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SIGNALING MECHANISMS INVOLVED IN THE BLOCK OF MAG'S INHIBITON OF AXONAL REGENERATION

by

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

2002

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/25/02
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ABSTRACT

Signaling Mechanisms Involved in the Block of MAG's Inhibition of Axonal Regeneration

by

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Myelin-associated glycoprotein (MAG) is a potent inhibitor of axonal regeneration. However, prior exposure of neurons to neurotrophin (priming) blocks inhibition by MAG *via* a cAMP-dependent mechanism. Here we characterize the receptors and the signaling components involved in the neurotrophin/cAMP effect. First we showed that during the priming process, neurotrophins signal through the Trk receptor. Inhibition of ERK (extracellular signal-regulated kinases) by the ERK kinase (MEK) inhibitors completely abrogates both the neurotrophin and the dbcAMP effect. In addition, we demonstrate that activation of ERK by BDNF (brain-derived neurotrophic factor) down-regulates phosphodiesterase 4 (PDE4) activity and elevates cAMP levels in a MEK-dependent manner. Minimal inhibition of PDE4 by its specific inhibitor rolipram restores BDNF/cAMP's ability to overcome inhibition by MAG in the presence of the MEK inhibitor. These results suggest BDNF inhibits PDE *via* activated ERK to elevate cAMP and overcome myelin inhibitors of regeneration.

The cAMP response element (CRE) -binding protein (CREB) is a transcription factor induced by a variety of stimuli, including growth factors and agents that increase

cAMP levels. CREB has been demonstrated to play important roles in neuronal survival and memory process. However, little is known about CREB in the process of axonal regeneration. Now we report that an inhibitor of transcription abrogates the neurotrophin/cAMP effect to overcome inhibition by MAG. We also show, in our system, that CREB is activated in response to dibutyryl cAMP and BDNF. A-CREB, a dominant-negative form of CREB that prevents wildtype CREB binding to DNA, abolishes the CRE-mediated gene expression and neurotrphin/cAMP effect on MAG. Furthermore, A-CREB prevents cAMP-dependent up-regulation of the enzyme Arg I, which has previously been shown to be essential for regulating neuronal regenerative capacity. Moreover, young dorsal root ganglion neurons that are promoted by MAG, when induced to express with A-CREB, switch their response to MAG to inhibition. These results suggest that activation of CREB, or its closely related family members, is involved in the downstream signaling by neurotrophin/cAMP to overcome MAG's inhibition.

ACKNOWLEDGMENTS

Upon completion of my studies for the degree of Doctor of Philosophy, I would like to take this opportunity to express my gratitude to everyone who helped me with my thesis study and writing of the dissertation.

First and most important of all, I would like to thank my mentor, Dr. Marie T. Filbin. Her passion and dedication to science is the greatest motivation to me. Under her instruction, I matured from a follower to an independent researcher. I was most fortunate to work in her lab and be on the leading edge of the field of neuroscience. Her kindness and fairness are the best things that can be given to a graduate student. Although Marie is an extremely busy person, she is always there to support and encourage me. She is not only a mentor, but also a loving mother figure to me. I owe so much to her. Kudos to our dear lab manager Willy. He is such a wonderful person and his arrival turned the lab around. I benefited a lot from his hard work and the many helpful discussions that I had with him. Thanks also to my partner, Elena. It's been a great joy to work with her. I learned and improved so much during the period that we worked together. I was also blessed to have had all of my good friends with me during my study: Kangwen, Zixuan, Noriko, Angela, Yang and Steven. Thank you all for your support and friendship. To former lab members, Wenhui, Marie Elena, Song, Yingjing, Dongming, Adijat, Jane, Susanna, Oliver, Holly and the current lab members, Marco, Tim, Hong, Mia, Aurelie, Vaciliki and Jianwei for bearing with me and helping me through all these years. Thanks should also go to grant administrators Winnie and Courtney, who make our lives and our

timely payment much easier. Special thanks to Tim for his kindness in proofreading my thesis manuscript. It is not an easy job and I greatly appreciate his help.

I would also like to thank Dr. Thomas Schmidt-Glenewinkel for admitting me into the program and giving me so much help during my study. Thanks also to Dr. Eckardt and Dr. Raps for their instructions and helps during my rotations. To my other committee members, Drs David Colman, David Foster and Tadmiri Venkatesh for their invaluable advice. Also thanks for all the support from the Biology Department of Hunter College. It was a great place to stay and study.

Finally, I would like to dedicate this thesis to my parents in China and my brother in Australia. Their unconditional love and support is the most important thing to me. I thank my parents for their sacrifice in letting their children go to fulfill their dreams. Without them, I could not be here today. And last, to my cousin Helene and her husband. Their support during my first years in the United States helped me greatly; thank you very much.

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ABBREVIATIONS

AC	adenylyl cyclase
rAPS	rat adaptor molecule containing PH and SH2 domains
Arg	arginase
ART	artemin
ATF-1	activating transcription factor 1
Bad	Bcl-2/Bcl-x-associated death promoter
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
bZIP	basic-domain-leucine zipper
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic AMP
CAP-23	cytoskeleton-associated protein-23
CBP	CREB-binding protein
cGMP	cyclic GMP
CHK	Csk homologous kinase
CHO	Chinese hamster ovary
CLC	cardiotrophin-like cytokine
CLF	cytokine-like factor
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRE	cAMP-responsive element

CREB	cAMP-responsive element-binding protein
CREM	cAMP response element modulator
CSPGs	chondroitin sulfate proteoglycans
CST	corticospinal tract
DAG	diacylglycerol
DARPP-32	dopamine and cAMP-regulated phosphoprotein
dbcAMP	dibutyryl cAMP
DMEM	Dulbecco's modified eagle's medium
DRB	5.6-dichloro-1-β-D-ribofuranosylbenzimidazole
DRG	dorsal root ganglion
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FRS-2	fibroblast growth factor receptor substrate 2
Gab-1	Grb-associated binder-1
GAP	GTPase-activating protein
GAP-43	growth-associated protein-43
GDNF	glial-derived neurotrophic factor
GEF	guanine nucleotide exchange factor
GFLs	GDNF family ligands
GFP	green fluorescent protein

GFR	GDNF family receptor
GPI	glycosylphosphatidylinositol
HRP	horse radish peroxidase
Ig	immunoglobulin
IL-6	interleukin-6
IP_3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrate
JAK	Janus tyrosine kinase
JIP	JNK interacting protein
JNK	Jun amino-terminal kinase
KID	kinase-inducible domain
KIM	kinase interaction motif
LIF	leukemia inhibitory factor
LRR	leucine-rich repeat
LTP	long-term potentiation
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MEKK	MEK kinase
MP1	MEK partner 1
NGF	nerve growth factor
NNT-1	novel neurotrophin-1
NT	neurotrophin

NTN	neurturin
OECs	olfactory ensheathing cells
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PDE	phosphodiesterase
PDK	3-phosphoinositide-dependent kinase
PI-PLC	phosphatidylinosito-specific phospholipase C
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase C-gamma
PLL	poly-L-lysine
PND	postnatal day
PNS	peripheral nervous system
PP-1	protein phosphatase-1
PSP	persephin
PTX	pertussis toxin
PVDF	polyvinylidene fluoride
p75NTR	p75 neurotrophin receptor
Ret	Rearranged in Transformation
Rsk	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate

Shc	SH2 domain containing protein
SHP-2	Src homology 2 phosphatase
SH2-B	Scr homology 2-B
SNT	suc-associated neurotrophic factor-induced tyrosine-phosphorylated target
SOS	son of sevenless
STAT	signal transducers and activators of transcription
TNF	tumor necrosis factor
Trk	tropomyosin-related kinase
Y	tyrosine

CHAPTER I

INTRODUCTION

1. Regeneration in the adult mammalian CNS

When axons in the adult brain and spinal cord are damaged they are unable to regenerate. Consequently, there is permanent functional impairment (Johnson, 1993; Schwab and Bartholdi, 1996). Thousands of years after the first written description of central nervous system (CNS) injury (compiled by Breasted, 1930), there is still no cure for patients suffering from CNS trauma. Not until the last decade have advances been made to shed light on the mechanisms which promote the regrowth of injured axons and provide some functional recovery in the adult mammalian CNS. The stimulation of regeneration in the CNS has been one of the major challenges for neurobiologists.

Mature axons in the CNS differ greatly in response to injury from those in the peripheral nervous system (PNS) and in the embryo. The reasons for the failure of axonal regeneration in the adult mammalian CNS can be grouped into two major categories: the extrinsic unfavorable environment and the intrinsic growth capacity of the neuron.

1.1 Extrinsic inhibitory environment of axon regeneration in the CNS

Axons in the mammalian CNS do not spontaneously regenerate following injury. In contrast, injured axons in the adult PNS do regenerate following axotomy (Fu and Gordon, 1997). However, it is clear that CNS axons are capable of regeneration when given a suitable environment (Aguayo *et al.*, 1981; Bray *et al.*, 1987). Injured CNS axons can extend long fibers through growth-permissive substrates such as myelin-free spinal cord (Savio and Schwab, 1990), peripheral nerve graft (David and Aguayo, 1981), or purified Schwann cell implants (Li and Raisman, 1994; Paino *et al.*, 1994). These

observations strongly suggest that the adult CNS environment actively inhibits axonal regeneration. Immediately after injury, at the lesion site, the distal tip of the injured axon encounters both secreted and membrane-bound myelin-associated inhibitors exposed by the damaged myelin (Fournier and Strittmatter, 2001). A number of developmental, repulsive guidance factors also play a role at the CNS lesion site (Qiu *et al.*, 2000). Later, formation of the glial scar seals the fate of these injured axons, prohibiting further regeneration. These multiple inhibitory influences (Table 1) in the environment contribute to the lack of spontaneous regeneration in the CNS.

Table 1 Inhibitors of axonal regeneration.

CNS myelin	Glial scar	Developmental axon repulsion
Nogo	Tenascin	Semaphorins/Collapsins
MAG	CSPG	Slits
Proteoglycans	Keratin	Netrins
Janusin/Restrictin	Semaphrin-3A	Ephrins
Arretin		

1.1.1 Myelin-associated inhibitors

Experiments aimed at removing myelin from the injured region in order to stimulate regeneration have demonstrated that inhibitory proteins in myelin are indeed an important barrier to axon regrowth in the CNS (Dyer *et al.*, 1998; Savio and Schwab, 1990). The fact that the loss of regeneration potential during development correlates roughly with the onset of myelination also supports this idea (Keirstead *et al.*, 1992). Inhibitors of regeneration have been proposed to be broadly distributed in myelin and white matter. Multiple inhibitors have been identified in CNS myelin such as Nogo

(Spillmann *et al.*, 1998), myelin-associated glycoprotein (MAG) (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994) and various proteoglycans (Niederost *et al.*, 1999).

1.1.1.1 Nogo

Identification of the growth inhibitory protein Nogo stemmed from the studies of the monoclonal antibody IN-1. Initial biochemical analysis revealed that the inhibitory activity of myelin enriched in the two protein fractions of 35 kDa (NI-35) and 250 kDa (NI-250), which exerted strong inhibitory effects on neurite outgrowth when used as substrates (Caroni and Schwab, 1988a). The monoclonal antibody IN-1 was then raised against purified NI-250 and recognizes both NI-250 and NI-35 (Caroni and Schwab, 1988b). Nogo cDNA, cloned by three groups (Chen *et al.*, 2000; GrandPre *et al.*, 2000; Prinjha *et al.*, 2000), was based on the peptide sequences derived from the purified bovine homolog of NI-250 (Spillmann *et al.*, 1998). The major transcripts (Nogo-A, -B, and –C) encoded by the Nogo gene are generated by both promoter usage of a single gene and alternative spicing (Figure 1.1). Nogo-A appears to be NI-250 and the size of Nogo-B suggests that it might be NI-35. The three transcripts share a common carboxy-terminal of 188 amino acids, and this region is homologous to the reticulon protein family. The carboxyl portion of the protein contains two hydrophobic sequences that are likely to form transmembrane domains, which are separated by an extracellular 66-amino-acid loop (GrandPre *et al.*, 2000). Both the amino-terminal domain (amino-Nogo) (Prinjha *et al.*, 2000) and the 66-amino-acid extracellular fragment (Nogo-66) have inhibitory activity (Fournier *et al.*, 2001). Unlike amino-Nogo, Nogo-66's effect appears to be neuron-specific and it induces growth-cone collapse in embryonic chick dorsal root

ganglion (DRG) neurons. The independent action of these two domains suggests a synergistic effect on axonal outgrowth.

The functional receptor for Nogo-66 (NgR) has been identified recently (Fournier *et al.*, 2001). The cDNA sequence for NgR encodes a 473 amino acid protein (Figure 1.1). The amino-terminal of NgR encodes a conventional translocation signal sequence, followed by eight leucine-rich repeat (LRR) motifs and an LRR carboxy-terminal motif (LRRCT). At the C terminus is a glycosylphosphatidylinositol (GPI) linkage. By analog of other GPI-linked receptors, it is likely that NgR is associated with a separate transmembrane protein to transduce signals. NgR is expressed predominantly in the brain and by a wide variety of CNS neurons. Although the functional receptor and signaling components of amino-Nogo have not been determined, it is speculated that amino-Nogo could possibly act by a mechanism independent of NgR since amino-Nogo interacts with diverse cell types and has a distinctive amino acid composition that is rich in proline and negative charges (Brittis and Flanagan, 2001).

Nogo is present in the myelinating cells of the CNS but not in the PNS. It is an important component of CNS myelin. Addition of the IN-1 antibody (Caroni and Schwab, 1988a) or the new antibodies raised against Nogo-A (Chen *et al.*, 2000) neutralized much of the *in vitro* inhibitory activity of CNS myelin, providing persuasive evidence of the inhibitory effect of Nogo. More importantly, *in vivo*, IN-1 treatment of rats with spinal cord injury induced an improvement in regeneration of corticospinal axons (Schnell and Schwab, 1990), as well as enhanced recovery of behavioral function in some cases (Bregman *et al.*, 1995). Despite considerable long-distance growth achieved after IN-1

treatment, at most, only 5-10% of axons regrew, suggesting that myelin is likely to contain inhibitors other than Nogo.

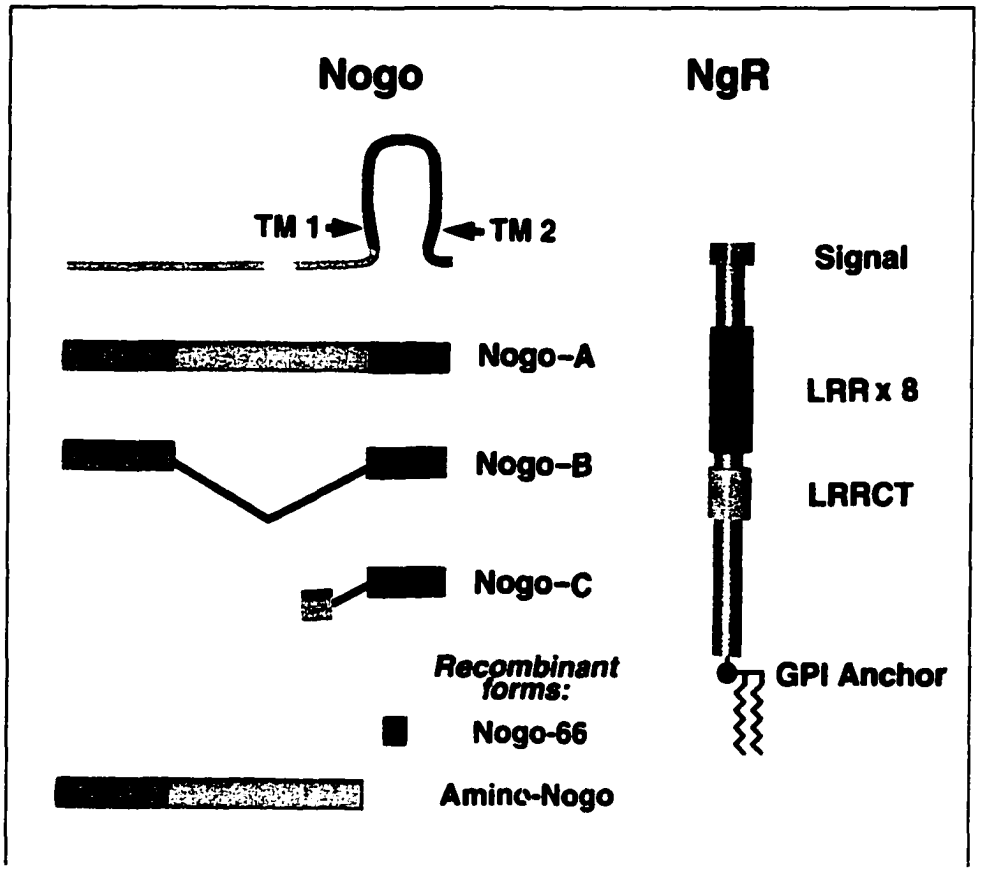


Figure 1.1 Structure of Nogo isoforms and the NgR. Three Nogo isoforms, Nogo-A, -B and –C are generated by alternative RNA splicing or promoter usage of a single gene. A model for Nogo membrane topology is illustrated at the top. The region common to all three isoforms is the two transmembrane domains (TM 1 and TM2) and the extracellular loop between them. Nogo-66 and amino-Nogo are recombinant fragments which show *in vitro* inhibitory activity. NgR is a receptor that mediates inhibition by Nogo-66. NgR contains a translocation signal sequence (Signal), eight Leucine-rich repeat motifs (LRR), an LRR carboxy-terminal motif (LRRCT), and a GPI anchor (Adapted from Brittis and Flanagan, 2001).

1.1.1.2 MAG

MAG was the first myelin-derived growth inhibitory protein to be identified (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). A member of the Siglec family,

MAG is a sialic acid-binding protein (Kelm *et al.*, 1994) containing five immunoglobin (Ig)-like domains in its extracellular sequence (Lai *et al.*, 1987; Salzer *et al.*, 1987). MAG is present in both CNS and PNS myelin. It is a bifunctional molecule, promoting young neurons and inhibiting older neurons in neurite outgrowth (DeBellard *et al.*, 1996; Mukhopadhyay *et al.*, 1994).

MAG is a very potent inhibitor of axonal growth *in vitro* (Filbin, 1995; Filbin, 1996; McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Tang *et al.*, 1997a; Tang *et al.*, 1997b). A soluble and proteolytic fragment of MAG, which encompasses the entire extracellular region, is released from damaged myelin and strongly inhibits neurite outgrowth (Tang *et al.*, 1997b). This fragment of MAG is likely to play an important role in preventing regeneration immediately after injury (Tang *et al.*, 2001). The extent to which MAG inhibits growth *in vivo* has been controversial, due to inconsistency of the studies performed with MAG knockout mice: one group was able to show a small improvement in regeneration in MAG -/- mice (Li *et al.*, 1996) but others observed no change (Bartsch *et al.*, 1995). Regardless of the differences between these studies, regeneration *in vivo* of these MAG -/- mice was poor, probably because of the presence of other inhibitors.

Despite conflicting findings about regeneration in the MAG -/- mice, two recent studies in the PNS demonstrated that MAG can inhibit axonal regeneration *in vivo*. The C57BL/Ola mice that undergo Wallerian degeneration very slowly exhibit impaired regeneration probably due to the presence of myelin debris (Brown *et al.*, 1992). Interestingly, improved regeneration was reported for C57BL/Ola mice that had been crossed with MAG -/- mice (Schafer *et al.*, 1996). Another study was the generation of

transgenic mice that express MAG under the control of p75 promoter (DeBellard *et al.*, 1999). After PNS injury, endogenous p75 is up-regulated and so too in these mice is expression of MAG by Schwann cells. In contrast, wild-type mice down-regulate MAG and other myelin proteins after injury. Significantly, PNS regeneration in these transgenic mice is retarded compared to their wildtype equivalent.

Even though the putative functional receptor for MAG is still elusive, studies have shown that MAG can bind to sialoglycoproteins (DeBellard *et al.*, 1996) and specific gangliosides bearing terminal α2–3-linked sialic acid such as GD1a and GT1b (Yang *et al.*, 1996a). Furthermore, antibody cross-linking of cell surface GT1b mimics the effect of MAG on neurite outgrowth (Vinson *et al.*, 2001). The MAG receptor may also be associated with a heterotrimeric G protein and can therefore affect the levels of second messengers in signaling cascades (Cai *et al.*, 1999). As with different binding and inhibition sites shown for Nogo (GrandPre *et al.*, 2001), in MAG the neuronal binding site and the inhibition site are separate (Filbin, 1996). Arg118 in the extracellular domain 3 of MAG is crucial for sialic acid binding because mutation of this site abolishes binding completely but has no effect on axon outgrowth (Tang *et al.*, 1997a). Nevertheless, the extracellular domains 4 and 5 are required for inhibition of neurite outgrowth (Cao and Filbin, unpublished data). Experiments are currently underway in our lab to identify the exact inhibition site(s) of MAG within these two domains.

1.1.1.3 Other myelin-associated inhibitors

Besides Nogo and MAG, there are a number of myelin-related or glial-derived inhibitory molecules present in the adult CNS. Several inhibitory proteoglycans that

associate with myelin were identified, although their role in myelin is not clearly understood. NG_2 proteoglycan expressed by the oligodendrocyte precursors (Dou and Levine, 1994; Levine and Nishiyama, 1996) and two chondroitin sulfate proteoglycans (CSPGs), brevican and versican present in purified CNS myelin (Niederost *et al.*, 1999) all exhibit potent inhibition of neurite outgrowth. In addition, Janusin/restrictin, a tenascin-related extracellular matrix molecule, is secreted mainly by oligodendrocytes and is repulsive towards growth cones when used as a substrate *in vitro* (Pesheva *et al.*, 1989). Finally, a preliminary report revealed an inhibitory molecule present in myelin called arretin (Xiao, 1997), but the identity of this molecule has not yet been described.

1.1.2 Glial scar

Following CNS injury, glia and other non-neuronal cells infiltrate the central area of necrosis (Ramon y Cajal, 1928). The fibrous glial scar in the lesion site usually takes weeks to be fully formed and is contributed to by several cell types. Reactive astrocytes undergo a morphological change by extending tightly interlinked processes. Meningeal cells invade the scar region to line the lesion cavity (Fawcett and Asher, 1999), coupled with invasion of blood vessels after injury of the spinal cord (Imperato-Kalmar *et al.*, 1997). In addition to forming a physical barrier to prevent injured axons from regrowing, the glial scar is also able to create a chemical barrier by the up-regulation of inhibitory proteins of axonal outgrowth, such as tenascin, keratin, semaphorin-3A and different types of CSPGs (Canning *et al.*, 1996; Davies *et al.*, 1999; Letourneau *et al.*, 1994; McKeon *et al.*, 1995; Pasterkamp *et al.*, 1999), within hours to days after injury. The

biochemical changes at the lesion site present a non-permissive environment for regeneration before the actual physical obstacle is fully shaped.

1.1.3 Other repulsive factors

Other potential inhibitors of axon regeneration are molecules initially identified as guidance factors that are expressed during development. The collapsins/semaphorins family of secreted and transmembrane glycoproteins function as chemorepellents during development (Kolodkin *et al.*, 1993; Luo *et al.*, 1993; Luo *et al.*, 1995). Among them, sema3A appears to play a role in regeneration of olfactory receptor neurons (Pasterkamp *et al.*, 1999; Williams-Hogarth *et al.*, 2000). In addition, Netrins are secreted by the floor plate, and guide commissural axons toward the ventral midline (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). They can induce either attractive or repulsive responses in different types of neurons *in vitro* (Hamelin *et al.*, 1993; Hedgecock *et al.*, 1990), or in the same neuron depending on the intracellular levels of cyclic nucleotide (Ming *et al.*, 1997). Similarly, slits are extracellular matrix proteins expressed by midline glia and prevent axons from crossing the midline during development (Brose *et al.*, 1999; Kidd *et al.*, 1999; Wang *et al.*, 1999). They can repel spinal motor, olfactory and hippocampal axons and collapse their growth cones *in vitro* (Li *et al.*, 1999; Nguyen Ba-Charvet *et al.*, 1999). Finally, ephrins bind to ephrin receptors and have been shown to be involved in axonal outgrowth and pathfinding (Holder *et al.*, 1998). EphB3 has been reported to be up-regulated in white matter astrocytes following injury (Miranda *et al.*, 1999). The role of all these guidance factors in regeneration has not yet been elucidated. However, the expression levels and their localization in injury models suggest a role in axonal

regeneration (Luo *et al.*, 1995; Madison *et al.*, 2000; Pasterkamp *et al.*, 1998a; Pasterkamp *et al.*, 1998b).

In summary, myelin-associated inhibitors in the damaged CNS, formation of a glial scar and existence of other repulsive molecules create hostile circumstances for axonal regeneration in the CNS. An interesting question concerning the unfavorable environment of the CNS is: is the normal CNS tissue inhibitory, or just the reactive area surrounding an injury? Work from Silver's group (Davies *et al.*, 1997) suggests an encouraging possibility that the normal, undamaged white matter in the adult CNS appears to be permissive, as long as the axons can get through the developing reactive area before it becomes inhibitory. However, to effectively promote regeneration of injured axons, we still need to overcome the non-permissive surroundings in the damaged CNS.

1.2 Intrinsic neuronal capacity

Adult CNS axons fail to upregulate some of the growth-associated proteins that are expressed during development and during successful regeneration (Fu and Gordon, 1997). Axon-growth-promoting cues such as neurotrophic factors, which mediate cell survival and neurite outgrowth during development, are often absent following injury (Davies, 1994). The intrinsic state of the neuron appears to dictate its response to the unfavorable environment of the damaged adult CNS.

Embryonic neurons, when transplanted into the adult spinal cord, undergo long-distance axon growth and presumably possess a different growth capacity from their adult

counterparts (Bates and Stelzner, 1993; Hasan *et al.*, 1993). The inability of adult CNS neurons to regenerate compared to young ones implies that at some time during developmentm, the CNS neurons lose the intrinsic capacity to regrow.

A model which provides important insights into the intrinsic mechanisms that prevent CNS regeneration is the damage to the axons of primary sensory neurons. The cell bodies of these neurons lie in the DRG and have axonal branches in the PNS and the CNS. The central and peripheral projections have different regenerative capacities after injury. Axotomy of peripheral nerves is followed by regeneration and target re-innervation, while lesion of the central projection results in a complete lack of regeneration (Ramon y Cajal, 1928). More strikingly, peripheral nerve injury changes the response of the DRG cell body. DRG neurons switch from neurotransmission to regeneration accompanied by dramatic changes in gene expression. These changes include the up-regulation of regeneration-associated immediate-early genes as well as induction of the growth-associated proteins (Andersen and Schreyer, 1999). In contrast, following lesions to the central branch of the DRG, the changes displayed by the peripheral injury are muted (Chong *et al.*, 1996). Thus the weak intrinsic regenerative capacity of CNS axons contributes to their failure to regenerate after axotomy. The association of a robust cell body response with regeneration has led to studies in which "conditioning" peripheral nerve lesions have been used in attempts to augment central regrowth following injury (Neumann and Woolf, 1999; Oudega *et al.*, 1994; Richardson and Issa, 1984; Richardson and Verge, 1986). Neumann and Woolf demonstrated that a prior, conditioning lesion to the peripheral branch of DRG neurons resulted in a remarkably robust, long distance regeneration in the lesioned CNS branch of the same

neurons (Neumann and Woolf, 1999). Impressively, a substantial number of fibers grew past the lesion in the spinal cord without the addition of agents that block myelin inhibitors (Figure 1.2). It seemed that the growth state of the DRG neurons had been altered by the peripheral-branch-conditioning lesion. As a result, the unfavorable environment no longer blocked the axonal growth of the CNS axons.

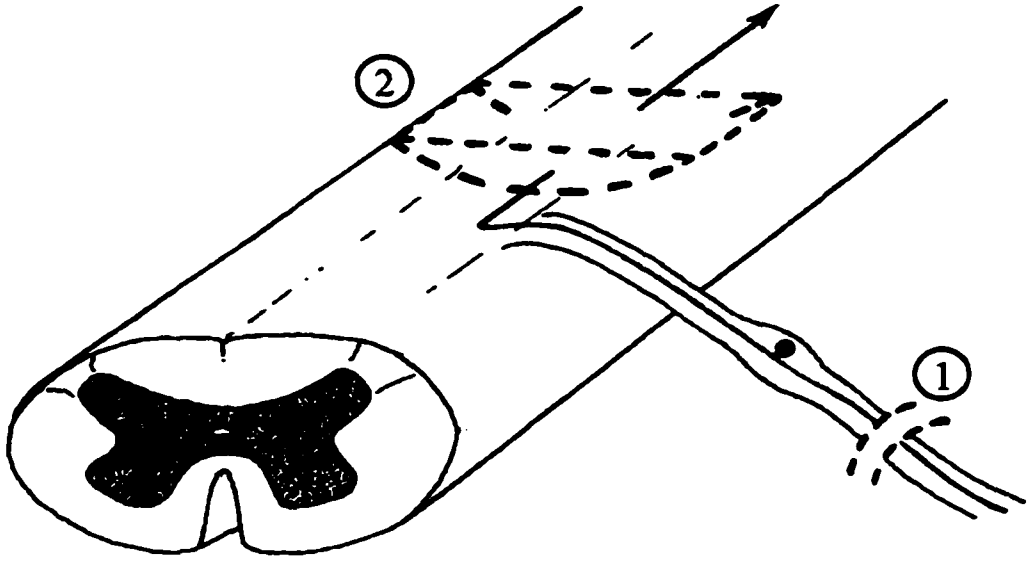


Figure 1.2 Sites of preconditioning peripheral branch lesion and subsequent central branch lesion in DRG nerves.
Preconditioning lesions were created in the sciatic nerve (L4-L6) (1) either simultaneously with, or 1 or 2 weeks prior to, a dorsal column transection at T6-T7 (2). Regeneration occurred through the dorsal column lesion site if the preconditioning lesion preceded the dorsal column lesion by 1 or 2 weeks; regrowth occurred only into the lesion if the preconditioning lesion was inflicted at the same time; no regeneration occurred without a pheripheral lesion. The dashed line shows the sites of lesions. (Adapted from Filbin, 1999)

In both embryonic neurons and those primed by a conditioning lesion, the state of second messengers or intracellular signaling molecules may differ from that in an injured adult neuron, and therefore may promote growth in an otherwise inhibitory environment.

