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INHIBITION OF PHOSPHODIESTERASE4'S IN CULTURE OR *IN VIVO* OVERCOMES INHIBITION OF REGENERATION BY MAG AND MYELIN.

by

Elena Nikulina

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Abstract

INHIBITION OF PHOSPHODIESTERASE4'S IN CULTURE OR *IN VIVO* OVERCOMES INHIBITION OF REGENERATION BY MAG AND MYELIN.
by

Elena Nikulina

Adviser: Professor Marie T. Filbin

Axons of the adult mammalian central nervous system (CNS) do not regenerate after injury. Previously, we showed that elevation of cAMP, either via db-cAMP or by priming neurons with BDNF prior to exposure to myelin, blocks inhibition of axonal regeneration by myelin associated glycoprotein (MAG) and myelin. Here, we assess the effects of elevating cAMP in neurons using rolipram, a specific inhibitor of phosphodiesterase4's (PDE4) (the enzymes that hydrolyze cAMP) on the ability of the neurons to grow in the presence of MAG and myelin. First, rolipram was added directly to the media of neurons growing on MAG, which partially overcame the inhibition by MAG. However, prior incubation of neurons with rolipram before exposure to MAG or myelin resulted in a complete block of inhibition by MAG and myelin. Second, we administered rolipram subcutaneously to animals prior to isolation of neurons used in the neurite outgrowth assay. We report here that after continuous, subcutaneous delivery of rolipram for one day with osmotic minipumps, DRG neurons were not inhibited by MAG or myelin when grown in culture, this block of inhibition was abrogated by inhibitors of protein kinase A (PKA) and transcription. After two days of rolipram treatment, growth of DRG neurons, both in presence and absence of inhibitors, was improved. This improved growth was PKA-independent and transcription-independent. This study reveals the potential

therapeutic value of specific inhibitors of PDE4 in encouraging axonal regeneration after injury.

Additionally, we report that downregulation of PDE4 activity can account for the elevation of cAMP levels in the course of priming neurons with BDNF. Total PDE activity in neurons decreases sharply after addition of BDNF. This decrease in PDE activity, specifically PDE4s, is ERK-dependent. Subsequent elevation of cAMP levels is dependent on activation of ERK. To our knowledge, this is the first time that ERK-dependent inactivation of PDE4 activity and subsequent elevation of cAMP levels have been demonstrated in primary neuronal cultures in response to BDNF.

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Abbreviations

AC	adenylyl cyclase
AP	alkaline phosphatase
Arg	arginase
ATF-3	activating transcription factor 3
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic AMP
cGMP	cyclic GMP
CHO	Chinese hamster ovary
CNS	central nervous system
CREB	cAMP-binding protein
db-cAMP	dibutyryl cAMP
DMEM	Dulbecco's modified Eagle's medium
DRB	5,6-dichloro-1-b-D-ribofuraanosylbenzimidazole
DRG	dorsal root ganglion
EGF	epidermal growth factor
ERK	extracellular-signal-regulated kinase
GAP	GTPase-activating protein
GAP-43	growth associated protein-43
GDNF	glial-derived neurotrophic factor
GEF	guanine nucleotide exchange factor

GPI	gkycosylphosphatitylinositol
Ig	immunoglobin
IL-6	interleukin-6
JAK	Janus tyrosine kinase
KIM	kinase interaction motif
LTP	long-term potentiation
MAG	myelin-associated protein
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MEKK	MEK kinase
MS	multiple sclerosis
NGF	nerve growth factor
NgR	Nogo receptor
NT	neurotrophin
OMgp	oligodendrocyte myelin glycoprotein
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PC12	phaeochromocytoma cell line
PDE	phosphodiesterase
PI-PLC	phosphatidylinosytol-specific phospholipase C
PKA	protein kinase A
PNS	peripheral nervous system
RTK	receptor tyrosine kinase

SDS	sodium dodecyl sulfate
Trk	tropomyosin-related kinase
ucr	upstream conserved region

Chapter I.

Introduction.

1. 1 Regeneration in the adult mammalian central nervous system.

The adult mammalian central nervous system (CNS) does not regenerate after injury. The original dogma was that adult neurons had lost their intrinsic ability to regenerate, however, several studies performed in the last two decades have disproved this theory. It was demonstrated that when a peripheral nerve segment was used as a "bridge", CNS neurons extended axons through the "bridge" for long distances (David and Aguayo, 1981). Function was not restored, since axons did not grow into the host CNS. When a permissive environment was provided, by grafting pieces of embryonic spinal cord into bisected, lower thoracic spinal cord of neonatal cats, neurons extended the axons into the permissive tissue of grafts, but not beyond into the injured CNS (Howland et al., 1995). This strongly suggested that the injured adult CNS environment inhibits axonal regeneration (Berry, 1982). Studies were then carried out in order to establish whether there are components of the CNS that actively inhibit growth of axons *in vitro* and it was confirmed that dissociated neurons grew only through explants of sciatic nerve (part of peripheral nervous system (PNS)), but failed to grow into optic nerve explants (CNS) (Schwab and Thoenen, 1985). It was reported by Crutcher and colleagues that explants of lumbar sympathetic ganglia, from embryonic chicks, grew on gray matter regions of fresh-frozen sections of mature rat brain and spinal cord; whereas major white matter tracts showed little or no support for neurite growth. These results are consistent with the presence of growth-inhibiting factors associated with CNS white matter (Crutcher, 1989), whilst further studies revealed that inhibitory properties of CNS myelin are associated with differentiated oligodendrocytes (Schwab and Caroni, 1988). Treatment of purified CNS myelin in culture with proteases abolished its inhibition of axonal growth (Caroni and Schwab, 1988b). Hence, it was

concluded that proteins associated with membranes of oligodendrocytes conferred their inhibitory properties upon them. Size fractionation of myelin proteins by SDS-PAGE revealed two highly nonpermissive, minor protein fractions of molecular weight 35 and 250-kD. Supplementation of permissive membrane protein fractions, such as liver, with small amounts of 35- or of 250-kD CNS myelin protein was sufficient to generate highly nonpermissive substrates. A monoclonal antibody was raised against these proteins and termed IN-1, which bound both to the 35 kd and 250 kd inhibitors and to the surface of differentiated cultured oligodendrocytes. (Caroni and Schwab, 1988a). Adsorption of CNS myelin in culture with IN-1 markedly reduced neurite outgrowth inhibition and injection of IN-1 into optic nerve explants allowed axons of co-cultured sensory and sympathetic neurons to grow into the explant. These experiments demonstrate that the nonpermissive properties of CNS myelin are associated with the inhibitory proteins expressed in myelin.

Another obstacle preventing regeneration, after injury to the CNS, is glal scar formation, which consists, predominantly, of reactive astrocytes (astrocytes that have changed in response to the CNS injury) and connective tissue elements. In the early 1950's it was considered to be an impenetrable, mechanical barrier to regenerating axons. Indeed, the structure of astrocytic scar tissue is a mass of tightly bound processes, and it has long been proposed that astrocytic scars might be inhibitory because it is physically impossible for axons to penetrate them. Recent studies have also demonstrated that reactive astrocytes synthesize molecules that are inhibitory to the growth of axons (McKeon et al., 1995)); the most intensely studied of which are tenascins (tenascin gene family of extracellular matrix glycoproteins) and proteoglycans (McKeon et al., 1991, Fitch and Silver, 1997). Hence, the glial scar appears to be both a mechanical barrier and a source of molecules that inhibit

growth of regenerating axons. Therefore, it can be concluded that the glial scar and myelin debris are the main factors that impede axonal regeneration after injury.

Developing strategies to promote regeneration in the CNS can be classified into three approaches, as follows: neutralizing myelin inhibitors of axonal growth, preventing the glial scar from forming, and changing the intrinsic state of neurons, so that they do not respond to myelin inhibitors.

There are three inhibitory molecules of the adult injured CNS identified to date. They are myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo (Spillmann et al., 1998; (Chen et al., 2000) and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002). Surprisingly, they were found to share at least one receptor complex.

1.1 Inhibitors of neurite outgrowth associated with myelin:

MAG

Myelin-associated glycoprotein (MAG) is a member of the SIGLEC (sialic acid-dependent immunoglobulin-like family member lectin) family; it is expressed by Schwann cells in the peripheral nervous system and by oligodendrocytes in the CNS. The extracellular portion of MAG contains five Ig-like domains. MAG is expressed very early during myelination (Willson et al., 1987), is localized at the inner rim of myelin sheaths (Stenberger et al., 1979; Trapp and Quarles, 1984) and in the paranodal regions of myelin sheaths (Trapp and Quarles, 1984; Martini and Schachner, 1986). It was proposed that MAG is involved in the initiation of myelination and in maintaining the axon-glia junction (Trapp et al., 1984). Interestingly, studies in MAG deficient mice initially did not detect any abnormalities in myelin formation (Li et al., 1994), however, older MAG -/- mice suffered from extensive

demyelination in the PNS, along with axonal degeneration (Fruttiger et al., 1995). Ultra structural analysis of CNS myelin from 8-month-old mice displayed symptoms similar to those described before as "dying-back oligodendrogliopathy" in diseases of toxic or immune-mediated demyelination, such as multiple sclerosis (Lassmann et al., 1997) .

Although MAG was initially identified as a promoter of axonal regeneration from neonatal dorsal root ganglion (DRG) neurons (Johnson et al., 1989), it was demonstrated later that MAG is a bifunctional molecule: depending on the age of the neurons, MAG either promotes or inhibits axonal growth (DeBellard et al., 1996; Mukhopadhyay et al., 1994). Axonal outgrowth from embryonic neurons is promoted by MAG. Axonal growth from postnatal cerebellar, hippocampal, superior cervical ganglion and spinal neurons is inhibited by MAG. Postnatal DRG neurons are promoted by MAG initially, but then sharply switch their response to MAG from promotion to inhibition at postnatal day 3 (Mukhopadhyay et al., 1994, McKerracher et al., 1994; DeBellard et al., 1996). It was also demonstrated that the proteolytic fragment of MAG, d-MAG, that consists of the entire extracellular domain of MAG is released from damaged CNS and inhibits neurite outgrowth (Tang et al., 1997).

Nogo

A monoclonal antibody, IN-1, was raised against one of the inhibitory components of myelin (Caroni and Schwab, 1988). It took ten years to identify and characterize the identity of the molecules recognized by the IN-1 antibody. As a result of a study performed by Schwab and colleagues, six partial-peptide sequences of a bovine inhibitory molecule, which is recognized by IN-1, were published (Spillmann et al., 1998). Two years later three groups independently identified a gene, designated Nogo, which encoded an inhibitory myelin protein (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

Nogo is a member of the reticulon family of proteins (GrandPre et al., 2000). The original member of this family, Reticulon1, is expressed exclusively in neuro-endocrine cells and when exogenously expressed in kidney cells, is localized in the endoplasmic reticulum (van de Velde et al., 1994). All known members of the reticulon family of proteins are localized in the endoplasmic reticulum, hence the name. All proteins of the reticulon family share a conserved 200 amino-acid residue region at the C-terminus that contains two hydrophobic domains, separated by a 66–residue hydrophilic segment (Roebroek et al., 1996). The three isoforms of Nogo discovered were designated Nogo-A, -B and –C. Nogo-A is a full-length protein and it has the strongest inhibitory activity in culture; it is localized in CNS myelin and is expressed by oligodendrocytes but not by Schwann cells. Nogo-B and Nogo-C are found in certain neurons and several non-neural tissues (Chen et al., 2000). Both the amino-terminus and C-terminus of Nogo-A are believed to be cytosolic and the intervening sequence of 66 amino acids, termed Nogo66, is proposed to be localized at the cell surface. Both the long amino-terminal region (Chen et al., 2000; Prinjha et al., 2000) and Nogo-66 (GrandPre et al., 2000) are inhibitory for neurite outgrowth. How the cytosolic portion of Nogo-A, which is not predicted to have any contact with neurons, could be inhibitory remains a controversial issue. It may be exposed after injury or Nogo-A may have more than one configuration in the membrane. Nogo-A was demonstrated to cause both growth cone collapse and inhibition of axonal outgrowth in vitro.

Oligodendrocyte myelin glycoprotein (OMgp).

Oligodendrocyte myelin glycoprotein, a 440 amino acid protein, is glycosyl-phosphatidyl-inositol (GPI)-linked to the cell membrane in myelinating oligodendrocytes and

is localized to the glial-axonal interface of myelinated axons (Habib et al., 1998; Mikol et al., 1993). When purified myelin was treated with phosphatidylinosytol-specific phospholipase C (PI-PLC), it was found that PI-PLC released proteins exhibited growth cone collapse activity. The protein responsible for that was identified as OMgp, which was found to induce the collapse of chick embryonic DRG growth cones and inhibited neurite outgrowth of rat cerebellar granule neurons from rats post natal day 7 (He et al., 2002; Wang et al., 2002b). It was reported recently that the protein fraction from bovine brain myelin (previously called arretin) contains predominantly OMgp and it has been confirmed that it strongly inhibits neurite outgrowth from different types of neurons (Kottis et al., 2002).

Nogo, MAG and OMgp share the same receptor.

Identification of the receptor is a crucial step in understanding the mechanisns of action of the signaling molecules and cascades of cellular events that are initiated by these molecules. To identify the receptor of Nogo, Strittmatter's group used an alkaline phosphatase (AP) fusion protein approach (Flanagan and Cheng, 2000). Nogo-66-placental alkaline phosphatase fusion protein (AP-Nogo-66) was constructed. It has been shown that AP-Nogo-66 binds to chick embryonic DRG neurons and induced growth cone collapse. When AP-Nogo-66 was used to screen a mouse brain cDNA expression library, only a few clones showed strong AP-Nogo-66 binding. Two independently isolated clones were almost identical to one another except for a 100 base pair extension of the 5'-untranslated region of one clone (Fournier et al., 2001). These two clones contained cDNA coding for a 473 amino acids protein and further analysis identified this protein as a receptor that mediates Nogo-66 inhibition of axonal outgrowth. Nogo-66 receptor (NgR) is a brain-specific, GPI-linked protein. It was demonstrated that NgR specifically binds to Nogo-66 and expression of the

NgR protein was sufficient to convert axonal growth cones of embryonic day 7 chick DRG neurons from Nogo-66 insensitive to Nogo-66 sensitive state, causing growth cone collapse.

Importantly, De Bellard and Filbin showed that binding of MAG to neurons occurred in a GPI-linked protein-dependent manner (unpublished results). Moreover it was demonstrated that a soluble, chimeric form of MAG, consisting of the extracellular domain of MAG fused to the Fc region of human IgG (MAG-Fc), specifically co-immunoprecipitates a number of surface proteins from different kinds of neurons. From a western blot of proteins co-immunoprecipitated with MAG-Fc, a band of 85 kD was clearly distinguished (De Bellard and Filbin, 1999), the molecular weight of which corresponds to the size of NgR. These studies started a series of experiments to prove that NgR is the functional receptor for MAG. In two papers published simultaneously, we and Strittmatter's group demonstrated that MAG and Nogo-66 share the same receptor (Domeniconi et al., 2002; Liu et al., 2002). In order to prove that NgR is a functional receptor for MAG, it had to be demonstrated that these two proteins bind to each other, and that by blocking each of these proteins with antibody the inhibition can be blocked. It was shown by Domeniconi and colleagues that MAG-Fc binds specifically to NgR expressing Chinese hamster ovary (CHO) cells in a GPI-linked protein dependent manner and conversely, NgR-AP binds specifically to MAG-expressing CHO cells. Furthermore, NgR antibody blocks inhibition of neurite outgrowth by MAG. When soluble NgR-AP was added to neurons growing on MAG, it blocked inhibition of neurite outgrowth by MAG in a dose-dependent manner. It was also demonstrated that MAG and Nogo-66 compete for binding to NgR (Domeniconi et al., 2002; Liu et al., 2002). Strittmatter's group sought to identify an associated transmembrane co-receptor component for NgR since NgR lacks an intracellular component. For this study soluble NgR-AP was

created and was used to screen an adult mouse brain cDNA expression library. Six out of ten positive pools contained the cDNA to the NgR itself. This possibly indicates strong cell-cell interaction between cells expressing NgR. It would, therefore be interesting to investigate if NgR-NgR interaction plays a role in the fasciculation of axons. One of the other clones contained the cDNA encoding MAG and further analysis confirmed that MAG binds with high affinity to NgR in a GPI- dependant manner. Chick, embryonic day 7 DRGs do not express NgR endogenously and they are insensitive to MAG and Nogo. When NgR was expressed in these neurons exogenously, both Nogo-66 and MAG induced growth cone collapse. Strittmatter's group reported that excess Nogo-66 does not interfere with MAG binding to NgR. In addition, it was demonstrated that peptide NEP1-40, derived from the amino-terminal fragment of Nogo-66 and previously demonstrated to block inhibitory effects of Nogo-66, does not affect the inhibitory action of MAG. It was concluded that MAG and Nogo-66 possess separate binding sites within the same domain of NgR (Liu et al., 2002). That last conclusion contradicts the results of our laboratory (Domeniconi et al., 2002) which strongly suggested that MAG and Nogo-66 compete for the same site or closely positioned sites and the matter requires further investigation.

It is particularly interesting that OMgp was also demonstrated to be a NgR ligand (Wang et al., 2002b). The finding that all three myelin inhibitors identified to date share the same receptor is consistent with the results previously published by Filbin's group; namely, that all treatments of neurons in vitro that overcome inhibition of axonal growth by MAG also overcome inhibition of axonal growth by myelin. There are still a lot of questions that remain to be answered: one of the most important is the identity of the co-receptor for NgR.

NgR is a GPI-linked protein, which does not span the plasma membrane. Hence, to transmit the signal it requires a transducing molecule or receptor complex that has trans-membrane and intracellular components. Several studies published recently, as well as unpublished data from our lab, identified the p75 neurotrophin receptor as the transducing partner of NgR for MAG, Nogo and OMgp signaling. Yamashita and colleagues reported that p75 is a signal transducing element for MAG (Yamashita et al., 2002). It was demonstrated in this study that DRG and cerebellar neurons from mice carrying a mutation in the p75 gene were not inhibited by MAG-Fc in culture. Furthermore, it was shown that MAG and p75 colocalized when MAG-Fc was added to neurons in culture. More recently, after NgR was found to be a receptor for MAG, two papers were published demonstrating that p75 binds NgR and that it is p75 that transduces the inhibitory effects of MAG, Nogo and OMgp. In the study of He and coworkers, it was shown that p75 and NgR could be immunoprecipitated together from both CHO cells when expressed exogenously and from rat cerebellar neurons (Wang et al., 2002a). Addition of MAG-Fc enhances the formation of the p75-NgR complex. Furthermore, fusion proteins consisting of the extracellular domain of p75 and alkaline phosphatase (p75-AP) specifically bind CHO cells expressing NgR. Importantly, it was also demonstrated that neurite outgrowth from DRG neurons, isolated from p75 knockout mice, were insensitive to the inhibition by Nogo, MAG, OMgp and purified myelin. When a fusion protein consisting of the extracellular portion of p75 and Fc region of human IgG (p75-Fc) was added to neurons, together with the myelin-associated inhibitors, neurite outgrowth inhibition by MAG, Nogo, OMgp and myelin was abrogated. When a truncated form of p75 lacking the intracellular domain was introduced into rat cerebellar neurons, the effect of myelin inhibitors was again abrogated. The authors concluded that p75 forms a receptor

complex with NgR to mediate the inhibitory effects of MAG, Nogo and OMgp. In the study published by Mu-ming Poo and coworkers, the role of p75 as the transdusing partner of NgR for MAG signaling was further confirmed (Wong et al., 2002). It was shown that an antibody against p75 abolished MAG- induced, repulsive turning of Xenopus axonal growth cones and disrupted co-immunoprecipitation of NgR and p75. Interestingly, immunohistochemical studies performed by this group revealed that p75 colocalizes with NgR, in rat embryonic day 14 neurons. At this developmental stage, our lab and others have found that axonal growth is not inhibited by MAG but is, in fact, promoted (DeBellard et al., 1996; Johnson et al., 1989). Further investigation is required to establish if the same NgR-p75 receptor complex is required to mediate both the inhibitory and growth-promoting effects of MAG.

