Down Regulation of Neuronatin by microRNA-151 Overcomes Inhibition of Axonal Growth by Myelin-based Inhibitors

by
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Abstract

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After injury, the axons of the adult central nervous system (CNS) fail to regenerate. This failure is due to the cellular environment and the neuronal response to that environment. One factor for environmentally-mediated axonal inhibition are the proteins that are present in myelin, such as myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp). Previously, our lab has shown that elevating the ubiquitous second messenger cyclic-adenosine monophosphate (cAMP) overcomes MAG/myelin inhibition. MicroRNAs (miRNA or miR), are small fragments of RNA that have been shown to bind to target mRNAs and regulate their translation. We hypothesized that miRNAs might be playing a role in the ability of cAMP to overcome MAG/myelin-based axonal growth inhibition. To investigate if miRNAs have a role in the cAMP effect we performed a miRNA microarray with cAMP treated vs. control dorsal root ganglion (DRG) neurons. One miRNA that increased more than two fold with cAMP treatment was miRNA-151. As previously stated, the axons of the mammalian CNS do not regenerate after injury. However, there is one situation in which they have been shown to spontaneously regenerate. DRG axons are bifurcated with one branch extending into the CNS and the other into the peripheral nervous system (PNS). Studies have shown that if a lesion is made to the PNS branch and then subsequently to the CNS

branch, the CNS branch will regenerate. This “conditioning lesion”-induced regeneration has been found to be dependent upon increased levels of cAMP. We next investigated whether like after treatment with cAMP, miR-151 was also increased after a peripheral conditioning lesion. We found similar significant increases in miR-151 levels in DRG neurons following a peripheral conditioning lesion. To determine a functional role for miR-151 in overcoming MAG/myelin-mediated neurite outgrowth inhibition we next performed overexpression and knockdown analyses of miR-151 and then subsequently subjected the neurons to a neurite outgrowth assay. Overexpression of pre-miR-151 in DRG neurons overcame MAG/myelin-mediated neurite outgrowth inhibition and conversely, knockdown of miR-151 with anti-miR-151 in DRG neurons attenuated the ability of db-cAMP to overcome MAG/myelin-mediated inhibition. To investigate the mechanism by which overexpression of miR-151 overcomes MAG/myelin-mediated inhibition we sought to identify miR-151 target mRNAs, using target prediction algorithms. One putative target was Neuronatin, a 9 kD transmembrane proteolipid protein with unknown neuronal function. We next wanted to assess if miR-151 could bind to the 3’UTR of Neuronatin and inhibit its translation. To test this, 293-T cells were co-transfected with miR-151 and a luciferase reporter gene fused to a wildtype or mutated Neuronatin 3’UTR. MiR-151 overexpression decreased the luciferase activity of the wildtype, but not the activity of the mutated Neuronatin 3’UTR, thus validating that Neuronatin is a miR-151 target. Likewise, we found that both treatment of DRG neurons with db-cAMP or overexpressing miR-151 led to a significant decrease in Neuronatin protein levels, while Neuronatin mRNA levels were unaffected. Finally, using siRNA we knocked-down Neuronatin in DRG neurons and then subjected the neurons to a neurite

outgrowth assay. Knockdown of Neuronatin led to a significant increase in total neurite length on both MAG-expressing CHO cells and purified myelin. Our findings suggest that the cAMP-induced miR-151 plays an important role in overcoming MAG/myelin-mediated axonal growth inhibition.

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Chapter I: Introduction

1.1 CNS Regenerative Failure

Injuries to the central nervous system have plagued humankind since the time of ancient civilizations. After injury, unlike the neurons of the peripheral nervous system, the neurons of the adult mammalian central nervous system fail to regenerate. The oldest known written record is thought to be from the Egyptian physician Imhotep, the author of the Edwin Smith Surgical Papyrus; the perhaps 5000 year old document describing patients with various spinal injuries, all of whom end with the same prognosis "An ailment not to be treated". Today, while the reason for the prognosis is understood, a treatment remains elusive. Since the work of Santiago Ramon y Cajal in the early 1900's it has been understood that changes in the environment surrounding the injury and the neuronal response to those changes are key factors in regeneration, "As soon as favorable experimental circumstances concur, however, the regenerative tendency [which is] latent in the fibers of the [central nervous system] is aroused and develops extraordinary strength" (Ramon y Cajal, 1928). Around the same period of time Ramon y Cajal also became the first person to hint that CNS myelin was inhibitory to neuronal regeneration. The first evidence to support Ramon y Cajal's theory came in 1981 from the work of David and Aguayo who showed that CNS neurons were indeed able to regrow if provided with a favorable substance, which in this case was peripheral nerve graft. In 1982, experiments from Martin Berry further supported the favorable environment theory when he showed that CNS axons could not regenerate in the presence of deconstructed myelin (Berry 1982). The non-permissive properties of myelin remained elusive until the late-eighties when Schwab and colleagues were able to extract from CNS myelin fractions two proteins with highly non-growth permissive substrate properties (Caroni and Schwab

1988). In the twenty years since these findings, myelin has been found to contain several molecules that have been identified as CNS regeneration inhibitors.

To date, myelin is known to contain three proteins that are thought to be main regeneration inhibitors: myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp) (McKerracher et al. 1994; Mukhopadhyay et al. 1994; GrandPre et al. 2000; Wang et al. 2002). All three proteins have been shown to be present in both the CNS and the PNS, they all have different structures and yet all three have been shown to have a point of convergence; they all mediate their effects by binding to a common receptor complex, the NgR-LINGO/TROY-$p75^{NTR}$ complex (Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002; Wong et al. 2002; Mi et al. 2004). Following injury and subsequent myelin fragmentation, all three proteins are exposed and able to bind to receptors on the neuronal surface. Why then does the PNS regenerate and the CNS not?

One factor mediating PNS regeneration is the phagocytotic Wallerian degeneration. Wallerian degeneration is a process by which myelin and axonal debris are phagocytosed by Schwann cells and macrophages, resulting in the clearing of growth inhibitors and after 7-14 days the eventual sprouting of the nerve segment (Vargas and Barres 2007). While Wallerian degeneration does occur in the CNS, it's an extremely slow process, lasting months or possibly years and still never completely "clearing" the debris. In addition to poor Wallerian degeneration the CNS also faces another barrier to

regeneration, the glial scar. Formed by the increase in reactive gliosis after injury, the glial scar represents both a physical and chemical barrier to regeneration.

1.2 Inhibitors of Axonal Regeneration in Myelin

One factor that contributes to lack of regeneration are the protein inhibitors present in myelin. In the PNS, myelin is responsible for giving neurons and tracts their characteristic white appearance. In the CNS, myelin is responsible for white matter and also functionally responsible for fast nerve conduction (Martenson, 1992). Simply put, myelin is an electrically insulating spiral of membrane extended by glial cells (oligodendrocytes in the CNS and Schwann cells in the PNS). This spiral of membrane serves as an insulator, increasing the speed of saltatory conduction and thus allowing for faster action potentials. In cases of disease or death of the myelin, the demyelination results in a loss of signal propagation and thus the eventual loss of neuronal motor and sensory function.

Myelin was originally thought to be a substance that was permissive to neuronal regeneration. In 1928, Ramon y Cajal became the first person to hint that myelin was inhibitory to neuronal regeneration (Ramon y Cajal, 1928). Since the work of Ramon y Cajal the non-permissive properties of myelin remained elusive until the late-eighties when Martin Schwab and colleagues were able to extract from CNS myelin fractions membrane proteins of 35 kD and 250 kD with highly nonpermissive substrate properties. They were then able to raise two monoclonal antibodies against these proteins. Antibodies IN-1 and IN-2 bound to both protein inhibitors and to the surface of

differentiated cultured oligodendrocytes (Caroni and Schwab 1988). Adsorption of nonpermissive CNS myelin or nonpermissive oligodendrocytes with either antibody greatly enhanced their substrate binding properties. Optic nerve explants treated with IN-1 or IN-2 resulted in axon growth of co-cultured sensory and sympathetic neurons (Caroni and Schwab 1988). However it took more than a decade before the antigen was identified. In 2000, three groups independently reported that twelve years after the discovery of the antibody, an antigen was found (Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000)

1.3 Inhibitors In Myelin

1.3.1 Nogo

Collective data showed that Nogo-A, an IN-1 antigen that is produced by oligodendrocytes, is a potent inhibitor of neurite outgrowth (Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000). Recognized by monoclonal antibody IN-1, and named Nogo by Schwab, Nogo was found to inhibit neurite outgrowth from dorsal root ganglia (DRG) neurons (Prinjha et al. 2000). The *Nogo* gene was found to encode three major protein products: Nogo-A, -B and –C (Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000). Nogo is a member of the reticulon family, proteins that usually associate with the endoplasmic reticulum. Nogo-A, is the isoform that is most commonly found on the surface of oligodendrocytes and at the innermost loop of the myelin membrane, and has two known inhibitory domains (Nogo-66 and amino-Nogo) (Chen et al. 2000). Nogo-66 is common to all three isoforms, whereas amino-Nogo is unique to Nogo-A. Nogo-A has

two different topologies: one with the N- and C- terminals located extracellularly and the other with them located intracellularly (GrandPre et al. 2000)

With its neurite outgrowth inhibition properties well established, Nogo quickly became a target protein for knockout analysis. In April of 2003, three groups independently published conflicting data on the effect of Nogo knockout. The Strittmater group analyzed mice with a nogo mutation that eliminated Nogo-A/B expression. They found that after spinal cord injury in young *nogo-A/B* $^{-/-}$ mice, corticospinal tract (CST) axons sprout extensively rostral to a transection and numerous fibers regenerate into distal cord segments. They also reported improved locomotor function in these *nogo-A/B* $^{-/-}$ mice (Kim et al. 2003). The Schwab group knocked out Nogo-A and noted with the knockout an extreme increase in Nogo-B. They found that two weeks after dorsal hemisection of the thoracic spinal cord, Nogo-A knockout mice displayed more CST fibers growing rostral to the lesion as compared to their wildtype littermates. They also reported that CST fibers caudal to the lesion were more frequent in the Nogo-A knockout animals (Simonen et al. 2003). These two studies had similar results and indicated a Nogo-A deletion alone is sufficient to induce axonal regeneration. However, in the same issue of Neuron, a third Nogo knockout study was published with conflicting results. The Tessier-Lavigne group investigated the inhibitory role of Nogo by generating two types of Nogo knockouts, Nogo-A/B$^{-/-}$ mice and Nogo-A/B/C$^{-/-}$ mice. Similar to the other Nogo knockout findings, they reported that the Nogo-A/B deficient myelin had reduced inhibitory activity in a neurite outgrowth assay *in vitro*. However, unlike the other findings, tracing of CST fibers after dorsal hemisection of the spinal cord did not reveal

an obvious increase in regeneration or sprouting in either mouse line, suggesting that Nogo deletion alone is not sufficient to induce axonal regeneration (Zheng et al. 2003).

1.3.2 Myelin-Associated Glycoprotein

Myelin-associated glycoprotein (MAG) is a 100 kD glycoprotein located in the periaxonal membranes of myelin forming oligodendrocytes and Schwann cells. MAG is known to be present in two isoforms, a 607 amino acid form termed L-MAG and a 563 amino acid form termed S-MAG. The two are identical and only differ in their cytoplasmic C-terminal domains generated by alternative splicing. MAG is a member of the Ig superfamily containing 5 extracellular Ig-like domains and was found to be a sialic acid-binding protein, binding gangliosides at arginine 118 in the first Ig domain (Kelm et al. 1994; Tang et al. 1997). Due to homology in the first four Ig-like domains, MAG has been categorized as a member of the Sialic-acid-binding Ig-like lectin (Siglec) family. In 1994, two independent laboratories discovered MAG to be an inhibitor of neurite outgrowth *in vitro* (McKerracher et al. 1994; Mukhopadhyay et al. 1994). With its inhibitory properties reported, MAG quickly became a target for knockout analyses. In 1995, Bartsch et al. published a study in which they used MAG deficient mice to test MAGs inhibitory properties both *in vitro* and *in vivo*. Using purified myelin from the MAG deficient mice they performed neurite elongation, cell spreading and growth cone collapse analyses and reported no significant difference compared to neurons grown on wildtype purified myelin. They also measured the extent of regeneration *in vivo* after either an optic nerve lesion or a dorsal column hemisection, and found no significant increase in regeneration in the MAG deficient mice as compared to the wildtype (Bartsch

et al. 1995). Thus, contradictory to the 1994 findings, they concluded that MAG was not a major inhibitor of neurite outgrowth. In 1996, Li et al. published a study indicating MAG as a neurite regeneration inhibitor both *in vitro* and *in vivo*. Using polystyrene beads coated with recombinant MAG (rMAG) they showed that 60% of hippocampal growth cones collapsed when they encountered the rMAG as compared to when the axons encountered a denatured rMAG. Although they did not see increased growth on MAG deficient purified myelin substrates, they did see a very small number of anterogradely labeled axons extending past the lesion site after a dorsal hemisection in MAG deficient mice. They concluded that MAG is an important inhibitor of neurite outgrowth and that the lack/poor regeneration observed was likely due to other protein inhibitors present in myelin (Li et al. 1996). Finally, in 1998 the Filbin group published evidence further supporting MAGs status as an inhibitor of neurite outgrowth. Using purified myelin from MAG deficient mice as a substrate they showed an increase in axonal length in both cerebellar and DRG neurons as compared to neurons grown on wildtype myelin substrate. They showed that by desialating neurons they were able to reverse the inhibitory effect of CNS myelin by 45% and also when non-MAG expressing Schwann cells were retrovirally induced to express MAG, axonal outgrowth and neurite branching were greatly inhibited (Shen et al. 1998).

1.3.3 Oligodendrocyte-Myelin Glycoprotein

Oligodendrocyte-myelin glycoprotein (OMgp) was first discovered to be inhibitory by He and colleagues in 2002 (Wang et al. 2002). OMgp is a glycosyl phosphatidylinositol (GPI) linked protein that contains a leucine rich repeat (LRR) domain followed by a C-

terminal domain rich in serine/threonine repeats (Mikol et al. 1990). The LRR in OMgp was found to be highly conserved amongst 14 mammalian species and using deletion mutagenesis it was shown that the LRR domain is responsible for a total loss of function in an *in vitro* expression system (Vourc'h et al. 2003). OMgp has been found to be expressed in low levels in oligodendrocytes and at high levels in various neurons showing particular enrichment in the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellum, the brainstem motor neurons and the anterior horn cells of the spinal cord (Habib et al. 1998). OMgp is a relatively minor component in myelin and has been shown to be localized at the paranodal region; it is also expressed in the PNS (Apostolski et al. 1994). Gene expression analyses have shown that OMgp is developmentally regulated; peaking in the late stages of myelination and it is thought to serve as a marker to arrest myelination (Vourc'h et al. 2003). Like Nogo and MAG before it, with its neurite outgrowth inhibitory properties established, OMgp quickly became a target for *in vivo* knockout analysis. In 2008 Ji et al, generated two $OMgp^{-/-}$ mice strains each from different genetic backgrounds. They then performed complete transection of the spinal cord or a milder dorsal hemisection. They found that the $OMgp^{-/-}$ on the mixed 129/Sv/C57BL/6 (129BL6) genetic background showed functional improvement as compared to the wildtype littermates. They also found that the myelin isolated from these knockout mice was significantly less inhibitory as compared to the myelin from the wildtype. However, mice from the C57BL/6 (BL6) genetic background showed neither functional recovery nor axonal sprouting following dorsal hemisection (Ji et al. 2008). In 2005, work from Colman and colleagues showed that OMgp is not localized to compact myelin but to oligodendroglia-like cells whose processes form a ring around the nodes of

Ranvier. They then observed the nodes of Ranvier in OMgp-null mice and found they exhibited collateral sprouting and were abnormally wide. They concluded that in the CNS OMgp may be playing a role in the regulation of axonal spouting by ensheathment of the node (Huang et al. 2005).

1.3.4 Ephrin-B3 and Sema4D

Searching for molecules that are known to act as repellants in vertebrate axon pathfinding, in 2005, Benson et al. identified another myelin-based inhibitory molecule, Ephrin-B3 (Benson et al. 2005). Previously known to function as a midline repellant for axons of the CST in mice, Ephrin-B3 was found to be expressed in postnatal myelinating oligodendrocytes, and neurite outgrowth assays showed that Ephrin-B3 myelin knockout mice had similar neurite extension on wildtype myelin as neurons from Nogo A/B knockout and MAG knockout mice (Benson et al. 2005).

In a similar study, another molecule previously known for regulating axonal pathfinding, Sema4D, was also found to be a myelin-based growth inhibitor. The semaphorins are one of the largest families of axon guidance molecules and are widely expressed in the mammalian nervous system (Raper 2000). Sema4D was shown to be selectively expressed in myelinating oligodendrotcytes in the postnatal mouse brain *in vitro* and *in vivo*, to be transiently upregulated after spinal cord lesion, and to be inhibitory to both DRG and cerebellar granular neurons in an *in vitro* immobilized substrate stripe assay (Moreau-Fauvarque et al. 2003).

1.4 Receptors for the Myelin-Based Inhibitors

1.4.1 Nogo-66 Receptor (NgR)

Each of the three myelin-based proteins mediate their inhibitory effects through binding to the same tripartite receptor complex, the GPI-linked Nogo receptor (NgR1). In 2001, Strittmatter and colleagues cloned a binding partner for Nogo and named it the Nogo receptor (NgR1) (Fournier et al. 2001). Strittmatter also showed that direct interaction of Nogo-66 with NgR1 was required to induce growth cone collapse (Fournier et al. 2001). In 2002, Strittmatter and colleagues, as well as Filbin and colleagues, identified MAG as an additional NgR1 ligand (Domeniconi et al. 2002; Liu et al. 2002). Also, in 2002, Zhigang He and colleagues discovered that OMgp was a third ligand for NgR1 (Wang et al. 2002). NgR1 is an 85 kD protein expressed on the surface of various neurons. It is a GPI-linked protein that contains a series of eight LRRs clustered in two groups and a unique C-terminal sequence (Fournier et al. 2001). Since NgR1 mediates the signal for all three known myelin inhibitory proteins, it would appear then that NgR would be a key factor in inhibiting regeneration. In 2004 the Strittmatter group showed that after dorsal hemisection or complete transection of the spinal cord, NgR knockout mice have some improved motor function due to partial regeneration of raphespinal and rubrospinal fibers. However, CST fibers do not regenerate (Kim et al. 2004). In support of the 2004 study, in 2005 Zheng et al. also showed that NgR knockout mice had no significant enhancement in regeneration of CST axons after dorsal hemisection as compared to their wildtype littermates (Zheng et al. 2005). Thus, like Nogo, MAG, and OMgp before it,

NgR was found to be only partially responsible for the lack of regeneration (Kim et al. 2004).

In 2003, two NgR homologs were discovered via database searches (Barton et al. 2003; Pignot et al. 2003). The proteins named NgRH1 or NgR2 and NgRH2 or NgR3, were homologous in their primary structure (eight LRR repeats with a C-terminal GPI sequence), biochemical properties and expression patterns to NgR (Pignot et al. 2003). Surprisingly, despite sharing the extensive sequence similarity with NgR, Barton et al. determined that NgR2 and NgR3 did not bind to Nogo, MAG, OMgp or NgR1 (Barton et al. 2003). Two years later, after performing several control affinity experiments; it was shown that NgR2 was a selective receptor for MAG (Venkatesh et al. 2005).

1.4.2 The p75 Neurotrophin Receptor

The GPI linked NgR receptor indicated an additional transmembrane protein was required to mediate the myelin inhibitory proteins signals into the interior of the neurons. In 2002, Zhigang He's group demonstrated that the widely known p75 neurotrophin receptor specifically interacts with NgR (Wang et al. 2002). First identified as a neurotrophin signaling receptor in 1992 by Barker and colleagues, $p75^{NTR}$ is a member of the tumor necrosis factor (TNF) family, and is a transmembrane transducing glycoprotein (Barker and Murphy 1992). In 2002, He's group showed that $p75^{NTR}$ knockout mice are no longer responsive to myelin, or the known myelin inhibitory proteins (Wang et al. 2002).

In further support, of $p75^{NTR}$ being required for NgR-mediated signaling, in 2002 three independent groups all published that when $p75^{NTR}$ is in complex with NgR1, the binding of Nogo-66, OMgp or MAG inhibits neurite outgrowth (Domeniconi et al. 2002; Wong et al. 2002; Yamashita et al. 2002). Up to this point it was known that the three protein inhibitors present in myelin bound to the GPI-linked NgR1 and that the $p75^{NTR}$ was the signal transducer for these proteins. What was not known was how the two came together to form a functional signal transducing unit.

1.4.3 Lingo and TROY

Lingo1, discovered by Pepinsky and colleagues in 2004, is a transmembrane protein that associates with both NgR1 and $p75^{NTR}$ to form a functional receptor for myelin derived inhibitors (Mi et al. 2004). Lingo1 is composed of 12 LRR motifs flanked by N and C-terminal capping domains and one Ig domain (Mi et al. 2004). In 2005, He and colleagues identified TROY (TAJ) as another transmembrane protein capable of transducing a ligand-mediated signal (Park et al. 2005; Shao et al. 2005). TROY is a member of the TNF receptor family and is expressed throughout the adult nervous system (Park et al. 2005; Shao et al. 2005). In neurons that do not express $p75^{NTR}$, it has been shown that TROY can form a receptor complex with NgR1 and Lingo1 mediating the inhibitory effects of the myelin proteins (Park et al. 2005; Shao et al. 2005).

1.4.4 PirB

As suggested by the knockout analyses on NgR, it was thought that besides NgR1, myelin based inhibitors must have another receptor to mediate their inhibitory effects. In 2008 Tessier-Lavigne and colleagues published a study identifying a second high affinity receptor for Nogo, MAG and OMgp, the paired immunoglobulin-like receptor B (PirB) (Atwal et al. 2008). Identified by expression clone screening of a human cDNA library, PirB was previously implicated in nervous system plasticity, and is the only mice homolog to the human leukocyte immunoglobulin-like receptor B2 (Atwal et al. 2008). By using both antibodies that interfered with the PirB-myelin inhibitors interactions, and mice that carried a loss of function *PirB* allele they were able to show that interfering with PirB partially rescues neurite inhibition by Nogo, MAG, OMgp and myelin (Atwal et al. 2008). Attempting to completely rescue neurite inhibition, they used NgR knockout mice and anti-PirB antibodies in combination and nearly completely reversed neurite inhibition in an *in vitro* assay (Atwal et al. 2008). While the mechanism by which PirB exerts its effects have not been elucidated, it would appear that PirB plays an important role in myelin mediated growth inhibition.

1.5 Signaling by Inhibitors

The actin cytoskeleton in animals is responsible for many aspects of cell life: motility, cytokinesis, phagocytosis, and intracellular transport to name a few. The actin cytoskeleton is composed of actin filaments and actin-binding proteins organized into

different structures to fulfill different roles. For example, actin stress fibers consist of long bundles of actin filaments that span the cell and are connected to the extracellular matrix by integrins and focal adhesion complexes (Small 1981). Focal adhesions are multi-molecular protein complexes that occur when actin stress-fibers are linked to integrins at the inner surface of the plasma membrane (Heath and Dunn 1978). Actin filaments are also found to form a highly compact meshwork that can be found at the leading edge of motile cells in lamellipodia (Small et al. 1995); and small bundles of actin filaments are also found at the ends of growth cones in neurons (O'Connor and Bentley 1993).

Rho, Rac and cdc42, are three members of the Rho-family of the ras-related superfamily of guanine-triphosphatases (GTPases) (Adams et al. 1990; Ridley and Hall 1992; Ridley et al. 1992). GTPases are regulatory hydrolases that cycle between two conformations – an active and an inactive form, GTP-bound and GDP-bound, respectively. GTPases are activated by guanine exchange factors (GEFs) and are inactivated by GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis, returning the GTPase to its GDP-bound form. In fibroblasts, Rho, Rac and cdc42, regulate the polymerization of actin to produce stress fibers, lamellipodia, and filopodia, respectively (Nobes and Hall 1995). Inhibition of RhoA or its downstream target, Rho-associated coiled-coil-containing protein kinase (ROCK), has been shown to promote axonal regeneration (Nakayama et al. 2000). Rho GTPases act as intracellular switch proteins that transduce signals from extracellular growth factors and aid in the assembly of focal adhesions and actin stress fibers (Ridley and Hall 1992). In its active, GTP bound state; RhoA promotes

actin polymerization, thereby promoting axonal growth cone collapse (Yamashita et al. 1999). In its inactive, GDP bound state RhoA is associated with Rho-Guanine nucleotide Dissociation Inhibitor (GDI), which inhibits nucleotide dissociation, thereby inhibiting axonal growth cone collapse (Sasaki and Takai 1998).

In 1999, Lisa McKerracher and colleagues showed that introduction of a dominant negative Rho into PC12 cells allowed the cells to extend extensive neurites on a MAG substrate. They also showed primary retinal ganglion cells (RGC) that had been treated with C3 (an enzyme from *Clostridium botulinum* that selectively ADP-ribosylates Rho) had neurite extension on MAG substrates to an extent similar to that observed on control substrates and that C3 treatment led to robust axonal regeneration after an *in vivo* optic nerve crush (Lehmann et al. 1999). In 2005, Filbin and colleagues showed that upon MAG binding to the receptor complex, $p75^{NTR}$ is first cleaved extracellularly by α-secretase, followed by a protein kinase C (PKC)-dependent intramembrane γ-secretase cleavage (Domeniconi et al. 2005). These proteolytic cleavages are necessary for both inhibition of neurite outgrowth and the activation of RhoA, (Domeniconi et al. 2005). It has been shown that $p75^{NTR}$ itself is the signal transducing element for MAG (Yamashita and Tohyama 2003). It is known that MAG activates RhoA by a $p75^{NTR}$ dependant mechanism, although the precise mechanism has yet to be fully elucidated (Domeniconi et al. 2005).

1.6 Overcoming the Myelin-induced Growth Inhibition

The main scope of our research is to find a cure for CNS injury. As aforementioned, it is understood that changes in the environment surrounding the injury and the neuronal response to those changes are key factors in promoting regeneration. Being that all three known protein inhibitors mediated their effects by binding to the same tripartite complex, the components of the receptor complex as well as the myelin-based inhibitors have all become attractive targets for potential intervention. There have been several different strategies taken to address overcoming myelin-induced inhibition including direct blocking of the inhibitors, intrinsic boosting of the growth potential of the neuron, blocking of the downstream effectors, knockout analyses and the blocking of transactivated receptors.

