

Glutamate Receptor Signaling is a Mediator of Neurite Outgrowth Inhibition by MAG

By

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Abstract

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Unlike the peripheral nervous system (PNS) or embryonic neurons, the adult mammalian central nervous system (CNS) does not spontaneously regenerate after injury. This is due, in part, to the presence of myelin-associated inhibitors, such as myelin-associated glycoprotein (MAG). Our lab has shown that elevation of intracellular cAMP blocks these inhibitors *in vitro* and *in vivo* in a transcription-dependent manner. Subsequent microarray analysis revealed that elevation of cAMP results in upregulation of Arginase I (Arg1), a key enzyme in the synthesis of polyamines. Our lab has demonstrated that administration of polyamines is sufficient to block MAG and myelin-induced inhibition of axonal outgrowth *in vitro* as well as enhance CNS axon regeneration *in vivo*. Others have shown that binding of polyamines to ionotropic glutamate receptors (iGluRs), which include N-methyl-D-aspartate receptors (NMDAR), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors (KAR), blocks their activity. In addition, it is well established that iGluR activity is disrupted following CNS injury. Thus, since polyamines overcome MAG/myelin-mediated inhibition and given that polyamines block iGluRs activity, we set out to examine the role iGluR-mediated signaling plays in the MAG and myelin inhibitory pathways. We found that blocking

NMDAR or AMPA/KA receptor activity with pharmacological antagonists was sufficient to block MAG-induced inhibition of neurite outgrowth in dorsal root ganglia (DRG) and hippocampal neurons (HNs). Likewise, exposure to iGluR agonists increased MAG-mediated inhibition of neurite outgrowth. To determine whether prior exposure of iGluR inhibitors or priming is sufficient to overcome MAG inhibition as in the case of polyamines, HNs were treated with iGluR antagonists 18 hours before exposing neurons to MAG. We found that priming with iGluR antagonists was not sufficient to overcome MAG-mediated inhibition, suggesting that GluR antagonists exert their effect by intercepting MAG inhibition signals, while not changing the ability of neurons to respond to MAG. Because polyamines require priming whereas iGluR antagonists have no effect when used to prime neurons, we therefore conclude that in our model system, polyamines do not overcome inhibition by blocking iGluR activity.

It is well established that upon activation, NMDARs produce influx of Ca^{2+} inside the cell, which activates Ca^{2+} -dependent kinases such as extracellular signal-regulated kinase (ERK) and conventional protein kinase C (PKC), both of which are important for synaptic plasticity, long term potentiation (LTP), and long term depression (LTD). Importantly, conventional PKC and epidermal growth factor receptor (EGFR)-regulated ERKs are also known to be activated in response to myelin inhibitors in a Ca^{2+} -dependent manner. Blocking PKCs or EGFRs is sufficient to overcome inhibition by myelin inhibitors *in vitro* and promote axonal regeneration *in vivo*. To establish which downstream target activation is affected in response to iGluR activity block as well as myelin, PKC and ERK signaling pathways were examined. We found that myelin induces robust ERK activation, and importantly, inhibiting iGluR activity was sufficient to block

ERK activation. Next, to determine whether myelin-induced ERK activation is inhibitory for neurite outgrowth, HNs were treated with the ERK inhibitor U0126 and then subjected neurite outgrowth assay in the presence of MAG. We found that blocking ERK was sufficient to overcome MAG-mediated inhibition in a dose dependent manner. Our findings demonstrate that exposure to myelin induces ERK activation and that ERK activation is regulated by iGluR activity. Since ERK is one of the main downstream targets activated by NMDAR-induced Ca^{2+} influx, our findings support the idea that exposure to myelin results in iGluR activation.

In synapses, repeated activation of NMDAR leads to long-term potentiation (LTP) through mechanisms involving the activation of PKC and ERK as well as rapid forward trafficking of NMDARs to the membrane. This is consistent with reports that PKC induces phosphorylation in the C-terminal of the NMDA NR1 subunit, leading to increased surface insertion of NMDAR channels. To determine whether myelin-associated inhibitors affect NMDAR trafficking, cortical neurons were treated with myelin and then the amounts of surface NMDARs was assessed. We found that myelin induced a rapid increase in surface NMDAR number. In addition, myelin treatment induced phosphorylation of ser890 and ser896 C-terminal NMDA NR1 subunits, which have been shown to promote forward NMDAR trafficking to the cell surface. Thus, our findings suggest that myelin-induced PKC activation promotes surface incorporation of NMDARs, which can potentially enhance NMDAR-mediated currents. Taken together, our results implicate iGluR activity as well as ERK signaling as new mediators of MAG/myelin-induced inhibition, offering new insights into the molecular mechanism of myelin-induced block of neurite outgrowth.

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Abbreviations

A AC adenylate cyclase ALS amyotrophic lateral sclerosis AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor	M MAG myelin-associated glycoprotein
B BDNF brain derived neurotrophic factor	N NEP1-40 Nogo extracellular peptide, residues 1-40 NGF nerve growth factor NTs neurotrophins NMDAR N-methyl-D-aspartate receptor
C C3 c3 transferase cAMP cyclic adenosine monophosphate CGN cerebellar granule neurons CHO Chinese hamster ovary CNS central nervous system CSPGs chondroitin sulfate proteoglycans CST corticospinal tract	O OMgp oligodendrocyte myelin glycoprotein OPs oligodendrocyte precursors
D dbcAMP dibutyryl cAMP DRG dorsal root ganglia	P p75^{NTR} p75 neurotrophin receptor PDE4 phosphodiesterase 4 PI phosphatidyl inositol PNS peripheral nervous system PirB paired immunoglobulin like receptor
E ECM extracellular matrix EGFR epidermal growth factor receptor ERK extracellular-signal regulated kinase ER endoplasmic reticulum	R RGCs Retinal ganglion cells RTK receptor tyrosine kinase
G IGluR glutamate receptor GAG glycosaminoglycan GFAP glial fibrillary acidic protein GPI glycosyl phosphatidyl inositol	S SCs Schwann cells SCI spinal cord injury Siglec sialic acid binding Ig-like lectin
H - I HN hippocampal neurons Ig immunoglobulin	T TNFR tumor necrosis factor receptor Trk tropomyosin kinase receptor
L LTP long term potentiation LTD long term depression	W WD Wallerian degeneration

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Chapter I: Introduction

1.1 Outcomes of CNS and PNS Injury

It has been known for centuries that following an injury, adult mammalian CNS axons fail to spontaneously regenerate, whereas repair of damaged PNS axons is quite common. The question which still remains is: what causes this loss of ability to regenerate after injury? It is well established that there are two main obstacles to regeneration after injury: inhibitors within myelin and the formation of the glial scar. The scar is formed mainly by astrocytes which become reactive after injury and form a dense structure. This scar presents both physical and chemical barrier to growth since many inhibitors are expressed on its extracellular surface (McKeon et al., 1991, Asher et al., 2001, Silver and Miller, 2004). However, since the scar takes several weeks to mature, inhibitors in myelin seems to be the major source of growth inhibition immediately after injury to the CNS.

One major difference between PNS and CNS is the sequence of events after injury. Injury to the PNS triggers extensive degenerative events collectively called Wallerian degeneration. Wallerian degeneration is a process of rapid clearing, including an influx of immune cells such as macrophages, which functions in the removal of myelin debris distal to the injury (Griffin et al., 1992). In addition, shortly after PNS injury, Schwann cell facilitation takes place, where Schwann cells start to proliferate and ultimately form guidance and growth promoting substrates, allowing the regenerating axons to reach their original targets (Fawcett and Keynes, 1990, Vargas and Barres, 2007). Wallerian degeneration in the PNS takes up to two weeks to complete, while in the CNS it is a very slow process lasting months to years (Griffin et al., 1992, George and Griffin, 1994). Furthermore, during CNS Wallerian degeneration, oligodendrocytes do

not clear myelin debris and peripheral macrophages cannot enter the injury site because the blood brain barrier (BBB) fails to become permeable (Barres et al., 1993, Vargas and Barres, 2007). Therefore, the failure to clear CNS myelin results in its prolonged presence at the injury site, creating an inhospitable environment for regenerating axons.

The inhibitory properties of the injured CNS environment were demonstrated by David and Aguayo in the early 1980s, when they showed that a lesion to the CNS results in an inhibitory environment that prevents axonal regrowth. However, if provided with a known favorable environment, such as peripheral nerve graft, injured CNS axons were able to regenerate, suggesting that the regenerative capacity of adult CNS axons is dictated by the environment (David and Aguayo, 1981). The following year, Martin Berry was the first to suggest that the products of myelin breakdown in the adult CNS can inhibit the regrowth of damaged axons (Berry, 1982). Furthermore, work by Schwab and colleagues demonstrated that immobilized CNS myelin, but not PNS myelin, inhibits neurite outgrowth (Schwab and Thoenen, 1985). Schwab and colleagues later generated a monoclonal antibody named IN-1, which neutralized myelin inhibition *in vitro* (Caroni and Schwab, 1988a). In addition, the important role of myelin-associated inhibitors in the failure of axon regeneration was confirmed by a study by David and colleagues where mice were immunized with myelin three weeks before their spinal cords were injured. When compared to controls that were immunized with liver tissue, mice that were immunized with myelin demonstrated considerable axon regeneration and enhanced functional recovery (Huang et al., 1999).

To date, three major inhibitors of axonal regeneration associated with myelin have been identified: myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-

myelin glycoprotein (OMgp) (Caroni and Schwab, 1988b, McKerracher et al., 1994, Mukhopadhyay et al., 1994, Wang et al., 2002b).

Although these inhibitors lack any structural homology or sequence similarity, it has been shown that all three myelin-associated molecules exert their inhibitory effect via interaction with the same receptor complex, consisting of NgR-Lingo-p75^{NTR}/Troy or paired-immunoglobulin-like-receptor (PirB) (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002c, Wong et al., 2002, Mi et al., 2004, Park et al., 2005, Shao et al., 2005). All three myelin inhibitory factors are expressed in the adult CNS and *in vitro* data demonstrated that each inhibitor sufficiently blocks regeneration following injury (McKerracher et al., 1994, Mukhopadhyay et al., 1994, Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000, Wang et al., 2002a).

1.2 Obstacles to CNS Regeneration

1.2.1 Inhibitors of Axonal Growth in Myelin

Myelin is a fatty sheath which wraps around axons, serving as an electrical insulator which increases the speed of electrical impulse propagation along axons. In the PNS, myelin is produced by Schwann cells with a single cell myelinating a single axon, while in the CNS, myelin is made by oligodendrocytes, with a single oligodendrocyte capable of myelinating many axons. In either system, myelin is essential for propagation of action potentials along the axons.

1.2.1.1 Nogo

The idea that CNS myelin was inhibitory for axonal regrowth in the mature CNS was first suggested by Berry and colleagues (Berry, 1982). Later on, the identification of Nogo as myelin inhibitor of regeneration started when Schwab and colleagues observed that the inhibitory activity of myelin was enriched in two protein fractions of myelin extracts, with molecular weights of 35kDa and 250kDa (Caroni and Schwab, 1988b). A monoclonal antibody, IN-1, raised against the 250kDa fraction was shown to reduce the inhibitory activity of CNS myelin *in vitro*, and to enhance regrowth of injured corticospinal tract (CST) axons *in vivo*, which was associated with some functional recovery (Schnell and Schwab, 1990, Bregman et al., 1995). It was not until the year 2000 that the IN-1 antigen was identified, independently cloned by three different groups, and named Nogo (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000).

There are three Nogo isoforms, Nogo-A, B and C, due to alternative splicing and promoter usage. All three isoforms have a common 188-amino-acid residue C-terminus homologous to reticulon protein family, proteins usually associated with the endoplasmic reticulum (ER) (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000). All three isoforms share a common C-terminal domain, homologous to the reticulon protein family. Nogo-A isoform is enriched in oligodendrocytes of adult CNS tissue, whereas Nogo-B and-C are expressed in other cells, such as neurons. Nogo-A expression was shown to be localized to the cell surface of oligodendrocytes where it can make contact with an axon (Chen et al., 2000, GrandPre et al., 2000, Huber et al., 2002).

Nogo-A is characterized by two inhibitory domains, Nogo-66 and amino-Nogo. Nogo-66, which is common to all Nogo isoforms, contains 66 amino acids in its extracellular region, which is sufficient to exert growth inhibitory activity *in vitro* (GrandPre et al., 2000). Amino Nogo is the second domain, which is localized near the N-terminal region and was shown to inhibit neurite outgrowth (Fournier et al., 2001).

In vivo and *in vitro* studies implicate Nogo-A as inhibitory to axon regrowth following injury. Nogo-A was shown to inhibit DRG neurite outgrowth and fibroblast spreading, both of which could be neutralized by either the IN-1 antibody or an antibody against the C-terminal domain of Nogo-A (Chen et al., 2000, GrandPre et al., 2000). *In vivo* delivery of anti-Nogo IN-1 monoclonal antibodies, which is directed against amino Nogo, via transplantation of IN-1 secreting hybridoma cells, resulted in enhanced axon regeneration and functional recovery following dorsal hemisection of the spinal cord (Schnell and Schwab, 1990).

In order to examine axonal regeneration *in vivo*, Nogo knockout mice were generated. The results, however, were contradictory. Whereas two groups reported limited cortical-spinal tract (CST) axon regeneration using Nogo A/B $-/-$ or Nogo A $-/-$ mice (Kim et al., 2003, Simonen et al., 2003), Tessier-Levigne's group, by generating Nogo A/B $-/-$ and Nogo A/B/C $-/-/-$, reported a lack of spinal regeneration in either line, following CST lesion (Zheng et al., 2003). Furthermore, four years later, the Steward group repeated the Strittmatter group's 2003 experiments, using the same mouse strain and observed no significant regeneration (Steward et al., 2007). It appears that the limited or lack of regeneration observed in the Nogo knockout mice might be attributed to the other myelin inhibitors that are still present.

1.2.1.2 Myelin - Associated Glycoprotein (MAG)

Myelin-associated glycoprotein (MAG) is a member of the immunoglobulin (Ig)-like family (Salzer et al., 1987, Salzer et al., 1990). It contains five extracellular Ig-like domains, a single transmembrane domain, and a short cytoplasmic domain (Salzer et al., 1990). MAG is a well characterized, myelin-specific protein that was shown to play a role in myelination as well as formation and maintenance of myelin-axon interaction (Quarles, 1983, Salzer et al., 1990, Trapp, 1990, Filbin, 1995). This idea is supported by studies indicating that in older MAG-deficient (MAG $-/-$) mice, products of the first myelination events are normal, however, they eventually acquire altered periaxonal architecture and an increase in axonal loss (Fruttiger et al., 1995).

MAG was the first myelin inhibitor to be identified by two independent studies, demonstrating that MAG inhibits neurite outgrowth *in vitro* (McKerracher et al., 1994,

Mukhopadhyay et al., 1994). CNS neurons cultured on monolayers of MAG-expressing cells displayed a drastic reduction in axonal growth as compared to neurons plated on control cells. A neuronal cell line, NG108 cells, also fail to extend neurites when plated on slides coated with myelin or MAG. (McKerracher et al., 1994, Mukhopadhyay et al., 1994).

MAG can bind sialic acid, which is a sugar moiety, with its extracellular domain, thus classifying it as sialic acid binding Ig-like lectin (Siglec) subgroup of immunoglobulins (Kelm et al., 1994). Consequently, MAG can bind gangliosides, sialic acid bearing glycosphingolipids, via arginine 118 (R118) in its first Ig domain (Kelm et al., 1994). It has been shown that this binding is not required for membrane localized MAG to inhibit neurite outgrowth (Tang et al., 1997). More recent results from our lab demonstrated that the inhibitory site of MAG is distinct from the sialic acid binding site and maps to the fifth Ig domain (Cao et al., 2007).

MAG is expressed in both PNS and CNS myelin, representing 1% and 0.1% of the total myelin proteins, respectively (Trapp, 1990). In the adult CNS, MAG is located exclusively in the periaxonal membrane of myelin where it comes in contact with the axon. This differs from the PNS myelin, where MAG is expressed in the paranodal regions, Schmidt-Lanterman incisures and outer mesaxon segments (Trapp et al., 1989).

MAG acts as a bifunctional molecule. Thus, depending of the age and type of neuron, MAG can either promote or inhibit axonal regeneration (Johnson et al., 1989, Salzer et al., 1990, Mukhopadhyay et al., 1994). MAG was shown to promote growth of embryonic neurons, but it inhibits outgrowth of postnatal neurons. An exception is DRG

neurons, which during the first postnatal week undergo a switch from promotion to inhibition (Bregman et al., 1993, Hasan et al., 1993, Cai et al., 1999).

MAG's role in axon regeneration *in vivo* is less clear. MAG^{-/-} mice lacked spinal axon regeneration as compared to wild type, while in another study, using a different strain of mice, MAG^{-/-} mice demonstrated enhanced axonal regeneration (Bartsch et al., 1995, Li et al., 1996). Our lab has shown that cerebellar, DRG and retinal neurons growing on MAG-deficient myelin substrate demonstrated enhanced neurite outgrowth (DeBellard et al., 1996). In conclusion, even though MAG is only one of several identified inhibitors of axonal regeneration, it contributes, at least partly, to the poor regeneration of CNS axons following injury.

The analysis of genetic deletion of MAG, however, provided insights into its role in the intact CNS. MAG^{-/-} mice exhibit delayed myelin formation, with the mature myelin exhibiting structural defects (Bartsch et al., 1995, Li et al., 1996).

1.2.1.3 OMgp

Oligodendrocyte myelin glycoprotein (OMgp) was identified in 2002 by Zhigang He's group as a third inhibitor of regeneration that is present in myelin (Wang et al., 2002b). Originally identified in the 1980s, OMgp is expressed in oligodendrocytes where it is localized to the paranodal loops, adjacent to the node of Ranvier. It is also found in CNS neurons as well as in the PNS (Mikol and Stefansson, 1988). OMgp is normally anchored to the membrane via a glycosyl phosphatidyl inositol (GPI) anchor (Mikol and Stefansson, 1988). OMgp is reported to be a minor component of CNS myelin, where its peak expression occurs at the latest stages of myelination (Habib et al., 1998, Vourc'h et al., 2003). OMgp function in non-injured animal is still unknown, although recent

observations suggest that OMgp could be involved in the regulation of oligodendrocytes growth (Kottis et al., 2002, Huang et al., 2005). Nonetheless, OMgp was shown to inhibit axonal regrowth *in vitro* in a manner similar to MAG or Nogo (Wang et al., 2002b).

1.2.1.4 Ephrin B3 and Semaphorin 4D

Molecular guidance cues play a major role in axonal pathfinding during development. However, expression of some repulsive guidance cues expression persists into adulthood and they have been shown to be present in myelin. Two examples are Ephrin B3 and Semaphorin 4D (sema4D). Both were shown to be expressed in postnatal oligodendrocytes and act as repulsive or inhibitory substrates for regenerating axons in the adult CNS (Benson et al., 2005). Myelin derived from ephrinB3 *-/-* mice is less inhibitory for neurite outgrowth than wild-type myelin (Benson et al., 2005). Similarly, expression of sema4D is sufficient to inhibit axonal growth from several neuronal types (Moreau-Fauvarque et al., 2003). Therefore, although many guidance cues that are involved in the formation of the nervous system become downregulated when development is complete, some guidance cues persist into adulthood and become upregulated in response to injury, exerting inhibitory effects.

1.2.2 Receptors for Inhibitors of Axonal Growth

1.2.2.1 Nogo Receptor (NgR)

Nogo receptor (NgR), as its name indicates, was first identified as a putative receptor for Nogo-66. Amino-Nogo, the second inhibitory domain, was not able to bind NgR in a protein binding screen (Fournier et al., 2001). Surprisingly, the two other identified inhibitors in myelin, MAG and OMgp, were soon reported to bind NgR as well, even though they share no sequence or structural homology with Nogo (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002a). Subsequent analysis of the crystal structure of the NgR ligand binding site revealed the presence of conserved aromatic residues in the ecodomain that can provide a degenerate ligand binding site, with each inhibitor choosing its preferred binding site (He et al., 2003).

NgR is expressed exclusively in CNS neurons (Fournier et al., 2001), which is consistent with its proposed function in axonal regrowth inhibition in the adult CNS following injury. In two independent studies, NgR was implicated in restricting developmental plasticity in intact CNS synapses (McGee et al., 2005, Lee et al., 2008). In the first study, deletion of NgR extended the duration of the critical period of ocular dominance plasticity within the visual cortex. The experimental paradigm in that study employs monocular deprivation or closure of one eye during a critical period timeline where the establishment of synaptic connections is experience-based, resulting in reduced responsiveness in the deprived eye, while inducing a relative shift in ocular dominance plasticity to the non-deprived eye. In wild type mice, the critical period of ocular

dominance plasticity is between postnatal day 19 to 32, while in NgR $-/-$ the critical period was still occurring at postnatal day 45 or 120, indicating that NgR-mediated signaling functions to restrict plasticity in the visual cortex (McGee et al., 2005). In the second study, loss of NgR was shown to affect synaptic plasticity, specifically, attenuating LTD in CA1 regions in the hippocampus (Lee et al., 2008). Furthermore, NgR1 was shown to be enriched in synapses, particularly in spines, and loss of NgR resulted in altered spine morphology, enriching a certain morphology found in immature rodent synapses (Lee et al., 2008).

NgR is a 473 amino acid GPI-linked protein. It lacks an intracellular component, which suggests that association with a coreceptor is needed in order to transduce the signal intracellularly. In initial studies, the removal of NgR GPI-anchor from axonal surfaces could reduce or abolish neuronal responses to myelin inhibitors, whereas, overexpression of NgR could confer responsiveness to these inhibitors in embryonic neurons, which are normally unresponsive (Fournier, 2001)

NgR was later termed NgR1 since two other NgR homologs were identified, NgR2 and NgR3 (Barton et al., 2003, Venkatesh et al., 2005). The Barton group reported that NgR2 and NgR3 do not interact with neither Nogo, MAG, nor OMgp, however, the Giger group showed that NgR2 can selectively bind MAG to mediate axonal inhibitory response (Barton et al., 2003, Venkatesh et al., 2005).

In order to further assess the *in vivo* role of NgR in axon regeneration, two independent groups generated NgR1 knockout mice. Analysis of regenerating CST axons revealed no CST regeneration in the NgR1 $-/-$ mice in both studies, while improved regeneration in raphespinal and rubrospinal tracts, associated with improved motor

function was seen in one of the studies (Kim et al., 2004b, Zheng et al., 2005). In addition, *in vitro*, DRG and cerebellar neurons from NgR $-/-$ mice were shown to be inhibited by Nogo or myelin substrate to the same extent as wild type neurons (Zheng et al., 2003). Overall, data from these studies suggest the presence of an additional receptor capable of binding myelin inhibitors to mediate inhibition of axon regeneration.

1.2.2.2 p75 Neurotrophin Receptor (p75^{NTR})

p75^{NTR}, originally identified as a low affinity neurotrophin receptor, is a transmembrane protein that belongs to the tumor necrosis factor receptor (TNFR) family (Chao et al., 1998). Since NgR lacks an intracellular domain to transduce signals into the cell, it requires a coreceptor. It was later demonstrated that p75^{NTR} coprecipitated with NgR upon treatment with myelin inhibitors (Wang et al., 2002c, Wong et al., 2002). Neurons isolated from p75^{NTR} $-/-$ mice or neurons overexpressing dominant negative p75^{NTR} demonstrated reduced neurite outgrowth inhibition by all three myelin-associated inhibitors, or by myelin in general (Wang et al., 2002c, Wong et al., 2002). Furthermore, neurons derived from mutant p75^{NTR} failed to activate the small GTPase RhoA, an important target for most inhibitors (Yamashita et al., 2002). Our lab later reported that MAG binding to NgR induces regulated intramembrane proteolysis of p75^{NTR}, an event that is necessary for Rho-A activation and axon growth inhibition (Domeniconi et al., 2005).

