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The Role of CaMKIV in the Neurotrophin-induced Priming Effect and the Characterization/Regeneration of ES-derived Motor Neurons

by
Tim Spencer

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

2005

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Abstract

The Role of CaMKIV in the Neurotrophin-induced Priming Effect and the Characterization/Regeneration of ES-derived Motor Neurons

by
Tim Spencer

Thesis Advisor: Dr. Marie T. Filbin

The lack of axonal regeneration observed in the adult mammalian central nervous system (CNS) is due, in part, to the presence of the myelin-associated inhibitors, such as myelin-associated glycoprotein (MAG). The inhibition induced by these molecules, or by myelin in general, can be blocked, however, via artificial (dibutyryl-cAMP) or signaling-induced (neurotrophin-treated) elevation of intracellular cAMP and modulation of the PKA and ERK pathways. We have previously shown that these effects are dependent on the activation of the cellular transcription factor, the cAMP response-element binding protein (CREB). Calcium/calmodulin-dependant kinase IV (CaMKIV) has been shown to act as a modulator of CREB activity and therefore, we set out to elucidate what role, if any, CaMK plays in the neurotrophin-induced block of MAG-mediated inhibition of neurite outgrowth. Here, we show that blocking CaMK with the pharmacological inhibitor KN-62 can abrogate the BDNF-induced phosphorylation of CREB as well as the reversal of MAG-mediated inhibition of neurite outgrowth from cerebellar (CN) and dorsal root ganglion (DRG) neurons but not in neurons treated with dibutyryl-cAMP.

In order to verify and attribute these findings specifically to the CaMKIV moiety, we generated adenoviral constructs containing constitutively-active and kinase-dead mutant forms of CaMKIV and infected primary neurons *in vitro* prior to exposure to MAG-expressing CHO cells or purified myelin. Our findings indicate that CaMKIV is a downstream effector in the BDNF-induced priming pathway. Furthermore, we show that blocking CaMK signaling has no effect on the elevation of endogenous cAMP levels which occurs in response to priming with BDNF suggesting that CaMKIV is not upstream from cAMP elevation and PKA activation, but rather constitutes a separate and parallel signaling pathway, leading to CREB phosphorylation and activation of transcription. Further evidence suggests that calcium influx from intracellular stores may be responsible for this induction of CaMKIV and its subsequent activity.

In addition, we have sought to examine a potential role for embryonic stem cell (ES)-derived motor neurons in "replacement" therapies following either spinal cord injury or onset of certain motor neuron degenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS). ES cells derived from mouse embryos can be induced to differentiate into motor neurons in culture and can be subsequently engrafted *in vivo*, thereby replacing motor neurons lost to disease or injury. However, these engrafted motor neurons must be able to extend axons through the CNS and PNS environments and synapse with their proper targets. Here, we report that these motor neurons are indeed inhibited by both myelin-associated glycoprotein (MAG) and purified myelin *in vitro*. In order to encourage axonal growth in these neurons, we investigated

whether we could modulate the same signaling pathways that we have shown to be involved in encouraging regeneration in endogenous neurons. Our findings suggest that ES-derived motor neurons are responsive to some components of the inhibition-blocking signaling pathway which leads to axonal regeneration, such as elevation of intracellular cAMP levels, priming with neurotrophins and increasing polyamine levels.

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Chapter I: Introduction

1.1 CNS Regenerative Failure

Since the time of the ancient Egyptian civilizations, it has been understood that following injury, the adult mammalian CNS fails to regrow cut or damaged axons whereas repair of damaged peripheral (PNS) nerve connections is quite common. Why *do* injured CNS neurons fail to regenerate? Do these neurons lose their intrinsic capacity to grow with development, or are there extrinsic factors which prevent repair? In order to answer these questions, one must look to the local CNS environment. The first evidence for this idea is indicated by the finding that, if provided with a "favorable" substrate, such as a peripheral nerve graft, injured CNS neurons are indeed able to regrow severed axons (David and Aguayo, 1981). Furthermore, in 1982, Martin Berry was the first to suggest that perhaps this lack of regeneration may not be entirely cell autonomous. Berry found that products of the breakdown of the myelin sheath, which are released after CNS injury, can inhibit the regrowth of damaged axons (Berry, 1982). In addition, several years later, work by Martin Schwab's group identified specific myelin membrane-associated protein fractions which displayed axonal growth inhibitory properties (Caroni and Schwab, 1988a). Since these seminal findings, several molecules, associated with myelin, have been found which may be primary factors in the lack of CNS regeneration.

In order for a molecule to be considered to be an inhibitor of CNS regeneration *in vivo*, it must, of course, be present in the adult mammalian CNS and be in a position to encounter an extending axon following neuronal injury and the associated damage. To date, three major inhibitors of axonal regeneration associated with myelin have been identified:

myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte-myelin glycoprotein (OMgp). Recent evidence from several labs has shown, interestingly, that all three of these molecules enact their effects via binding to the same receptors, the NgR-LINGO-$p75^{NTR}$ complex (Domeniconi et al., 2002; Liu et al., 2002; Mi et al., 2004; Wang et al., 2002a; Wong et al., 2002). All three of these inhibitory molecules appear to be present in the adult mammalian CNS and *in vitro* data suggests that they may each be sufficient to block regeneration after CNS injury (Chen et al., 2000; GrandPre et al., 2000; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Prinjha et al., 2000; Wang et al., 2002b). While their respective distribution and abundance in CNS myelin varies, all are present and able to bind receptor(s) on the neuronal surface following injury and myelin fragmentation. Interestingly, these molecules are also thought to be present in the PNS as well. Why, then, do PNS axons readily regenerate after injury while CNS axons do not? One hypothesis suggests that the process of rapid Wallerian degeneration—which includes an influx of immune responsive elements such as macrophages, which occurs after injury to the PNS, results in the clearing of myelin debris—results in the rapid removal of the myelin-associated inhibitors, thereby allowing regeneration. Evidence for this hypothesis can be seen in the C57BL/6WLD/OLA (Wld^S) mice. These Wallerian degeneration-slowed mice exhibit retarded regeneration following PNS injury (Bisby et al., 1995; Brown et al., 1991; Brown et al., 1992; Perry et al., 1991). Conversely, the immune-privileged CNS, myelin clearing proceeds much more slowly and, therefore, the inhibitors are still present and, indeed, more accessible, following injury.

In addition to the myelin-associated inhibitors, axonal regeneration can be blocked by the formation of a structure called the glial scar. This structure, indicated by the increase in reactive gliosis which occurs after injury to the CNS, forms both a chemical and physical barrier to regenerating axons. Furthermore, there also exist several molecules which act as repulsive or inhibitory guidance cues during development. While these molecules are able to induce growth cone collapse or turning in embryonic and adult neurons, it is not clear if these molecules are in fact active in the mature nervous system and so their physiological role as inhibitors of axonal regeneration remain questionable. These molecules and structures will be discussed in more detail later.

1.2 Inhibitors of Axonal Regeneration in Myelin

The myelin sheath is, simply put, a membranous extension which encircles an axon in a multilayered, concentric fashion. This structure serves as an "electrical insulator" which increases the velocity of conducted action potentials via a process called saltatory conduction. In the peripheral nervous system (PNS), myelin is produced by cytoplasmic extensions from Schwann cells with a single Schwann cell myelinating a single axon. In the CNS, however, myelination is performed by oligodendrocytes. Here, oligodendrocytes send out projections which, upon encountering an axon, begin the process of membrane extension and axonal envelopment. In the CNS, a single oligodendrocyte can myelinate many axons. In either system, however, myelin is essential for proper neuronal signal propagation. Myelination failure or disorders which result in demyelination, such as multiple sclerosis, invariably result in loss of effective sensory and motor function or death.

During the processes of myelination in both the CNS and PNS, changes occur in the expression and localization of myelin membrane proteins. The completion of myelination also correlates with a precipitous drop in axonal sprouting and growth (Kapfhammer and Schwab, 1994a; Kapfhammer and Schwab, 1994b; Keirstead et al., 1995; Savio and Schwab, 1990; Schwegler et al., 1995). This loss of growth potential may be attributed to the expression of the myelin-associated inhibitors of regeneration. Since these myelinated axons have already reached their targets prior to the final stages of myelination, it is believed that the aforementioned inhibitors may act to prevent inadvertent sprouting and improper synapse formation. While this blockage of axonal growth by the myelin-associated inhibitors is essential following the termination of development, it also has the unfortunate effect of blocking any attempts by damaged adult axons to regenerate after injury.

Despite the differences in cellular origin, however, both PNS and CNS myelin express the proteins associated with inhibition of axonal regeneration. The myelin-associated inhibitors all appear to be present in undamaged myelin and may have roles other than the block of regeneration or inadvertent sprouting. For example, myelin-associated glycoprotein (MAG) is found in the periaxonal surface of CNS myelin and is thought to be responsible for maintaining the 12-14nm space between myelin and the axonal membrane. In the CNS, MAG and Nogo have been shown to be present in the periaxonal internode and OMgp is believed to be enriched in the paranodal loops. However, following injury, all of these proteins may become exposed to any potentially regenerating axons as a result of fragmentation of the myelin.

1.3 Inhibitors In Myelin

1.3.1 Nogo

In the late 1980's several investigators reported that both CNS and PNS neurons could extend neurites through a sciatic nerve explant but failed to enter an explant from the optic nerve. Since the two explants differed only in their myelin producing cells, Schwann cells in the sciatic nerve and oligodendrocytes in the optic nerve, it was proposed that the oligodendrocytes were the deciding factor in the lack of neurite outgrowth seen within the CNS tracts and, subsequently, myelin was proposed as an inhibitor of regeneration (Berry, 1982). In a seminal study on this CNS-specific inhibition, Martin Schwab and colleagues at the University of Zurich examined CNS myelin's inhibitory properties and found that the main inhibitory components were membrane-bound and associated with the protein fraction of CNS myelin (Caroni and Schwab, 1988b). The inhibitory components of myelin could be recovered after separation in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as two minor myelin-associated proteins with relative molecular masses of 35kDa and 250kDa (then called neurite growth inhibitors NI-35 and NI-250, respectively) (Caroni and Schwab, 1988b).

When the same group generated a monoclonal antibody, IN-1, against the two proteins, they showed that the addition of IN-1 to cultures reduced the inhibitory activity of myelin (Caroni and Schwab, 1988a). Notably, IN-1 injected into the injured spinal cord resulted in regeneration of 5% of the damaged axons and improvements in functional recovery in injured adult rats (Bregman et al., 1995; Schnell and Schwab, 1990).

Using peptide sequences derived from the bovine homologue of NI-250 (Spillmann et al., 1998), three groups independently identified the IN-1 antigen(s) as products of the *Nogo* gene (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

Three Nogo isoforms, Nogo-A, -B, and –C, are encoded from a single gene by alternative splicing and/or promoter usage. In human and rat fetal tissue, Nogo-A mRNA is strongly expressed in the ventral spinal cord, dorsal root and autonomic ganglia. The same expression pattern is observed in the adult spinal cord and ganglia. High levels of Nogo-A message are present in oligodendrocytes, motor neurons and sensory ganglia neurons, but not in astrocytes or Schwann cells. Minor expression is also observed in developing muscle tissue (Josephson et al., 2001). Western blot analysis of adult tissue reveals that Nogo-A protein is present in brain and spinal cord, and at low levels in the testis and heart (Huber et al., 2002). After spinal cord injury, Nogo-A is up-regulated to a moderate degree (Wang et al., 2002c), whereas traumatic lesions to the cortex do not change Nogo-A expression (Huber et al., 2002). Nogo-B and Nogo-C have a much wider expression profile in neurons, skeletal muscle and various peripheral tissues.

The *nogo* products show a high degree of homology with the reticulon protein family (van de Velde et al., 1994) and contain a dilysine endoplasmic reticulum (ER) retention sequence. Interestingly, Nogo proteins are mostly localized to the ER with a small percentage present at the plasma membrane (GrandPre et al., 2000). Throughout the adult CNS, Nogo-A has been detected by confocal and electronimmuno-microscopy on oligodendrocyte processes in the periaxonal and outermost myelin membranes (Huber et al., 2002; Wang et al., 2002c). Furthermore, it has been suggested that Nogo-A may have two different membrane topologies, one in which both the N- and C-terminus are oriented cytoplasmically, and a second in which both termini are oriented extracellularly (Oertle et al., 2002).

All three Nogo isoforms contain a 66-amino acid extracellular region (Nogo-66) which displays neuron-specific growth inhibitory activity *in vitro* (GrandPre et al., 2000). An additional domain which is specific to Nogo-A, termed Amino-Nogo, has been localized to a 195-amino acid stretch near the N-terminus which was shown to inhibit neurite outgrowth as well as abrogate 3T3 fibroblast spreading (Fournier et al., 2001; Prinjha et al., 2002). Schwab's team generated a transgenic mouse expressing Nogo-A under the control of the Schwann cell-specific P0 promoter which is strongly induced 7 days post peripheral nerve injury at the onset of re-myelination (Gupta et al., 1988). These transgenic mice displayed an impaired recovery from sciatic nerve crush injury suggesting that Nogo-A was capable of inhibiting axonal growth *in vivo* (Pot et al., 2002).

Several groups using different strategies have created Nogo knockout mice but, at the present time, there is a lack of consensus on the effects of the Nogo deletion. Strittmatter and colleagues utilized a retroviral gene trap insertion technique in order to disrupt exon 3 (specific to Nogo-A) of the Nogo gene. The resultant mice, however, were found to lack both Nogo-A and Nogo-B with Nogo-C levels remaining unaffected (Kim et al., 2003). In this study, Strittmatter's group reports limited regeneration in several young animals, but these improvements appear to be lost as the animals age (Kim et al., 2003). Conversely, Tessier-Lavigne's group generated two different mouse models. In the first, they induced a deletion in the exon 1 region of the Nogo gene, thereby disrupting Nogo-

A and –B expression but without affecting Nogo-C expression. The second model featured a selected deletion of the C-terminal region which is common to all three isoforms, thereby eliminating all Nogo expression. In both of these mouse models, Tessier-Lavigne and colleagues failed to observe any improvements in axonal regeneration following injury (Zheng et al., 2003). Finally, Schwab and colleagues report another Nogo-deficient mouse model where selected disruption of the Nogo-A-specific exon (exon 3) resulted in a complete loss of Nogo-A expression. Surprisingly, however, this group also reported a robust, compensatory increase in Nogo-B expression. In regeneration studies performed on these mice, only limited axonal regeneration, and no improvements in functional recovery, was observed *in vivo* (Simonen et al., 2003). It appears likely that the limited/failed regeneration observed in these mice may be attributable to the other, still-present inhibitory proteins found in myelin. This idea is supported by the finding that inhibition of neurite outgrowth *in vitro* by Nogo-A-/- myelin is primarily reduced and these remaining, residual effects are completely abolished by application of anti-MAG antibodies (Kim et al., 2003).

1.3.2 Myelin-Associated Glycoprotein

Myelin-associated glycoprotein (MAG) is a member of the sialic acid binding Ig-like lectin (siglec) family of adhesion molecules (Siglec 4) (Crocker et al., 1998). MAG contains a short cytoplasmic domain and a single transmembrane region while its extracellular domain consists of five Ig-like domains (Lai et al., 1987; Salzer et al., 1987; Salzer et al., 1990). MAG expression is limited to the myelin forming cells, oligodendrocytes in the CNS and Schwann cells in the PNS, although with varying expression patterns within uncompacted myelin areas (Trapp, 1990; Trapp et al., 1989). In the CNS, MAG comprises 1% of the total myelin protein and it is localized solely to the periaxonal membrane in the internodal segments of the myelin sheath (Trapp, 1990). In the PNS, MAG is expressed in the paranodal regions, Schmidt-Lanterman incisures, and outer mesaxon segments, though it only represents 0.1% of the total PNS myelin protein (Trapp, 1990). Because of its molecular structure, which is closely related to that of N-CAM, and due to its localization, MAG is hypothesized to play a role in the stabilization of the axon-glia interface (Filbin, 1995; Quarles, 1983; Salzer et al., 1990; Trapp, 1990). This idea is supported by studies indicating that in older MAG-deficient (MAG -/-) mice, generated via homologous recombination-induced deletion of the MAG gene (Li et al., 1994; Montag et al., 1994), these mice exhibit normal products of the first myelination events however, they eventually acquire altered periaxonal architecture and an increase in axonal loss (Fruttiger et al., 1995).

In 1994, two investigators independently identified MAG as a major inhibitor of axonal growth *in vitro* (McKerracher et al., 1994; Mukhopadhyay et al., 1994). Primary CNS neurons cultured on monolayers of MAG-expressing cells display a drastic reduction in axonal growth as compared to neurons plated on control cells. NG108 cells (a neuronal cell line) also fail to extend neurites when plated on slides coated with myelin or MAG. Furthermore, a soluble, proteolytic fragment of MAG, consisting of the entire extracellular domain and found *in vivo*, was shown to inhibit neurite outgrowth *in vitro* and *in vivo* (Tang et al., 2001; Tang et al., 1997b). In an effort to further elucidate the role of MAG in the inhibition of axonal regeneration, several groups sought to compare neurite outgrowth on myelin purified from the MAG -/- mice. In these studies, two separate groups found that the MAG -/- myelin exhibited an impaired ability to inhibit axonal growth from primary neurons *in vitro* (Li et al., 1996; Shen et al., 1998) while a third group found that while neurite length from NG108 cells was improved, there was no effect on the growth of primary neurons (Bartsch et al., 1995). It is likely that this discrepancy in inhibitory ability may be due in part to the existence of the other now-identified myelin-associated inhibitors as well as due to the methods used for myelin preparation (Filbin, 1996).

However, further evidence supporting MAG activity *in vivo* was gathered using the mutant Wld^S mice. As mentioned earlier, the Wld^S mice exhibit very slow Wallerian degeneration following peripheral nerve injury with impaired axonal regeneration (Brown et al., 1991). The dramatic differences in the clearance of myelin debris could account, at least in part, for the difference in regenerating ability seen between CNS and PNS neurons. Furthermore, Martini and colleagues crossbred Wld^S mice with MAG -/- mice and studied axonal regrowth *in vivo* (Schafer et al., 1996). Following peripheral nerve injury, analysis of MAG-deficient/Wld^S mice revealed that the number of myelin sheets associated with re-growing axons doubled as compared to Wld^S mice (Schafer et al., 1996). These results further support the notion that MAG-deficient myelin is less inhibitory than the wild type myelin.

1.3.3 Oligodendrocyte-Myelin Glycoprotein

The latest addition to the group of myelin based inhibitors is oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b), a minor component of central nervous system myelin whose expression in development coincides closely with the caudal to rostral progression of CNS myelination (Habib et al., 1998; Vourc'h and Andres, 2004). OMgp is detectable by Western blot analysis in the brains of post-natal rats and its concentration peaks at the late stages of myelination (Habib et al., 1998; Vourc'h et al., 2003a). The majority of the protein is found in diverse groups of neurons, particularly in large projection neurons such as the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellum, motor neurons in the brainstem, and anterior horn cells of the spinal cord (Habib et al., 1998). However, OMgp is not confined to these cells and it is expressed in oligodendrocytes as well (Habib et al., 1998). Although its "normal" function is still unknown, recent observations suggest that OMgp could be involved in the regulation of oligodendrocytes growth as well as in the arrest of myelination or compaction of myelin. Regardless of this proposed function, it was also demonstrated to inhibit axonal growth *in vitro* (Kottis et al., 2002; Wang et al., 2002b). Originally identified in the late 1980s (Mikol and Stefansson, 1988), OMgp is an extracellular membrane protein anchored to the cell surface through a glycosylphosphatidyl-inositol (GPI) lipid intermediate and it contains a highly conserved leucine-rich repeat (LRR) domain which is necessary for proper receptor binding. The deletion of this LRR domain is responsible for a total loss of inhibitory function in an *in vitro* neurite outgrowth assay (Vourc'h et al., 2003b; Wang et al., 2002b).

1.4 RECEPTORS FOR THE MYELIN INHIBITORS

1.4.1 Nogo-66 Receptor (NgR)

The receptor for Nogo-66 (NgR) was identified using an alkaline phosphatase (AP) fusion protein to screen a mouse brain cDNA library transfected into COS-7 cells (Fournier et al., 2001). NgR is a 473 amino acid glycosylphosphatidyl-inositol (GPI)-anchored protein. Its globular structure consists of a translocation signal sequence, eight leucine-rich repeat (LRR) motifs, which are capped by N-terminal and C-terminal cysteine-rich modules (termed LRR-NT and LRR-CT segments, respectively), and a unique C-terminal region proximal to the anchoring site (Fournier et al., 2001). The functional specificity of NgR was demonstrated by a gain-of-function experiment in chick early embryonic retinal ganglion cells (RGC), which are insensitive to Nogo66-induced growth cone collapse. Viral-mediated NgR expression in embryonic day 7 (E7) RGCs renders these neurons sensitive to the Nogo-66 activity (Fournier et al., 2001). Also, a truncated, soluble NgR antagonizes Nogo66-dependent inhibition of neurite extension by E13 DRGs (Fournier et al., 2002). The expression pattern of NgR is consistent with a role in the inhibition of axonal regeneration. Transcripts are present predominantly in the adult and maturing brain where the NgR protein is found in a wide variety of neurons but not in oligodendrocytes (Hunt et al., 2002b). Expression is minimal prior to myelination and there are no detectable changes after trauma (Hunt et al., 2002b).

NgR functions as a common receptor mediating the inhibition of axonal growth by at least the three myelin based inhibitors discussed so far, Nogo-66, MAG and OMgp

(Domeniconi et al., 2002; Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). The additional Nogo-A domain Amino-Nogo does not seem to interact with NgR (Hunt et al., 2002a). Notably, the structurally unrelated ligands all bind to NgR with high affinities (Domeniconi et al., 2002; Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). Mutation analysis demonstrated that the receptor-ligand interaction is localized to the LRR motifs. The structural basis for the ligand recognition has been partially clarified by the resolution of NgR crystal structure (Barton et al., 2003; He et al., 2003). The multiple LRR motifs result in a concave groove which contains a putative degenerate binding site, hence accounting for the similar interactions with such different ligands. The structure also suggests a possible co-receptor binding site within the unique C-terminal region and the deletion studies support this idea.

Some groups have also reported that sialylated glycans are mediators of MAG inhibition of neurite outgrowth and that MAG inhibition is a result of carbohydrate recognition (Vinson et al., 2001; Vyas et al., 2002). MAG specifically binds gangliosides GT1b and GD1a, which are both expressed on the surface of MAG-responsive neurons. It is also known that multivalent IgM antibody cross-linking of cell surface GT1b mimics the effect of MAG (Vinson et al., 2001). However, other studies indicate that removal of sialic acid residues by sialidase treatment does not affect MAG's inhibitory activity (Tang et al., 1997a). Since the addition to outgrowth assays of IgM Fab fragments generated from either anti-GT1b or anti-GD1a antibodies mimics MAG inhibitory activity, it is likely that this interaction is not specific to a MAG pathway. Current data supports a model in which the recognition of sialylated glycans, while not essential for inhibition of neurite outgrowth by MAG, may result in a weak, transient activation of the inhibitory pathway, independent of the NgR-dependent effects. Furthermore, *in vivo*, the effects of ganglioside-mediated binding of MAG may serve to potentiate the inhibitory effects of the NgR-mediated pathway. One possible interpretation of these events is that MAG has two discrete functions: first, to hinder aberrant sprouting and generate structural axon-glia stability via interaction with gangliosides, and, second, to strongly inhibit outgrowth via interaction with a functional high-affinity receptor.

1.4.2 The p75 Neurotrophin Receptor

Due to the GPI-linked nature of NgR, the ability of this receptor to initiate a signaling pathway requires the presence of at least one co-receptor. Indeed, it was recently found that the inhibition of axonal elongation induced by all three of the myelin-associated inhibitors, Nogo-66, MAG and OMgp, depends on the association of NgR with the p75 neurotrophin receptor ($p75^{NTR}$) (Wang et al., 2002a; Wong et al., 2002). This is evidenced by the fact that cultured primary neurons from $p75^{NTR}$ null mice are insensitive to the activity of the myelin-associated inhibitors (Wang et al., 2002a). The extracellular domain of $p75^{NTR}$ contains four cysteine-rich motifs, which are necessary for NGF binding (Dostaler et al., 1996). Thus far, it is unclear if these motifs are also responsible for NgR binding although it should be noted that simultaneous treatment with NGF does not abrogate nor does it reduce myelin-mediated inhibition of growth from Trk-A negative neurons (Cai et al., 1999). The $p75^{NTR}$ intracellular domain does not have an intrinsic enzymatic activity and the signal transduction takes place through interaction with several adaptor proteins (Bandtlow and Dechant, 2004; Barker, 2004). The cytoplasmic segment of $p75^{NTR}$ contains a palmitoylation site, two TNFR-associated factor (TRAF) binding sites, a type II death domain, a small G-protein activating domain, and a PDZ domain-binding motif.

During early development $p75^{NTR}$ is expressed in a wide variety of cells within the CNS and PNS, as well as many non-neuronal tissues such as kidney, testis, lung and muscle (Ryffel and Mihatsch, 1993). Postnatally, $p75^{NTR}$ levels are reduced in most tissues and restricted to a narrower range of cells (Ryffel and Mihatsch, 1993). Trauma to the nervous system induces $p75^{NTR}$ expression in many cell types (Ebadi et al., 1997). After injury, increased mRNA and protein levels have been documented in motor, corticospinal and hippocampal neurons, as well as oligodendrocytes, Schwann and Purkinje cells, microglia and macrophages (Ebadi et al., 1997).

1.4.3 Lingo

A third molecule has been associated with the NgR/$p75^{NTR}$ complex. Lingo-1 is a human homologue of SLIT—a Drosophila axonal guidance molecule which binds the neuronal receptor Robo—which is highly expressed in CNS tissue and is undetectable in non-neuronal tissue (Mi et al., 2004). Like $p75^{NTR}$, its expression is up-regulated following trauma. The protein consists of 12 leucine-rich repeat (LRR) motifs, one Ig-like domain, a transmembrane domain and a short cytoplasmic tail (Mi et al., 2004). The cytoplasmic tail contains a canonical epidermal growth factor receptor-like tyrosine phosphorylation site. Lingo-1 interacts with both NgR and $p75^{NTR}$ and its absence reduces the inhibitory activity of myelin proteins. It has been demonstrated that concurrent expression of NgR,

$p75^{NTR}$ and Lingo-1 into the non-neuronal cell line COS-7 confers sensitivity to OMgp (Mi et al., 2004).

