SPATIAL REGULATION OF BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) EXPRESSION IN SENSORY NEURONS BY microRNA-206

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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LIST OF ABBREVIATIONS

UTR	Untranslated Region
DRG	Dorsal Root Ganglion
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
ddPCR	Droplet Digital Polymerase Chain Reaction
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
SRP	Signal Recognition Particle
NGF	Nerve Growth Factor
BDNF	Brain Derived Neurotrophic Factor
FISH	Fluorescence *In Situ* Hybridization
FRAP	Fluorescence Recovery After Photobleaching
DLR	Dual Luciferase Reporter
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
MBP	Myelin Basic Protein
PolyA	Polyadenylation
CDS	Coding Sequence
Myr	Myristoylation

ABSTRACT

The precise regulation of selective mRNA transport to its final destination and local translation is essential for normal neuronal function, morphological maintenance and regeneration after injury. Transcripts encoding brain-derived neurotrophic factor (BDNF) that regulates synaptic plasticity and memory are differentially localized in subcellular regions of hippocampal neurons. Given that differential distribution of the two different variants of *BDNF* mRNA's 3′ untranslated region (UTR) containing either a short (0.35kb) or a longer (2.9kb) form, I speculated that the longer 3′UTR variant of *BDNF* mRNA is specifically localizing in distal axons of neurons and its local translation is regulated by small non-coding RNAs in a spatially specific manner. Using RT-PCR and fluorescent recovery after photobleaching (FRAP) approaches, I showed that *BDNF* mRNA with the longer 3′UTR is endogenously present in distal axons of the sensory neurons, though the copy number is very low. Inconsistent with previous studies of dendrites of CNS neurons, the short 3′UTR variant of *BDNF* mRNA targets a reporter mRNA to distal axons of sensory neurons. Bioinformatics analysis for predicting microRNAs (miRNAs) targeting the 3′UTR of *BDNF* mRNA reveals that miRNA-206 may recognize and play regulatory roles in its gene expression. Results from target gene dual luciferase assay and overexpression of miR-206 in F-11 cells show that miR-206 differentially down-regulates *BDNF* mRNA

containing a longer 3′UTR variant from that with a short form by targeting the predicted target site residing in the distal segment the longer form of the 3′UTR. Western blots show that BDNF protein expression in DRG neurons (both cell body and axons) is decreased by 23% upon overexpression of miR-206. On the basis of these works presented here, I propose that different variants of *BDNF* mRNA that are differentially distributed to axons of sensory neurons are differentially regulated by miR-206.

Chapter 1

INTRODUCTION

1.1 Neurotrophins

Neurotrophins are secreted proteins that play crucial roles in the survival of neurons, growth of neuronal processes and synaptic plasticity (Poo et al., 2001; Volsosin et al., 2006; Skaper et al, 2012). They are synthesized and released by various cell types including neurons and glial cells (Korsching et al., 1993; Thoenen et al., 1995; Huang etal., 2001). There are four members of neurotrophin family characterized in mammals that are similar in sequence and structure; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Hallbook et al., 1999; Lindvall et al., 1992). Nerve growth factor (NGF) is the first and best characterized member of the neurotrophin family (Levi-Montalcini et al., 1987; Hallbook et al., 1999). NGF has been known to play a key role in preventing neuronal degeneration and in neuronal cell growth of severed sensory nerves in rats (Cao et al., 2014; Allen et al., 2011). From extensive studies of cellular/molecular mechanisms of NGF, we now know that almost all cell types internalize neurotrophins by receptor-mediated mechanism to exert their biological

cellular functions. Neurotrophins bind to two different classes of transmembrane receptor proteins, the tropomyosin receptor kinase (Trk) and the neurotrophin receptor p75 (Huang et al., 2009; Reichardt et al.,2006). The specificity of neurotrophin action is defined in part by the selective interaction of the neurotrophin with a specific member of the Trk receptor family. For example, NGF binds to TrkA, BDNF and NT-4 bind to TrkB with high affinity and NT-3 binds to TrkC (Numakawa et al., 2010; Vidal et al., 2015). The Trk receptor function is further modulated by p75 on several levels such as accessibility of neurotrophins through promotion of axonal growth and target innervation.

1.2 Brain Derived Neurotrophic Factor (BDNF)

Brain Derived Neurotrophic Factor (BDNF) was discovered by Barde et al. (1982) in pig brain. BDNF is known to be involved in various brain functions such as neuronal development, maintenance of synaptic plasticity and neuronal survival (Caputo et al., 2011; Anastasia and Hempstead., 2014). A decreased level of BDNF expression is associated with the pathophysiology of depression and loss of memory in transgenic mouse models (Nakajo et al., 2008; Angelucci et al., 2005) as well as neurological diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Zuccato and Cattaneo, 2009; Qin et al., 2016). Besides its effects on neurons, BDNF has other roles in non-neuronal cells. For example, regeneration of skeletal muscle after injury is hindered in *BDNF* knockout mice, indicating its role in muscle cells for growth and regeneration (Clow et al., 2010;

Mousavi et al., 2006). Therefore, manipulating expression level of BDNF has been proposed as a potential approach in tackling several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, and also promoting muscle differentiation (Nagahara and Tuszynski, 2006; Wu et al., 2016).

1.3 Protein Localization

Protein transport can take place by three different ways; gated transport, transmembrane transport or vesicular transport. In gated transport such as in nuclear pore complexes help to actively transport protein molecules from cytoplasm to nucleus and vise-versa. Whereas, in transmembrane transport proteins are transported from one membrane to another through the membrane bound transmembrane proteins. For example, proteins from cytoplasm to endoplasmic reticulum (ER) are transported in this manner. Besides, these pathways, proteins can also be transported in the form of vesicles from one compartment to another such as the transport of proteins from ER to Golgi apparatus. There are signal peptides present in the polypeptide that contains the protein localization signal and determines the precise choice of delivery pathway for the protein. (Alberts et al., 4th Edi; Cross et al., 2009; Kessel et al., 2011). Protein can also be transported to specific locations using motor proteins such as kinesin and dynein as cargoes that can help to deliver long range transports (Hirokawa et al., 2009; Gumy et al., 2013).

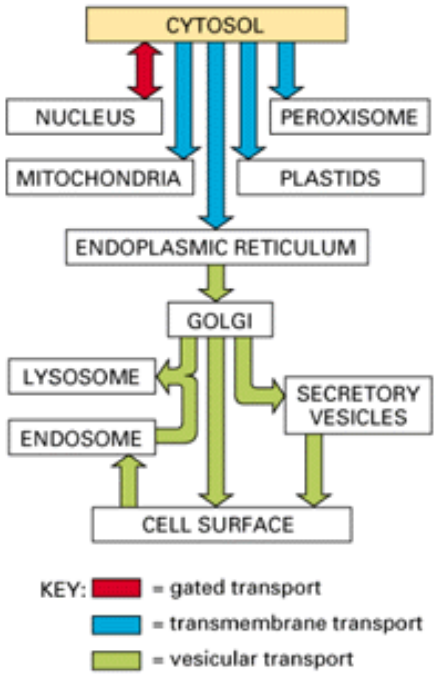

Figure 1.1: **Different protein transport pathways** (Alberts et al., Mol. Biology of cell, 4th Ed.). The pathways show how proteins are transported through different pathways depending upon the signal peptides present.

Protein localization to its final target site through these processes or by motor proteins has several drawbacks. For example, protein localization may be accomplished with less efficiency, as they have to pass through several steps to complete the delivery process. In addition, the protein transport by motor proteins also face a challenge as some processes like axons in neurons can extend over several meters long that could cause more time for axonal proteins to reach their destination (Millecamps and Julien, 2013; Morfini et al., 2012). Some proteins might have a short

half-life, leading to degradation before arriving to their final destination (Alvarez et al., 2000). In addition, excessive abnormal protein accumulation can also take place, which can be harmful to the cells. For example, overexpression of amyloid precursor protein (APP) is known to trigger Alzheimer's disease through aggregation of Amyloid beta (Aβ), forming amyloid plaques within brain (Murphy et al., 2010; Reiman, 2016).

Polarized cells such as neurons have mechanisms by which extremity of distal processes can locally respond autonomously from the cell body to overcome disadvantages from protein localization. Although some proteins are actively and selectively transported after translation or during translation, studies have shown selective mRNAs are transported to the subcellular regions of the cell and locally translated when needed (Wickramasinghe et al., 2015; Hirokawa, 2006). Regulation in the pathways of mRNA transport and local translation leads to spatial and temporal control of gene expression. Therefore, mRNA localization and local protein synthesis might be preferable to protein localization for providing proteins needed and asymmetric distribution of proteins in cells (Martin et al., 2009; Jansen, 2001).

1.4 mRNA Localization

Regulation of messenger RNA (mRNA) localization has several advantages over control of protein localization. For example, one mRNA molecule can serve as a

template for multiple rounds of translation. This makes mRNA localization more energy efficient and aid in rapidly repopulating distal processes with new proteins such as in neurons (Donnelly et al., 2010). Some proteins can act abnormally before they reach the target site, which can be prevented by mRNA localization. This is particularly important to determine cell fates and prevent from harmful proteins being synthesized in the incorrect location, such as myelin basic protein. mRNA localization provides the ability to fine tune expression of specific genes (Martin et al., 2009; Holt et al., 2009, Medioni et al., 2012).

mRNA molecules are transcribed in the nucleus and then are exported into the cytoplasm, where they are translated into proteins. However, not all mRNAs immediately undergo translation, some are selectively directed to specific subcellular areas before translation occurs. Recent studies show that, there mRNA localization signals called cis-elements in the mRNA which are recognized by trans-acting factors such as RNA binding proteins (RBPs). These cis-elements contain the mRNA localization signal analogous to the signal peptides in the proteins. This can result in an asymmetric distribution of proteins with localized activities in polarized cells or developing embryos (Fig 1.3) (Martin et al., 2009; Jansen, 2001; Shav-Tal and Singer, 2005).

