cAMP and Polyamines Overcome Inhibition by MAG by Activating Cdk5 via Increased Expression of p35 Regulated by Activation of eIF5A

by

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Abstract

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Damaged axons in adult mammalian central nervous system (CNS) are unable to regenerate after injury although axons in the peripheral nervous system (PNS) or embryonic CNS can. The inhibitory molecules associated with myelin are one of the major obstacles to successful axon regeneration in the adult mammalian CNS. To date, three inhibitors of regeneration have been identified in myelin: NogoA, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMpg) (Filbin, 2003). Interestingly, all these three ligands bind to the same receptor Nogo receptor (NgR) to mediate the inhibitory effect. $p75^{NTR}$ or TROY and Lingo-1(LRR and Ig domain-containing, Nogo Receptor interacting protein) are necessary components of the receptor complex as NgR is glycosyl phosphatidylinositol (GPI)-anchored and lacks a signaling domain (Wang et al., 2002a; Mi et al., 2004; Park et al., 2005; Shao et al., 2005). Activation of the receptor complex by myelin inhibitors activates the small GTPase RhoA resulting in rearrangement of the cytoskeleton and inhibition of axonal outgrowth (Hu and Strittmatter, 2004).

It has been shown in our lab that elevating intracellular levels of cyclic AMP (cAMP), either via application of a cAMP analog or by prior exposure to neurotrophins (NTs) can block the inhibition of axonal regeneration by MAG and myelin (Cai et al.,

1999; Cai et al., 2001; Qiu et al., 2002). Elevation of cAMP results in up-regulation of arginase I (ArgI) and subsequent synthesis of polyamines. Up-regulation of ArgI or priming with the polyamine putrescine or spermidine blocks the inhibition of axonal growth by MAG/myelin (Cai et al., 2002; Deng et al., 2009). Polyamines are known to have effects in regulating cytoskeleton organization in both the short term and the long term, but their downstream effectors have yet to be identified. Many studies have shown that Cyclin-dependent kinase 5 (Cdk5) is involved in neurite outgrowth and regulates the neuronal cytosekeleton, which prompted us to hypothesize that Cdk5 may play a role in blocking MAG/myelin-mediated inhibition.

Cdk5 is a multifunctional serine/threonine kinase and its activator, p35, is expressed only in the nervous system (Tsai et al., 1994). It has been shown that activity of Cdk5 is required for neurite elongation (Nikolic et al., 1996; Paglini et al., 1998; Li et al., 2000; Harada et al., 2001). Cdk5 phosphorylates cytoskeleton proteins and regulates the organization of all three cytoskeleton elements microfilaments, microtubules and intermediate filaments (Dhavan and Tsai, 2001). Here we show that Cdk5 is required for db-cAMP and putrescine to overcome inhibition. The effect of db-cAMP and putrescine in overcoming inhibition by MAG is abolished in the presence of a specific inhibitor of Cdk5, Roscovitine. Neurons infected with dominant negative Cdk5 HSV viruses are not able to overcome inhibition by MAG in the presence of db-cAMP or putrescine. Importantly, neurons infected with HSV viruses overexpressing p35, the neuronal specific activator for Cdk5, overcome MAG's inhibition. Moreover, db-cAMP and putrescine increase the expression of p35. This in turn induces the kinase activity of Cdk5. The up-regulation of p35 by putrescine is also reflected in the increased

distribution of p35 in neurites and growth cones. Furthermore, we show that putrescine up-regulates p35 protein by hypusine modification of eukaryotic Initiation Factor 5A (eIF5A), and this hypusination is necessary for putrescine to overcome inhibition by MAG. Our findings reveal a previously unknown mechanism by which polyamines encourage regeneration after CNS injury.

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Abbreviations

Arg	Arginase
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cAMP	cyclic AMP
Cdk5	cyclin dependent kinase 5
CGN	cerebellar granule neuron
CHO	Chinese hamster ovary
CNS	Central nervous system
CREB	cAMP-responsive element-binding protein
CSPGs	chondritin sulfate proteoglycans
dbc-AMP	dibutyl cAMP
DHS	deoxyhypusine synthase
DMEM	Dulbecco's modified eagle's medium
DOHH	deoxyhypusine hydroxylase
DRB	5,6-dicholro-1-β-D-ribofuranosylbenzimidazole
DRG	doral root ganglion
ECL	enhanced chemiluminescence
eIF5A	eukaryotic initiation factor 5A
ER	endoplamic reticulum
ERK	extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	fetal bovine serum

GC-7	N^1-guanyl-1,7-diaminoheptane
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic proteins
GFP	green fluorescent protein
GPI	glycosylphophatidylinositol
HRP	horse radish perosidase
Ig	immunoglobulin
Lingo-1	LRR and Ig domain-containing, Nogo Receptor-interating protein
IL-6	Interleukin 6
MAG	myelin associated glycoprotein
MAP	microtubule-associated protein
MF	microfilament
MT	microtubule
NF	neurofilament
NGF	nerve growth factor
NgR	Nogo receptor
NMDA	N-methyl-D-asparate
NOG	neurite outgrowth assay
NTs	Neurotrophins
NT-3	Neurotrophin-3
ODC	Ornithine decarboxylase
OEC	olfactory ensheathing cell

OMgp	Oligodendrocyte myelin glycoprotein
p75 NTR	p75 neurotrophin receptor
PAGE	poly-acryamide gel electrophoresis
PAK	p21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	Phosphadiesterase
PFU	Plaque forming units
PirB	paired immunoglobulin-like receptor B
PKA	protein kinase A
PKC	protein kinase C
PLL	Poly L-lysine
PND	post natal day
PNS	Peripheral nervous system
PUT	putrescine
RGC	retinal ganglion cell
SDS	Sodium dodecyl sulfate
SELEX	systematic evolution of ligands by exponential enrichment
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein

Chapter I: Introduction

Axon regeneration failure in adult mammalian CNS after injury

It is generally believed that axons in the adult mammalian CNS are incapable of regeneration after injury. Once damaged, the CNS axons that connect one neuron to the others can not regrow. In sharp contrast, the adult mammalian PNS readily regenerates after injury. The reasons for CNS regenerative failure have remained a mystery until recently. Our understanding of molecular mechanisms that control the behavior of injured CNS neurons has been quickly expanding in the past decade. Although fully restorative therapies for spinal cord and brain injury are not yet available, progress in this field has made axon regeneration therapies promising.

1.1 PNS versus CNS response after injury

The difference between the PNS and the CNS in their regenerative capacity is not because the peripheral myelin is non-inhibitory as myelin-associated inhibitors are present in the PNS and PNS myelin does inhibit axonal growth of neurons (Shen et al., 1998). Rather, the change in the PNS environment is responsible for regeneration. When peripheral nerves are cut, the axons distal to the lesion undergo a process of rapid Wallerian degeneration which normally involves the recruitment of macrophages to the lesion sites and the clearing of myelin debris (Brown et al., 1991). In addition, macrophages stimulate the proliferation and de-differentiation of Schwann cells. In de-differentiating Schwann cells, all myelin proteins are down-regulated while growth-promoting molecules are up-regulated, which thereby allows more regeneration (Fawcett

and Keynes, 1990). In contrast, these changes do not occur in the CNS. When axons are severed, Wallerian degeneration in CNS is a very slow process, taking months or years. Unlike Schwann cells in the PNS, oligodendrocytes in the CNS continue to express myelin proteins after injury and they do not engulf myelin debris including myelin-associated inhibitors. As a result, exposed myelin inhibitors block spontaneous regeneration of injured axons. Moreover, over time reactive astrocytes form a glial scar at the lesion site and act as additional barrier to axon regrowth (Silver and Miller, 2004).

1.2 Inhibitory environment of the injured CNS

One possible explanation for the regeneration failure in adult CNS is the decline in the intrinsic growth capacity of neurons. However, in the early 1980s, the pioneering work of David and Aguayo showed that injured axons can regrow if neurons are provided with a permissive environment. David and Aguayo used long peripheral nerve segments to make bridges between medulla oblongata and spinal cord to demonstrate that some injured axons can extend over long distances (30mm) in the peripheral nerve graft (David and Aguayo, 1981). Using a similar technique, Aguayo's group later also demonstrated that axons in the cerebral cortex and optic nerve could regenerate in a similar manner (Benfey and Aguayo, 1982; Vidal-Sanz et al., 1987). Furthermore, for neurons such as DRGs that innervate both the CNS and PNS, transection of the peripheral branch will regenerate, while the CNS which does not. These observations suggest that the CNS environment plays a key role in limiting regeneration. Now we know that the glial scar and inhibitors

within myelin are two major factors that contribute to inhibitory environment of the injured CNS.

1.2.1 The Glial Scar: Physical and biochemical barrier to axonal regeneration

Following CNS injury, the glial scar, which consists predominantly of reactive astrocytes and proteoglycans, forms as a result of glial reaction. Glial reaction, generally referred to as reactive astrocytosis, is an increase in number and size of astrocytes, a change in morphology marked by upregulation in intermediate filaments such as glial fibrillary acidic protein (GFAP) and an increase in expression of extracellular matrix components, most notably chondroitin sulphate proteoglycans (CSPGs). The glial scar was viewed as a mere physical barrier to axonal regeneration in the beginning, but later inhibitory molecules such as CSPGs were found in the scar as well. CSPGs, including aggrecan, brevican, neurocan, veriscan, phosphacan and NG2, are a family of preoteoglycans with a protein core linked to a sulphated glycosaminoglycan (GAG) chain by four sugar moieties (Morgenstern et al., 2002). CSPGs are very inhibitory for axonal regeneration both *in vitro* (Rudge and Silver, 1990; Snow et al., 1990; McKeon et al., 1991; Smith-Thomas et al., 1994; Niederost et al., 1999) and *in vivo* (Davies et al., 1999; Tang et al., 2003).The inhibitory activity of CSPGs depends on GAG components because removal of GAG chains by the enzyme Chondroitinase ABC eliminates inhibition and improves regeneration and functional recovery (Bradbury et al., 2002; Carulli et al., 2005). The mechanisms by which these CSPGs exert their inhibition are not clear. However, there is evidence to show that CSPGs activate the small GTPase, RhoA and this activation is necessary for their inhibitory effects (Borisoff et al., 2003; Monnier et al., 2003).

Moreover, two additional signal mediators have been found to be common to both CSPGs and myelin: protein kinase C (PKC) and epidermal growth factor receptor (EGFR) (Hasegawa et al., 2004; Sivasankaran et al., 2004; Koprivica et al., 2005). The signaling pathway will be discussed in detail later in this thesis.

In addition to CSPGs, there are also other molecules known to be upregulated in the glial scar depending on the severity of the lesion. In severe lesions, fibroblasts can invade the lesion core and induce the astrocytic expression of repulsive guidance cues such as Slit and ephrin-B as well as glypican 1 receptors, ephrin receptors EphB2 and EphA4 (Turnley and Bartlett, 1998; Bundesen et al., 2003; Hagino et al., 2003; Goldshmit et al., 2004; Silver and Miller, 2004). Meanwhile, the expression of repulsive SEMA3 by fibroblasts and SEMA3 receptor, neuropilin 1, by neurons that project to the injury site are both increased (Pasterkamp et al., 1999; De Winter et al., 2002; Silver and Miller, 2004). Together, these molecules contribute additional complexity to the glial scar and further prevent axon regeneration.

In addition to preventing regeneration, however, the glial scar forms to isolate the injury site and minimize the area of inflammation and cellular degeneration thereby preventing uncontrolled tissue damage (Faulkner et al., 2004). Therefore, the glial scar is important to the overall survival of the animal. Also the glial scar often takes several weeks to mature, thus myelin inhibitors present in myelin debris resulting from the injury pose the most immediate obstacle to regeneration (Huang et al., 1999).

1.2.2 Myelin-associated inhibitors

It was proposed by Ramon y Cajal that white matter could block regeneration in the CNS (Cajal, 1928). However, the first experimental data supporting the suggestion that myelin inhibits axonal regeneration did not come by until 50 years later. In 1982, Berry showed that myelin breakdown products released after CNS jury could inhibit axon regrowth (Berry, 1982). Several years later, Martin Schwab's group isolated two proteins, of 35 KD and 250 KD respectively, from CNS myelin and demonstrated that these proteins inhibited neurite outgrowth *in vitro* (Caroni and Schwab, 1988a). They subsequently generated a monoclonal antibody IN-1 against the two proteins and showed that addition of IN-1 could overcome myelin inhibition *in vitro* (Caroni and Schwab, 1988b). Notably, *in vivo*, administration of IN-1 enhanced regeneration of corticospinal axons and improved functional recovery after spinal cord injury (Schnell and Schwab, 1990, 1993; Bregman et al., 1995). Several myelin-associated inhibitors have been identified since these seminal findings.

1.2.2.1 Myelin-Associated Glycoprotein (MAG)

In 1994, MAG was the first myelin inhibitor to be described by two independent studies showing MAG was a potent inhibitor of neurite outgrowth in culture (McKerracher et al., 1994; Mukhopadhyay et al., 1994). When grown on MAG-expressing Chinese Hamster Ovary (CHO) cells, both postnatal cerebellar and DRG(older than P3) neurons displayed a dramatic reduction in neurite outgrowth compared to neurons plated on control CHO cells not expressing MAG. Application of an anti-MAG antibody reversed this inhibitory effect (Mukhopadhyay et al., 1994). Similarly, NG108 cells failed to extend neurites

when plated on a slide coated with myelin or MAG and immunodepletion of MAG from purified bovine CNS myelin extract strongly reduced the inhibitory activity of myelin (McKerracher et al., 1994). Furthermore, soluble forms of MAG, which consist of the entire extracellular domain, either recombinant MAG-Fc or dMAG, which is a proteolytic fragment of MAG and released from damaged myelin, were shown to inhibit neurite outgrowth (Tang et al., 1997b; Tang et al., 2001). MAG was also shown to induce growth cone collapse in hippocampal neurons (Li et al., 1996; Shibata et al., 1998).

Interestingly, MAG seems to be a bifuntional protein in that it is stimulatory for the neurite outgrowth from young neurons but it is inhibitory for older neurons (McKerracher et al., 1994; Mukhopadhyay et al., 1994; DeBellard et al., 1996). For example, MAG promotes neurite outgrowth form young postnatal DRG neurons and the transition form promotion to inhibition by MAG occurs sharply at P3-4 (DeBellard et al., 1996). Retinal Ganglion (RG) neurons and spinal neurons exhibit a similar response to MAG except that the promotion-to-inhibition transition occurs at birth instead of postnatally (Salzer et al., 1990; Turnley and Bartlett, 1998). Conversely, MAG inhibits neurite outgrowth from all postnatal neurons tested to date including retinal, hippocampal, superior cervical ganglion, spinal cord and DRG neurons older than P3 (Mukhopadhyay et al., 1994; DeBellard et al., 1996).

MAG is a member of the immunoglobulin (Ig) super family and it contains five extracellualr Ig-like domains, a single transmembrane region and a short cytoplasmic domain (Lai et al., 1987; Salzer et al., 1987; Salzer et al., 1990). MAG exists in two alternatively spliced forms, a large (L) and a small (S) form that differ only in their cytoplasmic sequences (Lai et al., 1987; Salzer et al., 1987).These two isoforms are 72

KDa and 67 KDa, respectively. However, when gylcosylated, they co-migrate on a SDS-PAGE as a single band at around 100KDa. MAG is also a sialic acid binding protein and is a member of sialic acid binding Ig-like lectin (Siglec) famlily of proteins (Siglec 4) (Kelm et al., 1994; Crocker et al., 1998). It binds specifically to the gangliosides GT1b and GD1a. The sialic acid binding site for MAG is localized to Arg 118 in the first Ig domain (Tang et al., 1997a). However, the sialic binding ability of MAG is not essential for it to exert inhibition because a truncated form of MAG, consisting of only MAG Ig – domain 1-3, can bind to neurons in a sialic acid-dependent manner but does not inhibit neurite outgrowth. Moreover, inhibition by membrane-bound MAG is unaffected by Arg118 mutation further suggesting that the inhibitory domain of MAG is distinct from sialic acid-binding site (Tang et al., 1997a). Indeed, recently it has been shown that the inhibition site on MAG is located to Ig domain 5 (Cao et al., 2007).

MAG is found in both the CNS and the PNS and it is expressed in the myelin-forming cells: oligodendrocytes in the CNS and Schwann cells in the PNS (Trapp et al., 1989; Trapp, 1990). In the CNS, MAG represents 1% of the total myelin protein and is localized solely to periaxonal membrane in the internodal segments of the myelin shealth (Trapp, 1990). In the PNS, MAG only represents 0.1% of the total myelin protein and is expressed in the paranodal regions, Schmit-Lanterman incisures, and outer mesaxon segments (Trapp, 1990). Because of its location next to the axon and its early expression during myelination, MAG has been suggested to play roles in myelin formation and stabilization (Trapp et al., 1989; Salzer et al., 1990; Trapp, 1990; Filbin, 1995). In the PNS, cell culture experiments show that MAG is involved in myelin initiation by Schwann cells (Owens and Bunge, 1989; Owens et al., 1990; Owens and Bunge, 1991).

But analysis of the MAG null mice demonstrates that MAG is required for the initiation of myelination only in the CNS (Schachner and Bartsch, 2000). However, in both the PNS and the CNS, MAG plays a role in the long-term maintenance of myelin (Schachner and Bartsch, 2000).

1.2.2.2 Nogo

The antigen of IN-1 antibody was finally identified in 2000 when three different groups independently cloned the Nogo protein using a partial sequence of IN-250 (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). There are three Nogo isforms, Nogo-A, B and C, due to alternative splicing and promoter usage. All three isoforms have a common 188-amino-acid residue C-terminus homologous to reticulon protein famlily, proteins usually associated with the endoplasmic reticulum (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). All three isoforms also contain a 66-amino-acid extracellular loop termed Nogo-66 (GrandPre et al., 2000). Nogo-A, the largest isoform, is mainly expressed in the CNS by oligodendrocyts and neurons and is localized to the inner and outer loops of myelin and on the surface of oligodendrocytes (Chen et al., 2000; GrandPre et al., 2000; Huber et al., 2002). Nogo-B is expressed in many tissues and cell types including neurons and Nogo-C is found largely outside the nervous system (Chen et al., 2000; GrandPre et al., 2000; Huber et al., 2002). Since Nogo-B and Nogo-C were not detectable in myelin-forming oligodendrocytes, most of the studies to date have focused on Nogo-A.

The inhibitory effect of Nogo has been mapped to Nogo-66, which also induces growth cone collapse from DRG neurons (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Nogo-A has an additional domain, termed amino-Nogo, localized to

a 195-amino-acid stretch near the N-terminus. Interestingly, amino-Nogo induces growth cone collapse, inhibits neurite outgrowth as well as abrogate 3T3 fibroblast spreading (Prinjha et al., 2000; Fournier et al., 2003). Nogo-A is believed to have two membrane topologies, one in which Nogo-66 is outside the cell and the N and C-terminus in the cytoplasmic compartment, and a second in which amino-Nogo and the C-terminus are extracelluar and Nogo-66 is inside the cell (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

1.2.2.3 Oligodendrocyte Myelin Glycoprotein (OMgp)

In 2002, OMgp was identified as a third myelin-associated inhibitor (Wang et al., 2002b). OMgp is a glycosylphosphatidyinositol (GPI)-linked protein with a leucine-rich repeat (LRR) domain and a serine/threonine region. It is a minor component in CNS myelin and it is believed to be localized largely to the paranodal loops, next to the node of Ranvier (Filbin, 2003). OMgp is expressed in both oligodendrocytes and neurons (Mikol and Stefansson, 1988; Mikol et al., 1990; Habib et al., 1998).The expression of OMgp is developmentally regulated in rat CNS with an increase after birth to until postnatal week 6 (Vourc'h et al., 2003). Like MAG and Nogo, it can inhibit neurite growth and cause growth cone collapse (Kottis et al., 2002; Wang et al., 2002b). Zhigang He’s group showed that OMgp inhibited neurite outgrowth and induced growth cone collpase from P7-9 rat cerebellar neurons, which was similar to Nogo-66 (Wang et al., 2002b). Kottis et al also showed that OMgp inhibited neurite growth from hippocampal neurons and induced growth cone collapse in DRG explants (Kottis et al., 2002). A recent study

suggests that OMgp may maintain the nodal structure by inhibiting collateral axon sprouting (Huang et al., 2005).

1.2.2.4 Sema4D and Ephrin-B3

Sema 4D and Ephrin-B3 are the latest proteins that have been shown to be inhibitory factors of neurite outgrowth in myelin. Semaphorins are a family of secreted or membrane-bound proteins, which are known to regulate axonal pathfinding. Secreted semaphorins such as Sema 3 are expressed by fibroblasts of the glial scar in several CNS lesion models (Pasterkamp and Verhaagen, 2001). Sema 4D, also called CD100, is a member of class 4 transmembrane semaphorins. It has been shown to induce growth cone collapse in CNS neurons (Swiercz et al., 2002). Moreau-Fauvarque et al. reported that Sema 4D was expressed selectively by oligodendrocytes and myelin and was strongly inhibitory for P6 DRG and P5 cerebellar granule neurons (Moreau-Fauvarque et al., 2003). Ephrin-B3 is also a repulsive guidance cue with a role in axon pathfinding. It repels corticospinal axons from the midline of spinal cord during development (Kullander et al., 2001). Recently, ephrin-B3 has been found to be expressed in CNS myelin and to inhibit neurite outgrowth for cerebellar and cortical neurons (Benson et al., 2005).

