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Messenger RNA Transport and Translation Regulated by the 3' UTRs of Dendritic mRNAs and Abnormal Alternative Splicing of Neuroligin1 in the Fmr1 KO Mouse Hippocampus

Tianhui Zhu

Graduate Center, City University of New York

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Messener RNA Transport and Translation Regulated by the 3' UTRs of Dendritic mRNAs and Abnormal Alternative Splicing of *Neuroligin1* in the *Fmr1* KO Mouse Hippocampus

by

Tianhui Zhu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2016
Messenger RNA Transport and Translation Regulated by the 3' UTRs of Dendritic mRNAs and Abnormal Alternative Splicing of *Neuroligin1* in the *Fmr1* KO Mouse

**Hippocampus**

by

**Tianhui Zhu**

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry to satisfy the dissertation requirement for the degree of Doctor of Philosophy.

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Date Executive Officer

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Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

Messenger RNA Transport and Translation Regulated by the 3' UTRs of Dendritic mRNAs and Abnormal Alternative Splicing of *Neuroligin1* in the *Fmr1* KO Mouse Hippocampus

by

Tianhui Zhu

Advisors: Dr. Paul Feinstein

Fragile X Syndrome (FXS) is one of the most commonly inherited mental retardations. It is caused by the loss of functional fragile X mental retardation protein (FMRP). Loss of functional FMRP is the most widespread single-gene cause of autism. The most prominent phenotype of FXS patients is an IQ ranging from 20 to 70. FMRP is an RNA binding protein, widely expressed in almost all tissues and highly expressed in brain. As a RNA binding protein, 85-90% of FMRP in the brain is associated with polyribosomes. Approximately 4% of total mRNA is associated with FMRP, which functions in the stability, transport and translational regulation of its targeted mRNAs.

The 3’ untranslated region (3’UTR) of mRNAs can be important for their subcellular localization and translational regulation. Many genes contain localization elements in their 3’UTRs that enable transcripts to localize to dendrites for site-specific translation. FMRP functions as a translational regulator in this process.

Local translation of certain proteins is crucial to synaptic plasticity. Synaptic plasticity is the ability of a synapse to regulate its strength over time in response to stimuli. It is the basis of learning and memory. The two forms of long-term plasticity are long-term depression (LTD) and long-term potentiation (LTP). FXS patients and *Fmr1* KO mice all show exaggerated LTD in the
hippocampus, which is the center of emotion, memory, and the autonomic nervous system. In addition, some types of abnormal LTP have been demonstrated in Fmr1 KO mice. These evidences indicate that FMRP plays an important role in synaptic plasticity through translational regulation of target mRNAs.

Neuroligin (NL) is a family of neural adhesion molecules. Neuroligins are transmembrane proteins located at the post-synaptic membrane and bind with pre-synaptic adhesion molecules known as NEUREXIN (NRX). Neuroligins function in synaptogenesis, synapse differentiation, and synapse maintenance. Importantly, they have been shown to be involved in autism and other cognitive diseases.

Both neuroligins and neurexins are products of alternative splicing. The recognition between neuroligins and neurexins is splice variant-dependent. This variant-specific binding triggers different downstream signals for synaptogenesis and synapse differentiation. The work presented here addresses the targeting of FMRP to the 3’ UTR of some dendritic mRNAs and their translational regulation. And for the first time, we demonstrate that FMRP is involved in the alternative splicing of mRNA. FRMP is found related to Neuroligin1 splicing in mice hippocampus.
ACKNOWLEDGMENT

Accomplishing this doctoral dissertation is the most significant challenge I have ever encountered in my life, in both academic aspect and life aspect. I was being offered kind and generous help during my study. It is a great pleasure to share my appreciation to the people who helped to make this achievement possible.

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I would like to thank my family for their understanding, patience, and support. Thank you all for endless love and tolerance so I could focus on my study. I wish I could have spent more time with you. This thesis is dedicated to my most beloved grandmother, who passed away during my PhD study.
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<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CaMKIIα</td>
<td>cam kinase II α</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CPE</td>
<td>cytoplasmic polyadenylation element</td>
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<tr>
<td>CPEB</td>
<td>CPE binding protein</td>
</tr>
<tr>
<td>DHPG</td>
<td>3, 5-dihydroxyphenylglycine</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vivo</em></td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FL</td>
<td>full length</td>
</tr>
<tr>
<td>FMRP</td>
<td>fragile X mental retardation protein</td>
</tr>
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<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>KH K</td>
<td>Homology domain</td>
</tr>
<tr>
<td>KHC</td>
<td>kinesin heavy chain</td>
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<tr>
<td>KLC</td>
<td>kinesin light chain</td>
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<tr>
<td>MAP2</td>
<td>microtubule associate protein 2</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose-binding protein</td>
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<td>NL</td>
<td>Neuroligin</td>
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<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>NRX</td>
<td>Neurexin</td>
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<td>PABP</td>
<td>poly (A) binding protein</td>
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<tr>
<td>PI</td>
<td>protease inhibitor</td>
</tr>
<tr>
<td>RBP</td>
<td>mRNA binding protein</td>
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<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RI</td>
<td>RNAase inhibitor</td>
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<td>RNP</td>
<td>ribonucleoprotein</td>
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<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>vGAT</td>
<td>vesicular GABA transporter</td>
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<td>vGLUT</td>
<td>vesicular glutamate transporter</td>
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<td>ZBP1</td>
<td>zipcode-binding protein</td>
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CHAPTER 1

INTRODUCTION
1.1 Introduction of autism

Autism is a neural development disorder characterized by impaired social interaction and communication, as well as repetitive and stereotyped behavior patterns. The term "autism" was first used by Swiss psychiatrist, Paul Eugen Bleuler, in the 1912 American Journal of Insanity (Greydanus & Toledo-Pereyra, 2012). The name is derived from the Greek word “autos” which means "self."

The Diagnostic and Statistical Manual of Mental Disorders (DSM), published by the American Psychiatric Association, is the standard classification of mental disorders used by mental health professionals in the United States. It contains a listing of diagnostic criteria for every psychiatric disorder recognized by the U.S. healthcare system. Based on the classification in DSM-5 (2013), autism is but one type of Autism Spectrum Disorder (ASD). The other two types of ASD are Asperger’s Syndrome, and pervasive developmental disorder not otherwise specified (PDD-NOS). Autism and mental retardation are not necessarily associated with each other. Mental retardation is a possible comorbid disorder of the autism spectrum at more severe level.

In 1943, Leo Kanner described three girls and eight boys between 2 and 8 years of age who exhibited an extreme preference for solitude and patterns such as repetitive behaviors, persistent interests, and deficits in both language and communication (Kanner, 1943). It was the first time that a male predominance was noticed in autism disorder. In 1944, Austrian pediatrician, Hans Asperger, published observations of patients who exhibited similar symptoms. Both researchers observed autistic traits in the families of affected individuals, which lead them to believe there was a possible genetic link with the disease.
In 1977, the first twin study was performed comparing autistic patients with their identical or fraternal twins. Identical twins develop from one fertilized ovum and are genetically identical, while fraternal twins develop from two different ova and have unidentical genomes. Results from twenty-one twin pairs revealed that the co-occurrence of autism in identical twins was much higher than in fraternal twins (Folstein & Rutter, 1977), indicating that genetic factors may play a crucial role in autism.

Although a great number of genes are associated with autism, a clear genetic basis has not been found. Current data, however, shows that 10-15% of autism cases are monogenic, arising from defects in a single gene. Studying these monogenic cases of autism is the best way to understand autism pathogenesis and cognition at the molecular and cellular level.

1.2 The Fragile X Syndrome (FXS) and FMRP

Data from the mid-1990’s show that 1.49% of the US population is affected by mental retardation and/or a developmental disability (Larson et al., 2001). Amongst all mental retardations, Fragile X Syndrome (FXS) is the most widespread single-gene cause of autism and the most frequent cause of inherited mental retardation. Martin and Bell studied several generations in one family and described members with intellectual deficits and social withdrawal. In addition, they were able to demonstrate a male-linked genetic pattern (J. P. Martin & Bell, 1943). Fragile X Syndrome occurs at a rate of 1:5,000 in males and a rate approximately half that in females (1/10,000) (Coffee et al., 2009). These rates may be underestimated since some reports show an even higher occurrence at 1:1,500 in males and 1:2,500 in females (Rousseau et al., 1994). Autism and FXS do not necessarily overlap with each other. The prevalence of autism
in FXS is 15-30% and the rate of FXS in total autism is 2-7% (Rogers, Wehner, & Hagerman, 2001) (Kaufmann et al., 2004) (Harris et al., 2008).

The most prominent symptom of FXS is mental retardation. Approximately 85% of males and 25-30% of females with full mutation FXS (full mutation will be talked soon) have a Full Scale Intelligence Quotient (FSIQ) lower than 70, with an average of 40 in males (Rousseau et al., 1994) (Merenstein et al., 1996) (Loesch, Huggins, & Hagerman, 2004) (Loesch et al., 2004). Other symptoms of FXS include short-term memory loss and defects in visuospatial ability (Crowe & Hay, 1990). The physical characteristics of affected people include a long narrow face, prominent forehead, jaw and ears, and enlarged testicles in male patients. There are also a variety of connective tissue abnormalities such as hyper-extensible finger joints, flat feet, soft skin and a high-arched palate (Chudley & Hagerman, 1987) (Lachiewicz & Dawson, 1994).

In the 1970s, an association between mental retardation and abnormal synapse dysmorphogenesis was first described (Marin-Padilla, 1972; Purpura, 1974). Dendritic spines display an immature phenotype in FXS patients, with the spines being longer and thinner compared to unaffected people (Bagni & Greenough, 2005). FXS patients also have high dendritic spine density. This abnormality in spine structure can be found in other types of ASD (Aldinger, Plummer, Qiu, & Levitt, 2011). This indicates a potential pathological basis of the disease. This neurological phenotype has led FXS research to focus on synapses, with an emphasis on synapse structure, function, and especially plasticity.

Fragile X Syndrome is caused by the loss of functional fragile X mental retardation protein 1 (FMRP). The “X” refers to the X chromosome where the gene is located. In 1991, a trinucleotide repeat expansion (CGG) was found in the 5’ UTR of the fragile-X mental retardation gene 1 (FMR1) in FXS patients. This repeat expansion leads to hypermethylation of
the \textit{FMRI} promoter and its resultant silencing (Fu et al., 1991) (Pieretti et al., 1991) (Verkerk et al., 1991). Unaffected individuals have between 5-54 CGG trinucleotide repeats in \textit{FMRI}, with an average of 30 repeats. Expansion of these repeats to 55-200 (pre-mutation) does not cause FXS but rather fragile X-associated tremor/ataxia syndrome (FXTAS), characterized by cognitive decline, autonomic dysfunction, ataxia, and Parkinsonism (Iwahashi et al., 2006). In females, decreased ovarian function can be present which is known as fragile X-associated primary ovarian insufficiency (FXPOI). This causes infertility and early menopause in some female pre-mutation carriers (Oostra & Willemsen, 2009). If the number of repeat units exceeds 200, it is referred to as a full mutation and results in hypermethylation and transcriptional silencing of \textit{FMRI} (Oberle et al., 1991). The severity of the disease varies based on the level of mutation. Transcriptional silencing of \textit{FMRI} is the primary cause of FXS. However, other mutations in \textit{FMRI} can also cause FXS. For example, one patient was found to have a point mutation leading to an amino acid change (I340N) in FMRP that changed protein function (De Boulle et al., 1993) (Y. Feng, Absher, et al., 1997). Another patient with an R138Q mutation in FMRP has also been identified (Collins et al., 2010).

In 1994, an \textit{Fmr1} knockout (KO) mouse was generated by the insertion of a neomycin cassette into exon 5 of the mouse \textit{Fmr1} gene (Consort, 1994). The \textit{Fmr1} KO mouse model has been an essential tool in the study of FMRP and FXS. Neurons from \textit{Fmr1} KO mice show highly similar phenotype as FXS patients, including long and thin dendritic spines (Galvez & Greenough, 2005) (Grossman, Aldridge, Weiler, & Greenough, 2006).

To understand the etiology of FXS, it is important to study the function of FMRP. FMRP is an RNA-binding protein (RBP) believed to be involved in RNA processing, transport and translational regulation, especially in dendrites (Santoro, Bray, & Warren, 2011) (Bhakar, Dolen,
& Bear, 2012). FMRP is widely expressed in virtually all cell types, with particularly high levels in neurons but low levels in glial cells (Y. Feng, Gutekunst, et al., 1997).

As an RNA binding protein, FMRP binds specific RNAs as opposed to having a general affinity for total RNA. Sequence analysis of FMRP has revealed several common RNA binding domains: two hnRNP K-homology (KH) domains known as KH1 and KH2, and one Arg-Gly-Gly (RGG) box (Figure 1-1) (Ashley, Wilkinson, Reines, & Warren, 1993). The KH domain binds to "kissing-complex" tertiary RNA motifs (Darnell et al., 2005). The RGG box binds G-quartet loops (Blackwell, Zhang, & Ceman, 2010) and SoSLIP (Sod1 mRNA Stem Loops Interacting with FMRP) motifs (E. G. Bechara et al., 2009). Messenger RNAs (mRNA) with U-rich sequences but no specific corresponding RNA binding domains in FMRP have also been reported as FMRP targets (Chen, Yun, Seto, Liu, & Toth, 2003) (Fahling et al., 2009).

The aforementioned point mutation leading to the amino acid change I340N in FMR1 affects the KH2 domain of FMRP and causes disease (De Boulle et al., 1993) (Y. Feng, Absher, et al., 1997). This indicates that the RNA binding ability of FMRP is important to its function. Although other RNA motifs have been identified as FMRP targets, no common FMRP targeting motif has been found (Darnell et al., 2011). Experiments using high-throughput sequencing combined with crosslinking immunoprecipitation (HITS-CLIP) found that FMRP can target mRNAs in their coding regions (66 %), 3' UTRs (19 %) and 5' UTRs (5 %) (Darnell et al., 2011). Interestingly, one FMRP target is its own gene product, FMR1 mRNA (C. T. Ashley, Jr. et al., 1993).

There are three highly homologous members in the FMRP RNA-binding protein family: FMRP, Fragile X-related proteins 1 and 2 (FXR1P and FXR2P, respectively) (Figure 1-2) (Tamanini et al., 2000) (Ascano et al., 2012). The expression of FMR1 is under the regulation of
alternative splicing (C. T. Ashley et al., 1993). The full-length isoform, isoform 1, encodes a 71 kDa protein. The major isoform, isoform 7, encodes a 69 kDa protein (Figure 1-2) (C. T. Ashley et al., 1993) (Verkerk et al., 1993) (Ascano et al., 2012). A stoichiometric study of FMRP-RNA binding with in vivo transcribed fetal human brain mRNAs estimated that FMRP targets 4% of total mRNAs (C. T. Ashley, Jr. et al., 1993). This data has been confirmed in mice by microarray (Brown et al., 2001) and by HITS-CLIP (Darnell et al., 2011).

The International Human Genome Sequencing Consortium (IHGSC) of the Human Genome Project (HGP) has placed an estimate of 20,000 to 25,000 genes in the human genome (International Human Genome Sequencing, 2004). It has been reported that 84% of all genes are expressed in the human brain (Hawrylycz et al., 2012). Based on these facts, FMRP should potentially target 670-840 genes. The HITS-CLIP study identified 842 targets in the mouse brain, which fits the original estimate (Darnell et al., 2011). These mRNA targets are physically bound by FMRP, not all of their translation are known to be regulated by FMRP. 28 of them are also candidates for autism as determined by the SFARI database of autism candidate genes. These include the well-studied autism genes such as NEUROLIGIN3, NEUREXIN1, and SHANK3 (http://gene.sfari.org). As more autism candidate genes are identified, there are likely to be more FMRP targets found in this pool such as the other two members of NEUREXIN family: NEUREXIN 2 and NEUREXIN 3. In a recent study, exome sequencing of 343 families with an ASD child showed that one-fifth of candidate autism genes with de novo mutations are FMRP targets (Iossifov et al., 2012). This surprising overlap strengthens the link between FMRP and autism.

1.3 FMRP and mRNA transport
For many years it was believed that mRNAs and their translation machinery were only found near the nucleus in the cell body. Classical dogma is that protein translation occurs in the soma. If proteins are required elsewhere, they will be transported to the destined subcellular locations. However, considering the morphology of neurons, with distant dendritic and axonal compartments, as well as the relatively slow speed of protein translation/transport from soma, researchers were forced to consider local translation as a mechanism neurons might use to work around these morphological challenges. Messenger RNA transport combined with local protein synthesis is a more responsive mechanism for cells to respond to extracellular signals than retrograde signaling followed by anterograde microtubule-dependent protein transport.

As visualization techniques advanced, mRNAs and components of the translational machinery were eventually identified in dendrites and axons (Figure 1-3) (Steward & Levy, 1982) (Koenig & Giuditta, 1999). Since one copy of mRNA can be used as template for several rounds of protein translation, local translation is economical for the cell. Furthermore, not only the mechanism of local translation provides a faster response to stimuli, but also functions in the establishment of cell polarity and the definition of cellular compartments (Besse & Ephrussi, 2008) (Becalska & Gavis, 2009).

Transport of mRNAs is a required component of local translation. Messenger RNAs do not diffuse randomly in cytoplasm but are sorted and transported to specific sites based on the requirements of their protein products. Instead of being transported as single molecules, mRNAs associate with RNA binding proteins to form cytoplasmic messenger ribonucleoprotein particles (mRNPs) complexes. There are three major types of mRNP complexes in the cytoplasm: stress granules (SGs), processing bodies (P-bodies) and ribonucleoprotein particles (RNPs) (Bramham & Wells, 2007).
Stress granules and processing bodies share some components. But in general, the major difference is that stress granules function in RNA storage, whereas P-bodies function in RNA degradation (Anderson & Kedersha, 2006). Stress granules appear in the mammalian cell cytoplasm when cells experience environmental stress such as UV, heat, or oxidation (Krichevsky & Kosik, 2001). Stress granules contain stalled translation pre-initiation complexes as well as many early translation initiation factors. Several well-known RNA binding proteins are found in stress granules, like FMRP, the cytoplasmic poly-A element binding protein (CPEB) (Napoli et al., 2008) and Staufen (Anderson & Kedersha, 2006). P-bodies are RNA granules that contain the mRNA decay machinery (Bashkirov, Scherthan, Solinger, Buerstedde, & Heyer, 1997).

RNPs are formed in the nucleus following RNA transcription and processing. To be specific, mature mRNA transcripts complex with RNA binding proteins to form messenger RNPs. Messenger RNPs are then exported to the cytoplasm where they are remodeled and delivered to their destinations (Figure 1-4). In neurons, mRNPs are found in both dendrites and axons and local translation occurs in both sites (Besse & Ephrussi, 2008).

Cytoskeletal and motor proteins are required for proper mRNP transport. Microtubules are oriented in distal dendrites with their minus ends at the proximal side and plus ends at the distal side. In proximal dendrites, microtubules exist in both orientations. All three types of motors (kinesin, dynein and myosin) are implicated in mRNA transport (Jansen, 2001). Kinesin family motor proteins associated with microtubules move from minus end to plus end, whereas dynein family motors move from plus end to minus end. In addition, there are myosin family motors that move on actin. The FMRP-induced mRNA transport in dendrites is dependent on microtubules, but not on actin (Antar, Dictenberg, Plociniak, Afroz, & Bassell, 2005).
In the majority of cases, however, RNAs are transported on microtubules by kinesins (Kiebler & Bassell, 2006). Kinesin superfamily proteins (KIFs) are classified into 15 kinesin families (Kinesin1 to Kinesin 13, Kinesin 14A and Kinesin 14B). The kinesin1 family contains KIF5A, KIF5B, and KIF5C. The KIF5s are motors for the anterograde transport of mRNPs in dendrites (from proximal cell body to distal dendrites) (Hirokawa, 2006). RNA granules directly interact with the KIF5 C-terminal tail (heavy chain) (Hirokawa & Takemura, 2005). The kinesin motor does not bind to mRNA directly. RNA binding proteins target cis element in mRNAs and serve as adaptors between RNA granules and kinesin. One example is the β-actin zip code binding protein 1 (ZBP1) which recognizes a 54 nucleotide sequence (so called zip code), in the 3' UTR of β-actin mRNA for transport to dendrites (Kislauskis, Zhu, & Singer, 1994).

Many studies have suggested an mRNA transport function for FMRP. Both nuclear localization signals (NLS) and nuclear export signals (NES) are found in FMRP (Eberhart, Malter, Feng, & Warren, 1996). FMRP also forms complexes with mRNAs and the kinesin light chain (KLC) of KIF5 (Dictenberg, Swanger, Antar, Singer, & Bassell, 2008). Taken together, these findings imply a nuclear-cytoplasmic shuttling function of FMRP, carrying target mRNAs out of nucleus.

FMRP interacts specifically with an mRNA nuclear export factor, nuclear RNA export factor 2 (NXF2) (Lai, Sakkas, & Huang, 2006). FMRP also binds to nuclear mRNAs, a nuclear exporter protein (Tap/NXF1), and transcribing pre-mRNAs (Kim, Bellini, & Ceman, 2009). Binding of FMRP with transcribing pre-mRNAs raises the possibility that FMRP may be involved in RNA processing. The missense mutation (R138Q) in the NLS of FMRP causes development delays in patients, indicating an important role for FMRP in the nucleus or in nuclear-cytoplasmic shuttling (Collins et al., 2010).
The majority of FMRP (95 %) is found in the cytoplasm where it localizes to the soma, axons, dendrites and post-synaptic sites (Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993). In the cytoplasm, FMRP has been visualized as part of dynamic RNA granules, trafficking to dendrites and axons (De Diego Otero et al., 2002) (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004) (Antar et al., 2005). FMRP was found to co-localize and co-sediment with Staufen (Ohashi et al., 2002) (Ferrari et al., 2007), which regulates mRNA transport to dendrites (Tang, Meulemans, Vazquez, Colaco, & Schuman, 2001).

In some cases, FMRP trafficking to dendrites can be stimulated by neuronal activities (Antar et al., 2004) (Gabel et al., 2004). Several specific mRNAs are transported to dendrites in response to neuronal activities (Mayford, Baranes, Podospanina, & Kandel, 1996). Surprisingly, FMRP-targeted mRNAs can be correctly localized to their destinations in the absence of FMRP (Bhakar et al., 2012). Thus, FMRP is more likely to be a passive passenger in mRNPs. Many RNA binding proteins possess the same feature and are thought to be dispensable for RNA transport, but required for granule assembly, stability and translation (Kiebler & Bassell, 2006).

The translation of mRNAs in FMRP-containing mRNPs is widely accepted to be repressed during transport. One convincing piece of evidence supporting this is that microtubule-associated FMRP is restricted to mRNPs that do not contain ribosomes (Wang et al., 2008). Stimulation by activity at the base of dendritic spines may signal a relief of translational repression (Sutton & Schuman, 2006).

1.4 Local translation and translational regulation by FMRP

In 1982, Steward and Levy visualized polyribosomes localized at the base of dendritic spines during synaptogenesis (Figure 1-3) (Steward & Levy, 1982). Synaptogenesis is the
formation of synapses between neurons. This observation strongly suggested that local translation of proteins is important for synaptogenesis. Together, the RNA binding ability of FMRP and its dendritic localization suggest that FMRP may regulate the local translation of its target mRNAs at the synapse.

Synaptic plasticity, the ability of a synapse to respond to stimulation by changing its strength, is an important aspect of learning and memory. It is associated with the local translation in neuron processes. To elucidate the possible roles of FMRP in synaptic plasticity, its association with polyribosomes was investigated. FMRP was found associated with polyribosomes by ultracentrifugation in a sucrose density gradient (Y. Feng, Absher, et al., 1997).

In general, FMRP is recognized as a translational repressor. *in vivo* experiments using recombinant FMRP added to rabbit reticulocyte lysates showed a dose-dependent reduction of brain mRNA translation, but not rabbit reticulocyte lysate mRNA (Li et al., 2001). A study comparing protein synthesis in wild type and *Fmr1* KO hippocampal slices showed a significant increase of basal translation in *Fmr1* KO mice compared to WT mice (Qin, Kang, Burlin, Jiang, & Smith, 2005) (Dolen et al., 2007). Some genes, such as hASH1 (human aschaete-scute homologue 1) (Fahling et al., 2009) and Sod1 (superoxide dismutase 1) (E. G. Bechara et al., 2009) (Bechara et al., 2009) exhibited enhanced FMRP-mediated translation. FMRP has also been shown to stimulate the translation of the K+ ion channel, Kv4.2, by binding to the 3’UTR of Kv4.2 mRNA (Gross, Yao, Pong, Jeromin, & Bassell, 2011).

Translational inhibition can be achieved both by blocking the initiation of ribosome assembly and stalling the elongation of actively translating ribosomes (Darnell et al., 2011). Binding of the translation initiation factor eIF4E to the 5’-7 methyl guanosine cap (5’ cap) of mRNA is the rate-limiting step of translation initiation ( Hiremath, Webb, & Rhoads, 1985).
(Duncan, Milburn, & Hershey, 1987). The finding that FMRP co-sediments with the cytoplasmic FMRP interacting protein (CYFIP1), the 80S ribosome complex and translation initiation factors suggests that FMRP can repress translation at the initiation phase (Napoli et al., 2008).

Considering that the majority of FMRP is found bound with polyribosomes, it is widely accepted that the primary way FMRP regulates translation is during the translation elongation step. Samples from the patient with the FMRP missense mutation showed that this mutation abolishes the interaction of FMRP with polyribosomes (Y. Feng, Absher, et al., 1997). The I304N mutation localizes in the second KH domain of FMRP, indicating that the RNA binding ability and association of FMRP in polyribosomes is crucial to the normal function of FMRP. The presence of FMRP in all three types of RNA granules also suggests that FMRP may be involved in translational inhibition in many phases (Shiina & Tokunaga, 2010).

FMRP also binds to RNAi/microRNA machinery proteins, such as endoribonuclease Dicer (Dicer, also called helicase with RNase motif) (Caudy, Myers, Hannon, & Hammond, 2002) (Ishizuka, Siomi, & Siomi, 2002) and Argonaute 2 (Ago2) (Jin et al., 2004) in Drosophila. Dicer is an endoribonuclease in the RNase III family that produces miRNA. Ago2 is the catalytic component of RNA-induced silencing complex (RISC). Besides the RNAi/microRNA machinery proteins, small RNA molecules like miR-125b and miR132 were found to associate with FMRP (Muddashetty et al., 2011). A region in non-coding RNA, brain cytoplasmic RNA1 (BC1), has been shown to bind CYFIP1 and translation initiation factors, providing one example of RNA based translational regulation by FMRP (Zalfa et al., 2003).