1.6.1 Blocking Inhibition with Antibodies and Peptides

In 1988, Caroni and Schwab were able to extract from CNS myelin fractions membrane proteins of 35 kD and 250 kD with highly nonpermissive substrate properties. They were then able to raise two monoclonal antibodies against these proteins, IN-1 and IN-2 (Caroni and Schwab 1988). Adsorption of nonpermissive CNS myelin or nonpermissive oligodendrocytes with either antibody greatly enhanced their substrate binding properties and optic nerve explants treated with IN-1 or IN-2 resulted in axon growth of co-cultured sensory and sympathetic neurons (Caroni and Schwab 1988). In 1990, Schnell and Schwab were able to intracereberally apply the monoclonal IN-1 antibody by implanting antibody-producing tumors. After tumor implantation complete transection of the CST

was performed and 2-3 weeks later regeneration was assessed. Antibody-induced neutralization of the myelin-based inhibitors led to robust regeneration. They found that animals who received the IN-1 regenerated axons more than 10 times the length of those animals who did not receive the antibody (Schnell and Schwab 1990). In a 1995 follow-up study they showed again that implanting hybridoma cells excreting the IN-1 antibody could lead to axon regeneration following a dorsal hemisection and also that this axonal regrowth was associated with recovery of locomotor function (Bregman et al. 1995).

In another antibody-mediated therapeutic approach, in 1999, David and colleagues immunized adult female mice with a homogenate of mouse spinal cord. Three weeks after the start of the injections the mice received a dorsal hemisection lesioning both CST tracts. For another three weeks the animals received twice weekly immunization injections, then were sacrificed and CST regeneration was assessed. Mice that received the immunizations showed extensive regeneration of the CST axons and in some cases recovered hind limb motor function. Furthermore, they found that when tissue culture substrate coated with CNS myelin was incubated overnight with antisera from immunized mice cerebellar neurons were able to extend neurites four times longer than when grown on CNS myelin alone. Thus, stimulating the animals own immune system to produce polyclonal antibodies that block the inhibitory proteins found in myelin promoted neurite outgrowth both *in vitro* and *in vivo* (Huang et al. 1999). However, this is not a feasible therapy because the immunization must occur before the injury.

In a peptide-mediated approach, Strittmatter and colleagues identified competitive antagonists of NgR derived from amino-terminal peptide fragments of Nogo-66. The Nogo-66 (1-40) antagonist peptide (NEP1-40) allowed for the assessment of the contribution of NgR ligands to the inhibitory properties of myelin. *In vitro* analysis showed that soluble NEP1-40 blocked Nogo-66/CNS myelin inhibition of axonal outgrowth and *in vivo* intrathecal delivery of NEP1-40 to rats with mid-thoracic spinal hemisection resulted in significant CST axon growth. Thus indicating that NgR peptide antagonists may have therapeutic potential (GrandPre et al. 2002). In a similar study, in 2002 Filbin and colleagues used a polyclonal NgR antibody to block NgR from binding to MAG and found that application of the antibody blocked inhibition of neurite outgrowth by MAG for both cerebellar and DRG neurons (Domeniconi et al. 2002).

Other peptide-mediated therapies have been investigated all with similar growth promoting results. Various groups assessed the contributions of the NgR/p75^{NTR}/LINGO complex using truncated or Fc-versions of the tripartite complex with the results indicating that intervention of the ligand-receptor interaction significantly promotes axonal regeneration *in vitro* (Liu et al. 2002; Wang et al. 2002; Mi et al. 2004).

1.6.2 cAMP

Dorsal root ganglion (DRG) cells are clusters of sensory neurons that carry information from the skin, muscles and joints of the limbs and trunk to the spinal cord (Kandel, 1991). DRG cells are located within the vertebral column immediately adjacent to the spinal cord. DRG cells are psuedo-unipolar, with one bifurcated axon branching into

both the central nervous column and into the peripheral nervous system (Kandel, 1991). The peripheral branch terminates in the skin, muscle or other tissue as a free nerve ending or in association with specialized receptors, while the central branch process enters the spinal cord (Martenson, 1992) where the axon branches and either terminates within the spinal grey matter or it ascends to nuclei at the junction of the spinal cord with the medulla (Kandel, 1991). In a seminal study by Neumann and Woolf it was shown that the CNS axon branch, thought to be unable to regenerate, can be 'primed' to grow if a lesion to the peripheral branch is made first, even in the absence of a peripheral nerve graft (Neumann and Woolf 1999).

This 'priming' or 'conditioning effect' has been shown to be dependent upon increased levels of the ubiquitous signaling messenger, cyclic-adenosine monophosphate (cAMP) and the effects of a conditioning lesion on dorsal column regeneration can be mimicked by elevating intracellular levels of cAMP (Neumann and Woolf 1999; Qiu et al. 2002). In 2002, Filbin and colleagues showed that MAG/myelin inhibition can be significantly reduced *in vitro* using a cAMP membrane permeable analogue dibutyryl-cAMP (db-cAMP) and that administered prior to a dorsal column hemisection, a single direct DRG injection of db-cAMP lead to extensive regeneration of the dorsal columns axons, in the absence of a peripheral lesion (Cai et al. 1999; Qiu et al. 2002). In a similar paradigm, in 2004, Hoffman and colleagues showed that a single intraocular injection of db-cAMP was sufficient to promote regeneration of RGC axons after an optic nerve crush (Monsul et al. 2004). Furthermore in 2004, Tuszynski and colleagues showed that in combination

with a cAMP injection, injection of the neurotrophin NT-3 led to clear axonal regeneration beyond spinal cord injury sites (Lu et al. 2004).

Elevating cAMP in neurons is known to overcome inhibition in two phases. The first (1-2 days post-injection) is rapid, transcription-independent and directly dependent on protein kinase A (PKA) activation. In the second phase (about 7 days post injection), the ability of db-cAMP to overcome MAG/myelin inhibition becomes transcription-dependent and PKA-independent (Qiu et al. 2002). cAMP requires activation of the transcription factor cAMP response element binding protein (CREB) to exert its effects on overcoming inhibition (Gao et al. 2004). It is proposed that a downstream signal transduction pathway involving cAMP, PKA, and the eventual phosphorylation of CREB, plays a major role in overcoming inhibition (Cai et al. 1999; Gao et al. 2004). Elevated levels of cAMP have also been implicated to overcome glial scar associated growth inhibition (Shearer et al. 2003).

The effect of MAG on neurons is dual. To date, all neurons tested seem to switch their responses to MAG with developmental age. The specific age at which the switch occurs seems to be dependent on the particular neuron. In some young neurons, DRG cells for example, at the age when they can spontaneously regenerate and are not inhibited by MAG/myelin, are found to have high levels of cAMP. However, as the neuron ages the amount of cAMP decreases and with that the ability to regenerate spontaneously and to grow in the presence of MAG/myelin (Cai et al. 2001). In rat DRG cells, MAG/myelin promotes neurite outgrowth until approximately post-natal day 5 (P5) (Cai et al. 1999).

After P5 there is a switch at which MAG/myelin begins to inhibit neurite outgrowth (Cai et al. 1999). While the molecular reasons behind the switch are not completely understood, it has been observed that this switch is directly related to the amount of endogenous cAMP (Cai et al. 1999).

1.6.3 Activating Alternative Pathways

Since the discovery of the myelin-based protein inhibitors the attempts to overcome the growth inhibition have mostly focused on neutralizing the environment. In 2008, Zhigang He and colleagues began to search for ways to intrinsically boost the neurons growth capabilities. Looking for evolutionarily conserved pathways that control cellular growth and size they generated conditional floxed mice for many genes including: the retinoblastoma gene product Rb, the transcription factor p53, a member of the Darfwin family of regulatory proteins Smad4, the double-stranded RNA cleaver Dicer, and the tumor suppressor phosphatase and tensin homolog (PTEN) and subjected the mice to the optic nerve crush model. Regeneration was assayed and the mice with the largest effects on both neuronal survival and axonal regeneration were those with the PTEN deletion (Park et al. 2008). PTEN functions as a tumor suppressor by negatively regulating the Phosphatidylinositol-3,4,5-trisphosphate (PIP3) /mammalian target of rapamycin (mTOR) pathway, a pathway which regulates many basic cellular properties including proliferation, survival, motility and angiogenesis (Chan 2004).

They next monitored the activity of the downstream effectors of the mTOR pathway and observed a global reduction in protein synthesis after axotomized-triggered stress in

wildtype RGC's. Rapamycin, a product of the bacterium *Streptomyces hygroscopicus*, is a known pharmacological inhibitor of protein translation. Thus, deletion of PTEN should lead to increased protein translation. Using RGC's from the PTEN deleted mice, they next assessed the mTOR activity before and after optic nerve crush. While they saw no significant increase in protein synthesis prior to injury, they were able to observe that the PTEN deleted mice maintained mTOR activity levels similar to those of uninjured wildtype neurons. They were able to conclude that activating the mTOR pathway was sufficient to promote both RGC survival and axon regeneration after an optic nerve crush (Park et al. 2008). These results showed an important role for the requirement of active protein synthesis to promote axon regeneration and functional recovery after CNS injury. Further elucidation of the mTOR and other growth promoting pathways may provide new targets for therapeutic intervention.

1.7 The Glial Scar

Astrocytes, microglia, and oligodendrocytes and their precursors make up the glial cells of the CNS. They all provide the CNS with the necessary structural and physiological support it needs, however, after injury, they are also responsible for walling off areas of damage to prevent further destruction, and thus in the process forming a physical barrier to axonal regeneration. This physical barrier named "the glial scar" is formed by microglia, macrophages and especially astrocytes that migrate to the site of injury and secrete chondroitin sulfate proteoglycans (CSPGs) (McKeon et al. 1999). During the glial

scar formation the astrocytes undergo a process called reactive astrogliosis in which they experience cellular hypertrophy and increases in glial fibrillary acidic protein (GFAP), the exact triggers for this transformation have yet to be elucidated (Fitch and Silver 2008). The secreted CSPGs are composed of core proteins of molecules made of multiple domains that affect their integration into the ECM or provide putative attachment or signaling functions and covalently linked chondroitin sulfate glycosaminoglycans (Galtrey and Fawcett 2007).

1.8 MicroRNA

1.8.1 Discovery

In C. elegans, a genetic pathway of heterochronic genes is responsible for the temporal fates of larval cells during development. In 1981, while experimenting with loss of function gene mutations (lf), it was discovered that the gene *lin-4* acts early in C. elegans larval development to effect the timing of developmental events in essentially all larval stages and that animals carrying a *lin-4* lf displayed reiterations of early fates at inappropriately late developmental stages (Chalfie et al. 1981). A few years later the same group showed that *lin-14* null animals display the opposite phenotype of *lin-4* lf animals, and that in wildtype animals a high level of *lin-14* activity is associated with early stages of development while a lower level of *lin-14* activity is observed in later stages (Ambros and Horvitz 1987). They hypothesized that the normal developmental progression depended on the *lin-4*-dependent decrease in *lin-14* activity. In 1991, the Ruvkun group validated that hypothesis by showing that the temporal down regulation of

lin-14 was indeed directly proportional to the levels of the *lin-4* gene (Arasu et al. 1991). In 1993, Wightman et al. showed that the *lin-14* transcripts are constant throughout development, thus indicating a post-transcriptional negative regulation of the LIN-14 protein (Wightman et al. 1993). Through loss and gain of function mutations, Wightman et al. were able to identify the 3'UTR of the *lin-14* transcript to be a necessary and sufficient cis-regulatory element of the LIN-14 protein level (Wightman et al. 1993). These experiments led to the theory that the *lin-4* gene product was responsible for the post-transcriptional regulation of the *lin-14* mRNA. In a seminal finding, in 1993 Lee et al. showed that the *lin-4* gene did not encode a protein but rather two small RNA transcripts of approximately 22 and 61 nucleotides that were complimentary to a repeated sequence in the *lin-14* 3'UTR (Lee et al. 1993). A few years later using P^{32} labeled lin-4 RNA probes, the Ruvkun group showed the physical RNA/RNA duplex interaction was causing the post-transcriptional negative regulation of *lin-14*(Ha et al. 1996). It took seven years before the next small RNA regulator, *let-7*, was discovered (Reinhart et al. 2000) and with its discovery homologues were soon identified in a wide range of animal species (Pasquinelli et al. 2000).

The term microRNA was first introduced in a series of Science articles in 2001 (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). In 2004 a microRNA registry was set up to catalogue and name the hundreds of microRNAs that had been indentified (Griffiths-Jones 2004). Since then, thousands of microRNAs have been shown to be evolutionarily conserved and involved in a broad range of regulatory functions in both vertebrates and invertebrates.

1.8.2 Biogenesis

The first event in the biogenesis of a microRNA is its initial transcription by either RNA polymerase II, characterized by the polyadenylation and capping (Cai et al. 2004; Lee et al. 2004) or RNA polymerase III (Borchert et al. 2006). MicroRNA transcript regulation is achieved at a few levels. One level of regulation is that RNA polymerase II and III are regulated differently and recognize specific promoter and terminator elements, and on a more specific level of expression, selected microRNAs have been shown to be dependent upon transcription factors or promoter methylation (Brueckner et al. 2007; Lujambio et al. 2008). The long primary transcripts (pri-miRNAs) that encode the microRNAs are usually thousands of nucleotides long and are sequentially cleaved by a series of cellular processes (Lee et al. 2002).

In mammals, the first cleavage is performed endonucleolytically by the RNAse III enzyme Drosha in combination with the double-stranded RNA-binding protein DiGeorge critical region 8 (DGCR8 also known as Pasha) (Lee et al. 2003). Together these two proteins have been termed the Microprocessor Complex (Denli et al. 2004). A typical mammalian pri-miRNA contains a hairpin stem of approximately 30 bases with single-stranded flanking regions. Drosha cleavage is initiated eleven base pairs away from the single-stranded/double stranded junction at the base of the hairpin stem and the resulting product of approximately 70 nucleotides long is referred to as a precursor microRNA (pre-miRNA) (Zeng and Cullen 2005). While the vast majority of microRNAs are made via Drosha/Pasha processing, in 2007 Ruby et al. showed an alternative pathway for microRNA biogenesis. Some debranched introns mimic the structural features of pre-

miRNAs and are processed directly by the spliceosome, bypassing the Microprocessor complex completely (Ruby et al. 2007). These intron-derived microRNAs are termed mirtrons and have been found in several species. Whether processed by Drosha/Pasha or the spliceosome the pre-miRNA is next exported out of the nucleus into the cytoplasm by Exportin-5 in complex with Ran-GTP (Yi et al. 2003; Bohnsack et al. 2004).

Once in the cytoplasm the pre-microRNA is recruited into a multi-protein complex called the RNA induced silencing complex (RISC) loading complex (RLC). The RLC consists of the RNase III Dicer, the double-stranded binding proteins Tar RNA binding protein (TRBP) and protein activator of PKR (PACT), and Argonaute-2 (Ago-2) (Gregory et al. 2005; Haase et al. 2005; Lee et al. 2006). Once the pre-microRNA has bound to the RLC the complex is considered loaded and the second cleavage is initiated. Dicer then cleaves off the hairpin loop of the pre-miRNA leaving behind an approximately 22 nucleotide long miRNA duplex with two nucleotides protruding as overhangs at each 3' end (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001).

After the second cleavage, in a process not yet completely elucidated, the resulting miRNA duplex is believed to disassociate from Dicer and its interactors in order to undergo an unwinding process. The double-stranded duplex is loaded onto an argonaute protein and unwound into the mature single-stranded form (referred to as the guide strand) and the complementary strand (referred to as the passenger strand or miRNA*) (Schwarz et al. 2003). This unwinding action has been shown to be facilitated by the cleaving of the passenger strand by Ago-2 and its believed that different helicases may

regulate the unwinding on a miRNA specific basis (Tomari et al. 2004; Matranga et al. 2005; Meister et al. 2005; Chu and Rana 2006; Robb and Rana 2007; Salzman et al. 2007).

1.8.3 Mechanism

Either strand of the miRNA duplex could give rise to the guide strand. However, usually only one strand is incorporated into the active RISC, the other is degraded (Schwarz et al. 2003). In 2003, Khvorova et al. identified thermodynamic stability as the determining factor. Using statistical analysis of the internal stability of published miRNA hairpin sequences, they identified the strand with the least stable base pair at its 5' end is the one chosen as the guide and thus loaded in to the RISC complex (Khvorova et al. 2003).

The RISC complex consists of proteins of the Argonaute family and various other proteins have been associated including members of the mammalian Gemin family, the putative RNA helicase armitage, and the DEAD box helicase RCK/p54 (Mourelatos et al. 2002; Tomari et al. 2004; Chu and Rana 2006). It is here in the RISC that the mature microRNA binds to its mRNA target. Although the exact mechanism of microRNA target recognition is not yet understood, it is known that the seed region of the guide strand (nucleotides 2-8 on the 5' end) must engage in 100 percent complementary Watson-Crick base pairing with the target mRNA (Lewis et al. 2003; Doench and Sharp 2004). Once the microRNA has bound to the target mRNA it is either translationally repressed or degraded. Which pathway is taken depends on the complementarity with which the

microRNA binds the mRNA. If the binding is at 100% complementary the mRNA is degraded, if it's less than 100%, the mRNAs translation is repressed (Zeng et al. 2003).

While the birth of the microRNA has been well characterized, its death is another story. There is evidence that microRNAs that do not induce degradation are reused (Hutvagner and Zamore 2002), however yet to be identified mechanisms determine the control of the microRNA half-life. It has been shown that microRNA repressed mRNAs are transferred to cytoplasmic RNA processing bodies called P bodies (Sen and Blau 2005). While, P bodies are thought of as mRNA decay centers and are known to be sites of mRNA degradation and storage, whether microRNAs are delivered to P bodies to be destroyed or their presence there is a secondary consequence of being attached to a repressed mRNA, has yet to be elucidated.

1.8.4 MicroRNA in the Mammalian Central Nervous System

Starting soon after their discovery in 1993, there was an explosion in the field of microRNA regulation. MicroRNAs in all cellular types were being shown to have diverse roles in nearly all cellular processes and there were multiple expression analysis studies indicating microRNA regulation in the CNS (Krichevsky et al. 2003; Kim et al. 2004; Sempere et al. 2004). To date there are an approximate 5000 microRNAs already catalogued in the MicroRNA registry, and fewer than 20 have had their roles in the CNS fully characterized.

1.8.4.1 Synaptic Plasticity

Evidence for mammalian neuronal microRNA regulation in synaptic plasticity started with the seminal finding of miR-134. In 2006, the Greenberg group identified miR-134 as a brain specific relievable regulator of dendritic spine (post-synaptic sites of excitatory synaptic transmission) development (Schratt et al. 2006). Using *in situ* hybridization they determined the subcellular localization of miR-134 to be in the dendrites of hippocampal neurons and enriched during the time of synaptic development. Through functional analyses they were able to show that overexpression of the microRNA led to decreased spine width, while knockdown had the converse effects and they were also able to identify a target of miR-134 to be Lim-domain-containing protein kinase 1 (Limk1). This finding was the first of its kind to identify a specific microRNA, its target and their role in mammalian synaptic plasticity.

In 2009 the Schratt group identified a cluster of microRNAs whose expression regulates activity-dependent dendritogenesis. The cluster of microRNAs, miR-379-410, were all robustly induced by both BDNF and KCl in rat hippocampal neurons. To determine how the miR cluster was being induced they devised a comparative genomic screen to identify activity-regulated transcription factors with potential binding sites upstream of the cluster. One transcription factor with multiple binding sites was Mef2, a transcription factor known for its negative regulation of synapse number in mature hippocampal neurons. Using ChIP and luciferase reporter assays they were able to determine that Mef2 activates the miR 379-410 cluster expression in response to neuronal activity by binding to a site upstream of the cluster. To address the physiological relevance of the microRNA

expression, they tested whether perturbation of the miR-379-410 cluster affected the ability of neurons to undergo dendritogenesis in response to activity. They found that both membrane depolarization via KCl and BDNF treatment increased the complexity of the dendritic tree. They were also able to show that anti-miR knockdown of some (but not all) of the microRNAs in the cluster was sufficient to block activity-dependent dendritogenesis. They further showed that knockdown of Mef2 led a to similar phenotype as microRNA loss of function. Using *in silico* target prediction models they were able to identify the RNA-binding protein and transcriptional repressor Pumilio2 (Pum2) as a putative target and then confirmed Pum2 as a target of one of the cluster miRs, miR-134, using luciferase reporter assays. Having already showed a role for miR-134 in synaptic plasticity, they now investigated whether Pum2 regulation by miR-134 was involved in activity-dependent dendritogenesis. Knocking down Pum2 with siRNA, the authors then observed if they could rescue the reduced dendrite complexity observed upon miR-134 inhibition in membrane depolarized neurons. They found that knockdown specifically rescued the miR-134 loss of function as compared to a siRNA control. From these experiments the authors concluded that miR-134 induced negative regulation of Pum2 is necessary for activity-dependent dendritogenesis (Fiore et al. 2009).

Their previous findings on microRNA regulation at the synapses led the Schratt group in 2009 to perform a large-scale expression profile study of synaptically enriched microRNAs. One microRNA that was found to be approximately two fold higher in synaptosomes was miR-138. To determine a functional role for miR-138 in synaptogenesis they performed knockdown and overexpression analyses. Inhibition of

miR-138 resulted in a significant increase in the spine volume of hippocampal neurons; conversely, overexpression of miR-138 resulted in decreased spine volume. The authors then identified and validated a target of miR-138 to be acyl-protein thioesterase 1 (APT1). While the expression and function of APT1 in the nervous system were uncharacterized, it was known that APT1 catalyses the removal of palitate, a lipid modification that has been implicated in the regulation of synaptic efficacy. To determine a physiological role for APT1 in synaptogenesis they performed functional analyses on APT1. Using siRNA they knocked down APT1 and monitored spine volume, finding a significant decrease in the size of the spines. Similarly, inhibition of APT1 enzymatic activity also led to a decrease in dendritic spine volume, indicating the requirement of the depalmitoylating activity in the APT1-dependent control of dendritic spine morphology.

The authors next knocked down miR-138 and looked to see if the resulting increase in APT1 led to an increase in dendritic spine morphology. They observed an increase in spine growth and concluded that APT1 activity is required for spine enlargement caused by miR-138 inhibition. To determine how miR-138 regulation of APT1 decreases spine size they looked for proteins that APT1 depalmitoylates. They focused on $G\alpha_{13}$ due to its implication in synaptic plasticity and because its palmitoylation is required for plasma membrane localization and Rho-dependent signaling. Through overexpression and knockdown analyses they were able to determine that membrane localization of $G\alpha_{13}$ counteracts the spine enlargement caused by miR-138 inhibition. Thus, they conclude that miR-138 might inhibit spine growth by increasing the membrane localization of $G\alpha_{13,}$ which results in elevated activity of the growth inhibitory RhoA pathway (Siegel et al. 2009).

1.8.4.2 Neural Differentiation & Cell Specification

The first evidence to suggest a role for microRNA regulation in neuronal differentiation came from studies on mouse P19 embryonal carcinoma cells that were induced to develop into neurons. In 2005, Wu and Belasco reported that miR-125 was targeting lin-28 mRNA and down-regulating its expression. They concluded that the regulation of lin-28 by miR-125 during that time in development suggested a role for miR-125 in mammalian neuronal differentiation, however, it would take an additional two years before its role was fully characterized (Wu and Belasco 2005). In 2006, Conaco et al. introduced similar evidence that microRNAs were playing a role in neuronal cell differentiation. Using Chromatin Immunopricipitation they were able to identify that the transcriptional repressor, RE1 silencing transcription factor (REST) was regulating the expression of a family of microRNAs including the brain specific miR-124a. When REST is in an active state in non-neuronal cells and neural progenitors, it inhibits miR-124a expression, allowing for the persistence of non-neuronal transcripts. As progenitors differentiate into mature neurons, REST becomes inactive, relieving its transcriptional repression and allowing for the non-neuronal transcripts to be degraded by miR-124a binding (Conaco et al. 2006).

In 2006, the Kosik group showed that miR-124a and miR-9 affect neural lineage differentiation in embryonic stem (ES) cell-derived cultures. Using loss and gain-of function analyses they showed that miR-124a and miR-9 when over expressed together led to substantial alteration of neural lineage differentiation markers. They hypothesized that miR-124a and miR-9 expression might be affecting signaling pathways and focused

on the signal transducer and activator of transcription 3 (STAT3) pathway due to its known role in neuronal development as a possible microRNA target. Through Western blot analysis they observed some miR-124a/miR-9 regulation-induced changes in STAT3 phosphorylation but were unable to provide conclusive evidence (Krichevsky et al. 2006). Interestingly, in the first *in vivo* work on miR-124, Cao et al. observed that neither knockdown of miR-124 with anti-miRs, nor overexpression of miR-124 with a cloned miR-124 expressing plasmid, affected neuronal differentiation in the chick neural tube. However, they did notice two miR-124 targets, laminin γ1 and integrin β1 were repressed upon neuronal differentiation and suggested that miR-124 is post-transcriptionally inhibiting their expression (Cao et al. 2007).

Following with the target studies, in 2007 the Lee group identified the phosphatase small C-terminal domain phosphatase 1 (SCP1) as a valid miR-124 target whose timely miR-induced suppression in critical for neurogenesis in the developing chick spinal cord (Visvanathan et al. 2007). As if miR-124 wasn't promiscuous enough, in 2007 the Maniatis group showed the RNA-binding protein PTBP1 to be a valid miR-124 target. PTPB1 was already known to encode a global repressor of nervous system-specific pre-mRNA splicing and they concluded that through PTPB1 repression miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing (Makeyev et al. 2007).

The subventricular zone (SVZ) is the largest germinal region in the adult mammalian brain. The stem cells in this region are astrocytes that give rise to dividing transit

amplifying cells, which generate into neuroblasts that differentiate into granule and periglomerular interneurons in the olfactory bulb. In 2009, the Doetsch group identified a microRNA that regulates adult neurogenesis in the SVZ. Using *in situ* hybridization and qRT-PCR the authors were able to determine that the brain-specific miR-124 is upregulated as cells transition from transit amplifying cells to neuroblasts and is further upregulated as neuroblasts exit the cell cycle. To assess the *in vitro* function of miR-124 in the SVZ they performed knockdown analyses on FACS purified SVZ cell populations. Knockdown of miR-124 led to an increase in dividing precursor cells (transit amplifying cells and neuroblasts), indicating that miR-124 expression is necessary for the cells to differentiate. Conversely, they found that overexpression of miR-124 promotes neuronal differentiation by causing a decrease in the number of dividing cells and an increase in the number of postmitotic neurons. To assess the *in vivo* function of miR-124 they performed knockdown and overexpression analyses on SVZ lineage progression. Similar to the *in vitro* findings, *in vivo* knockdown of miR-124 resulted in a decrease in postmitotic neurons in migratory chains and an increase in dividing cells, while overexpression of miR-124 resulted in an increase in the proportion of postmitotic neurons and a decrease in dividing precursors. It is known that the SVZ regenerates after anti-mitotic treatment in about one week. To determine if miR-124 had a role in this neuronal regeneration they investigated whether knockdown of miR-124 is required for neuronal differentiation itself or whether it regulates the timing of the progression along the SVZ lineage. They treated the cells with an anti-mitotic inhibitor for 6 days and then knocked-down miR-124. They found that knockdown of miR-124 delayed regeneration, but did not permanently block neuronal differentiation. To determine a target of miR-124

they performed *in silico* target prediction analysis and looked for targets that were known to be expressed in the SVZ and are involved in the progression of SVZ stem cell lineage. They performed luciferase reporter assays to validate a few miR-124 targets and choose Sox9 as one of particular interest for its role in the SVZ is unknown. To determine if miR-124 regulated Sox9 *in vitro* and *in vivo* they knocked-down miR-124 and analyzed the expression patterns of Sox9. As expected, *in vitro* knockdown of miR-124 led to an upregulation of Sox9 protein in dividing neuroblasts and *in vivo* knockdown led to an upregulation of Sox9 protein in migratory chains. Since the *in vivo* expression pattern of Sox9 indicated that it was down-regulated during neurogenesis they performed *in vitro* functional overexpression and knockdown analyses on Sox9. Overexpression of Sox9 resulted in an abolishment of neuronal differentiation. Conversely, Sox9 knockdown resulted in an increase in differentiation. Altogether, these results suggest that the miR-124 tightly regulated Sox9 protein levels are important in the SVZ stem cell lineage progression (Cheng et al. 2009).

