MAG and myelin block the ability of BDNF to overcome inhibition of axonal regeneration by inhibiting BDNF's activation of Rap1

by

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Abstract

MAG and myelin block the ability of BDNF to overcome inhibition of axonal regeneration by inhibiting BDNF's activation of Rap1

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It is well established that axons of the adult mammalian CNS do not regenerate if injured by trauma, or if afflicted by various neurodegenerative conditions. It is also well established that myelin, the insulating and protective membranous sheath around neurons, contains several proteins that act as inhibitors of neurite outgrowth, such as MAG, and damaged myelin is one of the factors limiting CNS regeneration after injury.

Because of their well established role in neuronal development, survival, and function, neurotrophins have been candidates for therapeutic intervention in the nervous system for many years, and have already been used in clinical trials for various neurological conditions.

We have previously shown that neurotrophins elevate cAMP. If the cAMP signaling cascade is initiated in neurons exposed to MAG and myelin, with the non-hydrolyzable analogue dibutyryl-cAMP, inhibition of neurite outgrowth by both is blocked *in vitro*. Importantly, we have shown that if cAMP is elevated in damaged neurons, nerve regeneration is promoted *in vivo*. We have also observed that neurotrophin-induced cAMP elevation is blocked in the presence of MAG, and neurotrophins are effective in overcoming MAG inhibition only by overnight pre-

exposure of neurons to them, a procedure we call “priming with neurotrophins”. Therefore, understanding the mechanism of MAG and myelin’s block of neurotrophin signaling could result in the design of therapeutics that target only specific molecules in the neurotrophin cascade, in order to potentiate only specific and desirable effects of neurotrophins, for specific types of injury or disease.

We have found that MAG and myelin block the BDNF activation of Rap1, one of the Ras-superfamily members activated in the neurotrophin cascade, which is also part of the cAMP signaling network, due to cAMP-activation of one of its upstream activators, EPAC. We have also shown that MAG and myelin treatment of neurons results in upregulation of the protein levels of the Rap1 inactivator Rap1GAP, which provides a mechanism for the block of Rap1 activation by BDNF. Additionally we have shown that priming of neurons with the EPAC-specific cAMP analogue 8-CPT-2Me-cAMP, like priming with neurotrophins, is sufficient to overcome inhibition by MAG. Moreover, infection of neurons with adenoviruses carrying wild type Rap1, or the RapGAP insensitive mutant Rap1F64A, improved growth on MAG and myelin. Infection of neurons with adenovirus carrying the Rap1 inactivator Rap1GAPI blocked the ability of neurons to extend neurites, demonstrating an essential role of the Rap1 family in regulating neurite outgrowth.

Besides its implications for therapeutic intervention after CNS trauma, the modulation of the neurotrophin cascade by MAG demonstrated here might be a new manifestation of the role of MAG and myelin in restricting plasticity in the intact CNS.

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Abbreviations

A

AC adenylate cyclase
ALS amyotrophic lateral sclerosis

B

BDNF brain derived neurotrophic factor

C

C3 c3 transferase
cAMP cyclic adenosine monophosphate
CGN cerebellar granule neurons
CHO Chinese hamster ovary
CNS central nervous system
CSPGs chondroitin sulfate proteoglycans
CST corticospinal tract

D

dbcAMP dibutyryl cAMP
DRG dorsal root ganglia

E

ECM extracellular matrix
EGFR epidermal growth factor receptor
ERK extracellular-signal regulated kinase

F

FOR-SCI Facilities of Research-SCI

G

GAG glycosaminoglycan
GCV ganciclovir
GFAP glial fibrillary acidic protein
GPI glycosyl phospatidyl inositol

I

Ig immunoglobulin

M

MAG myelin-associated glycoprotein

N

NEP1-40 Nogo extracellular peptide, residues1-40
NGF nerve growth factor
NT/s neurotrophin/s
NT-4 neurotrophin 4

O

OMgp oligodendrocyte myelin glycoprotein
OPs oligodendrocyte precursors

P

$p75^{NTR}$ p75 neurotrophin receptor
PC pheochromocytoma
PDE4 phosphodiesterase 4
PI phosphatidyl inositol
PNS peripheral nervous system

R

Ral-GDS-RBD Ral guanine nucleotide dissociation stimulator binding domain
RGCs Retinal ganglion cells
RTK receptor tyrosine kinase

S

SCG superior cervical ganglia
SCs Schwann cells
SCI spinal cord injury
Siglec sialic acid binding Ig-like lectin

T

TNFR tumor necrosis factor receptor
Trk tropomyosin kinase receptor

W

WD Wallerian degeneration

Chapter I: Introduction

1.1 Scope of our research: finding a cure for injuries in the central nervous system

The brain and spinal cord constitute the central nervous system (CNS). The nerves that project from the spinal cord outwards to innervate the limbs, skin, and all other organs constitute the peripheral nervous system (PNS). Both of these systems are composed of the neuronal cells and their supporters, the glial cells. Specialized glial cells, the myelinating glia, wrap around axons and form an insulating membranous sheath called myelin. The myelinating glial cells are different, however, in the two systems: in the PNS it is the Schwann cells (SCs) that myelinate axons and in the CNS it is the oligodendrocytes. The CNS contains two additional types of glia, astrocytes and microglia (Kim and de Vellis, 2005).

There are many disorders afflicting the CNS, such as Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and stroke, which currently have no cure. Injury to the CNS, such as head or spinal cord trauma, is also a problem, as there is no spontaneous axonal regeneration, and the current treatments are very limited. As a result there is a lifelong high cost of care for patients, as well as a high physical and psychological burden. In our lab we are studying CNS regeneration after injury, but the scientific consensus is that breakthroughs in this area will also find applications in other CNS disorders where neuronal degeneration occurs.

1.2 Spinal cord injury statistics and goals of current research

It is estimated that the annual incidence of spinal cord injury (SCI) in the U.S. is approximately 12,000 new cases each year. As of 2007, an estimated 255,702 people with SCI were living in the U.S, with an estimated lifetime health care cost between $494,145 and $3,059,184, depending on patient age upon injury and the severity of the injury (National Spinal Cord Injury Statistical Center, *www.spinalcord.uab.edu*). Currently, there is no treatment for SCI.

According to the National Institute of Neurological Disorders and Stroke (NINDS), there are four key principles in spinal cord repair: to protect surviving nerve cells from further damage, to replace damaged nerve cells, to stimulate the regeneration of axons and the proper connection to their targets, and to retrain neural circuits in order to restore body functions (http://www.ninds.nih.gov/disorders/sci/sci.htm). These are all areas of basic biological research and many scientists now agree that a SCI therapy will involve a combination of drugs that will simultaneously address all these principles, and thereby help improve the prognosis of the injury.

We do not know if paralysis will ever be completely cured, but even if surviving axons can be encouraged to grow and reach the next one or two spinal segments past the injured site, the benefits for the patient could be tremendous. Depending on the patient, such growth might restore the ability to breathe without a respirator or allow for bowel and bladder control (Yiu and He, 2006).

1.3 Regeneration in the CNS is possible

1.3.1 PNS regenerates after injury, but CNS does not

It has been known for a long time that the adult mammalian PNS can spontaneously repair itself after an injury, whereas the adult CNS cannot. Upon injury to a peripheral nerve, a clearance operation called Wallerian degeneration takes place within the Schwann cell basal laminar tubes, which facilitates regeneration of the damaged nerves (Vargas and Barres, 2007). During this process, the transected axons distal to the injury site degenerate and the Schwann cells not only break down their own myelin, but also phagocytose and digest the resulting myelin debris. They also secrete cytokines which recruit hematogenous macrophages to the site. The macrophages mediate the final removal of the myelin debris, and also phagocytose the axonal debris, before eventually migrating out of the laminar tube. The Wallerian degeneration process clears all the debris from the distal nerve segment, so that it can receive the regenerating axon sprouts coming from transected axons proximal to the injury site. If the debris persisted at this site, it would pose a barrier to the regenerating axons.

Another key event in PNS regeneration is the proliferation of Schwann cells, which begins a few days after injury. Schwann cells de-differentiate, down-regulate myelin proteins, and start dividing. They form columns named “bands of Bungner” which serve as a guidance and growth-promoting substrate, so that regenerating axons can navigate back to their previous pathways and original targets. Axons regenerate in contact with Schwann cells and eventually become re-myelinated by them [reviewed in (Vargas and Barres, 2007) and (Fawcett and Keynes, 1990)] .

In the mammalian PNS, Wallerian degeneration takes 7-14 days to complete, but in the CNS it is an extremely slow process lasting months or years, and this is one of the reasons that adult CNS regeneration does not occur spontaneously. The myelin debris present after CNS injury is now known to present not only a physical, but also a major biochemical barrier for regenerating axons. Myelin contains several proteins which inhibit axonal growth, therefore its prolonged presence at the CNS injury site creates an environment that is inhibitory to axonal growth.

1.3.2 Developmental loss of rapid axonal growth capacity in CNS neurons

Scientists had speculated that another reason for lack of regeneration was that adult CNS neurons simply do not have the ability to re-grow if severed, once CNS development is finished. This view, first proposed by Ramon y Cajal (Cajal, 1928), remained the dogma for several decades, until experiments performed in the early `80s provided evidence to the contrary.

It is true though that adult CNS neurons have a reduced intrinsic growth capacity, when compared to both peripheral and embryonic neurons. After all, the intrinsic programs controlling growth should not be the same in young and developing neurons, as in adult neurons that have already reached their targets. Barres and colleagues have reported that *in vitro*, embryonic day 20 (E20) retinal ganglion cells (RGCs) extend axons 10 times faster than postnatal day 8 (P8) RGCs (Goldberg et al., 2002).

In the literature, there are several examples of how regulation of proteins and pathways involved in growth of neuronal cells is altered, as development progresses. This altered regulation has been correlated with a reduced regenerative capability. For example, in RGCs' axons, a down-regulation of the inhibitor of apoptosis Bcl-2 was observed between E16 and E18, and this coincided with a reduced capacity to regenerate (Chen et al., 1997a). Another recently reported example is the suppression of the mammalian target of rapamycin (mTOR) pathway by the tumor suppressor PTEN in RGCs as development progresses, and also after optic nerve injury in the adult. mTOR regulates cellular growth and size, by regulating protein synthesis, and its downregulation by PTEN in neurons was suggested to contribute to a reduced ability to spontaneously regenerate (Park et al., 2008). In this study, Zhigang He's group used mice with a conditional PTEN deletion in an optic nerve crush injury model. The authors found that, after injury, there was increased RGC survival, and up to 10% of injured axons regenerated, with some of them extending for long distances. Based on these results, they suggested that enhancing protein synthesis in mature neurons will enhance regeneration, and targeting PTEN activity might be one way to achieve this.

Additionally, a developmental downregulation in the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP) has been proposed as yet another reason for the loss of axonal regeneration in the CNS. Our lab has found that with development, there is a decline in the neuronal endogenous level of cAMP, and this contributes to the neuronal susceptibility to myelin inhibitors, and thus to the inability to regenerate (Cai et al., 2001).

Overall, these and other studies have demonstrated that the intrinsic growth capacity of neurons is reduced as the animal develops and the neuronal connections mature. Thus, the correlation between a developmental switch in growth mode and a loss of regenerative ability in the CNS (Goldberg et al., 2002) is a factor that needs to be considered in the efforts to enhance CNS regeneration after injury (Park et al., 2008).

1.3.3 CNS neurons can regenerate when provided with a permissive environment

Despite their reduced intrinsic growth capacity, several experiments proved that CNS neurons, like PNS neurons, still possess some capacity to grow and regenerate. The paradigm to study CNS response to injury in many of these studies was to monitor regenerative responses after spinal cord injury in rodents.

In the ‘80s, Aguayo and his colleagues used transplantation experiments in rodents to demonstrate that the injured CNS environment plays a major role in preventing regeneration. In one of these early experiments, David and Aguayo used a segment of adult peripheral nerve to extra-spinally connect an adult rat spinal cord with its brainstem, thus creating a “bridge” between them. This grafting procedure itself created local damage to the spinal cord at the site of graft insertion. Upon staining, it was discovered that the injured CNS axons grew inside the PNS graft for long distances (3 cm in rats after 22-30 weeks). Anterogradely labeled axons within the graft entered the CNS for approximately 2 mm, and then stopped. The conclusion was that CNS neurons still possess an intrinsic capacity to re-grow after a lesion, and changing the environment of the lesion site to resemble that of the PNS can support regenerative growth (David and

Aguayo, 1981). The extrinsic environment, therefore, plays a major role in determining how neurons will respond after an injury.

Additional studies have established that besides peripheral nerves, there is yet another environment which can support spontaneous CNS regeneration, that of the embryonic CNS. After its complete transection, the spinal cord of newborn opossums, which because they are marsupial correspond to E13-E14 rat embryos, repairs itself and there is a remarkable functional recovery as well in these animals (Nicholls and Saunders, 1996). Also, after embryonic chick spinal cord transection prior to embryonic day 13 (E13), there is complete anatomical repair and functional recovery (Keirstead et al., 1992).

Results from the above studies, as well as others not mentioned here, prompted many scientists to start looking for cellular and molecular factors which inhibit regeneration within the injured adult CNS.

1.3.4 SCI regeneration without a transplant: the conditioning lesion effect

There are some neurons that have a physical presence in both CNS and PNS; primary sensory neurons in the dorsal root ganglia (DRG) are pseudo-unipolar cells, and possess both a peripheral and a central branch. These neurons were a very useful tool for scientists to compare PNS and CNS regenerative responses after injury, because their peripheral branch regenerates, whereas their central branch does not. Several studies took advantage of the DRG neuron morphology, to demonstrate that the same neuronal

cell can exhibit different growth responses after injury, highlighting once again the importance of the local environment in stimulating regeneration.

In one of these studies, when the central axons of DRG neurons were cut, located into dorsal column structures within the spinal cord and ascending towards the brain, they did not regenerate (Neumann and Woolf, 1999). When both their central and peripheral processes were lesioned at the same time, the dorsal column axons showed enhanced growth well into the lesion site, but they did not extend into the spinal cord rostral to the injury. However, when their peripheral axons were cut, and then a week later their central axons were cut, the latter regenerated robustly into the lesion site in 50% of the tested animals. These axons even extended beyond the lesion site, for several millimeters into intact spinal cord tissue, in both grey and white matter. This is significant, because as mentioned before, white matter contains inhibitors of axonal growth. When the peripheral axons were cut 2 weeks before the injury to the central axons, there was regeneration into grey matter beyond the lesion in 25% of the animals.

These results suggest that after a PNS injury, certain molecular signals are produced in the PNS, which stimulate the DRG neurons. These signals enhance the intrinsic growth state of the neurons, and induce a regenerative response by the DRGs. Surprisingly, the intrinsic changes that occur are also sufficient to enable the CNS axonal branch of the DRG neurons to regenerate, if cut after the peripheral injury. Such regeneration does not occur in the CNS under typical injury conditions. Because the PNS lesion “conditions” or “primes” the CNS to regenerate, this phenomenon was named the “conditioning lesion” effect (Neumann and Woolf, 1999).

The conditioning lesion experiments further demonstrated that an injured axon responds differently depending on its environment. Clearly, the pathophysiology of the injured CNS environment does not support but rather inhibits regeneration, and therefore the CNS must contain physical and molecular factors that limit regeneration. Furthermore, they indicated once again that CNS neurons do possess the intrinsic capacity to grow, as shown by others before in the transplantation experiments (David and Aguayo, 1981). More importantly though, this paradigm proved for the first time ever that it is possible to stimulate an innate growth program to overcome inhibition and accomplish neuronal regeneration within the hostile injured CNS, even without a permissive transplant.

1.4 Anatomy and physiology of SCI: Reasons for regeneration failure in the injured CNS milieu

Armed with the knowledge that CNS regeneration is possible, and in parallel to the transplantation experiments, several groups focused on identifying the CNS regeneration inhibitors at the cellular and molecular level. A deep understanding of the pathophysiology of CNS injury, as well as identification of the inhibitory factors and mechanisms is needed, for the eventual development of effective treatments. The hope was, and still is, that once identified, the inhibitors could be blocked. At the same time, CNS neurons could be encouraged to grow by somehow altering their intrinsic growth capacity (i.e. as in the conditioning lesion paradigm). Overall, this combinatorial approach might be a successful strategy to accomplish CNS regeneration.

After a SCI such as a contusive or a penetrating lesion, edema develops at the injury site and there is also expansion of damage to the intact tissue around the original lesion site, a phenomenon called “secondary damage”. The edema causes formation of a fluid-filled cavity at the epicenter of the impact [reviewed by (Schwab et al., 2006)].

There is also disruption of the local CNS structure. The oligodendrocytes are damaged, and the myelin sheath breaks down, creating myelin debris within the lesion site. In contrast to the PNS, the myelin remnants are cleared very slowly in the CNS, and they can persist at the lesion site for months after injury [reviewed by (Case and Tessier-Lavigne, 2005; Vargas and Barres, 2007)].

Immediately after injury, a glial scar starts to form around the lesion epicenter. Astrocytes are the major cell type contributing to scar formation, and therefore it is also referred to as the astroglial scar. The glial scar has a very dense and complex structure, consisting of many different cell types and extracellular matrix molecules. It takes weeks for the glial scar to mature, but now it is well understood that it poses both a physical and chemical barrier for regenerating axons.

The pathology of SCI is very complex, but numerous factors responsible for preventing the regeneration of axons have now been identified. A major part of the inhibition is due to proteins produced by the CNS glial cells. The myelin debris, the glial scar, and the extracellular matrix present within the injury site all contain molecules that have been identified as inhibitory to axonal outgrowth.

1.4.1 Inhibition of CNS axon regeneration by the glial scar

Injury to the CNS stimulates migration of local glia, namely astrocytes, microglia and oligodendrocyte precursors (OPs), to the impacted site. The fluid-filled cyst that forms is immediately surrounded by astrocytes which create a barrier in an effort to seal off the area and minimize inflammation. In cases where the blood-brain barrier is disrupted, there is also invasion by non-CNS cells such as fibroblasts, macrophages, and meningeal cells (Silver and Miller, 2004), (Yiu and He, 2006). At the same time, astrocytes, microglia and OPs undergo hypertrophic morphological changes [reviewed by (Rhodes and Fawcett, 2004)]. Their cell bodies enlarge, their processes thicken, and their gene expression is altered. The astrocytic processes become highly branched, and microglia and OPs also start to proliferate locally. Expression of several proteins is upregulated by these glia, and some of these have been shown to be inhibitory for axonal outgrowth. Eventually this network of "reactive" cells will create the dense glial scar, which experts agree, initially stabilizes the vulnerable CNS after an injury (Silver and Miller, 2004). Recruitment of these cell types is beneficial for the overall survival of the injured animal (Silver and Miller, 2004). For example, the role of reactive astrocytes was examined in various CNS injury models by selectively killing them. Selective ablation of astrocytes was achieved by the use of the antiviral drug ganciclovir (GCV) in transgenic mice that expressed the thymidine kinase gene of herpes simplex virus (HSV-Tk) under the control of the glial fibrillary acidic protein (GFAP) promoter. GFAP is an intermediate filament protein whose expression, restricted to astrocytes and related cells, is upregulated in reactive astrocytes after injury. Upon injury, administration of GCV to the transgenic animals will cause proliferating astrocytes expressing the HSV-Tk

transgene to die. This occurs because thymidine kinase will break down GCV into toxic nucleotide analogues which inhibit cell division and cause cell death (Bush et al., 1999). Using these mice, it was shown that in the absence of reactive astrocytes, the severity of SCI is increased due to reduced wound healing, absence of blood-brain barrier repair, increased inflammation, and degeneration of both neurons and oligodendrocytes (Faulkner et al., 2004).

Although the glial scar is initially beneficial for survival after an injury, it later presents an impenetrable barrier for regenerating axons. The barrier is not only physical but also molecular, as several proteins present in the scar are inhibitors of axonal growth. Astrocytes in particular upregulate the expression of several extracellular matrix (ECM) molecules, such as tenascin and proteoglycans [reviewed by (Rhodes and Fawcett, 2004)]. Proteoglycan molecules consist of a glycoprotein core to which sugar chains of glycosaminoglycan (GAG) are covalently attached. They are secreted into the ECM, and one particular class, the chondroitin sulfate proteoglycans (CSPGs) are a major component of the glial scar (Jones et al., 2003), and have been shown to be inhibitors of neurite outgrowth *in vitro* (Snow et al., 1990).

In addition to CSPGs and tenascin, other proteins present in the glial scar have been proposed to contribute to its growth inhibitory effects. These include semaphorin (Pasterkamp et al., 1999), slit (Hagino et al., 2003), ephrin, and ephrin receptor family members (Bundesen et al., 2003). These protein families have important roles in axon guidance during CNS development, but have been also shown to be upregulated by glial scar cell components after CNS injury, and have been implicated in regeneration failure [see also review by (Silver and Miller, 2004).

For example, the role of a tyrosine kinase receptor (RTK) for ephrin ligands, EphA4, in limiting regeneration has been described using EphA4-/- mice in a spinal hemisection injury model. When compared to wild type animals, EphA4-/- mice exhibited a lack of reactive gliosis, indicating that EphA4 has a role in astrocyte activation after injury. As a result, there was increased regeneration which resulted in functional recovery in these mice. Ephrins participate in both forward signaling through ephrin receptors on neighboring cells, and also reverse signaling into the cells that express them. The authors of this study suggested that it is probably the expression of Eph-B3, a ligand for EphA4 tyrosine kinase, on the neurons that likely mediates axonal outgrowth inhibition by binding to the EphA4 receptor on astrocytes (Goldshmit et al., 2004).

1.4.2 Inhibition of CNS axon regeneration by myelin

1.4.2.1 Experimental proof for the existence of myelin inhibitors

Berry was the first to hypothesize that myelin could inhibit axonal growth (Berry, 1982), but Martin Schwab's group in Switzerland was the first group to demonstrate experimentally that this indeed was the case. He was the first investigator to study the responses of individual, dissociated neurons, which were given the choice to grow into tissue explants of sciatic nerve (PNS) or optic nerve (CNS) in culture. He observed that axons never grew on the optic nerve explants, and always preferred the sciatic nerve explant (Schwab and Thoenen, 1985).

He subsequently tested astrocytes, oligodendrocytes, rat liver membranes and myelin preparations from rat PNS, rat CNS, chick CNS, trout CNS, and frog CNS, as substrates for fibroblast attachment and spreading, as well as neurite outgrowth (Schwab and Caroni, 1988), (Caroni and Schwab, 1988a). Cultured oligodendrocytes, as well as CNS myelin from rat and chick, were the only substrates found to be inhibitory for neurite outgrowth and fibroblast spreading (Caroni and Schwab, 1988b).

Schwab then aimed at identifying the inhibitory components in myelin membranes. He demonstrated that they were proteins, because protease treatment of the myelin eliminated its inhibitory effect on fibroblast spreading. He performed SDS-PAGE size fractionation of myelin proteins, extracted the protein fractions from the gel, reconstituted them in liposomes and found that the fractions containing 35 kDa and 250 kDa size proteins were inhibitory substrates for both fibroblast spreading and neurite extension from superior cervical ganglia (SCG) neurons (Caroni and Schwab, 1988b).

He then produced monoclonal antibodies against the 250 kDa fraction, and found that one of the antibodies produced, named IN-1, was able to neutralize CNS myelin non-permissiveness *in vitro*. The IN-1 antibody was able to bind to the 35 kD inhibitory myelin fraction as well, and neutralize its non-permissiveness for growth. Pretreatment of myelin with trypsin abolished IN-1 binding to it, therefore Schwab concluded that the IN-1 antigens were not glycolipids (Caroni and Schwab, 1988a). Nevertheless, cloning and identification of the IN-1 antibody antigen would take another decade.

Since these pioneering observations by Schwab, several proteins present in myelin have been shown to be inhibitors of neurite outgrowth *in vitro.* At the same time receptors and co-receptors for them, which transduce the inhibitory signals, have been

identified on the neuronal membrane. Upon injury, the oligodendrocytes forming the myelin sheath around axons are damaged, and myelin membranes are locally disrupted, resulting in their altered geometrical organization. The inhibitors present in the damaged myelin, which come in contact with neuronal growth cones that are presumably trying to regenerate after injury, present a major obstacle to axonal growth and regeneration.

In recent years the myelin proteins myelin-associated glycoprotein (MAG), nogo-A, oligodendrocyte myelin glycoprotein (OMgp), ephrin-B3, and sema4D have been shown to inhibit neurite outgrowth *in vitro*, and are considered to be potent inhibitors of axonal re-growth after axotomy *in vivo*.

1.4.2.2 MAG

MAG is a transmembrane myelin protein found in both CNS and PNS myelin sheaths, which contains about 30% by weight carbohydrate. It has one transmembrane domain, and its intracellular domain can be in a short (S-MAG), or a long (L-MAG) form due to mRNA splicing. It also contains five immunoglobulin (Ig) like domains in its extracellular domain, which classify MAG as belonging to the Ig superfamily. Via arginine 118 (R118) in its first Ig domain, the extracellular domain of MAG binds sialic acids (Tang et al., 1997), which are nine-carbon sugars that often are the outermost monosaccharides on the glycan chains of cell-surface glycoproteins and glycolipids (Crocker, 2002), (Varki and Varki, 2007). Therefore MAG belongs to the sialic acid binding Ig-like lectin (Siglec) subgroup of immunoglobulins, and is categorized as human Siglec-4a. The sialic acid bearing glycosphingolipids (also known as gangliosides) GD1a and GT1b, have been proposed to be functional ligands in MAG-mediated inhibition of

nerve regeneration (Vyas et al., 2002). However, it seems that binding to GT1b might be necessary for inhibition only when MAG is presented to neurons as a soluble molecule, or as an immobilized substrate, and not when MAG is within its native, cell membrane topology [see review by (Filbin, 2003)].

MAG is found in CNS and PNS myelin, and its abundance is estimated to be about 1% of all CNS myelin proteins, and 0.1% of PNS myelin proteins (Trapp, 1990). Immunocytochemical studies showed that MAG's localization is periaxonal, meaning that it is present in the portion of the myelin sheath that contacts the axon. It is also localized to the paranodes of Schwann cells, and to a lesser extent in paranodal regions of some areas of the CNS. More recently, Huang et al., (2005) also observed MAG localization in non-myelin derived membranes of the axoglial apparatus at the nodes of Ranvier.