1.5 Signaling By Inhibitors

Following the binding of each of the myelin-associated inhibitors to the NgR-$p75^{NTR}$-LINGO receptor complex, there is an induction of a signaling pathway which eventually leads to the blockage of neurite extension from damaged or naïve adult neurons. While the entire pathway and all of its constituent members have yet to be elucidated, some of the prime movers in this pathway have begun to reveal themselves.

It has been known for several years that the MAG-mediated block of axonal regeneration is dependent on the activity of the small GTPase, Rho A (Lehmann et al., 1999). Work from the lab of Lisa McKerracher has shown that blocking Rho A activity can promote axonal regeneration both in the presence of MAG or purified myelin *in vitro* or following the application of a CNS injury *in vivo* (Dergham et al., 2002; Lehmann et al., 1999; Winton et al., 2002). Furthermore, recent findings have also suggested that the activation of $p75^{NTR}$ upon binding of the receptor complex by MAG results in activation of Rho A (Wang et al., 2002a; Yamashita et al., 2002) in a PKC-dependent manner (Sivasankaran et al., 2004) and that this RhoA activation may work via the sequestration of the Rho-GDP dissociation inhibitor, Rho-GDI, by $p75^{NTR}$ following activation of the signaling complex (Yamashita and Tohyama, 2003).

Beside its role in transducing the inhibitory signal from myelin based inhibitors, $p75^{NTR}$ is involved a wide array of biological activities, requiring it to interact with multiple ligands, surface receptors and adaptor proteins (Bandtlow and Dechant, 2004; Barker, 2004; Dechant and Barde, 2002). Evidence indicates that $p75^{NTR}$ can bind all known neurotrophins with similar affinities as well as pro-neurotrophins (Lee et al., 2001). In addition, several non-neurotrophin ligands have been shown to associate with the receptor. Among these are the neurotoxic prion protein fragment PrP(26–106) (Della-Bianca et al., 2001) and the Aβ-peptide of the amyloid precursor protein (APP) (Perini et al., 2002). At the cell surface, $p75^{NTR}$ also associates with a growing number of membrane proteins. It can interact with itself to form homodimers, with gangliosides such as GT1b (Yamashita et al., 2002), the three Trk receptors and the ankyrin repeat–rich membrane spanning protein (ARMS) (Kong et al., 2001).

A member of the tumor necrosis factor (TNF) superfamily, $p75^{NTR}$ can regulate cell death and survival (Chao et al., 1998; Lee et al., 2001). Among the ligands that activate $p75^{NTR}$ cell death pathways, pro-neurotrophins seem to be the most effective (Lee et al., 2001). This function is carried out by novel interaction with Sortilin (Nykjaer et al., 2004). Regulation of life and death pathways in different contexts requires that $p75^{NTR}$ interacts with a plethora of cytoplasmic adaptors like the neurotrophin receptor interacting factors 1 and 2 (NRIF1 and NRIF2) (Casademunt et al., 1999), the IAP-binding protein neurotrophin receptor–interacting MAGE homologue (NRAGE or MAGED1) (Salehi et al., 2000), FAS-associated phosphatase 1 (FAP-1) (Irie et al., 1999), the $p75^{NTR}$-associated cell death executor (NADE) (Mukai et al., 2000), and several of the TNF receptor associated factors (TRAFs) (Ye et al., 1999). Furthermore, $p75^{NTR}$ interacts with caveolin (Bilderback et al., 1997) and with protein kinases such as the interleukin-1 receptor associated kinase (IRAK) (Mamidipudi et al., 2002) and the mitogen-activated protein kinases ERK1 and ERK2 (Volonte et al., 1993) and P38β2 (Wang et al., 2000).

Some of the adaptor proteins that interact with p75^{NTR} block cell-cycle progression when expressed in cultured cells. These adaptors include: the zinc finger proteins SC-1 (Chittka et al., 2004), NRIF1 and NRIF2, as well as NRAGE. Interestingly, a recent study indicates that p75^{NTR} can affect synaptic transmission between sympathetic neurons and cardiac myocytes (Yang et al., 2002). In this system, single neurons can release two different and "contrasting" neurotransmitters: norepinephrine, which increases the twitching frequency of myocytes, and acetylcholine, which has the opposite effect (Furshpan et al., 1976). While the addition of NGF to co-cultures increases the release of norepinephrine in a TrkA-dependent manner (Lockhart et al., 1997), the addition of BDNF increases inhibitory transmission by promoting acetylcholine release (Yang et al., 2002).

1.6 Overcoming the Myelin-induced Block of Regeneration

One of the most intriguing and encouraging aspects of the axonal regeneration paradigm is the aforementioned finding that all three of the myelin-associated inhibitors bind and exert their effects via the same receptor complex. This fact suggests a redundancy in the activity of these various inhibitory proteins and also provides a host of potential targets for therapeutic intervention to encourage axonal regeneration. If the binding or signaling of a single receptor complex can be compromised, it may be possible to permit sufficient regeneration in the adult mammalian CNS after injury, particularly prior to formation of the glial scar.

1.6.1 Blocking Inhibition with Antibodies and Peptides

The concept of inhibitor and receptor-specific targeting in regeneration-encouraging paradigms was born with the discovery of the NI-35 and NI-250-blocking antibody, IN-1 (Caroni and Schwab, 1988a). *In vivo* application of this inhibition-blocking antibody following CNS injury was shown to mediate moderate regeneration of injured fibers and this improved regeneration could be correlated with an increase in recovery of function (Bregman et al., 1995; Schnell and Schwab, 1990). Since this time, many studies have followed which sought to examine the effects on regeneration of blocking the myelin-associated inhibitors. One particularly interesting study found that if adult mice are immunized against myelin-associated protein components—including the myelin-associated inhibitors of regeneration—some regeneration is observed following dorsal column lesioning *in vivo*, and that this improved regeneration occurs in the absence of a cellular inflammatory response (Huang et al., 1999).

Another method for blocking the inhibitory effects of the myelin-associated inhibitors is the use of function-blocking antibodies to the receptor complex. Indeed, it has been shown that if the binding of the myelin inhibitors to NgR is blocked via addition of an anti-NgR antibody *in vitro*, axotomized neurons will extend long processes even on purified myelin (Domeniconi et al., 2002). Furthermore, recent work by several groups has shown that expression of small antagonistic peptides may also provide a potential avenue for intervention. Application of a small peptide which consists of the first forty amino acids of the Nogo-66 domain (NEP 1-40) will effectively bind to NgR and block the inhibitory signaling of the Nogo-66 inhibitor (GrandPre et al., 2002). Conversely, introduction of non-signaling peptide fragments of the receptor complex such as p75-Fc (Wang et al., 2002a), a truncated fragment of NgR (NgR-Ecto) (Liu et al., 2002) or LINGO-Fc (Mi et al., 2004) are all able to effectively compromise the binding and/or signaling of the inhibitor-receptor interaction, thereby promoting axonal regeneration *in vitro*. Further work may soon elucidate the efficiency of these therapies *in vivo* following injury.

The convergence of the inhibitory effects of all three inhibitors is consistent with findings that modulations of the activity or expression of certain intracellular signaling molecules can simultaneously abrogate the inhibitory effects of all the inhibitors. To this end, it may be possible to target some of the downstream effectors of the NgR-LINGO-p75^{NTR} signaling cascade and thereby block the functional effects of these inhibitors. One such method is via blockage of the small GTPase, RhoA. Evidence suggests that blocking Rho signaling can promote axonal regeneration both in the presence of all of the myelin-associated inhibitors *in vitro* and following injury to the adult CNS *in vivo*. Work from the McKerracher lab has shown that inactivation of Rho, via application of the exoenzyme C3 transferase, or blocking the signaling cascade via inhibition of the downstream effector, Rho-associated kinase (ROCK) can effectively block the axonal growth inhibitory effects of the myelin-associated inhibitors both *in vitro* and *in vivo* (Dergham et al., 2002; Lehmann et al., 1999; Winton et al., 2002).

In addition to the blockage of the inhibitors or the signaling receptor complex, it is also possible to encourage axonal regeneration via induction of changes in the intrinsic growth state of the neuron such that it no longer responds to the myelin-associated inhibitors. It has been well characterized for many years that the axons of embryonic (or, in a few cases, neonatal) neurons are not inhibited in their growth potential by myelin and its inhibitors. It has been surmised that this is a result in a difference in the intrinsic growth state of the neuron rather than a difference in the inhibitory components in the environment. Thus, the question becomes, what are the modulatory signals that differentiate the growth state of embryonic versus adult CNS neurons?

1.6.2 cAMP

An answer to this question can be found in the levels of one of the usual cellular "suspects": the ubiquitous signaling messenger, cyclic-adenosine monophosphate (cAMP). In the nervous system, cAMP signaling has been implicated in a variety of neuronal processes including memory and learning (Alberini et al., 1995; Frey et al., 1993; Wong et al., 1999; Wu et al., 1995), neurotransmitter modulation (Byrne and Kandel, 1996; Castellucci et al., 1980; Castellucci et al., 1982; Milner et al., 1998) and axonal growth cone turning and developmental guidance. Indeed, significant evidence exists to suggest that the levels of intracellular cAMP can modulate the turning and growth induction effects of guidance cues on an extending growth cone (Song et al., 1998; Song et al., 1997). This phenomenon will be examined in more detail later.

The role of cAMP levels and signaling during development, however, are not limited to the modulation of responses to guidance cues. As stated earlier, it is commonly accepted that embryonic neurons are able to extend long axons both during normal development or following axotomy at the embryonic stages. Therefore, not surprisingly, examinations into the role of cAMP perinatally have revealed that the cAMP levels of embryonic neurons are significantly higher than that of their adult counterparts and that this elevation can account for the ability of these neurons to extend axons in the inhibitory CNS environment (Cai et al., 2001). Furthermore, the loss of regenerative ability which occurs postnatally directly correlates with a precipitous decrease in endogenous cAMP levels. Even in neuronal types which retain their regenerative abilities in the neonatal stages, cAMP levels appear to remain high until the developmental switch occurs.

Thus, it is reasonable to assume that if the elevated cAMP levels observed in regeneration-competent embryonic neurons could be replicated in the adult animal, perhaps axonal regeneration and repair post-injury could indeed be possible. Recent evidence suggests that this may, in fact, be the case. Elevation of intracellular cAMP levels via addition of chemical analogs can indeed improve the regenerative capacity of post-natal neurons on an inhibitory substrate *in vitro*. Interestingly, it was also noted that pre-treatment of neurons with neurotrophins like brain-derived neurotrophic factor (BDNF), called "priming", can also mediate improved regeneration on myelin-associated inhibitor-containing substrates. Not surprisingly though, it was found that even this mechanism is mediated by the induction of elevated cAMP levels and the associated signaling (Cai et al., 1999). Furthermore, the pro-regenerative effects of cAMP elevation are not limited to abrogation of myelin-associated inhibitor signaling. The growth inhibitory properties of several glial scar-associated proteoglycans as well as certain repulsive guidance cues can be overcome by induction of cAMP elevation (Shearer et al., 2003).

Evidence for the regeneration-promoting effects of cAMP elevation exists in a variety of animal model systems as well. One commonly employed tool utilizes the easily observable retinal ganglion cells (RGCs) as a model for improving CNS regeneration. Indeed, evidence exists which suggests that application of chemical cAMP analogs (Monsul et al., 2004) or adenylyl-cyclase activators along with neurotrophic factors (Watanabe et al., 2003) can mediate improved regeneration of adult mammalian RGCs *in vivo*.

cAMP elevation has also been implicated in another regeneration paradigm, the conditioning lesion effect. Dorsal root ganglion neurons (DRG) are unique in that they possess two branches, one which extends into the spinal cord (the CNS branch) and another which extends into the periphery. It has been well established that if a "pre-conditioning" lesion is applied to the peripheral branch of a DRG neuron and, one day or one week later, a second lesion is introduced into the dorsal column (the CNS branch), CNS regeneration is observed. Regenerating axons will extend processes into a peripheral nerve graft at the lesion site (Richardson and Issa, 1984; Richardson and Verge, 1986) and even in the absence of such a graft, into and beyond the site of injury (Neumann and Woolf, 1999). This effect, as well, has been shown to be cAMP-dependent (Neumann et al., 2002; Qiu et al., 2002). In fact, a single injection of dibutyryl-cAMP (db-cAMP), a non-hydrolysable analog of cAMP, can mimic the conditioning lesion effect, even in the absence of the peripheral lesion (Qiu et al., 2002). Furthermore, a more recent study by the Tuszynski laboratory has demonstrated that the injection of db-cAMP into DRG neurons may be combined with other therapeutic techniques, such as injection of the neurotrophin NT-3 and bone marrow stromal cells directly into the lesion site to improve overall regeneration after a cervical spinal cord injury (Lu et al., 2004).

These findings have helped to elucidate an essential portion of the regeneration-invoking signaling pathway but, neither application of a pre-conditioning lesion, nor direct injection of db-cAMP are viable avenues for therapeutic intervention in human spinal cord injury patients. Therefore, more recent work has focused on less intrusive and post-injury applied methods for elevation neuronal cAMP levels. One such method for achieving this is to elevate cAMP levels by blocking its degradation. Recent evidence from several labs has indicated that application of the phosphodiesterase 4 inhibitor, rolipram, can effectively induce an increase in cAMP levels *in vitro* and post-injury *in vivo* and can improve regeneration and functional recovery, particularly when combined with other therapeutic methods (Nikulina et al., 2004; Pearse et al., 2004). The reason that rolipram is so attractive as a potential spinal cord injury therapeutic is due to the fact that it is blood-brain barrier permeable and, therefore, can be injected subcutaneously, thereby alleviating the need for intrusive surgery.

However, since cAMP has many roles in a multitude of signaling pathways in neuronal cells, the artificial elevation of cAMP alone may not be a valid therapeutic approach.

Therefore, it is essential to comprehend the entire complexity of the cAMP signaling pathway, particularly the downstream effectors that mediate this improved regenerative capacity. Understanding this signaling pathway may present yet another potential target for therapeutic intervention in the hopes of encouraging regeneration in the adult.

1.6.3 Downstream Effectors

It is well known that elevated cAMP levels result in an activation of the cAMP-dependant protein kinase, or protein kinase A (PKA)—and, indeed, both the improved regenerative growth observed following priming *in vitro* (Cai et al., 1999) and following a conditioning lesion *in vivo* are dependent on PKA activation (Neumann et al., 2002; Qiu et al., 2002)—but recent evidence suggests that another well-known signaling component may also be involved in the regeneration-promoting signal. Induction of the neurotrophin-induced elevation of cAMP also appears to be mediated by an extracellular-regulated kinase (ERK)-dependent process. Activation of ERK by neurotrophin binding results in a transient inhibition of the neuronal-enriched phosphodiesterase, PDE4. As mentioned above, inhibition of PDE4, results in an accumulation of cAMP, thereby increasing intracellular levels and inducing subsequent signaling modules. Blocking the activation of this mitogen-activated protein kinase (MAPK) abrogates the improved regeneration observed after cAMP elevation *in vitro* and therefore, it is an essential member of this regeneration-promoting signal (Gao et al., 2003).

In neurons, PKA and MAPK have many cellular targets, including activation of the cAMP-response element binding protein (CREB) and the subsequent induction of gene transcription. And, indeed, recently published evidence suggests that the phosphorylation and activation of CREB is an integral part of the cAMP-mediated axonal growth (Gao et al., In Press). This coincides with evidence which suggests that in the regenerative paradigms illustrated above, the cAMP-induced promotion of axonal growth is, initially, PKA dependent but later becomes independent of PKA activation (Qiu et al., 2002) and sensitive to pharmacological inhibitors of transcription (Cai et al., 2002). These facts, coupled with the latency of response observed in the conditioning lesion effect, suggest that cAMP-mediated genetic transcription is essential for axonal regeneration. Therefore, the question then becomes which genes are up-regulated in response to elevated cAMP levels and what are their roles in the axonal regeneration paradigm?

The induction of cAMP-mediated gene transcription often results in an upregulation of many immediate early gene products. However, only some of these products play a role in regeneration. Recently, evidence has emerged which suggests a role for a family of known cytoskeletal regulators, the polyamines. Activation or overexpression of the protein Arginase I, a key enzyme in the polyamine synthesis pathway, or application of the polyamine putrescine can induce improved growth on inhibitory substrates. Conversely, blocking the synthesis of these molecules can abrogate the cAMP-mediated regenerative increase (Cai et al., 2002). Furthermore, an upregulation of Arginase I temporally coincides with either conditioning-lesion (unpublished data) or artificially-induced elevated cAMP levels. The polyamines are known modulators of cytoskeletal rearrangement, a step which is ultimately necessary for the induction of axonal growth and regeneration, and it is this activity which makes the polyamines encouraging targets for therapeutic intervention following spinal cord injury.

Further elucidation of the signaling cascade and cytoskeletal targets which mediate the cAMP-induced axonal regeneration may soon follow, thereby providing new and, potentially, more specific targets for therapeutic intervention and induction of regeneration after injury *in vivo*.

1.7 Guidance Cues of Development

In the developing mammalian CNS, the neurons which will comprise the sensory and motor systems must extend their axons from the location of the cell body—often in the brain or spinal cord—to their eventual targets (which can be as far as hundreds of centimeters in humans) and form synaptic connections. As noted earlier, the embryonic CNS environment is not inhibitory toward these growing axons and, indeed, at this juncture, myelination has not begun in earnest and therefore, many of the myelin-associated inhibitors may not be present in abundance. However, these pioneering axonal processes do not find their proper targets on their own. How, then, do these developing axons determine the proper pathways and target locations? Once again, the local environment of the CNS provides the answer. While the aforementioned myelin-associated inhibitors are either not yet present or unable to affect axonal growth, there are many "cues" which act on each of these developing axons to "nudge" or "draw" them in the proper direction. These molecules are called developmental axonal guidance cues.

There are primarily four types of guidance "forces" found in the developing CNS: chemoattraction, chemorepulsion, contact attraction and contact repulsion.

The "contact" type of forces often work over short distances and are mediated by membrane or extracellular matrix (ECM)-bound molecules. This mode of guidance acts much like the large highway: the attractive cues often provide the "roadway" on which the axons grow and subsequently follow and the repulsive cues are the "guardrails", preventing any wayward axons from wandering off the "road".

The "chemo" forces are usually more long-range in their effects and are mediated by diffusible factors which bind receptors on the surface of the developing growth cone. Often, subtle gradients of these diffusible factors determine the growth mode and direction of these axonal processes: gradients of attractive cues will "pull" the growth cone toward the proper pathway while repulsive cues prevent growth into inappropriate regions or away from incorrect targets.

1.7.1 The Guidance-associated Molecules

To date, several molecules have been identified as potential developmental guidance cues. These molecules include the slits, ephrins, netrins and semaphorins. Each of these factors, while normally associated with one type of chemotactic modulation, has shown bifunctional properties under a variety of conditions. Each of these molecules is expressed during the initial stages of nervous system development and aids extending axons to assume the correct pathfinding mode and direction. To this end, these molecules may attract, repel or even inhibit the growth of these developing axons. With this in mind, one may ask whether or not these factors may play the same roles in the adult organism, specifically after CNS injury. In other words, can these guidance cues be another set of axonal regeneration inhibitors?

While the presence and effects of these molecules are undeniable in the developing nervous system, the question that remains unanswered is whether or not they are in fact present in the mature CNS and, if so, whether they can still induce growth modulation in a regenerating axon. To answer this question, we must first examine some of the more prominent guidance molecules.

1.7.1.1 Netrin

Netrins are bifunctional molecules, generally diffusible in nature, which can act to either attract or repel developing axons. Netrin binds to axonal processes via two receptor complexes: the DCC (Deleted in Colorectal Cancer) receptor or the UNC-5 receptors. The attractive or repellant nature of netrin binding is dependent on several factors, including receptor choice and intracellular levels of key signaling molecules. It has been shown that binding of netrin to the DCC receptor mediates the chemoattractive signal while UNC-5 receptor-binding results in a repulsive response. This receptor-mediated activation is coupled to downstream signaling components which subsequently generate the growth response. The attractive response of the growth cone to netrin signaling may be broken down into two components: the attractive turning response and the axonal growth response. The attractive component of this response appears to be mediated by the induction of a multifaceted signaling cascade involving the activation of phospholipase C (PLC) (Ming et al., 1999), MAP kinase (Forcet et al., 2002; Ming et al., 2002b), phosphatidylinositol 3-kinase (PI3K) (Ming et al., 1999) and the small GTPases Rac and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002). Conversely, axonal extension involves a calcium-dependent activation of the cellular phosphatase calcineurin, activation of the nuclear transcription factor NFAT and subsequent gene transcription (Graef et al., 2003). The UNC5-mediated signaling which results in growth cone repulsion, however, is less well understood. Following ligand binding the UNC5 receptor becomes phosphorylated via a mechanism which may involve the src family of kinases (Tong et al., 2001). What other downstream components are induced following this induction of signaling have yet to be elucidated.

The activity of netrin on an extending growth cone involves induction of highly regulated signaling molecules and therefore, not unexpectedly, intracellular levels of ubiquitous signaling molecules such as cAMP and calcium appear to be involved in determining this axonal response. High levels of either of these two molecules results in a chemoattractive response to netrin, whereas constitutively low levels results in axonal repulsion (Hong et al., 2000; Ming et al., 1997; Nishiyama et al., 2003; Song et al., 1998; Song et al., 1997). *In vitro*, modulation of these factors does, in fact, mediate axonal growth and turning as well as repulsion/inhibition. And while netrin and its receptors are constitutively expressed in the adult CNS, what role, if any, netrin plays in regeneration—or lack thereof—after injury is still unclear.

1.7.1.2 Semaphorins

The semaphorins are a family of either secreted or membrane-bound chemotactic molecules which have been shown to exhibit both repulsive and, under certain conditions, attractive properties for developing axons. Semaphorins have been classified into eight distinct subclasses. Class 1 and 2 are exclusive to the invertebrates. Among the vertebrate semaphorins, class 3 is a secreted protein while the semaphorin classes 4 to 7 are all linked to the membrane via GPI-anchors. In addition, there is a viral class of semaphorin which is also secreted.

The soluble (secreted) semaphorin molecules have been shown to bind to the neuropilin class of receptors, of which there are two, neuropilin-1 and neuropilin-2. Conversely, the membrane-bound semaphorin family members bind via the plexin receptors. The intracellular domain of the neuropilins, however, are very short and do not appear to be able to initiate signaling and therefore, they are unable to mediate the semaphorin-induced effects alone. Thus induction of the signaling cascade is dependent on the formation of a receptor complex which consists of a heterodimer of the neuropilin and plexin receptors. Indeed, the precise types of complexes formed upon binding may determine the binding specificity of these interactions. While differential dimerization patterns and ligand binding activates differing downstream effectors, the subsequent induction of Rho-dependent cytoskeletal rearrangement results in the repulsion of the axonal growth cone. However, one class of semaphorin, Sema7A, has been shown to bind the β1 integrin molecule, a cell adhesion-associated receptor. Upon binding to this receptor, an ERK-dependent pathway is initiated which leads to attraction and axonal outgrowth instead of repulsion.

While the role of these molecules in the guidance of developing axons is undeniable, recent evidence has also suggested that they may play a role in the inhibition of axonal regeneration after injury. The semaphorin family member, Sema3A, in particular, may be active in regulating regeneration after CNS injury. Secreted by both fibroblasts and neurons, Sema3A binds neuropilin1 on the neuronal cell surface and the complete receptor complex forms upon subsequent binding by the signaling co-receptor, plexinA1. Binding and formation of this ligand-receptor complex appears to be sufficient to induce growth cone collapse via a signaling cascade which involves activation of the collapsin response mediator protein (CRMP) and, not surprisingly, RhoA. Sema3A and the components of its receptor complex have all been shown to be expressed in the adult mammalian CNS after injury (Pasterkamp et al., 2001; Pasterkamp et al., 1998a; Pasterkamp et al., 1999; Pasterkamp and Verhaagen, 2001). In addition, it has been demonstrated that these molecules can inhibit axonal sprouting after injury and even induce cell death via a pro-apoptotic pathway. Interestingly, it has also been shown that Sema3A is downregulated in the PNS (an environment where axonal regeneration is readily observed) after injury and that levels remain low until regeneration is complete (Pasterkamp et al., 1998b). Thus, the semaphorins may indeed have a post-developmental role and may also participate in cell death and inhibition of axonal regeneration in the CNS after injury.

1.7.1.3 Slits

The Slits are a secreted family of proteins which were initially characterized modulators of embryonic patterning. In 1999, however, several investigators identified the slit proteins as repellant cues for developing commissural axons (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). The slit-induced axonal repulsion is mediated via binding to the Roundabout (robo) receptor family and have been shown to induce axonal repulsion in a several neuronal types (including commissural and retinal). Following ligand binding, the robo receptors have been shown to initiate a signaling cascade which includes activation of the mammalian Enabled (Mena) and, interestingly, modulation of a Rho GTPase family member. The proline-rich CC3 region of the robo receptor has been shown to bind a subfamily of Rho GAPs called srGAPs (Wong et al., 2001). Slit-induced growth cone repulsion appears to be mediated by the inhibition of the Rho GTPase, Cdc42, which results in modulation of actin dynamics and subsequent repulsive turning in the extending axon. In the adult CNS, both the slit ligands and their robo receptors have been observed and, indeed, may exhibit elevated levels as compared to embryonic expression (Marillat et al., 2002). Furthermore, recent studies suggest that slit2 is expressed in reactive astrocytes after injury (Hagino et al., 2003), thus indicating that these slit ligands may, in fact, be available at the right place and time to act as a regeneration inhibitor, but there is no evidence, as yet, to indicate that they do so.

1.7.1.4 Ephrins

The ephrins are yet another family of axon guidance molecules. The ephrins exist as membrane-bound proteins which bind the surface receptors, Eph-A and Eph-B. The ephrin ligands and the Eph receptors are expressed on both the neuronal and astrocytes cell membranes. The ephrins-A molecules are anchored to the cell membrane via a GPI linkage while the ephrins-B moieties include both transmembrane and intracellular domains which may be involved in "reverse" signaling. These molecules generally act to mediate a contact repulsive guidance cue, serving to direct developing axons away from inappropriate areas and toward their targets. However, evidence exists which indicates that in some circumstances, the ephrins may also induce growth cone adhesion and attraction (Knoll and Drescher, 2002).

