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Systemic Dexamethasone Administration Increases Septal Trk Autophosphorylation in Adult Rats via an Induction of Nerve Growth Factor

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SUMMARY

Nerve growth factor (NGF) maintains cholinergic neurons in various animal models of neurodegeneration and is thus a potential treatment for certain neurodegenerative disorders such as Alzheimer's disease. Because NGF does not cross the blood-brain barrier, we have proposed elevating endogenous levels of NGF in the central nervous system with small molecules that induce NGF expression, as an alternative strategy. The present studies were conducted to determine whether pharmacologically elevated levels of NGF are sufficient to cause subsequent stimulation of its high affinity receptor, as measured by increased levels of Trk phosphorylation. Dexamethasone (0.5–20 mg/kg, intraperitoneally) caused a time- and dose-dependent increase in NGF mRNA and NGF protein in the hippocampus and septum of adult male Sprague-Dawley rats. Exogenously administered NGF (1 μg, intracerebroventricularly) led to a rapid (30 min) and transient increase in Trk phosphorylation in the septum, which has high levels of NGF-specific TrkA. Similarly, dexamethasone led to an increase in Trk phosphorylation only within the septum. Dexamethasone-mediated Trk phosphorylation was dose and time dependent, with peak increases being observed 12 hr after injection, concurrently with peak increases in NGF protein. These data demonstrate an increase in activation of the high affinity NGF receptor with a compound that elevates levels of NGF in the central nervous system, and they support the strategy of discovering a pharmacological agent that induces NGF as a method for treating neurodegenerative disorders.

Neurotrophins are multifunctional protein growth factors that induce the differentiation or increase the survival of a wide variety of neurons in the developing and mature peripheral nervous system and CNS (1). Of the neurotrophins that have been identified, NGF was the first to be isolated and is thus the best characterized, with well defined functions in the mammalian brain (2). Developing and fully mature cholinergic neurons of the basal forebrain and the striatum respond to exogenously administered NGF by displaying elevated levels of enzymatic and immunohistochemical cholinergic markers, as well as neuronal hypertrophy (3–5). Moreover, NGF prevents degeneration and increases function in various animal models of cholinergic degeneration (2, 5).

Cholinergic neurons arising from the basal forebrain and projecting to the hippocampus and cortex are the most affected neuronal group in the pathology of Alzheimer's disease (6). Degeneration of this pathway is suggested to result in some of the memory loss associated with this disease state (2, 6). The utility of NGF in the treatment of Alzheimer's disease has been proposed on the basis of the effects of NGF on degenerating cholinergic neurons (2). However, neurotrophins are high molecular weight, highly charged proteins that do not penetrate the blood-brain barrier. On this basis, several laboratories, including our own, have proposed an alternative approach, which is to discover compounds that enter the brain and elevate endogenous levels of NGF (7–14).

A variety of structurally diverse compounds increase the levels of NGF in various cell culture systems and *in vivo*. Proteins such as basic fibroblast growth factor and interleukin-1β, as well as organic compounds including 4-methylcatechol, phorbol esters, and 1,25-dihydroxyvitamin D_3, induce NGF protein and mRNA levels with varying degrees of potency and by diverse mechanisms (7–13). *In vivo*, pharmacological elevation of NGF mRNA levels in the CNS of adult rats can be measured after an ICV administration of *N*-methyl-D-aspartate receptor agonists (14), 4-methylcatechol, interleukin-1β, or 1,25 dihydroxyvitamin D_3 (15, 16) or peripheral administration of DEX (17–20). These reports, however, fail to demonstrate whether NGF protein is increased after mRNA induction and whether induced NGF is elevated sufficiently to initiate an NGF-mediated biological event in the CNS. To address these issues, activation of a specific neurotrophin response needs to

ABBREVIATIONS: CNS, central nervous system; NGF, nerve growth factor; ICV, intracerebroventricular(ly); DEX, dexamethasone; BDNF, brain derived neurotrophic factor.

be demonstrated *in vivo* with an NGF-inducing compound, in order for this approach to be deemed an acceptable alternative to direct NGF administration in the CNS.

