The Role of Soluble Adenylyl Cyclase in the BDNF-Dependent Block of MAG/Myelin-Mediated Inhibition

by

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

2010

Approval

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

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Abstract

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In the adult mammalian central nervous system axons do not spontaneously regenerate following injury. This lack of axonal regeneration is partly due to the presence of inhibitory proteins, such as myelin-associated glycoprotein (MAG). Previously, we showed that elevating cyclic AMP (cAMP) by pretreating (priming) neurons with neurotrophins, such as brain-derived neurotrophic factor (BDNF), is sufficient to overcome the block of axonal outgrowth by MAG. Additionally, we demonstrated this BDNF-mediated effect to be PKA-, ERK-, calcium- and CREB-dependent. However, increasing cAMP levels in response to BDNF could be dependent on several factors. A balance between the production of cAMP by adenylyl cyclases and its degradation by PDEs will determine intracellular cAMP levels. Given that the source of the cAMP produced in response to BDNF is unknown, we sought to investigate which adenylyl cyclase is activated, transmembrane adenylyl cyclase (tmAC) or soluble adenylyl cyclase (sAC). tmACs and sAC differ in their spatial localization within the cell, structure and regulation. Our hypothesis is that the rise in cAMP in response to BDNF priming is partially dependent on sAC activation.

In this study, we have detected an isoform of sAC, somatic sAC, expressed in various postnatal rat primary neurons and demonstrated that specifically blocking sAC with the

pharmacological inhibitors, KH7 and OH-E or by knocking down sAC expression with siRNA, abolishes the ability of BDNF to overcome inhibition by MAG. Additionally, infection of primary neurons with a lentivirus that expresses sAC is sufficient to overcome the block of axonal growth by MAG and myelin *in vitro* and promotes optic nerve regeneration *in vivo*. As previously mentioned, priming with BDNF leads to ERK activation, which results in overcoming MAG-induced inhibition of neurite growth. We found that blocking sAC with pharmacological inhibitors blocked the BDNF-dependent phosphorylation of ERK whereas blocking tmAC had no effect on ERK activation by BDNF. Lastly, we sought to determine if alternative modes of sAC regulation exist, such as interactions with TrkB. Our data demonstrated that sAC does not associate with inactive or active TrkB receptors, yet does not rule out that other potential modes of regulation may exist. Taken together, our data suggest that sAC plays an integral role in BDNF signaling to overcome inhibition of axonal growth by MAG.

Acknowledgements

My list, in gratitude and thanks, of people that have given me their tremendous support and encouragement throughout my academic journey is a long one.

First and foremost, I would like to thank my mentor, Dr. Marie Filbin, for accepting me with open arms into her lab and supporting me wholeheartedly. She is one of the most intelligent and stylish women in science. I admire her strength, endurance, and ambition. She is a true inspiration to all young scientists, especially women aspiring to careers in science. But, most of all, I would like to thank her for choosing to stay at a city university with such a diverse and unique student body. She has helped make Hunter College a great research institution.

I would also like to thank my committee members, Dr. Venkatesh, Dr. Pereira, Dr. Rockwell and Dr. Levin. Dr. Pereira, who will always be "Dr. P" to me, was a wonderful undergraduate mentor and like a second mother to me. It was an instant bond. I am indebted to her for encouraging me everyday during my time as a technician to join the Ph.D. program. Thank you Dr. P! In my capacity working as her technician, Dr. Rockwell taught me so many techniques and the research skills that I still use today, I thank her greatly. To my future bosses, Dr. Levin and Dr. Buck, I thank them for all their support, advisement, and for generously providing me with most of the material I needed for my project. Mostly, I appreciate and thank them for their contagious optimism and enthusiasm. I am looking forward to an exciting and productive post-doctoral experience.

To the Filbin lab (my work family), I would like to thank Mustafa, Prospero, Jianwei, Aung, Najat, Chris, Rathna, Pete, Macro, Dinorah, Denise, Kiran, Zelda, John and Erica for all their support. I would especially like to thank Sarit and Huifang for their constant support and encouragement. We were like the three musketeers; we started and finished the program together. I couldn't have asked for better labmates! I would like to thank Sari for her patience and critical reading of my thesis; she's the best and brightest person I've had the good fortune to work with! A very special thank you to Elena, for sharing all the long nights together in the lab and for all her encouragement. And to Melissa, where do I

begin. I thank her for her love, support, encouragement, friendship and strength. She is a life-long friend.

To the former members of the Filbin lab: Tim, Barney, Zixuan, Kangwen, Dawn, Saranna, Mari, Marianna and Vasiliki, thanks for their support. Special thanks to Willy and Aline. I deeply thank Willy, my second mentor, for his support, encouragement and optimism. I deeply thank Aline for the many great things she brought to my life: this project, hope, inspiration, support, and encouragement. She is also a life-long friend.

A special thanks to members of the Levin/Buck lab: Alex, Lavos, Jonathan, Martin, Eric and Anna. I thank them for all their help and advisement. I look forward to working with them.

To Gail Smith from the AGEP/NSF program, thank you for partially funding me throughout my Ph.D. and for allowing me to attend many meetings and workshops. AGEP is a great minority-based program that exposes graduate students to the many opportunities available at several universities in the country. Most of all, it furthered my interest of becoming a professor, which I now definitively know is my passion.

I would like to thank all of my family and friends, they were all my rocks. They were all my inspiration and support. A special thanks to my dad, Catherine, Michell, Jen, Vanesa, Roxanne, Luena, Evan, Jessica, Christina, and Ceci. A very special thanks to my uncle Ernie who has been like a father to me. I thank him for all his love, support, generosity and encouragement.

Last, but certainly not least, I would like to thank my mom, my sister Sandra and my brother Frank. They are my biggest cheerleaders and my strongest supporters. I could never thank them enough. I thank them for their unconditional love and always believing in me, I couldn't have done it without them.

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Abbreviations

2-APB	2-Aminoethyl diphenyl borate
AC	adenylyl cyclase
ADP	adenosine diphosphate
AKAP	A-kinase anchoring protein
AP	alkaline phosphate
Arg-1	Arginase-1
ATP	adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cAMP	cyclic adenosine-3', 5'-monophosphate
C1	catalytic domain 1
C2	catalytic domain 2
C3	C3-ADP-ribosyltransferase
cDNA	complimentary deoxyribonucleic acid
CaM	calcium/calmodulin
CGN	cerebellar granule neuron
CHO	Chinese hamster ovary
CNS	Central nervous system
CREB	cAMP-responsive element-binding protein
CSPGs	chondritin sulfate proteoglycans
CST	corticospinal tract
dbcAMP	dibutyryl cAMP
ddAdo	2', 5' dideoxyadenosine
DMEM	Dulbecco's modified eagle's medium
DRB	5, 6-dicholro-1-β-D-ribfuranosylbenzimidazole
DRG	dorsal root ganglion
DSC	dorsal spinal cord

E	embryonic day
ESC	embryonic stem cells
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cells
FBS	fetal bovine serum
GAG	glycoaminoglycan
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
GC	guanylyl cyclase
GPCR	G-protein coupled receptors
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic proteins
GFP	green fluorescent protein
GPI	glycosylphophatidylinositol
GST	glutathione *S*-transferase
HN	hippocampal neurons
HRP	horseradish perosidase
hsAC	human soluble adenylyl cyclase
IF	immunofluorescene
IgG	immunoglobulin G
IL-6	Interleukin 6
IP	immunoprecipitation
IP3	inositol 1, 4, 5-triphosphate
IRES	internal ribose entry site
ITIM	immunoreceptor tyrosine-based inhibitory motifs
kDa	kiloDalton

KH7	2-(*H*-benzoimidazole-2-ylsulfanyl)-propionic acid (5-bromo-2-hydroxy-benzylidene)-hydrazide
K_m	Michaelis Menten constant
KO	knock out
LINGO-1	LRR and Ig domain-containing Nogo Receptor-interating protein
LRR	leucine rich repeat
m	meter
MAG	myelin associated glycoprotein
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MSC	marrow stromal cells
mTOR	mammalian target of rapamycin
MEK	MAPK kinase
MLN	myelin
MW	molecular weight
NGF	nerve growth factor
NgR	Nogo receptor
NMDA	N-methyl-D-asparate
NOG	neurite outgrowth assay
NTs	Neurotrophins
NT-3	Neurotrophin-3
OEC	olfactory bulb ensheathing cells
OH-E	2-hydrooxyestradiol
OMgp	Oligodendrocyte myelin glycoprotein
$p75^{NTR}$	p75 neurotrophin receptor
p75ICD	p75 intracellular domain
P	post-natal day
PACAP	pituitary adenylyl cyclase activating peptide
PAGE	poly-acryamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDE	phosphodiesterase
PIPσ	protein tyrosine phosphatase sigma
PirB	paired immunoglobulin-like receptor B
PKA	protein kinase A or cAMP-dependent protein kinase
PKC	protein kinase C
PLL	Poly L-lysine
PMSF	phenylmethylsulphonylfluoride
PND	post-natal day
PNS	Peripheral nervous system
PTEN	phosphate and tension homolog
PVDF	polyvinylidene
RIP	regulated intramembrane proteolysis
RGC	retinal ganglion cell
RNA	ribonucleic acid
ROCK	Rho-associated kinase
RT-PCR	reverse transcriptase polymerase chain reaction
sAC	soluble adenylyl cyclase
sAC_{fl}	sAC full-length
sAC_{t}	sAC truncated
SCI	spinal cord injury
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
Siglec	sialic acid binding IgG-like chain
siRNA	small interfering RNA
TBST	Tris-buffered saline tween
tmAC	transmembrane adenylyl cyclase
V_{max}	maximum velocity
XT-1	xylosyltransferase-1

Chapter I: Introduction

1.1 Failure of CNS Regeneration following Injury

It is well established that following injury, the central nervous system (CNS) is unable to spontaneously regenerate damaged axons, while the peripheral nervous system (PNS) can. Many factors can contribute to this difference, such as the extracellular environment and the intrinsic response of these injured neurons to molecules in their environment. In the last two decades, many studies have been focused on understanding the major obstacles that prevent axons in the CNS from regenerating. Two main events occur following CNS injury. The myelin sheath, which insulates axons, is damaged and myelin proteins are exposed, preventing axons from regenerating. Additionally, astrocytes undergo reactive astrogliosis to form both a chemical and physical barrier to growth by releasing inhibitory molecules and forming the glial scar. However, since the glial scar takes weeks or even months to fully develop, the inhibitory molecules in myelin are the major contributors to the failure of axonal regeneration in the CNS immediately following injury.

1.1.1 Regeneration in the Nervous System following Injury: CNS versus PNS

In order to achieve recovery following an injury to the nervous system, it will be critical to repair the injury, recover physiological functions and prevent further damage to the surrounding healthy tissue. The difference in the regenerative abilities of the PNS and CNS lies in their response to changes in their environment and the sequence of events that occur after injury. In the PNS, these changes in the environment facilitate regeneration. For centuries, it has been known that when a nerve is severed, gradual

degeneration of its distal end will occur (Vargas and Barres, 2007) and this process is known as Wallerian degeneration. In the PNS, a sequence of events occurs once the axon begins to degenerate: the myelin shealth breaks apart, macrophage infiltration occurs, and the blood nerve barrier becomes more permeable (Griffin et al., 1992). Also, at the distal end of the severed axon, rapid clearing of the myelin debris by Schwann cells and macrophages takes place (Fawcett and Keynes, 1990, Griffin et al., 1992). In the mammalian PNS, Wallerian degeneration occurs rather quickly, within 1 to 2 weeks following injury, whereas in the CNS it is a slow process that could take months to years (Vargas and Barres, 2007). Given that several proteins in myelin inhibit axon regeneration, failure to rapidly remove the myelin debris after injury contributes to the inability of CNS axons to regenerate. Additionally, in the CNS following injury, reactive astrocytes form a glial scar at the injury site and release inhibitory molecules (Silver and Miller, 2004). These events in the CNS result in a physical and chemical barrier to axonal regeneration.

1.2 Inhibitory Environment of the CNS after Injury

For centuries, since the ancient Egyptian civilization, it has been known that injuries to the CNS cannot be treated. In other words, once axons are damaged in the CNS, they will not spontaneously regenerate. This was first shown by Santiago Ramon y Cajal, when he demonstrated that following an injury, peripheral nerves were able to spontaneously regenerate, whereas in the brain and spinal cord, although severed axons had the capacity to grow short distances, this growth would abruptly stop (Ramon y Cajal, 1928). These

findings were the first indication that neuronal processes in the CNS have an intrinsic capacity for regeneration and suggested that factors in the environment surrounding the damaged axon may be impeding further growth. It was not until 1981 that the work of David and Aguayo showed that when a peripheral nerve graft was implanted into the spinal cord and brainstem to act as a "bridge", CNS axons were able to grow into the graft (David and Aguayo, 1981). Therefore, CNS axons are able to regenerate long distances if provided a permissive substrate to facilitate growth. This pivotal finding as well as others demonstrated that limited regeneration in the CNS was partially due to its environment. In 1982, Martin Berry was the first to suggest that dissociated myelin prevents damaged CNS axons from regenerating (Berry, 1982). Then in 1988, the Schwab group extracted 35- and 250-kD proteins from CNS myelin fractions that exhibit inhibitory properties to neurite growth *in vitro* (Caroni and Schwab, 1988b). Furthermore, in 1999 the David group illustrated the significant role myelin-associated inhibitors play on blocking axonal growth when mice were immunized by subcutaneous injection with homogenates of mouse spinal cord, which is rich in myelin, and after three weeks lesioned the corticospinal tracts (CST) (Huang et al., 1999). These mice displayed extensive regeneration of axons in the CST and recovery of motor function (Huang et al., 1999). In addition, antisera from the immunized mice blocked myelin-associated inhibitors to enhance neurite outgrowth *in vitro* (Huang et al., 1999).

1.2.1 Inhibitors in Myelin

Myelin is a fatty, membraneous structure that is tightly wrapped around the axon and increases the rate of signal propagation by acting as an electrical insulator. Myelin in the

CNS is produced by oligodrendrocytes, which can myelinate several axons simultaneously. In contrast, in the PNS, individual Schwann cells will myelinate one section of one axon. As previously mentioned, the work of several groups led to the discovery that myelin molecules contribute to the failure of CNS axons to regenerate following injury. Today, several myelin-associated inhibitors of regeneration have been identified.

1.2.1.1 Nogo

The first indication of an inhibitory component in myelin was provided when the IN-1 antibody was generated against a portion of myelin with high levels of inhibitory activity. These inhibitory components were the two proteins formally known as neurite growth inhibitors (NI), NI-35 and NI-250 (Caroni and Schwab, 1988b). When IN-1 was introduced into lesion site after injury, it improved neurite outgrowth *in vitro* (Caroni and Schwab, 1988a, b), and promoted CST axon regeneration *in vivo* (Schnell and Schwab, 1990). Ten years later, three groups cloned and identified the IN-1 antigen (Chen et al., 2000a, GrandPre et al., 2000, Prinjha et al., 2000), which was named Nogo. A single gene gives rise to three isoforms through alternative splicing and the use of different promoter regions. The three Nogo isoforms are Nogo A, B and C, but it is Nogo A that is mainly expressed in oligodendrocytes and neurons (Huber et al., 2002). Nogo B and C are expressed in various tissues, including neurons (Huber et al., 2002). However, Nogo C is primarily found outside the nervous system (Chen et al., 2000a, GrandPre et al., 2000, Huber et al., 2002). All three isoforms contain a common C-terminus, consisting of 188-amino acid residues similar to that of members of the reticulon family (Chen et al., 2000a, Prinjha et al., 2000, GrandPre et al., 2002). Nogo A contains two inhibitory

domains, amino-Nogo in the N-terminus, which is exclusive to Nogo A, and a 66-amino acid sequence, extracellular loop, called Nogo-66 (Chen et al., 2000a, GrandPre et al., 2000, Prinjha et al., 2000). Although both domains have been shown to block neurite extension and induce growth cone collapse *in vitro* (GrandPre et al., 2000, GrandPre et al., 2002, Oertle et al., 2003), amino-Nogo can also block fibroblast spreading (Chen et al., 2000a, Prinjha et al., 2002), which suggests that functional differences exist between the two inhibitory domains.

1.2.1.2 Myelin-Associated Glycoprotein

Myelin-associated glycoprotein (MAG) was identified more than two decades ago as a protein that has a role in the initiation and maintenance of myelin (Quarles, 1983), but it was not until 1994 that it was also shown to be capable of inhibiting neurite outgrowth (McKerracher et al., 1994, Mukhopadhyay et al., 1994). MAG is a member of the sialic acid binding IgG-like lectin (Siglec) family of adhesion proteins (Kelm et al., 1994, Crocker et al., 1998). It consists of five IgG-like extracellular domains, a single transmembrane region and a short cytoplasmic tail (Lai et al., 1987, Salzer et al., 1987, Salzer et al., 1990). There are two isoforms of MAG produced by alternative splicing, L-MAG and S-MAG, which contain the same sequence, except for their cytoplasmic domain (Lai et al., 1987, Salzer et al., 1987). Interestingly, MAG is exclusively located in myelin, especially in the periaxonal membranes and represents roughly 1% of all protein found in CNS (Trapp, 1990). However, in the PNS, MAG is found in the paranodial regions, Schmidt-Lanterman incisures and outer mesaxonal segments of the myelin shealth and accounts for approximately 0.1% of PNS myelin (Trapp, 1990).

In 1994, two groups independently showed that MAG blocks neurite outgrowth *in vitro* (McKerracher et al., 1994, Mukhopadhyay et al., 1994). Although it had been known since 1988 that proteins on myelin were inhibitory, MAG was the first inhibitor to be definitely identified (McKerracher et al., 1994, Mukhopadhyay et al., 1994). It was shown that when various CNS neurons, such as cerebellar granular neurons (CGN) and dorsal root ganglion (DRG) neurons, were grown on monolayers of MAG-expressing cells, neurite growth was strongly inhibited when compared to growth on control cells (Mukhopadhyay et al., 1994, DeBellard et al., 1996). Likewise, when the neuronal cell line, NG108, was plated on slides containing MAG or myelin, neurite extension was blocked (McKerracher et al., 1994).

Interestingly, given that MAG is a sialic acid binding protein, it was shown to interact with gangliosides, specifically GT1b and GD1a through its first IgG domain, which contains the essential residue, Arg 118 (R118) (Tang et al., 1997). However, this residue is not necessary for membrane-bound MAG to elicit its inhibitory effects on neurite growth (Tang et al., 1997). Mutating the R118 residue on membrane-bound MAG did not block inhibition, therefore suggesting that the sialic acid binding domain is distinct from the inhibitory domain (Tang et al., 1997). In fact, it has been recently shown that the fifth IgG domain of MAG contains the site necessary for inhibition (Cao et al., 2007).

MAG displays a bifunctional role, as it can either promote or inhibit neurite growth and this was shown to be dependent on the developmental stage of several types of neurons (Johnson et al., 1989, Salzer et al., 1990, Mukhopadhyay et al., 1994). Several studies have shown that MAG can potentiate growth of young neurons and inhibit growth in older neurons (Johnson et al., 1989, McKerracher et al., 1994, Mukhopadhyay et al.,

1994, DeBellard et al., 1996, Turnley and Bartlett, 1998). However, MAG is inhibitory for all postnatal neurons tested thus far, with the exception of DRG neurons postnatal day 1 to 3 (Mukhopadhyay et al., 1994, DeBellard et al., 1996).

In studies with MAG -/- mice, the phenotype observed was subtle and showed that the initiation of myelination, the formation of intact myelin sheaths and the integrity of myelin in the CNS was slightly impaired (Schachner and Bartsch, 2000). However, MAG is essential for the maintenance of myelin in both the PNS and CNS (Schachner and Bartsch, 2000). When CGN and DRG neurons were plated on a substrate of purified myelin from MAG -/- mice, they observed improved neurite growth as compared to myelin from wildtype mice (Bartsch et al., 1995, DeBellard et al., 1996, Li et al., 1996). Additionally in *in vivo* studies where the CST and optic nerve were injured in MAG -/- mice, they observed slightly enhanced axon regeneration (Bartsch et al., 1995, Li et al., 1996). Collectively, these findings illustrate that MAG plays an important role in the initiation of myelination and maintenance, however its role in axonal regeneration *in vivo* is less clear, suggesting that other molecules in myelin contribute to the inhibitory properties of myelin.

1.2.1.3 Oligodendrocyte-Myelin Glycoprotein

In 2002, oligodendrocyte-myelin glycoprotein (OMgp) was the third protein shown to be a myelin-associated inhibitor (Wang et al., 2002b). OMgp is a glycosyl phosphatidylinositol (GPI)-anchored protein containing a leucine rich repeat (LRR) domain and C-terminal serine/threonine rich domain (Mikol and Stefansson, 1988). It is expressed in several types of neurons as well as oligodendrocytes, specifically in the

paranodal loops of CNS myelin near the nodes of Ranvier (Mikol and Stefansson, 1988, Habib et al., 1998). Its expression is regulated during development in the rat CNS, where it is highly expressed after birth until approximately six weeks of age (Vourc'h et al., 2003). It was shown to block neurite outgrowth and induce growth cone collapse in cultured neurons (Kottis et al., 2002, Wang et al., 2002b). Additionally, it was shown that myelin extracted from OMgp -/- mice was significantly less inhibitory than myelin from control mice *in vitro* (Ji et al., 2008).

1.2.2.4 Sema4D and Ephrin-B3

During development, guidance cues are important factors that are required for proper axonal pathfinding. These guidance cues can be either attractive or repulsive to growth and some are present in myelin. Two factors that have been shown to inhibit growth are Sema4D and ephrin-3B (Moreau-Fauvarque et al., 2003, Benson et al., 2005). Semaphorins are a family of proteins that exist as membrane-bound or secreted proteins. They are expressed throughout the CNS and in particular, Sema4D was shown to be selectively expressed in oligodendrocytes and myelin (Moreau-Fauvarque et al., 2003). Following spinal cord injury (SCI), increased expression of Sema4D was detected in oligodendrocytes and *in vitro* studies showed that Sema4D strongly inhibited neurite growth of postnatal CGN and DRG neurons (Moreau-Fauvarque et al., 2003). Additionally, studies in primary hippocampal neurons showed that Sema4D induced growth cone collapse (Swiercz et al., 2002). During development, ephrin3B repels axons from the midline in the CST of mice; therefore its role in axonal pathfinding is repulsive.

Studies with postnatal primary neurons revealed that ephrin-3B is expressed in myelinating oligodendrocytes and blocks neurite outgrowth (Benson et al., 2005).

1.2.2 Receptors for Myelin-Associated Inhibitors

The identification of the myelin-based inhibitors shed light on how myelin blocks axonal regeneration, however determining which receptors they bind to will help us to understand its intracellular signaling events that lead to inhibition. Interestingly, Nogo, MAG and OMgp were shown to interact with common receptors to induce inhibition, yet these inhibitors share no sequence homology. To date, the aforementioned inhibitors bind to two receptors, NgR and PirB. Additional co-receptors are necessary to elicit intracellular signaling via NgR. The structure and function of these receptors will be discussed in detail below.

1.2.2.1 Nogo-66 Receptor

In 2001, the Strittmatter group identified a GPI-linked, leucine-rich repeat (LRR) glycoprotein that binds to Nogo-66 and named it the Nogo receptor (NgR) (Fournier et al., 2001). This was accomplished by using a soluble form of Nogo fused to alkaline phosphate (AP) and screening a mouse CNS expression library in COS-7 cells to assess binding (Fournier et al., 2001). Furthermore, direct binding of Nogo-66 to NgR was shown to mediate growth cone collapse *in vitro* (Fournier et al., 2001). Given that developing neurons are unresponsive to Nogo-66, it was shown that transfecting embryonic RGCs with NgR cDNA and subsequently exposing them to glutathione *S*-

transferase (GST)-Nogo-66 induced growth cone collapse (Fournier et al., 2001). Additionally, in embryonic DRG neurons treated with a specific phospholipase, PI-PLC, which removes GPI-linked proteins from the surface of plasma membranes, Nogo-66 was unable to induce growth cone collapse (Fournier et al., 2002). These results validated NgR as the GPI-anchored receptor for Nogo-66.

NgR is 473 amino-acid, 85 kDa protein that consists of eight LRR domains at the N-terminus and C-terminal rich in cysteines, followed by a unique C-terminus sequence (Fournier et al., 2001). It is expressed on the surface of several types of neurons (Fournier et al., 2001). In 2002, MAG and OMgp were also shown to bind to NgR, although there is no domain homology between these three inhibitory proteins (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002b). Furthermore, binding of the aforementioned inhibitors to NgR was shown to be necessary to induce inhibition of neurite growth *in vitro* (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002b). Interestingly, it was shown that the sialic acid binding domain on MAG was not required for interaction with NgR (Domeniconi et al., 2002, Liu et al., 2002).

In 2003, using database searches, two proteins were found to be homologous to NgR and were later named NgR2 and NgR3 (Barton et al., 2003, Pignot et al., 2003). Therefore, the NgR characterized by the Strittmatter group was designated NgR1. Initial analysis of their primary sequences, biochemical properties and expression pattern revealed that although these receptors are structurally similar neither MAG, OMgp nor Nogo bind to NgR2 and NgR3 (Barton et al., 2003). Then in 2005, MAG was shown to preferentially bind to NgR2 to induce inhibition in a sialic-acid-dependent manner (Venkatesh et al., 2005).

1.2.2.2 p75 Neurotrophin Receptor and TROY

Because NgR is a GPI-linked protein lacking an intracellular domain, it needs a binding partner to transduce a signaling response following ligand binding. In 2002, the p75 neurotrophin receptor ($p75^{NTR}$) was shown to associate with NgR (Wang et al., 2002a, Wong et al., 2002). $p75^{NTR}$ is a transmembrane protein that is part of the tumor necrosis factor (TNF) receptor family (Chao et al., 1998). Previous studies performed using neurons from $p75^{NTR}$-deficient mice showed that the inhibitory effect of MAG on neurite outgrowth is attenuated and therefore, that $p75^{NTR}$ is involved in mediating MAG-induced inhibition of neurite growth (Wang et al., 2002a, Yamashita et al., 2002). Co-immunoprecipation studies of MAG, $p75^{NTR}$, and NgR revealed that these molecules interact, confirming that $p75^{NTR}$ was a co-receptor and signal transducer for NgR (Wang et al., 2002a, Wong et al., 2002). In addition, it was shown that binding of MAG, Nogo or OMgp to this NgR-$p75^{NTR}$ complex lead to the inhibition of neurite outgrowth (Wang et al., 2002a, Wong et al., 2002).