The results from regeneration studies in p75^{NTR} $-/-$ mice, however, were contradictory. *In vitro*, DRG neurons from p75^{NTR} $-/-$ mice demonstrated improved

growth on myelin, while cerebellar neurons from $p75^{\text{NTR}} -/-$ mice were still inhibited by myelin (Zheng et al., 2005). Furthermore, *in vivo* studies in $p75^{\text{NTR}} -/-$ mice showed a lack of CST axon regeneration following a dorsal column lesion (Zheng et al., 2005).

In summary, although reports demonstrate that $p75^{\text{NTR}}$ expression is induced in response to injury (Ebadi et al., 1997), $p75^{\text{NTR}}$ may not be the only signal transducer following CNS injury since only a subpopulation of neurons, such as DRG neurons were shown to express $p75^{\text{NTR}}$ in the mature CNS (McMahon et al., 1994).

1.2.2.3 Troy / Taj

The lack of $p75^{\text{NTR}}$ expression in certain neuronal populations in the CNS, together with the observation that $p75^{\text{NTR}}$ knockout mice failed to regenerate after CNS injury, led to speculation that an alternate coreceptor or $p75^{\text{NTR}}$ homolog might exist (Zheng et al., 2005). Indeed, two groups independently demonstrated that Troy (also termed Taj), a member of the TNFR similar to $p75^{\text{NTR}}$, is broadly expressed in young and adult CNS neurons (Park et al., 2005, Shao et al., 2005). More importantly, Troy was shown to interact with NgR and substitute for $p75^{\text{NTR}}$ in the inhibitory receptor complex, activating Rho-A in response to myelin inhibitors. Moreover, genetic deletion or overexpression of a dominant negative form of Troy can also promote neurite outgrowth on a myelin substrate (Park et al., 2005, Shao et al., 2005)

1.2.2.4 Lingo-1

In 2005, Sha Mi's group identified Lingo-1 as an additional component of the NgR/ $p75^{\text{NTR}}$ receptor complex (Mi et al., 2004). Lingo-1 expression is specific to the CNS and is present after injury. It was shown to bind both NgR and $p75^{\text{NTR}}$, and it is

believed to bring ligand bound NgR and p75^{NTR} or Troy together (Mi et al., 2004). Deletion of Lingo-1 reduced the inhibitory activity of myelin inhibitors (Mi et al., 2004). Therefore, coexpression of all three components, NgR/ p75^{NTR}/ Lingo-1, is required to confer responsiveness to myelin components (Mi et al., 2004).

1.2.2.5 PirB

Since genetic deletion of NgR did not relieve neurite outgrowth inhibition by myelin *in vitro* or enhance regeneration of CST axons following injury *in vivo*, it implied that other receptors for these factors existed. Recently, the Tessier-Levigne group utilized expression cloning to screen a library of human genes in an attempt to find more potential binding partners for Nogo-66. Paired-Immunoglobulin-Like-Receptor or PirB was identified in the screen and was shown to bind Nogo-66, MAG and OMgp with high affinity (Atwal et al., 2008). Furthermore, blocking PirB reverses neurite outgrowth inhibition by myelin or Nogo for CNS neurons. Interestingly, blocking both NgR and PirB completely abolished myelin's ability to inhibit neurite outgrowth, indicating that both NgR and PirB are independently activated by myelin inhibitors, and block axon regrowth following injury to the CNS (Atwal et al., 2008).

In the intact CNS, PirB has been shown to be expressed throughout the brain, and like NgR, has been reported to restrict plasticity in the visual cortex (McGee et al., 2005, Syken et al., 2006). In mice lacking functional PirB, cortical-ocular dominance plasticity persisted throughout adulthood (postnatal day 110) rather than being terminated by postnatal day 32 (Syken et al., 2006). Taken together, PirB, like NgR, appears to be a

novel receptor that mediates regeneration block in the injured CNS while restricting plasticity in the intact CNS.

1.2.3 Signaling By Myelin-Associated Inhibitors

Inhibitory molecules of the adult CNS include myelin-associated inhibitors present in the myelin debris (MAG, Nogo-A, OMgp, EphB3 and Sema4D) and inhibitors in the glial scar (chondroitin sulfate proteoglycans or CSPGs) (McKerracher et al., 1994, Mukhopadhyay et al., 1994, Wang et al., 2002a, Silver and Miller, 2004)(Moreau-Fauvarque, 2003; Benson, 2005). Myelin-associated inhibitors bind to the NgR1-p75^{NTR}/Troy-Lingo receptor complex and/or to PirB. Binding of myelin or glial scar-associated inhibitors initiates an inhibitory signaling pathway which culminates in a rise in intracellular Ca²⁺ and the activation of the small GTPase RhoA (Bandtlow et al., 1993, Lehmann et al., 1999, Winton et al., 2002, Wong et al., 2002, Yamashita et al., 2002). RhoA activation has a key role in mediating inhibitory signaling both *in vitro* and *in vivo*. By regulating actin cytoskeleton rearrangement, RhoA activation results in growth cone collapse and inhibition of the growth of axons (Dergham et al., 2002, Winton et al., 2002).

The involvement of the second messenger Ca²⁺ signaling in mediating neurite outgrowth inhibition in response to inhibitors in myelin and the glial scar has been also reported. Specifically, elevation of intracellular Ca²⁺ ([Ca²⁺]_i) in response to application of MAG, Nogo and CSPGs has been observed (Hasegawa et al., 2004, Henley et al., 2004). Mu-Ming Poo and colleagues reported that inducing the release of low levels of

$[Ca^{2+}]_i$ from intracellular stores triggered by MAG application or by directly adding low levels of Ca^{2+} results in growth cone repulsion of embryonic spinal neurons, whereas, inducing high levels of $[Ca^{2+}]_i$ release via cAMP application or by directly adding high levels of Ca^{2+} results in growth cone attraction (Henley et al., 2004). Poo and colleagues also reported that the levels of $[Ca^{2+}]_i$ dictate whether netrin-1-induced turning will be attractive or repulsive. High level $[Ca^{2+}]_i$ triggered an attractive response, while low-level $[Ca^{2+}]_i$, triggered by blocking either Ca^{2+} influx through L-type channel or release from intracellular stores, led to a repulsive response (Hong et al., 2000).

In subsequent studies, inhibitors in myelin were shown to induce the activation of Ca^{2+} and Diacylglycerol (DAG)-sensitive protein kinase C ($PKC\alpha$, $-\beta$,) (Hasegawa et al., 2004, Sivasankaran et al., 2004). Furthermore, studies have shown that inhibiting inositol-tri-phosphate (IP3), which blocks release of $[Ca^{2+}]_i$ stores, does not overcome MAG mediated inhibition, which suggests that blocking IP3-induced $[Ca^{2+}]_i$ release is probably not sufficient to block PKC activation (Hasegawa et al., 2004, Sivasankaran et al., 2004).

Studies in our lab have shown that PKC is implicated in regulating $p75^{NTR}$ cleavage. MAG binding to NgR induces regulated intramembrane proteolysis of $p75^{NTR}$, an event that is necessary for Rho-A activation and axon growth inhibition. Furthermore, the cleavage of $p75^{NTR}$ is PKC-dependent (Domeniconi et al., 2005).

Importantly, Zhigang He's group has reported that the EGFR/ERK signaling pathway is activated in response to inhibitors in myelin and the glial scar and is regulated by Ca^{2+} signaling. Blocking EGFR kinase activity was overcome myelin-mediated

neurite outgrowth inhibition manner and promoted optic nerve regeneration (Koprivica et al., 2005).

1.2.4 Formation of the Glial Scar

1.2.4.1 Physical Barrier to Axon Regeneration

One of the main barriers to axonal regeneration is the development of glial scar, which is thought to form in an effort to seal off the wounded area and minimize inflammation (Silver and Miller, 2004, Yiu and He, 2006). Subsequently, various extracellular matrix molecules are deposited in and around the glial scar by proliferating astrocytes, as well as inflammatory cells. In more severe injuries to the CNS, where the meninges are disrupted, astrocytes become mixed with other cells, such as fibroblasts. Eventually, this network of reactive astrocytes creates a dense glial scar structure, presenting both a physical and biochemical barriers to the regenerating axons (Silver and Miller, 2004).

1.2.4.1 Inhibitors in the Glial Scar

While glial scar formation presents an impenetrable barrier for regenerating axons, it acts not only as a physical barrier but also as a reservoir of inhibitory molecules that were shown to inhibit axonal regrowth. One family of molecules of particular interest is chondroitin sulfate proteoglycans (CSPGs), which are extracellular matrix (ECM) proteins that can interact with cell adhesion molecules and integrins. CSPGs are considered a major inhibitory component of the glial scar and have been shown to

dramatically inhibit axonal outgrowth both *in vitro* and *in vivo* (Rudge and Silver, 1990, McKeon et al., 1991, Jones et al., 2002). CSPGs (aggrecan, brevican, neurocan, versican, phosphacan and NG2) are part of the proteoglycan family of molecules, and they are characterized by a protein core to which glycosaminoglycan (GAG) sugar chains are attached (Morgenstern et al., 2002). CSPGs are expressed by astrocytes and their expression is highly upregulated after CNS injury.

In more severe CNS lesions where the meninges are opened, astroglial cells become mixed with connective tissue elements such as fibroblasts, which have been shown to express repulsive axonal guidance molecules such as ephrins and semaphorins. Ephrins and their receptors, EphB2 play a major role in axonal pathfinding and are identified as axonal chemorepellents that were shown to inhibit axonal growth (Benson et al., 2005). Ephrins were shown to be expressed by reactive astrocytes, while EphB2 receptors were shown to be expressed by certain CNS neurons, so it is thought that interactions between the two cell types underlie some the inhibitory characteristics of the glial scar (Bundesen et al., 2003). Genetic deletion of EphA4, which is also expressed by astrocytes, was shown to reduce reactive astrocyte hypertrophy or gliosis and inhibition of axonal growth both *in vivo* and *in vitro* was reduced (Goldshmit et al., 2004). In addition, ephrin-B3 has been found to be expressed in CNS myelin and was shown to inhibit neurite outgrowth of cerebellar and cortical neurons (Benson et al., 2005).

The second class of axonal guidance cues is the semaphorins, which like ephrins, exhibit prominent repulsive action during early neuronal development. Sema3A is expressed by fibroblasts following CNS injury, while Sema4D, which is expressed by oligodendrocytes and myelin, inhibits neurite outgrowth of postnatal DRG and cerebellar

neurons (De Winter et al., 2002, Moreau-Fauvarque et al., 2003). Taken together, the dense structure and the associated inhibitors in the glial scar are responsible, at least partially, for the failure of axonal regeneration in the adult CNS following injury.

1.3 Strategies to Block Inhibitors in Myelin and the Glial Scar.

1.3.1 Blocking the Extrinsic Environment to Overcome Inhibition

1.3.1.1 Blocking myelin inhibitors

The observation that all three myelin inhibitors, MAG, Nogo-A and OMgp, interact with the same receptor complex was a very intriguing and encouraging finding. It suggests a redundancy in the signaling mediated by myelin-associated inhibitors and also provides potential targets for therapeutic intervention to encourage axonal regeneration.

Neutralizing myelin inhibitors by generating myelin-specific antibodies was one of the first approaches used to block myelin-associated inhibitors. Reports from two studies indicate that administration of the IN-1 antibody, which is raised against one of Nogo's inhibitory domains, Nogo-66, resulted in CST axon regeneration following spinal cord dorsal hemisection (Schnell and Schwab, 1990, Brosamle et al., 2000).

An additional approach to blocking Nogo signaling is by utilizing a peptide called NEP1-40 to block NgR. NEP-40 is a 40 amino acid antagonist peptide, corresponding to the Nogo-66 sequence. It blocks one of Nogo's ligand binding sites within NgR.

Application of NEP1-40 relieved myelin-induced inhibition of axonal outgrowth *in vitro* and improved regeneration *in vivo* (GrandPre et al., 2002, Li and Strittmatter, 2003). It remains to be elucidated whether the action of this peptide blocks binding of all three inhibitors.

As mentioned, genetic deletion of Nogo produced conflicting results, and the overall conclusion in the majority of the studies is that genetic deletion of Nogo does not result in CST axonal regeneration following injury (Simonen et al., 2003, Zheng et al., 2003, Kim et al., 2004a).

Genetic deletion of MAG did not improve axon regeneration of CST and RGC fibers following spinal cord dorsal hemisection or optic nerve crush, respectively (Bartsch et al., 1995). However, another study reported enhanced regeneration in the CST in MAG^{-/-} mice compared to wild type mice (Li et al., 1996).

1.3.1.2 Blocking Glial Scar Inhibitors

In culture, inhibition of neurite outgrowth has been observed in many types of CNS neurons when CSPGs were used as a substrate (Bovolenta et al., 1993a, b). The inhibition can be largely reversed with the enzyme chondroitinase ABC (ChABC), which digests the chondroitin sulfate glycosaminoglycan (CS-GAG) portion of CSPGs. These findings suggest that the GAG chain portion is crucial for the inhibition of neurite outgrowth by CSPGs. *In vivo*, intrathecal delivery of ChABC into the CST and nigrostriatal tract following injury resulted in increased regeneration in both tracts and enhanced CST functional recovery (Moon and Fawcett, 2001, Bradbury et al., 2002).

In more severe lesions to the CNS where the meninges are disrupted, fibroblasts, which express Sema3A, intermix with astroglia. Blocking Sema3A, whose expression is upregulated following CNS injury, promotes survival of RGCs but no axonal regeneration was observed (Shirvan et al., 2002). In summary, blocking glial scar inhibitors results in increased axonal regeneration, supporting the notion that inhibitors associated with the glial scar make a significant contribution to axonal growth inhibition following an injury.

1.3.2 Changing the Intrinsic State of Neurons to Overcome Inhibition

1.3.2.1 Blocking the Receptor for Myelin Inhibitors

Once the NgR-p75^{NTR}/Troy-Lingo-1 receptor complex was identified as the functional receptor for myelin inhibitors, the number of targets for therapeutic intervention increased dramatically. One could now block NgR, or block NgR from associating with its coreceptors p75^{NTR} / Troy or Lingo-1 to encourage regeneration.

Different strategies to block NgR activity and expression have yielded conflicting results. Blockade of NgR via viral infection of truncated mutant NgR led to increased RGC axon regeneration following optic nerve crush (Fischer et al., 2004). Furthermore, intrathecal administration of NgR-blocking peptides resulted in sprouting of CST and raphespinal fibers following spinal cord dorsal hemisection, and this was associated with improved functional recovery (Li et al., 2004, Li et al., 2005). On the other hand, genetic

deletion of NgR failed to promote CST regeneration following injury (Kim et al., 2004b; Zheng et al., 2005).

In vitro, neurons from p75^{NTR} -/- mice or dominant expression of p75^{NTR} overcame neurite outgrowth inhibition by all three myelin-associated inhibitors or by myelin in general. However, genetic deletion of p75^{NTR} did not promote axon regeneration in the CST or dorsal column following spinal cord injury (SCI) (Wang et al., 2002c, Wong et al., 2002, Song et al., 2004).

Thus, whereas blocking existing NgR induced *in vivo* axon regeneration, genetic deletion of NgR or its coreceptor p75^{NTR} had no effect on axon regeneration, raising the possibility that compensatory pathways and/or the presence of other functional receptors for myelin inhibitors exist.

Indeed, PirB was recently identified as an additional functional receptor for myelin inhibitors. *In vitro*, CNS neurons isolated from mutant PirB mice were less inhibited by myelin inhibitors. In addition, PirB antibody was able to partially block the inhibition by Nogo 66, MAG and total myelin both in cerebellar and DRG neurons. Interestingly, while blocking PirB alone was able to partially overcome inhibition, blocking both NgR and PirB completely abolished myelin's ability to inhibit neurite outgrowth, indicating that both NgR and PirB are independently activated by myelin inhibitors to block axon regrowth following injury to the CNS (Atwal et al., 2008). However, the importance of PirB in the axonal regeneration model *in vivo* has not yet been established.

1.3.2.2 Blocking Downstream Inhibitory Signaling Mediators

Considering the many ligands and receptors that mediate inhibition by CNS myelin and the glial scar, targeting individual components might not be the most efficient way to overcome these inhibitory influences. Instead, identifying an intracellular pathway that is common to multiple sources of inhibition could offer a better prospect for promoting axon regeneration.

So far, the best characterized pathway and the converging point for both myelin and glial scar inhibitors is the small GTPase RhoA. RhoA signaling, which is known to be a regulator of actin cytoskeleton dynamics, was reported to induce growth cone collapse and axon guidance repulsion (Hall, 1998, Maekawa et al., 1999).

In vitro, inhibiting RhoA activation by using the toxin C3 transferase, which hydrolyzes RhoA-GTP to RhoA-GDP, or dominant negative RhoA promotes axon outgrowth on inhibitory substrates. In addition, pharmacological inhibition of the RhoA effector, Rho associated kinase (ROCK), produced similar results (Lehmann et al., 1999, Dergham et al., 2002, Fournier et al., 2003). *In vivo* delivery of the RhoA inhibitor C3 transferase or ROCK inhibitor Y-27632 following SCI promotes CNS axon regeneration and improved functional recovery in several studies (Lehmann et al., 1999, Dergham et al., 2002, Fournier et al., 2003).

An additional signaling pathway that is common to both myelin and glial scar inhibitors is protein kinase C (PKC) activation. Pharmacological inhibition of conventional PKC isoforms (PKC α , β) attenuates neurite outgrowth inhibition and RhoA activation by both myelin and CSPGs (Hasegawa et al., 2004, Sivasankaran et al., 2004). *In vivo* administration of the PKC inhibitor Go6976 following spinal cord dorsal

hemisection resulted in dorsal column regeneration, but did not promote CST fiber regeneration (Sivasankaran et al., 2004).

Epidermal growth factor receptors (EGFRs) are an additional signaling pathway that was shown to contribute to neurite outgrowth inhibition by myelin and CSPGs. Myelin-associated inhibitors were shown to induce phosphorylation of EGFR in Ca^{2+} -dependent manner, whereas administration of EGFR inhibitor PD168393 resulted in enhanced axonal regrowth both *in vitro* and *in vivo* (Koprivica et al., 2005). However, the failure to detect EGFR in the NgR receptor complex, suggest trans-activation of EGFR by means of signaling pathways downstream of active NgR (Koprivica et al., 2005).

1.3.2.3 Activating Growth-Promoting Signaling: PTEN/mTOR Pathway, NTs, cAMP, and PAs

An alternative approach to encourage axonal regrowth following CNS injury is the manipulation of intrinsic growth control pathways which can be repressed in mature post-mitotic neurons (Weinberg et al, 2007).

It has been recently demonstrated by Zhigang He's group that the activation of the mammalian target of rapamycin (mTOR) pathway by a conditional deletion of its negative regulators phosphatase and tensin homologue (PTEN) or tuberous sclerosis complex 1 (TSC1), results in RGC axon regeneration following an optic nerve crush injury. Furthermore, in wild type adult mice mTOR activity is suppressed and as a result, protein synthesis is impaired in axotomized RGCs, which may block CNS axonal regeneration (Park et al., 2008).

Our lab has demonstrated that prior exposure of postnatal neurons to neurotrophins (NTs) (aka. Priming) is sufficient to block the inhibition by MAG or

myelin (Cai et al., 1999). If, however, NTs are added to neurons at the same time as myelin inhibitors, neurite outgrowth inhibition persists. Our lab also demonstrated that priming is mediated by the elevation of intracellular cyclic AMP (cAMP), since protein kinase A or (PKA) inhibitor co-treatment abolished the priming effect (Cai et al., 1999). *In vivo*, administration of NTs along with a permissive substrate, like fetal spinal cord, resulted in enhanced axonal regeneration (Bregman et al., 1997).

In order to determine if the priming effect can be mimicked by cAMP, a membrane permeable cAMP analogue, such as dibutyryl cAMP (dbcAMP) or 8-bromo-cAMP, was utilized and proved to be sufficient for blocking neurite outgrowth inhibition by MAG and myelin substrates (Cai et al., 1999). Our lab has also demonstrated that the endogenous level of cAMP correlates with the neuronal response to MAG/myelin. Young neurons, in which axonal growth is promoted by MAG/myelin, express high levels of endogenous cAMP; whereas older neurons of the same type are inhibited by MAG/myelin, express low levels of endogenous cAMP (DeBellard et al., 1996, Cai et al., 2001). Other reports demonstrated additional role for cAMP signaling as a modulator of growth cone turning, since the growth cone response can be reversed to repulsion by blocking cAMP-dependent protein kinase A (PKA) (Ming et al., 1997, Song et al., 1997, Song et al., 1998).

DRG neurons have axons projecting to the PNS (peripheral process) and axons projecting to CNS (central process). It was shown that lesioning of the peripheral sciatic nerve seven days prior to a central lesion, will enhance the central axons regeneration. This unique phenomenon is termed the conditioning lesion effect (Neumann and Woolf, 1999). The role of cAMP signaling in promoting axonal regeneration after a conditioning

lesion was also demonstrated by studies in our lab (Qiu et al., 2002). It was initially shown that one day after conditioning lesion, cAMP levels in DRG neurons increased by three-fold, blocking MAG and myelin's inhibition of neurite outgrowth in a PKA-dependent manner. One week following the conditioning lesion, cAMP levels return to control levels, but growth on MAG or myelin substrate is still enhanced and is now PKA-independent (Qiu et al., 2002). The observation that the conditioning lesion effect is PKA-dependent one day post-lesion suggests that the conditioning lesion effect is cAMP-dependent. This was confirmed by *in vivo* administration of dbcAMP into the DRG following a central DRG lesion, which resulted in the enhancement of central axon regeneration (Neumann and Woolf, 1999, Qiu et al., 2002). These studies demonstrate that the intracellular levels of cAMP dictate the regenerative capacity of severed CNS neurons.

Another way to increase intracellular cAMP is to inhibit its degradation. Our lab has shown that the inhibition of phosphodiesterase 4 (PDE4), a group of enzymes responsible for cAMP degradation, by administration of rolipram, resulted in enhanced axon growth *in vivo* and *in vitro* (Nikulina et al., 2004). Interestingly, in rolipram treated rats, attenuation of the glial scar was also observed, which alleviates its physical and biochemical barrier properties (Nikulina et al., 2004). Furthermore, rolipram administration resulted in significant axon sparing and promoted myelination when combined with peripheral Schwann cell grafts following spinal cord contusion injury (Pearse et al., 2004). A combinatorial treatment of subcutaneous delivery rolipram with local increase in cAMP at the growth cone spinal cord injury site, along with transplanted Schwann cells was shown to enhance axon regeneration and improve functional recovery,

indicating that combinatorial drug therapy can be more effective than administration of any drug or treatment alone (Pearse et al., 2004).

In order to determine if cAMP-mediated signaling induce changes in gene expression, a transcriptional inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) was used to block RNA polymerases. When DRB was included, the ability of either NTs priming or dbcAMP treatment to overcome MAG/myelin induced inhibition was completely abrogated, suggesting that NTs or cAMP effect is dependent on gene transcription (Cai et al., 2002). It was later demonstrated that cAMP response element binding protein (CREB), which can be activated by both NTs and cAMP treatment, is a critical transcription factor involved in modulating the regrowth capacity of mature neurons (Gao et al., 2004). It was shown that overexpressing a dominant negative CREB blocked the ability of NTs and cAMP to overcome MAG/myelin mediated inhibition, while expression of a constitutively active CREB resulted in enhanced neurite outgrowth in the presence of MAG/myelin inhibitors.