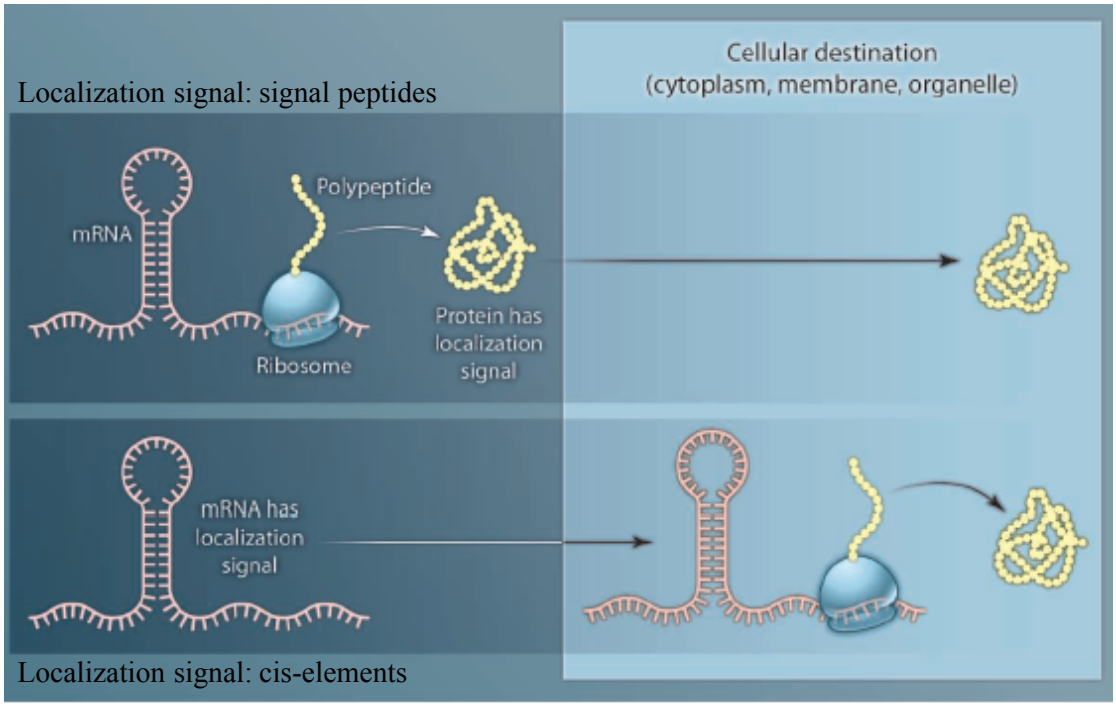

Figure 1.2: **Asymmetric distribution of proteins** (Ramanurthi et al., 2011)**:** The image shows the asymmetric distribution of proteins by two different mechanisms; protein localization or mRNA localization due to the presence of localization signals in them, signal peptides or *cis*-elements respectively.

mRNA localization is evolutionarily conserved because this mechanism is present in simple to more complex organisms. For example, during the budding process of yeasts, many RNAs are translocated from the mother to the daughter cells. *ASH1* mRNA localizes to the bud tip of the dividing cell and inhibits translation of *HO* (Homothallic switching endonuclease) in daughter cells through binding to *HO*

promoter. This ensures that mother and daughter cells have distinct mating types (Long et al., 1997; Takizawa et al., 1997). In *Drosophilla*, several mRNAs such as *bicoid*, *oskar*, and *nanos* localize to the anterior and posterior poles of the oocyte respectively, during oogenesis. This asymmetric localization of the mRNAs is responsible for early embryonic patterning (Berleth et al., 1988; Gilbert 2000; Johnstone and Lasko et al., 2001). Similarly, in the oocytes of the *Xenopus*, *VegT* is localized to the vegetal pole and is important for endodermal and mesodermal cell specification during embryo development (Heasman et al., 2001; King et al., 2005; Birsoy et al., 2006). *β-actin* mRNA in migrating fibroblasts is localized to the leading edge of the cells, which correlates with the elevated levels of β-actin protein in lamellipodia essential for directional cell motility (Condeelis and Singer et al., 2005; Liao et al., 2011; Bunnell et al., 2011).

In highly polarized mammalian cells such as neurons, localization of RNAs and local translation at specific subcellular regions have been shown to be integral part to the proper functioning of the cells. For example, *myelin basic protein* (*MBP*) mRNA in oligodendrocytes is transported to the distal processes, so the myelination can take place (Brophy et al., 1993; Waxman, 2006). MBP protein is a major component of the axon-wrapping myelin sheet and is a sticky protein that interacts with any cell membrane in the cell. In brain, local translation of mRNA at the growth cones help neurons to respond autonomously to the local environment (Lin and Holt et al., 2007; Martin and Zukin et al., 2006; Zukin et al., 2009; Jung et al., 2012). Translation of

BDNF mRNA is tightly regulated as BDNF is important for the survival and growth of the neurons (Cohen-Cory et al., 2010). Therefore, it is essential to understand the molecular mechanisms underlying regulation of *BDNF* mRNA localization and local translation.

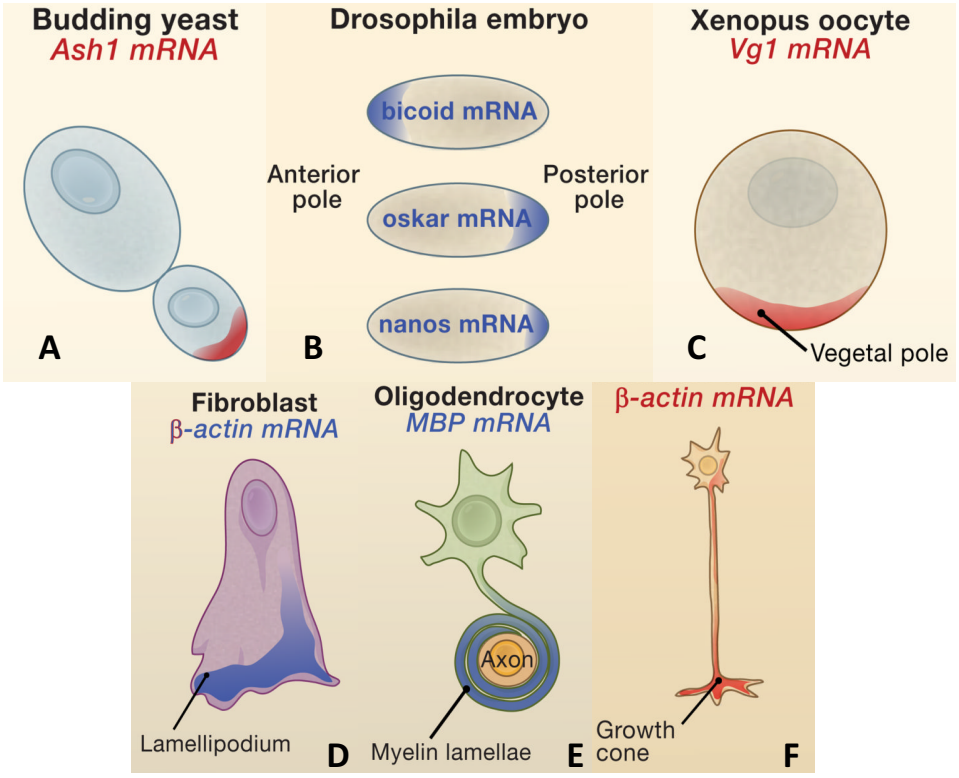

Figure 1.3: **mRNA localization is evolutionarily conserved.** (Adapted from Martin et al., 2009); **A.** *ASH1* mRNA localizes to the bud tip of the daughter cell in yeast; **B.** Different mRNAs localize in *Drosophila* embryos; anterior (*bicoid)* and posterior (*oskar* and *nanos);*

C. *Vg1* mRNA found to localize to the vegetal pole of Xenopus oocyte; **D.** *β-actin* localizes to lamellipodia; **E.** *Myelin basic protein* (*MBP*) mRNA localizes to myelinating axons; **F.** *β-actin* mRNA present in growth cones.

1.5 Regulation of localization and local translation of *BDNF* mRNA in neurons

The structure of *BDNF* gene is complex and contains multiple 5′ untranslated exons with 8 independent promoters conjugated to a coding exon (Figure 1.3). Multiple promoters enable a precise control of gene expression at transcriptional level. The coding exon consists of two alternate polyadenylation (PolyA) sites that lead to the generation of two different variants of *BDNF* transcript's 3′UTR, a short form (0.35kb) or a longer 3′UTR (2.9kb) (Timmusk et al., 1993; Greenberg et al., 2009; Lau et al., 2010). Lau et al. (2010) showed that upon seizure-induced activation, the *BDNF* mRNA containing a long 3′UTR specifically undergoes robust translational activation in the hippocampus. Taken together, these studies indicate that the 3′UTR of *BDNF* mRNA may play important roles both in the localization and the regulation of translation. Recently, small non-coding RNAs such as microRNAs (miRNAs) have emerged as key regulators of gene expression in the nervous system. The targeting sites at which miRNAs recognize and make base-pairing is often in their target transcript's 3′UTR.

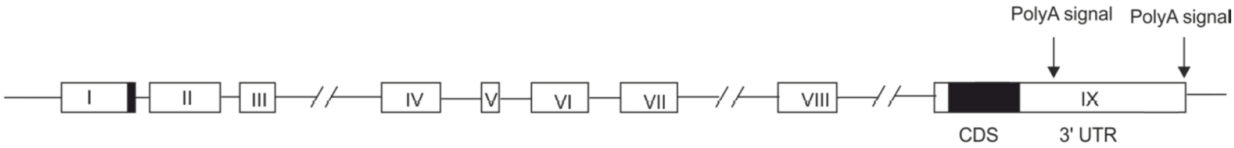

Figure 1.4: Schematic diagram of *BDNF* mRNA (Adapted from Matsushita et al., 1997 and Aid et al., 2007) This is a *BDNF* mRNA in mouse showing 8 different promoters, coding sequence and 3′UTR region with location of two polyA signal sites

An et al. (2008) previously showed that *BDNF* mRNA containing a longer 3′ untranslated region (UTR) localizes to the distal dendrites of mouse hippocampal neurons, whereas the presence of the shorter 3′UTR variant of *BDNF* mRNA is restricted to the soma. They also generated a mouse model carrying a truncated long 3′UTR of *BDNF* mRNA and found that the impairment of *BDNF* mRNA localization in dendrites results in defective pruning and enlargement of dendritic spines of hippocampal neurons. These studies indicate that *cis*-element(s) that control the mRNA localization into distal processes of neurons are present within the distal segment of the longer form of 3′UTR of *BDNF* mRNA. However, the possibility that the longer 3′UTR variant of *BDNF* mRNA is selectively localizing into distal axons of neurons has not been experimentally tested.

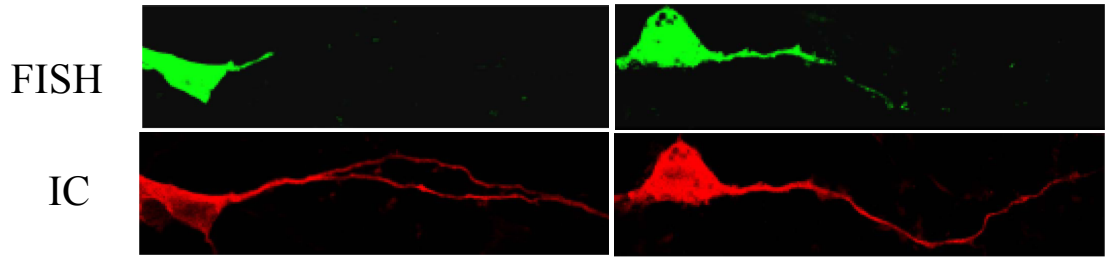

Figure 1.5: Localization of *BDNF* mRNA in hippocampal neurons by Fluorescence *in situ* hybridization (FISH) (Adapted from An et al., 2008). Images on the top left and top right indicate *BDNF* mRNA with a short 3′UTR and a long 3′UTR, respectively. Whereas, red indicate immunocytochemistry (IC) of neuronal processes with MAP2.

1.6 MicroRNAs (miRNAs)

miRNAs are a family of small non-coding RNAs that regulate gene expression by binding to the complementary sequences in their target mRNAs. They are about 22 nucleotides in length and have been recognized as a translational regulator (Bartel, 2004; Ambros et al., 2004; Wahid et al., 2010). During the microRNA biogenesis, several kilo base nucleotides of primary miRNA (pri-miRNA) miRNAs are synthesized from miRNA genes or non-coding genes by RNA polymerase II. Pri-miRNA is processed to generate 70-80 nucleotide length precursor miRNAs (pre-miRNAs) in the nucleus and then exported to the cytoplasm where it is cleaved by another ribonuclease called Dicer, resulting in a double stranded duplex miRNAs (Lee

et al., 2003; Lam et al., 2015). This duplex interacts with Argonaute (AGO) proteins in the cytosol forming a ribonucleoprotein complex called RNA induced silencing complex (RISC). Of the two strands, the passenger strand is degraded and the other strand (guide strand) guides the RISC complex to the complementary sequence in the target mRNA (Pratt and MacRae, 2009; Yoda et al., 2009). In animals, 6-8 complementary nucleotides matching at the 5′end of the guide strand of mature miRNA are essential for its regulatory roles in gene expression. This 6-8 nucleotide sequence is referred to as the seed region. Compared to animals, plants require close to perfect complementarity for the miRNA to control gene expression. One mRNA can be targeted by multiple miRNAs or an individual miRNA can target multiple mRNAs (Lewis et al, 2003; Schwab et al., 2005).

