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CHARACTERIZATION OF A ROLE FOR CYCLIC AMP (cAMP) IN BLOCKING INHIBITION OF AXONAL REGENERATION BY MYELIN-ASSOCIATED GLYCOPROTEIN (MAG) AND MYELIN

by

DONGMING CAI

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

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ABSTRACT

Identification of a Role for Cyclic AMP (cAMP) in Blocking Inhibition of Axonal Regeneration by Myelin-Associated Glycoprotein (MAG) and Myelin

by

Dongming Cai

Advisor: Professor Marie T. Filbin

Myelin-associated glycoprotein (MAG), a well-characterized glycoprotein found in both CNS and PNS myelin, has been shown to be a potent inhibitor of axonal outgrowth in culture and *in vivo*. Here, we demonstrated that the inhibition of MAG/myelin on neurite outgrowth is completely blocked if neurons are exposed to neurotrophins before they encounter MAG or myelin. This priming effect is via a cAMP-dependent pathway. In addition, if neurons are exposed to MAG/myelin and neurotrophins simultaneously but in the presence of a Gi inhibitor, pertussis toxin, the inhibition is blocked without the need to prime. Therefore, we proposed a model to explain the priming effect and possible signaling mechanism involved in MAG's inhibition on axonal outgrowth.

Next, we showed that the promotion of neurite growth from younger neurons by MAG/myelin as well as the spontaneous axonal regeneration *in vivo* after spinal cord transection in young animals is dependent on cAMP activation. In addition, there is a decrease in endogenous cAMP levels during development, which suggests that neuronal

cAMP levels dictate the developmental switch of neurons to inhibition. Furthermore, the molecular mechanism underlying the improved CNS axonal regeneration following a peripheral conditioning lesion was studied. It has been found that transection of the peripheral nerve branch of DRG neurons results in an increase in endogenous levels of cAMP, which can neutralize the effect of myelin inhibitors, and then allow injured neurons to grow through white matter. The application of cAMP directly into the neuronal cell body without any peripheral nerve lesion mimics the effect of a conditioning lesion on axonal regeneration.

Finally, the possible downstream effectors induced by cAMP were investigated. One signaling event is the up-regulation of arginase I, which in turn activates the pathway for the biosynthesis of polyamines and initiates the neuronal regenerative machinery. Blocking one step in the pathway of polyamine production, synthesis of putrescine from ornithine, with an inhibitor of ODC, abrogates cAMP-induced reversal of inhibition by MAG and myelin completely. Then, a modified model is proposed to explain the possible signal transduction mechanism involved in the neurotrophin/cAMP-induced axonal outgrowth on MAG/myelin.

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ABBREVIATIONS

AC	adenylyl cyclase
Arg	arginase
BDA	biotin dextran amine
BDNF	brain-derived neurotrophic factor
cAMP	cyclic AMP
CAP-23	cytoskeleton-associated protein
CHO	Chinese hamster ovary
cGMP	cyclic GMP
CNS	central nervous system
CRE	cAMP-responsive element
CREB	cAMP-responsive element-binding protein
CS-PGs	chondritin sulfate proteoglycans
DbcAMP	dibutyryl cAMP
DCC	*deleted in colorectal cancer*
DFMO	DL-α-difluoroemthylornithine
DMEM	Dulbecco's modified eagle's medium
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DREZ	dorsal root entry zone
DRG	dorsal root ganglion
ECM	extracellular matrix
EIA	enzymeimmunoassay

ER	endoplasmic reticulum
FGF	fibroblast growth factor
FCS	fetal calf serum
GAP-43	growth-associated proteins-43
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic proteins
GFP	green fluorescent protein
IEGs	immediate-early genes
Ig	immunoglobulin
IP_3	inositol 1,4,5-triphosphate
LTP	long-term potentiation
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
MLCK	myosin light chain kinase
MOI	multiplicity of infection
NGF	nerve growth factor
NG2 PG	NG2 proteoglycan
NMDA	N-methyl-D-aspartate
NTs	neurotrophins
NT-3	neurotrophin-3
ODC	ornithine decarboxylase
PCR	polymerase chain reaction
PDE	phosphodiesterase

PFU	plaque forming units
PKA	protein kinase A
PKG	protein kinase G
PLL	poly-L-lysine
PND	postnatal day
PNS	peripheral nervous system
PTX	pertussis toxin
RGC	retinal ganglion cell
RT-PCR	reverse transcription – polymerase chain reaction
SAM	s-adenosylmethonine
SCG	superior cervical ganglion
Sema III	semaphorins III
TN-R	tenascin R

CHAPTER I

INTRODUCTION

Adult mammalian central nervous system (CNS) neurons fail to regenerate after injury. Several factors are likely to contribute to the limited axonal regeneration of injured neurons, including the low intrinsic regenerative potential of adult CNS neurons (Fawcett, 1992), the absence of permissive trophic factors and the presence of inhibitory factors in the glial and extracellular environment surrounding damaged CNS neurons (Aubert et al., 1995). Nevertheless, when provided with a favorable environment, injured CNS neurons can regrow their axons, and can sometimes form synaptic connections with their original targets (David and Aguayo, 1981), which suggests that adult CNS neurons have not completely lost their regenerative capacity. Therefore, mainly non-permissive environmental factors in the adult CNS contribute to the failure of axonal growth after injury. Such factors include myelin-associated inhibitors of neurite growth (Schwab et al., 1993) and some properties of so called "reactive astrocytes" (McKeon et al., 1991). However, this unfavorable adult CNS environment does not prevent axonal growth from embryonic neurons (Shewan et al., 1995; Li and Raisman, 1993). It has been well documented that embryonic neurons are more plastic than their adult counterparts (Bregman and Goldberger, 1982; Davies et al., 1994; Bedi et al., 1992; Chen et al., 1995; Shewan et al., 1995; Fawcett et al., 1989), though the precise molecular determinants that regulate this change in regeneration and sprouting between young and old axons, remain unknown. Taken together, these observations indicate that to achieve successful axonal regeneration in the adult CNS, at least two lines of therapeutic avenues should be explored: neutralizing the inhibitory effects of CNS myelin and glial scar, and improving the intrinsic regenerative potential of neurons.

Regeneration in the Adult Mammalian CNS

It is believed that the adult CNS environment is unfavorable for growth. If provided with an appropriate environment, however, adult CNS neurons still possess the intrinsic mechanisms for axonal growth over long distances (David and Aguayo, 1981). Three lines of investigation support the idea that the CNS environment does not allow neurons to regenerate. First, the axons of many sensory neurons divide into two branches, one branch that remains in the peripheral nervous system (PNS) with the cell bodies and one that projects into the CNS. After transection, only the PNS branch is able to regenerate (Cajal, 1928). Second, grafting experiments show that CNS neurons will regenerate an axon when provided with the type of environment normally encountered by PNS neurons (David and Aguayo, 1981). Finally, PNS neurons, which normally regenerate very well, abruptly cease regenerative growth when directed into a CNS environment (Hall and Kent, 1987). The environmental factors that contribute to the non-permissiveness of the adult CNS will be discussed in the following sections.

1.1 Trophic Support and Growth Promotion by Neurotrophins

Upon damage of the CNS, the first challenge for injured neurons is survival (Aubert et al., 1995). Neurons need certain neurotrophic factors for survival and in order to reach their original targets (Stensaas et al., 1987; Davies et al., 1994; Mettling et al., 1995; Robinson et al., 1996). However, these factors are either distant or present in very low levels in the mature CNS (Kawaja et al., 1992), as opposed to the PNS, where they are either constitutively present or up-regulated after injury (Richardson and Ebendal, 1982; Whittemore et al., 1985; Heumann et al., 1987; Sandrock and Matthew, 1987). It has

been found that exogenously providing these neurotrophic factors to the injured CNS improves neuronal survival and axonal regeneration.

To date, numerous neurotrophic factors have been shown to exert neuroprotective effects in the developing and mature CNS, both *in vitro* and *in vivo* (Aubert et al., 1995). Studies show that brain-derived neurotrophic factor (BDNF) improves survival of retinal ganglion cells (RGC) (Meyer-Franke et al., 1995a; Shen et al., 1999) and neurotrophin-3 (NT-3) prevents axotomy-induced atrophy in rubrospinal neurons (Bregman et al., 1995). On the other hand, neurotrophic factors have also been shown to play an important role in promoting axonal regeneration (Schnell et al., 1994; Xu et al., 1995; Grill et al., 1997; Menei et al., 1998). By implanting peripheral nerve tissue into the vitreous body immediately after optic nerve crush, an extensive outgrowth of axons across the lesion site and into distal white matter was observed (Berry et al., 1996). It has been suggested that secretion of neurotrophic factors from grafted Schwann cells contribute to the improvement of regenerative abilities. Similarly, administration of BDNF and NT-3 into spinal cord lesion sites in the presence of embryonic transplants (Bregman et al., 1998), significantly improved the growth capacity of damaged host spinal cord axons.

Different types of neurons favor certain neurotrophic factors. Sensory neurons will regenerate their axons more vigorously in the presence of BDNF and nerve growth factor (NGF) (Lindsay, 1988), whereas retinal ganglion cells grow more vigorously in the presence of fibroblast growth factor (FGF) (Bahr et al., 1989). Recent studies have shown that in adult rats with injured dorsal roots, treatment with NGF, NT-3 and glial-derived

neurotrophic factor (GDNF), but not BDNF, resulted in selective regrowth of distinct subpopulations of damaged axons across the dorsal root entry zone (DREZ) and into the spinal cord (Ramer et al., 2000). This suggests the differential effects of neurotrophins in promoting axonal regeneration. Therefore, it is possible that the correct combination of neurotrophic factors applied to damaged neurons could both protect them from cell death or atrophy, as well as considerably enhance the vigour of their growth after axotomy.

1.2 Glial Scar: Physical and Chemical Barriers of Axonal Regeneration

The PNS regeneration is stimulated by Schwann cells producing NGF and other adhesive molecules (Scherer and Salzer, 1995). In contrast, following injury, CNS neurons encounter a non-permissive environment, including an astrogliotic barrier (McKeon et al., 1995), and myelin inhibitory molecules (Schwab et al., 1993). Damage to the central nervous system (CNS) results in a glial reaction, leading eventually to the formation of a glial scar. This 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 increased expression of glial fibrillary acidic proteins (GFAP) in astrocytes. It has been proposed that astrocytic scars are inhibitory because it is mechanically impossible for axons to penetrate them (Stensaas et al., 1987). On the other hand, studies have shown that reactive astrocytes probably upregulate molecules besides GFAP, such as chondritin sulfate proteoglycans (CS-PGs) and tenascin, which seem to negatively influence their ability to serve as a substrate for regenerating axons (Rudge and Silver, 1990; McKeon et al., 1991).

There is evidence to show that CS-PGs, which are important molecules in inhibiting axonal growth, are produced by astrocytes after CNS injury. Immunostaining and *in situ* hybridization have shown that there is a considerable increase in CS-PGs expression around CNS injury sites. In addition, axonal growth stops precisely where CS-PGs are deposited (Snow et al., 1990; McKeon et al., 1991; McKeon et al., 1995; Stichel et al., 1995). Recently, Davies et al. showed that transplanted adult DRG neurons were able to regenerate their axons in adult CNS white matter if implantation was as atraumatical as possible (Davies et al., 1997). However, if the expression of CS-PGs was increased around the transplant, the axons appeared to stop at this proteoglycan barrier (Davies et al., 1997). In various *in vitro* assays, several CS-PGs 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).

The glial scar which forms around the lesion site often takes weeks to complete. CNS axons, however, are still unable to regrow in the meantime, which suggests that there are other inhibitory factors present in the CNS which block axonal regeneration. Recent evidence also suggests that reactive astrocytes may provide a permissive substrate for axonal regeneration under certain conditions (Aubert et al., 1995). Studies found that reactive astrocytes, at least in the presence of NGF, may be a permissive substrate for axonal regeneration (Kajawa and Gage, 1991). Eagel and colleagues have demonstrated that NGF-responsive sprouting axons, from the nucleus basalis to the striatum of adult rats, can regrow despite being in close apposition with astrocytic processes (Eagle et al., 1995). Other *in vitro* analyses have shown that different astrocytic cell lines possess

varying abilities with respect to their influence on axonal regenerative capacity. They may either promote or inhibit neuronal regeneration, depending on which extracellular matrix (ECM) molecules are produced (Fok-Seang et al., 1995). 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.

1.3 Inhibitory Effect of Myelin on Axonal Regeneration

In vivo, a close relationship exists between the failure of axonal regeneration of CNS neurons and the appearance of myelin in their tracts (see review, Schwab et al., 1993). The possibility that oligodendrocytes and myelin are responsible for regeneration failure has been investigated in a series of experiments conducted both *in vitro* and *in vivo*. Cryostat sections of adult CNS tissue were shown to be non-permissive for neurite outgrowth, especially on densely myelinated areas (Carbonetto et al., 1987; Savio and Schwab, 1989; Crutcher, 1989). In addition, differentiated oligodendrocytes in culture and CNS myelin exhibited a strong inhibitory effect on neuronal adhesion and outgrowth, as well as fibroblast spreading (Caroni and Schwab, 1988a; Bandtlow et al., 1990). Finally, it has been shown that growth cones interacting with differentiated oligodendrocytes collapsed (Bandtlow et al., 1990).

In vivo experiments have demonstrated that preventing oligodendrocyte development and myelin formation via irradiation of neonatal spinal tracts allows adult corticospinal tract fibers to regenerate for 4.5-6mm distal to the lesion site. This is in contrast to the failure of control fibers to elongate more than 1mm (Savio and Schwab, 1990).

Furthermore, recent studies found that if mice were vaccinated against myelin three weeks before spinal cord injury, numerous axons regenerated across the lesion sites, and grew to up to two thirds of the length of the entire spinal cord, whereas no regeneration was observed in control injected mice (Huang et al., 1999). All these studies strongly suggest that CNS myelin is non-permissive for axonal outgrowth. Neutralization of the inhibitory effects of CNS myelin could, in theory, improve axonal regeneration dramatically.

Initially, because regeneration in the PNS proceeds after injury, it was assumed that only CNS myelin contained inhibitory components. However, it was found later that peripheral myelin does, in fact, inhibit axonal growth of both PNS and CNS neurons (David et al., 1995; Shen et al., 1998). However, while peripheral and central myelin seem to be equally inhibitory, PNS grafts support axon growth of CNS neurons whereas CNS tissue does not (Bahr and Przyrembel, 1995). This is most likely due to the effective removal of myelin debris following injury, the proliferation and de-differentiation of Schwann cells and the supplies of growth promoting molecules in the PNS (see review, Scherer and Salzer, 1995). These processes, however, do not occur in the CNS (see review, Hirsch and Bahr, 1999).

Compared to the CNS, the PNS environment is supportive of regeneration because Schwann cells provide suitable substrates for regrowing axons. When mammalian peripheral nerves are cut, the axons distal to the lesion undergo Wallerian degeneration (Waller, 1850). This is normally associated with the recruitment of macrophages to the

lesion site, which remove axonal and myelin debris (Stoll et al., 1989; Perry et al., 1987; Brown et al., 1991), and with the proliferation of Schwann cells (Salzer and Bunge, 1980). In the PNS, Schwann cells may be crucial for the regeneration of axons across the lesion site in injured nerves and for the correct orientation of growing axons (see review, Hirsch and Bahr, 1999). It is believed that the longitudinal alignment of the endoneurial tubes, the so called bands of Bungner (Bunger, 1891), and the Schwann cell's surface and matrix molecules associated with them in lesioned nerves (Salzer and Bunge, 1980), provide the basis of a guidance system for regenerating axons. Studies also showed that during regeneration, Schwann cells de-differentiate and down-regulate the expression of myelin-associated proteins (Scherer and Salzer, 1995). At the same time, growth-promoting molecules such as L-1 and N-CAM are up-regulated in Schwann cells after lesioning (Heumann et al., 1987; Martini et al., 1990; Meyer et al., 1992). In contrast, CNS axons do not regenerate perhaps because the above changes do not occur in the CNS.

1.3.1 Myelin Specific Inhibitors: Nogo, MAG, and Other Inhibitors

It is believed that the inhibitory effects of myelin on regeneration come from its array of myelin-associated neurite outgrowth inhibitors. Certain myelin components have been long identified as inhibitors of axonal growth (Schwab et al., 1993).

1.3.1.1 Nogo

Initial biochemical analysis revealed that part of the inhibitory activity of differentiated oligodendrocytes and CNS myelin in rats resided in the two protein fractions of 35 (NI-35) kDa and 250 (NI-250) kDa, which exerted strong neurite

outgrowth inhibition when used as culture substrates (Caroni and Schwab, 1988a). The subsequent production of the monoclonal antibody IN-1, raised against NI-250, was shown to reduce the inhibitory activity of CNS myelin, as well as facilitate the structural plasticity of a variety of lesioned fiber tracts (Caroni and Schwab, 1988b; Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995).

Based on the preliminary biochemical data on rat NI-35/250, the bovine homolog bNI-220 was purified to homogeneity (Spillmann et al., 1998). Purified bNI-220 is a strong inhibitor for neurite outgrowth of many neurons and neuronal cell lines. The inhibitory properties can be completely neutralized by monoclonal antibody IN-1 (Spillmann et al., 1998). Protein microsequence analysis of bNI-220 gave rise to six peptides that led to the cloning of the corresponding cDNA (Chen et al., 2000). The gene, called *nogo*, gives rise to three splice variants with Nogo-A as the largest transcript (Chen et al., 2000).

Three groups reported the identification of the novel gene, *nogo*, which encodes an inhibitory myelin protein, recognized by the IN-1 antibody (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Based on homology, the protein encoded by *nogo* was classed as the fourth member of the reticulon family, proteins associated primarily with the endoplasmic reticulum (ER) membrane (GrandPre et al., 2000; Prinjha et al., 2000). There are three alternatively spliced isoforms of *nogo*, designated Nogo-A, B and C. Nogo-A is mainly localized to CNS myelin, and is expressed by oligodendrocytes but not by Schwann cells (GrandPre et al., 2000). Nogo-B and -C are found in both CNS and

PNS myelin, as well as in certain neurons and several non-neural tissues (Chen et al., 2000). All three groups agree that Nogo-A protein is inhibitory on axonal regeneration in culture (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). However, there are controversies as to whether Nogo-B and -C are actually inhibitory, which Nogo-A domains are inhibitory and what configuration Nogo-A has in the membrane (Goldberg and Barres, 2000).

Since neutralization of Nogo effected by IN-1 antibody only promotes 5-10%, at most, of injured axons to grow across the lesion site (Schnell et al., 1994), it is reasonable to assume that there are other inhibitory molecules present in myelin. Studies of fractions from purified CNS myelin showed that two fractions exhibited neurite outgrowth inhibitory activity, one of which could be immunodepleted by an antibody against Myelin-Associated Glycoprotein (MAG) (McKerracher et al., 1994). At the same time, our lab showed that MAG potently inhibits neurite outgrowth from postnatal cerebellar neurons as well as older dorsal root ganglion (DRG) neurons (Mukhopadhyay et al., 1994). These results suggest that MAG may be another important inhibitor in CNS myelin.

1.3.1.2 Myelin-Associated Glycoprotein (MAG)

1.3.1.2.1 Structure and Location

The myelin-associated glycoprotein (MAG), relative to other myelin-specific proteins, is a quantitatively minor component of myelin sheaths, comprising 1% and 0.1% of total myelin protein in the CNS and PNS, respectively (Schachner and Bartsch,

2000). Since MAG is heavily glycosylated, it was first detected by the incorporation of radioactive sugar precursors into glycoproteins that were present in purified CNS myelin fractions (Quarles et al., 1973). This 100-kDa glycoprotein has five extracellular immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic domain that occurs as two different isoforms resulting from alternative mRNA splicing (see review, Quarles 1992). These two polypeptide isoforms (L-MAG and S-MAG) have different molecular masses, which are 72 kDa and 67 kDa, respectively. However, when glycosylated, these polypeptides co-migrate on a SDS-PAGE as a single, broad band of about 100 kDa. The difference in the molecular weights of these two isoforms is caused by the distinction between their cytoplasmic domains, where the 53 C-terminal amino acid sequence in its intracellular domain of L-MAG is replaced by a different sequence of 9 amino acids in S-MAG (see review, Quarles 1992).

In the rodent brain, the large and small isoforms are developmentally regulated, with a greater expression of L-MAG relative to S-MAG early in myelinogenesis and substantial amounts of both isoforms present in the adult stage (Quarles, 1997). Unlike in CNS, 95% or more of the MAG synthesized in the PNS appears to be the shorter isoform at all ages (Frail et al., 1985; Noronha et al., 1989; Tropak et al., 1988).

As a member of the immunoglobulin (Ig) superfamily, MAG has five Ig-like extracellular domains. The terminal domain (D1) of MAG folds as a V-type conformation, in contrast to the C2-type folding in the other domains of MAG (see review, Quarles 1992). The consequence of this conformation is the formation of an

unusual intrasheet disulfide linkage between the second and third cysteines in D1 as well as a interdomain disulfide linkage between the first cysteine in D1 and the fifth cysteine in D2 (see review, Quarles 1992). Subsequently, the tripeptide sequence Arg-Gly-Asp (RGD) in the interior of D1, an epitope known to be a binding site for some extracellular proteins to integrin-type receptors on the cell surface, is in a location of limited accessibility (see Figure 1.1). This is also consistent with the experimental observations that the anti-RGD antibody only recognizes denatured MAG (Pedraza et al., 1990) and that the RGD-containing peptide does not block the binding of MAG containing liposomes to axons (Sadoul et al., 1990). Therefore, the steric inaccessibility of the RGD sequence in MAG excludes the possibility of MAG binding to integrins via this sequence (Pedraza et al., 1990).

In addition, structural studies by transmission electron microscopy reveal a rod-like structure for the extracellular domain of MAG with a terminal globular part and two thin parallel arms (Fahrig et al., 1993). Thus, predominantly the third Ig-like domain (D3) and parts of the second (D2) and fourth (D4) domains would comprise the globular part of MAG, while Ig-like domains D1 and D5 as well as parts of the Ig-like domains D2 and D4, are located in the non-globular part (Fahrig et al., 1993).

Immunocytochemical localization of MAG on the cell surface of oligodendrocytes and Schwann cells has been performed both at the light and electron microscopy level (Bartsch et al., 1989; Martini and Schachner, 1986). It has been found that the surface of oligodendrocyte processes is already MAG-immunoreactive when axons are being

ensheathed (Bartsch et al., 1989; Trapp et al., 1989), whereas on myelinating Schwann cells, MAG becomes detectable only after Schwann cell processes have turned for approximately 1.5 loops around the axon (Martini and Schachner, 1986). After myelination, MAG is restricted to the periaxonal region of myelinated axons (Sternberger et al., 1979; Trapp and Quarles, 1982), and is expressed in the paranodal regions of myelin sheaths (Trapp and Quarles, 1982; Martini and Schachner, 1986). However, in PNS myelin sheaths, MAG is also present in Schmidt-Lanterman incisures, lateral loops, and the inner and outer mesaxons (Trapp et al., 1982; Quarles, 1997).

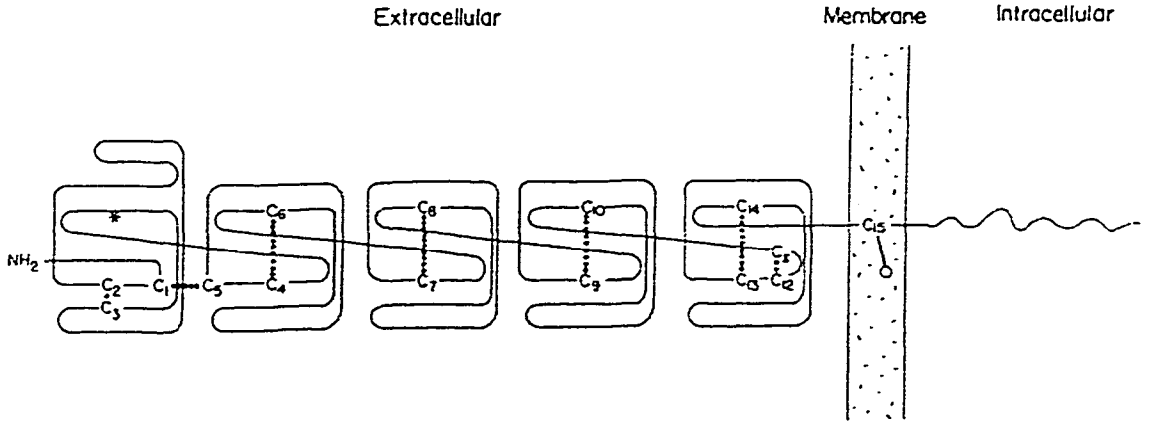


Fig.1.1 Model of the conformation of MAG. The predicted positions of cysteines and their disulfide linkages are indicated, including an interdomain disulfide linkage of first and fifth cysteine, and the intrasheet linkage of second and third linkage. The predicted position of the RGD sequence (*) of MAG within the first domain is also shown (Pedraza et al., 1990).

The localization of MAG in the periaxonal membranes of myelin-forming oligodendrocytes and Schwann cells suggests that it may function in glia-axon interactions. The functional role of MAG in the interaction of glia with neurons is further supported by the experiments showing that MAG affects the neural adhesion and neurite outgrowth function. For example, MAG-containing liposomes specifically bind to neurons (Poltorak et al., 1987; Johnson et al., 1989; Sadoul et al., 1990). In addition, it

has been found that neuron-oligodendrocyte adhesion as well as MAG-promoted DRG neurite extension could be blocked by anti-MAG antibodies (Poltorak et al., 1987).

1.3.1.2.2 The Function of MAG in Myelination

In addition to the particular location of MAG at the interface between axons and myelin sheaths, many *in vitro* and *in vivo* experiments have further suggested that one of the functional roles of MAG is in the initiation of myelination. In myelinating co-cultures of DRG neurons and Schwann cells, MAG was upregulated on myelinating Schwann cells relative to MAG expression on non-myelinating Schwann cells (Owens and Bunge, 1989). Furthermore, when Schwann cells were infected by a retrovirus expressing MAG antisense RNA, used to reduce levels of MAG expression, they failed to segregate large caliber neurites of co-cultured DRG neurons and myelination of DRG was impaired (Owens and Bunge, 1991). In contrast, when levels of MAG expression were increased in Schwann cells, initial investment of DRG neurites was accelerated (Owens et al., 1990).

Furthermore, analyzing the phenotype of MAG knockout mice generated by homologous recombination has suggested the involvement of MAG in the initiation of CNS myelination, the formation of morphologically intact myelin sheaths, and the maintenance of myelin (see review, Schachner and Bartsch, 2000). MAG null mutants display various subtle but not dramatic abnormalities in the CNS and degenerate with age in the PNS (Fujita et al., 1998). In MAG-/- mice, the formation of CNS myelin is significantly delayed (Li et al., 1994; Montag et al., 1994), while oligodendrocytic cytoplasmic collars of mature CNS myelin are frequently missing or reduced (see review,

Bartsch, 1996). Compact myelin of MAG-/- mice also contains an increased presence of cytoplasmic loops of oligodendrocytes. Studies have identified Fyn, a nonreceptor tyrosine kinase, as a signaling molecule downstream of L-MAG, and also implicated its involvement in the initiation of CNS myelination (Umemori et al., 1994). Furthermore, it has been shown that in MAG/Fyn double knockout mutant mice, a much more severe degree of hypomyelination in the CNS nerves is observed compared to MAG or Fyn single deficient mutants (Biffiger et al., 2000). Behavior deficits are also seen in the MAG/Fyn-deficient mice (Biffiger et al., 2000). Taken together, these observations suggest that MAG and its related signaling pathways likely participate in the initiation of CNS myelination.

In contrast, PNS myelin formation proceeds normally in MAG-deficient animals (Montag et al., 1994). However, analysis of the PNS of older MAG mutants revealed axonal and myelin degeneration with the presence of superfluous Schwann cell processes, indicating that MAG plays a role in maintaining PNS myelin integrity (Fruttiger et al., 1995). Recent studies also showed a correlation between the absence of MAG in the PNS with a reduced axonal caliber, decreased neurofilament spacing and reduced neurofilament phosphorylation (Yin et al., 1998). These data support a functional role for MAG in modulating the maturation and viability of myelinated axons (Yin et al., 1998).

In addition, a mutant mouse deficiency in L-MAG has recently been generated by introducing a stop codon into exon 13 of the MAG gene (Fujita et al., 1998). Exon 13 encodes the C-terminal portion of L-MAG, but not S-MAG. So mutant mice continue to

express S-MAG, but produce truncated L-MAG, lacking the unique cytoplasmic domain of L-MAG (see review, Schachner and Bartsch, 2000). Compared to the total MAG knockout mice, the CNS myelin of these animals displays most of the morphological abnormalities, whereas the PNS of the truncated L-MAG mutants appears normal. These studies have shown a differential role for the L-MAG isoform in CNS and PNS myelin. The cytoplasmic domain of L-MAG is necessary for the proper myelination of CNS, whereas S-MAG is sufficient to maintain the integrity of PNS myelin (Fujita et al., 1998).

1.3.1.2.3 Function of MAG in Axonal Regeneration

Like other members of the immunoglobulin (Ig)-superfamily such as L-1 and NCAM, MAG was originally found to be a neurite-outgrowth promoting molecule. However, recent studies suggest that MAG is also a potent inhibitor of axonal regeneration. While MAG promotes the neurite outgrowth from newborn dorsal root ganglion (DRG) neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994), it inhibits outgrowth from adult DRG neurons (Mukhopadhyay et al., 1994), a variety of other postnatal neurons (DeBellard et al., 1996), and a neuroblastoma cell line (McKerracher et al., 1994). Therefore, MAG is classed as a bifunctional molecule since it can either promote or inhibit neurite outgrowth depending on the age and type of neuron (Mukhopadhyay et al., 1994; DeBellard et al., 1996).

It has been found that neurite outgrowth from postnatal cerebellar and DRG (older than postnatal day 3) neurons plated on MAG-expressing CHO cells was significantly inhibited compared with neurons plated on control cells (Mukhopadhyay et al., 1994). A

partial decrease of this neurite growth-inhibitory activity could be achieved by the application of polyclonal anti-MAG antibodies (Mukhopadhyay et al., 1994). Similarly, NG108-15 cells did not extend neurites onto a substratum adsorbed with myelin or MAG. In addition, the inhibitory activity of purified bovine CNS myelin was reduced by more than 60% after immunodepletion of MAG from the extract (McKerracher et al., 1994).

Furthermore, Li and colleagues reported that 80% of growth cones collapse when neurons encountered MAG-coated beads. Moreover, 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). In addition, by infecting Schwann cells with a retroviral vector carrying MAG cDNA, it has been demonstrated that neurite outgrowth from cerebellar, DRG and superior cervical ganglion (SCG) neurons is inhibited by MAG-expressing Schwann cells (Shen et al., 1998). These MAG-expressing Schwann cells also inhibit neurite branching of DRG and SCG neurons, which implies a role for MAG in the control of collateral sprouting, another important determinant in axonal regeneration (Shen et al., 1998). Taken together, these data suggest that MAG is a potent inhibitory molecule instead of only a non-permissive molecule for growth.

Although the studies in cell culture indicate that MAG is a potent inhibitor of axonal regeneration, the results from *in vivo* studies of MAG-deficient mice remain controversial. Comparing the regeneration after spinal cord injury in MAG-/- mice to wildtype mice has resulted in conflicting findings. Li et al (1996) found not only longer

axons in MAG-/- mice but also more axons regenerating (Li et al., 1996), while Bartsch and colleagues found no difference between the MAG-/- and wildtype mice (Bartsch, 1996). Two possible theories may explain these contradictory results. First, different mouse strains were used in each group, which might result in different responses to injury. Second, each group used different methods of analysis. Li and colleagues measured the number and the length of all regenerating axons, while Bartsch et al only reported the length of one axon per animal, the longest (Li et al., 1996; Bartsch, 1996). The subtle differences in regeneration between MAG-/- and wildtype mice may not have been revealed by the latter method.

Despite these different observations *in vivo*, it is reasonable to think that the lack of an obvious improvement in axonal regeneration of MAG-/- mice might be due to redundancy of myelin specific inhibitors. It is quite unlikely that one inhibitor is solely responsible for the failure of axonal regeneration. Once one inhibitor like MAG has been knocked out, it is possible that another inhibitory molecule, either constitutively present or up-regulated in response, compensates for the reduced inhibitory effect by the absence of an individual inhibitor. For example, the application of IN-1 antibody to injured normal rats, allows only 5-10% of axons to regrow (Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995), whereas axonal regeneration in the myelin-immunized mice (Huang et al., 1999) was much more extensive by comparison (Filbin, 2000). Taken together, these observations suggest that in order to get significant improvement in axonal regeneration from MAG-/- mice, it will be necessary to block the inhibitory effects of all potential inhibitors simultaneously (Qiu et al., 2000).

In addition to studies *in vivo*, the neurite outgrowth on purified CNS myelin from MAG-/- and normal mice was compared. It was found that the neurite length of cerebellar, retinal or older DRG neurons on MAG-/- CNS myelin was about twice as long as on wildtype myelin (Shen et al., 1998). Similarly, Li et al observed an improvement in average neurite length from NG108-15 cells grown on MAG-/- CNS myelin, even though they reported the improvement was not statistically significant because of experimental variations (Li et al., 1996). However, Bartsch et al found no difference in outgrowth from cerebellar or DRG neurons on wildtype or MAG-/- myelin substrates (Bartsch, 1996). The possible explanation for the high variability in experimental results may be in the preparation of the myelin used as a substrate. It was observed that a large amount of MAG is converted to its soluble form, dMAG, within 30 minutes of incubation in the course of myelin purification (Sato et al., 1982). Therefore, MAG might be lost as dMAG during the substrate preparation (Filbin, 1996).

Despite these controversies surrounding MAG null mutants, recent studies with the double mutant mice that crossbreed C75BL/Wld^s with MAG-/-, demonstrate that MAG can inhibit axonal regeneration *in vivo*. As mentioned before, the PNS axon regenerates only after myelin is cleared (Stoll et al., 1989; Perry et al., 1987; Brown et al., 1991) and myelin-associated proteins are down-regulated by Schwann cells (Salzer and Bunge, 1980), a process called Wallerian degeneration. In the mutant mouse strain, C57BL/Wld^s, Wallerian degeneration is markedly delayed and myelin debris persists for weeks after axotomy, in contrast to a few days in wildtype mouse (Brown et al., 1992; Brown et al.,

1994). Consequently, regeneration in these mutants is impaired, especially in nerves with very few unmyelinated nerves such as phrenic nerves (Brown et al., 1994). However, upon breeding C57BL/Wld^s with MAG-/- mice, regeneration in these double mutant mice is increased (Schafer et al., 1996). Thus, the absence of MAG in these animals was sufficient to enhance PNS regeneration significantly.

In addition, transgenic mice in which expression of MAG is under the control of the p75 promoter, were developed (DeBellard et al., 1999). After injury, in these transgenic mice, expression of MAG in Schwann cells is upregulated along with endogeous p75, instead of being down-regulated as in normal mice. Interestingly, these transgenic mice show decreased PNS axonal regeneration following injury compared to their wildtype counterparts (DeBellard et al., 1999) Together, these observations suggest that MAG, when presented to an axon *in vivo*, can inhibit axonal regeneration.

1.3.1.2.4 The Binding Partner of MAG

Because of the functional importance of MAG in axonal regeneration and myelination, the next important question to ask is what is the identity of the putative axonal receptors with which MAG interacts. One clue to its identity emerged from the fact that MAG is a member of the Ig superfamily. The Ig superfamily members such as CD22 and sialoadhesin, are known to bind to sialic acid-containing oligosaccharides (Powell and Varki, 1995). Similarly, MAG has also been found to possess a sialic acid-dependent binding property.

Studies have shown that MAG binds specifically to a sialic-acid component on both cerebellar and DRG neurons at all postnatal ages, regardless of whether outgrowth is promoted or inhibited (Kelm et al., 1994). Desialyation of neurons prior to the outgrowth assay or inclusion of small sialic-acid-bearing oligosaccharides in the cultures, abolishes the binding of MAG to neurons (DeBellard et al., 1996). In addition, it has been shown that this sialic acid-dependent binding is trypsin-sensitive (DeBellard et al., 1996), which suggests this interaction between MAG and neurons is via a sialoglycoprotein. Moreover, it has been found that MAG's inhibitory or promoting effects on neurite outgrowth are reduced by desialyation, even though complete reversal is never accomplished (DeBellard et al., 1996). Studies with a truncated form of MAG, MAG domain 1 to 3 fused with Fc, also show that even though MAG (d1-3)-Fc binds to neurons in a sialic acid-dependent manner, it does not inhibit axonal growth (Kelm et al., 1994). This suggests that the sialic acid-dependent binding of MAG to neurons is only partially responsible for its inhibitory effects on axonal growth (Qiu et al., 2000).

Studies with MAG mutated at Arg118 further strengthen this hypothesis. By aligning the first Ig-like domain of MAG, sialadhesin and CD22, it was noted that Arg118 (R118) in the first Ig-domain of MAG corresponds to the arginine involved in sialic acid-dependent binding of CD22 and sialoadhesin (Tang et al., 1997a). It has been shown that mutation at this site abolishes the sialic acid-dependent binding of MAG to neurons completely (Tang et al., 1997a). In addition, the inhibition of neurite outgrowth by a soluble form of MAG is lost if this site is mutated. However, if MAG mutated at Arg118 is expressed in CHO cells or Schwann cells, the inhibitory effects of MAG are

unaffected; R118A MAG expressed by cells inhibits neurite outgrowth as effectively as wildtype MAG (Tang et al., 1997a). These data suggest that the inhibition of neurite outgrowth by soluble MAG-Fc requires the Arg118 binding site, but that sialic-acid binding alone is insufficient to effect inhibition of axonal regeneration when R118A MAG is expressed by cells (Tang et al., 1997a). Therefore, it is proposed that there are two neuronal recognition sites on MAG, one that binds one or more sialoglycoproteins, and another that is the neurite inhibition effecting site (Filbin, 1996).

In addition to binding sialoglycoproteins, MAG will also bind some gangliosides, such as GD1a and GT1b (Yang et al., 1996). The fact that MAG binds to oligosaccharides on both glycoproteins and glycolipids also indicates that there may be more than one binding site for MAG on the axolemma (Quarles, 1997). Regardless of what MAG interacts with, the interaction must activate second messenger systems in axons. Elucidation of downstream signaling pathways induced in neurons may be helpful for the studies with the MAG receptor or receptor complex.

1.3.1.3 Other Inhibitory Molecules

In addition to Nogo and MAG, there are a number of myelin-related or glial-derived inhibitory molecules present in the adult CNS, which also may contribute to the non-permissiveness of the environment.

1.3.1.3.1 Tenascin R

As a member of the tenascin family of molecules, Tenascin R (TN-R) is present in the extracellular matrix in the CNS, most abundantly in white matter (Schachner, 1994). It is

produced by oligodendrocytes during the onset and early stages of myelination and remains detectable in the adult (Xiao et al., 1998). It has also been shown that Tenascin-R is upregulated after injury (Fawcett and Asher, 1999). *In vitro* experiments demonstrate that Tenascin R is inhibitory to the axonal growth of several types of neurons such as cerebellar and hippocampal neurons (Wintergerst et al., 1997). This inhibition is mediated by interaction with a specific cell surface molecule, F3/11 (Pesheva et al., 1993). Interestingly, studies also show that if TN-R is coated as a uniform substrate, it can neutralize the inhibitory effect of the phosphacan-related molecules on neurite outgrowth (Xiao et al., 1997). In contrast, when TN-R is present as a sharp substrate boundary with a permissive substrate, it usually repels the growth cone and prevents neurite elongation (Pesheva et al., 1993). However, if in the presence of phosphacan-related proteoglycans, this growth cone repulsive effect of TN-R is abolished (Xiao et al., 1997). These observations suggest that Tenascin R can interact with various proteoglycans that modulate its inhibitory influence on neurite growth (Xiao et al., 1997).

1.3.1.3.2 Oligodendrocyte Precursors and NG2 Proteoglycan

Recently, the CNS has been shown to contain many small cells that stain with an antibody against NG2 proteoglycan (NG2 PG). These cells were identified as oligodendrocyte precursors (Levine and Nishiyama, 1996). NG2 proteoglycan is found all over the surface of these cells in developing and adult CNS, and has been found to be an inhibitor of axonal growth from sensory, cortical and cerebellar neurons (Dou and Levine, 1994; Dou and Levine, 1997; Levine and Nishiyama, 1996). It has been shown that after CNS injury, the oligodendrocyte precursors are recruited to the lesion site,

which then express several proteoglycans such as NG2 (Levine, 1994; Keirstead et al., 1998). NG2 represents only 0.2% of the proteoglycans produced by the oligodendrocyte precursor cells, but neutralization of NG2 with a blocking antibody removes most of the inhibitory activity of these cells. This suggests that NG2 is very potent in inhibiting axonal regeneration, at least *in vitro* (Fidler et al., 1999).