Given that $p75^{NTR}$ is only expressed in a subset of neurons in the adult nervous system, it was no surprise that another co-receptor for NgR exists. In 2005, TROY, another member of the TNF receptor family, was also found to be a co-receptor for NgR (Park et al., 2005, Shao et al., 2005). TROY is widely expressed in the nervous system in both postnatal and adult neurons (Park et al., 2005, Shao et al., 2005). Experiments using mutant TROY or soluble TROY in neurite outgrowth assays resulted in the block of myelin-associated inhibition of neurite outgrowth (Park et al., 2005, Shao et al., 2005). Interestingly, TROY was found to be functionally homologous to $p75^{NTR}$ with regard to myelin-induced inhibitory signaling (Park et al., 2005, Shao et al., 2005).

1.2.2.3 Lingo-1

Interestingly, the NgR and $p75^{NTR}$/TROY complex alone is not sufficient to induce myelin-associated inhibitory signaling. A third binding partner in this receptor complex, the transmembrane protein, LINGO-1, was shown to be necessary for transducing the inhibitory signal (Mi et al., 2004). LINGO-1 is highly expressed in rat CNS and consists of twelve extracellular LRR modules, an IgG domain, a transmembrane domain and a short cytoplasmic domain (Mi et al., 2004). It was shown that myelin inhibition was decreased in CGN transfected with a mutant human LINGO-1, (Mi et al., 2004).

1.2.2.4 PirB

Studies in NgR -/- mice revealed that a receptor other than NgR interacts with MAG, OMgp and Nogo to induce myelin-mediated inhibition (Kim et al., 2004, Zheng et al., 2005). Using expression cloning to screen a human cDNA library, it was shown that the aforementioned inhibitors interact with another receptor, paired immunoglobulin-like receptor B (PirB) (Atwal et al., 2008). It consists of six extracellular IgG-like repeats, a single transmembrane region and four cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM). Similar to NgR, PirB is expressed throughout the nervous system and was shown to negatively regulate plasticity in the visual cortex (McGee et al., 2005, Syken et al., 2006). It was shown that binding of MAG, Nogo and OMgp to PirB mediates inhibition of neurite outgrowth (Atwal et al., 2008). Eliminating PirB activity using anti-PirB or a PirB mutant mouse carrying a loss-of-function *PirB* allele (PirBTM), was shown to partially reduce inhibition mediated by MAG, OMgp and Nogo (Atwal et al., 2008). Collectively, these results validated that PirB is a functional receptor for

MAG, OMgp, and Nogo that mediates myelin inhibition. Interestingly, when CGN from NgR-/- mice were cultured with anti-PirB, it resulted in almost complete reversal of inhibition *in vitro* (Atwal et al., 2008), which suggest that these two receptors are responsible for the majority of inhibitory signaling by myelin. However, the intracellular signaling cascade leading to inhibition via PirB has yet to be elucidated.

1.2.3 Signaling by Myelin-Associated Inhibitors

Early on, it was shown that MAG- and myelin-induced inhibition was RhoA-dependent (Lehmann et al., 1999, Dergham et al., 2002, Winton et al., 2002). Rho GTPase, RhoA, is known to be involved in regulating the polymerization of actin filaments (Nikolic, 2002). Inactivation of RhoA by C3-ADP-ribosyltransferase (C3) in cultured cortical and retinal neurons plated on purified CNS myelin resulted in enhanced neurite growth *in vitro* and promoted axonal regeneration in the rat optic nerve and mouse spinal cord *in vivo* (Lehmann et al., 1999, Dergham et al., 2002). Studies have also shown that p75 ^{NTR} activates RhoA when MAG interacts with the NgR receptor complex and this activation via p75^{NTR} may result indirectly from the sequestering of Rho-GDP dissociation inhibitor, Rho-GDI (Nikolic, 2002, Wang et al., 2002a, Yamashita et al., 2002). In addition, work from several labs has indicated that blocking activation of RhoA or one of its downstream targets, Rho-associated kinase (ROCK), can improve neurite extension *in vitro* and regeneration following injury *in vivo* (Lehmann et al., 1999, Dergham et al., 2002, Winton et al., 2002, Fournier et al., 2003). Together, these results demonstrate that RhoA and its downstream signaling components are crucial factors that lead to the inhibitory effects of the myelin-associated inhibitors.

Several isoforms of protein kinase C (PKC) were also shown to be key signaling components that are activated by the myelin-associated inhibitors. Blocking PKC activity with pharmacological inhibitors or dominant negative PKCs improves axonal regeneration following a dorsal column injury *in vivo* (Sivasankaran et al., 2004). In 2005, our group showed that regulated intramembrane proteolysis (RIP) of $p75^{NTR}$ by α- and γ-secretases in a PKC-dependent manner is necessary to activate Rho GTPase, which blocks neurite extension in response to MAG (Domeniconi et al., 2005). Using γ-secretase inhibitors, RhoA activation was blocked and therefore, it was postulated that the product of this $p75^{NTR}$ cleavage, a 25kDa intracellular domain (p75ICD) leads to the activation of Rho A (Domeniconi et al., 2005). These results show that PKC also plays an important role in mediating myelin-based signaling.

In 2005, it was shown that an additional signaling cascade was activated in response to NgR stimulation (Koprivica et al., 2005). Binding of the myelin-associated inhibitors to NgR led to the transactivation of the epidermal growth factor receptor (EGFR) in a calcium-dependent manner (Koprivica et al., 2005). Using pharmacological inhibitors of EGFR, primary neurons grown on purified myelin exhibited improved neurite growth *in vitro* (Koprivica et al., 2005). These inhibitors promoted axonal regeneration in the optic nerve *in vivo* as well (Koprivica et al., 2005). In 2007, another study showed that in a weight-drop spinal cord injury model, application of EGFR inhibitor to the injury site led to significant functional recovery of locomotor, sensory, and bladder function (Erschbamer et al., 2007).

1.2.4 The Glial Scar

In the adult mammalian CNS, injury will induce tissue damage that creates an additional obstacle to axonal regeneration. As previously mentioned, astrocytes will undergo reactive astrogliosis following injury resulting in the release of inhibitory molecules and the formation of the glial scar. This response occurs to prevent the spread of potentially harmful factors, which can result in further tissue damage. Reactive astrogliosis causes hypertrophy and an increase in expression of the intermediate filament, glial fibrillary acidic protein (GFAP) as well as an increase in astrocyte proliferation (Silver and Miller, 2004). In addition, several extracellular matrix (ECM) proteins, such as chondroitin sulphate proteoglycans (CSPGs) are up-regulated in reactive astrocytes (Silver and Miller, 2004). In fact, CSPGs have been shown to block neurite growth *in vitro* (McKeon et al., 1991, Smith-Thomas et al., 1994, Niederost et al., 1999) and inhibit axonal regeneration *in vivo* (Davies et al., 1999). CSPGs belong to a family of proteoglycans, which contain a protein core connected by four sugar moieties to a sulphated glycosaminoglycan (GAG) chain that consists of repeating disaccharides (Silver and Miller, 2004). It was shown that removal of the GAG chain by the enzyme chondroitinase ABC resulted in enhanced regeneration and functional recovery following SCI (Bradbury et al., 2002). Like the myelin inhibitors, CSPGs mediate their inhibitory effects via the RhoA/ROCK signaling pathway (Dergham et al., 2002, Borisoff et al., 2003, Monnier et al., 2003). Additionally, CSPGs were also shown to activate PKC and EGFR (Sivasankaran et al., 2004, Koprivica et al., 2005). In 2009, the transmembrane protein, protein tyrosine phosphatase (PTPσ), was identified as a receptor for CPSGs that transduces inhibition of growth (Shen et al., 2009). DRG neurons from PTPσ null

mutant mice were shown to be less inhibited by CSPGs *in vitro* (Shen et al., 2009). Following SCI in PTPσ null mutant mice, they observed enhanced axon regeneration in areas rich in CSPGs *in vivo* (Shen et al., 2009). Collectively, these results demonstrate the importance of the glial scar and the degree to which it is inhibitory to regeneration, however the full development of the scar may take several weeks to months. For this reason, the myelin-associated inhibitors that are exposed immediately following injury are believed to be the major factors impeding axonal regeneration.

1.3 Strategies for Promoting Regeneration following Injury

Understanding the major obstacles that prevent CNS axonal regeneration made it possible to now explore ways to overcome them. With the knowledge that factors in the CNS environment, such as molecules in myelin and the glial scar prevent axonal regeneration, strategies can be tested to block their effects. Additionally, it is known that neurons have the intrinsic ability to regenerate axons, therefore enhancing these signals can block the aforementioned inhibitory effects and facilitate regeneration.

1.3.1 Overcoming the Myelin-Induced Block of Regeneration

1.3.1.1 Blocking Inhibition with Antibodies and Peptides

Given that MAG, OMgp and Nogo interact with the same receptors to elicit intracellular signaling cascades that inhibit axonal regeneration, attempts to neutralize the myelin-associated inhibitors were the first approaches used. As previously mentioned, two

antibodies, IN-1 and IN-2, were generated against two myelin fractions, NI-35 and NI-250 (Caroni and Schwab, 1988a, b). Injection of IN-1 or IN-2 into optic nerve explants resulted in the improved growth of co-cultured sensory and sympathetic neurons (Caroni and Schwab, 1988a). In 1990, hybridoma cells expressing IN-1 were injected into the cortices of rats 7 to 10 days before transecting the CST and this resulted in the enhanced regeneration of severed CST axons (Schnell and Schwab, 1990). In 1993, the same group transplanted embryonic spinal cord tissue and IN-1 into the lesion site of young rats following transection of the spinal cord and observed axonal extension (Schnell and Schwab, 1993). In 1995, these IN-1 expressing hydridoma cells were injected into the cortex and adult dorsal column lesions and CST lesions were performed simultaneously. This led to the regeneration of raphespinal, coeruleospinal and CST axons and the recovery of locomotion function (Bregman et al., 1995). As previously mentioned, another approach used to neutralize the myelin-associated inhibitors was to immunize mice with homogenates of mouse spinal cord, which resulted in robust regeneration of axons and functional motor recovery following dorsal hemisection (Huang et al., 1999). In addition, antisera from the immunized mice blocked myelin-associated inhibitors to enhance neurite outgrowth *in vitro* (Huang et al., 1999). However, using this approach to block myelin-induced inhibition requires the application of antibodies and myelin anti-sera before the injury occurs, therefore it may not be a useful therapeutic tool.

Another approach involved blocking the interaction between myelin-based inhibitors and their common receptor, NgR, via administration of a function-blocking NgR antibody or peptide. Studies showed that application of the peptide, NEP1-40, which consists of the first 40 amino acids of Nogo-66, blocked binding of Nogo-66 to NgR, resulting in an

improved growth response *in vitro* (GrandPre et al., 2002). Also, NEP 1-40 was introduced through intrathecal administration following a mid-thoracic spinal cord hemisection in rats and this resulted in extensive growth of CST axons and improved motor function (GrandPre et al., 2002). Using a polyclonal antibody against NgR to block NgR and MAG from interacting, it was shown that MAG-mediated inhibition was blocked in CGN and DRG neurons (Domeniconi et al., 2002). In addition, administration of function-blocking or non-signaling receptor fragments, such as $p75^{NTR}$Fc (Wang et al., 2002a), NgR-Ecto (Li et al., 2004, Li et al., 2005) and LINGO-Fc (Mi et al., 2004) resulted in the block of the receptor complexes, which led to enhanced axonal growth *in vitro*. In studies using NgR-Ecto, it also promoted axonal regeneration *in vivo* and improved spinal cord electrical conduction and locomotor recovery (Li et al., 2004).

1.3.1.2 Using Knockout Mice: Myelin-Associated Inhibitors and Receptors

Purified myelin from MAG-/- mice, was used as a substrate to analyze neurite extension, growth cone collapse and cell spreading (Bartsch et al., 1995). Results from these *in vitro* studies showed no significant improvement as compared to control myelin (Bartsch et al., 1995). In addition, following an optic nerve lesion or dorsal column hemisection of the CST in MAG -/- mice, little to no axonal regeneration was observed as compared to wildtype mice (Bartsch et al., 1995). However, in another studies using MAG-/- mice, there was a slight yet significant improvement in growth *in vitro* and axonal regeneration in the CST *in vivo* was observed (Li et al., 1996). These observations from MAG-/- mice

indicated that although MAG contributes significantly to the inhibition of axon growth associated with myelin, other inhibitory components in myelin exist.

In 2003, three independent studies using three different methods produced conflicting reports on the role of Nogo on inhibition (Kim et al., 2003, Simonen et al., 2003, Zheng et al., 2003). The Strittmatter group generated mice containing a Nogo mutation that eliminated NogoA and NogoB expression (NogoA/B -/- mice) (Kim et al., 2003). Following SCI in these mice, they observed that there was significant axonal sprouting in the CST rostal to the lesion and improved locomotor function as compared to control mice (Kim et al., 2003). The Schwab group generated Nogo-A knockout (KO) mice, which interestingly displayed an increase in NogoB expression in the CNS. Following dorsal hemisection in Nogo-A KO mice, enhanced CST axon regeneration was observed as compared to control (Simonen et al., 2003). These two groups demonstrated similar results, showing significant regeneration of CST axons following SCI using two different Nogo KO mice. The Tessier-Lavigne group generated two additional strains of Nogo KO mice: Nogo-A/B mutant mice and Nogo-A/B/C mutant mice. Using purified myelin from Nogo-A/B mutant mice, it was shown that inhibition of neurite outgrowth was decreased *in vitro* (Zheng et al., 2003). However, in both strains of mutant mice, no significant improvement in CST axonal regeneration was observed following dorsal hemisection (Zheng et al., 2003).

In OMgp -/- mice, an increase in collateral sprouting was observed at the nodes of Ranvier, which suggests that OMgp may function to prevent axonal sprouting (Huang et al., 2005). Recently, *in vivo* analysis of OMgp -/- mice on a mixed 129BL6 genetic background following complete transection or dorsal hemisection of the spinal cord

showed increased axonal regeneration and functional recovery compared to wildtype mice (Ji et al., 2008).

Two groups used NgR -/- mice to assess how NgR contributes to mediating inhibitory signals from myelin and blocking regeneration *in vivo* (Kim et al., 2004, Zheng et al., 2005). The Strittmatter group performed a dorsal hemisection or complete transection of the spinal cord in NgR -/- mice and found limited regeneration of raphespinal and rubrospinal fibers, but no regeneration of the CST. However, they did observe functional motor recovery (Kim et al., 2004). Similarly, the Tessier-Lavigne group did not detect any regenerating fibers in the CST following dorsal hemisection in NgR -/- mice (Zheng et al., 2005). These results demonstrated that another receptor, such as PirB, might be compensating for NgR. *In vivo* studies in PirB -/- mice as well as generating a PirB/NgR double knockout would further eludicate their role in axon regeneration.

Studies in $p75^{NTR}$ -/- mice showed no axonal regeneration in the descending CST (Song et al., 2004, Zheng et al., 2005) or in ascending sensory tracts following SCI (Song et al., 2004). Given that TROY also interacts with NgR and is a homolog of $p75^{NTR}$, it is to little surprise that studies in $p75^{NTR}$ -/- mice did not promote regeneration. Thus far, *in vivo* studies using TROY -/- mice have yet to be reported.

1.3.1.3 Activating Stimulatory Pathways: cAMP and PTEN/mTOR

Another approach to promoting regeneration is to block or reverse the inhibitory signals within the neurons, hence activating growth-promoting pathways. In the nervous system, cyclic-adenosine monophosphate (cAMP) and its downstream signaling pathway

modulates a number of neuronal processes, such as learning and memory (Frey et al., 1993, Wong et al., 1999), growth cone turning, and axonal guidance during development (Song et al., 1998). Our lab has shown that activation of the cAMP pathway blocks the inhibitory effects of myelin-associated inhibitors (Cai et al., 1999, Qiu et al., 2002). Our group also showed that cAMP levels are significantly higher in embryonic CNS neurons, which exhibit a spontaneous regenerative capability, than in adult neurons, which do not regenerate (Cai et al., 2001).

In DRG neurons, lesioning of the peripheral branch, one day or seven days prior to performing a dorsal column lesion, improves regeneration of the damaged CNS axons, a phenomenon termed the conditioning lesion effect (Richardson and Issa, 1984, Richardson and Verge, 1986, Neumann and Woolf, 1999). This effect was also found to be dependent on the elevation of intracellular cAMP levels (Neumann et al., 2002, Qiu et al., 2002). In fact, a single injection of a cAMP analog, db-cAMP directly into DRG is sufficient to mimic the effect of the conditioning lesion and induces extensive regeneration of dorsal column axons following injury (Neumann et al., 2002, Qiu et al., 2002). Similarly, another study showed that a single injection of db-cAMP intraocularly was also sufficient to promote RGC axonal regeneration following an optic nerve crush (Monsul et al., 2004).

In our studies, elevating intracellular cAMP levels in neurons was accomplished in three ways: directly through the application of dbcAMP; by blocking phosphodiesterases (PDEs), the enzymes which break down cAMP, with the PDE inhibitor, rolipram; or by pretreating with neurotrophins, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Cai et al., 1999, Gao et al., 2003, Nikulina et al., 2004).

Elevating cAMP by the aforementioned methods resulted in enhanced neurite outgrowth in the presence of MAG or myelin *in vitro*. In addition, application of rolipram promoted regeneration *in vivo*, improved functional recovery and was shown to decrease reactive astrogliosis following a lateral hemisection of the spinal cord (Nikulina et al., 2004). Activation of the cAMP pathway can clearly block the inhibitory effects of myelin on axonal regeneration, however understanding the downstream effectors that stimulate growth-promoting molecules must next be explored.

In an attempt to find alternative pathways to block myelin-mediated inhibition, the He group analyzed cell growth control genes using virus-induced *in vivo* conditional knockouts in RGCs (Park et al., 2008). Using several different strains, they found that mice with deletions in the tumor suppressor, phosphate and tension homolog (PTEN) in adult RGCs exhibited improved regeneration following optic nerve crush and enhanced neuronal survival (Park et al., 2008). PTEN negatively regulates the mammalian target of rapamycin (mTOR) pathway, which modulates several cellular activities, such as survival, proliferation, and cell growth by regulating cap-dependent protein translation initiation (Park et al., 2008, Park et al., 2010). Interestingly, they found that following axotomy of RGCs in wildtype mice, mTOR activity was decreased and new protein synthesis was reduced, which could contribute to the lack of regeneration (Park et al., 2008). To test if mTOR activation is necessary for the regenerative effects of PTEN deletions, the mTOR inhibitor rapamycin was administered and this resulted in reduced regeneration and RGC survival following injury (Park et al., 2008). Based on these results, they concluded that the activation of the mTOR pathway was sufficient to promote regeneration and survival following optic nerve injury.

1.3.1.4 Downstream Effectors of cAMP

Elevation of intracellular cAMP levels leads to the activation of cAMP-dependent protein kinase (PKA) and this activation is necessary for the enhanced regeneration detected when neurons are primed with neurotrophins (Cai et al., 1999) or receive a conditioning lesion (Neumann et al., 2002, Qiu et al., 2002). It was shown that priming CGN and DRG neurons with neurotrophins and a PKA inhibitor in the presence of MAG or myelin prevented growth, indicating that cAMP signaling is dependent on PKA to reverse myelin-induced inhibition (Cai et al., 1999). Since these events are PKA-dependent, the downstream effectors involved in encouraging regeneration must be identified. One of these targets, extracelluar signal-regulated kinase (ERK), is activated when neurons are primed with neurotrophins, such as BDNF (Gao et al., 2003). Activated ERK will in turn block PDE, possibly by phosphorylation, which ultimately leads to an increase in cAMP levels (Gao et al., 2003). Using a pharmacological inhibitor of ERK or dominant negative ERK, it was shown in CGN or DRG neurons that BDNF was unable to reverse MAG or myelin inhibition of neurite outgrowth *in vitro* (Gao et al., 2003). In addition, blocking PDE4 activity with rolipram also blocked inhibition induced by MAG or myelin in the absence of BDNF (Gao et al., 2003).

In an attempt to identify genes that are influenced by cAMP elevation, a microarray analysis, which was custom made with all known genes, followed by qRT-PCR was performed. Samples were obtained from rat P5 DRG neurons that were treated with dbcAMP and DRG neurons isolated from adult rats following a peripheral lesion. The results obtained from the microarray revealed that under both conditions, eleven genes were upregulated more than 2-fold and these results were later confirmed by qRT-PCR

(unpublished observation, Filbin lab). One of these upregulated genes, Arginase 1 (Arg1) was of great interest given that it is the key enzyme necessary for the synthesis of polyamines, which are involved in cytoskeleton formation and regulation (Cai et al., 2002). A long term effect of polyamines is the promotion of microtubule assembly by regulating the expression of key cytoskeletal proteins, β-actin and α-tubulin (Kaminska et al., 1992). These effects on the cytoskeleton may contribute to the ability of polyamines to improve axonal regeneration in response to MAG or myelin. Our lab has shown that application of polyamines is sufficient to block MAG or myelin inhibition of neurite outgrowth *in vitro* (Cai et al., 2002) and promote axonal regeneration *in vivo* (Deng et al., 2009). More importantly, Arg I expression increases when neurons are exposed to db-cAMP or BDNF and this upregulation is both PKA- and cAMP response element binding protein (CREB)-dependent (Cai et al., 2002, Gao et al., 2004). In addition, another gene upregulated and identified in the microarray, the cytokine interleukin-6 (IL-6), was shown to overcome MAG and myelin-induced inhibition of neurite outgrowth *in vitro* and was sufficient to enhance regeneration *in vivo* (Cao et al., 2006). However, IL-6 overcomes inhibition in a transcription-dependent, but PKA-independent manner (Cao et al., 2006).

As mentioned above, cAMP-induced block of inhibition is transcription-dependent. This was shown when neurons treated with transcription inhibitor 5, 6-dicholro-1-β-D-ribfuranosylbenzimidazole (DRB) prevented BDNF and dbcAMP from reversing MAG and myelin-induced inhibition of growth *in vitro* (Cai et al., 2002). Furthermore, it was shown that cAMP blocks inhibition in a CREB-dependent manner (Gao et al., 2004). Neurons infected with an adenovirus expressing a dominant-negative CREB were unable

to overcome MAG-mediated inhibition in the presence of dbcAMP or BDNF (Gao et al., 2004). In addition, using an adenovirus expressing a constitutively active CREB alone was sufficient to facilitate growth of DRG axons *in vitro* and promote regeneration *in vivo* following a dorsal column lesion (Gao et al., 2004).

1.3.2 Blocking Glial Scar Inhibitors

As previously discussed, several ECM proteins, such as CSPGs, are upregulated in reactive astrocytes following injury, and this contributes to the lack of CNS regeneration (Silver and Miller, 2004). Eliminating CSPG function by treating with chondroitinase ABC, which removes the GAG chain in CSPGs, resulted in enhanced regeneration *in vivo* and functional recovery following injury (Moon et al., 2001, Bradbury et al., 2002). In the first study, the nigrostriatal tract of adult rats was transected and chondroitinase ABC was delivered to the site of axotomy, which resulted in enhanced axonal regeneration (Moon et al., 2001). The next study showed that after SCI, chondroitinase ABC was intrathecally administered to the injury site and these animals displayed improved axonal regeneration of ascending sensory projections and descending CST fibers as well as exhibited improved locomotor functions (Bradbury et al., 2002). Although studies using chondroitinase ABC *in vivo* led to increased axonal regeneration, growth past the lesion site was limited. This may be due to the incomplete removal of GAG chains, which can lead to the partial block of growth (Lemons et al., 2003). In 2004, a DNA enzyme was generated to target the mRNA of the GAG chain initiating enzyme, xylosyltransferase-1 (XT-1), which is necessary to initiate glycosylation of the protein backbone of proteoglycans (Grimpe and Silver, 2004). Delivery of XT-1 to the

lesion site following a SCI resulted in significant axonal regeneration around the core of the lesion (Grimpe and Silver, 2004). In addition, blocking CSPG synthesis with decorin was shown to promote axon growth following acute stab injury to the adult rat spinal cord (Davies et al., 2004).

1.3.3 Providing a Permissive Environment: Cell Transplantation

Given that the CNS environment contains several inhibitory factors that limit regeneration following injury, providing a permissive environment could encourage growth. Several studies created favorable environments for growth by transplanting cells, such as Schwann cells (Xu et al., 1995), olfactory bulb enshealthing cells (OEC) (Navarro et al., 1999), fibroblasts (Liu et al., 1999), embryonic stems cells (ESC) (McDonald et al., 1999), and marrow stromal cells (MSC) (Hofstetter et al., 2002) into lesion sites to promote regeneration of damaged fibers. Schwann cell were seeded in guidance channels and grafted into transected rat spinal cords after SCI (Xu et al., 1995). One month after injury, myelinated fibers were found in these channels and there was extensive regeneration of both propriospinal and sensory axons in the spinal cord (Xu et al., 1995). A suspension of OECs was injected into the dorsal root entry zone following transection of the L3 to L6 dorsal roots and one month later, animals that received OEC transplants showed enhanced regeneration and functional recovery of sensory function (Navarro et al., 1999). Fibroblasts were infected with a retrovirus expressing BDNF and grafted into the lesion site after a partial cervical hemisection (Liu et al., 1999). One month after SCI, there was significant regeneration of fibers in the rubrospinal tracts and recovery of forelimb function (Liu et al., 1999). Neural differentiated mouse ESCs were

transplanted into the spinal cord of rats nine days after SCI and five weeks later were shown to have differentiated into astrocytes, oligodendrocytes and neurons (McDonald et al., 1999). More importantly, this resulted in improved hindlimb function (McDonald et al., 1999). Lastly, transplantation of MSCs into injured spinal cords resulted in the formation of bundles bridging the epicenter of the injury and improved cell survival and recovery (Hofstetter et al., 2002). These studies demonstrate that transplanting cells into lesion sites following injury not only creates permissive environments, but these transplanted cells also serve as bridges to faciliate growth past the lesion cysts. However, growth past the lesion site is limited, therefore a combination of therapies will be necessary to enhance axonal growth, which will allow axons to properly connect with their targets.

