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Activated CREB Is Sufficient to Overcome Inhibitors in Myelin and Promote Spinal Axon Regeneration In Vivo

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Summary

Inhibitors in myelin play a major role in preventing spontaneous axonal regeneration after CNS injury. Elevation of cAMP overcomes this inhibition, in a transcription-dependent manner, through the upregulation of *Arginase I* (*Arg I*) and increased synthesis of polyamines. Here, we show that the cAMP effect requires activation of the transcription factor cAMP response element binding protein (CREB) to overcome myelin inhibitors; a dominant-negative CREB abolishes the effect, and neurons expressing a constitutively active form of CREB are not inhibited. Activation of CREB is also required for cAMP to upregulate Arg I, and the ability of constitutively active CREB to overcome inhibition is blocked by an inhibitor of polyamine synthesis. Finally, expression of constitutively active CREB in DRG neurons is sufficient to promote regeneration of subsequently lesioned dorsal column axons. These results indicate that CREB plays a central role in overcoming myelin inhibitors and so encourages regeneration in vivo.

Introduction

The lack of CNS regeneration in adult mammals is largely attributable to the presence of an inhibitory environment. In general, there have been two approaches to encourage regeneration in vivo—change the environment by blocking/neutralizing inhibitors of regeneration (Bradbury et al., 2002; Bregman et al., 1995; GrandPre et al., 2002), or change the intrinsic growth state of the neuron/axon such that it no longer responds to an inhibitory environment (Lehmann et al., 1999; Neumann et al., 2002; Qiu et al., 2002). In an injured CNS, an axon that attempts regeneration faces both inhibitors of regeneration in myelin and a physical barrier presented by a glial scar (Filbin, 2003; Schwab and Bartholdi, 1996; Silver and Miller, 2004). To date, three inhibitors of regeneration have been identified in myelin-Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) (Filbin, 2003). Interestingly, MAG and OMgp, as well as at least one inhibitory domain of Nogo, interact with the same receptor complex to exert their inhibitory effect, suggesting redundancy among the inhibitors. This receptor complex consists of a GPI-linked protein named Nogo receptor (NgR) because it was originally identified as a binding partner for a 66 amino acid sequence of Nogo, predicted to be extracellular (Domeniconi et al., 2002; Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). The transducing component of this receptor complex was identified as the neurotrophin p75 receptor (p75NTR) (Wang et al., 2002a; Wong et al., 2002). Recently, a third component of this receptor complex has been described—a transmembrane protein called Lingo (Mi et al., 2004). As these three inhibitors all interact with at least one common receptor complex, the signal transduction pathway that results in inhibition of axonal growth must also be shared by all inhibitors. It follows, then, that activation of a parallel pathway that blocks the signaling of one inhibitor would do the same for all three inhibitors.

Previously, we (Cai et al., 1999) and others (Song et al., 1998) found that when cAMP is elevated, either with analogs or by priming neurons with a variety of neurotrophins, inhibition and repulsion by both MAG and by myelin in general are overcome. Importantly, elevation of cAMP by direct injection of db-cAMP into the cell body of dorsal root ganglion (DRG) neurons is sufficient to induce regeneration of subsequently lesioned spinal axons that emit from the same cell body as was injected (Neumann et al., 2002; Qiu et al., 2002). Without elevation of cAMP, these axons, like all spinal cord axons, fail to regenerate. The question then becomes how cAMP induces axons to grow through an inhibitory environment. We showed that both the db-cAMP and neurotrophin effect in overcoming inhibition are each transcription dependent. One gene that is upregulated in response to either treatment is for the enzyme Arginase I (*Arg I*), a key enzyme in the synthesis of polyamines. Indeed, overexpressing Arg I or addition of the polyamine putrescine to neurons in culture is each sufficient to overcome inhibition by MAG and myelin (Cai et al., 2002). The steps between elevation of cAMP (activation of PKA) and upregulation of *Arg I* and polyamines are not known.

In many other situations, activation of the transcription factor cAMP response element binding protein (CREB) is a requirement not only for upregulation of a variety of genes in response to elevated cAMP, but also in response to a variety of signal transduction pathways, not necessarily involving cAMP (Lonze and Ginty, 2002). To date, at least six CREB-like molecules have been identified, and they act by forming homo- or heterodimers, before binding to CRE sequences to regulate transcription (Mayr and Montminy, 2001; Montminy, 1997; Shaywitz and Greenberg, 1999). More than 100 genes have been reported to be upregulated following activation of either CREB or a closely related protein (Lonze and Ginty, 2002). Of these, different repertoires of gene

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expression have been implicated in different neuronal processes such as survival, synaptic plasticity, memory and learning, and drug habituation and addiction (Kandel, 2001; Lonze and Ginty, 2002; Nestler, 2001; Silva et al., 1998). Here, we show that activation of CREB, or a CREB-like molecule, is also required to overcome inhibition by MAG and myelin in response to either db-cAMP or the neurotrophin brain-derived neurotrophin factor (BDNF). Activation of CREB, alone, is sufficient to overcome inhibition by myelin and to promote spinal axon regeneration in vivo. Furthermore, activation of CREB is required for either db-cAMP or BDNF to upregulate *Arg I*. These results not only help us expand our understanding of the signaling pathway that allows cAMP to overcome inhibition by MAG and myelin and encourage regeneration in vivo, but they also reveal yet another system in which CREB activation appears to be essential, in this case axonal regeneration in an inhibitory environment.

Results

Dominant-Negative CREB Blocks the Ability of BDNF and db-cAMP to Overcome Inhibition by MAG

It has been established that the strength and the duration of activation of CREB varies depending on the extracellular stimulus and/or the signaling pathway that induces the activation (Lonze and Ginty, 2002). This in turn has been implicated in influencing what CREB-dependent genes are transcribed. Because we observe differences in the timing and also in the strength of the signal resulting from treatment with BDNF and db-cAMP in overcoming inhibition by MAG and myelin, we first wanted to compare the time course and the extent of activation of CREB in response to these two stimuli. To do this, cerebellar neurons were plated onto poly-l-lysine and incubated in the presence or absence of BDNF or db-cAMP for various times before being lysed and activation determined by assessing levels of Ser133-phosphorylated-CREB (phospho-CREB) by Western blotting. As shown before, both BDNF and db-cAMP induce activation of CREB. For BDNF, the peak of activation is between 15 and 30 min, and by 120 min activated CREB has returned to control levels. In contrast, for neurons treated with db-cAMP, by 5 min phospho-CREB is apparent, with activation peaking at about 60 min, but it is still strong at 120 min. Therefore, db-cAMP activates CREB by phosphorylation of Ser133 more robustly and for longer than BDNF (Figure 1).

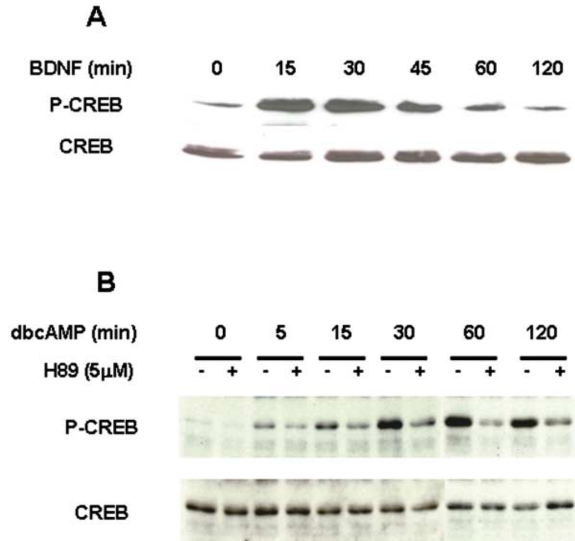

Figure 1. Activation of CREB in Response to BDNF and db-cAMP

Cerebellar neurons were plated on poly-l-lysine in the presence or absence of either BDNF (200 ng/ml) (A) or db-cAMP (1 mM), with and without the PKA inhibitor H89 (5 mM), and for various times as indicated (B) before being lysed, subjected to Western blotting, and stained for phospho-CREB (P-CREB). The blots were stripped and stained for total CREB. Similar results were obtained from at least three experiments.