Blocking inhibitory molecules of myelin improves regeneration in the CNS.

Since it was recognized that myelin of the adult, mammalian CNS inhibited axonal regeneration (Berry, 1982), several studies were performed in order to neutralize the inhibitors of myelin to promote regeneration in the injured CNS. In the pioneering work of Caroni and Schwab (Caroni and Schwab, 1988), the monoclonal antibody IN-1, which recognizes one of the inhibitory components of myelin, promotes regeneration when applied *in vivo* to a corticospinal lesion (Bregman et al., 1995; Schnell and Schwab, 1990; Schnell and Schwab, 1993). Subsequently, when the IN-1 antigen was identified as Nogo-A, a different approach was taken to promote regeneration by interfering with Nogo signaling. A competitive, antagonist peptide for NgR, derived from amino-terminal peptide fragments of Nogo-66, was characterized. The Nogo-66(1–40) antagonist peptide (NEP1–40) when administered to rats with spinal cord hemisection, promoted regeneration of the corticospinal tract and improved functional recovery (GrandPre et al., 2002).

In another study, in order to block all the inhibitors of CNS myelin, not just Nogo, mice were immunized with a CNS myelin preparation to promote the production of antibodies against all myelin components (Huang et al., 1999). In these myelin-immunized mice a large number of axons of the corticospinal tract were reported to regenerate after spinal cord lesion. In this study about 10 to 15% of the neurons regenerated, which is higher than the 5% of neurons regenerating in the studies with IN-1 antibody. Functional recovery was also detected in myelin-immunized mice after spinal cord lesion. For some reason only 54% of immunized mice had regenerating neurons and 58% had evidence of functional recovery. The authors suggest that the effect of prior immunization depended on the individual immune response of the animals.

One of the most controversial issues in the field of CNS regeneration is whether it is the myelin-associated inhibitors or proteoglycans of the glial scar that are the main obstacles to axonal regrowth after injury. The experiments of Schwab's and David's groups discussed above, demonstrate that blocking myelin inhibitors immediately after injury promotes regeneration in the CNS. Indeed, the glial scar takes some time to form after injury, but in contrast, myelin-associated inhibitors of axonal growth are present at the lesion site at the time of the injury. Moreover, it was demonstrated that MAG is released from damaged myelin, causing blockade of axonal regeneration (Tang et al., 1997). Therefore, the main obstacles to regeneration, immediately following injury, are inhibitors of axonal growth present in myelin.

1.2 The intrinsic growth state of neurons; role of cAMP.

Recently, the intrinsic response of neurons to myelin inhibitors has been the focus of several research groups (Broude et al., 1999; Cai et al., 2001; Neumann et al., 2002; Neumann

and Woolf, 1999). It is well-established that the embryonic CNS will spontaneously regenerate in vivo (Bates and Stelzner, 1993; Hasan et al., 1993). Embryonic neurons are not inhibited by myelin in culture (Shewan et al., 1995) and can extend long axons when transplanted into the adult CNS (Davies et al., 1994; Li and Raisman, 1993). In the study published by Filbin's group, the molecular mechanism that underlies the different responses of embryonic and adult neurons to the myelin inhibitors was characterized (Cai et al., 2001). It was demonstrated that endogenous levels of cAMP are higher in young neurons where axonal growth is not inhibited by myelin and MAG. Moreover, spontaneous regeneration in injured neonatal rat spinal cord *in vivo* was reduced by inhibition of PKA, the downstream effector of cAMP. These results imply that the developmental loss in the ability of axons to spontaneously regenerate is mediated by a decrease in endogenous neuronal cAMP levels. The role of endogenous cAMP levels was studied in a model of growth cone response to various guidance cues (Ming et al., 1997) and it has been shown that elevated cAMP changed the growth cone response to MAG from repulsion to attraction (Song et al., 1998). Recently, studies on the developmental switch in response of retinal axons to netrin-1, from attraction to repulsion, demonstrated that an intrinsic decrease in neuronal cAMP levels also underlies this switch (Shewan et al., 2002). The conclusion is that high cAMP levels determine the ability of young neurons to be unresponsive to inhibitors of axonal outgrowth associated with myelin and to allow regeneration after injury.

Studies performed by Filbin's group over the years, as well as the experiments of Woolf (Neumann and Woolf, 1999) and of Bregman (Broude et al., 1999), have suggested that the intrinsic state of neurons of the adult CNS can be changed such that they do not respond to inhibitors of myelin and can therefore regenerate. In the work of Filbin and

coworkers, it was demonstrated that the ability of neurons to regenerate is correlated with elevated levels of the second messenger, cAMP.

1.2.1 Fetal spinal cord transplants and exogenous neurotrophic support promotes regeneration after spinal cord injury.

In experiments carried out by Bregman's group, a piece of embryonic spinal cord tissue was grafted into injured, adult rat spinal cord. Neurotrophins, brain derived neurotrophic factor (BDNF) or neurotrophic factor- 3 (NT-3), were pumped into the graft (Broude et al., 1999). Extensive growth of regenerating axons beyond the lesion site was reported (Bregman. 1998). It was noted by Filbin that in these experiments growing axons were exposed to neurotrophins before they encountered the inhibitory molecules of the injured adult CNS. In the studies of Filbin's group, it was then shown that cerebellar or DRG neurons treated with BDNF or NT-3 in vitro for 6-18 hours (termed, priming with neurotrophins) before exposure to inhibitors of axonal outgrowth associated with myelin, are subsequently capable of growing in the presence of myelin inhibitors. It was found that levels of cAMP in neurons were elevated after priming with neurotrophins, and inhibitors of protein kinase A (PKA) blocked the priming effect of neurotrophins in overcoming inhibition by MAG and myelin (Cai et al., 1999).

1.2.2. The conditioning lesion effect - regeneration of dorsal column fibers after spinal cord injury and the role of cAMP.

Primary sensory neuronal cell bodies reside in the dorsal root ganglion and they have two branches, one of which extends to the PNS and spontaneously regenerates after injury. A second axon enters the CNS and does not regenerate after injury. It was shown that if the peripheral branch of DRG was lesioned one or two weeks prior to a central lesion, termed a conditioning lesion, growth of the central DRG axons into peripheral nerve grafts was augmented (Chong et al., 1999; Richardson et al., 1980; Richardson and Verge, 1987).

Neumann and Woolf studied the effects of a conditioning lesion without using a PNS bridge and they reported that injured fibers regenerate beneath and beyond the lesion in some animals, whereas in other animals a massive growth occurred, both along the plane of the lesion, as well as growth on the surface of the cord. A more modest outgrowth of regenerating fibers, beyond the lesion, into gray matter was also reported (Neumann and Woolf, 1999).

Results of a study published by Filbin and collegues demonstrated that cAMP plays a role in the ability of the central branch of DRG neurons to regenerate after a conditioning lesion. It was shown that the effect of a conditioning lesion has two phases (Qiu et al., 2002): in the early phase cAMP levels in DRGs are elevated and protein kinase A (PKA) inhibitors block improved regeneration; whilst in the later phase, one week after a peripheral conditioning lesion, growth of isolated DRG neurons in the presence of MAG was significantly promoted compared to the growth of DRG neurons from control animals. The late stage coincided with the return of cAMP levels back to control levels and inhibitors of PKA could not alter the improved growth. Importantly, injecting PKA inhibitor directly into the DRG simultaneously with conditioning lesion blocked improved growth on MAG when DRG neurons were isolated one day after the lesion and attenuated it at one week.

In two papers published simultaneously it was shown that the effect of a conditionning lesion can be mimicked by artificially elevating cAMP levels, in DRG neurons, 48 hours (Neumann et al., 2002) or one week (Qiu et al., 2002) prior to a dorsal column lesion, by injection directly into the DRG of the unhydrolizable analogue of cAMP, dibutyryl cAMP (db-cAMP). Axons of the lesioned central branch of the DRG injected with db-cAMP showed extensive growth beyond the lesion site.

It would appear that in all of these studies, where regeneration in the adult CNS was reported, cAMP levels were elevated. Moreover, data of Filbin and colleagues and Basbaum and colleagues demonstrated that it is not a mere coincidence or secondary effect, because when cAMP levels of DRG neurons are artificially elevated by injecting db-cAMP, neurons acquire the ability to regenerate spontaneously into the injured CNS (Neumann et al., 2002; Qiu et al., 2002). This led to a study of the effects of elevating cAMP levels in neurons by blocking the enzymes that break down cAMP – phosphodiesterases.

1.3 Phosphodiesterases.

Although cAMP was the first intracellular second messenger identified (Sutherland, 1970), our understanding of the complex system of enzymes that generate, regulate, detect and break down cAMP is far from complete. Mammalian cells express up to 9 isoforms of adenylyl cyclase, the enzyme that synthesizes cAMP in cells (Antoni, 2000; Hanoune and Defer, 2001; Patel et al., 2001). The main target of cAMP, PKA, consists of multiple forms of both regulatory and catalytic subunits. The existence of A-kinase anchoring proteins makes the system even more complex (Feliciello et al., 2001). The enzymes that catalyze the hydrolysis of cAMP, phosphodiesterases (PDE), are the only enzymes identified to date, that are used by cells to terminate and modulate the cAMP signal. PDEs constitute a diverse group of enzymes; the level of complexity of phosphodiesterases matches that of adenylyl cyclases, and probably even surpasses it. This is because PDEs provide the cells with an additional opportunity for crosstalk between different signaling pathways.

Cloning of phosphodiesterases began in 1976 when, in the laboratory of Seymour Benzer, Drosophila were screened for memory deficiency by their avoidance of an odorant

associated with electric shock. Three mutants called dunce, rutabaga and amnesiac were isolated (Dudai et al., 1976; Quinn et al., 1979) and interestingly, all three mutants exhibited impairment of the cAMP signaling pathway. It has been demonstrated that rutabaga is deficient in the Ca/calmodulin-dependant adenylyl cyclase (Dudai et al., 1983), amnesiac lacks a peptide transmitter that acts on adenylyl cyclase (Feany and Quinn, 1995) and dunce carries a mutation in cAMP specific PDE (Byers et al., 1981). In 1986, the dunce gene was cloned (Davis and Davidson, 1986) and it was found that this gene codes for 6 different RNAs as a result of alternative splicing. Researchers found the complexity of this gene remarkable. Today, the dunce gene remains one of the most complex genes in Drosophila, extending over 100 kb and comprising 17 exons. In 1989 the mammalian homologues of the dunce gene were cloned and characterized (Davis et al., 1989) and they were later proved to be members of the PDE4 family of enzymes; so named because in the process of purification these enzymes elute from a DEAE column in the fourth peak of activity. All enzymes of the PDE4 family are cAMP specific and they are inhibited by rolipram. From initial studies it was evident that the pattern of transcription and splicing of PDE4 changed with the animal's development (Davis et al., 1989). Two features were exceptional: the first was the extent of similarity of mammalian PDE4 to the Drosophila dunce gene, at 75%, showing that PDE4s are among the most conserved genes; the second feature was the complexity of the rat PDE4 genes. For example, the PDE 4A gene is 49 kb long and has 16 exons. Molecular cloning of PDE4 genes was the starting point for cloning of other families of PDEs. To date, the number of mammalian phosphodiesterases genes is 22, and these are subdivided into 12 different PDE families that differ in their specificity for substrates (cAMP, cGMP or both), regulatory properties, range of Km values and their inhibition by specific inhibitors. All PDE proteins

have a similar basic structure; the C-terminal part of PDEs is comprised of a catalytic "core", which is highly conserved even between members of different PDE families. The N-terminal region of PDEs contains regulatory domains that are unique to each family. Thus, Ca^{2+}/CaM binding domain of PDE1 confers upon the members of this family the property of being activated by Ca^{2+}. GAF domains first found in c*G*MP –specific phosphodiesterases, cyanobacterial *A*nabaena adenylyl cyclase, and the Escherichia coli transcriptional regulator *f*hlA and are named after the first letters of these proteins. The GAF domains of PDE2, PDE5, PDE6, PDE10 and PDE11 all bind cGMP. For PDE2 it was demonstrated that upon cGMP binding the activity of the enzyme is upregulated (Martinez et al., 2002; Martins et al., 1982). In the case of PDE4, upstream conserved regions (ucr) serve as regulators of enzymatic activity when phosphorylated by PKA and ERK (Hoffmann et al., 1999); in addition, NH_2-terminals of PDE4 determine sub-cellular location of the proteins, cytosolic or membrane-bound (Bolger et al., 1997). Importantly, splice variants of all PDE enzymes may contain different sets of these regulatory domains, therefore, each type of cell can synthesize certain sets of PDEs, which provides the cell with the means to create and maintain compartmentalized gradients of cAMP and cGMP in response to extracellular signals, as well as providing the cell with an additional opportunity to crosstalk between different signaling pathways.

Almost all PDEs are expressed, in varying amounts, within the nervous system, however, here we will focus on the effects of PDE4 inhibitors for the following reasons: 1) Activity of PDE4 is responsible for 70% of total cAMP PDE activity in the brain (Jin et al., 1999),

2) Experiments with inhibitors of PDE in different tissues demonstrated that only in neurons were cAMP levels elevated significantly after applying PDE4 specific inhibitors. In other tissues, a combination of inhibitors of different PDE families was required (Polson and Strada, 1996), suggesting that the relative contribution of PDE4 is not as high as in the nervous system. Therefore, it can be concluded that the enzymes of the PDE4 family (PDE 4 A, B, D) are the most abundant PDEs in the nervous system (Perez-Torres et al., 2000).

1.3.2 Phosphodiesterases type 4.

The PDE 4 family consists of four enzymes, three of which are expressed in the nervous system: PDE4A, PDE4B, PDE4D(Perez-Torres et al., 2000). These proteins are coded for by four genes. In humans they are located on chromosome 19 (PDE4A) (Horton et al., 1995), chromosome 19 (PDE4C) (Sullivan et al., 1999), chromosome 1 (PDE4B) and chromosome 5 (PDE4D) (Milatovich et al., 1994). Each gene has several distinct promoters, 18 or more exons and can code for up to 6 splice variants. All these proteins have a similar basic structure (Bolger et al., 1997).

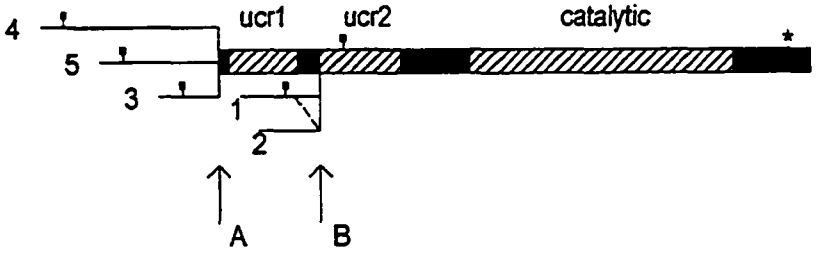


Figure1. Structure of mRNA transcripts from the human PDE4D gene (adapted from (Bolger et al., 1997).

All the PDE4 proteins contain a conserved catalytic domain at the COOH terminus and a choice of two upstream conserved regions (ucr1 and ucr2) at the amino terminus of the protein. Thus, PDE4 genes can generate long, short and supershort isoforms: long isoforms

have both ucr1 and ucr2, short isoforms have only the ucr2 (Bolger et al., 1993) and supershort isoforms of PDE4 contain a truncated ucr2 (Sullivan et al., 1998).

It was demonstrated that the histidine residues, which chelate the tightly bound, divalent cations (Zn^{2+} and Mg^{2+}) are essential for the catalytic activity of PDE4 (Xu et al., 2000). When the three-dimensional structure of PDE4 was studied, it was revealed that the catalytic core of the protein consists of three distinct subdomains, which together form a 440Å pocket, lined with hydrophobic and negatively charged groups. For PDE4B, histidines 238 and 274 coordinate the first tightly bound Zn^{2+} ion, whilst the second Me^{2+} ion is exchangeable (it could be Mg^{2+}) and is coordinated by his278 and his 307. When cAMP binds to this pocket, H_2O binds to the Me^{2+} ion, which acts as a nucleophile in a hydrolysis reaction; the Me^{2+} ions stabilize the transition state, while His 234 protonates the O3' leaving group. The fact that interaction of three subdomains is required to form a catalytically active enzyme implies that any conformational change of these subdomains, by imteractions with other proteins or by phosphorylation, could alter the activity of PDE4 (Houslay, 2001). The amino terminus of PDE4 may contain different sets of ucr's, which is one of the major factors that determines the level of activity of PDE4 and the mode of its regulation.

As well as the apparent impact on regulation of PDE4 activity, the amino terminus of the enzyme dictates the destination of proteins within cells; the most obvious difference between different PDE4 splice variants is their subcellular distribution. Long isoforms that possess both ucr1 and ucr2 (figure 1) are associated with membranes, whereas the short forms are usually cytosolic: the nervous system expresses mostly the long isoforms of these enzymes (Bolger et al., 1997). In conclusion, the various combinations of upstream conserved regions and the extreme amino termini, which are unique to each protein, target the

enzymes to their intended sub-cellular destination and confer on them their distinctive regulatory properties.

1.3.3 Regulation of the activity of PDE4 by phosphorylation.

PDEs activity in the cell is tightly regulated; one level of this regulation is via phosphorylation - it is generally accepted that all PDE4's are activated through phosphorylation of serine 13 and serine 54 by protein kinase A (PKA): as in the case of PDE4D (Hoffmann et al., 1998; Sette and Conti, 1996). Phosphorylation of serine 54 causes a 3-4 fold increase in the Vmax. When serine-54 is exchanged for asparagine, to mimic the negative charge of the phosphate group, activity of this mutant protein resembles that of the wild type enzyme when activated by PKA (Hoffmann et al., 1998). Phosphorylation of serine-54, located within ucr1, most likely disrupts interactions between ucr1 and ucr2 (Beard et al., 2000), resulting in negative feedback, and may be the mechanism that ensures that cAMP levels are kept within a narrow range of concentrations. This mechanism could provide the cell with a mean of terminating the signal that causes cAMP elevation, as well as the means of desensitization of the cell to signals that increase cAMP levels. Interestingly, it was recently proposed that the real purpose of existence of this mechanisms is to terminate the inhibitory effect of extracellular-signal-regulated kinase (ERK) phosphorylation on long PDE4B/C/D enzymes (Houslay, 2001).

The mitogen-activated protein kinase (MAPK) cascade is one of the most conserved of all eukaryotic signal transduction cascades and serves to transmit signals from cell surface receptors into the cell; extracellular signal-regulated kinase (ERK) was the first MAPK to be identified and as such is the founder of a family of kinases. The most studied MAPKs are ERK, JUN-aminoterminal kinase (JNK) and p38 MAPK, which all recognize a similar consensus motif as a substrate for phosphorylation: Pro-Xaa-(Ser/Thr)-Pro, however, this consensus is too short to provide specificity. It was observed that different MAPKs require

different docking sites, termed kinase interaction motifs (KIM), which are located 120-150 amino acids N-terminal from the phosphorylation sites. For ERK an additional specificity determinant was found: the Phe-Gln-Phe (or Phe-Xaa-Phe) motif, that is located 5-30 amino acids C-terminal from the Ser/Thr phosphorylation site, although not all of the ERK substrates possess this motif. Interestingly, PDE4B, PDE4C and PDE4D have both KIM and Phe-Gln-Phe motifs, located on the third subdomain of the catalytic unit, meaning that activity of PDE4 can be directly regulated by ERK. Several groups have reported that this is, in fact, the case (Hoffmann et al., 1999; MacKenzie et al., 2000).

