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Regulation of translation in neuronal function and development

By

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B.S., University of Georgia 1998

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An abstract of
a dissertation submitted to the Faculty of the
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Abstract

Regulation of translation in neuronal function and development

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Brain development and function requires sophisticated regulation of gene expression achieved by both transcriptional and post-transcriptional mechanisms. While transcriptional regulation can have long-term effects on gene expression, rapid and local changes in protein levels, far beyond the capabilities of transcription, are due to intricate mechanisms of post-transcriptional regulation and essential for brain function. However, key mechanisms underlying post-transcriptional regulation in neurons remain poorly understood. In this dissertation, the roles of both cis- and trans-acting factors controlling translation and subcellular localization of mRNA were explored during brain development and function. First we examined the ability of distinct 3' untranslated regions (3'UTRs), long or short, in differential regulation of translation and subcellular localization of transcripts encoding the brain-derived neurotrophic factor (BDNF) upon neuronal stimulation. We showed that the short 3'UTR restricts BDNF mRNA in neuronal soma and supports active BDNF translation while the long 3'UTR promotes dendritic localization and represses translation of BDNF mRNA at rest. We further demonstrated that, neuronal activation releases translational repression of the long 3'UTR BDNF mRNA, which is accompanied by a rapid activation of the tropomyosin kinase receptor B in the hippocampus.

We next explored the role of the fragile X mental retardation protein (FMRP), a selective RNA-binding protein, in hippocampal development. Loss of FMRP resulted in dysregulated translation of the microtubule associated protein 1B, which is associated with abnormal projection of mossy fiber axons in the neonatal hippocampus, which offers a comprehensive model for the functional consequence of translational dysregulation in abnormal neuronal network development due to FMRP deficiency. Lastly, in an effort to elucidate trans-acting factors mediating translational regulation of the long 3'UTR BDNF mRNA, we found that the long 3'UTR BDNF mRNA is associated with FMRP as well as a specific microRNA, miR-128. In addition, miR-128 suppresses translation of luciferase reporter in a BDNF long 3'UTR -dependent manner, and neuronal activation causes significant down-regulation of miR-128. Taken together, these data provide novel insights regarding cis- and trans-acting factors that control translation to accommodate brain development and function.

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List of Abbreviations

Activity-regulated cytoskeletal associated protein…….Arc

Afterdischarge duration…….....AD

Afterdischarge threshold……....ADT

Bovine growth hormone……BGH

Brain-derived neurotrophic factor……BDNF

Calcium/calmodulin kinase IIα…….....CAMKIIα

Ca^{++}, cyclic adenosine monophosphate responsive element binding protein……..……...CREB

Central nervous system……..…CNS

Cortex……...……Cx

Cytoplasmic polyadenylation element binding protein……..……...CPEB

Days in vitro……DIV

Dentate gyrus…….…...DG

Dentate gyrus granular cells……..DGC

Elongation factor 1a……..…EF1a

Endoplasmic reticulum…….ER

Eukaryotic translation initiation factor 2c……....eIF2c

Eukaryotic translation initiation factor 5α……...eIF5α

Fluorescent in situ hybridization…...FISH

Fragile X granules……..FXG

Fragile X mental retardation protein……..FMRP

Fragile X Syndrome……FXS

GluR2 interacting protein……..GRIP-1

G-protein coupled receptors……....GPCR

Green fluorescent protein……GFP

Hippocampus…….....….Hp

Human embryonic kidney……...HEK

Immunoprecipitation……..IP

Infrapyramidal bundle…….IFB

Long-term depression…….....LTD

Long-term modulation……...LTM

Long-term potentiation……..…....LTP

Mammalian target of rapamycin….mTOR

Matrix metalloprotease….....MMP

Messenger ribonucleoprotein…..…mRNP

Metabotropic glutamate receptor…mGluR

Methyl cytosine-binding protein...MECP2

Microtubule associated protein 1B……..…MAP1B

Microtubule associated protein 2…MAP2

Mossy fibers…….MF

Mushroom bodies…….MB

Nerve growth factor……..…....NGF

Neural-restrictive silencer element..NRSE

Neurotrophin-3……....NT3

Neurotrophin4/5……..NT4/5

Open reading frame……ORF

p75 Neurotrophin receptor……...p75NTR

Paraformaldehyde……...PFA

Postsynaptic density……PSD

Postsynaptic density protein 95…..PSD95

Potassium buffered saline……...PBS

Repressor element-1 silencing transcription factor…….…..REST

Ribonuclease protection assay……RPA

RNA-induced silencing complex…..RISC

RNA polymerase II……...Pol II

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

Status epilepticus…….…..SE

Superoxide dismutase 1……SOD1

Suprapyramidal bunde……SPB

Tetraethylammonium…….TEA

Tris buffered saline…….TBS

Tropomyosin-related kinase B receptor…….....TrkB

Untranslated region…….…...UTR

Zinc transporter 3……..ZnT3

Zip code binding protein……..…ZBP

CHAPTER 1: Introduction to Dissertation

1.1 Introduction: regulation of mRNA translation and localization governs neuronal development and plasticity.

Brain development and function requires precise control of gene expression. Neuronal development requires the establishment of sophisticated cellular polarity consisting of dendrites and axons, which shape the basis for the formation of numerous synapses necessary for neuron-to-neuron communication. Gene expression in the brain can be regulated transcriptionally, post-transcriptionally and/or post-translationally. While transcription provides a means to intricately regulate gene expression, it cannot completely accommodate the necessary requirement of temporal and spatial control needed for the function of brain neurons, especially the rapid synapse-specific neuronal activity in response to stimulation. Thus, post-transcriptional mechanisms for gene regulation play particularly important roles in controlling brain development and function as compared to other tissues.

The proper establishment of the neural network is critical for brain function in regards to learning and memory formation and is dependent upon increases in protein synthesis. In early work from the 1960's, intracerebral injection of puromycin, a translation inhibitor, caused a loss of both short-term and long-term memory formation in mice (Flexner et al., 1963; Flexner et al., 1962). Many years later, in an experiment by Miller and colleagues, a mouse lacking dendritic targeting and translation of the calcium/calmodulin-dependent kinase IIα (CAMKIIα) revealed deficits in spatial memory and fear conditioning (Miller et al., 2002). Taken together, these data not only show a necessity of increased protein synthesis for learning and memory but that the

proper spatial increase in protein synthesis is highly important. However, the mechanisms regulating local protein synthesis remain poorly understood. To this end, this dissertation addresses the translational regulation and functional consequences of two important proteins involved in synaptic plasticity and mental function, the brain-derived neurotrophic factor (BDNF) and the fragile X mental retardation protein (FMRP).

1.1.1 Activity-dependent protein synthesis and local mRNA translation in synaptic plasticity

How do changes in protein synthesis in the central nervous system (CNS) lead to the formation and storing of memory? It has long been postulated that use-dependent changes in synaptic strength or efficacy (chemical plasticity), and numbers of synapses in the functional circuitry (structural plasticity), are underlying mechanisms behind learning and memory (Abel et al., 1997; Kandel, 2001). Two major alterations in synaptic strength exist in the mammalian brain, long-term potentiation (LTP) and long-term depression (LTD), which can be induced chemically by glutamate or electrically by stimulating at high and low frequency, respectively (Sutton and Schuman, 2005). In addition, LTP can also be induced chemically by molecules such as BDNF and dopamine (Sutton and Schuman, 2005). LTP is a process of increasing synaptic strength and can last for varying amounts of time, from hours to weeks to even years (Abraham et al., 2002; Racine et al., 1983). LTP can be broken down into two different phases: early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP lasts ~1-2 hours and is mediated by post-translational modifications of existing proteins (Pfeiffer and Huber, 2006), independent of new protein synthesis. In contrast, L-LTP expression requires

gene transcription and is dependent on new protein synthesis (Frey et al., 1988; Sutton and Schuman, 2005). More recently, knockout of genes that negatively regulate translation like GCN2, an eukaryotic initiation factor 2α (eIF2α) kinase, were shown to enhance memory formation (Costa-Mattioli et al., 2005), suggesting a need for new protein synthesis in memory. Conversely, LTD is the process of decreasing synaptic strength, which also is dependent on rapid protein synthesis (Waung et al., 2008). In both cases of LTP and LTD, local protein synthesis at the postsynaptic sites is believed to be necessary for the altered synaptic strength. The first evidence that local protein synthesis is sufficient to support LTP was demonstrated by a seminal study from the Schuman lab, in which application of BDNF, known to induce LTP, to severed dendrites in hippocampal slices resulted in strengthened synaptic transmission which was blocked by protein synthesis inhibitors (Kang and Schuman, 1996). Likewise, (RS)-3,5-dihydroxyphenylglycine (DHPG) induction of LTD through activation of the group 1 metabotropic glutamate receptors (mGluRs) requires protein synthesis at the dendrites and not the cell body as evidenced by robust mGluR-LTD induction in isolated dendrites from the CA1 region of the hippocampus (Huber et al., 2000).

These studies demonstrate a need for new protein synthesis in dendrites to maintain both LTP and LTD, which poses the question of how to increase protein levels specifically in activated synapses. One possible solution came from electron microscopy studies in the early 1980's that demonstrated localization of polyribosomes at the base of dendritic spines of the granule cells of the dentate gyrus (Steward and Falk, 1985; Steward and Levy, 1982). Furthermore, synaptosome preparations, which isolate synaptic components away from the neuronal soma, were able to incorporate radiolabeled

amino acids into newly synthesized proteins, indicating active translation in the synaptic compartment (Rao and Steward, 1991; Torre and Steward, 1992; Weiler and Greenough, 1991). Moreover, upon neuronal activation, polyribosomes in the dendritic shafts are shifted into or beneath dendritic spines (Ostroff et al., 2002). These studies, together with the marked increase in the number of synapses containing polyribosomes, suggest increased protein synthesis specifically in activated synapses (Ostroff et al., 2002). Additionally, poly-adenylated mRNAs were detected in distal dendrites, near synaptic sites in cultured neurons (Bruckenstein et al., 1990; Kleiman et al., 1993), and in biochemical preparations of post synaptic density (Martone et al., 1996), further supporting the idea that localized protein synthesis may occur in distal dendrites and perhaps even near synaptic terminals to promote synapse-specific plasticity. In fact, a number of mRNAs encoding essential proteins for synaptic plasticity, represented by CAMKIIα (Blichenberg et al., 2001; Mori et al., 2000) and the activity-regulated cytoskeletal associated protein (Arc) (Kobayashi et al., 2005; Steward and Worley, 2001), were found localized to dendrites in hippocampal neurons in vivo and in culture. More importantly, neuronal activation results in an increase in dendritic translation of such synaptic proteins, including CAMKIIα, elongation factor 1a (EF1a), and Arc, which is predicted to underlie changes in synaptic plasticity (Huang et al., 2005; Steward and Halpain, 1999; Tsokas et al., 2005).

Many of the new translated synaptic proteins play important roles in regulating the surface expression of AMPA receptors, as well as the size and the number of dendritic spines, all of which are associated with changes during LTP and LTD. The induction of LTP results in increased surface expression of AMPA receptors, which in

turn heightens response to glutamate signaling thus leading to the strengthening of individual synapses (Bourne and Harris, 2008). Conversely, LTD decreases the surface expression of the AMPA receptor to reduce synaptic excitability (Bourne and Harris, 2008). In addition, the increased or decreased surface expression of AMPA receptors coincides directly with an increase or decrease in the size of the postsynaptic density (PSD) and a concomitant change in spine head size seen during LTP and LTD, respectively (Bourne and Harris, 2008). Treatment with anisomycin, a protein synthesis inhibitor, blocked spine enlargement during LTP (Fifkova et al., 1982; Kelleher et al., 2004), indicating the functional requirement of new protein synthesis for structural synaptic plasticity. Furthermore, LTP is also marked by an increase in spine number, while LTD causes a decrease in the number of spines (Bourne and Harris, 2008). Such vigorous remodeling of the neuronal network upon synaptic plasticity is an important mechanism for long-term modulation (LTM) that can last for days and even years, and is believed to be the basis for long-term memory.

1.1.2 Synaptic circuitry and function of hippocampal neurons

Its well-characterized synaptic circuits make the hippocampus an ideal model system to study synapse development and plasticity. The hippocampus is a part of the limbic system and is thought to be involved in long-term memory formation and spatial learning. The axons of the perforant pathway, which originate from neurons in layer II of the entorhinal cortex, synapse with the dendrites of the granular cells of the dentate gyrus (DGCs). The dentate gyrus (DG) serves as the "gatekeeper" of inputs into the hippocampus and is also the only brain region besides the subventricular zone that

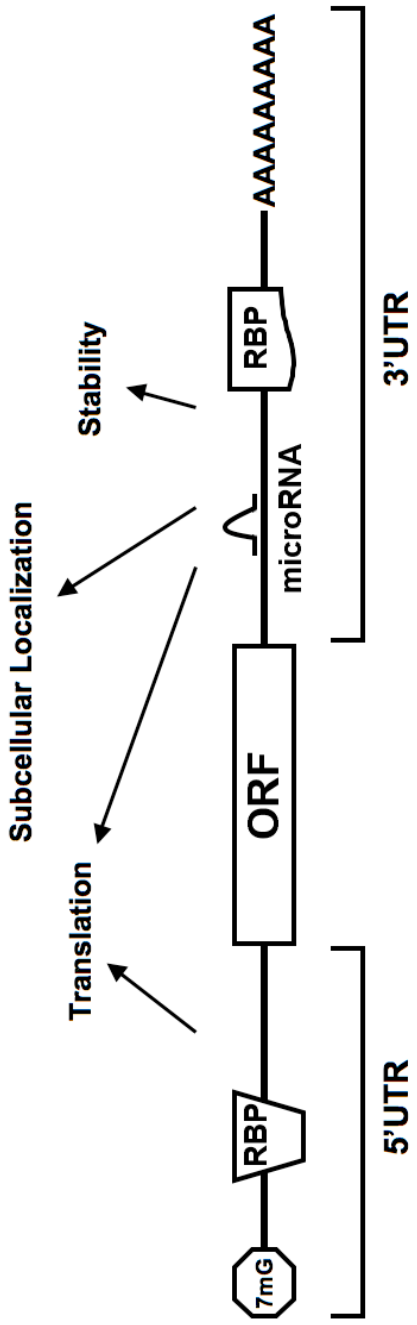
harbors neurogenesis in the adult brain (Ming and Song, 2005). During learning tasks and epileptogenesis, neurogenesis is markedly increased in the subgranular zone of DG (Ma et al., 2009). Reciprocally, ablation of neurogenesis causes abnormality in multiple learning and memory tasks associated with hippocampal function (Jessberger et al., 2009; Saxe et al., 2006). However, the exact role of increased neurogenesis in learning and memory remains unknown. Once the input is received by the DGCs, a signal is transmitted by the mossy fibers that are axonal projections from DGCs forming synapses with the dendrites of pyramidal cells in the CA3 region. Developmentally, the mossy fibers begin to form after birth with rapid growth occurring between postnatal day 5-9 (Amaral and Dent, 1981), while the connections with the dendritic spines of the CA3 pyramidal cells occur between postnatal day 10-15 giving rise to the "thorny excrescences" characteristic of mature mossy fibers (Stirling and Bliss, 1978). The CA3 pyramidal cells then project axons (Schaffer collaterals) to synapse on dendrites of pyramidal cells of the CA1 region. Finally the pyramidal cells of the CA1 region project back to the entorhinal cortex.

1.1.3 Cis- and trans-acting factors that control mRNA translation and localization

Cis-acting factors, particular nucleotide sequences contained within the transcripts, are essential for proper post-transcriptional regulation of mRNAs. Both the 5' and 3' untranslated regions (UTRs) as shown in Figure 1-1, can play important roles in regulating mRNA translation and subcellular localization. For example, the 3'UTR of CAMKIIα mRNA regulates the localization and translation efficiency of its transcript

Figure 1-1: Cis and trans-acting factors. Schematic illustrating the 5' and 3'UTRs ability to regulate translation, subcellular localization, and stability of mRNA through binding to different trans-acting factors such as RNA-binding proteins (RBP) and microRNA. ORF indicates the open reading frame and 7mG is the 7-methylguanosine cap.

Figure 1-1


AAAAAAAA
RBP
3'UTR
Stability
microRNA
Subcellular Localization
ORF
Translation
RBP
5'UTR
7mG

(Mayford et al., 1996). In fact, a mutant mouse lacking the localization element found in 3'UTR of CAMKIIα prevents the localization and subsequent local translation of CAMKIIα mRNA, leading to deficits in LTP (Miller et al., 2002). Similarly, many synaptically localized mRNAs; such as MAP2 (Blichenberg et al., 1999; Garner et al., 1988), Arc (Kobayashi et al., 2005; Steward and Worley, 2001) and β-actin (Eom et al., 2003; Zhang et al., 2001a), rely on localization sequences in their 3'UTRs to be transported into the dendrites. Recently, we showed that the long 3'UTR of BDNF transcript is sufficient for localization of BDNF mRNA to dendrites and essential for maintaining dendritic LTP (An et al., 2008), leading us to hypothesize that the long 3'UTR may regulate the translation of BDNF mRNA, possibly in a localized manner. In this dissertation, we tested the hypothesis that cis-acting mechanisms exist to differentially regulate the translatability of BDNF transcripts (Chapter 2).

The cis-acting elements located in the 5' and 3' UTRs act as binding sites to facilitate interaction with trans-acting factors, either proteins or non-coding RNAs (i.e. microRNAs), to regulate mRNA translation and/or subcellular localization. Examples of trans-acting factors are cytoplasmic polyadenylation element binding protein (CPEB), fragile X mental retardation protein (FMRP), and microRNAs. CPEB is a selective RNA binding protein that facilitates dendritic localization and induces translation of target mRNAs upon neuronal stimulation by binding to CPE sites in the 3'UTR and increasing the length of the poly-A tail (Atkins et al., 2004; Huang et al., 2003; Huang et al., 2002b). Likewise, FMRP is a selective RNA-binding protein that can repress the translation and promote subcellular localization of target mRNAs through binding to either the 5'UTR or 3'UTR (Feng, 2002; Jin and Warren, 2003). The function of FMRP-mediated translation

repression during development is the focus of Chapter 3 of this dissertation. Lastly, microRNA are a class of small non-coding RNAs that bind to the 3'UTR with imperfect sequence complementarity to repress translation of target mRNAs (Bartel, 2004). In Chapter 4, we examined the ability of microRNAs to repress the translation of BDNF mRNA.

1.2 Brain-Derived Neurotrophic Factor (BDNF) expression and the functional link to translational regulation

1.2.1 BDNF gene expression and post-translational processing

In 1982, BDNF was isolated from pig brains by Barde and colleagues as a novel neurotrophic factor capable of supporting the survival of cultured sensory neurons (Barde et al., 1982). BDNF is a member of the secreted neurotrophin family, including NGF, NT3, and NT4/5. These proteins are expressed in both the developing and mature brain (Hofer et al., 1990; Maisonpierre et al., 1990; Phillips et al., 1990). BDNF is widely expressed throughout CNS with the highest levels found in the hippocampus, neocortex, amygdala, and cerebellum (Hofer et al., 1990; Maisonpierre et al., 1990).

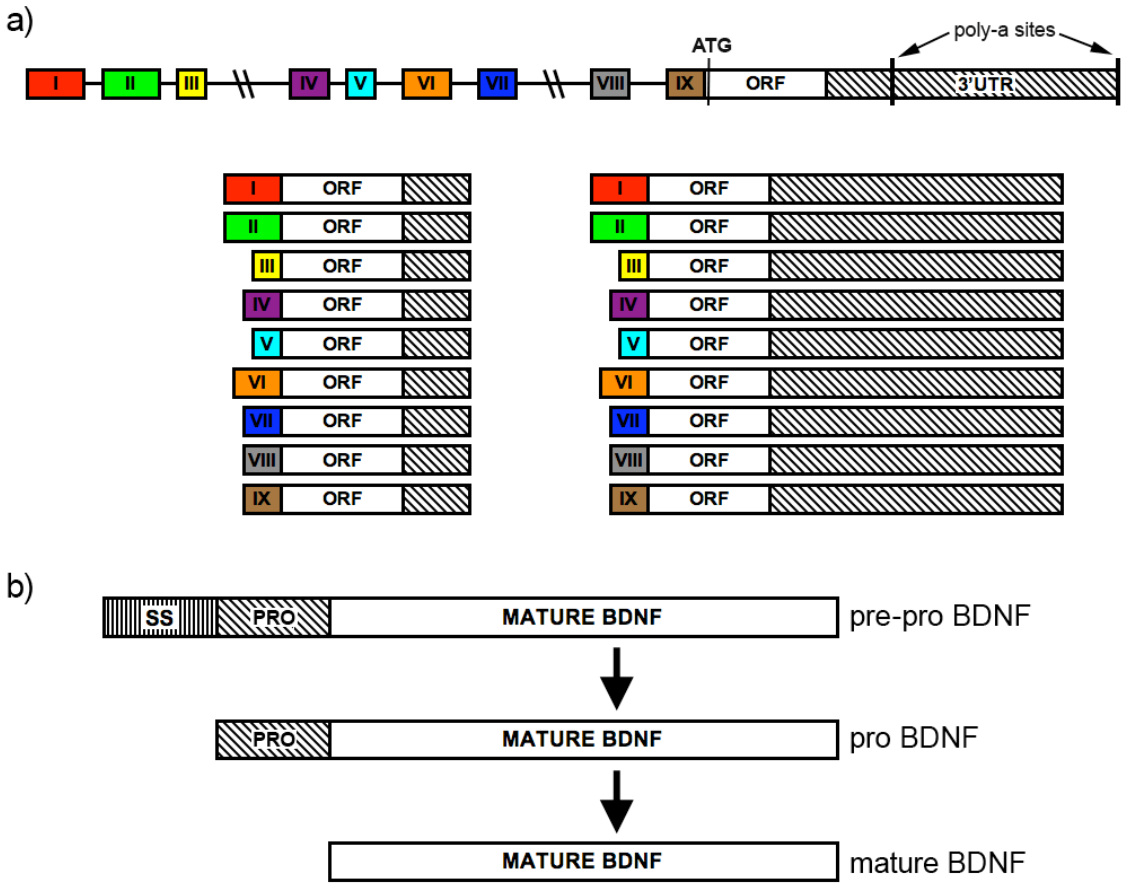
The BDNF gene is comprised of nine exons whose transcription can be driven by different promoters, which can be activated by various cellular and developmental cues (Aid et al., 2007; Liu et al., 2006; Liu et al., 2005b). For example, promoter VI displays robust mRNA expression in the heart and lung (Timmusk et al., 1993), which is not seen by any of the other promoters. Similarly, in the different brain regions, the promoters are preferentially activated. For example, promoter I is preferentially active in the cerebellum

and promoter IV in the hippocampus (Tian et al., 2009b). Once transcribed, the donor site of the upstream exon is spliced to the acceptor site ~20 base pairs upstream of the open reading frame and 3' UTR, which are found in exon IX (Figure 1-2a). The BDNF transcript is then processed at one of the two poly-adenylation sites giving rise to two distinct 3'UTRs; a short or long, 0.35kb and 2.85kb, respectively (Timmusk et al., 1995). A total number of 18 BDNF mRNA isoforms have been identified due to the various combination of the nine alternative 5'UTRs and the two 3'UTRs (Figure 1-2a), all of which encode the same BDNF protein.

The BDNF protein, like the rest of the neurotrophic factors, is initially translated as pre-pro-BDNF, which first requires the removal of the signal peptide upon sequestration in the endoplasmic reticulum (ER). From the ER, pro-BDNF is transported to the trans-Golgi network or immature secretory vesicles where it is further cleaved at the N-terminus to produce the mature form (Figure 1-2b), which is sorted into regulatory secreted vesicles for activity dependent BDNF secretion (Hartmann et al., 2001; Kohara et al., 2001; Kojima et al., 2001). As a consequence, neurons primarily release mature BDNF, although BDNF is also expressed in glia (Dai et al., 2001; Zafra et al., 1992). Emerging evidence indicates, however, that BDNF can be secreted by neurons in both the pro and mature BDNF forms; with pro-BDNF more abundantly released in developing mice and mature BDNF in adult mice (Nagappan et al., 2009; Yang et al., 2009). Importantly, released pro-BDNF can be cleaved in the synaptic cleft by extracellular proteases such as matrix metalloproteases (MMPs) and plasmin, whose activity is increased upon neuronal stimulation (Lee et al., 2001; Nagappan et al., 2009; Pang et al.,

Figure 1-2: BDNF gene structure and protein processing. a) Schematic of the BDNF gene structure, made up of nine exons (top panel). Exon IX contains the open reading frame and the 3'UTR which can be processed at two alternative polyadenylation sites, resulting in either a long or short 3'UTR. The transcription of each exon is driven by its own promoter, followed by splicing to the open reading frame. The bottom panel depicts the 18 potential transcripts generated by the BDNF gene. b) Schematic of the processing of the BDNF protein. Translation of BDNF mRNA initially yields a BDNF protein containing a signal sequence (SS), necessary for localization to the ER, and the proBDNF sequence. The SS is cleaved off in the ER leaving proBDNF, which is further cleaved to mature BDNF.

Figure 1-2


a)
ATG
poly-a sites
I
II
III
IV
V
VI
VII
VIII
IX
ORF
3'UTR
b)
SS
PRO
MATURE BDNF
pre-pro BDNF
pro BDNF
mature BDNF

2004), providing a mechanism for controlling mature BDNF expression and subsequent function.

1.2.2 BDNF signaling and physiological functions

Release of mature BDNF activates the high affinity tropomyosin-related kinase B (TrkB) receptor, whereas pro-BDNF binds to and activates the low affinity, promiscuous p75 neurotrophin receptor (p75NTR), which is common for all members of the neurotrophin family. p75NTR is a member of the tumor necrosis receptor superfamily (TNF). The p75NTR is composed of 4 cysteine rich motifs, which are responsible for neurotrophin binding, a single transmembrane region, and a cytoplasmic domain, which contains a "death" domain similar to other members of the tumor necrosis receptor superfamily (Underwood and Coulson, 2008). Unlike other neurotrophin receptors, p75NTR has no known enzymatic property, thus requiring adapter proteins to initiate signaling cascades. Binding of pro-BDNF to the p75NTR can initiate several different signaling cascades, which can lead to apoptosis (Bertrand et al., 2008; Teng et al., 2005), axonal pruning (Singh et al., 2008), and increased filiapodia length (Gehler et al., 2004) while negatively modulating dendritic spine number and complexity (Zagrebelsky et al., 2005). In this regard, activation of p75NTR may functionally antagonize the more common mature BDNF signaling through the high affinity TrkB receptor.

TrkB is a member of the tropomyosin-related kinase (Trk) receptor family and binds to a homodimer of mature BDNF. There are three major Trk receptors, namely TrkA, TrkB, and TrkC. Unlike p75NTR that can be activated by all neurotrophins, the Trk family receptors are highly specific for the corresponding ligands. NGF specifically

activates TrkA, BDNF and NT4 activate TrkB, and NT3 activates TrkC. Due to the low abundance of NT4 and its low affinity to TrkB, BDNF is the primary physiological ligand for TrkB. Binding of mature BDNF results in homodimerization of TrkB receptors followed by autophosphorylation of multiple tyrosine residues. Three major signaling cascades are activated upon BDNF binding to TrkB: 1) Ras pathway, 2) phosphotidyl-inositol 3 kinase (PI3K) pathway, and 3) phospholipase C-γ pathway (Lu, 2003b; Reichardt, 2006; Segal, 2003). The end consequence of these pathways is to increase cell survival, differentiation, and neurogenesis (Chan et al., 2008; Huang and Reichardt, 2001; Lipsky and Marini, 2007). Aside from the general growth factor role BDNF plays, both BDNF and TrkB proteins are localized to neuronal synapse (Tongiorgi et al., 1997), where they play key roles in governing synaptic formation and plasticity.

1.2.3 Role of BDNF in the synapse

In the synapse, BDNF enhances the growth of dendrites and axons (Danzer et al., 2002; Jin et al., 2003; Scharfman et al., 1999), facilitates dendritic spine maturation (Murphy et al., 1998), advances synapse formation (Vicario-Abejon et al., 1998), and promotes LTP (Minichiello et al., 2002; Patterson et al., 1996). It was recently demonstrated that activity-induced structural plasticity of individual dendritic spines is strongly dependent on BDNF action (Tanaka et al., 2008). Furthermore, BDNF facilitates synaptic transmission (Levine et al., 1998; Li et al., 1998; Tyler and Pozzo-Miller, 2001) and neurotransmitter receptor clustering (Elmariah et al., 2004), which has profound functional impacts in brain function, including learning/memory behavior (Lipsky and Marini, 2007; Rattiner et al., 2004). The positive reinforcement on synaptic

function and development are mediated by activation of the TrkB receptor. Interestingly, BDNF is involved in both protein synthesis dependent LTP, through activation of the mTOR pathway, and protein synthesis independent L-LTP (Pang et al., 2004). In contrast, signaling of p75NTR by pro-BDNF can facilitate NMDA-dependent hippocampal LTD (Woo et al., 2005), allowing for a bidirectional control of hippocampal synaptic plasticity.

Furthermore, mutations or knockouts of either BDNF or its receptors lead to improper synapse formation and function, ultimately leading to alterations in learning and memory. A mutation in pro-BDNF val66met, which prevents the sorting of BDNF into the regulated secretory vesicles, fails to localize BDNF to synapse, leading to impairment of hippocampal function and episodic memory (Egan et al., 2003). Likewise, mice with TrkB conditional knockout demonstrated a significant reduction in LTP and impaired spatial memory (Hennigan et al., 2009; Minichiello et al., 2002). In addition, a novel conditional knockout of TrkB receptor in specific regions of the hippocampus possessed fewer dendrites and enlarged dendritic spines, which indicates an important role for BDNF-TrkB signaling in neural network formation of the hippocampus (Danzer et al., 2008). In contrast, homozygous knock out of p75NTR resulted in enhanced LTP in the Schafer collateral synapse, and enhanced spatial memory (Barrett et al., 2010), which again demonstrates a bidirectional regulation of BDNF function in regulating synaptic plasticity.

1.2.4 Regulation of BDNF expression

1.2.4.1 Transcriptional regulation

Activity-dependent cleavage and secretion of BDNF allows for the instantaneous effects of BDNF. However, prolonged elevation of BDNF function requires increased expression of the BDNF gene. Upon neuronal stimulation, the levels of both BDNF mRNA and protein are dramatically increased (Tongiorgi et al., 2004). The complexity and multitude of promoters affords a mechanism by which transcription of the BDNF gene can be differentially controlled by various stimulation cues. For example, in the hippocampus, chemically-induced seizures upregulate the expression of transcripts containing exon I, II, and IV (Aid et al., 2007; Kokaia et al., 1994; Timmusk et al., 1993), while acute stress increases the transcription of exon IV BDNF transcripts (Molteni et al., 2009) and physical activity increases the expression of exon I BDNF transcripts (Russo-Neustadt et al., 2004; Russo-Neustadt et al., 2000). Of the nine different BDNF promoters, four (I, II, IV, VI) have been studied extensively in relation to activity-dependent regulation, with promoters I and IV showing the greatest increase in levels upon robust neuronal stimulation (Timmusk et al., 1994). Several transcription factors, including methyl cytosine-binding protein 2 (MECP2), repressor element-1 (RE-1)-silencing transcription factor (REST), and Ca^{++}/cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB), play important roles in regulating BDNF transcription driven by the individual promoters. Under basal conditions, MECP2, which binds to DNA with methylated cytosines, associates specifically to promoter IV, thus reducing the transcription from this promoter (Chen et al., 2003). Likewise, REST binds

to a neural-restrictive silencer element (NRSE)/RE-1 sequence of 21 nucleotides in the intron between promoters I and II, aiding to decrease transcription initiated by either promoter (Tabuchi et al., 1999; Timmusk et al., 1999). Upon neuronal activity, transcription from promoters I and IV of BDNF is derepressed through the release of REST and MECP2, respectively, by phosphorylation of the transcription factor and by reducing histone methylation, thus leading to activity-dependent increase in BDNF expression (Chen et al., 2003; Tian et al., 2009a; Tian et al., 2009b; Zhou et al., 2006).

Release of the transcription repressors MECP2 and REST is closely followed by binding of the transcription activator CREB (Tao et al., 1998). The phosphorylation of CREB, which promotes binding to CRE-sites in genomic DNA, is mediated by several different signaling processes; such as Ca^{++} influx through voltage gated Ca^{++} channels or ligand-gated channels (i.e. NMDA receptors), increased cAMP levels through G-protein coupled receptors (GPCRs), and neurotrophic activation of tyrosine kinase receptors, all of which are involved in neuronal activation (Alberini, 2009). Once phosphorylated, CREB activates transcription at promoters I and IV of the BDNF gene (Shieh et al., 1998; Tian et al., 2009a), thereby further enhancing activity-dependent transcription of BDNF mRNA. Despite the complex regulation occurring at the BDNF promoters in response to stimulation, the activity-dependent transcriptional regulation of BDNF cannot completely account for the known temporal and spatial regulation of BDNF. In particular, transcriptional regulation of BDNF cannot explain the synapse-specific modulation by BDNF. This raises an intriguing argument that post-transcriptional mechanisms may also play important roles in controlling BDNF protein expression in neurons.

1.2.4.2 Post-transcriptional regulation of BDNF mRNA

Post-transcriptional regulation of BDNF mRNA can occur at several stages: 1) stability, 2) translation, and 3) subcellular localization. Each of these regulatory mechanisms could be controlled by neuronal activity, providing a means for regulating BDNF during synaptic plasticity and neuronal development in addition to and possibly uncoupled from BDNF transcription. Very little is known about the stability of the individual BDNF mRNA transcripts, except that the long 3'UTR BDNF mRNA transcript has a shorter half-life ($t_{1/2}$= ~23 min) compared to that of the short 3'UTR BDNF transcripts ($t_{1/2}$= ~132 min) (Castren et al., 1998). The half-life of neither the short nor long 3'UTR BDNF transcripts was affected upon kainic acid induced neuronal stimulation, in cultured hippocampal neurons (Castren et al., 1998) . However, whether BDNF mRNA stability is differentially regulated in specific neuronal populations in response to activity changes still remains elusive at this point.

Just as is the case with stability, very little is known as to whether translation of BDNF mRNA is regulated to accommodate neuronal activity. The translatability of the BDNF mRNA was examined in a crude polysome fractionation obtained from rat brain followed by ribonuclease protection assay to detect exons I, II, IV, and VI as well as the long 3'UTR message (Timmusk et al., 1994). All the transcripts containing the four individual exons were more abundant in the polyribosomal fraction than in the total RNA fraction, while the long 3'UTR BDNF mRNA was reduced in the polyribosomal fraction as compared to total RNA. These data suggest that the long 3'UTR BDNF mRNA is poorly translated in the rat brain. Given that many dendritically localized mRNAs are transported as translationally dormant complexes with RNA-binding proteins (Huang et

al., 2002b; Huttelmaier et al., 2005; Kiebler and Bassell, 2006), translation suppression of the long 3'UTR BDNF mRNA might help to promote subcellular localization of BDNF mRNA and regulate expression of BDNF protein.

One way for BDNF to control synaptic function may be through transporting the BDNF mRNA into dendrites, which enables local synthesis of BDNF protein in or near activated synapses. Using radiolabeled in situ hybridization; Dugich-Djordjevic and colleagues discovered that BDNF mRNA showed intense staining at the border between the granular cells and molecular layers of the dentate gyrus after kainate-induced seizures, providing the first evidence of dendritic localization of the BDNF mRNA (Dugich-Djordjevic et al., 1992). However, this study was done after neuronal activation, leaving the question of what the subcellular localization is at rest compared to the neuronal-activity induced localization. In 1997, Tongiorgi and colleagues demonstrated the presence of BDNF mRNA in the proximal dendrites of cultured hippocampal neurons and that treatment with high concentration of KCl, which causes depolarization of neurons, resulted in accumulation of BDNF mRNA in the distal dendrites, suggesting that neuronal-activity mobilizes BDNF mRNA into dendrites (Tongiorgi et al., 1997). Furthermore, both physiological and robust seizure-induced neuronal activity show a similar ability to localize BDNF mRNA to the dendrites of hippocampal and cortical neurons in vivo (Capsoni et al., 1999; Tongiorgi et al., 2004). Interestingly, in addition to KCl and seizure-inducing chemicals (pilocarpine and kainate), BDNF-TrkB signaling is likewise capable of inducing dendritic localization of BDNF mRNA (Righi et al., 2000). Given the possibility of 18 different BDNF transcripts, the cis and/or trans-acting factors that mediate the subcellular localization remain unknown and provide enormous

complexity to achieve dendritic localization of the BDNF mRNA. Recently, the ability of different 5'UTRs, denoted by exon incorporation into the transcript, to regulate localization of the BDNF transcripts has been demonstrated such that exon I and IV (old III) are found primarily within the soma, whereas exon II and VI are distributed in both the soma and dendrites (Chiaruttini et al., 2008; Pattabiraman et al., 2005). Conversely, emerging evidence proposes that the long 3'UTR is sufficient to preferentially mediate dendritic localization of BDNF mRNA, while the short 3'UTR is sequestered in the neuronal soma (Chapter 2.2.1)(An et al., 2008). Regardless of the mechanism of transport, once in the dendrite, BDNF mRNA is able to associate with polyribosomes in the shaft and spines (Tongiorgi et al., 2004), suggesting the ability and necessity for localized BDNF protein synthesis.

Several lines of evidence suggest that BDNF production may be regulated at the level of translation (Katoh-Semba et al., 1999; Nanda and Mack, 2000; Timmusk et al., 1994). For example, following chemical-induce seizures, BDNF mRNA is robustly increased 1 hour and 8 hours after injection, while the BDNF protein levels remain unchanged (Nanda and Mack, 2000), suggesting translational repression of BDNF mRNA after PTZ treatment. Similarly, kainate-induced seizures show a robust increase of BDNF mRNA 3 hours after injection, which significantly declines at 8 hours, and returns to basal levels 24 hours later. However, the increase in BDNF protein level does not occur until 4 hours after kainate treatment, reaching peak expression 12 to 24 hours later, after the transient increase in mRNA levels has returned to basal levels (Katoh-Semba et al., 1999). Such differential temporal profiles of BDNF mRNA and protein suggest regulation either at the level of translation of BDNF mRNA or stability of the

BDNF protein. However, the ability of neuronal stimulation to modulate translation of BDNF mRNA and possible factors underlying translational regulation of BDNF remains unknown. In this dissertation, the ability of the distinct 3'UTRs of BDNF mRNA to differentially regulate BDNF mRNA translation in response to changes in neuronal activity is explored along with a potential role in seizure development (Chapter 2).

1.2.5 BDNF in disease

Given the multitude of BDNF functions and the complex regulation of its expression levels, abnormality in BDNF expression and BDNF-TrkB signaling could have direct consequences on brain function and development, manifesting in a wide range of neurological and neuropsychiatric diseases. Indeed, decreases in BDNF protein and mRNA levels have been associated with a wide variety of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Rett's Syndrome and Huntington's disease (Hu and Russek, 2008). In addition, the role of BDNF-TrkB signaling in epilepsy has been intensively explored. Epilepsy is defined as a brain disorder characterized by recurrent seizures. The hippocampus plays central roles in epileptogenesis, the process by which a normal brain becomes epileptic, and is the most extensively studied brain region for physiological as well as pathological plasticity (Joels, 2009; Zhao and Overstreet-Wadiche, 2008). The protein and mRNA levels of BDNF are rapidly and drastically increased upon seizure induction in the hippocampus (Katoh-Semba et al., 1999; Timmusk et al., 1993). Despite the trophic effect of BDNF in neuronal survival and synaptic formation, overexpression of BDNF in the mouse brain results in enhanced seizure severity and in spontaneous seizures (Croll et al., 1999).

Conversely, a decrease in BDNF levels in the heterozygote BDNF knockout mouse lessens seizure formation (Kokaia et al., 1995), suggesting overproduction of BDNF is an important factor for epileptogenesis. Interestingly, the loss of TrkB signaling in hippocampal DG and CA3 regions in a TrkB conditional knockout mouse completely abolishes kindling-induced seizure development (He et al., 2004), indicating a key role of TrkB signaling in epileptogenesis through the mossy fiber (MF) synapse. Structurally, sprouting of hippocampal MFs is observed in nearly all experimental models of epilepsy and in the epileptic human hippocampus (Sutula and Dudek, 2007). In addition, hippocampal neuron loss and ectopic generation of hyperexcitable DGCs integrated into the existing neural network are also commonly observed in epilepsy development (Dudek and Sutula, 2007). BDNF clearly promotes all the aforementioned pathological reconstruction observed in epileptogenesis (Koyama et al., 2004). By understanding molecular mechanisms that can regulate BDNF expression and/or TrkB signaling, potential therapeutic targets could be discovered epilepsy and other neurodegenerative disorders.