1.3.1.3.3 Proteoglycans in CNS Myelin: Versican and Brevican

It has been shown that there are two proteoglycan molecules, versican and brevican, present in purified CNS myelin, which in addition to Nogo and MAG, contribute to the neurite growth-inhibitory activity of myelin (Niederost et al., 1999). Immunohistochemical staining shows that these two molecules are on the surface of differentiated oligodendrocytes (Niederost et al., 1999). Like NG2, versican and brevican, whose levels of expression are increased in the adult brain compared to embryonic brain, can also be upregulated following injury (Fawcett and Asher, 1999). Moreover, it has been shown that *in vitro* neurite outgrowth of cerebellar and DRG neurons is inhibited by membrane fractions containing these two CS-PGs. Versican and brevican can also induce growth cone collapse, which is reversed by the inclusion of a proteoglycan synthesis inhibitor (Niederost et al., 1999).

In summary, a large number of myelin-related molecules mainly contribute to the failure of axonal regeneration in the adult CNS, which include cell surface inhibitory molecules, extracellular matrix molecules and a variety of proteoglycans. These myelin-associated inhibitors are important in the early stages of axonal regeneration, since glial

scar-derived inhibitors such as CS-PGs usually take time to be expressed in the injured site, and the scar takes even longer to form a physical barrier and "lock in" axons. In the meanwhile, there is a window of opportunity for axonal regeneration. To accomplish spontaneous CNS repair during this window period, it would be more practical to block all these inhibitors simultaneously rather than to neutralize the inhibitory effect of each individual inhibitory molecule. Using therapeutic approaches like vaccinating with CNS myelin against spinal cord injury may achieve this goal (Huang et al., 1999; Filbin, 2000).

1.4 Axonal Guidance Cues Effective during Development

After CNS damage, injured neurons initially face the challenge of maintaining survival and initiating axonal regrowth. Subsequently, the regenerating axons need to return to their original destinations. The specificity required for the proper pathway formation during development is thought to rely on a number of molecular guidance cues and mechanisms. After injury, regenerating axons may also use the same guidance cues to migrate back to their original targets. Analysis of the molecular basis of these guidance mechanisms during development has shown that the molecules involved often belong to distinct multigene families and that they can provide both short- and long-range attractive as well as repulsive cues (Tessier-Lavine and Goodman, 1996). Axons can be guided at short-range by contact-mediated mechanisms involving nondiffusible cell surface and ECM molecules, and also directed by long-range cues, which are diffusible chemotropic substances secreted by target cells at a distance such as netrins and semaphorins (Tessier-Lavigne and Goodman, 1996).

1.4.1 Netrins and their Receptors

The target-derived diffusible factors, netrins, were first indicated to mediate long-range chemoattractive actions of floor plate cells on commissural axons (Keynes and Cook, 1995a). Subseuqently, netrins were found to be bifunctional molecules, capable of attracting some axons while repelling others (Tessier-Lavigne and Goodman, 1996). Such differential neuronal responses to netrins are originally presumed to be dependent on the receptors expressed by the growth cone of different types of neurons. It has been shown that members of the *deleted in colorectal cancer* (DCC) subfamily of the Ig superfamily are components of receptors that mediate the attractive effects of netrins (Chan, 1996). On the other hand, UNC-5, a transmembrane protein that defines a distinct branch of the Ig superfamily, mediates repulsive actions of the netrin UNC-6 in *C. elegans* (Hamelin, 1993). Furthermore, studies with the turning responses of *Xenopus* spinal neurons to a netrin-1 gradient, show that the attractive turning response of these axons toward netrin-1 requires the DCC receptors, and converts to a repulsive response when an UNC-5 receptor is expressed in these neurons (Hong et al., 1999). It has also been suggested that the cytoplasmic domain of UNC-5 determines the response of growth cones to the netrin-1 signal, since a chimeric receptor consisting of a DCC ectoderm and an UNC-5 cytoplasmic domain mediates neuronal repulsive response to netrin-1 (Hong et al., 1999).

However, recent studies also showed that in culture, the same *Xenopus* spinal neuron axon exhibits opposing turning responses to certain extracellular gradients, like netrin-1 (Ming et al., 1997), depending on their intracellular cyclic AMP (cAMP) levels. By altering a cAMP-dependent pathway in the growth cone, neuronal responses to netrin-1

can be switched from attraction to repulsion and vise versa (Ming et al., 1997). Experiments further demonstrate that in the presence of laminin-1, netrin-mediated attraction is converted into repulsion since the cAMP level in neurons is lowered by laminin (Hopker et al., 1999). These results suggest that modulating neuronal cAMP levels or the activity of cAMP-dependent pathways, by ECM molecules as well as by pharmacological reagents, can switch the behavior of growth cones in response to certain diffusible guidance cues. In addition, recent studies have shown that the interaction between netrin-1 and an adenosine receptor A2b, a receptor able to stimulate the activity of adenylyl cyclase in response to the adenosine binding, modulates intracellular cAMP level and then mediates netrin-dependent attraction and/or axonal outgrowth (Corset et al., 2000). It has also been suggested that a receptor complex containing DCC and A2b may be required for the chemoattracting and growth-promoting functions of netrin-1 (Corset et al., 2000).

In addition, cyclic GMP (cGMP) has also been found to have dramatic effects on chemotropic (attractive/repulsive) actions mediated by certain guidance molecules such as semaphorins III (Sema III) (Song et al., 1998). Phosphalipase C-γ, phosphoinositode 3-kinase, and Ca^{++} also can play critical roles in regulating the turning responses (Ming et al., 1999). For example, by lowering the concentration of extracellular Ca^{++}, the turning responses of spinal neurons to netrin-1, BDNF or MAG, but not Sema III, are completely abolished (Song et al., 1998). Therefore, Poo and colleagues classified axonal guidance cues into two groups, depending on the cAMP/cGMP-dependency of growth cone turning responses as well as calcium-dependent/independent regulation in attractive/repulsive

reactions (Song et al., 1998). Together, these studies have begun to identify common signaling pathways that are utilized by different classes of axonal guidance cues.

1.4.2 Semaphorins/Collapsins

The repulsive turning of growth cones is thought to be an important guidance mechanism as well and result from a local, asymmetrical collapse of growth cones (Keynes and Cook, 1995b). Semaphorins/Collapsins are a large family of cell-surface and secreted proteins that function as chemorepellents or inhibitors (Tessier-Lavigne and Goodman, 1996). There are at least five different subtypes of semaphorins, including the secreted and transmembrane members. Among them, vertebrate Semaphorin III/Collapsin-1 has been shown to be a potent inducer of sensory growth cone collapse (Luo, 1993) and has been implied as a diffusible chemorepellent that patterns the sensory axon projections in the spinal cord (Tessier-Lavigne and Goodman, 1996).

Furthermore, studies have shown that the signaling molecule cGMP is a primary determinant of the different turning responses to SemaIII. In *Xenopus* spinal neurons, elevating intracellular cGMP levels could switch SemaIII-mediated growth cone repulsion into attraction (Song et al., 1998). Interestingly, recent studies that addressed the function of SemaIII in development of the cerebral cortex suggest that there is a gradient of SemaIII in the developing cortex, which directs axons to grow down the gradient to reach the subcortical white matter. In contrast, the apical dendrite of cortical cells extends up this gradient toward the pial surface. Correspondingly, it has been observed that soluble guanylyl cyclase in these neurons is distributed asymmetrically,

which then generates a difference in local concentrations of cGMP, -low in the cell body and high in the apical dendrite. High concentrations of cGMP in apical dendrites may contribute to attraction, as reagents that reduce cGMP levels or activity disrupt the response of dendrites to SemaIII (Polleux et al., 2000). Together, these studies illustrate one of the physiological settings in which cyclic nucleotides modulate neurite guidance (Strittmatter, 2000).

1.4.3 Nerve Growth Factor (NGF) Family of Neurotrophins

In addition to being trophic factors, the nerve growth factor (NGF) family of neurotrophins has been considered as chemotropic factors for years. Recent studies with double null mice of NGF and BAX, a pro-apoptotic signaling molecule that is associated with programmed cell death, suggest that NGF does contribute to guiding sensory axons to innervate their targets. All DRG neurons that normally die in the absence of NGF/Trk signaling survive if BAX is also eliminated. These neurons fail to innervate superficial cutaneous targets and express the biochemical markers characteristic of the nociceptive phenotypes (Patel et al., 2000). Several lines of evidence also suggest that NGF is involved in the regulation of axon terminal arborization within the target tissue (Thoenen, 1991). Furthermore, studies have shown that other NGF family neurotrophins such as BDNF and NT-3 function as chemotropic factors *in vitro* and may play a role in guidance during development (Kennedy and Tessier-Lavigne, 1995).

Recent studies by Poo and colleagues show that differences in cAMP-dependent activity in a *Xenopus* spinal neuron result in opposite turning responses of the growth

cone in response to a gradient of BDNF, but not to NT-3. A gradient of BDNF normally triggers an attractive response, whereas the same gradient induces a repulsive response when a PKA inhibitor is included (Song et al., 1997). Moreover, cAMP-dependent activity plays a role in regulating neuronal growth cone collapse induced by neurotrophins. For example, when embryonic *Xenopus* spinal neurons are first isolated and cultured within 6 hours, BDNF induces growth cone collapse and neurite retraction. This collapsing effect is cAMP-dependent since elevation of cAMP levels completely blocks the collapsing effect (Wang and Zheng, 1998). After longer period in culture, like 24 hours, when neurons grow toward BDNF, inhibition of PKA restores the collapsing action (Wang and Zheng, 1998) as well as blocks the chemoattraction (Song et al., 1997).

1.4.4 Slit Proteins and Robo

In flies and vertebrates, the midline acts as an important choice point for navigating axons, known as a source of both attractive and repulsive cues. Recently, Slit proteins have been identified as the midline repellents in *Drosophila* and mammal, which are expressed by midline glial cells and are responsible for preventing axons from crossing the midline inappropriately during development. Slit mutants have a collapsed midline phenotype, where axons extend towards the midline but, rather than crossing it, remain at the midline as one large axon fascicle (Tessier-Lavigne and Goodman, 1996).

In addition, studies have identified Robo as a transmembrane receptor and a member of the Ig superfamily that mediates repulsive responses to the repellent, Slit. Three lines of evidence from studies using *Drosophila* strongly implicate that Robo is the receptor

for a repellent factor made by midline cells (Brose and Tessier-Lavigne, 2000). First, the expression of Robo on axons correlates with the inability to cross the midline. For example, longitudinally projecting neurons, whose axons never cross the midline, express Robo on their axons from the start. On the other hand, commissural neurons, whose axons cross the midline once and then grow alongside it without recrossing, express Robo only after they have crossed the midline (Kidd et al., 1998). Second, in *robo* mutants, these axons cross and recross the midline freely. Finally, the phenotype of Robo mutants can be rescued by transgenic expression of Robo in neurons.

Later, analyzing phenotypes of *robo* and *slit* homozygote and heterzygote mutants suggests that Robo is the receptor of Slit proteins. *In vitro* binding assays further show that Slit and Robo interact with each other. Taken together, these studies demonstrate that Slit is indeed the midline repellent and the Robo ligand. A recent study has also identified that a Slit protein is a positive regulator of sensory axon elongation and branching in vertebrates (Brose and Tessier-Lavigne, 2000). Wang and colleagues developed an assay in which DRG neurons are isolated immediately before the stage at which branching starts and cultured and exposed to different factors. When these neurons are exposed to the amino-terminal cleavage fragment of bovine Slit2 (Slit2-N), axons show more complex, longer, and branched morphology (Wang, 1999). In addition, mRNAs for both Slit2 and Slit1, as well as for the receptor Robo2, are expressed at the appropriate time and place to play a role in regulating branching *in vivo* (Brose and Tessier-Lavigne, 2000).

1.5 Intrinsic Growth Capacity of Neurons

During development, two events are ongoing in neurons, which are the appearance of myelin and the loss of neuronal regenerative capacity. Many *in vivo* and *in vitro* observations suggest that immature mammalian CNS neurons possess the intrinsic capacity to repair themselves after injury (Cajal, 1928; Shewan et al., 1995). However, this growth capacity is lost during early developmental stages (Shewan et al., 1995). For example, mature CNS tissue, which does not support regrowth of its own damaged axons, does support axonal growth from embryonic CNS neurons implanted into the adult animals (Nicholls and Saunders, 1996; Fawcett, 1992). Human forebrain neuroblasts implanted into the lesioned striatum of adult rats extended axons along major myelinated fiber tracts for distance of up to 20mm (Wictorin et al., 1990; Wictorin et al., 1992). In contrast, adult CNS neurons fail to penetrate mature CNS tissue by more than 1mm (Vidal-Sanz et al., 1987).

Moreover, the consequences of CNS injury in young mammals are less harmful than lesions of the mature nervous system. CNS lesions in newborn animals not only resulted in a better regeneration of transected fibers but also in compensatory growth of lesion-spared recovery (Bregman and Goldberger, 1982, 1983c; Bregman et al., 1993). These processes are paralleled by a high degree of functional recovery (Bregman and Goldberger, 1982; Bregman and Goldberger, 1983c; Bregman et al., 1993).

The precise molecular determinants that regulate this change in the regenerative capacity of neurons remain 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). There are growth-associated genes that are typically expressed in developing neurons and reactivated in response to injury. These include GAP-43, cytoskeleton proteins and some immediate early genes (Fawcett, 1992; Schwab and Bartholdi, 1996). For example, in the retina and the rubrospinal pathway, only neurons that regenerate their axons into peripheral nerve grafts upregulate GAP-43 (Fawcett and Geller, 1998). In addition, Aigner et al. demonstrated that over-expression of GAP-43 in transgenic mice could promote sprouting of CNS neurons beyond the borders of their normal territory. Substantial axon regrowth was observed in these mice when axons of sensory neurons projecting into the CNS were cut (Aigner et al., 1995).

Similarly, studies from Chen et al. reported the involvement of the *bcl-2* gene, which is an anti-apoptotic proto-oncogene, in the developmental changes of growth and regeneration in retinal axons. They found that retinal ganglion cells express high levels of Bcl-2 protein only in early development. Longer-lasting overexpression of *bcl-2* gene leads to target innervation at a stage when neurons from control mice fail to do so. More importantly, after transection of adult retinal ganglion neuron axons *in vivo*, reinnervation of the superior colliculus took place in mice overexpressing *bcl-2*, while in controls, all axons stop sharply at the lesion site (Chen et al., 1997). However, these effects of Bcl-2 on axonal elongation did not appear to be a consequence of the inhibition of programmed cell death. When a protease inhibitor was added, the cell death of RGCs was prevented, but axonal elongation still did not occur (Chen et al., 1997).

Taken together, it has been suggested that certain developmental programs control the changes in the ability of neurons to extend axons. This could involve the sequential switching of genes in response to the environmental cues, which will determine the speed and extent of axonal growth following injury. The failure of axonal regeneration in adult CNS neurons is likely due to the decrease in intrinsic growth capacity in response to the environmental changes, from a permissive to a non-permissive environment. One way to improve axonal regeneration, would be to change the growth capacity of adult CNS neurons to that of young ones such that they are no longer inhibited by myelin and other inhibitory factors in the adult CNS environment.

1.6 Treatment in vivo

In general, taking the observations presented above together, to improve axonal regeneration of adult CNS neurons following injury allows for the exploration of at least two lines of therapeutic avenues: neutralizing the inhibitory effects of CNS myelin and the glial scar and improving the intrinsic growth capacity of adult neurons to overcome myelin inhibitors.

There are several therapeutic approaches already being used *in vivo* to improve CNS regeneration. They include the use of peripheral nerve bridges (Cajal, 1928; David and Aguayo, 1981; Cheng et al., 1996), Schwann cell grafts (Xu et al., 1995; Martin et al., 1996; Guest et al., 1997; Menei et al., 1998), embryonic spinal cord tissue transplant (Bregman et al., 1998), and olfactory ensheathing cell transplant (Ramon-Cueto et al., 1998; Li et al., 1997; Ramon-Cueto and Avila, 1998; Ramon-Cueto et al., 2000).

Therefore, application of a combination of therapeutic strategies might be able to achieve the successful axon regeneration in the adult CNS. Such applications may include providing neurotrophic factors, neutralizing the inhibitory effects of myelin-derived and glial scar-associated inhibitors and changing the intrinsic growth capacity of neurons to overcome myelin inhibitors (Qiu et al., 2000).

1.7 The Goals of the Work in this Thesis

As discussed before, the adult CNS neurons fail to regenerate after injury due to the non-permissive environment they encounter. One of these environmental factors is the CNS myelin that contains a number of inhibitory molecules which prevent damaged axons from regrowing. One of the myelin-specific molecules, MAG, has been identified as a potent inhibitor of neurite outgrowth in culture (Mukhopadhyay et al., 1994; McKerracher et al., 1994). In addition, MAG can also inhibit axonal regeneration *in vivo* (Schafer et al., 1996; DeBellard et al., 1999). Therefore, understanding the inhibitory mechanism(s) activated by MAG may reveal the therapeutic approaches which may be employed to improve CNS axonal regeneration after injury. However, the receptor or receptor complex of MAG on neurons remains unknown. How does MAG interact with its neuronal receptors? What signal transduction pathway does it induce in neurons? Which signaling molecule is critical in this pathway? What is the functional role of this signaling mechanism *in vivo*? Does MAG regulate the regenerative capacity of neurons at the transcriptional and translational levels? These questions are addressed as follows:

1. Priming neurons with neurotrophin blocks the inhibition of axonal growth by MAG, and the priming is via a cAMP-dependent mechanism (Chapter III).

2. The developmental switch in response of neurons to MAG and myelin has been studied in three different types of neurons and the cyclic AMP-dependency of this developmental switch is identified (Chapter IV).

3. The role for cyclic AMP in regulating the regenerative capacity of neurons in *in vivo* regeneration has been addressed (Chapter V).

4. The regulation of axonal regenerative ability by cAMP at the transcriptional and the translational level has been addressed (Chapter VI).

CHAPTER II

MATERIAL AND METHODS

2.1 Cell Culture Maintenance

Permanently transfected MAG-expressing and control CHO cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), proline (40mg/liter), glycine (7.5mg/liter), and thymidine (0.73mg/liter) at 37^0C in 5% CO_2. MTX was included into the media at the concentration of 1.0μM.

2.2 Isolation of Neurons

To isolate cerebellar, retinal, hippocampal or raphe spinal neurons, cerebellum, retina, hippocampus or the most caudal region of the raphe nuclei were removed from two to five rat or mouse pups at different postnatal days (DeBellard et al., 1996). Tissue was triturated in 6ml of 0.025% trypsin in PBS and incubated in 37^0C. For dorsal root ganglion (DRG) neurons, ganglia were removed from rat pups at different postnatal ages and incubated in 5ml of L15 containing 0.15% collagenase type I (Wortington) and 0.025% trypsin for 30 minutes to 2 hours at 37^0C. The ganglia were triturated with a fine polished Pasteur pipette.

Trypsinization was stopped by adding 10ml DMEM containing 10% FCS, and then the cells were centrifuged at 1000 rpm for 5 minutes. The isolated neurons were resuspended to a single-cell suspension in SATO medium (progesterone, 20nM; selenium, 30nM; putrescine 2μM; insulin, 5μg/ml; BSA, 4mg/ml; L-thyroxine, 0.1μg/ml; tri-iodo-thyronine, 0.08 μg/ml) [Doherty, 1990 #211]. After that, the cells were counted by hemacytometer.

2.3 Priming Neurons with Neurotrophic Factors

24-well tissue culture dishes (6cm) were coated with 16.6μg/ml poly-L-lysine (Sigma) for 30 minutes at room temperature. Excess poly-L-lysine was washed off with H_2O, and the plates were washed one more time. Isolated neurons in SATO were plated onto the poly-L-lysine coated dishes at 1x 10^6 cerebellar neurons/well or 0.5x10^6 DRG neurons/well. Where indicated, either BDNF, GDNF, or NGF (Sigma) was added at a concentration between 25 and 200 ng/ml in the presence or absence of a protein kinase A inhibitor, KT5720 (Calbiochem), at 200nM. After culture for various times, from 1 hour to 24 hours, the media was removed, neurons were washed with PBS, and lifted up with 0.1% trypsin. Then trypsinization was stopped by adding 10ml DMEM containing 10% FCS. Neurons were centrifuged at 1000rpm for 5 min, resuspended in SATO, and plated immediately onto either MAG-expressing or control CHO cells, or purified, immobilized myelin.

2.4 Preparation of Myelin

Myelin was purified from rat CNS white matter following 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 immediately as a substrate in the neurite outgrowth assay.

2.5 Neurite Outgrowth on Immobilized Myelin or Transfected Cells

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

0.5-1.0μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen et al., 1998). Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an 8-chamber tissue culture slide (Lab-Tek). The cerebellar, DRG, retinal, hippocampal or raphe spinal neurons were isolated and resuspended in Sato media as described previously. Then the neurite outgrowth assay was carried out by adding $5x10^4$ neurons to the immobilized myelin substrate or $2x10^4$ neurons to the CHO cell monolayers, either primed or not.

Where indicated, dbcAMP at 1mM, dbcGMP at 1mM, H89 at 5 μM, KT5720 at 200 nM, KT5823 at 1μM, Rp-cAMP at 20 μM, Sp-cAMP at 50 μM, DRB at 5 μM, DFMO at 1mM, Putrescine at 10 μM to 1 mM, BDNF, GDNF or NGF at 200 ng/ml, was added into the culture, or neurons were incubated with pertussis toxin at 2μg/ml for 14hr prior to the addition of neurotrophins.

After 16-18 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 GAP43 (1:4000, from R. Curtis and G. Wilkins Imperial College, London). Cells were washed three times with PBS-BSA (2%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit IgG (1:500, Amershem), washed three times, and then incubated with streptavidin-conjugated Texas Red (1:300, Amersham) 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 GAP43-positive neuron for the first 150-200 neurons encountered when scanning the slide in a systematic manner was determined by using an Oncor Image Analysis program.

For the raphe nuclei neurons, because the culture is not pure, cultures were also stained for serotonin. A goat serotonin antibody (DiaSorin) was used at 1:1000. For double staining, GAP43 antibody was detected with anti-rabbit Oregon Green conjugated secondary antibody and serotonin antibody was detected with anti-goat biotinylated secondary antibody followed by Texas Red-conjugated streptavidin. Only those neurons that were both GAP43 and serotonin positive were measured.

2.6 Binding of MAG-Fc to Neurons

Protein A Sepharose-purified Fc chimeras, either MAG-Fc or MUC18-Fc as a control (Kelm et al., 1994; Tang et al., 1997) were absorbed at 30μg/ml for 3hr at 37^0C to wells of microtiter plates (Dynatech) coated for 2 hr at 37^0C with anti-human IgG at 15μg/ml in 0.1M bicarbonate buffer (PH=9.6). Prior to the binding assay, neurons were vitally labeled with a fluorescence dye, calcein AM (Molecular Probes), by incubating 2x 10^6 neurons in 5ml of 10μM Calcein AM in PBS for 15 min at 37^0C before being washed and resuspended in PBS. A suspension (100μl) of vitally labeled neurons, containing 0.5-2x 10^5 cells, was added to each well and allowed to incubate for 1 hr at room temperature. The plates were washed three times with PBS applied to each well under gravity, and the fluorescence was measured in Fluroimager (Molecular Dynamics).

2.7 cAMP Immunoassay

For each cAMP assay, neurons were dissociated and cyclic AMP was measured by using a competitive immunoassay, according to the manufacturer's instruction (Amersham). 2 x 10^5 cerebellar, or 2 x 10^4 DRG neurons were plated per well of a 96-well dish. The neurons were first incubated with Sato media alone for at least 6 hr. Then either BDNF, GDNF, or NGF was added at 200ng/ml, with and without MAG-Fc at 30μg/ml as indicated, and incubated for a further 30 min. Where indicated, neurons were incubated with pertussis toxin at 2μg/ml for 1-2hr prior to the addition of neurotrophin or neurotrophin and MAG-Fc.

The assay we used to measure intracellular cAMP levels is the cAMP enzymeimmunoassay (EIA) system, available from Amersham. After lysing primed neurons by using specific lysis buffers, we transferred lysates into a 96-well plate which was precoated with secondary antibody and later immobilized the anti-cAMP antibodies. During the incubation time, unlabelled cAMP from samples competed with peroxidase-labelled cAMP for a limited number of binding sites on cAMP-specific antibody. At the end, the amount of peroxidase labelled cAMP bound to the antibody was determined by addition of specific substrate (TMB) and the resultant color read at certain wavelength in a microtitre plate spectrophotometer. The higher the OD reading from samples, the lower the cAMP levels in these samples. Figure 2.1 shows the whole procedure of cAMP enzymeimmunoassay (EIA) system in flow chart.

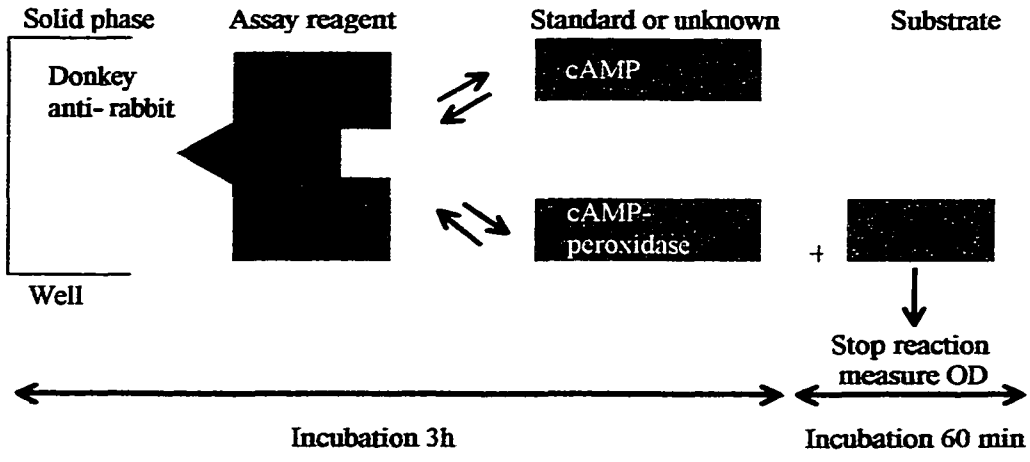


Fig.2.1 The Flow chart of the cAMP Enzymeimmunoassay (EIA) System.

For comparison of basal cAMP levels of DRG, RG and raphe spinal neurons from different developmental ages, the cAMP levels were measured as described previously with the following modifications. For each assay, neurons were dissociated, and then 1x 10^6 RG, or 2x 10^5 DRG or raphe spinal neurons at different developmental ages were lysed by lysis buffers provided (Amersham). Endogenous cAMP levels were measured immediately by using a competitive immunoassay. Each result was represented the means ±SEM of at least 4 experiments.

2.8 Immunofluorescence Staining for cAMP

cAMP immunostaining was performed on DRG cryosections with a rabbit polyclonal antiserum according to Wiemelt's method (Wiemelt et al., 1997). Isolated, intact dorsal root ganglia were fixed with acrolein for 1hr at room temperature, incubated in 20% sucrose until equilibrium, embedded with OCT compound (Tissue Tech) and cryosectioned to 5μm of thickness. The fixed DRG cryosections were washed in a quenching solution containing 1mg/ml glycine for 15 min, followed by 1% (w/v) sodium

cyanoborohydride for 15 min. Then the slides were stained by incubating them overnight at 4^0C in anti-cAMP antiserum (kindly provided by Dr. Arthur McMorris and Dr. Anthony Weimelt at Wilstar Institute in Philadelphia) diluted 1:50 in a solution containing 10% FCS and 0.5% triton-X 100. The binding of the primary antibody was then visualized by using the biotin-streptavidin system as described previously.

2.9 Spinal Cord Lesions and Transplants

Pups (postnatal 2-3 days) were anesthetized by hypothemia, and then under the sterile surgical conditions, an over-hemisection lesion was made at the T6 spinal cord level in all animals (N=36). The following experimental groups were examined: lesion plus H89 (N=10), lesion plus saline (N=10), lesion plus transplant and H89 (N=11), lesion plus transplant and saline (N=7). The saline or H89 (0.5mM) was delivered via gelfoam placed at the lesion or lesion plus transplant site.

2.10 Tracing and Immunohistochemistry

Two weeks after lesion, the anterograde tracer, biotin dextran amine (BDA, 10,000 MW, Molecular Probes Inc.), was used to study changes in the descending corticospinal tract fibers. Following anesthesia (choral hydrate, 400mg/kg), the sensorimotor cortex was exposed and a 10% solution of BDA in the sterile saline was injected into the cortex bilaterally (0.3-0.5ml per injection, 3ml total volume per motor cortex). Following the injections, the dura was covered with gelfoam soaked with saline, and then the overlaying skin was sutured.

Two weeks after the BDA injection, the rats were anesthetized with chloral hydrate and perfused transcardially with 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer. Tissue was passed through graded sucrose solutions, and prepared for immunohistochemistry. Cryostat sections were washed with phosphate buffer and incubated in an avidin-biotin-peroxidase complex for 90-120 minutes at room temperature (Vector Elite ABC kit, Burlingame, CA) and the BDA-filled axons were visualized by using a solution of diaminobenzibine tetrahydrochloride/nickel ammonium sulfate and hydrogen peroxide. Adjacent sections were processed immunocytochemically for visualization of serotonergic axons (Serotonin, DiaSorin Inc.). The extent of axonal growth was evaluated by individuals unaware of the experimental group to which the animals belonged.

2.11 In vivo Peripheral Conditioning Lesions

Postnatal day 18 rat pups were anesthetized by metaphane, 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 day or 1 week after the sciatic nerve transection. L4 and L5 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 described previously.

2.12 In vivo Injection of dbcAMP or PKA Inhibitor to DRG

Postnatal day 18 rat pups were anesthetized by metaphane, and then L4-5 dorsal root ganglia were exposed bilaterally by laminectomy. 4μl of 50mM dbcAMP, 4μl of 20mM PKA inhibitor H89, or 4μl of 0.9% saline were injected into the ganglia by using a glass micropipette over a period of 1 hour. Then the injected ganglia were isolated 1 day after or 7 days after the microinjection, and plated on the monolayers of CHO cells or immobilized purified CNS myelin. The Neurite outgrowth assay was carried out as described previously.

2.13 Immunodetection of Arginase I in Neurons Treated with BDNF or dbcAMP

Neurons were treated either with Sato alone, or Sato with 200ng/ml BDNF or 1 mM dbcAMP overnight, and then were lysed in RIPA buffer (50mM Tris-HCl, PH=7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, 1mM vanadate and the following antiproteases: 1μg/ml leupeptin, aprotinin and pepstatin). The cell lysates were kept on ice for 15 min and centrifuged at 14,000 x g for 10 min. Then the supernatant was immediately transferred to a fresh centrifuge tube and the protein concentration was measured with a Bio-Rad kit (Bio-Rad Labs, Hercules, CA). The normalized lysates were denatured at 95^0C for 5 min, after which they were subjected to SDS-PAGE in a 12% acrylamide gel. Then the proteins were transferred to nitrocellulose membrane and immunostained with a rabbit polyclonal antibody against arginase I at a 1:5000 dilution (kindly provided by Dr. Ratan at Harvard Medical School) by incubating overnight at 4^0C. Furthermore, the proteins were incubated with the secondary antibody, peroxidase-linked protein A, at 1:5000 for 1 hour (Boehringer Mannheim). Finally, the proteins were

detected by the enhanced chemiluminescence (ECL) detection system, according to the manufacturers' instructions (Amersham).

2.14 Detecting Arginase I Gene Expression from Primed Neurons

Total RNA was isolated from $2x10^6$ cerebellar neurons, which were either treated with BDNF at 200ng/ml or dbcAMP at 1mM overnight, by using RNeasy kit (Qiagen) following the manufacturers' instructions. Then cDNA was synthesized from total RNA by using random hexamers and a reverse transcriptional enzyme (Stratagene) at 37^0C for 2 hr. After that, ArgI cDNA was amplified through polymerase chain reaction (PCR) using the specific primers. These primers were synthesized from GibcoBRL. Finally, ArgI amplified cDNA samples was detected by running 1% agarose gel and staining with ethidium bromide.

Primer: Arg-197F (Acct.# N5682H09)

5' GTC CCC AAT GAC AGC CCC 3'

Primer: Arg-700R (Acct.# N5682H10)

5' CTT TTC TTC CTT CCC AGC AG 3'

2.15 Adenovirus-Mediated in vivo Gene Introduction into Neurons

Recombinant adenovirus containing Arginase I gene was kindly provided by Dr. Ratan at Harvard Medical School. Postnatal cerebellar neurons were isolated and plated down on the poly-L-lysine coated 6-well plates. Then neurons were infected by adenovirus containing arginase I, or GFP as control at a final concentration of 10^{10} PFU/ml (or MOI=100) and maintained within virus containing media for 1 hours. After

infection, media was washed away, and neurons were kept in Sato media for a further 2 days to recover and for gene expression. Then the infected neurons were plated onto MAG-expressing or control CHO cells to carry out the neurite outgrowth assay as described previously.

CHAPTER III

PRIMING NEURONS WITH NEUROTROPHINS BLOCKS THE INHIBITION OF AXONAL GROWTH BY MAG AND MYELIN

Introduction

In the adult mammalian central nervous system (CNS), injured neurons fail to regrow due to the non-permissive environment they encounter after damage. It is generally believed that the failure of axonal regeneration after injury is mainly due to the inhibitory molecules in the adult CNS (Johnson, 1993). Some of these inhibitors are myelin-specific or glial-derived molecules (Schwab et al., 1993). For example, Nogo is a myelin-related inhibitor which is recognized by the monoclonal antibody IN-1 (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). IN-1 can neutralize the inhibitory effects of Nogo and promote injured axons to regenerate (Caroni and Schwab, 1988b; Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995).

In addition to Nogo, myelin-associated glycoprotein (MAG), a well-characterized glycoprotein found in both CNS and PNS myelin, has been shown to be a potent inhibitor of axonal outgrowth (Mukhopadhyay et al., 1994; McKerracher et al., 1994). In culture, it is able to inhibit the neurite outgrowth of a wide variety of postnatal neurons, such as cerebellar neurons and adult dorsal root ganglion (DRG) neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996), as well as neurite elongation of neuroblastoma cells (McKerracher et al., 1994). Despite conflicting findings about regeneration in MAG-deficient mice (Li et al., 1996; Bartsch et al., 1995), two recent studies demonstrate that MAG, when presented to an axon *in vivo*, can inhibit axonal regeneration (Schafer et al., 1996; DeBellard et al., 1999). For example, in double mutant mice, the crossbreed of C57BL/Wld^{s} with MAG-/-, the absence of MAG can improve the impaired axonal

regeneration of C57BL/Wld^{s} mice, which is due to the delayed removal of myelin debris (Schafer et al., 1996). In contrast, in transgenic mice in which the expression of MAG is under the control of the p75 promoter, MAG in Schwann cells is upregulated with endogenous p75 after PNS injury. Consequently, regeneration is greatly retarded compared to their wildtype counterparts (DeBellard et al., 1999). Together, it has been shown that MAG, one of most likely many myelin-associated inhibitory molecules, can inhibit axonal growth in culture and *in vivo*.

Although MAG/myelin is inhibitory for axonal regeneration, a recent study reported extensive axonal growth into CNS white matter, distal to an injury site despite the absence of reagents that could neutralize the inhibitory effect of CNS myelin (Berry et al., 1996; Bregman et al., 1998). Bregman and colleagues have shown that when embryonic tissue is implanted into spinal cord lesion site, there is axonal growth into the embryonic tissue but not out into the host white matter beyond the lesion. However, when either of two neurotrophins, brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3), is pumped into the implanted embryonic tissue, injured axons not only grow into the implanted tissue, but they also extend long processes into the white matter beyond (see Figure 3.1) (Bregman et al., 1998). Independently, Berry et al. demonstrated that if a peripheral nerve explant was placed in the vitrous body of the retina after optic nerve lesion, the injured axons grew extensively into the white matter distal to the lesion site, even without coming into direct contact with the peripheral nerve explant (Berry et al., 1996). These observations suggest that some components secreted from peripheral nerve

explants, such as neurotrophins, affect the response of injured axons to CNS myelin, and somehow block its inhibitory effect.

However, the underlying signaling mechanism of this neurotrophin-mediated axonal regeneration remains unclear. So the goal of the studies in this chapter is to determine the signaling mechanism induced by neurotrophins that blocks the MAG and myelin-mediated inhibition of axonal regeneration after injury.

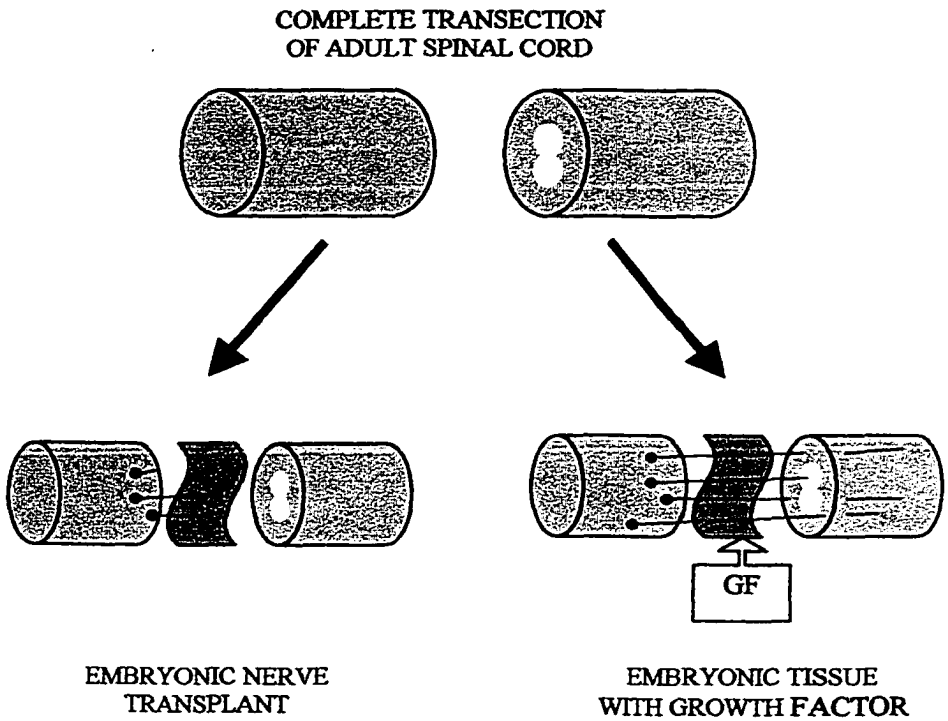


Fig.3.1. The Flow Chart of Studies in Axonal Regeneration Following Spinal Cord Transection in the Presence of Embryonic Nerve Transplant with or without Growth Factor (Bregman et al., 1998).

Results

Priming Neurons with Neurotrophins has a Blocking Effect on MAG and Myelin Inhibition

As described above, studies show that exposing injured axons to neurotrophins within an embryonic tissue transplant for a certain period of time, can improve axonal regeneration in transected adult spinal cord. To determine whether neurotrophins can reverse the inhibitory effect on neurite outgrowth by MAG, BDNF, GDNF or NGF were added individually at a concentration of 200ng/ml, to either postnatal cerebellar neurons or DRG neurons (older than postnatal day 4 - PND4) which were grown on monolayers of either MAG-expressing or control CHO cells. After 18 hours of incubation, cultured neurons were fixed and immunostained for GAP43. Then, the longest neurite from individual neurons was measured and the mean length of at least 150 neurons was assessed.

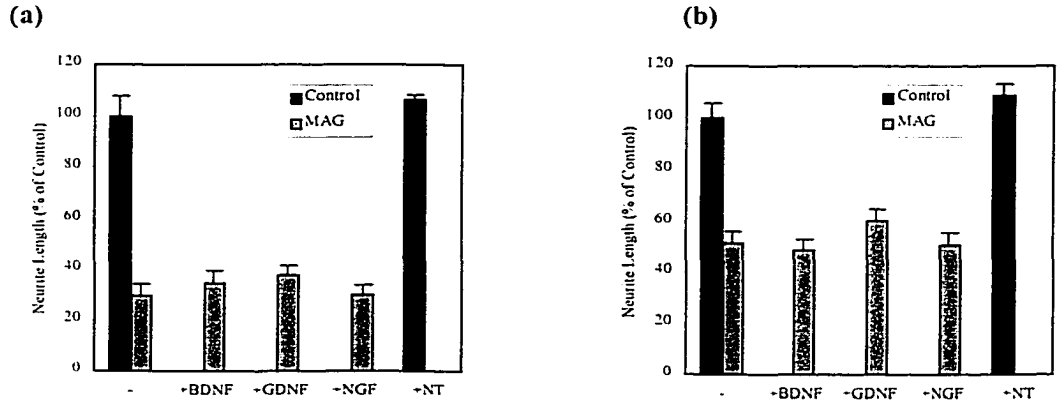


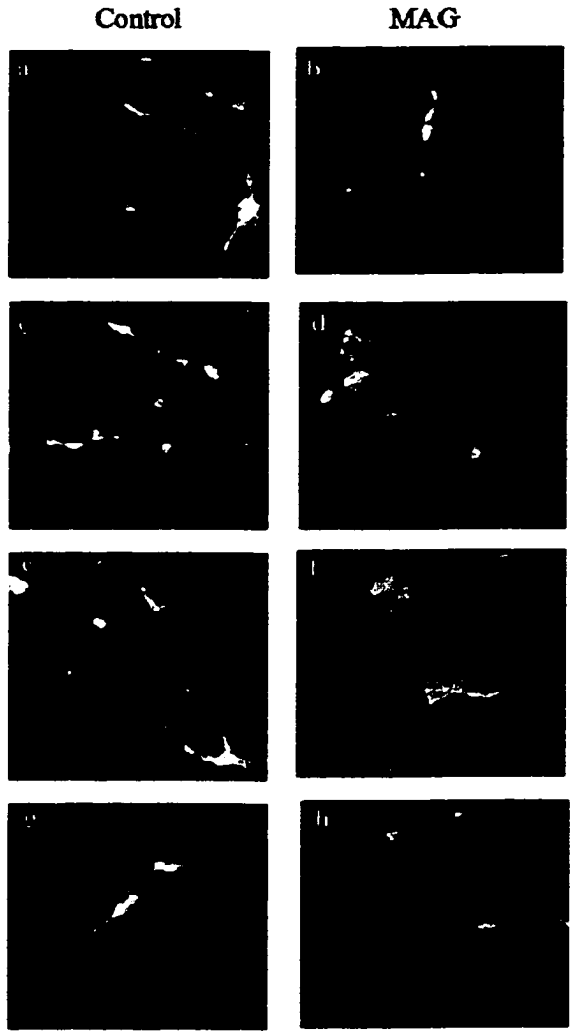
Fig.3.2. The Effect of Neurotrophins on Inhibition by MAG without Priming. Cerebellar (a) and older DRG (b) neurons were isolated and plated onto either MAG-expressing (stippled bars) or control (black bars) CHO monolayer cells. During the incubation time, BDNF, GDNF or NGF was added to culture at 200ng/ml individually. After 18 hours of incubation, neurons were fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (± SEM) for 150 neurons.