Various abnormalities have been reported in both glial cells and neurons in MAG-null mutant mice, but the overall phenotype resulting from MAG ablation is subtle (Schachner and Bartsch, 2000). In the CNS, MAG plays a role in myelin formation and maintenance of normal oligodendrocytes. In aging MAG-null mice there is degeneration of the oligodendrocyte processes surrounding axons, a phenomenon called dying-back oligodendrogliopathy (Lassmann et al., 1997). Quarles et al. also discovered decreased levels of several myelin proteins in these mice (CNPase, N-CAM, tubulin, Fyn, Na^+K^+ATPase), an indication of pathological changes which may result in oligodendroglial dystrophy or damage (Weiss et al., 2000). They proposed that these pathological changes might be the underlying cause of the oligodendrogliopathy observed

by Lassmann et al., and that MAG may be acting as a receptor that relays trophic signals from the neurons to the oligodendrocytes [also reviewed by (Quarles, 2007)].

In the PNS, MAG appears to have a role in the formation and maintenance of myelinated axons. It is known that myelination increases axonal caliber by a mechanism involving increased phosphorylation and spacing of neurofilaments. In the PNS of MAG-null mice, normal myelin forms during development; however, a neuropathy was observed at 9 and 16 months, which was also characterized by decreased axonal caliber and Wallerian degeneration of myelinated fibers (Yin et al., 1998). Loss of MAG in the nulls also resulted in reduced neurofilament phosporylation and spacing, as well as axonal atrophy in paranodal regions, suggesting that MAG modulates the caliber and viability of myelinated axons.

The discovery of these CNS and PNS abnormalities implies that MAG is involved in bi-directional signaling between neurons and glia [reviewed by (Schachner and Bartsch, 2000)]. It may be acting both as a ligand for a neuronal receptor(s) involved in maintenance of normal myelinated axons in the PNS, and also as a receptor on the glial membrane for a signal that is involved in the maintenance of normal oligodendrocytes [reviewed by (Quarles, 2007)].

In 1994, the Filbin lab, and in parallel McKerracher's group in Canada, proposed that MAG acts as an inhibitor of regeneration in the injured CNS (Mukhopadhyay et al., 1994), (McKerracher et al., 1994). Isolated cerebellar and DRG neurons that were grown on a substrate of MAG-expressing feeder cells exhibited about 70% shorter neurites than neurons growing on control cells. As previously mentioned, MAG binds sialic acids via the R118 residue in its first Ig domain, but this event is not required for membrane-

localized MAG to inhibit neurite outgrowth (Tang et al., 1997). It was later shown by our lab that the inhibitory site of MAG is distinct from its sialic acid binding site, and maps to MAG's fifth Ig domain (Cao et al., 2007).

That same year, MAG knockout mice became available, thus making it possible to test the role of MAG as a major inhibitor of regeneration *in vivo* (Bartsch et al., 1995). In their study, the authors found the same lack of regeneration in both $MAG^{+/+}$ and $MAG^{-/-}$ mice, when they examined the corticospinal (CST) and optic nerve tracts after injury. In the same study, the IN-1 antibody was applied to the $MAG^{-/-}$ mice, and the authors found improvement of axonal growth in both tracts, and to a similar extent in wild type and MAG mutant animals. Based on these results, they suggested that MAG does not play a major role in preventing axonal regeneration *in vivo*.

The following year, another group performed *in vivo r*egeneration studies of MAG null mice, which had a different genetic background from the null mice used in the first study (Li et al., 1996). They reported limited regeneration after spinal cord injury, with a very small number of CST axons extending past the lesion site. However, this latter observation is significant, because as will be discussed below, MAG is only one of several white matter inhibitors acting collectively. It is hard to estimate the relative percent contribution of each of the inhibitors to the total myelin inhibition effect *in vivo*, but the fact that some regeneration was observed in the MAG null mice suggests a role for MAG in it.

1.4.2.3 Nogo

The Nogo protein was independently cloned and identified as a myelin inhibitor in 2000 by three different groups (Chen et al., 2000), (GrandPre et al., 2000), (Prinjha et al., 2000). Three main Nogo isoforms are expressed in the CNS, Nogo-A, -B and –C. The Nogo-A isoform is enriched in oligodendrocytes, but none of the three isoforms are significantly expressed by Schwann cells (GrandPre et al., 2000). All three isoforms are also expressed by various neuronal subpopulations, as well as by several other non-neuronal tissues (Oertle and Schwab, 2003), (Hunt et al., 2003). They all contain an extracellular loop, named Nogo-66, which inhibits axonal growth (GrandPre et al., 2000), (Fournier and Strittmatter, 2001). Nogo-A, the longest of the isoforms, contains an additional inhibitory region, named Amino-Nogo, within its unique amino terminus. Amino-Nogo has been shown to exist both on the cell surface facing extracellularly, but also intracellularly, facing the cytoplasmic side of internal membranes. Because of these observations, Oertle et al., (2003) suggested that Nogo-A might exist in two different membrane topologies (Oertle et al., 2003). It has been shown to inhibit both axon outgrowth and fibroblast spreading *in vitro,* but the receptor/s mediating this function remain unknown (Oertle et al., 2003), (Fournier et al., 2001).

In 2003, regeneration studies in Nogo knockout mice were published by two of the original groups that cloned Nogo, as well as by Tessier-Lavigne and collegues. The three groups looked for regeneration in the CST using the same labeling technique, but three different results were reported. Schwab's group used a mouse strain that did not express the Nogo-A isoform, and also exhibited higher levels of Nogo-B expression, but unchanged Nogo-C expression. Some degree of CST regeneration was reported in these

mice (Simonen et al., 2003). Strittmatter's group, which used a mouse strain that lacked expression of both Nogo-A and Nogo-B isoforms, reported extensive regeneration of the CST of younger mice (< 9 weeks old) (Kim et al., 2003). Tessier-Lavigne's group used two mice strains in their study. In one strain both Nogo-A and –B were deleted, and in the other all three Nogo isoforms were deleted. This study reported no CST regeneration in either of the two strains (Zheng et al., 2003).

In all three studies, the mutant animals were early generation chimeras of the mouse strains Sv129 and C57BL/6, but with unknown proportions of each genetic background. Because of this, Schwab's group compared the effects of mouse strain genetic background on the penetrance of Nogo-A deletion in CST regeneration. Backcrossed breeding was used to obtain two Nogo-A knockout mouse strains with high purity (99%) strain backgrounds, C57BL/6 and Sv129. The assumption was that different inbred mouse strains differ phenotypically and genotypically, and these differences might alter their regenerative potential. Indeed, there was enhanced regeneration in the Nogo-A knockout of the Sv129 strain when compared to not only wild type animals of the same strain, but also compared to the Nogo-A knockouts of the C57BL/6 strain (Dimou et al., 2006). These experiments confirmed the assumption that differences in the genetic makeup of the animals used in the earlier studies could produce different regeneration responses. In humans, it is now well understood in the area of pharmacogenomics, that different individuals can have a different response to a specific treatment, a fact that also applies in the development of treatments for CNS regeneration.

To further investigate the reasons for the conflicting results, Steward and colleagues repeated the same experiments using the same mouse strains that Strittmatter's

group used when they observed extensive CST regeneration. The results were published four years later in the correspondence section of the journal Neuron (Steward et al., 2007). Steward proposed that the regeneration Strittmatter's group had observed was due to erroneous labeling, an assertion that the Strittmatter group rejected (Cafferty et al., 2007). Overall, the current perspective on the controversies regarding regeneration in the Nogo knockout mice can be summed up in the last sentence of Strittmatter's correspondence: "*Thus, strain, age, mutant allele, and lesion model all have an influence on the adult CNS axonal growth phenotype of mice lacking Nogo-A"*.

1.4.2.4. OMgp

A third protein, OMgp, was characterized as a myelin inhibitor by Zhigang He's group (Wang et al., 2002b). OMgp is anchored to the cell membrane extracellularly, through a glycosyl phosphatidylinositol (GPI) group, and was known to be expressed by oligodendrocytes (Mikol and Stefansson, 1988). It was identified as an inhibitor in an assay where phosphatidylinositol (PI) -containing proteins were cleaved from purified myelin by PI-specific phospholipase C treatment. The released proteins were able to induce collapse of embryonic day 13 (E13) chick DRG growth cones. One of the released proteins was OMgp. The authors purified recombinant polyhistidine-tagged OMgp and showed that it causes growth cone collapse in E13 chick DRG neurons, and inhibits neurite outgrowth of cultured cerebellar granule neurons.

Another study demonstrated that OMgp is expressed by oligodendrocyte-like cells that ensheath axons at the nodes of Ranvier. In this study, OMgp-/- mice exhibited abnormally wide node spacing, and also collateral sprouting from the nodes. These

results suggest that OMgp plays a role in normal node architecture and in preventing collateral sprouting from axons at the nodes of Ranvier (Huang et al., 2005).

1.4.2.5 Developmental guidance molecules as inhibitors of regeneration in myelin: Ephrin-B3 and Sema4D

EphrinB3 and Sema4D are repulsive guidance cues for axonal outgrowth during development, and apparently, they also play a role in myelin's regeneration blocking activity. They are expressed by oligodendrocytes, and have been shown to be components of the inhibitory activity of myelin *in vitro* (Benson et al., 2005; Moreau-Fauvarque et al., 2003).

1.4.3 Receptors for the myelin inhibitors

1.4.3.1 Nogo receptor family

The first receptor for a myelin inhibitor was identified in 2001. It was named Nogo receptor (now called NgR1), because the extracellular domain of Nogo-A protein, Nogo-66, was used as a bait to screen an expression library (Fournier et al., 2001). Amino-Nogo, the other inhibitory region of Nogo-A, is not a ligand for NgR1. Later, both MAG and OMgp were also identified as ligands of NgR1 (Liu et al., 2002; Wang et al., 2002b) (Domeniconi et al., 2002). Even though they share no sequence or structural similarity, all three inhibitors bind to this leucine-rich repeat receptor to inhibit neurite outgrowth. These findings raised hope that blocking the NgR1 receptor would result in

substantial *in vivo* regeneration, because the effect of several inhibitory signals would be blocked at once.

Like one of its ligands, OMgp, NgR1 is linked to the cell membrane via a GPI group and has no cytosolic domain. Several co-receptor molecules in complex with NgR1 have been identified though, which transduce the inhibitory signal into the cell. Two homologs of NgR1, the structurally related proteins NgR2 and NgR3 have also been discovered (Barton et al., 2003; Pignot et al., 2003). NgR2 is a selective receptor for MAG, and is able to transduce MAG's inhibitory signal (Venkatesh et al., 2005).

Interestingly, NgR1 and NgR2 were recently implicated in modulating inflammatory responses, specifically in macrophage clearance from injured peripheral nerves. As previously discussed, macrophages migrate into the distal segment of the injured nerve and clear up the cellular debris generated by injury. The repair process also requires that macrophages eventually leave the site, once their job is completed. Sam David's group found that the efflux of macrophages from the nerve into the endoneurial space is mediated by NgRs. Once phagocytosis of axon and myelin debris is completed, activated macrophages upregulate NgR1 and Ngr2. Thus, it appears that re-myelination of the regenerating axons by SCs provides the repulsive signals (*i.e.* MAG and other inhibitors) that macrophages need in order to be expelled away from the recovering nerve (Fry et al., 2007).

Two independent groups generated NgR1 knockout mice to assess its role in regeneration. No CST regeneration was observed in these mice. *In vitro*, DRG and cerebellar neurons from these mice were inhibited by myelin and Nogo-66 to the same degree as wild type mice (Zheng et al., 2005). Strittmatter's group, however, observed

regeneration of the raphespinal and rubrospinal tracts, and also a reduction of MAG-Fc – induced growth cone collapse in DRG neurons in their NgR1 null mice (Kim et al., 2004). Overall, these results suggested the presence of additional, unidentified receptors.

The emerging view is that NgR1 seems to confer the acute growth-cone collapsing effect of the three myelin inhibitors, but is not necessary for the chronic growth inhibition by myelin. A recent study showed that cerebellar granule neurons (CGN) (P7) from the $NgR1^{-/-}$ mouse are still inhibited by MAG, and DRG neurons (P25) from the $NgR1^{-/-}$ mouse are still inhibited byNogo66 and OMgp, when these are used as substrates for growth. The same persistence of inhibition by MAG was observed in E18.5 rat cortical neurons transfected with shRNA for NgR1. However, DRG neurons from the $NgR1^{-/-}$ mice exhibited reduced MAG-Fc- and OMgp-Fc-induced growth cone collapse when compared to wild type neurons (Chivatakarn et al., 2007). These results, combined with the results from Kim et al. (2004), provided additional evidence for the existence of additional receptors for the myelin inhibitors.

1.4.3.2 Co-receptors for NgR1

As soon as NgR1 was discovered, it was realized that co-receptors for it must exist, because the NgR protein does not contain an intracellular domain. The transmembrane protein p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor family (TNFR), was found to interact with NgR1, and proposed to be the signal transducing subunit of the receptor complex (Wang et al., 2002a).

The current model is that activation of the NgR1 receptor complex by the myelin inhibitors results in activation of the monomeric GTPase RhoA (Fournier et al., 2003; Yamashita et al., 2002), and that this event is required for inhibition of neurite outgrowth. RhoA activation is also $p75^{NTR}$ –dependent (Yamashita and Tohyama, 2003). Rho is a known mediator of extracellular signals to the actin cytoskeleton in the growth cone, and actin polymerization at the growth cone drives axonal extension (Hall, 1998). RhoA activation has been shown to mediate growth cone collapse and neurite retraction (Jalink et al., 1994), and it is considered a convergence point for both myelin inhibitors of axonal outgrowth, as well as CSPGs of the glial scar (Dergham et al., 2002).

In a study by Sha Mi and colleagues, when NgR1 and p75 were transfected in a non-neuronal cell line, and cells were stimulated with OMgp, Rho-A was not activated. This experiment indicated the existence of additional co-receptors, besides p75, in the functional NgR receptor complex. Another transmembrane protein called LINGO-1, which is selectively expressed in the brain, was found to bind both NgR1 and p75. If COS-7 cells are tranfected with NgR1, p75 and LINGO-1, and then stimulated with either MAG, Nogo-66 or OMgp, there is activation of Rho-A, an indication that a functional tripartite receptor complex is formed by the three tranfected proteins (Mi et al., 2004).

In neurons that do not express $p75^{NTR}$ in the adult CNS, TROY/TAJ, which is another member of the TNFR family, can interact with NgR1 and LINGO to form a functional receptor complex (Park et al., 2005; Shao et al., 2005).

The availability of $p75^{-/-}$ mice made it possible to examine the role of this co-receptor in regeneration. Neurite outgrowth of adult DRG neurons from the $p75^{-/-}$ mouse

was not inhibited by MAG-Fc. Furthermore, neurite outgrowth of $p75^{-/-}$ P7 CGN was not inhibited by Nogo-66, OMgp and myelin when used as immobilized substrates (Wang et al., 2002a). Another study, however, found that $p75^{-/-}$ CGN (P7) were still inhibited by myelin to the same extent as wild type, and that the CST tract of the $p75^{-/-}$ mice does not regenerate (Zheng et al., 2005). Similar results to this latter study were reported by Song *et al.*, (2004). In studies from this group, the p75 knockouts did not display any regeneration of the CST or of ascending sensory neurons in the injured dorsal column (Song et al., 2004). Collectively, these results once again pointed to the existence of more receptors for the inhibitors, besides the NgR1 receptor complex.

1.4.3.3 PirB

Recently, the suggestion that, besides NgR1, there must be other receptors for myelin inhibitors present on the neuronal membrane was validated. Tessier-Lavigne's group used expression cloning to screen a library of human cDNAs for binding partners of Nogo-66. They identified the human leukocyte Ig-like receptor B2 (LILRB2) as a receptor not only for Nogo66, but once again, MAG and OMgp as well (Atwal et al., 2008). The single mouse ortholog for LILRB2 is the paired immunoglobulin-like receptor B (PirB). His group also used mutant mice lacking functional PirB (PirBTM mice), (Syken et al., 2006) to test myelin inhibition *in vitro.* P7 CGNs from PirBTM mice were less inhibited by myelin, Nogo66, MAG, and OMgp, and DRG neurons from the same mice were less inhibited by myelin and Nogo-66.

It is surprising that history is repeating itself, because even though Nogo, MAG, and OMgp share no sequence similarities, they can all bind to two receptors which also

share no sequence similarities between them (Fig. 1.1). However, the unusual promiscuity of NgR and PirB might be an advantage in therapy design, because when both of these receptors are blocked, using cerebellar neurons from $NgR^{-/-}$ mice and adding an anti-PirB antibody, inhibition by myelin was completely blocked (Atwal et al., 2008). The CNS regeneration field is now eagerly anticipating the results of CST regeneration experiments in an NgR-PirB double knockout mouse [see (Filbin, 2008)].

1.4.3.4 EGFR and other calcium-activated signals

Nogo-66, OMgp, and CSPG stimulation of neonatal CGN can trigger activation of the epidermal growth factor receptor (EGFR) tyrosine kinase. The myelin inhibitory ligands do not bind directly to the EGFR, and the EGFR does not physically interact with either NgR or p75, as shown by cell surface binding and co-immunoprecipitation experiments. EGFR phosphorylation is a "trans-activation" event, which happens in a NgR-complex and Ca^{++} dependent manner. *In* vitro, EGFR inhibitors attenuate inhibition of certain types of neurons by myelin, Nogo-66, MAG and CSPG immobilized substrates. *In vivo,* EGFR inhibitors promoted regeneration of a small number of retinal ganglion axons beyond the lesion site in an optic nerve crush model (Koprivica et al., 2005). This latter study was not the first to implicate glial inhibitors in elevating the levels of the Ca^{++} second messenger. CSPGs and myelin components have been previously shown to elevate Ca^{++} (Bandtlow et al., 1993),(Snow et al., 1994) and MAG and Nogo have been shown to also activate protein kinase C (PKC) (Hasegawa et al., 2004). Our lab has further shown that a PKC- and γ-secretase dependent release of

the cytoplasmic domain of $p75^{NTR}$ is necessary for Rho activation and inhibition of neurite outgrowth (Domeniconi et al., 2005).

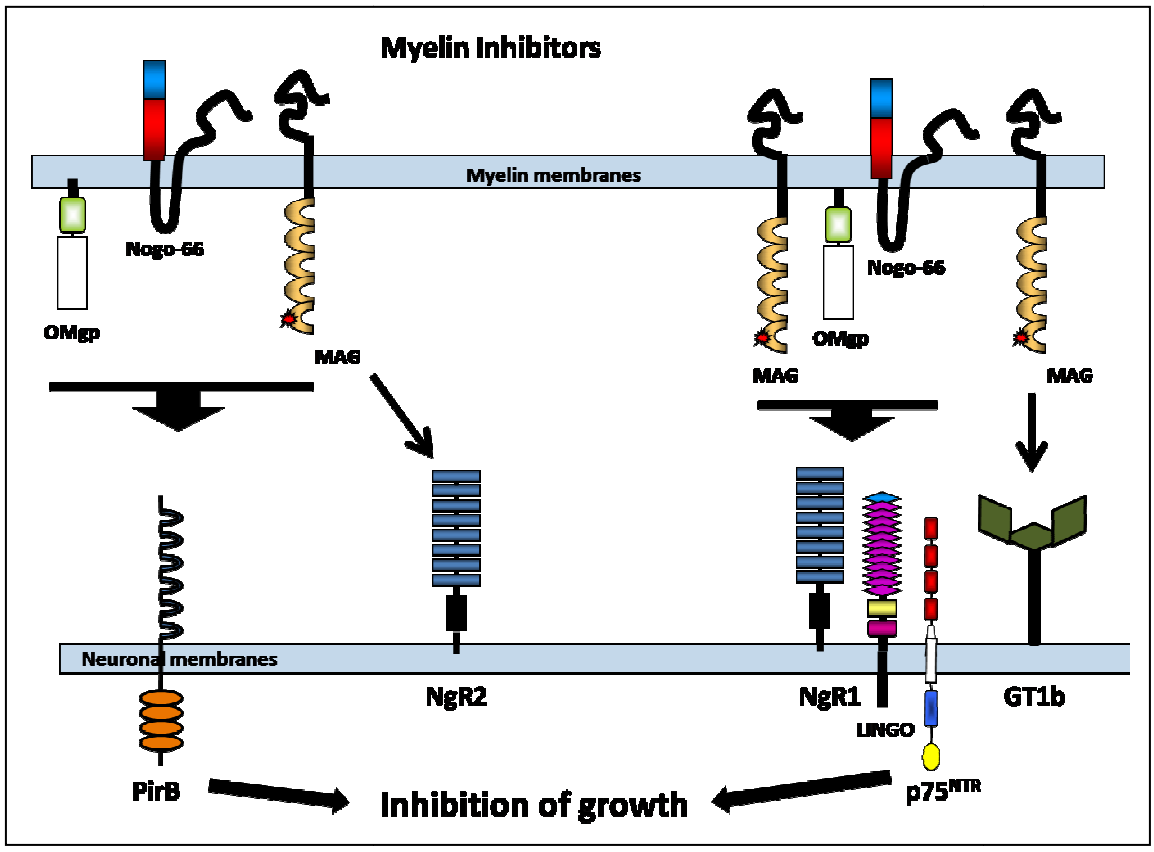


Figure 1.1. The three myelin inhibitors MAG, Nogo-66, and OMgp bind to common receptors to inhibit neurite outgrowth. All three inhibitors bind PirB, and also act through the NgR1-receptor complex in the inhibitory pathway. NgR2 is a selective receptor for MAG, able to transduce MAG's inhibitory signal. Binding of MAG to the ganglioside GT1b is necessary for inhibition when MAG is presented to neurons either in a soluble form, or as an immobilized substrate.

1.4.4 Physiological role of the myelin inhibitory signals

The vast majority of the studies of myelin inhibitor signaling have been performed in the context of CNS injury models, and our understanding of the role of the inhibitors in limiting CNS regeneration has dramatically increased. But there is also a need to define the normal, physiological role of these molecules in the developing and mature brain.

One of the roles appears to be the suppression of plasticity in the mature CNS in order to maintain integrity of the neural circuitry (McGee et al., 2005). One form of neuronal circuitry plasticity is "experience-driven". Experience-driven plasticity is very important for proper development of the brain's sensory systems, and for complex abilities like language and social behavior. However, there are established critical periods in animal development, beyond which such plasticity cannot occur (Kandel, Principles of Neural Science, 2000).

Interestingly, two recent studies demonstrated a role for NgR and PirB proteins in ending the critical period for experience-driven plasticity, and also in inhibiting plasticity thereafter. The experimental paradigm used in these studies was ocular dominance plasticity in the visual cortex of mice. In this paradigm, closure of one eye (monocular deprivation) for a few days during a postnatal critical period between 19 to 32 days shifts ocular dominance in the visual cortex to the open eye, resulting in loss of vision in the deprived eye. If monocular deprivation is imposed after the critical period or well into adulthood, no such shift occurs (Fagiolini et al., 1994), (Pizzorusso et al., 2002).

In mutant mice lacking NgR or NogoA/B, monocular deprivation on postnatal day 45, after the critical period had ended, resulted in an ocular dominance shift to the non-

deprived eye. A similar effect was observed when monocular deprivation was performed in adult four month old NgR-/- mice (McGee et al., 2005).

Similarly, in mutant mice lacking the PirB receptor, ocular dominance plasticity after monocular deprivation or after removal of one eye (monocular enucleation) was enhanced during the critical period compared with that of wild-type mice. The enhanced plasticity was detected by the method of Arc mRNA induction, an activity-regulated immediate-early gene (Tagawa et al., 2005), (Lyford et al., 1995). Visual stimulation induces Arc mRNA in the visual cortical neurons of the stimulated eye. If the visual stimulation is followed by *in situ* hybridization for Arc mRNA, the distribution of the neurons functionally connected to the stimulated eye in the visual cortex can be revealed (Syken et al., 2006). In the same study, enhanced ocular dominance plasticity after monocular enucleation was also detected in adult mice (P110), demonstrating that PirB restricts ocular dominance plasticity both during and after the critical period.

The results of both studies support the hypothesis that one of the physiological roles of the myelin inhibitors is to constrain neuronal plasticity, in order to stabilize the neural circuits which form during development (Sengpiel, 2005).

1.5 *In vivo* strategies to promote spinal cord regeneration

In parallel to the identification of the several factors involved in regeneration block, scientists begun to explore various strategies to overcome inhibition *in vivo* by blocking the inhibitors, their receptors, or their signaling effectors. Another strategy might be to alter the intrinsic growth dynamic of the neurons, so that they become insensitive to the inhibitory proteins in myelin.

1.5.1 *In vivo* interventions to block glial scar inhibitors: Infusion of ChABC

Chondroitinase ABC (ChABC) is a bacterial enzyme capable of degrading the GAG chains of many inhibitory proteoglycans in the glial scar. Its therapeutic potential was tested by intrathecal delivery in an adult rat model of SCI. After bilateral dorsal column lesion, treatment with this enzyme resulted in regeneration of CST axons, and also of ascending sensory axons. Even though the observed anatomical regeneration was limited, it resulted in functional recovery as measured by various sensory and motor tests (Bradbury et al., 2002).

1.5.2 *In vivo* interventions to block myelin inhibitors and their receptors

1.5.2.1 Use of knockout mice for MAG, Nogo, NgR, p75NTR

As mentioned earlier, mutant mice with genetic deletions of individual myelin inhibitors, or receptors for them, were used to demonstrate their role in the block of regeneration *in vivo.* Experiments with these knockout mice, however, had mixed outcomes, and limited success in promoting regeneration *in vivo.* The reason for this is probably redundancy of inhibitors and receptors, as well as compensation mechanisms in the mutant mice [discussed in (Yiu and He, 2006), (Xie and Zheng, 2008)]. Besides the use of knockouts, additional strategies have been used to neutralize the myelin inhibitory molecules and promote regeneration *in vivo*, and these are discussed below.

1.5.2.2 Use of the IN-1 antibody

Two years after the production of the IN-1 antibody, Schwab and colleagues reported the results of its use in a young rat (2-6 week old) *in vivo* regeneration model, employing complete transections of the CST. The presence of the IN-1 antibody (supplied by antibody-producing tumors intracerebrally) resulted in long-distance growth of labeled CST axons, up to 5-11 mm caudal to the lesion after 2-3 weeks post-injury. By contrast, in control animals the amount of growth did not exceed 1mm (Schnell and Schwab, 1990).

In a similar study, Schwab and colleagues used young adult rats (6-8 weeks old) in an overhemisection SCI model, with the same source of IN-1 as in the 1990 study. Once again, the presence of the IN-1 antibody promoted regeneration of not only corticospinal fibers, but also serotonergic and noradrenergic descending spinal axons, all caudal to the lesion. Importantly, the IN-1 treated animals also recovered some aspects of the lost locomotor function (Bregman et al., 1995).

