FRAGILE X MENTAL RETARDATION PROTEIN IS REQUIRED FOR CHEMICALLY-INDUCED LONG-TERM POTENTIATION OF THE HIPPOCAMPUS IN ADULT MICE

by

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Abstract

Fragile X syndrome (FXS) is caused by the lack of fragile X mental retardation protein (FMRP). The animal model of FXS, *Fmr1* knockout (KO) mice, shows impairment in hippocampus-dependent learning and memory. However, results for long-term potentiation (LTP), remain inconclusive in the hippocampus of *Fmr1* KO mice. Here, we demonstrate that FMRP is required for glycine-induced LTP (Gly-LTP) in the CA1 of hippocampus. The Gly-LTP requires activation of postsynaptic NMDA receptors and metabotropic glutamateric receptors, as well as the subsequent activation of extracellular signal-regulated kinase (ERK) 1/2. However, paired-pulse facilitation was not affected by glycine treatment. Our study provide evidences that FMRP participates in Gly-LTP by regulating the phosphorylation of ERK1/2, and that improper regulation of these signaling pathways may contribute to the learning and memory deficits observed in FXS.

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List of Appendices

AC1 -- adenylyl cyclase 1

ACC -- anterior cingulate cortex

AMPA -- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic

Arc -- activity-regulated, cytoskeletal-associated protein

BDNF -- brain-derived neurotrophic factor

CaMKII -- calcium/calmodulin-dependent protein kinase II

CaMKIV -- calcium/calmodulin-dependent kinase IV

cAMP -- cyclic adenosine monophosphate

CREB -- cAMP response element-binding protein

DHPG -- (RS)-3,5-dihydroxyphenylglycine

ERK -- extracellular signal-regulated kinase

FXS -- fragile X syndrome

FMR1 -- fragile X mental retardation 1

FMRP -- fragile X mental retardation protein

KO -- knockout

LTD -- long-term depression

LTP -- long-term potentiation

MAPK -- mitogen-activated protein kinase

mGluRs -- metabotropic glutamate receptors

NMDA -- N-methyl-D-aspartate

PKA -- protein kinase

PKC -- protein kinase C

WT -- wild-type

Chapter 1 Introduction and Background

1 Introduction and Background

1.1 Fragile X syndrome and fragile X mental retardation protein

1.1.1 Fragile X syndrome and fragile X mental retardation 1 gene

Fragile X syndrome (FXS) is the most frequent form of inherited mental retardation and often presents as an autism spectrum disorder, with approximately 1 in 4500 males and 1 in 9000 females affected (Garber *et al.* 2006, Garber *et al.* 2008, Huber 2006). FXS and fragile X-associated tremor/ataxia syndrome, as well as premature ovarian failure are all caused by the expansion of CGG-repeats in the 5' untranslated region of fragile X mental retardation 1 (*FMRI*) gene which encodes fragile X mental retardation protein (FMRP) (Eichler *et al.* 1994, Siomi *et al.* 1994). FXS is caused by a massive expansion of CGG beyond 200 repeats compared to normal alleles containing 30 repeats on average; and both fragile X-associated tremor/ataxia syndrome and premature ovarian failure are associated with premutation alleles (55–200 repeats) (Feng *et al.* 1995, Pieretti *et al.* 1991). Both abnormal alleles result in transcriptional dysregulation of the *FMRI* gene and therefore the abnormal expression of FMRP. Whereas FXS is almost exclusively caused by a complete transcriptional shutdown of the *FMRI* gene, the premutation-associated diseases are caused by excess transcript levels leading to — at least for fragile X-associated tremor/ataxia syndrome — toxic effects of repeat-containing mRNA (fig 1.1).

Mental retardation is the most prominent phenotype of FXS, with IQ values typically between 20 and 70. FXS patients generally exhibit social deficits, anxiety disorders, hyperactivity, hypersensitivity and repetitive behaviors (O'Donnell & Warren 2002). They have severe problems in performing tasks that require the central executive of working memory and their language and intellectual abilities develop slower compared to normal people (Hall *et al.* 2008). Some FXS patients may also exhibit autism phenotypes. Besides these mental disabilities, FXS patients show a spectrum of characteristic physical, including elongated face, large or protruding ears, flat feet, larger testicles in men, and low muscle tone. Some patients may also have poor motor coordination, and an increased incidence of epilepsy. Consistent with the neurological deficits seen in FXS, patients have significantly longer, immature spines and fewer short, mature spines than did controls. Spine density in these patients was also increased compared to

control males of the same age, suggesting a defect in spine maturation and elimination in fragile X patients (O'Donnell & Warren 2002).

Figure 1.1. FMR1 gene and FMRP. (From Bassell, G. J. and Warren, S. T. *Neuron*, 60, 201-214. 2008)

1.1.2 The mouse model for Fragile X syndrome

To further explore the role of *FMR1* gene in FXS and overcome the limitation of performing research in the brains of humans, the mouse model for FXS—*fmr1* knockout (KO) mouse was created in 1994 by the insertion of a neomycin cassette into exon 5 of the murine gene (Bakker *et al.* 1994). *FMR1* is highly conserved between humans and mouse with a nucleotide and amino acid identity of 95% and 97% respectively, including the CGG repeat in the 5' untranslated region (Ashley *et al.* 1993). Consistent with the phenotype of FXS in humans, the *Fmr1* KO mice recapitulate many of symptoms of FXS, including hyperactivity, seizures and macroorchidism. In behavior tests, *Fmr1* KO mice exhibit increased locomotor activity and reduced habituation in an open field, and increased susceptibility to audiogenic seizure. Additionally, mild learning deficits have been noted (Kooy 2003, Godfraind *et al.* 1996). Consistent with these behavioral deficits, our previous studies have reported that *Fmr1* KO mice are deficient at acquiring trace fear memory during training as well as in the

expression of trace fear memory during testing on the following day (Zhao *et al.* 2005). Besides these behavior abnormalities, *Fmr1* KO mice also have increased spine density and immature spine morphologies in hippocampus, neocortex, and cerebellum. These spine abnormalities are present in both young and adult *Fmr1* KO mice (Comery *et al.* 1997, Nimchinsky *et al.* 2001, Grossman *et al.* 2006). Spines are dynamic structures that can regulate many neurochemical events related to synaptic transmission and modulate synaptic efficacy. These pathology and anatomical studies performed on FXS patients and *Fmr1* KO mice suggest that the lack of FMRP expression may alter the development of synaptic connectivity and plasticity and therefore contribute to alterations in dendritic spine structure and the deficits of brain functions.

1.1.3 Fragile X mental retardation protein

FMRP, a 71 kD protein encoded by *FMR1* gene, is widely expressed in fetal and adult tissues, with the most abundant expression in brain and testes (fig 1.2). As an RNA-binding protein, FMRP associates with large polyribosomes as part of a messenger ribonucleoprotein complex and binds to as many as 400 different brain mRNA transcripts, including its own (Jin *et al.* 2004). It is founded that the complex of FMRP granules localize to both developing axons and dendrites, including filopodia, dendritic spines, and growth cones (Antar *et al.* 2005).

Figure 1.2. Structure model of FMRP.

FMRP is a highly conserved protein and contains a functional nuclear localization signal and a nuclear export signal, consistent with the observation that FMRP shuttles between the nucleocytoplasmic spaces (Eberhart *et al.* 1996). It is thought that FMRP is involved in translational control and could suppress translation both in vitro and in vivo and that most of the well-characterized targets of FMRP regulation are associated with cytoskeletal and synaptic organization. Previous studies showed that (RS)-3,5-dihydroxyphenylglycine (DHPG), the agonist of group I metabotropic glutamate receptors (mGluRs), could up-regulate the expression of FMRP in wild-type (WT) mice and induce increased long-term depression (LTD) in the hippocampus and the anterior cingulate cortex (ACC) in *Fmr1* KO mice, suggesting that the protein synthesis-dependent functions of mGluRs activation are exaggerated due to the lack of FMRP in fragile X syndrome (Huber *et al.* 2002, Wang *et al.* 2008b).