It was found that none of the neurotrophins tested had any effect on the inhibition of neurite outgrowth by MAG if they were added to the culture at the same time as the exposure to MAG. The neurite outgrowth from the postnatal cerebellar neurons and the older DRG neurons is still inhibited by about 70% and 50%, respectively, when grown on MAG-expressing compared to control cells, regardless of the presence of neurotrophins in the media (Fig. 3.2 a&b).

Reconsidering Bregman's *in vivo* experiments, it is observed that while the axons are growing through the embryonic transplant, they are being exposed to neurotrophins for several days. During this period, neurons are exposed to neurotrophins in the absence of any myelin-specific inhibitor. We, therefore, modified our usual neurite outgrowth assay and instead of adding neurotrophins directly to the culture at the same time as neurons are exposed to MAG, neurons were grown overnight on poly-L-lysine in the presence of neurotrophins. We term this process "priming with neurotrophins". After priming, we transferred these neurons to either MAG-expressing or control CHO cells. Here, we asked if prior exposure to neurotrophins affects the regenerative ability of neurons when subsequently grown on MAG.

For cerebellar neurons primed with BDNF or GDNF, but not NGF, before being exposed to MAG, the inhibition of axonal growth by MAG is completely blocked. Figure 3.3 shows that priming with BDNF or GDNF results in the same extent of neurite outgrowth on MAG as on the control cells (Fig.3.3 c-f). In contrast, neurons primed with NGF or without neurotrophins are still inhibited by MAG and the inhibition is about 70%, which is comparable to the degree of inhibition of non-primed cerebellar neurons

(Fig.3.3 a, b and g, h). This priming effect is specific to the MAG-induced inhibition, since improvement of axonal growth is only seen in neurons grown on MAG-expressing cells. There is no difference in neurite lengths for neurotrophin-treated and -untreated neurons subsequently grown on control CHO cells (Fig.3.3 i).



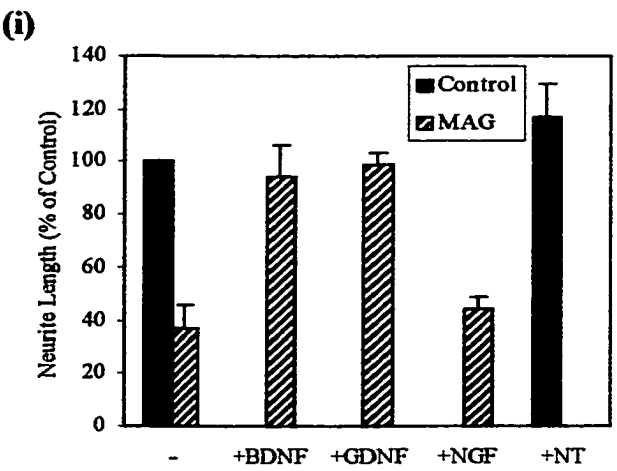
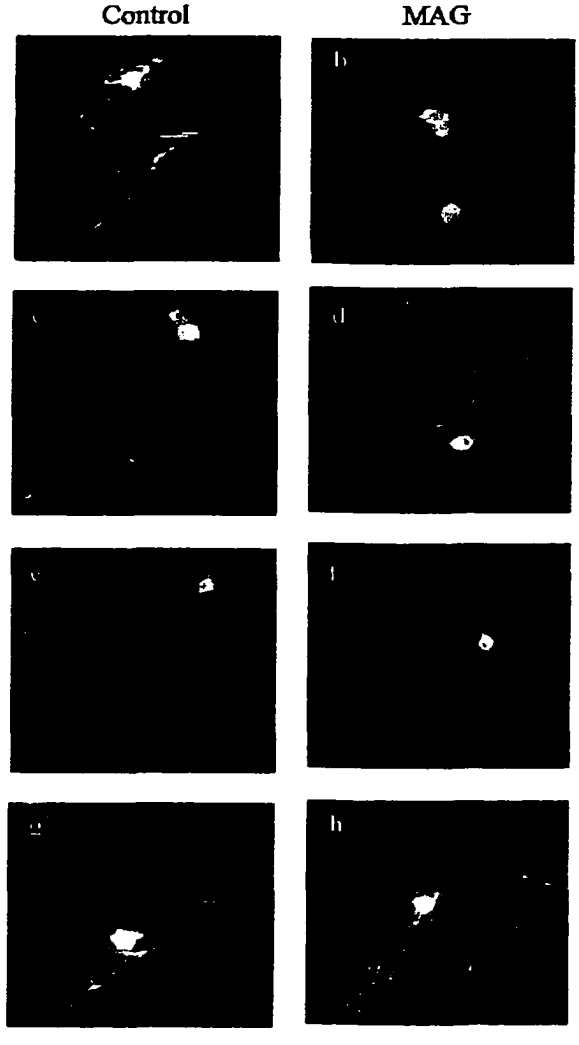


Fig.3.3 The Effect of Priming Cerebellar Neurons with Neurotrophins on Inhibition of Neurite Outgrowth by MAG. Dissociated postnatal cerebellar neurons are first primed overnight without neurotrophins or in the presence of BDNF, GDNF or NGF at 200ng/ml and then transferred to a monolayer of either MAG-expressing CHO cells (b,d,f,g) or control cells (a,c,e,g). After that, neurons are cultured for further 18 hours, and then fixed and immunostained for GAP43. Results in (i) show the mean length of the longest neurite per neuron (±SEM) for 150 neurons, grown on either control (solid bars) or MAG (stippled bars). Results are standardized to percentage of control, taken as neurite length from neurons primed without neurotrophin and then grown on control CHO cells. NT refers to neurons primed with either of three neurotrophins before subsequently being grown on control cells.

Priming experiments with older DRG neurons, however, show that any one of these three growth factors can completely block the inhibitory effects of MAG on neurite outgrowth (Fig. 3.4 d,f,h). Interestingly, priming different types of neurons with NGF shows different effects on the inhibition by MAG. At a concentration of 200ng/ml, NGF

does. not block the inhibitory effect of MAG on cerebellar neurons, whereas for DRG neurons, NGF can reverse the inhibition of neurite growth by MAG completely. Therefore, depending on the type of neurons, prior exposure to certain neurotrophins can specifically block the inhibitory effects of MAG.



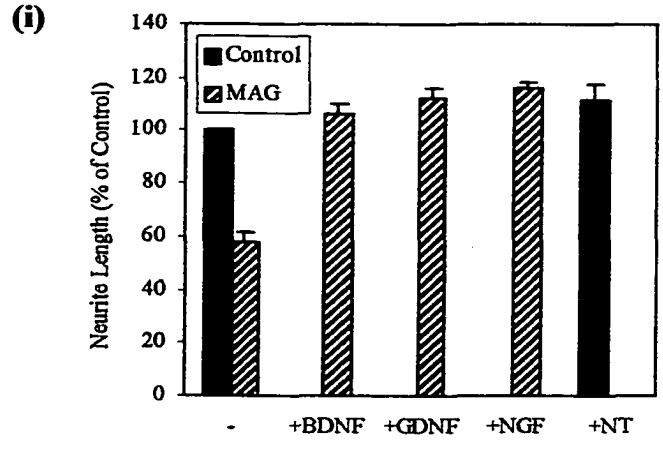


Fig.3.4 The Effect of Priming DRG Neurons with Neurotrophins on Inhibition of Neurite Outgrowth by MAG. Dissociated postnatal DRG neurons (>PND4) are first primed overnight without neurotrophins or in the presence of BDNF, GDNF or NGF at 200ng/ml and then transferred to a monolayer of either MAG-expressing CHO cells (b,d,f,g) or control cells (a,c,e,g). After that, neurons are cultured for a further 18 hours, and then fixed and immunostained for GAP43. Results in (i) show the mean length of the longest neurite per neuron (±SEM) for 150 neurons, grown on either control (solid bars) or MAG (stippled bars). Results are standardized to percentage of control, taken as neurite length from neurons primed without neurotrophin and then grown on control CHO cells. NT refers to neurons primed with either of three neurotrophins before subsequently grown on control cells.

CNS myelin is known to be inhibitory for axonal regeneration (Schwab et al., 1993). We have shown that priming neurons with neurotrophins can block the inhibitory effect on axonal outgrowth by MAG, a myelin-specific inhibitor. Now we would like to determine whether priming with neurotrophins is a general approach to block the

inhibitory properties of myelin. After purifying myelin from adult rat brain, CNS myelin was immobilized onto poly-L-lysine coated slides. The primed neurons were then cultured overnight on this substrate.

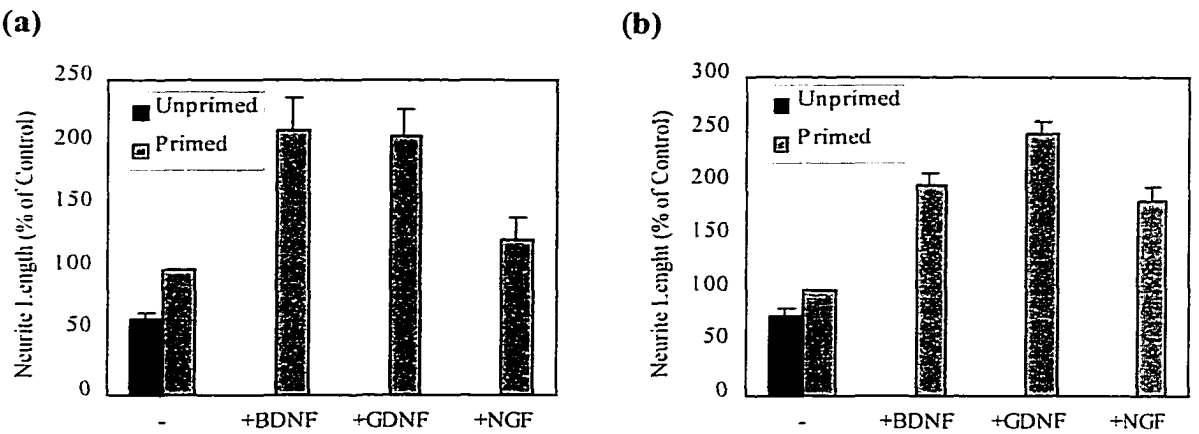


Fig.3.5 Priming Neurons with Neurotrophins Improves Axonal Outgrowth on Myelin. Dissociated cerebellar (a) or DRG (b) neurons were primed with or without neurotrophins and then transferred to immobilized myelin substrate (1μg/well) for further incubation. Neurons were fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (±sem) for 150 neurons either unprimed (solid bar) or primed (stippled bar) without neurotrophin or with various neurotrophins as indicated. Results are percent of control, with 100% control taken as growth of neurons primed in the absence of neurotrophin before subsequently being grown on myelin.

In figure 3.5 a & b, we show that neurons primed with neurotrophins extend much longer neurites on the myelin substrate than those primed without neurotrophins or non-primed neurons. Furthermore, by visual observation, we have found that neurons primed with neurotrophins show extensive branching compared to controls, which implies that priming not only improves axonal outgrowth but also promotes neurite sprouting. In addition, priming neurons with NGF has a similar effect on the inhibition of axonal outgrowth by myelin as it does on MAG's inhibition, depending on the type of neuron being primed (cerebellar versus DRG neurons). At a concentration of 200ng/ml, priming with NGF does not neutralize the inhibitory effect of myelin on cerebellar neurons, whereas extensive improvement of axonal growth of DRG neurons is observed after priming with NGF.

To establish further the specificity of the priming effect, we investigated the time-dependence and the dose-dependence of priming. Postnatal cerebellar neurons were primed in the presence of 200ng/ml BDNF, GDNF, and NGF, for different lengths of time, and then transferred to MAG-expressing CHO cells. Results are shown in figure 3.6a. For BDNF or GDNF, but not NGF, longer priming times increase the block of outgrowth inhibition by MAG. By six hours of priming with BDNF or GDNF the majority of MAG's inhibition (80%) is blocked, while 24 hours' priming reverses the inhibition completely. For NGF, priming for any length of time has little effect on MAG's inhibitory effect on axonal growth from cerebellar neurons.

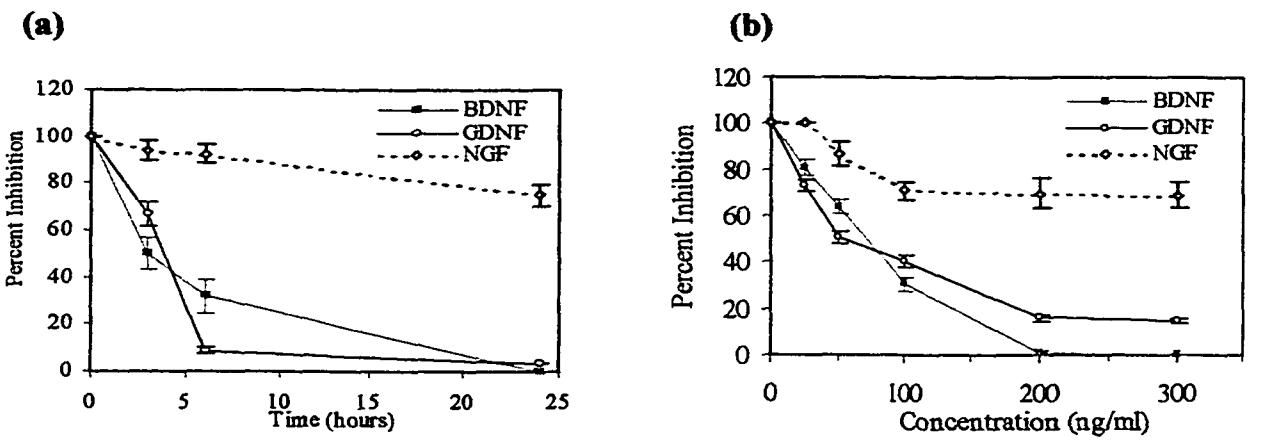


Fig.3.6 The Time-Dependence and Dose-Dependence of Priming Neurons with Various Neurotrophins. (a)Cerebellar neurons were primed for different times with BDNF (closed squares), GDNF (open circles), or NGF (dotted line), each at 200ng/ml, and then transferred to MAG-expressing or control CHO cells. (b)Neurons were primed overnight with different concentrations (0-300ng/ml) of BDNF (closed squares), GDNF (open circles), or NGF (dotted line) before being transferred to monolayers for further incubation. Then neurons were fixed and immunostained for GAP43. Results are represented as percent inhibition, while 100% inhibition is taken as neurite length of neurons primed without neurotrophins and then grown on MAG.

To determine if the priming effect is dose-dependent, cerebellar neurons were primed overnight with different concentrations of various neurotrophins. Figure 3.6b shows that as the concentration of the growth factors (BDNF or GDNF, but not NGF) increases, the block of MAG's inhibition also increases. At a concentration of 200ng/ml, either BDNF

or GDNF can completely neutralize the inhibition of axonal growth by MAG. But priming with NGF across a range of concentrations, has much less improvement on axonal growth on MAG-expressing CHO cells (20% at most in blocking the inhibition) than on neurite outgrowth after priming with the other two growth factors at corresponding concentrations.

In summary, these data suggest that priming cerebellar neurons with BDNF or GDNF, but not NGF, can block the inhibition of neurite outgrowth by MAG and myelin, while prior exposure of DRG neurons to any of these three neurotrophins has similar blocking effects. The priming effect is specific to block the inhibition of axonal growth by MAG as indicated by its time-dependence and dose-dependence. Next, we would like to investigate the underlying signaling mechanism initiated by priming with neurotrophins, which can interfere with the signal transduction pathway activated by MAG and myelin, and block its inhibitory effect on axonal regeneration.

Overcoming the Inhibition by MAG/Myelin via Priming is cAMP-dependent

In general, there are two possible mechanisms for how priming might overcome the inhibition by MAG and myelin. First, the putative receptors of MAG or myelin may be down-regulated on neurons primed with neurotrophins. Second, downstream signal transduction steps may be activated during priming and subsequently block the inhibitory pathway induced by MAG and myelin. If the first hypothesis is true, we would expect that there are fewer or no receptors available on neurons for MAG to bind to after priming with neurotrophin. We previously established a binding assay (DeBellard et al.,

1996; Tang et al., 1997a), in which recombinant MAG-Fc chimera (the extracellular domain of MAG fused to the Fc region of human IgG) is immobilized onto a 96-well plate, and then fluroscently-labeled neurons are allowed to bind. The number of bound neurons is counted by a Fluoroimager and compared to binding to a control Fc chimera (MUC18-Fc). MUC18 is another Ig superfamily member and like MAG, has 5 Ig domains in its extracellular region (Kelm et al., 1994).

As shown in Fig.3.7, priming either cerebellar or DRG neurons with any of these three neurotrophins has no effect on the ability of MAG-Fc to bind to neurons. The amount of MAG bound to primed neurons is the same as the amount bound to non-primed neurons. These results suggest that the surface expression of MAG receptors is unaffected by priming with neurotrophins. Therefore, the block of inhibition is unlikely to be due to the downregulation of the putative MAG receptor.

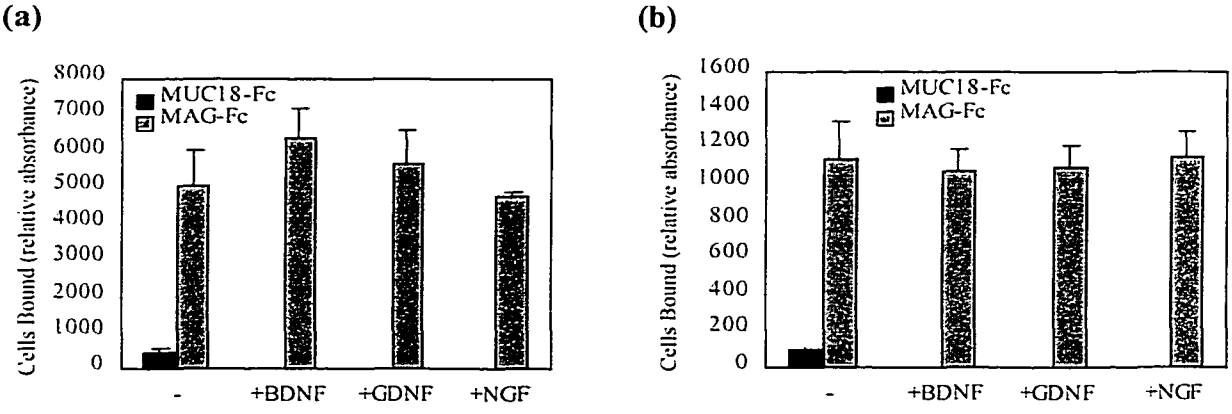


Fig.3.7 Binding of MAG-Fc to Neurons after Priming. Dissociated postnatal cerebellar (a) or DRG (b) neurons primed overnight with BDNF, GDNF, or NGF each at 200ng/ml, were vitally labeled with Cacein AM and then bound MAG-Fc (black bar) or MUC18-Fc (stippled bar) immobilized at 10μg/ml, on a 96-well microtiter plate. 100,000 neurons were added to each well and after incubation and washing, the number of cells bound was quantitated with a Fluoroimager. Results are from three experiments, each with 10 samples, and represent the mean ±SEM.

To assess the possibility that certain signal transduction mechanisms induced by priming might be involved in blocking the inhibition by MAG/myelin, a battery of

reagents known to block or activate various component of signaling pathways were tested. Previous investigation of signaling molecules involved in MAG's inhibition found that dibutyryl cAMP (dbcAMP), an analog of cAMP, reversed the inhibition of axonal growth completely without affecting neurite outgrowth on control CHO cells.

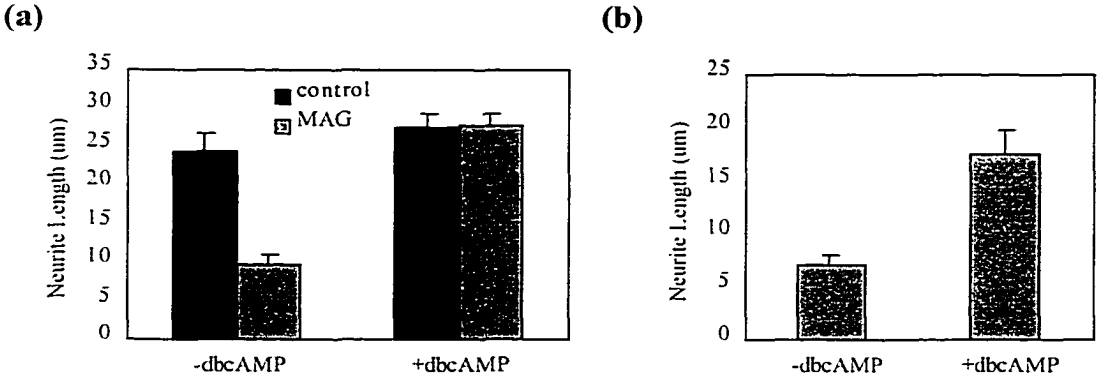


Fig.3.8 The Effect of Dibutyryl cAMP on Inhibition of Neurite Outgrowth by MAG and Myelin. Dissociated Cerebellar neurons were plated directly onto (a) monolayers of MAG-expressing (stippled bars) or control (black bars) CHO cells or (b) immobilized myelin substrate (1μg/ml) and cultured overnight with or without dbcAMP at 1mM. Neurons were fixed and then immunostained for GAP43.

In figure 3.8a, we show that adding dbcAMP directly to cerebellar neuronal cultures at a concentration of 1mM, can block the inhibition by MAG completely. Furthermore, at the same concentration, dbcAMP improves axonal growth on myelin to at least two times longer than untreated neurons (Fig. 3.8b). Similar to the blocking effect seen in cerebellar neurons, reversal of inhibition is also observed for axonal outgrowth of DRG neurons on MAG-expressing cells or on a CNS myelin substrate in the presence of 1mM dbcAMP (data shown in chapter IV).

Since artificially elevating cAMP levels in neurons with dbcAMP or priming neurons with specific neurotrophins can each block the inhibition by MAG and myelin, we asked whether the priming effect is cAMP-dependent. To investigate the cAMP-dependence of

priming, two experiments were carried out. First, intracellular cAMP levels of neurons primed with different neurotrophins were compared to the levels in untreated neurons. Second, since protein kinase A (PKA) is activated by elevation in cAMP levels, an inhibitor of PKA was included during priming to determine whether the blocking effect was abrogated.

To investigate whether specific neurotrophins increase neuronal cAMP levels, neurons primed with or without neurotrophin were lysed and intracellular cAMP levels were measured. Dissociated cerebellar neurons ($2x10^5$ per well) or DRG neurons ($5x10^4$ per well) are placed in a 96-well plate for 6 hr to let cells settle down and attach to the plate. Then, the neurons were cultured with BDNF, GDNF, or NGF for a further 30min. Following incubation, neuronal cAMP levels were measured immediately using an enzymeimmunoassay (EIA) system and compared to levels in neurons treated in the same way but without neurotrophins. Table 3.1 shows that the neuronal cAMP levels increase after being treated with specific neurotrophins and the values represent the increase relative to neurons incubated for the same length of time without neurotrophins. In cerebellar neurons, exposure to BDNF or GDNF, but not NGF, results in a two-fold increase in cAMP levels, while in DRG neurons, any of these three neurotrophins induces a similar elevation in cAMP levels.

As can be seen in Table 3.1, incubating neurons with neurotrophin, with the exception of cerebellar neurons with NGF, elevates neuronal cAMP levels at least two-fold relative to their basal levels, which are around 20-50 fmol/10^5 cerebellar neurons and 150-280

fmol/10^4 DRG neurons. Since neurotrophin can increase cAMP levels and such an increase may lead to activation of protein kinase A (PKA), we then determined whether activation of PKA during priming was involved in the block of inhibition. Therefore, a PKA inhibitor, KT5720 at 200nM, was included in the media during priming.

Neuron	Neurotrophin	Relative cAMP Levels
Cerebellar	-	1.0
Cerebellar	BDNF	2.1±0.26
Cerebellar	GDNF	1.98±0.15
Cerebellar	NGF	0.80±0.16
DRG	-	1.0
DRG	BDNF	1.80±0.22
DRG	GDNF	2.20±0.38
DRG	NGF	1.80±0.16

Table 3.1 Measurement of cAMP Levels in Neurons after Exposure to Neurotrophins. Dissociated cerebellar ($2x10^5$) or DRG ($5x10^4$) neurons were placed in 96-well plate. The neurons were cultured for 6 hours after which BDNF, GDNF, or NGF, each at 200ng/ml, was added into the media and incubated for a further 30 min. Following incubation, the cAMP levels were measured and results represent the fold-increase relative to neurons incubated for the same length of time but without treatment of neurotrophin.

In figure 3.9, it is shown that the addition of a PKA inhibitor (KT5720) along with neurotrophins during priming, completely abrogates the blocking effects on the inhibition by MAG. For cerebellar neurons, the priming effect of BDNF or GDNF is completely abrogated by adding KT5720 at 200nM during priming (Fig 3.9a). Similarly, the PKA inhibitor can completely abolish the priming effect of all three neurotrophins on axonal outgrowth from DRG neurons (Fig.3.9b). 100% of control length was taken as neurite

length from neurons primed without neurotrophins but with KT5720, and then grown on control CHO cells. However, priming with a PKA inhibitor alone has no effect on subsequent neurite growth on MAG. In addition, priming neurons with or without a PKA inhibitor, in the presence of neurotrophins, has no effect on neurite outgrowth on control CHO cells (Fig.3.9), which suggests that the PKA inhibitor specifically abrogates the priming effects of neurotrophins on MAG.

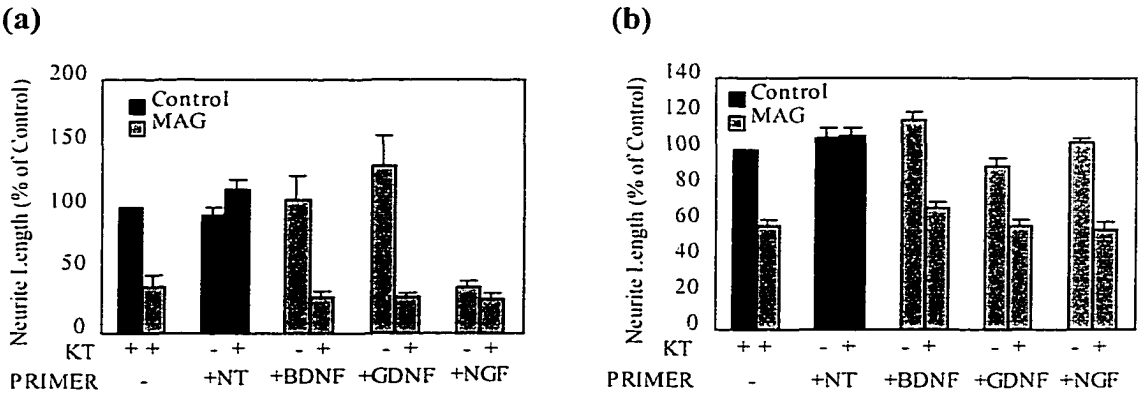


Fig.3.9 The Effect of Priming Neurons with Neurotrophins in the Presence of a PKA Inhibitor on Inhibition of Neurite Outgrowth by MAG. Dissociated postnatal cerebellar neurons (a) or DRG neurons were first primed overnight on poly-L-lysine without neurotrophins or in the presence of BDNF, GDNF or NGF, each at 200ng/ml, with or without the PKA inhibitor, KT5720 at 200nM as indicated before being transferred to either monolayers of MAG-expressing (stippled bars) or control (solid bars) CHO cells for further overnight culture. After incubation, neurons were fixed and immunostained for GAP43. Results show the mean of the longest neurite per neuron (±SEM) for 150 neurons. Results were standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells. NT (a) refers to neurons primed with either BDNF, GDNF, or NGF before being cultured on CHO cells.

Furthermore, it has been found that the presence of KT5720 during priming of either cerebellar or DRG neurons also prevents the neurotrophin block of inhibition by myelin (Fig.3.10). Myelin is as effective an inhibitor of axonal growth from cerebellar neurons (3.10a) or DRG neurons (3.10b) after being primed with neurotrophins in the presence of KT5720 as neurons that were never exposed to neurotrophins. In figure 3.10, results were standardized to percentage of control. 100% of control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on myelin.

Together, these results suggest that the increase in neuronal cAMP levels during prior exposure to neurotrophins is necessary to block the inhibitory effect of myelin-specific inhibitors on axonal growth. As shown previously, the addition of neurotrophins at the same time as the neurons are being exposed to MAG/myelin does not block the inhibitory effects. One reason why priming is required may be that MAG/myelin prevents the elevation of cAMP induced by neurotrophins. If neurons are exposed to neurotrophins before MAG/myelin, the neuronal cAMP levels can increase before they encounter the inhibitors. Then downstream signaling events can be activated that subsequently block the inhibitory pathway of MAG and myelin. To test this hypothesis, we further investigated which pathway was activated after myelin-specific inhibitors bound to their receptors on neurons, to prevent the increase of neuronal cAMP level induced by neurotrophins.

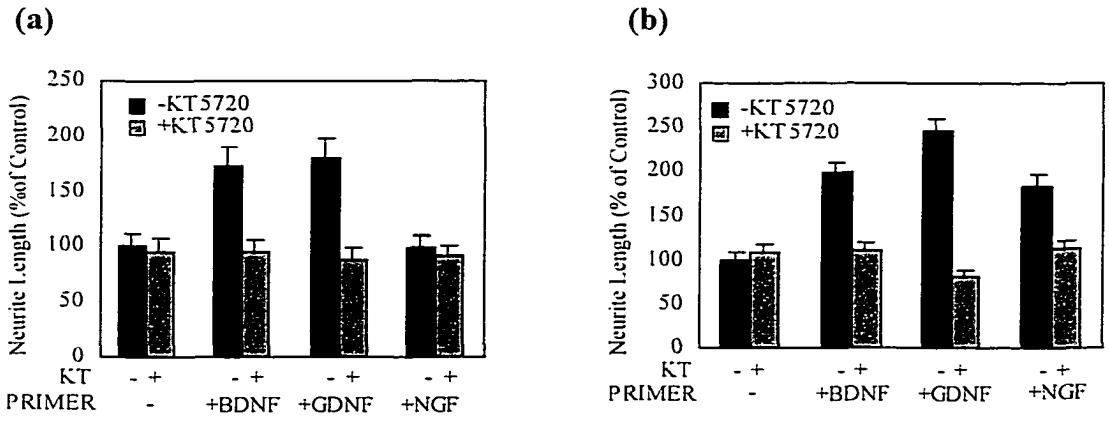


Fig.3.10 The Effect of Priming Neurons with Neurotrophins in the Presence of a PKA Inhibitor on Inhibition of Neurite Outgrowth by Myelin. Dissociated postnatal cerebellar neurons (a) or DRG neurons (b) were first primed overnight on poly-L-lysine without neurotrophins or in the presence of BDNF, GDNF or NGF, each at 200ng/ml, with (stippled bars) or without (solid bars) the PKA inhibitor, KT5720 at 200nM as indicated before being transferred to CNS myelin substrate for further overnight culture. After incubation, neurons were fixed and immunostained for GAP43. Results show the mean of the longest neurite per neuron (±SEM) for 150 neurons. Results were standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on myelin.

Involvement of Gi in Inhibition by MAG and Myelin

It is known that heterotrimeric GTP/GDP-binding proteins (G proteins) are major regulators of adenylyl cyclases (AC), which are key enzymes in cyclic AMP biosynthesis (Linder and Gilman, 1992). Non-overlapping regions exist on these enzymes which allow separate interaction with α_s, α_i, and $\beta\gamma$ subunits of G proteins (Linder and Gilman, 1992). Previous data suggest that MAG or myelin can prevent the activation of the cAMP pathway induced by culturing neurons with neurotrophins. Because if neurons are exposed to neurotrophins simultaneously when they encounter MAG/myelin, MAG still inhibits neurite outgrowth. However, pertussis toxin (PTX), a molecule that binds to and inactivates the heterotrimeric GTP-binding proteins, Gi and Go (Post and Brown, 1996), alone has no effect on MAG's inhibition of neurite outgrowth (Shen, unpublished data). One possible explanation is that MAG does not activate Gi to block the cAMP pathway and inhibit axonal growth. Another possibility is that there are two parallel pathways activated by MAG, of which activation of Gi to prevent the elevation of cAMP is just one and inhibition of axonal growth is the other. If the latter is true, blocking Gi on neurons first via the addition of PTX and then exposing neurons to neurotrophins simultaneously when they encounter MAG or myelin, should be able to restore axonal growth on myelin-specific inhibitors without priming.

To test this hypothesis, neurons were first cultured with PTX and then exposed to neurotrophins in the presence of MAG or myelin without neurotrophin priming. Results are shown in figure 3.11 and 3.12. It was found that PTX eliminated the need for priming to block the inhibition by MAG and myelin with neurotrophins. If neurons are first

cultured with PTX and then exposed to neurotrophin and MAG, BDNF or GDNF partially blocks the inhibitory effects of MAG (Fig.3.11a) in cerebellar neurons, while in DRG neurons, all three neurotrophins completely block the inhibition by MAG without priming (Fig.3.11b). Similar results were found in neurons treated with PTX and subsequently grown on myelin in the presence of neurotrophins (Fig.3.12). These data suggest the involvement of Gi in the signal transduction pathways activated by MAG and myelin.

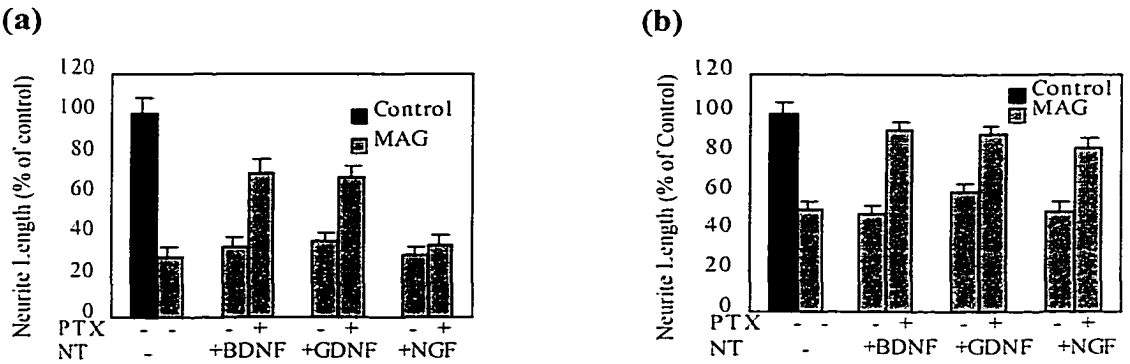


Fig.3.11 The Effect of Neurotrophins, without Priming, in the Presence of Pertussis Toxin on Inhibition of Neurite Outgrowth by MAG. Dissociated postnatal cerebellar neurons(a) or DRG neurons (b) were cultured for 1-2 hours in the presence of 2μg/ml pertussis toxin before being cultured overnight on a monolayer of either MAG-expressing (stippled bars) or control (solid bars) CHO cells and BDNF, GDNF or NGF, each at 200ng/ml was simultaneously added into the culture media. After overnight incubation, neurons were fixed and immunostained for GAP 43. Results show the mean length of the longest neurite per neuron (±SEM) for 150 neurons. Controls were taken as neurite length from neurons grown on control CHO cells in the absence of PTX and neurotrophins.

On the other hand, these results also strengthen the idea that when no Gi inhibitor is provided, priming with neurotrophins is required to block the inhibition by MAG. We showed previously that without priming, neurotrophins could not block the inhibition of axonal growth by MAG (Fig.3.1). MAG or myelin may activate a G protein which then prevents the neurotrophin-induced elevation of cAMP in neurons exposed to neurotrophins and myelin inhibitors simultaneously. Only when neurons are exposed to neurotrophins before they encounter myelin-specific inhibitors, can cAMP levels be

elevated and subsequent downstream signaling molecules activated to block the inhibitory pathway initiated by MAG.

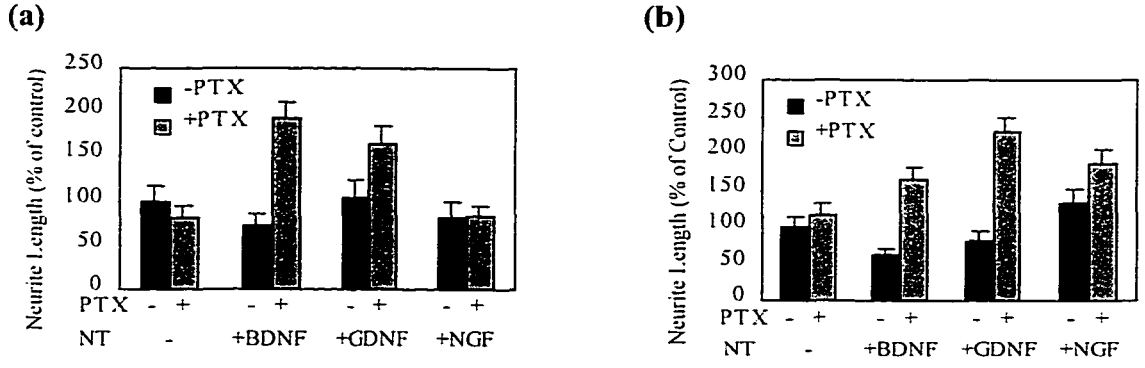


Fig.3.12 The Effect of Neurotrophins, without Priming, in the Presence of Pertussis Toxin on Inhibition of Neurite Outgrowth by Myelin. Dissociated postnatal cerebellar neurons (a) or DRG neurons (b) were cultured for 1-2 hours in the presence (stippled bars) or absence (solid bars) of 2μg/ml pertussis toxin before being cultured overnight on a substrate of CNS myelin and BDNF, GDNF or NGF, each at 200ng/ml was simultaneously added into the culture media. After overnight incubation, neurons were fixed and immunostained for GAP 43. Results show the mean length of the longest neurite per neuron (±SEM) for 150 neurons. Controls were taken as neurite length from neurons grown on myelin in the absence of PTX and neurotrophins.

To further support the suggestion of Gi's involvement in the block of cAMP elevation by MAG, neuronal cAMP levels were measured after exposure to neurotrophins and MAG-Fc, a soluble form of MAG which can effectively inhibit axonal regeneration (Tang et al., 1997a). The intracellular cAMP levels were also measured in neurons treated with neurotrophins, MAG-Fc, and PTX simultaneously. As shown in Table 3.2, MAG-Fc can prevent the neurotrophin-induced elevation of cAMP, which keeps cAMP around the neuronal basal level. Also, PTX neutralizes the blocking effect of MAG-Fc and rescues the neurotrophin-mediated elevation of cAMP. cAMP levels in neurons treated with any single reagent or combination, are compared to basal levels, which are cAMP levels in neurons without any treatment. The values represent relative amounts. It is shown that addition of MAG-Fc and PTX restores the neurotrophin-induced cAMP increases in neurons except NGF-treated cerebellar neurons.

Neurons	Treatment	Relative cAMP Levels (Fold)
Cerebellar	-	1.0
Cerebellar	BDNF / MAG-Fc	1.10±0.16
Cerebellar	BDNF / MAG-Fc / PTX	1.96±0.48
Cerebellar	GDNF / MAG-Fc	1.15±0.07
Cerebellar	GDNF / MAG-Fc / PTX	2.25±0.35
Cerebellar	NGF / MAG-Fc	1.02±0.07
Cerebellar	NGF / MAG-Fc / PTX	1.26±0.20
DRG	-	1.0
DRG	BDNF / MAG-Fc	1.23±0.17
DRG	BDNF / MAG-Fc / PTX	1.69±0.21
DRG	GDNF / MAG-Fc	1.13±0.06
DRG	GDNF / MAG-Fc /PTX	1.85±0.11
DRG	NGF / MAG-Fc	0.80±0.12
DRG	NGF / MAG-Fc / PTX	1.80±0.22

Table.3.2 Measurement of cAMP in Neurons with Different Treatments. Dissociated cerebellar or DRG neurons were plated in a 96-well plate. The neurons were first cultured without neurotrophins for at least 6 hours. Then, where indicated, neurotrophins were added at 200ng/ml with MAG-Fc at a concentration of 20μg/ml or neurons were incubated with 2μg/ml PTX prior to the addition of neurotrophin and MAG-Fc. Following incubation, the cAMP levels were measured and results represent the fold-increase relative to neurons incubated for the same length of time but without treatment of neurotrophin.