1.5.2.3 Blocking ligand access to NgR1

Since three major myelin inhibitors act via the common receptor NgR1, in theory, blocking NgR1 activation should relieve a considerable portion of myelin's inhibition of regeneration *in vivo.* Blocking signaling by a receptor can be achieved by designing molecules that bind the receptor and, without activating it, prevent access to it from its ligand (competitive antagonism). Another approach is to use soluble receptor, or small fragments of it, to saturate its ligand, thus blocking ligand binding to the real cellular

receptor (dominant negative). Both approaches have been used successfully to block NgR1 signaling in models of SCI.

A synthetic Nogo-66 peptide called NEP1-40 (Nogo extracellular peptide, residues 1-40) binds NgR1 with high affinity without activating it. In a competitive antagonism approach, this peptide was delivered acutely through an osmotic minipump at the site of a dorsal hemisection injury in rats. Significant CST and serotonergic raphespinal sprouting and axon growth occurred. This was accompanied by functional recovery, thus rendering NEP1-40 a potential therapeutic agent (GrandPre et al., 2002). In another study by the same group, similar results were reported after systemic NEP1-40 administration one week after injury, a therapeutic strategy with greater clinical feasibility that the acute intrathecal application in the original study (Li and Strittmatter, 2003).

In a more recent study, Steward et. al. repeated the NEP1-40 antagonist study, as part of the NIH FOR-SCI project(Facilities of Research-Spinal Cord Injury), which supports independent replication of promising findings, in order to promote their transition to translation (Steward et al., 2008). Key experiments published in Li and Strittmatter (2003) were repeated as closely as possible in the new study. The results though were different. The authors discovered that in control mice there is an innate capacity for CST regeneration, or sprouting, that had not been reported before. After injury, some axons can regenerate/sprout past the lesion, via the ventral column to reach segments caudal to the lesion site. This growth was observed in NEP1-40 treated animals with a greater incidence than in the control groups, suggesting that NEP1-40 treatment created a more conducive environment for it. Additionally, there was also a

trend of enhanced locomotor function at 7, 10 or 13 (but not 17 or 20) days post injury in a subgroup of both NEP1-40 and control, reversed NEP1-40 sequence-peptide treated animals. Overall though, the major finding was that the authors did not detect the enhanced regeneration of CST and serotonergic fibres, or the overall enhanced functional recovery that was reported previously (Steward et al., 2008), (Li and Strittmatter, 2003). The authors attributed their different experimental outcome to unknown experimental variables, which need to be identified in order to fully characterize the therapeutic potential of the NEP1-40 peptide.

A similar model system to the one used in testing NEP1-40 by GrandPre et al., (2002) was used by Strittmatter's group to test the dominant negative approach of NgR1 neutralization. In this study, the NgR(310) ecto-Fc peptide was applied, which corresponds to the first 310 amino acids of NgR1 fused to the rat IgG1 Fc domain (Li et al., 2004). This soluble peptide contains the ligand binding domains for Nogo, MAG and OMgp and was shown to prevent binding of all three ligands to NgR *in vitro*. *In vivo* use of the peptide resulted in significant regenerative growth in both the CST and the serotonergic raphespinal fiber systems, along with enhanced locomotor function. These results demonstrated the clinical potential in blocking NgR signaling.

1.5.3 Blocking RhoA GTPase signaling

As previously mentioned, RhoA signaling mediates inhibition by both myelin inhibitors (Lehmann et al., 1999) and CSPGs (Dergham et al., 2002), and thus, targeting the RhoA pathway allows disruption of several inhibitory signals at once. The effect of RhoA inhibition on regeneration was studied in both mouse and rat dorsal hemisection

models of SCI. RhoA can be blocked by the *Closteridium botulinum* enzyme C3 transferase (C3), which ADP-ribosylates Rho's effector domain. Administration of C3 via polymer release after thoracic dorsal over-hemisection in adult mice resulted in sprouting of CST axons into the lesion site and dorsal white matter, and produced functional recovery (Dergham et al., 2002). C3 was also administered intrathecaly, via an osmotic mini pump, after thoracic dorsal hemisection lesions in adult rats, but there was no regrowth of the CST fibers under these experimental conditions (Fournier et al., 2003). In these two studies, the effect of inhibiting Rho's downstream effector ROCK was also examined by employing the same paradigms used for C3 testing. In both the mouse and rat models, ROCK inhibition resulted in enhanced regeneration of the CST axons and functional recovery (Dergham et al., 2002; Fournier et al., 2003). These results provided evidence that inhibition of the Rho signaling pathway is a promising therapeutic target.

1.5.4 In vivo interventions to enhance neuronal intrinsic growth ability

1.5.4.1 Use of Neurotrophins in models of CNS injury and regeneration

It was soon realized that just providing a permissive environment might not be enough to stimulate regeneration of CNS axons. When embryonic spinal cord transplants were inserted to bridge a spinal cord lesion in adult rats, host axons grew into the transplant, but innervation of it was sparse, and the axons stopped growing at the immediate host-transplant border (Bregman et al., 1997). Bregman and colleagues hypothesized that while providing a conducive environment was important, neurons

might also need a growth stimulus in order to grow long distances within the permissive transplants, and also beyond, into host tissue caudal to the transplant.

Besides Wallerian degeneration, another difference between the PNS and CNS after injury is that in the PNS there is production of trophic molecules by neurons, Schwann cells, and fibroblasts, and also upregulation of receptors for these molecules by neurons. Among the upregulated molecules are brain derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) protein family, and also TrkC, the receptor for neurotrophin-3 (NT-3) (Plunet et al., 2002; Vargas and Barres, 2007).

It therefore seemed possible that NTs would be perfect candidates to provide this growth signal after CNS injury. Neurotrophins (NTs) exert various trophic effects and maintenance roles in the nervous system. It was hypothesized that if neurons are stimulated by NTs, their intrinsic growth capacity will be enhanced and CNS regeneration will be enhanced as well.

1.5.4.2 Transplants and neurotrophins after SCI result in a better outcome than transplants alone

When NTs were included along with fetal spinal cord transplantation acutely after injury, innervation of the transplant was greatly improved. There was increased density as well as increased length of axons in the transplants, which was not observed in the absence of NT treatment (Bregman et al., 1997). Additionally, 55% of the treated animals exhibited labeled serotonergic fibers in the host cord caudal to the transplant (Coumans et al., 2001).

In another set of experiments, Bregman's group delayed the transplant and NT treatment until 2 weeks after injury, after removing the scar tissue that had formed. Under these conditions, in 89% of the animals, axons not only grew into the transplant, but were able to advance beyond it, and enter non-permissive host spinal tissue caudal to the transplant. Axonal growth into the caudal host spinal cord was more dense, more branched, and greater in distance, compared to acute treatment. Importantly, the improved regeneration that was observed resulted in improved motor function (Coumans et al., 2001).

The results of these experiments demonstrated that treatment with NTs can increase the extent of axonal growth into permissive transplants, and also into host cord tissue, facilitating greater functional recovery. NTs therefore may be a useful treatment in CNS injury, because they stimulate axon growth into both a permissive and a non-permissive environment.

1.5.4.3 In vivo administration of the second messenger cAMP mimics the conditioning lesion effect and promotes regeneration

A peripheral injury enhances the intrinsic growth state of the neuron, so finding a way to mimic this effect might be a useful regenerative strategy. Indeed our lab discovered a way to manipulate neuronal signaling pathways *in vivo*, resulting in regeneration that mimics the conditioning lesion effect. This was accomplished by elevating neuronal cAMP levels. Elevating the intracellular concentration of cAMP can be accomplished *in vivo* by delivering its non-hydrolysable analogue dibutyryl cAMP (dbcAMP) to a particular structure in a living animal, or *in vitro* by adding it to cultured

neurons. To evaluate the drugs' effects in the *in vitro* experiments, we use a neurite outgrowth assay, in which isolated neurons are plated on MAG-expressing, as well as control monolayers of Chinese hamster ovary cells (CHO), or on a myelin substrate. Neurite length can then be measured after the addition of dbcAMP or other agents in the media, to assess their potential in overcoming inhibition by MAG/myelin.

It is worth mentioning that there is a wealth of literature about the role of cAMP in regulating neuronal function. This second messenger has been shown to be a differentiation signal in neuronal cells (Dworkin et al., 2009). It has also been shown to convert axonal repulsion by negative guidance cues to attraction (Ming et al., 1997), and is involved in neuronal plasticity and memory (Frey et al., 1993).

First, our lab demonstrated that the conditioning lesion effect is cAMP and PKA dependent (Qiu et al., 2002). The peripheral branch of DRG neurons was cut and then 1 day, or 1 week later, the DRG neurons were removed and cultured overnight on MAG expressing cells or myelin. The neurons were not inhibited under these conditions, indicating that a conditioning lesion results in neurons becoming insensitive to myelin inhibitors *in vitro*. However, if a PKA inhibitor is added to DRG neurons that have been removed one day after peripheral lesion, they are still inhibited by MAG and myelin. This indicates that the effect of the conditioning lesion in overcoming myelin inhibitors is cAMP and PKA dependent at 24 hours after lesion. If a PKA inhibitor is added to DRG neurons removed one week after peripheral lesion, a different effect is observed: neurons still overcome inhibition by MAG and myelin *in vitro*. This indicates that the peripheral injury effect in overcoming inhibition one week after lesion is no longer dependent on cAMP and PKA. Additionally, immunostaining DRGs for cAMP, and also measuring its

levels in DRG cells by a competitive immunoassay one day after injury revealed that the conditioning lesion had resulted in a three-fold elevation in cAMP levels. When analyzed one week after injury though, cAMP levels had dropped back to uninjured control levels.

Next our lab examined if the PKA-independent phase in the block of inhibition observed one week after peripheral lesion requires the cAMP and PKA signaling occurring after the peripheral lesion, or if these are independent phenomena. The PKA inhibitor H89 was delivered intrathecally after the conditioning lesion, either for 24 hours or for one week. PKA inhibition for 24 hours after peripheral lesion resulted in neurons being inhibited when cultured on MAG or myelin. PKA inhibition for one week after peripheral lesion also attenuated the block of MAG's inhibition observed after peripheral lesion. These experiments proved that the conditioning lesion effect in overcoming myelin inhibitors is PKA dependent, and thus established a role for cAMP in it.

In the same study, dbcAMP was injected into L5 DRG *in vivo*, and then a week later the T7 spinal cord segment was lesioned by bilateral dorsal column lesion (DCL). There was significant regeneration of the dorsal column axons into the lesion site and even beyond it, when these axons were visualized 1 or 2 weeks after injury (Qiu et al., 2002). Similar findings were reported in parallel by another group (Neumann et al., 2002).

Both groups' results suggest that the conditioning lesion effect is mediated by elevation of neuronal cAMP in the DRG cell bodies, which is caused by signals produced by the peripheral conditioning lesion. Several retrograde injury signals are known to mediate regeneration, but so far it is not known how these signals elevate cAMP in the

DRG cell bodies [reviewed in (Abe and Cavalli, 2008)]. The elevated cAMP levels alter the intrinsic growth capacity of the sensory neurons, so that they can now regenerate their CNS branch when it is lesioned. If this is what is happening in the body of an animal upon peripheral conditioning lesion, it makes sense that injection of cAMP was sufficient to mimic this effect.

Similar effects of elevated cAMP in CNS regeneration have also been reported in another injury model, the optic nerve crush. Intraocular injection of dbcAMP, both a day before, or a day after optic nerve crush in rats, resulted in regeneration of RGC axons up to 1 mm beyond the crush site (Monsul et al., 2004).

In terms of the therapeutic potential of these results, drugs that elevate cAMP levels already exist, such as the antidepressant rolipram, a phosphodiesterase 4 inhibitor which has already been used in several clinical trials (www.clinicaltrials.gov). Also, these results opened the way for studying the signaling pathways activated downstream of cAMP elevation, in order to understand how they enable CNS regeneration to occur.

1.5.5 Combinatorial therapies for CNS regeneration

There are many reasons why spontaneous regeneration does not occur in the CNS including: 1) reduced intrinsic growth capacity 2) lack of neurotrophic signals 3) presence of glial-derived inhibitory molecules, and 4) lack of permissive substrate for axonal extension into the injury site (Lu et al., 2004). Therefore, some groups have followed a combinatorial approach to address several of these factors at once, in the hope of propelling axonal extension into and beyond the injury site.

For example, Bunge and colleagues reported that after a thoracic contusion injury, a combination of SC transplants and cAMP elevation (via administration of the PDE4 inhibitor rolipram, or dbcAMP injection), promoted regeneration of serotonergic fibers and improved locomotion (Pearse et al., 2004).

In another example, Lu et. al., (2004) used three types of interventions in a C4 adult rat dorsal column lesion model: 1) injection of dbcAMP into L4 DRG 5 days before injury to stimulate growth signals in the soma, 2) implantation of autologous bone marrow stromal cells (MSC) in the lesion site to provide a supportive matrix for regeneration, together with NT-3 injection into the lesion site to attract axons in this milieu, and 3) additional injection of NT-3 1.5 mm rostral to the lesion site a week after injury, to attract regenerating axons into host spinal cord tissue. Various combinations of these treatments were used in 60 rats to ensure proper controls. One or three months later, animals were tested for functional recovery and axonal growth was visualized with appropriate labeling. Axonal growth through the lesion site and for up to 2 mm into host white matter rostral to the lesion site was observed only in the group that had received cAMP injections together with MSC implant and the two different NT-3 injections. Even though no functional recovery was observed, these results for the first time provided proof of principle that such an approach could enhance regeneration in the spinal cord.

1.6 Neurotrophin signaling pathways

Since NTs are being considered as potential therapeutic agents for various neurodegenerative diseases and CNS injuries (Blesch and Tuszynski, 2002), understanding their signaling pathways is of great importance.

The NTs BDNF, NT-3, nerve growth factor (NGF), and neurotrophin-4 (NT-4) are proteins that regulate many aspects of neuronal physiology such as differentiation, survival, process growth, modulation of neuronal function and even cell death (Huang and Reichardt, 2001, 2003; Kaplan and Miller, 2000).

By binding to their tropomyosin kinase receptors (Trks), neurotrophins induce receptor dimerization, which leads to cross-phosphorylation, generation of several phosphotyrosine residues, and further full activation of the Trk kinase domain. Various adaptor proteins may then be recruited to specific phosphotyrosines, in order to be phosphorylated and activated by the Trk kinase domain [reviewed by (Friedman and Greene, 1999) .

One of the proteins recruited to phospho-tyrosine 490 (pY490) is the linker protein Shc, which is then phosphorylated, resulting in the activation of two main signaling cascades: The Ras/ERK (Extracellular Signal Regulated Kinase) pathway which mediates differentiation, neurite outgrowth and synaptic plasticity, and the phosphatidyl inositol-3 (PI3)-kinase/AKT pathway which mediates survival. Through recruitment of phospholipase C-γ to pY785, another main pathway activated by neurotrophins is the PLC-γ1/Ca^{2+}/PKC pathway, which is involved in activity-dependent plasticity (Bibel and Barde, 2000; Chao, 2003; Huang and Reichardt, 2003).

The Ras/ERK pathway is activated when the linker protein Shc recruits Grb2. Grb2 is an adaptor protein that carries the Ras GDP/GTP exchange factor (GEF) SOS to the activated receptor, and thus to the membrane area. From there, SOS can activate its target Ras, and this leads to ERK activation through the Ras → c-Raf → MEK → ERK pathway. Activated ERK can now act in a negative feedback loop to shut down Ras

signaling, because ERK phosphorylates SOS resulting in block of Ras signals (Dong et al., 1996).

NT activate ERK through the Ras pathway transiently, for only a few minutes, but NT activation of ERK can be prolonged, lasting for hours. It has been proposed that another small GTPase cousin of Ras, Rap1, mediates the prolonged ERK activation observed after NT activation of the Trk receptors. Upon Trk activation, a membrane-anchored linker protein, FRS2, recruits the Crk adaptor protein, which then recruits the Rap1 activator C3G to the membrane area. C3G then activates Rap1 and this leads to sustained ERK activation through the Rap1→ B-Raf → MEK → ERK pathway. This pathway can only be activated in cells that express the Raf isoform B-Raf, such as neurons, and also requires Trk receptor internalization into endosomes (Kao et al., 2001; York et al., 2000; York et al., 1998).

1.7 Neurotrophins overcome myelin-mediated inhibition *in vitro* by elevating cAMP

1.7.1 Overcoming myelin-mediated inhibition of growth *in vitro* with neurotrophins and cAMP

As previously discussed, Bregman's experiments (Coumans et al., 2001) demonstrated that fetal transplants in combination with NTs resulted in regeneration in a SCI model. In a 1999 study our lab tested the hypothesis that treatment with NTs can block inhibition of neurite outgrowth by inhibitors present in myelin in general, and also specifically by MAG.

Using the neurite outgrowth assay, we have found that if added directly to neurons growing on myelin or MAG, NTs have no effect on growth (Cai et al., 1999). We then devised a "priming" protocol, where neurons are first treated with NTs overnight, and then are transferred on inhibitory substrates the next day. After this prior exposure to NTs, neurons are not inhibited my MAG or myelin. Importantly, these observations might explain what happened *in vivo*, in the course of the Bregman experiments, which used transplants in conjunction with NTs (Coumans et al., 2001). In these experiments the neurons grew into the transplant, a permissive environment, where they were "primed" with pumped NTs, before they encountered the host CNS inhibitory environment caudal to the transplant. When they reached the host CNS again, there was increased innervation of the host CNS compared to animals that were not treated with NTs. Thus priming with NTs allows neurons to overcome inhibition.

Investigation into the mechanism of NTs' blockade of inhibition revealed that they elevate cAMP by at least two-fold. However in the presence of MAG, NT-induced cAMP elevation was blocked (Fig. 1.2). Inhibition of PKA by KT5700 blocked the priming effect in overcoming MAG's inhibition, indicating that priming is PKA-dependent.

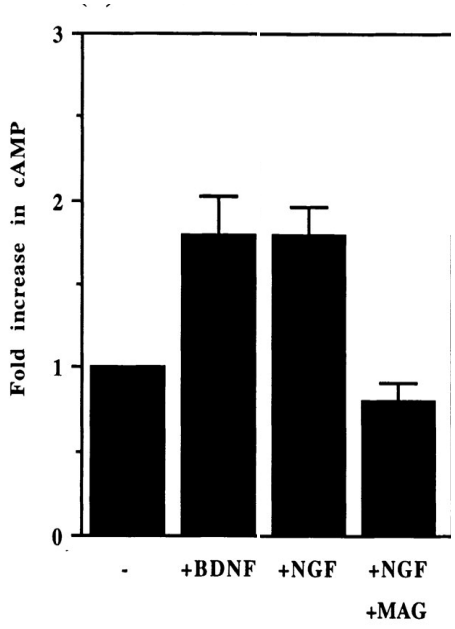


Figure 1.2 Measurements of cAMP in neurons after exposure to neurotrophins.

Dissociated primaryneurons (DRG) were placed in individual wells of a 96-well dish. The neurons were cultured for at least 6 hr after which time BDNF, or NGF, each at 200 ng/ml, was added as indicated and incubated for a further 30 min. Where indicated, neurotrophin was added with MAG-Fc (+MAG) at a concentration of 20 μg/ml. Following incubation, the cAMP levels were measured and compared to a standard. The results are the mean of between four and seven experiments, each done in quadruplicate. Results represent the fold-increase relative to neurons incubated for the same length of time but without the addition of neurotrophin [adapted from (Cai et al., 1999)].

It is possible for NT treatment to overcome inhibition by MAG directly, without overnight priming, if neurons are treated with pertussis toxin (PTX) for 1-2 hours before addition of NT and exposure to MAG. PTX blocks activation of the $G\alpha_{i/o}$ subunit of G proteins by their corresponding receptors (He et al., 2006). The activated $G\alpha_i$ subunit inhibits adenylate cyclases (AC) and blocks cAMP production. Importantly, PTX treatment alone does not block inhibition. These results suggest that besides the well-characterized, PTX-insensitive inhibitory pathway through NgR-Rho, MAG initiates

another, PTX-sensitive pathway to block elevation of cAMP by NTs (Cai et al., 1999; Gao et al., 2004).

The next step was to artificially elevate the intracellular levels of cAMP, and see if we could overcome inhibition. Indeed, addition of dbcAMP blocks inhibition by MAG and myelin *in vitro* directly, without priming. When we added dbcAMP to neurons growing on MAG CHO mololayers, inhibition was blocked and neurons grew on MAG-expressing CHO cells as well as they did on control monolayers (Cai et al., 1999).

Subsequently, we have combined the above results, to propose a model for the "priming effect" (Fig. 1.5). In this model, during priming, NTs acting through Trk receptors activate ERK for a prolonged period. ERK phosphorylates phosphodiesterase4 (PDE4), the enzyme that degrades cAMP, inhibiting its activity. As a result, the cAMP levels are elevated in the cell and PKA is activated (Gao et al., 2003). Downstream signaling cascades are then initiated which culminate in the block of MAG inhibition (Gao et al., 2003). If ERK activation is blocked using MEK inhibitors, both priming and direct addition of dbcAMP cannot overcome inhibition (Gao et al., 2003).

Because NTs do not block inhibition directly but only with priming, we hypothesized that perhaps myelin inhibitors intercept the NT signaling cascade at a specific molecular point, thus blocking the NT-induced cAMP elevation.

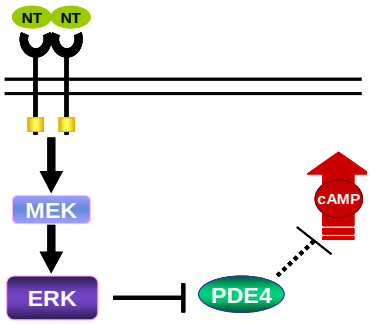


Figure 1.3 Proposed model for neurotrophin elevation of cAMP

Neurotrophins activate ERK which in turn inhibits PDE4, the enzyme that hydrolyzes cAMP. The intracellular concentration of cAMP can then rise to a required threshold and neurite outgrowth inhibition by MAG is blocked.

1.7.2 MAG and myelin block elevation of cAMP by neurotrophins

Surprisingly, as shown in figure 1.2, direct addition of dbcAMP does not overcome inhibition if at the same time ERK is inhibited. This sounds like a paradox, because in our proposed model to explain the priming effect (Fig. 1.3), ERK is operating upstream of cAMP, inhibiting PDE4 in order to elevate cAMP.

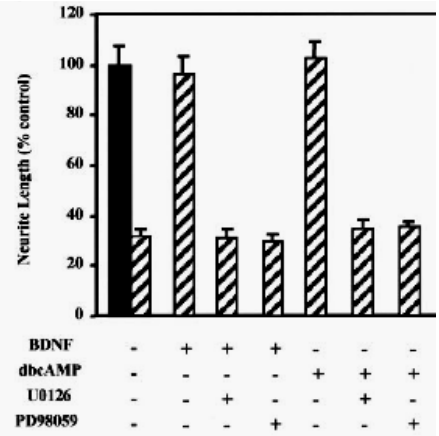


Figure 1.4 Inhibitors of ERK blocks the ability of BDNF and dbcAMP to overcome inhibition by MAG

Cerebellar neurons were primed with and without BDNF (200 ng/ml) before transfer to control CHO cells (black bars) or MAG-expressing CHO cells (stripped bars), or dbcAMP was added directly to the cultures at 1mM. Where indicated, the MEK inhibitors UO126 (5μM) or PD98059 (50μM) were included during priming or added directly to the cultures with dbcAMP. After overnight culture, the cells were fixed and immunostained for GAP43, and neurite length was measured [adapted from (Gao et al., 2003)].

Three factors need to be considered in order to explain these results:

i) even though addition of 1mM of the membrane-permeable cAMP is considered a fairly high concentration, the amount that actually enters neurons is unknown

ii) the cAMP threshold concentration needed to overcome inhibition is a combination of both the endogenously produced cAMP and the exogenously added dbcAMP levels

iii) PDE4 activity is regulated positively by PKA and negatively by ERK.

When dbcAMP is added to our cultures, the cAMP levels increase intracellularly, and its effector PKA is activated. PKA can phosphorylate PDE4 causing an increase in its activity. Since dbcAMP cannot be hydrolyzed, the incease in PDE4 activity does not affect the dbcAMP nucleotide pool in the cell. However, it is decreasing the endogenous cellular cAMP levels, and thus affecting the cAMP threshold needed to overcome inhibition.

After addition of dbcAMP to our cultures, ERK is also activated (Vossler et al., 1997), which can phosphorylate PDE4 and inhibit its activity, allowing cAMP levels to rise again. The total cAMP concentration (endogenous cAMP plus exogenous dbcAMP) can then reach a required threshold and block inhibition. There is a way though to by-pass the ERK requirement for the dbcAMP effect in overcoming inhibition, and this is by using 2 mM dbcAMP. The cAMP threshold needed is achieved in the neurons because of this high exogenous application of dbcAMP, and they can overcome inhibition even in the presence of ERK inhibitors (Gao et al., 2003).

The dual and opposing regulation of PDE4 by both PKA and ERK explains why ERK activity is necessary for both priming with NTs, and addition of dbcAMP to overcome inhibition. ERK then must be a key molecule in the "priming effect", and its activity is involved in the elevation of cAMP levels by NTs.

According to our model, if ERK activation is blocked, cAMP cannot be elevated to a certain threshold to overcome inhibition neither by NTs, nor by exogenous addition of dbcAMP. But if cAMP levels are elevated to a certain threshold by addition of dbcAMP, then we can overcome inhibition without a requirement for ERK activity. These observations once again suggest that, in the presence of MAG, NTs cannot elevate cAMP, which has been previously demonstrated by our lab via cAMP assays (Fig. 1.2), (Cai et al., 1999).

1.8 A hypothesis to explain why neurotrophins cannot elevate cAMP in the presence of MAG: involvement of Rap1

The question that arises in our priming studies with NTs is why BDNF cannot elevate cAMP and activate its downstream targets when myelin inhibitors are present. If NTs can elevate cAMP, and cAMP elevation blocks MAG inhibition, why then direct addition of BDNF to neurons growing on MAG cannot block inhibition? Why do we need to prime with NTs in order to overcome inhibition? We hypothesized that signaling by myelin inhibitors can block signaling pathways initiated by NTs.

Since NTs are being studied as potential therapeutic agents in models of CNS injury, understanding how myelin inhibitors block their signalling pathways will enable us to enhance their potential.

The PC12 cell line has served as a model system to study NT signaling for many years. It is of neuronal origin, derived from a rat pheochromocytoma (PC) of the adrenal medulla (Greene and Tischler, 1976). PC12 cells differentiate into a sympathetic neuron-like phenotype upon treatment with NGF. There is evidence from this model that activation of the small GTPase Rap1 (a close relative of the Ras GTPase) results in sustained ERK activation, an event that is sufficient for differentiation after NGF treatment (Vossler et al., 1997; Wu et al., 2001; York et al., 1998). Therefore we sought to examine the possibility that Rap1 GTPase might play an important role in the NT signaling cascade that allows neurons to overcome inhibition by myelin inhibitors.