1.8.4.3 Axonal Pathfinding & Growth

cAMP response element binding protein (CREB) has been shown to play diverse roles in many neuronal cellular aspects including plasticity, maturation, addiction, circadian rhymicity and additional studies have suggested a role for CREB in CNS morphogenesis. To gain insight into the molecular mechanisms that underlie CREB-regulated plasticity, in 2005 the Impey group developed a genome wide screen that profiled CREB binding sites. Through this screen they identified miR-132 as a putative CREB-responsive microRNA. They validated that CREB induced miR-132 expression using *in vivo* DNAse

I footprinting assays and qRT-PCR. While investigating a function for the CREB-induced miR-132 increase they indicated that prior experiments suggested that CREB is a critical regulator of NGF-induced axonal outgrowth in peripheral neurons (Lonze and Ginty 2002; Lonze et al. 2002) and thus performed functional neurite outgrowth assays. Overexpression of miR-132 led to a significant increase in total neurite length in cortical neurons on a growth permissive substrate as compared to cells transfected with GFP control. Furthermore, they found that knockdown of miR-132 with transfection of an anti-miR-132 attenuated neurite outgrowth, noting a marked reduction in both neurite length and total number of primary neurites on a growth permissive substrate (Vo et al. 2005). Using target prediction algorithms and a reporter plasmid assay they identified p250-GAP as a valid miR-132 target. Following the logic that miR-132 increases neurite outgrowth, they looked to see if knockdown of p250-GAP could lead to an increase in neurite outgrowth. Using a short hairpin RNA (shRNA) to repress p250-GAP, they observed a striking increase in neurite outgrowth on a growth permissive substrate as compared to a control shRNA (Vo et al. 2005). This study was the first evidence that microRNAs were playing a role in axonal outgrowth.

In 2008 the Impey group followed up on this study by further showing that miR-132 controls dendritic plasticity by down-regulating p250-GAP. Using hippocampal neurons they induced synaptic activity by bicuculline-mediated inhibition of $GABA_A$. Using Northern blot and qRT-PCR, they observed that bicuculline triggered a rapid increase in miR-132 expression. Having previously shown that miR-132 was CREB responsive, they now showed that blocking the Cam Kinase and ERK pathways blocked the ability of

bicuculline to induce miR-132 expression. Furthermore, Chromatin Immunoprecipitation analysis (ChIP) showed the direct binding of CREB to the miR-132 promoter. They next investigated whether miR-132 might mediate depolarization-induced dendritic growth, a process the CREB pathway has recently been implicated in (Redmond et al. 2002). Using knockdown and overexpression analyses, their data suggested that CREB positively regulated dendritic growth by controlling miR-132 transcription. Having previously identified p250-GAP as a miR-132 target, they sought to identify whether synaptic activity repressed p250-GAP expression. They found that treatment of hippocampal neurons with bicuculline led to a decrease in p250-GAP protein, but did not affect the mRNA levels. They next sought to determine if p250-GAP repression contributed to activity-mediated dendritic growth. Using a siRNA targeted to p250-GAP they observed a 50% increases in dendritic growth and branching. Conversely, overexpression of a miR-132 insensitive p250-GAP led to attenuated growth and branching. Since p250-GAP is known to inhibit Rho family GTPases *in vitro* (Nakazawa et al. 2003; Taniguchi et al. 2003), they sought to identify which Rho family GTPases participate in miR-132/p250-GAP regulated dendritic growth. They found that expression of a dominant-negative Rac, but not Cdc42, largely blocked miR-132 and siRNA-p250-GAP stimulated growth and branching. They concluded by suggesting that miR-132 and p250-GAP regulate dendritic growth by activating a Rac family GTPase and that this pathway might play a key role in activity-dependent structural and functional plasticity (Wayman et al. 2008).

MiR-124 has been shown to be widely expressed in the brain in both differentiating and differentiated neurons. With this in mind, the Turner group sought to identify a role for

miR-124 in the regulation of neurite outgrowth during neuronal differentiation. Using basic helix-loop-helix (bhlh) transcription factors to induce mouse P19 embryonal carcinoma cells to differentiate into neurons, they showed that miR-124 enhances neurite initiation and outgrowth, while knockdown of miR-124 delays neurite outgrowth. When miR-124 was expressed in uncommitted P19 cells they observed substantial changes in the cytoskeleton and protein levels of Rac1 and Cdc42. Overexpression of activated Rac and Cdc42 led to a decrease in miR-124-induced neurite outgrowth, leading them to the hypothesis that Rac1 and Cdc42 may function downstream of miR-124. They searched for potential miR-124 targets using a global screen and found multiple putative targets with known function in cytoskeletal regulation. Their results suggest that cytoskeletal regulation by miR-124 contributes to the control of neurite outgrowth in differentiating neurons (Yu et al. 2008).

Besides microRNA regulation, another mechanism of gene regulation is local protein translation, whereby proteins are translated extra-somatically. The first observation that neurites severed from their cell bodies could elongate was made by Shaw and Bray in 1977 (Shaw and Bray 1977). The finding of translation machinery and mRNA in dendrites (Tiedge and Brosius 1996) led to the mechanism of local translation being extensively studied in dendrites in the context of synaptic plasticity. Initially local protein synthesis in axons was thought not to occur because translational machinery could not be detected. However, in 1999 cytoskeletal mRNAs were detected in the axon (Eng et al. 1999) and later, by detecting *de novo* synthesis within individual axons using RNAs encoding visualizable reporters, the necessary translation components were shown to be

present (Brittis et al. 2002). The ability of axons to synthesize proteins locally is advantageous because it allows rapid changes in the growth cone granting the neuron immediate response to stimuli; therefore having an important role in neuronal plasticity, growth and function.

Mitochondria are crucial to the function of the distal axon. Neuronal development, maturation and synaptic plasticity all require energy through mitochondrial metabolism. In 2008 Aschrafi et al. showed that mitochondrial function is locally regulated in axons by miR-338, allowing for rapid changes in mitochondrial respiration (Aschrafi et al. 2008). Previously, Aschrafi et al. had reported that several nuclear-encoded mitochondrial mRNAs were present in the distal axons of rat sympathetic neurons and that their local translation plays a vital role in mitochondrial function and axonal maintenance (Hillefors et al. 2007). One such mRNA of interest was COXIV, a major component of the mitochondrial respiratory chain. COXIV is part of the inner mitochondrial membrane multi-protein complex cytochrome *c* oxidase (IV). Cytochrome *c* oxidase (IV) is the last complex in the mitochondrial respiratory chain transferring electrons to O_2 to form H_2O. Therefore COXIV plays a vital role in mitochondrial and presumably axonal function. Using *in silico* prediction models and luciferase reporter plasmid assays, they were able to validate COXIV as a miR-338 target (Aschrafi et al. 2008).

The authors next investigated the location of this neuronal specific miRNA using *in situ* hybridization and found it to be present in the distal axon, proximal axon and soma. To

assess the regulation of axonal levels of mature miR-338 mRNA, they performed overexpression and knockdown analyses using Campenot chambers. They found that when pre-miR-338 was transfected into the soma or distal axons there was a decrease in COXIV mRNA in that corresponding neuronal compartment. Conversely, they found that when knocking down miR-338 by transfecting anti-miR-338 into the distal axons they saw an increase in axonal COXIV mRNA. They postulated that the rapid increase in axonal COXIV mRNA levels after anti-miR-338 transfection supports their hypothesis that it results from local axonal miRNA regulation and not from transport of mitochondria from the cell soma. Their hypothesis was further supported with qRT-PCR analysis showing that the axonal mRNA levels of COXII, another protein needed for mitochondrial respiration, is unaffected by the miR-338 transfections. They next investigated the affects of miR-338 on mitochondrial activity. Using Campenot chambers to compartmentalize cultures they transfected the distal axons of superior cervical ganglia (SCG) neurons with pre-miR-338, anti-miR-338 or a control nontargeting pre-miR (pre-miR-NT). Using a redox-sensitive dye, they found the axons transfected with pre-miR-338 had significantly reduced oxygen consumption and those transfected with the anti-miR-338 had an approximately 50% increase as compared to the nontargeting miR-NT control. These observed changes in mitochondrial activity suggested a role for miR-338 in axonal mitochondrial respiration (Aschrafi et al. 2008).

Having shown that miR-338 can regulate axonal mitochondrial respiration, they next investigated how this impinged on axonal function. Mitochondrial respiration results in the production of energy (ATP). ATP is needed for vesicle cycling, and neurotransmitters

are taken up by vesicle endocytosis (Brodin et al. 1999). Hence, one way to measure axonal function is to look at neurotransmitter uptake. They assessed this by culturing SCG neurons in Campenot chambers and transfecting the distal axons with pre-miR-338 or anti-miR-338. The neurons were then incubated with tritium labeled norepinephrine (NE) and NE uptake was measured by spectrometry. Transfection of distal axons with pre-miR-338 led to a decrease in NE, while transfection with anti-miR-338 led to an increase in NE uptake. These results suggest that not only does miR-338 regulation have a direct impact on mitochondrial ATP synthesis and respiration but also on neurotransmitter uptake (Aschrafi et al. 2008). The Aschrafi et al. findings reveal a miRNA that regulates local axonal translation of a key protein in mitochondrial function which is crucial to the function of the distal axon.

1.8.4.4 Neuronal Diseases

The first evidence that microRNAs play a role in neurodegeneration came from a study by the Bonini group in 2006. Trying to identify a possible neurodegenerative role for microRNAs they tested whether genes critical for microRNA processing effected the toxicity induced by the spinocerebellar ataxia type 3 (SCA3) protein. They observed that when Dicer is knocked out, either using a null mutant in Drosophila or siRNA in human HeLa cells, it dramatically enhanced SCA3-induced neurodegeneration. While these studies weren't very in-depth or conclusive they did suggest a vital role for microRNA in preventing neurodegeneration (Bilen et al. 2006). In a similar finding, in 2007 the Greengard group showed that conditional Purkinje cell-specific ablation of Dicer leads to cerebellar degeneration, development of ataxia and eventual cell death. They concluded

that microRNA processing is essential to avoid neurodegeneration and thus microRNAs may have an involvement in neurodegenerative disorders (Schaefer et al. 2007).

Spinocerebellar ataxia type 1 (SCA1) is caused by an expansion of CAG repeats that encode polyglutamine (poly-Q) in ataxin 1 (ATXN1). These poly-Q expanded proteins are toxic and their accumulation leads to the eventual degeneration of Purkinje neurons. In 2008, the Zoghbi group showed that ATXN1 translation is simultaneously co-regulated by three different microRNAs: miR-19, miR-101 and miR-130 and that cumulative knockdown of these microRNAs enhanced the cytotoxicity of poly-Q expanded ATXN1. They concluded that mutations in microRNA genes or their targets 3'UTRs may be causing neurodegenerative phenotypes owing to the accumulation of ATXN1 (Lee et al. 2008).

Parkinson's disease (PD) is hallmarked by midbrain dopaminergic neuron (DN) degeneration. The first evidence of microRNA involvement in DN degeneration was from the Abeliovich group in 2007. They first generated conditional DN Dicer knockout mice and observed a progressive loss of midbrain DNs correlated with the loss of functional Dicer. To identify possible DN specific microRNAs they compared microRNA expression profiles of adult normal midbrain with the profiles of midbrain depleted of DNs and also cerebral cortex samples from Parkinson's disease patients. From a pool of over 200 microRNAs, one microRNA, miR-133b was specifically enriched in the midbrain, deficient in the PD patient samples and also deficient in two other DN deficiency models (adult Aphakia mice which are mutant in the transcription

factor Pitx3 and mice treated with a DN specific toxin). Since the Aphakia mice are almost deficient in miR-133b, and contain a mutated form of Pitx3, they hypothesized that miR-133b was a direct target of Pitx3. To confirm this they overexpressed Pitx3 in ES cultures and observed a correlative increase in miR-133b expression. They also observed that miR-133b reporter vector expression was inducible by Pitx3 in COS cells. In primary midbrain cultures, overexpression of miR-133b led to a decrease in Pitx3 protein and knockdown of miR-133b led to a significant induction of Pitx3. They proposed a model in which Pitx3 induces miR-133b expression, which in turn feeds back and negatively regulates Pitx3. Taken together the concluded that miR-133b regulates the maturation and function of midbrain DNs within a feedback circuit (Kim et al. 2007).

Alpha-Synuclein (α-Syn) accumulates as fibrillary aggregates and is a key player in PD hallmarked DN degeneration. Through *in silico* prediction algorithms, overexpression analysis and qRT-PCR, in 2009, the Mouradian group was able to show that miR-7 was a translational repressor of α-Syn. They further showed that knockdown of miR-7 in human dopaminergic neuroblastoma cell lines upregulated α-Syn expression. In the PD patient brain, overexpression of α-Syn and its PD causing mutant A53T, have been shown to increase the susceptibility of cells to oxidative stress. To investigate a role for miR-7 regulation in cytotoxicity they overexpressed either a mutated 3'UTR version of A53T or a wild-type then assayed for cytotoxicity upon the addition of miR-7 and hydrogen peroxide. They found that cells infected with the wild-type, which were able to bind miR-7, displayed a decreased sensitivity to cytotoxicity as compared to the cells infected with the mutant version. They concluded that miR-7 inhibits α-Syn expression thus protecting

against α-Syn-mediated proteosome impairment and susceptibility to oxidative stress (Junn et al. 2009).

Huntington's disease (HD) is a neurodegenerative diseases cause by a CAG expansion in the huntingtin (Htt) gene. The disease is characterized by the progressive loss of cortical and striatal neurons caused by transcriptional misregulation. In a style similar to Kim et al. 2007, in 2008 Johnson et al. compared microRNA expression profiles from normal and both murine models of and human HD patient brain samples. While they did find numerous neuronal specific microRNAs to be dysregulated in the HD samples, further studies were needed to elucidate which specific microRNAs are regulating Htt. Their analyses did suggest a possible involvement of microRNA regulation in HD (Johnson et al. 2008). A few months later, the Davidson group identified two microRNAs and their role in HD. RE1-silencing transcription factor (REST) is primarily sequestered in the cytoplasm through interaction with Htt and expressed at low levels in mature neurons. In patients with HD, htt fails to bind REST and thus REST translocates to the nucleus. Once in the nucleus REST recruits the corepressor CoREST and neuron-specific genes are inactivated. The Davidson group performed a microRNA microarray to evaluate the expression patterns of microRNAs in control versus HD patient brains. Using the microarray results they focused on two microRNAs miR-9 and miR-9* whose expression decreased during HD progression. Using *in silico* target prediction analyses they found putative targets of miR-9 and miR-9* to be REST and CoREST, respectively and then validated those targets using luciferase reporter plasmid assays. These results suggest a microRNA negative feedback loop maintains healthy REST and CoREST nuclear sequesterization and this loop is affected in HD patients (Packer et al. 2008).

MicroRNAs are also thought to be involved in Alzheimer's Disease (AD). In 2008, Cogswell et al. used qRT-PCR to identify regional and stage specific deregulation of miRNA expression in AD patient brains and cerebral spinal fluid. They hypothesized that these microRNA changes could be used as bio-markers to identify the onset of Alzheimer's (Cogswell et al. 2008). In 2009, the DeStrooper group identified a group of microRNAs that regulate Amyloid precursor protein (APP). Increased expression of APP leads to its proteolytic cleavage into the Aβ peptides that accumulate in the brain of AD patients and are the hallmark of the disease. Using *in silico* target prediction models and luciferase reporter plasmids, they were able to validate that APP is a target of miR-20a, miR-17-5p and miR-106b. The authors performed functional overexpression analyses and were able to show that APP is endogenously regulated by all the microRNAs in neuroblastoma mouse Neuro2A and human SK-N-SH cells. Using qRT-PCR the authors showed a developmental correlation between the microRNAs and APP expression during brain development. A dramatic down-regulation of the microRNAs was correlated with an up-regulation in APP protein, however, no variation was observed in the APP mRNA levels. The results suggest that microRNA regulation could have a strong role in the regulation of APP during neuronal development (Hebert et al. 2009).

Mutations in methyl CpG–binding protein 2 (MeCP2) are the hallmark of Rett syndrome, an X-linked neurodevelopmental disorder. The mouse model of Rett syndrome has a duplication of the MeCP2 gene and similar to humans with Rett syndrome, MeCP2 knock-in mice develop a progressive neurological phenotype correlated with the onset of MeCP2 expression. Previous studies have determined that a narrow range of MeCP2

expression is necessary for normal development. Having previously shown that miR-132 is induced by BDNF via CREB, Impey along with the Goodman group identified MeCP2 to be a valid miR-132 target. In rat cortical neurons they observed increased levels of MeCP2 and BDNF after miR-132 knockdown and also observed that MeCP2 knockout mice had reduced levels of BDNF and miR-132 *in vivo* compared to their wildtype littermates. They proposed a model in which BDNF induces miR-132 expression in a CREB dependent manner, and miR-132 translationally represses MeCP2, which itself induces BDNF. Taken together they concluded that CREB-induced miR-132 homeostatically regulated MeCP2 expression within a negative feedback circuit (Klein et al. 2007).

1.9 Goals of this Work

After injury the axons of the adult central nervous system fail to regenerate. This failure is due to both the cellular environment (myelin-based inhibitors) and the neuronal response to that environment. It has been shown that elevating endogenous cAMP can overcome myelin-based growth inhibition. However, if microRNA regulation plays a role in the ability of cAMP to overcome this inhibition has yet to be elucidated. And thus the goal of the following work is:

1) To identify and characterize the role, if any, of cAMP-responsive microRNA in the ability of cAMP to overcome myelin-based protein growth inhibition.
2) To identify and characterize putative microRNA targets and their role in the ability of cAMP to overcome myelin-based protein growth inhibition.

Chapter II: Materials and Methods

2.1 Tissue / Cell Culture

2.1.1 CHO Cells

2.1.1.1 Maintenance

Control or stably-transfected MAG-expressing Chinese Hamster Ovary (CHO) cells (Mukhopadhyay et al. 1994) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) which had been supplemented with 10% dialyzed fetal bovine serum (FBS, Gibco), 34.8mM L-Proline (Sigma), 10mM Glycine (Sigma), 300nM Thymidine (Sigma) and 2mM L-Glutamine (Gibco).at 37ºC in 5% CO_2. These cells were used as monolayer substrates in the neurite outgrowth assay (NOG) described below.

2.1.1.2 Monolayer Preparation

To prepare monolayers for the NOG assay, Permanox 8 well chamber slides (Lab-Tek) were coated with 16.6 µg/ml poly-L-lysine (>300kD; Sigma) for 30 minutes at room temperature. After incubation, poly-L-lysine was removed and the slides were treated with 10µg/ml fibronectin (Sigma) for at least 2 hours at 37ºC. Control or MAG-expressing CHO cells (passage number 3 – 20) were plated onto these slides at the following concentrations: control – 1.8 x 10^5 cells/ml; MAG – 1.8 x 10^5 cells/ml. The slides were then incubated overnight at 37ºC prior to the plating of primary neurons.

2.1.2 Isolation of Neurons

2.1.2.1 Dorsal Root Ganglia

To isolate dorsal root ganglia (DRG) neurons, 8-16 Long-Evans rats of P5-9 were sacrificed and the DRG collected on ice into 1ml of 0.1% Collagenase in Hank's Balanced Salt Solution (HBSS, Gibco) media. The DRGs were flicked gently and then incubated for 20 minutes at 37ºC. The media was then replaced with another ml of 0.1% Collagenase in HBSS, the neurons were gently flicked followed by another 20 minute incubation at 37 ºC. After incubation, the neurons were gently flicked then allowed to settle by gravity. The media was removed and the neurons were washed three times by adding 1ml HBSS plus Antibiotic-Antimyotic (AA, Gibco), inverting and flicking the tube, and allowing neurons to settle by gravity. After the last wash the supernatant was removed and replaced with 0.1x trypsin in HBSS-AA. 100µL of DNase I (1mg/ml, Worthington) was added to the mixture and the cells were thoroughly mixed by flicking, then incubated a further 15 minutes at 37ºC. Trypsinization was halted with DMEM media containing 10% serum. The cells were then triturated gently in HBSS-AA, first with a 1ml pipette, then with a p200. Dissociated DRGs were then spun three times in a microcentrifuge for 1 minute at 2K, triturating in between spins. Cells were then washed in SATO plating medium, counted and plated onto either 6-well dishes or 8 chamber slides.

2.1.3 Purified Myelin

2.1.3.1 Myelin preparation

The medulla from an adult rat brain was isolated and homogenized in a 0.25M sucrose solution containing a protease inhibitor cocktail (Cal Biochem) using a glass and teflon homogenizer. The homogenate is then mixed with a 2.55 M sucrose solution to create a 1.4M solution. This is then layered onto a 1.9M sucrose solution, followed by 0.85M and 0.25M solutions. The gradient is centrifuged at 40,000 rpm for 14 hours at 4ºC. Following centrifugation and separation, the extracted myelin is homogenized again in dH_2O and protease inhibitor, centrifuged at 14,000 rpm for 1 hour at 4ºC, resuspended in 10mM HEPES and triturated using 18.5 and 26.5 gauge needles.

2.1.3.2 Immobilized myelin substrates

8 chamber Permanox slides were coated with 100 µg/ml poly-L-lysine for 30 minutes at room temperature. After incubation, PLL was removed and washed once with dH_2O. Myelin was then plated at a concentration of 5µg/mL and then dried overnight in a vacuum chamber filled with Drierite dessicator. Slides were then used immediately or frozen at -80ºC for no more than 5 days prior to use.

2.1.4 Priming Neurons with cAMP

6-well tissue culture dishes were coated with 100µg/ml of poly-L-lysine for at least 30 minutes at room temperature. The wells were then washed once with dH_2O to remove excess PLL. Isolated dorsal root ganglion neurons were plated onto these dishes at a

concentration of approximately 1 x 10^6 cells/well. These neurons were then treated with 1mM db-cAMP. The neurons were then cultured overnight at 37ºC for 12-24 hours.

2.2 The Neurite Outgrowth Assay

2.2.1 Neurite Outgrowth Culture

Following dissociation, noted above, primary DRG neurons were plated onto the CHO cell monolayers or purified myelin substrate at a cell density of 5.0 x 10^3 cells per well (8 chamber slide). This co-culture was then incubated for 15 hours at 37ºC. Where indicated, these cells were also treated with 1mM db-cAMP (Cal-Biochem), 60nM Pre-rno-miR-151 (Ambion), 60nM Anti-rno-miR-151 (Ambion), 6 or 60nM Pre-miR-Cy3-Negative Control #1 (Ambion), 75nM Nnat siRNA (Santa Cruz) or 75nM scrambled control siRNA-A (Santa Cruz), during this period.

After incubation, the cultures were then fixed twice with 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with cold 100% methanol for 2 minutes. The cells were then blocked against non-specific binding with DMEM containing 10% serum for a minimum of 20 minutes at room temperature.

The neurons were then stained for the neuronal-specific marker βIII tubulin as follows. After blocking, the cultures were incubated overnight at 4ºC in a PBS-BSA (0.05%) solution containing a monoclonal anti-βIII tubulin antibody (Covance) at a dilution of

1:1000. After this incubation, the cultures were washed 3 times with PBS and then incubated at room temperature for 30 minutes in a PBS-BSA (0.05%) solution containing biotinylated donkey anti-mouse IgG at a 1:500 dilution. Alternatively, after the first incubation the cultures were washed 3 times with PBS-Tween and then incubated at room temperature for 60 minutes in a PBS-BSA (0.05%) solution containing the green-fluorescent dye conjugant Alexa Fluor 488® (Invitrogen) at a 1:1000 dilution. Finally, the cultures were washed 3 more times with PBS-Tween and then immobilized using the Permafluor (Immunon) and viewed under a fluorescent microscope.

2.2.2 Measuring and Quantification

In order to quantify the neurite outgrowth length from these treated neurons, the immunostained cultures were observed under a fluorescent microscope and the neurite length was measured using the Metamorph imaging quantification software. Briefly, the longest neurite from each of 50-200 βIII tubulin-positive neurons per well, selected systematically by progressive movement from one side of the well to the other, were traced onscreen and the mean neurite length was calculated using the software tools. Statistical analysis of the data obtained was performed using the GraphPad Prism software program.

2.3 Quantitative Real-Time PCR

2.3.1 Isolation of total mRNA

Total RNA was extracted from cultured DRG neurons (previously described) using mirVana™ (Ambion) miRNA isolation kit. Briefly stated, the neurons are lysed in a denaturing lysis solution and disrupted with a homogenate additive, followed by an organic extraction using Acid-Phenol Chloroform, the suspension is further purified over a glass-fiber filter and then eluted into RNAse free dH_2O.

2.3.2 qRT-PCR

qRT-PCR analysis was performed using mirVana™ (Ambion) qRT-PCR miRNA Detection Kit or RT2 First Strand Kit and qPCR Master Mixes (Superarray) and primer sets for normalization (U6snRNA(Ambion), β-II microglobulin (Superarray)) and rno-miR-151 (Ambion) or Neuronatin (Superarray). RNA was added at 1ng/μL for a total concentration of 10ng/reaction. Relative quantification analysis was performed on an Applied Biosystems 7300 RT-PCR system with the an initial denaturing block of 95ºC for 3 minutes, followed by 40 PCR cycles of 95ºC for 15 seconds, 60 ºC for 33 seconds. Relative differences were calculated using the comparative CT method. The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where $[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$.

2.4 Pre-miR, Anti-miR and siRNA Transfection

To make the Lipofecatmine liposomes, Pre-miR-151, Anti-rno-miR-151, Pre-miR-Cy3-Negative Control #1, Nnat siRNA (Santa Cruz) or Scrambled control siRNA-A (Santa Cruz) was diluted in 50µL of Opti-MEM I Medium (Invitrogen). 10µL of Lipofectamine 2000™ was mixed gently then diluted in 40µL of Opti-MEM, followed by a 5 minute incubation at room temperature. After the incubation the diluted RNA and the diluted Lipofectamine were combined, gently mixed and incubated for a minimum of 30 minutes at room temperature. The RNA containing liposome complexes were then added by droplets onto cultured DRG neurons at a final concentration of 6-75nM.

