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APPROACHING THE CONVERGENT POINT: DOWNSTREAM EFFECTORS OF CYCLIC AMP AND MAG ON CYTOSKELETON REORGANIZATION IN RAT PRIMARY NEURONS

by

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ABSTRACT

Approaching the Convergent Point: Downstream Effectors of cyclic AMP and MAG on Cytoskeleton Reorganization in Rat Primary Neurons

by

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Inhibitors of axonal outgrowth in myelin such as myelin-associated glycoprotein (MAG) are major impediments to regeneration after injury. Elevation of cyclic AMP (cAMP) induces up-regulation of Arginase I (ArgI) which resulted in synthesis of polyamines (putrescine, spermidine and spermine). Up-regulation of ArgI or supplements of putrescine are each sufficient to overcome the inhibition by MAG and myelin *in vitro*. It had been shown that a conditioning peripheral nerve lesion could alter the intrinsic growth state of dorsal root ganglia (DRG) neurons and increase regrowth of their central axons. We demonstrate here that both ArgI and polyamines levels increase after a conditioning lesion. If synthesis of polyamines is blocked by DFMO, an inhibitor for ornithine decarboxylase, along with a sciatic nerve lesion, the inhibition by MAG remained when the DRG neurons were removed and cultured. In addition, intrathecal delivery of putrescine, without a peripheral lesion, was sufficient to switch the response of these DRG neurons to MAG. Hence, activation of ArgI/polyamines pathway is required and sufficient for the conditioning peripheral lesion effects.

It has been suggested that different polyamines play different roles in biological processes. In this study, we characterized the effects of putrescine, spermidine and spermine in axonal outgrowth. The content of both putrescine and spermidine, but not spermine, was increased after incubation with dibutyryl cAMP, a cell permeable analogue of cAMP. Inhibition of spermidine synthase activity by its inhibitor - BCHS resulted in accumulation of putrescine. However, when putrescine is elevated by BCHS even in the presence of exogenous dbcAMP or putrescine, inhibition by MAG and myelin are not blocked. This suggests that putrescine alone was insufficient to overcome the inhibitory effects of MAG and myelin, and that its conversion to spermidine was required. Moreover, priming neurons with spermidine was sufficient to block the inhibition by MAG and myelin. In addition, intrathecal deliver of spermidine overcomes inhibition by MAG and myelin when DRG neurons are then cultured.

Cytoskeleton rearrangement must be the ultimate convergent point of these extracellular signals. We show here that simultaneously strengthening tubulin polymerization with taxol and destabilizing microfilaments with cytochalasin D resulted in improved neurite outgrowth on MAG and myelin in a dose-dependent manner. Tubulin is known to undergo several post-translational modifications that result in stable but not dynamic microtubules inside cells. Newly synthesized α-tubulin is tyrosinated while older, more stable tubulins are detyrosinated (or glutamylated) and/or acetylated. Taxol treatment resulted in a loss of tyrosinated-tubulin (Tyr-T) and strikingly increased the intensity of acetylated-tubulin (Acet-T). Both Tyr-T and Acet-T content are increased by dbcAMP treatment. However, if MAG-Fc, a soluble analog of MAG, is included in the cultures there is less Tyr-T. More importantly, the effect of myelin on tubulin modification is reversed by dbcAMP.

Cyclin-dependent kianse 5 (CDK5) is a multifunctional serine/threonine kinase. Activity of CDK5 has been shown to be critical for neurite elongation and cytoskeleton stability of axonal structures. The present study demonstrates that neurotrophins through activation of the Erk pathway induce expression of p35, the neuronal specific activator for CDK5. This in turn induces the kinase activity of CDK5. We show that elevated activity of CDK5 is required for neurotrophins, dbcAMP or polyamines to overcome inhibition by MAG and myelin.

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ABBREVIATIONS

Acet-T	acetylated α-tubulin
Arg	arginase
BCHS	bis-cyclohexylammonium sulphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic AMP
CB	cytochalasins B
CD	cytochalasins D
CDK5	cyclin dependent kinase 5
CHO	Chinese hamster ovary
CNS	central nervous system
CRE	cAMP-responsive element
CREB	cAMP-responsive element-binding protein
CSF	cerebrospinal fluid
CSPGs	chondritin sulfate proteoglycans
dbcAMP	dibutyryl cAMP
DFMO	DL-α-difluoroemthylornithine
DMEM	dulbecco's modified eagle's medium
DRB	5,6-dicholro-1-β-D-ribofuranosylbenzimidazole
DREZ	dorsal root entry zone
DRG	dorsal root ganglion

ECL	enhanced chemiluminescence
ECM	extracellular matrix
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GAP	GTPase-activating protein
GAP-43	growth associated protein 43
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
GDNF	glial cell-derived neurotrophic factor
GEF	guanine nucleotide exchange factor
GFAP	glial fibrillary acidic proteins
GFP	green fluorescent protein
Glu-T	polyglutamylated α-tubulin
GPI	glycosylphosphatidylinositol
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
Ig	immunoglobulin
Lingo-1	LRR and Ig domain-containing, Nogo Receptor-interacting protein
IL-6	interleukin 6
MAG	myelin associated glycoprotein
MAP	microtubule-associated protein
MF	microfilament
MT	microtubule

NF	neurofilament
NGF	nerve growth factor
NgR	Nogo receptor
NMDA	N-methyl-D-aspartate
NTs	neurotrophins
NT-3	neurotrophin-3
ODC	ornithine decarboxylase
OMgp	oligodendrocytes myelin glycoprotein
$p75^{NTR}$	p75 neurotrophin receptor
PAGE	poly-acryamide gel electrophoresis
PAK	p21 activate kinase
PAO	polyamine oxidase
PAs	polyamines
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphadiesterase
PFU	plaque forming units
PKA	protein kinase A
PKC	protein kinase C
PLL	poly L-lysine
PND	post natal day
PNS	peripheral nervous system
PTX	pertussis toxin

PUT	putrescine
RGCs	retinal ganglion cells
RT-PCR	reverse transcription-PCR
SAM	s-adenosylmethonine
SDS	sodium dodecyl sulfate
SPD	spermidine
SPM	spermine
SSAT	spermidine/spermine N 1 acetyltransferase
Tyr-T	tyrosinated α-tubulin

Chapter I

Introduction

1.1 Lack of Regeneration of CNS after Injury

It has been recognized for a long time that if axonal connections are damaged in the adult brain or spinal cord, they show an extremely limited ability to regenerate. The normal response to injury of axons in the brain and spinal cord is at best a short period of axonal sprouting followed by retraction and end-bulb formation (Fishman and Kelly, 1984; Ramon Y, 1991 [1928]; Houle and Jin, 2001). However, damage to the peripheral nervous system (PNS) is frequently reversible. In the PNS, regenerative sprouts often elongate and grow over long distances to reestablish functional connections. Neurons are able to regenerate and eventually restore functional connections of their peripheral axons. The reasons for this selective shutdown of regeneration in the adult central nervous system (CNS) are poorly understood.

1.1.1 Response after PNS Injury

One of the earliest events after peripheral nerve damage is the recruitment of circulating macrophages to the site of the lesion. The myelin debris is then cleared by these macrophages. In addition, macrophages stimulate the proliferation of Schwann cells in the vicinity of the lesion, probably by secreting mitogenic growth factors. Then, the proliferating schwann cells secrete several extracellular proteins, in particular laminin, which promote axon extension. Schwann cells also de-differentiate, becoming more permissive for regeneration by down-regulating expression of myelin proteins. PNS axons then can regenerate between these permissive schwann cells and the basal lamina (Fawcett and Keynes, 1990).

1.1.2 Response after CNS Injury

When the CNS is injured a set of glial responses occurs. This CNS injury response produces an environment that is inhibitory to axon regeneration, which finally leads to a glial scar, a physical barrier to further prevent regeneration. Unlike Schwann cells in the PNS, oligodendrocytes in the CNS continue to express myelin proteins after injury and they do not engulf myelin debris. Oligodendrocytes proliferate massively in all forms of CNS damage. They produce inhibitory proteoglycans, which prevent axon regeneration (Fawcett and Asher, 1999). Myelin debris, which includes inhibitors, is cleared away only very slowly (Schwab and Bartholdi, 1996; Fitch et al., 1999; Qiu et al., 2000).

In comparing the PNS and CNS after injury, the formation of the glial scar and inhibitors present in the myelin and myelin debris are two major obstacles that are responsible for the lack of regeneration of CNS.

1.2 Obstacles to Regeneration

1.2.1 Glial Scar Formation

The glial scar consists predominately of reactive astrocytes and proteoglycans. In severe lesions that open the meninges, astroglia become mixed with invading connective tissue elements. Glial reaction generally refers to reactive astrocytosis, which is an increase in the number and size of astrocytes, a change in astrocytic morphology, and an increase in expression of glial fibrillary acidic proteins (GFAP) by astrocytes. It has been proposed that astrocytic scars are inhibitory because it is mechanically impossible for axons to penetrate them (Stensaas et al., 1987). When regenerating axons encounter the scar, so-called dystrophic endbulbs form (Ramon Y, 1991 [1928]). On the other hand, in the early 1990s, studies have shown that reactive astrocytes up-regulate molecules such as chondritin sulfate proteoglycans (CSPGs) and tenascin, which seem to inhibit growth of regenerating axons (Rudge and Silver, 1990; McKeon et al., 1991). It has been shown by immunostaining and *in situ* hybridization that there is a considerable increase in CSPGs expression around CNS injury sites. Furthermore, axonal growth stops precisely at the site that CSPGs are deposited (Snow et al., 1990b; McKeon et al., 1991; Stichel et al., 1995). In addition, Davies and colleagues showed that transplanted adult DRG neurons were able to regenerate their axons in adult CNS white matter if implantation was as atraumatical as possible. However, the axons appeared to stop at this proteoglycans barrier if the expression of CSPGs was increased around the transplant (Davies et al., 1997). The results from *in vitro* assays are in agreement with the *in vivo* results. Several CSPGs have been shown to inhibit axonal growth or to inhibit molecular interactions that promote axonal growth (Friedlander et al., 1994; Retzler et al., 1996; Yamada et al., 1997).

Taken together, these observations suggest that the glial scar may be partially, but not solely, responsible for the failure of axonal regeneration in the CNS. The glial scar which forms around the lesion site often takes weeks to mature. CNS axons, however, are still unable to re-grow in the meantime, which suggests that inhibitors present in myelin and myelin debris is the major obstacle at the beginning.

1.2.2 Myelin/Oligodendrocytes Derived Inhibitors

The idea that CNS myelin was involved in the prevention of axonal regeneration in adult mammals was first suggested by Berry and colleagues (Berry, 1982). Subsequently the ability of CNS myelin to inhibit neurite outgrowth was studied in detail by Schwab and Caroni. In a series of landmark papers, they demonstrated that CNS myelin and oligodendrocytes were non-permissive to axonal growth (Caroni and Schwab, 1988b; Schwab and Caroni, 1988).

To date, three inhibitory components of myelin have been identified: myelin-associated glycoprotein (MAG), Nogo and most recently, oligodendrocytes-myelin glycoprotein (OMgp).

1.2.2.1 Myelin-Associated Glycoprotein (MAG)

Myelin-associated glycoprotein (MAG) is a well-characterized myelin-specific protein. MAG was first demonstrated to have a function in myelination and the formation and maintenance of myelin-axon interaction. Since 1990, there is increasing evidence that MAG is also a potent inhibitor of axonal regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994).

MAG is a member of the immunoglobulin (Ig) superfamily, and contains five Ig-like domains in its extracellular sequence (Lai et al., 1987; Salzer et al., 1987). It is expressed by Schwann cells in the PNS and by oligodendrocytes in the CNS. MAG is a component of both CNS and PNS myelin and represents 0.1 and 1% of the total myelin proteins in each of these membranes, respectively. In the adult CNS, MAG is located exclusively in the periaxonal oligodendroglial membranes of myelin internodes (Trapp et al., 1989). This restricted distribution differs from that in adult PNS myelin where MAG is also detected in membranes of the paranodal loops, Schmidt-Lanterman incisures and mesaxons (Quarles, 1989).

Although MAG was identified to be a neurite-outgrowth promoting molecule like other members of the Ig-superfamily, such as L-1 and NCAM (Johnson et al., 1989), in 1994, two independent studies suggested that MAG is also a potent inhibitor of axonal regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994).

Comparison of neurite outgrowth from neurons grown on a substrate of Chinese hamster ovary (CHO) cells transfected to express MAG versus untransfected CHO cells showed inhibition of 50-90% depending on the neuronal type and this inhibition is reversed by an anti-MAG antibody (Mukhopadhyay et al., 1994). McKerracher *et al* came to the same conclusion in the search for inhibitors in CNS myelin by fractionating solubilized CNS myelin proteins by DEAE chromatography. They found that a major peak of inhibitory activity corresponded to the elution profile of MAG. Immunodepletion of MAG from these inhibitory fractions removed neurite growth inhibition. In support of an active inhibitor of regeneration, growth cones of hippocampal neurons collapsed when they encounter beads coated with recombinant MAG (Li et al., 1996; Shibata et al., 1998). Futhermore, it has been shown that soluble forms of MAG, which contain the entire extracellular domain of MAG, either recombinant MAG-Fc or dMAG, which is proteolytically released from damaged myelin, could inhibit axonal growth from neurons (Tang et al., 1997b).

MAG is a bifunctional molecule. That is to say, depending on the age and type of neuron, MAG can either promote or inhibit axonal regeneration (Johnson et al., 1989; Salzer et al., 1990; Mukhopadhyay et al., 1994; Turnley and Bartlett, 1998). MAG promotes growth of embryonic neurons, yet inhibits regrowth for all postnatal neurons tested to date, with the exception of newborn DRG neurons, indicating that a switch in growth response to MAG from promotion to inhibition occurs during development. For DRG neurons, this switch occurs sharply at postnatal day 3-4. Taken together, embryonic neurons, in contrast to postnatal neurons in almost all circumstances, are not inhibited by myelin, either *in vivo* (Bregman et al., 1993; Davies, 1993; Hasan et al., 1993; Davies, 1994) or in culture (Shewan et al., 1995; Cai et al., 1999).

The potency of MAG as a neurite outgrowth inhibitor *in vivo* is less clear. Investigation of CNS axonal regeneration by Bartsch in MAG knockout (MAG-/-) mice suggests there is no difference in the regeneration capacity of damaged spinal axons of MAG-/- and wild type mice (Bartsch et al., 1995). However, using a different line of MAG deficient mice, another group reported more regenerating axons across the lesion site and longer process outgrowth (Li et al., 1996), although both groups observed an improvement in neurite length for NG108 cells grown on MAG-/- myelin substrate. Results from our lab have also shown improved process growth of cerebellar, DRG neurons and retinal neurons growing on MAG deficient myelin (DeBellard et al., 1996). In summary, all observations agree that MAG contributes, at least partly, to the poor regeneration of CNS after injury although further studies are still required to determine the contribution of MAG in this issue.

1.2.2.2 Nogo

Martin Schwab and his colleagues observed that the inhibitory activity of myelin was enriched in two protein fractions of myelin extraction, with molecular weights of 35kDa (NI-35) and 250kDa (NI-250) (Caroni and Schwab, 1988b). A monoclonal antibody IN-1, raised against purified NI-250 (Caroni and Schwab, 1988a) shown to reduce the inhibitory activity of CNS myelin, as well as to facilitate the structural plasticity of a variety of lesioned fiber tracts (Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995). A decade later, three groups, independently, cloned Nogo, the neuronal antigen(s) for IN-1 (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

There are three distinct isoforms of Nogo, A, B and C, which are generated by alternative splicing (Nogo-A/B) and promoter usage (Nogo-C). All three isoforms of Nogo share a common C-terminal domain of 188 amino acids, which is homologous to the reticulon protein family. Reticulon proteins are anchored to the endoplasmic reticulum (ER) *via* a C-terminal ER retention signal and relatively little is known about their function. Among Nogo A, B and C, Nogo A is the one that is expressed in oligodendrocytes, which express most of the myelin-derived inhibitors, while the other two are ubiquitously expressed, including by some neurons, so that NogoA received most attention among the three. Nogo A is expressed on the cell surface of oligodendrocytes, although much of it is likely to be retained in the ER. Moreover, it also exists on the innermost loop of the myelin membrane, where it could make contact with the axon (Huber et al., 2002; Wang et al., 2002c). Nogo-A has a unique amino (N) terminus and at least two inhibitory domains (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). One of these domains (Nogo-66) is carried by a 66-amino-acid sequence, which is common to all three isoforms, and the second domain (amino-Nogo) is unique to the Nogo-A-specific N terminus. The most common topology of Nogo-A in the membrane places Nogo-66 outside the cell, and the N- and C-terminals in the cytoplasmic compartment (Grandpre et al., 2000). A second topology has also been proposed whereby amino-Nogo is extracellular, and it has been suggested that the two different topologies might coexist simultaneously (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Huber et al., 2002).

In vivo and *in vitro* studies showed Nogo is inhibitory to re-growth after injury. Recombinant full-length Nogo A inhibits both 3T3 fibroblast spreading and DRG neurite outgrowth (Chen et al., 2000; GrandPre et al., 2000), which could be neutralized by either IN-1 or antibody against C-terminal portion of Nogo A. A more detailed study indicated that the extracellular Nogo-66 domain possesses strong inhibitory activity. It induces significant growth cone collapse in E13 chicken DRG explants and inhibits neurite outgrowth from PC12 cells and E12 chicken DRG neurons (GrandPre et al., 2000). The work of two groups (Chen et al., 2000; Prinjha et al., 2000) have shown that the amino-Nogo domain also carries inhibitory function. Rat DRG neurons fail to extend neurites into adult control rat optic nerves but do so into optic nerves partially neutralized by antiserum directed against this region.

There is discrepancy between the results from three different lines of Nogo knockout mice generated from three different labs (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). Two groups reported substantially improved regeneration after dorsal column lesion (Kim et al., 2003; Simonen et al., 2003) using Nogo A/B -/- or Nogo A -/- respectively. However, Tessier-Lavigne's group studied two lines of mutant mice, one lacking all three Nogo proteins, and another lacking Nogo A/B. Neither line had any significant improvement of axonal growth (Zheng et al., 2003).

1.2.2.3 Oligodendrocyte Myelin Glycoprotein (OMgp)

OMgp appears as a highly glycosylated polypeptide anchored in the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI)-link. OMgp was expressed not only by oligodendrocytes, but also at high levels in neurons. The precise localization of OMgp on oligodendrocytes and in white matter is not well characterized. However, Mikol and Stefansson (1988) observed an expression of OMgp near the node of Ranvier (Mikol et al., 1988).

OMgp is the most recently identified myelin associated inhibitor of axonal growth (Wang et al., 2002b). OMgp was detected in the fraction from bovine myelin treated with phosphatidylinositol-specific phospholipase C (PI-PLC). That released proteins which were shown to have growth cone collapsing and neurite outgrowth inhibitory activities. Furthermore, the inhibitory activity of OMgp is as strong as MAG or Nogo (Wang et al., 2002b) in *in vitro* assay. The contribution of OMgp in inhibiting axon regeneration *in vivo* has not yet been determined.

1.2.3 Receptor(s) of Myelin Derived Inhibitors

1.2.3.1 NgR

NgR was first identified as a putative receptor of Nogo-66, using interaction screening of a mouse brain cDNA expression library transfected into non-neuronal COS-7 cells (Fournier et al., 2001). NgR transcripts are expressed in a wide variety of CNS neurons but not in oligodendrocytes, which is consistent with its function in regulating axonal regeneration and plasticity in the adult CNS. Structurally, NgR is a GPI-linked protein with a conventional signal peptide at the N-terminus, which is followed by an eight leucine-rich-repeat (LRR) domain and an LRR C-terminal flanking domain. NgR and Nogo-66 were shown to bind directly to one another with high affinity (Fournier et al., 2001). Gain of function and loss of function assays were both employed to investigate the function of NgR as a receptor. E13 DRG neurons, which express NgR and respond strongly to Nogo-66 in a growth cone collapse assay, no longer, had this response once NgR was removed from the cell surface by PIPLC treatment. On the other hand, expression of NgR in E7 DRGs and E7 retina, which are insensitive to Nogo-66, renders the growth cones of this cell sensitive to Nogo-66 induced collapse. That is to say, NgR is being recognized as the receptor of Nogo-66 and is capable of mediating Nogo-66 signalling of axon outgrowth.

NgR lacks an intracellular component, suggesting that an associated transmembrane co-receptor subunit mediates intracellular signal transduction. In searching for this co-receptor by an expression cloning strategy using soluble alkaline phosphatase-linked NgR (AP-NgR) fusion protein to screen pools of adult mouse brain cDNA expression library transfected into COS-7 cells, to their surprise, instead of getting the co-receptor, Liu *et al.* identified another ligand for NgR, which turns out to be MAG (Liu et al., 2002). With the use of different binding and functional assays, they also observed the direct binding of MAG to NgR and the indispensability of NgR for the inhibitory activity of MAG and CNS myelin. In addition, they found that excess Nogo-66 or using selective Nogo-66 antagonist peptide NEP1-40 does not prevent MAG/NgR binding. This means that although MAG and Nogo-66 both bind to the LRRs of NgR, they appear to bind independently.

At the same time, independent of the findings stated above, Filbin's group reported similar results while searching for the binding partner of MAG (Domeniconi et al., 2002). Previously, we showed that a soluble, chimeric form of MAG, MAG-Fc (the extracellular domain of MAG fused to the Fc portion of human IgG), was able to specifically precipitate a number of neuronal surface proteins (DeBellard et al., 1996). A 80kD protein was precipitated, and was shown to be NgR. NgR binds specifically to a MAG-expressing cell line. Also, MAG precipitates NgR specifically from NgR-expressing CHO cells, cerebellar neurons, and DRG neurons, while a truncated, non-inhibitory form of MAG that binds neurons in a sialic acid-dependent manner does not. Importantly, soluble forms of NgR, as well as an antibody to NgR, or dominant-negative NgR can each block inhibition of neurite outgrowth by MAG. The only discrepancy is that our results showed that Nogo-66 and MAG compete with one another for the binding site on NgR.

Interestingly, He's group reported that OMgp also interacts with the NgR and it also requires interacting with NgR for its inhibitory effects (Wang et al., 2002b). In searching for the receptor of OMgp, they screened an expression library and identified the NgR as a high-affinity OMgp-binding protein. Cleavage of NgR and other GPI-linked proteins from the cell surface renders axons of dorsal root ganglia insensitive to OMgp. Conversely, introduction of exogenous NgR confers OMgp responsiveness to otherwise insensitive neurons. However, whether the binding of OMgp to NgR is competitive or additive to the binding of Nogo and MAG is not addressed.

The crystal structure of the ligand binding (leucine-rich repeat) domain of the human NgR has been reported (He et al., 2003), which partially shed light on this question. The structure reveals a conserved, putative ligand binding site within the concave groove of the NgR LRR. They propose that the presence of conserved aromatic residues in the curved ecto-domain of NgR could provide degenerate ligand binding sites with each ligand choosing its preferred binding site. Also, Barton et al showed that the N-terminal region of NgR is sufficient to bind each of these ligands (Barton et al., 2003). Therefore, although Nogo, MAG and OMgp lack sequence homologies, they all bind to the NgR, a GPI-linked cell surface molecule, with high affinity, to exert inhibition.

Recently, two NgR-related proteins, NgR2 and NgR3 have been identified (Barton et al., 2003; Venkatesh et al., 2005). Barton et al claimed that NgR2 and NgR3 do not bind Nogo, MAG, OMgp or NgR. However, Giger's group reported that NgR2 and NgR3 are expressed strongly in the adult brain and NgR2 acts selectively to mediate MAG inhibitory responses (Venkatesh et al., 2005). In addition, they thought the conflict comes from their usage of AP-MAG (Barton) instead of MAG-Fc (Giger) in the binding assay. The AP tagging might somehow interfere with their binding to NgR2.

However, NgR is a GPI-linked protein, which means it lacks an intracellular domain. That is to say, a transmembrane transducer that can bind to NgR is required to propagate the inhibitory signal delivered by the binding of myelin components to NgR.

1.2.3.2 p75

p75 was long known as a non-specific, low affinity neurotrophin receptor. Yamashita *et al* found that it is also the signal-transducing element for MAG in 2002, before the identification of NgR (Yamashita et al., 2002). A mutation in p75 abolished the inhibitory action of MAG in neurite outgrowth. And neurons from the mutant failed to activate a small GTPase Rho, an important intracellular target for most of the inhibitors to bring about inhibition, in the presence of MAG. They further demonstrated that MAG and p75 are co-localized although they do not directly bind to each other. Soon after NgR was found to be the receptor of three distinct myelin inhibitors, two groups show evidences that p75 is the binding partner of NgR and it acts as the signal transducer of the receptor complex. p75 performs direct association with NgR endogenously through its extracellular domain and is required for NgR-mediated signaling (Wang et al., 2002a). Furthermore, the inhibitory activities are attenuated by either blocking the interaction between NgR and p75 or transfecting the neurons with a truncated p75. Similar results were obtained by Poo's group, which showed that p75 and NgR form a receptor complex which mediates the repulsive signaling by MAG (Wong et al., 2002).

The results on the knockout mice of this receptor complex seem less promising. In *in vitro* assays, cerebellar neurons from either NgR knockout (NgR-/-) or p75 deficient (p75-/-) mice were still inhibited by myelin (Zheng et al., 2005).

Only DRGs from p75-/- showed improved growth on myelin (Zheng et al., 2005). After dorsal column lesion, no significant regeneration of corticospinal fibers was reported (Kim et al., 2004; Zheng et al., 2005) and only limited regeneration of raphespinal and rubrospinal fibers and certain degree of motor function recovery (Kim et al., 2004) in NgR-/-. In addition, in spinal cord-injured mice, however, depletion of functional p75 is not sufficient to improve the regenerative growth of descending corticospinal or ascending sensory neurons (Song et al., 2004).

Although many lines of evidence suggest that p75 expression is upregulated by injury (Ernfors et al., 1989; Roux et al., 1999; Andsberg et al., 2001), p75 still may not be the only signal transducer in the NgR receptor complex. It has already been shown that some neurons that are inhibited by myelin inhibitors, adult RGCs for instance, failed to immunostain with the p75 antibody (Ding et al., 2001). Immunohistochemical data showed that p75 was expressed in only a very small subset of ascending sensory axons but not in any corticospinal axons in the dorsal column of either normal or injured spinal cord. The possibility is the existence of other p75 homologs or other NgR co-receptor(s) to bring about inhibition.

