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**Studies on the role CREB as a mediator of neurotrophin-3 actions in
oligodendrocytes**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

| | |
|---------------|--|
| BDNF | brain-derived neurotrophic factor |
| bFGF | basic fibroblast growth factor |
| CamK | Ca ²⁺ -calmodulin-dependent kinases |
| CBP | CREB-binding protein |
| cdks | cyclin-dependent kinases |
| CDM | chemically defined medium |
| CNPase | 2',3'-cyclic nucleotide 3'-phosphodiesterase |
| CNS | central nervous system |
| CRE | cyclic AMP response element |
| CREB | cyclic AMP response element binding protein |
| CREM | cyclic AMP response element modulator |
| DAG | <i>sn</i> -1,2-diacylglycerol |
| db-cAMP | dibutyryl cyclic AMP |
| DMEM/Ham F-12 | Dulbecco's modified Eagle's medium |
| EAE | experimental autoimmune encephalomyelitis |
| E-NCAM | embryonic neural cell adhesion molecule |
| Erks | extracellular signal-regulated kinases |
| GC | galactocerebroside |

| | |
|----------|--|
| GFAP | glial fibrillary acidic protein |
| Grb2 | growth factor receptor-bound protein 2 |
| H-89 | PKA inhibitor |
| HBSS | Hanks' balanced salt solution |
| HRP | horse radish peroxidase |
| ICER | inducible cAMP early repressor |
| IGFs | insulin-like growth factors |
| KN-62 | CamK II, IV and V inhibitor |
| LY294002 | PI3 kinase inhibitor |
| MAG | myelin-associated glycoprotein |
| MAPK | mitogen activated protein kinase |
| MEK | MAPK/Erk kinase |
| MBP | myelin basic protein |
| MOG | myelin-oligodendrocyte glycoprotein |
| MS | multiple sclerosis |
| NCAM | neural cell adhesion molecule |
| NGF | nerve growth factor |
| NT-3 | neurotrophin-3 |
| NT-4/5 | neurotrophin-4/5 |
| NT-6 | neurotrophin-6 |
| O2-A | OLGs/ type-2 astrocytes |
| OLGs | oligodendrocytes |

| | |
|--------------|------------------------------------|
| PBS | phosphate buffer saline solution |
| PC12 | pheochromocytoma cell line |
| PCNA | proliferating cell nuclear antigen |
| PD098059 | MEK inhibitor |
| PDGF | platelet-derived growth factor |
| PI3K | phosphatidylinositol 3-kinase |
| PKA | cAMP-dependent protein kinase |
| PKC | protein kinase C |
| PNS | peripheral nervous system |
| PLC γ | phospholipase C gamma |
| PLP | proteolipid protein |
| RSK | ribosomal S6 kinase |
| SDS | sodium dodecyl sulfate |
| SH2 | Src homology 2 domain |
| Sos | son of sevenless protein |
| TCA | trichloroacetic acid |
| Trk | receptor tyrosine kinases |

ABSTRACT

STUDIES ON THE ROLE OF CREB AS A MEDIATOR OF NEUROTROPHIN-3 ACTION IN OLIGODENDROCYTES

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A thesis submitted in partial fulfillment of the requirements for the degree of M.S. at Virginia Commonwealth University.

Virginia Commonwealth University, 2000

Thesis Director: Carmen Sato-Bigbee, PhD., Assistant Professor, Department of Biochemistry and Molecular Biophysics

In the central nervous system (CNS), oligodendrocytes (OLGs) are the cells responsible for producing the myelin membrane which allows for the saltatory conduction of neuronal impulses. We have previously shown that CREB (cAMP response element binding protein), a transcription factor that belongs to a large family of bZip (basic leucine zipper) proteins, could be a mediator of neuronal signals that, coupled to different signal transduction pathways, may play different regulatory roles at specific stages of oligodendrocyte development. We have found before that, in committed OLGs, CREB activation by phosphorylation can be triggered by β -adrenergic stimulation and appears to play a role in the induction of OLG differentiation by cAMP. In contrast, in OLG precursor cells, CREB phosphorylation is stimulated by neuroligands that increase

calcium levels by a process that involves a mitogen activated protein kinase (MAPK)/protein kinase C (PKC) pathway. This observation suggested that, at this early developmental stage, CREB could play a role in regulating cell proliferation. In support of this hypothesis, we have now found that a rapid and dramatic stimulation of CREB phosphorylation is one of the earliest events that precedes the increase in cell proliferation that is observed when OLG precursors are treated with neurotrophin-3 (NT-3). Moreover, our present results also showed that down-regulation of CREB expression in the OLG precursors abolished the increase in cell proliferation that is observed when the cultures are treated with NT-3. Experiments in which CREB phosphorylation was investigated in the presence of different kinase inhibitors indicated that the activation of this transcription factor in the presence of NT-3 is mediated by the concerted action of MAPK- and PKC-dependent signal transduction pathways. Additional experiments using specific inhibitors of protein kinase A (PKA), Ca^{2+} -calmodulin-dependent kinase (CamK) and phosphatidylinositol 3-kinase (PI3K) pathways suggested that these kinases may not play a significant role in mediating CREB phosphorylation by NT-3. However, further studies are required for more conclusive results about these kinases. Thus, our present results support the idea that stimulation of OLG proliferation by NT-3 involves the CREB transcription factor and its activation by MAPK- and PKC-dependent signal transduction pathways.

INTRODUCTION

The myelin membrane

The myelin sheath is a highly modified and specialized membrane structure that wraps around axons and allows for the saltatory conduction of neuronal impulses. The myelin membrane surrounds the axons in a spiral fashion to form a multi-lamellar structure (Fig 1). In the central nervous system (CNS), myelin is formed by the extended plasma membrane of the oligodendrocytes (OLGs). In the peripheral nervous system (PNS), however, the Schwann cells synthesize this membrane. The myelin membrane and the areas of the axon that are myelinated are collectively called the internodes. These internodes are separated by regions of the axon that are bare and are known as the nodes of Ranvier (Raine, 1984a).

In the PNS, the myelin membrane of each internode is made by the Schwann cell. During development the Schwann cells migrate with the peripheral nerve fibers which begin to be myelinated after reaching the diameter of 1-2 μm . A single Schwann cell will then wrap around an axon as the Schwann cell's cytoplasmic ridge folds around the axon and underneath its own membrane on the other side. This expanding portion of the membrane, called the mesaxon, continues to concentrically encircle the axon as the cytoplasm is extruded, thus condensing the membrane surfaces and producing compact myelin. By this process, the cell body of the Schwann cell remains closely apposed to the

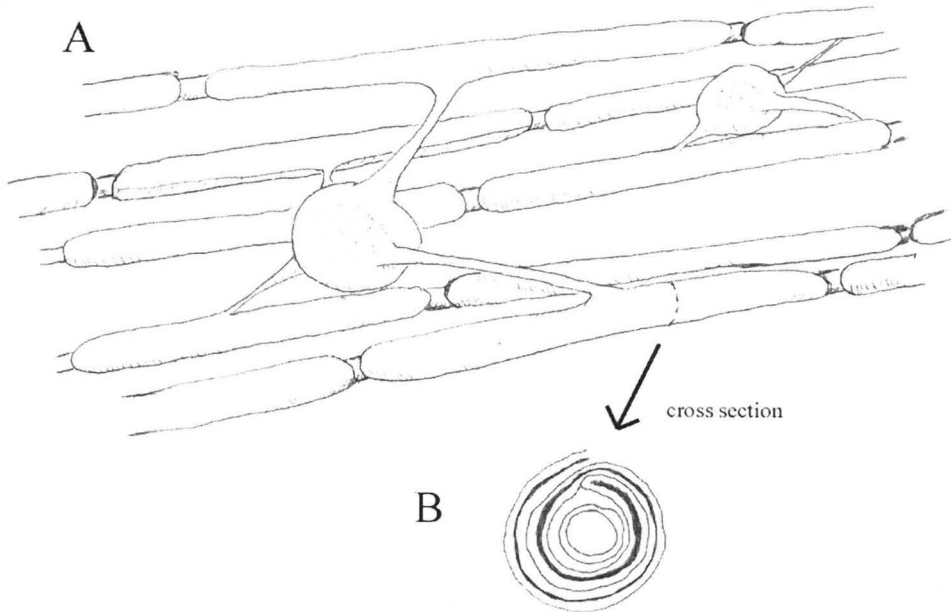


Figure 1. An illustration of OLGs myelinating axons in the CNS. (A) OLG cell body with many processes myelinating various axons. (B) Cross section of an axon with concentrically wrapped myelin membrane.

axon (Raine, 1984a).

The myelination process in the CNS by the OLGs is similar to that of the Schwann cell. However, the major difference is that OLGs have arborized processes that extend from their soma; it is the ends of these processes that come into contact with the axons forming the myelin membrane around them. By this process, each OLG in the CNS could myelinate 40 or more separate axons with its cell body being some distance away from the axons (Davidson et al., 1970).

The functional role of the myelin membrane, as an electrical insulator, was speculated as early as 1878 by Ranvier. Currently, it is accepted that the myelin membrane allows for the rapid form of neuronal impulse propagation known as saltatory conduction. In unmyelinated nerve fibers, the neuronal impulses are propagated in such a manner that a local circuit is created in the axonal membrane in which the resulting current travels by depolarizing the adjacent area of the membrane in a sequential and continuous fashion. However, during saltatory conduction, only the nodes of Ranvier participate in the depolarization while the myelin membrane insulates the rest of the axon. Furthermore, the sodium channels required for the depolarization are localized at the nodes of Ranvier (Waxman et al., 1993).

The presence of myelin not only saves energy by resulting in less sodium flux due to the localization of the sodium channels at the nodes of Ranvier, but also greatly

increases the conduction velocity of the neuronal impulse by producing a current that is saltatory. It also has space saving properties by decreasing the axonal diameter requirements which, in theory, should be proportional to the conduction velocity. To better understand this, if unmyelinated axons were to replace the myelinated ones, in order to maintain the same conduction velocity, the human spinal cord would have to be as thick as a tree trunk (Ritchie, 1984).

The myelin membrane is a particularly good insulator, not only because of its multi-lamellar structure, but also due to its composition, characterized by a high lipid to protein ratio, of about 80 to 20. Besides cholesterol, a major lipid component of myelin is cerebroside, also known as galactosylceramide. About one-fifth of these galactolipids also occur as sulfatides in which the 3-hydroxyl group on the galactose moiety of cerebroside is sulfated (Norton and Cammer, 1984). Because of their quantity, it was believed that these galactolipids were essential for myelin formation. However, a mouse knockout model lacking the last step in cerebroside biosynthesis revealed that the myelin formed was relatively normal and that these galactolipids perhaps played a role in myelin stability, not myelin formation (Coetzee et al., 1996). A minor component of myelin are gangliosides, which comprise approximately 0.1 to 0.3% of the total lipid. These are complex glycolipids in which the ceramide backbone is esterified to three or more sugar residues.