2.5 miR-151 Target Prediction

Two different target prediction algorithms were used to identify possible miR-151 mRNA targets: miRanda (www.microrna.org), and miRbase (http://microrna.sanger.ac.uk). Briefly, the current target prediction softwares work by using the miRanda algorithm (John et al. 2004). The current miRanda uses "dynamic programming alignment to identify highly complementary sites which are scored between 0 and 100, where 0 represents no complementarity and 100 complete complementary". The algorithm uses a weighted scoring system and rewards complementarity at the seed region which is the 5' end of the microRNA. Target sites that are deemed complementary are then passed through the Vienna RNA folding routines in order to estimate their thermodynamic stability. Finally all putative targets are checked to see whether the microRNA binding site is conserved in orthologous transcripts from other species.

2.6 Luciferase Reporter Assay

2.6.1 Neuronatin Plasmid Construction

To confirm putative targets for miR-151 we used the pMIR-REPORT™ System miRNA Expression Reporter Vector (Ambion, Figure 1). For analysis of the predicted target Neuronatin sites we synthesized complementary DNA oligonucleotides (Fisher Oligo) consisting of the wildtype or a mutated Neuronatin 3'UTR microRNA binding site flanked by single-stranded overhangs encoding restriction enzyme sites as shown below:

MT NNAT 3'UTR:
5' GAGCTCATCAGGTGCTCCTGTGCTTCTCGACCAGCATGGGAG '3
Sac1
3' TAGTCCACGAGGACACGAAGAGCTGGTCGTACCCTCTTCGAA '5
HindIII
UAGAUUGUAAGCUCCUGG

WT NNAT 3' UTR:
5' GAGCTCTGTGTCTCCCCGGCTAGATTGTAAGCTCCTGGAGACAGGGACC '3
Sac1
3' ACACAGAGGGCCGACTAACATTCGAGGACCTCTGTCCCTGGTTCGAA '5
HindIII

To grow the pMIR-REPORT plasmid pMIR-REPORT Luciferase 100mL of Luria Broth (LB) containing 100µg/mL carbenicillin was inoculated with 50µL of the supplied glycerol stock and incubated overnight in a shaking 37ºC incubator. After a minimum of 12 hours the plasmid DNA was purified from the culture using a Qiagen Midi Kit (Qiagen). 10µg of the pMIR-REPORT Luciferase DNA was then linearized overnight at

37 ºC with the restriction enzymes Sac1 (20,000U/mL, NEB) and HindIII (20,000U/mL, NEB) in Buffer 2 (NEB) containing 1% BSA.

To prepare the synthetic oligos for ligation 1 µg of sense and 1 µg of anti-sense were diluted in 30mM HEPES (pH 7.4) containing 100mM Potassium Acetate and 2mM Magnesium Acetate, heated to 90 ºC for 3 min, then incubated at 37 ºC for 1 hour. The insert was ligated into the pMIR-REPORT Luciferase using a 10-fold molar excess of oligo (70nM) to plasmid (7nM) with T4 DNA Ligase and 10X Ligase Reaction Buffer (Ambion) and incubated overnight at 16 ºC along with a no-insert ligation negative control. The ligated plasmid was then precipitated using 7.5M Ammonium Acetate and 100% EtOH and a 30 minute incubation at -20 ºC. After the incubation the plasmid was spun at 13k for 10 minutes and the resulting pellet was washed with 70% EtOH before being spun again at 13k for 5 minutes. The pellet was then lyophilized and resuspended in dH_20.

50µL of 10pg/µL Electrocompetent *E.coli* dαH5 cells (Invitrogen) were then transformed with the ligated plasmids by adding 10µL of the precipitated plasmid to the cells and electroporating the bacteria at 200Ohms/25µF/ 2.5KV using the Bio-Rad Gene Pulser. After electroporation the bacteria were recovered in 500µL or room temperature LB, transferred to 1.5mL eppendorf tubes and spun at 14k for 1 minute. The resulting pellet was resuspended in LB, spread onto LB-carbinicillin (100µg/mL) plates and incubated at 37 ºC overnight. A non transformed competent cell control was also included.

To identify clones with the insert, 10 colonies were chosen from both plates containing the wildtype and mutated inserts. The colonies were grown overnight in 2mL of LB-carbinicillin in a shaking 37 ºC incubator. The clones were then purified using a Qiagen Mini Kit (Qiagen), the DNA was digested ran on a 1% agarose gel to identify clones with the proper size insert. Selected clones were then grown overnight in 250mL LB-carbinicillin in a shaking 37 ºC incubator. After a minimum of 12 hours the plasmid DNA was purified from the culture using a Qiagen Maxi Kit (Qiagen).

2.6.2 Transfection of 293-T Cells

293-T cells (Human Embryonic Kidney cells were transformed by the stable integration of the Adenovirus E1 region) were grown in DMEM + 10% FBS (BRL) at 37 ºC in 6-well culture plates until 90% confluency. The plasmid DNA was then transfected into the cells using Lipofectamine 2000 (Invitrogen). To make the Lipofecatmine liposomes, 4µg of plasmid DNA was diluted in 50µL of Opti-MEM I Medium (Invitrogen). 10µL of Lipofectamine 2000™ was mixed gently then diluted in 40µL of Opti-MEM, followed by a 5 minute incubation at room temperature. After the incubation the diluted DNA and the diluted Lipofectamine were combined, gently mixed and incubated for a minimum of 30 minutes at room temperature. The DNA containing liposome complexes were then added by droplets onto the confluent 293-T.

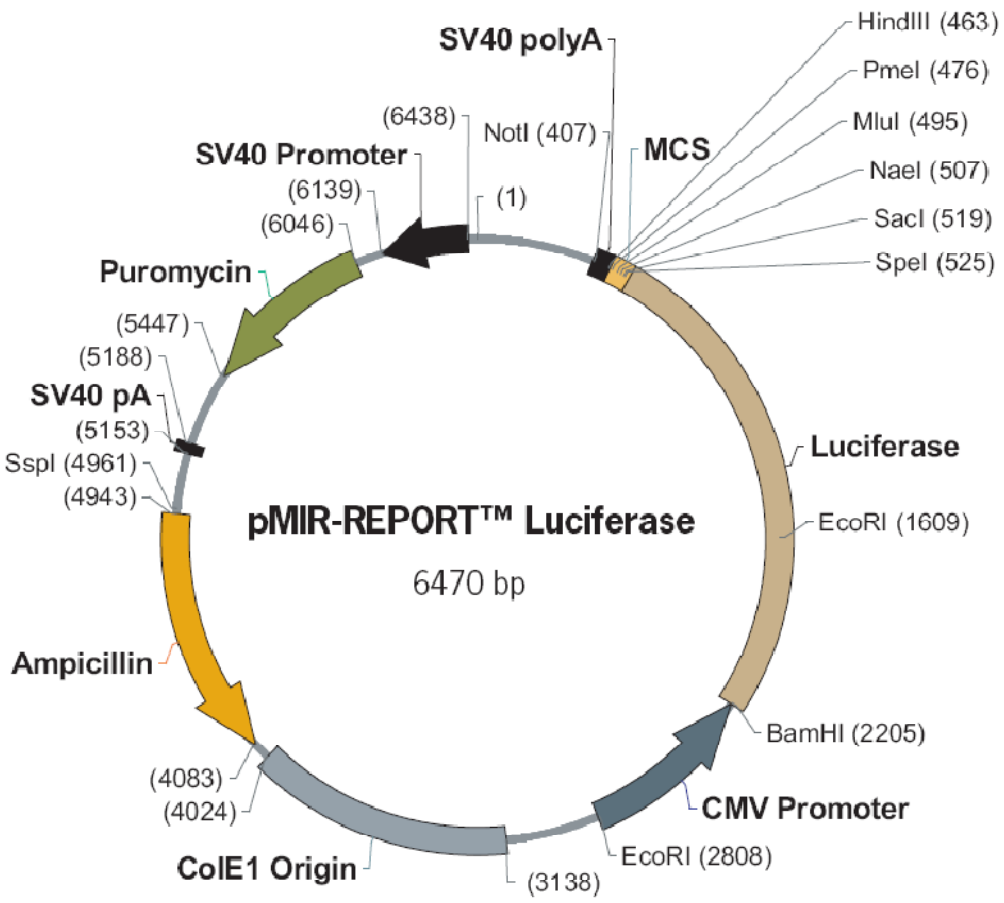


Figure 2 1: Schematic of pMIR-REPORT Luciferase (Ambion)

2.6.3 Luciferase Evaluation

After a minimum of 24 hours the transfected 293-T cells were lysed in 500µL Cell Extract Buffer (R&D Systems) for 10 minutes at room temperature. 500µL of Luciferase Substrate (Promega) was then added to the lysed cells and the Luciferase activity was measured using the Gemini EM microplate spectrofluorometer from Molecular Devices.

2.7 Western Blot

Total protein was isolated from DRG neurons using 1X RIPA Buffer (Pierce Biotechnology) with 1X Protease and Phosphatase Inhibitors (Cal Biochem). Cell lysate was separated by SDS-PAGE using 4-20% gradient gels (Thermo Scientific). The protein was transferred to a 0.2µM Immuno-Blot PVDF Membrane (Bio-Rad). Membranes were then blocked in 5% BSA in PBS-0.1% Tween-20 and probed with primary Neuronatin 1:1000 (Santa Cruz), Actin 1:5000(Cell Signaling), or FAK 1:1000 (Up State) antibody in blocking buffer overnight at 4°C. Membranes were washed three times in PBS with 0.1% Tween-20. Secondary anti-rabbit HRP-conjugated antibody (Cell Signaling) was diluted 1:2000 in blocking buffer and incubated with the membranes for 1 hr at room temperature. Membranes were then washed as before and developed using ECL (Amersham Biosciences).

2.8 *In situ* Hybridization

2.8.1 miR-151 *in situ*

DRG neurons (P5-P6) were dissociated as previously described, treated with or without 1or 2mM db-camp (Cal Biochem) and plated onto 100mg/mL PLL-coated 8 chamber Permanox glass slides at approximately 1.0 x 10^4 cells/well. After 24 hours the neurons were fixed with 4% PFA in DEPC-treated water (Ambion) for 15 minutes at room temperature. The spent PFA was discarded and fresh 4% PFA was applied for an additional 15 minutes at room temperature. The neurons were then permeabolized in

RNAse free PBS (Ambion) containing 0.4% Triton X-100 (USB) for 15 minutes at room temperature followed by 3 consecutive 5 minute PBS washes. After completely air-drying, 250pmol 5'-Digoxigenin-labeled LNA-miR-151 or scrambled 5'-Digoxigenin-labeled LNA control (Exiqon) in *in situ* hybridization buffer (Enzo) was added to the slides which were then heated to 60º for 5 minutes followed by a 15 hour incubation at 37ºC. The slides were washed in 0.2X SSC (Ambion) with 2% BSA (Sigma) shaking at 4ºC for 5 minutes.

The DIG-labeled probe was then detected by incubation of the slide with 100µL of antidigoxigenin/alkaline phosphatase (Roche) (1:150 dilution in PBS) for 30 minutes at 37ºC. The slides were washed in 500µL of SignaSure Wash Buffer (Enzo) for 1 minute at room temperature, followed by a 37ºC incubation with 50µL of NBT/BCIP Reaction Mixture (Enzo) and 50µL of AP Detection Reagent (Enzo) for 15 minutes to several hours. The slides are then placed in to dH_20 to stop the reaction.

2.8.2 Immunohistochemistry

Slides were blocked in PBS-0.1% Tween-20 with 5% BSA for a minimum of 1 hour. After blocking, the cultures were incubated overnight at 4ºC in a PBS-BSA (0.05%) solution containing a monoclonal anti-βIII tubulin antibody (Covance) at a dilution of 1:2000 or anti-Neuronatin antibody (Santa Cruz) at a dilution of 1:1000. After this incubation, the cultures were washed 3 times with PBS and then incubated at room temperature for 30 minutes in a PBS-BSA (0.05%) solution containing biotinylated donkey anti-mouse IgG at a 1:500 dilution. Alternatively, after the first incubation the

cultures were washed 3 times with PBS-Tween and then incubated at room temperature for 60 minutes in a PBS-BSA (0.05%) solution containing the green-fluorescent dye conjugant Alexa Fluor 488® (Invitrogen) at a 1:1000 dilution. Finally, the cultures were washed 3 more times with PBS-Tween and then immobilized using the Permafluor (Immunon) and viewed under a fluorescent microscope.

2.9 Peripheral Nerve Lesion

P64 adult rats were anesthetized by isofluorane, then a sciatic nerve was exposed at mid-thigh level and a ligature was firmly tightened around the nerve distal to its emergence to the greater sciatic notch. The nerve was transected distal to the ligature and the wound closed. The animals were sacrificed 24 hours after the sciatic nerve transection. L4, 5 and 6 dorsal root ganglia from the lesion side and contralateral control side were isolated respectively.

Chapter III: Identifying the cAMP-Responsive MicroRNA miR-151

3.1 Introduction

After injury the axons of the mammalian adult central nervous system fail to regenerate. This failure is due to the cellular environment and the neuronal response to that environment. One factor for the environmentally-mediated failure to regenerate is myelin. Myelin is an electrically insulating spiral of membrane produced by oligodendrocytes in the CNS and Schwann cells in the PNS. After injury, it's believed that the exposure of proteins present in the myelin lead to axonal growth inhibition. The main three proteins that have been identified to be growth inhibitors in myelin are MAG, Nogo, and OMgp (McKerracher et al. 1994; Mukhopadhyay et al. 1994; GrandPre et al. 2000; Wang et al. 2002). Although these three proteins share no sequence or structural similarity they were all found to mediate their inhibitory effects by binding to the same tripartite receptor, the Nogo Receptor/$p75^{NTR}$/LINGO complex (Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002; Wong et al. 2002; Mi et al. 2004) and more recently it has been shown that all three proteins can also mediate their inhibitory effects by binding to another receptor, PirB (Atwal et al. 2008). Methods for overcoming the myelin-based protein axonal growth inhibition have focused on ways to pacify the inhibitory environment and/or boost the intrinsic growth capabilities of the neuron. One way that has been shown to overcome MAG/myelin-based inhibition is through elevation of the ubiquitous second messenger, cAMP. Our lab has shown that elevation of cAMP is able to overcome inhibition on both a MAG-expressing monolayer and a purified myelin substrate (Cai et al. 1999; Qiu et al. 2002). As aforementioned, axons of the adult central nervous system do not

spontaneously regenerate. However, there has been one situation in which they have been found to do so. Dorsal root ganglion (DRG) neurons are pseudo-unipolar with one bifurcated axon branching into both the CNS and the PNS. If a lesion is made to the CNS branch, the axons do not regenerate. If a lesion is made to the PNS branch and then subsequently a lesion is made to the CNS branch, the neurons regenerate (Neumann and Woolf 1999). This primary PNS lesion is referred to as a "conditioning lesion" and our lab has shown that the conditioning lesion effect is directly dependent on increased cAMP (Neumann and Woolf 1999; Qiu et al. 2002).

Recently it has been shown that small pieces of RNA called microRNA are able to bind to target mRNAs and translationally repress the protein expression (Lee et al. 1993) (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Our lab performed a preliminary microRNA microarray with db-cAMP versus untreated DRG neurons and found 100's of microRNAs to be cAMP-responsive; and so we hypothesized that microRNA regulation may have a role in cAMPs ability to overcome MAG/myelin-mediated axonal growth inhibition. Therefore in order to further confirm the presence of the mature microRNA in DRG neurons and its cAMP responsiveness we performed *in situ* hybridization and qRT-PCR analyses.

3.2 Results

Our lab has shown that elevating endogenous levels of cAMP can mimic the effect of a conditioning lesion in promoting neurite outgrowth in the presence of MAG/myelin (Cai et al. 1999; Qiu et al. 2002; Atwal et al. 2008). MicroRNAs have been shown to have

various roles in neurons including: identity and differentiation, maturation, dendritic spine morphogenesis and development, and neurite outgrowth (Vo et al. 2005; Conaco et al. 2006; Schratt et al. 2006; Visvanathan et al. 2007). In order to determine if microRNAs were involved in the ability of db-cAMP to overcome MAG/myelin-mediated neurite outgrowth inhibition, we first needed to determine if microRNAs were responsive to cAMP. To answer this we performed a microRNA microarray with db-cAMP treated versus control DRG neurons. DRG neurons were treated +/- db-cAMP for 24 hours. The cells were lysed and the RNA was extracted. The microarray was performed using an N-code™ system microarray with all known mammalian microRNAs being screened (Cain, Hart & Filbin, unpublished data). Hundreds of microRNAs were shown to be cAMP responsive. One microRNA of interest that was shown to increase upon treatment with db-cAMP was microRNA-151 (miR-151). miR-151 was one of the most cAMP-responsive microRNAs and after performing some *in silico* analyses for putative mRNA targets using the algorithms Microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and MiRanda (http://www.microrna.org/microrna/home.do), we decided to investigate if miR-151 had a role in cAMPs ability to overcome myelin-based axonal growth inhibition.

To confirm the presence of mature miR-151 in DRG neurons we carried out *in situ hybridization* on post-natal day 5-6 neurons using 5'-Digoxigenin-labeled LNA-miR-151 or scrambled 5'-Digoxigenin-labeled LNA control probes. The DIG-labeled probe was detected by incubation of the slide with antidigoxigenin/alkaline phosphatase and then immunohistochemistry was performed for the detection of the neuronal marker β-III

Tubulin with the green-fluorescent dye conjugant Alexa Fluor 488® used as the secondary antibody. As shown in Figure 3.1, *in situ* hybridization confirmed that mature miR-151 was present in DRG neurons. No staining was observed in the control DRG neurons that were probed with a scrambled miR-151 probe.

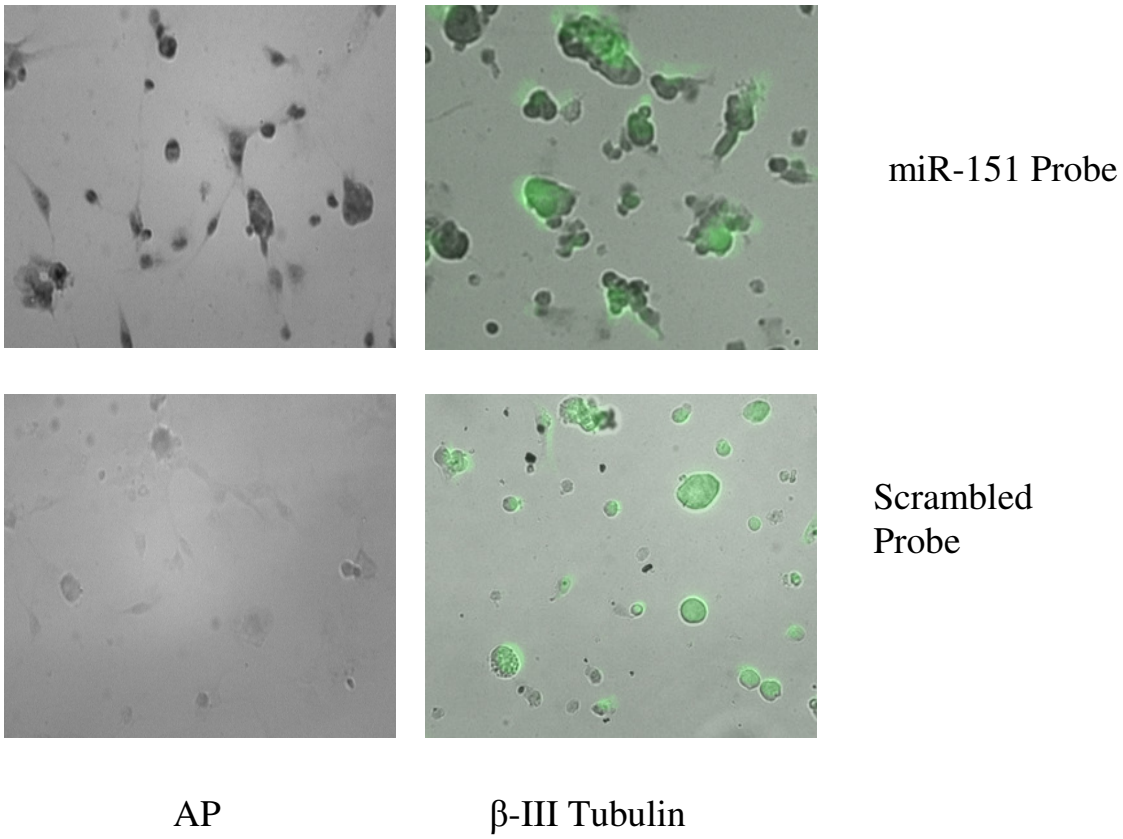


Figure 3.1: *In situ* hybridization of mature miR-151 in DRG neurons. DRG neurons were probed with a DIG-labeled mature miR-151 probe. No staining was observed when a scrambled probe was used. B-III Tubulin was used as a neuronal marker.

To confirm miR-151 is cAMP responsive we carried out quantitative real time-PCR (qRT-PCR) using primers that were targeted to the mature form of miR-151. DRG neurons from post-natal day 5-7 rats were isolated, dissociated and plated on PLL coated 6-well chamber plates in the presence or absence of 1mM db-cAMP. After 24 hours the neurons were lysed and the total RNA was collected and subjected to qRT-PCR. As shown in Figure 3.2, we found miR-151 was up-regulated in DRG neurons treated with db-cAMP as compared to the control untreated neurons (relative to the non-cAMP-responsive U6 small RNA housekeeping gene) ($P<0.005$).

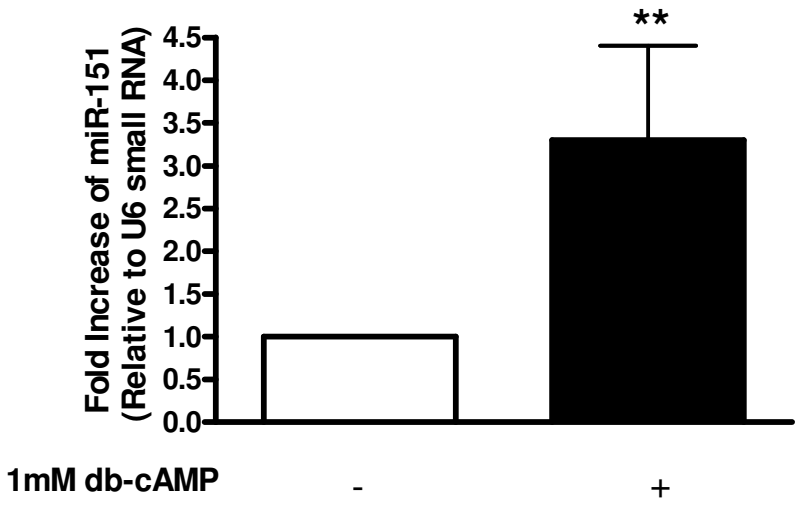
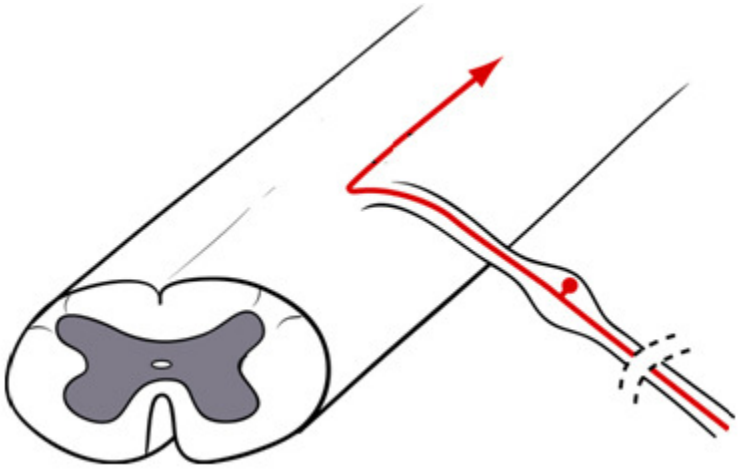


Figure **3.2: qRT-PCR Analysis of cAMP-induced miR-151 Increase**. **Fold increase of miR-151 (relative to U6 small RNA housekeeping gene) in P5-7 DRG neurons after treatment with db-cAMP for 24 hours as compared to the control untreated (P<0.005 Students two tailed t test, n=3).**

It is known that the CNS axon branch of the DRG neuron can be 'primed' to grow if a lesion to the peripheral branch is made first (Neumann and Woolf, 1999) (Figure 3.3). This 'priming' or 'conditioning effect' has been shown to be dependent upon increased levels of cAMP and the effects of a conditioning lesion on dorsal column regeneration can be mimicked by elevating levels of cAMP (Neumann et al. 2002; Qiu et al. 2002). Since miR-151 is increased after treatment with cAMP, we wanted to assess if miR-151 is likewise elevated after a conditioning lesion. We performed a conditioning lesion of the sciatic nerve in post-natal day 64 rats and allowed the rats to survive a further 24-28 hours after which the DRGs were removed from L4-6, homogenized, lysed, total RNA was extracted and qRT-PCR analysis was performed. As shown in Figure 3.4, miR-151 is up-regulated after a peripheral conditioning lesion as compared to the internal contralateral control ($P<0.005$). Thus, in DRG neurons, miR-151 expression is up-regulated by cAMP.



Sacrifice after 24 hours

↓

Isolate L4-6 Dorsal Root Ganglia

↓

Homogenize and Lyse

↓

Isolate total RNA

↓

qRT-PCR

Figure 3.3: Conditioning Lesion and Isolation of total RNA

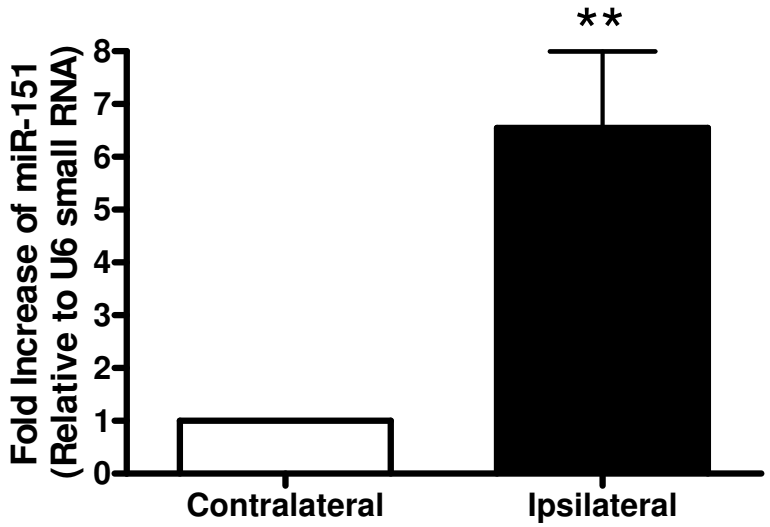


Figure 3.4: qRT-PCR Analysis of miR-151 in DRG Neurons After a Peripheral Conditioning Lesion. miR-151 is increased in vivo in DRG neurons after a peripheral conditioning lesion, as compared to the contralateral control (P<0.005, Students two tailed t test, n=3).