To determine if activation of CREB is required to overcome inhibition by MAG by either priming with BDNF or addition of db-cAMP, a dominant-negative CREB, *A-CREB*, was used. We first confirmed that in our hands *A-CREB* was indeed behaving in a dominant-negative fashion using a reporter gene system (data not shown). To assess the effect of *A-CREB* on neurite outgrowth, both cerebellar and DRG neurons were infected with the A-CREB-containing, wild-type CREB-containing, or control adenoviruses before being plated onto monolayers of MAG-expressing Chinese hamster ovary (CHO) cells or control CHO cells. Figure 2A shows a typical infected cerebellar neuron, expressing GFP, which is also positive for the neuronal marker GAP43. By only measuring neurites from neurons positive for both GFP and GAP43, we ensured that only infected neurons were included in the analysis. Figures 2B and 2C show, as we reported before, that for cerebellar and DRG neurons MAG inhibits neurite outgrowth by about 70% and 40%, respectively, regardless of whether they are infected with any of the viral constructs (Cai et al., 1999; Mukhopadhyay et al., 1994). Furthermore, the percent inhibition relative to control was the same regardless of whether we measured the longest neurite from each neuron or the total process length (data not shown). In sharp contrast, unlike uninfected or control-infected neurons, where db-cAMP blocks inhibition, it has no effect on inhibition by MAG of neurons expressing A-CREB. In a similar manner, the ability of priming with either BDNF or NGF to overcome inhibition is blocked by expression of *A-CREB* (Figure 2D). This is not a general effect on growth, as neurons expressing A-CREB are unaffected in their ability to extend long neurites on control CHO cells not expressing MAG. In other controls, infection with WT-CREB-containing virus or control virus has no effect on the ability of neurotrophins or db-cAMP to overcome inhibition by MAG (Figures 2B–2D). In addition, under the conditions used here, as assessed by comparing the number of live and dead cells under the various conditions, using probes that measure two recognized parameters of cell viability, calcein AM (live cells) and ethidium homodimer (dead cells) uptake, it was clear that expression of A-CREB has no effect on neuronal survival, regardless of the substrate used (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/44/4/609/DC1/).

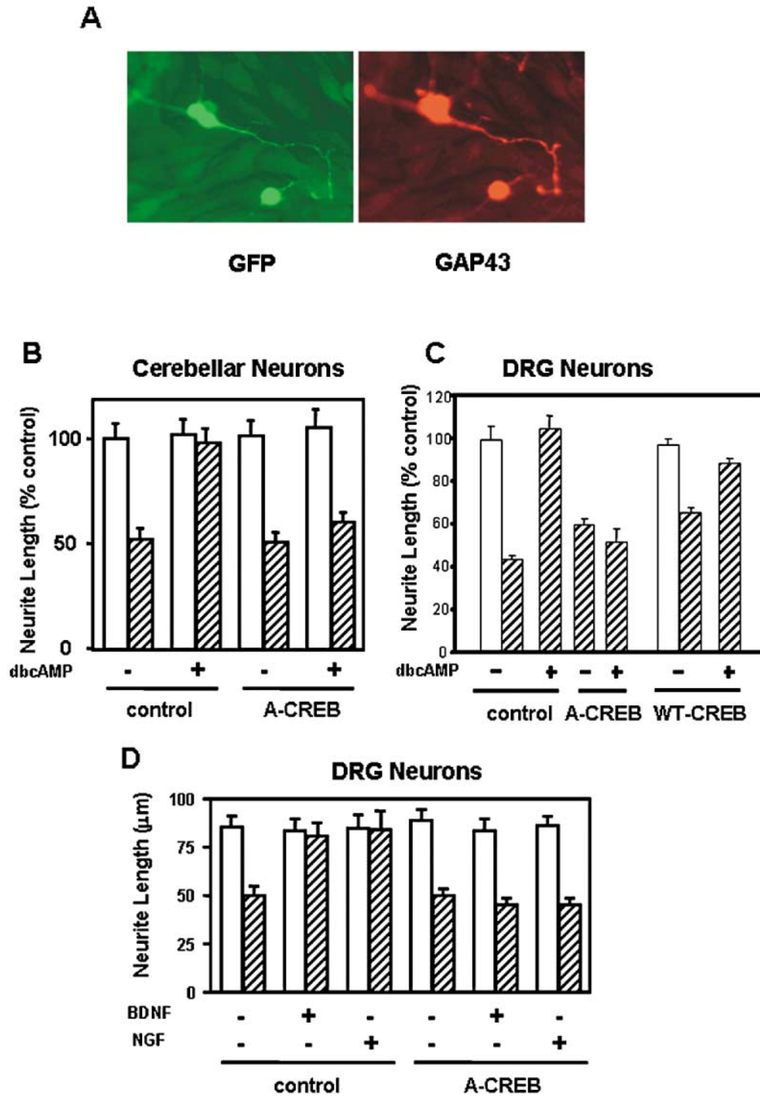

Figure 2. Dominant-Negative CREB Blocks the Ability of db-cAMP and BDNF to Overcome Inhibition by MAG

(A) A typical infected cerebellar neuron expressing GFP (left) and GAP43 (right).

(B)–(D) Cerebellar (P5–P7) or DRG (P5–P7) neurons were isolated and infected with either control Ad virus or A-CREB containing Ad virus and primed overnight with either BDNF or NGF (200 ng/ml) (D) before being transferred to either MAG-expressing (striped bars) or control CHO (white bars) cells, or db-cAMP was added directly to the cocultures (B and C). After overnight coculture, neurons were fixed and stained for GAP43 and viewed for GFP. In each experiment, the mean length of the longest GAP43-positive neurite for 180 to 200 neurons was measured (±SEM), for at least three separate experiments.

Neurons Expressing Constitutively Active CREB Are Not Inhibited by MAG

To address whether activation of CREB alone is sufficient to overcome inhibition by MAG, a constitutively active form of CREB, *VP16-CREB*, was used (Barco et al., 2002). The *VP16-CREB* chimeric cDNA was inserted into an adenovirus delivery vector that also contained the *GFP* cDNA. The expression of the transgene is regulated by the tTA/tetO system such that expression of the transgene is obtained only after coinfection with tTA-expressing adenovirus and in the absence of Dox. Figure 3A shows typical infected DRG neurons positive for GFP and the neuronal marker GAP43. Expression of the VP16-CREB protein in the infected neurons was confirmed by Western blotting, and Figure 3B shows that coinfection with the tTA-expressing adenovirus, in the absence of Dox, significantly increases VP16-CREB expression. Neurons infected with virus with or without the VP16-CREB were then cultured on either MAG-expressing or control CHO cells, in the absence of Dox. Again to ensure that only infected neurons were assessed, neurites were measured only from those neurons that were both GFP and GAP43 positive. Figure 3C shows that DRG neurons that express VP16-CREB are not inhibited by MAG, while control-infected neurons are inhibited to the same extent as we routinely find for uninfected neurons, by about 40%. These results suggest that activation of CREB is sufficient to change the neuron's response to MAG such that it is no longer inhibited.

Neurons from a Transgenic Mouse Expressing Constitutively Active CREB Are Not Inhibited by MAG and Myelin

Using this same constitutively active VP16-CREB and the same Tet-regulatory system, a transgenic mouse was made, in which the expression of the transgene is under the control of the CaMK II promoter (Barco et al., 2002). Expression of VP16-CREB was turned on by removal of Dox from the diet in mice 3 weeks old for 1 week before we started our experiments. However, these mice do not express the transgene in the neuronal types that we routinely use in our neurite outgrowth experiments, DRG and cerebellar neurons (Figure 4A