1.3 The fragile X mental retardation protein (FMRP) governs neuronal translation and brain function

1.3.1 Fragile X syndrome and the role of FMRP in translation regulation

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation affecting 1 in 4000 males and 1 in 8000 females (Jin and Warren, 2003; O'Donnell and Warren, 2002; Turner et al., 1996). Patients with FXS display cognitive

deficits, impaired social skills, anxiety, hyperarousal to sensory stimuli, and an increased susceptibility to seizures during childhood (Berry-Kravis, 2002; Garber et al., 2008; Musumeci et al., 1999; Sabaratnam et al., 2001). Since no effective therapy is available to cure this devastating disease, current therapies for FXS are aimed at managing the symptoms. Thus, understanding the cause and mechanisms underlying the disease are of vast importance. FXS results from the loss of function of the fragile X mental retardation protein (FMRP). In most cases of FXS, the loss of FMRP is due to the expansion of the CGG trinucleotide repeat in the 5'UTR of the fragile X gene. The normal population carries from 5 to 55 CGG repeats (Fu et al., 1991; Snow et al., 1993); while the full mutation arises when the number of repeats is greater than 200 (Fu et al., 1991; Snow et al., 1993). The fully expanded repeats result in DNA hypermethylation of the 5'UTR and CpG island in the FMR1 locus, leading to a subsequent transcriptional silencing of the gene (Sutcliffe et al., 1992).

FMRP is a selective RNA-binding protein that is expressed ubiquitously, with the highest levels found in the testis and brain (Devys et al., 1993). It has been shown that FMRP associates with about 4% of total brain mRNA, many of which encode key factors for neuronal development and synaptic plasticity, represented by post-synaptic density protein-95 (PSD-95), CAMKIIα, microtubule associated protein 1B (MAP1B), and Arc (Lu et al., 2004; Steward and Worley, 2001; Todd et al., 2003; Zalfa et al., 2007; Zalfa et al., 2003). The primary role of FMRP is to repress translation of its bound mRNA and to mediate neuronal activity-dependent translation, possibly via translational de-repression of its targets. Interestingly, FMRP follows its mRNA ligands to polyribosomes during translation (Corbin et al., 1997; Feng et al., 1997a; Khandjian et al., 2004; Stefani et al.,

2004). However, the precise mechanism by which FMRP regulates translation remains undefined.

FMRP is found primarily in the neuronal soma, but can shuttle in and out of the nucleus due to a nuclear localization signal (Eberhart et al., 1996). Nonetheless, FMRP does not appear to be necessary for nuclear export of its mRNA ligands. In addition, both FMRP and its message are found localized to dendrites and dendritic spines, suggesting a role of FMRP in local protein synthesis (Feng et al., 1997b). Recently, FMRP has been found to localize to axonal growth cones in cultured neurons (Antar et al., 2006) and developing neuropils of olfactory and hippocampal neurons in vivo (Christie et al., 2009) (more details discussed in Chapter 3). Taken together, these data suggest that FMRP can play an important role in both pre- and post- synaptic development via regulating translation.

Binding of mRNA by FMRP is potentially mediated through two different binding domains: an RGG box (denoted by repeats of an arginine-glycine-glycine motif), and heterogeneous nuclear ribonucleoprotein K-homology domains (KH domains). The RGG box interacts with a specific RNA structural element in vitro, called G-quartet (Brown et al., 2001; Darnell et al., 2001; Ramos et al., 2003), while the KH domains may interact with a distinct RNA structural motif known as a "kissing" complex (Darnell et al., 2005). Many of the FMRP's mRNA ligands contain a G-quartet element, but no mRNA targets of FMRP have been shown to contain the "kissing" element (Darnell et al., 2005). Interestingly, a patient with the point mutation, isoleucine-304 to asparagine (I304N), in the second KH domain of FMRP displayed the most severe form of FXS (Siomi et al., 1994), indicating a crucial role of the KH domain in FMRP function in

binding to both polyribosomes and target mRNAs (Feng et al., 1997a; Zang et al., 2009). Besides direct binding to the mRNA targets, both Drosophilia and mammalian FMRP were shown to associate with microRNAs as well as key components of the microRNA machinery, including Argonaute2, the p68 RNA helicase, and active Dicer (Jin P. 2004, Caudy AA 2002, Ishizuka A 2002), further implicating a role for FMRP to regulate the translation of target mRNAs, perhaps in collaboration with microRNAs.

Indeed, the protein levels of many of the mRNA targets of FMRP are abnormally elevated in the Fmr1 knockout (Fmr1 KO) mouse (Lu et al., 2004; Zalfa et al., 2003). One of the best-characterized FMRP targets is the mRNA encoding MAP1B, which stabilizes microtubules and plays key roles in axonal pathfinding and extension (Bouquet et al., 2004; Brugg et al., 1993). Importantly, MAP1B protein levels, but not MAP1B mRNA, are aberrantly elevated during neonatal hippocampal development in the Fmr1 KO mice (Lu et al., 2004), suggesting that FMRP represses the translation of MAP1B mRNA. Despite the increased steady state protein levels in Fmr1 KO mice, suggesting that FMRP is a translation repressor, a recent publication cited a novel function for FMRP as a positive modulator of translation of superoxide dismutase 1 (SOD1) (Bechara et al., 2009). How FMRP may exert opposing roles in modulating translation of distinct mRNAs and the functional significance of such diverse regulation in brain function is not understood.

Many mRNA targets of FMRP, such as CAMKII and PSD-95, are localized to dendrites (Blichenberg et al., 2001; Muddashetty et al., 2007). FMRP and target mRNAs form large granules with other proteins that can interact with the kinesin motor protein (Davidovic et al., 2007; Dictenberg et al., 2008), thereby facilitating transport of mRNAs

to distal dendrites in a translationally repressed state. Once FMRP has transported the target mRNA to the synapse, the translational repression can be released by activation of mGluR and other signaling pathways, leading to localized protein synthesis of selected mRNAs (Muddashetty et al., 2007; Todd et al., 2003; Volk et al., 2007). However, in Fmr1 KO mice, the basal steady state protein levels of FMRP targets, like PSD-95, CAMKIIα, MAP1B and Arc, are increased in the synapse without a subsequent increase in mRNA level, suggesting increased local translation at steady state (Liao et al., 2008; Lu et al., 2004; Muddashetty et al., 2007; Zalfa et al., 2003). Furthermore, subsequent activation of mGluRs can no longer induce local translation in Fmr1 KO mice (Muddashetty et al., 2007; Weiler et al., 2004). Combined, the increased basal protein levels in the synapse and the inability to induce local translation upon neuronal activity is believed to underlie the developmental and cognitive impairments in FXS.

1.3.2 FMRP in synaptic development and function

If FMRP is involved in regulating translation and transport of target mRNAs in dendrites and synapses, then loss of FMRP could have profound effects on synapse development and function. Indeed, one of the more profound pathological phenotypes of FXS patients and Fmr1 KO mice is an increase in the number of immature dendritic spines, characterized by longer and thinner spines with reduced spine heads as compared to that in normal subjects (Comery et al., 1997; Grossman et al., 2006; Irwin et al., 2000; Nimchinsky et al., 2001). While much of the focus has been on FMRP-induced changes in the post-synaptic morphology, recent evidence has implicated a potential role in pre-synaptic and axonal development. FMRP was found to localize to axonal growth cones

of cultured hippocampal neurons, along with one of its target mRNAs, MAP1B (Antar et al., 2006). In Drosophilia, loss of dFMR in the mushroom bodies (MB), the center of learning and memory in flies, increases the number of axonal branches, the length of axons and leads to abnormal synapse formation (Pan et al., 2004). In the developing mammalian brain, FMRP appears to localize in axons of hippocampal and olfactory neurons as larger granules, designated as fragile X granules (FXG) (Christie et al., 2009). However, the function that FMRP and/or the FXGs play in axonal development has not been reported. We show in Chapter 3 that FMRP and MAP1B mRNA colocalize to the axonal terminals of hippocampal mossy fibers during the critical window of mossy fiber projection and formation of synapses with CA3 dendrites. The loss of FMRP in the hippocampal mossy fibers leads to increased MAP1B protein and abnormal projection of the infrapyramidal bundle of the mossy fibers. This is the first evidence that indicates a role of FMRP in mediating the development of both the pre- and post-synaptic sides in mammalian brain.

Deficits in learning and memory are thought to arise from changes in synaptic plasticity. LTP and LTD are the commonly used physiological readouts for measuring synaptic plasticity (more details described in 1.1.2). Interestingly, Fragile X knockout mice display alterations in both forms of plasticity. The most widely studied abnormality in Fmr1 KO mice is the metabotropic glutamate receptor-dependent LTD (mGluR-LTD) in the CA1 region of the hippocampus. mGluR-LTD is protein synthesis dependent and mediated by a decrease in the surface expression of the AMPA receptor (Huber et al., 2000). Activation of group I-mGluRs by (RS)-3,5-dihydroxyphenylglycine (DHPG) increases the protein synthesis of dendritically localized mRNAs (Hou et al., 2006), many

of which are potential FMRP targets. Several of these mRNAs play important roles in AMPA receptor internalization. For example, MAP1B, an mRNA target of FMRP, interacts with the GluR2 interacting protein (GRIP-1), a protein that stabilizes AMPA receptor in the plasma membrane (Seog, 2004). The MAP1B protein levels and the MAP1B-GRIP-1 interaction are increased upon DHPG, an mGluR1 agonist, stimulation in wild type mice, which causes GRIP-1 to dissociate from AMPA receptors, promoting AMPA internalization (Davidkova and Carroll, 2007). The loss of FMRP in the synapse likely increases the levels of these "LTD proteins", represented by MAP1B, which are necessary for regulating the endocytosis of AMPA receptors. Indeed, loss of FMRP results in decreased surface expression of AMPA receptors (Nakamoto et al., 2007), which leads to enhanced mGluR-LTD in the CA1 region of the hippocampus (Huber et al., 2002). Interestingly, unlike normal mGluR-LTD, the enhanced mGluR LTD in Fmr1 KO mice does not require protein synthesis (Nosyreva and Huber, 2006), suggesting the importance of FMRP-mediated localized translation repression in regulating this form of synaptic plasticity.

Conversely, the reduction in cell surface expression of GluR1-containing AMPA receptors in the Fmr1 KO mice could attenuate LTP induction. In fact, LTP is completely abolished in the neocortex of Fmr1 KO mice (Wilson and Cox, 2007). Contrary to initial reports indicating no change in LTP in the hippocampus of Fmr1 KO mice (Godfraind et al., 1996; Huber et al., 2002), emerging evidence suggests an impairment of LTP induction in hippocampus of Fmr1 KO mice (Lauterborn et al., 2007; Shang et al., 2009; Zhang et al., 2009). The impairment of LTP in both the hippocampus and neocortex in Fmr1 KO mice could stem from the loss of surface expression of

GluR1-containing AMPA receptors through the loss of the Ras signaling cascade (Hu et al., 2008), which is important for insertion of AMPA receptors in the plasma membrane. Additionally, alterations in the synapse formation could play a key role in LTP induction. Aside from the appearance of immature dendrites in Fmr1 KO mice, the function of FMRP in synaptogenesis and neuronal network formation remains unclear and is explored further in Chapter 3.

1.4 MicroRNA in neurons

1.4.1 MicroRNA, a novel class of small non-coding RNA that controls brain development and function

MicroRNAs are small, noncoding RNAs (19-23 nucleotides) that function to repress translation and/or degrade target mRNAs through binding to the 3'UTR of the mRNA targets with imperfect sequence complementarity (Ambros, 2004; Bartel, 2004; Jing et al., 2005). Lengthy pri-precursor microRNAs are transcribed in the nucleus by RNA polymerase II (Lee et al., 2004) and then cleaved by Drosha, an RNase III family member, into the smaller precursor form (70-80nt, pre-microRNA) (Lee et al., 2003). The pre-microRNA is predicted to fold into a stem-loop structure that contains the microRNA within its stem, and is exported from the nucleus by exportin 5 (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, another RNase III family member, Dicer, further cleaves the pre-microRNA (Bartel, 2004), liberating the mature microRNA (19-23 nt). The mature microRNA is then incorporated into the RNA-induced silencing complex (RISC), thereby mediating translational suppression (Bartel, 2004). The mechanism used by microRNA to repress the translation of its target mRNAs

remains largely unknown. Several potential mechanisms; including blocking translation initiation (Kiriakidou et al., 2007; Pillai et al., 2005), preventing elongation (Maroney et al., 2006; Olsen and Ambros, 1999), and by deadenylation of the poly-a tail (Wakiyama et al., 2007; Wu et al., 2006), have been demonstrated, suggesting that the protein composition of the RISC complex might play a role in determining how translation is repressed.

Bioinformatic analysis suggests the existence of several hundred microRNAs encoded in the mammalian genome (Berezikov et al., 2005), many demonstrating developmentally regulated expression profile. In fact, the first two microRNAs discovered, lin-4 and let-7, are required for proper timing of *C. elegans* development (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). Let-7 is one of the many microRNAs that are highly expressed in the brain and promotes neuronal differentiation (Sempere et al., 2004; Zhao et al., 2010). It has been postulated that ~70% of detectable microRNAs are expressed in the brain and that close to half of those are only found in the brain, where their transcription is controlled during both development and neuronal activity, implicating a role for microRNA in neuronal differentiation, development, and function (Nelson et al., 2004; Sempere et al., 2004). Indeed, the lsy-6 microRNA has been shown to determine left-right chemosensory neuron development in *C. elegans* (Chang et al., 2004; Johnston and Hobert, 2003). Emerging evidence also demonstrates the ability of microRNA to regulate cell survival. Indeed the lack of microRNA production in conditional Dicer knockout mice is associated with increased apoptosis in the cortex during the early postnatal stages and microcephaly, or decreased head circumference (Davis et al., 2008).

1.4.2 MicroRNA in synaptic development and function

In situ hybridization studies and microarray analysis have demonstrated the presence of mature microRNAs within the neuronal synapse, suggesting a function for microRNAs in regulating synaptic development and function (Lugli et al., 2008; Siegel et al., 2009). In fact, a Dicer conditional knockout, which abolishes microRNA production, resulted in reduced dendritic branch elaboration and large increases in dendritic spine length with no concomitant change in spine density (Davis et al., 2008). In addition, the loss of Dicer leads to improper targeting of dendrite and axons in the *Drosophilia* olfactory projections neurons to the proper brain centers (Berdnik et al., 2008). While both of these approaches ablate microRNA production, several specific microRNAs have been implicated in dendritic spine formation (Impey et al., 2009; Schratt et al., 2006; Siegel et al., 2009). For example, miR-132 promotes neurite extension in both hippocampal and cortical neurons (Vo et al., 2005; Wayman et al., 2008). Furthermore miR-132 is necessary for dendritic spine formation in cultured hippocampal neurons (Impey et al., 2009). Contrary to the positive role miR-132 plays in dendritic spine development, miR-134, which is localized to dendrites, was found to decrease the volume of dendritic spines by repressing the translation of lim kinase, a protein that regulates actin filament dynamics (Schratt et al., 2006). Interestingly, stimulation of hippocampal neurons by BDNF leads to the de-repression of lim kinase translation that is mediated by miR-134 (Schratt et al., 2006), suggesting that microRNA-mediated translation repression could be alleviated upon neuronal stimulation. Curiously, emerging evidence indicates that not only are mature, but also precursor microRNAs are found in the synapse, along with Dicer and eIF2c, a component of the RISC complex (Lugli et al.,

2005; Lugli et al., 2008). Interestingly, Dicer localized to postsynaptic densities has no RNAse III enzymatic activity. However, NMDA-dependent Ca^{++} influx leads to the activation of calpain, a Ca^{++} dependent protease, which in turn cleaves Dicer to restore RNAseIII activity (Lugli et al., 2005). Taken together, these data indicate a function for microRNA in proper synapse formation and that neuronal activity could serve to further repress mRNA translation through local production of microRNAs or by alleviating the translation repression allowing for the synapse-specific protein synthesis.

1.5 Goals for Dissertation

The ability to regulate translation of a wide range of mRNAs is vastly important in the development and function of the CNS. Abnormalities in protein expression levels in response to neuronal activity changes resulting from dysfunction in the regulatory mechanisms that maintain mRNA translation can ultimately lead to cognitive deficits as well as a multitude of neurological and psychological diseases. Thus, understanding the mechanisms that control translation in neurons and the functional importance of such regulation in normal neuronal development and under pathological conditions is an important and challenging task, and will provide important insights for the treatment of brain disorders. To this end, my thesis project focuses on translational regulation of BDNF mRNA and the role of FMRP-mediated translation, two well-known molecules that govern normal brain development and function whose abnormalities are the cause of mental and cognitive disorders.

Traditionally, regulation of BDNF expression was thought to be mediated by intricate transcription driven by multiple different promoters. However, transcription

cannot fully account for the rapid, localized expression of BDNF, suggesting a role for post-transcriptional mechanisms to mediate the spatial and temporal regulation. All BDNF transcripts can be grouped into two pools based upon the size of its 3'UTR, either short or long (Timmusk et al., 1995). These distinct 3'UTRs provide an opportunity for unique posttranscriptional regulation of BDNF expression, which has been overlooked. We show here that the presence of cis-acting factors contained specifically within the long 3'UTR of BDNF mRNA promote dendritic localization and repress translation of BDNF mRNA, while the short 3'UTR BDNF mRNA is actively translated to maintain basal BDNF expression under resting conditions. Furthermore, neuronal activity results in the rapid translational derepression of the long 3'UTR BDNF mRNA to sustain BDNF protein levels in response to neuronal stimulation before the increase in transcription. The activity-dependent increase in translation of the long 3'UTR BDNF mRNA coincides with a robust and rapid activation of TrkB in the hippocampal mossy fibers. The activation of TrkB in the hippocampal mossy fiber is necessary for seizure development (He et al., 2004), posing the question of what role the activity-dependent translational derepression of the long 3'UTR BDNF mRNA might play in seizure formation. In mice lacking the long 3'UTR BDNF, seizure development is delayed, suggesting a role for activity-dependent BDNF expression in epileptogenesis. These data provide a novel mechanism by which the synthesis of the same protein is differentially regulated at rest and after neuronal activity.

Secondly, we examined the effects FMRP, a trans-acting repressor of translation, has on the development of the hippocampus. A hallmark morphological change in patients and mice lacking FMRP is an increase in the number of dendritic spines, which

are immature in appearance (Grossman et al., 2006; Irwin et al., 2001). This indicates a postsynaptic role for FMRP in synapse formation. However, the role of axonal localized FMRP (Antar et al., 2006; Christie et al., 2009) in synapse formation remains unknown. Here we show that FMRP represses the translation of MAP1B mRNA, with the loss of FMRP leading to increased MAP1B protein levels in the hippocampal mossy fibers (MF) during neonatal development. Subsequently, the loss of FMRP results in the increased length and abnormal projection of the axons comprising the infrapyramidal bundle, which is temporally associated which the increased MAP1B protein expression in MFs. Furthermore, loss of FMRP leads to increased zinc transporter 3 (ZnT3), a protein that pumps zinc into synaptic vesicles (Palmiter et al., 1996), levels in the axonal terminals of the mossy fibers, suggesting an increase in synaptic vesicle density. These data suggest a role of FMRP in synaptogenesis and neuronal network formation in the hippocampus.

Lastly, we aimed to understand possible trans-acting factors that mediate the translational repression/derepression of the long 3'UTR BDNF mRNA in response to neuronal activity level. FMRP and BDNF have very similar roles in neuronal development and synaptic plasticity, leaving the possibility that FMRP might repress the translation and promote the localization of the long 3'UTR BDNF mRNA. To this end, we show a preferential association of the long 3'UTR BDNF mRNA with FMRP in the mouse brain. Besides RNA-binding proteins, we also explored the possible involvement of microRNA in regulating BDNF translation via the long 3'UTR. We found a particular microRNA, miR-128, that has multiple predicted binding sites in the BDNF long 3'UTR and can specifically repress translation of a luciferase reporter containing the BDNF long 3'UTR. In addition, both physiological and pathological neuronal stimulation leads to a

decrease in the levels of miR-128, suggesting the possibility that this microRNA is involved in the repression/derepression of the long 3'UTR BDNF mRNA. Combined, these data suggest two mechanisms that might regulate the translation of the long 3'UTR BDNF mRNA.

CHAPTER 2: The distinct 3'UTRs differentially regulate subcellular localization and translation of BDNF mRNA in the hippocampus.

2.1 Introduction

Brain-derived neurotrophic factor (BDNF) is known to elicit a plethora of functions in the brain, ranging from neuronal survival and differentiation to circuit development and synaptic plasticity (Huang and Reichardt, 2001; Kuipers and Bramham, 2006; Lu et al., 2008; Segal, 2001; Wirth et al., 2003; Zheng and Quirion, 2004; Zhou et al., 2006). Abnormalities in BDNF function have been implicated in both neurological and psychiatric disorders (Binder et al., 2001; Lu and Martinowich, 2008; Martinowich and Lu, 2008; Martinowich et al., 2007). To accommodate such diverse functions, a variety of mechanisms have evolved that tightly control BDNF expression. Transcription of the BDNF gene can be initiated from nine distinct promoters in mammals, allowing for sophisticated regulation by divergent extracellular and developmental cues (Aid et al., 2007; Liu et al., 2006; Liu et al., 2005b). However, transcriptional regulation cannot completely account for changes in BDNF levels in relation to the diverse functions of BDNF in soma versus dendritic compartments. Moreover, the BDNF transcripts can be processed at two alternative polyadenylation sites in all tissues examined, giving rise to two pools of BDNF mRNAs that harbor either a short or a long 3'UTR of 0.35 kb and 2.85 kb in length respectively (Liu et al., 2006; Liu et al., 2005b). Each BDNF mRNA isoform encodes the same BDNF protein. However, the relative abundance of the short and long 3'UTR BDNF mRNAs differ in various brain regions (An et al., 2008), regardless which promoter is used for transcription. These different 3'UTRs in BDNF messages offer a mechanism to increase the capacity and complexity for regulation of

BDNF expression at posttranscriptional levels, such as translation and subcellular localization, which is beyond the traditional view of transcriptional regulation.

Hundreds of mRNAs are localized and translated in the dendrites of hippocampal and cortical neurons (Eberwine et al., 2001; Poon et al., 2006; Zhong et al., 2006). Several of these mRNA, such as the α subunit of the Ca^{2+}- and calmodulin- dependent protein kinase II (CAMKIIα), the activity regulated cytoskeleton associated protein (Arc) and the microtubule associated protein 2 (MAP2) contain cis-acting elements in their 3'UTRs, which are necessary to target the messages to dendrites for activity-dependent local translation (Blichenberg et al., 2001; Blichenberg et al., 1999; Kobayashi et al., 2005; Mori et al., 2000; Steward and Worley, 2001). Another mRNA localized to dendrites is the BDNF transcript (Righi et al., 2000; Tongiorgi et al., 2004; Tongiorgi et al., 1997). In fact, BDNF mRNA levels in dendrites and the maximal distance of BDNF mRNA from the cell body increase upon chemically-induced neuronal activity (Tongiorgi et al., 1997), revealing a possible mechanism to control the spatial expression of BDNF protein as a means of controlling localized BDNF function. Interestingly, upon neuronal activity the increased temporal or spatial expression levels of BDNF protein cannot be completely explained by increased levels of BDNF mRNA (Katoh-Semba et al., 1999; Nanda and Mack, 2000), which leads to the question of what cis-acting factors can mediate subcellular localization and translation of the BDNF message both at rest and after neuronal stimulation. The production of the two distinct BDNF 3'UTRs, long or short, leads to the hypothesis that the different 3'UTRs can uniquely regulate both the localization and translation of BDNF mRNA to control spatial and temporal expression of BDNF protein in response to neuronal activity.

In this study, we show that the long and short 3'UTRs play opposing roles in regulating the translation and localization of BDNF mRNA in the hippocampus. The long 3'UTR BDNF mRNA preferentially localizes to the dendrites while the short 3'UTR BDNF mRNA is restricted to the soma. Given that neuronal activity-dependent transcription is quite prominent for the BDNF gene, it is difficult to study whether neuronal activity can control BDNF translation. At basal conditions, the short 3'UTR BDNF mRNA is actively translated, serving as the primary source of BDNF production. In contrast, the long 3'UTR BDNF mRNA is translationally suppressed at rest, but undergoes robust translational activation upon neuronal stimulation. Moreover, the long BDNF 3'UTR is essential for the rapid activation of the tropomyosin-related tyrosine kinase B (TrkB) in hippocampal mossy fibers upon seizure induction. We further show in mutant mice lacking the BDNF long 3'UTR ($Bdnf^{klox/klox}$) and activity-induced BDNF translation, seizure development is significantly hampered. These results provide the first evidence for activity-dependent control of BDNF translation in TrkB activation and neuronal function.

2.2 Results

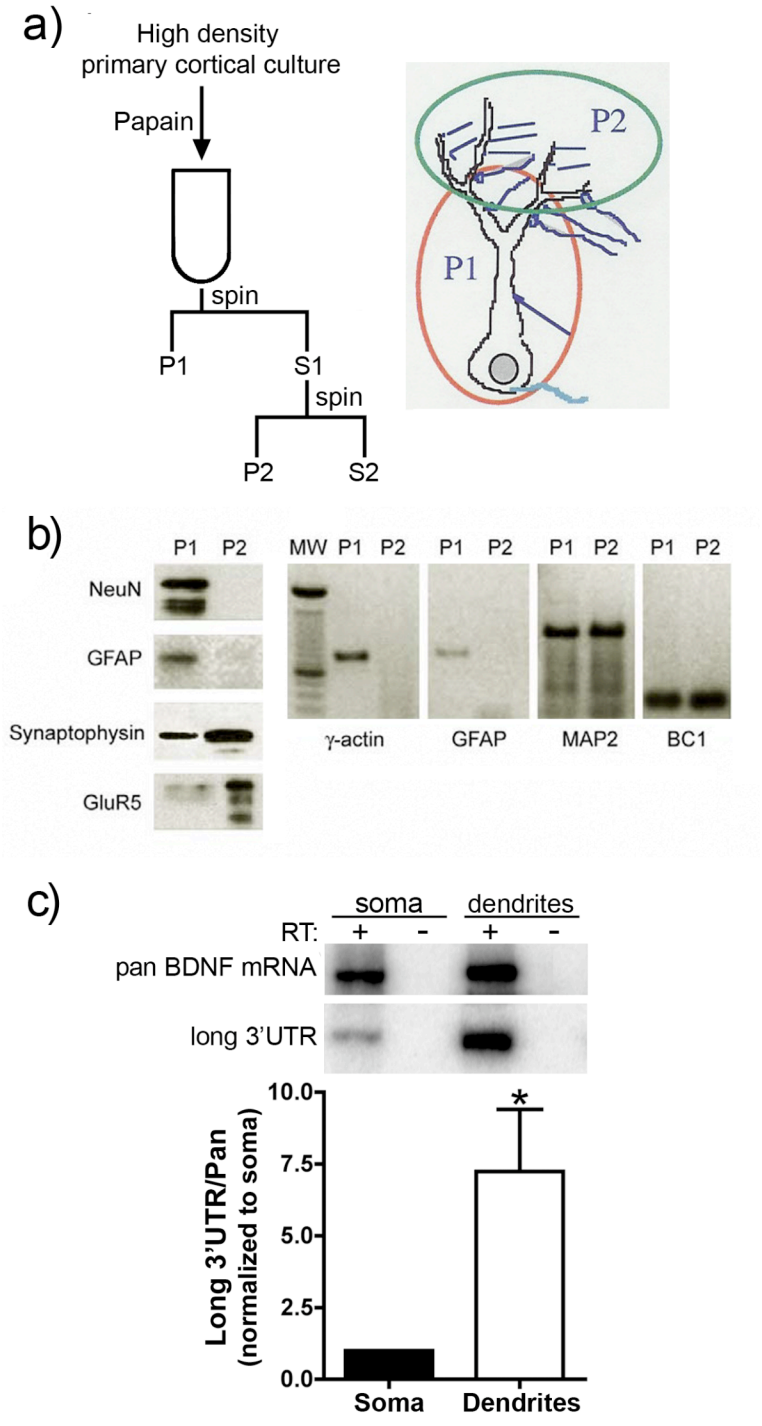
2.2.1 Long 3'UTR BDNF mRNA is preferentially localized to dendrites in primary cultured neurons

To assess the ability of the different BDNF 3'UTRs to control subcellular localization, we obtained total RNA isolated from the soma and neurite fractions, which contain both dendrites and axons, of a high-density culture of primary cortical neurons as

illustrated in Figure 2-1a. However, at this age of culture (DIV21), mRNA from the neurite fraction corresponds to mRNA localized to dendrites because the axons are mature and thus contain no mRNA. The quality of the fractionation was assessed by western blotting for specific protein markers, such as NeuN (soma only), synaptophysin (enriched in neurites), and GluR5 (highly enriched in neurites); and also by RT-PCR for specific mRNAs, i.e. γ-actin and GFAP (soma only), and MAP2 and BC1 RNA (soma and neurites) (Figure 2-1b). From these fractions, the relative levels of pan BDNF mRNA, which is an indication of total BDNF mRNA as measured by coding region primers, and the long 3'UTR BDNF mRNA were simultaneously measured by semi-quantitative, radiolabeled RT-PCR from both fractions (Figure 2-1c). Quantification of the ratio of long 3'UTR BDNF mRNA to pan BDNF mRNA demonstrates a 7-fold enrichment in dendrites compared to that in the soma (Figure 2-1c), suggesting a preferential localization of the long 3'UTR BDNF mRNA in the dendrites of cultured neurons. Further, An and colleagues confirmed our findings that revealed distinct roles of the long and short 3'UTRs in controlling the localization of reporter mRNAs fused to the long or short BDNF 3'UTR and the abundance of BDNF protein in neuronal soma and dendrites (An et al., 2008). In particular, the long 3'UTR is responsible for targeting the BDNF mRNA into neuronal dendrites, thus leading to the regulation of dendritic spine development and long-term potentiation (LTP) (An et al., 2008). Together, these findings raise another intriguing question as to whether the long and short 3'UTRs may differentially regulate BDNF translation in the somatal and dendritic compartments.

Figure 2-1: Preferential dendritic localization of the long 3'UTR BDNF mRNA. a) Schematic of cellular fractionation of high-density primary cortical neuronal cultures. The cultures are subjected to papain and manual disruption of the neuronal network, followed by sucrose fractionation and centrifugation to yield the soma fraction (P1) and the dendritic fraction (P2) (right panel). The left panel is a visual representation of the corresponding fractions. b) Purity of isolation technique was determined by western blot analysis (left) which shows the absence of the nuclear protein NeuN and the glial marker, GFAP but the presence of synaptophysin and GluR5 in the P2 fraction. The right panels show RT-PCR analysis revealing γ-actin and GFAP mRNA only in the soma fration, P1 whereas MAP2 and BC1 mRNA in both the P1 and P2 fractions. c) ^{32}P-semi-quantitative RT-PCR of the long 3'UTR BDNF mRNA and pan BDNF mRNA from total RNA isolated from the individual fractions (top panel), indicated in (a). The "-" RT was used as a negative control. The bottom panel shows the quantification of the ratio of the intensities of the radiolabeled PCR bands of the long 3'UTR BDNF mRNA to the pan BDNF mRNA, which indicates a higher fraction of the long 3'UTR BDNF mRNA in the neurites compared to the soma. (Student's t-test, $P<0.05$, $n=3$).

Figure 2-1

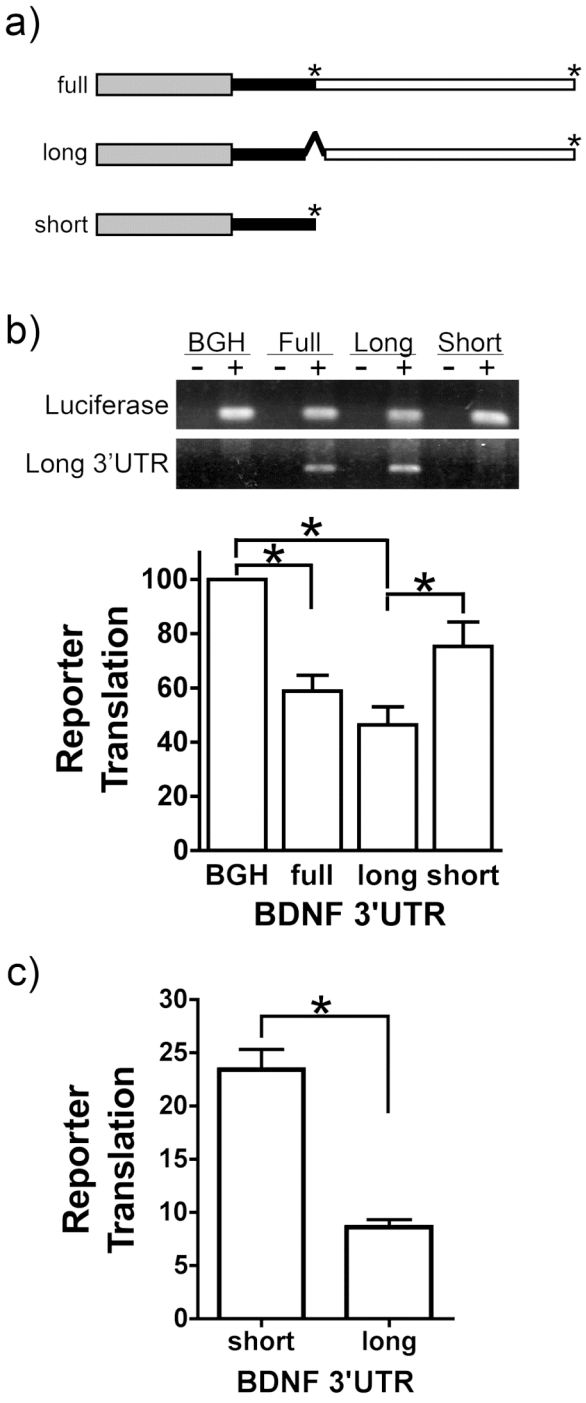

a)
High density
primary cortical culture
Papain
spin
P1
S1
spin
P2
S2
P2
P1
b)
P1 P2
NeuN
GFAP
Synaptophysin
GluR5
MW P1 P2
γ-actin
P1 P2
GFAP
P1 P2
MAP2
P1 P2
BC1
c)
soma
dendrites
RT: + - + -
pan BDNF mRNA
long 3'UTR
Long 3'UTR/Pan
(normalized to soma)
10.0
7.5
5.0
2.5
0.0
*
Soma
Dendrites

2.2.2 The long 3'UTR of BDNF mRNA is a bona fide cis-acting translation repressor at rest

To directly test whether the two distinct 3'UTRs may differentially influence translation, we constructed reporter plasmids in which the firefly luciferase coding region is fused with either the full length BDNF 3'UTR containing both polyadenylation sites (full), the long 3'UTR lacking the proximal polyadenylation site (long), or the short 3'UTR (short) (Figure 2-2a). The parental luciferase construct that carries the bovine growth hormone (BGH) 3'UTR for highly efficient translation was used as a reference in parallel experiments (Figure 2-2a). When transfected into the immortalized cortical neuron cell line CAD, the short BDNF 3'UTR construct displayed a high level of luciferase translation activity, similar to that of the highly active BGH 3'UTR construct (Figure 2-2b). In contrast, translation activity from the long BDNF 3'UTR construct was significantly lower as compared to that from either the short BDNF 3'UTR construct or the parental BGH 3'UTR construct ($p<0.05$, Figure 2-2b). The BDNF full length 3'UTR construct that can be processed at both polyadenylation sites mediated an intermediate level of luciferase translation (Figure 2-2b), further supporting the conclusion that the long 3'UTR mediates poor translation. Moreover, a significantly reduced level of reporter translation was observed from the BDNF long 3'UTR construct as compared to that from the short 3'UTR construct when expressed in primary cultured hippocampal neurons (Figure 2-2c). No differences in reporter mRNA expression level was detected in the long or the short BDNF 3'UTR transfected cells. These results indicate that the BDNF

Figure 2-2: The BDNF long 3'UTR is a bona fide cis-acting translation suppressor. a) Schematic of luciferase reporter constructs harboring full length BDNF 3'UTR, short BDNF 3'UTR, and a deletion mutant that only produces BDNF long 3'UTR. Asterisks indicate polyadenylation sites. b) Reporter constructs in (a) were transfected into CAD cells. RT-PCR shows the expression of the corresponding mRNAs with or without the long 3'UTR as expected (top panel). Expression levels of luciferase mRNAs were determined by qRT-PCR reading normalized to GAPDH mRNA. The luciferase activity was normalized to the qRT-PCR reading of reporter mRNA to evaluate translation of the aforementioned reporters and displayed in the bottom panel. Error bars in all figures indicate s.e.m. A significant reduction of reporter translation was observed from the reporter mRNAs that carry the BDNF long 3'UTR or full 3'UTR as compared to either the bovine growth hormone (BGH) 3'UTR or the short BDNF 3'UTR. ($P<0.05$ by One-way ANOVA analysis, * indicates $p<0.05$ between the indicated groups based on Tukey's analysis, n=4). c) Primary hippocampal neuron cultures (DIV3) were transfected with the firefly luciferase reporter constructs harboring either the short or long BDNF 3'UTR, shown in (a), along with pRL-TK that encodes the renilla luciferase. The firefly luciferase activity was normalized to the renilla luciferase activity in each transfection. Significantly reduced luciferase activity was detected from cells transfected with the BDNF long 3'UTR construct as compared to the BDNF short 3'UTR construct. (* indicates $p<0.05$ by Student's t-test, n=6).

Figure 2-2

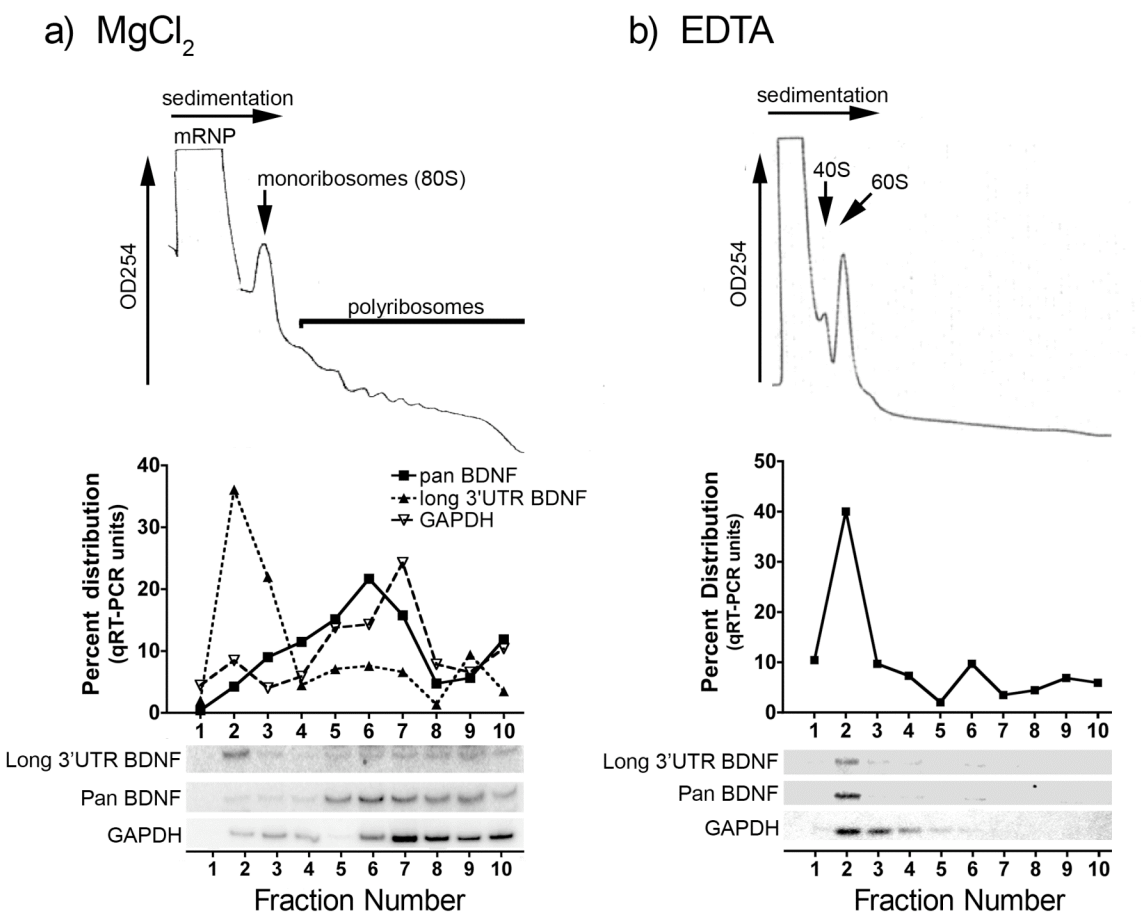

a)
full
long
short
b)
BGH
Full
Long
Short
- + - + - + - +
Luciferase
Long 3'UTR
Reporter Translation
100
80
60
40
20
0
BGH
full
long
short
BDNF 3'UTR
c)
Reporter Translation
30
25
20
15
10
5
0
short
long
BDNF 3'UTR

3'UTRs harbor distinct intrinsic cis-acting properties that differentially control translation in neurons.