The Ephs are tyrosine kinase receptors which, following ligand binding, autophosphorylate tyrosine residues in the intracellular domain which then become docking sites for the SH2-containing proteins. Like virtually all other growth cone inhibitory molecules, ephrins signaling appears to be mediated via activation of the small GTPase, RhoA. Ligand binding and induction of the signaling cascade results in the activation of a Rho guanine exchange factor (GEF) termed ephexin. This ephexin-mediated Rho activation, coupled with a simultaneous inactivation of both Rac and Cdc42, results in the modulation of the actin cytoskeleton and the subsequent repulsion or collapse of the extending growth cone (Wahl et al., 2000).

While their expression in the adult CNS has been confirmed (Bundesen et al., 2003; Janis et al., 1999; Miranda et al., 1999; Willson et al., 2002; Winslow et al., 1995), no evidence yet exists to indicate that they may in fact play a role in the inhibition of axonal regeneration after injury. Instead, it has been suggested that since the developmental expression patterns of the ephrins have been demonstrated to persist in the adult superior colliculus (Knoll et al., 2001; McLaughlin and O'Leary, 1999), the role of ephrins post-injury may be the same as during development: the precise guidance on growing axons toward their proper targets.

1.8 The Glial Scar

Injury to the adult mammalian nervous system often causes extensive damage to the spinal cord and surrounding tissue. As elucidated above, this can induce the release of many molecules from injured cells of the CNS, including the myelin-associated inhibitors of regeneration with the end result being a block of spontaneous regeneration by injured axons. However, the myelin-associated inhibitors are not the only factors which prevent regeneration. Following injury and the associated ancillary damage, one may observe the formation of a structure called the glial scar which forms both a physical and chemical barrier to regeneration *in vivo*.

During glial scar formation, astrocytes in the area of the lesion undergo a process which is referred to as "reactive gliosis". Hypertrophic reactive astrocytes will exhibit changes in both cellular morphology and in protein expression patterns (Barrett et al., 1981; Yang et al., 1994). One effect of these morphological changes is the formation of a sturdy, intransigent membrane which effectively forms a "wall" through which regenerating axons can not pass: the glial scar. This regeneration-inhibiting wall, however, can often take several weeks to fully form following injury.

In addition to the formation of this physical barrier, reactive astrocytes express a class of molecules called proteoglycans (Gallo et al., 1987). These extracellular-matrix molecules consist of a protein core connected to a sulphated glycosaminoglycan (GAG) chain containing repeating disaccharides. One group of these proteoglycans, called the chondroitin sulphate proteoglycans (CSPGs) have been shown to be up-regulated in the glial scar following injury to the CNS of adult animals (Jones et al., 2003; McKeon et al., 1999; Tang et al., 2003) and have also been implicated in axonal regenerative failure after injury both *in vitro* (McKeon et al., 1991; Niederost et al., 1999; Smith-Thomas et al., 1994) and *in vivo* (Davies et al., 1999). These reports are further supported by recent findings which have indicated that removal of the chondroitin sulfate GAG chains using the enzyme chondroitinase ABC improves regeneration and functional recovery after CNS injury (Bradbury et al., 2002). Interestingly, evidence from several groups suggest that the growth inhibitory effects of CSPGs in the glial scar appear to mediated via a signaling pathway which, like the NgR-LINGO-p75^{NTR}-induced pathway, utilizes the small GTPase, Rho and its downstream effector, ROCK (Borisoff et al., 2003; Dergham et al., 2002; Monnier et al., 2003). This finding has important implications for the development of potential therapeutics since it suggests a viable target for pharmacological or genetic intervention which may effectively block the regenerative inhibition induced by both the myelin-associated inhibitors as well as the glial scar proteoglycans.

1.9 Goals of this work

As stated earlier, axons from neurons of the adult mammalian CNS do not spontaneously regenerate after injury. However, if intracellular cAMP levels are elevated via any of several methods—application of the analogue db-cAMP, pre-treatment with neurotrophins or application of a pre-conditioning lesion—these neurons can regenerate long axons both *in vitro* and *in vivo*. While our lab has begun to sort out some of the timing and signaling components of these pathways, more molecular players may exist and so this goal of the following work is primarily two-fold:

1) To elucidate the timing of the specificity of cAMP-dependent signaling in both the neurotrophin-induced priming effect and in the conditioning lesion paradigm.

2) Given the relatively recent finding that BDNF treatment of cortical neurons can mediate a Calcium/Calmodulin-dependent Kinase IV(CaMKIV)-dependent activation of CREB (Finkbeiner et al., 1997), we sought to discover what role, if any, does CaMK have in the neurotrophin-induced block of MAG-mediated inhibition of axonal regeneration.

Chapter II: Materials and Methods

2.1 Tissue / Cell Culture

2.1.1 CHO Cells

2.1.1.1 Maintenance

Control or stably-transfected MAG-expressing Chinese Hamster Ovary (CHO) cells (Mukhopadhyay et al., 1994) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) which had been supplemented with 10% dialyzed fetal bovine serum (FBS, Gibco), 34.8mM L-Proline (Sigma), 10mM Glycine (Sigma), 300nM Thymidine (Sigma) and 2mM L-Glutamine (Gibco).at 37°C in 7.3% CO_2. These cells were used as monolayer substrates in the neurite outgrowth assay (NOG) described below.

2.1.1.2 Monolayer Preparation

To prepare monolayers for the NOG assay, Permanox 8 well chamber slides (Lab-Tek) were coated with 16.6 μg/ml poly-L-lysine (>300kD; Sigma) for 30 minutes at room temperature. After incubation, poly-L-lysine was removed and the slides were treated with 10μg/ml fibronectin (Sigma) for at least 2 hours at 37°C. Control of MAG-expressing CHO cells (passage number 3 – 20) were plated onto these slides at the following concentrations: control - 6 x 10^4 cells/well; MAG - 5.5 x 10^4 cells/well. The slides were then incubated overnight at 37°C prior to the plating of primary neurons.

2.1.2 Isolation of Neurons

2.1.2.1 Cerebellar Neurons

To isolate cerebellar neurons, 5-10 post-natal day 5-9 (P5-9) Long-Evans rats were sacrificed and the cerebellum was recovered in 6ml of 0.1x trypsin with 50μg/ml DNase I. The tissue was dissociated by trituration and incubation for 7-10 minutes at 37°C / 7.3%CO_2. Trypsinization was stopped using media with 10% serum after which the cells were washed once with DMEM. These isolated neurons were resuspended to a single-cell suspension in a modified Sato medium (DMEM; 10% Path-O-Cyte BSA; 20nM progesterone; 100mM putrescine; 30nM sodium selenite; 5μg/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), counted and plated.

2.1.2.2 Dorsal Root Ganglia

To isolate dorsal root ganglia (DRG) neurons, 8-16 Long-Evans rats of either P0-2 (for neurons not inhibited by MAG) or P5-9 (for MAG-inhibited neurons) were sacrificed and the DRG collected on ice into 4.5ml of 0.15% Collagenase in L-15 media. The DRG were triturated gently and then incubated for 30-90 minutes at 37°C. After incubation, 0.1x trypsin and 50μg/ml DNase I were added to the mixture and the cells were incubated a further 10 minutes at 37°C. In order to dissociate any remaining clusters, the cells were triturated gently in the trypsin solution. Trypsinization was halted with media containing 10% serum. Dissociated DRG were then washed once with DMEM and resuspended in

Sato media. In order to enrich for DRG neurons, cultures were pre-plated onto tissue culture-treated plastic dishes for 60-90 minutes at 37°C. After pre-plating, DRG were collected, counted and plated onto either 24-well dishes or 8 chamber slides.

2.1.3 Purified Myelin

2.1.3.1 Myelin preparation

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

2.1.3.2 Immobilized myelin substrates

8 chamber Permanox slides were coated with 16.6 μg/ml poly-L-lysine for 30 minutes at room temperature. After incubation, PLL was removed and washed once with dH_2O. Myelin was then plated at a concentration of 1 – 2 μg/well and then dried overnight in a vacuum chamber filled with Drierite dessicator. Slides were then used immediately or frozen at -80°C for no more than 5 days prior to use.

2.1.4 Priming Neurons with Neurotrophins

24-well tissue culture dishes were coated with 100µg/ml of poly-L-lysine for at least 30 minutes at room temperature. The wells were then washed once with DMEM to remove excess PLL. Isolated cerebellar or dorsal root ganglion neurons were plated onto these dishes at a concentration of approximately 1 x 10^6 cells/well. These neurons were then treated with either BDNF or GDNF (200 ng/ml) in the presence or absence of KN-62 (10µM), 2-Aminoethyl diphenyl borate (2-APB) (100µM), Nimodipine (10µM) or D(-)-2-Amino-5-phosphonopentanoic acid (APV) (100µM). The neurons were then cultured overnight at 37°C after which they were removed from the dish via trypsinization (0.4x trypsin for 10 minutes at 37°C). Trypsinization was stopped by 10% serum-containing media and the cells were collected, resuspended in fresh Sato, counted and plated onto either a purified myelin substrate or MAG-expressing CHO cells.

2.2 The Neurite Outgrowth Assay

2.2.1 Neurite Outgrowth Culture

Following dissociation, noted above, primary neurons were plated onto the CHO cell monolayers or purified myelin substrate at a cell density of 1.1 – 1.5 x 10^4 cells per well (8 chamber slide) for cerebellar neurons and 0.75 – 1 x 10^4 cells per well for DRG neurons. This co-culture was then incubated for 18 hours at 37°C. Where indicated, these cells were also treated with 1mM db-cAMP, 10μM KN-62 or 200ng/ml BDNF during this period.

After incubation, the cultures were then fixed with 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with cold 100% methanol for 2 minutes. The cells were then blocked against non-specific binding with DMEM containing 10% serum for a minimum of 20 minutes at room temperature.

The neurons were then stained for the neuronal-specific marker βIII tubulin as follows. After blocking, the cultures were incubated overnight at 4°C in a PBS-BSA (0.05%) solution containing a monoclonal anti-βIII tubulin antibody (Covance) at a dilution of 1:1000. After this incubation, the cultures were washed 3 times with PBS and then incubated at room temperature for 30 minutes in a PBS-BSA (0.05%) solution containing biotinylated donkey anti-mouse IgG at a 1:500 dilution. After this second incubation, the cultures were once again washed 3 times with PBS and further incubated 30 minutes at room temperature in a PBS-BSA solution containing Streptavidin-Texas Red at 1:500. Finally, the cultures were washed 3 more times with PBS and then immobilized using the Permafluor (Immunon) and viewed under a fluorescent microscope.

2.2.2 Measuring and Quantification

In order to quantify the neurite outgrowth length from these treated neurons, the immunostained cultures were observed under a fluorescent microscope and the neurite length was measured using the Simple PCI image quantification software. Briefly, the longest neurite from each of 150-200 βIII tubulin-positive neurons per well, selected systematically by progressive movement from one side of the well to the other, were traced onscreen and the mean neurite length was calculated using the software tools. Statistical analysis of the data obtained was performed using the SigmaStat software program.

2.3 CREB Activation/Phosphorylation

In order to observe activation of the cAMP-response element binding protein, we quantified phosphorylation of this protein at the serine 133 residue using specifically directed antibodies. Briefly, $\geq 5 \times 10^6$ cerebellar or DRG neurons were isolated and plated onto 6 well plates coated with 100μg/ml poly-L-lysine. These cells were incubated overnight at 37°C / 7%CO_2 to allow them to adhere to the plate. The following day, the neurons were starved by incubation in plain DMEM media for 2 hours at 37°C. This step was required to assure a standard baseline of CREB phosphorylation across all wells. Following starvation, the neurons were treated for 30 minutes at 37°C with either 1mM db-cAMP or 200ng/ml BDNF and in the presence or absence of 10μM KN-62, 10μM nimodipine, 100μM D(-)-2-Amino-5-phosphonopentanoic acid (APV) or 100μM 2-Aminoethyl diphenyl borate (2-APB). The neurons were then lysed in 200μl of hot lysis buffer and DNA was removed via centrifugation through a QIAshredder spin column (QIAGEN). Total protein concentrations for the samples were determined using the BCA Protein Assay Kit (Biorad).

To determine phosphorylation levels, the samples were separated via polyacrylamide gel electrophoresis (PAGE). Briefly, approximately 10-20μg of total protein per sample was loaded onto a 10% polyacrylamide gel and run at ~50mA per gel for 2 hours. The separated proteins were then transferred onto a PVDF membrane (Immobilon-P, Millipore) at either 50mV per gel for 2.5 hours at room temperature or 5mV overnight at 4°C. Following transfer, the blots were blocked with either 3% BSA or 5% dried milk in PBS-Tween20 (0.5%), washed and incubated with rabbit anti-phosphorylated CREB (Ser133) antibody (Cell Signaling) at a dilution of 1:1000 overnight at 4°C with shaking. After primary antibody binding, the blots were washed 3 times with PBS-Tween20 and incubated for 1 hour at room temperature with shaking in HRP-linked goat anti-rabbit IgG antibody (Cell Signaling) at a dilution of 1:2000. Visualization of the proteins was performed using the ECL chemiluminescence kit (Amersham).

2.4 cAMP Immunoassay

Cellular cAMP levels were quantified using the BIOMOL Format A cAMP "PLUS" EIA kit. Cerebellar or DRG neurons were isolated from rat pups as indicated above and plated onto 6 well plates coated with 100μg/ml poly-L-lysine. After allowing for adhesion to the plate overnight at 37°C, the neurons were starved for 2 hours in plain DMEM media and then treated for 30 minutes with 200ng/ml BDNF and/or 10μM KN-62. After the 30 minute experimental exposure time, cells were lysed with 0.1M HCl and cAMP quantification was conducted as indicated by the manufacturer. Briefly, a polyclonal antibody to cAMP is used to bind either cAMP from the sample or an alkaline phosphatase-linked cAMP competitor on a microtiter plate which has goat antibody to rabbit IgG covalently linked. A p-nitrophenyl phosphate substrate solution is then added to produce a colorimetric reaction with the alkaline phosphatase and the assay is quantified by microplate reading at 405nm. In this competitive assay, the intensity of the bound color displayed is inversely proportional to the amount of cAMP in the sample. Quantitation is achieved by comparison to a standard curve generated using prepared standards ranging from 0.078 to 20 pmol/ml.

2.5 Adenoviral Vector Prep and Infection

2.5.1 Preparation of CaMKIV Mutant Adenoviruses

The CaMKIV mutant constructs (Wild-type (WT); Kinase-dead (KD); Constitutively-active (CA)) were kindly provided by Dr. Talal Chatila of the Mattel Children's Hospital at UCLA. Viral vector construction was adapted from He et al., 1998. Briefly, each mutant construct was excised from the host plasmid (pSG5; Stratagene) with the enzyme Bgl II. The fragments were separated on a 1% agarose gel and the insert recovered by gel extraction using the QIAspin Gel Extraction Kit (QIAGEN). The mutant constructs were then subcloned into the pTRACK-CMV vector and introduced to XL-Blue (Stratagene) via electroporation. After expansion of these bacteria and collection of DNA via maxi-prep (QIAGEN), proper insertion of the gene was identified by restriction digest analysis. The isolated pTRACK-CMV with insert was then electroporated into a rec-positive bacterial line, BJ5183, which contain the pAdEasy1 vector. This vector contains all of the Ad5 genetic sequence with the exception of the E1 and E3 genes. After homologous recombination in the BJ5183 cells, the new vector, pAd-CMV+insert, was harvested. Virus production was achieved via transfection of each pAd-CMV (with respective inserts) into 293T cells which stably express the adenoviral E1 gene. The cells were cultured for 5 days at 37°C and virus was harvested from the supernatant (figure 2.1).

2.5.2 Infection of Primary Neurons

For the infection and priming of primary neurons, post-natal day 5-9 DRG neurons were dissociated as described above. These neurons were plated onto 100μg/ml poly-L-lysine-coated 24-well plates and incubated overnight in the presence of 100 Fluorescent units (Fu) / cell of either CaMKIV mutant-expressing (+GFP) or GFP alone control adenoviral vectors. These cultures were then "primed" with 200ng/ml BDNF overnight at 37°C and subsequently plated onto CHO cell monolayers, fixed and stained as indicated above. For the purposes of quantification, only cells that were positive for both GFP and βIII tubulin were counted and measured.

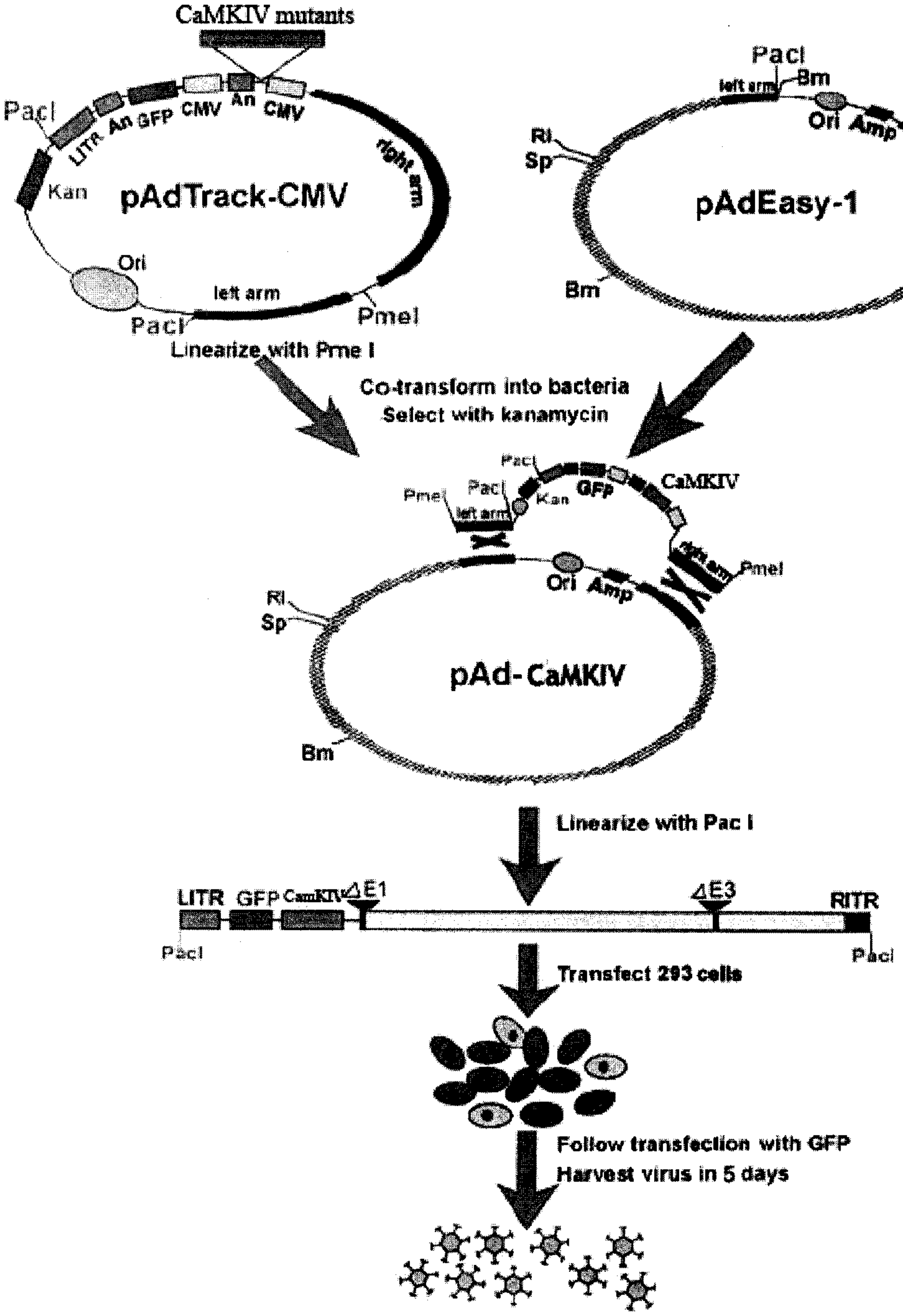

CaMKIV mutants
PacI
LITR
An
GFP
CMV
An
CMV
right arm
Kan
pAdTrack-CMV
Ori
left arm
PacI
PmeI
Linearize with Pme I
PacI
left arm
Bm
Ori
Amp
right arm
RI
Sp
pAdEasy-1
Bm
Co-transform into bacteria
Select with kanamycin
PacI
PmeI
PacI
left arm
Kan
GFP
CaMKIV
right arm
PmeI
Ori
Amp
RI
Sp
pAd-CaMKIV
Bm
Linearize with Pac I
LITR
GFP
CamKIV
ΔE1
ΔE3
RITR
PacI
PacI
Transfect 293 cells
Follow transfection with GFP
Harvest virus in 5 days

Figure 2.1: Production of the Adenoviral Vectors. CaMKIV mutant viral vectors were produced as indicated. The control virus was identical except that it lacked the inserted gene. All vectors contained GFP as the reporter. Adapted from (He et al., 1998).

2.6 Differentiation of Motor Neurons from Embryonic Stem Cells`

The transgenic mouse embryonic stems cells utilized in these studies were a gift from the laboratory of Thomas Jessell of Columbia University and all protocols illustrated here are modified from Wichterle et al., 2002.

2.6.1 Proliferation of ES Cells

In order to proliferate existing stocks of embryonic stem (ES) cells, ES cells were grown on a feeder layer of primary embryonic fibroblasts (PMEFs) (obtained from Specialty Media).

2.6.1.1 PMEF Culture

PMEFs were thawed quickly at 37°C and immediately resuspended in PMEF media (15% ES-qualified FBS, 2mM L-glutamine, 1X antibiotics, 1X nucleotides, 1X non-essential amino acids and 0.1mM 2-mercaptoethanol in ES-qualified DMEM—all from Specialty Media). Either T25 or T75 flasks were coated with 0.1% gelatin in water (Specialty Media) for 30 minutes at room temperature. After this incubation, the thawed PMEFs were plated onto the flasks and allowed to adhere and proliferate for approximately 3 days. Once the cells reached confluency, the PMEFs were then treated with 10mg/ml mitomycin-C (Sigma) for 2 hours at 37°C in order to inhibit further proliferation (inactivated PMEFs). After mitomycin-C treatment, PMEFs were washed twice with PBS and then incubated in PMEF media at 37°C / 5.0% CO_2 until needed.

2.6.1.2 ES Cell Proliferation Culture

ES cells were thawed quickly at 37°C and resuspended in ES Media (same components as PMEF media plus 1000 units/ml LIF-1 (Chemicon)). The ES cells were then plated onto a T25 flask containing a feeder layer of inactivated PMEFs. The coculture is then incubated at 37°C / 5.0% CO_2 for approximately 2 days (or until 80-90% confluent). After the desired confluency is reached, the culture is treated with 0.4X trypsin with 2mM EDTA for 1 minute at 37°C to detach the cells from the plate. After stopping trypsinization with ES media, the ES cell culture is then plated onto two T75 flasks containing feeder layers of inactivated PMEFs. This culture is allowed to proliferate a further 2-3 days at 37°C until confluency is reached. The culture is then treated with 0.4X trypsin with 2mM EDTA to detach the cells and then resuspended into 6-8 aliquots in ES freezing media (Specialty Media). Aliquots are frozen down using a isopropanol freezing chamber at -80°C and then stored until use in liquid nitrogen.

2.6.2 Differentiation of ES Cells

One to two vials of frozen ES cells were thawed quickly at 37°C and the cells were collected by centrifugation at ~800rpm. The ES cells were the resuspended in ES media and plated onto one (each) T25 flask which had been coated with 0.1% gelatin at room temperature for 30 minutes. The cells were then allowed to expand via incubation at 37°C / 5% CO_2 overnight. The following day, the media was replaced with fresh ES media. On day III, the ES cells were removed from the T25 flask via application of 0.4X trypsin with 2mM EDTA (~1 minute at 37°C). The cells were then collected, centrifuged and resuspended in DFNK media (20% DMEM; 20% F12 media-Gibco; 47% Neurobasal Media-Gibco; 1% Penicillin/Streptomycin; 10% Knockout Serum Replacement-Gibco; 2mM L-glutamine; 100μM 2-mercaptoethanol—all products were from Specialty Media, except where indicated otherwise). To enrich for the ES cell population and remove unnecessary PMEFs, the culture was then plated onto a T75 flask that had been coated with 0.1% gelatin for 45-60 minutes at 37°C, after which, the supernatant (including the unadhered ES cells) was collected, centrifuged and resuspended in 10ml DFNK. The ES cells were then counted using a hemocytometer and plated onto 60mm Petri dishes at a density of approximately $7.5 \times 10^5 - 1 \times 10^6$ cells per plate. The cells were then incubated once again overnight at 37°C. The following day, the newly-formed embryoid bodies (EBs) were collected, allowed to settle in a falcon tube by gravity, resuspended in fresh DFNK and replated onto new 60mm Petri dishes. They were then incubated again overnight at 37°C. On the first day of differentiation, the EBs were once again collected into falcon tubes, resuspended in fresh DFNK and replated onto new 60mm Petri dishes. However, to begin the differentiation process, 0.3μM retinoic acid (Sigma) and 0.3μM Sonic Hedgehog agonist (HhAg1.3-Curis, Inc.) were added to the culture. The differentiation process was then allowed to proceed for approximately five days at 37°C, with a single media change at day 3. By day 4-5, a large number of these ES cells had differentiated into post-mitotic motor neurons as indicated by expression of Hb9-driven GFP.

2.6.3 Neurite Outgrowth of ES-derived MNs

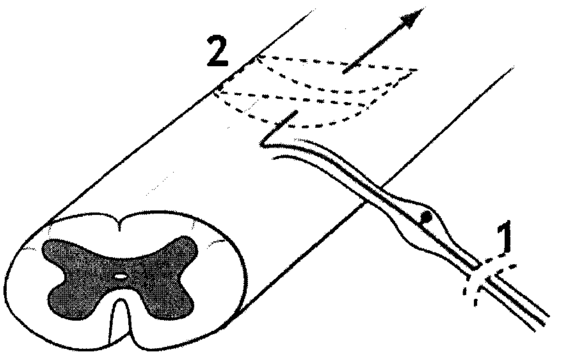
The motor neurons derived by the above-illustrated process were dissociated from the embryoid bodies using a papain dissociation kit (Worthington). Briefly, EBs were collected into a 15ml Falcon tube and allowed to settle by gravity. The media was removed and the EBs were resuspended in 1ml of papain + DNase with trituration. The cells were then incubated for 30-45 minutes at 37°C and triturated once more at the conclusion of incubation. The dissociated cells were then collected by centrifugation at ~300 x g at 4°C. The enzymatic reaction was then halted with addition of a solution of albumin inhibitor + DNase. The final collection of the dissociated cells was achieved via centrifugation through a gradient of albumin inhibitor for 6 minutes at 37°C. The cells were then resuspended in Sato media and counted using a hemocytometer.