1.2.3.3 Lingo-1 – Another Necessary Component of the Receptor Complex

Mi et al. identified a novel protein Lingo-1 (LRR and Ig domain-containing, Nogo Receptor-interacting protein), as an interactor of $p75^{NTR}$ and NgR (Mi et al., 2004). In reconstituted non-neuronal systems, NgR and p75 together are unable to activate RhoA. Lingo-1 is required to deliver the inhibitory signal. The Lingo-1-NgR-$p75^{NTR}$ complex is shown to confer the inhibitory effects on nerve cell regeneration of Nogo-66, OMgp, and MAG by activating the small guanosine triphosphatase (GTPase) RhoA.

Whether there are other components that exist in the receptor complex remains unknown. However, it is sufficient to say that NgR/p75/Lingo-1 as a complex is sufficient to bring about the inhibition by Nogo, MAG or OMgp. Taken together a putative model for inhibition becomes apparent: after CNS injury, myelin-associated inhibitors are exposed to injured neurons and bind to NgR. This ligand-receptor interaction enhanced the p75-NgR-Lingo-1 association and the transduction of inhibitory signals downstream into the cell to inhibit nerve regeneration (Figure 1.1).

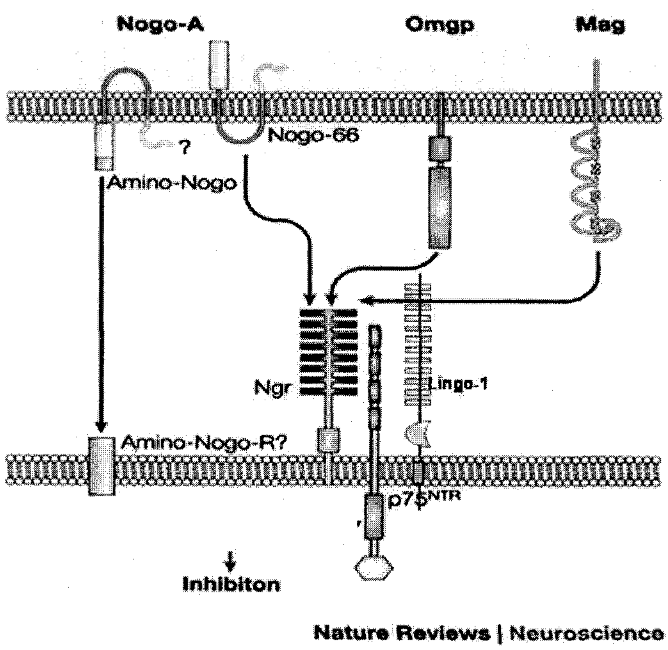


Figure 1.1: Diagram of Myelin-associated Inhibitors and Their Receptors
Schematic diagram showing signals induced by myelin-associated neurite growth inhibitors. MAG, Nogo66 and OMgp all bind with the Nogo66 receptor (NgR), which in turn interacts with p75 and Lingo-1. The activation of p75 transduces the inhibitory signals intracellularly and inhibits neurite outgrowth (adapted from (Filbin, 2003)).

1.2.4 Other Inhibitors Correlated with Axon Regeneration

Other than inhibitors derived by oligodendrocytes, there are also axon growth inhibitors derived by astrocytes or glial cells. For example, CSPG expression is upregulated in reactive astrocytes in lesions of the spinal cord (Snow et al., 1990a; Fitch and Silver, 1997). The regeneration of axons is inhibited when growth cones confront CSPG-rich astrocytes in a CNS lesion (Davies et al., 1997; Davies et al., 1999). *In vitro*, neurites will not grow over astrocytes expressing CSPG (Grierson et al., 1990; Meiners et al., 1995; Canning et al., 1996). It was recently reported that removal of glycosaminoglycan (GAG) chains from CSPGs *in vivo* resulted in improved regeneration and partial sensory recovery (Bradbury et al., 2002). No specific receptor for CSPG has been identified, although some proteoglycans, such as Versican, are thought to bind a potential CSPG receptor expressed on growth cone (Fawcett, 1997). NG2, which is a transmembrane CSPG expressed by glial cells in the adult CNS, and responds rapidly to CNS disruption (Levine et al., 2001). NG2 inhibition of neurite growth in culture is blocked by anti-NG2 antibodies (Dou and Levine, 1994; Chen et al., 2002; Ughrin et al., 2003). In the spinal cord, NG2 is produced after injury and it is in the right place at the right time to inhibit regeneration (Zhang et al., 2001; Jones et al., 2002). Furthermore, besides Nogo, MAG and OMgp, there are also a number of other molecules associated with myelin, such as axonal guidance molecules which have been suggested to be involved in axonal regeneration. For instance, collapsin-2, an inhibitory guidance cue active during development, was reported to be expressed by oligodendrocytes and present in both the gray and white matter in the spinal cord (Luo et al., 1995). In addition, netrins and slits, both of which are important guidance cues for axonal path-finding, have been shown to repel spinal motor, olfactory and hippocampal axons and collapse their growth cones *in* *vitro* (Li et al., 1996; Nguyen Ba-Charvet et al., 1999). Moreover, Janusin/restrictin, a tenascin-related ECM molecule, is also found to be secreted mainly by oligodendrocytes and exhibits repulsive activity towards growth cones when used as a substrate *in vitro* (Pesheva et al., 1989; Schachner et al., 1994).

1.3 Strategies to Block the Inhibitors in Myelin to Overcome Inhibition and Encourage Functional Recovery

1.3.1 Changing the Extrinsic Inhibitory Environment

1.3.1.1 Removing the Inhibitors

As mentioned above, CSPGs are inhibitory to axon growth *in vitro*, and regenerating axons stop at CSPG-rich regions *in vivo*. Removing CSPG glycosaminoglycan (GAG) chains by chondroitinase ABC attenuates CSPG inhibitory activity, which leads to increased axon regeneration (Bradbury et al., 2002). However, Chondroitinase ABC is unable to completely digest the GAG chains from the protein core, leaving an inhibitory "stub" carbohydrate behind. An alternative approach is suppression of CSPG core protein synthesis (Davies et al., 2004) or suppression of glycosaminoglycan chain synthesis (Grimpe and Silver, 2004).

For myelin-derived inhibitors, such as MAG, Nogo and OMgp, there are no attemtps to simply remove one or all to foster regeneration. That is because of their structural distinction, it does not seem to be a feasible approach. In addition, a soluble form of these inhibitors (Tang et al., 1997b; GrandPre et al., 2000) or even a domain, such as Nogo66, is sufficient to bring about inhibition (GrandPre et al., 2000).

The finding that all three inhibitors bind to the same receptor complex point out that blockage or deletion of the receptor might be a better strategy. However, as mentioned previously, no improvement of regeneration was observed in p75-/- (Song et al., 2004) and better regrowth of fiber in NgR-/- was observed only in rubrospinal and raphespinal tract (Kim et al., 2004) but not in corticospinal tract (Kim et al., 2004; Zheng et al., 2005). Also, Versican V2 and the central inhibitory domain of Nogo-A inhibit neurite growth *via* $p75^{NTR}$/NgR-independent pathways that converge at RhoA (Schweigreiter et al., 2004). These results indicated that there might be receptors other than NgR/p75 to bring about the inhibition.

1.3.1.2 Blocking Myelin-associated Inhibitors with Antibodies or Peptides

David and colleagues demonstrates that neutralizing myelin inhibitors with antibodies is a feasible approach (Huang et al., 1999). To date, this has only been attempted with one individual antibody, the IN-1 antibody, resulting in the regeneration of only 5-10% of axons (Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995). This approach can probably succeed only if all the myelin inhibitors are blocked simultaneously.

Before NgR was identified as receptor of MAG and OMgp, NEP1-40, a 40-amino-acid antagonist peptide corresponding to a Nogo-66 sequence, was shown to bind to the LRR ligand binding site on NgR and block Nogo-66 or CNS myelin inhibition of axonal outgrowth *in vitro* (GrandPre et al., 2002) and improved regeneration and better functional recovery *in vivo* (GrandPre et al., 2002; Li and Strittmatter, 2003). GrandPre *et al* then tried to conclude that Nogo is the main inhibitor at that time. However, we now know that all three inhibitors, competitive or not, bind to the LRR region of NgR. So, further investigation is needed to address whether this antagonist peptide also prevents the binding of MAG or OMgp to NgR.

The recent finding of the common receptor complex for the three known myelin inhibitors greatly increased the number of possible targets for therapeutic intervention using peptides or antibodies. Two obvious targets are: blocking the binding of ligands to NgR and blocking the interaction of NgR to p75/Lingo-1. Several observations reported lately suggest this is an effective approach. Blockade of NgR by viral infection of dorminant negative NgR in growth-sensitized retinal ganglia cells greatly enhances axon regeneration (Fischer et al., 2004). Administrating the soluble function-blocking NgR ectodomain to spinal cord injuried rat resulted in axonal sprouting of corticospinal and raphespinal fibers in NgR(310)ecto-Fc-treated animals correlates with improved spinal cord electrical conduction and improved locomotion (Li et al., 2004).

Another attempt was from our lab, utilizing two competitive antagonist peptides which were synthesized according to the sequence of MAG Ig domain 5, which carried the neurite outgrowth inhibitory site of MAG (Tang et al., 1997a). With the addition of individual antagonist peptides into neuron-myelin co-cultures, the inhibitory effect of myelin on neurite outgrowth was blocked. As a control, our lab also has data that suggests the addition of control Sialoadhesin-derived peptide has no effect on myelin's inhibition (Cao Z. unpublished data).

Although the crystal structure of NgR revealed that the C-terminal LRRCT of NgR is responsible for the NgR-p75 interaction (He et al., 2003), attempts to interrupt the interaction between NgR and p75 have not yet been reported. Agents targeted to this sequence would be the potential drug to encourage regeneration.

1.3.2 Changing the Intrinsic State of the Neurons

It is believed that the production of myelin and the loss of neuronal regeneration capacity coincide during development. Many *in vivo* and *in vitro* observations suggest that immature mammalian CNS neurons posses the intrinsic capacity to repair themselves after injury (Ramon Y, 1991 [1928]; Shewan et al., 1995). However, this growth capacity is lost during early developmental stages (Shewan et al., 1995). For instance, mature CNS tissue, which does not support re-growth of its own damaged axons, does support axonal growth from embryonic CNS neurons implanted into the adult animals (Fawcett, 1992; Nicholls and Saunders, 1996). The precise molecular determinants that regulate this change in the regenerative capacity of neurons remained unknown. It has been suggested that the switching of growth related genes during development might explain the changes in the ability of neurons to extend axons (Fawcett, 1992).

However, some early studies suggested adult CNS neurons could extend axonal processes within a permissive environment. In the early 1980's, several observations demonstrated that adult CNS neurons could form long projections through peripheral nerve grafts (Richardson et al., 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982). These results suggest that at least some matured CNS neurons still maintain the intrinsic growth capacity.

Regeneration of CNS *versus* PNS neurons in the DRG population provides important insights into this intrinsic mechanism(s). DRG neurons have two branches, one extends into the CNS and another extends peripherally. It has been well established that although the peripheral branch of DRG neurons readily regenerates after injury, their processes in the CNS fail to do so. But if the peripheral branch of DRG neurons are severed, followed by a second lesion to the central branch of the same nerve either at the same time or weeks later, there is extensive growth into the peripheral nerve grafts placed at the lesion site of the dorsal column (Richardson and Issa, 1984; Oudega et al., 1994). If the peripheral nerve lesion (conditioning lesion) is performed 1 week before the dorsal column transection, injured axons can regenerate across the injury site and grow into the tissue beyond even in the presence of a tissue graft (Neumann and Woolf, 1999). This suggests that the regenerative capacity of DRG neurons is certainly improved by first injuring to the peripheral branch, which seemingly improved the growth state of the DRG neurons.

1.3.2.1 Manipulating the Direct Inhibitory Signaling Pathway - Rho Inhibitor

The Rho family of small GTPasees, the best studied RhoA, Cdc42 and Rac1, act as molecular switches to drive growth and guidance by regulating F-actin dynamics (Sandvig et al., 2004). Indeed, Rho family GTPases orchestrate actin filament assembly and disassembly through the control of actin polymerization, branching, and depolymerization. The well-characterized intracellular signaling events that transduce repulsive effects of several axon guidance cues, such as Slits, Semaphorin and Ephrins, during neural development provide an excellent starting point for determining the basis of repulsive and inhibitory guidance in the mature nervous system. Coupling of the neurite-inhibitory molecule growth cone receptors to the Rho family of G-proteins is a common thread shared by Nogo, MAG, and OMgp to induce growth cone collapse. McKerracher's group investigated a strategy to block the neuronal response to growth-inhibitory signals by inhibiting the Rho GTPase signaling pathway. They report that injured axons re-grow directly on complex inhibitory substrates when Rho GTPase is inactivated by C3 convertase (Lehmann et al., 1999). Since C3 is largely membrane impermeable, once it was applied locally to the lesioned CNS, it cannot readily diffuse to other tissues (Dergham et al., 2002). When C3 is delivered intrathecally, it has no effect on regeneration *in vivo*. However, if Y27632, an inhibitor of Rho-associated protein kinase (ROCK), which is an effecter of Rho, was applied to the injury site, regeneration occurred (Fournier et al., 2003). It remains to be seen whether the effects of the new generation of C3 molecules, which are more membrane-permeable, can be similarly confined and effective.

1.3.2.2 Neurotrophins and Cyclic AMP

Instead of manipulating the direct inhibitory signaling pathway, the other approach would be activating parallel signaling pathways to overcome the inhibition. The work in our lab demonstrated that exposure of neurons to neurotrophins prior to encountering the myelin inhibitors (so called priming) blocks inhibition by MAG or myelin (Cai et al., 1999). However, if NTs are added directly to the neurons in the presence of pertussis toxin, a Gi/G0 inhibitor, inhibition is blocked without priming. This "priming" effect is mediated through elevation of endogenous cyclic AMP (cAMP), and can be abolished in the presence of a protein kinase A (PKA) inhibitor. By adding membrane-permeable analogs of cAMP, such as dibutyryl cAMP (dbcAMP) and 8-bromo cAMP, neurons can overcome the inhibitory effect of MAG, as well as other inhibitors in myelin, which mimic the priming effect (Cai et al., 1999). In addition, the endogenous levels of cAMP, are dramatically higher in young neurons in which axonal growth is promoted than in the same types of neurons that, when older, are inhibited by myelin-MAG (Cai et al., 2001). At the same time, Poo's group did a series of work to demonstrate that the response of a growth cone to many attractive guidance cues can be changed to repulsion by blocking the cAMP-dependent kinase, PKA (Ming et al., 1997; Song et al., 1997; Song et al., 1998; Mueller, 1999; Song and Poo, 1999).

We also showed that 1 day post-peripheral-lesion, DRG-cAMP levels triple and MAG/myelin no longer inhibit growth, an effect that is PKA dependent. By 1 week post-lesion, DRG-cAMP level returns to control, but growth on MAG/myelin improves and is now PKA independent (Qiu et al., 2002b). Injection of dbcAMP into the DRG following a central DRG lesion results in the promotion of central regeneration of these projections, which mimics completely a conditioning lesion (Qiu et al., 2002b). These results pointed out that neuronal cAMP level dictate the regenerative capacity of neurons.

On the other hand, instead of artificially increasing the cAMP level by adding the membrane permeable dbcAMP, the work of Nikulina *et al* has shown that we could inhibit the activity of phosphodiesterase 4 (PDE4), a group of enzymes responsible for cAMP degradation, by rolipram to achieve a similar result. In addition, in rolipram-treated animals, an attenuation of reactive gliosis was observed, which might further facilitate regrowth of the regenerating axons (Nikulina et al., 2004). Even more, rolipram produced significant axon sparing and promoted myelination when combined with schwann cell grafts after spinal cord contusion (Nikulina et al., 2004). Combination of rolipram with local increases in cAMP at the growth cone along with transplanted schwann cells further enhance this effect and led to significant improvement in locomotor function (Pearse et al., 2004).

1.3.2.3 Downstream Events of Up-regulation of cAMP

The effect of NTs and cAMP is transcription dependent (Cai et al., 2002). When a transcription inhibitor, 5,6-dicholro-1-β-D-ribofuranosylbenzimidazole (DRB), was included, the ability of either of these treatments to block inhibition by MAG/myelin is completely abrogated. Gao *et al* suggested that CREB (cAMP response element binding protein) is a critical transcription factor involved in modulation of the re-growth capacity of mature neurons (Gao et al., 2004). Identification and characterization of CREB stemmed from studies of cAMP stimulating target gene expression *via* a conserved cAMP-responsive element (CRE). Diverse extracellular stimuli, including NTs, activate CREB through multiple signaling cascades that converge to phosphorylate Ser133. We showed that priming neurons with NTs or treatment with dbcAMP activate CREB. Furthermore, a dominant negative CREB, A-CREB, blocks CRE-mediated gene expression and the NTs/cAMP effect on MAG. Moreover, by infecting the neurons with a constitutively active CREB, VP16-CREB, these neurons were no longer inhibited by MAG and myelin.

In searching for the genes that were being turned on after treatment with dbcAMP, microarray analysis was done by our lab in cooperation with Hart's group. RNA was harvested from neurons either treated with dbcAMP *in vitro* or at different time points after conditioning lesion, which was subjected to oligo chips and/or RT-PCR. Genes that significantly increased or decreased were selected (unpublished data, Cao Z.; Gao Y. *et al*). One of those genes that was significantly up-regulated is Arginase I (ArgI), a rate limiting enzyme for polyamines synthesis. It has been shown by us that ArgI expression increased 5-fold by NTs/dbcAMP treatment (Cai et al., 2002) and infection of A-CREB attenuated the effect of NTs/dbcAMP in elevation of ArgI. On the other hand, ArgI levels doubled in hippocampal neurons from transgenic mice expressing VP16-CREB comparing to control animal. Although the CRE site in the promoter region of ArgI might not be a "true" CRE site, this still implicated that the up-regulation of ArgI depends on phosphorylation of CREB directly or indirectly (Gao et al., 2004).

1.3.2.4 Arginase I and Polyamines

ArgI is a cytosolic isoform of arginase that is normally expressed in hepatocytes, which is thought to play a role in determining whether ornithine becomes converted to polyamines or to glutamate and proline (Kepka-Lenhart et al., 2000). It is one of the rate-limiting enzymes for *de novo* synthesis of polyamines. It has been demonstrated that the level of ArgI can be induced by cAMP (Wang et al., 1995; Gotoh et al., 1996; Morris et al., 1998) in macrophages as well as other tissues (Wu et al., 1996; Flynn and Wu, 1997).

Many studies had implicated polyamines (PAs) in regeneration. Gilad's group has demonstrated that following axotomy of neurons of the superior cervical sympathetic ganglion (SCG), administration of exogenous polyamines accelerates the reinnervation by these neurons of their target tissues (Dornay et al., 1986; Gilad and Gilad, 1988; Gilad et al., 1996). However, none of the above showed a definitive effect on overcoming myelin inhibitors. We showed that treating neurons with either dbcAMP or BDNF in culture resulted in up-regulation of ArgI and polyamines synthesis. Over-expression of ArgI by adenovirus or exogenous addition of polyamines is sufficient to overcome inhibition by MAG and myelin. Conversely, blocking either ArgI or the next enzyme, ornithine decarboxylase (ODC), in polyamine synthesis can abrogate the ability of dbcAMP or BDNF to overcome inhibition. Mimicking in mature DRGs the high endogenous ArgI levels present early in development can block the switch from MAG promotion to inhibition (Cai et al., 2002). However, how polyamines alter the growth inhibitory effect of MAG is still undetermined.

1.4 Our Working Model

Based on the results from our lab and others, a model is proposed to explain the possible signaling mechanism involved in how axonal regeneration is blocked by myelin inhibitors. First, after injury, neuronal growth cone were exposed to myelin inhibitors, such as MAG, Nogo and OMgp. These ligands bind to the same receptor, NgR, by associating with its transducer p75 and Lingo-1, the inhibitory signal was transduced to the neuron. Subsequently, neuronal outgrowth was blocked. On the other hand, if neurons were primed with neurotrophins, they activate Erk through the Trk receptors, Erk then inhibits PDE and so cAMP accumulates and PKA is activated. CREB was phosphorylated and then the phospho-CREB was translocated to nucleus and turned on transcription of certain genes. ArgI, the key enzyme for polyamine synthesis, is one of the genes being up-regulated. Elevation of polyamines seems to promote axonal regeneration and overcoming inhibition by MAG or myelin in general.

1.5 Goals of the Work in This Thesis

We know that up-regulation of ArgI or supplement of putrescine is sufficient to overcome the inhibition by MAG/myelin in our *in vitro* assay. Now we want to further demonstrate the importance of the ArgI/PAs pathway in the conditioning lesion model to see whether ArgI/PAs is required and sufficient to overcome inhibition by MAG and myelin (**Chapter III**). Furthermore, polyamines include putrescine, spermidine and spermine, which have been shown to have either common and/or distinct function in neurons. In **chapter IV**, I hope to elucidate if they are working in an additive or competitive fashion to overcome inhibition.

Extracellular cues exert their actions by engaging specific cell-surface receptors and activating downstream signaling pathways that ultimately lead to the cytoskeleton reorganizations that underlie various axonal behaviors. In **chapter V**, we tried to manipulate the polymerization and depolymerization of either microtubules or microfilaments by pharmacological drugs to elucidate the "right state" for neurite outgrowth. How cytoskeleton components were being regulated by either positive or negative signal(s) would also be addressed partially in this chapter.

The activity of cyclin dependent kinase 5 (CDK5) and its regulatory subunit p35 has been shown to be involved in regulation of cytoskeletal dynamics (Nikolic et al., 1998; Humbert et al., 2000) (Ishiguro et al., 1994; Pigino et al., 1997; Paglini et al., 1998). And its activity has been shown to associate with neurite outgrowth *in vitro* (Nikolic et al., 1996; Paglini et al., 1998). In **chapter VI**, we tried to elucidate a role of CDK5 in neurite outgrowth and overcome the inhibition of myelin inhibitors.

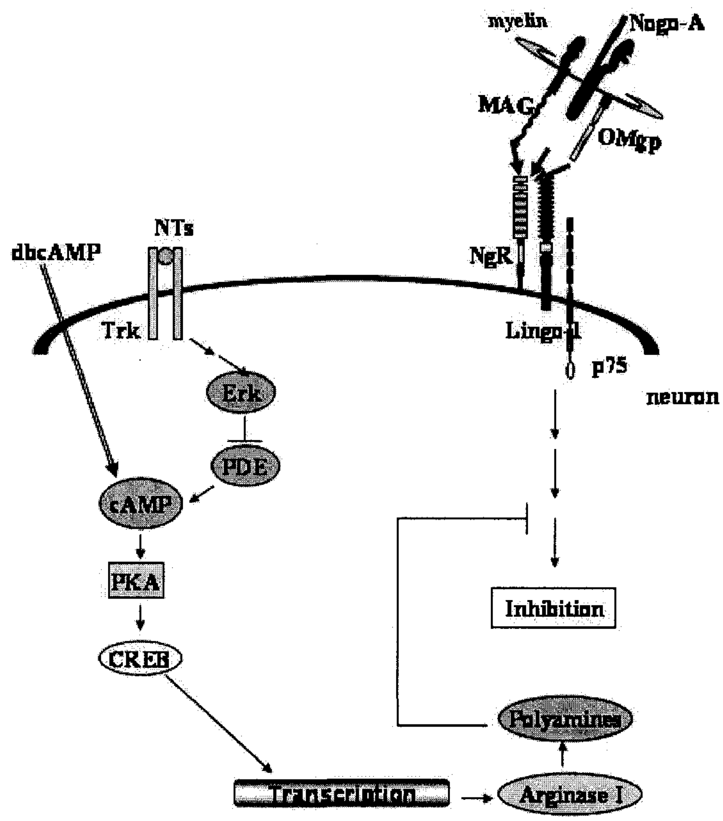


Figure 1.2 Working Model of Our Lab

Activation of NgR, p75 and Lingo-1 results in inhibition of axonal growth. Priming neurons with neurotrophins, which signal through tyrosine kinase receptor (Trk), can elevate endogenous cAMP through activation of Erk and inhibition of PDE activity. Alternatively we could elevate cAMP with non-hydrolysable analogues, such as dbcAMP. cAMP activates protein kinase A (PKA), which can affect inhibition directly by activating CREB, which triggered transcription of genes such as ArgI, resulting in an increase in synthesis of polyamines that blocks inhibition.

Chapter II

Methods and Materials

2.1 Cell Culture

Permanently transfected MAG-expressing and control CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technology) supplemented with 10% dialyzed Fetal Bovine Serum (FBS), praline (40mg/L), glycine (7.5mg/L), thymidine (0.73mg/L) and L-glutamine (0.29g/L) at 37°C in 7.3% CO_2.

2.2 Isolation of Primary Neurons

For cerebella neurons, both cerebellums were taken from each animal at the age from postnatal day 1 (PND1) to day 5 and dissociated in 6ml of 0.025% trypsin (Life Technology) by pipetting. 10ml DMEM containing 10% FBS was then added to terminate trypsinization. Cells were centrifuged at 1000 rpm for 5 min at 4°C and resuspended to a single-cell suspension in SATO (progesterone, 200nM; selenium, 224nM; putrescine 100μM; insulin, 4μg/ml; BSA, 0.35mg/ml; L-thyroxine, 0.4μg/ml; tri-iodo-thyronine, 0.34μg/ml) (Doherty et al., 1990). For DRG neurons, ganglia were removed from animals aging from PND5 to PND8 and incubated in 6ml of L15 media containing of 0.025% trypsin and 0.15% collagenase type I (Worthington) for 60-90 min at 37°C. The ganglia were triturated and trypsinization was stopped by adding 10ml of DMEM containing 10% FBS, centrifuged at 1000 rpm for 5 min and resuspended in SATO (DeBellard et al., 1996).

2.3 Priming Neurons

24-well plates (6cm) were coated with 16.6μg/ml PLL for 30min at room temperature. Isolated neurons in SATO were plated onto the PLL-coated well at a density of 1 $\times 10^6$ cells/well. Where indicated, either BDNF (200ng/ml), NGF

(200ng/ml), GDNF (200ng/ml), putrescine, spermidine, BCHS (200μM), Roscovitine (10μM) (all from Sigma) or DFMO, PD98059 (20μM) (Calbiochem), was added. After overnight incubation, the media was removed and neurons were washed with PBS and removed with 0.1% trypsin. Trysinization was stopped by adding 2ml DMEM containing 10% FBS. Neurons were centrifuged at 1,000rpm for 5min, resuspended in SATO and plated immediately onto either MAG-expressing or control CHO cells or myelin substrate.