1.2.3 Receptors for myelin-associated inhibitors

After identification of these myelin inhibitors, the next step followed was to find receptors on neurons that transduce the inhibitory signals. Surprisingly, although MAG, Nogo and OMgp share no sequence homology or structure similarity, they all bind to a

common receptor complex to mediate inhibition. This complex will be discussed as follows.

1.2.3.1 Nogo-66 Receptor (NgR)

Nogo-66 receptor (NgR) was identified by Strimatter and colleagues in 2001, using Nogo-66-AP, an alkaline phosphatase (AP) fusion protein of Nogo-66, to screen COS-7 cells transfected with a mouse brain cDNA library (Fournier et al., 2001). NgR is a 473-amino-acid, 85 KDa, GPI-linked protein consisting of a conventional signal sequence, an 8 LRR domain and an LRR C-terminal motif (LRR-CT) followed by a unique carboxy-teminal region proximal to the GPI anchor. The NgR mRNA is expressed by a variety of neurons in the brain, but undectable in white matter or oligodendrocytes (Fournier et al., 2001), which is consistent with its role in inhibiting axon regeneration. NgR binds to Nogo-66 with high affinity and its function as a receptor was demonstrated in both loss of function and gain of function assays (Fournier et al., 2001). E13 chick DRG neurons express NgR and respond strongly to Nogo-66 in a growth cone collapse assay. However, when NgR was removed from the cell surface with phosphatidylinositol-specific phospholipase C (PIPLC), Nogo 66 failed to induce growth cone collapse in chick E13 DRG neurons. Conversely, E7 chick RGC and DRG neurons are normally insensitive to Nogo-66 treatment due to the lack of expression of NgR at an early age. However, when NgR was introduced, they became responsive (Fournier et al., 2001).

One year later, both Filbin and Strimatter's groups discovered a second ligand for NgR. In an attempt to identify a signal-tranducing partner for NgR, Strimatter's group used NgR-AP to screen pools of COS-7 cells transfected with adult mouse brain cDNA

library. They were surprised to discover that MAG was a new ligand (Liu et al., 2002). They further demonstrated that MAG bound to NgR directly and that NgR was required for the inhibitory effect of MAG and myelin (Liu et al., 2002). Filbin's group reported similar results while searching for the MAG receptor. They showed that NgR bound to MAG-expressing cells and MAG-Fc precipitated NgR from NgR-expressing cells, cerebellar neurons, and DRG neurons. Importantly, a soluble form of NgR, a NgR antibody or a dominant negative NgR could each block MAG's inhibition (Domeniconi et al., 2002). Both groups showed the interaction between MAG and NgR was sialic acid-independent. The only discrepancy is whether MAG and Nogo-66 compete with each other for the binding site on NgR. One study reported that excess soluble Nogo-66 could block the binding of soluble MAG (Domeniconi et al., 2002) while the other study reported that neither excess Nogo-66 nor the Nogo extracellular peptide NEP1-40 reduced MAG binding to NgR (Liu et al., 2002).

Interestingly, during the same time, He's group not only identified OMgp as a third myelin inhibitor, but also showed that NgR was a functional receptor for OMgp (Wang et al., 2002b). Furthermore, they showed that OMgp and Nogo-66 could bind to an overlapping region on NgR and hence compete with one another (Wang et al., 2002b).

The crystal structure of NgR offers a possible explanation for the diversity of its ligands. NgR adopts an LRR module structure whose concave exterior surface contains a broad region of conserved aromatic residues, which could provide degenerate ligand binding sites with each ligand choosing its preferred site (Barton et al., 2003; Monnier et al., 2003).

Recently, a NgR-related protein, NgR2 was identified as another receptor for MAG (Venkatesh et al., 2005). NgR2 is expressed in adult rat brain and can bind to MAG to mediate inhibition, but this binding is sialic acid dependent (Venkatesh et al., 2005).

1.2.3.2 The p75 Neurotrophin Receptor and TROY

Because NgR is a GPI-linked protein and lacks an intracellular signaling domain, it must interact with other co-receptors to transduce the inhibitory signal across the membrane. Before the discovery of NgR as a common receptor for all three myelin inhibitors, Yamashita et al reported that p75 neurotrophin receptor was the transducer for MAG (Yamashita et al., 2002). They showed that DRG and cerebellar neurons isolated from p75-/- mice were not inhibited by MAG and p75 and MAG were co-precipitated from neurons although a direct interaction could not be shown. Wang et al further showed that p75 could be precipitated by MAG, Nogo-66 and OMgp, and NgR was present in these complexes. Neurons from p75-/- mice were not inhibited by each of the ligands and myelin in general. Also, the inhibitory activity of each individual inhibitor or myelin was abolished when p75 and NgR interaction was interrupted by soluble p75-Fc or a dominant negative NgR which lacks the binding site for p75. Moreover, overexpression of a truncated p75 lacking the intracellular domain in primary neurons attenuated the same set of inhibitory activities, suggesting p75 intracellular domain is required for NgR-p75 signaling (Wang et al., 2002a). Mu-ming Poo's group also observed similar results showing that p75 and NgR form a receptor complex that mediates repulsive signaling by MAG (Wong et al., 2002).

p75 is a member of tumor necrosis factor receptor (TNFR) superfamily, a class of type I transmembrane proteins bearing significant homology in their extracellular domains, which contain one or more cysteine-rich domains (CRDs) (Locksley et al., 2001; Dempsey et al., 2003). It was originally identified as the low-affinity neurotrophin receptor that has been suggested to play important roles in cell death signaling (Rabizadeh and Bredesen, 2003). Although p75 is highly expressed in the developing nervous system, it is only expressed in limited populations of mature neurons in the adult CNS, even after injury (Roux and Barker, 2002; Chao, 2003). For instance, adult cerebellar and cortical neurons do not express p75 and only 60-70% of adult DRG neurons express it (Park et al., 2005), yet these neurons are still inhibited by myelin suggesting that another receptor exists to substitute for p75 and transducer the inhibitory signal. Indeed, it was subsequently found independently by two labs that TROY (also known as TAJ), another member of the TNF receptor family, could also associate with NgR to form a functional complex and mediate myelin inhibition (Park et al., 2005; Shao et al., 2005). TROY is broadly expressed in postnatal and adult neurons and can bind to NgR to activate RhoA in the presence of myelin inhibitors. Soluble TROY and dominant negative TROY block the neurite outgrowth inhibition by myelin inhibitors. In addition, cerebellar and DRG neurons isolated from TROY-deficient mice are resistant to OMgp and Nogo-66 (Park et al., 2005; Shao et al., 2005).

1.2.3.3 Lingo-1

In 2004, a third molecule, Lingo-1 (LRR and Ig domain-cotaining, Nogo Receptor-interacting protein), was identified by Mi et al as a component of NgR and p75 receptor

complex. Lingo-1 is a nervous system specific transmembrane protein consisting of 12 LRR motifs, one Ig-like domain, a transmembrane domain and a short cytoplasmic tail (Mi et al., 2004). The cytoplasmic tail has a canonical epidermal growth factor receptor-like tyrosine phosphorylation site. Lingo-1 is highly expressed in rat brain with highest expression at postnatal day 1. In reconstituted non-neuronal systems, Lingo-1 interacts with NgR and p75 to activate RhoA, but binary combination of them can not activate RhoA. Expression of dominant negative Lingo-1 in cerebellar neurons or addition of soluble Lingo-1 attenuates myelin inhibition. Conversely, transfecting cerebellar neurons with full length Lingo-1 makes neurons more responsive to myelin (Mi et al., 2004).

1.2.3.4 PirB

In 2008, with the use of expression cloning Tessier-Lavigne's group identified another receptor for Nogo-66, human leukocyte immunoglobulin (Ig)-like receptor B2 (LILRB2), which has five homologous family members in humans (Atwal et al., 2008). In mice, there is only a single ortholog of LILRB2, paired immunoglobulin-like receptor B (PirB), which shares ~50% amino acid similarity with LILRB2 and contains six Ig-like repeats instead of four in LILRB proteins. Atwal et al first showed that, like NgR, PirB could also bind to Nogo66, MAG and OMgp. They then demonstrated that PirB was a functional receptor mediating myelin inhibition of neurite outgrowth using PirB antibody and PirB mutant mouse, PirBTM. PirB antibody was able to partially block the inhibition by Nogo 66, MAG and total myelin both in cerebellar neurons and DRG neurons. Neurons from PirBTM mice were less inhibited by Nogo66, MAG, OMgp and myelin. They further addressed whether PirB and NgR function together to mediate inhibition of

neurite outgrowth. NgR null cerebellar neurons were able to completely overcome inhibition by myelin in the presence of PirB antibody. Interestingly, the same combination only partially blocked inhibition by Nogo-66, suggesting there are additional receptors for Nogo-66. Together, these results establish PirB as a functional receptor for myelin inhibitors to block axon regeneration (Atwal et al., 2008). Future *in vivo* studies with NgR-PirB double knockout mice are required to fully determine the role of PirB in axonal regeneration. Also the mechanisms by which PirB signals to inhibit axon growth in response to myelin inhibitors have yet to be explored.

It is interesting that in both the PirB mutant mice and NgR null mice the critical period during which experience-driven plasticity of ocular dominance occurs is extended (McGee et al., 2005; Syken et al., 2006). In addition, removal of CSPG by chondroitinase-ABC can restore ocular dominance to the adult visual cortex (Pizzorusso et al., 2002). Therefore, one physiological role of myelin inhibitors and perhaps other inhibitory proteins that prevent axon regeneration is to constrain axon growth and sprouting to terminate ocular dominance plasticity.

1.2.4 Signaling by myelin-associated inhibitors

Even before the identification of NgR complex, it had been known that activation of RhoA mediates MAG and myelin-induced inhibition of neurite outgrowth (Lehmann et al., 1999; Kottis et al., 2002; Winton et al., 2002). Application of C3 transferase, which ADP-ribosylates and inhibits RhoA, can promote axon regeneration *in vivo* in the rat optic nerve (Lehmann et al., 1999) and mouse spinal cord (Dergham et al., 2002). Later findings have shown that p75 activates RhoA upon binding of MAG to the receptor

complex (Wang et al., 2002a; Yamashita et al., 2002) and that p75 may activate RhoA indirectly by the sequestration of Rho-GDP dissociation inhibitor, Rho-GDI (Hasegawa et al., 2004). RhoA-associated kinase (ROCK), a major RhoA effector, has also been implicated in myelin-mediated inhibition. Similar to C3, an inhibitor of ROCK called Y27632 can also block the inhibitory activity of myelin inhibitors (Fournier et al., 2003) (Dergham et al., 2002), which adds ROCK to the list of major downstream components of NgR complex signal.

Recently, Filbin's group showed that binding of MAG to NgR induced regulated intramembrane proteolysis (RIP) of p75 and the RIP was required for RhoA activation and neurite outgrowth inhibition (Domeniconi et al., 2005). Soluble MAG induces cleavage of the p75 extracellular domain (ECD) by α-secretase followed by a γ-secretase cleavage within the transmembrane domain of p75, which releases a 25 KDa intracellular domain (p75ICD). The resulting p75ICD is necessary to acitvate RhoA as inhibitors of γ -secretase block RhoA activation. In addition, γ -secretase but not a-secretase activity is dependent on PKC activation as PKC inhibitor blocks the generation of p75ICD but not ECD (Domeniconi et al., 2005). These findings further support a previous report by He's group that myelin-associated inhibitors and CSPGs block axon regeneration in a PKC-dependent manner (Sivasankaran et al., 2004).

EGFR is the latest protein to be implicated in the downstream signaling of NgR activation (Koprivica et al., 2005). EGFR can be phosphorylated by Nogo66 and OMgp and this activation is dependent on both calcium and the NgR complex. Inhibition of EGFR kinase activity by pharmacological inhibitors promotes neurite outgrowth on myelin, MAG and Nogo-66 and enhances regeneration of retinal ganglion cell axons in

optic nerve. However, activation of EGFR by its own ligand, EGF, does not inhibit neurite outgrowth indicating that EGFR activation is necessary but not sufficient to bring about inhibition and that NgR must activate a separate pathway to affect inhibition (Koprivica et al., 2005) . Furthermore, EGFR is not immunoprecipitated with NgR or p75 and does not bind to myelin inhibitors, suggesting that phosphorylation of EGFR is through transactivation by calcium. It should be noted that CSPGs also activate EGFR in a calcium-dependent manner, which is necessary for them to exert inhibition (Koprivica et al., 2005).

Strategies to promote regeneration after CNS injury

With progress in the understanding of the inhibitory mechanisms that repress axon extension, scientists have made attempts to promote axon regeneration in adult CNS after injury. In general, there are two approaches to encourage regeneration in the injured mammalian CNS: (a) to neutralize extrinsic inhibitory environments and (b) to change the intrinsic state of neurons such that they ignore the inhibitory signals.

1.3 Changing the inhibitory environment

1.3.1 Removing the glial scar inhibitors

As mentioned above, CSPGs are the crucial molecules in the scar that inhibit axon regeneration. Altering CSPGs levels using enzyme Chondroitinase ABC, which removes

the chondroitin sulfate GAG chains, improves axonal regeneration (Moon et al., 2001; Bradbury et al., 2002). Injection of Chondroitinase ABC into the area near lesioned nigrostriatal tract results in the growth of axons to the striatum (Moon et al., 2001). Intrathecal delivering the enzyme can promote regeneration and functional improvement after spinal cord injury (Bradbury et al., 2002). Alternatively, suppression of CSPGs core protein synthesis by decorin can promote axon growth across spinal cord injury in adult rats (Davies et al., 2004). In addition, administration of a DNA enzyme that interferes with GAG synthesis also enhances axonal regeneration (Grimpe and Silver, 2004).

1.3.2 Blocking myelin-associated inhibitors and their receptors

1.3.2.1 Using antibodies and peptides

The first strategy employed to neutralize myelin inhibitors was the use of the monoclonal antibody IN-1, raised against an antigen later identified as Nogo-A (Schnell and Schwab, 1990). Treatment with IN-1 antibody from hybridoma implants enhances regeneration of transected corticospinal axons (Schnell and Schwab, 1990, 1993; Bregman et al., 1995) and promotes functional recovery (Bregman et al., 1995; Thallmair et al., 1998; Merkler et al., 2001). In addition, delivery of a humanized Fab fragment of the IN-1 antibody with the use of the osmotic pumps also promotes regeneration of lesioned corticospinal tract (CST) fibers (Brosamle et al., 2000). The IN-1 antibody has also been shown to enhance regenerative growth in a number of other CNS lesion models (Cadelli and Schwab, 1991; Weibel et al., 1994; Raineteau et al., 1999; Papadopoulos et al., 2002; Tatagiba et al., 2002). Furthermore, vaccine approach may also be used to block multiple inhibitors in myelin. In one interesting study, mice were immunized with purified myelin and robust

regeneration of CST axons was observed after dorsal hemisection of the spinal cord in the immunized mice (Huang et al., 1999).

The finding that NogoA, MAG and OMgp bind to a common receptor complex NgR offers promising targets for intervention. For example, NEP1-40, a small peptide that consists of the first 40 residues of the Nogo-66 sequence, can compete with Nogo-66 for binding to NgR without exerting inhibition. Intrathecal delivery of NEP1-40 enhanced regeneration of CST fibers and functional recovery following spinal cord hemisection (GrandPre et al., 2002). In another study from the same group, subcutaneous treatment with NEP1-40 resulted in extensive growth of CST axons, sprouting of serotonergic fibers and enhanced locomotor recovery after spinal cord injury (Li and Strittmatter, 2003). Similarly, when a soluble function-blocking NgR ectodomain [aa27-310; NgR (310) ecto], was fused to Fc and delivered intrathecally or secreted from astrocytes in transgenic mice, it induced axonal sprouting of corticospinal and raphespinal fibers as well as improved spinal cord electrical conduction and locomotion after mid-thoracic dorsal over-hemisection (Li et al., 2004; Li et al., 2005). p75-Fc and Lingo-Fc have also been shown to block neuritie outgrowth inhibition *in vitro* (Wang et al., 2002a; Mi et al., 2004). However, there is no report about their function *in vivo*.

1.3.2.2 Knockout mice

MAG-deficient (MAG-/-) mice were first used to further elucidate the role of MAG in the inhibition of axonal regeneration in CNS. Although one study showed little or no detectable regeneration of optic nerve and CST fiber (Bartsch et al., 1995), another study indicated that there was a small but significant amount of regeneration in the CST in

MAG-/- mice compared to wild type mice (Li et al., 1996). The discrepancy between the two studies could be due to differences in the genetic background of the mutant mice. However, given the presence of glial scar and multiple inhibitors other than MAG in myelin, the lack of robust improvement of axonal regeneration in MAG-/- mice is not entirely surprising.

Nogo knockout mice have been generated to study axonal regeneration in the spinal cord by three different groups with different strategies and the results are controversial (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). Strittmatter's group disrupted the longest exon of Nogo-A with a retroviral gene trap insertion technique. The expression of Nogo-A and Nogo-B, but not Nogo-C, were eliminated as predicted. After spinal cord injury, these young adult Nogo-A/B-/- mice exhibited robust axon sprouting and regeneration as well as improved functional recovery (Kim et al., 2003). Schwab's group disrupted similar Nogo-A exon with conventional knockout approach. Surprisingly, they observed up-regulation of Nogo-B in the CNS. Nonetheless, these Nogo-A -/- mice did display enhanced regeneration after dorsal hemisection. Finally, Tessier-Lavigne's group generated two lines of mutant mice, one lacking Nogo-A and B and the other lacking all three Nogo isoforms. However, contrary to Stittmatter's report, they did not observe any spontaneous regeneration after CST injury. The varied *in vivo* outcomes could be attributable to age at the time of lesion, mouse strain background, surgical techniques, axonal tracing methodology and the nature of the mutant allele (Woolf, 2003). Nevertheless, these results emphasize that Nogo-A is only one of the factors that is important in restricting axon regeneration and functional recovery after CNS injury.

Two strains of mutant mice lacking NgR have been generated to assess the contribution of NgR to regeneration failure *in vivo*. Although, there was some regeneration in raphespinal tract and rubrospinal tract with some recovery of motor function after dorsal hemisection in one study (Kim et al., 2004), there was no CST regeneration in either study (Kim et al., 2004; Zheng et al., 2005), suggesting that additional receptors for MAG, Nogo-A, OMgp may exist. Indeed, as described above, PirB is another receptor for the three inhibitors and future *in vivo* studies with PirB knockout and NgR-PirB double knockout mice are needed to fully elucidate the role of PirB in axon regeneration.

Similar to NgR knockout mice, p75 knockout mice did not show promising results. There was no detectable regeneration in the CST of p75-/-mice after spinal cord injury (Song et al., 2004; Zheng et al., 2005), or enhanced sensory ascending axons and dorsal column fibers observed in p75 -/- mice (Song et al., 2004), again suggesting p75 may not be a converging molecule mediating inhibitory function of myelin inhibitors *in vivo*. The discovery of the p75 homolog, TROY, may help to explain the lack of regeneration in these mice. However, regeneration studies in TROY knockout mice *in vivo* have not been reported yet.

1.3.3 Cell transplantation to create more permissive environments

Since the CNS environment is very inhibitory for neuronal regeneration after injury, using cell transplantation to create a favorable environment has been explored. The cell types that have been used and found to achieve significant regeneration include Schwann cells (Xu et al., 1995), olfactory ensheathing cells (OECs) (Navarro et al., 1999),

fibroblasts expressing trophic factors (Liu et al., 1999), bone marrow stromal cells (BMSCs) (Hofstetter et al., 2002), and embryonic stem cells (McDonald et al., 1999). In addition to creating a permissive environment, cellular transplantation also serves as a bridge across lesion cysts or cavities or replaces dead cells by providing new neurons or myelinating cells. However, most neurons only extend axons within the grafts but never enter the inhibitory tissue beyond, which emphasizes the necessity of combinatorial approaches after CNS injury.

1.4 Improving the intrinsic growth capacity of neurons

Another approach to encouraging regeneration after injury is to alter intrinsic growth properties of mature neurons so that they no longer respond to the inhibitory environment. This can be divided into two strategies: interfering with the common cascade that signals inhibition or activating a parallel signaling pathway to overcome inhibition.

1.4.1 Blockage of RhoA small GTPase

As described earlier, PKC, EGFR and RhoA activation are convergent points in the inhibitory signaling pathway for both glial scar component CSPGs and myelin-associated inhibitors MAG, Nogo and OMgp. Interfering with these factors and their downstream effectors thus offers a great prospect for promoting axon regeneration. Intrathecal infusion of the PKC inhibitor Gö6976 after dorsal column hemisection promotes increased regeneration of dorsal column fibers (Sivasankaran et al., 2004). Application of

EGFR inhibitors such as PD168393 promotes dramatic retinal ganglion axon regeneration after optic nerve crush (Koprivica et al., 2005). It is not surprising that RhoA inactivation promotes regeneration, since RhoA is known to regulate the actin cytoskeleton (Jaffe and Hall, 2005). McKerraher's laboratory has shown that blocking RhoA with C3 transferase following optic nerve crush in adult rats allows severed axons to traverse the lesion to regrow in the distal white matter of the opitic nerve (Lehmann et al., 1999). More importantly, C3 delivered in a fibrin adhesive promotes long-distance regeneration of CST axons for up 12mm and functional recovery after spinal cord dorsal hemisection in adult mice (Dergham et al., 2002). Furthermore, blocking the RhoA downstream effector, ROCK, with Y27632 also enhances regeneration and sprouting of CST fibers and lomotor recovery (Dergham et al., 2002; Fournier et al., 2003).