In order to identify which genes are upregulated or downregulated in response to cAMP treatment, a microarray analysis was conducted, in which RNA was isolated from cAMP-treated or conditionally lesioned DRG neurons, reverse transcribed to cDNA and hybridized to a custom-made array. Among the genes that were upregulated was Arginase I (ArgI), the rate limiting enzyme in polyamine synthesis, which was also shown to be upregulated in response to NTs treatment, and downregulated in response to dominant negative CREB infection (Cai et al., 2002, Gao et al., 2004). Work from our lab then demonstrated that overexpression of ArgI by viral infection or exogenous administration of polyamines, is each sufficient to overcome neurite outgrowth inhibition

by MAG or myelin (Cai et al., 2002). Conversely, blocking the polyamine synthesizing enzymes, ArgI or the next enzyme in the polyamine synthesis, ornithine decarboxylase, can each abrogate the ability of cAMP or BDNF to block myelin inhibitors (Cai et al., 2002). Furthermore, similar to cAMP endogenous expression, ArgI expression is high in young neurons and decreases as neurons mature (Cai et al., 2002). Importantly, our lab has recently demonstrated that *in vivo* administration of the polyamine spermidine, which has been shown to be the main effector in blocking myelin inhibition, can promote RGC axon regeneration following optic nerve crush (Deng et al., 2009). Interestingly, during the late 1980s and mid 90s, several papers demonstrated that the administration of polyamines resulted in enhanced axon regeneration *in vivo*, but none of the studies characterized polyamines ability to overcome myelin inhibitors and their prominent role in the conditioning lesion effect (Gilad and Gilad, 1988, Gilad et al., 1996).

Polyamines (putrescine, spermidine and spermine) have long been associated with cell growth and development, cytoskeleton regulation, and interestingly, were shown to modulate glutamate receptors (iGluRs) (Lerma, 1992, Williams, 1997). Polyamines are thought to exert their role in part by direct interaction with acidic substances such as DNA, RNA, and proteins, thus affecting both transcription and translation machinery in the cell.

1.4 Ionotropic Glutamate Receptors

1.4.1 Background

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate the majority of excitatory neurotransmission in the CNS. Ligand-gated ion channels are abundantly expressed in the CNS as membrane proteins and their function relies on their ability to respond very rapidly to a transient release of a neurotransmitter, producing a change in the membrane potential of the postsynaptic cell.

Mammalian iGluR ion channels are encoded by eighteen genes that make up three major families, the N-methyl-D-aspartate receptors (NMDAR), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors (KAR) (Collingridge and Lester, 1989). NMDARs are encoded by seven genes and are thought to form tetramer structures with two mandatory NR1 subunits and two NR2 subunits (NR2A-D) or in some cases, NR3 (NR3A-B) (Dingledine et al., 1999, Chatterton et al., 2002). NMDARs subtypes are differentially expressed in the adult brain. Whereas NR1 and NR2A are ubiquitous, NR2B is restricted to the forebrain, NR2C to the cerebellum, and NR2D expression is restricted to a few numbers of cells in selective brain regions, such as the cortex (Kohr, 2006). AMPA receptors is encoded by four genes (GluR1 - GluR4), whereas KARs are encoded by five genes (GluR5 - GluR7 and KA1 - KA2). AMPA and KARs can form homomeric or heteromeric tetramer structures, while AMPA receptors can only form homomeric tetramers (Rosenmund et al., 1998).

Activation of NMDARs is a complex process. It requires the binding of an agonist, the neurotransmitter glutamate, which is transiently released from presynaptic terminals in an activity-dependent manner, as well as the binding of a coagonist, glycine,

which acts as a modulator and is present in the extracellular fluid at more constant level (Kuryatov et al., 1994, Laube et al., 1997). Electrophysiological studies have demonstrated the existence of two independent glycine binding sites on the NR1 subunits, as well as two independent glutamate binding sites on the NR2 subunits (Benveniste and Mayer, 1991, Clements and Westbrook, 1991). On the other hand, binding of glutamate alone is sufficient for AMPA/kainate receptor activation (Kleckner and Dingledine, 1988, Patneau and Mayer, 1990).

Structural analysis studies revealed that NMDARs are hetero-oligomeric ligand-gated cation channels, where each subunit has a large extracellular amino terminal region and a portion of this region (the S1 domain), together with an extracellular M3-M4 loop (the S2 domain), forms the agonist binding site. The co-agonist glycine binds NR1 subunit and in some cases, NR3, whereas glutamate binds NR2 (NR2A-NR2D) subunits (Cull-Candy et al., 2001) (Laube et al., 1997, Furukawa et al., 2005). In AMPA receptors, the glutamate agonist binding site is mapped to GluR0 and GluR2, while in kainate KARs, the agonist binding site is found on GluR5 and GluR6 (Armstrong et al., 1998, Panchenko et al., 2001, Mayer, 2005).

To regulate their activation, NMDAR ion channels are blocked in a voltage-dependent manner by extracellular Mg^{2+} , leading to a decreased conduction at resting potential, with a little or no current flow (Mayer and Westbrook, 1987). Thus, in addition to having agonists and coagonists bound, activation of NMDARs is activity-dependent, requiring action potentials in order to displace Mg^{2+} .

The regulation of the strength and timing of AMPA/kainate receptor synaptic transmission is based on the functional properties as well as the number of channels that

are clustered at synapses. Therefore, current through AMPA receptors regulates downstream processes like NMDARs activation and action potential generation.

Once activated by glutamate, AMPA/kainate channels cause Na^+ influx, K^+ efflux, while being impermeable to Ca^{2+} . A small subset of AMPA receptors, however, which lack the GluR2 subunit, have demonstrated increased Ca^{2+} permeability (Burnashev et al., 1992, Jonas et al., 1994). Activation of the AMPA channels results in a partial membrane depolarization, which is sufficient to lift the Mg^{2+} block, resulting in the activation of NMDARs. Once activated, NMDAR causes Na^+ and Ca^{2+} influx, and K^+ efflux. It is via this Ca^{2+} influx that NMDARs exert their intracellular physiological effects.

1.4.2 Glutamate Receptor Function in CNS

NMDARs play a central role in synaptic transmission and in mediating plasticity including long term potentiation (LTP), and long term depression (LTD), processes that are believed to underlie learning and memory (Malenka and Bear, 2004). NMDARs are also present in oligodendrocytes and astrocytes, as well as non-neuronal cells such as osteoclasts and chondrocytes, where their function is not well understood (Szczesniak et al., 2005, Verkhratsky and Kirchhoff, 2007, Ramage et al., 2008).

While it is well established that excessive NMDAR activity induces destructive effects, the survival of several neuronal types is dependent on physiological synaptic NMDAR activation (Ikonomidou and Turski, 2002, Hardingham and Bading, 2003). In the developing CNS, blocking NMDAR activity *in vivo* causes a decrease in the number of healthy cells and severe deterioration of the dentate gyrus morphology in first week

postnatal rat pups, as well as causing widespread cell death and abnormal wiring in the CNS during the period of synaptogenesis (Brenneman et al., 1990). In the adult CNS, NMDAR blockade can exacerbate neuronal loss when applied after traumatic brain injury and during ongoing neurodegeneration, and prevents the survival of newborn neurons in the adult dentate gyrus (Ikonomidou et al., 2000; Tashiro et al., 2006). Activation of Ca^{2+} -dependent pro-survival signaling such as PI3K/AKT kinase cascade, CRE cAMP-response element-binding protein (CREB) were shown to contribute to the pro-survival effects of NMDARs (Papadia et al., 2005; Lafon-Cazal et al., 2002; Hardingham et al., 2002). An example for CREB-induced target gene is the NT BDNF, which is known to promote survival of neurons (Hardingham et al., 2002). BDNF was shown to be upregulated by NMDAR activity, whereas blocking NMDAR activity *in vivo* reduces BDNF mRNA expression, and *in vitro* application of BDNF can rescue neurons from NMDAR antagonist-induced cell death (Tao et al., 1998; Hansen et al., 2004).

1.4.3 NMDA Receptor Pathological Role in CNS Injury and Disease

While iGluR-induced activity is essential to the normal function of the CNS, persistent activation by excessive levels of glutamate is thought to contribute to neuronal damage and cell death following CNS injury and in neurological disorders.

NMDARs were shown to cause cell death in many neuropathological conditions when intensely or chronically activated, and are thought to contribute to the aetiology of many chronic neurodegenerative disorders, such as Huntington disease, amyotrophic lateral sclerosis (ALS), Parkinson's and Alzheimer's diseases (Lipton and Rosenberg,

1994, Lancelot and Beal, 1998, Kaul et al., 2001, Chen and Lipton, 2006). It is thought that certain aspects of these disorders render neurons particularly vulnerable to endogenous NMDARs-mediated activity or unable to carry out normal glutamate homeostasis. Furthermore, in injured CNS tissue, neurons are exposed to a microenvironment that contains toxic factors, such as an imbalance in the levels of excitatory amino acids and ions, reactive oxygen species, and free radicals. Consequently, disturbance of extracellular glutamate levels or excessive stimulation of iGluRs results in excitotoxicity, which has been shown to be the primary cause of neuronal injury and death following acute trauma such as stroke, mechanical trauma or seizure (Olney et al., 1986, Dirnagl et al., 1999, Lipton, 2006)

In theory, any CNS disorder, in which neuronal loss is caused by glutamate-induced excitotoxicity has the potential to be treated by blocking NMDARs. Several NMDAR antagonists were proven to be neuroprotective agents in animal models of stroke, but failed in human clinical trials of stroke and traumatic brain injury (Hoyte et al., 2004, Lipton, 2007). It seems that the equivalent to neuroprotective doses in animal studies are very toxic to humans or produce serious side effects, such as hallucinations / psychosis or in some cases catatonia and anesthesia (Lipton, 2004, 2006). One of the reasons for having these adverse side effects is because the antagonists block all NMDAR-induced neurotransmission, including basal NMDAR activity, which is essential for neuronal homeostasis. Several antagonists that have been utilized were shown to block both pathological and basal activation of receptors. Examples include 2-amino-5-phosphonovalerate (APV), which blocks all channel subtypes regardless of their activation state, or MK-801, which irreversibly binds all open channels. An alternative

blocking approach is to preferentially inhibit the activation of excessively active receptors in a reversible manner while not disrupting basal NMDAR activity. This can be accomplished by utilizing low-affinity, reversible, subtype-specific activity blockers. Memantine is such a drug. It is the first NMDAR blocker that was clinically approved for the treatment of moderate to severe stages of Alzheimer's disease and vascular dementia, due to its neuroprotective efficacy and mild side effects (Lipton, 2006). Memantine is a low-affinity uncompetitive antagonist, which selectively targets persistently-activated NMDAR ion channels with faster reversibility. One of its off-targets, however, is its non-specific interactions with the serotonin receptor and nicotinic α -7 receptors (Chen and Lipton, 2006). On the other hand, a similar drug, Ifenprodil, which is shown to target only persistently activated NR1/NR2B containing NMDAR channels, was shown to be neuroprotective in animal stroke models with minimal side effects at maximal dose, but unfortunately, failed in clinical trials because its high affinity binding blocked normal NMDAR activation (Gotti et al., 1988, Kew et al., 1996, Gill et al., 2002). A different approach to avoid unwanted side effects is to ensure that NMDARs are only partially blocked. This can be achieved by selectively blocking the glycine binding site. Preclinical studies suggest that glycine site antagonists possess a better side effect profiles, although chemically, developing glycine-site antagonists has proven to be challenging (Leeson and Iversen, 1994, Tranquillini and Reggiani, 1999). Taken together, the dual role of NMDARs in normal and pathological functioning of the nervous system imposes important constraints on possible therapeutic strategies aimed at ameliorating neurological diseases. Nevertheless, the complexity of NMDAR activation presents many opportunities for developing modulatory drugs that will balance between blocking the

persistently-activated channels while sparing resting or low basal NMDAR ion channel activation which is critical for normal brain function.

1.4.4 Modulation of Glutamate Receptor Activity by Polyamines

Given that our lab has demonstrated that *in vivo* application of polyamines is sufficient to enhance axonal regeneration and promote neurite outgrowth in the presence of MAG/myelin *in vitro*, we were interested in exploring polyamines potential targets in order to elucidate the mechanism by which they block myelin inhibition.

Studies have reported that polyamines, specifically spermidine or spermine, can directly bind to iGluR channels, resulting in increased activity or its blockage. This depends on the state of the neuron, polyamines levels, or receptor subtype (Benveniste and Mayer, 1993, Williams, 1997). Electrophysiological analyses have shown that polyamines are able to block the activity of a subset of AMPA and kainate channels, (Usherwood and Blagbrough, 1991, Bowie and Mayer, 1995), while promoting activation or blockage of NMDAR channels, depending of NMDAR subtype, neuronal state, and polyamines levels (Williams et al., 1990, Lerma, 1992, Araneda et al., 1993, Araneda et al., 1999). Because polyamines can both overcome MAG/Myelin-associated inhibition and modulate iGluR activity, we were interested to determine whether blocking iGluR activity with antagonists will do the same.

It was reported previously that the polyamines binding site is located on NMDAR NR2B subunits (Williams, 1993). Therefore, in order to differentiate polyamines role in the context of NMDARs, the pharmacological NMDAR channel blocker, Ifenprodil was

utilized to specifically block the polyamine's binding site, which is located on NR2B subunits (Williams, 1993).

1.4.5 NMDAR-Induced MAPK/ERK Activation

It is well accepted that NMDARs are crucial for synaptic plasticity. NMDAR-dependent activity determines the Ca^{2+} concentration inside the postsynaptic cell, with moderate activity triggering LTD, whereas strong activation produces LTP (Mulkey and Malenka, 1992, Bliss and Collingridge, 1993, Malenka and Bear, 2004). In turn, activity-dependent Ca^{2+} influx triggers the activation of several protein kinases such as PKA, PKC and MAPK (Bliss and Collingridge, 1993, Yasuda et al., 2003, Thomas and Huganir, 2004). Activation of the MAPK signaling pathway is believed to be important for LTP induction (Thomas and Huganir, 2004). Furthermore, PKA and PKC activation are also shown to be important for LTP, while their inactivation has been shown to be central for LTD induction (Bliss and Collingridge, 1993, Daw et al., 2002, Yasuda et al., 2003, Thomas and Huganir, 2004).

Mitogen-activated protein kinase (MAPK) consists of three major members: extracellular signal-regulated kinase 1 and 2 (ERK), p38, and c-Jun N-terminal kinase, each is involved in a separate signaling pathway. While the activation of the ERK pathway was shown to regulate cell growth, survival and differentiation in the developing CNS (Pearson et al., 2001), several recent reports have implicated this cascade in the control of synaptic plasticity of the adult CNS. Neuronal MAPK/ERK signaling was reported to be directly activated in response to membrane depolarization or glutamatergic signaling (Fiore et al., 1993, Xia et al., 1996). ERK activation that is induced by these

stimuli is Ras and Ca^{2+} -dependent (Rosen et al., 1994). Furthermore, Ca^{2+} -dependent Ras guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which catalyze the conversion of Ras to an active or inactive form, respectively, were also identified, which suggests that Ras, an established activator of ERK is downstream of Ca^{2+} (Farnsworth et al., 1995, Walker et al., 2003). Therefore, Ca^{2+} -dependent MAPK/ERK activation is initiated by NMDARs or voltage-gated Ca^{2+} channels. It is not clear, however, how Ca^{2+} influx triggers Ras activation. The importance of MAPK signaling in synaptic plasticity was demonstrated by several studies where the use of MAPK/ERK pharmacological inhibitors, resulted in the block of a specific form of synaptic plasticity. For example, it has been shown that administration of an ERK inhibitor, PD 98059 or U0126, which blocks ERK's upstream activator MEK, blocked ERK activity and the induction of LTP in many forebrain regions such as the hippocampus CA1-CA3, dentate gyrus, cortex, and amygdala (Schafe et al., 2000, Di Cristo et al., 2001, Adams and Sweatt, 2002).

The ERKs have recently been shown to play an important role in memory consolidation. A new study suggests that in the hippocampus, there is circadian oscillation of ERK activation that is essential for persistent and stable memory-formation (Eckel-Mahan, Phan T 2008)

1.4.6 Activation of MAPK/ERK Signaling Following CNS Injury and in Neurodegenerative Diseases

As mentioned previously, our lab has demonstrated that NTs ability to block MAG/myelin-mediated neurite outgrowth inhibition is abolished when ERKs are inhibited, therefore suggesting that the NT effect is ERK-dependent (Gao et al., 2003). Moreover, administration of Rolipram, which blocks cAMP degradation by inhibiting PDE4, along with NTs and ERK inhibitors restored NTs ability to overcome neurite outgrowth inhibition by MAG/myelin, suggesting that activation of ERK triggers the inhibition of PDE4 (Gao et al., 2003). Administration of rolipram, which elevated cAMP levels, was sufficient to overcome MAG/myelin-mediated inhibition and promote regeneration following CNS injury (Nikulina et al., 2004).

Paradoxically, MAPK/ERK signaling was shown to be involved in the inhibitory pathway as well. It was reported that suppressing the kinase function of epidermal growth factor receptor (EGFR) overcomes neurite outgrowth inhibition by myelin and glial scar inhibitors *in vitro*, and promotes regeneration of injured optic nerve fibers *in vivo* (Koprivica et al., 2005). Epidermal growth factors (EGFs) are mitogenic factors that stimulate the proliferation of different cell types. EGF activates the EGFRs, which in turn, initiates intracellular signaling that culminates in the activation of ERKs. The He group also reported that myelin inhibitors triggered the activation of EGFRs in Ca^{2+} -dependent manner, and resulted in ERKs activation (Koprivica et al., 2005). The source of Ca^{2+} remains to be determined; it could be released from intracellular stores into the lumen or in the cytoplasm in response to activity via NMDAR and/or voltage-gated Ca^{2+} channels.

Reactivation of the EGFR/ERK pathway in astrocytes has been also shown to occur following injury in the adult CNS, causing quiescent astrocytes to become reactive astrocytes and form the scar (Miller, 2005). In addition, CNS and PNS injury-induced activation of MAPK pathways in glial cells and DRG neurons were reported to be necessary for the development and maintenance of neuropathic pain (Woolf and Salter, 2000, Watkins et al., 2001, DeLeo et al., 2004, Ji and Strichartz, 2004).

ERK activation has been also implicated in the pathogenesis of Alzheimer's disease. Abnormal activation of ERK is seen in degenerating neurons in the hippocampus and ERK was shown to be persistently activated by amyloid- β as well as inducing the activation of tau *in vitro*. The abnormal functions of Amyloid- β and tau is part of the phenotypic hallmark of the Alzheimer's disease (McShea et al., 1999, Rapoport and Ferreira, 2000, Holzer et al., 2001).

Taken together, depending on the cellular state, the different activators and spatial-temporal control, the MAPK/ERKs cascade can mediate growth, survival and synaptic plasticity in intact neurons, whereas CNS injury or disease can induce a pathological MAPK activation that might be detrimental for neuronal survival and function.

1.4.7 NMDA Receptors and PKC Signaling

Several studies have demonstrated the importance of protein kinase C (PKC) signaling in the induction of LTP and LTD. Various reports indicate that the activation of this pathway is important for LTP, while its inactivation is required for LTD (Malinow et

al., 1989, Bliss and Collingridge, 1993, Malenka and Nicoll, 1999). The exact role of PKC in synaptic plasticity as well as its mode of activation is the subject of intense controversy and conflicting results have been produced. During the last decade, however, more comprehensive reports regarding PKC function in synaptic plasticity have emerged, implicating it as a modulator of NMDAR trafficking to and from the synapse. This is accomplished in part by inducing phosphorylation of serine residues on the C-terminal cassette of NR1 (Tingley et al., 1997, Xiong et al., 1998, Zheng et al., 1999). Importantly, PKC-induced forward NMDAR trafficking to the membrane was demonstrated to be important for LTP, while reverse trafficking or internalization of NMDARs is important for LTD (Snyder et al., 2001, Grosshans et al., 2002). PKC-induced NMDAR trafficking will be discussed in more detail in the NMDAR trafficking section.

1.4.8 PKC Role in the Inhibition of CNS Axon Regeneration

There is ample evidence has accumulated regarding the conventional isoforms of protein kinase C (PKC- α , β) are one of the key components in the inhibitory signaling pathways initiated by myelin and CSPGs. The Yamashita and He groups have reported that inhibitors in myelin and the glial scar induce PKC activation (Hasegawa et al., 2004, Sivasankaran et al., 2004). Consequently, disrupting PKC activity, either pharmacologically or genetically, attenuated the ability of myelin and glial scar inhibitors to activate RhoA and inhibit neurite outgrowth (Hasegawa et al., 2004, Sivasankaran et al., 2004). Furthermore, blocking PKC activity *in vivo* resulted in axon regeneration following dorsal hemisection (Sivasankaran et al., 2004).

It was later shown by our lab that MAG induces regulated intramembrane proteolysis (RIP) of p75^{NTR}, which is required for inhibition of neurite outgrowth. Importantly, MAG-induced RIP is PKC-dependent (Domeniconi et al., 2005).

Taken together, activation of conventional Ca²⁺-sensitive PKCs by regeneration inhibitors triggers RhoA activation and p75^{NTR} cleavage, two central events in the signaling pathways that inhibit axonal regrowth, pointing to PKC as an important mediator of CNS regeneration failure.

1.4.9 Synaptic Plasticity Regulation by NMDA Receptor Trafficking

An emerging concept is that activity-dependent, bidirectional regulation of NMDAR trafficking by altering its phosphorylation state provides a dynamic and potentially powerful mechanism for the regulation of synaptic plasticity (Collingridge et al., 2004, Lee, 2006). New research provides support that synaptic NMDAR number and subunit composition changes dynamically in a cell-specific and synapse-specific manner during development and in response to neuronal activity or sensory experience (Lee, 2006).

During synaptogenesis, assembled NMDARs are targeted selectively to the postsynaptic side of glutamatergic synapses and appear together with AMPARs within hours of initial axodendritic contact (Friedman et al., 2000). Prior to synapse formation, NMDARs cycle in and out of the dendritic plasma membrane during pauses of movement along microtubules while trafficking to distal axons (Washbourne et al., 2004). By

contrast, in older animals, when most of the synapses have formed, NMDAR subunits are recruited more gradually to nascent synapses in receptor clusters (Bresler et al., 2004). Thus, synaptic recruitment of NMDARs might occur via distinct mechanisms at differing ages. At immature synapses, targeting of newly synthesized NMDARs is tightly regulated and follows subunit-specific rules. In embryonic hippocampal neuron synapses, NMDARs are primarily NR1-NR2B heterodimers, while during postnatal development there is a gradual switch to NR1-NR2A NMDAR heterodimers (Kohr, 2006). It is hypothesized that NR1-NR2A containing NMDARs promote fine-tuning in mature synapses as they display the fastest decay kinetics, resulting in shortening of NMDA-mediated synaptic current, which is known to affect synaptic plasticity (Sheng et al., 1994, Tovar and Westbrook, 1999).

Multimeric membrane proteins, such as NMDAR and AMPA/KAR are synthesized as individual subunits and assembled into a complex in the endoplasmic reticulum (ER). Their subsequent delivery to the synapse is regulated by localization signals that have been identified in the C-terminal tail of NR1 subunits (Standley et al., 2000, Scott et al., 2001). Longer NR1 subunits are retained in the ER due to the presence of ER retention signal in alternatively spliced C1 cassette. During receptor assembly, the binding of NR2 subunits masks the ER retention signal and can promote forward trafficking to the synapse (McIlhinney et al., 1996, Scott et al., 2001, Mu et al., 2003)). In addition, association with scaffolding proteins such as post-synaptic density 95 (PSD-95) was shown to promote forward trafficking of NMDARs from ER to post synaptic membrane (Losi et al., 2003, Prybylowski et al., 2005). SAP-102 is a scaffolding protein that was shown to interact with sec8, which is a component of the exocyst complex that

was shown to be involved in targeting vesicles to specific domains in the yeast's plasma membrane (Sans et al., 2003).