2.4 Neurite Outgrowth Assay on CHO Cell Monolayer

Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an 8-chamber tissue culture slide (Lab-Tek). Primary neurons were isolated and resuspended in Sato media as described previously (DeBellard et al., 1996). Neurite outgrowth assay was carried out by adding 1.5×10^4 neurons to the CHO cell monolayers. After 18-20 hours of incubation, the neurons were fixed for 30 min with 4% paraformaldehyde and permeabilized with ice-cold methanol for 2 min. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated overnight with a rabbit polyclonal antibody against the growth-associated protein GAP43 (1:4000, from R. Curtis and G. Wilkins Imperial College, London) diluted in PBS-5%BSA. Cells were washed three times with PBS and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit IgG (1:500, Amersham, Arlington Heights, IL), washed three times, and then incubated with streptavidin-conjugated Texas Red (1:300, Amersham, Arlington Heights, IL) for 45 min. After three more washes, the slides were mounted in Permafluor (Immunon) and viewed with a fluorescence microscope. The length of the longest neurite for each neuron for the first 180-200 neurons encountered when scanning the slide in a systematic manner was determined by using an Oncor image analysis program.

2.5 Neurite Outgrowth Assay on Immobilized L1-Fc Substrate

We have developed an assay in which the growth of isolated young cerebellar neurons on the substrate of the growth-promoting molecule L1 would be inhibited by the presence of wild type MAG-Fc (Tang et al., 1997a). An 8-chamber slide (Lab-Tek) was first coated with 16.6μg/ml poly-L-lysine (Sigma) in sterile water for 1 hr at room temperature, and then washed twice with freshly made 0.1M bicarbonate buffer. Wells were then incubated with anti-human Fc antibody (Sigma) 15μg/ml in 0.1M bicarbonate buffer for 2 hr at 37°C. After washing three times with DMEM, L1-Fc was immobilized onto anti-Fc by incubating 30μg/ml L1-Fc in the wells overnight at 37°C. Unbound L1-Fc was washed off with DMEM. Cerebellar granular neurons were suspended in SATO medium with 2% FBS into a concentration of $5x10^4$cells/well, and incubated with 25μg/ml of wild type or mutant MAG-Fc proteins at 37°C for 18 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

2.6 Myelin Preparation

Myelin was purified from rat CNS medulla following the Norton's protocol (Norton and Poduslo, 1973). After the final hypotonic shock, the membranes were centrifuged and resuspended in 10mM HEPES. Then the protein concentration of the preparation was determined (Biorad) and used as a substrate in the neurite outgrowth assay.

2.7 Neurite Outgrowth on Immobilized Myelin

For myelin membranes, wells of an 8-chamber tissue culture slide (Lab-Tek) were coated with 16.6μg/ml poly-l-lysine at room temperature for 1 hour. Rat CNS myelin at 0.5- 2.0μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen et al., 1998). $2x10^4$ cells/well of DRG or 5 $x10^4$ cerebellar neurons were suspended in Sato medium, and incubated in myelin-coated wells at 37°C for 24 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

2.8 Immunostaining of MAG Expressed on CHO Cells

Cells are grown overnight in chambers of 8-well tissue culture slides coated with poly-L-lysine (Sigma) and fibronectin (Sigma) before fixed with 4% paraformaldehyde for 15 min at room temperature. Cells will then be washed with PBS three times and incubated with monoclonal antibody 513 at 2μg/ml at 4°C overnight. After washing with cDMEM for three times, cells will be incubated with Oregon green goat anti-mouse IgG at room temperature for 1 hour, then will be washed twice with PBS before being mounted with Gel-mount (Biomeda) and viewed with a Zeiss fluorescence microscope.

2.9 Isolation of Total mRNA from Cultured DRG Neurons

DRG from PND5 rat pups were isolated and 50 x 10^4/well neurons were plated onto poly-l-lysine or purified myelin coated 24-well tissue culture plates. Dibutyryl-cAMP (1mM, Calbiochem) was then added to the culture medium and the cells were incubated for 18 hours at 37°C with 7.3% CO_2. The cells were then washed once with ice cold PBS and lysed in lysis buffer provided by RNeasy RNA isolation kit (QIAGEN). RNA was prepared *via* the RNeasy RNA isolation kit (QIAGEN), according to the manufacturers instructions.

2.10 *In vivo* Peripheral Conditioning Lesion

PND18-21 rat pups were anesthetized by isofluorane, then a sciatic nerve was exposed at mid-thigh level and a ligature was firmly tightened around the nerve distal to its emergence from the greater sciatic notch. The nerve was transected distal to the ligature and the wound closed. The animals were sacrificed 1-7 days after the sciatic nerve transection. L4,5 dorsal root ganglia from the lesion side and contralateral control side were isolated respectively, and then a neurite outgrowth assay was carried out on monolayers of CHO cells or immobilized purified CNS myelin as describle previously.

2.11 Intrathecal Administration

Osmotic mini-pumps (Alzet model 1007D) were filled with saline or drugs diluted in saline according to the instructions of the manufacturer. Minipumps were incubated in sterile saline solution (AmTech) at 37°C overnight or at least 6 hours. Rats at age of PND20-23 were anesthetized with isofluorane and inserted with one minipump in each animal. The spine of each animal was opened at L4-L6 through microsurgery and only the drug-delivering tip of each mini-pump was inserted into the space under dure membrane without injuring spinal cord. Operated animals were kept for 1day to 1 week before sacrificing. L4-L5 DRG neurons from each animal were isolated and underwent further neurite outgrowth procedures.

2.12 Detection of Gene Expression

Total RNA was isolated from L4, 5 DRG neurons after conditioning lesion or DRG neurons, which were dissociate from PND 5-8 pups and treated with BDNF (200 ng/ml), NGF (200 ng/ml) or db cAMP (1 mM) overnight. RNA was extracted using an RNeasy kit (Qiagen) following the manufacturers' instructions. cDNA was synthesized from total RNA using oligo dT and reverse transcriptional enzyme (stratagene) at 37°C for 1 hr. After which, ArgI cDNA was amplified using the following specific primers (Gibco BRL) that spanned nucleotide 197 to 700 of Arg I cDNA (Accession number NM017134): 5′ GTC CCC AAT GAC AGC CCC 3′ and 5′ CTT TTC TTC CTT CCC AGC AG 3′ using the polymerase chain reaction (PCR) at 35 cycles P35 cDNA was amplified using primers F: 5' GCTCTGCAG GGA TGT TAT CTC C 3' and R: CTT CTT GTC CTC CTG ACC ACT C 3' (Invitrogen). Amplification of GADPH from the same sample was used as control. GAPDH-Forward ATG GTG AAG GTC GGT GTG AAC G; Reward TGG TGA AGA CGC CAG TAG ACT C. PCR 30 cycles annealing temp 55°C 45 sec. DNA was detected in a 1% agarose gel, stained with ethidium bromide.

2. 13 Measurement of Polyamines

5×10^6 postnatal cerebellar neurons or 1×10^6 L4, 5 DRG neurons were plated onto poly L-lysine coated six-well plates. BDNF (200 ng/ml), dbcAMP (1 mM), or BCHS (200 μM) were added and incubated for various times as indicated. Cells were washed and lysed in 100 μl of 1.5 M $HClO_4$. Three lysates were pooled and the reaction was stopped by adding 150 μl of neutralizing buffer (2 M potassium bicarbonate). After centrifugation, polyamines in the supernatant were measured by HPLC carried out by a service (Dr. G. Wu, Department of Animal Science, Texas A&M University).

2.14 Immunodetection

Cerebellar or DRG neurons of different ages were lysed in 1X boiled sample buffer (62.5 mM Tris-HCl, pH6.8 and 2% SDS). The cell lysates were purified by Qiashredder (Qiagen) and centrifuged at 14,000 × g for 2 min. Protein concentration was measured with a Bio-Rad kit (Bio-Rad Labs, Hercules, CA). Normalized lysates were boiled for 5 min, after which they were subjected to SDS-PAGE in a 10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunostained with anti Arg I (polyclonal) (Esch et al., 1998) at 1:5,000, anti P35 (Santa Cruz) at 1:400, anti CDK5 (Santa Cruz)at 1:400, anti acetylated tubulin (Sigma) at 1:4,000, anti poly-glutaminated tubulin (Sigma) at 1:2,000, anti tyrosinated tubulin (Sigma) at 1:4,000 overnight at 4°C. Blots were incubated with anti-rabbit IgG-HRP secondary antibody (1:5,000) or anti-mouse IgG-HRP (1:1,000) at room temperature for 1 hour. Detection was with an enhanced chemiluminescence (ECL) detection system, according to the manufacturers' instructions (Amersham). The blot was stripped with stripping buffer (0.2M glycine, pH2.2, 1%Tween-20 and 0.1% SDS) for 1 hour at room temperature and reprobed with either anti actin (1:1,000) (Sigma), or anti β-tubulin (1:2,000) (Sigma) accordingly.

Chapter III

Arginase I / Polyamines Pathway is Involved in Conditioning Lesion and Regeneration

Introduction

It has been well established that although the peripheral branch of neurons such as DRG, readily regenerates after injury, their processes in the CNS fail to do so (Cajal, 1928). But if provided with a conditioning lesion (Figure 3.1) to the peripheral branch of DRG neurons, followed by a second lesion to the central branch of the same nerve, either at the same time or weeks later, there is extensive growth into peripheral nerve grafts placed at the lesion site of the dorsal column (Richardson and Issa, 1984; Oudega et al., 1994). If the peripheral nerve lesion is performed 1 week prior to a dorsal column transection, which severs the central branch of large diameter sensory axons, there is regeneration of fibers across the injury site and growth into the tissue beyond even in the absence of a tissue graft (Neumann and Woolf, 1999). This suggests that the regenerative capacity of DRG neurons is significantly improved by the first injury to the peripheral branch.

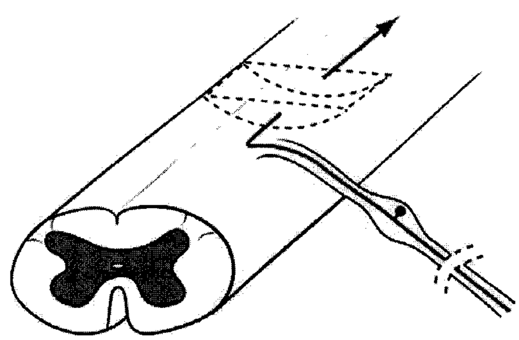


Figure 3.1 The Model of Preconditioning Peripheral Lesion and Subsequent Central Dorsal Column Lesion in DRG Nerves (Neumann and Woolf, 1999)

Our lab (Qiu et al., 2002b) and others (Neumann et al., 2002) have shown that cAMP signaling pathway plays an important role in promoting significant axonal regeneration in the CNS environment *in vivo*. We had shown that 1 day post-peripheral-lesion, DRG-cAMP levels tripled and MAG/myelin could no longer inhibit growth, an effect that is PKA dependent. By 1 week post-lesion, DRG-cAMP returns to control, but growth on MAG/myelin was improved and is now PKA independent (Qiu et al., 2002b). Injection of dbcAMP into the DRG following a central DRG lesion results in promotion of central regeneration of these projections, which mimics completely a conditioning lesion (Qiu et al., 2002b). Consistent with our results, Neumann *et al* demonstrated that injection of a membrane permeable cyclic AMP analog into the lumbar DRG markedly increases the regeneration of injured central sensory branches. These results point out that neuronal cAMP level can dictate the regenerative capacity of neurons.

In searching for the downstream player of cAMP, our lab used an *in vitro* assay, where several genes were up-regulated, in particular the enzyme, Arginase I (ArgI). ArgI has been characterized to degrade L-arginine to urea and ornithine, and then ornithine decarboxylase (ODC) converts ornithine to polyamines (Figure 3.2). ArgI and ODC are two rate-limiting enzymes for polyamine synthesis (Morris et al., 1998). It has been reported that both the expression of ArgI and the activity of ODC can be induced by elevated cAMP in liver and in macrophages (Wang et al., 1995; Gotoh et al., 1996; Morris et al., 1998). In addition, studies showed that ODC activity (Tetzlaff and Kreutzberg, 1985) as well as putrescine contents were increased following injury (Shohami et al., 1992; Henley et al., 1996; Rao et al., 1998). Furthermore, Gilad's group has demonstrated that early polyamine treatment accelerates regeneration of injured rat sciatic and facial nerve (Gilad and Gilad, 1988; Gilad et al., 1996), which implicated a role of polyamines in regeneration. However, the precise molecular mechanism remained unknown.

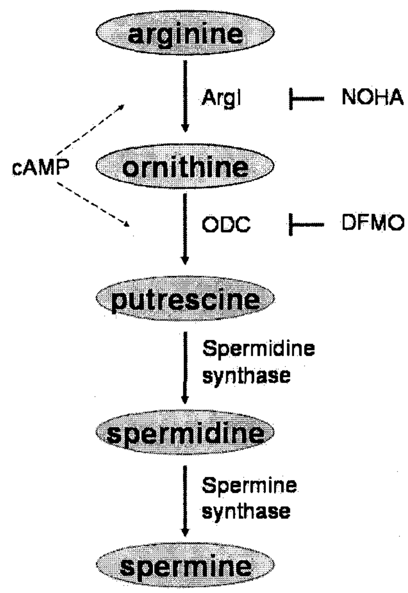


Figure 3.2 Biosynthesis of Polyamines

Along with our studies on a critical role of cAMP in regeneration (Cai et al., 1999; Cai et al., 2001), we showed that the effect of either cAMP or priming with neurotrophins (NTs) is transcription dependent. After treatment with dbcAMP, both the transcription and translation levels of ArgI were up regulated (Qiu et al., 2002a). In addition, it was found that if ArgI or ODC is blocked by the inhibitors N-(omega)–hydroxy-nor-L-arginine (NOHA) or DL-α-Difluoromethyl-ornithine (DFMO), respectively, the improved axonal growth on MAG/myelin induced by priming with BDNF or treating with dbcAMP is completely abrogated. Furthermore, if putrescine is presented with BDNF during priming, the priming effect of BDNF, which is abolished by NOHA or DFMO, is then restored. Interestingly, priming with putrescine alone is sufficient to block the inhibition by MAG/myelin completely. Up-regulation of ArgI through an adenvirus expression vector is sufficient to overcome the inhibition of axonal outgrowth by MAG. Also, the growth promoting effect of

MAG on young DRG neurons is blocked by either NOHA or DFMO. It is known that DRG neurons switch their axonal regeneration response to MAG and myelin from promotion to inhibition at postnatal day 3-4 (DeBellard et al., 1996). Consistent with this, we observe that the ArgI level is high in young neurons and decreases during development (Qiu et al., 2002a). Together, our results indicated that one mechanism whereby cAMP blocks inhibition of axonal regeneration by MAG/myelin is through up-regulation of ArgI and subsequent elevation of polyamine synthesis.

It has been demonstrated in our lab that the growth of dissociated DRG neurons on substrates of CNS myelin or MAG-expressing CHO cells parallels very well with the regenerative capacity of dorsal column fibers in the injured adult spinal cord. Therefore, the growth of adult DRG neurons *in vitro* may represent the regeneration of corresponding spinal cord axons *in vivo*. Here, we will address if ArgI/PAs pathway is involved in the improved growth capacity of DRG neurons as a consequence of a previous conditioning peripheral lesion, which resulted in elevation of endogenous cAMP (Cai et al., 2001).

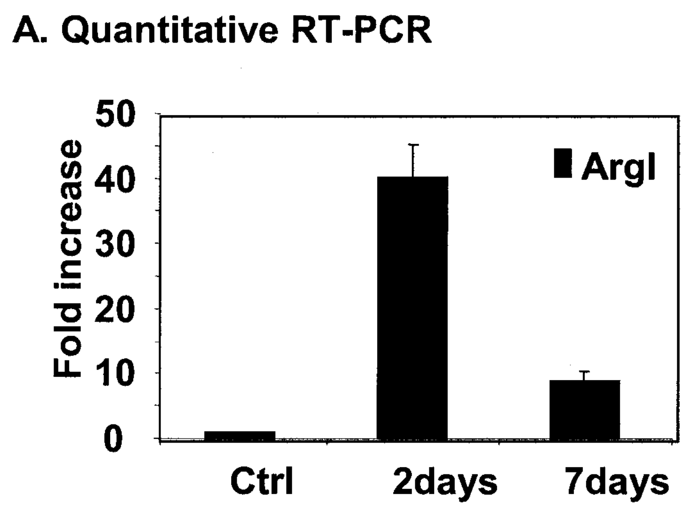
Results

ArgI and Polyamines Increased in Response to Conditioning Lesion

As we reported previously, after conditioning lesion, a three folds increase in cAMP level was observed at 1 day post sciatic nerve transection, and then the cAMP level went back to the basal level in 1 week (Cai 2001). Here, we want to address whether ArgI was up regulated in transcription and translation level after a peripheral lesion in response to elevated cAMP. Bilateral sciatic nerve transection was performed on 3-week old rats. After 1-7 day(s), DRGs located at lumber 4 and 5 (L4, 5) from both sides were isolated and lysed, and either RNA or protein was extracted. The extracted RNA was subjected to reverse-transcribed and then quantitative PCR (RT-PCR), using specific primers for ArgI. As can be seen in figure 3.3A, there is basal level expression of ArgI in DRGs. After a conditioning lesion, transcription of ArgI mRNA increased about 40-fold in two days and went down in 1 week, yet still was significantly higher than uninjured control (10 fold).

Futhermore, the expression of ArgI in injured L4, 5 DRGs was assessed at the protein level. At different time post sciatic nerve transaction L4, 5 DRGs were lysed and the extracted protein were subjected to SDS-PAGE before being transferred to membrane and immunostained with a polyclonal antibody against ArgI (the antibody was provided by Dr. Ratan from Harvard Medical School). Figure 3.3B showed that there is a band at the predicted molecular weight, 36kDa, for ArgI. ArgI expression was low in the uninjured control. One day post lesion, the amount of ArgI protein has increased significantly. By 2 days after lesion, ArgI levels were dramatically higher than the unlesioned control (Figure 3.3B) and stayed up for at least 1 week (data not shown). The same blot was stripped and re-probed with anti-actin as an internal loading control. And the actin level remained unchanged.

Therefore, these results indicated that there is a striking up-regulation of ArgI at both RNA and protein level after a conditioning lesion.



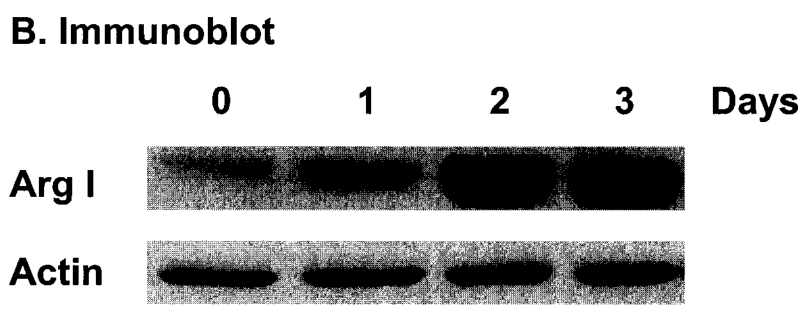


Figure 3.3 Transcription and Translation of ArgI went up after Conditioning Lesion.

The sciatic nerve bilateral transection was performed at PND19-21 rat. L4 and 5 DRGs were isolated after the indicated time. L4, 5 DRGs from uninjured animals were used as control. (A) RNA was obtained and subjected to RT-PCR using primers specific for ArgI. (B) Protein was extracted and equal amount of protein was loaded per sample to a 10% SDS-PAGE and immunostained with antibody for ArgI. The same blot was stripped and immunostained with antibody to actin as a loading control.

As mention earlier, ArgI is the rate-limiting enzyme for polyamines synthesis. A more direct approach to showing the alteration of polyamine metabolism was by measuring the content of polyamines correlated with the elevation of ArgI. To do this, L4, 5 DRGs were lysed at different time-points post-sciatic nerve lesion, then subject to HPLC assay (Done by Dr. Wu GuoYao at Texas A & M University). To our surprise, no change in polyamines levels was detected (data not shown). Since we know that the mRNA level of ArgI is high post-lesion in the ganglia, we thought the reason may lie in the way we collected the samples. In our experiment, we took the ganglia, which only included cell bodies of DRG neurons, instead of whole DRGs with its two branches to CNS or PNS. This interpretation of our observation is supported by that fact that polyamines were being axonally transported in regenerating axons to the lesion site, which is a distant from the cell bodies (Lindquist et al., 1985). So, we decided to culture the isolated DRG neurons overnight on poly-l-lysine coated plates to allow for the synthesis of polyamines. Then the samples were extracted and polyamines, putrescine, spermidine and spermine, were measured by HPLC respectively (Figure 3.4). The glutamate level for the sample was also measured and served as a protein control. At this time, a 2-fold increase was detected in both putrescine and spermidine at 2 days post lesion and remained significantly higher than uninjured control for up to 1 week. However, no significant change of spermine level was detected for up to 7 days after lesion.

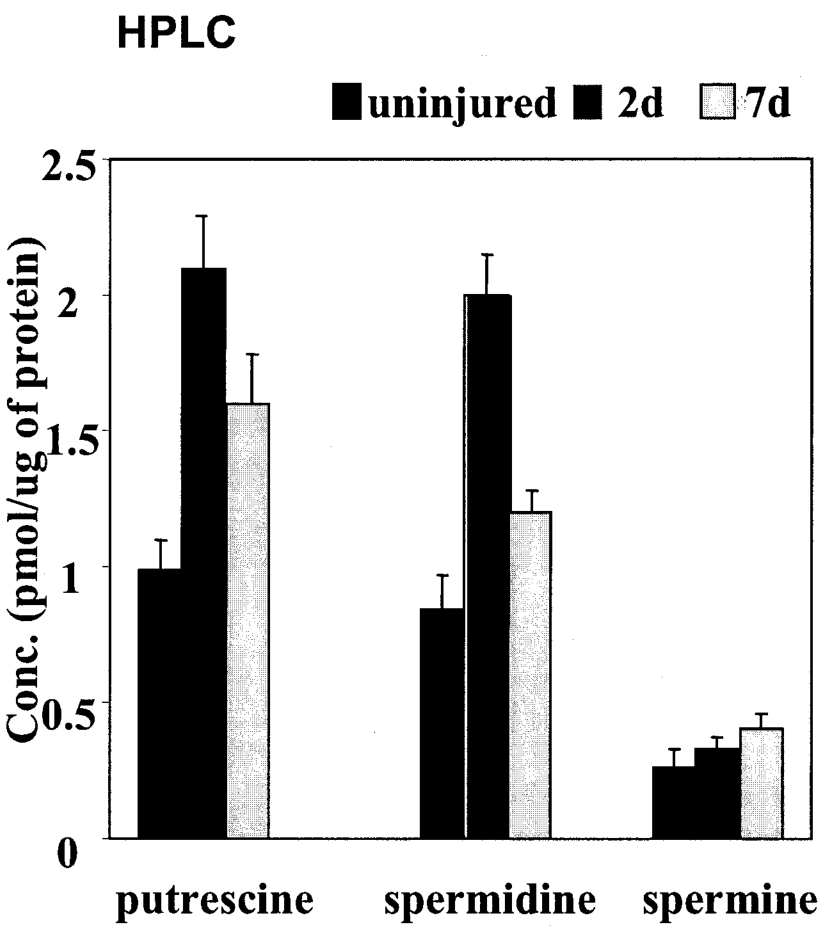


Figure 3.4 Synthesis of Polyamines is increased after Conditioning Lesion at Different Time Points.

L4, 5 DRGs from 2, or 7 day conditioning lesion rat were isolated and plated onto PLL coated plate. After overnight culture in SATO medium (Putrescine free), the cells were lysed and polyamines (putrescine, spermidine or spermine respectively) concentration in the lysate was measured by HPLC. Results are normalized by glutamate concentration for each sample and presented as the amount of polyamines per 1μg of glutamate. Each measurement was carried out in duplicate, at least two times.

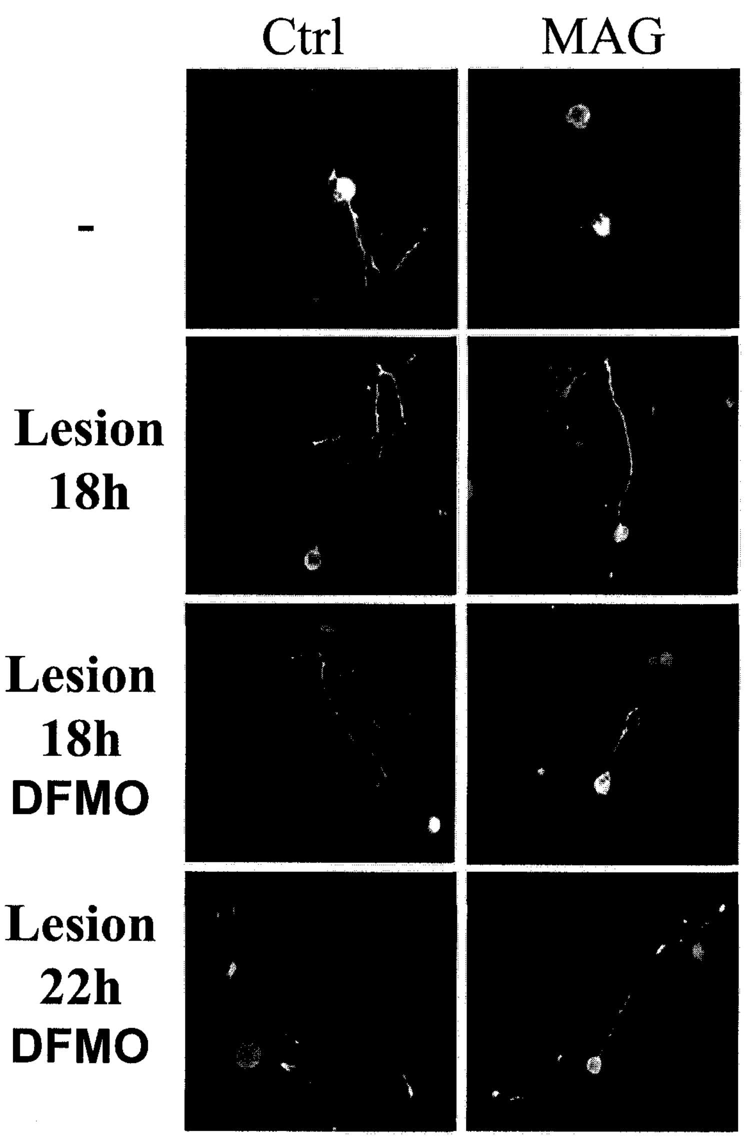
Polyamines are Required for the Conditioning Lesion Effect

Next, we want to determine if the ability of mature DRG neurons to grow on MAG after a conditioning peripheral lesion is dependent on ArgI/polyamines pathway. As we reported before (Mukhopadhyay et al., 1994), PND19-25 DRG neurons grown on CHO cells transfected to express MAG extend processes that are about 40% shorter than when grown on control CHO cells not expressing MAG. This inhibition by MAG is completely blocked if the peripheral branch of the DRG is lesioned 1 day prior to removal of the neurons from the animals and cultured on MAG (Figure3.5 A, B). Indeed, in addition to overcoming inhibition by MAG, there is a general improvement in growth as neurite length from these lesioned neurons on control CHO cells are 2 times longer than from uninjured nerve. DL-α-difluoromethylornithine (DFMO) is an irreversible inhibitor of the enzyme ornithine decarboxylase (ODC), which could almost deplete all three polyamines (Oredsson et al., 1980).While DFMO (5mM) was included in the culture, it abolished the conditioning lesion effect at 18 hours but was not able to block it 22 hours post lesion (Figure3.5 A, B). A similar study in the lab has also demonstrated that the improved growth on MAG can be block by DFMO at 16 hours post lesion, but cannot be blocked 1 week after lesion (Qiu J. unpublished data). In addition, DFMO has no effect on the neurite length of the uninjured control. This suggests improved growth after injury is initially dependent on polyamine synthesis but becomes independent with time. This may be due to the accumulation of polyamines reaching the peak/threshold 22h post lesion, which is sufficient to overcome inhibition.