It has been shown that the PDE4D3 enzyme possesses a docking site for ERK2, allowing it to bind to PDE4D3 resulting in and phosphorylation, in vitro (MacKenzie et al., 2000). Further studies by Houslay's group demonstrated that PDE4D3 is phosphorylated in vitro by ERK2 on Ser 579 (Hoffmann et al., 1999). PDE4D3 and PDE4D5, exogenously expressed in COS1 cells, were both phosphorylated by ERK2 when the cells were challenged with epidermal growth factor (EGF), which resulted in a two-fold decrease in activity of PDE4D3 and PDE4D5. The same result was obtained for PDE4D3 and PDE4D5 endogenously expressed in HEK 293 cells treated with EGF. In a more recent study from the same laboratory (Baillie et al., 2000), the authors clarified previous results on inactivation of PDE4 enzymes by phosphorylation through MAP kinases. They concluded that long isoforms of PDE4B and PDE4D, but not PDE4A, are inhibited by ERK-2, whereas short forms of these enzymes were not inhibited by ERK-2.

The inactivation of PDE4B, PDE4C and PDE4D by ERK-2 might be of functional relevance in neurons since neurons are highly enriched in long isoforms of PDE4B and PDE4D. Moreover, ERK plays a crucial role in neuronal differentiation, survival and memory

formation (Kaplan and Miller, 2000). Importantly, according to Houslay (Houslay,2001), PDE4 enzymes and the haematopoietic protein tyrosine phosphatase (He-PTP) are the only enzymes that have docking sites for ERK. In other proteins KIMs were found in the promoter regions. Hence in all other cases ERK does not regulate the activity of the proteins, but the levels of their expression.

1.3.4. Rolipram.

A well documented isozyme-selective inhibitor of PDE4s is rolipram (4-(3-(cyclopentoxy-4methoxyphenyl)-2-pyrrolidinone), which was developed by Schering AG in the late seventies(Karppanen et al., 1979).

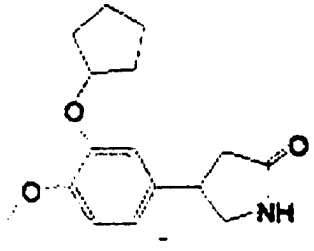


Figure 2. Structural formula of rolipram.

Rolipram underwent clinical trials to investigate its anti-depressant effects in Germany (Zeller et al., 1984), Italy (Bertolino et al., 1988) and Austria (Hebenstreit et al., 1989), during the 1980's. It's potential therapeutic effects as an anti-inflammatory agent (Francischi et al., 2000; Hogan et al., 2001), a memory improving agent (Bach et al., 1999; Barad et al., 1998) and as a sedative agent (Silvestre et al., 1999), were later investigated in animal studies. Studies of the memory-improving effects of rolipram in mice and aging rats, performed in Kandel's laboratory, used very low concentrations of the drug (0.1-3 μmol/kg). It was demonstrated that rolipram injected subcutaneously at a dose up to 1.0 μmol/kg does not significantly raise basal cAMP levels in the brain. However, when injected

subcutaneously 30 min before testing at a dose of 0.1μmol/kg, rolipram improved the performance of mice in a hippocampus-dependant memory task. Interestingly, higher doses of rolipram (3.0 μmol/kg), which did cause an increase in basal cAMP levels, did not have the same effect. At these low concentrations of rolipram, no side effects were reported (Barad et al., 1998).

The anti-inflammatory effect of rolipram has also been demonstrated: doses of 20μmol/kg prevented bone and cartilage destruction in a rat model of rheumatoid arthritis (Francischi et al., 2000); and in a rabbit model of infectious arthritis, infusions of rolipram led to a decreased white blood cell count, the elevation of which is symptomatic of inflammation (Hogan et al., 2001). The sedative effect of rolipram has also been shown in rats (at a concentrations 5-10 μmol/kg) (Silvestre et al., 1999), whilst a neuroprotective role has been demonstrated, by Block and coworkers in a model of excitotoxic neuronal damage caused by injection of quinolinic acid (Block et al., 2001). This study demonstrated that initiation of rolipram treatment 6 h after quinolinic acid injection, resulted in decreased neuronal damage compared to control animals. The combined neuroprotective and anti-inflammatory properties of rolipram make it a possible candidate for treatment of patients with multiple sclerosis (MS). Although the pathogenic mechanisms of demyelination and tissue damage in MS are not completely elucidated, two major characteristics of the disease could potentially be targeted by PDE4 inhibitors: these are the autoimmune and inflammatory nature of MS as well as the demyelination and subsequent hypoxia-like tissue injury (Lassmann, 2003; Sommer et al., 1997). Since rolipram was demonstrated to have both anti-inflammatory and neuro-protective effects, its potential as a treatment for multiple sclerosis is currently being investigated in stage two clinical trials.

Chapter II.

Materials and methods.

2.1 Isolation of neurons.

The cerebella from postnatal day 5 rat was dissociated by pipeting in 5 ml of 0.025% trypsin, incubated 10 min at 37°C. Trypsinization was stopped with 5 ml of Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% FBS. Cells were centrifuged at 1000 rpm for 5 min. The cells were resuspended to a single-cell suspension in 2 ml of Sato (Progesterone, 20nM, selenium,30 nM; putrescine, 100μM; insulin, 5 μg/ml; BSA, 4mg/ml; L-thyroxine, 0.1 mg/ml; tri-iodo-thyronine, 0.08 μg/ml) (Doherty et al., 1990). Neurons were plated on myelin covered slides or monolayers of CHO cells at 20 000 neurons per well. For older DRG neurons, ganglia were removed from two animals and incubated in 5 ml of Sato media containing 0.025% trypsin and 0.15% collagenase type I (Worthington) for 30 min at 37°C. The ganglia were triturated and trypsinization was stopped by adding 5 ml of DMEM containing 10% FBS, centrifuged at 800 rpm for 6 min, and resuspended in Sato (De Bellard et al., 1996).

2.1 Cell culture maintenance.

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

2.2 Preparation of myelin.

Myelin was purified as described previously (Norton and Poduslo, 1973) from rat CNS white matter. After the final hypotonic shock, the membranes were centrifuged and resuspended in

10 mM HEPES. The protein concentration of the preparation was determined (BioRad) and used immediately as a substrate in the neurite outgrowth assay.

2.4 Priming neurons with neurotrophins and rolipram.

Tissue culture dishes (6 cm) were coated 16.6 μg/ml with poly-l-lysine (Sigma) for 30 min at room temperature. Excess poly-l-lysine was washed off with H_2O. Isolated neurons in Sato were plated onto the poly-l-lysine-coated dishes at a density $1x10^6$ cells/dish. Where indicated, either BDNF, dbcAMP or rolipram (all from Sigma) was added at the indicated concentration in the presence or absence of a protein kinase A inhibitor, KT5720 (Calbiochem), MEK inhibitors, U0126 and PD98059 (Calbiochem). After overnight culture, the media was removed, neurons were washed with PBS, and removed with 0.1% trypsin with 5 mM EDTA. Trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS; neurons were centrifuged at 1000 rpm for 5 min, resuspended in Sato, and plated immediately onto either MAG-expressing CHO cells, control CHO cells, or purified, immobilized myelin.

2.5 Neurite outgrowth assay on immobilized myelin or transfected cells

For myelin membranes, wells of an eight-chamber tissue culture slide (Lab-Tek) were coated with 16.6 μg/ml poly-L-lysine at room temperature for 1 hr and washed with 0.1 M NaHCO3. Rat CNS myelin at 0.5–1 μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen et al., 1998). Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an eight-well tissue culture slide (Lab-Tek). The neurite outgrowth assay was carried out as described previously

(Mukhopadhyay et al., 1994 ; De Bellard et al., 1996 ; Tang et al., 1997b) by adding 2 x 10^4 cerebellar or DRG neurons, either primed or not, to the immobilized myelin substrate or to the CHO cell monolayers. Where indicated, db-cAMP at 1 mM, BDNF at 200ng/ml, rolipram at the indicated concentrations was added. After 16–18 hr of culture, the neurons were fixed for 30 min with 4% paraformaldehyde and made permeable with ice-cold methanol for 2 min. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated for 4 hr with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins, Imperial College, London). Cells were washed three times with PBS-BSA (2%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit Ig (1:500, Amersham), washed three times, and incubated with streptavidin-conjugated Texas Red (1:300, Amersham) for 45 min. After three more washes, the slides were mounted in Permafluor (Baxter) and viewed with a fluorescent microscope. The length of the longest neurite or the total process outgrowth for each GAP43-positive neuron for the first 180–200 neurons encountered when scanning the slide in a systematic manner was determined using an Oncor image analysis program. Using a video camera, the neuron image was projected to a computer screen. The longest neurite or all process were manually traced and quantified.

2.6 Phosphodiesterase activity assay.

For each assay, 5x10^6 cerebellar neurons were plated per well of a poly-l-lysine coated 6-well dish. The neurons were incubated overnight in Sato, then starved for 2hr in DMEM before the addition of BDNF with or without MEK inhibitor, U0126 (5 μM) for 5, 10, 30 or 60 min. The neurons were washed once with PBS and immediately lysed on ice and scraped using a rubber policeman in 100 μl of PDE buffer, 50 mM of Tris-HCl (pH7.5), 8.3 mM

$MgCl_2$, 1.7 mM EGTA, 3.75 mM of β-mercaptoethanol, proteinase inhibitors (Calbiochem) and phosphatase inhibitors. PDE activity was measured in lysates using a scintillation proximity assay, according to the manufacturer's instructions (Amersham). To determine the percent of rolipram-sensitive PDE activity in the lysates, 25 µl of lysate treated with BDNF was incubated with 100 µM rolipram for 15 min before measuring PDE activity.

2.7 cAMP assay.

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

2.8 Subcutaneous delivery of rolipram by injection.

Long Evans Hooded Rats post natal day 14 and 30 were from Harlan. Rolipram was dissolved in sterile DMSO (from Sigma) and sterile saline solution 0.9% (from AmTech) was added to adjust the doses to indicated. Rats were injected subcutaneously every 3 hours for

indicated period of time with insulin syringes (1CC U-100 from Beckton Dickinson). The volume of rolipram solution in each injection was 0.2ml.

2.9 Subcutaneous delivery of rolipram by minipump.

Minipumps (2001 from Alzet) were filled according to the instructions of the manufacturer with indicated concentrations of rolipram solution. Minipumps were preincubated overnight in sterile saline solution 0.9% (from AmTech) minipumps were inserted subcutaneously. Since rolipram is not soluble enough, two minipumps were used per each animal (Alzet 2001, pumping rate 1 μl/hour, nominal duration 1 week). The minipumps were inserted according to the instructions of the manufacturer. Briefly, rats were anesthesized with isofluorane, skin on the animal's back was shaved and washed with beta iodine and ethanol, incision was made, minipumps were inserted under the skin, the wound was closed with wound clips.

Chapter III.

The specific inhibitor of phosphodiesterase4's, rolipram, blocks inhibition of axonal outgrowth by MAG and myelin.

3.1 Introduction.

One of the most important discoveries in the field of neuroscience during the past two decades was the finding that adult mammalian CNS neurons do not intrinsically lack the ability to regenerate. When provided with a permissive environment by grafting fragments of peripheral nerve (David and Aguayo, 1981), or fragments of embryonic spinal cord (Reier et al., 1986; Tessler et al., 1988), injured CNS neurons extended long processes into the graft tissue but not beyond. It is believed that the inhibitory environment of the injured CNS prevents regeneration into the host lesioned spinal cord. Bregman and her colleagues have reported that when a piece of embryonic tissue is grafted into rat's leisioned spinal cord along with neurotrophins (BDNF or NT-3) being pumped into the graft, extensive regeneration of the lesioned axons is observed both into the graft and beyond it. In our lab the molecular mechanism that allows the axons to grow through the inhibitory environment of injured CNS was investigated (Cai et al, 1999). It was noted by Filbin that while growing through the graft regenerating axons were exposed to neurotrophins before they encountered myelin-associated inhibitors. When conditions of the experiment were reproduced in vitro, and neurons treated simultaneously with neurotrophins and inhibitory molecules- MAG or myelin, neurite outgrowth was still inhibited. However, when neurons were exposed to neurotrophins for 18 hours prior to exposure to MAG or myelin (termed priming), they are no longer inhibited by MAG or myelin. Moreover, it was found that the effect of priming with neurotrophins to block inhibition by MAG was abrogated when inhibitors of protein kinase A (PKA) were added to the media during priming. The intracellular levels of cAMP in the neurons were found to be significantly increased after priming with neurotrophins. Finally, it was reported that artificial elevation of cAMP levels in neurons with db-cAMP blocks inhibition of axonal

outgrowth by MAG and myelin. These results hold importance for several reasons. First, they demonstrate that priming with neurotrophins can block inhibition by MAG and myelin. Second, they also demonstrate that priming with neurotrophins is mediated through a PKA-dependent pathway. It is not clear yet if elevation of cAMP is required only for initiation of the pathway, or if elevated levels of cAMP is the necessary characteristic of the neurons that are not inhibited by MAG or myelin. Combined results of the experiments of priming with neurotrophins and experiments with db-cAMP suggest that cAMP plays both roles.

In contrast to neurotrophins, addition of db-cAMP to neurons overcomes inhibition of neurite outgrowth by MAG and myelin without requiring priming. This suggests that elevation of cAMP is enough to block inhibition by MAG. However the fact that the effect of db-cAMP is transcription-dependent indicates that cAMP, even in the absence of priming, is signaling the neurons to become insensitive to myelin inhibitors (To be discussed in chapter V).

An alternative approach to elevate cAMP in neurons is to block the process of cAMP degradation. The enzymes that hydrolyze cAMP are phosphodiesterases (PDE). It has been shown that the enzymes of the PDE4 family represent the major portion of total PDE activity in the nervous system (Jin et al, 1999; Survana and O'Donnell, 2002). All members of the PDE4 family are specifically inhibited by rolipram (Livi 1990, Rocque 1997). It was demonstrated by Kandel's group (Barad et al., 1998) that rolipram at a concentration of up to 0.3 μM did not significantly increase cAMP levels in hippocampal slices, but at a concentration 0.1 μM it significantly increased cAMP levels upon activation of adenylyl cyclases with the drug forskolin.

If elevation of intracellular cAMP can initiate pathways leading to the block of the inhibition of axonal growth by MAG and myelin, then inhibition of PDE4 by rolipram and, hence accumulation of cAMP, may be a novel approach to induce regeneration in the CNS. Therefore, as a first step, it must be established whether rolipram will improve neuronal outgrowth in culture if added directly to the media (similar to addition of db-cAMP) or if priming with rolipram will be required to overcome axonal inhibition by MAG and myelin. In addition, it is important to know how different populations of neurons will respond to treatment with rolipram.

We report here that inhibiting PDE4 activity in neurons by adding rolipram directly to the media, at a concentration of 0.5 μM, partially blocks the inhibition of neurite outgrowth by MAG from cerebellar neurons. Priming with rolipram at concentrations of 0.25-0.5 μM completely blocks inhibition of neurite outgrowth by MAG and myelin from cerebellar and DRG neurons in a dose-dependent manner.

3.2 Results.

3.2.1 Rolipram partially blocks inhibition of neurite outgrowth by MAG without priming.

It was demonstrated in our laboratory that neurite outgrowth from postnatal cerebellar neurons and from DRG neurons older then postnatal day 4 is inhibited when grown on monolayers of MAG-expressing CHO cells compared to control CHO cells (Cai et al., 1999; Mukhopadhyay et al., 1994). Artificial elevation of cAMP levels via addition of a non-hydrolyzable analogue of cAMP, db-cAMP, added directly to the neurons growing on the MAG-expressing cells overcomes inhibition by MAG (Cai et al., 1999). Here we ask if addition of the specific PDE4 inhibitor, rolipram, has the same effect. Cerebellar neurons from P5 rats were isolated as previously described (DeBellard et al., 1996) and plated onto a monolayer of CHO cells expressing MAG or a monolayer of control cells. Where indicated, dbcAMP at 1mM or rolipram at 0.1μM, 0.25μM, 0.5μM or 1.0μM was added to the media. After 18 hours of culture, neurons were fixed and stained with a rabbit polyclonal antibody against GAP43.

As can be seen in Fig.3, rolipram in the range of concentrations 0.25-1.0μM, partially blocks inhibition of axonal outgrowth by MAG. Compared to dbcAMP which blocks inhibition of axonal outgrowth by MAG completely, rolipram, at a concentration of 0.5 μM, blocks the inhibition of axonal outgrowth by MAG by approximately 80%. In addition, the effect of rolipram is dose-dependant.

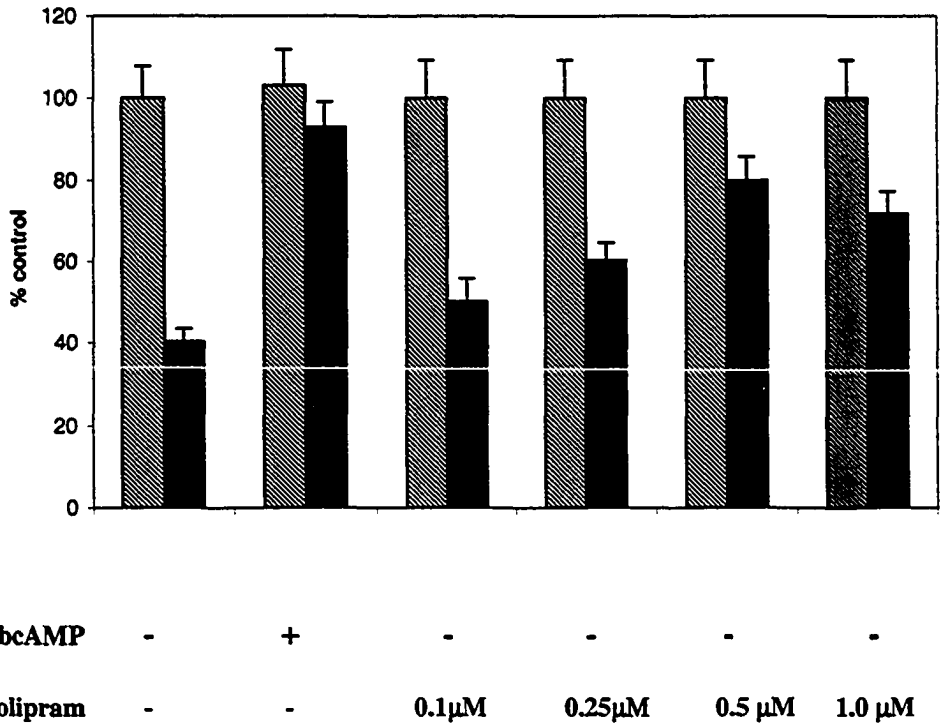


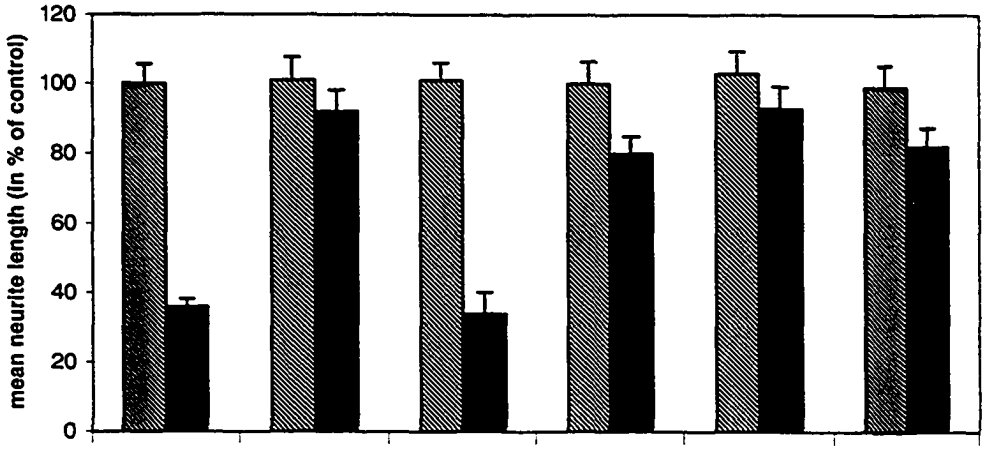
Figure 3. **Rolipram added to the media partially blocks inhibition of axonal outgrowth of cerebellar neurons by MAG.**
Dissociated cerebellar neurons were plated onto monolayers of either MAG-expressing cells (solid bars) or control CHO cells (striped bars) in the presence of dbcAMP at 1mM, or rolipram at 0.1– 1.0 μM (as indicated), and cultured overnight before being fixed and immunostained for GAP-43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of dbcAMP and rolipram.