Several recent studies have demonstrated that the intracellular levels of cyclic nucleotide can dictate the neuronal response to guidance cues and neurotrophic factors, and in particular, influence the ability of mature CNS neurons to initiate and maintain a regrowth response (Cai *et al.*, 2001; Cai *et al.*, 1999; Kim and Wu, 1996; Lohof *et al.*, 1992; Ming *et al.*, 1997; Song *et al.*, 1998; Song *et al.*, 1997). We have shown that MAG/myelin-dependent inhibition can be overcome by priming neurons with neurotrophins and that the effect is mediated by activating the cAMP-PKA (protein kinase A) pathway (Cai *et al.*, 1999). Moreover, the endogenous neuronal cAMP levels are elevated after a conditioning lesion. In addition, there are higher cAMP levels in embryonic neurons than in the adult (Cai *et al.*, 2001). Altogether, it seems that the state of intracellular molecules such as cyclic nucleotides may indeed dictate a neuron's growth response when it is exposed to inhibitory conditions.

In summary, the lack of axon regeneration in the adult mammalian CNS is due to both an extrinsic unfavorable environment and a poor intrinsic neuronal state. Inhibitors associated with myelin and the glial scar contribute largely to the non-permissive conditions that prevent injured axons to regrow. Meanwhile, the intrinsic neuronal state, especially the intracellular levels of cyclic nucleotides, may control the neuron's ability to extend axons in an inhibitory environment. Thus, for regeneration to be successful, we need to develop strategies addressing both problems and try to overcome these obstacles simultaneously.

1.3 Approaches to stimulate regeneration in the spinal cord

It is estimated that more than 250,000 Americans have spinal cord injuries, with 11,000 new cases every year (Berkowitz *et al.*, 1998). Although there are no effective clinical treatments for injuries beyond limiting the immediate effects of trauma with steroids, recent advances and new discoveries in animal models show that novel treatments for anatomical regeneration and functional recovery might soon emerge.

1.3.1 Blocking myelin-associated inhibitors with antibodies

Blocking myelin-associated inhibitors with antibodies to encourage axon regeneration in the CNS was first attempted by using the IN-1 antibody in the spinal cord (Schnell and Schwab, 1990). Hybridoma cells secreting the IN-1 antibody against high molecular weight myelin proteins, were implanted in the brain. By this approach, after spinal cord lesion, long-distance growth of corticospinal axons and improvements in functional recovery were observed. However, the number of axons that regenerated was quite small, only about 5-10%. This is likely to be because other inhibitors exist in the CNS and the effects of all these inhibitors are distinct from one another. The presence of any one can effectively inhibit growth. Nonetheless, neutralizing at least some of the inhibitors, in the case of using IN-1 antibody, can lead to improved growth (Bregman *et al.*, 1995).

More encouraging results were obtained by David and colleagues employing a novel vaccination approach (Huang *et al.*, 1999). They used the animal's own immune system to produce antibodies against all of the spinal cord derived inhibitors of regeneration. Adult mice were immunized over a 3-week period with a CNS myelin

preparation. Vaccination led to remarkably robust regeneration of corticospinal tract (CST) fibers in the immunized mice (Figure 1.3). Compared to application of the IN-1 antibody to a transected rat spinal cord, the distance of regeneration in this model is similar. But many more axons, about two-thirds of the entire spinal cord, regrew in the myelin-immunized mice. Future directions for this approach will be to immunize animal at the same time as the spinal cord injury. Alternatively, giving antibodies against all myelin inhibitors by passive immunization at the time of lesioning might compensate for the interim period required for endogenous antibody production, thus allowing axons to grow before glial scar formation.

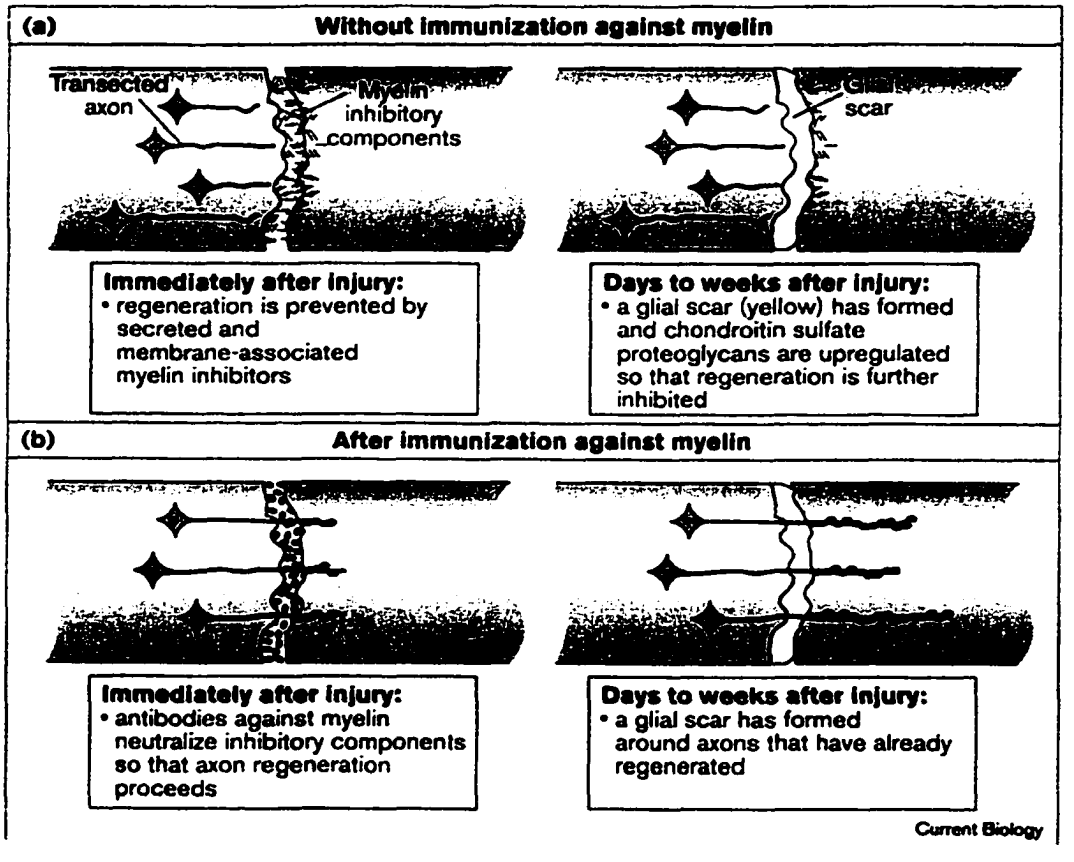


Figure 1.3 Axon regeneration in the spinal cord (a) without and (b) after immunization against myelin. As recently reported by Huang *et al.* (1999), immunization of mice against myelin allows axon regeneration over long distances; in the absence of immunization, no regeneration is seen. (Adapted from Filbin, 2000)

Besides blocking inhibition pathways to promote regeneration, neutralization of myelin inhibitors may promote functional recovery by enhancing CNS plasticity, forming new pathways which compensate for those lost after block of inhibition (Z'Graggen *et al.*, 2000). Together these results suggest that blocking myelin-associated inhibitors of regeneration exhibits great potential for treating humans with spinal cord injuries.

1.3.2 Altering the intrinsic state of neurons

Historically, strategies to overcome inhibition have focused on altering the inhibitory environment of the injured axon. However, the injured cell body may present a more accessible target for treatment following injury. Enhancing the intrinsic growth state of neurons to allow axons to grow directly on the inhibitory substrate of the CNS is a relatively new strategy.

Neurotrophins and growth-associated proteins are absent following injury in the adult CNS. By re-introducing these factors, which are normally present in development and critical for neurite outgrowth, we can improve the growth capacity of the damaged neurons. The use of infusion pumps, viral vector or gene therapy thechniques to deliver growth factors to injured neurons have had some notable success (Blesch *et al.*, 1999; Liu *et al.*, 1999; Ramer *et al.*, 2000). Additionally, co-expressing two major growth cone proteins, GAP-43 (growth-associated protein-43) and CAP-23 (cytoskeleton-associated protein-23), can elicit long axon extension by adult DRG neurons *in vitro* (Bomze *et al.*, 2001). More importantly, this expression triggers a 60-fold increase in regeneration of DRG axons in adult mice after spinal cord injury *in vivo*. Replacing key growth components, therefore, could be an effective way to stimulate regeneration of CNS axons.

Another approach to stimulate regeneration after injury in the CNS is to manipulate the intracellular neuronal signaling mechanisms to all growth cones, forcing them to ignore growth inhibitors and regrow directly on inhibitory substrates. Since myelin inhibitors may share common intracellular effectors, it is possible to use some antagonist to overcome the inhibitory effect of multiple myelin inhibitors. One possible common target is the Rho family of GTPases which are the intracellular regulators of growth cone collapse (Jin and Strittmatter, 1997; Kuhn *et al.*, 1999; Lehmann *et al.*, 1999). PC12 cells treated with C3 toxin to inactivate Rho, or transfected with dominant negative Rho were able to grow on inhibitory substrates. Moreover applying C3 directly in the lesion site of crushed adult rat optic nerves and treatment of C3 in injured adult mouse spinal cord produced regenerated axons traversing long distances (Lehmann *et al.*, 1999). Since cAMP is likely to inhibit the Rho pathway through PKA as shown for nonneuronal cells (Dong *et al.*, 1998; Lang *et al.*, 1996), inactivation of Rho is also in agreement with our recent findings on the role of cAMP in growth inhibitory signaling. Treating neurons with neurotrophins to increase intracellular cAMP levels before they encounter growth inhibitors, or increasing intracellular cAMP artificially, overcomes the inhibitory effect of MAG or myelin (Cai *et al.*, 1999). Direct application of cAMP analogs to neurons without a conditioning lesion can mimic the block of myelin inhibitors induced by a peripheral nerve transection when grown in culture and can also improve axonal regeneration of the damaged adult spinal cord *in vivo* (Qiu *et al.*, 2002). Discovery of the signals responsible for converting a neuron into a highly growing state in the inhibitory environment, such as cAMP and Rho, may provide a means to stimulate older neurons to regenerate after injury.

1.3.3 Transplantation

Spawned by Ramon y Cajal and Tello's classic peripheral nerve transplant experiment (Ramon y Cajal, 1928), success in achieving long distance regeneration in the CNS of adult animals had been demonstrated with a plethora of cellular grafts to replace the inhibitory CNS environment, such as Schwann cells (Guest *et al.*, 1997; Li and Raisman, 1994; Paino *et al.*, 1994; Tuszynski *et al.*, 1998; Xu *et al.*, 1995), fibroblast expressing trophic factors (Blesch *et al.*, 1999; Liu *et al.*, 1999; Nakahara *et al.*, 1996; Tuszynski *et al.*, 1994), fetal spinal cord transplants (Bregman *et al.*, 1993; Diener and Bregman, 1998), macrophages (Lazarov-Spiegler *et al.*, 1996), embryonic stem cells (McDonald *et al.*, 1999) and olfactory ensheathing cells (OECs) (Li *et al.*, 1997; Ramon-Cueto *et al.*, 2000; Ramon-Cueto *et al.*, 1998). Transplantation of cells to the damaged spinal cord serves as a bridge across lesion cavities and promotes repair. While transplantation is an excellent experimental model to study regeneration, most axons do not leave their favorable graft environment and enter their natural target region in the spinal cord. One of the more effective procedures for achieving regeneration in the CNS is the transplantation of cells expressing neurotrophins (Bregman *et al.*, 1998), most likely due to the priming effect that neurotrophins provide in overcoming the inhibitory effects of myelin (Cai *et al.*, 1999). Another case where axons extend long distances out of the initial transplant region is bridging grafts that "capped" both ends of a Schwann cell-containing tube graft with OECs to render closer the transition from the PNS to the CNS (Ramon-Cueto *et al.*, 1998). As a result, more damaged axons breached the graft-host boundary and regrew across the lesion site without contacting the glial scar. OECs are the principal glial cell of the peripheral olfactory system where axon growth occurs

not only in response to injury but also occurs as a normal physiological process throughout the lives of healthy individuals. OECs share properties with both Schwann cells and astrocytes (reviewed by Doucette, 1990). The OECs not only provide a permissive environment themselves but also reduce the degree of glial scarring, which together allow more regenerating axons to grow into and out of the graft (Ramon-Cueto *et al.*, 1998). The transplanted OECs also appear to accompany the regenerating axons into the host tissue, migrating further within the CNS than transplanted Schwann cells (Li *et al.*, 1998).

Advances in animal models of spinal cord injury suggest the potential for different strategies in regeneration and functional recovery. Because each of these approaches addresses one of several different parameters of regeneration, it might be reasonable to suppose that a combination of all strategies would result in substantial success in regeneration. Then, the next and more challenging step will be to guide the regenerating axons to their appropriate postsynaptic target to achieve successful functional recovery.

2. Neurotrophic factors

Neurotrophic factors are growth factors whose activity is restricted to neuronal cells. This distinguishes them from other growth factors (e.g. platelet-derived growth factor and fibroblast growth factor) which have pleiotropic activities and are often studied in systems other than the nervous system (Eckenstein, 1994; Patterson, 1992; Westermark and Heldin, 1993). Neurotrophic factors play a critical role in the

development and maintenance of the mammalian nervous system, exemplified by the members of the nerve growth factor (NGF) family, also known as "neurotrophins" (Barde, 1994; Levi-Montalcini, 1998), the glial-derived neurotrophic factor (GDNF) family ligands (GFLs) (Lin *et al.*, 1993) and ciliary neurotrophic factor (CNTF) (Sendtner *et al.*, 1994). All these neurotrophic factors contribute to neuronal survival and differentiation.

2.1 Neurotrophic factors and their receptors

Each of these neurotrophic factors exerts its biological activities through a different class of receptors. The NGF family signal through the Trk (tropomyosin-related kinase) family of tyrosine kinase receptor (Barbacid, 1994; Chao, 1992; Meakin and Shooter, 1992) and interact with a second receptor, p75 (Chao, 1994). Both GFLs (Lindsay and Yancopoulos, 1996; Mason, 1996) and CNTF signal through a multimeric receptor complex that consists of binding and signaling components.

2.1.1. The NGF family

NGF, the prototypic member of the family, was discovered in the 1940s and purified in the 1960s. A second member of the NGF family was identified in the 1980s and named the brain-derived neurotrophic factor (BDNF). The cloning of BDNF led within a short time to the cloning of neurotrophin-3 (NT-3) and NT-4/5, which are the final known mammalian members of the family. Members of the NGF family share a significant level of homology to one another and modulate generally similar types of biological responses, such as neuronal survival, differentiation, neurite outgrowth, sprouting, synaptic activity, gene regulation and mitogenesis (for reviews see

Chalazonitis, 1996; Conover and Yancopoulos, 1997; Levi-Montalcini, 1998; Lewin and Barde, 1996; Lu and Figurov, 1997; Rodriguez-Tebar *et al.*, 1991; Schuman, 1999).

The NGF family binds specifically to the three known Trk tyrosine kinase receptors, TrkA, TrkB and TrkC. Whereas NGF binds to TrkA, BDNF and NT4/5 interact with TrkB. NT-3 appears to bind to each of the Trk receptors, but its primary biological responses are mediated by TrkC (Barbacid, 1995; Ip and Yancopoulos, 1994). In addition, each of the NGF family of neurotrophins binds to the p75 neurotrophin receptor (p75NTR), a receptor structurally unrelated to the Trks and a member of the tumor necrosis factor receptor superfamily (Chao, 1994). Trks and p75NTR, often present on the same cell, coordinate and modulate the responses of neurons to neurotrophins. The functions of these two receptors vary markedly. While Trk receptors transmit positive signals such as enhanced survival and growth, p75NTR transmits both positive and negative signals depending on the co-existence of the Trk receptors. Trk and p75NTR either collaborate with or inhibit each other's actions to mediate neurotrophin effects (for reviews see Friedman and Greene, 1999; Kaplan and Stephens, 1994).

Generation of knockout mice carrying mutations in these genes also provides important evidence for the function of the Trk receptor and p75NTR *in vivo*. The striking similarities between the phenotypes of mice defective for each Trk receptor and its cognate neurotrophin, compellingly support the idea that Trk receptors mediate most, if not all, the biological activities of the neurotrophins (Snider, 1994). Mice defective for either NGF (Crowley *et al.*, 1994) or the TrkA receptor (Smeyne *et al.*, 1994) display severe sensory defects characterized by a complete loss of nociceptive activity. A similar correlation can be found between the phenotypes of mice deficient in NT-3 (Ernfors *et al.*,

1994b; Farinas *et al.*, 1994) and TrkC (Klein *et al.*, 1994) except that they exhibit normal nociception, but are defective in proprioception. TrkB mutant (Klein *et al.*, 1993) and BDNF-defective (Ernfors *et al.*, 1994a; Jones *et al.*, 1994) mice have short life spans. BDNF-defective mice also show defective movement coordination and balance. The phenotypic effects of these mutant mice in the CNS are not notable, probably due to the trophic redundancy in the CNS neurons (Snider, 1994). Meanwhile, genetic disruption of the p75NTR gene in mice revealed sensory and sympathetic defects, demonstrating that the p75NTR has an essential role in neuronal development (Lee *et al.*, 1992). However, these mice do not display the defects characteristic of mice lacking BDNF/NT-3, and their cognate receptors TrkB/TrkC. The observed defects in the p75 null mice appear to be limited to NGF-dependent neurons (Lee *et al.*, 1994), and these defects are much more restricted than those observed in either NGF or TrkA mutant mice. These indicate that p75NTR may not be involved in mediating the biological activity of all members of the NGF family *in vivo*.

Analysis of germ-line mutations in each member of the neurotrophin and Trk families demonstrates not only the necessity for both the neurotrophins and the Trks in the PNS, but also that each neurotrophin and its receptor support specific subpopulations of neurons (Conover and Yancopoulos, 1997). Cells that are responsive to NGF are quite limited, as opposed to the more numerous neuronal populations that are dependent upon BDNF, NT-3 and NT-4/5. During development, expression of *trkA* is limited to sensory and sympathetic neurons in the PNS and cholinergic neurons of the basal forebrain (Holtzman *et al.*, 1992; Martin-Zanca *et al.*, 1990), while more extensive CNS expression

is found for *trkB* and *trkC* (Barbacid, 1994). The p75NTR has a much wider distribution and is expressed on numerous cell types (Bothwell, 1991; Thomson *et al.*, 1988).

2.1.2 The GDNF family

GDNF was first discovered as a potent survival factor for midbrain dopaminergic neurons (Lin *et al.*, 1993). It promotes survival in a wide spectrum of neuronal cell types, which include motor, dopaminergic and adrenergic neurons of the CNS, autonomic, enteric and subsets of sensory neurons of the PNS and the epithelial cells of the ureteric bud and its branches (Arenas *et al.*, 1995; Buj-Bello *et al.*, 1995; Hearn *et al.*, 1998; Henderson *et al.*, 1994; Heuckeroth *et al.*, 1998; Lin *et al.*, 1993; Oppenheim *et al.*, 1995; Trupp *et al.*, 1995). GDNF is approximately 20 kDa in size and shows remarkable cross-species amino-acid sequence homology, with 93% identity between rat and human GDNF (Lin *et al.*, 1993).

The other three members in the GDNF family (or GFLs), neurturin (NTN), artemin (ART) and persephin (PSP), were discovered by research groups led by Drs. J. Milbrandt and E. M. Johnson. NTN was purified as a survival factor for sympathetic neurons (Kotzbauer *et al.*, 1996), after which ART (Baloh *et al.*, 1998b) and PSP (Milbrandt *et al.*, 1998) could be identified on the basis of sequence homology. GFLs are related to the transforming growth factor-β (TGF-β) superfamily, possessing seven conserved cysteine residues. NTN and ART have many neurotrophic effects similar to GDNF; they all support the survival of peripheral sympathetic and sensory neurons as well as midbrain dopamine neurons (Baloh *et al.*, 1998b; Horger *et al.*, 1998; Kotzbauer

et al., 1996). PSP is expressed at low levels in most tissues and supports CNS dopamine and motor neurons, but not peripheral neurons (Milbrandt *et al.*, 1998).

GDNF signals through a novel receptor complex (Lindsay and Yancopoulos, 1996; Mason, 1996; Olson, 1997). It consists of the transmembrane Ret (Rearranged in Transformation) receptor tyrosine kinase (Durbec *et al.*, 1996; Trupp *et al.*, 1996; Worby *et al.*, 1996) and a ligand-binding, GPI-linked component, the GDNF-family receptor α1 (GFRα1) (Jing *et al.*, 1996; Treanor *et al.*, 1996).

Like the NGF family, all four known GFLs have their own preferred coreceptors. NTN signals preferentially via GFRα2 receptor (Buj-Bello *et al.*, 1997; Klein *et al.*, 1997), while ART signals through GFRα3 (Baloh *et al.*, 1998a), a more distantly related GFR receptor. PSP has been reported to bind to GFRα4 (Enokido *et al.*, 1998), a receptor only identified thus far in chicken (Thompson *et al.*, 1998). Besides binding to its own preferred coreceptor, cross talk between different ligands and receptors is apparent (Baloh *et al.*, 1998a; Baloh *et al.*, 1998b; Buj-Bello *et al.*, 1997; Creedon *et al.*, 1997; Jing *et al.*, 1997; Sanicola *et al.*, 1997; Trupp *et al.*, 1998). GFRα1 seems to be the more promiscuous coreceptor, because it can also bind and mediate the survival effects of ART (Baloh *et al.*, 1998b), in addition to NTN and its preferred GDNF.

Mice lacking Ret (Durbec *et al.*, 1996; Schuchardt *et al.*, 1994), GDNF (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996), or GFRα1 (Cacalano *et al.*, 1998; Enomoto *et al.*, 1998) all die soon after birth and share a similar phenotype of kidney agenesis and absence of enteric neurons below the stomach. This suggests a tight coupling of GDNF and GFRα1 in Ret signaling *in vivo*. In the mice lacking different

GFLs, only minor phenotypic changes have been found in the CNS so far, supporting the idea of trophic redundancy for CNS neurons (Snider, 1994).

2.1.3 CNTF

CNTF was first identified as a trophic factor in chick eye and nerve extracts that supported the survival of ciliary ganglionic neurons *in vitro* (Ip and Yancopoulos, 1996; Segal and Greenberg, 1996). It is a cytosolic protein that lacks a consensus sequence for secretion and is consequently thought to be released only by damaged cells (Sendtner *et al.*, 1994; Stockli *et al.*, 1989). It promotes the differentiation and survival of a wide range of cell types in the peripheral and central nervous systems. CNTF prevents the degeneration of axotomized motor neurons (Curtis *et al.*, 1993) and attenuates the motor deficits found in different strains of mice with neuromuscular deficiencies (Mitsumoto *et al.*, 1994; Sendtner *et al.*, 1992). In addition to its activities in the nervous system, CNTF is trophic for denervated skeletal muscle (Helgren *et al.*, 1994) and regulates muscular strength in aging (Guillet *et al.*, 1999).

CNTF is related to the interleukin-6 (IL-6) family of cytokines. These cytokines share receptor components, resulting in some functional redundancy throughout the family (Taga and Kishimoto, 1997). The multimeric receptor complex of CNTF consists of a GPI-anchor protein as a binding subunit, also referred to as CNTFR (Davis *et al.*, 1991). The signaling subunits of the receptor complex include gp130 (Kishimoto *et al.*, 1992) and the β-subunit of the receptor for leukemia inhibitory factor (LIFRβ) (Gearing *et al.*, 1991). CNTFR confers selectivity for CNTF upon the receptor complex. Without CNTFR , the remaining two subunits constitute a receptor for LIF, whereas two gp130

subunits plus the IL-6 receptor form the functional receptor complex for interleukin-6. This scheme of mixing and matching various receptor subunits produces a range of receptor complexes from a relatively limited set of receptor components (Ip and Yancopoulos, 1996; Segal and Greenberg, 1996).

The existence of another CNTF-like factor was suggested by comparing the phenotypic consequences of disrupting CNTF versus CNTFR (DeChiara *et al.*, 1995). Although CNTF –/– mice appear largely normal except for a mild phenotypic change involving motor neuron degeneration, prevalent postnatally (Masu *et al.*, 1993), *CNTFR* disruption is devastating. Deletions in the motor nuclei cause perinatal death from the inability to suckle (Takahashi *et al.*, 1994). Recently, a new member of the IL-6 class of cytokines, cardiotrophin-like cytokine (CLC) or novel neurotrophin-1 (NNT-1) (Senaldi *et al.*, 1999), was identified (Elson *et al.*, 2000). CLC together with another secreted protein, cytokine-like factor-1 (CLF) (Elson *et al.*, 1998) form a complex and act as a ligand for the CNTF receptor complex. More importantly, CLC–CLF competes with CNTF for binding to the CNTF receptor and can promote the survival of embryonic motor neurons *in vitro*, a hallmark of the biological activity of CNTF (Elson *et al.*, 2000). Genetic disruption of CLF leads to perinatal death due to a suckling defect (Alexander *et al.*, 1999) which resembles the phenotype of the CNTFR knockout. This suggests that CLC-CLF is an excellent candidate for the elusive CNTF II.

2.2 Signal transduction of neurotrophic factors

The identification of intracellular signaling pathways for the NGF family of neurotrophins originated from studies in PC12 cells. PC12 cells are derived from a rat

pheochromocytoma and mimic the *in vivo* response to NGF, thus providing a model system for studying the NGF family (Greene and Tischler, 1976; Tischler and Greene, 1975). In recent years, studies using primary neurons and *in vivo* systems have unveiled a new stage for learning the signaling mechanisms employed by neurotrophins.

2.2.1 The NGF family

Early work on PC12 cells provided the initial evidence that NGF binds to the TrkA receptor and follows the general scheme established for the receptor tyrosine kinase. The binding of ligand to the receptor induces receptor dimerization and autophosphorylation (Jing *et al.*, 1992; Kaplan *et al.*, 1991a; Kaplan *et al.*, 1991b). Maximal activation of the TrkA receptor requires the initial autophosphorylation of tyrosine residues 670, 674 and 675 that lie within the kinase activation loop (Cunningham and Greene, 1998). The other two tyrosine (Y) residues, 490 and 785, are autophosphorylated after ligand binding and play direct roles in Trk signaling (Loeb *et al.*, 1994; Obermeier *et al.*, 1994; Stephens *et al.*, 1994) (Figure 1.4). Phosphorylation of the Trk receptor results in recruitment of different adapters, such as Shc (SH2 domain containing protein) / Grb / SOS (son of sevenless) (Dikic *et al.*, 1995; Obermeier *et al.*, 1994; Stephens *et al.*, 1994), FRS-2 (fibroblast growth factor receptor substrate 2) (Easton *et al.*, 1999; Kouhara *et al.*, 1997; Meakin and Shooter, 1992), rAPS (rat adaptor molecule containing PH and SH2 domains), SH2-B (Src homology 2-B) (Qian and Ginty, 2001), CHK (Csk homologous kinase) (Yamashita *et al.*, 1999a), SHP-2 (Src homology 2 phosphatase) (Hadari *et al.*, 1998), Crk (Ribon and Saltiel, 1996; Torres and Bogenmann, 1996), Gab-1 (Grb-associated binder-1) (Holgado-Madruga *et al.*, 1997) and IRS (insulin

receptor substrate)-1/2 (Yamada *et al.*, 1997) to the docking sites on the receptor. Shc and FRS-2 bind to phosphorylated Y490 of TrkA, while PLCγ (phospholipase C-gamma) and CHK bind to phosphorylated Y785 (Kaplan and Miller, 1997; Yamashita *et al.*, 1999a). A combination of different adapters determine the downstream signaling pathways mediated by neurotrophins (Figure 1.4). Thus far, several downstream cascades have been defined that play major roles in the neurotrophin signaling that regulates survival and neurite outgrowth in neuronal cells. Foremost among these is the Ras/Raf/MEK/MAPK (mitogen-activated protein kinase) pathway which plays a major role in mediating neuronal survival, neurite outgrowth and other responses to neurotrophins (for reviews see Chao *et al.*, 1998; Frade and Barde, 1998; Greene and Kaplan, 1995; Kaplan and Miller, 1997; Klesse and Parada, 1999; Segal and Greenberg, 1996). Additionally, a second pathway leading to sustained activation of MAPK or ERK (extracellular signal regulated kinase) involves Crk/C3G/Rap1/bRaf (Matsuda *et al.*, 1994; York *et al.*, 1998). Inclusion of this second pathway to activate the same signaling molecule ensures a robust and sustained elevation of ERK in response to neurotrophins as well as the possibility of subtle modulation. Another important downstream element in neurotrophin signaling is PI3K (phosphatidylinositol-3-kinase). Trk activates PI3K through the Ras and the Gab-1/IRS-1/IRS-2 family of adapter proteins (Holgado-Madruga *et al.*, 1997; Korhonen *et al.*, 1999; Yamada *et al.*, 1997). PI3K stimulates the activities of PDK (3-phosphoinositide-dependent kinase) 1/2, which in turn activate Akt (or protein kinase B) (Chan *et al.*, 1999; Datta *et al.*, 1999; Vanhaesebroeck and Alessi, 2000). The Ras/PI3K/Akt pathway is a major regulator of neuronal survival (Kaplan and Miller, 2000). Akt may suppress apoptosis directly by inhibiting the activities of

Forkhead or Bad (Bcl-2/Bcl-x-associated death promoter), the apoptotic pathways in primary neurons (Brunet *et al.*, 1999; Datta *et al.*, 1997; del Peso *et al.*, 1997; Paradis and Ruvkun, 1998). The third well-established downstream mediator of neurotrophin action is PLCγ which in turn affects intracellular calcium levels as well as PKC (protein kinase C) (Obermeier *et al.*, 1994; Stephens *et al.*, 1994). Other molecules such as SNT (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target) (Peng *et al.*, 1995; Rabin *et al.*, 1993) and Src family members may also contribute to neurotrophin signaling.