FMRP exists predominantly in its phosphorylated form (Narayanan et al., 2007). A serine residue (human ser500, murine ser499) is crucial to the phosphorylation of FMRP. The phosphorylation of this serine residue can trigger subsequent phosphorylation of specific nearby
residues and switch the translational regulation status of FMRP. Two FMRP mutants have been constructed to mimic phosphorylated FMRP (S499D) and dephosphorylated FMRP (S499A) (Figure 1-5) (Ceman, O'Donnell, et al., 2003). With the help of these mutants, it has been demonstrated that the phosphorylation status of FMRP does not affect its RNA binding ability but does affect its association with polyribosomes (Ceman, O'Donnell, et al., 2003) (Muddashetty et al., 2011). Wild-type FMRP and the S499D mutant show resistance to ribosome run-off after sodium azide treatment, representing stalled polyribosomes (Ceman, O'Donnell, et al., 2003). Unphosphorylated FMRP is associated with actively translating polyribosomes. The phosphorylation and dephosphorylation of FMRP are catalyzed by ribosomal S6 kinase (S6K) and protein phosphatase 2A (PP2A), respectively (Santoro et al., 2011). Thus, translation of FMRP target mRNAs may be regulated by phosphorylation and dephosphorylation via S6 and PP2A.

1.5 FMRP and synaptic plasticity

It is important for the correct protein to be expressed in the correct site at the right time in cells, especially to avoid ectopic expression of deleterious proteins. Local translation of specific mRNAs targeting at specific regions is important for spatial restriction of specific protein. Now it is widely accepted that specific mRNAs can be localized to dendrites and locally translated. In general, locally translated proteins can be classified into structural proteins (like actin) and regulatory proteins (like kinases and phosphatases). The translational regulation of these mRNAs can cause morphological changes in neurons like dendritic spine shape, and physiological changes like synaptic plasticity.
Synaptic plasticity is the foundation of learning and memory. It is the ability of synapses to change the strength of signal transmission between neurons, which bequeaths synapse “processing” power. Neurons would merely be connected “electrical wires” without synaptic plasticity. Based on timescales, plasticity can be classified as short-term (tens of milliseconds to minutes) and long-term (minutes or longer). Long-term potentiation (LTP) and long-term depression (LTD) are two types of long-term plasticity, which occur at excitatory synapses (Gerrow & Triller, 2010).

Cellular synapses can be classified as excitatory synapse and inhibitory synapse based on their ability to change the post-synaptic potential. This depends on the receptor type on the post-synaptic terminal. Excitatory synapses are a type of synapses in which an action potential (AP) from a pre-synaptic neuron depolarizes the post-synaptic neuron, raises the resting membrane potential towards the threshold potential, and eventually makes an action potential more likely to happen at the post-synaptic neuron. Inhibitory synapses do the opposite, lowering the membrane potential, making it more difficult for a post-synaptic neuron to fire an AP.

These two types of synapses can be identified based on the neurotransmitters used. In mammals, the major excitatory synapses in the central nervous system (CNS) are glutamatergic synapses. The major inhibitory synapses in the CNS are GABAergic synapses. The amino acid L-glutamate is the primary excitatory neurotransmitter in the mammalian CNS, but there are also catecholamines, which include epinephrine, norepinephrine and dopamine. In the peripheral nervous system (PNS), the most typical excitatory neurotransmitter is acetylcholine, which works at neuromuscular junctions.

There are two types of glutamate receptors: ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels, and metabotropic glutamate receptor (mGluRs). There are
three types of iGluRs: the N-methyl-D-aspartate receptor (NMDA receptor or NMDAR), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor or AMPAR) and kainate receptors with nomenclature based on their agonists (Ronald, Larsson, Anckarsater, & Lichtenstein, 2011). There are eight different types of mGluRs, labeled mGluR1 to mGluR8. They are classified into three groups, Group I, II, and III (Gp1, Gp2, Gp3) based on receptor structure and physiological activity (Ohashi et al., 2002). Group I mGluRs contain mGluR1 and mGluR5; Group II mGluRs contain mGluR2, mGluR3 and mGluR4; Group III mGluRs contain mGluR4, mGluR6, mGluR7 and mGluR8.

It is known that activation of the Gp1 mGluRs (mGluR1 and mGluR5) can stimulate translation in biochemical preparations of enriched cortical synapses (Weiler & Greenough, 1993). Polyribosomes can accumulate rapidly in dendrites after stimulation of metabotropic glutamate receptors (mGluRs) using the Gp1 mGluRs agonist 3, 5-dihydroxyphenylglycine (DHPG). Protein translation involving FMRP can also be triggered by DHPG at dendrites (Weiler et al., 1997). This rapid response of local translation to stimuli through mGluR is important for synaptic plasticity (Huber, Kayser, & Bear, 2000).

LTP was first observed in the hippocampus. This region has been extensively studied with regards to synaptic plasticity although both LTP and LTD are found in other regions too. The hippocampus is important for inhibition (for example, animals with hippocampal damage tend to be hyperactive (Nadel, 1975 #1807)), memory, and spatial recognition. The hippocampus is divided into several regions, such as dentate gyrus (DG) and Cornu Ammonis - horn of (the ancient Egyptian god) Amun. Cornu Ammonis is divided into four regions: CA1, CA2, CA3, CA4 (only CA1 and CA3 are shown in the figure) (Figure 1-6) (Riedemann, Patchev, Cho, & Almeida, 2010). The DG contains granule cells. The CA areas of hippocampus are full of
pyramidal neurons. The term “hippocampus proper” refers to the four CA regions. The term “hippocampal formation” refers to the hippocampus proper plus DG and subiculum. In the hippocampus, there are three principal information pathways. First, the perforant path from the entorhinal cortex to granule cells in the DG; second, the mossy fiber path (which is the axon of granules cells) from the granule cell of the DG to the pyramidal cells in the CA3; and third, the Schaffer collateral-commissural (SCC) path from the CA3 to CA1. LTD and LTP were induced by placing electrodes in the Schaffer collateral-commissural (SCC) pathway and recorded from the CA1 (Figure 1-6) (Riedemann et al., 2010).

Both LTP and LTD could be dependent on NMDARs or mGluRs. LTP is an enhancement of signal transmission between neurons. It can be stimulated by tetanic stimulation, which consists of high frequency stimulation. It is widely accepted that LTP is induced by increasing neurotransmitter release, or increasing AMPAR numbers on the post-synaptic terminal, or increasing the conductance of ion channels. The NMDAR-dependent LTP in the CA1 region of adult hippocampus is the most widely studied (Castillo, 2012).

There are three phases of LTP: LTP 1, LTP 2 and LTP 3. LTP 1 is known as early-form LTP (E-LTP), and is independent of protein translation. LTP 2 is dependent on protein translation. LTP 3 is dependent on both RNA transcription and protein translation. Together, LTP 2 and LTP 3 are called late LTP (L-LTP). In E-LTP, AMPARs become permeable to Na⁺ by L-glutamate stimulation. The Na⁺ influx results in excitatory post-synaptic potentials (EPSP), which release the Mg²⁺ block in NMDAR to make them permeable to Na⁺ and Ca²⁺. Ca²⁺ influx triggers downstream signaling in post-synaptic neuron. In E-LTP, the calcium-calmodulin dependent protein kinase II (CaMKII) is activated due to phosphorylation caused by an increase in Ca²⁺ concentration. Activated CamKII phosphorylates AMPARs to increase their conductance.
It also phosphorylates other proteins to induce AMPAR membrane insertion, which increase AMPAR density at the post-synaptic terminal. Late LTP is dependent on gene transcription and translation, which are stimulated by the extracellular signal regulated kinase (ERK). The ERK pathway can be activated by kinases like protein kinase C (PKC), protein kinase A (PKA), CamKII and phosphoinositide 3-kinase (PI-3K) (Malenka & Bear, 2004).

LTD is the weakening of CNS synapse strength, whereby the effectiveness of synaptic transmission is decreased on an activity-dependent basis (Santoro et al., 2011). There are many types of LTD depending on the type of neurons: in the CA1 region of hippocampus, there are NMDAR-dependent LTD and mGluR- dependent LTD. The change in membrane potential adjusts the concentration of Ca\(^{2+}\) in the cell following neurotransmitter release and determines whether the post-synaptic neuron will undergo LTP or LTD following stimulation. If Ca\(^{2+}\) concentrations is below threshold, activation of phosphatase dephosphorylates kinase, results in LTD. Further research found that not only the post-synaptic Ca\(^{2+}\) concentration but also the amplitude and duration of Ca\(^{2+}\) concentration elevation is essential to inducing of LTP or LTD differentially (Mizuno, Kanazawa, & Sakurai, 2001).

DHPG can trigger LTD via mGluR (Palmer, Irving, Seabrook, Jane, & Collingridge, 1997). This mGluR-LTD is a major form synaptic plasticity and is translation-dependent in dendrites (Huber et al., 2000). In contrast, NMDAR-dependent LTD is independent on translation. NMDAR-dependent LTD can be triggered by internalization of AMPARs, which is induced by stimulation of Gp1 mGluR using DHPG (Snyder et al., 2001). In the hippocampus, the major mGluR located on dendrites in the stratum radiatum is mGluR5, while mGluR1 localizes predominantly on cell bodies (Luscher & Huber, 2010). Knowing that FMRP translation is enhanced at dendrites by DHPG stimulation (Weiler et al., 1997) and that FMRP
happens to be a translation inhibitor, it is natural to suspect that FMRP is involved with mGluR5 and mGluR-LTD. Interestingly, DHPG-induced LTD is exaggerated in the hippocampus of *Fmr1* KO mice (Huber, Gallagher, Warren, & Bear, 2002).

FMRP is essential for translational activation following neurotransmitter release. The *Fmr1* KO mouse shows deficits in activating protein synthesis induced by DHPG (Weiler et al., 2004). Since FMRP is widely accepted as a translational repressor, FMRP targets may be over expressed in the absence of FMRP, including proteins responsible for AMPAR internalization. Excessive AMPAR internalization results in exaggerated LTD in *Fmr1* KO mice.

New protein synthesis is not necessary for mGluR-LTD. This could be because these LTD proteins are already present, or they are overexpressed. This significant discovery led to the “mGluR theory of FXS" (Figure 1-7) (Bear, Huber, & Warren, 2004). This theory was validated by reducing mGluRs in *Fmr1* KO mice by creating mGluR<sup>+</sup> heterozygous mice (Dolen et al., 2007). Antagonists of mGluR<sub>5</sub> can also rescue some deficits in *Fmr1* KO mice (de Vrij et al., 2008). Based on this theory, mGluR5 antagonists have been used in patients to treat autism. Some drugs have been in clinical trials since 2009 (http://clinicaltrials.gov). In conclusion, the symptoms of FXS may be caused by both exaggerated mGluR-LTD, and inability of enhancing local protein translation at post-synaptic terminal to respond new stimulations.

LTP in *Fmr1* KO mice was considered normal for years (Godfraind et al., 1996; Paradee et al., 1999). However, new protocols designed to induce LTP by glycine bath of hippocampal tissue slices shows that this form of LTP is decreased in *Fmr1* KO mice (Shang et al., 2009). One form of LTP is known as spike timing dependent long-term potentiation (STD-LTP). This STD-LTP does not require FMRP to be expressed, but the absence of FMRP results in a higher threshold (Meredith et al., 2007). Therefore, FMRP is involved in both LTD and LTP.
The HITS-CLIP result shows that 18 out of 58 NMDAR complex genes, 20 out of 32 mGluR5 complex genes, 1 out of 3 AMPAR complex genes are targets of FMRP (Darnell et al., 2011) (Sidorov, Auerbach, & Bear, 2013). Taken together, FMRP may be involved in synaptic plasticity induction, expression and maintenance in more than one pathway by translational regulation of “plasticity-related-proteins.”

1.6 Neurexin and Neuroligin

Synapse is the key site of information transmission between neurons. Most communications between neurons at synapse is achieved via neurotransmitters. Neurotransmitters are released by pre-synaptic neuron to activate ion channels or receptors on the post-synaptic terminal. The basis of this transmission is synaptogenesis and synapse maintenance. Synaptogenesis is the process of formation, specification and maturation of functional synapses between neurons. There are three steps involved in synaptogenesis: initial recognition, synaptic junction formation and final maturation (Sudhof, 2008). There are growing evidences to show the adhesion molecules at both pre-synaptic and post-synaptic terminals play important roles in synapse formation and synapse function. Neurexins (NRXs) and Neuroligins (NLs) are currently the most widely studied synaptic adhesion molecules. Neurexins localize to the pre-synaptic terminal and neuroligins are the corresponding ligands on the post-synaptic terminal (Ichtchenko et al., 1995). They form a trans-synaptic complex in the synaptic cleft.

Both Neurexin and Neuroligin contain several isoforms and all isoforms are under alternative splicing (Figure 1-8). To make it clear, in this thesis, isoforms are defined as different genes of the same family that are transcribed from different promoters and variants are defined as different alternative splicing products of one isoform. Neurexin was first found to be the
receptor of α-latrotoxin, which is a toxin from the black-widow spider (Ushkaryov, Petrenko, Geppert, & Sudhof, 1992). Neurexins are type 1 membrane proteins. There are three neurexin genes in mammals: NrxI, Nrx2, and Nrx3. Each neurexin gene encodes an α-form neurexin (longer isoform) and a β-form neurexin (shorter isoform) by transcription from different promoters (Tabuchi & Sudhof, 2002). Both α-NRX and β-NRX share the same C-terminus. In total, there are six types of neurexin isoforms in α-Nrx1, β-Nrx1, α-Nrx2, β-Nrx2, α-Nrx3, and β-Nrx3.

There are four Neuroligin isoforms (Nl1-4) in rodents and five NEUROLIGIN isoforms (NL1-5) in humans (Sudhof, 2008). NL1-3 are highly homogeneous and they are mainly expressed in CNS, while NL4 is expressed in both CNS and PNS (Hoon et al., 2011). NL1 preferentially localizes to excitatory synapses (Song, Ichtchenko, Sudhof, & Brose, 1999). NL2 preferentially localizes to inhibitory synapses (Varoqueaux, Jamain, & Brose, 2004). NL3 was found to localize to both types of synapses (Budreck & Scheiffele, 2007).

The alternative splicing diversity of Neurexin and Neuroligin provides the physical basis of recognition between pre-synaptic terminal and post-synaptic terminal. In Neurexin, there are five splice sites (SS 1-5) in α-Nrx, which can result in more than 1000 potential variants in different combination. There are two splice sites (SS 4, SS 5) in β-Nrx, which can produce four variants (Ichtchenko et al., 1995) (Shen et al., 2008). All Nl isoforms have a splice site A (SS A) and NlI has one additional splice site (SS B). It is widely accepted that there are four NlI variants: +A+B, +ΔAB, ΔA+B, and ΔAΔB. Delta (Δ) means the following splice site is missing. For example, the major variant of NlI, ΔA+B, contains splice site B but not splice site B. All the splice sites of neurexin and neuroligin are localized in the extracellular domain. Including or excluding the splice sites bequeaths binding specificity to NRX and NL. The major NRX binding
partner of NL1 is β-neurexin ΔS4 (Sudhof, 2008). Neurexins do not only bind to NLs, but also to dystroglycan and neuroexophilins (Craig & Kang, 2007).

Previous studies found that splice site B (SS B) of the NL1 variant is a key switch affecting NRX recognition. For NRX1 and NL1 interaction, NL1 variants with SS B bind specifically to β-NRX without SS 4 (not to α-NRX or β-NRX with SS 4). NL1 without SS B is able to bind all α and β-NRX variants, which is independent on SS 4 of NRX. NL2-4 behave in the same way as NL1 without SS B, since there is no SS B in these isoforms (Baudouin & Scheiffele, 2010).

The presence or absence of splice sites on NRX and NL makes possible the specific recognition between the two types of molecules. This molecular recognition and binding may further influence the recognition and binding between pre-synaptic terminal and post-synaptic terminals during synaptogenesis and may also influence synapse maintenance and signal transmission in mature synapses. Since SS B is only a 9 AA sequence, it is amazing that this short peptide can switch the recognition and binding affinity of NL1 with NRXs. Protein crystal structure shows that SS B of NL1 is on the binding interface with neurexin, while SS A of NL1 and SS 4 of NRX1 are nearby (Arac et al., 2007) (Figure 1-8). This is consistent with the key role these splice sites play in Neuroligin/Neurexin recognition and binding (Koehnke et al., 2008) (Shen et al., 2008). As the only NL isoform that contains SS B, NL1 is distinct from all the other NLs. It has different binding affinity with NRX and different functions. The SS A of all neuroligins regulates the binding to NRX with much smaller effect (Comoletti et al., 2006).

Co-culture experiments with non-neuronal cells expressing NL on cell membrane and neurons has shown formation of pre-synaptic terminals in neurons (Scheiffele, Fan, Choih, Fetter, & Serafini, 2000). Complimentary experiments with non-neuronal cells expressing neurexin co-
cultured with neurons showed formation of post-synaptic terminals in neurons at the position of contact with ectopically expressed neurexin (Graf, Zhang, Jin, Linhoff, & Craig, 2004; Takei et al., 2004). It has also been shown that overexpression of NL in cultured neurons increases synapse number (Dean et al., 2003) (Chih, Afridi, Clark, & Scheiffele, 2004) (Graf et al., 2004) (Prange, Wong, Gerrow, Wang, & El-Husseini, 2004) (Levinson et al., 2005) (Nam & Chen, 2005).

Overexpression of NL1 can increase excitatory synapse number (Prange et al., 2004) and overexpression of NL2 can increase inhibitory synapse number (Chih, Engelman, & Scheiffele, 2005). The balance of expression of distinct neuroligins regulate the excitatory/inhibitory balance (Levinson & El-Husseini, 2005). On the other side, RNAi knockdown of NL1-3 shows decreased synapse number (Chih et al., 2005). It suggests that NL plays a role in synapse formation. However, gene knockout experiments in mice show that knockout of any one of Nl1-3 is not fatal, only the triple knockout is fatal. Mice lacking Neuroligin1-3 died at birth because of breath failure. The analysis of the triple knockout neurons showed that the synapse number and morphology are all normal, but the electrophysiology is impaired (Varoqueaux et al., 2006). Taken together, these data suggest that Neuroligins are essential to synapse function but not to synapse formation (Chubykin et al., 2007; Varoqueaux et al., 2006).

Neurexins and Neuroligins all have a PDZ binding site in the intracellular part of the protein, binding and interacting with other PDZ-containing proteins like scaffold and signaling proteins. The name PDZ come from the first letter of post synaptic density protein 95 (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) (Kennedy, 1995). Neuroligin1 binds to PSD95 (Irie et al., 1997) and S-SCAM (Chen et al., 2000) by their PDZ binding domain in the post-synaptic terminal. These proteins relate NL1 to the glutamate
receptor recruitment mechanism directly or indirectly, affecting synaptic plasticity (Dean & Dresbach, 2006).

Changes in synapse activity by overexpression of Nl1 in cultured neurons were explained by the observed increases in synapse numbers (Graf et al., 2004) (Chih et al., 2005). However, more recent evidence showed that NL1 is actually involved in synaptic plasticity. NL1 plays a subtype-specific role in LTP in young CA1 and adult DG at the hippocampus. In fact, knocking out Nl1 changes the AMPAR/NMDAR ratio in neurons (Chubykin et al, 2007; Shipman, 2012), diminishing LTP in the CA1 region (Blundell et al., 2010) and completely eliminating LTP at DG cells (Shipman & Nicoll, 2012). However, NL3, which does not contain SS B, appears in excitatory synapses, and is not essential to support LTP in CA1 (Shipman & Nicoll, 2012). Overexpression of the extracellular domain of NL1 can enhance NMDAR-mediated current. The SS B of NL1 is essential for these effects. NL1 with SS B has a stronger synaptogenic phenotype (Boucard, Chubykin, Comoletti, Taylor, & Sudhof, 2005). It is more prone to form excitatory synapse (Chih, Gollan, & Scheiffele, 2006). The manipulation of NL1 levels could affect synapse number as well as the ratio of NMDAR/AMDA in synapses. The ratio of NMDAR/AMDA may affect Ca$^{2+}$ influx following neurotransmitter release and eventually affect synaptic plasticity. But the all-or-none results of the LTP deficit in the neuroligin KO mice shows that there is more than just the NMDAR change.

There has been accumulative evidence showing that NRXs and NLs are involved in autism. Copy number variation and mutation in Nl1, Nl3, Nl4 and Nr1-3 are associated with autism (Jamain et al., 2003) (J. Feng et al., 2006) (Sudhof, 2008). (http://gene.sfari.org). Nl1 KO mice also showed ASD like behavior (Chubykin et al., 2007) (Blundell et al., 2010).
Interestingly, \( Nl1 \), \( Nl2 \) and \( Nl3 \) and all three \( Nrxs \) (\( Nrxs1-3 \)) are FMRP targets (Dahlhaus & El-Husseini, 2010; Darnell et al., 2011).

1.7 FMRP and RNA processing

Although FMRP is localized to both the nucleus and cytoplasm, most research has been focused on its behavior and function in cytoplasm where it is predominantly located. However, the importance of a protein cannot be judged simply by its abundance. FMRP does not only bind mRNAs in the cytoplasm but also nucleus (Kim et al., 2009). Based on the fact that both NES and NLS exist in FMRP, FMRP has been observed shuttling in and out of nucleus. So one proposed behavior of FMRP is shuttling out of the nucleus with its target mRNA. However, the major focus of FMRP research has been on its role in the cytoplasm, especially in dendritic spines. It is possible FMRP is playing a different role in the nucleus, which happens before target RNAs reach the cytoplasm and certainly before cytoplasmic transport and translation. More and more discoveries about the role of FMRP in the nucleus have been made in recent years.

Binding to other proteins to form functional complexes fulfills the functions of FMRP. Finding a nuclear-localized FMRP binding protein would help to understand its functions in the nucleus. Several FMRP binding proteins such as FXR1P, FXR2P, nucleolin and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) have been discovered using co-immunoprecipitation (Ceman, Brown, & Warren, 1999). Recently, FMRP was reported as a chromatin binding protein which functions in the DNA damage response (DDR) (Alpatov et al., 2014).

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of RNA-binding proteins that bind to pre-mRNAs and are involved in RNA processing, including 5’ capping, 3’ polyadenylation, RNA editing and RNA splicing. One of the most abundant and studied
members of this family is hnRNP A1. It is a “Swiss army knife” playing a role in many processes involved in gene expression, like constitutive splicing, alternative splicing, and translation. It also plays a role in nuclear export, telomere maintenance and telomerase activity (Jean-Philippe, Paz, & Caputi, 2013).

The role of hnRNP in splicing is the most important and was the first of its functions discovered. RNA splicing is the removal of intron and joining of exons to form mature RNA. This can happen in tRNAs, rRNAs and mRNAs. Splicing is one major event in eukaryotic RNA processing. In mRNA, the splicing action is performed by the spliceosome, which is an RNA-protein complex composed of five small nuclear RNAs (snRNAs U1, U2, U4, U5, and U6) and several protein factors. The mechanism of splicing includes two nucleophilic attacks (Figure 1-9) (Patel & Steitz, 2003). Its function is fulfilled through cis-acting elements and trans-acting proteins (activators and repressors). Based on their effect and location, cis-acting elements can be classified as intronic splicing silencers (ISS), exonic splicing silencers (ESS), intronic splicing enhancers (ISE), and exonic splicing enhancers (ISE). The predominant splicing repressors are hnRNPs, such as hnRNP A1.

Most activator proteins are members of the serine-rich (SR) protein family that contain RNA recognition motifs and arginine and serine-rich (RS) domains (Matlin, Clark, & Smith, 2005). Alternative splicing is RNA splicing in which particular exons of a gene may be included or excluded from the same pre-mRNA to form different transcripts, which eventually translate to different proteins. There are several alternative splicing patterns that depend on one exon included or excluded (Figure 1-10). Alternative splicing increases the diversity of genome. For example, the same Nl and Nrx pre-mRNA can produce several variants. More than 80 % of human genes are alternatively spliced (Kampa et al., 2004). In fact, abnormal alternative splicing
can cause many diseases. For example, tauopathies are diseases caused by abnormal ratio of tau isoforms (I used variant in this thesis) produced from deregulated alternative splicing in the central nervous system (Tazi, Bakkour, & Stamm, 2009).

A nuclear protein reported to have interaction with the Drosophila fragile X homolog (dFMRI) is Drosophila adenosine-to-inosine RNA-editing enzyme (dADAR). Its activity is modulated by dFMRI (Bhogal et al., 2011). The dADAR is responsible for adenosine-to-inosine RNA editing (A-I editing). The inosine is recognized as guanosine by the cellular machinery due to a similarity in molecular structure (Figure 1-11) (Basilio, Wahba, Lengyel, Speyer, & Ochoa, 1962). RNA editing is an event whereby RNA molecules undergo nucleotide sequence changes after the RNA has been transcribed, hence the term “editing”. It is one process of eukaryotic pre-mRNA processing. It has been observed in eukaryotes, viruses, archaea and prokaryotes. In eukaryotic cells, it happens not only to mRNA but also to tRNA, rRNA, and even miRNA (Mehler & Mattick, 2007). There are generally two types of editing: by insertion or deletion, and by deamination (Gott & Emeson, 2000). Deamination can transform the cytidine base to uridine (C-U editing) or change adenosine to inosine (A-I editing). All of them change the primary sequence of RNA, which may affect any biological process involving RNA sequence or structure. By changing RNA sequence, RNA editing allows cells great molecular diversity.

A-I editing is the major RNA editing form in mammals (Danecek et al., 2012). All A-I editing occurs in a double stranded RNA (dsRNA) region. A-I editing is classified as a specific type and a promiscuous type. Specific editing occurs in short complexes like the double strand formed between intronic sequences and its complimentary exon. Promiscuous editing happens in long complexes. If an intronic sequence is involved to form the double strand, it means this editing process must happen prior to splicing or at least simultaneously. If it is a pre-splicing
procedure, then the nucleotide change at splice sites would affect splicing. Altered editing on the branch site or splice site could cause many types of effects on splicing, including abolished splicing or changed splice site choice (Figure 1-12) (Valente & Nishikura, 2005). If the changes in nucleotides happen in the coding region, it may alter the primary sequence of the encoded protein, introduce a stop codon producing a shorter peptide, or introduce additional ATGs, enabling the expression of a new transcript. Changes in the non-coding region may cause a corresponding effect based on the changes.

Since RNA editing can change the information encoded by the mRNA, it is not surprising that it has many physiological functions and related diseases. Many disorders are related to abnormal RNA editing (Maas, Kawahara, Tamburro, & Nishikura, 2006) (Slotkin & Nishikura, 2013). It has been shown that A-I editing is very prominent in nervous system (Bass, 2002) (Valente & Nishikura, 2005). RNA editing, especially A-I editing, participates in almost all aspects of development, homeostasis, synaptic plasticity, and neural network (Mehler & Mattick, 2007). Among the diseases caused by abnormal A-I editing, many of them are nervous system diseases. Some neurotransmitter receptors in CNS are regulated by A-I editing, like GluR2 of AMPAR. More than 99.9 % of GluR2s undergo A-I editing resulting in a Q to R change which makes sure the AMPAR permeability is low to Ca$^+$ at basal state (Barbon & Barlati, 2011). Inhibition of editing or knockout of ADAR2 causes epileptic seizures in transgenetic mice due to excessive Ca$^+$ infuse, and early death (Reenan, 2001). Research using deep sequencing to compare A-I editing sites in neurotypical and autistic postmortem cerebella showed a correlation between A-I editing and autism (Eran et al., 2013).