Together, these results suggest that MAG or myelin activates an inhibitory, pertussis toxin-sensitive, heterotrimeric G protein- most likely Gi- which in turn prevents any

elevation of cAMP that would otherwise be stimulated by neurotrophin binding. This is the reason why we need to prime neurons with neurotrophins to accomplish the block of inhibition by myelin-specific inhibitors.

Discussion

The results presented here show that although neurotrophins cannot overcome the inhibition of MAG/myelin on neurite outgrowth when they are added to neurons in the presence of the inhibitors, the inhibitory effect is completely blocked if neurons are exposed to neurotrophins before they encounter MAG or myelin (a process termed "priming"). For the neurotrophins used here, the priming effect is via a cAMP-dependent pathway. It has been shown that neurotrophin elevates intracellular cAMP levels and an inhibitor of PKA, downstream effector of cAMP, abrogates the block of inhibition when presented during priming. Finally, if neurons are exposed to MAG/myelin and neurotrophins simultaneously, but in the presence of the Gi inhibitor, pertussis toxin, the inhibition is blocked without the need to prime. Taken together, it is suggested that to accomplish axonal outgrowth in the presence of myelin-specific inhibitors, neuronal cAMP levels must be elevated and subsequently, PKA activated before neurons encounter myelin inhibitors. Based on these findings, we proposed a model to explain the priming effect and possible signaling mechanism involved in MAG's inhibition on axonal outgrowth. The model is shown in Figure 3.13 (Cai et al., 1999).

The model suggests that during priming, in the absence of MAG and myelin, neurotrophins bind to their specific receptors and initiate downstream signaling events,

one of which is elevation of cAMP. Subsequent activation of PKA, or other downstream signaling molecules, blocks the activation of the MAG/myelin inhibitory pathway. However, if neurons are exposed to neurotrophins and myelin inhibitors at the same time, endogenous cAMP levels are prevented from increasing because MAG activates Gi which prevents the elevation of cAMP. Under these conditions, inhibition persists. However, Gi activation is not required for the inhibition by myelin-specific inhibitors. Because adding pertussis toxin alone into culture, cannot block the inhibition by MAG and myelin (Fig.3.13). These data suggest that another parallel pathway exists to induce the inhibition of axonal regeneration by MAG/myelin binding to its receptor(s).

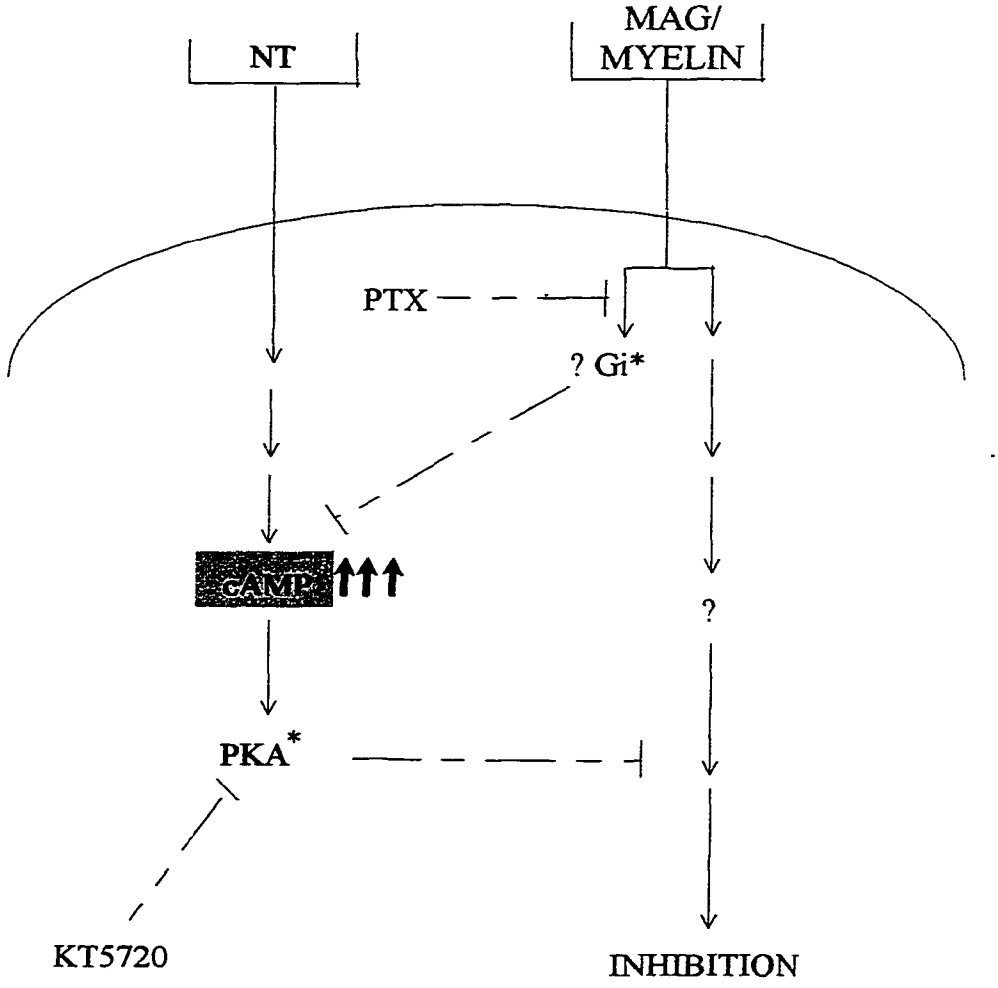


Fig.3.13 The Model of the Priming Mechanism (Cai et al., 1999).

This study offers a possible explanation for the two recent studies of axonal regeneration *in vivo* by Berry et al. (1996) and Bregman (1998). In both studies, extensive axonal regrowth into white matter, distal to the injury site, is found in the absence of reagents that neutralize the inhibitory effect of myelin. However, in both of these studies, either growth cones or cell bodies of neurons are exposed to growth factors before they encounter myelin. Therefore, based on our results, it is quite likely that in these *in vivo* experiments, the intracellular cAMP levels of injured neurons are altered when they are exposed to neurotrophins before they encounter myelin-specific inhibitors in the damaged white matter. This change may result in the block of myelin's inhibitory effect and then axonal regeneration in the adult CNS environment.

In Bregman's experiments, the neurons are exposed to either BDNF or NT-3 prior to encountering myelin. As we show, priming with BDNF blocks the inhibition by MAG/myelin through a cAMP-dependent pathway. In addition, some of our unpublished data demonstrates that priming with neurotrophin-3 (NT-3), another member of the Trk-family neurotrophins, can partially neutralize the inhibitory effects of myelin inhibitors, but through a cGMP-dependent instead of a cAMP-dependent pathway (Adebola, unpublished data). It has been found that the blocking effect of NT-3 can be abrogated by addition of PKG inhibitors, but not by PKA inhibitors. Interestingly, results obtained from studies of neurotropic behaviors by Poo and colleagues also suggest that the response of an axon to a particular guidance molecule may switch between repulsion and attraction by changes in the neuron's endogenous levels of cAMP or cGMP (Ming et al., 1997; Song et al., 1998). Similarly, they show that BDNF-mediated chemoattraction is

via a cAMP-dependent pathway, whereas the neurotropic effect of NT-3 is through a cGMP-dependent mechanism (Ming et al., 1997; Song et al., 1998). The possible explanation for the NT-3's partial block of inhibition by MAG might be that the step directly responsible for blocking inhibition is downstream from cAMP-dependent PKA activation. During priming, NT-3 activates a cGMP-dependent pathway that may share the same downstream signaling event with the cAMP pathway to exert the block of axonal inhibition by MAG. Therefore, elevating cAMP blocks inhibition of axonal regeneration, while other mechanisms, bypassing the cAMP step, may also block inhibition.

In the experiments of Berry and colleagues, the retinal ganglion cell body is exposed to a number of neurotrophins secreted by Schwann cells in the peripheral nerve grafts (Berry et al., 1996). Berry et al. suggest that receptor(s) for myelin inhibitors may be downregulated after neurons are exposed to these neurotrophins. However, we find that the neurotrophin-mediated block of inhibition is not due to the downregulation of MAG/myelin receptors, or through neurotrophin competitively binding to MAG/myelin receptors. First, there is no change in binding ability of MAG to its receptor(s) after priming with neurotrophins (shown in Fig.3.7). Second, addition of a PKA inhibitor with neurotrophins during priming, restores the inhibitory effects by MAG and myelin (Fig.3.9 & 3.10), which would only be possible if the receptors of MAG/myelin are not preoccupied by neurotrophins (Cai et al., 1999). Instead, we show that the neurotrophins BDNF, GDNF, and NGF activate PKA via elevation of cAMP and then initiate downstream signaling events to neutralize the inhibitory effect of MAG/myelin.

Our data suggest that the neurotrophin-mediated improvement of axonal regrowth is via the elevation of endogenous cAMP levels. However, the involvement of certain neurotrophin receptors in this signaling event need to be further investigated. In our experiments, members from two different neurotrophin families, Trk family and GDNF-like family, can each block inhibition by MAG and myelin. These two neurotrophin families activate different receptor systems, but converge on the same point, cyclic AMP. The differential effects of NGF on different types of neurons (cerebellar versus DRG neurons) strongly suggest that Trk receptors instead of p75, are involved in the priming mechanism of the Trk-family neurotrophins. Recent unpublished data from our lab further strengthens this idea by showing that the addition of a Trk-specific tyrosine kinase inhibitor (K252a) during priming with BDNF or NGF can abolish the priming effect completely, whereas a neutralizing antibody against p75 has no effect on priming (Gao and Filbin, unpublished data).

As an important signaling molecule, cyclic AMP, regulates several signaling pathways (Iyengar, 1996). Recent studies have highlighted the importance of intracellular cAMP as a gating element in a number of different signaling mechanisms. For example, cAMP regulates synaptic plasticity as well as neurotrophin-dependent survival in the central nervous system through its gating function (Iyengar, 1996). In addition, it was recently reported that differences in cAMP-dependent activity in a neuron might result in the opposite turning response of growth cones to the same guidance cue (Ming et al., 1997; Song et al., 1998). A gradient of BDNF normally triggers an attractive

turning response of the growth cone of *Xenopus* spinal neurons in culture, but the same gradient induces repulsive turning of these growth cones in the presence of Rp-cAMP or a PKA inhibitor (Song et al., 1997). This cAMP-dependent switch of the turning response is also found for the turning induced by netrin-1 (Ming et al., 1997), the axon guidance molecule secreted from the floor plate (Kennedy and Tessier-Lavigne, 1995). Blocking cAMP-dependent activity switches the chemoattractant response to a repellent one. Interestingly, MAG, when applied as a concentration gradient, also exerts chemorepellent effects on growth cones of *Xenopus* spinal neurons (Song et al., 1998). If cAMP levels are artificially increased, repulsion of growth cones by MAG is changed into attraction (Song et al., 1998). This result is consistent with ours and suggests that the signaling pathway induced by MAG is modulated by a cAMP-dependent activity. The evidence we have shown suggests that inhibition by MAG can only be achieved under conditions in which neuronal cAMP levels are low and PKA is inactivated.

The repulsive turning of growth cones is thought to result from a local, asymmetrical collapse of the growth cone. Recently, it was found that cAMP-dependent activity also plays a role in regulating growth cone collapse. Acutely applied BDNF induces rapid growth cone collapse and neurite retraction of embryonic *Xenopus* spinal neurons that were isolated and cultured for 6 hours and this collapsing effect is regulated by cAMP-dependent activity (Wang and Zheng, 1998). Elevation of intracellular cAMP levels completely blocks the collapsing effect, whereas inhibition of PKA potentiates the collapsing action. BDNF-induced growth cone collapse is only observed with neurons isolated and cultured for 6 hours but not those cultured for 24 hr. Because the inhibition

of PKA by Rp-cAMP restored the collapsing response of neurons in 24 hr-cultures, it is suggested that *Xenopus* spinal neurons may up-regulate their endogenous cAMP-dependent activity during their time in culture, leading to a blockade of their collapsing response to BDNF.

However, elevation of cAMP through ligand-receptor interaction is just a transient signaling event. Although the increases in intracellular cAMP levels are sustained for 30 to 60 min after the treatment with neurotrophins (Table 3.1), neuronal cAMP level after overnight incubation with neurotrophins is not different from the basal level. On the other hand, in the neurite outgrowth assay, priming reaches its maximal effect only after 6 hours of incubation (Fig.3.6a). Furthermore, experiments show that when neurons primed with neurotrophins for only 1 hour are kept in media without neurotrophins for another 5 hours before they encounter myelin inhibitors, block of inhibition is obtained similar to priming neurons with neurotrophins for 6 hours before grown on MAG/myelin (Spencer and Filbin, unpublished data). These data imply that following the elevation of cAMP, the activation of downstream signaling events in the absence of myelin inhibitors, is more important than cAMP elevation alone in blocking the inhibitory effect of MAG/myelin on axonal regeneration, though the initial cAMP increase is the key to blocking this inhibition. The signaling events following elevation of cAMP during the rest of the priming time even without neurotrophins, are quite likely to be involved with downstream gene transcription and protein synthesis. As proposed in the model (Fig.3.13), there is a protein X yet to be determined, a downstream effector in the MAG

inhibition pathway that is modulated by cAMP-dependent activity. Further investigation of this issue will be discussed in Chapter VI.

In summary, our results suggest that although the cAMP-dependent activity is not directly involved in the inhibition by MAG, it plays an important role in modulating the neuronal response to MAG and myelin. The effects of MAG and other myelin-specific inhibitors on axonal growth as either inhibitors/repellents or promoters/attractants can be determined by the endogenous levels of neuronal cAMP. The next step is to determine whether cAMP-dependent mechanisms can regulate neuronal responses to MAG/myelin during development.

Chapter IV

The Developmental Switch of Neuronal Response to MAG and Myelin is Under the Control of Cyclic AMP

Introduction

The regenerative capacity of neurons declines during development. After injury, embryonic or neonatal neurons can regrow axons and restore function, while adult neurons fail to do so. Changes in the environment during development might contribute to the switch of the regenerative capacity in neurons, such as the appearance of myelin at a late embryonic stage (Kapfhammer and Schwab, 1994). Studies *in vivo* and *in vitro* have shown that CNS myelin is inhibitory to axonal regeneration of adult neurons and this inhibitory effect is mainly due to myelin-specific or glial-derived inhibitors (Schwab et al., 1993; McKerracher et al., 1994; Mukhopadhyay et al., 1994). Results have also suggested that the axonal growth of embryonic or neonatal neurons is not impeded by CNS myelin either in culture (Fawcett et al., 1989; Bedi et al., 1992; Shewan et al., 1995; Chen et al., 1995) or *in vivo* (Wictorin et al., 1990; Wictorin et al., 1992; Li and Raisman, 1997; Davies et al., 1994). However, the molecular basis that regulates the differences between young and old neurons in response to myelin, as well as in general regenerative capacity, remains unclear.

MAG, a myelin-specific inhibitor, is a bifunctional molecule. Along with a developmental change in response to myelin, certain types of neurons show a developmental switch in response to MAG, from promotion to inhibition (DeBellard et al., 1996). For example, it has been shown that MAG inhibits axonal growth from adult DRG neurons (Mukhopadhyay et al., 1994). In contrast, it promotes neurite outgrowth from young postnatal DRG neurons (Mukhopadhyay et al., 1994; Johnson et al., 1989). This transition from promotion to inhibition of axonal growth by MAG in DRG neurons

occurs sharply at PND3-4 (DeBellard et al., 1996). Other types of neuron such as retinal ganglion (RG) neurons and spinal neurons exhibit a similar trend in response to MAG during development, except that the switch has occurred by birth instead of postnatally (Salzer et al., 1990; Turnley and Bartlett, 1998).

Recently, Poo and colleagues have shown that the turning response of a growth cone (attraction/repulsion) to certain guidance cues is cAMP-dependent and by modulating neuronal cAMP levels they can control the ultimate growth direction toward or away from a guidance signal (Song et al., 1998). In *Xenopus* spinal neurons, artificially increasing cAMP levels can switch the repulsive response to MAG into attraction (Song et al., 1998). Alternatively, blocking the activity of a cAMP pathway can convert the attraction by some guidance cues, like netrin-1, to repulsion (Ming et al., 1997). On the other hand, it has been described in the previous chapter that the inhibition of axonal growth by MAG and myelin can be blocked when neurons are exposed to neurotrophins before they encounter myelin inhibitors (a process termed "priming"). This priming effect is via activation of a cyclic AMP-dependent pathway. Also, artificially elevating neuronal cAMP levels by pharmacological reagents can reverse the inhibitory effect of MAG/myelin on axonal regeneration without priming (see chapter III and Cai et al., 1999). Together, these results suggest that the cAMP-dependent activity can regulate the neuronal turning response as well as the regenerative capacity.

So the question now raised is whether endogenous cAMP levels influence the switch of neuronal response to MAG/ myelin during development and subsequently affect the axonal regenerative capacity *in vivo*.

Results

Changes in Endogenous Cyclic AMP Levels Correlate with the Switch of Response to Myelin Inhibitors at Different Postnatal Ages of DRG Neurons

It has been shown that the response of DRG neurons to MAG switches from promotion to inhibition during development. This transition happens sharply at about PND 3/4 (DeBellard et al., 1996). Now, we want to determine whether DRG neurons have a similar age-related switch in response to myelin. CNS myelin was prepared as a substrate at 1μg of total protein/well in an 8-chamber slide, and DRG neurons from different postnatal ages were cultured overnight on this substrate. It was found that axonal outgrowth of DRG neurons from different ages is dramatically different, not only in axonal length, but also in neurite branching. As shown in figure 4.1a, DRG neurons from P1 extend relatively long neurites and branch extensively, whereas P5 DRG neurons grow either very short or no neurites. Quantitative results of neurite length match the visual observation (Fig.4.1b).

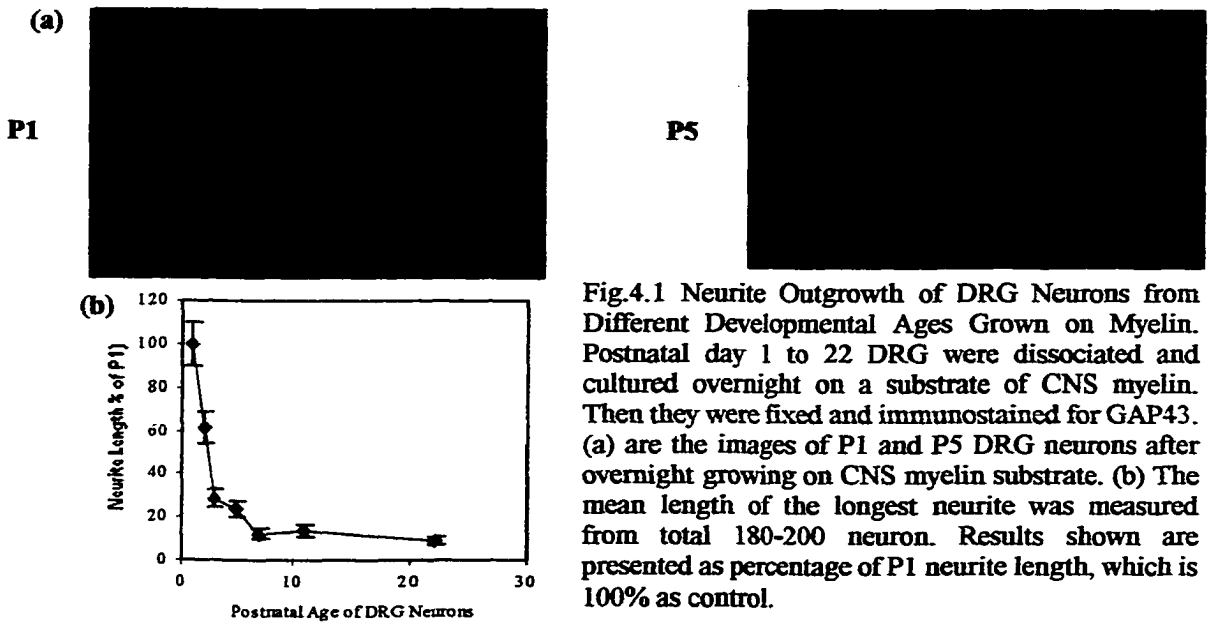


Fig.4.1 Neurite Outgrowth of DRG Neurons from Different Developmental Ages Grown on Myelin. Postnatal day 1 to 22 DRG were dissociated and cultured overnight on a substrate of CNS myelin. Then they were fixed and immunostained for GAP43. (a) are the images of P1 and P5 DRG neurons after overnight growing on CNS myelin substrate. (b) The mean length of the longest neurite was measured from total 180-200 neuron. Results shown are presented as percentage of P1 neurite length, which is 100% as control.

In figure 4.1b, the average length of the longest neurite from total 180-200 P1 DRG neurons is around 25 μm, which then shows diminished length with age. By P5, neurite length is about 5-fold shorter than neurites from P1 DRG. Poor growth on myelin persists through to adults. As a control for the CNS myelin substrate, neurons grown on a substrate of poly-L-lysine (PLL) were studied. It was found that there is no difference in neurite length between young and old DRG neurons grown on a PLL substrate. This suggests that the decrease in neurite outgrowth from DRG neurons during development is due to a switch of neuronal response to myelin inhibitors instead of a loss of intrinsic growth ability in general.

As described in the previous chapter, priming DRG neurons (older than PND4) with neurotrophins can block the inhibition of axonal outgrowth by MAG/myelin. This priming effect is via activation of a cAMP pathway. Artificially elevating neuronal cAMP levels can improve axonal regeneration of older DRG neurons on MAG and myelin. Therefore, it is reasonable to suggest that the better growth of young DRG neurons on MAG/myelin may be due to higher endogenous cAMP levels relative to older DRG neurons. To check whether the higher cAMP levels of young DRG neurons are required for promotion of neurite outgrowth by MAG/myelin, a downstream effector of cAMP, protein kinase A (PKA) was inhibited by pharmacological reagents to determine if the better outgrowth of young neurons can be abolished.

Figure 4.2 shows that the addition of the PKA inhibitors KT5720 at 200nM or Rp-cAMP at 20 μM during neurite outgrowth, eliminates the better outgrowth of P1 DRG

neurons on MAG completely without affecting the growth on control CHO cells. Moreover, the promotion of axonal growth by MAG (Fig.4.2a) specifically depends on cAMP levels because a PKG inhibitor, KT5823 at 1mM, has no effect on axonal outgrowth from P1 DRG neurons. Similar results have been seen for neurons grown on myelin as a substrate. However, none of these reagents have any effect on axonal growth on control CHO cells or neurons grown on a PLL-substrate (a control for CNS myelin substrate). Together, these results suggest that in DRG neurons, the promotion/inhibition of axonal regeneration by MAG and myelin is cAMP-dependent. In addition, the endogenous cAMP level regulates the neuronal response to myelin inhibitors during development.

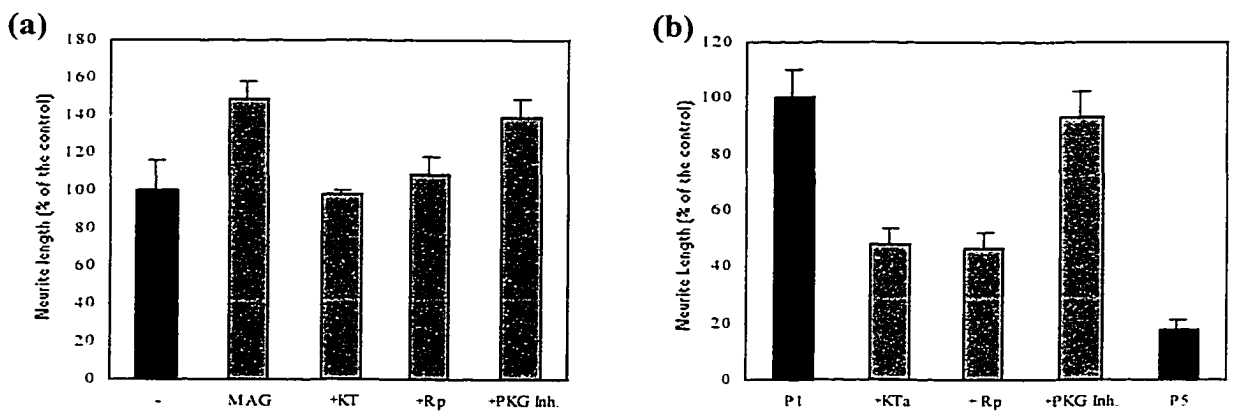


Fig.4.2. Neurite Outgrowth of P1 and P5 DRG Neurons on MAG or Myelin in the Presence of PKA or PKG Inhibitors. (a) P1 DRG neurons were dissociated and cultured overnight with or without PKA inhibitors, KT5720 (KT) or Rp-cAMP (Rp), or PKG inhibitor (PKG Inh.) on MAG-expressing CHO cells (stippled bars) or control CHO cells (solid bar). (b) P1 and P5 DRG neurons were dissociated and cultured overnight with (stippled bars) or without (solid bars) PKA or PKG inhibitors. Then neurons were fixed and immunostained for GAP43. The results were the mean length (±SEM) of the longest neurite of each neuron from 180-200 neurons. 100% values were taken as neurite lengths of P1 neurons grown on control CHO cells (a), or on myelin (b) without any treatment.

To strengthen the idea that the switch in DRG regenerative abilities correlates with changes in neuronal cAMP levels, endogenous cAMP from different postnatal ages of DRG neurons was measured. Moreover, sections through P0 and P5 intact DRG were immunostained *in situ* with a cAMP antibody (kindly provided by Dr. Arthur McMorris

and Dr. Anthony Weimelt at Wistar Institute). Figure 4.3b shows that during development, neuronal cAMP levels decrease dramatically, from about 20 x 10^{-4} fmol/neuron at P1 to 0.5 x 10^{-4} fmol/neuron at P3, then persist at these low levels to adult. Consistent with the measurement of cAMP levels, DRG immunostaining shows that younger DRG's are much brighter than older ones, which suggests that there is much more cAMP in P0 than in P5 DRG neurons (Fig.4.3a).

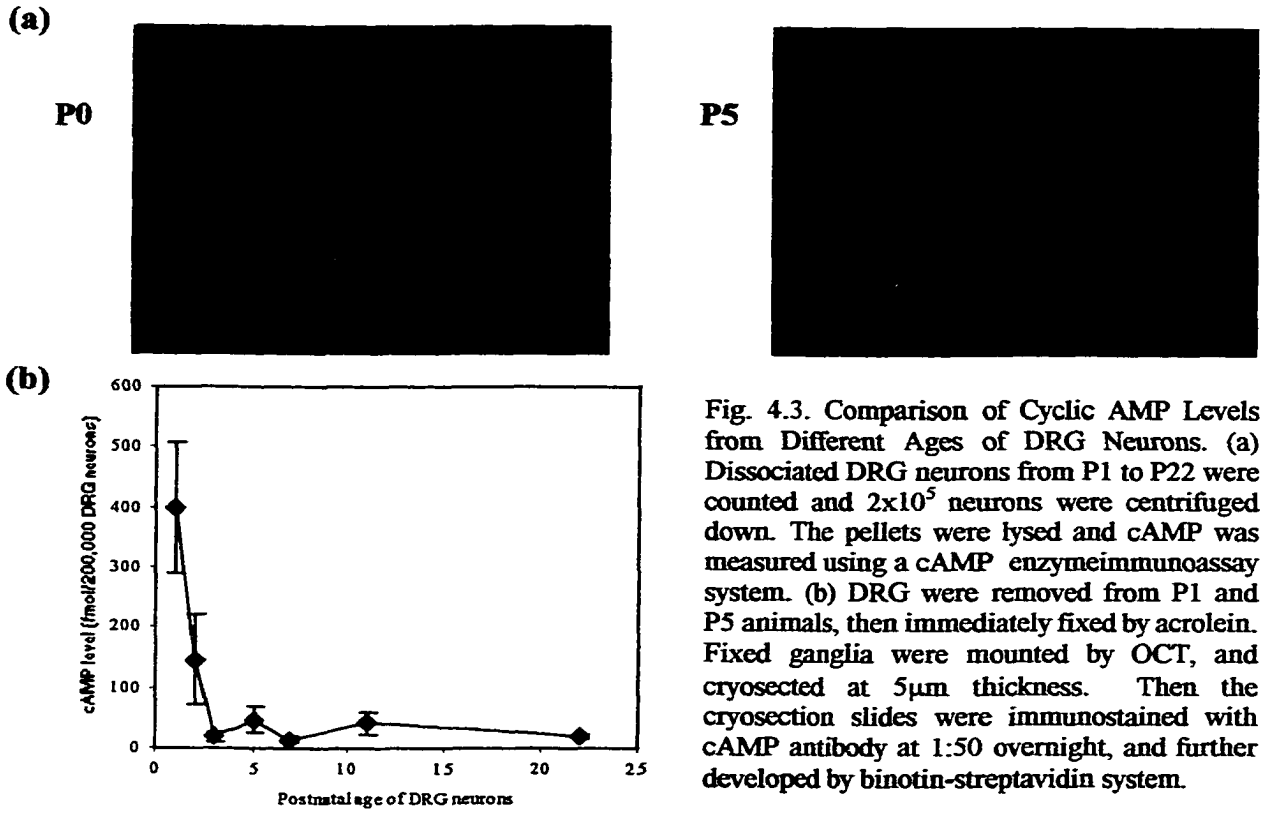


Fig. 4.3. Comparison of Cyclic AMP Levels from Different Ages of DRG Neurons. (a) Dissociated DRG neurons from P1 to P22 were counted and $2x10^5$ neurons were centrifuged down. The pellets were lysed and cAMP was measured using a cAMP enzymeimmunoassay system. (b) DRG were removed from P1 and P5 animals, then immediately fixed by acrolein. Fixed ganglia were mounted by OCT, and cryosected at 5μm thickness. Then the cryosection slides were immunostained with cAMP antibody at 1:50 overnight, and further developed by binotin-streptavidin system.

In summary, the results suggest that during the development of DRG neurons, the change in the endogenous cAMP level of neurons can explain the switch of neuronal response to MAG/myelin from promotion to inhibition. However, DRG neurons are just one type of neuron that switches in response to myelin inhibitors during development. The next question to answer is whether the decrease of cAMP levels during development is a general mechanism responsible for the loss of regenerative ability in the CNS.

Studies have shown that retinal ganglion (RG) neurons also switch their response to MAG during development, except that the transition happens at the embryonic stage instead of postnatally (Salzer et al., 1990). So we studied the axonal regeneration of RG neurons from different developmental stages grown on MAG and myelin. In addition, we compared the endogenous cAMP levels of embryonic RG neurons to cAMP of postnatal neurons.

A Decrease in cAMP Levels Dictates the Change in Regenerative Ability on MAG and Myelin during Development of Retinal Ganglion Neurons

Embryonic day 18 and postnatal retinal ganglion (RG) neurons were isolated and plated onto either MAG-expressing or control CHO cells or CNS myelin as a substrate (1μg/well) for overnight culture. It was found that embryonic RG neurons are promoted by MAG, whereas neurons at postnatal ages are inhibited when grown on MAG-expressing CHO cells (Fig. 4.4 a & b). Similarly, on a myelin substrate, axonal outgrowth of embryonic RG neurons is much longer than the neurite length of postnatal RG neurons (Fig. 4.5).

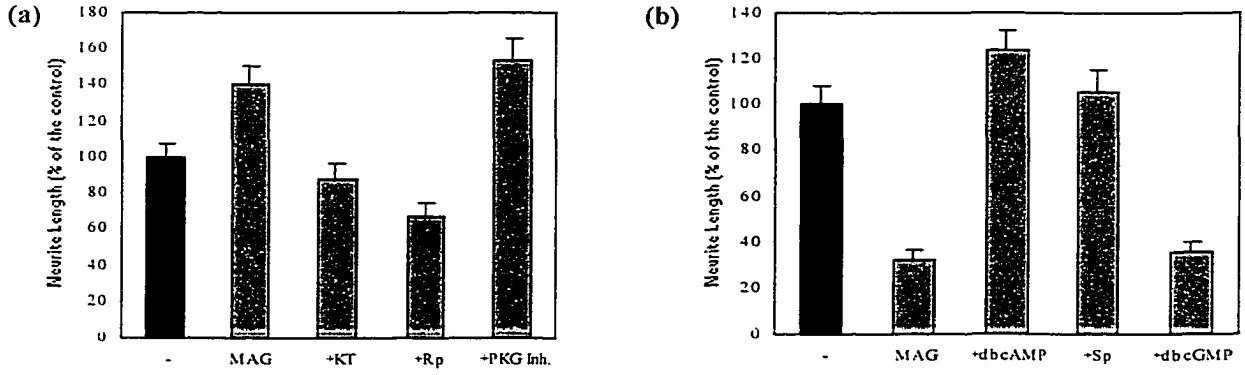
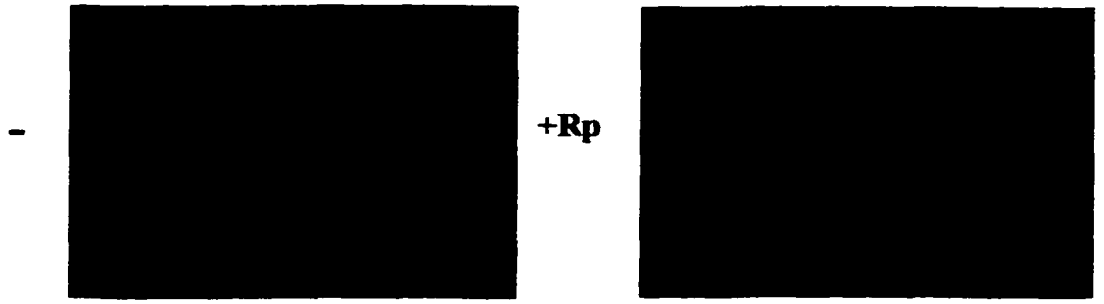


Fig.4.4. Neurite Outgrowth of E18 and P5 RG Neurons on MAG. (a) Embryonic day 18 RG neurons were dissociated and cultured overnight with or without PKA inhibitors, KT5720 (KT) or Rp-cAMP (Rp), or PKG inhibitor (PKG Inh.) on MAG-expressing CHO cells (stippled bars) or control CHO cells (solid bar). (b) P5 RG neurons were dissociated and cultured overnight with or without cAMP analogs dbcAMP or Sp-cAMP (Sp), or cGMP analog dbcGMP on MAG-expressing CHO cells (stippled bars) or control CHO cells

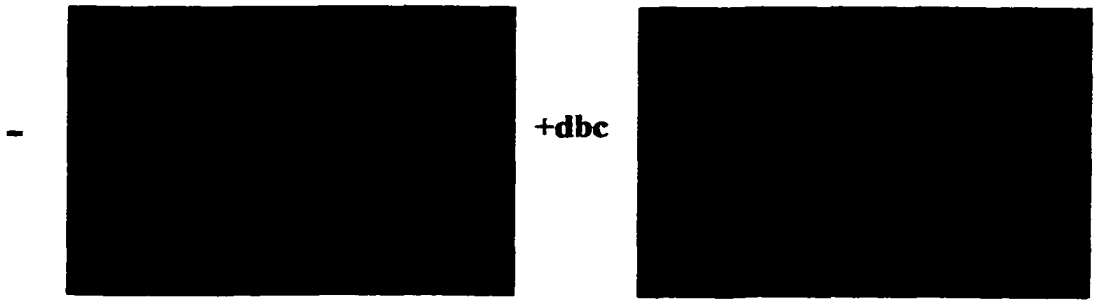
(black bars). Then neurons were fixed and immunostained for GAP43. The results were the mean (±SEM) of the longest neurite of each neuron from 180-200 neurons. 100% values were taken as the neurite lengths of E18 neurons (a) or P5 neurons (b) grown on control CHO cells without any treatment.

Furthermore, to determine cAMP-dependency of this developmental transition of response to MAG/myelin in RG neurons, neuronal cAMP levels or the activity of a down-stream effector was artificially changed to check whether the promoting/inhibiting effects on axonal growth by MAG/myelin could be altered.

(a) E18 RG Neurons on Myelin



(b) P5 RG Neurons on Myelin



(c)

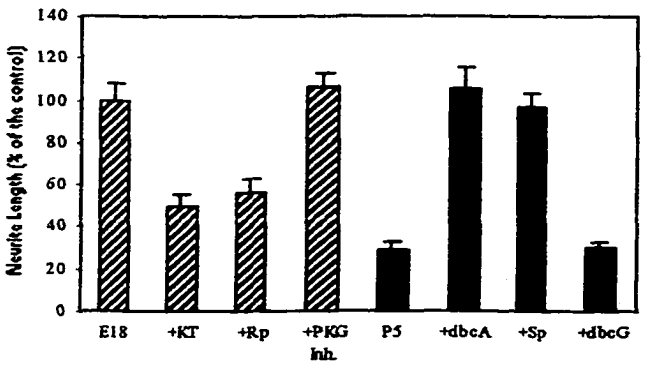


Fig.4.5. Neurite Outgrowth of RG Neurons from Different Ages on Myelin. Dissociated E18 and PND5 RG neurons were plated onto a CNS myelin substrate (1μg/well) for overnight incubation. Where indicated, KT 5720 (+KT), or Rp-cAMP (+Rp), or PKG inhibitor (+PKG Inh.) were included into E18 RG neuron cultures, while dbcAMP (+dbcA), or Sp-cAMP (+Sp), or dbcGMP (+dbcG) were inclued into p5 RG neuron cultures. Then neurons were fixed and immunostained for GAP43.

In figures 4.4 and 4.5, it is shown that addition of a protein kinase A inhibitor (KT5720 or Rp-cAMP), but not a protein kinase G inhibitor (KT5823), can block the promotion of axonal outgrowth of E18 RG neurons by MAG and myelin. On the other hand, artificially elevating cAMP levels by using cAMP analogs (dbcAMP or Sp-cAMP) in P5 RG neurons can improve the axonal regeneration of postnatal RG neurons on MAG/myelin. However, elevating cGMP levels by dbcGMP in P5 RG neurons fails to block the inhibition by MAG/myelin. These data strongly suggest that the cAMP level of RG neurons regulates the axonal regenerative capacity, similar to DRG neurons. In addition, the endogenous cAMP level of E18 retinal ganglion neurons was measured and compared to the level of neurons at postnatal ages. Results are shown in figure 4.6. The endogenous cAMP level of E18 RG neurons is approximately 1.5 x 10^4 fmol/cell, and then decreases to 0.5 x 10^4 fmol/cell at postnatal ages. This decrease in basal level of cAMP during development of RG neurons dictates the switch of neuronal response to MAG and myelin from promotion to inhibition.

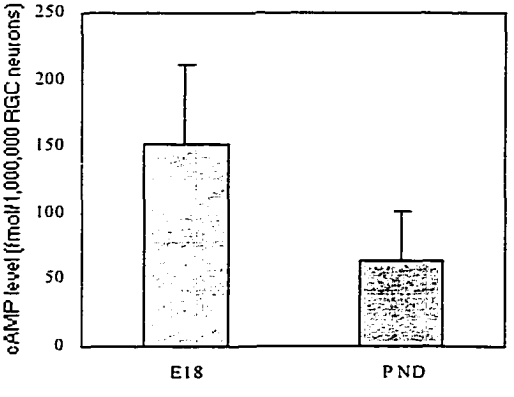


Fig.4.6. Comparison of Endogenous cAMP Levels in RG Neurons at Different Developmental Ages. 1 x 10^6 dissociated E18 or PND RG neurons were centrifuged down, and pellets were lysed by lysis buffers. The intracellular cAMP levels were measured by using a competitve immunoassay. Results shown are the mean (±SEM) of at least three experiments, each in sextuplet.

In summary, we have shown that in two types of neuron, (DRG and RG), basal level of cAMP drops dramatically during development, and this change in cAMP coincides with the switch of neuronal regenerative ability on MAG and myelin. Now, we would

like to determine the regulatory role of cAMP *in vivo* in the regenerative ability during neuronal development.

Regeneration of Neonatal Spinal Neurons in vivo and in Culture is cAMP-dependent

It is well known that the consequences of CNS injury in neonatal mammals are less harmful than lesions in the nervous system of adult animals. CNS lesions in newborn animals not only result in a better regeneration of transected fibers but also in compensatory growth of lesion-spared recovery. These processes are paralleled by a high degree of functional recovery (Bregman and Goldberger, 1982; Bregman and Goldberger, 1983a, b, c). For different types of spinal tract projections, there is a difference in the timing of when axons reach their target and myelination begins. For example, in rat at P1, some axons of the spinal tracts have already reached their targets and others are still in the process of elongating throughout the cord. Therefore, the functional recovery after neonatal spinal cord injury is due to regeneration of severed more mature axons, re-routing of late-developing axons and sprouting from lesion-spared axons (Bregman et al., 1989; Bregman et al., 1993). The window for this development plasticity is quite limited and exists for only the first couple of postnatal days. After that, there is a dramatic decrease in axonal regeneration after injury (Bregman et al., 1989). Based on the previous observations on DRG and RG neurons grown in culture, it is reasonable to suggest that the better *in vivo* regeneration in younger animals correlates with the high endogenous level of cAMP in spinal neurons. If this is the case, the better axonal regrowth in neonatal animals after injury should be abolished by applying a PKA inhibitor into the damaged spinal cord.