Further evidences from our lab showed that activation of group I mGluRs is linked to cyclic adenosine monophosphate (cAMP) -dependent protein kinase (PKA) through adenylyl cyclase 1 (AC1) and that AC1 and calcium/calmodulin-dependent kinase IV (CaMKIV) contribute to the upregulation of FMRP and the phosphorylation of cAMP response element-binding protein (CREB) induced by stimulating group I mGluRs (fig 1.3) (Wang *et al.* 2008b, Wang *et al.* 2009). Our lab also approved that FMRP acts as a key messenger in both NMDA receptor-dependent LTP and DA receptor-mediated facilitation in the forebrain area (Wang *et al.* 2008a). It is thought that the function of group I mGluRs activation require the translation of preexisting mRNA near active synapses and that mGluRs in the central nervous system contribute to higher brain functions including learning/memory, persistent pain, and mental disorders (Bear *et al.* 2004). Based on these observations, FMRP might be involved in the long term synaptic transmission and the *Fmr1* KO mice may have deficits in long-term potentiation (LTP) due to the lack of FMRP.

Figure 1.3. FMRP is downstream of the group I mGluR–CREB pathway in ACC neurons. (From Wang, H. *J Neurosci*, 28, 4385-4397. 2008)

1.2 LTP in *Fmr1* KO Mouse

1.2.1 Learning, memory and LTP

Synaptic plasticity is the basis of information storage in the brain. It enables the brain to store and use vast amounts of information in the form of learnt behaviors and conscious memories. LTP and LTD are two well studied models of synaptic plasticity that may serve as cellular substrates for cognitive behaviors such as learning and memory (Madison *et al.* 1991). LTP, which is characterized by a long-lasting increase in synaptic strength that is caused by a brief period of coordinated neuronal activity, has been most extensively studied in the hippocampus, a brain region long known to be essential for learning and memory. LTP is a fundamental property of the majority of excitatory synapses in the mammalian brain and shares many features with long-term memory. Therefore, it is widely considered one of the major cellular mechanisms that underlie learning and memory (Bliss & Collingridge 1993). As the opposing process, LTD is the weakening of a neuronal synapse that lasts from hours to days and it results

from either strong synaptic stimulation or persistent weak synaptic stimulation. In hippocampus, it is thought that LTD may be important for the clearing of old memory traces (Bear & Malenka 1994, Barrionuevo *et al.* 1980).

Most of the experimental works about LTP focus on excitatory synapses in the hippocampus, specifically on the synapses between the Schaffer collateral and commissural axons and the apical dendrites of CA1 pyramidal cells. In the CA1 region of the hippocampus, it is generally thought that the triggering of LTP requires synaptic activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors. The activation of NMDA receptors requires depolarization of the postsynaptic cell, which is usually accomplished experimentally by repetitive tetanic stimulation (high frequency stimulation) of synapses or by directly depolarizing the cell while continuing low-frequency synaptic activation (a pairing protocol) (Malenka & Nicoll 1999). For the early-phase LTP, it first requires the activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors, which provides the majority of inward current for generating synaptic responses. After the depolarization of the postsynaptic cell, NMDA receptors are activated and Mg^{2+} dissociates from its binding site within the NMDA receptor channel, allowing Ca^{2+} as well as Na^{+} to enter the dendritic spine. The rapid rise in intracellular calcium concentration triggers the short-lasting activation of several enzymes that mediate early-phase LTP. Among these enzymes, calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), PKA and mitogen-activated protein kinase (MAPK) play an important role in LTP (fig 1.4)(Sweatt 1999, Malenka & Nicoll 1999).

For the late-phase LTP, it requires gene transcription and new protein synthesis in the postsynaptic cell (Frey *et al.* 1996, Frey *et al.* 1988). If protein kinases, which are involved in early-phase LTP, were persistently activated, they might induce gene expression and protein synthesis and therefore cause late-phase LTP (Lynch 2004b). It is thought that MAPK, specifically the extracellular signal-regulated kinase (ERK) subfamily of MAPKs, may be the molecular link between early-phase LTP and late-phase LTP (Sweatt 1999). The activation of ERK could phosphorylate a number of cytoplasmic and nuclear molecules including the transcription factors, such as CREB. The phosphorylation of CREB may trigger the synthesis of proteins which are required for the maintenance of late-phase LTP (fig 1.4)(Malenka & Nicoll 1999, Lynch 2004b).

Figure 1.4. A schematic model of the molecular events contributing to the early and late phase of LTP in the Schaffer collateral pathway. (From Eric R. Kandel, *Principles of Neural Science*)

Based on the observation that patients with FXS and *Fmr1* KO mice have deficits in learning and memory, it is suggested that LTP, which is considered as the basis for long-term memory, might be impaired in the hippocampus of *Fmr1* KO mice. Besides, previous studies showed that the CaMKII and ERK pathways are dysregulated in the mGluRs dependent LTD process in the *Fmr1* KO mice (Hou *et al.* 2006, Kim *et al.* 2008, Napoli *et al.* 2008). It is possible that these molecular pathways might also be changed in the LTP process in the hippocampus of *Fmr1* KO mice due to the lack of FMRP.

1.2.2 Previous studies about LTP in *Fmr1* KO mice

Possibly reflecting their poor performance in some types of memory and learning, *Fmr1* KO mice show altered synaptic plasticity in brain areas involved in learning and memory. The *Fmr1* KO mice exhibit several behavioral traits similar to those of fragile X patients, as demonstrated by impaired performance in Morris water maze and trace fear memory tests (Kooy 2003, Godfraind *et al.* 1996, Zhao *et al.* 2005).

Consistent with these behavioral deficits, our previous studies have reported the loss of LTP in the ACC and the lateral amygdala (LA) in *Fmr1* KO mice (Zhao et al. 2005). Attenuated LTP was also found in other areas such as neocortex and somatosensory cortex (Table 1.1). However, in the hippocampus of fragile X mice, it is thought that there is no alteration in LTP but increased long term LTD instead (Godfraind et al. 1996, Huber et al. 2002, Li et al. 2002). Recently, Lauterborn and colleagues showed that a train of five theta bursts stimulation could induce decreased LTP in the hippocampus in *Fmr1* KO mice (Lauterborn et al. 2007). Besides, Hu and colleagues found that LTP in hippocampal CA1 pyramidal neurons was reduced in *Fmr1* KO mice by pairing protocol using whole-cell voltage-clamp technique in young animals (Hu et al. 2008).

Method	Region	Results	References
Tetanic stimulation	Hippocampus	No impairment in hippocampus	Godfraind et al., 1996
TBS (10 burst)	Hippocampus	No impairment in hippocampus	Paradee et al., 1999
Tetanic stimulation	Somatosensory Hippocampus	Reduced LTP in somatosensory but not in hippocampus	Li et al., 2002
TBS (10 burst)	Piriform Hippocampus	Reduced LTP in anterior piriform cortex (APC) from <i>Fmr1</i> KO mice, but not in hippocampus	Larson et al., 2005
Pairing protocol (80 pulses, 2 Hz, +30 mV)	ACC Lateral amygdale (LA)	Loss of LTP in the ACC and LA	Zhao et al., 2005
Tetanic stimulation	Neocortex	LTP was severely attenuated	Wilson et al., 2007
Spike-timing	Prefrontal cortical	LTP is not so much absent, but the threshold for induction is increased.	Meredith et al., 2007
TBS (5 burst)	Hippocampus	BDNF rescued impaired LTP in hippocampus	Lauterborn et al., 2007
Pairing protocol (200 pulses, 2 Hz, - 5 mV)	Hippocampus	LTP is reduced in hippocampal CA1 pyramidal neurons	Hu et al., 2008

Table 1.1. Previous studies on LTP in *Fmr1* KO mice

It is most likely different forms of LTP can be induced by various induction protocols, and different signaling pathways are involved. Based on these evidences, FMRP might play an important role in LTP but the mechanism of this pathway is still controversial. Other types of LTP are needed to performed in the hippocampus of *Fmr1* KO mice.