Myelin basic protein (MBP) is a major extrinsic membrane protein that exists

both in the CNS and PNS myelin, however, it is more abundant in the CNS myelin comprising about 30%-40% of the total protein (Lees and Brostoff, 1984). MBP exists on the cytoplasmic surface and is believed to play a role in myelin compaction forming a structure known as the major dense line resulting from the apposition of the cytoplasmic face of the plasma membrane after the extrusion of the cytoplasm. Evidence for this comes from studies of a line of mutant mice called shiverer (Chernoff, 1981). It was shown that MBP is specifically deficient in these mice (Dupouey et al., 1979), and that the major dense line in these animals is not evident, indicating uncompacted myelin (Privat et al., 1979).

There are several variants of MBP that range from 21.5 kDa to 14.1 kDa which result from alternative splicing of a single gene containing at least seven exons (Takahashi et al., 1985; Mentaberry et al., 1986; Roth et al., 1987; Newman et al., 1987). Although the functional roles of these variants are uncertain, changes in their ratios and differential expression suggest that some of these MBPs play a role during myelination. It has been suggested that the larger forms of MBP, 21.5 kDa and 17 kDa, which contain exon II may play an important role in the early period of myelin formation and/or OLG differentiation, since they are relatively more abundant at that time (Carson et al., 1983; Roth et al., 1987).

Proteolipid protein (PLP) is another major constituent of the CNS myelin, comprising about 50% of its total protein (Lees and Brostoff, 1984). Like MBP, PLP is

not exclusive to the CNS. However, in the PNS, PLP is expressed at much lower levels in the Schwann cells and is also restricted to the cell cytoplasm (Puckett et al., 1987). In the CNS, PLP serves as a 30 kDa integral membrane lipoprotein that probably participates in the formation of the intraperiod line, which results from the close apposition of the extra-cytoplasmic side of the adjacent plasma membranes in the concentric layers of myelin. Again, the evidence for this comes from a line of mutant mice called jimpy (Sidman, 1964) in which there are abnormalities in the intraperiod lines (Duncan et al., 1987).

Another protein component of myelin is the myelin-associated glycoprotein (MAG). Unlike MBP and PLP, MAG is expressed at low levels in the CNS and PNS myelin comprising about 1% of the total protein (Quarles, 1984) and is expressed at the periaxonal regions of the myelin sheath (Stemberger et al., 1979). MAG has a single transmembrane domain and five immunoglobulin-like domains, a structure similar to the one corresponding to the neural cell adhesion molecule (NCAM) (Salzer et al., 1987). In addition, treatment of cell cultures with anti-MAG antibodies blocks neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte adhesion (Poltorak et al., 1987). Thus, these observations support the notion that MAG serves as an adhesion molecule that may function in cell to cell signaling, perhaps directing the initiation of myelination. An interesting aspect of the cell to cell signaling properties of MAG is the fact that it may not only direct the myelination of an axon, but it may also control the growth of the axon itself as MAG is one of the molecules in the CNS that has been shown to inhibit neurite

outgrowth in tissue cultures (Mukhopadhyay et al., 1994).

Another minor protein component of CNS myelin is the myelin-oligodendrocyte glycoprotein (MOG) (Gardinier et al., 1992). Similar to MAG, MOG also contains an immunoglobulin-like domain. However, unlike MAG, MOG is localized on the surfaces of myelin and OLGs, which may indicate a function in transmitting extracellular information to the OLG interior. These protein components may play a role in certain pathologies of myelin. Much like MBP, MOG has been implicated as a target antigen in the autoimmune aspects of demyelinating neuropathies of the CNS such as multiple sclerosis.

Multiple sclerosis

Multiple sclerosis (MS) is a disease that currently afflicts an estimated 350,000 persons in the United States (Anderson et al., 1992) and is perhaps the most studied among a group of diseases of the CNS collectively known as demyelinating neuropathies characterized by a loss or damage of the myelin membrane. Consequently, one of the first neurological disabilities usually caused by MS is optic neuritis resulting in impaired vision. Symptoms can remit and relapse while recovery may become incomplete as the disease progresses. Further loss of neurological control may occur as sufferers of the disease possibly face being wheelchair bound or, in extreme cases, death.

Currently, it is believed that the etiology of MS involves a genetically predisposed individual who becomes stimulated, perhaps by viruses during the late childhood, to have immunological reactions against the antigens found in the CNS, in particular in the myelin membrane and/or OLGs (Sorenson et al., 1998). Mutant or abnormal genes have not been linked to MS, however, a susceptibility allele has been linked to both familial and sporadic MS. (Yaouanq et al., 1997).

Family studies have also revealed a genetic susceptibility component. First-degree relatives have a 20-fold increased risk compared to the population background (Sadovnick et al., 1993), while a non-related child adopted into MS families retains the population background level of developing MS. (Ebers et al., 1995). Also, siblings raised in separate households retain an equal chance of developing MS. (Sadovnick et al., 1996). Furthermore, the importance of genetics or perhaps other environmental factors is emphasized by the fact that Japan exhibits a lower occurrence of MS compared to other populations in similar latitudes (Hartung et al., 1990). Interestingly, if an individual moves from a high-risk area to a low risk area during childhood, he or she will acquire the low risk of developing MS. However, if that individual moves after adolescence, he or she retains the risk of the original location. (Kurtzke, 1977).

The idea that the onset of MS could be stimulated by viral infections stems from the notion that MS is an autoimmune/inflammatory disease. Support for this notion comes from studies of experimental autoimmune encephalomyelitis (EAE), an animal

model in which rats are immunized with components of the myelin membrane, i.e. myelin basic protein (MBP) (Raine, 1984b; Lassman, 1983). According to this model, T helper cells (CD4 positive, class II MHC restricted) recognize myelin antigens that are presented by cells of the macrophage lineage (microglia) and perhaps astrocytes resulting in inflammation that destroys myelin as well as damaging the OLGs.

It is believed that in humans these T cells are generated by processes of molecular mimicry in which viral or bacterial antigenic fragments closely resemble myelin components (Wucherpfennig et al., 1995). Furthermore, break down of the BBB is a characteristic component of MS pathogenesis, albeit it is unknown whether this is an initiating factor or a secondary event to inflammation. However, it has been shown that activated T cells are able to cross the BBB (Hickey, 1991) and is believed that they become resident in the CNS if they are specifically targeted to CNS antigens. The origins of the autoreactive T cells are not known, however the idea of molecular mimicry is supported by the studies finding that 129 bacterial and viral peptides were similar enough to MBP to trigger the activation of human T cell clones (Wucherpfennig et al., 1995). Lastly, although these mechanisms of inflammation in MS are conjectural, it has been shown that MS patients harbor autoreactive T cells (Hafler et al., 1985a; Hafler et al., 1985b; Allegretta et al., 1990; Allegretta et al., 1994).

Due to its complex pathogenesis, understanding and trying to find therapies for MS involves various research disciplines which include genetics, epidemiology,

neuropathology, immunology and virology. In this project, we have focused our attention on the signaling cascade mechanism(s) involved in regulating the proliferation of the OLGs. It is hoped that a better understanding of these mechanisms will eventually help to develop methods which will aid in replenishing the OLGs that are damaged and/or lost in the course of diseases like MS.

Oligodendrocyte development

OLGs continue to divide throughout life, although at a much slower rate than during CNS development (Kaplan and Hinds, 1980; McCarthy and Leblond, 1988). Therefore, one might assume that diseases like MS where OLGs are damaged and/or lost may be easily cured by simply replenishing the cells. Unfortunately, the healing process is neither so easy nor simple. However, knowing that these cells could potentially regenerate gives hope into the possibility of developing treatment for diseases such as MS. This has made the study of OLGs very important, because still little is known about the processes that regulate the proliferation and differentiation of these cells during normal CNS development.

OLGs originate in the late gestational and early postnatal period from multipotent neural stem cells which are present in the periventricular zone and can generate neuronal, astroglial and oligodendroglial progenitors (Davis and Temple, 1994; Marmur et al., 1998; Vescovi et al., 1999; Rogister et al., 1999; Tropepe et al., 1999). Cells of the OLG

lineage are initially identified in distinct regions of the ventricular and subventricular zone from where they migrate during differentiation (LeVine and Goldman 1988; Curtis et al., 1988; Warf et al., 1991). The different stages of differentiation can be distinguished by the sequential expression of different antigenic markers. Early proliferative precursors express the embryonic neural cell adhesion molecule (E-NCAM) (Hardy and Reynolds, 1991). These cells later develop into early OLG progenitors or O-2A cells which were originally identified in cultures of developing optic nerve.

The O-2A cells were appropriately named so because, depending on the culture conditions, they are able to differentiate into either OLGs or type-2 astrocytes. When cultured in serum-free, chemically defined medium (CDM), the O-2A cells develop into OLGs. However, when these cells are grown in medium containing 10% fetal bovine serum, they develop into cells called type-2 astrocytes which express glial fibrillary acidic protein (GFAP), an astrocytic marker (Raff, 1983). The existence of the type-2 astrocytes *in vivo* is controversial. Experiments in which O-2A cells were labeled, *in vitro*, with fast blue dye and transplanted into neonatal rat brain, showed that all labeled cells developed into OLGs (Espinosa de los Monteros et al., 1993). This observation indicates the necessity for careful extrapolations of data obtained from *in vitro* experiments to an *in vivo* environment.

The O-2A progenitors were first identified by using the A2B5 monoclonal antibody (Eisenbarth et al., 1979; Raff et al., 1983) known to react with several gangliosides (Kundu et al., 1983; Fredman et al., 1984; Majocha et al., 1989). These cells have a simple, bi- or tripolar morphology (Temple and Raff, 1986; Small et al., 1987),

express the intermediate filament vimentin (Raff et al., 1984), are very motile (Small et al., 1987) and proliferate rapidly with a cell cycle time of approximately 18 to 20 hrs (Noble et al., 1988). The O-2A cells later develop into the O4 positive progenitors characterized by their reactivity with the O4 antibody which recognizes cerebroside and sulfatides (Sommer and Schachner, 1982; Dubois-Dalcq, 1987). Further differentiation of these progenitors into the mature committed OLGs follows the orderly expression of galactocerebroside (GC) (Raff et al., 1979), the myelin enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (McMorris, 1983) and in a few days time, the myelin proteins MBP, PLP and MAG (Dubois-Dalcq et al., 1986).