3.2.2 Priming cerebellar neurons with rolipram overcomes inhibition of axonal growth by MAG and myelin.

Previously it has been shown that treating cerebellar neurons with neurotrophins (BDNF, NGF) for 6-18 hours prior to exposing them to the inhibitors of axonal outgrowth, allowed neurons to grow in the presence of MAG and myelin in vitro (Cai et al., 1999) and to regenerate in vivo (Broude et al., 1999). The levels of cAMP in these neurons were elevated after priming. As 100% block of regeneration was not achieved when rolipram was added directly to the media, we asked if priming with rolipram would be as effective in blocking inhibition of axonal outgrowth as priming with BDNF. Priming of neurons was performed as previously described (Cai et al., 1999). Briefly, isolated neurons were plated onto poly-l-lysine-coated dishes. Where indicated, either BDNF or rolipram was added. After culture for 18 hours, neurons were removed with trypsin and plated immediately either onto MAG-expressing CHO cells, control CHO cells, or purified, immobilized myelin. Priming of cerebellar neurons with rolipram at a concentration of 0.5 μM completely blocked inhibition of neurite outgrowth by MAG. The effect of rolipram is dose-dependant, which can be seen at Figure 4. At a concentration of 0.1 μM, it did not have any effect on the length of the neurons grown on MAG.

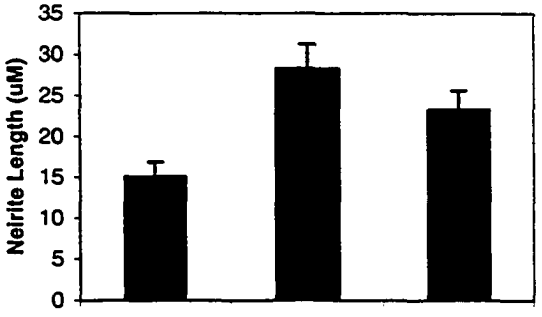
Myelin in general is inhibitory to axonal outgrowth. Besides known inhibitors such as MAG, Nogo and OMgp, myelin is likely to contain as yet unidentified inhibitors of axonal outgrowth. Therefore, it is important to determine if priming with rolipram overcomes the combined effects of myelin inhibitors. Similar to the results with BDNF, whereby inhibition of neurite outgrowth from cerebellar neurons by myelin is overcome (Cai et al, 1999), we show here that priming with rolipram improves neurite outgrowth on a substrate of purified myelin. Neurons

primed with rolipram extend neurites that are about two-fold longer then neurites of untreated neurons grown on myelin (Figure 4B).



BDNF	-	+	-	-	-	-
Rolipram	-	-	0.1μM	0.25μM	0.5μM	1.0 μM

A.



BDNF	-	+	-
Rolipram	-	-	0.25μM

B.

Figure 4. **Priming cerebellar neurons with rolipram blocks inhibition of neurite outgrowth by MAG and myelin.**

Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with BDNF at 200 ng/ml or rolipram at 0.25 μM, 0.5μM or 1.0 μM (as indicated) before being transferred to a monolayer either MAG-expressing cells (solid bars) or control CHO cells (striped bars) (A) or onto myelin covered slides (B) and cultured overnight before being fixed and immunostained for GAP-43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells.

3.2.3 Priming with rolipram overcomes inhibition by myelin of neurite outgrowth from DRG neurons.

In order to ensure that effects of rolipram are not restricted to only one type of neuron we primed dorsal root ganglion (DRG) neurons overnight with rolipram at the concentrations of 0.25μM and 0.5μM, and then transferred the neurons to slides coated with purified myelin. We asked if priming DRG neurons with rolipram could block the inhibitory properties of myelin. It was previously demonstrated that priming with BDNF significantly improved neurite outgrowth from DRG neurons on myelin (Cai et al., 1999). Therefore, here we used DRG neurons primed with BDNF as a positive control. We demonstrate that the lengths of neurites from DRG neurons primed with rolipram at a concentration 0.5 μM are twice as long as those of untreated neurons. The effect of priming with rolipram is comparable to the effect of priming with BDNF. The effect of rolipram is dose-dependent. Moreover, this dose-dependency has a bell-shape. While maximum effect was achieved at a concentration of rolipram 0.5 μM, priming with rolipram at a concentration of 0.1μM has no effect on inhibition of neurite outgrowth by myelin (result not shown). Priming with rolipram at concentrations greater then 1 μM results in a decrease in the length of neurites compared to neurons treated with the optimal concentration of rolipram. When concentrations of rolipram exceed 2μM, neurites of the treated neurons grow shorter than neurites of untreated neurons (results are not shown).

These data suggest that the inhibitory effects of myelin can be reversed by exposure of DRG neurons to the specific inhibitor of PDE4, rolipram.

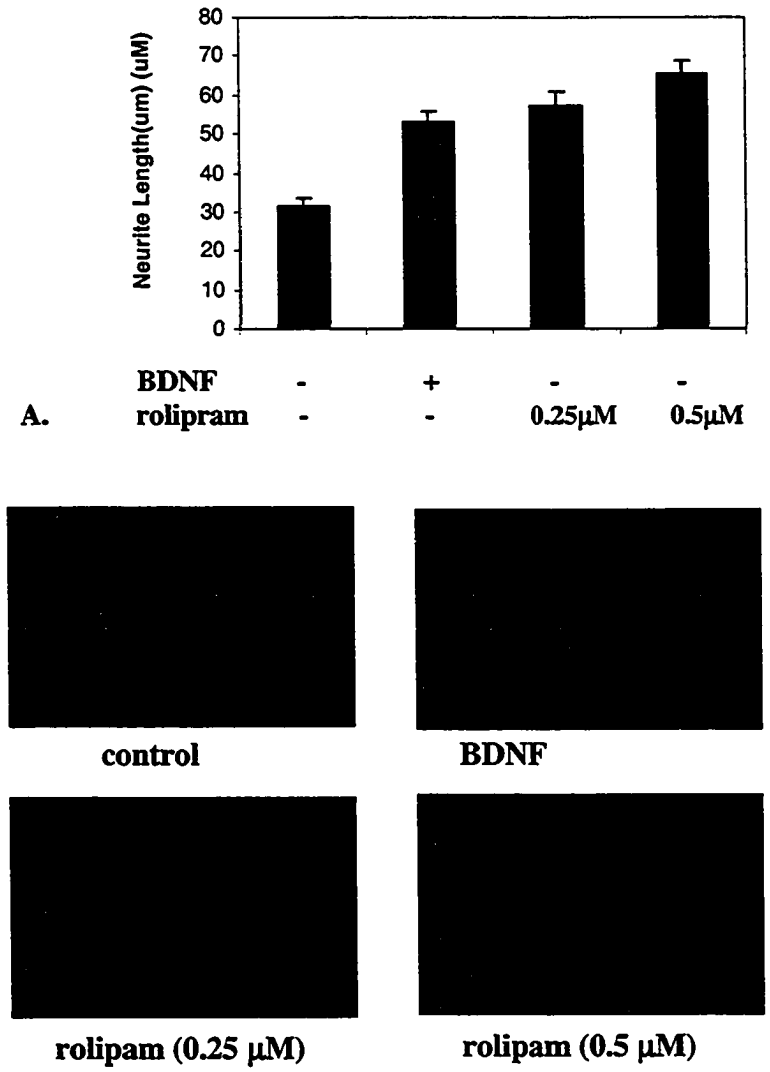


Figure 5. **Priming DRG neurons with rolipram blocks inhibition of axonal outgrowth by myelin.** Dissociated DRG neurons were first primed overnight on poly-l-lysine with BDNF at 200ng/ml or rolipram at 0.25μM or 0.5 μM as indicated before being transferred onto myelin covered slides and cultured overnight before being fixed and immunostained for GAP-43.

3.3 Discussion.

The results presented here demonstrate that rolipram, added directly to the media improves neurite outgrowth of cerebellar neurons growing on MAG-expressing cells, but under these conditions, rolipram does not completely block inhibition by MAG. It is possible that the inhibitory effects of MAG block a step in the accumulation of cAMP, therefore only blocking the degradation of cAMP by rolipram is not sufficient to allow endogenous cAMP to accumulate to a sufficient level to block inhibition by MAG. This suggests that the inhibitory signaling pathway initiated by MAG or other myelin-associated inhibitors overrides the BDNF or rolipram signaling pathways. This may be the reason why neurons must be primed with BDNF or rolipram. Currently, it is not clear at which point in the pathways initiated by MAG the effects of BDNF and rolipram are blocked. It was demonstrated in our lab (Cai et al., 1999) that addition of pertusis toxin allows BDNF to block inhibition of axonal growth by MAG without priming. Pertusis toxin is an inhibitor of the G_i family of G protein-coupled receptors. Upon binding of extracellular ligands, G_i inhibits adenylyl cyclase (Sprang, 1997). Recently another mechanism for the negative regulation of cAMP signaling by G_i was proposed. It was demonstrated that G_i directly interacts with the Rap1 GTPase-activating protein (Rap1GAP) (Jordan et al., 1999). Rap1GAP is a negative regulator of the Rap1 protein. Rap1 is a Ras- like GTPase, a member of Ras family of G proteins that cycle between GDP-bound inactive and GTP-bound active forms. Results reported by several groups suggest that Rap1 is a major activator of MAPK (also discussed in chapter 4) in the neurotrophin-induced signal transduction pathway (Wu et al., 2001; York et al., 1998). Rap1 has very low intrinsic GTPase activity, therefore, GTPase-activating proteins (GAPs) are important in the

GDP–GTP cycle because they induce GTP hydrolysis. Intriguingly, Rap1GAP is particulary abundant in the brain (Rubinfeld et al., 1991). It was proposed by Stork and co-workers that in the inactive state, G_i binds to Rap1GAP and sequesters it. Upon activation, Rap1GAP detaches from G_i and blocks activation of Rap1. The finding that addition of G_i inhibitor to neurons treated with BDNF eliminates the requirement of priming, together with our finding that MAPK activation plays a major role in the neurotrophin effect of blocking inhibition by MAG, strongly suggests that MAG and myelin activate G_i- Rap1GAP and in this way, block Rap1 activation of MAPK

On the other hand, priming neurons with rolipram prior to exposure to myelin inhibitors completely blocks inhibition by MAG and myelin. This indicates that in the absence of inhibitors, blocking the degradation of cAMP alone is enough to allow cAMP to accumulate to sufficient levels to initiate the pathway to bring the neurons to a state where they no longer respond to myelin inhibitors. Priming with rolipram is effective in overcoming inhibition by both MAG and myelin, which supports the notion that initiating the cAMP pathway is sufficient to block all inhibitors of axonal outgrowth in myelin. Moreover, others have suggested that rolipram blocks the inhibition of axonal outgrowth by proteoglycans (J.Silver, personal communication).

The effects of rolipram are dose-dependant, but unlike the effects of neurotrophins, it does not have a "plateau" concentration. We showed that the effects of neurotrophins to overcome inhibition by MAG and myelin are dose-dependant. Concentrations of neurotrophins above values that completely blocked inhibition did not change the response of the neurons to MAG and myelin (Cai et al., 1999). It was also reported by Barres group that neurotrophins increase the rate of axonal growth in a dose-dependant manner (Goldberg et al.,

2002). The rate of axonal growth increases with increasing concentrations of neurotrophins, and then reaches a maximum growth rate at the optimal dose of neurotrophins. Elevation of the concentration of neurotrophins above this did not change the rate of the axonal growth (Goldberg et al., 2002). However, unlike neurotrophic factors, rolipram does not have a "plateau" concentration. It would appear that concentrations that exceed the optimal level of rolipram needed for a complete block of inhibition are inhibitory to neurite growth and perhaps even toxic. This notion is further supported by *in vivo* experiments (to be discussed in chapter 4). These studies suggest that active PDE4's are vital to maintain a healthy state in neurons. It was demonstrated in frog's cardiocytes that PDE4's maintain the cAMP gradient of a cell (Antoni, 2000). These gradients are likely to be even more important for neurons since they are highly polarized.

We conclude here that rolipram, added directly to the media, improves axonal growth in the presence of MAG from cerebellar neurons in a dose-dependant manner. Priming cerebellar or DRG neurons with rolipram can completely block inhibition of neurite growth by MAG and myelin. What also makes rolipram an attractive candidate as a potential therapeutic agent for different pathologies of the nervous system is the fact that it can cross the blood-brain barrier and even accumulates in the CNS because it binds to the molecules of PDE4, which reside predominantly in the CNS. (To be discussed in the chapter 5).

Chapter IV.

Rolipram, delivered subcutaneously, blocks inhibition of neurite outgrowth by MAG and myelin and promotes regeneration in the CNS.

4.1 Introduction

Previously we showed that elevated levels of intracellular cAMP correlate with the ability of neurons to regenerate. It was demonstrated that high levels of endogenous cAMP in different types of embryonic neurons and in young DRG neurons account for the promotion of neurite outgrowth by MAG and myelin. The developmental decrease in cAMP levels coincide with a switch to the inhibition of regeneration by MAG and myelin (Cai et al., 2001).

A conditioning lesion of the peripheral axons of DRG allows subsequently lesioned central axons to regenerate in the lesioned dorsal columns (Newman and Wolf, 1998). This conditioning lesion effect appears to occur in two phases (Qui et al, 2002). In the early phase, 24 hours after a conditioning peripheral lesion, levels of cAMP in DRG neurons are elevated and PKA inhibitors can block improved growth on MAG and myelin in culture, as well as regeneration of the central axons in the dorsal columns. In the second phase levels of cAMP decrease to control levels. However, the growth of isolated DRG neurons in the presence of MAG and myelin is even better than in the first phase and this improved growth can not be blocked by PKA inhibitors. We and others (Qui et al, 2002; Neuman et al, 2002) have shown that the effects of both the early and the late phases of a conditioning lesion can be mimicked by direct injections of db-cAMP into DRGs. This finding is the first direct evidence showing that elevated cAMP does not merely coincide with the ability of the neurons to regenerate but rather dictates whether axons grow into the hostile environment of the injured CNS. These finding also demonstrated that the initial elevation of cAMP triggers the second phase of improved growth of axons, which is PKA independent.

Levels of cAMP in neurons not only dictate their ability to grow in the presence of myelin inhibitors, but also determine the response of the neuronal growth cones to

signals provided by extracellular cues (Song et al., 1998) as well as survival of the neurons (Hanson et al., 1998; Meyer-Franke et al., 1998). Levels of cAMP are under stringent regulation both on the level of synthesis as well as degradation. Phosphodiesterases are the only enzymes that hydrolyze cAMP. As discussed in the chapter 1, the PDE4 family of enzymes, which are all specifically inhibited by the drug rolipram, carry out the majority of PDE activity in neurons. Rolipram allows cAMP levels to be specifically increased in neurons. Since rolipram can cross the blood-brain barrier it can be applied systemically to increase cAMP levels in the CNS.

Rolipram was used in animal studies to assess its effectiveness as an antidepressant, an anti-inflammatory agent, a memory-improving agent and as a sedative agent. Memory-improving effects of rolipram were studied in Kandel's group (Bach et al., 1999; Barad et al., 1998). *In vitro* effects of rolipram in establishing long-term potentiation were carried out in parallel with *in vivo* studies of the behavioral effects of rolipram on the performance of mice in a hippocampus-dependant memory task. At concentrations of up to 0.3μM rolipram did not raise basal cAMP levels in hippocampal slices *in vitro*, however in the presence of 0.1 μM of rolipram a single train of titanic stimulation induced LTP in the hippocampal slices. Increased basal cAMP levels in hippocampal slices could be detected at a concentration of rolipram of 1.0-3 μM (Barad et al., 1998). When injected subcutaneously at a dose of 0.1μmol/kg, rolipram improved the performance of mice in a hippocampus-dependent memory task. Interestingly, higher doses of rolipram, 3 μmol/kg, which correlated to the concentration of the drug that caused an increase in basal cAMP levels in slices *in vitro*, did not have memory improving effects. No side effects of rolipram were reported at any of these concentrations (Barad et al., 1998).

The pharmacokinetics of rolipram studied in rats demonstrates that rolipram is able to pass the blood-brain barrier achieving concentrations in the cerebrospinal fluid twice those achieved in plasma (Krause and Kuhne, 1988). According to the results of Kandel's group, a dose of 0.1 μmol/kg rolipram yields a concentration of rolipram between 0.06 μM and 0.2 μM in brain tissue 30 min after treatment, based on a half-life of 1-3 hr and the observation that cerebrospinal fluid concentration was twice that in serum (Barad et al., 1998). Since the best results in our experiments *in vitro* indicate that priming neurons with rolipram is more effective in blocking inhibition of axonal growth by MAG and myelin than direct treatment with rolipram, we delivered rolipram to the animals for 24 hours to mimic the conditions of priming and then studied the effect of rolipram treatment on the ability of isolated neurons to grow in the presence of MAG and myelin in culture.

Here we present results showing that subcutaneous injection of rolipram into postnatal day 14 (P14) rats blocks inhibition of neurite outgrowth by MAG both from isolated DRG and cerebellar neurons in a dose-dependent manner. The same effect on DRG neurons was detected when postnatal day 30 (P30) rats were treated with rolipram. Importantly, we found that the most effective dose for P30 rats was higher than that for P14 rats. We conclude here that the most effective dose of rolipram in blocking the inhibition of axonal outgrowth by MAG is age-dependent. We found that after one day of treatment neurons were not inhibited by MAG or myelin anymore. After 2 days of treatment the general ability of the neurons to grow was significantly improved compared to the one day treatment yielding an effect that is very similar to that of a conditioning lesion. Moreover, this improved growth was PKA-independent.

Formation of the glial scar is another factor that contributes to the lack of regeneration in the CNS. The main components of the glial scar are reactive astrocytes and connective tissue elements that can serve as a scaffold for depositing various inhibitory molecules, such as proteoglycans (Fitch and Silver, 1997; McKeon et al., 1991). Importantly astrocytes cease to proliferate in response to elevated cAMP levels in cells. When cAMP was elevated, with db-cAMP, proliferation of C6 astrocytoma cells was attenuated (Dugan et al., 1999). The other major inhibitory factor that contributes to the glial scar formation is deposition of extracellular matrix proteins. Topical application of db-cAMP into the lesion site significantly reduces proliferation rate and extracellular matrix production capacity of invading fibroblasts (Hermann et al. 2001). The above results suggest that elevation of cAMP by inhibitors of PDE4 will, in addition to overcoming myelin inhibition, reduce or prevent formation of the glial scar after injury.