The discovery that the Trk family of receptor-linked tyrosine kinases are high affinity neurotrophin receptors (21–25) has facilitated research on neurotrophic function and has provided a means for detecting specific neurotrophin activity in culture and potentially *in vivo*. TrkA is a 140-kDa glycoprotein with high binding specificity for NGF (21, 22). Consistent with the function of NGF as a cholinergic neurotrophic factor, high levels of TrkA and its mRNA are localized to developing and mature cholinergic neurons in the septum and in the striatum (26, 27). In PC12 cells, NGF binding to TrkA evokes a highly specific, rapid, and easily measured receptor autophosphorylation that initiates a cascade of intracellular events that correlates with the differentiation and neurite outgrowth observed in these cells (21, 22). Although neurotrophin-mediated Trk phosphorylation has not been clearly demonstrated *in vivo*, it does occur in primary cultures of septal neurons (28), suggesting that it may be a potentially useful response to assess "downstream" biological effectiveness of neurotrophin induction in the CNS. On that basis, the present studies were conducted to determine 1) whether elevated levels of Trk phosphorylation can be used to assess increased neurotrophin activity, by testing whether exogenously adminstered NGF itself evokes an increase in this response in the CNS, and 2) whether systemically administered DEX, which is a known NGF-inducing compound, elicits similar Trk phosphorylation responses concomitantly with its ability to elevate NGF.

Materials and Methods

Acute *in vivo* studies. Male Sprague-Dawley rats (Charles River, Kingston, NY) weighing 175–200 g were used for all experiments. DEX, solubilized in saline/ethanol (1:1), was administered intraperitoneally at doses ranging from 0.05 mg/kg to 20 mg/kg. NGF at a dose of 1 μg was administered ICV as described previously (15, 16). Animals were sacrificed between 4 and 24 hr after DEX administration and between 30 min and 2 hr after NGF treatment. The hippocampus and septum were dissected, frozen on dry ice, and stored at −70° until ready for use. The septal region was a rectangular region of tissue that, when dissected, contained the septohippocampal nuclei, the lateral septal nuclei, the medial septal nuclei, and the horizontal and vertical limbs of the diagonal band.

RNA preparation. Total RNA from brain tissue was prepared by a standard guanidine thiocyanate precipitation procedure (15), using a commercial product (RNAzol B; Cinna/Biotecx, Houston, TX). The final RNA pellets were resuspended in 50–100 μl of H_2O. Final total RNA concentrations ranged between 1 and 2 μg/μl.

Probes. A cDNA complementary to the 3′ region of the rat NGF gene that codes for the mature form of its mRNA was used for the ribonuclease protection assays. A 450-base pair NGF cDNA was inserted into pGEM vector and linearized with *Nco*I; it is identical to a published sequence (29). A 250-base pair β-actin (Ambion) cDNA was used as a standard in our studies. cRNA riboprobes were generated from these cDNAs as described previously (15). When synthesized, >90% of each riboprobe was shown to be full length, as determined by visualization on a denaturing polyacrylamide gel.

Detection of NGF mRNAs. The ribonuclease protection assay was modified from a kit provided by Ambion (RPA II) and was used to detect NGF mRNA in rat brain after test compound treatment, as described previously (15, 30). Total RNA (10–40 μg) from the indicated brain region was dried with 3–4 × 10^5 cpm of NGF riboprobe and was hybridized at 43° for 18–24 hr. Hybridization samples also contained 1 × 10^5 cpm of a β-actin riboprobe. After hybridization the samples were digested with RNase for 1 hr. After RNase digestion the samples were precipitated and then isolated on a denaturing polyacrylamide gel. The radioactivity in isolated NGF and β-actin mRNA was visualized and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The ratio of the NGF signal to the β-actin signal was used to quantitate the changes in RNA levels.

NGF enzyme-linked immunosorbent assay. The selected brain regions were sonicated in 20 volumes of 1× homogenization buffer (250 mM Tris·HCl, 1 M NaCl, 50 mM $CaCl_2$, 0.5% Triton X-100, 0.25% sodium azide) and centrifuged at 10,000 × *g* for 15 min. The supernatants from the brain preparations were then assayed for NGF protein levels by a sensitive two-site immunoassay procedure (Boehringer-Mannheim), according to the protocol recommended by the manufacturer and described previously (13, 16). For brain samples, total protein levels were also determined by a standard Bio-Rad procedure and NGF levels were normalized to total protein amounts.