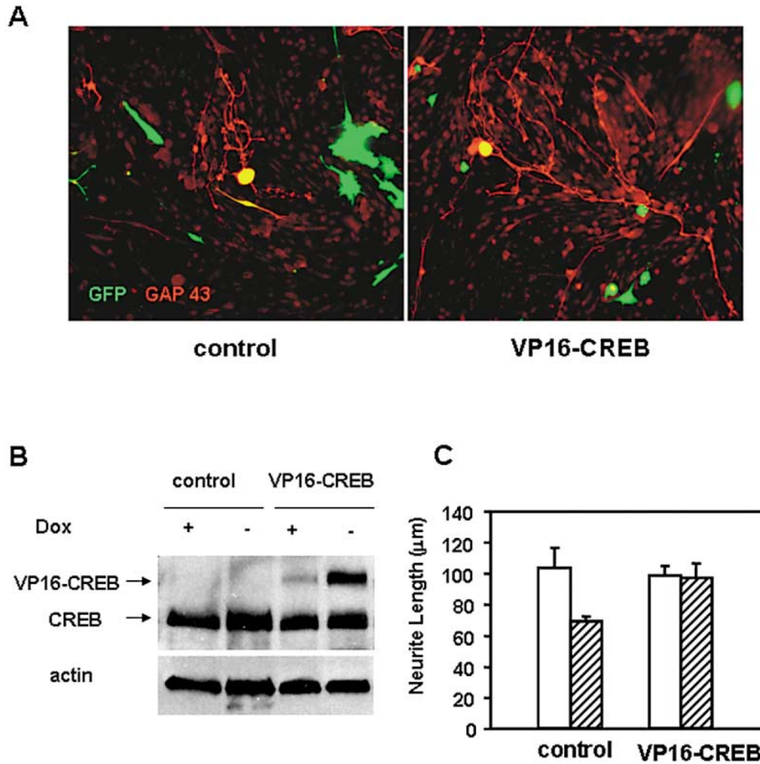

Figure 3. Expression of Constitutively Active CREB Is Sufficient to Block Inhibition by MAG

DRG neurons (P5–P7) were dissociated and infected with adenovirus vectors containing the cDNA for *VP16-CREB* and *GFP* or with the cDNA for *GFP* only. Twenty-four hours after infection, the neurons were either lysed and subjected to Western blotting or transferred to MAG-expressing CHO cells, control CHO cells, or myelin, before being fixed and stained for GAP43 and viewed for GFP. (A) Image of control-infected (left panel) and VP16-CREB-infected (right panel) neurons positive for both GFP and GAP43. (B) Western blot of neurons infected with *VP16-CREB* cDNA-containing virus or with control virus, grown with or without Dox (Tet-off) and stained for CREB. Arrows indicate the positions of VP16-CREB and wild-type CREB. Blots were stripped and immunostained for actin (bottom panel). (C) Measurement of neurite length from neurons infected with control virus (control) or with virus containing *VP16-CREB* cDNA (VP16-CREB), grown on either MAG-expressing CHO cells (striped bars) or control CHO cells (white bars), before being fixed and stained for GAP43. In each experiment, the mean length of the longest GAP43/GFP-positive neurite for 180 to 200 neurons was measured (±SEM). Each experiment was carried out at least three times.

and data not shown). Instead, we used hippocampal neurons from 1-month-old mice. Figure 4A shows that there is robust expression of the VP16-CREB transgene in hippocampal neurons, but no expression in DRG neurons, in mice 1 month old and 4 months old that were not fed Dox. Hippocampal neurons from 1-month-old mice were then used in the neurite outgrowth assay. Figure 4C shows that for neurons from transgenic mice expressing *VP16-CREB*, MAG has no effect on neurite outgrowth; neurite length is the same on MAG-expressing and on control CHO cells. Likewise, these hippocampal neurons expressing the *VP16-CREB* transgene are not inhibited by myelin (Figure 4B). In contrast, when the same transgenic mice are maintained on Dox and the VP16-CREB is not expressed, MAG inhibits neurite outgrowth by about 60%, and relative to growth on poly-l-lysine, myelin inhibits neurite outgrowth by about 70% (Figures 4B and 4C). Therefore, consistent with what we

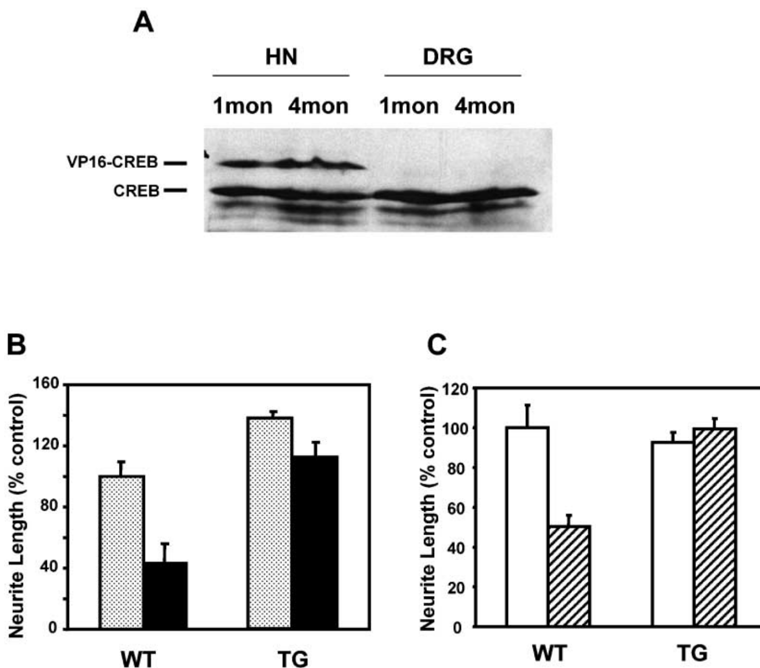

Figure 4. Neurons from a Transgenic Mouse Expressing Constitutively Active CREB Are Not Inhibited by MAG

Hippocampal or DRG neurons were removed from either 1-month-old or 4-month-old VP16-CREB transgenic (Tg) or wild-type (WT) mice and either lysed and subjected to Western blotting or transferred to MAG-expressing, control CHO cells or myelin as a substrate and cultured overnight before being fixed and stained for GAP43. (A) Western blot of lysates from hippocampal (HN) and DRG neurons from 1-month-old and 4-month-old transgenic mice, stained for CREB. Both VP16-CREB and WT-CREB are indicated. (B and C) Measurement of neurite outgrowth from hippocampal neurons from 1-month-old VP16-CREB transgenic (Tg) and wild-type mice, grown on (B) poly-l-lysine (stippled bars) or myelin (black bars) or (C) MAG-expressing CHO cells (striped bars) or control CHO cells (white bars). In each experiment, the mean length of the longest GAP43/GFP-positive neurite for 180 to 200 neurons was measured (±SEM). Each experiment was carried out at least three times.

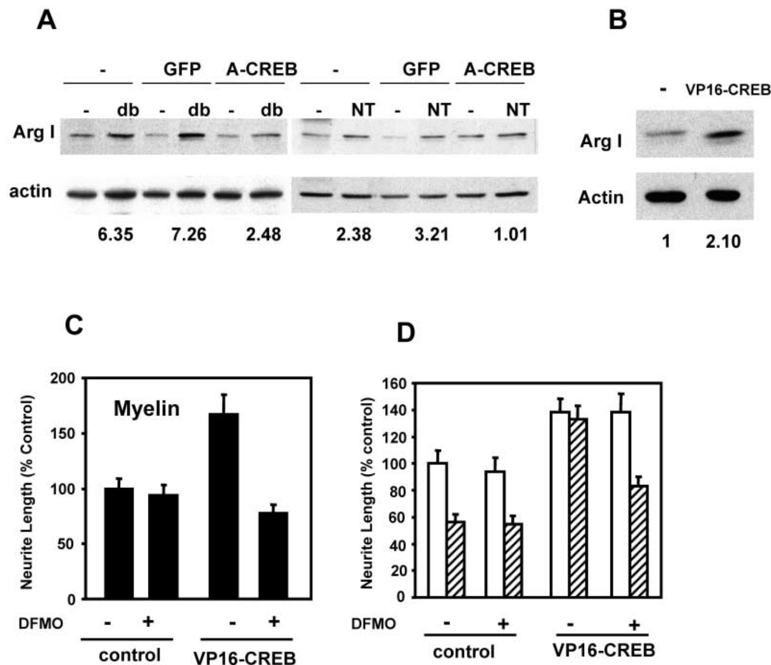

Figure 5. Expression of Arg I Is Regulated by CREB, and the Ability of Constitutively Active CREB to Overcome Inhibition Is Dependent on Polyamine Synthesis

(A and B) DRG neurons (P5–P7) were infected with adenovirus containing *A-CREB*, *VP16-CREB*, or only *GFP*. (A) Uninfected neurons (−), infected with only GFP (GFP) or with A-CREB, were incubated with 1 mM db-cAMP (db) or 200 ng/ml BDNF (NT) overnight before being lysed, or neurons infected with VP16-CREB (B) were lysed and subjected to Western blotting and stained for Arg I. Numbers at the bottom of each gel refer to the fold increase relative to control and standardized for loading to actin. (C and D) Measurement of neurite length from control-infected or VP16-CREB-infected DRG neurons, grown on (C) myelin or (D) control CHO cells (white bars) or MAG-expressing CHO cells (striped bars), with and without DFMO (5 mM) as indicated. In each experiment, the mean length of the longest GAP43/GFP-positive neurite for 180 to 200 neurons was measured (±SEM). Each experiment was carried out at least three times.

observed for neurons infected to express VP16-CREB in culture, expression of constitutively active CREB in vivo is sufficient to block inhibition by MAG and myelin when these neurons are placed in culture. We do not know, however, if VP16-CREB is preferentially affecting dendritic or axonal growth, as the hippocampal neurons are not in culture long enough to make the distinction. Even after 3 days in culture, under the conditions that were used here for hippocampal neurons from P30 mice all processes are positive for both MAP 2, a dendritic marker, and β III tubulin, a general neuronal marker, regardless of whether CREB is activated (data not shown; Dotti et al., 1988).