1.2.3 Glycine and chemically-induced LTP

Amino acids are among the most abundant of all neurotransmitters present within the central nervous system (CNS). Glycine is the major inhibitory neurotransmitter in the brainstem and spinal cord, where it participates in a variety of motor and sensory functions. Glycine is also present in the forebrain, where it has recently been shown to function as a coagonist of NMDA receptors (fig 1.5). It has a binding site on the NR1 subunit of the NMDA receptors and could promote the actions of glutamate in forebrain area (Schell 2004).

Fig 1.5. Glycine binding site on NMDA receptors.

Besides the binding site on NMDA receptors, there is another target of glycine in the forebrain. Glycine transporters (GLYT) can regulate and maintained the glycine concentrations around NMDA receptors to a sub-saturated level (Smith *et al.* 1992). There are two types of GLYT: GLYT1 and GLYT2. GLYT2 is mainly expressed in the spinal cord and brainstem, co-localizing with inhibitory glycine receptors

(Aragon & Lopez-Corcuera 2003), while GLYT1 is predominantly expressed in glia and neurons in forebrain areas and is associated with glutamatergic synapses (Cubelos *et al.* 2005).

There are also glycine receptors in the CNS. Glycine receptor is one of the most widely distributed inhibitory receptors in the central nervous system and has important roles in a variety of physiological processes, especially in mediating inhibitory neurotransmission in the spinal cord and brain stem (Lynch 2004a). The glycine receptors can be activated by a range of simple amino acids including glycine, β -alanine and taurine, and can be selectively blocked by the high-affinity competitive antagonist strychnine. Strychnine-sensitive glycine receptors are members of a family of ligand-gated ion channels.

Generally, it is thought that there are two kinds of LTP: the NMDA-dependent LTP and NMDA-independent LTP. The concentration of glycine in the synapse cleft could have effect on the excitability of NMDA receptors. When more glycine is binding to NMDA-receptors, it could activate the NMDA receptor and therefore induce LTP. However, when the glycine concentration is too high, it will result toxic effect on NMDA receptors and cause the loss of LTP (Martina *et al.* 2004). The binding site on NMDA receptors, glycine transporters, and glycine receptors are all glycine buffers in the CNS and could balance the glycine concentration. Through apply proper glycine in the hippocampus, LTP could be induced.

1.3 Extracellular signal-regulated kinase (ERK) pathways in *Fmr1* KO Mouse

1.3.1 Extracellular signal-regulated kinase (ERK) pathways

MAPK, a family of serine/threonine protein kinases, can transduce extracellular signals from cell surface receptors to the cell nucleus and therefore respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (Pearson *et al.* 2001). MAPK could also respond to growth factors such as brain-derived neurotrophic factor (BDNF) or nerve growth factor. Extracellular stimuli lead to activation of MAPK via a signaling cascade ("MAPK cascade") composed of MAPK, MAP kinase kinase (MKK, MEKK, or MAP2K), and MAP kinase kinase kinase (MKKK or MAP3K) (Chang & Karin 2001). MAPK family contains three distinct groups in mammals: ERK, c-Jun N-terminal kinases (JNKs) and p38 isoforms. Both JNK and p38 signaling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis (fig 1.6) (Pearson *et al.* 2001).

ERK pathways have several subgroups—ERK1/2, ERK 3/4, ERK5, ERK7/8. ERK1 is the 44 kD isoform of MAPK and ERK2 is the 42kD isoform of MAPK. ERK1 and ERK2 have 85% sequence identity and are considered to be functionally redundant. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens, could activate the ERK pathway. ERKs are known to activate transcription factors and some downstream protein kinases and involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells (Pearson et al. 2001). In the signaling pathways, distinct stimuli appear to selectively trigger the activation of a particular MAPK cascade and therefore the ERKs are activated only by MEK1/2.

Fig 1.6. MAPK signaling pathways.

1.3.2 The role of ERK in LTP

Recent evidence has shown that neuronal activity-dependent modulation of translation initiation factor activity by the ERK1/2 signaling pathway plays an important role in the establishment of late-phase LTP. ERK is the downstream target of cAMP signaling pathways and could be activated by membrane depolarization and activation of glutamate receptors (Impey *et al.* 1998, Davis *et al.* 2000, Wei & Zhuo

2008). It was showed that the activation of ERK2 is required for the NMDA-dependent LTP. The phosphorylation of p42 MAPK could activate CREB and then participate in high frequency-stimulated LTP (English & Sweatt 1996, Huang *et al.* 2000). In most cell signaling pathways, stimuli that trigger the ERK cascade activate both ERK1 and ERK2. However, in the NMDA-dependent LTP process, the activation of PKC and glutamate receptor could only lead to the activation of ERK2 but not ERK1 (English & Sweatt 1997, English & Sweatt 1996).

1.3.3 ERK and *Fmr1* KO mice

There are two distinct mechanisms for mRNA recognition with FMRP, the first involving direct mRNA binding mediated by G quartet structures in target mRNAs, and the second involving indirect binding to target mRNAs mediated by the noncoding dendritic RNA BC1 (Darnell *et al.* 2001, Zalfa *et al.* 2003). The BC1-dependent association of FMRP with several dendritic mRNAs, including α CaMKII and activity-regulated, cytoskeletal-associated protein (Arc), appears to result in their translational repression. Both the α CaMKII and Arc are involved in the LTP process and related to ERK; and therefore the interruption in the α CaMKII and Arc pathways due to the lack of FMRP in the *Fmr1* KO mice might inflect the ERK pathway and cause the change of LTP. Previous study showed that loss of FMRP could block the activation of ERK in DHPG-stimulated LTD in *Fmr1* KO mice (Kim *et al.* 2008, Hou *et al.* 2006). However, the possible relationship between FMRP and ERK in hippocampal LTP has not been reported. Future studies are needed to address the role of FMRP in the ERK pathways during the LTP process.

1.4 Research objectives

Different paradigms of stimulation may induce distinct LTP and different signaling pathways are involved in. High frequency stimulation and TBS (10 burst) are strong stimulus and therefore may mask the impairment caused by the loss of FMRP. So it might be possible to observe the changes of LTP in the hippocampus of *Fmr1* KO mice if a mild stimulation is used. In this project, we try to address whether other types of LTP is modified in the hippocampus of *Fmr1* KO mice. Also we intend to check through which molecular pathway FMRP is involved in the regulation of LTP.

In the present study, by using bath application of glycine, we were able to induce synaptic LTP in CA1 region of the hippocampal slices. We found that LTP induced by glycine application was reduced in

Fmr1 KO mice. The Gly-induced LTP was blocked by NMDA antagonist AP5 and mGluRs antagonist DL-AP3. However, the basal synaptic transmission and NMDA receptor mediated EPSCs remained unchanged in the hippocampus of *Fmr1* KO mice. This glycine treatment provided the opportunity to measure biochemical changes in potentiated hippocampal synapses. The immunoblotting results showed that p42 MAPK phosphorylation level was increased after application of glycine in WT mice but phosphorylation of p42 and p44 MAPK were decreased in *Fmr1* KO mice. The evidence that MEK inhibitor U0126 blocked Gly-LTP further confirms the involvement of MAPK pathway in Gly-LTP. Together with the recent reports of on the requirement of FMRP for activation of ERK activity by DHPG (Hou et al. 2006, Kim et al. 2008), these results suggest that FMRP may contribute to hippocampal learning and memory through direct or indirect regulation of ERK1/2 signaling pathways.

