentally regulated: from P8-P15, mGluR-LTD expression is presynaptic and from P21-P30 and beyond, mGluR-LTD expression is postsynaptic (Nosyreva & Huber, 2005). In hippocampus, both mGluR1 and mGluR5 are expressed, and these receptors are both in involved mGluR-LTD induction (Volk *et al.*, 2006). Neither MPEP nor LY367385 alone is sufficient to block mGluR-LTD – blockade of both mGluR1 and mGluR5 is necessary to abolish mGluR-LTD. Little is known about the role of intracellular mGluR5 in synaptic plasticity, but a recent report suggests that intracellular mGluR5 activation may be linked to upregulation of the important plasticity protein, Arc (Kumar *et al.*, 2012). In sum, we have briefly introduced mGluR-LTD and NMDAR-LTD and will emphasize in Chapter 4 that while these processes are mutually exclusive, mGluR5 and NMDA receptors are linked closely both by physical scaffolds and by physiologically relevant interactions.

1.3.4: Mouse visual cortex as a system for studying experience-dependent plasticity

Primary visual cortex (V1) has provided one of the best-studied models for studying experience-dependent plasticity. Binocular V1 in mouse has a well-defined laminar structure and receives input, via the LGN, from both eyes. Synaptic function in visual cortex has been typically studied in vivo using four techniques: (1) extracellular visually evoked potentials (VEPs), (2) extracellular unit recordings, (3) intracellular whole-cell recordings, and (4) calcium imaging. Using each of these methods, bidirectional changes in synaptic strength have been reported following either electrical stimulation or visual experience (Feldman, 2009, Smith *et al.*, 2009). Additionally, imaging of thalamocortical axons has also revealed structural plasticity associated with manipulations of visual experience (Coleman *et al.*, 2010). Here we briefly describe the current approaches available to study experience-dependent plasticity in mouse visual cortex. There is a large body of evidence suggesting that these forms of plasticity are

encoded through the mechanisms of LTP and LTD, and vary mechanistically by cortical layer (discussed in Chapter 4).

The visually evoked potential is a crude yet powerful measure of synaptic function that can be used to assess changes in synaptic strength over days and weeks. Typically, a single electrode is surgically implanted into layer IV of binocular visual cortex. Following recovery, awake mice are head-fixed and view simple stimuli on a computer screen that occupies their entire visual field. Broadly, the averaging of many single stimulus presentations results in a well-defined VEP, which is thought to be a good measure of mouse visual function. If the presented stimuli are outside the mouse range of visual detection, no VEP is evoked. VEP magnitude can be increased or decreased by experience. Stimulus-selective response potentiation (SRP) occurs when a mouse views the same stimulus over days, and is expressed by increased VEP magnitude (Cooke & Bear, 2010). Monocular deprivation (MD), similar to that in Hubel and Wiesel's classical experiments on kittens, induces decreases in VEP magnitude in the hemisphere contralateral to deprivation (Frenkel & Bear, 2004). As discussed fully in Chapter 4, SRP and MD induce plasticity which occurs through LTP and LTD-like mechanisms. In Chapter 4 we characterize how mGluR5 regulates in vivo and in vitro forms of synaptic plasticity in mouse visual cortex.

1.3.5: Summary

Metabotropic glutamate receptor 5, a group 1 mGluR, is positively coupled to synaptic protein synthesis through the ERK and mTOR signaling pathways. Mainly via regulation of protein synthesis, mGluR5 is required for multiple forms of synaptic plasticity. In Chapter 2, we discuss mechanisms of mGluR-dependent LTD in a mouse model of FX, and in Chapter 4, we assess how mGluR5 regulates synaptic and experience-dependent plasticity in wild-type visual cortex.

A

IMBECILITY   SCHOLARSHIPS   DIED IN INFANCY
MENTAL DEFICIENCY   DIED IN ASYLUM   TUBERCULOSIS
X SEEN

"Four of the five living children of IV 11 are now serving in the Army; V32, said to be very fat, is employed in a fish shop."

"V 48 is markedly schizoid, and has now reached a state of almost complete mental detachment from his surroundings, with mutism and unceasing stereotyped movements."

B

C
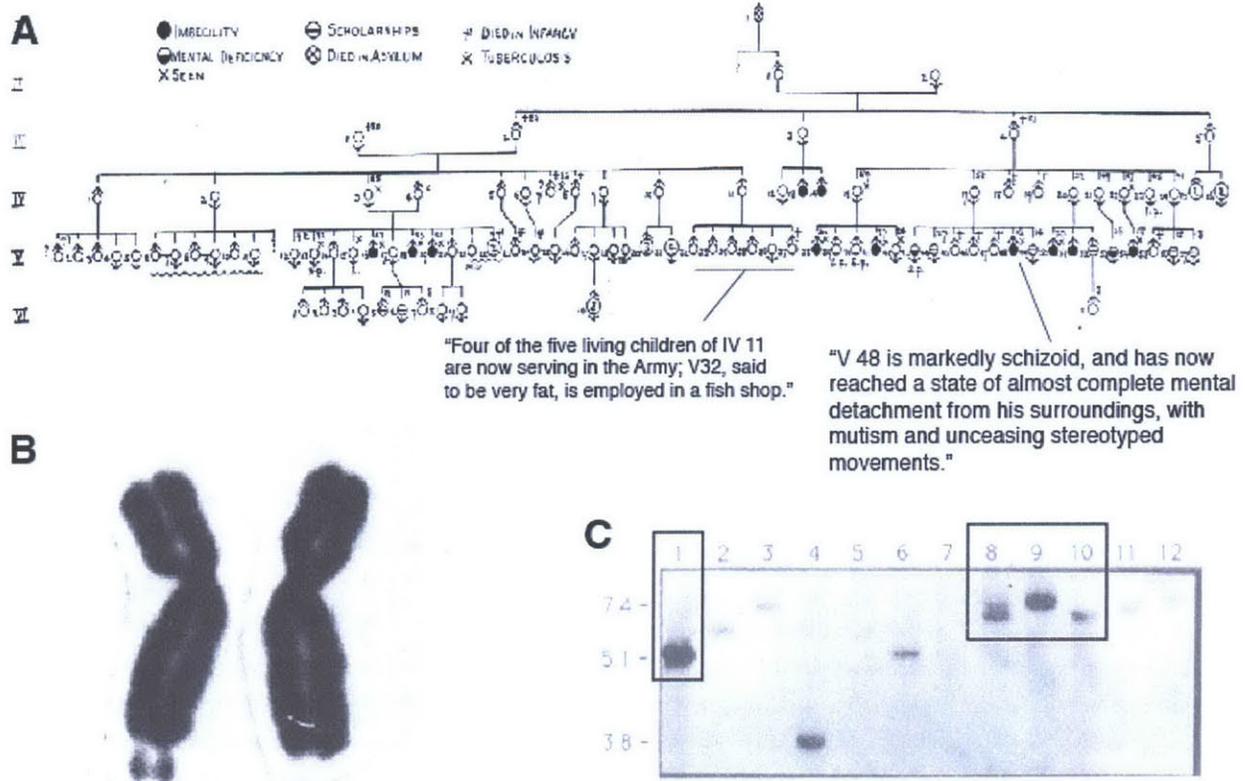
Figure 1.1: Historical characterization of Martin-Bell syndrome, an X-linked cause of intellectual disability.

(A) The original pedigree of one family with eleven cases of Martin-Bell X-linked intellectual disability. Adapted from Martin and Bell, 1943. The description of subject V 48 represents a likely early example of co-morbidity with autism. **(B)** The original report of "fragile X-linked mental retardation" shows a fragile breakpoint on one chromosome of a known FX carrier (Moore *et al.*, 1982). **(C)** The original report showing lengthened CGG repeats in the Xq27.3 chromosomal region in FX syndrome. Lane 1 shows a band representing normal CGG repeat length, and increased length of repeats in patients is best illustrated in lanes 8-10. Adapted from Verkerk *et al.*, 1991.

	Normal	Premutation	Full mutation
	$(CGG)_{n < 55}$	$(CGG)_{55 > n > 200}$	$(CGG)_{n > 200}$
Genotype			
Transcription (gene activity)			
FMR1 mRNA			
Translation (protein synthesis)			
FMRP			
Clinical phenotype	Normal	Emotional problems Premature ovarian failure Tremor/ataxia syndrome	Fragile X syndrome
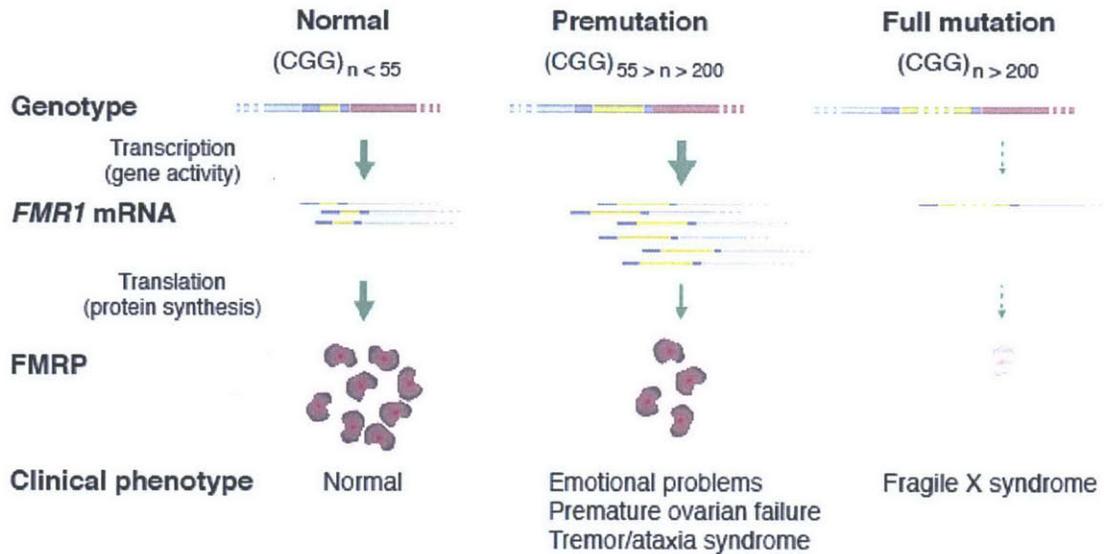
Figure 1.2: Conditions associated with CGG repeat at locus Xq27.3.

Individuals with fewer than 55 CGG repeats have normal transcription and translation of *FMR1* mRNA, normal levels of FMRP protein, and are unaffected. Individuals with FX premutation (~55-200 repeats) typically display increased transcription but decreased translation of *FMR1*, resulting in decreased levels of FMRP and partial phenotypes, including but not limited to Fragile X tremor/ataxia syndrome. Full mutation (>200 repeats) results in *Fmr1* gene silencing, resulting in a near-complete loss of FMRP and typical Fragile X phenotypes. (Adapted from Hagerman and Hagerman, 2002).

Category	Region	Fragile X Mouse Phenotype	Age	References
mGluR LTD	hippocampus	enhanced	P25-30	Huber et al., *PNAS*, 2002; Hou et al., *Neuron*, 2006; Bhattacharya et al., *Neuron*, 2012; Michalon et al., *Neuron*, 2012
mGluR LTD	hippocampus	does not require new protein synthesis	4-12 wk	Nosyreva and Huber, *J Neurophys*, 2006; Hou, et al., *Neuron*, 2006; Zang et al., *PLoS Genetics*, 2009
mGluR LTD	hippocampus	[*Fmr1* CGG premutation KI mouse] enhanced and not PS-dependent	P35-42	Iliff et al., *Hum Mol Genet*, 2012
mGluR LTD	cerebellum	enhanced	3-7 wk	Koekkoek et al., *Neuron*, 2005
mAChR LTD	hippocampus	enhanced and does not require new protein synthesis	3-7 wk	Volk et al., *J Neurosci*, 2007
LTP	hippocampus	NONE	20-26 wk; 8-10 wk; 3-12 month	Godfraind et al., *Am J Med Genet*, 1996; Li et al., *Mol Cell Neurosci*, 2002; Larson et al., *J Neurosci*, 2005
L-LTP	hippocampus	NONE	5-7 wk; 2-4 month	Paradee et al., *Neuroscience*, 1999; Zhang et al., *J Neurophys*, 2009
LTP	hippocampus	deficient	2 wk; 6-8 wk	Hu et al., *J Neurosci*, 2008; Shang et al., *J Neurochem*, 2009
LTP	hippocampus	deficient with weak stimulus; normal with strong stimulus	2-3 month	Lauterborn et al., *J Neurosci*, 2007
LTP	hippocampus	enhanced B-adrenergic-facilitated heterosynaptic LTP (PS-dependent)	3-4 month	Connor et al., *Learn Mem*, 2011
LTP priming	hippocampus	does not require new protein synthesis (mGluR-dependent)	6-10 wk	Auerbach and Bear, *J Neurophys*, 2010
LTP	anterior cingulate ctx	deficient	6-8 wk	Zhao et al., *J Neurosci*, 2005; Xu et al., *PLoS One*, 2012
LTP	anterior cingulate ctx	impaired facilitation of LTP by 5-HT2AR agonist	6-8 wk	Xu et al., *PLoS One*, 2012
LTP	somatosensory, temporal ctx	deficient	8-10 wk; 3 month	Li et al., *Mol Cell Neurosci*, 2002; Hayashi et al., *PNAS*, 2007
LTP	somatosensory ctx	delayed window for plasticity	P3-10	Harlow et al., *Neuron*, 2010
LTP	visual ctx	deficient (mGluR-dependent)	P13-25	Wilson and Cox, *Neuron*, 2007
LTP	anterior piriform ctx	deficient in aged mice; normal in 3-6 mo mice	6-18 month	Larson et al., *J Neurosci*, 2005
LTP	amygdala	impaired (mGluR-dependent)	6-8 wk; 3.5-6 mo	Zhao et al., *J Neurosci*, 2005; Suvrathan et al., *PNAS*, 2010
STD-LTP	somatosensory ctx	deficient with weak stimulus	P10-18	Desai et al., *J Neurophys*, 2006
STD-LTP	prefrontal ctx	deficient with weak stimulus; normal with strong stimulus	P14-23	Meredith et al., *Neuron*, 2007
homeostasis	hippocampus	deficient translation-dependent scaling	P6-7 slice culture	Soden and Chen, *J Neurosci*, 2010
homeostasis	hippocampus	normal transcription-dependent scaling	P6-7 slice culture	Soden and Chen, *J Neurosci*, 2010
experience-dependent	visual ctx (in vivo)	altered ocular dominance plasticity	LTD	Dolen et al., *Neuron*, 2007
experience-dependent	somatosensory ctx	deficient experience-dependent plasticity (induced by whisker trimming)	LTD	Bureau et al., *J Neurosci*, 2008

Figure 1.3: Fragile X mouse synaptic plasticity phenotypes.

Fragile X mouse models have multiple altered forms of synaptic plasticity across multiple brain regions. The majority of phenotypes were assessed in the *Fmr1* KO mouse which lacks FMRP.
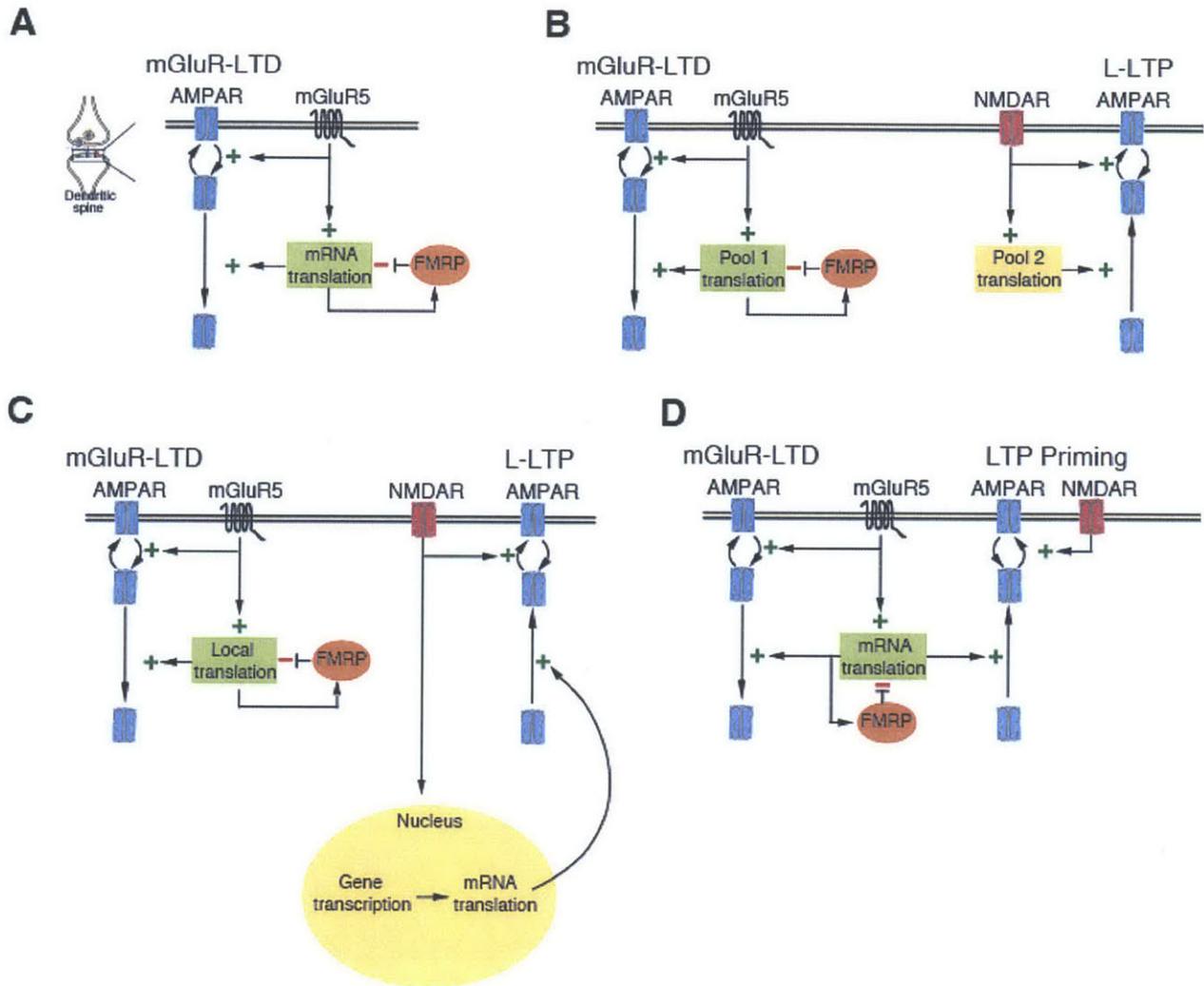
Figure 1.4: The role of FMRP in translation-dependent synaptic plasticity.

(A) FMRP and mGluR5 impose opposite regulation on the local mRNA translation required for mGluR-LTD expression. In the absence of FMRP, there is excessive protein synthesis and exaggerated LTD. **(B)** While FMRP is known to regulate the translation required for LTD, evidence suggests it is not involved in the expression of L-LTP. There may be different pools of mRNA available at the synapse that are differentially required for LTD versus LTP, and FMRP may specifically regulate the pool required for LTD. **(C)** FMRP is explicitly involved in the regulation of dendritically localized translation and may not regulate somatic translation. Consequently, FMRP may only impact forms of plasticity that require local translation, such as mGluR-LTD. **(D)** In addition to mGluR-LTD, FMRP regulates the protein synthesis involved in mGluR-dependent facilitation of LTP. This finding suggests that the proteins whose translation is controlled by FMRP may be involved in bi-directional maintenance of plasticity rather than being specific to LTD.
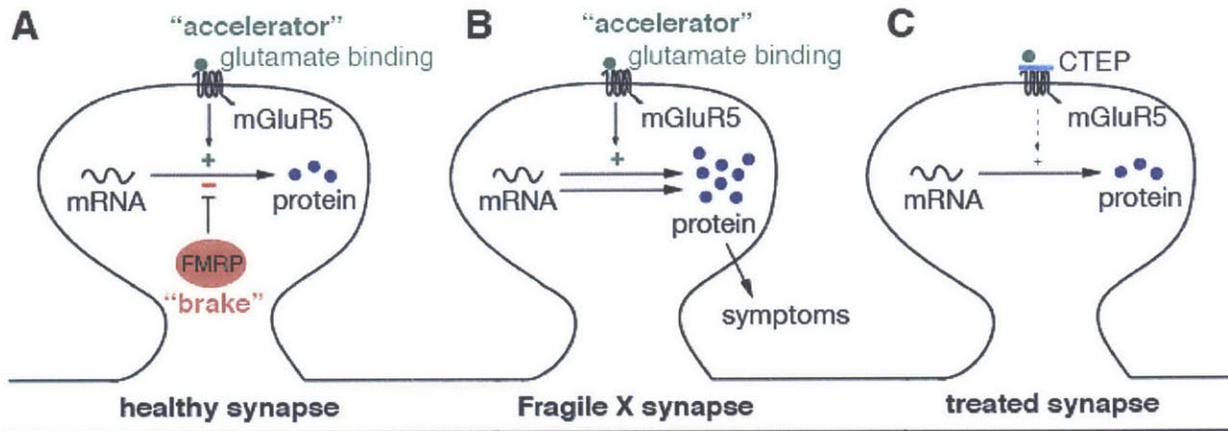
Figure 1.5: The mGluR theory of Fragile X informs treatment approaches.

(A) In the normal brain, mGluR5 activation promotes protein synthesis and FMRP inhibits protein synthesis. **(B)** In Fragile X (where FMRP is absent), unchecked mGluR5 activation leads to enhanced protein synthesis. **(C)** Downregulation of mGluR5-mediated signaling (illustrated here via CTEP) has potential to correct Fragile X phenotypes.
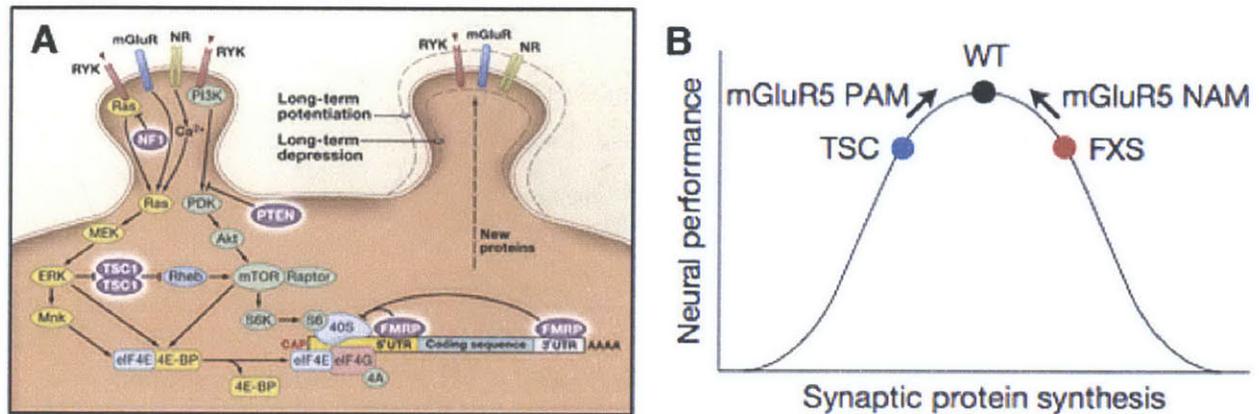
Figure 1.6: Altered synaptic protein synthesis is a shared feature of single-gene disorders associated with autism.

(A) Impaired function of synaptic proteins which regulate translation (in purple) is associated with multiple single-gene disorders associated with autism. Figure from Kelleher and Bear, *Cell*, 2008. **(B)** Bidirectional changes in synaptic protein synthesis (in TSC and FX) result in phenotypic convergence, suggesting that negative or positive regulation of mGluR5 could be a relevant treatment strategy for other causes of autism associated with dysregulated protein synthesis. Figure from Auerbach *et al., Nature*, 2011.
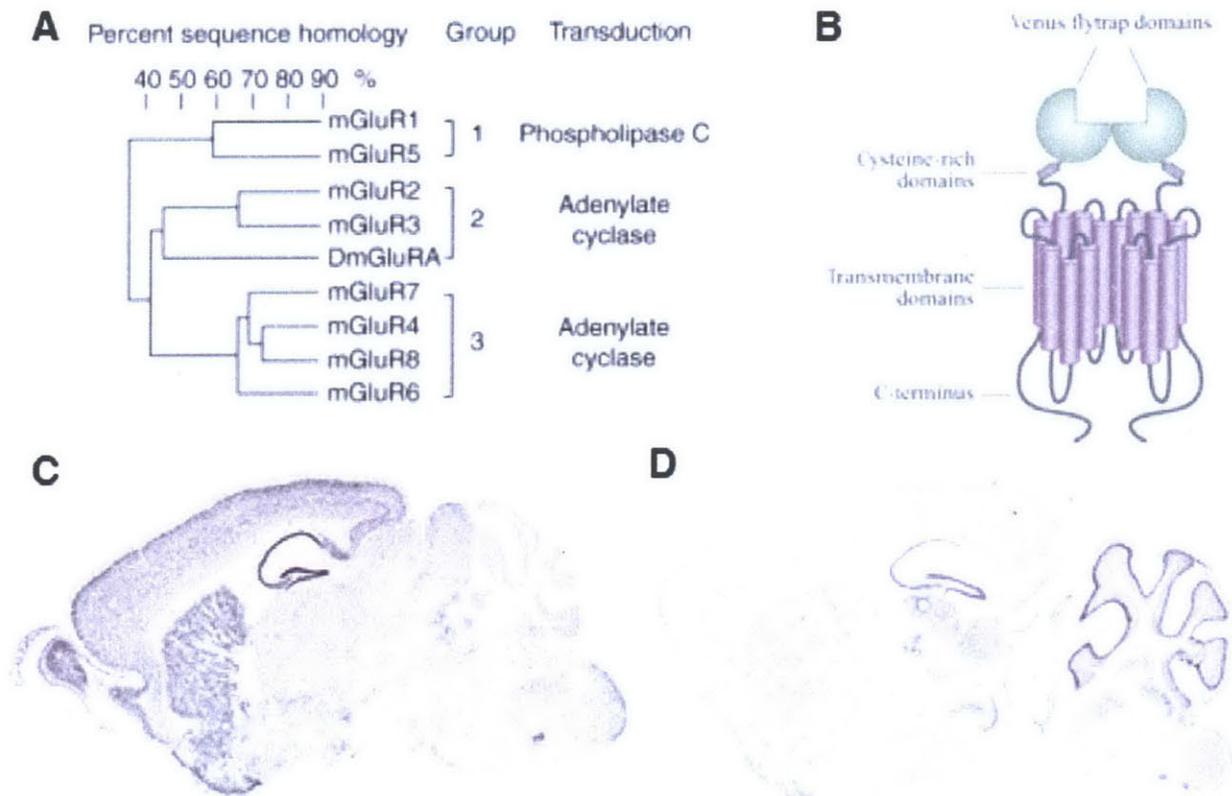
Figure 1.7: Taxonomy, structure, and localization of metabotropic glutamate receptors.

(A) Group 1 mGluRs include mGluR1 and mGluR5. **(B)** Metabotropic glutamate receptors have seven transmembrane domains, an extracellular glutamate binding site, and intracellular regions for G-protein coupling and associations with synaptic scaffolds and other downstream targets. **(C)** Sagittal section showing mGluR5 expression (enriched in cortex) and **(D)** mGluR1 expression (enriched in cerebellum) in mouse brain. Figures from Dolen *et al, Pharmacol. Ther.,* 2010, Niswender *et al., Annu. Rev. Pharmacol. Toxicol,* 2010, and the Allen Brain Atlas.
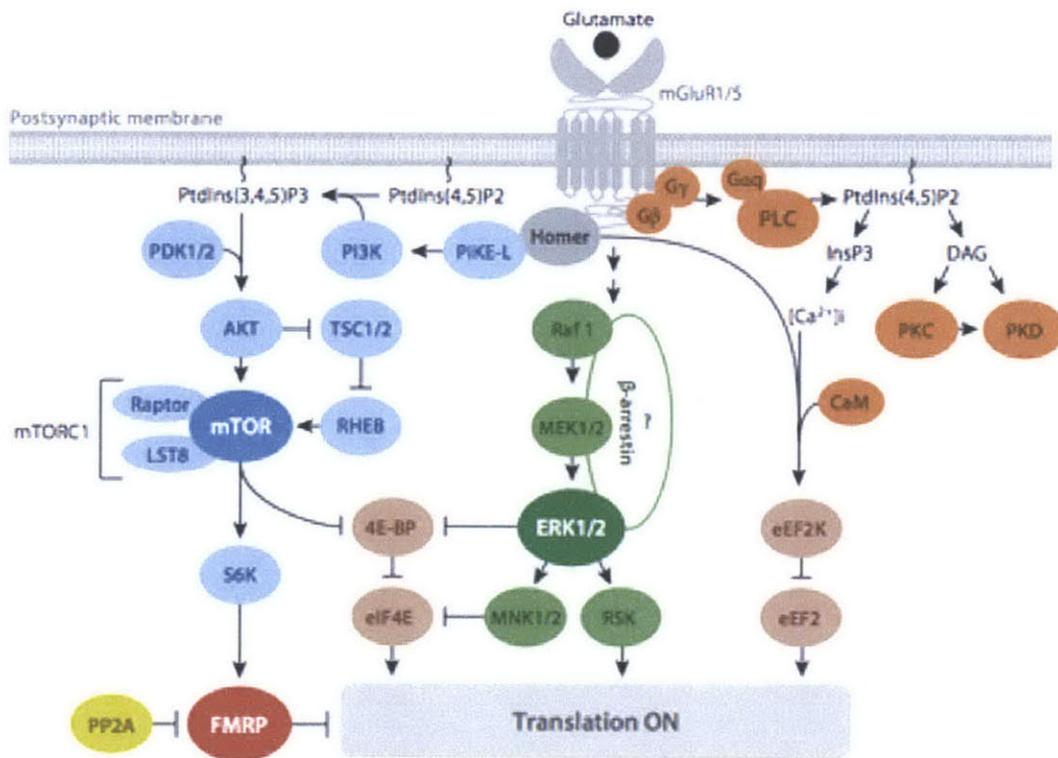
Figure 1.8: Intracellular pathways couple mGluR5 to synaptic protein synthesis.

Three main signaling pathways link mGluR5 to regulation of translation: the Akt/mTOR pathway (blue), the MEK1/2/ERK1/2 pathway (green), and canonical signaling pathways downstream of G_q (orange). Figure from Bhakar *et al., Annu. Rev. Neurosci.,* 2012.

Chapter 2

Chronic pharmacological mGluR5 inhibition corrects Fragile X in adult mice

2.1: Abstract

Fragile X is the most common form of inherited intellectual disability and autism. Previous studies have implicated mGluR5 in the pathogenesis of the disease, but a crucial unanswered question is if pharmacological mGluR5 inhibition is able to reverse an already established FX phenotype in mammals. Here we have used the novel, potent and selective mGluR5 inhibitor CTEP to address this issue. Acute CTEP treatment corrects elevated hippocampal LTD, protein synthesis and susceptibility to audiogenic seizures. Chronic treatment, inhibiting mGluR5 within a narrow receptor occupancy range of $80 \pm 5\%$, rescues learning and memory deficits, auditory hypersensitivity, aberrant dendritic spine density, and overactive ERK and mTOR signaling. This study shows that a comprehensive phenotype correction in FX is possible with pharmacological intervention starting in young adulthood after development of the phenotype. It is of great interest how these findings may translate into ongoing clinical research testing mGluR5 inhibitors in FX patients.

2.2: Introduction

2.2.1: CTEP as a tool for late-onset mGluR5 inhibition

Fragile X is a monogenic developmental disorder associated with a complex neuro-psychiatric phenotype (Schneider *et al.*, 2009). FX is caused by mutations in the fragile X mental retardation 1 (*FMR1*) gene, triggering partial or complete gene silencing and partial or complete lack of the fragile X mental retardation protein (FMRP) (Oostra & Willemsen, 2003).

It has been proposed that exaggerated consequences of mGluR5-mediated signaling in the absence of FMRP play a causal role in FX (Bear *et al.*, 2004). This theory is strongly supported by the finding that genetic reduction of mGluR5 expression is sufficient to correct a broad range of phenotypes in the *Fmr1* KO mouse (Dolen *et al.*, 2007). Additionally, a number of pharmacological studies have shown that short-acting mGluR5 inhibitors such as MPEP and fenobam can ameliorate Fragile X phenotypes in several evolutionarily distant animal models (Krueger & Bear, 2011).

Although these studies support the mGluR theory, they do not address the very important conceptual question whether mammalian Fragile X phenotypes can be prevented or reversed with late-onset mGluR5 inhibition. A failure to correct mutant phenotypes with treatment starting after symptom onset would suggest a missed critical period, and indicate that Fragile X is a terminally differentiated phenotype of altered brain development. On the other hand, amelioration of phenotypes with late treatment would support the notion that many problems are due to an ongoing imbalance in synaptic signaling, which can be substantially improved once the normal balance is restored (Figure 2.1). The genetic rescue experiments to date have not addressed this question because they are germ-line manipulations, present *in utero*. Neither have the pharmacological experiments to date been able to address this question in mammals because they have relied on compounds with a short duration of action. Experiments with acute drug treatment cannot explore the full therapeutic potential of mGluR5 antagonists in view of the chronic and developmental nature of FX.