1.3.4 Combinatorial Therapeutic Strategies

Thus far, it has been highlighted that several factors contribute to the failure of injured axons to regenerate. These include the presence of inhibitory molecules from myelin and reactive astrocytes, lack of permissive substrates in the CNS environment to facilitate growth past the lesion site, restricted intrinsic growth-promoting signals within injured cells and lastly, lack of neurotrophic support. Although neutralizing the inhibitory factors and stimulating intrinsic growth-promoting signals has resulted in promoting regeneration and improving motor function following injury, the degree of regeneration and recovery has been modest at best. Therefore, the goal of these studies was to not only enhance regeneration, but also facilitate proper axonal reinnervation to its targets. In 1997, the Bregman group performed a dorsal hemisection on adult rats and transplanted

embryonic (E) 14 spinal cord tissue. They also administered neurotrophins into the lesion site. Two months later, they observed an increase in regenerated axons as compared to neurotrophin administration alone (Bregman et al., 1997). These results indicated that neurotrophins not only influence growth in developing neurons, but also stimulate the intrinsic growth promoting capabilities of injured neurons. In addition, the same group performed a complete spinal cord transection and transplanted fetal tissue and neurotrophins into the lesion site at two different time points: immediately following transection (acute injury) or two to four weeks after transection (chronic transplants) (Coumans et al., 2001). Interestingly, chronic transplants displayed more extensive regeneration of the supraspinal tracts and greater recovery of motor function as compared to acute application (Coumans et al., 2001). Furthermore, fibers extended into the transplanted tissue and rostrally beyond the lesion site, which suggests that injured neurons have the ability to reinitiate growth signals (Coumans et al., 2001). In 2004, the Bunge group performed a spinal cord contusion injury and found that cAMP levels decreased in the rostal spinal cord, sensorimotor cortex and brainstem. Following injury, Schwann cells were tranplanted into the lesion site along with the PDE4 inhibitor, rolipram and this resulted in enhanced sparing and myelination of supraspinal and proprioceptive axons (Pearse et al., 2004). Furthermore, a combination of rolipram and a single injection of db-cAMP close to the graft of Schwann cells significantly improved regeneration of serotonergic fibers into and beyond the site of injury and locomotor function (Pearse et al., 2004). Also in 2004, the Tuszynski group injected dbcAMP into DRGs (preconditioning stimulus) five days before performing a complete transection in rat spinal cords. This was followed by grafting MSCs/NT-3 into the lesion site and

injection of NT-3 (postinjury stimulus) rostral to the lesion site. This combination of factors was administered to stimulate growth signals in the soma, to provide a permissive substrate for growth through the lesion and to attract axonal growth beyond the lesion site (Lu et al., 2004). Following a one to three month post-operative period, there was extensive regeneration of dorsal-column sensory axons into and beyond the lesion, however no significant functional recovery was observed (Lu et al., 2004). In 2008, the same group not only sought to facilitate growth beyond the lesion, but also to guide regenerated axons to their targets and establish new synapses. First, they performed a conditioning lesion of the sciatic nerve. Seven days later, the dorsal column was lesioned and MSCs and NT-3 were grafted into the lesion (Alto et al., 2009). In addition, lentiviruses expressing NT-3 were injected into the nucleus gracilis (appropriate target) and reticular nucleus (inappropriate target) immediately after grafting (Alto et al., 2009). The results showed that axons regenerated through the lesion site and into the brainstem (Alto et al., 2009). More importantly, NT-3 expression in the midbrain led to the regeneration of axons into the nucleus gracilis. These regenerating axons also formed axodendritic synapses with target neurons (Alto et al., 2009). This pivotal study demonstrated that chemotropic axon guidance factors, such as neurotrophins play an important role in facilitating target selection and synaptic formation by regenerating fibers following SCI.

1.4 Neurotrophin Signaling: Blocking MAG-Induced Inhibition by cAMP Elevation

Neurotrophins (NTs) such as BDNF and NGF have been widely studied in the nervous system, especially during development. NTs regulate a plethora of neuronal functions such as cell morphology, differentiation, synaptic plasticity, survival, and synaptic transmission (Boulanger and Poo, 1999). In order for NTs to mediate intracellular signaling cascades, NTs must bind to Trk receptors and induce dimerization, which leads to the autophosphorylation of several conserved tyrosines in the cytoplasmic portion of the receptor (Huang and Reichardt, 2003). These phosphorylated tyrosines create docking sites for numerous adaptor proteins containing PTB or SH2 domains. Interacting with these adaptor proteins will in turn activate a number of signaling pathways, such as Ras, PI 3-kinase, and PLCγ, which leads to the induction of gene expression, neuronal survival and neurite growth (Huang and Reichardt, 2003). Of particular interest is the Ras-Raf-ERK pathway, which is involved in long-term potentiation, synaptic plasticity, and survival of neurons (Kaplan and Miller, 2000). In RGCs, the MEK/ERK pathway has been implicated in promoting survival of these neurons following injury (Shen et al., 1999).

As previously discussed, we have shown that pre-treating or "priming" neurons with BDNF prior to being exposed to myelin-based inhibitors such as MAG, will block inhibition of growth by these myelin-based proteins in a cAMP-dependent manner (Cai et al., 1999). However, if neurons are treated directly with BDNF and immediately exposed to MAG or myelin, BDNF does not reverse inhibition (Cai et al., 1999). Interestingly, when CGNs were pretreated with pertussis toxin, then exposed simultaneously to BDNF

and MAG, BDNF was able to overcome inhibition without priming (Cai et al., 1999). Pertussis toxin inhibits the heterotrimeric G protein subunit, $G\alpha_i$, which is known to negatively regulate transmembrane adenylyl cyclases (Post and Brown, 1996). Therefore, these results suggested that MAG activates $G\alpha_i$, which potentially inhibits transmembrane adenylyl cyclases and in turn prevents BDNF from inducing cAMP production. For this reason, it was thought that if cAMP production was blocked, then BDNF must overcome MAG-mediated inhibition by blocking cAMP degradation. Subsequently, our lab showed that this was partially the case: BDNF binds to TrkB receptors and activates ERK, which results in the inactivation of PDEs, and reduced breakdown of cAMP (Gao et al., 2003). This results in the elevation of cAMP and the activation of PKA and CREB, which are necessary for the expression of regeneration-associated genes to block MAG/myelin-induced inhibition (Cai et al., 1999, Gao et al., 2004).

However, it is now well-established that not only is it important to regulate the degradation of cAMP, but the rate of its synthesis must also be controlled so that its levels can be elevated and induce the desired intracellular response (Bender and Beavo, 2006). This is of great importance given that in most tissues, PDE activity is higher than cyclase activity (Bender and Beavo, 2006). Therefore, it is essential to elucidate how cAMP is produced in response to BDNF. Since a balance between the production and degeneration of cAMP is necessary to establish intracellular cAMP levels that are sufficient to induce signaling cascades, it is important to explore the activation of adenylyl cyclase, the enzyme responsible for the synthesis of cAMP.

1.5 Adenylyl Cyclases

The synthesis of the second messenger, cyclic adenosine-3', 5'-monophosphate (cAMP), results from the catalysis of adenosine triphosphate (ATP) into cAMP and inorganic pyrophosphate. The enzymes responsible for this conversion are adenylyl cyclases. In mammals, there are two families of the Class III adenylyl cyclases that generate cAMP, transmembrane adenylyl cyclase (tmAC) and soluble adenylyl cyclase (sAC) (Kamenetsky et al., 2006). Although they are differentially regulated, their crystal structures show that the structure of the catalytic domains is conserved (Tang and Hurley, 1998, Geng et al., 2005). Both enzymes contain two structurally similar catalytic cores, C1 and C2, which dimerize to form an active cyclase (Tang and Hurley, 1998). The dimer interface, where the active site is located, contains the essential residues that are necessary for cyclase activity. Interestingly, analysis of their crystal structure reveals that these essential residues are located on only one site of the dimer center (Tesmer et al., 1997). Therefore, only one site of the dimer interface is active to catalyze ATP. Although the dimer appears symmetrically similar, the cores seem to asymmetrically dimerize or pseudo-heterodimerize making them functionally distinct. These structural and functional differences may account for their unique regulation, which will be discussed in detail below (Tesmer et al., 1997, Zhang et al., 1997). These enzymes also differ in their spatial distribution throughout the cell as well as their distribution in different tissues within the body (Hanoune and Defer, 2001).

1.5.1 Transmembrane Adenylyl Cyclase (tmAC)

Expression & Structure

tmACs are expressed from nine discrete genes (type I to type IX) which give rise to 9 similar transmembrane isoforms (AC1 to AC9) (Taussig and Gilman, 1995). Each tmAC is approximately 120 kDa and consists of a short cytoplasmic N-terminus, 2 hydrophobic domains (each containing a six transmembrane span) which separates two cytoplasmic loops that contain the two catalytic domains, designated $C1_a$ and $C2_a$ respectively (Taussig and Gilman, 1995). When $C1_a$ and $C2_a$ interact, they pseudo-heterodimerize to form an active cyclase. tmACs are located within the plasma membrane and the different subtypes display discrete patterns in their distribution inside cells as well as in their tissue distribution (Hanoune and Defer, 2001). However, distinguishing subcellular localization of each tmAC has been limited due to the lack of high quality antibodies (Hanoune and Defer, 2001). *In situ* hybridization studies revealed that each tmAC is present throughout the CNS, but individual isoforms are limited to certain areas within the CNS (Matsuoka et al., 1992, Furuyama et al., 1993, Mons et al., 1995). The tmACs that are highly expressed in the CNS are AC1, AC2 and AC8, which in mammals have been linked to synaptic plasticity. They are found in areas involved in learning and memory, such as the cerebral cortex, cerebellum and hippocampus (Mons and Cooper, 1995, Hanoune and Defer, 2001).

Regulation

In 1985, when the catalytic domains of tmACs were purified and characterized from both bovine brain cortex (Pfeuffer et al., 1985b) and rabbit myocardial membrane (Pfeuffer et

al., 1985a), it revealed the potential site of interaction of its regulators. Within the dimer interface of the catalytic domains, two distinct pockets are formed. ATP will bind to one pocket to be catalyzed to cAMP and the other, which is non-catalytic, will bind certain regulator molecules, such as forskolin (Linder and Schultz, 2008). Studies on the crystal structure of tmACs revealed that they are largely regulated by the displacement and reorganization of the catalytic domains in the dimer (Zhang et al., 1997). This displacement was shown to greatly enhance catalytic activity (Linder and Schultz, 2008).

Following their cloning, nearly all tmAC isoforms were found to be primarily regulated by $G_s\alpha$ and diterpene forskolin (Tang and Hurley, 1998). G proteins can positively or negatively regulate tmACs, through the subunits $G_s\alpha$ or $G_i\alpha$ respectively (Tang and Hurley, 1998). G protein-coupled receptors (GPCRs) are activated by ligands, such as hormones and neurotransmitters, that will in turn activate G-proteins, which consist of three subunits, Gα, Gβ and Gγ (Bridges and Lindsley, 2008). Upon activation, the inactive GDP form of Gα is converted to an active GTP Gα and dissociates from Gβ and Gγ (Bridges and Lindsley, 2008). $G_s\alpha$ was shown to interact with AC $C1_a$ and $C2_a$ at the non-catalytic pockets and resulted in the displacement of the dimer, by promoting closure of the catalytic site. On the other hand, it is not clear whether or not regulation by forskolin leads to the closure and displacement of the dimer (Linder and Schultz, 2008). Forskolin is a small molecule that is derived from the root of the plant *Coleus forskolhii* (Seamon and Daly, 1986) and was found to bind to a single site on the C_{1a}/C_{2a} heterodimer, in a hydrophobic patch at the dimer interface (Tang and Hurley, 1998). Analysis of its crystal structure reveals that forskolin may create a hydrophobic bridge

between the two monomers, $C1_a$ and $C2_a$ (Zhang et al., 1997). This hydrophobic bridge is believed to stabilize the dimer (Zhang et al., 1997).

To a lesser extent, certain tmACs are also regulated by calcium receptor protein calmodulin. Calcium/calmodulin (CaM) can directly regulate AC1 and AC3 by interacting with $C1_b$ and $C2_b$, which are regulatory regions located between $C1_a$ and $C2_a$ (Tang and Hurley, 1998). It can also indirectly regulate tmACs by modulating CaM kinase activity, specifically CaMK II (Kamenetsky et al., 2006); however, the precise mechanism of calmodulin activation is unknown (Tang and Hurley, 1998). Additionally, on a structural basis, it is challenging to elucidate their mechanism for activation of tmAC and to determine which types of calcium signals they respond to (Linder and Schultz, 2008).

1.5.2 Souble Adenylyl Cyclase (sAC)

Discovery, Expression & Structure

In 1975, Braun and Dods discovered an Mn^{2+}-sensitive, "soluble" adenylyl cyclase in sperm found in the seminiferous tubules and epididymis of rat testis. It was shown to be present in the seminiferous tubules of immature rats at early stages of spermatogenesis, specifically in spermatid cells until they develop into mature spermatozoa (Braun and Dods, 1975). After centrifugation, activity was detected in the cytosolic fractions (Braun and Dods, 1975). In 1981, this Mn^{2+}-dependent adenylyl cyclase was purified from cytosolic fractions of rat testis and was found to be 47-48 kDa (Gordeladze and Hansson, 1981). Furthermore in 1983, it was shown in several different mammalian sperm cells

that this cytosolic adenylyl cyclase was not activated by forskolin, which was the first indication that its regulation is different from that of tmACs (Forte et al., 1983). In 1999 the Levin and Buck group purified, cloned, and functionally expressed the cDNA that encoded soluble adenylyl cyclase (sAC) from rat testis (Buck et al., 1999). They found a full-length cDNA sequence, which was predicted to express a 187kDa protein. It was thought that this full-length form would undergo proteolytic processing to give rise to the catalytically active 48kDa "truncated" protein (Buck et al., 1999). Then in 2001, it was shown that these isoforms were derived from the alternative slicing of the sAC gene (Jaiswal and Conti, 2001).

In addition to the finding that sAC was present in cytosolic fractions isolated from rat testis, studies using HEK 293 cells stably transfected to express full-length sAC also revealed significant adenylyl cyclase activity in the pellet following centrifugation (Buck et al., 1999, Chen et al., 2000b). They showed by Western blot analysis that sAC was also highly present in the particulate fraction of various mammalian cells (Chen et al., 2000b). These results suggested that sAC is not only found in the cytosol, but also may be tethered to intracellular membranes or associated with membrane-bound molecules, such as receptors or anchoring proteins. Although sAC was widely studied in the male rodent reproductive system, RT-PCR showed that sAC is expressed in many other tissues, such as brain, liver and kidney (Sinclair et al., 2000).

It was not until 2005 that the Moe group cloned and characterized human sAC (hsAC) and also found it to be expressed in various tissues, such as brain, heart, liver and kidney (Geng et al., 2005). Using RT-PCR, they detected three splice variants and confirmed these results by sequence analysis (Geng et al., 2005). Western blotting revealed

isoforms at approximately 190- and 80-kDa from both the cytosolic and membrane fractions of several human-derived cell lines. Immunofluorescence showed that hsAC was ubiquitously expressed throughout the cell and interestingly, was found to co-localize with microtubules (Geng et al., 2005).

Most sAC isoforms contain two catalytic domains, C1 and C2, within the N-terminus which pseudo-heterodimerize to form active cyclases (Kamenetsky et al., 2006). It is believed that the active site within the catalytic dimer opens following substrate binding and subsequently closes to drive catalysis of ATP to produce cAMP and pyrophosphate (Steegborn et al., 2005, Kamenetsky et al., 2006). Interestingly, these catalytic domains were found to be more closely related to those of cyanobacteria and myxobacteria, than to those of mammalian tmACs (Buck et al., 1999). These bacteria contain multiple isoforms of ACs with a single catalytic domain. Comparisons of these single domains with the domains in sAC suggest that during evolution, fusion of distinct bacterial domains gave rise to the C1 and C2 domains in sAC (Buck et al., 1999).

Presently, three isoforms of sAC have been identified in rodents: sAC full-length (sACfl), sAC truncated (sACt) and somatic sAC. Both sACfl and sACt contain the two catalytic domains, C1 and C2, that form active cyclases but differ in their intrinsic specific activities (Kamenetsky et al., 2006). sACfl is a 187 kD protein that has an autoinhibitory domain 17kb downstream from the C2 catalytic domain (Chaloupka et al., 2006). This autoinhibitory domain gives sACfl lower activity compared to sACt (Chaloupka et al., 2006). sACt differs from sACfl in that it is a ~50 kD protein, which lacks exon 12 and has twenty times more activity (Chaloupka et al., 2006). Recently discovered somatic sAC lacks the C1 domain, contains a unique start site at exon 5 and is ~50 kD (Farrell et

al., 2008). Since all cyclases require both catalytic domains to be active, the mechanism for somatic sAC activity is unknown.

Regulation

As previously mentioned, both sACfl and sACt have been extensively studied in the male reproductive system and are required for sperm cell maturation and motility (Esposito et al., 2004, Hess et al., 2005). It was shown that these events are both cAMP- and bicarbonate-dependent (Garbers and Kopf, 1980, Garty and Salomon, 1987). Given that studies in human epididymal sperm showed that a combination of 50mM calcium and 50mM bicarbonate caused increased adenylyl cyclase activity in human ejaculated sperm (Rojas et al., 1993), it was plausible that both calcium and bicarbonate could potentially regulate sAC. In 2000, using a purified recombinant sAC, the Levin and Buck group demonstrated that bicarbonate increased sAC activity by seven fold and this response was not due to alterations in pH (Chen et al., 2000b). Furthermore, they showed that tmAC activity was insensitive to bicarbonate, illustrating that tmAC and sAC have uniquely different modes of regulation (Chen et al., 2000b). In 2003, biochemical and kinetic analysis on purified recombinant sAC revealed that bicarbonate regulates sAC by increasing the maximum velocity (V_{max}) of the enzyme (Litvin et al., 2003). Additionally, they found that calcium modulates sAC activity by lowering its Michaelis Menten constant (*Km*) for ATP-Mg^{2+}, which will in turn increase its affinity for substrate ATP-Mg^{2+}, independently of calmodulin (Litvin et al., 2003). Interestingly, bicarbonate and calcium were shown to activate sAC synergistically (Litvin et al., 2003).

Intracellular Distribution

Given that once cAMP is generated, it can only diffuse a short distance from ACs (Zippin et al., 2003) and that PDEs form barriers or "firewalls" that also limit its diffusion (Beavo et al., 1994), cAMP effector proteins must be located near their targets. Therefore, determining where sAC is localized intracellularly would clarify how multiple cAMP effector proteins are activated far from the plasma membrane. In various types of non-neuronal cells, sAC was shown to be expressed in specific subcellular domains, such as in the mitochondria, mid-bodies, microtubules, and nucleus (Zippin et al., 2003). Several of these distinct microdomains contain cAMP effector molecules such as the exchange protein activated by cAMP (EPAC) and protein kinase A (PKA). A subset of PKA targets are localized to the plasma membrane, while others are tethered within the cytoplasm by A kinase anchoring proteins (AKAPs) (Zippin et al., 2003). Also, in COS7 cells, bicarbonate-induced sAC activation was shown to mediate PKA-dependent activation of CREB in the nucleus (Zippin et al., 2004). This nuclear cAMP signaling microdomain is modulated by intrinsic cellular signals, such as local intracellular concentrations of bicarbonate and calcium (Zippin et al., 2004). In mitochondria isolated from mouse liver, the Manfredi group identified a mitochondria-sAC (mito-sAC) signaling cascade generated within the mitochondria that acts as a metabolic sensor to regulate oxidative phosphorylation (Acin-Perez et al., 2009). Additionally, studies in coronary endothelial cells showed that sAC modulated mitochondria-dependent apoptosis (Kumar et al., 2009).

1.6 Goal of this Work

In the adult mammalian CNS, axons do not spontaneously regenerate following injury. This lack of axonal regeneration is partly due to the presence of inhibitory proteins in myelin, such as MAG. Previously, we have shown that elevating cAMP by pretreating (priming) neurons with neurotrophins, such as BDNF, is sufficient to overcome the block of axonal outgrowth by MAG. This downstream BDNF effect is ERK-dependent, leading to the block of PDE4 and therefore preventing the conversion of cAMP to AMP. A balance between the production of cAMP by adenylyl cyclases and its degradation by PDEs will ultimately determine intracellular cAMP levels. Therefore, our goal is to determine if the rise in cAMP in response to BDNF priming is partially dependent on sAC activation. To accomplish this, I will:

1. Identify whether sAC is expressed in postnatal rat primary neurons.

2. Determine whether sAC plays an integral role in BDNF signaling to overcome inhibition of axonal growth by MAG.

Chapter II: Materials and Methods

2.1 Cell Culture: CHO cells and HEK 293

Control or stably-transfected MAG-expressing Chinese Hamster Ovary (CHO) cells (Mukhopadhyay et al., 1994) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) which had been supplemented with 10% dialyzed fetal bovine serum (FBS, Gibco), 34.8mM L-Proline (Sigma), 10mM Glycine (Sigma), 300nM Thymidine (Sigma) and 2mM L-Glutamine (Gibco) at 37ºC in 7.0% CO_2. These cells were used to make monolayers for the neurite outgrowth assay (NOG) described below.

Human Embryonic Kidney (HEK) 293 cells that were stably-transfected to express TrkB (Narisawa-Saito et al., 2002) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) which had been supplemented with 10% qualified fetal bovine serum (FBS, Gibco), 1X antibiotics/antimycotic (Gibco) and 2mM L-Glutamine (Gibco) at 37ºC in 7.0% CO_2. HEK 293 TrkB cells were used as a positive control for immunoprecipitations to show if sAC and TrkB interact.

2.2 Isolation of Neurons

Cerebellar Granular Neurons

Post-natal day 5-7 (P5-7) Long-Evans rats were sacrificed and the cerebellum were recovered in 2ml of Neurobasal media. The tissue was dissociated by incubation with 1mg of papain for 20-30 minutes at 37ºC / 7.0%CO_2. The media was removed and 2ml of fresh Neurobasal media was added with 1mg papain and 200µg of DNase 1, then incubated for an additional 20-30 minutes. Papainization was stopped using 100µg of soybean trypsin inhibitor for 2 minutes, washed three times with Neurobasal media,

triturated and passed through a 40 μm cell strainer, then centrifuged for 5 minutes at 2000 rpm. The isolated cerebellar granular neurons (CGN) were resuspended to a single-cell suspension in a modified Sato medium (DMEM; 10% Path-O-Cyte BSA; 20nM progesterone; 100mM putrescine; 30nM sodium selenite; 5mg/ml insulin; 80ng/ml tri-iodo-thyronine (T3); 10ng/ml thyroxine (T4); 118 U/ml penicillin; 118 mg/ml streptomycin; 295 ng/ml amphotericin B), counted and plated.

Dorsal Root Ganglia

P5-7 Long-Evans rats were sacrificed and the dorsal root ganglia (DRG) were collected on ice into 1ml tubes of 0.1% Collagenase in Hank's Balanced Salt Solution (HBSS, Gibco) buffer. The DRG were incubated for 20-30 minutes at 37ºC/7.0%CO_2 with gentle flicking of the tube every 10 minutes. DRG were allowed to settle in the tube and supernatant was removed and replaced with fresh of 0.1% Collagenase in HBSS buffer and 200μg of DNase 1, then incubated for an additional 20-30 minutes. After incubation, 0.1x trypsin and 50μg DNase I were added with fresh HBSS buffer and the cells were incubated a further 15 minutes at 37ºC/7.0%CO_2. Trypsinization was stopped with DMEM media containing 10% serum. The cells were triturated in HBSS with large pipette tip (P1000), then a smaller pipette tip (P200). Dissociated DRG were then washed three times with DMEM and resuspended in Sato media, counted, and plated accordingly.

Cortical Neurons

P0-P2 Long-Evans rats were sacrificed and cortices were collected on ice in Neurobasal media. The tissue was dissociated by incubation with 100μg of papain for 20-30 minutes at 37ºC / 7.0%CO_2. The media was removed and 2ml of fresh Neurobasal media was

added with 1mg papain and 200μg of DNase 1, then incubated for an additional 20-30 minutes. Papainization was stopped using 100μg of soybean trypsin inhibitor for 2 minutes, washed three times with Neurobasal media, triturated and passed through a 40 μm cell strainer, then centrifuged for 5 minutes at 2000 rpm. The isolated neurons were resuspended in 6 ml of Neurobasal media. The cells were resuspended and the single cell suspension was 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 manufacturer's manual from Optiprep™. The neuron-enriched suspension was diluted with plain neurobasal, and pelleted by centrifugation. The pellet containing dissociated neurons was then resuspended in Neurobasal media supplemented with B27 (Sigma), 1X antibiotics/antimycotic (Gibco) and 2mM L-Glutamine (Gibco), and neurons were counted and plated.