Chapter 2 Materials and Methods

2 Materials and Methods

2.1 Animals

For adult brain slice recordings, male mice were 6-8 weeks of age. WT and *Fmr1* KO mice of the FVB.129P2-*Fmr1*^{tm1Cgr} strain were used and were generously provided by Dr. W.T. Greenough. In this "sighted" strain, the *Pde6b* gene, which in a mutated form codes for retinal degeneration in FVB mice, has been selectively replaced by crossing with strains carrying the non-defective allele (Weiler *et al.* 2004). All mice were housed under a 12:12 light cycle with food and water provided ad libitum. The Animal Care and Use Committee of University of Toronto approved all mouse protocols.

2.2 Field potential recording

WT and *Fmr1* KO mice, 6-12 weeks of age, were anaesthetized with halothane and killed. The brain was then quickly removed and submerged in icy, oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 4.4 KCl, 1 NaH₂PO₄, 1 MgSO₄, 1.6 CaCl₂, 25 NaHCO₃, 10 Glucose, and 25 Sucrose. Slices were cut roughly perpendicular to the longitudinal axis of the hippocampus at a thickness of 350µm. Slices were placed on an interface recording chamber and allowed to recover for at least 2 hour prior to commencement of recording.

Through recording, oxygenate ACSF was infused at a rate of approximately 60ml/h. Additionally, warmed and humidified 95% O₂-5% CO₂ was blown into the infusing ACSF solution. Slices were maintained at 28±1°C. Glass electrodes filled with ACSF were used to record field activity. Extracellular signals were amplified with a differential AC amplifier. Stimulation pulses were generated using a stimulator and delivered through bipolar electrodes made of twisted nichrome wire. These field responses were evoked every 50s by stimulation Schaffer collateral-commissural fibers. Input-output curves were constructed by varying stimulus intensity between 10 and 60 V and measuring the fEPSP slope. Five responses were collected and averaged at each stimulation intensity. Paired-pulse curves were determined by stimulating the synapses with twin pulses at interpulse intervals of 25-150 ms. For LTP recording, stable field excitatory postsynaptic potentials (EPSPs) were evoked by adjusting stimulation intensity so

that a half-maximal fEPSP was elicited. The responses were sampled every 50s and averaged every 5min. The synaptic strength was calculated using the initial rising slope phase of the fEPSP to avoid contamination of the response by the population spike. Data were normalized with respect to the mean values of the responses in baseline period. Data were collected by Clampex 5.0 and analyzed using the pClamp 9.2 software. The amount of LTP in Figure 1C, Figure 2C and Figure 3C were measured by transmission for 50min after glycine treatment. For the statistical comparisons of Gly-LTP in two different groups, data were calculated after baseline recording, from 0min to 80min.

Drugs applied by addition to the ACSF included: glycine (EMD, Germany), D-2-Amino-5-phosphonovaleric acid (AP5, Sigma, USA), DL-2-Amino-3-phosphonopropionic acid (DL-AP3, Tocris, USA), picrotoxin (Sigma, USA), U0126 (Tocris, USA). The drugs were prepared as stock solution, stored frozen in the dark, and diluted to their final concentration in the perfusion solution immediately before use.

2.3 Patch-clamp recording

Coronal brain slices (350 μ m) containing hippocampus were prepared using standard methods (Wu *et al.* 2008). Slices were transferred to a submerged recovery chamber at room temperature for at least 1 hour before electrophysiological experiments, and were incubated with oxygenated (95 % O₂ and 5 % CO₂) artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgSO₄, 25 NaHCO₃, 1 NaH₂PO₄, and 10 glucose.

Whole-cell patch clamp recording experiments were performed in a recording chamber on the stage of an Olympus BX51WI microscope (Tokyo, Japan) with infrared DIC optics for visualization of whole-cell patch clamp recordings. The recording pipettes (3-5 M Ω) were filled with solution containing (in mM): 102 CsMeSO₃, 3.7 NaCl, 5 QX-314 chlorides, 5 TEA-Cl, 10 BAPTA, 0.2 EGTA, 20 HEPES, 0.3 Mg-ATP, and 0.3 Na₃-GTP (adjusted to pH 7.2 with CsOH). Excitatory postsynaptic currents (EPSCs) were recorded from hippocampal CA1 pyramidal neurons with an Axon 200B amplifier (Molecular devices, CA) and the stimulations were delivered by a bipolar tungsten stimulating electrode placed in striatum radium. Picrotoxin (100 μ M) was always present to block GABA_A receptor-mediated inhibitory synaptic currents. AMPA receptor-mediated EPSCs were induced by repetitive stimulations at 0.05 Hz, and neurons were voltage clamped at -70 mV (liquid junction potential corrected). The NMDA receptor-mediated EPSCs were recorded in the presence of CNQX (20 μ M) and evoked at 0.05 Hz. Neurons were voltage-clamped at 0 mV in the input-output relationship experiments of

NMDA receptor-mediated EPSCs. Data were discarded if access resistance changed by more than 15% during experiments. Data were collected and analyzed using the pClamp 9.2 software.

2.4 Western blot

Western blot was conducted as previously described (Wang et al. 2008a, Wang *et al.* 2008b).

Brain tissues were harvested and homogenized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1% SDS, 1X protease inhibitor cocktail (Sigma, MO), and 1X phosphatase inhibitor cocktail 1 and 2 (Sigma, MO). Protein was quantified by Bradford assay.

Electrophoresis of equal amounts of total protein was performed on SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes at 4°C for Western blot analysis. Membranes were probed with 1:1000 dilution of anti-FMRP (Chemicon, CA), 1:1000 dilution of anti-FMRP (rabbit polyclonal kindly provided from Dr. C. Bagni) (Ferrari *et al.* 2007), and 1:1000 dilution of anti phosphorylation p44/p42 MAPK and p44/p42 MAPK (Chemicon, CA). The membranes were incubated in the appropriate horseradish peroxidase-coupled secondary antibody diluted 1:3000 for 1 h followed by enhanced chemiluminescence (ECL) detection of the proteins with Western lightning chemiluminescence reagent plus (Perkin Elmer Life sciences, MA) according to the manufacturer's instructions. To verify equal loading, membranes were also probed with 1:3000 dilution of anti-actin antibody (Sigma, MO). The density of immuno-blots was measured using NIH ImageJ software.

2.5 Data analysis

Statistical comparisons were made using the paired t-test or One way or two way ANOVA (Student-Newmann-Keuls test was used for post hoc comparison). All data were presented as the mean $\pm$ S.E.M. In all cases, $p < 0.05$ is considered statistically significant.

Chapter 3

Results

3 Results

3.1 Glycine-induced LTP was decreased in *Fmr1* KO mice

Previous studies have shown that perfusion of the NMDA receptor co-agonist glycine could induce LTP in hippocampus cultured neurons and slice (Serulle *et al.* 2007, Wang *et al.* 2008c, Lu *et al.* 2001, Musleh *et al.* 1997). However, there are no reports of glycine-induced LTP in *Fmr1* KO mice and whether Gly-LTP in *Fmr1* KO mice is different from WT mice remains unknown, although several studies have found that LTP induced by high frequency stimulation and TBS in the hippocampus of *Fmr1* KO mice were almost unchanged, compared with the WT mice (Li *et al.* 2002, Godfraind *et al.* 1996, Paradee *et al.* 1999). In the present study, the hippocampus from 8-10 weeks old mice were separated and cut into 350µm slices. fEPSP was elicited in the CA1 region by stimulating the Schaffer commissural projections and glycine (1 mM) was applied in ACSF after at least 30 min of baseline recording. 30 min after glycine treatment, there was an obvious and progressive increase of fEPSP slope in both WT (361%) and *Fmr1* KO (213%) mice, compared to baseline fEPSP slope (fig 3.1B). However, the LTP was attenuated and the increase of fEPSP slope in *Fmr1* KO mice was smaller than the WT mice (WT, $n=7$; *Fmr1* KO $n=8$; $p < 0.01$, Two-way ANOVA, fig 3.1B and C). The fEPSP slopes increased gradually after glycine application and reached a plateau following washout with ACSF. However, the slope continued to be much larger in the WT mice than in the *Fmr1* KO mice. The Gly-LTP could last for more than an hour, and so the normalized fEPSP slope was measured during the last hour of the recording. In the *Fmr1* KO mice, the slope was attenuated when compared to WT mice (WT, $n=7$; *Fmr1* KO $n=8$; $p < 0.01$, t-test, fig 3.1C).