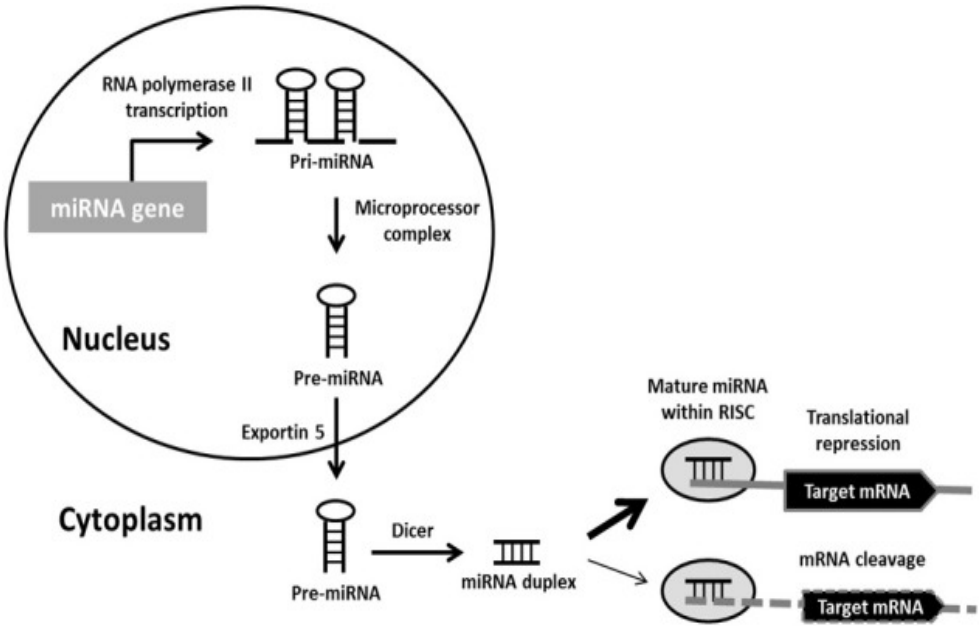

Figure 1.6: **miRNA biogenesis pathway** (Goodall et al., 2013; Biggar and Storey et al., 2011). Initial transcription of microRNAs to pri-miRNA involves the processing of pri-miRNA to pre-miRNA, which is exported to the cytoplasm. Pre-miRNAs are further processed to produce miRNA duplex from which matured miRNA is generated and loaded to RISC complex to exert translational inhibition.

Recently, Lee et al. (2012) showed that in the Alzheimer disease mouse model and postmortem brains of Alzheimer disease patients, BDNF protein was downregulated. Using microRNA target prediction logarithms, they found 13 microRNAs that have predicted binding sites in the 3'UTR of BDNF mRNA. Using quantitative real time PCR (qRT-PCR) they found that miR-206 was drastically upregulated in both transgenic mouse (12 months) and Alzheimer's disease patients when compared to controls, implicating that BDNF expression is regulated by miR-206 (Figure 1.6 and 1.7). Further, they identified 3 putative target sites of miRNA-206 in the 3′UTRs of *BDNF* mRNA using miRNA target prediction program (Lee et al., 2012).

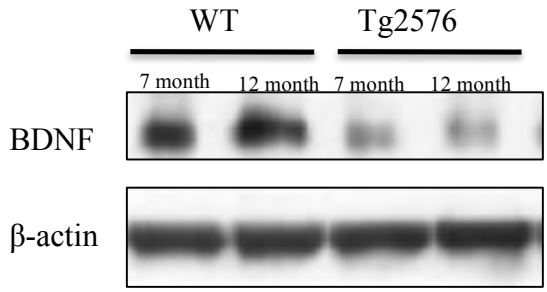

Figure 1.7: Decreased BDNF protein expression in Alzheimer's disease mouse model compared to wild type (Lee et al., 2012). Western blot of whole brain lysates from control and Alzheimer's disease mouse model (Tg2576) of different age groups showed a significant decrease in BDNF protein expression in disease model mouse.

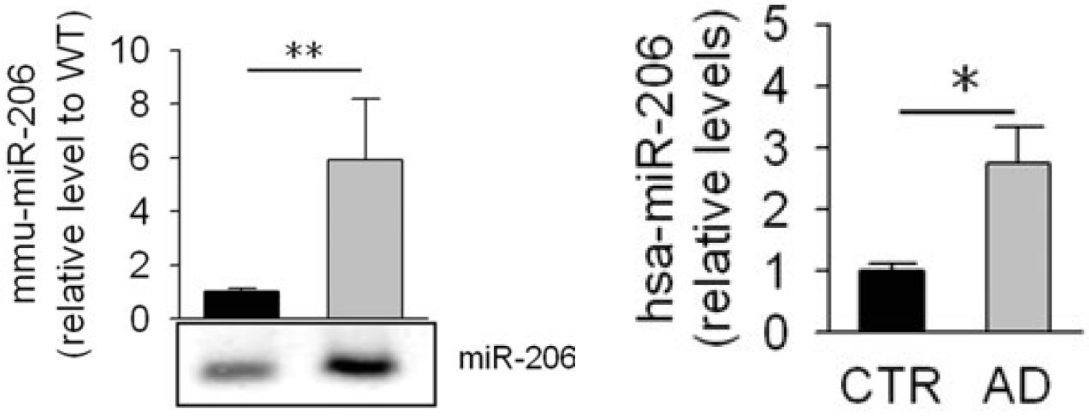

Figure 1.8: Quantitative Real time PCR of brain samples from Alzheimer's disease mouse model and patients showing an upregulation of miR-206 (Lee et al., 2012). Left panel shows 12-month old wild type compared Tg2576 (Alzheimer's disease mouse model). Whereas, right panel shows control patients compared to Alzheimer patient.

1.7 Hypothesis and Aims

Current studies have only shown that *BDNF* mRNA with a longer 3′UTR variant is localized to distal dendrites and *BDNF* mRNA with a short form 3′UTR is restricted to the cell bodies in the CNS neurons. However, it has not been studied experimentally whether *BDNF* mRNA with the longer 3′UTR variant is localized into distal axons of neurons.

Based on all these data including out preliminary studies, I hypothesize that *BDNF* mRNA with a longer 3′UTR variant is endogenously present in distal axons of the sensory neurons and is specifically regulated by the miR-206 at a post-transcriptional level. The hypothesis will be tested with the following Specific Aims:

Specific Aim 1:

To determine whether *BDNF* mRNA with a longer 3′UTR variant is present in distal axons of neuron.

Specific Aim 2:

To determine the target specificity of miR-206 on *BDNF* mRNA with the longer 3′UTR variant.

Chapter 2

MATERIALS AND METHODS

2.1 Plasmid Expression Constructs for Dual Luciferase Reporter Assay

Since the longer 3′UTR variant of *BDNF* mRNA is approximately 2.9 kb in length, I divided the 3′UTR into three 1 kb fragments with some overlapping sequences between them by designing 3 sets of forward and reverse primers accordingly for RT-PCR amplification. After RT-PCR for the three fragments, the amplified products were placed together in a reaction and RT-PCR was performed again using the forward primer containing NotI restriction enzyme site for the first fragment and the reverse primer containing XhoI restriction enzyme site for the last fragment to amplify the full length (longer) 3′UTR of *BDNF* mRNA. After double digestion with NotI and XhoI restriction enzymes, the 3′UTR of *BDNF* mRNA was cloned into Renilla reporter pre-digested with NotI and XhoI restriction enzymes to generate Renilla-full length 3′UTR of *BDNF* mRNA (Renilla-*BDNF*$^{\text{Full 3′UTR}}$).

Renilla reporters containing 3 predicted target sites within the 3′UTR of *BDNF* mRNA for miRNA-206 were generated by dimerization of the sense and antisense sequences of each of the target sites. Since the sense and antisense oligonucleotides were designed to contain sticky ends of NotI and XhoI, respectively, the dimerized

product could be directly ligated to Renilla reporter pre-digested with NotI and XhoI restriction enzymes (Renilla-target site #1^{miR206}, Renilla-target site #2^{miR206}, and Renilla-target site #3^{miR206}).

Targets #	Sense Sequence (5′→ 3′)	Antisense Sequence (5′ → 3′)
1	GGCCGCTGCGCACAACTTTAAAA GTCTGCATTACATTCCTCGATAC	TCGAGTATCGAGGAATGTAATGC AGACTTTTAAAGTTGTGCGCAGC
2	GGCCGCAAGCAAAAACAAAAATT TGAACCAAAACATTCCGTTTACC	TCGAGGTAAACGGAATGTTTTGG TTCAAATTTTTGTTTTTGCTTGC
3	GGCCGCGATGGAGGTGGGGAATG GTACTTGAGACATTCCTAAAGGC	TCGAGCCTTTAGGAATGTCTCAA GTACCATTCCCCACCTCCATCGC

Table 2.1: List of oligonucleotides used to prepare the dimerized miR-206 targets. The table shows the sense and anti-sense primer sequences to produce dimerized product with NotI and XhoI restriction sites. The underlined sequence represents the target sequence.

A.

Proximal (short) 3′UTR

B.

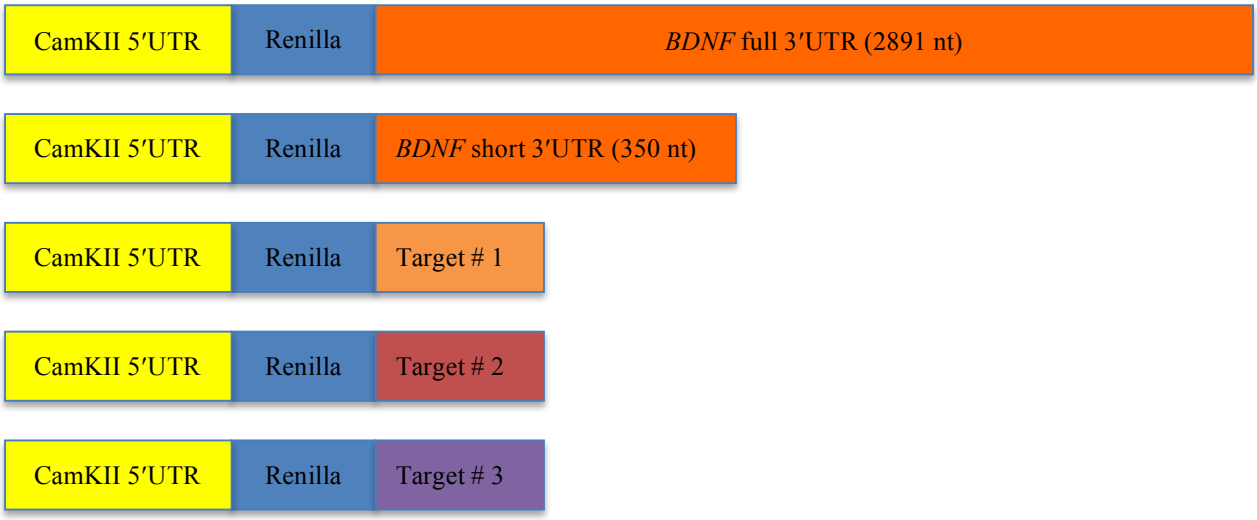

Figure 2.1: Schematic diagram of constructs with *BDNF* 3′UTR constructs.