In order to determine whether depletion of polyamines after sciatic nerve transection would abrogate the ability of DRG axons to regenerate, either ODC inhibitor, DFMO (0.5μmol/h), which equals to 1.1μg of DFMO per hour per gram of body weight, or saline was filled into a mini osmotic pump embedded under the skin and linked to cerebrospinal fluid (CSF) through a catheter, so that the solution was delivered to L4 and L5 level at the same time as the conditioning lesion was performed. After 1 day delivery of DFMO coinciding with the 1 day post-lesion, neurons extend shorter neurite on MAG expressing CHO cells than the non-expressing control (Figure 3.5B). That is to say, DFMO abrogated the improved growth on MAG and myelin by conditioning lesion. Again, DFMO had no effect on growth on control cells. As shown before, 1 week after conditioning lesion, in the absence of DFMO, there is a general improvement in growth. The neurite length doubled comparing to neurons from uninjured nerve. In figure 3.5B, delivery of DFMO for a week following the conditioning lesion attenuated the conditioning lesion effect on overcoming inhibition by MAG/myelin. The improved growth on MAG is about 30% less than growth of the same neurons on control CHO cells. In addition, the general improvement on growth in 1-week post-lesion neurons was not affected by the presence of DFMO.

Together, these results indicated that the elevation of polyamines is required for improved growth on MAG after conditioning lesion.

A.



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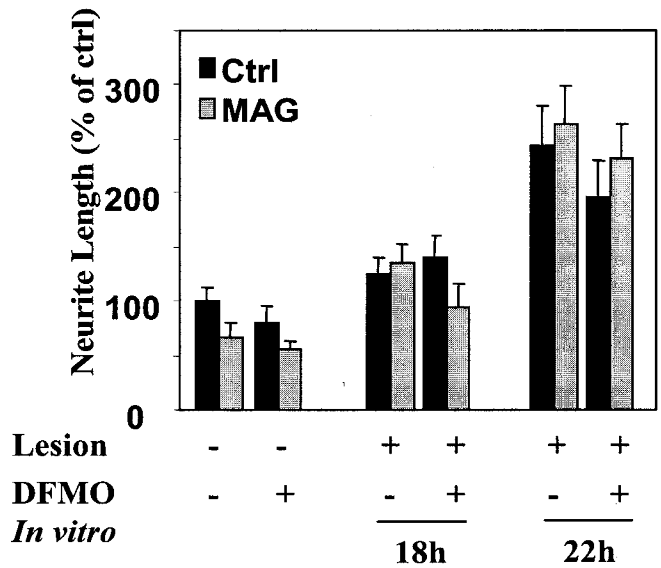

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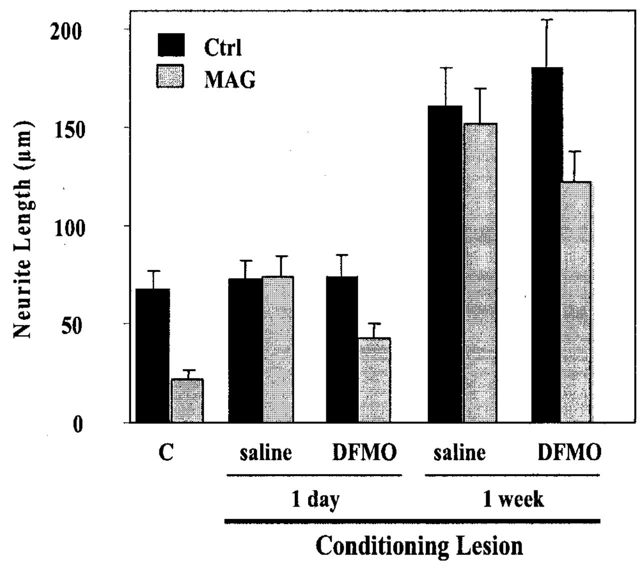


Figure 3.5 Conditioning Lesion Effect is DFMO Dependent at the Beginning and Becomes DFMO Independent with Time.

L4,5 DRG neurons were isolated from uninjured (control), 18h or 22h after conditioning lesion animals and then plated onto either MAG-expressing (blue bars) or control CHO cells (black bars) in the absence or presence of DFMO at 5mM. Neurons were cultured for a further 18hrs, then fixed and immunostained with anti-GAP43 (A). Results were quantitative by measuring the mean length of the longest GAP43-positive neurite for 180–200 neurons (± SEM) (B).

(C) Either DFMO (0.5μmol/h) or saline was intrathecal delivered into CSF with a sciatic nerves unilateral lesion and 1 day or 1 week later the L4, 5 DRG neurons were removed, dissociated and cultured overnight on either MAG expressing (blue bars) or control CHO cells (black bars). The neurons were fixed, stained and measured as mentioned above. C=uninjuried control.

Priming DRGs with Putrescine Overcomes Inhibition by MAG and Myelin in Culture

Although we knew that putrescine has a blocking effect on the inhibition by MAG/myelin in cerebellar neurons, its effect on DRGs remained unknown. So, before testing the impact of putrescine in conditioning lesion, we want to elucidate its effect on DRGs in culture. DRG neurons from post natal day 5 (PND5) pups were dissociated and incubated overnight with putrescine at different concentrations (100-400μM), so called priming, before being transferred to monolayer of CHO cells or CNS myelin. Consistent with what we had shown before, PND5 DRG neurons were inhibited by MAG compared to control CHO cells. Priming DRG neurons with putrescine is sufficient to block the inhibitory effect of MAG (figure 3.6 C, D) and has no significant increase in the growth on control cells (figure 3.6 A, B). The effect of putrescine is dose dependent. As can be seen from figure 3.6G, at 100μM, the growth on MAG was significantly improved. However, MAG still inhibited the neurite outgrowth by 20% compared to the control. Priming neurons with putrescine at 200μM is sufficient to block the inhibitory effect of MAG. At doses higher than 200μM, the blocking effect of putrescine on MAG saturates.

Furthermore, as growing evidence demonstrates that the three major myelin-associated inhibitors, MAG, Nogo-66 and OMgp, share the same neuronal receptor and the intracellular signaling pathway, we wanted to determine if putrescine would be able to block the inhibition by myelin in general. As reported before (DeBellard et al., 1996), DRG neurons from older animals extend either very short or no neurites at all when grown on myelin (Figure3.6E). In the presence of putrescine, however, neurons put out much longer processes on myelin (Figure 3.6F). Neurites are more than twice as long (Figure 3.6H). Furthermore, the effect of putrescine on improvement of neurite length is comparable to the effect of dbcAMP (Cai et al., 1999).

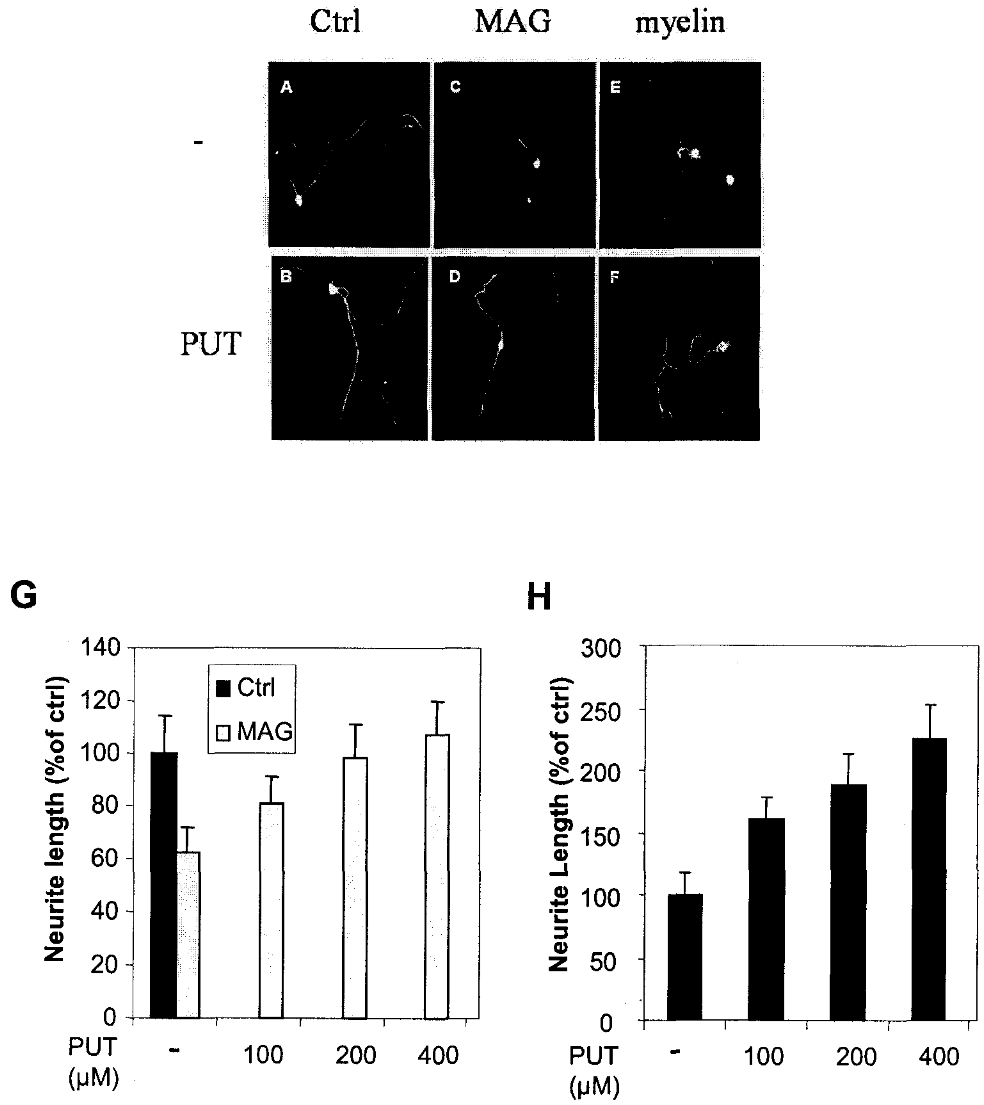


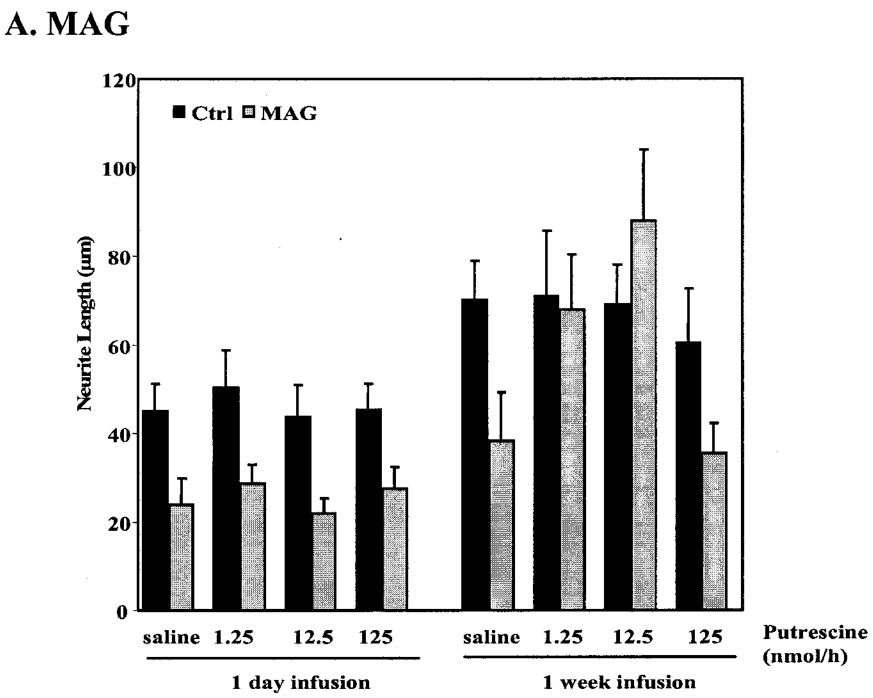
Figure 3.6 Priming DRG Neurons with Putrescine Overcomes the Inhibition by MAG and Myelin.

Dissociated PND5 DRG neurons are first primed overnight with or without the presence of putrescine (PUT) at the indicated concentration and then transferred to a monolayer of either MAG-expressing CHO cells (C, D), control CHO cells (A, B) or myelin (E, F). Neurons were cultured for16hrs, fixed and immunostained for GAP-43. Results in (G) and (H) showed the mean length of the longest neurite per neuron (±SEM) for 100-150 neurons, grown on either MAG or myelin respectively, data was presented as the neurite length percentage of the length of control.

Intrathecal Delivery of Putrescine Blocks Inhibition by MAG and Myelin

The question now raised is whether an increase in polyamines alone, without a peripheral lesion, is, sufficient to induce the ability to grow on MAG/myelin. To test this possibility, we delivered putrescine or saline intrathecally using an osmotic pump at the level of L4 and 5 to each postnatal day 35-40 rat to see whether it could allow axons to grow on MAG and myelin without the peripheral conditioning lesion.

Figure 3.7A shows that putrescine, in concentration range from 1.25nmol/h to 125nmol/h, which equals to 2.5 – 250ng /gram weight of animal /h, was now delivered for 1 day or 1 week before the removal of the DRGs from the animals. 1 day after infusion, at all three concentrations, MAG still inhibits growth. 1 week after infusion, however, inhibition by MAG was overcome by putrescine at 1.25nmol/h to 125nmol/h. Also, a general improvement of axonal growth was observed. At 125nmol/h putrescine, MAG inhibits neuronal extension again. This indicates the effect of putrescine is both time and dose dependent. Its effects augment at 1 week at 12.5nmol/h. To further establish the effect of putrescine on overcoming inhibitions, putrescine (12.5nmol/h) was intrathecal delivered to DRG neurons *in vivo* for 4 days before removing them for the neurite outgrowth assay on CNS myelin. Figure 3.7B showed that the neurite length of neurons from putrescine, delivered *in vivo,* doubled in comparing to the saline delivered control (70μm *vs* 27.97μm). The effect of intrathecal delivery of putrescine is comparable to the effect of either peripheral conditioning lesion or dbcAMP injection (Qiu et al., 2002b). Taken together, these results demonstrate that putrescine can induce an ability to overcome inhibitors in myelin.



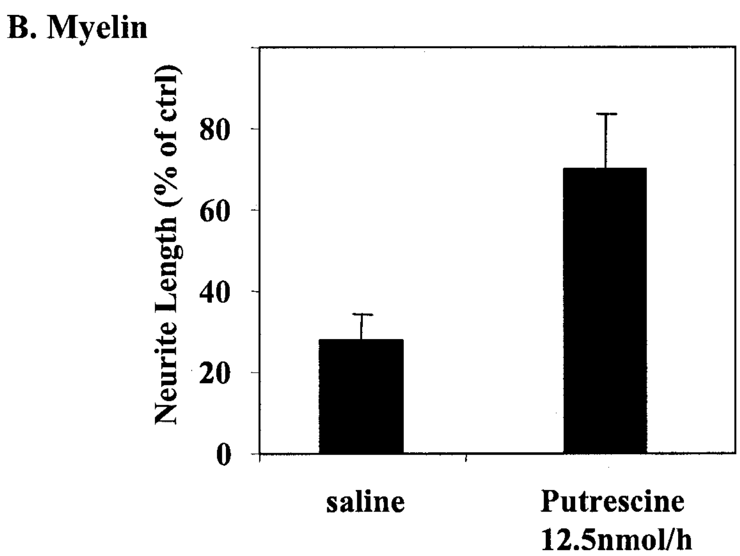


Figure 3.7 Intrathecal Delivery of Putrescine Overcomes Inhibition by MAG and Myelin.

Putrescine was delivered to cerebrospinal fluid (CSF) by an osmatic pump for 4 days. L4, 5 DRG neurons were dissociated and grown on either MAG expressing CHO (blue bars) or control CHO cells (black bars) (A) or purified CNS myelin (B). Axonal growth of putrescine delivered DRG neurons were compared to saline delivered control. After overnight incubation, neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

Discussion

This study elucidates the molecular consequences of a peripheral conditioning lesion that allows axons to grow through the hostile environment of the CNS and then to induce the same changes in all CNS axons tested to encourage them to regenerate. Following our findings that cAMP is a key player in this sequence of events (Cai et al., 1999; Cai et al., 2001; Qiu et al., 2002a), we demonstrated that the ArgI/PAs pathway was stimulated by elevated cAMP and polyamines in turn work as the downstream effector of cAMP to overcome myelin inhibition. The effects of a conditioning lesion are mediated at least partly by stimulation of cAMP levels and polyamines levels in sensory neurons.

We demonstrated here that ArgI/polyamines pathway plays a critical role in effecting regeneration *in vivo*. Stimulation of the ArgI/PAs pathway changed the intrinsic growth capacity of sensory ganglia neurons. Furthermore, incubation of DRG neurons with putrescine overnight or intrathecal delivery of putrescine for more than a day is sufficient to alter the neurons' response to MAG/myelin, which mimics the effect of either peripheral conditioning lesion or artificially elevating cAMP (Cai et al., 1999). Consistent with our results, Chu *et al* found that polyamines promote neurite regeneration following axotomy in cultured rat hippocampal neurons (Chu et al., 1995). In addition, it has been reported that exogenous polyamines can enhance neuronal survival and accelerate the rate of axon regeneration and functional recovery after sympathetic and sciatic nerve injuries (Gilad et al., 1996).

In support of what we observed, it had been shown that the activity of another rate-limiting enzyme for polyamines synthesis - ODC, which converts ornithine to putrescine, increased 8 hours after nerve transaction and reached maximum levels after 24 hours (3-fold increase) (Tetzlaff and Kreutzberg, 1985). In addition, ODC activity increased in the olfactory bulb after nerve section (Rochel and Margolis, 1980) and after spinal cord compression injury (Mautes et al., 1999). The increase of ODC activity, along with up-regulation of ArgI, could contribute to enhance polyamines metabolism as well.

Although we have interpreted our results as if they are due to stimulation of ArgI/PAs signaling in the neurons, we cannot exclude that the effect of either conditioning lesion or cAMP could have resulted from the response of nonneuronal cells, such as glia and Schwann cells, in the DRGs. Then, polyamines were up-regulated (Schreiber et al., 2004). There are several possibilities for these nonneuronal synthesis polyamines to bring about their effect to enhance neurite outgrowth. For example, Schwann cells have been clearly demonstrated to provide trophic support for neurons. Different cytokines, and neurotrophins (NTs), are produced and released by Schwann cells (Cornbrooks et al., 1983; Bunge, 1994; Torres et al., 2002). Although it has not been described in the literature, elevation of polyamines in Schwann cells might enhance the secretion of these trophic factors. Second, it might also be possible that polyamines are secreted by nonneuronal cells to the extracellular matrix and may be then taken up by neurons to exert their effect (Dot et al., 2004).

Base on the previous results (Cai et al., 2002), the effect of polyamines appears to be mediated at least partly, if not entirely, by its elevation in neurons. In that paper, by infecting neurons with ArgI expressed by adenoviruses, we clearly showed up-regulation of ArgI expression in neurons. Also, it has been shown that there are polyamine pools in motor, sensory, and autonomic neurons (Fujiwara et al., 1997). But, again, the polyamines synthesized by neurons could also be transported and secreted. For example, Ingoglia and colleagues founded that polyamines do not remain within regenerating axons following injury. They were transported axonally

(Ingoglia and Sturman, 1980) and transferred to cells surrounding the axons (Ingoglia et al., 1982). Hence, they could exert their effect by changing the behavior of the cells, such as macrophage and oligodendrocyte, in the lesion site. In a word, which cell type polyamines come from and how they exert their effects are still to be determined.

We suggested previously that a conditioning lesion improves growth in two phases (Qiu et al., 2002b). In the first phase, cAMP is elevated and improvement of growth on MAG and myelin is in a PKA dependent manner. In the second phase, cAMP level was back to control levels and growth on MAG and myelin increases and is PKA independent. Here, we showed that there is a transition from DFMO-dependent (16h) to DFMO-independent (1 day). This phenomenon might be due to a couple reasons. First, it might be because of stimulating the ArgI/PAs pathway for 1 day resulted in enough accumulation of polyamines to overcome inhibition. Second, a conditioning lesion activates more than one pathway, one or more of which might parallel the ArgI/PAs pathway and might be sufficient to promote growth on myelin. For example, data from our lab (Cao, 2003) indicated interleukin-6 (IL-6) is dramatically increased after lesion, and elevation of IL-6 levels by adding directly to the neuronal cultures overcomes the inhibition by MAG and myelin. Moreover, *in vivo* intrathecal or subcutaneous delivery of IL-6 promotes the regeneration of cultured adult DRG neurons to overcome MAG inhibition *in vitro* (Cao, 2003). In addition, it has been shown that the effect of IL-6 cannot be blocked by DFMO (Cao Z. unpublished data), which implicates that IL-6 overcomes inhibition in a pathway parallel to ArgI/PAs.

Observations by others also showed that regeneration of the frog sciatic nerves, along with daily injections of DFMO at the same time as crushing and continued throughout the regeneration period, resulted in reduced outgrowth of new sensory axons by about 30%. If DFMO injections started 2 days after crushing, the outgrowth distance did not differ from control values (Edbladh et al., 1990). These results are consistent with ours showing that there is a transition from dependence of ODC activity to independence of it. This indicates that DFMO influences the early onset of regeneration *in vivo*.

Furthermore, our results showed that if DFMO was infused for a week, along with a sciatic nerve lesion, the general growth promoting effect of a peripheral lesion remained. Neurons which grew in culture on MAG expressing monolayer were still inhibited. In contrast, the infusion of DFMO for a week does not change the neurite length on control CHO cells. This indicates a conditioning lesion initiates more than one event. Among them, one is to overcome inhibitors on myelin, which might be separated from the general growth promoting effect. However, the effect of polyamines seems specific to overcoming the inhibition by MAG/myelin.

In summary, our results provide evidence that the ArgI/PAs pathway is involved in a conditioning lesion. It works downstream of cAMP to bring about the effect of overcoming inhibition by MAG or myelin. In addition, putrescine enhances the growth capacity of injured axons to overcome inhibition. Putrescine may be a useful component of therapy to enhance axonal regeneration in the CNS.

Chapter IV

Spermidine, NOT Putrescine, is the Real Effecter among Polyamines in Overcoming Inhibition by MAG/Myelin

Introduction

Polyamines, namely putrescine, spermidine and spermine, are ubiquitous naturally occurring, small molecules that are positively charged at physiological Ph (Tabor and Tabor, 1984; Seiler and Bolkenius, 1985). Polyamine content is narrowly regulated by biosynthesis, degradation, uptake and transport systems. The *de novo* synthesis of polyamines (Figure 4.1) requires ornithine decarboxylase (ODC) to convert ornithine to putrescine. Subsequently then spermidine and spermine originate from the successive transfers of amino-propyl moiety of decarboxylated S-adenosylmethionine to the secondary amines of putrescine by spermidine synthase and spermine synthase respectively (Morgan, 1998). Also, spermine and spermidine can be retroconverted to spermidine and putrescine, respectively, by the polyamine inter-conversion pathway (Seiler et al., 1981; Grillo, 1985; Seiler, 1990).

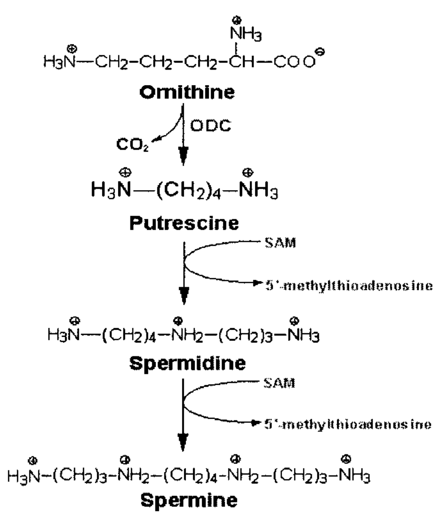


Figure 4.1 Biosynthesis of Polyamines

Although most studies of polyamines looked into their function in growth and differentiation (Fozard et al., 1980; Tabor and Tabor, 1984; Pegg, 1988), cumulative evidence indicates that polyamines serve specific functional roles in mature cells (Seiler, 1991; Gilad and Gilad, 1992). It is postulated that after trauma, the transient induction of polyamine metabolism is essential for neuronal survival (Gilad and Gilad, 1992, 2003).

