CAAT/Enhancer-binding Protein δ and cAMP-response Element-binding Protein Mediate Inducible Expression of the Nerve Growth Factor Gene in the Central Nervous System

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Nerve growth factor (NGF) synthesis in the rat cerebral cortex is induced by the β2-adrenergic receptor agonist clenbuterol (CLE). Because NGF is a crucial neurotrophic factor for basal forebrain cholinergic neurons, defining the mechanisms that regulate its transcription is important for developing therapeutic strategies to treat pathologies of these neurons. We previously showed that the transcription factor CCAAT/enhancer-binding protein (C/EBPδ) contributes to NGF gene regulation. Here we have further defined the function of C/EBPδ and identified a role for CAMP response element-binding protein (CREB) in NGF transcription. Inhibition of protein kinase A in C6-2B glioma cells suppressed CLE induction of an NGF promoter-reporter construct, whereas overexpression of protein kinase A increased NGF promoter activity, particularly in combination with C/EBPδ. A CRE-like site that binds CREB was identified in the proximal NGF promoter, and C/EBPδ and CREB were found to associate with the NGF promoter in vivo. Deletion of the CRE and/or C/EBP sites reduced CLE responsiveness of the promoter. In addition, ectopic expression of C/EBPδ in combination with CLE treatment increased endogenous NGF mRNA levels in C6-2B cells. C/EBPδ null mice showed complete loss of NGF induction in the cerebral cortex following CLE treatment, demonstrating a critical role for C/EBPδ in regulating β2-adrenergic receptor-mediated NGF expression in vivo. Thus, our findings demonstrate a critical role for C/EBPδ in regional expression of NGF in the brain and implicate CREB in CLE-induced NGF gene transcription.

Alzheimer disease is characterized by devastating changes in plasticity of basal forebrain cholinergic neurons and deficits in memory and other cognitive functions (1). Among the most important questions in Alzheimer disease pathobiology is whether it is possible to restore cholinergic neurotransmission and improve cognition in patients. A significant amount of data indicates that nerve growth factor (NGF) is a trophic factor for experimentally injured cholinergic neurons (reviewed in Ref. 2) and in Alzheimer disease patients (3). Thus, pharmacological compounds that increase the availability of NGF in the brain (4) would provide a useful therapeutic approach for the treatment of Alzheimer’s disease, as has been demonstrated for focal ischemia (5–7).

In the adult brain NGF is produced by cholinergic targets within the hippocampus and neocortex (8, 9). Various agents increase NGF expression in these areas, including glucocorticoid hormones (10–12) and the neurotransmitters glutamate (13, 14) and acetylcholine (15). Furthermore, activation of β2-adrenergic receptors (BARs) by treatment with the lipophilic agonist, clenbuterol (CLE), also leads to increased levels of NGF mRNA and protein in the rat cerebral cortex (16, 17). However, little is known about signal transduction and transcriptional events underlying the neuronal specific activation of the NGF gene. NGF gene transcription is induced by increases in cAMP and activation of the protein kinase A (PKA) signaling pathway (reviewed in Ref. 18). Among transcription factors that are cAMP-inducible, CCAAT/enhancer-binding protein δ (C/EBPδ) has been implicated in regulating NGF gene expression in the brain in response to BAR activation (19). C/EBPδ belongs to a family of six structurally and functionally related transcription factors (C/EBPα, -δ, -β, -γ, -ε, and -η) that regulate multiple aspects of cell function, including proliferation, differentiation, stress responses (20), and neuronal development (21). Several C/EBPs are expressed in specific brain structures. For example, C/EBPα mRNA is found in the murine hippocampus, cerebellum, and cortex (22), whereas C/EBPδ is widely expressed in the mouse central nervous system and is a downstream target of NGF receptor activation (23). Additionally, C/EBPδ and C/EBPε expression and DNA-binding activities are enhanced by stimulation of cAMP-dependent signaling pathways in rat hippocampal neurons, and both proteins have been implicated in learning and memory formation (24–26).