Because all BDNF mRNA isoforms encode the same BDNF protein, the influence of the individual 3'UTRs on BDNF translation *in vivo* cannot be assessed by simply measuring changes in BDNF protein expression. Therefore, we performed a linear sucrose gradient fractionation assay, a commonly used approach that assesses translation efficiency of an mRNA by measuring its ability to carry translating polyribosomes (Prendergast, 2003; Zalfa et al., 2006). Sedimentation of ribosome-free messenger ribonucleoprotein (mRNP) complexes (fraction 1-3), the 80S monoribosome (fraction 3) and polyribosomes engaged in translation elongation (fraction 4-10) in mouse hippocampal lysates are monitored by absorption at the wavelength of 254 nm (Figure 2-3a top panel). qRT-PCR analysis of RNA isolated from the individual fractions revealed that approximately half of the endogenous long 3'UTR BDNF mRNA was sequestered into translationally dormant mRNPs in the resting hippocampus, not associated with polyribosomes (predominantly in fraction 2, Figure 2-3a middle panel). It is known that a majority of the BDNF mRNA in the hippocampus carries the short 3'UTR (An et al., 2008). In hippocampal RNA isolated from the cytoplasmic extracts used for fractionation, the long 3'UTR BDNF mRNA comprised less than 20% of the pan BDNF mRNA (2.21 $\pm$ 0.59 femtograms of long 3'UTR BDNF mRNA and 11.65 $\pm$ 3.13 femtograms of pan BDNF mRNA in 1μg total RNA). Interestingly, in contrast to the long 3'UTR BDNF mRNA, the pan BDNF mRNA is predominantly associated with translating polyribosomes (fraction 4-10), similar to the actively translated GAPDH

Figure 2-3: Endogenous long 3'UTR BDNF mRNA is translationally repressed in mouse hippocampus. a) Cytoplasmic extracts from mouse hippocampi were prepared in the presence of $MgCl_2$ for linear sucrose gradient fractionation (15-45%) to separate translating polyribosomes (4-10) from non-translating components including dormant mRNPs, ribosome subunits and monoribosomes (fraction 1-3) monitored by absorption at OD254 (top panel). qRT-PCR was performed to determine the distribution of the pan BDNF mRNA, the long 3'UTR-BDNF mRNA and the house keeping GAPDH mRNA in each gradient fraction (middle panel). In addition, ^{32}P -labeled semi-quantitative RT-PCR products of the aforementioned mRNAs were electrophoresed on polyacrylamide gels and visualized by a phosphorimager (bottom panel). b) Hippocampal lysates were treated with EDTA to release mRNAs from polyribosomes to ribosome-free mRNPs. Note the disappearance of mono- and polyribosomes, and the accumulation of released 40S and 60S ribosome subunits on the linear sucrose gradient (top panel). qRT-PCR and ^{32}P-labeled semi-quantitative RT-PCR products for the pan BDNF mRNA, long 3'UTR mRNA, and GAPDH mRNA were indicated in the mid and bottom panels respectively.

Figure 2-3


a) MgCl2
b) EDTA
sedimentation
mRNP
monoribosomes (80S)
polyribosomes
OD254
40S
60S
Percent distribution (qRT-PCR units)
Percent Distribution (qRT-PCR units)
pan BDNF
long 3'UTR BDNF
GAPDH
Long 3'UTR BDNF
Pan BDNF
GAPDH
Fraction Number

mRNA (Figure 2-3a). Thus, unlike the translationally repressed long 3'UTR BDNF mRNA, the short 3'UTR BDNF mRNA, which comprises a majority of the pan-BDNF mRNA in hippocampus, must be engaged in active translation. EDTA-treatment, which dissociates all ribosomes into subunits, released both the long 3'UTR BDNF mRNA and pan BDNF mRNA into ribosome-free mRNP complexes (fraction 2, Figure 2-3b). The similar sedimentation of BDNF long 3'UTR complexes under physiological conditions and EDTA-treatment indicates that the long 3'UTR BDNF mRNA is indeed sequestered into translationally dormant mRNPs not associated with ribosomes (Figure 2-3a). The identification of the BDNF long 3'UTR mRNA in dormant mRNPs extends the previous observation showing a low abundance of this mRNA in crude polyribosome pellet (Timmusk et al., 1994), suggesting that the long 3'UTR may suppress translation initiation of BDNF. Semi-quantitative RT-PCR allows visualization of these mRNA species in the gradient fractions (Figure 2-3a and 2-3b, bottom panels), confirming the translation status of the aforementioned mRNAs. Together, these data suggest that while the short 3'UTR mediates active translation of BDNF mRNA at rest, the long 3'UTR is a bona fide cis-acting translation suppressor in vitro and in vivo.

2.2.3 Neuronal activity causes translation derepression of the long 3'UTR BDNF mRNA.

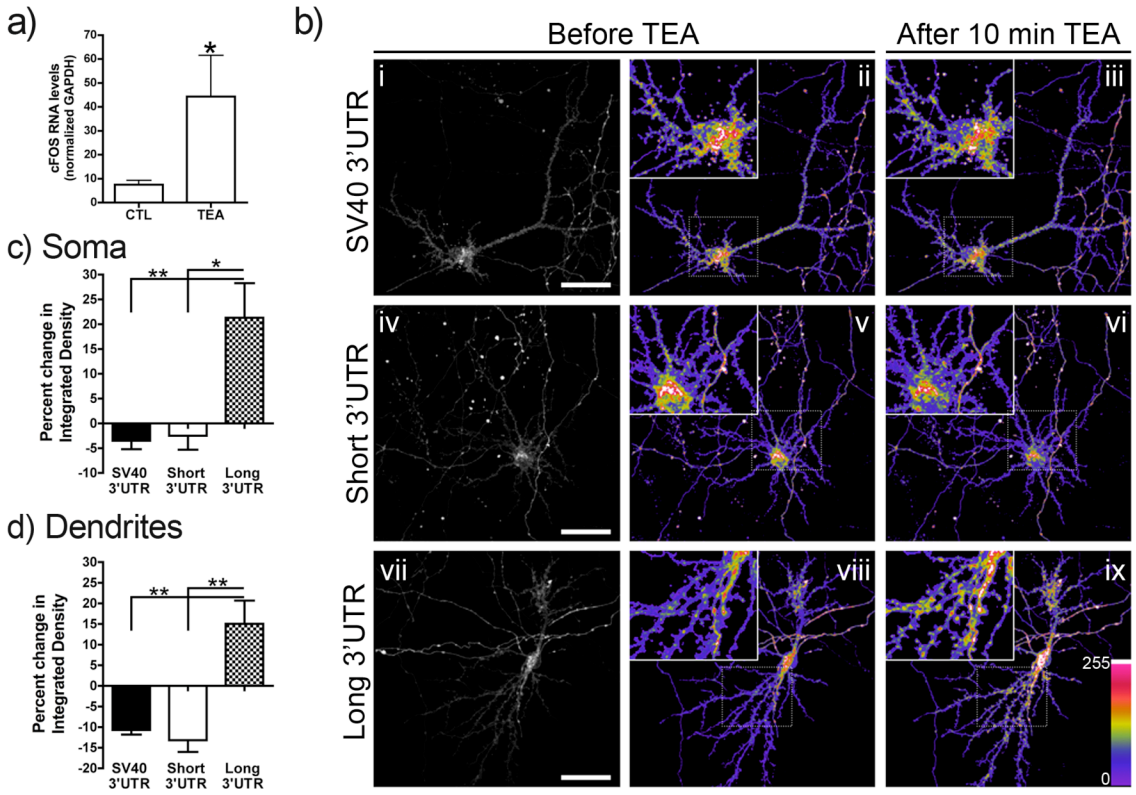
We next questioned how the distinct BDNF 3'UTRs govern reporter translation in response to neuronal activity changes. A reporter construct that encodes destabilized EGFP (d2EGFP) (Aakalu et al., 2001), which harbors an amino-terminal membrane insertion tag for limiting diffusion of newly synthesized d2EGFP from the site of

translation, was fused with the long or short BDNF 3'UTR, or the SV40 3'UTR and were individually transfected into primary cultured hippocampal neurons at 16 days in vitro (DIV 16). The shorter half-life of d2EGFP (2 hr) than that of standard EGFP (26 hr) is suitable for detecting rapid changes in protein synthesis. At DIV21 when the synaptic network was well formed, the culture was exposed to tetraethylammonium (TEA) for ten minutes, a well-established stimulation paradigm broadly used for inducing LTP in the hippocampus (Aniksztejn and Ben-Ari, 1991). Neuronal activation upon TEA-treatment was evidenced by the robust up-regulation of c-fos (Figure 2-4a). Interestingly, live cell laser confocal imaging demonstrated that TEA-treatment produced a rapid increase of d2EGFP expression as compared to that at the basal level before TEA-treatment in a BDNF long 3'UTR-dependent manner (Figure 2-4bvii-ix). In particular, the elevated d2EGFP expression from the BDNF long 3'UTR construct was clearly visualized in dendrites (inset of Figure 2-4bvii-ix), consistent with the long 3'UTR-mediated dendritic localization of BDNF mRNA (An et al., 2008) and potential local translation. Quantitative analysis indicated that TEA causes increased fluorescence intensity of d2EGFP reporter in both the soma and dendrites from cells transfected with the BDNF long 3'UTR construct (Fig 2-4c and d). In contrast, neither the short BDNF 3'UTR nor the SV40 3'UTR could mediate activity-dependent d2EGFP expression (Figure2-4bi-bvi, Fig 2-4c and d).

To delineate whether and how neuronal activation may regulate translation of endogenous BDNF mRNAs *in vivo*, we performed linear sucrose gradient fractionation of hippocampal lysates derived from pilocarpine-treated rats, a paradigm known to evoke

Figure 2-4: The long 3'UTR is sufficient for activity-induced expression of d2EGFP reporter construct. a) Increased cfos mRNA levels in primary cultured hippocampal neurons upon TEA-induced neuronal activity. The cfos mRNA level was determined by qRT-PCR and normalized to the qRT-PCR units for the GAPDH mRNA in each sample (Student's t-test: * indicates $P<0.05$; n=4). b) Laser confocal imaging of cultured hippocampal neurons (DIV21) expressing d2EGFP fused with the SV40 3'UTR (i-iii), the short (iv-vi) or long BDNF 3'UTR (vii-ix) was performed before and after 10 min exposure to 25 mM TEA. Each neuron was imaged by z-sectioning through the entire cell (10-15 optical slices) and projected into a 2D image (max intensity). For clarity, each cell is presented in three panels, with the first panel in grayscales and the other two in pseudocolors encoding fluorescent intensities (see the color bar). First and middle panel: before TEA (i, ii, iv, v, vii, viii); third panel: after 10 min TEA treatment (iii,vi,ix). The regions enclosed by dotted boxes are magnified (2X) and shown as insets. Scale bars = 40 um. c) and d) Quantification of the changes in d2EGFP reporter fluorescence after TEA treatment. The total integrated fluorescent density in the soma and dendrites of the same cell before and after 10 min exposure to TEA was measured. The % change of fluorescence was calculated by normalizing the integrated density after TEA-treatment against that in the same cell before TEA treatment and the results were displayed graphically for the soma (c) and dendrites (d). The decrease of fluorescence for reporters that carry the BDNF short 3'UTR and the SV40 3'UTR reflects photobleaching during confocal imaging. (One-way ANOVA analysis: $p<0.05$ for both soma and dendrites, n=4)

Figure 2-4

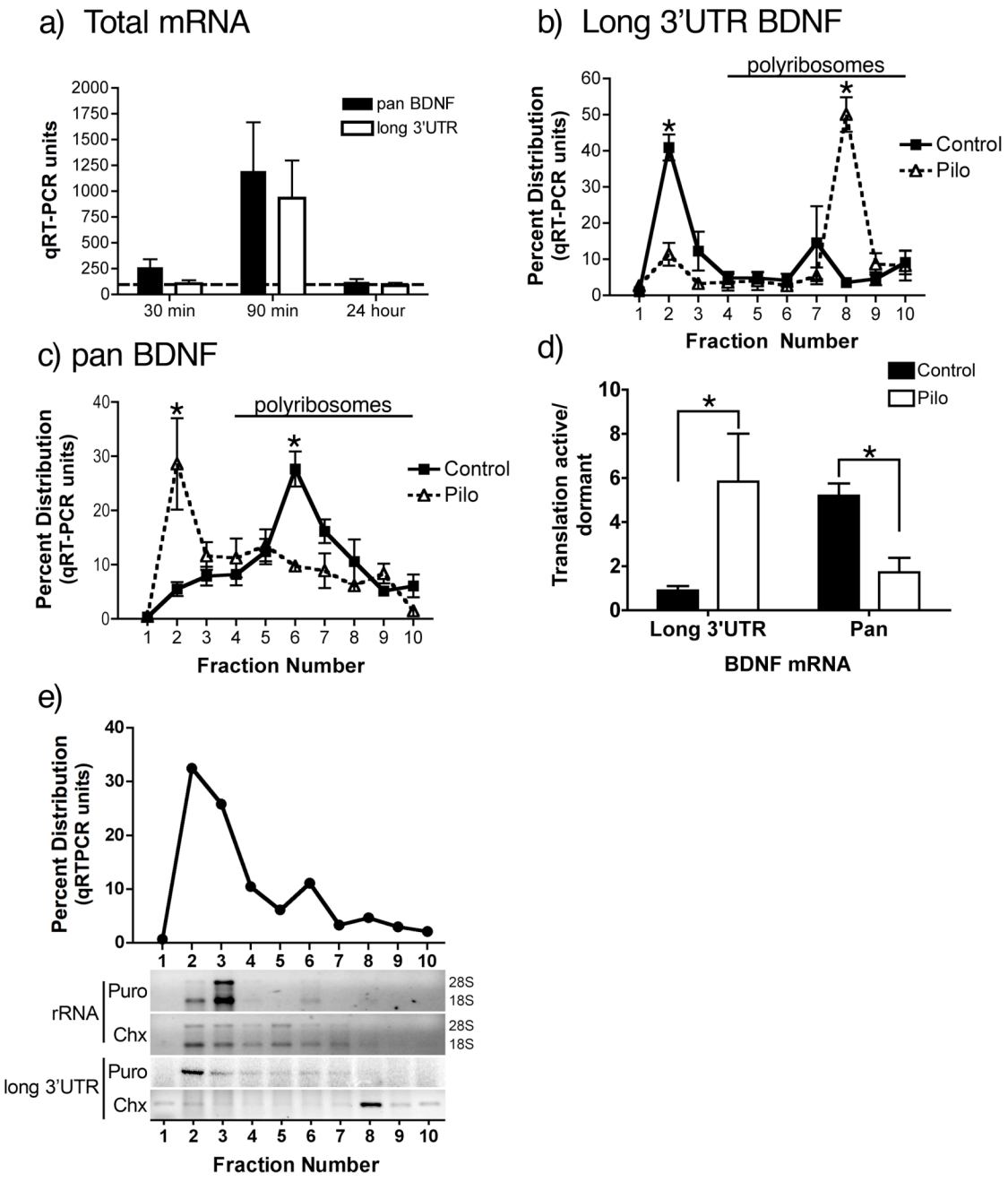

a)
cFOS RNA levels (normalized GAPDH)
CTL
TEA
c) Soma
Percent change in Integrated Density
SV40 3'UTR
Short 3'UTR
Long 3'UTR
d) Dendrites
Percent change in Integrated Density
SV40 3'UTR
Short 3'UTR
Long 3'UTR
b)
Before TEA
After 10 min TEA
SV40 3'UTR
Short 3'UTR
Long 3'UTR
i
ii
iii
iv
v
vi
vii
viii
ix
255
0

rapid and robust BDNF up-regulation (Tongiorgi et al., 2004). A single dose of pilocarpine reliably induces stage V seizure, characterized by rearing, loss of postural control and hind limb clonus (Racine, 1972). This is followed by increased seizure intensity, duration and frequency, designated as status epilepticus (SE). As shown in Figure 2-5a, both the long 3'UTR BDNF mRNA and the pan BDNF mRNA were markedly increased in animals that experienced 90 minutes SE upon pilocarpine-treatment. Consistent with previous reports (Katoh-Semba et al., 1999), BDNF mRNAs returned to basal levels 24 hours later. A slight increase of pan BDNF mRNA was detected 30 minutes after the initial onset of a stage V seizure, whereas no detectable changes were observed of the long 3'UTR BDNF mRNA (Figure 2-5a). However, prior to the drastic increase of the BDNF transcripts, a robust translation activation of the BDNF long 3'UTR mRNA was observed 30 min after the induction of stage V seizure (Figure 2-5b). Almost all the translationally suppressed BDNF long 3'UTR mRNA (fraction 1-3) was shifted to the polyribosomal fractions (fraction 4-10). In contrast, the pan BDNF mRNA, in which the majority harbors the short 3'UTR, was translationally repressed, evidenced by a decrease in polyribosome-association and a concomitant accumulation in the ribosome-free mRNPs (fraction 1-3, Figure 2-5c). The opposing responses mediated by the two 3'UTRs on BDNF translation upon neuronal activation in pilocarpine-induced seizure was further evidenced by the reciprocal changes in the ratio of polyribosome-associated and polyribosome-free complexes harboring the long 3'UTR BDNF mRNA or the pan BDNF mRNA (Figure 2-5d).

To further validate whether the long 3'UTR BDNF mRNA is indeed incorporated into actively translating polyribosomes in response to pilocarpine-induced neuronal

Figure 2-5: Pilocarpine-induced SE results in translational de-repression of BDNF specifically mediated by the long 3'UTR. a) Fold increase of steady state levels of pan BDNF mRNA and long 3'UTR BDNF mRNA in rat hippocampus 30 min, 90 min and 24 hours after pilocarpine-induced SE. BDNF mRNA levels in each sample were measured by qRT-PCR and normalized to that of GAPDH mRNA. Dotted line indicates average BDNF mRNA level in vehicle-treated rats, which was set at 100% for normalization. b-c) Polyribosome association profiles of long 3'UTR BDNF mRNA (b), and pan BDNF mRNA (c) as determined by linear sucrose gradient fractionation followed by qRT-PCR using hippocampal extracts isolated from control (closed squares) or pilocarpine-treated rats (open triangles) 30 min after seizure induction. d) Quantification of the ratio of BDNF mRNAs that harbor either the long or the short 3'UTR in actively translating polyribosomes (fraction 4-10) to that in translationally dormant complexes (fraction 1-3) in control and pilocarpine-treated rats 30 minutes after seizure induction. (Two-way ANOVA analysis: $p<0.05$, n=3,). e) Percent distribution of the long 3'UTR BDNF mRNA, as measured by qRT-PCR, derived from pilocarpine-treated rats on linear sucrose gradient after incubation with puromycin (top). Puromycin treatment results in dissociation of translating polyribosomes into subunits and monoribosomes, as indicated by distribution of 28S and 18S ribosomal RNAs detected by agarose gel electrophoresis after ethidium bromide-staining (middle). ^{32}P-labeled semi-quantitative RT-PCR products showing distribution of the long 3'UTR BDNF mRNA on linear sucrose gradient with and without puromycin-treatment (bottom).

Figure 2-5

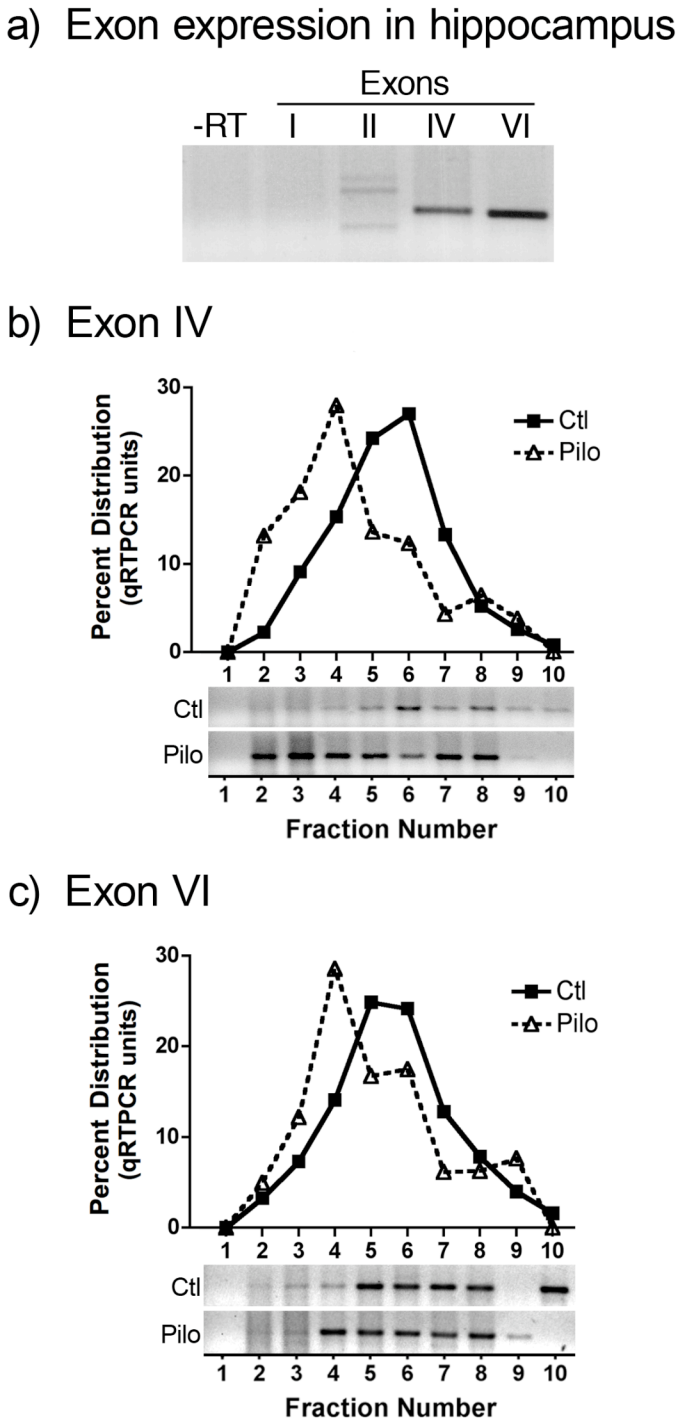

a) Total mRNA
qRT-PCR units
pan BDNF
long 3'UTR
30 min
90 min
24 hour
b) Long 3'UTR BDNF
polyribosomes
Percent Distribution (qRT-PCR units)
Control
Pilo
Fraction Number
c) pan BDNF
polyribosomes
Percent Distribution (qRT-PCR units)
Control
Pilo
Fraction Number
d)
Translation active/ dormant
Control
Pilo
Long 3'UTR
Pan
BDNF mRNA
e)
Percent Distribution (qRTPCR units)
rRNA
Puro
Chx
28S
18S
long 3'UTR
Puro
Chx
Fraction Number

activation, we performed linear sucrose gradient fraction after puromycin-treatment, which terminates translation elongation and specifically dissociates polyribosomes that are engaged in active translation elongation (Stefani et al., 2004; Theodorakis et al., 1988). As expected, puromycin-dependent polyribosome dissociation was evidenced by a shift of ribosomal RNAs from polyribosomal fractions (fraction 4-10) into fractions containing ribosome subunits and monoribosomes (fraction 2 and 3) (Figure 2-5e, middle panels). In response to puromycin-treatment, the BDNF long 3'UTR mRNA was also shifted into ribosome-free mRNPs (fraction 2 and 3, Figure 2-5e top and bottom panels). This result clearly indicates that pilocarpine-induced neuronal activation enhances the engagement of the long 3'UTR BDNF mRNA with actively translating polyribosomes.

Multiple promoters generate BDNF transcripts with distinct 5'UTRs (Aid et al., 2007; Liu et al., 2006), among which exon IV and VI transcripts were the most abundant in adult rat hippocampus (Figure 2-6a). Transcription of exon IV BDNF mRNA and dendritic localization of the exon VI BDNF mRNA are regulated by neuronal activity (Hong et al., 2008; Pattabiraman et al., 2005; Sakata et al., 2009). To examine whether the 5'UTRs encoded by exon IV and VI contribute to translational regulation, we examined polyribosome-association by the exon IV and VI transcripts; each includes the long and the short 3'UTR BDNF mRNA. At rest, these transcripts were predominantly associated with translating polyribosomes; only low levels were present in translationally dormant mRNP fractions (Figure 2-6b and 2-6c). Upon pilocarpine-induced seizure, the association of exon IV and VI transcripts with polyribosomes was decreased, accompanied by a concomitant increase in the mRNP fractions (Figure 2-6b). This was in contrast to the dramatically enhanced polyribosome-association of the long 3'UTR

Figure 2-6: The most prominent 5'UTRs of BDNF mRNA are not responsible for pilocarpine-induced translational de-repression. a) Semi-quantitative RT-PCR of the 5'UTRs in BDNF mRNA indicates that exon IV and VI are the major 5'UTRs expressed in the adult rat hippocampus. b-c) Polyribosomal profiles of exon IV (b), and exon VI (c) determined by linear sucrose gradient fractionation-qRT-PCR assay using hippocampal lysates from control or pilocarpine-treated rats 30 minutes after seizure induction (top). Semi-quantitative RT-PCR products for the exon IV and VI containing BDNF mRNA from sucrose gradients were displayed in the bottom panel.

Figure 2-6


a) Exon expression in hippocampus
Exons
-RT
I
II
IV
VI
b) Exon IV
Percent Distribution (qRTPCR units)
Ctl
Pilo
Fraction Number
c) Exon VI
Percent Distribution (qRTPCR units)
Ctl
Pilo
Fraction Number

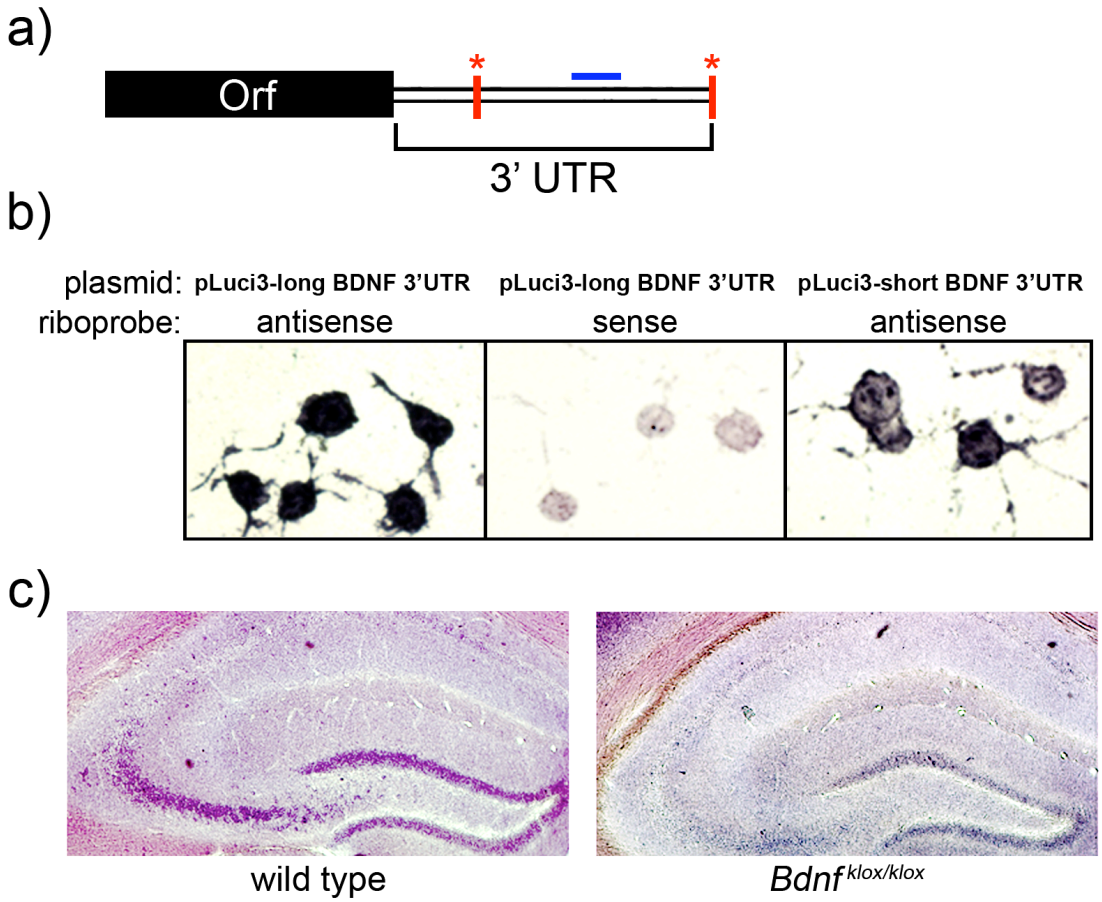
BDNF mRNA upon pilocarpine-induced SE (Figure 2-5b). BDNF mRNAs harboring other 5'UTRs were expressed at low levels and did not yield clear signals on the linear sucrose gradient. Hence, no specific 5'UTRs appear to be responsible for the activity-dependent translational de-repression of BDNF. The decreased polyribosome-association by exon IV and VI transcripts upon pilocarpine-induced seizure recapitulates that from the pan BDNF mRNA that comprises primarily of the short BDNF mRNA, most likely due to the translational suppression mediated by the BDNF short 3'UTR (Figure 2-5c).

2.2.4 The BDNF long 3'UTR is essential for seizure-induced rapid TrkB activation in hippocampal mossy fibers

BDNF is the primary neurotrophin that activates the TrkB receptor in the CNS (Lu, 2003a). Consistent with this, the BDNF protein is enriched in the hippocampal mossy fiber (MF) tract, which are the axons extending from the granular cells of the dentate gyrus and synapse with the dendrites of the pyramidal cells of the CA3 region where BDNF is released in an activity-dependent manner (Danzer and McNamara, 2004). We then examined the regions of the hippocampus that express the long 3'UTR BDNF mRNA to determine possible sites of action for the activity-dependent long 3'UTR-mediated translation de-repression of BDNF mRNA. To this end, we performed in situ hybridization using a BDNF long 3'UTR specific riboprobe (Figure 2-7a). To test the specificity of our BDNF long 3'UTR riboprobe, we performed in situ hybridization on CAD cells transfected with the luciferase reporter constructs fused with the long or short 3'UTRs. As expected the antisense long 3'UTR probe resulted in increased staining of the CAD cells transfected with the long 3'UTR reporter construct and not the short

Figure 2-7: Hippocampal localization of long 3'UTR BDNF mRNA. a) Schematic of long 3'UTR specific riboprobe (blue). The * indicate polyadenylation sites. b) In situ hybridization with the long 3'UTR riboprobe in (a) of CAD cells transfected with luciferase reporter constructs containing the long or short BDNF 3'UTR. The left panel shows robust staining of CAD cells transfected with the long 3'UTR reporter construct using the antisense long 3'UTR riboprobe. The sense long 3'UTR riboprobe, which was used as a negative control, showed no staining in long 3'UTR transfected CAD cells (middle panel). To show specificity, CAD cells transfected with the short 3'UTR reporter construct showed minimal staining, which was due to endogenous long 3'UTR BDNF mRNA present in the CAD cells (right panel). c) In situ hybridization of wild type and $Bdnf^{klox/klox}$ brain slices with a long 3'UTR BDNF mRNA specific riboprobe in (a). Through the alkaline phosphatase staining, as indicated by purple color, the long 3'UTR BDNF mRNA is highly enriched within the dentate gyrus granular cells and CA3 pyramidal cells of the hippocampus.

Figure 2-7


a)
Orf
3' UTR
b)
plasmid: pLuci3-long BDNF 3'UTR pLuci3-long BDNF 3'UTR pLuci3-short BDNF 3'UTR
riboprobe: antisense sense antisense
c)
wild type
Bdnf klox/klox

3'UTR reporter construct, which should detect only the endogenous levels of the long 3'UTR BDNF mRNA in CAD cells (Figure 2-7b, right and left panels). As a negative control, the long 3'UTR sense probe was used resulting in the detection of no signal in CAD cells expressing the long 3'UTR reporter construct (Figure 2-7b, middle panel), demonstrating that our long 3'UTR riboprobe is specific for the long 3'UTR BDNF mRNA. Using the BDNF long 3'UTR specific antisense probe, in situ hybridization showed an enrichment of the long 3'UTR BDNF mRNA in the granular cells of the dentate gyrus and pyramidal cells of the CA3 region of wild type mice, which was ablated in the $Bdnf^{klox/klox}$ mice (Figure 2-7c). This data indicates a possible role of long 3'UTR-mediated translation regulation in activity-dependent signaling between the CA3 pyramidal cells and the dentate gyrus granular cells, which are connected via the hippocampal mossy fiber tract.

Interestingly, in the control rats, minimal TrkB activation, measured by a specific anti-phospho-TrkB antibody pY816 (Bath et al., 2008; Jeanneteau et al., 2008) (Figure 2-8a, top panel), was detected at the MF synapses. Consistent with the previous report (Jeanneteau et al., 2008), pY816 signals were also clearly detected in the subgranular zone of DGCs (Figure 2-8b). Remarkably, when translation de-repression of BDNF was induced from the long 3'UTR mRNA 30 min after pilocarpine-induced seizure (Figure 2-5b), a robust activation of TrkB was observed in the MFs (Figure 2-8a, bottom panel). At this time point, translation from the short 3'UTR BDNF mRNA was largely repressed (Figure 2-5c). Similarly, a robust but transient TrkB activation was detected in the MFs 30 minutes after kindling-induced seizures in wild type mice, which declined to a low

Figure 2-8: Increased p-TrkB staining in pilocarpine treated rats. a) Immunofluorescent staining indicates a robust increase of pY816-TrkB (green) in the hippocampal mossy fibers (MF) in a pilocarpine-treated rat 30 minutes after achieving stage V seizure as compared to that in the control rat. b) Immunofluorescent staining of pY816-TrkB (green) can be clearly detected in the subgranular layer (SGL) of the dentate granule cells (DGCs). For both (a) and (b), DAPI (blue) staining marks nuclei.

Figure 2-8

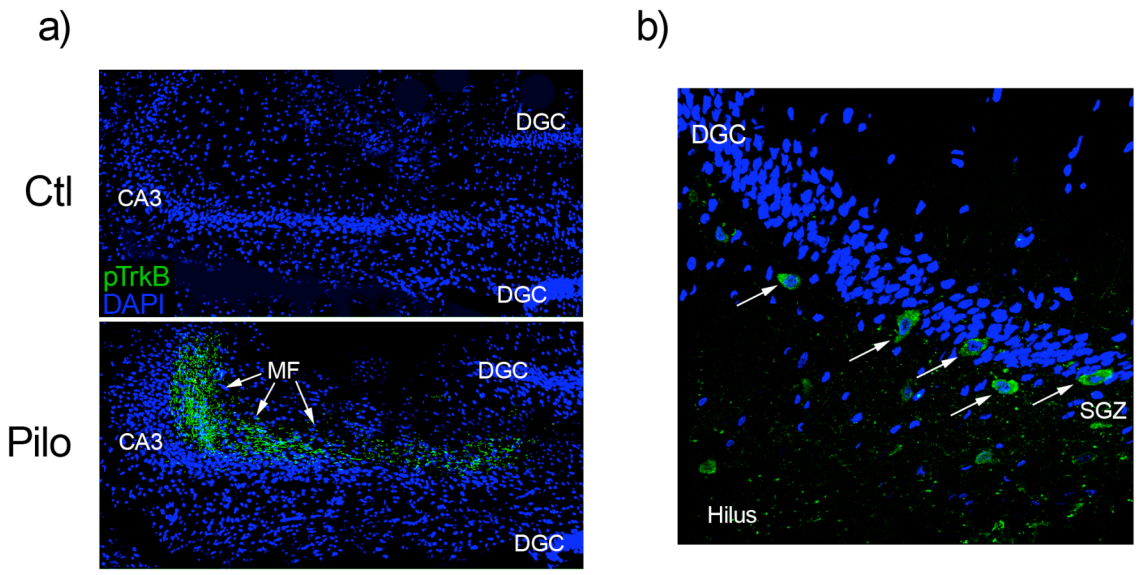
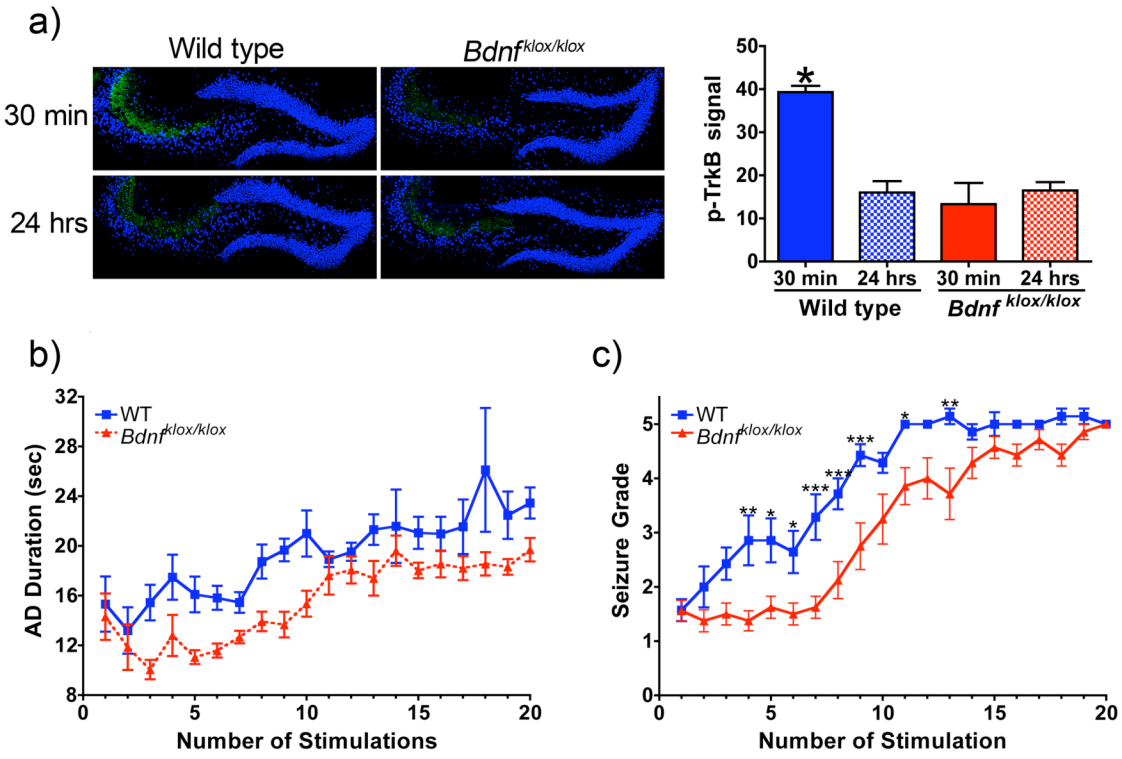


Figure 2-9: Loss of the BDNF long 3'UTR in *Bdnf*$^{klox/klox}$ mutant mice attenuates activity-stimulated TrkB activation and delays kindling development. a) Immunofluorescent staining of pY816-TrkB in wild type and *Bdnf*$^{klox/klox}$ mouse hippocampi. A robust increase in p-TrkB signals was detected in the mossy fiber track of the wild type animal 30 min after the last kindling induction, which is ablated in the *Bdnf*$^{klox/klox}$ mice. Twenty-four hours after the last kindling, pY816-TrkB declines to a low level in wild-type mice, which is comparable to that in the *Bdnf*$^{klox/klox}$ mice. Quantitative analysis of the p-TrkB signaling in the CA3 by Image-J was graphically displayed on the right (n=4). (One-way ANOVA analysis: $p<0.05$.) b) The growth of afterdischarge duration during kindling development is slowed in *Bdnf*$^{klox/klox}$ (red squares) compared to wild type (blue diamonds) mice. c) The growth of seizure severity during kindling development is delayed in *Bdnf*$^{klox/klox}$ mice (red squares) compared to that in wild type mice (blue diamonds). (Two-way ANOVA analysis: * - indicates $p<0.05$, ** - indicates $p<0.01$, and *** - indicates $p<0.001$).