In conclusion, FMRP may get involved in RNA editing through ADAR2, get involved in RNA splicing through hnRNP A1, or get involved in RNA splicing indirectly through ADAR2.
It is possible that there are other hnRNPs and RNA editing enzymes regulated by FMRP directly or indirectly.
1.8 Specific aims

The experiments described in this thesis have following specific aims:

First, study the protein-RNA interaction FMRP and its potential target $Fmr1$ 3’ UTR. Investigate the subcellular localization of $Fmr1$ 3’ UTR. At the translation level, study the translational regulation of FMRP through $Fmr1$ 3’ UTR and local translation regulated through $Fmr1$ 3’ UTR.

Second, study the protein-RNA interaction FMRP and its potential target $Nl1$ 3’ UTR. Investigate the subcellular localization of $Nl$ 3’ UTR. Eventually, At the translation level, study the translational regulation of FMRP through $Nl1$ 3’ UTR and local translation regulated through $Nl1$ 3’ UTR.

Third, analyze the abnormal alternative pattern of $Nl1$ mRNA in $Fmr1$ KO hippocampus. Study the role FMRP plays in alternative splicing of $Nl1$ mRNA. Rescue abnormal neuron properties of $Fmr1$ KO hippocampus with specific $Nl1$ variant.
Chapter 1 Figures

**Figure 1-1  FMRP schematic**
The FMRP schematic, showing the NLS and NES of FMRP, also the three RNA binding domains: KH1, KH2, and RGG box. (Cheever & Ceman, 2009). RNA 15(3): 362-366.

**Figure 1-2  FMR1-family proteins**
The FMR1 family of proteins contains FMR1, FXR1 and FXR2. They all contain three RNA binding domains: two hnRNP K-homology (KH) domains (blue), and one arginine–glycine-rich (RG/RGG) box (yellow). The full-length FMR1 isoforms 1 (iso1) (71 kDa) and predominant isoform 7 (iso7) (69 kDa) vary by the presence of exon 12 (black) within KH2. I304N mutation localizes in KH2 (red star). (Ascano et al., 2012). Nature 492(7429): 382-386.

**Figure 1-3  Polyribosomes clusters at the base of dendritic spines**
Dendritic and axonal RNAs are transported in mammalian neurons. Yellow arrow stands for retrograde signal triggered by transsynaptic stimuli, sending from synapse to cell body and nucleus. RNPs are assembled in the cytoplasm and transported along cytoskeleton. RNPs get recruited at target site and then locally translated. (Tiedge, Bloom, & Richter, 1999). Science 283(5399): 186-187.

**Figure 1-4** RNA transport and local translation in neuron

**Figure 1-5** FMRP phosphorylation and translational regulation

(A) FMRP phosphorylation and translational regulation. Phosphorylated FMRP bind target mRNA and repress its translation. Dephosphorylated FMRP release binding to target mRNA and release translation. (B) Schematic of the domain structure of FMRP showing phosphorylation site. Serine is shown in bold. Arrows point to the four possible sites of phosphorylation. (Ceman, O'Donnell, et al., 2003). Hum Mol Genet 12(24): 3295-3305.
**Figure 1-6  Hippocampus, LTP and LTD**
Schematic representation of induction and recording of long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD can be induced by electrodes placed in the Schaffer collateral-commissural (SCC) pathway and recorded from the CA1 subfield as shown in upper panel. Lower panel shows excitatory post-synaptic potentials (EPSP) of LTD and LTP (Riedemann et al., 2010). Riedemann, T., et al. (2010). *Mol Brain* 3: 2.

**Figure 1-7  mGluR theory of FXS**
Activation of group I mGluRs stimulate translation of FMRP target mRNAs while FMRP inhibit FMRP targets translation. Absence of FMRP increase FMRP targets translation, which will cause FXS. Antagonist or Gp1 mGluR decrease target mRNA transcription translation. (Dolen, Carpenter, Ocain, & Bear, 2010). Pharmacol Ther 127(1): 78-93.
Figure 1-8  Neuroligin-Neurexin binding and alternative splicing of Neuroligin
Upper panel shows splice site A (including A1 and A2) and B of NL1. Upper right shows the isoelectric point of A1 (positive) and A2 (negative). The structure of NL1 and β-NRX1 shows their interacting interface. Splice sites are shown in yellow. Modified from (Dean & Dresbach, 2006). Trends Neurosci 29(1): 21-29.
Biochemical mechanism of splicing

Splicing reaction is done by two nucleophilic attack reactions between nucleotides. The 2’ OH of branch point nucleotide in the intron perform nucleophilic attacks on the first nucleotide of the intron on the 5’ to form a lariat intermediate (lariat means loop). Then the 3’ OH of the 5’ exon attacks the last nucleotide of intron at the 3’ splice site. Finally the exons get joined and the intron get released (Patel & Steitz, 2003). Nat Rev Mol Cell Biol, 4(12), 960-970. doi: 10.1038/nrm1259.

Alternative splicing patterns

Figure 1-11  A-I editing and recognition of I as G

Figure 1-12  RNA editing in splice site alters splicing
(A) Regular splicing without editing. (B) Splicing is abolished when RNA editing removes branch site. (C) 5’ alternative editing site is created. (D) 3’ alternative editing site is created. (E) Splicing is abolished when 3’ splice site is destroyed. (F) Created splice site is destroyed. (Valente & Nishikura, 2005). Prog Nucleic Acid Res Mol Biol 79: 299-338.
CHAPTER 2

MATERIALS AND METHODS
\section*{2.1 Hippocampal neuron culture}

Following mouse strains were purchased from the Jackson Laboratory: WT (FVB.129P2-Pde6b+Tyrc-ch/AntJ) and \textit{Fmr1}-KO (FVB.129P2-Pde6b+Tyrc-ch \textit{Fmr1}tm1Cgr/J). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed in the Hunter College Animal Facility, approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC). Mice were raised in a controlled environment.

Hippocampal cell cultures were prepared from P0 (postnatal) mice. Whole brains were removed from newborn pups and the hippocampi from 5~7 mice were dissected. Hippocampal tissue was digested in plating medium (MEM, 10\% FBS, 10 mM HEPES, 33 mM glucose) with trypsin for 15 min at 37\(^\circ\)C. Cells were gently dispersed with a glass Pasteur pipette (opening narrowed to proper size by flame before use). Dispersed hippocampal neurons were then plated at low density (25,000 cells/cm\(^2\)) on poly-L-lysine pre-coated (0.1 mg/ml) glass chambers (Thermo) or coverslips. Cultures were incubated at 37\(^\circ\)C with 4 \% CO\(_2\). Plating media was replaced with maintenance medium (astrocyte-conditioned Neurobasal/B27/GlutaMAX) 2 hours after plating. AraC (1-\beta-D-arabinofuranosylcytosine) was added to a final concentration of 2.5 \(\mu\)M two days after plating to curb glial proliferation. Transfection was performed on cultured neurons on DIV (day \textit{in vivo}) 6~7.

\section*{2.2 Plasmid construction}

The dual luciferase vector pmirGLO (Promega) is designed to evaluate microRNA activity on target 3‘ UTRs. The pmirGLO-\textit{Fmr1} 3‘ UTR vector and pmirGLO-\textit{Nl1} 3‘ UTR vector were constructed by cloning the \textit{Fmr1} 3‘ UTR and \textit{Nl1} 3‘ UTR from cDNA of the
appropriate (KO or WT) mouse brain into the SacI and SalI sites of the multiple cloning site (MCS) of pmirGLO, downstream of Firefly luciferase. The flag-FMRP construct was prepared by adding a FLAG tag to the 5’ of mouse FMRP and cloning the flag-FMRP into pcDNA3 vector. Vector pBS+ and pBS- were constructed by replacing CMV promoter of pcDNA3.1+ and pcDNA3.1- by the Synapsin promoter cloned from mouse genomic DNA. The “+” and “-” vector have that the same multiple cloning sites in reverse orientations. The dual synapsin promoter vector, pDS- vector was constructed based on pBS- vector by adding another synapsin promoter. The multiple cloning site was split into two parts: MCS1 and MCS2. The SV40 polyA signal sequence from pmirGLO vector was added to downstream of MCS1. A bovine growth hormone polyadenylation signal sequence (BHG polyA) was added to downstream of MCS2. These two polyA signal sequence were used to stop transcription of the first and the second transcript respectively. Vector pDS_GFP-FMRP_LifeAct-TagRFP was constructed by cloning GFP-FMRP into MCS1 and LifeAct-TagRFP into MCS2.

The MCP-MS2 system was used to visualize mRNA subcellular localization (Figure 2-1). Some changes were made to the traditional system. The MCP-TagRFP vector in both pcDNA3.1+ and pBS+ were constructed to increase signal/noise ratio instead of GFP. The MS2 vector backbone used for MS2 vector construction was derived from the Rous sarcoma virus (RSV) promoter-LacZ-MS2-β-actin 3’ UTR vector. The MS2-Fmr1 3’ UTR vector, MS2-Nl1 3’ UTR vector and MS2-Gapdh 5’UTR-CDS-3’ UTR vector were constructed by replacing the β-actin 3’ UTR to make new constructs. The myristoylated-destabilized-TagRFP vector was constructed by adding a myristoylation consensus sequence at the 5’ end of TagRFP. A destabilization signal was the added to the 3’. Next, Fmr1 3’UTR or Nl1 3’ UTR were added to
the MCS at downstream of the coding sequence (myr-RFP-destabilization AA). Plasmids were amplified in *E. coli* and extracted using PerfectPrep EndoFree Plasmid Maxi Kit (5 Prime).

### 2.3 Neuron transfection

Neuron culture medium was exchanged with pre-warmed antibiotic free conditioned medium at least 2 hours before transfection. For each chamber or coverslip (one well of a 12-well plate), 2~2.5 μg plasmid was diluted in 50 μl Opti-MEM (Life Technologies) with 0.75 μl Plus reagent (Life Technologies). This was mixed with 2.25 μl Lipofectamine LTX diluted in 50 μl Opti-MEM, incubated for 5 min at RT, and plated.

Stealth siRNAs (400 nM) targeting mouse *Fmr1* were transfected into neuronal cultures in 12 well plates. Lipofectamine RNAiMAX (Life Technologies) was used for siRNA transfection as per instructions.

### 2.4 Immunoprecipitation (IP) and western blot

HEK293 cells were trypsinized and split into 6 well plates to ensure a density of 70~80 % at the time of transfection on the second day. Co-transfection was performed using LipoD293TM DNA *in vivo* Transfection Reagent (SignaGen® Laboratories) as per instructions. The following combinations were used: FLAG-FMRP and pcDNA3, FLAG-FMRP and pmirGLO (blank vector), FLAG-FMRP and pmirGLO-*Fmr1* 3’ UTR, FLAG-FMRP and pmirGLO-*Nl1* 3’ UTR. Cells were incubated for 36-48 hours. Following incubation, cells were washed with phosphate buffered saline (PBS) and treated with IP buffer (50 mM Tris-Cl [pH 7.3], 100 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 5 % glycerol, 0.1 % Triton X-100, protease inhibitor, and RNase Inhibitors) on ice for 15 min and followed by 4℃ centrifugation (16,000 X g) for 10 minutes.
Each supernatant was immune-precipitated using ANTI-FLAG® M2 magnetic beads overnight at 4° C. Beads were washed 3 times with 10 bead volumes of IP buffer. Beads were then split into two equal halves. One half was resolved in 2X SDS-PAGE loading buffer, heated at 95 °C and the supernatant was electrophoresed on a 4-12 % gradient Bis-Tris gels. Gels were transferred to nitrocellulose membranes with iBlot® 7-Minute Blotting System (Life Technologies). The membrane was probed with mouse anti-FLAG antibody (mouse, clone M2) and scanned with ODYSSEY® Infrared Imaging System (LI-COR Bioscience). Quantification was performed using Image J (NIH). The other half of the sample was treated with Trizol (Life Technologies) to extract mRNA for reverse transcription and PCR. Taq DNA Polymerase (New England Biolabs) was used for amplification. The cDNA derived from the IP was used to detect/amplify Firefly luciferase, Renilla luciferase and the N19 region of Fmr1 by PCR. NL1 antibody (clone 4C12) (SYSY, Synaptic Systems) was used to probe NL1 in synaptosomes from hippocampus.

2.5 Live imaging and Fluorescence recovery after photobleaching (FRAP)

All transfections for live imaging were performed on DIV 6-7 hippocampal neurons. In the MS2-MCP experiment, the two constructs (MCP-RFP and MS2-Fmr1 3’ UTR, or MCP-RFP and MS2-Nl1 3’ UTR, or MCP-RFP and MS2-Gapdh mRNA) were transfected with MCP-RFP and MS2-3’-UTR at a 1:9 ratio to maximize mRNA labeling efficiency based on our experience. Triple plasmid transfections of GFP-FMRP, MCP-RFP and MS2-3’-UTR were done with a ratio of 1:1:9. Two plasmids transfection of Cerulean and myr-deTagRFP vectors were performed at a ratio of 1:9. For all DHPG treatments, neuronal cultures were treated with 100 µM (RS)-3, 5-DHPG (TOCRIS Bioscience) for 15 minutes. Because of slight physiological differences
between (RS)-3, 5-DHPG and (S)-3, 5-DHPG, we used (RS)-3, 5-DHPG (Wisniewski et al., 2002). For all brain-derived neurotrophic factor (BDNF) treatment, neuronal cultures were treated with 50 ng/ml BNDF for 1h. Differential interference contrast (DIC) images were taken to visualize live cell morphology.

All live images were captured using a Nikon ECLIPSE Ti inverted fluorescence microscope with following lenses: Nikon 40 X/1.30 Plan Fluor Oil object lens (DIC H/N2 ∞/0.17 WD 0.2), Nikon 60 X/1.49 Apo TIRF Oil object lens (∞/0.13-0.21 DIC N2), Nikon 100 X/1.49 Apo TIRF Oil object lens (∞/0.13-0.20 DIC N2).

For live imaging experiments, transfected neuron chambers were scanned with the 40 X lens to localize transfected neurons. 60 X and 100 X lenses were used for live imaging. Laser was provided by a Laser Diode Control Unit (LDCU8) (Power Technology). Lasers were adjusted through MicroPoint (Andor). Images and videos were deconvolved with AutoQuant (Media Cybernetics) before quantification to enhance the quality of images.

2.6 Artificial synapse formation assay

HEK293 cell culture was trypsinized and split into 24 well plates with HEK culture medium (DMEM with 6 mM L-glutamine and 10% BGS). Transfection of the Neurexin-GFP vector was performed on the second day at a density of 70~80 % using LipoD293TM DNA in vivo Transfection Reagent (SignaGen® Laboratories) as per instructions. The corresponding neuronal culture for co-culture experiments was transfected on the same day with the Neuroligin (A2B) vector, PSD95-TagRFP vector, the MS2-MCP system, or deTagRFP-3’ UTR plasmids.

Co-cultures were prepared on the third day (one day after transfection). Neurexin-GFP transfected HEK293 cells were briefly treated with trypsin to get cells off bottom without too
much digestion of the NEUREXIN-GFP on the HEK cell surface. Pre-warmed HEK cell culture medium was used to neutralize trypsin. Cells were centrifuged at room temperature at 100 x g for 1 min. Then trypsin containing medium was removed and cells were diluted with pre-warmed neurobasal medium (GIBCO, Life Technologies). Neurobasal medium (200 μl) was used to dilute cells from one well of a 24 well plate and 100 μl of the diluted HEK cells were added to a chamber of transfected hippocampal neurons. Images and videos were taken either immediately after co-culture started, or one hour later depending on the aim of experiment.

2.7 NEUREXIN-FC staining and surface GluA2 staining

Rattus Neurexin1 beta was cloned into a FC-IgG vector in a scheme of hemagglutinin of Influenza A virus-Rattus Neurexin1 beta-hFc IgG in the plasmid with psecTag2 vector (Life Technologies) backbone. The hemagglutinin of Influenza A virus at N terminal of protein is capable to secrete the recombinant protein out of cell. Human Fc region (fragment crystallizable region) is the tail region of an antibody, which is capable to bind protein A (a 56 kDa surface protein originally found in the cell wall of the bacterium Staphylococcus aureus). HEK293 cells were split and cultured in 100 mm Nunclon® cell culture dishes. Transfection was performed the second day at a density of 70~80 % by calcium phosphate transfection method. Medium was collected and replaced with fresh culture medium every day since transfection for 3 days. Hepes (pH 7.4, 1.0 M) was added to collected medium with a ratio 1:40 to a final concentration 25 mM and preserved in 4 °C. 3~5 collections were done before purification. Collected medium was centrifuged in 50 ml polypropylene tubes at 3000 rpm, 15 min, 4 °C to pellet suspended cells. The supernatant was then filtered by 0.45 μm cellulose acetate (CA, membranes provide fast flow rates and low protein binding and are good for filtering cell culture media), polystyrene
nonpyrogenic filter system (Corning) (CA). Protein A Dynabeads magnetic beads (Life Technologies) was washed with fresh culture medium before incubation with filtrated medium. 0.5~0.7 µl Protein A magnetic beads were added to 1 ml filtrated medium and rotated in 50 ml polypropylene tubes in 4°C cold room overnight. The beads were concentrated by spinning down at 700 rpm, 4 min, 4°C and purified from medium by magnet. The beads were washed 4 times with 1 ml Hepes (pH 7.4, 25 mM) on ice and suspended to final concentration as 33 % bead volume-buffer volume in ice-cold Hepes (pH 7.4, 25 mM). The beads with NEUREXIN-FC were preserved in 4°C. To dilute NEUREXIN-FC from the beads, 100 µl glycine buffer (pH 2.2, 100 mM) was used on 100 µl beads. The concentration of eluted NEUREXIN-FC was measured with a Qubit fluorometer as per instructions (Life Technologies).

2.8 Immuno-Fluorescence and (IF) Fluorescent In Situ Hybridization (FISH)

For IF imaging, neurons were fixed on the day after transfection in fixative (4 % paraformaldehyde (PFA) in 1 X PBS with 4 % glucose) for 20 minutes at room temperature. PBS-T (1X PBS, 0.1 % Triton X-100) was used to penetrate cell membranes. Then neurons were blocked with blocking buffer PBS-TB (PBS-T, 3 % BSA). Primary and secondary antibodies were diluted in blocking buffer. Neurons were incubated with diluted antibodies for 40 min to 1h and 20-30 min respectively. Rinsing was performed 3 times using PBS-T between primary antibody and secondary antibody incubations. After staining, coverslips were mounted with ProLong® Gold Antifade Reagent overnight. The following antibodies were used in the IF: MAP2 (mouse, clone HM-2, Sigma), synapsin I (rabbit; S 193, Sigma), Vglut1 (rabbit, BNP1, Synaptic Systems) and VGat (rabbit, 131002, Synaptic Systems).
Neurons were transfected with MS2-NlI 3’ UTR for the FISH experiment. IF was done before FISH following the same procedure as mentioned. An additional 4 % PFA fixation was performed to fix antibodies before doing FISH. Anti-sense MS2 probes were used to detect MS2-mRNA subcellular location following a previously described protocol (Bassell et al., 1998). Probes (30 ng) were used per slide. Slides were incubated at 37°C for 5 hours before rinsing. The coverslip was mounted similarly to those in IF.

All imaging of immunofluorescence slides were captured using Nikon ECLIPSE TE200-U inverted fluorescence microscope with a Nikon 60 X 1.40 Oil Plan Apo VC lens. Imaging was performed by using NIS-elements. Images were deconvolved with AutoQuant (Media Cybernetics). Quantification Analysis of Fluorescence images was performed using NIS-elements software (Nikon).

2.9 PCR

Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used for cloning. Taq DNA Polymerase (New England Biolabs) was used to perform semi-quantitative PCR. 1 kb Plus DNA Ladder (Life Technologies) was used as a marker in all DNA agarose gel electrophoresis experiments. Quantification of DNA bands was performed using Image J (NIH).

2.10 Luciferase assay

Luciferase assays in HEK293 cells were performed by transfecting HEK293 cells with pmirGLO (blank) and pmirGLO_Fmr1 3’ UTR vector. HEK293 cells were split into 48 well plates to ensure a 70-80 % density the next day. Transfection was performed with LipoD293TM DNA in vivo Transfection Reagent (SignaGen® Laboratories) as per instructions. Cells were
lysed with lysis buffer included in the kit and collected 24 h after transfection. Luciferase assays were performed using the Dual-Glo Luciferase Assay System (Promega) and GloMax 96 Microplate Luminometer (Promega). Firefly luciferase and Renilla luciferase amounts were reported. The ratio of Firefly luciferase to Renilla luciferase was calculated.

For all DHPG treatments, neuronal cultures were treated with 100 µM (RS)-3, 5-DHPG (TOCRIS Bioscience) for 15 minutes. Hippocampal neurons were cultured on poly-L-lysine coated 24 well plates. Plasmid constructs of pDS-LUC and pDS with different 3’ UTRs were transfected on DIV 6-7 with Lipofectamine LTX (Life Technologies) as described previously. Neurons were lysed and collected for luciferase assays as described previously. Luciferase assay was performed as described previously. Firefly luciferase and Renilla luciferase amounts were recorded. The ratio of Firefly luciferase to Renilla luciferase was calculated.

In the analysis of luciferase assay, any translational effect on the target 3’ UTR will be reflected by the amount of firefly luciferase. The Renilla luciferase serves as an internal control. Compared to the blank pmirGLO vector which serves as a internal control and the ratio of Firefly luciferase to Renilla luciferase shows the translational effect on the target 3’ UTR.
**Figure 2-1  MS2-MCP system**
(1) DNA and RNA polymerase in nucleus. (Bemben et al.) Tandem repeat of MS2 fused to mRNA of interest. (3) MCP-GFP with a NLS is translated in cytoplasm and accumulate in nucleus, also lower the concentration of MCP-GFP in the cytoplasm. (4) MCP-GFP bind MS2-mRNA of interest and export to cytoplasm. (5-6) MCP-GFP + MS2-mRNA of interest complex localize to the biological destination of mRNA of interest. (7) The complex got dissembled mRNA of interest get degraded. (Weil, Parton, & Davis, 2010). Trends Cell Biol 20(7): 380-390.
CHAPTER 3

FMRP BINDS TO THE 3’UTR OF FMR1 TO REGULATE MRNA TRANSLATION IN THE DENDRITES OF HIPPOCAMPAL NEURONS
3.1 Introduction

The fragile X mental retardation protein (FMRP) binds its own mRNA with high affinity (C. T. Ashley, Jr. et al., 1993). Previous reports focused on an FMRP binding sequence located in the coding region of \textit{Fmr1} mRNA, specifically a G-quartet motif within an Arg-Gly-Gly (RGG) box (Schaeffer et al., 2001). A subdomain named N19 (1470-1896 nt, the 427 last nucleotides of the coding region of \textit{FMR1} mRNA) derived from the \textit{FMR1} mRNA which corresponds to the RGG box is often used as a positive control for FMRP targeting and binding (Schaeffer et al., 2001) (E. Bechara et al., 2007).

However, for many mRNAs, the 3’ untranslated region (3’ UTR) is the target of RNA binding proteins that facilitate mRNA transport and translational regulation. Many genes have \textit{cis}-acting localization signals in their 3’ UTRs (K. C. Martin & Ephrussi, 2009). One example is the 3’ UTR of \(\beta\)-\textit{actin}, which contains a 54 nt “zipcode” localization element. This sequence contains enough information to guide the entire transcript to its destination (Kislauskis et al., 1994). It is arguable whether the 3’ UTR of \textit{Fmr1} serves as a major binding site for FMRP (Brown et al., 1998) (Sung, Conti, Currie, Brown, & Denman, 2000) (Schaeffer et al., 2001). Besides this question, there remains some other questions: Does the 3’UTR of \textit{Fmr1} localize the mRNA to specific region in the neuron? If it does, is the translation regulated by FMRP? Is the translation globally or locally in dendrites? Moreover, how does FMRP regulate the translation upon stimulation?

The technique of imaging RNA subcellular localization has advanced over the years. Fluorescent in situ hybridization (FISH) was routinely used to visualize RNA molecules in fixed cells. With the development of green fluorescent protein (GFP) fusion proteins, researchers can visualize proteins in live cells. The visualization of RNA in live cells using GFP is possible, but
this requires an adaptor to tether the RNA to the GFP reporter. Interestingly, RNA binding proteins containing specific RNA targeting motifs fit the requirements of a tether.

The MS2-MCP system was developed to detect the subcellular localization of target RNAs in living cells. It has been used in hippocampal neurons for the live imaging of mRNA granules (Rook, Lu, & Kosik, 2000). This two plasmid system consists of one MCP-GFP plasmid expressing GFP fused the MS2 capsid protein (MCP), and a MS2-RNA plasmid expressing the target RNA sequence fused with several repeats of a stem-loop structure RNA derived from bacteriophage MS2 (Bertrand et al., 1998). MCP is capable of binding to the stem-loop structure of MS2 mRNA. Independent of where the target MS2-RNA fusion transcript localizes, the MCP-GFP fusion protein is able to bind the MS2 stem loops of RNA. This stem loop binding of MCP-GFP allows the entire complex to be visible in cells, depicting the location of target RNA molecules. Recently, MS2-MCP has been used to visualize single molecule of RNA in live mouse brain (Park et al., 2014).

The MS2-MCP system is implemented by transfecting live cells with the MS2 plasmid and the MCP-GFP plasmid. Live imaging is possible using this method, which is a great advantage compared to FISH (Fluorescent In Situ Hybridization), in which cells must be fixed and provide only static images. Although a strong signal may be obtained by microinjection of \textit{in vivo} synthesized and fluorescently labeled RNA into living cells, the MS2-MCP delivery causes less trauma to cells because the microinjection uses a needle to penetrate the plasma membrane and inject exogenous material into cells. The MS2-MCP system uses much milder plasmids transfection. The endogenous RNA transcription and protein translation machinery provide signals. In addition, microinjection can sometimes deliver too much injected RNA to the cell, which may overwhelm RNA processing and transporting mechanisms and lead to false positive
results. Lastly, the MS2-MCP method provides cells with more “natural” mRNAs since it is transcribed in nucleus by endogenous RNA polymerase II, modified by adding a 5’ m7G cap and a 3’ poly A tail, also correctly folded. Since the secondary and tertiary structure of RNA is important to its function, proper RNA folding is crucial. Since RNA expressed in MS2-MCP system experiences the same mRNA transcription, RNA processing, modification and transport as endogenous mRNA, the MS2-MCP system can highly mimic endogenous mRNA expression. This gentle delivery method for mRNAs into live cells is a great advantage for in vivo study.

Here we show that FMRP binds to the 3’ UTR of Fmr1 in HEK293 cells. We also show that the Fmr1 3’ UTR is integrated into ribonucleoprotein (RNP) granules that localize to dendrites of hippocampal neuron. The FMRP co-localizes with the Fmr1 3’ UTR in these RNP granules and co-transport to hippocampal processes. Using the luciferase assay, we found that the translation of luciferase reporter is regulated through the Fmr1 3’ UTR by FMRP. We have also observed local translation of myristoylated-destabilized-RFP reporter fused with Fmr1 3’UTR expressing in dendrites by Fluorescence recovery after photobleaching (FRAP).