It has been shown by our lab that priming neurons with putrescine is sufficient to block the inhibitory effect of MAG or myelin on axonal outgrowth (Qiu et al., 2002a). Also, the results from Chapter III indicated that ArgI/polyamines pathway is downstream of cAMP and putrescine is required and sufficient for the improved growth capacity of DRGs after a peripheral lesion. We noticed from our polyamine measurement data that only putrescine and spermidine, but not spermine were elevated in response to dbcAMP (Qiu et al., 2002a) or a conditioning lesion (Figure 3.4). The question raised is whether all three polyamines bring about their effect(s) through a common mechanism? In searching the literature for possible answers, we came to the conclusion; so far, there is no agreement on this issue.

Chu and colleagues observed the effect of putrescine, spermidine and spermine on promoting axonal elongation following axonal injury of cultured rat hippocampal neurons (Chu et al., 1995). They discovered that the effects of these three polyamines were very similar, and no additive effect was observed when maximally effective concentrations of polyamines were added together, suggesting that they acted through a common mechanism (Chu et al., 1995). Also, all exogenously administered polyamines have been reported to promote functional recovery of autonomic motor neurons after an axonal crush (Dornay et al., 1986) and enhance survival of sympathetic neurons after axonal trauma (Gilad and Gilad, 1988).

In rat cerebellar granule neurons, all three polyamines prevented cell death induced by high KCl concentration (Harada and Sugimoto, 1997).

On the other hand, it has been shown lately that each polyamine also has different functions, although those specific functions are still obscure. For example, Yuan and colleagues showed that putrescine, by itself, is not essential for migration and growth of intestinal epithelial cells, but it seems that conversion into spermidine and/or spermine is required (Yuan et al., 2000).

More importantly, several observations reported a difference in polyamine response after trauma (Adibhatla et al., 2002; Gilad and Gilad, 2003). During maturation, the CNS, unlike the PNS, undergoes changes which result in the expression of a so called "incomplete polyamine response" after trauma. This may be due to the change of activity of two key enzymes in polyamine metabolism, namely, an increase in production through ODC and a decrease in metabolism through S-adenosylmethionine decarboxylase activity (Kleihues et al., 1975; Dienel et al., 1985) or the activation of the interconversion pathway through the polyamine oxidase (PAO) (Dogan et al., 1999b; Dogan et al., 1999a). Induction of this partial polyamine response after injury results in a sustained accumulation of putrescine, whereas the conversion of putrescine to spermidine and spermine is inhibited. Looking at normal physiological concentrations, spermidine and spermine in the brain are normally high, while that of putrescine is very low (Seiler and Bolkenius, 1985). It was suggested previously that a persistent stress-induced accumulation of putrescine may be harmful (Paschen et al., 1988). However, administration of spermidine or spermine to normalize polyamine levels could alleviate the detrimental effect of putrescine (Hatcher et al., 2003).

Accumulating evidence shows different function of individual polyamines in proliferation, differentiation, survival and injury. For instance, among the polyamines presumably spermidine is the most important in the regulation of polyamine homeostasis (Seiler et al., 1996). Depletion of putrescine and spermidine at normal spermine concentration prevents cells from growing (Pegg, 1988; Seiler and Dezeure, 1990; Morgan, 1998). Putrescine and spermidine, but not spermine, are preferentially released by the injured brain (Seiler et al., 1996). Spermine has survival-promoting effects at a very low concentration, but spermidine and putrescine showed no significant effect on neuronal survival (Chu et al., 1994). Putrescine but not spermidine or spermine stimulated neurogenesis, while spermidine and spermine but not putrescine stimulated neuronal differentiation in the house cricket (Cayre et al., 2001). Intracellular or extracellular spermine has been found to induce block and modulation of a number of types of ion channels (Williams, 1997a), but spermidine and putrescine are less likely to be able to do that. In a word, these findings indicated that all three polyamines might work in a different fashion.

It's obvious that if neurons were primed with putrescine, using available enzymes for polyamines' synthesis and inter-conversion in the cell, putrescine could be converted to spermidine and then spermine. Ingoglia's group reported, on the basis of isotope transport studies, approximately 84% of the injected putrescine was converted by cells in the ventral spinal cord to spermidine, with minor amounts being converted to spermine (Lindquist et al., 1985). Other groups have observed that daily injection of either spermine or spermidine enhances regeneration of peripheral somatic nerves (Sebille and Bondoux-Jahan, 1980; Kauppila et al., 1988; Kauppila, 1992).

Based upon our findings in chapter III that ArgI/PAs pathway played a critical role in neuronal regeneration, it would be interesting to further clarify the distinct role that each polyamine plays in overcoming inhibition by MAG and myelin in general. In the following study, a pharmacological approach was used to block the conversion from putrescine to spermidine, which resulted in the accumulation of putrescine. The effect of putrescine in overcoming inhibition by MAG/myelin was abolished when its conversion to spermidine was blocked. Furthermore, priming neurons or intrathecal delivery of spermidine for more than 1 day could alter the neurons' response to MAG and myelin.

Results

An Inhibitor of Spermidine Synthase Effectively Blocks the Conversion of Putrescine to Spermidine

Spermidine synthase forms spermidine by transfer of aminopropyl residues from decarboxylated S-adenosylmethionine onto putrescine (figure 4.1). To determine whether putrescine by itself is sufficient to overcome the inhibition by MAG and myelin, bis-cyclohexylammonium sulphate (BCHS), an inhibitor of spermidine synthase was used to block the conversion of putrescine to spermidine (Caruso et al., 1992; Caruso et al., 1994). First, the efficiency of BCHS in blocking the conversion of putrescine to spermidine in the cells was assessed by measuring the polyamines' concentration after incubating neurons with or without dbcAMP or BCHS. In figure 4.2, PND5 cerebellar neurons were cultured overnight in the presence of either 1mM dbcAMP, 200μM BCHS or both before extracting with perchloric acid and subjected to HPLC. As reported before, both putrescine and spermidine level doubled by overnight incubation with dbcAMP (Qiu et al., 2002a). Overnight incubation of neurons with BCHS resulted in a 5-fold increase in putrescine, as we expected. In addition, if BCHS was co-cultured with dbcAMP, BCHS blocked the effect of dbcAMP in the aspect of spermidine elevation. That is to say, incubation of neurons with BCHS leads to accumulation of putrescine in the cells, with or without the presence of dbcAMP, and no significant change of either spermidine or spermine content was detected.

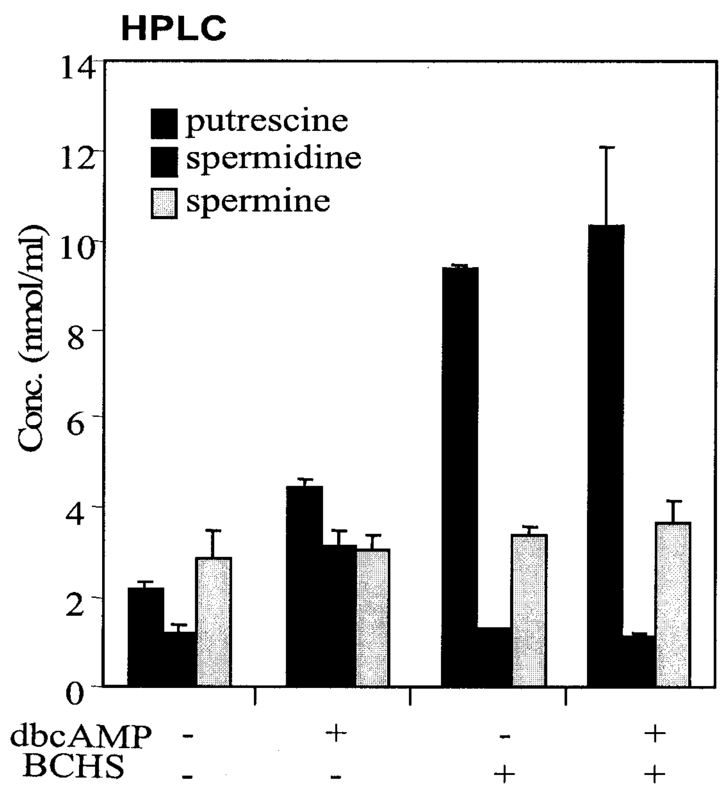
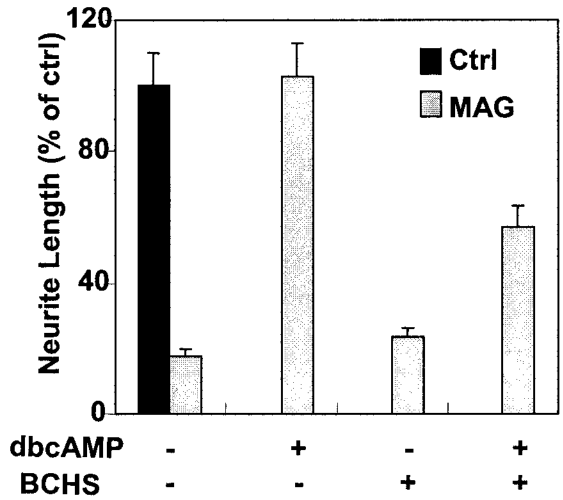


Figure 4.2 Incubating Neurons Overnight with Spermidine Synthase Inhibitor – BCHS Leads to Accumulation of Putrescine.
Cerebellar neurons (PND5) were plated onto poly-L-lysine coated plates and treated with dbcAMP (1mM), BCHS (200μM), or both for 18h and then lysed and putrescine, spermidine or spermine content was measured by HPLC. Each measurement was carried out in triplicate, at least two times.

BCHS Abrogated the Effect of dbcAMP and BDNF in Overcoming Inhibition by MAG and Myelin

Priming neurons with putrescine is sufficient to overcome the inhibition by MAG or myelin in general (Qiu et al., 2002a). However, the machinery for polyamine interconversion is available to almost all cells (Tabor and Tabor, 1984; Pegg, 1988), meaning that priming neurons with putrescine could result in the formation of other polyamines. The exogenously applied putrescine most likely would be converted to spermidine and further to spermine (Sturman et al., 1976). Here, we want to determine whether the conversion of putrescine to spermidine is a step required to bring about the ability of putrescine to overcome inhibition by MAG and myelin. We proposed that if putrescine is the effector in this process, it would be reasonable to speculate that increasing putrescine levels by incubating neurons with BCHS would overcome inhibition by MAG, which is the same effect as priming neurons with putrescine to extend longer neurite on MAG expressing cells. In figure 4.3, as we showed before, the growth of the neurites on MAG-expressing CHO cells is 60%-80% shorter compared to the control CHO cells not expressing MAG. The presence of 1mM dbcAMP or priming neurons with 200ng/ml BDNF completely overcomes the inhibition by MAG (Cai et al., 1999). However, BCHS (200μM), priming or non-priming, alone has not altered the neurite length on MAG-expressing cells (figure 4.3 A, B). Furthermore, while BCHS was added along with dbcAMP or included in the priming with BDNF, it partially abrogated the effect of both dbcAMP and BDNF in overcoming MAG's inhibition, with neurite length on MAG expressing cells being 40-50% shorter than control. Therefore, elevation of putrescine alone, without changing the level of spermidine, is insufficient to overcome the inhibition by MAG. That is to say, the conversion of putrescine to spermidine is required.

A



B

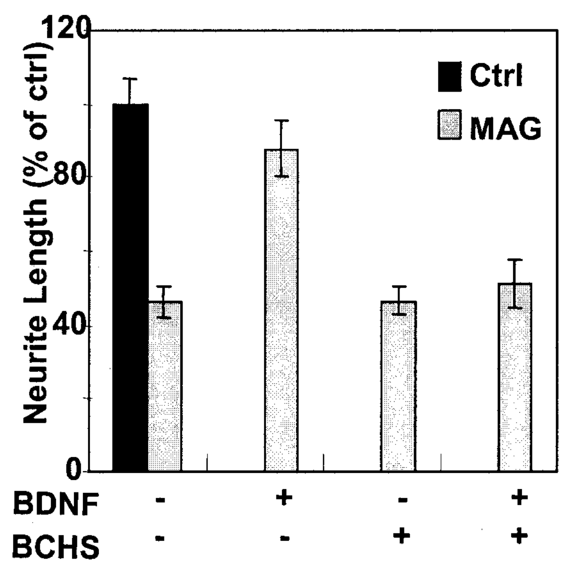


Figure 4.3 BCHS Abolished the Effect of Either dbcAMP or BDNF in Overcoming Inhibition by MAG.

Dissociated cerebellar neurons (PND5) were plated (A) directly with dbcAMP 1mM BCHS 200μM or both; (B) incubated with BDNF 200ng/ml, BCHS 200μM or both overnight before transferring onto monolayer of MAG-expressing (blue bars) or control (black bars) CHO cells and cultured overnight. Neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

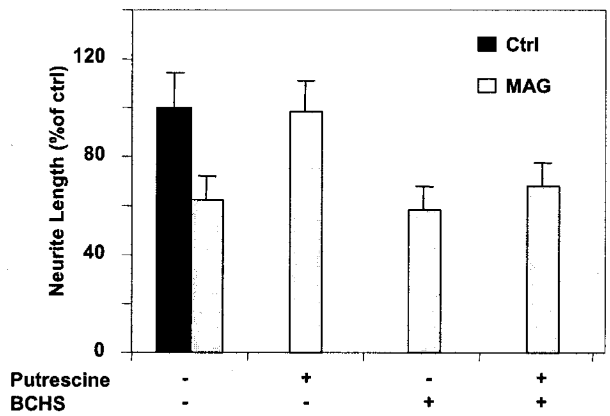
BCHS Blocks the Effect of Putrescine

The *in vitro* results presented above indicated that elevation of putrescine solely, but not spermidine and spermine, could not overcome inhibition of MAG. Next, we want to determine if we exogenously applied putrescine and together with the spermidine synthase inhibitor, BCHS, would this also block the improved neurite growth on MAG or myelin. To test this, BCHS was included along with putrescine while priming DRG neurons for the neurite outgrowth assay. As we had shown before, priming neurons with putrescine completely overcomes the inhibition by MAG. Priming neurons with BCHS does not change the neurite length on MAG expressing CHO cells. When BCHS was included along with putrescine, it abrogated the effect of putrescine in overcoming inhibition by MAG (Figure 4.4A). The same sets of experiments were carried out on CNS myelin. Consistent with the results with MAG, neurons extended short neurites on myelin. Neurite length was doubled by priming neurons with putrescine. However, priming with putrescine no longer promoted neurite outgrowth in the presence of BCHS (Figure 4.4B).

To further elucidate that elevation of spermidine is a critical step to bring about the effect of intrathecal administration of putrescine, 10nmol/h BCHS (30ng/g/h) was delivered, alone or along with putrescine, utilizing an osmotic pump to adult rats for 4 days before the removal of L4 and 5 DRGs and then grown on either MAG expressing or control CHO cells. As we demonstrated in the previous chapter, intrathecal delivery of putrescine can mimic the peripheral conditioning lesion and allows the DRG neurons to overcome inhibition by MAG and by myelin in general. In figure 4.5, MAG inhibits neurite outgrowth of either saline or BCHS delivered DRGs. If BCHS was delivered together with putrescine, it blocks the effect of putrescine both in overcoming MAG's inhibition and in improvement of general growth capacity completely.

These results suggest that elevation of spermidine, but not putrescine, is important in overcoming the inhibition by MAG or myelin and improved the growth capacity in both permissive and inhibitory environments *in vitro*.

A. MAG



B. Myelin

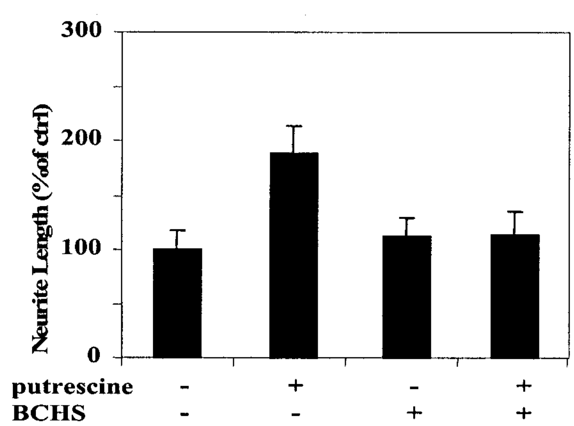


Figure 4.4 Priming Effect of Putrescine can be blocked by BCHS in Culture.
DRG neurons were dissociated and plated onto PLL-coated 24-well plates in the presence of putrescine 200μM, BCHS 200μM or both. After overnight incubation, neurons from each treatment were trypsinized and transferred onto monolayers of MAG-expressing (blue bars) or control (black bars) CHO cells (A) or myelin (B). Neurons were fixed and immunostained with anti-GAP43 antibody. The longest

neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

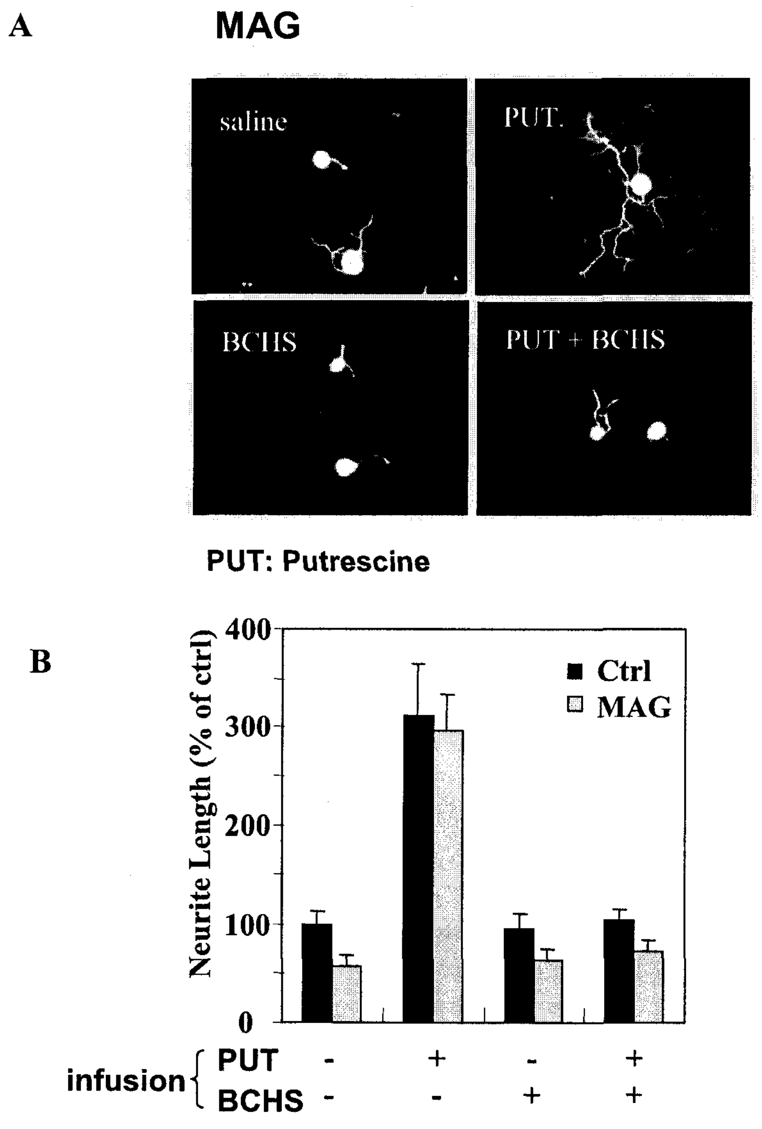


Figure 4.5 Infusion of BCHS along with Putrescine abolished the Effect of Putrescine in Overcoming Inhibition by MAG.

Saline, putrescine (12.5nmol/h), BCHS (10nmol/h) or both was put into a mini osmotic pump and delivered intrathecally for 4 days before the neurons from L4 and L5 DRGs were removed and grew on either control or MAG expressing CHO. (A) Neurons were fixed and immunostained with anti-GAP43 of control or putrescine or BCHS administrated adult DRG neurons grown on MAG-expressing CHO cell monolayer. (B) Quantification of neurite extension of saline, putrescine or BCHS administered adult DRG neurons. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

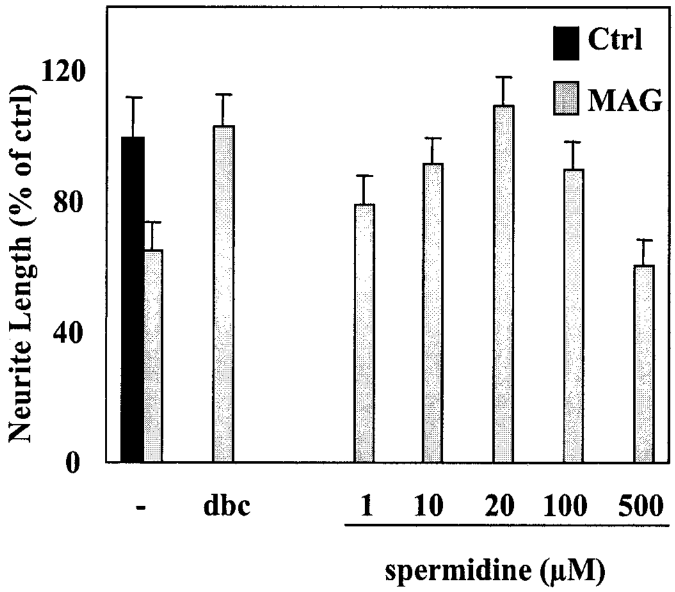
Priming Neurons with Spermidine Overcomes Inhibition by MAG and Myelin in a Dose-dependent Manner

The question now is whether or not priming neurons with spermidine can overcome the inhibition by MAG and myelin. PND5 DRG neurons were primed with spermidine at different concentrations (1-500μM). Then the neurons were transferred and grown on MAG-expressing cells, control cells, and on myelin. As can be seen in figure 4.6A, priming neurons with spermidine can indeed block the inhibition by both MAG and myelin in a dose-dependent manner, with the maximum block of inhibition reached at a concentration around 20μM. Spermidine alone had no significant effect on neurite outgrowth from neurons grown on control cells or on poly L-lysine (results not shown).

Likewise, growth on myelin improved in a dose-dependent manner (Figure 4.6B), at a concentration of 20μM, neurites were about twice as long if neurons were primed with spermidine. This is even greater than that observed when db cAMP is added directly to the media or the neurons were primed with BDNF (Cai et al., 1999).

Furthermore, if BCHS was included in the priming with spermidine, the effect of spermidine in overcoming inhibition of MAG remained (Figure 4.7). In a word, elevation of spermidine is required and sufficient to overcome inhibition by MAG and myelin in general.

A. MAG



B. Myelin

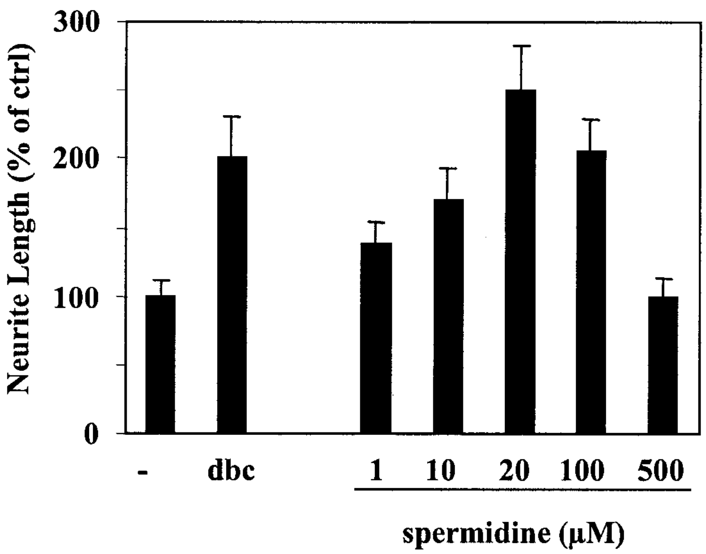


Figure 4.6 Priming Neurons with Spermidine Overcome Inhibition by MAG and Myelin in a Dose-dependent Manner.

DRG neurons were dissociated and plated onto PLL-coated 24 well plates in the presence of different concentration of spermidine (1-500μM). After overnight incubation, neurons from each treatment were trypsinized and transferred onto (A) monolayers of MAG-expressing (blue bars) or control (black bars) CHO cells or (B) purified CNS myelin and cultured overnight. Neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained. dbc:dbcAMP.

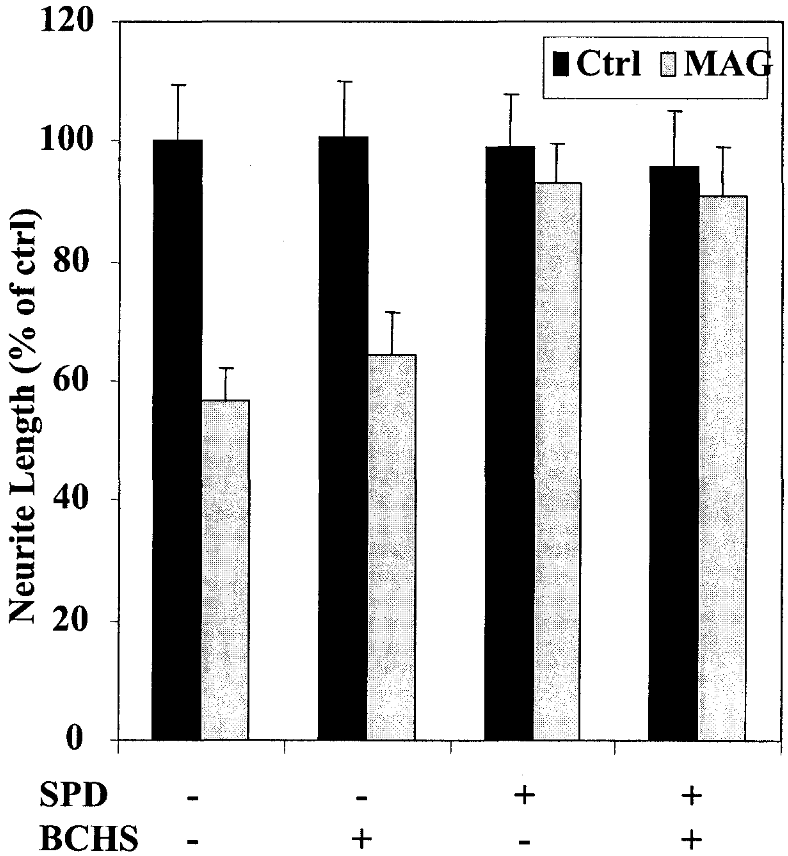


Figure 4.7 BCHS cannot block the Effect of Spermidine *in vitro*

DRG neurons were dissociated and plated onto PLL-coated 24 well plates in the presence of spermidine 20μM, BCHS 200μM or both. After overnight incubation, neurons from each treatment were trypsinized and transferred onto monolayers of MAG-expressing (blue bars) or control (black bars) CHO cells. Neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained. SPD: spermidine

Intrathecal Delivery of Spermidine is Sufficient to Overcome Inhibition by MAG and Myelin

It has been demonstrated in our lab that the growth of dissociated adult DRG neurons on substrates of CNS myelin or MAG-expressing CHO cells parallels very well the regenerative capacity of dorsal column fibers in the injured adult spinal cord. Therefore, the growth of adult DRG neurons *in vitro* may represent the regeneration of corresponding spinal cord axons *in vivo*. In the previous study, we established that the abrogation of MAG-mediated neurite outgrowth inhibition by putrescine requires its conversion to spermidine. Based on this, we next assessed the potential role of spermidine in the regeneration of adult DRG neurons *ex vivo*. To test this, each postnatal day 35-40 rat was implanted with an osmotic mini-pump filled with 25nmol/h spermidine, which equals 40ng per gram of body weight per hour, or saline. Spermidine was delivered directly into the CSF with an attached cannula inserted beneath the dura membrane between L4 and L5. After 4 days delivery, DRGs located at L4 and L5 from both sides were removed, dissociated and grown overnight on a monolayer of MAG expressing or control CHO cells (figure 4.8A), or myelin (figure 4.8B). Figure 4.8 shows that there is extensive neurite outgrowth in DRG neurons taken from spermidine treated animals, overcoming the inhibition by MAG and improving growth on myelin. In addition, comparing the neurite length of DRG neurons from saline treated to spermidine treated rats; the latter neurons bear much longer neurites on control CHO cells. This means, in addition to overcoming inhibitors of regeneration in myelin, there is a general promotion of growth, which mimics the effect of conditioning lesion, dbcAMP injection or putrescine treatment.