1.4.2 Neurotrophins and cAMP

Besides interfering with the inhibitory signaling cascade, increasing the activity of stimulatory pathways also enhances neuronal regenerative potential. Neurotrophins are known to promote the survival and maintenance of the injured neurons in the mammalian CNS, but they can not directly overcome inhibition by myelin. In 1999, our lab showed that neurons exposed to neurotrophins overnight (priming) before encountering the myelin inhibitors could overcome inhibition by MAG and myelin (Cai et al., 1999). This priming effect is mediated by increased cAMP levels and can be abolished in the presence of a PKA inhibitor. Addition of a cAMP analog such as dibutyryl cAMP (db-cAMP) or 8-bromo cAMP blocked inhibition by MAG and myelin (Cai et al., 1999). Consistent with this, elevation of cAMP changes the response of the growth cone from

repulsion to attraction when either soluble MAG or myelin is presented in an asymmetric gradient (Song et al., 1998). In 2001, our lab also demonstrated that endogenous cAMP levels are high in young neurons in which axon growth is promoted by MAG and myelin and this cAMP level drops precipitously with development at a time that correlates with the onset of the inhibitory response to MAG and myelin (Cai et al., 2001). More importantly, injection of db-cAMP directly to DRG cell body promotes regeneration of spinal dorsal column axons (Neumann et al., 2002; Qiu et al., 2002). Furthermore, cAMP elevation has also been implicated in the conditioning lesion paradigm. It has been well established that if a lesion is applied to the peripheral branch of a DRG neuron and, one day or one week later, a second lesion is introduced to the dorsal column, CNS regeneration occurs (Neumann and Woolf, 1999). This effect of the conditioning lesion has been shown to be cAMP-dependent and can be mimicked by single injection of db-cAMP into DRG's *in vivo* (Neumann et al., 2002; Qiu et al., 2002). One day after sciatic nerve transection, cAMP levels in DRG neurons triple, and these neurons are able to overcome inhibition by MAG/myelin, an effect that is PKA dependent. By 1 week post-lesion, cAMP levels return to baseline, but neurite outgrowth on MAG/myelin improves and now is PKA independent (Qiu et al., 2002). These results imply the cAMP can alter the growth state of neurons and activation of the cAMP pathway may be a useful tool to stimulate regeneration.

To activate the cAMP pathway, there are several ways to increase cAMP levels: by prior exposure of neurons to neurotrophins, such as NGF, BDNF and GDNF or treatment with membrane permeable, non-hydrolysable cAMP analogue such as db-cAMP (Cai et al., 1999; Neumann et al., 2002; Qiu et al., 2002); with forskolin (which

activates adenylyl cycalse) (Gao et al., 2003); and by inhibiting phosphodiesterases (PDEs), the enzymes that degrade cAMP (Nikulina et al., 2004). The work from our lab has shown that application of PDE4 inhibitor, rolipram, improved regeneration and functional recovery (Nikulina et al., 2004). In addition, rolipram attenuated reactive gliosis, which might further facilitate regrowth of axons (Nikulina et al., 2004) . Initially developed as an antidepressant, rolipram has become an attractive potential spinal cord injury therapeutic because it is able to cross blood-brain barrier and can be administered subcutaneously (Hannila and Filbin, 2008).

Since cAMP is a cellular second messenger and has many roles in a variety of signaling pathways, the artificial elevation of cAMP alone may not be an optimal therapeutic approach. Therefore, understanding the downstream consequences of cAMP signaling that overcome inhibition may present other potential targets for therapeutic intervention to encourage regeneration in adult CNS.

1.4.3 Downstream effectors of cAMP

It is well known that elevation of cAMP results in the activation of PKA, a cAMP effector. Indeed, inhibition of PKA blocks the effects of a conditioning lesion or priming with neurotrophins (Cai et al., 1999; Qiu et al., 2002). The effect of cAMP is transcription dependent, as 5,6-dicholro-1-β-D-ribofuranosylbenzimidazole (DRB), a transcription inhibitor, abrogates the effect of db-cAMP in overcoming inhibition (Cai et al., 2002). In 2004, Gao et al showed that activation of the transcription factor cAMP response element binding protein (CREB) is necessary to overcome inhibition. This activation is PKA dependent as CREB phosphorylation is reduced when PKA inhibitor is

present together with db-cAMP. Adenoviral expression of dominant negative CREB, A-CREB, blocked the ability of db-cAMP to overcome inhibition by MAG, whereas constitutively active CREB, VP16-CREB, overcame MAG's inhibition without db-cAMP. Moreover, injection of VP- CREB promoted axonal regeneration after dorsal column lesion (Gao et al., 2004). Therefore, it would be interesting to identify cAMP elevation regulated genes responsible for overcoming inhibition, and to reveal the underlying mechanisms that cAMP promotes axon regeneration.

Our lab has identified several cAMP-regulated genes that play roles in regeneration including arginase I (ArgI) and the cytokine interleukin-6 (IL-6). Microarray analysis with mRNA from DRG neurons after db-cAMP treatment or conditioning peripheral lesion revealed robust increased expression of IL-6 (Cao et al., 2006). *In vitro*, recombinant IL-6 could overcome inhibition by MAG and myelin. Importantly, intrathecal delivery of IL-6 to adult rats after a dorsal column lesion promoted axonal regeneration. In addition, this IL-6 effect was transcription dependent and was through activation of the classic IL-6 receptor complex IL-6R/gp130, which resulted in activation of the JAK signaling cascade (Cao et al., 2006). However, further downstream targets of this signaling pathway remains to be determined. Cao and colleagues also noted that 11 genes such as ArgI, neuropeptides Y, CREM (cAMP response element modulator) and VGF (nerve growth factor-induciblbe growth factor) were up-regulated and 4 genes were down-regulated in response to db-cAMP. These genes merit further study in the axonal regeneration paradigm and some of them are currently ongoing projects in our lab.

1.4.4 Arginase I and polyamines

ArgI was the first cAMP-regulated gene to be shown to play a role in overcoming inhibition by MAG/myelin. ArgI is a rate-limiting enzyme in the synthesis of polyamines which include putrescine, spermidine and spermine (Kepka-Lenhart et al., 2000; Li et al., 2001). In 2002, we showed that ArgI expression and polyamine putrescine synthesis were increased in neurons in response to db-cAMP or BDNF (Cai et al., 2002). Either overexpression of ArgI or exogenous addition of putrescine was sufficient to overcome inhibition by MAG/myelin. Conversely, blocking ArgI or the next enzyme in the polyamine synthesis, ornithine decarboxylase (ODC), abrogated the ability of db-cAMP or BDNF to overcome inhibition (Cai et al., 2002). Interestingly, ArgI protein level in DRG neurons drops dramatically between P3 and P5, which coincides with the decrease in endogenous cAMP levels and the switch from promotion to inhibition by MAG and myelin. Over-expressing ArgI in older DRG neurons blocks this switch (Cai et al., 2002).

Recently, Deng et al showed that a peripheral conditioning lesion also induced an increase in ArgI expression and synthesis of the polyamines putrescine and spermidine (Deng et al., 2009). The conditioning lesion effect in overcoming inhibition by MAG was initially dependent on ongoing polyamine synthesis but with time became independent of ongoing synthesis. Importantly, putrescine, delivered either in culture or in intrathecally, must be converted to spermidine to overcome inhibition by MAG. Spermidine overcame inhibition by MAG and myelin *in vitro* and intrathecally delivery of spermidine mimicked the conditioning lesion effect when neurons were cultured. Furthermore, spermidine promoted optic nerve regeneration *in vivo* (Deng et al., 2009). These results suggest that spermidine is a more specific treatment for spinal cord or brain injury.

1.5 Combination of therapeutic strategies

Although neutralizing the extrinsic inhibitory environment or changing the intrinsic state of neurons has individually resulted in increased regeneration and functional recovery after CNS injury, the number of regenerating neurons is generally small and functional recovery is modest, which suggests that targeting multiple factors might be a better treatment for axon regeneration. Indeed, growing evidence has shown that combination therapies can be undertaken to induce more optimal regeneration. For example, reducing Nogo signaling or inactivating Rho A combined with inflammation-induced stimulation of the intrinsic growth capacity of retinal ganglion cells promotes optic nerve regeneration (Fischer et al., 2004a; Fischer et al., 2004b). Notably, combining rolipram with db-cAMP and Schwann cell transplantation enhances axonal sparing and myelination, promotes growth of serotonergic fibers into and beyond grafts and significantly improves locomotor function (Pearse et al., 2004). Similarly, a combination of db-cAMP, NT-3 and bone marrow stromal cells has been shown to promote axonal regeneration into and beyond sites of spinal cord injury (Lu et al., 2004). Results from these combinatorial studies clearly lend encouragement to the possibility that clinically successful regeneration can be achieved in the injured CNS and development of future effective combinatory treatments is a necessary step toward a cure after CNS injury.

Goals of this work

Our lab has shown that elevating intracellular cAMP levels can overcome inhibition by MAG and myelin both *in vitro* and *in vivo*. Elevation of cAMP results in up-regulation of

arginase I (ArgI) and subsequent synthesis of polyamines. Over-expression of ArgI or priming with the polyamine putrescine or spermidine blocks the inhibition of axonal growth by MAG/myelin. Putrescine must be converted to spermidine to overcome inhibition by MAG. Polyamines are known to have a plethora effects on neurons such as regulating cytoskeleton organization (Kaminska et al., 1992; Williams, 1997; Banan et al., 1998), binding to ion channels (Williams, 1997) and modifying proteins post-translationally (Chakraborty et al., 1987; Huang et al., 2007). The goal of my research is to identify the mechanisms by which polyamines bring about this regeneration effect. Therefore, this thesis set out to answer the following two questions:

1) What is the downstream target for polyamines to overcome inhibition?

2) How do polyamines regulate this target to exert their effect?

Chapter II: Materials and Methods

2.1 Cell culture

Control or stably-transfected MAG-expressing Chinese Hamster Ovary (CHO) cells (Mukhopadhyay et al., 1994) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) which had been supplemented with 10% dialyzed fetal bovine serum (FBS, Gibco), 40mg/L L-Proline (Sigma), 7.5mg/L Glycine (Sigma), 0.73mg/L Thymidine (Sigma) and 0.29g/L L-Glutamine (Gibco).at 37ºC in 7.3% CO_2. These cells were used as monolayer substrates in the neurite outgrowth assay (NOG).

2.2 Preparation of monolayers

Permanox 8 well chamber slides (Lab-Tek) were coated with 20 µg/ml poly-L-lysine (Sigma) for 30 minutes at room temperature. After incubation, poly-L-lysine was removed and the slides were treated with 20 µg/ml fibronectin (Sigma) for at least 2 hours at 37ºC. Monolayers of control and MAG-expressing CHO cells (passage number 5-20) were plated in the individual chambers of an 8-well tissue culture slide (Lab-Tek) at the following concentrations: control-CHO: 6 x 10^4 cells/well; MAG-CHO: 5.5 x 10^4 cells/well. The slides were then incubated overnight at 37º C and grown to confluency, prior to the plating of primary neurons on top of the CHO monolayers.

2.3 Isolation of neurons

For cerebellar neurons, cerebellums were taken from each animal at the age from postnatal day 5 (P5) to day 7 and dissociated in 6ml of 0.025% trypsin (Life Technology)

by pipetting. 10ml DMEM containing 10% FBS was then added to terminate trypsinization. Cells were centrifuged at 1000 rpm for 5 minutes at 4°C and resuspended to a single-cell suspension in SATO (progesterone, 200nM; selenium, 224nM; insulin, 4μg/ml; BSA, 0.35mg/ml; L-thyroxine, 0.4μg/ml; tri-iodo-thyronine, 0.34μg/ml). For DRG neurons, ganglia were removed from animals aging from P5 to P8 and incubated in 6ml of L15 media containing of 0.025% trypsin and 0.15% collagenase type I (Worthington) for 60-90 min at 37°C. The ganglia were triturated and trypsinization was stopped by adding 10ml of DMEM containing 10% FBS, centrifuged at 1000 rpm for 5 minutes and resuspended in SATO (DeBellard et al., 1996). For Cortical neurons, Long-Evans rats of P0-P2 were sacrificed, and the cotices were collected on ice in plain neurobasal media. Papain was added to the collection media at a final concentration of 0.5 mg/ml, and tissues were incubated at 37ºC for 20 minutes. After incubation, media was aspirated, and then the procedure was repeated once more. Papain was inhibited by the addition of soybean trypsin inhibitor. After 2 minutes, media was aspirated, and the tissue was washed 2 times with plain neurobasal media. Plain neurobasal was added again, and tissue was triturated and passed through a 40 μm cell strainer. 6 ml of this single cell suspension were then loaded on top of a gradient, consisting of four layers of Optiprep™ working solution (30% w/v iodixanol, 0.425% NaCl, 5 mM MOPS-NaOH, pH 7.4) of densities 1.057, 1.043, 1.036 and 1.029 g/ml (listed from bottom of the tube to top of the tube). Neurons were then centrifuged at 1900 rpm for 15 minutes at room temperature. Fractions containing enriched populations of neurons were isolated according to the directions of the Optiprep™ Application Sheets, diluted with plain

neurobasal, and pelleted by centrifugation. The pellet containing dissociated neurons was then resuspended in neurobasal media, and neurons were counted and plated.

2.4 Priming neurons

24-well plates were coated with 20μg/ml PLL for 30 minutes at room temperature. Isolated neurons in SATO were plated onto the PLL-coated well at a density of 1 $x10^6$ cells/well. Where indicated, either, putrescine, Roscovitine (10μM) (all from Sigma), GC-7 or DRB, cycloheximide, was added. After overnight incubation, the media was removed and neurons were washed with PBS and removed with 0.1% trypsin. Trysinization was stopped by adding 2ml DMEM containing 10% FBS. Neurons were centrifuged at 1,000 rpm for 5 minutes, resuspended in SATO and plated immediately onto either MAG-expressing or control CHO cells or myelin substrate.

2.5 Preparation of myelin

Myelin was purified from rat CNS medulla following the Norton's protocol (Norton and Poduslo, 1973). Briefly, The medulla from an adult rat brain is isolated and homogenized in a 0.25M sucrose solution containing a protease inhibitor cocktail (CalBiochem) using a glass and Teflon homogenizer. The homogenate is then mixed with a 2.55 M sucrose solution to create a 1.4 M solution. This is then layered onto a 1.9 M solution, followed by 0.85 M and 0.25 M solutions. The gradient is centrifuged at 40,000 rpm for 14 hours at 4ºC. Following centrifugation and separation, the extracted myelin is homogenized again in dH_2O and protease inhibitor, centrifuged at 14,000 rpm for 1 hour at 4ºC,

resuspended in 10mM HEPES, and triturated using 18.5 and 26.5 gauge needles. Then the protein concentration of the preparation was determined (Biorad) and used as a substrate in the neurite outgrowth assay.

2.6 Neurite outgrowth assay on CHO cells or immobilized myelin

Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an 8-chamber tissue culture slide (Lab-Tek). Primary neurons were isolated and resuspended in Sato media as described previously (DeBellard et al., 1996). Neurite outgrowth assay was carried out by adding $1.5x10^4$ neurons to the CHO cell monolayers. After 18-22 hours of incubation, the neurons were fixed for 30 minutes with 4% paraformaldehyde and permeabilized with ice-cold methanol for 2 minutes. The cells were then blocked for 30 minutes with DMEM containing 10% FCS and incubated overnight with a rabbit monoclonal antibody against the β-III-tubulin (1:1000, Covance) diluted in PBS-5%BSA. Cells were washed three times with PBS and then incubated for 30 minutes at room temperature with a biotinylated donkey anti-mouse IgG (1:500, Amersham, Arlington Heights, IL), washed three times, and then incubated with streptavidin-conjugated Texas Red (1:300, Amersham, Arlington Heights, IL) for 45 minutes. After three more washes, the slides were mounted in Permafluor (Immunon) and viewed with a fluorescence microscope. The length of the longest neurite for each neuron for the first 200-400 neurons encountered when scanning the slide in a systematic manner was determined by using an Oncor or MetaMorph image analysis program.

For myelin membranes, wells of an 8-chamber tissue culture slide (Lab-Tek) were coated with 20 μg/ml poly-l-lysine at room temperature for 1 hour. Rat CNS myelin at 0.5-2.0μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen et al., 1998). $2x10^4$ cells/well of DRG or $5 x10^4$ cerebellar neurons were suspended in Sato medium, and incubated in myelin-coated wells at 37°C for 24 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

2.7 Neurite outgrowth assay on immobilized L1-Fc substrate

We have developed an assay in which the growth of isolated young cerebellar neurons on the substrate of the growth-promoting molecule L1 would be inhibited by the presence of wild type MAG-Fc (Tang et al., 1997a). An 8-chamber slide (Lab-Tek) was first coated with 20 μg/ml poly-L-lysine (Sigma) in sterile water for 1 hour at room temperature, and then washed twice with freshly made 0.1M bicarbonate buffer. Wells were then incubated with anti-human Fc antibody (Sigma) 15 μg/ml in 0.1M bicarbonate buffer for 2 hours at 37°C. After washing three times with DMEM, L1-Fc was immobilized onto anti-Fc by incubating 30 μg/ml L1-Fc in the wells overnight at 37°C. Unbound L1-Fc was washed off with DMEM. Cerebellar granular neurons were suspended in SATO medium with 2% FBS into a concentration of $5x10^4$ cells/well, and incubated with 25 μg/ml of wild type or mutant MAG-Fc proteins at 37°C for 18 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

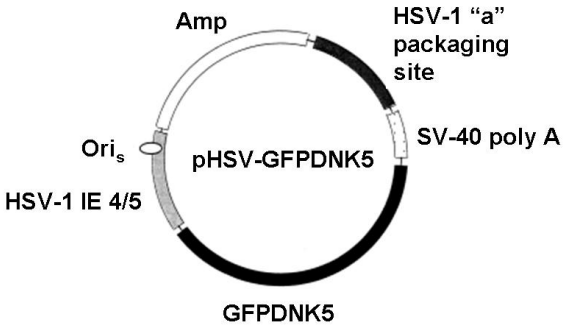
2.8 Western blotting

Cerebellar or DRG neurons were lysed in 1X RIPA lysis buffer (Upstate) supplemented with phosphatase inhibitors (1mM Na3VO4, 1mM NaF) and proteinase inhibitors (1mM EDTA, 1mM PMSF, and aprotinin, leupeptin, pepstatin 1ug/ml each). Protein concentration was measured with a Bio-Rad kit. Normalized lysates were boiled for 5 minutes, after which they were subjected to SDS-PAGE in a 10% polyacrylamide gel. Then protein was transferred to nitrocellulose membrane. Membranes were then blocked in 5% nonfat dry milk in PBS-0.1%Tween-20 and probed with either a p35 antibody (Santa Cruz) , a Cdk5 antibody (Santa Cruz), a phospho-ERK antibody (cell signaling), a DHS antibody (Santa Cruz) or eIF5A antibody (BD Biosciences) in blocking buffer overnight at 4° C. Membranes were washed three times in PBS with 0.1% Tween-20. Secondary goat anti-rabbit HRP-conjugated antibody or Donkey anti-goat HRP-conjugated antibody (cell signaling) was diluted 1:2000 in blocking buffer and incubated with the membranes for 1 hour at room temperature. Membranes were then washed as before and developed using ECL detection system, according to the manufacturers' instructions (Amersham Biosciences). Blots were later stripped with stripping buffer (Pierece) for 30 minutes at room temperature and reprobed as indicated.

2.9 HSV infection of neurons

$1X10^{6}$ DRG neurons were infected with herpes simplex viruses (HSV) containing dominant negative Cdk5-GFP (DNK5), p35 or lacZ gene at a final concentration of 10^{7} PFU/ml and maintained in virus-containing media overnight. Neurons were then transferred directly to monolayers of CHO cells and neurite outgrowth assay was carried

out as described above. Alternatively, infected neurons were lysed in 1X RIPA buffer and subjected to Western blot analysis.



Map of pHSV-DNK5 (DN-CDK5)

2.10 Cdk5 kinase assay

Cell lysates were immunoprecipitated with anti-CDK5 antibody overnight at 4^0C and then 3 hours with protein G Sepharose beads. The beads were washed twice with RIPA lysis buffer and once with kinase reaction buffer (50mM HEPES [pH 7.55], 10mM MgCl2, 1mM DTT, phosphotase and protease inhibitors). The washed beads were mixed with 10ug of histone H1 in kinase reaction buffer with 2μCi γ-32P ATP for 30 minutes at 30^0C. The reaction was stopped by sample buffer (62.5 mM Tris-HCl [pH6.8] and 2% SDS). The samples were subject to SDS-PAGE and autoradiography.

2.11 Immunostaining of neurons

Isolated cortical neurons were plated onto poly-l-lysine-coated chambers of 8 well tissue culture slide and incubated overnight with or without putrescine treatment. The cells were then fixed and double stained with p35 (1:50, rabbit polyclonal, Santa Cruz) and Cdk5 (1:100, mouse monoclonal, Upstate) or eIF5A (1:100, goat polyclonal, Santa Cruz) and β-III-tubulin (1:1,000, mouse monoclonal, Covance) antibodies overnight at 4^0C, followed by a 1h incubation with an anti-rabbit (or anti-goat) IgG-Texas Red and an anti-mouse IgG-FITC secondary antibody.