Figure 3.1. Glycine-induced LTP was decreased in the hippocampus of *Fmr1* KO mice.

A, Sample traces show evoked potential changes (top) and the long term potentiation (bottom) induced by glycine in WT and *Fmr1* KO mice. The Gly-LTP lasts more than one hour.

B, Application of glycine (1 mM) induced LTP in WT and *Fmr1* KO mice. Glycine was treated for 30 minutes and Gly-LTP was reduced in *Fmr1* KO mice, compared with WT mice (WT, $n=7$; *Fmr1* KO, $n=8$; $p < 0.01$, Two-way ANOVA).

C, Average value of fEPSP percentage in the maintenance phase of Gly-LTP (recordings after glycine application) was calculated (WT, $n=7$; *Fmr1* KO, $n=8$; $p < 0.01$, t-test).

fEPSP slopes were normalized by average value of baseline slopes. * $p < 0.05$, ** $p < 0.01$.

3.2 Gly-LTP and neurotransmitter receptors

3.2.1 Gly-LTP is NMDA receptor and mGluR dependent

The induction of LTP normally requires the activation of NMDA receptors and a rise in postsynaptic Ca^{2+} . However, not all forms of LTP are NMDA receptor-dependent (Nicoll & Malenka 1995, Bliss & Collingridge 1993). To investigate whether activation of NMDA receptors is involved in Gly-LTP, AP5 (100 μM), a selective NMDA receptor antagonist, was applied during glycine perfusion. We found that AP5 significantly reduced LTP in WT mice (glycine + AP5, $n=6$; glycine, $n=7$; $p < 0.01$, Two-way ANOVA, fig 3.2A). The percentage of fEPSP slope in WT mice was decreased to $154 \pm 4\%$ when AP5 was added to the glycine application solution (fig 3.2C), suggesting that Gly-LTP is NMDA receptor-dependent.

It has been previously reported that the DHPG-induced LTD was enhanced in the hippocampal Schaffer collateral synapses in *Fmr1* KO mice (Huber et al. 2002). Moreover, our previous work showed that activation of group 1 mGluR could up-regulate FMRP in ACC neurons through the Ca^{2+} -dependent signaling pathways in adult mice (Wang *et al.* 2008b). This suggests that group 1 mGluR might be involved in the reduced Gly-LTP observed in the hippocampus of *Fmr1* KO mice. To test this, Gly-LTP was recorded in the presence of DL-AP3 (300 μM), the selective antagonist of group 1 mGluR. We found that DL-AP3 blocked Gly-LTP in WT mice (glycine + DL-AP3 $n=5$; glycine, $n=7$; $p < 0.01$, Two-way ANOVA, fig 3.2B and C).

Similar experiments were conducted in the hippocampus of *Fmr1* KO mice. AP5 failed to produce further inhibition in *Fmr1* KO mice (glycine + AP5, $n=7$; glycine, $n=8$; $p > 0.05$, Two-way ANOVA, fig 3.3A). However, application of DL-AP3 slightly reduced the fEPSP slope in the hippocampus of *Fmr1* KO mice (glycine + DL-AP3, $n=7$; glycine, $n=8$; $p < 0.05$, Two-way ANOVA, fig 3.3 B), although the effect was not as great as in the WT mice. Taken together, these results indicate that Gly-LTP is NMDA receptor- and group 1 mGluR-dependent, and that these two signaling cascades are improperly regulated in *Fmr1* KO mice.

Figure 3.2. Gly-LTP was blocked by AP5 and DL-AP3 in the hippocampus of WT mice.

A, Application of AP5 (100 μ M), NMDA receptor antagonist, blocked Gly-LTP in WT mice (glycine + AP5, $n=6$; glycine, $n=7$, $p < 0.01$, Two-way ANOVA). AP5 was applied during glycine treatment. Sample traces of baseline and traces after AP5 application with glycine in WT mice were showed (top).

B, Application of DL-AP3 (300 μ M), mGluR antagonist, blocked Gly-LTP in WT mice (glycine + DL-AP3, $n=5$; glycine, $n=7$, $p < 0.01$, Two-way ANOVA). DL-AP3 was applied during glycine treatment. Sample traces of baseline and traces after DL-AP3 application with glycine in WT mice were showed (top).

C, Summary of the effects of AP5 and DL-AP3 on Gly-LTP in WT mice (glycine, $n=7$; glycine + AP5, $n=6$; glycine + DL-AP3, $n=5$).

fEPSP slopes were normalized by average value of baseline slopes. * $p < 0.05$, ** $p < 0.01$.

Figure 3.3. AP5 had no effect on Gly-LTP and DL-AP3 reduced Gly-LTP slightly in the hippocampus of *Fmr1* KO mice.

A, Application of AP5 (100 μ M) with glycine did not affect Gly-LTP in *Fmr1* KO mice (glycine + AP5, $n=7$; glycine, $n=8$, $p > 0.05$, Two-way ANOVA). Sample traces of baseline and traces after AP5 application with glycine in *Fmr1* KO mice were showed (top).

B, Gly-LTP in *Fmr1* KO mice was slightly reduced by DL-AP3 (300 μ M) application. DL-AP3 was applied during glycine treatment (glycine + DL-AP3, $n=7$; glycine, $n=8$, $p < 0.05$, Two-way ANOVA). Sample traces of baseline and traces after DL-AP3 application with glycine in *Fmr1* KO mice were showed (top).

C, Summary of the effects of AP5 and DL-AP3 on Gly-LTP in the hippocampus of *Fmr1* KO mice (glycine, $n=8$; glycine + AP5, $n=7$; glycine + DL-AP3, $n=7$).

fEPSP slopes were normalized by average value of baseline slopes. * $p < 0.05$, ** $p < 0.01$.

3.2.2 GABA receptors are not involved in Gly-LTP

GABA_A receptor-mediated inhibition plays an important role in regulating regional activity and in modulating the induction of synaptic plasticity (Steele & Mauk 1999). Recently, it was reported that picrotoxin, a GABA_A receptor antagonist, could rescue the memory deficits in aging mice (Yoshiike *et al.* 2008). To investigate whether GABA_A receptor-mediated inhibition is involved in Gly-LTP and whether picrotoxin could restore the decreased Gly-LTP observed in *Fmr1* KO mice, picrotoxin (100 μ M) was applied with glycine during the induction phase. We found that in the WT mice, the fEPSP slope after adding picrotoxin was not changed (glycine + picrotoxin, $n=6$; glycine, $n=6$; $p > 0.05$, Two-way ANOVA, fig 3.4A and B), which suggests that GABA receptors do not contribute to the Gly-LTP. Furthermore, picrotoxin treatment had no effect on Gly-LTP in the hippocampus of *Fmr1* KO mice (glycine + picrotoxin, $n=8$; glycine, $n=8$; $p > 0.05$, Two-way ANOVA, fig 3.4A and C).

Figure 3.4. Picrotoxin treatment had no effect on Gly-LTP in hippocampus.

A, Sample traces showed evoked potential changes after treatment with picrotoxin and glycine in WT and *Fmr1* KO mice.