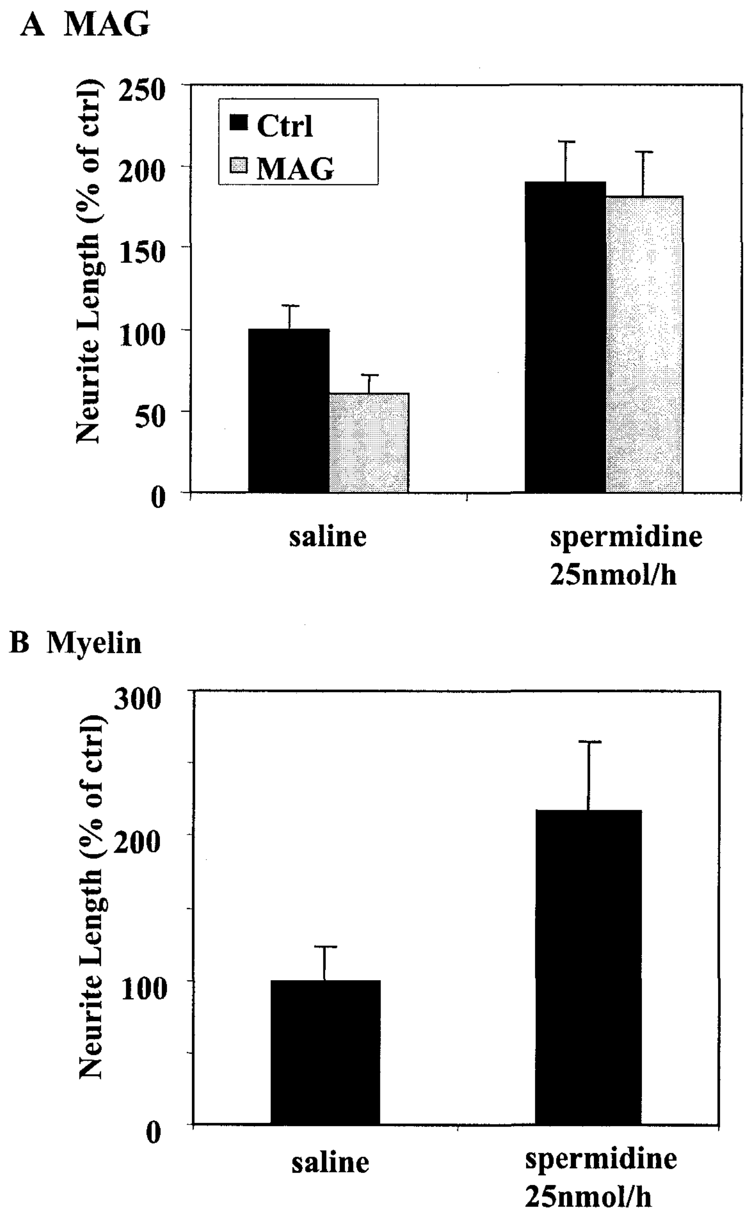


Figure 4.8 Intrathecal Delivery of Spermidine Overcomes Inhibition by MAG and Myelin.

Spermidine at 25nmol/h was delivered to CSF by an osmotic pump for 4 days. L4 and L5 DRG neurons were dissociated and grown on either MAG expressing CHO or control CHO cells (A) or purified CNS myelin (B). Axonal growth of spermidine delivered DRG neurons were compared to saline delivered control. After overnight incubation, neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

Discussion

Previously we showed that increasing the levels of cAMP and subsequent ArgI/PAs elevation is critical for overcoming inhibition by MAG and myelin (Cai et al., 1999; Qiu et al., 2002a). Along with the work in our lab, data in this chapter highlighted the importance of spermidine in overcoming inhibition by MAG and myelin. The effect of putrescine mainly, if not solely, comes from it conversion to spermidine. If the synthesis of higher polyamines, spermidine and spermine, are blocked, the inhibition of MAG remained. In addition, priming or infusing animals intrathecally with spermidine is sufficient to promote neurite outgrowth on MAG or myelin.

In our experiments, BCHS was used to elevate the endogenous putrescine and block the conversion of putrescine to spermidine. Although there are many factors involved in regulation of spermidine content in the cells, the HPLC data (figure 4.2) clearly indicated that BCHS is an effective inhibitor and it is sufficient for this purpose. Consistent with us, Caruso *et al* noticed administration of BCHS to *in vitro* cultures of chick embryo fibroblasts caused a decrease in cellular spermidine levels and an increase in putrescine (Caruso et al., 1992). The effect of putrescine, added exogenously, is blocked completely by BCHS (figure 4.4 and 4.5). The specificity of BCHS has not been addressed in our hands and has not been reported. Based on the similarity of spermidine synthase and spermine synthase, it might also inhibit spermine synthase. However, the ability of spermidine to overcome inhibition remained (figure 4.7) and the lack of change of spermine levels (figure 4.2) in the presence of BCHS argued against its non-specificity.

To date, we indicated that elevation of putrescine and spermidine by dbcAMP, BDNF, a conditioning lesion or exogenous application of putrescine can alter the response of neurons to MAG/myelin. In all the experiments with polyamines we performed, however, no significant change in spermine level was detected. Consistent with our findings, Boeshore *et al* observed a marked increase in the level of spermidine and its precursor putrescine, but not spermine following axotomy of a sympathetic ganglion (Boeshore et al., 2004). However, it has been reported that spermine promotes regeneration of injured axons of cultured rat hippocampal neurons (Chu et al., 1995) and injection of spermine accelerated motor function recovery (Sebille and Bondoux-Jahan, 1980; Kauppila et al., 1988). It was reported that the interconversion pathway was active in their experiment. The effect of spermine that they observed might come from spermidine. Polyamines, in particular spermine, had been reported to be responsible for the intrinsic gating and rectification of strong inward rectifier K^+ channels by directly plugging the ion channel pore and modulation of NMDA receptors (Williams, 1997b). So far, no evidence showed any link between activities of these channels and axonal regeneration. One other possibility is that the change in spermine level is restricted to certain regions such as the growth cone, which was not detected by an overall assay. In a word, whether or not spermine is critical in overcoming inhibition by MAG remains a question to be addressed.

What would be the target(s) for polyamines to exert their effect? One of the possibilities is that polyamines may exert their effects on the cytoskeleton integrity. Kaminska *et al* showed that inhibitors of polyamine biosynthesis prevent mitogen-induced accumulation of mRNAs encoding β-actin and α-tubulin in mouse splenocytes (Kaminska et al., 1992). Caruso *et al* observed that BCHS provoked alterations in fibroblasts' morphology, a marked disorganization of microfilaments and changes in microtubule network structure. They suggested spermidine played a role in microtubule and microfilament organization (Caruso et al., 1994). Although no morphological changes of neurons with the addition of polyamines or their inhibitors, DFMO or BCHS, was observed in our experimental conditions, we cannot rule out the possibility that there still might be differences in the neuroskeleton arrangement by these treatments.

Other targets of polyamines include transcription and translation. For example, eIF5A, a translational initiation factor involved in the turnover of mRNA (Kemper et al., 1976), contains a unique spermidine-dependent hypusine modification on a specific lysine residue (Cooper et al., 1983). Thus, spermidine may play a role in gene expression through degradation of inactive mRNAs (Igarashi and Kashiwagi, 2000).

By testing the effects of several synthetic analogues of either putrescine or spermine, Abe *et al* found that the butanediamine moiety is essential for the activity (Abe et al., 1997). Lately, Jarvinen and colleagues studied the metabolically stable polyamine derivatives (Jarvinen et al., 2004) α-methylspermine and bis- α-methylspermine. Both were readily converted to α-spermidine and spermidine in liver. This conversion could be blocked by PAO inhibitors and spermidine/spermine-N1-acetyltransferase (SSAT) inhibitors. And both of them can overcome the proliferative block of liver regeneration by DFMO. In addition, two reports pointed out that spermidine, but not spermine, has a critical role in the regeneration of liver (Alhonen et al., 2002; Rasanen et al., 2002), which supports the importance of spermidine in liver regeneration.

Whether or not polyamines can cross the blood brain barrier is still a matter of debate. Shin *et al* showed that transport of putrescine is mainly due to passive diffusion (Shin et al., 1985). The blood brain barrier restricts the exchange of spermidine and spermine between blood and brain (Shin et al., 1985), however, putrescine is taken up by the brain and rapidly transformed within the brain into spermidine and spermine (Fischer et al., 1972). To avoid this issue, in our experiments polyamines and inhibitors of polyamines synthesis were all delivered intrathecally.

In conclusion, this study suggests that among the three polyamines the elevation of spermidine is critical in overcoming inhibition by MAG and myelin. This might facilitate the search for downstream effectors of polyamines to foster better growth on myelin. Also, it might shed some light on the potential development of therapeutic strategies to encourage axonal regeneration after CNS injury *in vivo* by developing a stable, blood brain barrier permeable analogue of spermidine.

Chapter V

Role of Microfilaments and Microtubules in Overcoming Inhibition by Myelin-Associated Glycoprotein

Introduction

The cell's cytoskeleton is a continuously changing fabric of protein filaments underneath the cell membrane. Beyond giving a cell mechanical strength, the cytoskeleton is the actual executor of most biological events that require a change in a cell's shape or motility. The cytoskeleton is the major intracellular determinant of neuronal morphology and is required for fundamental processes during the development and maintenance of a neuron. It is composed of three elements: microtubules (MT), microfilaments (MF) and intermediate filaments.

Elongating axons terminate in a protuberance called the growth cone. Growth cones have three main regions (figure 5.1): the central core, filopodia and lamellipodia. The central core is rich in MT. Projecting from the body are long slender extensions called filopodia. These rod-like, actin-rich, membrane-limited structures are highly motile. The sensory capability of the growth cone depends in large part on its filopodia. Between the filopodia are lamellipodia, which are also motile and give the growth cone its characteristic ruffled appearance (Kandel et al., 1991).

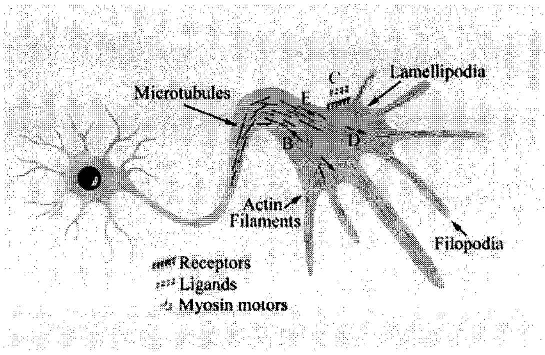


Figure 5.1 Growth Cone. (Goldberg, 2003)

There are several steps in movement of the growth cone and axonal outgrowth. First, a filopodium contacts an adhesive surface/cue and contracts, thus pulling the growth cone forward. Actin filaments assemble at the leading edge of a filopodium, disassemble at the trailing edge, and interact with myosin along the way (Forscher and Smith, 1988). Force generated by the retrograde flow of actin and its subsequent polymerization pushes the filopodium forward. The actin polymers are linked to adhesion molecules on the plasma membrane. Second, the combined action of all the motors creates an actin poor space into which the MT from the central core advance (Lin and Forscher, 1995). Third, the MTs form a bundle, and the cytoplasm collapses around them to create a new length of axonal shaft.

Actin filaments exhibit a retrograde flow that moves filaments from the peripheral edge of the growth cone, where actin is assembled, to the central domain of the growth cone, where actin is disassembled. The balance of anterograde polymerization and retrograde retraction determines the advance of these actin-rich structures in the peripheral domain (Lin et al., 1996). However, whether disruption of MFs in the growth cone facilitates outgrowth is a matter of debate. It has been reported that growth cones treated with cytochalasins, a group of MF destabilization drugs, *in vivo* and *in vitro* advance extremely slowly and have abnormal morphologies that lack lamellipodia and filopodia (Schindelholz and Reber, 1999). Some groups indicated that cytochalasins paralyze normal growth cone motility and inhibit axonal elongation (Letourneau et al., 1987). Other groups have found that the disruption of the cortical actin network is the pivotal step in process formation (Baorto et al., 1992). It has been suggested that particles larger than 7nm cannot penetrate into actin filament structures such as stress fibers and the leading edge of fibroblasts (Luby-Phelps et al., 1986; Luby-Phelps et al., 1987; Luby-Phelps and Taylor, 1988). This is consistent with the concept that structures as large as microtubules (25μm) cannot assemble in regions rich in actin filaments because the three-dimensional mesh is too small. Given these findings, MT assembly and growth cone movement would then likely require the disruption of MF. Shelanski's group has indicated that dbcAMP can induce the formation of processes in cultured primary astrocytes and that cytochalasin B can mimic the effect of dbcAMP (Baorto et al., 1992). They then introduced the "spring-loaded" hypothesis – the assembly of MT is counterbalanced by a physical barrier formed by F-actin in the cell cortex. In addition, neuro-2a cells form thin, unbranched neurites when treated simultaneously with taxol, a MT stabilizer and cytochalasin D (CD), a MF destabilizer (Spero and Roisen, 1985).

Tubulin is known to undergo several post-translational modifications, such as glutamylation (or detyrosination) and acetylation, that accumulate in stable but not dynamic MTs. Newly assembled MTs are concentrated in the proximal (soma) and distal (growth cone) regions of growing axons (Brown et al., 1992). Axonal MTs are composite, consisting of two distinct domains that differ in their content of tyrosinated α-tubulin (Tyr-T). One domain is poor in Tyr-T and is situated at the minus end of the MT. The other domain is rich in Tyr-T and extends from the plus end of the Tyr-T-poor domain to the end of the MT (Baas and Black, 1990; Brown et al., 1993). Glutamylation was initially found associated with detyrosinated α-tubulin (Edde et al., 1990). However, elevated glutamylated α-tubulin (Glu-T) does not stabilize the MT and the mechanism for the stability of Glu-T is not known (Infante et al., 2000). Another modification, acetylation, is very common and acetylated tubulin (Acet-T) is present in stable and ordered arrays of MTs. Cytoplasmic MTs containing Acet-T were found to be more resistant than other cellular MTs to the effects of antimitotic drugs (LeDizet and Piperno, 1986). The distribution of Acet-T between

Tyr-T-poor and Tyr-T-rich domains was somewhat complementary to that of Tyr-T. Specifically, the Tyr-T poor domain stained relatively brightly for Acet-T, whereas the Tyr-T rich domain stained relatively poorly for Acet-T (Brown et al., 1993).

We showed that artificially elevating cyclic AMP levels in cultured neurons by adding dbcAMP or priming neurons with neurotrophins (NTs) can completely overcome the inhibition by MAG and myelin (Cai et al., 1999). It has been reported that cAMP induces a decrease in MF, and a stabilization of MT in cytotoxic T lymphocytes (Valitutti et al., 1993). Also, cAMP induces expression of the catalytic subunit of PKA resulting in a dramatic increase in cellular MT polymer content (Gradin et al., 1998). Downstream of cAMP, activation of the ArgI/PAs pathway or exogenous application of polyamines is sufficient for improved neurite outgrowth on inhibitory substrates (Cai et al., 2002). Although the physiological functions of polyamines are still not well understood, it has been shown by many groups that polyamines are involved in cytoskeleton structure. Studies in cell types other than neurons show that polyamines are essential for the organization of cytoskeletal proteins such as actin and trypomyosin (Kaminska et al., 1992; McCormack et al., 1994; McCormack et al., 1999). At low concentrations, polyamines induce polymerization of muscle actin (Nishida et al., 1987). Evidence also suggests that polyamines influence the distribution of MT during cell damage *in vivo* (Kaminska et al., 1992; Banan et al., 1998). Kaminska and colleagues found that both spermine and putrescine induced increases in expression of genes coding for major cytoskeletal proteins and that inhibitors of polyamine biosynthesis affect the expression of genes encoding the cytoskeletal proteins-β-actin and α-tubulin in mouse splentocytes (Kaminska et al., 1992). Pohjanpelto *et al* observed the disappearance of 90% of the actin filament bundles and the lack of assembly of MTs in DFMO-treated CHO cells (Pohjanpelto et al., 1981). These findings suggest mechanisms through which cAMP or polyamines may exert their effect on cytoskeletal integrity.

On the other hand, it has been shown that MAG and other myelin-derived inhibitors activate Rho GTPase through p75 (Yamashita and Tohyama, 2003; Gallo and Letourneau, 2004; Sandvig et al., 2004), the signal transducer in the receptor complex. In neurons, RhoA increases myosin II activity and promotes growth cone collapse and axon retraction by regulating MF dynamics (Katoh et al., 1998; Wahl et al., 2000; Jurney et al., 2002). The influence of MAG on MT is less clear. It had been shown by Quarles' group that in DRG neurons MAG increased CDK5 activity, which in turn enhanced phosphorylation of MAP1B (Dashiell et al., 2002).

The cytoskeletal reorganization in neurons is the final point of convergence between the MAG/myelin-mediated inhibitory pathways and the neurotrophins/cAMP-induced axonal outgrowth pathways. The balance between the negative effects of MAG/myelin and the positive effects of NTs/cAMP/PAs on the cytoskeleton may determine neuronal regenerative ability. Based on available evidence stated above, especially the "spring-loaded" hypothesis, we proposed that to inhibit growth, MAG/myelin induces stabilization of MFs and destabilization of MTs, in contrast, NTs/cAMP/PAs may destabilize MFs and stabilize MTs, which will overcome inhibition by MAG and myelin.

Results

Taxol and CD Promoted Neurite Outgrowth in the Presence of MAG-Fc

To determine if simultaneously strengthening polymerization of MT and destabilization of MF is sufficient to allow better growth on inhibitory substrates, the neurite outgrowth assay was performed with postnatal cerebellar neurons in the presence or absence of taxol (Parekh and Simpkins, 1997), which binds to the N-terminal region of β-tubulin and promotes the formation of highly stable MT that resist depolymerization, and CD (Wodnicka et al., 1992), which is cell permeable fungal toxin that disrupts actin MF. In order to have the appropriate control, dissociated primary neurons were treated with L1-Fc protein, a cell adhesion molecule that has been shown to promote neurite outgrowth from a variety of neurons (Doherty et al., 1995). L1-Fc was immobilized on a 8-chamber slide pre-coated with anti-Fc antibody. Then isolated cerebellar neurons from PND2 rats were plated onto immobilized L1-Fc with MAG-Fc proteins, a soluble form of MAG, included in the neuronal culture. Previously, we showed that MAG-Fc can inhibit neurite outgrowth in a dose-dependent manner. In figure 5.2B, at a concentration of 40μg/ml of MAG-Fc, inhibition was approximately 70% of growth, which is comparable to what we have observed in the past with our soluble neurite outgrowth assay (DeBellard et al., 1996). Neither taxol nor CD could overcome the inhibitory effect of MAG-Fc. With taxol and CD added together along with MAG-Fc, cerebellar neurons extended one or two thick, unbranched neurite(s) (figure 5.2A). MAG can no longer inhibit the neurite outgrowth and the neurite length is even longer than control (Figure 5.2).

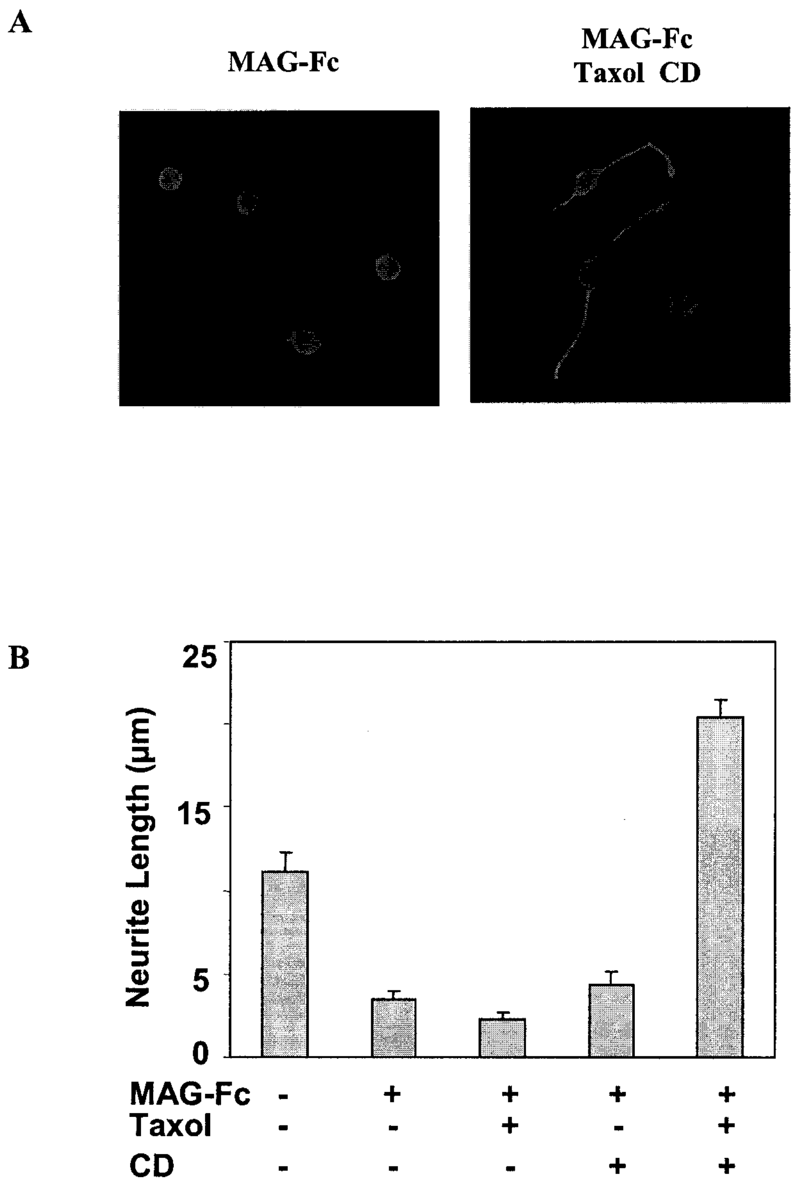


Figure 5.2 Adding Taxol and CD can Completely Overcome Inhibition by MAG-Fc. Addition of Either Taxol or CD Alone has No Effect on Inhibition.
Cerebellar neurons from PND2 animals were cultured on a substrate of the growth promoting molecule, L1-Fc, and either MAG-Fc or a control Fc-chimera were added each at a concentration of 40μg/ml. Where indicated taxol (1μM) and cytochalasin D (4μM) were added. (A) Neurons were stained with GAP43 and photographed. (B) Results represent the measurement of the longest neurite of 180-200 neurons.

The Effect of either Taxol or CD is Dose-dependent

To further establish the specificity of either taxol or CD, we investigated their dose-dependent effect. Postnatal cerebellar neurons were dissociated and plated on top of immobilized L1-Fc with or without the presence of MAG-Fc in the medium. In figure 5.3A, different concentrations (0.01μM-1μM) of taxol were included along with 2μM CD. Neurite length was dependent on the concentration of taxol, at less then 0.1μM; it does not have a significant effect. As the concentration of taxol increases, the neurite length on MAG increases. At a concentration higher than 0.1μM, it reaches a plateau (figure 5.3A). On the other hand, if the concentration of taxol was kept constant (1μM) and the concentration of CD varied, improvement of growth was also dependent on the dose of CD. At a concentration higher than 1μM, the effect of CD saturated (figure 5.3B).

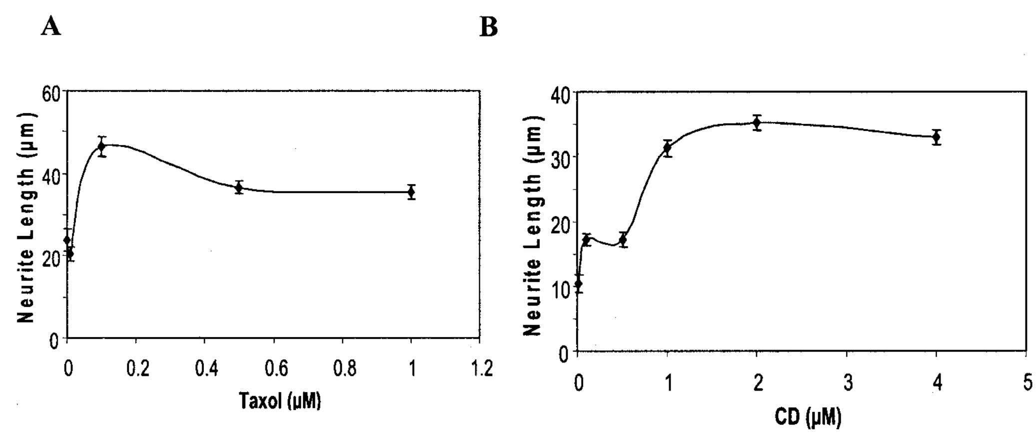


Figure 5.3 The Dose-dependent Effect of either Taxol or CD on Neurite Outgrowth
Cerebellar neurons from PND2 animals were cultured on a substrate of the growth promoting molecule, L1-Fc, and either MAG-Fc or a control Fc-chimera were added each at a concentration of 40 μg/ml. (A) Varied concentrations of taxol and constant cytochalasin D (2μM) or (B) different concentrations of CD and constant taxol (1μM) were added along with MAG-Fc into the medium. Neurons were incubated overnight, then fixed and immunostained for GAP43.