A. *BDNF* mRNA with predicted miR-206 target sites.

B. *BDNF* mRNA with a full 3′UTR (Renilla-*BDNF*$^{\text{Full 3′UTR}}$), a short 3′UTR (Renilla-*BDNF*$^{\text{short 3′UTR}}$) and three predicted miR-206 target sites in *BDNF* 3′UTR (Renilla-target#1$^{\text{miR206}}$, Renilla target#2$^{\text{miR206}}$, and Renilla-target#3$^{\text{miR206}}$).

2.2 Dual Luciferase Reporter (DLR) Assay

We used F-11 cells (derived from the fusion between mouse neuroblastoma cell line N18TG2 with embryonic rat DRG neurons) for *in vitro* assay of miRNA target validation experiments. Briefly, F-11 cells were plated at a density of 8,000 cells/cm^2 on 6 cm culture dishes with DMEM/F12 (50/50) media (Cellgrow, Manassas, VA) containing 10% Fetal Bovine Serum (Gibco, Carlsbad, CA), 2 mM L-Glutamine and 1X Penicillin/Streptomycin (Cellgrow, Manassas, VA) filtered through 0.22 μm filter. Cells were co-transfected with internal control (Firefly plasmid) and experimental plasmids (Renilla-*BDNF*$^{\text{Full 3′UTR}}$ or Renilla-*BDNF*$^{\text{Short 3′UTR}}$) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacture's protocol. 24 hrs after the first transfection, the F-11 cells were replated at a density of 5,000 cells/cm^2 in a 24 well plate and incubated for 24 hrs. The cells were transfected again with either scrambled miRNA, Firefly duplex, Renilla duplex, miR-206 mimic or pRNAT- pre-miR-206-AcGFP using Lipofectamine 2000. Duplex is an siRNA control for the effective knockdown of luciferase gene (Renilla or Firefly). MicroRNA mimics are chemically modified miRNA-like RNA that is designed to copy the functionality of mature endogenous miRNA upon transfection.

Luciferase assays were performed in F-11 cells using the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI). In DLR assay, both Firefly and Renilla luciferase activities were measured sequentially from the same cell lysate.

The Firefly luciferase was measured first by adding Luciferase Assay Reagent II (LARII), followed by measuring Renilla luciferase activity consecutively in the same reaction by adding Stop and Glo reagent. The luminescence signal of the experimental report (Renilla) was normalized to that of control (Firefly) to minimize experimental variability between replicates.

2.3 Axoplasmic RNA Extraction from Sciatic Nerve and RNA Quantification

Animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC), and the experiments were conducted under the IACUC at Alfred I. duPont Hospital for Children. 150-225 g male *Sprague Dawley* rats were euthanized by asphyxiation with CO_2 using compressed sources of gas. Once euthanized the sciatic nerves were surgically removed from both sides of the rat and carcasses were disposed by the regulations. A mechanical squeezing method was used to isolate the axoplasm from the collected rat sciatic nerve. Briefly, the sciatic nerves were cleaned from the surrounding connective tissues using ultra fine forceps in 1XPBS solution. Sciatic nerve was cut into ~10 mm length segments using a surgical blade. Then, the axoplasm was carefully squeezed manually using a pestle fit into a 1.5 mL microcentrifuge tube containing TRIzol (Invitrogen, Carlsbad, CA) on ice. Nucleic acids were separated from proteins and other cellular components by centrifugation. To separate RNA from DNA, acidic phenol/chloroform (pH 5.5,

ThermoFisher, Boston, MA) was used. The purified RNA was then dissolved in nuclease free water and stored at -80°C until use.

The quantity of RNA extracted was determined by the VersaFluoro fluorometer using RiboGreen (Invitrogen, Carlsbad, CA). Briefly, standard dilutions containing 16S ribosomal RNA (rRNA) were prepared to generate a standard curve to calculate the RNA concentration. TE buffer was dispensed in microcentrifuge tubes followed by addition of ribosomal RNA to generate a range of standard is from 0 - 200 ng/mL (Table 2.2). Then, ribogreen solution (2,000 fold diluted in TE buffer) was added to each tube to make a total of 200 µL assay volume and transferred to cuvettes. The fluorescence reading was obtained at 485 - 495 nm excitation and 515-525 nm emission filters.

Standard (ng/mL)	**rRNA (µL)**	**TE buffer (µL)**
200	20	80
100	10	90
80	8	92
40	4	96
20	2	98
10	1	99
0	0	100

Table 2.2: **Dilutions used for RiboGreen assay.** A standard RiboGreen plot was made followed by linear equation and used to calculate the concentration of the unknown axoplasmic RNA.

2.4 cDNA Synthesis and Axoplasm RNA Purity

500 ng of RNA was reverse transcribed to cDNA using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) as per manufacturer's instructions. To confirm purity of the axoplasmic RNA extracted, extended RT-PCR method (X40 cycles) was utilized to check contaminations from cell bodies and non-neuronal cells including glial cells. I considered the RNA as highly enriched axoplasmic RNA and used for further studies only when axoplasmic RNA samples were not detected for cell body restricted *microtubule-associated protein 2* (*MAP2*) and *H1 histone family member 0* (*H1F0*), non-neuronal *glial fibrillary acid protein* (*GFAP*) and *Receptor tyrosine-protein kinase ErbB family-3* (*ErbB3*) mRNAs. In addition, the presence of *β-actin* mRNA in the sample was indicative of the presence of mRNA in the extraction.

Primers	Forward Primer (5′→ 3′)	Reverse Primer (5′ → 3′)	Purpose
ErbB3	CCCTTGAGAGTATCCACTTTGG	TTCTTTAAAGGTTGGGCGAATA	Glial cell/Non-neuronal cells
GFAP	TCAACGTTAAGCTAGCCCTGGACA	TCGCATCTGGAGGTTGGAGAAAGT	
MAP2	CCTCTGGCTTCTGATATT	TTCCTCTTTGCTCTCTATT	Cell body
H1F0	GGCCAAGGCCGCCAAGAAGT	GGCGCCCACCCCTTTGGTTT	
β-actin	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	mRNA

Table 2.3: **List of primers used to check for the axoplasm purity.** These are the primers used to check for cell body and glial cell contamination in the axoplasm samples.

After the purity analysis of the isolated axoplasmic RNA, the presence of *BDNF* mRNA with a longer 3′UTR was determined using RT-PCR with specific primers for the longer 3′UTR variant.

Primers	Forward Primer (5′→ 3′)	Reverse Primer (5′→ 3′)
BDNF Short 3′UTR	ATACAGTACAGTGGTTCTAC	CCACAACATTATCGAGGA
BDNF Long 3′UTR	GGAGTAGGGATGGAGAAA	CCAGTCATCTGATTGGATT
BDNF CDS	GAACTACCCAATCGTATG	GTACATACACAGGAAGTG
GAPDH	GACAACTTTGTGAAGCTCAAT	GGGCCTCTCTCTTGCTCTCA

Table 2.4: List of primers used to test the presence of *BDNF* mRNA with a longer 3′UTR variant in axoplasm. These primer sets were used to check for the presence of BDNF mRNA with long 3'UTR, BDNF mRNA coding sequence and GAPDH for qRT-PCR.

2.5 Fluorescence Recovery after Photobleaching (FRAP)

A eGFP reporter construct containing a longer 3′UTR of *BDNF* mRNA (^{myr}eGFP-*BDNF*$^{Full\ 3'UTR}$) and a mCherry reporter construct containing a short 3′UTR of *BDNF* mRNA (^{myr}mCherry-*BDNF*$^{Short\ 3'UTR}$) were generated.

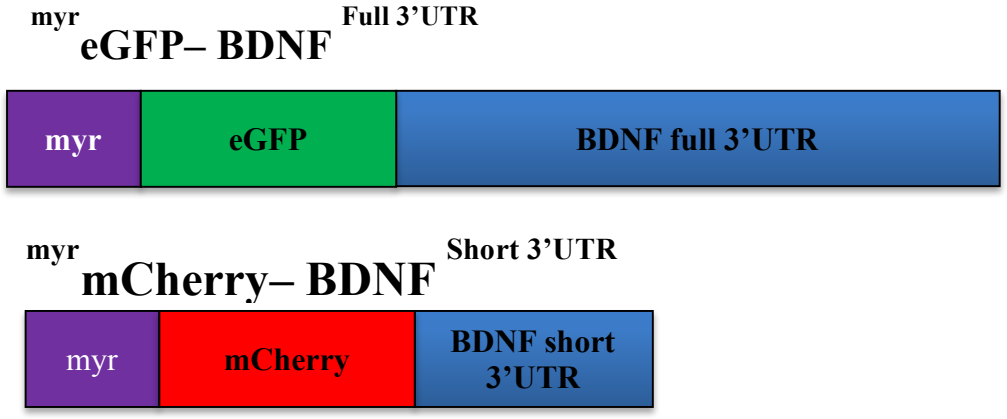

Figure 2.2: **Reporter constructs used for FRAP.** Schematics of ^{myr}eGFP and ^{myr}mCherry reporter constructs used to identify localization cis-elements within the 3'UTR of BDNF mRNA.

Myristoylation tag (myr) was added to the eGFP or mCherry to limit cellular free diffusion as myr tag will target the newly synthesized reporter protein to adjacent membrane. Dorsal Root Ganglion (DRG) neurons were isolated from adult male *Sprague Dawley*. The collected DRGs were dissociated using collagenase followed by trituration using fire polished glass pipettes. DNA exonuclease I (DNase I) was added to further aid the dissociation process. Once the cells were in a single cell suspension, they were washed in DRG culture media (DMEM/F-12 containing 5% horse serum, 5% fetal bovine serum, 1X HEPES, 2 mM L-Glutamine, 1X N1 supplement and 10 µM Ara C) 3 times to remove cell debris. Then, they were transfected with either ^{myr}eGFP-*BDNF*$^{Full\ 3'UTR}$, ^{myr}mCherry-*BDNF*$^{Short\ 3'UTR}$ construct, or both, using the 4D Nucleofactor system (Lonza, Hopkinton, MA) as per manufacturer's instructions. The

transfected DRG neurons were dispensed in culture media dropwise on glass-bottom 35 mm culture dishes that were doubly coated with poly-l-lysine and laminin.

Transfected DRG cultures were analyzed 48-72 hr after transfection for intra-axonal eGFP or mCherry fluorescence. A Zeiss LSM 880 confocal microscope fitted with an environment chamber was used for photobleaching and monitoring recovery of the fluorescence. Axons longer than 400 μm in length from the cell body were intentionally selected for FRAP experiments to minimize diffusion of eGFP or mCherry from the cell body during recovery. Terminal axons were imaged every 30 sec for 10 min with a 488 nm or a 594 nm laser to establish a baseline fluorescence intensity of eGFP or mCherry, respectively (prebleach). A region of interest (ROI) of terminal axons was exposed to 100% power of the 488 nm or the 594 laser for 35 repeats for photobleaching (bleaching). The recovery of fluorescence within the photobleached ROI was then monitored by acquiring images every 60 sec under the same 488 nm or 594 nm laser sets as in the prebleach for a total of 30 min period (postbleach). Cyclohexamide (100 μg/mL) was added as a translational inhibitor to ensure that fluorescence recovery depends on local protein synthesis. Cyclohexamide interferes with the translocation step in the protein synthesis, i.e., blockade of the translational elongation.