To investigate a possible mechanism for the cAMP-induced miR-151 expression we sought to identify putative miR-151 transciption sites. After retrieving the rat miR-151 accession number from the microRNA registry (www.miRbase.org) we entered the accession number (MI0000647) into the Ensembl Genome Browser (http://www.ensembl.org/index.html) and searched the rat genome for alignment. One genomic alignment was found, mapping to one gene, Focal Adhesion Kinase (FAK) also known as protein tyrosine kinase 2 (ptk2) (Figure 3.5).

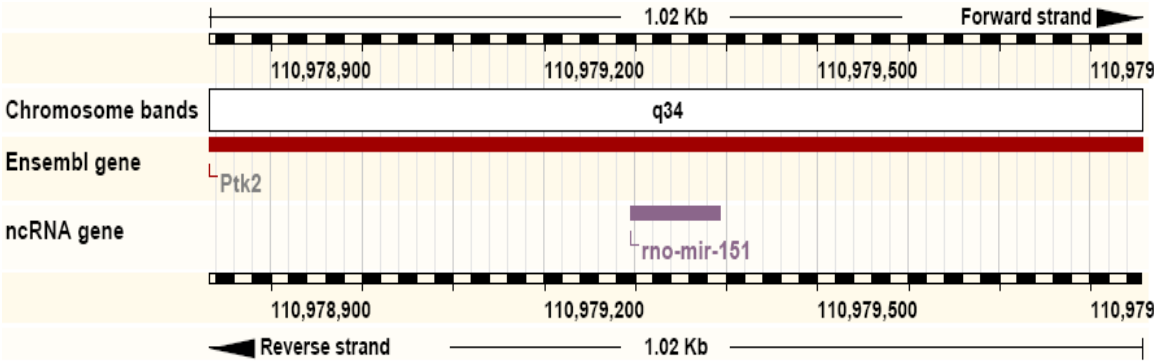


Figure 3.5: miR-151 is Encoded in the Gene for FAK. microRNA-151 aligned along the rat genome at one position, as an intronic sequence in the gene for FAK (ptk2). (http://www.ensembl.org/Rattus_norvegicus)

Thus, miR-151 is a mirton, a microRNA transcribed from an intron in a gene (FAK). When splicing occurs the adjacent two exons are combined and the microRNA is released (Winter et al. 2009) (Figure 3.6). Since cAMP is known to activate the transcription factor, cAMP response element binding protein (CREB), we next looked to see if the 5' sequence of FAK contained any known cAMP response element (CRE) binding sites. The 5' UTR of FAK was found to contain two half CRE sites (Figure 3.7).

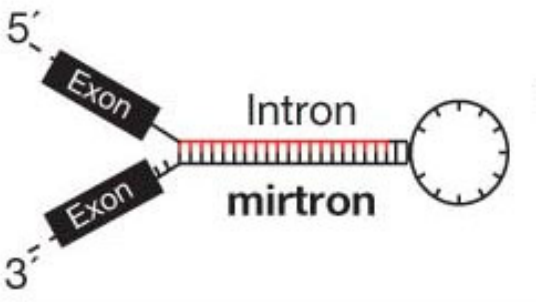


Figure 3.6: Mirtron Schematic. MicroRNAs that are transcribed in intron are referred to as mirtrons. A mirtron occurs when the length and hairpin structure of a released intron resembles that of a pre-miRNA. (Adapted from Winter et al.,2009)

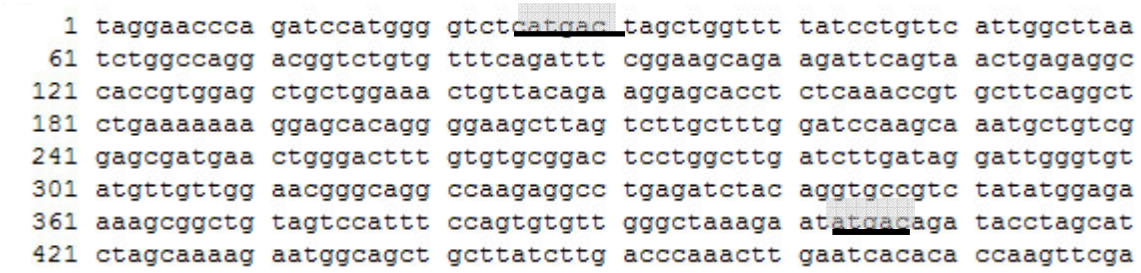


Figure 3.7: The 5'UTR of FAK Contains Two Putative CREs. One possible mechanism for cAMP-induced miR-151 expression is through CREB-mediated miR-151 expression. The 5'UTR of FAK contains two putative CREB binding sites (ATGAC), underlined and highlighted in grey.

One possible mechanism for the cAMP-induced miR-151 may be through cAMP activation of CREB which binds to the CRE sites in the FAK gene and initiates transcription of the gene and thus the microRNA. To determine if cAMP was inducing FAK expression we performed qRT-PCR anaylsis on db-cAMP treated versus untreated DRG neurons. As shown in Figure 3.8, FAK mRNA levels are upregulated after treatment with db-cAMP relative to a non cAMP-responsive U6 small RNA housekeeping gene. Our findings presented here suggest that miR-151 is present in DRG neurons, is cAMP-responsive with a possible mechanism for this increase being through its increased expression via CREB, and is also upregulated after a conditionng lesion.

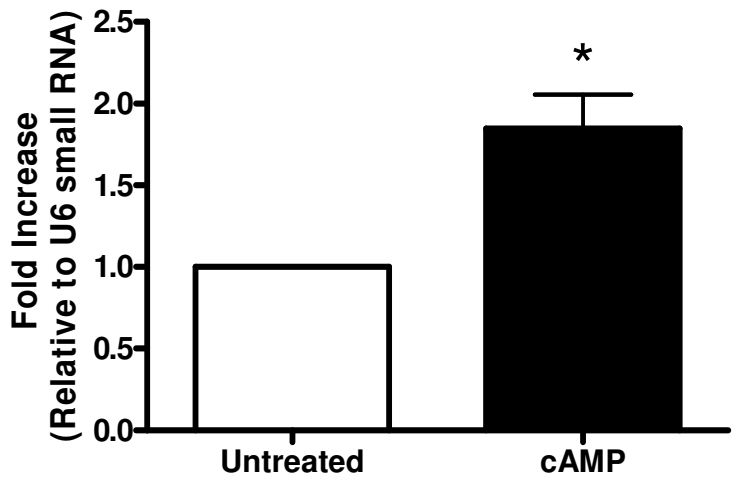


Figure 3.8: FAK mRNA is cAMP -Responsive. Fold increase of FAK mRNA (relative to U6 small RNA housekeeping gene) in P5-7 DRG neurons after treatment with 1mM db-cAMP for 24 hours as compared to the control untreated (P<0.05 Students two tailed t test, n=3).

3.3 Discussion

The findings outlined in this chapter provide some intriguing insight into a possible role for microRNA regulation in cAMPs ability to overcome MAG/myelin-mediated axonal growth inhibition. With the data obtained here, we can begin to hypothesize that cAMP is altering microRNA expression, both *in vitro* and *in vivo* (after a conditioning lesion) and that this alteration may have an effect on overcoming MAG/myelin-mediated axonal growth inhibition. It has been shown that cAMP activates the transcription factor CREB and that CREB induces expression of genes via binding to CRE sites and initiating transcription. In 2009, the Ghosh group showed that transcription factors promoted

microRNA expression when they published that the transcription factor Mef2 directly binds to the promoter of a microRNA cluster (a series of microRNAs induced by the same promoter, including miR-132) and induces their expression (Fiore et al. 2009). In previous work published by the Impey group, it has been shown that another transcription factor, CREB, directly binds to the miR-132 promoter and induces its expression (Wayman et al. 2008). Mir-132 has also been previously shown by the Impey group to be BDNF-responsive and targeting p250-GAP. They also showed that the miR-132 induced translational repression of p250-GAP results in increased neurite outgrowth on a permissive substrate (Vo et al. 2005). The work described here paints a very similar picture, cAMP activates CREB, which in turn induces FAK mRNA expression and thus, inducing miR-151 production from a FAK intron. Interestingly, while we have shown that FAK mRNA is cAMP-responsive, unpublished data from our lab has suggested that FAK protein levels are not cAMP-responsive (Martinez and Filbin, unpublished). This mRNA specific increase suggests that cAMP may induce transcription of FAK mRNA only to produce the hidden microRNA, and not the expression of FAK protein. This possibility of gene transcription specifically for the production of a microRNA adds another dimension to the complexity of post-transcriptional gene regulation.

As our lab has shown, increased levels of cAMP promote axonal growth on an inhibitory substrate and in an inhibitory environment (Cai et al. 1999; Qiu et al. 2002). The work described here suggests that in DRG neurons microRNA production, specifically miR-151, is elevated after either treatment with cAMP or a peripheral conditioning lesion. In the next few chapters we will determine if this cAMP-induced microRNA upregulation

might be playing a role in the ability of cAMP to overcome MAG/myelin-mediated axonal growth inhibition.

Chapter IV: Determining the Functional Role of miR-151 in Overcoming MAG/Myelin-Mediated Neurite Outgrowth Inhibition

4.1 Introduction

After injury the axons of the adult mammalian central nervous system fail to regenerate. To date, there have been three major growth inhibitory proteins identified in myelin, MAG, Nogo, and OMgp (McKerracher et al. 1994; Mukhopadhyay et al. 1994; GrandPre et al. 2000; Wang et al. 2002). It is believed that after injury the exposure of the neuron to these myelin-based inhibitory proteins is the first barrier to axonal regeneration. In the Filbin lab we focus on ways to enhance axonal regeneration after spinal cord injury. One way we test for the ability of a compound or molecule to overcome MAG-myelin-based inhibition is through a neurite outgrowth assay. In this assay we take primary neurons, triturate them into single neuronal suspensions, then treat them with a compound/molecule of interest before plating them onto either a permissive substrate of Chinese Hamster Ovary (CHO) cells, or a non-growth permissive substrate of CHO cells that have been stably transfected to express MAG (alternatively we plate the neurons onto a permissive substrate of poly-L-lysine and a non-growth permissive substrate of purified myelin). Using this assay, one way that our lab has found to overcome the MAG/myelin-based axonal growth inhibition is through elevation of cAMP (Cai et al. 1999; Qiu et al. 2002).

Recently, small fragments of RNA called microRNA have been shown to bind to target mRNA and inhibit its translation. Our lab performed a microRNA microarray identifying

hundreds of cAMP-responsive microRNAs (Cain, Hart & Filbin, unpublished data). From that microarray, we chose one of the most cAMP-responsive microRNAs, miR-151, to further characterize. As shown in the previous chapter, mature miR-151 is expressed in DRG neurons and is upregulated by cAMP treatment and after a conditioning lesion, presumably by cAMP activation of the transcription factor CREB. While identification of a cAMP-responsive microRNA is interesting, if the microRNA has no function in overcoming MAG/myelin-mediated axonal growth inhibition, then we needed to focus on another cAMP-responsive microRNA. So, to determine if miR-151 has a role in the ability of cAMP to overcome MAG/myelin-mediated axonal growth inhibition we performed functional overexpression and knockdown analyses on miR-151 in DRG neurons that were subsequently subjected to a neurite outgrowth assay.

4.2 Results

Our expression analysis suggests that because miR-151 expression is elevated after both treatment with cAMP and a peripheral conditioning lesion, miR-151 may play a role in overcoming MAG/myelin-mediated axonal growth inhibition. To assess if miR-151 does play a role in overcoming MAG/myelin-mediated inhibition, we carried out microRNA overexpression studies on DRG neurons subjected to a neurite outgrowth assay (Figure 4.1). To overexpress the miR-151 we used chemically modified mature miR-151 mimics. These miRNAs are double-stranded RNA molecules that are easily transfected into cells due to their small size. Using Lipofecatmine, we were able to transfect a Cy3-labeled control miR (a control microRNA mimic with a Cy3 molecule attached to the 5'end, that has been extensively studied and found to have no known cellular effects) into DRG

neurons and calculated a percent of transfection of approximately 60%. Cy3-labeled control miR transfection can be seen in Figure 4.2. However, since the miR-151 mimic is not labeled, in order to ascertain which neurons were transfected with the microRNA, we co-transfected the miR-151 mimic along with the Cy3-labeled control miR at a ratio of 10:1 and only counted the neurons that were transfected with the visible Cy3.

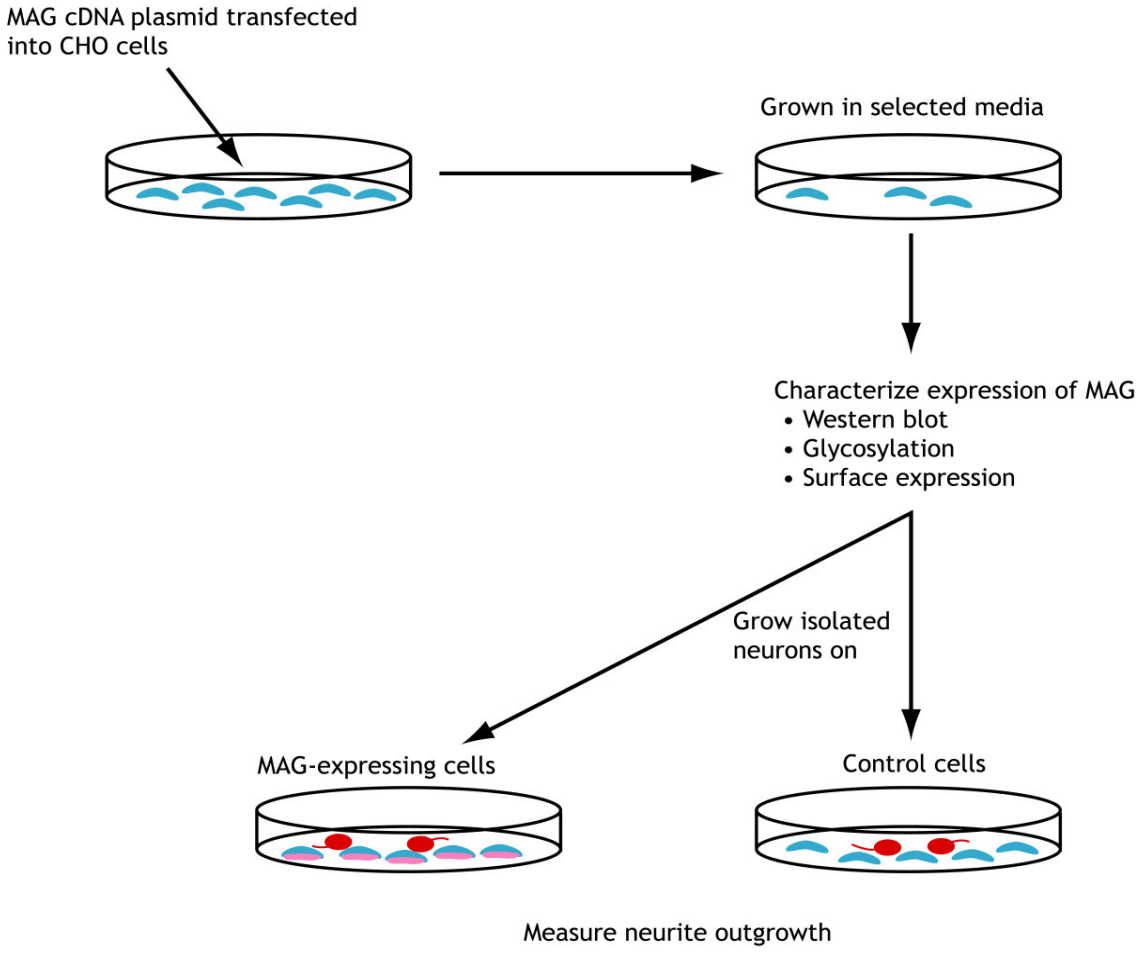


Figure 4.1: Schematic Representation of the Generation of MAG-expressing CHO cells and the Standard Neurite Outgrowth Assay. Alternatively neurons are plated on a non-growth permissive purified myelin substrate or a permissive poly-L-lysine substrate.

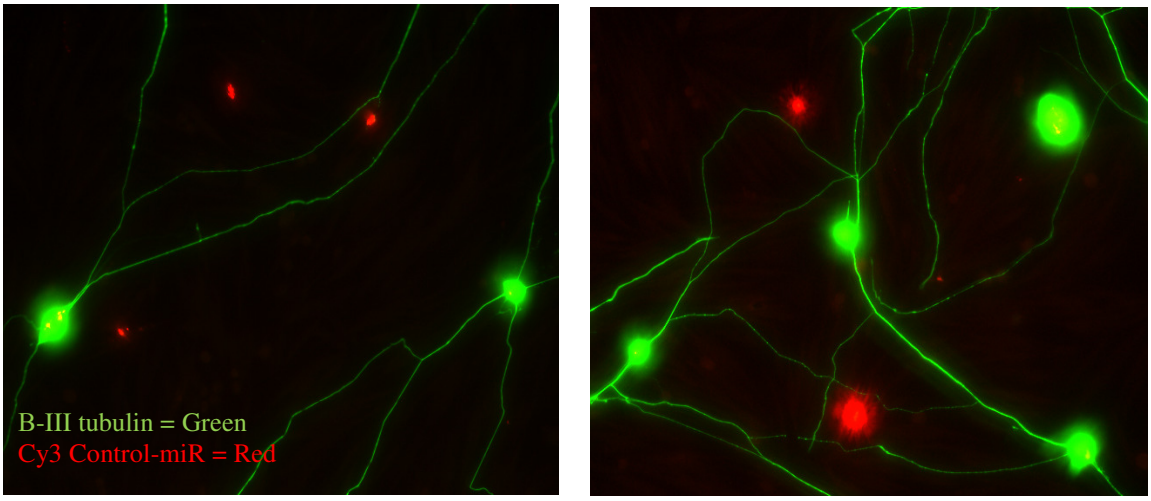


Figure 4.2: DRG Neurons are Transfected with the Cy3-labeled Control-miR. DRG neurons were plated on ploy-L-lysine then Lipofectamine-transfected with 60nM of Control-miR. Neurons were then fixed and stained for the neuronal marker β-III tubulin, and a secondary antibody of AlexaFluor 488. Cy3 was visualized using immunofluorescence microscopy.

DRG neurons (postnatal day 5-7) were dissociated and plated on poly-L-lysine coated 6 well chamber plates and incubated for 12 hours. After incubation the neurons underwent a Lipofeactamine-mediated co-transfection with chemically modified mature miR-151 mimics (60nM) and/or a Cy3-labeled control miR (60/6nM) and were then harvested and plated on CHO or MAG-expressing CHO monolayers and incubated for 15 hours. Figures 4.3 and 4.4 show that miR-151 overexpression results in an approximately 25% increase in neurite length on MAG-expressing CHO cells (relative to DRG neurons transfected with only a Cy3-labeled chemically modified control miR) ($P<0.05$). The miR-151-induced growth of DRG neurons observed on the MAG-expressing CHO cells is equivalent to the growth on control CHO cells not expressing MAG. Whereas the DRG

neurons that were transfected with the control microRNA were inhibited on the MAG-expressing CHO cells but exhibited normal growth on the control CHO cells.

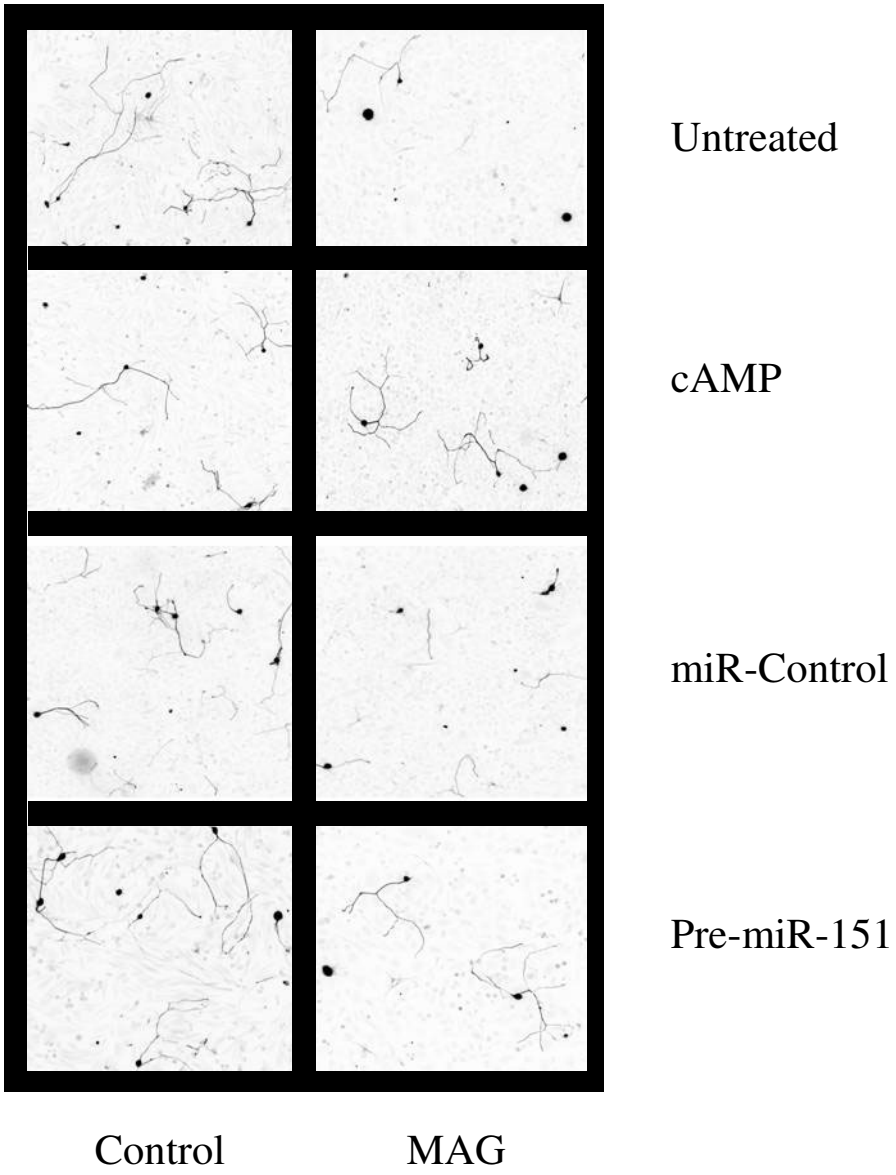


Figure 4.3: Overexpression of miR-151 Overcomes MAG-mediated Axonal Growth Inhibition. DRG neurons were transfected with 60nM Pre-miR-151 or 60nM Control-miR or 1mM db-cAMP and then subjected to a neurite outgrowth assay. Overexpression of Pre-miR-151 led to a significant increase in neurite length on MAG-expressing CHO cell monolayers as compared with a control-miR.

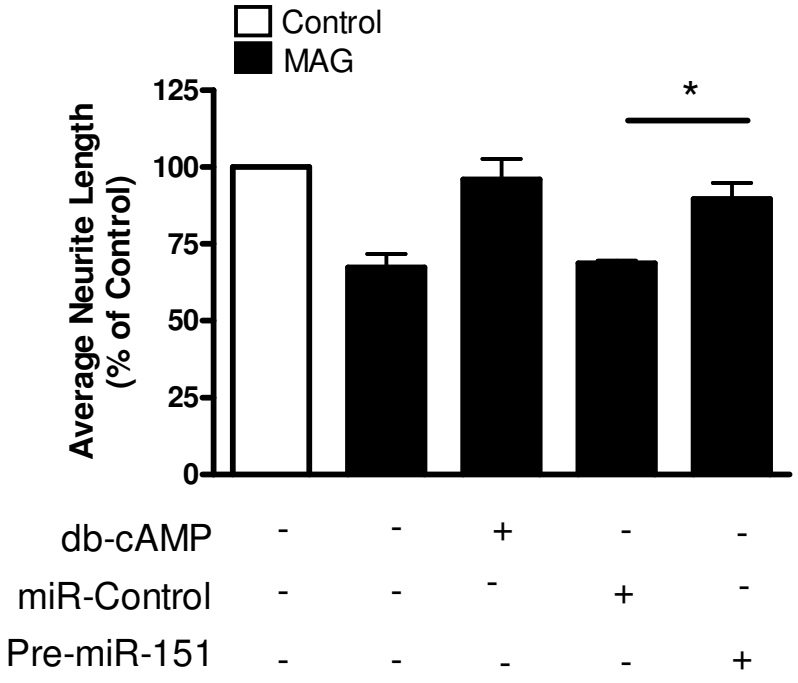


Figure 4.4: miR-151 Overexpression Overcomes MAG Inhibition. DRG neurons were transfected with 60nM Pre-miR-151 or 60nM Control-miR or 1mM db-cAMP and then subjected to a neurite outgrowth assay. Overexpression of Pre-miR-151 led to a significant increase in neurite length on MAG-expressing CHO cell monolayers as compared with a control-miR. Growth of the Pre-miR-151 transfected neurons on MAG-CHO was equivalent to the neurons treated with db-cAMP ($P<0.05$, 1 way ANOVA, Tukey Post test, n=3).

Similar results were obtained when pre-miR-151 transfected neurons were subjected to a neurite outgrowth assay on a purified myelin substrate (Figure 4.5), with miR-151 overexpression resulting in an approximately 25% increase in neurite length on myelin (relative to DRG neurons transfected with a Cy3-labeled control miR ($P<0.05$)). The miR-151-induced growth of DRG neurons observed on the myelin is equivalent to the growth on control PLL. Whereas the DRG neurons that were transfected with the control microRNA had no effect on either inhibition by myelin or on control growth. Thus, overexpression of miR-151 in DRG neurons overcomes inhibition by MAG/myelin.