The insertion of NMDARs into the cell membrane is tightly regulated during development and occurs in response to synaptic activity and sensory experience. Phosphorylation is a well characterized means for regulating receptor trafficking and in the case of NMDARs, there is evidence that PKA and PKC are involved. Phosphorylation of NR1 near the ER retention signal in the C1 cassette by PKC (ser890 and ser896) and PKA (ser897) was shown to promote NMDAR trafficking to the plasma membrane in non-neuronal cells over a number of hours (Scott et al., 2001, Scott et al., 2003). On a much shorter timescale, activation of PKC enhanced NMDAR channel opening and promoted its trafficking to the cell surface of hippocampal neurons within minutes (Lan et al., 2001). The differences in timescales suggest that the slower trafficking is mediated by the secretory pathway, whereas the rapid receptor insertion is shown to occur through SNARE-dependent exocytosis (Lan et al., 2001). Recently, it was shown that PKC also phosphorylates the SNARE protein, synaptosomal-associated protein of 25kDa (SNAP-25) at ser187 to promote PKC-induced NMDAR insertion to cell surface in cortical neurons (Lau et al., 2010). Since ER export is the rate-limiting step for surface delivery, these findings emphasize the importance of trafficking checkpoints in controlling the supply of NMDARs to the synapse (Lodish et al., 1983). In addition, chronically blocking neuronal activity promotes synaptic clustering of NMDARs in a PKA-dependent manner, and these clusters can be dispersed from the synaptic site by PKC treatment (Rao and Craig, 1997, Fong et al., 2002).

NMDAR endocytosis is tightly regulated and governed by subunit-specific rules. Receptor internalization is a fundamental mechanism by which neurons regulate intracellular signaling, synapse maturation, and synaptic strength, and it is mediated by interactions between tyrosine-based internalization motifs contained within the NR2 C-terminal and clathrin adapter proteins (AP2) to promote clathrin-mediated endocytosis to recycling endosomes (Lavezzari et al., 2004). NMDARs also contain conserved membrane proximal endocytic motifs within the NR1 and NR2 subunits, which drive post synaptic density (PSD) internalization to degradative endosomes (Scott et al., 2004). Furthermore, the level of activity at synapses can modulate synaptic plasticity by driving changes in the composition of proteins via ubiquitin-dependent removal of synaptic proteins and their subsequent degradation, which in turn can augment or suppress synaptic signaling via MAPK/ERK and PKC (Ehlers, 2003).

Regulation of NMDAR-dependent internalization was shown, also to be determined by activity-dependent tyrosine phosphorylation. For example, NR2B-dependent phosphorylation of tyr1472 by Fyn kinase was shown to suppress NMDAR internalization, while mutation in that site induced internalization (Prybylowski et al., 2005). Similarly, stabilization of NR2A containing receptors at the cell surface depends on tyrosine phosphorylation, while tyrosine dephosphorylation (tyr842) triggers NMDAR internalization (Vissel et al., 2001).

Furthermore, the presence of specialized endocytic zones in regions lateral to synapses were described, indicating that lateral diffusion or transport away from a synapse, where receptors are tightly anchored, is needed for endocytosis to occur (Blanpied et al., 2002). Indeed, NMDARs were shown to also move laterally in the

plasma membrane between synaptic and extrasynaptic sites in an activity and phosphorylation-dependent manner, which decreases with neuronal maturation (Tovar and Westbrook, 2002, Groc et al., 2006).

Various studies indicate that activity-dependent alterations in NMDAR trafficking might provide a powerful mechanism for regulating synaptic plasticity. In one study, NMDAR-mediated Ca^{2+} influx triggers responses which involve PKC and the tyrosine kinase Src, resulting in increased forward trafficking of NR2A-containing NMDARs to synapses during LTP (Grosshans et al., 2002). The mechanism is consistent with PKC's reported role in enhancing channel opening and promoting insertion of NMDARs. Conversely, during LTD, NMDAR-induced Ca^{2+} influx triggers various responses, which trigger actin depolymerization, resulting in NMDAR internalization; a process which is thought to be mediated by lateral movement to extrasynaptic site, where internalization takes place (Morishita et al., 2005).

In another form of synaptic plasticity, metaplasticity, or activity-induced tuning of synaptic plasticity, previous synaptic activity or other external stimuli can influence the occurrence of subsequent synaptic plasticity, where the level of activity determines the weight of synaptic strength and excitability level. Thus, bidirectional, activity-dependent alteration in NMDA synaptic strength can modulate this homeostatic plasticity. Accordingly, it was reported that chronic activity blockade of NMDARs enhances surface expression number of NR2A/B NMDARs and spine density in synapses in a PKA-dependent manner (Rao and Craig, 1997, Liao et al., 1999). Chronic activity blockade also increases spine density *in vivo* in the lateral geniculate nucleus projection neurons and generates silent synapses. These synapses are characterized by an increasing

NMDA:AMPA ratio, leading to predominant expression of NMDARs (Marie et al., 2005).

In addition to altering NMDAR number, activity can also regulate NMDAR synaptic strength by modifying alternative RNA splicing of the C-terminal cassette of NR1 encoding regions to yield either C1, C2, or C2' splice variant. Evidence indicates that these domains within the C-terminal contain trafficking and targeting signals that direct the delivery, maintenance and stabilization of NMDARs at synapses (Carrol and Zukin, 2002; Wenthold and Prybylowski, 2003). Chronic activity was demonstrated to promote splicing of the C2 cassette of NR1 subunit, while chronic activity blockade favors splicing of the C2' cassette, which contains a coat protein complex II (COPII) recognition site that was shown to bind nascent NMDARs. Consequently, association of receptors with COPII promotes ER exit and accelerates forward trafficking to synaptic sites (Mu et al., 2003). These findings provide a mechanistic and temporal link between synaptic activity and surface delivery of NMDARs, but how alterations in activity are detected by the RNA splicing machinery is unknown.

1.5 Goals of the Work in This Thesis

Polyamines proposed role in enhancing axon regeneration and their reported capability of blocking iGluR activity, led to our hypothesis that polyamines block iGluRs to promote regeneration. In addition, parallels in the myelin inhibitory and NMDAR downstream signaling pathways, led to our assessment of iGluR's potential role in modulating neurite outgrowth inhibition mediated by MAG/myelin-associated inhibitors.

To determine if iGluRs play a role in MAG-mediated inhibition, the effects of pharmacological inhibitors and activators of NMDA and AMPA/KAR are examined in our neurite outgrowth assay (**chapter 2.1**). I will also examine whether prior exposure (priming) with iGluR antagonists is sufficient to overcome MAG-mediated inhibition.

Since priming with polyamines and protein translation are required for polyamines to overcome MAG-mediated inhibition, we hypothesize that iGluR antagonists exert their effect by intercepting MAG inhibitory signaling. It was demonstrated that Ca^{2+} -dependent activation of PKC and EGFR/ERK mediate myelin-inhibition of axonal regeneration. Importantly, activation of iGluR also leads to PKC and ERK activation. Thus, assessment of ERK activation in response to inhibitors in myelin and/or iGluR antagonists is the focus of **chapter 2.2**. Importantly, I will examine the role of ERK in our neurite outgrowth assay.

Finally, since PKC plays an important role in mediating MAG signaling and since PKC is a known regulator of NMDAR forward trafficking, I will examine whether NMDARs trafficking and PKC-mediated serine phosphorylation is altered by myelin treatment (**chapter 2.3**).

Chapter II Results

2.1 The Role of iGluR Activity in MAG-Induced Inhibition of Neurite Outgrowth

2.1.1 Blocking iGluR Ion Channel Activity Overcomes Neurite Outgrowth Inhibition by MAG

Since it was demonstrated by our lab that polyamines are sufficient to promote neurite outgrowth in the presence of inhibitory substrates such as MAG or myelin *in vitro* and promote axonal regeneration *in vivo*, we began to explore polyamine's potential targets (Cai et al., 2002, Deng et al., 2009). Polyamines are polycation molecules that have been shown to regulate many aspects of cellular function, such as DNA and RNA stabilization, cytoskeleton rearrangement, protein translation, and the activity of ionotropic glutamate receptors (iGluRs) (Igarashi and Kashiwagi, 2000; Williams, 1997). If polyamines overcome inhibition by blocking iGluR activity, inhibiting iGluR activity should do the same. The first question that we asked was: is blockade of iGluR signaling sufficient to overcome MAG-mediated inhibition? To answer this question postnatal day 1 (P1) hippocampal neurons or P5 dorsal ganglia (DRG) neurons were isolated and grown *in vitro* on a substrate of control or MAG-expressing CHO cells in the presence or absence of pharmacological iGluR antagonists. We found that application of either NMDAR or AMPA/KAR antagonist, APV or CNQX, respectively, completely blocked MAG-induced inhibition of neurite outgrowth in P5 DRG neurons (Fig. 2.1A, C) or postnatal day-1 (P1) hippocampal neurons (HNs) (Fig.2.2A-D). Furthermore, applying both NMDAR and AMPA/KAR blockers in P5 DRG neurons did not enhance neurite outgrowth on MAG substrate beyond that observed with each antagonist alone (Fig. 2.1A, C), which might suggest a common mechanism of action, namely blocking NMDAR activity. This might also explain why blocking either NMDAR or AMPA/KAR

activity is sufficient to overcome MAG-mediated inhibition. Given that NMDAR activation requires depolarization, which can be generated by AMPA/KAR activation, inhibiting AMPA/KAR activity would prevent NMDAR from being activated.

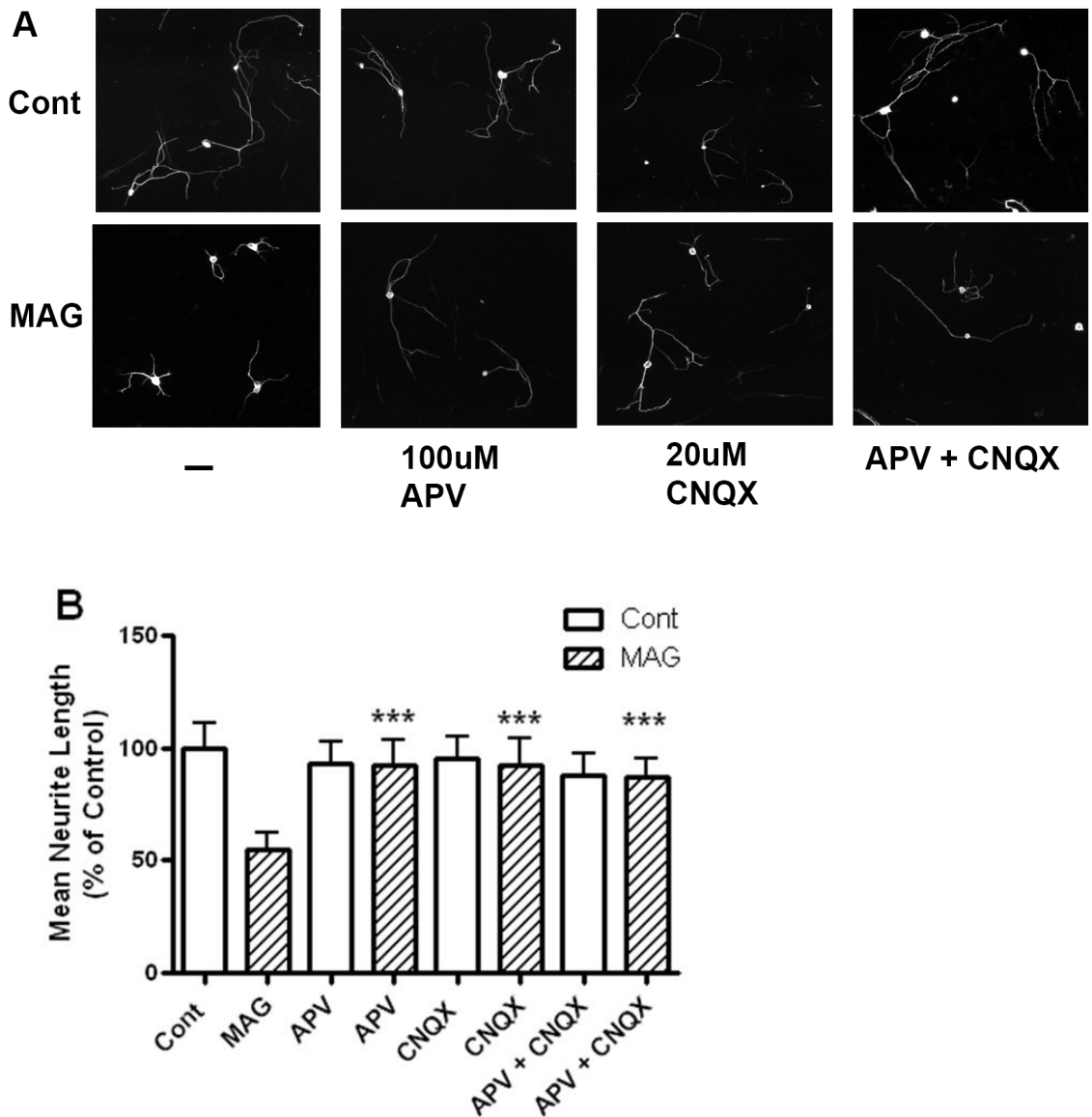


Figure 2.1: Pharmacological inhibition of iGluR is sufficient to overcome MAG – mediated inhibition of neurite outgrowth. Dissected postnatal day 5 or P5 dorsal root ganglia (DRG) (A) neurons were plated overnight on MAG (striped bar) or control (white bar) expressing CHO monolayers in the presence or absence of NMDAR blocker, APV and/or AMPA/Kainate receptor antagonist, CNQX, after which the cells were fixed and immunostained with the neuronal marker β -III-tubulin. (B) Quantitation of neurite outgrowth - Results show the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 200 DRG neurons. *** Statistically significant different from neurite length on control MAG-expressing cells, $p \leq 0.001$, one-way ANOVA. (B, n=5)

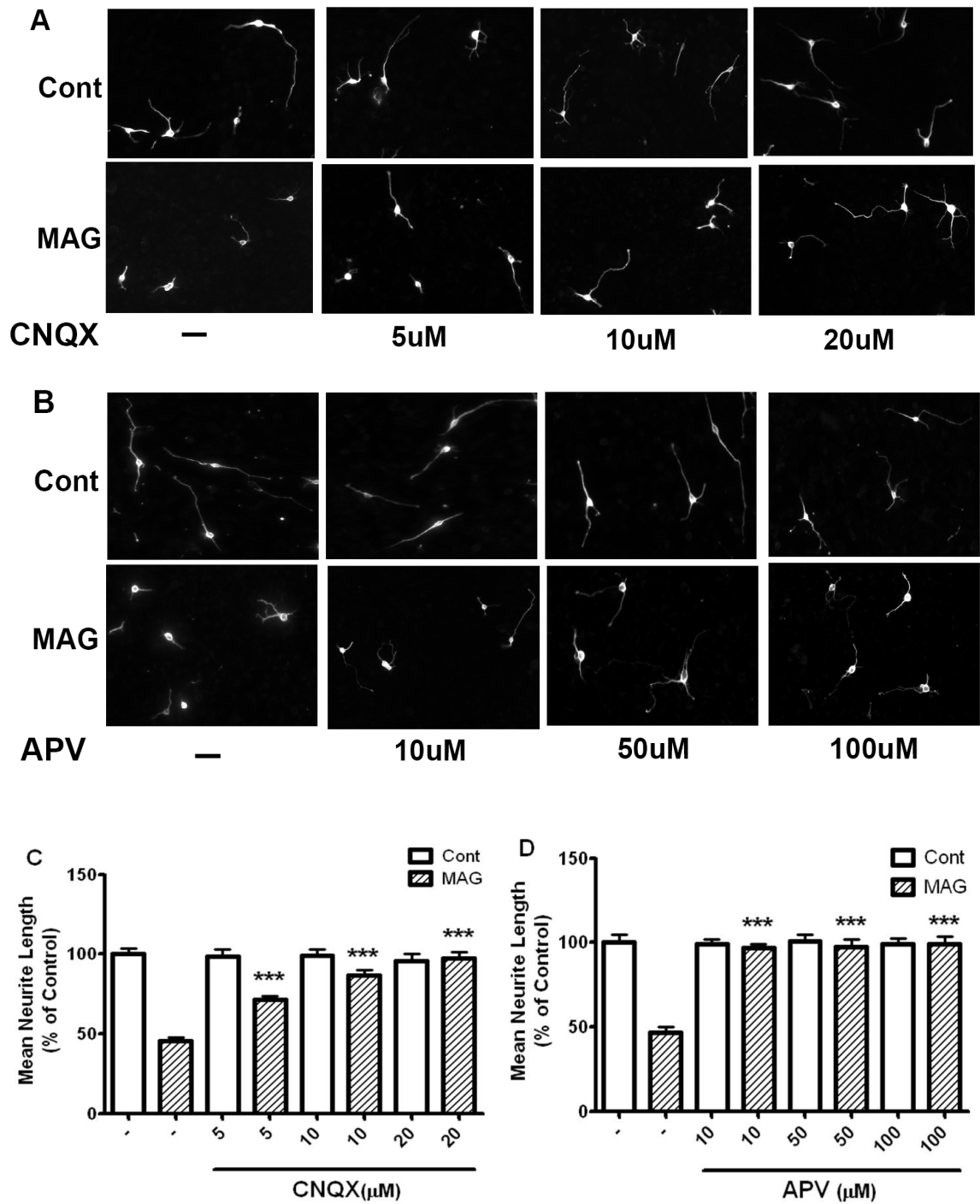


Figure 2.2: Administration of various NMDAR or AMPA/Kainate activity blockers overcomes MAG-mediated inhibition in a dose-dependent manner. (A, B) Dissected P1 HNs were plated overnight on CHO monolayers in the presence or absence of different concentrations of NMDAR antagonists: APV (B, D), MK-801 (E), Ifenprodil (F), or AMPA/Kainate receptor antagonist, CNQX, (A, C). (C-F) Quantitation of neurite outgrowth. Results show the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 300-400 neurons. *** Statistically significant different from neurite length on control MAG-expressing cells, $p \leq 0.001$, * $p \leq 0.05$ one-way ANOVA (C, D, $n=3$; E, F, $n=2$).

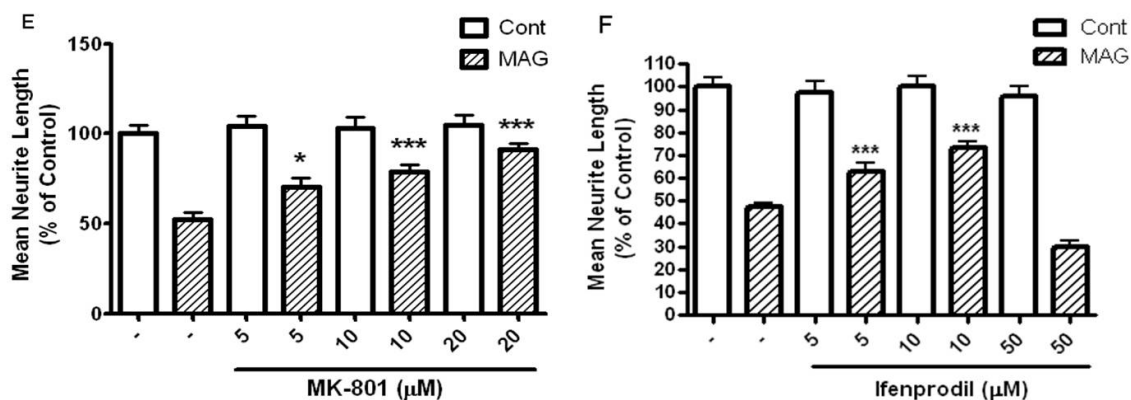


Figure 2.2: Administration of various NMDAR or AMPA/Kainate activity blockers overcomes MAG-mediated inhibition in a dose-dependent manner. (A, B) Dissected P1 HNs were plated overnight on CHO monolayers in the presence or absence of different concentrations of NMDAR antagonists: APV (B, D), MK-801 (E), Ifenprodil (F), or AMPA/Kainate receptor antagonist, CNQX, (A, C). (C-F) Quantitation of neurite outgrowth. Results show the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 300-400 neurons. *** Statistically significant different from neurite length on control MAG-expressing cells, $p \leq 0.001$, * $p \leq 0.05$ one-way ANOVA (C, D, $n=3$; E, F, $n=2$).

Since many pharmacological antagonists exert non-specific effects, we tested two additional NMDAR antagonists with different mechanisms of action and examined their effects in our neurite outgrowth assay. The first antagonist was MK-801, which blocks the activity of activated, open NMDAR channels, and the second one was Ifenprodil, which specifically binds to the same site as polyamines, located on the NMDAR NR2B subunit. As shown in figure 2.2, administration of each of these NMDAR antagonists was sufficient to overcome MAG-mediated inhibition. Thus, our results suggest that activation of NMDARs by MAG is required for neurite outgrowth inhibition. Interestingly, blocking NR2B-containing NMDAR at polyamine site only partially overcomes MAG-mediated inhibition, which implies that polyamines may weakly block NR2B-containing NMDAR but this blockade is not sufficient to completely overcome MAG-mediated inhibition. An alternative explanation is that only subpopulations of NMDARs in our neuronal cultures express NR2B subunits, which are blocked by

Ifenprodil. However, it is unlikely since it has been reported that the majority of NMDARs express NR2B subunits at early postnatal age and gradually switch expression to NR2A-containing receptors. Nevertheless, expression of NR2B needs to be examined in P1 HNs before any conclusions are drawn.

We observed a dose-dependent response with the NMDAR blocker MK-801 and the AMPA/Kainate blocker CNQX, while exposure to the NMDAR blocker, APV, completely blocked MAG-mediated inhibition at the lowest concentration. This might indicate that the applied concentration (10uM) was not low enough to reach a dose-dependent response.

2.1.2 Application of iGluR Antagonists to Young DRG Neurons induces a Switch from Neurite Outgrowth Promotion to Inhibition by MAG

While all postnatal CNS neurons tested to date demonstrate dramatic neurite outgrowth inhibition when plated in the presence of MAG, DRG neurons are unique in the sense that they are not inhibited by MAG or myelin until postnatal day 5 (P5) (Johnson et al., 1989, DeBellard et al., 1996, Cai et al., 2001). Interestingly, as indicated in figure 2.3, the presence of NMDAR antagonists (APV), non-NMDAR antagonists (CNQX), or the polyamine spermidine, resulted in inhibition of neurite outgrowth on MAG, whereas neurite outgrowth in non-treated DRG neurons was not inhibited by MAG. Therefore, our findings suggest that iGluR-induced activity or its immediate consequence, Ca^{2+} influx, is important for the response of young DRG neurons to MAG

and blocking iGluR activity induces a switch from increased outgrowth to inhibitory response once iGluR activity is blocked.