In the current study, we used a new pharmacological tool, CTEP, a selective, orally bioavailable and long acting mGluR5 inhibitor (Lindemann *et al.*, 2011) (Figure 2.2) to test whether chronic pharmacological mGluR5 inhibition can reverse FX phenotypes in a fully developed brain. We chose to start treatment at an age of 4 - 5 weeks when the mouse brain development is anatomically complete but highly plastic, and when all FX phenotypes relevant for the study are established. Our results show that chronic, one month treatment of young adult *Fmr1* KO mice with an mGluR5 inhibitor rescues a broad range of phenotypes, including learning deficits, hyperreactivity to sensory stimuli, and increased dendritic spine density in the cortex. Our data also reveal correction of elevated sensitivity to epilepsy, excessive protein synthesis, long-term depression, activity of signaling pathways, and an amelioration of macroorchidism. Taken together, the data suggest beneficial effects in a wide range of symptoms and a disease-modifying potential for mGluR5 inhibitors in FX.

2.2.2: Lovastatin as a tool for ERK inhibition

In addition, pathological changes observed in FX are believed to stem in part from an elevation of basal protein synthesis downstream of an extracellular signal-regulated kinase (ERK1/2) signaling pathway (Osterweil *et al.*, 2010). ERK1/2, a key member of the larger MAP kinase (MAPK) signaling pathway, is located downstream of mGluR5 and upstream of protein synthesis. At the head of this intracellular cascade lies the small GTPase Ras. The Ras/MAPK pathway is a major regulator of cell growth, and thus a strong inhibitor of Ras-ERK1/2 would have deleterious consequences on the developing brain. However, a previous study reported that lovastatin, an HMG-CoA reductase inhibitor in widespread use for the treatment of hypercholesterolemia in both children and adults, could correct cognitive deficits caused by excess Ras activity in the mouse model of neurofibromatosis type 1 (Li *et al.*, 2005). Lovastatin can achieve a mild reduction in Ras-ERK1/2 activation by interfering with the recruitment of Ras to the membrane, a process that is required to transition from the inactive GDP-bound form to the active GTP-bound form (Kloog *et al.*, 1999, Schafer *et al.*, 1989). The interaction of Ras with the membrane requires the posttranslational addition of a farnesyl group to the

C terminus and lovastatin inhibits Ras farnesylation by targeting the upstream mevalonate pathway (Figure 2.15A) (Li *et al.*, 2005, Mendola & Backer, 1990, Schafer *et al.*, 1989). We therefore wondered whether lovastatin could prevent pathological changes in FX that lie downstream of excessive ERK-mediated protein synthesis. Specifically, we investigated the effects of lovastatin on hippocampal mGluR-LTD in *Fmr1* knockout mice, as part of a larger study which tested other electrophysiological and behavioral phenotypes associated with FX and epilepsy (Osterweil *et al.*, 2013).

2.3: Results

2.3.1: CTEP enables chronic pharmacological inhibition of mGluR5 in mice

CTEP is a novel potent, selective, and orally bioavailable mGluR5 inhibitor with a unique long half-life of approximately 18 hr in mice (Figure 2.3A)(Lindemann *et al.*, 2011). In vivo receptor occupancy measurements with the tracer [^{3}H]-ABP688 (Hintermann *et al.*, 2007) revealed 50% mGluR5 occupancy (EC$_{50}$) by 12.1 ng/ml plasma and 75.0 ng/g brain concentration of CTEP, respectively (Figure 2.3B). A regimen of one dose of 2 mg/kg CTEP p.o. per 48 h achieved uninterrupted mGluR5 occupancy. The minimal (trough level) drug exposure reached after 2 weeks of treatment was 98 ± 14 ng/ml in plasma and 215 ± 28 ng/g in brain (Figure 2.3C), corresponding to an estimated mean receptor occupancy level of 81% with a peak to trough range of 85% - 77% (Figure 2.3D).

2.3.2: CTEP corrects excessive protein synthesis in the hippocampus of Fmr1 KO mice

FMRP binds hundreds of mRNAs *in vivo* and represses their translation (Darnell *et al.*, 2001). Accordingly, at the core of FX pathophysiology is an elevated rate of protein synthesis (Dolen *et al.*, 2007, Osterweil *et al.*, 2010, Qin *et al.*, 2005). This phenotype was confirmed by measuring [^{35}S]-methionine/cysteine incorporation in acute hippocampal slices (*Fmr1* KO = 115 ± 7% of WT, p<0.05; Figure 2.4A,B). As previously shown with MPEP (Osterweil *et al.*, 2010), bath application of CTEP (10 μM) corrected

the elevated protein synthesis rate in *Fmr1* KO hippocampal slices (*Fmr1* KO = 104.9 ± 10% of WT) without affecting WT.

2.3.3: Correction of elevated mGluR-LTD in the hippocampus following in vivo CTEP administration

Fmr1 KO mice show elevated Gp1 mGluR-dependent LTD (Huber *et al.*, 2002) which can be corrected by genetic reduction of mGluR5 expression levels (Dolen *et al.*, 2007) but not by bath application of MPEP (Volk *et al.*, 2006). We therefore determined if *in vivo* administration of CTEP could reduce levels of LTD *ex vivo* in the *Fmr1* KO hippocampus to WT values. WT and KO animals (P25-30) received a single dose of CTEP (2 mg/kg, s.c.) or vehicle 24 h prior to euthanasia and hippocampal slice preparation. We found that Gp1 mGluR-mediated hippocampal LTD was elevated in vehicle-treated *Fmr1* KO mice compared to WT (WT/vehicle: 84.6 ± 2.4%; KO/vehicle: 76.1 ± 2.5%; $p<0.05$; Figure 2.5A,B) and was normalized by a single dose of CTEP (KO/vehicle vs. KO/CTEP: 86.9 ±3.3%; $p<0.01$).

CTEP treatment also reduced the maximum transient depression (MTD) to DHPG, which represents an electrophysiological readout of Gp1 mGluR activation. After 4 week chronic dosing, MTD was strongly suppressed by CTEP (KO/vehicle: 57.1 ± 2.2%; KO/CTEP: 33.2 ± 2.6%; $p<0.01$; summary in Figure 2.5C,D, raw data in Figure 2.5B,C), even more so than after a single dose (KO/vehicle: 62.9 ± 3.0% vs. KO/CTEP: 49.4 ±4.6%; $p<0.05$), showing that the drug efficacy is maintained throughout chronic treatment.

2.3.4: mGluR-LTD in Fmr1 KO mice remains protein synthesis-independent following CTEP treatment

In *Fmr1* KO mice, in addition to the magnitude of mGluR-LTD being increased, its expression is qualitatively different. Typically, mGluR-LTD requires protein synthesis for its induction and expression in wild-type hippocampus (Huber *et al.*, 2000). However, mGluR-LTD in *Fmr1* KO hippocampus can occur independent of new protein synthesis (Nosyreva & Huber, 2006), presumably because proteins necessary for LTD induction

are already present at higher levels due to increased basal protein synthesis. Therefore we tested whether *in vivo* CTEP treatment would return the protein synthesis-dependence to LTD *ex vivo* in *Fmr1* KO mice. First we confirmed that in WT mice (P25-30), bath application of the protein synthesis inhibitor cycloheximide (60 μm) does significantly reduce the magnitude of mGluR-LTD (Figure 2.6A; WT/ACSF: 80.2 ± 4.1%, n=9, WT/CHX: 90.7 ± 3.7%, n=10; p<.05). Following both vehicle and CTEP treatment, LTD magnitude is not affected by cycloheximide in *Fmr1* KO slices (Figure 2.6B,D; KO/vehicle/ACSF: 77.2 ± 3.7%, n=11, KO/vehicle/CHX: 75.9 ± 3.6%, n=13, KO/CTEP/ACSF: 87.5 ± 3.7%, n=17, KO/CTEP/CHX: 87.2 ± 3.1%, n=12). CTEP-treated mice do show a reduction in both LTD and MTD as previously reported (two-way ANOVA: main effect of CTEP treatment on LTD, p<.05 ; main effect of CTEP treatment on MTD, p<.05), confirming the efficacy of CTEP in this independent experimental group. We then tested whether four weeks of chronic CTEP treatment would be sufficient to return protein synthesis-dependence to LTD. In both vehicle and CTEP-treated mice, LTD magnitude is unchanged in the presence of a protein synthesis inhibitor (Figure 2.6C,D, n=9-12). Figures 2.5D and 2.6C-D summarize the same chronic experimental groups, whereas Figures 2.5B and 2.6A-B summarize different acutely treated groups.

2.3.5: Correction of learning and memory deficits

Cognitive impairment is a core symptom in FX. We confirmed that *Fmr1* KO mice exhibit deficits in inhibitory avoidance (Figure 2.7). Vehicle-treated *Fmr1* KO mice showed significantly reduced latencies to enter the dark compartment compared to vehicle-treated WT littermates 6 h after conditioning and during all extinction trials (6 h p=0.0186, 24 h p=0.0095, and 48 h p=0.0582; Figure 2.7B-D). There was no difference in the pain threshold between *Fmr1* KO and WT mice (data not shown, see Figure 2E of Michalon, Sidorov, *et al.*, 2012).

Chronic treatment fully rescued the memory deficit in the IA paradigm, with CTEP-treated *Fmr1* KO mice exhibiting latencies to enter the dark compartment similar to vehicle-treated WT mice at all test sessions. Correspondingly, CTEP-treated *Fmr1*

KO mice exhibited significantly more avoidance than vehicle-treated *Fmr1* KO mice (6 h p=0.0817, 24 h p=0.0016 and 48 h p=0.0007).

2.3.6: Correction of hypersensitivity to sensory stimuli

FX patients frequently present a hypersensitivity to sensory stimuli (Miller *et al.*, 1999), mirrored in *Fmr1* KO mice by a hypersensitivity to low intensity auditory stimuli (Nielsen *et al.*, 2002). The whole body startle response to short auditory stimuli of moderate intensity (6 to 24 dB over background) was measured in chronically treated *Fmr1* KO and WT mice. The elevated startle response of *Fmr1* KO mice compared to WT mice was fully corrected by chronic CTEP treatment (genotype effect: p=0.029; treatment effect: p=0.035; Figure 2.8A). Treatment with CTEP had no effect on the response of WT animals. There was no potential bias between the experimental groups due to body weight (Figure 2.8B).

2.3.7: Correction of elevated locomotor activity

Hyperactivity is frequently observed in FX patients, a symptom that is reproduced in *Fmr1* KO mice (Consortium, 1994). In the open field test, vehicle-treated *Fmr1* KO mice exhibited elevated novelty-induced locomotor activity compared to vehicle-treated WT mice at the age of 2 and 5 months (2 months p<0.001, 5 months p=0.014; Figure 2.9A-B). The increased locomotor activity was corrected after 17 weeks (treatment effect p=0.009; KO/CTEP vs. KO/vehicle at 2 min p<0.001, 4 min p=0.06; Figure 2.9B) but not after 5 weeks (Figure 2A) of chronic CTEP treatment.

2.3.8: Correction of increased susceptibility to audiogenic seizures

FX patients have increased rates of epilepsy, and this is reflected in *Fmr1* KO mice by an increased susceptibility to audiogenic seizures (AGS) (Musumeci *et al.*, 2000, Musumeci *et al.*, 1999). Drug-naive *Fmr1* KO mice presented an elevated seizure response to intense auditory stimuli (120 dB) compared to WT littermates on both C57BL/6 and FVB genetic background. This hypersensitivity to AGS was fully corrected by a single dose of CTEP administrated 4 h before testing (Figure 2.10). These results

are consistent with the previously reported anti-convulsant activity of other mGluR5 antagonists in *Fmr1* KO mice (Qiu *et al.*, 2009, Yan *et al.*, 2005). However, chronic CTEP dosing (2 mg/kg p.o., every 48 hours for one week) did not correct hypersensitivity to AGS in *Fmr1* KO mice (Figure 2.11). These results are consistent with previous reports of tolerance to chronic MPEP treatment in AGS assays (Yan *et al.*, 2005).

2.3.9: Correction of the dendritic spine phenotype in the visual cortex

Increased dendritic spine density was reported in post-mortem analysis of FX patient brain tissue (Irwin *et al.*, 2001), and can be observed in *Fmr1* KO mice (Galvez & Greenough, 2005). Vehicle-treated *Fmr1* KO animals showed a significantly higher spine density in pyramidal neurons of the binocular visual cortex compared to vehicle-treated WT animals in basal but not apical dendrites (KO/vehicle vs. WT/vehicle: segments 50 μm p=0.029, 75 μm p=0.030, Figure 2.12). Chronic treatment with CTEP corrected this phenotype, reducing spine density in *Fmr1* KO animals to WT levels. In basal dendrites, spine density in CTEP-treated KO animals was significantly lower than vehicle-treated KO animals (25 μm p=0.009, 50 μm p=0.002, 75 μm p=0.022). In WT animals, CTEP treatment had no significant effect on the spine density.

2.3.10: Correction of abnormal intracellular signaling in the cerebral cortex

The ERK and mTOR signaling pathways have been implicated in the coupling of mGluR5 to the synaptic protein synthesis machinery (Banko *et al.*, 2006, Gallagher *et al.*, 2004). The basal activity levels of ERK and mTOR in cortex of mice chronically treated with CTEP and vehicle were analyzed by semi-quantitative phospho-specific Western blots. The phosphorylation of ERK1/2 at Thr202 and Tyr204, and the autophosphorylation of mTOR at Ser2481 correspond to the active forms of these kinases (Dalby *et al.*, 1998, Soliman *et al.*, 2010).

The ERK phosphorylation level was significantly higher in cortex of vehicle-treated *Fmr1* KO animals compared to vehicle-treated WT littermates (KO/vehicle: 122.9 ± 9.3% of WT/vehicle; p=0.010; Figure 2.13A,E). Chronic treatment with CTEP

specifically reduced the elevated ERK activity in *Fmr1* KO cortex (KO/CTEP: 89.5 ±6.5% of WT/vehicle, KO/CTEP vs. KO/vehicle p=0.0012) with no effect on ERK activity in WT cortex. Chronic CTEP treatment also triggered a modest increase of the total ERK expression level in *Fmr1* KO mice compared to vehicle-treated KO animals (KO/CTEP: 109.0 ± 6.5%, KO/vehicle: 95.1 ± 6.0%; p=0.013; Figure 2.13B).

The mTOR phosphorylation level was non-significantly increased in vehicle-treated *Fmr1* KO animals compared to vehicle-treated WT littermates (KO-vehicle: 109.1 ± 5.0% of WT-vehicle; p=0.13; Figure 2.13C,F). Chronic CTEP treatment significantly reduced the mTOR phosphorylation level specifically in *Fmr1* KO mice and not in WT animals (KO/CTEP: 92.0 ± 4.6% of WT/vehicle; KO/CTEP vs. KO/vehicle p=0.006). mTOR expression levels were similar in WT and KO animals and were unchanged by treatment (Figure 2.13D).

2.3.11: Partial correction of macroorchidism upon chronic treatment

The postadolescent macroorchidism observed in FX patients is reflected in elevated testis weight in *Fmr1* KO mice (Consortium, 1994). Testis weight was monitored starting with drug naive 5 week old mice throughout 17 weeks of chronic treatment with CTEP and vehicle. *Fmr1* KO mice presented significantly increased testis weight compared to WT animals at all adult ages (effect size: +32.8 mg, p<0.001; Figure 2.14A), which was partially corrected upon chronic treatment (effect size: -13.5 mg, p<0.001). No significant differences in plasma levels of testosterone (Figure 2.14B) and progesterone (Figure 2.13C) were observed between genotypes and treatment groups.

2.3.12: Absence of chronic treatment effect on motor coordination and general fitness of the animals

Chronic treatment was well tolerated by the animals independent of the genotype. There was a minimal reduction in body weight gain and a modest decrease in body temperature of 0.5°C on average in animals receiving chronic CTEP treatment compared to vehicle in both genotypes (data not shown, see Michalon, Sidorov, *et al.*,

2012, Figure S1A-B). Chronic drug treatment for 4 weeks had no effect on the rotarod performance (data not shown, see Michalon, Sidorov, *et al.*, 2012, Figure S1C). A small but significantly reduced grip strength of vehicle treated Fmr1 KO compared to WT mice, and of CTEP-treated mice of both genotypes compared to vehicle-treated WT mice was observed (data not shown, see Michalon, Sidorov, *et al.*, 2012, Figure S1D). A modified version of the Irwin battery of simple neurological and observational measures (Irwin, 1968) did not reveal any noticeable alteration in the general fitness of the animals resulting from the mutation or the treatment (data not shown, see Michalon, Sidorov, *et al.*, 2012, Table S1).

2.3.13: Lovastatin corrects altered pathophysiology in Fragile X

Lovastatin, an FDA-approved HMG-CoA reductase inhibitor, indirectly inhibits ERK1/2 signaling via upstream inhibition of the mevalonate pathway and prevents Ras from binding to the membrane and becoming active (Figure 2.15A). We therefore examined the effect of lovastatin on mGluR-LTD in hippocampal area CA1 of WT and *Fmr1* KO mice. Slices were incubated in vehicle or 50 µM lovastatin, extracellular field potentials were recorded in area CA1 in response to Schaffer collateral stimulation, and mGluR-LTD was induced with a 5 min bath application of 50 µM DHPG. We found that lovastatin corrects the exaggerated mGluR-LTD observed in the *Fmr1* KO to WT levels (WT/veh versus KO/veh, p=.005, KO/veh versus KO/lova, p<.001) but had no significant effect on mGluR-LTD in the WT (WT/veh versus WT/lova, p=0.869; Figure 2.15B,C).

2.3.14: UO126 reduces mGluR-LTD magnitude in Fmr1 KO mice

In addition to reducing ERK1/2 signaling, lovastatin has other targets and effects, notably reducing cholesterol. To assess whether lovastatin's effect on mGluR-LTD was due to downregulation of ERK1/2 signaling, we assessed mGluR-LTD in *Fmr1* KO hippocampal slices treated either with the MEK1/2-ERK1/2 inhibitor U0126 or with vehicle. We found that 20 µM U0126 but not 5 µM U0126 significantly reduced the magnitude of mGluR-LTD in *Fmr1* KO slices (p<.05, Figure 2.16).

2.3.15: Lovastatin corrects excess protein synthesis and prevents epileptogenesis in Fmr1 KO mice

The above LTD experiments (sections 2.3.13-14) were done in the context of a larger study investigating the effectiveness of lovastatin in correcting electrophysiological and behavioral phenotypes in the *Fmr1* KO mouse, especially those linked to epilepsy (Osterweil *et al.*, 2013). This broader study, led by Dr. Emily Osterweil (MIT) and supported by Dr. Alexander Chubykin (MIT) and Dr. Shih-Chieh Chuang (SUNY Downstate Medical Center), found that lovastatin corrects excess protein synthesis, prevents audiogenic seizures, blocks mGluR-mediated epileptiform bursting, and normalizes increased excitability in the visual cortex of *Fmr1* KO mice.

2.4: Discussion

2.4.1: CTEP treatment beginning in young adulthood corrects FX phenotypes

This study assessed the therapeutic potential of chronic pharmacological mGluR5 inhibition in a mouse model of FX, with treatment starting in young adulthood. The study became possible with the discovery of the novel mGluR5 inhibitor CTEP (Lindemann *et al.*, 2011), enabling continuous mGluR5 inhibition with a receptor occupancy of ca. 81 ± 4% (Figure 2.3). Acute treatment with CTEP rescued elevated protein synthesis in hippocampal slices, and single-dose administration *in vivo* normalized LTD *ex vivo* and suppressed the audiogenic seizure phenotype. Four weeks of chronic CTEP treatment starting at the age of five weeks reversed the learning and memory deficit in the inhibitory avoidance test (Figure 2.7), the hypersensitivity to auditory stimuli (Figure 2.8), the increased dendritic spine density in the primary visual cortex (Figure 2.12), and the elevated ERK and mTOR activities in the cortex *of Fmr1* KO mice (Figure 2.13). Chronic CTEP treatment for 17 weeks also corrected elevated locomotor activity (Figure 2.9) and partially reversed macroorchidism without affecting testosterone and progesterone plasma levels (Figure 2.14). For some measures (e.g., elevated protein synthesis, auditory hypersensitivity, basal dendrite spine density, and ERK phosphorylation), the corrective effect of CTEP was specific for the KO, whereas

for others (e.g., LTD, inhibitory avoidance, and locomotor activity) CTEP treatment also had a proportional effect on WT. Regardless, CTEP treatment moved Fragile X phenotypes closer to the untreated WT values for all these measures. The important and therapeutically relevant conclusion is that a broad spectrum of FX phenotypes — biochemical, structural, and behavioral — can be improved with treatment onset in young adults.

Our results are in good agreement with the comprehensive phenotypic rescue obtained by genetic reduction of mGluR5 expression level (Dolen *et al.*, 2007). A limitation of the genetic approach, however, was that mGluR5 expression levels were reduced at the earliest stage of embryonic development and thus may prevent the development of phenotypes rather than correct them. With respect to pharmacological mGluR5 inhibition, a study by Su et al. (2011) reported a rescue of increased dendritic spine density in cortical neurons *in vivo* by two weeks of MPEP administration when treatment started at birth, but not when treatment started in 6 week old animals (Su *et al.*, 2011). All other experiments reporting correction of the increased spine density phenotype with mGluR5 antagonists (MPEP, fenobam and AFQ056) were limited to *in vitro* experiments on primary cultured neurons (De Vrij *et al.*, 2008, Levenga *et al.*, 2011). In contrast to the results of Su *et al.* (2011), our data show that starting treatment immediately after birth is not a requirement; instead, chronic treatment starting in young adulthood can reverse an established phenotype. The difference in the outcomes of these experiments could be due to the duration of treatment (2 vs. 4 weeks), continuous or fluctuating receptor inhibition achieved with a long versus short half-life molecules (CTEP and MPEP), respectively, as well as the targeted mGluR5 receptor occupancy range, which is not available for previous studies.

Previous studies have noted impaired inhibitory avoidance acquisition and exaggerated extinction in the *Fmr1* KO (Dolen *et al.*, 2007, Yuskaitis *et al.*, 2010). Consistent with findings in KO (Dolen *et al.*, 2007) and WT (Xu *et al.*, 2009) mice, we found that chronic mGluR5 inhibition retarded memory extinction. We were surprised to discover, however, that long-term CTEP treatment also increased acquisition in both genotypes. We speculate that metaplasticity after chronic partial mGluR5 inhibition

promotes the synaptic modifications that accompany inhibitory avoidance acquisition (Whitlock *et al.*, 2006).

FX patients frequently present a hypersensitivity to sensory stimuli (Hagerman, 1996) and a deficit in the prepulse inhibition (PPI) of the startle response (Frankland *et al.*, 2004). In *Fmr1* KO mice, correction of the increased PPI by acute MPEP administration could be demonstrated based on eye blink response (De Vrij *et al.*, 2008) but not by measuring whole body startle response (Thomas *et al.*, 2011). The interpretation of these PPI results in mice is confounded because *Fmr1* KO compared to WT mice show a reduced whole body startle in response to loud (>110 dB) auditory stimuli, but an elevated whole body startle response to low intensity auditory stimuli (<90 dB) (Nielsen *et al.*, 2002). With this knowledge, we studied the elevated whole body startle response in *Fmr1* KO compared to WT mice to low intensity stimuli which was fully corrected by chronic CTEP treatment (Figure 2.8).

To better understand the molecular underpinning of the treatment effects, ERK and mTOR phosphorylation were studied in the cortex of adult animals after chronic CTEP treatment. ERK is an important component of the signaling cascade downstream of Gp1-mGlu receptors, and ERK inhibition is sufficient to normalize the elevated protein synthesis rate in *Fmr1* KO hippocampus sections and to suppress seizures (Chuang *et al.*, 2005, Osterweil *et al.*, 2010). Like ERK, mTOR is an important regulator of protein synthesis and also modulates Gp1-mGluR dependent hippocampal LTD (Hou & Klann, 2004). In *Fmr1* KO mice, the level of mTOR activity is elevated in some preparations and unresponsive to mGluR1/5 activation (Osterweil *et al.*, 2010, Sharma *et al.*, 2010). These observations suggest that the normalization of ERK and mTOR activity in *Fmr1* KO mice by chronic CTEP treatment is likely an integral part of the cellular mechanism by which mGluR5 inhibitors correct the altered hippocampal LTD, elevated AGS susceptibility, and deficient learning and memory in FX.

2.4.2: Lovastatin normalizes hippocampal physiology in Fmr1 KO mice

Targeted downregulation of ERK1/2 signaling with lovastatin is an alternative approach to correct enhanced protein synthesis, pathophysiology, and behavior in *Fmr1*

KO mice (Osterweil *et al.*, 2013). This approach has particular clinical relevance because lovastatin is already approved for use in children and adults for cholesterol reduction. We found that bath application of lovastatin selectively reduces the magnitude of mGluR-LTD in *Fmr1* KO hippocampal slices, correcting enhanced LTD back to WT levels (Figure 2.15). This result was quite surprising and prompts further study of the mechanism by which ERK1/2 regulates mGluR-LTD in *Fmr1* KO mice. We know that lovastatin corrects enhanced protein synthesis in *Fmr1* KO mice (Osterweil *et al.*, 2013), but it is unlikely that lovastatin's correction of enhanced LTD is through its role as an inhibitor of translation, because direct inhibition of protein synthesis does not reduce the magnitude of mGluR-LTD in *Fmr1* KO mice (Nosyreva & Huber, 2006) (Figure 2.6B). Additionally, inhibition of protein synthesis does reduce the magnitude of mGluR-LTD in WT mice (Huber *et al.*, 2000) (Figure 2.6A) but lovastatin does not (Figure 2.15). This suggests lovastatin may be affecting LTD through an alternate mechanism not involving downregulation of protein synthesis (Figure 2.17). This hypothesis is plausible given the numerous known substrates of ERK1/2 (Roskoski, 2012).

2.4.3: Summary

Taken together, our data provide evidence for the potential of mGluR5 inhibitors to correct a broad range of complex behavioral, cellular and neuroanatomical phenotypes closely related to patients' symptoms in *Fmr1* KO mice (Figure 2.18). The data further reveal that a pharmacological correction of key FX phenotypes can be achieved with treatment beginning in young adulthood after anatomically completed brain development. The breadth of phenotypes addressed and degree of normalization by mGluR5 inhibition supports the expectation that mGluR5 inhibitors might have the ability to change the developmental trajectory of FX patients and thus could hold the potential for disease modification. Additionally, these results suggest that FX is in part a disease of acute synaptopathy rather than altered development. Currently, several mGluR5 inhibitors are under clinical examination in FX. It will be of great interest to see if the clinical phenotype can be addressed in a similar broad fashion and with a similar

magnitude as suggested by the preclinical data. Taken together with the clinical potential of lovastatin (Osterweil *et al.*, 2013), it appears that there may be promise in targeting multiple steps in the pathways linking group 1 mGluRs to local protein synthesis in Fragile X.

2.5: Methods

2.5.1: Animals

Wild-type and *Fmr1* knockout mice (Consortium, 1994) were initially obtained from The Jackson Laboratory, and maintained on congenic C57BL/6J and FVB genetic backgrounds, respectively, by mating heterozygous $Fmr1^{0/x}$ females and WT males. Mice on a congenic C57BL/6J genetic background were used throughout except for audiogenic seizures experiments where FVB and C57BL/6 mice were used. Animals were maintained in a 12:12 h light:dark cycle, with lights on at 6 am and experiments conducted during the light phase. Genotyping was performed as described (Dolen *et al.*, 2007). Animal housing an experimental procedures were in line with ethical and legal guidelines, and were approved by local veterinary authorities. All experiments were conducted with experimenters blind to genotype and drug treatment.

2.5.2: Drug treatment

CTEP was synthesized at Roche and formulated as a microsuspension in vehicle (0.9% NaCl, 0.3% Tween-80). Chronic treatment consisted of once per 48 h dosing at 2 mg/kg per os (p.o.) in a volume of 10 ml/kg. In animals younger than P30, acute dosing (for LTD and AGS experiments) was s.c.

2.5.3: Characterization of CTEP pharmacological properties

The concentration of CTEP in the plasma or brain of treated animals was determined using a combined HPLC/MS method as described in Lindemann et al. (2011). In vivo mGluR5 receptor occupancy was evaluated with [^{3}H]-ABP688 as described in Lindemann et al. (2011). Simulation of the plasma levels and receptor

occupancy during chronic dosing was performed in GastroPlus software version 6.1 (Simulations Plus, Inc., Lancaster, CA, USA) using a compartmental pharmacokinetic model linked to an Emax model for occupancy estimation. The plasma pharmacokinetics for multiple dosing was simulated with a model fit to single dose data and verified to match the sparse concentration measurements made during the chronic study. The occupancy model used parameters estimated from plasma levels and occupancies measured in the in vivo binding study (Emax = 92%, EC_{50} = 12.1 ng/ml).

2.5.4: Metabolic labeling

Metabolic labeling was performed essentially as described in Osterweil et al. (2010). Briefly, 500 μm thick hippocampal slices were prepared from 4-week old animals and incubated in 32.5°C in ACSF (124 mM NaCl, 3mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$ and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2). Following a 3.5 h recovery period, actinomycin D at a final concentration of 25 μm and either CTEP at a final concentration of 10 μm or vehicle were added, and the slices were incubated for 30 min. A mix of [^{35}S]-labeled amino acids (Express protein labeling mix, Perkin-Elmer) was added to the bath at a concentration of 9.5 μM (11 μCi/ml). Slices were incubated for 30 min after which the incorporation of radioactive amino acids was stopped by transferring the slices into ice-cold ACSF. The sections were homogenized in protein lysis buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% TX100 and protease inhibitor (Roche, Complete), and unincorporated amino acids were removed by precipitating proteins in the homogenate with trichloroacetic acid. The incorporated radioactivity was measured by liquid scintillation counting with quench correction and normalized to protein concentration and to the specific radioactivity of the reaction medium.

2.5.5: Electrophysiology

Fmr1 KO and wild-type littermate controls were treated acutely (s.c., 24 hours before euthanasia) or chronically (every 48 hours p.o. for 4-5 weeks) with CTEP or vehicle, or lovastatin or vehicle (0.05% DMSO), as noted. Where noted, cycloheximide

or DMSO vehicle was bath-applied for the duration of recordings. 350 μm thick transverse hippocampal slices were prepared in ice-cold dissection buffer (in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 75 sucrose, 10 dextrose, 1.3 ascorbic acid), and the CA3 region was removed. Slices were left to recover for >3 hours at 32°C in ACSF (in mM: 124 NaCl, 5 KCl, 1.24 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$) before recordings. Extracellular field potentials were recorded in stratum radiatum of CA1 in response to Schaffer collateral stimulation. Evoked responses (initial slope) were measured every 30 seconds for a 20 minute baseline, and 50 μM DHPG ((RS)-3,5-dihydroxyphenylglycine) was applied for 5 minutes to induce LTD. Experiments where baselines drifted more than 5% over 20 minutes were excluded. Maximal transient depression (MTD) for a slice was defined as the time point post-DHPG application with the greatest depression within each individual slice. P25-P30 mice were used for acute experiments, and P58-P65 mice were used for chronic experiments. For clarity of presentation, each two points (one minute) were averaged together and represented as a single point.

2.5.6: Inhibitory avoidance

The inhibitory avoidance learning and extinction test, a single trial aversive conditioning followed by combined test and extinction sessions, was performed in computer-controlled shuttle boxes (San Diego Instruments, USA). All sessions started with a 90 s habituation to the lit compartment before the door separating the two compartments opened. Once the animal entered the dark compartment, the door was closed and the animal received a single electric foot-shock of 0.5 mA intensity and 2 s duration. Experimental sessions consisted in measuring the latency to enter the dark compartment 6 h, 24 h and 48 h after conditioning. The 6 h and 24 h test sessions were combined with extinction sessions during which the animals were enclosed in the dark compartment for 200 s but did not receive a foot-shock.

2.5.7: Whole body startle response to auditory stimuli

The whole body startle response to low intensity auditory stimuli was measured using startle response boxes (SR-LAB, San Diego Instruments). After 5 min habituation to the boxes, mice were presented with white sound pulses of 20 ms duration and moderate intensity (72 (+6), 78 (+12), 84 (+18), 90 (+24) dB over a white background noise at 66 dB). 32 pulses (8 per intensity) were presented in random order with variable inter-trial intervals (10 to 20 s).

2.5.8: Locomotor activity

Spontaneous locomotor activity was recorded during 10 min in large plexiglas chambers (40 x 40 cm) equipped with an automated video tracking system (Ethovision, Noldus).