Trk phosphorylation assay. The Trk immunoprecipitation assay was conducted as described previously, with slight modifications for brain tissue (22). Rabbit anti-Trk antiserum that recognizes TrkA, -B, and -C was prepared using the 16-amino acid carboxyl-terminal peptide fragment of human TrkA. The hippocampus or basal forebrain from NGF- or DEX-treated animals was homogenized with a Dounce homogenizer in 500 μl of RIPA buffer (10 mM Tris, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 158 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM orthovanadate). The samples were centrifuged at 11,000 × *g* for 30 min. One milligram of total protein from the supernatant was immunoprecipitated overnight at 4° with Trk antibody and Protein A-Sepharose. Immunoprecipitated Trk protein was separated on a 7.5% polyacrylamide gel and transferred to 0.45-μm nitrocellulose membranes. The blot was incubated with antiphosphotyrosine antiserum (Upstate Biotechnology, Inc., Lake Placid, NY) and then with anti-mouse IgG-horseradish peroxidase conjugate. The ECL detection system (Amersham) was used to visualize phosphorylated Trk. Bands were quantified by measuring the density of each band by image analysis on a Bioquant System IV (R-M Biometrics, Nashville, TN).

Statistics. All data were expressed as percentage of control unless otherwise indicated. Values are the average ± standard deviation for at least six animals. Control values were considered to be 100%. Data were analyzed by analysis of variance and differences between treatments were determined by *post hoc* Tukey's test. Means were considered significantly different at $p < 0.05$.

Results

Time course and dose-response studies of DEX-mediated NGF mRNA and protein induction. Peripherally administered DEX was shown previously to induce mRNA in the hippocampus and cortex, where it was accompanied by a slight increase in NGF protein (17, 18). However, in each of these previous studies only a single dose of DEX was used. To establish the optimal conditions for determing whether DEX-mediated NGF induction in the brain evokes NGF-mediated activity, time course and dose-response studies were conducted and NGF mRNA and protein levels were assessed after systemic injection. NGF levels were assessed in the septum and hippocampus because these are the regions in which NGF-responsive neurons originate and to which they project, respectively.

NGF mRNA was measured at various times after administration of 5 mg/kg DEX, a dose shown to elevate CNS levels of NGF (17). As seen in Fig. 1, A and B, the highest levels of NGF mRNA occurred by 8 hr after administration. NGF mRNA returned to control levels by 24 hr after injection. Induction occurred in hippocampus and septum with similar time courses.