Previous studies suggested that C/EBPδ may function as a region-specific regulator of NGF gene transcription in the brain (19). Stimuli known to increase NGF synthesis enhanced C/EBPδ DNA-binding activity in a glial cell line and in rat brain, and C/EBPδ was shown to transactivate a NGF promoter-reporter construct in glioma cells. Using DNase I footprinting analysis, C/EBPδ was found to interact with a sequence spanning nucleotides −90 to −59 of the NGF promoter (19). However, C/EBPδ alone is probably insufficient to mediate the induction of NGF in response to BAR activation. Indeed, inspection of the NGF promoter sequence revealed a putative cAMP-response element (CRE) residing within the C/EBPδ footprint (19). The presence of a CRE-like site within the NGF promoter suggests that CRE-binding protein (CREB) may function together with C/EBPδ to regulate NGF promoter activity. CREB is ubiquitously and constitutively expressed throughout the central nervous system, and CREB activation in the
brain in response to cAMP-induced signaling has been well documented (reviewed in Ref. 27). In this study we provide evidence that C/EBPβ is a central component of NGF transcriptional induction following BAR stimulation. Our results suggest that CREB also participates in the induction of NGF gene transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids**—C6-2B rat glioma cells were maintained as previously described (28). pNFG-luc (615/+50) has been described (19). pNFG-luc (615/+16) was generated in similar fashion and used as a template for production of the 5′ deletion mutant series (pNFG–100/+16 through pNFG–60/+16) (19). pNFG-C/EBP (5′-TGTTAC-CAGG), -CRE (5′-GGTACCGA), and -C/EBP CRE mutant promoter-reporter constructs were produced by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s directions. pCDNA-C/EBPβ was derived from pMEX-C/EBPβ (29) by digesting the pMEX construct with EcoRI and HindIII and inserting the 800-bp mouse C/EBPβ coding region fragment into pCDNA3.1(−). An expression construct for PKA (pPKA-wt) has been previously described (30) and was kindly provided by Christian Trautwein.

**Transient Transactivation Assay**—C6-2B cells were transiently transfected with FuguNe2 6 transcription reagent (Roche Applied Science, Indianapolis, IN). Transfections were carried out using 0.1 µg of pNFG promoter-reporter constructs, 0.1 µg of pCDNA or pCDNA-C/EBPβ expression constructs, 0.5 µg of pPKA-wt, and 0.2 µg of pRSV-β-galactosidase reporter plasmid as an internal control. Twenty-four hours following transfection, cells were washed with phosphate-buffered saline and placed in serum-free medium. Cell lysates were analyzed for luciferase activity 48 h post-transfection, cells were washed with phosphate-buffered saline and placed in serum-free medium 24 h before the experiment. Where indicated, cells underwent 3-h stimulation with 10 µM CRE. RNase protection assay was carried out using the rat neurotrophin probe set, rNT-1 (Multi-Probe RNase Protection Assay System, BD Biosciences). Probe labeling and hybridization were carried out per the manufacturer’s instructions. Briefly, 40 µg of RNA was hybridized overnight at 56 °C with 1×6 cm probe/sample. After hybridization, samples were digested with RNase for 45 min at 30 °C and precipitated for 30 min in a dry ice/EtOH bath. Pellets were resuspended in 3 µl of loading buffer and analyzed on 6% polyacrylamide sequencing gel. Densitometric scanning was used to normalize protected fragments against glyceraldehyde-3-phosphate dehydrogenase or L32 internal control probes.

**RNase Protection assay** was used to determine the relative levels of c-Fos mRNA in mouse brain samples (see below). The assay was performed with a 404-base 32P-labeled mouse c-Fos cRNA as described (19). [32P]Cyclophilin cRNA was used as a reference to correct for RNA loading (19).

**Immunoblotting**—Transfected C6-2B cells were harvested in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl), and 30 µg of cell lysate was fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose (Schleicher and Schuell), and filters were blocked in Tris-buffered saline with 0.02% Tween 20 and 5% nonfat milk. The membrane was incubated for 1 h with primary antibody against C/EBPβ (C-22) or C/EBPβ (C-19) (1:1000, Santa Cruz Biotechnology), washed, and incubated with anti-rabbit-conjugated horseradish peroxidase antibody (1:20,000, Promega). Signals were detected by enhanced chemiluminescence using Super Signal reagent (Pierce) according to the manufacturer’s directions.