The factors that control the proliferation and differentiation of these cells have been the topic of intense scrutiny. It has been shown that platelet-derived growth factor (PDGF) is one of the major mitogens (Noble et al., 1988; Richardson et al., 1988), as well as a chemoattractant (Armstrong et al., 1991) for OLG progenitors. Type-I astrocytes and neurons produce PDGF (Yeh, et al., 1991; Sasahara et al., 1991), which supports the idea for a regulatory role on OLG proliferation and differentiation *in vivo*.

PDGF is a dimer of a cationic glycoprotein (30 kDa) and in the human, there are two distinct, but related polypeptides called A and B chains (Betsholtz et al., 1986). PDGF A transcripts were found in the type-I astrocyte (Richardson et al., 1988) and compared to other dimers, the AA homodimer appears to be the most potent mitogen for O-2A cells compared to other dimers (Pringle et al., 1989). There are two classes of

PDGF receptors, the A form which can bind all three dimers, and the B form which has high affinity for the BB dimer, but low affinity for the AB (Hart et al., 1988; Heldin et al., 1988). Radiolabeled PDGF binding assays suggest that O-2A cells have the A type PDGF receptor (Hart et al., 1989). Interestingly, in the continued presence of PDGF, the O-2A precursors stop proliferating and acquire characteristics of mature OLGs (Noble et al., 1988; Richardson et al., 1988). However, this is not due to receptor loss, for the O-2A progenitor cells continue to express the PDGF alpha receptor (Hart et al., 1989).

Basic fibroblast growth factor (bFGF) has also been shown to be mitogenic for OLGs (Eccleston and Silberberg, 1985; Besnard et al., 1989) as well as astrocytes (Pettman et al., 1985; Kniss and Burry, 1988). bFGF not only causes the O-2A cells to undergo a high rate of proliferation (Noble et al., 1988), it also maintains high levels of PDGF receptors on the O-2A precursors (McKinnon et al., 1990). An interesting observation is the lack of MBP transcripts after treatment with bFGF (McKinnon et al., 1990), which may suggest an inhibitory role for bFGF on myelin gene expression and/or OLG differentiation. Consequently, the combination of PDGF and bFGF causes O-2A precursor proliferation for long periods without differentiation (Bögler, et al., 1990).

It has been shown that insulin and insulin-like growth factors (IGFs) are essential for the development of OLGs *in vitro* (van der Pal et al., 1988). A role of these factors *in vivo* have come from the fact that transcripts for insulin-like growth factors, IGF-I and IGF-II have been found in the CNS, with the highest levels of gene expression coinciding

with early neuronal development (E14-18) (Rotwein et al., 1988). IGF-I and IGF-II receptors have been detected in rodent brain as well as cultured astrocytes, OLGs, and O-2A progenitor cells (Gammeltoft et al., 1985; Balloti et al., 1987; Ocrant et al., 1988; McMorris and Furlanetto, 1989). The importance of these factors have been shown in that low levels of IGF levels result in hypomyelination *in vivo* (Phillips and Vaffilopoulou-Sellin, 1979; Wiggins, 1982). On the other hand, increased IGF expression is associated with increased myelination (Carson et al., 1988, McMorris et al., 1990).

OLGs contain high levels of iron, suggesting an important relationship between iron and the state/function of the OLGs (Connor and Menzies, 1995). Consequently, it has been shown that transferrin, an iron mobilizing protein is an essential factor for myelination by OLGs (Espinosa de los Monteros et al., 1999). Iron plays a direct role in lipid and cholesterol biosynthesis by acting as a required co-factor and may have a protective function by regulating oxidative stress (Connor and Menzies, 1996). The importance in understanding the combined information from the effects of all of these factors is seen in the advent of a chemically defined medium giving optimal conditions to grow OLGs in culture, thus enabling the further elucidation of factors that may regulate OLG development.

CREB, a transcription factor highly expressed in developing OLGs

Previous studies from this laboratory have shown that developing OLGs express

elevated levels of a transcription factor known as CREB (cyclic AMP response element binding protein) (Sato-Bigbee and Yu, 1993; Sato-Bigbee et al., 1994 Sato-Bigbee and DeVries, 1996).

CREB belongs to a large family of transcription factors characterized by the presence of a basic leucine zipper dimerization domain and their binding to a consensus nucleotide sequence TGACGTCA. This sequence is known as CRE (cyclic AMP response element) and it is present in the promoter region of cAMP and Ca²⁺ responsive genes (Montminy et al., 1990; Sheng et al., 1991). The ability of CREB to activate transcription is positively regulated by phosphorylation at a region called the P-box, specifically, a serine residue at position 133.

CREB is encoded by a large gene that generates several alternatively spliced transcripts (Hoeffler et al., 1990). However, the 43 kDa CREB and a form known as Δ CREB, which is missing the alternatively spliced 14 amino-acid segment called the α -region (Yamamoto et al., 1990), appears to be the predominant isoforms expressed in most of the tissues tested. Our previous results indicated that the CREB protein expressed in the OLGs contains the α -region (Sato-Bigbee et al., 1994). This is particularly important because this region interacts cooperatively with the phosphorylation motif which as indicated above is required for transcriptional activation. Moreover, it has been shown that the potency of Δ CREB as a transcriptional activator is 10-fold lower than that of CREB (Yamamoto et al., 1990).

Initially, CREB phosphorylation at Ser¹³³ was attributed to cAMP-dependent protein kinase (PKA) (Gonzalez and Montminy 1989). Thus, factors which elevate cAMP levels may result in the activation of PKA and the subsequent phosphorylation of CREB, leading to gene activation. However, later evidence indicated that CREB could be phosphorylated at Ser¹³³ by several other kinases including Ca²⁺-calmodulin-dependent kinases (CamK) (Sheng et al., 1991), protein kinase C (PKC) (Xie and Rothstein, 1995), and the ribosomal S6 kinase (RSK) (Xing et al., 1996), which as described later, is a target of the MAPK (mitogen activated protein kinase) pathway.

In addition to the P-box, other structurally important domains in CREB are the glutamine rich regions flanking the P-box which are believed to play a role in interacting with other components of the transcription machinery (Gonzalez et al., 1991). Of these two glutamine-rich domains (Q1 and Q2) that flank the P-box, Q2 appears to have more of a significant role in the activation of transcription, for the deletion of Q2 dramatically reduces CREB function (Brindle et al., 1993). Furthermore, CREB also requires a cofactor called CBP (CREB-binding protein). CBP is a 265 kDa protein that interacts with the phosphorylated P-box of CREB (Chrivia et al., 1993). The phosphorylation of Ser¹³³ on CREB promotes the binding of CBP, which mediates CREB interaction with the RNA polymerase II complex. For full activity however, it was demonstrated that the glutamine-rich region was necessary for its role in interacting with the general transcription factor TFIID (Nakajima et al., 1997).

As far as the negative regulation is concerned, it appears that the main mechanism attenuating transcriptional activation by CREB is dephosphorylation. CREB is dephosphorylated *in vivo* by protein phosphatase-1 (Hagiwara et al., 1992) and protein phosphatase-2 appears to have some activity as well (Wadzinski et al., 1993). Although the capacity of CREB to activate transcription is regulated by its phosphorylation, other factors can also have effects in regulating the function of this protein. While CREB stimulates transcription, certain forms of another transcription factor called CREM (cAMP response element modulator) work as competitive repressors (Foulkes et al., 1991), as does the ICER (inducible cAMP early repressor) proteins (Molina et al., 1993).

The CREM gene also generates a large family of alternatively spliced isoforms. CREM alpha, beta and gamma function as antagonists of CREB-induced transcription either by binding to CRE sites as homodimers or heterodimers, thus blocking activator binding to the CRE. This is possible due to the fact that structurally, these CREM isoforms are very similar to CREB as they also bind to the CRE sequence, but lack the glutamine rich domains required for transcriptional activation (Foulkes et al., 1991).

ICER, a truncated CREM product (Stehle et al., 1993; Molina et al., 1993), is transcribed from an alternative promoter within an intron of the CREM gene and acts as a powerful repressor of CREB-induced transcription. ICER, which actually negatively autoregulates its own promoter (Molina et al., 1993), is able to heterodimerize with the CREM proteins as well as with CREB (Stehle et al., 1993). Thus, the fact that many of

these different CRE-binding factors are able to heterodimerize with each other (Hai et al., 1989) makes the study of these proteins and their mechanism of action very complicated.

Possible role of CREB in developing OLGs

Studies from this laboratory in which CREB expression was analyzed in OLGs directly isolated from rat brain at different stages of development (Sato-Bigbee and Yu, 1993; Sato-Bigbee et al., 1994) or in neonatal OLGs that were allowed to differentiate in vitro (Sato-Bigbee and DeVries, 1996), indicated that this protein is highly expressed in cells that are still MBP negative and have a typical morphology of immature OLGs. However, CREB expression decreases to background levels of detection in cells that express MBP and exhibit highly branched processes characteristic of mature OLGs. This pattern of expression suggested that in the OLGs, CREB plays a role in a developmental window that precedes the period of active myelination.

While the activity of transcription factors like CREB occurs in the nucleus of the cell, the stimuli which activate such transcription factors are usually extracellular in origin. Thus, interaction of these external stimuli with the cells by means of specific ligand-receptor mechanisms that are coupled to different signal transduction cascades, are able to elicit changes in gene activity that regulate cell development.

Recent results from this laboratory have suggested that CREB could be a mediator of neuronal signals that, coupled to different signal transduction pathways, may play

different roles at specific stages of OLG development (Sato-Bigbee et al., 1999a). We have found before that in committed OLGs, CREB activation by phosphorylation at Ser¹³³ can be triggered by the beta-adrenergic agonist isoproterenol, which is known to increase cAMP levels in these cells. In this regard, we have previously found that treatment of young but already committed OLGs with db-cAMP (a cell permeable analogue of cAMP) resulted in stimulation of MBP expression and cell process outgrowth. However, this stimulation was not observed in cells in which the expression of CREB was inhibited by transfecting the cells with an antisense oligonucleotide directed against CREB mRNA. Thus, these results indicated that CREB plays a crucial role in the stimulation of OLG differentiation by cAMP (Sato-Bigbee and DeVries, 1996). Based on these observations, it is possible to hypothesize that in young, but already “committed” OLGs, beta adrenergic stimulation followed by PKA activation and CREB phosphorylation could be at least one of the signals triggering the final stages of OLG maturation.