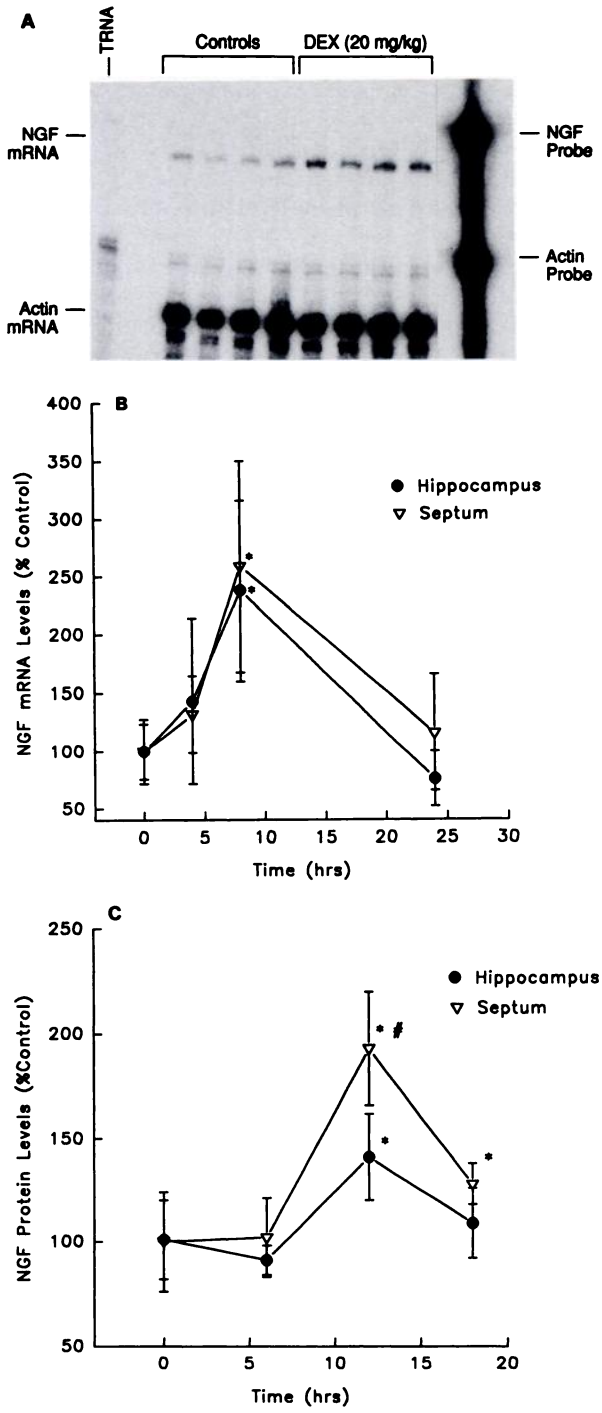

Fig. 1. Time course study of DEX-mediated increases in NGF mRNA and NGF protein. DEX was injected at a dose of 5 mg/kg and the animals were sacrified 6, 12, or 18 hr after injection. The hippocampus and septum were assessed for NGF mRNA levels by ribonuclease protection assay, as described in Materials and Methods. A, Representative Phosphorimager results, demonstrating DEX-mediated induction of NGF mRNA in the hippocampus 6 hr after injection. B, Graph of cumulative data for NGF mRNA increases with DEX over time. C, Graph of NGF protein induction. Base-line levels of NGF protein were 101 ± 11 and 53 ± 7 pg/mg of protein in the hippocampus and septum, respectively. All values are expressed as percentage of control (mean ± standard deviation). *, Statistically significantly ($p < 0.05$) different from corresponding control; #, significantly different from hippocampus.

Concurrent studies assessing NGF protein were conducted to correlate induction of mRNA with that of protein. As seen in Fig. 1C, DEX-mediated elevations in NGF protein also occurred in a time-dependent fashion, in the same regions, with peak elevations being observed 12 hr after injection. Smaller but significant increases in NGF protein were observed in the septum 18 hr after administration.

To determine the optimal doses for NGF induction, DEX was administered at doses ranging from 0.05 mg/kg to 20 mg/kg. For NGF mRNA and protein analysis the animals were sacrificed 6 hr and 12 hr after injection, respectively, time points at which these parameters were elevated to maximal levels. NGF mRNA levels, as measured by RNase protection analysis, increased in a dose-dependent fashion in the hippocampus and septum (Fig. 2A). Maximal induction (approximately 2-fold) occurred at the two highest doses (5 and 20 mg/