B, Application of picrotoxin (100 μ M), antagonist of GABA_A receptors, did not change the Gly-LTP in WT mice (glycine+picrotoxin $n=6$; glycine, $n=6$, $p > 0.05$, Two-way ANOVA). Picrotoxin was applied during the treatment of glycine.

C, Application of picrotoxin (100 μ M) did not affect the Gly-LTP in *Fmr1* KO mice (glycine+picrotoxin $n=8$; glycine, $n=8$, $p > 0.05$, Two-way ANOVA). Picrotoxin was applied during the treatment of glycine.

fEPSP slopes were normalized by average value of baseline slopes.

3.3 Basal synaptic transmission and NMDA receptor-mediated EPSCs in *Fmr1* KO mice

3.3.1 Presynaptic mechanisms are not involved in Gly-LTP

Both presynaptic and postsynaptic mechanisms may contribute to the expression of LTP. The presynaptic expression of LTP is generally thought to be accompanied by alterations in the paired-pulse facilitation (PPF) (Nicoll & Malenka 1995). To test whether presynaptic changes are involved in the Gly-LTP, PPF was measured before and after glycine application. The results showed that PPF induced at five different intervals did not differ after 30 min glycine application in WT mice ($n=8$, $p > 0.05$, Two-way ANOVA; fig 3.5A). This indicates that presynaptic mechanisms may not contribute to Gly-LTP.

3.3.2 Normal synaptic transmission in *Fmr1* KO mice

FMRP is believed to be important in the regulation of protein synthesis needed for synaptic function in synapses by binding with mRNAs (Bassell & Warren 2008). Experiments were conducted to examine whether the reduced LTP in *Fmr1* KO mice is due to the altered basal synaptic transmission. The input-output relationship was observed in field recording and there were no significant differences between WT and *Fmr1* KO mice at various stimulus intensities (WT, $n=10$; *Fmr1* KO, $n=8$; $p > 0.05$, two-way ANOVA; fig 3.5B). Therefore, the loss of FMRP had no effect on fEPSP amplitude under different stimulus intensities.

3.3.3 NMDA receptor-mediated EPSCs are unchanged in *Fmr1* KO mice

To further explore the synaptic mechanism behind the reduced Gly-LTP in *Fmr1* KO mice, the voltage dependence of NMDA receptor-mediated currents in hippocampus were tested by whole cell patch-clamp recording. We recorded synaptic responses in voltage-clamp mode over a range of membrane potentials from -60 mV to +60 mV and found that the I-V relationship of the NMDA receptor-mediated EPSCs in *Fmr1* KO mice was similar to the I-V relationship in WT mice (WT, $n=5$; *Fmr1* KO, $n=5$; $p > 0.05$, Two-way ANOVA; fig 3.5C and D). Consistent with previous studies, the input-output curve in field recording and NMDA-mediated EPSCs did not change between WT mice and *Fmr1* KO mice in the hippocampus, indicating that there are no

significant differences in basal synaptic transmission (Zhao et al. 2005, Larson *et al.* 2005, Hu *et al.* 2008).

Figure 3.5. PPF and synaptic transmission in the hippocampus of WT and *Fmr1* KO mice.

A, Paired-pulse facilitation (PPF) at five different intervals did not differ after glycine (1 mM) application for 30 minutes ($n=8$, $p > 0.05$, Two-way ANOVA). Sample traces show PPF at 50ms interval, before and after glycine treatment, in the hippocampus of WT mice (top).

B, Input-output curves for fEPSP slope in the hippocampus of WT and *Fmr1* KO mice did not change (WT, $n=10$; *Fmr1* KO, $n=8$, $p > 0.05$, Two-way ANOVA). Sample traces show potential changes evoked by 10-60 mV stimulation in the hippocampus of WT and *Fmr1* KO mice (top).

C, Sample traces show NMDA receptors mediated-EPSCs evoked at holding potentials from -60 mV to +60 mV in the hippocampus of WT and *Fmr1* KO mice.

D, Current-voltage plots for NMDA receptors mediated-EPSCs in hippocampus did not differ between WT mice and *Fmr1* KO mice (WT, $n=5$ slices/3 mice; *Fmr1* KO, $n=5$ slice/3 mice, $p > 0.05$, Two-way ANOVA).

3.4 The expression of FMRP was decreased after glycine application

Our previous study showed that the expression of FMRP was increased in a time-dependent manner after group 1 mGluRs were activated by DHPG in the ACC (Wang et al. 2008b). In the present study, we wanted to examine whether FMRP is involved in NMDA receptor- and mGluR-dependent Gly-LTP. The samples were collected during the glycine application period as well as during the washout period and the expression level of FMRP was measured by immunoblotting analysis. The hippocampus slice was cut relatively perpendicular to the longitudinal axis of the hippocampus at a thickness of 350um and only the CA1 area was taken for immunoblotting analysis (fig 3.6A). We found that the expression of FMRP began to decrease 15 min after glycine (1 mM) application ($109 \pm 18\%$ at 5min; $93 \pm 8\%$ at 15min, $p < 0.05$; $77 \pm 9\%$ at 30 min, $p < 0.05$; compared to control level, $N=9$, t-test, fig 3.6C) and continued to decrease after glycine was washed out ($78 \pm 11\%$ at 5min, $p < 0.05$; $65 \pm 8\%$ at 15min, $p < 0.01$; $55 \pm 5\%$ at 30 min, $p < 0.01$; compared to control level, $N=7$, t-test, fig 3.6C). This indicates that glycine treatment can reduce the expression of FMRP in the hippocampus of WT mice, further supporting the idea that FMRP is required for Gly-LTP.

Figure 3.6. FMRP expression level in hippocampus was decreased after application with glycine.

A, Diagram of a sample slice (350 μm) collected from hippocampus and a model for preparation of slices.

B, Representative Western blot showed the expression level of FMRP.

C, The expression of FMRP began to decrease 15min after glycine (1mM) treatment and kept a lower level during the maintenance phase of Gly-LTP, after glycine was washed out (glycine treatment period $N=9$, washout period $N=7$).

Data were normalized by the control values. * $p < 0.05$, ** $p < 0.01$, compared to control.

3.5 Phosphorylation of ERK1/2 was involved in Gly-LTP

The glycine-induced LTP is NMDA receptor-dependent, suggesting that signaling pathways downstream of NMDA receptors may contribute to Gly-LTP in the hippocampus. The MAPK signaling pathways include five distinct groups: MAPK-ERK1/2, MAPK-p38, MAPK-JNK, MAPK-ERK3/4, and MAPK-ERK5 (Widmann et al. 1999). ERK, a downstream target of NMDA receptors and cAMP signaling pathways, plays an important role in the regulation of synaptic plasticity, learning and memory in the adult brain (Impey et al. 1999). Our previous studies showed that the ERK1/2 (p42/44 MAPK) signaling pathway is involved in the induction of cingulate LTP (Toyoda et al. 2007).