Interestingly, we have found that at an earlier developmental stage, when the cells are still “immature” OLG precursors, CREB phosphorylation is stimulated by the cholinergic agonist carbachol, glutamate and ATP; all neuroligands that increase Ca²⁺ levels in the cells. In this case, CREB phosphorylation involved the action of a MAPK/PKC pathway. These latter results suggested that at this early developmental stage, CREB could play a role in regulating cell proliferation. This hypothesis is based on the observation that the MAPK pathway in OLGs is stimulated by neurotrophin-3 (NT-3), PDGF, and bFGF (Bhat and Zang, 1996; Cohen et al., 1996a), all factors known

to stimulate OLG proliferation (Bögler et al., 1990; McKinnon et al., 1990; Barres et al., 1993, 1994). Thus, based on the results described above, we decided to test whether CREB could be a mediator of NT-3 action in OLGs.

Neurotrophins and their signaling cascade pathway

NT-3 is a member of a family of closely related peptide factors known as neurotrophins. Neurotrophins also include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5) (Snider et al., 1989; Eide et al., 1993) and NT-6 (Gotz et al., 1994). These factors bind to and activate the Trk family of receptor tyrosine kinases. TrkA, TrkB, and TrkC are the receptors for NGF, BDNF and NT-3, respectively (Thoenen, 1991). TrkB also serves as a receptor for NT-4/5 (Bothwell, 1991). In the nervous system, neurotrophins affect a wide range of biological responses which include proliferation, differentiation, and survival of neuroblasts (Confort et al., 1991; Diccico-Bloom et al., 1993) as well as the survival and development of neurons (Levi-Montalcini, 1987; Ghosh et al., 1994). The differentiation effects elicited by neurotrophins include enhanced neurite outgrowth (Segal et al., 1995), alterations in the electrophysiological properties of neurons as well as enhanced synaptic transmission (Levine et al., 1995a; Levine et al., 1995b), and determination of the neuronal cell fate (Sieber-Blum, 1991).

Neurotrophins are also known to affect the glial cells as they regulate the function

of cultured microglia (Nakajima et al., 1998), and the morphological behavior of astrocytes *in vitro* (Hutton et al., 1995). *In vitro* experiments have shown that NT-3 in particular appears to be important for OLG proliferation, development, and survival (Barres et al., 1994; Kumar et al., 1998). *In vivo* results from Kahn et al. (1999) showed that knockout mice lacking the TrkC receptor or NT-3 resulted in fewer progenitor cells as well as attenuated expression of OLG specific markers. Moreover, it has been shown that transplantation of NT-3 and BDNF producing fibroblasts into contused rat spinal cord ameliorated the axonal and myelin damage after spinal cord injury (McTigue et al., 1998). Furthermore, Heinrich et al. (1999) recently demonstrated that NT-3 aids in the early differentiation of OLGs in rat cortical cultures.

The mechanisms of action of neurotrophins are still controversial as they seem to include the concerted actions of different signal transduction pathways, and this complexity may explain the variety of effects that these factors are able to elicit.

The best studied example is the action of NGF on the rat pheochromocytoma cell line PC12 (Kaplan et al., 1991). The binding of NGF to its receptor TrkA causes receptor dimerization and the activation of the intrinsic tyrosine kinase activity of TrkA (Jing et al., 1992). Once the receptor is activated by auto-phosphorylation, the phosphotyrosines and the nearby amino acids of the receptor act as recognition sites for effector molecules that contain the Src homology 2 (SH2) domain. Among the proteins that have the SH2 domains are the enzymes phospholipase C gamma (PLC γ), phosphatidylinositol 3-

kinase (PI3 kinase) (Stephens et al., 1994; Obermeier et al., 1993), and the adapter protein Shc (Stephens et al., 1994).

The MAPK pathway, critical for the NGF induction of PC12 cell differentiation, is set into motion when the Shc adapter protein binds to its recognition site on the activated TrkA receptor. Shc then becomes phosphorylated by the receptor tyrosine kinase and serves as an adapter for yet another SH2 containing protein known as Grb2 (growth factor receptor-bound protein 2) (Rozakis-Adcock et al., 1992). Grb2 has yet another structural motif called the Src homology 3 (SH3) domain (Lowenstein et al., 1992), which mediates its association with the GTP exchange factor Sos (son of sevenless protein). Sos then activates a membrane bound G protein called Ras by exchanging GDP for GTP on Ras

(McCormick, 1994). The activated, GTP-bound Ras then interacts and activates the serine-threonine kinase Raf (Moodie et al., 1993), which in turn phosphorylates a dual specificity threonine/tyrosine kinase called MEK (MAPK/Erk kinase) (Jaiswal et al., 1994). The substrates for MEK are MAPK1 and MAPK2 (mitogen-activated protein kinases 1 and 2, also known as Erks (extracellular signal-regulated kinases) (Crews et al., 1992). Once the MAPKs are activated, they are translocated into the nucleus (Chen et al., 1992) where they phosphorylate several transcription factors as well as other kinases, i.e. the ribosomal S6 kinases (Rsk) (Chen et al., 1993). It appears that the role of Rsks is to phosphorylate other transcription factors, one of which is CREB (Xing et al., 1996).

(Fig. 2).

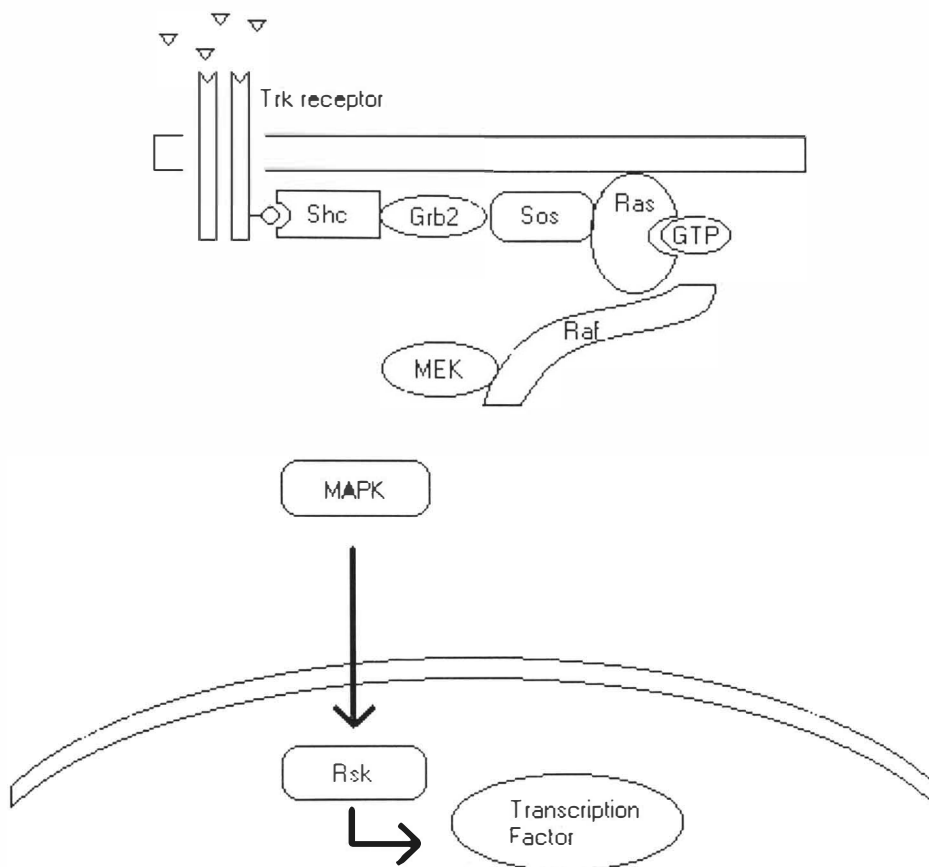


Figure 2. The NGF stimulated MAPK pathway as described in the PC12 cells. This figure illustrates the proteins involved in activating the MAPK pathway in the PC12 cells after NGF treatment.

The importance of CREB in the NGF induction of neuronal differentiation has been demonstrated by Bonni et al. (1995). However, a similar role for CREB in other cells of the CNS has never been described before. Thus, in the present study, we have investigated the possible role of CREB as a mediator of NT-3 actions in OLG precursor cells.

MATERIALS AND METHODS

Isolation and culture of oligodendrocytes. OLGs were isolated from 2-day-old Sprague-Dawley rat cerebrum by using a Percoll (Sigma Chemical Co., St Louis, Mo) gradient according to the method of Berti-Mattera et al. (1984) with minor modifications (Sato-Bigbee et al., 1999a). The cerebra are minced and then dissociated in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution (HBSS), 25 mM HEPES (pH 7.2), 1 mg/ml glucose, 0.1 mg/ml DNase and 1mg/ml acetyltrypsin. After incubation for 45 minutes at 37 °C, the tissue is forced through a 74 µm pore size nylon mesh and the resulting cell suspension is mixed with 1.5 vol. isosmotic Percoll and centrifuged at 30,000 x g for 15 minutes. The band corresponding to the OLGs and their precursor cells is collected, washed with HBSS, and the final cell suspension incubated for 30 minutes on tissue culture-treated Petri dishes to allow the attachment of residual microglial and astrocyte contamination (~5-10%). The dishes were then gently swirled for 10 seconds and the non-adherent cells plated in 24-well plates (1x10⁶ cells/well) previously coated with reduced-growth factor Matrigel (Becton Dickinson, NJ, USA) (10 µl/well). Cells were grown in chemically-defined medium (CDM) [Dulbecco's modified Eagle's medium (DMEM)/Ham F-12 medium (1:1, vol/vol) supplemented with 1 mg/ml bovine serum albumin, 50 µg/ml transferrin, 5 mg/ml insulin, 30 nM sodium selenite, 0.11 mg/ml sodium pyruvate, 10 nM biotin, 2 µM hydrocortisone, 15 nM triiodothyronine, 50 units/ml penicillin, and 50 µg/ml streptomycin] at 37 °C in 5% CO₂. Cultures prepared in

this way are comprised of OLG precursor cells that are either bipolar or have several simple processes and can be labeled with the O₄ (Sommer and Schachner, 1981) and /or the A2B5 (Levi et al., 1987) antibodies. Astrocyte contamination, as judged by staining with anti-gial fibrillary acidic protein antibody, was < 5%. Neuronal contamination as determined by staining with anti-neurofilament antibody was < 1%.