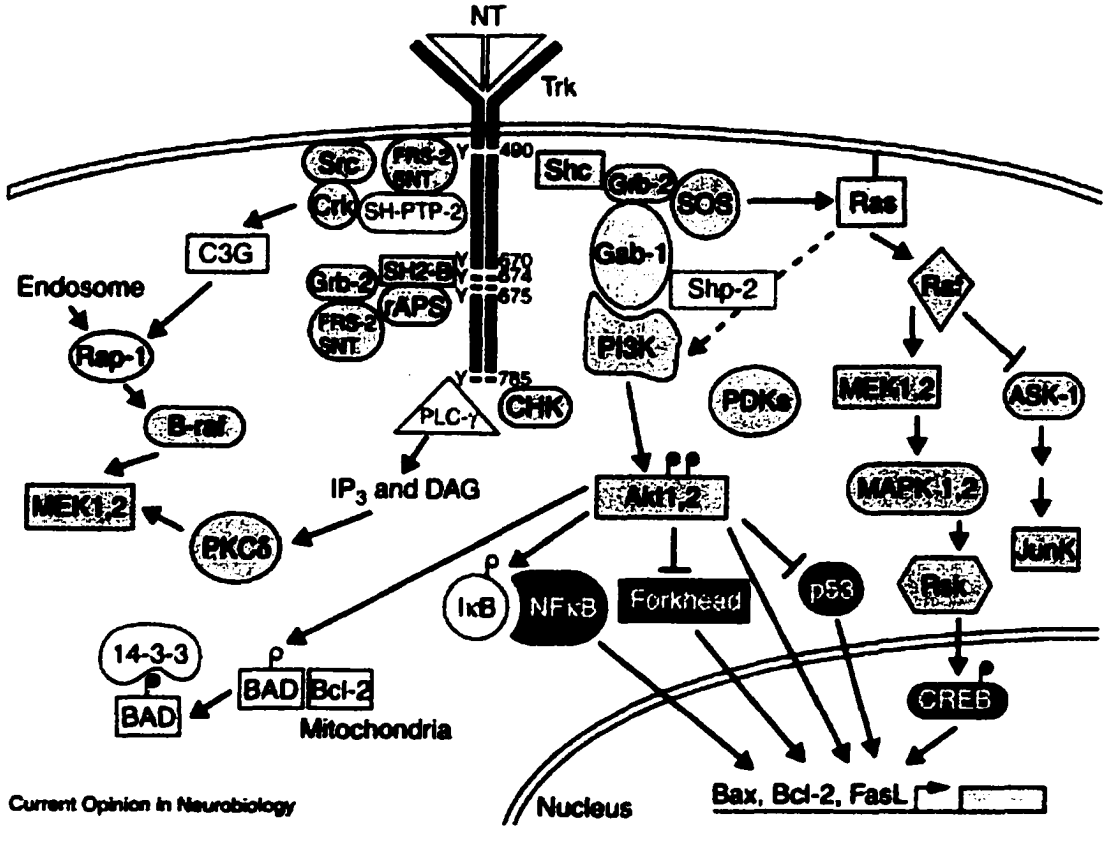


Figure 1.4 Diagram of neurotrophin signal transduction pathways mediated by Trk receptors. The different pathways are described in the text. The nomenclature for tyrosine residues of Trk receptors is based on the sequence of human TrkA. (Adapted from Patapoutian and Reichardt, 2001)

In addition to the Trk receptor, all members of the NGF family bind to p75NTR (Chao, 1994). An understanding of the p75NTR's physiological role and underlying signaling mechanisms has been lagging. Not until the past year was a consensus reached regarding the signaling pathway activated by p75NTR and its potential biological functions. p75NTR can interact directly with Trk (Bibel *et al.*, 1999). The signaling capacity and biological role of p75NTR is a function of cellular Trk activation status (Friedman and Greene, 1999; Kaplan and Miller, 2000).

Ligand-dependent activation of p75NTR has been shown to mediate neuronal apoptosis in a large number of primary neural cells in a Trk-independent fashion. In these cases, Trk is either absent, inactive or suboptimally activated (Bamji *et al.*, 1998; Barrett, 2000; Casaccia-Bonnefil *et al.*, 1996; Davey and Davies, 1998; Sedel *et al.*, 1999; Soilu-Hanninen *et al.*, 1999). This p75NTR apoptotic pathway involves the JNK (Jun amino-terminal kinase)-p53-Bax pathway (Aloyz *et al.*, 1998; Yoon *et al.*, 1998). p75NTR is also capable of interacting with the small GTPase Rho, an important factor in regulating cytoskeletal structure (Yamashita *et al.*, 1999b). When Trk receptors are present in the cells, activation of Trk silences p75NTR apoptotic signaling (Bamji *et al.*, 1998; Davey and Davies, 1998; Yoon *et al.*, 1998). Interestingly, p75NTR interacts with different Trk receptors to modulate neurotrophin actions. For example, coexpression of p75 with TrkA increases high-affinity binding of NGF (Hempstead *et al.*, 1991) and influences the ability of NGF to stimulate TrkA in PC12 cells (Barker and Shooter, 1994). p75NTR is essential for maintaining the specificity of neuronal survival responses to different neurotrophins (Chao, 1994; Kimpinski *et al.*, 1999). In other words, p75NTR collaborates with different Trks to mediate axonal growth either positively or negatively, depending

on the proportion of unliganded to liganded p75NTR present in the local microenvironment.

2.2.2 The GDNF family

Binding of GFLs to Ret and GFRα induces Ret phosphorylation (Durbec *et al.*, 1996; Jing *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996), and hence the activation of several intracellular pathways similar to those induced by the NGF family. The Ras-MAPK pathway appears to be necessary for neuronal survival and neurite outgrowth (Creedon *et al.*, 1997; van Weering and Bos, 1997). PI3K signaling (possibly independent of its downstream substrate, the Ser/Thr-kinase Akt) is required for GDNF-induced formation of large lamellipodia, which are implicated in neuritogenesis (van Weering and Bos, 1997) and differentiation of cultured dopaminergic neurons (Pong *et al.*, 1998). GDNF can also activate the JNK pathway via Rho/Rac-related small GTPase, such as Cdc42 (Chiariello *et al.*, 1998).

GDNF can promote survival and Fos activation in cell lines expressing GFRα1 but lacking Ret (Trupp *et al.*, 1999). In postnatal rat cochlear neurons, which express *Gfra*1 but lack detectable Ret (Ylikoski *et al.*, 1998), GFRα1 receptors located in the lipid raft, recruit and activate Src-type kinase upon GDNF binding. Src kinases, in turn, phosphorylate phospholipase C, leading to the production of IP_3 and release of Ca^{2+} from intracellular pools (Airaksinen *et al.*, 1999). The Ret-independent signaling by GDNF may play a role postnatally in brain plasticity or in Schwann cells after nerve lesion as an autocrine signaling mechanism to promote regeneration.

Signaling of GDNF sometimes requires other trophic factors. Recently, TGF-β was shown to be a necessary cofactor for the trophic effects of GDNF in a variety of neurons *in vitro* (Krieglstein *et al.*, 1998) and also *in vivo* (Schober *et al.*, 1999). However, the mechanism of action and the *in vivo* physiological relevance of TGF-β in Ret signaling are unknown. Interestingly, promotion of the survival of axotomized corticospinal neurons by GDNF appears to require endogenous BDNF. This suggests cross-talk between GDNF and BDNF signaling in regeneration and possibly in other types of neuronal plasticity (Giehl *et al.*, 1998).

2.2.3 CNTF

Because the transmembrane subunits of CNTF are common to the cytokine receptor complexes, the down-stream effects of receptor activation by CNTF are often similar, if not identical, to cytokine signaling (Ip and Yancopoulos, 1996; Segal and Greenberg, 1996). The association between CNTF and CNTFR leads to recruitment and dimerization of gp130 and LIFR, which in turn induces downstream signaling events including activation of the Janus tyrosine kinase (JAK)/STAT (Signal Transducers and Activators of Transcritption) pathway (Heinrich *et al.*, 1998). The activated CNTF receptor complex preferentially phosphorylates STAT3 at residue Y705 and, to a lesser extent, STAT1 at Y701, thus promoting homodimerization or heterodimerization of STAT3 and STAT1 and the subsequent nuclear translocation of dimerized STAT proteins (Bonni *et al.*, 1993; Darnell *et al.*, 1994; Wegenka *et al.*, 1993). CNTF can also induce MAPK activation via recruiment of SHP2 to tyrosin-phosphorylated gp130, an interaction between SHP-2 and membrane-assocaited Ras and a subsequent stimulation

of ras-MAPK signaling (Chin *et al.*, 1997; Kim and Baumann, 1999). MAPK activation was shown to downregulate STAT3-mediated transcription (Jain *et al.*, 1998; Sengupta *et al.*, 1998). Cross-talk between these two pathways is likely to be involved in mediating the effects of CNTF in promoting neuronal survival and determining cell fate during development (Bonni *et al.*, 1997).

The complexity of neurotrophin signaling reflects the variety of responses they are able to promote and the evolution of a varied repertoire of possible signaling elements in different cell types. Despite the different structures of the receptors, neurotrophic factors utilize increased tyrosine phosphorylation of cellular substrates to mediate neuronal cell survival. Some signaling mechanisms activated by the different families of neurotrophic factors share a high degree of similarity, especially the signal transduction pathways activated by the NGF family through the Trk receptor, and those activated by GDNF through Ret. Erk and PI3K seem to be the key components for mediating neuronal survival and differentiation upon activation by neurotrophic factors. More importantly, the mode by which neurotrophins can elicit different biological responses by activating similar signaling subunits is still a subject of intense study.

2.3 Neurotrophins in axon regeneration

Following injury in the adult mammalian CNS, the axons are exposed to insufficient quantities of neurotrophins to maintain survival for the entire axotomized population (Kawaja *et al.*, 1992), as opposed to in the PNS where neurotrophins are either constitutively present or upregulated after injury (Heumann *et al.*, 1987;

Richardson and Ebendal, 1982; Sandrock and Matthew, 1987; Whittemore *et al.*, 1985). One reason for the inadequate level of neurotrophins may be the lack of autocrine/paracrine support due to the comparatively low expression of neurotrophins detected after injury (Kobayashi *et al.*, 1996; Stilwell *et al.*, 1997). The damaged neurons require neurotrophins for their survival and hence, to reach their original targets. Thus, treatment with neurotrophins has been shown to enhance regeneration and the expression of growth-associated molecules (Blesch and Tuszynski, 2001; Blesch *et al.*, 1999).

Neurotrophins have been shown to exert neuroprotective effects in the developing and mature CNS (Aubert *et al.*, 1995) and to promote neurite outgrowth *in vitro* (Gavazzi *et al.*, 1999). The potential regeneration-promoting effects of neurotrophic factors have also been investigated *in vivo*. Anatomical studies have shown that neurotrophic factors promote regeneration of dorsal root axons into the spinal cord whether delivered in fibrin glue (Iwaya *et al.*, 1999), via adenoviral vectors (Zhang *et al.*, 1998) or intrathecally (Oudega and Hagg, 1999; Ramer *et al.*, 2000). Neurotrophic factors also promote regeneration following dorsal column lesions (Bradbury *et al.*, 1999; Oudega and Hagg, 1999). However, regenerating dorsal column axons do not reach their normal targets, suggesting long-distance axon growth needs to be guided by molecules other than neurotrophins. Moreover, it has been suggested that secretion of neurotrophic factors from grafted Schwann cells contributes to the improvement of regenerative ability of retinal ganglion axons severed in the optic nerve (Berry *et al.*, 1996). Similarly, administration of BDNF and NT-3 into spinal cord lesion sites in the presence of embryonic transplants significantly improves the growth capacity of damaged host spinal cord axons (Bregman, 1998).

Perhaps one of the best models to explain the effect of neurotrophins in regeneration was proposed by our lab (Cai *et al.*, 1999). We reported that although neurotrophins could not overcome MAG/myelin's inhibition of neurite outgrowth when added directly to neurons in the presence of the inhibitors, the inhibitory effect was blocked if neurons were pre-incubated (a process we term "priming") with neurotrophins before encountering MAG/myelin. Significantly, priming neurons with neurotrophins elevated the endogenous cAMP levels. Furthermore, artificially elevating cAMP with dibutyryl cAMP also overcomes the inhibition by MAG and myelin. The effect of priming is dependent on pre-exposure to neurotrophins because MAG/myelin can act through heterotrimeric G-proteins to inhibit neurotrophic stimulation of the cAMP-PKA pathway (Figure 1.5).

Considering the large number of candidate inhibitors present in the injured CNS, it may ultimately be more effective to modulate intracellular pathways to axon responsiveness rather than trying to block them extracellularly. It is reasonable to speculate that cAMP, cGMP and other signaling pathways converge at a common downstream intracellular target to control the growth cone and ultimately the neurite outgrowth response. The identification of common intracellular substrates and second messengers for different repulsive molecules suggests the possibility that a single treatment may by capable of combating multiple inhibitory influences.

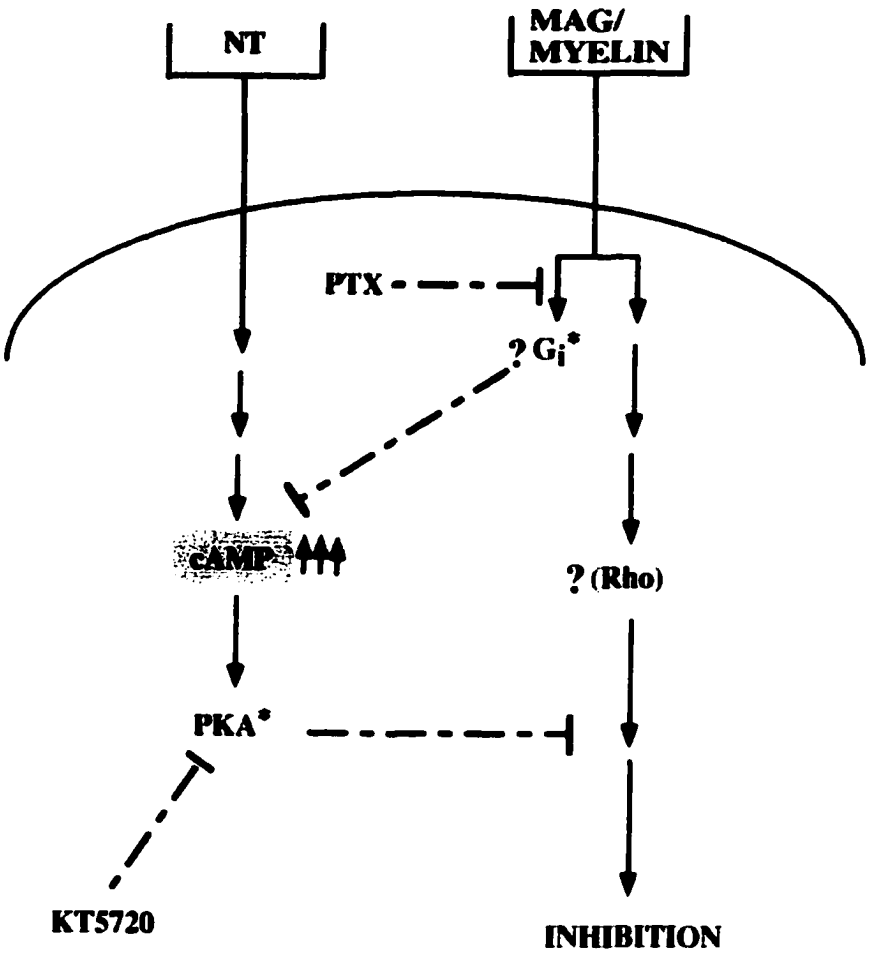


Figure 1.5 Model to explain how priming with neurotrophins blocks inhibition by MAG and myelin. During priming, neurotrophins (NT) interact with a surface neuronal receptor inducing an increase in neuronal cAMP, which in turn activates protein kinase A (PKA). Activation of PKA or some as yet unidentified downstream signal then blocks subsequent inhibition by MAG or myelin, perhaps by inactivating the small GTPase, Rho. If, however, neurotrophin is added to the neuron at the same time as exposure to MAG or myelin, cAMP is prevented form increasing by MAG/myelin activation of a pertusis toxin (PTX)-sensitive G protein (Gi*) and so inhibition of axonal regeneration is not blocked. G protein activation by MAG or myelin has no direct effect on inhibition of axonal regeneration by MAG or myelin. (Adapted from Cai *et al.*, 1999)

3. Goals of the work in this thesis

As discussed above, the adult mammalian CNS does not regenerate after injury due to the non-permissive environment and the poor intrinsic growth capacity of the

injured neuron. One of the inhibitory environmental factors is the myelin-specific inhibitor MAG (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). However, when neurons are first exposed to particular neurotrophins before encountering MAG or myelin, their axonal growth is no longer inhibited (Cai *et al.*, 1999). The effect of priming with neurotrophins is mediated through activation of the cAMP-dependent pathway, because priming has no effect when it is carried out in the presence of an inhibitor of PKA. It seems that priming with neurotrophins is so effective that it blocks all the major myelin-specific inhibitors at once. Therefore, understanding the signaling mechanisms activated by neurotrophins which result in the overcoming of MAG/myelin's inhibition may reveal a therapeutic approach to improving CNS axon regeneration after injury. However, what receptors do neurotrophins interact with to mediate the priming effect? What signal transduction pathways does it induce afterward? What signaling molecules are critical in this pathway? How do neurotrophins elevate endogenous levels of cAMP? Is priming with neurotrophins regulated at the transcriptional levels? All of these questions will be addressed in the following chapters:

1. Receptors involved in the block of MAG's inhibition by priming with neurotrophins (Chapter III).

2. Inhibition of ERK activity blocks both the neurotrophin and the cAMP effect (Chapter IV).

3. Direct inhibition of PDE blocks the MEK inhibitor effect on BDNF and cAMP (Chapter V).

4. Activation of CREB is required to block MAG's inhibition (Chapter VI)

CHAPTER II

METHODS AND MATERIALS

1. Cell culture maintenance

Permanently transfected MAG-expressing and control CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technology) supplemented with 10% dialyzed fetal calf serum (FCS), proline (40 mg/liter), glycine (7.5 mg/liter), thymidine (0.73 mg/liter) and L-glutamine (0.29 g/liter) at 37°C in 7.3% CO_2. COS-1 cells were maintained in DMEM supplemented with 10% qualified FCS.

2. Isolation of neurons

The cerebella from two animals was combined in 5 ml of 0.025% trypsin, triturated and incubated for 10 min at 37°C. DMEM (5 ml) containing 10% FCS was added and cells were centrifuged at 800 rpm for 6 min. The cells were resuspended to a single-cell suspension in 2ml of Sato (progesterone, 20 nM; selenium, 30 nM; putrescine, 100 μM; insulin, 5 μg/ml; BSA, 4 mg/ml; L-thyroxine, 0.1 μg/ml; tri-iodo-thyronine, 0.08 μg/ml) (Doherty *et al.*, 1990). For older DRG neurons, ganglia were removed from two animals and incubated in 5 ml of Sato media containing 0.25% trypsin and 0.15% collagenase type I (Worthington) for 30 min at 37°C. The ganglia were triturated and trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS, centrifuged at 800 rpm for 6 min and resuspended in Sato (De Bellard *et al.*, 1996).

3. Priming neurons with neurotrophic factors

24-well plates (6cm) were coated with 16.6 μg/ml poly-l-lysine (Sigma) for 30 min at room temperature. Excess poly-l-lysine was washed off with dH_2O. Isolated neurons in Sato were plated onto the poly-l-lysine-coated wells at a density of $1x10^6$

cells/well. Where indicated, either BDNF, GDNF, NGF, rolipram (all from Sigma) or forskolin (Calbiochem), was added in the presence or absence of either Trk inhibitor, K252a (Calbiochem), functional blocking antibody of p75 (kindly provided by Dr. Moses V. Chao), PKA inhibitor, KT-5720 (Calbiochem), MEK inhibitors, U0126 and PD98059 (Calbiochem) or Akt inhibitor, LY294002 (Calbiochem). After overnight culture, the media was removed, neurons were washed with PBS and removed with 0.1% trypsin. Trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS; neurons were centrifuged at 800 rpm for 6 min, resuspended in Sato and plated immediately onto either MAG-expressing CHO cells or control CHO cells.

4. Neurite outgrowth assay

Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an eight-well tissue culture slide (Lab-Tek). The neurite outgrowth assay was carried out as described previously (Mukhopadhyay *et al.*, 1994; De Bellard *et al.*, 1996; Tang *et al.*, 1997) by adding $2x10^4$ cerebellar or DRG neurons, either primed or not to the CHO cell monolayers. Where indicated, dbcAMP at 1 mM was added to the neurons. After 16-18 hr of culture, 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 for 4 hr 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, Amersham), washed three times and incubated with streptavidin-conjugated

Texas Red (1:300, Amersham) for 45 min. After three more washes, the slides were mounted in Permafluor (Baxter) and viewed with a fluorescent microscope. The length of the longest neurite or the total process outgrowth for each GAP-43-positive neuron for the first 100 neurons encountered when scanning the slide in a systematic manner was determined using an Oncor image analysis program. Using a video camera, the neuron image was projected to a computer screen. The longest neurite or all process was traced and quantitated.

5. Western blot analysis

Neurons were isolated and cultured overnight in Sato as described above. Prior to different treatments, neurons were starved for 2 hours in plain DMEM. Treatments include addition of either BDNF (200 ng/ml) or NGF (200 ng/ml) in the presence or absence of Trk receptor inhibitor, K-252a (50 nM); addition of BDNF in the presence or absence of either MEK inhibitors, PD98059 (50 μM) or U0126 (5 μM) or PI3K inhibitor, LY294002 (20 μM) or CaMK inhibitor, KN-62 (10 μM); addition of dbcAMP (1 mM) in the presence or absence of either MEK inhibitor, U0126 or PKA inhibitors, H89 (5 μM) or KT-2750 (200 nM). All inhibitors mentioned above were purchased from Calbiochem except KN-62 is from Sigma. After treatment, cells were washed once with PBS and lysed with boiled sample buffer (62.5 mM Tris-HCl, pH6.8 and 2% SDS) which included phosphatase inhibitors (20 mM NaF, 1 mM Na_3VO_4 and 10 mM β-glycerophosphate, all from Sigma). Samples were run through DNA shredders (Qiagen) first, then measured by a DC protein assay (Bio-Rad). For detection of ERK, Akt, CREB, 20 μg, 30 μg or 50 μg of protein from the samples were loaded respectively and separated by 10% SDS-PAGE.

For detection of Trk, 50 μg of samples were separated by 5% SDS-PAGE. After electrophoresis, gels were transferred to PVDF membranes (NEN) and probed with either a phospho-Trk (Tyr 674/675) antibody (1:750), a phospho-Erk (Thr 202/Tyr 204) antibody (1:2000), a phospho-Akt (Ser 473) antibody (1:1000) or a phospho-CREB (Ser 133) antibody (1: 1000) overnight at 4 °C. Blots were incubated with an anti-rabbit IgG-HRP secondary antibody (1:5000) for 1hr and detection was performed using ECL (Amersham). The same blot was later stripped with stripping buffer (0.2 M glycine, pH2.2, 1% Tween-20 and 0.1% SDS) for 1hr at room temperature and re-probed with either a Trk antibody (1:1000), an Erk antibody (1:2000), an Akt antibody (1:1000) or a CREB antibody (1:2000), accordingly. All antibodies mentioned above were purchased from Cell signaling.

6. Treatment of neurons with PI-PLC

2 x 10^6 cerebellar neurons were resuspended in 1ml of PBS. 0.4U of PI-PLC (ICN) was added to the cell suspension. After incubation for 45 min in a rotator at 37 °C, cells were centrifuged down, resuspended in 1 ml of Sato media and used for priming with neurotrophic factors as described above.

7. PDE assay

For each assay, 5x10^6 cerebellar neurons were plated per well of a poly-l-lysine coated 6-well dish. The neurons were incubated overnight in Sato, then starved for 2hr in plain DMEM before the addition of BDNF with or without MEK inhibitor, U0126 (5 μM) for a time course of 5 min, 10 min, 30 min or 60 min respectively. The neurons were

washed once with PBS and immediately lysed on ice using a rubber policeman in 100 µl of PDE buffer, 50 mM of Tris-HCl (pH7.5), 8.3mM $MgCl_2$, 1.7 mM EGTA, 3.75 mM of β-mercaptoethanol, proteinase inhibitors (Calbiochem) and phosphatase inhibitors. PDE activity was meansured in lysates using a scintillation proximity assay, according to the manufacturer's instructions (Amersham). Also, 25 µl of lysate treated with BDNF was incubated with 100 µM rolipram for 15 min before proceeded to PDE activity measurement to determine the amount of rolipram-sensiteve PDE activity among the total PDE activity in the lysates.

8. cAMP assay

For each assay, $2x10^6$ cerebellar neurons (usually P5-P7) were plated per well of a poly-l-lysine coated 24-well plate. Each sample was done in duplicate. The neurons were incubated overnight in Sato media. Then, BDNF at 200 ng/ml was added with or without the MEK inhibitor, U0126 at 5 µM and incubated for a further 30 min. Neurons were carefully washed once with PBS and lysed in 250 µl of 0.1N HCl by incubating first at – 20 °C for 15 min then at room temperature for 15 min and also by vigorous pipetting. Cell lysates were collected and centrifuged at 13,000 rpm for 15 min at room temperature. 10 µl of supernatant was used to perform a DC protein assay (Bio-Rad). The remaining supernatant was then acetylated and cyclic AMP was measured using an enzyme immunoassay, according to the manufacturer's instructions (BioMol).

9. Adenovirus-mediated gene introduction into neurons

Recombinant adenovirus containing A-CREB was kindly provided by Dr. Charels Vinson from National Cancer Institute. This adenovirus was modified to express both A-CREB and GFP proteins under the CMV promoter (He *et al.*, 1998). Cerebellar and DRG neurons were isolated and plated down on the poly-L-lysine coated 24-well plates. Then neurons were infected by adenovirus containing A-CREB, or control virus at a final concentration of 10^{10} PFU/ml (or MOI=100) and maintained in virus-containing media for 1 hour. After infection, media was washed away and the neurons were kept in Sato media overnight for gene expression. Infected neurons were then either primed with neurotrophins or directly plated onto transfected CHO cells with dbcAMP to carry out the neurite outgrowth assay as described previously.

10. Transfection of COS-1 cells with GenePORTER

$3x10^5$ COS-1 cells per well of a 6-well plate were plated on the day before transfection. Cells were incubated with 0.25 ml of adenovirus solution (10^{10} PFU/ml) for one hour before proceeding to transfection. According to the manufacturer's protocol (Gene Therapy System), 4 μg of pCRE-Luc DNA (Stratagene) per well was used for each transfection. DNA was first diluted with new DNA diluent B and incubated for 5 min. Then, the hydrated GenePORTER 2 reagent was diluted with plain DMEM and mixed with the DNA solution. Following 5-10 min incubation at room temperature, the GenePORTER 2/DNA complexes were formed and added directly to the cells in serum-free DMEM. After incubation with the GenePORTER 2/DNA complexes for 5 hr at 37 °C, one volume of DMEM containing 20% FCS was added. 24 hrs post-transfection, the media was changed to DMEM containing 2% FCS in the presence or absence of dbcAMP

at 1mM and incubated for a further 24 hrs. Cells were then lysed and ready for the luciferase assay.

11. Luciferase assay

Transfected cells were washed once with PBS and lysed with 200 µl of 1x cell lysis buffer, provided by the Luciferase Assay Kit (Stratagene), for 15 min, swirling occasionally. Cell lysate was collected and centrifuged for 5 min at 12,000 rpm. Supernatant was transferred to a new tube and the protein concentration was determined by a DC protein assay (Bio-Rad). 20 µl of supernatant was added to 100 µl of the luciferase substrate-assay buffer mixture. Luciferase activity was assayed immediately in a 96-well plate using an integration time of 10 seconds with a Labsystems Luminoskan RT 1.3 (Ascent Research) and normalized according to the protein concentration in different samples.

12. Immunodetection of Arginase I in infected DRG neurons treated with neurotrophins or dbcAMP

8 x 10^5 DRG neurons in duplicate were either infected with A-CREB-containing adenovirus, control virus or nothing for 1hr and incubated overnight to achieve viral gene expression. Neurons were then treated either with Sato alone, Sato with a combination of neurotrophins (200 ng/ml of NGF, 200 ng/ml of BDNF and 200 ng/ml of GDNF) or 1 mM of dbcAMP for 26-28 hr and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, 1 mM vanadate and the following antiproteases: 1 µg/ml leupeptin, aprotinin and pepstatin). The cell lysates were

kept on ice for 30 min with constant vortexing and centrifuged at 14,000 x g for 10 min. The supernatant was collected and the protein concentration was measured with a DC protein assay (Bio-Rad). 30 μg of the normalized lysate was boiled for 5 min and subjected to a 12% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and immunostained with a rabbit polycolonal antibody against arginase I (kindly provided by Dr. Ratan at Harvard Medical School), diluted at 1:5000, overnight at 4 °C. The blots were then incubated with an anti-rabbit IgG-HRP secondary antibody (Cell signaling) for 1 hr. Arg I expression was detected by an ECL detection system (Amersham). The same blot was stripped as described previously and probed with an antibody for actin (Sigma) to confirm equal loading. The expression levels of the proteins were quantitated by a FluorImage system.