2.12 siRNA

siRNA sequences for the sense strand of the central 19 or 21-nt double-stranded region were derived from rat DHS gene: GCCCAUAAGAACCACAUAC and rat eIF5A gene: AAAGGAAUGAUUUCCAGCUGA. The scrambled sequences were CCAUUAAACCCGGCCAAAA and CGUUAUGACGAAACAGAAGUU, respectively. The siRNA duplexes with a thiol on the sense strand were synthesized and HPLC purified (Dharmacon). Annealed siRNA duplexes were resuspended in the RNAase-free water. An equimolar ratio of Penetratin I (Q-Biogene) was added and the mixture was heated to 65^0C for 15min and further incubated at 37^0C for 1 hour. The coupled siRNAs were then added the cultured cerebellar neurons at the concentration of 300nM and the neurons were cultured for further 24-28 hours, after which neurons were lysed and subjected to western blot analysis, or transferred onto monolayers of CHO cells for neurite outgrowth assay as described.

2.13 Isolation of total mRNA from cultured Neurons

Cerebellar or DRG neurons from PND5 rat pups were isolated and 50 x 10^4/well neurons were plated onto poly-l-lysine or purified myelin coated 24-well tissue culture plates. Dibutyryl-cAMP (1mM, Calbiochem) or putrescine (100µM, Sigma) was then added to the culture medium and the cells were incubated for different time period at 37°C with 7.3% CO_2.

The cells were then washed once with ice cold PBS and lysed in lysis buffer provided by RNeasy RNA isolation kit (QIAGEN). RNA was prepared *via* the RNeasy RNA isolation kit (QIAGEN), according to the manufacturers instructions.

2.14 Detection of gene expression

Total RNA was isolated cerebellar or DRG neurons treated with db cAMP (1 mM) or putrescine (100µM) overnight. RNA was extracted using an RNeasy kit (Qiagen) following the manufacturers' instructions. cDNA was synthesized from total RNA using oligo dT and reverse transcriptional enzyme (stratagene) at 37°C for 1 hour. After which, p35 cDNA was amplified using primers F: 5’ GCTCTGCAG GGA TGT TAT CTC C 3’ and R: CTT CTT GTC CTC CTG ACC ACT C 3’ (Invitrogen) using the polymerase chain reaction (PCR) at 30 cycles. Amplification of GADPH from the same sample was used as control. GAPDH-Forward ATG GTG AAG GTC GGT GTG AAC G; Reward TGG TGA AGA CGC CAG TAG ACT C. PCR 30 cycles annealing temp 55°C 45 seconds. DNA was detected in a 1% agarose gel, stained with ethidium bromide.

2.15 Peripheral conditioning lesion

P28 rats were anesthetized by isofluorane, then a sciatic nerve was exposed at mid-thigh level and a ligature was firmly tightened around the nerve distal to its emergence from the greater sciatic notch. The nerve was transected distal to the ligature and the wound closed. The animals were sacrificed 18 hours-7 days after the sciatic nerve transection. L4,5 dorsal root ganglia from the lesion side and contralateral control side were isolated respectively lysed and subjected to western blotting .

2.15 Statistical analysis

Prism GraphPad software was used to perform one-way ANOVA, followed by multiple comparisons using the Turkey's test.

Chapter III: Cyclin Dependent Kinase 5 (Cdk5) Activity is Required for cAMP and Putrescine to Overcome Inhibition by MAG

3.1 Introduction

Previous work from our lab has shown that elevating intracellular cAMP levels can overcome inhibition by MAG and myelin both *in vitro* and *in vivo* (Cai et al., 1999; Qiu et al., 2002). One consequence of elevated cAMP is the up-regulation of arginase I (ArgI) and subsequent synthesis of polyamines (Cai et al., 2002). Over-expression of ArgI or priming with the polyamines putrescine or spermidine blocks the inhibition of axonal growth by MAG and myelin (Cai et al., 2002; Deng et al., 2009). We also have shown that putrescine must be converted to spermidine to overcome inhibition by MAG and that spermidine is sufficient to promote optic nerve regeneration *in vivo* (Deng et al., 2009). All signals, either extrinsic or intrinsic, ultimately bring about their effect by regulating cytoskeleton dynamics. However, little is known about the mechanism(s) whereby cAMP and polyamines regulate cytoskeletal organization to overcome inhibition. Accumulating evidence suggests that cyclin dependent kinase 5 (Cdk5) might be one such regulator for the cAMP/polyamines to rearrange the cytoskeleton and thus bring about regeneration.

Cdk5, a 33KD protein, known as a neuronal Cdc2-like kinase, is a member of the proline-directed serine/threonine Cdk family. Cdk5 was initially identified from bovine brain by its sequence homology to human Cdc2 (Lew et al., 1992; Meyerson et al., 1992). Unlike other Cdks which are regulated by cyclins and traditionally involved in regulating cell cycle progression, Cdk5 is not activated by cyclins and does not participate in cell cycle regulation (Lew and Wang, 1995). Although Cdk5 is ubiquitously expressed in mammalian tissues, its highest expression and associated kinase activity are detected only in the nervous system (Tsai et al., 1993). This is due to its non-cyclin activator, p35 or its isoform p39, whose expression is found almost

exclusively in post-mitotic neurons (Tsai et al., 1994; Tang et al., 1995). Interestingly, although both p35 and p39 have little sequence similarity to the cyclins, they can adopt a cyclin-like tertiary structure (Tarricone et al., 2001).

Cdk5 is fundamental for CNS development. Mutant $Cdk5^{-/-}$ mice die in the perinatal period and show widespread disruptions in neuronal layering of many brain structures including the cerebral cortex, hippocampus and cerebellum (Ohshima et al., 1996). Mice lacking p35 show an inverted layering of cortical neurons similar to that seen in the $Cdk5^{-/-}$ mice (Chae et al., 1997). p35-mutant mice also show defects in fasciculation of some prominent axon tracts (Kwon et al., 1999). These results support a role for Cdk5/p35 in neuronal migration and axon guidance.

Another major role for Cdk5 is the regulation of neurite outgrowth. Dominant negative Cdk5 or antisense p35 treatment in cultured rat E17 cortical neurons inhibits neurite outgrowth, which is rescued by coexpression of the wild-type proteins (Nikolic et al., 1996). Cdk5 or p35 antisense oligonucleotides suppress laminin-induced axonal elongation in primary cultures of cerebellar macroneurons (Pigino et al., 1997; Paglini et al., 1998). In immortalized rat hippocampal cells, microinjection of vectors containing p35 cDNA promotes neurite outgrowth and a dominant negative Cdk5 blocks both p35-induced neurite extension as well as basic fibroblast factor-induced neuronal differentiation (Xiong et al., 1997). In PC12 cells, NGF induces strong, sustained expression of p35 through activation of the ERK pathway and inhibition of Cdk5 either with its pharmacological inhibitor Roscovitine or by transfection with dominant negative Cdk5 blocks neurite outgrowth induced by NGF (Harada et al., 2001). Soluble GFRα1, a GPI-anchored receptor for GDNF, prolongs GDNF-mediated

activation of Cdk5, an event required for GFRα1-induced neurite outgrowth and axon guidance in sensory and sympathetic neurons (Ledda et al., 2002). Inhibition of Cdk5 activity either by Roscovitine or with a dominant negative Cdk5 caused a dramatic decrease in retinoic acid -induced neurite outgrowth in human neuroblastoma SK-N-BE (2) C cells (Lee and Kim, 2004). In Paju cells, another human neuroblatoma cell line, small interfering RNA targeting Cdk5 abrogates interferon γ (IFN-γ)-induced neurite outgrowth (Song et al., 2005). Cdk5 has been also reported to be involved in BDNF-stimulated dendritic growth in hippocampal neruons (Cheung et al., 2007). Importantly, Cdk5 protein levels and kinase activity are elevated in regenerating axons of facial motor neurons after nerve crush (Namgung et al., 2004). Administration of Cdk5 inhibitors, Roscovitine or Olomoucine, into the crushed nerves results in retardation of nerve fiber regrowth (Namgung et al., 2004).

Other roles of Cdk5 include the regulation of synaptic transmission, neurodegenerative disease and neuronal survival (Dhavan and Tsai, 2001; Cheung and Ip, 2004). Several synaptic proteins such as synapsin 1, MUNC18, amphiphysin, PSD-95 and NMDA receptors have been shown to be Cdk5 substrates (Matsubara et al., 1996; Shuang et al., 1998; Fletcher et al., 1999; Floyd et al., 2001; Wang et al., 2003; Morabito et al., 2004). The increased sensitivity of p35 knockout mice to seizure also indicates that Cdk5 is involved in synaptic functions (Chae et al., 1997). Transgenic mice studies have shown that aberrantly increased Cdk5 activity tiggers pathological events leading to neurodegeration and neurofibrillary tangles (Cruz et al., 2003). $Cdk5^{-/-}$ mice show perinatal lethality as well as swelling of cell soma and nuclear margination

in the brainstem and spinal cord neurons, suggesting that Cdk5 actitvity is indispensable for neuronal survival during development (Ohshima et al., 1996).

Although Cdk5 has been implicated in a plethora of processes in the nervous system with major roles in neuronal migration, axon guidance and neurite outgrowth, whether it is involved in axonal regeneration in the CNS has not been addressed previously. In this study we sought to investigate whether Cdk5 plays a role in overcoming inhibition by MAG/myelin.

3.2 Results

Cdk5 Activity is Required for both db-cAMP and Putrescine to Overcome Inhibition by MAG

Previously we showed that elevation of cAMP with analogues such as db-cAMP could overcome inhibition by MAG and myelin (Cai et al., 1999). To investigate whether Cdk5 activity has a role in overcoming inhibition, a specific Cdk5 inhibitor Roscovitine was used in the neurite outgrowth assay. Figure1B and 1C show that consistent with results we reported before, for cerebellar and DRG neurons, MAG inhibits neurite outgrowth by about 70% and 40%, respectively; db-cAMP blocks inhibition by MAG. In contrast, however, in the presence of Roscovitine, the ability of db-cAMP to overcome inhibition is abolished and MAG still inhibits neurite outgrowth. Roscovitine alone has no effect on the neurite outgrowth of neurons growing on both control and MAG-expressing CHO cells, indicating the basal neurite outgrowth does not require

Cdk5 activation. These results indicate that the effect of db-cAMP depends on Cdk5 activity suggesting that Cdk5 serves as a downstream effector of the cAMP signaling pathway.

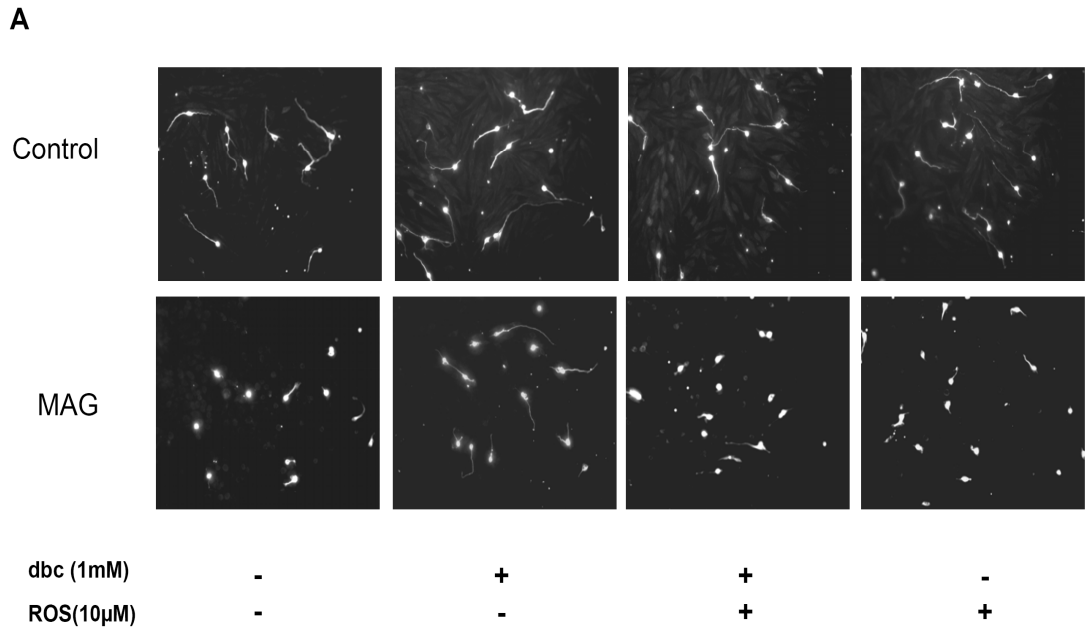


Figure 1A. Inhibition of Cdk5 activity abrogates the effect of dbcAMP in overcoming inhibition by MAG
Cerebellar neurons were isolated and added directly to MAG-expressing CHO cells or control CHO cells in the presence of db-cAMP (dbc), after which they were cultured overnight, fixed and immunostained with β-III-tubulin. Representative images of cerebellar neurons grown on CHO monolayers under various conditions as indicated.

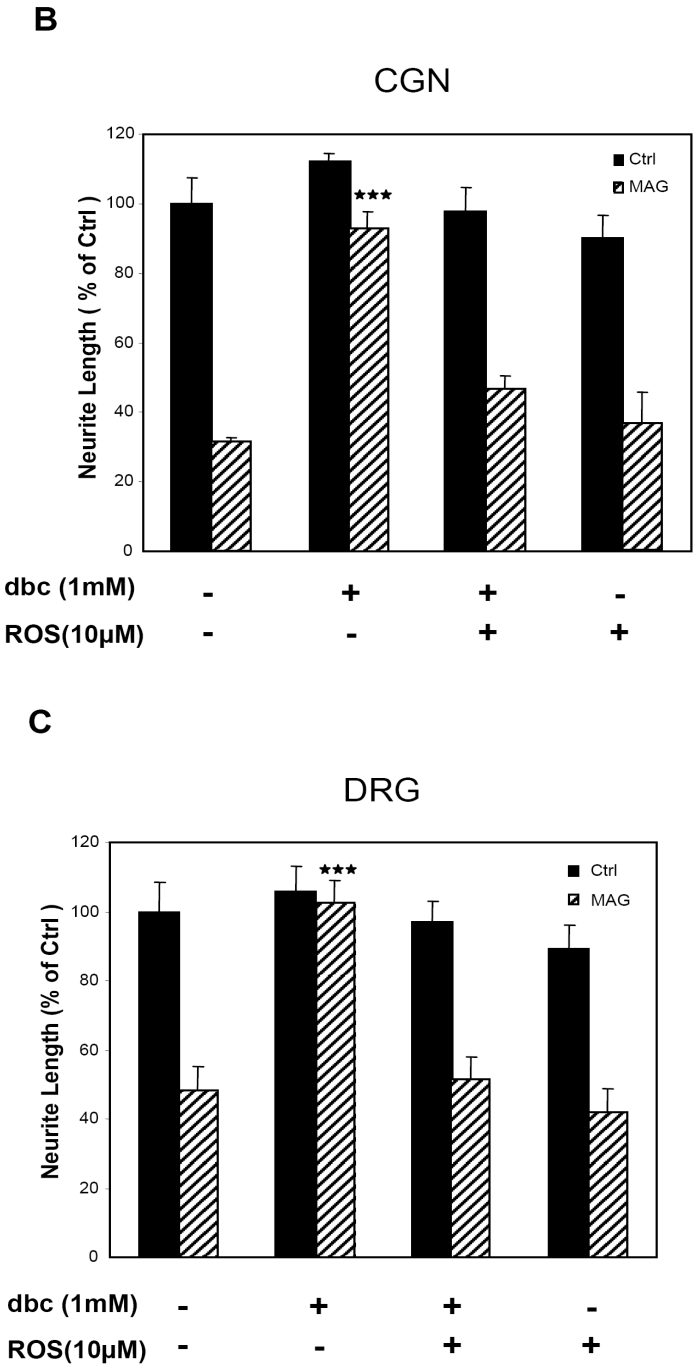


Figure 1B and 1C. Inhibition of Cdk5 activity abrogates the effect of dbcAMP in overcoming inhibition by MAG and myelin Cerebellar or DRG neurons (P5-P7) were isolated and added directly to MAG-expressing CHO cells or control CHO cells in the presence of db-cAMP (dbc), with or without Roscovitine (Ros) as indicated , after which they were cultured overnight, fixed and immunostained with β-III-tubulin. The mean length of the longest neurite for 200-400 neurons was measured (±SEM). (***P<0.001, compared with neurite length on MAG-CHO cells, N=3).

We also showed previously that a consequence of elevated cAMP is increased expression of ArgI and increased synthesis of polyamines and that priming neurons with the polyamine putrescine blocks inhibition by MAG and myelin (Cai et al., 2002). We also showed that putrescine must be converted to spermidine, which is available readily in cells to bring about these effects and spermidine is sufficient to promote optic nerve regeneration in vivo (Deng et al., 2009). Now we wanted to determine if activation of Cdk5 by db-cAMP is upstream or downstream from the increased synthesis of polyamines. We used putrescine throughout these experiments because it is more stable on the bench than spermidine. We primed cerebellar neurons with putrescine in the presence or absence of Roscovitine overnight before plating on control or MAG-expressing CHO cells. Figure 1D shows that putrescine overcomes inhibition by MAG, Roscovitine also abrogates the priming effect of putrescine. Similar results were found when DRG neurons were primed with putrescine and Roscovitine before being transferred on MAG or myelin (Figure 1E and 1F). These results demonstrate that activation of Cdk5 is downstream from elevation of polyamines in response to db-cAMP and that Cdk5 activity is required for polyamines to overcome inhibition by MAG and myelin.

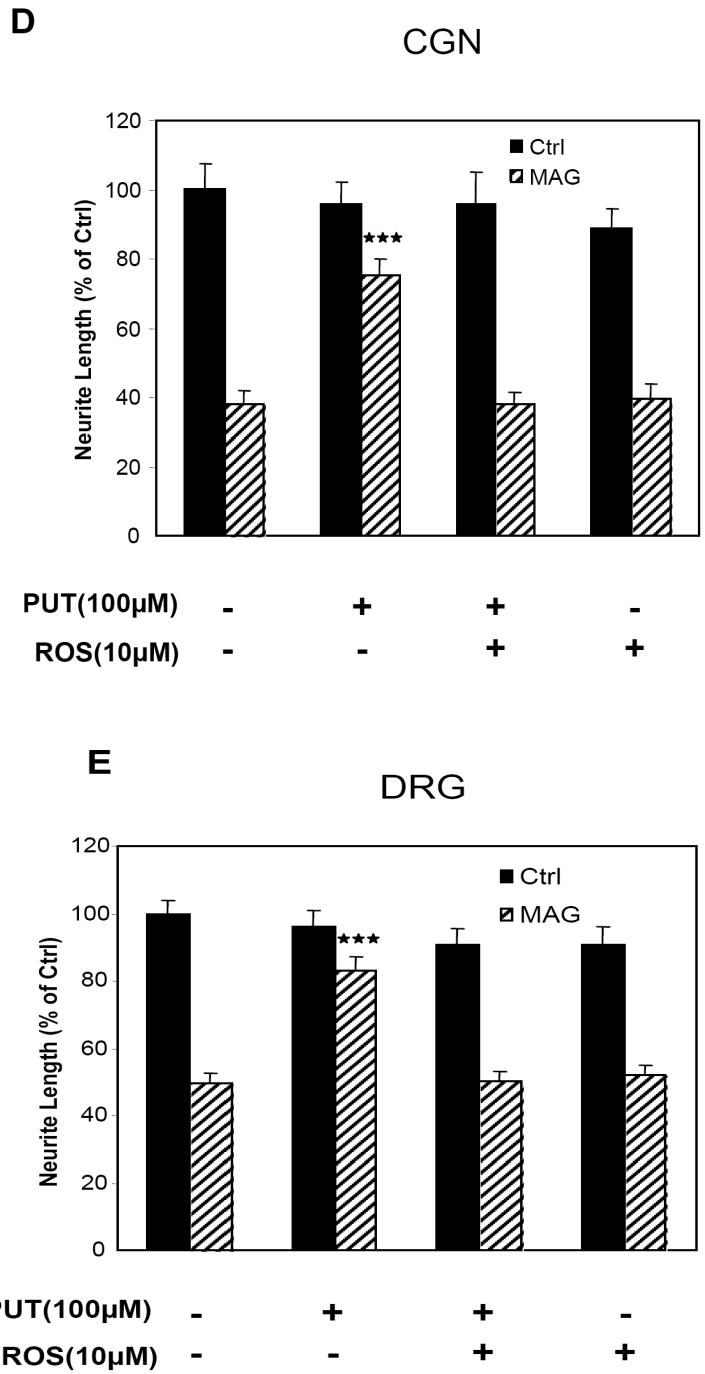


Figure1D and 1E. Inhibition of Cdk5 activity abrogates the effect of putrescine in overcoming inhibition by MAG Cerebellar or DRG neurons (P5-P7) were isolated and primed overnight with putrescine (PUT) in the presence or abasence of Roscovitine (Ros) as indicated. Neurons were then transferred onto MAG-expressing CHO cells or control CHO cells and cultured overnight, after which they were fixed and immunostained with β-III-tubulin. The mean length of the longest neurite for 200-400 neurons was measured (±SEM). (***P<0.001, compared with neurite length on MAG-CHO cells, N=3).