**Chromatin Immunoprecipitation**—ChIP assays were performed essentially as described previously (31). Briefly, sub-confluent cells were placed in serum-free medium 24 h before the experiment. Where indicated, CRE (10 µM) was added 3 h prior to harvest. Cells (15-cm dish per ChIP assay) were cross-linked with 1% formaldehyde for 10 min at room temperature and washed with phosphate-buffered saline, and nuclei were purified as described previously (32). Nuclei were resuspended in lysis buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.1) and sonicated to obtain DNA fragments below 1000 bp. Immunoprecipitation was performed using 1 µg of the following antibodies: C/EBPβ C-terminal (C-22, Santa Cruz Biotechnology), CREB-1 C-terminal peptide antibody (C-21, Santa Cruz Biotechnology), or CREB-1 polyclonal antiserum (generously provided by David Ginty). For control
Analysis of NGF mRNA Expression in WT and C/EBP Knock-out Mice—C/EBPα and C/EBPβ knock-out mice have been described (24, 33). Animals received saline or CLE intraperitoneally and were sacrificed by cervical dislocation at various times after the injection. The brain was removed, and brain areas were dissected on ice, frozen on dry ice, and stored at −80 °C until further processing. NGF mRNA levels in mice were determined by Northern blot analysis as previously described (34, 35). In brief, total RNA was extracted and size-fractionated by agarose/formaldehyde gel electrophoresis. RNA was transferred to nylon membranes (Hybond-XL, Amersham Biosciences) and hybridized overnight at 65 °C in hybridization buffer (50% formamide, 5× SSC (1× SSC is 0.15 M NaCl/15 mm sodium citrate), 0.2% (w/v) polyvinylpyrrolidone, 20 mM EDTA, pH 8.1, 1% (w/v) SDS), containing 32P-labeled NGF cRNA (specific activity, ~6 × 10^6 cpm/mg of RNA). The probe contained 721 bases of the rat NGF coding region (36); NGF cDNA was generated by digesting the plasmid with EcoRI. Linearized plasmid was used as a template for in vitro transcription using T3 polymerase (Promega). Blots were washed once for 15 min at room temperature in 2× SSC buffer containing 0.1% SDS, washed three times for 15 min each at 68 °C in 0.1% SSC buffer containing 0.1% SDS, and then exposed to x-ray film with Hyperscreen (Amersham Biosciences). After development, blots were stripped and re-hybridized with [32P]cyclophilin cRNA as a standard to control for RNA loading.

Relative levels of NGF mRNA are expressed as arbitrary units and were calculated by measuring the optical density of the NGF mRNA band on the autoradiograph analyzed by a densitometer (Bio-Rad GS-710, Bio-Rad) normalized to cyclophilin, as previously described (17). Animals were sacrificed by cervical dislocation at various times after the injection. The brain was removed, and brain areas were dissected on ice, frozen on dry ice, and stored at −80 °C until further processing. NGF mRNA levels in mice were determined by Northern blot analysis as previously described (34, 35). In brief, total RNA was extracted and size-fractionated by agarose/formaldehyde gel electrophoresis. RNA was transferred to nylon membranes (Hybond-XL, Amersham Biosciences) and hybridized overnight at 65 °C in hybridization buffer (50% formamide, 5× SSC (1× SSC is 0.15 M NaCl/15 mm sodium citrate), 0.2% (w/v) polyvinylpyrrolidone, 20 mM EDTA, pH 8.1, 1% (w/v) SDS), containing 32P-labeled NGF cRNA (specific activity, ~6 × 10^6 cpm/mg of RNA). The probe contained 721 bases of the rat NGF coding region (36); NGF cDNA was generated by digesting the plasmid with EcoRI. Linearized plasmid was used as a template for in vitro transcription using T3 polymerase (Promega). Blots were washed once for 15 min at room temperature in 2× SSC buffer containing 0.1% SDS, washed three times for 15 min each at 68 °C in 0.1% SSC buffer containing 0.1% SDS, and then exposed to x-ray film with Hyperscreen (Amersham Biosciences). After development, blots were stripped and re-hybridized with [32P]cyclophilin cRNA as a standard to control for RNA loading.