Figure 2-9


a)
Wild type
Bdnf klox/klox
30 min
24 hrs
p-TrkB signal
50
40
30
20
10
0
*
30 min
24 hrs
30 min
24 hrs
Wild type
Bdnf klox/klox
b)
WT
Bdnf klox/klox
AD Duration (sec)
32
28
24
20
16
12
8
0
5
10
15
20
Number of Stimulations
c)
WT
Bdnf klox/klox
Seizure Grade
5
4
3
2
1
0
**
*
*
***
***
***
*
**
0
5
10
15
20
Number of Stimulation

level 24 hours later (Figure 2-9a). In contrast, mutant mice that lost the BDNF long 3'UTR (*Bdnf*$^{klox/klox}$) (An et al., 2008) and thus lack the activity-stimulated BDNF translation, failed to exhibit rapid synaptic TrkB activation in the MFs (Figure 2-9a), despite the successful induction of full scale seizure (Figure 2-9c). However, *Bdnf*$^{klox/klox}$ mice express normal levels of BDNF protein at rest (An et al., 2008), suggesting that the rapid TrkB activation in the MF synapse requires the functional long 3'UTR-mediated, activity-dependent BDNF translation for TrkB signaling during kindling-induced seizures.

Besides the critical role of TrkB signaling in neuronal survival and synaptic plasticity (Huang and Reichardt, 2001), repetitive activation of TrkB in hippocampal MFs is predicted to be a critical underlying mechanism for epileptogenesis (Danzer et al., 2004; He et al., 2004). We then examined whether and how the impairment of activity-dependent BDNF translation by the long 3'UTR may impact kindling-induced epileptogenesis in the *Bdnf*$^{klox/klox}$ mice. It is important to point out that only the rapid TrkB activation was affected in the *Bdnf*$^{klox/klox}$ mutant mice, because the pY816 signal intensity 24 hours after kindling induced seizure was comparable to that in wild-type controls (Figure 2-9a). Nonetheless, the electrical stimulation-enhanced afterdischarge duration (AD) during kindling was reduced in the *Bdnf*$^{klox/klox}$ mutant (Figure 2-9b). Moreover, kindling-induced seizure development was significantly attenuated (Figure 2-9c), resulting in a delay of full scale seizure in the *Bdnf*$^{klox/klox}$ mutant. These results reveal a functional role of neuronal activity-dependent, long 3'UTR-mediated BDNF translation in TrkB signaling. Furthermore, the delayed seizure development upon electrical stimulation in the *Bdnf*$^{klox/klox}$ mutant mice suggests that activity-dependent BDNF

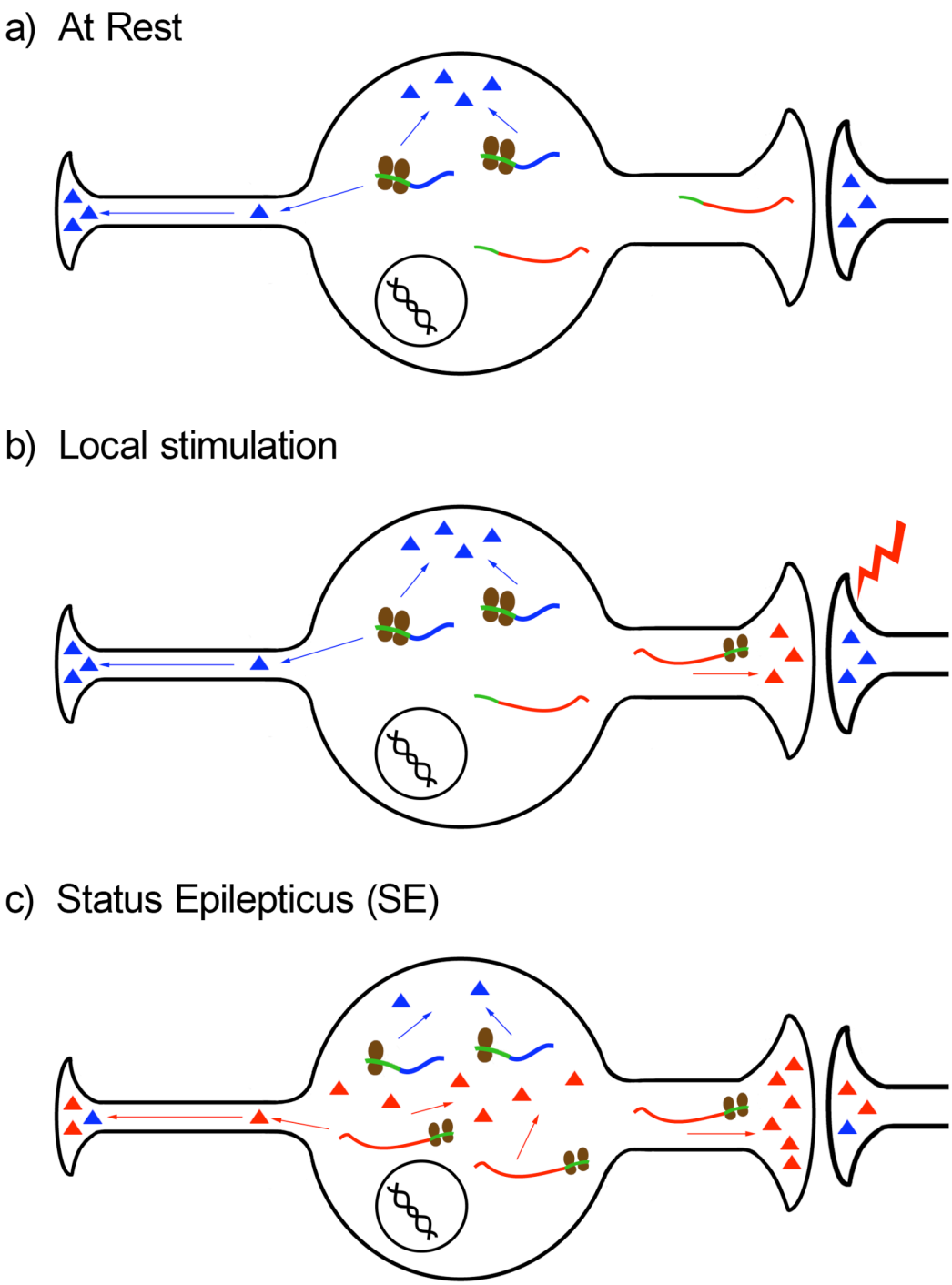
translation immediately upon kindling induction may have an important impact in the pathogenesis of epilepsy.

2.3 Discussion

Our studies indicate that the long and short 3'UTRs harbor distinct abilities to govern BDNF mRNA localization and translation (Figure 2-1 to 2-3). In addition, our results provide the first example that alternative 3'UTRs can differentially control translation of the same protein in response to rapid neuronal activity changes. Further, we show that the long 3'UTR mediates activity-dependent translational de-repression of BDNF in vivo as well as activity-stimulated reporter translation in primary cultured hippocampal neurons (Figure 2-4 and 2-5), a finding that may have important implications in normal synaptic plasticity and pathogenesis of epilepsy. Considering the fact that the short 3'UTR restricts the BDNF mRNA to the neuronal soma whereas the long 3'UTR can target the BDNF mRNA to the dendrites (Figure 2-1) (An et al., 2008), our current results suggest the following model (Figure 2-10). Under physiological conditions (Figure 2-10a and 2-10b), the short 3'UTR mediates active BDNF translation in the soma, which supports constitutive BDNF release and a basal level of TrkB activation. In contrast, a significant fraction of the long 3'UTR BDNF mRNA is translationally repressed as dormant mRNPs, which can be transported to dendrites (Figure 2-1) (An et al., 2008) for local translation of BDNF in response to synapse-specific stimulation under physiological conditions, such as LTP in apical dendrites (An et al., 2008). However, upon robust neuronal activation, the BDNF long 3'UTR imparts a rapid translation de-repression in both soma and dendrites while the short 3'UTR

Figure 2-10: A model for somatodendritic BDNF translation mediated by the two distinct 3'UTRs. a) At resting conditions, the short 3'UTR BDNF mRNA (blue line) is restricted to neuronal soma and actively translated, which is the primary source for BDNF protein production for constitutive release as well as for storage in synaptic vesicles (blue triangles). In contrast, the long 3'UTR containing BDNF mRNA (red lines) is translationally repressed in the soma and is present mainly in translationally dormant mRNP complexes, which can be transported to dendrites. The green region indicates the BDNF coding region. b) Upon synaptic stimulation, the dendritically localized long 3'UTR BDNF mRNA undergoes translational de-repression to produce BDNF protein (red triangles) at the selected postsynaptic sites. Such synapse-restricted activity unlikely affects active translation of the short 3'UTR BDNF mRNA in the soma. c) Seizure-induced robust neuronal activation leads to translational reduction of BDNF from the short 3'UTR in the soma. At the same time, a global translation de-repression occurs on the long 3'UTR BDNF mRNA in both the soma and dendrites, which may be important against seizure-induced insult. However, repetitive and/or prolonged translation de-repression of BDNF from the long 3'UTR may lead to pathological over-activation of neuronal circuitry in the hippocampus, which may contribute to epileptogenesis.

Figure 2-10



BDNF is translationally repressed (Figure 2-10b and 2-10c). Hence, the long 3'UTR mRNA may serve as the primary source of BDNF production upon the onset of seizure. However, repetitive translation de-repression from the BDNF long 3'UTR mRNA in chronic seizure paradigms may lead to aberrant somatodendritic BDNF production/secretion and pathological over-activation of TrkB, represented by that in the MF pathway (Figure 2-9a) that contributes to epileptogenesis (Figure 2-9c). Thus, the long BDNF 3'UTR-mediated translation regulation has dual significance in both physiological and pathological plasticity.

The activity-dependent translational de-repression of BDNF via the long 3'UTR was rapid and robust (Figure 2-4 and 2-5b). Moreover, the BDNF long 3'UTR is sufficient for mediating translation suppression at rest and activity-stimulated translation of reporter genes (Figure 2-2 and 2-4). In pilocarpine-induced seizure, activity-dependent translational regulation of BDNF occurred before a detectable increase of the long 3'UTR mRNA (Figure 2-5a). Although the long 3'UTR BDNF mRNA can be deposited into dendrites, a majority can still be detected in the soma (An et al., 2008). Thus, translation de-repression of BDNF most likely occurs in both soma and dendrites upon robust neuronal activation. Considering that the BDNF mRNA is promptly localized into dendrites upon seizure induction (Chiaruttini et al., 2008; Tongiorgi et al., 2004) and the prolonged up-regulation of BDNF protein after transcription of BDNF declines to basal levels (Mhyre and Applegate, 2003), the translation de-repression mediated by the long 3'UTR in seizure paradigms may be sustained, perhaps in the dendritic compartment of a selected neuronal population that are tagged for long term plasticity.

Besides the long 3'UTR, the 5'UTRs encoding Exon II and VI are also thought to direct dendritic localization of the BDNF mRNAs, whereas Exon I and IV BDNF transcripts are largely retained within the soma (Chiaruttini et al., 2008). All the alternatively expressed 5'UTRs can be linked with either the short or and long BDNF mRNA (Timmusk et al., 1993; Timmusk et al., 1994). Exon IV and VI BDNF mRNAs are the most abundant in the resting adult rat hippocampus (Figure 2-6a), but neither is responsible for the activity evoked translation de-repression of BDNF upon pilocarpine-induced seizure (Figure 2-6b and 2-6c). This is consistent with the observation that the BDNF long 3'UTR is sufficient for mediating synaptic activity-dependent translation of the reporter gene in the TEA stimulation paradigm without BDNF 5'UTRs (Figure 2-4bvii-ix). However, whether and how 5'UTRs may facilitate the long 3'UTR-dependent translation regulation and/or dendritic localization still needs to be elucidated by future studies.

The fact that the long 3'UTR mediates dendritic localization of BDNF mRNA (Figure 2-1) (An et al., 2008), translation suppression of BDNF protein at rest, and activity-dependent BDNF translation (Figure 2-3 to 2-5) suggests that these posttranscriptional regulations may be tightly linked. Interestingly, a number of mRNAs are transported to dendrites in the form of translationally repressed granules, represented by the β-actin mRNA and the CamKIIα mRNA (Huang et al., 2002b; Huttelmaier et al., 2005; Kiebler and Bassell, 2006). It is believed that these translationally dormant mRNAs will be activated locally upon synaptic stimuli (Bassell and Kelic, 2004). Moreover, selective RNA-binding proteins that are known to suppress translation can facilitate dendritic localization of their target mRNAs, represented by the zip code-

binding protein (ZBP1) (Huttelmaier et al., 2005) and the cytoplasmic polyadenylation element (CPE)-binding protein (CPEB) (Huang et al., 2002b; Wu et al., 1998). Whether translation suppression of BDNF in the soma by the long 3'UTR may facilitate dendritic localization of this BDNF mRNA is an intriguing possibility to be explored. In addition, whether the same or distinct elements in the long 3'UTR are responsible for dendritic localization and translation regulation, and what trans-acting factors may coordinate dendritic transport and translation regulation of the BDNF mRNA, are the next challenges for future studies.

Sophisticated mechanisms that regulate BDNF expression eventually converge on TrkB activation, which is the key for governing normal synaptic plasticity and neurogenesis (Huang and Reichardt, 2001). Deficiency of BDNF and TrkB signaling is implicated in a number of learning/memory disorders and cognitive diseases (Buckley et al., 2007; Lu and Martinowich, 2008; Pillai, 2008). However, pathological TrkB over-activation, particularly in the hippocampal MFs, may trigger the development of epilepsy in the kindling model (He et al., 2004). Indeed, selective elimination of TrkB specifically from dentate gyrus and CA1 in hippocampus completely abolished kindling-induced seizure (He et al., 2004). In addition, conditionally removing the BDNF gene from the aforementioned hippocampal regions (conditional $BDNF^{-/-}$) causes a moderate delay of kindling development, despite the potential functional complementation by increased NT3 expression (He et al., 2004).

Unlike the conditional $BDNF^{-/-}$ mutant, mice lacking the long 3'UTR in the $BDNF^{klox/klox}$ mutant produce similar levels of total BDNF protein in the hippocampus as compared to that in wild type controls (An et al., 2008). However, these mutants fail to

achieve the rapid TrkB activation upon kindling-induced full-scale seizure (Figure 2-9a). It is known that TrkB activation can be achieved via BDNF-independent mechanisms, such as zinc-mediated transactivation of TrkB (Huang et al., 2008). However, zinc transporter 3 (ZnT3), the driving forcing for zinc deposition into presynaptic vesicles (Cole et al., 1999; Palmiter et al., 1996), is expressed at normal levels in the MFs of $BDNF^{klox/klox}$ mutant (data not shown). Thus, the defect in TrkB activation in MF synapses of the $BDNF^{klox/klox}$ mutant (Figure 2-9a) is unlikely due to deficiency of presynaptic zinc. Rather, TrkB activation in MFs most likely requires immediate and perhaps also sustained BDNF translation upon seizure induction, which cannot be replaced by BDNF-independent mechanisms. In fact, the moderate delay of kindling development in the $BDNF^{klox/klox}$ mutant is similar to the phenotype in the mutant that lost the entire BDNF gene in dentate gyrus and CA3 (He et al., 2004), suggesting the functional importance of activity-dependent regulation of BDNF via the long 3' UTR and the immediate TrkB activation in epilepsy development. Whether TrkB activation is triggered by BDNF translated in the presynaptic dentate gyrus granular cells or the postsynaptic CA3 neurons, or both, still remain elusive. The loss of long 3'UTR-mediated regulation of BDNF may also cause defects in neural network formation and perhaps aberrant storage/release of BDNF upon activation, which could also contribute to the impairment of TrkB signaling. Nonetheless, the delayed kindling development in the $BDNF^{klox/klox}$ mutant (Figure 2-9c) suggests that the long 3'UTR BDNF transcript is a key factor contributing to the pathogenesis of epilepsy. Taken together, translational regulation mediated by the distinct BDNF 3'UTRs provides a novel means for precise control of BDNF expression in response to neuronal activity changes. Such a regulatory

mechanism could be employed by the increasing numbers of neuronal mRNAs identified that harbor alternative 3'UTRs, including the GluR2 mRNA (Irier et al., 2009), which can govern somatodendritic translation of the same encoded protein in order to accommodate sophisticated neuronal function in response to various extracellular stimuli.

2.4 Materials and Methods

Constructs

The luciferase coding sequence from the pGL3-basic vector (Promega) was inserted into the pcDNA3 vector (Invitrogen), designated pLuci3. The short 3'UTR including the proximal BDNF polyadenylation site (750bp-1113bp) was generated by PCR amplification from mouse genomic DNA and inserted between the Xho and Xba sites downstream of the luciferase coding region in pLuci3. The 2.9 kb full length 3'UTR was subcloned into pLuci3 between the same restriction sites. The long 3'UTR lacking the proximal polyadenylation site was generated by PCR amplification of two fragments, 750bp-1013bp and 1147bp to 3604bp, from mouse genomic DNA and sequentially inserted into pLuci3. Fragments containing the aforementioned 3'UTRs were subcloned into the d2EGFP vector individually (kindly proved by Dr. Bassell) (Aakalu et al., 2001).

Cells, transfection, and luciferase assay

CAD cells were maintained in DMEM/F12 (Cellgro) containing 10% FBS (Atlanta Biologicals) as previously described (Wang et al., 2008). The cells were cultured in 24 well dishes and then transfected with 200 ng of each of the aforementioned firefly luciferase reporter constructs and with 100ng of pTK-Renilla plasmid (Promega) to ensure comparable transfection efficiency using Lipofectamine 2000 (Invitrogen).

Twenty-four hours after transfection, the cells were harvested per the manufacturers protocol. Briefly, the cells were washed once with PBS and then incubated with 100μL of 1X passive lysis buffer (Promega) at room temperature for 15 min on a rotating shaker. Then 10μL of cell lysates were subjected to dual luciferase assay per manufacturer's protocol (Promega). The 10μL of cell lysate was aliquoted in duplicate into a white 96-well flat bottom microtiter plate. To read luminescence, 50μL of Luciferase Assay Reagent II (LARII, Promega) was injected into well followed by 5s integration time then firefly luciferase activity was measured in a Mediators phL luminometer. Immediately after firefly reading, 50μL of Stop&Glo reagent, which quenches firefly activity and measures renilla luciferase activity, (Promega) was injected followed by 5s integration time and then renilla activity was quantified by the Mediators phL luminometer. An aliquot of the cells was used to prepare total RNA with TRIzol (Invitrogen). After RNA isolation but before qRT-PCR, the total RNA was subjected to DNAse (Promega) treatment for 30 min at 37°C, to remove any genomic or plasmid DNA, followed by phenol chloroform extraction and ethanol precipitation. Translation activity from each construct was estimated by normalizing the firefly luciferase readings to firefly luciferase mRNA levels determined by qRT-PCR.

Primary hippocampal neurons were isolated from E18 Sprague Dawley rats, as previously described (Banker and Cowan, 1977) and plated on poly-L-lysine coated 24-well dishes. The cells were co-transfected at DIV3 using 4μL of Lipofectamine 2000 (Invitrogen) with 700ng of firefly luciferase reporter constructs along with 300ng of renilla luciferase plasmid, pRL-TK (Promega), to control for transfection efficiency. Cell lysates were harvested 24 hours after transfection as described above. The cell lysates

were subjected to dual luciferase assay (Promega) in a 20/20n luminometer (Turner Biosystems) as above except the samples were tested in microcentrifuge tubes instead of 96-well plate, all volumes and times remained the same. The firefly luciferase activity was normalized to that of the renilla luciferase and statistical analysis was performed as described in the corresponding figure legends.

Dendrite fractionation assay

Three week-old high-density cultures of rat cortical neurons were prepared as previous described (Torre and Steward, 1992) and treated with papain (0.1 mg/mL) for 5 minutes at room temperature. The cultures were rinsed 3 times with PBS and covered with 3 ml/dish of a buffer containing 10 mM Tris-HCl (pH 7.4), 0.5 mM EGTA and 0.35 M sucrose, and passed through a wide bore pipette to break down the network. The fractions were pooled and mixed 1:1 with a 0.8 M sucrose solution prepared in the same buffer and centrifuged at 1,500 x g for 10 min. The pellet (P1) contains cell bodies and probably large dendritic fragments. The supernatant was recovered and centrifuged at 28,000 x g for 10 min to produce a pellet (P2), which was free of somatic contamination and enriched in pre- and post-synaptic structures. The total RNA was then isolated for the individual fractions, soma and dendrites, by Trizol (Invitrogen) per manufacturer's protocol. Then approximately 5% and 100% of total RNA isolated from the soma fraction and the dendrite fraction, respectively, were reverse transcribed followed by semi-quantitative PCR using ^{32}P-labeled primers specific for the long 3'UTR or the coding region, described below. The ^{32}P radioactivity was determined based on phosphorimager reading.

Pilocarpine induced seizure

Adult male Sprague Dawley rats (Charles River Labs, Wilmington, MA), 40-50 days of age and 200-250 gram body weight, were used in all experiments. Animals were treated according to NIH regulations under the approval of the Emory University IACUC. Status epilepticus (SE) was induced in these rats as previously described (Huang et al., 2002a). Briefly, rats were injected with a mixture of methylscopolamine and terbutaline (2.5 mg/kg i.p.). After 20 min, rats were injected with pilocarpine HCl (380-400 mg/kg s.c.) or an equivalent volume of saline. Pilocarpine reliably induced stage V seizures, characterized by distinct motor behaviors including forelimb clonus, loss of postural control, rearing and falling. Animals presenting these behaviors with increased seizure intensity, duration and frequency 20- 40 min after the injection of pilocarpine were declared to be in SE. The rats were anesthetized with isoflurane before having head removed by guillotine. The hippocampi were then dissected from control or pilocarpine-treated rats 30 minutes after the initial on set of stage V seizure and processed for sucrose gradient analysis as described below. Additional rats were processed 90 min or 24 hr after achieving SE.

Linear sucrose gradient fractionation

Hippocampal tissues were homogenized and lysed in gradient buffer (20 mM Tris pH7.5, 100mM KCl, 5mM $MgCl_2$) containing 100 μg/ml cycloheximide to arrest polyribosome migration and then lysed with 1% Triton X-100. Cytoplasmic extracts were loaded on 15–45% (wt/vol) sucrose gradient, centrifuged at 39,000 rpm in a SW41 rotor for 60 min at 4°C and fractionated as previously described(Feng et al., 1997a). To dissociate polyribosomes, EDTA-treated lysate was centrifuged on a parallel gradient lacking $MgCl_2$ but containing 1 mM EDTA. For puromycin treatment, the hippocampi

were briefly homogenized in gradient buffer lacking cycloheximide but containing 2mM puromycin (Sigma) and incubated for 30min at room temperature followed by incubation on ice for 2 hours. The hippocampal suspension was then lysed by the addition of 1% Triton X-100 and processed as described above. After linear sucrose gradient fractionation, total RNA was extracted from each fraction by phenol-chloroform extraction then divided into 3 equal fractions to which 1/10 of the volume of 3M sodium acetate and 1ml of 100% ethanol was added and incubated at -80°C overnight. The samples were centrifuged at 14000rpm for 30 minutes, and then washed with 75% ethanol. The resulting RNA pellets were air-dried and resuspended in 10μL of RNAse-free dH_2O. cDNA was then made from the entire 10μL of RNA using the superscript reverse transcriptase II kit (Invitrogen) before performing either real time or semi-quantitative RT-PCR described below.

Tetraethylammonium (TEA)-evoked neuronal activation.

Primary hippocampal neurons from E18 Sprague Dawley rats cultured on coverslips (Banker and Cowan, 1977) were transfected using CalPhos Mammalian Transfection Kit (Clontech) with d2EGFP reporter constructs fused with either the SV40 3'UTR, the BDNF short 3'UTR, or the BDNF long 3'UTR on DIV 16 and imaged on DIV 21. Before imaging, neurons were changed from culture medium to HEPES buffered recording solution (HBS) (140mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1.5mM $MgCl_2$, 10mM glucose, 25mM HEPES, pH 7.4). Coverslips with cells were mounted onto a heating chamber and warmed to 37°C during imaging. Images were acquired using a Nikon C1 laser scanning confocal system on a Nikon inverted microscope. The first image was taken while neurons were incubated in HBS before being changed to chemical long term

potentiation (cLTP) medium (140mM NaCl, 5mM KCl, 5mM $CaCl_2$, 0.1mM $MgCl_2$, 10mM glucose, 25mM HEPES, pH 7.4 and 25mM TEA); a second image was then taken 10 minutes after incubation with cLTP medium. To reliably detect any changes in fluorescence, each cell was imaged before and after cLTP with the exact same parameters (laser power, pinhole size, gain, contrast, and section number).

Kindling

A twisted bipolar stainless steel stimulation/recording electrode was implanted in the right olfactory bulb using the following coordinates with the confluens sinuum as reference: 1.0 mm anterior, 1.0 mm right, and 1.2 mm ventral from dura. The surgery was performed under avertin (tribromoethanol 1.9%; 0.25 ml/g BW; i.p.) anesthesia. After a 7 day recovery period the kindling procedure was started with determination of the after discharge threshold (ADT), i.e. the lowest stimulation intensity at which an electrographic seizure > 5 sec can be elicited. The biphasic constant current stimulation consisted of a 1s train of 1ms single pulses applied at 60 Hz. The starting current for the ADT determination was 20 mA and subsequent increases were done in 20 mA steps. No significant difference in ADT was detected in the wt and the *BNDF* $^{klox/klox}$ mutant groups. Thereafter, two daily stimulations at least 7 hrs apart, were given at the ADT current intensity. Evaluation of the afterdischarge durations (AD) in response to stimulation was performed offline and blinded for the genotype. In addition, EEG and behavioral seizures were observed and recorded. Behavioral seizures were scored according to a modified seizure classification scale (Racine et al., 1972) as follows: Stage 1: arrest and facial clonus; stage 2: head nodding; stage 3: unilateral forelimb clonus; stage 4: rearing and bilateral fore limb clonus; stage 5: rearing, loss of postural control, fore and hind limb

clonus; stage 6: running and/or bouncing. Animals were stimulated until reaching a fully kindled stage (criterion: 10 stage 5 seizures). The mice were sacrificed for further processing in immunohistochemistry at either 30 min or 24 hrs after the last seizure, with the genotype in each animal blinded until all the images were quantified.

Immunocytochemistry

pTrkB staining: Brain slices were blocked in PBS containing 0.01% Triton X-100, 2% BSA and 1mM sodium orthovanadate (Sigma) for 1 hour before being incubated overnight at 4˚C with p-TrkB antibody (1:200) in blocking solution. After washing 3X10 minutes in PBS containing 1mM sodium orthovanadate, the slices were incubated with anti-rabbit-FITC (1:1000 from Jackson Immunoresearch) in PBS containing 1mM sodium orthovanadate at room temperature for 1 hour. After washing 3X10 minutes with PBS containing 1mM sodium orthovanadate, the brain slices were mounted with a DAPI-containing mounting solution. Fluorescent signals were detected using the Zeiss (Oberkochen, Germany) LSM 510 confocal microscopic imaging system. The fluorescent intensity was measured using ImageJ (NIH).

In situ hybridization

The probe for in situ hybridization was constructed by isolating a 350 bp fragment of the long 3'UTR BDNF mRNA using mouse hippocampal cDNA as a template for PCR using BDNF long specific primers described below. The resulting fragment was cloned into pDrive (Qiagen) and sequenced for orientation and fidelity of probe. The vector containing the fragment was linearized by XhoI for T7 transcription (antisense strand – sense probe) or by PstI for SP6 promoter (sense strand – antisense probe). The

linearized plasmid was then in vitro transcribed with digoxigenin labeled rUTP using T7 or SP6 polymerases (Stratagene) following manufacturer's protocol.

In situ hybridization was performed on 30 μm thick brain slices from both wild type and *Bdnf*$^{klox/klox}$ mice as previously described (Tongiorgi et al., 1998) with some minor modifications. Slices were incubated in 4% paraformaldehyde (PFA) at room temperature for 3 hours. Then the slices were washed 2X5 min in PBST (1XPBS + 0.1% Tween-20) and then quickly with deionized H_2O (dH_2O), before permeablization with 2.3% Na meta-periodate (Sigma) in dH_2O for 5 min at room temperature. Followed by a quick wash with dH_2O, then incubated with 1% NaBH4 (Sigma) in 0.1M Tris pH 7.5 for 10 min at room temperature. After 2X3 min washes with PBST, the slices were treated with 8μg/mL of proteinase K in PBST for 15 min at room temperature and then washed 2X5 min in PBST. The slices were fixed in 4% PFA for 5 min at room temperature and then washed 3X10 min with PBST. After these steps, the slices were prehybridized by incubating at 55°C in hybridization buffer (20mM Tris pH 7.5, 1mM EDTA, 1X Denhardt's solution, 300mM NaCl, 0.5mg/mg salmon sperm DNA, 0.5 mg/ml yeast tRNA, and 50% formamide) for 1 hour. The brain slices were then hybridized overnight at 55°C in hybridization buffer containing 10% dextran sulfate and 100 ng/mL of the full length sense or antisense digoxigenin labeled riboprobes, described above. The next day the slices were washed as follows: 1) 2X 30 min in 2X SSCT (2XSSC +0.1% Tween-20) containing 50% formamide at 55°C. 2) 1X 20 min in 2X SSCT at 55°C. 3) 1X 30 min in 0.2X SSCT at 60°C. 4) 1X 30 min in 0.1X SSCT at 60°C. 5) 3X 5 min in TBS (50mM Tris pH 7.5 and 150mM NaCl). After these washes, the slices were blocked in 1X blocking solution (Roche), diluted in TBS, for 2.5 hours at room temperature. The slices

were incubated overnight at 4°C with anti-digoxigenin-IgG (Roche, 1:500) in blocking solution. The next day, slices were washed 5X 10 min with TBST (TBS with 0.1% Tween-20) at room temperature followed by incubation with mouse IgG coupled to alkaline phosphatase (Jackson, 1:2000). The slices were then washed 4X 10 min in TBST and 1X 10 min in TBST containing 1mM levamisole (Sigma), to block endogenous alkaline phosphatase activity. The signal was detected by incubating the slices with one step NBT/BCIP (4-nitro blue tetrazolium/5-brom-4-chloro-3-indolyl-phosphate) (Promega), until a purple color was observed. After washing the slices 3X 5min in TE buffer (10mM Tris pH 7.5, 1mM EDTA) to stop the reaction, they were mounted onto slides using AquaPolymont (Polysciences, Inc.) and covered with a glass cover slip. Images of the stained hippocampus were taken with Retica digital camera attached to light microscope.

RT-PCR and real-time RT-PCR

Total RNA extracted by Trizol was quantified by OD260 reading and quality was confirmed by ethidium bromide-stained agarose gel electrophoresis prior to RT-PCR. Reverse transcription was performed using 2μg of total RNA with random primers (Promega) and Superscript II RNaseH-reverse transcriptase (Invitrogen), followed by PCR analysis. The following primers were used to detect pan BDNF mRNA: 5'-tggctgacactttgagcac-3' (forward), 5'-ccagccaattctctttttgc-3' (reverse); BDNF long 3'UTR mRNA: 5'-tggcctaacagtgtttgcag-3' (forward), 5'-ggatttgagtgtggttctcc-3' (reverse). Previously published primers were used to detect BDNF mRNAs that carry various 5' exons (Aid et al., 2007) and GAPDH mRNA was detected as described previously (Zhao et al., 2006).

Semi-quantitative PCR was performed by ^{32}P-radiolabeling of the forward primer by T4 Polynucleotide kinase (NEB). Briefly, 5 pmoles of the forward primer was incubated with ^{32}P-γATP and 10 units of T4 Polynucleotide Kinase for 20 min at 37°C. The reaction was stopped by heat-inactivation for 10 min at 65°C. PCR was performed by adding 0.5μL of ^{32}P-labeled primer to the standard reaction mixture. Normal PCR conditions were used with 20 cycles of amplification. Then 10μL of reaction was run on a 12% polyacrylamide gel, which was then exposed to phosphoscreen. The radiolabeled signal was then detected by Typhoon phosphoimager and signal quantified by using the Qimager program.

Real time PCR was performed using iCYCLER (BioRad) and SYBR-green master mix (NEB), per manufacturer's protocol. Basically, 5μL of template was mixed with 5μL of 2.5μM mixed primer set then 10μL of 2X reaction buffer was added. Standard curves were established for all primers using reverse transcribed cDNA from serial dilutions of a 50 ng/μL mouse hippocampal total RNA. The following primer pairs were used for real-time detection: pan BDNF: 5'-gccgcaaacatgtctatgagggtt-3' (forward), 5'-ttggcctttggataccgggacttt-3' (reverse); long 3'UTR BDNF: 5'-caggaggaatttctgagtggcca-3' (forward), 5'-gcagaaggcctaagcaacttgaca-3' (reverse); GAPDH: 5'-cacagtcaaggctgagaatgggaa-3' (forward), 5'-gtggttcacacccatcacaaacatg-3' (reverse); firefly luciferase: 5'-aagattcaaagtgcgctgctggtg-3' (forward), 5'-ccgcttccccgacttccttagag-3' (reverse), and cfos: 5'-agaaggggcaaagtagagcag-3' (forward), 5'-cgcagacttctcgtcttcaagt-3' (reverse).

Chapter 3: Deficiency of the fragile X mental retardation protein results in dysregulated MAP1B translation and aberrant mossy fiber projection during hippocampal development

3.1 Introduction

Fragile X Syndrome (FXS), the most common inherited form of mental retardation, results from the loss of the fragile X mental retardation protein (FMRP) (Bardoni and Mandel, 2002; Jin and Warren, 2003; O'Donnell and Warren, 2002). FMRP is a selective RNA-binding protein highly expressed in brain neurons (Devys et al., 1993), transported with its mRNA ligands into dendrites and developing axonal growth cones, and known to regulate translation of its target mRNAs (Antar et al., 2006; Miyashiro et al., 2003; Muddashetty et al., 2007). Translational dysregulation in the somatodendritic compartments, as a result of lacking functional FMRP, is believed to underlie the pleotropic clinical phenotypes in FXS patients, including learning and memory deficits, autism, and increased susceptibility to epilepsy since early childhood (Berry-Kravis, 2002; Garber et al., 2008; Musumeci et al., 1999; Sabaratnam et al., 2001). Close to 400 mRNA targets have been identified for FMRP, many of which play important roles in neuronal development and synaptic plasticity (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003; Zalfa et al., 2003). Indeed, FMRP-dependent protein synthesis in response to neuronal stimulation is demonstrated to be essential for governing normal metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD) in mouse hippocampus (Huber et al., 2000). In addition, the lack of FMRP causes defects in dendritic spine maturation in FXS patients as well as Fmr1 knockout (Fmr1 KO) mice (Comery et al., 1997; Grossman et al., 2006; Irwin et al., 2000; Irwin et al., 2001; Nimchinsky et al., 2001; Reiss et al., 1991), indicating the functional importance of FMRP in controlling synaptic development. Moreover,

emerging evidence also suggests a pre-synaptic role of FMRP (Antar et al., 2006; Christie et al., 2009), which conceivably may play equally important functions in controlling synapse formation and function as shown in a Drosophila model of FXS (Gatto and Broadie, 2008; Michel et al., 2004; Pan et al., 2004). However, whether and how FMRP indeed governs axonal development in the mammalian brain has not been demonstrated. Moreover, no comprehensive model is currently available to connect dysregulated translation of FMRP mRNA targets to synaptic development.

An extensively characterized mRNA target of FMRP encodes the brain specific microtubule associated protein (MAP) 1B, which is the first MAP expressed in the embryonic brain (Ma et al., 1997; Ohyu et al., 1997) and plays key roles in early neuronal development (Dehmelt and Halpain, 2004; Gonzalez-Billault et al., 2004; Gordon-Weeks and Fischer, 2000). Vigorous up-regulation of MAP1B drives active neuronal processes extension (Gonzalez-Billault et al., 2002; Goold and Gordon-Weeks, 2001; Gordon-Weeks and Fischer, 2000), followed by a gradual decline of MAP1B when robust axonal growth is largely completed and synapse formation begins (Lu et al., 2004; Ma et al., 1997). Precise production of MAP1B is crucial for proper neuronal network formation, likely via modulating microtubule dynamics. Indeed, MAP1B deficiency results in a range of deficits in axonal extension and path finding (Bouquet et al., 2004; Brugg et al., 1993; Edelmann et al., 1996; Gonzalez-Billault et al., 2001; Meixner et al., 2000; Takei et al., 2000), and over production of MAP1B in fragile X mouse neurons leads to aberrant microtubule stability (Lu et al., 2004). However, whether and how FMRP indeed represses MAP1B translation, and the functional consequence of MAP1B dysregulation in brain development due to FMRP deficiency still remains elusive.

Here we show that acute knockdown of FMRP by siRNA increased MAP1B translation in culture and significantly enhanced neurite extension. In addition, the lack of FMRP results in abnormal up-regulation of MAP1B protein in the mossy fibers (MFs), the projection of axons from the dentate gyrus granular cells that form synapses with CA3 dendrites. Importantly, elevated MAP1B in Fmr1 KO MFs is accompanied by aberrant MF projection and significantly increased deposition of the zinc transporter 3 (ZnT3) in MFs. Taken together these data provide the first evidence that FMRP plays important roles in axonal development in the mammalian brain. The temporal association of MF developmental abnormality with MAP1B dysregulation suggests that MAP1B is a pathologic target contributing to the abnormality in early neuronal network development due to the loss of FMRP.