Because of its role in prolonged ERK activation, we hypothesized that Rap1 activation is a key molecule for maintaining the threshold of cAMP levels needed to overcome inhibition. If active Rap1 is responsible for prolonged activation of ERK, and ERK inhibits PDE4, then the intracellular cAMP concentration will gradually increase and eventually reach a threshold that can block MAG's inhibition. Because NTs cannot elevate cAMP levels in the presence of MAG, we hypothesized that MAG signals to inactivate Rap1.

1.9 Rap1 background

Rap1 (Ras-proximate-1) is a member of the Rap family of small G proteins, which has one additional closely related member, Rap2. They both belong to the Ras superfamily of monomeric GTPases. Rap1 was discovered in 1989 as a revertant of fibroblast transformation. v-K-ras-transformed fibroblasts were tranfected with a normal human fibroblast cDNA library. From the flat revertants a cDNA clone was isolated and

found to encode the Rap1 small GTPase protein. Therefore the initial proposed role for Rap1 was inhibition of Ras signaling (Kitayama et al., 1989). Rap1 shows 50% amino acid homology to the Ras small GTPase protein and almost identical effector domains. The intrinsic GTPase activity of Rap1 is much lower than Ras though, due to the presence of a threonine residue at amino acid position 61, instead of a glutamine present in most other GTPases (Bos et al., 1997).

There are two Rap1 isoforms, Rap1a and Rap1b, which are 95% identical. A geranylgeranyl group is attached to Rap1 after its synthesis at its C-terminus, and this modification is responsible for membrane attachment. Rap1 localization varies in different cell types. It has been found to be on the cell membrane (in platelets and neutrophils), in the perinuclear region, and also on various membranous organelles such as late endosomes (in fibroblast, pC12 cells and DRG neurons) (Bos et al., 2001; Bos et al., 1997, York, 2000 #126)}.

1.9.1 Rap1 functions

1.9.1.1 Rap1 activation mediates the sustained ERK activation induced by NGF: studies in the cell line PC12

As already mentioned, treatment of PC12 cells with NGF, which acts through the tyrosine kinase receptor TrkA, results in differentiation into a sympathetic neuron-like phenotype and ERK activation is necessary for this phenomenon (Cowley et al., 1994).

If PC12 are treated with Epidermal Growth Factor (EGF), which like NGF, also acts through tyrosine kinase receptors, they start to proliferate and ERK activation is also

necessary for this phenomenon (Cowley et al., 1994; Stork, 2005). Even though NGF and EGF signaling in PC12 cells share many common signal transduction events (i.e. activation of Ras, ERK), the final outcome is very different, namely neuronal differentiation versus proliferation.

It was proposed that signal specificity by NGF and EGF in this model is achieved by induction of different temporal modes of ERK activation by each factor. NGF signaling results in a prolonged state of ERK activation, which lasts for hours, whereas EGF signaling results in a transient ERK activation which lasts for only minutes. The prolonged phase of activation results in neuronal differentiation, and the transient phase results in proliferation (Traverse et al., 1992), (Pang et al., 1995).

Stork's group proposed that the small GTPase Rap1 mediates the sustained ERK activation induced by NGF, which is considered to be required for neuronal differentiation. Dissecting into the mechanism of sustained ERK activation by this group revealed that NGF activates Rap1 (York et al., 1998). NGF activation of ERK is composed of two activation phases, one transient, which lasts only a couple of minutes, and a prolonged which lasts for hours. The transient phase involves Trk→Shc→ Grb2-SOS → Ras→ Raf-1 → ERK. The prolonged phase of activation is the result of recruitment of CrkII/CrkL and activation of the GEF C3G which activates Rap1. Rap1 then, in a PKA-dependent but Ras-independent fashion, activates the B-Raf kinase which activates ERK for a prolonged period (Arevalo and Wu, 2006).

The MAPK signaling cascade and the cAMP second messenger system are ancient signal transduction modules, conserved by evolution and present in all eukaryotes (Houslay and Kolch, 2000). It has been recognized for a while that there is cross-talk

between these two systems. In some cell types cAMP elevation inhibits growth factor-stimulated ERK activation and in others it promotes it (Houslay and Kolch, 2000). In neurons cAMP elevation activates ERK, and the underlying mechanism has also been demonstrated in the neuronal cell line PC12. PC12 cell neuronal differentiation can also be induced by cell-permeable analogues of cAMP (Yao et al., 1995). It has been known that cAMP elevation activates ERK in PC12 cells (Frodin et al., 1994). In 1997 Philip Stork's group proposed a mechanism to explain how cAMP activates ERK in PC12 cells, in the absence of growth factor signals (Vossler et al., 1997). According to their findings, cAMP elevation activates both PKA and Rap1, and Rap1 is an activator of B-Raf, a MEK for ERK. They thus proposed a model where, in cells that express the Raf isoform B-Raf, elevated cAMP can activate ERK in a Rap1, PKA and B-Raf-dependent, but Ras-independent pathway.

In cerebellar neurons cAMP activation of ERK is PKA-, Src-, Ras-, and PI3K-dependent. Additionally, cAMP activation of ERK is also Rap1dependent but as shown by Stork's group, only for the limited fraction of ERK recruited to membranes (Obara et al., 2007).

1.9.1.2 Knockout models for Rap1 and its regulators

In Drosophila, loss of function Rap1 mutations are embryonic lethal (Hariharan et al., 1991), and Jeon et al. also reported they were unable to obtain a Rap1 knockout in *Dictyostelium discoideum* (Jeon et al., 2007), indicating the essential role of Rap1 in these organisms [see also review by (Caron, 2003)]. Zwartkruis et. al., have generated Rap1a knockout mice, which are viable and fertile, but have defects in cell adhesion in

certain immune cell types (Duchniewicz et al., 2006). Rap1b knockout mice were also generated, and were characterized by bleeding defects (Chrzanowska-Wodnicka et al., 2005) . Eric Kandel's group has also generated a conditional forebrain-specific Rap1 knockout, and found that Rap1 is required for certain cAMP-dependent forms of synaptic plasticity (Morozov et al., 2003). Other studies have examined the effects of genetic disruption of various Rap1 regulators. Knockout mice for the activator C3G (C3G-/-) die before embryonic day 7.5 (Ohba et al., 2001), and knockout mice for another activator, RA-GEF-1, also die in the mid-gestation period (Wei et al., 2007). Deletion of the inactivator Rap1GAP in *Drosophila* does not result in phenotypic abnormalities (Chen et al., 1997b), but overexpression of Rap1GAP causes a rough-eye phenotype.

1.9.2 Regulation of Rap1 activity

All small GTPases are often described as being "molecular switches". They are in an active state when GTP is bound, and return to an inactive state as soon as GTP is hydrolyzed to GDP due to their intrinsic GTPase activity. Binding of GTP is enhanced by regulators called Guanine-nucleotide Exchange Factors (GEFs) and the hydrolysis of GTP is enhanced by regulators called GTP-ase Activating Proteins (GAPs). Therefore, signaling from their specific GEFs turns small G proteins on, whereas signaling from GAPs turns them off.

1.9.2.1 RapGEFs

Most small G proteins, including Rap1, have very high affinities for either GDP or GTP and thus once bound, these nucleotides have a slow dissociation rate. But in order for small G proteins to be activated in response to a signal, they need to be able to quickly release the bound GDP, and exchange it for GTP. Activated GEFs bind G-proteins, and catalyze the release of the bound nucleotide, whether it is GDP or GTP. This eventually results in activation of a certain pool of a small G protein in the cell, because GTP, whose levels are about ten times higher intracellularly than GDP, can then bind and turn the G protein on (Bos and Wittinghofer, Cell 2007).

GEFs for Rap1 can be activated by many diverse signals, leading to Rap1 activation. Such signals have been described emanating from tyrosine kinase receptors, heterotrimeric G-protein-coupled receptors, cell adhesion molecules and cytokine receptors. Second messengers generated by these receptors, such as cAMP, Ca^{++}, and DAG, have all been linked to activation of various Rap1 GEFs.

Activation of Rap1 appears to be a common event in signal transduction by many diverse cellular stimuli, suggesting a role of Rap1 in diverse cellular functions. As discussed at the end of the previous chapter, in PC12 cells NGF induces a sustained Rap1 activation, which in turn mediates the sustained ERK activation induced by NGF (Stork, 2005; York et al., 1998). One mode of Rap1 GEF activation through Trk involves recruitment of Crk SH3-domain-binding guanine Gucleotide releasing factor (C3G). C3G associates with the SH3 domain of Crk adaptor proteins. The Crk-C3G complex is recruited to activated Trks, where C3G is phosphorylated and activated, resulting in activation of Rap1 (Bos et al., 2001). Neurotrophin activation of Rap1 and ERK has been

shown to be dependent on PI3K and endocytosis. In both PC12 cells and DRG neurons inhibition of PI3K blocked endocytosis of NGF-activated TrkA receptors, and also blocked activation of Rap1. As a consequence, ERK was not activated by NGF in the presence of PI3K inhibitors (York et al., 2000).

1.9.2.2 RapGAPs

Several RapGAPs proteins have been discovered, which enhance Rap1's low intrinsic GTPase activity. These include Rap1GAPI, Rap1GAPII, SPA-1, tuberin, SPAR and SPAR2 (Spilker et al., 2008). Recent studies have demonstrated that a variety of extracellular signals regulate RapGAPs . For example, SPAR contains a PDZ domain that allows it to associate with the C-terminal PDZ-binding motif of the activated EphA4 tyrosine kinase receptor. Upon Ephrin stimulation this receptor causes growth cone collapse, an effect mediated through SPAR inactivation of Rap1 (Richter et al., 2007). Another mechanism of RapGAP regulation has been described for Rap1GAPII. Iyengar's lab has shown that the activation of the Gαo/i-coupled CB1 cannabinoid receptor causes the proteasomal degradation of Rap1GAPII, and thus activation of Rap1. In these studies however it is the unactivated form of the Gα subunit that stimulates Rap1GAPII degradation (Jordan et al., 2005).

1.9.2.4 Rap1 regulation by cAMP

cAMP regulates Rap1 activity by activating Rap GEFs. In 1998, two new membrane-associated GEFs for Rap1were discovered, EPAC1 and EPAC2 (de Rooij et al., 1998), (Kawasaki et al., 1998), (de Rooij et al., 2000). These proteins are cAMP sensors, and can be activated through cAMP elevations in the cell. Their discovery confirmed previous predictions that there must be more cAMP effectors than described, because it had been observed that some cAMP effects were not dependent on the effectors known to date. The cAMP affinity of EPAC1 and the PKA holoenzyme are similar (with a K_d of 2.8 and 2.9 µM respectively), indicating that factors other than affinity might determine which of the two become activated in cellular compartments with elevated cAMP levels (Dao et al., 2006).

Epac's regulatory region contains one (EPAC1) or two (EPAC2) cAMP binding domains (CNB) and their catalytic region contains a CDC25-homology domain responsible for the guanine-nucleotide-exchange activity towards Rap1. In the absence of cAMP, these proteins are "folded" in such a way that their regulatory region covers their catalytic region, creating "autoinhibition". When cAMP levels rise, cAMP binds to the CNB domains, autoinhibition is relieved and EPACs can then facilitate Rap1 activation (Bos, 2006).

In 2002 a cAMP analogue was developed, 8-pCPT-2'-Me-cAMP, which can specifically activate EPACs without activating PKA; therefore by using this analogue, it is possible to distinguish between PKA- or EPAC-Rap1-mediated cAMP effects. In this new analogue, the proton of the 2'OH group of the ribose of cAMP is replaced by a methyl (-CH3) group and a proton at the 8' position of the base by a 4-chlorophenylthio

(pCPT) group. These modifications make this synthetic nucleotide capable of being a very potent agonist of EPAC proteins without activating PKA (Bos, 2006), (Enserink et al., 2002). The ability of this analogue to activate the PKA regulatory subunits I and II *in vitro* was tested, when the latter are used at physiological concentrations (300nM). 8-pCPT-2'-Me-cAMP used at 1mM concentration achieved less that 25% activation of either PKA subunit in these *in vitro* kinase activity experiments (Christensen et al., 2003). When the same concentration of 8-pCPT-2'-Me-cAMP was used in intact cell experiments though, no PKA activation was observed. It was concluded that even high concentrations of this analogue are insufficient to activate PKA *in vivo* (Enserink et al., 2002).

The creators of 8-pCPT-2Me-cAMP challenged the role of Rap1 as a mediator of cAMP regulation of ERKs, which by consequence challenges the role of Rap1 as mediating the sustained ERK activation in neurotrophin signaling (Springett et al., 2004). They used CHO cells to test the belief that cAMP activates ERK in a Rap1-dependent manner. It is known that in the CHO cells cAMP activates ERK, so they stimulated these cells with both 8-Br-cAMP (which activates both PKA and EPAC) and also with 8CPT-2Me-cAMP (which is EPAC-specific) and looked for ERK activation. They found that only 8-Br-cAMP activated ERK and CREB (a common PKA substrate), and they concluded that cAMP-induced Rap1 activation is not sufficient to activate ERK. A later study published by a German group also used discriminating cAMP analogues to treat PC12 cells and study ERK activation and differentiation (Kiermayer et al., 2005). They found that the PKA-selective cAMP analogue 6-Benz-cAMP activates ERK rapidly but transiently, and the EPAC-specific cAMP analogue 2-Me-cAMP activated ERK slightly,

but in a sustained fashion. Another analogue, CPT-cAMP, which activates both PKA and EPAC, caused a robust and sustained ERK activation. Additionally, they found that the PKA-selective analogue elicits only proliferation of PC12 cells, whereas analogues that activate both PKA and EPAC elicit neurite outgrowth. Because of these conflicting results, whether 8-CPT-2Me-cAMP can couple activation of Rap1 to activation of ERK remains somewhat controversial.

Additionally, Rap1 has been shown to be a target of PKA phosphorylation at serine 179 in its carboxy terminus (Altschuler and Lapetina, 1993). This event is not necessary for Rap1 activation by cAMP, as the latter can occur via EPAC (Bos, 1998). However, PKA phosphorylation of Rap1 appears to be involved in the sustained ERK activation stimulated by NGF and cAMP in PC12 cells (Yao et al., 1998).

An alternative mechanism of cAMP regulation of Rap1 is via RapGAPs. It is intriguing that Rap1GAP, like Rap1 itself, is a target of PKA phosphorylation. The carboxy-terminal of Rap1GAP contains sites of phosphorylation by serine/threonine kinases. In initial studies, no changes in Rap1GAP activity were detected after its phosphorylation *in vitro*, and therefore the significance of phosphorylation for its regulation was not understood (Polakis et al., 1992) (Rubinfeld et al., 1992). Recently though, it was shown in Neuro-2a cells that PKA phosphorylation of Rap1GAP at serines 441 and 499 results in higher levels of Rap1 activation (McAvoy et al., 2009). In the same study, NMDA-induced de-phosphorylation of Rap1GAP resulted in lower levels of Rap1 activation in lysates from striatal slices. In the same study, in cultured hippocampal neurons, overexpression of a wild type Rap1GAP, which leads to lower Rap1GTP levels, resulted in formation of spines with larger heads compared control. This effect was

potentiated further by overexpression of the Rap1GAP mutant Ser-441/499Ala, which mimics Rap1GAP's phosphorylated state. The authors therefore proposed that PKA phosphorylation of Rap1GAP controls dendritic spine morphology because it results in higher levels of active Rap1.

1.10 What is the mechanism of MAG's blockade of neurotrophin signaling?

The role of Rap1 in the NT signaling cascade is well documented in different models. Therefore we decided to investigate the role of Rap1 in the priming effect of BDNF, which overcomes inhibition by MAG.

Our priming results suggest that NTs cannot elevate cAMP when myelin inhibitors are present. cAMP activates ERK in neuronal cells and Rap1 has been proposed to be responsible for the sustained mode of ERK activation. If Rap1 plays a role in neuronal differentiation induced by neurotrophins, then activation of Rap1 might also be a requirement in order for NT signaling to overcome inhibition.

We propose that Rap1 is a signaling node where MAG and myelin block NT signaling. Since Rap1 is essentially a molecular switch because of its GTPase identity, we will examine the possibility of myelin inhibitors affecting the activation state of Rap1, thereby interfering with the signaling cascades that neurotrophins initiate. Understanding the molecular mechanism by which MAG/myelin block NT signaling will enable us to devise ways to eliminate the need for priming, therefore providing new methods of therapeutic intervention.

Chapter II: Results

2.1 Activation of Rap1 by BDNF is blocked by MAG and myelin

One way to overcome MAG and myelin inhibition *in vitro* is by overnight priming of neurons with NTs, before the neurons come in contact with these inhibitors (Cai et al., 1999). During priming, Trks activate ERK in a sustained manner, which then inhibits the cAMP degrading enzyme PDE4, allowing for the accumulation of cAMP, and block of MAG's inhibition (Gao et al., 2003). Direct addition of NTs in the presence of MAG or myelin does not overcome inhibition (Cai et al., 1999), suggesting that MAG intercepts NT signaling.

Studies in the PC12 model of NT signaling have suggested that Rap1 is responsible for the sustained ERK activation by NTs, and therefore acts as a key molecule in the induction of a neuronal-like phenotype in these cells upon NGF treatment (York et al., 1998).

By combining the above observations, we hypothesized that Rap1 might be one signaling node where MAG inhibits NT signaling, thus preventing NTs from overcoming inhibition directly, resulting in the need for overnight priming.

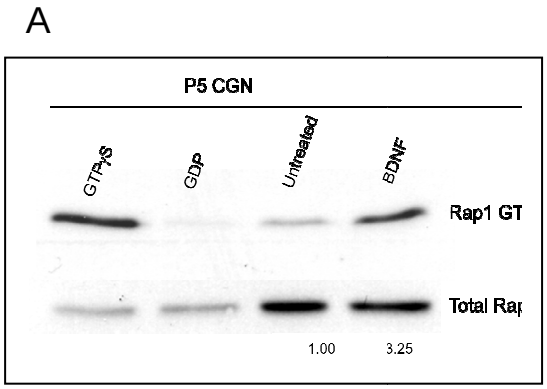
Therefore, we examined Rap1 activation by BDNF in BDNF-responsive neuronal cultures, both in the absence and presence of MAG or myelin. For this we used a biochemical assay, which enables us to precipitate active, GTP-bound Rap1 by utilizing beads coupled to one of its downstream effectors, the Ral guanine-nucleotide dissociation stimulator binding domain (Ral-GDS-RBD), (Franke et al., 1997). After treatment of cells with Rap1 activating agents, a pool of Rap1GTP is generated in cells. Some of this Rap1GTP is free, and some becomes engaged with downstream effectors. The Rap1 assay allows the precipitation of activated Rap1 in response to such treatments. Ral-

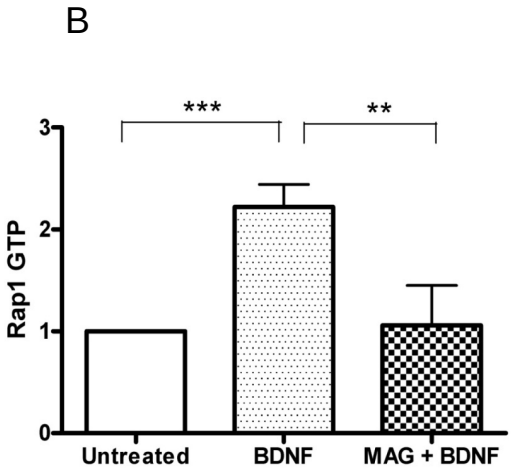
GDS-RBD binds Rap1GTP with a very high affinity (K_D=10 nM), more than any other known Rap1 effector (Linnemann et al., 2002), and therefore it can compete with other downstream effectors, and sequester Rap1GTP. For example, the affinity of Rap1GTP binding to another effector, AF6-RBD, is much lower (K_D=250 nM) than the one for Ral-GDS-RBD (Linnemann et al., 1999). Besides the Rap1 activation assay, similar pull-down assays were developed for other small GTPases, like Ras and Rho, which utilize their known specific effectors, and have become a common tool in studying the regulation of these proteins.

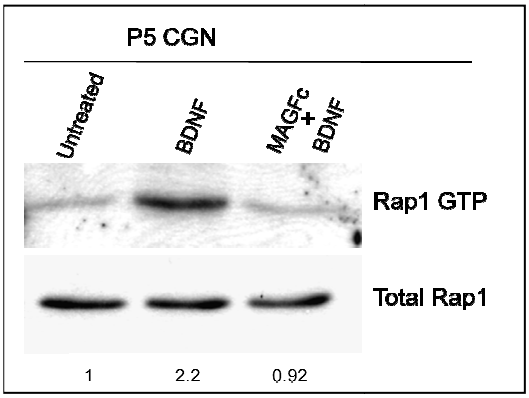
Primary cultures of neonatal rat cerebellar granule cells were starved overnight, and Rap1 activation was examined after stimulating the cells with BDNF (200ng/ml), and also after loading cell lysates with either GTPγS as a positive control, or GDP as a negative control (Fig. 2.1). To examine if MAG-Fc and myelin can block BDNF activation of Rap1, we also pre-treated cells with these inhibitors by adding soluble MAG-Fc (Fig. 2.1, B), or homogenized myelin membrane preparation (Fig. 2.1,C) into the culture media, prior to stimulation with BDNF. We hypothesized that pre-treatment with the inhibitors will initiate signaling that will inhibit the neurotrophin cascade. Under these conditions, BDNF activated Rap1, but this activation was attenuated when cells were pre-treated with either MAG-Fc, or myelin, before BDNF stimulation. These data indicate that Rap1 is indeed a signaling node downstream of Trk receptors, where MAG and myelin are able to inhibit the NT signaling cascade, and block it from overcoming inhibition directly.

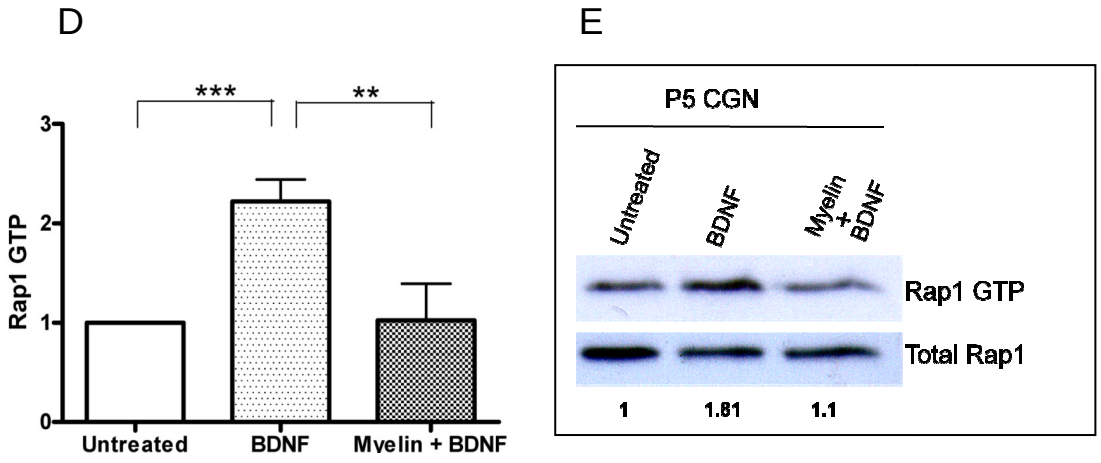
Figure 2.1. MAG and myelin block activation of Rap1 by BDNF.

(A, C, E) Representative western blots showing Rap1 activation. Neonatal cerebellar granule cells (P5-P6) cultured on PLL were starved, and then either left untreated, or treated with BDNF alone (200ng/ml) for 20 min, or pre-treated with MAG-Fc (20μg/ml) for 20 min (C) or myelin (20μg/ml) for 20 min (E), before BDNF treatment. As a positive and negative control, untreated cell lysate was loaded with either GTPγS (100 μM) or GDP (1mM) for 30 min before the Rap1 pull-down (A). Activated Rap1 was precipitated using the protocol and reagents of a Rap1 activation assay kit.
(B and D) Quantitative analysis of Rap1 activation from multiple experiments, where Rap1GTP levels were normalized to the total Rap1 levels in the input lysate (n=10 for BDNF, n=4 for MAGFc + BDNF, and n=3 for Myelin + BDNF).









2.2 Activation of Rap1 by an EPAC-specific analogue is blocked by MAG and myelin, suggesting involvement of RapGAPs

Next, we examined the mechanism of MAG's block of Rap1 activation. Because of its GTPase identity, Rap1 is subject to a dual mode of regulation both by exchange factors and GTPase activating proteins, and in theory, MAG could be regulating Rap1 via either pathway. It could be blocking activation of Rap1 exchange factors in the neurotrophin cascade, and therefore blocking Rap1 activation by BDNF. Another alternative would be that MAG activates RapGAPs, resulting in Rap1 inactivation after it has been activated by exchange factors.

To distinguish between these two possibilities, we used the biochemical Rap1 activation assay in an experimental setting where Rap1 activation by one of its known regulators, EPAC, cannot be intercepted. EPAC is a cAMP sensor protein and an

exchange factor for Rap1. In our experimental setting, Rap1 activation is provided by EPAC, by addition of the EPAC-specific cAMP analogue 8-pCPT-2Me-cAMP, which does not activate PKA. Upon cAMP binding, EPAC's autoinhibition is relieved, and the catalytic region responsible for the GEF activity towards Rap1 is exposed.

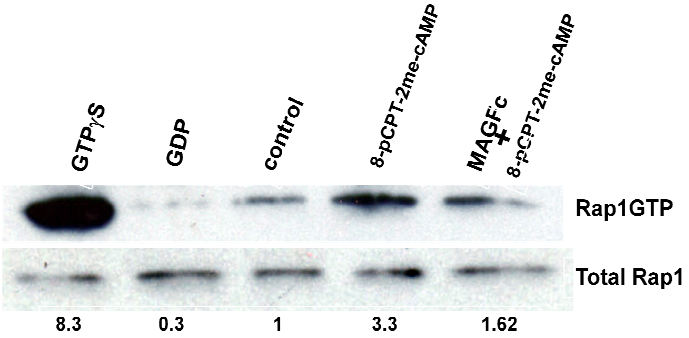
The cAMP sensor proteins EPAC, and the PKA regulatory subunit, which is the presumed ancestor of the cAMP-binding site of EPACs (Dao et al., 2006), are both activated by changing levels of cAMP, in an allosteric fashion. As far as we are aware, the only known regulator of EPAC's guanine exchange factor activity is the binding of cAMP, and proteins that inactivate the cAMP-activated EPAC (or the cAMP-activated regulatory subunit of PKA for that matter) have not been identified to date. Therefore by using 8-pCPT-2ME-cAMP, we ensure that we activate EPAC's GEF action towards Rap1. Thus, if Rap1 activation is still blocked by MAG under these conditions, it is because of increased RapGAP activity. If Rap1 activation by EPAC is not blocked by MAG in this experimental setting, it indicates that MAG does not activate RapGAPs. This would strongly suggest that MAG blocks the BDNF activation of Rap1 by interfering with activation of Rap GEF(s) in the NT signaling pathway.