2.6 Dorsal Root Ganglion (DRG) culture in the Boyden Chamber to Isolate Neuronal Processes.

The Boyden chamber is a porous cell culture insert, separating the cell culture dish into two compartments. The size of the pores (only a few microns in diameter) allows only axons traverse through the pore to reach the undersurface of the membrane (axonal compartment) and restrict the larger cell bodies to the upper surface of the insert (cell body compartment). By physically scraping the insert, I was able to separate cell bodies from the axonal processes of DRG neurons and obtained a pure separation of axons. Briefly, 8 μm tissue culture inserts were placed in 6 multiwell culture plates and coated with poly-L-lysine (50 μg/mL in working solution, Sigma, St. Louis, MO) for an hour at 37°C. The wells and inserts were washed with autoclaved water and air dried in room temperature for 20 min. Then, the inserts were coated with laminin (10 μg/mL working solution, Millipore, Billerica, MA) prepared in cold 1XPBS and placed in a rocker at 4°C overnight.

DRG neurons were isolated from adult rats, as described in the previous section. They were dissociated in collagenase for a total of 30 min at 37°C followed by trituration using fire polished pipettes. In addition, DNase I (1 μg/mL) was added

to facilitate the dissociation of DRG neurons to single cell suspension. The single cell suspension was washed multiple times with DRG culture media containing 5% horse serum, 5% fetal bovine serum, 1X HEPES, 2 mM L-Glutamine, 1X N1 supplement and 10 μM Ara C in DMEM/F-12. Then, the cells were co-transfected with either empty pRNAT-miR-AcGFP control plus miR control mimic, or pRNAT -miR-206-AcGFP plus miR-206 mimic using the 4D Nucleofactor system (Lonza, Hopkinton, MA) as per manufacturer's instructions. The purpose of using the pRNAT plasmid in addition to miR mimic for transfection experiments was to provide continuous supply of the miRNA within the transfected DRG neurons. Immediately after the transfection, the cells were plated onto the tissue inserts and incubate for 3 days in culture at 37°C with 5% CO_2.

Axonal total proteins were isolated first by scraping the bottom surface of the membrane with a cell scraper and dispersed in modified Radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethanesulfonyl fluoride (PMSF), a protease cocktail and a phosphatase inhibitor. Cell body proteins were processed by scraping the upper membrane. The lysates were rotated for 15min in cold room and briefly spun down before running the gel.

2.7 Western Blot

Lysates of cell body or axonal fractions were mixed with 5X loading buffer (denaturing buffer), boiled for 5 min and placed on ice immediately to denature the proteins until ready to load. In general, 10 μL of all cell body lysate and 60 μL and axonal samples were loaded onto 12% SDS-PAGE gels. The proteins were separated by running the gels at 60 V until the dye front reaches the running gel and then increased to 100 V before stopping the electrophoresis run when the dye front reaches the bottom of the gel. The proteins fractionated on 12% PAGE gels were transferred onto PVDF membranes at 15 mA for 30 min in transfer buffer at 4°C. The membrane was then blocked in 5% milk in tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) for 1 hr at room temperature. Then, the membrane was probed first with anti-BDNF (rabbit polyclonal, 1:1000 dilution, Bioss, Woburn, MA) in 5% milk in TBS-T overnight at 4°C on a shaker. After washing extensively in TBS-T , the membrane was incubated in a secondary antibody conjugated to horseradish peroxidase (HRP) [Goat α-Rabbit HRP (1:3000 dilution)] in 5% milk in TBS-T for 1 hr at room temperature. The HRP activity was visualized using Amersham enhanced chemiluminescence prime western blotting detection reagent (GE Healthcare, Piscataway, NJ) that uses luminol as a substrate for HRP. After developing the membrane to X-ray film, the membrane was extensively washed and probed with anti-GAPDH (1:3000 dilution, Cell Signaling, Beverly, MA) as previously described. GAPDH served as an internal control for normalization.

2.8 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

500 ng of RNA was reverse transcribed to cDNA using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) as per manufacturer's instructions. The RNA samples were analyzed for cell bodies and non-neuronal cells including glial cell contamination using extend RT-PCR as described in previous section (2.4). Using SYBER Green PCR Master Mix (QIAGEN, Hilden, Germany), 1ng of enriched axoplasmic RNA were amplified for target transcripts (Table 2.4) in a total volume of 20 μL per well. All the samples were assayed in triplicates. 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to perform qRT-PCR in a 384 well plate. The instrument settings used was a PCR activation at 95°C for 15 min and 40 cycles of denaturation at 94°C for 15 sec, annealing at 50°C for 15 sec and extension at 72°C for 30 sec. Threshold cycle (C_T) values from each BDNF samples was normalized to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. Also, BDNF coding sequence specific primers were used to observe the presence of total *BDNF* mRNA.

2.9 Droplet Digital Polymerase Chain Reaction (ddPCR)

Droplet digital PCR (ddPCR) is a technological refinement of conventional PCR method that uses a water-oil emulsion droplet system to partition a PCR sample into nanoliter-size samples and encapsulate into oil droplets. The protocols were

followed as per manufacturer's instructions (Bio-Rad, Hercules, CA). Briefly, the samples were prepared similar to the real time PCR assay. The sample reaction was then placed in a droplet generator system that partitions the reaction into 20,000 nanoliter sized droplets, which is the essential aspect of the technology as compared to the traditional PCR. After amplification in the compatible thermocycler, a droplet reader counted the fluorescent positive and negative droplets to determine the concentration of the starting target molecule in units of copies/µL input. ddPCR provides absolute quantification of target DNA copies per input samples without the need of running standard curves. In addition to the absolute quantification, ddPCR enables to detect very low abundant gene with sensitivity and precision. Thus, this technique was used to calculate the copy numbers of *BDNF* mRNA in the axoplasmic RNA samples.

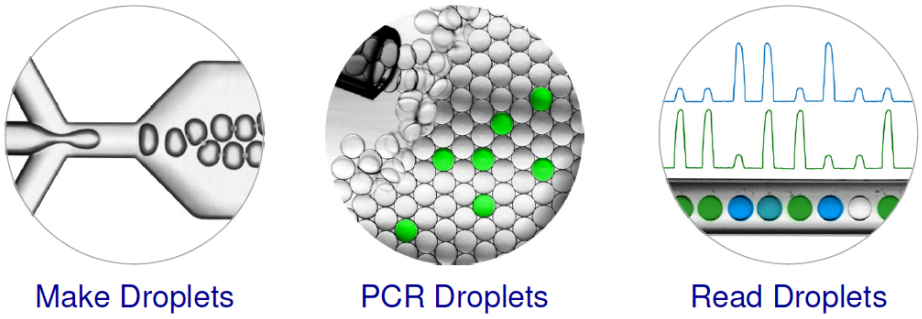

Figure 2.3: Droplet digital PCR methodology (Bio-Rad, Hercules, CA)**.** The diagram shows the droplet generation of sample using droplet generator

system, PCR reaction of the droplets and droplet readouts counting droplets with or without fluorescent signal.

Chapter 3

RESULTS

3.1 *BDNF* mRNA with a long 3′UTR is present in distal axons of adult rat sensory neurons

I hypothesized that *BDNF* mRNA with a long 3′UTR is present in the distal axons of sensory neurons. Sciatic nerve from adult rats was used as the source of axoplasm of distal axons of sensory neurons. After extended RT-PCR was carried out to determine the purity of the axoplasmic RNA for glial and cell body contamination, these samples were checked for the presence of *BDNF* mRNA with long 3′UTR in the distal axons of sensory neurons using RT-PCR. Detection of PCR products for *H1F0* and *MAP2* genes presented cell body contaminations, and that for *GFAP* and *ErbB3* transcripts pointed glial cell contamination. *β-actin* was included to confirm the presence of mRNA in the extracted samples. RNA from whole tissue lysates of rat brain was also included to test the primer sets, showing positive PCR amplification for all genes tested

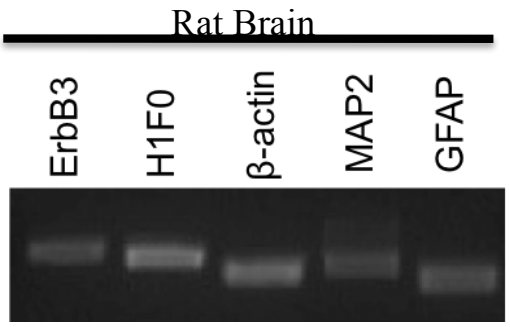

Figure 3.1: 1% agarose gel with extended RT-PCR products for mRNA extracted from whole tissue lysates of rat brain with primer sets tested.

Axoplasmic RNA samples were considered to be highly enriched when the extended RT-PCR showed a positive PCR product only for *β-actin* and none for other primer sets examined (Fig 3.2). I found that axoplasmic RNA showed a positive PCR product for *β-actin* primer set, but no PCR products were observed after amplification using other primer sets. This indicated a highly enriched axoplasmic RNA.

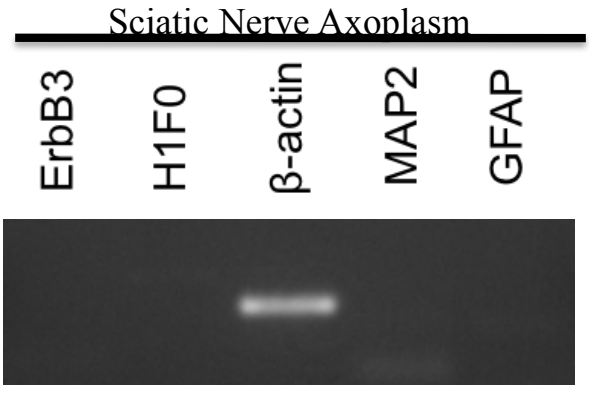

Figure 3.2: 1% agarose gel with extended RT-PCR products of mRNA extracted from sciatic nerve.

After determining the purity, axoplasmic RNA extracted from sciatic nerve axons was used to examine the presence of *BDNF* mRNA by RT-PCR. Additional to the axoplasmic RNA, I included RNAs extracted from rat brain and DRG. The RT-PCR products showed that endogenous *BDNF* mRNA is present in distal axons of neurons.