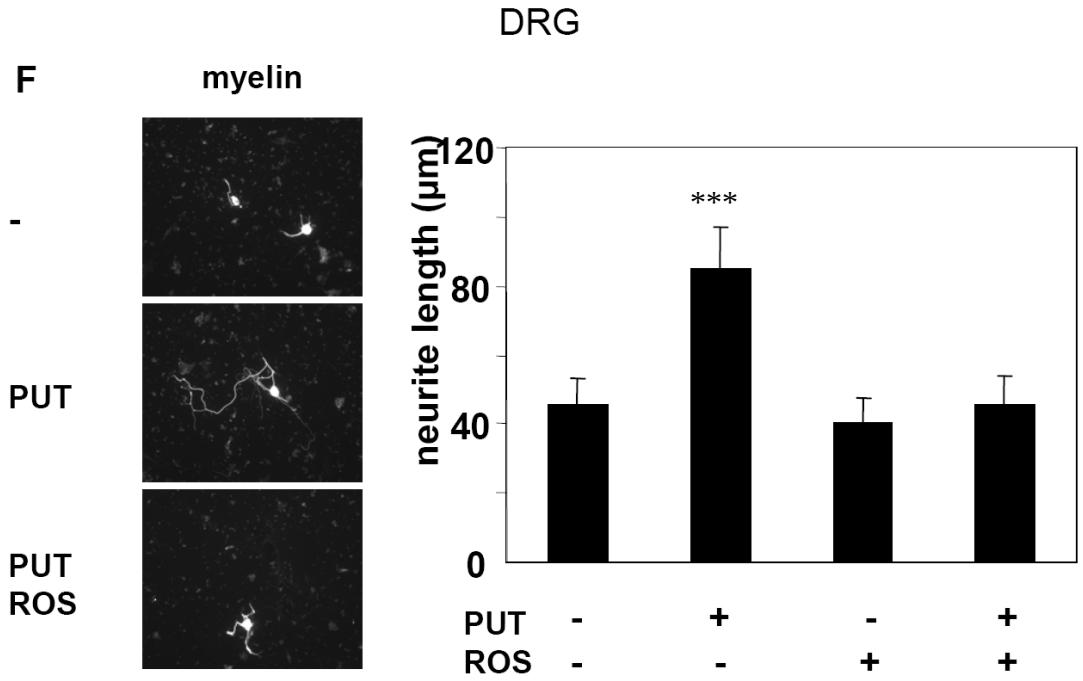


Figure 1F. **Inhibition of Cdk5 activity abrogates the effect putrescine in overcoming inhibition by myelin**

DRG neurons (P5-P7) were isolated and primed overnight with putrescine (PUT) in the presence or abasence of Roscovitine (Ros) as indicated. Neurons were then transferred onto myelin and cultured overnight, after which they were fixed and immunostained with β-III-tubulin. The mean length of the longest neurite for 200-400 neurons was measured (±SEM). (***P<0.001, compared with neurite length on all other three treatments, N=3).

To further confirm that Cdk5 activity is necessary for db-cAMP and putrescine to overcome the inhibitory effect of MAG, we used herpes simplex viruses (HSVs) containing GFP-linked dominant negative Cdk5 (DNK5), p35 or lacZ gene. We infected DRG neurons with these HSVs overnight and then performed neurite outgrowth assays. Meanwhile, we analysed the lysates by western blotting with antibodies to each gene product to ensure robust expression (Figure 2B). In contrast to HSV-lacZ infected neurons (control) where db-cAMP or putrescine blocks inhibition, HSV-DNK5 infected neurons are unable to overcome inhibition by MAG even if db-cAMP or putrescine is added (Figure2C and 2D). This is consistent with the effect of the Cdk5 inhibitor Roscovitine. Importantly, neurons over-expressing p35 are not inhibited by MAG (Figure 2C). These results not only further confirm that Cdk5 activity is required to overcome inhibition, but also suggest that activation of Cdk5 by p35 is sufficient to change the neuron's response to MAG such that it is no longer inhibited.

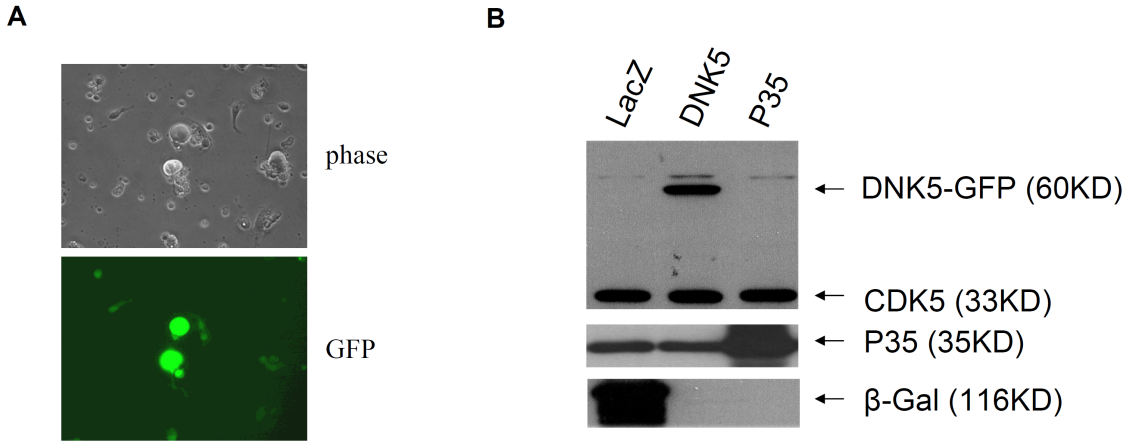


Figure 2A and 2B. Cdk5 activity is required in overcoming inhibition by MAG and over-expression of p35 blocks the inhibitory effect of MAG
Expression of DNK5, constitutively active p35 and β-Gal after HSV infection. Twenty-four hours after infection, the neurons were lysed and subjected to Western blotting.

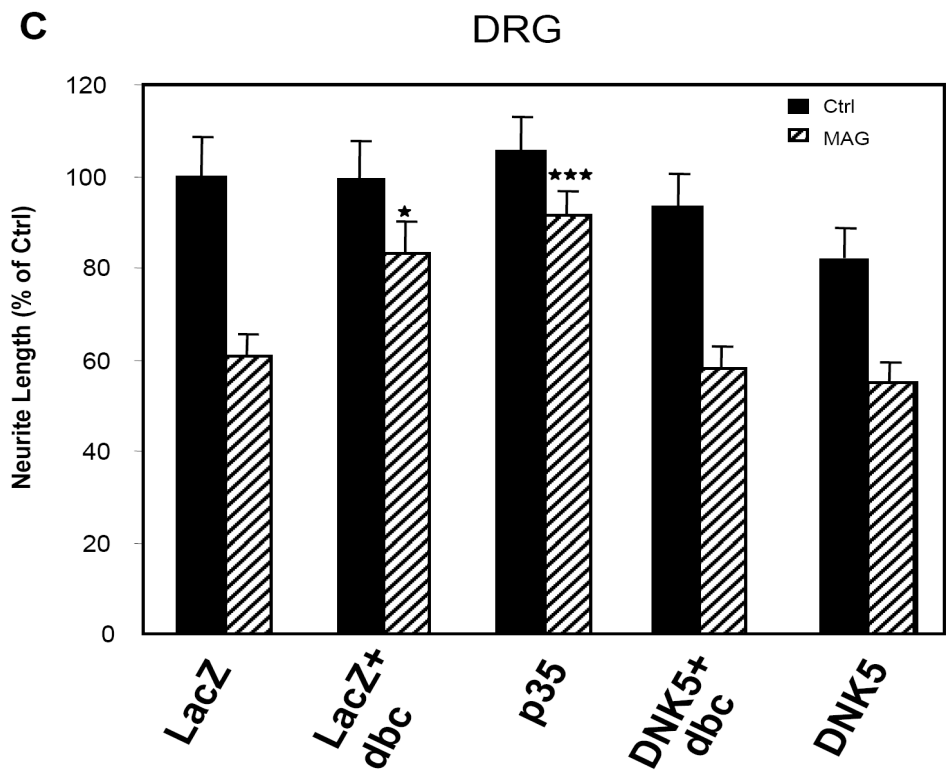


Figure 2C. Cdk5 activity is required in overcoming inhibition by MAG and over-expression of p35 blocks the inhibitory effect of MAG

Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from DRG neurons (P5) infected with HSV-lacZ, HSV-p35 or HSV-DNK5 in the presence or absence of dbc-AMP (dbc), as indicated. The mean length of the longest neurite for 180 to 200 neurons was measured (±SEM). (***P<0.001, *P<0.05, compared with neurite length on MAG-CHO cells, N=3).

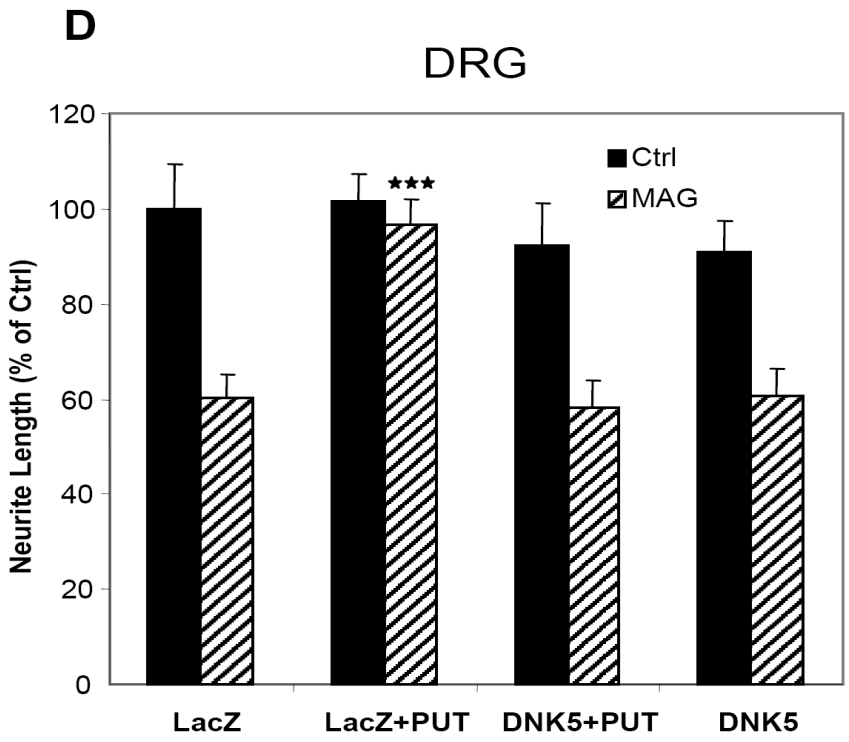


Figure 2D. Cdk5 activity is required for putrescine to overcome inhibition by MAG
Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from DRG neurons (P5) infected with HSV-lacZ, HSV-p35 or HSV-DNK5 in the presence or absence of putrescine (PUT). The mean length of the longest neurite for 180 to 200 neurons was measured (±SEM). (***P<0.001, compared with neurite length on MAG-CHO cells, N=3).

3.3 Discussion

In this chapter, we present data which suggest a role for Cdk5 in the cAMP and polyamine-mediated ability to overcome inhibition by MAG and myelin. We show that blocking Cdk5 activity either through its specific inhibitor Roscovitine or by a dominant negative mutant Cdk5 (DNK5) abolishes the ability of db-cAMP and the polyamine putrescine to overcome inhibition in both cerebellar and DRG neurons. In addition, we show that when p35 is over-expressed, DRG neurons are no longer inhibited by MAG. These results demonstrate that activation of Cdk5 is necessary for both db-cAMP and putrescine to overcome inhibition and that activation of Cdk5 by over-expression of p35 is sufficient to block inhibition. To our knowledge, this is first evidence that links the polyamines to Cdk5 activity, pointing out a possible mechanism for polyamines to overcome inhibition by MAG and myelin. However, this is not surprising since Cdk5 is a multi-functional protein that has been implicated in axon guidance, neuronal migration and neurite outgrowth.

How does Cdk5 exert its effect on overcoming inhibition? The answer may lie in the specific substrate(s) that Cdk5 targets. Cdk5 phosphorylates many proteins and regulates the organization of all three cytoskeleton elements: microfilaments, microtubules (MT) and intermediate filaments (Dhavan and Tsai, 2001).

Cdk5 substrates include many proteins related to actin dynamics, such as CaMKII, Cables, p21-activated kinase 1 (PAK1), amphiphysin and synapin I (Matsubara et al., 1996; Nikolic et al., 1998; Zukerberg et al., 2000; Floyd et al., 2001; Dhavan et al., 2002). Members of the Rho family GTPases are key regulators of the actin cytoskeleton and are directly implicated in axon guidance, neurite outgrowth and

cell motility (Hall, 1998; Luo, 2000). PAK1 is present in the Rac-p35/Cdk5 complexes and becomes phosphorylated and inhibited by Cdk5/p35 in a Rac-dependent manner. This phosphorylation of PAK1 by Cdk/p35 is likely to have an impact on the dynamics of the reorganization of the actin cytoskeleton in neurons, promoting neuronal migration and neurite outgrowth (Nikolic et al., 1998). WASP (Wiskott-Aldrich syndrome protein)-family verprolin-homologous protein (WAVE) subfamily WAVE1 and WAVE2 have been recently identified as Cdk5 substrates (Kim et al., 2006; Miyamoto et al., 2008). WAVE proteins constitutively regulate the formation of lamellipodia and membrane ruffling through the Rho family small GTPase Rac (Miki et al., 1998). Cdk5 phosphorylates WAVE1 at multiples sites, resulting in an increase in dendritic spine formation in hippocampal neurons (Kim et al., 2006). Cdk5 phosphorylation of WAVE2 mediates oligodendrocyte precursor cell migration induced by PDGF (Miyamoto et al., 2008). It is possible that Cdk5 overcomes inhibition by regulating these proteins involved in actin dynamics.

Like actin filaments, microtubule dynamics are influenced by their associated proteins. Cdk5 is abundant in microtubule preparations and can be purified from bovine brain (Ishiguro et al., 1992; Sobue et al., 2000). Several microtubule-associated proteins (MAPs) including MAP2, MAP1B and Tau are substrates of Cdk5 (Paglini and Caceres, 2001). Phosphoryalation of MAP1B by Cdk5 in cerebellar macroneurons is related to neurite extension induced by laminin (Pigino et al., 1997; Paglini et al., 1998). MAP1B phosphorylation by Cdk5 is required for Netrin 1-mediated chemoattration and migration in neurons (del Rio et al., 2004). Cdk5 plays a crucial role in elongation and maintenance of axons as well as stability and steering of the growth cone by

phosphorylating MAP1B (Hahn et al., 2005). Cdk5 phosphorylates Tau, a neuron-specific MAP, reduces the binding of Tau to microtubules and inhibits microtubule assembly (Dhavan and Tsai, 2001). Interestingly, in cerebellar neurons, phosphorylated MAP1B, but not phosphorylated Tau, goes up after putrescine treatment and Roscovitine blocks this MAP1B phosphorylation, indicating that Cdk5 can differentially phosporylate its substates to exert its effects and MAP1B might be one of the targets necessary for putrescine-induced ability to overcome inhibition (He and Filbin, unpublished data). In addition, Cdk5 phosphorylates Nudel, a protein that associates with dynein. Cdk5 phosphorylation of Nudel could regulate specific dynein functions that play a role in neuronal migration and axon growth (Niethammer et al., 2000). Focal adhesion kinase (FAK) is also a physiological substrate of Cdk5 (Xie et al., 2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization and neuronal migration (Xie et al., 2003). Furthermore, Doublecortin (Dcx) is phosphorylated by Cdk5 and this phosphorylation regulates neuronal migration through an effect on microtubule dynamics (Tanaka et al., 2004). Although migration and axon outgrowth are not the same processes, the mechanisms underlying them can be shared. Thus, Cdk5 may overcome inhibition by MAG via regulating the functions of any of these proteins.

Cdk5 also phosphorylates the intermediate and heavy chain of neurofilaments (NFM and NFH) *in vitro* (Li et al., 2000). It has been proposed that phosphorylation of the NFH and NFM tail domains increase the total negative charges and the lateral extension of neurofilament side arms resulting in increased neurofilament spacing and axonal caliber (Li et al., 2000). The role of neurofilament phosphorylation in axonal

outgrowth is not clear. However, it may help stabilize axonal structures (Shea and Beermann, 1994).

The importance of Cdk5 in the regulation of the cytoskeleton is also emphasized by the studies that link Cdk5 to several diseases, including Alzheimer's disease (Ahlijanian et al., 2000; Lee et al., 2000), amyotrophic lateral sclerosis (Nguyen et al., 2001), and Parkinson's disease (Brion and Couck, 1995). Cdk5 activity is associated with hyperphosphorylation of tau or neurofilament proteins in these diseases. However, in all these cases, Cdk5 is de-regulated and hyperactivated probably due to the cleavage of p35 into p25 and mislocation of p25 in the cells (Dhavan and Tsai, 2001), which we will discuss in more detail in the next chapter.

It should also be noted that Goshima's group has reported that Cdk5 is involved in Sema3A and ephrin-A5 signaling (Sasaki et al., 2002; Cheng et al., 2003; Uchida et al., 2005). They showed that Fyn, a member of the Src family of nonreceptor tyrosine kinase, increases Cdk5 activity via Tyr15 phosphorylation and thus mediates Sema3A-induced growth cone collapse in DRG neurons (Sasaki et al., 2002). They further showed that collapsin response mediator protein-2 (CRMP2), a phosphoprotein involved in microtubule dynamics (Gu and Ihara, 2000; Fukata et al., 2002), is the Cdk5 target that is required for Sema3A signaling (Uchida et al., 2005). In another paper, they reported that ephrin-A5 induces phosphorylation of Cdk5 at Tyr15 thereby increasing Cdk5 activity, which in turn phosphorylates Tau leading to growth cone collapse in RGCs (Cheng et al., 2003). Additionly, Ip's group reported that Cdk5 phosphorylation at Tyr15 regulates ephrin-A1- EphA4-mediated dendritic spine retraction by stimulating ephexin's activity towards RhoA (Fu et al., 2007). Therefore,

activation of Cdk5 which modulates cytoskeletal dynamics, can also result in growth growth cone collapse. This seems to be contradictory to the general role of Cdk5 in the neurite outgrowth, but several reasons can account for this discrepancy. First, growth cone collapse is acute occurring within minutes and activation of Cdk5 is transient and restricted to certain areas of the growth cones, for example, partially collapsed, but not fully extended, growth cones (Sasaki et al., 2002). Second, Sema3A and ephrin can only transduce their signals to Cdk5 after activation of Src family kinases or EphA tyrosine kinases, indicating Tyr15 phosphorylation is important for the signaling. In addition to facilitating Cdk5 activity, phosphorylated Tyr15 may regulate Cdk5 subcellular localization thereby regulating specific substrates for growth cone collapse. Moreover, in these studies, Cdk5 is not the only pathway that is required. It may be in concert with other effectors such as Rho kinase to regulate cytoskeletal reorganization (Cheng et al., 2003). Therefore, depending on the ligand-receptor interaction, Cdk5 can mediate different signaling cascades for axon guidance.

Future studies are needed to identify the target(s) of Cdk5 activated by cAMP and polyamines to overcome inhibition. But another important question raised immediately in this chapter is: how is Cdk5 activity regulated? Currently, this is not well understood because most studies have been focused on the downstream targets of Cdk5, as discussed above. The elucidation of how cAMP and polyamines activate Cdk5 is the subject of the following chapters of study.

Chapter IV: cAMP and Putrescine Increase Cdk5 Activity by Up-regulating p35 levels

4.1 Introduction

From the results of chapter III, we know that the Cdk5 activity is required for db-cAMP and polyamines to overcome inhibition by MAG and myelin. In addition, overexpression of p35 is sufficient to block the inhibitory effect of MAG. Therefore, db-cAMP and polyamines must activate Cdk5 to overcome inhibition. In this chapter we will elucidate how cAMP and polyamines lead to increased Cdk5 activity.

The upregulation of Cdk5 activity can be achieved through upregulation of Cdk5 levels itself or through elevation of p35 levels. The increase in Cdk5 levels has been implicated in the dopaminergic signaling pathway (Bibb et al., 2001). Administration of repeated electroconvulsive seizures (ECS), an effective antidepressant treatment, induces transcription factor δFosB expression resulting in increased Cdk5 expression (Chen et al., 2000). Chronic cocaine treatment also increases the levels of δFosB and Cdk5 (Bibb et al., 2001). In addition, overexpression of δFosB in stable C6 glioma cells induces expression from the Cdk5 promotor (Chen et al., 2000).

p35 is the major neuronal-specific Cdk5 regulator which activates Cdk5 upon direct binding (Lew et al., 1994; Tsai et al., 1994). It has been reported that laminin stimulates p35 expression, increases Cdk5 activity and accelerates the redistribution of Cdk5/p35 to the axonal growth cone to induce axon elongation (Pigino et al., 1997; Paglini et al., 1998). Li et al showed that laminin interaction with the integrin receptor $\alpha_1\beta_1$ induced upregultion of p35 and activation of Cdk5 leading to neurite outgrowth in differentiated human SH-SY5Y cells (Li et al., 2000), but how the integrin receptor upregulates p35 is not clear. Harada et al showed that in PC12 cells, NGF induced

strong, sustained expression of p35 through activation of the ERK/Egr1 pathway. They also showed that treatment of cerebellar granule cells from neonatal rats with BDNF for 24 hrs activated ERK, increased the amounts of p35 mRNA and protein and the kinase activity of Cdk5 (Harada et al., 2001). In addition, Desbarats et al showed that crosslinking Fas, which is also known as CD95 or Apo-1, a member of the tumor-necrosis factor receptor (TNFR) superfamily, on DRG neurons induced neurite outgrowth through sustained activation of the ERK pathway and the consequent upregulation of p35 (Desbarats et al., 2003). Lee and Kim showed that retinoic acid treatment of SK-N-BE (2) C human neuroblastoma cells increased expression of p35 through ERK pathway (Lee and Kim, 2004). Finally, Song et al showed that IFN-γ induced neurite outgrowth in human neuroblastoma Paju cells by up-regulation of p35 which is via activation of ERK pathway (Song et al, 2005).