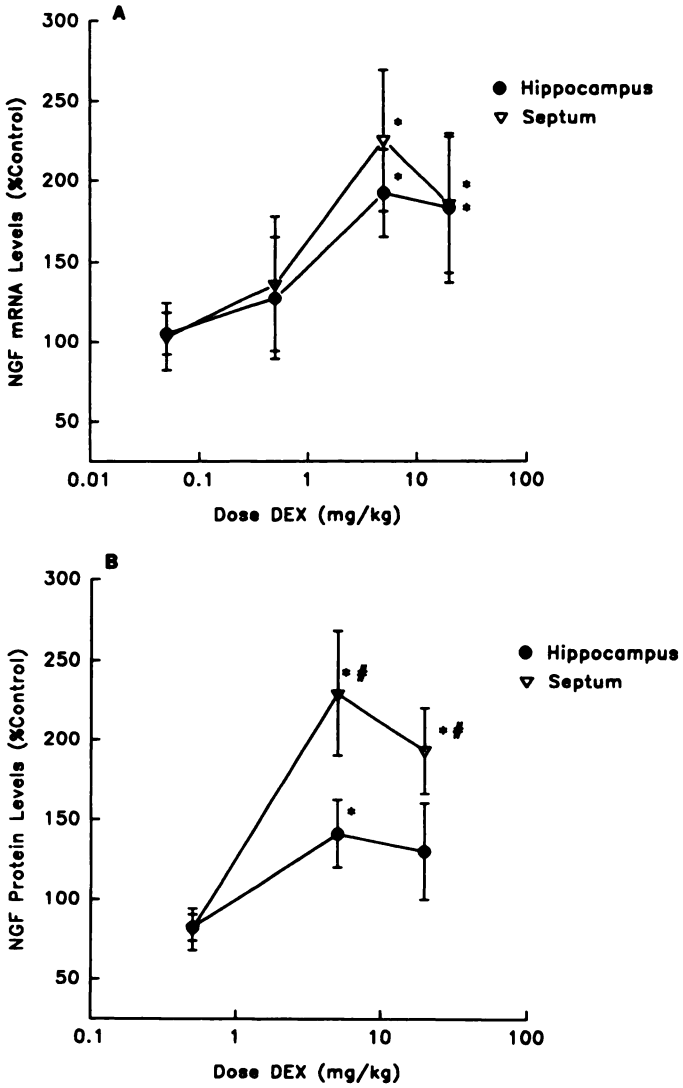

Fig. 2. Dose-response study of DEX effects on NGF mRNA and NGF protein. DEX was injected intraperitoneally at doses ranging from 0.05 mg/kg to 20 mg/kg, and the animals were sacrificed 6 hr or 12 hr after injection, for NGF mRNA and NGF protein analyses, respectively. A, NGF mRNA levels. B, NGF protein levels. Values were calculated directly from Phosphorimager results. All values are expressed as percentage of control (mean ± standard deviation). *, Statistically significantly ($p < 0.05$) different from corresponding control; #, significantly different from hippocampus.

kg) in both brain regions, with half-maximal elevations occurring between 1 and 5 mg/kg. Similar results were observed in the cortex (data not shown). In contrast to the elevations in NGF levels, and similar to reported data (17), BDNF mRNA levels were significantly decreased (by 25%) in the hippocampus at doses (5–20 mg/kg) and at a time (6 hr) at which NGF mRNA levels were maximally induced (data not shown). As seen in Fig. 2B, DEX at maximally inducing doses elevated NGF protein levels by 1.4- and 2.2-fold in the hippocampus and the septum, respectively. The doses that induced NGF protein were identical to the doses that induced NGF mRNA, with maximal elevations occurring at 5–20 mg/kg and half-maximal elevations occurring at 1–5 mg/kg.

Characterization of Trk phosphorylation *in vivo*. To study NGF-mediated responsiveness *in vivo*, we have modified an assay that was originally designed to measure NGF receptor activation in cultured cells to assess NGF-mediated Trk phosphorylation in the CNS. As seen in Fig. 3, the identity of the band corresponding to phosphorylated Trk was confirmed by demonstration of an identically migrating band in PC12 cells after NGF treatment and by the ability of the Trk peptide fragment to compete with and eliminate the band representing phosphorylated Trk. Furthermore, incubation in the absence of tissue or in the absence of primary antibody effectively eliminated the observed signal.

In our studies, NGF (1 μg) administered ICV elicited similar 3-fold increases in Trk phosphorylation in the septum at 0.5 and 2 hr, but not 6 hr, after injection (Fig. 4). Elevations in Trk phosphorylation were not apparent in the hippocampus at any time point after NGF injection.

DEX-mediated Trk phosphorylation in the septum. The ability of NGF induced by DEX to stimulate phosphorylation of Trk receptors in the brain was examined using the conditions for optimal NGF induction established above. When animals were treated with DEX under maximally NGF-inducing conditions (20 mg/kg; 12 hr after injection), Trk phosphorylation was observed exclusively in the septum (Fig. 5). A time course study demonstrated that this response was detectable at 12 hr after DEX treatment, but not at 6 or 18 hr, which paralleled the induction of NGF protein in this region (Fig. 6). Similarly to ICV administered NGF, there was no observable increase in Trk phosphorylation in the hippocampus. Densitometric analysis of the band representing Trk indicated that