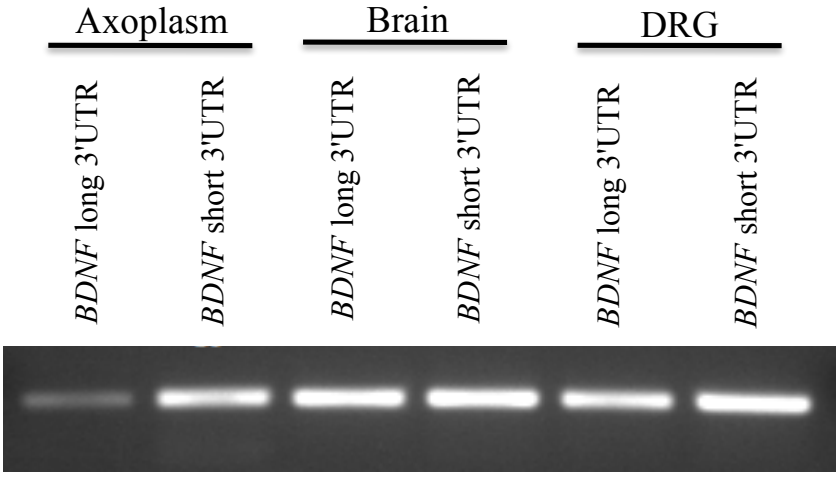

Figure 3.3: 1% agarose gel with RT-PCR products of RNAs extracted from sciatic nerve axoplasm, rat brain and DRG, indicating the presence of *BDNF* mRNA

However, the relative intensity of *BDNF* mRNA with a long 3′UTR was lower compared to that of *BDNF* mRNA with a short 3′UTR. To quantify the relative levels

of two different variants of *BDNF* mRNA's 3′UTR, a short form and a longer 3′UTR, in sciatic nerve axons, I utilized quantitative real time polymerase chain reaction (qRT-PCR) approach. In qRT-PCR assay, threshold cycle (C_T) represents the cycle number at which the fluorescence signal generated from a PCR reaction becomes significantly above the background fluorescence intensity. Therefore, the threshold cycle (C_T) is inversely proportional to the amount of the gene of interest. Further, since the short 3′UTR form of *BDNF* mRNA is contained within the longer 3′UTR variant, any primers designed to *BDNF* mRNA with the short 3′UTR should simultaneously amplify both variants. That is, PCR using the short 3′UTR primers should amplify the short + longer 3′UTR variants of *BDNF* mRNA, and PCR using specific primer for the longer 3′UTR should amplify only the longer variant. The equation to calculate the amount of short form from the qRT-PCR approach was as follows:

$$\text{Short 3′UTR variant of } BDNF \text{ mRNA} = 2^{-C_T\ (\text{Short+Longer 3′UTRs})} - 2^{-C_T\ (\text{Longer 3′UTR})}$$

However, qRT-PCR failed to yield valid C_T values for quantification of *BDNF* mRNA with a longer 3′UTR in the axoplasmic RNA samples (the C_T value as high as 35-40, or even undetectable), indicating very low copy number of endogenous *BDNF* mRNA with a longer 3′UTR. Therefore, I advanced to use droplet digital PCR (ddPCR) technology. ddPCR is a highly sensitive technique compared to qRT-PCR as

it can detect low copy numbers and rare allele. It directly counts the number of target molecules rather than relying on reference standards and endogenous controls. I compared the copy number of endogenous *BDNF* mRNA with a longer 3′UTR and *BDNF* coding sequence in cell body (DRGs) and axons (Sciatic nerve). Results from ddPCR revealed that there was significantly lower copy number of endogenous *BDNF* mRNA with a longer 3′UTR in distal axons by ~20 times lower compared to the cell body per ng of RNA.

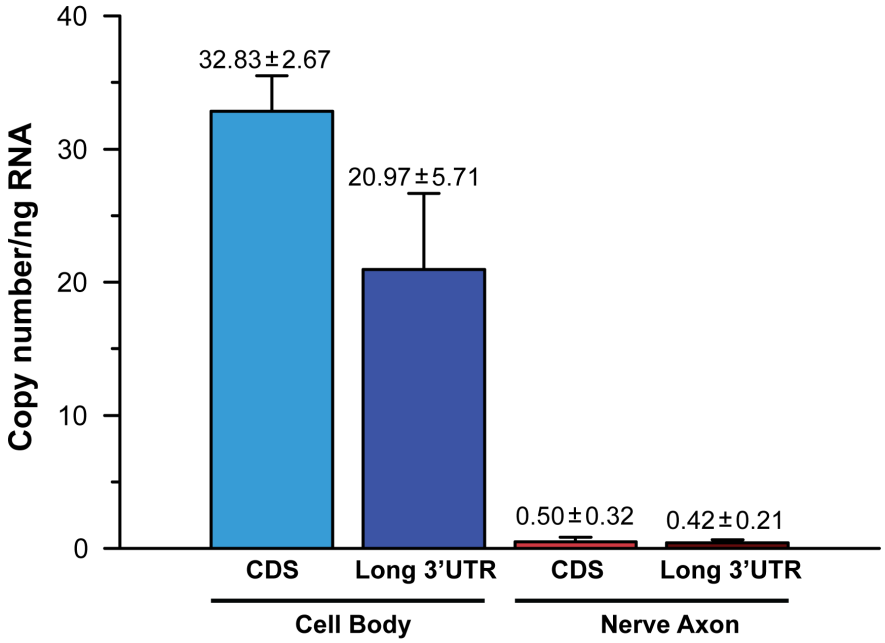

Figure 3.4: Droplet digital PCR data showing accurate copy number of endogenous *BDNF* mRNA with long 3′UTR in cell body (DRG) and axoplasm (from Sciatic nerve). *CDS stands for coding sequence for BDNF mRNA

3.2 Reporter with a long 3′UTR localized to distal axons of neurons undergoes local translation

Dorsal Root Ganglia (DRG) neurons were isolated from adult male Sprague Dawley and transfected with either ^{myr}eGFP-*BDNF*$^{Full\ 3'UTR}$ or ^{myr}mCherry-*BDNF*$^{Short\ 3'UTR}$ construct, using the 4D Nucleofactor system (Lonza, Hopkinton, MA) as per manufacturer's instructions. FRAP analysis showed that distal axons of DRG neurons transfected with ^{myr}eGFP-*BDNF*$^{Full\ 3'UTR}$ reporter was significantly recovered from photobleaching by 4 min (Figure 3.5). Further, this recovery was significantly blocked by addition of cyclohexamide, translational inhibitor. All these results indicate that a longer 3′UTR of BDNF mRNA drives the reporter mRNA to distal axons of neurons where local translation of localized reporter mRNA occurs.

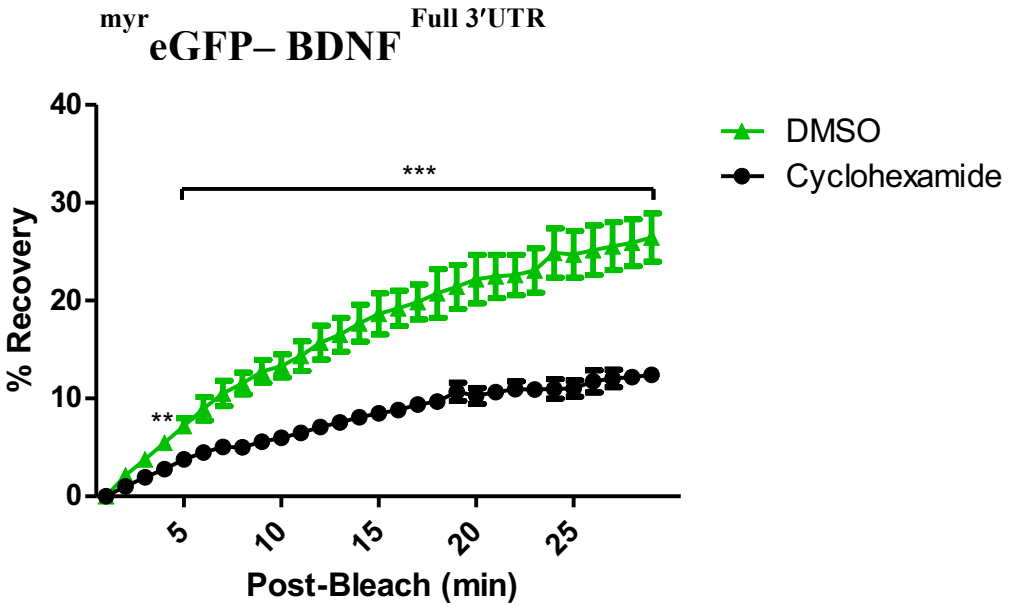

Figure 3.5: FRAP data analysis of ^{myr}eGFP reporter with a long 3'UTR of BDNF mRNA. DRG neurons transfected with construct ^{myr}eGFP reporter containing a full length 3′UTR of *BDNF* mRNA (long 3′UTR) is localized in distal axons of neurons and locally translated. Two-way ANOVA with repeated measures was used to determine any significance differences in relative fluorescence recovery between treatments (** $p < 0.01$ and *** $p < 0.001$ for indicated time points *vs.* t=0 by one-way ANOVA with Bonferroni post-hoc test). n = 7

Unexpectedly, I also observed a statistically significant recovery of fluorescence after photobleaching by 4 min in the axons of DRG neurons transfected with ^{myr}mCherry-*BDNF*$^{Short\ 3'UTR}$ construct (Figure 3.6). This recovery was

significantly blocked by addition of cyclohexamide, similar to the FRAP experiments with ^{myr}eGFP-*BDNF*$^{Full\ 3'UTR}$ reporter, indicating that ^{myr}mCherry reporter mRNA containing a short 3′UTR of *BDNF* mRNA showed the protein synthesis dependent recovery after photobleaching.

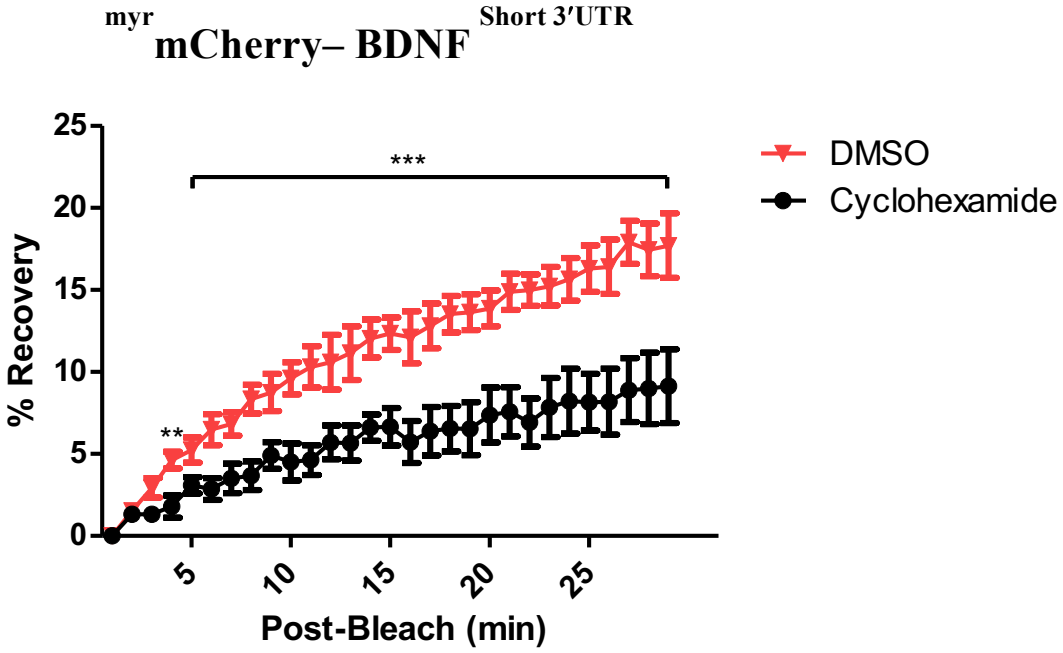

Figure 3.6: FRAP data analysis of ^{myr}mCherry reporter with a short 3'UTR of BDNF mRNA. DRG neurons transfected with construct ^{myr}mCherry reporter containing a short 3′UTR of *BDNF* mRNA is localized in distal axons of neurons and locally translated in axons of DRG. Two-way ANOVA with repeated measure was used to determine any significance differences in relative fluorescence recovery between treatments (** $p <$

0.01 and *** $p < 0.001$ for indicated time points *vs.* t=0 by one-way ANOVA with Bonferroni post-hoc test). n = 7

3.3 A longer 3′UTR variant of *BDNF* mRNA is involved in miR-206-mediated regulation of gene expression

I hypothesized that *BDNF* mRNA variant with a longer 3′UTR variant present in distal axons of sensory neurons is specifically regulated by miR-206. Recent studies suggested that the intra-axonal translation of localized mRNA is precisely regulated by specific miRNAs that recognize their target sequences present in 3′UTR of the transcripts (Besse and Ephrussi, 2008; Xing and Bassell, 2012). Since bioinformatics analysis for predicting miRNAs targeting the 3′UTR of *BDNF* mRNA revealed that miRNA-206 may contribute to the regulation of *BDNF* expression, I first performed DLR assay on F-11 cells that were co-transfected with internal control Firefly plasmid and experimental Renilla plasmids containing either a full 3′UTR or a short 3′UTR form of *BDNF* mRNA. 24 hrs after the reporter transfection, cells were transfected again with miR-206 mimic to test whether overexpression of miR-206 affected on expression of the reporter construct. MicroRNA-206 mimic is chemically modified miRNA-like RNA that provide the functionality of mature endogenous miRNA-206.