Our data, demonstrating that treatment with rolipram in vivo blocks inhibition of neurite outgrowth by MAG and myelin of isolated neurons in culture, together with the evidence that elevated cAMP in astrocytes could prevent the glial scar formation, indicate that rolipram is an excellent candidate for promoting regeneration in the injured CNS.

4.2 Results.

4.2.1. Subcutaneous injection of rolipram into P14 rats blocks inhibition of neurite outgrowth by MAG from isolated cerebellar and DRG neurons.

In the first set of experiments we sought to find out if delivering rolipram subcutaneously would have an effect on the ability of neurons, when isolated from treated animals, to grow in the presence of MAG. Neurons isolated from the animals injected with saline/DMSO solution served as a control. (In a separate set of the experiments we established that DMSO, a solvent of rolipram did not affect the ability of the neurons to grow on either control CHO cells or on MAG-expressing CHO cells, when mixed with saline in appropriate concentrations (results are not shown)). Neurons isolated from animals injected with saline/DMSO solution and grown in the presence of db-cAMP, served as a positive control. We injected rolipram, or control saline/DMSO solution, into P14 rats every 3 hours for 24 hours and measured neurite outgrowth of isolated cerebellar and DRG neurons in the presence or absence of MAG. We found that the treatment of P14 rats with rolipram, delivered by subcutaneous injections, completely blocked inhibition of neurite outgrowth from isolated cerebellar and DRG neurons isolated from treated animals. The effect of rolipram is dose-dependent (fig. 6) and the most effective dose of rolipram, in treated P14 rats, is 40 nmol/kg. Injection of rolipram for 24 hours did not affect the neurite length of neurons grown on control cells.

Animals tolerated the rolipram treatment well. No side effects were detected at the concentrations that effectively blocked the inhibition of neurite outgrowth by MAG. It is especially important to note this because in the preliminary set of experiments the same doses of rolipram caused limb-tremor in P5 rats (results not shown).

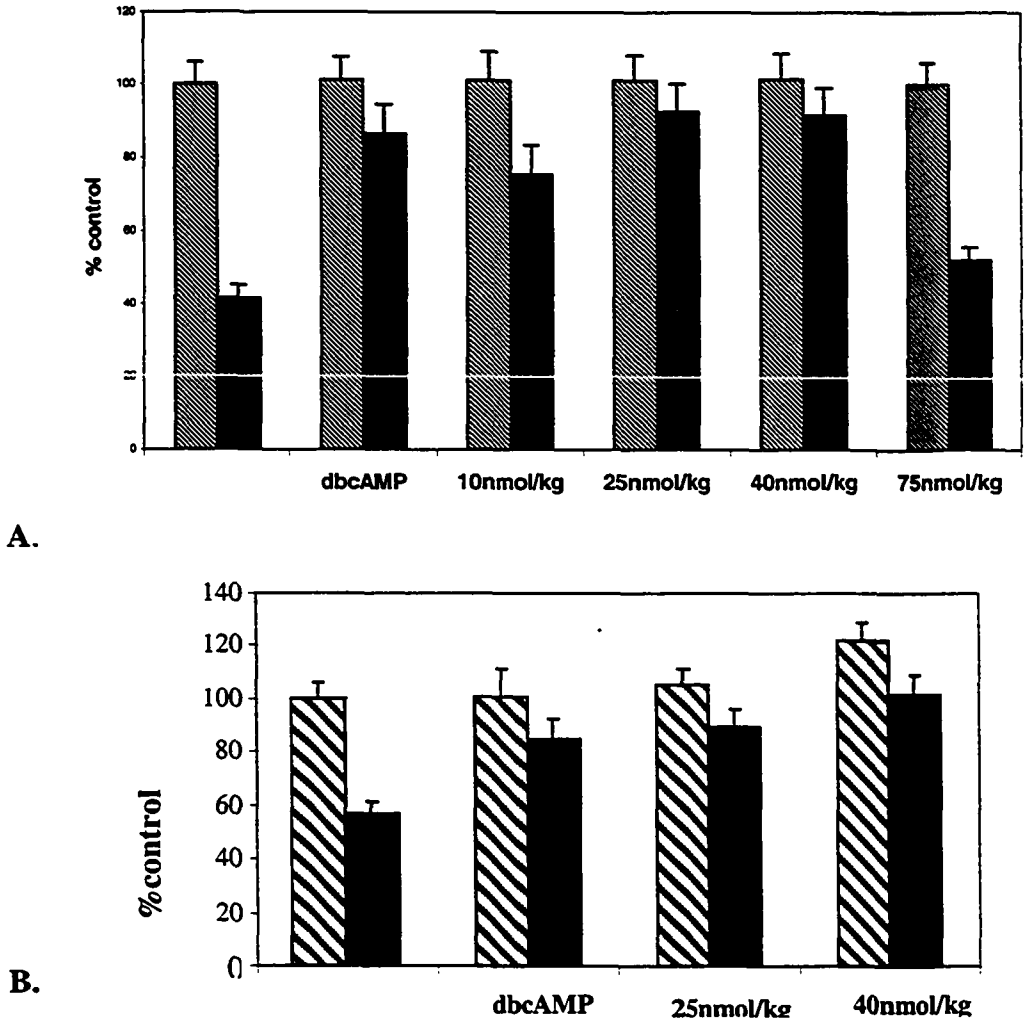


Figure 6. Subcutaneous injections of rolipram into P14 rats block the inhibition of neurite outgrowth by MAG.

Cerebellar neurons (A) and DRG neurons(B) were isolated from animals treated with rolipram injections (every 3 hours for 24 hours at the indicated doses). Neurons were dissociated before being transferred to a monolayer of either MAG-expressing cells (solid bars) or control CHO cells (striped bars) and cultured overnight before being fixed and immunostained for GAP-43. Neurite length was then measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons.

4.2.2 Subcutaneous injection of rolipram into P30 rats blocks inhibition of neurite outgrowth by MAG from isolated DRG neurons.

In order to assess the possible therapeutic effect of rolipram on the promotion of CNS regeneration, its effects need to be studied using a rat model of dorsal column lesion. In all existing "standard" models, the rats used are older then P30, which is why the second series of our experiments sought to find the most effective concentration of rolipram for P30 animals (Fig. 7). Neurons isolated from animals injected with saline/DMSO solution served as a control. We injected rolipram, or saline/DMSO solution, into P30 rats every 3 hours for 24 hours at doses ranging from 0.1 μmol/kg to 2.0μmol/kg. The animals were then sacrificed and the DRG neurons were isolated and plated onto either monolayers of control CHO cells or CHO cells expressing MAG. After 20 hours of incubation the neurons were fixed and stained with GAP43. We found that treatment of P30 rats with rolipram, delivered by subcutaneous injections, completely blocked inhibition of neurite outgrowth from the isolated DRG neurons and that this effect was dose-dependent (Fig. 7). The most effective dose of rolipram injected in P30 rats was 0.5 μmol/kg. Injection of rolipram for 24 hours did not affect neurite length from neurons grown on control cells. The results described here are the average results taken from 3 independent experiments. In each experiment at least two animals were injected with the same dose of rolipram.

We also found the most effective dose of rolipram for P30 rats, which blocked inhibition of neurite outgrowth by MAG, 0.5 μmol/kg, was higher than the most effective dose for P14 rats (0.04 μmol/kg). We conclude here that most effective doses of rolipram are age-dependant. The possible reason for this effect is different pattern of expression of PDE4

at different stages of animal development (Davis et al., 1989). No side effects of rolipram were detected at these doses.

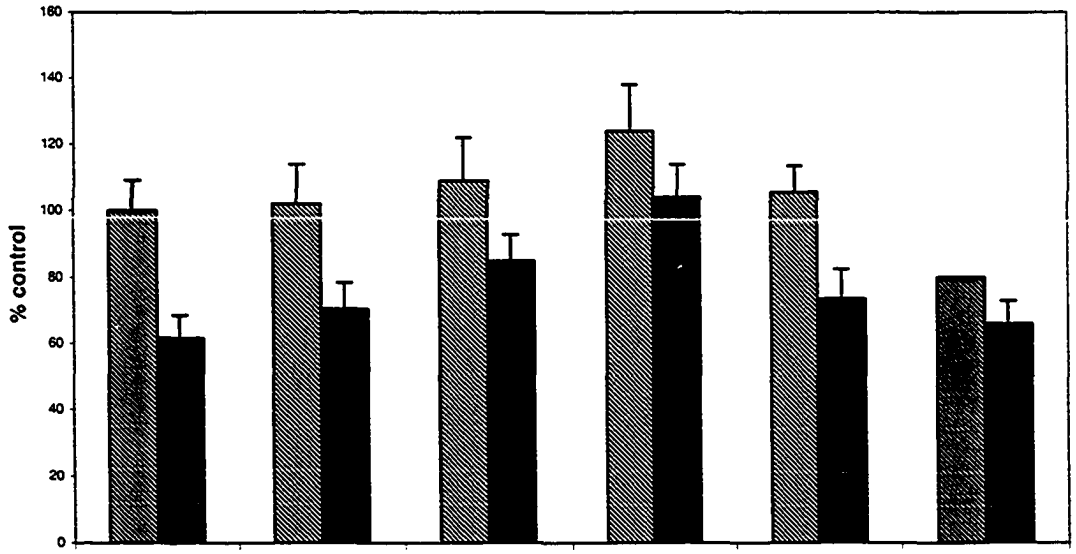


Figure 7: Subcutaneous injections of rolipram, in P30 rats, blocks the *in vitro* inhibition of neurite growth by MAG.

A single dose of rolipram was injected subcutaneously into P30 rats every 3 hours for 24 hours. The doses of rolipram ranged from 0.1 μmol/kg to 2 μmol/kg. Control animals were injected with a mixture of DMSO and saline following the same schedule. The DRG neurons from treated animals were isolated and plated onto a monolayer of MAG expressing CHO cells (solid bars) or onto a monolayer of control CHO cells (striped bars). Neurons were then cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites. The length of the longest neurite per neuron , from 180-200 neurons, was measured and the results show are the average length +/- SEM, expressed as a percentage of the length of the neurite growth on control CHO cells.

4.2.3 Time course of rolipram effects when delivered by subcutaneous injection.

In former experiments, we reproduced the conditions of priming that were previously studied in culture (see chapter III), whilst in the latter set of experiments we elucidated the time course of the effects of rolipram treatment. In order to do this we used the most effective dose of rolipram for P30 rats (0.5 μmol/kg), as determined in the previous experiment. The first group of animals received two injections of rolipram, three hours apart, then, 18 hours later DRG neurons were isolated and plated onto monolayers of either control CHO cells or CHO cells expressing MAG. The results of this experiment are labeled "6 hours" on the Fig. 8. The second group of animals received an injection of rolipram every 3 hours, for 9 hours, and DRG neurons were isolated one hour after the last injection. The results of this experiment are marked "10 hours" on the Fig.8. The third group was injected with rolipram every 3 hours for 24 hours. DRG neurons were isolated one hour after the last injection. The results of this experiment are labeled "24 hours" on the Fig.8. In addition, two groups of animals were each injected for either 2 or 3 days diurnally, the results are labeled as "48 hours" and "72 hours", respectively. These experiments show that the effects of rolipram are indeed time-dependent (Fig.8). The MAG- induced inhibition of neurite outgrowth was only partially blocked in DRG neurons isolated from animals treated with rolipram for less then 24 hours. However, treatment of rats with rolipram for 24 hours proved to be the most effective in blocking MAG inhibition. There were no clear benefits in treating the animals with rolipram for 48 and 72 hours, compared with the treatment for 24 hours. However, it is technically difficult to inject animals every 3 hours for 3 days. Later, we repeated this experiment with constant delivery of rolipram using mini-pumps and found that

the effects of rolipram on blocking inhibition by MAG and in improving the general growing capacity of DRG neurons are indeed time-dependant.

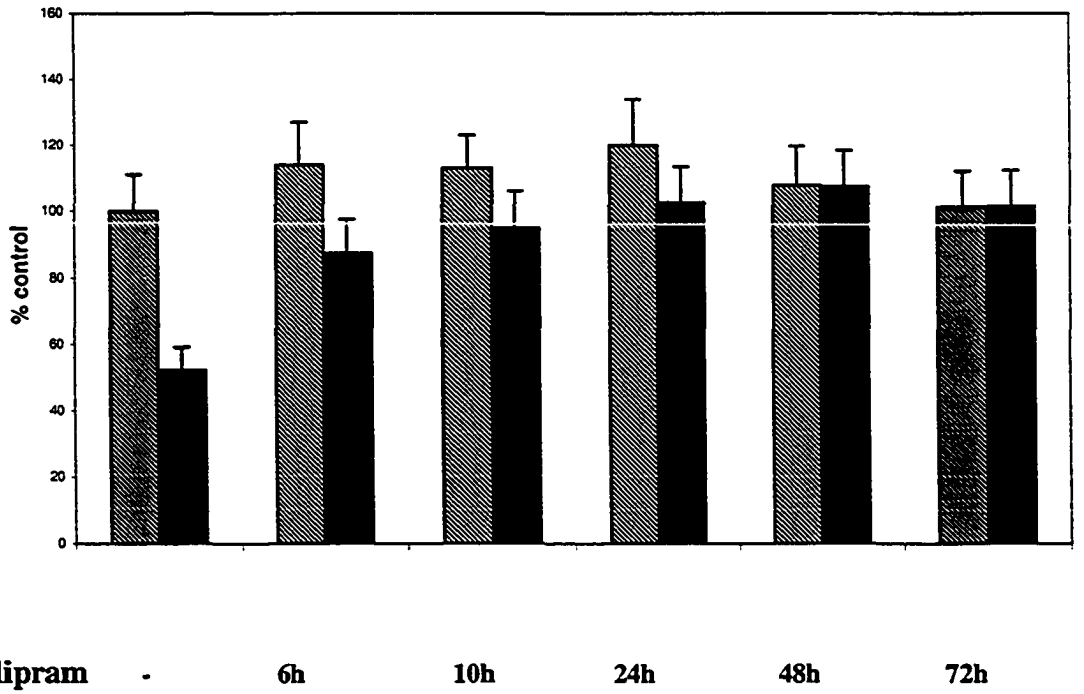
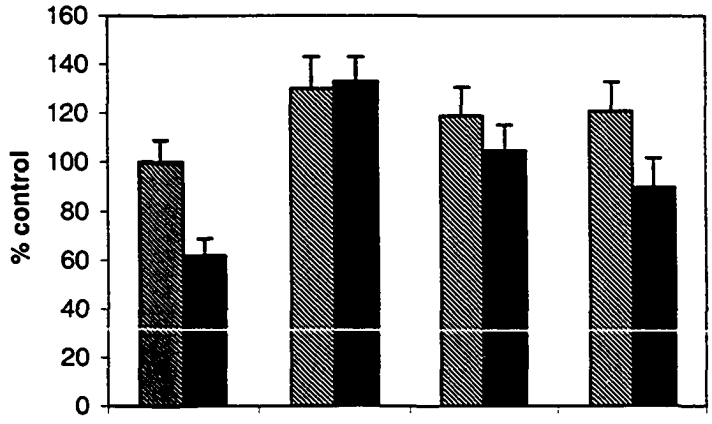


Figure 8. The time couse of rolipram effects in blocking inhibition by MAG following subcutaneous injections

Rolipram was dissolved in DMSO and sterile saline was added to adjust the concentrations of the dose to 0.5 μmol/kg. Rolipram was delivered by subcutaneous injection for indicated time period. Control animals were injected with a mixture of DMSO and saline following the same schedule and isolated DRG neurons were plated onto a monolayer of MAG expressing CHO cells (solid bars) or onto a monolayer of control CHO cells (striped bars). Neurons were cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites. The length of the longest neurite per neuron, from random 180-200 neurons was measured and the results show are the average length +/- SEM, expressed as a percentage of growth of the neurons isolated from control animals on control CHO cells, without dbcAMP or rolipram.

4.2.4 Rolipram delivered by minipump blocks inhibition of neurite outgrowth by MAG.

The delivery of rolipram by minipump establishes a stable concentration of the drug in the body. This is particularly important in the case of rolipram, since, as we have shown in the experiments *in vitro*, its effects depend very much on concentration. In the following series of the experiments we used minipumps to deliver rolipram subcutaneously into P30 rats. Since rolipram is not soluble enough to deliver sufficient dose of the drug using one minipump, two minipumps were used per animal, producing combined flow rate of 2μl/hour. Minipumps were placed under the skin of the animal's backs according to the instructions of the manufacturer. The concentrations of rolipram were adjusted to deliver the following doses of rolipram 0.4 μmol/kg/h, 0.5 μmol/kg/h or 0.7 μmol/kg/h. After 24 hours of treatment, DRG neurons were isolated and plated onto monolayers of CHO cells expressing MAG or control CHO cells. Minipumps with appropriate mixtures of DMSO and saline were inserted subcutaneously as controls. There was no detectable difference in neurite outgrowth of isolated DRG neurons between control animals and untreated animals (results are not shown). The experiment showed that the effects of rolipram, when delivered by minipump, are dose-dependant and the most effective dose is 0.4 μmol/kg/hr. No side-effects were observed following 24 hours of treatment (Fig. 9).



rolipram - 0.4μmol/kg/h 0.5 μmol/kg/h 0.7 μmol/kg/h

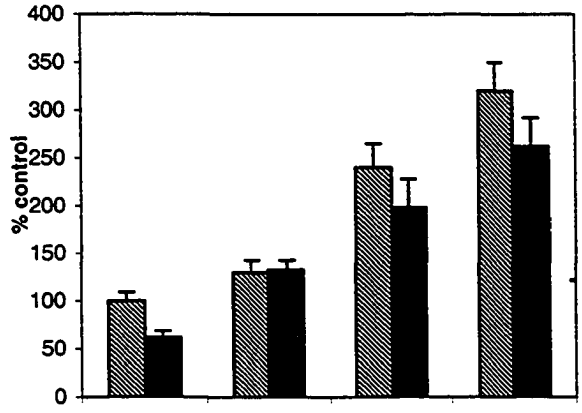
Figure 9. Rolipram, delivered subcutaneously by minipump, blocks inhibition of neurite outgrowth by MAG, in vitro.

Rolipram was delivered subcutaneously with ALZET 2001 minipumps. After 24 hours of treatment, DRG neurons were isolated and plated onto a monolayer of MAG expressing CHO cells (solid bars) or control CHO cells (striped bars). Neurons were then cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites. The length of the longest neurite per neuron, from random 180-200 neurons was measured and the results show the average neurite length +/- SEM, expressed as a percentage of neurite growth, from neurons isolated from control animals.