Priming and/or neurite outgrowth of these dissociated motor neurons was then carried out as indicated above for primary neurons. Quantification, however, did not require staining with βIII tubulin. Instead, only GFP-positive neurons were measured and quantified.

Chapter III: Timing the cAMP/PKA-dependent Signaling in Overcoming Myelin-induced Inhibition

3.1 Introduction

Neurons of the adult mammalian central nervous system fail to regenerate damaged axons after injury. This is believed to be due to the several factors including the presence of myelin-associated inhibitory proteins and formation of the glial scar, which results in both a physical and chemical barrier to regeneration. These inhibitory proteins include Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), myelin-associated glycoprotein (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and oligodendrocyte-myelin glycoprotein (Kottis et al., 2002; Wang et al., 2002b) from myelin and proteoglycans molecules of the glial scar (Davies et al., 1999; McKeon et al., 1991; Niederost et al., 1999; Smith-Thomas et al., 1994). Methods for overcoming the inhibition provided by these molecules have centered around two distinct approaches. The first involves attempting to block either the binding of the inhibitors to their receptor(s) (Bradbury et al., 2002; Fournier et al., 2002; GrandPre et al., 2002; Wang et al., 2002a) or the signaling which results from this binding (Borisoff et al., 2003; Dergham et al., 2002; Dubreuil et al., 2003; Fournier et al., 2003; Lehmann et al., 1999; Monnier et al., 2003; Winton et al., 2002). The second approach attempts to change the intrinsic growth state of the neuron such that it no longer responds to the inhibitory substrate. On such approach, pioneered by our laboratory, has shown that elevation of intracellular cAMP levels, either via application of chemical analogues or via priming with neurotrophins, can effectively block the inhibition of axonal regeneration induced by the myelin-associated inhibitory proteins (Cai et al., 1999). Furthermore, our lab and others have found that in situations where spontaneous CNS axonal regeneration is observed (e.g. embryonically or following a pre-conditioning lesion), the levels of endogenous cAMP are significantly higher than in situations where regeneration is not observed (Cai et al., 2001; Neumann et al., 2002; Qiu et al., 2002). Furthermore, this hypothesis was verified by the finding that a single injection of db-cAMP can effectively mimic the pre-conditioning lesion even in the absence of such a lesion (Qiu et al., 2002). Interestingly, though, while CNS regeneration observed one week after a conditioning lesion is improved compared to one day post-lesion, the levels of endogenous cAMP appear to have returned to control levels by this time (Figure 3.1).



16 hours Later:

***In vivo*: Regeneration into dorsal column lesion site evident**

***In vitro*: Inhibition of neurite outgrowth by MAG/myelin is overcome and is cAMP dependent**

7 Days Later:

***In vivo*: Regeneration into and beyond dorsal column lesion site**

***In vitro*: Inhibition of neurite outgrowth by MAG/myelin is overcome and is cAMP independent**

Figure 3.1: The Conditioning Lesion Effect. Lesioning of the peripheral branch (1) of DRG neurons either one day or one week prior to performing a central branch lesion (2) can significantly increase the ability of these neurons to regenerate their CNS-extending axons.

In addition, it is now known that this elevation of cAMP levels results in gene transcription in a CREB-dependent manner (Gao et al., 2004), and so we hypothesize that after induction of cAMP elevation, the downstream effectors of this pathway may be active only during a relatively short period of time following induction of either the conditioning lesion paradigm or during neurotrophin-induced priming.

Therefore, in order to further illustrate and elucidate the precise timing of these events, we performed time course observations of cAMP levels and downstream signaling post-lesion and PKA-dependence during priming.

3.2 Results

While a conditioning lesion itself is not a viable therapeutic approach to encouraging CNS regeneration in humans, the recent finding by our lab elevation of cAMP levels are one mediating factor in this effect allows us to look more carefully into targeting one of the signaling components active in the conditioning lesion paradigm. However, in order to investigate this treatment as a possible therapeutic, we first needed to understand the precise timing of certain signaling events post-conditioning injury so that we might be able to mimic them in designing a potential therapeutic approach.

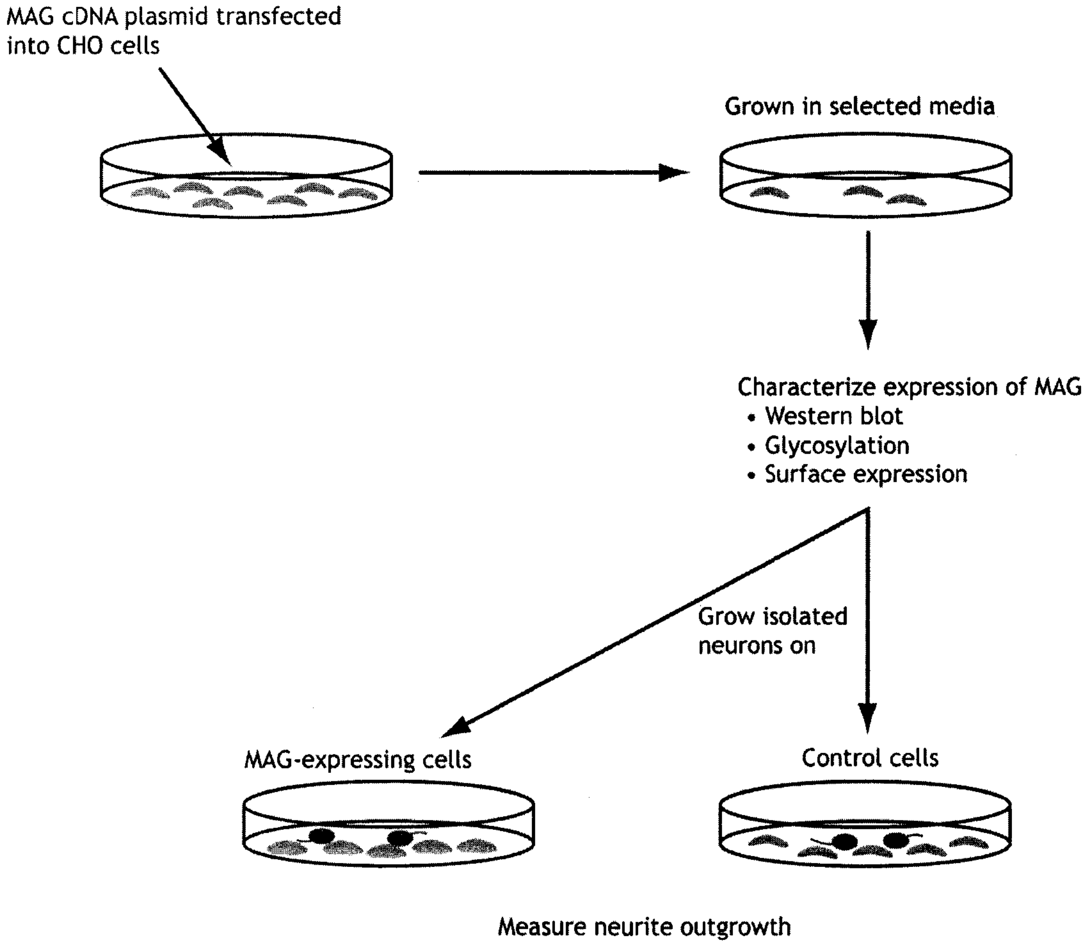

MAG cDNA plasmid transfected into CHO cells
Grown in selected media
Characterize expression of MAG
• Western blot
• Glycosylation
• Surface expression
Grow isolated neurons on
MAG-expressing cells
Control cells
Measure neurite outgrowth

Figure 3.2: 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.

The first question that we asked here was: at what time post-injury does the improved axonal regeneration become independent of both PKA activation and transcriptional upregulation? To answer this, we performed a unilateral lesion to sciatic nerve of post-natal day 20-23 rats, waited between 12-32 hours, sacrificed the animals and collected the dorsal root ganglia from the L4-5 region. The collected DRG were dissociated and grown *in vitro* on a substrate of MAG-expressing CHO cells in the presence or absence of pharmacological inhibitors and neurite outgrowth was quantified (figure 3.2). As indicated in figure 3.3, we found that the improved neurite growth on the inhibitory substrate is blocked by application of an inhibitor of PKA (H89) at any time point prior to 18 hours, but this effect is no longer responsive to PKA inhibition after this time.

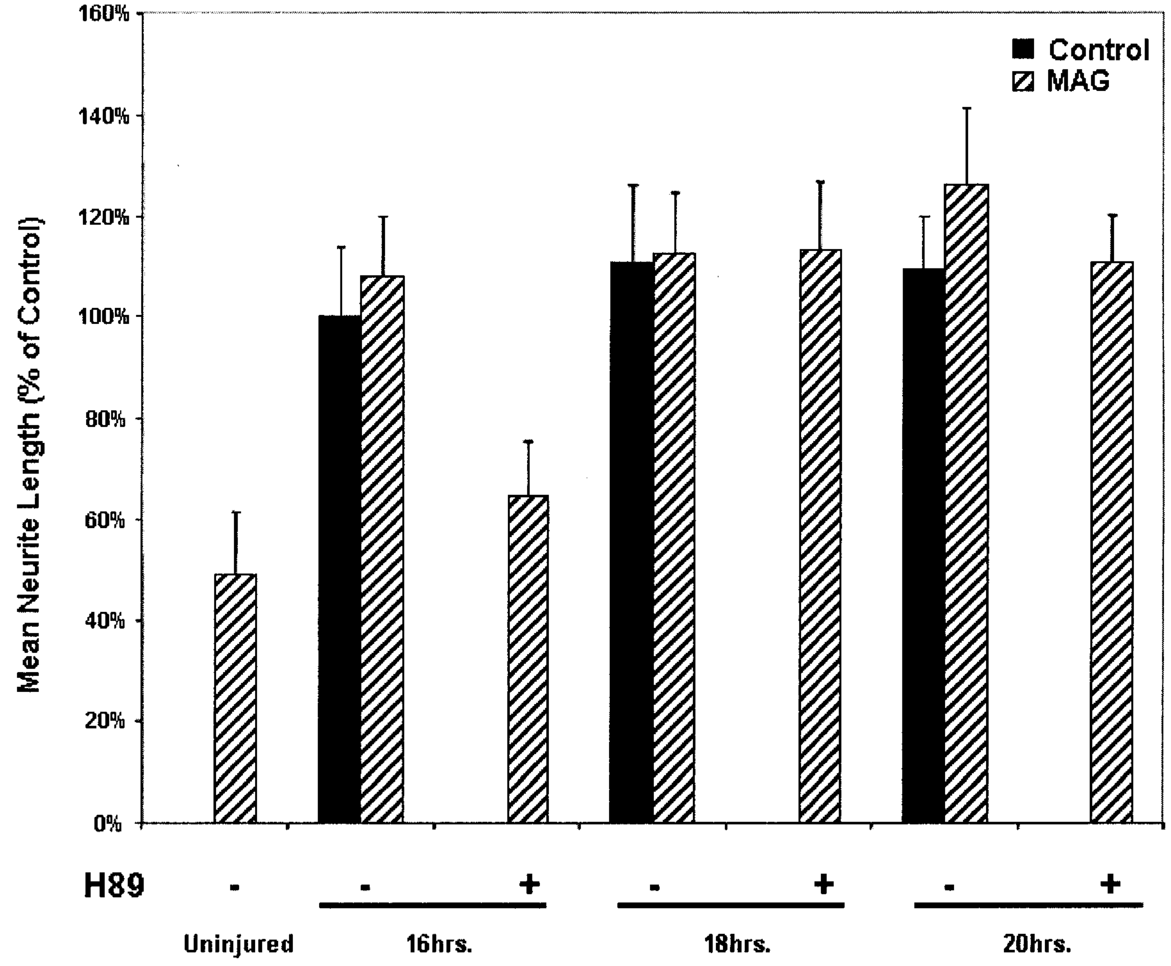


Figure 3.3: Time Course of PKA Dependency in the Conditioning Lesion Effect. The sciatic nerve of post-natal day 20 rats was lesioned and the L4-5 DRG were collected, dissociated and plated onto CHO cell monolayers at the time points indicated (post-lesion) in the presence or absence of the PKA inhibitor, H89 (5μM).

Furthermore, the dependence of this effect on cellular transcription appears to follow a similar time course: application of an RNA polymerase inhibitor, DRB, only significantly blocks the improved neurite outgrowth prior to about 18 hours post-lesion (figure 3.4). If this PKA and transcriptional dependency appears to be active during the first 18 hours post-injury, it would also seem logical that the elevated cAMP levels should behave in a similar manner.

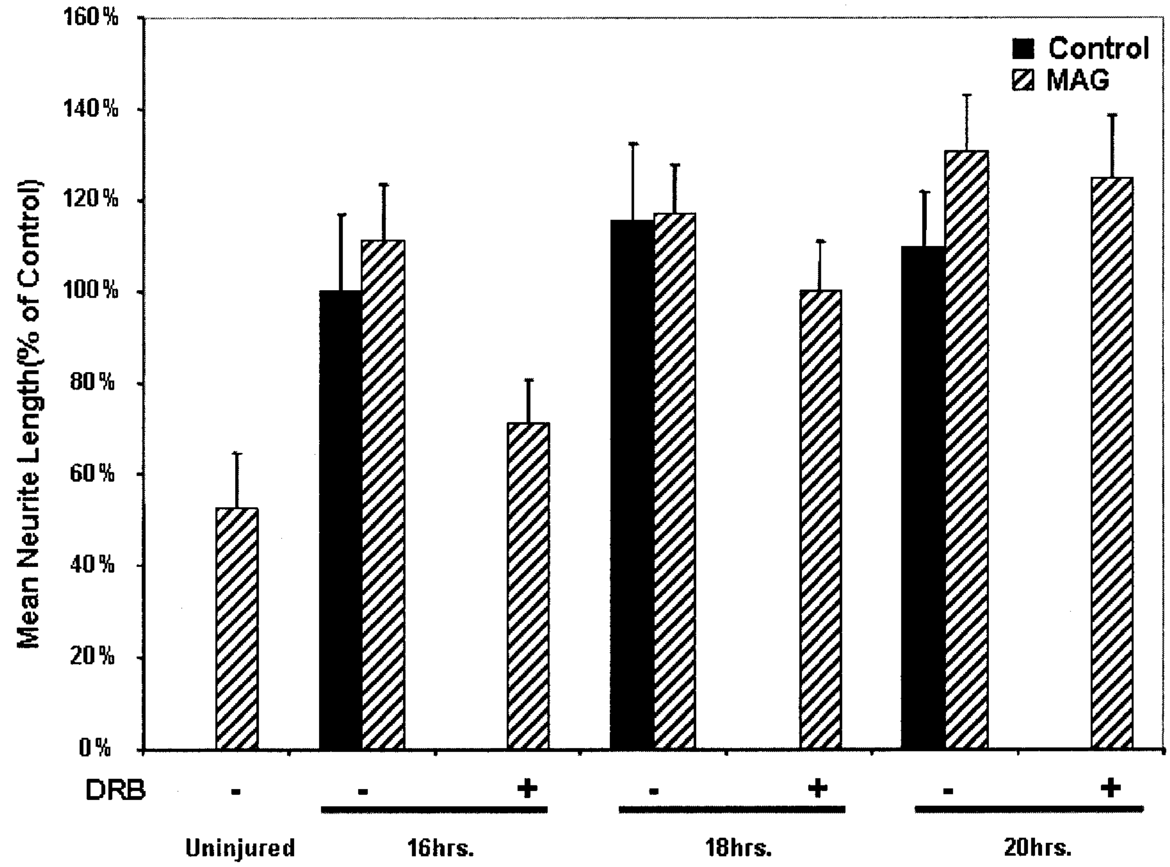


Figure 3.4: The Transcriptional Dependency of the Conditioning Lesion Effect. The sciatic nerve of post-natal day 20 rats was lesioned and the L4-5 DRG were collected, dissociated and plated onto CHO cell monolayers at the time points indicated (post-lesion) in the presence or absence of the RNA Polymerase inhibitor, DRB (5μM).

To test this, we once again performed a conditioning lesion of the sciatic nerve in post-natal day 20-23 rats and allowed the rats to survive a further 14-28 hours after which the DRG were removed from L4-5, homogenized in 0.1M HCl and the cAMP levels were immediately quantified using an competitive immunoassay (BIOMOL) (figure 3.5). As indicated in figure 3.6, the cAMP levels of the injured DRG are elevated as compared to the contralateral control at early time points, but return to control levels by 18 hours post-lesion. This corresponds nicely with the previous findings for both PKA and

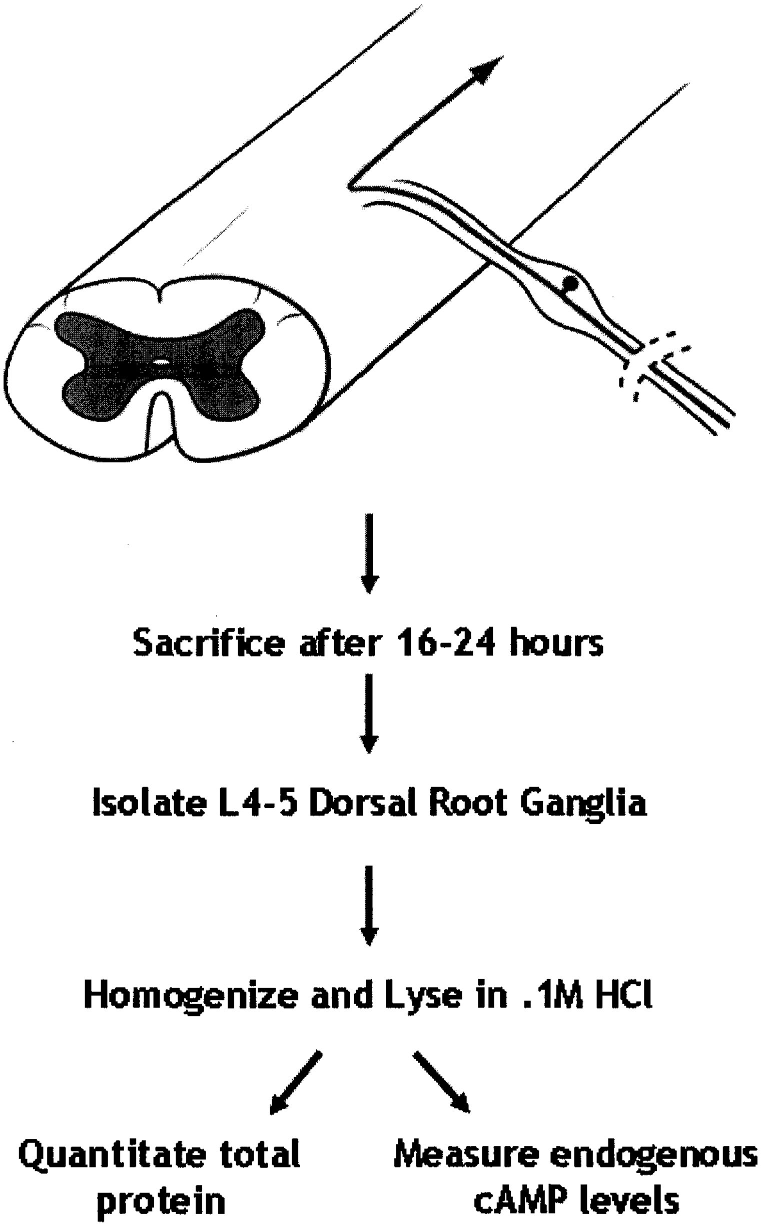

Sacrifice after 16-24 hours
Isolate L4-5 Dorsal Root Ganglia
Homogenize and Lyse in .1M HCl
Quantitate total protein
Measure endogenous cAMP levels

Figure 3.5: Conditioning Lesion and Collection of DRG.

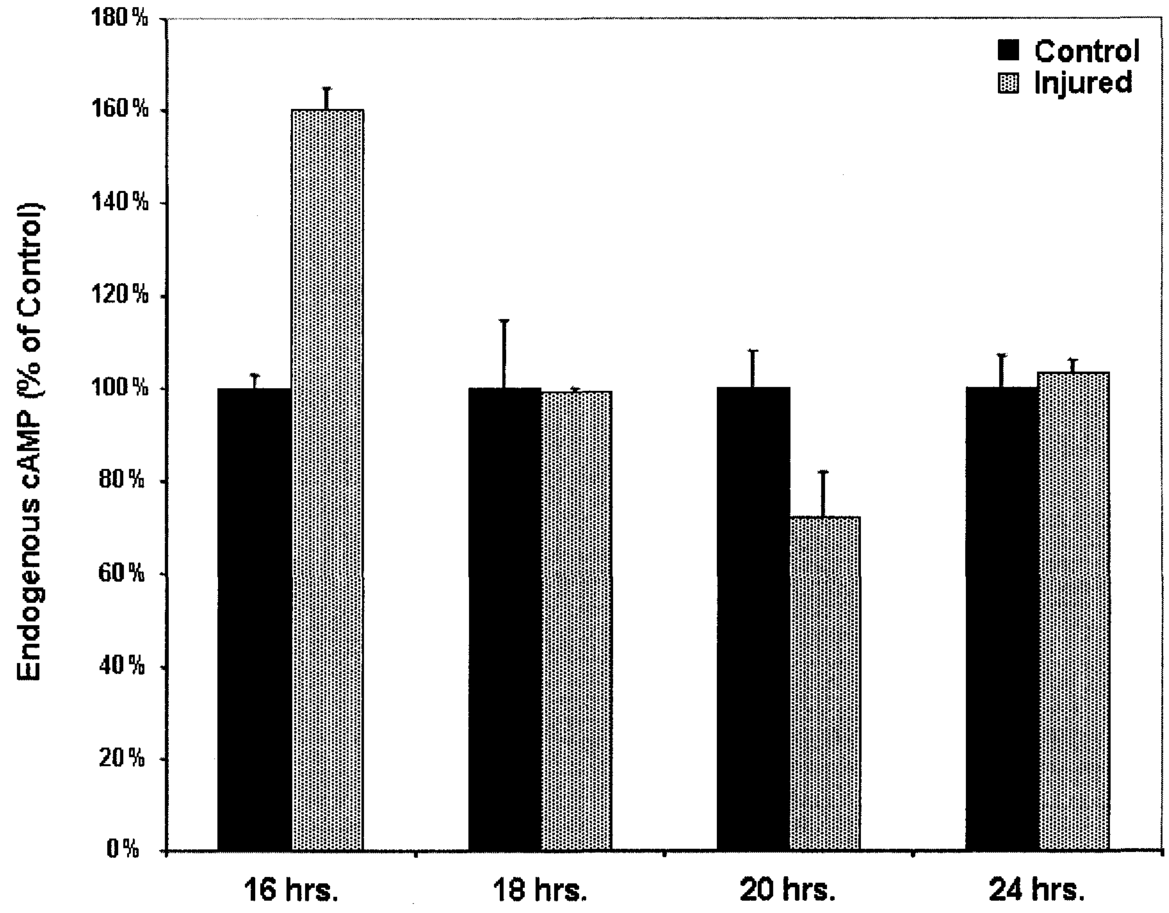


Figure 3.6: Endogenous cAMP Levels After a Conditioning Lesion. The sciatic nerve of post-natal day 20-23 rats was lesioned and the L4-5 DRG were collected, lysed in 0.1M HCl and the cAMP levels measured via competitive immunoassay. DRG were collected at the time indicated (post-lesion) and cAMP levels were quantitated immediately. Controls were taken from contralateral side of the same animals.

transcriptional dependence. With these results in mind, we also sought to elucidate some of the genes which are upregulated during situations where axonal inhibition is overcome (i.e. after treatment with db-cAMP and following a conditioning lesion). To do this, we treated dissociated primary neurons with db-cAMP in vitro or performed a sciatic nerve conditioning lesion in vivo for 18 hours (the time at which cAMP, PKA and transcription dependence has ceased), collected mRNA and performed microarray analysis (in collaboration with Jason Carmel and Ron Hart at Rutgers University). The microarray chips utilized contained over 5000 custom, 70-nucleotide oligos which were sequences from genes which have been shown previously to be regulated in response to spinal cord injury, cAMP modulation and inflammation. Table 3.1 illustrates some the genes which were most highly regulated as measured by both the microarray analysis and Q-RT-PCR. For the purposes of our study, we looked for genes that were either up or down-regulated by approximately 2-fold or more in both experimental scenarios. In these categories, we found 11 genes that were upregulated in common, including Arginase I, an enzyme which we had already found to be involved in cAMP-mediated axonal regeneration (Cai et al., 2002) and IL-6, whose role and effects were further investigated by Zixuan Cao from our lab (Cao et al., in preparation). The remaining up and down regulated genes are still be investigated by our lab for their relevance and contribution to axonal regeneration.

Gene	Q-RT-PCR Fold Change	Microarray Fold Change	Q-RT-PCR t-test	Microarray t-test
interleukin-6	15.3	1.9	0.002	0.008
secretory leukocyte protease inhibitor (SLPI)	8.5	3.9	0.005	0.008
fibulin 5	6.9	2.1	0.004	0.014
superoxide dimutase 3	4.4	3.8	0.003	0.018
early growth response	2.9	2.0	0.023	0.018
nerve growth factor inducible protein (VGF)	2.5	2.4	0.002	0.053
insulin-like growth factor binding protein 5 protease	2.0	2.6	0.003	0.040
lactate dehydrogenase A	1.9	3.1	0.005	0.005
Arginase I	1.7	2.1	0.01	0.07
rat metallothionein-i (mt-1)	1.7	2.6	0.005	0.016
NMDA receptor glutamate-binding subunit	1.3	2.2	0.048	0.008
insulin-like growth factor-binding protein 5 (Igfbp5)	-2.1	-2.9	0.002	0.00001
rat chemoattractant protein-1 (MCP-1)	-2.9	-4.1	0.011	0.030
growth accentuating protein 43	-3.5	-2.8	0.008	0.004
activating transcription factor 3 (Atf3)	-3.8	-2.4	0.001	0.007

Table 3.1: Genes which were Highly Regulated in both the db-cAMP-treated and Conditioning Lesion Scenarios.