Ratio of Renilla signal to Firefly signal was calculated and normalized to that of the cells transfected with scrambled miRNAs (negative control). F-11 cell

transfected with Renilla plasmid containing a full 3′UTR of *BDNF* mRNA showed a significant downregulation of Renilla expression by ~40% by overexpression of miR-206. In contrast, no significant change in Renilla activity was observed when F-11 cells were transfected with reporter containing a short 3′UTR form of *BDNF* mRNA.

These results strongly support the hypothesis that the *BDNF* mRNA with a longer 3′UTR variant, which is endogenously present in the distal axon of the sensory neurons, is regulated post-transcriptionally by miR-206.

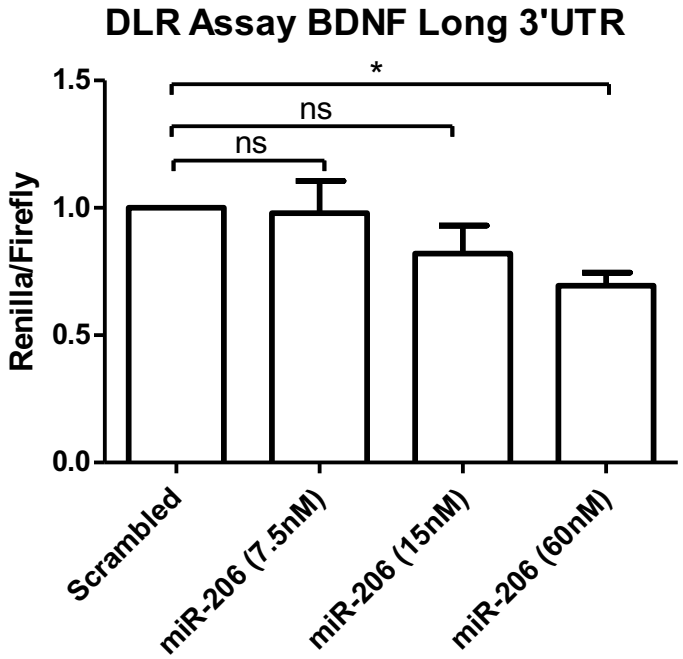

Figure 3.7: DLR Assay of F-11 cell transfected with Renilla construct containing BDNF long 3'UTR. DLR Assay showing significant

downregulation in Renilla activity by miR-206. *p<0.05, Student t test, n=3. ns: not significant (p>0.05)

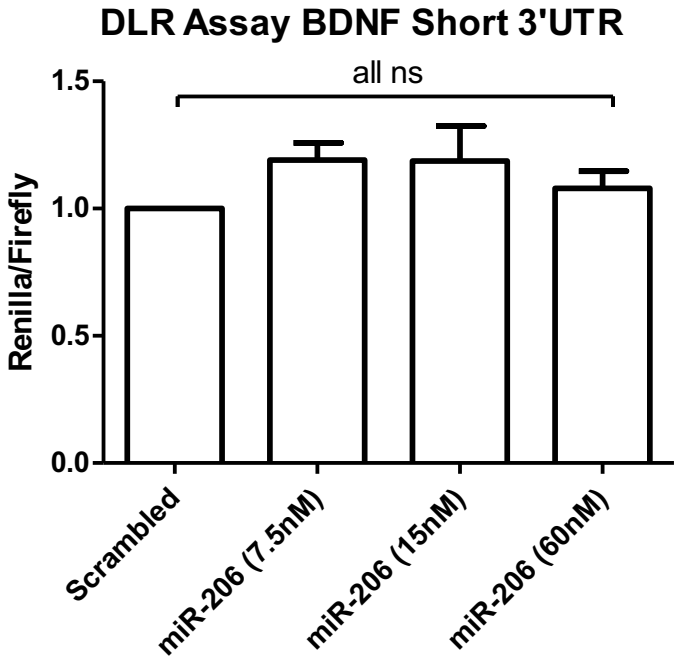

Figure 3.8: DLR Assay of F-11 cell transfected with Renilla construct containing BDNF short 3'UTR. DLR Assay showing no significant change in Renilla activity by miR-206. Student t test, n=3. ns: not significant (p>0.05)

3.4 miR-206 specifically recognizes and targets the predicted target site # 3 within the 3'UTR of *BDNF* mRNA to regulate BDNF expression.

Bioinformatics analyses of *BDNF* transcript predicted 3 different target sites within the 3'UTR that could be recognized and targeted by miR-206. To further map out which site(s) are biologically targeted by miR-206, F-11 cells were co-transfected with internal control Firefly plasmid and experimental plasmids containing either predicted target site #1 (Renilla-target site #1^{miR206}), #2 (Renilla-target site #2^{miR206}), or #3 (Renilla-target site #3^{miR206}). 24hr after the initial transfection, the cells were transfected with pRNAT- pre-miR-206-AcGFP. The precursor miR-206 construct was used to overexpress pre-miR-206 that ultimately and continuously gives rise to mature miR-206 for a longer period of time. Ratio of Renilla/Firefly was calculated and normalized to that of the cell transfected with negative control (pRNAT-AcGFP-scramble miRNA). Results from DLR assay revealed that miR-206 target site #3 was specifically involved in the regulation of *BDNF* expression by miR-206, because the normalized ratio of Renilla to Firefly in F-11 cells transfected with Renilla plasmid containing the miR-206 target site #3 sequence in the 3'UTR of *BDNF* mRNA was significantly downregulated by ~45% with overexpression of miR-206. However, no significant change in Renilla activity was observed when F-11 cells were transfected with reporter construct containing miR-206 target site # 1 and 2 sequences in the 3'UTR *BDNF* mRNA. These results strongly suggest that the predicted target site #3

of miR-206 that is exclusively present in the longer 3'UTR of *BDNF* mRNA is involved in the regulation of *BDNF* expression (Fig 3.9).

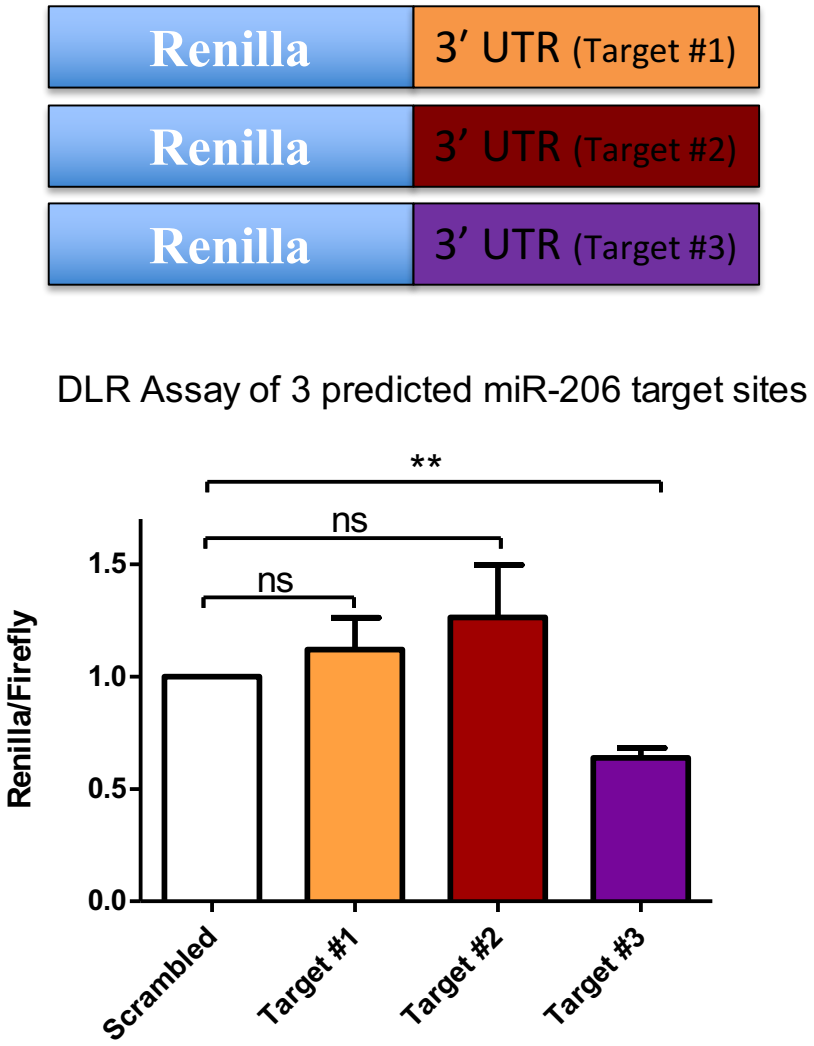

Figure 3.9: DLR Assay of 3 predicted miR-206 target sites. To test which of the three miR-206 predicted target sites was involved in the *BDNF*

expression regulation, DLR assay was performed. Upon analysis, Student's t-test showed that F-11 cells transfected with Renilla-target site #3^{miR206} construct showed significant down-regulation by overexpression of miR-206. **p<0.005, n= 4, ns: not significant (p>0.05)

3.5 Overexpression of miR-206 in DRG neurons results in a significant decrease in BDNF protein expression.

DRG neurons co-transfected with pRNAT- miR-AcGFP control plus mimic control, or pRNAT- miR-206-AcGFP plus miR-206 mimic were cultured for 3 days on 8 μm tissue culture inserts doubly coated with poly-L-lysine and laminin. Using both miR-206 mimic and miR-206 precursor construct insures overexpression of matured miR-206. Although miR-206 mimic results in overexpression of mature miR-206, it has a relatively short half-life. To extend overexpression effect, construct containing precursor miR-206 (pRNAT- pre-miR-206-AcGFP) was included in these experiments. The axonal lysate and cell body lysate prepared in cell lysis buffer were separated in 12% SDS-PAGE gels and transferred to PVDF membranes for western blot analysis. I found that overexpression of miR-206 in DRG neurons caused a noticeable decrease in BDNF protein expression as shown in figure 4.0.