3.2 Results

3.2.1 FMRP represses translation of MAP1B mRNA

Previous studies clearly indicated that FMRP associates with the MAP1B mRNA in the brain and the lack of FMRP in Fmr1 KO neurons results in elevated MAP1B protein expression without altering the levels of MAP1B mRNA (Brown et al., 2001; Darnell et al., 2001; Lu et al., 2004; Zalfa et al., 2003; Zhang et al., 2001b). However, direct evidence for FMRP repression of MAP1B translation has not been demonstrated. To address this question, we chose to acutely knock down FMRP, thus avoiding potential adaptations in Fmr1 KO neurons that may indirectly affect MAP1B translation. The immortalized cortical neuron cell line CAD is an ideal model system, which harbors high levels of FMRP and MAP1B expression as seen in embryonic neurons, can be transfected

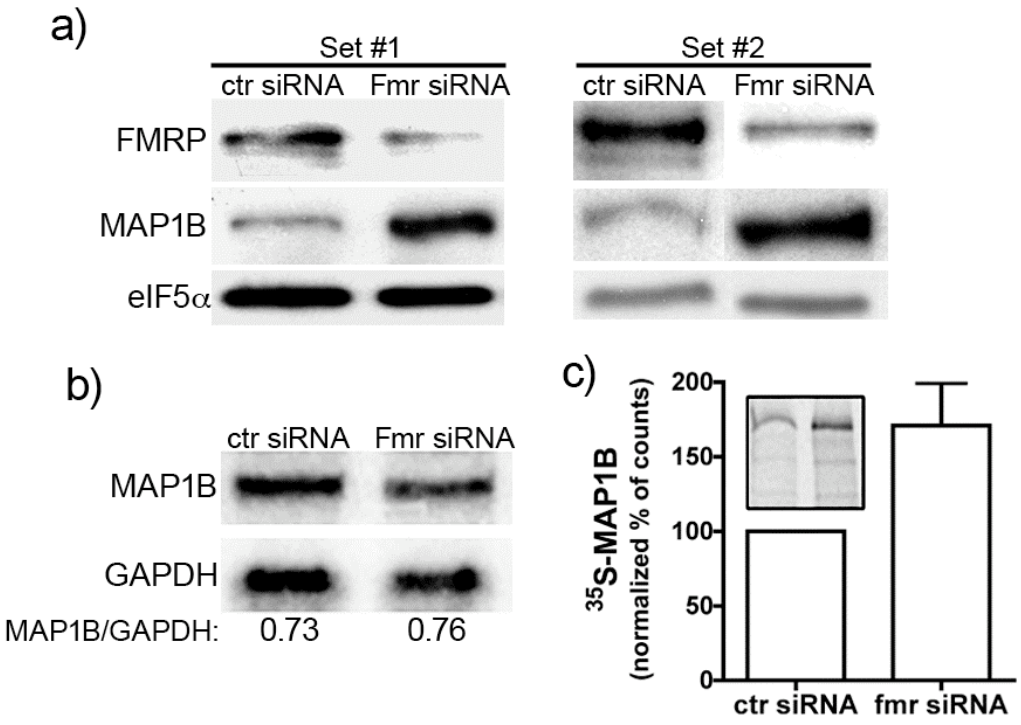
with high efficiency and induced for rapid neurite outgrowth in a synchronized manner. As shown in Figure 3-1a, Fmr1 siRNA-treatment, by two distinct siRNAs, markedly reduced FMRP protein levels and increased MAP1B protein expression at the steady state as compared to that in cells treated with a negative control siRNA that harbors no sequence complementarity with any mammalian mRNA (Figure 3-1a). No change in MAP1B mRNA levels was detected in the Fmr1 siRNA-treated cells by quantitative RNase protection analysis (Figure 3-1b). Furthermore, we directly measured the function of FMRP on translation of MAP1B based on metabolic labeling. In this experiment, ^{35}S-Met pulse-labeled MAP1B in Fmr1 siRNA-treated cells and negative siRNA-treated cells were immunoprecipitated and quantified by scintillation count (Figure 3-1c). A significant increase of the newly synthesized MAP1B protein was detected in response to FMRP knockdown (Student's t-test, $P<0.01$, $n=4$), providing direct evidence that FMRP indeed repress translation of MAP1B.

3.2.2 FMRP siRNA promotes neurite extension and resistance to neurite retraction in response to microtubule disruption in cultured cells.

Considering the role of MAP1B in promoting neurite growth, we next questioned whether FMRP knockdown might influence neurite development. Fmr1 siRNA or the negative control siRNA was transfected into proliferating CAD cells along with a plasmid encoding the green fluorescent protein (GFP) to mark transfected cells, which routinely yields ~50-75% cells expressing GFP. Cells were induced for differentiation 24 hrs after transfection, and images of GFP-positive cells in random fields at various

Figure 3-1: FMRP represses translation of MAP1B mRNA. a) Western blot analysis showing knockdown of FMRP by two different siRNAs leads to increased levels of MAP1B expression. eIF5α was used as a loading control. b) RPA analysis illustrates that fmr siRNA does not alter MAP1B mRNA levels. The MAP1B mRNA level was normalized to that of GAPDH and the average result was depicted underneath of the representative image. c) Metabolic labeling of Fmr1 siRNA treated cells indicates increased translation of MAP1B mRNA. Immunoprecipitation of labeled MAP1B was quantified by scintillation counts (Student t-test: * indicates $p<0.01$, $n=4$). The inset shows a representative image of ^{35}S-MAP1B on SDS-PAGE.

Figure 3-1


a)
Set #1
ctr siRNA
Fmr siRNA
Set #2
ctr siRNA
Fmr siRNA
FMRP
MAP1B
eIF5α
b)
ctr siRNA
Fmr siRNA
MAP1B
GAPDH
MAP1B/GAPDH:
0.73
0.76
c)
35S-MAP1B
(normalized % of counts)
200
150
100
50
0
ctr siRNA
fmr siRNA

time points of differentiation were captured for quantitative analysis of total neurite length, longest neurite and number of neurites.

As shown in Figure 3-2a, Fmr1 siRNA-treated cells, marked by GFP, harbor longer neurites. Quantitative analysis revealed that the total neurite length in Fmr1 siRNA-treated cells was increased 31% and 75% after 24 and 65 hours of differentiation, respectively (Figure 3-2b). In addition, a 42% and 75% increase after 24 and 65 hours, respectively, was also observed when measuring the longest neurite of Fmr1 siRNA-treated cells as compared to that in control siRNA-treated cells (Figure 3-2c). Furthermore, the number of protruding neurites was moderately reduced in the Fmr siRNA treated cells than control siRNA treated (Figure 3-2d). These results clearly indicate that knocking down FMRP leads to increased neurite extension.

Since MAP1B stabilizes microtubules (Takemura et al., 1992; Tint et al., 2005) and FMRP knockdown increases MAP1B protein levels (Figure 3-1a), we tested whether FMRP knockdown may attenuate neurite retraction in response to microtubule disruption. Considering the fact that MAP1B is particularly effective in protecting microtubules from depolymerization caused by nocodazole (Noiges et al., 2002), a commonly used microtubule destabilization reagent, we exposed Fmr1 siRNA-treated and control siRNA-treated cells that had undergone 65 hrs of differentiation to nocodazole in parallel experiments. As shown in Figure 3-3a, obvious neurite shortening was detected in nocodazole-treated cells as compared to mock-treated cells. Interestingly, nocodazole caused more than 40% of control siRNA-treated cells to lose their neurites, whereas only ~10% of Fmr1 siRNA-treated cells lost neurites. Quantitative analysis further revealed that in control siRNA-treated cells the neurite length was shortened 56% after nocodazole

Figure 3-2: Knockdown of FMRP by siRNA increases neurite extension in differentiating CAD cells. a) Representative light images of CAD cells transfected with either control (ctr) siRNA or Fmr siRNA at 24 hours after differentiation. b) Transfection of Fmr siRNA into CAD cells significantly increases the total neurite length at 24, 36, and 65 hours after differentiation as compared to that in ctr siRNA treated cells (Student's t-test, * indicates $p<0.05$ and **$p<0.01$). c) Knock down of FMRP in CAD cells by siRNA increases the length of the longest neurite upon induced differentiation for 36 or 65 hours compared to ctr siRNA (Student's t-test was performed on each time point, *indicates $p<0.05$ and ***indicates $p<0.001$). For (b) and (c), three independent experiments were conducted. More than 70 cells from randomly selected fields were measured in each experiment. d) The number of neurites in control siRNA-treated and Fmr siRNA-treated cells. Results represent measurement from three independent experiments, each contain more than 50 randomly selected cells that were subjected to neurite counting (Two-way ANOVA, $p>0.05$).

Figure 3-2

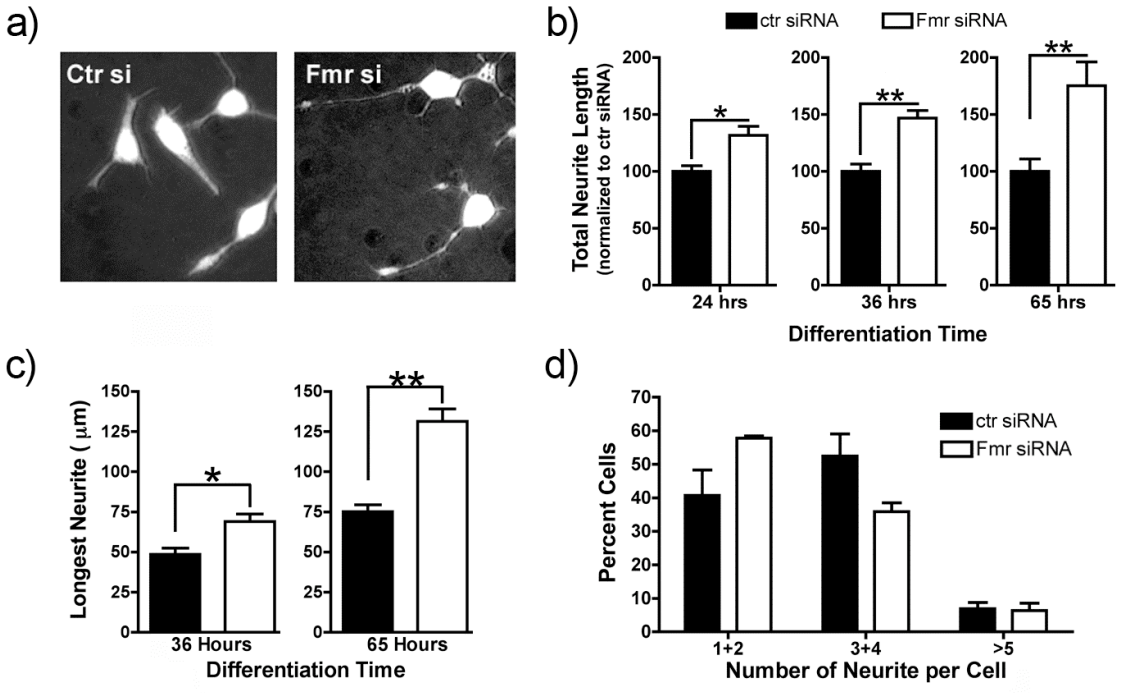
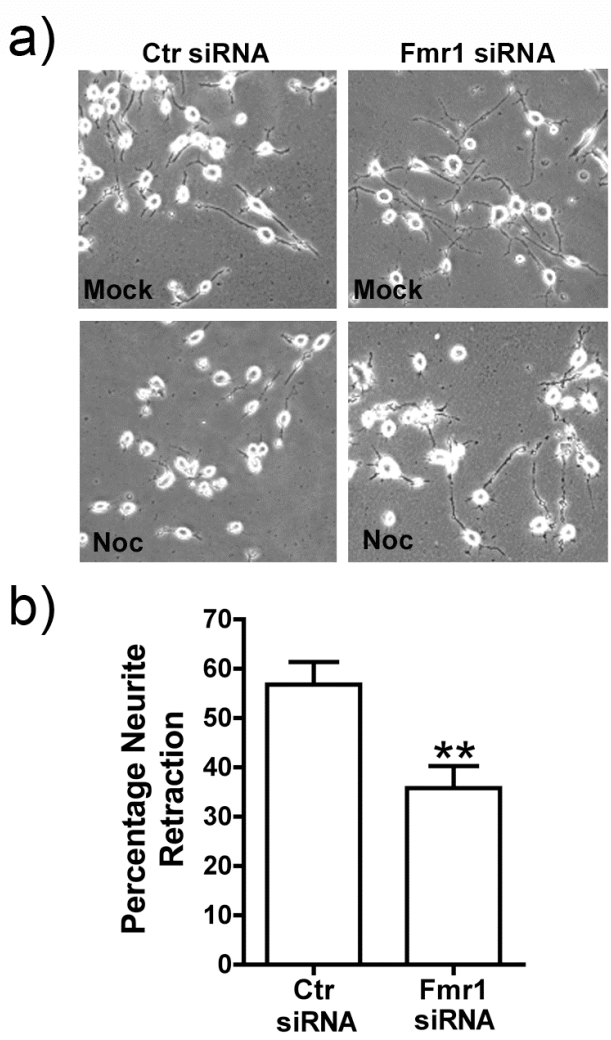

a)
Ctr si
Fmr si
b)
ctr siRNA
Fmr siRNA
Total Neurite Length
(normalized to ctr siRNA)
200
150
100
50
0
*
**
**
24 hrs
36 hrs
65 hrs
Differentiation Time
c)
Longest Neurite (μm)
150
125
100
75
50
25
0
*
**
36 Hours
65 Hours
Differentiation Time
d)
Percent Cells
70
60
50
40
30
20
10
0
ctr siRNA
Fmr siRNA
1+2
3+4
>5
Number of Neurite per Cell

Figure 3-3: Knocking down FMRP by siRNA leads to increased resistance to neurite retraction caused by microtubule depolymerization. a) Control- and Fmr1-siRNA-treated cells that underwent 65 hrs of differentiation were exposed to mock-treatment or 60 nM of nocodazole (Noc) for 4 hrs. b) Quantification of percentage of neurite retraction in control-siRNA-treated and Fmr1-siRNA-treated cells in response to nocodazole-treatment, calculated based on comparison with the average total neurite length in the corresponding mock-treated cells. More than 50 randomly selected cells were measured in each group. (** indicates $P<0.01$ by student t-test)

Figure 3-3


a)
Ctr siRNA
Fmr1 siRNA
Mock
Mock
Noc
Noc
b)
Percentage Neurite Retraction
70
60
50
40
30
20
10
0
**
Ctr siRNA
Fmr1 siRNA

treatment, whereas in Fmr1 siRNA-treated cells the neurite length was reduced 36% (Figure 3-3b). Therefore, knocking down FMRP expression significantly increased the resistance to neurite shortening in response to microtubule depolymerization, a predicted consequence of elevated MAP1B production.

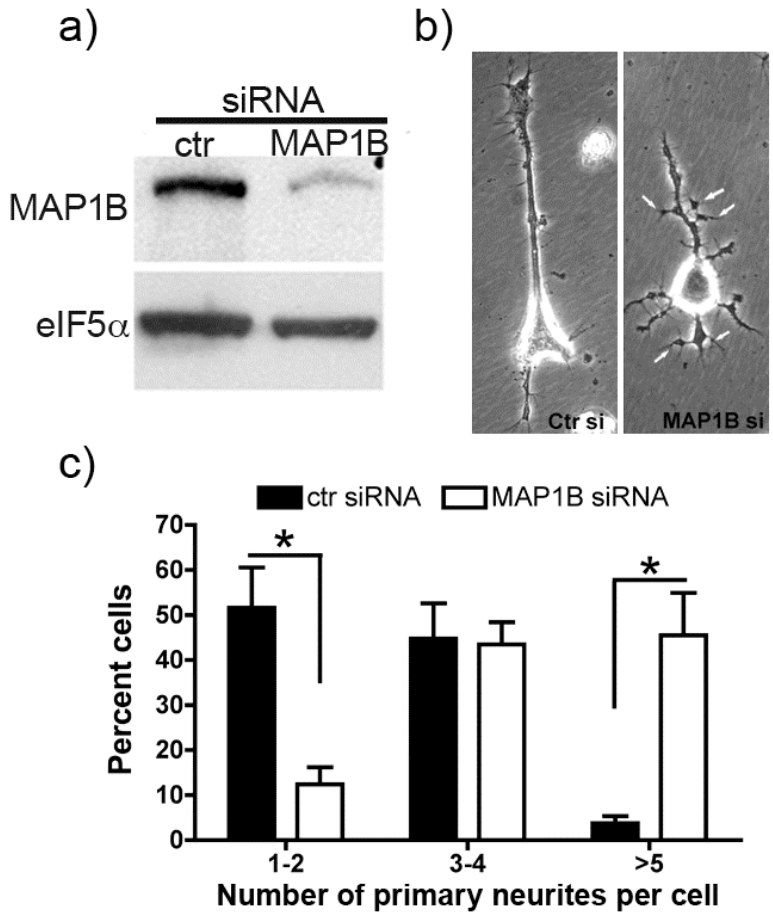
3.2.3 Knocking down MAP1B expression results in aberrant neurite protrusion in culture.

To understand the function of MAP1B in neuritogenesis, we designed a double stranded small interfering RNA that is predicted to knockdown MAP1B expression by RNA interference (RNAi). This siRNA was transfected into proliferating CAD cells together with a plasmid encoding the green fluorescent protein (GFP) to monitor transfection efficiency. In addition, a negative control siRNA that does not harbor sequence complementarities with any mammalian mRNA was employed in parallel transfection. 24 hrs after transfection, cells were induced for differentiation, and images of GFP-positive cells in random fields at various differentiation stages were captured for quantitative analysis.

As shown in Figure 3-4a, the MAP1B siRNA clearly leads to reduced MAP1B protein expression as compared to that in control siRNA-treated cells. The translation initiation factor 5α (eIF5α) was used as a loading control because it was abundantly expressed at steady levels during CAD cells differentiation. Interestingly, reduction of MAP1B did not ablate neurite outgrowth. Instead, many cells aberrantly protrude more short primary neurites after 24 hrs of differentiation (Figure 3-4b). Quantitative analysis revealed that more than 50% of MAP1B siRNA-treated cells harbor 5-8 primary neurites,

Figure 3-4: Knocking down MAP1B by siRNA results in aberrantly increased neurite protrusion. a) Immunoblot analysis detects a marked reduction of MAP1B by siRNA. 24 hrs after transfection, cells were induced for differentiation for 24 hrs before harvested for immunoblot analysis. The translation initiation factor 5α (eIF5α) was used as a loading control. b) Representative image of control- or MAP1B-siRNA-tretaed cells after 60 hrs of differentiation. The increased protrusions of filiapodia from primary neurites are marked by the white arrows in the MAP1B-siRNA-treated cell. c) Quantitative analysis of neurite numbers in control-siRNA-treated cells or MAP1B-siRNA-tretaed cells. More than 70 cells from randomly selected fields in three independent experiments were analyzed. Percentage of cells carrying various numbers of neurite was calculated and graphically displayed. (Two-way ANOVA, * indicates $p<0.05$).

Figure 3-4


a)
siRNA
ctr
MAP1B
MAP1B
eIF5α
b)
Ctr si
MAP1B si
c)
ctr siRNA
MAP1B siRNA
Percent cells
70
60
50
40
30
20
10
0
*
*
1-2
3-4
>5
Number of primary neurites per cell

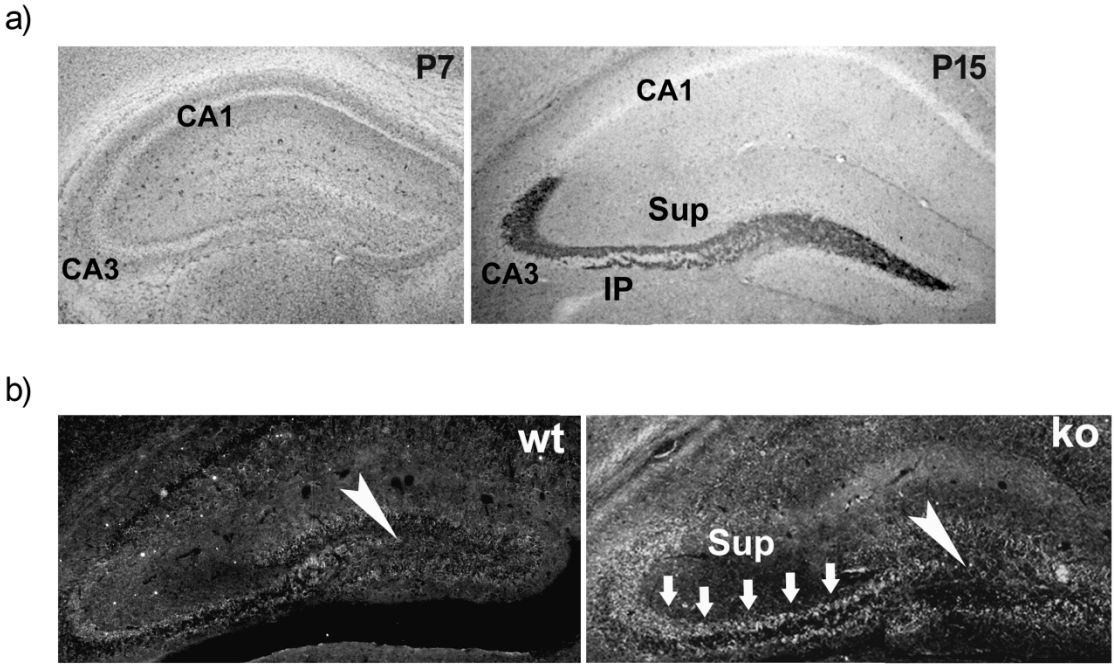
whereas in control siRNA-treated cells greater than 90% of cells harbor no more then 4 primary neurites (Figure 3-4c). Neurites in MAP1B siRNA-treated cells were able to extend, however with more filopodia protrusions as compared to that in control siRNA-treated cells (Figure 3-4b), consistent with a previous report showing increased growth cones in primary cultured neurons derived from a hypomorphous MAP1B mutant (Gonzalez-Billault et al., 2001).

3.2.4 Loss of FMRP leads to aberrantly elevated MAP1B protein levels in hippocampal mossy fiber axons during neonatal development

Development of the hippocampal MF axons starts during the first few postnatal days in mice (Stirling and Bliss, 1978). Between postnatal days 5-9 (P5-9), MF axons are rapidly projected from the DGCs, passing the hilus and approaching the CA3 area (Amaral and Dent, 1981). By P15, MF buttons have formed synapses with CA3 pyramidal neurons and spikes can be elicited in the CA3 dendrites (Bliss et al., 1974). Maturation of MF terminals is indicated by the deposition of zinc into synaptic vesicles (Slomianka and Geneser, 1997), which can be detected by Timm staining, the classical method for zinc detection in the MF tracks (Danscher, 1981; Danscher and Zimmer, 1978). As shown in Figure 3-5a, no Timm staining was observed at P7 in normal hippocampus. However, robust Timm signals were clearly detected at the age of P15, indicating vigorous maturation of the MF terminals in the second postnatal week. Our previous studies showed that in P7 Fmr1 KO hippocampus, abnormal increase of MAP1B protein was mainly detected in the hilus where MFs just begin to form (Lu et al., 2004). At the age of P15 when MFs are fully projected to the CA3 region, MAP1B

Figure 3-5: Abnormally elevated MAP1B protein in the hippocampal mossy fibers of P15 Fmr1 KO mice. a) Timm staining of wild type hippocampus at postnatal day 7 shows little to no zinc deposition. However, the P15 wild type hippocampus demonstrates increased zinc in the suprapyramidal (Sup) and infrapyramidal (IP) mossy fiber projections emanating from the dentate gyrus to the CA3 of the hippocampus, indicating synapse formation and maturation. b) Immunostaining of MAP1B in hippocampus from wild type and Fmr1 KO mice at P15. MAP1B expression is demonstrated at low levels in the hilus (arrowhead), with a majority of staining in the suprapyramidal mossy fiber bundle (arrows), with a marked increase in the Fmr1 KO mouse.

Figure 3-5



declined to low levels in the hilus, but became concentrated in the suprapyramidal (SPB) and infrapyramidal (IFB) bundles of the MF tracts (Figure 3-5b, left panel), suggesting a role of MAP1B in extension and maturation of MF axons. Importantly, it was the MF bundles that harbor elevated MAP1B expression in the Fmr1 KO mice at this age (Figure 3-5b, right panel). Thus, in the hippocampus the MF track is the major location for dysregulated MAP1B as a result of FMRP deficiency.

3.2.5 Colocalization of FMRP and MAP1B mRNA in the mossy fiber terminals

Previous studies showed that FMRP could be detected in axonal growth cones in cultured neurons (Antar et al., 2006) and in the CA3 stratum lucidem of the hippocampus (Christie et al., 2009), raising a hypothesis that FMRP may regulate translation locally within the developing axons. To explore whether FMRP and MAP1B mRNA can be colocalized to hippocampal MF axons, we performed immunofluorescent staining for FMRP simultaneously with fluorescent in situ hybridization (FISH) for MAP1B mRNA in P15 hippocampus. The specificity for FMRP detection (red, Figure 3-6a,i) was confirmed by the complete absence of signal in Fmr1 KO controls (Figure 3-6a, ii and v). Consistent with previous reports, a majority of FMRP was detected in the somata of CA3 neurons. However, colocalization of FMRP with zinc transporter 3 (ZnT3, green), which is responsible for loading free zinc into synaptic vesicles (Palmiter et al., 1996) thereby marking the MF axonal terminals, can be clearly detected in the stratum lucidem of the hippocampal CA3 region (Figure 3-6a, iii) as well as in the SPB and IFB along the CA4 neurons (Figure 3-6a, iv). The colocalized signal of FMRP and ZnT3s in the aforementioned regions can be determined using the Coloc Software from Imaris. In

Figure 3-6: Colocalization of FMRP and MAP1B mRNA in axonal terminals. a) Immunostaining of FMRP (red) localizes with the synaptic vesicle targeted zinc transporter 3 (ZnT3, green) in the CA3 stratum lucidem (sl, part i, iii) and also the suprapyramidal (SPB) and infrapyramidal (IFB) bundles (iv). Fmr1 KO mice slices were used as negative control for FMRP staining (ii, v). b) Colocalization of FMRP (red) and MAP1B mRNA (green) in the CA3 region of mouse hippocampus (i). Colocalization of FMRP and MAP1B mRNA with calbindin (axonal marker, purple, ii) in the axonal terminals in the stratum lucidem. Magnified image shows colocalization of FMRP-MAP1B mRNA-calbindin (yellow arrows, iii) of a section of the stratum lucidem (ii).

Figure 3-6

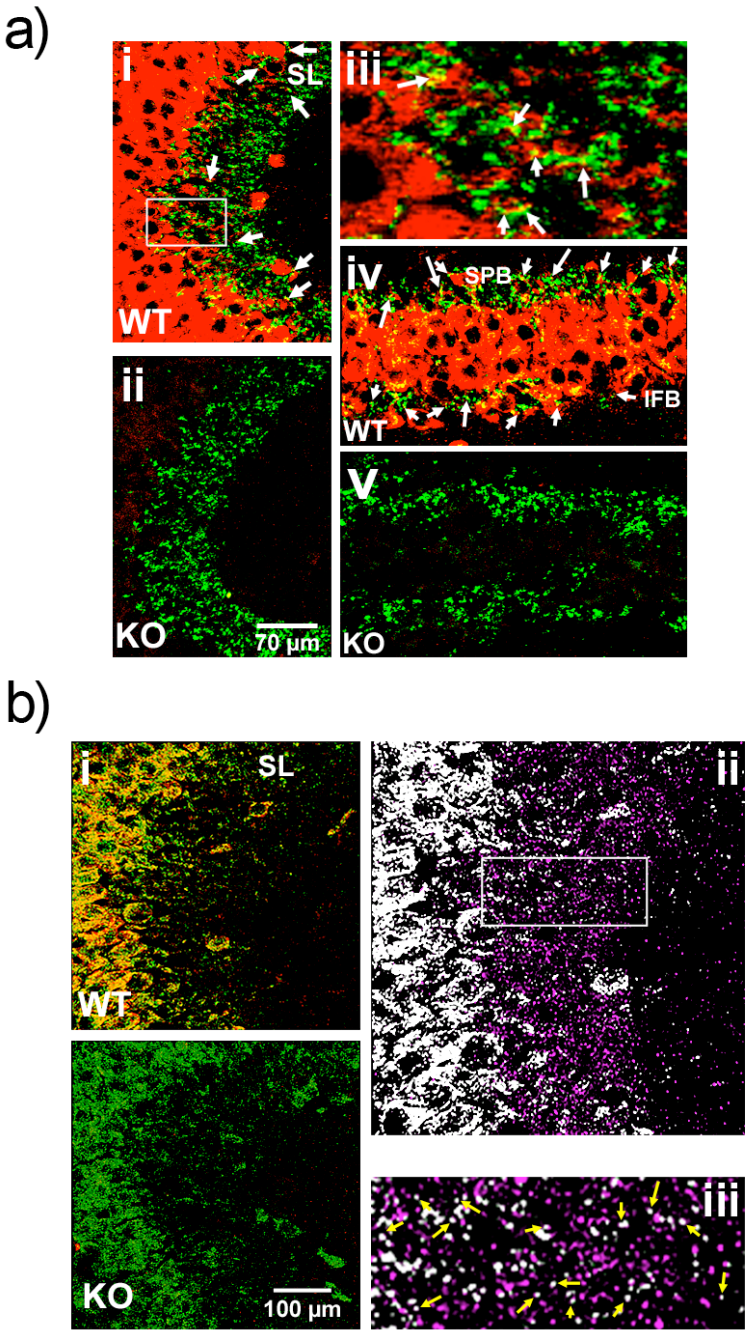
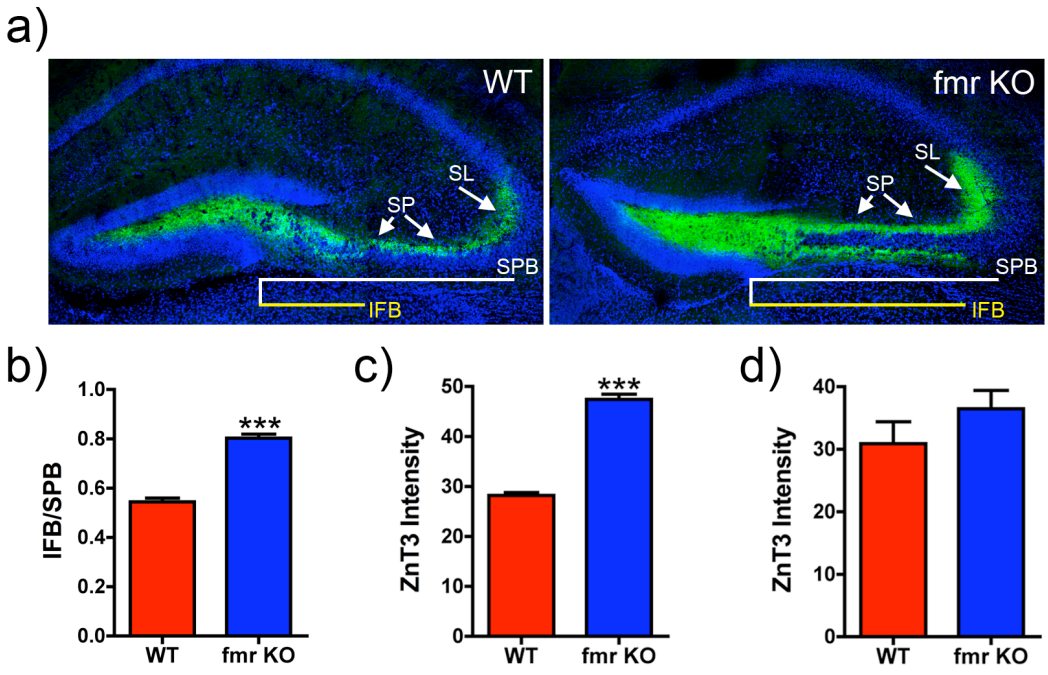


Figure 3-7: Overprojection of hippocampal mossy fibers with increased ZnT3 in Fmr1 KO mice. a) Staining of the hippocampus of P15 wild type and Fmr1 KO mice with ZnT3 (green) indicates overprojection of infrapyramidal bundle (IFB) in Fmr1 KO mice (DAPI – blue). The white line represents measurement of the suprapyramidal bundles (SPB) and yellow line the measurement for the IFB used for quantification in (B). b) Quantification of the increased ratio of IFB length normalized to that of SPB in Fmr1 KO mice compared to wild type. (Student's t-test, *** indicates $P<0.001$, n=5). c) Quantification of increased staining of ZnT3 in the stratum lucidem (SL) of the CA3 region in the fmr1 KO mice. (Student's t-test, *** indicates $P<0.001$, n=5). d) Measurement of ZnT3 staining in the suprapyramidal (SBP) bundle above CA4 shows no significant change between wild type and Fmr1 KO mice. (Student's t-test, $p>0.05$, n=5).

Figure 3-7


a)
WT
SL
SP
SPB
IFB
fmr KO
SL
SP
SPB
IFB
b)
IFB/SPB
1.0
0.8
0.6
0.4
0.2
0.0
***
WT
fmr KO
c)
ZnT3 Intensity
50
40
30
20
10
0
***
WT
fmr KO
d)
ZnT3 Intensity
40
30
20
10
0
WT
fmr KO

addition, colocalization of the MAP1B mRNA FISH signal (green) with FMRP (red) can be detected in the CA3 soma as well as in the MFs in the CA3 stratum lucidem (Figure 3-6b, i). Furthermore, the extracted colocalization of FMRP and MAP1B mRNA signals (white, Figure 3-6b, ii) was superimposed to MF axons marked by calbindin (pink, Figure 3-6b, ii) (Abraham et al., 2009), which shows colocalized FMRP and MAP1B mRNA in the MF terminals (yellow arrows, Figure 3-6b, iii). These data suggest a possibility that FMRP may also regulate local translation of MAP1B in the developing MFs, which in turn controls MF projection and synapse formation.

3.2.6 Abnormal projection of MFs and increased ZnT3 in the mossy fibers of Fmr1 KO mice during hippocampal development

The well-known role of MAP1B in controlling axon extension and path finding (Bouquet et al., 2004; Brugg et al., 1993; Edelmann et al., 1996; Gonzalez-Billault et al., 2001; Meixner et al., 2000; Takei et al., 2000) and the aberrantly increased MAP1B in Fmr1 KO MFs (3.2.5) led us to explore whether Fmr1 KO mice display abnormalities in MF development. P11-15 is a particularly interesting time window as it is the peak for synapse formation between MFs and CA3 dendrites (Amaral and Dent, 1981). The ratio of the length in the IFB and the SPB is a well accepted measurement to examine abnormal MF projection (Bagri et al., 2003), which is then employed to examine MF projection marked by immunofluorescence of ZnT3 in wt and Fmr1 KO mice in parallel. As shown in Figure 3-7a, abnormal appearance of the IFB is observed in a P15 Fmr1 KO mouse as compared to that in the wt littermate, characterized by the inability of the IFB to cross the CA4 soma layer and overprojection toward CA3 stratum pyramidal. Results

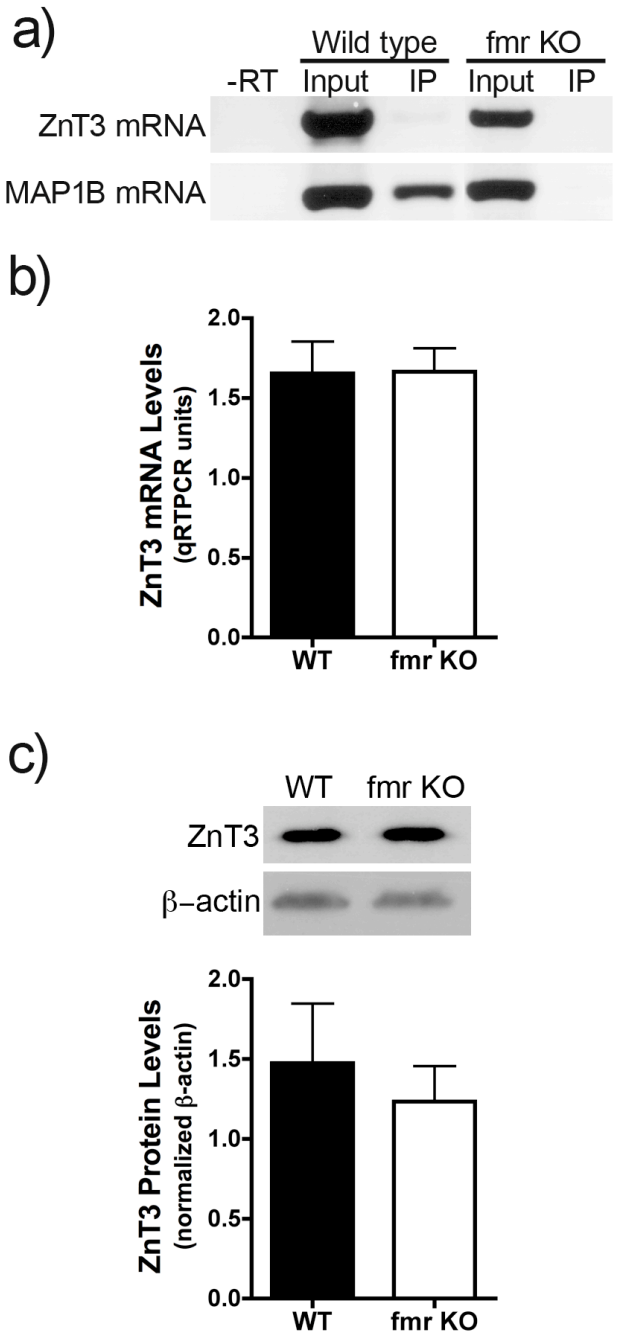
derived from multiple independent experiments clearly showed an increase in the ratio of IFB length to SPB length in the Fmr1 KO mice as compared to that in wt controls at P15 (Figure 3-7b). A similar trend of IFB over projection was also observed at the age of P11, although not reaching statistical significance (data not shown). This result is consistent with the known function of MAP1B in promoting axonal extension and the enhanced MAP1B translation and neurite growth as a result of FMRP deficiency (3.2.1 -3.2.3).

Besides the aberrant MF projection, we were intrigued to find that ZnT3 staining intensity was significantly increased in Fmr1 KO MFs as compared to that in wt controls. At the age of P15, the increased ZnT3 signal is significant in the stratum lucidem of Fmr1 KO mice (Figure 3-7c, d), which is the location for synapse formation by these MF axons. Consistent with the temporal profile of MF development, a similar level of ZnT3 intensity was detected in the SPB above CA4 at P11 (data not shown). Considering the fact that ZnT3 is responsible for zinc deposition into presynaptic vesicles, the increased ZnT3 expression in Fmr1 KO MFs most likely will lead to increased zinc release upon presynaptic activation.

We next questioned whether the ZnT3 mRNA is a potential target of FMRP and whether the increase of ZnT3 in MFs is due to a general up-regulation of ZnT3 protein expression in the Fmr1 KO hippocampus. As shown in (Figure 3-8a), the ZnT3 mRNA does not associate with the immunoprecipitated-FMRP complexes isolated from P15 wt hippocampus. In addition, the loss of FMRP does not affect the global expression of either ZnT3 mRNA (Figure 3-8b) or protein (Figure 3-8c) in the Fmr1 KO hippocampus. Thus, the specific increase of ZnT3 in the Fmr1 KO MF tracts is most likely a result of over-growth of MFs or increased presynaptic vesicles similar to what was reported in the

Figure 3-8: Loss of FMRP does not effect global ZnT3 mRNA or protein levels. a) RT-PCR analysis of ZnT3 and MAP1B mRNA from total input lysate (input) and immunoprecipitated FMRP-RNA complexes (IP). A reaction lacking reverse transcriptase (-RT) was performed as a negative control. b) Real time RT-PCR quantification of total mRNA isolated from hippocampus of P15 wild type and fmr1 KO mice demonstrates no change in ZnT3 mRNA levels. (Student's t-test, $P>0.05$. wt n=3, ko n=5). c) Western blot analysis for ZnT3 of hippocampal lysates from P15 wild type and Fmr1 KO mice (top). β-actin was used as a loading control. Quantification of ZnT3 protein levels normalized to β-actin shows no change in ZnT3 expression levels between wild type and Fmr1 KO mice (bottom). (Student's t-test, $P>0.05$, wt n=3, ko n=5).

Figure 3-8


a)
Wild type
fmr KO
-RT
Input
IP
Input
IP
ZnT3 mRNA
MAP1B mRNA
b)
ZnT3 mRNA Levels
(qRTPCR units)
2.0
1.5
1.0
0.5
0.0
WT
fmr KO
c)
WT
fmr KO
ZnT3
β–actin
ZnT3 Protein Levels
(normalized β-actin)
2.0
1.5
1.0
0.5
0.0
WT
fmr KO

axonal bouton of dfmr1 flies (Pan et al., 2004). Considering the role of zinc in activating MMP- 9, which in turn suppresses dendritic spine maturation, the increased ZnT3 in Fmr1 KO MFs potentially contributes to the delayed dendritic spine maturation and aberrant synapse formation (Bilousova et al., 2009). Furthermore, over-projection of the MFs in Fmr1 KO mice may provide the pathological structural base for the epileptiform in CA3 (Chuang et al., 2005), reminiscent to the well-known malformation of neurocircuitry designated as MF sprouting in epileptogenesis.

3.3 Discussion

Our studies directly demonstrated that FMRP indeed acts to suppress MAP1B translation, and FMRP deficiency causes elevated MAP1B production. The colocalization of FMRP with MAP1B mRNA in the developing MFs further suggests the possibility for FMRP to control local MAP1B translation. Consistent with the role of MAP1B in promoting neuronal processes extension and axonal path finding, increased MAP1B production as a result of FMRP deficiency is accompanied by over-growth of neurites in culture and abnormal projection of hippocampal MF axons. Finally, the increase of ZnT3 in the Fmr1 KO MFs and the abnormality in MF projection provide an explanation of the defects in synaptic maturation at CA3 dendrites in Fmr1 KO mice. Taken together, these findings provide the first evidence that FMRP indeed governs presynaptic development in the mammalian brain, which is an important factor in synaptic development and the pathogenesis of FXS.