Taxol and CD Overcome the Inhibition by MAG and Myelin

To further elucidate the effect of taxol and CD in improving axonal outgrowth, we tested these inhibitors with MAG and myelin. In this experiment, instead of soluble MAG, MAG was expressed by CHO cells and was present at the membrane, as it would be presented by oligodendrocytes *in vivo*. Considering that both taxol and CD could influence the cytoskeleton of CHO cells and in turn, it might affect the presence of MAG at the membrane, we had to check for expression of MAG on the surface of CHO cells in the presence of these inhibitors. To do that, either MAG expressing or control CHO cells were cultured overnight in the presence of 1μM taxol and 2μM CD in the media and then immunostained with 513, a specific antibody for MAG. Figure 5.4A shows that MAG was still strongly expressed at the surface of MAG expressing CHO cells, although the morphology of these cells was slightly changed. Control CHO cells did not stain with 513 at all. In figure 5.4B, neurite outgrowth of cerebellar neurons was strongly inhibited by MAG. In the presence of Taxol, neurite length on MAG and even on control CHO cells was significantly shorter. CD alone had no significant effect on growth on either MAG or control. However, if taxol and CD were both included, the inhibition of axonal growth by MAG was completely blocked.

CNS myelin is known to be inhibitory for axonal regeneration. As we have shown before, dbcAMP and putrescine promoted axonal growth on MAG and also on myelin substrate. Now we would like to determine whether taxol and CD can block the inhibitory properties of myelin. The same neurite outgrowth experiment was carried out on adult CNS myelin. As can be seen in figure 5.5A, most neurons either have no neurites or bear very short neurons when grown on myelin. However, in the presence of taxol and CD, most neurons extend long processes. The mean length of the neurites is more than 4 times greater than control (figure 5.5 B).

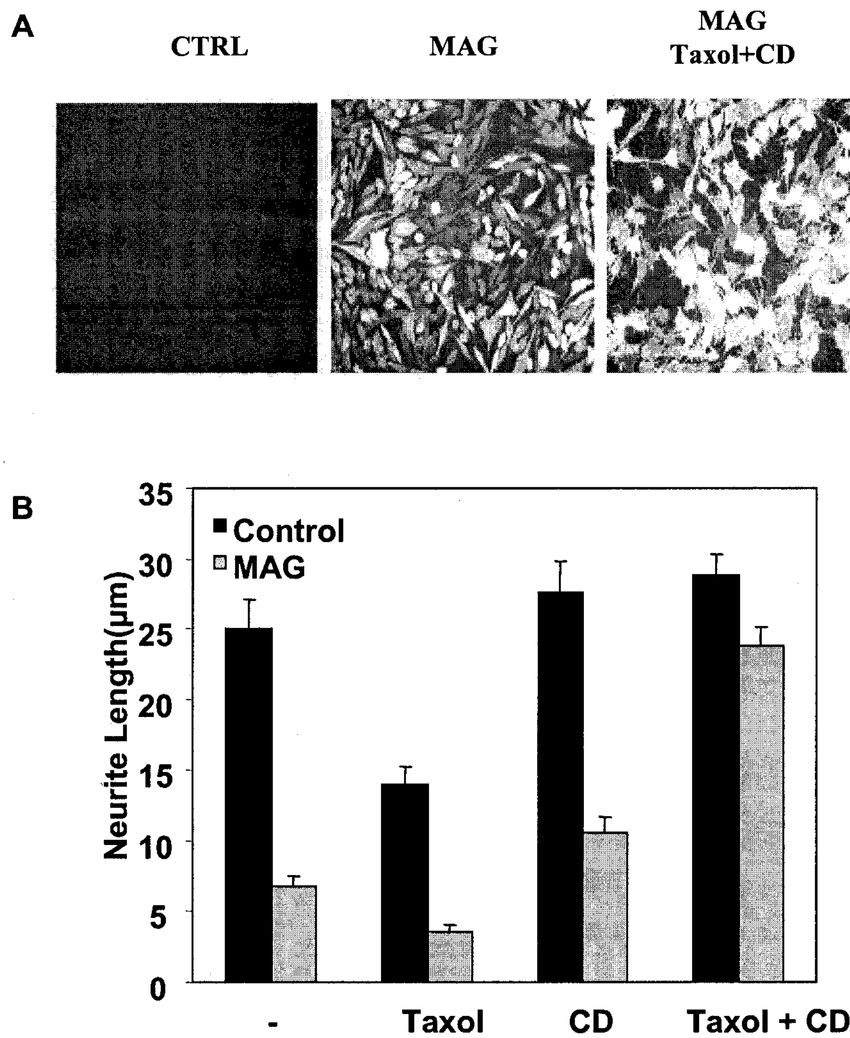
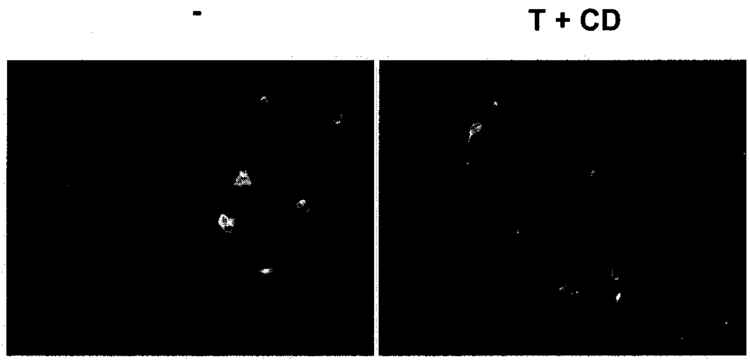


Figure 5.4 Taxol and CD Improve Axonal Outgrowth on MAG
Cerebellar neurons from PND3 animals were dissociated and cultured on either control CHO cells (black bar) or MAG-expressing CHO cells (blue bar) in the presence of taxol (1µM), cytochalasin D (2 µM) or both, as indicated. (A) CHO cell monolayer was stained with anti-MAG antibody and photographed. (B) Neurons were fixed and immunostained for GAP43. Results are the mean of 180-200 neurite lengths +/- sem.

A. Myelin



B

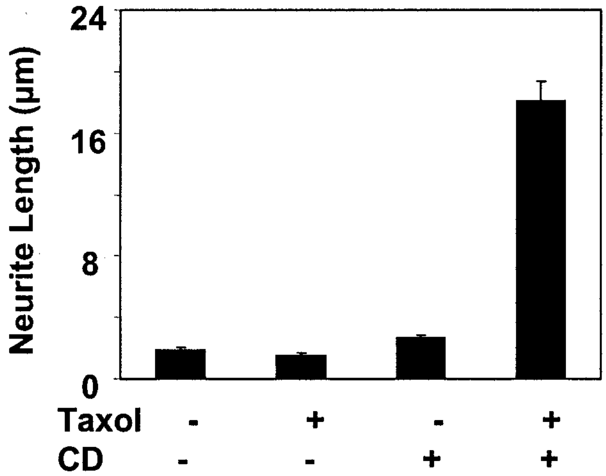


Figure 5.5 Myelin's Inhibition was abolished in the Presence of Taxol and CD
Cerebellar neurons from PND3 animals were dissociated, plated on adult CNS myelin (1μg/well) and cultured overnight in the presence of taxol (T) and CD. (A) Neurons were stained with GAP43 and photographed. (B) Results represent the measurement of the longest neurite of 180-200 neurons.

Prior Exposure of Neurons to Taxol and CD has No Effect on Inhibition by MAG

The effect of either taxol (Collins, 1991; Theiss and Meller, 2000) or CD (Stevenson and Begg, 1994) is reversible. To elucidate that the effect of taxol/CD comes from their direct binding to the cytoskeleton instead of evoking downstream events to foster better growth on inhibitory substrates, neurons were incubated with taxol and CD on poly-l-lysine coated plates overnight (priming) before being transferred to a monolayer of MAG expressing CHO (figure 5.6) or myelin (data not shown). So, the drugs would be removed in the process of transferring. As shown in figure 5.6, the inhibition of MAG remained. That is to say that taxol and CD have to be present while the neurons are exposed to inhibitory substrates to exert their effects.

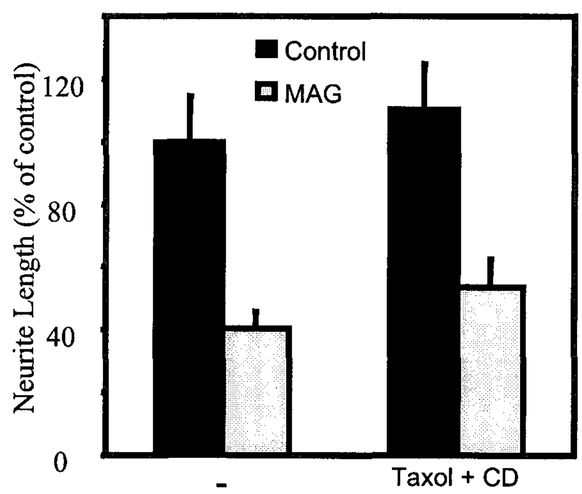


Figure 5.6 Prior Exposure of Neurons to Taxol and CD has no effect on Overcoming Inhibition by MAG
Cerebellar neurons were primed with or without taxol and CD overnight on poly-l-lysine coated plates and then transferred to control or MAG-expressing cells. Neurite length for 180-200 neurons was measured (± SEM). Results represented as percentage of control.

Microtubules Modification by Taxol and/or CD Treatment

Tubulin in axonal MT can be post-translationally modified (Brown et al., 1993). It has been established that unassembled or newly assembled tubulin in cells is tyrosinated, and that this tubulin is converted to detyrosinated tubulin (or glutamylated tubulin, Glu-T) after it assembles into MTs in a time-dependent manner (Gundersen et al., 1987). As well as Tyr-T and Glu-T, there is acetylation of α-tubulin (Acet-T). It had been shown that acetylated α-tubulin is present in various MT structures and may have a role in stabilizing the structure of all MTs (Piperno et al., 1987). Generally speaking, young (newly assembled) MTs contain more tyrosinated tubulin (Tyr-T) than older (more stable) MTs (Brown et al., 1992; Brown et al., 1993). Taxol induces MT assembly and formation of MT bundles (Schiff and Horwitz, 1980; Parness and Horwitz, 1981). Cytoplasmic MT, which normally disassemble and assemble at high rates, in the presence of taxol bind the drug and are stabilized (Schiff and Horwitz, 1980). The α-tubulin becomes acetylated in the presence of taxol in all cytoplasmic MT of 3T3 and HeLa cells (Piperno et al., 1987) or in cortical neurons (Mansfield and Gordon-Weeks, 1991).

To determine whether there is a change in tubulin modification with taxol and/or CD treatment in our system, cerebellar neurons were cultured overnight on either poly-l-lysine or myelin coated wells in the presence of taxol, CD or both for 18 hours. The protein samples were extracted with sample buffer and subjected to SDS-PAGE. As can be seen in figure 5.7, Tyr-T, Glu-T and Acet-T signal were detected in neurons grown on either PLL or myelin. Taxol resulted in a loss of Tyr-T and strikingly increased the intensity of Glu-T and Acet-T (figure5.7A), which is consistent with the results shown by others (Mansfield and Gordon-Weeks, 1991) in cortical neuron culture. There was less staining of all three modified tubulin in CD-treated neurons. When both were presented to the neurons, the staining pattern mimics the treatment with taxol alone. If the same experiment was performed on myelin instead of PLL coated plate, in the presence of taxol there was an overall reduction in Tyr-T and Glu-T immunoreactivity and an increase of Acet-T immunoreactivity (figure 5.7B). CD does not alter all three modified tubulin components alone nor does it change the pattern exhibited by a combination with taxol-treatment. These results suggested that upon stabilization of MTs by taxol binding, newly synthesis tubulins (Tyr-T) was recruited to MTs and remained in the stabilized state (Acet-T).

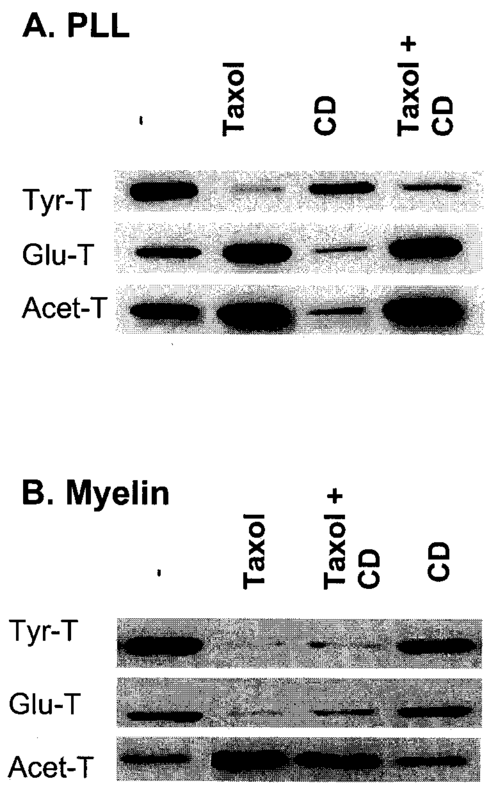


Figure 5.7 The Effect of Taxol and CD on Tubulin Modification
Cerebellar neurons (PND5) were isolated and plated onto (A) pll-coated plate or (B) myelin-coated plate and cultured overnight in the presence of taxol, CD or both. 5μg total protein was loaded to each lane. The same blot was probed and stripped with Tyrosinated-tubulin (Tyr-T), Glutamylated-tubulin (Glu-T) or Acetylated-tubulin (Acet-T) in turn.

The Effect of MAG and/or dbcAMP on Tubulin Modification

In neurons, the change in tubulin modification in response to cAMP or MAG/myelin has never been shown. So, it would be interesting to reveal a pattern of tubule modification as an indicator for neurite outgrowth.

Cerebellar neurons were dissociated and cultured overnight on PLL coated plates and then either 1mM dbcAMP or MAG-Fc (40μg/ml) was added to the medium for the indicated time before the samples were harvested and subjected to 10% SDS-PAGE. Upon dbcAMP treatment, the level of Tyr-T increased 3-fold in 0.5 h and is sustained for up to 6 h. Slight elevation of Acet-T and β-T was also detected with dbcAMP treatment. In the presence of MAG-Fc, however, the total amount of Tyr-T exhibits a 3-fold decrease (Figure 5.8 A, B), and an increase in Acet-T levels started at 2h and maintained high levels for up to 6h. The content of both Glu-T and β tubulin (β -T) remained the same. The amount of GADPH was stained as the loading control, and no significant change in both blots were observed. In addition, when the same blot was stripped and reprobed with actin, we didn't observe significant up-regulation of it in either dbcAMP or MAG-Fc treated neurons for up to 6h. If dbcAMP was present while neurons were plated onto myelin coated wells, dbcAMP reversed the effect of myelin which deceased Tyr-T levels (figure 5.8 C).

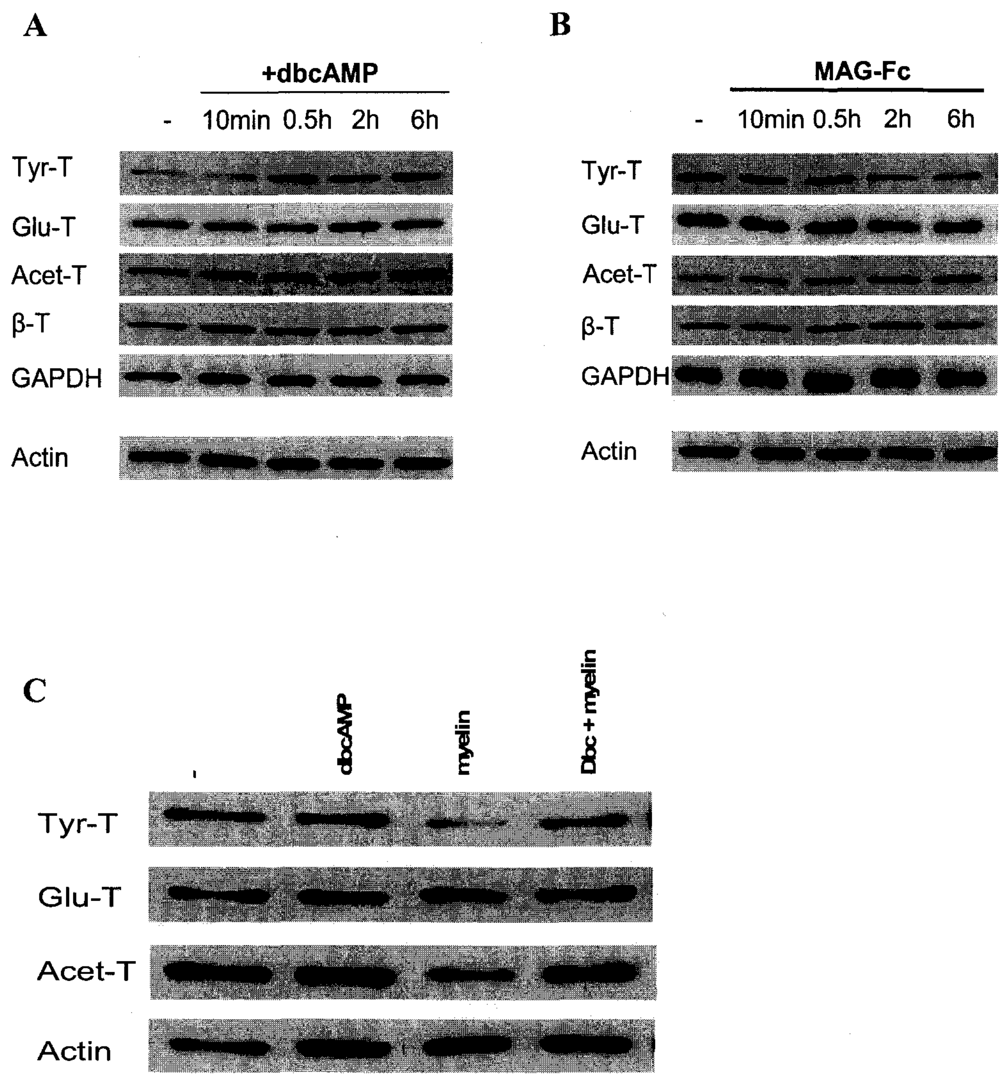


Figure 5.8 The Effect of MAG and/or dbcAMP on Tubulin Modification.
PND5 cerebellar neurons were dissociated, cultured overnight and incubated with dbcAMP (A), MAG-Fc (B) for different times indicated or dbcAMP/±myelin for 6 hours (C). Neurons were lysed and equal amounts of protein were loaded into each lane. The same blot was probed with Tyr-T, Glu-T Acet-T, β-T, GADPH or actin as indicated.

Discussion

We have examined the roles of MTs and MFs in neurite outgrowth by chemically modifying their interactions in neurons. Neurons exposed to taxol, an agent that promotes MT polymerization and stabilization, formed short or no neurites in overnight culture. Similarly, neurons exposed to CD, an agent which promotes MF depolymerization, did not improve neurite outgrowth on MAG/myelin as well. However, while taxol and CD were present simultaneously, cerebellar neurons extended long neurites regardless of growth on inhibitory or control substrate. These results support our hypothesis that F-actin forms a physical barrier in the central region of the growth cones to prevent the assembly of MTs, if MFs are destabilized and MTs are stabilized, inhibition by MAG/myelin will be overcome.

In our neurite outgrowth assay, taxol alone inhibited neurite outgrowth, whereas taxol along with CD promoted neurite elongation. It was suggested that taxol inhibits neurite elongation because unregulated polymerization disrupts MT movement in neurites and inhibits the push of MTs to the neurite tip (Letourneau and Ressler, 1984). Consistent with our result, it has been reported that inhibition of neurite elongation by taxol is abolished by levels of CB that substantially disrupt actin networks in chick DRG neurons (Letourneau et al., 1987). In addition, the unique extension of neurites in taxol and CB may reflect the combined effects of a disrupted actin network, reduced neuritic tensions, translocation of stable MT bundles and specialized neuronal cytoskeleton (Letourneau et al., 1987).

Morphological alteration of neurites after taxol and CD treatment was observed. Neurites lacked typical growth cones, were packed densely with MTs and were deficient in MFs. The neurite also appeared to be thick and no turning was observed compared to the control. No significant difference on neurite length was observed growing neurons on L1-Fc, MAG-expressing / control CHO cells, PLL or myelin. This means that the neurite length is dependent on the permissiveness of the substrate and time in culture. The extension rate of the neurite may be independent of the presence of inhibitory substrates. In support of what we observed, others reported that both growth cone attraction and repulsion induced by diffusible cues in culture can be completely blocked by inhibition of growth cone dynamics (Buck and Zheng, 2002). Also they showed that local MT stabilization by taxol induces growth cone turning toward the site of application (Buck and Zheng, 2002).

We showed that Acet-T is the dominant type in the presence of taxol. Others have also reported that when order and stabilization among all cytoplasmic MTs is created in the presence of taxol, the acetylation of α-tubulin is extended to the whole MT population (Piperno et al., 1987). However, synthesis of tubulin is still required for continuous elongation of neurites. And this newly made tubulin most likely would be Tyr-T. Yet, with taxol treatment, Tyr-T level decreased to a very low level in our overall immunoblot assay. Arregui *et al* suggested that after long incubation periods in the presence of taxol, the Tyr staining disappeared from the axon but remained in the cell body (Arregui et al., 1991). So, it would be reasonable to speculate that this "new" tubulins (Tyr-T) would be recruited to MT soon after synthesis, stabilized by taxol binding and become acetylated.

Although tubulin is known to undergo several post-translational modifications that accumulate in stable but not dynamic MTs inside cells, the function of these modifications is poorly understood. For instance, Edde *et al* thought that Glu-T is important in the interactions of tubulin with microtubule-associated proteins (MAPs) (Edde et al., 1990). Tubulin acetylation reduces cell motility and reduces MT stability (Hubbert et al., 2002). Importantly, it was suggested that MTs probably are stabilized by an interaction with unknown cellular elements and then are acetylated on the α-tubulin (Piperno et al., 1987). Also, Gelfand and Bershadsky indicated that these modifications are correlated with, but do not appear to cause, an increase in the stability of the MT to destabilizing treatments *in vivo* (Gelfand and Bershadsky, 1991). That is to say, the switch from Tyr-T to more stable Acet-T or Glu-T is the result, but not the cause of MT stabilization. Our results suggested that cAMP treated neurons had more newly synthesized tubulins (Tyr-T) and also stabilized the tubulins that incorporated into MT (Glu-T or Acet-T). In contrast, Tyr-T was decrease in the presence of MAG. These results parallel the observations that cAMP leads to more dynamic growth cones (Kim and Wu, 1996; Wang and Zheng, 1998), and MAG results in growth cone collapse and inhibition of axonal growth (Li et al., 1996; Tang et al., 1997c), which indicated a less active growth status. More importantly, the effect of MAG on the switch of modified tubulin could be reversed by dbcAMP. Our neurite outgrowth results show that dbcAMP overcomes the inhibition by MAG/myelin (Cai et al., 1999) as well.

The most prominent cytoskeletal change that occurs in response to dbcAMP is a rearrangement of actin filaments characterized by a loss of cortical F-actin staining and the appearance of actin filament staining at the tips of the processes (Baorto et al., 1992). To support our hypothesis, it would be a convincing result if we could immunohistochemically stain MF at the growth cone in response to either exogenous cAMP/PAs or MAG/myelin. Our prelimilary results from culture hippocampal neurons indicated lose of F-actin in the presence of dbcAMP and stronger actin filament staining in MAG-treated neurons (data not shown). However, we have technical difficulty obtaining a convincing picture of the growth cones to quantitate the intensity of staining.

In summary, we suggest here that simultaneously strengthening polymerization of MTs and depolymerization of MFs in neurons results in improved neurite outgrowth. These results help to narrow down the target proteins in the search for molecules that target MTs and/or MFs dynamics in response to either positive (NTs/cAMP/PAs) or negative (MAG/myelin) signals.

Chapter VI

Activation of Cyclin Dependent Kinase 5 is required in Overcoming inhibition by MAG and Myelin

Introduction

We showed that elevating neuronal cAMP, either with a cAMP analogue or by pre-incubating with various NTs, overcomes inhibition by MAG and myelin *in vitro* (Cai et al., 1999). We have established that neuronal cAMP levels are an indicator of the intrinsic growth state of neurons, which in turn dictates the neuronal response to the CNS environment and consequently regulates the ability of injured CNS neurons to regenerate (Cai et al., 1999; Cai et al., 2001; Qiu et al., 2002b). All extrinsic signals, either NTs or MAG, ultimately bring about their effect by regulating cytoskeleton dynamics. So, it would be interesting to manipulate the activity of protein(s) that directly regulate the dynamics of MTs and MFs and investigate their effect on neurite extension. Recent findings indicate that cyclin-dependent kinase 5 (CDK5) might be a candidate. Activity of CDK5 is critical for neurite elongation (Nikolic et al., 1996; Paglini et al., 1998; Li et al., 2000; Harada et al., 2001). More importantly, CDK5 phosphorylates neuronal cytoskeleton proteins, which may provide stability to axonal structures (Shea and Beermann, 1994).

CDK5 is a multifunctional serine/threonine kinase. It was originally isolated on the basis of its close primary sequence homology to the human cdk2, the prototype of the cyclin-dependent kinases (CDKs) (Lew et al., 1992; Pines, 1996). In contrast to other CDKs, CDK5 appears not to be expressed in dividing cells in the developing brain (Tsai et al., 1993). Expression of CDK5 is concentrated in axons of postmitotic neurons. CDK5 activity is restricted to the nervous system because its activator, p35, is expressed only in the nervous system (Tsai et al., 1994; Humbert et al., 2000). Mice lacking p35 display defects in cortical and hippocampal lamination as well as fasciculation of the axon fibers (Chae et al., 1997). In the cortex of p35 knock-out mice, the layering of cortical neurons is inverted, because neurons born later cannot migrate past their predecessors (Chae et al., 1997).