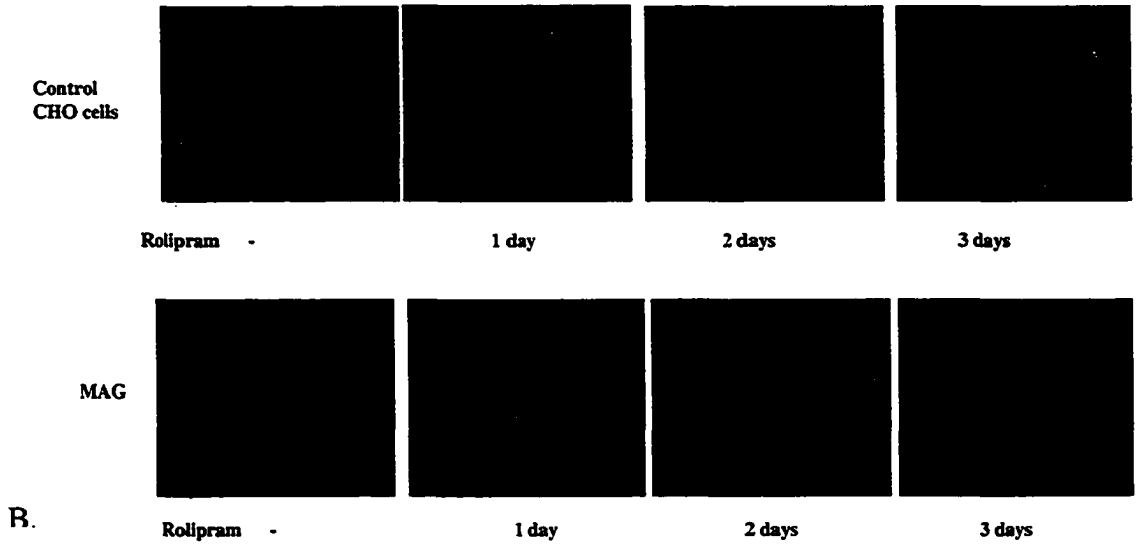
4.2.5 Prolonged treatment with rolipram improved the general growth capacity of the neurons.

In the following series of experiments, we studied the effects of prolonged treatment with rolipram on the ability of DRG neurons, isolated from treated animals, to grow in the presence of MAG. We inserted minipumps that delivered optimum concentration of rolipram as determined by previous experiments (0.4μmol/kg/h).

After one, two and three days of continuous rolipram delivery, DRG neurons were isolated and plated onto monolayers of CHO cells expressing MAG and control CHO cells. The results show that after one day of treatment neurite growth of isolated neurons was no longer inhibited by MAG, and after two and three days of treatment the DRG neurons isolated from treated animals grew much longer on monolayers of both control CHO cells and MAG expressing cells (Fig.10).



A. Rolipram - 24 hours 48 hours 72 hours



B.

Figure 10. Rolipram delivered subcutaneously by minipumps blocks inhibition of neurite outgrowth by MAG, *in vitro*. Time course.

Rolipram was delivered subcutaneously by ALZET 2001 minipumps, with a combined flow rate of 2ul/hour. Rolipram was dissolved in DMSO and sterile saline was added so that an adjusted dose of 0.4 μM/kg/hr was administered. After 1 day, 2 days and 3 days of treatment DRG neurons were isolated and plated onto monolayers of MAG expressing CHO cells (solid bars) or control CHO cells (striped bars). The neurons were cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites. (B). The length of the longest neurite per neuron, from random 180-200 neurons was measured and the results show the average length +/- SEM, expressed as a percentage of growth of the neurons isolated from control animals (A).

4.2.6 Rolipram delivered subcutaneously via minipump, blocks inhibition of neurite outgrowth by myelin.

Myelin associate glycoprotein (MAG) is one of three known inhibitors of neurite outgrowth present in myelin, and although it was recently demonstrated that two other inhibitors found in myelin, Nogo and OMgp, share the same receptor as MAG, it is possible that there are other, as yet unidentified, inhibitory molecules. Here we demonstrate that the treatment of rats with rolipram blocks the general myelin induced inhibition of neurite outgrowth. DRG neurons isolated from animals treated with rolipram delivered by minipump for one and two days (we used the most effective dose, as determined by previous experiments, 0.4μmol/kg/hour) were plated onto slides coated with myelin. We found that DRG neurons isolated after one day after rolipram treatment were able to extend processes on myelin, unlike DRG neurons from the control animals. DRG neurons isolated after two days of treatment with rolipram grew even better on myelin. (Fig 11).

Figure 11. Rolipram, delivered subcutaneously by minipump, blocks inhibition of neuronal outgrowth by myelin, *in vitro*. Time course. Rolipram was delivered subcutaneously by ALZET 2001 minipumps, with a combined flow rate of 2µl/hour. Rolipram was dissolved in DMSO and sterile saline was added so that an adjusted dose of 0.4 µmol/kg/hr was administered. After 1 day and 2 days of treatment DRG neurons were isolated and plated onto myelin coated slides. Neurons were cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites Minipumps with saline/DMSO solution were inserted subcutaneously into the control animals for the same period of time as minipump with rolipram.

4.2.7 Effects of different doses of rolipram in Fisher rats.

In our laboratory, Long Evans hooded rats are routinely used experimentally. In other laboratories different strains of laboratory rat are used. The most common strain used in regeneration experiments being Fisher rats, which have a lower body weight. Therefore, to determine if different strains of rat have a different optimal dose of rolipram, we found the most effective dose of rolipram for Fisher rats. In this series of experiments 6 week old female Fisher rats were used, with the most effective dose for these animals, 0.3 μmol/kg/hour. This is slightly lower than the most effective dose for Long Evans rats (Fig.12). We conclude here that the effects of rolipram on blocking inhibition by MAG and myelin, is highly sensitive to the dose of rolipram. Therefore, for each strain and age of the animals the correct dose must be determined.

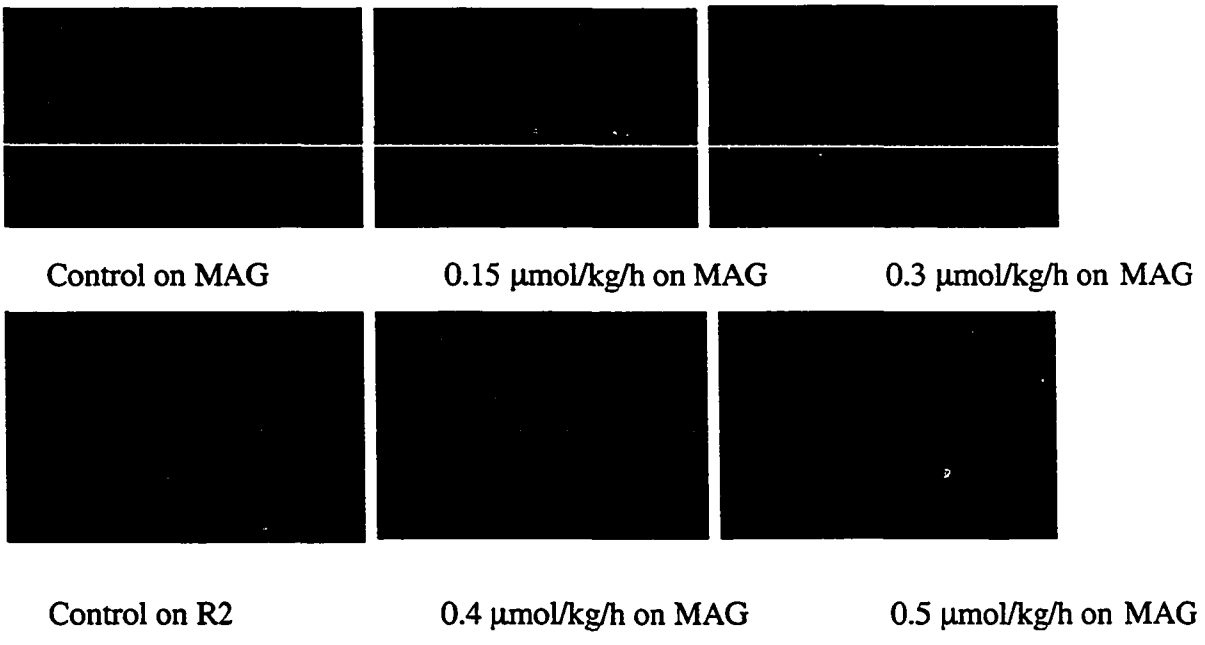


Figure 12. Rolipram delivered subcutaneously to the Fisher rats by minipumps blocks inhibition of neurite outgrowth by MAG *in vitro*. Rolipram was delivered subcutaneously with ALZET 2001 minipumps, with a combined flow rate of 2μl/hour. Rolipram was dissolved in DMSO and sterile saline was added so that adjusted doses of 0.15,0.3,0.4 and 0.5 μmol/kg/hour were administered. After 2 days of treatment DRG neurons were isolated and plated onto MAG expressing or control CHO cells. Neurons were cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites Minipumps with saline/DMSO solution were inserted subcutaneously into the control animals for the same period of time as minipumps with rolipram.

4.2.8 After two days of rolipram treatment improved neurite outgrowth from isolated DRG neurons becomes PKA- and transcription-independent.

In conditionally lesioned DRG neurons and in DRG neurons injected with db-cAMP two phases of improved growth are observed. During the first phase, DRG neurons are not inhibited by MAG and myelin but this block of inhibition is abrogated when inhibitors of PKA or transcription are added to the media. In contrast, in the second phase, these inhibitors are unable to abrogate the block of inhibition by MAG or myelin(Qiu et al., 2002).

Here we show that rolipram, when delivered subcutaneously to rats induces both the early PKA- and transcription-dependent phase of growth, as well as the late PKA- and transcription-independent phase of blockade of MAG's inhibition of neurite outgrowth. Postnatal day 30 rats were treated with rolipram, delivered by minipump at a dose of 0.4 μmol/kg/hour. After 1, 2 or 7 days of treatment DRG neurons were isolated and plated onto monolayers of either MAG-expressing CHO cells or control CHO cells. Where indicated, the specific inhibitor of PKA, KT 5720 at 200nM, or the transcription inhibitor, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at a concentration of 7 μM, was added to the media for the duration of the 18 hour culture period. As can be seen on Fig.13, after 1 days of rolipram treatment addition of PKA and transcription inhibitors abrogate the effects of rolipram treatment, the isolated DRG neurons are inhibited by MAG. In contrast, after two days of treatment (results not shown) and after 7 days of treatment, the improved growth of the neurons on MAG expressing cells is not affected by the presence of PKA and transcription inhibitors.

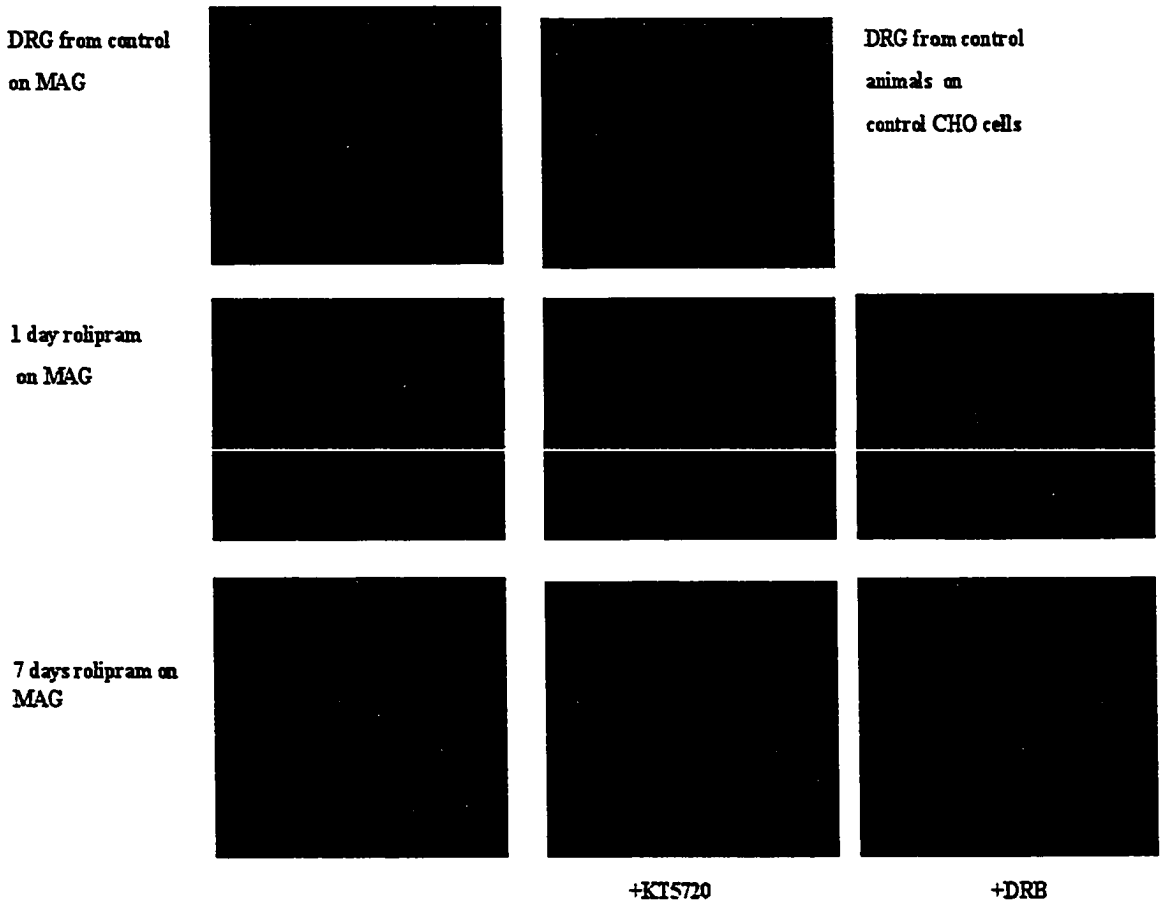


Figure 13. Blockade of inhibition of neurite outgrowth by MAG after 1 day of treatment with rolipram is PKA and transcription dependent but after two days it becomes PKA and transcription independent. Rolipram was delivered subcutaneously by ALZET 2001 minipumps, with a combined flow rate of 2µl/hour. Rolipram was dissolved in DMSO and sterile saline was added so that an adjusted dose of 0.4 µmol/kg/hr was administered. After 1 day and 7 days of treatment DRG neurons were isolated and plated onto monolayers of MAG expressing CHO cells or control CHO cells (not shown). Where indicated, inhibitor of PKA, KT 5720 (200nM) or inhibitor of transcription, DRB (7µM) was added to the media. Neurons were cultured overnight before being fixed and immunostained for GAP43 in order to visualize the neurites. Minipumps with saline/DMSO solution were inserted subcutaneously into the control animals for the same period of time as minipumps with rolipram.

4.2.9 Side effects of prolonged rolipram treatment.

Postnatal day 30 rats tolerated treatment with rolipram well. We did not notice any side effects following a short period of treatment. However, the behavior of rats treated for one week was different from that of their untreated littermates. They were more active and they did not gain bodyweight, in contrast with their control littermates, which gained 15-25 gram in 7 days. This must be considered a side effect, since normally rats of this age grow fast. No other behavioral abnormalities were observed.

4.3 Discussion.

In the present study, we show that raising cAMP levels in neurons using a specific inhibitor of PDE4, rolipram, blocks the inhibitory effects of MAG and myelin, resulting in the improved ability of the CNS neurons to grow in the presence of MAG and myelin. This data further supports the hypothesis that was developed by Filbin and coworkers over the years. Elevated cAMP levels in neurons, during development (Cai et al., 2001)or as a result of treatment with neurotrophins or cAMP analogues (Cai et al., 1999) dictates the ability of neurons to extend neurites in the presence of MAG and myelin in culture and to regenerate *in vivo* (Qiu et al., 2002).

We demonstrated that rolipram, when delivered subcutaneously, blocks the inhibition of neurite outgrowth from cerebellar and DRG neurons from P14 rats as well as DRG's from P30 rats. The effects of rolipram are dose-dependant and time-dependant. We also determined that the most effective dose of rolipram for younger animals is lower than for older ones. Therefore, the effects of rolipram are also age-dependent. This conclusion is in accordance with previously described changes in PDE4 expression, during development (Davis et al., 1989; Zhang et al., 1999).

Our data demonstrate that treatment of animals with the specific inhibitor of PDE4, rolipram, when delivered subcutaneously for one day, resulted in complete block of inhibition of axonal growth by MAG and myelin. Prolonged treatment with rolipram significantly promoted (up to three fold) the general ability of DRG neurons to grow, both in the absence and in the presence of myelin and MAG. The effects of rolipram treatment closely resemble the effects seen with the conditioning lesion (Neumann and Woolf, 1999)

and artificial elevation of cAMP levels by injection of db-cAMP into DRG (Neumann et al., 2002; Qiu et al., 2002). Similar to these two models, the effects of rolipram follow a two-stage time course. During the first stage, approximately 18 hours after the beginning of treatment, neurons are no longer inhibited by MAG, but inhibitors of PKA or transcription, when added to the media, abrogated rolipram-induced block of axonal inhibition by MAG. However, after two days of treatment these inhibitors no longer affect the improved growth on MAG and myelin. Thus, we have very good reasons to believe that treatment with rolipram will be as effective in promoting regeneration of lesioned axons as a conditioning lesion or dbcAMP injections. Furthermore, it is highly likely that treatment with rolipram will be more effective because the effects of rolipram are not restricted to prelesioned axons or to specific DRGs injected before the lesion.

Since publication of the paper on the effects of a conditioning lesion on the ability of DRG neurons to regenerate into the CNS, this model has been extensively studied. The methodology of these studies is tedious. To begin with, the sciatic nerve has to be cut, then, only the L4, L5 and L6 DRG's can be collected from each animal. If treatment with rolipram is proven to be as effective, in the promotion of axon regeneration, as conditioning lesion; the task of studying the molecular processes involved in potentiation of regeneration would be much simplified.

It is an ongoing debate in the field of CNS regeneration, whether inhibitors associated with damaged myelin or the proteoglycans of forming glial scar, are the major impediments of regeneration in the damaged CNS. Successful experimental models of regeneration such as the conditioning lesion model, injection of dbcAMP and preimmunization with myelin proteins serve as an indicator that, at least during the initial stage, disrupted myelin is the

main obstacle to regeneration. This then suggests that there is a window of opportunity before formation of the mature glial scar blocks the possibility of regeneration completely. Now it appears that this window of opportunity became wider. From the publication of other groups it seems clear that elevated cAMP levels can block the proliferation of astrocytes (Dugan et al., 1999) and fibroblasts (Hermann et al, 2001), which are the main contributers to the process of glial scar formation. The experiments of Bregmann and coworkers support this notion. They reported that in animals treated with rolipram, less staining for glial marker GFAP was observed. This could indicate that the glial scar formation is impeded by rolipram treatment.

Few treatments have been attempted in humans after spinal cord injury, largely because there is usually some residual function, as a result of spared axons. Surgeons therefore are reluctant to attempt any intervention at the injury site, in order to avoid further damage, causing the loss of what little function may remain. Most of developing strategies to cure spinal cord injuries involve local interventions at the site of injury. For example, grafts of Schwann cells (Guest et al., 1997), grafts of ensheathing glial cells (Li et al., 1997; Ramon-Cueto et al., 1998) and grafts of genetically modified cells expressing neurotrophins (Menei et al., 1998; Tuszynski et al., 1998). However, because of the above-mentioned reasons, these strategies will be difficult to implement. It is especially difficult in the first hours after injury when the amount of damage is particularly hard to estimate, but on the other hand it is very important to start treatment in the initial hours after injury before glial scar formation. An ideal treatment would then be one that is minimally invasive, preferably one that could be administered sub-cutaneously or intravenously, with a drug that is capable of crossing the blood brain barrier and reaching the site of injury. In this regard treatment

with rolipram, if proven to be clinically effective, is an extremely promising approach to the treatment of spinal cord injuries.

Chapter V.

BDNF transiently downregulates activity of PDE4 and elevates cAMP levels in neurons in a MEK-dependent manner.