2.3 Priming Neurons

24-well tissue culture plates were coated with 100mg/ml of poly-L-lysine (PLL) for at least 30 minutes at room temperature. The wells were then washed once with double distilled water to remove excess PLL. Isolated CGN or cortical neurons were plated onto each well at a concentration of approximately 1 x 10^6 cells/well. These neurons were then pretreated with sAC inhibitors, KH7 (1μM) or OH-E (1μM), tmAC inhibitor, ddAdo (50 μM) for 1 hour, and then treated ± with BDNF (200ng/ml) in SATO. The neurons were

then cultured for 15-17 hrs at 37ºC/7.0%CO_2 before they were removed from the dish via trypsinization (0.4x trypsin) for 10 minutes at 37ºC /7.0%CO_2. Trypsinization was stopped by 10% serum-containing media (dDMEM) and the cells were collected, resuspended in fresh Sato, counted and plated onto either a purified myelin substrate or MAG-expressing CHO cells.

2.4 Purified Myelin: Preparation and Use

Preparation

The medulla from an adult rat brain is isolated and homogenized in a 0.25M sucrose solution containing a protease inhibitor cocktail (CalBiochem) using a glass and teflon homogenizer. The homogenate is then mixed with a 2.55 M sucrose solution to create a 1.4M solution. This is then layered onto a 1.9M sucrose solution, followed by 0.85M and 0.25M solutions. The gradient is centrifuged at 40,000 rpm for 14 hours at 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.

Immobilized on Slides

8 chamber glass slides were coated with 100µg/ml PLL for 30 minutes at room temperature. After incubation, PLL was removed and washed once with dH_2O. Myelin was then plated at a concentration of 2 mg/well and then dried overnight in a vacuum chamber filled with Drierite dessicator. Slides were then used immediately.

2.5 Neurite Outgrowth Assay

Monolayers

8-well tissue culture glass slide (Lab-Tek) were coated with 20μg/ml of PLL at room temperature for 30 minutes and then coated with 10 μg/ml of fibronectin at 37°C/7.0%CO_2 for 2 hours. Confluent monolayers of control or MAG-expressing CHO cells are grown on the slides at 37°C/7.0%CO_2 overnight.

Myelin Slides

8-well tissue culture glass slide (Lab-Tek) were coated with 100μg/ml of PLL at room temperature for 30 minutes. Rat CNS myelin (see Chapter 2.4) at 2 μg total protein/well are dried overnight onto the coated wells and used as a substrate.

Cell Culture for Assay

Where indicated, isolated P5-P7 CGN at 1.0 X 10^6/ml were pretreated with sAC inhibitors, KH7 (1μM) or OH-E (1μM), tmAC inhibitor, ddAdo (50 μM) for 1 hour, then treated ± with BDNF (200ng/ml) for 15-17 hours in SATO at 37°C/7.0%CO_2. Alternatively, P1-P2 cortical neurons at 2.5 X 10^4/ml were infected with LV-LacZ or LV-sACt. Neurons are transferred and plated onto purified myelin or CHO monolayers at a cell density of 2.0-3.0 X 10^4/well and 1.0-1.5 X 10^4/well, respectively. After 22 (CHO monolayers) or 26 (myelin) hours, the cultures are fixed twice with 4% paraformaldehyde for 15 minutes. The slides were then permeablized with ice-cold methanol for 2 minutes and blocked with DMEM containing 10% FBS for 1 hour. The neurons were immunostained with β-III tubulin, a neuron-specific marker, at a dilution of 1:1000 in

PBS/0.05%BSA at 4°C overnight. Following incubation, slides were washed 3 times with 1X PBS and incubated at room temperature with a biotinylated donkey anti-mouse IgG in PBS/0.05%BSA at a 1:500 dilution for 1 hour at room temperature. The slides were washed 3 times and probed for Streptavidin-Texas Red at 1:500 in PBS/0.05%BSA. Alternatively, following β-III tubulin incubation, slides were stained with donkey anti-mouse Alexa Fluor® 568 red fluorescent antibodies at 1:1000 in PBS/0.05%BSA for 1 hour at room temperature. Finally, the cultures were washed 3 times and then immobilized using Permafluor mounting media (Immunon) and viewed under a fluorescent microscope.

Analysis

The length of the longest neurite for each β-III tubulin-positive neuron for 500-800 neurons was scanned and quantified using the Metamorph imaging analysis software. Briefly, the longest neurite from each β-III tubulin-positive neuron (500-800 neurons per well), selected systematically by progressive movement from one side of the well to the other, were traced onscreen and the mean neurite length was calculated using the software tools. Statistical analysis of the data obtained was performed using the Graphpad Prism software program.

2.6 Immunostaining of Neurons

8-well tissue culture glass slide (Lab-Tek) were coated with 100μg/ml of PLL at room temperature for 30 minutes. P5-7 CGN, P0-2 cortical neurons or P5-P7 DRG (see Chapter 2.2 for isolation) were plated at density of 6.7 X 10^4/ml (CGN and cortical

neurons) and 3.3 X 10^4/ml (DRG) and incubated at 37°C/7.0%CO_2 overnight. The cultures were fixed twice with 4% paraformaldehyde for 15 minutes each, then permeablized with ice-cold methanol for 2 minutes. The slides were then blocked with dilution buffer (25mM Tris-HCL pH 7.2, NaCL 300mM, Triton X-100 0.3%, BSA 0.5mg/ml, Thimerisol 0.01%) and 5% normal goat serum for 1 hour. After 3 washes with 1X PBS, the slides were double or triple stained with a combination of the following antibodies: monoclonal sAC antibodies, clone R21 (exon 5, 1:100), clone R37 (exon 11, 1:200), and clone R40 (exon 2, 1:100), and anti-βIII tubulin (1:1000, for neurons), and/or palloidin Alexa Fluor® 568 (1:1000, for action) in dilution buffer at 4°C overnight. Following incubation, slides were washed 3 times and probed with various Alexa Fluor® fluorescent antibodies at 1:1000 in dilution buffer for 1 hour at room temperature. The slides were then washed again 3 times and immobilized using Permafluor mounting media (Immunon) and viewed under a fluorescent microscope.

2.7 Immunoprecipitation (IP)

10 cm tissue culture plates were coated with 100mg/ml of poly-L-lysine for at least 30 minutes at room temperature. P5-7 CGN, P0-2 cortical neurons or P5-P7 DRG (see Chapter 2.2 for isolation) were plated at a density of 3.0 X 10^6/ml (CGN and cortical neurons) and 2.0 X 10^6/ml (DRG) and incubated at 37°C/7.0%CO_2 overnight. Note, the following procedures were performed on ice. The plated cells were washed 3 times with ice-cold 1X PBS with 100mM Na_3VO_4, then cells were lysed with 150µl of lysis buffer in the presence of phosphatase and protease inhibitors (1X RIPA: 50 mM Tris, 150 mM NaCl, 0.4 mM EDTA, 0.1 mM DTT, and 1 M PMSF, 10 mg/ml aprotinin, 10 mg/ml

leupeptin, 1% NP40, 2mM imidazole, 1nM NaF, 1mM Na_3VO_4, 1mM Na_2MoO_4, and 1mM $C_4H_8Na_2O_8$) (1:10 w/v). Samples were lysed on ice for 30 minutes, with vortexing every 10 minutes within that time. Homogenates were then centrifuged at top speed for 10 minutes at 4°C. The protein concentration of the supernatant fractions were determined (BioRad) and an aliquot saved at 4°C for Western blot analysis ('pre-IP lysate'). Equivalent protein amounts (200-400 μg/sample) from different supernatants were precleared by incubation with protein G beads (Amersham Pharmacia, 100 ml of 50% bead slurry) overnight at 4°C. Samples were centrifuged at top speed for 10 minutes, and the supernatant was collected into fresh tubes. Clarified lysates were incubated with specific anti-sAC antibodies (R37 or R40) or control, mouse IgG at a concentration of 2-4 μg antibody/sample for 4 hours at 4°C. Immune complexes were collected on protein G beads (100 μl of 50% bead slurry/ sample) and incubated for 1 hour. Beads were collected by centrifugation, and an aliquot of the supernatant was collected for Western blot analysis (post-IP supernatant). Beads were washed 3 times with lysis buffer, then 80 μl of 1X Laemmli Tris-Glycine SDS-PAGE denaturing, reducing sample buffer was added and samples were stored at -80°C for western blotting.

2.8 Western Blotting

For IP samples, tubes were thawed at room temperature and 5% b-mercaptoethanol with added to each sample, briefly spun, and an aliquot were used for SDS/PAGE. Proteins were transferred to PVDF membranes, which were blocked in 5% milk in TBST (1XTBS and 0.01% Tween 20) for 1 hour at room temperature, rinsed once with TBST and incubated with biotinylated mAb R21 (1:1000 in TBST) overnight at 4°C. Membranes

were rinsed in TBST and incubated with an HRP-conjugated streptavidin (1:2000 in TBST, Amersham) for 1 hour at room temperature. Bands were visualized using enhanced chemiluminescence (Pierce Co.).

For ERK expression studies, P5-7 CN (see Chapter 2.2 for isolation) were plated on 6 well plated at a density of 5×10^6 CGN/well at 37°C/7.0%CO_2 overnight. The cells were starved in DMEM for 6-8 hours then pretreated with KH7 (50μM) and tmAC inhibitor, ddAdo (50 μM) for 30 minutes before treating with BDNF (200ng/ml) for an additional 10 minutes. Note, the following procedures were performed on ice. Neurons were washed twice with ice-cold 1X PBS, then lysed with 75μl of lysis buffer as indicated above (see Chapter 2.7). Protein concentration was measured with a Bio-Rad kit. Normalized lysates in sample buffer were boiled for 5 minutes and 40μg protein was loaded per well onto a 10% polyacrylamide gel. Separated proteins were then transferred onto a PVDF membrane (BioRad), blocked in 5% milk in TBST for 1 hour at room temperature and washed 3 times for 5 minutes with TBST. The blots were then incubated with rabbit monoclonal anti-phospho p44/42 (pERK) antibody (1:1000; Cell Signaling), total p44/42 (ERK) (1:1000; Cell Signaling) and actin (1:2000) in 5% BSA/TBS overnight at 4°C. Blots were washed 3 times with TBST and incubated for 1hour at room temperature with shaking in HRP-linked goat anti-rabbit IgG-HRP antibody (Cell Signaling) at a dilution of 1:2000. Visualization of the proteins was performed using the ECL chemiluminescence kit (Amersham).

2.9 Lentivirus Production

Lentivirus production and titering was performed using the Virapower Lentiviral production kit according to the directions of the manufacturer and was generously provided to us by Dr. Levin and Dr. Buck from Weill Medical College of Cornell University. Briefly, cDNAs encoding sAC (*B*50 kDa) or LacZ were cloned into the pLenti/D-TOPO vector (CMV promoter), and virus was generated in the 293FT viral packaging cell line. These lentiviruses were used to infect neurons, followed by NOG analysis and injected into the optic nerve following an optic nerve crush for *in vivo* studies.

2.10 RT-PCR

P5-7 CGN, P0-2 cortical neurons or P5-P7 DRG (see Chapter 2.2 for isolation) were plated in 24 well tissue culture plates coated with 100μg of PLL at a density of 1.0 X 10^6/ml (CGN and cortical neurons) and 8.0 X 10^5/ml (DRG) and incubated at 37°C/7.0%CO_2 overnight. Media was removed from plate and cells were lysed in lysis buffer provided by RNeasy RNA isolation kit (QIAGEN) according to the manufacturers instructions. Total RNA was quantified spectrophotometrically and at least 2 mg of total RNA was used to generate polyA$^+$ RNA using the Micro Poly (A) Purist Kit according to manufacturer's protocol (Ambion). Purified polyA$^+$ RNA was resuspended in RNase/DNase-free water and used to synthesize cDNA with oligo dT and reverse transcriptional enzyme (Stratagene) at 37°C for 1 hour. After which, sAC cDNA was amplified using primers, forward (F): 5' ATG AGT GCC CGA AGG CAG GAA TTA CAG 3' (exon 1) and reverse (R): 5' TGC TCT CTG ATC CGG AAT CCT 3' (exon 5)

or F: 5' TTG ATG TTT AAA GAG CAA GAC AAA GCA G 3' (C2) and R: 5' CAG CAA TAT TGA CCT TTT GGC C 3' (C2) (Invitrogen) using the polymerase chain reaction (PCR, Stratagene). Amplification of GADPH from the same sample was used as control and primers used were F: 5' ATG GTG AAG GTC GGT GTG AAC G 3'and 5'TGG TGA AGA CGC CAG TAG ACT C 3'. PCR was performed according to manufacturer's protocol, briefly annealing temperature was 59°C for 30 seconds for 40 cycles (Stratagene). DNA was detected in a 1.5% agarose gel, stained with ethidium bromide.

2.11 siRNA

siRNA sequences for the sense strand of the central 19 nt double-stranded region were derived from rat sAC gene (exon 5): CCAAGUGUAUGGCCUUCAU and scrambled sequences: AUAUAUAUCUGUCGCGCGG. 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 (300nM) were then added to cultured CGN for 24 hours, after which neurons were treated with BDNF (200ng/ml) for an additional 15-17 hours at 37°C/7.0%CO_2 overnight. Neurons were then transferred onto monolayers of CHO cells for neurite outgrowth assay as described (see Chapter 2.5).

2.12 Optic Nerve Crush

Procedure

The optic nerve crush and the intraocular injection were performed as described previously (Leon et al., 2000) as follows. Male Fischer rats (250 –300 g) were deeply anesthetized with isofluorane. The optic nerve was surgically exposed, the dural sheath surrounding the optic nerve was carefully incised, and the nerve was crushed with #5 jewelers' forceps for 10 seconds. The surgical site was sutured closed, and preservation of the central retinal artery was verified by direct ophthalmoscopy for signs of ischemic damage; animals showing signs of ischemic damage were excluded from the study. Immediately after the nerve crush, 5-10 μl of LacZ expressing lentivirus (LV) or sACt expressing LV was injected intraocularly via a pulled-glass pipette using a nano- injector (WPI). Recovery of postoperative animals was observed, and they were then individually housed with *ad libitum* access to food and water. Two weeks after surgery, animal were deeply anesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) and transcardially perfused with 200 ml of heparinized saline (1000 U/l) and 300 ml of 4% PFA. The optic nerve was dissected out, post-fixed in 4% PFA overnight, and cryoprotected in 30% sucrose in Tris-buffered saline (TBS). Additionally the lens of each eye was examined for injury (opaque eyes) at the time of removal and nerves from eyes exhibiting such injury were excluded from the study.

Immunohistochemistry and image analysis

Frozen serial sections (20-30μm) were cut from the optic nerves described above and immunofluorescent labeling was performed as follows: sections were washed 4x with

TBS or PBS, blocked for 1 hour with TBS plus 0.2% Triton X-100 and 5% normal goat serum or dilution buffer with 5% normal goat serum and then incubated with sheep anti-GAP-43 primary antibody (gift from L. Benowitz, Children's Hospital, Boston, MA), diluted 1:1000 in blocking buffer, overnight at 4°C. Sections were then washed four times with TBS or PBS and incubated with goat anti-sheep conjugated to FITC or donkey anti-sheep Alexa Fluor® 488, diluted 1:500 or 1:750, respectively, in blocking buffer, for 1 hour at room temperature. Finally, sections were washed four times with TBS or PBS and coverslipped with aqueous mounting medium, permaflour, before imaging. Sections were scanned and quantitated using MetaMorph acquisition software. The distance traversed by the three longest GAP-43-positive axons, relative to the distal edge of the lesion, was measured for each animal. Three animals for sACt and two animals for LacZ were included in each group. Prism GraphPad software was used to perform one-way ANOVA, followed by multiple comparisons using the Tukey's procedure.

Chapter III: Expression of sAC in Post-Natal Neurons

3.1 Introduction

Following injury, spontaneous axonal regeneration in the adult mammalian central nervous system (CNS) does not occur. This inability to grow is partially due to the formation of the glial scar, which creates a physical and chemical barrier to regeneration, and to the release of inhibitory molecules found in CNS myelin which act as a molecular barrier (Filbin, 2003). These inhibitory proteins, such as myelin-associated glycoprotein (MAG), will block axonal growth by binding to the NgR, Lingo and p75NTR/Troy receptor complex, which leads to RhoA activation and the rearrangement of the axonal cytoskeleton (Filbin, 2003).

When cAMP levels are increased either by direct application of dbcAMP or by pretreating neurons with brain-derived neurotrophic factor (BDNF), myelin-induced inhibition of axonal outgrowth is blocked (Cai et al., 1999). BDNF binds to TrkB receptors which homodimerize and trigger an intracellular signaling cascade, leading to the activation of ERK (Gao et al., 2003). Once ERK is phosphorylated, it will in turn, phosphorylate phosphodiesterase 4 (PDE4) and inactivate it, thereby blocking the degradation of cAMP (Gao et al., 2003). This will lead to an increase in cAMP, however the source of the cAMP produced in response to BDNF is unknown.

The synthesis of cAMP is accomplished by the activation of adenylyl cyclases that convert ATP into cAMP and pyrophosphate (Kamenetsky et al., 2006). There are two forms of adenylyl cyclases: transmembrane adenylyl cyclase (tmAC) and soluble adenylyl cyclase (sAC) (Figure 3.1). tmAC is exclusively localized to the plasma membrane whereas sAC is ubiquitously expressed throughout the cell and is found in

discrete microdomains where cAMP effector molecules are found (Zippin et al., 2003, Kamenetsky et al., 2006). cAMP produced by tmAC is limited to the plasma membrane, thereby restricting the activation of cAMP effector molecules to those that are close to the plasma membrane. Whereas, sAC activation in response to BDNF in neurons would allow for a greater spatial distribution of cAMP, allowing cAMP effectors located at further distances from the plasma membrane to be activated.

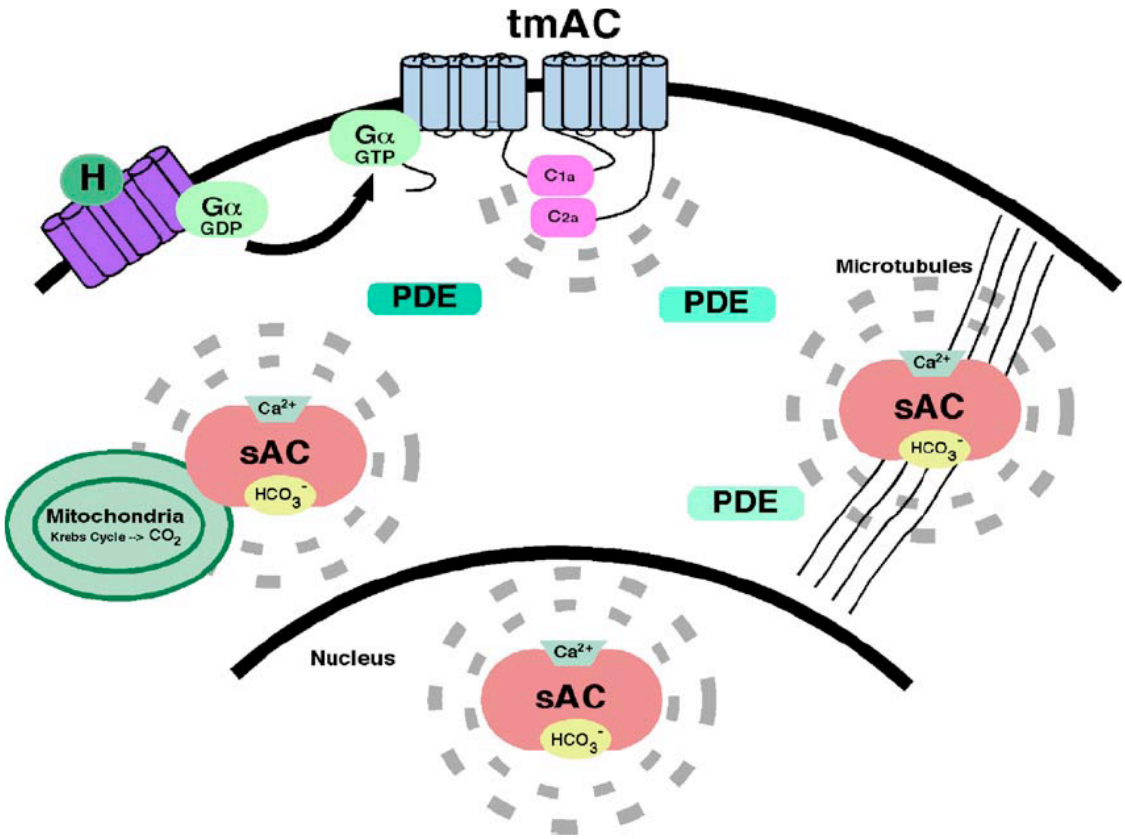

Figure 3.1: Schematic representation of cAMP signaling. The ubiquitous second messager cAMP is produced by ACs, tmAC and sAC. When cAMP is formed, it will diffuse, act on and become degraded near its targets. H refers to receptor-activating hormones and the broken lines represent the diffusion of cAMP produced from ACs (Kamenetsky et al., 2006).

There are multiple isoforms of sAC that are formed from the alternative splicing of a single gene and these isoforms are uniquely regulated by bicarbonate (Chen et al., 2000b) and calcium (Jaiswal and Conti, 2003, Litvin et al., 2003). sAC contains 2 catalytic domains, C1 and C2, which pseudo-heterodimerize to form active cyclases (Kamenetsky et al., 2006). Thus far, there are three known isoforms of sAC in rodents: sAC full-length (sACfl), sAC truncated (sACt), and somatic sAC (Figure 3.2). Both sACfl and sACt have been extensively studied in the male reproductive system and are required for sperm cell maturation and motility (Esposito et al., 2004, Hess et al., 2005). Newly identified somatic sAC does not contain the C1 domain, and it is not known how somatic sAC is activated (Farrell et al., 2008).

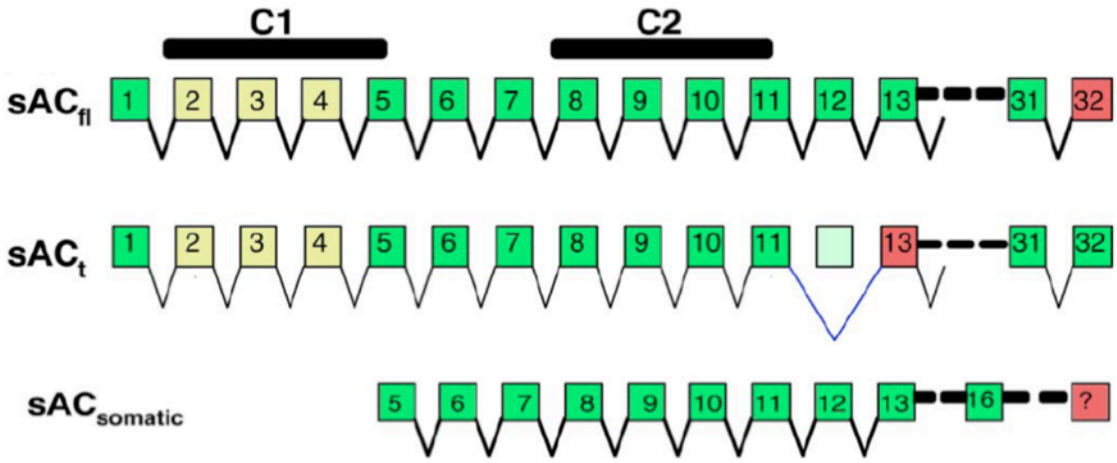

Figure 3.2: Schematic of identified sAC isoforms. Three isoforms of sAC have been identified in rodents: sACfl, sACt and somatic sAC. The numbered boxes represent exons. C1 and C2 indicate the catalytic domains. Red exons are stop codons. Yellow exons denote exons removed from Sacy^{tm1Lex} allele for the generation of transgenic mice (adapted from Farrell et al., 2008).

In 2006, the Jaffrey group was the first to show sAC expression and activation in the axons and growth cones of developing neurons (Wu et al., 2006). Using

immunohistochemistry and immunofluorescence, they showed that sAC was present in embryonic day (E) 14-15 dorsal root ganglia (DRG) neuronal cultures, E15 dorsal spinal cord explants and E18 hippocampal neurons (HN). They also showed that netrin-1-induced cAMP elevation is sAC-dependent and results in promoting axonal extension and growth cone expansion, events that are critical for axonal pathfinding in developing neurons (Wu et al., 2006). Since sAC was detected and shown to be activated in mammalian developing neurons, we propose that it is present throughout development and into adulthood.

Therefore, in order to investigate if sAC is an important component of the BDNF-signaling pathway, we first need to verify its expression in post-natal neuronal cultures.

3.2 Results

To investigate whether sAC is expressed in post-natal primary neurons, total RNA was extracted from post-natal day (P) 5-7 cerebellar granular neurons (CGN), DRG and P0-2 cortical neurons. Following RNA isolation, each sample was poly-A purified and reverse transcription was performed to produce cDNA for polymerase chain reaction (PCR). Two different sets of primers were used to amplify sAC transcripts (Figure 3.3 A). As predicted, testis contains sAC transcripts with both C1 and C2 domains (Figure 3.3 B), which are components of sACfl and sACt isoforms. In CGN, DRG and cortical neurons, amplification of sAC transcripts containing the C2 domain, but not the C1 domain was observed (Figure 3.3 B). Thus far, somatic sAC is the only sAC isoform that lacks the C1 domain. Therefore, our findings show that sAC transcripts are expressed in rat primary

post-natal neurons and suggest that somatic sAC is the predominant sAC isoform expressed in neurons.