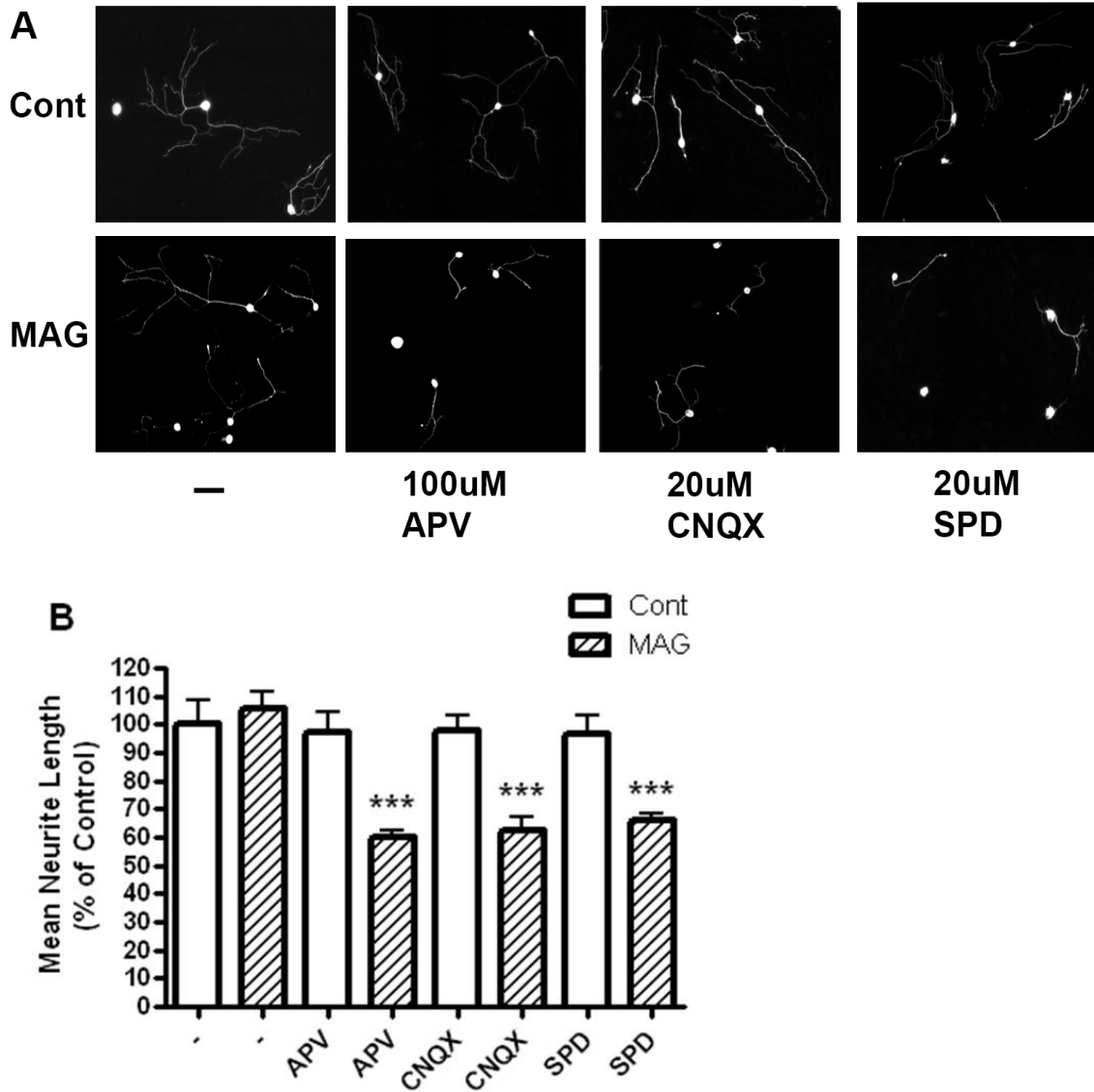


Figure 2.3: Administration of iGluR antagonists or PAs induces a switch from neurite outgrowth to inhibition by MAG. (A) Dissected P1 DRG neurons were plated overnight on MAG or control expressing-CHO monolayers in the presence or absence NMDAR antagonists, APV (100uM), AMPA/Kainate receptor antagonists, CNQX (20uM), or the polyamine spermidine (SPD, 20uM). (B) Quantitative Analysis - results indicate the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 100-200 neurons. *** Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.001$, one-way ANOVA ($n=4$).

2.1.3 Activation of iGluR Ion Channel Potentiates MAG - Mediated Inhibition

Since we observed that pharmacological inhibition of iGluRs is sufficient to overcome MAG-mediated inhibition in P1 HNs and P5 DRG neurons, our next step was to determine whether agonist-induced activation of iGluRs can affect MAG's inhibition of neurite outgrowth. To determine non-toxic concentrations of glutamate and NMDA, Fura-2 Ca^{2+} assays and live/dead assays were conducted by Dr. Peter Bradley in our lab. In the first assay he measured intracellular calcium in P1 HNs in response to differing concentrations of NMDA or glutamate. In the second assay, cell viability was analyzed in the presence of different concentrations of NMDA or glutamate. He demonstrated that in P1 HNs, increases in Ca^{2+} in response to 10-50 μM NMDA or glutamate did not result in cell death (data is not shown). We found that administration of either iGluR agonist, glutamate (Fig. 2.4A, C) or NMDAR-specific agonist, NMDA (Fig. 2.4B), increased MAG-mediated inhibition of neurite outgrowth, resulting in 20% decrease in neurite length as compared to non-treated cells. Furthermore, agonist-induced activation of NMDAR and iGluR resulted in a small but statistically significant decrease in neurite length on control monolayers (Fig. 2.4A,B), which strengthens the idea that chronically increasing iGluR activity, especially via NMDAR, is inhibitory for neurite outgrowth. Interestingly, we found that glutamate-induced iGluR activation produced more dramatic inhibition than NMDA (Fig. 2.4B, C). A possible explanation might be that while glutamate activates both NMDAR and AMPA/KAR, NMDA activates only the NMDARs. Consequently, activating all iGluRs is likely to lead to increased activity or currents, which can lead to activation of other channels, such as voltage-gated Ca^{2+}

channels. That might result in a stronger inhibitory response as compared to activating NMDARs alone.

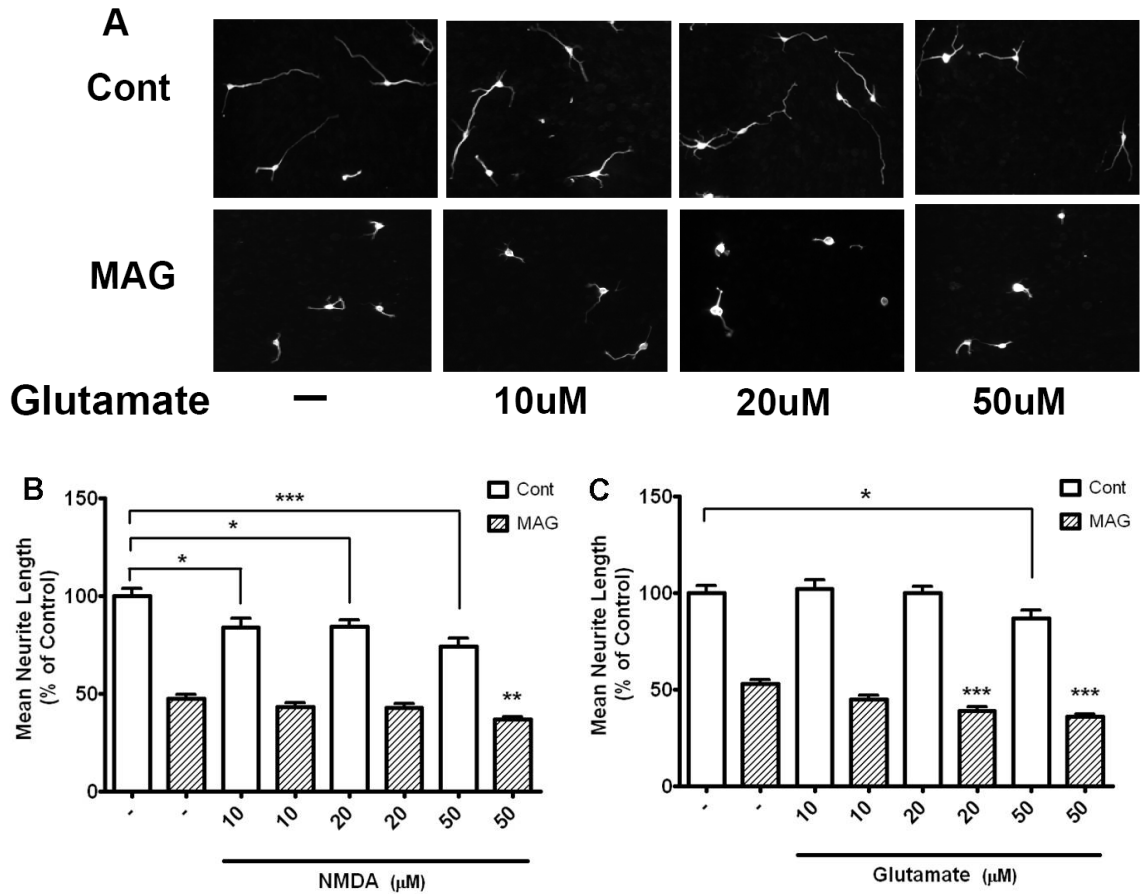


Figure 2.4: Application of Glutamate receptor agonists increase neurite outgrowth inhibition by MAG. Dissected P1 HNs were plated overnight on CHO monolayers in the presence or absence of differing dosages (as indicated above) of iGluR agonist, glutamate, (A,C) or NMDAR specific agonist, NMDA, (B). Results show the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 300-400 neurons. *** Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.001$, ** $p \leq 0.01$ one-way ANOVA, followed by Tukey's multiple comparisons test.

2.1.4 Priming With iGluR Antagonists Does Not Overcome MAG-Mediated Inhibition

To examine the mechanism by which iGluR blockers overcome inhibition, we next asked whether priming with iGluR antagonists can change the intrinsic response of neurons to MAG, as in the case of neurotrophins (NTs) such as BDNF. P1 HNs were primed with iGluR antagonists and then neurons were used in the neurite outgrowth assay. As indicated in figure 2.5, priming with APV or CNQX was not sufficient to overcome MAG-mediated inhibition, while priming with BDNF completely blocked MAG-mediated inhibition.

Our findings demonstrate that priming with iGluR antagonists does not block inhibition of neurite outgrowth by MAG, while direct application of iGluR antagonists effectively overcomes inhibition. This suggests that iGluR antagonists exert their effect by intercepting MAG inhibition signaling, while not changing the ability of neurons to respond to MAG. It is, therefore, unlikely that blocking the iGluR effect would be transcription dependent.

The reason we began examining iGluRs role in MAG inhibition was because our lab has shown that polyamines effectively block MAG-mediated inhibition, which we hypothesized to be mediated in part by blocking iGluR activity, given that others have demonstrated that polyamines can block iGluRs by directly binding to it. However, we found that polyamines are very effective when neurons are pretreated overnight prior to MAG/myelin exposure, whereas adding polyamines at the same time as MAG/myelin exert partial effects. In addition, polyamines exert a variety of effects inside the cells and our lab has shown that polyamines are involved in initiating translation, thus, inhibiting protein translation blocks polyamines ability to overcome MAG-mediated inhibition.

Conversely, our results demonstrate that iGluR antagonist cannot overcome MAG-mediated inhibition when added prior to exposure to MAG, but effectively block MAG's inhibition when added at the same time. Taken together, we conclude that although iGluR antagonists and polyamines efficiently block MAG-mediated inhibition, it is likely they each act via separate mechanisms. Although reports that polyamines can block iGluRs together with our data showing that blocking polyamines binding site can partially overcome inhibition may suggest that blocking polyamines binding sites might have a role in overcoming MAG's inhibition, is consistent with data from our lab demonstrated that neurons exposed to polyamines at the same time as MAG partially blocked neurite outgrowth in the presence of MAG. Since we do not know whether polyamines exert their effects inside or outside the cell, and the reports indicate that extracellular polyamines block iGluR activation, whereas intracellular polyamines potentiate their activation. In conclusion, we do not believe that polyamines block the iGluR activation as their primary mechanism of blocking MAG/myelin-induced inhibition.

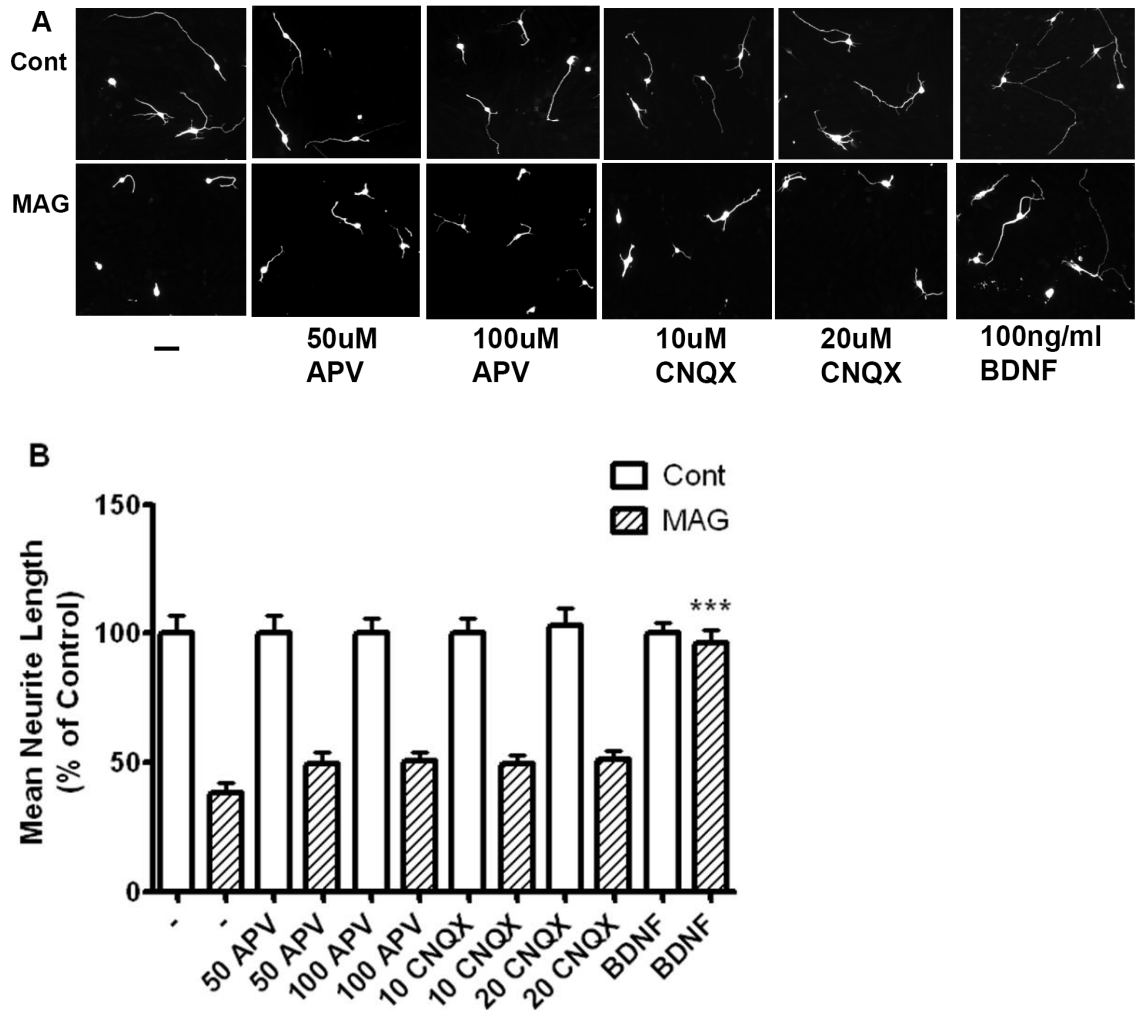


Figure 2.5: Priming with iGluR antagonists is not sufficient to block MAG – mediated inhibition.

(A) Representative images of HNs plated on CHO monolayers. Dissected P1 HNs were plated on poly-L-Lysine plates overnight (18 hours) in the presence or absence of NMDAR or AMPA/Kainate antagonists, APV (50 or 100 μ M) and CNQX (10 or 20 μ M), respectively, after which, cells were trypsinized and transferred onto CHO monolayers for neurite outgrowth assay.

(B), Quantitation of neurite outgrowth. Results show the mean length of the longest neurite per neuron ($\pm$ SEM) for approximately 300-400 neurons. *** Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.001$, one-way ANOVA ($n=4$)

2.2 Investigating a Potential Cross Talk between NMDARs and MAG/Myelin-Inhibitory Signaling Pathways

After establishing that pharmacological inhibition of NMDAR-induced Ca^{2+} influx efficiently blocks neurite outgrowth inhibition by MAG, our next goal was to identify downstream targets that are responsible for mediating iGluR activity blocker

effects. Our first step was to identify a common pathway that is regulated by either myelin-associated inhibitors or NMDARs, and subsequently, to examine potential cross-talk between myelin and NMDARs. A common pathway is the MAPK/ERK signaling pathway. Activation of NMDARs induces ERK activation during synaptic plasticity and ERK was also shown to be activated in response to inhibitors in myelin through a trans-activation of EGFRs (Koprivica et al., 2005).

2.2.1 Application of Ionotropic iGluR Antagonists Attenuates Myelin-Induced MAPK/ERK Activation

In order to examine ERK's role in MAG/myelin inhibition, we first needed to determine whether exposure to these inhibitors promotes its activation. Dissociated P6 cerebellar neurons were plated overnight on poly-L-lysine (PLL), serum starved for 8 hours, and then treated with myelin for 60 minutes. As indicated in figure 2.6A, application of myelin induces robust phosphorylation of ERK. However, myelin-induced ERK activation did not culminate in CREB activation (Fig. 2.7A, B), whereas dbcAMP treatment induced CREB activation after 60 minutes (Fig. 2.7A) and persisted overnight (Fig. 2.7B). The next step was to determine whether myelin-induced ERK activation can be blocked in the presence of iGluR activity inhibitors. P6 cerebellar neurons were treated with NMDAR-specific antagonist, APV or MK-801, AMPA/Kainate receptor antagonist CNQX, or ERK inhibitor, U0126, 30 minutes prior to myelin exposure. Cells were then lysed and proteins were analyzed by Western blotting. We found that myelin-induced ERK activation is significantly reduced in the presence of GluR antagonists. Subsequent quantitation demonstrated that application of myelin induced a nearly four-fold increase in pERK, whereas application of myelin plus NMDAR or AMPA/KAR

antagonists resulted in a two-fold increase in pERK, indicating that pERK was reduced 50% in the presence of iGluR antagonists. Thus, our findings here demonstrate that myelin-induced ERK activation is, at least partly, dependent on iGluR activity.

After observing that myelin induces a rapid ERK phosphorylation, we next characterized its duration by determining whether ERK activation is short-lived or sustained after overnight treatment. P6 CGN were treated with myelin or cAMP for 18 hours and subsequently lysed and subjected to Western blot analysis. As shown in figure 2.6D, overnight dbcAMP exposure induced a persistent ERK activation, while no ERK activation was detected after overnight exposure to myelin. Thus, myelin induced-ERK activation is relatively short-lived, while cAMP, which is the downstream effector of NTs, induced a more persistent ERK activation. In addition, we also found that myelin-induced ERK activation did not induce cAMP response-element binding protein (CREB) activation (Fig. 2.7A), whereas dbcAMP induced CREB phosphorylation within 60 minutes of treatment, which persisted after overnight cAMP treatment (Fig. 2.7A, B). Taken together, it seems contradictory that myelin, which induces outgrowth inhibition, and cAMP, which overcomes inhibition, both result in ERK activation. Nevertheless, it is likely that the nature of ERK-mediated response as well as its downstream targets may depend on its upstream activator. In our model, NT or cAMP-induced ERK activation is sustained for many hours and culminates in CREB activation to stimulate transcription of CREB-regulated genes, whereas, myelin-induced ERK activation is short-lived and does not stimulate CREB phosphorylation.

To confirm that NMDAR activity leads to ERK phosphorylation in our model system, NMDAR activity was chemically induced in P6 CGN by using the synthetic

agonist, NMDA, after which MAPK/ERKs activation was assessed. As indicated in figure 2.6C, we found that application of 50uM NMDA was sufficient to activate ERKs after 60 minutes, which is comparable to ERK activation seen with myelin.

2.2.2 Inhibition of MAPK/ERK Pathway Blocks MAG – Mediated Inhibition

After demonstrating that myelin induces ERK activation, which can be attenuated by blocking iGluR activity, we next determined whether ERK is important for MAG inhibition. The He group has shown that myelin-associated inhibitors trans-activate EGFR in an ERK-dependent manner (Koprivica et al., 2005). However, although they demonstrated that blocking EGFR efficiently overcome neurite outgrowth inhibition by myelin-associated inhibitors, ERK's specific role in mediating neurite outgrowth inhibition was never addressed (Koprivica et al., 2005). To determine whether ERKs play a role in MAG inhibition, P1 HN were treated with an ERK inhibitor, U0126 and subjected to a neurite outgrowth assay. As shown in figure 2.8, blocking ERK was sufficient to promote neurite outgrowth in the presence of MAG in a dose-dependent manner. Thus, our finding supports the notion that the ERK pathway is necessary for MAG-mediated inhibition

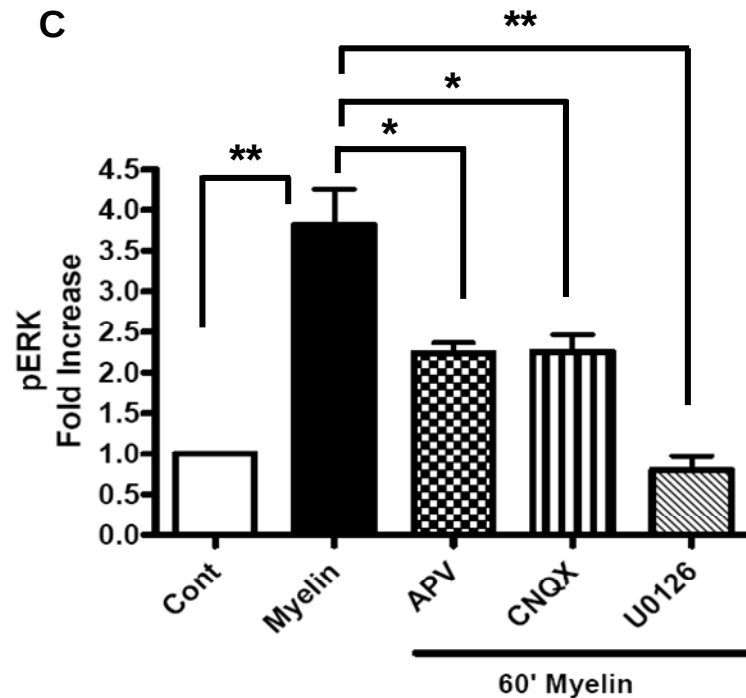
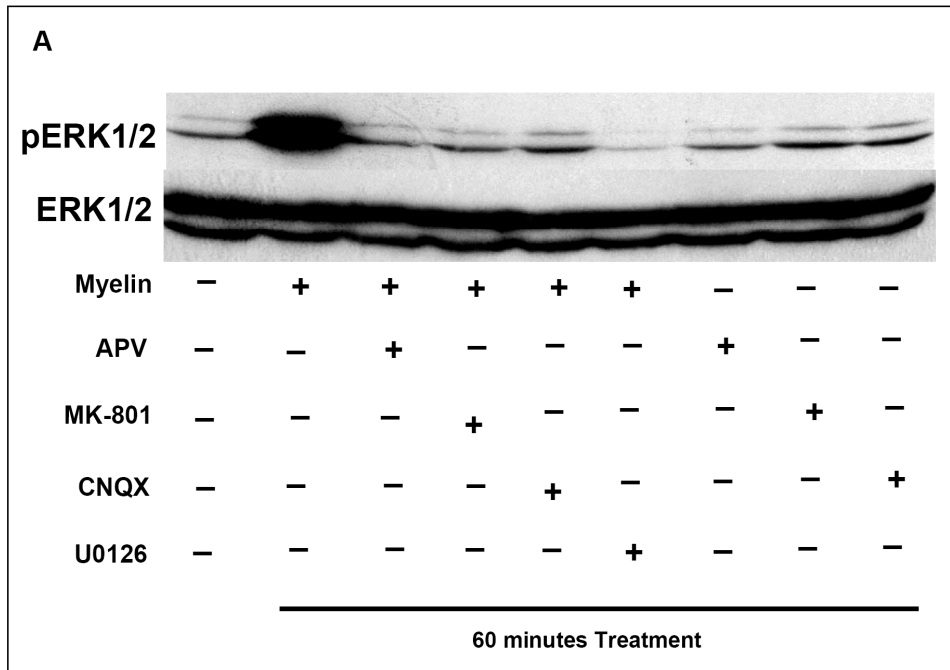