In addition to the conditioning-lesion effect, another method for improving axonal growth in the presence of an inhibitory substrate is the elevation of intracellular cAMP levels via the "priming" of neurons with neurotrophins. In order to compare the timing and signaling of these two methods of encouraging regeneration, we also sought to determine the timing of the neurotrophin-dependency during priming. To do this, primary cerebellar neurons dissociated from post-natal day 5-9 rat pups were cultured in the presence or absence of 200ng/ml BDNF for varying periods of time after which, the neurotrophin was removed and the cells were allowed to grow in culture for the remainder of the 24 hour time period before being transferred onto the CHO cell monolayers for the neurite outgrowth quantification (figure 3.7A). Our findings indicate that while 1 hour or 3 hours in the presence of BDNF is sufficient to induce an improvement in axonal growth in the presence of MAG, it is at 6+ hours that the BDNF effect best mimics the overnight application of neurotrophin (figure 3.8). To further elucidate the timing of this effect and to see if we can further reduce the time necessary for priming, we repeated these experiments while shortening the total incubation time to 6 hours (figure 3.7B). Surprisingly, however, we found that neurons incubated for only 6 hours before exposure to MAG are unable to be primed by neurotrophins, even if they “see” the BDNF for the full 6 hours (figure 3.9). This finding implies that these primary neurons require a period of time after induction of the BDNF-induced signaling in order to mediate a block of the MAG-induced inhibition of neurite outgrowth.

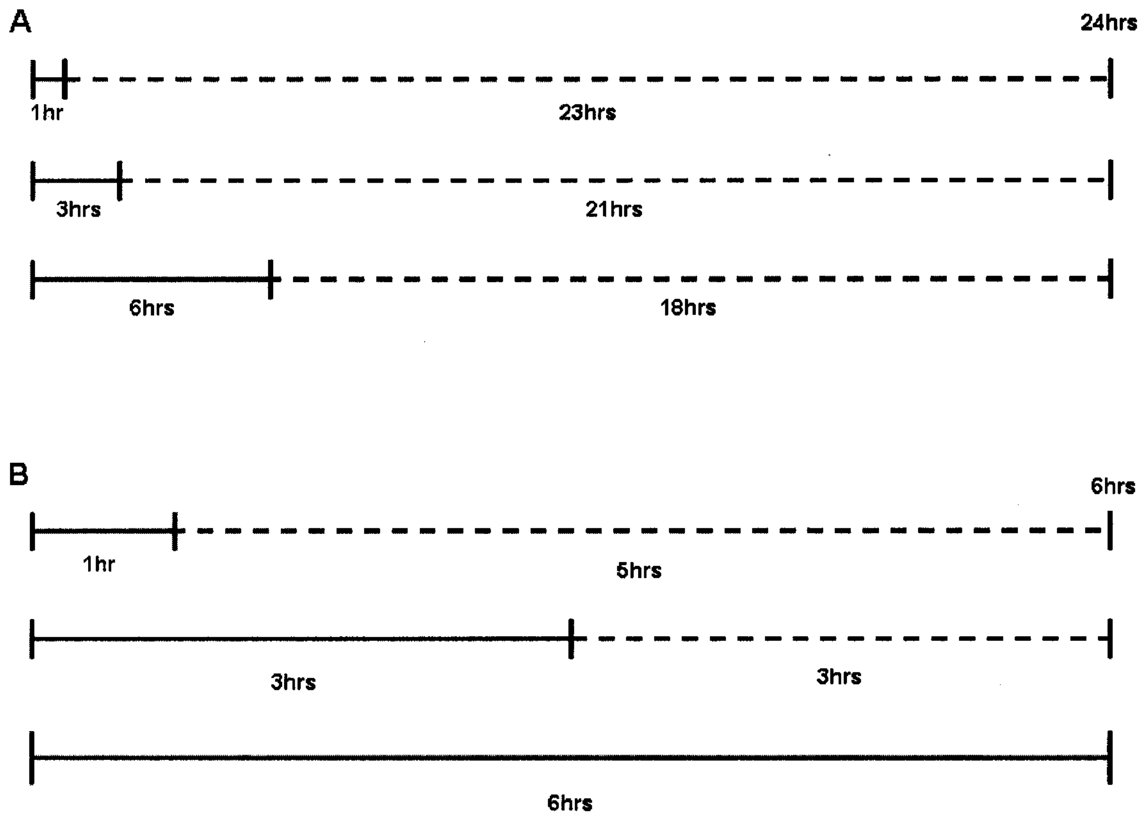

A
24hrs
1hr
23hrs
3hrs
21hrs
6hrs
18hrs
B
6hrs
1hr
5hrs
3hrs
3hrs
6hrs

Figure 3.7: Schematic of experimental design for timing the dependency of neurotrophins in the priming effect. 200ng/ml BDNF was applied to the neurons at time=0 and left in the culture for the time indicated below the solid bar. After the treatment period, the neurotrophin-containing media was removed and replaced with neurotrophin-free media and the neurons were incubated for the further time indicated below the dashed bar. The total time of priming was either 24 hours (A) or 6 hours (B).

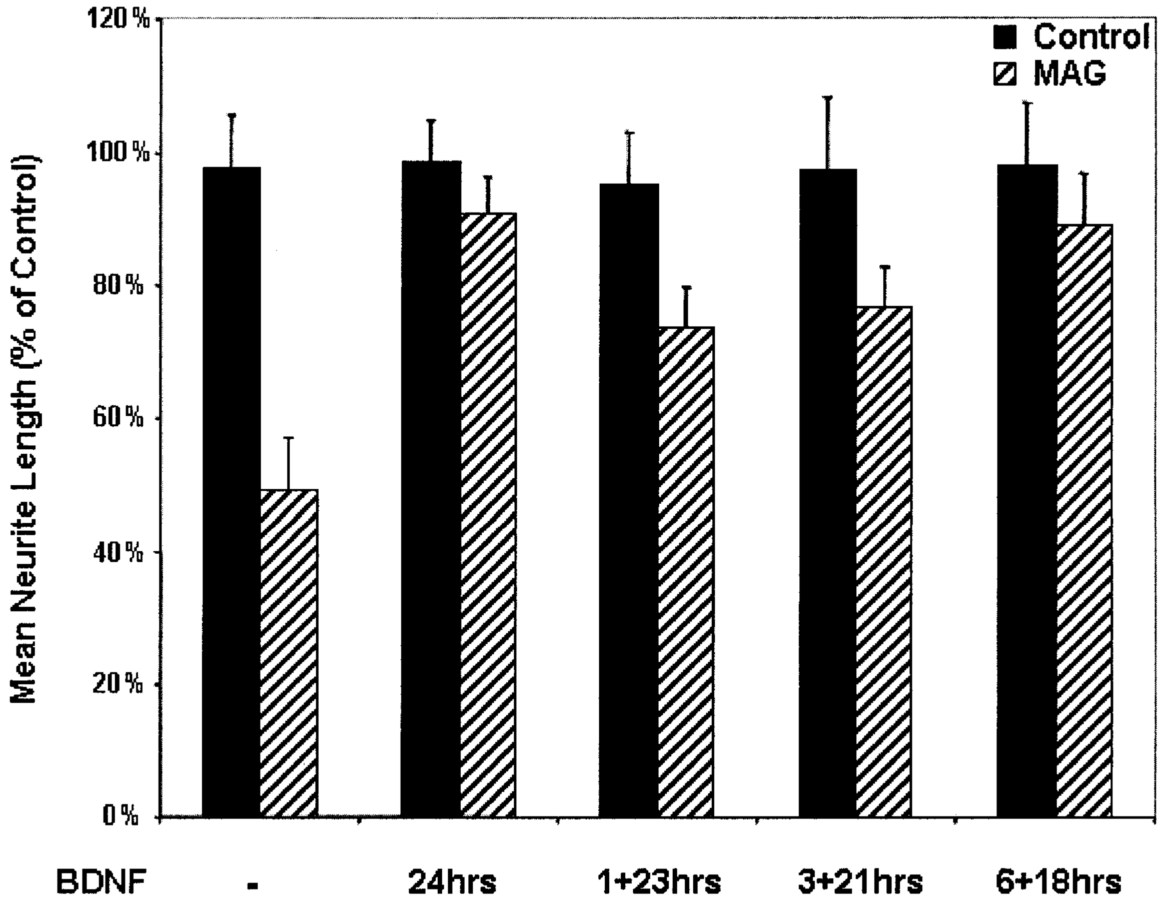


Figure 3.8: Timing the Neurotrophin Dependency During Priming (24hrs). P5-9 cerebellar neurons were primed on poly-L-lysine-coated dishes for 24 hours. Exposure to BDNF is indicated by the first number (in hours) after which neurotrophin was removed and the neurons were incubated for the remainder of the 24 hour time period (indicated by the second number) before being plated onto the CHO cell monolayers.

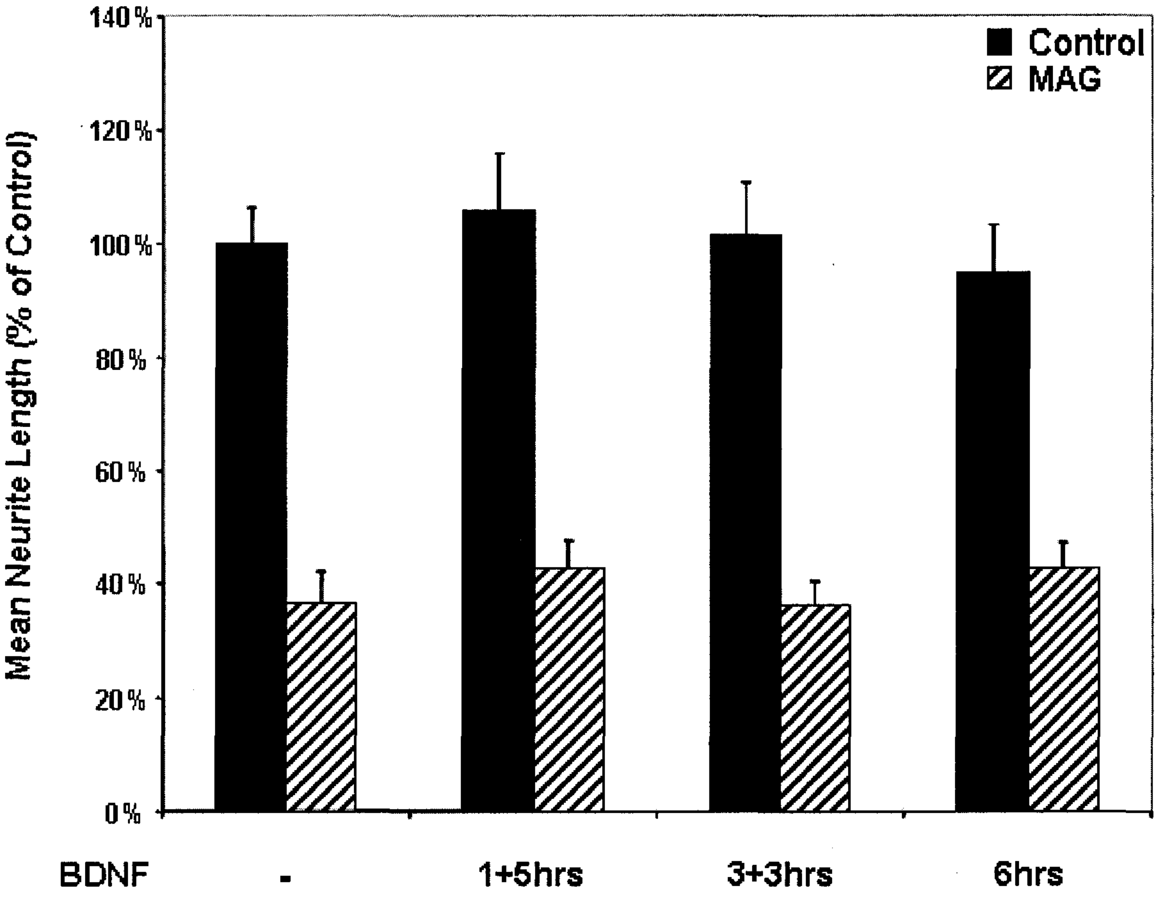


Figure 3.9: Timing the Neurotrophin Dependency During Priming (6hrs). P5-9 cerebellar neurons were primed on poly-L-lysine-coated dishes for 6 hours. Exposure to BDNF is indicated by the first number (in hours) after which neurotrophin was removed and the neurons were incubated for the remainder of the 6 hour time period (indicated by the second number) before being plated onto the CHO cell monolayers.

3.3 Discussion

The findings outlined in this chapter provide some intriguing insight into the timing of the events involved in the molecular signaling of both the pre-conditioning lesion and priming effects. With the data obtained here, we can begin to piece together the timing of some of the downstream signaling events induced by the conditioning lesion: elevation of cAMP levels occurs fairly early following a conditioning lesion (Qiu and Filbin, unpublished data) and remain high until at least 16 hours post-lesion. When these cAMP levels begin to drop by 18 hours, so, too, does the dependence on PKA signaling and the subsequently induced upregulation of gene transcription.

In previous work published by the lab of Pate Skene, it has been suggested that neurons in culture may exhibit growth in two stages: a "branching" stage which may be reminiscent of the synaptic plasticity and reorganization which may be observed even in adult animals and an "elongating" stage which may be more similar to axonal regeneration (Smith and Skene, 1997). In this work, Smith and Skene found that while naïve isolated neurons appear to exhibit "branching" growth for the first 20 hours *in vitro* before switching to "elongation", neurons that have first had a conditioning-lesion applied 2-7 days before isolation appear to enter the "elongation" stage almost immediately after plating. Interestingly, though, if naïve neurons are incubated with a reversible inhibitor of transcription for 12 hours, the switch to the "elongating" stage is shifted by 16 hours (from 20 hours to 36 hours in culture), implying that there is a minimum transcriptional requirement for "pure" growth, even in the absence of inhibitors. In addition, this work indicates that neurons which have been treated with a conditioning lesion for at least 2 days are independent of transcription. Thus, the work we present here contributes a more precise understanding of the timing—particularly during the initial stages (first 24 hours)—of the aforementioned signaling events following conditioning lesion and, more importantly, addresses these ideas in the context of the neurons' responsiveness to an inhibitory substrate as opposed to analyzing simple axonal growth on a neutral substrate as was done in the study noted above.

These findings have allowed us to both plan the timing of therapeutic intervention with respect to cAMP elevation as well as investigate precisely which genes are modulated by this effect by performing microarray analysis at the precise point at which transcriptional regulation ceases.

In addition, the investigations into the precise timing of the BDNF-induced priming effect have further supported findings that downstream signaling events are required to occur prior to the onset of the inhibitory signaling induced by the myelin-associated inhibitors of regeneration. While we know from the work of myself and Ying Gao (Gao et al., 2004) that the application of neurotrophins to primary neurons can induce the initial stages of overcoming axonal growth inhibition by 5-30 minutes, these findings suggest that the actual induction of the downstream events which are necessary for axonal regeneration require at least 16-18 hours after the induction of the signaling cascade for the their effects to be fully observed.

Chapter IV: Inhibition of Calcium/Calmodulin Kinase IV Can Block CREB Activation and Priming by BDNF

4.1 Introduction

Neurons that are injured following damage to the adult mammalian nervous system are often unable to regenerate to their axons. This is due, in part, to the presence of the myelin-associated inhibitors of regeneration. Previous work from our lab and others has shown that one of these inhibitors is Myelin-associated Glycoprotein (MAG) and that exposing primary neurons to MAG *in vitro* is sufficient to block the growth of their axons (McKerracher et al., 1994; Mukhopadhyay et al., 1994). Subsequent studies have also shown that it is possible to overcome MAG and the other inhibitory molecules found in myelin by elevating intracellular cAMP levels, both *in vitro* (Cai et al., 1999) and *in vivo* (Neumann et al., 2002; Qiu et al., 2002). This elevation of cAMP levels can be accomplished in several ways: application of the chemical analogue, dibutyryl-cAMP (db-cAMP), pre-treatment with neurotrophins (priming) or inhibition of the phosphodiesterase enzyme PDE4 with the pharmacological inhibitor, rolipram. In addition, each of these treatments alone is sufficient to block the MAG and myelin-mediated inhibition of neurite growth (Cai et al., 1999; Neumann et al., 2002; Nikulina et al., 2004; Qiu et al., 2002). With respect to the design of potential therapeutic techniques, the signaling of neurotrophins during the priming effect is of particular interest. Since neurotrophins are already present in the adult CNS both before and after injury, it is essential that we understand the signaling cascade which leads to elevation of cAMP levels and activation of the downstream effectors. Recent work from our lab has begun to elucidate some of the components of this signaling cascade and key participants in the downstream activities which result in improved axonal regeneration. Furthermore, the neurotrophin-induced priming effect induces phosphorylation and activation of the known cellular transcription factor, cAMP response element binding protein (CREB) in a protein kinase A (PKA) (Cai et al., 1999) and extracellular signal-regulated kinase (ERK) (Gao et al., 2003)-dependent manner (figure 4.1). In addition, activity of both of these enzymes is required for the db-cAMP-induced improvement of axonal growth. While blocking either of these enzymes can abrogate CREB phosphorylation and activation, neither was sufficient alone or in combination to completely block this effect. Therefore, we sought to determine if any other portions of this signaling cascade remained undiscovered.

Previous work from the lab of Michael Greenberg had shown that cortical neurons, when treated with BDNF, exhibited elevated CREB phosphorylation levels and that this effect was directly dependent on the activity of the enzyme calcium/calmodulin-dependent kinase IV (CaMKIV) (Finkbeiner et al., 1997). In addition, activity-dependent CaMKIV activation can induce increases in dendritic growth both *in vitro* and *in vivo* (Redmond et al., 2002). Although this was observed in a different system and under a different set of criteria, we sought to identify whether this signaling cascade was also activated during the priming of primary neurons by BDNF.

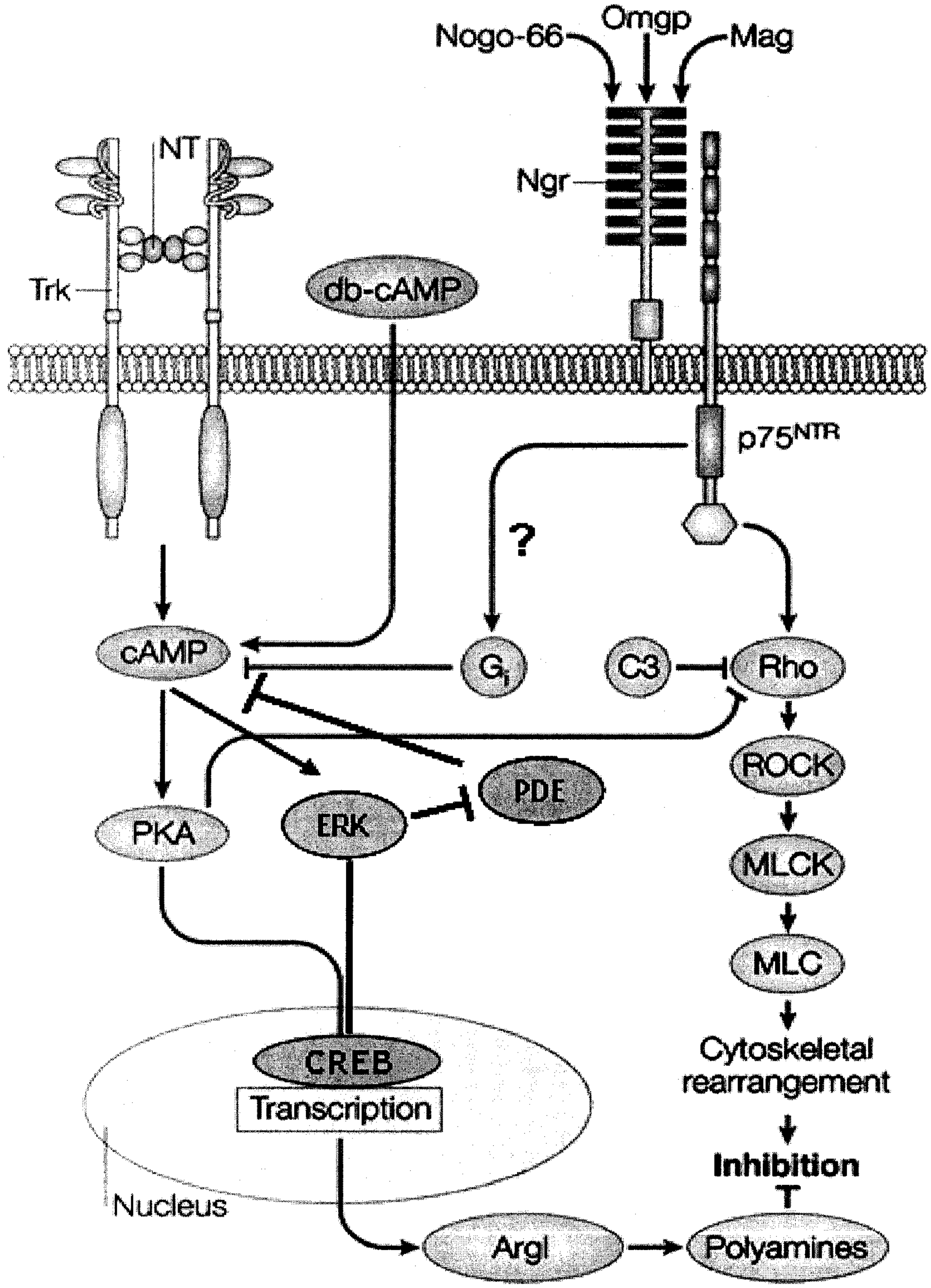

Nogo-66
Omgp
Mag
NT
Ngr
Trk
db-cAMP
p75NTR
?
cAMP
G_i
C3
Rho
ROCK
PKA
ERK
PDE
MLCK
MLC
Cytoskeletal rearrangement
Inhibition
CREB
Transcription
Nucleus
ArgI
Polyamines

Figure 4.1: Dueling Signaling Pathways. This diagram indicates some of the proposed signaling components of both the MAG/Myelin-mediated inhibition of axonal regeneration as well as the neurotrophin/cAMP block of this inhibition (adapted from Filbin, 2003).

4.2 Results

In order to identify whether the cellular enzyme CaMK plays any role in the neurotrophin-induced priming effect, cerebellar neurons taken from post-natal day 5-9 rat pups were plated onto poly-L-lysine and treated with 200ng/ml BDNF for 30 minutes either in the presence or absence of a pharmacological CaMK inhibitor, KN-62. The subsequent CREB activation was analyzed by quantification of its relative phosphorylation at residue serine 133. After treatment, the neurons were lysed and the collected cellular protein was separated on a polyacrylamide gel and the level of CREB phosphorylation was analyzed via Western blot analysis using a phospho-specific antibody. As indicated in figure 4.2, our findings show that while CaMK inhibition alone has no effect on basal levels, addition of KN-62 in the presence of treatment with BDNF is indeed sufficient to abrogate CREB phosphorylation levels in these neurons. This is, of course, not surprising given the findings of Finkbeiner et al., 1997, noted above. However, to determine whether this pathway is in fact relevant to the BDNF priming effect, we isolated cerebellar neurons once again, primed them with BDNF in the presence or absence of the inhibitor, KN-62, and then performed the neurite outgrowth assay. In agreement with the CREB phosphorylation data, we found that blocking the activity of CaMK does indeed interfere with the BDNF-induced priming effect (figure 4.3).

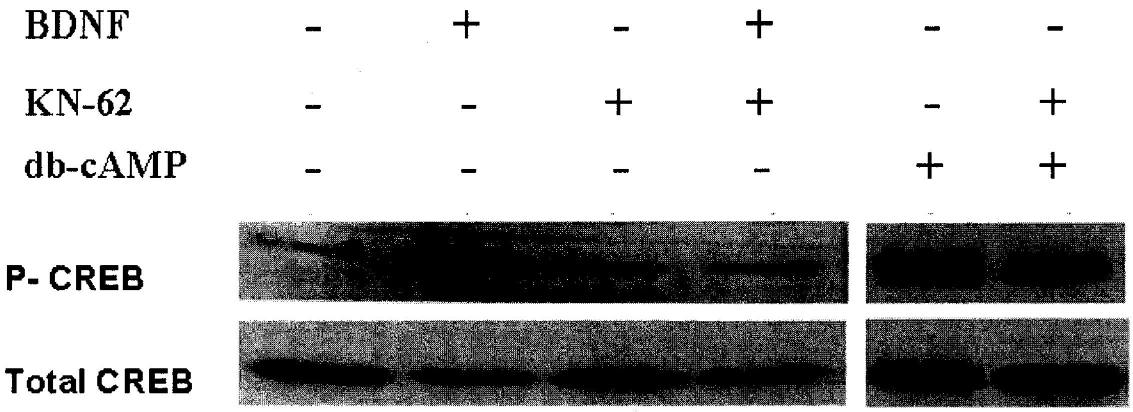


Figure 4.2: Phosphorylation of CREB via Treatment with cAMP-elevating Agents. P5-9 rat cerebellar neurons were treated for 30 minutes with either BDNF or db-cAMP in the presence or absence of 10μM KN-62. Cells were then lysed and phosphorylated CREB was identified by PAGE and Western blot analysis.