CREB Regulates the Expression of Arginase I to Overcome Inhibition

Previously, we reported that upregulation of Arg I, a key enzyme in the synthesis of polyamines, by either db-cAMP or priming with BDNF is sufficient to overcome inhibition by MAG and myelin. We also showed that blocking the polyamine pathway blocked the ability of upregulated Arg I to overcome inhibition and that exogenous polyamines, alone, could block inhibition (Cai et al., 2002). To determine if the increase in Arg I expression is dependent on activation of CREB, neurons expressing A-CREB were treated with either BDNF or db-cAMP, and then Western blots of the cell lysates were immunostained for Arg I. Here, we demonstrate that A-CREB blocks the upregulation of expression of Arg I in response to either db-cAMP or BDNF. Figure 5A shows a Western blot of lysates from uninfected, control-infected, and A-CREB-infected DRG neurons immunostained for Arg I protein. We reported before that db-cAMP and BDNF induce a 6- to 7-fold increase and 2- to 3-fold increase in Arg I expression, respectively. Now, we show that this holds true for both uninfected and control-infected neurons (Figure 5A). However, when infected with a virus containing A-CREB cDNA, the increase in Arg I expression is lost completely for BDNF treatment and more than halved in neurons treated with db-cAMP (Figure 5A). These results show that dominant-negative CREB blocks expression of one of the proteins that we have shown to be an effector of cAMP in overcoming inhibition of neurite outgrowth by MAG.

To complement these observations with dominant-negative CREB, we next asked if *Arg I* was upregulated in neurons infected with constitutively active VP16-CREB. Figure 5B is a Western blot for Arg I and shows that, in neurons infected with virus containing *VP16-CREB*, expression of Arg I has more than doubled. Results are standardized to a protein that does not change expression in the presence of VP16-CREB. This suggests that activation of CREB is sufficient to induce an upregulation in expression of Arg I, but is this what is responsible for activated CREB's ability to overcome inhibition by MAG? To address this question, we included in the neurite outgrowth assay an inhibitor of the Arg I-dependent polyamine synthesis pathway. The agent, DFMO, is an inhibitor of the enzyme following Arg I in the pathway, ornithine decarboxylase (ODC). Previously, we showed that DFMO blocked the ability of db-cAMP and priming with BDNF to overcome inhibition by MAG. Figures 5C and 5D show that the ability of neurons expressing VP16-CREB to grow on MAG or myelin was blocked when DFMO was included in the cultures, suggesting that indeed it is the upregulation of Arg I and synthesis of polyamines by constitutively active CREB that overcomes inhibition by MAG.

High Levels of Activated CREB Are Required to Overcome Inhibition by MAG

Neurotrophin Trk receptors, and not p75NTR, are required for the priming effect to overcome inhibition (Gao et al., 2003). Upon interaction with Trk receptors, neurotrophins such as BDNF can activate a number of differ-

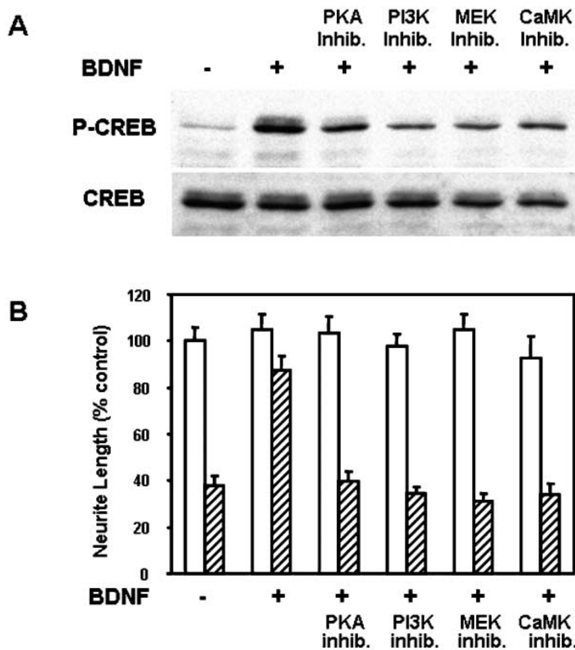

Figure 6. Activation of at Least Four Pathways by BDNF Is Required to Reach the Level of Activation of CREB Necessary to Overcome Inhibition by MAG

Cerebellar neurons (P5) were dissociated and primed for 30 min (A) or overnight (B), with and without BDNF (200 ng/ml), in the presence or absence of each of the following inhibitors: PKA inhibitor (200 nM KT5720), PI3K inhibitor (20 μM LY294002), MEK inhibitor (5 μM U0126), or CaMK inhibitor (10 μM KN62) as indicated, before being lysed and subjected to Western blotting or transferred to either MAG-expressing CHO cells or control CHO cells. (A) Western blot stained for phospho-CREB (P-CREB) and then stripped and stained for CREB. (B) Measurement of neurite length from neurons grown on MAG-CHO cells (striped bars) or on control CHO cells (white bars) overnight before being fixed and stained for GAP43. In each experiment, the mean length of the longest neurite for 180 to 200 neurons was measured (±SEM). Each experiment was carried out at least three times.

ent signaling cascades, each of which results in activation of CREB and transcription of a variety of genes (Kaplan and Miller, 2000). To determine which of these signaling pathways is required for the priming effect of BDNF, the effect of inhibitors of these different pathways was assessed on both phosphorylation of CREB and the ability of priming with BDNF to overcome inhibition. When inhibitors of protein kinase A (H89), PI3K (LY), Erk (U0126), or CaM kinase (KN62) were each included during priming with BDNF, activation of CREB was reduced in each case but not completely abolished (Figure 6A). This suggests that it is the additive effect of these signaling cascades that accounts for the final activation of CREB in response to BDNF. To assess the effect of these various inhibitors on the ability of BDNF to block inhibition by MAG, cerebellar neurons were primed with BDNF, with and without the inhibitors, before being cultured on MAG-expressing or control CHO cells. Figure 6B shows that, when inhibitors of the PKA, PI3K, Erk, or CaMK pathways are each included during priming, the ability of BDNF to block MAG's inhibition is completely abrogated. Therefore, those inhibitors that attenuate BDNF's activation of CREB each block completely the ability of BDNF to overcome inhibition by MAG. We ensured the specificity of the inhibitors by showing that neither the PI3K nor the CaMK inhibitors had any effect on the phosphorylation of Erk by BDNF (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/44/4/609/DC1/). None of these inhibitors affected neurite outgrowth on control CHO cells that did not express MAG, neither when they were included during priming with BDNF nor when they were added directly to the cocultures along with db-cAMP. This indicates that, although growth induced on the control CHO cells may involve activation of these pathways, simultaneous activation of all these pathways is not essential for this neurite outgrowth.

Previously, we showed that, at a concentration of 1 mM db-cAMP, activation of the Mek/Erk pathway as well as the PKA pathway is required to overcome inhibition, while at 2 mM db-cAMP only activation of PKA is required (Gao et al., 2003). Figure 7A shows that this is also true for db-cAMP-induced activation of CREB. At 1 mM db-cAMP, activation of CREB is reduced, and the ability to overcome inhibition is blocked by either a PKA or a Mek/Erk inhibitor but not by inhibitors of PI3K or CaMK (Figures 7A and 7B). However, at 2 mM db-cAMP, a Mek/Erk inhibitor has no effect on the phosphorylation of CREB (Figure 7C) or on its ability to overcome inhibition (Gao et al., 2003). Together, these results suggest that a relatively high level of activated CREB is required to overcome inhibition by MAG. For priming with BDNF, the level is reached by activation of four pathways simultaneously. For lower concentrations of db-cAMP (1 mM), activation of both PKA and Mek/Erk pathways are required, while at higher db-cAMP (2 mM), activation of the PKA pathway is strong enough alone to reach the requirement of CREB activation to overcome inhibition.