Now, we test if the spontaneous regeneration in neonatal animals is cAMP-dependent. In collaboration with Dr. Bregman from Georgetown, P2-3 rat pups' spinal cord were lesioned by over-hemisection (all axons in one half of the spinal cord are lesioned along with all dorsal column axons) at T6 level and the PKA inhibitor H89 was applied. It has been shown previously that implantation of embryonic spinal cord tissue into the lesion site enhances both survival and regeneration at P3 and P8 but does not alter the general outcome; at P3, spinal axons grow into and through the implanted tissue while at P8, axons grow only a short distance into the implanted tissue but never out into the distant host tissue (Bregman, 1987a; Bregman et al., 1989). Therefore, H89 was applied via gelform either directly to the lesion site or to the embryonic grafted tissue. After 2 weeks, corticospinal axons were labeled by anterograde tracing methods and raphespinal tract axons by immunostaining for serotonin.

The axonal growth of H89-treated animals is compared to the animals treated with saline. Results from a typical experiment for corticopsinal axons are shown in figure 4.7. It is found that corticospinal neurons regenerate their axons after injury at P2-3. However, in most of the animals treated with H89 (n=21), a visual difference from control (n=17) was immediately apparent, both in the length and in the number of corticospinal and raphespinal axons at the lesion or lesion plus transplant site. In H89-treated animals, the axonal growth characteristic of developmental plasticity was significantly attenuated in both the lesion only and the lesion plus transplant animals. The dense axonal growth charateristic of early lesions was decreased dramatically in 18 of the

21 H89-treated animals (Fig.4.7 A-D; arrows in D point to the little regeneration that is apparent in the H89-treated animals). Similar results were observed in raphespinal neurons by immunostaining serotonin to trace the axons. These results suggest that the spontaneous axonal regeneration in younger animals is cAMP-dependent.

As described above, the timing for target innervation and myelination of various spinal tracts is different. For example, in the rat at P1, most raphespinal axons have reached their targets at all spinal cord levels and myelination has begun, while corticospinal tract axons are still elongating toward their target (Bregman et al., 1989; Bregman et al., 1993). At this time in the rat spinal cord, some spinal tracts have begun to myelinate and the putative myelin inhibitor, MAG, is expressed at the initial stages of myelination (Quarles, 1983). Therefore, when the neonatal spinal cord is transected, injured raphespinal neurons will encounter MAG. However, these young raphespinal axons can regenerate without being inhibited by MAG. This *in vivo* spontaneous regeneration can be blocked by a PKA inhibitor, which strongly suggests the cAMP-dependency of the regenerative capacity in young spinal neurons.

Furthermore, the studies of raphespinal neurons grown in culture strengthen the idea of a correlation of neurite outgrowth ability with activity of a cAMP pathway. Figure 4.8 shows that raphespinal neurons from different postnatal ages show a developmental switch in response to MAG and myelin, as is the case for two other types of neuron, DRG and RG. Neurite outgrowth of P0/P1 raphespinal neurons is not inhibited by MAG and myelin, and addition of a PKA inhibitor can block the better growth of these young

neurons (Fig.4.8 a and c). In contrast, axonal growth of P4-8 raphespinal neurons is inhibited by MAG/myelin, while artificially elevating cAMP blocks this inhibition completely (Fig.4.8 b and c).

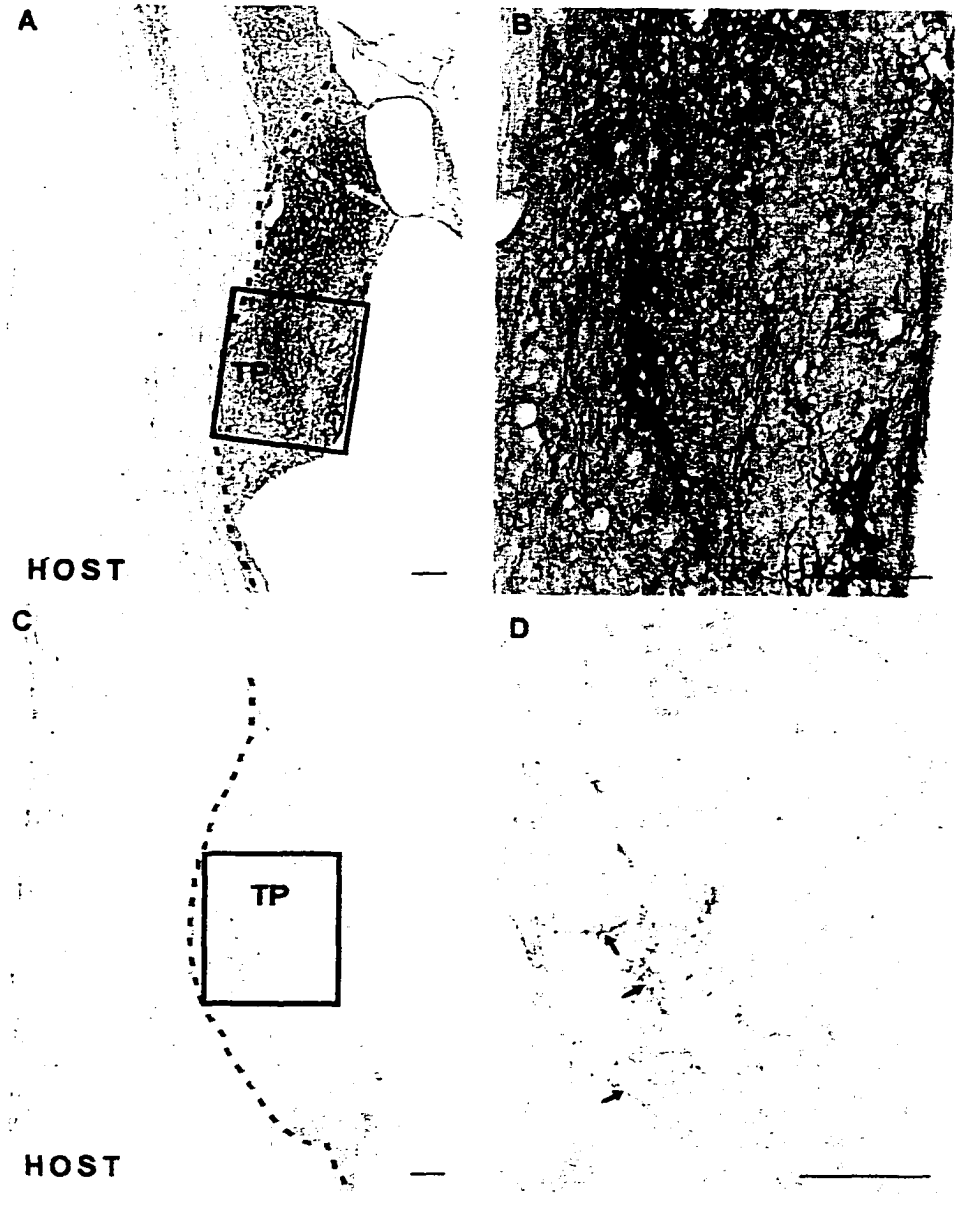


Fig.4.7. *In vivo* Injection of PKA Inhibitor H89 into Neonatal Spinal Cord Blocks the Regeneration after Transection. P2-3 rat pups were taken to make an over-hemisection at T6 of spinal cord level. The experimental groups are embryonic tissue transplants into lesion sites with H89 (n=11) (C&D) or embryonic tissues with saline (n=7) (A&B). The H89 at 0.5 mM or saline was delivered via gelfoam placed at lesion site. Two weeks later, the anterograde tracer biotin dextran amine (BDA) was injected into the sensorimotor cortex to trace the descending corticospinal tract fibers. After another two weeks, the rats were perfused and tissues were prepared for immunohistochemistry. BDA-positive axons were visualized by using a solution of diaminobenzidine tetrahydrochloride/nickel ammonium sulfate and hydrogen peroxide. (Data from Dr. Bregman's lab at Georgetown).

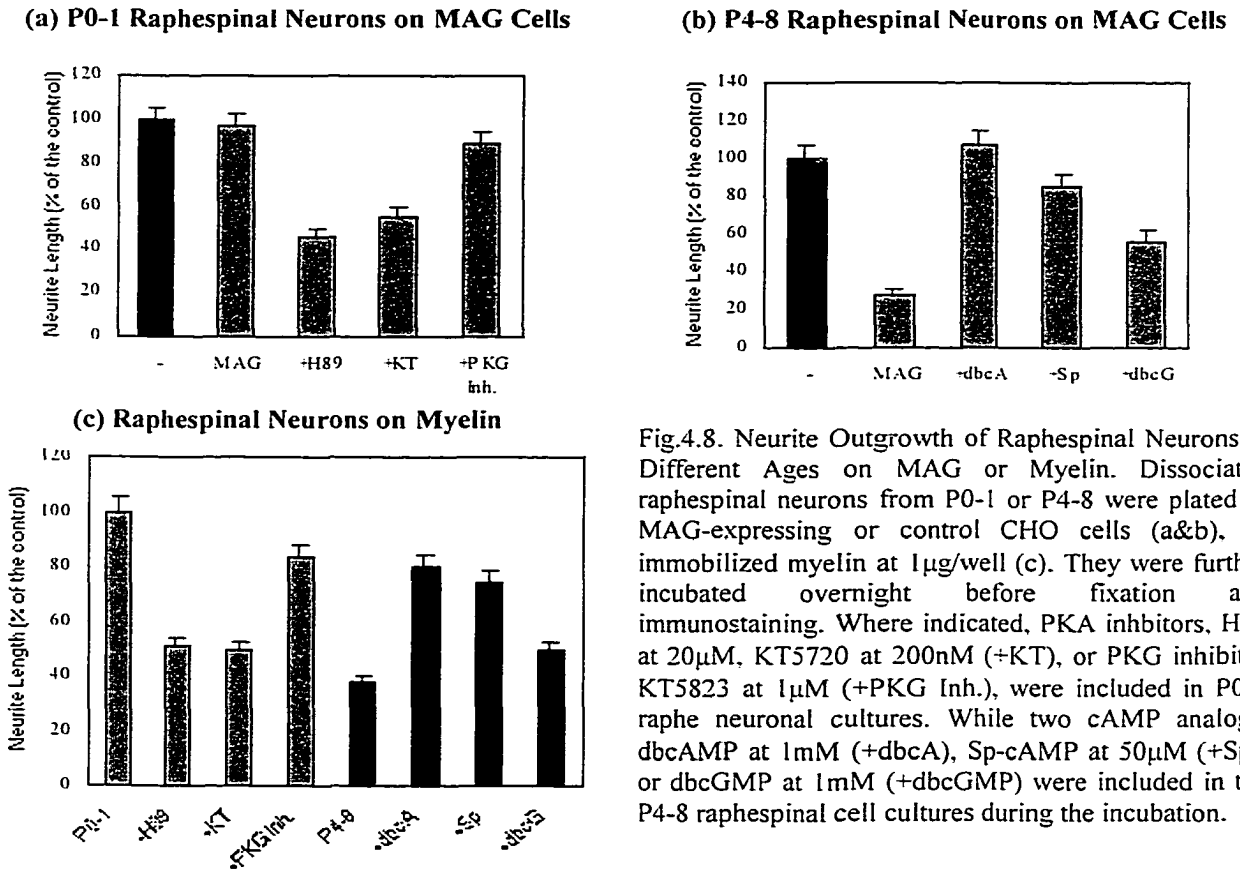


Fig.4.8. Neurite Outgrowth of Raphespinal Neurons at Different Ages on MAG or Myelin. Dissociated raphespinal neurons from P0-1 or P4-8 were plated to MAG-expressing or control CHO cells (a&b), or immobilized myelin at 1μg/well (c). They were further incubated overnight before fixation and immunostaining. Where indicated, PKA inhbitors, H89 at 20μM, KT5720 at 200nM (+KT), or PKG inhibitor KT5823 at 1μM (+PKG Inh.), were included in P0-1 raphe neuronal cultures. While two cAMP analogs, dbcAMP at 1mM (+dbcA), Sp-cAMP at 50μM (+Sp), or dbcGMP at 1mM (+dbcGMP) were included in the P4-8 raphespinal cell cultures during the incubation.

In addition, the measurement of endogenous cAMP levels in raphespinal neurons shows that there is a two-fold decrease in cAMP levels from postnatal day 0/1 to day 3/8 (Fig. 4.9). The basal levels of cAMP in P0-1 raphespinal neurons are around 12.5 x 10^{-1} fmol/ neuron, whereas in P3-8 the cAMP levels decrease to around 5.5 x 10^{-1} fmol/ neuron. Therefore, like DRG and RG neurons, raphespinal neurons switch their response to MAG and myelin during development and this switch is closely matched with their cAMP-dependent ability to regenerate in culture and *in vivo*.

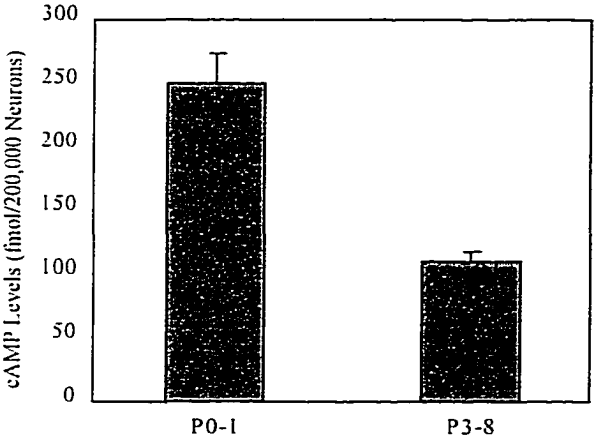


Fig.4.9. The Measurement of cAMP Levels from Different Postnatal Ages of Raphespinal Neurons. Dissociated raphespinal neurons from raphe nuclei at P0-1 or at P3-8 were lysed by lysis buffers provided from company (Amersham). The amounts of cAMP were determined by using cAMP enzymeimmunoassay (EIA) system.

In conclusion, *in vitro* and *in vivo* studies with three types of neuron suggest that the changes in endogenous cAMP levels of neurons regulate the switch in neuronal response to myelin and their *in vivo* regenerative capacity during development.

Discussion

The results presented in this chapter show that there is a difference in regenerative capacity in response to MAG and myelin between young and adult neurons. During development, the neuronal response to MAG/myelin switches from promotion to inhibition. However, the underlying molecular mechanism that regulates this developmental switch has never been described. Our data demonstrate that there is a decrease in the endogenous cAMP levels of neurons during development, which is responsible for the switch of axonal regenerative capacity as well as neuronal response to MAG and myelin. It has been shown that artificially elevating cAMP can prevent inhibition of axonal regeneration by MAG/myelin in older neurons whereas inhibiting the

activation of the cAMP downstream effector PKA can block promotion by MAG/myelin in younger neurons. Consistent with these observations in culture, the spontaneous regeneration *in vivo* after spinal cord transection in young animals is also dependent on cAMP activation. Therefore, we suggest that changes in neuronal cAMP levels dictate the developmental loss in axonal regenerative ability.

Our data suggest that cAMP is one of the molecular determinants responsible for the developmental switch of neuronal regenerative capacity. Artificially elevating cAMP levels or activity of PKA in adult neurons may switch their regenerative capacity to those of young neurons and support axonal regeneration after injury. It has been shown that the physiological changes in cAMP levels during development are sufficient to effect the switch of neuronal response to MAG/myelin from promotion to inhibition. When cAMP is high in neurons, neurons are able to regenerate because their response to MAG/myelin is promotion. In adult neurons, cAMP levels are low, which prevents axonal regrowth since neurons interpret signals from myelin as inhibitory. Therefore, the regenerative capacity of neurons during development is under the control of cAMP.

A number of previous studies have shown the gating function of cAMP during development: the regulation of synaptic plasticity (Iyengar, 1996), ion channel modulation (Zimmerman, 1995), and control of neurotrophin-dependent survival of neurons (Meyer-Franke et al., 1995a). For example, in long-term potentiation (LTP), the regulation of synaptic responses by cAMP is through inhibition of protein phosphatases. Hence, a "gate" is opened that allows signals for LTP to be transmitted and enhances the

synaptic responses to subsequent stimuli (Iyengar, 1996). However the inhibition of protein phosphatases by a cAMP pathway itself does not enhance synaptic responses (Iyengar, 1996). Others have also shown that synaptic potentiation induced by brain-derived neurotrophic factor (BDNF) is prevented by blocking cAMP signaling. However, activation of cAMP pathway alone is ineffective in modifying synaptic efficacy but greatly enhances the potentiation effect of BDNF. Thus, the synaptic actions of BDNF are gated by cAMP. It suggests that activity and other coincident signals that modulate cAMP levels may specify the action of secreted neurotrophins on developing nerve terminals (Boulanger and Poo, 1999).

Studies from other groups have also shown that in many species, cellular cAMP is a crucial determinant of embryonic development. It has been shown that the ambient cAMP levels, and hence the basal PKA activity, plays a critical role in the formation of the compound eye, wing and leg of *Drosophila* by regulating the activity of the morphogen Hedgedog (Blair, 1995; Pan and Rubin, 1995; Jiang and Struhl, 1995). Interestingly, it has been found that the basal activities of different adenylyl cyclase isoforms can contribute to very different intracellular cAMP levels. For example, AC2 has 10 to 25-fold greater activity than AC6 (Pieroni et al., 1995). It has long been noted that lowering cAMP levels can trigger either the proliferation of certain cells such as RAT-1 fibroblasts (van Corven et al., 1989) or the onset of the addicted state in neurons (Collier, 1980; Nestler, 1992). The lowered cAMP levels present during these processes might be a result of decreases in the activity of the preexisting adenylyl cyclases (ACs) or from up-regulation of different subtypes of adenylyl cyclases that have lower basal

activities (Pieroni et al., 1995). Therefore, the changes in neuronal cAMP levels during development, which are responsible for the neuronal switch of response to MAG/myelin in axonal regeneration, may be due to similar regulatory mechanisms modulated by adenylyl cyclases.

Moreover, it has also been demonstrated that phosphodiesterases (PDEs) in the nervous system undergo certain developmental changes in order for the cAMP signal transduction system to function properly (Zhang et al., 1999; Iona et al., 1998). For example, PDE4 activity in the olfactory bulb and hippocampus was found to be lower at birth in comparison to adult levels. Similarly, the V_{max} for cAMP hydrolysis by PDE4 was shown to be lower at birth when compared to adult levels in the cerebral cortex, cerebellum and neostriatum (Zhang et al., 1999). Therefore, these changes in ACs or PDEs converge to control cellular cAMP levels, and subsequently modulate different functions of cAMP.

In addition, protein kinase A (PKA), an important enzyme targeted by cAMP, can also affect the "free" levels of cAMP and the isoform composition of the enzyme affects the threshold level of cAMP required for its activation (Cummings et al., 1996; Brandon et al., 1998). Binding of cAMP to PKA regulatory (R) subunits induces the release of the catalytic (C) subunits, which then activates the downstream signaling (Brandon et al., 1997). Different isoforms of PKA regulatory subunits have different binding affinities for cAMP. The change in expression levels of different PKA isoforms may modulate the threshold of cAMP level required for downstream PKA activation. For example, in RIIβ

subunit knockout mice, RIα and RIβ subunits, which can be activated at low cAMP levels, are increased in their expression levels to compensate (Cummings et al., 1996; Brandon et al., 1998). Therefore, the threshold cAMP levels for PKA activation in these knockout mice may be lower than the cAMP levels required in their wildtype counterparts. Interestingly, it has been found in RIIβ subunit knockout mice that there is no switch of neuronal response to MAG from promotion to inhibition in DRG neurons during development (Qiu and Filbin, unpublished data). DRG neurons from these mice are not inhibited by MAG generally. It is possible that the low levels of cAMP in older DRG neurons are too low to activate PKA in wildtype neurons and so MAG inhibits neurite outgrowth. However, these low cAMP levels can still activate PKA enzyme containing the RIα and RIβ subunits in RIIβ-/- neurons and so MAG never inhibits neurite outgrowth from DRG neurons from these animals at any age. Alternatively, the absence of RIIβ may affect the "free" levels of cAMP through a feedback mechanism and impair the developmental decrease in cAMP level. Therefore, without the developmental changes in cAMP levels, there is probably no switch of neuronal response to myelin during the development of RIIβ-/- mice. So a complete study with the expression level and activity of different isoforms of cAMP-dependent enzymes, as well as measurement of cAMP levels from different developmental ages would help us to understand the mechanism that regulates the axonal outgrowth capacity in these RIIβ knockout mice.

Comparison of endogenous cAMP levels in three different types of neuron show that the lower basal level of cAMP that effects inhibition by MAG/myelin is similar for DRG and RG neurons (0.5×10^{-4} fmol/neuron) whereas it is much higher for raphe neurons (5

x 10^{-4} fmol/neuron). The cAMP levels in RG neurons that result in promotion of neurite outgrowth by MAG/myelin (1.5 x 10^{-4} fmol/neuron) are even lower than the levels in raphe neurons that cause an inhibitory response (5.5 x 10^{-4} fmol/neuron). This is unlikely to be due to the differences in sizes amongst the various neuronal types as DRG neurons are larger than raphespinal neurons. Alternatively, the threshold level of cAMP that induces a switch of response to MAG/myelin may be different for different types of neurons, which may in turn be due to distinct expression levels of different isoforms of PKA subunits in different neuronal types.

In addition to cAMP, cGMP has been shown to regulate neuronal attractive/repulsive responses to guidance molecules such as Sema3A and NT-3. Recent studies have also demonstrated that in pyramidal neurons, axons are repelled by Sema3A, whereas dendrites in the same neurons are attracted by Sema3A (Polleux et al., 2000). It has been found that asymmetric distribution of guanylate cyclase, which is the enzyme that synthesizes cGMP, is responsible for this phenomena, which results in low cGMP levels in axons and high levels in dendrites. As mentioned in the previous chapter, it has been shown that addition of dbcGMP directly without priming cannot block the inhibition of axonal growth by MAG and myelin. However, priming neurons with NT-3, which activates the cGMP pathway, can improve axonal regeneration on myelin specific inhibitors (Cai et al., 1999). Similarly, priming neurons with dbcGMP has the same blocking effect on MAG's inhibition (Adebola and Filbin, unpublished data). Together, these data suggest that the neuronal cGMP level has a functional importance in regulating axonal growth capacity in response to MAG/myelin. It is reasonable to suggest that

elevation of cGMP levels by priming with either dbcGMP or NT-3 may activate downstream effectors, which can also be activated by the elevation of cAMP. Currently under investigation in the lab is the identification of the downstream effectors responsible for the improved axonal regenerative capacity following the activation of cAMP or cGMP pathways. However, it should be noted that cAMP levels, but not cGMP levels, are sufficient to determine the developmental switch of neuronal response to MAG/myelin. Because blocking the activation of cGMP pathway by inclusion of a PKG inhibitor, has no effect on axonal growth on myelin inhibitors from young neurons (Fig.4.2).

As a second messenger, cAMP has been shown to regulate a striking number of physiological processes by altering the basic pattern of gene expression. A lot of evidence has demonstrated that PKA is required for cAMP to stimulate transcription. It has been shown that cAMP levels and the activity of PKA regulate neuronal regenerative capacity. It is reasonable to suggest that this cAMP-dependent regulation is related to gene transcription as well as protein synthesis. It has been found that adding inhibitors of transcription or protein synthesis to culture, the block of inhibition by cAMP can be completely abrogated (see data in Chapter VI). The changes in cAMP levels during development may also affect the activity of transcription factors as well as protein synthesis modulators, and consequently control the regenerative capacity of neurons. The regulation by cAMP of transcription and translation may be responsible for its persistent effect on axonal regeneration rather than just a transient effect on growth cone dynamic responses to guidance cues.

It should also be noted that the regulatory effect of cAMP on neuronal regenerative capacity is not simply a survival effect because elevated cAMP does not affect axonal growth on control cells. Although studies have shown that elevation of neuronal cAMP levels can enhance neuronal trophic responsiveness and thus improve cell survival, in some types of neurons, such as RG neurons, elevated cAMP alone may not be sufficient to improve survival (Meyer-Franke et al., 1995a; Shen et al., 1999). In addition, in mice over-expressing bcl-2, an anti-apoptotic protein, improved survival but not improved axonal regeneration is observed (Chen et al., 1997).

In summary, the results shown in this chapter represent the first step towards a molecular understanding of the switch during development that results in the inhibition of axonal regeneration by myelin and consequently the inability to regenerate *in vivo*. The neuronal response to myelin inhibitors is dictated by the neuron's endogenous level of cAMP. If cAMP does play a major role in the control of neuronal regenerative capacity during development, the next question is to determine if cAMP is important in axonal regeneration following injury.

Chapter V

The Regulatory Role of cAMP in Axonal Regeneration Following Injury

Introduction

In the adult CNS, neurons fail to regenerate after injury. However, providing a permissive environment for growth by means of a Schwann cell graft (Li et al., 1994; Xu et al., 1995), an olfactory ensheathing glia transplant (Li et al., 1997), or an embryonic tissue explant (Bregman et al., 1989) does improve axonal regeneration of injured CNS neurons. Neutralizing the inhibitory effects of myelin-specific inhibitors also enables lesioned axons to regrow and reestablish the same function (Schnell and Schwab, 1990; Schnell et al., 1994; Bregman et al., 1995; Huang et al., 1999).

It has also 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 DRG neurons are provided with a conditioning lesion to the peripheral branch, followed by a second lesion to the central branch of the same nerve either at the same time or weeks later, there is more extensive growth into peripheral nerve grafts placed at the lesion site of the dorsal column than without the conditioning lesion (Richardson and Issa, 1984; Oudega et al., 1994). However, there is no growth into the host tissue beyond.

Recently, studies by Neumann and Woolf extended these observations by demonstrating that a conditioning lesion to the peripheral branch of DRG neurons improves the regenerative capacity of their central branch and allows transected dorsal column axons to regrow in the non-permissive CNS environment after injury in the absence of a peripheral nerve graft (see figure 5.1) (Neumann and Woolf, 1999).

Simultaneous lesions in the peripheral and central branches of DRG neurons only allowed axons to regrow into the lesion site of the dorsal column but not beyond. However, when the conditioning lesion in the peripheral nerve was performed 1 week before the dorsal column transection, injured axons regenerated across the injury site and into the tissue beyond (Neumann and Woolf, 1999). Some of the regenerating axons even grew into the host white matter. This suggests that the regenerative capacity of DRG neurons is certainly improved by the first injury in the peripheral branch. However, the molecular mechanism underlying this improved axonal regeneration has not been described. It is possible that following the conditioning lesion, the receptors of myelin-specific or glial-derived inhibitory molecules are down-regulated, which consequently allows axonal regeneration in the CNS. Alternatively, activation of signaling pathway(s) following peripheral conditioning lesion may result in the changes in neuronal response to myelin and other glial-derived inhibitors and then neurons are no longer inhibited by the non-permissive CNS environment (Qiu et al., 2000).

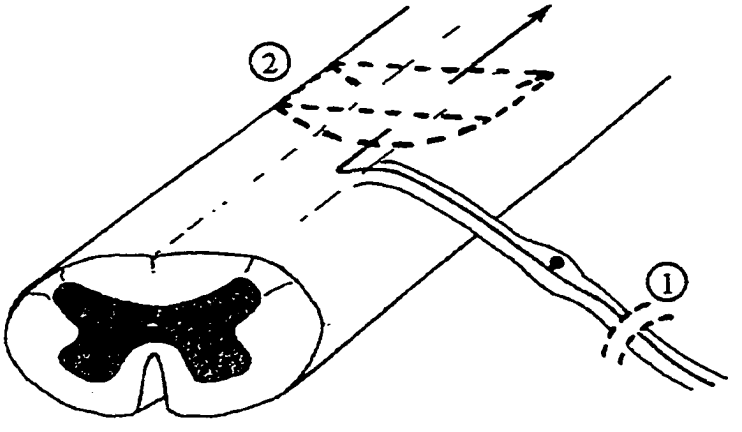


Fig.5.1 The Model of Preconditioning Peripheral Branch Injury and Subsequent Central Dorsal Column Lesion in DRG Nerves [from Neuron 1999 (23), 2-4].

In the previous chapter, it was shown that elevation of cAMP by priming with neurotrophins can block the inhibition of axonal growth by MAG and myelin. Endogenous cAMP levels also regulate the neuronal response to MAG/myelin during development. Therefore, it is reasonable to suggest that cAMP may play a role in regulating this improved nerve regeneration following peripheral conditioning lesion. To investigate this idea, the sciatic nerve was transected and then injured L4-5 DRG neurons were isolated at different times post-injury. Axonal outgrowth on MAG or myelin was assessed and the endogenous cAMP level of injured neurons was compared to their contralateral controls.

Results

16 hours or 1week after sciatic nerve transection, L4-5 dorsal root ganglia were isolated. First, the cAMP levels of injured L4-5 DRG neurons were compared to the levels of contralateral controls. It was found that 16 hours post-injury, the cAMP levels of injured DRG neurons increased by at least two-fold. By 1week post-injury, there was no difference in cAMP levels between injured neurons and controls.

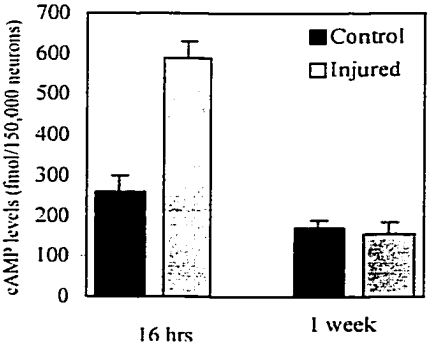


Fig.5.2 Changes in cAMP Level in Response to Peripheral Conditioning Lesions. The peripheral transection of sciatic nerves was performed at PND18 rats. After 16 hours or 1 week of injury, L4-5 DRGs from injured side were dissociated and cAMP levels of neurons were measured to compare with levels in DRGs from the contralateral control side. The cAMP levels were quantitated by using enzymeimmunoassay (EIA) system.

Figure 5.2 shows that the endogenous cAMP levels of injured DRG neurons exhibit a more than two-fold increase at 16 hours post-lesion. However, if cAMP levels of contralateral controls are compared to basal cAMP levels in adult DRG neurons shown in the previous chapter, at 16 x 10^{-4} fmol/neuron and 0.5 x 10^{-4} fmol/neuron respectively, the levels are quite different. The factor(s) responsible for this elevation of basal levels of cAMP at contralateral uninjured controls following injury remains unknown. Nevertheless, elevation of cAMP levels in injured neurons is much more dramatic than that in control ones at 16 hours post-injury. But this change in cAMP levels is not sustained for one week following injury. As shown in figure 5.2, the cAMP levels of injured DRG neurons decline to control cAMP levels by 1week post-injury.

The neurite outgrowth capacity on MAG/myelin from injured L4-5 DRG neurons was compared to the contralateral uninjured controls at 16 hours or one week after sciatic nerve transection. We found that 16 hours after peripheral conditioning lesions, there is an increase in axonal growth on MAG and myelin (Fig.5.3b), while 1week post-injury, axonal growth on MAG/myelin becomes even more extensive (Fig.5.3c). To investigate the involvement of cAMP in the improved growth following conditioning lesions, a PKA or PKG inhibitor was included in the culture media. Figure 5.3 shows the axonal outgrowth of injured DRG neurons from 16 hours or 1week post-lesion subsequently grown on myelin with or without a PKA inhibitor.

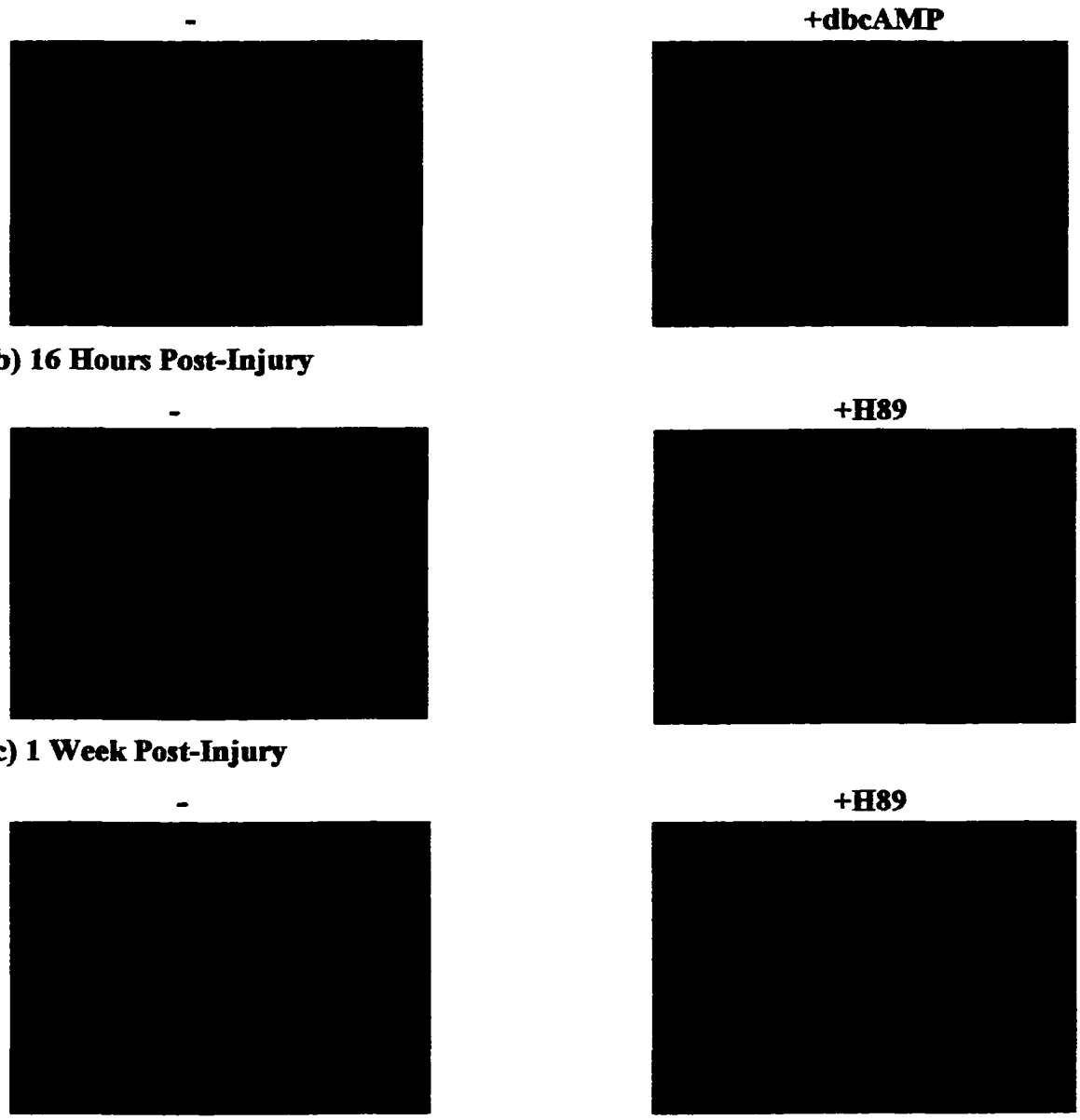


Fig.5.3. Images of Neurite Outgrowth of DRG Neurons from Injury and Control on Myelin Substrate. Unoperated neurons treated with or without dbcAMP at 1 mM (a), dissociated injured neurons 16 hours after lesions treated with or without PKA inhibitor H89 at 50μM (b), dissociated injured neurons 1 week after lesions treated with or without H89 at 50μM (c), were shown on CNS myelin substrate after overnight growth.

Figure 5.4 is the quantitation of neurite outgrowth on MAG and myelin from DRG neurons isolated at 16 hours or 1week post-injury. 16 hours after injury, DRG neurons are not inhibited by MAG/myelin in culture but a PKA inhibitor (H89 or KT5720), and not a

PKG inhibitor, blocks this improved axonal growth completely (Fig.5.3b and Fig.5.4). However, 1week after injury, axonal growth on MAG or myelin is extensively increased but this growth cannot be blocked by addition of a PKA inhibitor (Fig.5.3c and Fig.5.4). This suggests that the inhibition of neurite outgrowth by MAG/myelin is overcome by a conditioning sciatic nerve transection, which is similar to the Neumann et al. *in vivo* observation. This blocking effect is dependent on the cAMP signaling pathway at 16 hours but not 1 week post-injury.

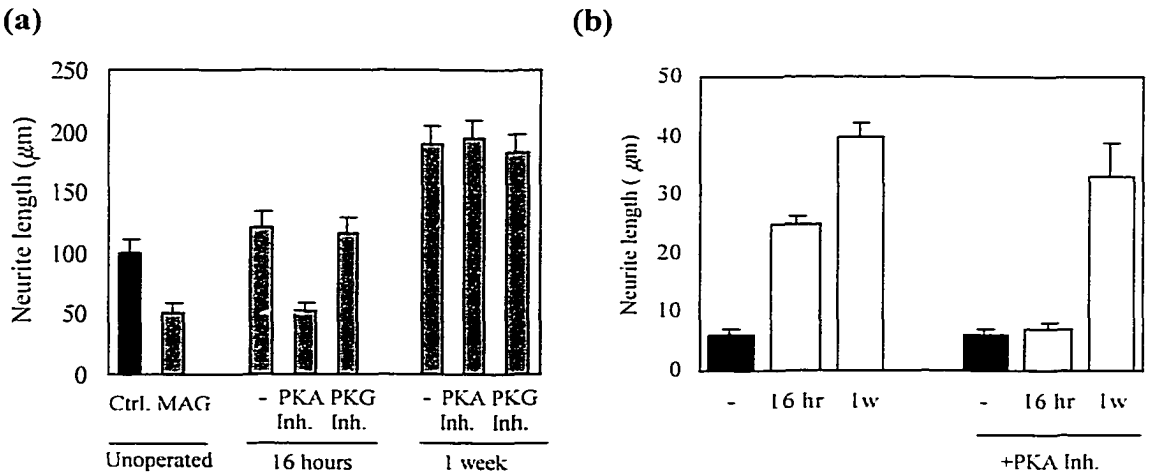


Fig.5.4 DRG Neurons from Conditioning Lesions or Contralateral Controls Grown on MAG or Myelin. 16 hours or 1 week after sciatic nerve transection, isolated L4-5 DRG neurons from either the injured side or contralateral control, were plated on either MAG-expressing CHO or control CHO cells (a), or purified CNS myelin (b). Where indicated, a PKA inhibitor, either H89 at 50μM, or KT5720 at 200nM, or PKG inhibitor KT5823 at 1μM, was included into culture during neurite outgrowth assay. After overnight incubation, neurons were fixed and immunostained for GAP43. The longest neurite from each neuron was measured and the average of neurite length from 180-200 neurons was obtained by using the Oncorimage analysis system.

Neumann et al. found *in vivo* that if the dorsal column is lesioned immediately after a peripheral conditioning lesion there is no regeneration in the CNS. However, if the sciatic nerve is transected 1 or 2 weeks prior to a dorsal column injury, there is extensive axonal regeneration into and across the lesion site (Neumann and Woolf, 1999). Our data in culture shows that 16 hours after a peripheral conditioning lesion, there is an increase in axonal growth on MAG and myelin. One week post-injury, axonal growth on

MAG/myelin is much more extensive than at 16 hours post-injury. In addition, axonal growth at 16 hours post-injury is cAMP-dependent, whereas it changes into a cAMP-independent growth by 1week post-injury. Therefore, axonal regeneration switches from cAMP-dependent growth to cAMP-independent growth. Following a peripheral conditioning lesion, there is an elevation of cAMP in the injured neuron cell body (shown in Fig.5.1), which could activate downstream signals and allow axonal regeneration in the presence of myelin-specific inhibitors. If this is so, the activation of the downstream signaling mechanisms after elevation of cAMP could lead to axonal regeneration. The next questions to be answered are: 1) When does cAMP-dependent axonal regeneration change into cAMP-independent growth between 16 hours and 1week post-injury? The investigation of a detailed time course of cAMP-dependent/independent growth post-injury is currently ongoing in the lab. 2) Whether the initial increase in cAMP (within 16 hours) is responsible for the more extensive axonal regeneration at 1week post-injury?