CDK5 activity has been shown to be essential for neurite outgrowth. Inactivation of CDK5 in cultured cortical or cerebellar neurons inhibits neurite outgrowth (Nikolic et al., 1996; Paglini et al., 1998). In immortalized hippocampal pyramidal cells, p35 is necessary for promoting neurite outgrowth in response to basic fibroblast growth factor (Xiong et al., 1997). In addition, Harada *et al* suggested that the NGF-induced kinase activity of CDK5 is required for neurite outgrowth in PC12 cells (Harada et al., 2001). Importantly, accumulating evidence suggests that CDK5 kinase activity is increased following ischemia, neuronal diseases and nerve injury (Green et al., 1997; Green et al., 1998; Namgung et al., 2004). The p35 protein was also found to be associated with increased CDK5 activity in regenerating peripheral nerves. Furthermore, administration of CDK5 inhibitors into the crushed nerves resulted in decreases in CDK5 kinase activity in nerves and retardation of nerve fiber regrowth (Namgung et al., 2004).

Neuronal specific cytoskeletal proteins are reported to serve as good substrates for CDK5/p35 complex. Among known or suspected CDK5 substrates are microtubule-associated proteins (MAPs), including tau, MAP2 and the dynein-associated protein, Nudel (Dhavan and Tsai, 2001). CDK5 substrates also include a long list of proteins related to actin dynamics, such as CaMKII, Cables, Pak1, amphiphysin, and synapsin 1. In addition, CDK5 can use two of three neuronal intermediate filament subunits, NF-H and NF-M, as substrates (Dhavan and Tsai, 2001).

CDK5 is associated with the MTs and can be purified from bovine brain MTs (Ishiguro et al., 1992; Sobue et al., 2000). Phosphorylation of MAP1b by CDK5 in cerebellar macroneurons is implicated in neurite extension (Pigino et al., 1997; Paglini et al., 1998). Also, phosphorylation of tau, a neuron specific MAP, by CDK5 reduces the binding of tau to MTs and inhibits the ability of tau to promote MT assembly (Evans et al., 2000). CDK5 suppression reduced axonal elongation and decreased the phosphorylation status of MAP1b, as well as its binding to MTs (Pigino et al., 1997) In addition, Paglini *et al* showed that laminin stimulates MAP1b phosphorylation and axonal elongation by regulating the expression and subcellular distribution of p35 in cerebellar macroneurons (Paglini et al., 1998).

As to its effects on MFs, overexpression of p35 in fibroblasts induces pronounced actin reorganization and the appearance of lamellipodia and filopodia (Nikolic et al., 1998; Humbert et al., 2000). p21-activated kinase (PAK), one of the effectors of Rac1 and Cdc42, is implicated in actin polymerization (Sells et al., 1997). CDK5 down-regulated PAK1 activity in a Rac-dependent manner (Nikolic et al., 1998). This modification of PAK1 is likely to regulate actin cytoskeleton dynamics in neurons, promoting neurons migration and neurite outgrowth.

Although there is growing evidence suggesting that CDK5 activity is required for neurite outgrowth, whether or not CDK5 is involved in the priming with NTs to overcome inhibition by MAG and myelin has never been shown. Based on the findings mentioned above, we speculated a role for CDK5 in overcoming inhibition by MAG and myelin.

Results

Effects of dbcAMP or BDNF in Overcoming Inhibition by MAG are Dependent on the Activity of CDK5

First, we want to determine if activity of CDK5 is required for the effect of either dbcAMP treatment or priming with BDNF to overcome inhibition by MAG. DRG neurons from PND5 rats were isolated and plated onto either MAG-expressing or control CHO cells for overnight culture in the presence of dbcAMP and/or roscovitine (2-(1-ethyl-2- hydroxyethylamino)-6-benzylamino-9-isopropylpurine), a potent, membrane-permeable, specific CDK5 inhibitor in neurons (Meijer et al., 1997; Planchais et al., 1997). Roscovitine is widely used as a specific inhibitor of CDK5 activity (Mgbonyebi et al., 1999; Lee and Kim, 2004). In our neurite outgrowth assay (figure 6.1), the neurite length on control CHO was slightly decreased in the presence of roscovitine but the growth on MAG-expressing cells was still significantly shorter. If roscovitine was presence along with dbcAMP, it abrogated the effect of dbcAMP in overcoming the inhibition by MAG (figure 6.1 A). Similar results were obtained by priming neurons with neurotrophins (NTs) in the presence or absence of roscovitine (figure 6.1 B). Roscovitine attenuated the effect of priming with NTs in overcoming MAG's inhibition.

These results suggested that the effect of dbcAMP or NTs was dependent on the activity of CDK5. CDK5 might serve as a downstream effector of NTs/cAMP signal.

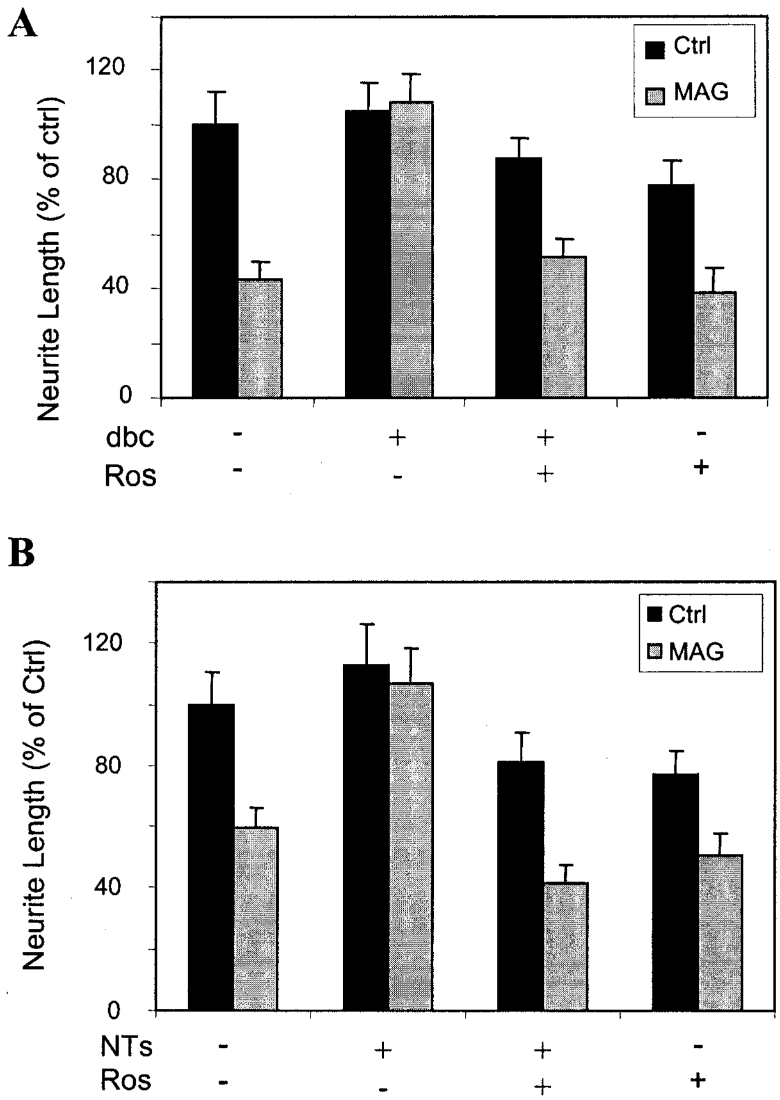


Figure 6.1 Inhibition of the Activity of CDK5 blocked the Effect of either dbcAMP or BDNF in Overcoming Inhibition by MAG
DRGs from PND5 pups were plated directly (A) with or without either dbcAMP (dbc) 1mM / Roscovitine (Ros) 10μM or (B) primed with or without NTs (BDNF, GDNF, NGF at 200ng/ml) / Ros 10μM onto monolayers of MAG-expressing (blue bars) or control (black bars) CHO cells and cultured overnight. Neurons were fixed and immunostained with anti-GAP43 antibody. The longest neurite from each neuron was measured and the average neurite length from 180-200 neurons was obtained.

Roscovitine abolished the Effect of Putrescine

We suggested that the ArgI/PAs pathway is downstream of cAMP signaling and it is required and sufficient to overcome MAG's inhibition ((Cai et al., 2002); Chapter III). Here, we are asking whether or not the effect of PAs also depends on CDK5 activity. DRG neurons were dissociated and primed overnight with putrescine at 200μM and/or roscovitine at 5μM before being transferred to a monolayer of CHO cells or CNS myelin. Consistent with what we had shown before, PND5 DRG neurons were inhibited by MAG compared to control CHO cells. Priming DRG neurons with putrescine is sufficient to block the inhibitory effect of MAG (figure 6.2 A). If roscovitine is included along with putrescine in priming, the effect of putrescine is diminished. Similar results were obtained when neurite outgrowth was performed on myelin (figure 6.2 B, C).

Furthermore, when putrescine was delivered through an osmotic pump to adult rats for 7 days, after which L4 and L5 DRGs were isolated and grown on either MAG expressing or control CHO cells, they were no longer inhibited by MAG. However, when roscovitine was included in the neuron cultures and incubated overnight before fixing and staining for GAP-43 positive neurons. As we demonstrated in the previous chapter, intrathecal delivery of putrescine can mimic the peripheral conditioning lesion and allows the DRG neurons to overcome inhibition by MAG and by myelin in general. In figure 6.2D, MAG inhibits growth of neurites of neurons from saline-delivered rat but not putrescine-delivered animals. If roscovitine is added *in vitro* to the *in vivo* putrescine-treated neurons, it blocks the effect of putrescine both in overcoming MAG's inhibition and in improvement of the general growth capacity completely. These results suggested that CDK5 might be downstream of polyamines.

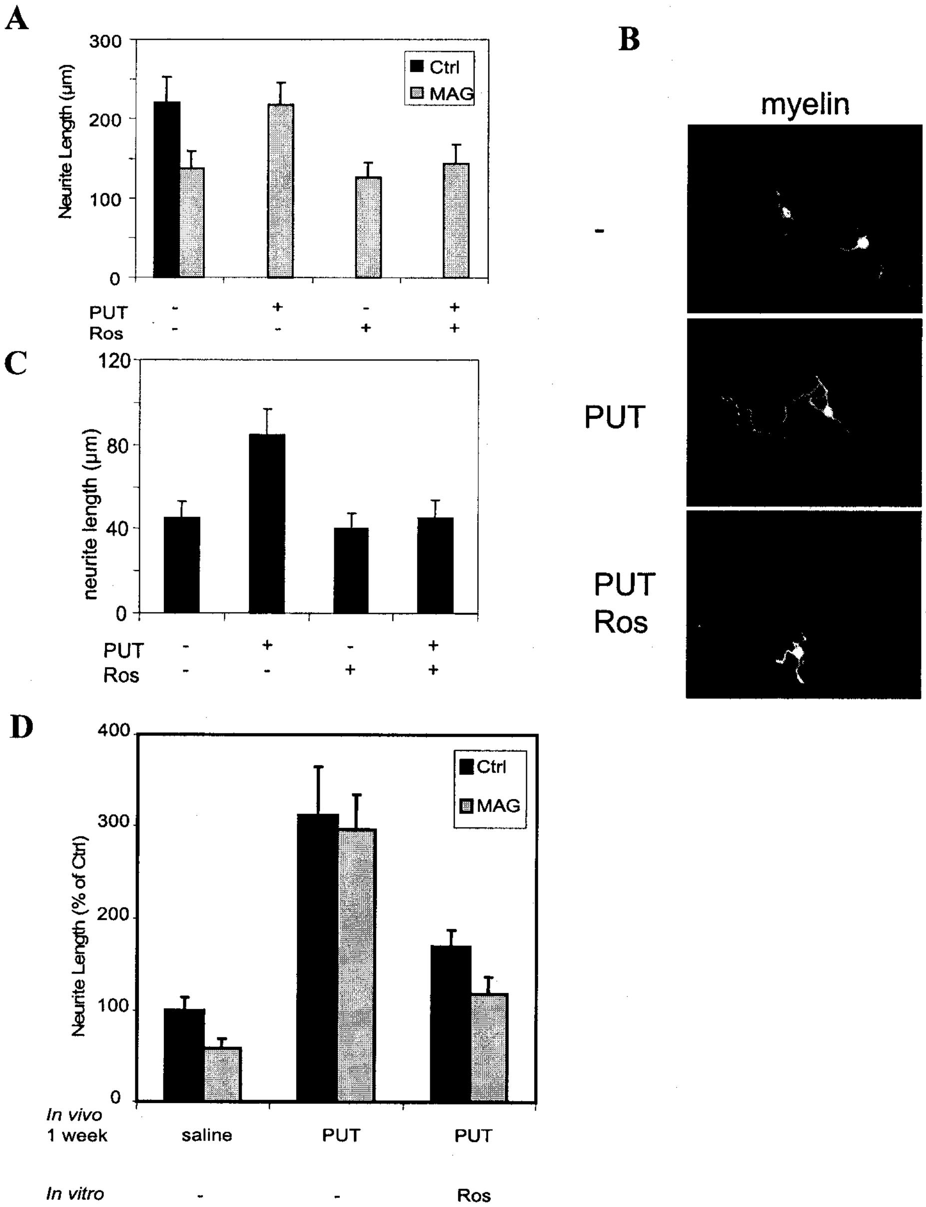


Figure 6.2 Activity of CDK5 is required to bring About the Effect of Putrescine DRG neurons are primed overnight with or without putrescine (PUT) at 200µM, and/or 5µM roscovitine (Ros) then transferred to a monolayer of either MAG-expressing (blue bar) or control CHO cells (black bar) (A) or myelin (B, C). Neurons were cultured for 16hrs, fixed and immunostained for GAP-43. Data was presented as the neurite length percentage of the length of control. (D) Either saline or putrescine (12.5nmol/h) was put into an osmotic pump and delivered intrathecally for 7 days before the neurons from L4 and L5 DRGs were removed and grown on CHO monolayer ± Ros (5µM).

The Expression of p35 increased in Response to NTs

It has been reported (Harada et al., 2001) that in PC12 cells, treatment with NGF induces strong, sustained expression of p35 through activation of the Erk pathway. p35 in turn induced kinase activity of CDK5, which is required for NGF-induced neurite outgrowth (Harada et al., 2001). Induction of CDK5/p35 was also through the Erk pathway in neuroblastoma cells (Lee and Kim, 2004). Data from our lab had shown that activation of Erk in response to either dbcAMP or BDNF treatment to cerebellar neurons is required to overcome inhibition by MAG (Gao et al., 2003). To determine whether CDK5/p35 was up-regulated in mRNA and protein level, and whether it related to the activation of Erk, DRG neurons from postnatal day 5 pups were isolated and cultured on poly-l-lysine coated plates overnight. Then NGF was added into the culture medium at 200ng/ml for the indicated time period and either RNA or protein was extracted. The extracted RNA was subjected to RT-PCR, using specific primers for p35 or GAPDH as a control. As can be seen in figure 6.3A, p35 is expressed in DRGs and upon treatment with NGF, the transcription of p35 mRNA increased with the peak at 10min, returning to basal level in an hour. In addition, the translation of p35 protein after NGF treatment was assessed. Primary DRG neurons were cultured overnight before being treated with NGF for 1-6 hour(s). Then the samples were harvested and subjected to 10% SDS-PAGE. The blot was first probed with anti-phospho-Erk, then stripped and reprobed with antibodies against p35 and CDK5 respectively. In figure 6.3B, phospho-Erk levels increased within 1 hour and sustained high levels for up to 6 hours in the presence of NGF. Protein levels of p35 were also significantly increased (1.5-fold at 1h, 2-fold at 3h and 3-fold at 6h) at this time period, while CDK5 levels remained constant. Consistent with our findings, it has been reported that CDK5 was constitutively expressed at high levels in adult brain and the availability of p35 appears to be the primary determinant for CDK5 activity (Qi et al., 1995; Tokuoka et al., 2000).

To further elucidate if the increase in p35 translation is depended on the Erk pathway, an Erk kinase inhibitor, PD98059 was included. Phospho-Erk levels in neurons from PD98059 (20 μM) treated DRG neurons dropped to a level lower than control even in the presence of NTs (figure 6.3 C). At the same time, the up-regulation of p35 was blocked. Again, there are no changes in CDK5 content.

These results indicated that the induction of p35 protein by NTs is Erk activity dependent.

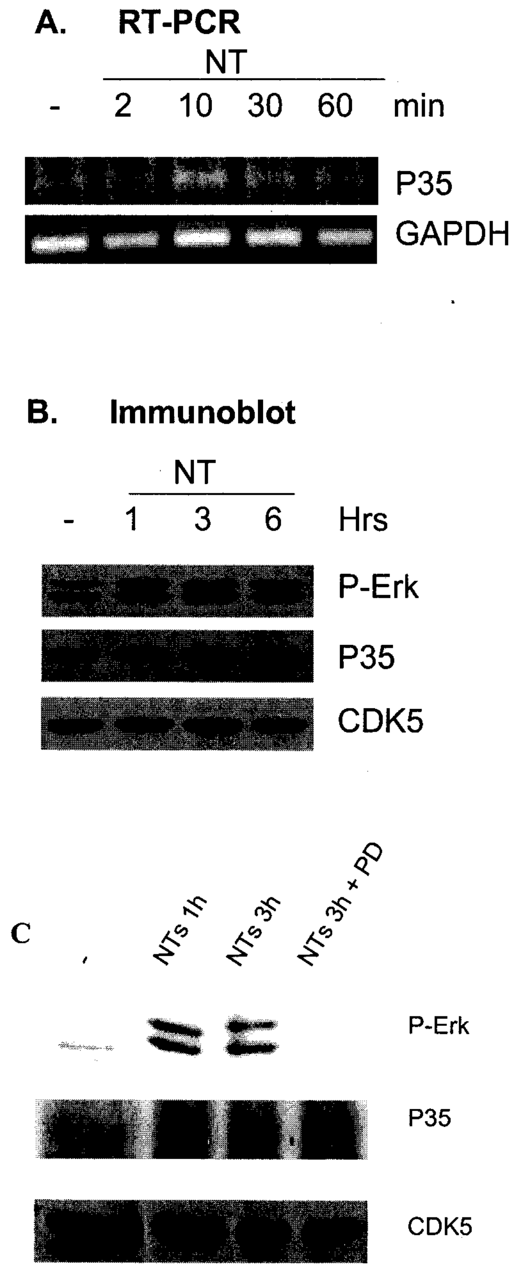


Figure 6.3 Elevation of p35 Expression in Response to NTs is Dependent on Activation of Erk.
PND5 DRGs were isolated and plated onto poly-l-lysine coated plated overnight. NGF (200ng/ml) was included at the time indicated. Then either mRNA or protein was extracted. (A) The mRNA samples were reverse transcripts and the products were subjected to PCR using specific primer for p35 or GAPDH respectively. (B) NGF (200ng/ml) treatment for 1, 3 or 6 hours. Equal amount of protein (5μg) was loaded into each lane and the blot was probed with phospho-ERK (P-Erk), p35 or CDK5 antibody. (C) NGF (200ng/ml) incubated for 1 or 3 hours with or without the presence of Erk kinase inhibitor – PD98059 (PD) (20μM) as indicated.

Discussion

The present study demonstrates that NTs through activation of the Erk pathway induced expression of p35, the neuronal specific activator of CDK5. This in turn induced the kinase activity of CDK5, and the elevated activity of CDK5 is required for NTs, dbcAMP or polyamines in overcoming inhibition by MAG and myelin.

Several reports have proposed a link between activation of Erk and CDK5 activities. For example, in cortical neurons, BDNF increases the activity of both Erk and CDK5 (Tokuoka et al., 2000). Increases in Erk activity and p35 expression have also been observed after ischemia in brain (Alessandrini et al., 1999; Hayashi et al., 1999; Park and Koh, 1999; Lee and Kim, 2004). Lee *et al* have shown that the elevation of CDK5 and p35 are closely regulated by the Erk pathway during retinoic acid induced neuronal differentiation (Lee and Kim, 2004). Furthermore, crosslinking Fas, the receptor for tumor necrosis factor, on primary sensory neurons induces neurite growth through sustained activation of the ERK pathway and the consequent upregulation of p35 (Desbarats et al., 2003). Although our lab (Gao et al., 2003) had implicated that the effect of NTs and dbcAMP in overcoming inhibition by MAG is Erk activity dependent, the link between NTs and CDK5 activity to growth on inhibitory substrate has not been shown before. Here, we suggeste that NTs induce activation of Erk, which in turn leads to up-regulation of p35. And the elevated p35 results in increased activity of CDK5 and improvement of neurite outgrowth on MAG or myelin.

We showed that polyamines are required and sufficient to overcome inhibition by MAG and myelin ((Cai et al., 2002); Chapter III). As an extension of this finding, we demonstrated here, that the ability of polyamines to overcome inhibition is also dependent on the activity of CDK5 (figure 6.2). With this result in hand, it seems that CDK5 works as a downstream effector of polyamines. So far, this is the first evidence that relates elevation of polyamines and CDK5 activity. It has been shown by others that over-expression of p35 and CDK5 results in longer neurites (Nikolic et al., 1996; Xiong et al., 1997). Whether over-expression of p35 and CDK5 would be sufficient to promote neurite outgrowth on inhibitory substrate remained to be elusived.

The transcription factor, Egr-1 has been reported to mediate the elevation of p35 (Harada et al., 2001; Lee and Kim, 2004). Activation of Erk by NGF induces the expression of Egr-1, which in turn induces p35 by binding to its three Egr-1-binding sites in the promoter region (Lee and Kim, 2004). Up-regulation of p35 at both transcription and translation levels was detected in DRG neurons treated with NGF (figure 6.3), and this elevation was Erk activity dependent. It is possible that the enhanced expression or binding of Egr-1 to the promoter region of p35 by activation of Erk was involved.

Taken collectively, this study indicates for the first time that CDK5/p35 is a downstream effector of NTs, cAMP and ArgI/PAs pathway and plays a role in improving neurite outgrowth on inhibitory substrates, most likely by modifying the cytoskeleton.

In conclusion, based on the results from all these chapters, a revised model is proposed to explain the possible signaling mechanism involved in the block of inhibition by MAG of axonal regeneration (figure 6.4). When neurons are primed with neurotrophins, they activate Erk through the Trk receptors, Erk then inhibits PDE and so cAMP accumulates and PKA is activated. PKA, probably, directly affects cytoskeleton organization and blocks the inhibitory pathway. In addition, CREB is phosphorylated and transcription of certain genes is turned on. Arginase I, the key enzyme in polyamine synthesis, is one of the genes up-regulated. As a consequence, the concentration of both putrescine and spermidine increases. The elevation of spermidine is required and sufficient to block the inhibition by MAG and myelin. In addition, the activation of Erk by NTs increases the transcription and translation of p35, an activator of CDK5. We suggest that CDK5 is a downstream effector of cAMP and polyamines in overcoming inhibition by MAG and acts by modifying F-actin and microtubule stability simultaneously.

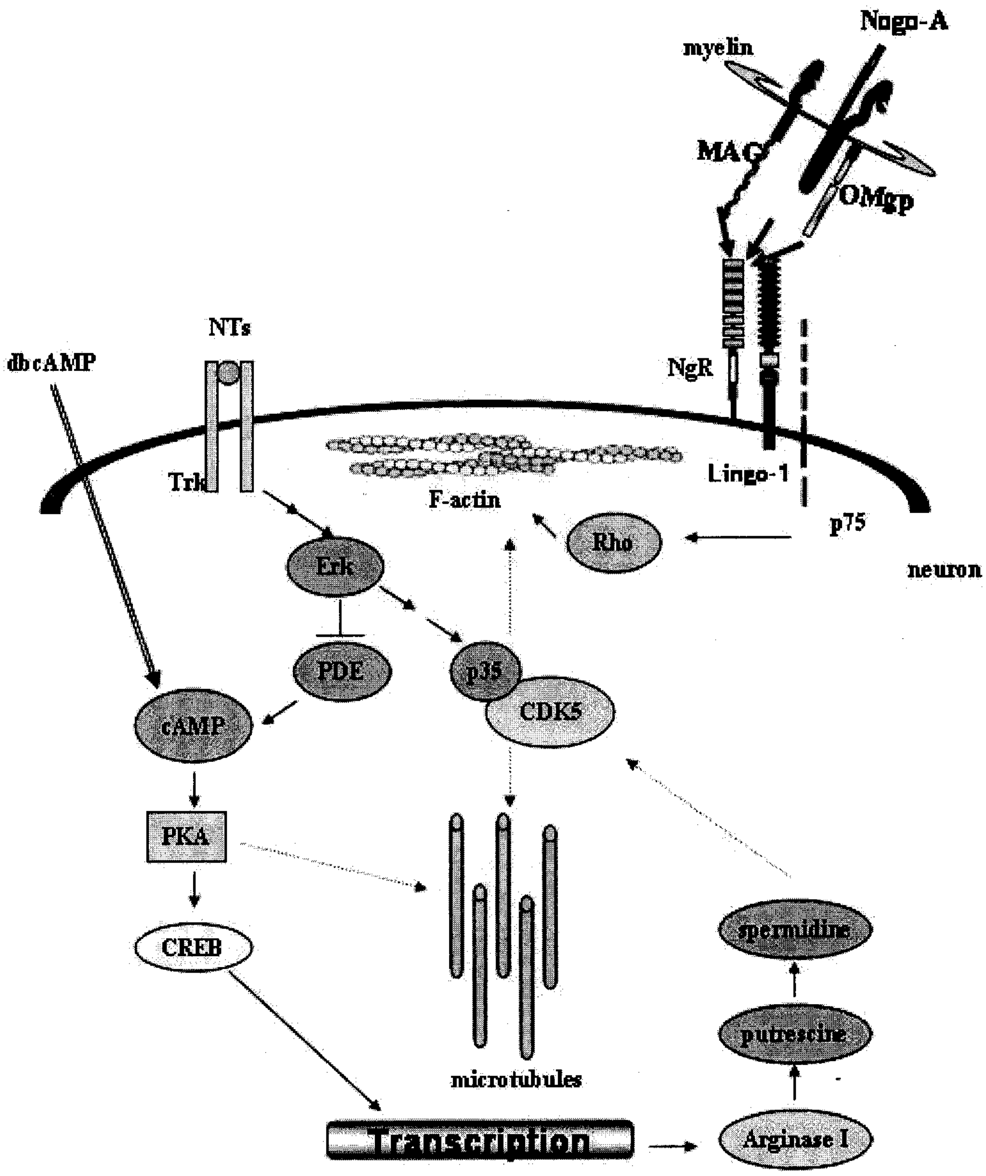


Figure 6.4 Signaling Mechanism Involved in the Block of Inhibition by MAG and Myelin of Axonal Regeneration.

Chapter VII

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