CHAPTER III

RECEPTORS INVOLVED IN THE BLOCK OF MAG'S INHIBITION BY PRIMING WITH NEUROTROPHINS

Introduction

Neurotrophic factors are survival and differentiation factors that exert profound effects in the nervous systems (Barde, 1994; Levi-Montalcini, 1987). Exemplified by the nerve growth factor (NGF) family (neurotrophins), the glial-derived neurotrophic factor (GDNF) family ligands (GFLs) and ciliary neurotrophic factor (CNTF), neurotrophic factors are unique in that they do not exist in *Drosophila melanogaster* and *Caenorhabditis elegans*, even though other polypeptide growth factors, such as epidermal growth factor, fibroblast growth factor and insulin are conserved in these species (Lee et al., 2001). Neurotrophic factors are essential for the development of the vertebrate nervous system and have been proposed as therapeutic agents for the treatment of neurodegenerative disorders and nerve injury.

Among them, the neurotrophins, which consist of NGF, brain derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5, associate as non-covalent homodimers in a biological active form. They are target-derived trophic factors active on distinct sets of neurons and restricted in duration during development (Davies, 1994). The NGF family of neurotrophic factors is unusal among other growth factors in that it activates two different receptor classes, the Trk tyrosine kinase receptors and the p75 neurotrophin receptor (p75NTR) to regulate growth, development, survival and repair of the nervous system. These receptors can either collaborate with or inhibit each other's action to mediate the neurotrophic effects (Kaplan and Miller, 2000).

Trk is a single-chain member of the receptor tyrosine kinase superfamily. It contains a combination of cell-adhesion motifs in the extracellular domains, with three tandem leucine-rich motifs flanked by two cysteine clusters in its amino terminus and

two immunoglobulin (Ig)-C2 domains in the more membrane-proximal region (Figure 3.1). The second Ig-C2 domain has been shown to mediate binding to the ligand (Urfer et al., 1998). The extracellular domains of the Trk receptors also contain multiple sites for glycosylation, implied to regulate appropriate transmembrane localization of Trks and to inhibit their spontaneous activation (Watson et al., 1999). The intracellular portion of Trk receptors consists a tyrosine kinase domain with a small interruption and a short cytoplasmic tail.

Three vertebrate *trk* receptor genes have been identified, TrkA, B and C (Barbacid, 1994). Trk receptors are activated by neurotrophins and mediate almost all of the survival-promoting activities of the NGF family, with NGF activating TrkA, BDNF and NT-4/5 activating TrkB and NT-3 activating TrkC. In addition, NT-3 can also activate TrkA and TrkB in certain cellular contexts. These different Trk receptors are thought to behave similarly since each member exhibits very high conservation in their intracellular domains. Molecular and biochemical analyses also revealed numerous splice variants of Trk receptors. Of those, the most prevalent appears to be truncated forms of TrkB and TrkC which are expressed at considerable levels in the mature brain (Barbacid, 1995). These truncations are intracellular and include the kinase domain, rendering the receptors nonfunctional, at least with respect to tyrosine kinase activity. The truncated receptors are proposed to act as dominant-negative molecules that spatially restrict neurotrophin signaling to defined domains within a neuron, and upregulate in expression in adjacent glia cells to sequester neurotrophin for neuron when injury occurs (Friedman and Greene, 1999).

Neurotrophin binding to Trk receptors results in receptor dimerization and kinase activation (Barbacid, 1995; Ip and Yancopoulos, 1994). Phosphorylation of the conserved tyrosine residues in the cytoplasmic domain activates the kinase and stimulates signaling by creating docking sites for adaptor proteins coupled to intracellular cascades. As a result, Trk receptors utilize a complex set of substrates and adapter proteins to activate defined secondary signaling cascades required for neurotrophin-promoted neuronal differentiation, plasticity and survival.

p75NTR is a universal receptor for neurotrophins and a member of the tumor necrosis factor (TNF) receptor/Fas/CD40 superfamily (Chao, 1994; Smith et al., 1994). p75NTR is a transmembrane glycoprotein, containing specific conserved structural elements including four negatively-charged, cysteine-rich repeats in the extracellular domain and a unique cytoplasmic domain which is highly conserved among species (Figure 3.1). There are no sequence similarities between the Trk and p75 receptors, in either the ligand binding or cytoplasmic domains. p75NTR has the additional capacity to regulate Trk's affinity for its cognate ligand (Lee et al., 1992). The mechanism of p75 receptor signaling is not fully understood. However, it has been implied that when the two receptors are coexpressed, p75NTR appears to modify Trk signaling (Friedman and Greene, 1999). In the absence of Trk receptors, p75NTR can generate, in specific cell populations, a death signal, including the induction of NFκB (Carter et al., 1996), the hydrolysis of sphingomyelin to ceramide (Dobrowsky et al., 1995) and apoptotic activity.

For the other family of neurotrophic factors, both GDNF and CNTF signal through a receptor complex composed of glycosylphosphatidylinositol (GPI) - linked binding and transmembrane signaling components. In the case of GDNF, the GPI-

anchored GFRα and the Ret tyrosine kinase receptor form the functional receptor complex (Lindsay and Yancopoulos, 1996) (Figure 3.1). Despite the difference in their receptor systems, neurotrophic factors all utilize increased tyrosine phosphorylation of cellular substrates to mediate neuronal cell survival.

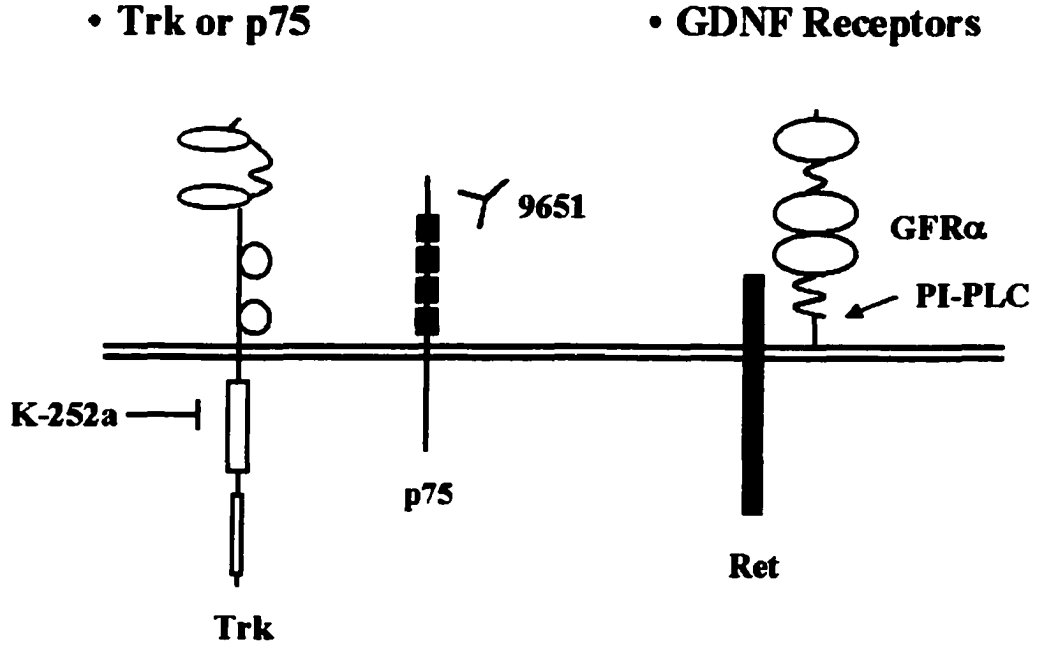


Figure 3.1 Schematic diagram of receptor structure of the NGF family and GDNF.

In the mammalian central nervous system (CNS), mature axons do not regenerate after injury (Johnson, 1993; Schwab and Bartholdi, 1996). One of the main factors for this lack of regeneration is the existence of inhibitors in myelin, such as MAG (myelin-associated glycoprotein) (McKerracher et al., 1994; Mukhopadhyay et al., 1994). We have shown previously that inhibition by MAG/myelin can be blocked if neurons are primed by exposure to neurotrophins before encountering inhibitors of axon regeneration

(Cai et al., 1999). This priming process to block MAG/myelin's inhibition is cAMP-dependent. However, what type of neurotrophin receptors mediates this priming effect is unkown. Hence the goal in this chapter is to identify the receptors involved in the block of MAG's inhibition by priming with neurotrophins.

Results

As we have reported before, neurite outgrowth from neonatal cerebellar neurons and from older DRG neurons is inhibited by about 60% and 40%, respectively, when grown on monolayer of MAG-expressing cells compared to control cells (Cai *et al.*, 1999; Mukhopadhyay *et al.*, 1994). When neurotrophins were added at a concentration of 200 ng/ml to either cerebellar (GDNF or BDNF but not NGF) or older DRG neurons (GDNF, BDNF or NGF) for overnight culturing before being plated onto MAG-expressing cells, inhibition is completely blocked. Adding neurotrophin at the same time as exposure to MAG has no effect on inhibition. In addition, we showed that neurotrophin blocks MAG's inhibition by activating PKA via elevation of cAMP. Artificial elevation of cAMP levels via addition of the non-hydrolyzable analog, dibutyryl cAMP (dbcAMP), also overcomes inhibition by MAG/myelin (Figure 3.2). However, which receptor mediates the effect of neurotrophin to block MAG's inhibition has not yet been determined.

There are two types of receptors identified for the NGF neurotrophin family, Trk and p75. Trk is a high-affinity tyrosine kinase receptor and only binds to its cognate neurotrophin. For expample, NGF specifically binds TrkA, while BDNF binds TrkB. In contrast, p75 can bind to all neurotrophins. Here, we wanted to determine if, for BDNF

and NGF, this effect is mediated by the Trk receptors or p75NTR. Since cerebellar neurons express p75NTR but not TrkA (Holtzman *et al.*, 1992; Martin-Zanca *et al.*, 1990) and NGF did not overcome inhibition by MAG/myelin of neurite outgrowth from cerebellar neurons, it suggests that the effect was not mediated by p75NTR. To confirm this possibility, a selective inhibitor of the Trk receptor and a functionally blocking antibody for p75 were employed in the experiments.

Figure 3.2 The effect of priming cerebellar neurons with neurotrophins on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine without neurotrophin (-) or with BDNF, GDNF, or NGF, each at 200 ng/ml, before being trypsinized and transferred to a monolayer of either MAG-expressing CHO cells or control CHO cells. For dbcAMP treatment, dissociated cerebellar neurons were added directly to the monolayer in the presence of 1mM dbcAMP, without priming. All cells were grown overnight on the monolayer before being fixed and immunostained for GAP43.

1. The p75 receptor is not involved in neurotrophin's block of inhibition by MAG

9651, a function blocking polyclonal antibody raised against the extracellular domain of p75, was applied to assess if p75 is involved in neurotrophins' block of inhibition by MAG. Rat cerebellar neurons were primed with BDNF in the presence of 9651 at a concentration that blocks the binding of BDNF to p75 (Huber and Chao, 1995) and at a higher concentration (Figure 3.3a). Addition of the p75 antibody did not change

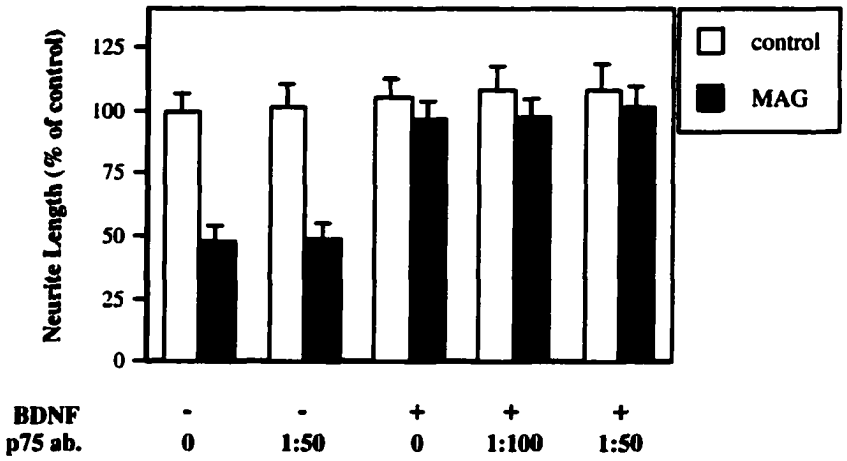
BDNF's ability to block MAG's inhibition. The antibody alone did not affect neurite outgrowth or inhibition by MAG. A similar result was obtained using DRG neurons (Figure 3.3b). The p75 antibody has no effect on the priming of DRG neurons with either BDNF or NGF. This suggests that the NGF neurotrophin family does not exert its effect through p75NTR, but most likely via the Trk receptor.

2. The Trk receptor is required for priming with neurotrophins

To determine if Trk receptors are involved in the BDNF and NGF effect on inhibition by MAG, the alkaloid K-252a was used. K-252a is a potent inhibitor of NGF-induced biological responses. Phosphorylation events initiated by Trk receptors can be inhibited by K-252a in a dose-dependent manner both *in vivo* and *in vitro*. The inhibitory effect of K-252a is likely to be exerted upon the Trk tyrosine kinase domain (Berg et al., 1992; Koizumi et al., 1988).

Before testing K-252a in the neurite outgrowth assay, we examined whether the Trk receptors were indeed activated within 5 minutes by BDNF or NGF and that K-252a blocked their activation. Figure 3.4 shows that BDNF phosphorylates TrkB in cerebellar neurons and NGF activates TrkA in DRG neurons. When 50 nM of K-252a is included in the media with either neurotrophin, this phosphorylation is blocked.

a) Cerebellar Neurons



b) DRG Neurons

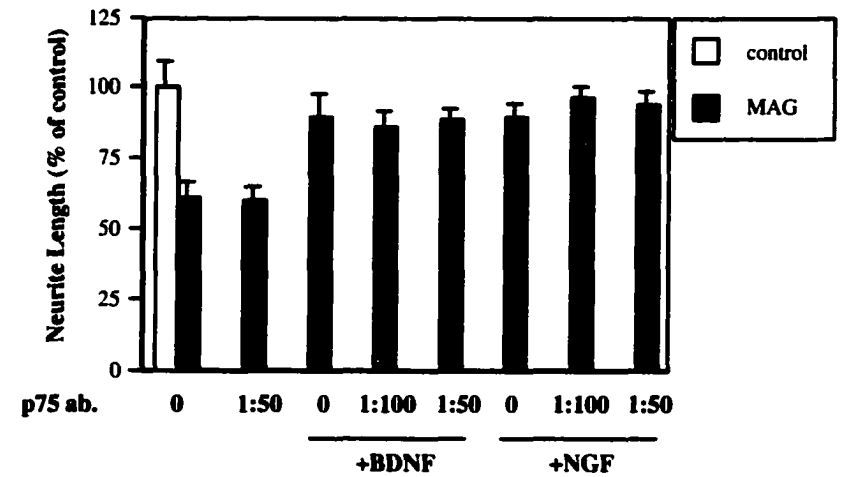


Figure 3.3 The effect of priming neurons with neurotrophins in the presence of a p75 antibody on subsequent inhibition of neurite outgrowth by MAG.
Dissociated a) cerebellar neurons, or b) older DRG neurons, were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF or NGF, each at 200 ng/ml, with or without a p75 antibody (1:50 or 1:100 dilution) as indicated. The neurons were then transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

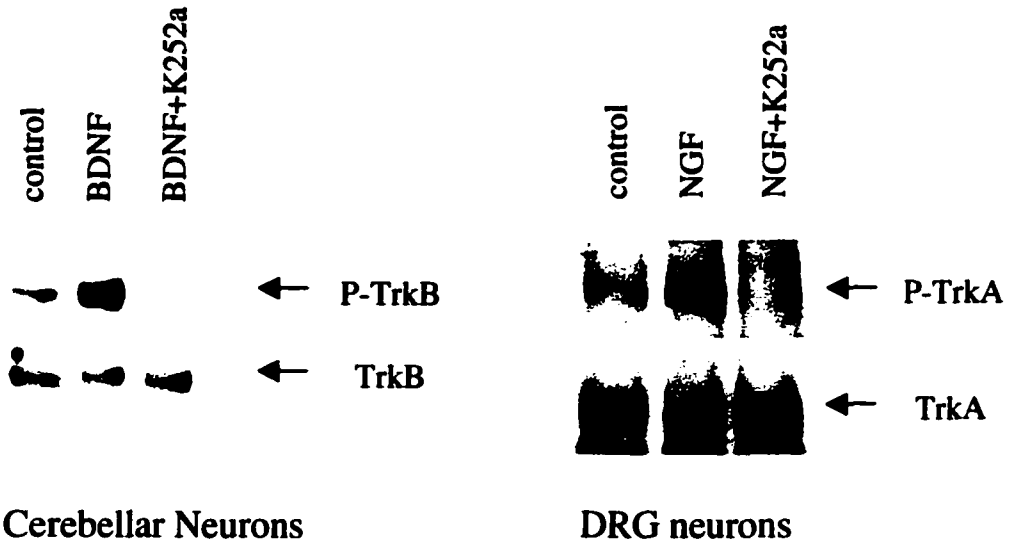


Figure 3.4 Phosphorylation of Trk receptors in neurons after treatment with neurotrophins in the presence or absence of the Trk inhibitor, K-252a.
Dissociated neurons were treated with specific neurotrophin at 200 ng/ml for 5 min; either cerebellar neurons with BDNF or DRG neurons with NGF, in the presence or absence of the Trk receptor inhibitor K-252a, at 50 nM. Cells were then lysed in boiled 1X SDS sample buffer with phosphatease inhibitors. 50 μg of proteins were subjected to 5% SDS-PAGE before being transferred to PVDF membrane and immunostained with an antibody against phosphorylated Tyr 674/675 of Trk receptor. The same blots were later stripped and probed with an antibody against total Trk proteins to confirm equal loading.

When K-252a is included in the media during priming of cerebellar neurons with BDNF, it affects BDNF's ability to block MAG's inhibition in a dose-dependent manner, with 50 nM K-252a completely abrogating the block of MAG's inhibition by BDNF (Figure 3.5). K-252a alone does not affect neurite outgrowth or MAG's inhibition.

The effect of K-252a is specific for the Trk receptor because K-252a has no effect on the ability of GDNF to induce a block of inhibition by MAG (Figure 3.6a). Also priming cerebellar neurons (which lack the TrkA receptor) with NGF, with or without K-

252a, had no effect on inhibition by MAG. In contrast, when K-252a was present during priming of DRG neurons with NGF, the block of MAG's inhibition by NGF was completely abrogated (Figure 3.6b).

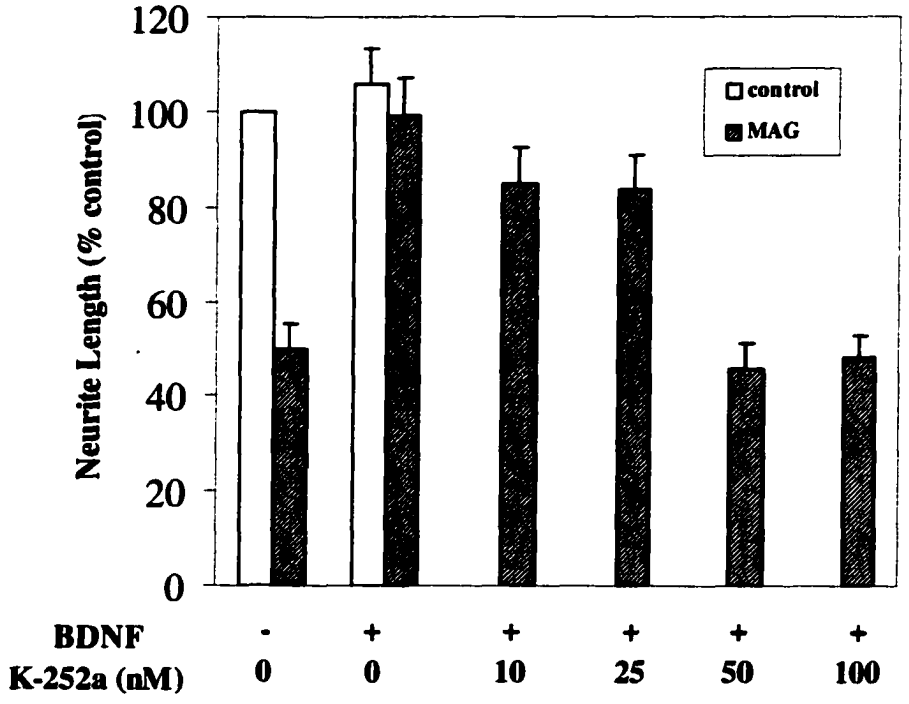
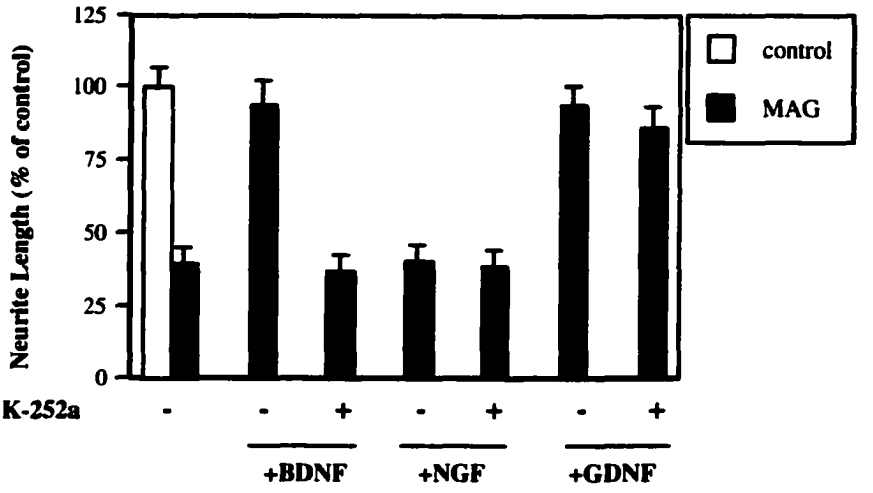


Figure 3.5 The effect of priming cerebellar neurons with BDNF in the presence of differening concentrations of K-252a.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF, at 200 ng/ml, with or without various concentrations (10-100 nM) of K-252a as indicated. After which they were transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

a) Cerbellar Neurons



b) DRG Neurons

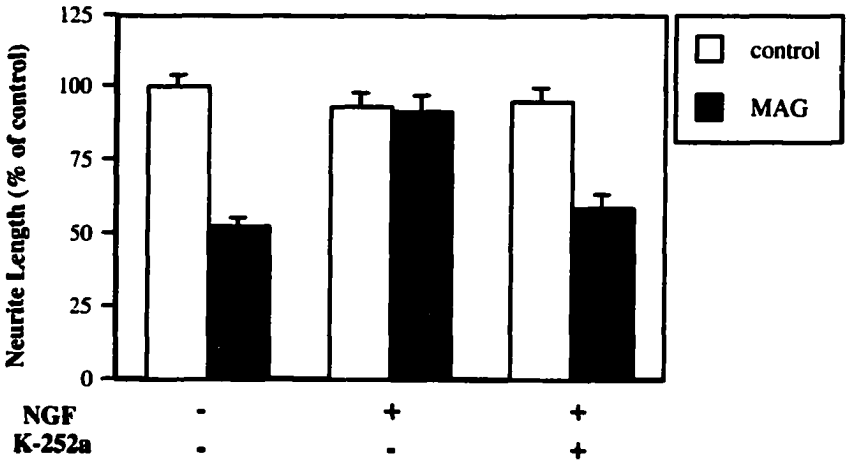


Figure 3.6 The effect of priming neurons with neurotrophins in the presence of the Trk receptor inhibitor on subsequent inhibition of neurite outgrowth by MAG.
Dissociated a) cerebellar neurons, or b) older DRG neurons, were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF, GDNF or NGF, each at 200 ng/ml, with and without the Trk inhibitor K-252a at 50 nM as indicated. The neurons were then transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

K-252a is capable of inhibiting other kinases, including PKA, at higher concentrations. Although the lack of effect of K-252a on priming by GDNF is a strong indication that the effect is via specific inhibition of Trk kinase, it is possible that the drug is inhibiting other kinases. PKA, in particular, the activation of which is required to overcome inhibition of MAG, is one potential target. Addition of the PKA inhibitor, KT5720, abolished the priming effect of BDNF (Cai et al., 1999). Therefore, we wanted to ensure the effect of K-252a was not through non-specific inhibition of PKA. To test this possibility, K-252a was included in the cultures where dbcAMP was added. Regardless of the presence of K-252a, dbcAMP overcomes inhibition by MAG, while the PKA inhibitor, KT5720, was able to block the dbcAMP effect (Figure 3.7). Together these results demonstrate that BDNF and NGF are exerting their effects by activating the TrkB and TrkA receptors, respectively, but not through p75NTR.

3. The GPI-linked receptor is involved in priming with GDNF

GDNF signals through a receptor complex distinct from the Trk receptors of the NGF family. This GDNF receptor complex includes GFRα, a GPI-linked cell surface receptor, and the Ret protein tyrosine kinase. GDNF binds to GFRα and signals either through Ret or independent of Ret. To test the role of GFRα in priming with GDNF, a phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves all GPI-linked membrane proteins, was used. After treatment with PI-PLC, priming with GDNF has no effect on inhibition by MAG, while BDNF still promotes the PI-PLC treated neurons to overcome inhibition by MAG. Basal neurite outgrowth of the PI-PLC treated neurons is

not affected (Figure 3.8). This indicates that priming with GDNF requires the GPI-linked GFRα receptor.

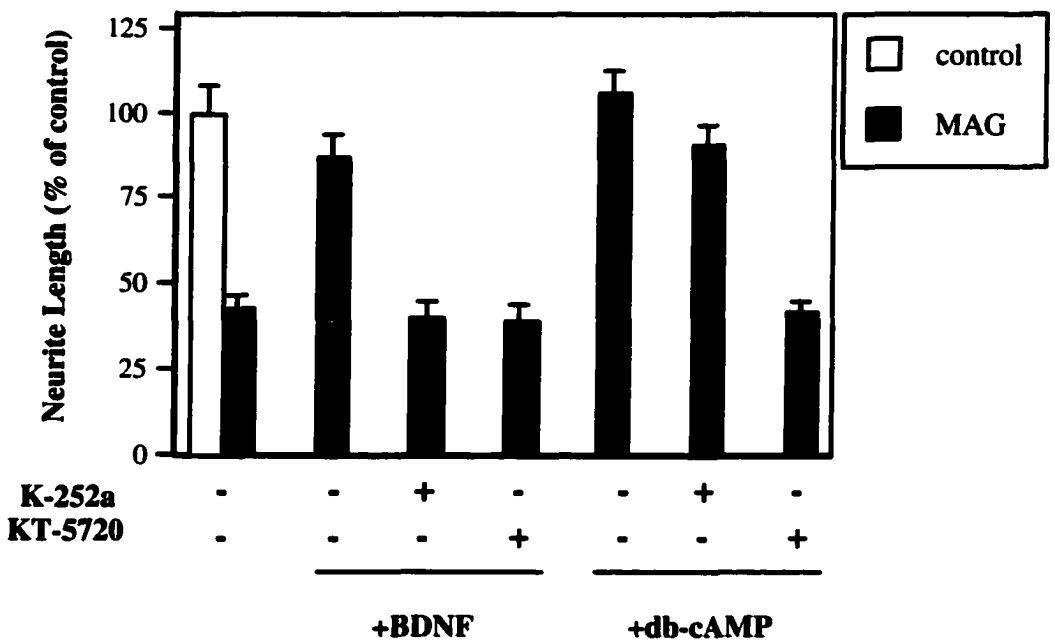


Figure 3.7 The effect of Trk inhibitor on priming cerebellar neurons with BDNF, or neurite outgrowth in the presence of dbcAMP.
Dissociated cerebellar neurons were first primed overnight with BDNF (200 ng/ml), in the presence of either Trk inhibitor K-252a (50 nM) or PKA inhibitor KT5720 (200 nM), before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture. Alternatively, cerebellar neurons were directly grown on a monolayer with dbcAMP (1mM) in the presence of either K-252a, or KT5720. Cells were then fixed and immunostained for GAP43. Results show the mean length of the longest neurite per neuron (+/- sem). Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

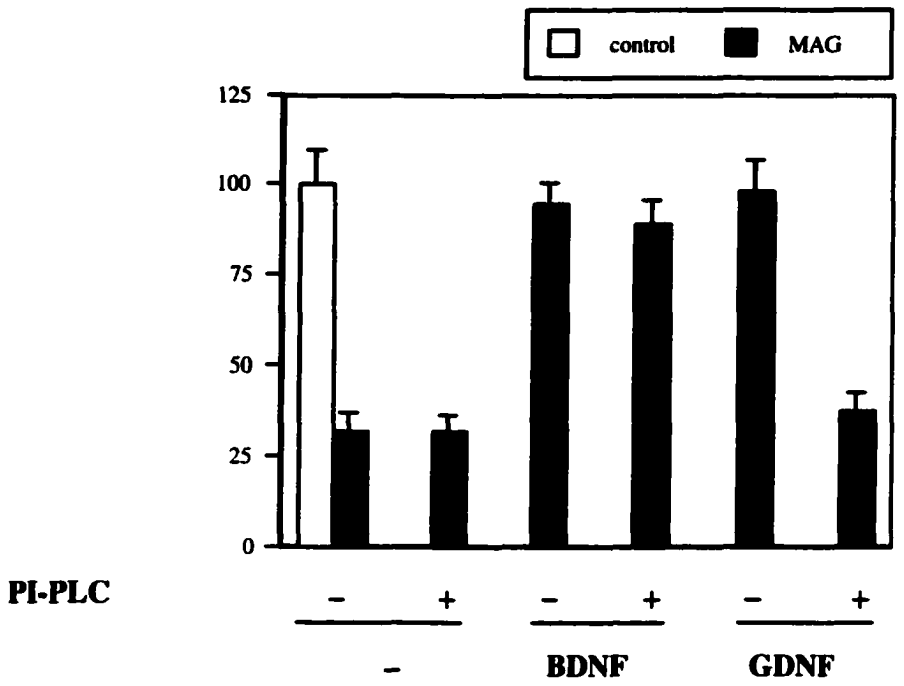


Figure 3.8 The effect of priming neurons with neurotrophins after treatment with PI-PLC on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were treated with PI-PLC (0.4 U/ml) and then primed overnight in the presence of BDNF or GDNF, each at 200 ng/ml as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

Discussion

The NGF family of neurotrophins can signal through either the Trk receptors or p75NTR. Previously, we reported that NGF had no effect on MAG/myelin inhibition of cerebellar neurons, which express p75NTR but not the TrkA receptor. This indicates that the priming effect of the NGF family is via the Trk receptor and not p75NTR. Here, we confirmed this suggestion by using a specific Trk inhibitor and an antibody blocking neurotrophins binding to p75NTR. We showed that the p75 antibody does not interfere

with the neurotrophins' ability to mediate a block of MAG's inhibition. However, addition of the Trk receptor inhibitor completely abrogates the priming effect. The specificity of this inhibitor was shown by its inability to affect either the dbcAMP effect or that of GDNF. The inability of the Trk inhibitor to influence dbcAMP's function also suggests that the cAMP/PKA pathway is downstream from the Trk receptors.