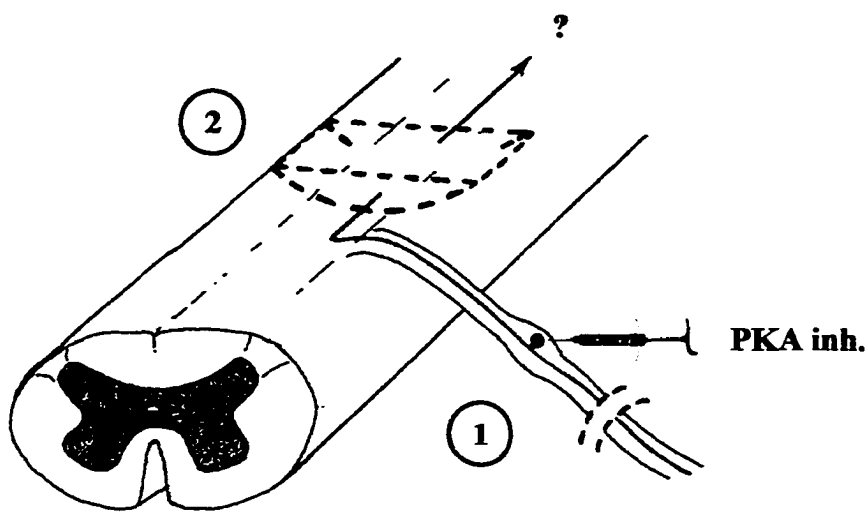


Fig.5.5 Scheme of Injection of PKA Inhibitiors into DRG with Sciatic Nerve Transection and Subsequent Central Dorsal Column Injury. Step 1 is the injection of H89 2ml at 20mM or saline 2ml to L4-5 DRG by using a glass micropipette over a period of 1 hour. At the same time, sciatic nerve transection was made to the animals. 1 day or 1 week later, we isolated injured and injected DRG neurons to analyze their neurite outgrowth abilities. Step 2, performed 1 or 2 weeks later, invovled dorsal column transection and the regeneration of injured fibers following conditioning lesions and injection, was investigated.

If the improvement of axonal growth on MAG/myelin at 16 hours or 1week post-injury is due to the intial elevation of cAMP, then providing a PKA inhibitor to the DRG *in vivo* at the same time as the peripheral nerve is transected should block the improved axonal growth on MAG/myelin. Figure 5.5 shows the experimental scheme. The PKA inhibitor, H89, is injected into L4 and 5 DRGs using a micropippette at the same time as the sciatic nerve is transected (Fig.5.5-1). The control injection of saline is applied at the same time as the peripheral nerve transection. 16 hours or 1 week after injection and lesion, neurons were isolated and grown on MAG-expressing CHO cells or CNS myelin substrate. Neurite outgrowth of injured neurons after PKA inhibitor injection was analyzed to see whether the improved axonal growth after a conditioning lesion is eliminated by blocking PKA activation *in vivo*.

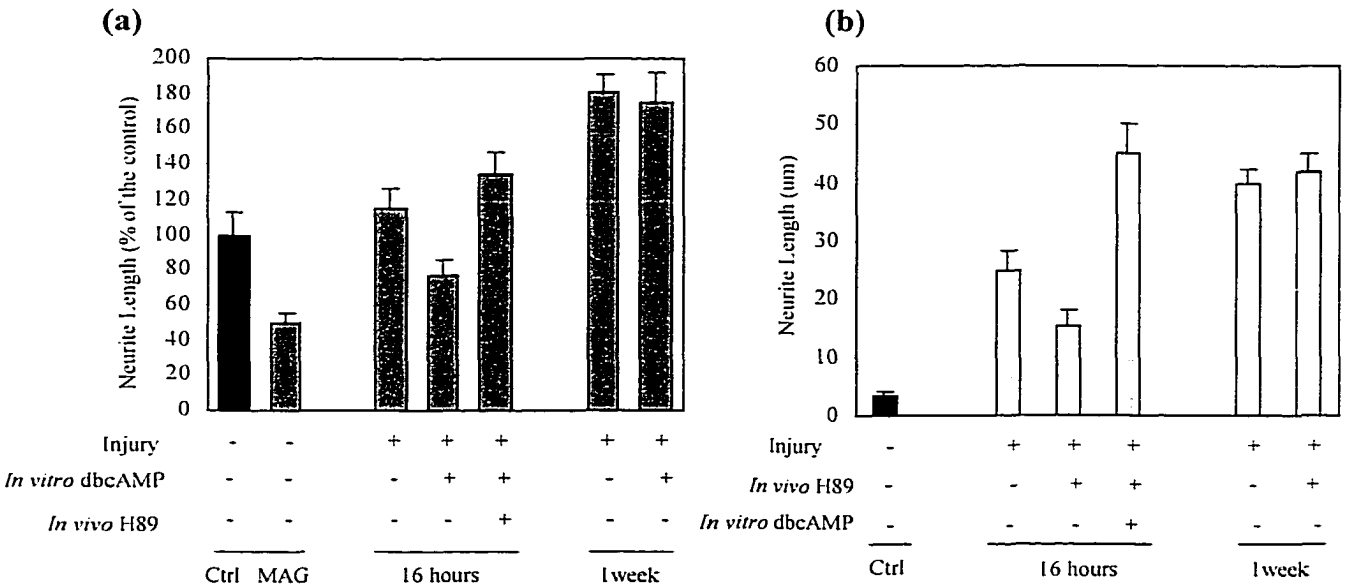


Fig.5.6. DRG Neurons from 16 Hours or 1Week Post Injection of H89 and a Conditioning Lesion Subsequently Grown on MAG or Myelin. A PKA inhibitor H89 (50mM) was injected into DRG by micropipette technique at the same time as sciatic nerve transection. 16 hours or 1 week after sciatic nerve transection and H89 *in vivo* injection, L4-5 DRG neurons were dissociated and plated on either MAG-expressing CHO or control CHO cells (a), or purified CNS myelin (b). Axonal outgrowth of injured and injected DRG neurons was compared to DRG neurons from unoperated animals. During the incubation time of the neurite outgrowth assay, dbcAMP at 1mM was included into culture where indicated. 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 by using the Oncorimage analysis system.

Figure 5.6 shows axonal growth of injected and injured DRG neurons on MAG/myelin compared to controls (mock injection with injury or injury alone). We found that injection of a PKA inhibitor (H89) *in vivo*, can partially block the increased axonal regeneration on MAG observed at 16 hours but not at 1week after peripheral nerve injury. The blocked growth capacity at 16 hours after injection can be restored by adding dbcAMP in culture during neurite outgrowth, demonstrating that the blocking effect of H89 is not due to the toxicity of drug.

However, a single injection of the PKA inhibitor H89 did not block the extensive improved growth on MAG/myelin 1week post-injury. As shown in figure 5.6, axonal growth is almost the same as without injection of H89 (injury only). This may be because just one injection of the drug can diffuse quickly into the tissue beyond and thereby does not persist long enough to block the activation of the cAMP pathway immediately following a peripheral nerve injury. To efficiently block cAMP activation it may be necessary to keep the PKA inhibitor in contact with the injured DRG cell bodies for a much longer period of time post-injury.

Together, these results suggest that cAMP plays a regulatory role in this improved growth on MAG/myelin after a conditioning lesion. However, there is a time-dependent change in axonal growth capacity in response to MAG/myelin after a conditioning lesion. Growth from 1week post-lesion DRG neurons is much better and is cAMP-independent as apposed to 16 hours post-lesion (shown in Fig.5.3 and Fig.5.4). It is reasonable to

suggest that elevation of cAMP induced by a conditioning lesion may activate downstream transcriptional and translational machinery. The expression levels of targeted genes are then upregulated or down-regulated which subsequently affect axonal regenerative capacity. Once the downstream transcriptional/translational machinery is turned on, it may be able to regulate axonal regeneration even in the absence of cAMP elevation.

Nevertheless, the activation of a cAMP pathway induced by a conditioning lesion is likely to be the molecular event responsible for the improved axonal regenerative capacity on myelin-specific inhibitors in culture as well as the regrowth of damaged dorsal spinal axons *in vivo*. Therefore, artificially elevating cAMP in DRG cell bodies *in vivo* should mimic the effect of a conditioning lesion on axonal regeneration in culture and *in vivo*.

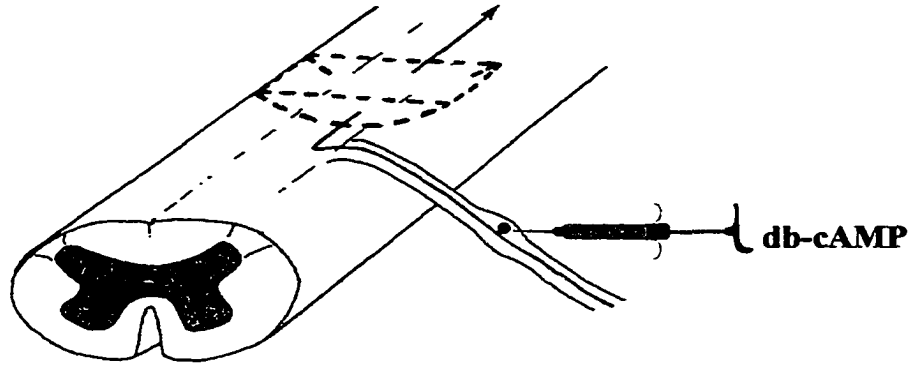


Fig.5.7 Scheme of Injection of dbcAMP into DRG and Subsequent Central Dorsal Column Injury. The injection of dbcAMP at 50mM or saline into L4-5 DRG by using a glass micropipette over a period of 1 hour. 16 hours or 1 week later, injected DRG neurons were isolated to analyze their neurite outgrowth capacity in response to MAG/myelin in culture. Or 1 or 2 weeks later, dorsal column transection was performed and the regeneration of injured fibers following injection would be investigated.

Dibutyryl cAMP was injected at 50mM into L4-5 DRGs without a peripheral nerve transection. 16 hours or 1week after injection, injected DRG neurons were isolated and the axonal growth capacity on MAG and myelin was studied. Results are shown in figure

5.8. In addition, *in vivo* dorsal column axons were transected after the injection of dbcAMP to check whether elevation of cAMP in DRG cell bodies could improve axonal regeneration in the adult CNS. Figure 5.7 shows the scheme of this experiment. Figure 5.8 shows that 16 hours or 1week after *in vivo* injection of dbcAMP into DRGs without a peripheral nerve lesion, the inhibition of axonal growth by MAG (a) or myelin (b) is completely overcome.

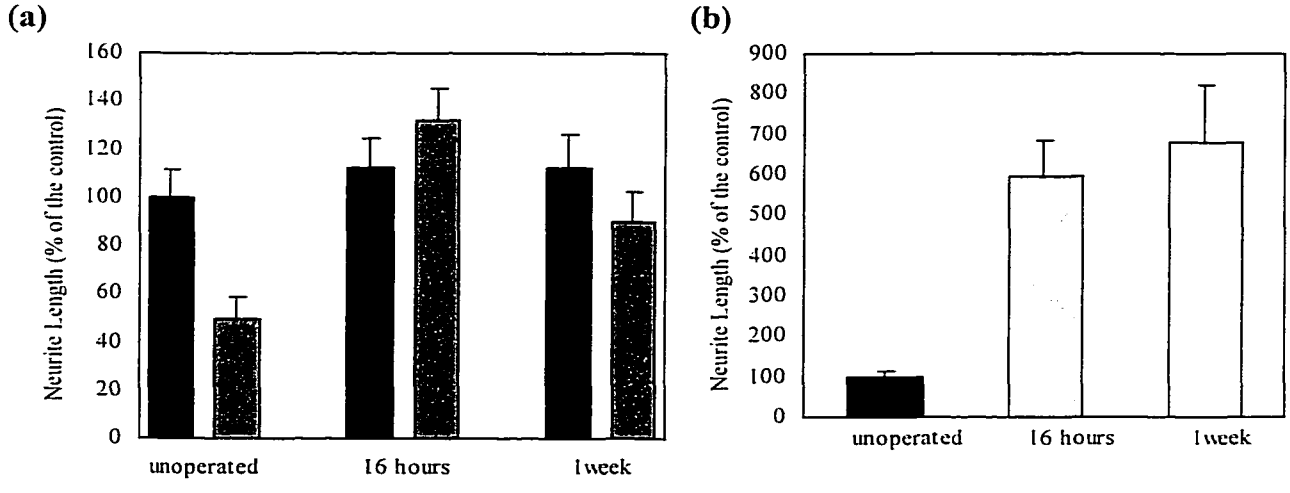


Fig.5.8. Neurite Outgrowth of DRG Neurons 16 hours or 1week after Injection of dbcAMP then Grown on MAG or Myelin. Dibutyryl-cAMP at 50mM in 2ml was injected into L4-5 DRGs using a glass micropippette over a period of 1 hour. 16 hours or 1week after injection, injected DRGs were isolated and plated onto MAG-expressing CHO (stippled bars in a) or control CHO cells (black bars in a), or a CNS myelin substrate (b). Axonal growth of injected neurons was compared to DRGs from unoperated animals. After overnight incubation, neurons were fixed and immunostained with anti-GAP43 antibody. Then, the longest neurite from each neuron was measured and the average of neurite length from 180-200 neurons was obtained by using the Oncorimage analysis system.

However, the improvement of axonal growth on MAG/myelin 1week after injection is not much different from the growth at 16 hours post-injection, which is different from the improved growth induced by a conditioning lesion at 16 hours and 1week post-injury, where there is a time-dependent increase in growth. The possible explanation is that the effect of a single injection of dbcAMP may be not strong enough to sustain and activate downstream machinery completely. To maintain a regenerative state, cAMP may have to be kept at a higher level for a certain period of time.

In addition, Dr. Bregman at Georgetown University studied the *in vivo* axonal regeneration of transected dorsal column axons after a single injection of dbcAMP into L5 DRGs. The experimental scheme is shown in Figure 5.7. As demonstrated in figure 5.9, after a single injection of dbcAMP into L5 DRGs, *in vivo* axonal regeneration of transected dorsal column fibers is improved dramatically compared to saline-injected animals. In the mid-lesion site of the dorsal column (fig.5.9b), there are quite a few injured axons regrowing across the damaged adult spinal cord, while none are seen in saline-injected animals.

In summary, these results suggest that following a peripheral nerve transection, there are changes in neurite growth capacity in response to MAG and myelin in culture as well as improvement of axonal regeneration of the damaged spinal cord *in vivo*. These are associated with the elevation of cAMP in injured DRG cell bodies and subsequent activation of downstream signaling pathways. Directly applying cAMP analogs to neurons without a conditioning lesion, can mimic the blocking effect on myelin-specific inhibitors induced by a peripheral nerve transection in culture and also improve the axonal regeneration of the damaged adult spinal cord *in vivo*.

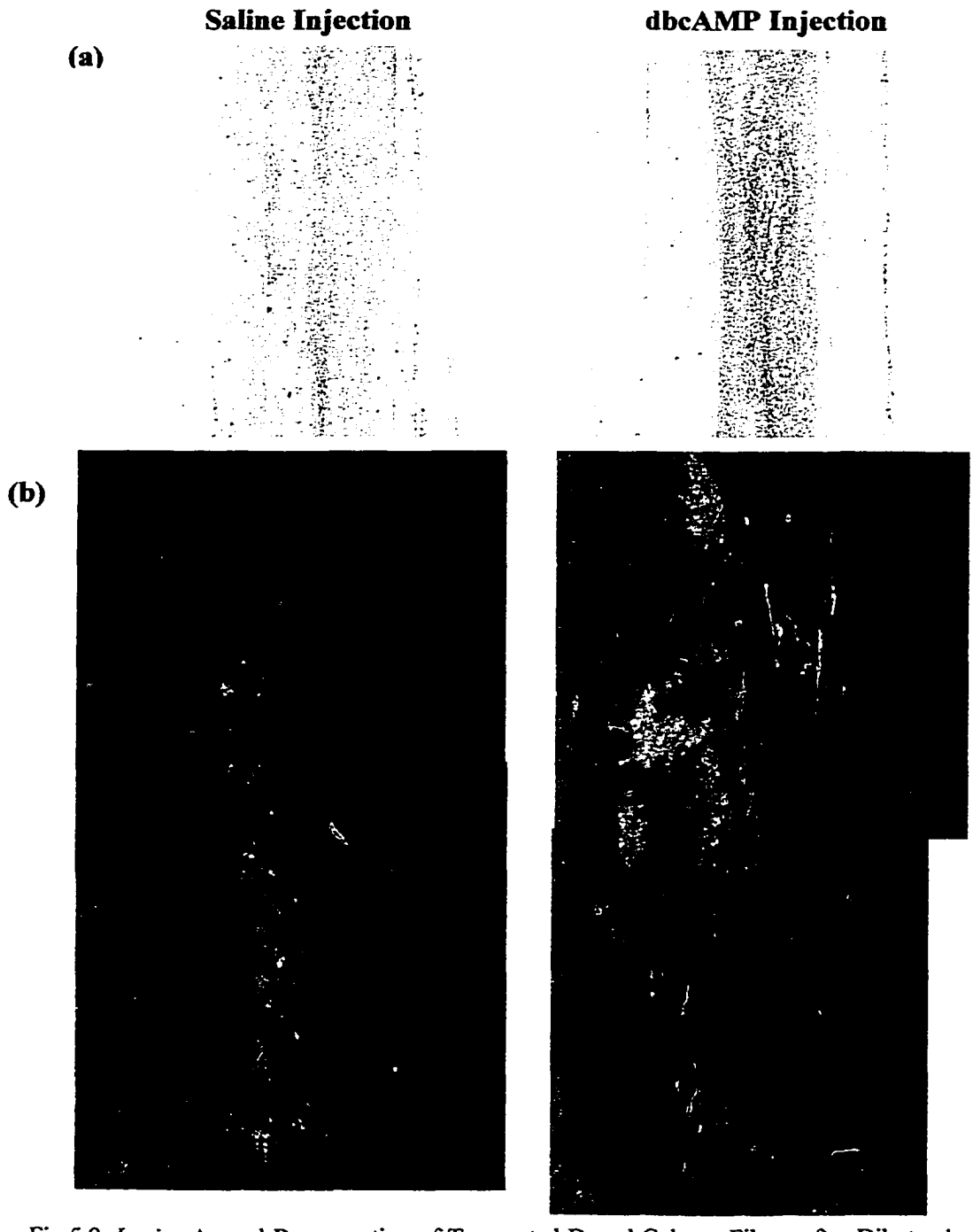


Fig.5.9. *In vivo* Axonal Regeneration of Transected Dorsal Column Fibers after Dibutyryl cAMP Injection into L5 DRGs. Dibutyryl cAMP at 50mM in 2ml was injected into left L5 DRGs using a glass micropippette over a period of 1 hour. Then 1 week later, dorsal column axons were transected at the T6 spinal cord level. Two weeks after lesion, the tracer cholera toxin HRP (1%) was injected into the sciatic nerve to study the changes in the ascending dorsal column fibers. After another two weeks, the rats were perfused and tissues prepared for immunohistochemistry using the modified techniques of Herzog and Bosamle (1997) and Veenman et al. (1992). Figure (a) shows the low power images through dorsal column lesion. Saline-injected animals were compared to the dbcAMP-injected animals. Figure (b) shows the magnified images of regenerating fibers in mid-lesion site. The upper of the images is in the rostral side, while the bottom of the images is in the caudal side. (Experiments done by Dr. Bregman's lab in Georgetown.)

Discussion

The results presented in this chapter suggest a molecular mechanism underlying the improved CNS axonal regeneration following a peripheral conditioning lesion. It has been shown that transection of the peripheral nerve branch of DRG neurons results in an increase in endogenous levels of cAMP, which are comparable to the endogenous cAMP levels in young neurons. Therefore, high cAMP can neutralize the effect of myelin inhibitors or effectively switch the response of axons to myelin inhibitors from inhibition to promotion, allowing them to grow through white matter. It has also been suggested that the effect of a conditioning lesion on both increased growth capacity and the ability to grow through myelin inhibitors can account for the pattern of regrowth of these dorsal spinal axons in the damaged spinal cord. The application of cAMP directly into the neuronal cell body without any peripheral nerve lesion, mimics the effect of a conditioning lesion on axonal regeneration. Together, this evidence suggests that cAMP plays a major role in improved nerve regeneration in damaged CNS tissue, which implies a possible molecular approach to encouraging CNS axons to regrow after injury.

Collective evidence suggests that the effects of cAMP seems to be mediated by PKA (Ming et al., 1997; Song et al., 1997; Cai et al., 1999), whose substrates include inositol 1,4,5-triphosphate (IP_3) receptors, as well as a number of cytoskeleton-associated proteins. For example, PKA was reported to play a role in regulating the activity of RhoA, a member of the small GTP-binding proteins involved in regulating cytoskeleton. RhoA can be specifically phosphorylated by PKA at Ser-188. This phosphorylation does

not affect the ability of RhoA to bind to guanine nucleotides nor does it modify its intrinsic GTPase activity, but results in a decreased affinity for its downstream effectors, as well as in the translocation of membrane-associated RhoA into the cytosol and thereby terminating RhoA signaling (Lang et al., 1996). More recently, it was reported that inactivation of Rho signaling pathways can overcome the inhibition by MAG/myelin and promote CNS axonal regeneration *in vivo* (Lehmann et al., 1999; Dergham et al., 2000). Additionally, it has been reported that myosin light chain kinase (MLCK) is another target of PKA. Phosphorylation of MLCK by PKA decreases its affinity for calcium and calmodulin and inhibits its activity (Tansey et al., 1994).

There is an abundance of evidence to support the idea that to maintain and complete the successful repair of injured *Aplysia* sensory axons, signals must be retrogradely transported from the injury site to the nucleus of neuronal cell body and thereby elicit changes in transcription (Ambron and Walters, 1996). Results show that the second messenger cAMP regulates a striking number of physiological processes, including metabolism, proliferation and neuronal signaling, by altering the basic pattern of gene expression (Montminy, 1997). For example, in long-term potentiation (LTP) of *Aplysia* sensory neurons, a brief exposure to serotonin is accompanied by elevated cAMP, which keeps the catalytic subunit of PKA dissociated and stimulation of numerous substrates. Surprisingly, there is a persistent phosphorylation of the same proteins for a long time after stimulation, even when cAMP is no longer elevated. Both LTP and the accompanying phosphorylation are dependent on transcription and protein synthesis (Schulman, 1991). Therefore, it is reasonable to speculate that the injury-induced

elevation of cAMP may lead to transcriptional regulation and subsequent protein synthesis, thereby improving the axonal growth capacity after injury. Preliminary studies in our lab have found that addition of a transcription inhibitor into culture can block the improved axonal growth induced by a conditioning lesion in the early stages. Subsequently, the growth is switched to a transcription-independent mechanism (Spencer and Filbin, unpublished data). If the cAMP-induced axonal regeneration does require transcriptional activation in the early stage, one candidate that may be involved in this transcription-dependent regulation is CREB (cAMP-responsive element-binding protein), which binds to the cAMP-responsive-element (CRE) promoter sites on target genes such as immediate-early genes (IEGs) and regulates downstream gene activation.

CREB functions as a stimulus-induced transcriptional activator, which is critical for a variety of cell processes, including differentiation, proliferation and adaptive responses (Shaywitz and Greenberg, 1999). It has also been shown to play a key role in regulation of neuronal survival (Walton and Dragunow, 2000). Phosphorylation of CREB promotes the survival of many cells *in vitro*, while activation of CREB initiates a neuroprotective program *in vivo*. Studies from our lab have found that if a dominant-negative form of CREB is introduced into neurons, the blocking effect of cAMP on the inhibition of axonal growth by MAG is abolished (Gao and Filbin, unpublished data). This suggests that CREB is at least one downstream transcriptional factor stimulated by cAMP in the blocking of inhibition of axonal growth by myelin inhibitors.

If activation of CREB is required for the improved axonal regeneration induced by cAMP, the next step is to find out its target genes and related proteins. One candidate molecule which may result in the improved growth is the growth-associated protein GAP-43, which is an abundant component of axonal growth cones and its expression widely correlates with successful axonal regeneration. It has been shown that expression of GAP-43 in injured neurons is up-regulated after peripheral nerve injury, but remains suppressed in damaged CNS neurons following injury (Skene and Willard, 1981; Skene et al., 1986; Skene, 1989). It has also been found that GAP-43 is up-regulated in spinal axons after a conditioning lesion. However, in transgenic mice overexpressing GAP-43, there is only enhanced sprouting in axon terminals but no improved regeneration in injured axons (Neumann and Woolf, 1999). This suggests that GAP-43 alone may not be sufficient to trigger axon regeneration. There may be other molecules also involved in cAMP-induced axonal regeneration. For example, recent studies show that co-expressing two growth cone proteins, GAP-43 and CAP-23 (cytoskeleton-associated protein), can promote axonal growth of adult DRG neurons both *in vitro* and *in vivo* (Bomze et al., 2001). Despite studies of proteins that may correlate with the regenerative capacity of neurons, it has also been shown that the expression of c-jun, a known immediate early gene target of CREB, is upregulated in response to injury to DRG peripheral axons (Broude et al., 1997; Kenney and Kocsis, 1997).

As shown in the results, after a peripheral branch lesion, the neuronal cAMP levels are elevated to change their growth capacity such that the inhibitors in the CNS environment no longer block the growth of CNS axons. However, whether the cAMP-

induced improvement of axonal regeneration is via the regulation of gene transcription and protein synthesis remains unknown. Preliminary data demonstrate that 1week after a conditioning lesion, the improved growth from injured DRG neurons on MAG/myelin is no longer abolished by a transcriptional inhibitor. This suggests that elevation of cAMP may activate downstream machinery and then up-regulate/down-regulate the expression of its target genes. Subsequently, the regulation of neuronal growth capacity becomes independent of cAMP as well as the transcriptional machinery. Then, it is necessary to determine whether the initial elevation of cAMP induced by a conditioning lesion is responsible for the extensive growth at 1week post-injury or if the improved growth is due to activation of a parallel signaling mechanism other than cAMP pathway.

If elevation of cAMP is the key signaling event in this improved axonal regeneration, blocking the cAMP pathway simultaneous to peripheral nerve transection should be able to abrogate this improved growth at 1week post-lesion. However, results show that a single injection of a PKA inhibitor (H89) into DRG can not block the improved growth on MAG/myelin at 1week post-injury (Figure 5.6). This may be due to the insufficiency of blocking by just one injection of a PKA inhibitor, H89. To successfully block the improved axonal growth at 1week post-injury, multiple injections of H89 or continuously providing DRGs with a PKA inhibitor by administration of a minipump filled with H89 during the 1week time period, may be necessary. The investigation of these issues is currently ongoing in the lab.

In addition, we would like to determine if cAMP is involved *in vivo* in axonal regeneration of injured dorsal column fibers into the white matter following a conditioning lesion. 1 or 2 weeks after application of H89 into L4-5 DRGs and a conditioning lesion in sciatic nerves, dorsal column axons were transected (Fig.5.5-2) to check whether the improved axonal regeneration induced by a conditioning lesion can be blocked by inhibiting the cAMP downstream effector, PKA, *in vivo*. These experiments are currently under investigation by the lab of our collaborator, Dr. Bregman at Gerogetown University.

Finally, it is not practical to apply a conditioning peripheral nerve injury to encourage growth of spinal axons. However, administration of a cAMP analog directly into the neuronal cell body can improve axonal growth of DRG neurons in culture as well as *in vivo*, which suggests a molecular mechanism to promote regrowth of injured axons. So the next questions to be addressed are: 1) How long must neurons be exposed to an elevated cAMP condition in order to obtain a more extensive regeneration than that observed following a single injection? 2) What are the downstream effector molecules in the cAMP-induced axonal regeneration? 3) Is this regulation by cAMP dependent on gene transcription and protein synthesis? 4) Can cAMP be administered at the same time as the spinal cord injury? 5) After axons grow across the lesion site, how can they grow back to their original destination? Answers to these questions would lead to a rational approach to encouraging damaged CNS axons to regrow *in vivo*.

Chapter VI

Up-regulation of Arginase I in Polyamine Biosynthesis Pathway is Involved in Cyclic AMP-Induced Axonal Regeneration on MAG/Myelin

Introduction

We have shown that priming neurons with neurotrophins elevates the cytosolic cAMP levels and activates PKA, which subsequently blocks the inhibition of axonal outgrowth by MAG and myelin (Cai et al., 1999). Therefore, it is believed that the intrinsic state (e.g. the intracellular cAMP levels) of neurons is altered by priming with neurotrophins, which affects the neuronal growth response to myelin inhibitors and so neurons fail to interpret signals in myelin as inhibitory (Qiu et al., 2000). Consistent with the view that the neuronal response to myelin inhibitors is dictated by the endogenous levels of cAMP, we have shown that the switch from promotion to inhibition by MAG/myelin during development is mediated by a developmentally regulated decrease in endogenous neuronal cAMP levels (Cai et al., 2001). In addition, it was found that elevation of cAMP following a conditioning lesion of the sciatic nerve can neutralize the inhibitory effect of MAG/myelin or switch the neuronal response to myelin inhibitors from inhibition to promotion, thereby allowing them to regrow in culture and regenerate *in vivo* (Qiu et al., 2001).

Therefore, the activity of a cAMP pathway may play an important role in the regulation of axonal regeneration. However, the downstream effectors induced by elevation of cAMP that are directly responsible for the improved neuronal outgrowth on MAG and myelin still remain unknown. One candidate may be polyamines. Previously, it was found that the polyamines, putrescine, spermidine and spermine, are ubiquitously distributed in eukaryotic tissues, and are abundant in the mammalian nervous system

(Shaskan and Snyder, 1973; Shaw and Pateman, 1973). Importantly, it has been shown that in the nervous system, polyamines promote neurite regeneration of injured axons of cultured rat hippocampal neurons (Chu et al., 1995). In addition, exogenously providing polyamines can accelerate axonal regeneration and enhance functional recovery of rat injured sciatic and facial nerves (Dornay et al., 1986; Kauppila et al., 1988; Kauppila, 1992). It has also been reported that polyamines enhance survival of sympathetic neurons after axonal injury (Gilad and Gilad, 1988). In addition, polyamines have been shown to be important in controlling the development of nervous tissue (Slotkin and Bartolome, 1986). For example, it is found that polyamines are necessary for axonogenesis and synaptogenesis in neonatal rat nervous system (Slotkin et al., 1982). Most interestingly, it has been demonstrated that the biosynthesis of polyamines is enhanced in neurons treated with certain substances such as cAMP (Morris and Slotkin, 1985) and nerve growth factor (MacDonnell et al., 1977). Therefore, it is reasonable to suggest that polyamines may be the downstream molecules in the cAMP pathway responsible for the regulation of neuronal regenerative capacity.

There are three enzymes, arginase (Arg), ornithine decarboxylase (ODC) and s-adenosylmethonine (SAM) decarboxylase, which are the rate limiting enzymes for polyamine production (see figure 6.1). Interestingly, it has also been demonstrated that the expression of Arg and ODC can be induced by cAMP (Gotoh et al., 1996; Morris et al., 1998; Wang et al., 1995) in macrophages as well as other tissues (Wu et al., 1996; Flynn and Wu, 1997). So, the questions asked in this chapter are: 1) Does cAMP induce the activation or up-regulation of arginase and ODC expression levels in the nervous

system as it does in other tissues? 2) Is the up-regulation of arginase and subsequent elevation of polyamine biosynthesis important in cAMP-induced improvement of axonal outgrowth on MAG/myelin? 3) Do the endogenous levels of ArgI correlate with the neuronal regenerative capacity during development?

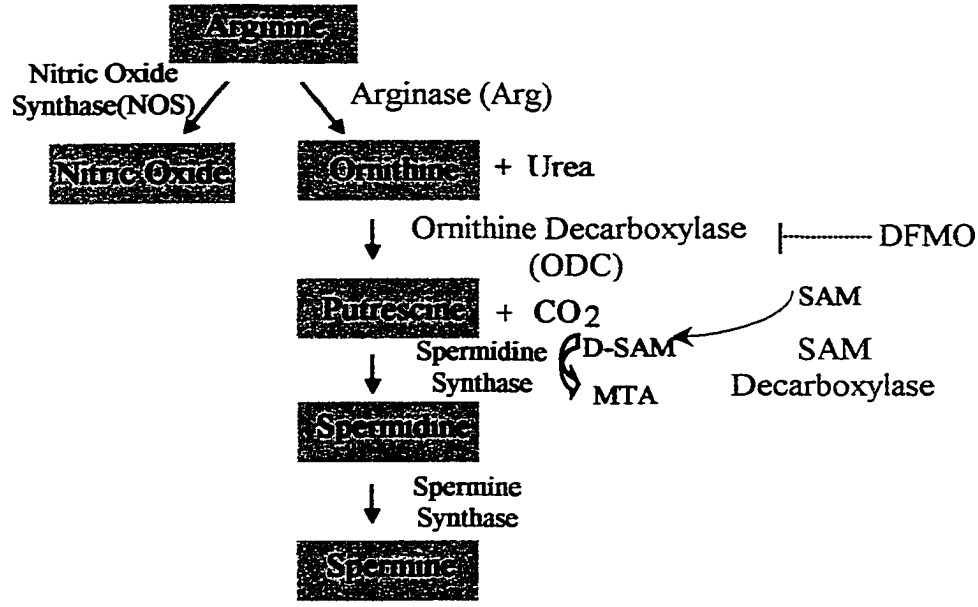


Fig.6.1. Biosynthesis of the Polyamines. (Slotkin and Bartolome, 1986)

Results

The cAMP-induced Axonal Growth on MAG/Myelin is transcription-dependent

First, we wanted to determine if the cAMP-induced block of inhibition of axonal outgrowth by MAG/myelin is transcription-dependent. A transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at 5μM was included in neuronal cultures in the presence of dbcAMP at 1mM. Alternatively, neurons were plated onto poly-L-lysine and cultured overnight with BDNF at 200ng/ml in the presence or absence of DRB, before being transferred to a monolayer of MAG-expressing or control CHO cells.

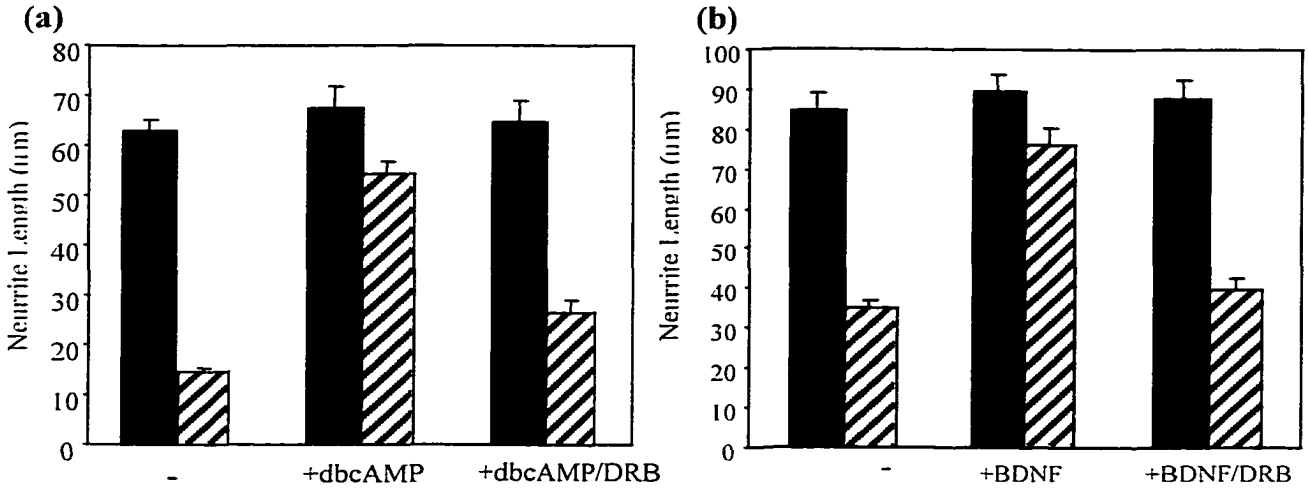


Fig.6.2. The Improved Axonal Growth on MAG Induced by Priming Neurons with BDNF or Culturing with dbcAMP is Transcription-dependent. (a) Isolated cerebellar neurons were plated at 20,000 neurons per well onto a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (black bars) and cultured overnight before being fixed and imuunostained for GAP43. The length of the longest neurite per neuron from 180-200 neurons was measured and results are the average length±SEM. Where indicated, dbcAMP at 1mM in the presence or absence of DRB at 5μM was added to the culture. (b) $1x10^6$ cerebellar neurons were plated onto poly-L-lysine and cultured overnight with BDNF at 200ng/ml, in the presence or absence of DRB at 5μM, before being transferred to the CHO cell monlayers.

Figure 6.2 shows that, as before (Cai et al., 1999), either addition of dbcAMP (a) directly to the neurons growing on the MAG-expressing CHO cells or prior exposure of the neurons to BDNF (b), blocks the inhibition by MAG. However, if an inhibitor of transcription, DRB, is included in the cultures, neither dbcAMP nor BDNF blocks the inhibition; MAG still inhibits axonal outgrowth. Therefore, the ability of both dbcAMP and BDNF to block inhibition by MAG is dependent on transcription. Certain proteins must be synthesized for the dbcAMP and BDNF block of inhibition to occur.

Furthermore, when similar experiments were carried out using purified CNS myelin as a substrate rather than MAG-expressing CHO cells, the same results were obtained. The presence of a transcriptional inhibitor completely abrogates the block of myelin's inhibition by addition of dbcAMP directly into culture or priming with BDNF. Results

Results are shown in figure 6.3 a and b. Therefore, dbcAMP and BDNF-induced reversal of inhibition by myelin in general is transcription-dependent.

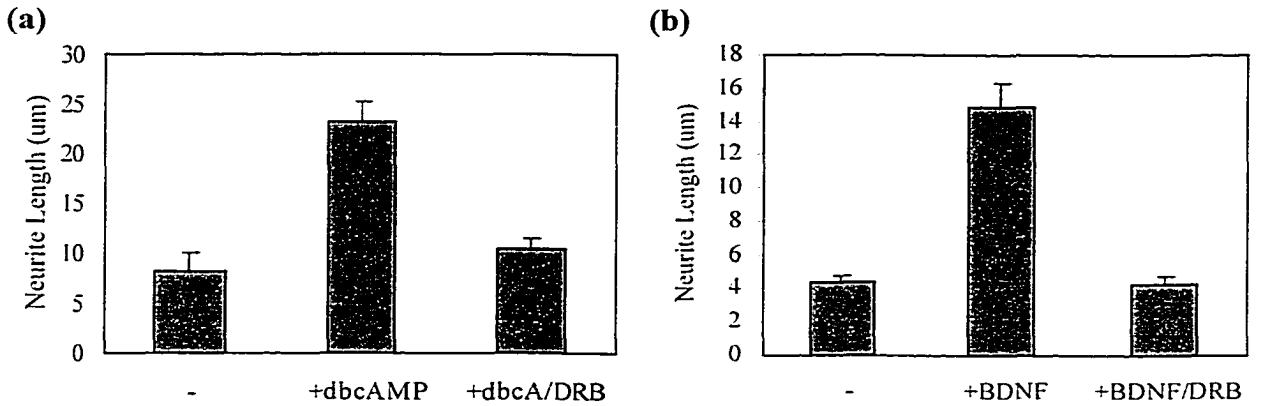


Figure 6.3 The Improved Axonal Growth on Myelin Induced by Culturing with dbcAMP or Priming Neurons with BDNF is Transcription-dependent. (a) Isolated cerebellar neurons were plated at 20,000 neurons per well onto a substrate of purified CNS myelin and cultured overnight before being fixed and imuunostained for GAP43. The length of the longest neurite per neuron from 180-200 neurons was measured and results are the average length ±SEM. Where indicated, dbcAMP at 1mM in the presence or absence of DRB at 5μM was added to the culture. (b) $1x10^6$ cerebellar neurons were plated onto poly-L-lysine and cultured overnight with BDNF at 200ng/ml, in the presence or absence of DRB at 5μM, before being transferred to the CNS myelin substrate.

ArginaseI is Up-regulated by Elevation of cAMP

The next step is to determine which target genes are up-regulated by elevated cAMP following treatment with dbcAMP or priming with BDNF. As mentioned before, it has been shown that polyamines can induce injured axons to regrow (Dornay et al., 1986; Wong and Mattox, 1991; Chu et al., 1995; Gilad et al., 1996). If the synthesis of polyamines is important in the cAMP-induced block of inhibition by MAG/myelin, it is quite likely that the expression levels of the rate-limiting enzymes in polyamine biosynthesis are coordinately up-regulated or the enzymes' activities are increased. The biosynthesis enzymes, arginase (Arg), ornithine decarboxylase (ODC) and s-adenosylmethionine decarboxylase (SAM), are three rate-limiting enzymes for polyamine production (see Fig.6.1). To determine if there is an up-regulation of arginase expression

following elevation of cAMP, cerebellar neurons were exposed to either BDNF at 200ng/ml or dbcAMP at 1mM overnight before being lysed, and either RNA or protein was extracted. The extracted RNA was reverse-transcribed and then subjected to semi-quantitative PCR (RT-PCR), using primers specific for the enzyme arginase I, an isoform of arginase abundant in liver tissue but barely detected in other tissues. Figure 6.4 shows the result of RT-PCR. It demonstrates that there is detectable ArgI in cerebellar neurons, although not as abundant as in liver. After exposure of these neurons to either BDNF (lane 2) or dbcAMP (lane 3), a two-fold increase of ArgI RNA levels is apparent (Fig.6.4).

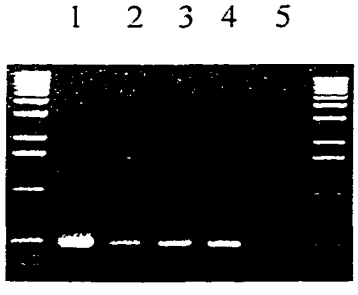


Fig.6.4. Priming Neurons with BDNF or dbcAMP Results in an Up-regulation of Arginase I in RNA Levels. RNA, isolated from cerebellar neurons without treatment (lane 2), from cerebellar neurons exposed to either BDNF (lane 3), or dbcAMP (lane 4), was reverse transcribed and then subjected to semi-quantitative PCR, using primers specifc for arginase I. As a positive control, RNA isolated from liver tissue was RT-PCR by using the same primers (Lane 1). Lane 5 is a blank control to check whether there is sample contamination.