Using this setting, we were able to detect Rap1 activation by 8-pCPT-2me-cAMP used at a concentration of 0.5 mM (Fig. 2.2). However, when cells were pre-treated with MAG-Fc, stimulation with 8-CPT-2Me-cAMP did not result in Rap1 activation. This result indicates that MAG is able to interfere with pharmacological Rap1 activation by its exchange factor EPAC, and suggests that it does so by increasing RapGAP activity.

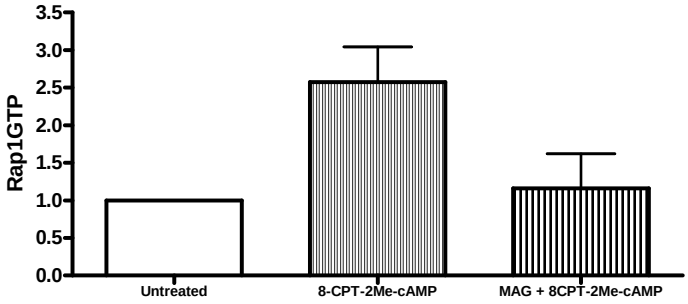
Figure 2.2 MAG-Fc blocks activation of Rap1 by 8-pCPT-2me-cAMP, a specific activator of EPAC.

(A) Representative western blots showing Rap1 activation. Neonatal cerebellar granule cells (P5-P6) cultured on PLL were starved, and then either left untreated (control), treated with 8-pCPT-2Me-cAMP (0.5 mM), or pre-treated with MAG-Fc (20μg/ml) for 20 min before treatment with 8-pCPT-2Me-cAMP. As a positive and negative control, untreated lysate was loaded with either GTPγS (100 μM) or GDP (1mM) for 30 min before the Rap1 pull-down. Activated Rap1 was precipitated using the protocol and reagents of a Rap1 activation assay kit.
(B) Rap1GTP quantitation of multiple experiments shown in (C), where Rap1GTP levels were normalized to the total Rap1 levels in the input lysate.

A



B



C

	Rap1GTP levels	
Neuron Type	8-CPT-2Me-cAMP vs. Untreated	MAGFc + 8-CPT-2Me-cAMP vs. Untreated
Cereb	1.7	0.7
Cereb	2.72	
Cereb	3.3	1.62

2.3 Priming neurons with an EPAC-specific cAMP analogue prior to exposure to MAG overcomes inhibition

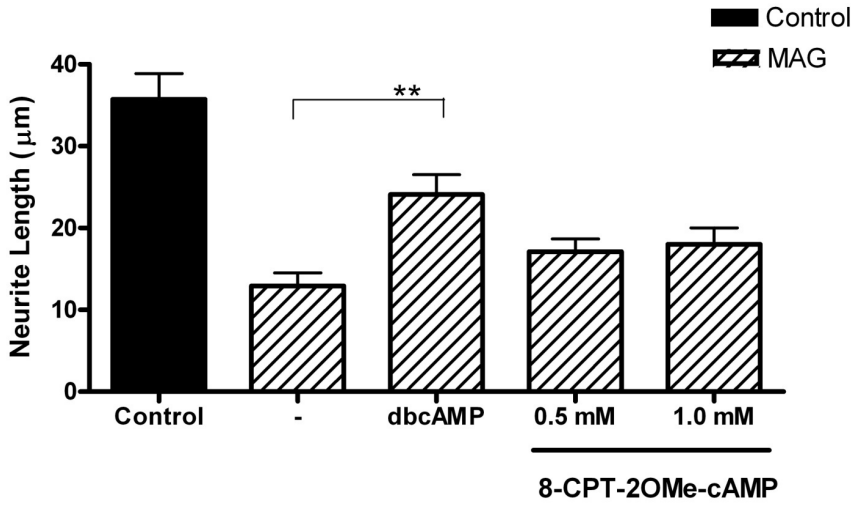
Previously, our lab has shown that elevating cAMP in neurons can overcome MAG and myelin inhibition of neurite outgrowth (Cai et al., 1999). The cAMP threshold to overcome inhibition can be achieved either by overnight treatment of neurons with neurotrophins, or by direct addition of the non-hydrolyzable analogue dbcAMP to them. cAMP has several intracellular receptors, and our lab has previously shown that overcoming inhibition is PKA-dependent (Cai et al., 1999). However, other cAMP receptors might be acting in synergy with PKA in overcoming inhibition after addition of dbcAMP.

EPAC has been recently shown by Murray et. al. to mediate several cAMP effects in neurons, including neurite outgrowth and growth-cone turning (Murray and Shewan, 2008). In this latter study, it was also demonstrated that EPAC activation significantly improved growth of DRG neurons on inhibitory CNS tissue *in vitro.* We decided to screen this EPAC-specific cAMP analogue in our neurite outgrowth assay, to test if addition of it on neurons growing on MAG is sufficient to overcome inhibition. So far in our study, we have shown that BDNF activates Rap1, which is a known ERK activator. Additionally, stimulation with BDNF results in sustained ERK activation, which in turn causes a rise in cAMP that overcomes inhibition. Based on these observations, we hypothesized that activation of Rap1 alone could be sufficient to overcome inhibition.

Activation of Rap1 by 8-pCPT-2Me-cAMP did not overcome MAG's inhibition in our neurite outgrowth assay, when this analogue was added to dissociated neurons immediately following their transfer onto MAG monolayers (Fig. 2.3). This result is

consistent with our earlier observation that MAG blocks activation of Rap1 by this analogue in the biochemical Rap1 activation assay.

Figure 2.3. The EPAC-specific cAMP analogue 8-CPT-2Me-cAMP has no effect on MAG's inhibition when added directly to neurons growing on MAG.
Dissociated cerebellar neurons (P5-P6) were isolated and added directly to monolayers of MAG-expressing CHO cells (stripped bars) or control CHO cells (black bars) in the presence of dcAMP (1mM), or the EPAC-specific cAMP analogue 8-CPT-2Me-cAMP at the indicated concentrations, after which they were cultured overnight, fixed, and immunostained for the neuronal marker β–tubulinIII. Results in graph show the mean length of the longest neurite per neuron. Bar graph shows quantitation of neurite outgrowth, based on the mean length of the longest neurite per neuron (±SEM) for approximately 100-150 neurons (** Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.01$, one-way ANOVA, followed by Tukey's Multiple Comparison Test).

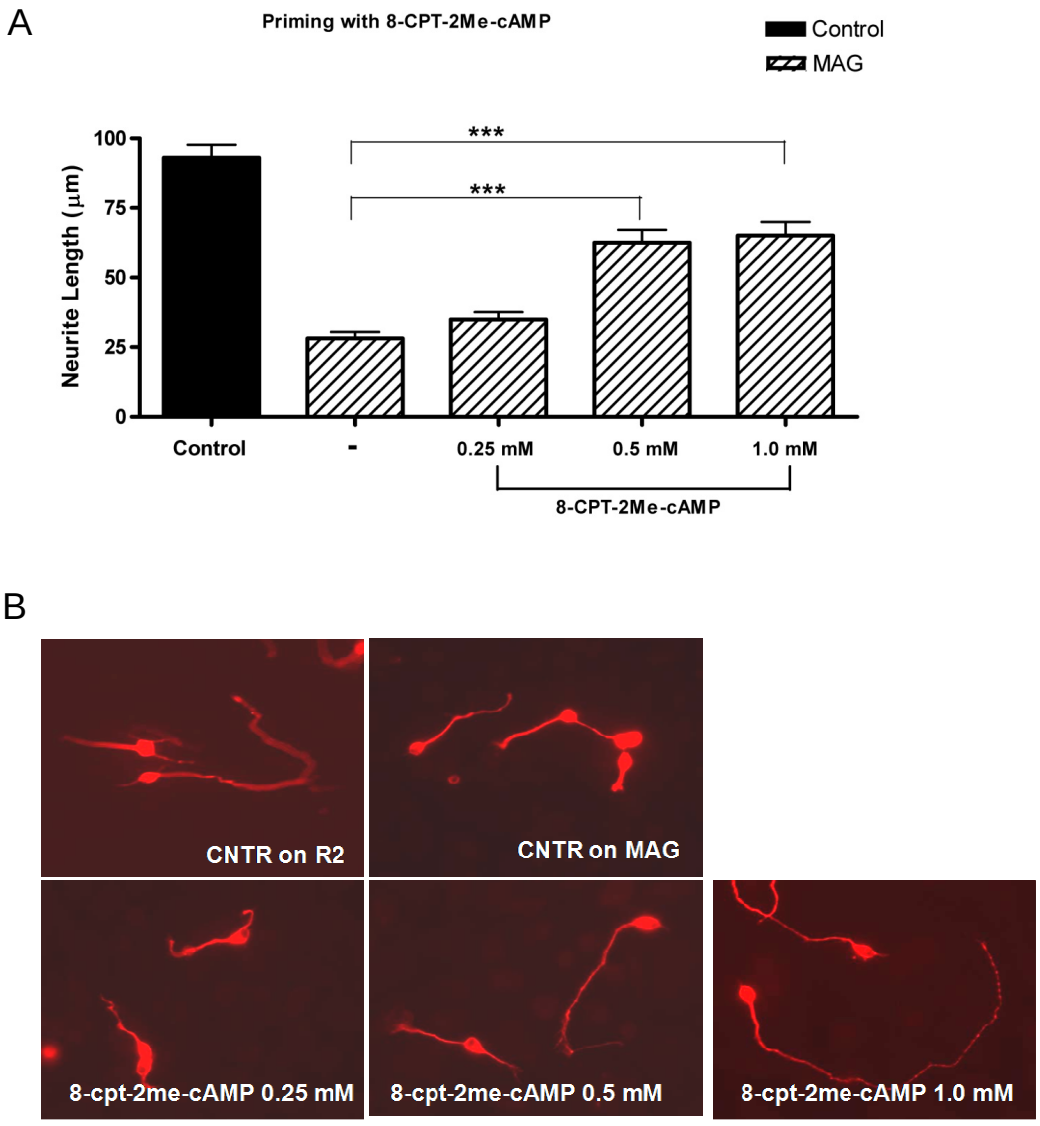


However, according to past observations in our lab (Cai et al., 1999), sometimes certain agents can block MAG's inhibition if neurons are pretreated or primed with them, but do not do so when added directly, because MAG is blocking their downstream signaling pathways. Discovery of such agents is still of value though, because if we prevent MAG from blocking them, we increase the list of agents with clinical potential. Therefore next, we tested whether overnight priming of neurons with the EPAC-specific cAMP analogue, before exposure of the neurons to MAG-expressing monolayers, could

overcome inhibition. We hypothesized that Rap1 activation alone, during overnight priming in the absence of MAG, might be sufficient to initiate signaling cascades that will allow for block of inhibition. For example, in PC12 cells, cAMP activates ERK through Rap1 activation of the MEK B-Raf (Vossler et al., 1997).

Indeed overnight "priming" with 8-CPT-2Me-cAMP, like overnight priming with neurotrophins, was able to block MAG inhibition in a dose dependent manner (Fig. 2.4). This result indicates that Rap1 stimulation alone during overnight priming with 8-pCPT-2Me-cAMP is sufficient to overcome inhibition. Priming with the EPAC/Rap1 activator 8-pCPT-2Me-cAMP, therefore, mimics the effect of priming with BDNF in overcoming inhibition. Our lab has demonstrated before that the BDNF priming effect is PKA-, ERK-, and CREB-dependent (Gao et al., 2004). Because Rap1 is a signaling effector of the NT pathway, it will be interesting to examine if there are common signaling pathways activated during priming with BDNF and 8-pCPT-2Me-cAMP. Are the downstream effectors of BDNF required for the priming effect (i.e. PKA, ERK, CREB) also effectors of the 8-pCPT-2Me-cAMP priming effect? Or does 8-pCPT-2Me priming utilize alternative pathways to overcome inhibition? These are all important questions that have not been addressed yet, and require further investigation.

Figure 2.4. Priming neurons with the EPAC-specific analogue 8-CPT-2ME-cAMP overcomes inhibition by MAG in a dose-dependent manner.
Dissociated cerebellar neurons (P5-P6) were incubated overnight with 8-CPT-2Me-cAMP at the indicated concentrations, before being trypsinized and transferred to a monolayer of either MAG-expressing CHO cells (striped bars), or control CHO cells (black bars) for further overnight culture, after which the cells were fixed and immunostained with the neuronal marker β–tubulin III. (A) Quantitation of neurite outgrowth. Results show the mean length of the longest neurite per neuron (±SEM) for approximately 150-200 neurons. *** Statistically significantly different from neurite length on control MAG-expressing cells, $p \leq 0.001$, one-way ANOVA, followed by Tukey's Multiple Comparison Test. (B) Representative images of neurons grown on the various monolayers of CHO cells as indicated.



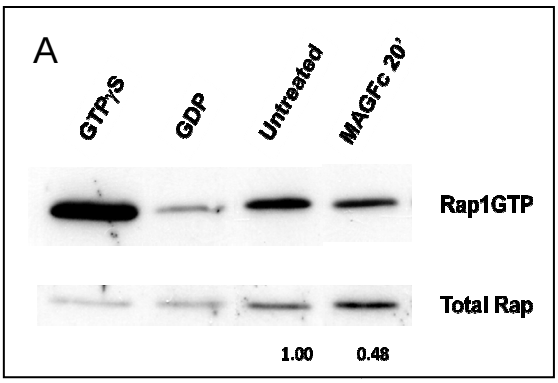
2.4 MAG and myelin treatment of neurons growing in culture reduces the basal levels of Rap1 activation

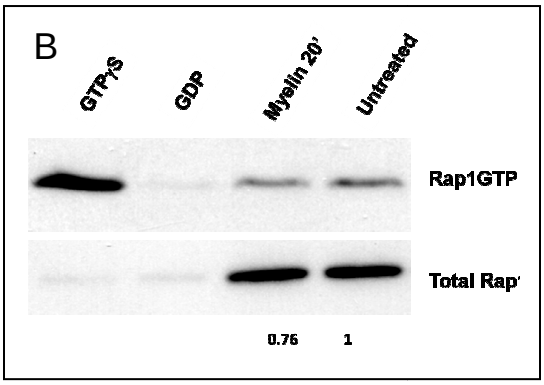
The previous experiment suggested to us that treating neurons with MAG results in increased RapGAP activity. If MAG is increasing Rap1GAP activity, this might be also reflected by decreased basal levels of Rap1GTP in neuronal cultures treated with MAG. Therefore, using the biochemical Rap1 assay, we next examined if treatment with MAG or myelin will have an inhibitory effect on the basal levels of Rap1 activation in neurons growing in culture on a PLL substrate. MAG-Fc, or a homogenized myelin preparation, was added to neuronal cultures, and active Rap1 was precipitated as described above. Both treatments resulted in a modest reduction of the basal Rap1GTP levels (Fig. 2.5), further indicating that MAG and myelin can attenuate Rap1 activity.

This result further supports our proposal of MAG increasing RapGAP activity. By doing so, MAG not only limits Rap1 activation by Rap1-activating agents such as BDNF and 8-pCPT-2Me-cAMP, but also reduces the endogenous basal levels of Rap1 activation in neurons growing in culture.

Figure 2.5. MAG and myelin treatment attenuates the basal levels of Rap1GTP.

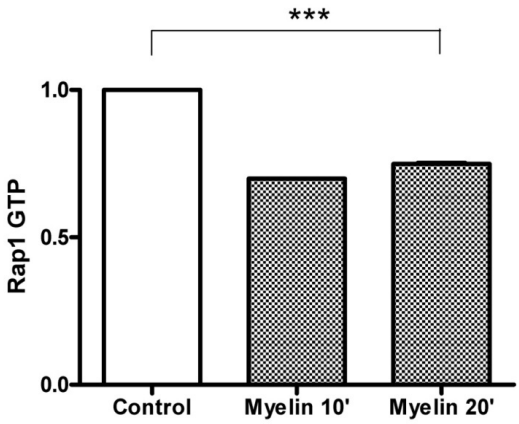
(A and B) Representative western blots showing Rap1GTP levels in cerebellar neurons upon treatments. Neonatal primary neuronal cultures (P5 Cerebellar or P1 Cortical) were starved and then either left untreated, or treated with soluble MAG (MAG-Fc , 20μg/ml), (A), or treated with myelin (20μg/ml), (B). As a positive and negative control, untreated lysate was loaded with either GTPγS (100 μM) or GDP (1mM) for 30 min before the Rap1 pull-down. Activated Rap1 was precipitated using the protocol and reagents of a Rap1 activation assay kit.
(C and D) (Left) Tables showing Rap1GTP levels obtained by densitometry in several experiments, and (Right) Quantitative analysis of Rap1 activation from multiple experiments, where Rap1GTP levels were normalized to the total Rap1 levels in the input lysate (n=1 for myelin 10 min, n=3 for myelin 20 min, n=1 for MAGFc in cerebellar, and n=2 for MAGFc in Cortical neurons.





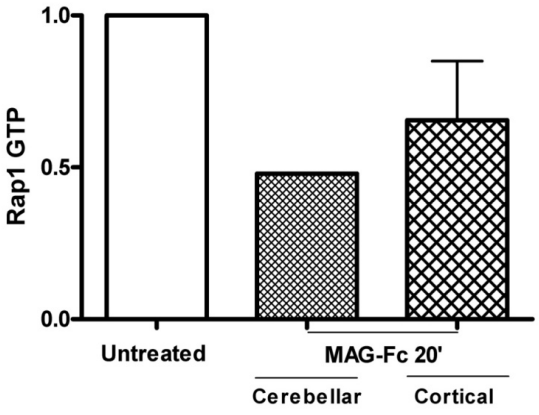
C

	Rap1GTP Levels Vs. Untreated	
Neuron Type	Myelin 10′	Myelin 20′
Cereb	0.70	
Cereb		0.76
Cereb P6		0.74
Cereb		0.75



D

Neuron Type	Rap1 GTP Levels vs. Untreated MAGFc 20′
Cereb P6	0.48
Cortic P1	0.46
Cortic P1	0.85

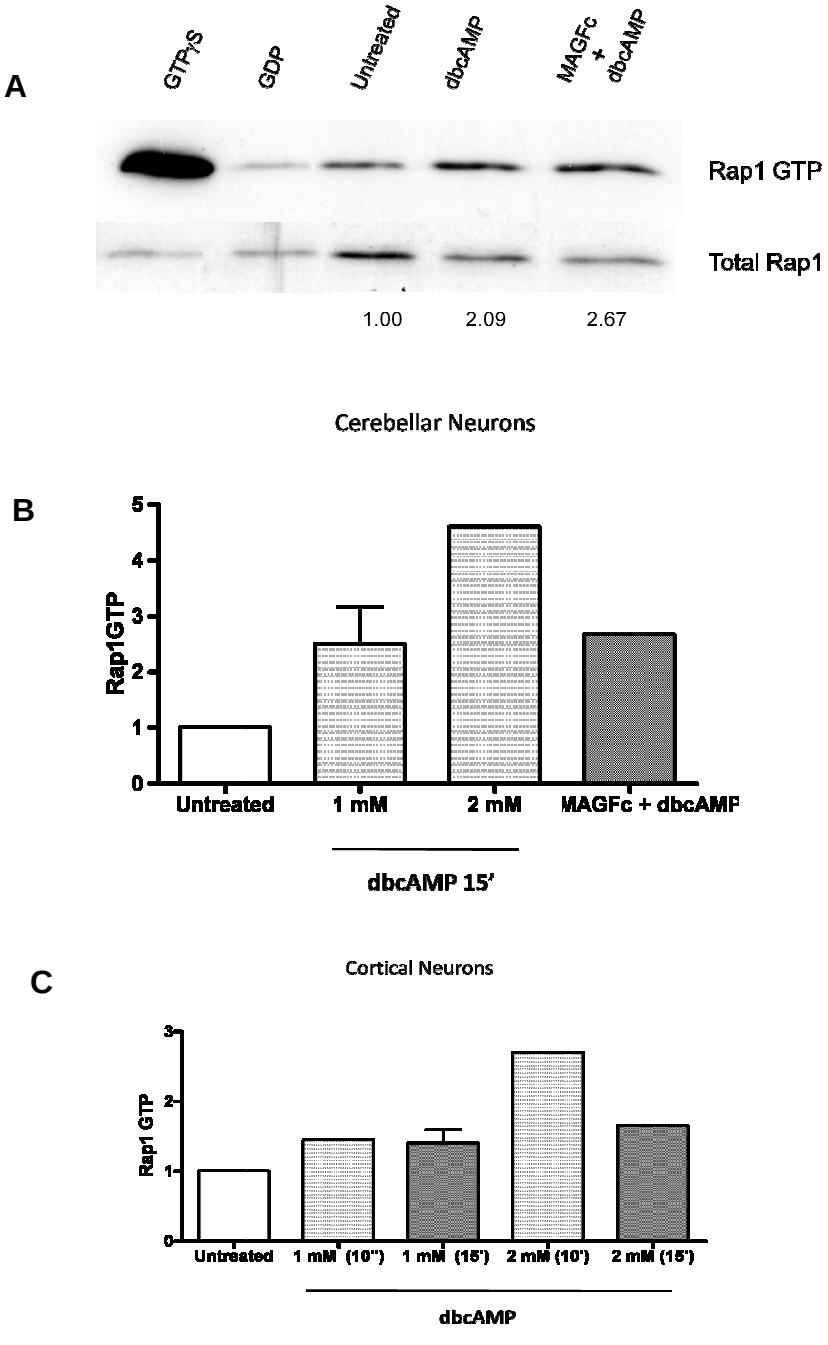


2.5 Activation of Rap1 by dbcAMP is not blocked by MAG

So far we have shown that both BDNF, a cAMP-elevating agent, as well as the EPAC-specific cAMP analogue 8-pCPT-2Me-cAMP activate Rap1, and that MAG blocks Rap1 activation by both of these agents. Our lab has demonstrated previously that when cAMP is elevated in neurons, via treatment with the analogue dbcAMP, they can overcome MAG and myelin inhibition directly, without overnight priming with this analogue. Therefore we wanted to examine if Rap1 activation occurs in the cAMP pathway. We next tested biochemically if cAMP elevation, via addition of dbcAMP, results in Rap1 activation in our model, and if MAG has any effect on it.

Treatment of neuronal cultures with dbcAMP for 15 min (1 mM) activated Rap1 in the biochemical Rap1 assay (Fig. 2.6). Contrary to what we observed when we treated with BDNF and the EPAC-specific analogue though, Rap1 activation by dbcAMP occurs even when cells were first pretreated with MAG-Fc, prior to stimulation with dbcAMP. In the context of NT signaling, addition of the dbcAMP analogue blocks MAG's inhibition of growth directly, without priming, because it is acting downstream of the signaling step where MAG blocks the cAMP elevation by NTs. Here, our result also demonstrates that dbcAMP acts downstream of where MAG interferes with Rap1 activation. The observation that MAG does not inactivate Rap1 that has been activated by dbcAMP addition indicates that dbcAMP signaling can override the inhibitory effects of MAG-Fc on Rap1 activation. Additionally, our results here also suggest that dbcAMP not only activates Rap1, but, like others have recently shown, it inhibits RapGAPs.

Figure 2.6. MAGFc does not block activation of Rap1 by dbcAMP. (A) Representative western blot showing Rap1 activation in cellebelar granule cells. Cultures were starved and then either left untreated (control), treated with dbcAMP (1mM, 15 min), or pre-treated with MAGFc (20μg/ml) for 30 min before treatment with dbcAMP. As a positive and negative control, untreated lysate was loaded with either GTPγS (100 μM) or GDP (1mM) for 30 min before the Rap1 pull-down. Activated Rap1 was precipitated using the protocol and reagents of a Rap1 activation assay kit. (B and C) Rap1 GTP quantitation of multiple experiments in cerebellar and cortical neonatal cultures. Bar graphs show Rap1GTP quantitation, where Rap1GTP levels were normalized to the total Rap1 levels in the input lysate. For cerebelar neurons: n=3 for dbcAMP 1mM, n=1 for dbcAMP 2mM, and n=1 for MAGFc + dbcAMP (1mM). For cortical neurons, n=1 for all treatments.



2.6 Infection of neurons with virus carrying a Rap1 mutant which cannot be inactivated by Rap1GAPs overcomes MAG's inhibition

In our experiments above, we observed that activation of Rap1 via the EPAC specific activator 8-pCPT-2Me-cAMP can overcome inhibition, but only if the neurons are primed overnight with it. Thus, activation of Rap1 is sufficient to overcome inhibition with priming.

We decided to further test the idea that Rap1 activation is sufficient to overcome inhibition in the neurite outgrowth assay by overexpressing a Rap1 mutant, Rap1F64A, which cannot be inactivated by RapGAPs. Once this mutant Rap1 is activated, it will remain in the GTP-bound form, and therefore its use resembles a constitutively active approach (Brinkmann et al., 2002). The use of Rap1F64A can bypass MAG's inactivation of Rap1, because even if MAG activates RapGAPs, GTP-bound Rap1F64A is RapGAP-insensitive.