Myelin Attenuates the Activation of CREB by BDNF but Not by db-cAMP to below the Level Required to Overcome Inhibition by MAG

Previously, we showed that to overcome inhibition neurons needed to be exposed to neurotrophins before they encountered the inhibitor. In contrast, prior exposure of the neuron to db-cAMP was not required to overcome inhibition (Cai et al., 1999). Our working hypothesis is that MAG and myelin activate a heterotrimeric G protein Gi/Go, which inhibits adenylate cyclase, the enzyme that synthesizes cAMP, and so attenuates cAMP accumulation in response to neurotrophin. Therefore, cAMP must be allowed to accumulate and trigger downstream pathways before adenylate cyclase is inhibited by interaction with MAG/myelin. On the other hand, when cAMP is artificially elevated by addition of db-cAMP, it is independent of adenylate cyclase, and so priming is not required. Here, we sought to determine if the ability of BDNF and db-cAMP to activate CREB is affected by the presence of inhibitor, and for this we used myelin. Cerebellar neurons were cultured on either a substrate of poly-l-lysine or purified myelin, in the presence or absence of either BDNF or db-cAMP. After 30 min, they were fixed and stained for phospho-CREB or subjected to Western blotting for phospho-CREB. As can be seen in Figure 8, when cerebellar neurons are cultured on

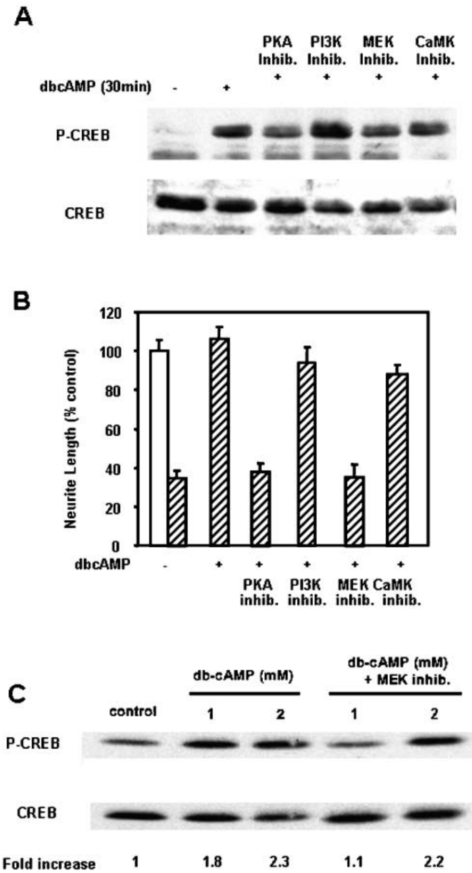

Figure 7. At High db-cAMP Concentrations, Activation of Only PKA Is Sufficient to Reach the Level of Activation of CREB Needed to Overcome Inhibition by MAG

Cerebellar neurons (P5) were dissociated and cultured with db-cAMP at 1 mM (A) or 1 or 2 mM (C), for 30 min (A and C) in the presence or absence of the inhibitors as indicated in Figure 6 before being lysed and subjected to Western blot, or neurons were cultured overnight on MAG-CHO or control CHO cells in the presence of db-cAMP and/or inhibitors as indicated. (A and C) Western blots stained for phospho-CREB (P-CREB) and stripped and stained for CREB. Fold increase in (C) refers to increase relative to control, standardized to total CREB per lane. (B) Measurement of neurite length as described in Figure 6.

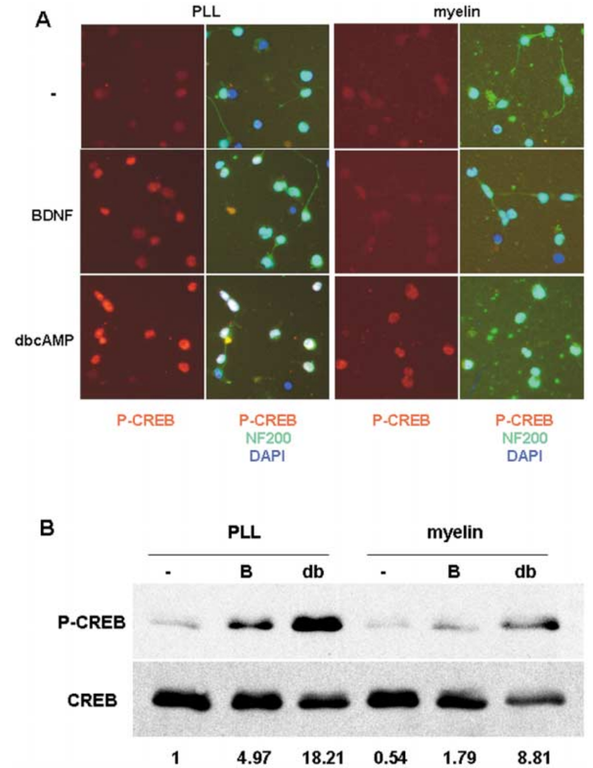

Figure 8. Myelin Reduces the Level of Activation of CREB to Background for BDNF but Not for db-cAMP

Cerebellar neurons (P5) were dissociated and plated onto either poly-l-lysine (PLL) or myelin and cultured for 30 min in the presence or absence of BDNF (200 ng/ml) or db-cAMP (1 mM), as indicated, before being fixed and stained for Phospho-CREB, NF200, and DAPI (A) or lysed, subjected to Western blotting, and stained for phospho-CREB and then stripped and stained for CREB. Numbers at the bottom of (B) refer to the fold increase in phospho-CREB relative to control neurons on PLL without BDNF or db-cAMP, standardized to total CREB.

poly-l-lysine activated CREB is barely detectable, but when BDNF or db-cAMP is added, phospho-CREB is readily detected. Consistent with what we observed from Western staining (Figure 1), the signal for phospho-CREB is stronger for neurons treated with db-cAMP than for those treated with BDNF. When neurons are cultured on myelin, in the absence of stimulus, again phospho-CREB is barely detectable. However, when BDNF is added to neurons on myelin, unlike when it is added to neurons on poly-l-lysine, the increase in phospho-CREB was greatly reduced, while addition of db-cAMP still induces strong activation of CREB. As a control, myelin has no effect on the ability of BDNF to activate Erk (Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/44/4/609/DC1/). Therefore, at both the immunocytochemical level and as estimated from Western blotting, the presence of myelin blocks almost completely the BDNF-induced but not the db-cAMP-induced activation of CREB.

Expression of Activated CREB Is Sufficient to Promote Spinal Axon Regeneration In Vivo

Previously, we and others reported that if db-cAMP is injected directly into DRG cell bodies, and the dorsal column axons of those same neurons are lesioned either 2 days or 1 week later, there is significant regeneration of these central axons (Neumann et al., 2002; Qiu et al., 2002). To assess if activation of CREB can achieve the same effect, *VP16-CREB*-containing adenovirus was injected into L4 DRG, 4 days before the dorsal column axons were lesioned. Figure 9A shows that at 4 days postinjection, a considerable number of DRG neurons are infected, as they are positive for the GFP reporter and the neuronal marker β-III tubulin. Importantly, these neurons also express the transgene, as they are positive for VP16-protein (Figure 9A). Hence, at the time of dorsal column lesion, at 4 days postinjection, a proportion of DRG neurons are expressing constitutively active CREB. As a control, DRGs were injected with control adenovirus expressing only the *GFP* reporter. The animals were