One dilemma regarding regulation of the neurotrophins' priming effect by Trk receptors is the relatively high concentration of neurotrophin (200 ng/ml) required to achieve optimal regenerative capability, as compared to the low concentration (50 ng/ml) needed for survival. Furthermore, the priming effect of neurotropohins is dependent on the cAMP/PKA pathway, which has never been shown to be directly activated by Trk receptors. This prompted the suggestion that the priming effect we observed could be Trk independent. However, we clearly show here that priming with neurotrophins in order to block inhibition by MAG requires activation of the Trk receptors. One explanation for this controversy could be the existence of other binding partners for Trk receptors. New Trk interactors, besides p75NTR, have been identified, such as components of cytoplasmic dynein (Yano et al., 2001) and the PDZ-containing GIPC proteins (Lou et al., 2001). These proteins are suggested to be involved in targeting the neurotrophin receptors to their intracellular destinations during important cellular processes such as retrograde transport, recycling, axonal and dendritic localization and synapse formation. Interaction of Trk receptors with these proteins may change Trk's affinity for its cognate ligand. Thus, more neurotrophins may be needed for a complex process such as regeneration, which requires multiple actions of retrograde transport, synapse formation and other neuronal responses, than for a relatively simple survival effect.

Second, Trk mediated survival and regenerative pathways may be different. Neurotrophin binding to Trk receptors results in receptor dimerization, kinase activation and adaptor proteins coupling to the receptors. Different subsets of adaptor proteins dictate the response to neurotrophin. For example, whether neurotrophin application results in a proliferation- or differentiation-inducing response seems to depend on whether there is a transient or prolonged activation of the ERK pathway (Grewal et al., 1999), which is mediated by different adaptor proteins. Moreover, the survival effect is cAMP-PKA independent, while the cAMP pathway is required to block MAG/myelin's inhibition. Although the Trk receptor may not activate cAMP/PKA directly, it is likely that under many circumstances, cross-talk between different pathways and activation of the cAMP pathway occurs in response to neurotrophins.

The transfer of Trk receptors to various membrane compartments also controls the efficiency of Trk receptor interaction with adaptors and the ability of the receptors to activate specific signaling molecules localized to specific membrane compartments (Saragovi et al., 1998; York et al., 2000). Importantly, in some CNS neurons, Trk receptors appear to be largely sequestered in intracellular vesicles (Meyer-Franke et al., 1998). A second signal, such as cAMP or Ca^{2+}, is required for efficient insertion of receptors into the surface membrane. For Trk responsiveness, these neurons may have to be incorporated into a signaling network that, through induction of second messengers, makes possible the transport of Trk receptors to the cell surface.

Finally, for another family of neurotrophic factors, priming with GDNF signals through its GFRα receptor. Although there is no similarity between the NGF and GDNF family receptor systems, they all seem to use similar cellular substrates to achieve the

same goal. How the evolutionally different neurotrophic factors elicit the same biological response by activating similar signaling subunits is still a subject of intense study.

In summary, our results suggest that the NGF family exerts its effects to overcome MAG's inhibition by activating Trk receptors and not through p75NTR; whereas GDNF requires GFRα to block the inhibition of MAG on axonal regeneration. The next step will be to determine what signaling pathway is involved in this priming process, downstream from the Trk receptors.

CHAPTER IV

INHIBITION OF ERK ACTIVITY BLOCKS BOTH THE NEUROTROPHIN AND THE cAMP EFFECT

Introduction

Signal transduction networks allow cells to perceive changes in the extracellular environment and to mount an appropriate response. Mitogen-activated protein kinase (MAPK) cascades are among the most thoroughly studied signal transduction systems. They are evolutionarily conserved in all eucaryotes and play a key role in a diverse array of cellular programs. MAPK cascades are typically organized into a three-kinase architecture consisting of a MAPK, a MAPK activator (MEK, or MAPK kinase) and a MEK activator (MEKK, MEK kinase). Transmission of signals is achieved by sequential phosphorylation and activation of the components specific to a respective cascade. In mammalian systems, the MAPK cascade includes the extracellular signal-regulated kinase (ERK), the Jun amino-terminal kinase (JNK) and p38 MAPK. Among them, ERK-1, -2 (often designated p44 and p42 MAPK) are important regulators of neuronal function, including synaptic plasticity, long-term potentiation and survival (Atkins *et al.*, 1998; Blum *et al.*, 1999; English and Sweatt, 1997; Grewal *et al.*, 1999; Martin *et al.*, 1997; Schaeffer and Weber, 1999). These actions of ERK have been best examined in the context of growth factor signaling via receptor tyrosine kinases (RTKs) (van der Geer *et al.*, 1994).

The archetypal ERK cascade involves the activation of the small GTPase, Ras, and the kinases Raf and MEK (van der Geer *et al.*, 1994). Recent studies have identified a novel mechanism for sustained activation of ERK, which involves the PKA-dependent activation of the Ras-related small G-protein Rap1, and the subsequent activation of B-Raf, a Raf isoform (Yao *et al.*, 1998; York *et al.*, 1998). Given that B-Raf is highly localized to neurons, the Rap1-dependent pathway may be unique to the nervous system.

Moreover, Ras and Rap1 activities are tightly regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The activity of GEFs is generally controlled by their recruitment from the cytoplasm to multiprotein complexes at the cell membrane initiated by the binding of neurotrophic factors to RTKs (Pawson and Scott, 1997). RTKs recruiting of particular adaptor molecules and the subsequent association of adaptors with either Ras- or Rap-GEFs determine ERK signal specificity. Recently, a new class of GEFs has been found, distinct from the RTK/adaptor-associated GEFs, that are activated by direct binding to second messengers such as calcium, cAMP and DAG (diacylglycerol) (de Rooij *et al.*, 1998; Ebinu *et al.*, 1998; Kawasaki *et al.*, 1998a; Kawasaki *et al.*, 1998b). The new GEFs each display very different and restricted CNS expression patterns, suggesting that they may act as distinct regulators of neuronal signaling. Opposite to GEFs, GAPs mediate the inhibition of Ras and Rap1 signaling, providing a different means to regulate ERK cascades.

Regulation of ERK signaling specificity involves targeting, activation, translocation and subsequent stimulation of specific targets. First, scaffold proteins involved in facilitating the assembly of enzyme-substrate complexes regulate MAPK targeting. MP1, a MEK binding partner, promotes specific complex formation between MEK1 and ERK1 (Schaeffer *et al.*, 1998), whereas JIP (JNK interacting protein) acts as a scaffold protein for members of the JNK signaling cascade (Whitmarsh *et al.*, 1998). These scaffold proteins, together with adaptors, mediate the proper targeting of signaling components in the MAPK family. Second, in extremely polarized cells such as neurons, the subcellular localization of ERK affects its activity and function. Hence, relocalization of ERK from neuronal processes to the soma is an important aspect of ERK signal

propagation in the polarized neurons (Martin *et al.*, 1997). This relocalization of signaling molecules is proposed to act through receptor activation which promotes internalization and formation of an intracellular vesicle (Grimes *et al.*, 1997). Finally, a major role for ERK lies in the regulation of gene expression. ERK can activate the transcription factor CREB via stimulation of the CREB kinase, RSK (Xing *et al.*, 1996). This action is a major downstream target of both neurotrophins and second messengers. In many instances, activation of transcription factors also requires translocation of ERK from the cytoplasm to the nucleus. Mechanisms leading to ERK translocation are still unclear, but they appear to require both ERK phosphorylation and dimerization. Interestingly, PKA has been shown to be required for the nuclear translocation of activated ERK (Impey *et al.*, 1998). In summary, the multitude of ERK regulation patterns enables neurons to integrate various signals and achieve specific neuronal responses.

While much attention has focused on the trophic actions of neurotrophic factors, it has become apparent that ERK can play multiple roles in the activity-dependent regulation of neuronal functions which involve the integration of second-messenger system, such as calcium, cAMP and DAG (Grewal *et al.*, 1999; Houslay and Kolch, 2000). In the last chapter, we noted that the tyrosine receptor kinase, Trk is involved in the block of MAG's inhibition by priming with neurotrophins. Being an important pathway downstream of neurotrophin signaling, it will be interesting to see if ERK also participates in the cAMP-dependent priming pathway activated by neurotrophins to overcome inhibition by MAG.

Results

The ERK pathway is one of the most well studied signal transduction modules in eukaryotes. Binding of neurotrophins to Trk receptors has been shown to robustly activate ERK in neurons. This activation is crucial for many neuronal processes such as survival, differentiation and memory. The importance of ERK in these neuronal processes and identification of the involvement of Trk receptors in the priming pathway suggest that ERK may play a key role in the pathway that leads to the priming of neurons with neurotrophins to block inhibition of axonal regeneration by MAG/myelin.

1. Incubation with BDNF, but not dbcAMP activates ERK in cerebellar neurons

To determine if ERK is activated under the conditions used here by BDNF and dbcAMP, cerebellar neurons were treated with either BDNF or dbcAMP for various lengths of time before being lysed, subjected to SDS-PAGE and western blotting. The membranes were stained with an antibody against the activated (phosphorylated) ERK. Figure 4.1 shows that incubation of cerebellar neurons with BDNF activates both ERK-1 and ERK-2 (p44 and p42, respectively). They are markedly phosphorylated within 10 min and remain activated for up to 2 hr. A MEK inhibitor, PD98059, reduces ERK activity dramatically (below even basal level). When the same membrane is stained for total ERK, it is observed that equal amounts of protein were loaded on the gel and that only the phosphorylation state of ERK is changed. In contrast, we found that dbcAMP barely affects the activity of ERK even after 2 hrs of treatment (Figure 4.2). Phosphorylation levels of ERK remain at basal, which differs dramatically from the

robust activation of ERK by BDNF (Figure 4.1). Therefore, BDNF, but not dbcAMP, activates ERK in cerebellar neurons.

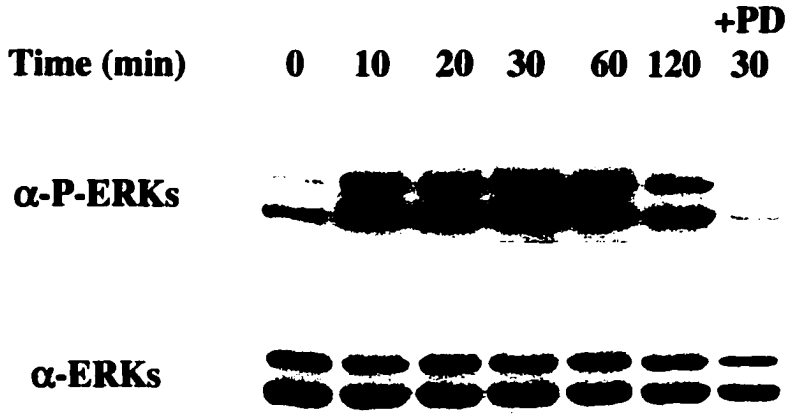


Figure 4.1 Phosphorylation of ERK in cerebellar neurons after treatment with BDNF in the presence or absence of a MEK inhibitor.
Dissociated cerebellar neurons were treated with BDNF at 200 ng/ml for differing lengths of time, up to 120 min. The MEK inhibitor, PD98059 (50 μM), was added to cells incubated with BDNF for 30 min. Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 20 μg of proteins were subjected to 10% SDS-PAGE before being transferred to a PVDF membrane and immunostained with an antibody against phospho-ERK (Thr 202/Tyr 204). The same blot was later stripped and probed with an antibody against total ERK to confirm equal loading.

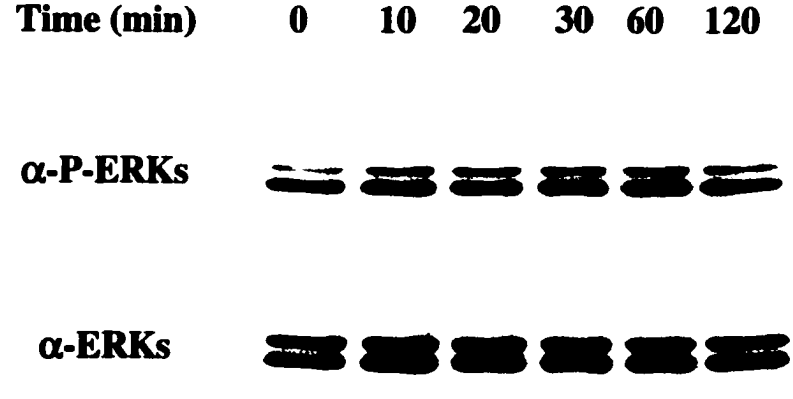


Figure 4.2 Phosphorylation of ERK in cerebellar neurons after treatment with dbcAMP.
Dissociated cerebellar neurons were treated with dbcAMP at 1 mM for up to 120 min. Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 20 μg of proteins were subjected to 10% SDS-PAGE before being transferred to a PVDF membrane and immunostained with an antibody against phospho-ERK (Thr 202/Tyr 204). The same blot was later stripped and probed with an antibody against total ERK to confirm equal loading.

Moreover, to test if PKA activity is required for activation of ERK by BDNF, an inhibitor of PKA was included during BDNF incubation. BDNF still activates ERK for up to one hour regardless of the presence of a PKA inhibitor (Figure 4.3). Hence, cAMP/PKA neither activates ERK, nor is it required for BDNF mediated elevation of ERK activity through Trk.

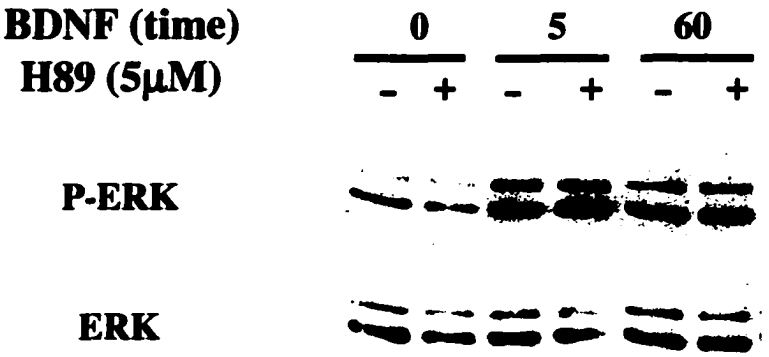


Figure 4.3 Phosphorylation of ERK in cerebellar neurons after treatment with dbcAMP in the presence of a PKA inhibitor.
Dissociated cerebellar neurons were treated with dbcAMP at 1 mM in the presence or absence of the PKA inhibitor H89 (5 μM), for either 5 or 60 mins. Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 20 μg of proteins were subjected to 10% SDS-PAGE before being transferred to a PVDF membrane and immunostained with an antibody against phospho-ERK (Thr 202/Tyr 204). The same blot was later stripped and probed with an antibody against total ERK to confirm equal loading.

2. Inhibition of ERK blocks both the BDNF and dbcAMP effect on inhibition by MAG

To assess if activation of ERK is required for BDNF to overcome inhibition by MAG, priming with BDNF was carried out in the presence of two different MEK inhibitors, leading to inactivation of ERK. The inhibitor PD98059 (50 μM) blocks access of MEK to activating enzymes, while U0126 (5 μM) is noncompetitive with respect to

MEK substrates. Addition of these MEK inhibitors during the priming of cerebellar neurons with BDNF prevents BDNF's ability to block inhibition by MAG (Figure 4.4). This result indicates that ERK activity is required for priming with neurotrophins. Surprisingly, in the neurite outgrowth assay both inhibitors completely abrogate dbcAMP's ability at 1 mM to block MAG's inhibition (Figure 4.5). Neither U0126 nor PD98059 alone have any effect on neurite outgrowth on MAG expressing-CHO cells or control cells (Figure 4.5). Hence, inhibitors of MEK block both the BDNF and dbcAMP effect on inhibition by MAG but only BDNF activates ERK.

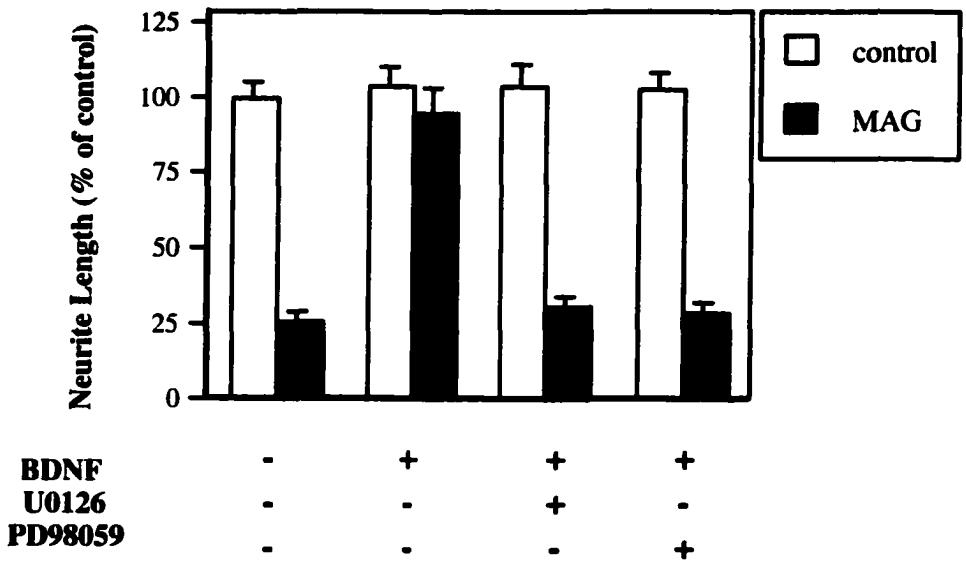


Figure 4.4 The effect of priming cerebellar neurons with BDNF in the presence of MEK inhibitors on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of BDNF, at 200 ng/ml, with or without either MEK inhibitor PD98059 (50 μM) or U0126 (5 μM) as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The culture was then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

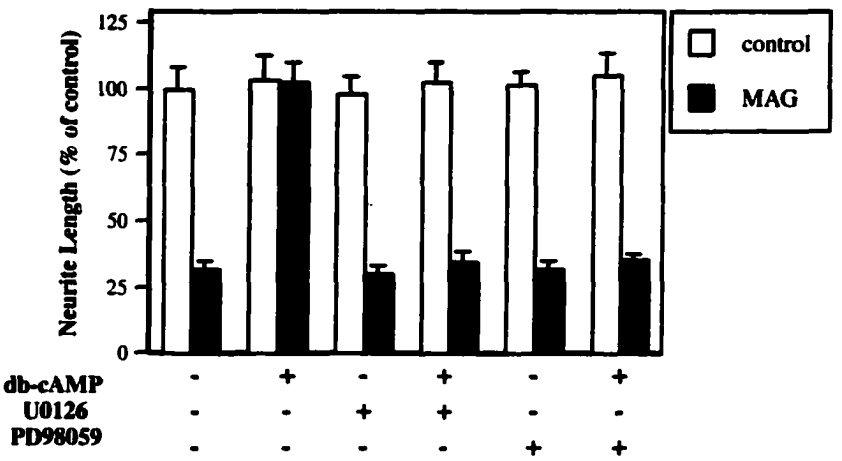


Figure 4.5 The effect of dbcAMP in the presence of MEK inhibitors on inhibition by MAG of neurite outgrowth from cerebellar neurons.
Dissociated cerebellar neurons were plated onto monolayers of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) in the presence of dbcAMP, at 1 mM, with or without either MEK inhibitor, PD98059 (20 μM) or U0126 (1 μM) as indicated, and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of neurotrophin.

To determine if the effect of MEK inhibition on the ability of dbcAMP to overcome inhibition by MAG is dependent on dbcAMP concentration, the dbcAMP concentration in the neurite outgrowth assay was varied from 0.2-4 mM in the presence or absence of 20 μM of MEK inhibitor PD98059. The length of neurons on MAG-expressing CHO cells was measured and expressed as a percentage of neurite length grown on control cells. As can be seen in Figure 4.6, and as we have reported before, dbcAMP overcomes inhibition by MAG in a dose dependent manner and at 1 mM, inhibition by MAG is completely blocked (Cai *et al.*, 1999). However, when a MEK inhibitor is included, the dose response curve for dbcAMP is shifted to the right.

Consistent with what is shown in figure 4.5, in the presence of PD98059, dbcAMP at 1 mM has little effect on inhibition by MAG. It is not until a concentration of 2-3 mM does dbcAMP completely block inhibition by MAG in the presence of a MEK inhibitor. This suggests that the MEK inhibitor decreases the endogenous levels of cAMP. At lower levels of endogenous cAMP, more added non-hydrolyzable cAMP analog is needed to reach the threshold required to block MAG's inhibition. Since dbcAMP does not activate ERK, addition of the MEK inhibitor only results in inhibiting the basal level of ERK activity, which seems sufficient to affect the ability of dbcAMP to reverse MAG's inhibition.

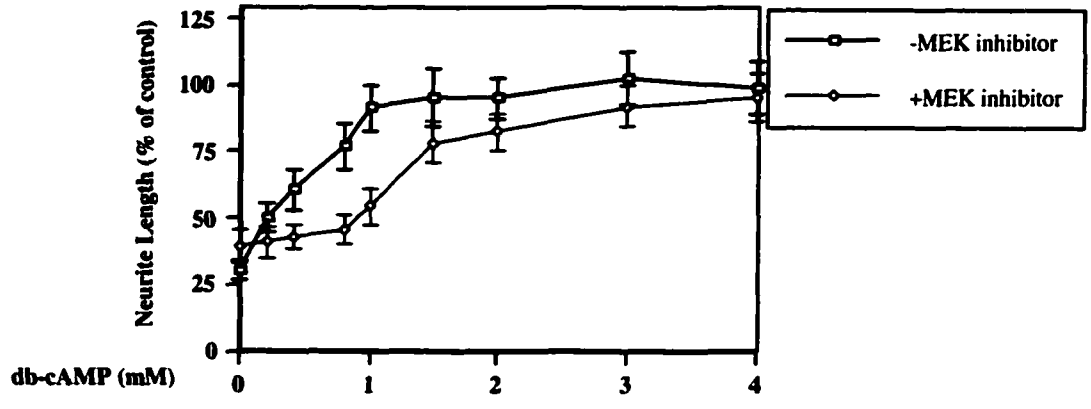


Figure 4.6 The effect of differening concentrations of dbcAMP in the presence or absence of a MEK inhibitor on inhibition of neurite outgrowth from cerebellar neurons by MAG.
Dissociated cerebellar neurons were plated onto monolayers of MAG-expressing CHO cells in the presence of differing concentrations of dbcAMP, varied from 0.2-4 mM, with or without the MEK inhibitor, PD98059 (20 μM), and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of dbcAMP.

3. Inhibition of PI3K blocks the BDNF, but not the dbcAMP effect on MAG

A second important downstream element in neurotrophin signaling via the Trk receptor is PI3K (phosphatidylinositol-3-kinase). PI3K activity stimulates PDK (3-phosphoinositide-dependent kinase), which in turn activates Akt (Chan *et al.*, 1999; Datta *et al.*, 1999; Vanhaesebroeck and Alessi, 2000). Both PI3K/Akt and MEK/ERK are the major effectors of neurotrophin and Ras-activated survival (Kaplan and Miller, 2000). We have shown that ERK is required for BDNF/cAMP to overcome inhibition by MAG. Considering that PI3K plays a similar role as ERK in neurotrophin signaling, it is possible that PI3K/Akt also participates in the block of MAG's inhibition by BDNF. To test this possibility, a specific inhibitor of PI3K, LY294002 was employed in the following experiments. We showed that in cerebellar neurons, BDNF activates Akt within 10 minutes and for up to 2 hours. LY294002 blocks the phosphorylation of Akt when included in the media with BDNF (Figure 4.7). As suspected, the PI3K inhibitor also abrogates the ability of BDNF to overcome inhibition by MAG (Figure 4.8). Interestingly, unlike ERK, PI3K does not seem to be involved in the block of inhibition by dbcAMP. In the presence of the PI3K inhibitor, dbcAMP still promotes neurite outgrowth (Figure 4.9). In contrast, the MEK inhibitors completely abolish the dbcAMP effect (Figure 4.5). Therefore, inhibition of either MEK/ERK or PI3K/Akt activity blocks the BDNF effect on inhibition by MAG. However, only inhibition of MEK/ERK affects cAMP's ability to promote axon regeneration. These suggest that there is cross-talk between the ERK and the cAMP pathway. This cross-talk is unique and important for both neurotrophin and cAMP in overcoming inhibition by MAG.

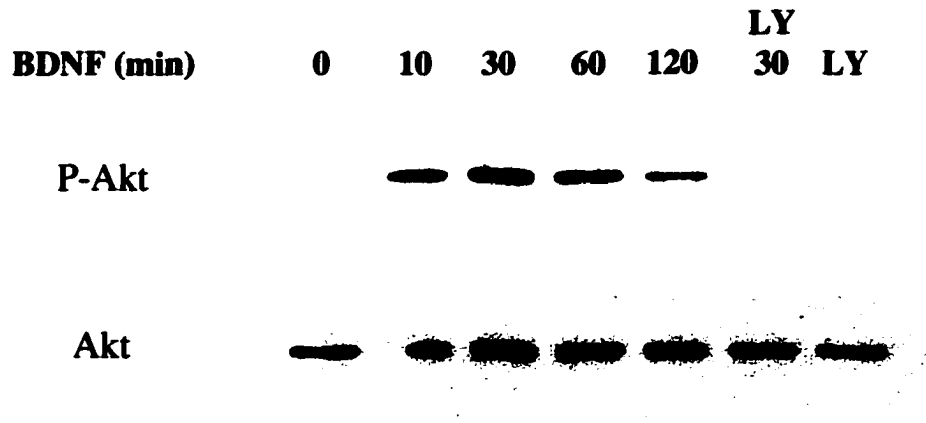


Figure 4.7 Phosphorylation of Akt in cerebellar neurons after treatment with BDNF in the presence or absence of a PI3K inhibitor.
Dissociated cerebellar neurons were treated with BDNF at 200 ng/ml for different lengths of time up to 120 min. The PI3K inhibitor LY294002 (20 μM) was added to cells incubated with BDNF for 30 min. Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 20 μg of proteins were subjected to 10% SDS-PAGE before being transferred to a PVDF membrane and immunostained with an antibody against phospho-Akt (Ser 473). The same blot was later stripped and probed with an antibody against total Akt to confirm equal loading.