It is important to note that fragile X patients and Fmr1 KO mice only display mild neural pathologies, which to this point are characterized by deficits in dendritic spine maturation. It has been proposed that the functional consequence of lacking FMRP in neuronal development is partly compensated by the FXRPs, which are closely related family members of FMRP (Kaufmann et al., 2002). In this regard, the rapid knockdown of FMRP by RNAi and the synchronized induction of neuritogenesis in CAD cells presumably maximized the effect of FMRP on neuritogenesis. However, since FMRP binds multiple mRNA targets (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003), it is conceivable that the enhanced neurite extension caused by FMRP RNAi is mediated by additional mechanisms besides elevated MAP1B production.

On the other hand, FMRP-mediated translational suppression of MAP1B offers a counteracting mechanism to govern the precise production of MAP1B during neurite development, as indicated by the elevated MAP1B translation in response to RNAi-mediated FMRP knockdown (Figure 3-1a). This is a direct extension of our previous observation that FMRP is required to inhibit MAP1B translation during the developmentally programmed decline of MAP1B (Lu et al., 2004), which suggests that FMRP-mediated translation suppression also occurs during MAP1B up-regulation in the early phase of neuronal development. The role of FMRP in suppressing MAP1B translation appears to be evolutionally conserved, since FMRP deficiency in Drosophila causes abnormally elevated expression of the MAP1B homologue Futsch, synaptic overgrowth and aberrant neuronal network elaboration (Pan et al., 2004; Zhang et al., 2001b).

In fact, the FMRP-mediated changes in both dendrites and axons could alter the wiring of the neuronal network in the hippocampus. The mossy fiber axons begin to develop and extend shortly after birth (Stirling and Bliss, 1978). However, rapid growth of the mossy fibers occurs between postnatal days 5-9 (Amaral and Dent, 1981), which could be further increased in the absence of FMRP. Interestingly, synaptic connections between the mossy fibers (presynaptic side) and the dendrites of the CA3 occur between P10 and P15 (Stirling and Bliss, 1978), leading to the question of what effects overprojection of the mossy fibers has on synaptogenesis and neuronal network formation.

An intriguing result that came from this study was the increased levels of ZnT3 in the stratum lucidem of the CA3. Since ZnT3 primarily localizes to synaptic vesicles (Palmiter et al., 1996), the increased levels of ZnT3 could indicate an increase in synaptic vesicles. This leads to the question of whether the increased vesicles are indicative of an increased number of axons originating from the dentate gyrus or increase in the vesicle pool. In Drosphilia, the loss of dFMRP results in an enlargement of the presynaptic bouton and an increase in the accumulation of synaptic vesicles (Pan et al., 2004). The presence of more ZnT3 in the axonal terminals of the hippocampal mossy fibers (Figure 3-7c) could lead to increased loading of zinc into synaptic vesicles and/or an increase in the overall number of zinc containing vesicles. Either scenario demonstrates the ability to increase zinc release into the synaptic cleft upon neuronal activity, where it acts as a neuromodulator affecting the function of a wide range of ion channels and receptors (Bitanihirwe and Cunningham, 2009). The changes in levels of zinc could have profound effects on both physiological and pathological plasticity seen in Fmr1 KO mice.

Increasingly, activation of the tropomyosin kinase receptor B (TrkB) has been found to be important in both forms of plasticity (Hennigan et al., 2009; Minichiello et al., 2002). Emerging evidence demonstrates activation of TrkB by zinc causes an increase in long-term potentiation of the mossy fiber-CA3 synapse independent of BDNF, the primary ligand for TrkB receptors (Huang et al., 2008). Activation of TrkB is required for epileptogenesis (He et al., 2004), but activation is not completely reliant on BDNF as evident in BDNF knockout mice that exhibit only a delay not abolishment in kindling-induced seizure development (He et al., 2004), suggesting the presence of additional ligands to mediate TrkB activation. Thus the potentially increased release of zinc in Fmr1 KO mice could lead to robust and prolonged activation of TrkB, possibly inducing seizure formation that is a symptom of children with FXS (Berry-Kravis, 2002; Musumeci et al., 1999; Sabaratnam et al., 2001). It would be of great interest to further explore the activation of TrkB in the hippocampal mossy fiber of Fmr1 KO mice, as a means of understanding seizure development in the absence of FMRP.

3.4 Materials and Methods

Cell culture, imaging, transfection and lysate

The CAD cell line was maintained for proliferation in DMEM/F12 containing 8-10% FBS (Invitrogen). Differentiation was induced by switching proliferating cells to a chemically defined media as described previously (Qi et al., 1997). For whole cell lysate preparation, CAD cells were harvested before being subjected to sonication in 1X Laemmli buffer (Li et al., 2000). Images of differentiating cells were captured using the

Olympus 1X51 inverted fluorescent microscope equipped with a Retiga monochrome cooled digital camera.

Animals and Tissue Collection

WT and Fmr1 KO mice were raised at the Emory University animal facility and treated in accordance with National Institutes of Health regulations and under approval of the Emory University Institutional Animal Care and Use Committee. WT and Fmr1 KO littermates were produced by breeding heterozygous females with Fmr1 KO males in congenic background of C57BL6. The genotype of each animal was initially mapped by PCR (Lu et al., 2004) and confirmed by immunoblot analysis of FMRP. The mice were anesthetized by isoflurane and sacrificed by cervical dislocation. The brains were dissected on ice to collect the hippocampus, followed by total RNA isolation using TRIzol extraction (Invitrogen), or subjected to preparation of whole tissue lysate (Wang et al., 2004). For staining, the mice were perfused in 4% paraformaldehyde then were further processed for immunofluorescent studies.

siRNA transfection and measurement of neurite extension

Double stranded siRNA (100 nM) against mouse Fmr1 (set #1 – the sense strand sequence is 5'-AUGAAAAGAGCCUUGCUGGtt-3' and antisense strand is 5'-CCAGCAAGGCUCUUUUCAUtt-3'; set #2 – chemically modified double-stranded RNA that was pre-designed by Invitrogen), mouse MAP1B (the sense strand sequence is 5'-GGAACUCAAACUUUUCGUAtt-3' and antisense strand is 5'-UACGAAAAGUUUGAGUUCCtt-3'), or the negative control siRNA #2 (Ambion) was co-transfected with 4μg of pEGFP-C2 (Clontech) respectively into proliferating CAD cells at 50-60% confluence using Lipofectamine 2000 (Invitrogen). 24 hours after transfection, cells were

washed twice with PBS and then exposed to a chemically defined media to induce differentiation. Fluorescent images of a large number of randomly selected cells at various time points of differentiation were captured from parallel cultures and the neurite length was measured and calculated using the ImagePro Plus software (C-Square).

Western Blot Analysis

SDS-PAGE immunoblot analysis was performed using standard procedures (Lu et al., 2004). Briefly, the blots were subjected to Ponceau S stain to ensure equal loading followed by blocking in 10% milk in PBST (1X PBS + 0.1% Tween-20) for 1 hr at room temperature. The blot was then incubated with primary in 2% milk in PBST at the following dilutions for 2 hours at room temperature. The primary antibodies were diluted as following: the antibody against eIF5a (1:5,000) was purchased from Santa Cruz; the antibodies against MAP1B (1:50,000) were characterized in previous reports (Ma et al., 1997; Tint et al., 2005; Tint et al., 1998); the ZnT3 antibody was kindly provided by Dr. Palmiter (Palmiter et al., 1996); and the 1C3 monoclonal antibody against FMRP (1:2,000) was produced in house from the cell line generously provided by J-L Mandel. Next, the blots were washed 3 X 10 min in PBST then incubated with HRP conjugated secondary antibody (1:5000, Jackson Immunology)_in 2% milk for 30 min at room temperature. The blots were washed again 3 X 10 min with PBST, then incubated with ECL reagent and exposed to film.

Immunostaining and fluorescent in situ hybridization (FISH)

Fluorescent *in situ* hybridization on coronal brain sections to detect MAP1B mRNA was performed as described previously with digoxigenin-labeled riboprobes (Muddashetty et al., 2007). Briefly, MAP1B antisense and sense riboprobes were

generated by *in vitro* transcription using digoxigenin-labeled UTP. As a template for *in vitro* transcription, a cDNA fragment of MAP1B (NM_008634.1, nt 142-1484) was synthesized by PCR and cloned into the *Xho*I/*Eco*RI sites of pcDNA3 (*Invitrogen*). Following hybridization, riboprobes were incubated with peroxidase-conjugated anti-digoxigenin antibody (sheep, Roche), anti-FMRP antibody (mouse, 2F5-1, gift from J. Darnell) and anti-Calbindin antibody (rabbit, *Cell Signaling Technology*). FMRP- and Calbindin-specific signals were detected with Cy3-coupled mouse, and Cy5-coupled rabbit antibodies, respectively. Peroxidase activity, i.e. digoxigenin-specific signals, was detected by subsequent fluorescein-linked tyramide signal amplification (TSA-Plus Fluorescein system, PerkinElmer). Images were acquired using a Zeiss LSM510 confocal microscope, deconvolved with AutoQuant X (*Media Cybernetics*), and are displayed as flattened z-stacks. Co-localization channels were built with Imaris Coloc software (*Bitplane*).

Mossy fiber staining

Brain slices were blocked in PBS containing 0.01% Tween 20, and 3% donkey serum for 30 minutes at room temperature before incubating overnight at 4˚C with ZnT3 antibody (1:130, rabbit, kindly provided by Dr. Palmiter (Palmiter et al., 1996) in blocking solution containing 5% BSA. After washing 3X10 minutes in PBST (PBS containing 0.5% Tween 20), the slices were incubated with anti-rabbit IgG coupled with Alexa 488 (Invitrogen, 1:250) in PBST for 1 hour at room temperature. The slices were washed 3X10 minutes in PBS and then counterstained with DAPI and fixed. Fluorescence was detected using an Olympus IX-51 fluorescent microscope, and images were captured with a Retica digital camera. MAP1B staining was performed as

previously described (Lu et al., 2004). Timm staining was performed as previously described (Danscher, 1981).

RNA extraction and analysis

Total RNA was extracted using Trizol according to the manufacture's protocol (Invitrogen). The quantity of RNA from each sample was determined by OD260 reading and further confirmed by ethidium bromide stained agarose gel electrophoresis. RPA was performed using MAP1B probe as described in our previous report (Lu et al., 2004). Increasing amount of total RNA input was used in test experiments to ensure the quantitative capacity of RPA for each probe. For RT-PCR, reverse transcription was performed using 1μg of total RNA with random primers (Promega) and Superscript II RNaseH-reverse transcriptase (Invitrogen), followed by PCR analysis, using the following primers ZnT3: 5'-atcctcctgtacctggcctt-3' (forward), 5'- gatggagatcatgggttgct-3' (reverse); and MAP1B: 5'- cctggagtgacccaggcagtag-3' (forward), 5'-ggactcagatccaagaaggggagg-3' (reverse).

Real time PCR was performed using iCYCLER (BioRad) and SYBR-green master mix (NEB), per manufacturer's protocol. Basically, 5μL of template was mixed with 5μL of 2.5μM mixed primer set then 10μL of 2X reaction buffer was added. Standard curves were established for all primers using reverse transcribed cDNA from serial dilutions of a 50 ng/μL mouse hippocampal total RNA. The following primer pairs were used for real-time detection ZnT3: 5'- agtgactatcacattgaggcgggt-3' (forward), 5'-attgggtatccatgcccttcctct-3' (reverse); and GAPDH: 5'-cacagtcaaggctgagaatgggaa-3' (forward), 5'-gtggttcacacccatcacaaacatg-3' (reverse)

^{35}S-pulse labeling and immunoprecipitation (IP)

After 24 hours of induced differentiation, siRNA-treated cells were pulse-labeled by ^{35}S-Met (60 μCi) in DMEM/F12 containing 2mM Met and Cys for 30 min, washed extensively with PBS to remove unincorporated ^{35}S-Met before subjected to lysis in 100 mM Tris pH7.5, 200mM NaCl, 1% Triton X-100 with protease inhibitors. An aliquot of the pre-cleared cytoplasmic extract (input for IP) was used for scintillation counting to determine the total translation level. MAP1B IP was performed using the polyclonal antibody against MAP1B (Ma et al., 1997). An aliquot of the immunoprecipitate was used for scintillation counting and the rest was visualized by a phosphorimager on SDS-PAGE. The scintillation counts of MAP1B was normalized to that of the total counts in the input in each sample. The specificity of IP was confirmed by a marked reduction of MAP1B on SDS-PAGE in response to MAP1B siRNA-treatment (data not shown).

Chapter 4: Trans-acting factors potentially involved in regulating the translation of the long 3'UTR BDNF mRNA

4.1 Introduction

The brain-derived neurotrophic factor (BDNF) transcripts carry either a long (2.85kb) or a short (0.35kb) 3' untranslated region (3'UTR), as a result of alternative polyadenylation site usage (Timmusk et al., 1995). Since all BDNF transcripts encode for the same protein, the distinct 3'UTRs conceivably may provide a different means of post-transcriptional regulation at the level of translation and subcellular localization of BDNF mRNA to further increase the complexity of regulating BDNF expression. In support of this idea, our recent data demonstrates that the long 3'UTR mediates translation suppression of BDNF at rest while the short 3'UTR supports active BDNF translation. However, upon robust neuronal activation, the long 3'UTR BDNF mRNA undergoes a translational de-repression while the short 3'UTR BDNF mRNA becomes translationally repressed. However, trans-acting factors that specifically regulate the long 3'UTR-mediated translation repression/de-repression of BDNF mRNA have never been explored.

One interesting idea is whether the fragile X mental retardation protein (FMRP), is involved in translation regulation of BDNF. FMRP is a selective RNA-binding protein known to repress translation of its bound mRNAs, localize to dendrites with its mRNA ligands, and is essential for neuronal activity-dependent translation in various plasticity and learning paradigms (Antar and Bassell, 2003; Bassell and Kelic, 2004; Bear et al., 2004; Feng, 2002; Jin et al., 2004a; Jin and Warren, 2003). Although BDNF mRNA has not been reported to bind FMRP directly, none of the previous reports specifically examined the long 3'UTR BDNF transcript. Nonetheless, several lines of evidence

suggest an overlapping and perhaps cross talk between the function of BDNF and FMRP. In fact, application of BDNF to Fmr1 KO hippocampal slices restored LTP to wild type levels (Lauterborn et al., 2007), suggesting that BDNF might be a downstream target of FMRP-mediated translation repression.

Besides RNA-binding proteins, emerging evidence indicates that microRNAs can also control neuronal translation (Fiore et al., 2008; Schratt et al., 2006). MicroRNA are small, noncoding RNAs of 19-23 nucleotides in length that function to repress translation and/or degrade target mRNAs through binding to the 3'UTR of target mRNAs with imperfect complementarity (Ambros, 2004; Bartel, 2004; Jing et al., 2005). Transcription of pri-pre-microRNAs is performed by RNA polymerase II (Pol II), after which the pri-pre-microRNA is cleaved to pre-microRNA by Drosha, an RNaseIII enzyme, before being exported from the nucleus to the cytoplasm by exportin 5 (Bohnsack et al., 2004; Lee et al., 2003; Lee et al., 2004; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, the pre-microRNA (70-80 nucleotides) is cleaved by another RNase III enzyme, Dicer, to produce mature microRNA (19-22 nucleotides) (Bartel, 2004). The mature microRNA is then loaded into the RNA-induced silencing complex (RISC), which then binds to its target mRNA (Bartel, 2004). Currently, there are estimated to be more than 800 microRNAs encoded within the human genome (Berezikov et al., 2005). Many of these are expressed and developmentally regulated in neurons (Nelson et al., 2004; Sempere et al., 2004), suggesting a role for microRNA in neuronal development. Emerging evidence also demonstrates the functional importance of microRNA in controlling dendritic morphology associated with synaptic plasticity (Impey et al., 2009; Schratt et al., 2006; Vo et al., 2005; Wayman et al., 2008). In addition, increased synaptic signaling is able to

release microRNA-mediated translational repression in activated synapses (Schratt et al., 2006). Thus, whether microRNAs could mediate the translational repression and subsequent activity-dependent derepression of the long 3'UTR BDNF mRNA is an intriguing possibility to be explored.

Here we show that the long 3'UTR BDNF mRNA preferentially associates with the FMRP complex isolated from mouse brain. In addition, the BDNF long 3'UTR contains multiple predicted binding sites for a brain-specific microRNA, miR-128. Furthermore, miR-128 is able to repress translation of a luciferase reporter in a BDNF long 3'UTR dependent manner while having no effects on the BDNF short 3'UTR. Finally, the expression levels of miR-128 are rapidly reduced upon both physiological and pathological neuronal stimulation, suggesting a mechanism for the activity-dependent release of translational repression of the long 3'UTR BDNF mRNA. These results provide the first evidence that FMRP and/or miR-128 are two potential mechanisms in regulating the neuronal activity-dependent translational of BDNF specifically via the long 3'UTR.

4.2 Results

4.2.1 FMRP preferentially associates with the long 3'UTR BDNF mRNA.

Our previous data leads to the question of what molecular mechanism is responsible for the translational repression of BDNF mRNA mediated by the long 3'UTR. Considering the potential functional overlap between activity-dependent translation of BDNF and the role of FMRP in regulating translation during synaptic

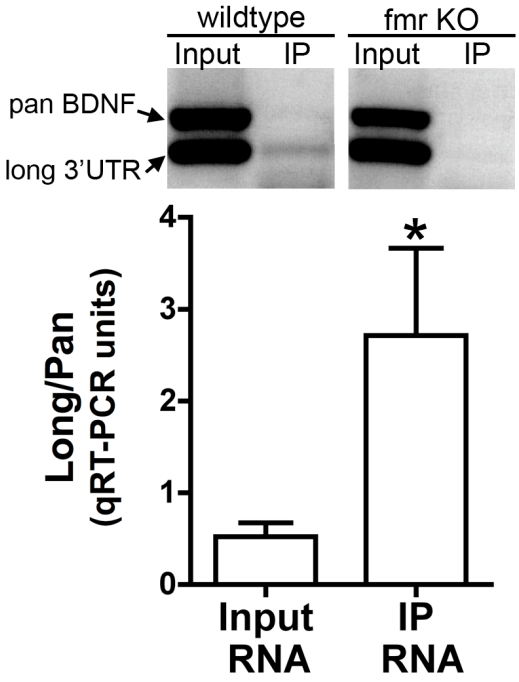
plasticity, we explored whether the long 3'UTR BDNF mRNA is a ligand for FMRP in the brain. In support of this idea, both the pan and long 3'UTR BDNF mRNA were detected in immunoprecipitated FMRP complexes from wild type mice at postnatal day 7 by RT-PCR as shown in Figure 4-1 (top panel). As a negative control, no BDNF mRNA was detected in immunoprecipitated mRNA from age matched Fmr1 KO brains (Figure 4-1, top panel). To further address whether the short and long BDNF mRNA may differentially associate with FMRP mRNP complex, the ratio of the long 3'UTR BDNF mRNA to pan BDNF mRNA was determined by qRT-PCR analysis using total RNA isolated from input lysates as well as that from immunoprecipitated FMRP complexes. As shown in Figure 4-1 (bottom panel), a higher ratio of the long 3'UTR BDNF mRNA to the pan BDNF mRNA was detected in the FMRP complex as compared to that in the total input RNA. Such a relative enrichment of the BDNF long 3'UTR mRNA suggests that FMRP may preferentially associate with the BDNF long 3'UTR. Whether FMRP is involved in repressing translation of the long 3'UTR BDNF mRNA, promoting dendritic localization of BDNF mRNA, and involved in the activity-dependent translation de-repression as shown in Chapter 2 are intriguing possibilities to be addressed by future studies.

4.2.2 miR-128 specifically represses translation of luciferase reporter that harbors the BDNF long 3'UTR.

We next explored the possibility whether microRNAs may target the long but not the short 3'UTR of BDNF thus contributing to the differential translation mediated by the two distinct 3'UTRs. Using the Miranda algorithm from Memorial Sloan Kettering with

Figure 4-1: The long 3'UTR BDNF mRNA associates with FMRP in mouse brain lysates. Input and FMRP immunoprecipitated (IP) RNA from wild type and Fmr1 KO mice was isolated from P7 mice. RT-PCR was performed on the isolated RNA with primers to detect pan BDNF mRNA and long 3'UTR BDNF mRNA (top panel). Both the pan and long 3'UTR BDNF mRNA were detected in immunoprecipitated FMRP complexes after 35 cycles of PCR reaction. Real-time RT-PCR detected a significant increase in the ratio of long 3'UTR BDNF mRNA to pan BDNF mRNA in the IP RNA compared to the input, suggesting that FMRP specifically associates with the long 3'UTR BDNF mRNA (Student's t-test, * indicates $p<0.05$, $n=3$).

Figure 4-1

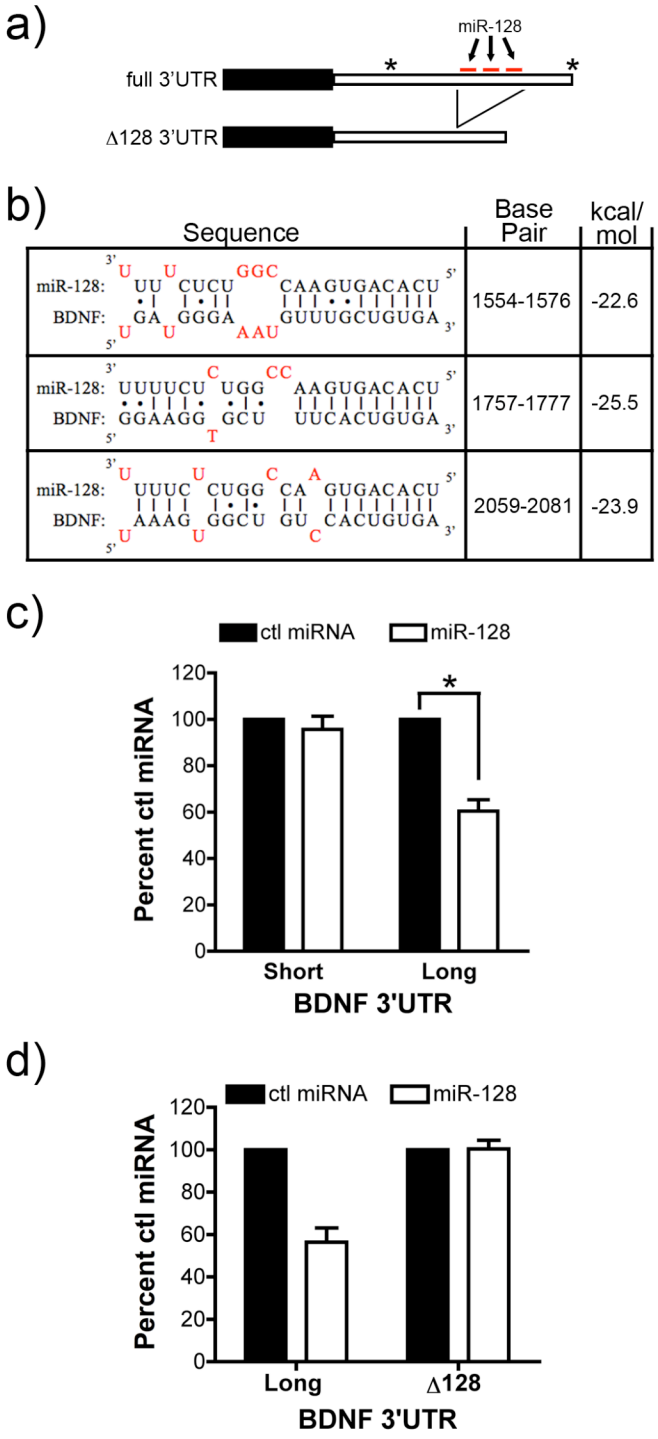

wildtype
fmr KO
Input
IP
Input
IP
pan BDNF
long 3'UTR
Long/Pan
(qRT-PCR units)
4
3
2
1
0
*
Input
RNA
IP
RNA

the criteria of an free energy cut-off at -15.0 kcal/mol and a perfect complementary seed region (John et al., 2004), we identified three potential binding sites for miR-128 specifically contained within the BDNF long 3'UTR (Figure 4-2a and b). These predicted miR128 sites were highly conserved between mouse, rat and human. No binding sites for miR-128 can be predicted in the short 3'UTR or coding region of BDNF mRNA.

To test whether miR-128 can specifically repress translation via the long but not the short 3'UTR of BDNF, we synthesized 5'-7-methylguanosine capped firefly luciferase reporter mRNAs containing the long 3'UTR or short 3'UTR of BDNF (described in Chapter 2.2) by *in vitro* transcription. The long or short 3'UTR luciferase-reporter mRNA was co-transfected either with synthetic miR-128 or a scrambled negative control microRNA, which contains no sequence homology to any mammalian gene, into an immortal cortical neuronal cell line, CAD. *In vitro* transcribed renilla luciferase mRNA was included in each transfection to monitor transfection efficiency. We measured the activity of both firefly and renilla luciferase 6 hours after transfection. As shown in Figure 4-2c, miR-128 caused a significant reduction in the translation efficiency of the long 3'UTR reporter mRNA compared to the negative control, as measured by normalizing the firefly luciferase activity to the levels of firefly luciferase mRNA. In contrast, miR-128 had no effect on the luciferase activity of the short 3'UTR reporter mRNA compared to that in the negative control microRNA-transfected cells (Figure 4-2c). Moreover, removal of the region containing the miR-128 binding sites in the long 3'UTR luciferase reporter construct completely alleviated the ability of miR-128 to repress luciferase reporter expression from the Δ128 construct (Figure 4-2d). These

Figure 4-2: miR-128 represses luciferase expression via the BDNF long 3'UTR. a) Schematic showing predicted binding sites for miR-128 specifically contained within the BDNF long 3'UTR luciferase reporter construct and deletion of this region in the Δ128 long 3'UTR reporter construct. b) Sequences and free energies, as measured by RNAhybrid, for the predicted binding sites for miR-128 in the mouse long 3'UTR of BDNF mRNA. The base pairs indicate the location of each binding site from the stop codon (position 0) of the BDNF open reading frame. c) Cotransfection of CAD cells with the control microRNA or miR-128 double stranded RNA and either the long or short 3'UTR reporter. The firefly luciferase activity was normalized to the firefly luciferase mRNA levels, measured by qRT-PCR. The normalized firefly luciferase levels were then taken as the percentage of the control microRNA. A significant reduction in luciferase activity was observed by the long 3'UTR reporter in the presence of miR-128 compared to control microRNA. The short 3'UTR reporter was not affected by the co-transfection of miR-128. (One-Way ANOVA, * indicates a $p<0.05$ between the indicated groups by tukey analysis, n=3). d) HEK cell stably expressing miR-128 or miR-23, which was used as a negative control, were transfected with luciferase reporter constructs containing the BDNF long 3'UTR or the BDNF Δ128 long 3'UTR, along with pRL-TK that encodes the renilla luciferase. The firefly luciferase activity was normalized to the renilla luciferase activity in each transfection then graphed as the percentage of control microRNA. miR-128 reduced luciferase activity in the cells transfected with the BDNF long 3'UTR reporter construct. This reduction was alleviated in the cells transfected with the BDNF Δ128 long 3'UTR reporter construct (n=2).

Figure 4-2


a)
miR-128
full 3'UTR
Δ128 3'UTR
b)
Sequence
Base Pair
kcal/ mol
miR-128:
BDNF:
1554-1576
-22.6
1757-1777
-25.5
2059-2081
-23.9
c)
ctl miRNA
miR-128
Percent ctl miRNA
120
100
80
60
40
20
0
Short
Long
BDNF 3'UTR
d)
ctl miRNA
miR-128
Percent ctl miRNA
120
100
80
60
40
20
0
Long
Δ128
BDNF 3'UTR

results provide the initial evidence that miR-128 can repress translation of the luciferase reporter specifically via the BDNF long 3'UTR.

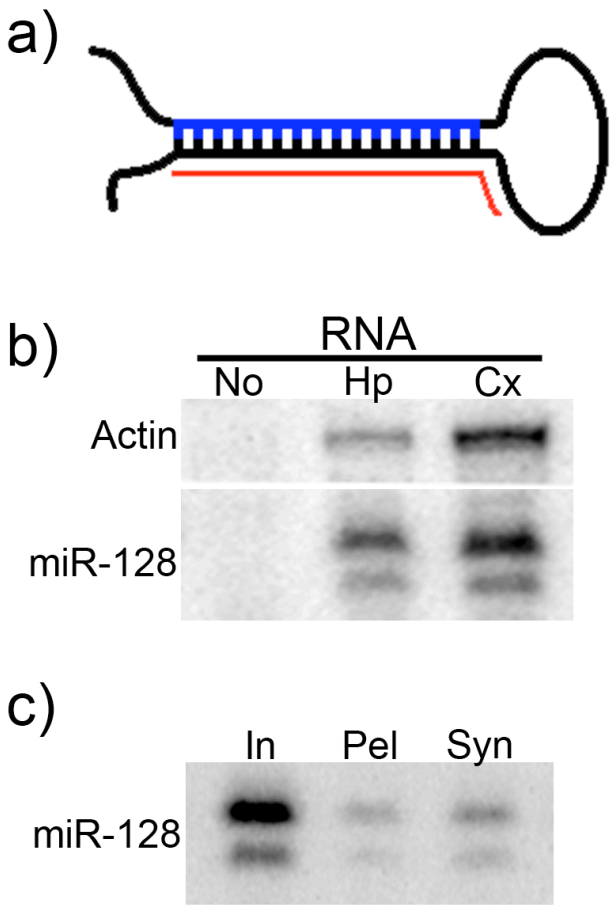
4.2.3 Expression of miR-128 in the brain

BDNF protein and mRNA isoforms are expressed in a variety of brain regions (Aid et al., 2007; Ernfors et al., 1990a; Ernfors et al., 1990b). The long 3'UTR BDNF mRNA is found highly expressed in the mouse cortex and hippocampus (An J.J. Cell 2008). To determine whether miR128 displays overlapping expression profiles with the BDNF transcripts, we developed a quantitative ribonuclease protection assay (RPA) with a probe specific for miR-128 (Figure 4-3a). Using this assay, we demonstrated expression of miR-128 in both the mouse hippocampus and cortex (Figure 4-3b). This data biochemically confirms in situ hybridization data showing miR-128 expression in the mouse hippocampus (Bak et al., 2008) , with an extensively spatial overlap with the long 3'UTR BDNF mRNA (Chapter 2).

Within neurons, BDNF mRNA is localized to the somatic compartment as well as dendrites and synapses, which is stimulated by neuronal activation (Tongiorgi et al., 2004; Tongiorgi et al., 1997), suggesting possible regulation of local dendritic BDNF protein synthesis. The ability of the long 3'UTR BDNF mRNA to preferentially localize to neuronal dendrites and the functional requirement of the long 3'UTR in dendritic spine maturation and LTP formation (An JJ Cell 2008) further support this hypothesis, Furthermore, the machinery necessary for microRNA function has also been found localized to the synapse (Lugli et al., 2005; Lugli et al., 2008) and emerging evidence implicates a role for microRNA in synapse development and function

Figure 4-3: Localization of miR-128. a) Schematic of the ribonuclease protection assay probe (red) used to detect miR-128. Blue indicates the mature microRNA. Red indicates probe designed for RPA. b) RPA probing for miR-128 and actin mRNA levels isolated from total mouse hippocampus (Hp) and cortex (Cx) RNA. A reaction containing no RNA was used a negative control for probe. c) RPA of RNA isolated from pellet, comprised of neuronal soma, (pel) and synaptic (syn) fractions after performing crude synaptoneurosome preparation from mouse hippocampus. Input (In) represents total RNA before synaptoneurosome prep.

Figure 4-3



(Schratt, 2009; Smalheiser and Lugli, 2009). Thus, we examined whether miR128 can also be detected in synaptic preparations isolated from mouse hippocampus. Indeed, miR-128 can be clearly detected in synaptic fractions using our RPA assay (Figure 4-3c), suggesting the possibility for miR-128 to regulate translation of the long 3'UTR BDNF mRNA in dendrites.

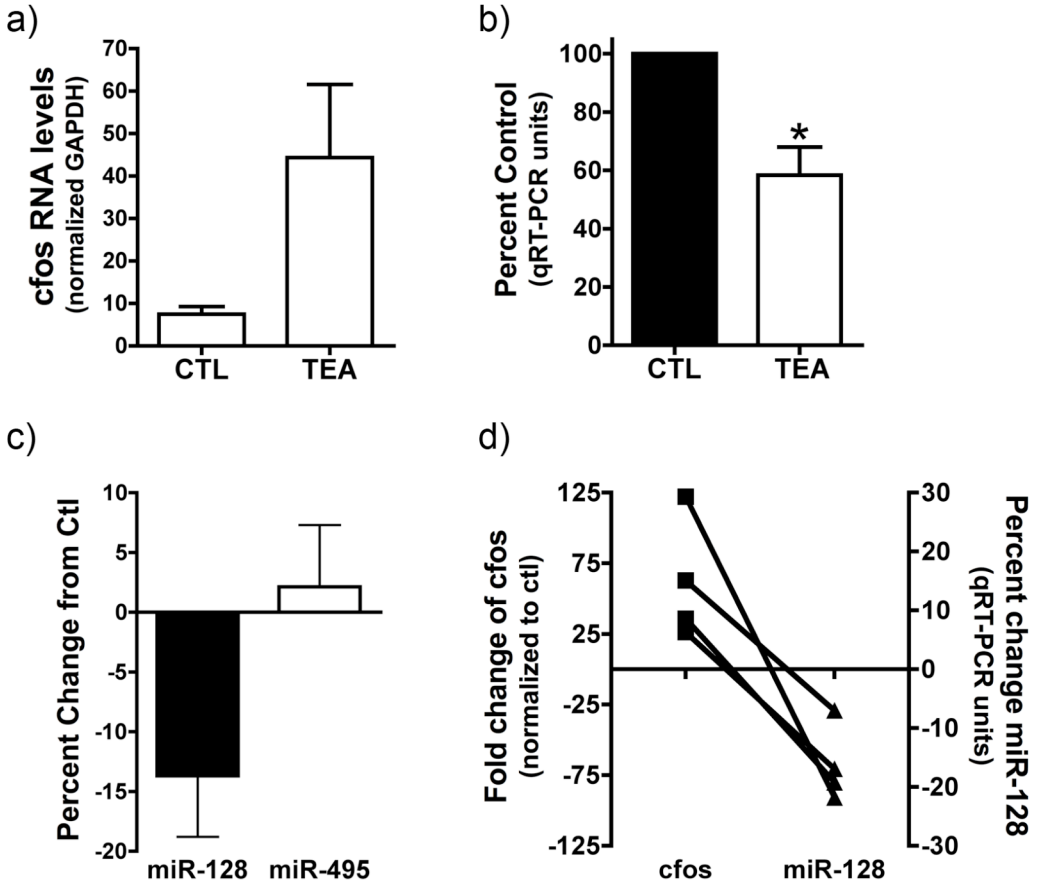
4.2.4 Down-regulation of miR-128 level upon neuronal stimulation

We show that upon neuronal stimulation, the translational repression mediated by the long 3'UTR BDNF mRNA is released (Chapter 2). Considering that neuronal activation can release microRNA-mediated translational repression in dendrites (Schratt et al., 2006), we examined the effects of both physiological and pathological neuronal stimulation on the expression levels of miR-128. First, we cultured hippocampal neurons for 21 days until the synaptic network is well formed, and exposed the cultures to tetraethylammonium (TEA) under a well-established stimulation paradigm for inducing LTP (Aniksztejn and Ben-Ari, 1991). Neuronal activation upon TEA-treatment was evidenced by the rapid and robust up-regulation of c-fos ten min after TEA-treatment (Figure 4-4a). In this paradigm, we showed that translation of a GFP reporter can be stimulated in a BDNF long 3'UTR-dependent manner (Chapter 2). Interestingly, increased neuronal activity by TEA led to a significant decrease in the levels of mature miR-128, as measured by real-time RT-PCR (Figure 4-4b).

Similarly, a single dose of pilocarpine, which reliably induces stage V seizure and causes a de-repression of the long 3'UTR BDNF mRNA (Chapter 2-2-4), resulted in a significant decrease in the levels of mature miR-128 (Figure 4-4c). Moreover, the c-fos

Figure 4-4: Neuronal activation results in a rapid decrease of miR-128 levels in the hippocampus. a) Primary hippocampal neurons at DIV21 were treated with TEA for 10 minutes. Neuronal activation by TEA was demonstrated by the robust increase in cfos mRNA as measured by qRT-PCR. b) Total RNA was isolated and qRT-PCR was performed to detect miR-128 and U6 rRNA, which was used a loading control. The miR-128 levels were normalized to U6 rRNA. A significant decrease in mature miR-128 was observed upon treatment with TEA compared to control. (Student's t-test, * indicates $p<0.05$, n=3). c) Total RNA was isolated from rat hippocampus 30 minutes after pilocarpine-induced seizures. The mature miR-128 and miR-495 levels were measured by qRT-PCR and normalized to U6 rRNA and graphed as the percent change of saline injected rats. A significant decrease is observed for miR-128 upon pilocarpine-induced seizures compared to saline treated rats. No change was observed in the levels of miR-495 upon pilocarpine-induced seizures. d) Levels of cfos were measured by qRT-PCR, then normalized to GAPDH and shown as fold change over control rats. An inverse correlation was demonstrated between the cfos levels and the miR-128 levels as measured in (c), suggesting the degree of neuronal activation affects the reduction of miR-128.

Figure 4-4


a)
cfos RNA levels
(normalized GAPDH)
70
60
50
40
30
20
10
0
CTL
TEA
b)
Percent Control
(qRT-PCR units)
100
80
60
40
20
0
*
CTL
TEA
c)
Percent Change from Ctl
10
5
0
-5
-10
-15
-20
miR-128
miR-495
d)
Fold change of cfos
(normalized to ctl)
125
75
25
-25
-75
-125
Percent change miR-128
(qRT-PCR units)
30
20
10
0
-10
-20
-30
cfos
miR-128

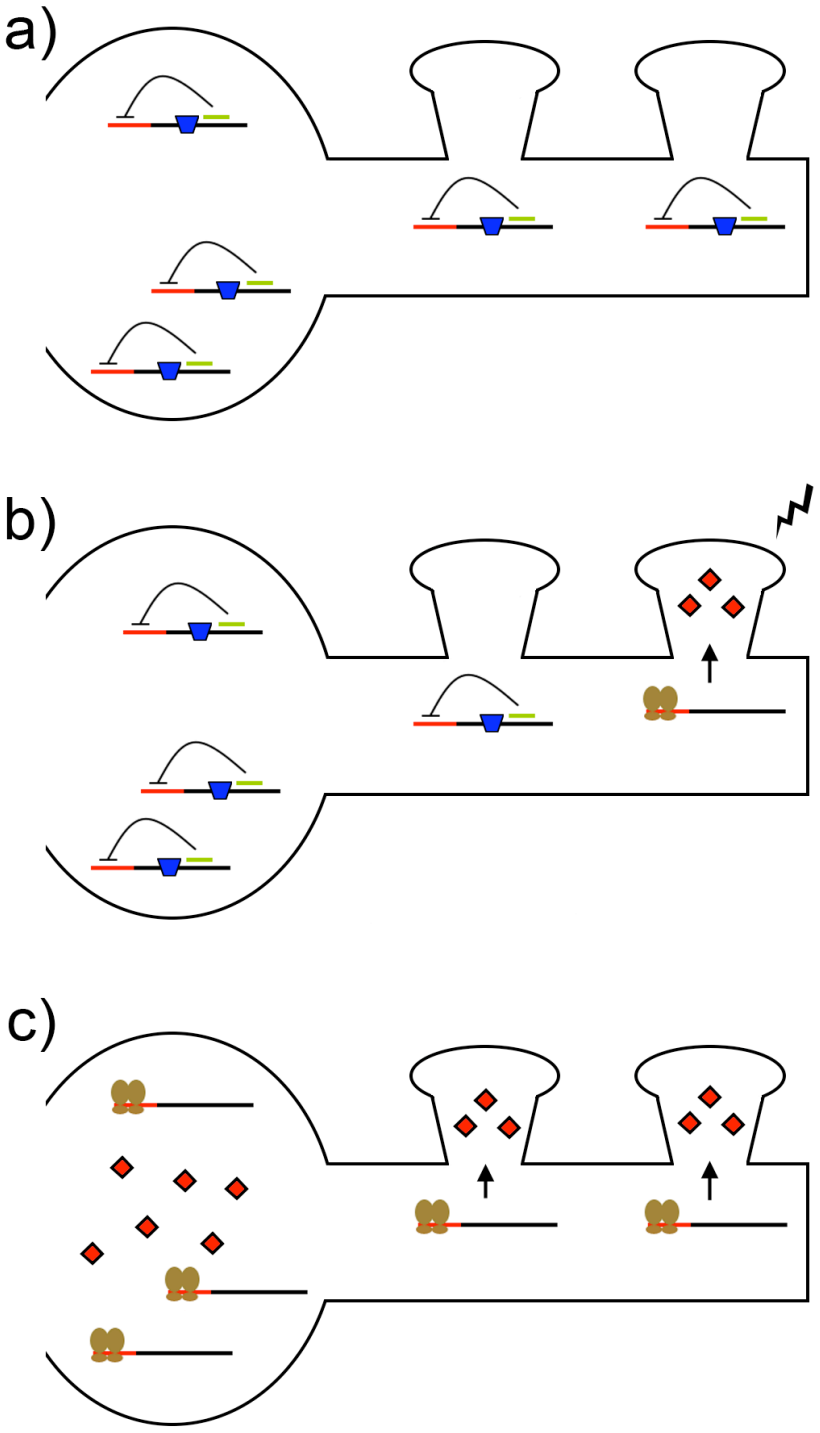
mRNA level was inversely correlated with that of miR-128 in the pilocarpine induced seizure paradigm (Figure 4-4d), suggesting that miR-128 down-regulation indeed depends on the level of neuronal activation. In contrast to the activity-dependent down-regulation of miR128, miR-495, an additional microRNA predicted to specifically target the long 3'UTR BDNF mRNA, did not decrease upon neuronal activity (Figure 4-4c). Taken together, these data suggest a possible role of miR-128 in the rapid translational de-repression of long 3'UTR BDNF mRNA upon neuronal activation.