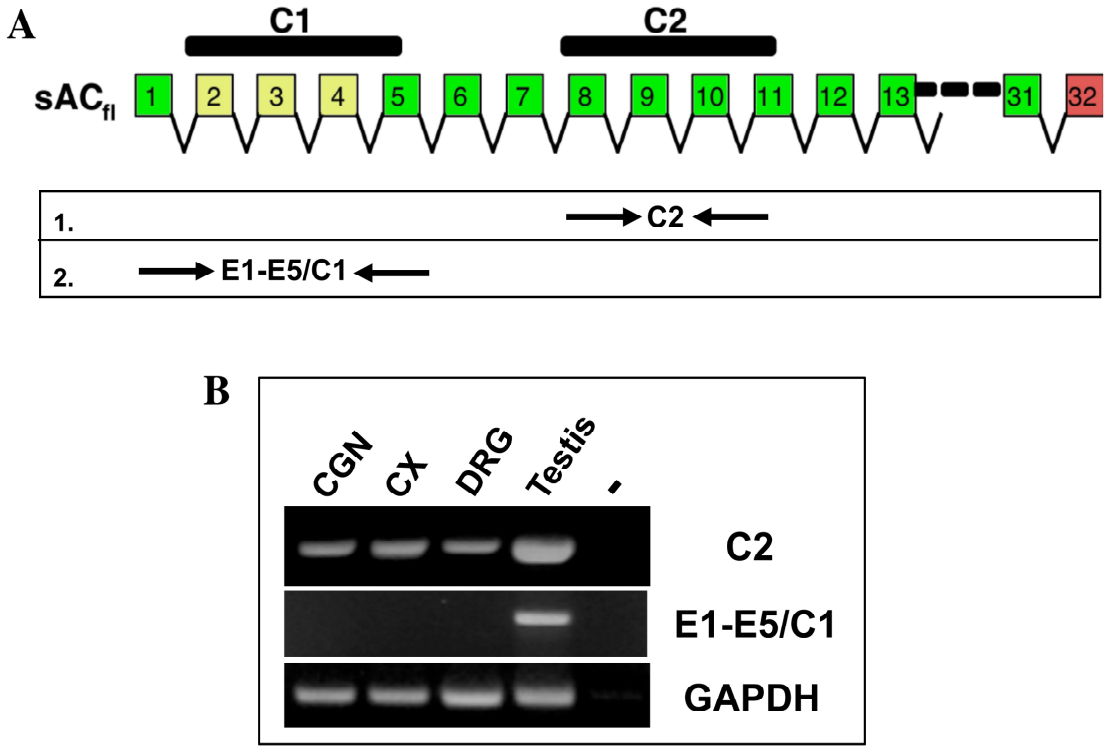

Figure 3.3: Expression of sAC transcripts in rat primary neurons. sAC primers were designed to detect transcripts containing the C1 domain of sAC and transcripts containing the C2 domain of sAC (A, modified schematic from Farell at al., 2008). Using RT-PCR, sAC transcripts containing the C2 domain, but not the C1 domain were detected in CGN and DRG, with low expression in cortical neurons (B). sAC transcripts containing both C1 and C2 domains were observed in testis (B). GAPDH was used as a control (B).

Next, we sought to demonstrate that sAC protein is also expressed in post-natal rat primary neurons. Lysates from P5-7 CGN, DRG and P0-2 cortical neuronal cultures were immunoprecipitated using monoclonal sAC antibody R37, which recognizes epitopes within the C2 domain (exon 11) of sAC, and then subjected to Western blotting with a biotinylated R21 sAC antibody, which recognizes epitopes at the end of the C1 domain (exon 5) of sAC (Figure 3.4 A). This was done for two reasons: to increase specificity of

the immunoreactive bands, as there are many potential unidentified isoforms of sAC, and to avoid detection of the R37 IgG. Since sACt and somatic sAC are approximately 50 kDa, the R37 IgG could prevent detection of those sAC isoforms, given that the heavy chain of IgG is also 50kDa. As expected, sAC was detected at ~50kD in CGN, DRG and cortical neurons (Figure 3.4 B and C). To verify that the biotinylated R21 antibody is specifically detecting sAC and the staining is not merely an artifact from the streptavidin secondary antibody, the Western blots were stripped of the primary biotinylated R21 antibody and reprobed for R37 (Figure 3.4 B and C). The results illustrate that sAC is present in pre-IP lysates and absent from post-IP lysates (Figure 3.4 B and C arrows). It also shows that the non-specific bands observed on the Western blots probed with biotinylated R21 antibody are probably products of streptavidin binding (Figure 3.4 B and C). To determine which isoform of sAC was detected, lysates from CGN were immunoprecipitated using monoclonal sAC antibody R40, which detects epitopes in the upstream region of the C1 domain (exon 2) of sAC (Figure 3.4 A). Our data illustrate that sAC can not be immunoprecipitated using R40 and further suggests that the sAC isoform detected is somatic sAC (Figure 3.4 D).

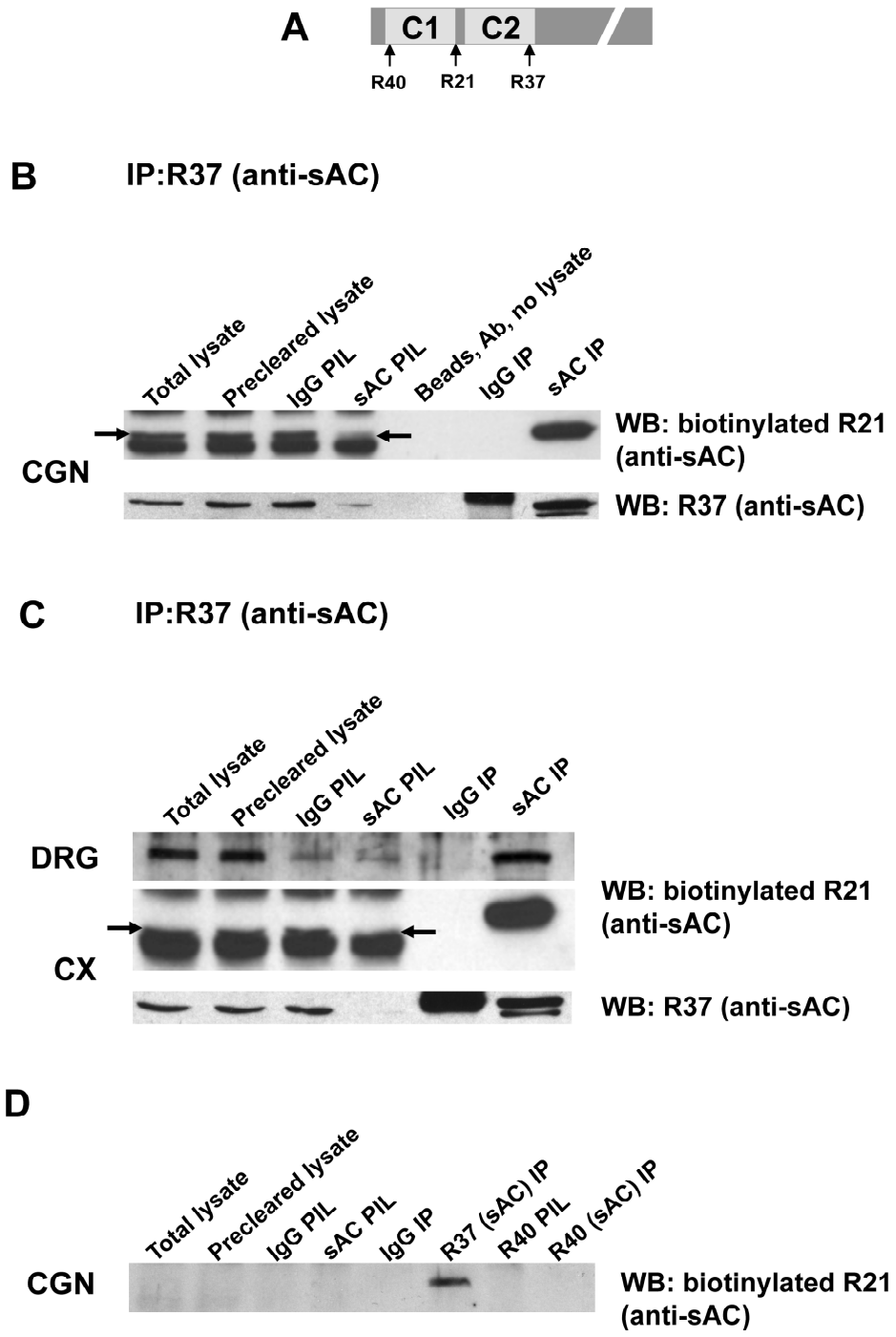

Figure 3.4: Detection of sAC protein in rat primary neurons. Schematic of sAC illustrating epitopes recognized by various monoclonal sAC antibodies (A): R40 (exon 2), R21 (exon 5), and R37 (exon 11). Total lysate was collected from P5-7 CGN (B, D), P0-2 cortical and P5-7 DRG (C). sAC was immunoprecipitated (IP) using R37 followed by Western blotting using a biotinylated R21 antibody (B-D). sAC was detected at ~50kD when lysates were IP with R37 (B-D), but not R40 (D). Arrows indicate sAC protein in pre-IP lysates versus post-IP lysates (PIL) lysates.

To further verify expression of sAC and to observe spatial localization of sAC in rat post-natal primary neurons, we isolated P5-7 CGN, DRG and P0-2 cortical neurons for immunofluorescence. First, neurons were plated on a permissive substrate of poly-L-lysine (PLL) and fibronectin, then cultured overnight. Following fixation and permeabilization, neurons were double stained with various monoclonal sAC antibodies and a neuron-specific marker, β-III tubulin (Tuj-1). Figure 3.5 (B and C) and 3.6 (B and C) show sAC expression in the soma and neurites of CGN, DRG and cortical neurons when immunostained with anti-sAC, R21 and R37, but not with R40. Since sAC was not detected using anti-sAC R40, this suggests that CGN, DRG and cortical neurons contain an isoform of sAC that lacks the C1 domain: somatic sAC. As a control, cultures were also stained with secondary antibody alone to eliminate the possibility of non-specific binding by the secondary fluorescent antibodies. Interestingly, in cortical neurons (Figure 3.6 B-D) a punctate distribution of sAC labeling was observed, especially in the neurites and growth cones (Figure 3.6 B-C). To confirm that this is indeed punctate distribution and not blebbing of the neurite, an indication of axonal degeneration, we looked at a higher magnification of the neurite. Figure 3.6 D illustrates that the β-III tubulin staining is continuous throughout the fiber and therefore, not blebbing of the neurite whereas sAC staining is clearly punctate.

Collectively, these results confirm that sAC is expressed in CGN, DRG and cortical neurons and that the predominant isoform expressed is somatic sAC.

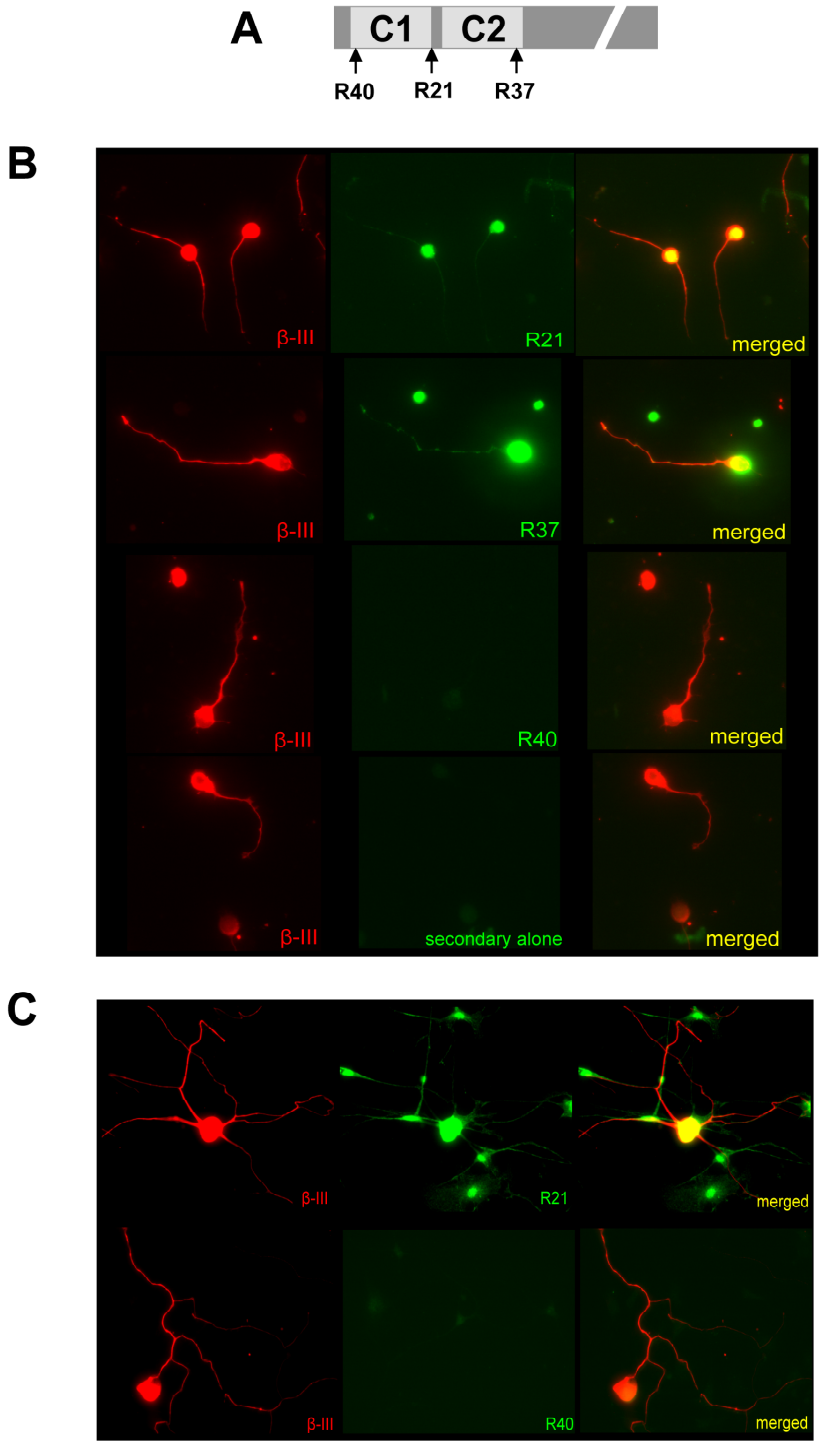

Figure 3.5: Expression of sAC in rat primary neurons. Schematic of sAC illustrating isotopes recognized by various monoclonal sAC antibodies (A): R40 (exon 2), R21 (exon 5), and R37 (exon 11). Immunofluorescence for sAC in P5-7 CGN (B) and DRG neurons (C). Dissociated neurons were labeled with various monoclonal sAC antibodies (green; R21, R37, and R40) and anti-βIII tubulin (red). sAC is ubiquitously expressed throughout the cell body and neurites of CGN and DRG when labeled with R21 and R37, but not R40.

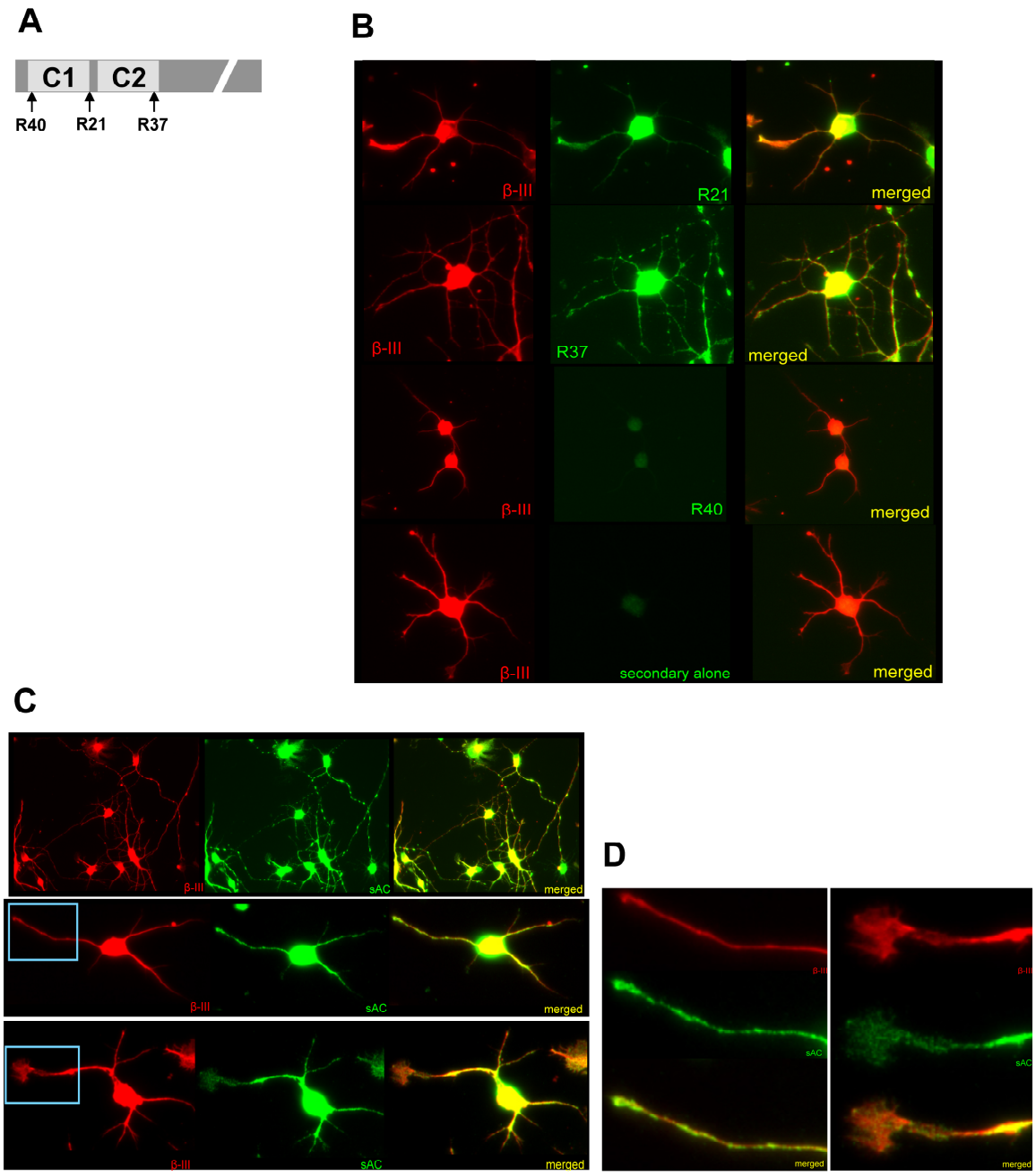

Figure 3.6: sAC expression and spatial distribution in rat cortical primary neurons. Schematic of sAC illustrating isotopes recognized by various monoclonal sAC antibodies (A): R40 (exon 2), R21 (exon 5), and R37 (exon 11). Immunofluorescence for sAC in P0-2 cortical neurons (B-D). Dissociated cortical neurons were labeled with various monoclonal sAC antibodies (green; R21, R37, and R40) and anti-βIII tubulin (red). sAC is ubiquitously expressed throughout the cell body and neurites of cortical neurons when labeled with R21 and R37, but not R40 (B). A punctate distribution of sAC labeling was observed in cortical neurons (C), especially in the neurites and growth cones (D). D represents the neurite and growth cone from blue boxes in C at higher magnification.

3.3 Discussion

We have demonstrated that sAC is expressed in post-natal CGN, cortical and DRG neurons (Figure 3.3-3.5). It was of great importance to verify sAC expression in post-natal primary neurons due to controversy within the netrin-1 field concerning its expression. Although the Jaffrey group showed that sAC is expressed and activated by netrin-1 in embryonic neurons for axonal outgrowth and growth cone elaboration (Wu et al., 2006), in 2008 the Kennedy group disputed this claim (Moore et al., 2008). According to Kennedy, netrin-1 does not induce an increase in cAMP levels and furthermore sAC is, at best, weakly expressed in the CNS. Using a sAC knockout (KO) mouse ($Sacy^{tm1Lex}/Sacy^{tm1Lex}$) containing an internal ribose entry site (IRES)-LacZ/neomycin cassette that replaces exons 2-4, which deletes the sequence that encodes a portion of the C1 domain of sAC, the Kennedy group showed that axonal pathfinding in these animals is normal (Moore et al., 2008). The differences in the results obtained by both groups may lie in their use of different experimental designs and techniques. Jaffrey showed expression of sAC using immunohistochemistry and immunofluorescence whereas Kennedy used RT-PCR analysis. For functional studies, Jaffrey used pharmacological inhibitors and siRNA for sAC and Kennedy used the aforementioned sAC KO mice. Therefore, which group is correct in their analysis of sAC-mediated netrin-1 signaling? In late 2008, the Levin and Buck group showed that $Sacy^{tm1Lex}/Sacy^{tm1Lex}$ KO mice do express sAC protein and furthermore, sAC from KO brain extracts is activate (Farrell et al., 2008). Since this knockout mouse was developed based on the knowledge that germ cells express two isoforms of sAC (sACfl and sACt) and given that these animal are sterile, it is possible that they may exhibit exclusively a

germ cell phenotype. Through their analysis of sAC protein expressed in these $Sacy^{tm1Lex}/Sacy^{tm1Lex}$ KO mice, Levin and Buck discovered a previously unidentified isoform of sAC, somatic sAC. In conclusion, using the $Sacy^{tm1Lex}/Sacy^{tm1Lex}$ to study netrin-1 mediated axonal pathfinding may not be the best tool for this study.

As previously discussed, presently there are three isoforms of sAC identified in rodents, sAC full-length (sACfl), sAC truncated (sACt) and somatic sAC. Both sACfl and sACt contain 2 catalytic domains, C1 and C2, that form active cyclases but differ in their intrinsic specific activities (Kamenetsky et al., 2006). sACfl is a 187 kD protein that has an autoinhibitory domain 17kb downstream from C2 catalytic domain (Chaloupka et al., 2006). This autoinhibitory domain gives sACfl lower activity compared to sACt (Chaloupka et al., 2006). sACt differs from sACfl in that it is a ~50 kD protein, which lacks exon 12 and has twenty times more activity (Chaloupka et al., 2006). Recently discovered somatic sAC lacks the C1 domain, contains a unique start site at exon 5 and is ~50 kD (Farrell et al., 2008). Since all cyclases require both catalytic domains to be active, the mechanism for somatic sAC activity is unknown. Given that our results suggest that somatic sAC is the predominant isoform present in neurons and data from the $Sacy^{tm1Lex}/Sacy^{tm1Lex}$ KO mice supports that there is sAC activity in brain extracts from these mice (Farrell et al., 2008), then how can somatic sAC be active without a C1 domain? Is there another cyclase that is compensating for the missing C1 domain? In order to answer this question, we need to evaluate the nucleotide cyclase family. Guanylyl cyclase (GC) is the enzyme that converts GTP into cGMP, which is involved in many signaling pathways, including those for synaptic transmission and long-term potentiation (Denninger and Marletta, 1999, Koesling, 1999). Like ACs, there are also

two forms of GCs, a membrane-bound form, particulate GC (pGC) and a cytoplasmic form, soluble GC (sGC) (Denninger and Marletta, 1999) . sGCs also contain two catalytic domains, α1 (or α2) and β1 (or β2) that heterodimerize to form an active cyclase (Koesling, 1999). Interestingly, the sGC α and β catalytic domains reside on separate polypeptide chains. In contrast, sAC catalytic domains C1 and C2 reside on one polypeptide chain (Kamenetsky et al., 2006). Given the sequence and structural similarities between sAC and sGC (Winger et al., 2008), one possibility is that the α subunit of sGC, which functionally corresponds to the C1 domain of sAC, could heterodimerize with the C2 domain in somatic sAC to form a functional, active cyclase. Currently, the Levin and Buck group are testing this hypothesis to determine first, if α1 (or α2) and C2 interact and second, if their interaction leads to a functional enzyme to produce cAMP.

We also demonstrate the sAC is expressed in the soma and neurites of CGN, DRG and cortical neurons (Figure 3.4 and 3.5). Interestingly, in cortical neurons we observed a punctate distribution of sAC within the neurites and growth cones (Figure 3.5). In various types of non-neuronal cells, sAC was shown to be expressed in specific subcellular domains, such as in the mitochondria, mid-bodies, microtubules and nucleus (Zippin et al., 2003). Several of these distinct microdomains contain cAMP effector molecules such as the exchange protein activated by cAMP (EPAC) and protein kinase A (PKA). A subset of PKA targets are localized to the plasma membrane, while others are tethered within the cytoplasm by A kinase anchoring proteins (AKAPs) (Michel and Scott, 2002). Once cAMP is generated, it can only diffuse a short distance from ACs (Zippin et al., 2003) and therefore cAMP effector proteins must be near their targets. In the

postsynaptic densities of neurons, AKAP 79/150 has been implicated as an important scaffolding protein that tethers PKA, which regulates glutamate receptors, specifically AMPARs during long-term depression and long-term potentiation (Dell'Acqua et al., 2006). Also, AKAP 9 (Yotiao), which is expressed in the cortex and cerebellum near neuromuscular junctions, anchors molecules such as, PKA, IP3 and the NR1 subunit of NMDARs (Dessauer, 2009). More importantly, recent studies of the aforementioned AKAPs focus on AKAP-organized signaling complexes that are involved in actin cytoskeletal dynamics regulated by cAMP and calcium (Dell'Acqua et al., 2006). These findings are of great interest to the field of axonal regeneration given that lack of axonal growth is ultimately due to cytoskeletal rearrangement. Given the fact that sAC is found in discrete microdomains, it would be interesting to determine if sAC associates with AKAP-organized signaling complexes. Also, in COS7 cells, bicarbonate-induced sAC activation was shown to mediate PKA-dependent activation of CREB in the nucleus (Zippin et al., 2004). This nuclear cAMP signaling microdomain is modulated by intrinsic cellular signals, such as local intracellular concentrations of bicarbonate and calcium (Zippin et al., 2004). Our previous studies have shown that CREB activation is sufficient for overcoming MAG/myelin-induced inhibition of neurite outgrowth *in vitro* and promoting regeneration *in vivo* (Gao et al., 2004). It is conceivable that sAC is also located in nuclear microdomains in neurons to induce PKA/CREB-dependent expression of the regeneration-associated genes that are necessary to promote axonal growth.

In conclusion, our findings verify that sAC is expressed in post-natal rat primary neurons. Next, we are interested in determining whether sAC plays a role in the BDNF signaling cascade that blocks MAG/myelin-mediated inhibition of axonal growth.