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RESULTS

Ectopic Expression of C/EBPα in Combination with CLE Treatment Increases Endogenous NGF mRNA Levels—We previously showed that the lipophilic BAR agonist CLE (37) increases C/EBPβ expression in vitro and in vivo (19). To examine the role of C/EBPβ in BAR-mediated activation of the endogenous NGF gene, we analyzed NGF mRNA levels in cells overexpressing C/EBPβ. C6-2B glioma cells were transfected with expression constructs for C/EBPβ or C/EBPα, a C/EBP family member that does not transactivate the NGF promoter (19). Following transfection, cells were left untreated or stimulated with CLE for 3 h, RNA was prepared, and NGF transcripts were analyzed by ribonuclease protection assays. In the absence of BAR activation, expression of C/EBPα or C/EBPβ had no effect on NGF mRNA levels (Fig. 1A). However, overexpression of C/EBPβ combined with CLE stimulation resulted in a 4.7-fold increase in NGF mRNA expression. In contrast, CLE treatment of cells expressing either C/EBPα or C/EBPβ with the empty vector caused only a 2-fold increase in NGF mRNA accumulation. Western blot analysis of lysates from C/EBPα or C/EBPβ transfected cells demonstrated comparable expression levels under both stimulated and unstimulated conditions (Fig. 1B). These data allow several conclusions. First, C/EBPα promotes expression of the endogenous NGF gene. Second, C/EBPα activation of NGF expression is specific, because C/EBPβ had no effect on NGF mRNA levels. Third, C/EBPα-mediated activation of the endogenous NGF gene requires an additional event that is induced by BAR stimulation.

C/EBPβ and CLE Stimulate Transcription from the NGF Promoter—To further investigate the role of C/EBPβ in BAR-mediated NGF promoter activity, we performed reporter assays in C6-2B cells transfected with a fragment of the NGF promoter fused to luciferase (pNGF-luc −615/+50). Treatment of the cells with CLE for 6 h resulted in a 3.5-fold increase in reporter activity compared with unstimulated cells (Fig. 2A). Cotransfection of a C/EBPα expression vector elicited a similar level of induction. Overexpression of C/EBPβ combined with CLE treatment yielded a further increase in luciferase activity compared with either alone. Thus, both BAR stimulation and C/EBPβ overexpression activate the NGF promoter in reporter assays, supporting the idea that C/EBPβ acts as a downstream effector of BAR signaling in regulating NGF gene transcription.

C/EBPβ and PKA Cooperate to Activate the NGF Promoter—The data of Fig. 1 suggest that a CLE-induced event is required for C/EBPβ to transactivate the NGF promoter. BAR stimulation activates adenyl cyclase, which causes elevation of intracellular cAMP levels and subsequent activation of PKA (38). To examine the possible requirement for PKA activation on CLE- and C/EBPβ-induced NGF promoter activity, we performed transient transcription assays in the presence of H89, a chemical inhibitor of PKA. H89 modestly decreased C/EBPβ-induced transcription (~40%) but had no significant effect on vector-transfected
cells (Fig. 2B). H89 also diminished NGF promoter activity in CLE-stimulated cells transfected with C/EBPβ (45% decrease) or the empty vector (35% reduction) (Fig. 2B). Thus, PKA signaling may contribute to NGF gene activation by CLE and C/EBPβ.

To address the relationship between C/EBPβ and PKA in regulating NGF promoter activation, we tested the effect of overexpressing PKA alone or in combination with C/EBPβ. Transfection of the catalytic subunit of PKA in C6-2B cells elicited similar levels of NGF-luc activity compared with overexpression of C/EBPβ, PKA, or both. The -fold induction was determined as described above and represents the mean ± S.E. of three independent experiments performed in duplicate.

To determine whether endogenous CREB binds to NGF-CRE, C6-2B cells were exposed to CLE or dibutyryl cAMP (Bt2cAMP, a cell-permeable analogue of cAMP) for 5 and 15 min. Nuclear extracts were prepared and analyzed by EMSA to assess CREB binding to the NGF-CRE probe. Recombinant CREB protein was incubated with oligonucleotide probes bearing a canonical CRE (Fig. 3A, lane 1) or the NGF-CRE-like element (Fig. 3A, lane 2). EMSA revealed that recombinant CREB binds to the NGF-CRE probe, which shares a half-site with the consensus CRE motif (Fig. 3A). CREB binding to the NGF-CRE probe was efficiently competed by 100-fold excesses of either the unlabeled NGF-CRE oligonucleotide, the entire NGF C/EBPβ-CRE region (NGF-CRE-60), or the consensus CRE sequence (cCREB) (lanes 3–5).