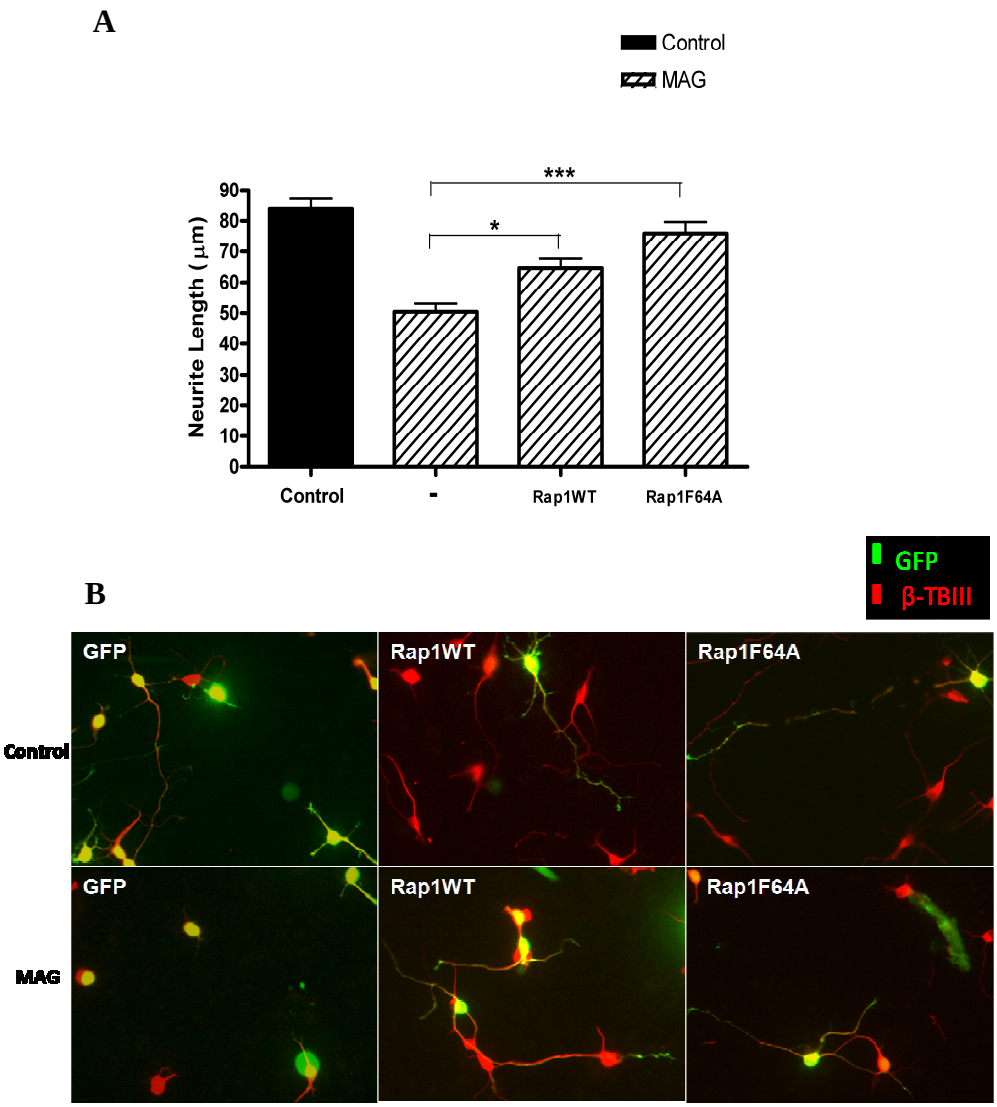
We inserted the Rap1A wild type (Rap1WT) and the Rap1F64A mutant genes into GFP-expressing adenoviruses, so that cells infected with these viruses can be identified by their fluorescent green color. There was an interesting observation in the course of constructing these two adenoviruses. In order to titrate the viruses, COS cells need to be infected with them, and by consequence, the transgenes carried by them are overexpressed in these cells. Overexpression of the control GFP-carrying virus did not have any effect on the morphology of the COS cells, but we observed some interesting phenotypes once we infected COS with the two Rap1 adenoviral constructs. Overexpression of both the Rap1A and Rap1F64 proteins resulted in morphological changes of the COS cells. COS cells overexpressing Rap1WT appeared to have formed

protrusions in their periphery. The effect was more prominent after infection with the Rap1F64A virus, and COS cells overexpressing this mutant extended long processes which had a neurite-like morphology (data not shown).

We used these adenoviral constructs to infect hippocampal and DRG primary neurons overnight, and then transferred the infected neurons on CHO cell monolayers, and myelin respectively. Infection of the neurons with both wild type Rap1A and Rap1F64 viruses overcame inhibition by MAG (Fig. 2.7, A and B), and myelin (Fig. 2.7, C and D).

Figure 2.7. Overexpression of wild type Rap1 and the RapGAP-insensitive Rap1 mutant Rap1F64A in neurons blocks inhibition my MAG and myelin.

Hippocampal or DRG neurons were isolated and infected with GFP-expressing adenovirus vectors carrying wild type Rap1 (Rap1WT) or a Rap1 mutant that is Rap1GAP-insensitive (Rap1F64A). 24 hrs after infection, the neurons were transferred to MAG-expessing CHO monolayers, or to a myelin substrate, before being fixed and immunostained for the neuronal marker β–tubulin III.
(A and C) Quantitation of neurite outgrowth. Results show the mean length of the longest neurite per neuron (±SEM) for approximately 50 neurons that are positive for both GFP and β-tubulinIII. *($p \leq 0.05$),and *** ($p \leq 0.001$) indicate statistically significant difference from neurite length of control GFP infected neurons, using one-way Anova, followed by Tukey's Multiple Comparison Test.
(B and D) Representative images of infected DRG neurons growing on myelin.



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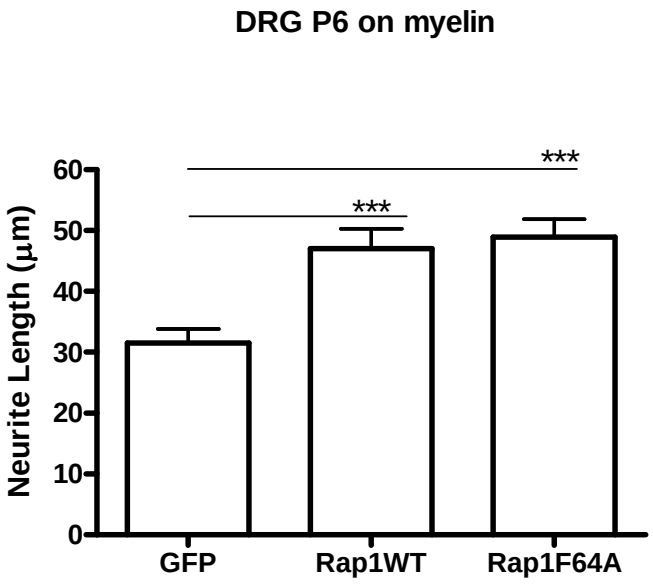

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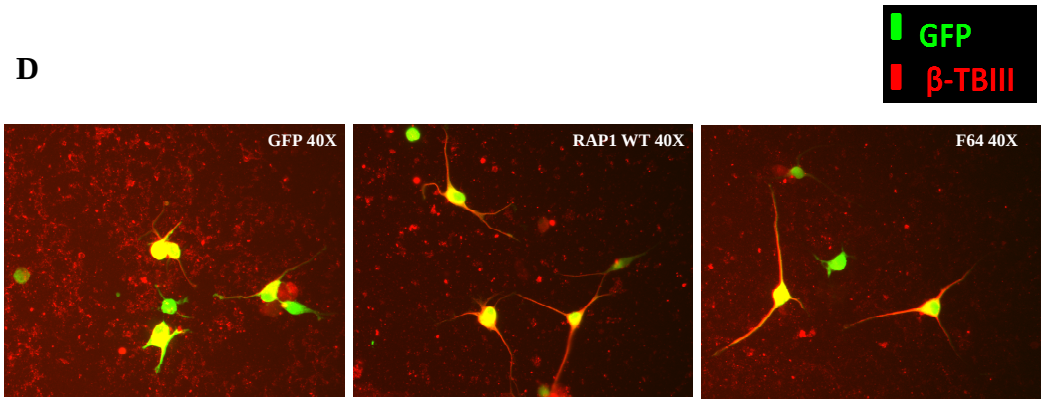


One of the questions that arises is the mechanism of block of MAG inhibition by overexpression of both wild type Rap1, as well as Rap1F64A mutant, in the absence of any signal that activates Rap1. In theory, when we overexpress Rap1A, the levels of this small GTPase increase in the cells, and it is known that GTP is about ten times more abundant within cells than GDP. Because Rap1 protein levels increase, there should be

more Rap1 molecules in the GTP- bound active state at any given moment, compared to cells that contain basal levels of active Rap1. If Rap1WT protein is produced at higher levels via adenoviral infection, the pool of endogenous RapGAP proteins activated by MAG might not be sufficient to inactivate it. This mechanism should work in the same way in the case of Rap1F64A overexpression, with the additional advantage that this mutant cannot be inactivated by RapGAPs, which are presumably activated by MAG. Therefore in the case of Rap1F64 the pool of Rap1 activated by overexpression should be even larger. So theoretically, by overexpressing these constructs, there should be an augmented pool of active Rap1 in the cell, which might be sufficient to signal the block of MAG's inhibition. Even though our data suggests that MAG activates RapGAPs, RapGAP inactivation of Rap1 is not an efficient mechanism to reduce Rap1GTP levels when the Rap1 protein is overexpressed. Rap1 overexpression via adenoviruses can overcome MAG inhibition, because the endogenous levels of Rap1GAP proteins activated by MAG is not high enough to provide negative regulation on the higher levels of Rap1GTP accomplished via adenoviral overexpression.

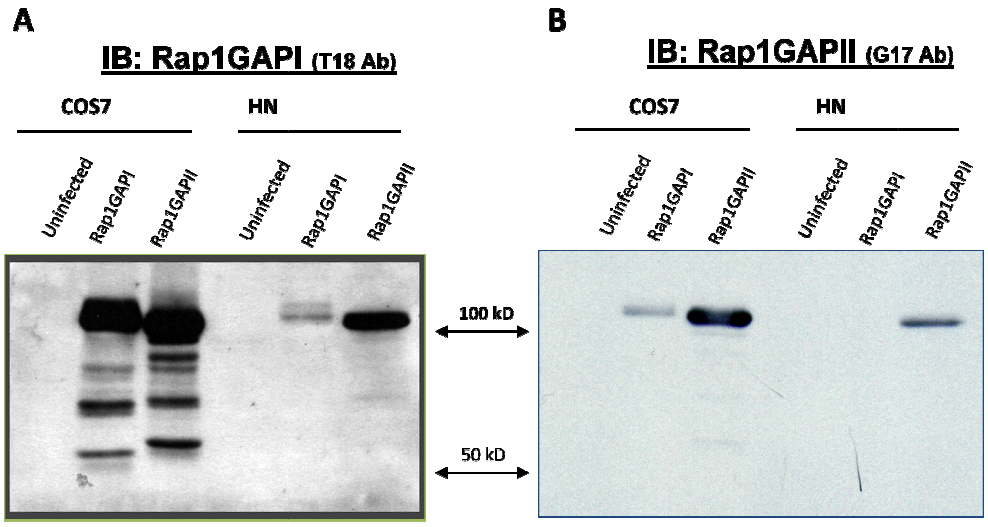
As far as the downstream mechanism of Rap1's overexpression blocking inhibition, there are several downstream Rap1 targets that may be operating in concert. One possibility is that Rap1 activation is elevating cAMP through ERK-mediated inhibition of PDE4. Another possibility is that some pool of Rap1 might be acting on the actin cytoskeleton by inactivating Rho (Cullere et al., 2005), and activating Rac (Arthur et al., 2004), (Bos, 2005), or even promoting increased substrate adhesion by activating integrins (Bos et al., 2003). A combination of all these events might be involved in the block of inhibition by Rap1 overexpression.

2.7 Overexpression of Rap1GAPI and Rap1GAPII in Cos7 cells and primary neurons

As shown before, the specific EPAC activator 8-pCPT-2me-cAMP activates Rap1, but this activation is blocked in the presence of MAGFc, presumably due to MAG activating RapGAPs. If MAG is indeed activating Rap1GAPs, then neurons overexpressing Rap1GAP proteins via an adenovirus should be strongly inhibited by MAG. By overexpressing Rap1GAPs, we augment the pool of RapGAP molecules that MAG can potentially activate, compared to neurons infected with a control virus.

We obtained adenoviruses carrying each of the two Rap1GAP isoforms, Rap1GAPI and Rap1GAPII, and we infected neurons with them to test this hypothesis. To confirm the overexpression of Rap1GAP proteins via the adenoviruses, we carried out infections in both a COS cell line, and also in neonatal (P0) rat hippocampal cultures. The T-18 antibody recognizes both Rap1GAPI and Rap1GAPII proteins (epitope mapping near the N-terminus of Rap1GAP of human origin). As can be seen in figure 8A, using this antibody we detect robust overexpression of a protein around 100Kd, which we presume is Rap1GAP, in both COS and primary hippocampal neurons. The G-17 antibody specifically recognizes Rap1GAPII protein (epitope mapping near the N-terminus of Rap1GAPII of human origin). As can be seen in figure 8B, using this antibody we detected overexpression of Rap1GAPII protein in both COS and primary neurons. These results demonstrate that infection with the adenoviruses carrying the Rap1GAPI and Rap1GAPII constructs results in overexpression of these two protein isoforms.

Figure 2.8. Overexpression of Rap1GAPI and Rap1GAPII in COS cells and primary neurons.
Cos7 cells and dissociated hippocampal neurons (P0) were infected with adenoviral vectors containing the cDNA for Rap1GAPI (A), or Rap1GAPII (B). 48 hrs after infection, cells were lysed, and 60 µg of each lysate was subjected to Western blotting. Blots were probed with antibodies specific for each Rap1GAP isoform. Numbers indicate the position of the molecular weight markers.



2.8 Ovexpression of Rap1GAPI protein mimics MAG's inhibitory effect on neurite outgrowth

RapGAP proteins stimulate Rap1's intrinsic GTPase activity, so we decided to use Rap1GAP overexpression to test the effects of Rap1 inactivation in primary neurons growing in culture. To examine the effects of Rap1GAPI overexpression on neurite outgrowth, we cultured primary hippocampal and DRG neurons on poly-L-lysine overnight, and the next day we infected them with Rap1GAPI-carrying adenovirus, as well as control GFP, Rap1WT, and Rap1F64 viruses as controls. After overnight

incubation to allow for transgene expression, we transferred neurons either to permissive substrates of poly-L-lysine and fibronectin (PLL+FN), (Fig. 2.9), or control (R2) CHO cell monolayers (Fig. 2.10, and Fig. 2.11), and also to an inhibitory substrate of MAG-expressing CHO monolayers (Fig. 2.10, and Fig. 2.11). We cultured the neurons overnight, and then the next day we assessed their growth by immunofluorescence.

GFP-, Rap1WT-, and Rap1F64-infected neurons were able to grow neurites under these conditions, which were longer on both (PLL+FN), and control CHO monolayers, as compared to the neurites grown on the inhibitory MAG CHO monolayer substrate, as described above (see Fig. 2.7). To our surprise though, Rap1GAPI-infected neurons did not extend long neurites on either permissive or non-permissive (MAG CHO) substrates (Figures 2.9, 2.10, 2.11).

From these observations we concluded that overexpression of Rap1GAPI virus has a general inhibitory effect on neurite outgrowth, indicating that Rap1 GTPase function is necessary for process outgrowth and neurite extension in neurons.

Figure 2.9. Effects of Rap1 and Rap1GAPI overexpression in the morphology of hipocampal neurons (HN).

A) Effects of Rap1 and Rap1GAPI overexpression on the moprphology of HN 24 hr after infection. Gradient-isolated P1 HN were grown overnight on PLL, and the next day, they were infected with control adenovirus-GFP, or adenovirus-GFP carrying the indicated transgenes. Cultures were incubated overnight to allow for expression of viral proteins, and the following day living GFP-expressing cells were visualized under a fluorescent microscope.
B) Next, infected cells were trypsinized, transferred on a PLL-FN substrate, and allowed to grow overnight. 24 hours after transfer on the new substrate, living GFP-expressing cells were visualized again to assess how transgene expression affected process outgrowth on the permissive substrate.

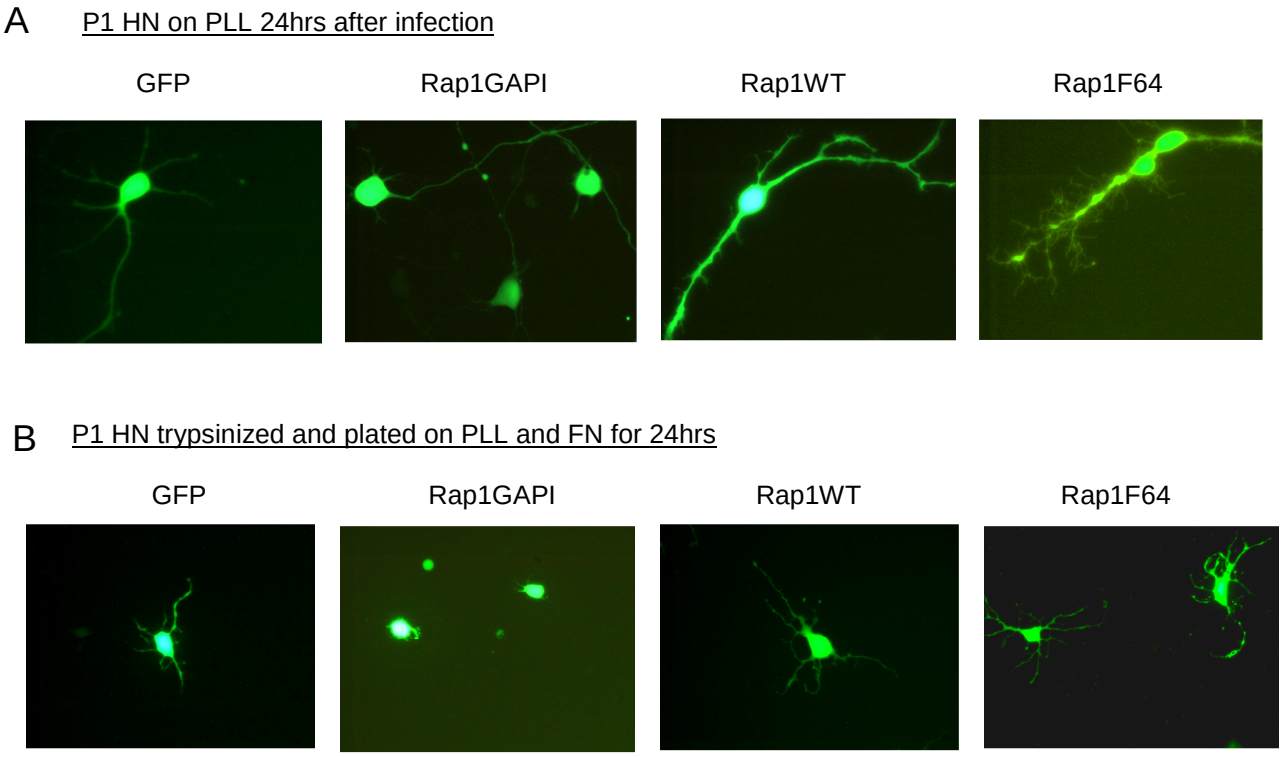


Figure 2.10. Effects of Rap1 and Rap1GAPI overexpression in HN growing on CHO cell monolayers.

Gradient-isolated P1 HN were grown overnight on PLL, and the next day they were infected with control adenovirus-GFP, or adenovirus-GFP carrying Rap1GAPI. Cultures were incubated overnight to allow for expression of viral proteins, and the following day infected cells were trypsinized, and transferred to control (R2) or MAG-expressing CHO cell monolayers, and allowed to grow overnight. 20 hours after transfer on the monolayers cells were fixed and immunostained with neuronal specific β−tubulinIII antibody. Immunofluorescence was then performed to visualize the infected neurons (both GFP and β−TBIII positive, yellow color) and assess how transgene expression affected their growth on the monolayers.
(A) Quantitation of neurite outgrowth of Rap1GAPI infected neurons. Results show the mean length of the longest neurite per neuron (±SEM) for approximately 50 neurons that are positive for both GFP and β-tubulinIII. *** indicate statistically significant difference ($p \leq 0.001$) from neurite length of GFP infected neurons on either control or MAG monolayers , using one-way Anova, followed by Tukey's Multiple Comparison Test. (B) Representative picture showing infected neurons on Control CHO cells.

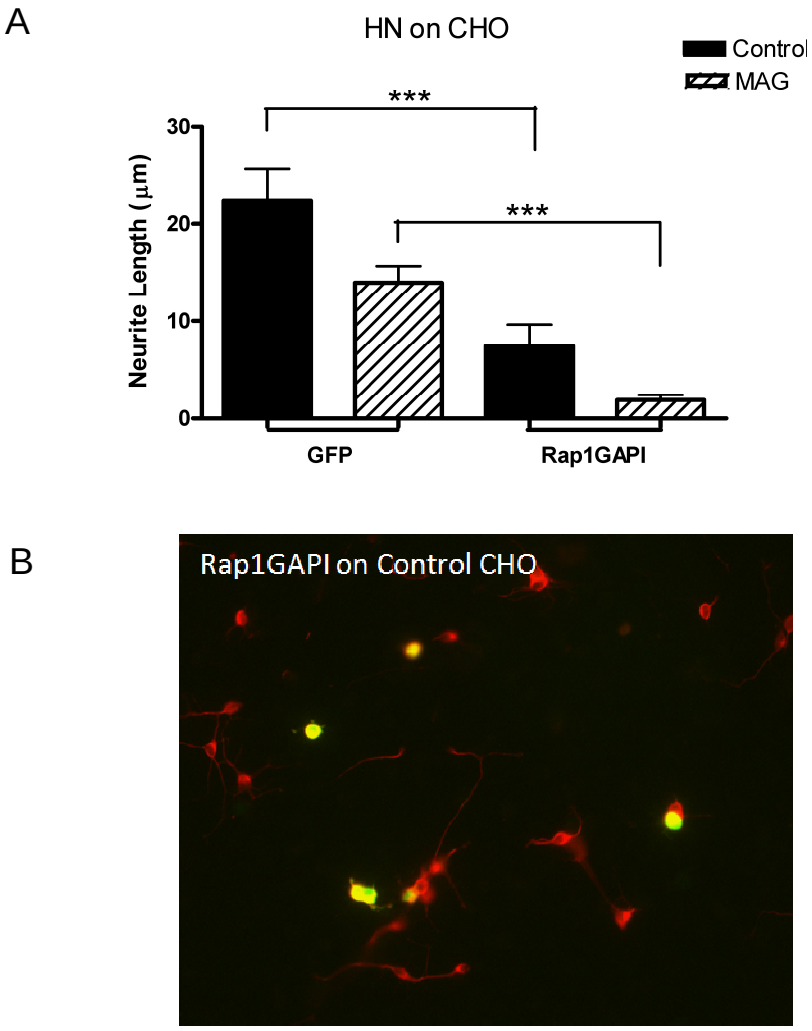
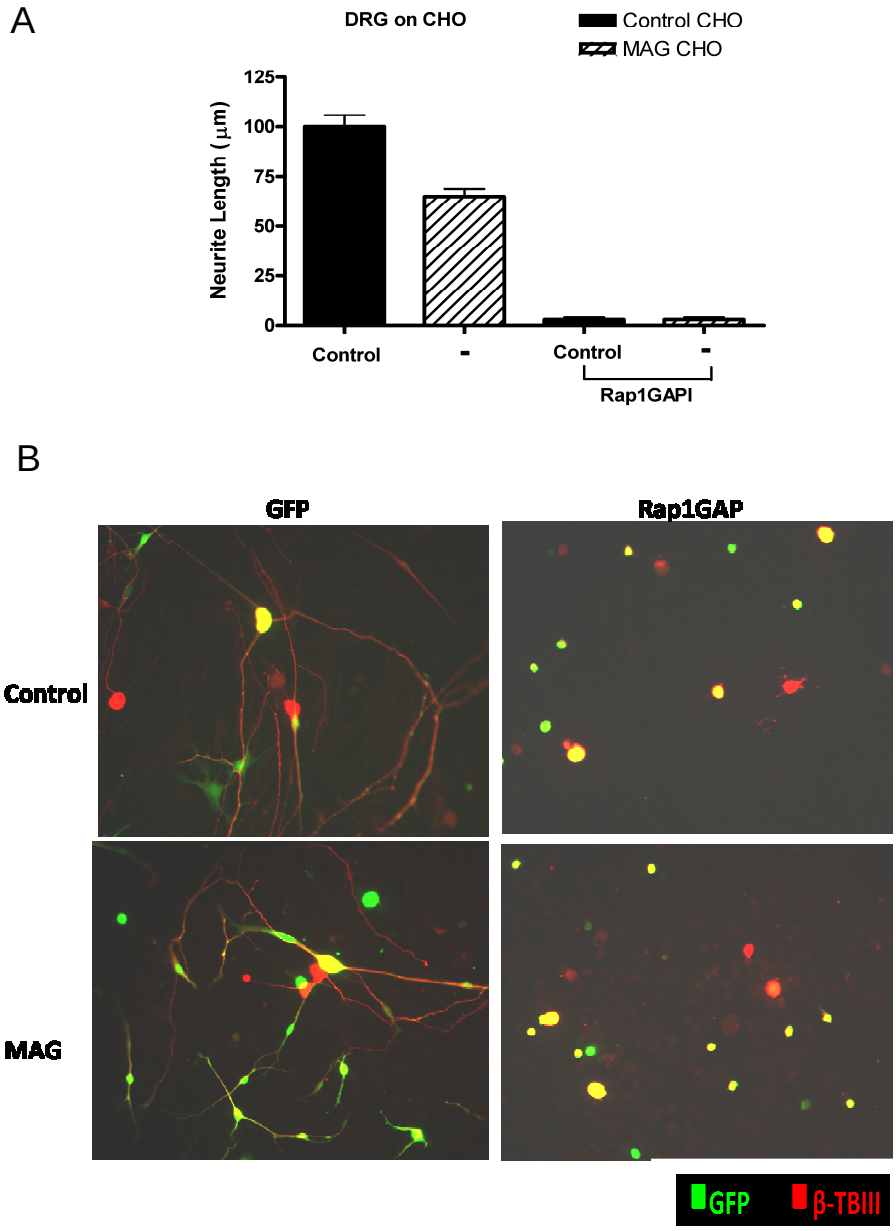


Figure 2.11. Effects of Rap1GAPI overexpression in DRG neurons growing on CHO cell monolayers.