Chapter IV: Inhibition of sAC and tmACs: Effects on BDNF-Dependent Block of Myelin-Mediated Inhibition

4.1 Introduction

It is established that when neurons are pre-treated or "primed" with BDNF prior to being exposed to myelin-based inhibitors such as MAG, inhibition of growth by these myelin-based proteins is blocked (Cai et al., 1999). However, if neurons are treated directly with BDNF and immediately exposed to MAG or myelin, BDNF does not reverse inhibition (Cai et al., 1999). BDNF binds to Trk B receptors and mediates the activation of ERK, which results in the inactivation of phosphodiesterases (PDEs), hence preventing the breakdown of cAMP (Gao et al., 2003). This results in the elevation of cAMP that leads to the activation of PKA and CREB, which are necessary for the expression of regeneration-associated genes to block MAG/myelin-induced inhibition (Cai et al., 1999, Gao et al., 2004). However, it has yet to be elucidated how cAMP is produced in response to BDNF. Since a balance between the production of cAMP and its degradation is necessary to establish intracellular cAMP levels that are sufficient to induce signaling cascades, it is important to explore the activation of adenylyl cyclase, the enzyme responsible for the synthesis of cAMP.

In this study, we will investigate which adenylyl cyclase is producing cAMP in the presence of BDNF. There are two forms of adenylyl cyclases, transmembrane adenylyl cyclase (tmAC) and soluble adenylyl cyclase (sAC). Although their catalytic domains are structurally homologous, they differ in their expression, spatial distribution and regulation (Kamenetsky et al., 2006). tmACs are encoded by nine distinct genes (type I to type IX), which gives rise to nine different isoforms (AC1 to AC9). They are located in the plasma membrane and their activity is regulated by forskolin and heterotrimeric G

proteins (Hanoune and Defer, 2001). In contrast, there are multiple isoforms of sAC that are formed from the alternative splicing of a single gene and these isoforms are uniquely regulated by bicarbonate and calcium (Kamenetsky et al., 2006). sAC is ubiquitously expressed throughout the cell and is found in discrete microdomains where cAMP effector molecules are found (Zippin et al., 2003). tmAC and sAC contain 2 catalytic domains, C1 and C2, which pseudo-heterodimerize to form active cyclases (Kamenetsky et al., 2006). tmACs are activated by G proteins and studies have shown that Trk receptors can be activated by G-protein coupled receptors (GCPR) in the absence of neurotrophins, however a link between Trk activation, G-proteins and tmAC activation has yet to be shown (Lee and Chao, 2001, Lee et al., 2002). Therefore, our focus is to explore the role of sAC activation in BDNF-mediated reversal of MAG/myelin-mediated inhibition.

4.2 Results

In order to determine if sAC increases cAMP levels in response to BDNF, we need to block sAC activity and analyze neurite growth using our neurite outgrowth assay (Figure 4.1). P5-7 CGN were isolated, plated on poly-L-lysine (PLL) and primed with 200ng/ml BDNF in the presence and absence of sAC-specific inhibitors, 2-(*H*-benzoimidazole-2-ylsulfanyl)-propionic acid (5-bromo-2-hydroxy-benzylidene)-hydrazide (KH7) or 2-hydrooxyestradiol (OH-E) for 15-17 hours. Using a combinatorial chemical compound library of small lipophilic molecules, KH7 was found to selectively inhibit sAC activity by blocking its active site (Hess et al., 2005). Comparatively, OH-E blocks cyclase activity by interacting with a hydrophobic patch at the dimer center resulting in a

distorted active site and preventing binding of ATP (Steegborn et al., 2005). Following priming, neurons were transferred onto slides containing a monolayer of stably-transfected MAG-expressing CHO cells or control CHO cells stably-transfected with a control construct. Alternatively, cells were transferred onto a substrate of purified CNS myelin or poly-L-lysine (PLL). Under the two aforementioned conditions, neurons were plated on a non-permissive substrate (MAG-CHO or myelin) and a permissive substrate (control CHO or PLL) to evaluate growth in response to various treatments (Figure 4.1). Our results show that CGN, treated with 1μM KH7 (Figure 4.2) or 1μM OH-E (Figure 4.3) and 200ng/ml BDNF, were unable to extend neurites on MAG-expressing CHO cells (Figure 4.2 B and 4.3 B) or on purified myelin (Figure 4.2 C and 4.3 C) whereas CGN primed with BDNF alone do extend neurites, as expected. These results demonstrate that the effect of BDNF in overcoming inhibition is sAC-dependent.

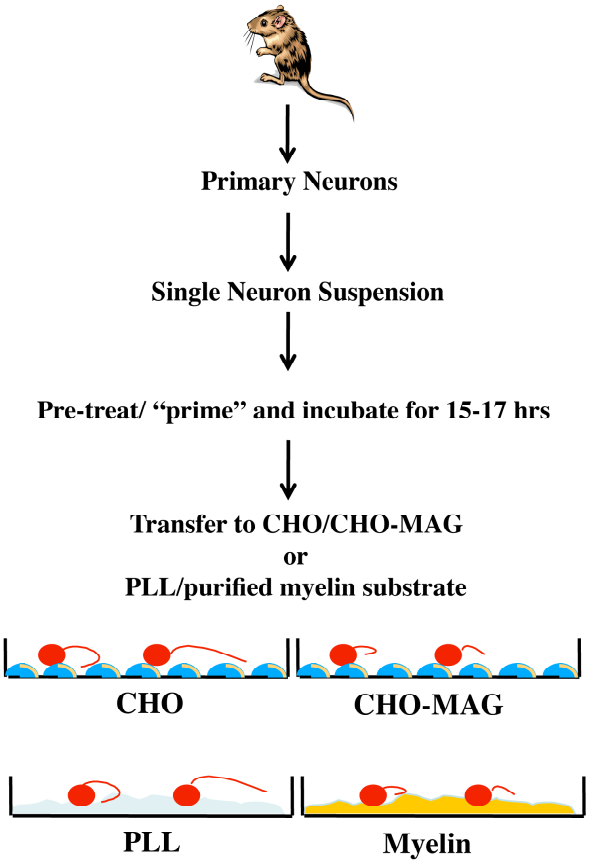

Figure 4.1: Schematic of Neurite Outgrowth Assay. Long-Evans rats were sacrificed and the desired brain tissue, such as the cerebellum was removed and dissociated into a single cell suspension. The neurons were then plated on PLL and pre-treated or "primed" with pharmacological inhibitors ± BDNF for 15-17 hrs at 37°C/7%CO_2. Following priming, neurons were transferred to slides containing a monolayer of MAG-expressing CHO cells or control cells and alternatively, onto a substrate of purified myelin or control PLL. The slides are then incubated at 37°C/7%CO_2 for 22 hrs (CHO) and 26 hrs (myelin), before fixation, staining with β-III tubulin (neuronal marker) and mounting with permafluor mounting media. The average of the longest neurite was determined using Metamorph imaging analysis system and statistical analysis was performed using Graphpad Prism.

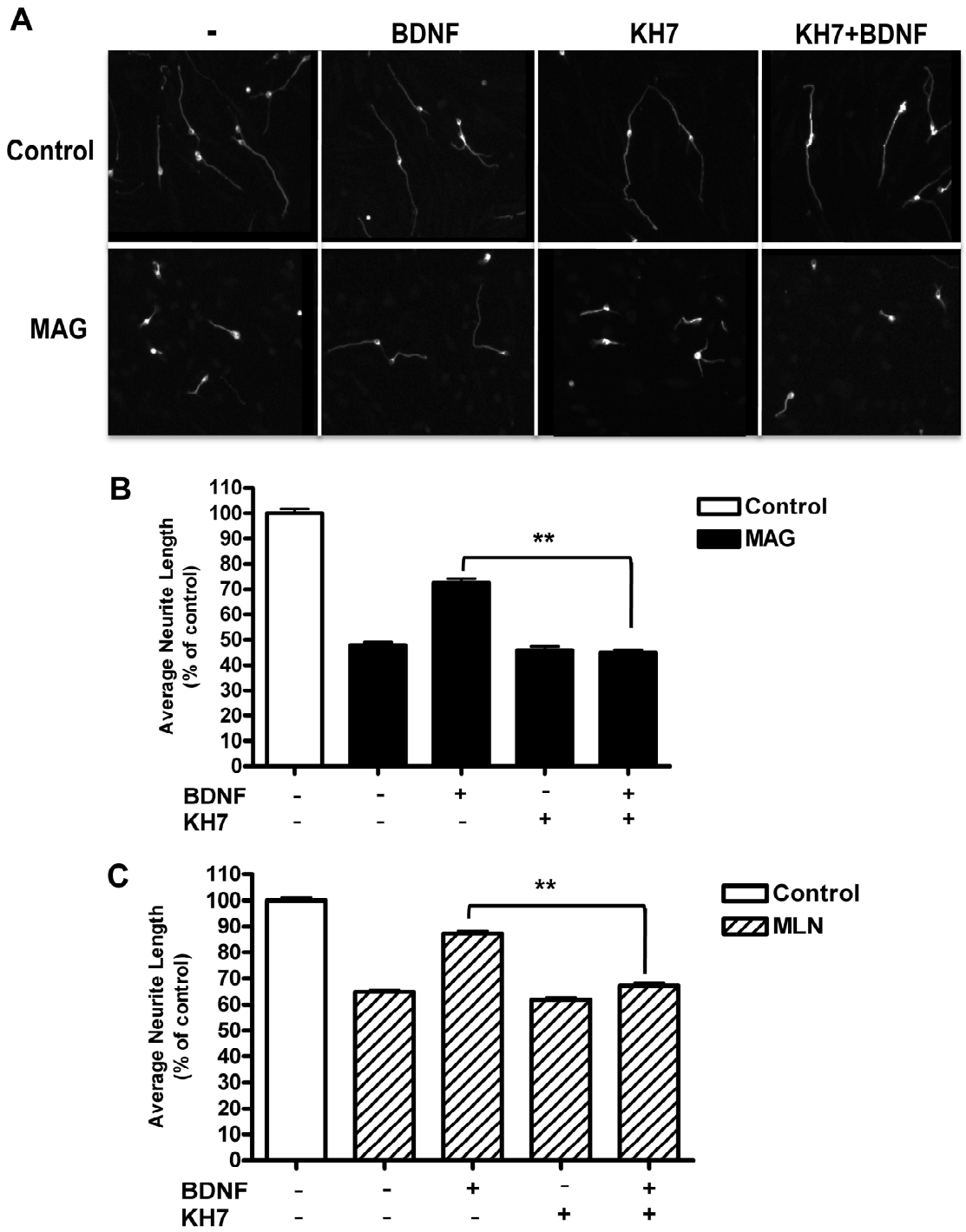

Figure 4.2: Reversal of MAG-induced inhibition by BDNF is blocked in the presence of sAC inhibitor, KH7. P5-7 CGN were isolated and treated with a sAC-specific inhibitor, KH7 ± BDNF for 15-17 hrs, before being transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells for 22 hours (B) or onto purified myelin (MLN) or PLL control for 26 hours (C). Neurons were fixed and stained for βIII tubulin. (A) Representative images of neurons growing on control and MAG-CHO cells under various conditions. Results represent the percentage of the average length of the longest neurite over the control from 500-800 neurons ±SEM. **P<0.01.

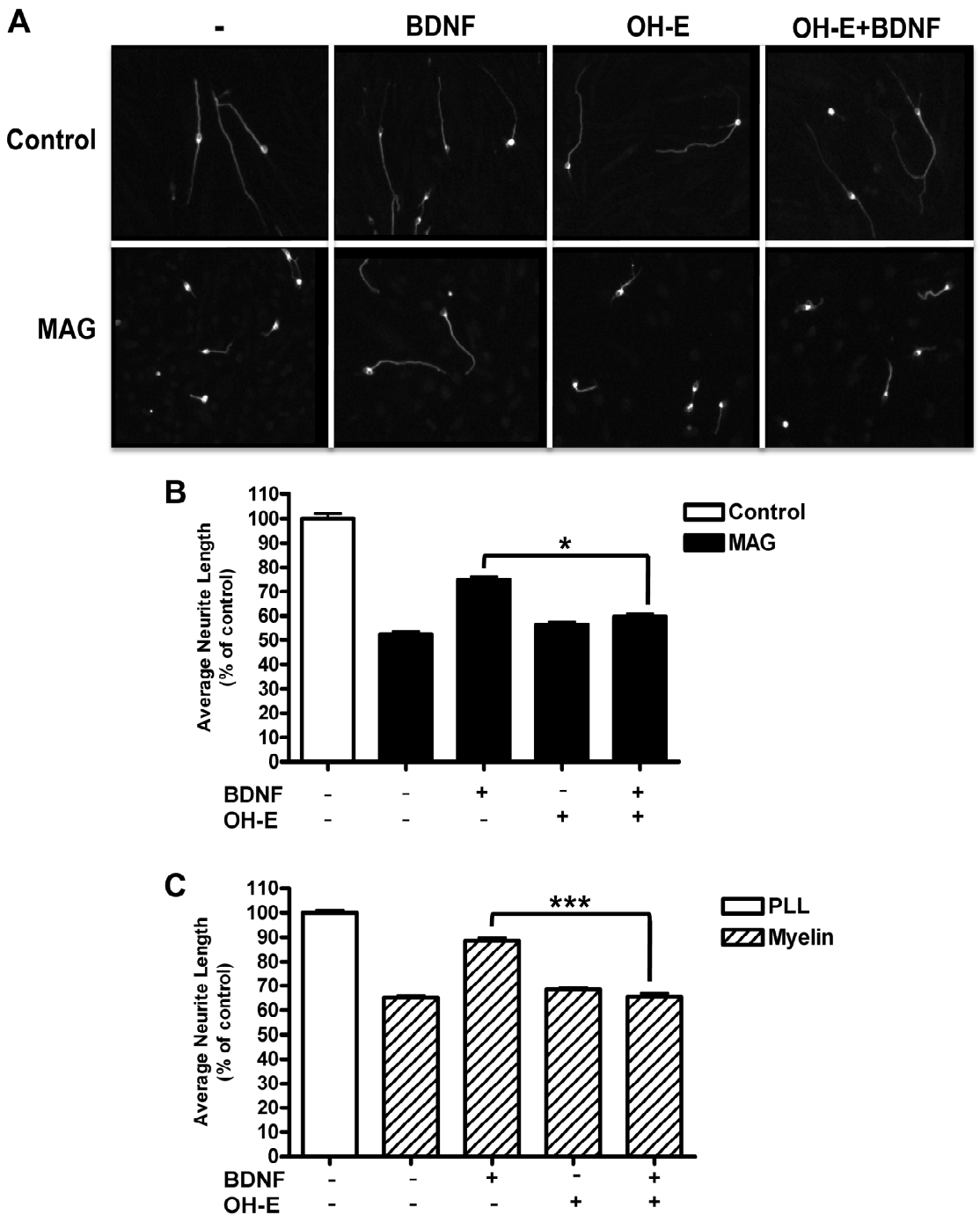

Figure 4.3: Inhibition of sAC by OH-E abrogates BDNF-dependent block of MAG-mediated inhibition. P5-7 CGN were isolated and treated with a sAC-specific inhibitor, OH-E ± BDNF for 15-17 hrs, before being transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells for 22 hours (B) or onto purified myelin or PLL control for 26 hours (C). Neurons were fixed and stained for βIII tubulin. (A) Representative images of neurons growing on control and MAG-CHO cells under various conditions. Results represent the percentage of the average length of the longest neurite over the control from 500-800 neurons ±SEM. *P<0.05, ***P<0.001.

To further validate that sAC is needed to elevate cAMP in response to BDNF, we knocked down sAC expression using siRNA directed against exon 5 of sAC. The method used to introduce the sAC siRNA into primary neurons was to covalently couple the siRNA to the small penetrating peptide Penetratin-1. Once within the cytoplasm, which provides a naturally reducing environment, the disulfide bond will be cleaved and the siRNA is released. This method of siRNA delivery is ideal for primary neurons because it allows for rapid delivery with very high efficiency and very low toxicity (Davidson et al., 2004). P5 CGN were isolated and plated on PLL overnight. Fresh media was added along with 100, 250 and 350 nM of sAC siRNA and 250 nM of scrambled siRNA as a control, then BDNF was added 9 hours later and the cells were incubated for an additional 15 hours for a total of 24 hours at 37°C. First, we verified that sAC was knocked down by the sAC siRNA by extracting total cell lysate and immunoprecipitating with sAC antibody (R37), and then subjected to Western blotting with a biotinylated (R21) sAC antibody. The results show that 250 nM of siRNA is sufficient to knock down sAC expression (Figure 4.1 A). Lastly, treated cells were transferred onto monolayers of CHO cells and subjected to the neurite outgrowth assay. Figure 4.4 B shows that when sAC is knocked down in a dose-dependent manner, CGN are unable to extend neurites even in the presence of BDNF. This result is consistent with the results observed with pharmacological inhibitors for sAC. Taken together, our data verify that sAC is required for the BDNF-dependent block of MAG-mediated inhibition.

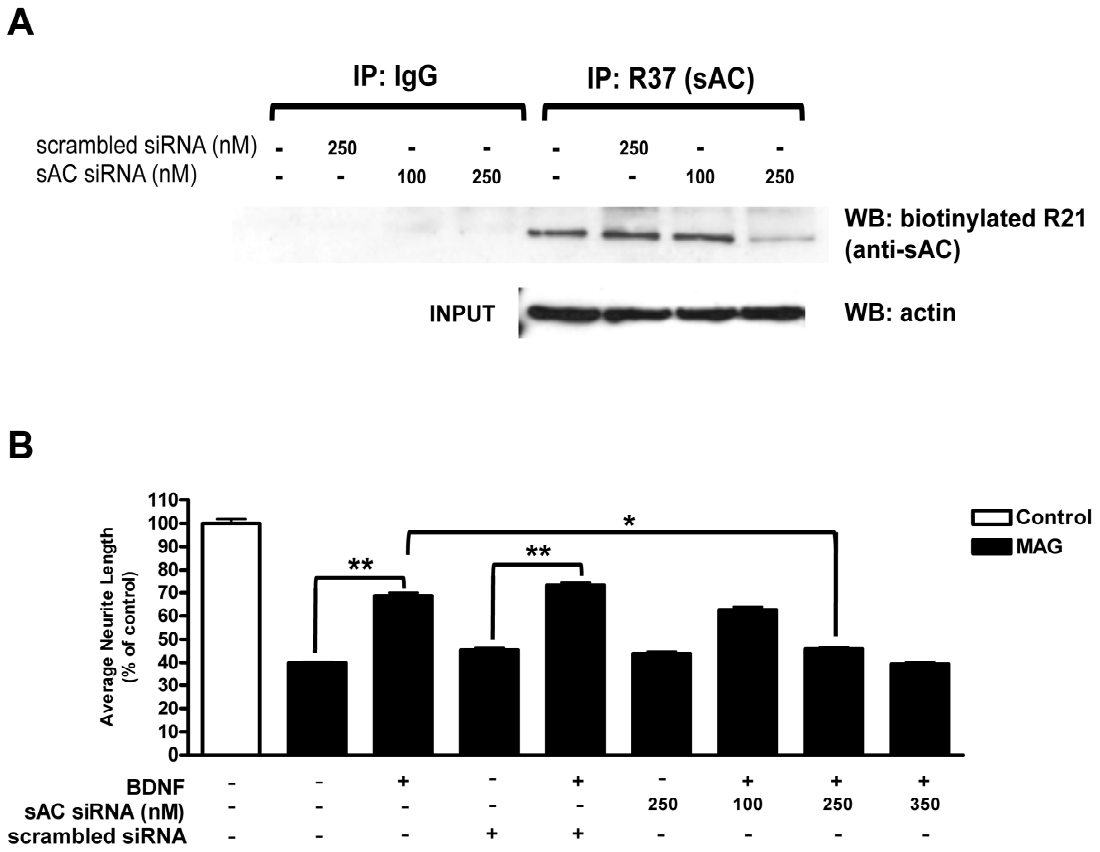

Figure 4.4 Knocking down sAC abrogates BDNF-dependent block of MAG-mediated inhibition. P5 CGN were isolated and plated on PLL overnight. Fresh SATO containing scrambled siRNA (250nM) or various concentrations of sAC siRNA were added. 9 hours later, BDNF was added and incubated for a total of 24 hrs. Total lysate was collected for the input (loading control) and sAC was immunoprecipitated (IP) using R37 followed by Western blotting using a biotinylated R21 antibody (A). In addition, the cells were transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells for 22 hours (B). Results represent the percentage of the average length of the longest neurite over the control from 150-300 neurons ±SEM. *P<0.05, **P<0.01.

To determine if tmACs are involved in the BDNF priming effect, tmACs were inactivated with a tmAC-selective inhibitor, 2', 5' dideoxyadenosine (ddAdo). Also known as a P-site inhibitor, ddAdo is a nucleotide analog that binds to the active site, thereby blocking ATP binding (Schlicker et al., 2008). P5-7 CGN were isolated and treated with 200ng/ml of BDNF in the presence and absence of 50μM ddAdo and subjected to the neurite outgrowth assay as previously described. Unlike sAC inhibition, blocking tmACs had no significant effect on the ability of BDNF to overcome MAG- or myelin-induced inhibition of neurite outgrowth (Figure 4.5 B and C). Therefore, tmAC activation is not essential for the BDNF priming effect.

Together, these results illustrate that activation of sAC, but not tmAC is necessary for the BDNF-mediated block of inhibition of neurite outgrowth.

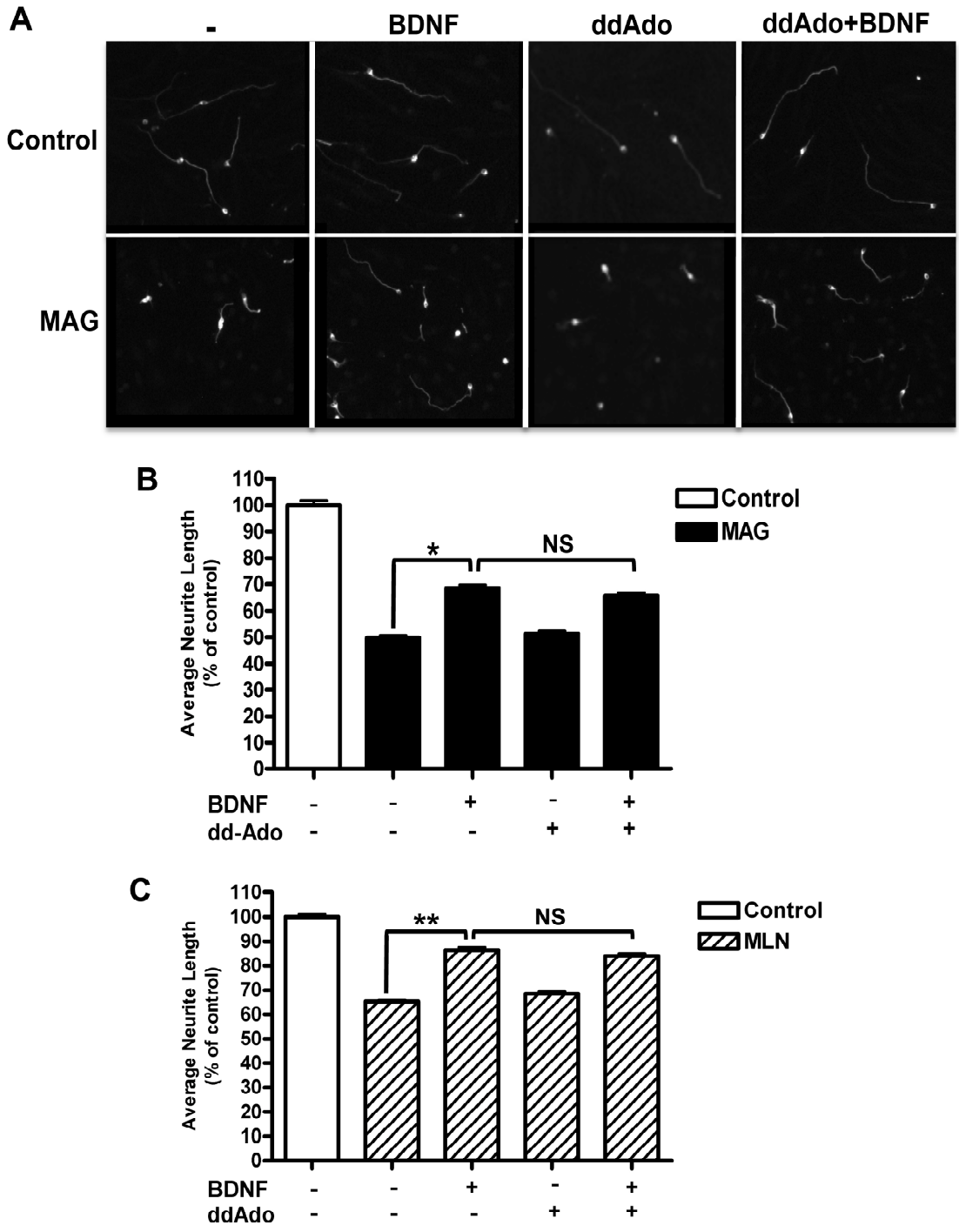

Figure 4.5: Priming neurons with BDNF overcomes MAG inhibition even in the presence of tmAC inhibitor. P5-6 CGN were isolated and treated with tmAC inhibitor, ddAdo, ± BDNF for 15-17 hrs, before being transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells for 22 hours (B) or onto purified myelin (MLN) or PLL control for 26 hours (C). Neurons were fixed and stained for βIII tubulin. (A) Representative images of neurons growing on control and MAG-CHO cells under various conditions. Neurons were fixed and stained for βIII tubulin. Results represent the percentage of the average length of the longest neurite over the control from 500-800 neurons ±SEM. *P<0.05, **P<0.01, NS: not significant.

4.3 Discussion

In the studies in this chapter, we show that the increase in cAMP in response to BDNF, which is sufficient to block MAG/myelin-mediated inhibition of neurite growth, is sAC-dependent. It is established that not only is it important to regulate the degradation of a second messenger, such as cAMP, but also to control the rate of its synthesis to effectively elevate its levels so that it can induce the desired intracellular response (Bender and Beavo, 2006). This is of great importance given that in most tissues, PDE activity is higher than cyclase activity (Bender and Beavo, 2006).