The activity of Cdk5 can also be regulated by phosphorylation (Dhavan and Tsai, 2001; Smith and Tsai, 2002). Tyr15 on Cdk5 can be phosphorylated by the non-receptor tyrosine kinase c-Abl, and this phosphorylation of Cdk5 is stimulatory and increases Cdk kinase activity (Zukerberg et al., 2000). Fyn, a member of the Src family of nonreceptor tyrosine kinases, also can facilitate Cdk5 activity mediated through Tyr15 phosphorylation of Cdk5 (Sasaki et al., 2002; Miyamoto et al., 2008). One report suggests that phosphorylation of Ser159 on Cdk5 augments Cdk5 activity (Sharma et al., 1999), but several reports indicate that this phosphorylation is dispensable (Qi et al., 1995; Poon et al., 1997; Tarricone et al., 2001).

Given the role of Cdk5 in the cAMP/polyamines pathway that we report in Chapter III, it is necessary to know how dbcAMP and polyamines regulate Cdk5

activity. Therefore, in this chapter, we will carry out experiments to see whether dbcAMP and putrescine increase Cdk5, p35 and phosphoratled Tyr 15 levels.

4.2 Results

dbcAMP and Putrescine Increase Cdk5 activity by upregulating p35 levels

To determine if the levels of p35 protein increase in response to db-cAMP and polyamines, cerebellar neurons were treated with dbcAMP or putrescine for different periods as indicated before being lysed and subjected to western blot. Figure 3A shows that the p35 protein levels increase within 3 hours following dbcAMP treatment and persist for up to 24 hours, while Cdk5 protein levels remain constant. More importantly, treatment of neurons with putrescine leads to dramatic expression of p35. Within 1 hour, putrescine induces 1.5-fold increase in p35 levels and by 3 hours after treatment, more than 2-fold increase is observed and sustained overnight, whereas levels of expression of Cdk5 are unchanged in the same neurons treated with putrescine for the same times (Figure3B).

We then immunoprecipitated endogenous Cdk5 with anti-Cdk5 antibody in cerebellar neurons following treatment of putrescine and performed an *in vitro* kinase assay using Histone H1 as a substrate. Figure 3C shows that after 1 hour of incubation of neurons with putrescine there is an increase in phosphorylation of Histone H1 by the immunoprecitpated Cdk5. After 3 hours of incubation, this phosphorylation is greater after which it plateaus but is sustained overnight as well. The kinase assay further verifies that putrescine indeed activates Cdk5 and it increases Cdk5 activity to a similar

extent as the upregulation of p35 (Figure 3C), which is in agreement with the previous finding that Cdk5 activity is dependent on its activator, p35 (Nikolic et al., 1996; Chae et al., 1997; Harada et al., 2001). These results not only demonstrate that cAMP and polyamines can enhance Cdk5 activity, but also indicate that polyamines are more efficient to activate Cdk5 and therefore might be more specific targets than cAMP to encourage regeneration.

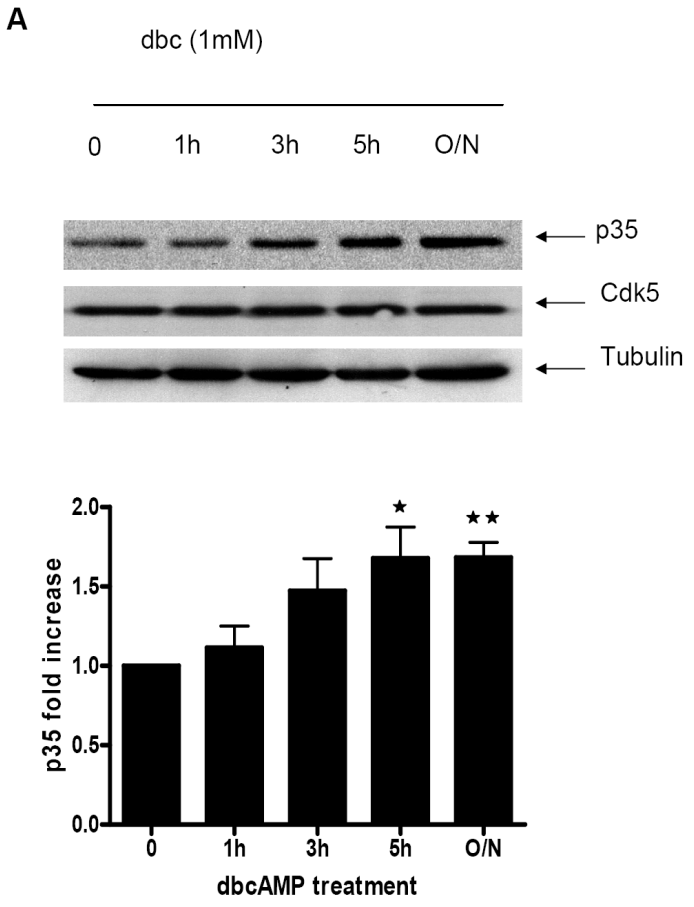


Figure 3A. **dbcAMP increases p35 protein levels** Cerebellar neurons (P5) were treated with dbcAMP for different time periods as indicated. Lysates were immunoblotted against p35, Cdk5 and tubulin as loading control. Lower panel: Statistic analysis from 3-5 experiments. (**p<0.01, *p<0.05, compared with control)

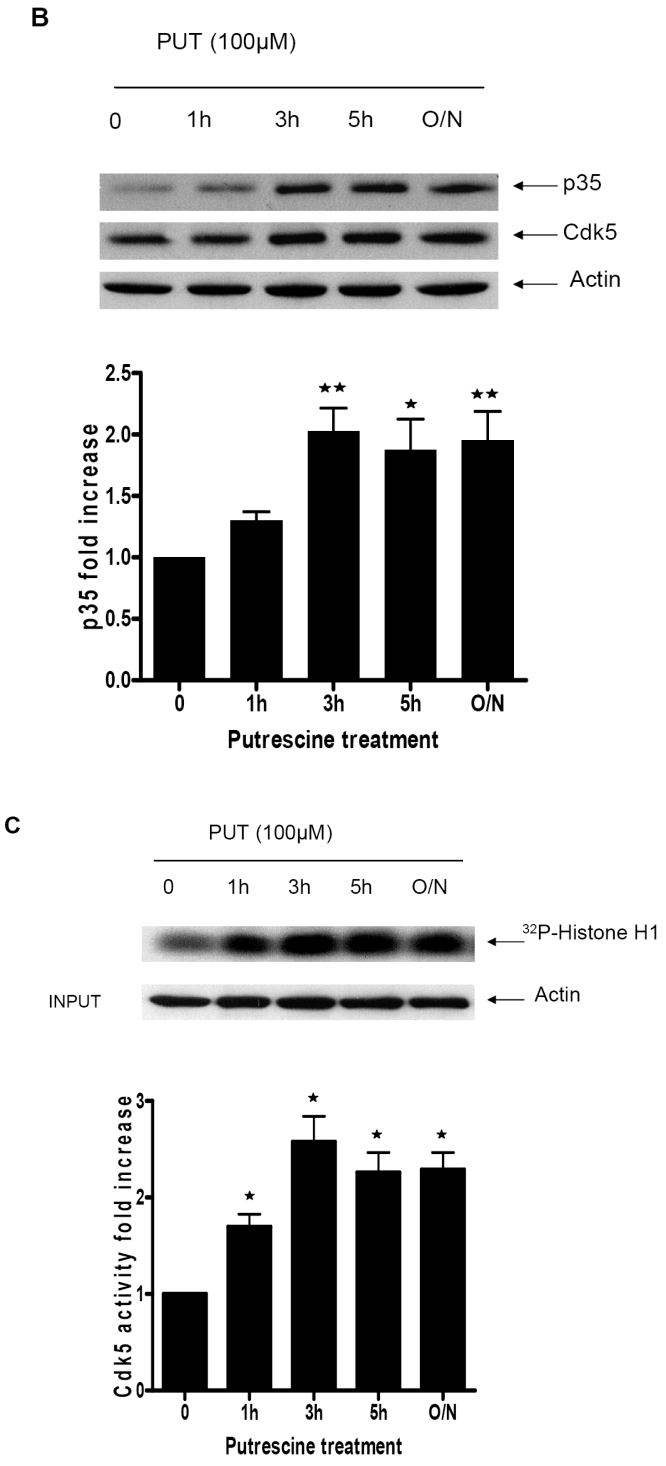


Figure 3B and 3C. **Putrescine increases Cdk5 activity by up-regulating p35 levels** (B) Cerebellar neurons (P5) were treated with putrescine for different time periods as indicated. Lysates were immunoblotted against p35, Cdk5 and actin as loading control. (C) Lysates from cerebellar neurons (P5) were immunoprecipitated with Cdk5 antibody and subjected to *in vitro* kinase assay using histone H1 as a substrate. Lower panel: Statistic analysis from 3-5 experiments. (**p<0.01, *p<0.05, compared with control)

p35 Protein is Distributed to Neurites and Growth Cones by Putrescine Treatment

It has been shown that in rat E17-18 cortical neurons, the subcellular localization of p35 was similar to that of Cdk5 with staining in soma and the entire length of neurites (Nikolic et al., 1996). To assess if putrescine can influence the distribution of p35 protein, we stained the P1 cortical neurons for p35 and Cdk5 after treatment of neurons with or without putrescine for 20 hours. We also used cortical neurons because they extend well defined processes and growth cone in culture. Figure 4 shows that p35 is clearly visible in the cell bodies but barely detectable in the neurites and growth cones of control neurons, not treated with putrescine. Cdk5 is present in both cell bodies and neurites in these control neurons. Following putrescine treatment, however, p35 staining is markedly up-regulated in neurites (arrowheads) with staining more evident in the growth cones (arrows). Cdk5 is colocalized with p35 and its staining is unchanged by putrescine treatment. These results not only confirm the up-regulation of p35 by putrescine, but also indicate that putrescine can increase the distribution of p35 to neurites and growth cones where it can interact with and activate resident Cdk5 to affect growth.

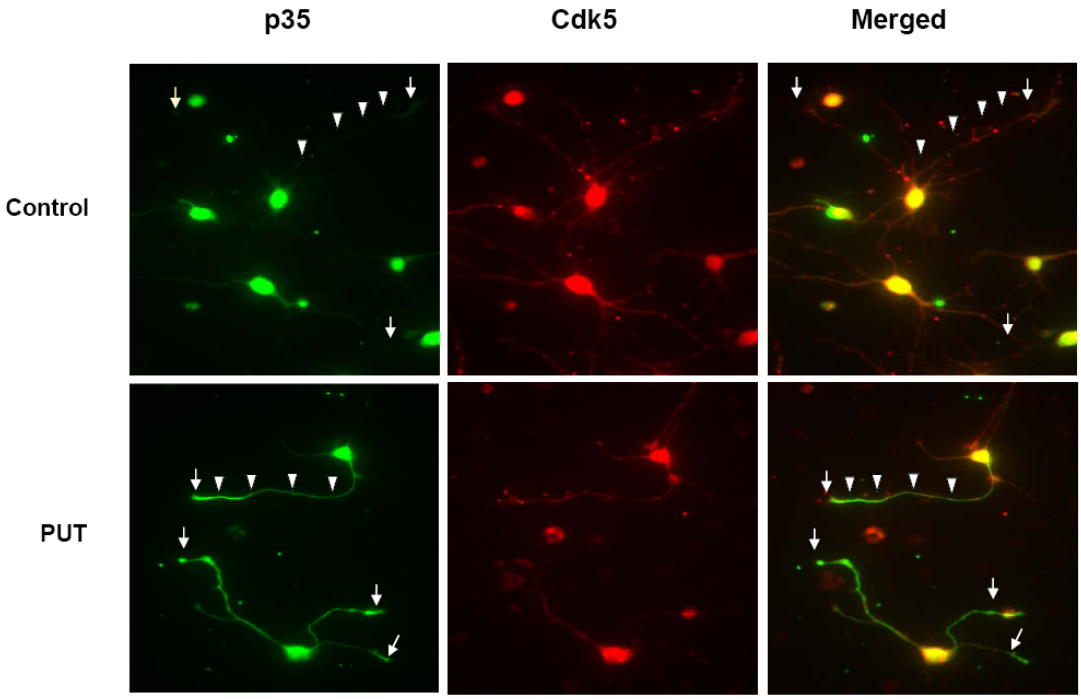


Figure 4. p35 protein is distributed to neurites and growth cones by putrescine treatment Cortical neurons (P1) were treated with or without putrescine (PUT, 100 μM) overnight. Neurons were fixed and immunostained with anti-p35 and anti-Cdk5 antibodies (arrow heads: neurites; arrows: growth cone).

4.3 Discussion

In this chapter, we show that db-cAMP and the polyamine putrescine, putrescine in particular, activate Cdk5 by upregulating expression of p35 protein, but not expression of Cdk5 protein itself. We show this through both western blot and immunostaining. db-cAMP increases p35 within 3 hours while putrescine treatment results in p35 expression even faster (within 1hour) and more dramatically, which is consistent with the report that putrescine acts downstream from cAMP in overcoming inhibition (Cai et al., 2002). This is the first time to demonstrate that putrescine increases p35 expression. As described in the introduction, very few extracellular stimuli have been reported to increase p35 protein levels, now putrescine is added to the list: laminin, NGF, BDNF, Fas, Retinoic acid, IFN-γ and putrescine, all of which are shown to increase neurite growth. Interestingly, among these, ERK/Egr1 is the only known pathway to upregulate p35. This raises an important question: does putrescine upregulate p35 through the same pathway? If not, what would be the mechanism for putrescine to induce p35 expression?

Although Tyr15 phosphorylation on Cdk5 is a common mechanism for facilitation of Cdk5 activitiy, we did not detect any changes of p-Tyr15 levels of Cdk5 upon putrescine treatment in cerebellar neurons (data not shown), indicating activation of Cdk5 by putrescine is not through Tyr15 phosphorylation. Indeed, putrescine has not been shown to associate with any tyrosine kinase.

p39 is an isoform of p35, which shows 57% amino acid sequence identity to p35 (Tang et al., 1995). Like p35, p39 associates with and activates Cdk5 (Tang et al., 1995; Humbert et al., 2000). However, most studies have been performed with Cdk5 complexed with p35 and little is known about the functions of p39. p39 deficient mice lack obvious

phenotype (Ko et al., 2001), indicating that p35 can compensate for the function of p39. Moreover, during development, the kinase activity of Cdk5 correlates well with the expression pattern of p35, but not with that of p39 (Wu et al., 2000). So far, no stimulus except for retinoic acid (but only after 21 days together with aggregation) has been reported to upregulate p39 (Fu et al., 2002). We did not check whether the levels of p39 are altered by putrescine due to unavailability of p39 antibodies. However, the fact that putrescine upregluates p35 and overexpresson of p35 in neurons is sufficient to overcome inhibition by MAG clearly demonstrates that it is p35 that functions in this paradigm to activate Cdk5 thereby affecting neurite outgrowth on the inhibitory substrate.

p25 is a proteolytic product of p35 by calpain (Kusakawa et al., 2000; Lee et al., 2000). It contains all the elements necessary for Cdk5 binding and activates Cdk5 *in vitro* and *in vivo* (Qi et al., 1995; Poon et al., 1997). Intertestingly, p25 has a substantially longer half-life than p35, which is very unstable with a half life of 20-30 min (Patrick et al., 1998). In addition, p25 lacks the amino-terminal myristoylation site of p35 and is concentrated in the cell body and nucleus, while p35 is present in the membrane. Therefore, generation of p25 is believed to disrupt the normal regulation of Cdk5 causing hyperactivation and mislocation of Cdk5 (Dhavan and Tsai, 2001). It has been reported that p25 is produced only by neurotoxic insults, H_2O_2, glutamate, maitotoxin and ionomycin treatments and that p25 levels are elevated in some neurodegenerative diseases (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000; Nguyen et al., 2001). In our experiments, treatment of neurons with putrescine did not produce p25 as we used a p35 antibody that recognizes p25 as well, but we did not see the 25kD band, which also indicates putrescine is not neurotoxic. With the increase in p35 levels following

putrescine treatments, neurons are in a good intrinsic state, thereby overcoming inhibition by MAG and myelin. That is to say, putrescine produces enough p35 protein which recruits and activates Cdk5 in the right place at the right time so that Cdk5 can phosphorylate the right target(s) to overcome the inhibitory substrate. This is in agreement with the report from Desbarats et al showing that Fas engagement induced expression of p35 thereby leading to regeneration in primary neurons and accelerated functional recovery after nerve injury *in vivo* (Desbarats et al., 2003). Although occuring in the PNS, it emphasizes the critical role of p35 in the regeneration.

In summary, results from this chapter together with the previous chapter show for the first time that putrescine activates Cdk5 by upregulating p35 protein levels and this Cdk5 activation is required for overcoming MAG/myelin inhibition in the CNS. Now an important question is: what is the underlying mechanism for putrescine to upregulate p35 protein? In the next chapter, we will sort out the answer for this novel discovery.

Chapter V: Activation of eukaryotic Initiation Factor 5A (eIF5A) is Involved in Putrescine-Mediated Up-regulation of p35 and Overcoming Inhibition by MAG

5.1 Introduction

We have shown in previous chapters that the polyamine putrescine increases expression of p35 protein thereby activating Cdk5 to overcome inhibition by MAG and myelin. Our results, for the first time, demonstrate that putrescine induces p35 expression. Now an immediate question raised is how does putrescine induce p35? Does putrescine do so through the known Erk/Egr1pathway? If not, what is the alternative pathway?

Polyamines have been implicated in many cellular processes including maintenance of DNA structure, RNA processing, transcription and translation (Childs et al., 2003). The polyamine spermidine modifies the eIF5A precursor by adding a 4-aminobutyl group (putrescine group) to form mature hypusinated eIF5A , a process referred to hypusination, and thus mediate the functions of eIF5A (Zanelli and Valentini, 2007). Importantly, in 2007 Huang et al showed that NGF induced expression of ArgI and consequent hypusination of eIF5A in PC12 cells. Prevention of hypusine formation blocked NGF-mediated neurite outgrowth and survival of PC12 cells. Inhibition of hypusine formation also inhibited neuronal process extension in hippocampal neurons (Huang et al., 2007). These findings, together with our previous results that neurotrophins and cAMP overcome inhibition through induction of ArgI with synthesis of polyamines, prompted us to investigate whether eIF5A plays a role in polyamine-induced upregulation of p35 and overcoming inhibition by MAG.

5.2 Results

The Increases in p35 levels in Response to Putrescine is not Dependent on ERK and Transcription, but Dependent on Translation

To see whether putrescine-induced upregulation of p35 is also through the ERK pathway, we assessed whether putrescine induced the activation of ERK in cerebellar neurons. Figure 5A shows that unlike BDNF, which induces robust ERK phosphorylation within half an hour, putrescine does not affect ERK activity at anytime for up to 6 hours while p35 level is steadily upregulated. Importantly, when the MEK inhibitor UO126 is included along with putrescine even for 6 hours, putrescine is still able to upregulate p35 when compared to control or UO126 treatment alone. These results indicate that induction of p35 by putrescine is not through ERK pathway and is likely transcription independent. To test this possibility, we used 5,6-dichloro-1-b-D-ribo-furanosyl-benzimidazole (DRB), an inhibitor of transcription, to assess whether the increased expression of p35 in cerebellar neurons in response to putrescine requires transcription. Figure 5B shows that as reported above, after overnight incubation with putrescine there is a significant increase in p35 protein in cerebellar neurons. However, addition of DRB, at a concentration that is known to block transcription, has no effect on the putrescine-induced increase in p35 levels, suggesting that putrescine stimulates the expression of p35 mainly by a post-transcriptional mechanism. RT-PCR results verify that putrescine treatment has no effect on the total p35 mRNA level in cerebellar neurons (data not shown). We then used a translation inhibitor, cycloheximide, to examine whether it can affect the p35 levels induced by putrescine. Figure 5C shows that cycloheximide not only completely eliminates the p35 protein induced by

putrescine, but also wipes out the basal level of p35. This is consistent with a rapid turnover of p35 protein (Patrick et al., 1998) which must be controlled at the level of translation. These results demonstrate that putrescine increases the levels of p35 in cerebellar neurons in a transcription and Erk-independent, but a translation-dependent manner.

Correlated with western blot results, data from neurite outgrowth assays (Figure5D) show that, as reported before, the effect of dbcAMP in overcoming inhibition is transcription dependent because the transcription inhibitor DRB abolishes its effect. However, DRB at the same concentration is not able to block the putrescine effect suggesting the ability of putrescine to overcome inhibition is transcription independent. In contrast, when we used the translation inhibitor cycloheximide, the priming effect of putrescine is lost, indicating the requirement of translation for putrescine to overcome inhibition.

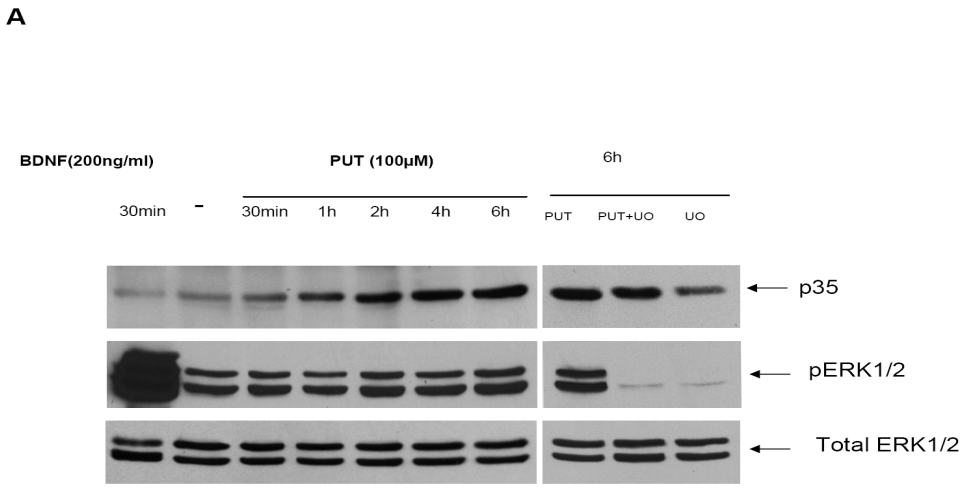


Figure 5A. Putrescine increases p35 protein in an ERK-independent manner Cerebellar neurons (P5) were treated with putrescine and BDNF for different time period, as indicated. Lysates were immunoblotted against phospho-ERK, total ERK and p35.