Furthermore, the expression of ArgI in cerebellar neurons was assessed at the protein level. After exposure of cerebellar neurons to either dbcAMP or BDNF for various times, neurons were lysed and the extracted proteins were subjected to SDS-PAGE before being transferred to membrane and immunostained with a polyclonal antibody against arginaseI (The antibodies were kindly provided by Dr. Ratan from Harvard Medical School). As can be seen in figure 6.5, in the absence of either BDNF or dbcAMP, ArgI is barely detectable in cerebellar neurons (arrows). After 1hour exposure of cerebellar neurons to either dbcAMP (a) or BDNF (b), the amount of ArgI proteins has increased at least two-fold. This increase peaks around 3 hours post-treatment, and is sustained for 21 hours. By 24 hours after either treatment, the levels of ArgI proteins begin to decline. Interestingly,

there is a doublet bands of protein (Fig.6.5) detected in cerebellar neurons at the predicted molecular weight, 36Kda for ArgI, whereas usually only one protein band is detected in liver (Esch et al., 1998) using the same antibody against ArgI. It is possible that there is more than one isoform of ArgI in the nervous system. Alternatively, ArgI in cerebelluar neurons may have a phosphorylated form. It should be noted that as shown in the western blot, there is another protein detected by this polyclonal antibody against ArgI at around 60Kda. The identity of this protein is not known. However, there is no difference in the expression level of this protein with or without treatment of BDNF or dbcAMP, which is a perfect internal loading control for ArgI.

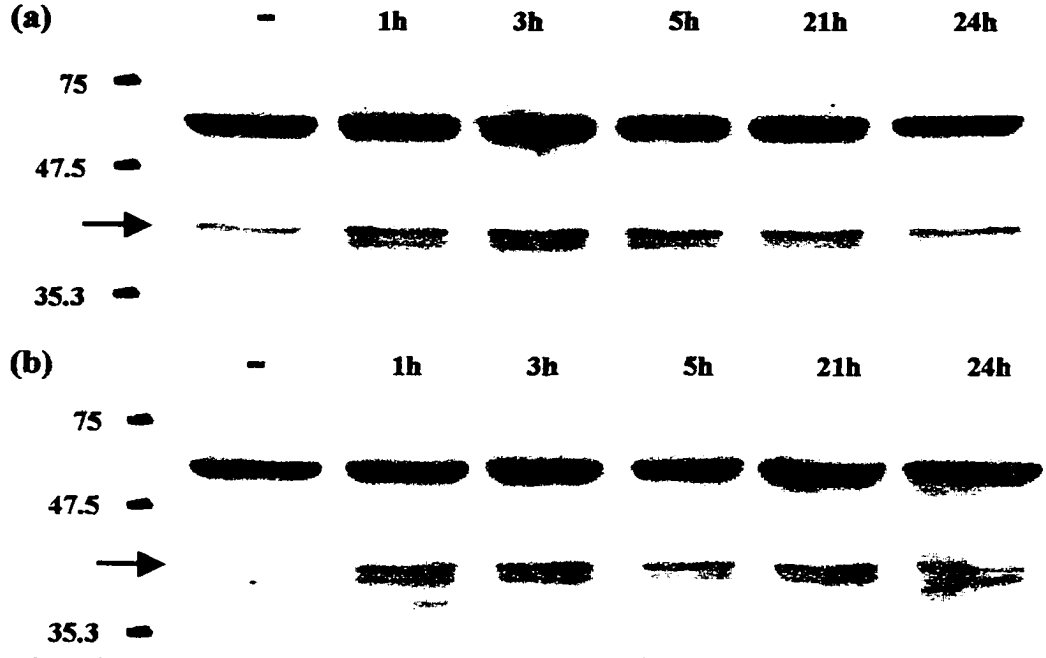


Fig.6.5. The Detection of ArgI in Cerebellar Neurons after Treating with dbcAMP or BDNF for Various Time Periods. The cerebellar neurons were exposed to either dbcAMP at 1mM (a) or BNDF at 200ng/ml (b) for 0, 1hr, 3hr, 5hr, 21hr, or 24hr, and then lysed in the presence of protease inhibitors. The 23μg of total proteins were subjected to 12% SDS-PAGE before being transferred to nitrocellulose membrane and immunostained with antibody to ArgI.

Therefore, these results suggest that exposure of cerebellar neurons to either dbcAMP or BDNF, each of which increase cAMP levels, results in an up-regulation of the enzyme ArgI at both the RNA and the protein level.

Next, we wanted to determine if the up-regulation of ArgI induced by BDNF or dbcAMP is important in the cAMP-mediated block of inhibition by MAG/myelin. An irreversible inhibitor of the enzyme ornithine decarboxylase (ODC), DL-α-difluoromethylornithine (DFMO), was included with BDNF or dbcAMP to assess if the cAMP-induced block of inhibition can be abrogated if one step downstream from arginase in the biosynthesis of polyamine is blocked (see Fig.6.1). As shown in figure 6.6, addition of dbcAMP blocks the inhibition by MAG and myelin as before. However, when the ODC inhibitor, DFMO is included, dbcAMP has no effect on the inhibition by MAG/myelin; MAG and myelin each still inhibit axonal outgrowth. DFMO alone has no effect on inhibition by MAG/myelin, nor on neurite growth on control CHO cells.

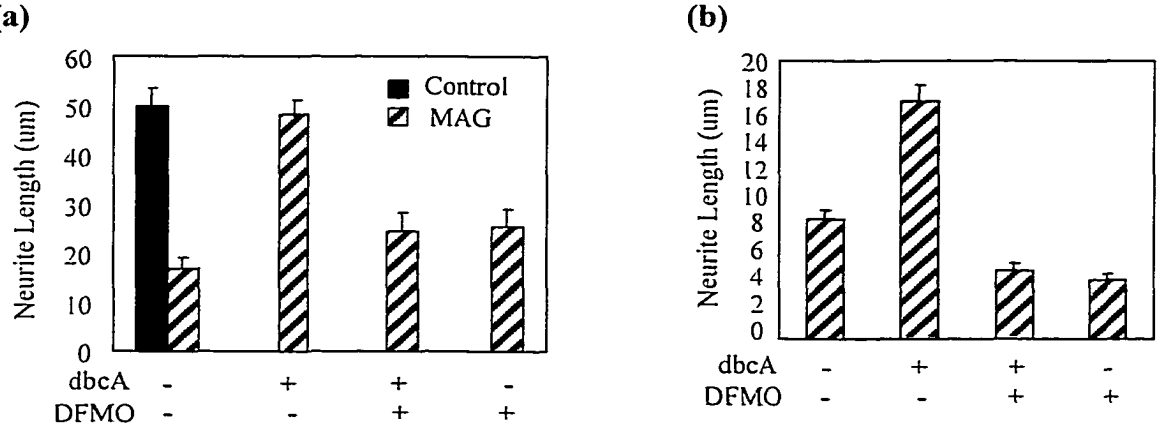


Figure 6.6 The ODC Inhibitor, DFMO, Abrogates the Blocking Effect of dbcAMP on the Inhibition of Axonal Outgrowth by MAG and Myelin. Cerebellar neurons were plated onto either MAG-expressing CHO (striped bars) or control CHO (black bars) cells (a) or a substrate of purified CNS myelin (b), in the presence or absence of dbcAMP at 1mM and/or DFMO (1mM), and cultured overnight before being fixed and immunostained for GAP43. Then the longest neurite from at least 180-200 neurons was measured. Results are the mean neurite length ±SEM.

In addition, DFMO was included while priming cerebellar neurons with BDNF at 200ng/ml in the overnight cultures, with or without putrescine at 10μM. Neurons were then transferred to either MAG-expressing or control CHO cells or a substrate of purified CNS myelin. Figure 6.7 shows that the presence of DFMO during priming completely

abrogates the blocking effect of BDNF on the inhibition by MAG (a) and myelin (b). That is to say, when DFMO was added with BDNF, there was no block of inhibition. However, when putrescine was added at 10 μM along with DFMO, BDNF still blocked the inhibition of axonal growth by MAG/myelin. Therefore, in the presence of putrescine, the blocking effect of BDNF on inhibition by MAG/myelin, which is eliminated by an ODC inhibitor, DFMO, can be restored. Next, we wanted to determine if priming with putrescine alone has a similar blocking effect as BDNF on inhibition by MAG/myelin.

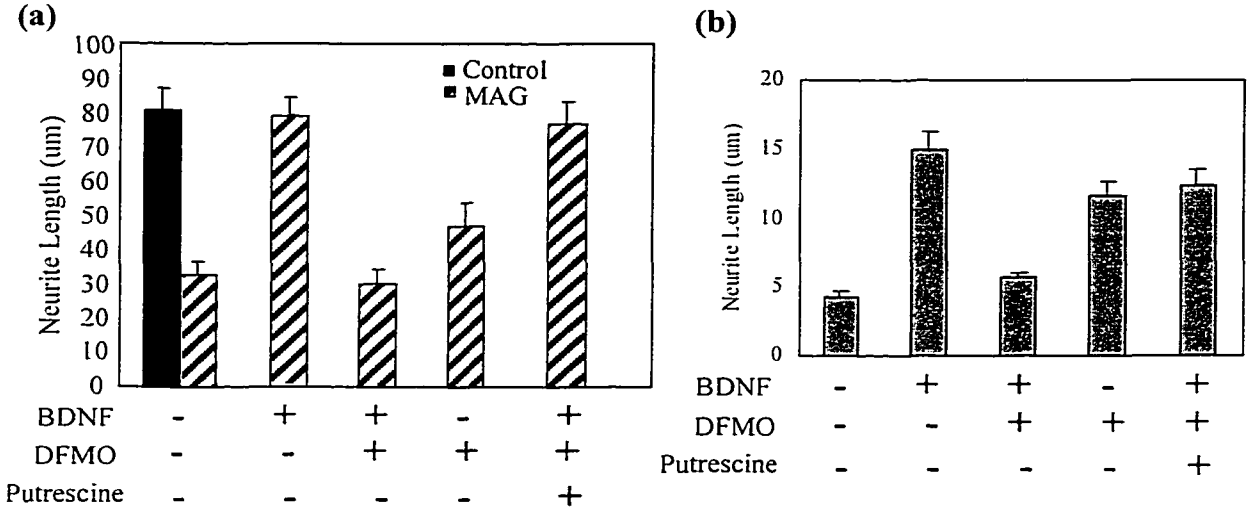


Fig.6.7 The Inhibitor of ODC Blocks the Priming Effects of BDNF on the Inhibition by MAG/Myelin and Putrescine Restores it. Cerebellar neurons were primed overnight with or without BDNF (200 ng/ml), in the presence or absence of DFMO (1mM), with or without putrescine at 10 μM, before being transferred to (a) either MAG-expressing CHO (striped bars) or control CHO (black bars) or (b) a substrate of purified CNS myelin. After further incubation, neurons were fixed and immunostained for GAP43. The longest neurite from each neuron for 180-200 neurons were measured and results are shown as the mean length ±SEM.

To determine whether putrescine alone has a blocking effect on the inhibition by MAG/myelin, neurons were primed with putrescine at different concentrations. Figure 6.8a shows that priming neurons with putrescine in a concentration range of between 10 and 25μM is sufficient to block the inhibitory effect of MAG on axonal outgrowth. At doses higher than 25μM, the blocking effect of putrescine on MAG saturates. On CNS myelin, priming neurons with putrescine at 10μM shows at least a two-fold increase in

axonal outgrowth, which is similar to the growth from neurons primed with BDNF at 200ng/ml (Fig.6.8b). If priming with putrescine at a concentration higher than 10μM, the axonal growth on myelin increases even more dramatically. The blocking effect on myelin by priming with putrescine saturates at the concentration of 50μM. These results are presented as the percentage of control, where 100% is the average neurite length of neurons primed with Sato media only and subsequently grown on control CHO cells (Fig. 6.8a) or on CNS myelin substrate (Fig. 6.8b).

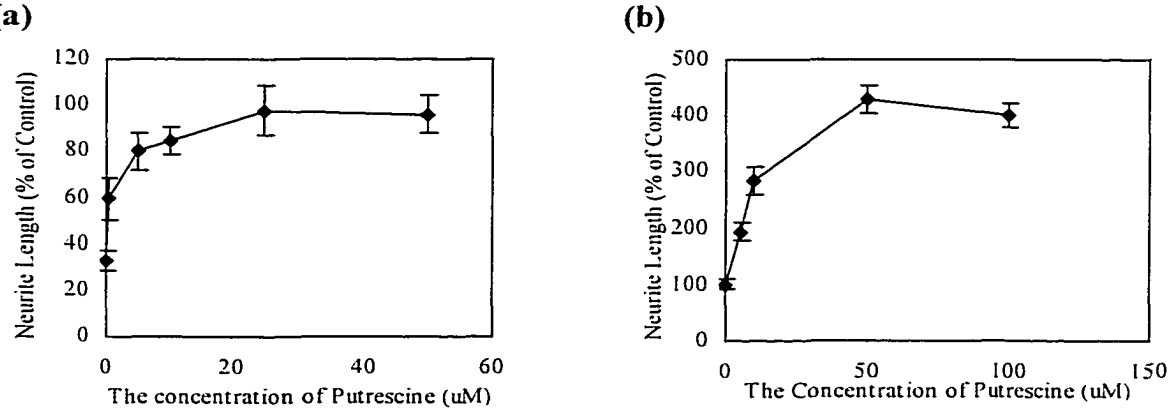


Figure 6.8 Priming Neurons with Different Concentrations of Putrescine and Subsequently Grown on MAG and Myelin. Cerebellar neurons were primed overnight with putrescine at different concentrations from 0-100μM. Then primed neurons were transferred to either MAG-expressing or control CHO cells (a) or a substrate of CNS myelin (b) for further incubation. After that, neurons were fixed and immunostained for GAP-43. The longest neurite from each neuron was measured for 180-200 neurons. Results are shown as the percentage of control. (a) 100% is the average neurite length of neurons primed without putrescine and subsequently grown on control CHO cells. (b) 100% is the average neurite length of neurons primed without putrescine and subsequently grown on myelin substrate.

Furthermore, to determine whether it is necessary to prime neurons with putrescince in order to completely block inhibition by MAG and myelin, cerebellar neurons were cultured with different concentrations of putrescine when grown directly on MAG-expressing CHO cells or a CNS myelin substrate. It is found that addition of putrescine without priming only partially blocks the inhibition of axonal growth by MAG/myelin. In the concentration range of between 15μM and 100μM, putrescine's blocking effect on the inhibition by MAG/myelin reaches a plateau at about 64% reversal.

So far, our data suggest that in the cAMP-mediated axonal regeneration on MAG/myelin, elevation of cAMP induces the up-regulation of arginase I at both the transcriptional and translational levels. In addition, it is found that if the enzyme ODC, downstream from ArgI in polyamine biosynthesis, is blocked by DFMO, the improved axonal growth on MAG/myelin induced by priming with BDNF or by treating with dbcAMP is completely abrogated. Furthermore, in the presence of putrescine at 10μM with BDNF during priming, the abolished BDNF's blocking effect by DFMO is restored. Priming with putrescine alone is sufficient to block the inhibition by MAG/myelin completely, and this blocking effect is dose-dependent.

Up-regulation of ArginaseI Blocks the Inhibition of Neurite Outgrowth by MAG

The next step was to determine whether the up-regulation of arginase I is sufficient to overcome the inhibition of axonal growth by MAG. Cerebellar neurons were infected with adenoviral vector encoding ArgI and green fluorescent protein (GFP) cDNAs. Controls are neurons infected with adenoviral vector encoding GFP cDNAs only. Therefore, those adenovirus-infected neurons were expressing the green fluorescent protein (GFP), which is a marker to distinguish positively infected cells from the others. After 1 hour of infection, neurons were incubated with Sato media for another 24-48 hrs in order for the neurons to recover. Neurons were then transferred to MAG-expressing or control CHO cells for neurite outgrowth before being immunostained. Since the virus infects glial cells as well as neurons, we identified GFP-positive neurons by immunostaining for GAP-43.

Fig.6.9a shows a neuron that is positively immunostained for GFP and simultaneously, for GAP-43. Figure 6.9b is a SDS-PAGE immunoblotted with an ArgI antibody to check the expression levels of ArgI after adenovirus infection. After infection, there is an abundant expression of adenoviral-mediated ArgI in cerebellar neurons at 37Kda, whereas none is detected in GFP-adenovirus infected cells. However, the endogenous levels of ArgI are undetectable in GFP-adenovirus infected neurons (Fig.6.9b the left lane). It may be that the total proteins loaded here are very low (5μg) if compared to the amount (23μg) at which we can detect the endogenous ArgI in cerebellar neurons.

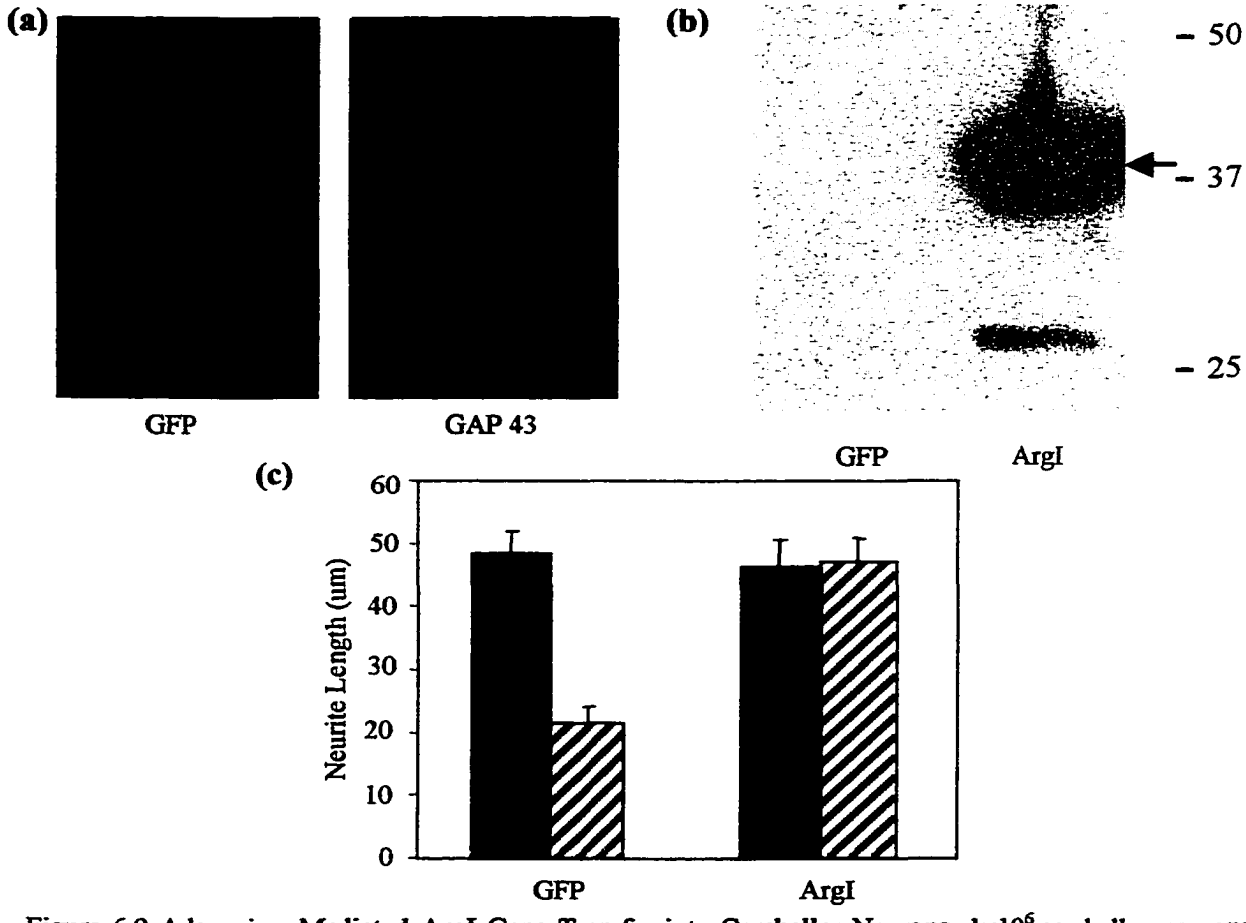


Figure 6.9 Adenovirus-Mediated ArgI Gene Transfer into Cerebellar Neurons. $1x10^6$ cerebellar neurons were plated into a 24-well plate and infected with adenovirus containing ArgI-GFP or GFP alone. After overnight culture, infected neurons were transferred to MAG-expressing CHO (stippled bars) or control CHO (black bars) cells for neurite outgrowth. Then neurons were fixed and immunostained for GAP-43. The neurons with double staining of GAP-43 and GFP are considered as positively infected ones, and the

length of the longest neurite per neuron was measured for 120-150 neurons. Figure (a) shows the ArgI-positive infected neurons are double-stained with α-GAP43 and α-GFP. (b) The Western blot of 5μg of protein lysates from neurons infected with ArgI-adenovirus and GFP only. (c) Results are shown as the average neurite length ±SEM of GFP-infected or ArgI-infected neurons grown on MAG.

Furthermore, we compared the axonal outgrowth ability of ArgI-adenovirus infected neurons to the growth of controls (neurons infected with GFP-adenovirus) on MAG-expressing or control CHO cells. As shown in figure 6.9c, axonal outgrowth from cerebellar neurons expressing the adenoviral-ArgI cDNAs are not inhibited by MAG. Therefore, the expression of arginase I is sufficient to overcome the inhibition of axonal outgrowth by MAG. This block of inhibition induced by ArgI gene transfer is specific because control-infected neurons (GFP-adenovirus) are still inhibited by MAG.

So far, we have shown that elevation of cAMP induces the up-regulation of arginaseI. Also, that the over-expression of ArgI alone is sufficient to overcome the inhibition by MAG. In the previous chapters, we showed that the endogenous cAMP levels correlate with the neuronal regenerative capacity during development and after injury. Therefore, it is reasonable to suggest that the endogenous expression levels of ArgI in neurons may also correlate with the neuronal regenerative capacity. DRG neurons from different postnatal days (between P0 and P7) were isolated and total proteins were extracted. The endogenous ArgI level at different postnatal ages was estimated by western blot.

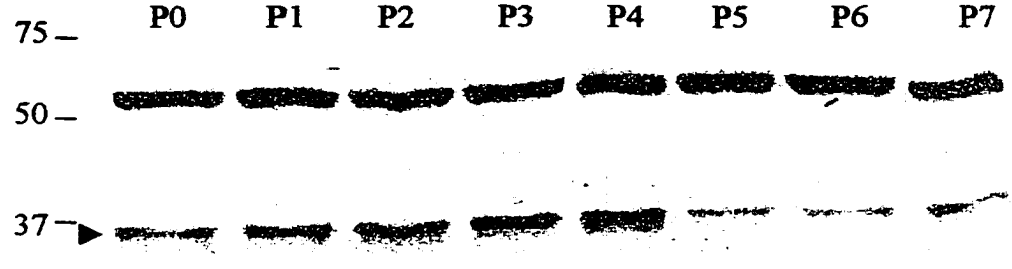


Figure 6.10 The Expression Level of Arginase I in DRG Neurons from Different Developmental Ages. DRG neurons from P0-P7 were isolated and lysed with RIPA buffer. The 32μg of total proteins were

subjected to 12% SDS-PAGE before being transferred to nitrocellulose membrane and immunostained with antibody against ArgI.

It was found that the ArgI protein levels in DRG neurons at P0-P4 (Fig.6.10) are more abundant compared to the level in postnatal cerebellar neurons (Fig.6.5). However, there is a sharp decrease in ArgI protein levels in DRG neurons at P4/5 and this low level persists to later postnatal ages. The studies in the previous chapters showed that changes in endogenous cAMP level coincide with this developmental switch in response to MAG/myelin. This change in cAMP level dictates the neuronal switch of response to MAG/myelin. Now, our data suggest that endogenous ArgI expression levels in DRG neurons have a similar trend of decrease as does cAMP during development. The next question is if different ArgI levels in DRG neurons from different ages affect their growth ability on MAG.

It is known that DRG neurons switch their axonal regenerative response to MAG and myelin from promotion to inhibition during development. This transition happens sharply at postnatal day 3-4 (DeBellard et al., 1996). First, to determine if the promotion of axonal growth from P0-1 DRG neurons by MAG can be blocked if a step downstream from arginase in the synthesis of polyamine is blocked. P0/1 DRG neurons were isolated and cultured on MAG-expressing CHO cells in the presence of an irreversible ODC inhibitor, DFMO. The results are shown in figure 6.11. It was found that in the presence of the ODC inhibitor DFMO, the promotion of P0-1 DRG neurons by MAG is completely abolished. The P0-1 DRG neurons are even slightly inhibited by MAG. In addition, DFMO alone has no effect on inhibition by MAG, nor on neurite growth on

control CHO cells. Therefore, in young DRG neurons, the block of a step downstream from arginase can switch the neuronal response to MAG from promotion to inhibition.

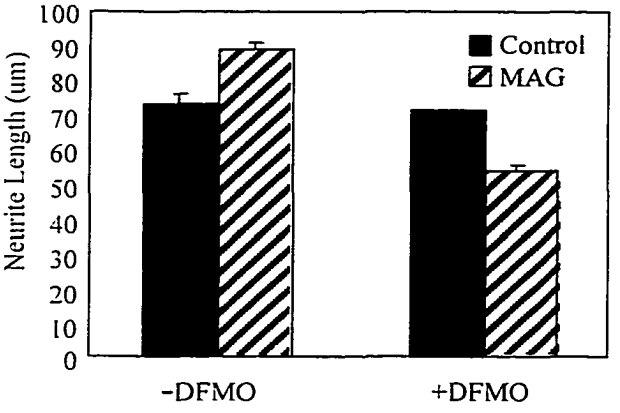


Figure 6.11 Neurite Outgrowth from P1 DRG Neurons in the Presence of an ODC Inhibitor, DFMO. P1 DRG neurons were isolated and plated on MAG-expressing (stippled bars) or control (black bars) CHO cells, with or without DFMO at 1mM. After overnight incubation, neurons were fixed and immunostained for GAP-43. The longest neurite per neuron was measured and the results were shown as the average neurite length ±SEM.

Next, we wanted to determine if an increase in the expression level of ArgI in older DRG neurons is sufficient to block MAG's inhibitory effect on axonal growth from these neurons. The experiment of adenoviral vector-mediated ArgI gene transfer was carried out in older DRG neurons (PND7). After infection and overnight culture to allow expression, neurons were transferred to either MAG-expressing or control CHO cells. Figure 6.12 shows that if exogenously providing DRG neurons with the ArgI cDNAs, neurons are no longer inhibited by MAG. The neuronal outgrowth capacity on MAG is significantly improved by over-expressing ArgI.

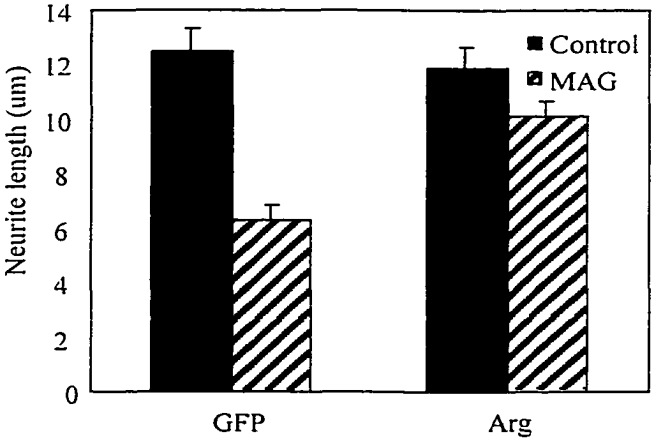


Figure 6.12 Adenovirus-Mediated ArgI Gene Infection in P7 DRG Neurons Subsequently Grown on MAG. $0.5x10^6$ postnatal day 5 DRG neurons were plated in 24-well plate and infected with adenovirus containing ArgI-GFP or GFP alone. After overnight culture, infected neurons were transferred to MAG-expressing

CHO (stippled bars) or control CHO (black bars) cells for neurite outgrowth. Then neurons were fixed and immunostained for GAP-43. The neurons with double staining of GAP-43 and GFP are considered as positively infected ones, and the length of the longest neurite per neuron was measured for 120-150 neurons. Results are shown as the average neurite length ±SEM of GFP-infected or ArgI-infected neurons grown on MAG.

Together, these results suggest that the expression levels of ArgI in neurons correlate with the axonal outgrowth capacity in response to MAG. In young neurons, endogenous cAMP and ArgI levels are high, axonal growth from these neurons is promoted by MAG. However during development, there is a decrease in the ArgI expression levels in neurons, which coincide with the changes in endogenous cAMP levels, and subsequently neurons at this stage are inhibited by MAG. Therefore, the changes in cAMP levels, as well as ArgI expression levels of neurons, dictate the developmental switch of neuronal responses to MAG.

Discussion

The results in this chapter suggest that the improved axonal regeneration on MAG/myelin induced by priming with neurotrophins or treating with dbcAMP is transcription-dependent. One downstream signaling event in this cAMP-induced axonal regeneration is the up-regulation of arginase I, which, in turn, activates the pathway for the biosynthesis of polyamines and initiates the neuronal regenerative machinery. Blocking one step in the pathway of polyamine production, synthesis of putrescine from ornithine, with an inhibitor of ODC, abrogates cAMP-induced reversal of inhibition by MAG and myelin completely. These results indicate that one mechanism whereby cAMP blocks inhibition of axonal regeneration by MAG/myelin is through up-regulation of arginase I and subsequent elevation of polyamine synthesis. A model is proposed to

explain the possible signal transduction mechanism involved in the cAMP-induced axonal outgrowth on MAG/myelin (see fig. 6.13).

As shown in figure 6.13, as we proposed before, priming neurons with neurotrophins (NT) elevates neuronal cAMP levels and thereby activates the downstream effector, PKA. Furthermore, the data presented in this chapter suggest that activation of PKA may subsequently turn on certain transcriptional/translational machinery and thereby targeted genes are transcribed and translated. The synthesis of one target protein that is up-regulated through this cAMP-induced regulatory mechanism appears to be arginase I (ArgI). Subsequently, the up-regulation of the ArgI expression level by cAMP can increase the polyamine biosynthesis and thereby improve neurite outgrowth ability. Recent studies in the lab have also demonstrated that the activation of Trk receptors during priming with neurotrophins is mainly responsible for the improved axonal outgrowth on MAG/myelin. In addition, we found that phosphorylation of MAP kinase (MAPK) and hence down-regulation of phosphodiesterase (PDE) are involved in this neurotrophin-induced elevation of cAMP (Gao and Filbin, unpublished data). Preliminary data also suggests that CREB is likely to be involved in the mechanism of cAMP-induced transcriptional/translational regulation (Gao and Filbin, unpublished data).

Interestingly, it has already been shown that expression of ArgI, ODC and SAM are induced by cAMP analogs through PKA activation in macrophages (Gotoh et al., 1996; Morris et al., 1998; Wang et al., 1995; Corraliza et al., 1997) as well as in other tissues (Wu et al., 1996; Flynn and Wu, 1997). In addition, it has been found that one

consequence of the interaction of the neurotrophin NGF with its target cells such as PC12 cells is an induction of ODC (Guroff and Dickens, 1983). However, it is the first time, to our knowledge, that a liver-specific enzyme ArgI has been shown to be up-regulated in neurons by treating with neurotrophin BDNF or the cAMP analog dbcAMP. ArgI is a cytosolic isoform of arginase that is normally expressed in hepatocytes, whereas ArgII is mitochondrial and expressed at low levels in many cell types. The different subcellular localization of the two isoforms is thought to play a role in determining whether ornithine becomes converted to polyamines or to glutamate and proline (Kepka-Lenhart et al., 2000). Consistent with this view, we showed that the endogenous levels of ArgI in postnatal cerebellum and older DRG neurons are low even though the ArgI levels in young DRG neurons are quite abundant. Nevertheless, elevation of cAMP in these neurons (postnatal cerebellum and older DRG neurons) can increase ArgI's expression level tremendously. Furthermore, artificially up-regulating the ArgI expression level in neurons through exogenous arginase I gene transfer with an adenoviral vector can block the inhibition of axonal growth by MAG completely. Therefore, because it is known that if inhibitors of regeneration present in myelin are blocked immediately after injury, regeneration can occur (Filbin, 2000), artificially up-regulating arginase I in neurons after injury *in vivo* is likely to allow regeneration to occur.

Moreover, our results suggest that endogenous arginase I expression levels may control the neuronal regenerative capacity during development. It is shown that the endogenous levels of ArgI differ from neurons at different developmental stages, which in DRG neurons are high in early postnatal ages then decline to low levels at later

postnatal stages. The changes in ArgI expression levels are likely to be the consequences of a decrease in cAMP levels during development (results shown in chapter IV), which are subsequently responsible for the neuronal switch of response to MAG/myelin from promotion to inhibition. Others have shown that the ODC/polyamine system controls the development of nervous tissue. In the developing brain, neurons are required to maintain higher levels of ODC as well as polyamines compared to cells in the adult. Interfering with the polyamine pathway by treating with DFMO, impairs developmental processes in neurons such as migration, axonogenesis and synaptogenesis, as well as cytoarchitechtual organization of brain tissues (Slotkin and Bartolome, 1986). Together, these findings suggest that cAMP-ArgI/ODC-dependent mechanisms play an important role in regulating the development of the nervous system as well as axonal outgrowth after injury.

The regulation of the expression of ArgI, ODC and SAM (three rate-limiting enzymes in polyamine production) in neurons by cAMP may be achieved by transcriptional, translational and/or post-translational mechanisms. It should be noted that there are cAMP-responsive element (CRE) sites in the promoters for ODC and SAM. Therefore, cAMP-responsive molecules or cAMP-related kinases/phosphotases may activate a putative transcription factor like CREB or a collection of transcription factors, which coordinately up-regulate the expression of these enzyme genes and thus increase polyamine synthesis. Additionally, the regulation may be translational, by controlling the process of the translation of ArgI and ODC messenger RNA. For example, it is known that the translational initiation factor eif4 is involved in the translational regulation of the

ODC message (Veress et al., 2000; Shantz and Pegg, 1999; Shantz et al., 1996b; Shantz et al., 1996a). Alternatively, cAMP elevation may induce a post-translational regulation by activation of some proteasome inhibitors, which may increase the half-life of these enzymes and potentially elevate polyamine synthesis.

No matter what regulatory mechanism is involved, up-regulation of arginase eventually induces polyamine synthesis. It has been shown that polyamines are highly concentrated in the mammalian nervous system (Shaskan et al., 1973; Shaskan and Synder, 1973; Shaw and Pateman, 1973) and exert regeneration-promoting effects on cultured rat hippocampal (Abe et al., 1997; Chu et al., 1995) and sympathetic neurons (Dornay et al., 1986). *In vivo* studies also show that polyamines can enhance functional recovery after facial nerve injury (Wong and Mattox, 1991; Gilad et al., 1996) as well as sciatic nerve trauma (Kauppila et al., 1988; Kauppila, 1992). Furthermore, it has been demonstrated that during regeneration of injured peripheral nerves, axonal transport and transcellular transfer of polyamines are increased significantly (Lindquist et al., 1985) The results from our studies further suggest that polyamines play an important role in blocking the inhibition of axonal regeneration by MAG and myelin.

However, the signaling mechanism that allows polyamines to promote injured axonal regrowth remains unknown. Recent studies in cell types other than neurons show that polyamines are essential for the organization of cytoskeletal proteins such as actin and trypomyosin (McCormack et al., 1994; McCormack et al., 1999; Kaminska et al., 1992). Evidence also suggests that polyamines influence the distribution of microtubules during

damage *in vivo* (Banan et al., 1998; Kaminska et al., 1992). These findings suggest mechanisms through which polyamines may exert their effect on the cytoskeleton intergrity. As we proposed in our model (fig.6.13), the cytoskeletal reorganization in neurons may be the final point of convergence between the MAG/myelin-mediated inhibitory pathways and the neurotrophin/cAMP-induced axonal outgrowth pathways. The balance between the negative effects by MAG/myelin and positive effects of neurotrophin/cAMP/polyamines on cytoskeleton proteins may determine neuronal regenerative capacity.

In addition, polyamines are known to interact with the *N*-methyl-D-aspartate (NMDA) type glutamate receptors and potentiate NMDA receptor-mediated responses such as ion channel function (Ransom and Stec, 1988; Sacaan and Johnson, 1990a, b; Williams et al., 1989). Therefore, the regeneration-promoting effect of polyamines may be also involved in controlling calcium influx by enhancing the NMDA receptor channel function (shown in fig. 6.13). It has been shown that calcium is involved in regulation of a wide range of cellular activities, including growth cone motility as well as neuronal differentiation (Hong et al., 2000; Wang and Zheng, 1998; Gu and Spitzer, 1995). Preliminary data suggests that calcium may play a regulatory role in the cAMP-induced axonal regeneration in response to MAG/myelin (Spencer and Filbin, unpublished data). Although studies from some groups show that the structural requirements of polyamines for promoting axonal regeneration are quite different from those for enhancing NMDA receptor channel function (Abe et al., 1997), it is possible that the regulation of calcium influx by polyamines may indirectly strengthen its regeneration-promoting effects.

Interestingly, to completely overcome the inhibition of axonal outgrowth by MAG/myelin, it is still necessary to prime neurons with putrescine, one of the polyamines. Without priming, putrescine can only partially reverse the inhibitory effects of MAG/myelin. However, it is not necessary to prime neurons with the cAMP analog, dbcAMP to overcome the inhibition by MAG/myelin completely. It may be due to that dbcAMP is a non-hydrolyzed reagent which may affect the axonal outgrowth persistently. Alternatively, dbcAMP may activate and/or inactivate some signaling events other than polyamine synthesis when neurons encounter MAG/myelin, which subsequently eliminates the need to prime. It has been reported that PKA plays a role in regulating the activity of RhoA, which is critical in mediating cytoskeleton reorganization and cellular morphological changes (Bradke and Dotti, 1999; Gallo and Letourneau, 1998; Hall, 1998). More recently, it was reported that inactivation of Rho signaling pathways can overcome the inhibition by MAG/myelin and promote CNS axonal regeneration *in vivo* (Lehmann et al., 1999; Dergham et al., 2000). As proposed in the model (fig.6.14), MAG/myelin may directly activate/inactivate some signaling molecules like RhoA, which are responsible for cytoskeleton disorganization. However, treating neurons with dbcAMP in the presence of myelin inhibitors, may block this effect and simultaneously increase neuronal polyamine production to reorganize cytoskeleton proteins and therefore, regeneration occurs. Without priming, putrescine alone may not be sufficient to overcome the inhibition since putrescine has to compete with the negative effects of myelin inhibitors on cytoskeleton organization, which are otherwise being blocked by dbcAMP. However, during priming, putrescine may be able to initiate certain

regulatory mechanisms and thereby activate the expression of target genes and modulate the synthesis of target proteins such as cytoskeleton proteins to facilitate axonal regeneration on MAG/myelin. It is suggested that polyamines can regulate expression of certain genes through modulation of acetylation and phosphorylation of specific histones, associated with ribosomal and transfer RNA, and enhancement of polypeptide synthesis (Slotkin and Bartolome, 1986). The investigation of the transcriptional/translational regulation induced by priming with putrescine is currently ongoing in the lab.

In summary, studies in this chapter investigate the possible downstream effectors in the cAMP pathway that are important in the improved axonal regeneration on MAG/myelin. It also provides a possible therapeutic treatment for adult nervous system injuries. It is known that the small molecules like polyamines are able to cross the blood-brain barrier easily, as well as transport into the cell nucleus. Therefore, instead of injecting cAMP analogs into the injured neuronal cell body, it may be more practical to treat injured animals with polyamines, intravenously or subcutaneously, to accomplish a similar degree of improvement in axonal regeneration as cAMP does. The next step is to determine if different polyamines have differential effects on axonal outgrowth. If this is the case, a combination of different polyamines may be able to improve axonal regeneration even more than treatment with a single polyamine.