5.1 Introduction.

In the damaged adult mammalian CNS myelin is a strong inhibitor of axonal regeneration. It inhibits axonal outgrowth of adult neurons both in vivo and in vitro. It was demonstrated by Filbin's group (Cai et al., 1999), that cerebellar neurons incubated overnight (priming) in the presence of BDNF or glial-derieved neurotrophic factor (GDNF), were no longer inhibited by MAG or myelin. The same effect was demonstrated for DRG neurons incubated overnight with BDNF, GDNF or nerve growth factor (NGF). Importantly, the mechanism for the priming with neurotrophins was suggested. It was shown that the cAMP levels of the neurons were elevated within 30 minutes of treatment with neurotrophins, and if inhibitors of PKA were added to the media the priming effect was completely blocked. Artificial elevation of cAMP levels via addition of db-cAMP also blocks inhibition of axonal outgrowth by MAG and myelin. Interestingly, priming was not required for abrogating the inhibitory effect of MAG or myelin when neurons were treated with db-cAMP. Our laboratory has begun to study the mechanism involved in the block of MAG or myelin inhibition via the priming of neurons with neurotrophins.

In recent studies conducted in our laboratory (Gao et al, submitted), it became evident that BDNF, in the course of priming, transduces its effects via the high affinity BDNF receptor TrkB and not through the p75 receptor. An initial indication that the effect was not via p75 came from the observation that NGF has no effect on the MAG/myelin-induced inhibition of cerebellar neurons (Cai et al., 1999), which express p75 but not TrkA (Holtzman et al., 1992; Martin-Zanca et al., 1990). Furthermore, addition of a p75 antibody, at a concentration known to block neurotrophin binding (Huber and Chao, 1995) had no effect on priming with NGF or BDNF. When activation of the Trk receptors is blocked with the Trk-

specific kinase inhibitor, K-252a, the priming effect is abrogated. The fact that the TrkB receptor is phosphorylated in response to treatment with BDNF further supports the notion that the priming effect is mediated through the Trk receptors. In the same set of experiments, it was found that activation of a MAPK, ERK1/2, plays a crucial role in the induction of the priming effect. Within 10 min of BDNF treatment, ERK is phosphorylated and this activation is sustained for at lease 120 min. The effect is specific because an inhibitor of MEK, which is responsible for ERK activation, blocks this phosphorylation (Gao et al, submitted).

Elevation of cAMP following BDNF treatment, however, could not be explained in terms of the Trk-family tyrosine kinase receptor known signaling pathways (Kaplan and Miller;2002). Since it was previously reported that activation of the MAP kinase pathway by tyrosine kinase receptors could result in inactivation of PDE4 and subsequent elevation of cAMP levels (Baillie et al., 2000; Hoffmann et al., 1999; MacKenzie et al., 2000), we decided to investigate if these same interactions take place during priming with BDNF. The PDE4 subfamily represents the most abundant PDEs in neuronal tissue (~70%) (Jin et al., 1999). They are cAMP-specific and are specifically inhibited by the drug, rolipram (Houslay and Kolch, 2000). Long splice isoforms of PDE4B and PDE4D, but not of PDE4A, are inhibited by ERK2. Short isoforms of these enzymes, however are not inhibited by ERK (Houslay M.D.,2001). Importantly, neurons are highly enriched in long isoforms of PDE4B and PDE4D(Bolger et al., 1997). Thus, the phenomena discovered in non-neuronal cells, namely, PDE4 inactivation and subsequent elevation of cAMP levels by ERK, could be especially important for neurons. Here, we present data that suggests that the total activity of PDE is transiently decreased in the course of BDNF treatment in an ERK-dependent manner. Moreover, the rolipram–insensitive portion of total PDE activity in the neurons remains

unchanged during a one-hour period, which suggests that PDE4 is solely responsible for this effect.

These findings imply that inhibition of PDE4 allows cAMP to increase and initiates the pathway, which leads to a block of inhibition of neurite outgrowth by MAG or myelin. The effect of ERK on the cAMP signaling pathway is a novel cross-talk mechanism in neurons. It is especially important for the understanding of mechanisms that allow neurons treated with neurotrophins to overcome inhibition by MAG and myelin, since activation of both the cAMP and MAPK signaling pathways were proven to be necessary.

5.2 Results.

5.2.1 The abrogation of the priming effect of BDNF, by MEK inhibitors, is overcome by low concentrations of rolipram.

It has been shown in our laboratory (Gao et al, submitted) that when inhibitors of MEK are added to the media during priming with neurotrophins, the block of inhibition by MAG is abrogated. We decided to check if application of a low concentration of the PDE4 inhibitor, rolipram, (priming with which by itself at this low concentration is not efficient to block inhibition by MAG), could restore the priming effect of BDNF blocked by the MAP kinase inhibitor. Neurons were primed as described above. Where indicated, BDNF at 200ng/ml, MAP kinase inhibitor (U0126) at 1 μM and rolipram at 0.1μM were added to the media while priming.

As can be seen on the Fig. 14, neurons incubated with BDNF overnight were not inhibited by MAG. When the MEK inhibitor, U0126 was added to the media during priming, the effect of BDNF was abrogated. Priming overnight with rolipram at a low concentration (0.1μM) alone did not improve neurite outgrowth in the presence of MAG. However, when neurons were primed with BDNF, MAP kinase inhibitor and this low concentration of rolipram, inhibition of the neuronal outgrowth by MAG was blocked. We conclude that rolipram at this low concentration restores the priming effect of BDNF blocked by the MAP kinase inhibitor.

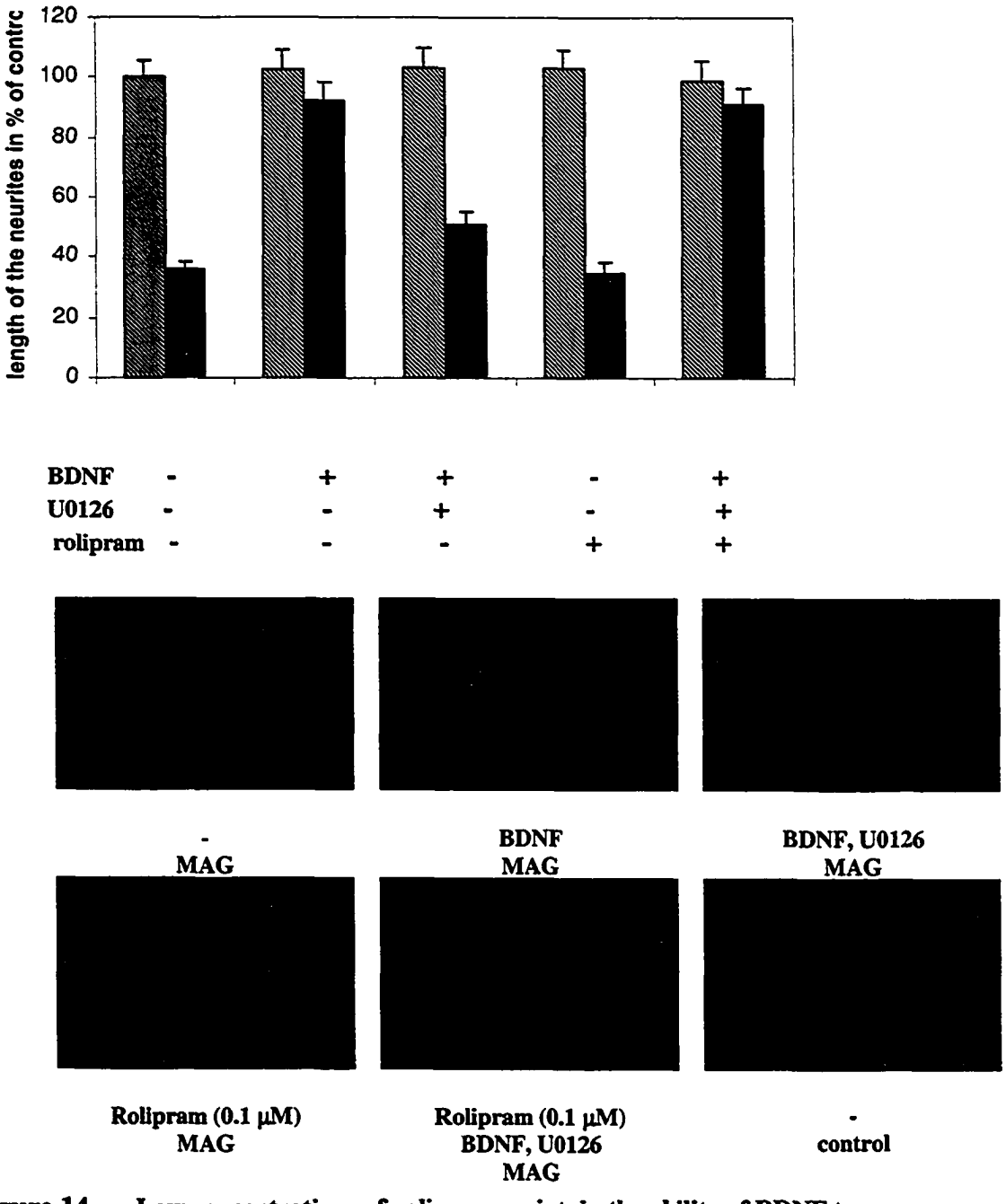


Figure 14. Low concentrations of rolipram maintain the ability of BDNF to overcome inhibition by MAG in the presence of an ERK inhibitor.

Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with BDNF at 200 ng/ml in the presence or absence of MEK inhibitor U0126 at 1μM and with or without rolipram at 0.1μM, as indicated, before being transferred to a monolayer of either MAG-expressing (solid bars) or control CHO cells (striped bars). Cells were cultured overnight before being fixed and immunostained for GAP-43. Neurite length was measured. Results are standardized to percentage of control. Control was taken as average neurite length from neurons grown on control CHO cells in the absence of BDNF and inhibitors.

5.2.2 The abrogation of the effect of db-cAMP, by MEK inhibitors, is overcome by low concentrations of rolipram.

Importantly, MEK inhibitors abrogate the ability of db-cAMP to block inhibition by MAG. It was demonstrated by Stork's group that elevation of cAMP by forskolin or hormones causes activation of ERK (Stork and Schmitt, 2002).

We addressed the question of whether a low dose of the PDE4 inhibitor rolipram, at a concentration of 0.1 μM, (which by itself did not block the inhibition of neurite outgrowth by MAG) will restore the effect of db-cAMP abrogated by MEK inhibitor.

Neurons were treated as described above. Where indicated, db-cAMP at 1mM, rolipram at 0.1μM and MAP kinase inhibitor (U0126) at a concentration of 1μM were added directly to the media. As it is shown in Fig.15, db-cAMP at 1mM completely blocks inhibition of neurite outgrowth by MAG. The inhibitor of MEK, U 0126 at 1μM abrogates the effects of db-cAMP and as before, rolipram, at a low concentration of 0.1 μM when added alone did not improve neurite outgrowth in the presence of MAG. However when this low concentration of rolipram was added to the media together with db-cAMP and the MEK inhibitor, the neurons were not inhibited by MAG. The ability of db-cAMP to block inbhibition by MAG was restored.

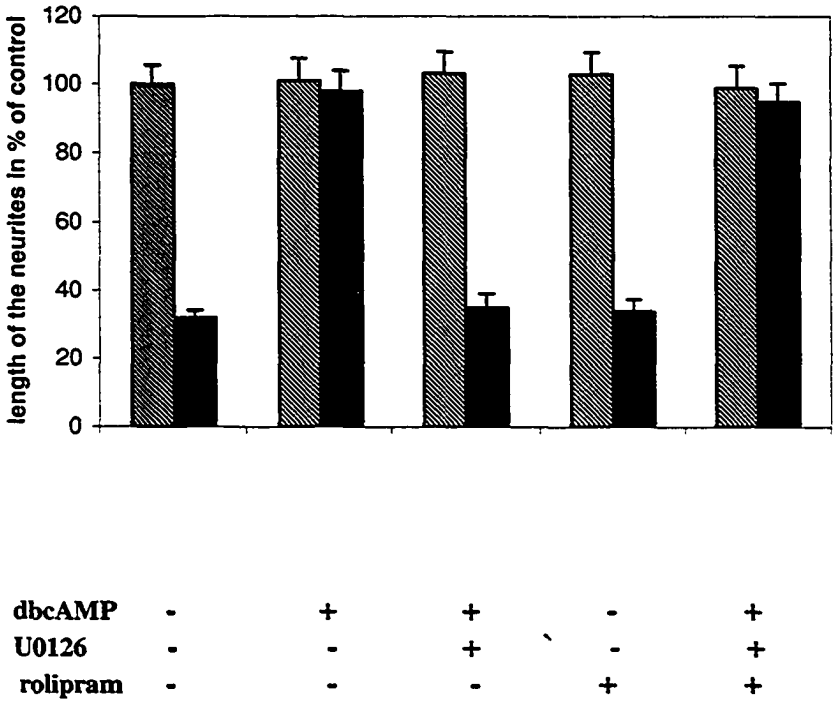


Figure 15. Low concentrations of rolipram maintain the ability of dbcAMP to overcome inhibition by MAG in the presence of an ERK inhibitor.

Dissociated cerebellar neurons were plated onto a monolayer of either MAG-expressing (solid bars) or control CHO cells (striped bars). Where indicated, dbcAMP at 1mM, the MEK inhibitor U0126 at 1 μM and rolipram at 0.1μM were added to the media. Neurons were cultured overnight before being fixed and immunostained for GAP-43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for random 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of dbcAMP and inhibitors.

5.2.3 Priming neurons with rolipram blocks inhibition by MAG and is not affected by ERK inhibitors.

If inactivation of PDE4 by MAP kinase is a direct downstream effect of priming with BDNF, then direct inhibition of PDE4 by rolipram should mimic the activation of the MAP kinase pathway by neurotrophins and inhibitors of MAPK should not block its effect. We assessed if MAPK inhibitors could abrogate priming with rolipram to block MAG inhibition of neurite outgrowth. As it is shown in Fig 16, priming with rolipram of cerebellar neurons at a concentration of 0.5 μM, was as effective as priming with BDNF in blocking inhibition by MAG. Although addition of the inhibitor of MEK, U0126 at 1 μM, does not block the priming effect of rolipram on inhibition of neurite outgrowth by MAG, it does abrogate the priming effect of BDNF. This result strongly supports our model, namely, that initial activation of ERK by tyrosine kinase receptor Trk B causes inactivation of PDE4 and subsequent elevation of cAMP. The fact that MAPK inhibitors do not block the effects of rolipram on MAG inhibition suggests that PDE4 is a downstream target of MAPK.

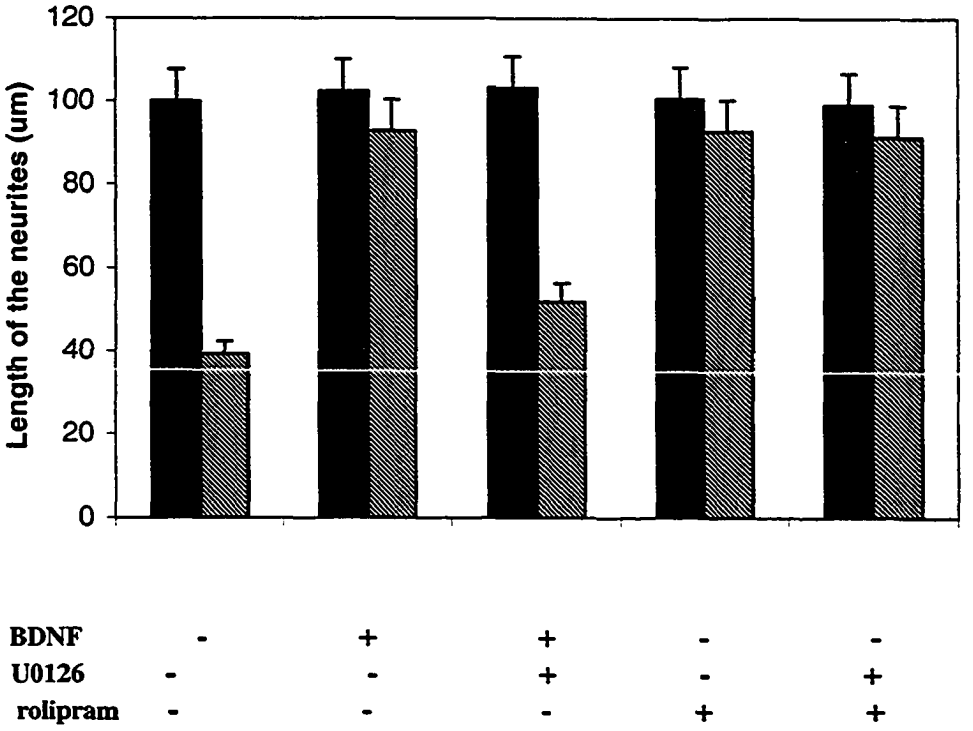


Figure 16. MEK inhibitor does not abrogate the priming of cerebellar neurons with rolipram to overcome inhibition by MAG.

Dissociated cerebellar neurons were first primed overnight on poly-l-lysine with rolipram at 0.5 μM or BDNF at 200 ng/ml in the presence or absence of MEK inhibitor U0126 at 1 μM, as indicated, before being transferred onto a monolayer of either MAG-expressing (solid bars) or control CHO cells (striped bars), and cultured overnight before being fixed and immunostained for GAP-43. Neurite length was measured. Results show the mean length of the longest neurite per neuron (+/- sem) for 100 individual neurons. Results are standardized to percentage of control. Control was taken as neurite length from neurons grown on control CHO cells in the absence of BDNF and inhibitors.

5.2.4 BDNF inhibits PDE activity and elevates cAMP in a MEK-dependent manner.

To determine if BDNF affects PDE activity directly, cerebellar neurons were treated with BDNF, with and without the MEK inhibitor U0126. After varying lengths of time, the cells were lysed and the PDE activity was measured. As can be seen in Fig. 17a, within 5 minutes of treatment with BDNF, the PDE activity in cerebellar neurons has decreased by about 35%. This decrease is sustained for almost 1 hour. In sharp contrast, when the neurons are exposed to BDNF in the presence of U0126, there is no decrease in the PDE activity; activity is the same as in control, untreated neurons. However, this assay measures total PDE activity. As reported by others, we show that about 70-80% of the activity in neurons is rolipram-sensitive, PDE4 activity. In order to differentiate between the two, we examined the effect of BDNF on non-PDE4 isoforms. The non-PDE4 activity (20-30% of total PDE activity) does not decrease in the presence of BDNF. Therefore, the decrease in PDE activity in response to BDNF is entirely due to an effect on PDE4. Therefore, treatment of neurons with BDNF results in an inhibition of PDE4 which can be blocked by a MEK inhibitor.

Previously, we showed that BDNF increases the endogenous levels of cAMP in cerebellar neurons. Now, we wanted to determine if this increase in cAMP was MEK/ERK-dependent. Cerebellar neurons were treated with BDNF with or without the MEK inhibitor, U0126. As can be seen in Fig. 17b and as we had reported before (Cai et al., 1999), after BDNF treatment, the levels of cAMP in cerebellar neurons almost doubled. However, when U0126 is included, this increase in cAMP does not occur. Together, these results show, first, that BDNF can inhibit PDE4 activity and, second, that both the BDNF-induced inhibition of PDE4 and the subsequent increase in cAMP are MEK-dependent.