### 3.2 FMRP binds Fmr1 mRNA in its 3’ UTR

We wanted to study the interaction of FMRP and the 3’ UTR of Fmr1 without the influence of the Fmr1 5’ UTR and coding region. This is primarily because the coding region of Fmr1 has been previously identified as an FMRP target (C. T. Ashley, Jr. et al., 1993). The mouse Fmr1 coding region was cloned to pcDNA3 to express FMRP (Figure 3-1, A). The mouse Fmr1 3’ UTR was cloned from wild type mouse brain cDNA to pmirGLO vector (Promega) (Figure 3-1, B and C). The pmirGLO vector is a dual luciferase reporter vector designed for 3’ UTR study. The amount of two different luciferases, Firefly luciferase and Renilla luciferase, can
be quantified differentially in the luciferase assay. The dual promoter vector was implemented through a dual promoter design. The human phosphoglycerate kinase (PGK) promoter drives the expression of Firefly luciferase which is at upstream of a multiple cloning site (MCS). The Fmr1 3’ UTR was cloned to the multipole cloning site. An SV40 late poly (A) signal sequence is downstream of the MCS to efficiently terminate transcription by the PGK promoter. The second promoter is an SV40 early enhancer/promoter that drives the expression of Renilla luciferase. There is a synthetic polyadenylation signal at the 3’ end of Renilla luciferase to terminate transcription. The two transcripts, Firefly luciferase_Fmr1 3’ UTR mRNA and Renilla luciferase mRNA are expressed independently.

To begin our experiments, we first wanted to confirm the expression of our plasmid constructs. The pmirGLO vector containing a control vector with no insert (pmirGLO, Figure 3-1, B) or the Fmr1 3’ UTR (Figure 3-1, C) were co-transfected with Flag-FRMP (Figure 3-1, A) into HEK239T cells. Flag-FRMP was also transfected with blank pcDNA3 as a positive control of FMRP-RNA binding since it reportedly targets the coding region of its own transcript.

RNA from cell lysates was extracted from cells 24 hours after transfection for RT-PCR to confirm the expression of Firefly luciferase, Renilla luciferase and N19 region of Fmr1 mRNA (Figure 3-1, D). Primer pairs produced a 500 bp amplicon for Firefly luciferase (Lanes 1 and 3), a 308 bp product for Renilla luciferase (Lanes 2 and 4) and a 379 bp product for N19 of Fmr1 (Lane 5). This result confirmed the expression of our plasmid constructs, also shows that our primers are specific, producing a single band of the expected size for each amplification.

To determine if FMRP binds to the Fmr1 3’ UTR, Flag-FMRP and its targeting RNAs were immunoprecipitated from lysate of transfected HEK293 cell using magnetic beads
conjugated with anti-FLAG antibody. RNA was extracted from the beads for RT-PCR using the same primers described above (Figure 3-1, E).

The N19 fragment of Fmr1, which has been previously identified as an FMRP target, is robustly amplified from IP samples derived from Flag-FMRP transfection alone (Figure 3-1, Lane 5). This confirms that the coding sequence of Fmr1 is a target of FMRP in this experiment, which proves the validity of the system. The co-transfection of the empty control vector pmirGLO and Flag-FMRP produced no visible bands for either Firefly or Renilla luciferase (Lanes 3 and 4). This is an expected result since neither luciferase transcripts interact with FMRP and therefore would not be pulled down. Only when the Fmr1 3’UTR is present in the pmirGLO vector does Flag-FMRP bind and co-immunoprecipitate with Firefly luciferase mRNA (Figure 3-1, Lane 1). As shown in Lane 4, a signal for Renilla luciferase is absent after the pull-down of Flag-FMRP under these conditions, since Renilla luciferase is not a target of FMRP (Figure 3-1, Lane 2).

FMRP is widely expressed in almost all cell and tissue types. We attempted to exclude endogenous FMRP in HEK293 cells by using FLAG-tagged FMRP and anti-FLAG antibody instead of FMRP antibody. To assess the efficiency of our pull-downs, we performed western blot for FLAG-FMRP under the conditions of our IP binding experiments (Figure 3-1, F). Using anti-FLAG antibody, we could detect FLAG-FMRP in our input samples and following IP under all conditions tested.

Taken together, these results strongly suggest that FMRP binds the 3’UTR of Fmr1. We expressed Flag-FMRP and the 3’UTR of Fmr1 fused to Firefly luciferase in HEK293 cells and performed IPs using anti-FLAG antibody. By extracting RNA from the pull-down samples, preparing cDNA and using specific primer sets for PCR, we were able to detect the Firefly
luciferase amplicon only when the 3'UTR of Fmr1 was present. When the same procedure was performed on samples where no Fmr1 3’UTR was present, no bands were observed upon amplification. This result is specific since all of our constructs are expressed in these cells and our primer sets produce strong, single bands for each for transcript prior to IP. These results demonstrate that FMRP specifically binds the Fmr1 3’ UTR HEK293 cells.

3.3 Dendritic localization of Fmr1 3’ UTR and co-transport of Fmr1 3’ UTR with FMRP in processes

Fmr1 3’ UTR was cloned into MS2 vector to replace the β-actin sequence in the original vector we have got (Figure 3-2, A). TagRFP was cloned to replace GFP in MCP-GFP vector to make MCP-RFP because it provides a better signal/noise ratio under the microscope for this experiment. TagRFP also has relative stable photostability which allows certain amount bleaching time for Fluorescence Recovery After Photobleaching (FRAP) experiment. A Nuclear localization signal (NLS) in the MCP-RFP construct can accumulate MCP-RFP in the nucleus to minimize the free MCP-RFP in the cytoplasm, since MCP-RFP not binding to MS2-target 3’ UTR RNA would be transported to nucleus. This design is necessary, without the NLS, the RNA binding MCP-RFP would be insignificant because the whole cell would be filled with free MCP-RFP. Hippocampal neurons were cultured in L-Lysine-coated glass chamber. The two constructs (MCP-RFP and MS2-Fmr1 3’ UTR) were transfected on days-in-vitro (DIV) 6-7 at a 1:9 ratio to maximize mRNA labeling. Photos and videos were taken 18~24 hours after transfection. Results showed RFP highlighted granules localized in dendrites (Figure 3-2, B), while there were much less RFP highlighted granules shown in dendrites with only MCP-RFP transfection without MS2-Fmr1 3’ UTR (Figure 3-2, C). No matter the RFP positive granule numbers or overall RFP
signal detected in neuron processes is significantly higher in the co-transfection than the MCP-RFP only transfection (Figure 3-2, D, E). Although there was no dendritic marker used in this live imaging experiment, fixed transfected neurons were stained by dendritic marker MAP2 (microtubule-associated protein 2) to show their dendrite identity (unpublished data). Plus in this live imaging result, typical pyramidal neuron contains one axon and several dendrites. It is obvious there are many cell processes (dendrites and axon) containing the moving RFP highlighted granules. So Fmr1 3’ UTR transport in dendrites although axon is not excluded in this experiment. Videos were taken to show RNP granules containing Fmr1 3’ UTR transporting in dendrites (unpublished data). Not all mRNA localize to dendrites, for example, Gapdh mRNA localizes in neuron soma specifically (Tubing et al., 2010). Here we cloned full Gapdh mRNA (including 5’ UTR, CDS and 3’ UTR) to MS2 vector to make MS2-Gapdh vector as a control. The transfection of MS2-Gapdh vector with MCP-RFP vector shows Gapdh mRNA is retained in the soma as expected (Figure 3-2, F).

In order to visualize the Protein-RNA interaction of FMRP and Fmr1 3’ UTR in neuron, pcDNA3_GFP-FMRP vector was co-transfected with MCP-MS2 system. If FMRP binds Fmr1 3’ UTR granule, the co-localization of them should be visible (GFP-FMRP in green, Fmr1 3’ UTR in red by MCP-RFP). Triple transfection of GFP-FMRP, MCP-RFP and MS2-CamKII 3’ UTR (a known FMRP target, dendritic localized) on days-in-vitro (DIV) 6-7 hippocampal neurons showed that granules containing both FMRP (green) and CamKII 3’ UTR (red) localized in dendrites (Figure 3-3, A). Yellow color shows the co-localization in the merged photo of Cy3 (red) and GFP (green). The green and red particle in the image does not completely overlap with each other in some granules. This is because the image was taken from live neurons so the particles may be moving when the photo was taken. It takes time for the microscope to switch its
Cy3 and GFP laser. So when the second fluoresce was taken, the granule may has already moved. This delayed image will produce a final merged photo of multi-channel like the live imaging photos presented in this thesis, which looks like incomplete overlap in merged photo. Then we used this validated method on *Fmr1* 3’ UTR (Figure 3-3, B). Many GFP-FMRP granules localize at the base of spine or the base of dendrite branch, where the translation machinery localizes (Figure 3-3, B). The result shows *Fmr1* 3’ UTR is transported to dendrites, and co-localizes with FMRP in granules at dendrites. But the co-localization of GFP-FMRP and *Fmr1* 3’ UTR in the static photo is possibly a result of independent transport and eventually co-localization at the destination. Time lapse frames show they are co-transported (Figure 3-3, C, D). GFP-FMRP transfection with MS2-β-actin 3’ UTR (not an FMRP target) with MCP-RFP showed many GFP-FMRP granules do not localize with β-actin 3’ UTR granules (Figure 3-3, E).

### 3.4 Translational regulation through *Fmr1* 3’ UTR

It is necessary to figure out how translation is regulated through *Fmr1* 3’ UTR, and whether or not FMRP regulate translation through *Fmr1* 3’ UTR since the binding has been proved by previous results. The pmirGLO vector is a dual luciferase assay vector designed to quantify translational regulation through 3’ UTR inserted MCS. Luciferase assay of pmirGLO_3’ UTR was performed in HEK293 cells with co-transfection of GFP-FMRP (Hawrylycz et al.), GFP-FMRP (S499D) and GFP-FMRP (S499A). These mutants do not show significant difference in binding affinity to some known FMRP targets (Ceman, O'Donnell, et al., 2003). The normalized expression ratio of luciferase assay is calculated as the formula in Figure 3-5, C. Since all results have been normalized to expression ratio of pmirGLO in the same type of transfection, the normalized expression ratio represents the translational regulation done
through the 3’ UTR. The differences between columns show the translational regulation of FMRP on reporter gene through target 3’ UTR. It shows the FMRP has a decrease of reporter translation, and both the S499D and S499A have a stronger inhibition effect, just the phosphorylation mimic mutant S499D have the strongest inhibition (Figure 3-5, D).

To investigate translational regulation through the target 3’ UTR, most luciferase assays are performed in cell lines like HEK293 cells. But FMRP is the widely expressed in almost all cell types in most organs. So the endogenous FMRP in HEK293 cells will influence the results, although the co-transfected vector expressing FMRP with a strong CMV promoter leads to a dominance of exogenous FMRP in transfected cells. To reveal the relative translational regulation through Fmrl 3’ UTR in the presence or absence of FMRP, WT and Fmr1 KO mice hippocampal neurons were used for this experiment. We did transfection and luciferase assay on WT and Fmr1 KO hippocampal neurons with pmirGLO vector and pmirGLO-Fmr1 3’ UTRs. But the repeats in one condition show very different ratio. One well (of 24 well plate) with the same treatment could give a ratio ten times different to other wells. The reason could be that the universal promoter PGK and SV40 of pmirGLO vector can express in both neurons and glia cells in the cell culture, while there are always glia cells in the neuron culture although AraC was applied to limit glia proliferation. Both reporters (Firefly luciferase and Renilla luciferase) using universal promoters (PGK and SV40) can be expressed in transfected glia cells, which disturbs the results since we only focus on the neurons. Considering the relative big size of glia cells, the luciferase assay reading contributed by glia cells could be significant. The transfection efficiency varies from well to well, the translational regulation varies in neurons and glia cells, then the reading of Firefly luciferase/Renilla luciferase ratio could be very different from one well to another.
In order to measure the FMRP translation in neurons only, a neuron specific expression system was established to restrict luciferase assay to neurons only. SYNAPSIN-1 (SYN1) is a pre-synaptic protein which is involved in neural transmitter release (Evergren, Benfenati, & Shupliakov, 2007). Synapsin promoter has been found to be active in neuron only but not glia cells (Kugler, Kilic, & Bahr, 2003). Neuron specific vectors (pBS3.1- and pBS3.1+) were constructed by cloning mouse Synapsin promoter from mouse genomic DNA to replace CMV promoter in pcDNA3.1- and pcDNA3.1+ vectors o make pBS+ and pBS- vectors. LifeAct is a 17-amino-acid peptide, which is able to bind filamentous actin (F-actin) in eukaryotic cells (Riedl et al., 2008). It was fused to fluorescent protein to highlight the morphology of transfected neurons for live imaging. A pBS_LifeAct-TagRFP vector was constructed by cloning LifeAct-TagRFP into the Synapsin promoter vector pBS+. TagRFP is the brightest monomeric red fluorescent protein available by the time of this experiment (148% brightness of EGFP), possessing excitation/emission peak at 555 and 584 nm. Multimeric fluorescent proteins are even brighter but very toxic to cells. TagRFP was generated from wild-type RFP (from sea anemone *Entacmaea quadricolor*). A co-transfection of hippocampal neuron (which always has glia cells in the culture) with pBS_LifeAct-TagRFP and pEGFP has been done to show the neuron specific expression by *Synapsin* promoter (Figure 3-4, E). Immunofluorescent staining of MAP2 (neuron dendritic marker) distinguishes neuron from glia. Fluorescent imaging shows the Synapsin promoter exclusively expresses LifeAct-TagRFP in neurons, while CMV promoter expresses in both neuron and glia cells.

In order to perform neuron specific luciferase assay, pDS vector (dual Synapsin promoter) was designed to express two genes simultaneously in neuron (Figure 3-4, A). It is a neuronal version of pmirGLO vector which used backbone of pcDNA3.1- with some key elements from
pmirGLO (SV40 pA, BGH pA) and mus Synapsin promoter. Vector pDS_GFP-FMRP_LifeAct-TagRFP was constructed to test the validity of this vector (Figure 3-4, B). Both GFP-FMRP and LifeAct-TagRFP were expressed in neuron by this single vector (Figure 3-4, C, D), but not in glia cells.

A pDS-LUC (dual Synapsin promoter-Luciferase) vector was constructed by cloning Firefly luciferase and Renilla luciferase at downstream of the first and second Synapsin promoter of pDS vector separately (Figure 3-5, A). Vector pDS-LUC_Fmr1 3’ UTR was constructed by cloning Fmr1 3’ UTR to the MCS1 following the first Synapsin promoter, and Renilla luciferase following the second Synpasin promoter (Figure 3-5, B). Neuron specific luciferase assay of Fmr1 3’ UTR was performed by transfecting WT hippocampal neurons and Fmr1 KO hippocampal neurons with empty pDS-LUC vector and pDS-LUC_Fmr1 3’ UTR vector on DIV 6~7. Cultures were collected 24h later for luciferase assay. Synapsin promoters restrict the expression of both Firefly luciferase and Renilla luciferase in neuron cells, excluding the effect of glia cells. DHPG stimulation was applied for 15 min on neuron culture in stimulation experiment before collection. Signals of Firefly luciferase was normalized by Renilla luciferase within sample first, then this Firefly/Renilla ratio of Fmr1 3’ UTR data were normalized to the Firefly/Renilla ratio of pDS-LUC blank vector in the same way as the pmirGLO luciferase assay in HEK293 (Figure 3-5, C).

Neuron specific luciferase assay of Fmr1 3’ UTR showed the translation of reporters is regulated through Fmr1 3’ UTR in a FMRP dependent manner (Figure 3-5, E). The relative translational regulation of Firefly luciferase is enhanced in WT neurons after DHPG stimulation, while there is no such increase in Fmr1 KO neurons. The absence of FMRP may make the cells “insensitive” to DHPG stimulation. The basal level expression regulated through Fmr1 3’ UTR is
higher in KO neurons than WT neurons, because the translation is relieved due to the lack of FMRP.

3.5 deTagRFP-Fmr1 3’ UTR expression in neurons and FRAP of deTagRFP-Fmr1 3’ UTR in dendrite

After mRNA has arrived at its destination in dendrite, local translation may occur when proper stimulation is applied to the translation machinery. Local translation is crucial to synaptic plasticity. The luciferase assay by using dual Synapsin promoter in hippocampal neurons shows global translational regulation through Fmr1 3’ UTR. Since the dendritic localization of Fmr1 3’ UTR and translation regulation by FMRP has all been proved, the next step is to look into the translation of reporter genes regulated through Fmr1 3’ UTR locally in dendrites.

A myristoylated-destabilized-RFP-based translation reporter was constructed to perform Fluorescence Recovery After Photobleaching (FRAP) experiments to show local translation. The N-myristoylation consensus AA sequence would bind plasma membrane, so its diffusion in the cytoplasm can be reduced. A destabilized peptide from ornithine decarboxylase which is a target of protein degradation machinery is added to C terminal (Marcora, Cejas, Gonzalez, Carrillo, & Algranati, 2010) (Figure 3-6, A). New fluorescence appeared at the bleached site represents newly translate protein at local (Figure 3-6, B). Myristoylated-destabilized-TagRFP (myr-deTagRFP) vectors with target 3’ UTRs were constructed by cloning the target 3’ UTRs to the MCS at downstream of the myr-deTagRFP, just like myr-deTagRFP-Fmr1 3’ UTR (Figure 3-6, A). Myr-deTagRFP_Nl 3’ UTR, Myr-deTagRFP_CamK II 3’ UTR, and Myr-deTagRFP_β-actin 3’ UTR were constructed in the same way.
HEK293 cells were transfected with regular TagRFP vector and the myr-deTagRFP vectors with target 3’ UTRs (Figure 3-6, C). All vectors have the same pcDNA3 backbone, the same CMV promoter. It is obvious that the all myristoylated-destabilized-TagRFP express much less fluorescence. HEK293 cells were transfected by the same protocol discussed before. RFP fluorescence of single cell was quantified and compared (Figure 3-6, D). Except the destabilization AA sequence strongly decreased the TagRFP amount, we can also tell differences between myr-deTagRFP vectors with different 3’ UTR (Figure 3-6, D). Compared to blank myr-deTagRFP, Fmr1 3’ UTR, Nl 3’ UTR and CamK II 3’ UTR all decreased myr-deTagRFP level while β-actin 3’ UTR did not significantly change myr-deTagRFP expression level.

The myr-deTagRFP blank or myr-deTagRFP-Fmr1 3’ UTR vector was co-transfected with pcDNA-Cerulean to DIV6-7 hippocampal neurons. Live imaging was done ~48 hrs after transfection. Photos of the same neurons were taken before and after stimulation with brain-derived neurotrophic factor (BDNF). Both myr-deTagRFP and Cerulean expression got increased in both WT and Fmr1 KO neurons by BDNF stimulation (Figure 3-6, E). The normalized expression ratio was calculated in a way similar to luciferase assay, all ratios from myr-deTagRFP-Fmr1 3’ UTR (TagRFP/Cerulean) were normalized to myr-deTagRFP ratios (TagRFP/Cerulean). Cell body and neuron processes were calculated separately (Figure 3-6, F). In the cell body, the deTagRFP-Fmr1 3’ UTR has a high expression in KO neurons at the basal state. After BDNF stimulation, there is no significant relative expression change in WT neurons, but a decrease in KO neurons. In the cell processes, the other trends are similar, however, the TagRFP also decreases in WT after stimulation.

For the FRAP experiment, once a transfected cell was identified, images were acquired for 1min (~01 min) and then a region of a dendrite was photobleached by 405nm light for 2 sec
and a photo was taken per minute (Figure 3-6, G). Local translation in this region was assayed by observing recovery of fluorescence for >30 min after photobleaching. Time lapse images of a representative experiment of myr-deTagRFP-\textit{Fmr1} 3’ UTR with BDNF treatment and deTagRFP blank vector with BDNF treatment were taken. Five frames of each bleaching condition are presented by rainbow contrast pictures, representative of before bleaching, after bleaching and the recovery. The hot spots are the local translation sites, pointed by arrows. The hot spots of myr-deTagRFP-\textit{Fmr1} 3’ UTR were bleached out but recovered in 30 min. There are no such hot spots in myr-deTagRFP blank vector transfected neurons and the fluorescent just smear back, without showing up as hot spots.

The analysis presented by heat map is shown for the corresponding FRAP experiment (Figure 3-6, G, bottom panel). X axis represents location along the dendrite in the time lapse photo, Y axis represents time. In myr-deTagRFP-\textit{Fmr1} 3’ UTR with BDNF treatment, the hot spot recovered after photobleaching. In the blank myr-deTagRFP FRAP experiment; the hot spot was bleached permanently in the experiment.

### 3.6 Conclusion

The results presented here show that the \textit{Fmr1} 3’ UTR can be pulled down by FLAG-FMRP from lysate of co-transfected HEK293 cells. With the help of the non-invasive MS2-MCP system, we observed the \textit{Fmr1} 3’ UTR RNP granules transported and localized in dendrites, while MCP-RFP accumulated in the nucleus and the Gapdh mRNA stayed in the soma. We also observed RNP granules containing both \textit{Fmr1} 3’ UTR and GFP-FMRP transporting in dendrites in the co-transection experiment. Most of the \textit{Fmr1} 3’ UTR signal observed in dendrites co-existed with GFP-FMRP signal in the same granule, which transports in the neuron processes.
These data do not provide solid evidence of direct binding between Fmr1 3’ UTR and FMRP. To investigate whether they bind directly to each other, UV-cross linking must be performed in further research.

Although this RNA-protein binding could be direct or indirect, we did observe translational regulation of reporter gene trough Fmr1 3’ UTR by FMRP. The relative translation level of reporter regulated through target 3’ UTR was quantified by a dual luciferase assay system. The translational regulation of wt GFP-FMRP and its phosphorylation/non-phosphorylation mimic mutants on Fmr1 3’ UTR was tested on HEK293 cells.

GFP-FMRP decreased the relative expression level of reporter through Fmr1 3’ UTR the most among all FMRP plasmids. This is consistent with the widely accepted idea that FMRP is a translational repressor. The translational regulation of FMRP on its target gene, at least partially, is regulated through its phosphorylation. The key phosphorylation of S499 in the murine FMRP will lead to the phosphorylation of several amino acids nearby. The phosphorylation mimic mutant GFP-FMRP (S499D) and the non-phosphorylation mimic mutant GFP-FMRP (S499A) both showed a stronger inhibition on relative expression of the reporter with Fmr1 3’UTR. S499D mutant gave the strongest inhibition as expected because phosphorylated FMRP is the inhibition form. The even lower expression of the non-phosphorylated form S499A compared to wt GFP-FMRP was unexpected, because it is the relief form. This could be due to the lack of translational regulation because of the inability to change phosphorylation status, while FMRP has also been reported as translation enhancer in some cases.

To exclude the influence of endogenous FMRP in the HEK293 cells, we wanted to perform the luciferase assay on WT and Fmr1 KO hippocampal neurons. A neuron specific dual luciferase vector was invented using Synaptic promoter. The neuron specific promoter made it
possible to perform neuron specific luciferase assay excluding the influence of glia cells. Result showed the relative expression level of reporter with Fmr1 3’ UTR had higher expression in Fmr1 KO neurons. The lack of FMRP regulation relieved the translation of reporter with Fmr1 3’ UTR. This is consistent with the results in the HEK293 cells, in which transfection without FMRP resulted in higher reporter expression. After DHPG stimulation, there was an increase of reporter expression in WT neurons, but no change in KO neurons. Our explanation of this result is the Fmr1 KO mouse lost the regulation by FMRP through Gp1 mGluR pathway.

To further look into the local translation regulated through Fmr1 3’ UTR, myr-deTagRFP vector was constructed to visualize new protein synthesis in dendrites. The global translation of myr-deTagRFP was examined first in HEK293 cells. Most of these target 3’ UTRs decreased reporter translation except β-actin 3’ UTR due to its nature as a housekeeping gene. Plus it is widely accepted that β-actin is not a FMRP target since no such finding has been published so far. The amount of myr-deTagRFP in the neurons cell body and cell processes were quantified separately. BDNF enhanced translation in general. We saw the basal level relative expression of reporter with Fmr1 3’ UTR was lower in WT, no matter the cell body or the processes, since the FMRP in WT can inhibit translation of reporter with Fmr1 3’ UTR. After BDNF stimulation, the relative expression cell body did not change in WT but greatly decreased in Fmr1 KO. The myr-deTagRFP level in Fmr1 KO decreased after BDNF stimulation in both WT and KO in processes.

To quantify the local expression of reporter, FRAP experiment was performed in cell processes. Although myristoylation consensus AA sequence has been added to the N terminal of TagRFP, there is tremendous level of diffusion of the myr-deTagRFP after bleaching, either in the cytoplasm or on the cell membrane. The diffusion happened fast. The myr-deTagRFP started to refill the bleached region in seconds after the reporter was bleached. So what we looked into
were the hot spots, which are the potential translation sites in dendrites. The neurons were treated with BDNF to increase translation in general. The hot spots of myr-deTagRFP-Fmr1 3’ UTR got recovered within 30 min after bleaching. While in neurons transfected with myr-deTagRFP blank vector, there were not so many hot spots visible in neuron processes and the fluorescence did not recover after bleaching.
Figure 3-1  FMRP binds *Fmr1* 3' UTR in HEK293 cells

(A) Plasmid map of Flag-FMRP. *Fmr1* coding region was cloned from wt mouse brain cDNA. A flag tag was added to the 5' to the FMRP coding region.

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**Figure 3-1**  
**FMRP binds** *Fmr1* **3' UTR in HEK293 cells**

(A) **Plasmid map of Flag-FMRP.** *Fmr1* coding region was cloned from wt mouse brain cDNA. A flag tag was added to the 5' to the FMRP coding region.
(B) **Plasmid map of pmirGLO.** The blank pmirGLO vector (Promega). The PGK promoter express the Firefly luciferase, the SV40 promoter/enhancer express the Renilla luciferase.

(C) **Plasmid map of pmirGLO-**Fmr1 3’ UTR. The Fmr1 3’ UTR was cloned from wt Fmr1 3’ UTR to the MCS of pmirGLO vector at downstream of Firefly luciferase.

(D) **PCR confirmation for expression of transfected constructs in HEK293 cells.** Following transfection of plasmid combinations into HEK293 cells, RNA was extracted for RT-PCR. Following amplicon were Firefly luciferase (FF; 500 bp), Renilla luciferase (REN; 308 bp) and N19 in the Fmr1 coding sequence (379 bp). In each case, primer pairs produced single bands of the expected size.

(E) **PCR of FLAG-FMRP associated RNAs from pull downs of HEK293 transfected cells.** Plasmid combinations were transfected into HEK293 cells and Flag-FMRP was immunoprecipitated using anti-Flag antibody conjugated to magnetic beads. RNA was extracted from beads, cDNA prepared and used for PCR with primer sets for Firefly luciferase, Renilla luciferase and N19. A strong signal for the N19 amplicon was obtained from Flag-FMRP single transfections (Lane 5). N19 represents the coding region of Fmr1. When samples were prepared from cells transfected with empty pmirGLO plasmid and Flag-FMRP, no signal was obtained for either Firefly (FF) or Renilla (REN) luciferases (Lanes 3 and 4). When pmirGLO containing the 3’UTR of Fmr1 was co-transfected with Flag-FMRP, a band was observed for Firefly luciferase (Lane 1) but not Renilla luciferase (Lane 2) due to the inability of the Renilla transcript and FMRP to interact.