Effect of NT-3 on CREB and MAPK phosphorylation. After one day in culture, OLGs were incubated for various times in CDM with or without 50 ng/ml human recombinant NT-3 (Pepro Tech Inc., Rocky Hill, NJ). After incubation, the cells were rinsed with ice-cold DMEM and processed for western blot analysis to determine the relative levels of total CREB, phosphorylated CREB and MAPK as described below. In experiments aimed to determine the role of different protein kinases, the cells were pre-incubated for 10 minutes in the presence of the following specific kinase inhibitors: 50 μ M PD098059 (MEK inhibitor); 10 μ M chelerythrine (PKC inhibitor), 0.5 μ M H-89 (PKA inhibitor), 30 μ M KN-62 (CamK II, IV and V inhibitor), or 10 mM LY294002 (PI3 kinase inhibitor). All inhibitors were obtained from Calbiochem (San Diego, CA). After this, culture were incubated for 15 minutes in the presence or absence of both NT-3 and kinase inhibitor. Inhibitor concentrations are higher than IC₅₀ values for the purified enzymes, but are in agreement with the concentrations previously used by us and other investigators to specifically inhibit these kinases in cell culture systems (Balboa and Insel, 1995; Campenot et al., 1994; Maurer et al., 1996; Muthalif et al., 1996; Sato-Bigbee et al., 1999b).

Western blot analysis. OLG cultures containing equivalent number of cells per well were lysed in 100 μ l of 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol. The samples were frozen and stored at -70°C until required. Fifteen μ l samples were subjected to SDS-polyacrylamide gel electrophoresis in 12% acrylamide gels and the proteins were then electrotransferred to nitrocellulose membranes. The membranes were then subjected to immunoblot analysis. Non-specific antibody binding to the blots was blocked by incubation in buffer containing 10 mM Na_2HPO_4 , 2.7 mM KCl, 137 mM NaCl (PBS); 3% non-fat dry milk; 0.05% Tween 20 (pH 7.4) (blocking solution), for 1 hour at room temperature. The blots were then incubated overnight with either a total CREB (phosphorylated and non-phosphorylated protein) anti-CREB monoclonal antibody (dil. 1:500) (Santa Cruz Biotech., Santa Cruz, CA) or a phosphorylated CREB antibody which recognizes CREB when phosphorylated at Ser¹³³ (Ginty et al., 1993) (dil. 1:1000) (Upstate Biotech. Inc., Lake Placid, NY). Phosphorylated MAPKs were detected using an antibody that specifically recognizes p42 and p44 MAPKs when phosphorylated at Tyr²⁰⁴ (dil. 1:1000) (Santa Cruz Biotech.). The blots were then incubated with the appropriate secondary horse radish peroxidase (HRP)-conjugated antibody (monoclonal anti-mouse IgG (dil. 1:2000) for total CREB and MAPK; polyclonal anti-rabbit IgG (dil. 1:1000) for phosphorylated CREB). After two 5 minute rinses of the blots with PBS containing 0.05% Tween 20 and three 5 minute rinses in PBS, the immunoreactive bands were revealed by a chemiluminescence reaction with SuperSignal Ultra reagents (Pierce, Rockford, IL). The relative amount of immunoreactive protein in each band was

determined by scanning densitometric analysis of the X-ray films.

Diacylglycerol Assay. OLG cultures were incubated for 10 minutes in CDM with or without 50 ng/ml NT-3. At the end of the incubation, the cultures were rinsed with ice-cold DMEM, transferred to ice, and the cells rapidly scraped off the plates and homogenized in phosphate buffer saline solution (PBS). Aliquots of the cell lysates were used to determine the concentration of *sn*-1,2-diacylglycerol (DAG) using an assay kit from Amersham (Arlington Heights, IL), according to the manufacturer's recommendations. This assay utilizes E. Coli DAG kinase and allows the quantitative conversion of the DAG present in the cells to [³²P]phosphatidic acid in the presence of [³²P]- γ -ATP.

Inhibition of CREB protein expression. CREB protein synthesis was inhibited by using a deoxyoligonucleotide directed against CREB mRNA as previously reported (Sato-Bigbee and DeVries, 1996) with minor modifications. Deoxyoligonucleotides corresponding to the CREB-1 sequence (Gonzalez et al., 1989a) in the antisense (5'-GC TCC AGA CTC CAT GGT CAT-3') and sense (5'-ATC ACC ATG GAC TCT GAA GC-3') orientations, spanning the initiation codon to nucleotide 20, were prepared by Ransom Hill Bioscience (Ramona, CA). Transfection was carried out by using Lipofectamine Plus™ reagent (GIBCO BRL, Gaithersburg, MD). Sense or antisense oligonucleotides (1 μ g/well) were incubated for 15 minutes with 5 μ l Plus reagent followed by 15 minutes with 1.25 μ l Lipofectamine. Cells were then incubated overnight with the

oligonucleotide mixture in DMEM:HAM F-12 (1:1 v/v). CREB expression after transfection was assessed by western blot analysis using anti-total CREB antibody as described above.

Proliferation assay. After transfection, the medium was replaced by CDM containing 0.5 $\mu\text{Ci/ml}$ [^3H]thymidine (75Ci/mmol, Amersham), in the presence or absence of 50 ng/ml NT-3. At the end of an 18 hour incubation period, the cultures were washed three times with ice-cold PBS and the cells were solubilized in 500 μl 0.2N NaOH. The DNA was precipitated with 20% trichloroacetic acid (TCA) and the pellet was washed four times with 5 % TCA. After solubilization of the pellet by incubation with 70% perchloric acid at 37 °C for 1 hour, the radioactivity was determined by liquid scintillation counting.

Statistical analysis. Statistical analysis was performed by one-way ANOVA. Differences were considered statistically significant when p values were < 0.05.

RESULTS

Treatment of neonatal OLG precursor cells with NT-3 results in stimulation of CREB phosphorylation.

As indicated before, previous results from this laboratory indicated that CREB phosphorylation in “immature” OLG precursors could be regulated by agents which stimulate a MAPK pathway. In order to investigate whether NT-3, which is known to activate MAPK in OLGs, could also regulate CREB activation in these cells, cultures of OLG precursors were incubated for various times in chemically defined medium containing 50 ng/ml NT-3. At the end of each incubation time, the cells were lysed and the levels of CREB phosphorylation were investigated by western blot analysis. In these experiments, we have used an antibody that specifically recognizes CREB only when phosphorylated at Ser¹³³. As indicated before, phosphorylation of CREB at Ser¹³³ is a requirement for this transcription factor to activate transcription.

Figure 3 shows that NT-3 treatment results in a rapid increase in the levels of CREB phosphorylation reaching a peak at 15 minutes and remaining elevated even after 45 minutes of incubation time. Consequently, future experiments investigating the signaling pathways leading to CREB activation utilized 15 minute incubation times.

To demonstrate that these results signified a true increase in CREB phosphorylation levels, as opposed to increased expression of the CREB protein itself,

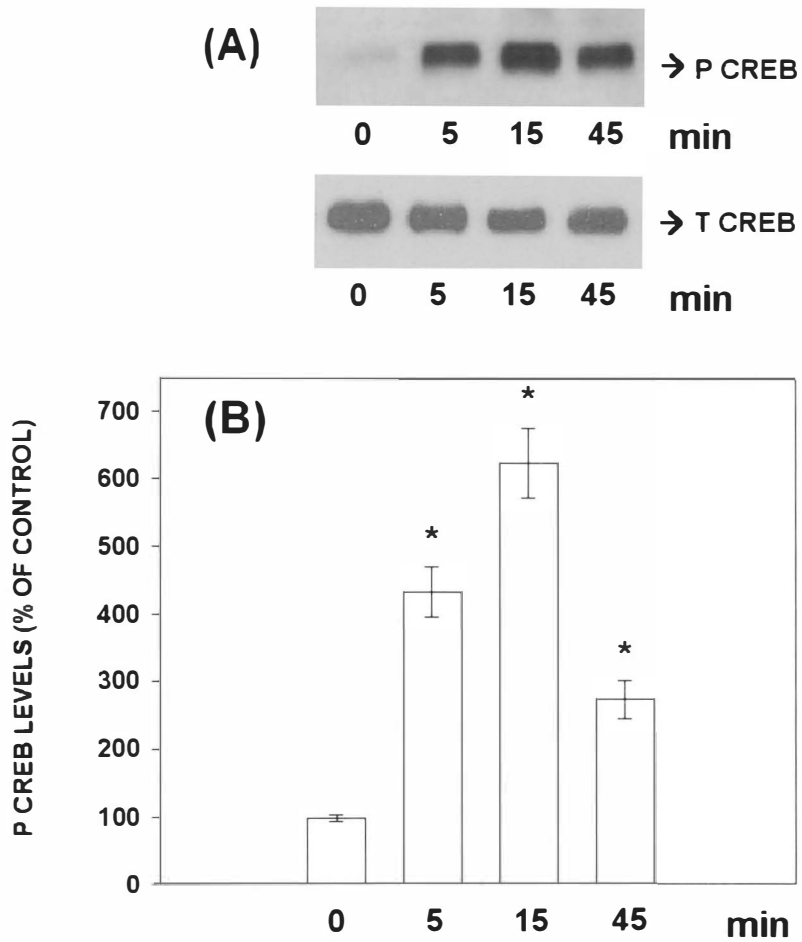


Figure 3. Treatment of OLG precursors with NT-3 results in stimulation of CREB phosphorylation.

Figure 3. Treatment of OLG precursors with NT-3 results in stimulation of CREB phosphorylation. After 1 day in culture, OLGs were incubated for various times in the presence of 50 ng/ml NT-3. Phosphorylated CREB (P CREB) was detected by western blot analysis with an antibody that specifically recognizes CREB when phosphorylated at Ser¹³³. Total CREB was detected using an antibody that detects both phosphorylated and non-phosphorylated CREB. (A) representative western blot, each lane corresponds to 10 μ g of cell lysate protein. (B) P CREB levels were determined by scanning densitometry of the bands. The results are expressed as % of the control values in the absence of NT-3 and represent the mean \pm SEM from 3-4 independent experiments. *0 min vs. 5, 15, 45 min: $p < 0.001$.

parallel western blots were performed using an antibody that recognizes total CREB protein levels, this is both the phosphorylated and non-phosphorylated forms of CREB. As shown in figure 3, incubation of the cultures in the presence of NT-3 did not affect the levels of total CREB protein, indicating that the observed increase in phospho-CREB levels directly reflect increased phosphorylation.

The MAPK and PKC pathways play a significant role in the NT-3 dependent stimulation of CREB phosphorylation.

Based on the results described above we decided to investigate the signal transduction pathway(s) mediating the stimulation of CREB phosphorylation in the cells treated with NT-3.