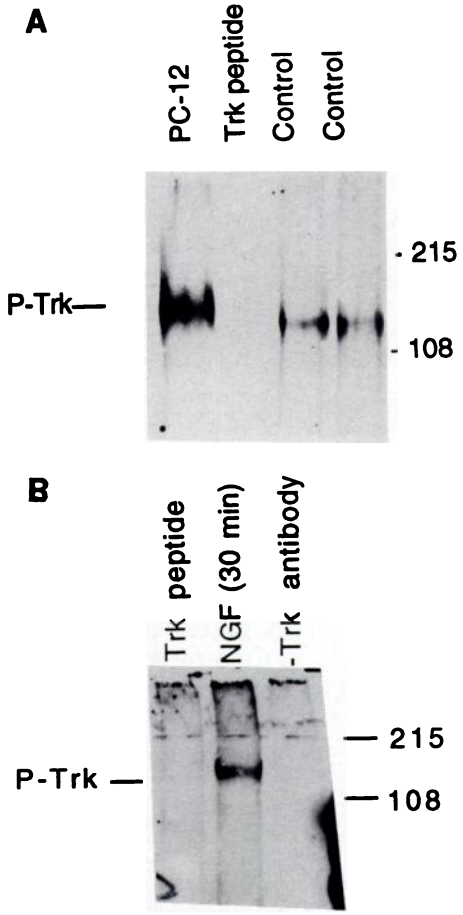

Fig. 3. Characterization of Trk phosphorylation *in vivo*. Trk immunoprecipitation and detection of phosphorylated Trk were conducted as described in Materials and Methods. A, Autoradiograph of phosphorylated Trk (*P-Trk*) from NGF-treated PC12 cells and septal brain homogenates (from untreated rats) preincubated in the presence or absence of Trk peptide fragment. B, Trk immunoprecipitation from septal brain homogenates (from NGF-treated rats) preincubated with Trk peptide fragment or in the absence of Trk antibody.

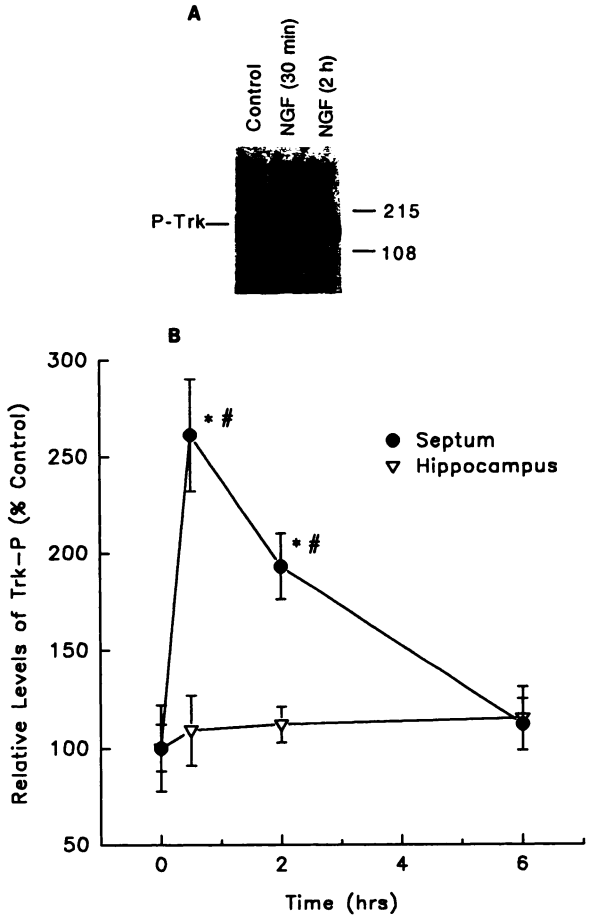

Fig. 4. NGF elevation of Trk phosphorylation in the septum. NGF was injected ICV and the animals were sacrified 30 min or 2 hr later. The septum and hippocampus were assessed for levels of phosphorylated Trk (*P-Trk*) as described in Materials and Methods. A, NGF-mediated Trk phosphorylation in the septum 30 min or 2 hr after injection. B, Graph of cumulative densitometric values (mean ± standard deviation). *, Statistically significant ($p < 0.05$) difference from control value for corresponding brain region; #, significant difference ($p < 0.05$) from hippocampus value at the same time.