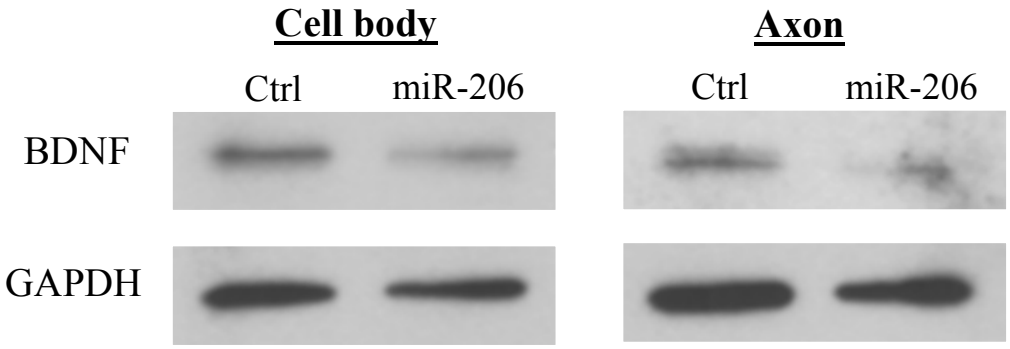

Figure 4.1: **Western blot analysis.** The blot was performed on cell lysates obtained from transfected DRG neurons cultured for 3 days using primary antibodies for BDNF and GAPDH.

Densitometry was carried out with *ImageJ* image processing software to quantitatively compare the intensity of the bands observed on developed blots. The band intensities of BDNF protein for both cell body (DRG) and axon were normalized to loading control GAPDH protein intensity. Results from densitometry analysis showed ~33% reduction in BDNF protein expression in both cell body and axons when miR-206 was overexpressed as compared to control transfected with scrambled miRNA. These results strongly suggested that miR-206 negatively regulates BDNF protein expression in sensory neurons.

Chapter 4

DISCUSSION

BDNF is involved in various functions such as neuronal development, neuronal survival, and maintenance of synaptic plasticity (Caputo et al., 2011; Balaratnasingm et al., 2012). Two alternate polyadenylation (PolyA) sites present in the coding exon lead to the generation of two different 3'UTR variants of *BDNF* transcript, a short form (0.35kb) and a longer 3'UTR (2.9kb) (Timmusk et al., 1993; Greenberg et al., 2009; Lau et al., 2010). An et al. (2008) previously showed that the longer 3'UTR variant of *BDNF* mRNA localizes into the dendrites of mouse hippocampal neurons, whereas the short 3'UTR variant is restricted to the soma. This study indicates that *cis*-element(s) that control the mRNA localization into distal processes of neurons are present within the distal segment of the longer form of 3'UTR of *BDNF* mRNA. However, the possibility that the longer 3'UTR variant of *BDNF* mRNA is selectively localized into distal axons of neurons has not been tested. In the present study, I was able to show the first evidence that *BDNF* mRNA with a long 3'UTR is present in distal axons of adult rat sensory neurons (Figure 3.3). It is noteworthy that since the whole sequences of the shorter 3'UTR variant of *BDNF* mRNA is completely included within the longer 3'UTR variant of *BDNF* mRNA, one

cannot exclusively distinguish the short 3'UTR variant from the longer variant using PCR method. However, the band intensities of RT-PCR products between the shorter and longer variants were considerably different, suggesting that at least a subset of the longer 3'UTR variant of *BDNF* mRNA is selectively localized into distal axons of sensory neurons.

Transcripts that are specifically localized in distal axons of a neuron account for less than 5% of the total mRNA that are present in the neuron (Eng et al., 1999; Jung et al., 2012, Gumy et al., 2011). Thus, the endogenous level of *BDNF* mRNA with a long 3'UTR in distal axons of neurons would be much lower than that in the cell body. My attempts to quantify the relative level of endogenous *BDNF* mRNA in distal axons using qRT-PCR approach was not successful, since the C_T values were either too high or undetectable. The threshold cycle (C_T) values being relatively high or absent indicate very low copy number of endogenous *BDNF* mRNA with a longer 3'UTR. Consistent with this data interpretation, I found that the distal axons of neurons have low copy number of *BDNF* mRNA with a longer 3'UTR compared to the cell body when ddPCR methodology was utilized (Figure 3.4). An et al. (2008) previously showed that *BDNF* mRNA containing a shorter 3'UTR variant is restricted to the soma by using FRAP and FISH methods and suggested that the localization *cis*-element(s) reside within the distal segment of the longer 3'UTR of BDNF mRNA.

Inconsistent with this previous report, FRAP experiments with DRG neurons transfected with ^{myr}mCherry-BDNF$^{Short\ 3'UTR}$ showed a significant recovery after photobleaching (Figure 3.6). Further, cyclohexamide treatment demonstrated that this recovery is local protein synthesis-dependent. Several previous studies suggest the presence of multiple *cis*-elements within the 3'UTR of transcripts that have differential degree of targeting capability (Vuppalanchi et al.,2010; Lau et al., 2010). Thus, it is plausible that BDNF mRNA might have multiple *cis*-acting motifs within the 3'UTR including the sequences of the shorter 3'UTR. Future studies are essential to verify the degree of localization capability between motifs in the 3'UTR.

MicroRNAs (miRNAs) are short non-coding RNAs that regulate translation of mRNAs by binding to their target sequences usually within the 3'UTR in a sequence-dependent manner. DLR assays and western blots showed that expression of *BDNF* mRNA is negatively regulated by miR-206. In addition, consistent with previous studies led by Lee et al. (2012) and Miura et al (2011) identified 3 separate predicted target sites of miR-206 in the 3'UTR of *BDNF* mRNA using multiple algorithms. Among these three predicted target sites for miR-206 present in the 3'UTR of *BDNF* mRNA, only target site # 3 present in the distal portion of the longer 3'UTR of *BDNF* mRNA is involved in the regulation of BDNF expression (Figure 3.9). Given that both the short and the longer 3'UTR variants of *BDNF* mRNA are present in distal

axons, and presumably that the shorter variant is less efficient for localization than the longer form, miRNA-206 may be able to distinguish the longer 3'UTR of *BDNF* mRNA from the short form in the process of translational control in distal axons. It is not clear yet why these two 3'UTR variants of *BDNF* mRNA are localized to distal axons in different efficiency and regulated independently by miR-206. However, it is possible that the longer 3'UTR form of *BDNF* transcript is encoding a protein that has novel non-neurotrophin functions. It may act locally within distal axons to influence axonal properties, similar to the intermediate filament protein lamin B2 in distal axons of Xenopus retinal ganglion neurons (Yoon et al, 2012). Alternatively, the newly synthesized protein that is translated from the longer variant of *BDNF* mRNA within axons could act as a retrograde injury signal, as previous studies showed (Hanz et al, 2003; Yudin et al., 2008; Cox et al., 2008, Ji et al., 2012, Harrington et al., 2013). A previous study by Lau et al (2010) has showed that *BDNF* mRNA with a longer 3'UTR specifically mediates activity-dependent translation while BDNF mRNA with a short 3'UTR is suppressed after induced seizures. Notably, retrograde injury signals locally synthesized can allow local signals at the distal processes of neurons to induce robust cell body responses. A major challenge in understanding whether locally synthesized BDNF protein in axons has biological roles in neurons is to monitor the trafficking of locally synthesized protein in axons.

Lastly, it is estimated that more than 50% of mammalian transcripts contain alternative poly(A) sites that can lead to different 3'UTR variants (Tian et al.,2005).

Although several previous studies showed that different variants of the same transcript are differentially transported to axons suggesting the presence of sequences or elements conferring axonal targeting within the 3'UTR, it is not clear how translation of these variants is differentially regulated from the corresponding transcript with a shorter 3'UTR. My studies present here may explain the possibility that variant-specific sequences contain miRNA-binding sites or sites for RNA-binding proteins. Interestingly, the Papalopulu group (Dajas-Ballador et al., 2012) demonstrated that miR-9 regulates translation of axonal Map1b mRNA to control axonal extension and branching that are BDNF-dependent.

In conclusion, the BDNF mRNA with longer 3'UTR is differentially regulated in distal axons of sensory neurons by miR-206 compared to that of BDNF mRNA with short 3'UTR. This provides a unique ability to precisely control the temporal and spatial regulation of BDNF protein upon neuronal activation. This will be beneficial in several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Multiple sclerosis where low level of BDNF has been observed. Therefore, understanding the regulative effect of miR-206 in BDNF expression could be potentially therapeutic.

FUTURE DIRECTIONS

The follow up studies would be to understand and recognize the *cis*-elements that are present in the 3'UTR of BDNF mRNA. This could possibly be done by removing the segments of mRNA and replacing it with different segment. This will help to understand the importance and the efficiency of these *cis*-elements in the localization of BDNF mRNA. Next, future directions would be to find *trans*-acting factors (RNA binding proteins) which bind to the *cis*-elements present in 3'UTR of BDNF mRNA. This will be achieved by RNA pull-down assays.

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APPENDIX

IACUC PERMISSION

Nemours.

Institutional Animal Care and Use Committee
1600 Rockland Road
Wilmington, DE 19803
p (302) 651-6826 f (302)-651-6881
IACUC@nemours.org

MEMORANDUM

DATE: February 18, 2016

TO: Soonmoon Yoo, Ph.D.

FROM: Paul T. Fawcett, PhD

SUBJECT: Axonal precursor microRNAs in regenerating nerve

The Institutional Animal Care and Use Committee (IACUC) have reviewed the submitted annual review on the above referenced project and the following decision has been made:

Action: **approved**

Date of Action: February 18, 2016

Approval Period: February 18, 2016 through February 17, 2017

Approved Number of Animals: 135

Please submit your Biosafety Classification form electronically to the Alfred I. duPont Hospital for Children Institutional Biosafety Committee via the link:

http://www.nemours.org/pediatric-research/approval/biosafety-committee.html

Please note that the study cannot begin until the Office of Regulatory Compliance in Research Administration has received all approvals.

Please maintain this approval with your project records. A tally of the number of animals approved and the number ordered for the project will be maintained in the Life Science Center. If changes occur in your protocol or if you require more animals than approved, and amendment to your protocol will need to be submitted for consideration

If you have any questions regarding this memorandum, please contact Paul T. Fawcett, Ph.D. at x 6776 or email: pfawcett@nemours.org.

Nemours

Institutional Animal Care and Use Committee
1600 Rockland Road
Wilmington, DE 19803
p (302) 651-6826 f (302)-651-6881
IACUC@nemours.org

MEMORANDUM

DATE: April 20, 2015

TO: Soonmoon Yoo, Ph.D.

FROM: Paul T. Fawcett, PhD

SUBJECT: Temporal regulation of localized mRNA translation in regenerating axons

The Institutional Animal Care and Use Committee (IACUC) have reviewed the submitted annual review on the above referenced project and the following decision has been made:

Action: Approved

Date of Action: April 20, 2015

Approval Period: April 20, 2015 through April 19, 2016

Approved Number of Animals: 126

Please submit your Biosafety Classification form electronically to the Alfred I. duPont Hospital for Children Institutional Biosafety Committee via the link:

http://www.nemours.org/research/committee/ibc.html

Please note that the study cannot begin until the Office of Regulatory Compliance in Research Administration has received all approvals.

Please maintain this approval with your project records. A tally of the number of animals approved and the number ordered for the project will be maintained in the Life Science Center. If changes occur in your protocol or if you require more animals than approved, and amendment to your protocol will need to be submitted for consideration

If you have any questions regarding this memorandum, please contact Paul T. Fawcett, Ph.D. at x 6776 or email: pfawcett@nemours.org.