Figure 2.6 Myelin-induced phosphorylation of ERKs is blocked by Glutamate receptor activity inhibitors. (A, C, D) Representative Western blots showing pERK/2 (top band) and total ERK1/2 (bottom band) protein levels derived from isolated P6 Cerebellar Granule neurons (CGN) that were cultured on PLL for 24 hrs, serum starved for 8hrs and treated with myelin (20ug/ml) for 60' (A), or overnight (D) in the presence or absence of NMDAR antagonists, APV (100uM), MK-801 (20uM), AMPA/Kainate inhibitor, CNQX (20uM), or positive control MEK inhibitor, U0126 (5uM) for 30min prior to myelin treatment (A) or overnight (D). (C) ERK1/2 Activation in response to agonist NMDA (10, 50uM) as positive control. (B) Representative graph of quantitative analysis of ERK1/2 phosphorylation fold increase (A) that were normalized with control non-treated (T-test ** $p \leq 0.01$; * $p \leq 0.05$; myelin, $n=6$; APV, $n=4$; CNQX, $n=3$; U0126, $n=4$). (D, $n=3$)

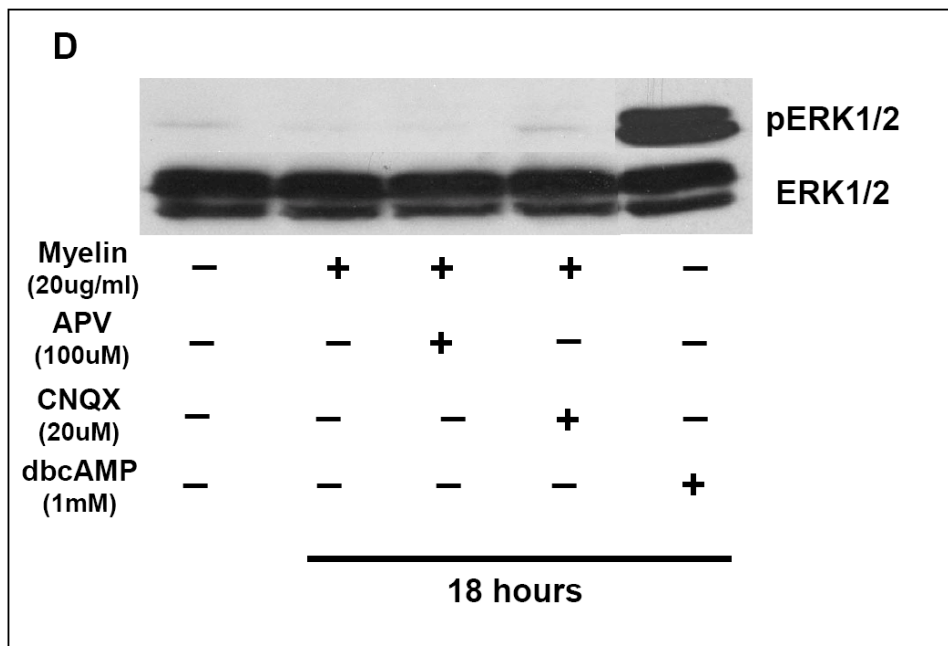
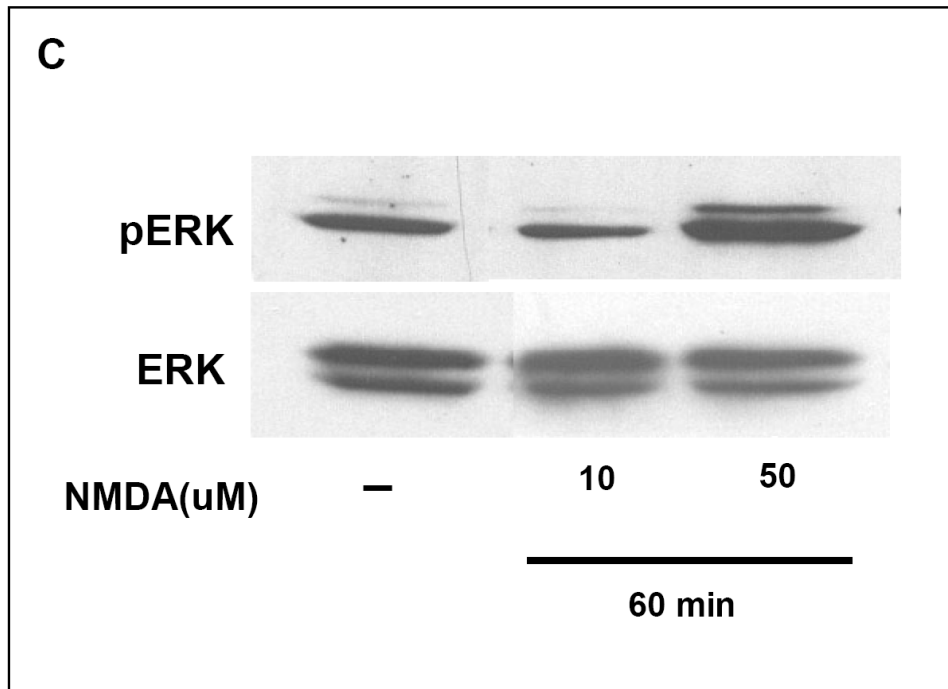


Figure 2.6 Myelin-induced phosphorylation of ERKs is blocked by Glutamate receptor activity inhibitors. (A, C, D) Representative Western blots showing pERK/2 (top band) and total ERK1/2 (bottom band) protein levels derived from isolated P6 Cerebellar Granule neurons (CGN) that were cultured on PLL for 24 hrs, serum starved for 8hrs and treated with myelin (20ug/ml) for 60' (A), or overnight (D) in the presence or absence of NMDAR antagonists, APV (100uM), MK-801 (20uM), AMPA/Kainate inhibitor, CNQX (20uM), or positive control MEK inhibitor, U0126 (5uM) for 30min prior to myelin treatment (A) or overnight (D). (C) ERK1/2 Activation in response to agonist NMDA (10, 50uM) as positive control. (B) Representative graph of quantitative analysis of ERK1/2 phosphorylation fold increase (A) that were normalized with control non-treated (T-test ** $p \leq 0.01$; * $p \leq 0.05$; myelin, $n=6$; APV, $n=4$; CNQX, $n=3$; U0126, $n=4$). (D, $n=3$)

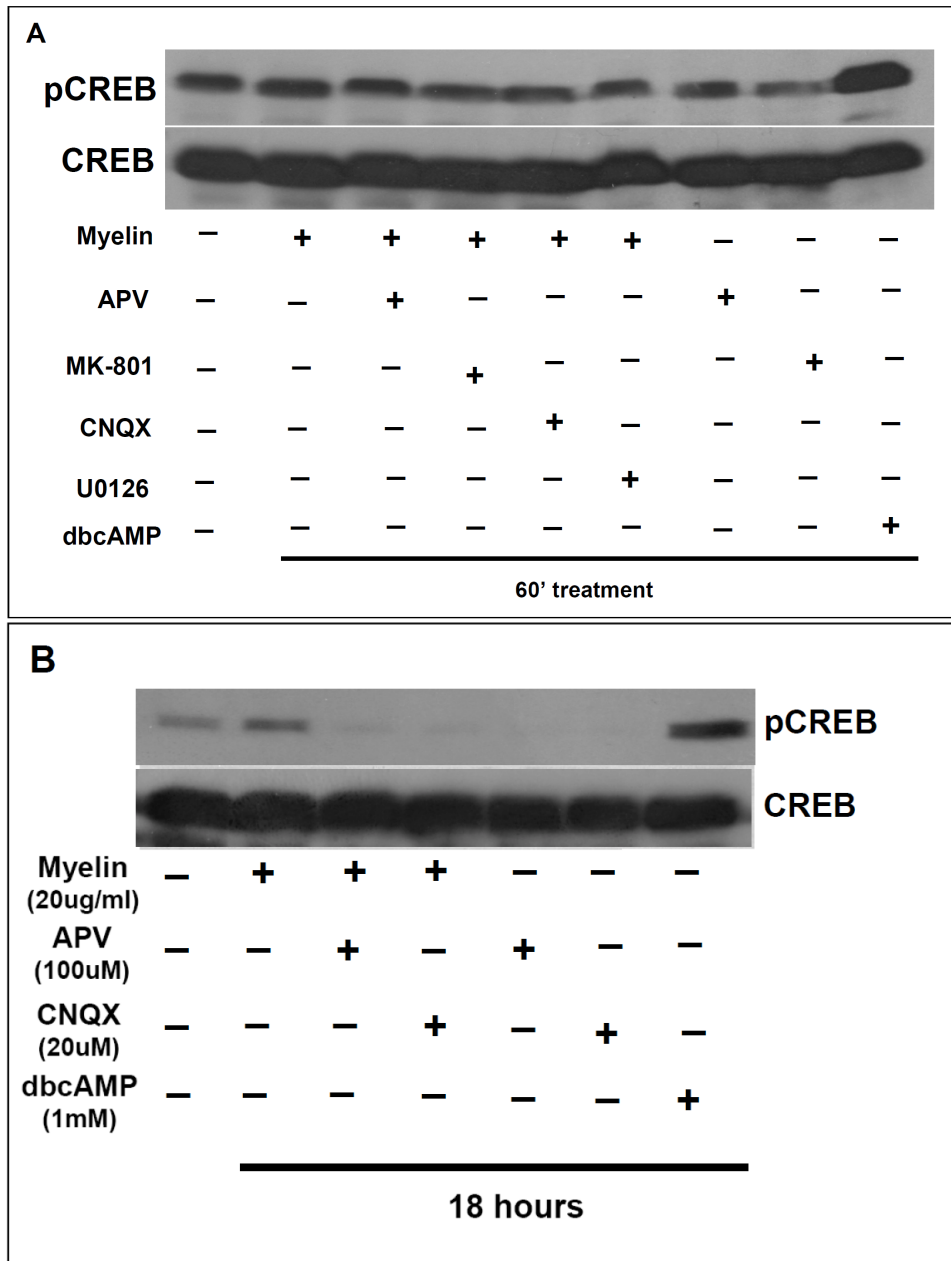


Figure 2.7 Myelin-induced phosphorylation of ERK does not induce CREB activation. Representative Western blot showing pCREB (top band) and total CREB (bottom band) protein levels derived from isolated P6 Cerebellar Granule neurons (CGN) that were cultured on PLL for 24 hrs, serum starved for 8hrs and treated with myelin (20ug/ml) for 60' (A) or overnight (B) in the presence or absence of NMDAR antagonists, APV (100uM), MK-801 (20uM), AMPA/Kainate inhibitor, CNQX (20uM), MEK inhibitor, U0126 (5uM), or positive control dbcAMP (1mM). (A, B n=2)

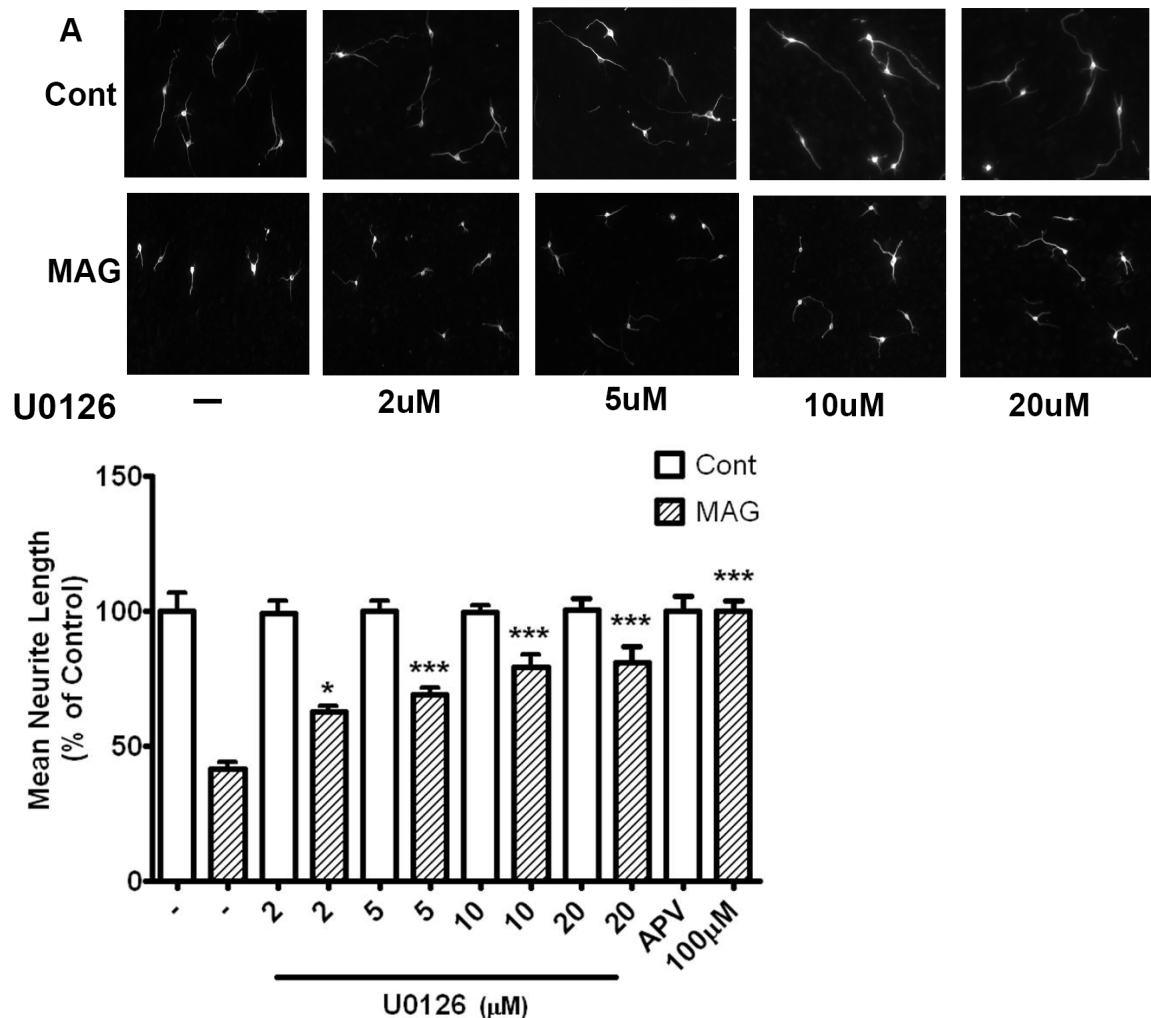


Figure 2.8 Pharmacological inhibition of ERK is sufficient to block MAG-mediated inhibition in a dose-dependent manner. Dissected P1 HNs were plated on CHO-MAG or CHO-control substrate in the presence or absence of various concentrations of the MEK inhibitor, U0126 or NMDAR antagonist, APV (100uM) for 20hrs. (A), Representative images of HNs plated on CHO monolayers. (B) Quantitative Analysis of mean number of the longest neurite per neuron (***) Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.001$, * $p \leq 0.05$, one-way ANOVA ($n=2$).

2.3 Characterizing NMDAR Trafficking in Response to Inhibitors in Myelin

Dynamic trafficking of NMDARs to and from the synapse was shown to be an important component of synaptic plasticity. For example, LTP is associated with increased synaptic incorporation of NMDARs, while LTD is associated with the removal of NMDARs from synapses (Grosshans et al., 2002, Montgomery et al., 2005).

Alternatively, studies have shown that chronic activity induces the internalization of NMDARs whereas chronic block of activity accelerates forward trafficking of NMDARs to the cell surface (Rao and Craig, 1997). Among the many kinases activated in response to synaptic plasticity such as LTP, PKC was shown to regulate NMDAR trafficking (Lin et al., 2006). PKC activation was shown to induce forward trafficking of NMDAR to synapses as well as increasing channel opening (Lan et al., 2001). Part of PKC's mechanism to increase forward NMDAR trafficking to the cell surface is by phosphorylating ser890 and ser896 at the C1-terminal cassette endoplasmic reticulum (ER) retention signal domain within the NR1 subunit (Tingley et al., 1997, Scott et al., 2001). Since PKC is also activated by myelin-associated inhibitors and transduces the inhibitory signal by regulating the cleavage of p75^{NTR} and RhoA activation, we examined whether exposure to myelin can modulate NMDAR trafficking in primary neurons.

2.3.1 Myelin Treatment Induces NMDAR Phosphorylation and Increased Surface Expression

It is well established that inhibitors in myelin promote the activation of several isoforms of PKC, including the conventional PKCs, and their activation is required for myelin-induced inhibition *in vitro* or blocking axonal regeneration *in vivo* (Hasegawa et al., 2004, Sivasankaran et al., 2004). Keeping these results in mind, we examined whether myelin treatment can stimulate NR1 forward trafficking and induce ser890 and ser896 phosphorylation within the C1-terminal cassette of NR1. P1 cortical neurons (CNs) were cultured for 2 days (2DIV), representing the short-term cultures (Fig. 2.9A) or for 7 days (7DIV), representing the long-term cultures (Fig. 2.9C). The short-term CN cultures were used to mimic the nerve outgrowth (NOG) conditions, while the long-term cultures were

used to represent more mature neurons, which extend long neurites and are likely to form synapses. While using 1 day CN cultures, rather than 2 day cultures, better reflects the NOG conditions, detection difficulties in the biotinylation assay with 1 day cultures convinced us to switch to 2 day CN cultures. After 2DIV or 7DIV, CNs were treated with 10-20 μ g/ml myelin concentrations for 10 or 20 minutes. We used these time points because PKC was shown to be activated 10 minutes after addition of myelin inhibitor, while ser890/896 phosphorylation was shown to take place 20-30 min after activating PKC with phorbol myristate acetate (PMA) (Koprivica et al., 2005). Thus, PMA was utilized as our positive control. Following treatments, neuronal cell surface proteins were tagged with biotin, after which cells were lysed, membrane fractions were isolated, and biotin-tagged proteins were pulled down with neutravidin beads and subjected to Western blot analysis. As shown in figure 2.9A, in the 2DIV CN cultures, NR1 subunit was robustly phosphorylated at ser890 within 10 minutes of myelin treatment, and it is sustained and even increased after 20 minutes regardless of myelin concentration (Fig. 2.9B). Similarly, in the 7DIV CN cultures, NR1 subunits were phosphorylated at ser890 in response to myelin. Importantly, we found that myelin promoted an increase in surface NR1 number in both 2DIV and 7DIV cortical cultures (Fig. 2.9A, C). Interestingly, while NR1 levels in the 2DIV samples are dramatically increased after 10 minutes and are sustained after 20 minute myelin treatment (Fig. 2.9A), surface NR1 protein levels in 7DIV samples do not increase until after 20 minutes of myelin treatment (Fig. 2.9C). The 10 minute lag seen in the 7DIV cultures might be explained by the possibility that NR1 needs to travel longer distances from the ER to the cell surface, given that these cultures have longer neurites which are enriched with surface NMDARs. Quantification of NR1

in 2DIV cultures indicates a two-fold increase in surface NR1 following myelin or PMA treatment (Fig. 3.0A). We next examined whether increased surface NMDAR number persists following overnight or chronic myelin treatment. We performed a receptor insertion assay, which is used to detect newly inserted receptors on the cell surface. P1 CNs were cultured for 2 days, after which all surface proteins were treated with sulfo-NHS-acetate, which irreversibly binds to all surface proteins at the same site where biotin binds, thus preventing subsequent biotin tagging of surface proteins. After 8-12 hours of cell surface acetate incubation, cells were treated with myelin, surface proteins were tagged with biotin, which represents only the newly inserted proteins, and then, membrane fractions were isolated and analyzed on a Western blot. As indicated in figure 3.0B, chronic myelin treatment, like acute myelin treatment, induced a two fold increase in surface NR1 subunit levels, which indicates that the increased forward trafficking to the cell surface is not a transient phenomenon.

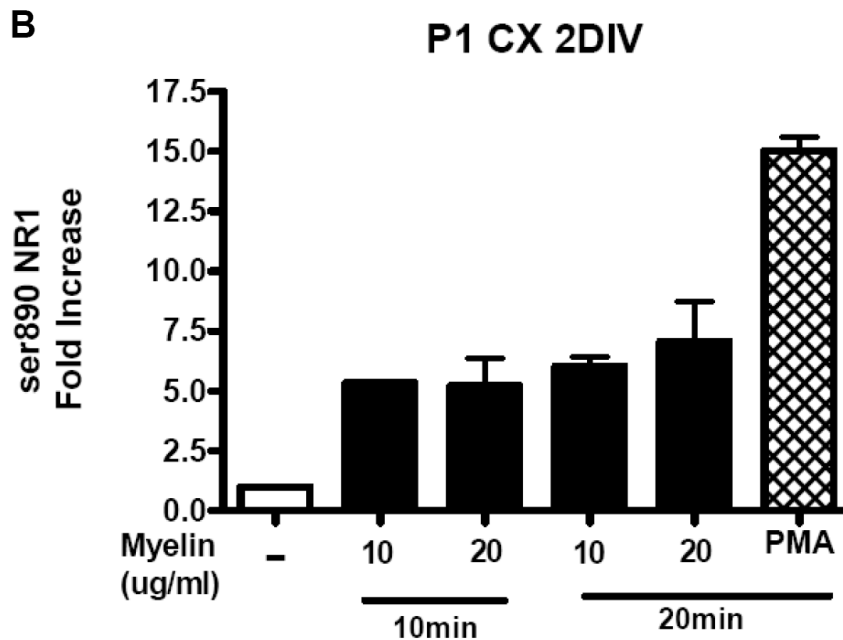
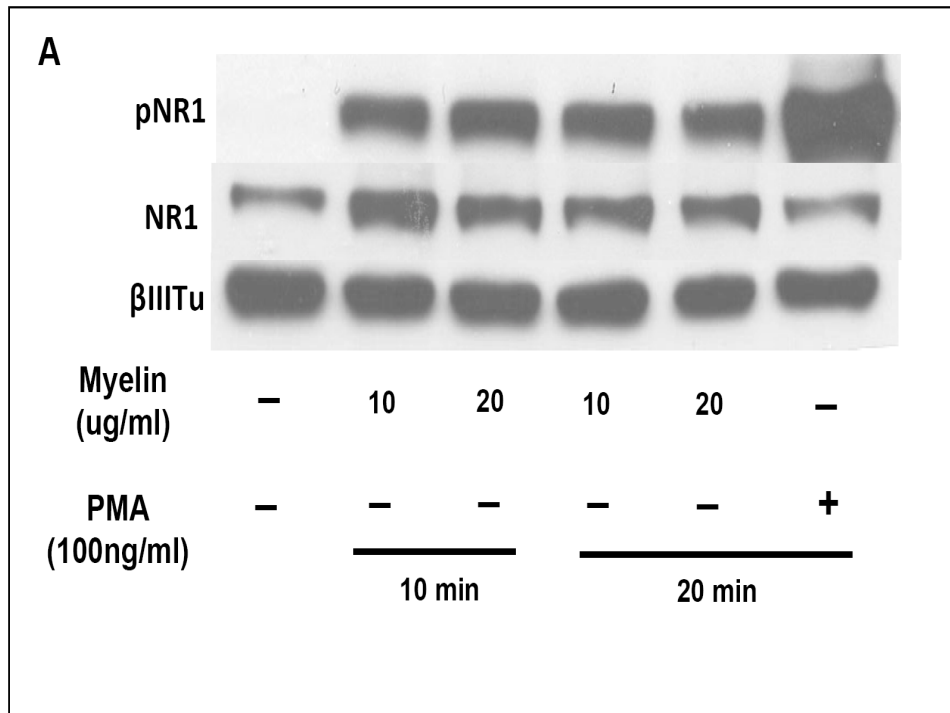


Figure 2.9: Myelin treatment induces serine 890 NR1 phosphorylation and increased surface NR1 levels. (A, C) Representative Western blot analysis of membrane levels of ser890 pNR1 and total NR1 proteins derived from P1 cortical neurons (CX) cultured for 2days (A) or 7days (C), treated with myelin (10 or 20ug/ml) for 10 or 20min or with PKC activator phorbol ester (PMA, 100ng/ml) for 20min., after which cells were biotinylated, lysed, and biotinylated membrane proteins were pulled down and separated on a acrylamide gel. (B, D), Quantitative analysis of ser890 pNR1 fold increase as normalized with non-treated controls (T-test ** statistically different from non-treated control $p \leq 0.01$; * $p \leq 0.05$; (B) $n=2$; (D) $n=3$).