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Identification of NGF Promoter Sequences Mediating Activation by C/EBPβ and CLE—We next sought to identify sequences within the NGF promoter that are necessary for induction by C/EBPβ and CLE. Candidate sequences include the CRE element and a C/EBPβ binding site in the −90/−60 region previously identified by DNase footprinting (19). We performed transient transactivation assays on a series of 5′ deletion mutants driving luciferase (shown in Fig. 4A). The reporter constructs did not include the AP-1 site located at +35, because C/EBPβ transactivation of the promoter is independent of this element (19). Truncation of the NGF promoter to −100 (NGF−100/+16) caused a 7-fold increase in basal promoter activity and a 5-fold augmentation of CLE-induced activity compared with the full-length promoter (Fig. 4B). These data demonstrate the existence of a novel repressive element located within the −615/−100 interval. Constructs bearing deletions to −85 (NGF−85/+16) or −72 (NGF−72/+16) also showed increased basal and CLE-induced promoter activity relative to the full-length promoter. Deletion to −60 (NGF−60/+16) reduced this increase in basal activity and diminished CLE responsiveness to less than 2-fold over the unstimulated level (Fig. 4B). Because the CRE is located between −72 and −60, these findings suggest that the NGF-CRE element plays an important role in CLE-induced transcription.

We also used the deletion constructs to examine sequence requirements for stimulation by C/EBPβ and/or PKA (Fig. 4C). As was seen with CLE treatment, the NGF−100/+16 construct showed 2- to 3-fold increases in C/EBPβ, PKA, or C/EBPβ + PKA-induced luciferase activity compared with the −615/+16 construct. In contrast, activation of NGF−85/+16 or NGF−72/+16 by C/EBPβ or PKA was comparable to that of the full-length promoter. Because deletion to −72 removes the putative C/EBP binding site, it was surprising that responsiveness to C/EBPβ was not compromised. It is possible that promoter activation by C/EBPβ in the absence of the putative C/EBP binding site occurs through direct C/EBPβ binding to the NGF-CRE region, consistent with previous observations that C/EBPβ has some affinity for CRE-like elements (39). Notably, resection to −60 abrogated or substantially impaired responses to C/EBPβ, PKA, and C/EBPβ + PKA (Fig. 4C). These data implicate the NGF-CRE motif as an important element regulating inducible transcription from the NGF promoter.

C/EBPβ and CREB Bind to the NGF Promoter in Vivo—We next used chromatin immunoprecipitation (ChIP) experiments to determine whether C/EBPβ and CREB are associated with the NGF promoter in cells. ChIP assays using a C/EBPβ antibody and either of two CREB antiseras demonstrated binding of both transcription factors to the NGF promoter region in C6-2B cells (Fig. 5). Binding to the promoter was similar in the absence or presence of CLE stimulation. Specificity was demonstrated by decreased ChIP signals when blocking peptides were included in the immunoprecipitation reactions or when a negative con-
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FIGURE 6. Mutation of the CRE or C/EBP sites impairs NGF promoter activation. Transient transactivation assays of NGF promoter-reporter constructs bearing CRE and/or C/EBP site mutations. A, promoter-reporter activity of CRE or C/EBP mutant constructs in C6-2B cells with or without CLE stimulation (6 h). CLE-induced luciferase activity of the NGF CRE + C/EBP double mutant. B, effect of promoter mutations on responses to PKA, C/EBPβ, or PKA plus C/EBPβ. The -fold induction of luciferase activity in A–C was determined as described in Fig. 3 and is calculated relative to the wild-type unstimulated construct (NGF – 615/+16 WT) plus pcDNA. Data are the mean ± S.E. of three independent experiments performed in duplicate. D, EMSA using recombinant CREB and C/EBPβ. Binding of CREB-bZIP to a consensus CRE probe (cCRE) was challenged with unlabeled WT or mutant NGF-CRE oligonucleotides or cCRE (left panel). A similar experiment using recombinant full-length C/EBP protein and C/EBPβ probe is shown in the middle panel. Comp., competitor. In the right panel, labeled WT and mutant NGF-CRE probes were analyzed for binding to CREB-bZIP.

Control antibody (IgG) was used. Based on these criteria, positive signals were obtained for C/EBPβ and CREB binding to the NGF promoter and to a positive control, the IL-6 promoter, which has a well characterized C/EBP site (40). Binding was not apparent using a negative control sequence from the β2-microglobulin gene (31). This amplicon exhibited higher background PCR signals that were generally not diminished by the blocking peptides, although a weakly positive signal was suggested with the competing peptide for the CREB #1 antiserum. Collectively, the data of Fig. 5 indicate that C/EBPβ and CREB are associated with the chromatin-embedded NGF promoter even in the absence of an inducing signal.