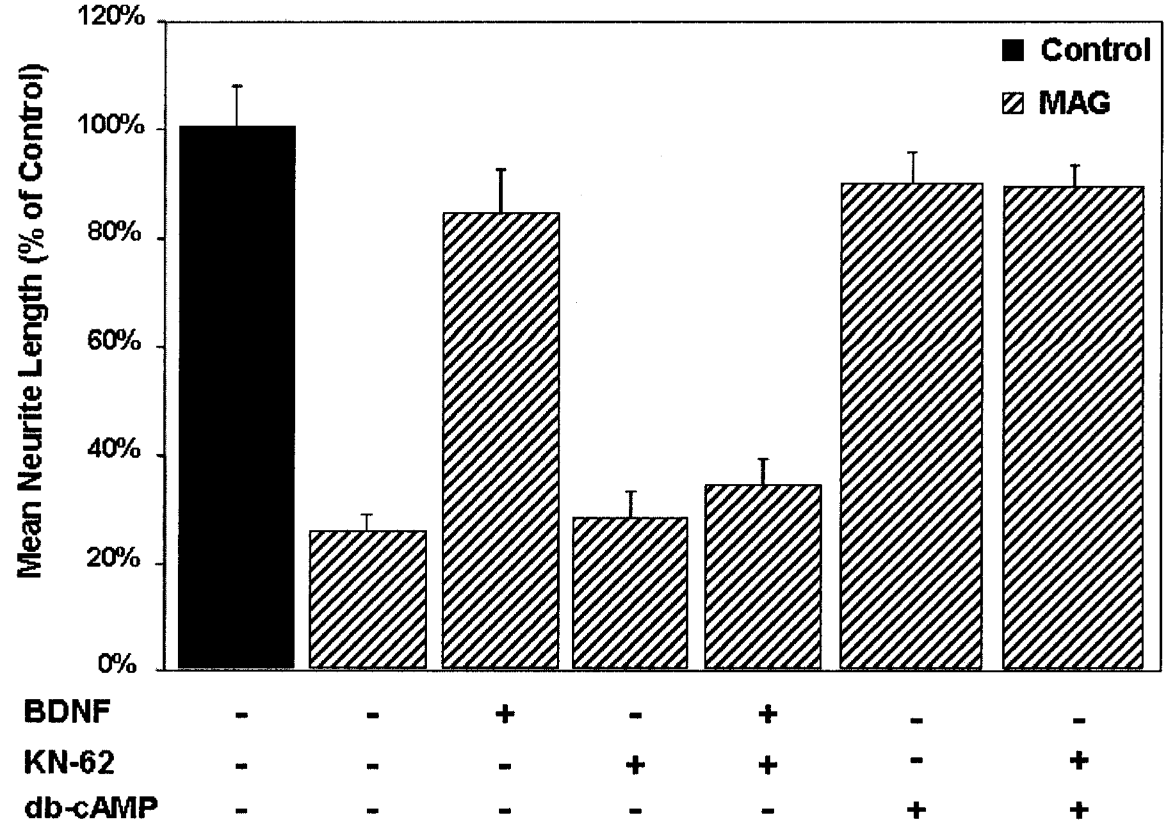


Figure 4.3: CaMK Signaling is Necessary for the BDNF-induced Priming Effect but not the db-cAMP Effect. P5-9 rat neurons were isolated and either primed with BDNF and/or KN-62 overnight or plated directly onto the CHO cell monolayers and treated with db-cAMP and/or KN-62. Neurons were stained for βIII tubulin and the length of the longest neurite per neuron was measured for 150-200 neurons.

These findings suggest that CaMK activation is indeed necessary for priming of the neurons, but how and where does this fairly promiscuous enzyme fit in the signaling pathway? In order to begin answering this question, we first sought to investigate whether CaMK is also necessary for the specific cAMP-dependent regeneration-promoting signaling pathway. For this purpose, we performed the same CREB phosphorylation assay illustrated above to examine if inhibition of CaMK was also sufficient to block the effects of direct cAMP elevation via addition of the chemical analogue, db-cAMP. Cerebellar neurons were once again isolated from post-natal rat pups and treated with 1mM db-cAMP in the presence or absence of KN-62, lysed, separated by PAGE and probed for phosphorylated CREB. To our surprise, however, the inhibition of CaMK was, in fact, not able to block or even reduce the induction of CREB phosphorylation by this method (figure 4.2). To confirm this finding and to determine if this has functional relevance to the cAMP-dependent axonal regeneration, we performed the neurite outgrowth assay in the presence of db-cAMP and the CaMK inhibitor. As expected from the CREB phosphorylation data, application of KN-62 had no effect on the regeneration-promoting effects of db-cAMP (figure 4.3).

This intriguing finding led us to further investigate whether inhibition of CaMK had any effect on axonal outgrowth in a scenario where cAMP levels were naturally elevated. Embryonic CNS neurons are known to be able to spontaneously regenerate axons *in vitro* or *in vivo* following injury. Previous work from our lab has shown that the reason for this spontaneous regeneration is that embryonic neurons have much higher endogenous cAMP levels than do their post-natal counterparts and that these elevate levels temporally correspond with this switch in responsiveness to the myelin-associated inhibitors (Cai et al., 2001). For most neurons, this switch has occurred by birth, however, dorsal root ganglion neurons switch their responsiveness (and show a decrease in endogenous cAMP levels) postnatally, at about day 3 or 4 after birth. Therefore, to test whether CaMK inhibition can block the improved *in vitro* axonal outgrowth of these perinatal neurons, we isolated and dissociated DRG neurons from post-natal day 1 rat pups and plated them onto MAG-expressing CHO cells in the presence or absence of CaMK inhibitor. As indicated in figure 4.4, inhibition of CaMK signaling had no effect on the observed growth of DRG axons in the presence of the inhibitor.

While these experiments provide evidence to suggest that a calcium/calmodulin-dependent kinase is indeed involved in the neurotrophin-induced priming of primary neurons, it remains unclear if this action is specific to the enzyme CaMKIV. The pharmacological inhibitor that had been used thus far, KN-62, is not specific in its activity for CaMKIV, as it potently inhibits both CaMKII and CaMKIV. Both of these enzymes can be found in adult CNS neurons and could potentially account for the observed effects. In order to distinguish if CaMKIV is in fact the CaMK moiety which is mediating the priming effect, we generated adenoviral vectors containing one of the following: a control vector, a CaMKIV dominant-negative mutant (kinase-dead) and a constitutively-active CaMKIV mutant. For each of these vectors, green fluorescent protein (GFP) was used as the reporter (figure 4.5).

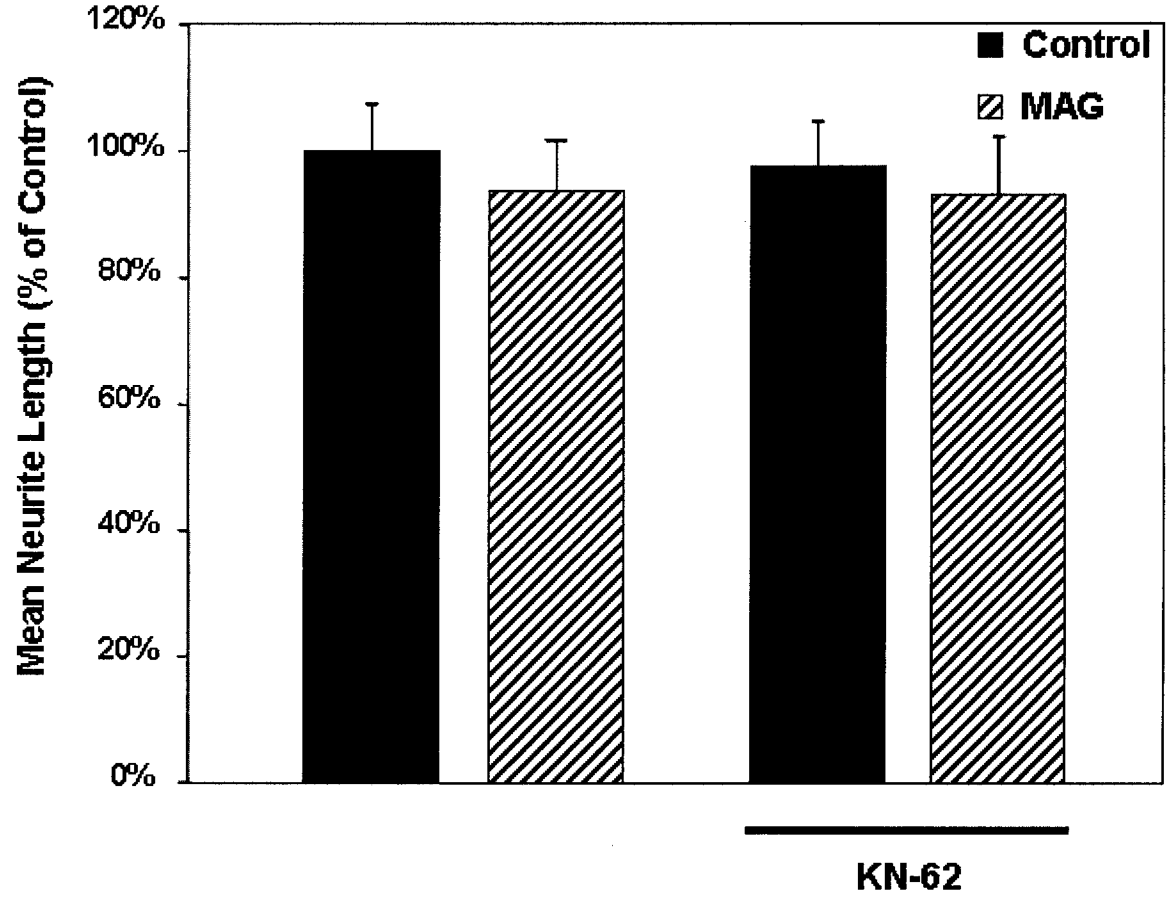


Figure 4.4: CaMK Signaling is Not Necessary for Regeneration in Young Neurons. P0-1 rat DRG were isolated and plated onto the CHO cell monolayers in the presence or absence of 10μM KN-62. Neurons were stained for βIII tubulin and the length of the longest neurite per neuron was measured for 150-200 neurons.

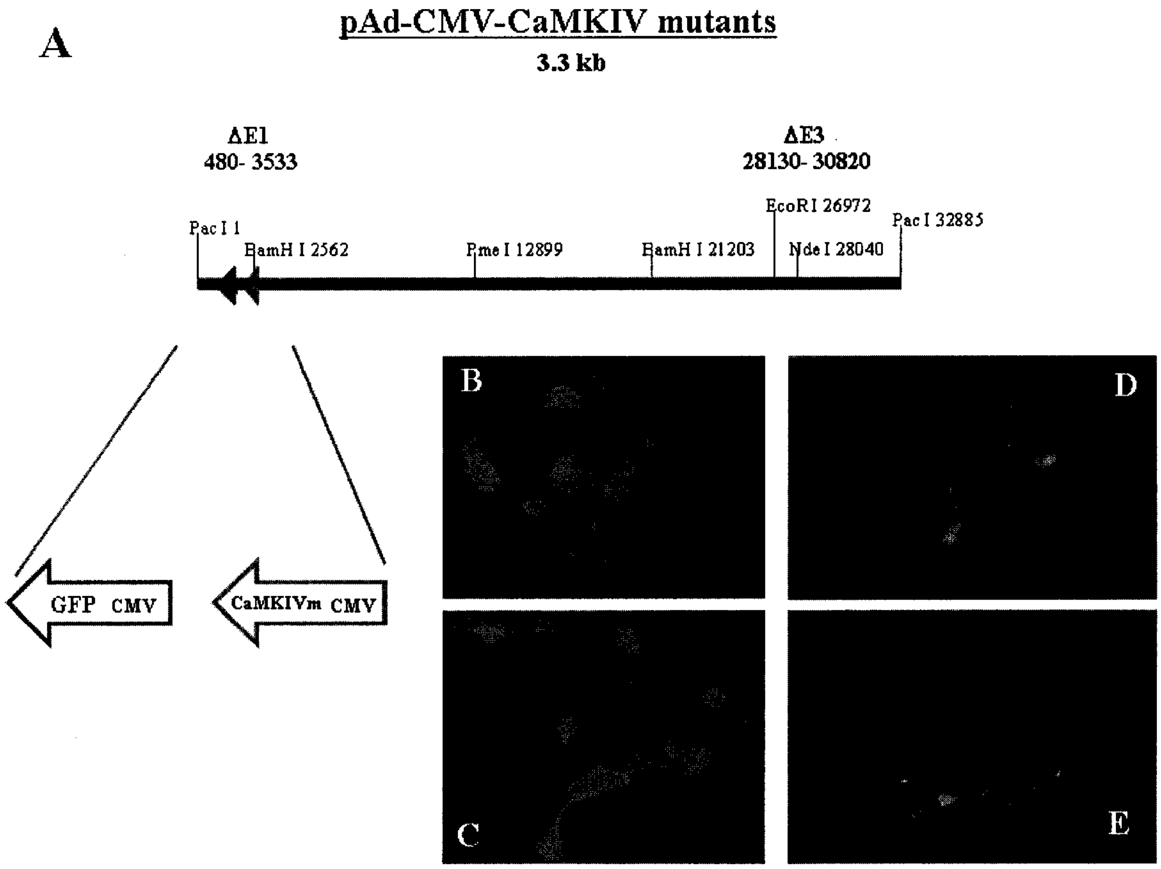

A
pAd-CMV-CaMKIV mutants
3.3 kb
ΔE1
480- 3533
ΔE3
28130- 30820
EcoRI 26972
PacI 32885
PacI 1
BamH I 2562
Pme I 12899
BamH I 21203
NdeI 28040
GFP CMV
CaMKIVm CMV
B
D
C
E

Figure 4.5: The CaMKIV Mutant Adenoviral Vector. The pAd-CMV-CaMKIV vector consists of a 3.3kb plasmid which contains CaMKIV mutant constructs, GFP (both under CMV promotion) and the Ad5 genome minus the E1 and E3 genes (A). Infection of these viruses was first performed in COS cells and GFP expression observed for both GFP control virus (B) and CaMKIV mutant virus (C). Finally, infection of neurons was performed in P5-9 DRGs for both GFP controls (D) and CaMKIV mutant virus (E).

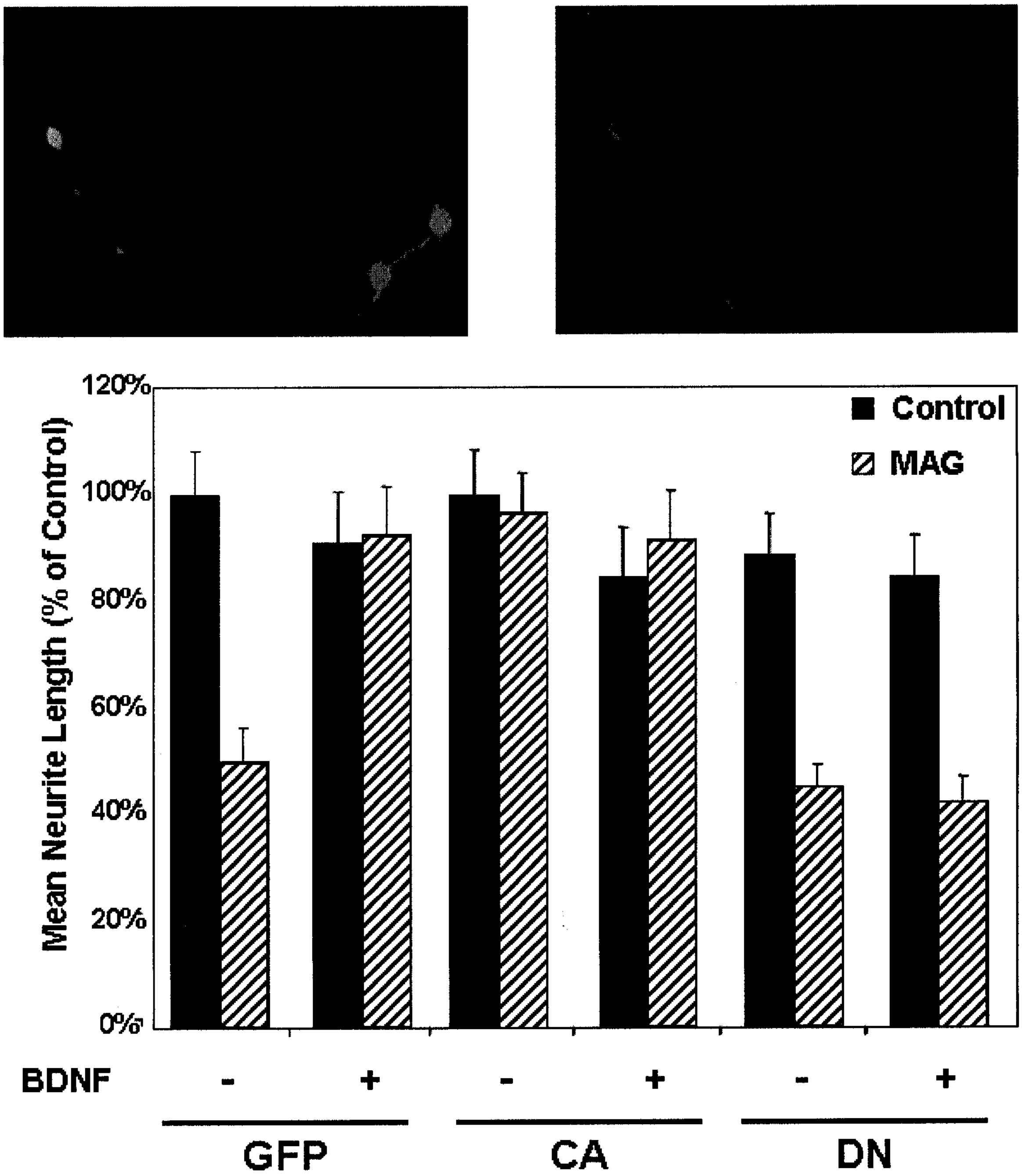


Figure 4.6: CaMKIV Activity is Necessary for the Neurotrophin-induced Priming Effect. P5-9 DRG neurons were isolated and plated onto poly-L-lysine-coated plates and infected with 100 Fu/cell of either control (GFP alone) or constitutively-active (CA) or dominant negative (DN) CaMKIV-containing adenoviral vector. After infection and incubation to allow for gene expression, the neurons were primed with 200ng/ml BDNF as indicated, plated onto CHO cell monolayers and stained for βIII tubulin. The length of the longest neurite per neuron was measured for 150-200 double stained neurons.

DRG neurons from post-natal day 5-8 rat pups were collected, dissociated, plated onto poly-L-lysine-coated plates and infected with the viral vector. After allowing for infection and expression of the exogenous protein, these DRG were then primed with 200ng/ml BDNF overnight and then plated onto MAG-expression CHO cells. Infection of DRG neurons with the control (GFP alone) virus had no effect of either inhibition by MAG or on control growth (figure 4.6). However, neurons infected by the CaMKIV dominant-negative-containing virus failed to grow long neurites on MAG, even following priming (figure 4.6). This confirms that the CaMK enzyme that is mediating the neurotrophin-induced priming effect is indeed CaMKIV. Interestingly, as further indicated in figure 4.6, neurons infected with the constitutively-active CaMKIV mutant were able to grow long processes on MAG even in the absence of priming with neurotrophins or db-cAMP .

Thus, it seems evident that the activity of CaMKIV is necessary for the neurotrophin-induced priming effect, but is dispensable once cAMP levels have already been elevated.

4.3 Discussion

In this chapter we present data which suggests a role for Calcium/Calmodulin-dependent kinase IV (CaMKIV) in the BDNF, but not db-cAMP, induced phosphorylation and activation of CREB and the resultant block of MAG and myelin-mediated inhibition of neurite outgrowth. Previous work from our lab has shown that blocking either PKA or

ERK also blocks both the neurotrophin priming-induced and db-cAMP-induced CREB phosphorylation and block of axonal growth inhibition, suggesting that their effects are downstream of the signaling step which results in the elevation of endogenous cAMP levels. However, CaMKIV inhibition has no effect on these phenomena once cAMP levels have been artificially elevated (via application of db-cAMP). Furthermore, while the discovery that a dominant-negative CaMKIV mutant blocks the priming effect was expected given the pharmacological inhibitor data, the finding that a constitutively-active mutant CaMKIV can effectively mimic the priming effect, even in the absence of exogenous treatment was significantly more surprising. One hypothesis for why a constitutively-active mutant may be sufficient to mimic priming may lie in the unique nature of the CaMKIV enzyme. CaMKIV can be activated by either Ca^{++}-Calmodulin binding, CaMKK-mediated phosphorylation or both. Activation by means of CaMKK-mediated phosphorylation can lead to prolonged and increased CaMKIV activation, even in the absence of elevated calcium levels and may be responsible in certain cellular circumstances to induce gene transcription (Soderling, 1999; Tokumitsu et al., 1995). In addition, it has been proposed that autophosphorylation by CaMKIV may mediate the availability of CaMKIV as a substrate for CaMKK (Chatila et al., 1996). Thus, overexpression of a constitutively-active CaMKIV moiety may mimic this calcium-independent activation and signaling which may facilitate further activation of endogenous CaMKIV by CaMKK due to relief of inhibition by autophosphorylation.

These findings concur with the work of Finkbeiner and colleagues who found that CREB activation and transcription can be induced in cortical neurons by application of BDNF and that this effect is CaMKIV dependent (Finkbeiner et al., 1997). Furthermore, this previously published work also suggests that the BDNF-induced signaling may bifurcate and regulate both CaMKIV and ERK-dependent signaling, as we find is also the case in the neurotrophin-induced priming effect.

Interestingly, results from our lab have shown that if we block any one of the PKA, ERK or CaMKIV signaling pathways, we lose the priming effect (but only PKA and ERK are necessary for the cAMP-specific effect). Therefore, we propose that it may be possible that a "threshold" level of CREB activation and subsequent transcriptional upregulation may be necessary for the observed effect to proceed. Thus, consistently activating one of these pathways—such as with the use of the non-hydrolysable db-cAMP or with the constitutively-active CaMKIV—may, alone, be sufficient to induce the improved axonal growth in the presence of inhibitors that we observe following priming with neurotrophins.

These findings, while surprising, provide some insight into the precise "location" of CaMKIV in this regeneration-promoting signaling pathway and opens the door for further investigation of this pathway: it is not, as is the case with PKA and ERK, downstream of cAMP, so therefore, it must be either upstream or working in parallel. The elucidation of precisely where CaMKIV fits is the subject of the following chapters of study.

Chapter V: Characterizing the CaMKIV Signaling Cascade

5.1 Introduction

Pre-treatment of primary neurons with neurotrophins is sufficient to overcome the axonal growth inhibition induced by the myelin-associated inhibitors in a PKA, ERK and, as shown by the results of the previous chapter, CaMKIV-dependent manner. PKA is a direct downstream effector of elevated cAMP levels and recent work by our lab has shown that ERK can act as both a CREB activator as well as an inhibitor of phosphodiesterase 4 (Gao et al., 2003). However, the finding that CaMKIV inhibition fails to block the db-cAMP-induced axonal growth on MAG begs the question: where, precisely, does CaMKIV fit in the neurotrophin-induced priming pathway?

The calcium/calmodulin-dependant kinases (CaMKs) have been shown to mediate many molecular signaling events in the CNS (figure 5.1) including: activation of certain adenylyl cyclases (Shaywitz and Greenberg, 1999; Wong et al., 1999), phosphorylation and activation of the cAMP response element binding protein (CREB) (Bito et al., 1996; Matthews et al., 1994; Sun et al., 1994), induction of MAPK signaling (Enslen et al., 1996) and activation of the transcriptional coactivator, CREB binding protein (CBP) (Chawla et al., 1998; Hu et al., 1999). These mechanisms are believed to mediate a variety of neuronal/brain functions such as synaptic transmission (Hardingham et al., 2001b), long-term potentiation (Bach et al., 1995; Giese et al., 1998; Mayford et al., 1996; Silva et al., 1992a; Silva et al., 1992b), synaptic plasticity and, of course, learning and memory (Shaywitz and Greenberg, 1999) and all of these mechanisms may be relevant in the neurotrophin-induced priming affect. Thus, in order to simplify the identification of the role of CaMK in this signaling cascade, we sought to answer whether or not CaMK is necessary for the induction of the known downstream components cAMP, ERK and CREB.

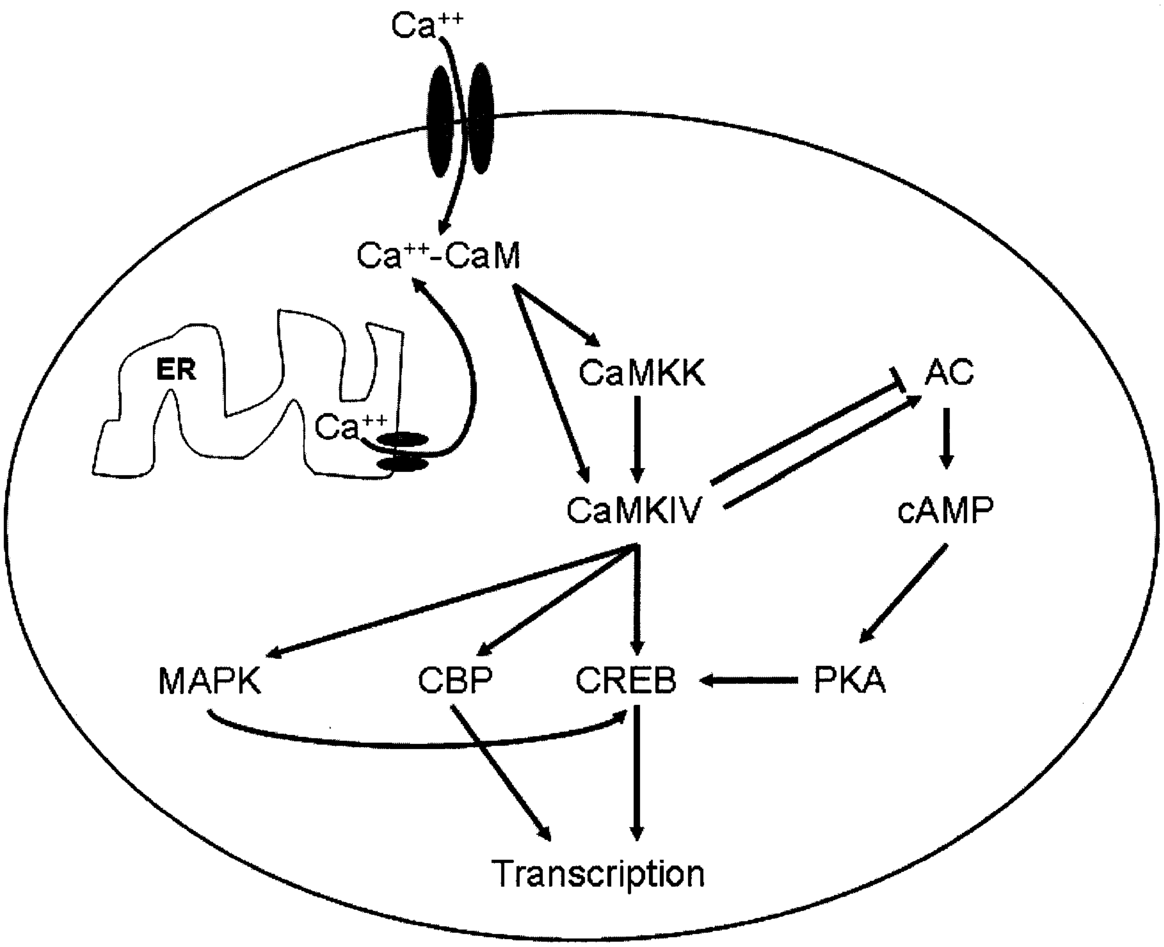


Figure 5.1: The Diversity of CaMKIV Signaling. Calcium-Calmodulin-dependent Kinase IV has been shown to have many potential downstream targets and while its effectors appear to be diverse, they all seem to converge at the induction of gene transcription in the nucleus. Adapted from (Soderling, 1999).