(F) **Western blot of FLAG-FMRP.** A Western blot using anti-FLAG antibody shows significant amounts of FLAG-FMRP is pulled down under each of our IP conditions.
A MCP-TagRFP
UbC NLS MCP RFP
MS2-Fmr1 3' UTR
RSV lacZ Fmr1 3' UTR
8×MS2
MS2-Fmr1 3' UTR count
MCP Only
0.00
0.02
0.04
0.06
MS2-Fmr1 3' UTR sum/length
MCP Only
0
10000
20000
30000
40000
50000
Average Light Intensity (AU)/um²
Average granule number /um²

B LifeAct-Cerulean
LifeAct-Cerulean
MS2-Fmr1 3' UTR
MCP-RFP

C

D

E

****
Figure 3-2  The Fmr1 3’UTR localizes to dendrites and co-transport with FMRP
(A) Schematic of the MS2-Fmr1 3’ UTR vector and the MCP-TagRFP. The MS2-Fmr1 3’ UTR vector was constructed by placing the Fmr1 3’ UTR downstream of the MS2 stem-loop sequence (8 repeats). MCP-TagRFP vector was constructed by replacing GFP in MCP-GFP with RFP. The two plasmids were co-transfected into wild-type hippocampal neurons at DIV 6-7. Ubiquitin C promoter, (UbC) and Rous sarcoma virus promoter, (RSV).
(B) Live image of MS2-Fmr1 3’ UTR localization hippocampal neuron dendrites. Representative image of hippocampal neurons transfected with MS2-Fmr1 3’ UTR vector and MCP-TagRFP. LifeAct-Cerulean was co-transfected to highlight neuron morphology. Image shows RFP positive granules in cell bodies and localized to dendrites. Photo shows merge of Cy3 (RFP, red) channel and CFP (cyan) channel. Rectangular region is a magnified selected region. Scale bar is 10 μm.
(C) MCP-RFP transfection without MS2 vector. Representative image of hippocampal neuron transfected with MCP-RFP vector and LifeAct-Cerulean. Most MCP-RFP accumulates in the cell body. Rectangular region is a magnified selected region. Scale bar is 10 μm.
(D) Quantification of RNA granules in dendrites. Graph reflect the mean values of RNA granule numbers in dendrites of >12 cells and at least 3-5 processes per neuron (±SEM). ****p < 0.0001 (Student’s t test).
(E) Quantification of Cy3 light intensity in dendrites. Graph reflects the mean value of light intensity in processes of >12 cells at least 3-5 processes per neuron (±SEM). ****p < 0.0001 (Student’s t test).
(F) MS2-Gapdh mRNA does not localize to hippocampal processes. Representative image of a hippocampal neuron transfected with MS2-Gapdh vector as a control for localization (live imaging). Gapdh mRNA stays in soma and does not localize to processes. Rectangular region is a magnified selected region. Photo shows merge of Cy3 (red) channel and DIC channel. Scale bar is 5 μm.
Figure 3-3  FMRP is transported and co-localized with the Fmr1 3’ UTR

(A) FMRP-GFP co-localizes with the MS2 CamK2 3’ UTR. Hippocampal neurons were co-transfected with GFP-FMRP, MS2-CamK2 3’UTR and MCP-TagRFP. Upper left panel shows Cy3 signal, upper right panel shows FITC signal and bottom left are merged images. The merged image shows most GFP-FMRP positive granules co-localized with CamK2 3’UTR granules in neuronal processes. Bottom left is the DIC channel. Arrows point to granules in which GFP-FMRP and Fmr1-3’ UTR co-localize. Scale bar is 5 μm.

(B) GFP-FMRP co-localizes with Fmr1 3’ UTR in dendrites. Hippocampal neurons were co-transfected with GFP-FMRP, MS2-Fmr1 3’ UTR, MCP-TagRFP and LifeAct-Cerulean on DIV 6-7. Results show most Fmr1 3’ UTR granules are co-localized with GFP-FMRP granules in neuronal processes. Lower right photo shows a merge of Cy3, GFP, CFP, and DIC channels. Scale bar is 5 μm.

(C) Co-transport of MS2-Fmr1 3’ UTR and GFP-FMRP. Hippocampal neurons were co-transfected with GFP-FMRP, MS2-Fmr1 3’ UTR and MCP-TagRFP on DIV 6-7. Time-lapse photography was performed the second day. Timeframe photos show co-transport of GFP-FMRP and Fmr1-3’ UTR granules in hippocampal processes. Scale bar is 2 μm.

(D) Velocity of Fmr1 3’ UTR/GFP-FMRP granules. Moving left is arbitrarily assigned as positive. Each bar is granule velocity in μm/s, calculated as the distance moved between two frames divided by the time duration between two frames.

(E) β-actin-3’ UTR does not co-localize with GFP-FMRP. Hippocampal neurons were co-transfected with GFP-FMRP, MS2-β-actin-3’ UTR, and MCP-TagRFP on DIV 6-7. Representative photo show they do not co-localize. Scale bar is 2 μm.
Figure 3-4  Neuron specific synapsin-promoter vector
(A) Plasmid map of pDS vector. pDS vector was constructed by cloning two Synapsin promoters to the pBS- backbone.
(B) Plasmid map of pDS_GFP-FMRP_LifeAct-TagRFP. pDS_GFP-FMRP_LifeAct-TagRFP was constructed by cloning GFP-FMRP and LifeAct-TagRFP to downstream of the two Synapsin promoter separately.
(C) **pDS_GFP-FMRP_LifeAct-TagRFP express in hippocampal neuron.** pDs_GFP-FMRP_LifeAct-TagRFP transfected neuron expressing both GFP-FMRP and LifeAct-TagRFP independently. Scale bar is 10 μm.

(D) **Subcellular localization of GFP-FMRP granule.** A region of neuron in (C) shows preferential subcellular localization of GFP-FMRP granule to the base of spine or process branch.

(E) **Synapsin promoter is neuron specific (IF).** Hippocampal neurons were co-transfected with pCDNA3_Cerulean (CMV promoter) and pBS+_LifeAct-TagRFP (Synapsin promoter) one DIV6. Neurons were fixed for immunofluorescence staining of MAP2. Both neuron and glia cells are expressing Cerulean (first frame, green, YFP channel). But only neuron is expressing LifeAct-TagRFP (second frame, red, CY3 channel). Dendritic marker MAP2 staining highlights neurons only (frame3, blue, Cy5 channel). Only the transfected neurons were pointed by arrows. Scale bar is 10μm.
Translational regulation through *Fmr1* 3’ UTR

(A) Plasmid MAP of pDS-LUC vector. pDS-LUC vector was constructed by cloning Firefly luciferase and Renilla luciferase to downstream of the first Synapsin promoter and second Synapsin promoter separately.

(B) Plasmid MAP of pDS-LUC_ Fmr1 3’ UTR. The *Fmr1* 3’ UTR was cloned to the downstream of the Firefly luciferase.
(C) **Normalized expression formula of luciferase assay.** Each normalized expression is calculated as Firefly luciferase/Renilla luciferase in each transfection. The final normalized expression ratio is calculated as ratio of normalized pDS-target 3’ UTR expression ratio/normalized pDS blank vector.

(D) **Normalized expression ratio of luciferase assay in HEK293 cells.** Luciferase assay was performed by transfecting HEK293 cells using pmirGLO or pmirGLO_Fm1 3’ UTR with pcDNA3 (control) GFP-FMRP (Hawrylycz et al.), GFP-FMRP-S499D, and GFP-FMRP-S499A respectively to HEK293 cells. Cells were collected for luciferase assay 24h after transfection. Results shows mean of relative expression of pmirGLO_Fm1 3’ UTR were normalized to pmirGLO vector expression, n=3 (±SEM)). * p<0.05, *** p<0.001, n=3, (±SEM) (Student’s t test).

(E) **Neuron specific luciferase assay of Fmr1 3’ UTR.** WT and Fmr1 KO hippocampal neurons were transfected with pDS-LUC vector and pDS-LUC_Fmr1 3’ UTR on DIV 6-7. Neurons were collected 24h after transfection for luciferase assay. Results shows mean of relative expression of pDS-LUC_Fmr1 3’ UTR normalized to pDS-LUC vector, n=3 (±SEM). Stimulated samples were treated with DHPG 20 min before collection. ns (non-significant) p≥0.05, ** p<0.01 (Student’s t test).
A

pcDNA3.1+ Myr-deTagRFP

Myristoylation sequence

Destabilizing sequence

B

Myristoylated N-terminus destabilization C-terminus

3' UTR

C

TagRFP

Myr-deTagRFP -blank

Myr-deTagRFP -Fmr1 3' UTR
E

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myr-deTagRFP - Fmr1 3' UTR</td>
<td>Cerulean</td>
<td>Cerulean</td>
</tr>
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</table>
| No treatment | ![Image of neurons with Myr-deTagRFP-Fmr1 3' UTR in WT and KO conditions.](image)
| BDNF       | ![Image of neurons with Myr-deTagRFP-Fmr1 3' UTR in WT and KO conditions.](image)

F

**Cell body**

![Bar chart showing relative expression ratio for WT Fmr1KO Fmr1BDNF in cell body.](image)

**Processes**

![Bar chart showing relative expression ratio for WT Fmr1KO Fmr1BDNF in processes.](image)
Figure 3-6  Global and local translation of deTagRFP-\(Fmr1\) 3’ UTR
(A) Plasmid map of pcDNA3.1+ Myr-deTagRFP vector. Myr-deTagRFP was constructed by fusing myristoylation consensus sequence to 5’ of TagRFP and destabilizing sequence to 3’. \(Fmr1\) 3’ UTR was placed downstream of myr-deTagRFP.
(B) Model of Myr-deTagRFP local expression.
(C) Expression of Myr-deTagRFP and regular TagRFP in HEK293 cells. Representative photos of HEK293 cells transfected with pcDNA-TagRFP, Myr-deTagRFP-blank, Myr-deTagRFP-Fmr1 3’ UTR, Myr-deTagRFP-Nl1 3’ UTR, Myr-deTagRFP-CamKII 3’ UTR, Myr-deTagRFP-β-actin 3’ UTR vectors. Upper panel is taken with Cy3 filter, lower panel is merge of Cy3 and DIC channel. Scale bar is 10μm.

(D) Comparison of myr-deTagRFP and regular TagRFP expression in HEK293 cells. Quantification of average light intensity of TagRFP vectors. Results shows mean of more than 10 cells (±SEM). ns (non-significant) p≥0.05, *p<0.05, **p<0.01 (Student’s t test).

(E) Expression of myr-deTagRFP and myr-deTagRFP-Fmr1 3’ UTR in hippocampal neuron. myr-deTagRFP or myr-deTagRFP-Fmr1 3’ UTR vector was co-transfected with pcDNA_Cerulean. Neuron culture was stimulated with 50ng/ml BDNF 1h before imaging. Scale bar is 10μm.

(F) Relative expression ratio of myr-deTagRFP Fmr1 3’ UTR in neuron cell body and processes. Light intensity ratio TagRFP/Cerulean of myr-deTagRFP Fmr1 3’UTR was normalized to myr-deTagRFP blank vector. Results shows mean of more than 12 neurons, 3-4 dendrites per neuron (±SEM). ns (non-significant) p≥0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (Student’s t test).

(G) FRAP of myr-deTagRFP-Fmr1 3’UTR and myr-deTagRFP blank vector. 405nm laser was applied to chosen region of dendrites in transfected neuron. Fluorescence recovery was recorded and presented as rainbow photos. In deTagRFP-Fmr1 3’ UTR transfected neuron, bleached region has hot spot recovered. Representative photos on 1 min before bleach, 1 min, 11 min, 21 min, 31 min and 36 min after bleaching are on the left. The recorded recovery of hot spot started in 11 min after bleaching. There is no such hot spot recovery in deTagRFP blank vector transfected neuron (representative photos on the right). Lower panel shows accumulative recovery over length of process during the whole FRAP experiment. X axis represent length and position of the whole process, Y axis represents time. Red arrows point to the bleaching point, which was performed on 00 min.
CHAPTER 4

FMRP BINDS TO THE 3’UTR OF *NEUROLIGIN1* TO REGULATE mRNA TRANSLATION IN THE DENDRITES OF HIPPOCAMPAL NEURONS
4.1 Introduction

Pre-synaptically localized neurexins (NRX) and post-synaptically localized neuroligins (NL) form a trans-synaptic complex between the pre-synaptic and post-synaptic terminals (Ichtchenko, Nguyen, & Sudhof, 1996). The recognition and binding between the two molecules are crucial to synaptogenesis, synapse maturation, differentiation, and maintenance. Mutation or copy number variations in some members of the neurexin and neuroligin families, specifically Nl1, Nl3, Nl4 and Nrx1-3, are related to autism (Jamain et al., 2003). In addition, some neurexin variants are FMRP targets as shown by high throughput sequencing crosslinking immunoprecipitation (HITS-CLIP) (Darnell et al., 2011).

Nl1 mRNA was not identified as an FMRP target by HITS-CLIP but by other method, which predicted G quartet regions in the 5’ UTR of Nl1 as potential FMRP target sequences (Dahlhaus & El-Husseini, 2010). We found two quadruplex forming G-rich sequences (QGRS) in the Nl1 3’ UTR using QGRS Mapper, a QGRS prediction program (http://bioinformatics.ramapo.edu/QGRS/index.php) (Figure 4-1, A). Similar experiments to those described in Chapter 3 were designed to assess FMRP-Nl1 3’ UTR binding, subcellular localization of the Nl1 3’ UTR, translational regulation by FMRP through the Nl1 3’ UTR and local translation of reporter-Nl1 3’ UTR in dendrites.

4.2 FMRP binds Nl1 3’ UTR in its 3’ UTR

To assess whether FMRP binds Nl1 3’UTR, we cloned the Nl1 3’ UTR from mouse brain cDNA and inserted it into the pmirGLO vector (Figure 4-1, B). This construct, pmirGLO-Nl1 3’ UTR, was co-transfected with Flag-FMRP into HEK293 cells for co-immunoprecipitation to pull down mRNA. Because the Nl1 3’UTR was transcribed with Firefly luciferase (FF) by the PGK
promoter, and the Renilla luciferase (Ren) was transcribed by the SV40 promoter, we can assess the presence of \textit{Nli} 3’UTR by amplifying each luciferase with PCR. HEK293 cells were co-transfected with both pmiRGLO-\textit{Nli} 3’UTR and Flag-FMRP (lane 1 and 2). A band of approximately 500 bp was amplified using FF primers (amplifying Firefly luciferase) (Figure 4-1, C, lane 1). The Ren primers (amplifying Renilla luciferase, 308bp amplicon if amplified, lane 2) amplified no band. In the co-transfection of the empty pmirGLO vector with Flag-FMRP (Lanes 3 and 4), neither amplicon was amplified. Our positive control for FMRP binding, the \textit{Fmr1} N19 sequence, produces a strong band at the expected size (Lane 5). This experiment demonstrates that FMRP binds the \textit{Nli} 3’UTR.

4.3 Dendritic localization of \textit{Nli} 3’ UTR and its co-localization/co-transport with FMRP

We used the MCP-MS2 system to determine subcellular localization of the \textit{Nli} 3’ UTR. The \textit{Nli} 3’ UTR was cloned into the MS2 vector and co-transfected into hippocampal neurons with MCP-TagRFP (described previously in Chapter2 material and methods, also in Chapter3). RNP granules containing \textit{Nli} 3’ UTR localized in pyramidal neuron processes. Time-lapse images showed moving granules (Figure 4-2, A). The moving velocity of a representative granule was measured (Figure 4-2, B). The measured granule shows both retrograde and anterograde movement, but in general it is an anterograde to the terminal of dendrites.

The dendritic localization of the \textit{Nli} 3’UTR was confirmed using fluorescent \textit{in situ} hybridization (FISH). Anti-sense MS2 probes was used on MCP-GFP/MS2-\textit{Nli} 3’UTR transfected neurons. MS2-\textit{Nli} 3’ UTR co-localize with MCP-GFP in dendrites (Figure 4-2, C). Dendrites were highlighted by dendritic marker microtubule-associated protein 2 (MAP2).
Co-transfection of MS2-Nl1 3’ UTR, MCP-TagRFP and GFP-FMRP shows co-localization of Nl1 3’ UTR and GFP-FMRP in RNP granules (Figure 4-3, A). Time-lapse frames show co-transport of Nl1 3’ UTR and GFP-FMRP over time (Figure 4-3, B). The arrow points to one representative moving granule at different time points.

Phosphorylation and dephosphorylation of FMRP is a key switch of its translational regulation function. Two FMRP phosphorylation mutants FMRP (S499A) and FMRP (S499D) were constructed to mimic dephosphorylated and phosphorylated FMRP at ser499 (Ceman, O'Donnell, et al., 2003). The phosphorylation and dephosphorylation of this amino acid triggers the phosphorylation/dephosphorylation of nearby sites (496S, 501T, and 503S) that are outside of the RNA binding domains of FMRP (KH1 206-280, KH2 281-422, RGG 534-552).

To determine if the phosphorylation status of FMRP at ser499 affects its RNA binding ability, we co-transfected hippocampal neurons with the FMRP mutants and both MS2-MCP constructs (MS2-Nl1 3’ UTR and MCP-TagRFP). Our results show that the phosphorylation status of FMRP does not affect its binding, co-localization or co-transport with the Nl1 3’ UTR (Figure 4-3, C).

### 4.4 Post-synaptic localization of Nl1 3’ UTR

To assess the subcellular localization of Nl1 3’ UTR in dendrites, we used a marker of post-synaptic density, post-synaptic density protein 95 (PSD95). Post-synaptic density protein 95 specifically localizes to the post-synaptic terminal of excitatory synapses. We co-transfected PSD95-GFP with the Nl1 3’ UTR MS2-MCP system. Merged Cy3, GFP and DIC images show co-localization of Nl1 3’ UTR, FMRP and PSD95 in granules localized to the post-synaptic terminal (Figure 4-4).
Neurexin (NRX) is an adhesion molecule at pre-synaptic terminal that binds to Neuroligin (NL) at post-synaptic terminal. Neuroligins expressed in non-neuronal cells can trigger pre-synaptic development (Scheiffele et al., 2000). The reverse experiment of NRX expressed on non-neuronal cells co-cultured with neurons can also induce post-synaptic development (Graf et al., 2004).

We performed an artificial synapse formation assay to set up a method for further research on new synapse. Neurons were co-transfected with NL1-RFP construct (NL1 coding sequence, +A2+B variant) and the LifeAct-Cerulean vector (LA-Cerulean) (Figure 4-5, A). LifeAct highlighted the morphology of the neuron by binding to F-actin. The transfected neurons were co-cultured with HEK293 cells transfected with NRX-GFP. Artificial synapses formed between HEK293 cells expressing NRX-GFP and neurons expressing both LA-Cerulean and NL1-RFP (pointed by arrow in Figure 4-5, A). The neurexins on HEK293 cells bind to neuroligin molecules on neurons and trigger formation of artificial synapses in minutes. The NRX-GFP accumulates at the presynapse on HEK293 cells, while NL1-RFP and LifeAct-Cerulean accumulate at the postsynapse on neurons. The accumulation of LifeAct-Cerulean at the post synaptic terminal of the artificial synapse represents the accumulation of F-Actin. It makes sense that there are more structural proteins accumulated in new synapses.

To determine the similarity between artificial synapses and real synapses, the artificial synapse formation assay was performed with neurons transfected with the PSD95-RFP vector and NL1-Cerulean. These neurons were then co-cultured with HEK293 cells transfected with Neurexin-GFP. PSD95-RFP accumulates at the post-synaptic terminal of the artificial synapse (Figure 4-5, B). The appearance of PSD95 at the post-synaptic terminal is a sign of its similarity compared to real synapse. Neurons treated with the translation inhibitor anisomycin before co-
culture, are still able to form artificial synapses (Figure 4-5, C). This suggests that artificial synapse formation is not translation-dependent.

With the knowledge of synaptic localization of NL1 protein and the dendritic localization of \textit{Nl1} mRNA, it is natural to further investigate whether the \textit{Nl1} 3’UTR is also localized to synapses for local translation. Artificial synapse formation assay was performed with neurons transfected with the \textit{Nl1} 3’ UTR MS2 system and LifeAct-Cerulean. \textit{Nl1} 3’UTR (red) accumulated at the postsynapse of artificial synapse (Figure 4-6 A). Time-lapse images were captured after NRX-GFP transfected HEK293 cells were co-cultured with transfected neurons (Figure 4-6, B). Arrows point to the locations of artificial synapses where \textit{Nl1} 3’ UTR get accumulated at the post-synaptic terminal over time (Figure 4-6, C).

Co-culture of HEK293 cells transfected with myr-GFP does not trigger the formation of an artificial synapse and no subsequent accumulation of \textit{Nl1} 3’ UTR (Figure 4-6, D). As control experiments, the same assay was performed using MS2-\textit{Gapdh} mRNA (whole mRNA containing 5’ UTR, CDS and 3’ UTR) and MS2-\textit{CamK2} 3’ UTR (Figure 4-6, E). \textit{Gapdh} mRNA is known to majorly stay in cell body, \textit{CamK2} 3’ UTR is a known FMRP target, and also localizes to dendrites (Mayford et al., 1996). The top panel shows the merge of images from the Cy3 channel (MS2-MCP), CFP channel (NL1-Cerulean), and GFP channel (NRX-GFP in HEK293). The bottom panel shows Cy3 only. The quantification of the region of interest (Lapidus et al.) shows the \textit{Gapdh} mRNA and \textit{CamK2} 3’ UTR do not concentrate at artificial synapses like the \textit{Nl1} 3’ UTR (Figure 4-6, F). Since the arbitrary identification of ROI could be inaccurate, the NL1-Cerulean signal was used to normalize the data (Figure 4-6, G), which shows that \textit{Gapdh} mRNAs do not concentrate at artificial synapses.
Our assumption was that the more NL1-Cerulean signal found at the artificial synapse, the bigger the postsynapses are, and therefore the more the synaptic mRNA present. Normalization of data represents mRNAs accumulated at artificial synapse normalized to artificial synapse amount (NL1-Cerulean). Whether or not CamK2 3’ UTR is accumulated in the artificial synapse may be arguable, but Gapdh mRNA does not accumulate at the artificial synapse. Therefore, we suggest that the Nl1 3’ UTR is transported and accumulates at the post-synaptic terminal of artificial synapses.

4.5 Translation regulated through Nl1 3’ UTR

To determine how translation is regulated through the Nl1 3’ UTR, a neuron-specific luciferase assay was performed. Dual synapsin luciferase vector pDS-LUC-Nl1 3’ UTR was constructed by cloning Nl1 3’UTR to pDS-LUC vector (Figure 4-7, A). Transfection and a luciferase assay were performed in the same way as the Fmr1 3’ UTR luciferase assay described previously (Chapter 3). The Firefly luciferase/Renilla luciferase ratio of pDS-LUC-Nl1 3’ UTR was normalized to the Firefly luciferase/Renilla luciferase ratio of pDS-LUC blank vector (Figure 3-5, C). The normalized expression ratio shows the translational regulation through Nl1 3’ UTR. Results show the basal levels of translation regulated through the Nl1 3’ UTR are the same in wild type and Fmr1 KO mouse hippocampal neurons (Figure 4-7, B). There is a significant increase with DHPG stimulation in wild type neurons while a decrease in KO.

To examine local translation, we performed a Fluorescence Recovery After Photobleaching (FRAP) experiment using a myristoylated-destabilized-RFP-based translation reporter (deTagRFP) as previous described (Chapter3). Myristoylation allows the protein to bind the membrane near its translation site thereby reduces diffusion in cytoplasm. The destabilized
peptide is a target of the protein degradation machinery. Therefore, any new fluorescence that appears at the bleached site represents new locally translated protein.

We constructed a myristoylated-destabilized-RFP-Nl1 3’ UTR vector (Figure 4-8, A). Neurons were transfected with myr-deTagRFP-Nl1 3’ UTR vector and LA-Cerulean. Immunofluorescent photos of the same neuron were taken before and after BDNF treatment. After BDNF treatment, both the deTagRFP signal and Cerulean signal increased. Relative expression is expressed as the ratio of deTagRFP-Nl1 3’ UTR/Cerulean normalized by ratio of deTagRFP/Cerulean, similar to the calculation of luciferase assay. The predominant expression of reporters was localized in cell bodies and the locally translated reporter in processes stay where they are translated. Therefore, cell bodies and processes were quantified and analyzed respectively (Figure 4-8, C and D). There is no significant difference in cell bodies between wild type and Fmr1 KO hippocampal neurons. However, there is more myr-deTagRFP expression in the Fmr1 KO processes (Figure 4-8, D). The relative expression in both wild type and Fmr1 KO neurons are decreased by BDNF treatment (Figure 4-8, C, D).

Fragile X mental retardation proteins are believed to be a major a translation repressor, although little is known about the mechanism of translational regulation. The unphosphorylated FMRP is associated with actively translating polyribosomes while a fraction of phosphorylated FMRP associates with stalled polyribosomes (Ceman, Zhang, Johnson, & Warren, 2003). This result suggests that translational regulation by FMRP is controlled by switching its phosphorylation status.

To examine if the phosphorylation status of FMRP affects its RNA binding ability under physiological conditions, the two FMRP mutants, S499A (the phosphorylation inhibition mutant) and S499D (the phosphorylation mimic) were used (Figure 4-9, A). The deTagRFP density in the
dendrites was quantified and compared (Figure 4-9, B). The mean deTagRFP intensity in
dendrites is much lower in S499D FMRP co-transfection. This further confirmed that FMRP
regulate translation through Nl1 3’ UTR.

4.6 Local translation regulated through Nl1 3’ UTR

With the knowledge of Nl1 3’ UTR dendritic/synaptic localization, further experiment
was performed to confirm its local translation. Hippocampal neurons of WT mice were
transfected with myr-deTagRFP vectors, Cerulean, and PSD95-GFP on DIV 6-7 and imaged
approximately 48 hrs later. The myr-deTagRFP-Gapdh vector with Gapdh (5’ UTR-CDS-3’
UTR) which localizes to the cell body, was used as a control. PSD 95-GFP is a post-synaptic
terminal marker of excitatory synapse. Regions where deTagRFP is expressed at PSD 95-GFP
positive sites were chosen for FRAP since PSD 95-GFP positive sites are the potential post-
synaptic terminal. The chosen region of a dendrite was photobleached by 405 nm light for 0.5
sec. Images were acquired before (time point -01 min) and after bleaching (00 min to 30 min).
Local translation in this region was assayed by observing recovery of fluorescence after
photobleaching. Experiment with the Nl1 3’-UTR untreated, treated with BDNF, protein
translation inhibitor Cycloheximide (CHX, inhibit protein translation by interfering with the
translocation step in protein synthesis) and with BDNF+CHX are indicated. Representative
FRAP images before bleaching, after bleaching and 30 min after bleaching are shown by
rainbow contrast pictures, (Figure 4-10, A). The hotspots in deRFP-Gapdh did not recover after
bleaching (Figure 4-10, B). The hotspots in deRFP-Nl1 3’ UTR got recovered. When deRFP-Nl1
3’ UTR was treated with BDNF, there is a robust recovery. But when the neurons are treated
with CHX (50 nM) for 1h before FRAP, the recovery of deTagRFP hotspots was inhibited, regardless whether BDNF was present or not (Figure 4-10, C).

Myr-deTagRFP-Nl1 3’ UTR was co-transfected with PSD95-GFP to WT hippocampal neurons on ~DIV 7 and images were taken 24~48 h after transfection (Figure 4-11). Arrows point to the co-localization of deTagRFP with PSD95-GFP, representing local translation of myr-deTagRFP at post-synaptic terminal. Therefore, the Nl1 3’ UTR is sufficient to guide the transcript to post-synaptic terminal, then the mRNA with Nl1 3’ UTR is locally translated there.