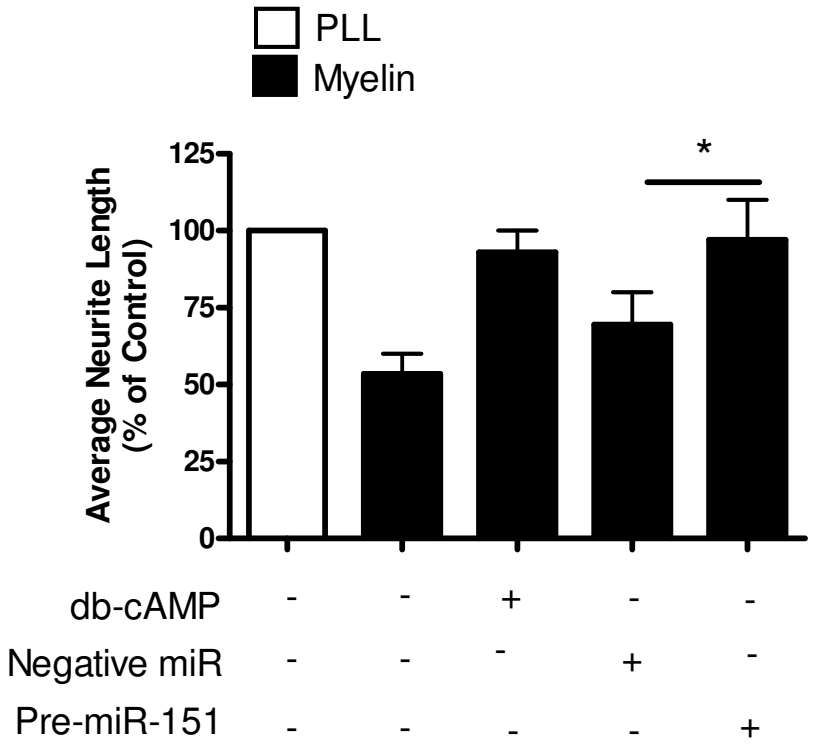


Figure 4.5: miR-151 Overexpression Overcomes Myelin Inhibition. DRG neurons were transfected with 60nM Pre-miR-151 or 60nM Control-miR or 1mM db-cAMP and then subjected to a neurite outgrowth assay. Overexpression of Pre-miR-151 led to a significant increase in neurite length on a purified myelin substrate as compared with a control-miR. Growth of the Pre-miR-151 transfected neurons on myelin was equivalent to the neurons treated with db-cAMP ($P<0.05$, 1 way ANOVA, Tukey Post test, n=3).

To further confirm a functional role for miR-151 in overcoming MAG/myelin-mediated axonal growth inhibition, we conversely knocked-down miR-151 in DRG neurons using a chemically modified single-stranded miR-151 inhibitor (Anti-miR-151). These Anti-microRNAs are similar to the Pre-miR mimics and thus we used the same co-transfection with a Cy3-labeled control miR strategy to ascertain a transfection efficiency of approximately 60%. Again, since the Anti-miR-151 is not labeled, in order to ascertain which neurons were transfected with the Anti-microRNA, we co-transfected the Anti-miR-151 along with the Cy3-labeled control miR at a rate of 10:1 and only counted the neurons that were transfected with the visible Cy3. After transfection with the Anti-miR-

151 and/or Cy3-labeled control miR, the neurons were subsequently subjected to neurite outgrowth analysis (Figure 4.7). As shown in Figure 4.6, treatment of DRG neurons with Anti-miR-151 decreased miR-151 levels in a dose-dependent manner, as determined by qRT-PCR.

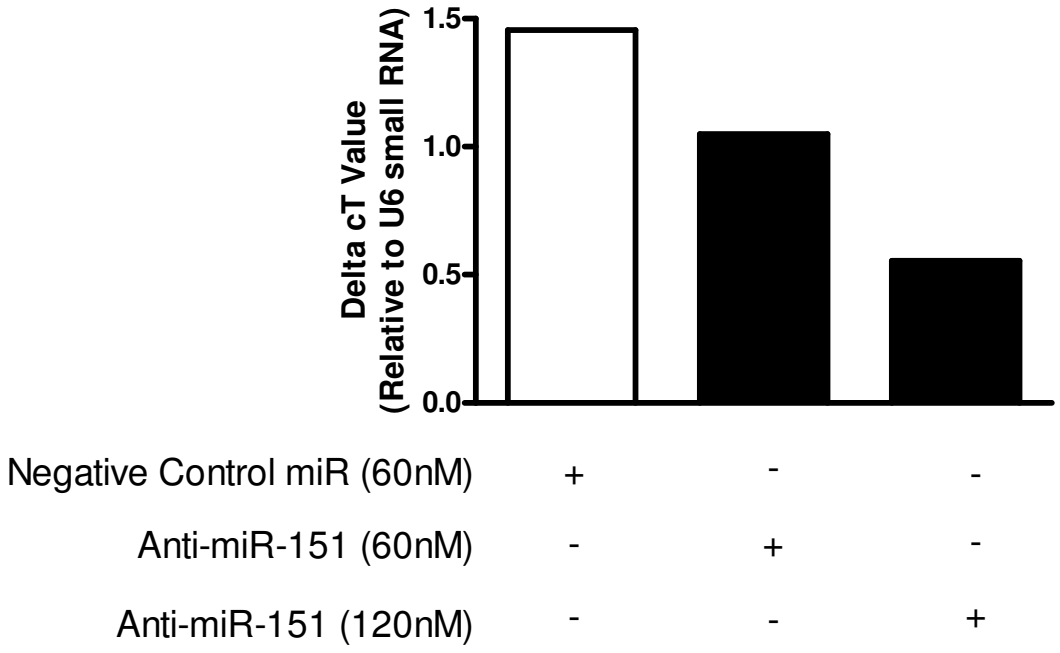


Figure 4.6: Treatment of DRG Neurons with Anti-miR-151 Decreases miR-151. DRG neurons were Lipofectamine-transfected with either Anti-miR-151 or a control-miR and then subjected to qRT-PCR. Treatment with Anti-miR-151 decreased miR-151 levels in a dose-dependent manner.

As shown in Figure 4.7, miR-151 knock-down abrogated the affect of db-cAMP to overcome MAG-mediated neurite outgrowth inhibition by approximately 25% (relative to neurons transfected with a Cy3-labeled control miR ($P<0.05$)). The anti-miR-151-induced abrogation of the db-cAMP effect on growth of DRG neurons observed on the MAG-expressing CHO cells is almost equivalent to the growth on MAG-expressing CHO cells not treated with db-cAMP. Whereas the DRG neurons that were transfected

with the control microRNA had no effect on either inhibition by the MAG-CHO or on control growth.

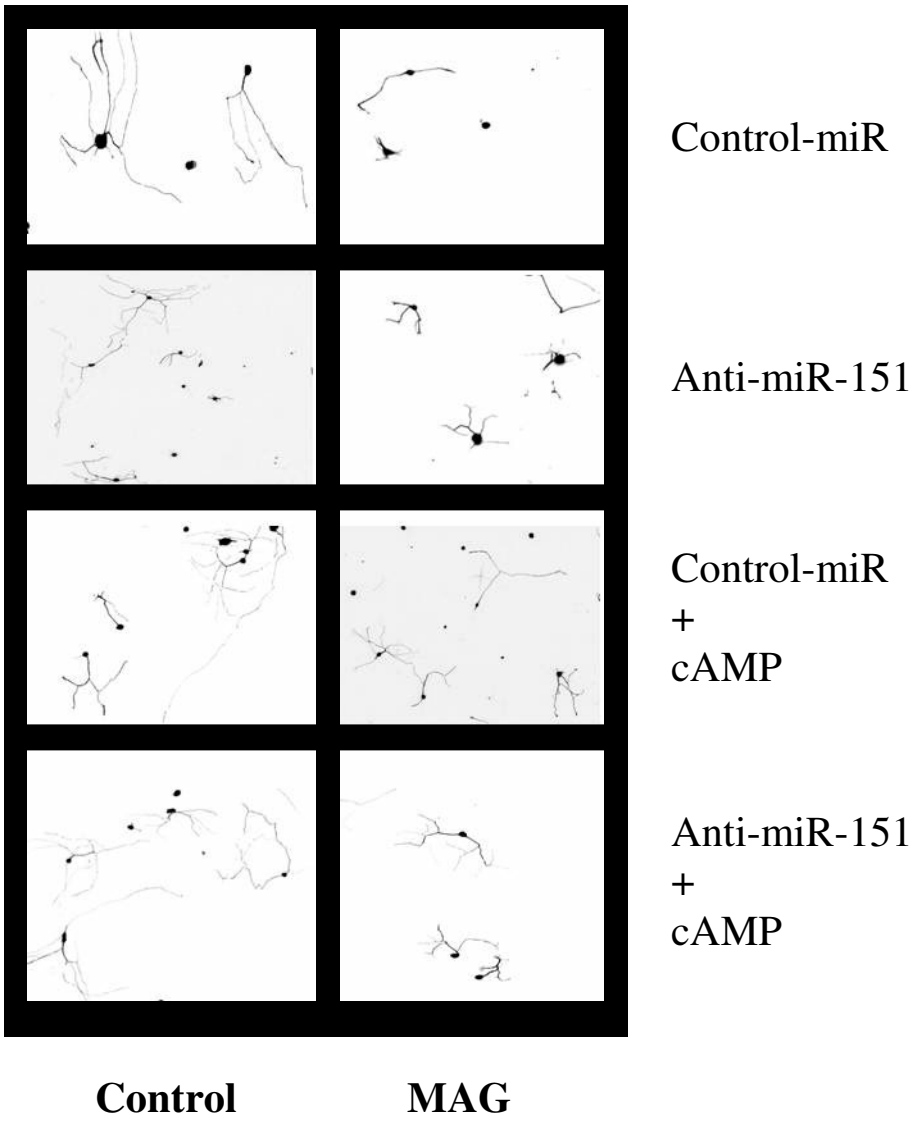


Figure 4.7: Knockdown of miR-151 Abrogates cAMPs Ability to Overcome MAG-mediated Axonal Growth Inhibition. DRG neurons were treated with 1mM db-cAMP and transfected with 60nM Anti-miR-151 or 60nM Control-miR. Treatment with cAMP overcame the MAG-mediated inhibition, with the Control-miR having no effect on the growth on MAG or control. However, treatment with db-cAMP failed to overcome the MAG-mediated inhibition when miR-151 was knocked-down. Thus, Knockdown of miR-151 by expression of Anti-miR-151 impaired the ability of cAMP to overcome MAG-mediated inhibition as compared to the Control-miR. ($p<0.05$, Students t test, two-tailed, n=3).

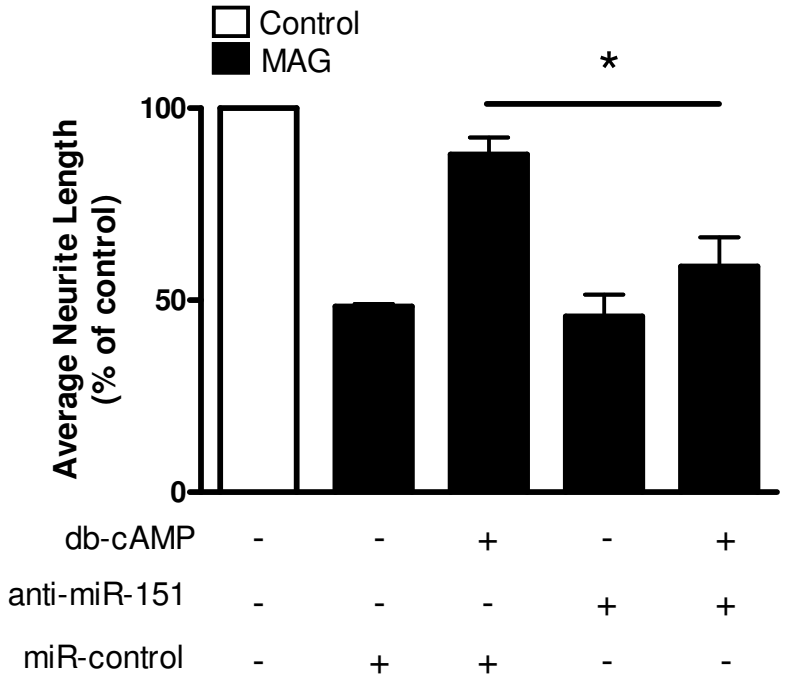


Figure 4.8: Knockdown of miR-151 Abrogates cAMPs Ability to Overcome MAG-mediated Axonal Growth Inhibition. DRG neurons were treated with 1mM db-cAMP and transfected with 60nM Anti-miR-151 or 60nM Control-miR. Treatment with cAMP overcame the MAG-mediated inhibition, with the Control-miR having no effect on the growth on MAG or control. However, treatment with db-cAMP failed to overcome the MAG-mediated inhibition when miR-151 was knocked-down. Thus, knockdown of miR-151 by expression of Anti-miR-151 impaired the ability of cAMP to overcome MAG-mediated inhibition as compared to the Control-miR. ($p<0.05$, Students t test, two-tailed, n=3).

Similar results were obtained when miR-151 was knocked-down in db-cAMP treated neurons which were subsequently subjected to a neurite outgrowth assay on a purified myelin substrate (Figure 4.8) with miR-151 knockdown resulting in an approximately 15% decrease in neurite length on myelin (relative to cAMP treated DRG neurons transfected with a control miR). Treatment with cAMP overcame the myelin-mediated inhibition, with the Control-miR having no effect on the growth on myelin or control. However, treatment with db-cAMP failed to overcome the myelin-mediated inhibition

when miR-151 was knocked-down ($P<0.05$). Together, these gain-of-function and loss-of-function analyses indicate that miR-151 has an important role in the db-cAMP effect of overcoming MAG/myelin-mediated neurite outgrowth inhibition.

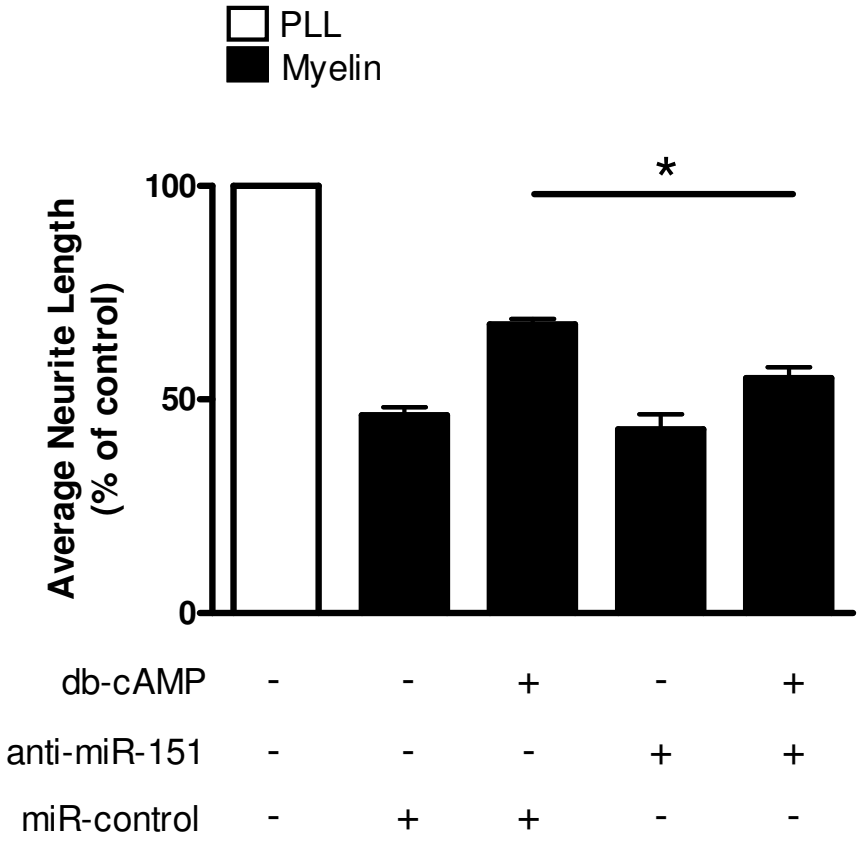


Figure 4.9: Knockdown of miR-151 Abrogates cAMPs Ability to Overcome Myelin Inhibition. Treatment with cAMP overcame the myelin-mediated inhibition, with the Control-miR having no effect on the growth on MAG or control. However, treatment with db-cAMP failed to overcome the myelin-mediated inhibition when miR-151 was knocked-down. ($P<0.05$, 1 way ANOVA, Tukey Post test, n=3).

4.3 Discussion

In this chapter we present data showing that overexpression of the cAMP-responsive miR-151 overcomes MAG/myelin-mediated axonal growth inhibition and conversely, knockdown of miR-151 abrogates cAMPs ability to overcome inhibition. Previously, our lab has shown that activating CREB is sufficient to overcome MAG/myelin-mediated inhibition (Gao et al. 2004). In the previous chapter we proposed a possible mechanism in which the cAMP-induced activation of CREB leads to increased miR-151 synthesis. If activation of CREB is sufficient to overcome inhibition through the upregulation of genes that are involved in overcoming MAG/myelin-inhibition, is it not possible that activation of CREB could lead to overcoming inhibition through the upregulation of a microRNA that represses translation of proteins that are growth inhibiting? While the idea that alteration of the levels of a small piece of RNA may be sufficient to overcome MAG/myelin-mediated inhibition may seem surprising, there have been plenty of instances in which the alteration of microRNA levels changes the intrinsic capabilities of a neuron. Specifically, there have been many published instances in which overexpression of a microRNA leads to the intrinsic alteration of neurons. For example, in 2005 the Impey group showed that overexpression of miR-132 led to an increase in total neurite length and branching on a permissive substrate (Vo et al. 2005); in 2006 the Greenberg group showed that overexpression of miR-134 led to an increase in dendritic spine width (Schratt et al. 2006); in 2008 the Kaplan group showed that overexpression of miR-338 led to a decrease in mitochondrial activity (which is crucial to the function of the distal axon) (Aschrafi et al. 2008); in 2009 the Schratt group showed that overexpression of miR-138 led to a decrease in dendritic spine volume (Siegel et al.

2009); in 2009 the Doetsch group showed that overexpression of miR-124 both *in vitro* and *in vivo* was sufficient to promote neuronal differentiation (Cheng et al. 2009). In all of the above mentioned studies it was shown that the microRNAs impact on the neuron was directly related to the target mRNA of the microRNA. MicroRNAs work by translationally repressing target proteins, and one microRNA can putatively bind to multiple targets. Thus, it is possible that overexpression of miR-151 overcomes MAG/myelin-mediated inhibition by targeting multiple proteins in a synergistic approach and this idea will be discussed in more detail in a chapter five.

In this chapter we showed that knockdown of miR-151 is sufficient to abrogate the cAMP effect on overcoming MAG/myelin-mediated axonal growth inhibition. How does knockdown of miR-151 abrogate the cAMP effect? It has been shown that cAMP overcomes MAG/myelin inhibition by activation of four parallel signaling pathways all of which converge at the activation of CREB: PKA, CamK, PI3K, and ERK (Liu and Snider 2001; Cai et al. 2002; Gao et al. 2004; Spencer et al. 2008). Furthermore it has been shown that if the signaling of anyone of these pathways is disrupted, then the ability of cAMP to overcome MAG/myelin-mediated axonal growth inhibition is abrogated (Cai et al. 1999; Gao et al. 2003; Gao et al. 2004; Spencer et al. 2008). One hypothesis for why knockdown of miR-151 abrogates the cAMP effect may lie in the mRNA targets. miR-151 may be regulating the expression of proteins that are necessary for the activation of CREB. Previous work from our lab has suggested that CREB phosphorylation must reach a threshold in order for it to overcome inhibition (Gao et al.

2004). If miR-151 stops the expression of a protein that abates the necessary threshold, then this might be how knockdown of miR-151 is sufficient to abrogate the cAMP effect.

Chapter V: Identifying and Validating Neuronatin as a miR-151 target

5.1 Introduction

MicroRNAs are small pieces of RNA that bind to target mRNAs and repress their translation. Computational analysis predicts that each microRNA has hundreds of putative targets. In the previous chapters we have identified a cAMP-responsive microRNA that is overexpression results in overcoming MAG/myelin-mediated axonal growth inhibition. In this chapter we will define a mechanism for how miR-151 overcomes MAG/myelin-mediated inhibition.

There are many miR/mRNA prediction algorithms available online and they all work in a similar manner to predict microRNA targets. Briefly, they scan the predicted microRNA against the selected genome and rate possible targets based upon predicted thermodynamic stability, UTR conservation, and number of predicted miRNA sites (Figure 5.1). Once the target predictions have been made the user can navigate through the list based on values such as energy, p-value, conservation and miRNA sites.

After performing a miRNA target search and identifying putative targets the next step is to validate these targets. There have been a few different approaches to target validation but they all center around a similar assay. A reporter plasmid has the 3'UTR of interest, or a 3'UTR of interest that has been mutated at the predicted microRNA binding site cloned into the multiple cloning site. These plasmids are then expressed in a cell line and the microRNA of interest is co-expressed. If the mRNA is a target of the microRNA then

upon microRNA expression there should be a detectable decrease in reporter expression. If, however, the microRNA cannot bind due to a mutation in the binding site, then addition of the microRNA should have no effect on the reporter expression. In this chapter we will attempt to validate a miR-151 target in both the 293-T cell line and in primary neuronal cultures.

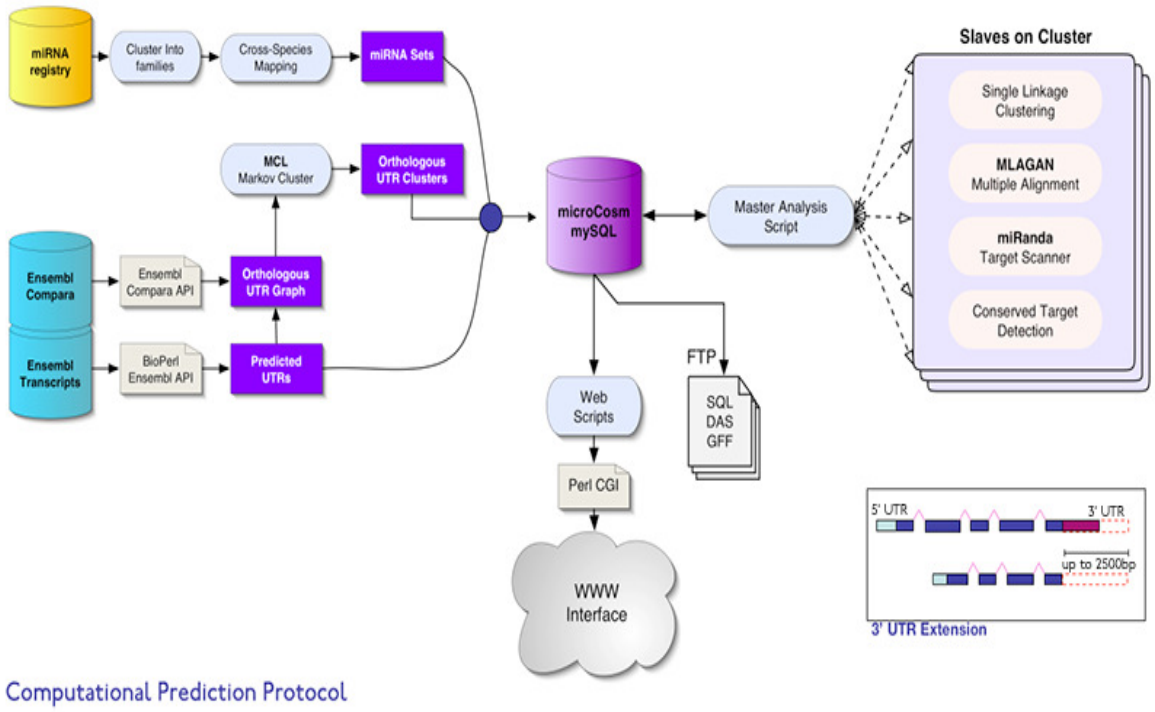


Figure 5.1: Computation Prediction Protocol of miRANDA. Briefly, a MicroRNA is entered into the MicroRNA Registry. That MicroRNA is then clustered into a family and put into a set. The MicroRNA is then scanned against the Ensembl genome of choice and based on certain alignment criteria computation target prediction is made.

5.2 Results

As previously stated, miRNAs have numerous putative target mRNA's. To investigate the mechanism by which overexpression of miR-151 overcomes MAG-mediated inhibition; we sought to identify miR-151 target mRNAs using the target prediction algorithm miRANDA (http://microrna.sanger.ac.uk) (Figure 5.1).

Among the predicted targets the one with the highest predicted thermodynamic duplex stability was Neuronatin (Nnat), a transmembrane proteolipid protein with relatively unknown neuronal function. Neuronatin was first discovered via molecular cloning in 1994 by Joseph, Dou and Tsang. They found that Nnat mRNA was highly expressed in the brain and observed selected expression of this mRNA in late fetal and early postnatal brain development, followed by a decrease in adulthood and senescence (Joseph et al. 1994). In 1995, the same group further characterized Nnat by showing that there are two alternatively spliced forms of the Nnat mRNA an α and a β, both with the same open reading frame. Both forms are identical except the α form encodes a protein of 81aa, while the β encodes a protein of 51aa. RT-PCR analysis showed that the mRNA for the α form first appeared at E7, while the β did not appear until E11 (Joseph et al. 1995). In 1996, Dou and Joseph determined the structure and organization of the human Nnat gene to span 3973 bases and contain three exons and two introns, with the α form including all three exons, and the β form missing the middle exon (Dou and Joseph 1996). Also in 1996, Dou and Joseph cloned the human Nnat gene and localized it to chromosome-20q11.2-12. They showed that the deduced protein has two distinct domains, a hydrophobic N terminal and a basic C terminal rich in arginine residues. Both the amino acid sequence and the secondary structure (as shown in Figure 5.2) of this amphipathic

polypeptide exhibited homology to PMP1 and phosopholamban, members of the proteolipid class of proteins which function as regulatory subunits of membrane channels, though this role has not been described for Neuronatin.

Joseph et al. went on to study Nnat expression in PC12 cells and was able to show that Nnat mRNA was abundant in PC12 cells, and that treatment with NGF (which results in neuronal differentiation) was associated with a decrease in Nnat mRNA, possibly providing a basis for a role for Nnat in neuronal growth and differentiation (Joseph et al. 1996). In 2005, Chu and Tsai showed that siRNA-mediated knockdown of Nnat in NIT cells (a pancreatic-β cell line) resulted in a reduction of the ability of the cells to respond to glucose (Chu and Tsai 2005). In 2006, Park and Hong showed that treatment of DRG neurons with artemin (a member of the glial derived neurotrophic factor family) led to a decrease in Nnat protein levels and with that an increase in the total neurite length and branching of DRG neurons on a permissive substrate was observed (Park and Hong 2006). In 2008, Joe et al. overexpressed both Nnat isoforms in pancreatic β-cells and found that they were primarily localized in the endoplasmic reticulum, and that their expressions increased insulin secretion by increasing intracellular calcium levels (Joe et al. 2008). Also in 2008, Mzhavia et al. showed that Nnat was expressed in mouse aorta endothelial cells. When they overexpressed Nnat in human aortic endothelial cells (HAEC's) using an Adenovirus-Nnat they observed an increase in expression of a panel of nuclear factor-κB (NF- κB)-regulated genes. They further showed that Nnat activation of NF- κB gene expression was occurring through stimulation of p38, Jun NH2-terminal kinase, extracellular signal-related kinase, and AKT kinase phosphorylation and that

PI3K and p38 inhibitors could prevent the Nnat-mediated NF-κB induced gene expression (Mzhavia et al. 2008).