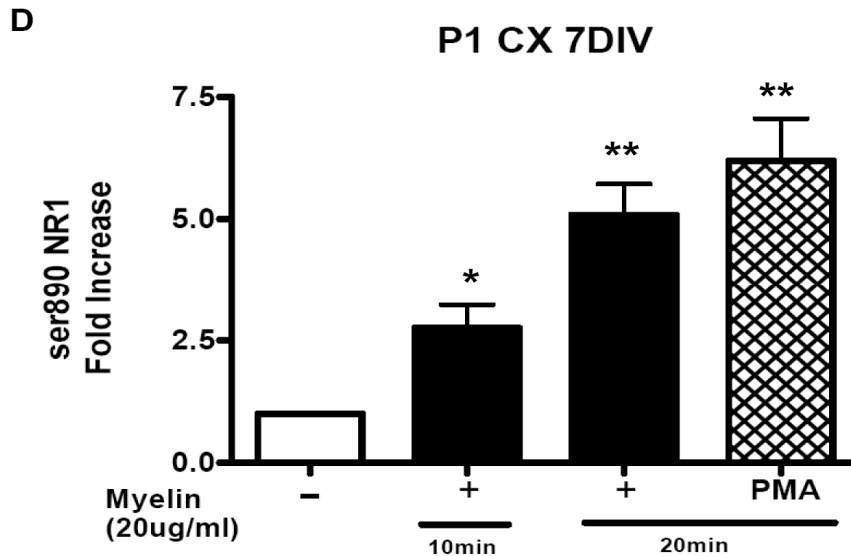
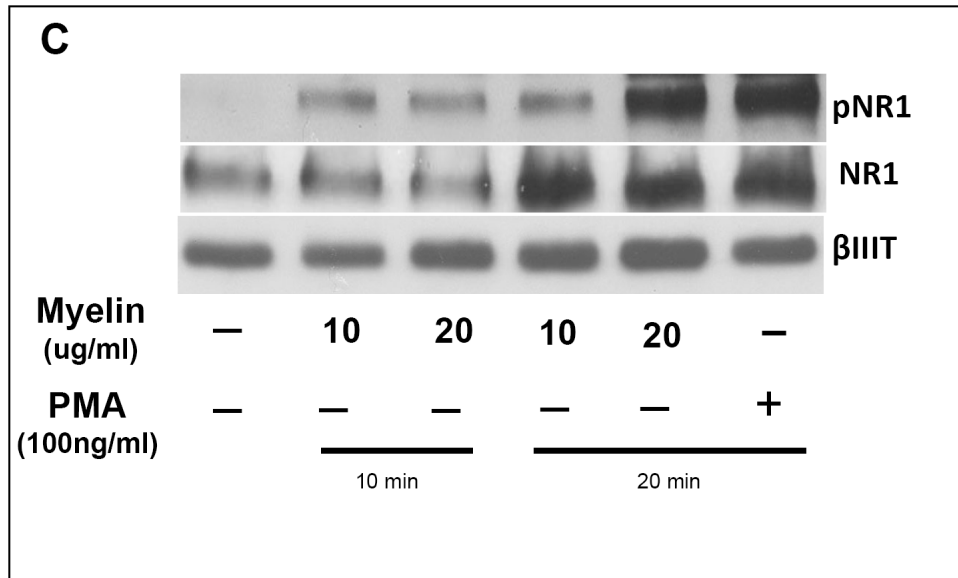


Figure 2.9: Myelin treatment induces serine 890 NR1 phosphorylation and increased surface NR1 levels. (A, C) Representative Western blot analysis of membrane levels of ser890 pNR1 and total NR1 proteins derived from P1 cortical neurons (CX) cultured for 2days (A) or 7days (C), treated with myelin (10 or 20ug/ml) for 10 or 20min or with PKC activator phorbol ester (PMA, 100ng/ml) for 20min., after which cells were biotinylated, lysed, and biotinylated membrane proteins were pulled down and separated on a acrylamide gel. (B, D), Quantitative analysis of ser890 pNR1 fold increase as normalized with non-treated controls (T-test ** statistically different from non-treated control $p \leq 0.01$; * $p \leq 0.05$; (B) $n=2$; (D) $n=3$).

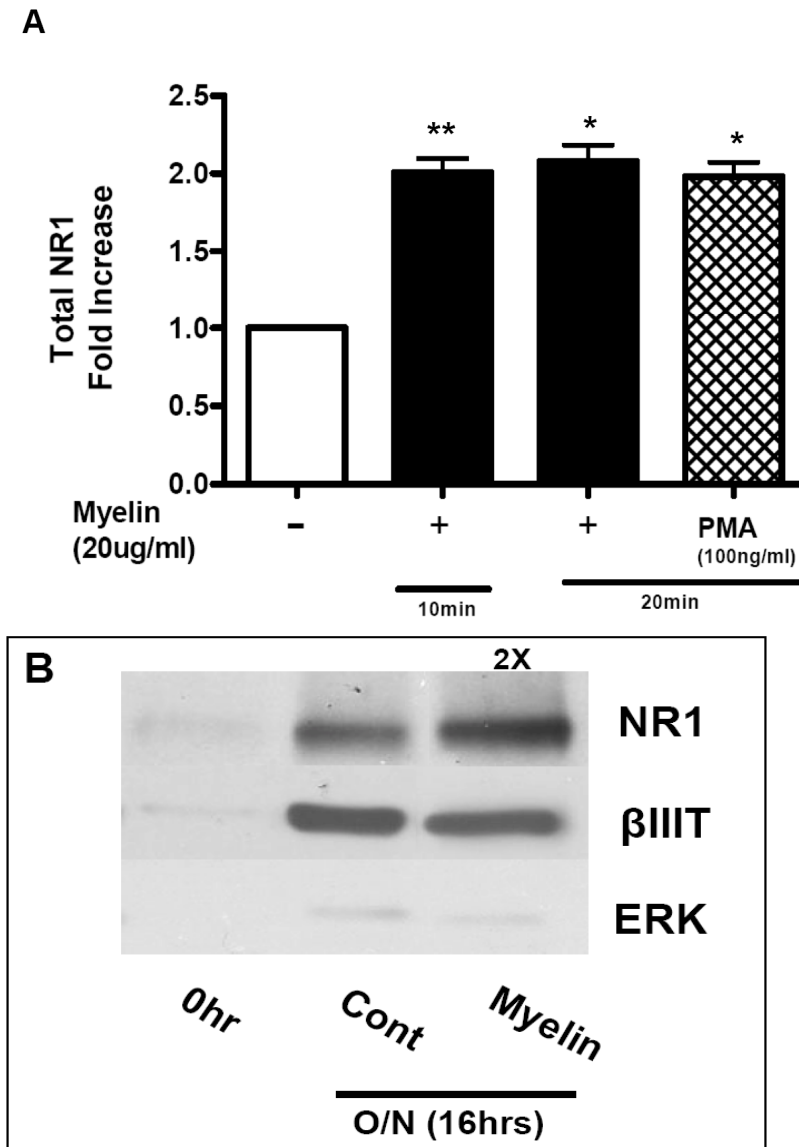


Figure 3.0: Myelin inhibitors induce increased surface NMDAR number. (A) Quantitative analysis of fold increase of total NR1 levels as compared to non-treated controls P1 CX 2DIV (T-test ** statistically different from non-treated control $p \leq 0.01$; * $p \leq 0.05$; 10' myelin, $n=3$; 20' myelin, $n=4$, PMA, $n=4$). (B), Western blot representation of newly inserted surface NR1 following overnight myelin (10ug/ml) incubation. (B, $n=2$)

Ser896 is an additional site within the C1-domain of NR1, which was shown to be phosphorylated by PKC in the ER, triggering ER release of NR1 receptors (Scott and Ehlers, 2001; Scott and Ehlers, 2003). Thus, phosphorylation of this site and/or ser890 promotes NMDAR forward trafficking to cell surface. We therefore analyzed total cell

lysates from P1 CNs 2DIV that were treated with differing concentrations of myelin for 10 or 20 minutes. As indicated in figure 3.1, myelin induced a 1.5-2 fold increase in pser896 NR1 following 10 minutes of treatment, and this persisted 20 minutes after treatment. Taken together, our findings in this section demonstrate that myelin or PMA induces robust C1-terminal NR1 subunit phosphorylation at ser890 and ser896 residues. Phosphorylation of these sites is likely to promote forward trafficking of NMDAR to the cell surface, which, in turn, likely increases the magnitude of NMDAR signals, given that the number of surface receptors is increased. In addition, our observations that myelin or PMA exerts similar effects, and given that both PMA and myelin-associated inhibitors activate PKCs, it is likely that myelin induces ser890/896 phosphorylation and increases in surface levels of NMDARs by activating PKCs.

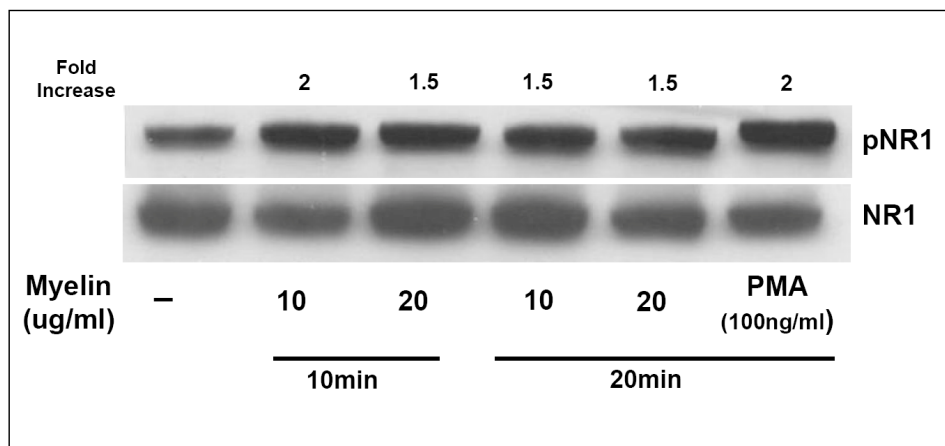


Figure 3.1: Myelin treatment induces increase in ser896 NMDAR NR1 phosphorylation in P1 HN lysates. Representative Western blot analysis of total lysate levels of ser896 pNR1 and total NR1 proteins derived from P1 HN cultured for 2days, treated with myelin (10 or 20 μ g/ml) for 10 or 20min or with PKC activator phorbol myristate acetate (PMA, 100ng/ml) for 20min., after which cells were biotinylated, lysed, and samples of the total lysate was separated on an SDS-PAGE gel. (n=2)

Chapter III: Discussion

Our findings here demonstrate that chronically blocking iGluR activity is sufficient to overcome MAG-mediated inhibition, whereas chronically activating them slightly increases the inhibition by MAG. Thus, our findings suggest that chronic iGluR activity mediates a portion of MAG's inhibitory effect. Furthermore, our observation that myelin-induced ERK activation is attenuated by blocking iGluR activity, suggests that myelin inhibitors are likely to trigger NMDAR-mediated signaling pathways, such as ERK. Considering the detrimental consequences iGluR hyperactivity exerts following CNS injury and based on our findings here, we conclude that inhibitors in myelin may induce an unbalanced iGluR activity which is triggered by increased surface NMDARs (Olney et al., 1986, Dirnagl et al., 1999, Lipton, 2006). Our observations here add important insight into the mechanisms of myelin's inhibitory role.

Chronically blocking iGluR activity, which overcomes MAG mediated inhibition, is likely to bring about two potential outcomes. The first is decreasing NMDAR-induced Ca^{2+} influx and its subsequent signaling, through ERK. The second outcome is changing the trafficking of iGluRs to and from the cell surface (Wentholt et al., 2003, Lau and Zukin, 2007).

A report demonstrating that myelin-associated inhibitors activate the EGFR signaling pathway was the first indication that ERK, one of the downstream targets of the EGFR pathway, can be an important mediator of CNS regeneration failure. It was shown that suppressing EGFR kinase activity was sufficient to overcome neurite outgrowth inhibition by the myelin inhibitors Nogo-66 and OMgp in a Ca^{2+} -dependent manner, indicating that the presence of increased intracellular Ca^{2+} is also important for mediating

EGFR-dependent neurite outgrowth inhibition (Koprivica et al., 2005). In addition, it was shown that growth cone repulsion by chondroitin sulfate proteoglycans (CSPGs), which are regeneration inhibitors that are present in the glial scar, is mediated by ERK as well as EGFRs. They demonstrated that CSPGs inhibitory effects in cerebellar neurons were reduced in the presence of ERK or EGFR pharmacological inhibitors (Kaneko et al., 2007). Our findings here demonstrate that application of myelin induces a robust activation of ERK. Importantly, we found that myelin-induced ERK activation is regulated in part by iGluR activity, since inhibiting NMDAR or AMPA/KR activity blocks about half of ERK's phosphorylation. While it is likely that part of the ERK activation observed is mediated by EGFR, our findings suggest that a significant portion of ERK activation is iGluR-dependent. Furthermore, we found that pharmacological inhibition of ERK is sufficient to overcome MAG-mediated inhibition of neurite outgrowth, which is the first evidence that ERK can mediate myelin-associated inhibitory signaling.

Our findings regarding ERK's potential inhibitory role were surprising given that ERK signaling is important for cell growth and survival as well as synaptic plasticity in the mature CNS (Thomas and Huganir, 2004). Moreover, our lab has previously shown that ERK is one of the downstream signaling targets that are activated in response to neurotrophins (NTs). Pharmacological inhibition of ERK efficiently blocked the BDNF priming effect (Gao et al., 2003). Given that ERK is a ubiquitous molecule, one that is involved in many aspects of cellular function, one cannot easily identify its targets. However, it is likely that the opposing actions mediated by ERK activation might result from different upstream activators as well as different spatio-temporal regulation of ERK.

The first difference we observed here is that BDNF-induced ERK activation is sustained for 18 hours, whereas myelin induced ERK activation is absent after overnight treatment. Importantly, we found that myelin-induced ERK activation did not culminate in CREB phosphorylation, while dbcAMP-induced ERK activation induced CREB phosphorylation. Our lab has shown that the ability of BDNF to overcome MAG/myelin-mediated inhibition is transcription and priming-dependent (Gao et al., 2003). Thus, BDNF-induced ERK activation, which probably takes place within or near the cell body, rapidly reaches the nucleus to phosphorylate CREB, which in turn, induces transcription of certain genes. Conversely, we hypothesize that myelin-induced ERK activation contributes to local signaling within the axon or synapse, given that pERK does not activate CREB and the observations that pERK is short-lived as compared to long-term activation in response to BDNF. Activity-induced ERK activation can induce transcription and translation with repeated bursts of neuronal activity. These cause global activation of ERK throughout the neuron, and can then lead to the activation of transcription factors in the nucleus (Wiegert et al., 2007). Interestingly, repeated activation of ERK is essential for structural plasticity and dendritic spine formation. This activation results from repeated depolarization and is dependent on protein synthesis, while transient ERK activation does not yield dendritic spine formation because it is probably insufficient for triggering protein synthesis (Wu et al., 2001). In summary, sustained or repeated ERK activation is suggested to trigger gene transcription and translation, which is required for synaptic plasticity events. Whereas myelin-induced ERK activation, although dramatic, is not sustained for many hours and does not induce

CREB activation, which may indicate that the effects of ERKs are mediated by interactions with downstream targets rather than through gene transcription.

A potential downstream target for ERK is described by Malinow and colleagues (2000). They have shown that ERK activation led to a tonic increase in synaptic AMPARs response, which is thought to be important for LTP. Their findings demonstrate that constitutively-active Ras, which promotes ERK activity, increased synaptic insertion and activity of AMPARs in hippocampal slices. In addition, following LTP-inducing stimuli, pERK immunoreactivity developed rapidly in both dendritic and somatic regions of hippocampal neurons, indicating that ERK substrates might be present throughout the neuron (Davis et al., 2000). ERK's important role in mediating LTP was demonstrated when increased AMPAR incorporation and neurotransmission as well as LTP induction were blocked with ERK inhibitors (Schafe et al., 2000, Di Cristo et al., 2001, Adams and Sweatt, 2002, Zhu et al., 2002). Interestingly, MEK inhibitors block LTP relatively rapidly - within 20-30 minutes following LTP induction (English and Sweatt, 1997, Ohno et al., 2001). This short period is unlikely to be sufficient for translocation of ERK from distal dendrites to the nucleus, followed by transcription, translation, and trafficking of new gene product to potentiated synapses. Since myelin induces a dramatic ERK activation that occurs within this time period, it is possible that myelin-induced ERK activation promotes an increase in AMPAR incorporation and currents. The results from this study are consistent with our findings that NMDARs are rapidly trafficked to the cell surface following myelin treatment. Consequently, given that myelin induces ERK activation that is partly regulated by iGluR activity, it is possible that ERK's role in neurite outgrowth inhibition is mediated by increasing AMPAR synaptic currents.

AMPA currents, in turn, will provide the depolarization needed to activate NMDARs, resulting in continuous NMDAR neurotransmission, which we have shown to be inhibitory for neurite outgrowth.

It has been shown that activation of conventional PKCs by myelin inhibitors triggers RhoA activation and p75^{NTR} cleavage. Since these events are central to axonal regrowth inhibition, this points to PKC as an important mediator of CNS regeneration failure (Hasegawa et al., 2004, Sivasankaran et al., 2004, Domeniconi et al., 2005). Our findings demonstrate that exposure to myelin results in a rapid increase in surface NMDARs as well as increased serine 890 and 896 phosphorylation within the C1 cassette of the NMDA NR1 subunit. Our findings are consistent with reports that activation of PKC induces an increase in surface NMDARs (Lan et al., 2001). A mechanism for PKC-induced increases in surface NMDARs was demonstrated in reports where PMA-induced PKC activation led to NR1 C1-terminal cassette ser890/896 phosphorylation (Scott And ehlers 2003; Tingley 1997). Our findings suggest that exposure to myelin, which induces PKC activation, triggers ser890/896 phosphorylation in the NMDAR NR1 C-terminal cassette. Importantly, we found that myelin treatment triggers a rapid increase in surface NMDARs that is likely to be a consequence of PKC-induced ser890/896 phosphorylation. These findings point to the possibility that the regulation of NMDAR trafficking may be due, in part, to the suppression of the NMDAR ER retention signal via phosphorylation. It should be noted that the data regarding PKC-induced NMDAR trafficking is controversial. While a number of studies report that PKC-induced ser890/896 phosphorylation promotes forward trafficking of NMDARs to the cell surface, others have shown that PKC-induced ser890 and/or ser896 phosphorylation leads

to the dispersion of NMDAR clusters (Tingley et al., 1997, Fong et al., 2002, Lau and Zukin, 2007). In order to confirm whether myelin-induced ser890/896 phosphorylation is responsible for the increase in surface NMDARs, one would need to mutate serine 890 and 896 sites and quantify increases in cell surface NMDARs in response to myelin treatment. In addition, although we found that PKC activation by PMA induces robust ser890/896 phosphorylation as well as increased surface NMDAR number, we need to confirm that PKC is responsible, by blocking PKC and examining ser890/896 phosphorylation and surface NMDAR number. Our findings demonstrate that newly inserted surface NMDARs persisted after chronic myelin treatment, indicating that NMDAR forward trafficking is not a transient phenomenon. The magnitude of NMDAR-mediated currents is determined in part by the number of receptors on the cell surface. Thus, since myelin promotes an increase in surface NMDAR number, which is likely to increase the magnitude of NMDAR currents, reducing or blocking the insertion of NMDAR at the cell surface will reduce the number of receptors on the surface, which can decrease the magnitude of NMDAR-mediated activity. This, in turn, will decrease the intracellular signaling triggered by NMDAR-induced Ca^{2+} influx, leading to block of inhibition by myelin-associated inhibitors. Consequently, regulating iGluR trafficking is an important tool for regulating the number of receptors being activated on the surface, given that improper surface NMDAR expression and signaling plays a fundamental role in the development of many disorders including stroke and neurodegenerative disease (Cull-Candy et al., 2001). Nonetheless, we first need to utilize the Fura-2 Ca^{2+} assay or an electrophysiological approach to confirm that myelin-induced increases in surface NMDAR leads to increases in the magnitude of iGluR activity.

A number of studies have demonstrated that forward trafficking of NMDARs to synapses and activation of kinases such as PKC and ERKs are events that are required for synaptic plasticity (Bliss and Collingridge, 1993, Yasuda et al., 2003, Thomas and Huganir, 2004). Our findings demonstrate that activation of PKC, ERKs and increased surface NMDARs are downstream events occurring in response to myelin treatment. It seems then, that there are a number of parallels between signaling occurring in response to myelin-associated inhibitors and signaling that induces synaptic plasticity. First, NgR and PirB, which are the receptors activated by myelin-associated inhibitors, are also shown to regulate synaptic plasticity by restricting plasticity in the visual cortex during the time termed the critical period (Filbin, 2003, McGee et al., 2005, Syken et al., 2006, Atwal et al., 2008). Furthermore, the Giger group reported the loss of NgR changes the magnitude of LTP and LTD responses. It is therefore tempting to speculate the possibility that myelin-induced activation of NgR and PirB receptors might mimic NMDAR-mediated synaptic plasticity in response to injury conditions. The disruption of intact myelin following CNS injury, which exposes several inhibitory elements to a new environment, where inappropriate interactions with the lesioned axons take place and since all the players mentioned here have an important role in synaptic plasticity; it is possible that myelin inhibitors induce chronic activity response, which is reminiscent of synaptic plasticity.

Our data demonstrate that P1 DRG neurons, which are not inhibited by MAG at this age (DeBellard et al., 1996), switch to inhibition upon exposure to iGluR antagonists. The opposing effects mediated by iGluR antagonists in younger versus older DRG neurons indicates that the intracellular state of the neurons differs in younger versus older

DRG neurons. A potential difference might be the intracellular Ca^{2+} levels and its subsequent signaling. Data to support this was provided by Yamashita's group and our lab. They showed that MAG-induced neurite outgrowth promotion in young DRG neurons depends on IP_3 signaling, which regulates release of Ca^{2+} from intracellular ER stores (Hasegawa et al., 2004). They demonstrated that when the IP_3 signaling pathway is inhibited with the drug XestC, MAG-induced neurite outgrowth response was switched to inhibition in young DRG neurons, while inhibiting IP_3R in postnatal cerebellar neurons, which are inhibited by MAG, had no effect (Hasegawa et al., 2004). Similarly, our lab has shown that blocking PKA with the drug KT5720 in P1 DRG neurons, resulted in a switch to neurite outgrowth inhibition, whereas inhibition of PKA in older DRG neurons had no effect on MAG-induced inhibition (Cai et al., 1999, Cai et al., 2001). Together, our results suggest that similar to IP_3 and PKA, blocking iGluR-mediated signaling induces neurite outgrowth inhibition in young DRG neurons. However, while inhibiting IP_3R and PKA have no effect in postnatal neurons that are inhibited by MAG, blocking NMDAR or AMPA/KAR efficiently overcomes MAG's inhibition. This may indicate that NMDAR-induced Ca^{2+} influx and its subsequent signaling might be an important determinant in the response to MAG in younger versus older DRG neurons.