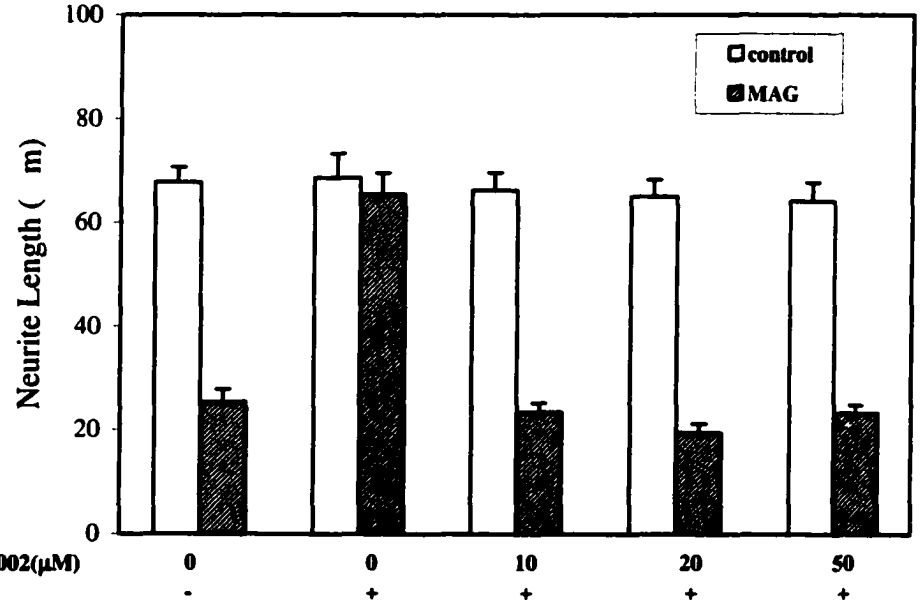


Figure 4.8 The effect of priming cerebellar neurons with BDNF in the presence of a PI3K inhibitor on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of BDNF at 200 ng/ml, in the presence or absence of 10-50 μM of PI3K inhibitor LY294002 as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The culture was then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

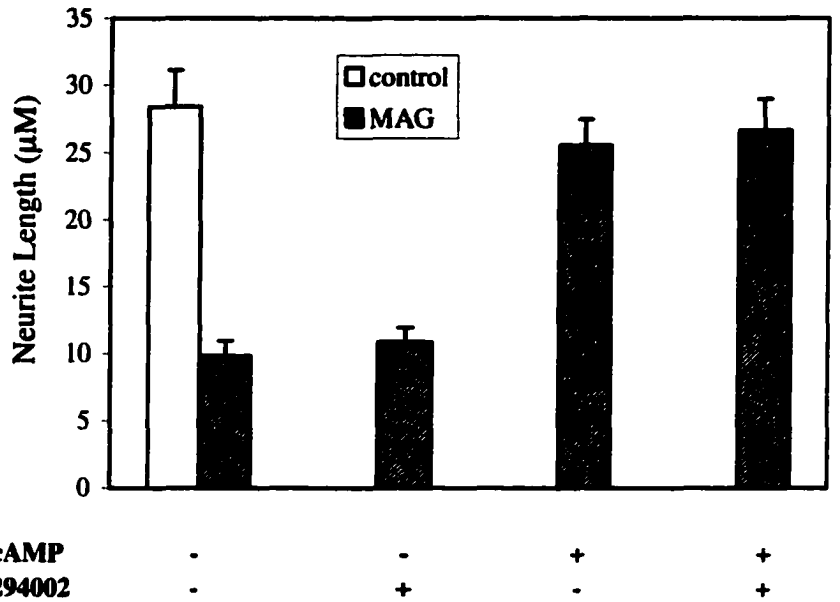


Figure 4.9 The effect of dbcAMP in the presence of a PI3K inhibior on inhibition by MAG of neurite outgrowth from cerebellar neurons.
Dissociated cerebellar neurons were plated onto monolayers of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) in the presence of dbcAMP, at 1 mM, with or without the PI3K inhibitor LY294002 (20 μM), as indicated, and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

4. Inhibition of ERK blocks the effect of GDNF on MAG

GDNF overcomes MAG's inhibition of neurite outgrowth as effectively as the NGF family of neurotrophins (Cai *et al.*, 1999). ERK also appears to be necessary for neuronal survival and neurite outgrowth mediated by GDNF (Creedon *et al.*, 1997; van Weering and Bos, 1997). To assess if activtion of ERK is required for GDNF to overcome inhibition by MAG, priming with GDNF was carried out in the presence of either U0126 or PD98059. Both MEK inhibitors completely block the ability of GDNF to overcome inhibition by MAG (Figure 4.10). Hence, similar to the NGF family of neurotrophins, inhibition of ERK blocks the effect of GDNF on MAG.

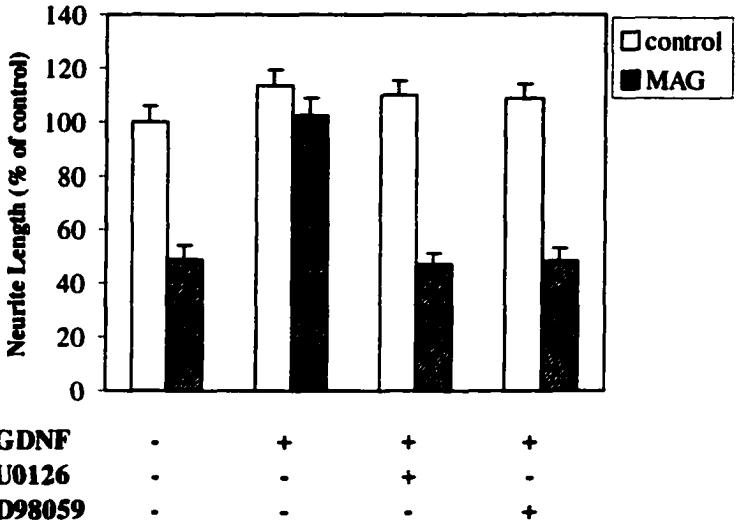


Figure 4.10 The effect of priming cerebellar neurons with GDNF in the presence of MEK inhibitors on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with no neurotrophin or in the presence of GDNF, at 200 ng/ml, with or without either MEK inhibitor PD98059 (50 μM) or U0126 (5 μM) as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The cultures were then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed without neurotrophin and subsequently grown on control CHO cells.

Discussion

We have shown in the last chapter that the effect of priming with neurotrophins to block MAG's inhibition of neurite outgrowth is mediated by Trk receptors. Thus far, several downstream cascades have been shown to play major roles in RTK-regulated neurotrophin signaling. Foremost among these is the ERK pathway. Here, we show that ERK is not only required for the BDNF effect but also the dbcAMP effect on inhibition by MAG. Both results with BDNF and dbcAMP indicate that there is a set activation threshold for cAMP/PKA required to overcome inhibition.

For BDNF treatment, it is clear that activation of ERK is required to trigger a cascade of downstream events to overcome inhibition by MAG. Since ERK

phosphorylation, mediated by BDNF, does not alter in the presence of a PKA inhibitor (Figure 4.3), it is likely that cAMP/PKA is downstream of ERK activation in the beginning of the priming process. Once activated, ERK is capable of initiating signals leading to elevation of cAMP levels and PKA activation. Recent advances also identified novel pathways that link second-messenger systems to the regulation of ERK signaling via the small G proteins Ras and Rap1. It has been reported in PC12 cells, there is a switch from the initial Ras-dependent, transient activation of ERK to the Rap1-dependent, sustained ERK activity (York *et al.*, 1998). Rap1 can be controlled by different GEFs, which are in turn regulated by multiple secondary messengers such as cAMP, calcium and DAG (Grewal *et al.*, 1999). Rap1 maintains a long period of ERK activation by forming a stable complex with B-Raf, a neuronal specific isoform of Raf. This switch from Ras to Rap1 could result in a prolonged increase in cAMP.

Although ERK is necessary for the BDNF-mediated block of inhibition, it is unlikely that it is the only pathway triggered by neurotrophins. For example, as the other major regulator of neuronal survival, PI3K is also found to be required for BDNF to overcome inhibition by MAG (Figure 4.8). Therefore, pathways activated by neurotrophins are likely to be stimulated in parallel, with each contributing some measure to overcoming inhibition. Since blocking any one pathway can diminish the neurotrophin effect, each pathway is possibly activated minimally which combined reach a threshold required to overcome inhibition. Therefore, inhibition can only be overcome when all pathways become active. The relative contribution of each pathway to the regenerative effect has not been elucidated. However, the prolonged elevation and amplification of

signals through cross-talk of the different pathways may prove to be critical for the later stage of the priming process.

Although required, activation of ERK appears not to be sufficient for overcoming inhibition by MAG. As shown previously, inhibition of PKA can abrogate the BDNF induced block of MAG's inhibition (Cai *et al.*, 1999). However, stimulation of ERK is not affected by the PKA inhibitor. Nevertheless, we can not exclude the possibility that PKA might have effects on the ERK pathway other than phosphorylation (activation) of ERK. PKA might play an important role in translocating the activated form of ERK from the cytoplasm to the nucleus (Impey *et al.*, 1998), thus making possible the activation of downstream transcription factors.

For the dbcAMP effect, it was surprising to find that although dbcAMP did not activate ERK, inhibition of ERK blocked the ability of dbcAMP to overcome inhibition by MAG. Moreover, in the presence of an ERK inhibitor, the dose-response curve of dbcAMP to overcome inhibition is shifted to the right. For example, in the absence of the ERK inhibitor, 1 mM of dbcAMP blocks inhibition by MAG completely, while only by about 50% when the inhibitor is added. A complete block of MAG's inhibition in the presence of the MEK inhibitor only occurs at 2-3 mM of dbcAMP (Figure 4.6). These results suggest that the neuronal cAMP levels are a combination of dbcAMP and the basal levels of endogenous cAMP, which together reach a threshold required to overcome inhibition by MAG. There is a set activation threshold and addition of 1 mM dbcAMP "just" reaches it. But why does a MEK inhibitor block the dbcAMP effect while dbcAMP has no effect on activation of ERK? It has been reported in other systems that activated ERK can inhibit the enzyme phosphodiesterase which is responsible for the degradation

of cAMP and results in an increase in cAMP levels in the cells. Therefore, if activation of ERK is inhibited, the basal levels of endogenous cAMP should decrease. Consequently, the threshold for regeneration is not reached and inhibition by MAG still occurs.

Moreover, the cAMP effect is only abolished by the inhibition of ERK but not PI3K (Figure 4.9), suggesting that the cross-talk for ERK and cAMP in neurons is unique and not shared by other effectors of neurotrophins.

Both cAMP and ERK can activate one common downstream target in the nucleus: the transcription factor CREB. PKA activation in the extranuclear region of the cell leads to translocation of the free PKA catalytic unit to the nucleus, whereupon it can phosphorylate CREB family members. ERK activates CREB through RSK (ribosomal S-6 kinase), with which it binds through a KIM (kinase interaction motif) docking site (Shaywitz and Greenberg, 1999). While CREB activation is often regarded as an index of PKA action, it can also provide an index of ERK activation. The significance of the overlap between PKA and ERK with regard to activating CREB is unclear. However, it will be interesting to determine if CREB is the common readout for regenerative ability in the neuron either through priming with neurotrophins or elevation of cAMP levels.

Although GDNF signals through a separate receptor system, it shares a similar mechanism to block MAG's inhibition with the NGF family of neurotrphins (Cai et al., 1999). They all require activation of ERK to overcome inhibition by MAG. This shows again that evolutionally different neurotrophic factors are capable of eliciting the same biological responses by activating similar signaling subunits.

In conclusion, our results indicate that activation of either ERK or PI3K is necessary for BDNF to block MAG's inhibition. Moreover, basal activity of ERK in our

system may be involved in cAMP's ability to overcome inhibition by MAG. The next step will be to determine how activation of ERK affects the levels of intracellular cAMP. This will be done by exploring the possibility of ERK influencing cAMP levels through inhibition of PDE, as has been suggested by others in transfected cells from outside the nervous system (Hoffmann *et al.*, 1999; MacKenzie *et al.*, 2000).

CHAPTER V

DIRECT INHIBITION OF PDE BLOCKS THE MEK INHIBITOR EFFECT ON BDNF AND cAMP

Introduction

cAMP is an important intracellular second messenger. It is involved in mediating the action of a host of processes in specialized cells, including from control of metabolic events, muscle contraction, secretion and memory formation (Houslay and Milligan, 1997). The level of endogenous cAMP is tightly regulated via synthesis by adenylyl cyclases and degradation by phosphodiesterases (PDE). The complexity of the cAMP synthetic machinery is exemplified by the existence of at least 10 forms of adenylyl cyclases able to generate cAMP. These enzymes are not functionally redundant, but rather, are related to requirements of defined regulation and intracellular localization (Sunahara *et al.*, 1996). More impressive is the machinery for cAMP degradation. For, at the present time, at least 15 genes encoding over 30 different PDE isoforms have been described (Conti and Jin, 1999; Soderling and Beavo, 2000). PDE enzymes can be found either as soluble cytosolic species or targeted to interact with particular subcellular membranes and other proteins (Houslay and Milligan, 1997; Houslay *et al.*, 1998), suggesting a fundamental role in defining compartmentalized signaling reactions.

Recent studies have highlighted the importance of intracellular cAMP as a gating element in a number of different signaling mechanisms in neurons. For example, cAMP regulates synaptic plasticity as well as neurotrophin-dependent survival in the CNS through its gating function (Iyengar, 1996). In addition, it was recently reported that the turning response of a growth cone 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 (Ming *et al.*, 1997; Song *et al.*, 1998). Moreover, we reported that the inhibition of axonal growth by MAG and myelin can be blocked when

neurons are primed with neurotrophins before they encounter myelin inhibitors. This priming effect is via activation of a cAMP-dependent pathway. Artificially elevating neuronal cAMP levels by pharmacological reagents can reverse the inhibitory effect of MAG/myelin on axonal regeneration without priming (Cai *et al.*, 1999). Furthermore, from previous chapters, we showed that the priming effect of neurotrophins is mediated by Trk receptors and requires activation of ERK. Thus, the question has been raised as to how an important second messenger, cAMP integrates into other signal systems, specifically the ERK pathway initiated by extracellular growth factors.

When the charting of the ERK pathway was successfully accomplished some 10 years ago, it laid open a seamless connection between different pathways. First came the seminal discovery that elevation of cAMP in fibroblasts and vascular smooth muscle cells inhibits ERK activation by growth factor (Burgering *et al.*, 1993; Cook and McCormick, 1993; Graves *et al.*, 1993). This inhibition was identified to target Raf-1 and require the action of PKA. However, evidence steadily accumulated that cAMP exerts controlling effects on the ERK signaling pathway in a cell-type specific fashion. cAMP seemingly promotes ERK activity in several other cell types, including neuronal cells where ERK closely participates in cAMP-regulated processes like long-term potentiation. Differentiation of PC12 cells appears to be mediated by cAMP through stimulation of MEK (Yao *et al.*, 1998). Alternatively, cAMP/PKA plays a more direct role by affecting ERK inactivation and subcellular localization. It has been shown recently that tyrosyl phosphatases specifically regulating ERK are phosphorylated by PKA and PKA prevents their binding to ERK (Blanco-Aparicio *et al.*, 1999; Saxena *et al.*, 1999). Thus, PKA

activation synergizes and even sustains ERK activation by inhibiting its dephosphorylation.

Very recently it has been shown that members of the multigene PDE4 can serve as ERK substrates (Hoffmann *et al.*, 1999; MacKenzie *et al.*, 2000). PDE4 is cAMP specific and characterized by its ability to be inhibited by the compound rolipram. The PDE4 family accounts for about 70% of the total PDE activity in neurons. This is in contrast to other tissues, where the relative contribution of PDE4 is much less. Moreover, the disruption of a closely related PDE4 member *dunc* PDE in *drosophila* causes learning and memory defects. The specific inhibitor of PDE4, rolipram, has also been used in clinical trial as an antidepressant agent (Houslay *et al.*, 1998). These observations indicate the importance of PDE4 in the nervous system. Recently, PDE4 has been shown to serve as a substrate for ERK in transfected COS cells (Hoffmann *et al.*, 1999; MacKenzie *et al.*, 2000). Activity of PDE4 is inhibited through phosphorylation by ERK upon EGF (epidermal growth factor) activation. As a result, the intracellular level of cAMP increases. This provides a direct means of how activation of ERK by a tyrosine kinase receptor affects the cAMP-dependent pathway.

Results from Chapter IV imply that inhibition of ERK activity affects endogenous cAMP levels since more exogenous cAMP analog is needed to reach a set threshold for regeneration. Now, in this chapter, we examine if activation of ERK will indeed inhibit activity of PDE in primary neurons, and how this inhibition will affect the endogenous levels of cAMP.

Results

1. BDNF inhibits PDE4 activity and elevates cAMP in a MEK-dependent manner

To demonstrate that BDNF affects PDE activity directly, cerebellar neurons were treated with BDNF in the presence or absence of the MEK inhibitor U0126. After various lengths of time, the cells were lysed and the PDE activity measured. We found that the activity of PDE was reduced by about 35% after BDNF treatment, within 5 minutes. This decrease is sustained for about half an hour. The PDE activity returns nearly to basal levels after about an hour, most likely due to a feedback response of PKA activating PDE (MacKenzie *et al.*, 2000). In sharp contrast, no reduction was detected for total PDE activity if cells were treated with BDNF in the presence of the MEK inhibitor. Activity is the same as in control, untreated neurons (Figure 5.1). However, this assay measures total PDE activity. As reported by others, ERK specifically inhibits type 4 PDEs (MacKenzie *et al.*, 2000). In order to determine the percentage of non-type 4 PDE in cerebellar neurons, we treated aliquots of the same BDNF-incubated lysates with specific PDE4 inhibitor rolipram before measuring total PDE activity. We observed that 60-70% of the activity is rolipram-sensitive PDE4 activity in cerebellar neurons, consistent with PDE4 being the dominant type of PDE in the nervous system (Figure 5.1). Treatment with neurotrophin does not change the percentage of non-PDE4 activity. Therefore, the decrease in PDE activity in response to BDNF is entirely due to an effect on PDE4. Treatment of cerebellar neurons with BDNF results in an inhibition of PDE4 and a MEK inhibitor blocks this effect.

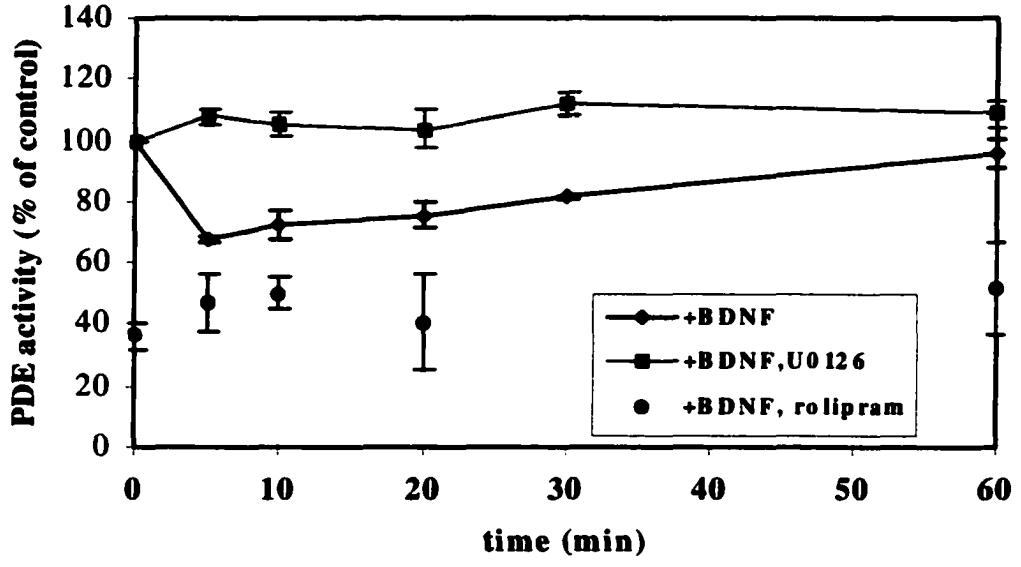


Figure 5.1 PDE activity in cerebellar neurons treated with BDNF in the absence or presence of a MEK inhibitor.
Dissociated cerebellar neurons were challenged with BDNF, at 200 ng/ml, in the presence or absence of a MEK inhibitor U0126 (5 μM) as indicated, and lysed at the indicated times for the determination of PDE activity. In addition, aliquots of the same BDNF-treated cell lysates were incubated with rolipram (100 μM) for a further 15 min before PDE assay to determine the percentage of rolipram insensitive PDE in the lysates. Results show the mean PDE activity (+/- sem). Results are standardized to percentage of control. Control was taken as PDE activity from untreated neurons.

Previously we showed that BDNF treatment increased the endogenous levels of cAMP in cerebellar neurons (Cai *et al.*, 1999). Now, we want to determine if this increase in cAMP is MEK/ERK dependent. Cerebellar neurons were treated with BDNF in the presence or absence of the MEK inhibitor U0126 for half an hour. As we had reported before, after BDNF treatment, the levels of cAMP in cerebellar neurons nearly double. However, when U0126 is included along with BDNF, this increase in cAMP levels does not occur (Figure 5.2). More importantly, the MEK inhibitor alone does not affect endogenous cAMP levels. Together these results show that, first, BDNF inhibits PDE4 activity and, second, that both the BDNF-induced inhibition of PDE4 and increase in

cAMP are MEK-dependent. This indicates that BDNF-mediated inhibition of PDE4 and the consequent elevation of cAMP levels, are mediated by activity of ERK.

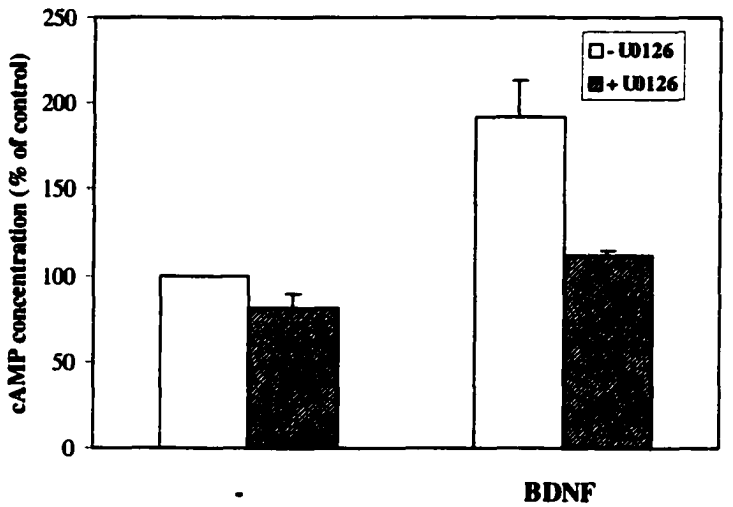


Figure 5.2 Measurement of cAMP concentration in cerebellar neurons after exposure to BDNF in the presence or absence of a MEK inhibitor.
Dissociated cerebellar neurons were plated in individual wells of a 24-well plate. The neuron were cultured overnight after which BDNF, at 200 ng/ml, was added in the presence or absence of a MEK inhibitor, U0126 (5 μM) as indicated for a further 30 min. Following incubation, the cAMP levels were measured and compared to a standard. The results are the mean of 3 separate experiments, each done in quadruplicate. Results are standardized to percentage of control. Control was taken as the cAMP concentration in untreated neurons.

2. Direct inhibition of PDE4 blocks the MEK inhibitor effect on BDNF treatment

We reasoned that if MEK/ERK activation is having its effect through inhibition of PDE4, then direct inhibition of PDE4 should block the effect of a MEK inhibitor on inhibition by MAG. Hence, we used a specific inhibitor of PDE4, rolipram, to restore the BDNF effect on MAG's inhibition in the presence of a MEK inhibitor.

Since inactivation of PDE4 increases the intracellular cAMP level and priming with neurotrophin also results in elevation of endogenous cAMP, incubation with

rolipram should have a similar consequence as priming neurons with neurotrophin. Indeed, when neurons were primed with rolipram at a concentration of 0.5 μM before being plated on MAG-expressing cells, they extended neurites comparable in length to those primed with BDNF (Figure 5.3). However at a lower dose of rolipram (0.1 μM), intracellular cAMP level is not elevated high enough to reach the threshold to reverse MAG's inhibition on neurite outgrowth.

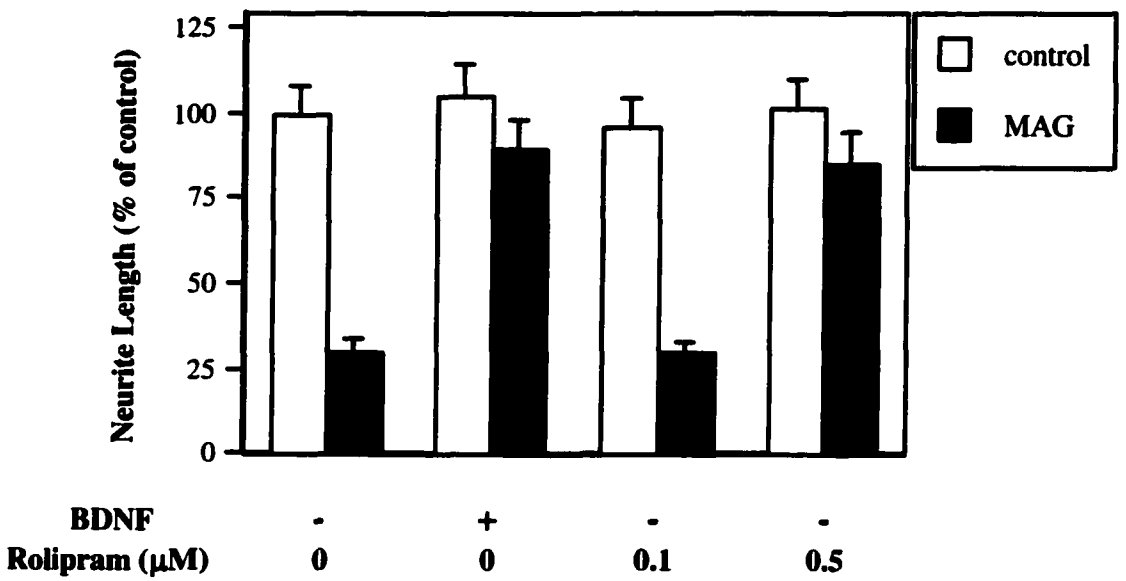


Figure 5.3 The effect of priming cerebellar neurons with BDNF and differing concentrations of rolipram on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of BDNF at 200 ng/ml, or with either 0.1 μM or 0.5 μM of rolipram as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The culture was then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed with nothing and subsequently grown on control CHO cells.

If inactivation of PDE4 by ERK is a direct downstream effect of priming with BDNF, inhibition of PDE4 by rolipram should mimic the activation of ERK and reverse the effect of the MEK inhibitor. Here, we applied a low dose of rolipram (0.1 μM) which, by itself, is unable to overcome MAG's inhibition. As shown before, the MEK inhibitor U0126 abolishes the priming effect of BDNF (Figure 5.4). However, when rolipram at a low concentration is included in the culture with U0126 during priming with BDNF, the ability to overcome inhibition by MAG is restored.

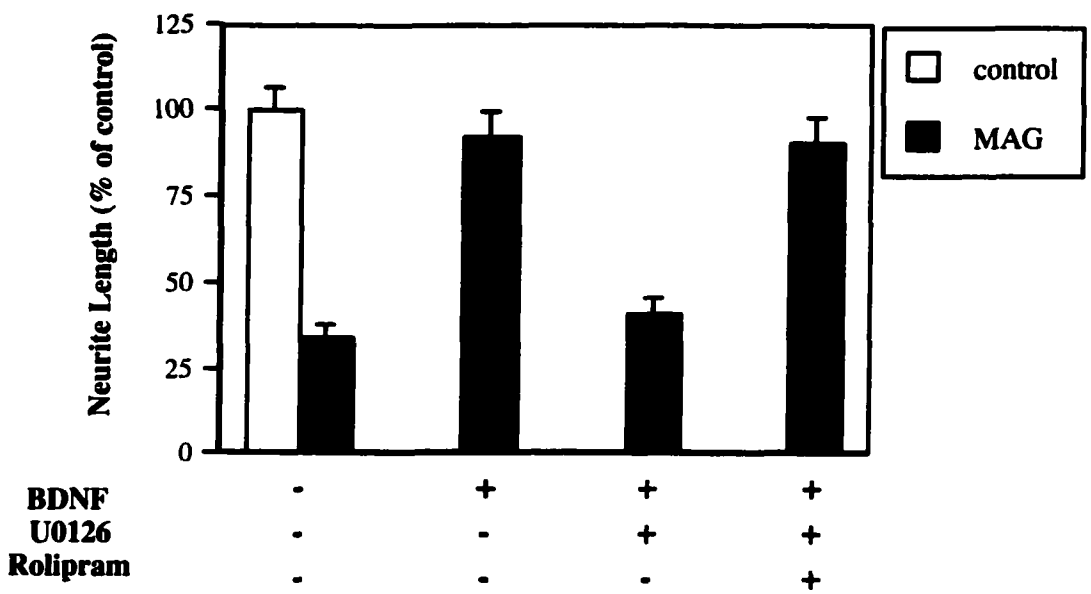


Figure 5.4 The effect of rolipram to block the MEK inhibitor effect on priming with BDNF. Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of BDNF at 200 ng/ml, with or without 0.1 μM of rolipram or MEK inhibitor U0126 at 5 μM as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture. The culture was fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed with nothing and subsequently grown on control CHO cells.

3. Direct inhibition of PDE4 blocks the MEK inhibitor effect on cAMP

Besides rolipram blocking the MEK inhibitor effect on BDNF, a similar result was obtained when a cAMP analog, dbcAMP (1 mM) was used to overcome MAG's inhibition. The MEK inhibitor U0126 blocks dbcAMP's effect on regeneration, most likely due to inhibition of basal levels of ERK activity. This impediment can be reversed by adding 0.1 μM of rolipram when incubating neurons with dbcAMP in the presence of U0126 (Figure 5.5). These neurons were no longer inhibited by MAG and extend neurites as long as those where dbcAMP was added alone.

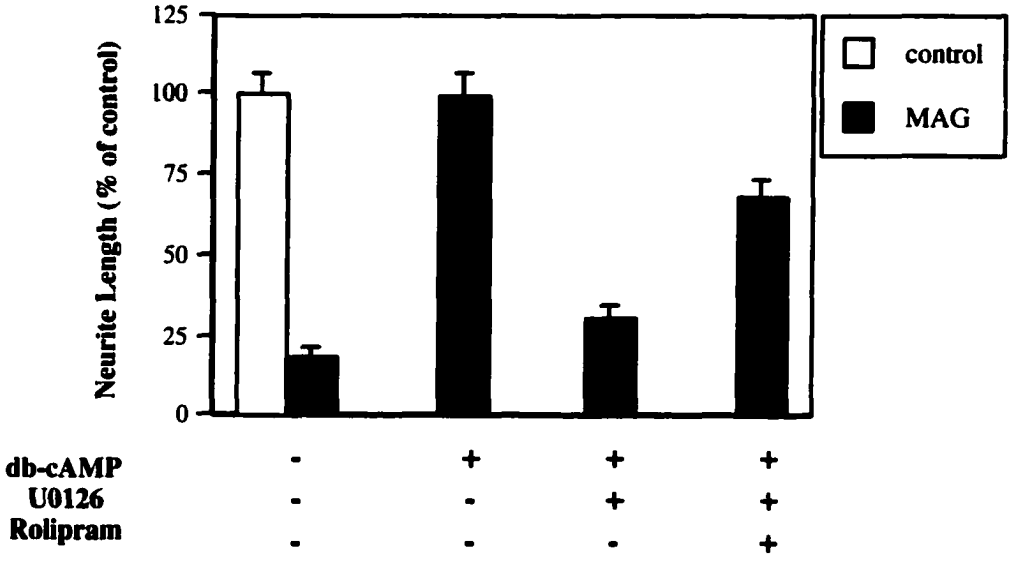


Figure 5.5 The effect of rolipram on the block of the MEK inhibitor effect on dbcAMP to overcome inhibition by MAG.
Dissociated cerebellar neurons were plated onto monolayers of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) in the presence of dbcAMP, at 1 mM, with or without MEK inhibitor U0126 (1 μM) or rolipram (0.1 μM) as indicated, and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of neurotrophin and inhibitors.

dbcAMP is a non-hydrolyzable analog of cAMP. Its physiological efficiency is very low compared to endogenous cAMP, thus only artificially elevating cAMP levels. To confirm that rolipram is able to block the MEK inhibitor effect on cAMP, another reagent that elevates the endogenous levels of cAMP, forskolin, was tested for its ability to overcome inhibition by MAG. Forskolin is an activator of adenylate cyclase, the enzyme responsible for sythesis of endogenous cAMP. Prior exposure of neurons to 1 μM of forskolin completely abrogates MAG's inhibition and has a similar effect on neurite outgrowth as addition of dbcAMP (Figure 5.6). At concentrations over 1 μM, forskolin becomes toxic and affects cell survival. As has been shown for BDNF and dbcAMP, a MEK inhibitor also abolishes forskolin's (1 μM) effect on neurite outgrowth. Addition of a low dose of rolipram overcomes the effect of the MEK inhibitor and forskolin again promotes neurite outgrowth in the presence of MAG (Figure. 5.7).