To investigate whether the ERK signaling pathway contributes to the reduced Gly-LTP in *Fmr1* KO mice, we observed the effect of glycine (1 mM) treatment on the expression and phosphorylation of ERK1/2 in the hippocampus slices. We found that the phosphorylation of p42 MAPK at threonine 202 site was triggered in the hippocampus of WT mice during bath application of glycine ($131 \pm 9\%$ at 5 min, $p < 0.01$; $136 \pm 16\%$ at 15 min, $p < 0.05$; $147 \pm 7\%$ at 30 min, $p < 0.01$; compared to control level, $N=6$, t-test, fig 3.7E). However, p42 MAPK was dephosphorylated during glycine application period in the hippocampus of *Fmr1* KO mice ($81 \pm 5\%$ at 5 min, $p < 0.05$; $78 \pm 10\%$ at 15 min, $p < 0.05$; $68 \pm 7\%$ at 30 min, $p < 0.01$; compared to control level, $N=7$, t-test, fig 3.7E). Surprisingly, the glycine treatment had no effect on the total protein level of p42 MAPK in the hippocampus of WT and *Fmr1* KO mice ($N=3$ for each group, $p > 0.05$, t-test, fig 3.7F). In contrast, the application of glycine did not change the phosphorylation level and the total protein level of p44 MAPK in the WT mice ($N=6$ for p-p44 MAPK, $N=3$ for p44 MAPK, $p > 0.05$, t-test, fig 3.7C and D). Interestingly, the phosphorylation of p44 MAPK in the *Fmr1* KO mice was decreased after glycine application ($83 \pm 5\%$ at 5 min, $p < 0.05$; $70 \pm 15\%$ at 15 min, $p < 0.05$; $60 \pm 5\%$ at 30 min, $p < 0.01$; compared to control level, $N=7$, t-test, fig 3.7C), although bath application of glycine had no effect on the expression of p44 MAPK in the hippocampus of *Fmr1* KO mice ($N=3$, $p > 0.05$, t-test, fig 3.7D). The data indicates that the p42 MAPK is downstream of cAMP pathways resulting in activation following glycine treatment and that the phosphorylation of ERK1/2 was improperly regulated in the hippocampus of *Fmr1* KO mice.

To further confirm that the phosphorylation of ERK1/2 is required for the Gly-LTP, we tested the effect of MEK inhibitor U0126 on Gly-LTP in the hippocampus of WT mice. U0126 (50 μ M) was applied with glycine treatment during field potential recording and the Gly-LTP was severely reduced by U0126 application (glycine + U0126, $n=6$; glycine, $n=7$; $p < 0.01$, Two-way ANOVA, fig 3.8A). This is consistent with our previous finding that MAPK and MEK inhibitors can block the LTP in the ACC (Toyoda et al. 2007). Taken together, these data suggest that the phosphorylation of ERK1/2 is involved in Gly-LTP, and that FMRP is important for the phosphorylation of ERK1/2

Figure 3.7. Phosphorylation of ERK1/2 was improperly regulated during glycine treatment in the hippocampus of *Fmr1* KO mice.

A, Representative Western blot showed the phosphorylation of ERK1/2 in the hippocampus of WT and *Fmr1* KO mice. Samples were collected at 5 min, 15 min and 30 min during glycine application.

B, Representative Western blot showed the expression of ERK1/2 in the hippocampus of WT and *Fmr1* KO mice. Samples were collected at 5 min, 15 min and 30 min during glycine application.

C, Application of glycine (1 mM) did not affect the phosphorylation of p44 MAPK in WT mice ($N=6$). Phosphorylation of p44 MAPK was decrease by glycine (1mM) treatment in *Fmr1* KO mice ($N=7$).

D, Application of glycine (1 mM) did not affect the expression of p44 MAPK in the hippocampus of WT and *Fmr1* KO mice ($N=3$ each group).

E, Application of glycine (1 mM) triggered the phosphorylation of p42 MAPK in the hippocampus of WT mice ($N=6$). But p42 MAPK was dephosphorylated in *Fmr1* KO mice by glycine treatment ($N=7$).

F, Application of glycine (1 mM) did not affect the expression of p42 MAPK in the hippocampus of WT and *Fmr1* KO mice ($N=3$).

Data were normalized by the control values. * $p < 0.05$, ** $p < 0.01$, compared to control; # $p < 0.05$, ## $p < 0.01$, compared to WT mice.

Figure 3.8. Gly-LTP was blocked by MEK inhibitor U0126 in the hippocampus of WT mice.

Application of U0126 (50 μ M) during glycine treatment reduced Gly-LTP in WT mice (glycine + U0126, $n=6$; glycine, $n=7$, $p < 0.01$, Two-way ANOVA). Sample traces of baseline and traces after U0126 plus glycine treatment in WT mice were showed (top).

fEPSP slopes were normalized by average value of baseline slopes.

Chapter 4 Discussion

4 Discussion

4.1 Conclusions

Absence of FMRP leads to increased spine density and immature spine morphologies in the hippocampus, neocortex, and cerebellum, suggesting that the lack of FMRP may have some negative effect on synaptic plasticity and learning (Weiler & Greenough 1999). Our previous work showed that trace fear memory was impaired and LTP in the ACC and the LA was abolished in *Fmr1* KO mice (Zhao et al. 2005).

Although numerous studies have focused on hippocampal LTP in *Fmr1* KO mice, with the exception of Lauterborn and colleagues who induced attenuated LTP by using a train of five theta bursts, generally no significant difference between WT mice and *Fmr1* KO mice was found (Li et al. 2002, Godfraind et al. 1996, Lauterborn et al. 2007, Larson *et al.* 2005). The variability seen in LTP may be due to different stimulation paradigms and different forms of LTP present in hippocampus; as Lauterborn and colleagues showed that a train of ten theta bursts induced nearly similar LTP in WT mice and *Fmr1* KO mice (Lauterborn et al. 2007). However, the mechanisms involved in the reduction of LTP in the hippocampus of *Fmr1* KO mice still remain to be elucidated.

4.2 Glycine-induced LTP

Application of glycine, which has a binding site on the NR1 subunit of the NMDA receptors, was first used to induce LTP in rat hippocampus in field recording, and this Gly-LTP could be blocked by ketamine (Shahi *et al.* 1993, Kew *et al.* 1998). Furthermore, Gly-LTP may share similar mechanisms with TBS-induced LTP, which could be occluded by glycine application (Musleh et al. 1997). Generally, it is thought that NMDA receptor is involved in Gly-LTP; but there are evidences that suggest the structural and functional modifications of AMPA receptors as well as the glycine transporter type 1 also participate in Gly-LTP (Musleh et al. 1997, Lu et al. 2001, Shahi et al. 1993, Martina *et al.* 2004). Here, we confirmed that 30min application of 1mM glycine could induce LTP in the hippocampus of WT mice for more than 1 hour. This induction of Gly-LTP could be blocked by bath application of NMDA receptor antagonist AP5. It is possible that other receptors, such as glycine and glycine receptors might also involved in

the Gly-LTP through regulating glycine concentration in the synapse clefts. Previous studies showed that the antagonist of glycine receptor, strychnine, could increase the Gly-LTP if proper dose of strychnine is chosen. Besides, it is reported that sarcosine, the compete antagonist of GLYT1, could increase Gly-LTP (Igartua *et al.* 2007). Under normal conditions, the concentration of glycine in the cerebrospinal fluid has been estimated to be in the low micromolar range (Westergren *et al.* 1994). When the glycine concentration is increased, the NMDA receptors are activated. However, blockade of GLYT1 or direct application of glycine at concentrations that exceed the level of saturation of the NMDA receptor glycine site result in reduced NMDA receptor currents and no increase in LTP magnitude (Martina *et al.* 2004).

In addition, we found that group 1 mGluR antagonist DL-AP3 could reduce the Gly-LTP, suggesting that mGluR might also contribute to the induction of Gly-LTP. Gly-LTP may provide a unique way to study long term synaptic transmissions while at the same time measuring chemical changes in transgenic mice models that mimic human mental disorders.