While Ca^{2+} signaling in response to MAG is not well understood in young neurons that are not inhibited by MAG, such as P1 DRG neurons, postnatal neurons that are inhibited by MAG demonstrate a moderate intracellular Ca^{2+} release from ER stores when treated with MAG, which, in turn, triggers growth cone repulsion and inhibition of neurite outgrowth (Hasegawa et al., 2004, Henley et al., 2004). However, data regarding intracellular Ca^{2+} role in inhibition are contradictory. In one report it was demonstrated

that postnatal cerebellar neurons treated with the Ca^{2+} chelator, BAPTA, which prevents elevation of intracellular Ca^{2+} , exhibited reduced MAG-induced growth cone repulsion (Henley et al., 2004). Conversely, although blocking intracellular Ca^{2+} release with XestC in postnatal cerebellar neurons reduced intracellular Ca^{2+} increase by MAG, it was not sufficient to overcome MAG-mediated inhibition (Hasegawa et al., 2004, Henley et al., 2004). A possible explanation is that BAPTA depletes extracellular and intracellular Ca^{2+} levels. Thus, other intracellular Ca^{2+} receptors, such as the ryanodine receptors, might release sufficient amount of Ca^{2+} to induce inhibition. Since there is sufficient evidence to support MAG as inducer of growth-cone collapse by elevating intracellular Ca^{2+} , we hypothesize that blocking NMDAR-induced Ca^{2+} influx might diminish Ca^{2+} release from intracellular ER stores. This is consistent with reports demonstrating that activity-induced Ca^{2+} influx can trigger Ca^{2+} release from internal stores in CNS neurons in a process called Ca^{2+} -induced Ca^{2+} release (CICR) (Alford et al., 1993, Shmigol et al., 1995, Jacobs and Meyer, 1997). In addition, it is also possible that blocking iGluR-induced Ca^{2+} influx disrupts the activation of Ca^{2+} -sensitive signaling molecules such as conventional PKCs, ERKs, both of which are shown to promote inhibition (Sivasankaran et al., 2002, Koprivica et al., 2005). In younger DRG neurons the scenario is likely to be different because of increased PKA and IP_3 activity, whereas PKC activity is low, which is correlated with increased neurite outgrowth in the presence of MAG (Hasegawa et al., 2004, Henley et al., 2004). Thus, differences in intracellular Ca^{2+} levels in younger versus older DRG neurons might be responsible for the differential response to MAG. Consequently, pharmacological inhibition of iGluR activity in young DRG neurons might lead to a decrease in cytoplasmic Ca^{2+} , resulting in a switch from neurite outgrowth

promotion to inhibition by MAG. A similar phenomenon, where intracellular Ca^{2+} levels dictate chemoattraction versus chemorepulsion is described by Poo and colleagues, where chemoattraction to netrins can be switched to chemorepulsion by lowering intracellular Ca^{2+} levels (Hopker et al., 1999). To confirm whether this scenario is the case, one would need to conduct Fura-2 Ca^{2+} assays in younger versus older DRG neurons and compare the intracellular Ca^{2+} response to MAG/myelin in the presence or absence of iGluR antagonists.

Although we did not investigate whether blocking excitatory activity can modulate calcium/calmodulin-dependent protein kinase II (CaMKII) activity, a body of evidence suggests that CaMKII activity and function is largely dependent on NMDAR-mediated currents. Our lab has shown that activation of CaMKs, specifically CaMKIV, is required for BDNF-induced priming. Furthermore, overexpression of a constitutively active form of CaMKIV was sufficient to block MAG-induced inhibition (Spencer et al., 2008). The role of CaMKII, however, was not addressed. It is well established that CaMKII plays a key role in mediating synaptic plasticity and its activation is regulated by NMDAR-induced Ca^{2+} influx (Silva et al., 1992, Malenka and Nicoll, 1999, Frankland et al., 2001, Malinow and Malenka, 2002). CaMKII holoenzyme is composed of 6-12 subunits, mainly α and β subunits, which are endogenously expressed in 3:1 ratio, respectively (Miller and Kennedy, 1985, Thiagarajan et al., 2002). CaMKII holoenzymes which predominantly express CaMKII α subunit exhibit low affinity to Ca^{2+} /CaM and are therefore activated in response to high Ca^{2+} /CaM, which occurs during LTP. CaMKII β , on the other hand, exhibits high affinity to Ca^{2+} /CaM and can be therefore activated under basal conditions, when Ca^{2+} /CaM levels are low. CaMKII α and β subunits are not

only activated in a differential manner, but also carry out separate functions. Activation of CaMKII α leads to an increase in number as well as the conductivity of AMPARs, whereas CaMKII β activation was shown to promote neurite extension, dendritic arborization, and synapse formation. Its role in regulating dendritic morphology is correlated with its interactions with the actin cytoskeleton to control filopodia extension. Interestingly, CaMKII α and β levels are inversely regulated in response to activity. Of particular interest, overnight blockade of NMDAR-mediated activity with APV was shown to induce a decrease in CaMKII α subunit levels, whereas blockade of AMPAR-mediated activity triggered an increased expression in CaMKII β subunits (Thiagarajan et al., 2002). Consequently, pharmacological block of either NMDAR or AMPAR activity led to a shift in α : β ratio from 3:1 to 1:1, enriching β subunit expression levels. It has been shown that pharmacological or genetic disruption of CaMKII β subunit, not CaMKII α , in cultured HNs disrupts filopodia motility, dendritic extension as well as synapse formation (Fink et al., 2003). We therefore speculate that pharmacological block of iGluR-induced activity may trigger a shift in CaMKII α : β ratio, upregulating CaMKII β expression. A greater number of CaMKII enzymes containing β subunits, in turn, can promote filopodia extension, which is controlled by the actin cytoskeleton, leading to enhanced neurite extension in the presence of MAG. How can CaMKII be activated in conditions of decreased activity, where intracellular Ca^{2+} /CaM levels are low? A potential explanation is derived from a study which has shown that the levels of Ca^{2+} /CaM in non-stimulated neurons is sufficient to mediate CaMKII β -dependent dendritic extension and motility, suggesting that the morphological function of CaMKII β only requires relatively weak Ca^{2+} /CaM signals (Fink et al., 2003). CaMKII activation

requires Ca^{2+} /CaM binding, which is likely to be present under basal conditions in unstimulated neurons. To examine whether CaMKII activity is important for overcoming MAG-mediated neurite outgrowth inhibition, one would need to examine whether pharmacological or genetic disruption of CaMKII can block the ability of iGluR antagonist to overcome MAG-mediated inhibition, and whether its overexpression is sufficient to overcome MAG-mediated inhibition. In addition, analyzing CaMKII α and β expression patterns and their subsequential ratio in response to chronic blockade of iGluR activity, in the presence or absence of MAG/myelin is needed in order to determine whether their levels are regulated by iGluR activity.

The results presented here propose a new role for iGluR channels as mediators of neurite outgrowth inhibition in response to myelin-associated inhibitors. This is the first report in which iGluR channels are implicated in mediating MAG/myelin-induced inhibition by activating ERK as well as promoting the forward trafficking of NMDAR to the cell surface.

Chapter IV: Methods and Materials

4.1 CHO Cell monolayers

4.1.1 Maintenance

Control or stably-transfected MAG-expressing Chinese Hamster Ovary (CHO) cells were used as monolayer substrates in the neurite outgrowth assay (NOG) described below. CHO cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco), which had been supplemented with 10% dialyzed fetal bovine serum (FBS, Gibco), 34.8 mM L-Proline (Sigma), 10 mM Glycine (Sigma), 300 nM Thymidine (Sigma), and 2 mM L-Glutamine (Gibco) at 37°C in 7.3% CO₂ (Mukhopadhyay et al., 1994).

4.1.2 Monolayer Preparation

Permanox or glass 8 well chamber slides (Lab-Tek) were precoated with 20.0 µg/ml poly-L-lysine (Sigma) for 30 minutes at room temperature, after which, poly-L-lysine was removed and the slides were treated with 20 µg/ml fibronectin (Sigma) for 2 hours at 37°C. Control and MAG-expressing CHO cells were then plated in each chamber of an 8-well tissue culture slide (Lab-Tek) at the following concentrations: control at $4-6 \times 10^4$ cells/well; MAG at $4-5.5 \times 10^4$ cells/well. The slides were incubated overnight at 37° C and grown to confluency after which CNS neurons were added for 16-24 hours.

4.2 Myelin purification

The medulla from an adult (30-60 days) rat brain was isolated and homogenized in a 0.25M sucrose solution containing protease inhibitors cocktail (CalBiochem) using a

glass and Teflon homogenizer. The homogenate was then mixed with a 2.55 M sucrose solution to create a 1.4 M solution. This is then layered onto a 1.9 M solution, followed by 0.85 M and 0.25 M solutions. The gradient was centrifuged at 40,000 rpm for 14 hours at 4°C. Following centrifugation, myelin layer was transferred into a 10ml Beckman tube, where it was homogenized again with dH₂O and protease inhibitors, centrifuged at 14,000 rpm for 1 hour at 4°C, resuspended in 10mM HEPES, extensively triturated using 5ml insulin syringe, and assayed for its protein concentration.

4.3 Primary Cultures

4.3.1 Hippocampal and Cortical neurons

Hippocampi or cortices were collected from postnatal day 1-2 (P1-P2) long-evans rats and placed on ice cold plain neurobasal media. The digestive enzyme, papain (Worthington), was added at a final concentration of 0.5 mg/ml for 20 minutes at 37°C, after which fresh papain solution was added for a second 20 minutes incubation. Two 2 minutes washes with Soybean-trypsin inhibitor (Sigma) were done in order to inactivate the papain. Tissues were then washed three times with plain neurobasal media, triturated with 2ml pipette for 10-15 times, passed through 40 µm cell strainer and 6 ml a single cell suspension were then loaded on top of a gradient, consisting of four layers of OptiprepTM working solution (30%w/v iodixanol, 0.425% NaCl, 5 mM MOPS-NaOH, pH 7.4) of densities 1.057, 1.043, 1.036 and 1.029 g/ml (listed from bottom of the tube to top of the tube). Neurons were then centrifuged at 1900 rpm for 15 minutes at room

temperature. Fractions containing enriched populations of neurons were isolated (below 3ml line, according to the directions of the OptiprepTM manual), diluted with plain neurobasal, and centrifuged at 1-200 rpm, 4C, for 5 minutes. The pellet containing dissociated neurons was then resuspended in supplemented (B-27, L-Glut, and antibiotic mix) neurobasal media (S-NB) and neurons were counted and plated on 10cm dishes, 6-well dishes or 8 chamber slides.

After initial plating in 10cm plates, media was replaced within two hours. Cells were then left in 37C incubator for either 2days or for 7days cultures; S-NB media was replaced at day 4.

4.3.2 Cerebellar Neurons

P5-7 Long-Evans rat's cerebellum was collected in 6ml of 0.025% trypsin with 100µg/ml DNase I. The tissue was dissociated by triturating 10-15 times, followed by 7-10 minutes 37°C incubation. Trypsin was inactivated with 10% serum/DMEM, after which cells were passed through a 40 µm cell-strainer, and pelleted by 5min, 4°C, 100-200rpm centrifugation. The pellet was then resuspended to a single-cell suspension in a modified Sato medium (DMEM; Path-O-Cyte BSA; 20nM progesterone; 100mM putrescine; 30nM sodium selenite; 5 µg/ml insulin, 80ng/ml tri-iodo-thyronine (T3); 10ng/ml thyroxine (T4); 118 U/ml penicillin; 118 µg/ml streptomycin; 295 ng/ml amphotericin B) or supplemented neurobasal medium, counted and plated on either 6-well dishes or 8 chamber slides.

4.3.3 Dorsal Root Ganglia (DRG) Neurons

DRG neurons were collected from P1 or P5 long-evans rats and placed into 4.5 ml ice-cold 0.15% Collagenase/L-15 or Hanks media. DRGs were then gently triturated for 10-15 times, incubated for 45 minutes at 37°C, after which, 0.025% trypsin and 50µg/ml DNaseI were added for 10 minutes at 37°C, after which cells were triturated again in order to dissociate any remaining clumps. 10% serum/DMEM was then added to inactivate trypsin, DRG neurons washed with DMEM, resuspended in Sato media, counted and plated onto either 6-well dishes or 8 chamber slides.

4.4 Priming Neurons with NTs or IGluR Antagonists

6-well plates were coated with 100 µg/ml of poly-L-lysine for 60 minutes at 37°C. The wells were then washed twice with PBS and once with DMEM to remove excess PLL. Isolated cerebellar neurons were then plated at concentrations of approximately $1-2 \times 10^6$ cells/well. Neurons were then treated with either BDNF (200 ng/ml), or NMDAR antagonist, APV (100uM), AMPA/Kainate receptor antagonist, CNQX (20uM). The neurons were then cultured overnight (18hours) at 37°C, after which cells were trypsinized (0.4x trypsin for 5-10 minutes at 37°C). 10% serum-containing media was then added and cells were collected, resuspended in fresh Sato, counted and plated onto control or MAG-expressing CHO cells for neurite outgrowth assay (NOG).

4.5 Neurite Outgrowth Assay

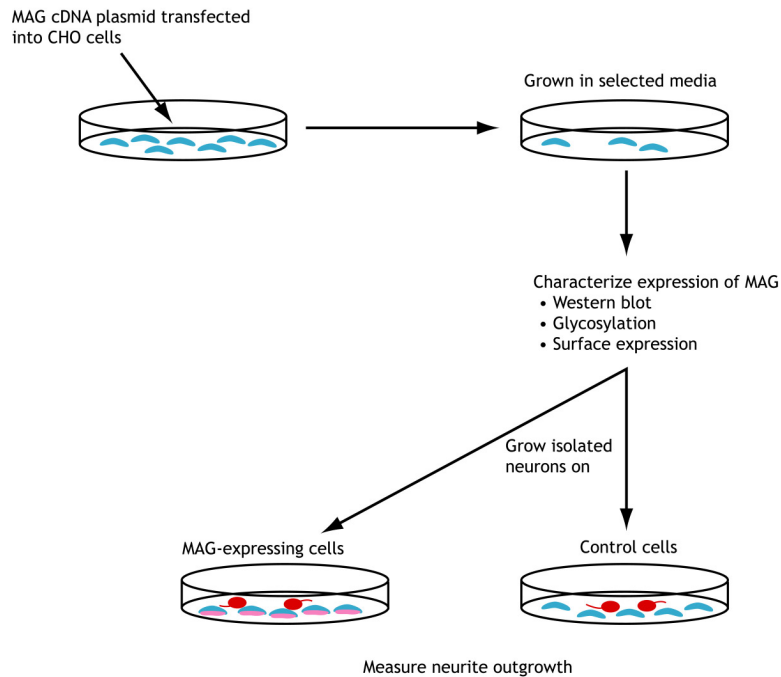


Figure 4.1: Schematic representation of the generation of MAG-expressing CHO cells and the standard Neurite Outgrowth Assay. When priming is performed, the neurons are first exposed to neurotrophins for 18-24 hours prior to plating on the CHO cell monolayers.

Following dissociation of primary neurons, cells are counted, mixed with iGluR antagonist or agonist, and plated onto CHO cell monolayers substrate at a cell density of 1.5×10^4 cells per well (8 chamber slide) for hippocampal neurons and $0.75\text{--}1 \times 10^4$ cells per well for DRG neurons. Plated cells were then incubated for 16 hours for DRG neurons or 18-22 hours for HNs, at 37°C . Cells were then fixed with 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with ice cold methanol for 2 minutes, and blocked with 10% serum/DMEM for 60 minutes at room temperature. Neurons were then stained for the neuronal-specific marker β -tubulin-III (monoclonal, 1:1000, Covance), in a PBS-BSA (0.05%), overnight at 4°C . The next day,

chamber-slides were washed 3 times with PBS, and 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. After that, chamber-slides were washed 3 times with PBS and incubated 30 minutes at room temperature in a PBS-BSA solution containing Streptavidin-Texas Red at 1:500. Chamber-slides were washed 3 more times with PBS and then mounted with coverslips on Permafluor (Immunon) containing slides, left to dry overnight and then analyzed under a fluorescent microscope. In order to quantify neurite outgrowth length, the mean neurite length of the longest neurite was measured using the simple PCI or Metamorph image quantification software (200-300 neurons per well). Statistical and data analyses were performed using the GraphPrism software program.

4.6 Cell Surface Biotinylation Assay

P1 cortical neurons were isolated and then plated on 10cm culture dish that was pre-coated with 100ug/ml PLL at density of $15\text{-}20 \times 10^6$ cells per plate for 2 days or $6\text{-}8 \times 10^6$ cells for 7 days at 37C. Cells were then treated with either myelin (10 or 20ug/ml) or phorbol ester (PMA, 100ng/ml) for either 10 or 20 minutes. Following treatments, cells were washed 3 times with (RT, 10C, and 4C) artificial cerebral spinal fluid (aCSF, phenol-red free Hanks' balanced salt solution, Gibco, 1mM MgCl_2 , 2mM CaCl_2 , and glucose, equilibrated with O_2CO_2). Cells were then incubated with cold 1.5mg/ml Sulfo-NHS-biotin tag (Pierce) in aCSF for 20 minutes at 4C, gently swirling every 5 minutes. This cell impermeable reagent covalently conjugates biotin to primary amine groups of surface proteins, coupling the biotin to proteins via a reversible disulfide linkage. Unbound biotin was then quenched with cold 50-100mM Glycine in aCSF (rinsed once

then twice washed for 10-15 minutes). Cells were then scraped into a lysis buffer (50mM tris pH 7.4, 2mM EDTA, 2mM EGTA, Roche 'Total EDTA' protease inhibitor cocktail, and 100X Sigma phosphatase inhibitor cocktail). Lysates were then sonicated for 30 seconds on ice and 10% of the lysates were collected for total protein lysate analysis. Remaining of lysates were then loaded into 2ml Beckman ultracentrifuge tube, capped and placed inside Ti90 rotor for centrifugation at 36000 rpm for 20 minutes. Membrane pellet was then resuspended in precipitation buffer (PB=lysis buffer plus 10mM NaCl), 0.2% of SDS was added and samples were incubated at 60C for 5 minutes. 1% Triton-X-100 was added, after which, samples were sonicated briefly on ice, and spun again for 20 minutes at 36000rpm to remove insoluble materials. Supernatant was then collected, assayed for protein concentration (BCA Assay, Pierce) and mixed with 50-100ul slurry of BSA-blocked Ultralink-neutravidin beads (Pierce). Samples were rotated in cold room overnight, after which, bound beads were washed with PB+1% Triton X-100 and centrifuged for 2-3 minutes at 8000 rpm, 4C, repeating wash/spin 4-5 times. Biotinylated samples were then eluted by boiling samples with 40ul Laemmli buffer. Subsequently, eluted proteins are resolved by SDS-PAGE in a 8% gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. Detection of ser890 and ser896 NR1 (cell signaling), total NR1 (Santa Cruz), or beta-III-Tubulin (Covance) protein levels followed.

4.6.1 Cell Surface Insertion Assay

P1 cortical neurons were collected and plated on 10cm dishes (precoated with 100ug/ml PLL) at density of $15-20 \times 10^6$ cells/plate. Neurons were cultured for 2-3 days, after which cells were washed 3 times with (RT, 10C, and 4C) aCSF. Cells were then incubated with cold 1.5mg/ml sulfo-NHS-Acetate (Pierce) in 0.1M sodium phosphate

buffer/150mM NaCl for 20 minutes at 4C, gently swirling every 5 minutes. Second fresh acetate solution was added for 20 additional minutes. The acetate tag covalently binds to the primary amine groups of surface proteins; therefore once bound, the amine group is irreversibly capped with an acyl group, preventing subsequent biotin tagging on the acyl-capped surface proteins. Unbound acetate was then quenched with cold 100mM Glycine in aCSF (rinsed once then twice washed for 10-15 minutes). Cells were then washed with plain NB, and incubated with S-NB in the presence or absence of myelin (10ug/ml) for 8 hours. As an assay control, one plate, termed 0 hours, was tagged with biotin immediately after acetate treatment, lysed and surface proteins were isolated according to the protocol above. Following 8 hours incubation, cells were biotinylated according to the protocol above. Since biotin cannot bind to the existing acetate-tagged surface proteins, the biotin-tagged surface protein are newly inserted to the surface.

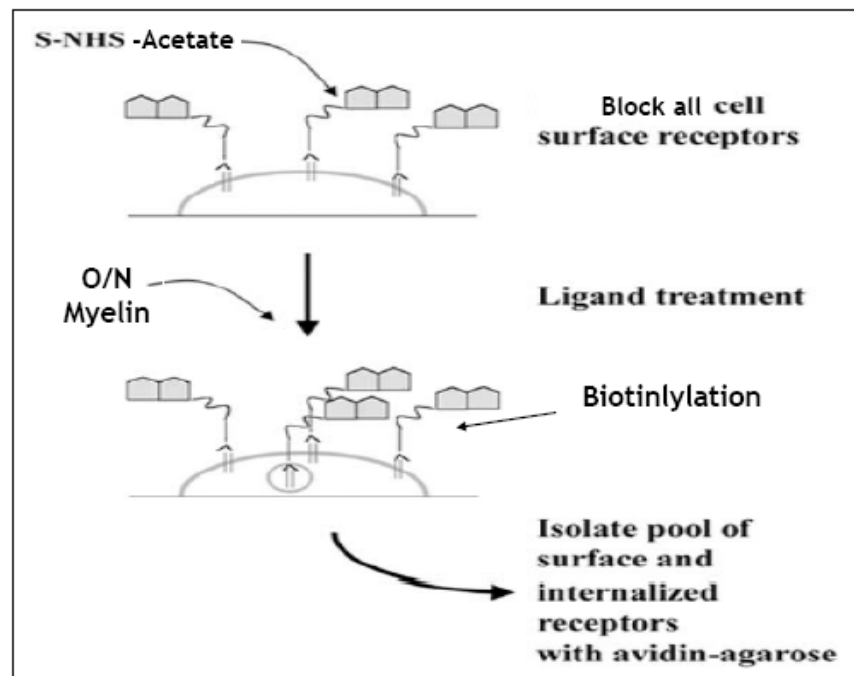


Figure 4.2: Schematic Representation of cell-surface Insertion Assay. When biotinylation alone is performed, treatment with myelin (10 or 20ug/ml) takes place 10 or 20 minutes prior to surface biotinylation.

4.7 Western Blot Analysis of Myelin and IGluR Antagonists

P6 cerebellar granule neurons (CGNs) were plated on 6-well plates, which were pre-coated with 100ug/ml PLL, at density of $3-5 \times 10^6$ cells/well and incubated for 24 hours at 37C. The next day, cells were serum-starved for 8 hours, treated with myelin (20ug/ml) or cAMP (1mM) for 60 minutes, or overnight (18hours) in the presence or absence of NMDAR antagonists, APV (100uM), MK-801 (20uM), AMPA/Kainate inhibitor, CNQX (20uM), or positive control MEK inhibitor, U0126 (5uM) for 30min prior to myelin treatment, or with NMDA agonist (20-50uM) for 10 or 60 minutes. Cells were then washed with cold PBS and scraped to a RIPA lysis cocktail buffer (Upstate) supplemented with protease and phosphatase inhibitors (Sigma), incubated on ice for 45 minutes, while vortexing samples every 10-15 minutes. Samples were then centrifuged for 15 minutes at 13000rpm at 4C, after which supernatant was collected, assayed for protein concentration (Modified Lowry Assay, BD biosciences) and loaded into a 10% acrylamide gel for Western blot analysis. pERK and total ERKs-specific antibodies were utilized for protein detection (Cell signaling).

Chapter IV: References

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