4.7 Local translation at artificial synapse

Neurons transfected with myr-deTagRFP-Nl1 3’ UTR (deR-Nl1 3’U) and LifeAct-Cerulean (LA-CRL) were co-cultured with Neurexin-GFP transfected to HEK293cells (Figure 4-12, A). The arrow points to an artificial synapse site formed between HEK293 cell and hippocampal neuron, where local translation of deTagRFP occurred. The time represents time passed since the start of co-culturing. A region of artificial synapses was chosen for quantification. Quantification of this region shows an increased amount of deTagRFP at this site, compared to a dendrite in the nearby area (Figure 4-12, B). The light intensity of the nearby dendrite area did not change significantly, while the intensity of hot spot was increased. Another example shows local translation at the tip of a dendrite/spine which is the post-synaptic terminal of the artificial synapse (Figure 4-12, C). The deTagRFP blank vector shows much less deTagRFP at artificial synapse (Figure 4-12, D). Anisomycin is a translation inhibitor interfering with protein synthesis by inhibiting peptidyl transferase or the 80S ribosome system. Neurons transfected with LifeAct-Cerulean and Nl1-RFP (coding sequence) were treated with anisomycin 1 h before co-culturing with Neurexin-GFP transfected HEK293 cells. The artificial synapse
formation was not affected as proved before (Figure 4-5, C). The local translation of myr-deTagRFP-\(Nl1\) 3’ UTR at post-synaptic terminal was inhibited by anisomycin. This further proved that the dendritic hotspot in Figure 4-12 A, C were local translated instead of diffused from cytoplasm. The relative expression level of myr-deTagRFP in myr-deTagRFP-\(Nl1\) 3’ UTR transfected vector was significantly reduced by anisomycin treatment (Figure 4-12, F). The translation inhibitor anisomycin is not supposed to affect RNP transport/accumulation, so the difference is caused by local translation.

### 4.8 Conclusion

The results presented here indicate the functions of \(Nl1\) 3’ UTR in both mRNA transport and translational regulation. \(Nl1\) 3’ UTR was pulled down by FLAG-FMRP from lysate of co-transfected HEK293 cells, despite this interaction could be direct or indirect. Using MS2-MCP system, we observed \(Nl1\) 3’ UTR RNP granules localized to dendrites and synapses. FMRP co-transported with \(Nl1\) 3’ UTR in mRNP granules to dendrites. The phosphorylation of FMRP at S499 had no effect on the RNA binding ability of FMRP with \(Nl1\) 3’ UTR in neuron. With the help of artificial synapse formation assay, we observed accumulation of MS2-\(Nl1\) 3’ UTR to artificial synapse over time.

The Synapsin promoter luciferase vector enable us to perform neuron specific luciferase assay with \(Nl1\) 3’ UTR in mice hippocampal neurons. Result shows the relative expression level of reporter with \(Nl1\) 3’ UTR has similar level of expression at basal state. But with DHPG stimulation, the relative expression of reporter with \(Nl1\) 3’ UTR got increased in WT, while it got decreased in \(Frm1\) KO hippocampal neurons. The lack of FMRP regulation resulted in the lack
of positive response of *Nl1* 3’ UTR to DHPG stimulation in reporter translation. The luciferase assay provide a view of global translational regulation through *Nl1* 3’ UTR within neuron.

Presumably, mRNAs move to their destination in dendrites, local translation occurs there under regulation. With the myristoylated-destabilized-RFP-based translation reporter myr-deTagRFP, we captured clear evidence of this process. Artificial synapse formation assay showed local translation of reporter deTagRFP on site over time. Translation inhibitors effectively inhibited the recovery of deTagRFP in FRAP experiment, also inhibited formation of hotspots in the artificial synapse. The two phosphorylation mutant S499A and S499D showed relieving or repressing of translation of myr-deTagRFP reporter as expected. The same experiment on *Fmr1* 3’ UTR does not show hot spot of local translation triggered by artificial synapse formation (data not shown).
Chapter 4 Figures

A

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Figure 4-1  FMRP binds Nl1 3’ UTR in HEK293 cells

(A) QGRS (Quadruplex forming G-Rich Sequences) prediction of Nl1 3’ UTR. QGRS prediction of two G-rich region was predicted using QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS/index.php).

(B) Plasmid map of pmirGLO-Nl1 3’ UTR. Nl1 3’ UTR was cloned from cDNA of whole mouse brain to pmirGLO vector MCS between SacI and SalI.

(C) PCR detecting mRNA pulled down in anti-FLAG full down. HEK293 cells were co-transfected with pmirGLO-Nl1 3’ UTR/pmirGLO vectors and pcDNA3_FLAG-FMRP for IP. RNA were extracted from beads after IP for RT-PCR. Expected Firefly luciferase amplicon is 500 bp, N19 amplicon is 379 bp, Renilla amplicon is 308 bp. PCR shows Firefly luciferase-Nl1 3’ UTR mRNA was pulled down (lane 1), but not Firefly luciferase mRNA itself (lane 3). Positive control N19 region of Fmr1 3’ UTR was pulled down as expected (lane 5). Renilla luciferase mRNA was not pulled down as expected (lane 2 and lane 4). See Figure 3-1 for primer test.
Figure 4-2  
*Nl1* 3’ UTR transport in dendrites 
(A) *Nl1* 3’ UTR granule moving in hippocampal neuron processes. Hippocampal neurons were co-transfected with RSV-MS2-*Nl1* 3’ UTR and MCP-TagRFP on DIV 6-7. Live imaging was done the second day. Timeframe photos of representative neuron processes show transport of *Nl1*-3’ UTR granules in processes. Arrow points to the moving granule. Scale bar is 5μm.
(B) **MS2-\textit{NII} 3’UTR moving velocity.** Moving velocity of the \textit{NII} 3’ UTR granule in (A) was measured. Left in (A) was assigned as positive.

(C) **FISH of MS2-\textit{NII} 3’ UTR transfected neuron.** Hippocampal neurons were co-transfected with RSV-MS2-\textit{NII} 3’ UTR and MCP-TagRFP on DIV 6-7. Neurons were fixed for FISH the second day. MAP2-Rab-Cy5 was used to stain neuron dendrites. MS2 probe was used to detect MS2-\textit{NII} 3’ UTR mRNA. Probed dendritic MS2-\textit{NII} 3’ UTR was pointed by arrows in the merge photo. Scale bar is 5 \( \mu \text{m}. \)
A

B

C

Aspartic acid
GFP-FMRP(S499D)
GFP-FMRP(S499A)
MS2-Nl1 3' UTR
LifeAct-Cerulean
Figure 4-3  Co-localization and co-transport of NII 3’ UTR with FMRP
(A) Co-localization of MS2-NII 3’ UTR and GFP-FMRP. WT hippocampal neurons were co-transfected with RSV-MS2-NII 3’ UTR, MCP-TagRFP and GFP-FMRP on DIV 6-7. Live imaging was done the second day. Representative photo of merged Cy3, GFP, and DIC channels shows co-localization of GFP-FMRP and NII 3’ UTR in granules, pointed by arrows. Scale bar is 2μm.
(B) Co-transport of MS2-NII 3’ UTR and GFP-FMRP. Time frames of video taken from transfection in (A) shows co-transport of MS2-NII 3’ UTR and GFP-FMRP neuron processes. Scale bar is 2μm.
(C) FMRP phosphorylation mutations does not affect their co-localization with MS2-NII 3’ UTR. WT hippocampal neurons were co-transfected with RSV-MS2-NII 3’ UTR, MCP-TagRFP, LifeAct-Cerulean and GFP-FMRP (wt, or S499D, or S499A) on DIV 6-7. Photos were taken the second day. Arrows point to the GFP-FMRP (wt, or S499D, or S499A) and NII 3’ UTR co-localized granules in cell processes. Scale bar is 5μm.

Figure 4-4  Post-synaptic localization of NII 3’ UTR
Hippocampal neurons were co-transfected with RSV-MS2-NII 3’ UTR, MCP-TagRFP and PSD 95-GFP on DIV 6-7. Live imaging was done the second day. Representative photo of merged Cy3, GFP, and DIC channels shows co-localization of GFP-FMRP and NII 3’ UTR in granules, pointed by arrows. Co-transfection of MS2-NII 3’ UTR, MCP-RFP and PSD 95-GFP in hippocampal neuron. Co-localization of PSD95-GFP and MS2-NII 3’ UTR localize to post synaptic terminal of excitatory synapse. Scale bar is 2μm.
Figure 4-5  Artificial synapse formation assay

(A) Artificial synapse formation assay. HEK293 cells expressing NRX-GFP were co-cultured with hippocampal neurons transfected with pDS_JhNL1-mRFP_LifeAct-Cerulean for 1h. Arrow points to artificial synapse formed between HEK293 cell and neuron. NRX-GFP accumulates in the pre-synaptic terminal (first frame, green). Both LA-Cerulean (frame 2, blue) and NL1-RFP
(frame 3, red) accumulates on the post-synaptic terminal. The arrow in merge photo (frame 4) points to artificial synapse formed between HEK293 and neuron. Scale bar is 2μm.

(B) **Artificial synapse formation assay with PSD95-RFP transfected neuron.** HEK293 cells transfected with NRX-GFP were co-cultured with neuron transfected with NL1-Cerulean and PSD95-TagRFP. NRX-GFP accumulates in the pre-synaptic terminal (first frame, green). Both NL1-Cerulean (frame 2, blue) and post-synaptic marker PSD95-RFP (frame 3, red) accumulates on the post-synaptic terminal. The arrow in merge photo (frame 4) points to artificial synapse formed between HEK293 and neuron. Scale bar is 2 μm.

(C) **Artificial synapse formation assay is protein translation independent.** Neurons were treated with 50μm anisomycin 1h before artificial synapse assay as (A). Protein translation inhibitor anisomycin does not affect artificial synapse formation. The arrow in merge photo (frame 4) points to artificial synapse formed between HEK293 and neuron in the presence of anisomycin. Scale bar is 2 μm.
Figure A shows images of HEK cells, neurons, and a combination of both.

Figure B presents time-lapse images for NRX-GFP, NL1-Cerulean, MS2-Nl1 3' UTR, and their merged images at 1 min, 20 min, 39 min, and 57 min.

Figure C illustrates the average light intensity (AU)/um² over time (min), with data points and error bars indicating variability.

Figure D displays images of MS2-Nl1 3' UTR and the merge.
**Figure 4-6**  *Nl1* 3’ UTR accumulate at artificial synapse

(A) *MS2-Nl1* 3’ UTR accumulate at artificial synapse. HEK293 cells expressing NRX-GFP were co-cultured with hippocampal neurons transfected with MCP-TagRFP and *MS2-Nl1* 3’ UTR for 1h. Artificial synapse formed between HEK293 (frame 1, green) and neuron (frame2, blue). MS2-Nl1 3’ UTR accumulated in post-synaptic terminal (frame 3, red). Arrow points to the MS2-Nl1 3’ UTR accumulated at the artificial synapse (frame 4, merge). Scale bar is 2μm.

(B) Time frame of *MS2-Nl1* 3’ UTR accumulate at artificial synapse. A time frame video was taken in the same transfection in (A). MS2-Nl1 3’ UTR signal (red) increases at artificial synapse over time. Arrows points to artificial synapse formed between HEK293 cells and neuron. Scale bar is 2μm.

(C) Quantification of *MS2-Nl1* 3’ UTR accumulated at artificial synapse over time in (B). Results show mean intensity of Nl1 3’ UTR signal at artificial synapse pointed in (B) over time (+SEM).

(D) Myr-GFP does not trigger MS2 accumulation. HEK293 cells transfected with myr-GFP (myristoylation consensus sequence-GFP) does not trigger formation of artificial synapse and
therefore does not cause accumulation of \textit{Nl1} 3’ UTR at the site the neuron in touch with HEK cells. Scale bar is 5μm.

\textbf{(E) Comparison of \textit{Nl1} 3’ UTR, CamK2-3’ UTR and Gapdh-mRNA accumulation at artificial synapse.} Scale bar is 5μm. HEK293 cells expressing NRX-GFP were co-cultured with hippocampal neurons transfected with MCP-TagRFP and MS2-\textit{Nl1} 3’ UTR, or MS2-\textit{CamK2}-3’ UTR, or \textit{Gapdh}-3’ UTR for 1h. Top frames show merge of HEK293 cells expressing NRX-GFP (green), MS2-RNA (red) and NL1-CRL (NL1-Cerulean, blue). Bottom frames show MS2-RNA only. Scale bar is 2μm.

\textbf{(F) Quantification of target mRNA at artificial synapse.} Results shows mean MS2-RNA signal at artificial synapse in (E) (±SEM). ****p< 0.0001 (Student’s t test).

\textbf{(G) Quantification of mRNA (relative) at artificial synapse.} Results shows mean MS2-RNA signal (normalized to NL1-Cerulean) at artificial synapse in (E) (±SEM). ns (non-significant) p≥0.05, ***p< 0.001 (Student’s t test).
Figure 4-7 Neuron specific luciferase assay of Nl1 3’ UTR.

(A) Plasmid map of pDS-LUC_Nl1 3’ UTR vector. Nl1 3’ UTR was cloned to pDS-LUC vector after the Firefly luciferase.

(B) Neuron specific luciferase assay of Nl1 3’ UTR. Hippocampal neurons were transfected with pDS-LUC_Nl1 3’ UTR on DIV 6-7. Transfected neurons were collected 24h after transfection for luciferase assay. Results shows mean of relative expression level of Firefly luciferase regulated through Nl1 3’ UTR in WT and Fmr1 KO hippocampal neurons, in the absence or presence of DHPG, n=4 (± SEM). For DHPG treated sample, neuron culture was treated with 100 μM (RS)-3, 5-DHPG for 20min before collection. ns (non-significant) p≥ 0.05, *p< 0.05, **p< 0.01 (Student’s t test).
**Figure 4-8**  myr-deTagRFP-Nl1 3’ UTR expression in HEK293 and neuron  
(A) **Plasmid map of pcDNA3.1+Myr-deTagRFP_Nl1 3’ UTR vector.** Myr-deTagRFP was constructed by fusing myristoylation consensus sequence to 5’ of TagRFP and destabilizing sequence to 3’ Nl1 3’ UTR was placed downstream of myr-deTagRFP.  
(B) **Myr-deTagRFP_Nl1 3’UTR expression in hippocampal neuron.** WT and Fmr1 KO hippocampal neurons were transfected with pcDNA3.1+Myr-deTagRFP_Nl1 3’ UTR and pcDNA-Cerulean. Photo of the same neuron was taken before and after BDNF stimulation. Top frames show before stimulation, lower panel shows after BDNF stimulation. Scale bar is 10 μm.  
(C) (D) **Relative expression ratio of myr-deTagRFP Nl1 3’ UTR in neuron cell body and processes.** Light intensity ratio TagRFP/Cerulean of myr-deTagRFP Fmr1 3’UTR was normalized to myr-deTagRFP blank vector. Results shows mean of more than 12 neurons, 3-4 dendrites per neuron (±SEM). ns (non-significant) p≥0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (Student’s t test).
Figure 4-9  FMRP mutants in translational regulation

(A) Phosphorylation status of FMRP regulate deTagRFP-Neu 3’ UTR expression. Hippocampal neurons were co-transfected with LA-Cerulean, myr-deTagRFP-Fmr1 3'UTR and GFP-FMRP S499A (phosphorylation mimic mutant) or S499D (phosphorylation inhibition mutant) on DIV 6-7. Live imaging was done the second day. Representative photo of CFP, GFP, Cy3, and merged channels shows the expression of reporter deTagRFP in neuron processes with either S499A or S499D. Scale bar is 2μm.

(B) Relative expression level of deTagRFP with GFP-FMRP S499A and S499D. Relative expression of deTagRFP in (A) was quantified. Results shows mean of relative expression of deTagRFP normalized to GFP-FMRP (S499A) or GFP-FMRP (S499D) (±SEM). More than 11 neurons, 2-3 processes per neuron were quantified. ** p<0.01 (Student’s t test). Scale bar is 2μm.
Figure 4-10  FRAP of deTagRFP-Nl1 3’ UTR

(A) FRAP experiment of deTagRFP with different 3’ UTRs and different treatment. Hippocampal neurons were transfected with PSD95-GFP, Cerulean, myr-deTagRFP-Gpadh mRNA or myr-deTagRFP-Nl1 3’ UTR on DIV 6-7. BDNF and CHX were used to treat neuron culture of myr-deTagRFP-Nl1 3’ UTR transfection before FRAP. 405nm laser was applied to chosen region of dendrites in transfected neuron. Fluorescence recovery was recorded and presented as rainbow photos. In myr-deTagRFP-Nl1 3’ UTR transfected neuron, bleached region has hot spot recovered. Representative photos on 1 min before bleach are shown as merged image of CFP channel, GFP channel, and Cy3 channel (top row). Hot spots with both PSD 95-GFP and deTagRFP were chosen for bleaching, which are pointed by arrows. Representative photos on 1 min before bleach, 1 min and 30 min after bleaching are shown from the second row to the fourth row as rainbow photo. The recorded recovery of hot spots were observed in myr-deTagRFP-Nl1 3’ UTR transfection (second column) and myr-deTagRFP-Nl1 3’ UTR transection treated with BDNF (fourth column). There was no such hot spot recovery in myr-deTagRFP-Gpadh mRNA vector (first column). The recovery was inhibited in myr-deTagRFP-Nl1 3’ UTR treated with protein translation inhibitor CHX, or myr-deTagRFP-Nl1 3’ UTR transfection treated with both BDNF and CHX (third and fifth column).

(B) (C) deTagRFP fluorescence recovery of FRAP experiment overtime. Graph shows accumulative recovery of more than 8 hot spots over time of (A) normalized to the fluorescence on the hotspot before bleaching (±SEM). X axis represent time, Y axis represent deTagRFP fluorescence. *p<0.05, **p<0.01 (Two way ANOVA).
Figure 4-11  Post-synaptic expression of deTagRFP-Nl1 3’ UTR
Hippocampal neurons were transfected with myr-deTagRFP 3’ UTR and post-synaptic marker PSD 95-GFP on DIV 6. Live images were taken the second day. Arrows point to synapse where deTagRFP expresses. Scale bar is 5μm.
Figure 4-12  Local translation of myr-deTagRFP N11 3’ UTR at artificial synapse
(A) **Time frame of myr-deTagRFP-Nl1 3’ UTR local translation at artificial synapse.** Artificial synapse formation assay trigger local translation of myr-deTagRFP-Nl1 3’ UTR. HEK293 cells transfected NRX-GFP were cultured with hippocampal neuron transfected with myr-deTagRFP and LACRL (LifeAct-Cerulean). Local translation of myr-deTagRFP-Nl1 3’ UTR was triggered at artificial synapse formed between HEK cell and neuron. The arrow points to a representative region have local translation. Scale bar is 2 μm.

(B) **Quantification of translation at artificial synapse in (B) overtime.** Cy3 intensity of two positions in (A) was quantified overtime (72 min). The myr-deTagRFP local translation of myr-deTagRFP-Nl1 3’ UTR in the red box where HEK293 cell expressing NRX-GFP increases over time.

(C) **Local translation of myr-deTagRFP at artificial synapse.** The same experiment like 60min after co-culturing. Scale bar is 2μm.

(D) **Artificial synapse formation assay with deTagRFP blank vector.** Artificial synapse formation assay was performed with HEK293 cells transfected with NRX-GFP and hippocampal neuron transfected with myr-deTagRFP blank vector. Representative photo shows limited expression of deTagRFP at artificial synapse. Scale bar is 2μm.

(E) **Local translation of myr-deTagRFP-Nl1 3’ UTR is translation dependent.** Protein synthesis inhibitor anisomycin inhibited local translation at artificial synapse. Neurons were treated with 50 μM anisomycin 1h before artificial synapse assay before artificial synapse.

(F) **Comparison of local translation at artificial synapse in regular luciferase and anisomycin treated one.** Results shows relative deTagRFP expression normalized to LifeAct-Cerulean at artificial synapse region (±SEM). 55 artificial synapse regions were measured. ****p < 0.0001 (Student’s t test).
CHAPTER 5

ABNORMAL *NEUROLIGIN1* MRNA ALTERNATIVE SPLICING IN *FMR1* KO MICE

HIPPOCAMPUS
5.1 Introduction

Neuroligins (NL) are a family of neural adhesion molecules (Sudhof, 2008). Located at the post-synaptic membrane, they bind to a pre-synaptic counterpart, Neurexins (NRX) (Ichtchenko et al., 1995). There are four neuroligin isoforms in rodents (Nl1-Nl4) and five isoforms in humans (NL1-NL5) (Baudouin & Scheiffele, 2010). It is suggested that alternative splicing of neuroligins produces variants that bind differentially to variants of neurexins (Ichtchenko et al., 1995) (Boucard et al., 2005). To clarify, the isoforms here represent different genes transcribed from different promoter, the variants represents alternative splicing products of the same pre-mRNA.

Four neuroligin alternative splice variants derived from two splice sites (A and B) have been reported: +A+B, +A∆B, ∆A+B, and ∆A∆B (Ichtchenko et al., 1995). The major splice variant is ∆A+B, which contains only splice site B. Splice site B of NL1 is a key switch for NL1 variants binding to NRX variants (Boucard et al., 2005). Neuroligin 1 with or without splice site B has different binding ability to different Neurexin variants. The regulation of RNA alternative splicing may affect the balance between different variants, which may further affect synapse formation and functions.

There are two variations of splice site A of Nl1, A1 and A2 (Bolliger et al., 2008). Most reports have used A2 to represent splice site A and ignored splice site A1. Both A1 and A2 are 60 nucleotides in length and are named in the order of their orientation in the pre-mRNA, with A1 closer to the 5’ end. The A1 variant translates to VKRISKECARKPGKKICRKG and the A2 variant translates to GPLTKKHTDDLGDNDGAEDE (Bolliger et al., 2008).

The short peptide of splice site A1 and A2 have different charges under physiological condition. Peptide of A1 is basic, while peptide of A2 is acidic (Figure 1-8). Since recognition
and binding between NL and NRX is charge dependent (Koehnke et al., 2010), spice site A1 and A2 may play different roles in synapse formation, maintenance, and signal transduction. Although spice site B functions in NRX-NL binding, the function of spice site A has not been elucidated.

We wanted to know if there is a difference in NL variants in wild type versus Fmr1 KO synaptosomes. Difference of NL1 abundance in WT and KO synaptosomes were assayed with Western blot (Figure 5-1). Synaptosomes were prepared from the hippocampus of wild type (P14) and Fmr1 KO mice. In the wild type sample, we observed one band migrating at approximately 117 kDa. However, the Fmr1 KO sample produced two bands (unpublished data by Valerie Drouet). Previous reports from Drosophila research describe an interaction between dFMRP and the RNA editing protein, dADAR (Bhogal et al., 2011). This was the first report of FMRP function in the nucleus and first report of FMRP function on RNA processing. Although RNA editing and RNA alternative splicing are different (see Chapter 1), the difference of NL1 western blot between WT and Fmr1 KO gives a possibility that FMRP may play a role in Nl1 RNA processing. Since Nl1 is under regulation of alternative splicing, the second band in our western blot could be a shorter variant (~95 kDa) (Figure 5-1).

To test the hypothesis that abnormal alternative splicing of Nl1 produces the unknown band observed in western blot, Nl1 alternative splicing variants were examined at the RNA level in the hippocampus of wild type and Fmr1 KO mice.

5.2 Abnormal alternative splicing in Fmr1 KO mice

We designed primers (named F and R) to amplify variants of Nl1 alternative splicing (Figure 5-2, A). The expected products and length of expected products were shown based on the
schematic of *Nl1* gene. *Nl1* variants were amplified from cDNA of wild type (P14) mouse hippocampus by RT-PCR with F and R primers. Three bands were found between 396-510 bp (DNA ladder not shown) as expected (Figure 5-2, B). The three bands were cut and cloned to TOPO TA vector for sequencing. Sequencing of the smallest band was confirmed to be the major *Nl1* variant, ΔA+B. The largest band was confirmed to be a combination of +A1+B and +A2+B variants. All the attempts to sequence the middle band failed. But our calculations show that the size of the middle band fits the expectation of the +AΔB variant (472bp).

The same F and R primers were used for the *Nl1* variants pattern with cDNA derived from the hippocampus of WT and *Fmr1* KO mice at P7, P14 and P21 (postnatal 7, 14, 21) (Figure 5-2, C). Only at P14, but not P7 or P21, we observed a significantly increased amount of the larger band (representing the +A+B variant) in the *Fmr1* KO, and a decrease in the smaller band (ΔA+B) in the *Fmr1* KO compared to wild type (Figure 5-2, C and D). This result suggests that the abnormal ratio of *Nl1* variants changes during development and P14 is a crucial stage. The expression of the middle band +AΔB (including +A1ΔB and +A2ΔB) are similar in wild type and *Fmr1* KO mice (Figure 5-2, E).

It is obvious that the absence of FMRP is the possible cause of the *Nl1* variant difference in P14 mouse hippocampus between WT and *Fmr1* KO mouse. To prove the connection of FMRP to this phenomenon FMRP in wild type neurons was knocked down to mimic the *Fmr1* KO neurons. *Fmr1* siRNAs was transfected to wild type hippocampal neurons on DIV 6-7. Approximately 85% of FMRP was knocked down at 48 h post-transfection (Figure 5-2, F and G). Only two bands instead of three were amplified in PCR of neuron culture samples (Figure 5-2 H). This pattern differs from the three bands in amplifications performed using hippocampus and may be the result of different environments of neurons *in vivo* and *in vitro*. The smallest band
was confirmed by sequencing to be the ∆A+B variant. However, the abundance of the largest band was too low to sequence. Nevertheless, we were able to see a change in the pattern of Nll variants, presented as a top band/bottom band ratio (Figure 5-2, H and I). In Fmr1 KO hippocampus on P14, the top band/bottom band ratio is higher, which means the major variant is +A+B (Figure 5-2, C and D). In the FMRP RNAi experiment, the top band/bottom band ratio is increased in wild type hippocampal neurons (Figure 5-2, H and I). These results show that knockdown of FMRP using siRNAs mimics the variant pattern of Nll in the Fmr1 KO hippocampus.

Next, we asked if the presence of FMRP could rescue the PCR pattern of Nll variants observed in Fmr1 KO neurons. We transfected hippocampal neurons of Fmr1 KO mice (P0) with a Flag-tagged FMRP vector. F/R primers were used to access the Nll variant pattern. The top band/bottom band ratio is decreased with Flag-FMRP transfection (Figure 5-2, J and K). Thus, the introduction of FMRP into the Fmr1 KO neurons resulted in PCR pattern that mimicked the Nll variant expression pattern observed in wild-type hippocampus at P14. The FMRP expression in Fmr1 KO neuron rescued the Fmr1 KO neuron to make the Nll variant pattern more normal.

5.3 PCR of Nll +A+B variants

As shown in previous results, we observed more +A+B expression (top band) in the Fmr1 KO hippocampus than in wild type at P14. Since +A+B is comprised of both +A1+B and +A2+B, we designed primers (A1, A2 and B) to examine which one of these variations contributes more to the elevated level of variant +A+B (Figure 5-2, C). Results show that the level of +A1+B in P14 Fmr1 KO hippocampus increased while the level of +A2+B decreased
(Figure 5-3, B and C). So the increased +A+B expression in the *Fmr1* KO hippocampus at P14 is due to increased amounts of the +A1+B variant.