Neurotrophins (NTs) such as BDNF and nerve growth factor (NGF) have been widely studied in the nervous system, especially during development. NTs regulate a plethora of neuronal functions such as cell morphology, differentiation, synaptic plasticity, survival, and synaptic transmission (Boulanger and Poo, 1999). Similarly, cAMP regulates many aspects of the aforementioned functions, such as synaptic plasticity and transmission (Frey et al., 1993). Interestingly, the Mu-ming Poo group showed that BDNF-mediated synaptic potentiation in developing neuromuscular synapses is regulated in a cAMP-dependent manner (Boulanger and Poo, 1999). Therefore, cAMP is required to modulate downstream signaling and activity by BDNF, which resembles the events that occur in our priming effect.

In order for NTs to mediate intracellular signaling cascades, NTs must bind to Trk receptors and induce dimerization, which leads to the autophosphorylation of several conserved tyrosines in the cytoplasmic portion of the receptor. (Huang and Reichardt, 2003). These phosphorylated tyrosines create docking sites for numerous adaptor proteins

containing PTB or SH2 domains. Interacting with these adaptor proteins will in turn activate a number of signaling pathways, such as Ras, PI 3-kinase and PLCγ leading to the induction of gene expression, neuronal survival and neurite growth (Huang and Reichardt, 2003). Of particular interest is the Ras-Raf-ERK pathway, which is involved in long-term potentiation, synaptic plasticity, and survival of neurons (Kaplan and Miller, 2000). In RGCs, the MEK/ERK pathway has been implicated in promoting survival of these neurons following injury (Shen et al., 1999). Similarly, we have shown that overcoming MAG-induced inhibition in response to BDNF is ERK-dependent (Gao et al., 2003).

Our results indicate that BDNF induces cAMP production via activation of sAC. This is consistent with previous studies from the Levin and Buck group, which show that in order to differentiate PC12 cells into sympathetic-like neurons, NGF signals through Trk receptors to activate Rap1 in a sAC-dependent manner (Stessin et al., 2006). They also show that sAC activation is dependent on NGF-induced increase in intracellular calcium. Previous work from our lab illustrates that in primary neurons, BDNF induces an increase in intracellular calcium via IP3 channels and this increase in calcium was necessary for the BDNF-dependent block of MAG/myelin-mediated inhibition of neurite growth (Spencer et al., 2008). To further validate the activation of sAC by BDNF, CGN were treated with sAC-specific inhibitor, KH7, in the presence and absence of BDNF. Using a cAMP ELISA, cAMP accumulation was analyzed and the results show that BDNF leads to an increase in cAMP, yet in the presence of KH7 it did not. These results illustrate that the elevation of cAMP mediated by BDNF is sAC-dependent (unpublished observation, A. Stessin from Levin/Buck lab, Weill Medical College of Cornell University).

The primary goal of this study was to determine which adenylyl cyclase is activated in response to BDNF. As previously mentioned, tmAC are largely regulated by the heterotrimeric G proteins (Taussig and Gilman, 1995). G-proteins consist of three subunits, Gα, Gβ and Gγ and are tethered to G-protein coupled receptors (GPCRs) when inactive. There are four subtypes of Gα: $G\alpha_s$ (stimulatory), $G\alpha_I$ (inhibitory), $G\alpha_q$, and $G\alpha_{12}$ (Bridges and Lindsley, 2008). When GPCRs are activated by extracellular ligands, the inactive GDP form of Gα is converted to an active GTP Gα and dissociates from Gβ and Gγ (Bridges and Lindsley, 2008). $G\alpha_s$ can then interact with downstream effectors, such as tmACs. In order for these G proteins to be activated, GPCRs must interact with ligands, such as hormones or neurotransmitters. GPCRs are not limited to only G protein activation; they are also able to activate other membrane receptors, such as neurotrophin receptors. Could the activation of neurotrophin receptors by GPCRs lead to tmAC activation?

It has been shown that Trk can be transactivated by GPCRs, adenosineA_2 and PAC1, the receptor for pituitary adenylate cyclase-activating polypeptide (PACAP), in the absence of neurotrophins (Lee and Chao, 2001, Lee et al., 2002). In order for these two GCPRs to be activated, they require binding by ligands adenosine and PACAP, respectively (Huang and Reichardt, 2003). Therefore, in the absence of these ligands, Trk transactivation cannot occur nor can activation of G-proteins. Moreover, tmACs are not activated by the aforementioned mechanism. Furthermore, since our studies show that BDNF must bind to Trk to induce the intracellular signaling cascade necessary for blocking MAG-mediated inhibition (Gao et al., 2003), it is not surprising that blocking tmACs merely

attenuated, but had no significant effect on BDNFs ability to overcome MAG-induced inhibition.

In conclusion, we have illustrated for the first time that activation of sAC is required for BDNF to elevate cAMP to block MAG/myelin-induced inhibition of neurite outgrowth. Conversely, tmACs are not necessary for BDNF signaling, suggesting that sAC is solely responsible for the generation of cAMP in response to BDNF.

Chapter V: Overexpression of sAC to Overcome Inhibition by Myelin and Promote Axonal Regeneration

5.1 Introduction

Elevating intracellular cAMP levels in neurons can be accomplished in multiple ways: such as through the application of a cAMP analog, dbcAMP; by blocking phosphodiesterases (PDE), the enzymes which break down cAMP, with the PDE inhibitor, rolipram; or by priming with BDNF or GDNF (Cai et al., 1999, Nikulina et al., 2004). Our laboratory also showed that cAMP levels are significantly higher in embryonic CNS neurons, which exhibit a spontaneous regenerative capability, than in adult neurons, which do not regenerate (Cai et al., 2001). It has been revealed that in DRG neurons, lesioning of the peripheral branch seven days prior to performing a dorsal column lesion (Figure 5.1), improves regeneration of the damaged CNS axons, a phenomenon termed the conditioning lesion effect (Richardson and Issa, 1984, Richardson and Verge, 1986, Neumann and Woolf, 1999). Evidence suggests that this effect is dependent on elevation of intracellular cAMP levels (Neumann et al., 2002, Qiu et al., 2002). In fact, a single injection of db-cAMP directly into the ganglion is sufficient to mimic the effect of the conditioning lesion (Neumann et al., 2002, Qiu et al., 2002). In addition, work from our group—as well as others—has shown that treatment with rolipram, which blocks PDE 4, can elevate cAMP levels which in turn enhances neurite outgrowth *in vitro* and regeneration following injury *in vivo* (Nikulina et al., 2004, Pearse et al., 2004). Neurons primed overnight with BDNF prior to exposure to the myelin inhibitory substrate will transiently inhibit PDE in an ERK-dependent manner. This elevates cAMP levels and induces regeneration after injury (Gao et al., 2003). Therefore, activation of the cAMP pathway can reverse the MAG or myelin inhibitory effect on axonal growth.

Here, our goal is to determine if overexpressing sAC to elevate cAMP levels is sufficient to overcome MAG/myelin-mediated inhibition of neurite growth and promote axonal regeneration.

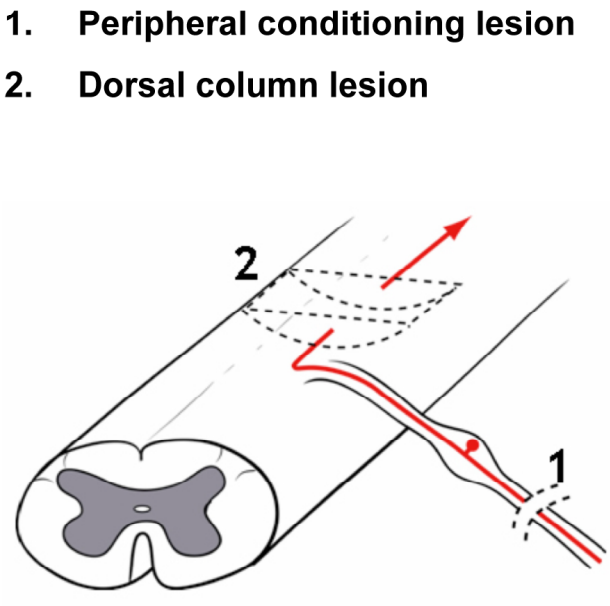

Figure 5.1: Schematic of the conditioning lesion effect. Following a dorsal column lesion (2), damaged axons are unable to regenerate. But if the sciatic nerve containing peripheral processes (1) of DRG neurons (red) is transected prior to dorsal column lesioning, spinal axons will sponaneously regenerate. Evidence suggests that this conditioning effect of the peripheral lesion is both cAMP- and CREB-dependent (modified from Filbin 1999).

5.2 Results

In order to determine if overexpressing sAC is sufficient to overcome MAG/myelin-mediated inhibition of neurite outgrowth, P5-7 CGN or P0-2 cortical neurons were infected with lentiviruses that express active sAC (sACt) or LacZ. These viruses were produced using the Virapower Lentiviral production kit by our collaborators, Dr. Levin and Dr. Buck at Weill Cornell. Briefly, cDNAs encoding sACt or LacZ were cloned into the pLenti/D-TOPO vector and the viruses were generated in the 293FT viral packaging cell line. Following infection, CGN or cortical neurons were transferred onto monolayers of MAG-expressing CHO cells or purified CNS myelin and subjected to the neurite growth assay. Figures 5.2 and 5.3 show that infecting CGN or cortical neurons with sACt-expressing lentivirus was sufficient to block MAG- (Figure 5.2A and 5.3A) or myelin- (Figure 5.2B and 5.3B) induced inhibition as compared to neurons infected with the control LacZ-expressing lentivirus (Figure 5.2 and 5.3). These results suggest that overexpression of sAC alone is sufficient to elevate intracellular cAMP levels and reverse MAG- and myelin-induced block of neurite growth.

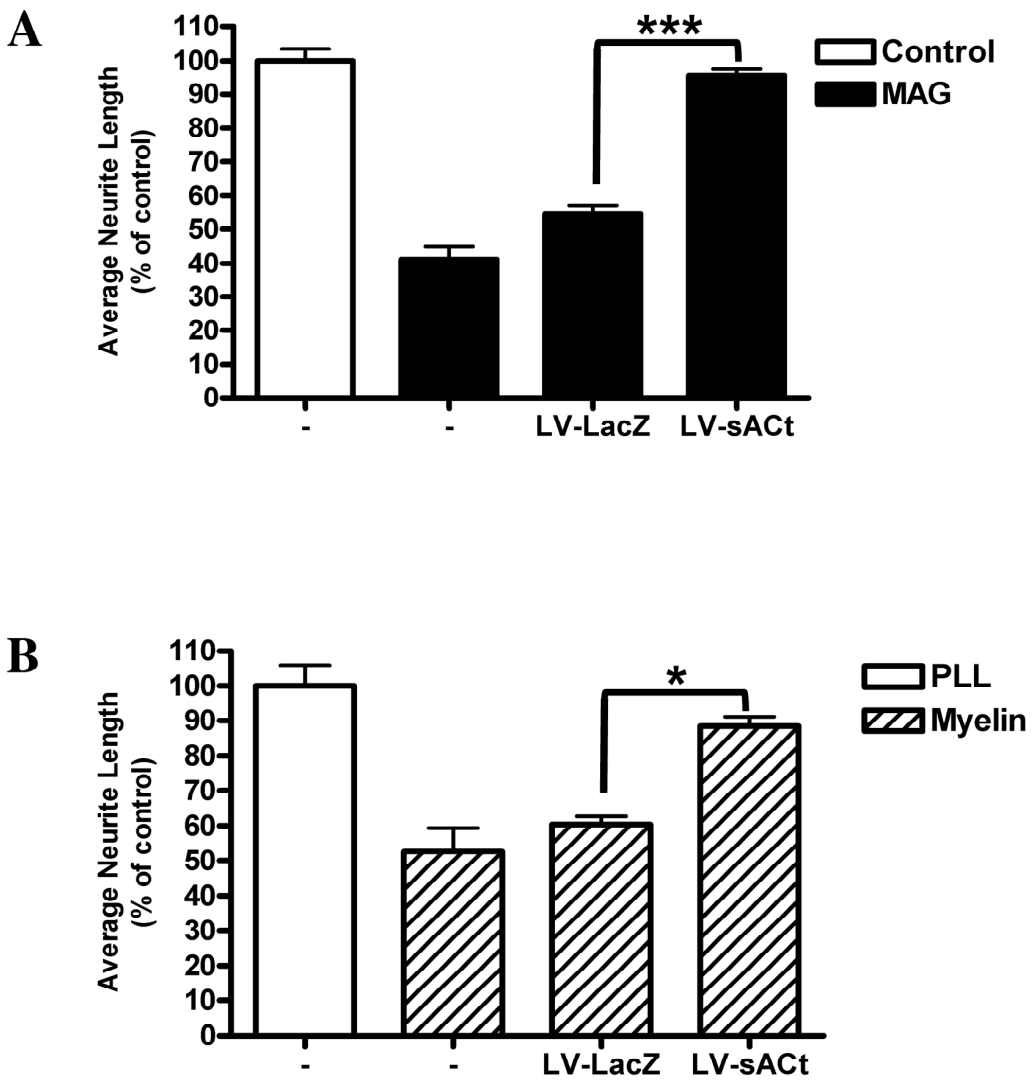

Figure 5.2: Expression of exogenous sAC in CGN promotes neurite outgrowth in the presence of MAG or myelin *in vitro*. P5-7 CGN were isolated and infected with a lentivirus containing sACt (LV-sAC) or LacZ-expressing lentivirus (LV-LacZ) as a control, then transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells (A), poly-L-lysine (PLL), or purified myelin (B). Neurons were fixed and stained for βIII tubulin. Results represent the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05, ***P<0.001. Statement of contribution: work done by A. Campana.

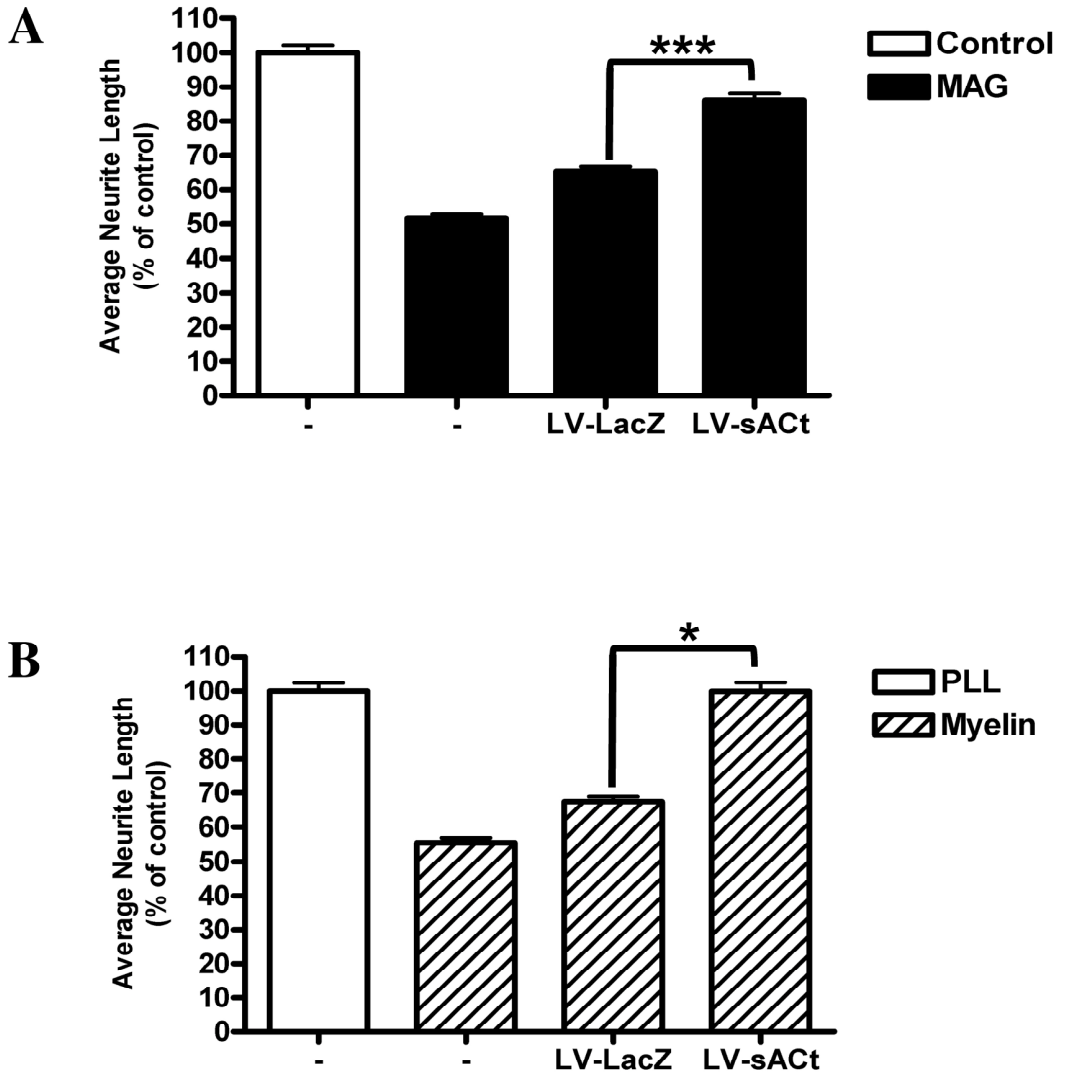

Figure 5.3: Overexpression of sAC in cortical neurons promotes neurite outgrowth in the presence of MAG or myelin *in vitro*. P0-2 cortical neurons were isolated and infected with a lentivirus containing sACt (LV-sAC) or LacZ-expressing lentivirus (LV-LacZ) as a control, then transferred onto monolayers of either MAG-expressing CHO cells or control CHO cells (A), poly-L-lysine (PLL), or purified myelin (B). Neurons were fixed and stained for βIII tubulin. Results represent the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05, ***P<0.001. Statement of contribution: work done by A. Campana.

To assess whether exogenous expression of sAC is sufficient to promote regeneration of CNS axons *in vivo*, we used an optic nerve crush model. In this model, adult rat retinal ganglion cell axons, which form the optic nerve, were crushed. Immediately after, rats were injected intraocularly with 5-10μl of either sAC-expressing lentivirus or LacZ-expressing lentivirus. Each injection was done without injuring the lens, which if injured, could cause inflammation and inadvertently result in promoting optic nerve regeneration (Leon et al., 2000). Two weeks after the crush and lentiviral injections, the animals were sacrificed by transcardial perfusion, the optic nerves were removed, post fixed, cryoprotected, sectioned and stained for GAP-43, which is a specific marker for injured and regenerating fibers. In animals injected with sAC-expressing lentivirus (Figure 5.4B), regenerating fibers (boxes and arrows) are seen beyond the site of injury (*) whereas animals injected with control LacZ-expressing lentivirus (Figure 5.4A) do not have any regenerating fibers. Regenerated axons in animals infected with the sAC-expressing lentivirus were observed up to 3mm beyond the site of injury (Figure 5.5A) and exhibited robust growth about 500μm beyond the injury site (Figure 5.5B) as compared to animals infected with control LacZ-expressing lentivirus, which showed little to no growth (Figure 5.4A and Figure 5.5A, B). These results show that overexpression of sAC is sufficient to promote axon regeneration *in vivo*.

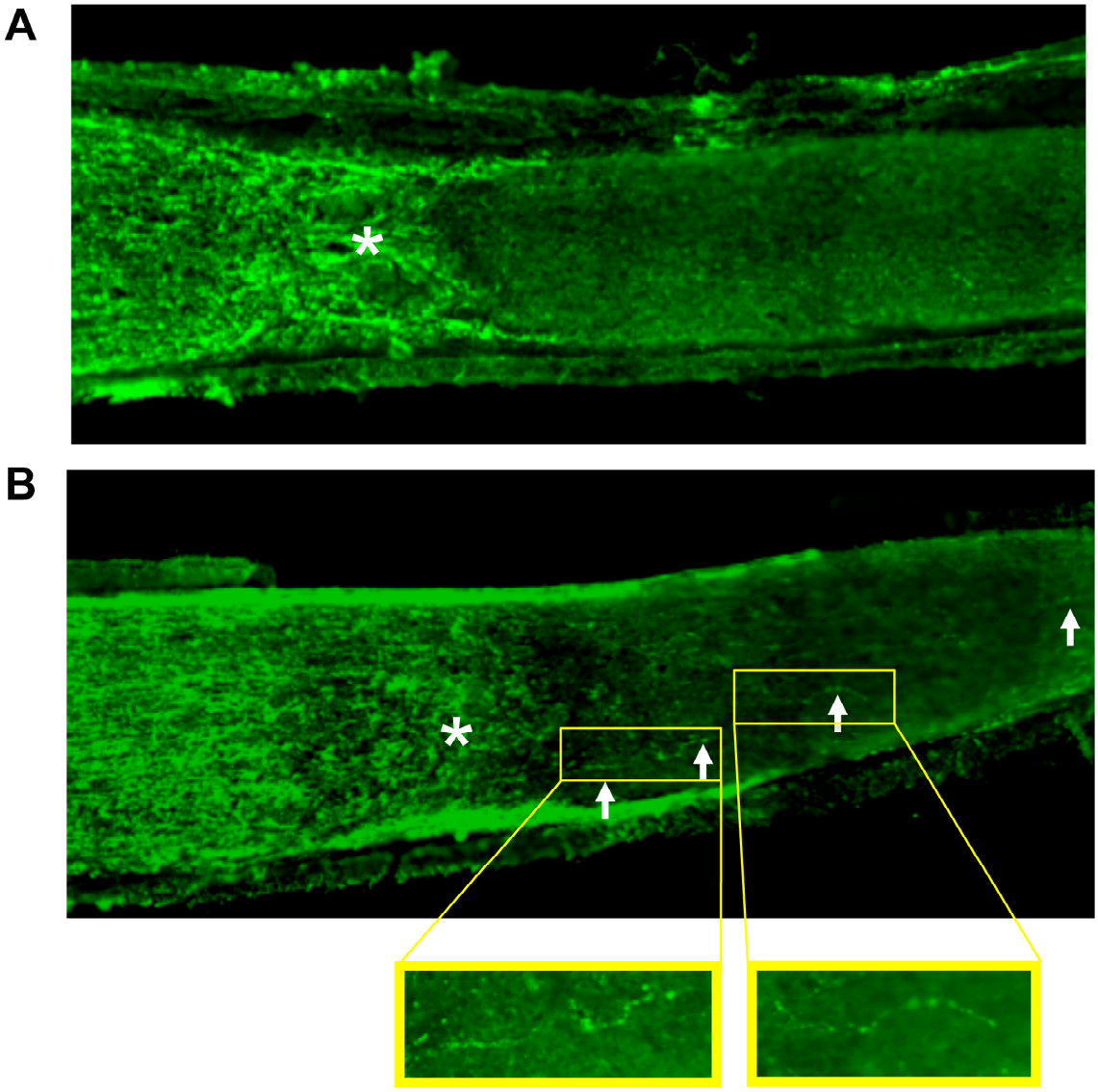

Figure 5.4: Overexpression of sAC is sufficient to promote axonal regeneration *in vivo*. The right optic nerves of adult Fisher rats (200-250g) were exposed and crushed 2mm behind the eye. sACt-expressing lentivirus (LV-sAC) (B) or LacZ-expressing lentivirus (LV-LacZ) (A) was injected into the vitreous chamber of the right eye (performed by S. Hannila and M. Siddiq). Animals were sacrificed after a two-week post-surgical survival period, then optic nerves were sectioned and immunostained for GAP-43 (performed by S. Husband and A. Campana). Representative images from a LV-LacZ (A) and a LV-sACt (B) infected animal. Boxed areas illustrate higher magnification of regenerating axons (arrows). Asterisk (*) indicates the injury site.

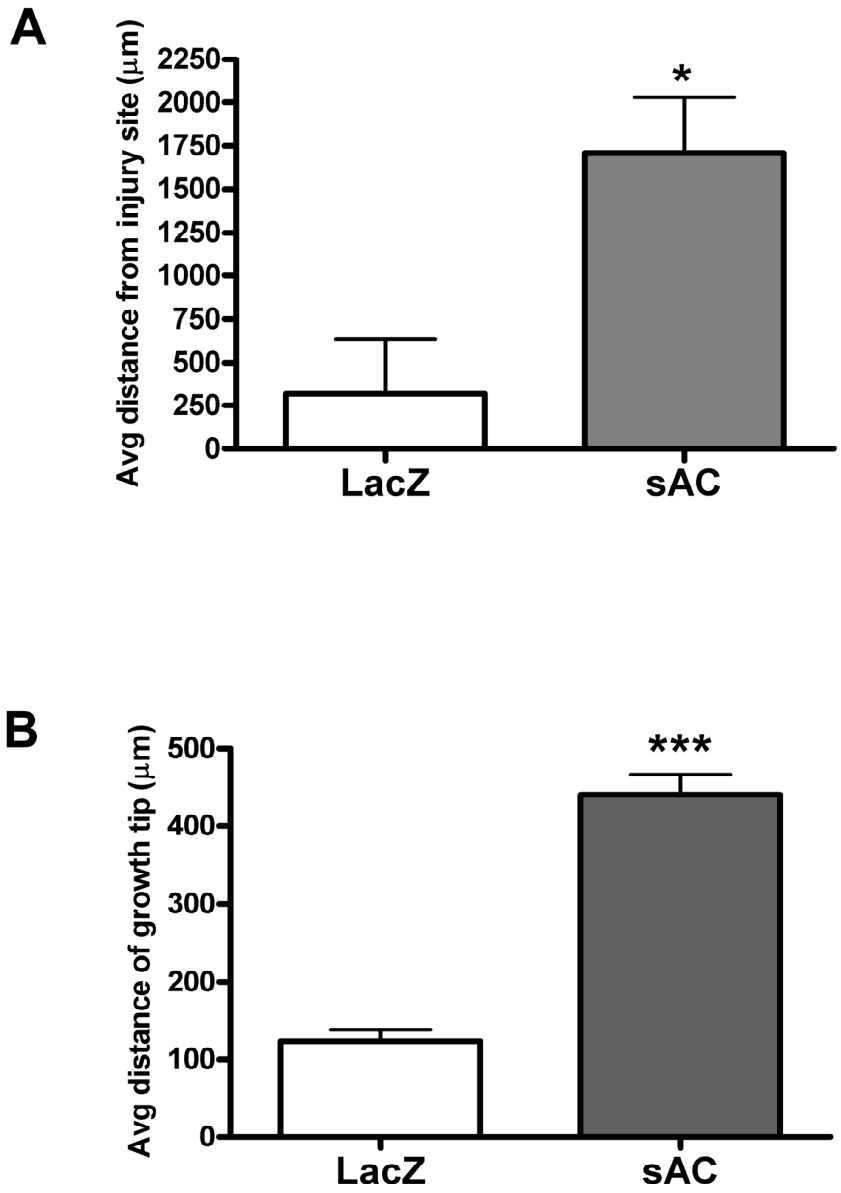

Figure 5.5: Expression of exogenous sAC is sufficient to promote axonal regeneration *in vivo*. The right optic nerves of adult rats were exposed, crushed 2mm behind the eye and sACt-expressing lentivirus (LV-sAC) or LacZ-expressing lentivirus (LV-LacZ) was delivered intraocularly (performed by S. Hannila and M. Siddiq). Animals were sacrificed after a two-week post-surgical survival period, then optic nerves were sectioned (performed by S. Husband and A. Campana) and immunostained for GAP-43. The distance of regeneration beyond the injury site was measured for the three longest axons for each animal (A). Alternatively, the distance of the growth tip of robust growth beyond the injury site was measured for each animal (B). *P<0.05, ***P<0.001.