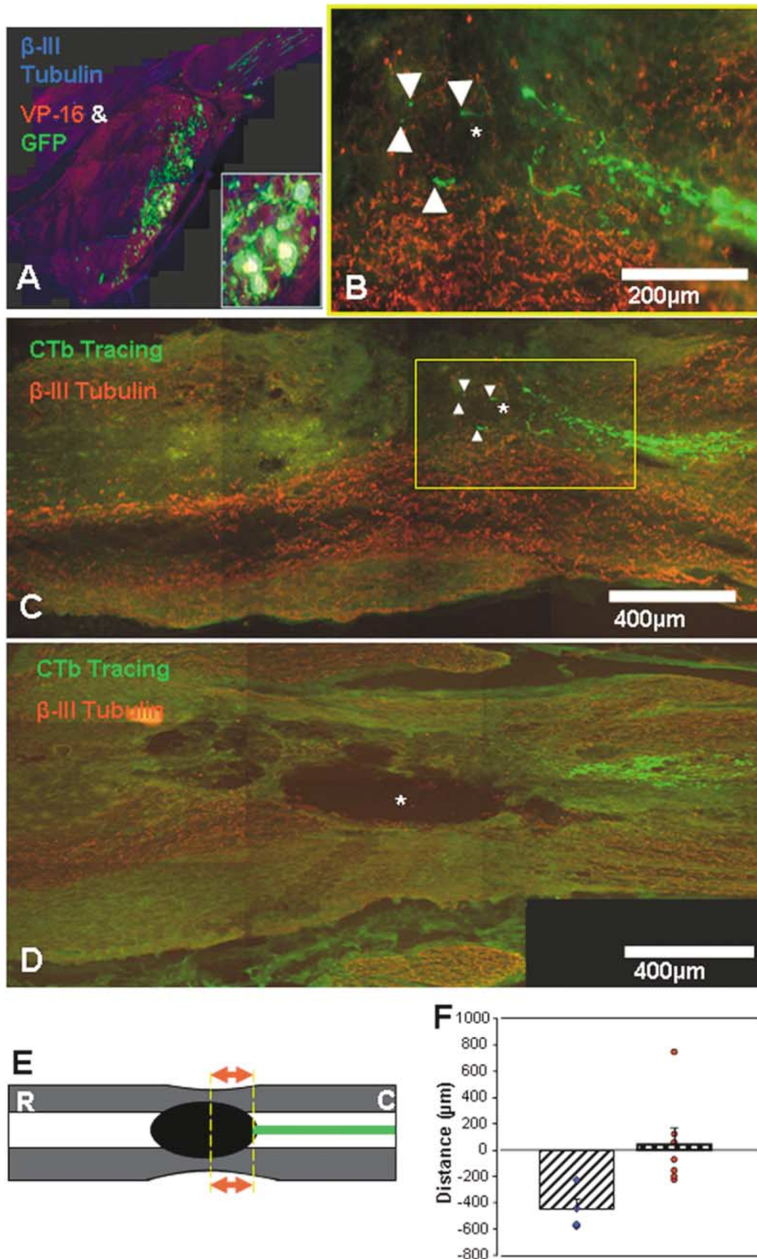

Figure 9. Expression of Constitutively Active CREB by DRG Neurons Is Sufficient to Promote Regeneration of Lesioned Dorsal Column Axons

The L4 DRG of adult rats was injected with either VP-16 CREB-containing virus ($n = 8$) or control virus expressing only GFP ($n = 5$). Four days later, either the animals were sacrificed and the DRGs were sectioned and stained for VP-16 and β-III tubulin, as well as monitored for expression of GFP (A), or the dorsal column was lesioned at T6–T7 (B–D). Before being sacrificed 28 days later, axons were transganglionically traced with cholera toxin B subunit (CTb). Serial 20 μm longitudinal sections were stained for CTb and β-III tubulin. (B) and (C) are a VP-16 CREB-treated animal, and (B) is a magnification of the boxed area in (C). Arrowheads point to axons that have almost traversed the lesion site. (D) is a control animal. The asterisks in (B)–(D) represent the center of the lesion. (E) and (F) are the measurements of the distance regenerated from the caudal side of the lesion, with the approximate center of the lesion as zero, such that any value greater than zero indicates regeneration beyond the lesion center (E). (F) is the distance regenerated by the VP-16 CREB-treated animals (circles; $n = 7$) and the control animals (diamonds; $n = 4$). The two groups are significantly different, with $p < 0.05$.

left for 4 weeks to allow axonal regeneration to occur. Four days before sacrifice, the dorsal column axons were transganglionically traced by back filling with the B subunit of cholera toxin (CTb), injected into the sciatic nerve. Figures 9B and 9C show the lesion site of a typical animal injected with VP-16 CREB-containing adenovirus, and axons that have grown up to and into the lesion site are clearly visible. A few have almost reached the rostral boundary of the lesion (Figures 9B and 9C, arrowheads). In sharp contrast, in control animals CTb-traced axons were only very rarely observed close to the lesion; in the majority of cases, as is usual, the axons had retracted from the caudal boundary of the lesion (Figure 9D). When the distance that axons had regenerated from the caudal edge of the lesion (where the penumbra begins) was measured, the average for the VP16-CREB animals was about 600 μm, while for the control animals the average was less than 200 μm (Figures 9E and 9F). In the majority of the VP16-CREB animals, axons had grown into the lesion site and beyond the lesion center, while only rarely were axons observed in the lesion site of control animals. At most, the axons in the control animals reached the caudal edge of the penumbra but never entered the lesion site proper. Furthermore, we also showed that there was no difference in survival/apoptosis after a dorsal column lesion of neurons infected with either the control virus or the *VP-16 CREB* virus. Animals were injected with virus into the DRG, and 2 days later the dorsal column axons were lesioned. After a further 2 days, the animals were sacrificed, and DRGs were assessed for expression of both GFP and β III tubulin to ensure that only infected neurons were

being counted, as well as for Tunel staining to identify dying cells. Out of 500 neurons observed in each group, only 38 infected with the *VP16-CREB* virus and 21 infected with the control virus appeared to be dying (data not shown). This indicates that any effect of VP16-CREB on regeneration is not an effect on improving survival. These results indicate that expression of constitutively active CREB is sufficient to promote spinal axon regeneration in vivo.

Discussion

It has already been established that elevation of cAMP can overcome inhibition by MAG and myelin to encourage spinal axon regeneration in a transcription-dependent manner (Cai et al., 1999, 2002; Song et al., 1998). Now, we include activation of CREB, or a closely related family member, as a necessary step in this signaling pathway. Our working model is, then, that in response to elevated cAMP, CREB is activated, and the enzyme Arg I is upregulated, resulting in an increase in polyamine synthesis. Polyamines, in turn, are necessary and sufficient to overcome inhibition and encourage regeneration in vivo (Filbin, 2003). All the results presented here are consistent with and support our developing model—a dominant-negative CREB blocks the ability of db-cAMP or BDNF to induce an upregulation of Arg I and to overcome inhibition by MAG/myelin; a constitutively active CREB is sufficient to induce upregulation of Arg I and to overcome inhibition by MAG/myelin, an effect blocked if the polyamine synthesis pathway is blocked. Importantly, expression of activated CREB in DRGs in vivo is sufficient to promote regeneration of subsequently lesioned dorsal column axons. Identifying activation of CREB as a key step in this process is crucial, because it provides an important putative target for therapeutic intervention to enable axonal growth through the hostile environment of a damaged, adult CNS. Indeed, given the central role that CREB appears to play in other systems, it is reasonable to expect that manipulation of CREB's activity may turn out to be pivotal in encouraging regeneration.

The effect of expressing constitutively active CREB in DRG cell bodies mimics the effects of a peripheral conditioning lesion on dorsal column regeneration. Many studies have reported that if the peripheral branch of DRG neurons is lesioned before the same neurons' central branch axons in the dorsal column are lesioned, these spinal axons will spontaneously regenerate (Neumann and Woolf, 1999; Richardson and Issa, 1984). We and others have shown this effect to be cAMP dependent (Neumann et al., 2002; Qiu et al., 2002). Although significant, the regrowth that we observe in response to expression of activated CREB in DRGs is not as robust as the conditioning lesion effect on dorsal column regeneration. This is due to a number of reasons. First, only a fraction of the DRG neurons are infected with the virus. The animals do not tolerate multiple DRG injections, so we were limited in the amount of virus we could deliver in a single injection to a single DRG. In contrast, lesioning the sciatic nerve conditions all the dorsal column axons emitting from L3, L4, and L5. Second, although we show expression of the transgene, we do not know how robust the expression is relative to what is required to maximize the effect. Third, a conditioning lesion is most effective if it is performed 1 week prior to the central lesion (Neumann and Woolf, 1999). We do not know when the optimal time is to activate CREB prior to a dorsal column lesion. Despite all these caveats, we still observe significant and obvious regrowth of dorsal column axons in animals expressing VP16-CREB and not in animals injected with the control virus.