2.5.9: Audiogenic seizure

Susceptibility to audiogenic seizure was tested in *Fmr1* KO and WT animals on the C57BL/6J and FVB genetic background. C57BL/6J mice were tested between P18 and P22, and FVB mice were tested between P30 and P60. For acute experiments, all animals received vehicle or CTEP at 2 mg/kg (p.o. in FVB, s.c. in C57BL/6J) 4 h before testing. For chronic experiments, all animals were treated with three doses of CTEP or vehicle, every 48 hours, with the final dose 24 h before resting. Following 1 min habituation to the behavioral chamber, animals were exposed to 120 dB sound emitted by a personal alarm siren (modified personal alarm, Radioshack model 49-1010, powered from a DC converter). Seizures were scored for incidence during 2 min or until animals reached one of the AGS endpoint (status epilepticus, respiratory arrest, death).

2.5.10: Golgi analysis

Golgi staining was performed in collaboration with Frimorfo Ltd. (Marly, Switzerland) on unfixed whole brains essentially as described (Gibb & Kolb, 1998). Analyses were performed on apical and basal dendrites of the layer 3 pyramidal neurons in the binocular region of primary visual cortex, and spine density was counted

per segments of 25 μm, beginning at the branching point of the first branch, as described (Dolen *et al.*, 2007).

2.5.11: Western-blot analysis

Cortices were rapidly dissected in ice-cold ACSF, snap-frozen in isobutane on dry-ice and stored at -80°C until processing. Samples were homogenized with a glass Dounce homogenizer in ice-cold modified RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche, Complete and PhosSTOP). Protein concentrations were determined with the BCA assay (Pierce) and adjusted for all samples. Aliquots of proteins were subjected to SDS-PAGE (NuPAGE 4-12% (ERK) and 3-8% (mTOR), Invitrogen), transferred to a nitrocellulose membrane (iBlot, Invitrogen) and processed for incubation with primary antibodies, followed by secondary antibodies labeled with infra-red sensitive fluorescent dyes, and scanned (Odyssey imager, LI-COR). The following antibodies and dilutions were used: from Cell Signaling Technology, anti-phospho-p44/42 MAPK (ERK1,2) (Thr202/Tyr204) (1/4000), anti-p44/42 MAPK (ERK1,2) (1/4000), anti-phospho-mTOR (Ser2481)(1/500), anti-mTOR (1/2000); from Sigma-Aldrich, anti-GAPDH (1/4000), anti-beta-actin (1/4000); from Rockland, anti-mouse IgG (IRDye-800DX), anti-rabbit IgG (IRDye-700DX). Signal intensity was quantified with Odyssey imager software.

2.5.12: Testis weight and hormone levels

Testis weight was determined from freshly dissected, unfixed tissue samples, and hormone plasma levels were measured using immunoassays from Cayman Chemicals (Tallinn, Estonia) following the manufacturer's instructions.

2.5.13: Neurological assessment, motor coordination and grip strength test

The neurological assessment including rotarod and grip strength tests of mice was performed as described (Higgins *et al.*, 2001).

2.5.14: Statistics

Data were analyzed with two-way ANOVA with genotype and treatment as independent factors and repeated measures as covariate when appropriate. Post-hoc tests were used to compare groups only if the global analysis indicated a statistically significant ($p < 0.05$) main effect or a significant interaction. A post-hoc Bonferroni test was applied to LTD data, and protected post-hoc Fisher test was used for all other experiments. AGS experiments were analyzed with nonparametric statistics for small sample size (Fisher exact test). Student's t-test was used when only two conditions were tested in LTD experiments.
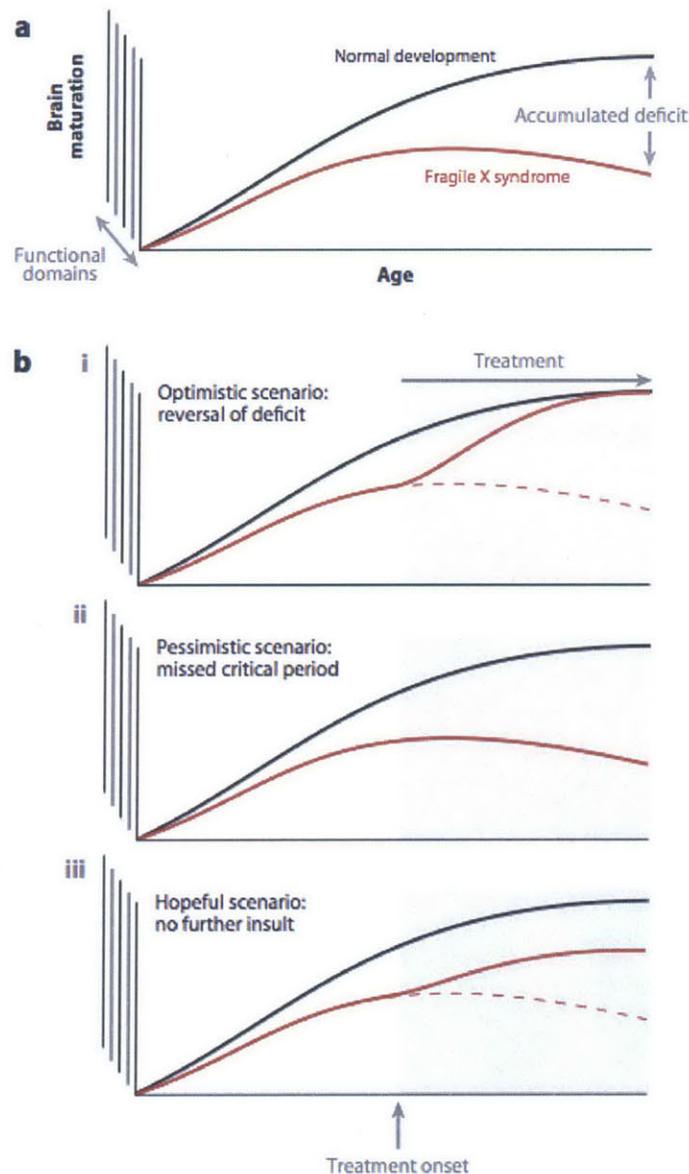
Figure 2.1: Prospects for the pharmacological treatment of Fragile X.

(A) Deficits accumulate across multiple functional domains over the course of development in FX. **(B)** Chronic treatment beginning in adulthood could reveal one of many results: (i) a complete reversal of FX phenotypes, suggesting that FX is purely a disease of acute synaptopathy, (ii) no correction of FX phenotypes, suggesting that FX is purely a disease of altered development with a critical period for treatment, or (iii) prevention of worsening of symptoms. It is also possible that the degree of improvement will vary by the domain of symptoms or phenotypes. Figure from Krueger and Bear, *Annu. Rev. Med.,* 2011.
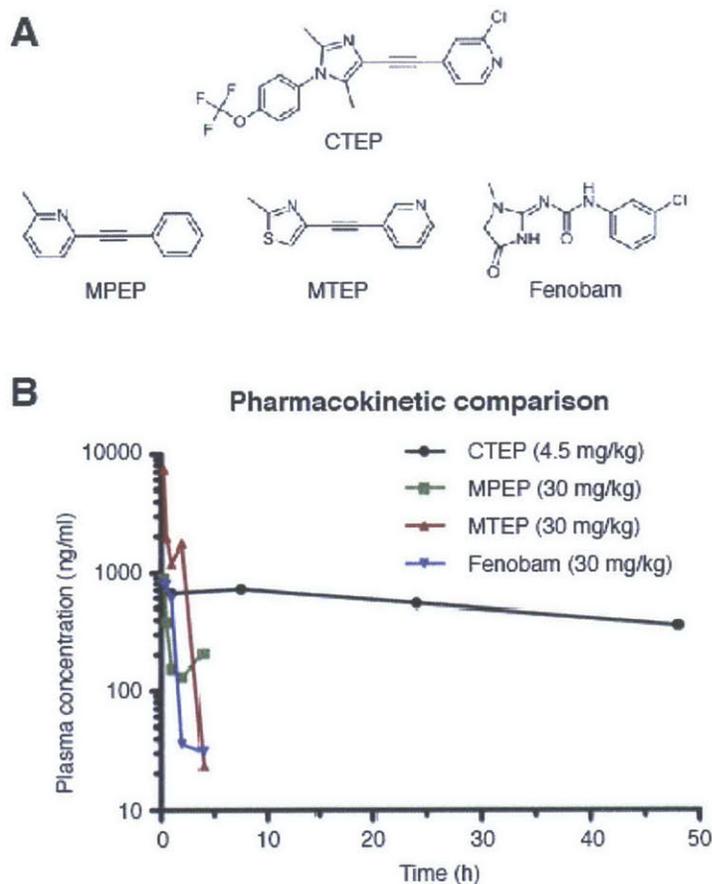
Figure 2.2: CTEP is a long-lasting mGluR5 inhibitor.

(A) Chemical structures of CTEP, MPEP, MTEP and fenobam. **(B)** Pharmacokinetic profiles of CTEP, MPEP, MTEP and fenobam in plasma in C57BL/6 mice after a single dose administered orally. Figure adapted from Lindemann *et al.*, 2011.
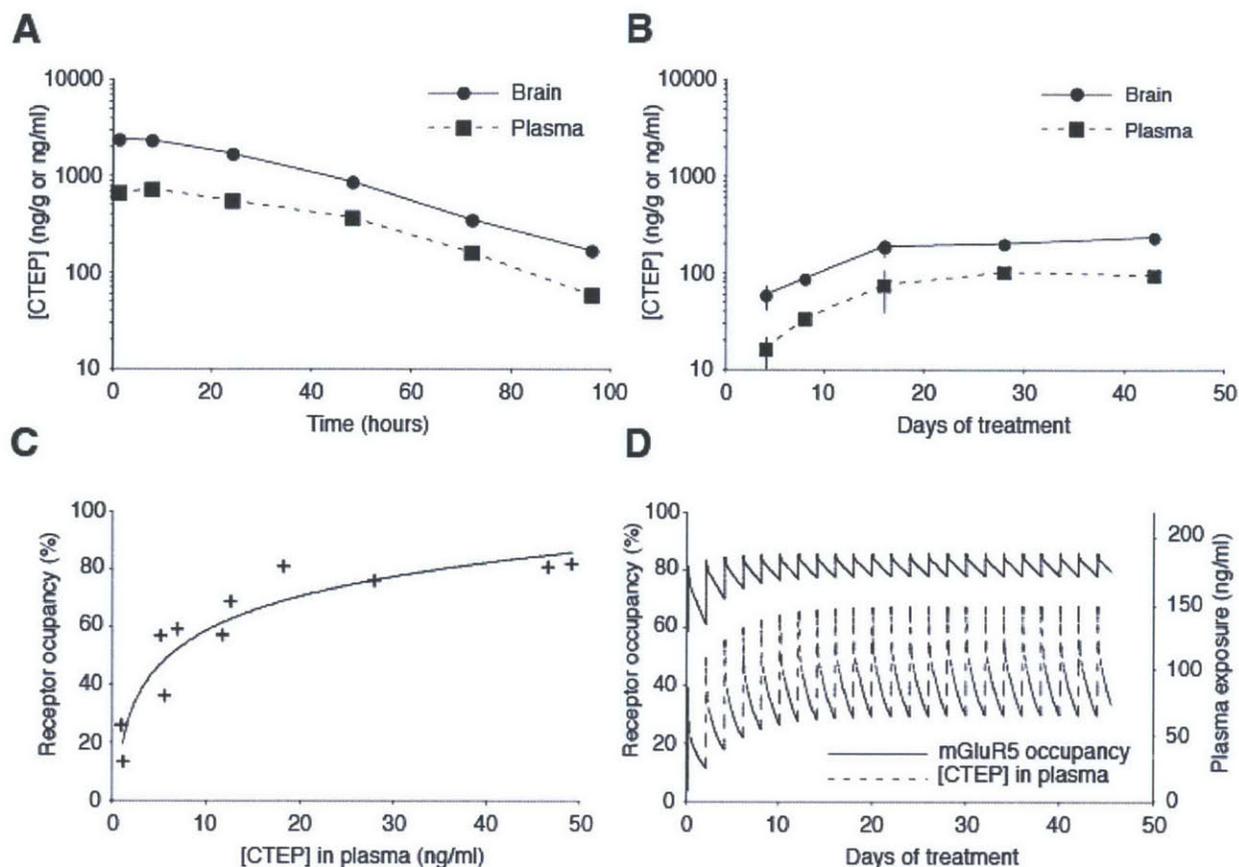
Figure 2.3: CTEP is suitable for chronic dosing in *Fmr1* KO mice.

(A) Drug exposure following a single oral dose of CTEP at 4.5 mg/kg; 2 mice per time point. **(B)** mGluR5 receptor occupancy in vivo as a function of drug exposure; 11 mice. **(C)** Drug exposure monitoring during chronic dosing at 2 mg/kg/48h p.o. Samples were collected 48 h after each drug administration and thus reveal the minimal levels of drug exposure; 2-4 mice per time point. **(D)** Simulation of mGluR5 receptor occupancy during the course of 6 week chronic treatment at 2 mg/kg/48h p.o. This dosing regimen achieved sustained receptor occupancy of 81 ± 4%. (Experiments conducted by Aubin Michalon and colleagues, Roche).
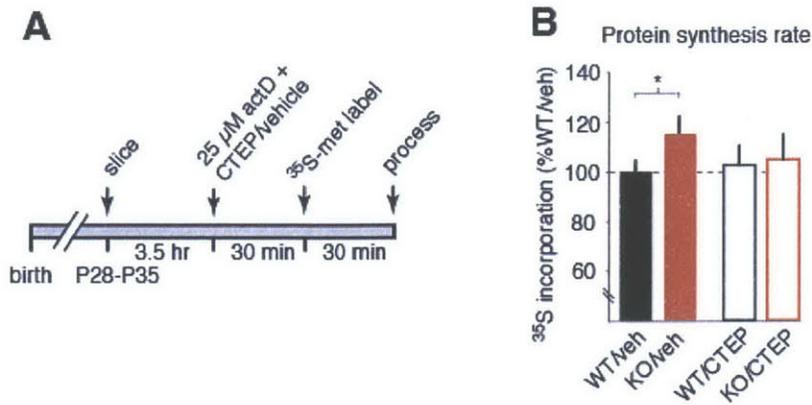
Figure 2.4: Acute CTEP corrects enhanced hippocampal protein synthesis.

(A) Timeline of the protein synthesis assay measuring ^{35}S-Met/Cys incorporation in hippocampus sections in vitro. **(B)** Protein synthesis rate in WT and *Fmr1* KO slices, in presence or absence of CTEP in the bath; mean ± SEM of 9-11 animals per group with 2 slices per animal and drug treatment; *p<0.05. (Experiments conducted by Aubin Michalon and colleagues, Roche).
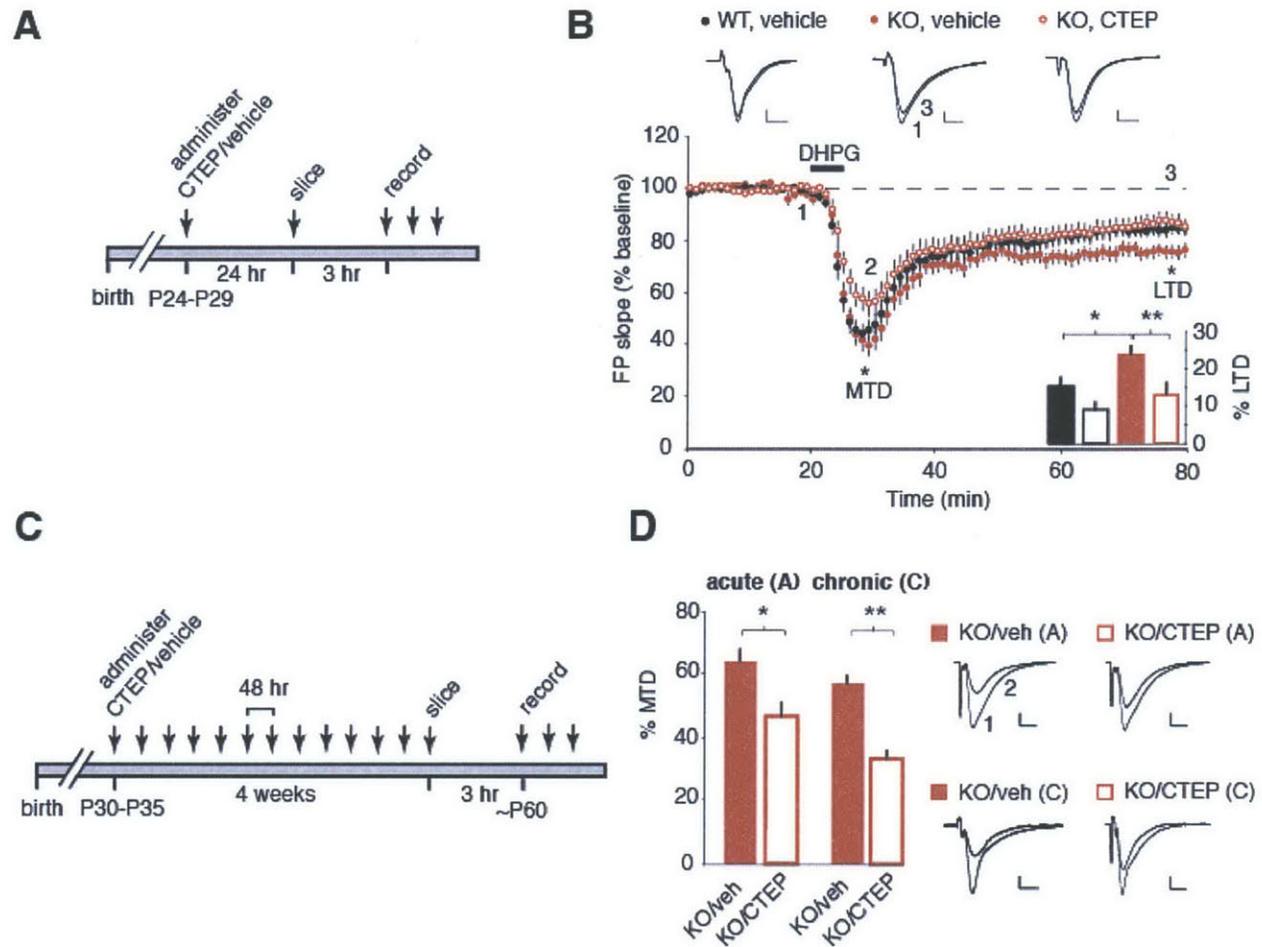
Figure 2.5: CTEP corrects enhanced LTD and reduces maximal transient depression.

(A) Timeline of acute s.c. treatment with CTEP or vehicle 24 h before dissection and LTD induction. **(B)** Gp1-mGluR LTD was enhanced in *Fmr1* KO, and was rescued by a single CTEP treatment. Two-way ANOVA revealed a significant effect of genotype (p<0.05) and treatment (p<0.01), but no interaction. Insert: post-hoc tests showed a significant LTD enhancement in KO/vehicle vs. WT/vehicle slices (*p<0.05), a significant rescue by CTEP (**p<0.01), and no significant effect of CTEP in WT slices (p=0.14). **(C)** Timeline of chronic treatment schedule (2 mg/kg/48h p.o) beginning at the age of 4-5 weeks. **(D)** The maximal transient depression (MTD) induced by DHPG was significantly reduced by both acute (*p<0.05) and chronic CTEP treatment (**p<0.01) in KO slices, showing that the drug efficacy is maintained throughout chronic treatment. For (C,D) mean ± SEM of 14-18 slices per condition.
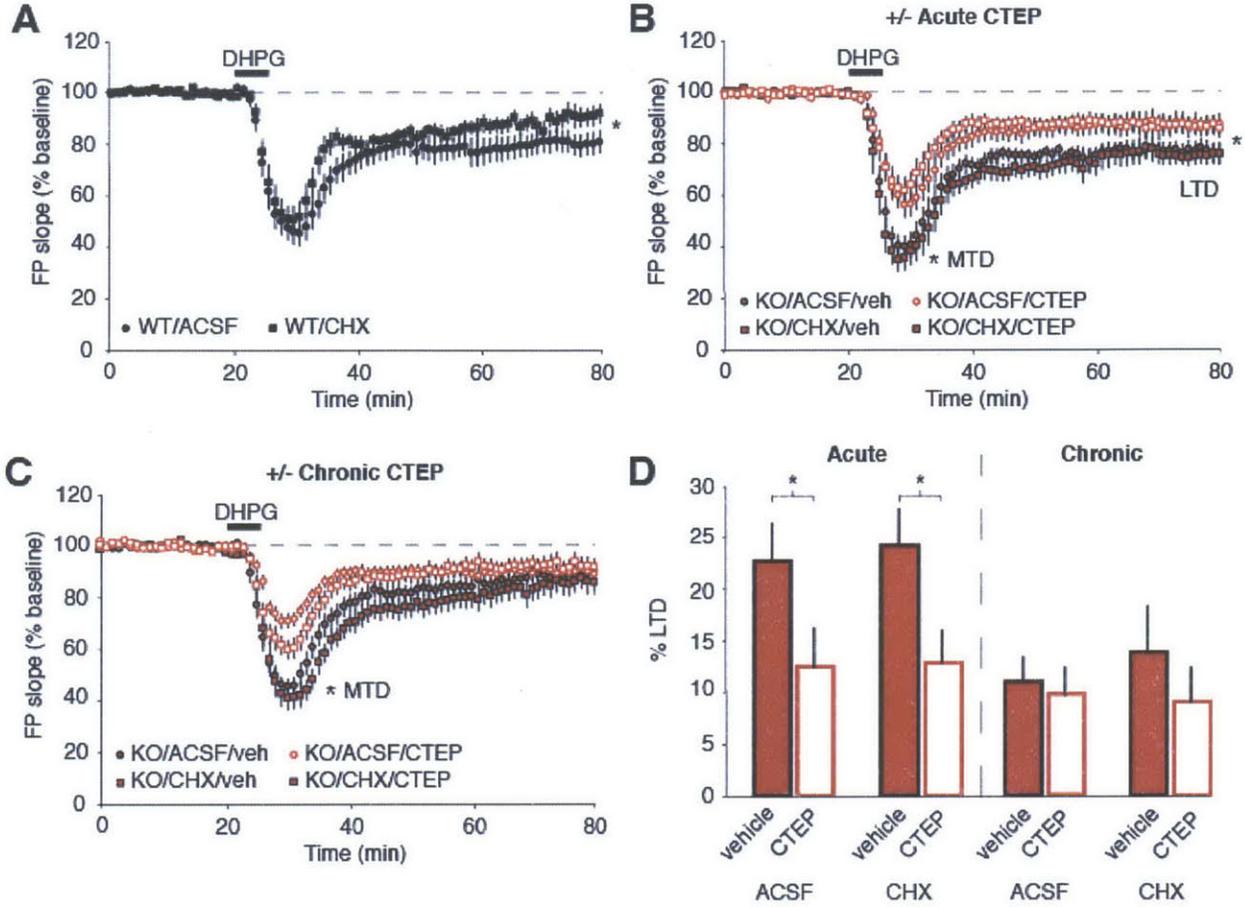
Figure 2.6: Protein synthesis independence of mGluR-LTD in *Fmr1 KO* mice is unchanged following CTEP administration.

(A) mGluR-LTD magnitude is reduced by the protein synthesis inhibitor cycloheximide (*$p<.05$). **(B)** *Fmr1* KO mice show mGluR-LTD which persists with normal magnitude in the presence of cycloheximide. Acute CTEP treatment reduces MTD (*$p<.05$) and LTD (*$p<.05$) magnitude, but does not alter LTD's protein synthesis-independence. **(C)** Chronic CTEP treatment reduces MTD (*$p<.05$) but LTD remains protein synthesis-independent in *Fmr1* KO mice. **(D)** Summary of LTD magnitude in *Fmr1* KO mice ± acute and chronic CTEP and ± cycloheximide.
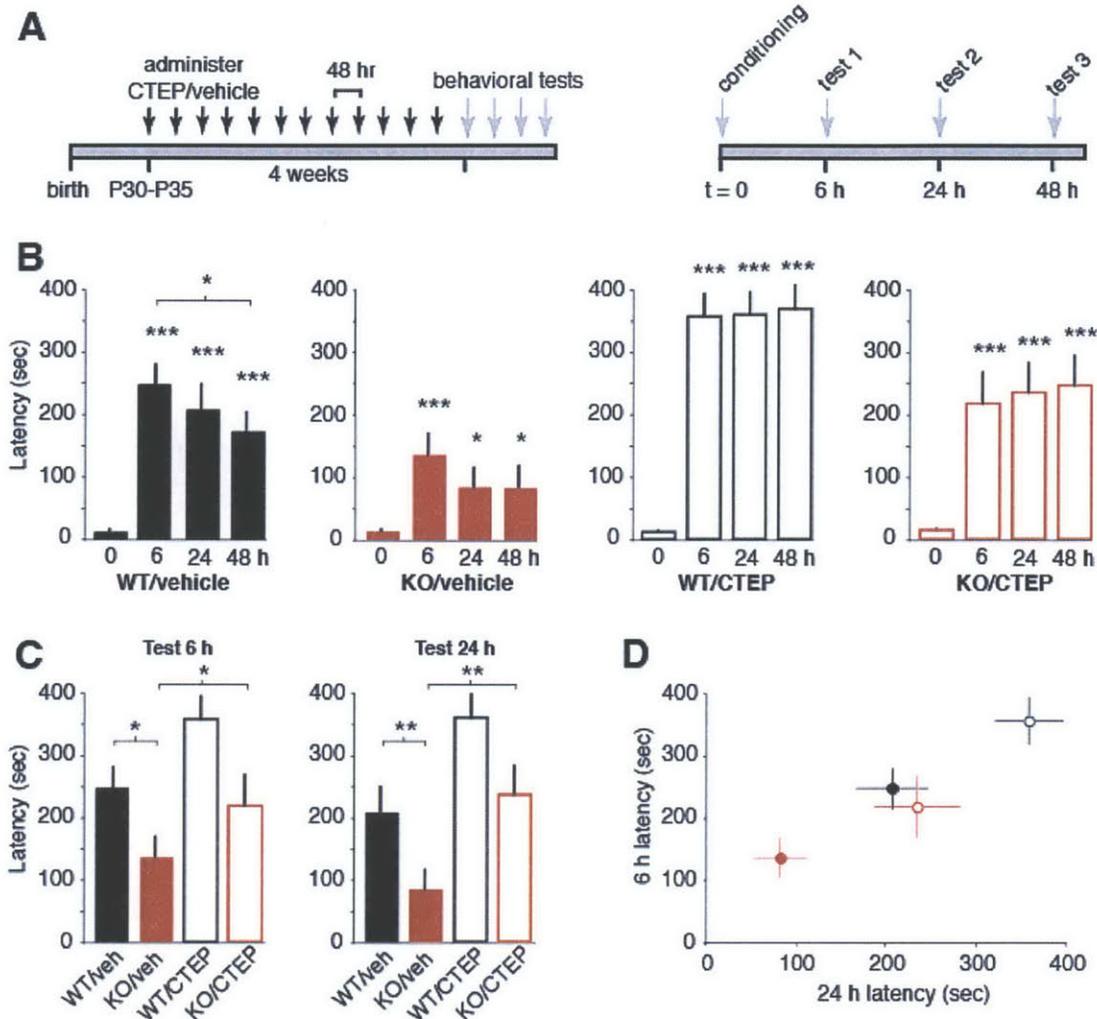
Figure 2.7: Pharmacological correction of learning and memory deficit in *Fmr1* KO mice.

(A) Timeline of chronic dosing prior to behavioral evaluation and the inhibitory avoidance and extinction (IAE) tests. **(B)** All experimental groups exhibited a significant increase in latency following the conditioning session (different from t=0 *p<0.05; **p<0.01, ***p<0.001) and WT/vehicle animals also showed significant memory extinction (different from t=6 h +p<0.05). **(C)** Comparison of latency across experimental groups at 6 and 24 h test sessions: KO/vehicle vs. WT/vehicle: *p<0.05, **p<0.01; KO/CTEP vs. KO/vehicle: °p<0.1, ++p<0.01. **(D)** Multivariate analysis of latency at 6 vs. 24 h: The learning deficit observed in KO/vehicle mice was fully compensated by treatment, and the effect of treatment was similar in WT and *Fmr1* KO mice. For B, C and D: mean ±SEM of 15-16 mice per group. (Experiments conducted by Aubin Michalon and colleagues, Roche).
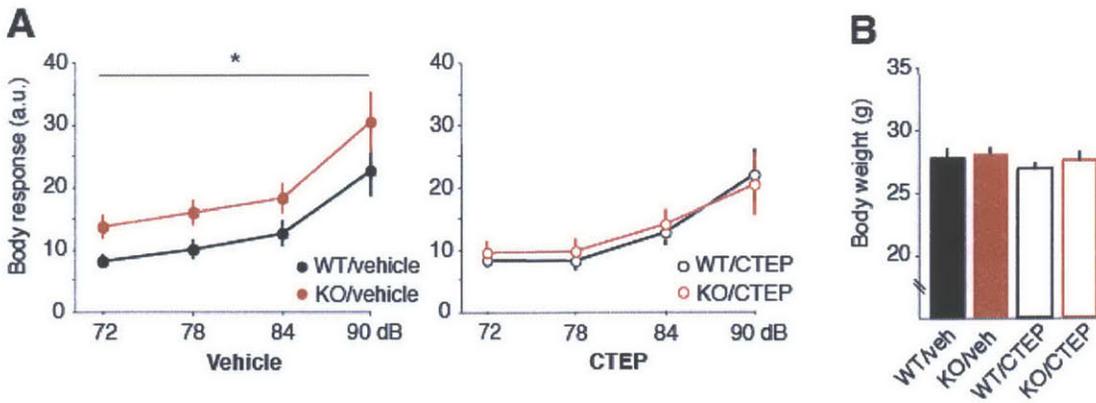
Figure 2.8: Pharmacological correction of hypersensitivity to auditory stimuli.

(A) Sensitivity to auditory stimuli: Mice were exposed to short auditory stimuli at 72 (+6), 78 (+12), 84 (+18), 90 (+24) dB over a white background noise at 66 dB, and the whole body startle response was recorded. Genotype effect *$p < 0.05$; treatment effect $p < 0.05$; mean ± SEM of 13-16 mice per group, with 8 presentations of each sound intensity. **(B)** Body weight: there was no significant difference in body weight between the experimental groups on the day of the whole body startle response experiment; mean ± SD of 13-16 mice per group. (Experiments conducted by Aubin Michalon and colleagues, Roche).
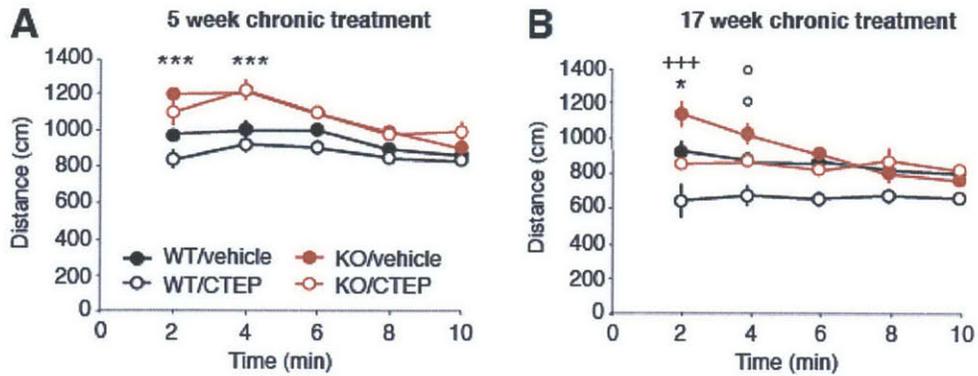
Figure 2.9: Correction of elevated locomotor activity.

Locomotor activity in the open-field: *Fmr1* KO mice exhibited elevated novelty-induced activity compared to WT mice at the age of 2 months **(A)** and 5 months **(B)**. Correction of the hyperactivity phenotype was observed after 17 weeks **(B)** of chronic treatment but not after 5 weeks (H). KO/vehicle vs. WT/vehicle °p<0.1, *p<0.05, ***p<0.001; KO/CTEP vs. KO/vehicle +p<0.05, ++p<0.01; mean ±SEM of 16-17 (A) and 13-15 (B) mice per group. (Experiments conducted by Aubin Michalon and colleagues, Roche).

A

C57/BL6 P18-P22
FVB P30-P60

administer CTEP/vehicle

habituate to chamber

4 hr   1 min   2 min   120 dB

seizure
no seizure

KO/veh   KO/CTEP
WT/veh   WT/CTEP

B C57/BL6

C57/BL6	wild running	seizure	death	incidence
WT/vehicle	1/14	0/14	0/14	7%
WT/CTEP	0/12	0/11	0/12	0%
KO/vehicle	8/19	3/19	2/19	42% ‡
KO/CTEP	1/15	1/15	0/15	7% *

C FVB

FVB	wild running	seizure	death	incidence
WT/vehicle	1/13	1/13	0/13	8%
WT/CTEP	0/12	0/12	0/12	0%
KO/vehicle	12/17	10/17	5/17	71% ‡
KO/CTEP	5/16	5/16	0/16	31% *

KO/veh   KO/CTEP
WT/veh   WT/CTEP

Figure 2.10: **Pharmacological rescue of elevated susceptibility for audiogenic seizure.**

(A) Timeline showing single dose of CTEP or vehicle, s.c., 4 hours prior to AGS testing. *Fmr1* KO mice display an elevated susceptibility to audiogenic seizure, in both C57BL/6 **(B)** and FVB **(C)** genetic backgrounds, which was rescued by acute administration of CTEP (4 h before testing, 2 mg/kg, s.c. or p.o.). The tables present for each experimental group the number of mice exhibiting wild running-jumping, tonic-clonic seizures or death, compared to the total number of animals tested. Pairwise group comparisons with the Fisher exact test: different from WT-vehicle ‡$p < 0.05$, different from KO-vehicle *$p < 0.05$.