5.3 Discussion

In the studies in this chapter, we have demonstrated that overexpressing sAC is sufficient to overcome MAG/myelin-induced inhibition of neurite growth *in vitro* and promote CNS axonal regeneration *in vivo*. Previously, we established three ways to elevate intracellular cAMP levels in neurons: direct application of dbcAMP, blocking PDEs with the PDE4 inhibitor, rolipram and by priming neurons with BDNF. Here, we have established a fourth way, by introducing exogenous sAC.

In the nervous system, cAMP and the downstream signaling pathway it activates modulates a number of neuronal processes, such as learning and memory (Frey et al., 1993, Wong et al., 1999), growth cone turning, and axonal guidance during development (Song et al., 1998). In developing neurons, nerve growth is regulated by attractive cues, such as netrins and neurotrophins as well as repulsive cues such as semaphorins (Song et al., 1997, Song et al., 1998). Studies in embryonic *Xenopus* spinal neurons show that cAMP converts repulsion of these factors into attraction, resulting in growth cone extension (Song et al., 1998). Recently, the Mu-ming Poo group showed that cAMP was also important for the differentiation of neuronal processes (Shelly et al., 2010). They showed that in undifferentiated rat embryonic hippocampal neurons, localized cAMP promoted axon formation by activating PKA and PDE3. Furthermore, cAMP acts as an antagonist to dendrite formation and was shown to decrease in all other neurites in the same cell (Shelly et al., 2010). This decrease in cAMP along with the activation of other factors will cause the remaining neurites to become dendrites.

Interestingly, we found that in embryonic neurons, intracellular cAMP levels are considerably higher than in postnatal or adult neurons, thereby facilitating axonal growth in the myelinated regions of the CNS (Cai et al., 2001). We also show that endogenous cAMP levels are high in post-natal neurons of the dorsal root ganglion (DRG) until post-natal day (P) 3-4, and then they drastically decline by P5 (Cai et al., 2001). Furthermore, P1-4 DRGs are able to extend neurites on MAG-expressing CHO cells whereas P5 and older DRGs can not, illustrating the importance of elevating intracellular cAMP levels to promote growth when neurons are in inhibitory environments.

Given that administration of sAC alone is sufficient to increase intracellular cAMP to overcome MAG/myelin-mediated inhibition of neurite growth *in vitro* and promote axonal CNS regeneration *in vivo*, our findings suggest that sAC can be considered as a novel therapeutic target to develop drugs to treat injuries, such as spinal cord injuries and traumatic brain injuries. In order to validate this, we need to develop an assay to screen for activators of sAC. This could be accomplished by using a Tri-institutional (Weill Cornell, Sloan Kettering Institute, and Rockefeller University) combinatorial chemical library (purchased from Chemical Diversity) of small molecules. The goal would be to find a small molecule that could cross the blood brain barrier and activate the cAMP pathway to block inhibitors of axonal regeneration and promote growth. Currently, this is our goal in collaboration with Dr. Levin and Dr. Buck from Weill Medical College of Cornell University.

The advantage of activating sAC as opposed to activating tmACs for therapeutic intervention in CNS injuries is that activation and subsequent production of cAMP is not limited to cAMP targets near the plasma membrane. As discussed previously, sAC was

shown to be found in microdomains, for instance in the nucleus and mitochondria where cAMP effectors, such as PKA are localized (Zippin et al., 2003). Given that reversal of MAG-induced inhibition is both PKA- and CREB-dependent (Cai et al., 1999, Gao et al., 2004), it would be ideal for sAC to be activated in these microdomains so that expression of regenerative-associated genes can be induced. Also, we showed that sAC is expressed in the growth cones of cortical neurons (Chapter 3). Studies have shown that cAMP modulates the transport of mRNAs or mRNA-containing ribonucleoprotein complexes (mRNPs) into neuronal processes (Bassell et al., 1998). Therefore, it is conceivable that activation of sAC to elevate cAMP may mediate the regulation of axonal protein translation or mRNA localization in regenerating axons to facilitate growth. Furthermore, studies have also shown that PKA-mediated dissociation of RhoA from the membrane to the cytosol separates it from its effector molecules (Lang et al., 1996). Thus, it is possible that sAC-mediated elevation of cAMP in the growth cone can lead to PKA activation. This may result in the direct phosphorylation of RhoA, which would block actin rearrangement and prevent growth cone collapse.

Here, we established an alternative way of inducing cAMP production to promote neurite outgrowth in culture and axonal regeneration *in vivo* in a sAC-dependent manner. These results shed light on sAC as a potential target for therapeutic intervention and we will attempt to pursue the development of sAC activators for the treatment of various CNS injuries.

Chapter VI: sAC Interactions and Effects on BDNF-Induced Activation of Downstream Effectors

6.1 Introduction

Increasing cAMP levels in neurons leads to the activation of PKA and this activation is necessary for the enhanced neurite outgrowth detected when neurons are primed with neurotrophins *in vitro* (Cai et al., 1999). PKA is also necessary after performing a conditioning lesion to encourage regeneration *in vivo* (Neumann et al., 2002, Qiu et al., 2002). Since these events are PKA-dependent, the downstream effectors involved in encouraging regeneration must be identified. When neurons are primed with BDNF, one of its targets, ERK, is activated and this results in the inhibition of PDE (possibly by phosphorylation), which ultimately leads to an increase in cAMP levels (Gao et al., 2003). This elevation of cAMP and subsequent activation of PKA and ERK induces transcription of several regeneration-associated genes by CREB, which is necessary to reverse the inhibitory effects of MAG/myelin on neurite growth (Gao et al., 2004). In our paradigm, the mechanism underlying BDNF-mediated activation of ERK via cAMP has yet to be elucidated.

There are several ways in which the cAMP pathway could trigger the activation of ERK and its downstream signaling pathway. This activation could occur in a PKA-dependent manner, such as through activation of the small G protein, Ras (Dumaz and Marais, 2005). Transactivation of Trk receptors by neurotrophins will recruit Ras through adaptor proteins, such as Grb and Sos (Huang and Reichardt, 2003). Subsequently, Ras will stimulate serine/threonine kinases, such as Raf-1, which ultimately leads to the activation of ERK (Vossler et al., 1997). Alternatively, this activation could occur in a PKA-independent manner, such as by activating the exchange protein activated by cAMP

(EPAC), which is a GTP exchange factor (Dumaz and Marais, 2005). Interestingly, the activation of the small G-protein, Rap1 by cAMP could be mediated by either PKA or EPAC (Impey et al., 1999). Also, PKA-induced release of calcium from intracellular stores leads to the activation of Rap1. This will in turn activate B-Raf to phoshophorylate ERK, which will induce CREB phosphorylation (Zanassi et al., 2001). Therefore, our hypothesis is that when priming with BDNF, ERK is activated in a sAC-dependent manner.

Additionally, it would be interesting to assess whether alternative modes of activation exist for sAC, such as an interaction between Trks, specifically TrkB, and sAC in response to BDNF. Does Trk recruit sAC upon transactivation? Is sAC somehow tethered to Trks and does it dissociate upon TrkB transactivation by BDNF? Could the formation of a Trk/sAC complex lead to the phosphorylation of sAC? Currently, it is unknown if sAC can be phosphorylated and if so, would phosphorylation lead to its activation? Therefore, we will first investigate whether sAC and Trks form a complex and if so, how this association is affected by BDNF-induced activation.

6.2 Results

To determine if sAC is necessary for the activation of ERK in response to BDNF, P5-6 CGN were isolated and plated on PLL overnight at 37°C. The cells were starved for 6 hours in plain DMEM before they were pretreated with sAC inhibitor, KH7 (50μM) and/or tmAC inhibitor, ddAdo (50μM) for 30 minutes, then treated with BDNF for an additional 10 minutes. Next, equal amounts of protein were subjected to SDS-PAGE

followed by Western blotting with antibodies against phosphorylated ERK (pERK), total ERK, and actin. As expected, treating with BDNF led to ERK activation and this activation was blocked when sAC was inhibited (Figure 6.1 A and B). Blocking tmACs, however, had no effect on the ability of BDNF to activate ERK. These results show that ERK activation downstream of BDNF is sAC-dependent, not tmAC- dependent.

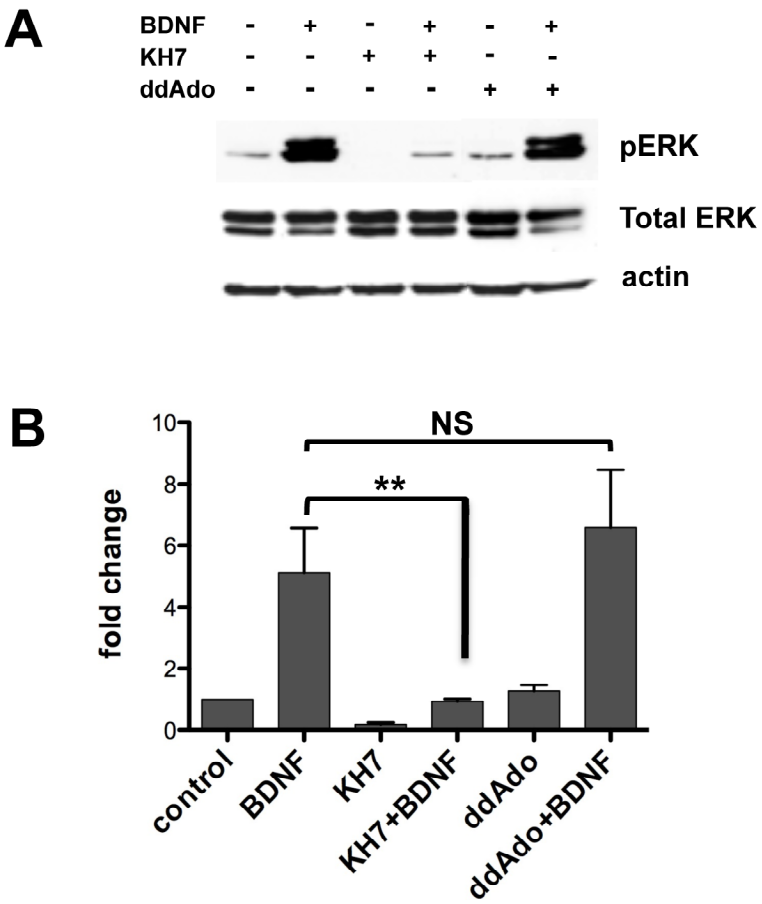

Figure 6.1: BDNF-induced activation of ERK is blocked in the presence of sAC inhibitor. P5-6 CGN were pretreated with sAC inhibitor, KH7 and tmAC inhibitor, ddAdo for 30 mins, and then treated with BDNF for an additional 15 mins. Cells were lysed and subjected to Western blotting using antibodies against pERK, total ERK and actin (A). Fold changes between treatments and statistical analysis (B). **P<0.01, NS: not significant.

To investigate if sAC and TrkB directly interact to form a complex, P5-6 CGN were isolated and plated overnight at 37°C. Additionally, HEK 293 cells stably transfected to express TrkB were plated overnight at 37°C. Both CGN and 293 TrkB cells were starved for 6 hours in plain DMEM and treated with BDNF for 15 mins. Lysates from CGN were immunoprecipitated with R37 monoclonal antibody against sAC and total lysates from the 293 TrkB cells were then subjected to Western blotting with a pan-Trk antibody, which recognizes Trk A, B, and C as well as antibodies against phosphorylated TrkB (pTrkB) and sAC (R37). Figure 6.2 shows that in 293 TrkB cells, Trk is approximately 140kDa (top arrow) and when these cells are treated with BDNF, TrkB is activated as expected (middle arrow). In CGN immunoprecipitated with sAC in the presence or absence of BDNF, there is a band roughly at 110kDa, which presumably corresponds to glycosylated Trk species (top panel, sAC IP). However, a band is not detected when the blot is probed with pTrkB (middle panel, sAC IP). These results suggest that sAC may interact with Trk receptors, yet there is no difference in its interaction in the presence or absence of BDNF. More importantly, sAC does not interact with activated TrkB following BDNF treatment.

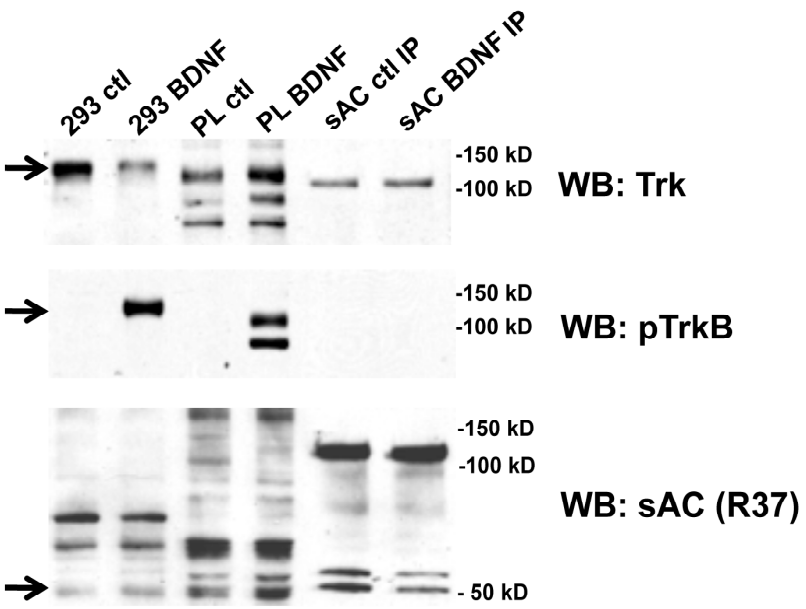

Figure 6.2: TrkB does not associate with sAC in the presence or absence of BDNF. Total lysate was collected from P5-7 CGN and TrkB-expressing 293 cells following BDNF treatment for 15 mins. sAC was immunoprecipitated (IP) using R37 followed by Western blotting using total Trk, pTrkB and sAC (R37) antibodies. Arrows indicate Trk or TrkB at 140kDa (two upper blots) and sAC at ~50kDa (lower blot). PL=pre-IP lysates.

6.3 Discussion

Our results demonstrate that ERK activation in response to BDNF is sAC-dependent. The activation of ERK and its downstream effectors regulate gene expression and morphology to mediate cell proliferation, survival, migration, differentiation, and memory formation (Impey et al., 1999, Schlessinger, 2000). Previously, we have shown that BDNF-induced ERK-activation leads to cAMP elevation, which is essential for reversing MAG/myelin-mediated inhibition of axonal growth (Gao et al., 2003). We have also shown that BDNF-dependent release of intracellular calcium through IP3 channels is also required for overcoming the inhibitory effects of MAG (Spencer et al., 2008). Given the importance and diversity of ERK signaling, our goal is to have a better understanding of the downstream signaling pathways and effectors involved in the BDNF-induced activation of ERK and elevation of cAMP, which leads to the block of inhibition.

Recent work from our laboratory as well as from our collaborators show that Rap1 is activated in response to BDNF (unpublished observations, E. Nikulina and V. Gkioka from Filbin lab and A. Stessin from Levin/Buck lab, Weill Medical College of Cornell University). Additionally, they show that overexpressing Rap1 in primary neurons was sufficient to improve neurite outgrowth on MAG and myelin (unpublished observation E. Nikulina and V. Gkioka). Furthermore, the Levin and Buck group showed that BDNF-induced Rap1 activation was sAC-dependent (unpublished observation, A. Stessin from Levin/Buck lab, Weill Medical College of Cornell University). In PC12 cells, they also showed that NGF-induced Rap1 activation was calcium- and sAC-dependent (Stessin et al., 2006).

Collectively, these results show that following priming with BDNF, Rap1 and ERK are activated in a sAC-dependent manner. Further studies are necessary to elucidate if other signaling molecules, such as EPAC or increased calcium levels, are involved in activating Rap1 and subsequently ERK. Given that sAC was shown to be localized in microdomains where cAMP effector molecules, such as PKA and EPAC are located (Zippin et al., 2003), it is possible that sAC could activate the aforementioned molecules, leading to Rap1 activation in response to BDNF. Alternatively, sAC-dependent activation of PKA may regulate Rap1 activity by phosphorylating Rap1GAP, which inactivates Rap 1 under physiological conditions (McAvoy et al., 2009). Studies in striatal spiny neurons showed that PKA-mediated phosphorylation of Rap1GAP at Ser-441 and Ser-499 leads to its inhibition and consequently increased Rap1 activity (McAvoy et al., 2009).

In addition, our results show that sAC does not interact with TrkB, suggesting that activated TrkB does not directly regulate sAC. In contrast, the Levin and Buck lab observed that sAC and Trk interact, however it was not shown in response to activation by neurotrophins (unpublished observation, A. Stessin from Levin/Buck lab, Weill Medical College of Cornell University). We show that sAC and TrkB did not form a complex in the presence or absence of BDNF, although it may interact with other Trks. Given these results, it is not logical to further investigate sAC activation via Trk transactivation and phosphorylation. It is conceivable that additional modes of sAC activation exist, which could be explored in future studies.

Summary

Following injury, spontaneous axonal regeneration in the adult mammalian CNS does not occur. This lack of growth is partially due to the formation of a glial scar, which creates a physical and chemical barrier to regeneration and partly due to the release of inhibitory molecules found in CNS myelin, which also act as a molecular barrier. These inhibitory proteins, such as MAG block axonal growth by binding to the NgR, Lingo and $p75^{NTR}$/Troy receptor complex. This leads to RhoGTPase activation, which will in turn result in the rearrangement of the actin cytoskeleton.

When cAMP levels are increased either by direct application of a nonhydrolyzable analog, such as dbcAMP or by pretreating neurons with BDNF, inhibition of axonal growth is blocked. BDNF binds to TrkB receptors, leading to the activation of ERK, which will in turn inactivate PDE4, thereby blocking the degradation of cAMP. This will lead to an increase in cAMP, however the source of the cAMP produced in response to BDNF is unknown. The synthesis of cAMP is accomplished by the activation of adenylyl cyclases that covert ATP into cAMP and pyrophosphate. Two forms of adenylyl cyclases exist: transmembrane adenylyl cyclase (tmAC) and soluble adenylyl cyclase (sAC).

In this study, we investigated which adenylyl cyclase is activated in response to BDNF and blocks myelin-mediated inhibition. tmACs and sAC differ in their spatial localization within the cell, structure, and regulation. Once cAMP is produced, it will diffuse only a short distance within the cell. Since tmACs are exclusively localized to the plasma membrane and sAC is ubiquitously expressed throughout the cell, sAC activation by BDNF in neurons would result in a greater distribution of cAMP. This would allow

cAMP effectors located at further distances from the plasma membrane to be activated. Therefore, our focus was to explore the role of sAC activation in BDNF-mediated reversal of MAG/myelin-mediated inhibition.

First, we verified that sAC is expressed in postnatal primary neurons. Using RT-PCR, immunofluorescence and immunoprecipation studies, we showed that sAC is expressed in CGN, cortical, and DRG neurons. Our results show that sAC is expressed throughout all the aforementioned neurons and suggest that somatic sAC is the predominant isoform present.

Next, we investigated whether sAC activation is necessary to block MAG/myelin-mediated inhibition by BDNF. Using pharmacological inhibitors to block sAC activity or a siRNA construct to knock down sAC, we found that blocking sAC prevents the BDNF-induced reversal of MAG/myelin-mediated inhibition. Conversely, while inhibiting tmACs attenuated BDNF's ability to overcome inhibition, it was not sufficient to completely block its effect. Additionally, we showed that sAC overexpression is sufficient to overcome inhibition by myelin inhibitors *in vitro* and promote axonal regeneration *in vivo*. We demonstrated this by infecting neurons with a sAC-expressing lentivirus and subjecting them to a neurite outgrowth assay. For *in vivo* studies, the aforementioned virus was used in an optic nerve crush model.

Lastly, we were interested in whether sAC activation was directly involved in the activation of downstream effectors in the BDNF signaling pathway. As previously mentioned, priming with BDNF leads to ERK activation, which results in overcoming MAG-induced inhibition of neurite growth. We found that blocking sAC with

pharmacological inhibitors blocked the BDNF-dependent phosphorylation of ERK and blocking tmAC had no effect on ERK activation by BDNF. Therefore, sAC is necessary for ERK activation in BDNF-signaling cascade, leading to the block of MAG/myelin-induced inhibition of neurite growth. We also sought to determine if alternative modes of sAC regulation exist, such as interactions with TrkB. Our data demonstrated that sAC does not associate with inactive or active TrkB receptors, yet does not rule out that other potential modes of regulation may exist.

In summary, our proposed mechanism for sAC activity in response to BDNF is as follows. Activation of TrkB by BDNF leads to the elevation of intracellular calcium via IP3. Calcium may result in the activation of sAC, which leads to the elevation of cAMP as well as to the activation of ERK. Inactivation of PDE4 by activated ERK, results in further elevations of cAMP. Simultaneously, elevated cAMP allows for the activation of PKA, which will in turn phosphorylate CREB for the transcription of the regenerative-associated genes that will block inhibition caused by MAG (Figure S1).

Collectively, our findings demonstrate that sAC plays an integral role in BDNF signaling and can be considered as a possible therapeutic tool to promote regeneration in the CNS.

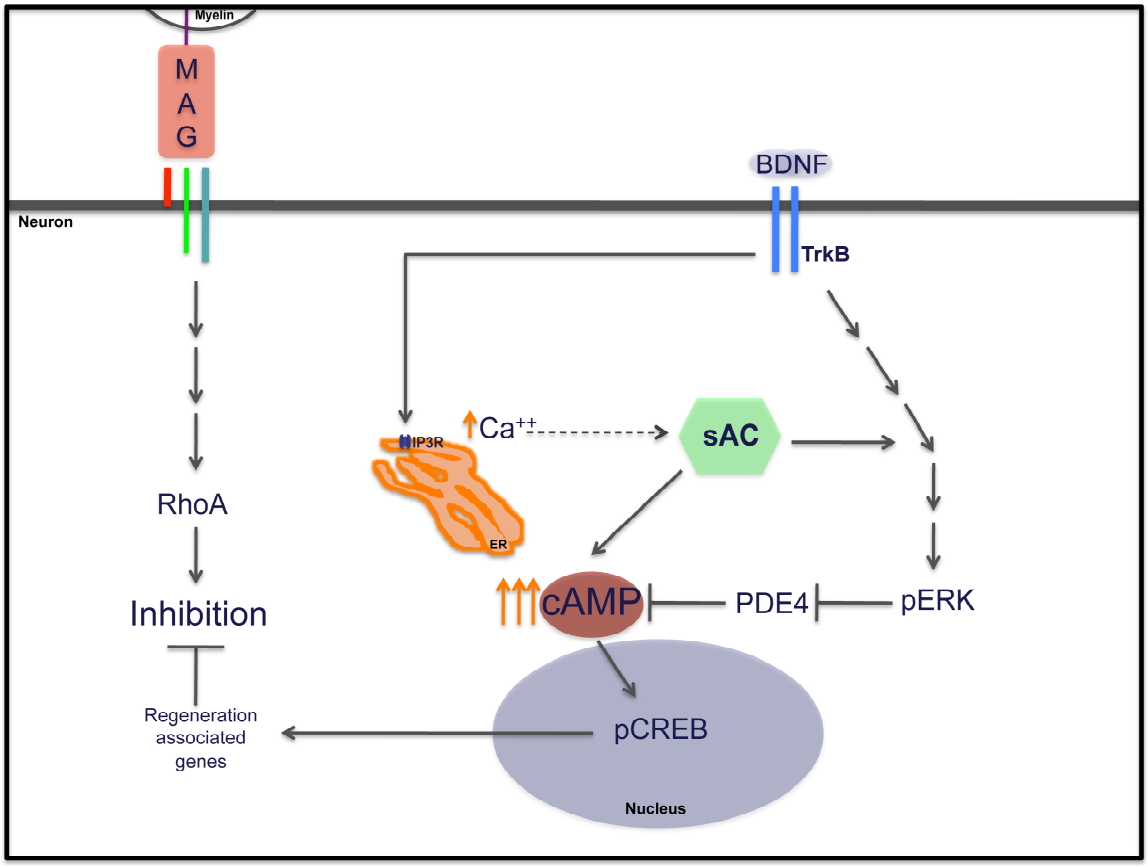

Figure S1: Schematic for the proposed mechanism for sAC activity in response to BDNF.

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