These results suggest that inhibition of MEK in the presence of BDNF, dbcAMP or forskolin allows for the lowering of the level of cAMP to just below the threshold required to overcome inhibition by MAG. Therefore, a small increase in endogenous cAMP, through minimal inhibition of PDE4, is sufficient to once again raise the neuronal cAMP levels above this threshold level.

Finally, from both the neurite outgrowth functional assay and the biochemical assay of PDE activity and cAMP measurement, we showed that activation of ERK by neurotrophin in cerebellar neurons inhibits PDE4 activity and, in turn, elevates intracellular cAMP levels. This is a novel mechanism that suggest ERK regulates the cAMP pathway in the nervous system.

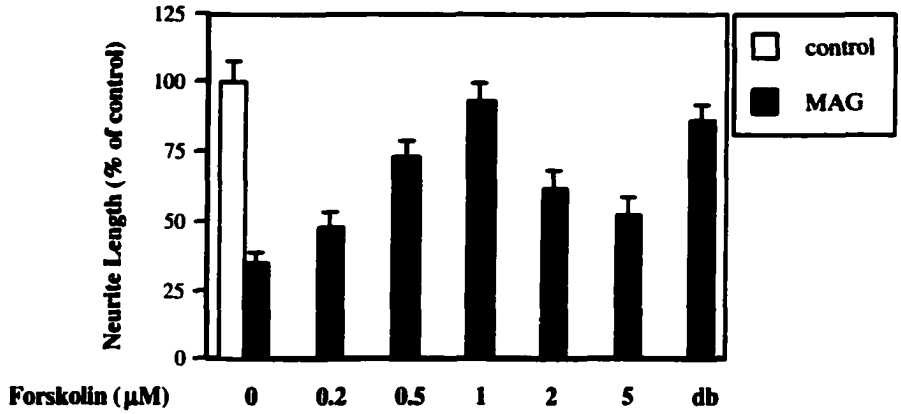


Figure 5.6 The effect of priming cerebellar neurons with forskolin on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of 0.2-5 μM of forskolin as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture. The culture was then fixed and immunostained for GAP43. Neurons were also grown on MAG-expressing CHO cells in the presence of dbcAMP (1 mM). Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed with nothing and subsequently grown on control CHO cells.

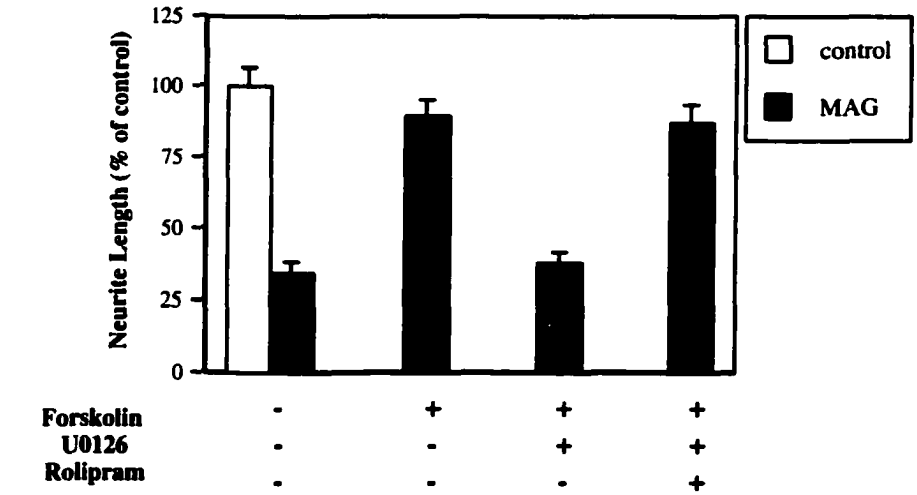


Figure 5.7 The effect of rolipram to block the MEK inhibitor effect on priming with forskolin.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of forskolin at 1 μM, with or without 0.1 μM of rolipram or MEK inhibitor U0126 at 5 μM as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bar) for further overnight culture. The culture was then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons primed with nothing and subsequently grown on control CHO cells.

Discussion

We reported before that neurotrophins were able to overcome inhibition by MAG in a cAMP/PKA dependent manner (Cai *et al.*, 1999). Activation of the Trk receptors and their downstream effector ERK, which are not classically regarded as being involved in the cAMP pathway, are required in this priming process (Chapter III and IV). Here we demonstrate that cross-talk between these two pathways is responsible for the neurotrophin-induced increase in cAMP that overcomes inhibition. We also show that ERK inhibits PDE4 which allows cAMP to accumulate and PKA to be activated. To our knowledge, this is the first time that activation of ERK has been shown to activate a cAMP-dependent pathways in neurons.

PDE4 is the most abundant PDE in neurons, accounting for about 70% of total PDE activity. PDE4 specifically breaks down endogenous cAMP to ATP and 5'-AMP. It is a key enzyme in regulating endogenous cAMP levels in the nervous system. As reported by others, PDE4 is strongly inhibited by ERK in transfected cells (MacKenzie *et al.*, 2000) and as shown, BDNF appears not to affect the level of activation of non-type 4 PDE in neurons (Figure 5.1). Hence, if we eliminate the contribution of the non-type 4 PDE from the total PDEs, there is a dramatic decrease in PDE4 activity (approximately 50%) in cerebellar neurons treated with BDNF (Figure 5.1). Although the level of PDE activity returns to normal after 1 hour, most likely due to feedback regulation by PKA, the dramatic drop in PDE activity within 5 minutes provides the initiation of a signal that leads to transient increase of cAMP levels, which may be sufficient to induce the downstream events which eventually results in axon regeneration.

The results with BDNF, dbcAMP and forskolin all indicate that there is a defined activation threshold for cAMP that is required to overcome inhibition. A subtle change in the level of endogenous cAMP greatly affects the ability of neurons to regenerate. Hence, addition of the PDE4 inhibitor, rolipram at a concentration that alone does not overcome inhibition, presumably because the cAMP threshold is not reached, is able to restore the BDNF and cAMP effects when a MEK/ERK inhibitor is present.

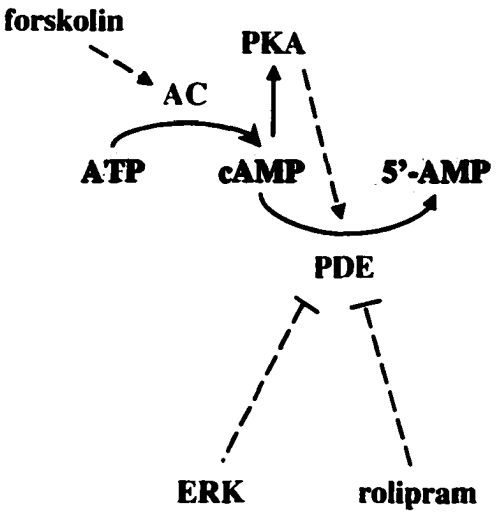


Figure 5.8 Cross-talk between the ERK and cAMP signaling systems via PDE.
Endogenous cAMP level is tightly regulated by adenylyl cyclases (AC) and phosphodiesterase (PDE). forskolin activates AC and increases endogenous cAMP levels; while ERK or rolipram elevates cAMP by inhibiting PDE. When cAMP level is up-regulated, it activates protein kinase A (PKA), which in turn inhibits PDE through a feedback loop.

cAMP is a key second messenger in all cell types. However, to our knowledge, stimulation of the cAMP pathway is a novel signaling mechanism for neurotrophins. Since most of the neurotrophin-mediated activities, such as survival, are cAMP/PKA independent, it is of particular interest that priming with neurotrophins to overcome inhibition by MAG/myelin is different from the other neurotrophin-mediated activities. Here, in our system, neurotrophins are not simply promoting neurite extension but, more importantly, are also overcoming the effect of inhibitors in myelin. It is possible that

signaling through the cAMP pathway may also be used by neurotrophins for other, as yet unidentified effects.

The interaction between the cAMP and ERK signaling systems is of pivotal importance. The very complexity of these signaling systems makes resolving the connections served to integrate cellular responses through these pathways extreamely challenging. However, molecular dissection of the integration of different signaling pathways will allow us to seek out the common downstream targets and provide a more rational and direct means to manipulate the pathway. In our case, this will be to find the most effective way to overcome inhibition and encourage axons to regenerate.

One point to bear in mind in analyzing different signaling systems is to take account of the set of molecular players involved, their isoforms and intracellular location. In trying to reconcile studies performed in different cell types, it is crucial to identify the isoforms of the signaling components, as well as, whether the intracellular organization of these components generates compartmentalized responses. The best example here is the study of cross-talk between the cAMP and ERK pathways. Even though cAMP has been shown to inhibit ERK in some cell types, cAMP mostly synergizes the ERK pathway in neurons (reviewed in Houslay and Kolch, 2000). This distinction between different cell types is, most likely, due to the existence of different isoforms of the signaling components in the ERK pathway, such as Rap1 and B-Raf in the nervous system. In addition, different PDE isoenzymes targeted to specific intracellular sites, offer the potential for differential regulation of cAMP levels by ERK, in discrete intracellular domains. Moreover, coupled feedback and feedforward loops may affect the output of signals in appropriate temporal windows. Thus, changes in individual

components of signaling systems such as cAMP, are transient and geared to trigger a defined cascade of responses with time as they pass over set activation thresholds.

Finally, since the cAMP and ERK signaling pathways both converge on CREB and are involved in the block of MAG's inhibition, the next step in this study would be to determine if CREB plays a role in blocking MAG's inhibition on neurite outgrowth by neurotrophin/cAMP.

CHAPTER VI

ACTIVATION OF CREB IS REQUIRED TO BLOCK MAG'S INHIBITION

Introduction

The cyclic AMP response element binding protein (CREB) is critical for a variety of cellular processes, including proliferation, differentiation, hormonal control of metabolic processes and learning and memory (Hanson and Reshef, 1997; Shaywitz and Greenberg, 1999; Silva *et al.*, 1998). Identification and characterization of CREB stemmed from studies of cAMP stimulating target gene expression via a conserved cAMP-responsive element (CRE). The CRE consists of an eight base-pair palindromic sequence (TGACGTCA) and is typically found within 100 nucleotides of the TATA box (Comb *et al.*, 1986; Montminy *et al.*, 1986; Short *et al.*, 1986). The palindromic CRE can also be separated into two CGTCA motifs, which may be configured on the same or on opposite strands to function cooperatively in response to stimuli (Fink *et al.*, 1988). The CRE was subsequently shown to bind CREB, a 43 kDa phosphoprotein (Montminy and Bilezikjian, 1987). Characterization of CREB lead to the discovery of two other, highly related family members: activating transcription factor 1 (ATF-1) (Hai *et al.*, 1989) and cAMP response element modulator (CREM) (Foulkes *et al.*, 1991). The CREB family members are capable of activating the transcription of target genes in response to a diverse array of stimuli. However, alternatively spliced isoforms of CREM either function as inhibitors or activators of CRE-mediated transcription (Shaywitz and Greenberg, 1999).

CREB belongs to a large family of bZIP (basic-domain-leucine zipper) transcription factors, including mammalian c-Fos, c-Jun and c-Myc. The family members of the bZIP transcription factors dimerize via the C-terminal leucine zipper and bind to a DNA target sequence through a lysine- and arginine-rich basic domain just amino-

terminal to the leucine zipper (Dwarki *et al.*, 1990). CREB, CREM and ATF-1 are able to form homodimers or heterodimers with each other. However, the CREB homodimers bind to the CRE with a longer half-life (Kobayashi and Kawakami, 1995). The N-terminus of CREB contains a modular activation domain that contains the kinase-inducible domain (KID) (de Groot *et al.*, 1994; Gonzalez *et al.*, 1991) and two glutamine-rich domains, Q1 and Q2, flanking the KID (Laoide *et al.*, 1993; Nakajima *et al.*, 1997).

Diverse extracellular stimuli activate CREB through multiple signaling cascades that converge to phosphorylate a critical residue, Ser133. Moreover, CREB activity and specificity can be further modulated by phosphorylation of additional sites on CREB or of proteins associated with CREB. Ser133 is located within the 60-residue region of the KID, which encompasses multiple potential phosphorylation sites for various protein kinases (Gonzalez *et al.*, 1991). The KID element is both necessary and sufficient for signal-induced activation of CREB (Brindle *et al.*, 1993; Quinn, 1993).

The CREB-binding protein (CBP) is associated with CREB in a phosphorylation-dependent manner. The N terminus KIX (KID interaction) domain of CBP binds to the KID domain of CREB (Chrivia *et al.*, 1993). CBP serves as a molecular bridge that allows upstream transcription factors, such as CREB, to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box (Barlev *et al.*, 1995; Berger *et al.*, 1992). In addition, CBP possesses an intrinsic histone acetyltransferase activity and is able to alter chromatin structure and make the DNA template more accessible to the transcriptional machinery (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996; Yang *et al.*, 1996b). Therefore, CBP contributes to the CREB-mediated transcription by recruiting Pol II and affecting chromatin structure.

The use of dominant-negative mutants of CREB has facilitated investigation of how CREB is involved in transcription in response to a variety of biological stimuli. There are three dominant-negative mutants of CREB available to date: M1, K-CREB and A-CREB. M1 is a non-phosphorylatable CREB, due to the mutation of Ser133 to Ala, thereby inhibiting CREB action by competitively binding to CRE and preventing access to wild-type CREB and other CRE-binding factors (Gonzalez and Montminy, 1989). K-CREB is unable to bind to CRE because of a mutation within the DNA-binding domain. However, K-CREB is able to dimerize with wildtype CREB family members and blocks gene activation by decreasing endogenous CREB family members interacting with the CRE (Walton *et al.*, 1992). Finally, A-CREB, in which basic residues within the bZIP domain have been mutated to acidic residues, also functions by heterodimerizing with endogenous CREB family members and preventing their binding to the CRE. Moreover, heterodimers of A-CREB and wild-type CREB are more stable than homodimers of either CREB or A-CREB, thereby ensuring that A-CREB and CREB will interact preferentially within a cell (Ahn *et al.*, 1998).

Diverse extracellular stimuli, such as neurotrophic factors and second messengers like cAMP and calcium, activate CREB at Ser133 (Figure 6.1). In most cells, the primary target of cAMP is the cAMP-dependent protein kinase A (PKA). Upon binding to cAMP, the catalytic subunits of PKA are released and translocated to the nucleus to phosphorylate CREB (Hagiwara *et al.*, 1993). As a pleiotropic second messenger, calcium activates a variety of signal transduction pathways. Calcium-enhanced kinases capable of phosphorylating CREB include: CaMK (calcium/calmodulin-dependent protein kinase), RSK (ribosomal S6 kinase), PKC (protein kinase C) and PKA (Shaywitz

and Greenberg, 1999). On the other hand, the neurotrophin-induced CREB kinases include RSK, a downstream target of the Ras/ERK pathway, MAPK (p38)-activated protein kinase-2 (MAPKAP-2), CaMK and possibly, Akt. Despite the common feature of phosphorylating Ser133, the mechanism by which CREB activates transcription varies and depends on the stimulus. In some cases, signaling pathways target additional sites on CREB or proteins associated with CREB, permitting CREB to regulate distinct programs of gene expression under different conditions of stimulation. The observation that diverse extracellular stimuli regulate CREB functions indicates that CREB and related family members play critical roles in development and adaptive responses that require stimulus-dependent transcription.

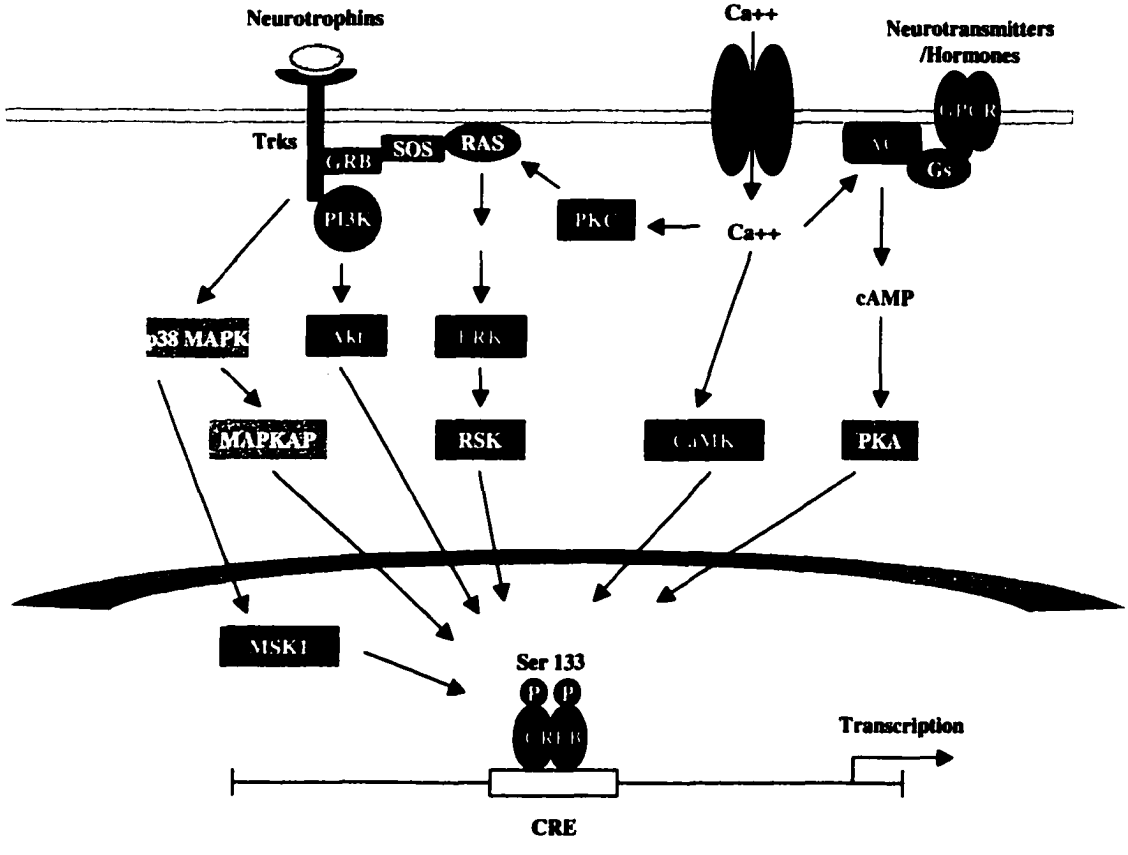


Figure 6.1 CREB signaling pathway.

Inhibitors of regeneration in myelin such as MAG (myelin-associated glycoprotein) play an important role in preventing growth after injury. As reported previously, neurotrophins overcome inhibition of axon regeneration by MAG/myelin via a cAMP-dependent mechanism (Cai *et al.*, 1999). Since CREB has been shown to be activated by both neurotrophins and cAMP, we want to determine if CREB also plays a role in axon regeneration, which possesses overlapping and similar features to CREB-dependent survival and memory formation.

Results

1. An inhibitor of transcription abrogates BDNF/cAMP's ability to overcome inhibition by MAG

Before exploring a role for a transcription factor (specifically, CREB) in axon regeneration, we want to ensure that priming neurons with neurotrophins or incubating with dbcAMP to block MAG's inhibition is transcription-dependent. Indeed, when the transcription inhibitor DRB (5,6-Dichloro-1-β-D-ribofuransylbenzimidazole) is included, BDNF no longer blocks MAG's inhibition (figure 6.2). The effect of dbcAMP on regeneration is also abolished by DRB (figure 6.3). The inhibitor alone does not affect neurite outgrowth and MAG's inhibition. Therefore, both the BDNF and dbcAMP effects on MAG are transcription dependent.

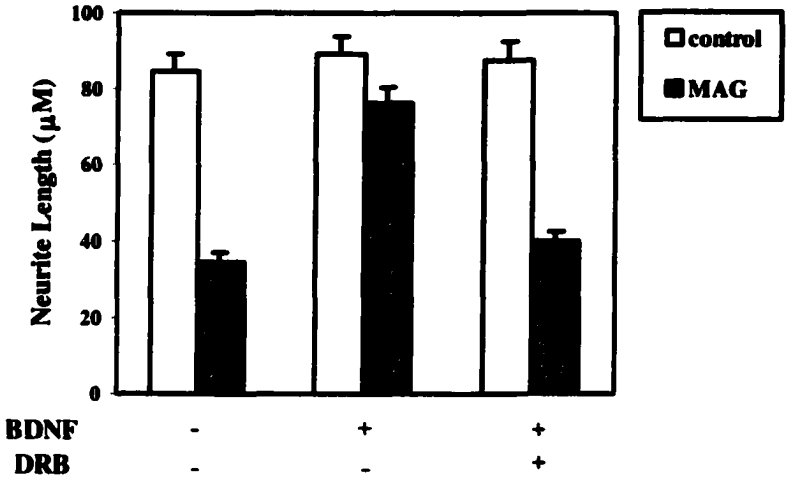


Figure 6.2 The effect of priming cerebellar neurons with BDNF in the presence of a transcription inhibitor on subsequent inhibition of neurite outgrowth by MAG.
Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with nothing or in the presence of BDNF at 200 ng/ml, in the presence or absence of 5 μM of DRB as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The cultures were then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

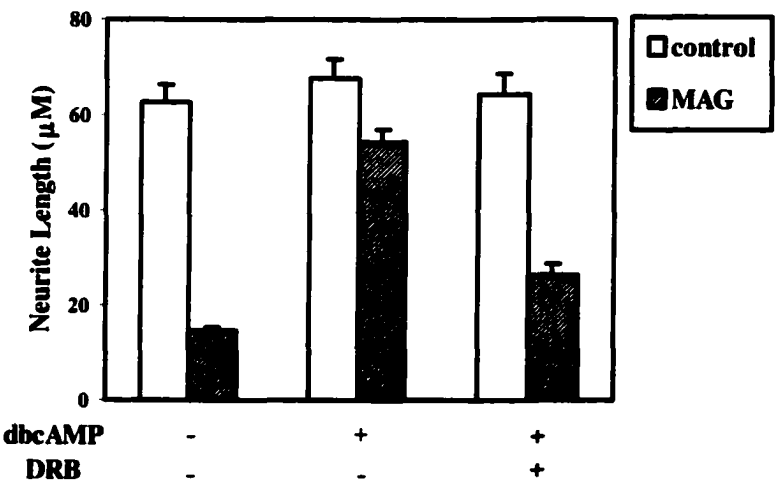


Figure 6.3 The effect of dbcAMP in the presence of a transcription inhibitor on inhibition by MAG of neurite outgrowth from cerebellar neurons.
Dissociated cerebellar neurons were plated onto a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) and cultured overnight in the presence of dbcAMP at 1 mM, with or without 5 μM of DRB as indicated. The cultures were then fixed and immunostained for GAP43, and the neurites were measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

2. CREB is activated in response to BDNF/cAMP in neurons

The ability to easily monitor phosphorylation of CREB at Ser133 *in vitro* has proved to be important in identifying the signaling pathways that trigger CREB phosphorylation and activation. To determine if CREB is activated under the conditions used here by BDNF, cerebellar neurons were treated with BDNF for various lengths of time before being lysed and subjected to Western blotting with an antibody against phospho-Ser133 of CREB. We show that incubation of neurons with BDNF results in activation of CREB (Figure 6.4). It is markedly phosphorylated within 15 min, reaches the highest level at 30 min, and goes back to basal levels after 2 hours.

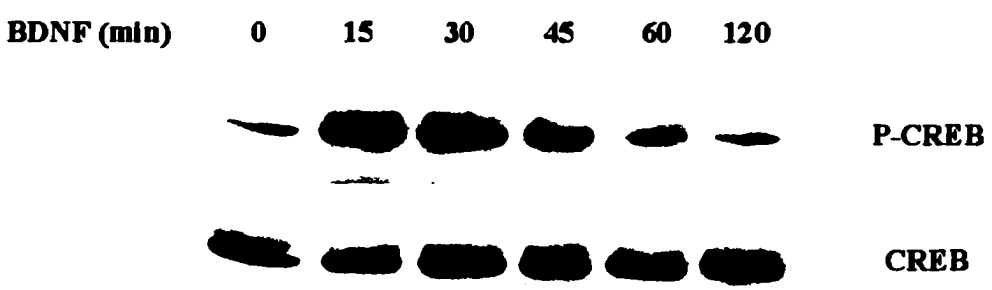


Figure 6.4 Phosphorylation of CREB in cerebellar neurons after treatment with BDNF.
Dissociated cerebellar neurons were treated with BDNF at 200 ng/ml for different lengths of time, up to 120 min. Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 50 μg of proteins were subjected to 10% SDS-PAGE before being transferred to PVDF membrane and immunostained with an antibody against phospho-CREB (Ser 133). The same blot was later stripped and probed with an antibody against total CREB to confirm equal loading.

BDNF-induced CREB phosphorylation may involve the Ras/ERK, calcium/CaMK, cAMP/PKA and possibly PI3K/Akt pathways (Figure 6.1). Indeed, inhibitors of either MEK/ERK, PKA, CaMK or PI3K partially inhibit CREB phosphorylation (Figure 6.5), as has been reported by others. Moreover, we also showed

that ERK (chapter IV), PI3K (Chapter IV), PKA (Cai *et al*, 1999) and CaMK (unpublished data from T. Spencer) are required for priming with neurotrophin to block MAG's inhibition. Thus, it is likely that many of the pathways leading to CREB activation play a role during priming with BDNF and CREB activation by all of these pathways could be the parameter that controls the outcome of upstream actions.

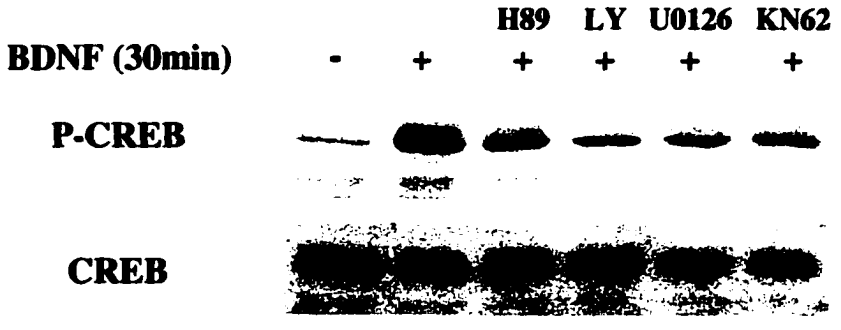


Figure 6.5 Phosphorylation of CREB after treatment with BDNF in the presence of different inhibitors. Dissociated cerebellar neurons were treated with BDNF at 200 ng/ml for 30 min in the presence of either nothing, or MEK inhibitor U0126 (5 μM), or PKA inhibitor H89 (5 μM), or CaMK inhibitor KN62 (10 μM), or Akt inhibitor LY294002 (20 μM). Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 50 μg of proteins were subjected to 10% SDS-PAGE before being transferred to PVDF membrane and immunostained with an antibody against phospho-CREB (Ser 133). The same blot was later stripped and probed with an antibody against total CREB to confirm equal loading.

Activation of CREB by dbcAMP is sustained longer than by BDNF. CREB is activated within 5 min and still heavily phosphorylated after 2 hr (Figure 6.6). Compared to the complex downstream signaling components capable of activating CREB, the primary target of cAMP elvation is PKA. Therefore, phosphorylation of CREB induced by dbcAMP is attenuated by the PKA inhibitor H89 (Figure 6.6). However, this block of CREB activation is not complete. There are still very low levels of phospho-CREB when H89 is present, indicating the existence of signaling molecules other than PKA activating CREB when stimulated by dbcAMP.

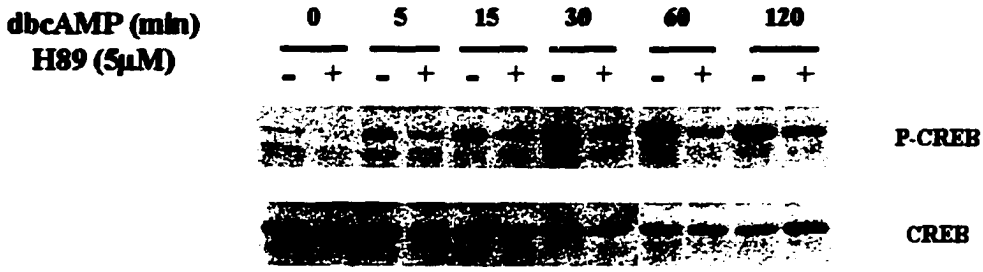


Figure 6.6 Phosphorylation of CREB after treatment with dbcAMP in the presence of a PKA inhibitor. Dissociated cerebellar neurons were treated with dbcAMP at 1 mM for different lengths of time, up to 2 hr, with or without the PKA inhibitor H89 (5 μM). Neurons were then lysed in boiled 1X SDS sample buffer with phosphatase inhibitors. 50 μg of proteins were subjected to 10% SDS-PAGE before being transferred to a PVDF membrane and immunostained with an antibody against phospho-CREB (Ser 133). The same blot was later stripped and probed with an antibody against total CREB to confirm equal loading.