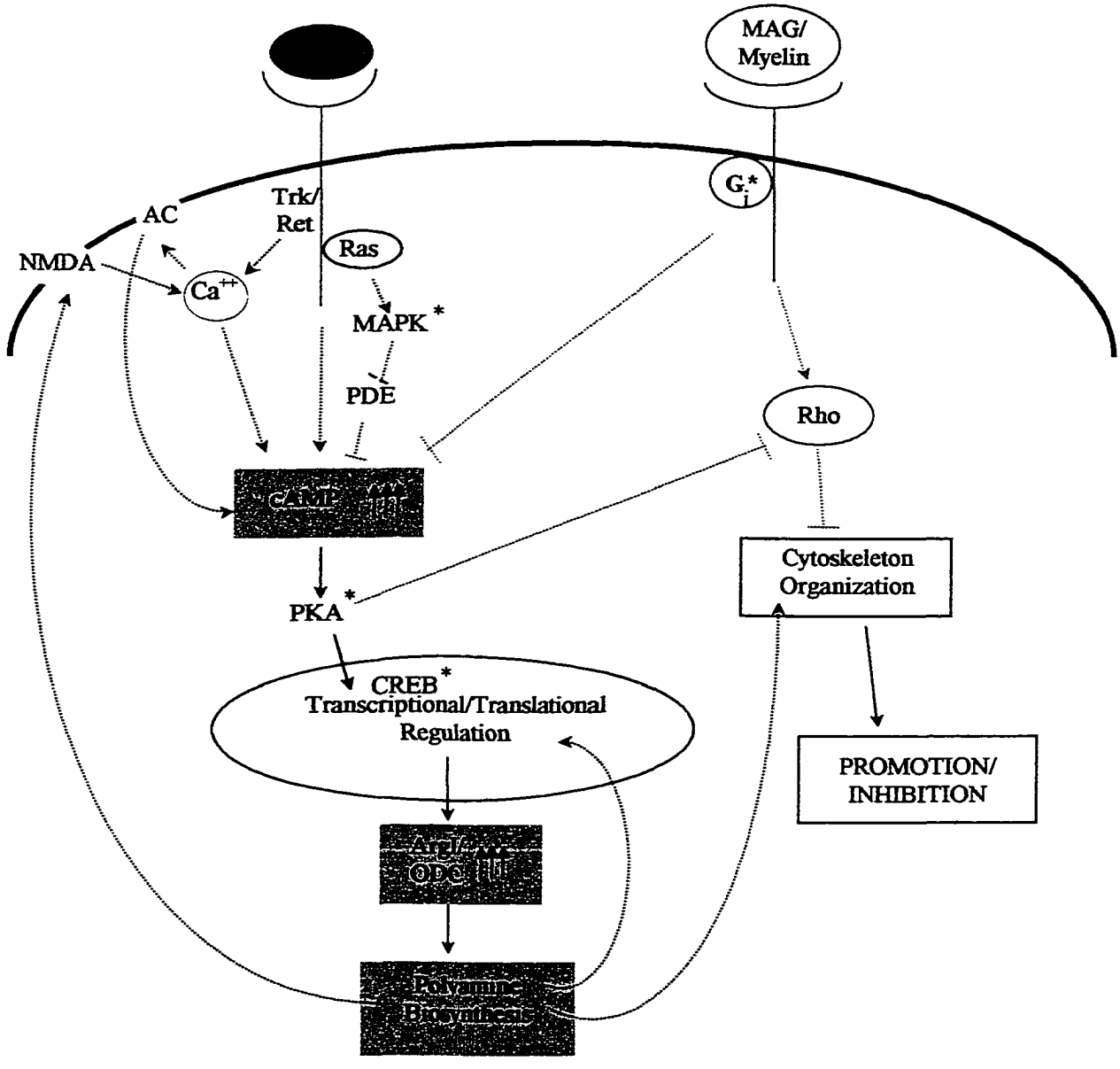


Figure 6.13 The Model of The Signal Transduction Mechanism in the cAMP-Induced Axonal Regeneration on MAG/Myelin.

Chapter VII

References

Abe K, Chu P, Shirahata A, Samejima K, Saito H (1997) Structural requirement for axonal regeneration-promoting effect of polyamines in cultured rat hippocampal neurons. Brain Res 766:281-284.

Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, Brenner HR, Caroni P (1995) Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell 83:269-278.

Ambron RT, Walters ET (1996) Priming events and retrograde injury signals. A new perspective on the cellular and molecular biology of nerve regeneration. Mol Neurobiol 13:61-79.

Aubert I, Ridet JL, Gage FH (1995) Regeneration in the adult mammalian CNS: guided by development. Curr Opin Neurobiol 5:625-635.

Bahr M, Przyrembel C (1995) Myelin from peripheral and central nervous system is a nonpermissive substrate for retinal ganglion cell axons. Exp Neurol 134:87-93.

Bahr M, Vanselow J, Thanos S (1989) Ability of adult rat ganglion cells to regrow axons in vitro can be influenced by fibroblast growth factor and gangliosides. Neurosci Lett 96:197-201.

Banan A, McCormack SA, Johnson LR (1998) Polyamines are required for microtubule formation during gastric mucosal healing. Am J Physiol 274:G879-885.

Bandtlow C, Zachleder T, Schwab ME (1990) Oligodendrocytes arrest neurite growth by contact inhibition. J Neurosci 10:3837-3848.

Bartsch U (1996) Myelination and axonal regeneration in the central nervous system of mice deficient in the myelin-associated glycoprotein. J Neurocytol 25:303-313.

Bartsch U, Kirchhoff F, Schachner M (1989) Immunohistological localization of the adhesion molecules L1, N-CAM, and MAG in the developing and adult optic nerve of mice. J Comp Neurol 284:451-462.

Bartsch U, Bandtlow CE, Schnell L, Bartsch S, Spillmann AA, Rubin BP, Hillenbrand R, Montag D, Schwab ME, Schachner M (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. Neuron 15:1375-1381.

Bedi KS, Winter J, Berry M, Cohen J (1992) Adult rat dorsal root ganglion neurons extend neurites on predegenerated but not on normal peripheral nerve *in vitro*. Eur J Neurosci 4:193-200.

Berry M, Carlile J, Hunter A (1996) Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. J Neurocytol 25:147-170.

Biffiger K, Bartsch S, Montag D, Aguzzi A, Schachner M, Bartsch U (2000) Severe hypomyelination of the murine CNS in the absence of myelin- associated glycoprotein and fyn tyrosine kinase [In Process Citation]. J Neurosci 20:7430-7437.

Blair SS (1995) Developmental biology. Hedgehog digs up an old friend. Nature 373:656-657.

Boulanger L, Poo M (1999) Gating of BDNF-induced synaptic potentiation by cAMP. Science 284:1982-1984.

Bradke F, Dotti CG (1999) The role of local actin instability in axon formation. Science 283:1931-1934.

Brandon EP, Idzerda RL, McKnight GS (1997) PKA isoforms, neural pathways , and behaviour: making the connections. Curr Opin Neurobiol 7:397-403.

Brandon EP, Logue SF, Adams MR, Qi M, Sullivan SP, Matsumoto AM, Dorsa DM, Wehner JM, McKnight GS, Idzerda RL (1998) Defective motor behavior and neural gene expression in RIIbeta-protein kinase A mutant mice. J Neurosci 18: 3639-3649.

Bregman BS, Goldberger ME (1982) Anatomical plasticity and sparing of function after spinal cord damage in neonatal cats. Science 217:553-555.

Bregman BS, Goldberger ME (1983a) Infant lesion effect: III. Anatomical correlates of sparing and recovery of function after spinal cord damage in newborn and adult cats. Brain Res 285:137-154.

Bregman BS, Goldberger ME (1983b) Infant lesion effect: II. Sparing and recovery of function after spinal cord damage in newborn and adult cats. Brain Res 285:119-135.

Bregman BS, Goldberger ME (1983c) Infant lesion effect: I. Development of motor behavior following neonatal spinal cord damage in cats. Brain Res 285:103-117.

Bregman BS, Kunkel-Bagden E, McAtee M, O'Neill A (1989) Extension of the critical period for developmental plasticity of the corticospinal pathway. J Comp Neurol 282:355-370.

Bregman BS, Broude E, McAtee M, Kelley MS (1998) Transplants and neurotrophic factors prevent atrophy of mature CNS neurons after spinal cord injury. Exp Neurol 149:13-27.

Bregman BS, Kunkel-Bagden E, Reier PJ, Dai HN, McAtee M, Gao D (1993) Recovery of function after spinal cord injury: mechanisms underlying transplant-mediated recovery of function differ after spinal cord injury in newborn and adult rats. Exp Neurol 123:3-16.

Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors [see comments]. Nature 378:498-501.

Bregman BS (1998) Transplants and neurotrophins increase regeneration and recovery of function after spinal cord injury. In Frontiers of Neural Development, K. Uyemura, ed. (Tokyo: Springer-Verlag).

Brose K, Tessier-Lavigne M (2000) Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol 10:95-102.

Broude E, McAtee M, Kelley MS, Bregman BS (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. Exp Neurol 148:367-377.

Brown MC, Lunn ER, Perry VH (1992) Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. J Neurobiol 23:521-536.

Brown MC, Perry VH, Hunt SP, Lapper SR (1994) Further studies on motor and sensory nerve regeneration in mice with delayed Wallerian degeneration. Eur J Neurosci 6:420-428.

Brown MC, Perry VH, Lunn ER, Gordon S, Heumann R (1991) Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. Neuron 6:359-370.

Cajal SR (1928) In Regeneration and Degeneration in the Nervous System. Oxford University Press: Oxford.

Cai D, Shen Y, De Bellard M, Tang S, Filbin MT (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22:89-101.

Cai D, Qiu J, Cao Z, McAtee M, Bregman BS, Filbin MT (2001) Neuronal cyclic AMP controls the developemntal loss in ability of axons to regenerate. Submitted to J Neurosci

Carbonetto S, Evans D, Cochard P (1987) Nerve fiber growth in culture on tissue substrata from central and peripheral nervous systems. J Neurosci 7:610-620.

Caroni P, Schwab ME (1988a) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J Cell Biol 106:1281-1288.

Caroni P, Schwab ME (1988b) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1:85-96.

Chen DF, Jhaveri S, Schneider GE (1995) Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. Proc Natl Acad Sci U S A 92:7287-7291.

Chen DF, Schneider GE, Martinou JC, Tonegawa S (1997) Bcl-2 promotes regeneration of severed axons in mammalian CNS [see comments]. Nature 385:434-439.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1 [see comments]. Nature 403:434-439.

Cheng H, Cao Y, Olson L (1996) Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function [see comments]. Science 273:510-513.

Chu PJ, Saito H, Abe K (1995) Polyamines promote regeneration of injured axons of cultured rat hippocampal neurons. Brain Res 673:233-241.

Collier HO (1980) Cellular site of opiate dependence. Nature 283:625-629.

Corraliza IM, Modolell M, Fereber E, Soler G (1997) Involvement of protein kinase A in the induction of arginase in murine bone marrow-derived macrophages. Biochim Biophys Acta 1334: 123-8.

Corset V, Nguyen-Ba-Charvet KT, Forcet C, Moyse E, Chedotal A, Mehlen P (2000) Netrin-1-mediated axon outgrowth and cAMP production requires interaction with adenosine A2b receptor [In Process Citation]. Nature 407:747-750.

Crutcher KA (1989) Tissue sections from the mature rat brain and spinal cord as substrates for neurite outgrowth in vitro: extensive growth on gray matter but little growth on white matter. Exp Neurol 104:39-54.

Cummings DE, Brandon EP, Planas JV, Motamed K, Iszerda RL, McKnight GS (1996) Genetically lean mice result fromtargeted disruption of the RIIbeta subunit of protein kinase A [see comments], Nature 382:622-626.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214:931-933.

David S, Braun PE, Jackson DL, Kottis V, McKerracher L (1995) Laminin overrides the inhibitory effects of peripheral nervous system and central nervous system myelin-derived inhibitors of neurite growth. J Neurosci Res 42:594-602.

Davies SJ, Field PM, Raisman G (1994) Long interfascicular axon growth from embryonic neurons transplanted into adult myelinated tracts. J Neurosci 14:1596-1612.

Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J (1997) Regeneration of adult axons in white matter tracts of the central nervous system. Nature 390:680-683.

DeBellard ME, Tang S, Mukhopadhyay G, Shen YJ, Filbin MT (1996) Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. Mol Cell Neurosci 7:89-101.

DeBellard ME, Weerth S, Griffin J, Filbin MT (1999) Myelin-associated glycoprotein (MAG) expressed from the p75 promoter retards PNS regeneration. Soc Neurosci (Abstr.) 29:402

Dergham P, Rossignol S, McKerracher L (2000) Inactivation of Rho GTPase stimulates axon regeneration in transected spinal cord of adult mice. Soc. Neurosci (Abstr.) 30:203

Dornay M, Gilad VH, Shiler I, Gilad GM (1986) Early polyamine treatment accelerates regeneration of rat sympathetic neurons. Exp Neurol 92:665-674.

Dou CL, Levine JM (1994) Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. J Neurosci 14:7616-7628.

Dou CL, Levine JM (1997) Identification of a neuronal cell surface receptor for a growth inhibitory chondroitin sulfate proteoglycan (NG2). J Neurochem 68:1021-1030.

Esch F, Lin KI, Hills A, Zaman K, Baraban JM, Chatterjee S, Rubin L, Ash DE, Ratan RR (1998) Purification of a multipotent antideath activity from bovine liver and its identification as arginase: nitric oxide-independent inhibition of neuronal apoptosis. J Neurosci 18:4083-4095.

Fahrig T, Probstmeier R, Spiess E, Meyer-Franke A, Kirchhoff F, Drescher B, Schachner M (1993) Functional topography of the myelin-associated glycoprotein. I. Mapping of domains by electron microscopy. Eur J Neurosci 5:1118-1126.

Fawcett JW (1992) Intrinsic neuronal determinants of regeneration. Trends Neurosci 15:5-8.

Fawcett JW, Geller HM (1998) Regeneration in the CNS: optimism mounts [see comments]. Trends Neurosci 21:179-180.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain Res Bull 49:377-391.

Fawcett JW, Housden E, Smith-Thomas L, Meyer RL (1989) The growth of axons in three-dimensional astrocyte cultures. Dev Biol 135:449-458.

Fidler PS, Schuette K, Asher RA, Dobbertin A, Thornton SR, Calle-Patino Y, Muir E, Levine JM, Geller HM, Rogers JH, Faissner A, Fawcett JW (1999) Comparing astrocytic cell lines that are inhibitory or permissive for axon growth: the major axon-inhibitory proteoglycan is NG2. J Neurosci 19:8778-8788.

Filbin MT (1996) The muddle with MAG. Mol Cell Neurosci 8:84-92.

Filbin MT (2000) Axon regeneration: Vaccinating against spinal cord injury. Curr Biol 10:R100-103.

Flynn NE, Wu G (1997) Enhanced metabolism of arginine and glutamine in enterocytes of cortisol-treated pigs. Am J Physiol 272:G474-480.

Fok-Seang J, Smith-Thomas LC, Meiners S, Muir E, Du JS, Housden E, Johnson AR, Faissner A, Geller HM, Keynes RJ, et al. (1995) An analysis of astrocytic cell lines with different abilities to promote axon growth. Brain Res 689:207-223.

Frail DE, Webster HD, Braun PE (1985) Developmental expression of the myelin-associated glycoprotein in the peripheral nervous system is different from that in the central nervous system. J Neurochem 45:1308-1310.

Friedlander DR, Milev P, Karthikeyan L, Margolis RK, Margolis RU, Grumet M (1994) The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. J Cell Biol 125:669-680.

Fruttiger M, Montag D, Schachner M, Martini R (1995) Crucial role for the myelin-associated glycoprotein in the maintenance of axon-myelin integrity. Eur J Neurosci 7:511-515.

Fujita N, Kemper A, Dupree J, Nakayasu H, Bartsch U, Schachner M, Maeda N, Suzuki K, Popko B (1998) The cytoplasmic domain of the large myelin-associated glycoprotein isoform is needed for proper CNS but not peripheral nervous system myelination. J Neurosci 18:1970-1978.

Gallo G, Letourneau PC (1998) Axon guidance: GTPases help axons reach their targets. Curr Biol 8:R80-82.

Gilad GM, Gilad VH (1988) Early polyamine treatment enhances survival of sympathetic neurons after postnatal axonal injury or immunosympathectomy. Brain Res 466:175-181.

Gilad VH, Tetzlaff WG, Rabey JM, Gilad GM (1996) Accelerated recovery following polyamines and aminoguanidine treatment after facial nerve injury in rats. Brain Res 724:141-144.

Goldberg JL, Barres BA (2000) Nogo in nerve regeneration [news; comment]. Nature 403:369-370.

Gotoh T, Sonoki T, Nagasaki A, Terada K, Takiguchi M, Mori M (1996) Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. FEBS Lett 395:119-122.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.

Grill R, Murai K, Blesch A, Gage FH, Tuszynski MH (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. J Neurosci 17:5560-5572.

Gu X, Spitzer NC (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{++} transients. Nature 375:784-787.

Guest JD, Rao A, Olson L, Bunge MB, Bunge RP (1997) The ability of human Schwann cell grafts to promote regeneration in the transected nude rat spinal cord. Exp Neurol 148:502-522.

Guroff G, Dickens G (1983) The effects of nerve growth factor on polyamine metabolism in PC12 cells. J Neurochem 40:1271-1277.

Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279:509-514.

Hall SM, Kent AP (1987) The response of regenerating peripheral neurites to a grafted optic nerve. J Neurocytol 16:317-331.

Heumann R, Lindholm D, Bandtlow C, Meyer M, Radeke MJ, Misko TP, Shooter E, Thoenen H (1987) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. Proc Natl Acad Sci U S A 84:8735-8739.

Hirsch S, Bahr M (1999) Growth promoting and inhibitory effects of glial cells in the mammalian nervous system. Adv Exp Med Biol 468:199-205.

Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion [see comments]. Cell 97:927-941.

Hong K, Nishiyama M, Henley J, Tessier-Lavigne M, Poo MM (2000) Calcium signaling in the guidance of nerve growth by netrin-1. Nature 384:166-169.

Hopker VH, Shewan D, Tessier-Lavigne M, Poo M, Holt C (1999) Growth-cone attraction to netrin-1 is converted to repulsion by laminin- 1. Nature 401:69-73.

Huang DW, McKerracher L, Braun PE, David S (1999) A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. Neuron 24:639-647.

Iona S, Cuomo M, Bushnik T, Naro F, Sette C, Hess M, Shelton ER, Conti M (1998) Characterization of the rolipram-sensitive, cyclic AMP-specific phosphodiesterases: identification and differential expression of immunologically distinct forms in the rat brain. Mol Pharmacol 53:23-32.

Iyengar R (1996) Gating by cyclic AMP: expanded role for an old signaling pathway. Science 271:461-463.

Jiang J, Struhl G (1995) Protein kinase A and hedgehog signaling in Drosophila limb development. Cell 80:563-572.

Johnson AR (1993) Contact inhibition in the failure of mammalian CNS axonal regeneration. Bioessays 15:807-813.

Johnson PW, Abramow-Newerly W, Seilheimer B, Sadoul R, Tropak MB, Arquint M, Dunn RJ, Schachner M, Roder JC (1989) Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. Neuron 3:377-385.

Kaminska B, Kaczmarek L, Grzelakowska-Sztabert B (1992) Inhibitors of polyamine biosynthesis affect the expression of genes encoding cytoskeletal proteins. FEBS Lett 304:198-200.

Kapfhammer JP, Schwab ME (1994) Inverse patterns of myelination and GAP-43 expression in the adult CNS: neurite growth inhibitors as regulators of neuronal plasticity? J Comp Neurol 340:194-206.

Kauppila T (1992) Polyamines enhance recovery after sciatic nerve trauma in the rat. Brain Res 575:299-303.

Kauppila T, Stenberg D, Porkka-Heiskanen T (1988) Putative stimulants for functional recovery after neural trauma: only spermine was effective. Exp Neurol 99:50-58.

Keirstead HS, Levine JM, Blakemore WF (1998) Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. Glia 22:161-170.

Kelm S, Pelz A, Schauer R, Filbin MT, Tang S, de Bellard ME, Schnaar RL, Mahoney JA, Hartnell A, Bradfield P, et al. (1994) Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. Curr Biol 4:965-972.

Kennedy TE, Tessier-Lavigne M (1995) Guidance and induction of branch formation in developing axons by target-derived diffusible factors. Curr Opin Neurobiol 5:83-90.

Kenney AM, Kocsis JD (1997) Timing of c-jun protein induction in lumbar dorsal root ganglia after sciatic nerve transection varies with lesion distance. Brain Res 751:90-95.

Kepka-Lenhart D, Mistry SK, Wu G, Morris SM (2000) Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? Am J Physiol Regul Integr Comp Physiol 279:R2237-2242.

Keynes R, Cook GM (1995a) Axon guidance molecules. Cell 83:161-169.

Keynes RJ, Cook GM (1995b) Repulsive and inhibitory signals. Curr Opin Neurobiol 5:75-82.

Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G (1998) Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205-215.

Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J (1996) Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. Embo J 15:510-519.

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19:7537-7547.

Levine JM (1994) Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. J Neurosci 14:4716-4730.

Levine JM, Nishiyama A (1996) The NG2 chondroitin sulfate proteoglycan: a multifunctional proteoglycan associated with immature cells. Perspect Dev Neurobiol 3:245-259.

Li C, Tropak MB, Gerlai R, Clapoff S, Abramow-Newerly W, Trapp B, Peterson A, Roder J (1994) Myelination in the absence of myelin-associated glycoprotein. Nature 369:747-750.

Li M, Shibata A, Li C, Braun PE, McKerracher L, Roder J, Kater SB, David S (1996) Myelin-associated glycoprotein inhibits neurite/axon growth and causes growth cone collapse. J Neurosci Res 46:404-414.

Li Y, Raisman G (1993) Long axon growth from embryonic neurons transplanted into myelinated tracts of the adult rat spinal cord. Brain Res 629:115-127.

Li Y, Raisman G (1997) Integration of transplanted cultured Schwann cells into the long myelinated fiber tracts of the adult spinal cord. Exp Neurol 145:397-411.

Li Y, Field PM, Raisman G (1997) Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells [see comments]. Science 277:2000-2002.

Linder ME, Gilman AG (1992) G proteins. Sci Am 267:56-61, 64-55.

Lindquist TD, Sturman JA, Gould RM, Ingoglia NA (1985) Axonal transport of polyamines in intact and regenerating axons of the rat sciatic nerve. J Neurochem 44:1913-1919.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394-2405.

MacDonnell PC, Nagaiah K, Lakshmanan J, Guroff G (1977) Nerve growth factor increases activity of ornithine decarboxylase in superior cervical ganglia of young rats. Proc Natl Acad Sci U S A 74:4681-4684.

Martin D, Robe P, Franzen R, Delree P, Schoenen J, Stevenaert A, Moonen G (1996) Effects of Schwann cell transplantation in a contusion model of rat spinal cord injury. J Neurosci Res 45:588-597.

Martini R, Schachner M (1986) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. J Cell Biol 103:2439-2448.

Martini R, Schachner M, Faissner A (1990) Enhanced expression of the extracellular matrix molecule J1/tenascin in the regenerating adult mouse sciatic nerve. J Neurocytol 19:601-616.

McCormack SA, Wang JY, Johnson LR (1994) Polyamine deficiency causes reorganization of F-actin and tropomyosin in IEC-6 cells. Am J Physiol 267:C715-722.

McCormack SA, Ray RM, Blanner PM, Johnson LR (1999) Polyamine depletion alters the relationship of F-actin, G-actin, and thymosin beta4 in migrating IEC-6 cells. Am J Physiol 276:C459-468.

McKeon RJ, Hoke A, Silver J (1995) Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. Exp Neurol 136:32-43.

McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci 11:3398-3411.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin- derived inhibitor of neurite growth. Neuron 13:805-811.

Menei P, Montero-Menei C, Whittemore SR, Bunge RP, Bunge MB (1998) Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. Eur J Neurosci 10:607-621.

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119:45-54.

Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995a) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15:805-819.

Meyer-Franke A, Tropak MB, Roder JC, Fischer P, Beyreuther K, Probstmeier R, Schachner M (1995b) Functional topography of myelin-associated glycoprotein. II. Mapping of domains on molecular fragments. J Neurosci Res 41:311-323.

Ming G, Song H, Berninger B, Inagaki N, Tessier-Lavigne M, Poo M (1999) Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. Neuron 23:139-148.

Ming GL, Song HJ, Berninger B, Holt CE, Tessier-Lavigne M, Poo MM (1997) cAMP-dependent growth cone guidance by netrin-1. Neuron 19:1225-1235.

Montag D, Giese KP, Bartsch U, Martini R, Lang Y, Bluthmann H, Karthigasan J, Kirschner DA, Wintergerst ES, Nave KA, et al. (1994) Mice deficient for the

myelin-associated glycoprotein show subtle abnormalities in myelin. Neuron 13:229-246.

Montminy M (1997) Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807-822.

Morris G, Slotkin TA (1985) Beta-2 adrenergic control of ornithine decarboxylase activity in brain regions of the developing rat. J Pharmacol Exp Ther 233:141-147.

Morris SM, Kepka-Lenhart D, Chen LC (1998) Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol 275:E740-747.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Nestler EJ (1992) Molecular mechanisms of drug addiction. J Neurosci 12:2439-2450.

Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury [see comments]. Neuron 23:83-91.

Nicholls J, Saunders N (1996) Regeneration of immature mammalian spinal cord after injury. Trends Neurosci 19:229-234.

Niederost BP, Zimmermann DR, Schwab ME, Bandtlow CE (1999) Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans. J Neurosci 19:8979-8989.

Noronha AB, Hammer JA, Lai C, Kiel M, Milner RJ, Sutcliffe JG, Quarles RH (1989) Myelin-associated glycoprotein (MAG) and rat brain-specific IB236 protein: mapping of epitopes and demonstration of immunological identity. J Mol Neurosci 1:159-170.

Norton WT and Podulso SE, (1973) Myelination in rat brain: method of myelin isolation. J Neurochem 21: 749-751.

Oudega M, Varon S, Hagg T (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: promoting effects of conditioning lesion and graft predegeneration. Exp Neurol 129:194-206.

Owens GC, Bunge RP (1989) Evidence for an early role for myelin-associated glycoprotein in the process of myelination. Glia 2: 119-128.

Owens GC, Bunge RP (1991) Schwann cells infected with a recombinant retrovirus expressing myelin- associated glycoprotein antisense RNA do not form myelin. Neuron 7:565-575.

Owens GC, Boyd CJ, Bunge RP, Salzer JL (1990) Expression of recombinant myelin-associated glycoprotein in primary Schwann cells promotes the initial investment of axons by myelinating Schwann cells. J Cell Biol 111:1171-1182.

Pan D, Rubin GM (1995) cAMP-dependent protein kinase and hedgehog act antagonistically in regulating decapentaplegic transcription in Drosophila imaginal discs. Cell 80:543-552.

Patel TD, Jackman A, Rice FL, Kucera J, Snider WD (2000) Development of sensory neurons in the absence of NGF/TrkA signaling in vivo [see comments]. Neuron 25:345-357.

Pedraza L, Owens GC, Green LA, Salzer JL (1990) The myelin-associated glycoproteins: membrane disposition, evidence of a novel disulfide linkage between immunoglobulin-like domains, and posttranslational palmitylation. J Cell Biol 111:2651-2661.

Perry VH, Brown MC, Gordon S (1987) The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. J Exp Med 165:1218-1223.

Pesheva P, Gennarini G, Goridis C, Schachner M (1993) The F3/11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1-160/180. Neuron 10:69-82.

Pieroni JP, Harry A, Chen J, Jacobowitz O, Magnusson RP, Iyengar R (1995) Distinct characteristics of the basal activities of adenylyl cyclases 2 and 6. J Biol Chem 270:21368-21373.

Polleux F, Morrow T, Ghosh A (2000) Semaphorin 3A is a chemoattractant for cortical apical dendrites [see comments]. Nature 404:567-573.

Poltorak M, Sadoul R, Keilhauer G, Landa C, Fahrig T, Schachner M (1987) Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. J Cell Biol 105:1893-1899.

Post GR, Brown JH (1996) G protein-coupled receptors and signaling pathways regulating growth responses. Faseb J 10:741-749.

Powell LD, Varki A (1995) I-type lectins. J Biol Chem 270:14243-14246.

Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, Michalovich D, Simmons DL, Walsh FS (2000) Inhibitor of neurite outgrowth in humans [see comments]. Nature 403:383-384.

Qiu J, Cai D, Filbin MT (2000) Glial inhibition of nerve regeneration in the mature mammalian CNS. Glia 29:166-174.

Quarles RH (1997) Glycoproteins of myelin sheaths. J Mol Neurosci 8:1-12.

Quarles RH, Colman DR, Salzer JL, Trapp BD (1992) Myelin-assocaited glycoprotein: structure -function relationships and involvement in neurological diseases. Myelin: Biology and Chemistry, Martensom RE ed. pp:413-448.

Quarles RH, Everly JL, Brady RO (1973) Myelin-associated glycoprotein: a developmental change. Brain Res 58:506-509.

Ramer MS, Priestley JV, McMahon SB (2000) Functional regeneration of sensory axons into the adult spinal cord [see comments]. Nature 403:312-316.

Ramon-Cueto A, Avila J (1998) Olfactory ensheathing glia: properties and function. Brain Res Bull 46:175-187.

Ramon-Cueto A, Plant GW, Avila J, Bunge MB (1998) Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. J Neurosci 18:3803-3815.

Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J (2000) Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25:425-435.

Ransom RW, Stec NL (1988) Cooperative modulation of [3H]MK-801 binding to the N-methyl-D- aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. J Neurochem 51:830-836.

Retzler C, Gohring W, Rauch U (1996) Analysis of neurocan structures interacting with the neural cell adhesion molecule N-CAM. J Biol Chem 271:27304-27310.

Richardson PM, Ebendal T (1982) Nerve growth activities in rat peripheral nerve. Brain Res 246:57-64.

Richardson PM, Issa VM (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309:791-793.

Robinson M, Adu J, Davies AM (1996) Timing and regulation of trkB and BDNF mRNA expression in placode- derived sensory neurons and their targets. Eur J Neurosci 8:2399-2406.

Rudge JS, Silver J (1990) Inhibition of neurite outgrowth on astroglial scars in vitro. J Neurosci 10:3594-3603.

Sacaan AI, Johnson KM (1990a) Characterization of the stimulatory and inhibitory effects of polyamines on [3H]N-(1-[thienyl]cyclohexyl) piperidine binding to the N- methyl-D-aspartate receptor ionophore complex. Mol Pharmacol 37:572-577.

Sacaan AI, Johnson KM (1990b) Spermidine reverses arcaine's inhibition of N-methyl-D-aspartate- induced hippocampal [3H]norepinephrine release. J Pharmacol Exp Ther 255:1060-1063.

Sadoul R, Fahrig T, Bartsch U, Schachner M (1990) Binding properties of liposomes containing the myelin-associated glycoprotein MAG to neural cell cultures. J Neurosci Res 25:1-13.

Salzer JL, Bunge RP (1980) Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. J Cell Biol 84:739-752.

Salzer JL, Pedraza L, Brown M, Struyk A, Afar D, Bell J (1990) Structure and function of the myelin-associated glycoproteins. Ann N Y Acad Sci 605:302-312.

Sandrock AW, Jr., Matthew WD (1987) Identification of a peripheral nerve neurite growth-promoting activity by development and use of an in vitro bioassay. Proc Natl Acad Sci U S A 84:6934-6938.

Sato S, Quarles RH, Brady RO (1982) Susceptibility of the myelin-associated glycoprotein and basic protein to a neutral protease in highly purified myelin from human and rat brain. J Neurochem 39:97-105.

Savio T, Schwab ME (1989) Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. J Neurosci 9:1126-1133.

Savio T, Schwab ME (1990) Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. Proc Natl Acad Sci U S A 87:4130-4133.

Schachner M (1994) Neural recognition molecules in disease and regeneration. Curr Opin Neurobiol 4:726-734.

Schachner M, Bartsch U (2000) Multiple functions of the myelin-associated glycoprotein MAG (siglec- 4a) in formation and maintenance of myelin. Glia 29:154-165.

Schafer M, Fruttiger M, Montag D, Schachner M, Martini R (1996) Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. Neuron 16:1107-1113.

Schnell L, Schwab ME (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343:269-272.

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion [see comments]. Nature 367:170-173.

Schulman H (1991) Serine/threonine kinases in the nervous system. Curr Opin Neurobiol 1:43-52.

Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76:319-370.

Schwab ME, Kapfhammer JP, Bandtlow CE (1993) Inhibitors of neurite growth. Annu Rev Neurosci 16:565-595.

Shantz LM, Pegg AE (1999) Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. Int J Biochem Cell Biol 31:107-122.

Shantz LM, Hu RH, Pegg AE (1996a) Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. Cancer Res 56:3265-3269.

Shantz LM, Coleman CS, Pegg AE (1996b) Expression of an ornithine decarboxylase dominant-negative mutant reverses eukaryotic initiation factor 4E-induced cell transformation. Cancer Res 56:5136-5140.

Shaskan EG, Snyder SH (1973) Polyamine turnover in different regions of rat brain. J Neurochem 20:1453-1460.

Shaskan EG, Haraszti JH, Snyder SH (1973) Polyamines: developmental alterations in regional disposition and metabolism in rat brain. J Neurochem 20: 1443-1452.

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821-861.

Shaw GG, Pateman AJ (1973) The regional distribution of the polyamines spermidine and spermine in brain. J Neurochem 20:1225-1230.

Shen S, Wiemelt AP, McMorris FA, Barres BA (1999) Retinal ganglion cells lose trophic responsiveness after axotomy. Neuron 23:285-295.

Shen YJ, DeBellard ME, Salzer JL, Roder J, Filbin MT (1998) Myelin-associated glycoprotein in myelin and expressed by Schwann cells inhibits axonal regeneration and branching. Mol Cell Neurosci 12:79-91.

Shewan D, Berry M, Cohen J (1995) Extensive regeneration in vitro by early embryonic neurons on immature and adult CNS tissue. J Neurosci 15:2057-2062.

Shewan D, Berry M, Bedi K, Cohen J (1993) Embryonic optic nerve tissue fails to support neurite outgrowth by central and peripheral neurons *in vitro*. Eur j Neurosci 5:809-817.

Skene JH (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Skene JH, Willard M (1981) Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96-103.

Skene JH, Jacobson RD, Snipes GJ, McGuire CB, Norden JJ, Freeman JA (1986) A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. Science 233:783-786.

Slotkin TA, Bartolome J (1986) Role of ornithine decarboxylase and the polyamines in nervous system development: a review. Brain Res Bull 17:307-320.

Slotkin TA, Grignolo A, Whitmore WL, Lerea L, Trepanier PA, Barnes GA, Weigel SJ, Seidler FJ, Bartolome J (1982) Impaired development of central and peripheral catecholamine neurotransmitter systems in preweanling rats treated with alpha-difluoromethylornithine, a specific irreversible inhibitor of ornithine decarboxylase. J Pharmacol Exp Ther 222:746-751.

Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J (1990) Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. Exp Neurol 109:111-130.

Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo M (1998) Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides [see comments]. Science 281:1515-1518.

Song HJ, Ming GL, Poo MM (1997) cAMP-induced switching in turning direction of nerve growth cones [published erratum appears in Nature 1997 Sep 25;389(6649):412]. Nature 388:275-279.

Spillmann AA, Bandtlow CE, Lottspeich F, Keller F, Schwab ME (1998) Identification and characterization of a bovine neurite growth inhibitor (bNI-220). J Biol Chem 273:19283-19293.

Stensaas LJ, Partlow LM, Burgess PR, Horch KW (1987) Inhibition of regeneration: the ultrastructure of reactive astrocytes and abortive axon terminals in the transition zone of the dorsal root. Prog Brain Res 71:457-468.

Sternberger NH, Quarles RH, Itoyama Y, Webster HD (1979) Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rat. Proc Natl Acad Sci U S A 76:1510-1514.

Stichel CC, Kappler J, Junghans U, Koops A, Kresse H, Muller HW (1995) Differential expression of the small chondroitin/dermatan sulfate proteoglycans decorin and biglycan after injury of the adult rat brain. Brain Res 704:263-274.

Stoll G, Trapp BD, Griffin JW (1989) Macrophage function during Wallerian degeneration of rat optic nerve: clearance of degenerating myelin and Ia expression. J Neurosci 9:2327-2335.

Strittmatter SM (2000) Dendrites go up, axons go down [news; comment]. Nature 404:557, 559.

Tang S, Shen YJ, DeBellard ME, Mukhopadhyay G, Salzer JL, Crocker PR, Filbin MT (1997a) Myelin-associated glycoprotein interacts with neurons via a sialic acid binding site at ARG118 and a distinct neurite inhibition site. J Cell Biol 138:1355-1366.

Tang S, Woodhall RW, Shen YJ, deBellard ME, Saffell JL, Doherty P, Walsh FS, Filbin MT (1997b) Soluble Myelin-Associated Glycoprotein (MAG) Found in Vivo Inhibits Axonal Regeneration. Mol Cell Neurosci 9:333-346.

Tansey MG, Luby-Phelps K, Kamm KE, Stull JT (1994) Ca(2+)-dependent phosphorylation of myosin light chain kinase decreases the Ca2+ sensitivity of light chain phosphorylation within smooth muscle cells. J Biol Chem 269:9912-9920.

Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274:1123-1133.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165-170.

Trapp BD, Quarles RH (1982) Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. J Cell Biol 92:877-882.

Trapp BD, Andrews SB, Wong A, O'Connell M, Griffin JW (1989) Co-localization of the myelin-associated glycoprotein and the microfilament components, F-actin and spectrin, in Schwann cells of myelinated nerve fibres. J Neurocytol 18:47-60.

Trapp BD, Webster HD, Johnson D, Quarles RH, Cohen SR, Murray MR (1982) Myelin formation in rotation-mediated aggregating cell cultures: immunocytochemical, electron microscopic, and biochemical observations. J Neurosci 2:986-993.

Tropak MB, Johnson PW, Dunn RJ, Roder JC (1988) Differential splicing of MAG transcripts during CNS and PNS development. Brain Res 464:143-155.

Turnley AM, Bartlett PF (1998) MAG and MOG enhance neurite outgrowth of embryonic mouse spinal cord neurons. Neuroreport 9:1987-1990.

Umemori H, Sato S, Yagi T, Aizawa S, Yamamoto T (1994) Initial events of myelination involve Fyn tyrosine kinase signalling. Nature 367:572-576.

van Corven EJ, Groenink A, Jalink K, Eichholtz T, Moolenaar WH (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. Cell 59:45-54.

Veress I, Haghighi S, Pulkka A, Pajunen A (2000) Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs. Biochem J 346 Pt 1:185-191.

Vidal-Sanz M, Bray GM, Villegas-Perez MP, Thanos S, Aguayo AJ (1987) Axonal regeneration and synapse formation in the superior colliculus by retinal ganglion cells in the adult rat. J Neurosci 7:2894-2909.

Walton MR, Dragunow I (2000) Is CREB a key to neuronal survival? Trends Neurosci 23:48-53.

Wang Q, Zheng JQ (1998) cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. J Neurosci 18:4973-4984.

Wang WW, Jenkinson CP, Griscavage JM, Kern RM, Arabolos NS, Byrns RE, Cederbaum SD, Ignarro LJ (1995) Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. Biochem Biophys Res Commun 210:1009-1016.

Whittemore SR, Nieto-Sampedro M, Needels DL, Cotman CW (1985) Neuronotrophic factors for mammalian brain neurons: injury induction in neonatal, adult and aged rat brain. Brain Res 352:169-178.

Wictorin K, Brundin P, Gustavii B, Lindvall O, Bjorklund A (1990) Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. Nature 347:556-558.

Wictorin K, Brundin P, Sauer H, Lindvall O, Bjorklund A (1992) Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. J Comp Neurol 323:475-494.

Wiemelt AP, Engleka MJ, Skorupa AF, McMorris FA (1997) Immunochemical visualization and quantitation of cyclic AMP in single cells. J Biol Chem 272:31489-31495.

Williams K, Romano C, Molinoff PB (1989) Effects of polyamines on the binding of [3H]MK-801 to the N-methyl-D- aspartate receptor: pharmacological evidence for the existence of a polyamine recognition site. Mol Pharmacol 36:575-581.

Wintergerst ES, Bartsch U, Batini C, Schachner M (1997) Changes in the expression of the extracellular matrix molecules tenascin-C and tenascin-R after 3-acetylpyridine-induced lesion of the olivocerebellar system of the adult rat. Eur J Neurosci 9:424-434.

Wong BJ, Mattox DE (1991) The effects of polyamines and polyamine inhibitors on rat sciatic and facial nerve regeneration. Exp Neurol 111:263-266.

Wu G, Knabe DA, Flynn NE, Yan W, Flynn SP (1996) Arginine degradation in developing porcine enterocytes. Am J Physiol 271:G913-919.

Xiao ZC, Bartsch U, Margolis RK, Rougon G, Montag D, Schachner M (1997) Isolation of a tenascin-R binding protein from mouse brain membranes. A phosphacan-related chondroitin sulfate proteoglycan. J Biol Chem 272:32092-32101.

Xiao ZC, Revest JM, Laeng P, Rougon G, Schachner M, Montag D (1998) Defasciculation of neurites is mediated by tenascin-R and its neuronal receptor F3/11. J Neurosci Res 52:390-404.

Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB (1995) A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol 134:261-272.

Yamada H, Fredette B, Shitara K, Hagihara K, Miura R, Ranscht B, Stallcup WB, Yamaguchi Y (1997) The brain chondroitin sulfate proteoglycan brevican associates with astrocytes ensheathing cerebellar glomeruli and inhibits neurite outgrowth from granule neurons. J Neurosci 17:7784-7795.

Yang LJ, Zeller CB, Shaper NL, Kiso M, Hasegawa A, Shapiro RE, Schnaar RL (1996) Gangliosides are neuronal ligands for myelin-associated glycoprotein. Proc Natl Acad Sci U S A 93:814-818.

Yin X, Crawford TO, Griffin JW, Tu P, Lee VM, Li C, Roder J, Trapp BD (1998) Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. J Neurosci 18:1953-1962.

Zhang K, Farooqui SM, O'Donnell JM (1999) Ontogeny of rolipram-sensitive, low-K(m), cyclic AMP-specific phosphodiesterase in rat brain. Brain Res Dev Brain Res 112:11-19.

Zimmerman AL (1995) Cyclic nucleotide gated channels. Curr Opin Neurobiol 5:296-303.