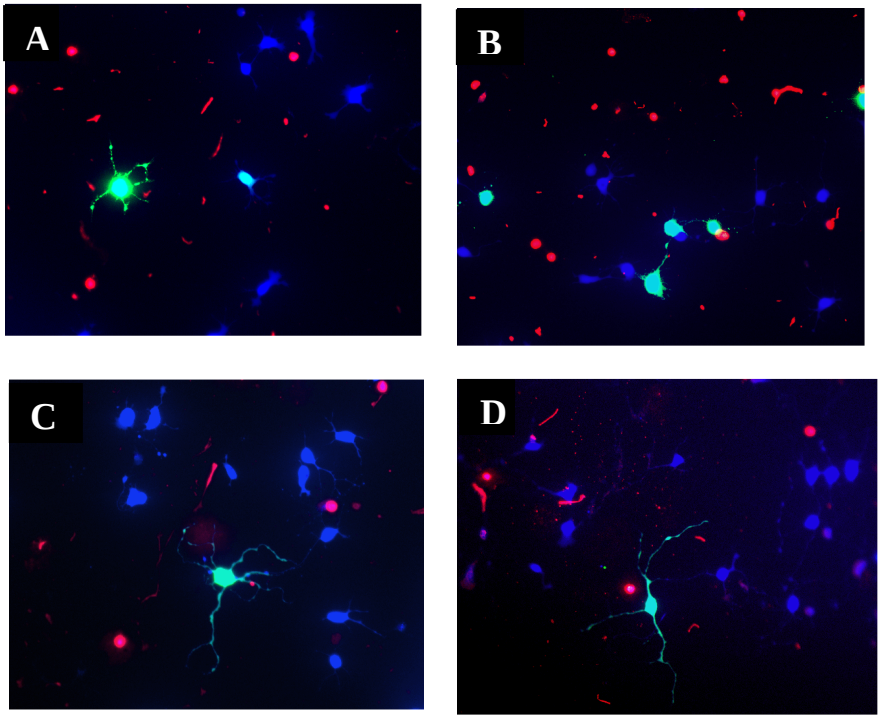
P5 DRG neurons were grown overnight on PLL, and the next day they were infected with control adenovirus-GFP, or adenovirus-GFP carrying Rap1GAPI. Cultures were incubated overnight to allow for expression of viral proteins, and the following day infected cells were trypsinized, and transferred to control (R2) or MAG-expressing CHO cell monolayers, and allowed to grow overnight. 20 hours after transfer on the monolayers cells were fixed and immunostained with neuronal specific β–tubulinIII antibody. Immunofluorescence was then performed to visualize the infected neurons (both GFP and β–TBIII positive, yellow color) and assess how transgene expression affected their growth on the monolayers. (A) Quantitation of neurite outgrowth of Rap1GAPI infected neurons. Results show the mean length of the longest neurite per neuron (±SEM) for approximately 50 neurons that are positive for both GFP and β-tubulinIII. (B) Representative picture showing infected neurons on Control CHO cells.



Because Rap1GAPI-overexpressing neurons could not extend long neurites regardless of the permissiveness of the substrate they were allowed to grow on, we next tested the viability of these neurons. We wanted to exclude the possibility that Rap1GAPI infected neurons are dying, and therefore unable to grow processes. To test this, we used the dye probes Ethidium homodimer-1 (EthD-1) and Calcein blue AM to label dead cells and live cells respectively in our adenovirus-infected neuronal cultures. The red dye EthD-1 is a cell death indicator, since it only penetrates cells with damaged plasma membranes, a recognized parameter of compromised viability. Another known indicator of cell viability is the activity of intracellular esterases. Calcein blue AM is a cell-permeant esterase substrate that, upon cleavage, is converted into a blue fluorescent product that is retained by live cells with intact plasma membranes. In order to assess the viability of infected cells, gradient-purified P0 HN were cultured overnight on poly-L-lysine, and then the next day they were infected with adenoviruses. The infected cultures were incubated overnight, and the following day cells were transferred to a PLL+FN substrate, to allow them to grow again overnight, while they are expressing the viral transgenes. The next day viability was assessed using EthD-1 and Calcein blue AM (Fig. 13). GFP-, Rap1WT-, and Rap1F64- infected cells are alive, as indicated by their blue color, and have extended processes. Rap1GAPI-infected cell are also alive, but severely impaired in their ability to put out processes when compared to cells infected with Ad-Rap1WT and Ad-Rap1F64, suggesting that this phenomemon is not due to toxic effects of the adenoviral infection itself, but rather a specific effect of the overexpressed transgene.

Figure 2.12. Cells infected with Rap1GAPI adenovirus are alive.

Assessment of cell viability via fluorescent microscopy imaging of P0 HN cultures infected with Ad-GFP (A), Ad-Rap1GAPI (B), Ad-Rap1WT (C), and Ad-Rap1F64 (D). Dissociated P0 HN cultures were plated on poly-L-lysine, and one day later they were infected with indicated adenoviral constructs. After overnight incubation to allow for viral transgene expression, cells were trypsinized, and transferred to a substrate of PLL+FN. Next, cells were incubated overnight to allow for growth, and the next day they were assessed for viability, using the markers EtdD-1 and Calcein blue AM. Red color: staining of dead cells with EthD-1. Blue color: Staining of living cells with Calcein blue AM. Cyan: GFP-expressing living cells.

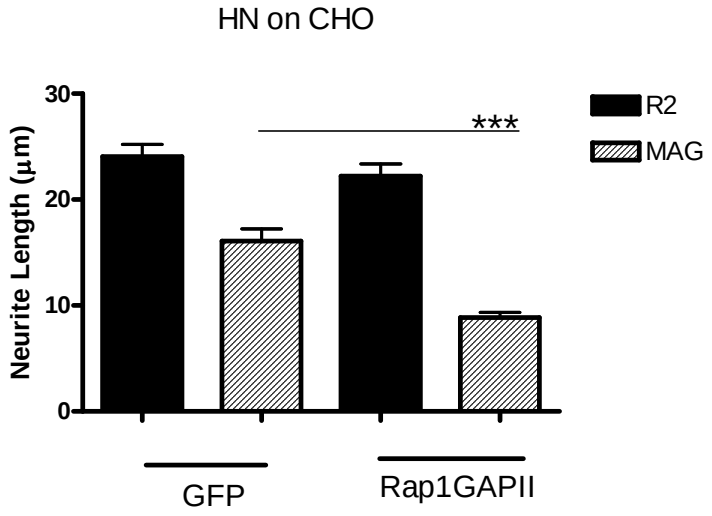


Neurons infected with Rap1GAPII can extent neurites when growing on a permissive substrate, but when growing in the presence of MAG, their neurites are much shorter that the ones in control cells, and MAG inhibition is potentiated (observations by Dr. E. Nikulina, see Fig. 2.13). This suggests that the presence of MAG augments Rap1GAPII activity. It also implies that, unlike the pool of overexpressed Rap1GAPI

protein, the pool of overexpressed Rap1GAPII protein is mostly inactive when cells grow on a permissive substrate, but in the presence of MAG, some portion of it becomes activated and inhibits outgrowth. Overexpression of Rap1GAPI though results in different effects that overexpression of its isoform Rap1GAPII: when expressed in neurons, it does not allow neurons to extend neurites, both on permissive and non-permissive substrate. This implies that the pool of overexpressed Rap1GAPI is in an active state, regardless of the presence of MAG.

Figure 2.13. Effects of Rap1GAPII overexpression in HN growing on CHO cell monolayers.

Quantitation of neurite outgrowth of Rap1GAPII infected neurons. Gradient-isolated P1 HN were grown overnight on PLL, and then the next day were infected with control adenovirus-GFP, or an adenovirus carrying Rap1GAPII. Cultures were incubated overnight to allow for expression of viral proteins, and the following day infected cells were trypsinized, and transferred on control (R2) and MAG-expressing CHO cell monolayers, and allowed to grow overnight. 20 hours after transfer to the monolayers, cells were fixed and immunostained with neuronal specific β–tubulinIII antibody, as well as with an antibody against Rap1GAPII. Bar graphs show the mean length of the longest neurite per neuron (±SEM) for approximately 50 neurons that are positive for both GFP and β-tubulinIII, or enhanced Rap1GAPII staining, as a marker for overexpression and β-tubulinIII. *** indicate statistically significant difference ($p \leq 0.001$) from neurite length of control GFP infected neurons on MAG monolayers , using one-way Anova, followed by Tukey's Multiple Comparison Test.



2.9 MAG and myelin upregulate Rap1GAP protein levels.

Pharmacological activation of EPAC in the presence of MAG did not result in Rap1 activation, and from this result we hypothesized that MAG is activating Rap1GAPs. There are several reports in the literature describing extracellular signal regulation of Rap1 activity via changes in the subcellular localization of Rap1GAPs . In one study the authors found that inactive $G\alpha_o$ sequesters Rap1GAP, and prevents it from interacting with Rap1 (Jordan et al., 1999). They suggested that upon its activation, $G\alpha_o$ would release Rap1GAP, which could then inhibit Rap1. Another report in PC12 cells has documented that upon $G\alpha_z$ activation, Rap1GAP was recruited from a cytosolic location to the membrane, and this resulted in a downregulation of Rap1 signaling (Meng and Casey, 2002). Thus, it is far from clear so far how Rap1GAP can associate with various $G\alpha$ subunits to regulate Rap1 activity.

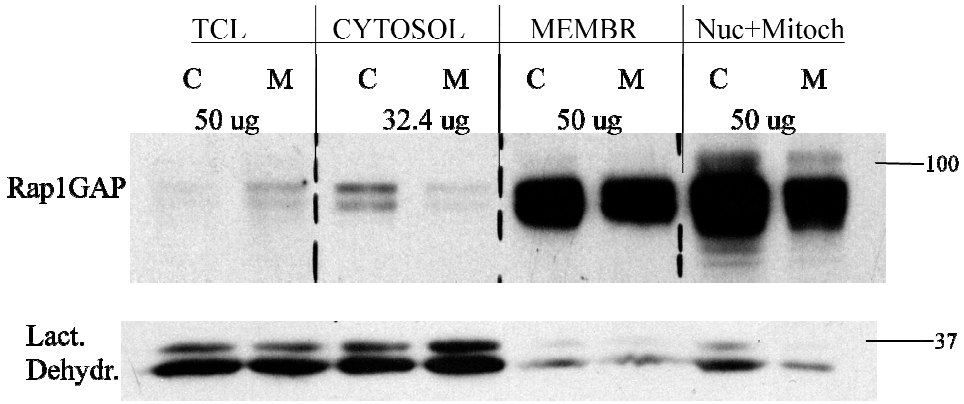
Therefore we decided to test the hypothesis that upon MAG treatment, Rap1GAP protein is either a) recruited from the cytosol to the membrane, where it will be in the appropriate cellular locale to act upon Rap1, or b) initially associated with membranes and inactive, Rap1GAP upon MAG treatment, will be released from the membrane to inactivate Rap1. Both of these modes of Rap1GAP regulation have been reported in the literature.

To check these possibilities, we performed subcellular fractionation, but we were unable to demonstrate a reproducible effect of MAG in Rap1GAP localization. However, in these subcellular fractionation experiments, we observed that Rap1GAP is primarily localized to the membrane, regardless of the presence of MAG, as can be seen in figure

2.14. Both Rap1GAP isoforms, Rap1GAP1 and the longer Rap1GAPII, appear as two bands a bit lower that the 100 kDa marker, consistent with Rap1GAPs' molecular weight being 85/95 kDa (Upstate/Millipore Certificate of Analysis of Rap1GAP antibody). The Rap1GAP bands emit a much stronger signal in the membrane and in the "Nuclear+Mitochondrial" fraction.

Figure 2.14. Rap1GAP localizes mostly in membranes.

P2 cortical neuronal cultures were left untreated as a control (C), or were treated with 20 μg/ml MAGFc for 15 min (M), and then subcellular fractionation was performed. Total cell lysate (TCL), cytosolic (CYTOSOL), membrane (MEMBR) and combined nuclear and mitochondrial (Nuc+Mitoch) fractions were run in a Western blot. The blot was probed with anti-Rap1GAP antibody, and then stipped and probed for the cytosolic protein lactate dehydrogenase. Numbers indicate the position of the molecular weight standard.



Nevertheless, during the course of the subcellular fractionation studies after MAG treatment we observed that the levels of Rap1GAP protein in the total cell lysate (TCL) were upregulated in response to MAG treatment. To further investigate this, we

performed a time-course with MAG-Fc treatment, and we were able to reproduce this observation, and detected Rap1GAP upregulation at 10 and 20 minutes after MAG treatment (Fig. 15, A and C). We were also able to observe upregulation of Rap1GAP levels after 20 min of myelin treatment (Fig. 15, B and D). This is an important observation, because upregulation of Rap1GAP at the protein level could be the underlying mechanism by which MAG/myelin block activation of Rap1. Finally, based on our results in the present study and on the existing Rap1 literature, a model of how we believe MAG blocks activation of Rap1 and inhibits cAMP elevation by NTs can be seen in figure 2.16.

Figure 2.15. MAG and myelin upregulate Rap1GAP protein levels.

(A and C) Representative western blots indicating Rap1GAP upregulation upon treatments. Primary rat neonatal neurons (P1-P3, cortical) were treated with soluble MAG (MAGFc) (A), or myelin (B) for the indicated intervals, and then mechanically disrupted with a hand-driven Dounce homogenizer. Detergent was added to the lysates, and Rap1GAP levels were detected by western blotting. The blots were then stripped and re-probed for the neuronal marker β−TBIII, or for actin.

(C and D) Quantification of Rap1GAP levels is represented as fold-increase upon MAG or myelin stimulation, where Rap1GAP levels were normalized to their respective loading controls (n=8 for MAG 10 min, n=6 for MAG 20 min, and n=3 for myelin 20 min).

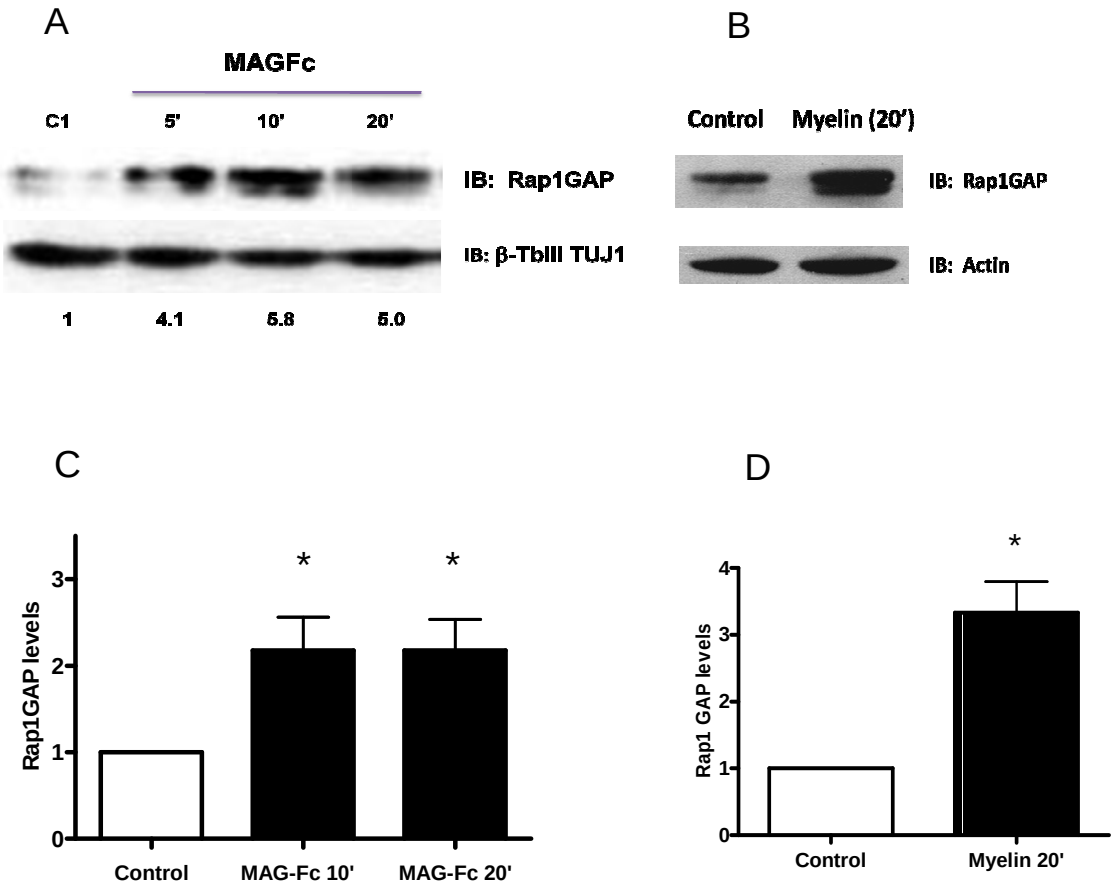
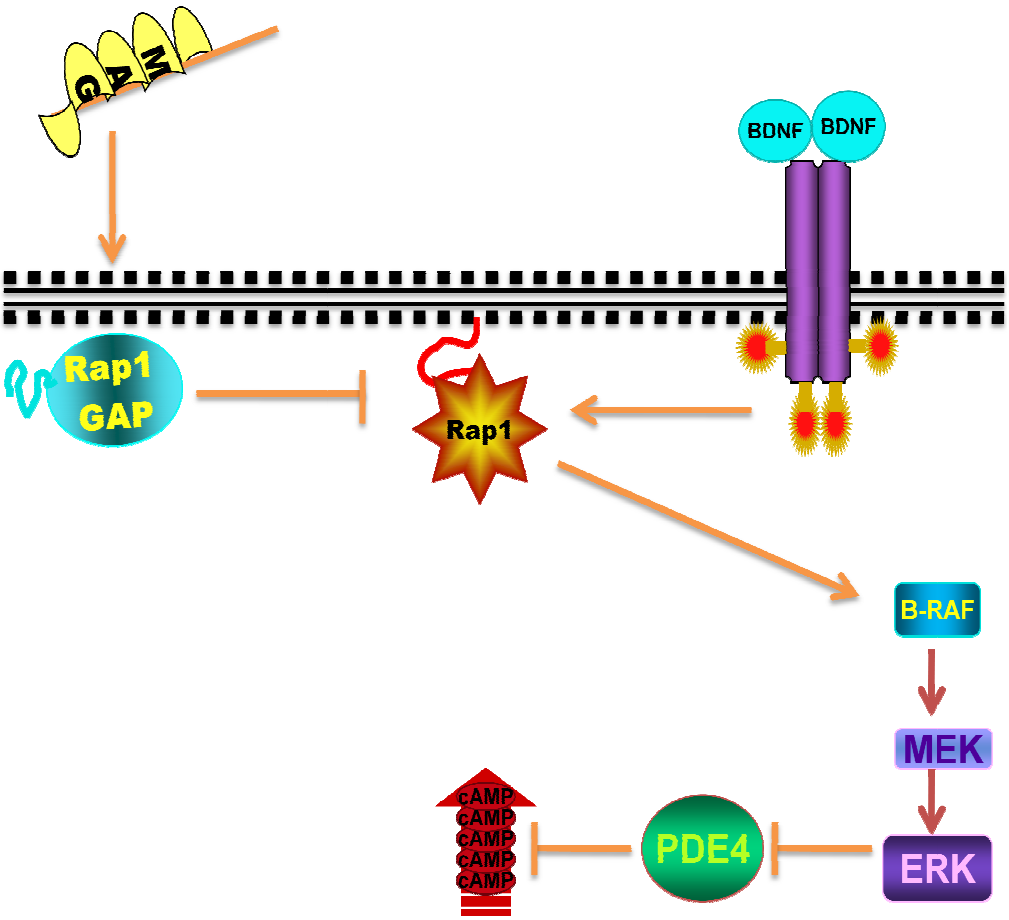


Figure 2.16. Model explaining MAG's inhibition of Rap1 activation by BDNF, leading to block of cAMP elevation by neurotrophins.

Neurotrophins activate Rap1, which can couple to ERK activation through B-Raf. ERK activation can lead to cAMP elevation, through PDE4 inhibition. Rap1 activation by BDNF is inhibited in the presence of MAG. Therefore, we propose:
i) MAG inhibits BDNF activation of Rap1, because it upregulates Rap1GAP protein levels, thus possibly resulting in increased GAP activity towards Rap1, and
ii) inhibition of Rap1 activation by BDNF might be the signaling node where MAG inhibits the ability of NT to elevate cAMP, due to Rap1's involvement in a feedback loop of cAMP elevation , through ERK-dependent PDE4 inhibition.



Chapter III: Discussion

Neurotrophins have been used *in vivo*, in several CNS disease/injury models to stimulate the intrinsic growth state of neurons and facilitate regeneration (Hollis et al., 2009), (Nagahara et al., 2009). So far, administration of NTs in spinal cord injury models has resulted in limited regeneration of certain neuronal populations (Tuszynski et al., 1996), (Ramer et al., 2002), (Zhou and Shine, 2003), [see also review by (Lu and Tuszynski, 2008). Therefore, in order to improve their therapeutic potential, we need to understand the factors that prevent NT from unleashing their maximal effects on nervous system repair and regeneration after injury.

Previously in our lab, we have used neurotrophins *in vitro* as agents for overcoming inhibition by myelin inhibitors, in our neurite outgrowth assay. We have found that direct addition of NTs to neurons plated on MAG or myelin does not relieve inhibition, but priming of neurons with various NTs, and subsequent transfer of them to inhibitory substrates overcomes inhibition. This was the first observation by our lab supporting the hypothesis that myelin inhibitors block neurotrophin signaling. In the same publication, Cai et. al., (1999) provided further evidence for this hypothesis. They utilized a competitive immunoassay method to demonstrate that MAG blocks cAMP elevation by neurotrophins. These findings lead to the proposal of the following model of how priming with NTs works in overcoming inhibition: NTs elevate cAMP via ERK-dependent inhibition of the cAMP-degrading enzyme PDE4, and the resulting cAMP elevation initiates signaling that overcomes inhibition. Here, we dissect further into the "priming" mechanism, and propose the involvement of Rap1.

Via a biochemical Rap1 activation assay, we demonstrated that BDNF activates Rap1, and MAG and myelin block this activation. Based on this observation, we hypothesized that by blocking Rap1 activation, MAG inhibits NT signaling.

Activation of Rap1 in the canonical NT signaling cascade is known to occur through recruitment of the RapGEF C3G to activated Trks receptors in the membrane area (Gotoh et al., 1995; York et al., 1998). It was also shown, in the PC12 cell NT signaling model, that Rap1 activation by NGF is a necessary upstream event in the sustained ERK activation observed after NGF stimulation (York et al., 1998). It was later shown by our lab, in neuronal cultures, that ERK activation by NTs results in increased cAMP levels, through ERK-dependent inhibition of PDE4. The MEK inhibitor UO126 blocked cAMP elevation by NTs, mimicing MAG's block of cAMP elevation by NTs. By combining these observations, a possible scenario would be that by blocking Rap1 activation in the NT cascade, MAG is inhibiting the actions of the Rap1 effector ERK, which is required for NTs to elevate cAMP, as shown by our lab. Thus, even though BDNF is a cAMP-elevating agent, it cannot elevate cAMP in the presence of MAG, and as a result, BDNF cannot overcome inhibition when added together with MAG. BDNF only overcomes inhibition via priming, in the absence of MAG, where all the effects of this NT on neurons can take place unimpeded.

Based on these observations, we are proposing the following model regarding Rap1's role in the NT signaling network that causes elevation of cAMP: an initial Rap1 activation by NTs, provided by C3G recruitement to activated Trks, is further reinforced for a longer period of time via the following positive feedback cycle: activation of Rap1→ERK→PDE4 pathway causes incremental elevations of cAMP, which then feed

back to Rap1, through the cAMP-binding Rap1 GEF, EPAC, resulting in Rap1's activation being sustained for a longer period. Rap1 then continues to activate ERK, resulting in cAMP elevations in the cellular microdomains where these events take place (see model in Fig. 2.15). This hypothesis also helps explain the sustained ERK activation in the NT pathway: since Rap1's activation is reinforced by the incremental cAMP elevations of a positive feedback cycle through EPAC, Rap1 continues to activate ERK in a sustained fashion.

This model provides us with a way to explain the MAG blockade of cAMP elevation by NTs that has been previously observed by Cai et. al., (1999) in our lab; by blocking Rap1, MAG is inhibiting the positive feedback loop that involves the players Rap1-ERK-PDE4 in elevating cAMP levels after NT treatment. This scenario explains why NTs do not overcome inhibition directly, but only if we pre-treat neurons with them, before culturing them on MAG or myelin. When NTs are added together with MAG or myelin, MAG and myelin block the cAMP elevation, through inhibition of Rap1. Therefore, NT effects are blocked, and inhibition persists. If we prime neurons with NTs though, in the absence of inhibitors, Rap1 is activated, and cAMP can accumulate to the required threshold through ERK-dependent PDE4 inhibition. This results in the initiation of signaling cascades that overcome inhibition, past the point where MAG can block the signal.

Additional findings in the literature support our hypothesis. It has been demonstrated, in hippocampal neurons, that Rap1 couples cAMP signaling to a membrane-associated pool of ERK (Morozov et al., 2003). Also, Li et. al., have demonstrated that, in growth factor signaling, activated EPAC2 can be recruited to the

plasma membrane by Ras, to activate a specific plasma membrane pool of Rap1 (Li et al., 2006). Therefore future experiments to validate our proposal include examining membrane-associated ERK activation in a time-course of NT treatment in the presence of MAG. Additionally, we could inhibit Rap1 via RapGAP overexpression, and then specifically examine membrane-associated ERK activation after BDNF stimulation. If our hypothesis is correct, we should then see MAG blocking ERK activation in this particular cellular locale.

We next examined Rap1 activation by EPAC biochemicaly, in the presence of MAG. In the activation-specific biochemical Rap1 assay, 8-pCPT-2Me-cAMP activated Rap1, and MAG inhibited this activation. There are two possible ways for MAG to block Rap1 activation by 8-CPT-2Me-cAMP: either by inactivating EPACs or by activating Rap1GAPs. Proteins that inactivate the cAMP receptors EPAC, or the PKA R subunit, have not been identified to date, in the best of our knowledge. Regulation of these cAMP sensors by cyclic nucleotides happens in an allosteric and reversible fashion, which is solely dependent on the increased levels of the nucleotides (Dao et al., 2006). It is known from the literature that 8-pCPT-2Me-cAMP is a very potent activator of EPAC; once bound to it, EPAC changes conformation, exposing its GEF domain which enhances Rap's exchange of GDP for GTP, thus switching Rap1 "on". Therefore, the source of Rap1's inactivation in our setting is very unlikely to be MAG inhibiting EPAC activation. We concluded that even though 8-pCPT-2Me-cAMP activates Rap1 through EPAC, Rap1 is rapidly inactivated by RapGAPs. Therefore, our results strongly suggest that MAG inactivates Rap1 via GEF-independent pathways, which involve Rap1GAPs.

Cellular elevation of cAMP affects several downstream effectors of this ubiquitous second messenger, one of the most widely studied being PKA. Indeed, Cai et. al. (1999) in our lab have shown that the "priming effect" in overcoming MAG's inhibition is, initially, PKA dependent. Here we examined the potential role of another cAMP receptor, the Rap1 activator EPAC, in overcoming MAG inhibition. Interestingly, an EPAC requirement for regeneration in an *in vitro* model has recently been reported by others. Murray and Shewan showed that EPAC activation by 8-CPT-2Me-cAMP in DRG neurons enhanced neurite outgrowth on a permissive substrate, induced growth cone turning, and also enhanced growth of both neonatal and adult DRGs on spinal cord sections. Using a specific siRNA approach, they also demonstrated an EPAC requirement for all these effects. Therefore, they proposed that EPAC plays an important role in the effects of cAMP on axonal growth, guidance and regeneration, and that it may serve as a most selective target than global cAMP elevation for therapeutic intervention (Murray and Shewan, 2008). In our *in vitro* model, treatment of neurons with the EPAC specific activator 8-CPT-2Me-cAMP, like treatment with neurotrophins, did not overcome MAG inhibition when added directly to neurons transferred to a MAG substrate. However, overnight priming of neurons with 8CPT-2Me-cAMP, like priming with NTs, overcomes MAG's inhibition.