4.3 Discussion

Our studies suggested possible trans-acting factors that regulate the translation status of the long 3'UTR BDNF mRNA. We first identified the ability of FMRP to preferentially associate with the long 3'UTR BDNF mRNA, which provides an intriguing clue that FMRP may be involved in translation regulation of BDNF via the long 3'UTR. We also identified a microRNA, miR-128, that specifically represses the translation of luciferase reporter through the BDNF long 3'UTR (Figure 4-2 a-c), most likely at the predicted miR-128 binding sites. Furthermore, the expression levels of mature miR-128 decrease rapidly upon neuronal activation (Figure 4-4), suggesting a possible involvement of miR-128 in the activity-dependent translational derepression of the long 3'UTR BDNF mRNA (Chapter 2). Based on these data, we propose the following model (Figure 4-5): under resting conditions, the long 3'UTR BDNF mRNA is specifically bound by trans-acting factors represented by FMRP and miR128, and sequestered from active translation. Considering the fact that majority of the dendritic BDNF mRNA

Figure 4-5: Model. a) At rest, the long 3'UTR BDNF mRNA (black and red line) interacts with either FMRP (blue trapazoid) or miR-128 (green line) leading to repression of BDNF translation and dendritic localization. b) Upon normal physiological stimulation, the FMRP-mediated or miR-128-mediated repression of the long 3'UTR BDNF mRNA is released allowing for increased BDNF protein (red diamonds) synthesis in the activated synapse. While the long 3'UTR BDNF mRNA remains suppressed in the remaining quiet synapses. c) However, upon pathological stimulation as seen during seizure induction, there is a loss of FMRP/miR-128 repression of the long 3'UTR BDNF mRNA throughout the neuron, leading to increased levels of BDNF protein and malfunction of BDNF signaling.

Figure 4-5



harbors the long 3'UTR (Chapter 2), upon physiological level of neuronal activity, the reduction in the levels of miR-128 could lead to increased local translation of BDNF protein within the dendrites, which could help promote LTP in specific synapses close to the sites of local BDNF production (Chapter 2, An JJ Cell 2008). On the other hand, robust neuronal activity exhibited by seizure induction could lead to a global translation derepression of the long 3'UTR BDNF mRNA in both soma and dendrites. Such regulation conceivably may serve as the primary source of BDNF production upon the onset of seizure, considering the fact that translation from the short BDNF mRNA is largely suppressed.

Pilocarpine-induced robust neuronal activation leads to the rapid reduction of miR-128 while having no effect on miR-495 expression levels (Figure 4-3), suggesting some specificity as to which microRNAs respond to neuronal activity. Each microRNA is predicted to bind to and regulate multiple target mRNAs. Based on the Targetscan algorithm designed to predict target mRNAs for a given microRNA, approximately 681 different target mRNAs are predicted to have binding sites for miR-128. Thus the reduction of miR-128 (Figure 4-3), while potentially effecting the protein expression of BDNF via the long 3'UTR, could also alter the translation of a multitude of other mRNAs, especially upon synaptic stimulation; thus potentially being a general mechanism for regulating synaptic development and plasticity.

It is thought that mRNAs, such as β-actin mRNA and the CamKIIα mRNA, are transported to dendrites as translationally repressed granules; where synaptic stimulation will result in local protein synthesis (Bassell and Kelic, 2004; Huang et al., 2002b; Huttelmaier et al., 2005; Kiebler and Bassell, 2006). Moreover, selective RNA-binding

proteins that are known to suppress translation can facilitate dendritic localization of their target mRNAs, represented by the zip code-binding protein (ZBP1) (Huttelmaier et al., 2005) and the cytoplasmic polyadenylation element (CPE)-binding protein (CPEB) (Huang et al., 2002b; Wu et al., 1998). Thus, an interesting question to be addressed in the future regards whether the microRNA- and/or FMRP-mediated translation repression of the long 3'UTR BDNF mRNA (Figure 4-1 and 4-2) may help to promote dendritic localization of BDNF mRNA (An et al., 2008).

A related question concerns whether and how FMRP and miR-128 work independently or in coordination with each other. Immunoprecipitation of FMRP demonstrated the association of FMRP with microRNA and some of the components of the microRNA-processing pathway; including Argonaute2, the p68 RNA helicase, and active Dicer (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004b). Interestingly, recent data also shows that FMRP is a microRNA acceptor protein after cleavage by Dicer, facilitating the annealing of the microRNA to its target mRNA (Plante et al., 2006). In fact, recent evidence demonstrated an enrichment of miR-128 in FMRP-immunoprecipitates from 3 month old wild type mouse brains compared to that of age-matched Fmr1 KO mouse brains (Edbauer et al., 2010). Combined, these data lead to the question of whether the tripartite binding of FMRP, miR-128 and the long 3'UTR BDNF mRNA is required for the long 3'UTR-mediated translational repression BDNF mRNA at rest which needs to be further explored.

Interestingly, both microRNA components and FMRP have been localized to structures known as processing bodies, or P-bodies (Barbee et al., 2006; Cougot et al., 2008; Liu et al., 2005a; Sen and Blau, 2005). P-bodies are cytoplasmic granules that are

involved in translational repression and mRNA decay (Parker and Sheth, 2007). Recently, P-bodies have been found in the neuronal soma and at the base of dendritic spines (Vessey et al., 2006; Zeitelhofer et al., 2008a). The granules are composed of proteins important in translation repression and mRNA decay as well as a subset of mRNAs (Parker and Sheth, 2007). It has been proposed that P-bodies are a site of storage of translationally repressed mRNAs, thus preventing degradation of the mRNA (Zeitelhofer et al., 2008b), until a signal dissembles the P-bodies releasing the mRNA to be translated. Emerging evidence demonstrates that chemically induced neuronal activation results in a reduction of the dendritic P-bodies (Zeitelhofer et al., 2008a), suggesting a release of translational repression and subsequent increase in protein synthesis upon neuronal stimulation. Thus binding of miR-128 and FMRP could promote the incorporation of the long 3'UTR BDNF mRNA into translationally repressed dendritic P-bodies for storage until neuronal activation releases the long 3'UTR BDNF mRNA from the P-bodies to be actively translated in a synapse specific manner.

4.4 Materials and Methods

Constructs

The luciferase coding sequence from the pGL3-basic vector (Promega) was inserted into the pcDNA3 vector (Invitrogen), designated pLuci3. The short 3'UTR including the proximal BDNF polyadenylation site (750bp-1113bp) was generated by PCR amplification from mouse genomic DNA and inserted between the Xho and Xba sites downstream of the luciferase coding region in pLuci3. The 2.9 kb full length 3'UTR

was subcloned into pLuci3 between the same restriction sites. The long 3'UTR lacking the proximal polyadenylation site was generated by PCR amplification of two fragments, 750bp-1013bp and 1147bp to 3604bp, from mouse genomic DNA and sequentially inserted into pLuci3. The Δ128 long 3'UTR luciferase construct, which deleted the base pairs 1554 to 2080 from the start of the 3'UTR, was generated by site directed mutagenesis (Stratagene) using the following primers: 5' – caataatgatattaatgcagcagactca gggagtgaagatacca – 3' (forward) and 5' – tggtatcttcactccctgagtctgctgcattaatatcattattg – 3' (reverse). The construct was verified by sequencing. The pTk-Ren vector (Promega) was used as transfection control and as template for *in vitro* transcription of renilla mRNA.

To construct the RPA probe, two complementary primers containing the mature miR-128 sequence with an additional 10 nucleotides of the pre-miR-128a stem-loop structure along with a 5'-A overhang. Primer sequences are 5' – ggtttacatcacagtgaaccgg tctctttttcagctgcA – 3' (forward) and 5' – gcagctgaaaaagagaccggttcactgtgatgtaaaccA – 3' (reverse). The primers were annealed by incubating at 95°C for 5 minutes then allowed to cool to room temperature slowly. The annealed primers were then subcloned into the pDrive vector. Once inserted the pDrive-miR128 was sequenced to determine orientation with respect to T7 and SP6 promoters. The β-actin RPA probe vector was purchased from Ambion.

Cell Culture, transfection and luciferase assay

CAD cells were maintained in DMEM/F12 (Cellgro) containing 10% FBS (Atlanta Biologicals) as previously described (Wang et al., 2008). The CAD cells were transfected with *in vitro* transcribed RNA corresponding to the long 3'UTR and short

3'UTR luciferase reporter constructs. The *in vitro* transcribed RNA was made from XbaI linearized reporter constructs harboring the long or short 3'UTR. The pTK-Ren, renilla plasmid was linearized with BamHI to generate *in vitro* synthesized mRNA to serve as transfection control. Once linearized, 5' capped RNA was made using mMessage mMachine kit (Ambion) following manufacturers protocol. Briefly, 1μg of linearized DNA was incubated for 2 hours at 37°C with T7 polymerase, followed by treatment with DNAse to remove template. After DNAse treatment, the in vitro transcribed RNA was recovered by LiCl precipitation. The concentration and purity of in vitro transcribed RNA was measured using the Biochip platform. Once made, equal moles (0.337 pmoles) of long and short 3'UTR reporter RNA and 100ng of renilla RNA were transfected by TransMessenger Transfection Reagent (Qiagen) into 24 well dishes of CAD cells with either 100nM of miR-128 siRNA or scrambled siRNA. The RNA was incubated for 2 hours and then the media removed and replaced with complete growth media. The cells were harvested 4 hours after media change and luciferase assay performed according to below.

HEK cells were maintained in DMEM (Cellgro) containing 10% FBS (Atlanta Biologicals) and pencillin/streptomycin at 37°C in 5% CO_2. HEK cells stably expressing either miR-128 or miR-23 were generated by selecting with puromycin following transfection by Lipofectamine 2000 (Invitrogen) with either miR-128 pEF1 or miR-23 pEF1 constructs. The HEK cells were cultured in 24 well dishes and then transfected with 100ng of firefly luciferase reporter constructs containing BDNF long 3'UTR or Δ128 long 3'UTR with 50ng of pTK-Renilla plasmid (Promega) to ensure comparable transfection efficiency using Lipofectamine 2000 (Invitrogen). Twenty-four hours after

transfection, the cells were harvested per manufacturers protocol and luciferase activity measured.

The cells were washed once with PBS and then incubated with 100μL of 1X passive lysis buffer (Promega) at room temperature for 15 mins on rotating shaker. Then 10μL of cell lysates were subjected to dual luciferase assay (Promega) in a Mediators phL luminometer as described in Chapter 2.4. An aliquot of the cells was used to prepare total RNA with TRIzol (Invitrogen). Translation activity from each construct was determined by normalizing the firefly luciferase readings to firefly luciferase mRNA levels determined by qRT-PCR.

FMRP Immunoprecipitation

The cerebrums of P7 Fmr1 KO mice and wt control were homogenized in lysis buffer (Brown et al., 2001) and subjected to high-speed centrifugation as previously described (Lu et al., 2004). Immunoprecipitation was performed using the 7G-1-1 monoclonal antibody against FMRP. The RNA from immunoprecipitates was isolated by phenol-chloroform extraction followed by ethanol precipitation. Then 1μg of input RNA and the entire isolated IP RNA sample was used for RT-PCR, as described below.

Ribonuclease Protection Assay (RPA)

To make the radiolabeled RPA probe, the miR-128 and β-actin probe constructs described above were linearized by digestion with PstI and HinfI, respectively, and purified by phenol/chloroform extraction followed by ethanol precipitation. The linearized DNA was then *in vitro* transcribed (Stratagene) in the presence of ^{32}P-UTP to generate the anti-sense radiolabeled probe. RPA was carried out by following the *mirVana* miRNA detection kit protocol (Ambion). Briefly, 1μL of each radiolabeled

probe was mixed with 2μg of total RNA, 10μL of 2X hybridization buffer, 5μg of yeast tRNA, and then incubated at 95°C for 3 min followed by hybridization overnight at 42°C. The following day, the remaining unhybridized RNA and probe were digested by an RNAse A/T1 mixture for 30 min at 37°C and then the RNA was precipitated and run on a 15% denaturing acrylamide gel. The RNA protected radiolabeled-probe signal was detected by phosphoimager (Typhoon).

Pilocarpine-induced neuronal activity

Adult male Sprague Dawley rats (Charles River Labs, Wilmington, MA), 40-50 days of age and 200-250 gram body weight, were used in all experiments. Animals were treated according to NIH regulations under the approval of the Emory University IACUC. Status epilepticus (SE) was induced in these rats as previously described (Huang et al., 2002a). Briefly, rats were injected with a mixture of methylscopolamine and terbutaline (2.5 mg/kg i.p.). After 20 min, rats were injected with pilocarpine HCl (380-400 mg/kg s.c.) or an equivalent volume of saline. Pilocarpine reliably induced stage V seizures, characterized by distinct motor behaviors including forelimb clonus, loss of postural control, rearing and falling. Animals presenting these behaviors with increased seizure intensity, duration and frequency 20- 40 min after the injection of pilocarpine were declared to be in SE. The rats were anesthetized with isoflurane before having head removed by guillotine. The hippocampi were then dissected from control or pilocarpine-treated rats 30 minutes after the initial on set of stage V seizure and total RNA was isolated by Trizol.

Tetraethylammonium (TEA)-evoked neuronal activation

Primary hippocampal neurons from E18 Sprague Dawley rats were cultured in 60mm dishes (Banker and Cowan, 1977). At DIV21, the neurons were then incubated in chemical long-term potentiation medium (140mM NaCl, 5mM KCl, 5mM $CaCl_2$, 0.1mM $MgCl_2$, 10mM glucose, 25mM HEPES, pH 7.4 and 25mM TEA) for 10 minutes at 37°C. The media was then changed from chemical LTP medium to complete Neurobasal media and incubated for an additional 10 minutes at 37°C. The control neurons were maintained in complete Neurobasal media until ready to be harvested. After washing two times in PBS, the cells were harvested in 500μL of TRIzol to isolate total RNA.

RNA isolation and real-time RT-PCR

Total RNA extracted by Trizol was quantified by OD260 reading and further confirmed by ethidium bromide-stained agarose gel electrophoresis prior to RT-PCR. Reverse transcription was performed in 20μL final volume using 1μg of total RNA with random primers (Promega) and Superscript II RNaseH-reverse transcriptase (Invitrogen), followed by real-time PCR analysis. Real time PCR was performed using iCYCLER (BioRad) and SYBR-green master mix (NEB), per manufacturer's protocol. Basically, 5μL of a 1:5 dilution of the RT reaction was mixed with 5μL of 2.5μM mixed primer set then 10μL of 2X reaction buffer was added. Standard curves were established for all primers using reverse transcribed cDNA from serial dilutions of a 50 ng/μL mouse hippocampal total RNA. The following primer pairs were used for real-time detection: GAPDH: 5'-cacagtcaaggctgagaatgggaa-3' (forward), 5'-gtggttcacacccatcacaaacatg-3' (reverse); pan BDNF: 5'-gccgcaaacatgtctatgagggtt-3' (forward), 5'-ttggcctttggataccgggacttt-3' (reverse); long 3'UTR BDNF: 5'-caggaggaatttctgagtggcca-3'

(forward), 5'-gcagaaggcctaagcaacttgaca-3' (reverse); firefly luciferase: 5'-aagattcaaagtgcgctgctggtg-3' (forward), 5'-ccgcttccccgacttccttagag-3' (reverse), and cfos: 5'-agaaggggcaaagtagagcag-3' (forward), 5'-cgcagacttctcgtcttcaagt-3' (reverse).

MicroRNA real-time RT-PCR

The levels of microRNA were measured from total RNA isolated from control and stimulated samples by Taqman microRNA assay (Applied Biosystems Inc.), per manufacturer's protocol. Briefly, total RNA was diluted to 2 ng/μL in dH_2O, then 5μL (10ng) was added to a final reaction volume of 15μL, which was used for reverse transcription by Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems Inc.) with primer specific for the desired microRNA (miR-128) or loading (U6 rRNA). For the Taqman assay, 1.5μL of cDNA template along with 1μL of microRNA specific primer mix was used per 20μL reaction. The real-time PCR was performed in triplicate in a 96-well plate using 7500 Fast Real-Time PCR system (ABI). The data was analyzed by taken $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{(microRNA)} - Ct_{(U6)}$. Statistical significance was determined by performing a Student's t-test with a $p < 0.05$.

Chapter 5: Conclusion and Future Directions

In conclusion, we show the preferential ability of the distinct 3'UTRs to regulate the subcellular localization and translation of BDNF mRNA in an activity dependent manner, which may be controlled by the trans-acting factors, microRNA and FMRP, two pathways involved in synapse formation and synaptic plasticity (Huber et al., 2002; Impey et al., 2009; Schratt et al., 2006; Shang et al., 2009; Wayman et al., 2008). In addition, we show a novel role for FMRP in regulating axonal development in the hippocampus, providing the first evidence for the essential role of FMRP in presynaptic development, which should have critical influence on dendritic spine maturation.

In the first section of this thesis, we demonstrated that distinct 3'UTRs of BDNF, which are generated by alternative polyadenylation site usage, play opposing roles in controlling dendritic localization and translation of BDNF in response to neuronal activity changes. We show that the short 3'UTR BDNF mRNA is critical for maintaining basal BDNF protein production within the neuronal soma. In contrast, the long 3'UTR BDNF mRNA is translationally repressed at rest, but upon robust neuronal activity becomes actively translated, suggesting that the long 3'UTR-dependent BDNF translation serves as the major source for BDNF protein production during the insult of seizure in hippocampal neurons. In addition, dendritic localization and the activity-dependent translation of the long 3'UTR BDNF transcript offers a possible mechanism underlying increased BDNF protein production during changes in synaptic plasticity. However, the cis-acting elements responsible for this means of regulation are unknown and will require further exploration.

It is important to point out that more than 50% of all human genes contain multiple polyadenylation sites, giving rise to distinct 3'UTRs (Tian et al., 2005). However, whether and how these alternative 3'UTRs control the expression of the same encoded protein in response to extracellular and developmental signals remains unknown. Thus, our data offers a model whereby alternative polyadenylation site usage can provide a layer of complexity to regulate the differential expression of the same encoded protein. Interestingly, the gene encoding TrkB produces transcripts with distinct 3'UTRs, long and short, based on polyadenylation site usage, similar to the BDNF gene. TrkB mRNA, like BDNF mRNA, is transported to distal dendrites upon neuronal stimulation, followed by an increase in TrkB protein levels (Tongiorgi et al., 1997). This poses the question whether the distinct 3'UTRs in TrkB transcripts may differentially promote subcellular localization and translation of TrkB mRNA upon neurocircuitry stimulation. Furthermore, it is worthwhile to consider that the alternative 3'UTRs in TrkB and BDNF may be regulated in a coordinate manner to promote simultaneous increase in BDNF-TrkB signaling in the synapse during neuronal activation.

In the second part of this dissertation, we explored the function of FMRP in controlling translation of MAP1B in early neuronal processes and neural network development. We provide the first direct evidence that FMRP represses the translation of MAP1B mRNA. Further, we show abnormally elevated levels of MAP1B protein in the hippocampal mossy fibers of Fmr1 KO mice, where FMRP and MAP1B mRNA are colocalized. The loss of FMRP-mediated translation repression leads to increased neurite extension in an immortal neuronal-like cell line and overprojection of the axons that make up the infrapyramidal bundle of the hippocampal mossy fibers in mice. Given that

FMRP has a multitude of mRNA ligands, the role that the loss of FMRP-mediated translational repression of MAP1B plays in aberrant hippocampal mossy fibers development in Fmr1 KO mice needs further exploration through the use of mice whose MAP1B protein levels have been genetically decreased ($MAP1B^{+/-}$), which should correct over expression of MAP1B cause by the lack of FMRP.

Furthermore, in conjunction with the abnormally increased MAP1B protein and mossy fiber overprojection, the zinc transporter 3 (ZnT3) expression levels are increased in Fmr1 KO mice, which could signal a concomitant increase in the release of free zinc into synaptic cleft of the axonal terminals in the hippocampal mossy fibers (Palmiter et al., 1996). Although direct evidence of increased zinc release in Fmr1 KO needs to be provided by future studies, the increase of ZnT3 most likely leads to an increase in the amount of zinc released into the synapse of Fmr1 KO mice following neuronal stimulation, which could have a multitude of effects on synaptic formation and function. Interestingly, zinc is required for activation of the matrix metalloproteinases, enzymes that cleave proteins of the extracellular matrix and play a role in dendritic spine development (Bilousova et al., 2006; Tian et al., 2007). Recent evidence demonstrates increased expression and activity levels of the matrix metalloproteinase-9 (MMP-9) in Fmr1 KO mice (Bilousova et al., 2009). Inhibition of MMP-9 activity in Fmr1 KO mice rescues the immature dendritic spine abnormalities seen in FXS brains (Bilousova et al., 2009), suggesting that increased zinc may potentiate MMP-9's pathological effect in synapse formation. Together, these data propose a novel role for FMRP in the proper targeting and development of axons, and further suggests a role for FMRP in both pre- and post synaptic abnormalities common in FXS.

The link between cis-acting factors and the specific trans-acting factors necessary for post-transcriptional regulation is poorly understood. An interesting link between the BDNF and FMRP pathways was suggested by our preliminary data that FMRP associates specifically with the long 3'UTR BDNF mRNA, suggesting that FMRP might plays a role in regulating BDNF translation to accommodate neuronal activity changes and synaptic plasticity. Thus if FMRP preferentially regulates translation of the long 3'UTR BDNF mRNA upon neuronal activation, the loss of FMRP could impair the activity-dependent BDNF protein synthesis mediated by the long 3'UTR, potentially altering synaptic plasticity in Fmr1 KO mice. In addition, the loss of FMRP could yield an increase in the steady state levels of BDNF protein throughout the cell, which could cause overactivation of TrkB signaling. Interestingly, both BDNF and zinc can independently activate TrkB in the mossy fibers of the hippocampus (Huang et al., 2008; Nagappan and Lu, 2005). The potential for persistent, overactivation of the TrkB signaling pathway in the mossy fibers of Fmr1 KO mice could play important roles in seizure development. Interestingly, overexpression of TrkB in mice leads to more severe chemically induced status epilepticus (Lahteinen et al., 2004). In contrast, mice lacking TrkB signaling specifically in the dentate gyrus-CA3 regions exhibits complete abolishment of kindling induced seizure development (He et al., 2004). Thus, increased TrkB signaling in Fmr1 KO, potentially mediated by increased free synaptic zinc and/or BDNF release, could lead to increased susceptibility to seizure development, commonly associated with FXS children (Berry-Kravis, 2002).

In addition to the possible FMRP-mediated translation regulation of the long 3'UTR BDNF mRNA, bioinformatics analysis has indicated hypothetical microRNA

binding sites for miR-128 specifically for the long 3'UTR BDNF mRNA and not the short 3'UTR BDNF mRNA or coding region. miR-128 is a brain enriched microRNA and is indeed able to repress the translation efficiency of a luciferase reporter fused to the BDNF long 3'UTR, which is mediated by the region containing the miR-128 binding sites, but not the short 3'UTR. Emerging evidence has indicated a role for microRNA in synaptic plasticity by acting as a translation switch in synapses in response to neuronal activity (Schratt et al., 2006). In the case of both physiological and pathological neuronal stimulation, the levels of mature miR-128 are significantly reduced, suggesting a loss of miR-128 mediated translation repression of the long 3'UTR BDNF mRNA after seizure induction. Mature miR-128 was observed in the isolated synaptic fraction suggesting a possible mechanism whereby miR-128 potentially regulates the translation of the BDNF mRNA via the long 3'UTR in dendrites and/or synapses, contributing to the function of BDNF in synaptic plasticity. Recently, miR-128 was reported to be associated with FMRP complexes in the brain (Edbauer et al., 2010). This data suggests that either FMRP and miR-128 can act independently to regulate the expression of BDNF through the long 3'UTR or an intriguing possibility for the formation of a tripartite complex between FMRP, miR-128, and the long 3'UTR BDNF mRNA to mediate the BDNF protein production during neuronal activity. Precise mechanism for FMRP and miR128 in regulating BDNF translation, and whether dysregulation of BDNF-TrkB signaling in FXS are important questions that warrant rigorous investigation by future studies.

Chapter 6: References

Aakalu G, Smith WB, Nguyen N, Jiang C and Schuman EM (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30**(2):489-502.

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER and Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* **88**(5):615-626.

Abraham H, Veszpremi B, Kravjak A, Kovacs K, Gomori E and Seress L (2009) Ontogeny of calbindin immunoreactivity in the human hippocampal formation with a special emphasis on granule cells of the dentate gyrus. *Int J Dev Neurosci* **27**(2):115-127.

Abraham WC, Logan B, Greenwood JM and Dragunow M (2002) Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. *J Neurosci* **22**(21):9626-9634.

Aid T, Kazantseva A, Piirsoo M, Palm K and Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. *J Neurosci Res* **85**(3):525-535.

Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev* **89**(1):121-145.

Amaral DG and Dent JA (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J Comp Neurol* **195**(1):51-86.

Ambros V (2004) The functions of animal microRNAs. *Nature* **431**(7006):350-355.

An JJ, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B and Xu B (2008) Distinct Role of Long 3' UTR BDNF mRNA in Spine

Morphology and Synaptic Plasticity in Hippocampal Neurons. *Cell* **134**(1):175-187.

Aniksztejn L and Ben-Ari Y (1991) Novel form of long-term potentiation produced by a K+ channel blocker in the hippocampus. *Nature* **349**(6304):67-69.

Antar LN and Bassell GJ (2003) Sunrise at the synapse: the FMRP mRNP shaping the synaptic interface. *Neuron* **37**(4):555-558.

Antar LN, Li C, Zhang H, Carroll RC and Bassell GJ (2006) Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. *Mol Cell Neurosci* **32**(1-2):37-48.

Atkins CM, Nozaki N, Shigeri Y and Soderling TR (2004) Cytoplasmic polyadenylation element binding protein-dependent protein synthesis is regulated by calcium/calmodulin-dependent protein kinase II. *J Neurosci* **24**(22):5193-5201.

Bagri A, Cheng HJ, Yaron A, Pleasure SJ and Tessier-Lavigne M (2003) Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. *Cell* **113**(3):285-299.

Bak M, Silahtaroglu A, Moller M, Christensen M, Rath MF, Skryabin B, Tommerup N and Kauppinen S (2008) MicroRNA expression in the adult mouse central nervous system. *RNA* **14**(3):432-444.

Banker GA and Cowan WM (1977) Rat hippocampal neurons in dispersed cell culture. *Brain Res* **126**(3):397-342.

Barbee SA, Estes PS, Cziko AM, Hillebrand J, Luedeman RA, Coller JM, Johnson N, Howlett IC, Geng C, Ueda R, Brand AH, Newbury SF, Wilhelm JE, Levine RB, Nakamura A, Parker R and Ramaswami M (2006) Staufen- and FMRP-containing

neuronal RNPs are structurally and functionally related to somatic P bodies. *Neuron* **52**(6):997-1009.

Barde YA, Edgar D and Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. *EMBO J* **1**(5):549-553.

Bardoni B and Mandel JL (2002) Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. *Curr Opin Genet Dev* **12**(3):284-293.

Barrett GL, Reid CA, Tsafoulis C, Zhu W, Williams DA, Paolini AG, Trieu J and Murphy M (2010) Enhanced spatial memory and hippocampal long-term potentiation in p75 neurotrophin receptor knockout mice. *Hippocampus* **20**(1):145-152.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2):281-297.

Bassell GJ and Kelic S (2004) Binding proteins for mRNA localization and local translation, and their dysfunction in genetic neurological disease. *Curr Opin Neurobiol* **14**(5):574-581.

Bath KG, Mandairon N, Jing D, Rajagopal R, Kapoor R, Chen ZY, Khan T, Proenca CC, Kraemer R, Cleland TA, Hempstead BL, Chao MV and Lee FS (2008) Variant brain-derived neurotrophic factor (Val66Met) alters adult olfactory bulb neurogenesis and spontaneous olfactory discrimination. *J Neurosci* **28**(10):2383-2393.

Bear MF, Huber KM and Warren ST (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* **27**(7):370-377.

Bechara EG, Didiot MC, Melko M, Davidovic L, Bensaid M, Martin P, Castets M, Pognonec P, Khandjian EW, Moine H and Bardoni B (2009) A novel function for fragile X mental retardation protein in translational activation. *PLoS Biol* **7**(1):e16.

Berdnik D, Fan AP, Potter CJ and Luo L (2008) MicroRNA processing pathway regulates olfactory neuron morphogenesis. *Curr Biol* **18**(22):1754-1759.

Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH and Cuppen E (2005) Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* **120**(1):21-24.

Berry-Kravis E (2002) Epilepsy in fragile X syndrome. *Dev Med Child Neurol* **44**(11):724-728.

Bertrand MJ, Kenchappa RS, Andrieu D, Leclercq-Smekens M, Nguyen HN, Carter BD, Muscatelli F, Barker PA and De Backer O (2008) NRAGE, a p75NTR adaptor protein, is required for developmental apoptosis in vivo. *Cell Death Differ* **15**(12):1921-1929.

Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW and Ethell IM (2009) Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet* **46**(2):94-102.

Bilousova TV, Rusakov DA, Ethell DW and Ethell IM (2006) Matrix metalloproteinase-7 disrupts dendritic spines in hippocampal neurons through NMDA receptor activation. *J Neurochem* **97**(1):44-56.

Binder DK, Croll SD, Gall CM and Scharfman HE (2001) BDNF and epilepsy: too much of a good thing? *Trends Neurosci* **24**(1):47-53.

Bitanihirwe BK and Cunningham MG (2009) Zinc: the brain's dark horse. *Synapse* **63**(11):1029-1049.

Blichenberg A, Rehbein M, Muller R, Garner CC, Richter D and Kindler S (2001) Identification of a cis-acting dendritic targeting element in the mRNA encoding the alpha subunit of Ca2+/calmodulin-dependent protein kinase II. *Eur J Neurosci* **13**(10):1881-1888.

Blichenberg A, Schwanke B, Rehbein M, Garner CC, Richter D and Kindler S (1999) Identification of a cis-acting dendritic targeting element in MAP2 mRNAs. *J Neurosci* **19**(20):8818-8829.

Bliss TV, Chung SH and Stirling RV (1974) Proceedings: Structural and functional development of the mossy fibre system in the hippocampus of the post-natal rat. *J Physiol* **239**(2):92P-94P.

Bohnsack MT, Czaplinski K and Gorlich D (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**(2):185-191.

Bouquet C, Soares S, von Boxberg Y, Ravaille-Veron M, Propst F and Nothias F (2004) Microtubule-associated protein 1B controls directionality of growth cone migration and axonal branching in regeneration of adult dorsal root ganglia neurons. *J Neurosci* **24**(32):7204-7213.

Bourne JN and Harris KM (2008) Balancing structure and function at hippocampal dendritic spines. *Annu Rev Neurosci* **31**:47-67.

Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, Jin X, Feng Y, Wilkinson KD, Keene JD, Darnell RB and Warren ST (2001) Microarray

identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* **107**(4):477-487.

Bruckenstein DA, Lein PJ, Higgins D and Fremeau RT, Jr. (1990) Distinct spatial localization of specific mRNAs in cultured sympathetic neurons. *Neuron* **5**(6):809-819.

Brugg B, Reddy D and Matus A (1993) Attenuation of microtubule-associated protein 1B expression by antisense oligodeoxynucleotides inhibits initiation of neurite outgrowth. *Neuroscience* **52**(3):489-496.

Buckley PF, Mahadik S, Pillai A and Terry A, Jr. (2007) Neurotrophins and schizophrenia. *Schizophr Res* **94**(1-3):1-11.

Capsoni S, Tongiorgi E, Cattaneo A and Domenici L (1999) Differential regulation of brain-derived neurotrophic factor messenger RNA cellular expression in the adult rat visual cortex. *Neuroscience* **93**(3):1033-1040.

Castren E, Berninger B, Leingartner A and Lindholm D (1998) Regulation of brain-derived neurotrophic factor mRNA levels in hippocampus by neuronal activity. *Prog Brain Res* **117**:57-64.

Caudy AA, Myers M, Hannon GJ and Hammond SM (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev* **16**(19):2491-2496.

Chan JP, Cordeira J, Calderon GA, Iyer LK and Rios M (2008) Depletion of central BDNF in mice impedes terminal differentiation of new granule neurons in the adult hippocampus. *Mol Cell Neurosci* **39**(3):372-383.

Chang S, Johnston RJ, Jr., Frokjaer-Jensen C, Lockery S and Hobert O (2004) MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. *Nature* **430**(7001):785-789.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R and Greenberg ME (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* **302**(5646):885-889.

Chiaruttini C, Sonego M, Baj G, Simonato M and Tongiorgi E (2008) BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae. *Mol Cell Neurosci* **37**(1):11-19.

Christie SB, Akins MR, Schwob JE and Fallon JR (2009) The FXG: a presynaptic fragile X granule expressed in a subset of developing brain circuits. *J Neurosci* **29**(5):1514-1524.

Chuang SC, Zhao W, Bauchwitz R, Yan Q, Bianchi R and Wong RK (2005) Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. *J Neurosci* **25**(35):8048-8055.

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA and Palmiter RD (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. *Proc Natl Acad Sci U S A* **96**(4):1716-1721.

Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ and Greenough WT (1997) Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc Natl Acad Sci U S A* **94**(10):5401-5404.

Corbin F, Bouillon M, Fortin A, Morin S, Rousseau F and Khandjian EW (1997) The fragile X mental retardation protein is associated with poly(A)+ mRNA in actively translating polyribosomes. *Hum Mol Genet* **6**(9):1465-1472.

Costa-Mattioli M, Gobert D, Harding H, Herdy B, Azzi M, Bruno M, Bidinosti M, Ben Mamou C, Marcinkiewicz E, Yoshida M, Imataka H, Cuello AC, Seidah N, Sossin W, Lacaille JC, Ron D, Nader K and Sonenberg N (2005) Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature* **436**(7054):1166-1173.

Cougot N, Bhattacharyya SN, Tapia-Arancibia L, Bordonne R, Filipowicz W, Bertrand E and Rage F (2008) Dendrites of mammalian neurons contain specialized P-body-like structures that respond to neuronal activation. *J Neurosci* **28**(51):13793-13804.

Croll SD, Suri C, Compton DL, Simmons MV, Yancopoulos GD, Lindsay RM, Wiegand SJ, Rudge JS and Scharfman HE (1999) Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. *Neuroscience* **93**(4):1491-1506.

Dai X, Qu P and Dreyfus CF (2001) Neuronal signals regulate neurotrophin expression in oligodendrocytes of the basal forebrain. *Glia* **34**(3):234-239.

Danscher G (1981) Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. *Histochemistry* **71**(1):1-16.

Danscher G and Zimmer J (1978) An improved Timm sulphide silver method for light and electron microscopic localization of heavy metals in biological tissues. *Histochemistry* **55**(1):27-40.

Danzer SC, Crooks KR, Lo DC and McNamara JO (2002) Increased expression of brain-derived neurotrophic factor induces formation of basal dendrites and axonal branching in dentate granule cells in hippocampal explant cultures. *J Neurosci* **22**(22):9754-9763.

Danzer SC, He X and McNamara JO (2004) Ontogeny of seizure-induced increases in BDNF immunoreactivity and TrkB receptor activation in rat hippocampus. *Hippocampus* **14**(3):345-355.

Danzer SC, Kotloski RJ, Walter C, Hughes M and McNamara JO (2008) Altered morphology of hippocampal dentate granule cell presynaptic and postsynaptic terminals following conditional deletion of TrkB. *Hippocampus* **18**(7):668-678.

Danzer SC and McNamara JO (2004) Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. *J Neurosci* **24**(50):11346-11355.

Darnell JC, Fraser CE, Mostovetsky O, Stefani G, Jones TA, Eddy SR and Darnell RB (2005) Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes. *Genes Dev* **19**(8):903-918.

Darnell JC, Jensen KB, Jin P, Brown V, Warren ST and Darnell RB (2001) Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* **107**(4):489-499.

Davidkova G and Carroll RC (2007) Characterization of the role of microtubule-associated protein 1B in metabotropic glutamate receptor-mediated endocytosis of AMPA receptors in hippocampus. *J Neurosci* **27**(48):13273-13278.

Davidovic L, Jaglin XH, Lepagnol-Bestel AM, Tremblay S, Simonneau M, Bardoni B and Khandjian EW (2007) The fragile X mental retardation protein is a molecular adaptor between the neurospecific KIF3C kinesin and dendritic RNA granules. *Hum Mol Genet* **16**(24):3047-3058.

Davis TH, Cuellar TL, Koch SM, Barker AJ, Harfe BD, McManus MT and Ullian EM (2008) Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. *J Neurosci* **28**(17):4322-4330.

Dehmelt L and Halpain S (2004) Actin and microtubules in neurite initiation: are MAPs the missing link? *J Neurobiol* **58**(1):18-33.

Devys D, Lutz Y, Rouyer N, Bellocq JP and Mandel JL (1993) The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nat Genet* **4**(4):335-340.

Dictenberg JB, Swanger SA, Antar LN, Singer RH and Bassell GJ (2008) A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome. *Dev Cell* **14**(6):926-939.

Dudek FE and Sutula TP (2007) Epileptogenesis in the dentate gyrus: a critical perspective. *Prog Brain Res* **163**:755-773.

Dugich-Djordjevic MM, Tocco G, Willoughby DA, Najm I, Pasinetti G, Thompson RF, Baudry M, Lapchak PA and Hefti F (1992) BDNF mRNA expression in the developing rat brain following kainic acid-induced seizure activity. *Neuron* **8**(6):1127-1138.

Eberhart DE, Malter HE, Feng Y and Warren ST (1996) The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. *Hum Mol Genet* **5**(8):1083-1091.

Eberwine J, Miyashiro K, Kacharmina JE and Job C (2001) Local translation of classes of mRNAs that are targeted to neuronal dendrites. *Proc Natl Acad Sci U S A* **98**(13):7080-7085.

Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, Tada T, Dolan BM, Sharp PA and Sheng M (2010) Regulation of Synaptic Structure and Function by FMRP-Associated MicroRNAs miR-125b and miR-132. *Neuron* **65**(3):373-384.

Edelmann W, Zervas M, Costello P, Roback L, Fischer I, Hammarback JA, Cowan N, Davies P, Wainer B and Kucherlapati R (1996) Neuronal abnormalities in microtubule-associated protein 1B mutant mice. *Proc Natl Acad Sci U S A* **93**(3):1270-1275.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B and Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* **112**(2):257-269.

Elmariah SB, Crumling MA, Parsons TD and Balice-Gordon RJ (2004) Postsynaptic TrkB-mediated signaling modulates excitatory and inhibitory neurotransmitter receptor clustering at hippocampal synapses. *J Neurosci* **24**(10):2380-2393.