A Timeline showing chronic one week dosing of CTEP or vehicle, s.c., in FVB background. **(B)** Increased susceptibility to audiogenic seizures in *Fmr1* KO mice is not corrected by chronic CTEP. Pairwise group comparisons with the Fisher exact test: different from WT-vehicle *p<0.05.

The figure contains the following table:

FVB	wild running	seizure	death	incidence
WT/vehicle	2/39	2/39	1/39	5%
WT/CTEP	4/36	4/36	0/36	11%
KO/vehicle	13/31	12/31	3/31	42%*
KO/CTEP	20/35	19/35	8/35	57%*

Figure 2.11: Tolerance to chronic CTEP in audiogenic seizure domain.

(A) Timeline showing chronic one week dosing of CTEP or vehicle, s.c., in FVB background. **(B)** Increased susceptibility to audiogenic seizures in *Fmr1* KO mice is not corrected by chronic CTEP. Pairwise group comparisons with the Fisher exact test: different from WT-vehicle *p<0.05.
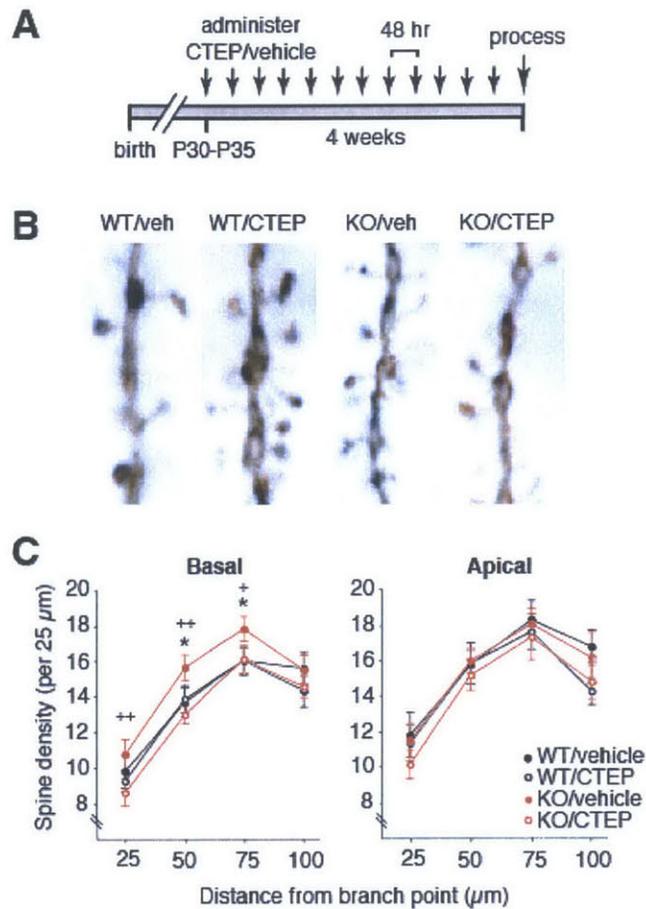
Figure 2.12: Pharmacological correction of elevated dendritic spine density.

(A) Timeline of chronic dosing morphological and biochemical analysis. (B) Representative images of Golgi stained neurons in the primary visual cortex; each segment shown is 10 μm long. (C) Spine density was increased in basal but not apical dendrites of KO/vehicle compared to WT/vehicle littermates (*p<0.05), and normalized in chronically treated KO animals (KO/CTEP s. KO/vehicle: +p<0.05, ++p<0.01). Mean ±SEM of 10 mice per group, with 3 dendrites on 3 different neurons counted per animal. (Experiments conducted by Aubin Michalon and colleagues, Roche).
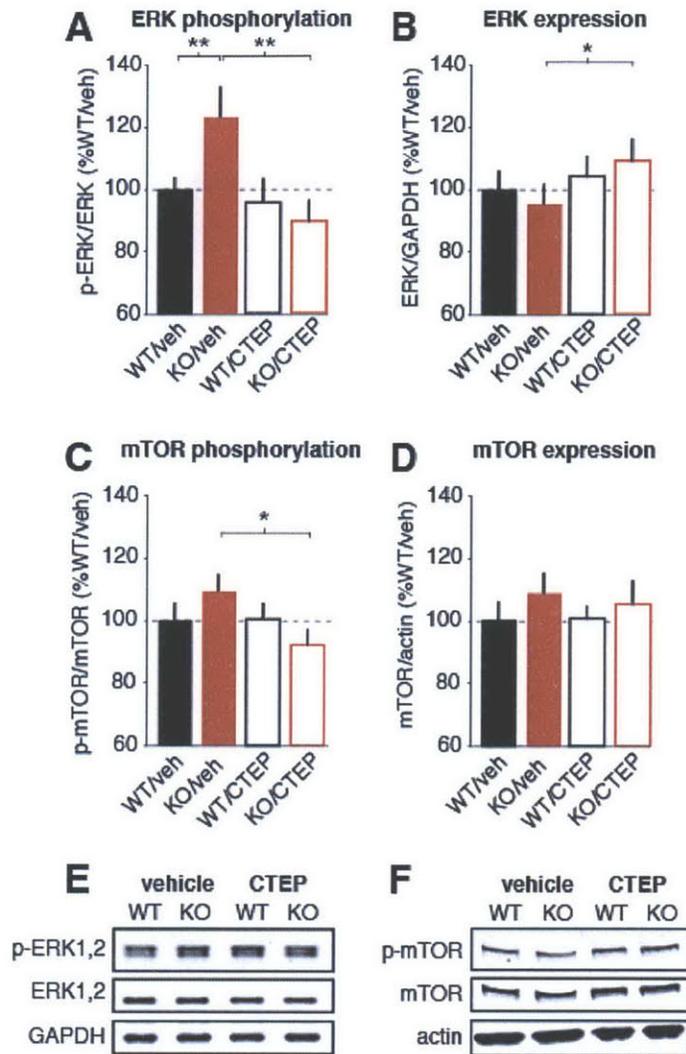
Figure 2.13: Pharmacological correction of altered intracellular signaling.

(A-D) Quantification of phosphorylation and expression levels of ERK and mTOR in cortical extracts collected from chronically treated animals. **(A)** Elevated ERK1,2 phosphorylation (Thr202/Tyr204) in KO/vehicle mice was corrected in chronically treated KO mice (**$p<0.01$). **(B)** Treatment also increased ERK expression level in KO animals (*$p<0.05$). **(C)** Similarly, mTOR phosphorylation (Ser2481) levels were significantly decreased in chronically treated KO mice compared to vehicle-treated *Fmr1* KO mice (*$p<0.05$). **(D)** mTOR expression levels were not altered in *Fmr1* KO mice, and not altered upon chronic treatment. For C and D, mean ± SEM of 11 mice per group with triplicate measurements. **(E, F)** Typical western-blot results. (Experiments conducted by Aubin Michalon and colleagues, Roche).
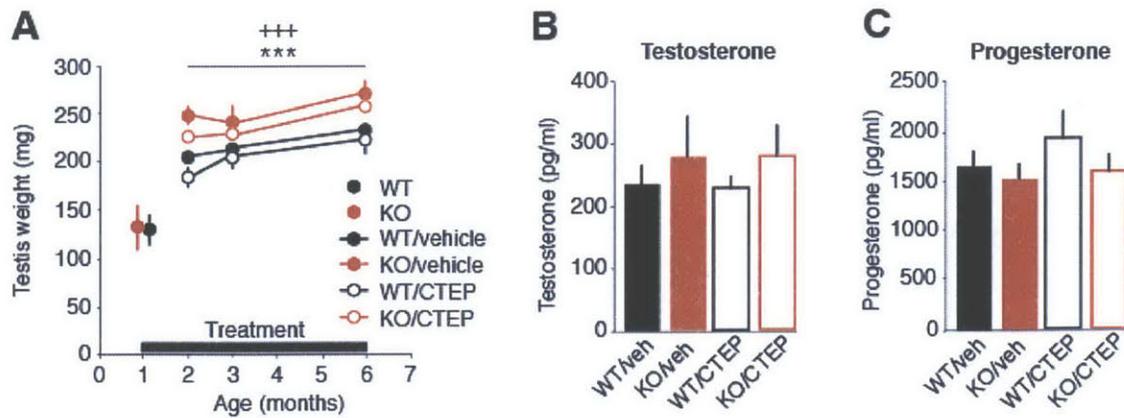
Figure 2.14: Pharmacological amelioration of macroorchidism.

(A) Testis weight. Adult *Fmr1* KO mice presented an increased testis weight compared to WT mice (genotype effect ***$p < 0.001$), what was partially corrected (~40% correction) upon chronic treatment (treatment effect +++$p < 0.001$). There was no significant genotype x treatment interaction. Mean ± SD of 9-12 mice per age and per group. **(B, C)** Testosterone and progesterone levels were determined in the plasma of animals submitted to 5 months of chronic treatment. For both hormones, the levels were similar in WT and *Fmr1* KO animals and were not affected by treatment. Mean ± SEM of 7-10 mice per group and duplicate measurements. (Experiments conducted by Aubin Michalon and colleagues, Roche).
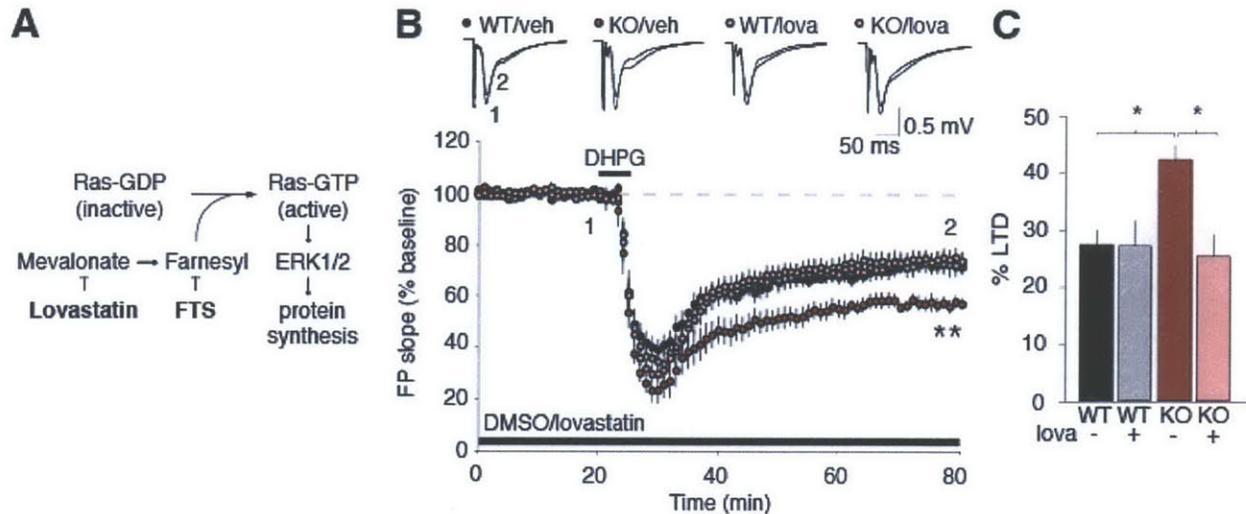
Figure 2.15: Lovastatin corrects exaggerated mGluR-LTD in the *Fmr1* KO hippocampus.

(A) Lovastatin indirectly downregulates ERK1/2 signaling, and protein synthesis, by inhibiting the mevalonate pathway. This prevents Ras from binding to the membrane and shifting from its inactive GDP-bound form to its active GTP-bound form. **(B)** In the presence of vehicle, greater LTD is observed in the *Fmr1* KO versus WT (WT/veh: 72.5 ± 2.5%, KO/veh: 57.5 ± 2.5%, **p=.005, n=9-10). Fifty micromolar lovastatin significantly reduces LTD magnitude in the *Fmr1* KO to WT levels (WT/lova: 72.7 ± 4.4%, KO/lova: 74.5 ± 3.4%, KO/veh versus KO/lova **p<.001, n=11-13) but has no significant effect on LTD in the WT (WT/veh versus WT/lova, p=0.869). Field potential traces are averages of all experiments and were taken at times indicated by numerals. **(C)** Lovastatin significantly reduces LTD magnitude in the *Fmr1* KO to WT levels (ANOVA genotype x treatment. *p=.021). n = animals. Error bars represent SEM.
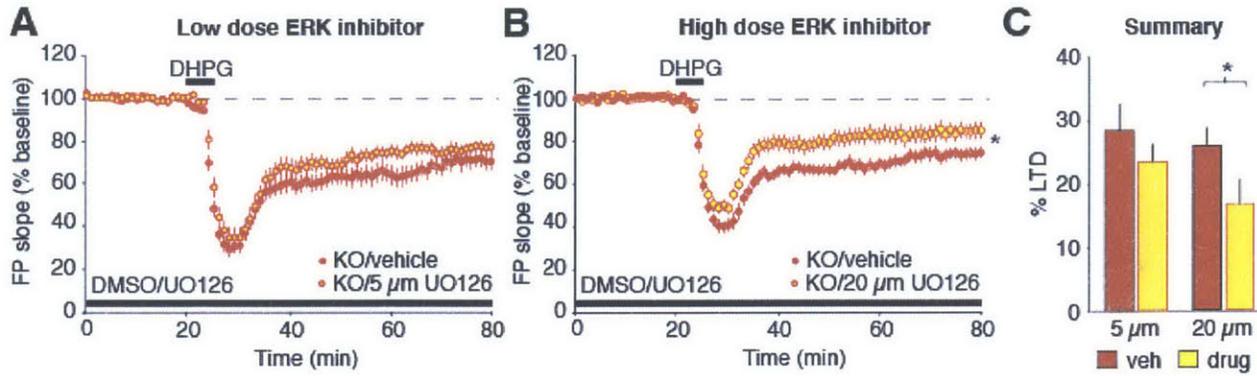
Figure 2.16: UO126 decreases the magnitude of mGluR-LTD in *Fmr1* KO hippocampus.

(A) Bath application of 5 μM U0126 does not reduce mGluR-LTD magnitude in *Fmr1* KO mice (KO/veh: 71.6 ± 4.2%, KO/U0126: 76.5 ± 2.7%, p=.431, n=10). **(B)** Bath application of 20 μM U0126 significantly reduces LTD magnitude (KO/veh: 74.2 ± 2.7%, KO/U0126: 83.3 ± 3.8%, *p<.05, n=13). **(C)** Summary. n = animals. Error bars represent SEM.
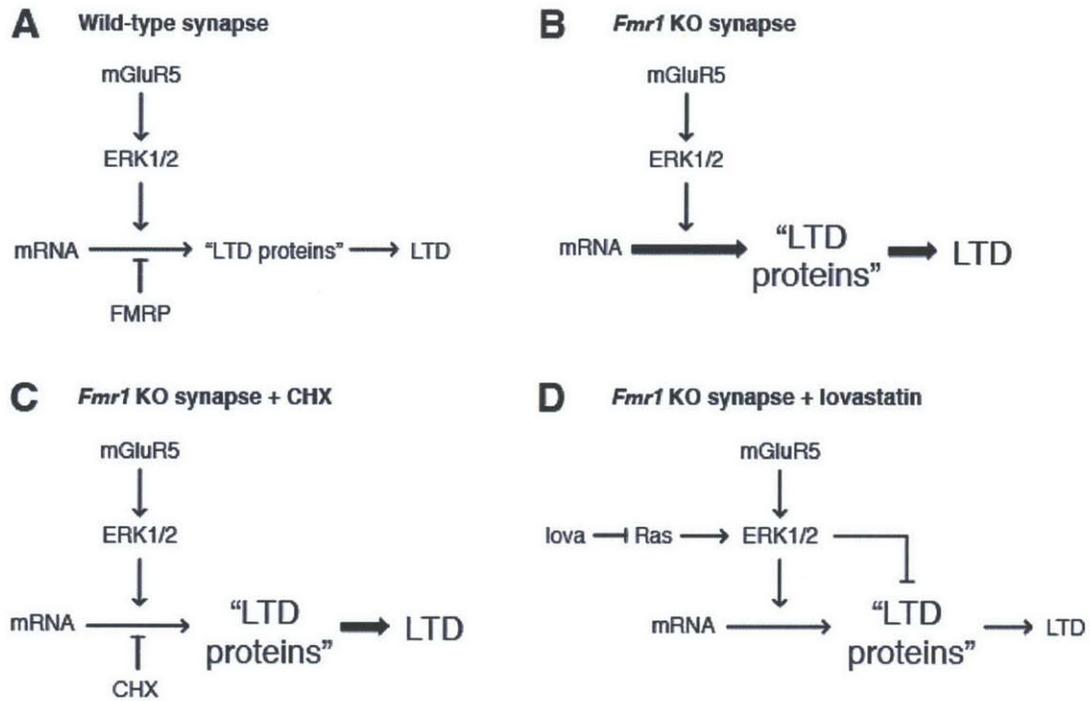
Figure 2.17: A model of lovastatin's mechanistic influence on mGluR-LTD in *Fmr1* KO mice.

(A) In wild-type synapses, FMRP and mGluR5-ERK1/2 signaling have functionally opposing effects on local protein synthesis, resulting in normal synthesis of proteins required for LTD and normal LTD magnitude. **(B)** In *Fmr1* KO synapses, loss of FMRP results in increased protein synthesis and increased LTD. **(C)** The enhancement of LTD in *Fmr1* KO synapses persists in the presence of a protein synthesis inhibitor, likely because candidate "LTD proteins" (e.g. Arc, STEP, MAP1B) are already over-synthesized. **(D)** *Fmr1* KO synapses treated with lovastatin show a correction of enhanced LTD to WT levels. If lovastatin was acting through ERK1/2 purely as a protein synthesis inhibitor, we would expect to see no effect, as is seen with cycloheximide. Therefore we propose that lovastatin, through ERK1/2, may also be inhibiting the action or function of already-synthesized "LTD proteins" and thus reducing LTD magnitude through a mechanism separate from its role in downregulating protein synthesis.

Phenotype	*Fmr1* KO	Treatment	Result
Protein synthesis	↑	acute	✓
mGluR-LTD	↑	acute	✓
Audiogenic seizures	↑	acute	✓
		chronic	---
Spine density	↑	chronic	✓
Acoustic startle	↑	chronic	✓
IA acquisition	↓	chronic	✓
ERK signaling	↑	chronic	✓
Macroorchidism	↑	chronic	✓
Open field	↑	chronic (4 wk)	---
		chronic+ (17 wk)	✓

Figure 2.18: Summary of pharmacological correction of *Fmr1* KO phenotypes via CTEP.

Chapter 3

Extinction of an instrumental response: a cognitive behavioral assay in Fmr1 knockout mice

3.1: Abstract

Fragile X is the most common genetic cause of intellectual disability and autism. Previous studies have showed that partial inhibition of metabotropic glutamate receptor signaling is sufficient to correct behavioral phenotypes in a mouse model of Fragile X, including audiogenic seizures, open field hyperactivity, and social behavior. These phenotypes model well the epilepsy (15%), hyperactivity (20%) and autism (30%) that are co-morbid with Fragile X in human patients (Schneider *et al.*, 2009). Identifying reliable and robust mouse phenotypes to model cognitive impairments is critical considering the 90% co-morbidity of Fragile X and intellectual disability. Recent work characterized a five-choice visuospatial discrimination assay testing cognitive flexibility, in which Fragile X model mice show impairments associated with decreases in synaptic proteins in prefrontal cortex (Krueger *et al.*, 2011). In this study, we sought to determine whether instrumental extinction, another process requiring prefrontal cortex, is altered in Fragile X model mice, and whether downregulation of metabotropic glutamate receptor signaling pathways is sufficient to correct both visuospatial discrimination and extinction phenotypes. We report that pharmacological intervention can correct operant acquisition but not visuospatial discrimination deficits in Fragile X model mice. In addition, instrumental extinction is consistently exaggerated in Fragile X model mice, yet this phenotype is not corrected by approaches targeting metabotropic glutamate receptor signaling. This work describes a novel behavioral extinction assay to model impaired cognition in mouse models of neurodevelopmental disorders, and provides surprising evidence that extinction is exaggerated in the Fragile X mouse model.

3.2: Introduction

3.2.1: Importance of modeling cognitive impairments in Fmr1 KO mice

The *Fmr1* knockout mouse has well-characterized behavioral deficits consistent with presentation of FX in humans. Specifically, FX has high comorbidity with epilepsy (~15%), hyperactivity (~20%), and autism (~30-60%) (Schneider *et al.*, 2009). Consistent with these clinical findings, *Fmr1* KO mice show increased seizure susceptibility (Yan *et al.*, 2004), open-field activity (Consortium, 1994, Mineur *et al.*, 2002), and social deficits (Oddi *et al.*, 2013). Cognitive impairments in *Fmr1* KO mice have been comparatively understudied, considering the ~90% comorbidity between FX and intellectual disability (Schneider *et al.*, 2009). *Fmr1* KO mice show learning deficits in multiple contexts, such as spatial learning, inhibitory avoidance, and sustained attention (D'hooge *et al.*, 1997, Dolen *et al.*, 2007, Kramvis *et al.*, 2013, Michalon *et al.*, 2012, Moon *et al.*, 2006). However, apart from conflicting reports of attentional dysfunction (Kramvis *et al.*, 2013, Krueger *et al.*, 2011, Moon *et al.*, 2006), the majority of learning tests in *Fmr1* KO mice are not sufficient to model cognitive dysfunction mediated by prefrontal cortex.

3.2.2: Visuospatial discrimination and instrumental extinction are cognitive processes mediated by PFC

Fmr1 KO mice show impairments in a visuospatial discrimination task which are consistent with deficits in cognitive flexibility, and this impairment is linked to altered synaptic protein levels in prefrontal cortex (PFC) (Krueger *et al.*, 2011). Extinction of operant behaviors is also an active cognitive process (Bouton, 2002) and relies on top-down control of PFC, independent of the nature of the reinforcer (Cleva *et al.*, 2010, Millan *et al.*, 2011, Rebec & Sun, 2005). Substantial evidence suggests mGluR5 is involved in regulating PFC-mediated operant extinction (Chesworth *et al.*, 2013, Cleva *et al.*, 2011, Gass & Olive, 2009, Kufahl *et al.*, 2012), and extinction of hippocampally-encoded inhibitory avoidance is altered in *Fmr1* KO mice (Dolen *et al.*, 2007). As

mGluR5-mediated processes are involved in FX pathogenesis (Bear *et al.*, 2004), we were thus motivated to study instrumental extinction in *Fmr1* KO mice.

3.2.3: Targeting mGluR5 to correct cognitive phenotypes

Enhanced synaptic protein synthesis in FX is likely to be pathogenic, and pharmacological approaches to downregulate protein synthesis are currently being evaluated in clinical trials based on their success in *Fmr1* KO mice (Krueger & Bear, 2011). Specifically, downregulation of mGluR5 or ERK1/2 signaling is sufficient to normalize protein synthesis and behavior in *Fmr1* KO mice (see Chapter 2) (Dolen *et al.*, 2007, Michalon *et al.*, 2013, Michalon *et al.*, 2012, Osterweil *et al.*, 2013, Yan *et al.*, 2005). We sought to determine whether downregulation of these signaling pathways could also correct altered visuospatial discrimination and instrumental extinction in *Fmr1* KO mice. Overall, visuospatial discrimination and instrumental extinction provide powerful behavioral assays in which to screen drugs in mouse models of Fragile X and other neurodevelopmental disorders associated with cognitive impairments and intellectual disability.

3.3: Results

3.3.1: Pharmacological correction of delayed operant acquisition in Fmr1 KO mice

Fmr1 KO mice show deficits in a visuospatial discrimination task which are consistent with impairments in cognitive flexibility (Krueger *et al.*, 2011). In this assay, mice are placed in an operant conditioning chamber with five nose-poke apertures and a food magazine (Figure 3.1A). Mice are trained on a visuospatial discrimination in two phases. In phase 1, all nosepoke apertures are illuminated and a response in any illuminated aperture results in reward delivery. In phase 2, only one aperture is randomly illuminated, and only a response in this aperture results in food reward. Acquisition of phase 2, but not phase 1, is delayed in *Fmr1* KO mice, indicating that these mice may be impaired in the ability to flexibly respond to changing reward contingencies (Krueger *et al.*, 2011). We asked whether two interventions could correct this phenotype: (1) genetic cross with *Grm5*$^{+/-}$ mice (heterozygous for deletion of mGluR5) and (2) chronic administration of lovastatin for four weeks prior to testing, which has been shown to indirectly reduce ERK1/2 signaling. We quantified the number of training days required to reach criterion for each phase (15 correct trials on two consecutive days for phase 1 and 15 correct trials on two consecutive days with over 50% correct for phase 2, see Methods).

Genetic cross of *Fmr1*$^{+/-}$ females and *Grm5*$^{+/-}$ males produced male offspring of four experimental genotypes: *Fmr1* WT/*Grm5*$^{+/+}$, *Fmr1* KO/*Grm5*$^{+/+}$, *Fmr1* WT/*Grm5*$^{+/-}$, and *Fmr1* KO/*Grm5*$^{+/-}$. There was no effect of *Fmr1* genotype or *Grm5* genotype on phase 1 acquisition in this group (Figure 3.1B; n = 32-45; two-way ANOVA: main effect of *Fmr1* genotype, $F_{1,133}$ = 1.634, p = .203; main effect of *Gmr5* genotype, $F_{1,133}$ < 1; *Fmr1* x *Grm5* interaction, $F_{1,133}$ < 1). Additionally, we saw a trend but no significant effect of *Fmr1* genotype on phase 2 acquisition (Figure 3.1C; two-way ANOVA: main effect of *Fmr1* genotype, $F_{1,149}$ = 3.591, p = .060). There was no significant interaction between *Fmr1* and *Grm5* genotype ($F_{1,149}$ = 1.678, p = .197).

Lovastatin, an indirect downregulator of ERK1/2 signaling, has been shown to correct audiogenic seizures as well as altered synaptic plasticity and excitability in *Fmr1*

KO mice (Osterweil *et al.*, 2013). Chronic lovastatin dosing in food ("lovachow") also corrects seizures in *Fmr1* KO mice, but this approach has not yet been tested on cognitive deficits. Following four weeks of lovachow or vehicle and concurrent with active dosing (Figure 3.1A), *Fmr1* KO mice surprisingly showed a delay in phase 1 operant acquisition not previously reported (Figure 3.1D; n = 24-38; two-way ANOVA: main effect of *Fmr1* genotype, $F_{1,131} = 6.889$, p = .010). This delay in days to criterion was corrected by chronic lovastatin dosing (genotype x drug interaction, $F_{1,131} = 4.990$, p = .027). Post-hoc analysis revealed significant differences between WT/vehicle and KO/vehicle groups (p<.001), as well as KO/vehicle and KO/lovachow groups (p<.05). There was no concurrent delay in phase 2 acquisition in *Fmr1* KO mice and no effect of lovastatin (Figure 3.1E; two-way ANOVA: main effect of genotype, $F_{1,119} < 1$; main effect of drug treatment, $F_{1,119} < 1$; genotype x treatment interaction, $F_{1,119} < 1$).

3.3.2: Chronic CTEP impairs operant acquisition

Evidence that chronic CTEP administration can correct numerous physiological and behavioral phenotypes in *Fmr1* KO mice prompted study of how similar treatment would affect phase 1 (operant acquisition) and phase 2 (visuospatial discrimination) learning. We treated *Fmr1* KO and WT mice with either CTEP or vehicle for 4+ weeks, beginning at P30, and lasting through phase 1 and phase 2 testing. CTEP treatment delayed phase 1 acquisition, independent of *Fmr1* genotype (Figure 3.2; two-way ANOVA, main effect of treatment, p<.001). There was no effect of genotype (p = .890) or genotype x treatment interaction (p = .443). There was no effect of *Fmr1* genotype (p = .084), treatment (p = .082), or genotype x treatment interaction (p = .727) on phase 2 acquisition. However, these results are difficult to interpret given prior impairments in phase 1.

3.3.3: Instrumental extinction is exaggerated in Fmr1 KO mice

While there was no statistically significant effect of the $Grm5^{+/-}$ genotype in the visuospatial discrimination task, we observed an interesting trend that might indicate an amelioration of cognitive function by mGluR5 reduction. To further investigate this

possibility, we decided to expand our analysis of phenotypes related to PFC function in the *Fmr1* KO mice. Due to previous reports of altered extinction of a fear response (Dolen *et al.*, 2007), we tested whether extinction of an appetitive operant response is also altered in *Fmr1* KO mice (Figure 3.3A; n = 16 per group). In this experiment, mice were first trained on a two-choice instrumental response, in which one aperture was illuminated and active, while the other was not illuminated and inactive. Acquisition of this task was normal in *Fmr1* KO mice, as measured by days to criterion (Figure 3.3B; Student's t-test, p = .657). In addition, absolute performance during the final five days of acquisition was similar between WT and *Fmr1* KO mice (Figure 3.3C; Student's t-test, p = .906, Figure 3.3D; Student's t-test on "ACQ", p=.456). Subsequently, mice received extinction training, during which no reward was delivered following a response in the illuminated or non-illuminated aperture. Surprisingly, we found that *Fmr1* KO mice displayed exaggerated extinction learning (Figure 3.3D; repeated measures two-way ANOVA, main effect of genotype, $F_{1,30}$ = 12.679, p = .001; genotype x day interaction, $F4,120$ = 8.080, p = .001; post-hoc tests: D1, p = .001, D2, p = .034, D3, p = .023, D4, p = .020, D5, p = .625). We normalized correct responses during extinction by animal to the last five days of acquisition to account for individual differences in basal response rate and found similar results (Figure 3.3E; repeated measures two-way ANOVA, main effect of genotype, $F_{1,30}$ = 7.906, p = .009; genotype/day interaction, $F4,120$ = 5.982, p = .004; post-hoc tests: D1, p = .005, D2, p = .071, D3, p = .093, D4, p = .053, D5, p = .188). The number of incorrect responses also decreased over days independent of genotype (Figures 3.3F-G, repeated measures two-way ANOVAs, main effect of day, $F_{4,120}$ = 9.822, p<.001 for raw data; $F_{4,120}$ = 5.513, p<.001 for normalized data). However, there was no effect of genotype ($F_{1,30}$ = 1.815, p = .188 raw and $F_{1,30}$ < 1 normalized) and no interaction between genotype and day ($F_{4,120}$ = 1.551, p = .204 raw and $F_{4,120}$ = 1.277, p = .150 normalized).

3.3.4: Inhibition of mGluR5 and ERK signaling pathways is insufficient to correct exaggerated instrumental extinction in Fmr1 KO mice

We next tested whether 50% genetic reduction of mGluR5 or chronic inhibition of ERK1/2 with lovastatin would correct exaggerated instrumental extinction in *Fmr1* KO mice. There was no effect of *Fmr1* genotype on acquisition in either the *Fmr1/Grm5* cross (Figure 3.4A; two-way ANOVA, main effect of *Fmr1* genotype, $F_{1,53} = 3.248$, p = .077 and Figure 3.4B; two-way ANOVA, main effect of *Fmr1* genotype, $F_{1,53} < 1$) or the *Fmr1* ± lovastatin groups (Figure 3.4C; two-way ANOVA, main effect of *Fmr1* genotype, $F_{1,37} = 1.029$, p = .317 and Figure 3.4D; two-way ANOVA, main effect of *Fmr1* genotype, $F_{1,37} < 1$). Chronic lovastatin treatment did improve acquisition, independent of genotype (Figure 3.4C; two-way ANOVA, main effect of drug, $F_{1,37} = 10.065$, p = .003, but no genotype x drug interaction, $F_{1,37} = 2.298$, p = .138).

During extinction training, *Fmr1/Grm5* cross cohorts (n = 14-15) showed a significant reduction in responding at the correct aperture independent of genotype (Figure 3.4E; repeated measures three-way ANOVA: main effect of day, $F_{2,100} = 108.429$, p<.001), but there was no main effect of either *Fmr1* or *Grm5* genotype ($F_{1,50} < 1$), no *Fmr1/Grm5* interaction ($F_{1,10} = 3.119$, p = .084), and no *Fmr1/Grm5/*day interaction ($F_{2,100} < 1$). Interestingly, there was a significant effect of *Fmr1* genotype on responding at the incorrect aperture (Figure 3.4F; repeated measures three-way ANOVA, main effect of *Fmr1* genotype, $F_{1,50} = 7.440$, p = .009), but no correction by *Grm5* cross (*Fmr1* x *Grm5* interaction, $F_{1,50} < 1$, *Fmr1* x *Grm5* x day interaction, $F_{1,50} < 1$). There was no difference in basal response rates during acquisition between genotypes (two-way ANOVA: main effect of *Fmr1* genotype, $F_{1,57} < 1$; main effect of *Grm5* genotype, $F_{1,57} < 1$; *Fmr1* x *Grm5* interaction, $F_{1,57} < 1$).