4.3 FMRP and hippocampal LTP

As an mRNA binding protein, FMRP is believed to associate with translating polyribosomes as well as regulating protein synthesis in the brain (Feng *et al.* 1997, Darnell *et al.* 2001). FXS patients have numerous neurological deficits, including developmental delay, attention deficit and hyperactivity disorder, and learning difficulties (Bassell & Warren 2008). *Fmr1* KO mice have abnormal dendritic spine density and morphologies in the hippocampus, neocortex, and cerebellum (Weiler & Greenough 1999). The synaptic transmission in *Fmr1* KO mice may be attenuated due to improper regulation of protein synthesis in absence of FMRP. Reduced LTP was observed in the somatosensory cortex and neocortex of *Fmr1* KO mice by tetanic stimulation (Li *et al.* 2002, Wilson & Cox 2007). A recent study has shown an age-dependent impairment of LTP, which was induced by 10 burst TBS in anterior piriform cortex of *Fmr1* KO mice older than 6 months (Larson *et al.* 2005). Additionally, our previous work showed that LTP induced by pairing presynaptic stimulation with postsynaptic depolarization was decreased in the ACC and the lateral amygdala of *Fmr1* KO mice (Zhao *et al.* 2005). The impairment of LTP was observed in different areas of the brain, with the exception of hippocampus, by distinct paradigms of stimulation protocols and the age-dependent attenuation effect was missing in hippocampus as well (Paradee *et al.* 1999, Godfraind *et al.* 1996, Larson *et al.* 2005). However, a recent study using a different stimulation protocol conducted by Lauterborn and colleagues demonstrated attenuation of LTP in the hippocampus of *Fmr1* KO mice (Lauterborn *et al.* 2007). This indicates that FMRP plays an

important role in LTP, but that different stimulation protocols are needed to study LTP in the hippocampus of *Fmr1* KO mice.

In the present study, we found that chemically-induced LTP, by glycine application (1mM), was decreased in the hippocampus of *Fmr1* KO mice. Several kinds of stimulation protocols in field potential recording could elicit LTP in the hippocampus; however, the biochemical cascades activated by different LTP protocols might be distinct, even though the synaptic responses may be similar in amplitude and stability. Additionally, slight deficits in LTP may be overcome by strong stimulation. The age-dependent LTP impairment could be observed by theta-frequency stimulation (5Hz) but not by 30 or 70 Hz high frequency stimulation (Tombaugh *et al.* 2002). LTP was unaffected in the hippocampus of *Fmr1* KO mice when using 10 burst TBS (Li *et al.* 2002, Larson *et al.* 2005). However, when the stimulation intensity was lowered, LTP was attenuated in the hippocampus of *Fmr1* KO mice, compared to WT mice (Lauterborn *et al.* 2007). Glycine application is a modest way for inducing LTP and shares the similar mechanism as TBS (Musleh *et al.* 1997). Therefore, we observed attenuation of glycine-induced LTP in the hippocampus of *Fmr1* KO mice. It may be possible that the deficits in LTP caused by the loss of FMR1 gene were overcome by intense afferent stimulation. This hypothesis is supported by the finding that spike timing dependent LTP at 5ms time window was strongly reduced in prefrontal cortex of *Fmr1* KO mice, and that this decrease could be reversed by stronger stimulation (Meredith *et al.* 2007). It may be that FMRP is involved in certain types of learning and memory processes that share similar pathways with Gly-LTP. Identifying the role of FMRP in Gly-LTP could help elucidate the mechanisms involved in impaired learning and memory of FXS patients.

It has been previously reported that activation of group 1 mGluR could facilitate FMRP protein synthesis and that DHPG could induce enhanced LTD in the hippocampus of *Fmr1* KO mice, as well as that FMRP might regulate downstream of mGluR (Weiler & Greenough 1999, Huber *et al.* 2002, Bear *et al.* 2004). This DHPG-induced LTD (mGluR-LTD) is NMDA-receptor-independent and has distinct mechanisms compared with NMDA-receptor-dependent LTD (Bear *et al.* 2004). One of the major distinctions is that mGluR-LTD requires the rapid translation of preexisting mRNA in the postsynaptic dendrites (Huber *et al.* 2000). As an mRNA binding protein, FMRP and *Fmr1* mRNA were localized in granules throughout dendrites and within spines. It was found that mGluR-LTD induced increased expression of FMRP in the CA1 area of hippocampus (Hou *et al.* 2006). Furthermore, our recent study found that activation of group 1 mGluR by DHPG could up-regulated FMRP in the ACC neurons of adult mice (Wang *et al.* 2008b). On the other hand, it has been proved that the mGluRs are required for the induction

and maintenance of LTP and that when the stimulation is mild the activation of mGluRs is critical for the NMDA-receptor-dependent LTP (Neyman & Manahan-Vaughan 2008). Our results showed that the group 1 mGluR antagonist DL-AP3 could block Gly-LTP in WT mice but not in *Fmr1* KO mice. This is consistent with previous findings that tetanic stimuli induced attenuated LTP in the neocortex of *Fmr1* KO mice and mGluR5 mediated synaptic plasticity was absent in the visual cortex area of *Fmr1* KO mice (Wilson & Cox 2007). During the mGluR-LTD process, there is increased mGluR-mediated activity in *Fmr1* KO mice and the activation of mGluR can up-regulate the expression of FMRP in WT mice. However, in the Gly-LTP process, the mGluR-mediated activity was decreased in *Fmr1* KO mice and the expression of FMRP was down-regulated in WT mice after glycine application. Therefore, we suggest that FMRP, as the downstream of mGluR, contribute to both the LTD and LTP process but through distinct signaling pathways. The increased mGluR-LTD and decreased Gly-LTP in *Fmr1* KO mice are both due to the improper regulation of FMRP pathways, which are caused by the loss of *Fmr1* gene.

4.4 ERK signaling pathway and FMRP

ERK1/2 (p42/p44 MAPK), two main MAPK isoforms that are widely expressed in post-mitotic neurons in the mammalian nervous system (Boulton *et al.* 1991), is downstream of NMDA receptors and could be activated by stimulation of glutamate receptors in the hippocampus (Kurino *et al.* 1995, English & Sweatt 1996, Wang *et al.* 2001). The phosphorylation of ERK1/2, which may be through the activation of PKA and PKC pathways, could lead to the phosphorylation and transactivation of CREB, thereby participating in long-term potentiation of neurons (Davis *et al.* 2000, Huang *et al.* 2000). Our previous studies have shown that application of forskolin in the superficial dorsal horn in mice could induce the enhancement of MAPK immunoreactivity while inhibitors of MAPK could block LTP in the dorsal horn and neurons in the ACC (Wei *et al.* 2006, Toyoda *et al.* 2007). Furthermore, phosphorylation level of ERK has been found to be increased in WT mice after DHPG application while remaining unchanged in *Fmr1* KO mice (Hou *et al.* 2006, Kim *et al.* 2008). There are also reports that histamine can stimulate the Ras-MEK-ERK pathway via potentiation of NMDA response and therefore increased phosphorylation of ERK1/2 in CA1 area in both WT and *Fmr1* KO mice. However, the increase was smaller in *Fmr1* KO mice compared with WT mice (Hu *et al.* 2008). This indicates that the ERK1/2 signaling cascade is regulated improperly in *Fmr1* KO mice.

Consistent with these findings, we also observed that p42 MAPK was activated by glycine treatment in the hippocampus of WT mice through immunoblotting. However, p42 MAPK began to be dephosphorylated in the hippocampus of *Fmr1* KO mice after glycine application for 5min. In addition, the field potential recording results showed that the MEK inhibitor U0126 could severely blocked the Gly-LTP in the hippocampus of WT mice. To our knowledge, this is the first time to show that activation of ERK1/2 contributes to Gly-LTP in the hippocampus and that the improper regulation of ERK1/2 phosphorylation, due to loss of FMRP, leads to the reduced Gly-LTP in the hippocampus of *Fmr1* KO mice.

4.5 Significance of Gly-LTP in *Fmr1* KO mice

In summary, we showed that glycine-induced LTP was attenuated in the hippocampus of *Fmr1* KO mice. It is possible that this decreased LTP is related to certain deficits in learning and memory of fragile X patients. FMRP may contribute to glycine-induced LTP by regulating the phosphorylation of p42 MAPK. This chemically-induced LTP offers an additional method to study the changes in long-term synaptic transmission as well as possible signaling cascade changes involved in transgenic mice models that mimic human mental retardation disorders.

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