For this purpose, cultures of OLG precursor cells were incubated in medium containing NT-3 in the presence or absence of cell permeable specific kinase inhibitors. The possible role of a MAPK pathway was studied by co-incubation of the cells in the presence of PD098059. This compound inhibits MEK, the kinase that phosphorylates and activates MAPK. As shown in figure 4, incubation in the presence of this inhibitor decreases the NT-3 dependent stimulation of CREB phosphorylation by about 23%.

Interestingly, incubation in the presence of chelerythrine, a specific inhibitor of PKC, also resulted in a significant reduction (~ 36%) in the NT-3 dependent stimulation of CREB phosphorylation. Most importantly, we observed that CREB phosphorylation in

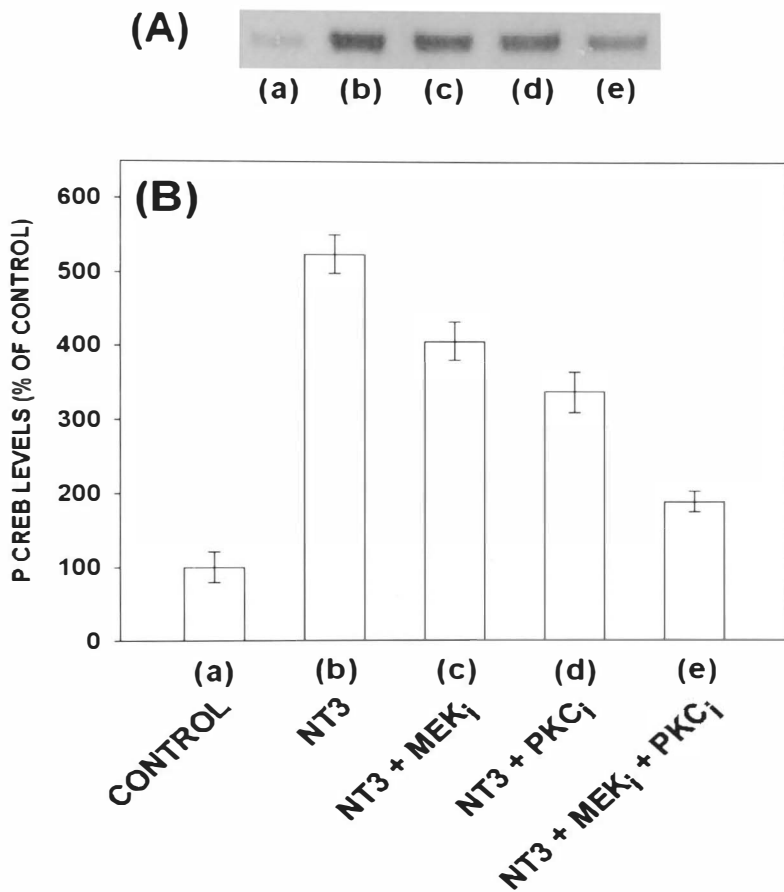


Figure 4. The NT-3 dependent stimulation of CREB phosphorylation in OLGs involves the action of MAPK- and PKC- signaling pathways.

Figure 4. The NT-3 dependent stimulation of CREB phosphorylation in OLGs involves the action of MAPK- and PKC -signaling pathways. After 1 day in culture, the cells were preincubated for 10 minutes under the following conditions: (a) and (b): medium alone; (c) 50 μ M PD098059 (MEK inhibitor); (d) 10 μ M chelerythrine (PKC inhibitor); or (e) 50 μ M MEK inhibitor + 10 μ M PKC inhibitor. Cultures were then incubated for 15 minutes in (a) medium alone (control); (b) 50 ng/ml NT-3; (c) 50 ng/ml NT-3 + 50 μ M MEK inhibitor; (d) 50 ng/ml NT-3 + 10 μ M PKC inhibitor; or (e) 50 ng/ml NT-3 + 50 μ M MEK inhibitor + 10 μ M PKC inhibitor. After incubation, phosphorylated CREB (P CREB) levels were determined by western blot analysis. (A) representative western blot, each lane corresponding to 10 μ g of cell lysate protein. (B) P CREB levels are expressed as % of the controls in the absence of NT-3 and represent the mean \pm SEM from 3-5 independent experiments. Control vs. NT-3: $p<0.001$; NT-3 vs. NT-3 + MEK inhibitor: $p<0.05$; NT-3 vs. NT-3 + PKC inhibitor: $p<0.05$; NT-3 vs. NT-3 + MEK inhibitor + PKC inhibitor: $p<0.001$.

the presence of NT-3 was dramatically decreased, by about 70%, when the MEK and PKC inhibitors were used simultaneously. This observation suggested the possibility of a concerted mechanism between the MAPK and PKC pathways.

To test this possibility, we investigated the role of both MEK and PKC in mediating the activation of MAPK by NT-3. In these experiments MAPK phosphorylation was investigated by western blot analysis using an antibody that specifically recognizes MAPK 42 and MAPK 44 when phosphorylated at Tyr²⁰⁴.

Our data showed that incubation in the presence of NT-3 resulted in a dramatic increase in the phosphorylation of MAPK. Inhibiting MEK, however, decreased this stimulation by about 50%. Interestingly, inhibition of PKC also reduced MAPK phosphorylation in the presence of NT-3 by ~ 50%, suggesting that, in addition to MEK, PKC also plays an integral role in the pathway that leads to the activation of MAPK by NT-3. Further evidence for a concerted mechanism is supported by the observation that incubation in the presence of both MEK and PKC inhibitors completely blocked the NT-3 mediated stimulation of MAPK phosphorylation. (Fig. 5).

To further support the participation of a PKC activity in the pathways triggered by NT-3, we investigated whether incubation with this neurotrophin could affect the levels of possible activators of PKC. As shown in figure 6, treatment of the cells with NT-3 resulted in a significant increase in the intracellular levels of diacylglycerol (DAG). Thus, it may be possible to speculate that this increase in DAG results in PKC activation;

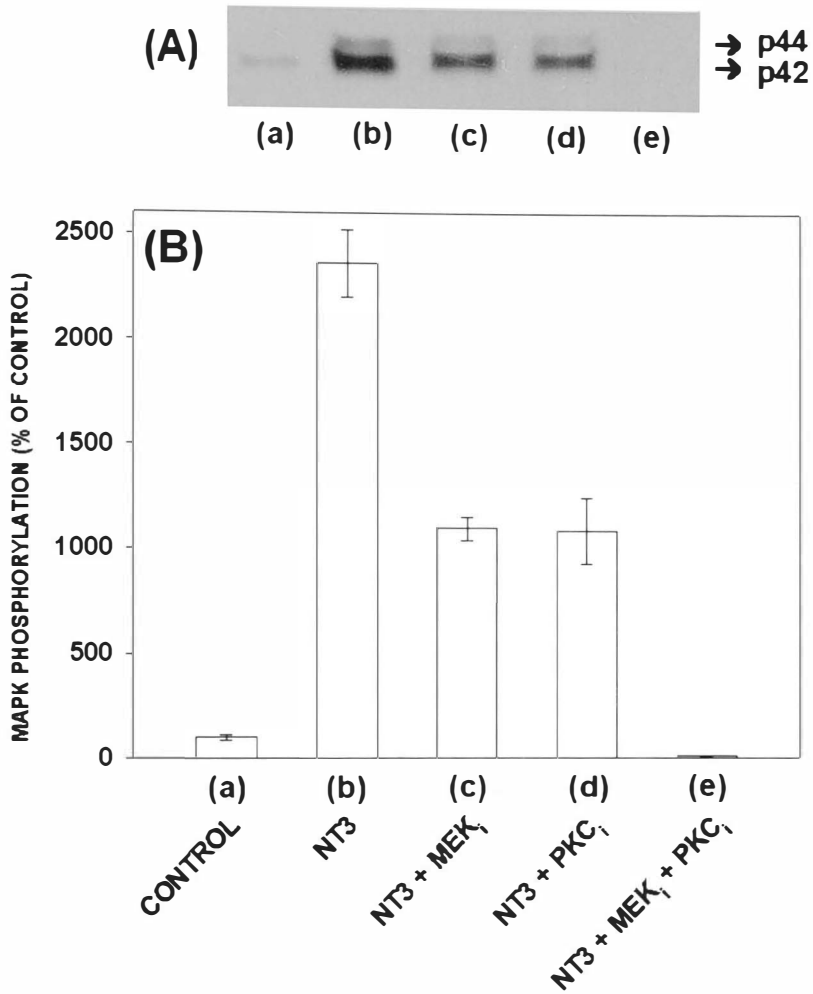


Figure 5. MAPK activation in OLGs treated with NT-3 is coupled to both MEK and PKC activities.

Figure 5. MAPK activation in OLGs treated with NT-3 is coupled to both MEK and PKC activities. After 1 day in culture, cells were pre-incubated for 10 minutes under the following conditions: (a) and (b): medium alone; (c) 50 μ M PD098059 (MEK inhibitor); (d) 10 μ M chelerythrine (PKC inhibitor); or (e) 50 μ M MEK inhibitor + 10 μ M PKC inhibitor. Cultures were then incubated for 15 minutes in (a) medium alone ; (b) 50 ng/ml NT-3; (c) 50 ng/ml NT-3 + 50 μ M MEK inhibitor; (d) 50 ng/ml NT-3 + 10 μ M PKC inhibitor; or (e) 50 ng/ml NT-3 + 50 μ M MEK inhibitor + 10 μ M PKC inhibitor. Western blot analysis was then used to detect p42 and p44 MAPK Tyr phosphorylation. (A) representative western blot, each lane corresponding to 10 μ g of cell lysate protein. (B) levels of phosphorylated MAPK were expressed as % of the control values in the absence of NT-3 and represent the mean \pm SEM from 3 independent experiments. Control vs. NT-3: $p < 0.001$; NT-3 vs. NT-3 + MEK inhibitor: $p < 0.001$; NT-3 vs. NT-3 + PKC inhibitor: $p < 0.02$; NT-3 vs. NT-3 + MEK inhibitor + PKC inhibitor: $p < 0.001$.