5.2 Results

Data from the previous chapter suggests that CaMK is indeed responsible for CREB phosphorylation and activation, but is this a direct or indirect effect? One potential target for CaMK signaling is adenylyl cyclase, the enzyme which produces cAMP. Perhaps CaMK-induced activation of adenylyl cyclase, when coupled with ERK-dependent PDE

4 inhibition, leads to the observed increase of intracellular cAMP levels after treatment with neurotrophins. To answer this question, we isolated cerebellar and DRG neurons from post-natal day 5-9 rat pups, treated them with 200ng/ml BDNF in the presence or absence of the CaMK inhibitor, KN-62, for 30 minutes, lysed them and quantitated the cAMP levels using a competitive immunoassay (BIOMOL). As indicated in figure 5.2, inhibition of CaMK signaling via simultaneous application of 10μM of the inhibitor KN-62 was not sufficient to completely block the BDNF-induced elevation of cAMP levels. While a slight effect may be observed here, it must be noted that the induction of cAMP was still significantly higher than in the untreated controls.

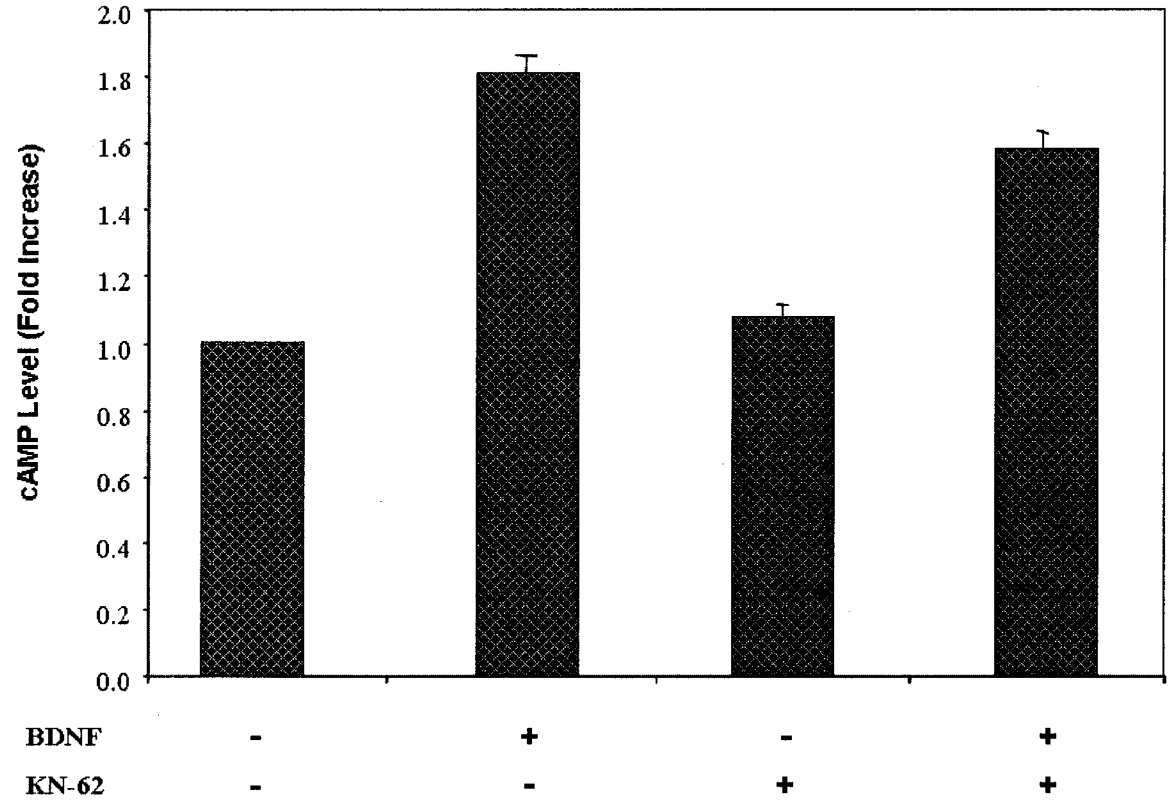


Figure 5.2: CaMK Activity is Not Necessary for the BDNF-induced Elevation of cAMP. P5-9 rat DRG neurons were isolated and treated with 200ng/ml BDNF in the presence or absence of 10μM KN-62 for 30 minutes prior to lysis in 0.1M HCl. Endogenous cAMP levels were assayed immediately using the BIOMOL competitive immunoassay kit.

While CaMK does not appear to affect the cAMP elevation it is perhaps possible that its role lies in the activation of ERK signaling. To address this possibility, we treated primary neurons with 200ng/ml BDNF once again, lysed the cells and analyzed, via Western blot, the induction of ERK phosphorylation. Figure 5.3 shows that in response to BDNF treatment, there is an increase in total ERK phosphorylation, but this effect is not abrogated by application of the CaMK inhibitor, KN-62. Thus, the role of CaMK appears not to be either the induction of adenylyl cyclase activation or other means of cAMP elevation, nor does it appear to be mediating the activation of ERK signaling.

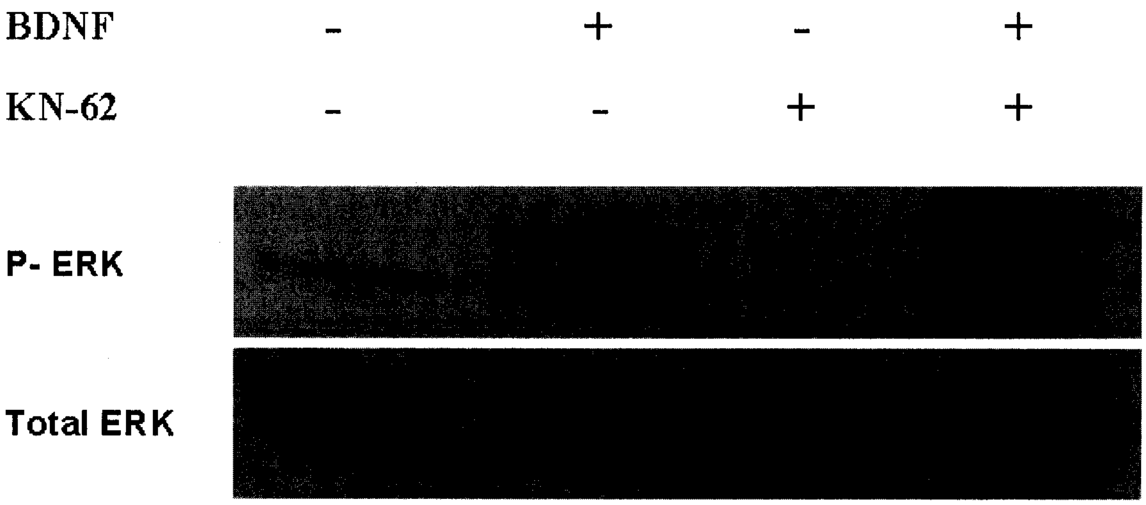


Figure 5.3: CaMK Inhibition has no Effect on ERK Phosphorylation by BDNF. P5-9 rat cerebellar neurons were isolated and treated with 200ng/ml BDNF in the presence or absence of 10μM KN-62 for 30 minutes. After treatment, cells were lysed and protein was separated by PAGE and phosphorylated ERK levels were determined by Western blot analysis.

If, as the findings of the past two chapters suggest, CaMKIV is neither downstream nor upstream of cAMP elevation, then it must lie in a parallel, but still necessary, signaling pathway. With this being the case, we sought to examine precisely how CaMKIV is activated in response to treatment of neurons with neurotrophins. The first question we asked is if intracellular calcium levels are in fact altered by treatment of DRG neurons with BDNF. In addition, it has been previously shown that the mode of avenue of calcium flux can often determine the intracellular signaling response (Berridge, 1998; Hardingham et al., 2001a; Hardingham et al., 2001b). First, in order to verify if treatment of primary DRG neurons does, in fact, induce calcium flux, we, in collaboration with Dr. George Holz at NYU Medical School, treated DRG neurons with 200ng/ml of BDNF and observed the intracellular calcium flux via Fura-2 imaging. As indicated in figure 5.4, it is evident that there is a significant increase in intracellular calcium levels in response to BDNF that exhibits a longer latency and persistence than does induction of voltage-gated calcium flux (as observed in KCl treated neurons), suggesting a signaling-induced mechanism, perhaps from intracellular stores.

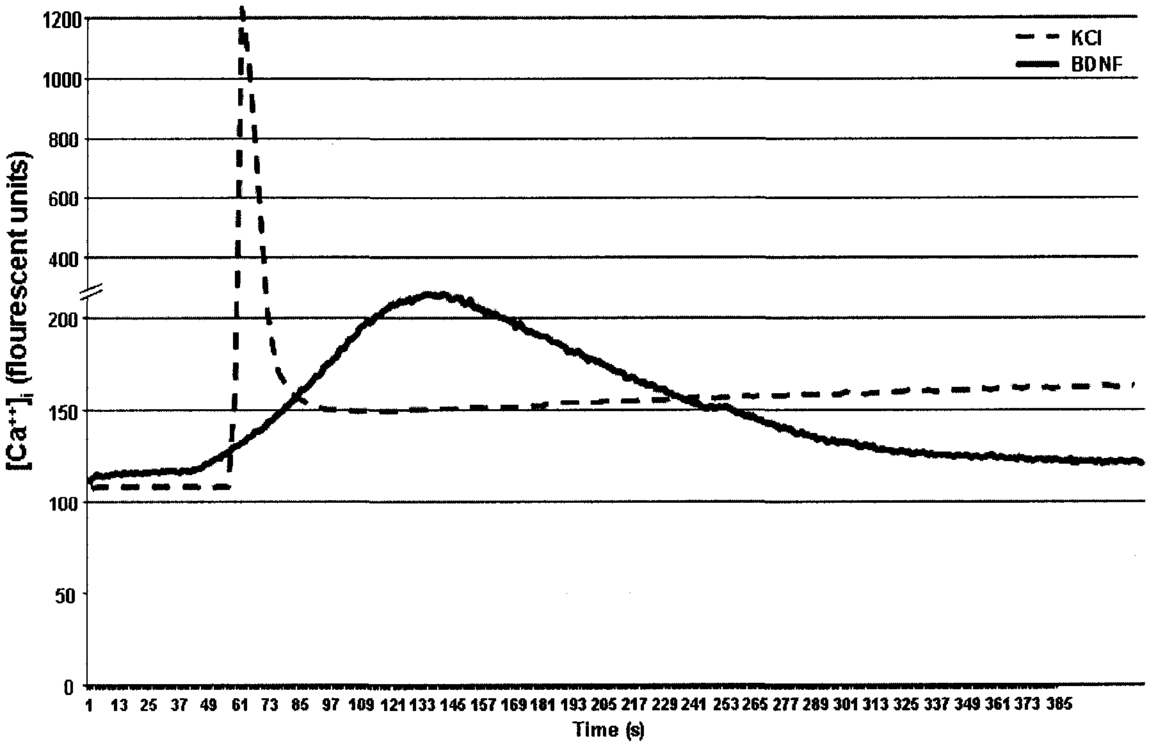


Figure 5.4: BDNF Induces Intracellular Calcium Flux with Long Latency in Primary DRG Neurons. P5-9 rat DRG were isolated, plated onto glass coverslips and loaded with Fura-2 for 1 hour at room temperature. Treatment with 50mM KCl or 200ng/ml BDNF was applied immediately prior to calcium level measurements.

To further this possibility and to elucidate specifically which mode of calcium flux is responsible for the activation of CaMKIV, DRG neurons from post-natal day 5-9 rat pups were primed once again with 200ng/ml BDNF in the presence or absence of inhibitors of calcium flux. In agreement with the calcium imaging data, we found that of the inhibitors tested, only 2-Aminoethyl diphenyl borate (2-APB), an inhibitor of calcium release from IP_3 channels, was able to block both the CREB phosphorylation (figure 5.5) and the improvement of axonal regeneration *in vitro* (figure 5.6) which results from BDNF treatment.

BDNF	-	+	+	+	+
2-APB (IP$_3$)	-	-	+	-	-
APV (NMDA)	-	-	-	+	-
Nimodipine (L-Type)	-	-	-	-	+

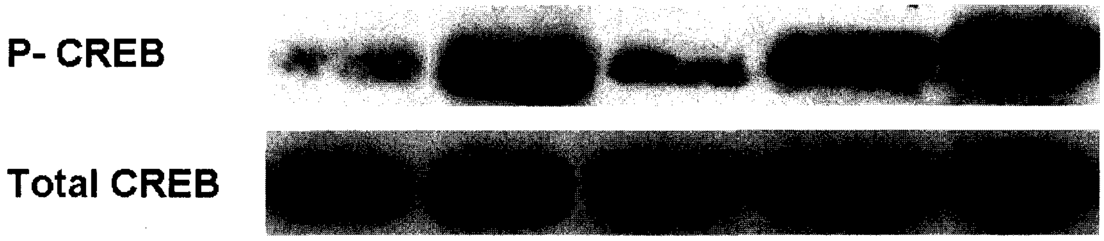
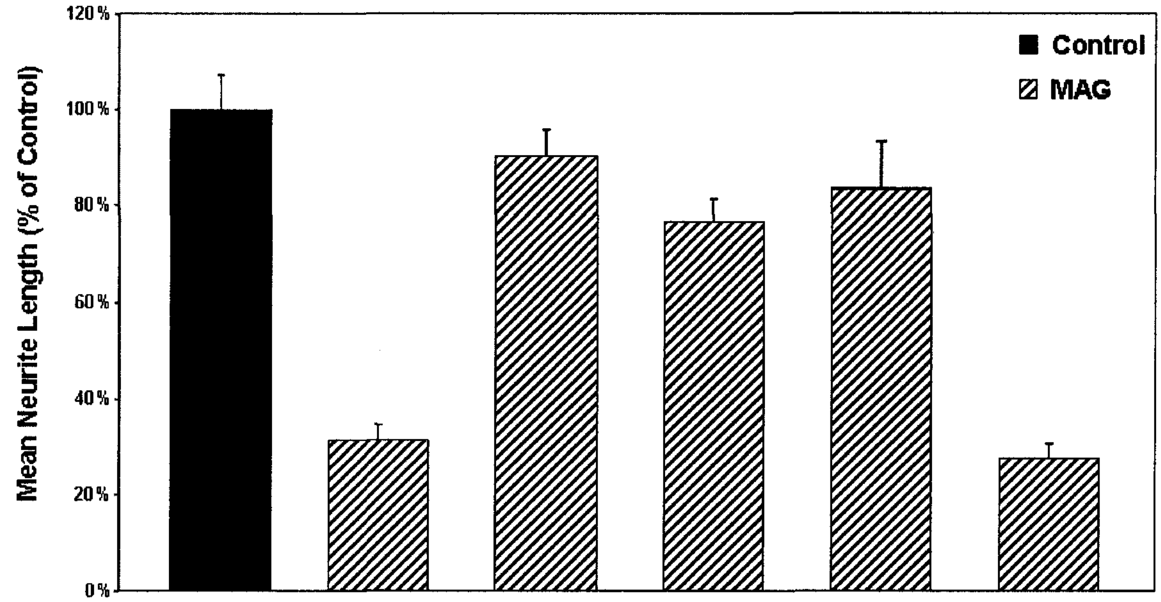


Figure 5.5: Effect of Blocking Calcium Flux on the BDNF-induced CREB Phosphorylation. P5-9 rat cerebellar neurons were isolated and treated with 200ng/ml BDNF in the presence or absence of each of the calcium flux inhibitors indicated (the mode of flux that each inhibits is indicated in the parentheses) for 30 minutes prior to lysis. Protein was separated by PAGE and phosphorylated CREB levels were determined by Western blot analysis.



BDNF	-	-	+	+	+	+
APV	-	-	-	+	-	-
Nimodipine	-	-	-	-	+	-
2-APB	-	-	-	-	-	+

Figure 5.6: Effect of Blocking Calcium Flux on the BDNF-induced Priming Effect. P5-9 rat DRG were isolated and primed with 200ng/ml BDNF in the presence or absence of each of the calcium flux inhibitors indicated. The primed neurons were then transferred to CHO cell monolayers and incubated a further 18 hours. Neurons were fixed and stained for βIII tubulin and the length of the longest neurite per neuron was measured for 150-200 neurons.

5.3 Discussion

The induction of the improved axonal growth on the inhibitory substrate observed in neurons which have been primed with neurotrophins appears to occur in two stages. The first is a rapid induction of the primary signaling components such as cAMP, PKA and ERK. To this list, we can now add the calcium/calmodulin-dependent kinase. However, while we believe we know precisely how and where these other components fit in the signaling cascade, the role of CaMKIV appears to be a bit more enigmatic. It does not seem to mediate the BDNF-induced elevation of cAMP levels. And while there appeared to be a slight decrease in total cAMP levels in KN-62 treated cells as compared to BDNF treatment alone, we can surely conclude that CaMKIV activation is not solely or even significantly responsible for the observed increase in cAMP levels. In addition, CaMKIV activation in response to BDNF is not necessary for the phosphorylation and activation of the extracellular signal regulated kinase (ERK), itself a necessary step in the activation of CREB, cAMP elevation and the block of MAG-mediated inhibition of axonal regeneration. Thus, while we have not yet pinpointed the precise position and role of CaMKIV in the neurotrophin-induced priming effect, we can say that it is not upstream of cAMP elevation and nor is it downstream of cAMP elevation, but rather, must exist in a separate and parallel pathway which leads from BDNF binding to the Trk B receptor to the phosphorylation and activation of CREB and the subsequent induction of transcription. We can, however, say that the induction of calcium flux in response to treatment of neurons with BDNF is dependent on flux from intracellular stores and that this pathway is necessary for both CREB phosphorylation and the improved axonal growth on an inhibitory substrate (figure 5.7). This mode of flux appears to be in agreement with previous work from Michael Greenberg's lab which suggests that treatment of cortical neurons with BDNF can induce CREB activation in a PLCγ-dependent manner (Finkbeiner et al., 1997). If indeed PLCγ is activated and necessary in the priming paradigm, we would then expect calcium flux to be via IP_3 channel-mediated release from intracellular stores.

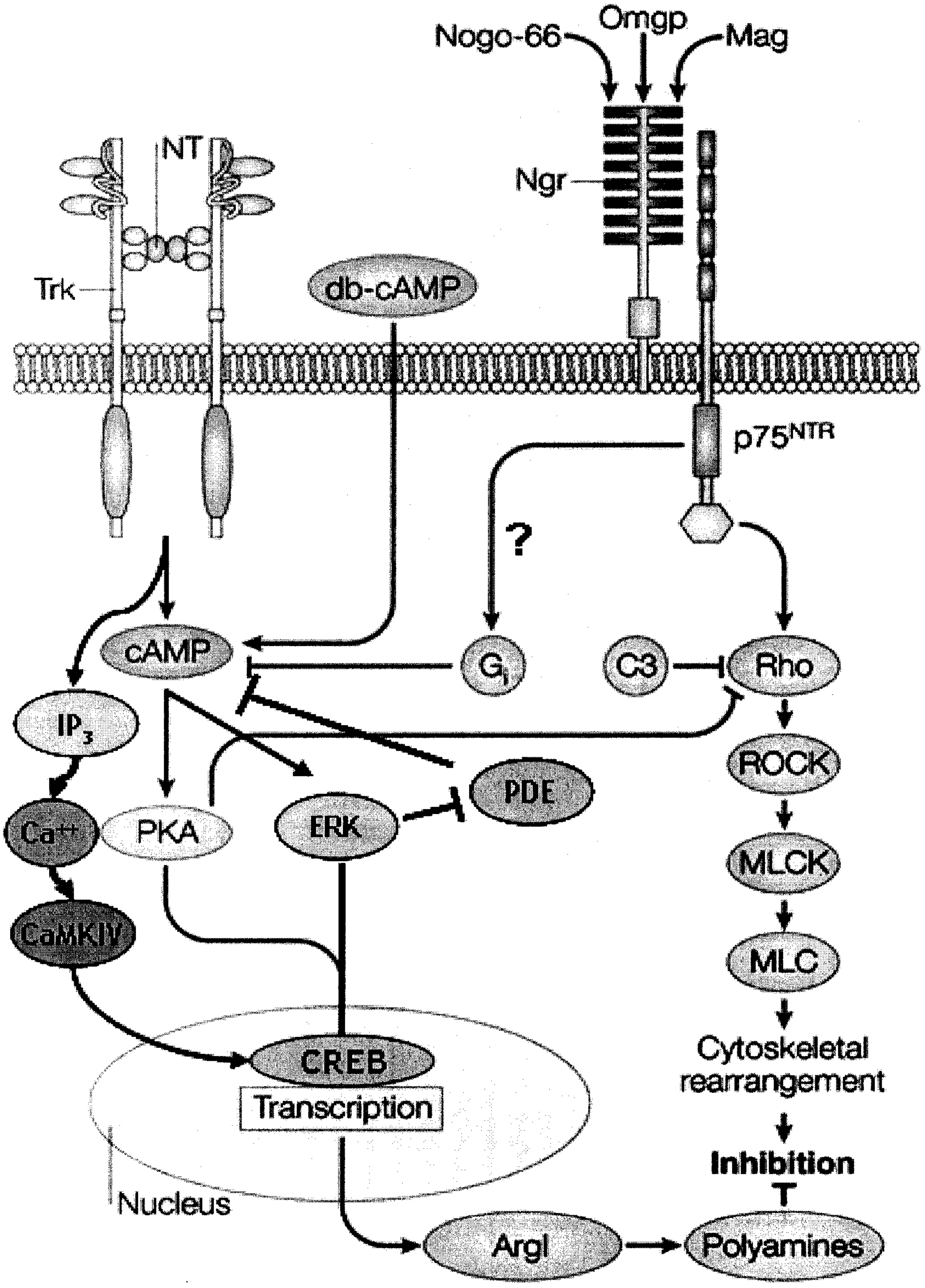

Nogo-66
Omgp
Mag
NT
Ngr
Trk
db-cAMP
p75^{NTR}
?
cAMP
G_i
C3
Rho
IP$_3$
ROCK
PDE
Ca++
PKA
ERK
MLCK
CaMKIV
MLC
Cytoskeletal rearrangement
CREB
Transcription
Inhibition
Nucleus
Argl
Polyamines

Figure 5.7: The "New" Priming Signaling Cascade. The neurotrophin-induced priming effect involves several key signaling components, including release of calcium from intracellular stores and activation of CaMKIV.

Recently published work by Henley and colleagues suggests that MAG, when acting as a repulsive axonal guidance cue, can also induce calcium flux from intracellular stores (Henley et al., 2004). While this may suggest a theoretical contradiction, there are several key differences in the mode of activity of these two phenomena. Firstly, Henley et al find that this flux occurs exclusively in the growth cone and is limited to the side of the cone nearest the MAG gradient and, thus, appears to be a localized effect more likely to regulate cytoskeletal rearrangement than cellular signaling. Furthermore, growth cone guidance effects occur on the order of minutes rather than hours as is the case with axonal regeneration and thus, it is likely that the guidance effects are acute and may be independent of gene transcription. While the difference in the inhibitory and guidance effects of MAG on axons appears to be that of acute versus chronic responses, both depend on intracellular calcium levels and cAMP concentrations (Henley et al., 2004; Hong et al., 2000; Ming et al., 1997; Ming et al., 2002a; Song et al., 1997) and, thus, may indeed be related phenomena.

Ongoing work in the lab will address precisely which signaling elements are involved in inducing this effect. My current efforts are centering on the most obvious target, the aforementioned enzyme phospholipase C, which is a known mediator of the IP_3-dependent signaling pathway.

Chapter VI: Characterization of Embryonic Stem Cell-derived Motor Neurons and Inhibition by MAG

6.1 Introduction

Injuries to the adult mammalian CNS which result from either insult or diseases such as Amyotrophic Lateral Sclerosis (ALS) often result in a selective loss of motor neurons and pathways. Many current studies focus on elucidating the mechanisms associated with this selective neuronal loss. However, once disease onset has begun or after CNS injury, it is also important to investigate methods by which the damaged motor pathways can be repaired or replaced. Recent advances in the isolation, culturing and engrafting of embryonic stem cells have suggested a new avenue for therapeutic intervention in the treatment of these maladies, neuronal replacement. Work from the lab of Thomas Jessell suggests that this may indeed be possible. ES cells derived from mouse embryos can be induced to differentiate into motor neurons in culture (Wichterle et al., 2002) and can be subsequently engrafted *in vivo*. However, the replacement of lost neurons is only one step in achieving complete functional recovery. These engrafted MNs must be able to extend axons through the CNS and PNS environments and synapse with their proper targets.

It is known that damaged axons fail to regenerate following injury to the adult mammalian CNS. This is due, in part, to the presence of the inhibitors of regeneration which are found in CNS and PNS myelin. While regeneration is common in the PNS, this may be due to the removal of myelin debris which occurs post-injury and not due to differences in the components of CNS and PNS myelin. The retarded regeneration observed in the Wld^S mice, a mouse which exhibits slowed Wallerian degeneration (Brown et al., 1992), suggests that this may, in fact, be the case. In order to investigate the potential of therapeutic motor neuron replacement, we first wanted to address whether motor neurons derived from ES cells were inhibited by the myelin-associated inhibitors of regeneration in the same way that both native and cultured sensory neurons are. Evidence from our laboratory indicates that these MNs are, in fact, inhibited by both myelin-associated glycoprotein (MAG) and purified myelin *in vitro*. Therefore, we propose that in order for the engrafting of ES cell-derived MNs to be used as a therapy for the replacement of motor neurons lost to ALS onset, they must be encouraged to regenerate their axons in the inhibitory environment of CNS and uncleared PNS myelin. Currently, there are two approaches to overcoming inhibitors in myelin and encouraging regeneration *in vitro* and *in vivo* being utilized. The first method involves negating the inhibitory environmental signals by blocking the individual inhibitors or their common receptor with antibodies and/or peptides. The second involves changing the intrinsic growth state of the neuron such that it no longer recognizes the environment as inhibitory (reviewed in Spencer and Filbin, 2004). In examining this second approach, previous work from our lab (Qiu et al., 2002) and others (Neumann et al., 2002) has shown that elevating intracellular cAMP levels or activation of a downstream component of the signaling pathway which is subsequently induced (e.g. Arginase I- (Cai et al., 2002) allow sensory neurons to regenerate both *in vitro* and *in vivo*.