The +A1+B and +A2+B variant pattern the FMRP RNAi experiment and *Fmr1* KO neuron rescue experiment should be consistent with the hippocampus result. The Nl1 variant PCR pattern showed the same trend as P14 hippocampus (Figure 5-3, D-G). The FMRP RNAi modified the variant pattern more like FMRP KO, while the rescue by expression of FMRP did the opposite.

*Nl2* is a known FMRP target. There is only splice site A in Nl2 which result in two variants, *Nl2* containing splice site A (*Nl2* (A)) and without splice site A (*Nl2* (ΔA)). Adding together, it is called *Nl2* (total). There is no significant *Nl2* (A)/total *Nl2* (total) ratio difference between WT and Fmr1 KO at P7, P14 or P21 (Figure 5-4).

### 5.4 Discovery of new alternative splicing variant of *Nl1* +SSA1+SSA2+SSB

The variant PCR experiments with primer A1 as forward primer and primer B as reverse primer shows an unknown band in wild type P14 hippocampus if the bands are properly separated on an agarose gel (Figure 5-5, A). The extra band was isolated for sequencing. It turned out to be a new variant of *Nl1* +A1+A2+B (Figure 5-5, B). This is a novel variant and we report it here for the first time (Figure 5-5, C). This variant only showed up in hippocampus of WT mice but not in *Fmr1* KO at P14 (Figure 5-5, A). It is also slightly visible in the Figure 5-3 D, top panel.

### 5.5 Neuron rescue by *Nl1* variant expression
If the absence of FMRP can result in the “abnormal” splicing pattern of Nl1, and abnormal synapse size and number, maybe there is connection between the two defects. Since Nl1 is synaptic adhesion molecules, it is possible that abnormal Nl1 variant pattern contributes to the abnormal synapse size and number more or less. Synapsin is a pre-synaptic marker for the synapse. Wild type and Fmr1 KO hippocampal neurons were stained with anti-synapsin antibody to label the synapse (Figure 5-6, A). We observed smaller synapse sizes and numbers in Fmr1 KO neurons compared to wild type (Figure 5-6, B and C).

Previous results show that there is less ΔA+B in Fmr1 KO hippocampus compared to wild type (Figure 5-2, C and D), and less +A2+B in Fmr1 KO compared to wild type (Figure 5-3, B and C). So if the lower level ΔA+B and +A2+B in Fmr1 KO hippocampus is the cause of the abnormal synapse size and number, expression of these two Nl1 variants in Fmr1 KO neuron may be able to rescue the anomalies. Fmr1 KO hippocampal neurons were transfected with Nl1 variants ΔA+B or +A2+B to determine if synapse number and size could be rescued (Figure 5-6, A). Both vectors increased synapse number in Fmr1 KO neurons, with +A2+B having a greater effect than ΔA+B (Figure 5-6, B). However, only ΔA+B increased synapse size (Figure 5-6, C).

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is composed of four types of subunits, GluA1-4, also called Glutamate receptor 1-4 (GluR1-4) (Shi et al., 1999). According to previous reports, the internalization of AMPAR is increased in FXS because absence of FMRP relieves the translation of FMRP targets. FMRP targets include proteins responsible for AMPAR receptor internalization (Sidorov et al., 2013). Previous research reported misregulated expression of GluA2 in Fmr1 KO neuron in response to stimulation but not at basal state (Muddashetty, Kelic, Gross, Xu, & Bassell, 2007).
To further look into the roles these two \( Nl1 \) variants \( \Delta A+B \) or \(+A2+B \) playing in neurons, we looked into if the expression of these two variants could rescue the surface AMPARs, since only the surface AMPARs are the “functional” receptors. We stained surface GluA2 and internal GluA2 to compare wild type and \( Fmr1 \) KO hippocampal neurons (Figure 5-7, A). Our results show less surface GluA2 on \( Fmr1 \) KO neurons and neither the expression \( \Delta A+B \) nor \(+A2+B \) rescues the amount of surface GluA2 (Figure 5-7, B). In the \( Nl1 \) \(+A2+B \) variant transfection there is even less surface GluA2 (Figure 5-7, B). Internal GluA2 amounts do not appear to be affected in \( Fmr1 \) KO neurons, and \( Nl1 \) variant transfection does not change internal GluA2 amounts (Figure 5-7, B). Therefore, it may not be the decreased amounts of \( \Delta A+B \) or \(+A2+B \) in \( Fmr1 \) KO neurons that account for decreased AMPAR on the neuron surface.

### 5.6 \( \beta\)-NRX-FC staining of WT and \( Fmr1 \) KO hippocampal neurons.

Neuroligins are the major target of \( \beta\)-NRX in dendrites. Since previous results show the \( Nl1 \) variant patterns are different in WT and \( Fmr1 \) KO hippocampal neurons, the neurexin binding to the post-synaptic terminal should be affected. Therefore \( \beta\)-NRX binding on the surface of wild type and \( Fmr1 \) KO neurons was accessed. A \( \beta\)-\( Nrx \)-FC protein was made to stain WT and \( Fmr1 \) KO neurons. \( \beta\)-\( Nrx \) binds to Nls on the post-synaptic terminal on dendrites. The peptide called fragment crystallizable region of antibody (FC) can recognize and bound protein A. Fluorescence labeled protein A can be used to stain neurons bound by \( \beta\)-\( Nrx \)-FC (Figure 5-8, A). \( \beta\)-\( Nrx \)-FC vector was constructed and transfected to HEK293 cells. A signal peptide at the N-terminus \( \beta\)-\( Nrx \)-FC allowed the protein to be secreted into the medium. The medium containing secreted \( \beta\)-NRX-FC was collected with protein A magnetic beads and then eluted from the beads for neuronal surface staining. After WT and \( Fmr1 \) KO hippocampal neurons were incubated with
β-NRX-FC, fluorescent Protein A was used to label β-NRX-FC on the neuron. Wild type and \textit{Fmr1} KO neurons were examined for the fluorescent hotspot size and numbers. Results showed protein A-stained hotspots in WT and \textit{Fmr1} KO neurons (Figure 5-8, B). There is less hotspots in \textit{Fmr1} KO neurons (Figure 5-8, C), and these hotspots are also smaller in \textit{Fmr1} KO neurons (Figure 5-8, D). Therefore, the absence of FMRP affects NRX binding the targeting proteins on the post-synaptic terminal, including Neuroligins.

5.7 Conclusion

For the first time, FMRP was found to be involved in RNA splicing, although there needs further research to know the mechanism, like whether the involvement is direct or indirect. An abnormal \textit{Nl1} alternative splicing pattern was found in hippocampus of P14 \textit{Fmr1} KO mice. Since it is only found in hippocampus on P14 but not P7 or P21, so this abnormal alternative splicing pattern of \textit{Nl1} is development dependent. In the \textit{Nl1} splicing PCR, the \textit{Fmr1} KO shows an elevated level of +A+B variant (+A1+B and +A2+B) and a decreased level of $\Delta A+B$ variant. To further validate that this abnormal pattern is caused by the absence of FMRP, FMRP in WT hippocampal neuron was knocked down by RNAi to mimic the \textit{Fmr1} KO neuron, and \textit{Fmr1} KO neuron was rescued by expressing FMRP in \textit{Fmr1} KO neuron to mimic WT neuron. Although the \textit{Nl1} variant PCR pattern were different in neuron culture and hippocampus, and the knockdown and rescue experiment both showed the same trend in WT hippocampus and \textit{Fmr1} KO hippocampus. FMRP decreased the largest band (+A+B, including both +A1+B and +A2+B) in hippocampus and top band (unknown, too tiny to separate for sequencing) in neuron culture (RNAi knockdown and FMRP over expression); FMRP increased the smallest band $\Delta A+B$ in both hippocampus and neuron culture (RNAi knockdown and FMRP over expression). On the
contrary, the known FMRP target Nl2 splicing was not affected in Fmr1 KO mice hippocampus (Figure 5-4).

To further investigate which +A+B (+A1+B or +A2+B) contributed to the +A+B increase in Fmr1 KO hippocampus, variant specific primers were used to amplify +A1+B and +A2+B variants of Nl1 (Figure 5-3). The +A1+B variant increased in Fmr1 KO mice hippocampus, while the +A2+B variant decreased. RNAi knockdown of FMRP increased +A1+B and decreases the amount of +A2+B (Figure 5-3, D and E). So the knockdown of FMRP made the neuron more like “Fmr1 KO”. In the Fmr1 KO neurons transfected with FMRP, the +A1+B variant was decreased, while the +A2+B was increased. Overexpression of FMRP turned the variant PCR pattern to more “wild-type” (Figure 5-3, F and G). All these evidence showed that FMRP affected alternative splicing of Nl1, changed the balance of different variants of Nl1.

A new alternative splicing variant was found when A1/B primers were used for +A1+B variant. Extra cycles in PCR and proper agarose gel running are needed to separate the new band from +A1+B. The new band is much dimmer but bigger than the +A1+B band. Sequencing of the new band shows it is +A1+A2+B. This variant was not reported before probably because of its low abundance and such tiny difference in length from the other variants. Abundance and importance may not be positively related. Although the abundance of this variant is low, it may be crucial to normal physiology since this variant was not found in KO hippocampus at P14. Sample from hippocampus of younger or older mice was not tested to detect this new variant. This variant is slightly visible in PCR of +A1+B of WT neuron culture (Figure 5-3, D). All possible alternative splicing variants are presented in Figure 5-9.

The size and number of Synapsin hot spot in Fmr1 KO hippocampal neurons are fewer and smaller compared to WT. Since the amount of +A2+B and ΔA+B Nl1 variants in Fmr1 KO
hippocampus were decreased, it is possible the lack of these two *Nl1* variants (+A2+B and ΔA+B) caused the anomalies in *Fmr1* KO neurons, at least partially. Both *Nl1* (+A2+B) and *Nl1* (ΔA+B) were used to transfect *Fmr1* KO hippocampal neurons to compare to WT. The pre-synaptic marker Synapsin staining shows less synapses and smaller synapse in *Fmr1* KO neurons. Both +A2+B and ΔA+B can increase Synapsin stained hot spots. NL1 +A2+B have stronger effect than NL1 ΔA+B in increasing synapse number. Only +A2+B can rescue synapse size.

There is less surface GluA2 in *Fmr1* KO neurons compared to WT, while there is no significant difference in internal GluA2. The surface GluA2 amount and surface/internal GluA2 ratio could not be rescued by expression of NL +A2+B and ΔA+B two variants.

The β-NRX-FC staining of wild type and *Fmr1* KO mice shows smaller and fewer hotspot size and numbers in *Fmr1* KO hippocampal neurons. This means the NRX binding proteins on the post-synaptic terminal is reduced in size and number, possibly NLs.
Chapter 5 Figures

**Figure 5-1**  **NL1 expression in synaptosomes of hippocampus**

Western blot using NL1 and DM1α antibodies were used to probe target proteins (P14 WT and *Fmr1* KO mice). One band is observed in the wild type samples while two bands are observed in the *Fmr1* KO samples (unpublished data, Valerie Drouet).
Figure 5-2  Nl1 primer design, PCR of A1B A2B
(A) Schematic of *Neuroligin1* and primers designed to amplify splice variants. Primers at upstream and downstream of splice site A and B were designed to amplify all possible alternative splicing variants of *Nl1*. Expected amplification products of *Nl1* variant PCR and expected length are presented.

(B) PCR products of *Nl1* variants from wild type (P14) hippocampus. Sequencing confirms the top band is +A+B (+A1+B and +A2+B) variant; middle band has the expected size of +AΔB (including +A1ΔB and +A2ΔB); lower band is confirmed as ΔA+B. The ΔAΔB variant was not detected.

(C) PCR of *Nl1* variants from mouse hippocampus at different ages. RNA was extracted from P7, P14 and P21 mouse hippocampus from wild type and *Fmr1* KO mice for cDNA synthesis. Semi-quantitative PCR was performed to assess the relative expression of each variant normalized to β-actin.

(D) Quantitation of top band/bottom band ratio in (C). Results show mean ratio of top band to bottom band. There is a significant difference between the top/bottom band ratios in hippocampus at P1 (n=3, ±SEM, * p<0.05) (Student’s t test).

(E) Relative expression of middle band. Results show relative expression of middle band, normalized to β-actin in (C) (n=3, ±SEM) (non-significant, p≥0.05) (Student’s t test).

(F) siRNA knock down of FMRP in hippocampal neurons. Wild type hippocampal neurons were transfected *Fmr1* siRNA to knock down FMRP. Western blot shows FMRP and β-actin expression in FMRP knock down samples compared to negative control (wild type neurons transfected with scrambled siRNA).

(G) Quantification of FMRP knockdown. Approximately 85% of FMRP was knocked down using siRNAs.

(H) *Nl1* PCR variants in wild type hippocampal neuron with FMRP knocked down. Two bands are observed in variant PCRs of hippocampal neurons in culture when FMRP knocked down by siRNAs. β-actin was amplified as internal control.

(I) Quantification of PCR product in (H). Results show mean mRNA amount of top band and bottom band, which are normalized to β-actin (n=3, ±SEM, ** p<0.01) (Student’s t test).

(J) *Nl1* variant PCR of *Fmr1* KO neuron transfected with Flag-FMRP vector. Cultured hippocampal neurons (P0) culture were transfected with Flag-FMRP at DIV 6-7. *Nl1* variant PCR shows the top band/bottom band ratio is decreased by FMRP overexpression (n=3, ±SEM, ** p<0.01) (Student’s t test).
Figure 5-3  *Nl1* A1B A2B PCR

(A) Primer design for *Nl1* +A+B variants. Primers were designed to detect +A1+B and +A2+B variants of +A+B. The expected PCR product is 427 bp.

(B) *Nl1* variant PCR of +A1+B and +A2+B using wild type and Fmr1 KO hippocampal RNA at P14. B-actin was amplified as an internal control.

(C) Quantitation of the relative expression of variants +A1+B and +A2+B in (B). Results show mean relative RNA amount of variant +A1+B and variant +A2+B in wild type and Fmr1-KO (P14) hippocampus, normalized to β-actin RNA (n=3, ±SEM, * p<0.05) (Student’s t test).

(D) *Nl1* variant PCR of +A1+B and +A2+B in WT hippocampal neurons with FMRP knocked down.

(E) Relative expression of variants +A1+B and +A2+B in (D). Results show mean relative RNA amounts of variant +A1+B and variant +A2+B in wild type hippocampal neurons and wild type l neurons with Fmr1 knocked down (n=3, ±SEM, * p<0.05) (Student’s t test).

(F) *Nl1* PCR variants of +A1+B and +A2+B in Fmr1 KO hippocampal neurons and Fmr1 KO hippocampal neurons transfected with an FMRP expression vector.
(G) Relative expression of variants +A1+B and +A2+B in (F). Results show mean relative RNA amount of variant +A1+B and variant +A2+B in (F) normalized to $\beta$-actin (n=3, ±SEM, *p<0.05) (Student’s t test).
Figure 5-4  *Nl2* variants are not affected in *Fmr1* KO mice

(A) **Variant PCR primer design for *Nl2* variants quantification.** *Nl2* has only one alternative splice site A, which could be present or absent in the mature mRNA. Primers were designed in the SS A and downstream for variant detection. Primers of *Nl2* amplifying the total *Nl2* are presented.

(B) **Variant PCR of *Nl2* variant *Nl2* (A) and total *Nl2*.** Primers in (A) were used to detect variant *Nl2* (A) that contains SS A. Another pair of primers out of splice site was used to detect total mRNA amount of *Nl2*.

(C) **Relative expression of *Nl2* in (B).** Results shows mean of the relative amount of *Nl2*+A variant in *Nl2* (total) (n=3, ±SEM) (non-significant, p≥0.05) (Student’s t test). There is no significant difference in *Nl2* mRNA variants between WT and *Fmr1* KO on P7, P14 and P21.
Figure 5-5  New variant: A1A2B

(A) Discovery of new alternative splicing variant in *Nl1*. *Nl1* Variant PCR with A1 forward primer and B reverse primer shows a different pattern compared to P14 WT and *Fmr1* KO hippocampus. There was only one band in KO hippocampus (+A1+B) but there is one unknown band in WT hippocampus (around 50 bp longer).

(B) Sequencing results of top band in (A). Sequencing result of the unknown band (Washbourne et al.) in (A) shows a transcript with both A1 and A2 next to each other.

(C) New *Nl1* variant +A1+A2+B schematic. The arrangement of SS A1, SS A2 and SS B are presented as an explanation of the unknown band in (A).
Figure 5-6  *Nl1* variants expression affect synapse number and size differently

(A) *Fmr1* KO neuron synapse rescue experiment (IF). Different neuroligin variants were transfected to *Fmr1* KO hippocampal neurons to rescue Synapsin I staining puncta number. IF staining was performed to hippocampal neuron of WT, *Fmr1* KO, and *Fmr1* KO transfected with empty vector (pcDNA3), or with *Nl1*ΔA+B variant vector, or with +A2+B variant vector. Presynaptic marker Synapsin I antibody was used to probe synapses, and dendritic marker MAP2 antibody was used to label dendrites.

(B) **Quantification of synapse number in (A).** Both ΔA+B and +A2+B can rescue the synapse number, but to different level. Results shows mean of average synapse number of more than 25 neurons, 2-4 dendrites per neuron (±SEM). Both *Nl1* ΔA+B and +A2+B variants can save the synapse number in *Fmr1* KO hippocampal neuron. ns (non-significant) p≥0.05, *p < 0.05, **p < 0.01, ****p < 0.0001, (Student’s t test).

(C) **Quantification of synapse size in (A).** Results shows mean of average synapse size of more than 25 neurons, 2-4 dendrites per neuron (±SEM). Only +A2+B variant can save the synapse size. ns (non-significant) p≥0.05, **p < 0.01, ***p < 0.001, (Student’s t test).
**Figure 5-7** *Nl1* variants affect surface GluA2 amount

(A) Surface GluA2 staining of *Fmr1* KO neuron is rescue by *Nl1* variants (IF). IF of surface/internal GluA2 in WT and *Fmr1* KO hippocampal neurons shows amount of GluA2 on the surface on the dendrites and in the dendrites. There are KO neurons transfected with *Nl1* variant ∆A+B or *Nl1* variant +A2+B. Dendritic marker MAP2 was used to highlight dendrites.

(B) External GluA2 density in (A). Results show the mean intensity of GluA2 on the surface of dendrites (±SEM). Surface GluA2 in KO hippocampal neurons is a significantly lower than in WT. ∆A+B variant expression does not change the external GluA2 amount, while +A2+B variant expression even decrease the GluA2 amount. More than 13 neurons, 4–6 dendrites per neuron were quantified. *p < 0.05, **p < 0.01 (Student’s t test).

(C) Internal GluA2 density in (A). There is no significant difference between KO and the others (±SEM) (Student’s t test).

(D) Ratio of external GluA2 to internal GluA2 in (A). There is a significant decrease in +A2+B variant transfected neuron (±SEM). **p < 0.0001 (Student’s t test).
Figure 5-8  NRX-FC staining of WT and KO neuron
(A) β-NRX-FC /protein A staining design. β-NRX-FC construct was made to produce β-NRX-FC from HEK293 cells. Overexpressed β-NRX-FC was purified for the protein A staining.
(C) Mean NRX-FC hotspot number difference in WT and KO hippocampal neuron. More than 20 neurons, 2-4 neuron processes were taken. (±SEM)**p < 0.01 (Student’s t test).
(D) Average NRX-FC hotspot area difference in WT and KO hippocampal neuron. More than 20 neurons, 2-4 neuron processes were taken. (±SEM)**p < 0.01 (Student’s t test).
All possible variants of *Neuroligin1*

All possible variants of *Nl1* alternative splicing. Possible variant +A1+A2+B, +A1+A2 and +A1ΔB have not been reported yet. +A1+A2+B were discovered for the first time in this thesis. +A2+B, +A2ΔB, ΔA+B and ΔAΔB were the variants reported frequently. ΔA+B is the predominant variant.
CHAPTER 6

DISCUSSION
Fragile X Syndrome (FXS) is the most widespread single-gene cause of autism and most frequent genetic cause of mental retardation, caused by the loss of functional fragile X mental retardation protein 1 (FMRP). FMRP is widely expressed in virtually all cells types, with an especially high level in neuronal cells in the brain and in the testis (Sethna, Moon, & Wang, 2014). This means FMRP has general functions in all cell types, and plays special roles in neurons. As a RNA binding protein, FMRP potentially regulates many target genes in many ways and plays a critical role in the neuron. Increasing amount of evidence show that FMRP is like a “Swiss army knife” in neurons, which plays multiple roles in gene expression, translational regulation and signal transduction. The research on FMRP in the past two decades has led to significant advances in understanding the molecular events in FXS. Despite the growing data achieved on the roles FMRP plays in physiology and in neuronal plasticity, details regarding behaviors of FMRP remain to be elucidated.

The majority of FXS is caused by the increase of the CGG trinucleotide repeat in the 5’ untranslated regions (5' UTR) of fragile X mental retardation 1 (FMRI) gene. The abnormal increased CGG repeats, especially the full mutation with more than 200 repeats, cause hypermethylation of that region. Eventually the hypermethylation diminishes FMRP expression at the transcription level (Fu et al., 1991) (Pieretti et al., 1991) (Bell et al., 1991). Other types of FXS include mutation in key positions of FMRP. One patient has an I340N mutation in the K Homology 2 (KH2) domain of FMRP which is one of the three RNA binding domains in FMRP (De Boulle et al., 1993) (Y. Feng, Absher, et al., 1997). Another patient has a R138Q mutation in the nuclear export signals (NES) of FMRP (Collins et al., 2010). These two mutations give hints about the key roles FMRP may play. One is its RNA binding ability, which is well known and
widely studied. The other is the functions of FMRP in the nucleus, which has been underestimated in the past.

The majority of FMRP research has focused on its roles in the cytoplasm in the past, probably because FMRP predominantly localizes in the cytoplasm. FMRP is widely recognized as a RNA binding protein. RNA binding proteins are important to control functions of target RNAs, like RNA transcription, RNA processing (including splicing and RNA editing), nuclear export, mRNA stability and translational regulation, almost all aspects of RNA during its whole life. FMRP is involved in the life of its target mRNAs in many ways, including its transport, stability and especially translational regulation (Sethna et al., 2014). The absence of FMRP causes many abnormal phenomena in neurons and the nervous system. From the morphological point of view, FXS patients and Fmr1 KO mice both show abnormal dendritic spine shape and spine numbers (Bagni & Greenough, 2005). From the physiological point of view, the absence of FMRP causes exaggerated long-term depression (LTD), one type of long-term plasticity important for learning and memory (Huber et al., 2002). From the molecular point of view, the transports of some FMRP target mRNAs are altered and the protein translations of these targets are abnormal. The mGluR theory of FXS explained the possible roles FMRP plays in synaptic plasticity (Bear et al., 2004). Based on this theory, loss of FMRP relieves translation of “LTD” proteins and results in the exaggerated LTD in the hippocampus of Fmr1 KO mice. One interesting target is its own mRNA Fmr1 (C. T. Ashley, Jr. et al., 1993), so we chose it as one research target in this thesis. Many FMRP targets are involved in autism, like Neuroligin (Nl) and Neurexin (Nrx). Neuroligin1 is another target studied in thesis because of its involvement in autism.
This thesis is devoted to explore FMRP function in both the cytoplasm and the nucleus. In the cytoplasm aspect, this thesis studied the 3’ UTR of Fmr1 and Nl1 because the 3’ UTR is usually a critical target of mRNA regulation. At the mRNA level, we confirmed the binding of target 3’ UTRs by FMRP. Then we studied the subcellular localization of target 3’ UTRs. At the protein level, we studied the FMRP translational regulation on reporter genes with target 3’ UTR and local translation of reporters with target 3’ UTRs. In the nucleus aspect, we discovered an abnormal alternative splicing pattern related to FMRP and studied the changes of Nl1 variants caused by the absence of FMRP.

**FMRP and Fmr1 3’ UTR**

In this study, we have presented evidence that FMRP binds the 3’ UTR of Fmr1, and regulates translation of reporters with the Fmr1 3’ UTR. The Fmr1 3’ UTR transported to dendrites of hippocampal neurons and a reporter with the Fmr1 3’ UTR was locally translated in dendrites.

We confirmed the proposed binding of FMRP to Fmr1 3’ UTR. Fmr1 mRNA is the target of its own protein FMRP. FMRP may target more than one region in its target mRNA, either in the coding or untranslated region. A purine quartet motif in the Fmr1 coding region is known to be bound by FMRP (Schaeffer et al., 2001). The N19 segment containing this region was used as the positive control of FMRP target in this thesis. But whether Fmr1 3’ UTR is a target of FMRP is arguable. It was reported as an FMRP target achieved by UV cross-linking (Brown et al., 1998). Another report proposed that the major FMRP binding site (FBS) only localizes in the RGG box in the 3’ of the Fmr1 coding region, not in its 3’ UTR (Schaeffer et al., 2001). Our immunoprecipitation (IP) result shows that Fmr1 3’ UTR was pulled down by
FLAG-FMRP from HEK239 cell lysate (Figure 3-1). This result is not achieved by using UV cross-linking, so the interaction between FMRP and *Fmr1* 3’UTR may be either direct or indirect. Further experiments in this thesis were designed to investigate *Fmr1* 3’UTR behaviors and regulation on it by FMRP based on the knowledge of this interaction.

To study FMRP’s role in translational regulation, we studied the effects of FMRP on reporter genes carrying the *Fmr1* 3’ UTR, as well as its effect on global translation in HEK293 cells. Previous publications showed that the phosphorylation status of FMRP may be a switch of its translational regulation, with phosphorylated FMRP being associated with stalled ribosomes, and dephosphorylated FMRP associated with active translation (Ceman, O'Donnell, et al., 2003). The luciferase assay of *Fmr1* 3’ UTR in HEK293 cells with transfection of FMRP or FMRP phosphorylated mimic mutant S499D or FMRP dephosphorylated mimic mutant S499A showed phosphorylation status of FMRP can adjust translational regulation activity of FMRP. A strong inhibition of reporter translation by FMRP (S499D) was expected, and the data showed that in fact, FMRP (S499D) strongly inhibited reporter gene translation (Figure 3-5, D). Although FMRP (S499A) which is the dephosphorylated mimic mutant, showed higher translation of the reporter gene than FMRP (S499D), it showed decreased translation compared to WT FMRP, in contrast to our initial expectations. We believe it is the lack of regulation ability of FMRP (S499A) hindered the expression of reporter luciferase. In other words, functional FMRP with regulation ability through phosphorylation/dephosphorylation is important to target gene expression. That is why we believe the best description of FMRP is as a translation regulator instead of an inhibitor, because it is not only repressing translation under all circumstances, but also can upregulate translation sometimes. The optimal regulation would allow the proper gene expressed at the right time in the right place responding to specific stimuli with the proper
strength. We used a myristoylated-destabilized-TagRFP reporter to visualize local translation, which can also provide another view of global translation by measuring RFP fluorescence. In HEK293 cells, we observed a decrease in myr-deTagRFP expression with all 3’ UTRs we tested (Fmr1, Nl1, CanK2) except β-Actin 3’ UTR (Figure 3-6, C and D).