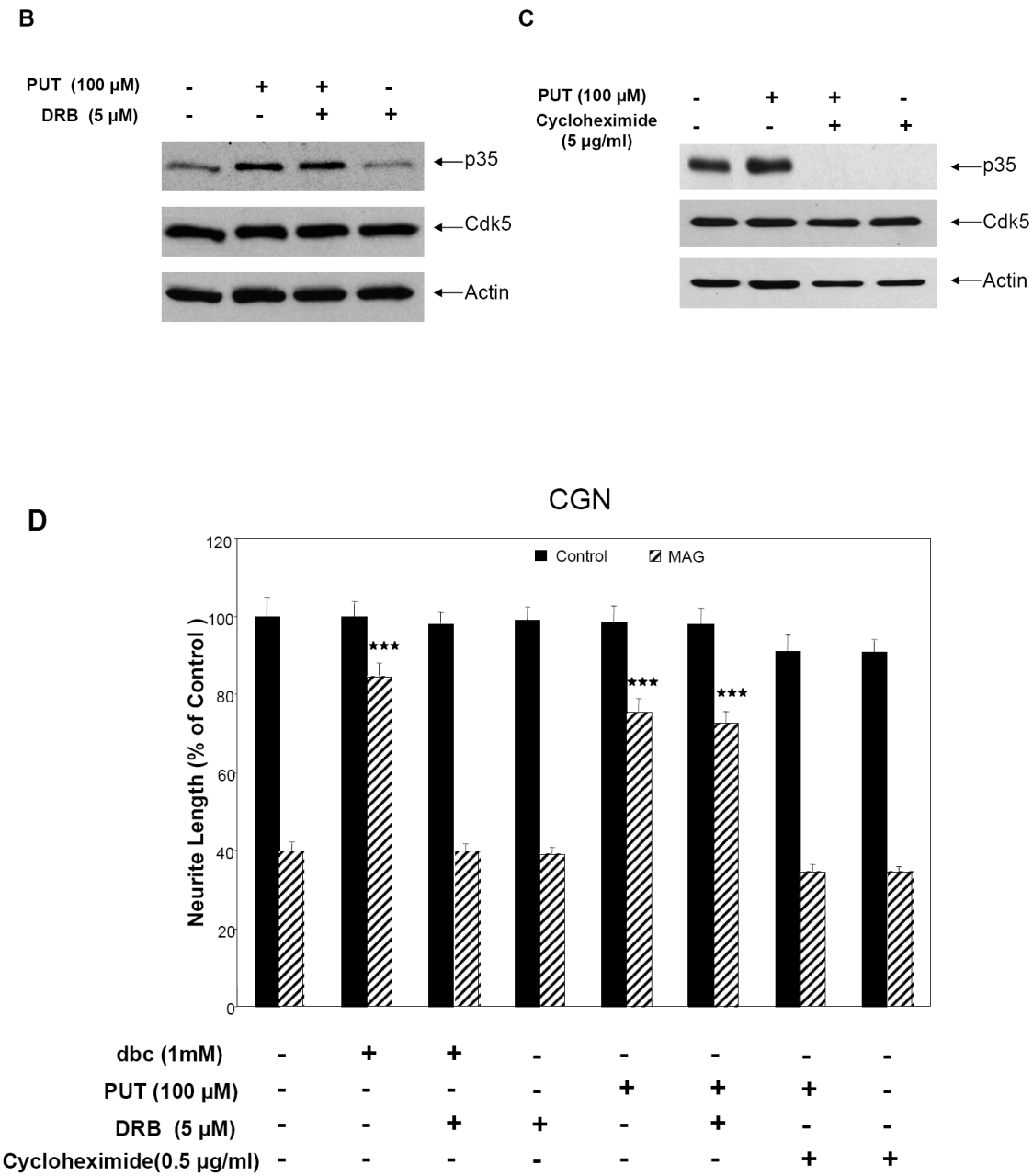


Figure 5B, 5C and 5D. Putrescine increases p35 protein in a transcription-independent but translation-dependent manner (B and C**)** Cerebellar neurons (P5) were treated with putrescine, DRB and cycloheximide overnight as indicated. Lysates were immunoblotted against p35, Cdk5 and actin. (D) Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from primed cerebellar neurons as indicated. The mean length of the longest neurite for about 400 neurons was measured (±SEM). (***P<0.001, compared with neurite length on MAG-CHO cells, N=3).

The Increases in p35 levels in Response to Putrescine is Dependent on eIF5A hypusination

As mentioned above, it was reported that the ability of NGF to induce neurite outgrowth and survival of PC12 cells required the polyamine-induced hypusination of eIF5A, the only known protein to contain the unique polyamine-derived amino acid hypusine, a modification that is essential for eIF5A function. eIF5A is a 17kD acidic protein, highly conserved throughout eukaryotes. Hypusine modification of eIF5A, called hypusination, is completed by 2 sequential steps: first, deoxyhypusine synthase (DHS) transfers 4-aminobutyl group of spermidine to an amino group of a specific lysine in the eIF5A precursor to generate an eIF5A intermediate, which is then hydroxylated by deoxyhypusine hydrolxylase (DOHH) to form mature eIF5A. Importantly, we had previously shown that to overcome inhibition by MAG and to promote axonal regeneration *in vivo*, putrescine must be converted to spermidine, which occurs readily in neurons. Therefore, it is conceivable that when neurons are treated with putrescine, more spermidine will participate in the modification of eIF5A thereby more functional eIF5A in neurons. To assess if hypusination of eIF5A was required for putrescine to increase p35, we used the potent DHS inhibitor N-guanyl-1,7-diaminoheptane (GC-7) to prevent eIF5A hypusination in cerebellar neurons. Figure 6A shows that GC-7 blocks the putrescine-induced increase in p35 but has no effect on the levels of expression of Cdk5 itself. The effect of the DHS inhibitor was verified using DHS RNA interference coupled with the small penetrating peptide Penetratin1 (Pen1-siRNA). It has been shown by Troy's group that conjugates of siRNA and penetratin1 can be used to rapidly deliver siRNA to primary cultures of neurons with very high efficiency and very low toxicity

(Davidson TJ et al., 2004). Cerebellar neurons were treated with 300nM of Pen1-siRNA overnight, and cells were then lysed and subjected to western blotting. When DHS is knocked down with siRNA, putrescine no longer induces an increase in expression of p35 (Figure 6B). Scrambled DHS siRNA has no effect on the putrescine-induced increase in p35 (Figure6C). Taken together, these results demonstrate that the upregulation of p35 by putrescine is dependent on eIF5A hypusination.

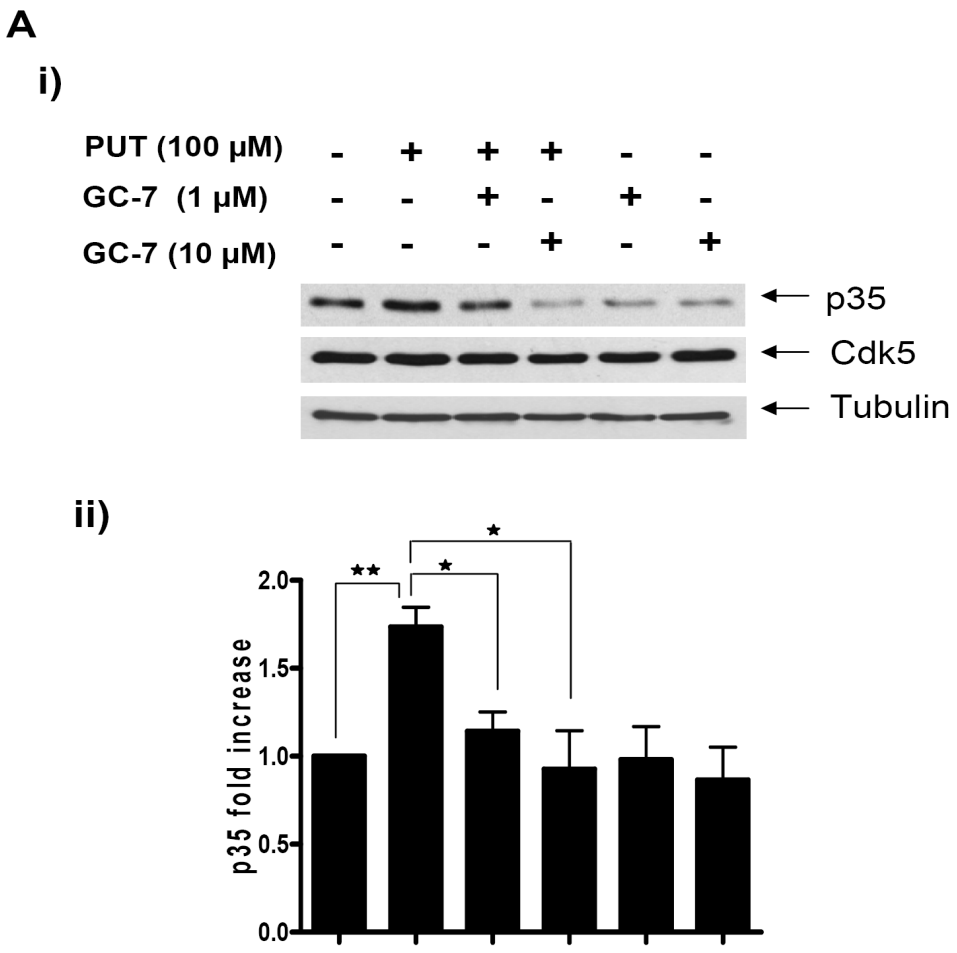


Figure 6A. Inhibition of hypusination blocks putrescine-induced upregulation of p35 protein i) Cerebellar neurons were treated overnight with putrescine and GC-7 at the concentrations as indicated. Cells were lysed and subjected to western blotting using antibodies agaist p35, Cdk5 and tubulin. ii) Statistical analysis from 3-5 experiments. (**p<0.01, *p<0.05).

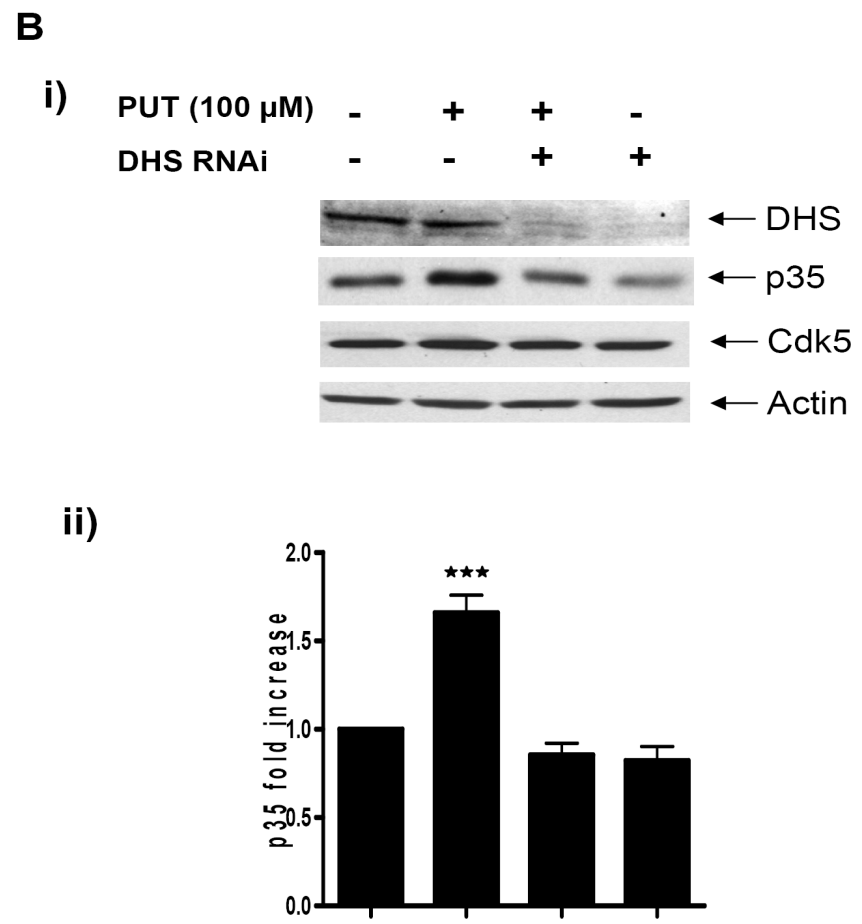


Figure 6B. Inhibition of hypusination blocks putrescine-induced upregulation of p35 protein i) Cerebellar neurons were treated with putrescine and/or DHS siRNA (300 nM) overnight. Cells were lysed and subjected to western blotting using antibodies against DHS, p35, Cdk5 and actin. ii) Statistical analysis from 3-5 experiments. (***$p<0.001$, compared with other three treatments).

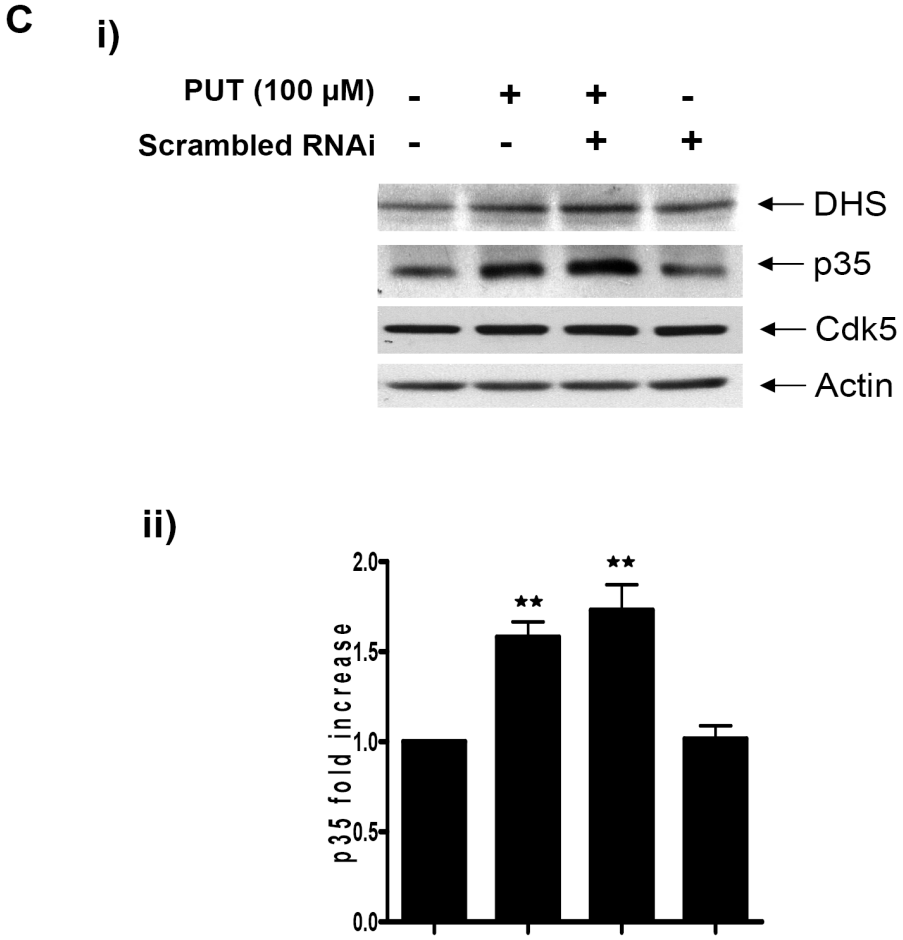


Figure 6C. Inhibition of hypusination blocks putrescine-induced upregulation of p35 protein

i) Cerebellar neurons were treated with putresicne and/or scrambled siRNA (300 nM) overnight. Cells were lysed and subjected to western blotting using antibodies agaist DHS, p35, Cdk5 and actin. ii) Statistical analysis from 3-5 experiments. (**p<0.01, compared with control and scramled siRNA alone treatments).

eIF5A hypusination is required for polyamines to overcome inhibition by MAG

We then assessed the requirement of hypusinated eIF5A for putrescine-mediated overcoming inhibition by MAG. Cerebellar neurons were primed with putrescine in the presence or absence of GC-7 overnight before being transferred onto control or MAG-expressing CHO cells. In the presence of GC-7, the ability of putrescine to overcome inhibition by MAG is abrogated. GC-7 alone has no effect on neurite outgrowth (Figure 7A). Similar effects are also seen in cerebellar neurons treated with DHS siRNA (Figure7B). These results further demonstrate that putrescine overcomes inhibition by modifying eIF5A hypusination and subsequently upregulating p35 and Cdk5 activity.

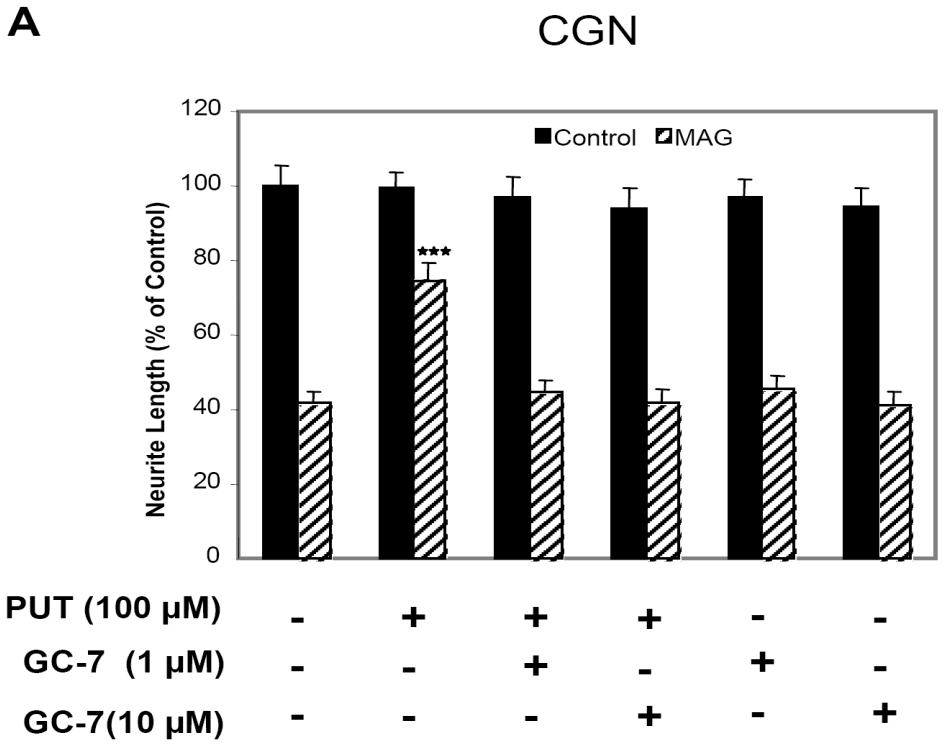


Figure 7A. eIF5A hypusination is required for polyamines to overcome inhibition by MAG

Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from cerebellar neurons (P5) primed with putrescine in the presence of GC-7. The mean length of the longest neurite for about 400 neurons was measured (±SEM). (***P<0.001, compared with neurite length on MAG-CHO cells, N=3).

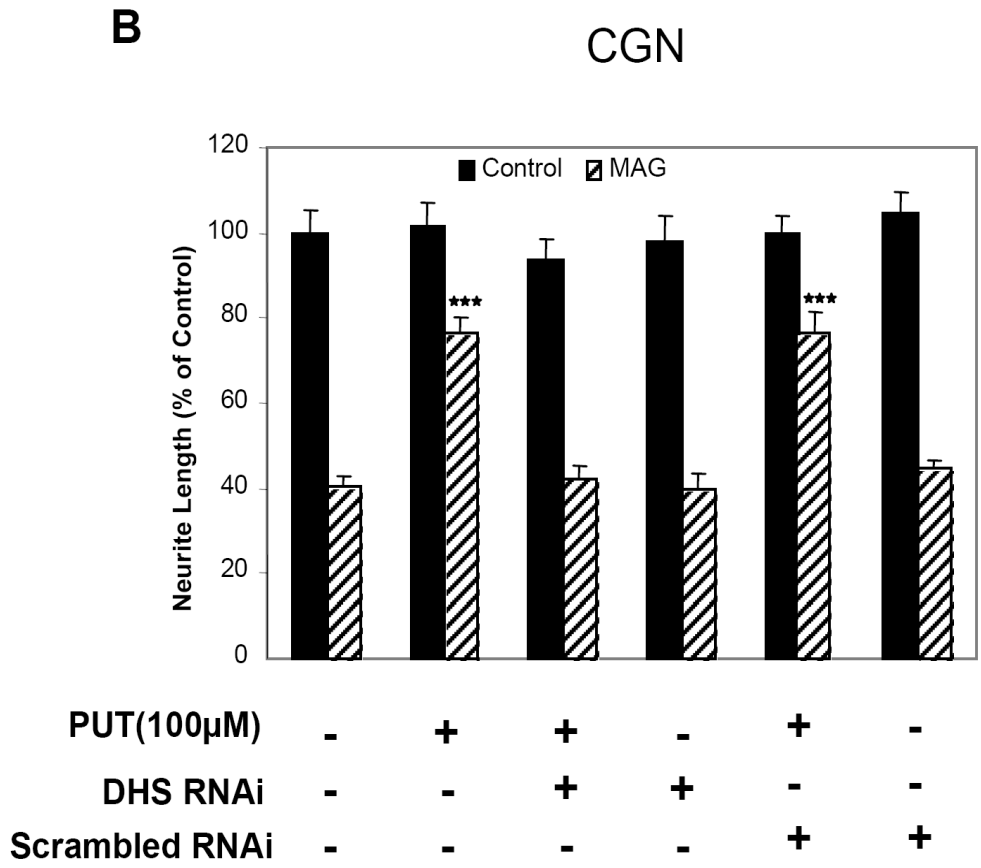


Figure 7B. eIF5A hypusination is required for polyamines to overcome inhibition by MAG

Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from cerebellar neurons (P5) primed with putrescine in the presence of DHS siRNA (300 nM) or scrambled siRNA (300 nM). The mean length of the longest neurite for about 400 neurons was measured (±SEM). (***P<0.001, compared with neurite length of other treatments on MAG-CHO cells, N=3).