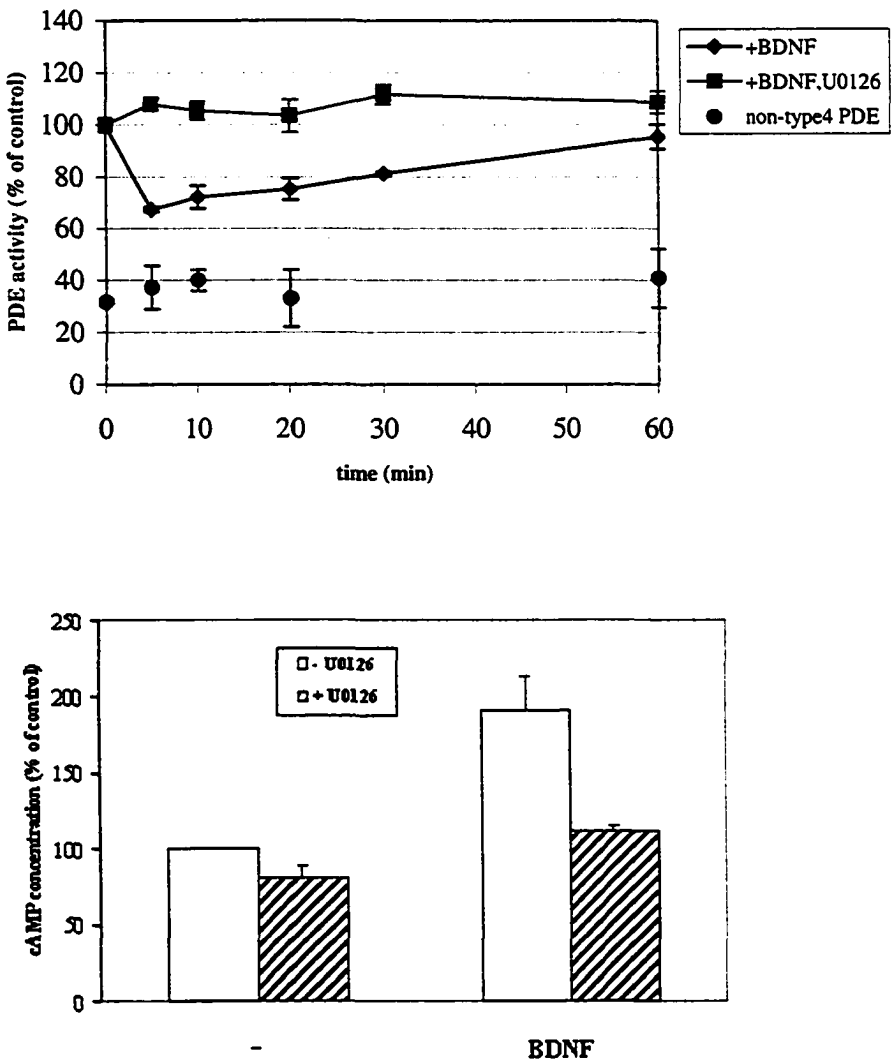


Figure 17: BDNF induces a transient inhibition of PDE4 and increases cAMP in an Erk-dependent manner:

a) Cerebellar neurons were incubated with 200 ng/ml BDNF, alone (filled squares) or in the presence of U0126 , 1μm, (open squares), or in the presence of 0.5 μM rolipram, (filled circles) for various times before being lysed and PDE activity measured. Results are expressed as a percentage of PDE activity in untreated neurons (+/- SE). For each experiment, samples were performed in triplicate and the experiments were repeated at least 3 times. (b) cAMP levels were measured using a competitive immunoassay after plating 200,000 dissociated cerebellar neurons per well and treating with and without BDNF, 200 ng/ml, in the presence (dark bars) or absence (white bars) of U0126 at 1 μM for 30 min. Results are the mean (+/- se) of at least 6 experiments, each carried out in quadruplet.

5.3 Discussion.

Neurons of the adult mammalian CNS can change their intrinsic ability to grow in the presence of inhibitors in damaged myelin. It was shown by Filbin and coworkers that a variety of neurotrophins were able to overcome inhibition of neurite outgrowth by MAG and myelin in a cAMP/PKA-dependent manner (Cai et al., 1999). In the course of further investigation, it was found that activation of Trk receptors, as well as ERK is crucial for the priming of neurons with neurotrophins (Gao et al., submitted). Activation of Trk receptors, and the MAPK pathway are the well characterized signaling pathway of neurotrophins (Kaplan and Miller, 2000), but the mechanisms by which neurotrophins could elevate cAMP levels in the neurons had not been documented before. Here, we show that it is cross-talk between two pathways that is responsible for the neurotrophin-induced, Trk-dependent, increase in cAMP that overcomes inhibition. As has been shown before, ERK is activated in response to neurotrophins, but now rather than signaling directly to transcription factors such as cAMP-response-element binding protein (CREB), we show that activated ERK also inhibits PDE and so increases cAMP levels and PKA is in turn activated. To our knowledge, this is the first time that activation of ERK has been shown to activate cAMP-dependent pathways in neurons.

We demonstrate here, both pharmacologically and biochemically, that downregulation of PDE4 activity by activated ERK is a crucial step in the BDNF transduction pathway in the priming of neurons. When an inhibitor of MAPK, U0126, was added to the media during priming with BDNF to block the inhibition of neurite outgrowth

by MAG, the priming was abrogated. When the specific inhibitor of PDE4, rolipram, at a low concentration that alone was unable to prime neurons to overcome inhibition by MAG, was added together with the MAPK inhibitor, the priming effect of BDNF was completely restored. Even more surprising to us was the finding that MAPK inhibitors were able to block the effects of db-cAMP. The same results were observed when instead of db-cAMP we used forskolin, a drug, that by stimulation of adenylyl cyclases raises the level of cAMP in cells. Consistent with the results of the experiments with db-cAMP, when inhibitors of MEK were added together with forskolin, the block of MAG inhibition was abrogated. Rolipram at a concentration of 0.1 μM restored the ability of forskolin to block inhibition by MAG in the presence of MEK inhibitors (results are not shown). The fact that a low concentration of rolipram was able to restore both the effects of db-cAMP and forskolin further supported the idea that the cross-talk between the MAPK and cAMP pathways occurs via a regulation of PDE4 activity. It was demonstrated by several groups that ERK is activated when forskolin or hormones are used to increase cAMP levels (Stork and Schmitt, 2002). This is suggested to occur when elevated cAMP activates the cAMP-dependent, PKA-independent G-protein exchange factors, Epac-1 and Epac-2, which in turn activate the Rap1/B-raf pathway, resulting in sustained activation of ERK (de Rooij et al., 2000; Grewal et al., 1999). We did not detect significant activation of ERK in response to db-cAMP treatment, or when we raised the levels of cAMP in neurons using forskolin. Although at the concentration of forskolin used in the experiments of Stork's group we could indeed detect activation of ERK, as can be seen in Fig.18 (Gao et al, submitted). Furthermore, as we reported before, db cAMP overcomes inhibition by MAG in a dose-dependent manner. At a concentration of 1 mM, inhibition by MAG is completely blocked. However, when the MEK inhibitor U0126 is

included in the assay, the dose response curve for db cAMP is shifted to the right (Fig. 19). In the presence of U0126, db cAMP at 1mM has little effect on inhibition by MAG. It is not until a concentration of 1.5-2 mM that db cAMP completely blocks inhibition by MAG in the presence of the MEK inhibitor (Fig. 11). Importantly, it was demonstrated by Hoffman and colleagues, that upon phosphorylation by PKA, PDE4D increases its specific activity and heightens its sensitivity to inhibition by rolipram. It was reported that the IC_{50} value of PDE4D, phosphorylated by PKA, was decreased about six-fold to the level of 0.1 μM (Hoffmann et al., 1998). This data suggest that rolipram at a low concentration of 0.1μM is not sufficient to inhibit PDE4's in their unphosphorylated state but at this concentration it efficiently inhibits PKA-activated PDE4. Activity and rolipram sensitivity of PDE4 phosphorylated by both PKA and by ERK are similar to those of unstimulated PDE4. These results suggest that at a concentration of 1 μM db cAMP, it is both the added db cAMP and the basal levels of endogenous cAMP that together reach a threshold required to overcome inhibition by MAG. When a MEK inhibitor is added, it blocks downregulation of PDE4 by MAPK, PDE4 become activated by PKA and combined cAMP levels decrease, and, as a result, the pathway to block MAG inhibition can not be initiated. Low concentrations of rolipram effectively inhibit PKA-activated conformation of PDE4, allowing cAMP levels to elevate and initiate the pathway to block inhibition by MAG.

Importantly, priming with rolipram at a concentration of 0.5μM, is not blocked by MAPK inhibitors, further supporting the notion that downregulation of activity of PDE4 is downstream of MAPK.

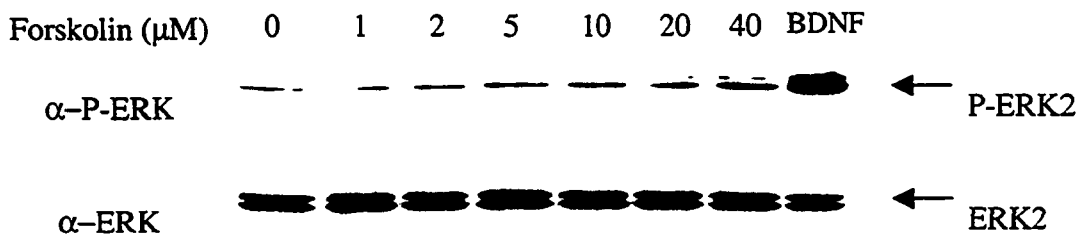


Figure 18. Forskolin activates ERK in a dose-dependent manner: Cerebellar neurons were incubated with forskolin at the concentrations from 0μM to 40 μM; for 20 min before being lysed and subjected to western blotting and probed for activated ERK (P-ERKs). Lysate of neurons incubated with BDNF at 200ng/ml and treated in the same manner serves as a positive control. The blots were stripped and re-probed for total ERK.

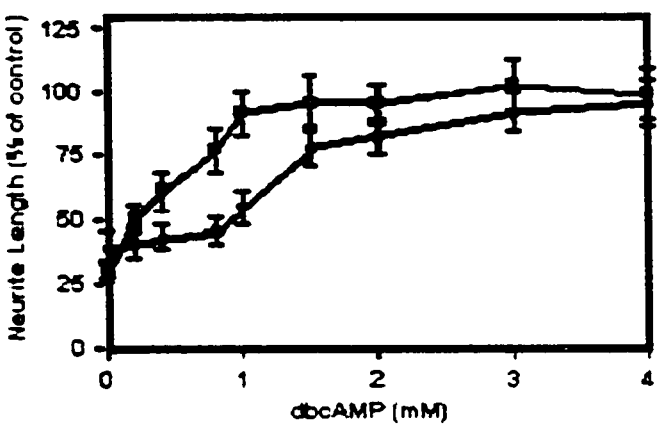


Figure 19: A threshold of cAMP must be reached to overcome inhibition by MAG.

Db-cAMP was added directly to cultures of cerebellar neurons (0.1-4 mM) with (filled squares) or without (closed squares) MEK inhibitor,U0126 at 1μM, before being transferred to MAG-expressing CHO cells (stripped bars) or control CHO cells (white bars). After overnight culture the cells were fixed and immunostained for GAP43. Results are the mean length of the longest neurite per neuron (+/- sem) for random 180-200 individual neurons, standardized to percentage of length on control CHO cells.

Here, we report that total PDE activity in the neurons following BDNF treatment decreases quite sharply, within the first 5 minutes after addition of BDNF. This decrease in PDE activity is dependent on activation of ERK. Addition of a specific inhibitor of ERK, U0126, completely blocks the effect of BDNF on PDE activity in neurons. The PDE4 subfamily of PDE's, is responsible for this effect, because we found that the rolipram-insensitive portion of total PDE activity is not changed by BDNF treatment. Direct measurements of cAMP levels showed that the levels of cAMP were increased twofold in response to BDNF, consistent with the data presented previously by our laboratory(Cai et al., 1999). We demonstrate for the first time that this elevation of cAMP levels is dependant on activation of ERK.

The mechanism we describe here was first proposed for non-neuronal cells and was studied extensively in vitro and in cell lines exogenously expressing different isoforms of PDE4 (Baillie et al., 2000; Hoffmann et al., 1998; MacKenzie et al., 2000). To our knowledge, this is the first time that ERK-dependant inactivation of PDE4 activity and subsequent elevation of cAMP levels has been demonstrated in primary neuronal cultures. To understand the transduction mechanisms of neuronal cells, this link is especially important. Interestingly, it can be an underlying mechanism for the switch from Ras-c-Raf dependant activation of ERK to Rap1-B-Raf-dependent activation of ERK, which occurs during neurotrophin signaling in the phaeochromocytoma cell line (PC12) differentiation model. Treatment of the PC12 cells with nerve growth factor (NGF) leads to differentiation of the cells into a phenotype that resembles sympathetic neurons. In the course of studies of NGF-

induced PC12 cell differentiation, it was found that ERK is activated initially by the Ras –c-Raf pathway, but sustained activation of ERK was dependant on activation of the Rap-1-B-Raf pathway (Kao et al., 2001; Wu et al., 2001; York et al., 1998).

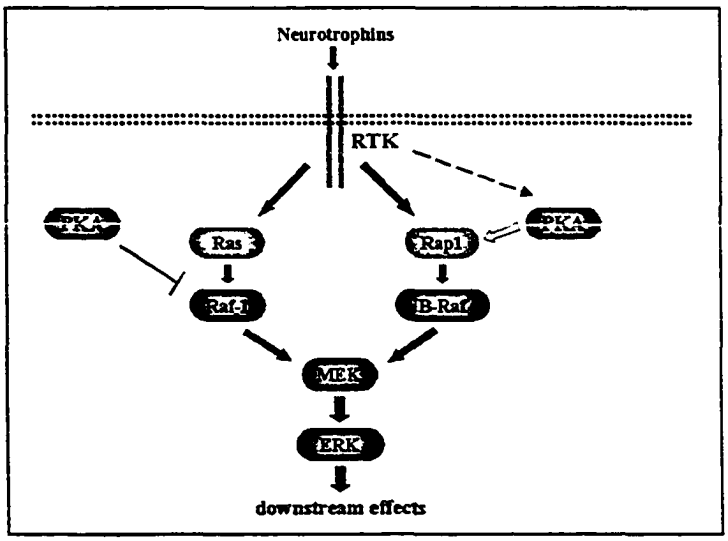


Figure 20. Ras- and Rap-1dependent ERK signaling cascades. (Adapted from (Grewal et al., 1999)

Intriguingly, Rap1 was first identified as a suppressor of the Ras-induced transformation of NIH3T3 cells (Kitayama et al., 1989). It inhibits, in a cAMP-dependant manner, activation of the Ras-c-Raf –ERK pathway. It was recently shown that Rap1 can act as a positive regulator of ERK in cells where B-Raf is present (Catling et al., 1994). It was also demonstrated that B-Raf is a primary MEK activator in the brain (Wu et al., 2001). Furthermore, sensory and motoneurons from B--Raf-deficient mice do not respond to neurotrophic factors for their survival (Wiese et al., 2001). Rap1, the only identified activator of B-Raf, is a member of Ras-family of small GTPases, and functions as a molecular switch that, when activated, shuts off the Ras-c-Raf activation of ERK and turns on B-Raf-dependant activation of ERK. Kinetic studies are consistent with consecutive activation of

Ras-cRaf and Rap1-B-Raf pathways during NGF-induced signal transduction pathways (Kao et al., 2001; Wu et al., 2001). The mechanism that activates Rap1in the course of NGF-induced PC12 differentiation is still unidentified. Importantly, Rap-1 is phosphorylated and subsequently activated by PKA (Vossler et al.,1997; York et al.,1998). Rap1 can be also activated in a PKA-independent but cAMP-dependant manner by guanine nucleotide exchange factors directly activated by cAMP, Epac1 and Epac2. Importantly, Epac2 has been found to be highly enriched in neurons (de Rooij et al., 1998; Kawasaki et al., 1998). We propose here that downregulation of activity of PDE4 by MAPK, which is activated by "classical tyrosine kinase receptor cascade" through Ras-c-Raf, and the subsequent elevation of cAMP levels causes activation of the Rap1-B-Raf pathway and inhibition of the Ras-c-Raf pathway. Unlike the Ras-c-Raf pathway, the Rap1-B-Raf pathway is not inhibited by elevation of cAMP, therefore causing sustained activation of ERK that could last for one hour after the receptor was initially activated.

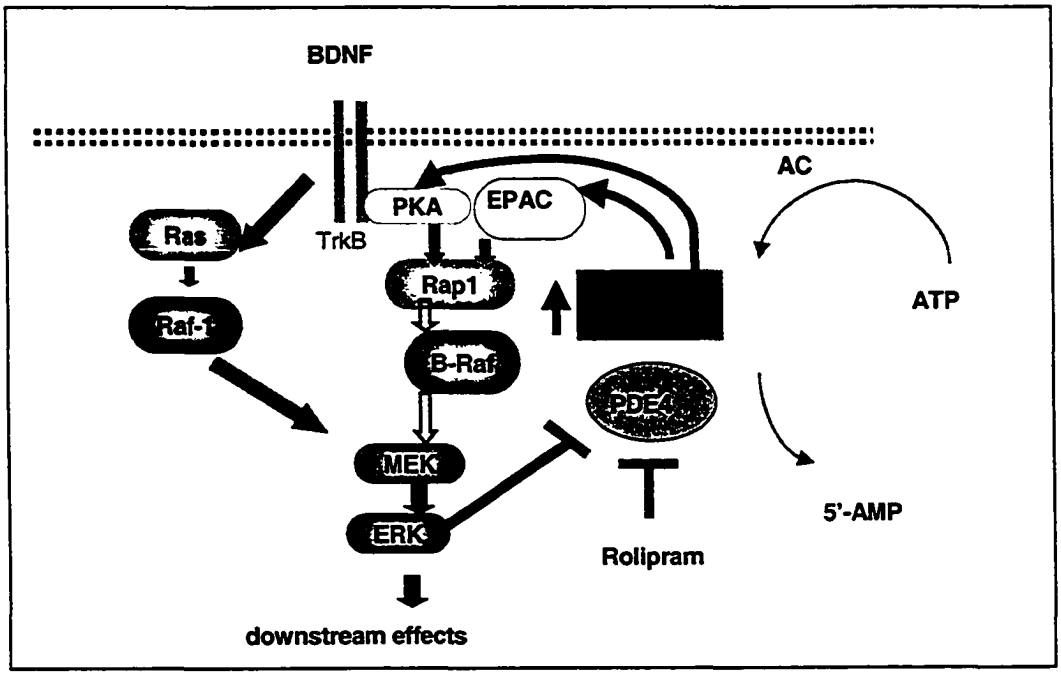


Figure 21. Activation of TrkB-Ras-Raf-1-ERK pathway causes transient downrwgulation of PDE4, elevation of cAMP and subsequent prolonged activation of ERK .

We propose the following model to explain our results (Fig.21). Upon activation of the TrkB receptor by its ligand, BDNF, the TrkB-Ras-c-RAF pathway activates and downregulates activity of PDE4. It leads to elevation of the cAMP level in neurons, which activates the exchange factor for Rap1, EPAC. Subsequently, Rap1in its active, GTP-bound state activates B-Raf, which results in prolonged activation of ERK and downstream effects, such as phosphorylation of CREB and transcription of the genes that confer upon neurons an ability to grow in the presence of myelin inhibitors. This model explains the previously reported elevation of neuronal cAMP level in response to neurotrophins (Cai et al., 1998). It also explains an underlying mechanism for the reported switch from early Ras-cRaf activation of ERK to the later and prolonged Rap1-B-Raf dependent activation of ERK in the course of PC12 cells differentiation upon NGF treatment (Wu et al., 2001).

Chapter VI.

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