Thus, to examine the viability of utilizing motor neurons differentiated from embryonic stem cells as a potential "replacement" therapy in the CNS, we sought to characterize these neurons with respect to their signaling similarities to endogenous neurons and their regenerative potential when exposed to the myelin-associated inhibitors of axonal regeneration.

6.2 Results

The first step in determining the value of using ES-derived motor neurons is to investigate whether these cells are actually inhibited by MAG and/or myelin. To do this, we utilized embryonic stem cells derived from a transgenic mouse which expresses green fluorescent protein (GFP) under the Hb9 promoter (a ventral spinal motor neuron-specific marker). ES cells were grown and differentiated according to the protocol described by Wichterle and colleagues (Wichterle et al., 2002) (figure 6.1). Differentiated motor neurons were identified by GFP expression. These motor neurons were then dissociated and plated onto substrates of MAG-expressing CHO cells or purified myelin and analyzed for axonal extension. In these experiments, we found that motor neurons derived from ES cells are indeed inhibited by both MAG and myelin (figure 6.2). While these neurons do appear to be inhibited by myelin, we also asked whether this may be working via the same receptor complex as is seen for primary neurons. Indeed, these motor neurons do appear to express the primary inhibitory receptor, NgR, on the cell surface following differentiation (figure 6.3).

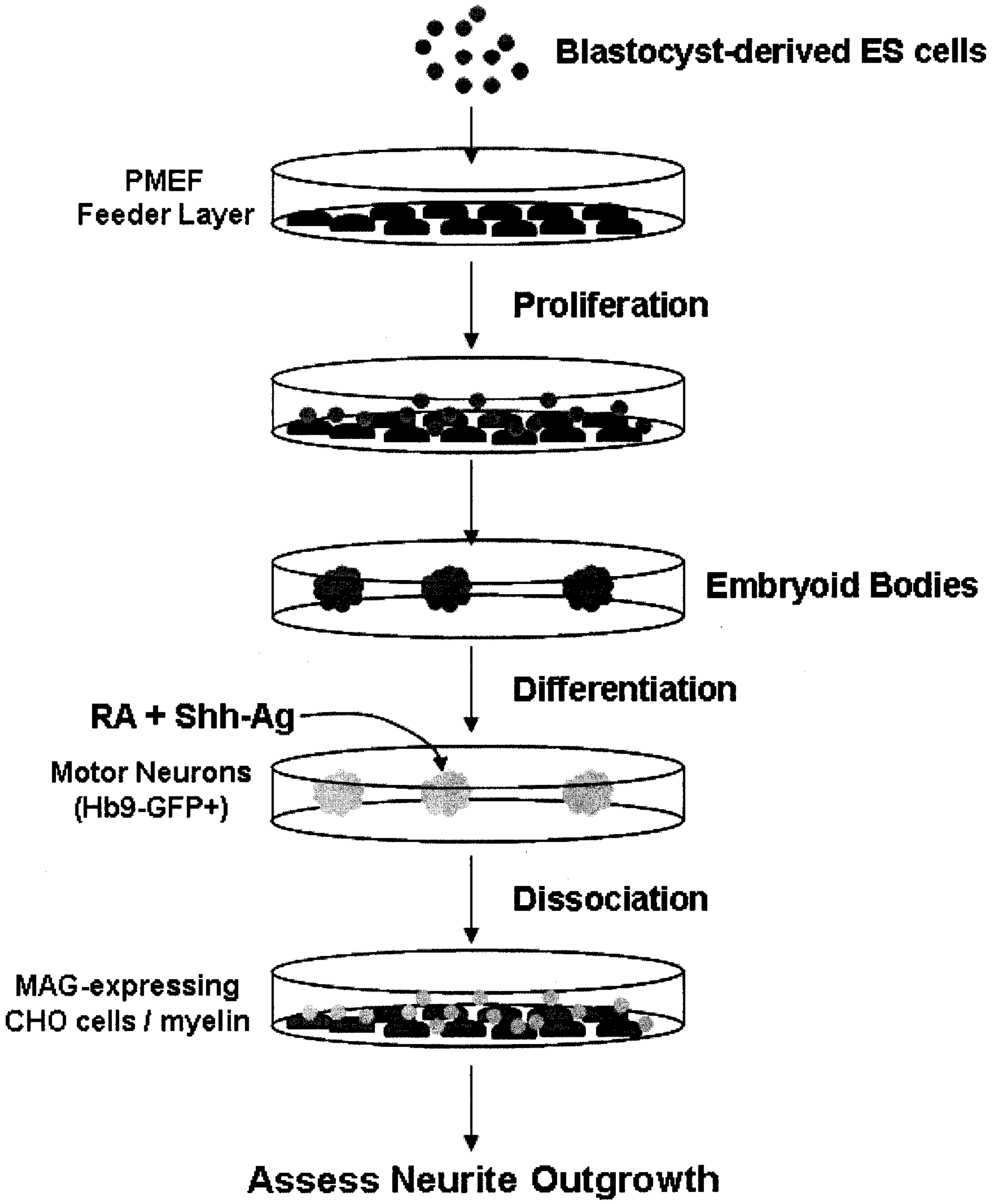

Blastocyst-derived ES cells
PMEF
Feeder Layer
Proliferation
Embryoid Bodies
Differentiation
RA + Shh-Ag
Motor Neurons
(Hb9-GFP+)
Dissociation
MAG-expressing
CHO cells / myelin
Assess Neurite Outgrowth

Figure 6.1: Proliferation and Differentiation of ES-derived Motor Neurons. Embryonic stem cells from transgenic mice which express GFP under Hb9 promotion were cultured as described by Wichterle et al., 2002. Differentiation was achieved via application of 0.3μM retinoic acid (RA) (Sigma) and 0.3μM sonic hedgehog agonist (Shh-Ag) (Curis) for 5 days. After differentiation, the embryoid bodies were dissociated and plated onto either MAG-expressing CHO cells or purified myelin and neurite outgrowth was assessed as previously indicated.

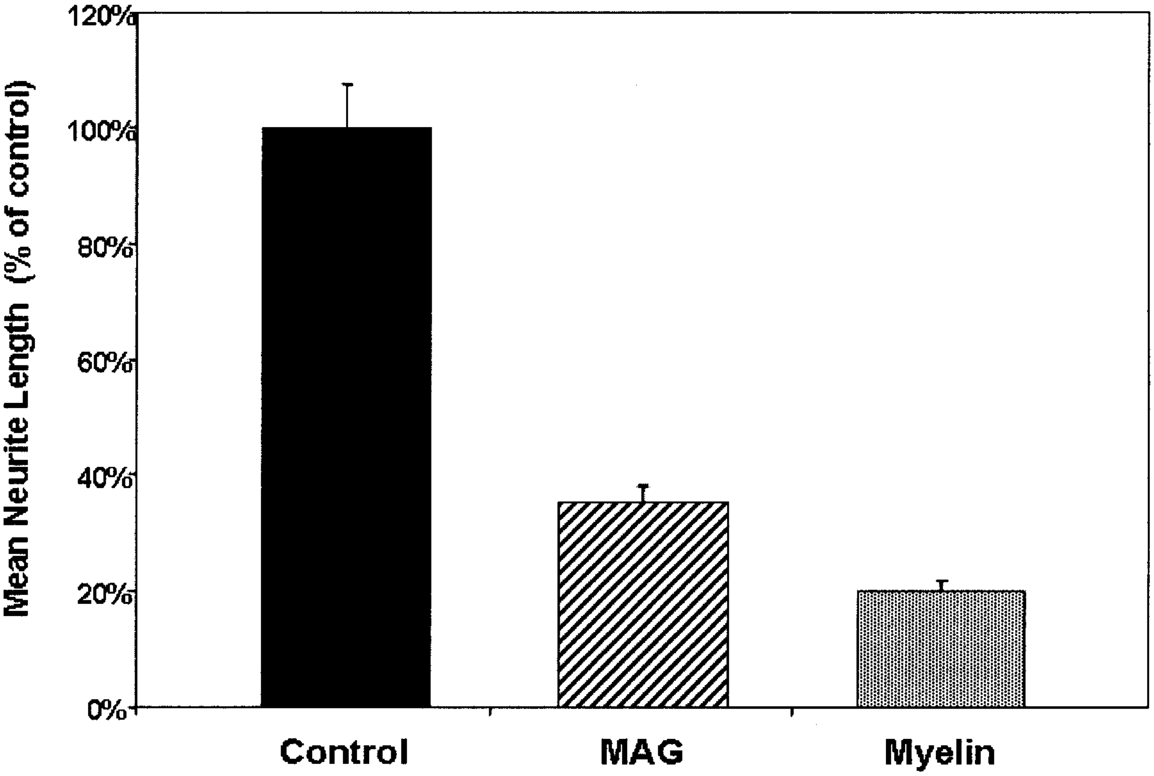


Figure 6.2: ES-derived Motor Neurons are Inhibited by MAG and Myelin. Following differentiation, motor neurons were plated onto either control or MAG-expressing CHO cells or 1μg myelin and allowed to extend neurites for 18 hours. After fixation, Hb9-positive motor neurons were identified by GFP expression and the length of the longest neurite per neuron was measured for 150-200 neurons.

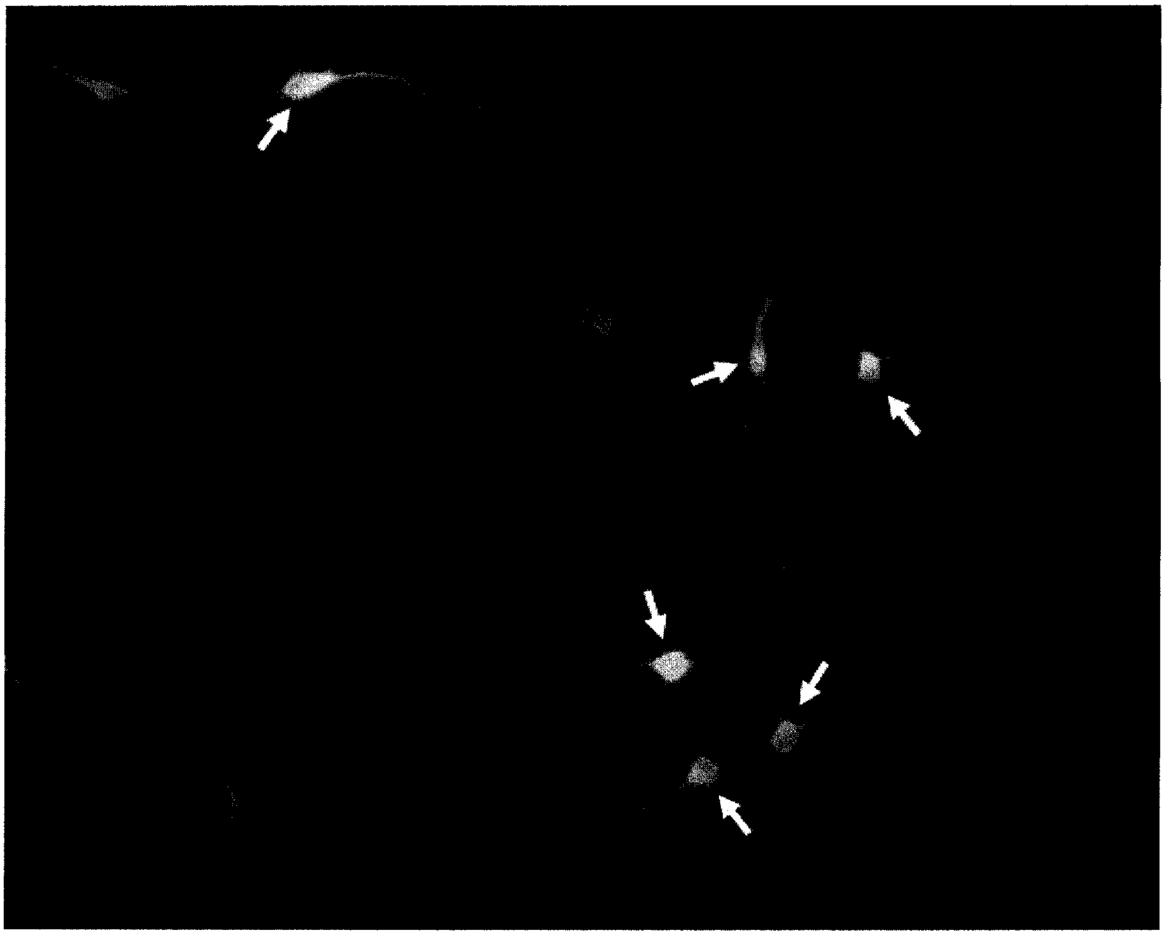


Figure 6.3: ES-derived motor neurons express the MAG/Nogo-66/OMgp Receptor NgR. After differentiation, motor neurons were plated onto poly-l-lysine and fibronectin-coated wells and allowed to extend neurites for approximately 24 hours. NgR expression was observed via immunostaining using a polyclonal anti-NgR antibody (Santa Cruz Biotechnology) Arrows indicate neurons double stained for GFP (Hb9+ cells) and Texas Red (NgR+ cells).

Since the neurite outgrowth from these neurons is inhibited by MAG in the same way as it is for primary post-natal neurons, we asked whether axonal extension from these ES-derived motor neurons also can be rescued by elevating intracellular cAMP levels. To do this, we performed a neurite outgrowth assay with differentiated motor neurons in the presence of db-cAMP, the non-hydrolysable cAMP analogue. As indicated in figure 6.4, we found that treatment of these ES-derived motor neurons with 1mM db-cAMP is sufficient to overcome the inhibitory effects of MAG *in vitro*. Figure 6.4 also suggests that there may be a slight dose-dependent effect as 10mM db-cAMP appears to induce better neurite outgrowth on MAG than does 1mM or 5mM. However, we believe that this is a result in an increase in efficacy rather than improved growth as the average lengths of motor neurons with neurites does not increase, but rather, *more* motor neurons have neurites in the 10mM treated condition than in the 1mM or 5mM. This finding suggests that the same signaling pathways which mediate the action and reversal of MAG-mediated inhibition in endogenous post-natal neurons may also be active in these *in vitro*-differentiated neurons.

To elucidate whether these pathways are indeed active in these neurons, we primed ES-derived motor neurons in the presence of either BDNF (figure 6.5) or the polyamines putrescine or spermidine (figure 6.6) and examined the ensuing neurite outgrowth on MAG. Not surprisingly, we found that each of these treatments is indeed sufficient to overcome the inhibition of neurite outgrowth by MAG, just as is seen in primary neurons.

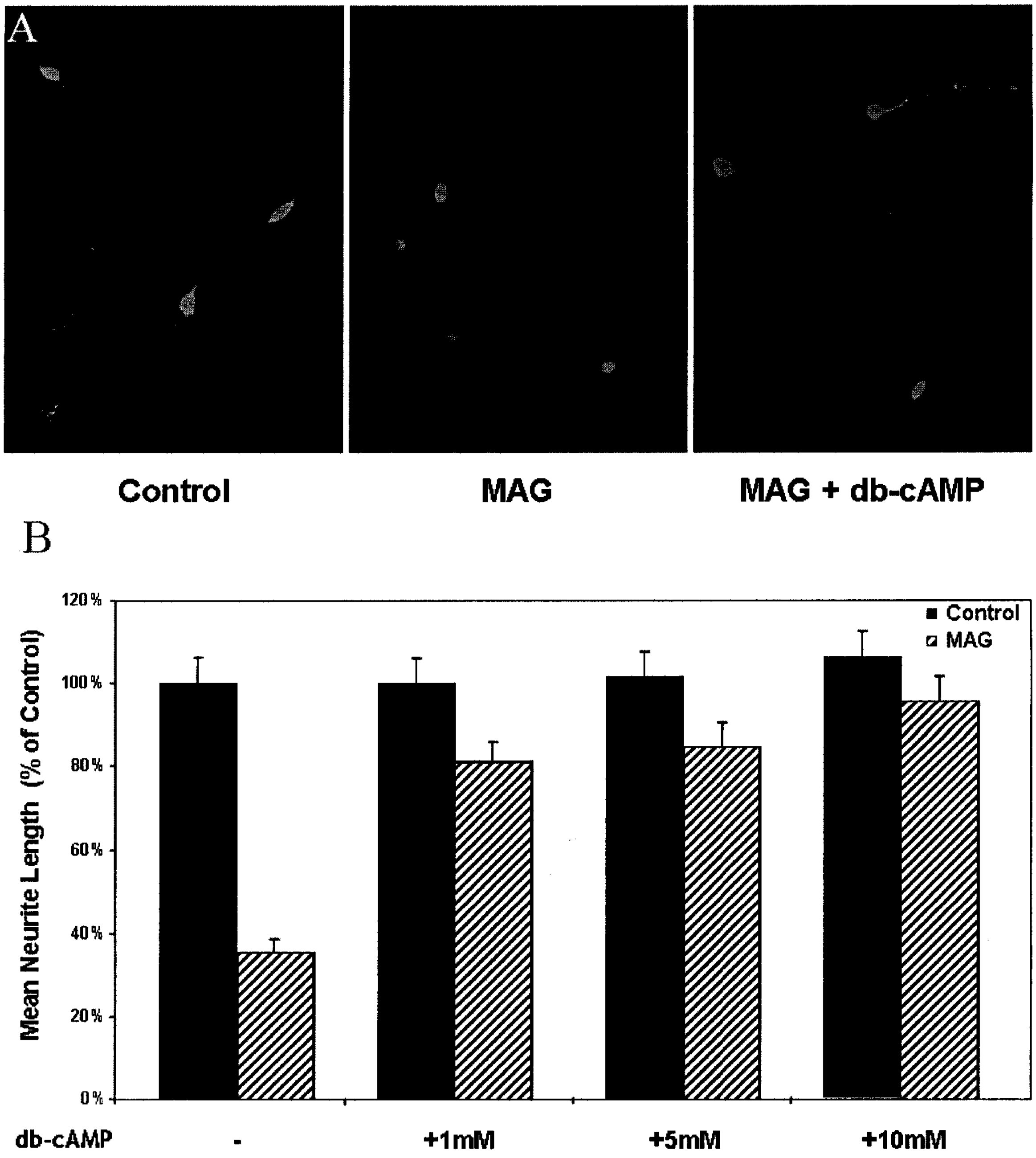


Figure 6.4: Elevation of cAMP Levels can Overcome Inhibition by MAG in ES-derived Motor Neurons. **Following differentiation, ES-derived motor neurons were plated onto control or MAG-expressing CHO cells in the presence or absence of differing concentrations of db-cAMP. After allowing for neurite extension and fixation, the length of the longest neurite per neuron was measured for 150-200 GFP-positive motor neurons.**

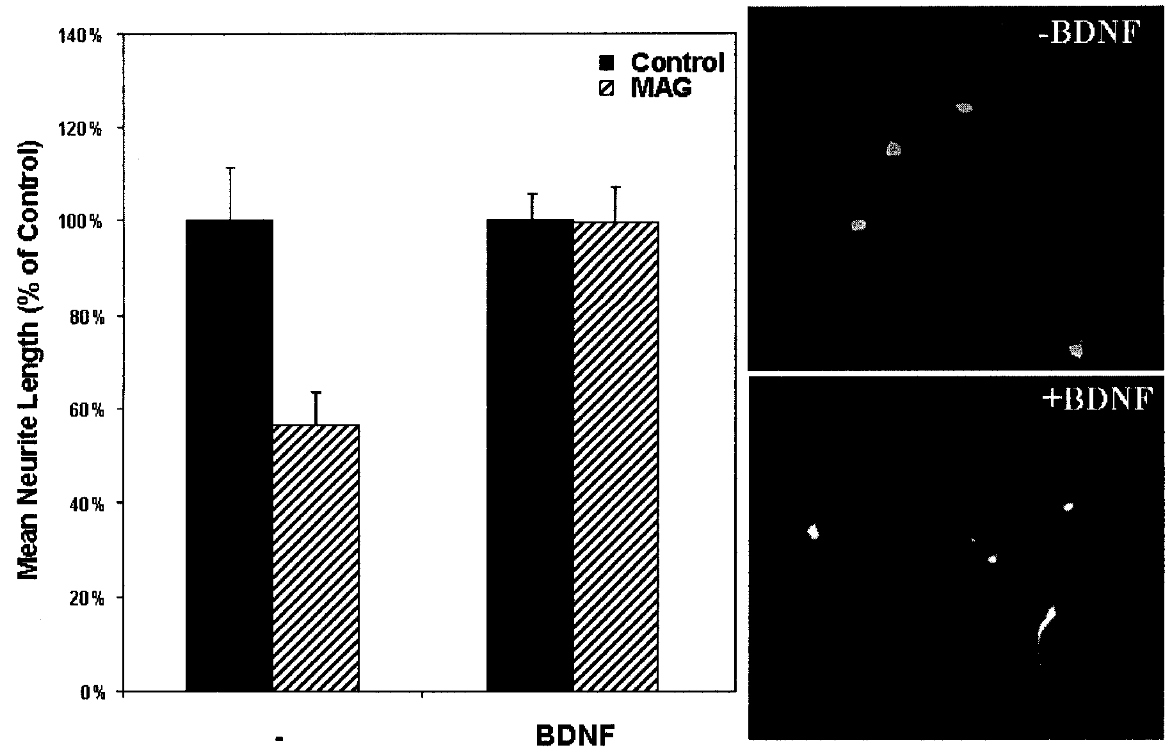


Figure 6.5: ES-derived Motor Neurons can be Primed with BDNF. After differentiation, ES-derived motor neurons were plated onto poly-l-lysine-coated dishes and primed with or without 200ng/ml BDNF. After the priming period, motor neurons were transferred to control or MAG-expressing CHO cell monolayers and incubated a further 18 hours to allow for axonal extension. After fixation, the length of the longest neurite per neuron was measured for 150-200 GFP-positive motor neurons.

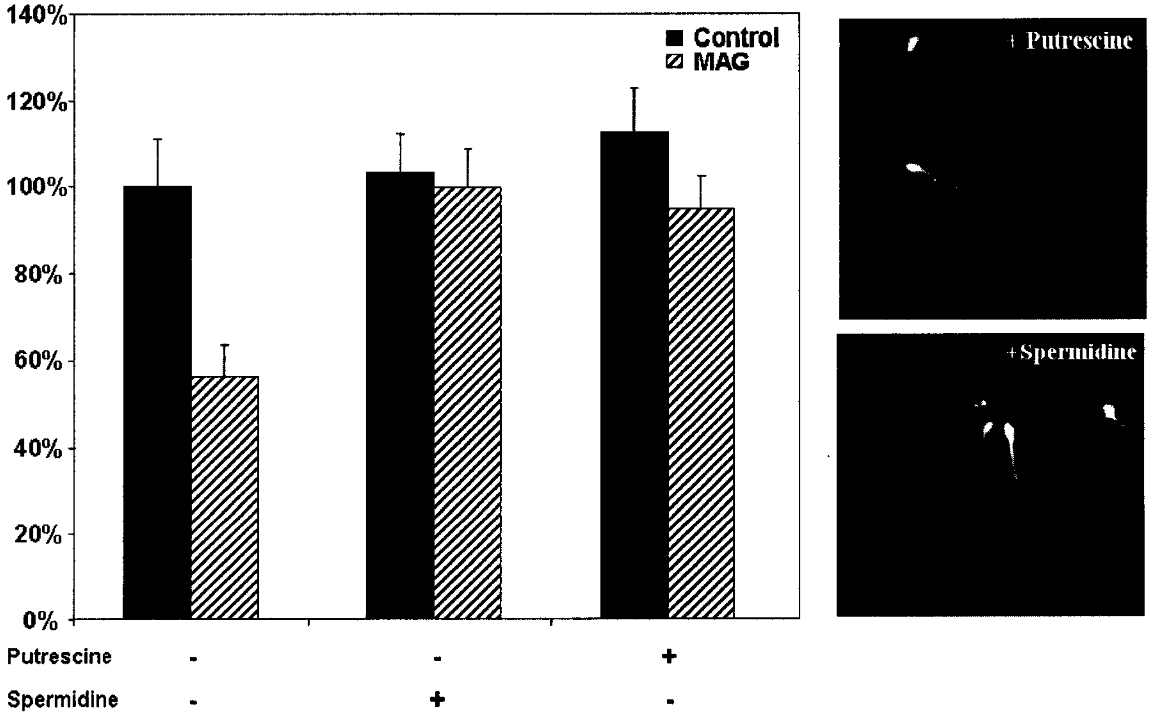


Figure 6.6: ES-derived Motor Neurons are also Responsive to Treatment with Polyamines. Following differentiation, ES-derived motor neurons were plated onto poly-l-lysine-coated dishes and treated with either 100μM putrescine or 50μM spermidine. After the treatment period, the cells were transferred to control or MAG-expressing CHO cell monolayers and incubated a further 18 hours to allow for neurite extension. After fixation, the length of the longest neurite per neuron was measured for 150-200 GFP-positive motor neurons.

6.3 Discussion

Adult mammalian CNS axons fail to regenerate damaged axons after injury due, in part, to the presence of the myelin-associated inhibitors. This fact also appears to be true for motor neurons derived from embryonic stem cells, as evidenced by the data noted above and some recently published work from Douglas Kerr's lab (Harper et al., 2004). This is particularly surprising as it is well-known that axonal regeneration from embryonic neurons is not inhibited by myelin. Thus, it appears that while these ES-derived motor neurons are indeed derived from embryonic cells, they no longer behave as embryonic neurons, at least with respect to their regenerative potential. Therefore, in order for these neurons to be used as a viable "neuronal replacement" therapy, they must be encouraged to extend axons into and through the inhibitory environments of the mammalian CNS and PNS.

In studies illustrated here, we find that the ES-derived motor neurons are, like their endogenous post-natal counterparts, responsive to both cAMP elevation (via application of db-cAMP or priming with neurotrophins) and to activation of the downstream signaling pathway which leads to improved regeneration. This further suggests that the treatments and therapies that we are currently pursuing for improving axonal regeneration of endogenous neurons after CNS injury may also be applicable to the axonal extension of these differentiated motor neurons following engrafting *in vivo*.

Furthermore, one benefit to the use of "cell culture-derived" neurons such as these is that they may be easily genetically modified prior to engrafting (as evidenced by our initial viral vector approaches outlined above) such that invasive, exogenous treatment may not be necessary following engrafting. These studies are currently ongoing in the lab and future work will investigate further the properties and identity of the signaling molecules involved in this improved axonal extension as well as fine tuning the application of the viral vector application and *in vivo* engrafting after both CNS injury and onset of motor neuron-specific disease.

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