Knockdown of eIF5A ablolishes the ability of putrescine to increase p35 and overcome inhibition by MAG

To complement the studies with the inhibition of hypusination, we wanted to demonstrate that it was indeed eIF5A that was required for the polyamine-induced increase in p35 expression and thus the ability to overcome inhibition. Figure 8A shows that indeed eIF5A is present in neurons and when immunostained, eIF5A is detected in both the neuronal cell body and processes. Figure 8B shows that when eIF5A is knocked down with siRNA, putrescine has no effect on the levels of expression of p35. Likewise, the putrescine-induced abitlity to overcome inhibition is lost (Figure 8C). Scrambled siRNA has no effect. Together, these results show that hypusination of eIF5A is required for both the polyamine-induced activation of Cdk5 and the ability to overcome inhibition by MAG. Figure 8D shows that penetratin-linked siRNA is taken up by neurons after 3hrs.

A

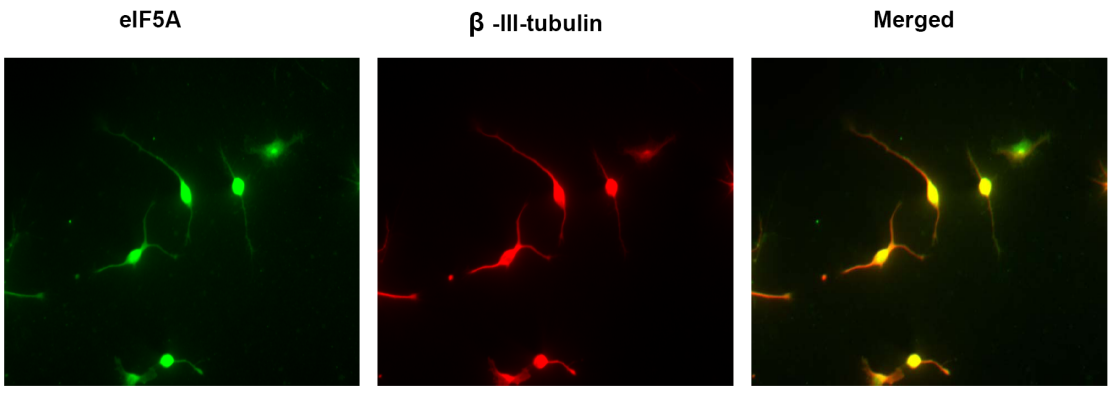


Figure 8A. eIF5A protein is present in neurons Cortical neurons (P1) were isolated and grown overnight. Neurons were fixed and immunostained with anti-eIF5A and anti-β-III-tubulin antibodies.

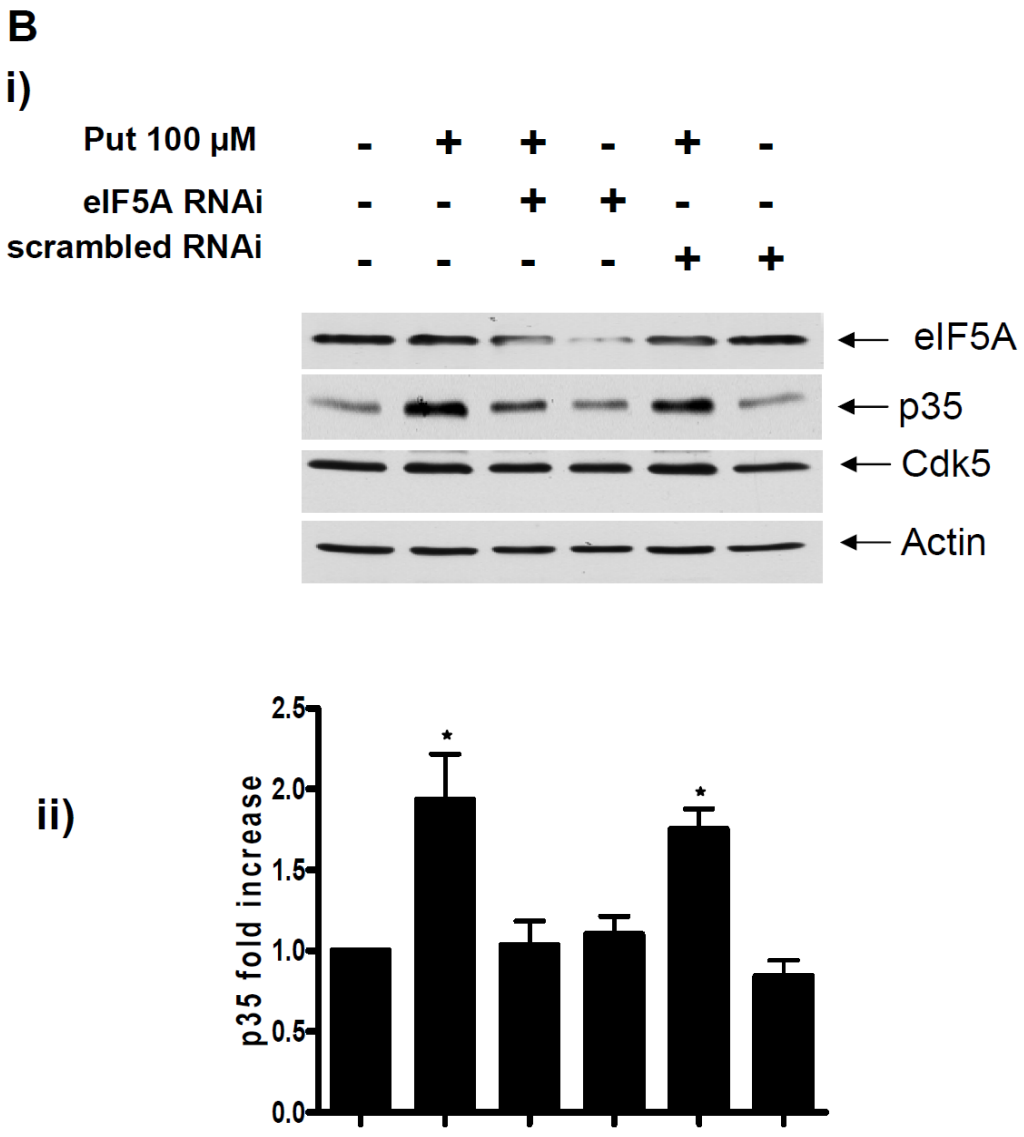


Figure 8B. Knockdown of eIF5A abolishes the ability of putrescine to increase p35
i) Cerebellar neurons were treated with putresicne and/or DHS siRNA (300 nM) or scrambled siRNA (300 nM) overnight. Cells were lysed and subjected to western blotting using antibodies agaist eIF5A, p35, Cdk5 and actin. ii) Statistical analysis from 3-5 experiments. (*$p<0.05$, compared with other treatments).

C

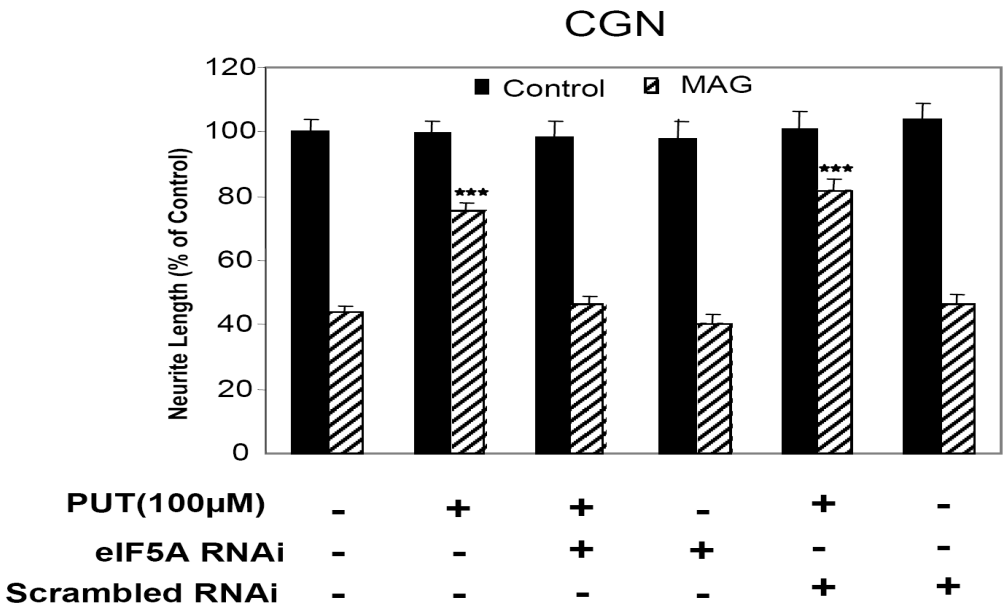


Figure 8C. Knockdown of eIF5A abolishes the ability of putrescine to overcome inhibition by MAG

Quantitation of neurite outgrowth on either MAG-expressing CHO cells or control CHO cells from cerebellar neurons (P5) primed with putrescine in the presence of eIF5A siRNA (300 nM) or scrambled siRNA (300 nM). The mean length of the longest neurite for about 400 neurons was measured (±SEM). (***P<0.001, compared with neurite length of other treatments on MAG-CHO cells, N=3).

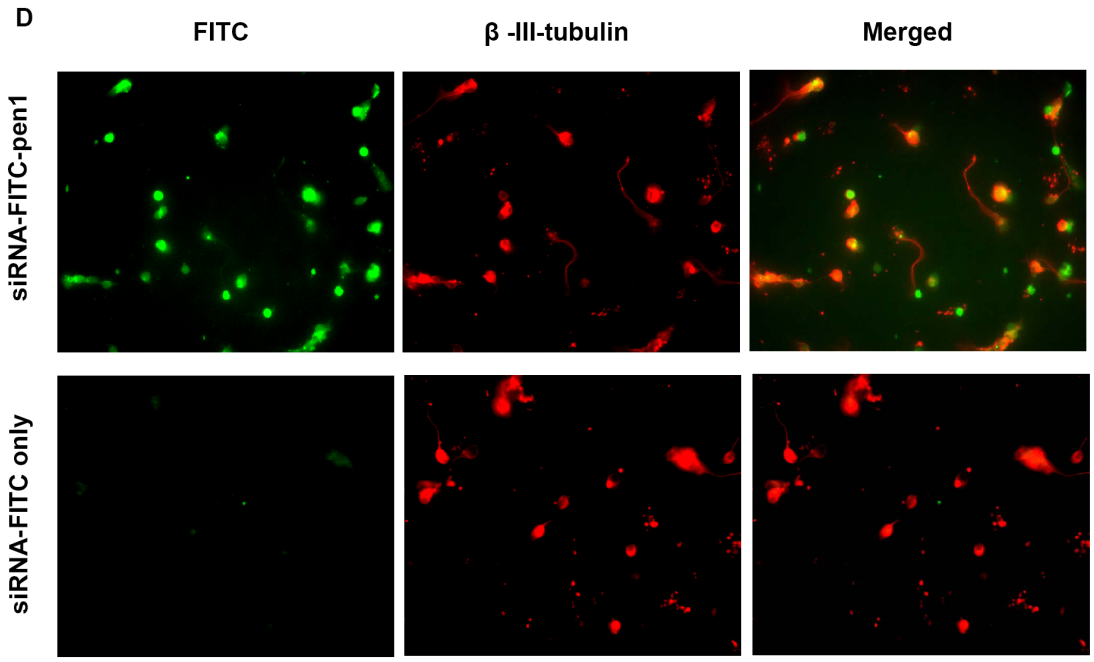


Figure8D. Neuronal uptake of siRNA
Cerebellar neurons were isolated and treated fluorescein-labled siRNA (siRNA-FITC) coupled with/without penetratin for 3h, after which cells were fixed and stained with β-III-tubulin.

5.3 Discussion

In this chapter, we further explored the mechanism by which putrescine upregulates p35 to activate Cdk5 thereby overcoming inhibition by MAG. We demonstrate that, unlike a known pathway in which p35 expression is through Erk activation, the increase in p35 expression in response to putrescine is Erk independent and moreover, transcription independent. Both the blockade of Erk activation and transcription do not affect the levels of p35 induced by putrescine. On other hand, the putrescine effect is dependent on translation. When a translation inhibitor cycloheximide is used, it blocks the ability of putrescine to increase p35 and overcome inhibition. These results also rule out the possibility that putrescine increases p35 levels by preventing p35 degradation as it fails to maintain the basal level of p35 when protein synthesis is blocked. Indeed, we further demonstrate that the eukaryotic eIF5A is involved in the putrescine effect.

eIF5A is a small, acidic protein which contains 154 amino acids and is highly conserved throughout eukaryotes (Smit-McBride et al., 1989; Park, 2006). eIF5A is the only known cellular protein that contains the unique polyamine-derived amino acid hypusine (hydroxyputrescine-lysine) (Cooper et al., 1983). Hypusine is essential for eIF5A function and is formed from spermidine by sequential action of two enzymes: DHS and DOHH. eIF5A was originally identified based on its ability to stimulate methionyl-puromycin synthesis in a model assay for translation initiation (Benne and Hershey, 1978). However, recently it has been found to associate with translating ribosomes and promote translation elongation in yeast (Jao and Chen, 2006; Zanelli et al., 2006; Gregio et al., 2009; Saini et al., 2009). Saini et al have proposed that eIF5A is a universally conserved translation elongation factor (Saini et al., 2009). eIF5A plays an

important role in cell proliferation in yeast and mammalian cells (Cooper et al., 1982; Gerner et al., 1986; Schnier et al., 1991; Wohl et al., 1993). Other eIF5A functions include its role as: a cellular cofactor of HIV-1REV (Ruhl et al., 1993), a factor involved in nuclear transport (Lipowsky et al., 2000) and mRNA turnover (Zuk and Jacobson, 1998; Valentini et al., 2002), and a role of eIF5A in cell wall integrity and actin polarity (Zanelli and Valentini, 2005).

Although eIF5A is a multi-functional protein, our data suggest that hypusinated eIF5A is involved in the translation of p35 protein induced by putrescine to overcome MAG/myelin inhibition. We show that blocking hypusination both by a DHS inhibitor or DHS siRNA abolishes the effect of putrescine on p35 levels and neurite outgrowth. We also show that knockdown of eIF5A itself has the same effect. These findings are novel for the following reasons: first, although eIF5A has a role in translation, depletion of this factor in yeast resulted in only a modest decrease in total protein synthesis (Kang and Hershey, 1994). Therefore, eIF5A was proposed to be involved in the translation of a specific subset of mRNAs. However, no reports have been available to reveal a specific gene expression affected by eIF5A at the translational level. Here, we provide the first evidence that p35 protein translation can be affected by eIF5A. Using the method of systematic evolution of ligands by exponential enrichment (SELEX) to identify the squence specificity of the potential eIF5A RNA targets, Xu and Chen reported that eIF-5A interacted with RNAs which share a high a sequence homology characterized by two motifs: UAACCA and AAUGUCACAC and that the hypusine residue was critical for this sequence-specific binding (Xu and Chen, 2001). Interestingly, p35mRNA also has sequences GAACCA and AAGGCCACAC. Therefore, eIF5A may enhance p35

translation through this specific binding. Second, we discover a novel pathway for the upregulation of p35. Instead of being regulated by the Erk pathway and at transcriptional level, p35 expression induced by putrescine is translation and eIF5A dependent. Third, despite abundant studies on the function of eIF5A in yeast and mammalian cells, few have been done in neurons. Our study present the data that eIF5A is present in cerebellar and cortical neurons and that eIF5A plays an important role in the induction of p35 protein by putrescine and the ability of putrescine to promote neurite outgrowth. Last, but most importantly, we identify a mechanism that polyamines are triggering to bring about the regeneration effect (Figure 9): inside the neuron, the polyamine putrescine is readily converted to spermidine and then spermidine modifies a specific lysine in eIF5A precursor to form mature eIF5A. As a result, more p35 proteins are translated thereby activating more Cdk5 which is required for overcoming inhibition by MAG.

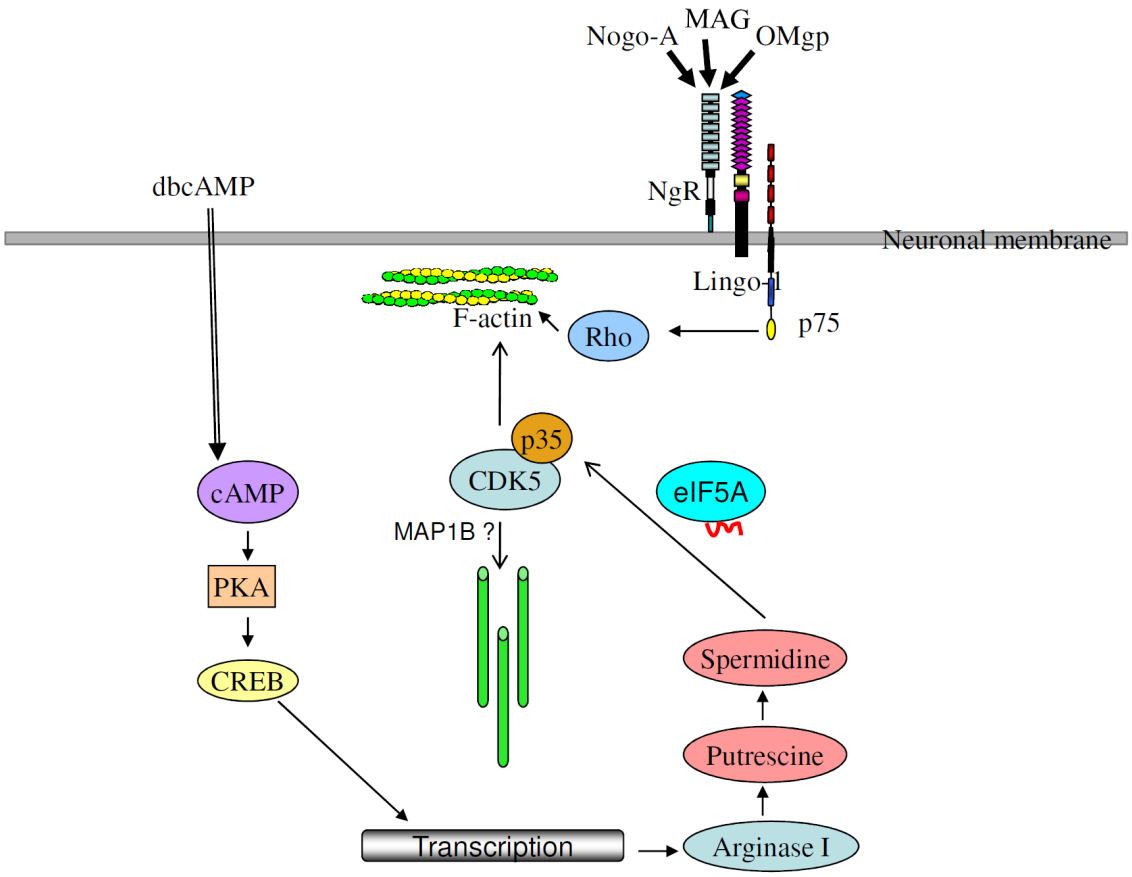


Figure 9. Model of signaling pathway that polyamines overcome inhibition by MAG.

In summary, this thesis presents the following results: Cdk5 activity is required for dbcAMP and putrescine to overcome inhibition by MAG and over-expression of p35 is sufficient to block the inhibiton; dbcAMP and putrescine increase Cdk5 activity by upregulating p35 levels; the increase in p35 levels in response to putrescine is ERK and

transcription independent, but translation dependent; the putrescine-induced translation of p35 is dependent on eIF5A hypusination; eIF5A hypusination is required for putrescine to overcome inhibition by MAG; knockdown of eIF5A with RNAi abolishes the ability of putrescine to increase p35 and overcome inhibition by MAG. Therefore, this body of work represents the first time that the polyamine putrescine increases p35 expression, that eIF5A enhances the translation of p35 in a hypusine-dependent manner and that Cdk5 is involved in overcoming inhibition in an inhibitory environment. The ultimate goal of the research in our lab is to identify possible targets to encourage regeneration. Could eIF5A and p35 be potential targets for *in vivo* therapeutics? Currently the lab is performing *in vivo* study in an optic nerve crush model, in which p35 is being overexpressed by the injection of HSV-p35 after crush and axonal regeneration is being assessed. This study together with future work on the downstream targets of Cdk5 could lead to new treatments for brain and spinal cord injury.

Chapter VI: References

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