During extinction training, lovastatin or vehicle-treated cohorts (n = 9-12), showed a significant reduction in responding at the correct aperture independent of drug treatment, and this was significantly exaggerated in *Fmr1* KO mice (Figure 3.4G; repeated measures three-way ANOVA, main effect of *Fmr1* genotype, $F_{1,37} = 10.175$, p = .003). Chronic lovastatin treatment does not correct exaggerated extinction (main effect of drug, $F_{1,37} < 1$; genotype x drug interaction, $F_{1,37} < 1$; no significant drug x day

or genotype x drug x day interactions). No significant effect of *Fmr1* genotype on responding at the incorrect aperture was observed (Figure 3.4D; three-way repeated measures ANOVA, main effect of *Fmr1* genotype, $F_{1,37} < 1$). However, lovastatin did alter responding at the incorrect aperture, independent of genotype ($F_{1,37} = 5.885$, p = .020). There was no significant genotype x treatment, genotype x day, treatment x day, or genotype x treatment x day interaction. There was no difference in basal response rates during acquisition between genotypes (two-way ANOVA: main effect of *Fmr1* genotype, $F_{1,38} = 3.484$, p = .070; main effect of *Gmr5* genotype, $F_{1,38} < 1$; *Fmr1* x *Grm5* interaction, $F_{1,38} < 1$).

3.4: Discussion

Development of reliable and robust assays to model high-level cognitive deficits in FX and other disorders would provide a valuable system in which to test the efficacy of pharmacological treatments in mice. Here, we describe a novel form of instrumental extinction learning that is altered in *Fmr1* KO mice in parallel with visuospatial discrimination (Krueger *et al.*, 2011).

3.4.1: Fmr1 KO mice display mirror symmetric alterations in visuospatial discrimination and instrumental extinction

We were intrigued to find that *Fmr1* KO mice display exaggerated instrumental extinction (Figure 3.3), while at the same time showing impaired visuospatial discrimination. A key element of impaired visuospatial discrimination in *Fmr1* KO mice is that it is accompanied by perseverative behavior and a lack of cognitive flexibility (Kramvis *et al.*, 2013, Krueger *et al.*, 2011). Cognitive inflexibility - the inability to modify behavior with changing rules - has been reported in many human neuropsychiatric disorders and mouse models and involves dopaminergic signaling in both PFC and striatum (Klanker *et al.*, 2013). Extinction of a learned instrumental behavior is different from switching rules (i.e. between acquisition phase 1 and phase 2), and thus it is not entirely surprising that extinction is exaggerated while visuospatial discrimination is impaired.

Exaggerated extinction of inhibitory avoidance, a learning test whose acquisition is hippocampally encoded, has been previously reported in *Fmr1* KO mice (Dolen *et al.*, 2007). In PFC, extinction of operant drug-seeking behavior is regulated by mGluR5. Specifically, positive allosteric modulation of mGluR5 results in exaggerated extinction for multiple reinforcers and mGluR5 KO mice have delayed extinction (Chesworth *et al.*, 2013, Cleva *et al.*, 2011, Gass & Olive, 2009, Kufahl *et al.*, 2012). Assuming that food rewards and drug rewards have similar saliency and involve similar circuits, our results would be consistent with enhanced mGlu5 signaling. In *Fmr1* KO mice, levels of mGluR5 protein are normal. However, in the absence of the translational repressor FMRP, protein synthesis downstream of mGluR5 is enhanced in *Fmr1* KO mice. ERK1/2 signaling, linking mGluR5 to protein synthesis, may also be upregulated in *Fmr1* KO mice (Michalon *et al.*, 2012) but see (Osterweil *et al.*, 2010). It is therefore a reasonable hypothesis that upregulation of pathways downstream of mGluR5 may account for exaggerated instrumental extinction in *Fmr1* KO mice. In addressing this hypothesis, it will be critical to understand whether this form of instrumental extinction requires protein synthesis.

3.4.2: Limited effectiveness of Grm5 cross and lovastatin in correcting cognitive phenotypes

Downregulation of mGluR5 and ERK1/2 signaling was not sufficient to correct altered extinction in *Fmr1* KO mice (Figure 3.4). However, a trend towards improvement in visuospatial discrimination was seen in the *Fmr1* KO/*Grm5*^{+/-} cross (Figure 3.1C). Additionally, ERK1/2 inhibition with lovastatin corrected a surprising impairment in operant acquisition (Figure 3.1E). Taking a broader view, the cognitive deficits seen in FX patients are complex, multimodal, and impossible to model by a single mouse behavior. It is likely that mechanisms for learning of visuospatial discrimination and extinction of instrumental responses vary within PFC, and it is therefore not surprising that treatment strategies vary in their effectiveness depending on the cognitive test. Indeed, in designing clinical trials where properly defined

endpoints are crucial, it is imperative to understand which cognitive behaviors may be most amenable to treatment by which drugs.

Based on our results, it is likely that instrumental extinction and visuospatial discrimination are equally robust assays for altered PFC-mediated behavior in *Fmr1* KO mice. In one of our visuospatial discrimination groups (*Grm5* cross), we saw a trend towards previously reported delayed phase 2 acquisition in *Fmr1* KO mice (p=.06). The lack of a statistically significant extinction phenotype in *Fmr1* KO mice in *Fmr1/Grm5* cross cohorts was likely due to a similar slight exaggeration in *Grm5* heterozygotes, not surprising considering known behavioral effects of mGlu5 inhibition in other contexts (Simonyi *et al.*, 2010). Had we not tested the *Grm5* cross concurrently, the difference between *Fmr1* KO and wild-type mice would have been statistically significant, as previously reported (Krueger *et al.*, 2011).

3.4.3: Lovastatin and CTEP affect operant acquisition

In cohorts treated ± lovastatin, we saw no phase 2 (visuospatial discrimination) phenotype but did see a surprising phase 1 (operant acquisition) delay, and correction, which both have not been previously reported (Figure 3.1). Experiments were always done blind to genotype, using littermate controls, and under similar conditions. It is likely that the formulation of vehicle and lovachow subtely altered either metabolism or the saliency of the reward (a different type of food pellet), and therefore motivation, in these cohorts. In addition, we reported that lovastatin improved instrumental acquisition, independent of *Fmr1* genotype, using a two-choice assay (Figure 3.4). Taken together, these results represent unexpected effects of lovastatin on operant acquisition and prompt further study.

In addition, we found that chronic CTEP treatment impairs operant acquisition, measured by phase 1 of the five-choice assay (Figure 3.2). By comparison, genetic mGluR5 knockdown had no effect on operant acquisition in either WT or *Fmr1* KO mice. We hypothesize that these effects of CTEP may be related to the known role of mGluR5 antagonists as anxiolytics (Spooren *et al.*, 2000). Given the frequent testing and handling of subjects, any baseline differences in overall anxiety could be manifested as

either improvements or impairments in phase 1 acquisition. While reduced anxiety would be beneficial for Fragile X patients, it could be a confounding variable in assessing the effects of mGluR5 antagonists on highly sensitive mouse behavior. We await future characterization of CTEP's anxiolytic properties compared to what is known about MPEP and MTEP.

3.4.4: Limits to mouse models in assessing cognitive impairments

Ultimately, developing robust assays for altered cognition is critical in *Fmr1* KO mice since cognitive impairments are at the core of FX. The availability to measure multiple *Fmr1* KO phenotypes under similar conditions will allow for potential drug screens, the results of which may provide insights into the proper endpoints to be considered for clinical trials in FX and other neurodevelopmental disorders associated with intellectual disability. However, there is inherent variability in both experimental results and interpretations of cognitive mouse behavioral assays. The recent development of *Fmr1* knockout rats should help expand the avenues for assessing subtle cognitive impairments. Despite these caveats, we should not understate the importance of measuring prefrontal-mediated cognitive behaviors in rodent models of Fragile X, intellectual disability, and autism. Measures of impaired cognition in humans and by proxy, defined endpoints for clinical studies, have been equally difficult to interpret and agree upon. We hope that isolation and correction of simplified rodent phenotypes will help in defining the most pharmacologically tractable and disease-relevant endpoints.

3.5: Methods

3.5.1: Subjects

Fmr1 KO and *Grm5$^{+/-}$* mice were originally obtained from Jackson Labs and were bred at MIT on a C57BL/6 background. Mice were maintained on a 12:12 h light:dark cycle. Experimental cohorts consisted of male mice that were aged 2-4 months at the time of experiments. All experiments were performed blind to genotype using littermate controls. All procedures were approved by the MIT Committee on Animal Care.

3.5.2: Behavioral apparatus

All experiments were performed using operant conditioning chambers (Med Associates) which had five nose-poke apertures on one wall of the chamber and a pellet dispenser and magazine for delivering food rewards (20 mg dustless precision pellets, BioServ) on the opposite wall. A house light over the food magazine permanently illuminated the chamber. Stimulus lights inside the nosepoke apertures could be controlled individually to provide visual cues during acquisition and extinction, as noted.

3.5.3: Food restriction

In all experiments, daily food restriction began after P60. During food restriction, the body weight of subjects was maintained at 85-90% free-feeding weight by providing unrestricted access to food for 1.5-2 hours per day immediately following daily experimental testing. Mice did not have access to food for the remainder of the day. The duration and time of feeding were kept consistent within each cohort to avoid motivation becoming a confounding variable. In some cases (where noted), experiments were conducted only 5 or 6 days per week. In these cases, ad libitum access to food was allowed on non-experimental days.

3.5.4: Acquisition of a five-choice visuospatial discrimination

Our five-choice visuospatial discrimination task was based on Krueger *et al.*, *PNAS*, 2011. All training sessions lasted 15 minutes. **Magazine training:** Subjects

were placed in the behavioral apparatus and each entry into the food magazine was reinforced by one food pellet on a fixed-time 10-s schedule. Subjects received two consecutive days of magazine training. **Phase 1:** During phase 1 of acquisition, all five nosepoke apertures were illuminated and active. To receive a reward, the subject had to respond in any illuminated aperture ("correct" response) and then return to the magazine for reward. Rewards were delivered on a fixed FR1 schedule. Illumination of apertures temporarily ceased during reward delivery until the reward was retrieved. On the first two days of phase 1, all mice were "primed" to respond in the apertures by placing a food pellet in each of the five apertures before the beginning of the session. In addition, any mouse recording less than five correct responses for two days in a row would be primed on the following day. To reach criterion and advance to the next training phase, mice were required to complete 15 trials within the 15 minute session on two consecutive days. **Phase 2:** During phase 2, only one aperture was illuminated at random on each trial. To receive a reward, the subject had to respond in the illuminated aperture ("correct" response) and then return to the magazine. "Incorrect" responses were defined as any response into a non-illuminated aperture and had no programmed consequence. To reach criterion and complete the task, mice had to complete 15 or more trials and achieve a 50% or greater success rate (defined as correct responses / [correct responses + incorrect responses]) on two consecutive days. Mice were excluded if they failed to reach criterion on phase 2 within 20 days. Experiments were conducted 6-7 days per week. All procedures were consistent with Krueger *et al.*, *PNAS*, 2011.

3.5.5: Acquisition and extinction of an instrumental response.

Three of the five nosepoke apertures (the outer two apertures and the center aperture) were closed using metal plugs (Med Associates), leaving only two open. **Magazine training:** Two days of magazine training occurred as described above. **Acquisition:** One of the two apertures was illuminated, and food rewards were delivered via the magazine following a correct response. Illumination of apertures temporarily ceased during reward delivery. The same aperture was illuminated on each

trial within a session, and between sessions over days. Half of subjects had the left aperture illuminated and half had the right aperture illuminated. To encourage responding, rewards were delivered on a fixed FR1 schedule for the first 10 rewards, then on a variable VR2 schedule (randomly requiring either 1, 2, or 3 responses) for the following rewards. To reach criterion and advance to extinction training, mice were required to complete 15 trials with a greater than 75% success rate on five consecutive days. **Extinction:** For 3-5 consecutive days following acquisition, extinction sessions were conducted in which no rewards were delivered following a response in either aperture. **Groups:** *Fmr1* KO/WT cohorts were trained 7 days per week. *Fmr1* KO/*Grm5*$^{+/-}$ and *Fmr1* KO ± lovastatin cohorts were trained 5 days per week. Extinction training was always conducted on three consecutive days following at least two consecutive days of acquisition. Therefore subjects in *Fmr1* KO/*Grm5*$^{+/-}$ and *Fmr1* KO ± lovastatin cohorts were trained to criterion for a range of 5-9 days to enable three consecutive extinction days following the final two acquisition days.

3.5.6: Lovastatin and CTEP treatment

Lovastatin or vehicle was formulated in the food at a dose of 10 mg/kg/day, as published (Osterweil *et al.*, 2013, Yamada *et al.*, 2000). "Lovachow" was administered for one month prior to the beginning of experiments, from P30-P60. During experiments, lovachow was used for the 1.5-2 hour unrestricted feeding. Lovastatin or vehicle were not present in reward pellets. CTEP or vehicle was applied chronically, every 48 h, for 4+ weeks from P30 through the duration of experiments. For more detail, see section 2.5.2.

3.5.7: Statistics

Where noted, extinction data (plotted as correct responses or incorrect responses) was normalized within animal to the last five days of acquisition averaged together. Two-way ANOVA was used to analyze five-choice and two-choice acquisition data where a 2x2 experimental design was used (*Fmr1* genotype x *Grm5* genotype and *Fmr1* genotype x lovastatin treatment). Student's t-test was used to compare two-

choice acquisition where two experimental groups were used (*Fmr1* KO and WT). To analyze extinction data, either a two-factor or three-factor repeated measures ANOVA was used. The repeated measure was days of extinction, one factor was *Fmr1* genotype, and where applicable, the other factor was either *Grm5* genotype or lovastatin treatment. Student's t-test or two-way ANOVA was used to compare raw baseline responding between groups. Post-hoc Bonferroni tests were used. Outliers greater than 2 standard deviations from the mean were excluded. N represents number of animals and error bars are ± SEM.
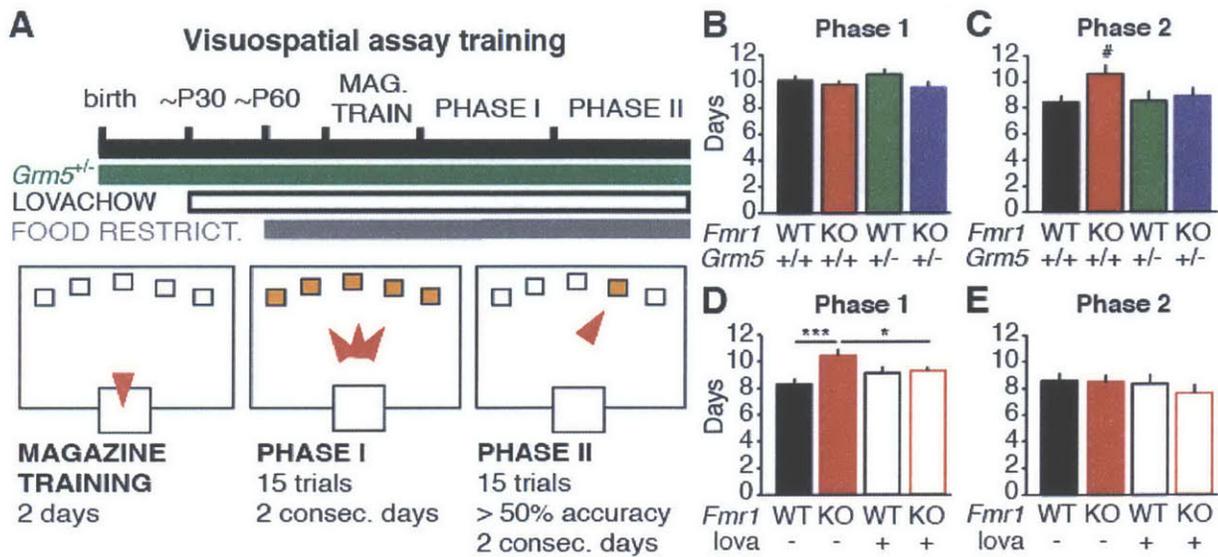
Figure 3.1: Pharmacological correction of delayed operant acquisition in Fmr1 KO mice.

(A) Schematic showing timing of treatment strategies relative to food restriction and two phases of training on a five-choice visuospatial discrimination task. **(B)** Phase 1 acquisition is normal in *Fmr1/Grm5* cross. **(C)** There is a trend towards delayed acquisition of phase 2 as a function of *Fmr1* genotype (p=.06, n=32-45). There is not a statistically significant interaction between *Fmr1* and *Grm5* genotypes (WT/*Grm5*$^{+/+}$: 8.4 +/- 0.5 days to acquisition, KO/*Grm5*$^{+/+}$: 10.6 +/- 0.7 days, KO/*Grm5*$^{+/-}$: 8.9 +/- 0.7 days). **(D)** In cohorts treated chronically with lovachow or similarly formulated vehicle chow, *Fmr1* KO mice show impaired phase 1 acquisition (WT/vehicle: 8.3 +/- 0.4 days, KO/vehicle: 10.4 +/- 0.5 days, ***p<.001), which is corrected by lovastatin treatment (KO/lova: 9.3 +/- 0.3 days, *p<.05). **(E)** Lovachow/vehicle cohorts show no effect of genotype or drug treatment on phase 2 acquisition.
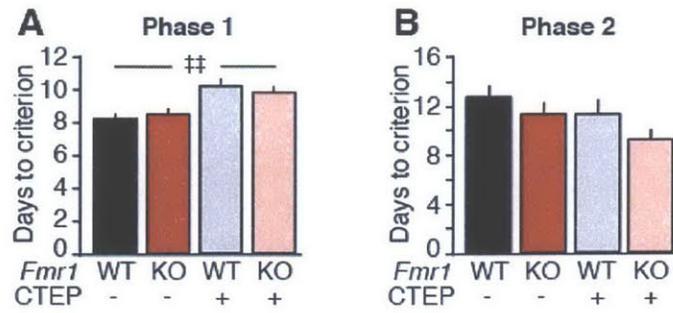
Figure 3.2: Chronic CTEP impairs operant acquisition.

(A) Chronic CTEP treatment impairs phase 1 operant acquisition, independent of genotype (‡‡p < .001; WT/vehicle: 8.3 +/- 0.3 days to acquisition, WT/CTEP: 10.2 +/- 0.5 days, KO/vehicle: 8.5 +/- 0.4 days, KO/CTEP: 9.9 +/- 0.4 days), but has no effect on **(B)** phase 2 visuospatial discrimination (WT/vehicle: 12.7 +/- 1.0 days, WT/CTEP: 11.3 +/- 1.2 days, KO/vehicle: 11.3 +/- 0.9 days, KO/CTEP: 9.3 +/- 0.9 days; n = 24 – 32).
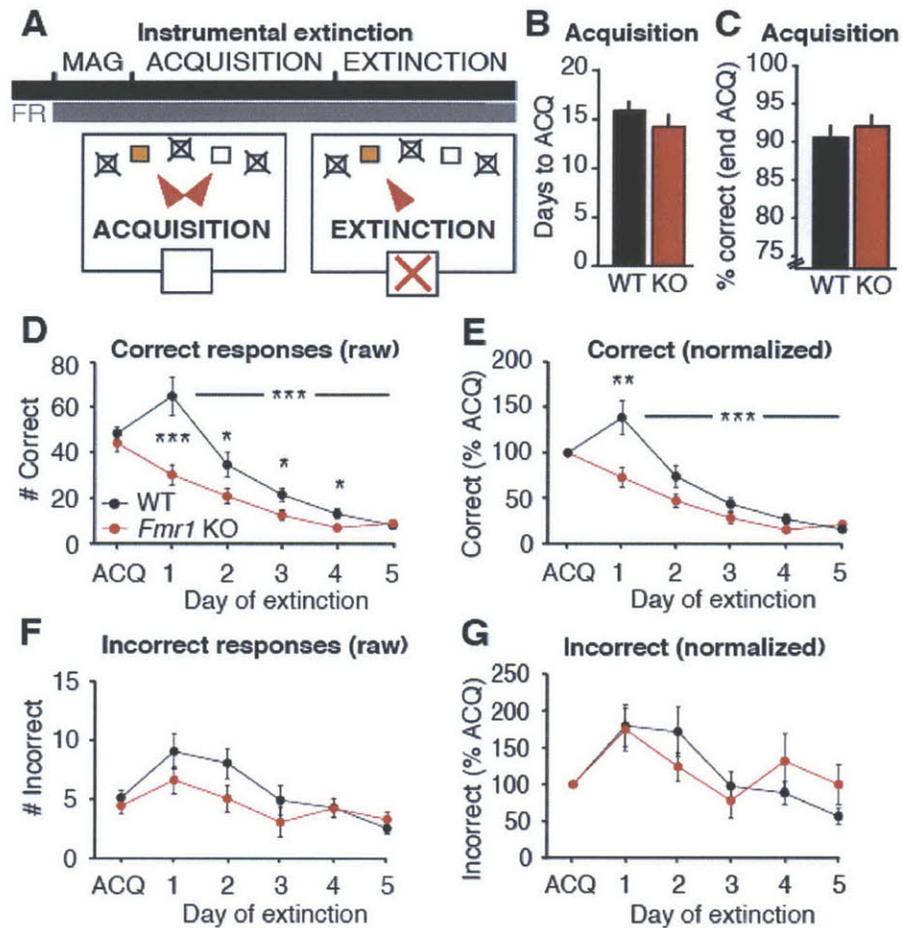
Figure 3.3: Instrumental extinction is exaggerated in Fmr1 KO mice.

(A) Schematic shows a test with one acquisition phase, followed by five days of extinction. **(B)** Days to acquisition and **(C)** performance during acquisition are normal in *Fmr1* KO mice. **(D)** Extinction of correct responding is significantly exaggerated in *Fmr1* KO mice (***p=.001, repeated measures two-way ANOVA), and post-hoc tests show significant exaggeration at days 1-4 (*p<.05). **(E)** Normalization reveals significantly exaggerated extinction in *Fmr1* KO mice (***p=.001), and post-hoc tests show a significant exaggeration at day 1 (**p<.01). **(F-G)** There is no effect of *Fmr1* genotype on incorrect responses.
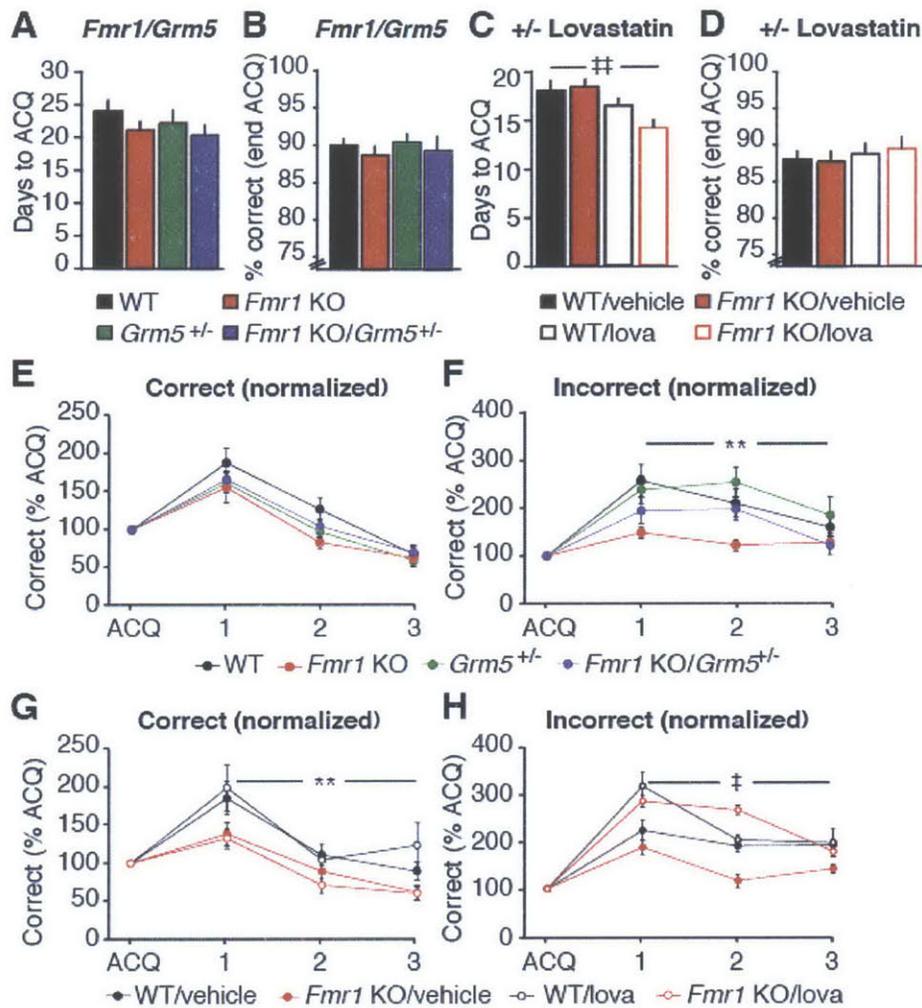
Figure 3.4: Inhibition of mGluR5 and ERK signaling pathways is insufficient to correct exaggerated instrumental extinction in *Fmr1* KO mice.

(A) Days to acquisition and **(B)** performance during acquisition are normal in *Fmr1* KO mice in *Fmr1/Grm5* cross groups. **(C)** In lovastatin/vehicle-treated groups, days to acquisition is normal in *Fmr1* KO mice, but there is a significant improvement in performance with lovastatin treatment, independent of genotype (‡‡$p<.01$). **(D)** In lovastatin/vehicle treated-groups, performance during acquisition is normal in *Fmr1* KO mice. **(E)** In *Fmr1/Grm5* cross cohorts, there is no significant effect of *Fmr1* genotype on extinction, and no *Fmr1/Grm5* interaction. **(F)** Effect of Fmr1 genotype on incorrect responding (**$p<.01$). **(G)** Extinction of an instrumental response is significant exaggerated in *Fmr1* KO mice independent of lovachow treatment (**$p<.01$). **(H)** Effect of lovachow treatment on incorrect responding (‡$p<.05$). There is no effect of *Fmr1* genotype and no genotype x drug interaction.

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Chapter 4

mGluR5 regulates NMDAR-dependent forms of synaptic plasticity in mouse visual cortex

4.1: Abstract

Long-term depression (LTD) of synaptic transmission occurs through multiple mechanisms in mouse visual cortex, which vary depending on the induction protocol and layer of cortex. Generally, LTD that is induced by low frequency stimulation (LFS) requires N-methyl-D-aspartic acid receptor (NMDAR) activation but not group 1 metabotropic glutamate receptors (mGluRs), and LTD that is induced by group 1 mGluR activation does not require NMDA receptors. Following a report that ocular dominance plasticity is deficient in mGluR5 knockdown mice (Dolen et al., 2007), we tested whether visual cortical LTD was also altered in these animals. We report that LFS-induced, NMDAR-dependent LTD is reduced specifically in layer IV of visual cortex in mGluR5 knockout mice, as well as in wild-type mice treated chronically with an mGluR5 antagonist. However, LTD induction is normal in the presence of acute mGluR5 antagonism in wild-type mice, suggesting an important difference between acute and chronic mGluR5 function. In vivo, monocular deprivation results in experience-driven weakening of synaptic strength, which occurs through similar mechanisms as LTD in vitro. We report that this ocular dominance plasticity is impaired following chronic mGluR5 inhibition. This study shows that specifically in layer IV, chronic but not acute downregulation of mGluR5 signaling has important consequences for forms of NMDAR-dependent plasticity in vitro and in vivo.

4.2: Introduction

4.2.1: NMDAR-LTD vs. mGluR-LTD

Long-term depression (LTD) is a process by which certain patterns of electrical or chemical stimulation can reduce the efficacy of synaptic transmission over a period of hours or more (Dudek & Bear, 1992, Kemp & Bashir, 1999, Palmer et al., 1997). Induction and expression mechanisms of LTD in mice have been well-studied and depend on age, region of the brain, and induction protocol used (Jiang et al., 2007, Nosyreva & Huber, 2005).

Two well-described methods of LTD induction involve activation of NMDA receptors or group 1 mGluRs, which include mGluR1 and mGluR5 (Figure 4.1). Low-frequency stimulation (LFS) of input fibers at 1 Hz for 15 minutes induces NMDAR-dependent LTD in the hippocampus and visual cortex (Dudek & Bear, 1992, Kirkwood & Bear, 1994). Bath application of the group 1 mGluR agonist DHPG or a paired-pulse LFS induction protocol leads to mGluR-dependent LTD in the hippocampus (Kemp & Bashir, 1999, Palmer et al., 1997). Expression of NMDAR-LTD and mGluR-LTD are similar: both forms result in AMPAR internalization (Carroll et al., 1999, Luscher et al., 1999, Man et al., 2000, Snyder et al., 2001, Xiao et al., 2001). However, their induction mechanisms vary: mainly, mGluR-LTD requires protein synthesis whereas NMDAR-LTD does not (Huber et al., 2000). Importantly, NMDAR-LTD induction persists with inhibition of group 1 mGluRs (Sawtell et al., 1999), and mGluR-LTD induction persists in the presence of NMDA receptor inhibition (Huber et al., 2001). These forms of synaptic weakening are thought to be exclusive and do not occlude each other (Huber et al., 2001, Oliet et al., 1997). Here, we report a form of LTD in layer IV of mouse visual cortex that is induced by 1 Hz LFS and is NMDAR-dependent, but is absent or reduced in mGluR5 knockdown mice ($Grm5^{-/-}$ and $Grm5^{+/-}$) and following chronic mGluR5 inhibition with CTEP.

4.2.2: Layer-specificity of LTD induction mechanisms in visual cortex

Within visual cortex, LTD induction and expression mechanisms vary by cortical layer (Crozier *et al.*, 2007, Daw *et al.*, 2004, Jiang *et al.*, 2007). In visual cortex, two LTD paradigms have been well-studied: stimulation of layer IV and recording in layer II/III ("layer II/III LTD") and stimulation of white matter and recording in layer IV ("layer IV LTD"). Induction of both layer II/III and layer IV LTD by low-frequency stimulation requires NMDA receptors (Crozier *et al.*, 2007). Layer IV LTD, like hippocampal NMDAR-LTD, requires internalization of AMPA receptors. Postsynaptic loading of a peptide (G2CT) that blocks AMPAR internalization abolishes LTD in layer IV, but not layer II/III (Crozier *et al.*, 2007, Yoon *et al.*, 2009). In contrast, LTD in layer II/III requires activation of endocannabinoid receptor CB1 and may be expressed presynaptically (Crozier *et al.*, 2007).

4.2.3: Ocular dominance plasticity occurs through LTD-like mechanisms

Ocular dominance plasticity is a form of experience-dependent plasticity where mice deprived of input to one eye for a period of days show altered responses to visual stimuli once vision is restored (Gordon & Stryker, 1996). Following three days of monocular deprivation (MD), responses evoked by presentation of a stimulus to the deprived eye decrease (Frenkel & Bear, 2004), and this deprived-eye depression is thought to occur through LTD-like mechanisms in mouse visual cortex (Heynen *et al.*, 2003, Yoon *et al.*, 2009). Specifically, ocular dominance plasticity is NMDAR-dependent (Bear *et al.*, 1990, Sawtell *et al.*, 2003), involves similar AMPA receptor phosphorylation and internalization as in LTD (Heynen *et al.*, 2003, Yoon *et al.*, 2009), and prior monocular deprivation occludes further LTD induction (Heynen *et al.*, 2003).

During MD experiments, visually evoked potentials (VEPs) are typically measured by implanting a recording electrode to depths of ~450 μm below the cortical surface, which corresponds to thalamorecipient layer IV. Thus reported similarities between deprived-eye depression and layer IV LTD are likely specific to layer IV. Indeed, viral administration of the G2CT peptide abolishes both deprived-eye depression and LFS-LTD in layer IV (Yoon *et al.*, 2009). However, the cannabinoid

receptor CB1 antagonist AM251 blocks both LTD and OD plasticity in layer II/III but not layer IV (Crozier *et al.*, 2007, Liu *et al.*, 2008). Therefore there is good evidence that LTD and deprived-eye depression in layer IV share mechanisms and are distinct from parallel mechanisms for depression in layer II/III.

Mice with 50% of normal mGluR5 expression ($Grm5^{+/-}$) have impaired deprived-eye depression (Dolen *et al.*, 2007) (Figure 4.2). We tested whether there is a corresponding deficit in visual cortical LTD in these animals. We report a deficit in LFS-induced LTD in layer IV of $Grm5^{-/-}$ and $Grm5^{+/-}$ mice that may correspond to the ocular dominance plasticity deficits reported by Dolen and colleagues (*Neuron*, 2007). This LTD deficit is seen in $Grm5^{-/-}$ and $Grm5^{+/-}$ slices as well as wild-type slices treated chronically for one week with an mGluR5 antagonist. However, normal LTD persists in wild-type slices treated acutely with an mGluR5 antagonist, suggesting that acute mGluR5 signaling is not necessary for the induction and expression of NMDAR-dependent LTD. Rather, the mGluR5 protein or its downstream signal is needed chronically or during development for the normal induction of layer IV NMDAR-dependent LTD in visual cortex. In parallel, we report impaired deprived-eye depression following chronic mGluR5 antagonism. Together, these findings provide surprising evidence that chronic or developmental mGluR5 signaling is required for induction of NMDA receptor-dependent forms of plasticity in vitro and in vivo.