3. A dominant-negative form of CREB (A-CREB) abolishes the CRE-mediated gene expression

To study CREB activation and function, we used a CREB mutant (A-CREB) in which basic residues within the bZIP domain were mutated to acidic residues. The mutated acidic patch of A-CREB is believed to mimic DNA and bind to the basic region of a wild-type CREB partner, thereby preventing their interaction with the CRE (Ahn *et al.*, 1998). In addition, heterodimers of A-CREB and wildtype CREB are orders of magnitude more stable than homodimers of either CREB or A-CREB, ensuring that A-CREB and CREB interact preferentially within a cell. (Figure 6.7).

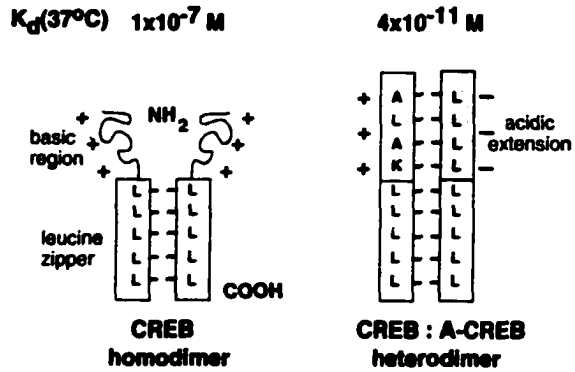


Figure 6.7 A schematic representation of an A-CREB-CREB dimer. The left panel shows a CREB homodimer with unhelical basic regions. The right panel shows a heterodimer of CREB and A-CREB resulting in an α-helical formation in the basic region. (adapted from Ahn *et al.*, 1998)

The original adenovirus carrying A-CREB was kindly provided by Dr. C. Vinson from NIH. In order for easy detection of infected cells, we modified the adenovirus construct by inserting the GFP (green fluorescent protein) gene as a marker into the virus genome. Therefore the modified adenovirus, when infected into a host cell, expresses both A-CREB and GFP proteins. Viruses were then introduced into primary neurons and cultured overnight to allow viral gene expression. The infected neurons were identified by both expression of GFP and staining for GAP43 (a neuronal marker) (Figure 6.8).

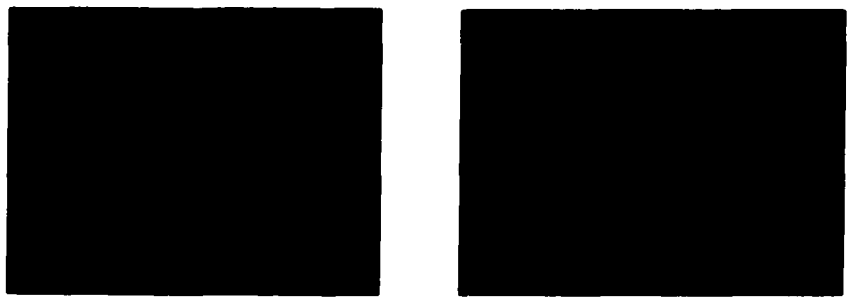


Figure 6.8 Fluorescent images of infected cerebellar neurons with A-CREB.
1x 10^6 cerebellar neurons were plated into each well of a 24-well plate and infected with adenovirus containing A-CREB-GFP or GFP alone. After 1hr infection and overnight culture, infected neurons were transferred to CHO cells for neurite outgrowth. Neurons were fixed and immunostained for GAP43. The cells expressing both GFP (left) and GAP-43 (right) are considered positively infected.

Assuming expression of A-CREB by the existence of GFP, we still need to confirm that the modified adenovirus containing A-CREB is functional. To test it, we first introduced a CRE-luciferase reporter gene and checked whether A-CREB can block the CRE-mediated luciferase expression. We infected COS-1 cells with either nothing, or virus containing A-CREB, or control virus expressing GFP alone. Infected cells were then transfected with a CRE-luciferase reporter and later challenged with dbcAMP. dbcAMP up-regulates the CRE-mediated luciferase expression in cells without infection

and cells infected with control virus expressing only GFP. In contrast, A-CREB reduces, by half, the ability of dbcAMP to induce luciferase activity via the CRE (Figure 6.9).

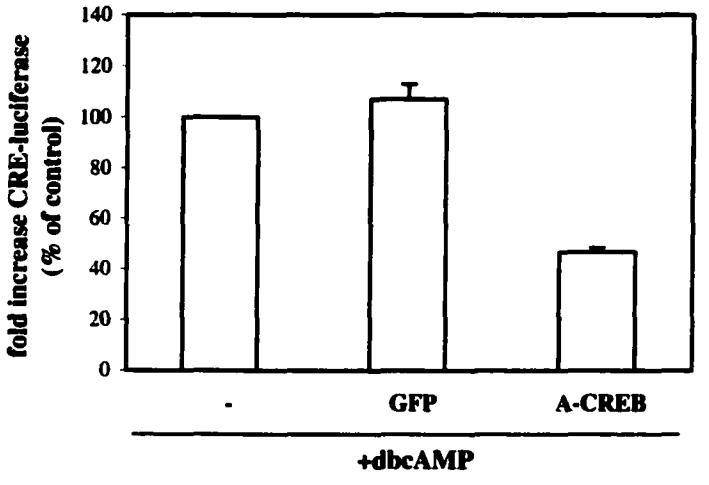


Figure 6.9 Expression of CRE reporter stimulated by cAMP in COS-1 cells infected with A-CREB. COS-1 cells were infected with either virus expressing A-CREB or control virus (GFP) or nothing (-) as indicated, then transfected with CRE-luciferase reporter overnight before incubated with dbcAMP (1 mM) for 24 hr. Cells were lysed and measured for luciferase activity. Results show the mean fold increase of luciferase activity by dbcAMP. Results are standardized to percentage of control. Control was taken as fold increase of luciferase activity by dbcAMP in cells without infection.

Moreover, in primary DRG neurons, A-CREB abolishes the endogenous Arginase (Arg) I expression after treatment with either dbcAMP (Figure 6.10) or neurotrophins (Figure 6.11). Arg I is the enzyme responsible for initiating the biosynthesis of polyamines. Overexpressing Arg I and addition of polyamines can overcome inhibition by MAG (Cai *et al.*, 2002). In addition, there is a CRE element in the Arg I promoter region (Ohtake *et al.*, 1988), indicating that Arg I is one of the downstream targets of CREB activation. When neurons are challenged with dbcAMP, expression of Arg I is increased by about 6 fold. In contrast, introduction of A-CREB results in up-regulation of Arg I of only 2.5 fold, while the control virus expressing only GFP has no effect on neurotrophins/cAMP's ability to stimulate Arg I expression (Figure 6.10).

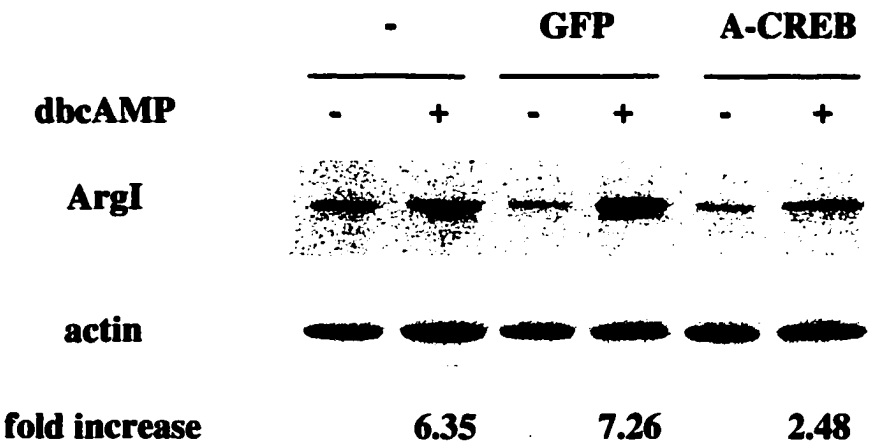


Figure 6.10 Expression of Arg I in A-CREB infected DRGs after treatment with dbcAMP.
The dissociated DRG neurons were infected with either nothing (-), or control virus (GFP), or virus containing A-CREB as indicated, then exposed to dbcAMP (1 mM) for 28 hr before lysed in RIPA buffer. 30 μg of total proteins were subjected to 12% SDS-PAGE before being transferred to nitrocellulose membrane and immunostained with an antibody against Arg I. The same blot was later stripped and probed with an antibody against actin to confirm equal loading. The expression levels of proteins were quantitated by a FluoImage system.

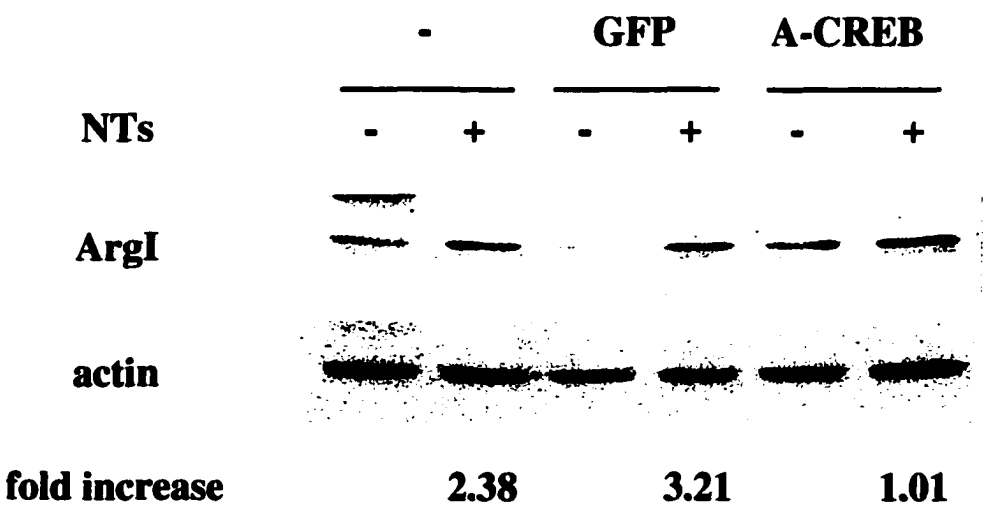


Figure 6.11 Expression of Arg I in A-CREB infected DRGs after treatment with neurotrophins.
The dissociated DRG neurons were infected with either nothing (-), or control virus (GFP), or virus containing A-CREB as indicated, then exposed to a combination of neurotrophins (200 ng/ml of NGF, 200 ng/ml of BDNF and 200 ng/ml of GDNF) for 28 hr before lysed in RIPA buffer. 30 μg of total proteins were subjected to 12% SDS-PAGE before being transferred to nitrocellulose membrane and immunostained with an antibody against Arg I. The same blot was later stripped and probed with an antibody against actin to confirm equal loading. The expression levels of proteins were quantitated by a FluorImage system.

In summary, A-CREB blocks both the exogenous CRE reporter gene expression in COS-1 cells mediated by cAMP and the endogenous Arg I expression in primary DRG neurons stimulated by neurotrophin/cAMP. These results confirm that the modified A-CREB is functional and ready to be used for studies of CREB in axon regeneration.

4. A-CREB abolishes neurotrophin/cAMP's ability to overcome inhibition by MAG

To explore a role for CREB in axon regeneration, primary neurons were infected overnight with either control virus or adenovirus expressing A-CREB, and then either primed with neurotrphins before being transferred to a monolayer of MAG-expressing CHO cells or directly plated to the monolayer in the presence of dbcAMP. Neurotrophin/cAMP is able to overcome MAG's inhibition on neurons infected with control virus only expressing GFP proteins. However, introduction of the A-CREB viral protein into primary neurons completely abrogates the neurotrophin and the dbcAMP effects on MAG-mediated inhibition (Figure 6.12 and Figure 6.13). Virus infection alone does not affect neurite outgrowth or MAG's inhibition in the absence of either neurotrophins or dbcAMP. Therefore, A-CREB abolishes both the neurotrophin and dbcAMP effects, suggesting that CREB is required for both priming with neurotrophins and incubating with dbcAMP to promote axon outgrowth in the presence of MAG.

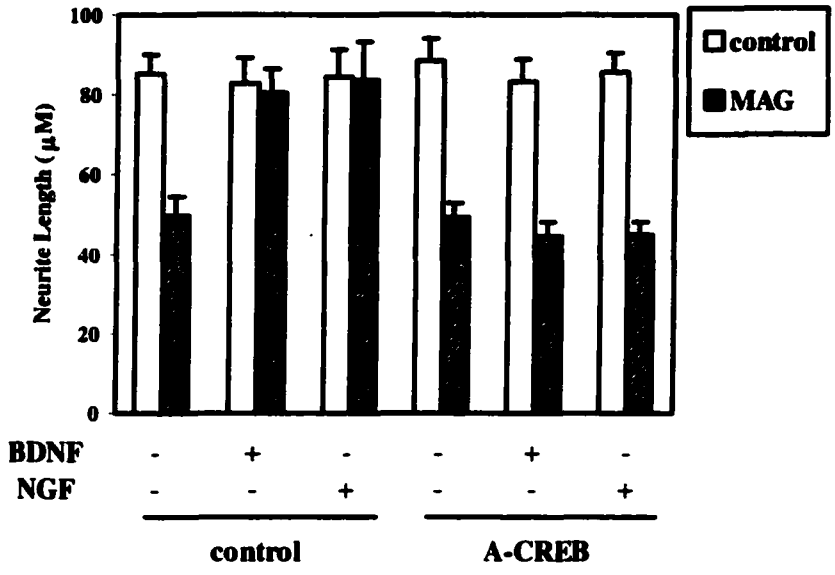


Figure 6.12 The effect of priming A-CREB infected DRG neurons with neurotrophins on subsequent inhibition of neurite outgrowth by MAG.
Dissociated P5-7 DRG neurons were first infected with either control virus or virus expressing A-CREB, then primed overnight on poly-l-lysine with no neurotrophin or in the presence of wither BDNF or NGF, both at 200 ng/ml as indicated, before being transferred to a monolayer of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) for further overnight culture. The cultures were then fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

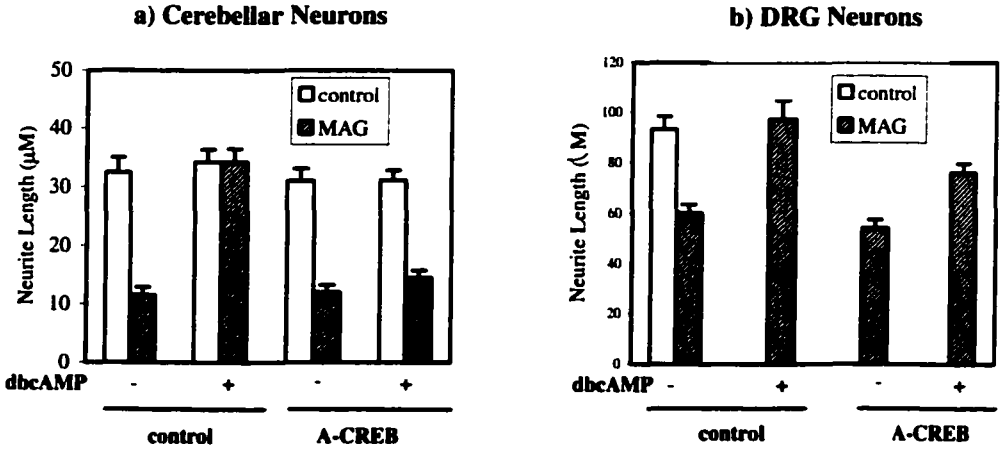


Figure 6.13 The effect of dbcAMP on inhibition by MAG of neurite outgrowth from neurons infected with A-CREB.
Dissociated a) cerebellar neurons, or b) DRG neurons were infected with either control virus or virus expressing A-CREB before plated onto monolayers of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) in the presence of dbcAMP at 1 mM as indicated, and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

5. Young DRG neurons, when infected with A-CREB, switch their response to MAG from promotion to inhibition

We have shown that the endogenous cAMP levels correlate with the neuronal regenerative capacity during development and after injury (Cai *et al.*, 2001). Since CREB activation is often regarded as an index of cAMP/PKA action, it is reasonable to suggest that the endogenous levels of activated CREB in neurons may also correlate with the neuronal regenerative capacity. It is known that DRG neurons switch their axonal regenerative response to MAG/myelin from promotion to inhibition during development (Mukhopadhyay *et al.*, 1994). MAG promotes neurite outgrowth from DRG neurons up to P3-P4, after which it inhibits regeneration. Here, when P1 DRG neurons were infected with a control virus expressing only GFP, they are not inhibited by MAG. In contrast, these same P1 DRG neurons when expressing A-CREB are strongly inhibited by MAG (Figure 6.14). Therefore, in young DRG neurons, blocking CREB activity can switch the neuronal response to MAG from promotion to inhibition. Activation of CREB is required for the switch of neurons with development from promotion of axonal regeneration to inhibition by MAG/myelin.

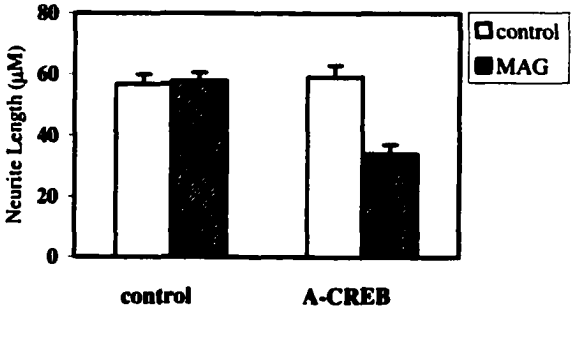


Figure 6.14 Neurite outgrowth of P1 DRG neurons infected with A-CREB.
Dissociated P1 DRG neurons were infected with either control virus or virus expressing A-CREB before plated onto monolayers of either MAG-expressing CHO cells (striped bars) or control CHO cells (solid bars) as indicated, and cultured overnight before being fixed and immunostained for GAP43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

Discussion

CREB is a well-characterized stimulus-induced transcription factor (Shaywitz and Greenberg, 1999). Activated CREB turns on transcription in response to a variety of stimuli, including growth factors and agents that increase cAMP levels. CREB has been demonstrated to play important roles in neuronal survival and memory processing (reviewed in Finkbeiner, 2000; Frank and Greenberg, 1994). However, little is known about CREB in the process of axonal regeneration. Here we report that an inhibitor of transcription abrogates BDNF/cAMP's ability to overcome inhibition by MAG. We also show that CREB is phosphorylated in response to dbcAMP and BDNF. A dominant-negative form of CREB (A-CREB) that prevents wildtype CREB binding DNA, blocks CRE-mediated gene expression and the neurotrphin/cAMP effect on MAG-mediated inhibition. Moreover, young DRG neurons that are promoted by MAG, when infected with A-CREB, switch their response to MAG to inhibition. Since CREB, ATF-1 and CREM share almost identical bZIP domains, A-CREB would be expected to interfere with both ATF-1 and CREM-dependent transcription when it is overexpressed in our infected neurons. Therefore, these results suggest that activated CREB, or its closely related family members, is involved in the downstream signaling of neurotrophin/cAMP to overcome MAG's inhibition.

Axon regeneration is viewed as the culmination of a series of temporally distinct but overlapping phases (Ambron and Walters, 1996). Within each phase, specific signals enter the nucleus to prime the cell for the arrival of subsequent signals. Successful axon regeneration requires the signals from the site of injury reach the nucleus to elicit changes in transcription. We showed that the neurotrophin/cAMP effect to block inhibition by

MAG is transcription-dependent. Specifically, CREB has been shown here to be important for the neurotrophin/cAMP effect. However, it is not yet determined in which phase of axon regeneration that CREB participates. In addition, we cannot exclude the possibility that other transcription factors are needed in different phases to promote axonal growth. Therefore, identification of various transcription factors critical in each phase of axon regeneration is necessary and will give us a better understanding of how injured axons achieve successful regeneration.

Regulation of CREB activation is complicated and involves many factors. First, the duration of phosphorylation at Ser133 determines the efficacy with which CREB induces gene expression. The more sustained the period of CREB phosphorylation, the more likely CREB will induce CRE-mediated transcription. Duration of CREB phosphorylation appears to be different in response to BDNF and cAMP. dbcAMP maintains a substantial activation of CREB for up to two hours (Figure 6.6), while at the same time after BDNF treatment, phosphorylation is already reduced (Figure 6.5). The difference in the time courses of CREB phosphorylation might be simply because dbcAMP is a non-hydrolyzable cAMP analog and will transmit signals persistently. In addition, it is widely accepted that the temporal pattern of CREB activation is determined by the negative regulation of the CREB phosphatases. It was reported that several serine/threonine phosphatases could mediate dephosphorylation of Ser133 (Cohen, 1989). Two okadaic-acid-sensitive phophatases, protein phosphatase-1 (PP-1) and PP-2A, have also been shown to be CREB phosphatases (Hagiwara *et al.*, 1992; Wadzinski *et al.*, 1993). The discrepancy of BDNF and dbcAMP in stimulating CREB activity is likely accounted for by the presence of a particular factor, dopamine and cAMP-regulated

phosphoprotein (DARPP-32). DARPP-32 is activated by PKA phosphorylation. Once phosphorylated, DARPP-32 inhibits PP-1 (Cohen, 1989). Therefore, the presence of DARPP-32 enables a cell to respond to dbcAMP but not BDNF with sustained CREB phosphorylation. As a result, sustained elevation of CREB activity enables efficient transcription of regenerative genes and probably one of the keys to successful axon regeneration.

Moreover, CREB is capable of being phosphorylated at Ser133 by many signaling pathways, which include calcium/CaMK, cAMP/PKA, PI3K/Akt and ERK/Rsk cascades. However, for activation of CREB-dependent transcription, other kinases contribute to the regulation of CREB activity by phosphorylating residues within the KID. Specifically, protein kinase C (Yamamoto *et al.*, 1988), glycogen synthase kinase III (Woodgett, 1994) and casein kinase II (Bullock and Habener, 1998) can all phosphorylate residues within the KID and may affect CREB function. In addition, regulation of CREB activity involves other promoter-bound factors, such as CBP, acting as a transcriptional adaptors linking Ser133-phosphorylated CREB to the basal transcription machinery (Barlev *et al.*, 1995; Berger *et al.*, 1992). Different phosphorylation events of CREB and the existence of the CREB adapters appear to regulate the ability of CREB to induce expression of specific target genes in response to particular stimuli. In this way, CREB is able to adjust specific cellular responses to meet specific stimuli. Consequently, the ability of CREB to be activated by various signaling pathways allows it to function as a sensitive barometer of environmental change. One challenge for the future in the studies of CREB will be to elucidate additional mechanisms that allow CREB to function with both sensitivity and specificity.

CREB turns on certain transcription/translational machineries and hence, targeted genes are transcribed and translated. One of the CREB-mediated target proteins that is elevated by neurotrophin/cAMP is Arg I (Figure 6.10). Arg I has been shown to be a neuronal anti-apoptotic factor (Esch *et al.*, 1998). It initiates polyamine synthesis by catalyzing the hydrolysis of arginine to ornithine and urea. Ornithine is then converted in the cytosol to the polyamine putrescine by the action of ornithine decarboxylase (ODC); putrescine is converted to two other polyamines, spermindine and spermine (Slotkin and Bartolome, 1986). These polyamines have been shown to be highly concentrated in the mammalian nervous system (Shaskan and Snyder, 1973; Shaw and Pateman, 1973) and exert regeneration-promoting effects on cultured neurons (Dornay *et al.*, 1986; Kauppila, 1992; Kauppila *et al.*, 1988). Studies *in vivo* also indicate that polyamines can enhance functional recovery after facial nerve injury as well as sciatic nerve trauma. Moreover, we revealed that either over-expression of Arg I or exogenous polyamines are each sufficient to overcome the inhibition by MAG/myelin (Cai *et al.*, 2002). Like Arg I, the other two rate-limiting enzymes in polyamine production (ODC and S-adenosylmethionine decarboxylase) also contain the CRE sites in their promoter regions (Pulkka *et al.*, 1991; van Kranen *et al.*, 1987). This suggests that neurotrophin/cAMP regulates polyamine synthesis by a CREB-dependent mechanism. Interestingly, polyamines are essential for the organization of the cytoskeleton and influence the distribution of microtubules during damage *in vivo* (Banan *et al.*, 1998; Kaminska *et al.*, 1992; McCormack *et al.*, 1994). Thus, the ability of polyamines to promote injured axons to regrow could be via a direct effect on the cytoskeleton. In addition, polyamines are known to interact with the N-methyl-D-aspartate (NMDA) receptor and control calcium

influx (Ransom and Stec, 1988; Sacaan and Johnson, 1990; Williams *et al.*, 1989). Given the role of glutamate and its receptors in synaptic plasticity, which also involves process outgrowth, it is reasonable to propose that polyamines might overcome inhibition by affecting these channels or receptors. Finally, Arg I/polyamines is only one of the downstream targets of CREB in regulating neuronal regenerative capacity. Identification of all other targets and characterization of their function may provide further insight into a more potential therapeutic cure for spinal cord injury.

In conclusion, based on the results from all these chapters, a model is proposed to explain the possible signaling mechanism involved in the block of MAG's inhibition of axonal regeneration (Figure 6.15). First, neurotrophins activate ERK through the Trk receptors. ERK then inhibits PDE and so cAMP accumulates and PKA is activated. It is possible that PKA affects axonal growth through a direct action on the cytoskeleton. In addition, PKA, with ERK and other kinases activated by neurotrophins, might indirectly influence axon regeneration through activation of the transcription factor CREB. In turn, CREB up-regulates transcription of specific factors capable of mediating the block of inhibition by MAG/myelin. One of these factors is Arg I, the key enzyme for polyamine synthesis. Elevation of polyamines seems to promote axonal regeneration through regulating cytoskeleton structure and thereby overcoming inhibition by MAG/myelin.

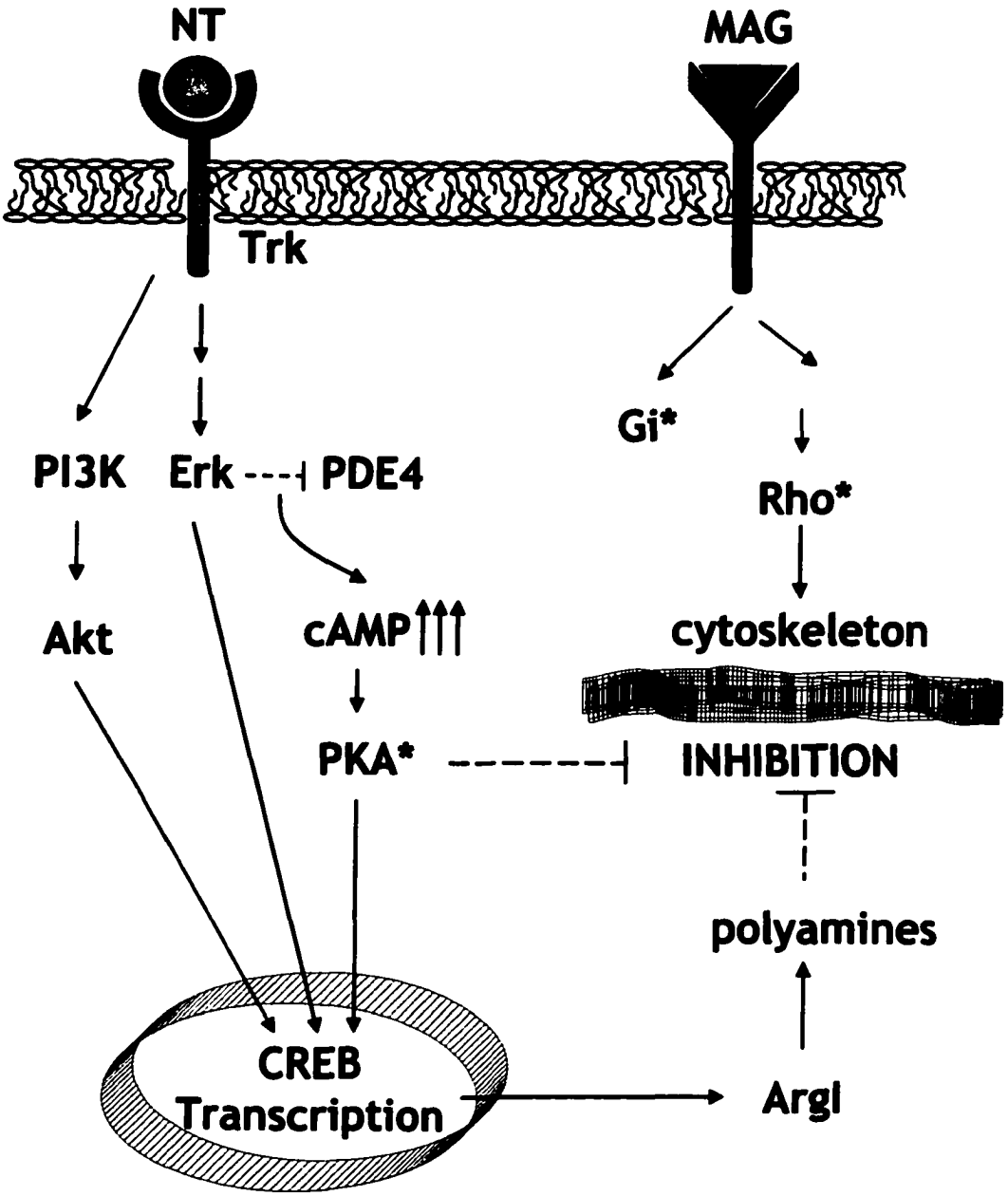


Figure 6.15 Model to explain how priming with neurotrophins blocks inhibition by MAG/myelin.

CHAPTER VII

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