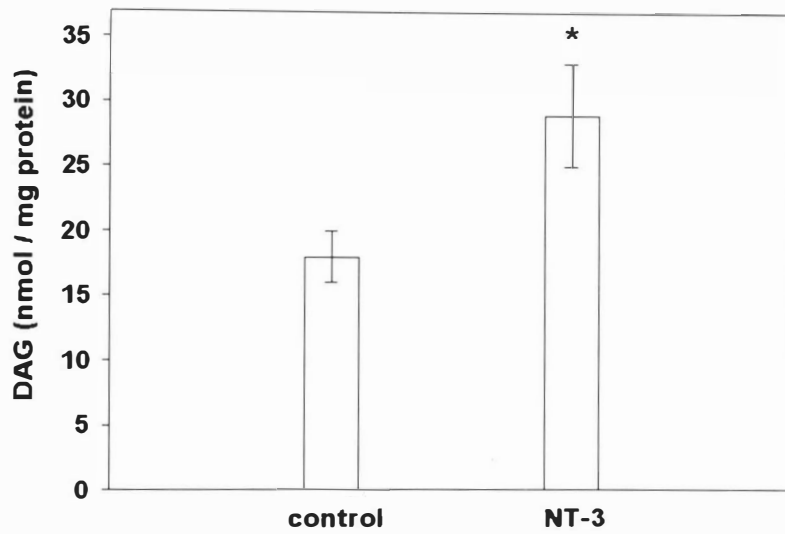


Figure 6. Treatment of OLG precursors with NT-3 results in increased levels of diacylglycerol (DAG).

Figure 6. Treatment of OLG precursors with NT-3 results in increased levels of diacylglycerol (DAG). After 1 day in culture, cells were pre-incubated for 10 minutes in the presence or absence of 50 ng/ml NT-3. At the end of the incubation, DAG concentration in the cells (5×10^6 cells/sample) was determined as indicated under "Methods." The results represent the mean \pm SEM from 3 independent determinations.
* Control vs. NT-3: $p < 0.05$.

this step being one of the events mediating the action of NT-3 on OLGs.

Since the inhibition of both MEK and PKC drastically decreased, but not completely abolished CREB phosphorylation, it is possible that other kinases may also play a minor role in mediating CREB activation by NT-3. In order to identify other possible kinase(s) involved in this stimulation, we carried out additional inhibition studies.

The possible role of PKA was investigated by co-incubation of the cells in the presence of its specific inhibitor H-89. On the other hand, the possible role of CamK and PI3-kinase was studied by treatment with their specific inhibitors KN-62 and LY294002, respectively. Figure 7 suggests that these kinases may not play a significant role in mediating CREB phosphorylation by NT-3. However, further studies are required for more conclusive results about these kinases.

Thus, the question still remains open as whether there may be a yet unidentified kinase(s) that may play a minor role in mediating the phosphorylation of CREB under NT-3 stimulation.

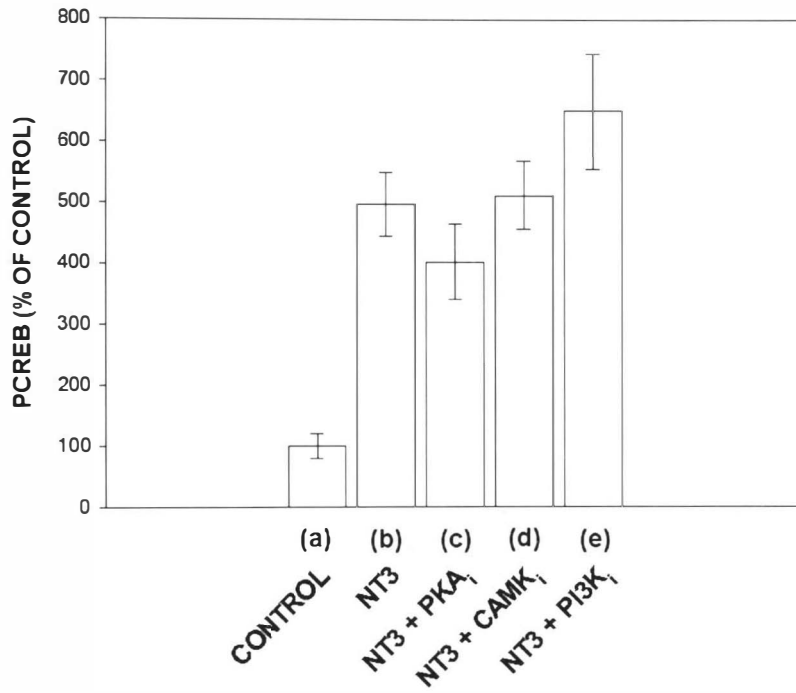


Figure 7. The NT-3 dependent stimulation of CREB phosphorylation in OLGs does not appear to involve the PKA, CamK, nor the PI3K pathways.

Figure 7. The NT-3 dependent stimulation of CREB phosphorylation in OLGs does not appear to involve the PKA, CamK, nor the PI3K pathways. After 1 day in culture, the cells were preincubated for 10 minutes under the following conditions: (a) and (b): medium alone; (c) 0.5 μ M H-89 (PKA inhibitor); (d) 30 μ M KN-62 (CamK II, IV and V inhibitor); (e) 10 μ M LY294002 (PI3 kinase inhibitor). Cultures were then incubated for 15 minutes in (a) medium alone (control); (b) 50 ng/ml NT-3; (c) 50 ng/ml NT-3 + 0.5 μ M PKA inhibitor; (d) 50 ng/ml NT-3 + 30 μ M CamK inhibitor; (e) 50 ng/ml NT-3 + 10 μ M PI3 kinase inhibitor. After incubation, phosphorylated CREB (P CREB) levels were determined by western blot analysis. P CREB levels are expressed as % of the controls in the absence of NT-3 and represent the mean \pm SEM from 2-3 independent experiments with each experiment having a minimum of 2 individual samples. NT-3 vs. NT-3 + PKA inhibitor: not significant; NT-3 vs. NT-3 + CamK inhibitor: not significant; NT-3 vs. NT-3 + PI3K inhibitor: not significant.

Inhibition of CREB expression abolishes the NT-3 dependent stimulation of DNA synthesis in OLGs.

As described before, results from different laboratories have indicated that NT-3 stimulates the proliferation of OLG precursor cells *in vitro* as well as *in vivo* (Barres et al., 1994; McTigue et al., 1998; Kumar et al., 1998). Thus, the results described above raise the question of whether CREB could play a role in mediating that stimulation.

To test this possibility, CREB expression in the OLG cultures was inhibited by using an antisense oligodeoxynucleotide sequence directed against CREB mRNA. For this, an oligonucleotide probe corresponding to the CREB sequence was prepared in the antisense (5'-GC TCC AGA GTC CAT GGT CAT-3') and sense (5'-ATG ACC ATG GAC TCT GGA GC-3') orientations, spanning the initiation codon to nucleotide 20. Control cultures were treated with a CREB sense oligodeoxynucleotide. In these experiments, the uptake of the oligonucleotides was facilitated by using a cationic liposome preparation. All conditions, including cell number, concentrations of oligonucleotides and transfection reagents, and incubation times were optimized to reach maximal levels of inhibition of CREB expression. The effectiveness of treatment with the antisense construct in inhibiting CREB expression was evaluated by western blot analysis. In these experiments, we use an antibody that recognizes total CREB, both phosphorylated and non-phosphorylated forms.

As shown in figure 8, the expression of CREB was drastically reduced in the

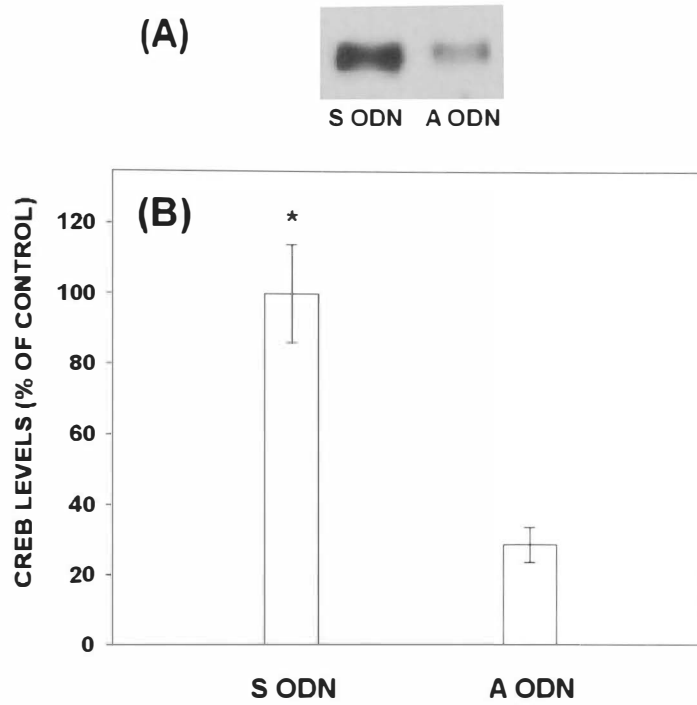


Figure 8. Inhibition of CREB expression in OLG cultures.

Figure 8. Inhibition of CREB expression in OLG cultures. CREB expression was blocked by transfection with an antisense oligodeoxynucleotide (A ODN) directed against CREB mRNA, as indicated under “Methods.” Control cultures were treated in a similar manner but in the presence of the corresponding construct in the sense orientation (S ODN). CREB expression after transfection was determined by western blot analysis with an antibody that recognizes both phosphorylated and non-phosphorylated CREB. (A) representative western blot, each lane corresponding to 10 μ g of cell lysate protein. (B) CREB levels are expressed as % of the values corresponding to the control cells (S ODN) and represent the mean \pm SEM from 4 experiments.

* S ODN vs. A ODN: $p < 0.001$.

antisense-treated cells compared to the sense strand treated control cells.

[³H]thymidine incorporation into DNA was then used to assess the effect of NT-3 on the proliferation of OLGs expressing either normal (sense-treated cells) or reduced (antisense-treated) CREB protein levels.

As shown in figure 9, incubation of the sense-treated OLG precursor cells with NT-3 resulted in a significant stimulation of DNA synthesis. However, this stimulation in DNA synthesis after NT-3 incubation was not seen in the antisense-treated cultures which, as shown before, expressed very low levels of CREB. Furthermore, CREB does not seem to have an important role as a regulator of the basal levels of DNA synthesis, for inhibition of CREB expression in cultures without NT-3 treatment did not appear to have any significant effects on [³H]thymidine incorporations. Altogether, these results support the idea that CREB is an important mediator in the stimulation of OLG proliferation by NT-3.