4.3: Results

4.3.1: LFS-induced LTD in layer IV of visual cortex is absent with genetic mGluR5 knockdown

We electrically stimulated white matter of visual cortical slices with a standard 1 Hz, 900 pulse LFS protocol and recorded extracellular field potentials from thalamorecipient layer IV in P21-P30 mice. We found that LTD was deficient in $Grm5^{-/-}$ and $Grm5^{+/-}$ slices compared to wild-type littermate controls (Figure 4.3A; one-way ANOVA: p = .012; post-hoc tests: WT vs. $Grm5^{-/-}$, p < .05; WT vs. $Grm5^{+/-}$, p < .05). There was not a statistically significant different between LTD magnitude in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice (p = .45). The deficit in LFS-LTD was restricted to layer IV. LTD induced by stimulation of layer IV and recording in layer II/III was not different in $Grm5^{-/-}$ or $Grm5^{+/-}$ slices as compared to WT slices (Figure 4.3B; one-way ANOVA: p = .64). This is in agreement with previous reports of normal layer II/III LFS-LTD in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice (Sawtell *et al.*, 1999).

4.3.2: LFS-induced LTD in layer IV of wild-type mice has characteristics of canonical NMDAR-dependent LTD

The deficit in layer IV LTD seen in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice was surprising and noteworthy as LFS, not DHPG treatment, was used for induction. We wanted to test whether this LTD is indeed canonical NMDAR-dependent LTD and not mGluR-LTD. We asked whether LFS-induced LTD in layer IV requires NMDAR activation by bath-applying 50 μM D-2-amino-5-phosphonopentanoic acid (APV), an NMDAR antagonist, to wild-type slices. Further, we tested whether it is protein synthesis-dependent by bath-applying 60 μM cycloheximide, an inhibitor of translation. Layer IV LFS-LTD is blocked in wild-type slices by APV but not cycloheximide (Figure 4.4, p<.05), confirming that its induction mechanisms are consistent with canonical NMDAR-dependent LTD.

4.3.3: Acute mGluR antagonism does not alter LFS-induced LTD in layer IV

Previous reports suggest that in visual cortex layer II/III, NMDA receptor-dependent LTD persists in the presence of group 1 mGluR antagonists (Sawtell *et al.*, 1999). However, this has not been shown specifically in layer IV. We asked whether acute inhibition of mGluR5 with the antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) in WT mice would phenocopy the LTD deficit seen in layer IV of $Grm5^{-/-}$ and $Grm5^{+/-}$ mice. Bath application of 10 µM MPEP had no effect on LFS-induced LTD in layer IV of wild-type mice (Figure 4.5A). In hippocampus, mGluR-LTD induced by DHPG requires both mGluR5 and mGluR1 activation (Volk *et al.*, 2006), so we tested whether inhibition of both mGluR5 with MPEP and mGluR1 with 100 µM LY367385 would alter the expression of LFS-induced LTD in layer IV. We found that normal LFS-LTD was induced in the presence of acute mGluR1 and mGluR5 inhibition (Figure 4.5A). This shows that while chronic mGluR5 expression is necessary for LFS-induced LTD in layer IV, acute activation of mGluR5 is not.

4.3.4: Chronic mGluR antagonism in WT mice reduces layer IV LTD

The finding that genetic knockdown of mGluR5 blocks NMDAR-LTD in layer IV but acute mGluR5 inhibition does not led to two potential hypotheses of how mGluR5 regulates NMDAR-LTD: (1) physical tethering of the mGluR5 protein to synaptic scaffolds is required for LTD induction or (2) mGluR5-mediated signaling during a sensitive developmental window is required for later LTD. The intracellular C-tail of mGluR5 binds to the synaptic protein Homer (Xiao *et al.*, 1998), and this interaction is required for induction of mGluR-LTD (Ronesi *et al.*, 2012). Homer binds to Shank, which associates with PSD-95 as part of a larger synaptic scaffold (Tu *et al.*, 1999). Thus mGluR5 is structurally linked to NMDA receptors through Homer, Shank, and PSD-95, and disruption of this physical interaction is a reasonable hypothesis for impaired LFS-LTD in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice. Indeed, proper PSD-95 function is critical for both LTP and LTD (Migaud *et al.*, 1998, Xu, 2011, Xu *et al.*, 2008). To assess whether genetic knockdown of mGluR5 impairs NMDAR-LTD via destruction of structural scaffolds at the synapse, we inhibited mGluR5 chronically for 7-11 days from

P14 until slice recording at P21-P25 (Figure 4.5B). This was accomplished with s.c. injections of CTEP (2 mg/kg), a highly specific, long-lasting mGluR5 antagonist. This dosing schedule achieves chronic uninterrupted inhibition of mGluR5 signaling by ~75% (Lindemann *et al.*, 2011). If inhibition of mGluR5 signaling is not sufficient to phenocopy NMDAR-LTD deficits, it would suggest that the physical association of mGluR5 with synaptic scaffolds may be required for normal LTD induction. Chronic administration of CTEP significantly reduced the magnitude of LTD in layer IV of visual cortex in wild-type mice (Figure 4.5C; student's t-test, *p < .05). Acute CTEP, similar to MPEP, had no effect on ex vivo LTD magnitude (Figures 4.5B,D). Thus chronic mGluR5-mediated signaling during a developmental window, rather than physical presence of the receptor, is required for expression of NMDAR-LTD.

4.3.5: Chronic mGluR5 antagonism in WT mice impairs ocular dominance plasticity

Following three days of monocular deprivation, wild-type mice display decreased VEP magnitude in the hemisphere contralateral to eye closure (Frenkel & Bear, 2004). $Grm5^{+/-}$ mice have impaired deprived-eye depression (Dolen *et al.*, 2007). We tested whether chronic mGluR5 antagonism with CTEP would be sufficient to impair OD plasticity in wild-type mice in parallel with its impairment of NMDAR-LTD in vitro (Figure 4.5). Mice were administered CTEP chronically, beginning at P21 and throughout the duration of monocular deprivation, which began at P28 and lasted for three days (Figure 4.6A). There was a significant effect of CTEP on the magnitude of deprived-eye (contralateral) depression (two-way repeated measures ANOVA, MD x treatment interaction, p = .0195) and on the magnitude of the contra/ipsi ratio shift, (p = .0143, n = 9-14), compared to vehicle treatment (Figures 4.6B-D).

Acute CTEP treatment in wild-type mice may transiently increase the magnitude of both monocular and binocular VEPs for roughly 24-48 hours following injection (Figure 4.7; two-way repeated measures ANOVAs, day x treatment interaction, p<.001 for binocular VEPs, p<.001 for contralateral VEPs, and p<.001 for ipsilateral VEPs). The ratio between contralateral and ipsilateral VEPs is preserved following CTEP

treatment. However, baseline (pre-MD) contralateral and ipsilateral VEP magnitude was comparable between chronic vehicle and CTEP-treated animals (Figure 4.6B-C). Therefore acute CTEP effects on baseline VEP magnitude were likely not relevant to our MD experiments.

4.3.6: Grm5$^{-/-}$ but not Grm5$^{+/-}$ mice display impaired stimulus-specific response potentiation

We wanted to assess whether the requirement for chronic mGluR5 signaling is limited to induction of synaptic depression in vitro and deprived-eye depression in vivo, or is generalized to other forms of NMDAR-dependent plasticity. Due to the extremely limited window for LTP induction and its limited magnitude in layer IV of visual cortex (Jiang *et al.*, 2007), we began by testing whether stimulus-specific response potentiation (SRP) is impaired in vivo in Grm5$^{+/-}$ and Grm5$^{-/-}$ mice. SRP is an experience-dependent form of synaptic strengthening in visual cortex which is NMDAR-dependent and occurs through LTP-like mechanisms (Cooke & Bear, 2010, Cooke & Bear, 2012, Frenkel *et al.*, 2006). During SRP, repeated exposure to a visual stimulus (Figure 4.8A) potentiates VEPs evoked by this familiar stimulus but not by a novel stimulus. SRP can be measured and compared between genotypes in two ways: (1) by assessing growth of VEP magnitude over six of familiar stimulus presentation and (2) by assessing the ability to distinguish novel from familiar stimuli on test day 6. In both measures, Grm5$^{-/-}$ mice show impaired SRP compared to WT and Grm5$^{+/-}$ mice, and Grm5$^{+/-}$ mice express normal SRP (Figure 4.8B-D).

There is a significant effect of Grm5 genotype on SRP expression, measured by growth of VEP magnitude over days (Figure 4.8B-C; two-way repeated measures ANOVA, genotype x day interaction, $p = .011$). There is also a significant effect of Grm5 genotype on the ability to distinguish between familiar and novel stimulus on day 6 of testing (Figure 4.8D; one-way ANOVA, $p = .001$). WT mice, Grm5$^{+/-}$ mice, and Grm5$^{-/-}$ mice can all distinguish novel from familiar stimuli on day 6 (paired t-tests: $p<.001$ for WT and Grm5$^{+/-}$, $p=.042$ for Grm5$^{-/-}$). However, the ability to distinguish familiar from novel is significantly impaired between Grm5$^{-/-}$ mice and both WT ($p = .005$) and Grm5$^{+/-}$

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mice (p = .001) (Familiar-to-novel ratios on test day 6: WT: 2.67 +/- 0.22, $Grm5^{+/-}$: 3.11

+/- 0.46, $Grm5^{-/-}$: 1.33 +/- 0.13). There is no significant difference between WT and

$Grm5^{+/-}$ mice (p = .864). Baseline day 1 VEP magnitude is increased in $Grm5^{-/-}$ mice

(Figure 4.8B; p<.05) but animal by animal assessment suggests that SRP impairment in

$Grm5^{-/-}$ mice regardless of day 1 VEP magnitude (Figure 4.8E). Additionally,

normalization of VEPs to day 1 within animal reveals similar impairments in SRP in

$Grm5^{-/-}$ mice (data not shown). In sum, SRP is impaired in $Grm5^{-/-}$ but not $Grm5^{+/-}$ mice,

measured both by the ability to distinguish familiar from novel stimulus on day 6, and by

growth of VEPs from days 1 to 6.

4.3.7: Chronic CTEP does not affect stimulus-specific response potentiation

Chronic CTEP treatment resulted in parallel LTD deficits in vitro and impaired in

deprived-eye depression in vivo (Figures 4.5-4.6). The finding that $Grm5^{-/-}$ but not

$Grm5^{+/-}$ mice show deficient SRP prompted study of CTEP's effects on SRP induction in

wild-type mice. Mice were treated chronically every 48 h with CTEP or vehicle,

beginning at P21 and continuing throughout the duration of six-day SRP from P30-P35

(Figure 4.9A). There was no difference in SRP magnitude between vehicle and CTEP-

treated mice (Figures 4.9B-D; repeated measures two-way ANOVA, treatment x day

interaction, p = .329) and no difference in the ability to discriminate novel from familiar

stimulus on test day (Student's t-test, p = .57). Both vehicle and CTEP-treated groups

had significant SRP and were able to discriminate novel from familiar stimuli on test day

(p<.001). Thus normal SRP persists in both CTEP-treated WT mice and $Grm5^{+/-}$ mice.

4.3.8: Visual acuity and contrast sensitivity are normal in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice

Impaired SRP and previously reported deficient deprived-eye depression in

$Grm5^{+/-}$ mice (Dolen et al., 2007) prompted study of the effects of mGluR5 knockdown

on baseline vision. Following SRP, novel stimuli were used to assess acuity and

contrast sensitivity across Grm5 genotypes (Figure 4.8A). There was no effect of

mGluR5 knockdown on VEP magnitude across a range of spatial frequencies (Figures

4.10A-B) and contrasts (Figures 4.10C-D; repeated measures two-way ANOVA). The

enhancement in baseline VEP magnitude seen on day 1 of SRP experiments in $Grm5^{-/-}$ mice is not seen in either acuity or contrast sessions at 0.05 cycles per degree and 100% contrast.

4.3.9: Pairing-induced LFS-LTD is impaired in $Grm5^{-/-}$ mice

To investigate the mechanism by which chronic mGluR5 signaling regulates NMDAR-dependent plasticity, it is critical to use a preparation in which phenotypes can be assessed at the single-cell level. Therefore we isolated and recorded from layer IV neurons, and first tested whether LFS-LTD is impaired in mGluR5 knockdown mice using this preparation. Voltage-clamp of single layer IV neurons allowed for induction of LFS-LTD with a pairing protocol. White matter stimulation was paired with depolarization of isolated neurons from -70 mV to -45 mV at 1Hz for 5 minutes. This protocol is standard to induce NMDAR-dependent LTD in visual cortex (Crozier *et al.*, 2007). There is a significant effect of mGluR5 knockdown on paired LFS-LTD induction (Figure 4.11; p = .04, one-way ANOVA). LTD is significantly impaired in $Grm5^{-/-}$ mice compared to WT mice and compared to $Grm5^{+/-}$ mice (p<.05, post-hoc tests) but not in $Grm5^{+/-}$ mice compared to wild-type. Persistence of deficient LTD at the single-cell level in $Grm5^{-/-}$ mice prompted further study of the mechanisms involved using similar recording techniques.

4.3.10: Gross NMDA receptor function is normal in $Grm5^{+/-}$ and $Grm5^{-/-}$ mice

$Grm5^{-/-}$ or $Grm5^{+/-}$ mice show deficits in LFS-LTD, deprived-eye depression, and SRP, all NMDAR-dependent forms of plasticity. To begin to understand the mechanism by which chronic mGluR5 signaling is required for NMDAR-dependent plasticity, we asked whether NMDA receptors are functionally impaired in $Grm5^{-/-}$ or $Grm5^{+/-}$ mice. First, we confirmed that basal synaptic transmission, driven mainly by AMPA receptor-mediated currents, is normal in both genotypes, as measured by input/output functions obtained prior to LTD recordings (Figures 4.12A-B; two-way repeated measures ANOVAs, stimulation intensity x genotype interactions, p = .985 and p = .628). Given that basal transmission was normal, we used AMPA/NMDA ratio as a way to test

NMDAR function in mGluR5 knockdown mice. As previously described (Myme *et al.*, 2003, Yoon *et al.*, 2009), AMPA and NMDA-mediated currents were isolated in layer IV neurons. There was no change in AMPA/NMDA ratio in $Grm5^{+/-}$ or $Grm5^{-/-}$ mice compared to wild-type littermate controls (Figure 4.12C; one-way ANOVA, p = .990). Additionally, biochemical measurement of NR1, an obligatory NMDA receptor subunit, showed no significant differences between wild-type, $Grm5^{+/-}$, and $Grm5^{-/-}$ visual cortical slices (Figure 4.12D; one-way ANOVA, p = .766). As a positive control, the same slices did show significantly decreased mGluR5 expression as a function of genotype (Figure 4.12D; one-way ANOVA, p<.001).

4.3.11: Gross inhibition is normal in $Grm5^{+/-}$ and $Grm5^{-/-}$ mice

In some contexts, inhibition during LFS prevents stable LTD induction (Dudek & Friedlander, 1996, Perrett *et al.*, 2001). Thus we hypothesized that altered levels of inhibition may impair LTD and OD plasticity in $Grm5^{+/-}$ and $Grm5^{-/-}$ mice. To assess relative levels of inhibition, we measured evoked IPSCs and EPSCs within individual layer IV neurons in response to varying intensities of white matter stimulation (Dong *et al.*, 2004) (Figure 4.13A). There was no significant change in IPSC (Figure 4.13B; two-way repeated measures ANOVA, main effect of genotype, p = .546), EPSC (Figure 4.13C, p = .464), or I/E ratio (Figure 4.13D, p = .076) as a function of *Grm5* genotype.

4.4: Discussion

Our data suggest that genetic knockdown or chronic inhibition of mGluR5 is sufficient to impair LFS-induced, NMDAR-dependent LTD in layer IV of visual cortex. This finding is noteworthy because induction of LFS-induced LTD, unlike DHPG-induced LTD, does not require acute activation of group 1 mGluRs. We confirmed that LFS-induced LTD persists in wild-type slice in the presence of acute mGluR5 inhibition with either MPEP or CTEP.

4.4.1: mGluR5 and NMDAR-mediated signaling interact to regulate synaptic plasticity

This is not the first report of a typically NMDAR-dependent form of plasticity that is altered in the mGluR5 knockout mouse. $Grm5^{-/-}$ mice also show deficient long-term potentiation in CA1 of mouse hippocampus (Jia *et al.*, 1998, Lu *et al.*, 1997) and altered thalamocortical plasticity and NMDAR-mediated synaptic function in barrel cortex (She *et al.*, 2009). Though in a different region of cortex, this study also sees differences in layer IV of the mGluR5 knockout – although they find deficient LTP and enhanced LTD in this region. Additionally, some forms of plasticity may require activation of both mGluR5 and NMDA receptors (Goh & Manahan-Vaughan, 2013, Kotecha *et al.*, 2003). In other contexts, activation of group 1 mGluRs with DHPG induces long-term depression of NMDAR-mediated EPSCs (Ireland & Abraham, 2009). In sum, mGluR5 and NMDA receptors are linked not only structurally via Homer, Shank, and PSD-95 (Tu *et al.*, 1999, Xiao *et al.*, 1998), but also functionally, especially in the context of synaptic plasticity. Because the mechanisms underlying synaptic plasticity vary as a function of induction protocol, age, brain region, and cortical layer, our results and discussion of potential mechanisms will be limited to layer IV of mouse visual cortex.

4.4.2: Layer-specificity in LFS-LTD and parallel plasticity mechanisms in vivo

It is not entirely surprising that mGluR5 is necessary for LFS-induced LTD in layer IV but not layer II/III. Studies of visual cortical slices (Crozier *et al.*, 2007) and in vivo visual cortex (Liu *et al.*, 2008, Yoon *et al.*, 2009) suggest that LTD and OD plasticity occur by different mechanisms in different layers. Specifically, LTD and OD plasticity in layer IV but not II/III depend on AMPA receptor endocytosis, and LTD and OD plasticity in layer II/III but not layer IV depend on cannabinoid receptors. The specific deficit we report in layer IV LFS-induced LTD is of particular interest because layer IV is the principal thalamorecipient layer, and LTD of thalamocortical synapses are thought to underlie forms of experience-dependent plasticity in visual cortex. Deficits in ocular dominance plasticity seen in $Grm5^{+/-}$ mice (Dolen *et al.*, 2007) were measured using visually evoked potentials in layer IV, and our work suggests a corresponding deficit in vitro. In parallel, we also found that chronic mGluR5 antagonism impairs ocular dominance plasticity in wild-type mice. Previous work in rat visual cortex shows that acute mGluR5 antagonism does not affect LFS-induced LTD in layer IV in vivo (Tsanov & Manahan-Vaughan, 2009), and we are currently testing whether acute CTEP treatment impairs OD plasticity. We hypothesize that acute CTEP treatment will have no effect. Overall, we have not shown definitively that requirements for mGluR5 in LTD and OD plasticity are linked, but convincing evidence does suggest that generally, deprived-eye depression occurs through LTD-like mechanisms (Heynen *et al.*, 2003, Yoon *et al.*, 2009).

4.4.3: Requirements for chronic mGluR5 signaling in regulation of LTD and OD plasticity

The finding that chronic CTEP treatment impairs LFS-LTD induction and OD plasticity implies that chronic mGluR5-mediated signaling during a developmental window, rather than physical presence of the receptor, is required for NMDAR-LTD induction. In our studies, 7+ days of CTEP administration began at P14 (for slice experiments) or at P21 (for in vivo experiments). From our studies, we were not able to establish whether the chronic nature of mGluR5 signaling or its timing during

development is critical to regulate plasticity. Future experiments should be designed to address this important question. It will be necessary to treat wild-type mice chronically with CTEP during adulthood and ask whether adult forms of NMDAR-dependent plasticity are impaired. Careful thought must go into the design of these experiments, as juvenile and adult forms of plasticity are quite different in visual cortex (Hensch, 2005).

4.4.4: Requirements for chronic mGluR5 signaling in regulation of other forms of NMDAR-dependent plasticity

Tight mechanistic coupling of LTD and deprived-eye depression in layer IV of visual cortex led us to ask whether chronic mGluR5 signaling is also required for other forms of NMDAR-dependent plasticity. SRP and LTP are also tightly coupled (Cooke & Bear, 2010), and induction of both processes requires NMDAR activation (Frenkel *et al.*, 2006, Kirkwood & Bear, 1994). We therefore assessed the effects of genetic mGluR5 knockdown and chronic mGluR5 inhibition on SRP. We found that $Grm5^{-/-}$ mice have impaired SRP, but $Grm5^{+/-}$ mice do not and chronic CTEP treatment has no effect. This is the only form of plasticity we report that is impaired only in $Grm5^{-/-}$ mice and not $Grm5^{+/-}$ mice or with CTEP treatment. Based on these results, it is unclear whether chronic mGluR5 signaling is required for forms of NMDAR-dependent plasticity beyond synaptic weakening. In parallel, we plan to test LTP in vitro. In visual cortex layer IV, the developmental window for LTP induction occurs earlier than LTD. LTP is almost entirely absent by P21 (Jiang *et al.*, 2007), whereas LTD can be induced from P21-P30. This complicates interpretations of LTP experiments in relation to LTD and drove our interest in beginning with SRP. In addition, use of extracellular layer IV field potentials results in low-magnitude LTP from P18-P21 (maximally ~10-15%, unpublished observations). Our finding that pairing-induced intracellular LTD is impaired in $Grm5^{-/-}$ mice (Figure 4.11) provides us with a useful system in which to test pairing-induced LTP, which can be more reliably induced, in parallel. Based on our SRP results, it is unclear whether chronic mGluR5 signaling is required for forms of NMDAR-dependent

plasticity beyond synaptic weakening. The ability to directly compare paired LTD and LTP will be important in addressing this question.

4.4.5: Towards a mechanism linking mGluR5, NMDA receptors, and induction of plasticity

Previous reports of acute interactions between mGluR5, NMDA receptors, and induction of various forms of synaptic plasticity (section 4.4.1) provide a useful background but do not provide a sufficient mechanistic explanation for our results. It is unlikely that acute interactions between NMDARs and mGluR5 account for the impaired plasticity we report with chronic but not acute mGluR5 downregulation. Since we describe three forms of impaired NMDAR-dependent plasticity, we first asked whether genetic mGluR5 knockdown impairs gross NMDA receptor function. We found normal AMPA/NMDA ratio and normal NR1 protein levels along with normal basal synaptic transmission in layer IV neurons (Figure 4.12). Knowing that altered levels of inhibition can affect LTD induction, we then asked whether the balance of excitation and inhibition was impaired in layer IV neurons. We found normal evoked IPSC and EPSC magnitude in $Grm5^{-/-}$ and $Grm5^{+/-}$ mice (Figure 4.13).

A third possibility is that the lack of mGluR5 signaling during development alters synapses in a way that makes them impervious to an LFS induction protocol. We hypothesize that NMDA receptor function may subtely impaired by a change in NR2 subunit composition in the absence of mGluR5 signaling. NMDA receptors are typically diheteromeric, including two NR2 subunits (NR2A-2D) in addition to the obligatory NR1 subunit (Cull-Candy *et al.*, 2001, Mcbain & Mayer, 1994). It is the nature of the NR2 subunits which regulates the conductance of NMDA receptors, and therefore, their functional consequences when activated (Monyer *et al.*, 1992, Vicini *et al.*, 1998). Specifically, the C-terminal tails of the NR2 subunits may define their functional roles, especially in the context of synaptic plasticity (Foster *et al.*, 2010). NR2 expression is tightly regulated by development and experience within visual cortex. NR2B expression peaks in the first postnatal week and is replaced by NR2A during the course of development (Monyer *et al.*, 1994, Sheng *et al.*, 1994). The NR2B-to-2A shift requires

visual input: it can be delayed by dark rearing and rapidly induced by eye opening (Carmignoto & Vicini, 1992, Lu & Constantine-Paton, 2004, Philpot *et al.*, 2001, Quinlan *et al.*, 1999).

Substantial evidence indicates that the relative levels of NR2A and NR2B in visual cortex have important consequences for the induction of NMDAR-dependent plasticity. Specifically, these subunits regulate the threshold for plasticity induction (Θ_m) in visual cortex (Figure 4.14). NR2A knockout mice display no LTD at using a 1 Hz stimulation frequency but LTD can be induced using lower-frequency stimulation and LTP induction is normal (Philpot *et al.*, 2007). These results indicate a "leftward" shift in Θ_m in the absence of NR2A. We hypothesize that increased relative levels of NR2B, as a result of chronic mGluR5 downregulation during development, might account for our reported deficits in layer IV LTD. Indeed, recent work has shown that mGluR5 signaling during development tightly regulates the NR2B-to-NR2A shift. In visual cortex as well as hippocampus, *Grm5*^{-/-} mice show enhanced NR2B expression during development (Matta *et al.*, 2011). Therefore we hypothesize that mGluR5 regulates plasticity in visual cortex via regulation of the developmental NR2B-to-NR2A shift. We plan to test this hypothesis using standard electrophysiological measures of NR2 function in layer IV visual cortical neurons (see Future Directions), where previous work has assessed only layer II/III (Matta *et al.*, 2011).

4.4.6: Consequences for Fragile X-targeted therapies

As mGluR5 antagonists are being considered as potential therapeutics for the treatment of Fragile X (see Chapter 2), it is critical to understand any potential side effects of CTEP treatment. We report that chronic CTEP treatment at a dose of 2 mg/kg impairs LFS-LTD and OD plasticity in visual cortex (Figures 4.5-4.6). It is possible that titration of dosage may reveal a window in which FX phenotype correction is possible without alterations to plasticity in visual cortex. Importantly, it is not well known whether CTEP affects plasticity similarly in visual cortex of *Fmr1* knockout and wild-type mice. Given the varied effects of FMRP on synaptic plasticity (see Chapter 1), it is possible that *Fmr1* KO mice may have altered baseline function in visual cortex, and that the

reported effects on plasticity in wild-type mice may be harmless, beneficial, or irrelevant in FX model mice. Further work must be done to characterize plasticity in visual cortex of *Fmr1* KO mice and assess the effects of CTEP treatment (see Future Directions). Overall, the behavioral consequences of our reported impairments in synaptic plasticity are not well known. Inhibition of mGluR5 has numerous known behavioral consequences, from impairments in learning and memory (Simonyi *et al.*, 2010) to anxiolytic effects (Spooren *et al.*, 2000). However, known side effects in mouse models have not prevented clinical trials of mGluR5 antagonists in Fragile X human patients (Krueger & Bear, 2011). If side effects do prove to be problematic, lovastatin provides another promising avenue for treatment in humans, as it has limited side effects and is already widely used.

In sum, this study describes parallel in vitro and in vivo forms of canonically NMDAR-dependent decreases in synaptic strength which are impaired by chronic downregulation of mGluR5 signaling. We hypothesize that mGluR5's known role in regulating the developmental shift between NR2B and NR2A subunits may underlie the plasticity deficits we report and future experiments will be designed to understand this mechanism.

4.5: Methods

4.5.1: Animals

Male and female $Grm5^{+/-}$ mice were bred on a C57BL/6 background, yielding littermates with three genotypes: $Grm5^{-/-}$, $Grm5^{+/-}$, and $Grm5^{+/+}$. All experiments were performed by an experimenter blind to genotype. Animals were group housed and kept on a 12 hour light/dark cycle, and all procedures were approved by MIT's Animal Care and Use Committee in conjuction with NIH guidelines.

4.5.2: Slice preparation

Visual cortical slices (350 μm thickness) were prepared from male C57BL/6 mice. For field potential LTD experiments, P21-P30 mice were used. For intracellular LTD experiments, P18-P25 mice were used. Following anesthesia using isofluorane inhalation and rapid decapitation, slices were made in standard high-sucrose dissection buffer containing (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 75 sucrose, 10 dextrose, 1.3 ascorbic acid. Slices were left to recover for 15 minutes at 32°C, then 1 hour at room temperature, in carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.23 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$ before recordings began.

4.5.3: In vivo drug treatment

CTEP was synthesized at Roche and formulated as a microsuspension in vehicle (0.9% NaCl, 0.3% Tween-80). Chronic treatment consisted of once per 48 h dosing at 2 mg/kg (s.c.), as described previously (Lindemann et al., 2011). For LTD experiments, chronic treatment began at P14 and continued through slice preparation, which occurred at P21-P25. For acute CTEP LTD experiments, a single dose was administered 3 hours prior to slice preparation. For SRP experiments, chronic treatment began at P21 and continued through the duration of SRP. All experiments were conducted using interleaved littermate controls and blind to CTEP or vehicle treatment.

4.5.4: Extracellular field potential recordings

Extracellular field potential recordings were conducted using an interface chamber, maintained at 30-32°C, with carbogenated ACSF flow at ~1.5 mL/min. All drugs (APV, 50 µM, cycloheximide, 60 µM, MPEP, 10 µM, LY367385, 100 µM) were included directly in the bath solution for the duration of recording sessions. A bipolar stimulating electrode was placed either in white matter or layer IV, and a glass recording pipette was placed either in layer IV or layer II/III, as noted, and extracellular field potentials were recorded. Input/output functions were calculated by adjusting the intensity of stimulation every 15 seconds, and the level of stimulation selected for baseline recordings was that which produced a field potential 40-50% of maximal response amplitude. 15 minutes of baseline was then collected at 0.03 Hz, and recordings were included only when baseline drifted by less than 5%. Following baseline, LTD was induced by low-frequency stimulation (1 Hz for 15 minutes), followed by 45 minutes of post-induction baseline at 0.03 Hz. For statistical analysis, the last 5 minutes of baseline and post-LFS were averaged and compared to determine the magnitude of LTD. Data acquisition and analysis were performed on a personal computer running pClamp 9.2 (Molecular Devices, Sunnyvale, CA).

4.5.5: Intracellular voltage-clamp recordings

Intracellular recordings were conducted using a submersion chamber, maintained at 30-32°C, with carbogenated ACSF flow at ~1.5 mL/min. Pipettes were pulled from thick-walled borosilicate glass. Somatic whole-cell voltage-clamp recordings were obtained from star pyramids in layer 4 identified using a Nikon E600FN microscope equipped with IR-DIC optics. Intracellular recording solution for LTD experiments contained, in mM: 103 d-gluconic acid, 103 CsOH, 5 QX-314-Cl, 0.2 EGTA, 5 TEA-Cl, 20 HEPES, 2.8 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine. Intracellular recording solution for AMPA/NMDA ratio and IPSC/EPSC experiments contained, in mM: 102 cesium gluconate, 20 HEPES, 0.2 EGTA, 3.7 NaCl, 5 TEA-Cl, 5 QX-314-Cl, 4 Mg-ATP, and 0.3 Na-GTP. Osmolarity was 290-300 mOsm and pH 7.2-7.3. When filled with internal solution, pipette resistances were ~5 MΩ. Pipette seal resistances

were >1 GΩ and pipette capacitive transients were minimized prior to breakthrough. Only cells with series resistance <40MΩ were included in this study. Layer 4 EPSCs were elicited with a concentric bipolar stimulating electrode (FHC, Bowdoin, ME) placed in white matter.

For pairing LTD experiments, stimulation intensity was adjusted to evoke a baseline current amplitude of 60-70% of the maximum response. Test stimuli (100 μsec) were delivered every 30 seconds. The test holding potential was –70 mV. LFS-LTD was induced by pairing 1-Hz presynaptic stimulation with a 100-msec postsynaptic-step depolarization from –70 to –45 mV for 300 pulses. Each presynaptic stimulation occurred midway (50 msec) into the step depolarization. Series resistance was monitored by measuring the peak of the capacitive transient elicited by a 5-mV hyperpolarizing pulse from –70 to –75 mV, and experiments were discarded if this value changed by more than 30% during the recording.

For AMPA/NMDA current recordings, bath solution was ACSF as described above, except with 4 mM Mg^{2+} and 4 mM Ca^{2+} to reduce polysynaptic activity, 50 μM picrotoxin to isolate excitatory responses, and 1 μM glycine. AMPA and mixed AMPA/NMDA currents were evoked by white matter stimulation and AMPA/NMDA ratio was calculated as described (Myme et al., 2003). Briefly, white matter was stimulated at an intensity that evoked responses of approximately 100 pA and evoked responses were measured in the patched neuron at -70 mV (AMPA-only response) and +40 mV (AMPA and NMDA combined response). Five traces, collected at 30-second intervals, were averaged at each holding potential. AMPA/NMDA ratio was calculated by dividing the amplitude of the AMPA-only response at +40 (the component within a 1 ms window of the -70 mV peak) by the amplitude of the NMDA-only response measured in a 10 ms window beginning 50 ms after stimulation. (See Figure 4.13C).

EPSCs and IPSCs were evoked by white matter stimulation at varying intensities as described (Dong et al., 2004). Briefly, EPSCs were recorded at -60 mV (the reversal potential for inhibitory responses) and IPSCs were recorded at 0 mV (the reversal potential for excitatory responses). Five responses were averaged at each stimulus

strength and holding potential, beginning at the threshold to induce a response ("T").
(See Figure 4.14A).