Directed Mutation of the C/EBP or CRE-like Sites within the Proximal NGF Promoter Impairs Induction by C/EBPβ and CLE—To further demonstrate that the C/EBP and CRE-like motifs are required for NGF promoter activation, we generated luciferase reporter constructs using the –615/+16 promoter fragment in which the two binding sites were mutated individually or in combination. The mutant constructs were analyzed for induction by CLE, C/EBPβ, and/or PKA. Mutation of either the CRE or C/EBP sites resulted in a ~40% reduction in CLE-induced promoter activity [Fig. 6A]. However, mutation of both binding sites together did not further diminish promoter activity (Fig. 6B). C/EBPβ- and/or PKA-induced activation of the mutant promoter constructs was decreased to a similar extent (~50%) compared with the WT promoter (Fig. 6C). The reduction in promoter activity when the C/EBP or CRE sites are mutated indicates that both motifs are important for maximal induction of NGF gene transcription.

To confirm that these mutations disrupted binding of CREB and C/EBPβ, we performed competition DNA-binding assays using recombinant proteins and consensus CRE or C/EBP site probes. Binding of each protein to its cognate probe was competed by an excess of WT NGF-CRE or NGF-C/EBP oligonucleotides but not by their respective mutant sequences (Fig. 6D, left and middle panels). Also, 32P-labeled NGF-CRE probe bound to CREB while the mNGF-CRE probe did not (right panel). Thus, the point mutations effectively eliminate binding of CREB and C/EBPβ.

C/EBPβ Knock-out Mice Are Defective for CLE-induced NGF mRNA Expression in the Cortex—Previous studies using rats showed that intraperitoneal injection of CLE results in 2- to 3-fold increase of NGF mRNA and protein specifically in the rat cerebral cortex (16, 17). This increase correlates with elevated levels of C/EBPβ DNA-binding activity in the same brain region (19). To definitively test the role of C/EBPβ in NGF transcription in vivo, we examined cortical NGF mRNA levels after CLE treatment of wild-type (WT) or C/EBPβ knock-out (KO) mice (Fig. 7, upper panel). A slight decrease in NGF mRNA levels was observed in saline-treated C/EBPβ KO animals compared with age-matched WT animals, although this effect was very minor when NGF expression was normalized to cyclophilin levels. Thus, C/EBPβ...
is not required for basal NGF expression in the cortex. We also measured normalized NGF mRNA levels in WT and mutant animals at various times following CLE treatment (Fig. 7, lower panel). CLE elicited a time-dependent increase in NGF mRNA in WT mice beginning at 5 h, which returned to basal levels after 18 h. In contrast, no induction of NGF mRNA was observed for C/EBPβ KO animals at any time examined. CLE-induced BAR signaling in C/EBPβ KO animals was confirmed by RNase protection analysis of c-Fos mRNA, which is also induced by CLE treatment (19). CLE caused a 2-fold increase in cortical c-Fos mRNA expression in both WT and C/EBPβ KO mice within 1 h after treatment (data not shown). Thus, C/EBPβ deficiency does not cause a general impairment of BAR activation or downstream signaling.

To examine whether other C/EBP family members might contribute to NGF regulation, we performed a similar experiment using C/EBPβ null mice. CLE caused a ~2.5-fold increase in NGF mRNA levels in both WT and C/EBPβ KO animals (Fig. 8). This result, together with the complete lack of NGF induction observed for C/EBPδ null mice, demonstrates that C/EBPβ selectively regulates NGF gene expression in vivo.

**DISCUSSION**

Although C/EBP protein function has been studied extensively in a variety of tissues such as liver and adipose, much less is known about the role of these transcription factors in modulating neurotransmitter-induced changes in gene expression in the CNS. In the present study we have further defined the role of C/EBPβ in regulating neurotrophin expression by demonstrating that C/EBPβ is a critical component of BAR-induced NGF gene transcription. Our observations in C6-2B glioma cells and, more importantly, in vivo using C/EBPδ null mice, establish C/EBPβ as an essential transcription factor in cAMP-mediated regulation of NGF expression. We show that C/EBPβ is specific, because C/EBPβ, a closely related family member whose expression is also increased by CLE stimulation (19), does not affect NGF mRNA induction in vitro or in vivo.