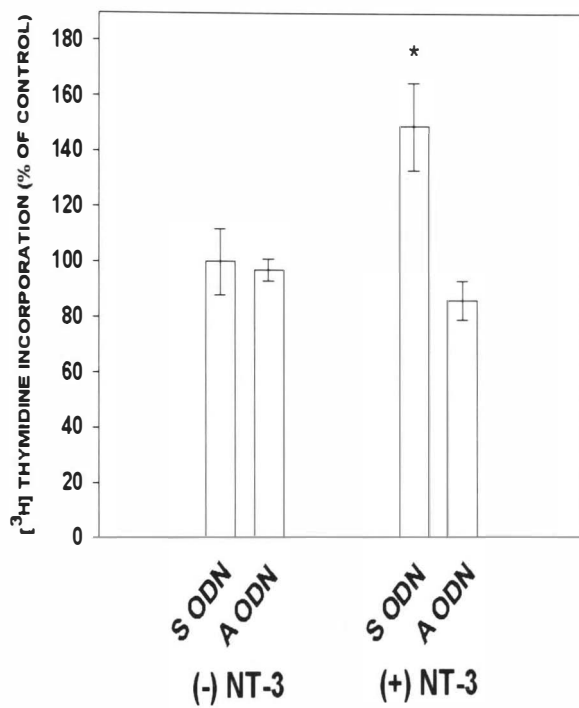


Figure 9. Inhibition of CREB expression abolished the NT-3 dependent stimulation of DNA synthesis in OLGs.

Figure 9. Inhibition of CREB expression abolished the NT-3 dependent stimulation of DNA synthesis in OLGs. CREB expression was inhibited by transfection with CREB antisense oligodeoxynucleotide (A ODN). Control cells were treated with the corresponding sense construct (S ODN). Eighteen hours after transfection, the cell cultures were incubated for 18 hours in medium containing [³H] thymidine in the presence or absence of 50 ng/ml NT-3. [³H] thymidine incorporation into DNA was determined as described under "Methods." The results are expressed as % of the values corresponding to the cells treated with S ODN in the absence of NT-3 (controls) and represent the mean±SEM from 5 experiments.

* S ODN vs. A ODN + NT-3: $p < 0.02$.

DISCUSSION

Results from Finkbeiner et al. (1997) indicated that in neurons, CREB is a key regulator in the induction of gene expression by BDNF, suggesting that CREB plays an important role in mediating neurotrophin responses in those cells.

However to our knowledge, a similar role for CREB in other cells of the CNS has not been studied before. Our present results provide the first evidence that this transcription factor also plays a crucial role in mediating the action of another neurotrophin, NT-3, in OLG precursor cells.

Our studies demonstrated that treatment of OLG precursors with NT-3 results in a rapid and dramatic stimulation of CREB phosphorylation. Moreover, inhibition of CREB expression in the OLG precursors abolished the stimulation of DNA synthesis that is observed when the cells are incubated with NT-3. Altogether, these results suggest that CREB activation is an important step in the signaling pathway(s) that triggered by NT-3, result in stimulation of OLG proliferation.

Our results indicated that in the OLGs, the stimulation of CREB phosphorylation by NT-3 appears to require the concerted action of MAPK- and PKC-mediated pathways. Based on the results previously reported by Xing et al. (1996), we could speculate that the most likely mechanism linking MAPK to CREB phosphorylation when the OLGs are treated with NT-3, is a MAPK-dependent activation of Rsk2 which could in turn phosphorylate CREB.

As indicated above we have found that, in addition to a MAPK pathway, CREB

phosphorylation in OLGs treated with NT-3 also involves a PKC activity. This is in contrast with the observation from Finkbeiner et al. (1997) showing that in neurons, the stimulation of CREB phosphorylation by BDNF is mediated by MAPK- and CamK-dependent pathways. Consistent with the involvement of a CamK, these authors have found that treatment of cortical neurons with BDNF results in a slowly developing but sustained increase in cytosolic Ca^{2+} levels. To support a role for PKC in our cells, we have found that NT-3 was able to elicit an increase in the concentration of DAG. This increase in DAG could result in PKC activation.

In support of this possibility, our present results suggested that in the presence of NT-3, a PKC activity is involved in mediating not only CREB phosphorylation but also the activation of MAPK by Tyr phosphorylation. In this regard, results from different laboratories have previously shown a role for PKC in the activation of MAPK in OLGs in response to different conditions, including muscarinic receptor stimulation (Larocca and Almazan, 1997; Pende et al., 1997), activation of glutamate receptor channels (Pende et al., 1997), and platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Bhat and Zhang, 1996).

PKC could directly phosphorylate Raf, which could then activate MEK resulting in MAPK phosphorylation (Kolch et al., 1993; Marquardt et al., 1994; Ueda et al., 1996). However, PKC may also modulate the MAPK pathway by different mechanisms in different cell types. It has been suggested that sites of PKC action, other than Raf activation, are likely to be effective at different points of the MAPK pathway as well (Cobb and Goldsmith, 1995).

Miranti et al. (1999) demonstrated that the alpha, delta and epsilon isoforms of PKC led to the activation of p42 MAPK by possibly regulating the Tyr phosphorylation of Shc in Cos 7 cells. Moreover, others have demonstrated that PKC can lead to the activation of MAPKs independent of many of the factors in the MAPK pathway, i.e. Ras, Raf, and MEK (Ueda et al., 1996; Chao et al., 1994; Grammar et al., 1997). However, the direct role that PKC plays in such cascades has yet to be elucidated.

Thus, it appears that the site where PKC is involved in the MAPK pathway may be variable. In our experiments, we have seen that MAPK phosphorylation is completely abolished only when both MEK and PKC are inhibited simultaneously. On the other hand, individual inhibition of either MEK or PKC only decreased the phosphorylation by 50%. These observations suggest that PKC is not working upstream of MEK because then the inhibition of MEK alone should produce the same result as inhibiting both MEK and PKC simultaneously. Furthermore, we know, by the antibody used in the western blots, that the MAPKs are phosphorylated at a Tyr residue. Thus, the possibility of a direct phosphorylation of MAPK by PKC is eliminated by the fact that PKC is a serine/threonine kinase. Thus, the possibility exists of a yet unidentified step which may link PKC with MAPK activation by Tyr phosphorylation.

Further complications in studying PKC signaling pathways stem from reports indicating the existence of several isoforms and differential cellular distributions of PKC (Slepko et al., 1999). It appears that in the OLGs, PKC isotypes are differentially expressed according to developmental stages. Asotra and Macklin (1994) reported that in

O-2A progenitors, only the Ca²⁺-independent PKC-delta, -epsilon and -zeta forms are expressed while other isotypes can be detected at later stages. The particular isoform of PKC appears to be critical in determining the physiological function effected by the kinase since Corbit et al. (1999) reported that the aid of PKC delta in MAPK activation in PC12 cells is related to neurogenic functions, but not to a mitogenic response. Studies investigating PKC activation have shown that PKC can be calcium dependent as well as calcium independent (Huang et al., 1993). Ohmichi et al. (1993) demonstrated that NGF stimulation of PC12 cells, which also stimulated the production of DAG, selectively activated the calcium-insensitive epsilon isoform of PKC. This report is similar to our findings in that NT-3 stimulation of OLG precursor cells resulted in elevated levels of DAG, which may activate PKC. Thus, further experiments are necessary to determine the precise mechanism by which PKC could stimulate MAPK activation in the OLGs in the presence of NT-3 and how these two signaling pathways interact to mediate the NT-3 dependent stimulation of CREB phosphorylation in the OLGs.

Previous results from this laboratory suggested that in committed OLGs, CREB plays an important role being at least one of the mediators in the stimulation of OLG differentiation by cAMP (Sato-Bigbee and DeVries, 1996). However, later studies investigating the regulation of CREB phosphorylation along OLG maturation raised the possibility that this transcription factor may play different roles by mediating the action of different signaling pathways at specific stages of cell differentiation (Sato-Bigbee et al., 1999a). Our present results support the idea that in the immature OLG precursors, CREB

plays an important role in transducing signals, which like NT-3, may regulate cell proliferation. In effect, previous results have shown that CREB phosphorylation in OLG precursors can also be stimulated by a MAPK pathway triggered by PDGF, bFGF (Pende et al., 1997) and the cholinergic agonist carbachol (Pende et al., 1997; Sato-Bigbee et al., 1999a); all factors known to promote OLG proliferation (Bögler et al., 1990; McKinnon et al., 1990; Cohen et al., 1996b). Thus, it is possible to hypothesize that CREB could be a common mediator of signals which, by activating the MAPK pathway results in CREB phosphorylation and stimulation of OLG proliferation. Therefore, we are currently focusing on identifying the gene(s) that may be regulated by CREB mediating the stimulation of OLG proliferation.

Our preliminary results indicated that treatment of OLGs with NT-3 also results in increased levels of c-fos (Sato-Bigbee et al., 1999a), a protein for which elevated expression has been linked to OLG proliferation (Bhat et al., 1992; Cohen et al., 1996b). It has been shown that in PC12 cells, CREB interacts with other transcription factors mediating the stimulation of c-fos expression by NGF (Bonni et al., 1995); and in neurons CREB by itself can mediate the up-regulation of c-fos expression by BDNF (Finkbeiner et al., 1997). Thus, CREB could in part mediate the up-regulation of c-fos expression that we have observed in the OLGs treated with NT-3. Interestingly, it is possible to hypothesize that CREB itself and c-fos could simulate the expression of several proteins that are crucial for cell proliferation to occur. One of these proteins is the proliferating cell nuclear antigen (PCNA), an essential factor for DNA polymerase. It has been shown that PCNA promoter activity in interleukin 2-stimulated T lymphocytes largely depends

on the presence of tandem CREB binding sites (Huang et al., 1994). Moreover, Lee and Mathews (1997) demonstrated that CREB acts as a transcriptional coactivator capable of mediating the induction of human PCNA promoter by the adenovirus E1A oncoprotein. Another possibility is that CREB may be involved in the regulation of genes encoding cyclins. Cyclins comprise a family of proteins which interact with and activate a series of kinases known as cyclin-dependent kinases or cdks (Pines, 1993). In recent years it has been shown that cdks catalyze phosphorylation events which are critical for the regulation of eukaryotic cell proliferation (Norbury and Nurse, 1992; Pines, 1993). In this regard, studies in human fibroblasts and muscle cells suggested that CREB and c-fos could play an important role in the cell cycle regulation of cyclin A expression (Desdouets et al., 1995; Sylvester et al., 1998). In addition, studies of cyclin D gene promoter characterization and regulation suggested a role for CREB and c-fos in cyclin D expression (Yang et al., 1996; Jun et al., 1997; Brown et al., 1998). However, the possibility of similar roles for CREB or c-fos in the OLG has not yet been investigated.

In summary, our present results indicated that CREB phosphorylation is at least one of the down-stream consequences of the NT-3 dependent activation of MAPK and PKC signaling pathways in OLG precursor cells. Moreover, CREB appears to play a crucial role in the stimulation of OLG proliferation by NT-3. Further experiments would determine whether these mechanisms are also operational *in vivo*. A better understanding of these regulatory systems and their final targets should provide important clues to design strategies to stimulate OLG proliferation and remyelination after demyelinating lesions of the CNS.

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VITA