4.5.6: In vivo electrophysiology

Tungsten electrodes were implanted 450 μM below the brain surface in binocular V1. Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine i.p., and a local anesthetic of 1% lidocaine hydrochloride was injected over the scalp. For all experiments, subjects habituated to the recording apparatus for two consecutive days prior to beginning of testing. C57BL/6 mice received either CTEP or vehicle injection every 48h for 1 week prior to and during monocular deprivation (MD). Visually-evoked potentials driven by the contralateral and ipsilateral eye were recorded before and after MD, which occurred between P28-31. Visual stimuli consisted of full-field sine-wave gratings of 100% contrast, square reversing at 1Hz, and presented at 0.05 cycles/degree. A novel stimulus was presented after MD.

During SRP experiments, C57BL/6 mice received either CTEP or vehicle injection every 48h for 1 week before beginning daily SRP sessions, where noted. CTEP-treated mice began SRP at P30; mice on the Grm5 background began SRP between P30-P60. During SRP, a familiar stimulus was presented consecutively for six days, and on the sixth testing day, a novel stimulus was interleaved during the same testing session. For all MD and SRP experiments, a single session consisted of four blocks of 100 presentations presented at 1 Hz with a 30 second inter-block interval. VEP amplitude was quantified by measuring peak-to-trough amplitude. For more detail, implantation and stimulus presentation procedures were based on Frenkel et al., Neuron, 2006.

4.5.7: Western blotting

All Western blotting (Figure 4.12D) was done by Emily Osterweil as published (Osterweil et al., 2010), using visual cortical slices and antibodies specific to NR1 And mGluR5 protein.

4.5.8: Statistics

To determine if there were significant differences between groups, one-way ANOVA was used, followed by post-hoc Student-Newman-Keuls tests. For experiments comparing only two conditions (e.g. CTEP vs. vehicle), Student's t-test was used. For monocular deprivation experiments, two-way repeated measures ANOVA was used with treatment and time as factors. For SRP data in *Grm5* background, repeated measures two-way ANOVA was used with genotype and test day as factors to compare VEP magnitudes over days. To assess the effect of genotype on novel versus familiar discrimination on test day 6, one-way ANOVA was used on familiar/novel ratios with post-hoc tests to compare between genotypes. Within-genotype comparison of novel and familiar VEPs on test day 6 was done using post-hoc paired t-tests, as indicated. For SRP data comparing CTEP and vehicle treatment, similar tests were used. For all LTD experiments, n represents number of animals. 1-3 slice recordings were averaged together per animal. For all in vivo experiments, n represents number of animals. For SRP experiments, 1-2 hemispheres were averaged together per animal. For all figures, * indicates $p < .05$ and error bars indicate SEM. Outliers more than two standard deviations from the mean were excluded.

mGluR-LTD   **NMDAR-LTD**

DHPG,
PP-LFS   LFS

mGluR5   AMPARs   NMDAR

Post-synaptic
membrane

mRNA translation
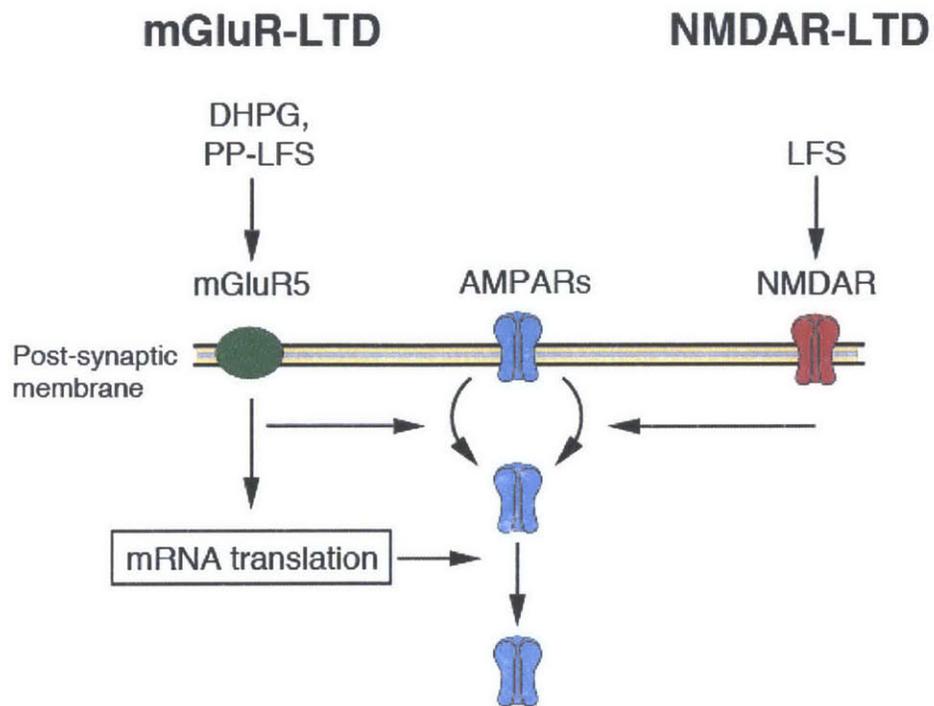
Figure 4.1: Distinct mechanisms of mGluR-LTD and NMDAR-LTD induction.

In hippocampal slices, mGluR-LTD can be induced by application of DHPG or by paired-pulse low frequency stimulation (pairs of pulses with a 50 ms interstimulus interval), and requires local protein synthesis. NMDAR-LTD can be induced by low-frequency stimulation and does not require protein synthesis. Both mGluR-LTD and NMDAR-LTD expression is maintained by internalization of AMPA receptors.
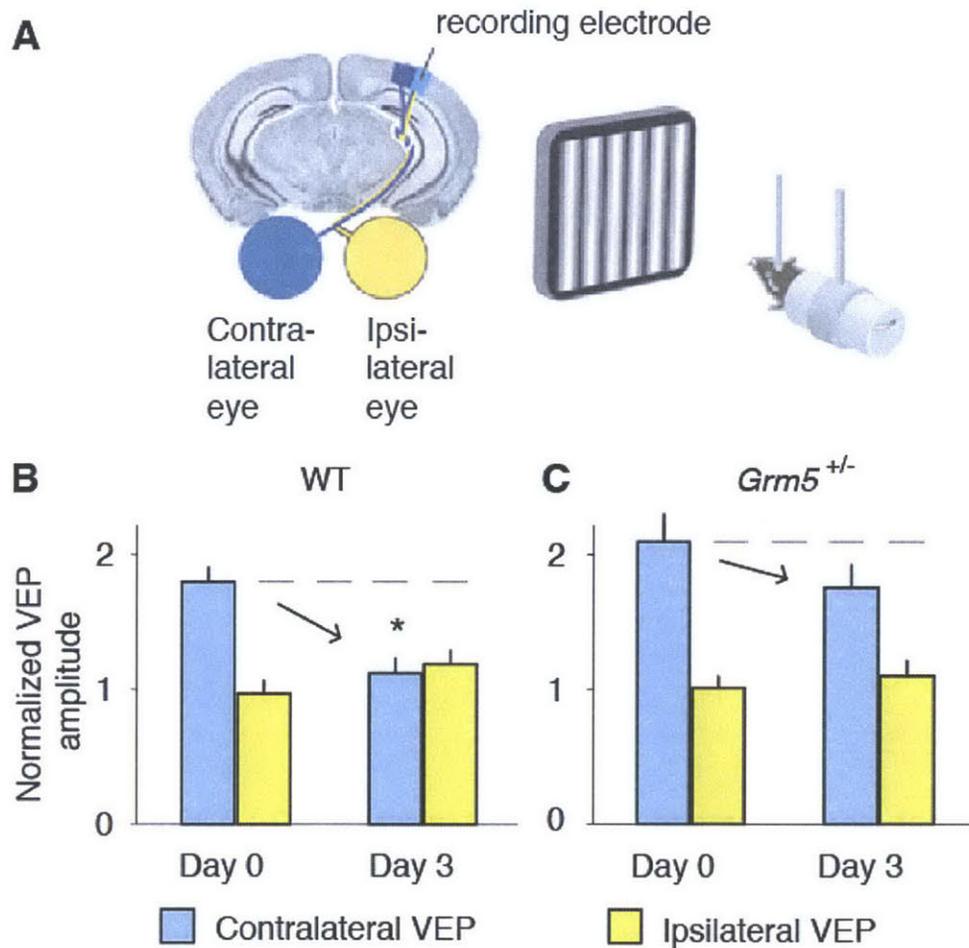
Figure 4.2: Deficient deprived-eye depression in *Grm5*^{+/-} mice.

(A) In a typical ocular dominance plasticity recording paradigm, awake, head-fixed view a drifting, phase-reversing grating. Electrodes implanted into the monocular region of visual cortex record visually evoked potentials in each hemisphere. An eye occluder is used to isolate responses in each hemisphere driven solely by input through either the eye contralateral or ipsilateral to electrode implant. VEPs are recorded once at baseline, then once following three days of monocular deprivation induced by eyelid closure. A novel stimulus orientation is used following eye-opening to avoid stimulus-specific response potentiation. **(B)** In wild-type mice, three day monocular deprivation induces an ocular dominance shift which is driven primarily by depression of contralaerally-driven VEPs (*p<.05). **(C)** *Grm5*^{+/-} mice display deficient deprived-eye depression. Adapted from Dolen *et al., Neuron*, 2007.
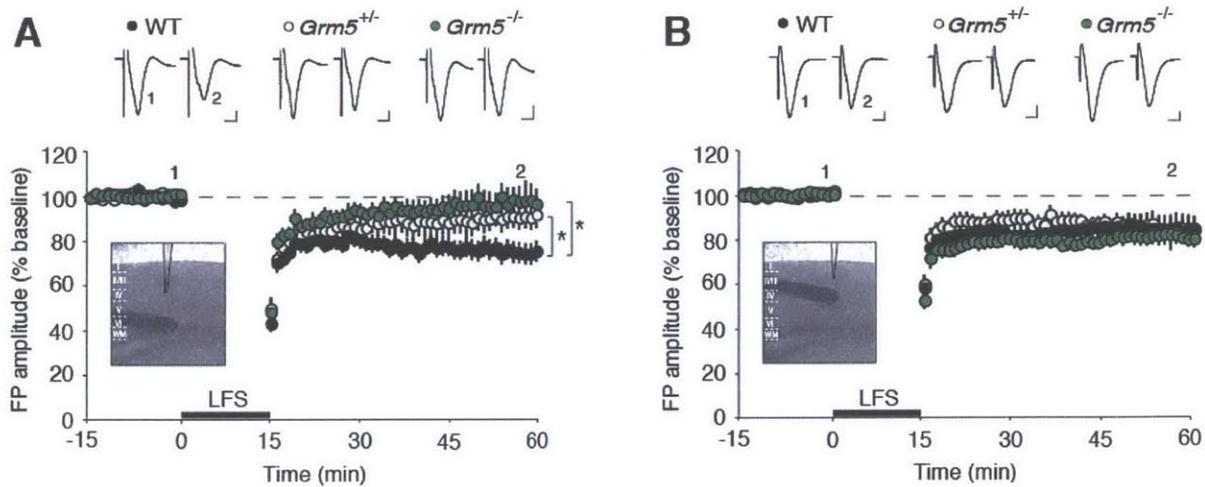
Figure 4.3: *Grm5*^{-/-} and *Grm5*^{+/-} mice have deficient LFS-induced LTD in layer IV of visual cortex.

(A) LTD induced by stimulation of white matter and recording in layer IV is significantly reduced in *Grm5*^{-/-} and *Grm5*^{+/-} mice (*p<.05). WT: 74.6 ± 3.9% of baseline, n = 8 animals (17 slices); *Grm5*^{-/-}: 97.2 ± 6.6%, n = 6 (13 slices); *Grm5*^{+/-}: 90.8 +/- 4.8%, n = 6 (9 slices). **(B)** The magnitude of LTD is similar in layer II/III across genotypes. WT: 83.9 +/- 5.5%, n = 6 (11 slices); *Grm5*^{-/-}: 80.9 +/- 5.9%, n = 7 (13 slices); *Grm5*^{+/-}: 83.5 +/- 4.3%, n = 4 (11 slices). Scale bars: 0.2 mV, 5 ms. Displayed traces are averaged across all experiments.
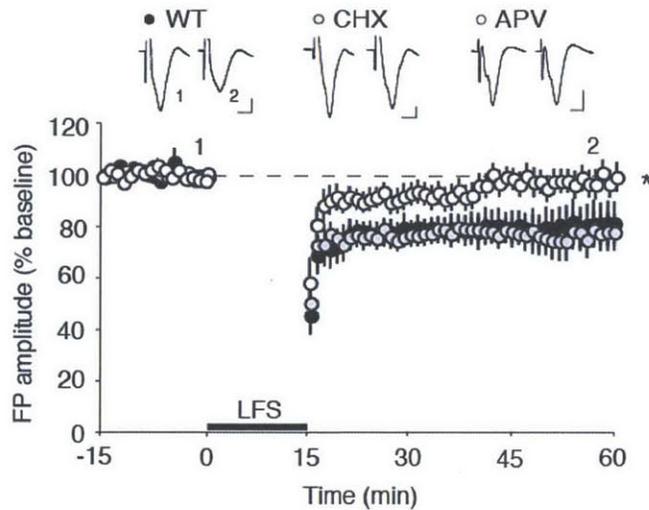
Figure 4.4: LFS-induced LTD in layer IV is NMDAR-dependent and not protein synthesis-dependent in wild-type mice.

Bath application of 50 μM APV significantly reduces LTD magnitude (*p<.05), and bath application of 60 μM cycloheximide has no significant effect. WT: 80.2 +/- 8.0%, n = 4 (9 slices); APV: 97.2 +/- 6.4%, n = 5 (8 slices); cycloheximide: 77.2 +/- 6.8%, n = 6 (10 slices). Scale bars: 0.2 mV, 50 ms. Displayed traces are averaged across all experiments.
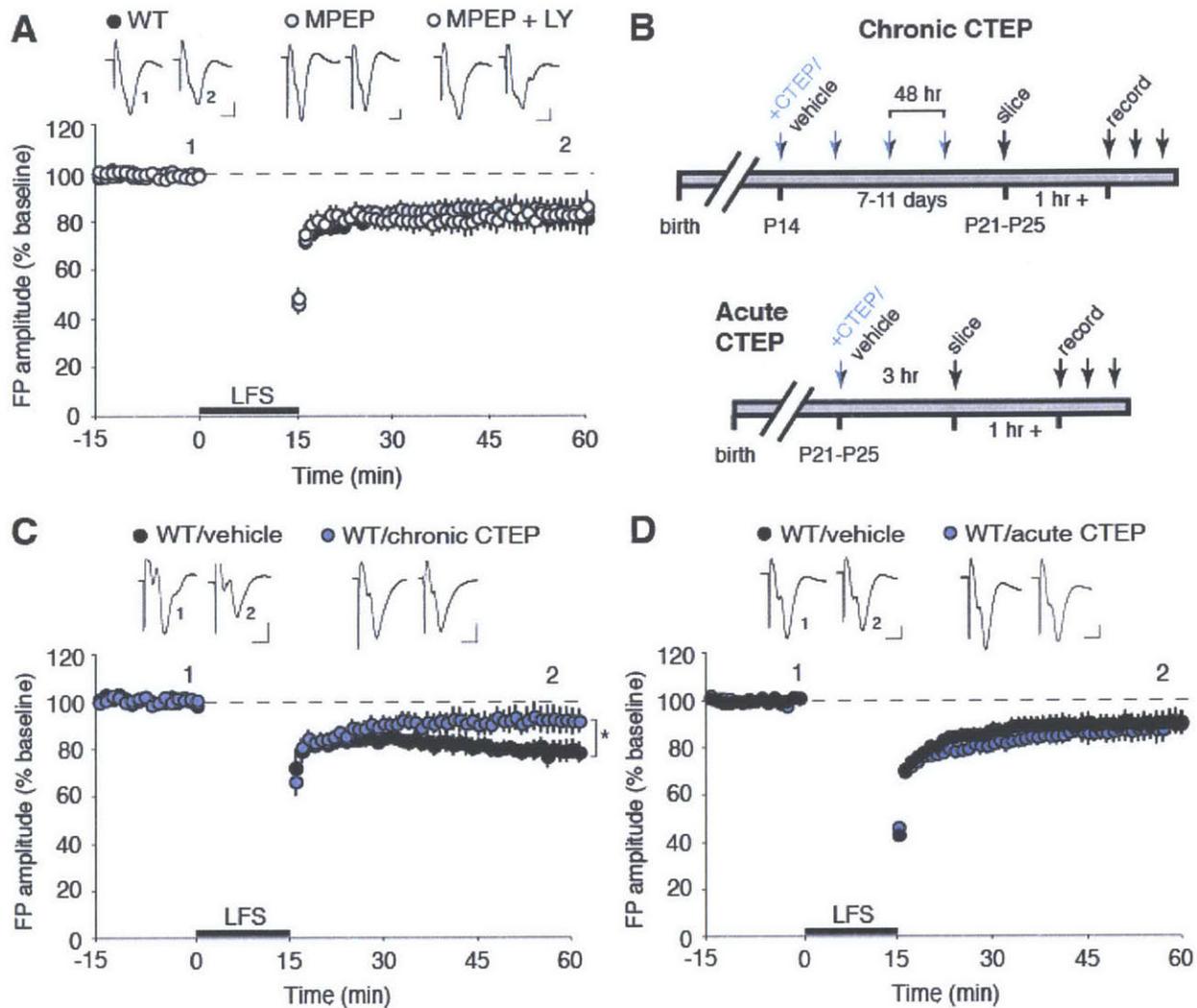
Figure 4.5: Chronic but not acute mGluR5 inhibition reduces the magnitude of LFS-induced LTD in layer IV in wild-type mice.

(A) Bath application of mGluR5 or mGluR5 + mGluR1 inhibitors has no effect on LTD magnitude in layer IV. WT: 82.8 +/- 6.0%, n = 13 (18 slices); MPEP: 84.8 +/- 5.0%, n = 5 (11 slices); MPEP + LY367385: 84.4 +/- 6.2%, n = 6 (13 slices). **(B)** Schematic showing chronic and acute CTEP dosing schedules. **(C)** Chronic CTEP reduces LTD magnitude in wild-type slices (*p<.05). WT/vehicle: 77.3 +/- 4.1%, n = 7 (13 slices); WT/CTEP: 91.8 +/- 5.0%, n = 9 (13 slices). **(D)** Acute CTEP does not alter LTD ex vivo in wild-type slices. WT/vehicle: 89.4 +/- 4.1%, n = 9 (9 slices); WT/CTEP: 88.5 +/- 5.1%, n = 8 (8 slices). Scale bars: 0.2 mV, 50 ms.
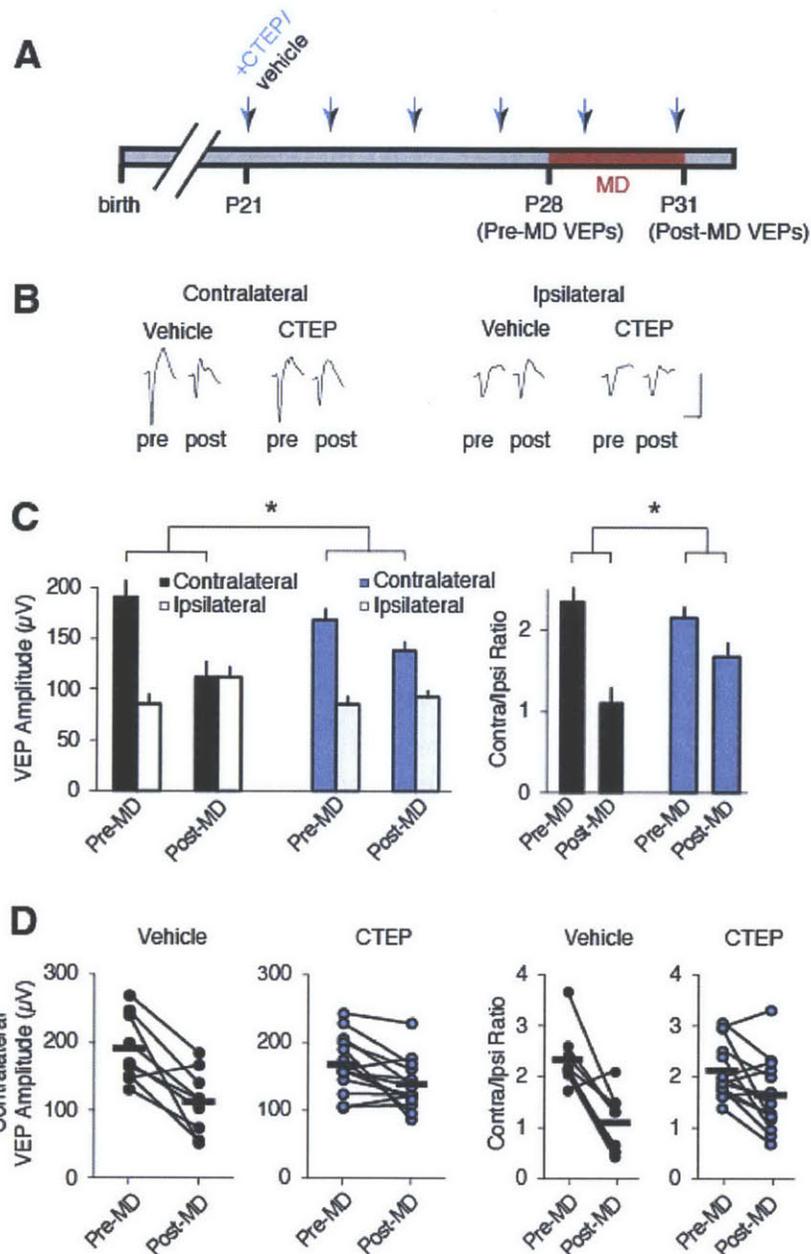
Figure 4.6: Chronic CTEP treatment impairs deprived-eye depression in wild-type mice.

(A) Schematic illustrating chronic CTEP or vehicle treatment beginning P21 and lasting throughout the duration of 3-day MD (P28-P31). **(B)** Averaged traces across all experiments, pre- and post-MD. Significant depression of VEP magnitude is evident in the hemisphere contralateral to the deprived eye (vehicle), and is impaired with CTEP treatment. There is no change in VEP magnitude in the ipsilateral hemisphere. Scale bar: 100 µV, 100 ms. **(C)** Summary of and **(D)** raw effects of monocular deprivation in CTEP and vehicle-treated mice. (Experiments performed by Eitan Kaplan.)
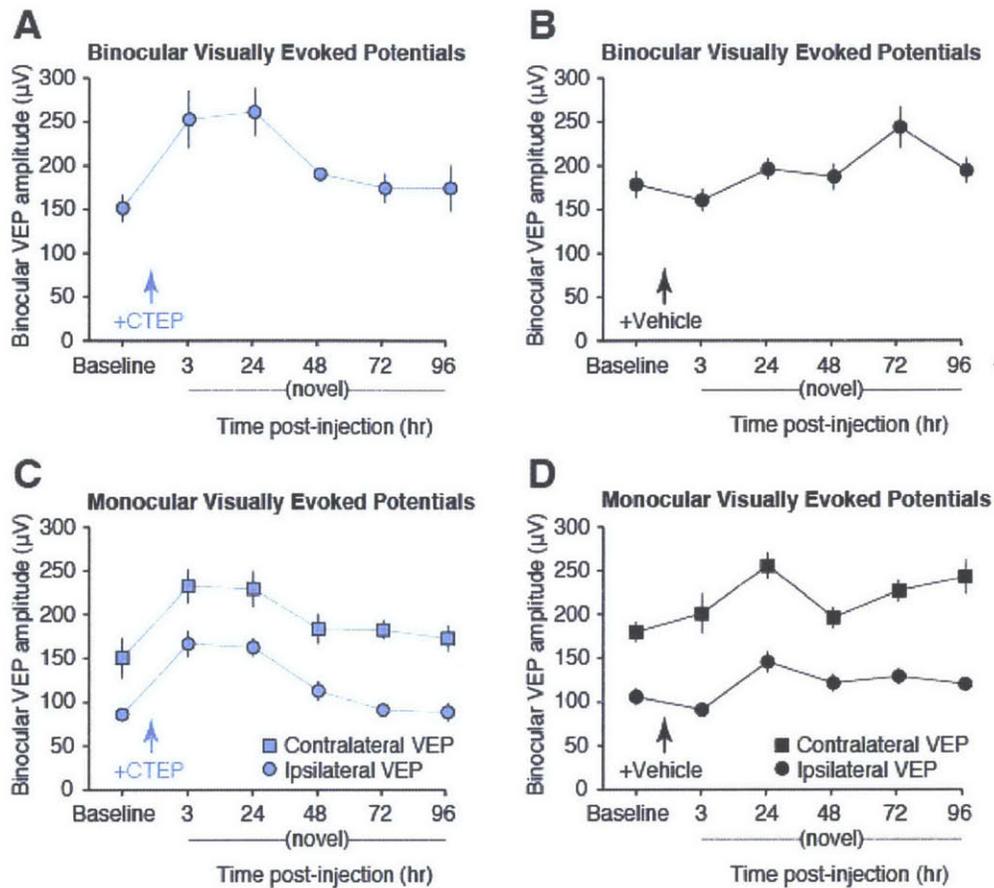
Figure 4.7: CTEP acutely affects amplitude of visually evoked potentials.

In parallel, binocular and monocular VEPs were measured. Following baseline VEP measurement, CTEP or vehicle is injected, followed by VEP measurements at 3, 24, 48, 72, and 96 hours post-injection. Each session a novel orientation is presented to avoid effects of SRP. **(A)** CTEP transiently increases binocular VEP amplitude compared to **(B)** vehicle. **(C)** CTEP transiently increases monocular VEP amplitude (both VEPs driven by contralateral and ipsilateral eye input), compared to **(D)** vehicle (n = 5 animals). (Experiments performed by Eitan Kaplan.)
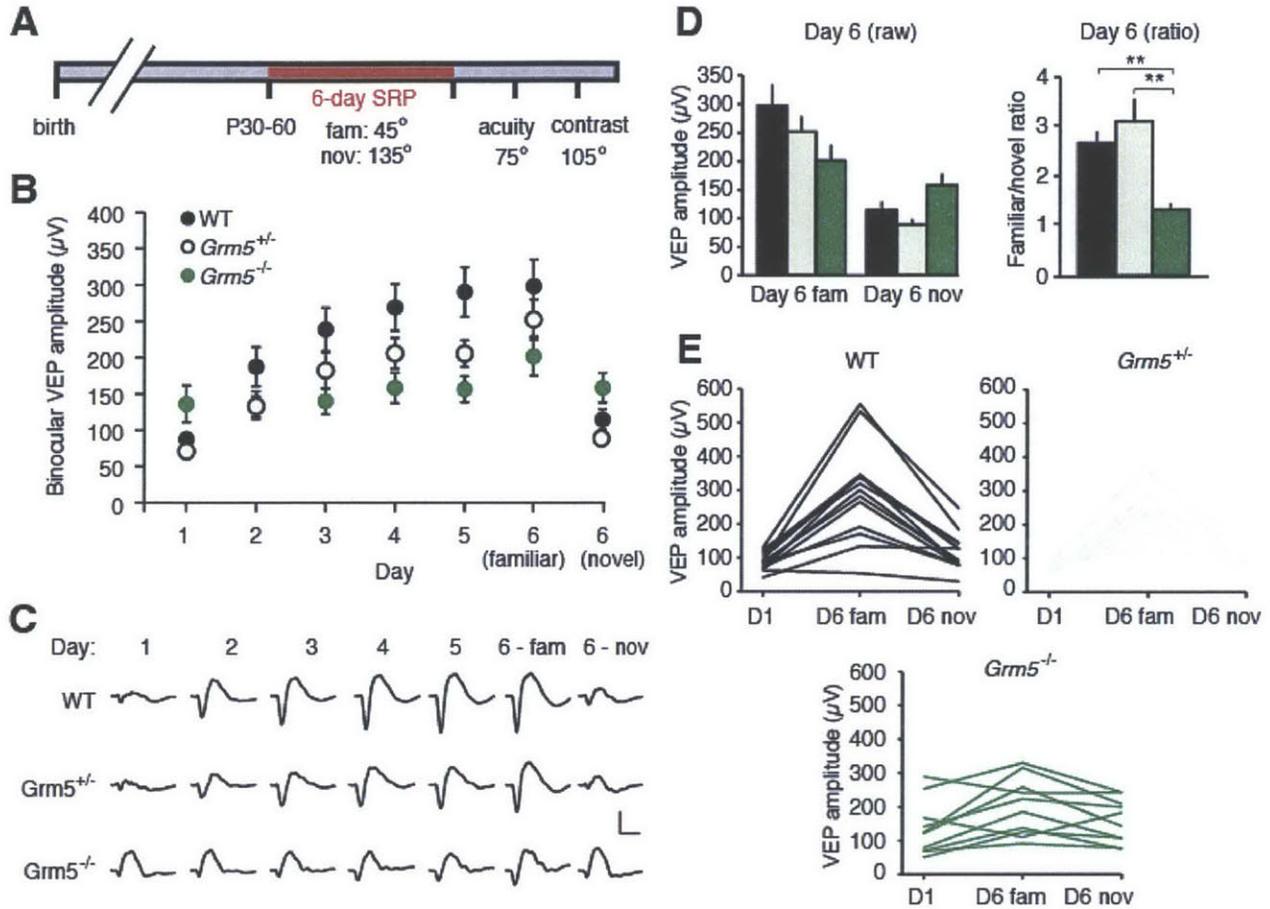
Figure 4.8: _Grm5⁻/⁻_ but not _Grm5⁺/⁻_ mice display impaired binocular SRP.

(A) SRP was induced by presentation of a familiar stimulus (45°) on six consecutive days, followed by interleaved presentation of a novel stimulus (135°) on test day 6. Measurements of visual acuity were conducted using novel stimulus orientations on test days following SRP (see Figure 4.10). **(B)** SRP is significantly impaired in _Grm5⁻/⁻_ mice. **(C)** Raw VEP traces across days show impairment of SRP in _Grm5⁻/⁻_ mice, as well as a VEP that looks qualitatively different. Scale bar: 100 ms, 100 μV. **(D)** Summary of SRP, comparing VEP magnitude between familiar and novel stimuli on test day 6. _Grm5⁻/⁻_ mice show significant impairments in distinguishing familiar from novel stimulus (**p<.01). **(E)** Line plots showing day 1 VEP, day 6 familiar VEP, and day 6 novel VEP within each animal. Impaired SRP in _Grm5⁻/⁻_ mice appears to be independent of the initially enhanced VEP magnitude on day 1.
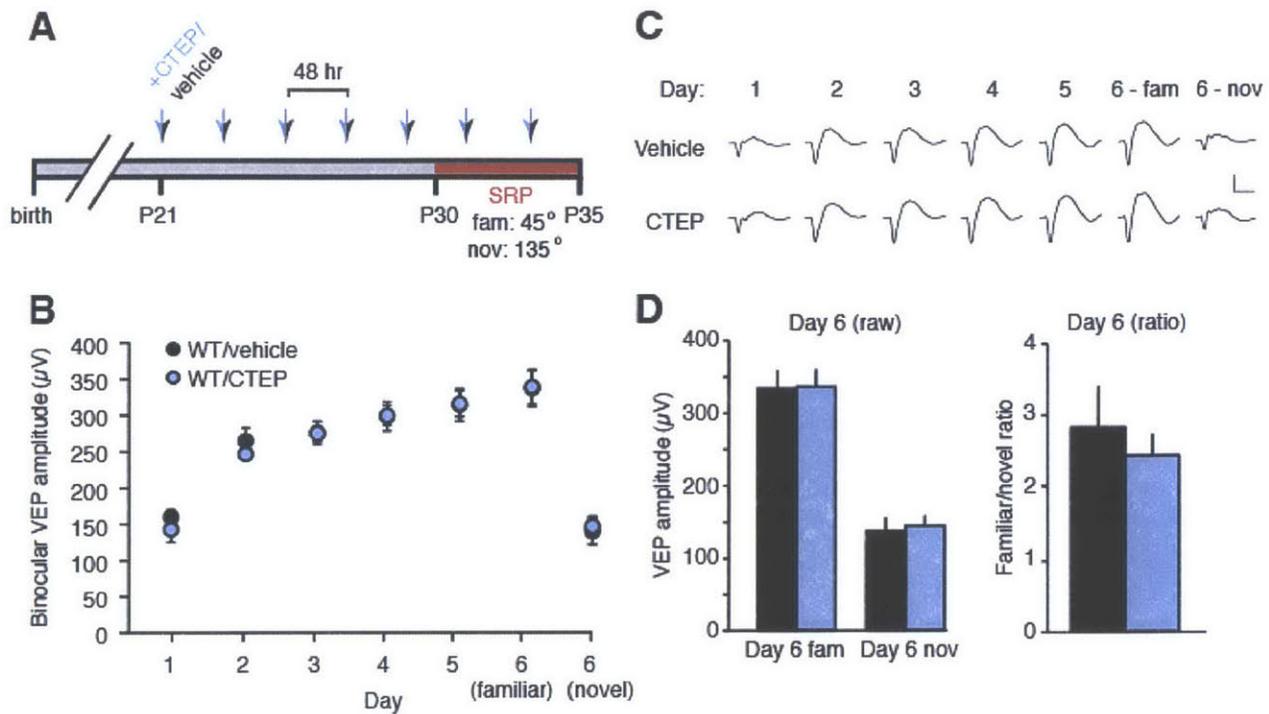
Figure 4.9: Chronic CTEP treatment does not affect SRP in wild-type mice.