Eom T, Antar LN, Singer RH and Bassell GJ (2003) Localization of a beta-actin messenger ribonucleoprotein complex with zipcode-binding protein modulates the density of dendritic filopodia and filopodial synapses. *J Neurosci* **23**(32):10433-10444.

Ernfors P, Ibanez CF, Ebendal T, Olson L and Persson H (1990a) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc Natl Acad Sci U S A* **87**(14):5454-5458.

Ernfors P, Wetmore C, Olson L and Persson H (1990b) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* **5**(4):511-526.

Feng Y (2002) Fragile X mental retardation: misregulation of protein synthesis in the developing brain? *Microsc Res Tech* **57**(3):145-147.

Feng Y, Absher D, Eberhart DE, Brown V, Malter HE and Warren ST (1997a) FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. *Mol Cell* **1**(1):109-118.

Feng Y, Gutekunst CA, Eberhart DE, Yi H, Warren ST and Hersch SM (1997b) Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. *J Neurosci* **17**(5):1539-1547.

Fifkova E, Anderson CL, Young SJ and Van Harreveld A (1982) Effect of anisomycin on stimulation-induced changes in dendritic spines of the dentate granule cells. *J Neurocytol* **11**(2):183-210.

Fiore R, Siegel G and Schratt G (2008) MicroRNA function in neuronal development, plasticity and disease. *Biochim Biophys Acta* **1779**(8):471-478.

Flexner JB, Flexner LB and Stellar E (1963) Memory in mice as affected by intracerebral puromycin. *Science* **141**:57-59.

Flexner JB, Flexner LB, Stellar E, De La Haba G and Roberts RB (1962) Inhibition of protein synthesis in brain and learning and memory following puromycin. *J Neurochem* **9**:595-605.

Frey U, Krug M, Reymann KG and Matthies H (1988) Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Res* **452**(1-2):57-65.

Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ, Fenwick RG, Jr., Warren ST and et al. (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* **67**(6):1047-1058.

Garber KB, Visootsak J and Warren ST (2008) Fragile X syndrome. *Eur J Hum Genet* **16**(6):666-672.

Garner CC, Tucker RP and Matus A (1988) Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature* **336**(6200):674-677.

Gatto CL and Broadie K (2008) Temporal requirements of the fragile X mental retardation protein in the regulation of synaptic structure. *Development* **135**(15):2637-2648.

Gehler S, Shaw AE, Sarmiere PD, Bamburg JR and Letourneau PC (2004) Brain-derived neurotrophic factor regulation of retinal growth cone filopodial dynamics is mediated through actin depolymerizing factor/cofilin. *J Neurosci* **24**(47):10741-10749.

Godfraind JM, Reyniers E, De Boulle K, D'Hooge R, De Deyn PP, Bakker CE, Oostra BA, Kooy RF and Willems PJ (1996) Long-term potentiation in the hippocampus of fragile X knockout mice. *Am J Med Genet* **64**(2):246-251.

Gonzalez-Billault C, Avila J and Caceres A (2001) Evidence for the role of MAP1B in axon formation. *Mol Biol Cell* **12**(7):2087-2098.

Gonzalez-Billault C, Jimenez-Mateos EM, Caceres A, Diaz-Nido J, Wandosell F and Avila J (2004) Microtubule-associated protein 1B function during normal development, regeneration, and pathological conditions in the nervous system. *J Neurobiol* **58**(1):48-59.

Gonzalez-Billault C, Owen R, Gordon-Weeks PR and Avila J (2002) Microtubule-associated protein 1B is involved in the initial stages of axonogenesis in peripheral nervous system cultured neurons. *Brain Res* **943**(1):56-67.

Goold RG and Gordon-Weeks PR (2001) Microtubule-associated protein 1B phosphorylation by glycogen synthase kinase 3beta is induced during PC12 cell differentiation. *J Cell Sci* **114**(Pt 23):4273-4284.

Gordon-Weeks PR and Fischer I (2000) MAP1B expression and microtubule stability in growing and regenerating axons. *Microsc Res Tech* **48**(2):63-74.

Grossman AW, Elisseou NM, McKinney BC and Greenough WT (2006) Hippocampal pyramidal cells in adult Fmr1 knockout mice exhibit an immature-appearing profile of dendritic spines. *Brain Res* **1084**(1):158-164.

Hartmann M, Heumann R and Lessmann V (2001) Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J* **20**(21):5887-5897.

He XP, Kotloski R, Nef S, Luikart BW, Parada LF and McNamara JO (2004) Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. *Neuron* **43**(1):31-42.

Hennigan A, Callaghan CK, Kealy J, Rouine J and Kelly AM (2009) Deficits in LTP and recognition memory in the genetically hypertensive rat are associated with decreased expression of neurotrophic factors and their receptors in the dentate gyrus. *Behav Brain Res* **197**(2):371-377.

Hofer M, Pagliusi SR, Hohn A, Leibrock J and Barde YA (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J* **9**(8):2459-2464.

Hong EJ, McCord AE and Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. *Neuron* **60**(4):610-624.

Hou L, Antion MD, Hu D, Spencer CM, Paylor R and Klann E (2006) Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. *Neuron* **51**(4):441-454.

Hu H, Qin Y, Bochorishvili G, Zhu Y, van Aelst L and Zhu JJ (2008) Ras signaling mechanisms underlying impaired GluR1-dependent plasticity associated with fragile X syndrome. *J Neurosci* **28**(31):7847-7862.

Hu Y and Russek SJ (2008) BDNF and the diseased nervous system: a delicate balance between adaptive and pathological processes of gene regulation. *J Neurochem* **105**(1):1-17.

Huang EJ and Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* **24**:677-736.

Huang F, Chotiner JK and Steward O (2005) The mRNA for elongation factor 1alpha is localized in dendrites and translated in response to treatments that induce long-term depression. *J Neurosci* **25**(31):7199-7209.

Huang Y, Doherty JJ and Dingledine R (2002a) Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. *J Neurosci* **22**(19):8422-8428.

Huang YS, Carson JH, Barbarese E and Richter JD (2003) Facilitation of dendritic mRNA transport by CPEB. *Genes Dev* **17**(5):638-653.

Huang YS, Jung MY, Sarkissian M and Richter JD (2002b) N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. *EMBO J* **21**(9):2139-2148.

Huang YZ, Pan E, Xiong ZQ and McNamara JO (2008) Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. *Neuron* **57**(4):546-558.

Huber KM, Gallagher SM, Warren ST and Bear MF (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A* **99**(11):7746-7750.

Huber KM, Kayser MS and Bear MF (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* **288**(5469):1254-1257.

Huttelmaier S, Zenklusen D, Lederer M, Dictenberg J, Lorenz M, Meng X, Bassell GJ, Condeelis J and Singer RH (2005) Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature* **438**(7067):512-515.

Impey S, Davare M, Lasiek A, Fortin D, Ando H, Varlamova O, Obrietan K, Soderling TR, Goodman RH and Wayman GA (2009) An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. *Mol Cell Neurosci*.

Irier HA, Shaw R, Lau A, Feng Y and Dingledine R (2009) Translational regulation of GluR2 mRNAs in rat hippocampus by alternative 3' untranslated regions. *J Neurochem* **109**(2):584-594.

Irwin SA, Galvez R and Greenough WT (2000) Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cereb Cortex* **10**(10):1038-1044.

Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, Larsen BP, Kooy F, Willems PJ, Cras P, Kozlowski PB, Swain RA, Weiler IJ and Greenough WT

(2001) Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet* **98**(2):161-167.

Ishizuka A, Siomi MC and Siomi H (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev* **16**(19):2497-2508.

Jeanneteau F, Garabedian MJ and Chao MV (2008) Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proc Natl Acad Sci U S A* **105**(12):4862-4867.

Jessberger S, Clark RE, Broadbent NJ, Clemenson GD, Jr., Consiglio A, Lie DC, Squire LR and Gage FH (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learn Mem* **16**(2):147-154.

Jin P, Alisch RS and Warren ST (2004a) RNA and microRNAs in fragile X mental retardation. *Nat Cell Biol* **6**(11):1048-1053.

Jin P and Warren ST (2003) New insights into fragile X syndrome: from molecules to neurobehaviors. *Trends Biochem Sci* **28**(3):152-158.

Jin P, Zarnescu DC, Ceman S, Nakamoto M, Mowrey J, Jongens TA, Nelson DL, Moses K and Warren ST (2004b) Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat Neurosci* **7**(2):113-117.

Jin X, Hu H, Mathers PH and Agmon A (2003) Brain-derived neurotrophic factor mediates activity-dependent dendritic growth in nonpyramidal neocortical interneurons in developing organotypic cultures. *J Neurosci* **23**(13):5662-5673.

Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H and Han J (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120**(5):623-634.

Joels M (2009) Stress, the hippocampus, and epilepsy. *Epilepsia* **50**(4):586-597.

John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS (2004) Human MicroRNA targets. *PLoS Biol* **2**(11):e363.

Johnston RJ and Hobert O (2003) A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. *Nature* **426**(6968):845-849.

Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**(5544):1030-1038.

Kang H and Schuman EM (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science* **273**(5280):1402-1406.

Katoh-Semba R, Takeuchi IK, Inaguma Y, Ito H and Kato K (1999) Brain-derived neurotrophic factor, nerve growth and neurotrophin-3 selected regions of the rat brain following kainic acid-induced seizure activity. *Neurosci Res* **35**(1):19-29.

Kaufmann WE, Cohen S, Sun HT and Ho G (2002) Molecular phenotype of Fragile X syndrome: FMRP, FXRPs, and protein targets. *Microsc Res Tech* **57**(3):135-144.

Kelleher RJ, 3rd, Govindarajan A and Tonegawa S (2004) Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron* **44**(1):59-73.

Khandjian EW, Huot ME, Tremblay S, Davidovic L, Mazroui R and Bardoni B (2004) Biochemical evidence for the association of fragile X mental retardation protein

with brain polyribosomal ribonucleoparticles. *Proc Natl Acad Sci U S A* **101**(36):13357-13362.

Kiebler MA and Bassell GJ (2006) Neuronal RNA granules: movers and makers. *Neuron* **51**(6):685-690.

Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT and Mourelatos Z (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* **129**(6):1141-1151.

Kleiman R, Banker G and Steward O (1993) Subcellular distribution of rRNA and poly(A) RNA in hippocampal neurons in culture. *Brain Res Mol Brain Res* **20**(4):305-312.

Kobayashi H, Yamamoto S, Maruo T and Murakami F (2005) Identification of a cis-acting element required for dendritic targeting of activity-regulated cytoskeleton-associated protein mRNA. *Eur J Neurosci* **22**(12):2977-2984.

Kohara K, Kitamura A, Morishima M and Tsumoto T (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. *Science* **291**(5512):2419-2423.

Kojima M, Takei N, Numakawa T, Ishikawa Y, Suzuki S, Matsumoto T, Katoh-Semba R, Nawa H and Hatanaka H (2001) Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. *J Neurosci Res* **64**(1):1-10.

Kokaia M, Ernfors P, Kokaia Z, Elmer E, Jaenisch R and Lindvall O (1995) Suppressed epileptogenesis in BDNF mutant mice. *Exp Neurol* **133**(2):215-224.

Kokaia Z, Metsis M, Kokaia M, Bengzon J, Elmer E, Smith ML, Timmusk T, Siesjo BK, Persson H and Lindvall O (1994) Brain insults in rats induce increased expression of the BDNF gene through differential use of multiple promoters. *Eur J Neurosci* **6**(4):587-596.

Koyama R, Yamada MK, Fujisawa S, Katoh-Semba R, Matsuki N and Ikegaya Y (2004) Brain-derived neurotrophic factor induces hyperexcitable reentrant circuits in the dentate gyrus. *J Neurosci* **24**(33):7215-7224.

Kuipers SD and Bramham CR (2006) Brain-derived neurotrophic factor mechanisms and function in adult synaptic plasticity: new insights and implications for therapy. *Curr Opin Drug Discov Devel* **9**(5):580-586.

Lahteinen S, Pitkanen A, Knuuttila J, Toronen P and Castren E (2004) Brain-derived neurotrophic factor signaling modifies hippocampal gene expression during epileptogenesis in transgenic mice. *Eur J Neurosci* **19**(12):3245-3254.

Lauterborn JC, Rex CS, Kramar E, Chen LY, Pandyarajan V, Lynch G and Gall CM (2007) Brain-derived neurotrophic factor rescues synaptic plasticity in a mouse model of fragile X syndrome. *J Neurosci* **27**(40):10685-10694.

Lee R, Kermani P, Teng KK and Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. *Science* **294**(5548):1945-1948.

Lee RC, Feinbaum RL and Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**(5):843-854.

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S and Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**(6956):415-419.

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH and Kim VN (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* **23**(20):4051-4060.

Levine ES, Crozier RA, Black IB and Plummer MR (1998) Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. *Proc Natl Acad Sci U S A* **95**(17):10235-10239.

Li YX, Zhang Y, Lester HA, Schuman EM and Davidson N (1998) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. *J Neurosci* **18**(24):10231-10240.

Li Z, Zhang Y, Li D and Feng Y (2000) Destabilization and mislocalization of myelin basic protein mRNAs in quaking dysmyelination lacking the QKI RNA-binding proteins. *J Neurosci* **20**(13):4944-4953.

Liao L, Park SK, Xu T, Vanderklish P and Yates JR, 3rd (2008) Quantitative proteomic analysis of primary neurons reveals diverse changes in synaptic protein content in fmr1 knockout mice. *Proc Natl Acad Sci U S A* **105**(40):15281-15286.

Lipsky RH and Marini AM (2007) Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. *Ann N Y Acad Sci* **1122**:130-143.

Liu J, Valencia-Sanchez MA, Hannon GJ and Parker R (2005a) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**(7):719-723.

Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y and Uhl GR (2006) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain research* **1067**(1):1-12.

Liu QR, Walther D, Drgon T, Polesskaya O, Lesnick TG, Strain KJ, de Andrade M, Bower JH, Maraganore DM and Uhl GR (2005b) Human brain derived neurotrophic factor (BDNF) genes, splicing patterns, and assessments of associations with substance abuse and Parkinson's Disease. *Am J Med Genet B Neuropsychiatr Genet* **134B**(1):93-103.

Lu B (2003a) BDNF and activity-dependent synaptic modulation. *Learn Mem* **10**(2):86-98.

Lu B (2003b) Pro-region of neurotrophins: role in synaptic modulation. *Neuron* **39**(5):735-738.

Lu B and Martinowich K (2008) Cell biology of BDNF and its relevance to schizophrenia. *Novartis Found Symp* **289**:119-129; discussion 129-135, 193-115.

Lu R, Wang H, Liang Z, Ku L, O'Donnell W T, Li W, Warren ST and Feng Y (2004) The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. *Proc Natl Acad Sci U S A* **101**(42):15201-15206.

Lu Y, Christian K and Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiol Learn Mem* **89**(3):312-323.

Lugli G, Larson J, Martone ME, Jones Y and Smalheiser NR (2005) Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J Neurochem* **94**(4):896-905.

Lugli G, Torvik VI, Larson J and Smalheiser NR (2008) Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain. *J Neurochem* **106**(2):650-661.

Lund E, Guttinger S, Calado A, Dahlberg JE and Kutay U (2004) Nuclear export of microRNA precursors. *Science* **303**(5654):95-98.

Ma D, Nothias F, Boyne LJ and Fischer I (1997) Differential regulation of microtubule-associated protein 1B (MAP1B) in rat CNS and PNS during development. *J Neurosci Res* **49**(3):319-332.

Ma DK, Kim WR, Ming GL and Song H (2009) Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. *Ann N Y Acad Sci* **1170**:664-673.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM and Yancopoulos GD (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* **5**(4):501-509.

Maroney PA, Yu Y, Fisher J and Nilsen TW (2006) Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat Struct Mol Biol* **13**(12):1102-1107.

Martinowich K and Lu B (2008) Interaction between BDNF and serotonin: role in mood disorders. *Neuropsychopharmacology* **33**(1):73-83.

Martinowich K, Manji H and Lu B (2007) New insights into BDNF function in depression and anxiety. *Nat Neurosci* **10**(9):1089-1093.

Martone ME, Pollock JA, Jones YZ and Ellisman MH (1996) Ultrastructural localization of dendritic messenger RNA in adult rat hippocampus. *J Neurosci* **16**(23):7437-7446.

Mayford M, Baranes D, Podsypanina K and Kandel ER (1996) The 3'-untranslated region of CaMKII alpha is a cis-acting signal for the localization and translation of mRNA in dendrites. *Proc Natl Acad Sci U S A* **93**(23):13250-13255.

Meixner A, Haverkamp S, Wassle H, Fuhrer S, Thalhammer J, Kropf N, Bittner RE, Lassmann H, Wiche G and Propst F (2000) MAP1B is required for axon guidance and Is involved in the development of the central and peripheral nervous system. *J Cell Biol* **151**(6):1169-1178.

Mhyre TR and Applegate CD (2003) Persistent regional increases in brain-derived neurotrophic factor in the flurothyl model of epileptogenesis are dependent upon the kindling status of the animal. *Neuroscience* **121**(4):1031-1045.

Michel CI, Kraft R and Restifo LL (2004) Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. *J Neurosci* **24**(25):5798-5809.

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME and Mayford M (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron* **36**(3):507-519.

Ming GL and Song H (2005) Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci* **28**:223-250.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R and Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron* **36**(1):121-137.

Miyashiro KY, Beckel-Mitchener A, Purk TP, Becker KG, Barret T, Liu L, Carbonetto S, Weiler IJ, Greenough WT and Eberwine J (2003) RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. *Neuron* **37**(3):417-431.

Molteni R, Calabrese F, Cattaneo A, Mancini M, Gennarelli M, Racagni G and Riva MA (2009) Acute stress responsiveness of the neurotrophin BDNF in the rat hippocampus is modulated by chronic treatment with the antidepressant duloxetine. *Neuropsychopharmacology* **34**(6):1523-1532.

Mori Y, Imaizumi K, Katayama T, Yoneda T and Tohyama M (2000) Two cis-acting elements in the 3' untranslated region of alpha-CaMKII regulate its dendritic targeting. *Nat Neurosci* **3**(11):1079-1084.

Muddashetty RS, Kelic S, Gross C, Xu M and Bassell GJ (2007) Dysregulated metabotropic glutamate receptor-dependent translation of AMPA receptor and postsynaptic density-95 mRNAs at synapses in a mouse model of fragile X syndrome. *J Neurosci* **27**(20):5338-5348.

Murphy DD, Cole NB and Segal M (1998) Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons. *Proc Natl Acad Sci U S A* **95**(19):11412-11417.

Musumeci SA, Hagerman RJ, Ferri R, Bosco P, Dalla Bernardina B, Tassinari CA, De Sarro GB and Elia M (1999) Epilepsy and EEG findings in males with fragile X syndrome. *Epilepsia* **40**(8):1092-1099.

Nagappan G and Lu B (2005) Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. *Trends Neurosci* **28**(9):464-471.

Nagappan G, Zaitsev E, Senatorov VV, Jr., Yang J, Hempstead BL and Lu B (2009) Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. *Proc Natl Acad Sci U S A* **106**(4):1267-1272.

Nakamoto M, Nalavadi V, Epstein MP, Narayanan U, Bassell GJ and Warren ST (2007) Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. *Proc Natl Acad Sci U S A* **104**(39):15537-15542.

Nanda SA and Mack KJ (2000) Seizures and sensory stimulation result in different patterns of brain derived neurotrophic factor protein expression in the barrel cortex and hippocampus. *Brain Res Mol Brain Res* **78**(1-2):1-14.

Nelson PT, Hatzigeorgiou AG and Mourelatos Z (2004) miRNP:mRNA association in polyribosomes in a human neuronal cell line. *RNA* **10**(3):387-394.

Nimchinsky EA, Oberlander AM and Svoboda K (2001) Abnormal development of dendritic spines in FMR1 knock-out mice. *J Neurosci* **21**(14):5139-5146.

Noiges R, Eichinger R, Kutschera W, Fischer I, Nemeth Z, Wiche G and Propst F (2002) Microtubule-associated protein 1A (MAP1A) and MAP1B: light chains determine distinct functional properties. *J Neurosci* **22**(6):2106-2114.

Nosyreva ED and Huber KM (2006) Metabotropic receptor-dependent long-term depression persists in the absence of protein synthesis in the mouse model of fragile X syndrome. *J Neurophysiol* **95**(5):3291-3295.

O'Donnell WT and Warren ST (2002) A decade of molecular studies of fragile X syndrome. *Annu Rev Neurosci* **25**:315-338.

Ohyu J, Yamanouchi H and Takashima S (1997) Immunohistochemical study of microtubule-associated protein 5 (MAP5) expression in the developing human brain. *Brain Dev* **19**(8):541-546.

Olsen PH and Ambros V (1999) The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol* **216**(2):671-680.

Ostroff LE, Fiala JC, Allwardt B and Harris KM (2002) Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. *Neuron* **35**(3):535-545.

Palmiter RD, Cole TB, Quaife CJ and Findley SD (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. *Proc Natl Acad Sci U S A* **93**(25):14934-14939.

Pan L, Zhang YQ, Woodruff E and Broadie K (2004) The Drosophila fragile X gene negatively regulates neuronal elaboration and synaptic differentiation. *Curr Biol* **14**(20):1863-1870.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL and Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* **306**(5695):487-491.

Parker R and Sheth U (2007) P bodies and the control of mRNA translation and degradation. *Mol Cell* **25**(5):635-646.

Pattabiraman PP, Tropea D, Chiaruttini C, Tongiorgi E, Cattaneo A and Domenici L (2005) Neuronal activity regulates the developmental expression and subcellular localization of cortical BDNF mRNA isoforms in vivo. *Mol Cell Neurosci* **28**(3):556-570.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC and Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16**(6):1137-1145.

Pfeiffer BE and Huber KM (2006) Current advances in local protein synthesis and synaptic plasticity. *J Neurosci* **26**(27):7147-7150.

Phillips HS, Hains JM, Laramee GR, Rosenthal A and Winslow JW (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. *Science* **250**(4978):290-294.

Pillai A (2008) Brain-derived neurotropic factor/TrkB signaling in the pathogenesis and novel pharmacotherapy of schizophrenia. *Neurosignals* **16**(2-3):183-193.

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E and Filipowicz W (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**(5740):1573-1576.

Plante I, Davidovic L, Ouellet DL, Gobeil LA, Tremblay S, Khandjian EW and Provost P (2006) Dicer-Derived MicroRNAs Are Utilized by the Fragile X Mental Retardation Protein for Assembly on Target RNAs. *J Biomed Biotechnol* **2006**(4):64347.

Poon MM, Choi SH, Jamieson CA, Geschwind DH and Martin KC (2006) Identification of process-localized mRNAs from cultured rodent hippocampal neurons. *J Neurosci* **26**(51):13390-13399.

Prendergast GC (2003) Signal transduction: putting translation before transcription. *Cancer Cell* **4**(4):244-245.

Qi Y, Wang JK, McMillian M and Chikaraishi DM (1997) Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. *J Neurosci* **17**(4):1217-1225.

Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* **32**(3):281-294.

Racine RJ, Gartner JG and Burnham WM (1972) Epileptiform activity and neural plasticity in limbic structures. *Brain research* **47**(1):262-268.

Racine RJ, Milgram NW and Hafner S (1983) Long-term potentiation phenomena in the rat limbic forebrain. *Brain Res* **260**(2):217-231.

Ramos A, Hollingworth D and Pastore A (2003) G-quartet-dependent recognition between the FMRP RGG box and RNA. *RNA* **9**(10):1198-1207.

Rao A and Steward O (1991) Evidence that protein constituents of postsynaptic membrane specializations are locally synthesized: analysis of proteins synthesized within synaptosomes. *J Neurosci* **11**(9):2881-2895.

Rattiner LM, Davis M and Ressler KJ (2004) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. *Learn Mem* **11**(6):727-731.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci* **361**(1473):1545-1564.

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR and Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**(6772):901-906.

Reiss AL, Aylward E, Freund LS, Joshi PK and Bryan RN (1991) Neuroanatomy of fragile X syndrome: the posterior fossa. *Ann Neurol* **29**(1):26-32.

Righi M, Tongiorgi E and Cattaneo A (2000) Brain-derived neurotrophic factor (BDNF) induces dendritic targeting of BDNF and tyrosine kinase B mRNAs in hippocampal neurons through a phosphatidylinositol-3 kinase-dependent pathway. *J Neurosci* **20**(9):3165-3174.

Russo-Neustadt AA, Alejandre H, Garcia C, Ivy AS and Chen MJ (2004) Hippocampal brain-derived neurotrophic factor expression following treatment with reboxetine, citalopram, and physical exercise. *Neuropsychopharmacology* **29**(12):2189-2199.

Russo-Neustadt AA, Beard RC, Huang YM and Cotman CW (2000) Physical activity and antidepressant treatment potentiate the expression of specific brain-derived neurotrophic factor transcripts in the rat hippocampus. *Neuroscience* **101**(2):305-312.

Sabaratnam M, Vroegop PG and Gangadharan SK (2001) Epilepsy and EEG findings in 18 males with fragile X syndrome. *Seizure* **10**(1):60-63.

Sakata K, Woo NH, Martinowich K, Greene JS, Schloesser RJ, Shen L and Lu B (2009) Critical role of promoter IV-driven BDNF transcription in GABAergic

transmission and synaptic plasticity in the prefrontal cortex. *Proc Natl Acad Sci U S A* **106**(14):5942-5947.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R and Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A* **103**(46):17501-17506.

Scharfman HE, Goodman JH and Sollas AL (1999) Actions of brain-derived neurotrophic factor in slices from rats with spontaneous seizures and mossy fiber sprouting in the dentate gyrus. *J Neurosci* **19**(13):5619-5631.

Schratt G (2009) microRNAs at the synapse. *Nat Rev Neurosci* **10**(12):842-849.

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M and Greenberg ME (2006) A brain-specific microRNA regulates dendritic spine development. *Nature* **439**(7074):283-289.

Segal R (2001) Neurotrophins: which way did they go? *Sci STKE* **2001**(84):PE1.

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* **26**:299-330.

Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E and Ambros V (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* **5**(3):R13.

Sen GL and Blau HM (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol* **7**(6):633-636.

Seog DH (2004) Glutamate receptor-interacting protein 1 protein binds to the microtubule-associated protein. *Biosci Biotechnol Biochem* **68**(8):1808-1810.

Shang Y, Wang H, Mercaldo V, Li X, Chen T and Zhuo M (2009) Fragile X mental retardation protein is required for chemically-induced long-term potentiation of the hippocampus in adult mice. *J Neurochem* **111**(3):635-646.

Shieh PB, Hu SC, Bobb K, Timmusk T and Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* **20**(4):727-740.

Siegel G, Obernosterer G, Fiore R, Oehmen M, Bicker S, Christensen M, Khudayberdiev S, Leuschner PF, Busch CJ, Kane C, Hubel K, Dekker F, Hedberg C, Rengarajan B, Drepper C, Waldmann H, Kauppinen S, Greenberg ME, Draguhn A, Rehmsmeier M, Martinez J and Schratt GM (2009) A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. *Nat Cell Biol* **11**(6):705-716.

Singh KK, Park KJ, Hong EJ, Kramer BM, Greenberg ME, Kaplan DR and Miller FD (2008) Developmental axon pruning mediated by BDNF-p75NTR-dependent axon degeneration. *Nat Neurosci* **11**(6):649-658.

Siomi H, Choi M, Siomi MC, Nussbaum RL and Dreyfuss G (1994) Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. *Cell* **77**(1):33-39.

Slomianka L and Geneser FA (1997) Postnatal development of zinc-containing cells and neuropil in the hippocampal region of the mouse. *Hippocampus* **7**(3):321-340.

Smalheiser NR and Lugli G (2009) microRNA regulation of synaptic plasticity. *Neuromolecular Med* **11**(3):133-140.

Snow K, Doud LK, Hagerman R, Pergolizzi RG, Erster SH and Thibodeau SN (1993) Analysis of a CGG sequence at the FMR-1 locus in fragile X families and in the general population. *Am J Hum Genet* **53**(6):1217-1228.

Stefani G, Fraser CE, Darnell JC and Darnell RB (2004) Fragile X mental retardation protein is associated with translating polyribosomes in neuronal cells. *J Neurosci* **24**(33):7272-7276.

Steward O and Falk PM (1985) Polyribosomes under developing spine synapses: growth specializations of dendrites at sites of synaptogenesis. *J Neurosci Res* **13**(1-2):75-88.

Steward O and Halpain S (1999) Lamina-specific synaptic activation causes domain-specific alterations in dendritic immunostaining for MAP2 and CAM kinase II. *J Neurosci* **19**(18):7834-7845.

Steward O and Levy WB (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J Neurosci* **2**(3):284-291.

Steward O and Worley PF (2001) A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. *Proc Natl Acad Sci U S A* **98**(13):7062-7068.

Stirling RV and Bliss TV (1978) Hippocampal mossy fiber development at the ultrastructural level. *Prog Brain Res* **48**:191-198.

Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D and Warren ST (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum Mol Genet* **1**(6):397-400.

Sutton MA and Schuman EM (2005) Local translational control in dendrites and its role in long-term synaptic plasticity. *J Neurobiol* **64**(1):116-131.

Sutula TP and Dudek FE (2007) Unmasking recurrent excitation generated by mossy fiber sprouting in the epileptic dentate gyrus: an emergent property of a complex system. *Prog Brain Res* **163**:541-563.

Tabuchi A, Nakatani C, Nakaoka R, Naruse Y, Kojima T, Mori N and Tsuda M (1999) Silencer-mediated repression and non-mediated activation of BDNF and c-fos gene promoters in primary glial or neuronal cells. *Biochem Biophys Res Commun* **261**(2):233-237.

Takei Y, Teng J, Harada A and Hirokawa N (2000) Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *J Cell Biol* **150**(5):989-1000.

Takemura R, Okabe S, Umeyama T, Kanai Y, Cowan NJ and Hirokawa N (1992) Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. *J Cell Sci* **103 (Pt 4)**:953-964.

Tanaka J, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GC and Kasai H (2008) Protein synthesis and neurotrophin-dependent structural plasticity of single dendritic spines. *Science* **319**(5870):1683-1687.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ and Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**(4):709-726.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A and Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *J Neurosci* **25**(22):5455-5463.

Theodorakis NG, Banerji SS and Morimoto RI (1988) HSP70 mRNA translation in chicken reticulocytes is regulated at the level of elongation. *J Biol Chem* **263**(28):14579-14585.

Tian B, Hu J, Zhang H and Lutz CS (2005) A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Res* **33**(1):201-212.

Tian F, Hu XZ, Wu X, Jiang H, Pan H, Marini AM and Lipsky RH (2009a) Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of Bdnf gene promoter 1. *J Neurochem* **109**(5):1375-1388.

Tian F, Marini AM and Lipsky RH (2009b) NMDA receptor activation induces differential epigenetic modification of Bdnf promoters in hippocampal neurons. *Amino Acids*.

Tian L, Stefanidakis M, Ning L, Van Lint P, Nyman-Huttunen H, Libert C, Itohara S, Mishina M, Rauvala H and Gahmberg CG (2007) Activation of NMDA receptors promotes dendritic spine development through MMP-mediated ICAM-5 cleavage. *J Cell Biol* **178**(4):687-700.

Timmusk T, Lendahl U, Funakoshi H, Arenas E, Persson H and Metsis M (1995) Identification of brain-derived neurotrophic factor promoter regions mediating tissue-specific, axotomy-, and neuronal activity-induced expression in transgenic mice. *J Cell Biol* **128**(1-2):185-199.

Timmusk T, Palm K, Lendahl U and Metsis M (1999) Brain-derived neurotrophic factor expression in vivo is under the control of neuron-restrictive silencer element. *J Biol Chem* **274**(2):1078-1084.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M and Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* **10**(3):475-489.

Timmusk T, Persson H and Metsis M (1994) Analysis of transcriptional initiation and translatability of brain-derived neurotrophic factor mRNAs in the rat brain. *Neurosci Lett* **177**(1-2):27-31.

Tint I, Fischer I and Black M (2005) Acute inactivation of MAP1b in growing sympathetic neurons destabilizes axonal microtubules. *Cell Motil Cytoskeleton* **60**(1):48-65.

Tint I, Slaughter T, Fischer I and Black MM (1998) Acute inactivation of tau has no effect on dynamics of microtubules in growing axons of cultured sympathetic neurons. *J Neurosci* **18**(21):8660-8673.

Todd PK, Mack KJ and Malter JS (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc Natl Acad Sci U S A* **100**(24):14374-14378.

Tongiorgi E, Armellin M, Giulianini PG, Bregola G, Zucchini S, Paradiso B, Steward O, Cattaneo A and Simonato M (2004) Brain-derived neurotrophic factor mRNA and protein are targeted to discrete dendritic laminas by events that trigger epileptogenesis. *J Neurosci* **24**(30):6842-6852.

Tongiorgi E, Righi M and Cattaneo A (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. *J Neurosci* **17**(24):9492-9505.

Tongiorgi E, Righi M and Cattaneo A (1998) A non-radioactive in situ hybridization method that does not require RNAse-free conditions. *J Neurosci Methods* **85**(2):129-139.

Torre ER and Steward O (1992) Demonstration of local protein synthesis within dendrites using a new cell culture system that permits the isolation of living axons and dendrites from their cell bodies. *J Neurosci* **12**(3):762-772.

Tsokas P, Grace EA, Chan P, Ma T, Sealfon SC, Iyengar R, Landau EM and Blitzer RD (2005) Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-term potentiation. *J Neurosci* **25**(24):5833-5843.

Turner G, Webb T, Wake S and Robinson H (1996) Prevalence of fragile X syndrome. *Am J Med Genet* **64**(1):196-197.

Tyler WJ and Pozzo-Miller LD (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* **21**(12):4249-4258.

Underwood CK and Coulson EJ (2008) The p75 neurotrophin receptor. *Int J Biochem Cell Biol* **40**(9):1664-1668.

Vessey JP, Vaccani A, Xie Y, Dahm R, Karra D, Kiebler MA and Macchi P (2006) Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. *J Neurosci* **26**(24):6496-6508.

Vicario-Abejon C, Collin C, McKay RD and Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J Neurosci* **18**(18):7256-7271.

Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH and Impey S (2005) A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc Natl Acad Sci U S A* **102**(45):16426-16431.

Volk LJ, Pfeiffer BE, Gibson JR and Huber KM (2007) Multiple Gq-coupled receptors converge on a common protein synthesis-dependent long-term depression that is affected in fragile X syndrome mental retardation. *J Neurosci* **27**(43):11624-11634.

Wakiyama M, Takimoto K, Ohara O and Yokoyama S (2007) Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes Dev* **21**(15):1857-1862.

Wang H, Dictenberg JB, Ku L, Li W, Bassell GJ and Feng Y (2008) Dynamic association of the fragile X mental retardation protein as a messenger ribonucleoprotein between microtubules and polyribosomes. *Mol Biol Cell* **19**(1):105-114.

Wang H, Ku L, Osterhout DJ, Li W, Ahmadian A, Liang Z and Feng Y (2004) Developmentally-programmed FMRP expression in oligodendrocytes: a potential role of FMRP in regulating translation in oligodendroglia progenitors. *Hum Mol Genet* **13**(1):79-89.

Waung MW, Pfeiffer BE, Nosyreva ED, Ronesi JA and Huber KM (2008) Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* **59**(1):84-97.

Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng HY, Marks D, Obrietan K, Soderling TR, Goodman RH and Impey S (2008) An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. *Proc Natl Acad Sci U S A* **105**(26):9093-9098.

Weiler IJ and Greenough WT (1991) Potassium ion stimulation triggers protein translation in synaptoneurosomal polyribosomes. *Mol Cell Neurosci* **2**(4):305-314.

Weiler IJ, Spangler CC, Klintsova AY, Grossman AW, Kim SH, Bertaina-Anglade V, Khaliq H, de Vries FE, Lambers FA, Hatia F, Base CK and Greenough WT (2004) Fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses. *Proc Natl Acad Sci U S A* **101**(50):17504-17509.

Wightman B, Ha I and Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75**(5):855-862.

Wilson BM and Cox CL (2007) Absence of metabotropic glutamate receptor-mediated plasticity in the neocortex of fragile X mice. *Proc Natl Acad Sci U S A* **104**(7):2454-2459.

Wirth MJ, Brun A, Grabert J, Patz S and Wahle P (2003) Accelerated dendritic development of rat cortical pyramidal cells and interneurons after biolistic transfection with BDNF and NT4/5. *Development* **130**(23):5827-5838.

Woo NH, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL and Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nat Neurosci* **8**(8):1069-1077.

Wu L, Fan J and Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A* **103**(11):4034-4039.

Wu L, Wells D, Tay J, Mendis D, Abbott MA, Barnitt A, Quinlan E, Heynen A, Fallon JR and Richter JD (1998) CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. *Neuron* **21**(5):1129-1139.

Yang J, Siao CJ, Nagappan G, Marinic T, Jing D, McGrath K, Chen ZY, Mark W, Tessarollo L, Lee FS, Lu B and Hempstead BL (2009) Neuronal release of proBDNF. *Nat Neurosci* **12**(2):113-115.

Yi R, Qin Y, Macara IG and Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**(24):3011-3016.

Zafra F, Lindholm D, Castren E, Hartikka J and Thoenen H (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci* **12**(12):4793-4799.

Zagrebelsky M, Holz A, Dechant G, Barde YA, Bonhoeffer T and Korte M (2005) The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons. *J Neurosci* **25**(43):9989-9999.

Zalfa F, Achsel T and Bagni C (2006) mRNPs, polysomes or granules: FMRP in neuronal protein synthesis. *Curr Opin Neurobiol* **16**(3):265-269.

Zalfa F, Eleuteri B, Dickson KS, Mercaldo V, De Rubeis S, di Penta A, Tabolacci E, Chiurazzi P, Neri G, Grant SG and Bagni C (2007) A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. *Nat Neurosci* **10**(5):578-587.

Zalfa F, Giorgi M, Primerano B, Moro A, Di Penta A, Reis S, Oostra B and Bagni C (2003) The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell* **112**(3):317-327.

Zang JB, Nosyreva ED, Spencer CM, Volk LJ, Musunuru K, Zhong R, Stone EF, Yuva-Paylor LA, Huber KM, Paylor R, Darnell JC and Darnell RB (2009) A mouse model of the human Fragile X syndrome I304N mutation. *PLoS Genet* **5**(12):e1000758.

Zeitelhofer M, Karra D, Macchi P, Tolino M, Thomas S, Schwarz M, Kiebler M and Dahm R (2008a) Dynamic interaction between P-bodies and transport ribonucleoprotein particles in dendrites of mature hippocampal neurons. *J Neurosci* **28**(30):7555-7562.

Zeitelhofer M, Macchi P and Dahm R (2008b) Perplexing bodies: The putative roles of P-bodies in neurons. *RNA Biol* **5**(4):244-248.

Zhang HL, Eom T, Oleynikov Y, Shenoy SM, Liebelt DA, Dictenberg JB, Singer RH and Bassell GJ (2001a) Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility. *Neuron* **31**(2):261-275.

Zhang J, Hou L, Klann E and Nelson DL (2009) Altered hippocampal synaptic plasticity in the FMR1 gene family knockout mouse models. *J Neurophysiol* **101**(5):2572-2580.

Zhang YQ, Bailey AM, Matthies HJ, Renden RB, Smith MA, Speese SD, Rubin GM and Broadie K (2001b) Drosophila fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell* **107**(5):591-603.

Zhao C, Sun G, Li S, Lang MF, Yang S, Li W and Shi Y (2010) MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. *Proc Natl Acad Sci U S A* **107**(5):1876-1881.

Zhao CS and Overstreet-Wadiche L (2008) Integration of adult generated neurons during epileptogenesis. *Epilepsia* **49 Suppl 5**:3-12.

Zhao L, Tian D, Xia M, Macklin WB and Feng Y (2006) Rescuing qkV dysmyelination by a single isoform of the selective RNA-binding protein QKI. *J Neurosci* **26**(44):11278-11286.

Zheng WH and Quirion R (2004) Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival. *J Neurochem* **89**(4):844-852.

Zhong J, Zhang T and Bloch LM (2006) Dendritic mRNAs encode diversified functionalities in hippocampal pyramidal neurons. *BMC Neurosci* **7**:17.

Zhou Z, Hong EJ, Cohen S, Zhao WN, Ho HY, Schmidt L, Chen WG, Lin Y, Savner E, Griffith EC, Hu L, Steen JA, Weitz CJ and Greenberg ME (2006) Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron* **52**(2):255-269.