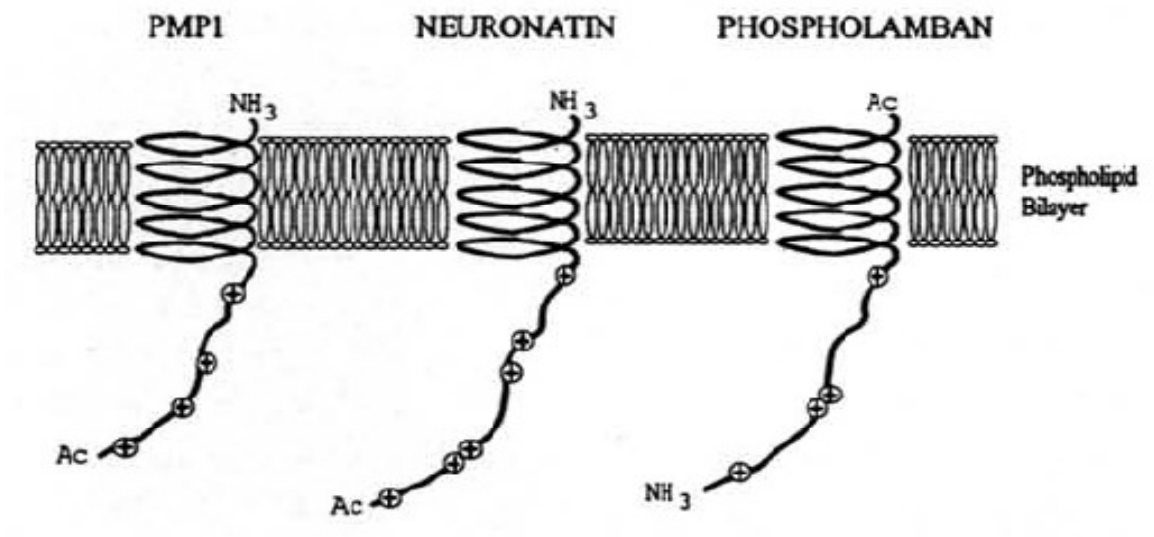


Figure 5.2: Predicted Structure of the Neuronatin Protein. Adapted from (Dou and Joseph 1996)

The first thing we wanted to investigate was if Nnat was expressed in postnatal neurons at a detectable level. To address this we performed Western blot analysis on different neuronal and glial cell populations using an antibody that detects both forms of Neuronatin. As shown in Figure 5.3, Neuronatin protein is expressed in P5 DRG and cerebellar granular neurons (CGN), in the glial cell populations of oligodendrocytes and astrocytes but was not present in Schwann cells. All of our previously performed work had been performed on DRG neurons. Since DRGs are a mixed population of cells containing both glial and neuronal cells, that Neuronatin is not expressed in Schwann

cells is very promising because it indicates that any Neuronatin results we observe are occurring in the neurons .

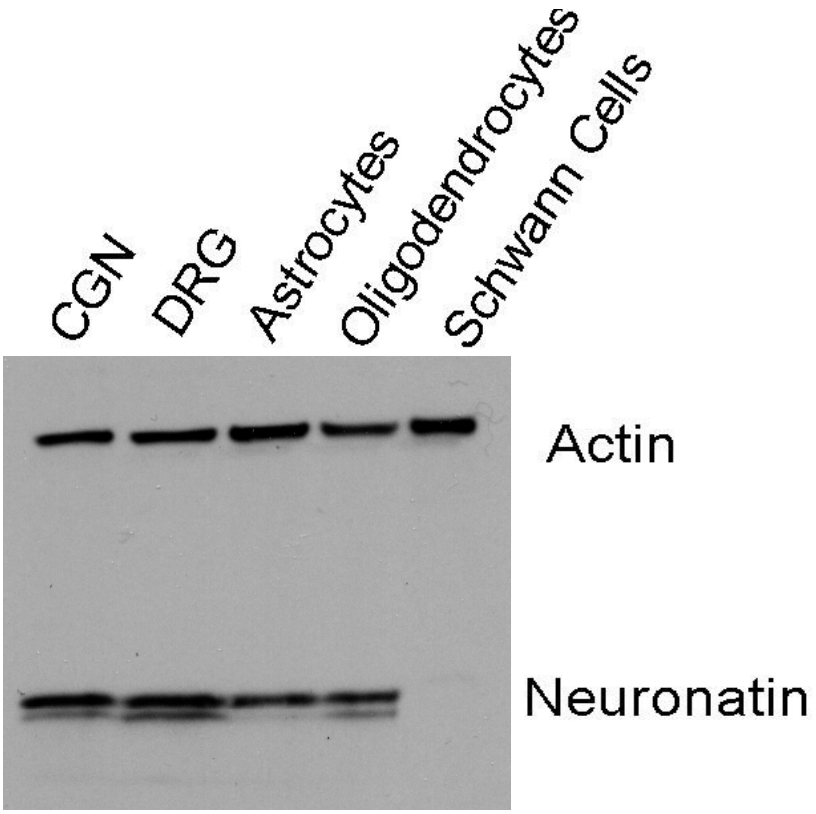


Figure 5.3: Neuronatin is Not Expressed in Schwann cells. Cerebellar granular neurons, dorsal root ganglion neurons, astrocytes, oligodendrocytes and Schwann cells were pleated on poly-L-lysine for 24 hours, then lysed and subjected to Western blotting (20µg) and stained for Neuronatin and Actin. Neuronatin was present in all cell types except Schwann cells.

To validate that Nnat is a target of miR-151 we performed a luciferase reporter assay. A luciferase reporter gene was fused to either a wildtype or mutated Nnat 3'UTR (Figure 5.4), the plasmids (Figure 5.5) were then co-transfected along with Pre-miR-151 mimic into 293-T cells and the luciferase activity was measured.

miR-151: 3' UGAUCUGACACUCGAGGAGCU 5'

Nnat 3'UTR WT: 5' AGGUGCUCCUGUGCUUCUCGA 3'

Nnat 3'UTR MUT: 5' GCUAGAUUGUAAGCUCCUGGA 3'

Figure 5.4: miR-151/Neuronatin Sequence Alignment. Sequence of miR-151, its predicted Nnat 3'UTR binding site and the mutated Nnat 3'UTR miR binding site. Mutations are highlighted in grey.

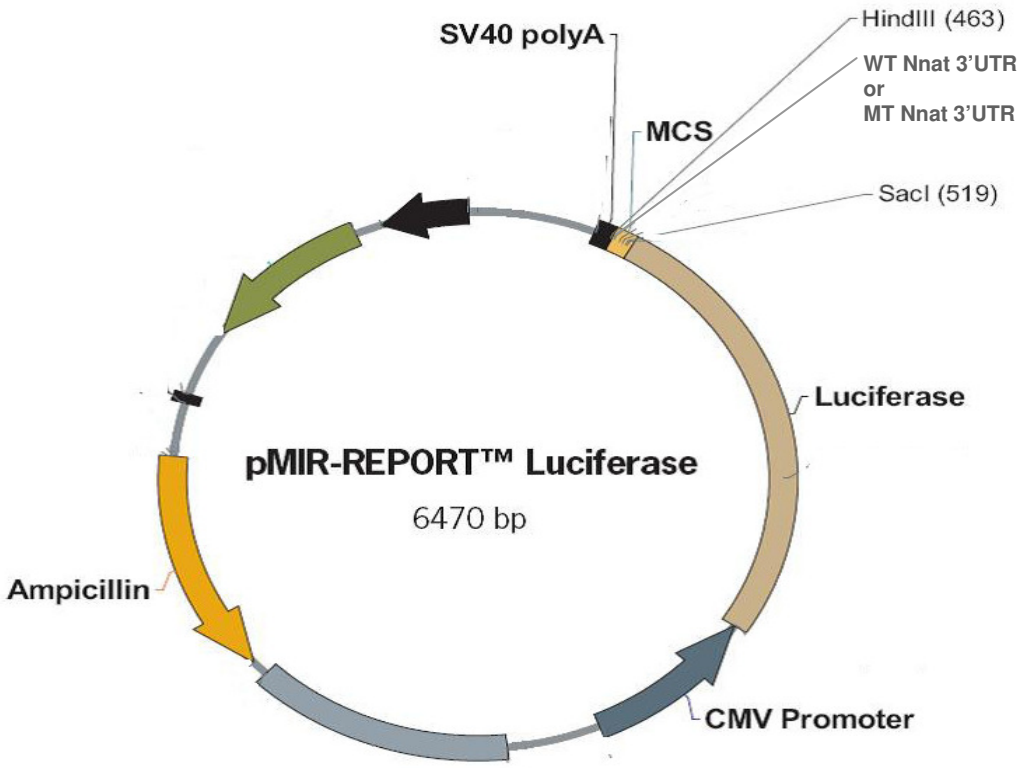


Figure 5.5: pMIR-REPORT Luciferase with cloned WT or MT Nnat 3'UTR.

As indicated in Figure 5.6, Lipofectamine-mediated Pre-miR-151 overexpression (60nM) decreased the luciferase expression of the wildtype by approximately 25% but had no effect on the Luciferase expression in the mutated Nnat control construct, where the microRNA could not bind and repress the translation ($P<0.05$), thus validating that Nnat is a miR-151 target.

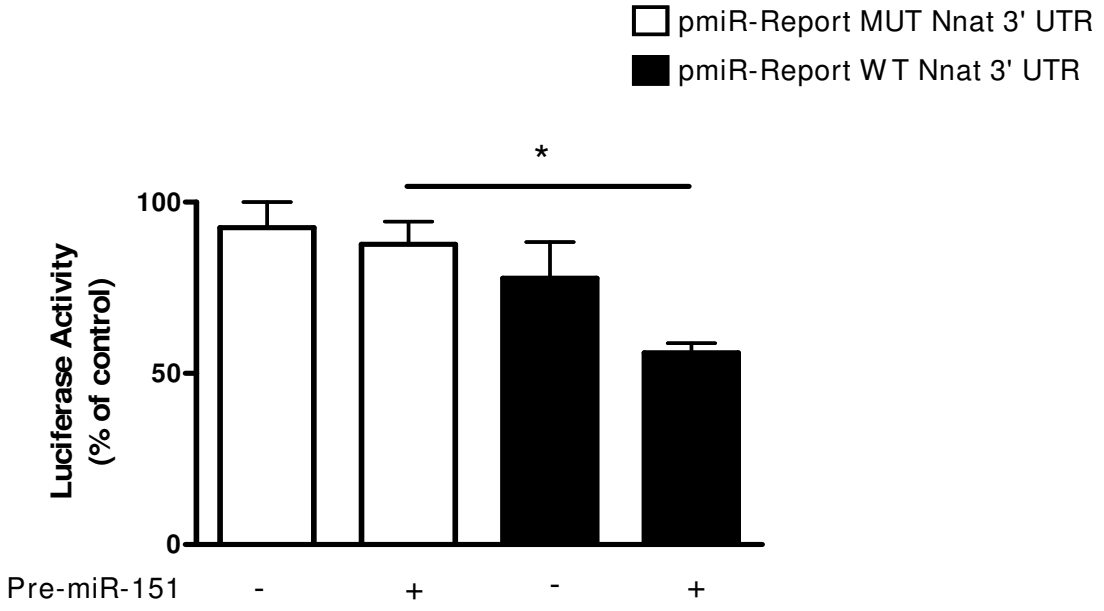


Figure 5.6: Neuronatin is a miR-151 Target. Neuronatin 3'UTR (wt or mutant) luciferase constructs were transfected into 293-T cells and Pre-miR-151 (60nM) was expressed. Normalized sensor luciferase activity is shown as a percentage of the control ($p<0.05$, Students one-tailed t test, n=3). Addition of Pre-miR-151 decreased the wildtype Luciferase activity by ~25% but had no effect on the Luciferase expression of the control mutated Nnat 3'UTR construct.

We next wanted to investigate if Nnat is a target of miR-151 in neurons. To do this we analyzed the expression of Nnat protein after miR-151 overexpression in DRG neurons. The hypothesis was that if miR-151 is binding to Nnat mRNA and decreasing its expression, then overexpression of miR-151 should decrease the endogenous levels of

Neuronatin. As indicated in Figure 5.7, miR-151 markedly reduced Nnat protein levels in DRG neurons (relative to the Control miR treated (P<0.05)).

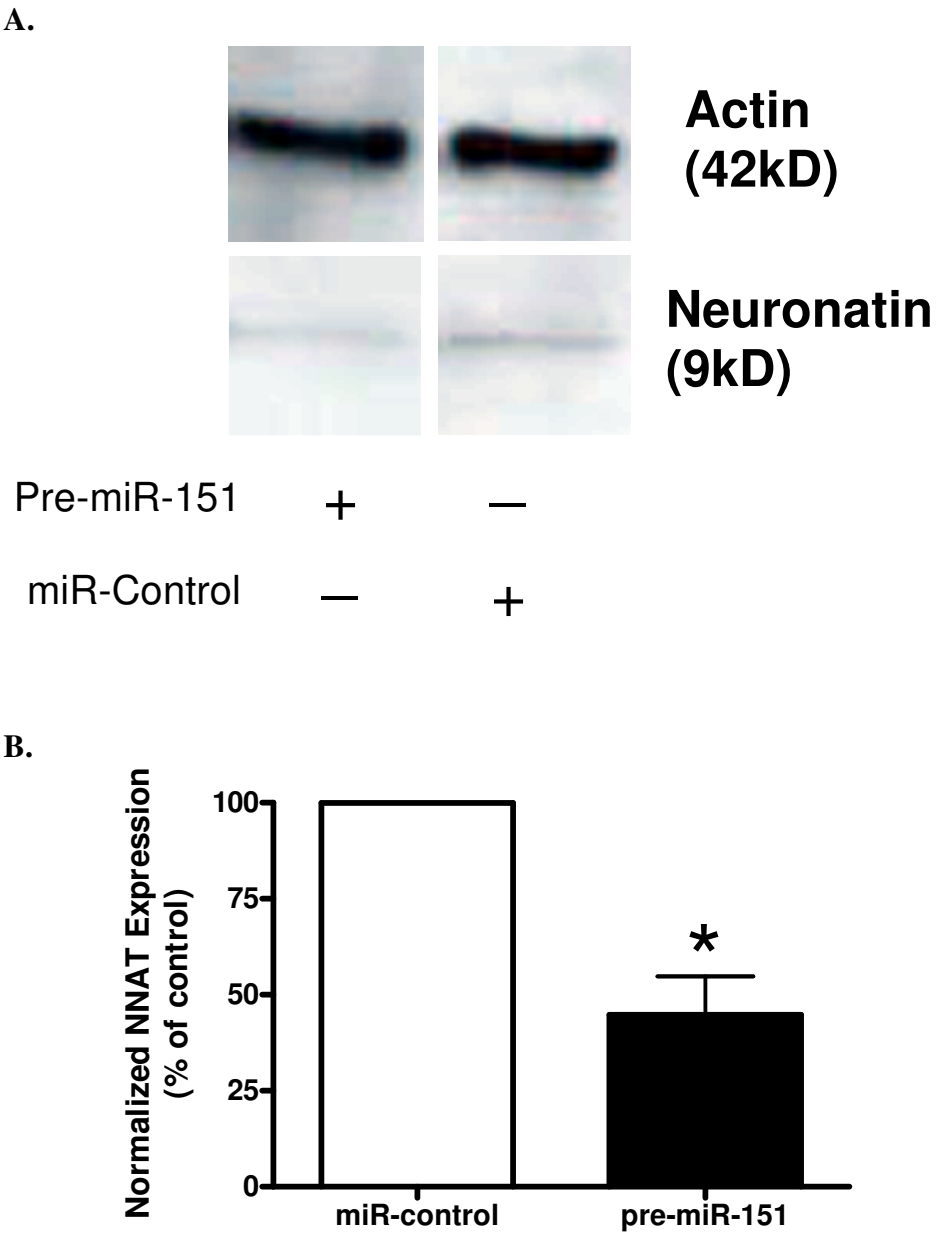
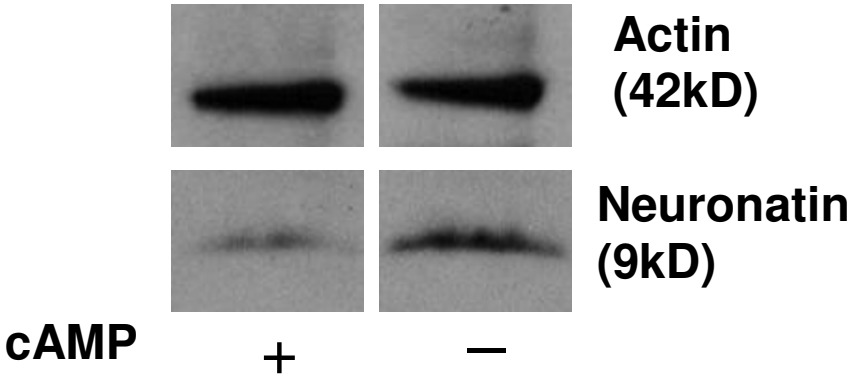


Figure 5.7: miR-151 Decreases Neuronatin Protein Levels in DRG neurons. A) DRG neurons were plated on poly-L-lysine, treated with either Pre-miR-151 (60nM) or miR-control (60nM) for 24 hours, neurons were then lysed and subjected to Western blotting (10µg). Treatment withPre-miR-151 reduced Nnat protein levels by ~50% as compared to Nnat protein levels in neurons treated with the miR-control. B) Quantification of Pre-miR-151 induced Nnat knockdown as a % of Nnat levels in neurons treated with miR-control (p<0.05, Students two tailed t test, n=3).

Thinking along the same line, we next wanted to investigate it cAMP treatment could decrease Neuronatin protein levels. In chapter one we showed that treatment with cAMP induces miR-151 expression, here in chapter 5 we have shown that miR-151 is binding to Neuronatin and decreasing its expression. Thus, if cAMP is increasing miR-151 and miR-151 is reducing Neuronatin expression then treatment of DRG neurons with cAMP should lead to a decrease in Neuronatin protein levels. As indicated in Figure 5.8 we found that when DRG neurons are treated with db-cAMP, Nnat protein levels were reduced by approximately 25% (relative to the untreated control DRG neurons) ($P<0.05$).

A.



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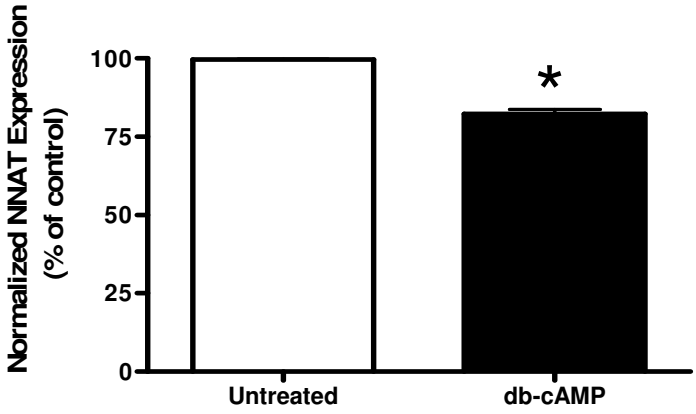

Figure 5.8: Treatment with db-cAMP Decreases Nnat Protein Levels. A) DRG neurons were plated on poly-L-lysine, treated +/- 1mM db-cAMP for 24 hours, neurons were then lysed and subjected to Western blotting (10µg). Treatment with db-cAMP reduced Nnat protein levels by ~25% as compared to Nnat protein levels in untreated DRG neurons. B) Quantification of db-cAMP induced Nnat knockdown as a percentage of Nnat levels in untreated DRG neurons ($p<0.05$, Students two tailed t test, n=3).

To further elucidate that the observed cAMP-induced Neuronatin protein decrease was occurring at the post-transcriptional level we determined if cAMP treatment had any effect on the Neuronatin mRNA levels. We treated DRG neurons with db-cAMP and performed a qRT-PCR assaying for Neuronatin mRNA. No significant change was seen in Nnat mRNA levels in DRG neurons after db-cAMP treatment (Figure 5.9), further supporting the hypothesis of miR-151 post transcriptional Nnat regulation.

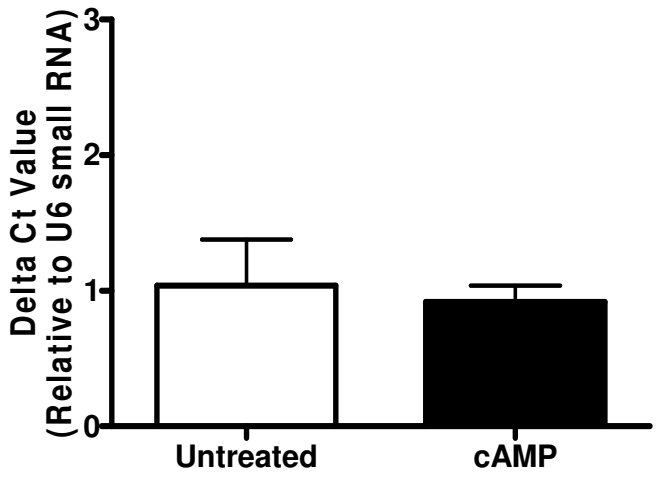

Figure 5.9: Neuronatin mRNA is Not cAMP-Responsive. Neuronatin mRNA levels were unaffected in DRG neurons treated with db-cAMP for 24 hours as compared to the control untreated (n=3).

5.3 Discussion

In this chapter we show that Neuronatin is a valid miR-151 target and that overexpression of miR-151 or treatment with db-cAMP leads to a decrease in Neuronatin protein levels. Previously our lab has shown that increasing intracellular levels of cAMP overcomes MAG/myelin-mediated axonal growth inhibition (Cai et al. 1999; Qiu et al. 2002). Until recently, the approach taken to understand how cAMP overcomes this inhibition has been to identify mRNA/proteins that are increased with cAMP treatment. The logic being that if increased levels of cAMP overcome inhibition via intrinsically boosting the regenerative capability of the neuron then cAMP must be increasing production of proteins that are growth-promoting. The work described here is the first time that we have looked for proteins that are decreased upon treatment with cAMP. Since microRNA regulation is a relatively new field, there have been no studies aimed at identifying microRNAs that are involved in intrinsically boosting the neurons regenerative capabilities in a non-permissive growth environment. Having in previous chapters already focused on one cAMP-responsive microRNA, miR-151, in this chapter we sought to identify a miR-151 target that may be involved in overcoming MAG/myelin axonal growth inhibition. miR-151, like all other microRNAs, has hundreds of putative targets. Although we only validate Neuronatin as a target it is highly likely that miR-151 is regulating other proteins involved in neurite outgrowth. Neuronatin had the lowest predicted energy score, thus was predicted to require the least amount of energy to form the duplex, making it a very attractive candidate for validation. Other predicted mRNA targets included in the top 25 on the list are: castor homolog 1 (castor is a zinc finger transcription factor that controls cell fate within neuroblast cell lineages in Drosophila)

(Liu et al. 2006); Pou4f2 (a transcription factor that controls retinal ganglion cell differentiation) (Mao et al. 2008); 1-acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransferase, zeta) (AGPAT6) (a lysophosphatidic acid acyltransferase that catalyzes the conversion of lysophosphatidic acid to phosphatidic acid (Chen et al. 2008); protein kinase c-theta (PRKCQ) (a member of the serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol and is required for the activation of the transcription factor NF-κB) (Kane et al. 2002); and contactin-4 presursor (Cntn4) (a GPI-anchored neuronal membrane protein that functions as a cell adhesion molecule). It is possible that miR-151 is having such an effect on overcoming MAG/myelin-mediated axonal growth inhibition by translationally repressing a group of proteins that combine for a synergistic approach. If this is indeed happening then these other proteins listed here may also be having a role in axonal growth. Cntn4 is an extremely interesting target due to its role in cell adhesion and inflammatory response. Cntn4 has been shown to regulate tumor necrosis factor receptor 1 (TNFR1) exosome-like vesicle release by acting as a guanine nucleotide exchange protein that activates class 1 ADP-ribosylation factors, and as an A kinase anchoring protein for RIIβ (a subunit of PKA which modulates the constitutive and cAMP-induced release of TNFR1 exosome-like vesicles) (Islam et al. 2008). TNFR1, also called the death receptor, is considered the major TNFR1 receptor; it is released into the extracellular space where it binds TNF and regulates its activity. TNF is a cytokine that induces inflammation, apoptosis and innate-immune responses through the NF-κB, and JNK pathways (Vallabhapurapu and Karin 2009). If miR-151 is regulating the translation of Cntn4, then treatment with cAMP would decrease the expression of Cntn4,

which would lead to less TNFR1 release and thus less expression of the NF- κB regulated genes which have a negative effect on neurite outgrowth (de Freitas et al. 2002; Furuno and Nakanishi 2006).

One result of interest in this chapter was the reduction of Nnat protein levels induced by both treatment with Pre-miR-151 and db-cAMP. Overexpression of Pre-miR-151 led to an approximately 50% reduction, while we only observed a reduction of 25% upon treatment with db-cAMP. In both experiments the lysate was taken 24 hours after treatment and while the 25% reduction seen in the db-cAMP treated is significant it is important to note the discrepancy. The difference in the knockdown is most likely due to the time that is needed for the microRNA biosynthesis. Pre-miR-151 is a mature miR-151 mimic. When added to a cell it is thought to be immediately incorporated into the RISC where it finds and represses its target mRNA. In contrast, when adding db-cAMP to a neuron first the microRNA has to be induced by CREB, then processed in the nucleus, then exported into the cytoplasm, then processed in the cytoplasm, then incorporated into the RISC. Thus, the processing that must occur in order for db-cAMP to effect Nnat translation most likely accounts for the difference in observed knockdown.

As previously stated our lab has shown that in rat DRG neurons, MAG/myelin promotes neurite outgrowth until approximately post-natal day 5 (P5) (Cai et al. 1999). After P5 there is a switch at which MAG/myelin begins to inhibit neurite outgrowth (Cai et al. 1999) and while the molecular reasons behind the switch are not completely understood, it has been observed that this switch is directly related to the amount of endogenous

cAMP (Cai et al. 1999). In adipocytes, increases in Nnat protein levels have been shown to enhance CREB activation through changes in intracellular calcium levels (Suh et al. 2005); in neurons it has been shown that Nnat mRNA levels are highly expressed embryonically and taper off to a baseline level with age (Joseph et al. 1994) and in PC12 cells it was shown that treatment with NGF led to a decrease in Nnat mRNA levels (Joseph et al. 1996). Could miR-151 be sequestering Nnat mRNA in P-bodies or RNP's waiting for the extracellular stimuli to relieve the repression and thus alter intracellular calcium levels to modulating growth cone guidance? While it has been shown in chapter one that in whole DRG neurons miR-151 is upregulated with cAMP treatment, unpublished work from our lab has indicated that in neurites mature miR-151 is down-regulated with cAMP treatment (Cain & Filbin, unpublished). This idea raises some interesting questions. Is mature miR-151 down-regulated in neurites after treatment with cAMP because it is being degraded? Or is it down-regulated because it is already bound to a target mRNA and sequestered in a P-body or RNP and thus undetectable in the neurite? If the later proves true then miR-151 might be locally repressing translation of Nnat in order to control dynamic local calcium regulation and this idea will be discussed in more detail in the following chapter.