The results are intriguing because they also suggest that a threshold of activation of CREB is required to overcome inhibition. For BDNF, where the cumulative activation of CREB through at least four pathways is not as robust or as prolonged as the activation seen with db-cAMP, activation of all four pathways is required to reach the putative threshold. In contrast, even at 1 mM db-cAMP, a strong activation of CREB is observed, and it is sustained for longer than that for the BDNF response. However, at 1 mM db-cAMP, this strong response is a consequence of the combined activation of CREB through the PKA and Mek/Erk pathways; inhibitors of each of these pathways attenuate the activation of CREB by 1 mM db-cAMP and block its ability to overcome inhibition. By contrast, at 2 mM db-cAMP, inhibition of the Mek/Erk pathway has no effect on activation of CREB or on blocking inhibition. The effect at 2 mM db-cAMP is completely dependent on the PKA pathway, consistent with what we have reported previously, that cAMP/PKA must reach a threshold of activation to overcome inhibition (Gao et al., 2003).

It is of note that, although during priming signaling by BDNF is required to subsequently overcome inhibition by MAG, this same BDNF signaling is not essential for growth when neurons are subsequently grown on control CHO cells. This is apparent because if activation of either PI3-K or Mek, which has been shown to be necessary for process outgrowth in response to neurotrophins (Liu and Snider, 2001), is blocked during priming with BDNF, it blocks the ability of BDNF to overcome inhibition but has no effect on neurite outgrowth from neurons subsequently grown on control CHO cells. This can be explained as follows. First, BDNF is only present during priming, and this priming has no effect on neurite length from neurons subsequently grown on control cells in the absence of BDNF. Second, the favorable environment created by the CHO monolayer accounts for the good growth on control CHO cells, which is likely to result in activation of numerous signaling pathways. This in turn allows for redundancy when any one pathway, such as PI3-K or Mek, is blocked, therefore growth proceeds.

Although we have shown that upregulation of Arg I is dependent on activation of CREB, we do not know if this effect is direct or indirect. The Arg I promoter region does not appear to contain the consensus, palindrome sequence TGACGTCA, which binds CREB with high affinity. Rather, it carries a variant CRE half-site, which can be functional but, because of lower affinity of binding, would not be as effective as the full CRE palindrome sequence (Craig et al., 2001; Fink et al., 1988; Yamamoto et al., 1988). If indeed the effect of CREB on Arg I expression is via direct interaction with its promoter, then a half-site rather than a full site may be why a relatively

high level of activated CREB is required to drive expression and overcome inhibition.

CREB family members have been shown to be involved in numerous aspects of neuronal function, from survival to learning and memory (Kandel, 2001; Lonze and Ginty, 2002). How is such a range of effects brought about by activation of this single transcription factor family? It is possible, as shown here and as proposed by others, that the strength and/or the duration of CREB activation by different stimuli dictate the resulting effect. For example, short, weak activation could result in a survival effect, while a much stronger and longer activation is required to overcome inhibitors in myelin. The fact that a differential effect arises from the strength of CREB binding is supported by the observation that the effect on survival of neurotrophins, unlike overcoming myelin inhibitors, is independent of cAMP/PKA (Boniece and Wagner, 1993; Rydel and Greene, 1988). The implication is that, although neurotrophins do indeed elevate cAMP and activate PKA, this pathway is not needed to reach the threshold of activation of CREB required for survival; activation of the Erk pathway is sufficient, because a lower threshold of activated CREB is required. This hypothesis in turn implies that different genes are turned on depending on the strength and duration of the CREB signal. Although Arg I has been shown to be an antiapoptotic agent (Esch et al., 1998), this function is not a consequence of neurotrophin signaling. It remains to be seen, however, whether or not blocking the synthesis of polyamines has any effect on the survival effects of neurotrophins.

Alternatively, it is possible that at the molecular level CREB regulation is the same but that the cellular and extracellular milieu dictate how the neuron can respond. For example, to achieve regeneration, CREB must activate a growth program and overcome inhibition. Likewise, for processes such as memory and learning, synapses must be strengthened by process outgrowth (Castellucci et al., 1970; Kupfermann et al., 1970). Mechanistically, there are many similarities between what is required structurally to encourage growth during regeneration and growth associated with plasticity. Indeed, for the model of learning and memory long-term potentiation (LTP), many of the steps shown to be involved are similar to what we describe here—cAMP/PKA, CREB, and BDNF are involved in both (see Kandel, 2001). In addition, polyamine synthesis has been shown to play a role in the modulation of synaptic transmission (Aizenman et al., 2002).

There are at least two other mechanisms that can contribute to the specificity of the response to CREB activation. First, in addition to phosphorylation at Ser133, CREB can also be phosphorylated at Ser129, -142, and -143 (Kornhauser et al., 2002). Different combinations of phosphorylation of these serines appear to occur in response to different stimuli. This in turn may influence which coactivators or corepressors associate with CREB, which then leads to differential transcription. In the study presented here, we have only assessed the phosphorylation state of Ser133. The extent of its phosphorylation is consistent with the threshold of activation of CREB that we propose is necessary to overcome inhibition. It is possible, however, that one or more of the other serines known to contribute to the activation state of CREB are also required along with a threshold level of Ser133 to be phosphorylated in order to overcome inhibition by MAG and encourage regeneration. Second, there are at least six CREB-like molecules that have been identified to date (Lonze and Ginty, 2002). Although the antibody we use to assess activation is specific for phospho-Ser133 CREB, the dominant-negative and constitutively active constructs we use are not (Ahn et al., 1998; Barco et al., 2002). That is to say, the dominant-negative A-CREB can block the function of a number of CREB family members. Likewise, VP16-CREB activity is not confined to CREB but can affect transcriptional regulation by other family members. The specificity of the antibody we use for phospho-Ser133 CREB lends confidence that the namesake of the family is involved in overcoming inhibition in response to cAMP; it is also highly likely that other family members also play a role.

Finally, although we have shown that upregulation of Arg I is necessary and sufficient to overcome inhibition by MAG, the fact that CREB regulates numerous genes suggests that it is possible and highly likely that other upregulated proteins can also affect regeneration. The important point here is that there are likely to be other parallel pathways that are activated that can also overcome inhibition and promote regeneration, which would broaden the spectrum of possible targets for therapeutic intervention. In summary, we have identified CREB as a central player in the ability of cAMP to overcome inhibition by MAG, and in promoting spinal axon regeneration in vivo, hence placing it in a pivotal position as a therapeutic target to encourage regeneration in vivo.

Experimental Procedures

Neurite Outgrowth Assay

For myelin membranes (Norton and Poduslo, 1973), 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. Rat CNS myelin at 0.5–1.0 μg total protein/well was dried overnight onto the coated wells and used as a substrate (Mukhopadhyay et al., 1994; Shen et al., 1998). Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an 8-well tissue culture slide (Lab-Tek). Where indicated, isolated cerebellar (P3–P7) and DRG (P5–P8) neurons in Sato (200 nM progesterone, 224 nM selenium, 4 μg/ml insulin, 0.35 mg/ml BSA, 0.4 μg/ml L-thyroxine, 0.34 μg/ml tri-iodo-thyronine) were plated on poly-l-lysine and primed overnight with either BDNF or NGF (200 ng/ml) (Sigma), in the presence or absence of either PKA inhibitor KT-5720 (200 nM), PI3K inhibitor LY294002 (20 μM), MEK inhibitor U0126 (5 μM), or CaMK inhibitor KN62 (10 μM) (Calbiochem) before being transferred to myelin or the CHO cells. The neurite outgrowth assay was carried out as described previously (Mukhopadhyay et al., 1994) with the following modifications: 5×10^4 neurons were plated onto immobilized myelin, and 2×10^4 neurons were used for the CHO cell monolayers. Where indicated, 5 mM DFMO and 1 mM db-cAMP with or without 200 nM PKA inhibitor KT-5720, 20 μM PI3K inhibitor LY294002, 5 μM MEK inhibitor U0126, or 10 μM CaMK inhibitor KN62 were added to the cultures. After 16–18 hr, the cultures were fixed and immunostained for GAP43 as described previously (Mukhopadhyay et al., 1994). The length of the longest neurite or the total process length for each GAP-43-positive neuron for the first 200 neurons encountered when scanning the slide in a systematic manner was determined using an Oncor image analysis program. The percentage change was the same regardless of the method of analysis.