We invented a neuron specific luciferase system for a neuron specific translation assay and investigated the translational regulation of FMRP on Fmr1 3’ UTR in hippocampal neurons. Since FMRP is widely expressed in almost all cell types, the influence of endogenous FMRP from HEK293 cells can not be avoided in the HEK293 luciferase assay. The best platform to differentiate the expression regulated through target 3’ UTR in the presence or absence of FMRP are WT and Fmr1 KO mouse hippocampal neurons. To exclude the influence of glia cells in the neuronal culture which can also express FMRP, a neuron specific dual Synapsin promoter vector was invented. The dual Synapsin promoter luciferase vector enables luciferase assay in neuron cells specifically without interference from the highly abundant glial cells routinely exist in the neuron cultures. At the basal level, the reporter with Fmr1 3’ UTR has a higher expression level in Fmr1 KO neurons compared to WT (Figure 3-5, E). The lack of FMRP regulation relieves the translational suppression of its target gene in Fmr1 KO neuron. With DHPG stimulation, reporter gene Firefly luciferase expression is increased in WT hippocampal neuron but the Fmr1 KO neuron does not respond to the stimulation. This suggests that the stimulation of Fmr1 3’ UTR translation by DHPG is fulfilled through FMRP. This is the FMRP translational regulation mentioned before, instead of just translation inhibitor.

We observed the dendritic localization of MS2-Fmr1 3’ UTR RNP granules, with the help of the non-invasive mRNA labelling method, the MS2-MCP system. This result shows the Fmr1 3’ UTR contains enough information to guide the transport of the whole reporter transcript
to the proper destination, just like the zip-code sequence in the β-Actin 3’UTR. The Fmr1 3’ UTR was co-transported in neuronal processes with FMRP, which is consistent with the previous pull down result showing that FMRP binds Fmr1 3’ UTR. One important role of 3’ UTR of many genes is targeting mRNA to specific subcellular compartments. For decades, it was believed that mRNAs are translated in the cell body near the nucleus, and then mature proteins were transported to their destination and execute their function there. After the discovery of translational machinery at the base of dendritic spines (Steward & Levy, 1982), evidence accumulated shows the existence of local translation and the significant roles local translation plays in cell, especially in neurons. The theory of synaptic tagging proposed that neural signals at synapses cause subsequent plasticity-related product trafficking that is essential for synaptic plasticity (Frey & Morris, 1998), and the transport of mRNAs to synapses fulfills the physical requirement of local translation.

We observed local translation of reporters carrying the Fmr1 3’ UTR. FMRP mediated local translation can be activated in dendrites in response to stimulation (Weiler et al., 1997). The luciferase assay can only provide a result of the global translational regulation in the whole cell, which is mostly contributed by the cell body. Thus, to focus on local translation in synapses that may be regulated through target 3’ UTRs, we used fluorescence recovery after photobleaching (FRAP) on neurons transfected with myristoylated-destabilized-TagRFP with Fmr1 3’ UTR. Results showed new protein was locally synthesized in the dendrites after photo bleaching of pre-existing TagRFP. Since FMRP plays an important role in local translation, local translation of FMRP itself is critical because new FMRP translated locally could regulate its targets mRNA translation on site immediately.
FMRP and *Neuroligin1* 3’ UTR

In this study, we have presented the evidence that *Nl1* 3’ UTR was transported to dendrites and post-synaptic terminals of hippocampal neurons, and that reporters with *Nl1* 3’ UTR are locally translated in dendrites and post-synaptic terminals; we also showed that FMRP binds the *Nl1* 3’ UTR, and regulates translation of reporters with the *Nl1* 3’ UTR.

There are several reasons why we became interested in *Nl1*. NEUROLIGIN (NL) is a family of post-synaptic terminal transmembrane proteins, which recognize and bind to their pre-synaptic counterpart NEUREXIN (NRX). One important function of NLs and NRXs is that they recognize each other, bind to each other, and trigger synapse formation (Sudhof, 2008) (Scheiffele et al., 2000). Since the mRNA of several *Nl* and *Nrx* have already been proved to be FMRP targets, and several *Nl* and *Nrx* isoforms have been found to be involved in autism (Jamain et al., 2003) (J. Feng et al., 2006) (Sudhof, 2008), it is possible that FMRP influences *Nls* in some pathway to cause autism. So either abnormal *Nls* themselves or absence of FMRP regulation on *Nls* could have pathogenic consequences. Over expression of eIF4E or relief of eIF4E (by knocking out of the eukaryotic translation initiation factor 4E-binding protein 2, 4E-BP2) can cause overexpression of Neuroligin, which breaks the excitation-to-inhibition balance and also causes Autism Spectrum Disorders (ASD) like phenotypes (Gkogkas et al., 2012). FMRP has been shown to repress translation through facilitating eIF4E binding (Napoli et al., 2008). These findings suggest a link between FMRP and NL1 in ASD, which makes it important to clarify the role of FMRP in regulating *Nl1* expression, particularly its regulation on local translation of *Nl1* in the post-synaptic terminal.

We predicted and confirmed the binding of FMRP to the *Nl1* 3’ UTR. *Nl1* was not found as a target of FMRP in a high-throughput sequencing combined with crosslinking
immunoprecipitation (HITS-CLIP) experiment, in which \textit{Nl2}, \textit{Nl3} and all three \textit{Nrxs} were found to be FMRP targets (Darnell et al., 2011). However, it was found to be an FRMP target in another report (Dahlhaus & El-Husseini, 2010). We found two G-rich region in \textit{Nl1} 3’ UTR by QGRS (Quadruplex forming G-Rich Sequences) prediction, which are potential FMRP binding regions. In fact, in our studies, \textit{Nl1} 3’UTR was pulled down in the same immunoprecipitation (IP) experiment as \textit{Fmr1} 3’ UTR in HEK293 cells by FLAG-FMRP. This result narrowed down the FMRP target region in \textit{Nl1} to its 3’ UTR, although we do not know whether this binding is direct or indirect.

We studied the localization of \textit{Nl1} 3’ UTR in hippocampal neurons, and found that it localized to dendrites and synapses. Using the MS2-MCP system, we observed dendritic localization of the \textit{Nl1} 3’ UTR (Figure 4-2) and its co-transport with FMRP (Figure 4-3). As we expected, the phosphorylation mutants of FMRP (S499A, S499D) did not interfere with the dendritic localization of \textit{Nl1} 3’ UTR and the co-transport of FMRP with \textit{Nl1} 3’ UTR. Since NL1 protein localized to post-synaptic terminals, we suspect that NL1 may be translated on site at where it is needed. Local translation of a gene requires the localization of its mRNA transported to its destination first. To narrow down the subcellular localization of \textit{Nl1} 3’ UTR, co-transfection of neurons with the MS2-MCP system and post-synaptic marker post synaptic density protein 95 (PSD95-GFP) showed that some \textit{Nl1} 3’ UTR granules in fact co-localize with PSD95 in post-synaptic terminals (Figure 4-4), showing that the 3’ UTR of \textit{Nl1} is sufficient to localize a reporter mRNA to functional post-synaptic terminals. Artificial synapse formation assay also showed \textit{Nl1} 3’ UTR accumulation at the post-synaptic terminal of artificial synapses (Figure 4-6).
In FRAP experiments using the myr-deTagRFP local translation reporter, we found that the *Nl1* 3’ UTR was sufficient to enable local translation of the reporter in post-synaptic terminals (Figure 4-10). As expected, the translation inhibitor CHX inhibited the recovery in fluorescence following photobleaching, indicating that the observed increase in fluorescence under normal conditions (no CHX) was in fact due to local translation. We found that local translation of myr-deTagRFP-*Nl1* 3’ UTR can also be triggered in the post-synaptic terminal of artificial synapses (Figure 4-12). However, we performed the same artificial synapse formation assay with myr-deTagRFP-*Fmr1* 3’ UTR, but no local translation was observed (unpublished data). This means that the *Fmr1* 3’ UTR and *Nl1* 3’ UTR are differentially regulated in terms of local translation. We proposed that at the synapse, NLs are needed between pre-synaptic and post-synaptic terminals, especially at new synapses. So the mRNAs for these synaptic proteins are likely transported to the appropriate destination. Then they simply wait for signals that will promote local translation, such as a new synapse forming. Although FMRP was reported to be locally translated in dendrites in response to stimulation of Gp1 mGlur, apparently this type of stimulation is different from the signals of new synapse formation. The new synapse needs protein synthesis of many genes especially structural proteins, and it may not need FMRP, which may inhibit translation of many genes at the new synapse, explaining our observed results.

We also investigated the role of the *Nl1* 3’ UTR on general translational regulation and in FMRP-specific regulation. To study the effects of this 3’ UTR on general translational regulation of reporter mRNAs, we used the myr-deTagRFP translation reporter in HEK293 cells (Figure 3-6). The results show that the *Nl1* 3’UTR repressed translation of the reporter, compared to the blank reporter. To exclude the effects of endogenous FMRP in HEK293 and glia cells, the effects of the *Nl1* 3’ UTR on translational regulation was studied with our neuron specific dual
luciferase system in WT and Fmr1 KO hippocampal neurons (Figure 4-7). The experiment shows that reporter expression is similar between WT and Fmr1 KO mouse hippocampal neurons at the basal state. Interestingly, translation was increased in WT neurons after DHPG stimulation but not in Fmr1 KO neurons, suggesting that Nl1 3’ UTR may be downstream of Gp1 mGluR signaling regulated by FMRP. The translational regulation pattern of Nl1 3’ UTR and Fmr1 3’ UTR are different. In the basal state, the Fmr1 3’ UTR showed a higher level of translation in Fmr1 KO neurons, while the basal level of expression of the reporter with Nl1 3’ UTR is similar in WT and Fmr1 KO neurons (Figure 3-5) (Figure 4-7). This difference could represent the different nature of FMRP and NEUROLIGIN proteins. For FMRP, it may require a relatively constant basal level of expression to regulate local translation at post-synaptic terminals. New synapse formation is unlikely to trigger local translation of FMRP, because elevated expression of many genes including FMRP targets are required as building blocks for new synapse. Expression of FMRP may enhance repression of these genes. For NL1, more NL1 is needed for synapse formation, maturation and enhancement after recognition between pre-synaptic and post-synaptic terminals, so more NL1 will be translated, probably locally at the base of spines. So the basal level expression regulated through Nl1 3’ UTR between WT and Fmr1 KO neurons are similar, since there is no stimulation like new synapse formation. In other words, FMRP is constantly needed to maintain homeostasis while NL1 is more needed during synaptogenesis. The difference of transitional regulation through Fmr1 3’ UTR and Nl1 3’ UTR reflect the difference in the role of their proteins. Synaptic genes like Nl1 may be regulated as the model in Figure 6-1.

Taken together, our results of FMRP and its targets provide a new role of Nl1 3’ UTR and Fmr1 3’ UTR in mRNA transport and translation regulation. We showed that FMRP interacts
with the Nl1 and Fmr1 3’ UTRs by immunoprecipitation from cell lysates. We also showed that
the *Nl1* 3’ UTR and *Fmr1* 3’ UTR contain enough localization information to guide the mRNAs
to dendrites, and specifically to post-synaptic terminals in the case of *Nl1*. Further, FMRP was
co-transported with both *Nl1* 3’ UTR and *Fmr1* 3’ UTR in mRNP granules to dendrite, but the
phosphorylation status of FMRP had no effect on this co-transport. After the mRNAs arrive to
their destination in dendrites, local translation could happen there under regulation. The hotspot
of deTagRFP has shown local translation in the dendrites, especially in the post-synaptic
terminal.

**The role of FMRP in splicing:**

In this study, we discovered an abnormal alternative splicing pattern of *Nl1* mRNA in
*Fmr1* KO hippocampus and studied the *Nl1* variant changes in the absence of FMRP. This
research was triggered by an extra band found in western blots for NL1 from synaptosomes
prepared from hippocampus at P14 compared to WT (Figure 5-1). With the knowledge that there
are several *Nl1* alternative splicing variants, the extra band in western blot was thought to be a
product of alternative splicing in *Fmr1* KO mouse hippocampus.

We found an abnormal *Nl1* splicing pattern hippocampus in *Fmr1* KO. *Nl1* variant
specific RT-PCR was performed on the mRNA from P7, P14 and P21 hippocampi of WT and
*Fmr1* KO mice (Figure 5-2, C). Based on previous reports and all possible combination, there
should be 6 *Nl1* variants with 4 different lengths: +A1+B and +A2+B (the longest variants with
the same length), +A1ΔB and +A2ΔB (the second longest variants with the same length), ΔA+B
(the third longest variant, the most abundant variant) and ΔΔB (the shortest variant). The
ΔΔB variant reported as one of 4 *Nl1* variants is either too low in abundance to be detected in
the PCR or too small to be separated from variant ΔA+B, since the difference between ΔA+B and ΔAΔB is the 27nt splice site B. Although splice site A1 (SS A1, VKRISKECARKPGKKICRKG) and splice site A2 (SS A2, GPLTKKHTDDLGNDGAEDE) have been reported in Nl1 as well as Nl3 (Koehnke et al., 2010) (Bollinger, 2008 #561), most reports of Nl1 just used SS A in the description of Nl1 (using SS A2 sequence). Only very few reports used SS A1 as SS A (Comoletti et al., 2003), or presented both SS A1 and SS A2. SS A2 was used as SS A most of the time probably because the abundance of +A2+B is more than +A1+B in our neuron culture, although the abundance are similar in hippocampus at P14 (Figure 5-2, Figure 5-3). The variant pattern difference of Nl1 mRNA in hippocampus was only observed on the P14, so this phenomenon is development dependent. Although the Nl1 variant pattern returns to normal in Fmr1 KO hippocampus at P21, we believe that the aberrant variant expression may have irreparable consequences on hippocampal development. The variant pattern change in the Fmr1 KO hippocampus was increase of the longest variants +A+B (containing both +A1+B and +A2+B) and decrease of the third longest variant (ΔA+B). In contrast, the variant expression pattern of the FMRP target Nl2 was not affected, so this change is Nl1 specific. To be more specific, in P14 Fmr1 KO hippocampus, the mRNA abundance of +A1+B variant increased, while the mRNA abundance of +A2+B and ΔA+B decreased. The ΔA+B is the major variant of Nl1 mRNA based on previous publications, and we found this to be the case in WT hippocampus in our experiment but not in Fmr1 KO hippocampus at P14.

The difference in variant mRNA pattern can not perfectly explain the extra band in the western blot of Fmr1 KO hippocampus based on the mRNA difference. Although more mRNA of one gene does not necessarily mean more protein will be translated, we still assume the ΔA+B NL1 is the major form NL1 protein in this case (Figure 5-1). The extra band (95kD) found in
western blot of *Fmr1* KO hippocampus synaptosomes is smaller in molecular weight than the major band (117kD). If this smaller band in western blot is a translation product of a shorter mRNA variant because of decreased number of amino acids, the size differences in mRNA should be much more than splice site A (SS A) (60 bp). But the increased variant in *Fmr1* KO is +A1+B, decreased variant is ΔA+B, The difference is 60 bp SS A1. The difference between ΔA+B and ΔAΔB is the 9AA splice site B, which is less than 1kD. The difference between the major band (117 kD) and the unexpected extra band (95 kD) in western blot of *Fmr1* KO hippocampus is 22 kD. So the different in AA can not explain the difference. Although the extra band in *Fmr1* KO hippocampus is the reason we started this research and we did find anomalies in alternative splicing at the mRNA level, we still have no evidence to show this extra band is caused directly by abnormal alternative splicing.

Another possible explanation of the extra band in western blot is protein modification. It has been shown that NL1 has two bands in western blot due to glycosylation and the ratio of these two bands changes during development (Comoletti et al., 2003). It is possible that the extra band we saw in *Fmr1* KO is not glycosylated while the major band with bigger molecule weight is glycosylated. We predicted an N-glycosylation consensus sequences in SS B, which may explain the extra band in western blot. The hypothesis is that *Fmr1* KO hippocampus on P14 contains certain amount of ΔΑΔΒ variant while WT does not. Thus, *Nl1* variant ΔΑΔΒ without SS B does not contain this N-glycosylation consensus sequences, and they migrate with a lower molecular weight than variants which contain SS B. This is just one hypothesis we got from the western blot and variant specific PCR of *Nl1*. There are still questions that can not be answered by this hypothesis, such as the fact that we observed +ΑΔΒ (including +Α1ΔΒ and +Α2ΔΒ) variants by PCR which do not contain the SS B. If our hypnosis is correct, this variant without
SS B should not contain the N-glycosylation in SS B and appears as a band lower than the major band in the western blot of synaptogenesis of hippocampus. But we did not see this band in the western blot of WT (Figure 5-1). So there is still a lot work to do to relate the variant pattern difference in mRNA and the protein difference in western blot between WT and Fmr1 KO hippocampus, especially at P14.

We found that Nl1 variants have different functional roles during synaptogenesis, and that expression of specific Nl1 variants can rescue some defects of Fmr1 KO hippocampal neurons. Previous reports found more excitatory synapses in WT than KO, and opposite for inhibitory synapses in the hippocampus (Dahlhaus & El-Husseini, 2010). Over expression of FMRP in Fmr1 KO hippocampal neurons decrease synapsin puncta (Pfeiffer & Huber, 2007). In our results, Fmr1 KO hippocampal neuron shows less synapsin puncta (Figure 5-6). Since variants +A2+B and ΔA+B were decreased in Fmr1 KO hippocampus, expressing them may correct some anomalies in Fmr1 KO. Some properties of Fmr1 KO neurons were rescued by expression of some of these variants (Figure 5-6). Another difference is the abundance of Nl1 variant +A1+B, which was increased in Fmr1 KO hippocampus. The splice site SS B is a key switch for recognizing and binding NRX (Baudouin & Scheiffele, 2010). It is already known the interaction between NL and NRX is charge dependent (Koehnke et al., 2010), suggesting that these two NL1 variants may have different physiological roles during neuronal development (SS A1 is positive, SS A2 is negative) (Figure 1-8). More experiments need to be done to show what difference these two splice sites (SS A1 and SS A2) have in physiology.

We also discovered a new splicing variant of Nl1: +A1+A2+B in WT hippocampus at P14. We did not check other age. This is the first time this variant was discovered. There was no evidence of the existence or function of this variant probably because of its low abundance.
(Figure 5-5). More experiments need to be done to understand its function. It is interesting this variant was not found in Fmr1 KO hippocampus at P14. Higher abundance does not necessarily mean bigger significance. It is possible the absence of this variant is a cause of anomalies in Fmr1 KO hippocampal neurons, at least partially. Further research are needed to investigate the physiological significance of this variant.

Our results clearly relate the N11 variant pattern differences in hippocampus of WT and Fmr1 KO mice to FMRP (Figure 5-2, Figure 5-3). We have 3 hypotheses to explain the difference in N11 variants caused by FMRP: splicing regulation, mRNA stability control or FMRP regulation on transcription factors.

Splicing regulation: The NLS and NES enable FMRP to localize in both the nucleus and cytoplasm. Most research about FMRP in the past has focused on its roles in the cytoplasm and its roles in RNA biding, RNA transport, translational regulation. Not much has been done on its roles in the nucleus. Among these limited research of FMRP in the nucleus, FMRP has been found to bind pre-mRNA in nucleus when it is transcribed (Kim et al., 2009). Drosophila fragile X homolog (dFMR1) has been shown to get involved in RNA editing in the nucleus (Bhogal et al., 2011). One recent report found FMRP gets involved in the DNA damage response (Alpatov et al., 2014). Most heterogeneous nuclear ribonucleoproteins (hnRNPs) localize and function in the nucleus. It is currently not known whether the binding of FMRP-hnRNP occurs in the nucleus or cytoplasm. Considering FMRP partially localizes in the nucleus and its RNA binding ability, it is possible that FMRP function in nucleus and may have a role in RNA processing. Our finding suggests FMRP is involved in splicing of specific gene. Adding together, we think FMRP plays a more important role in nucleus than we thought.
Stability control of mRNA: FMRP has been shown to be involved in the regulation of PSD-95 and NfxI mRNA stability in the hippocampus (Zalfa et al., 2007). The 4 variants of NfxI share the same 3' UTR and most of the coding sequence. But the variants differ a lot in the 5' UTR which is 1.3 kb in variants +A+B (+A1+B and +A2+B) and around 400 bp in the others, based on the information of NfxI in National Center for Biotechnology Information (NCBI). Details of which variant has which 5'UTR is not known. The NfxI 5' UTR is a potential regulation target for regulating stability of the mRNA. If FMRP target different NfxI 5' UTR hence regulate the stability of NfxI variants differently, then the actual amount of one variant would be changed depending on the absence or presence of FMRP, which explains our observation.

FMRP regulation on transcription factors: FMRP also targets mRNAs of several transcription factors (Darnell et al., 2011). It is not so clear what is the physiological meaning of the translational regulation by FMRP on nuclear proteins, since the proteins translated from most of its targets localize in cytoplasm. Apparently, the abundance of these proteins may affect RNA processing activities.

Further directions:

The thesis focused on the research of FMRP and Neuroligin1. We have found that FMRP has an important role in cytoplasmic mRNA transport and translation, and discovered a role for FMRP in the nucleus as well. Although we have a lot of discoveries on their functions and behaviors, there are still a lot need to do in the future to understand the roles they play in FXS and cognition.
We have found that FMRP has an important role in cytoplasmic mRNA transport and translation, and discovered a role for FMRP in the nucleus as well. It is unclear whether FMRP interactions with target mRNAs are continuous from the nucleus until their final destination in the cytoplasm, but the importance of FMRP’s nuclear role has certainly been underestimated in the past. A role for FMRP in the nucleus would be of importance due to the following reasons.

First, FMRP may affect splicing on other genes besides *Nl1*. If FMRP is involved in alternative splicing, this event would happen before its involvement regulating target mRNA stability, transport and translation. Thus, the earlier and the more upstream control is exerted on a gene, the more important this regulation is for the target gene. Considering that more than 80% of human genes undergo alternative splicing, it is possible that at least part of the pathogenicity of FXS is a result of the abnormal alternative splicing. If this hypothesis is true, then there will be many genes with altered splicing patterns in the absence of FMRP. To study this hypothesis, more genes should be tested for the abnormal splicing, like the known FMRP targets, and especially ones with alternative splicing, like other *Nl* isoforms besides *Nl1*, all *Nrxs* isoforms and *Fmr1* itself. If the alternative splicing of other genes is also changed in *Fmr1* KO mice, this would strengthen the argument that FMRP has an important role in alternative splicing. In fact, this may lead to a new era of FMRP research focusing on its roles in the nucleus and a new theory of how loss of functional FMRP causes the disease, namely the “altered splicing theory of FXS”.

Second, previous reports showed that *Drosophila FMR1* was involved in dADAR RNA editing, but it is unknown whether FMRP is also involved in RNA editing in mammals. If it is, whether or not this influence in RNA editing would affect splicing by editing critical splice sites for recognition by splicing machinery should be studied. The first experiments to test this
hypothesis should focus on determining whether FMRP and ADAR (and other RNA editing enzymes) physically interact via immunoprecipitation. It is possible that FMRP may play a role in alternative splicing through RNA editing: if a key splice site is edited through RNA editing by FMRP directly or indirectly, then the splicing pattern would be changed. If this is the case, carefully scrutinizing the \textit{Nll} DNA sequence and linking it to the mature variants will help to determine which type of RNA editing happening in \textit{Neuroligin1}. After all, there is also other types of RNA editing besides A-I conversion, and there are specific enzyme responsible for each type of editing.

Third, it is still unclear how the life cycle of FMRP looks like. It is possible the journey of FMRP and its targets start in the nucleus. So far, there is no solid evidence showing FMRP and its target mRNAs exporting from the nucleus to the cytoplasm. If this event is observed, then we can know more about when and where FMRP begins to get involved in RNA binding and gene expression regulation. New microscopic technology and RNA labeling technique may help us to observe this process.

In the cytoplasm phase, we have captured the transport of \textit{Nll} 3’ UTR mRNAs to synapses and the co-transport of mRNAs with FMRP. We have not yet visualized an mRNP containing both mRNA and FMRP moving to synapses, an important step towards understanding activity-dependent mRNA transport during synaptic plasticity. It seems that stimulation in the form of synapse formation is enough to trigger \textit{Nll} expression in WT but not in \textit{Fmr1} KO neurons (unpublished data). Using different combinations of fluorescent proteins in live imaging experiments can help us to visualize the co-transport of FMRP with its targets to synapses upon stimulation. We found that the \textit{Fmr1} and \textit{Nll} 3’ UTR are localized to dendrites, and that the \textit{Nll} 3’ UTR is specifically localized to synapses. Similar to the case of the zip-code region in the β-
Actin 3’ UTR, it would be interesting to study the 3’ UTRs of *Fmr1* and *Nl1* in more detail to identify specific sequences that are responsible for dendritic/synaptic targeting. In this thesis, the phosphorylation of FMRP did not interfere with the basal dendritic localization of *Nl1* 3’ UTR. However, previous reports provided evidence that the phosphorylation of FMRP affects the dynamics of RNP granules of some of its targets under stimulation. Thus, it’s possible that stimulation may cause FMRP-containing RNPs to slow down and anchor at synapses upon synaptic stimulation. This would require techniques to robustly image and analyze moving RNP granules containing *Fmr1* and *Nl1* 3’ UTR.

In the translational regulation aspect, we have observed that FMRP regulates global translation of target mRNAs, and that 3’ UTRs are sufficient to allow local. The next step would be to study in detail the role of FMRP in regulating local translation of mRNAs through regulation of their 3’ UTR – both in basal and stimulated conditions. It seems the stimulation like synapse formation is enough to trigger *Nl1* expression in WT but not in *Fmr1* KO (unpublished data).

The FMRP in the dendrites can be classified as soma translated and local translated. It is translated immediately in dendrites after stimulation and we also observed FMRP transported to dendrites. We believe there are differences between FMRP translated from different site. They may target different mRNAs and regulate translation differently. It would be to tell the difference between them.

In the Neuroligin research, we found a new splice variant +A1+A2+B, also we found the expression of variants +A1+B, +A2+B and ΔA+B are different in WT and *Fmr1* KO mice. It is unknown how these variants differ in neuron physiology like synaptogenesis and synaptic plasticity. More experiments are needed to distinguish the functions of these *Nl1* variants. The
physiological difference between \textit{Nli} variants may further explain the roles of FMRP in FXS. Also, we still do not know whether the changes in mRNA variants are the cause of the difference of proteins in western blot of synaptosomes from hippocampus of WT and \textit{Fmr1} KO mouse. We do not know the physiological effect caused by the protein bands either. For the splicing part, we found \textit{Nli} variant +A2+B abundance increased in \textit{Fmr1} KO hippocampus. To understand the function of this variant, overexpression of this variant in WT should be able to switch the neuron to be more like \textit{Fmr1} KO neurons.
**Figure 6-1  FMRP targets local translation model**

Stimulation like new synapse formation or neurotransmitter on the post-synaptic terminal can send retrograde signals to cell body and trigger anterograde co-transport of target mRNA and FMRP to base of spine. FMRP is regulated by local stimulation through phosphorylation and dephosphorylation. When FMRP is dephosphorylated, the target gene is locally translated at the post-synaptic terminal.
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