It has been shown in the PC12 model that stimulation with an EPAC-specific analogue results in sustained ERK1/2 activation in these cells (Kiermayer et al., 2005). Therefore, a possible explanation of how priming with 8-pCPT-2Me-cAMP overcomes inhibition is that after its activation by 8-CPT-2Me-cAMP, EPAC activates Rap1 which then recruits B-Raf (a MEKK for ERK), and thus ERK is activated, which then inhibits

PDE4. The cAMP concentration increases in cellular microdomains, which could result in PKA activation, and further downstream signaling that blocks inhibition. EPAC proteins contain membrane localization domains, which might contribute to the specificity of effector pathways that are activated by Rap1 (Wang et al., 2006). EPAC1 has been shown to have a perinuclear expression pattern in COS-7cells (Qiao et al., 2002), and Wang et. al., (2006), working in neuroendocrine cell lines, have proposed that because of its cellular localization, EPAC1 is unable to couple to ERKs. Quilliam, on the other hand, has shown that EPAC2 can be translocated to the plasma membrane and activate Rap1 there, but only as a response to signals that both elevate cAMP and activate Ras (Li et al., 2006). Since in our priming experiments with 8-CPT-2Me-cAMP we did not activate Ras, our result suggests that in our model, which uses primary neuronal cultures, there might be other modes for specific EPAC activation by a cAMP analogue to couple to Rap1. Additionally, Rap1 is also involved in several other cellular functions, such as adhesion and cytoskeletal regulation, which might play a role in overcoming inhibition after Rap1 activation by 8-pCPT-2Me-cAMP. These signaling events occur during overnight priming with this analogue only, but not when the analogue is added to neurons in the presence of MAG. Even though 8-CPT-2Me-cAMP activates Rap1 via EPAC, in the presence of MAG this activation is blocked, as we have shown in the biochemical Rap1 assay, presumably because MAG activates RapGAPs. The observation that Rap1 activation by the EPAC-specific cAMP analogue is sufficient to overcome inhibition by overnight priming suggests an important role for Rap1 in the BDNF/dbcAMP effect of overcoming MAG and myelin inhibition.

Our experiments with the cAMP analogue 8CPT-2me-cAMP suggested to us that MAG signals to inactivate Rap1 via activation of RapGAPs. In support of this hypothesis, we have found that treatment of neurons with MAG results in upregulation of the protein levels of Rap1GAP. This observation provides us with a mechanism for MAG to inactivate Rap1: the increased Rap1GAP levels result in augmentation of Rap1's intrinsic GTPase activity, which leads to lower Rap1GTP levels.

This is not the first time that inhibitors of neurite outgrowth have been shown to negatively regulate Rap1 activity. EphA4, a tyrosine kinase receptor for the repulsive guidance cues Ephrins, is involved in axonal pathfinding, growth cone collapse, regulation of dendritic spine morphology (Murai et al., 2003), and has also been implicated in the CNS regeneration block (Goldshmit et al., 2004). Richter et. al., showed that Ephrin-A-dependent growth cone collapse required Rap1 inactivation. They also showed that the growth cone collapse effect of EphA4, is mediated by the spine associated RapGAP (SPAR) (Richter et al., 2007). EphA4 contains a PDZ-binding motif in its C terminus, and when stimulated by ephrins, it associates with the SPAR PDZ domain, resulting in inactivation of Rap1 and Rap2. So in this study, activation of a receptor implicated in lack of CNS regeneration resulted in Rap1 inactivation via recruitment of a Rap GAP to it. Interestingly, the myelin inhibitor ephrin-B3 also acts through the EphA4 RTK to cause inhibition (Benson et al., 2005).

In another recent study however, which examined MAG's involvement in neuronal survival, it was reported that Rap1 was activated by soluble MAG in rat P7 cerebellar neurons (Taniguchi et al., 2008). The authors found that this activation was p75-dependent, because they did nor observe Rap1 activation by MAG in cerebellar

neurons from $p75^{-/-}$ mice. Activation of Rap1 by MAG in this paradigm was shown to be required for neuronal survival stimulated by MAG, but at the same time the authors reported that the Rap1 activation they observed in response to MAG was not necessary for inhibition of neurite outgrowth. There are differences in the Rap1 activation assay protocols between our study and the Taniguchi study, which might account for the conflicting results. We performed the Rap1 assay according to the protocol provided in the commercial assay kit, and we use the lysis buffer provided. In the Taniguchi study however, they use a modified lysis buffer in the Rap1 activation assay. They use only 150 mM NaCl, which is significantly lower than the 0.5 mM that the commercial kit specifies for this assay. The lower salt concentration used in this study might be allowing non-specific binding of Rap1GDP to the Ral-GDS effector domain. Additionally, the investigators also added EDTA (1mM) to their lysis buffer, which is also not specified by the kit, and it is not reported in published reports of the Rap1 activation assay protocol. In fact, EDTA is a chelating agent, which is added in the Rap1 assay only in the positive and negative control lysates, in order to facilitate loading with GTPγS, or GDP. Magnesium present in the lysate inhibits nucleotide exchange by small GTPases, and thus stabilizes the binding of the GTP or GDP on the G-protein. Addition of EDTA results in chelation of the magnesium, which then allows for rapid nucleotide exchange by G-proteins. Therefore, addition of EDTA in the lysate might allow for loading of Rap1 with the endogenous GTP, which is normally present in about a ten-fold excess in cells, relative to GDP. The last difference between our study and the Taniguchi study is in the observed levels of Rap1 activation, relative to control. We noted that the Rap1GTP levels the authors observed in response to the commercial MAG-Fc in their

study are extremely high. Even though the fold-increase of Rap1GTP compared to the untreated control is not specified in their representative western blot, the levels seem several units of magnitude higher than the untreated control levels. We have never observed such high levels of Rap1 activation, even when we treat neurons with neurotrophin. In our experiments, we consistently observe a 2-3-fold increase of Rap1GTP levels, when we activated Rap1 with BDNF or the EPAC activator 8-CPT-2M-cAMP. Additionally, such modest increase in Rap1 GTP levels is what many other studies have previously reported in response to Rap1-activating agents, both in neurons and cell lines (McAvoy et al., 2009; Richter et al., 2007; Xie et al., 2005), and neither of these referenced studies uses EDTA in the Rap1 assay lysis buffer. However, in order to fully investigate the discrepancies between the Taniguchi study and our study, we need to repeat the Rap1 activation in response to MAG, using Taniguchi's modified Rap1 lysis buffer.

We are currently investigating the precise mechanism by which MAG increases the levels of Rap1GAP protein. Rap1GAP exists in two splice variants, Rap1GAPI and Rap1GAPII (also known as Rap1GAP1a and Rap1GAP1b) (Willard et al., 2007). Rap1GAPII protein levels have been demonstrated to be under proteasomal control in different paradigms (Tsygankova et al., 2004), in one of them through a pathway that involved interactions with $G\alpha_{i/O}$ subunits (Jordan et al., 2005). Rap1GAPII, which is the longer isoform, contains a full GoLoco motif in its amino terminus, whereas the shorter Rap1GAPI contains only a truncated GoLoco motif. This motif is a conserved 18-36 amino-acid sequence which allows proteins that contain it to interact with $G\alpha_i$- and/or G_O- protein subunits of heterotrimeric G proteins, preferentially when the latter are in the

inactive, GDP-bound form (Kimple et al., 2002). One possibility therefore is that upon MAG treatment, Rap1GAP levels are stabilized through a mechanism involving a $G\alpha_{i/O}$ interaction.

Infection of neurons with adenoviruses carrying either wild type Rap1, or a RapGAP insensitive Rap1 mutant was sufficient to overcome inhibition, when infected neurons were transferred to CHO MAG monolayer. In this experiment, the exogenous expression of Rap1 via adenoviruses is the only means of augmenting the cellular pool of Rap1GTP, and no additional stimulus is provided.

Using the Rap1 activation assay, we also found that dbcAMP activated Rap1, but unlike BDNF and 8-pCPT-2Me-cAMP, dbcAMP activation of Rap1was not inhibited by MAG. Even though, according to our hypothesis, MAG is activating RapGAPs, dbcAMP signaling was able to override MAG's attenuation of Rap1GTP levels. Therefore, this result suggested to us that dbcAMP both activates Rap1 and disables RapGAPs.

Because cAMP overcomes inhibition directly, it is possible that cAMP effector signaling inhibits the putative activation of Rap1GAPs by MAG. Indeed, searching into the literature, we have found examples of cAMP-mediated Rap1 regulation, which might help us explain our observation that Rap1 activation by dbcAMP cannot be blocked by MAG. cAMP elevation in cells can activate Rap1 through EPAC, but also activates PKA. It has been known for a long time that PKA phosphorylates Rap1 on serine residues in its carboxy-terminus (Altschuler and Lapetina, 1993; Rundell et al., 2004), but the effects of PKA phosphorylation on Rap1 activity are not well understood (Rundell et al., 2004). It has also been known that PKA phosphorylates Rap1GAP. In a recent

publication, Rap1GAP was identified as a major PKA substrate in the striatum, specifically on residues Ser-441 and Ser-499 (McAvoy et al., 2009). In the same study it was shown in striatal slices that treatment with the cAMP-elevating agent forskolin resulted in Rap1GAP phosphorylation on these residues, and this resulted in higher levels of active Rap1. The authors concluded that PKA phosphorylation of Rap1GAP leads to its inhibition, and thus it provides an alternative mode of control of Rap1 activity in these neurons. It is possible therefore in our paradigm that addition of dbcAMP causes Rap1 activation via EPAC, accompanied by a concomitant decrease in Rap1GAP's activity through PKA phosphorylation. Therefore, cAMP elevation would prevent Rap inactivation by Rap1GAP proteins activated by MAG.

Several of our observations fit in this model: MAG activates Rap1GAP, therefore inhibits Rap1 activation by BDNF and 8-pCPT-2Me-cAMP, resulting in the need to prime in order to overcome inhibition by these two agents. Even though BDNF is a cAMP-elevating agent, it cannot elevate cAMP in the presence of MAG because MAG activates RapGAPs. Rap1 activation by dbcAMP though cannot be inhibited by MAG because activated PKA inhibits Rap1GAP by phosphorylating it, as recently shown by others (McAvoy et al., 2009).

Even though Rap1 is activated in the cAMP pathway, we have yet to determine if Rap1 activation is necessary for the dbcAMP effect in overcoming inhibition. The observation that specific EPAC activation was sufficient to overcome inhibition by priming suggests that activation of Rap1 is an important component of the cAMP effect in blocking MAG inhibition. To determine if Rap1 is required for this effect though, we need to perform neurite outgrowth after priming with BDNF, or after direct addition of

dbcAMP, while at the same time knocking down Rap1. If Rap1 is necessary for the dbcAMP effect, blocking Rap1GAPs might be one of the reasons why dbcAMP overcomes inhibition directly, without the need to prime neurons with it. Therefore blocking Rap1GAPs might be a possible target of therapeutic interventions after CNS injury. Additionally, future experiments need to address whether knocking down Rap1GAPs will enable neurons to overcome inhibition by MAG, and also whether Rap1GAP knockdown will enable BDNF to overcome inhibition directly, without the need to prime.

We identified Rap1 as a signaling node of the neurotrophin pathway that is blocked by MAG, via MAG-upregulation of the protein levels of Rap1GAPs. We do not know yet if this MAG effect occurs via the two known receptors for myelin inhibitors, the NgR complex and PirB. If it is independent of them, it will indicate the existence of additional receptors for MAG to the ones known to date.

Our observation that MAG and myelin block BDNF signaling might be another manifestation of the emerging physiological role of the myelin inhibitors in limiting plasticity in the intact CNS, as proposed before by Strittmatter's group (McGee et al., 2005). Our data support the existence of a dual system for plasticity control, where neurotrophins stimulate neuronal plasticity and synapse formation, and inhibitors in myelin, like MAG, signal to limit it. Such a system might provide the nervous system with the means of temporally and spatially regulating plasticity, allowing limited amounts of it to occur at the synapses, where there is no myelin present, but also restricting it at places where growing or developing neurons contact myelin, therefore preventing aberrant sprouting. Additionally, besides the periaxonal myelin sheath, MAG has also

been shown to localize in non-myelin derived membranes of the axoglial apparatus at the nodes of Ranvier (Huang et al., 2005), together with OMgp and other factors. The presence of these proteins in the CNS nodal vicinity might serve to inhibit axonal sprouting from the nodes of Ranvier, a phenomenon that has been documented to occur at the PNS after injury.

Controlling neuronal process outgrowth and collateral sprouting in the CNS is an emerging physiological function of the myelin inhibitors, which presents a disadvantage in the case of a CNS injury. It is known that after an injury neurotrophins are upregulated in the CNS, but we show here that myelin inhibitors can interfere with their pathways. Our observations point to Rap1 inactivation by MAG as yet another target for therapeutic intervention in CNS injury, which might enhance the effects of neurotrophins on injured neurons.

Chapter IV: Materials and Methods

4.1 CHO Cell monolayers

4.1.1 Maintenance

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), 34.8 mM L-Proline (Sigma), 10 mM Glycine (Sigma), 300 nM Thymidine (Sigma), and 2 mM L-Glutamine (Gibco) at 37ºC in 7.3% CO_2. These cells were used as monolayer substrates in the neurite outgrowth assay (NOG), described below.

4.1.2 Monolayer Preparation

To prepare monolayers for the NOG assay, Permanox 8 well chamber slides (Lab-Tek) were coated with 20.0 µg/ml poly-L-lysine (Sigma) for 30 minutes at room temperature. After incubation, poly-L-lysine was removed and the slides were treated with 20 µg/ml fibronectin (Sigma) for at least 2 hours at 37ºC. Monolayers of control and MAG-expressing CHO cells (passage number 4-20) were plated in the individual chambers of an 8-well tissue culture slide (Lab-Tek) at the following concentrations: control-6 x 10^4 cells/well; MAG-5.5 x 10^4 cells/well. The slides were then incubated overnight at 37º C and grown to confluency, prior to the plating of primary neurons on top of the CHO-cell monolayers.

4.2 Preparation of myelin substrates

4.2.1 Myelin purification

The medulla from an adult rat brain is isolated and homogenized in a 0.25M sucrose solution containing a protease inhibitor cocktail (CalBiochem) using a glass and Teflon homogenizer. The homogenate is then mixed with a 2.55 M sucrose solution to create a 1.4 M solution. This is then layered onto a 1.9 M solution, followed by 0.85 M and 0.25 M solutions. The gradient is centrifuged at 40,000 rpm for 14 hours at 4ºC. Following centrifugation and separation, the extracted myelin is homogenized again in dH_2O and protease inhibitor, centrifuged at 14,000 rpm for 1 hour at 4ºC , resuspended in 10mM HEPES, and triturated using 18.5 and 26.5 gauge needles.

4.2.2 Immobilized myelin substrates

8-chamber Permanox slides were coated with 20.0 µg/ml poly-L-lysine for 30 minutes at room temperature. After incubation, PLL was removed and washed once with dH_2O. Myelin was then plated at various concentrations (1-4 µg/well) and then dried overnight in a vacuum chamber filled with Drierite dessicator. Slides were then used immediately.

4.3 Isolation of primary neurons

4.3.1 Cerebellar Neurons

To isolate cerebellar neurons, post-natal day 5-9 (P5-9) Long-Evans rats were sacrificed and the cerebellum was recovered in 6ml of 0.025% trypsin with 50 µg/ml DNaseI. The tissue was dissociated by trituration and incubation for 7-10 minutes at 37ºC / 7.3% CO_2. Trypsinization was stopped using media with 10% serum, after which the cells were

strained through a 40 µm cell-strainer, and pelleted by centrifugation. The isolated neurons were then resuspended to a single-cell suspension in a modified Sato medium (DMEM; Path-O-Cyte BSA; 20nM progesterone; 100mM putrescine; 30nM sodium selenite; 5 µg/ml insulin, 80ng/ml tri-iodo-thyronine (T3); 10ng/ml thyroxine (T4); 118 U/ml penicillin; 118 µg/ml streptomycin; 295 ng/ml amphotericin B), counted and plated.

4.3.2 Dorsal Root Ganglia neurons

To isolate dorsal root ganglia (DRG) neurons, Long-Evans rats of P5-7 were sacrificed and the DRG collected on ice into 4.5 ml of 0.15% Collagenase in L-15 media. The DRG were triturated gently and then incubated for 45 minutes at 37ºC. After incubation, 0.025% trypsin and 50 µg/ml DNaseI were added to the mixture, and the cells were incubated a further 10 minutes at 37ºC. In order to dissociate any remaining clusters, the cells were triturated gently in the trypsin solution. Trypsinization was halted with media containing 10% serum. Dissociated DRG were then washed once with DMEM and resuspended in Sato media, counted and plated onto either 24-well dishes or 8 chamber slides.

4.3.3 Gradient purification of Hippocampal and Cortical neurons

To isolate hippocampal or cortical neurons, Long-Evans rats of P0-P2 were sacrificed, and the hippocampi or cotices were collected on ice in plain neurobasal media. Papain was added to the collection media at a final concentration of 0.5 mg/ml, and tissues were incubated at 37ºC for 20 minutes. After incubation, media was aspirated, and then the procedure was repeated once more. After incubation, papain was inhibited by the

addition of soybean trypsin inhibitor. After 2 minutes, media was aspirated, and the tissue was washed 2 times with plain neurobasal media. Plain neurobasal was added again, and tissue was triturated and passed through a 40 µm cell strainer. 6 ml of this single cell suspension were then loaded on top of a gradient, consisting of four layers of Optiprep™ working solution (30%w/v iodixanol, 0.425% NaCl, 5 mM MOPS-NaOH, pH 7.4) of densities 1.057, 1.043, 1.036 and 1.029 g/ml (listed from bottom of the tube to top of the tube). Neurons were then centrifuged at1900 rpm for 15 minutes at room temperature. Fractions containing enriched populations of neurons were isolated according to the directions of the Optiprep™ Application Sheets, diluted with plain neurobasal, and pelleted by centrifugation. The pellet containing dissociated neurons was then resuspended in neurobasal media, and neurons were counted and plated.

4.4 Priming neurons with neurotrophins and 8CPT-2Me.

24-well culture dishes were coated with 100 µg/ml of poly-L-lysine for at least 30 minutes at room temperature. The wells were then washed once with DMEM to remove excess PLL. Isolated cerebellar neurons were plated onto these dishes at a concentration of approximately 1 x 10^6 cells/well. These neurons were then treated with either BDNF (200 ng/ml), and 8CPT-2OMe (0.5 and 1 mM). The neurons were then cultured overnight at 37º C after which they were removed from the dish via trypsinization (0.4x trypsin for 10 minutes at 37ºC). Trypsinization was stopped by 10% serum-containing media and the cells were collected, resuspended in fresh Sato, counted and plated onto either a purified myelin substrate or MAG-expressing CHO cells.

4.5 Neurite outgrowth assay

Following dissociation, noted above, primary neurons were plated onto the CHO cell monolayers or purified myelin substrate at a cell density of 1.5 x 10^4 cells per well (8 chamber slide) for cerebellar neurons and 0.75-1 x 10^4cells per well for DRG neurons. This co-culture was then incubated for 18-20 hours at 37ºC. After incubation, the cultures were then fixed with 4% paraformaldehyde for 30 minutes at room temperature and permeabilized with cold 100% methanol for 2 minutes. The cells were then blocked against non-specific binding with DMEM containing 10% serum for a minimum of 20 minutes at room temperature. The neurons were then stained for the neuronal-specific marker β-tubulin-III as follows: After blocking, the cultures were incubated overnight at 4ºC in a PBS-BSA (0.05%) solution containing a monoclonal anti-βIII tubulin antibody (Covance) at a dilution of 1:1000. After this incubation, the cultures were washed 3 times with PBS, and then incubated at room temperature for 30 minutes in a PBS-BSA (0.05%) solution containing biotinylated donkey anti-mouse IgG at a 1:500 dilution. After this second incubation, the cultures were once again washed 3 times with PBS and further incubated 30 minutes at room temperature in a PBS-BSA solution containing Streptavidin-Texas Red at 1:500. Finally, the cultures were washed 3 more times with PBS and then immobilized using Permafluor mounting media (Immunon) and viewed under a fluorescent microscope. In order to quantify the neurite outgrowth length from these treated neurons, the immunostained cultures were observed under a fluorescent microscope and the neurite length was measured using the Simple PCI or Metamorph image quantification software. Briefly, the longest neurite from each β-tubulinIII positive neuron (100-150 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 GraphPrism software program.

4.6 Rap1 activation assay

The assay was performed using the protocol and reagents of the commercially available Rap1 Activation Assay Kit (Millipore, #17-321), which detects the active, GTP-bound form of Rap1 by exploiting the differential affinity of Rap1GTP and RAP1GDP for the Rap binding domain of its downstream effector RalGDS (Ral Guanine- nucleotide Dissociation Stimulator). Briefly, primary neurons are isolated and cultured overnight (25-40 x 10^6 cells per plate, covered with 100 µg PLL) as noted above, and double plates are used for each treatment. The next day cells are starved, stimulated with indicated agents, and subsequently lysed in lysis buffer (100mM Tris-HCl, pH 7.4, 1 M NaCl, 2% NP-40, 5 mM $MgCl_2$,10% glycerol, PMSF (0.1M), protease and phosphatase inhibitor cocktail (1X, CalBiochem)) . Activated Rap1 (Rap1GTP) is precipitated by incubating the lysate with Ral GDS-RBD (RalGDS Rap Binding Domain) glutathione-agarose beads for 45 minutes. The beads are then collected by centrifugation, and the affinity precipitate is washed three times with washing buffer, and eluted with Laemmli sample buffer. Subsequently, eluted proteins are resolved by SDS-PAGE in a 15% gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. Affinity-purified activated Rap1 was detected after overnight incubation at 4º with rabbit polyclonal anti-Rap1 antibody (1:500-1:1000, Millipore, #07-916), followed by incubation with the secondary

antibody anti-rabbit IgG conjugated to horseradish peroxidase (1:2000, Cell Signaling #7074).

4.7 Western blotting for Rap1GAP

Primary gradient-purified rat cortical neurons (P0-P3) were treated with soluble MAG (MAGFc), or myelin for the indicated intervals, and then mechanically disrupted by ~50 strokes with a hand-driven Dounce homogenizer in homogenization buffer (0.25 M Sucrose, 10 mM Tris pH 8.00, 1 mM $MgCl_2$, 1 mM EDTA, with protease and phosphatase inhibitors. Detergent (1% NP-40) and DNaseI (0.05 µg/µl) were then added to the homogenate, and Rap1GAP levels were detected by western blotting, using anti-Rap1GAP antibody (1:2000, Upstate/Millipore, #04-413). The blot was then stripped and re-probed for the neuronal marker β–TBIII (1:30,000).

4.8 Subcellular fractionation

Subcellular fractionation were performed for the most part as described in (Samuels et al., 2007). Primary gradient purified rat cortical neurons (P1-P3) were treated with MAG-Fc(20µg/ml), and then mechanically disrupted by ~50 strokes with a hand-driven Dounce homogenizer in homogenization buffer (0.25 M Sucrose, 10 mM Tris pH 8.00, 1 mM $MgCl_2$, 1 mM EDTA, with protease and phosphatase inhibitors). Lysates were spun at 800xg for 10 min at 4º, and the resulting pellet was resuspended in lysis solution as "nuclear". The supernatant was re-spun at 800xg and the resulting pellet, "*nuclear*" was

combined with "*nuclear*". Supernatant was then spun at 5,000xg for 15 min at 4ºC, resulting in "mitochondrial". Supernatant was then spun at 100,000xg for one hour at 4ºC, resulting in the "*membrane*" fraction. The final supernatant was the cytosolic fraction. Prior to running on SDS-PAGE, the "*nuclear+nuclear*" and the "*mitochondrial*" fractions were combined and treated with DNaseI. Fractions were run in an 8% gel, and the blot were probed with anti-Rap1GAP, and then stipped and probed for the cytosolic protein lactate dehydrogenase (abcam, ab52488).

4.9 Adenoviral infection of neuronal cultures

The Rap1 expression constructs of Rap1 wt and Rap1F64A used in the construction of adenoviral vectors were kindly provided from the laboratory of Dr. Oliver Daumke, and the Rap1GAPI construct was generously provided by Dr. P.J. Casey, Duke University Medical Center). Recombinant adenovirus construction was carried out by Dr. W. Mellado and K. Etesami, using a protocol adapted from He et al., (1998). Briefly, each construct was excised from the host plasmid, and the fragments were subcloned into pTRACK CMV, and then inserted by homologous recombination into pAdeasy-1. The viral preparation and purification was carried out as previously described (He et al., 1998). Primary neurons were isolated and plated on poly-L-lysine coated 24-well plates. Neurons were infected maintained in virus-containing media for 2 hrs, after which they were washed and the media replaced. After overnight culture to allow for transgene expression, neurons were transferred directly to monolayers of CHO cells or myelin, and incubated as described above. Neurite length was measured only for those neurons that were both GAP43 and GFP positive, so that only infected neurons were assessed.

4.10 Assessment of cell viability

Dissociated hippocampal neurons were plated in a 24-well slide covered with 50 µg/well poly-L-lysine, and the next day they were infected with adenoviruses-GFP carrying Rap1wt, Rap1F64, Rap1GAPI, as well as control virus. After 24 hour incubation, cells were transferred to PLL + FN covered 8-well slides (20,000/well), and were incubated again overnight. The next day, cells were incubated with EthD and Calcein blue AM for 30 min, after which the staining solution was removed, and then slides were mounted with Aqua Poly-Mount and with a glass cover. Next, cells were visualized with fluorescence microscopy. EthD-1 (Ethidium homodimer-1) cannot permeate the intact plasma membrane of live cells, but can pass through the damaged membrane of dead cells and bind to nucleic acids, undergoing a multi-fold enhancement of fluorescence to produce a bright red color. The cell-permeant esterase substrate Calcein blue AM is a cell-permeant esterase substrate that, upon cleavage, is converted into a blue fluorescent product that is retained by live cells with intact plasma membranes, and therefore acts as a marker for viability.

Reagents

8-pCPT-2'-Me-cAMP (8-CPT-OMe), (Sigma-Aldrich, #C8988), BDNF (Sigma-Aldrich, B-3795**,** Adenosine3',5'-cyclic Monophosphate, N^6**,**$O^{2'}$ **–**Dibutyryl-, Sodium Salt (dbcAMP), (CALBIOCHEM, #28745).

Chapter V: References

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