Our results also support a role for CREB, operating in conjunction with C/EBPβ, to achieve full promoter activation. Transcription from an NGF reporter construct was synergistically induced by PKA and C/EBPβ, suggesting that C/EBPβ and CREB may function together to induce NGF expression. EMSA studies demonstrate that activated CREB binds to the CRE-like element in vitro, and CREB is associated with the NGF promoter in vivo. Additional experiments have ruled out binding of two other CREB/ATF family members, activating transcription factors 1 and 2 (ATF1 and ATF2), to the NGF-CRE probe (data not shown), indicating that CREB mediates transcriptional activation via this cis-regulatory element.

CREB acts synergistically with other transcription factors to activate the brain-derived neurotrophic factor gene during neuronal survival and adaptive responses (41, 42). Interestingly, we have observed increases in endogenous brain-derived neurotrophic factor expression in C6-2B cells following CLE stimulation and C/EBPβ overexpression (data not shown). Thus, C/EBPβ and CREB may function in a combinatorial fashion to regulate neurotransmitter-induced transcription of neurotrophin genes through BAR signaling.

Another explanation for enhanced NGF promoter activity in response to PKA is direct activation of C/EBPβ. Phosphorylation and activation of C/EBPβ by PKA has been proposed previously, although the site of modification was not identified (43). Our observations tend to support PKA regulation of C/EBPβ activity, because PKA enhanced the ability of C/EBPβ to transactivate an artificial C/EBP-responsive reporter construct. Thus, we favor a model in which PKA signaling stimulates NGF transcription through modification and activation of both C/EBPβ and CREB. It remains to be determined whether collaboration of these proteins occurs via direct physical interaction or by some other means. Physical interaction of CREB with another C/EBP family member, C/EBPβ, has been demonstrated for regulation of the c-Fos promoter in C6 glioma cells (44), supporting the possibility of a direct association mechanism. Additionally, recent studies demonstrate interaction between C/EBPβ and the coactivator, CREB-binding protein, in osteoblasts. This observation suggests a possible mechanism involving CREB-binding protein as a tether that binds C/EBPβ and CREB and facilitates their interaction (43).

Interestingly, 5′ deletions that remove the putative C/EBPβ site in the proximal NGF promoter did not impact promoter activity in transient transactivation assays. In contrast, deletion to −60, which also elimi-
lates the CRE site, abolished promoter activation by CLE, C/EBPβ, PKA, or combined C/EBPβ and PKA overexpression. Similarly, point mutations that disrupt both sites reduced but did not entirely abolish promoter activity. Several explanations could account for these results. Recombinant C/EBPβ binding to the NGF promoter protects the region spanning −90/−59 (19). However, the putative C/EBP binding site within this region (Fig. 4A) is a non-canonical sequence and does not bind C/EBPβ with high affinity. Therefore, although this sequence appears to be important for full promoter activity, additional C/EBP binding sites may exist within the −615/+16 region. Further studies are required to identify these putative sites. Alternatively, C/EBPβ-mediated promoter activation could occur indirectly through binding of C/EBPβ to CREB, thereby enhancing the transcriptional potential of CREB. Finally, the CRE-like element is located within the C/EBPβ footprint, raising the possibility that C/EBPβ has some affinity for the CRE sequence and promotes transcription via this cis element. Additional transcription factors may also be involved; however, in vivo studies demonstrate an absolute requirement for C/EBPβ in CLE-mediated NGF expression (Fig. 7).

C/EBPβ has been shown to regulate expression of inflammatory mediators and pro-inflammatory cytokines (45–47), and the importance of CREB family members in promoting neuronal survival following oxidative stress, neurotransmitter toxicity, and ischemic tolerance has been well documented (48–50). Moreover, studies using animals lacking CREB or its related family member, CRE modulator, have established CREB-dependent transcription as a critical feature of neuronal survival pathways (51). We have identified C/EBPβ and CREB as key regulators of cellular responses to BAR stimulation. Thus, while C/EBPβ and CREB can participate in controlling inflammation in the brain, they may also be involved in the regulation of neuronal plasticity. Indeed, it has been shown that activation of BAR by norepinephrine affects the cellular mechanisms typically associated with various aspects of learning and memory (52–54). Thus, we propose that the functions of C/EBPβ and CREB in activating NGF gene expression represent one means of modulating neuronal plasticity in response to neurotransmitters. It is now important to establish whether noradrenergic regulation of NGF biosynthesis is a viable strategy to prevent cell death at early stages of chronic neurodegenerative diseases.

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