Recombinant Adenovirus

Recombinant adenovirus containing *A-CREB* was constructed by subcloning *CREB* dominant-negative cDNA (provided by Dr. Charles Vinson, National Cancer Institute) into pTRACK CMV and then inserting it by homologous recombination into pAdeasy-1 (He et al., 1998). The viral preparation and purification was carried out as described (He et al., 1998). Recombinant adenovirus *VP16-CREB*, fusion protein between HSV *VP16* (aa 363 to 490), and *CREB* (aas 88 to 341) (Barco et al., 2002) was constructed using the Tet-Off system from Clontech. Cerebellar or DRG neurons were isolated and plated on the poly-l-lysine-coated 24-well plates. Neurons were infected with adenovirus at a final concentration of 10^{10} PFU/ml (or MOI = 100) and maintained in virus-containing media for 1 hr, after which they were washed and the media replaced. After overnight culture to allow expression of the transgene, neurons were either primed with neurotrophins or transferred directly to monolayers of CHO cells and incubated as described above. Neurite length was measured only from those neurons that were both GAP43 and GFP positive; in this way only infected neurons were assessed. Alternatively, infected neurons were lysed in sample buffer and subjected to Western blot analysis.

Western Blot Analysis

Neurons were starved for 1 hr in DMEM and then incubated for different times with combinations of BDNF (200 ng/ml), NGF (200 ng/ml), db-cAMP (1–2 mM), PKA inhibitors H89 (5 μM) and KT-2750 (200 nM), PI3K inhibitor LY294002 (20 μM), MEK inhibitors PD98059 (50 μM) and U0126 (5 μM), or CaMK inhibitor KN62 (10 μM) (Calbiochem) as indicated. Neurons were then washed with PBS and lysed in boiled sample buffer (62.5 mM Tris-HCl [pH 6.8] and 2% SDS), which included phosphatase inhibitors, 20 mM NaF, 1 mM Na_3VO_4, and 10 mM α-glycerophosphate (Sigma). Samples were run through DNA shredders (Qiagen), and protein was measured by a Lowry-based protein assay (BioRad). Fifty micrograms of protein for detection of CREB and 20 μg of protein for detection of Arg I were loaded and separated by 10% SDS-PAGE. After electrophoresis, gels were transferred to PVDF membranes (NEN) and probed with either a phospho-CREB (Ser133) antibody (Cell Signaling), a CREB antibody (Cell Signaling), an actin antibody (Sigma), or an Arg I antibody (kindly provided by Dr. Rajiv R. Ratan, Harvard Medical School) overnight at 4°C. Blots were incubated with an anti-rabbit IgG-HRP secondary antibody (1:5000) for 1 hr, and detection was performed using ECL (Amersham). Some blots were later stripped with stripping buffer (0.2 M glycine [pH 2.2], 1% Tween-20, and 0.1% SDS) for 1 hr at room temperature and reprobed as indicated. The expression levels of proteins were quantitated by a FluorImage system.

Isolation of Neurons from Transgenic Mice

Bitransgenic mice were generated by crossing *pCaMKII-tTA* mice and *tetO-VP16-CREB* transgenics (Barco et al., 2002). For all experiments, Dox was administrated at 40 mg/kg of food and removed 1 week before experimentation. Hippocampal and DRG neurons were dissociated and plated onto a tissue culture dish and incubated overnight. Unadhered debris was gently washed off by PBS, and the neurons were transferred to monolayers of CHO cells and incubated as described above. Neurite length was measured only from those neurons that were both GAP43 and GFP positive; in this way only infected neurons were assessed. Alternatively, hippocampus and DRG were homogenized in sample buffer and prepared for Western blot analysis.

Immunostaining of Neurons

Isolated cerebellar neurons (5×10^4) were plated onto immobilized myelin or poly-l-lysine-coated chambers of an 8-well tissue culture slide and incubated overnight. After 1 hr starvation with DMEM, either BDNF (200 ng/ml) or db-cAMP (1 mM) was added to the culture and incubated for 30 min. The cells were then fixed and double stained with a rabbit anti phospho-CREB antibody (Cell Signaling) and a mouse anti-NF200 antibody (Sigma) overnight at 4°C, followed by a 1 hr incubation with an anti-rabbit IgG-Texas Red and an anti-mouse IgG-FITC secondary antibody. DAPI was added in the last wash to counterstain nuclei.

Surgical Procedures

Female Long-Evans rats, 8 weeks old, were used; nine animals were injected with a type 3 recombinant adenovirus expressing VP-CREB (Barco et al., 2002), and six were injected with control adenovirus, expressing only GFP under a CMV promoter. DRG injections were carried out as described previously. Briefly, the L3–L4 vertebrae were exposed, and the left L4 DRG was then unilaterally exposed by a partial laminectomy. Injection of the virus was carried out with a stereotaxic frame (David Kopf Instruments), using a micropipette with an OD of ~50 μm, inserted to a depth of ~20 μm (Qiu et al., 2002). Two microliters of the viral solution (2×10^{11} pfu, VP16-CREB Ad; 3×10^{10} pfu, control Ad) was infused at a rate of 0.5 μl/min, and the micropipette was left in place for a further 2 min before removal, to facilitate diffusion of the virus within the DRG and prevent "leakage." The overlying muscle was then sutured back in place, and the skin was closed using wound clips. Four days later, a dorsal column lesion was carried out as described previously (Qiu et al., 2002), at T6–T7 in seven of the constitutively active CREB-expressing animals and five of the control virus-infected animals. After 24 days, they were transganglionically traced with a 4 μl injection of 1% biotinylated cholera toxin B subunit (List Biologicals), delivered by Hamilton syringe, into the left sciatic nerve (10 mm proximal to the Tibial/Peroneal nerve bifurcation), which was then crushed to facilitate penetration of the tracer into the axons. Four days later, under deep anesthesia, animals were transcardially perfused with 300 ml of PBS, containing 1000 U/l of heparin (Sigma-Aldrich) and 250 ml of ice-cold 4% paraformaldehyde, in PBS. The thoracic spinal cord was then removed and postfixed in 4% paraformaldehyde for 50 min, before being cryoprotected in 10% sucrose solution (in PBS) for 2 hr and 20% sucrose (in PBS) overnight. Samples were then placed in OCT embedding media, appropriately aligned, and frozen for sectioning using dry ice.

Two animals from each group were transcardially perfused 4 days after injection of the viral solution to evaluate expression of the virus at the time of the dorsal column lesion in other animals. The lumbar spinal cord and dorsal nerve roots were then removed and prepared for sectioning according to the above method.

Immunofluorescent Staining

Thoracic spinal cord samples were cut into 20 μm thick longitudinal sections using a Jung Frigocut 2800N cryostat, collected onto Fisher Brand Superfrost Plus slides. Briefly, sections were washed in PBS and then incubated in blocking media (PBS with 2.5% BSA, 0.5% Triton X-100, and 2% normal goat serum) for 1 hr. Slides were then incubated with goat α-Choleragenoid (1:2000; List Biologicals), 2.5% BSA, and 2% Triton X-100, overnight at 4°C. They were then washed and blocked with 2% normal donkey serum for 1 hr. Sections were then incubated either with biotinylated donkey α-goat (1:200; Santa Cruz Biotechnology) alone or in combination with mouse α-βIII tubulin (1:1000; Covance) in PBS containing 2.5% BSA and 2% Triton X-100, for 2 hr. They were then washed, before being incubated with Fluorescein-conjugated Streptavidin (1:200; Amersham), and either Texas red-conjugated sheep α-mouse (1:200; Amersham) or mouse α-Glial Fibrillary Acidic Protein (Cy-3 Conjugate; 1:400; Sigma) in PBS containing 2.5% BSA and 2% Triton X-100, for 1 hr. Immunofluorescence was visualized under a Nikon E400 microscope, and images were captured with a Hamamatsu C4742-95 camera, using Simple PCI. Once the images were montaged, an image of a grid, obtained from a hemocytometer under exactly the same imaging conditions, was then superimposed on the reconstructed picture and was used for obtaining measurements.

Measurements

The section with the furthest penetration of CTb-labeled axons into the lesion site was selected for each animal and used for the measurements.

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