Chapter VI: Determining the Role of Neuronatin in MAG/Myelin-Mediated Neurite Outgrowth Inhibition

6.1 Introduction

After injury the axons of the adult mammalian central nervous system fail to regenerate. This failure has been attributed to myelin-based growth inhibitory proteins (McKerracher et al. 1994; Mukhopadhyay et al. 1994; GrandPre et al. 2000; Wang et al. 2002). One way that our lab has shown to overcome the myelin-based growth inhibitors is through elevation of the ubiquitous second messenger cAMP (Cai et al. 1999). In the previous chapters we have shown that treatment of DRG neurons with db-cAMP leads to the increased expression of microRNA-151 possibly through CREB-induced transcription. We have also shown that overexpression of miR-151 in DRG neurons is sufficient to overcome MAG/myelin-based axonal growth inhibition and conversely, knockdown of miR-151 abrogates db-cAMPs ability to overcome the MAG/myelin-based inhibition. We have identified and validated Neuronatin as a target of miR-151 and showed that both treatment with db-cAMP and overexpression of miR-151 led to a significant reduction in Nnat protein levels. In this chapter we will determine if knockdown of the miR-151 target, Neuronatin, is sufficient to overcome MAG/myelin-based axonal growth inhibition.

6.2 Results

To determine a role for the db-cAMP induced decrease in Nnat in overcoming MAG/myelin-mediated neurite outgrowth inhibition we evaluated the effect of Nnat knockdown in DRG neurons subjected to a neurite outgrowth assay. To do this we used a

commercially available siRNA that is itself a cocktail of three different siRNA all targeted to the 3'UTR of Neuronatin. To determine the appropriate concentration of siRNA needed in order to achieve significant knockdown we Lipofecatmine-transfected the siRNA cocktail or a scrambled control siRNA, that has no known target or cellular effects, into DRG neurons at two different concentrations and assayed for Nnat knockdown. As shown in Figure 6.1, treatment of DRG neurons with 75nM of Nnat siRNA led to an approximately 40% reduction in Nnat protein, as compared to the Nnat protein levels in DRG neurons that were treated with 75nM of a control scrambled siRNA. However, as also indicated in Fig. 6.1 there was no observed significant knockdown of Nnat when the DRG neurons were treated with 150nM of Nnat siRNA. This is most likely due to death of the neurons that were successfully transfected with the 150nM Nnat siRNA because, overall in the neurons treated with 150nM siRNA we saw greater cell death and less total protein lysate was recovered. Thus, all siRNA knockdown experiments were carried out using a final concentration of 75nM siRNA.

To determine if Neuronatin knockdown is sufficient to overcome MAG/myelin-based axonal growth inhibition we Lipofecatmine-cotransfected 75nM of Nnat siRNA and a Cy3-labeled control-miR at a ratio of 10:1 (as described previously, in order to visualize which cells had been transfected) into DRG neurons. After 24 hours, the neurons were harvested and subjected to a neurite outgrowth assay. As shown in Figures 6.2 and 6.3 siRNA-mediated knockdown of Nnat led to an approximately 40% increase in increase in neurite length on MAG-expressing CHO cells, relative to DRG neurons transfected with

only a scrambled siRNA control. Transfection of the neurons with the control siRNA had no effect on either inhibition by MAG or control growth ($P<0.05$).

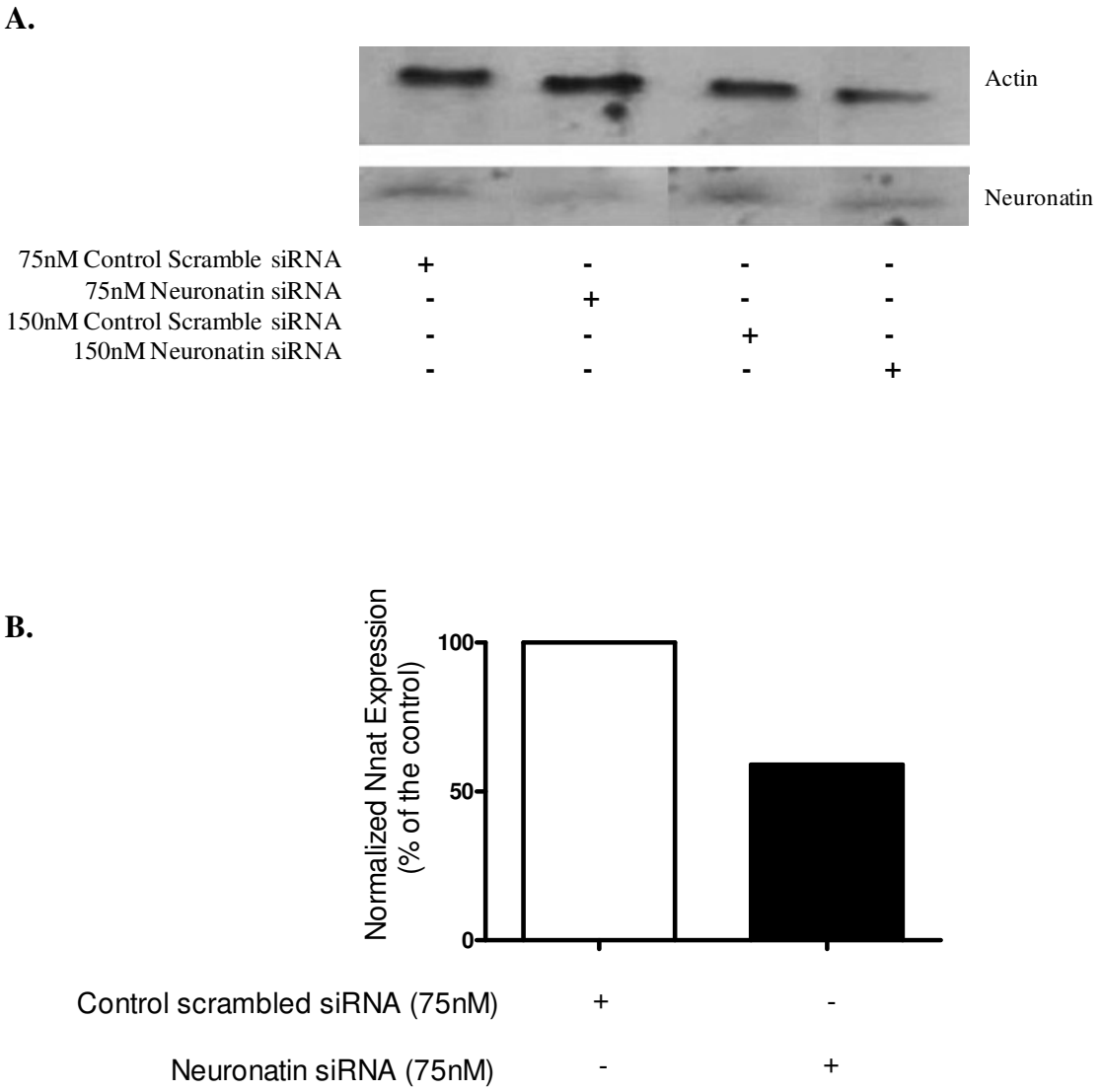

Figure 6.1: Neuronatin is Knocked-down by Nnat siRNA. A) DRG neurons were plated on poly-L-lysine and transfected with 75nM or 150nM Nnat siRNA or Control Scramble siRNA. After 24 hours the neurons were lysed and the total protein was subjected to Western blotting (10µg) with a Neuronatin antibody. Significant knockdown was observed in the neurons treated with 75nM but not 150nM Nnat siRNA. B) Quantification of 75nM Nnat siRNA knockdown. Transfection with Nnat siRNA reduced Nnat expression by ~40%.

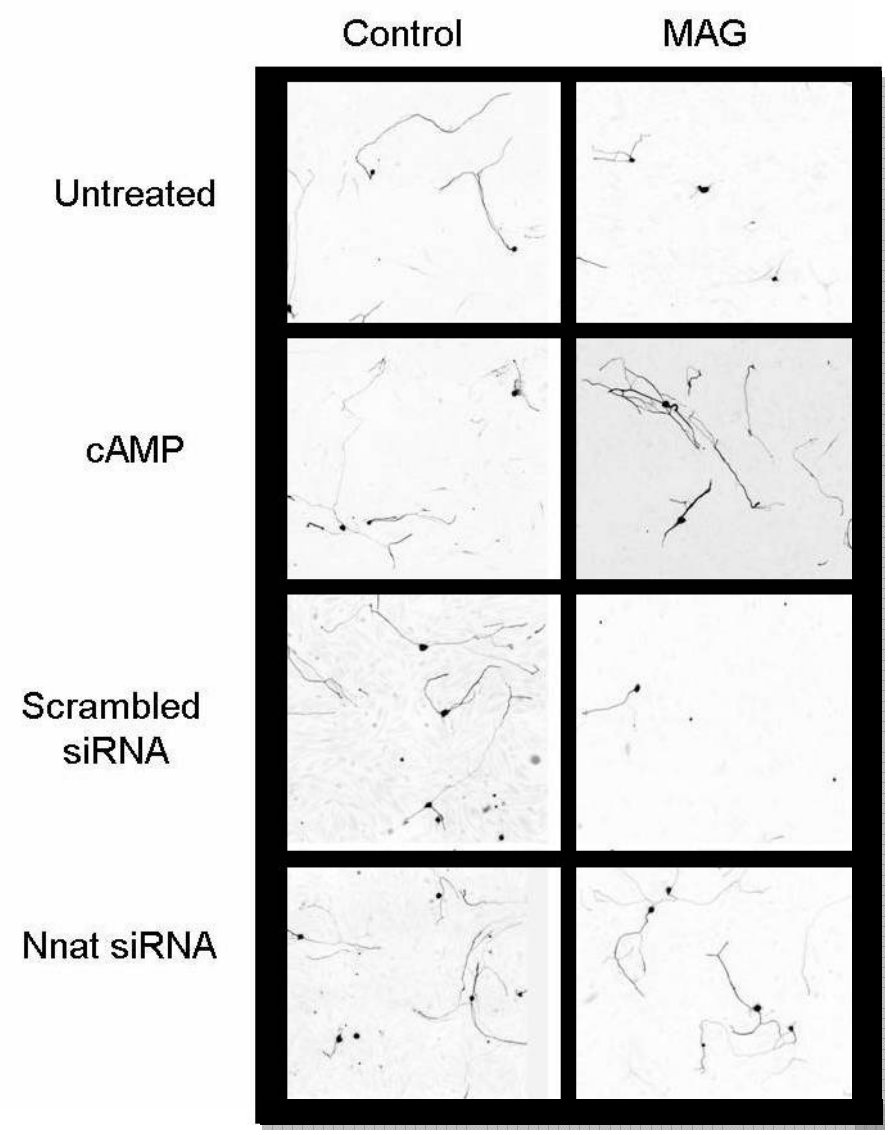

Figure 6.2: Knockdown of Neuronatin Overcomes MAG-mediated Axonal Growth Inhibition. DRG neurons were transfected with 75nM Nnat siRNA or 75nM Control Scrambled siRNA or treated with 1mM db-cAMP and then subjected to a neurite outgrowth assay. Knockdown of Nnat led to a significant increase in neurite length on MAG-expressing CHO cell monolayers as compared with a Control Scrambled siRNA. Transfection of DRG neurons with the control scrambled siRNA had no effect on either the inhibition by MAG or on control growth.

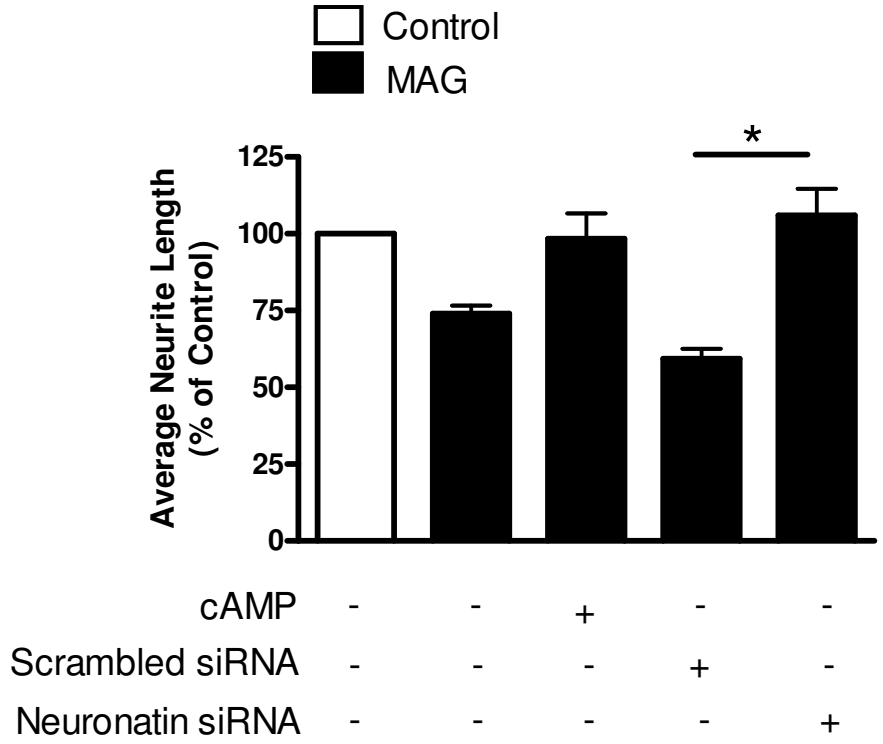

Figure 6.3: Neuronatin Knockdown Overcomes MAG Inhibition. DRG neurons were transfected with 75nM Nnat siRNA or 75nM Control Scrambled siRNA or treated with 1mM db-cAMP and then subjected to a neurite outgrowth assay. Knockdown of Nnat led to a significant increase in neurite length on MAG-expressing CHO cell monolayers as compared with a Control Scrambled siRNA. Nnat siRNA-induced growth on MAG was similar to the growth of db-cAMP treated neurons on MAG. Transfection of DRG neurons with the control scrambled siRNA had no effect on either the inhibition by MAG or on control growth. ($p<0.05$, n=3).

Similar growth results were obtained with DRG neurons that had undergone siRNA-mediated Nnat knockdown and were subsequently subjected to a neurite outgrowth assay on a purified myelin substrate (Figure 6.4) with Nnat knockdown resulting in an approximately 25% increase in neurite length on myelin (relative to DRG neurons transfected with a scrambled siRNA control ($P<0.05$).

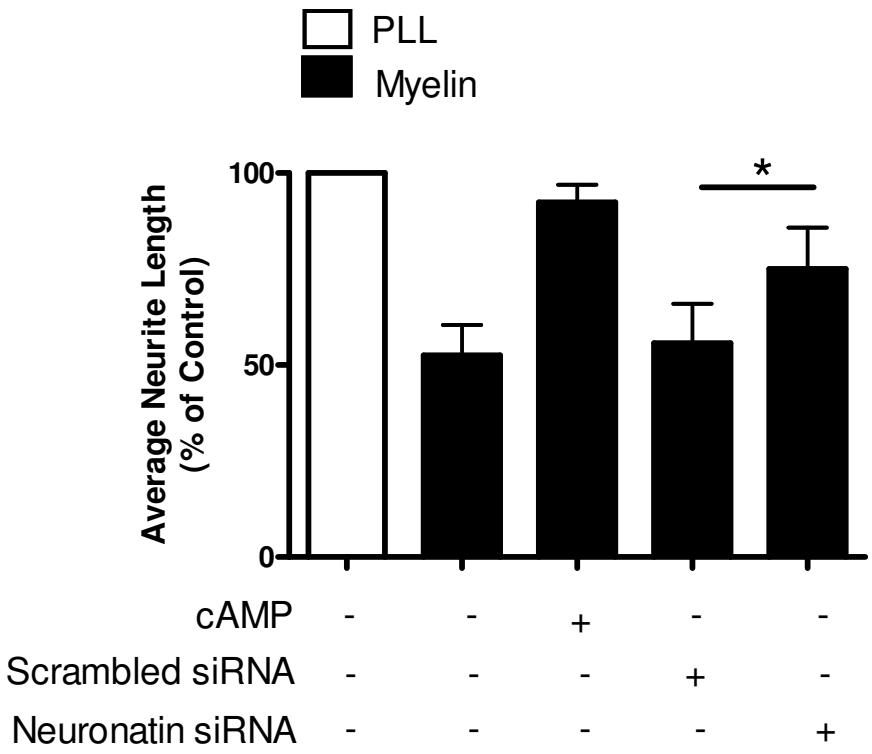

Figure 6.4: Neuronatin Knockdown Overcomes Myelin Inhibition. DRG neurons were transfected with 75nM Nnat siRNA or 75nM Control Scrambled siRNA or treated with 1mM db-cAMP and then subjected to a neurite outgrowth assay. Knockdown of Nnat led to a significant increase in neurite length on myelin as compared with a Control Scrambled siRNA. Nnat siRNA-induced growth on myelin was similar to the growth of db-cAMP treated neurons on myelin. Transfection of DRG neurons with the Control Scrambled siRNA had no effect on either the inhibition by myelin or on control growth. ($p<0.05$, $n=3$).

6.3 Discussion

In this chapter we have shown that knockdown of Neuronatin is sufficient to overcome MAG/myelin-based neurite outgrowth inhibition. Previously our lab has shown that elevation of cAMP is sufficient to overcome MAG/myelin-based axonal growth inhibition (Cai et al. 1999; Qiu et al. 2002) and also that it is possible to overcome MAG/myelin inhibition by stimulating the downstream effectors of cAMP, even in the absence of cAMP (Cai et al. 2002). In the previous chapters we have described a role for the cAMP- induced biosynthesis of miR-151 in translationally repressing the protein Neuronatin. In this chapter we have knocked-down Neuronatin and shown that inhibiting the translation of Neuronatin is sufficient to overcome MAG/myelin-based axonal growth inhibition even in the absence of cAMP. Thus, miR-151 is a downstream effector of cAMP that is capable of overcoming MAG/myelin-based neurite out growth inhibition by repressing the translation of Neuronatin.

The research shown in this body of work leads us to some important questions. How does the miR-151 translational repression of Neuronatin overcome myelin-based growth inhibition? What is the role of Neuronatin in axonal growth/inhibition? Could miR-151 or Neuronatin be potential drug targets for *in vivo* therapeutics?

How does the miR-151 translational repression of Neuronatin overcome myelin-based growth inhibition? When considering the goals and aims of this research this is a fundamental question. To understand how Neuronatin overcomes inhibition we must first try to understand the physiology of Neuronatin. We currently have developed two

hypotheses as to what knockdown of Neuronatin may be doing to stimulate growth in an inhibitory environment. Neuronatin has been reported to share about 50% sequence homology with two other proteolipid proteins that function as regulatory subunits of ion channels, PMP1 and phosholamban (a H^+-ATPase and a Ca^{2+} -ATPase, respectively) (Dou and Joseph 1996). In a 2005 paper by Suh et al. the authors overexpressed Nnat in 3T3-L1 cells (an adipocyte cell line) and found that overexpression led to enhanced activation of CREB through increasing the intracellular free calcium levels (Suh et al. 2005). They next investigated if Neuronatin, as its sequence homology would suggest, was a regulatory subunit of an endoplasmic reticulum (ER) Ca^{2+}-ATPase. To test this they expressed a Nnat-GFP in 3T3-L1 cells and looked under immunofluorescent microscopy to see if it co-localized with the ER marker BiP. Unfortunately, while they state that Nnat is retained in the ER, the images they show are not convincing and since they did not perform any further biochemical analyses a physical interaction of Neuronatin in the ER has yet to be shown. However, if Nnat does reside in the ER and is a regulatory subunit of a Ca^{2+}-ATPase, then knockdown of Nnat might lead to dysregulation of the intracellular calcium homeostasis. It has been shown that calcium dynamics modulate growth cone guidance (Gomez and Zheng 2006) and thus, one hypothesis is that knockdown of Nnat overcomes myelin-based growth inhibition possibly by altering the intracellular calcium dynamics. To test this hypothesis we have designed a set of experiments that include the immunoprecipitation of Nnat with the ER-specific BiP, and also conducting calcium electrophysiology analysis on neurons that have been treated with Nnat siRNA. Interestingly, if overexpression of Nnat leads to enhanced CREB activation, and CREB activation leads to increased miR-151, and miR-

151 binds to and translationally represses Nnat, then Nnat regulation may be part of a microRNA-regulated negative feedback loop (Fig. 6.5). Negative feedback loops are common in microRNA pathways (Kim et al. 2007; Klein et al. 2007; Packer et al. 2008), where multiple levels of regulation are needed in order to keep the cellular homeostasis. Perhaps this CREB/Neuronatin/miR-151 negative feedback loop is necessary to maintain calcium homeostasis. However, the complexity of calcium dynamics and their modulation are beyond the scope of this discussion (Gomez and Zheng 2006).

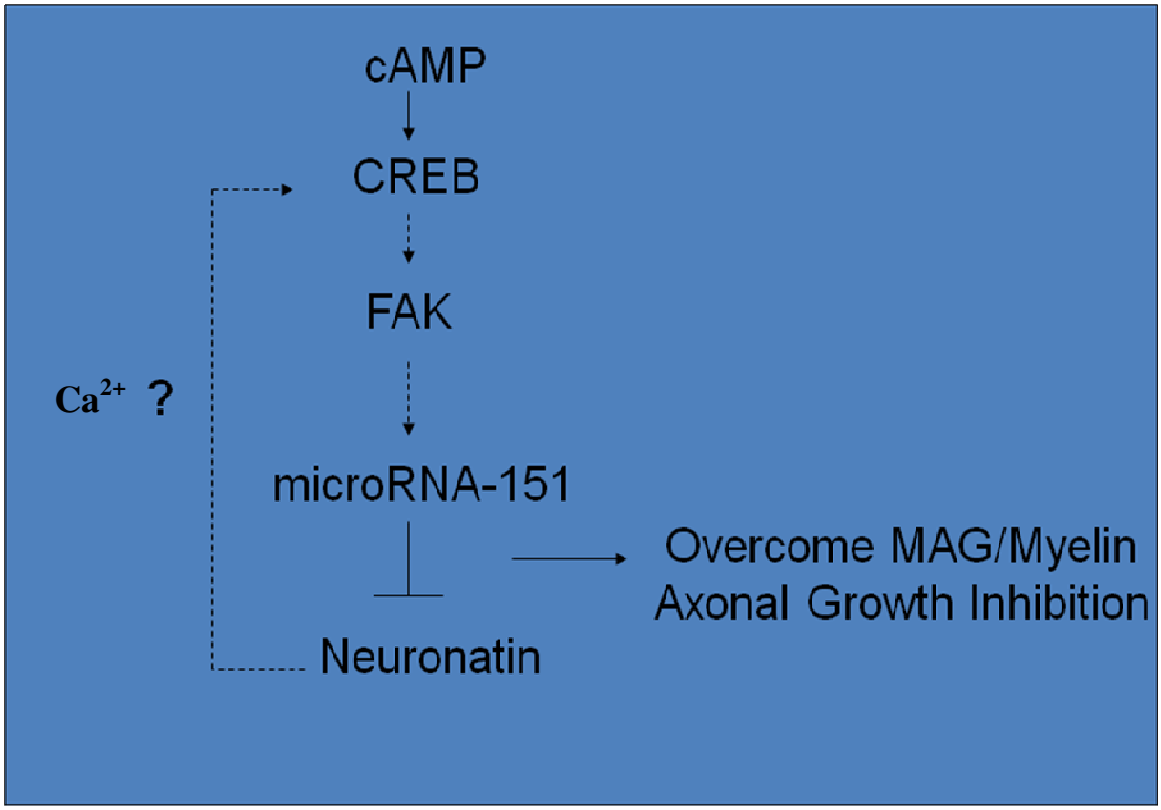

Figure 6.5: Proposed Hypothesis of CREB/miR-151/Neuronatin Feedback Loop. Previously our lab has shown that cAMP activates the transcription factor CREB. We have predicted that CREB is binding to the CRE sites in FAK mRNA and inducing its transcription. It has been predicted that miR-151 is produced from an intron in FAK. We have shown here that miR-151 is targeting Nnat mRNA and stopping its translation. Work from Suh et al. 2005 suggests that Nnat is enhancing the activation of CREB. CREB activation would produce more of the microRNA-151 which would regulate Nnat. Thus, creating a negative feedback loop.

A second hypothesis as to how knockdown of Neuronatin overcomes MAG/myelin-based neurite outgrowth inhibition involves the transcription factor NF-κB. In 2008, Mzhavia et al. showed that when they overexpressed Nnat in HAEC's using an Adenovirus-Nnat they observed an increase in expression of a panel of NF-κB-regulated genes including inflammatory cytokines, chemokines and cell adhesion molecules. Using biochemical analysis they were able to show that Nnat activation of NF-κB gene expression was mediated not through TNFα but rather through p38, Jun NH_2 terminal kinase, extracellular signal-related kinase, and AKT kinase. Interestingly, unpublished work from our lab has shown that cAMP is down-regulating the expression of NF-κB regulated genes by inducing the expression of secretory leukoprotease inhibitor (SLPI) (Hannila & Filbin, unpublished). Treatment with db-cAMP has been observed to cause the translocation of SLPI to the nucleus where its acts as a transcriptional repressor by binding to the TNFα promoter and stopping its transcription by NF-κB and we have shown that overexpression of SLPI is sufficient to overcome MAG/myelin inhibition both *in vitro* and *in vivo* (Hannila & Filbin, unpublished). Thus, similar to SLPI, another way in which knockdown of Nnat may be overcoming MAG/myelin-mediated axonal growth inhibition is through stopping the expression of NF-κB regulated genes.

What is the role of Neuronatin in axonal growth/inhibition? This too is an interesting question and was not addressed in this body of work. In order to fully understand if expression of Neuronatin leads to neurite growth inhibition, we must see if expression of Nnat inhibits growth. One way to test this is by overexpressing Nnat in neurons and measuring the total growth on a permissive substrate as compared to neurons that were

not transfected. Alternatively, in the Nnat knockdown neurite outgrowth analyses that we performed it would be convincing to see that overexpression of Nnat could rescue the MAG/myelin-mediated axonal growth inhibition. In the labs future, these experiments will be addressed.

Could miR-151 or Neuronatin be potential drug targets for *in vivo* therapeutics? The ultimate goal of this research is to identify possible targets for therapeutic intervention in patients of spinal cord injuries. This body of work represents the first time that a microRNA, miR-151, has been implicated in promoting neurite outgrowth in an inhibitory environment and also the first time that Neuronatin has been shown to play a role in inhibition of axonal growth. Currently the lab is beginning *in vivo* studies in two paradigms, an optic nerve crush and a dorsal column lesion, in which miR-151 is being overexpressed after crush/lesion and axonal regeneration is being assessed. These studies are ongoing in the lab and future work will further investigate the possibility of these novel targets being used for therapeutic drug studies which could lead to new treatments for spinal cord injury.

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