(A) Six-day SRP was induced beginning on P30 following chronic CTEP or vehicle treatment, which began at P21. (B) SRP magnitude is not different between CTEP and vehicle-treated wild-type mice. (C) Raw VEP traces across days show expression of SRP. Scale bar: 100 ms, 100 μV. (D) Summary of SRP, comparing VEP magnitude between familiar and novel stimuli on test day 6.
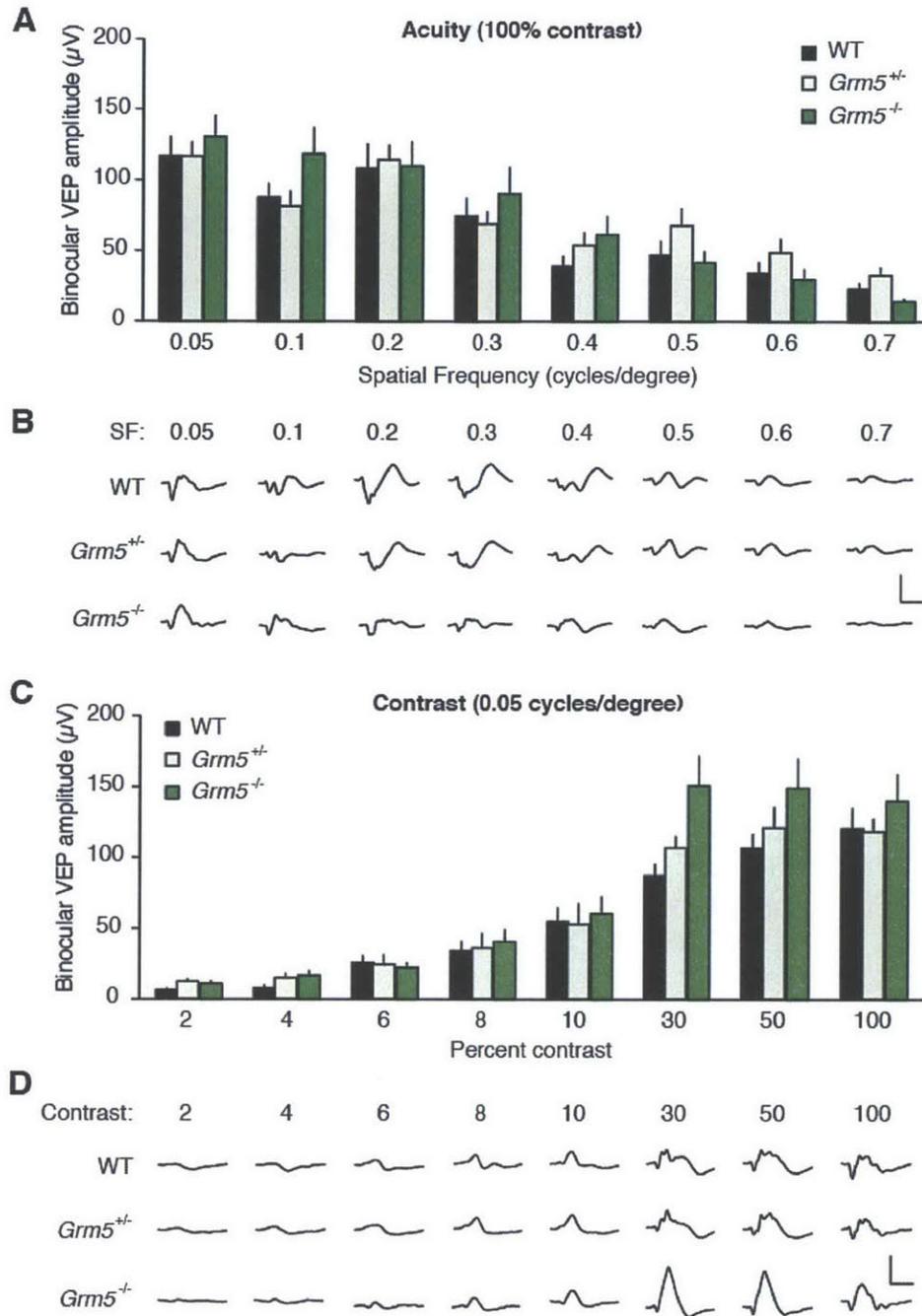
Figure 4.10: *Grm5⁻ᐟ⁻* and *Grm5⁺ᐟ⁻* mice have normal visual acuity and contrast sensitivity.

During acuity and contrast sensitivity testing, two blocks of 100 presentation of each spatial frequency or contrast were interleaved randomly within a single test session. **(A)** *Grm5⁻ᐟ⁻* and *Grm5⁺ᐟ⁻* mice have normal visual acuity. **(B)** Averaged traces from all mice. **(C)** *Grm5⁻ᐟ⁻* and *Grm5⁺ᐟ⁻* mice have normal contrast sensitivity. **(D)** Averaged traces from all mice. Scale bars: 100 ms, 100 µV.
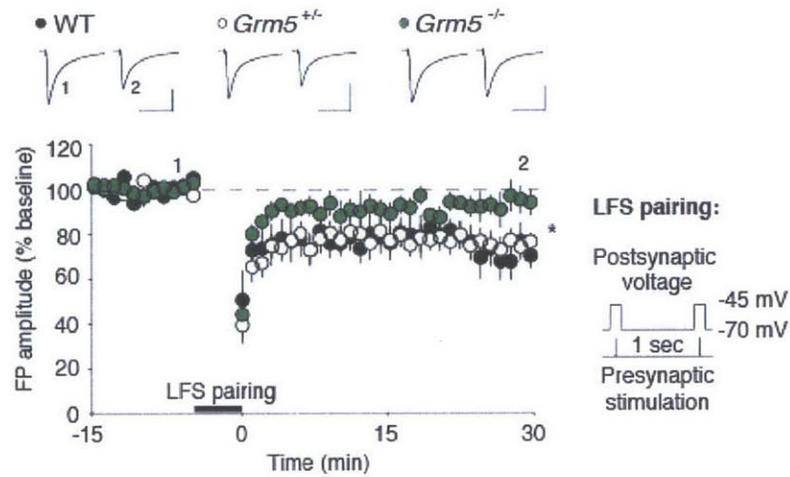
Figure 4.11: LFS-LTD induced by paired white matter stimulation and postsynaptic depolarization is impaired in layer IV of *Grm5*$^{-/-}$ mice.

LTD is induced by pairing presynaptic white matter stimulation with postsynaptic depolarization (see inset and Methods). Paired LTD is impaired in *Grm5*$^{-/-}$ mice compared to WT and *Grm5*$^{+/-}$ (*$p<.05$). WT: 72.1 +/- 7.5%, n = 7 (7 cells); *Grm5*$^{+/-}$: 73.5 +/- 4.7%, n = 8 (8 cells); *Grm5*$^{-/-}$: 93.1 +/- 6.1%, n = 6 (8 cells). (Experiments performed by Stephanie Tagliatela).
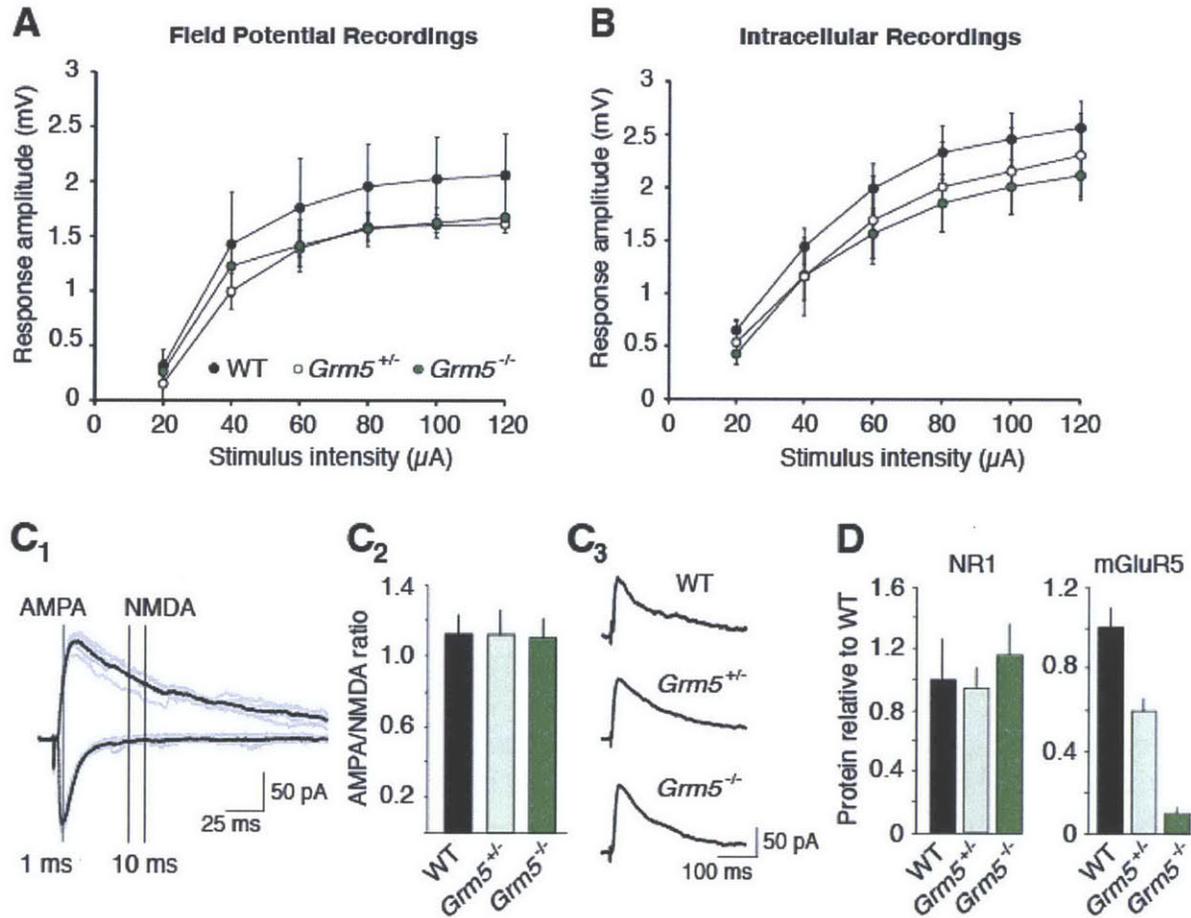
Figure 4.12: Gross NMDAR function is normal in *Grm5⁻ᐟ⁻* and *Grm5⁺ᐟ⁻* mice.

Input/output functions from **(A)** field potential LTD experiments and **(B)** intracellular paired LTD experiments show no change in basal synaptic transmission in *Grm5⁺ᐟ⁻* or *Grm5⁻ᐟ⁻* mice compared to wild-type. **(C1)** AMPA/NMDA ratio in layer IV is calculated by comparing AMPA-only responses to NMDA-only responses. The AMPA-only component of the response at +40 mV is taken from a 1 ms window corresponding to the peak at -70 mV, and the NMDA-only response is taken from a 10 ms window at +40 mV where no AMPA response is present (see Methods). The AMPA/NMDA ratio is normal in *Grm5⁺ᐟ⁻* and *Grm5⁻ᐟ⁻* neurons. **(C2)** Summary. WT: 1.12 +/- 0.10; *Grm5⁺ᐟ⁻*: 1.12 +/- 0.11; *Grm5⁻ᐟ⁻*: 1.10 +/- 0.14; n = 8-11 cells. **(C3)** Example traces. **(D)** Levels of NR1 protein are normal in *Grm5⁺ᐟ⁻* and *Grm5⁻ᐟ⁻* visual cortical slices (n = 11 animals, 1 slice per animal). WT: 100.0 +/- 23.8% of WT; *Grm5⁺ᐟ⁻*: 93.8 +/- 14.2% of WT; *Grm5⁻ᐟ⁻*: 115.1 +/- 20.5% of WT. Levels of mGluR5 protein are reduced as expected (n = 6, 1 slice per animal). WT: 100.0 +/- 10.4% of WT; *Grm5⁺ᐟ⁻*: 59.9 +/- 5.2% of WT; *Grm5⁻ᐟ⁻*: 9.8 +/- 3.2% of WT. (Experiments in Figure 4.12D were conducted by Emily Osterweil.)
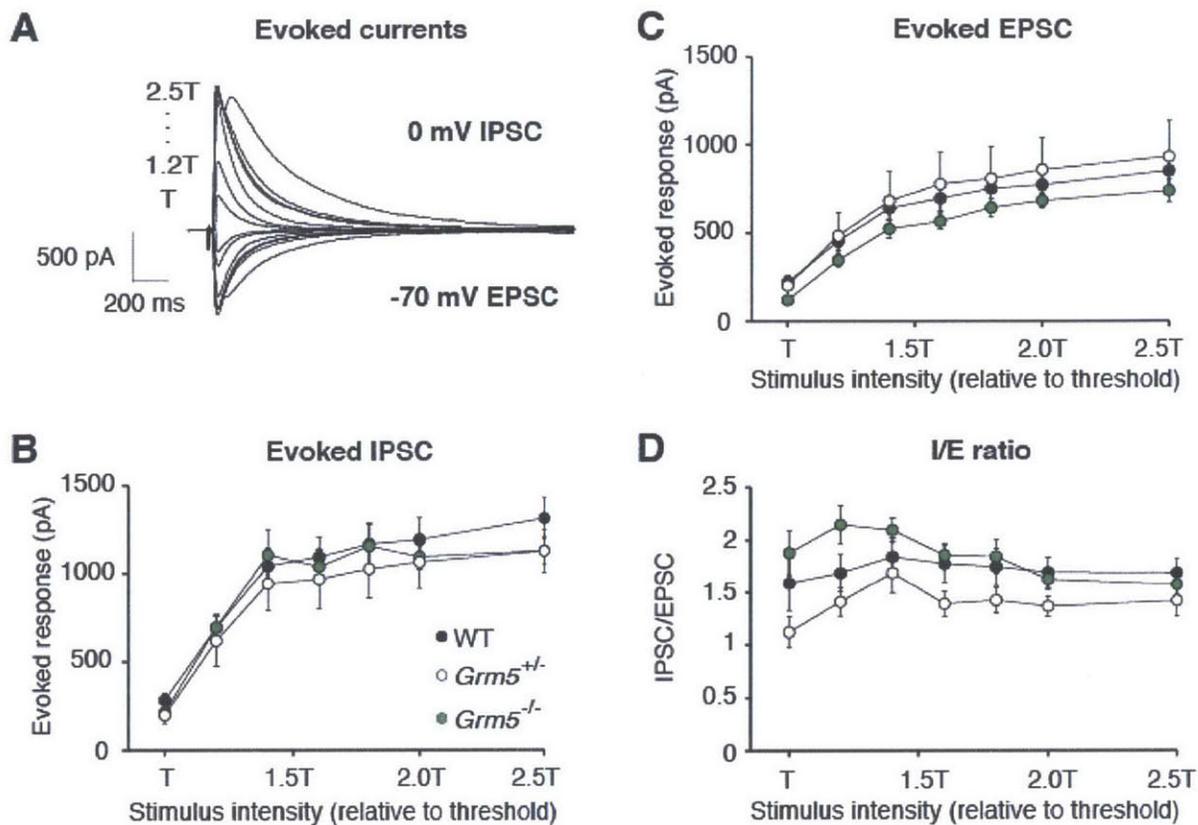
Figure 4.13: Relative inhibition is normal in *Grm5⁻/⁻* and *Grm5⁺/⁻* layer IV neurons.

(A) Evoked IPSCs and EPSCs were isolated in layer IV neurons by holding cells at 0 mV and -70 mV, respectively. White matter stimulation yielded a threshold "T" stimulation intensity required to evoke responses in layer IV. The amplitude of evoked IPSCs and EPSCs were recorded as a function of stimulation intensity (relative to threshold). There was no effect of Grm5 genotype on **(B)** evoked IPSC amplitude, **(C)** evoked EPSC amplitude, or **(D)** IPSC/EPSC ratio (n = 9-13 cells).
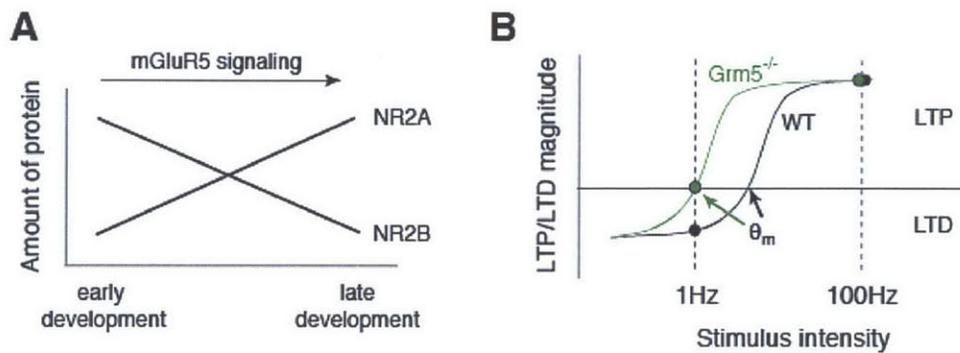
Figure 4.14: A hypothesis for mGluR5 regulating NMDAR-dependent plasticity via NR2A and NR2B during development.

(A) mGluR5 signaling is required for the normal developmental shift from NR2B to NR2A subunits. Simplified diagram based on data from Matta *et al., Neuron,* 2011. **(B)** NR2A knockout mice show impaired LTD with 1 Hz stimulation and a "leftward" shift in θ_m (Philpot *et al.*, 2007). We hypothesize that *Grm5*$^{-/-}$ mice may mimic NR2A knockout mice.

Chapter 5

Implications and Future Directions

5.1: Introduction

Enhanced hippocampal mGluR-LTD at Fragile X synapses likely does not cause the majority of FX symptoms such as intellectual disability and autism. However, the initial discovery of enhanced LTD (Huber *et al.*, 2002) has proven to be a critical readout of altered protein synthesis in *Fmr1* KO mice. It is quite likely that enhanced protein synthesis in Fragile X due to the loss of the translational repressor FMRP is indeed disease-causing, and treatment strategies aimed at correcting local protein synthesis have proven successful in mouse models. Genetic downregulation of mGluR5 signaling, which is positively couple to translation, corrected protein synthesis as well as numerous disease phenotypes in the *Fmr1* KO mouse (Dolen *et al.*, 2007). We have expanded on this approach by testing the timing of intervention required to correct FX phenotypes (Chapter 2). We reported that administration of the mGluR5 antagonist CTEP beginning in young adulthood was sufficient to correct enhanced LTD, protein synthesis, seizures, behavior, and altered signaling downstream of mGluR5. Additionally, we have reported that inhibition of the ERK1/2 signaling pathway with lovastatin is sufficient to correct enhanced LTD in *Fmr1* KO mice. We provided a model proposing that ERK1/2 may regulate LTD by multiple mechanisms in addition to regulation of protein synthesis (Chapter 2). In Chapter 3, we developed a novel instrumental extinction assay to assess cognitive impairments in *Fmr1* KO mice, and found that downregulation of mGluR5 and ERK1/2 signaling were not sufficient to correct exaggerated extinction. In Chapter 4, we assessed the role of mGluR5 in regulating synaptic plasticity in wild-type visual cortex. We found that chronic mGluR5 signaling is required for multiple forms of canonically NMDAR-dependent plasticity, including LTD, ocular dominance plasticity, and to an extent, stimulus-selective response potentiation. These studies both enhance our basic understanding of plasticity mechanisms in layer IV of mouse visual cortex and emphasize the need to understand the consequences of using chronic mGluR5 antagonism to treat Fragile X in humans.

5.2: Fragile X: disease or syndrome?

Following the pioneering work of Gul Dolen and colleagues, we aimed to assess whether mGluR5 antagonism beginning in young adulthood (~P30) would be sufficient to correct FX mouse phenotypes. This question was not just practical in designing clinical treatments, but a basic question underlying the nature of Fragile X. To what extent is Fragile X a disease of altered development and to what extent is it a disease of acute synaptopathology? Our results – correction of numerous phenotypes – suggest that FX is in part a disorder of acutely altered synapses. However, this is not an either/or question and developmental changes have been reported in the *Fmr1* KO mouse, especially in barrel cortex (Till *et al.*, 2012).

Synaptic and behavioral impairments in the FX mouse model are correctable beginning in adulthood, after their presentation. While the terms "syndrome" and "disease" are often used interchangeably, the former often describes two things: (1) a set of symptoms with unknown etiology and/or (2) a chronic, untreatable condition. The cause of Fragile X has been known for some time to be expanded CGG repeats at a "fragile" locus on the X chromosome, which results in hypermethylation, gene silencing, and loss of FMRP. Additionally, this and other work suggest that symptoms of FX may be treatable following their presentation. Therefore given the rapid progress in the field, we have generally referred to Fragile X as a disease or condition rather than "Fragile X syndrome".

5.3: Towards biology-based diagnosis and treatment of autism

Unlike Fragile X, autism is not a disease. Rather, autism is a collection of common symptoms with unknown etiology for the vast majority of cases. It seems likely that there are hundreds of autism risk genes, and that "autism" really should be best defined as a group of symptoms that are associated with any of the hundreds of yet-to-be discovered diseases caused by these genetic factors. Thus autism itself is no more

a disease than a fever is a disease; both are symptoms of an underlying condition. Recent evidence using single-gene models of autism suggest that some cell and circuit-level phenotypes may be common in models of autism and may provide a link between genes and behavior. As discussed in section 1.2.7, a complete one-to-one mapping of autism risk genes onto specific "autisms" represents an immense challenge. Finding common cell and circuit-level impairments associated with autism is critical to understanding what is truly causal of symptoms and in designing treatments.

5.3.1: Protein synthesis in autism

The maintenance and regulation of proper synaptic protein levels is critical for normal synaptic function and synaptic plasticity (Cajigas *et al.*, 2010). *Fmr1* knockout mice display consistently increased synaptic protein synthesis (Osterweil *et al.*, 2010, Qin *et al.*, 2005). The nature and function of these proteins is an area of active study. In addition to the candidate "LTD proteins" identified in Chapter 1, recent work has show that FMRP regulates over 800 mRNAs which code for proteins with a wide range of functions (Darnell *et al.*, 2011). Altered synaptic protein synthesis is a shared feature of single-gene disorders associated with autism (Figure 1.6A) (Kelleher & Bear, 2008, Zoghbi & Bear, 2012). Tuberous sclerosis is an especially interesting case because TSC knockout mice display decreased synaptic protein synthesis which can be corrected by positive allosteric modulation of mGluR5, yet similar autistic phenotypes to FX (Figure 1.6B) (Auerbach *et al.*, 2011). The juxtaposition of Fragile X and tuberous sclerosis seem to define an axis of synaptic pathophysiology, whereby bidirectional alterations in synaptic protein synthesis converge upon altered synaptic function. Based on this hypothesis, it would be incredibly useful to categorize existing causes of autism as either "Fragile X-like" (i.e. increased synaptic protein synthesis), "TSC-like" (i.e. decreased synaptic protein synthesis), or realistically, "other" for the causes that are likely to fall outside this simplistic model. Categorizing "autisms" along this spectrum may eventually help clinicians deliver personalized drug treatments. Where CTEP may be helpful for Fragile X, it certainly could be equally damaging for tuberous sclerosis or a similar disorder with decreased synaptic protein synthesis. In the long-term, if this

model holds up and many autisms are characterized by altered protein synthesis, it would be useful to be able to measure protein synthesis levels in real-time in a patient. Development of this technology is currently underway using PET scanning (Veronese *et al.*, 2012) and provides promise for individualized medicine in autistic patient populations. In addition to identification of basal protein synthesis levels, this approach would allow for a physiological readout of drug function. The simplest first experiment might be to measure protein synthesis in FX patients before and after treatments with an mGluR5 antagonist or lovastatin to ask whether these drugs have similar physiological consequences in mouse and man. Next one could correlate changes in protein synthesis with behavioral improvement, just as a previous study has correlated *FMR1* methylation state with behavioral improvement after AFQ056 treatment (Levenga *et al.*, 2011).

5.3.2: Excitatory/inhibitory imbalance in autism

A unifying pathogenic feature in autism is elevation in the ratio of excitatory to inhibitory (E/I) neurotransmission in cortical microcircuits (Rubenstein & Merzenich, 2003). Many causative alleles in autism encode ion channels and synaptic proteins that may perturb cortical E/I balance (Hayashi & Mcmahon, 2002). Furthermore, epilepsy, an extreme manifestation of elevated E/I ratio, is frequently comorbid with autism spectrum disorders. Manipulations that elevate the E/I ratio in the prefrontal cortex result in social and cognitive dysfunction in mice (Dani *et al.*, 2005), further supporting the hypothesis that E/I imbalance may contribute to autism spectrum disorders. Indeed, numerous mouse models of autism exhibit altered E/I balance (Dani *et al.*, 2005, Etherton *et al.*, 2009, Gkogkas *et al.*, 2013, Tabuchi *et al.*, 2007, Walllace *et al.*, 2012). Many of these mouse models also have altered synaptic protein synthesis, suggesting that potential "candidate proteins" may regulate E/I balance. Specifically in Fragile X, *Fmr1* knockout mice display increased up states and hyperexcitability (Chuang *et al.*, 2005, Gibson *et al.*, 2008, Osterweil *et al.*, 2013) as well as impaired GABAergic function (Olmos-Serrano *et al.*, 2010, Paluszkiewicz *et al.*, 2011a, Paluszkiewicz *et al.*, 2011b). Thus disruptions in protein synthesis resulting in altered excitatory/inhibitory

balance may underlie multiple forms of autism. Further work must be done to characterize the exact nature of E/I imbalance within specific microcircuits and in multiple brain regions. Characterization of the candidate proteins involved in these processes will help in this process and it seems likely that the potassium channel Kv4.2 is one of these candidate proteins in FX (Chapter 1). FMRP's regulation of Kv4.2 translation will have direct consequences for E/I balance and indirect consequences for synaptic plasticity, as discussed. It seems likely that E/I imbalance may be directly relevant for epilepsy in FX, but further work must be done to determine if and how E/I imbalance relates to other behavioral phenotypes.

5.3.3: Linking synapses, cells, circuits, and behavior

In Fragile X as well as other causes of autism, a major challenge will be to connect synaptic, cellular, and circuit-level phenotypes with behavior. Broadly, the use of region and cell-type specific methods to re-introduce FMRP as well as to inhibit mGluR5 will be critical in identifying neural correlates of specific behaviors. One particularly tractable example is the link between E/I imbalance and epilepsy, but even "E/I imbalance" is a broad term which encompasses diverse mechanisms. In Fragile X alone, we have described multiple forms of altered excitability in hippocampus as well as neocortex. Hyperexcitability in hippocampal CA3 has long been studied as a model for epilepsy (Wong et al., 2004). Indeed, Fmr1 KO mice display enhanced epileptiform discharges in CA3 (Chuang et al., 2005) which were corrected by lovastatin admnistration (Osterweil et al., 2013). To solidify the link between CA3 hyperexcitability and epilepsy in FX, it would be useful in the future to test whether CA3-specific re-expression of FMRP or CA3-specific mGluR/ERK antagonism is sufficient to prevent seizures in Fmr1 KO mice. Beyond FX, other mouse models of autism show hyperexcitability in CA3 linked with seizures (Calfa et al., 2011). One could directly compare CA3 excitability in FX model mice and other mouse models of autism which do not have seizures. We would hypothesize that those mouse models which do not show seizures may have normal CA3 function. In sum, we have discussed CA3 excitability and seizures because both the cellular phenotype and the behavioral phenotype have

been widely reported in mouse models of autism, and they are likely to be linked. For many other behavioral phenotypes, the cellular and circuit-level causes are less well-understood but will be even more important to study in the long term. For example, it would be a stretch to say at this stage that we can map a behavioral phenotype onto dendritic spine abnormalities or map a direct neural correlate of cognitive deficits in the *Fmr1* KO mouse.

In Chapter 3, we introduced an instrumental extinction assay and reported that *Fmr1* KO mice display exaggerated extinction. This type of learning is an active process and is known to require prefrontal cortex, and a detailed characterization of prefrontal function between wild-type and *Fmr1* KO mice would be informative as a future direction. Already, Dilja Krueger has shown that a number of synaptic proteins are downregulated in PFC of *Fmr1* KO mice and that levels of these proteins are correlated with performance on a similar visuospatial discrimination assay (Krueger *et al.*, 2011). These proteins included NR1, NR2A, and NR2B. Thus impairments in prefrontal NMDA receptor function may underlie some cognitive impairments seen in *Fmr1* KO mice. Future work should address whether similar dysfunction also correlates with performance on the instrumental extinction assay. In addition, we hope that instrumental extinction and visuospatial discrimination can be tested in parallel and compared between other mouse models of autism. We hypothesize that these phenotypes in the *Fmr1* KO mouse are a model of impaired executive function and intellectual disability, and are not necessarily at the core of the autistic phenotype. Therefore we would hypothesize that other mouse models of intellectual disability may show similar cognitive phenotypes to the *Fmr1* KO, but that if a model of "pure autism" were developed, it may not show impairments on these assays. Overall, intellectual disability, while at the core of FX, remains difficult to properly model and assess in mice. We hope that rat models of Fragile X will allow us to better model complex behavior in the future.

5.4: mGluR5 regulates plasticity in visual cortex

In addition to its role in regulating synaptic protein synthesis in the context of Fragile X, mGluR5 is critical for forms of plasticity in visual cortex. We have shown that chronic but not acute mGluR5 signaling is required for multiple forms of NMDAR-dependent plasticity in vitro and in vivo and likely limited to layer IV. We have described numerous reports of direct mGluR5-NMDAR interactions, both structurally and functionally (Chapter 4). However, because acute mGluR5 antagonism does not impair plasticity, it suggests that these interactions are likely not a proximal mechanistic explanation for our results. Future work should be aimed at addressing the mechanism by which genetic mGluR5 knockdown or chronic CTEP treatment impairs forms of NMDAR-dependent plasticity in visual cortex.

In both a slice preparation and in vivo, chronic CTEP treatment phenocopies mGluR5 genetic knockdown. Chronic CTEP from P14-P21+ impairs LFS-LTD and chronic CTEP from P21-P28+ impairs ocular dominance plasticity in wild-type mice. Both of these treatments last 7-10 days and their timing was designed to target the ideal window for LFS-LTD (P21-P25) and OD plasticity (P28-P31). However, our experiments were not able to dissect which component of mGluR5 signaling is more important for later plasticity: (a) its duration, or (b) its timing. Ideally, we would be able to distinguish between these two hypotheses by comparing the effects of chronic CTEP in development and in adulthood. However, OD plasticity is not expressed through deprived-eye depression in adult mice (Lena Khibnik), and LTD in layer IV is difficult to induce beyond P30 (Jiang *et al.*, 2007). Stimulus-specific response potentiation can be induced in adulthood and could potentially be an avenue to test the duration versus timing question. However, we reported that chronic CTEP treatment does not impair SRP beginning at P30.

We have hypothesized that a deficient NR2B to NR2A subunit switch as a result of developmental mGluR5 inhibition may account for the impairments in LTD and OD plasticity that we report. A 2011 study showed that mGluR5 is indeed required for the

normal developmental shift from NR2B to NR2A in hippocampus and layer II/III of visual cortex; however, this prediction has not yet been tested directly in layer IV (Matta *et al.*, 2011). We plan to test this prediction experimentally by measuring the kinetics of isolated NMDA currents in layer IV neurons. NR2A currents are known to have faster kinetics than NR2B currents (Townsend *et al.*, 2004, Vicini *et al.*, 1998). Additionally, we plan to measure sensitivity to ifenprodil, an NR2B-specific antagonist. If *Grm5*$^{-/-}$ mice indeed show enhanced NR2B relative to NR2A in layer IV, this would suggest that the timing of mGluR5 inhibition is more important than its duration. We would then predict that inhibition of mGluR5 beginning after the NR2B-to-NR2A shift is complete would have little effect on forms of synaptic plasticity in visual cortex.

5.5: Conclusions

FMRP and mGluR5 act in functional opposition at the synapse to regulate protein synthesis. We have demonstrated that inhibition of mGluR5 beginning in young adulthood is sufficient to correct numerous Fragile X phenotypes in mouse, suggesting that FX is in part a disease of acutely altered synapses. In parallel, we have demonstrated that chronic mGluR5 signaling is necessary for forms of plasticity in visual cortex which are canonically NMDA receptor-dependent. Thus a study with direct translational relevance has also shed light on the basic biology underlying Fragile X, and a basic study has provided caution for translating CTEP's potential side effects as well as its benefits. Future work will be aimed at understanding the mechanisms linking mGluR5 to NMDAR-dependent plasticity and at understanding common cell and circuit-level phenotypes underlying multiple mouse models of autism and intellectual disability.

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