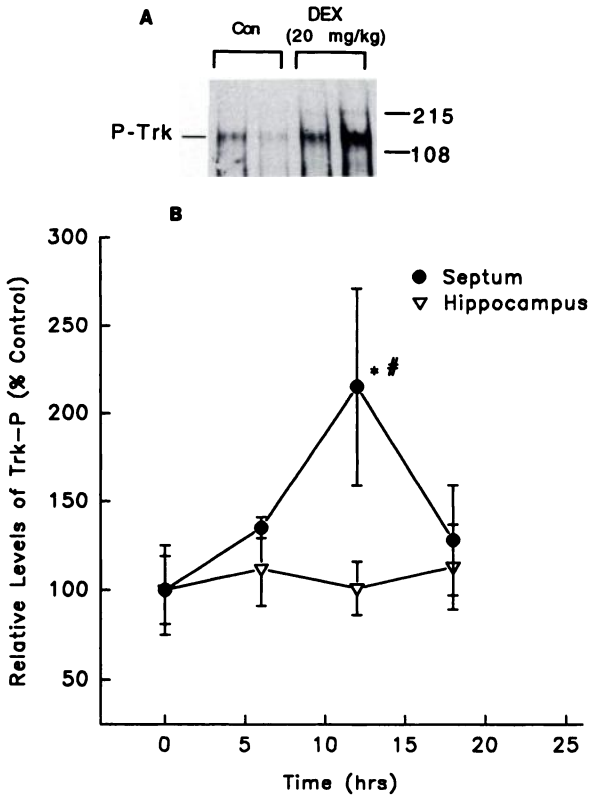

Fig. 5. DEX elevation of Trk phosphorylation in the septum. DEX was injected intraperitoneally at a dose of 20 mg/kg and the animals were sacrificed 6, 12, or 18 hr later. The septum and hippocampus were assessed for levels of phosphorylated Trk (*P-Trk*) as described in Materials and Methods. A, Representative autoradiograph demonstrating DEX-mediated Trk phosphorylation in the septum 12 hr after injection. *Con*, control. B, Graph of cumulative densitometric values (mean ± standard deviation). *, Statistically significant ($p < 0.05$) difference from control value for corresponding brain region; #, significant difference ($p < 0.05$) from hippocampus value at the same time.

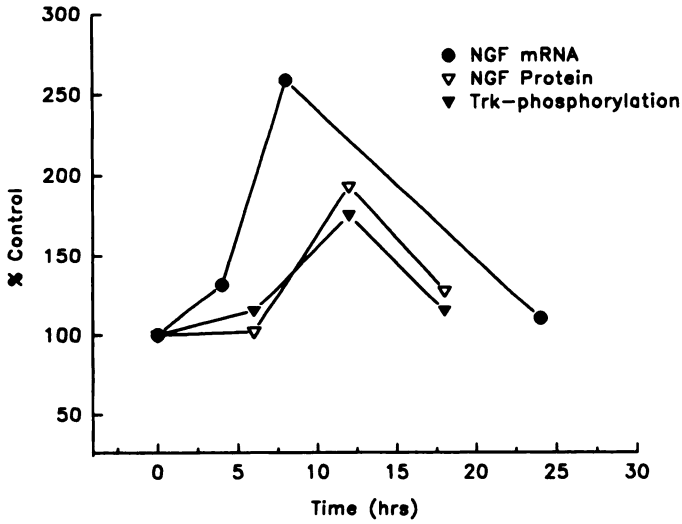

Fig. 6. Composite graph of the time course of DEX-mediated effects in the septum. Values are from Fig. 1, 2, and 5 showing DEX-mediated effects on levels of NGF mRNA, NGF protein, and Trk phosphorylation in the septum. Values are expressed as percentage of respective control.

DEX caused an approximately 2-fold increase in levels of Trk phosphorylation in the septum, which was slightly smaller than that observed with NGF.

Discussion

The neurotrophic effects of NGF on compromised cholinergic neurons within the mature CNS are well established. Thus, NGF is a proposed therapeutic treatment for certain neurodegenerative disorders such as Alzheimer's disease (2). A number of compounds elevate levels of NGF within the CNS, and this process is a proposed alternative strategy for exploiting the potential effects of NGF in neurodegenerative disease. The present studies expand on previous findings of induction of NGF in the CNS by providing the first evidence that up-regulated levels of NGF are capable of initiating a biochemical event in the CNS, as measured by increased activation of the high affinity NGF receptor.

Although DEX regulates the expression of a number of genes in the CNS (31), β-actin mRNA levels were unchanged and BDNF mRNA levels were decreased (data not shown) at all doses at which NGF levels were increased. These data suggest that DEX does not cause a general up-regulation of gene expression, especially neurotrophin gene expression, *in vivo* at the doses used in this study. The cell types in which NGF was induced are not clear from these results. *In situ* hybridization studies indicate that most endogenous NGF mRNA is localized to neurons within the adult brain (32). Astrocytes are capable of producing NGF *in vivo* (33) and probably are a source of pharmacologically elevated NGF after adminstration of certain compounds (15, 16). DEX decreases NGF levels in cultured astrocytes but elevates NGF in primary cultured hippocampal neurons (20), suggesting that neurons are the likely source of elevated NGF in these studies.

Cholinergic neurons that originate in the basal forebrain are proposed to receive support from both distal target regions and locally derived NGF (30, 32, 34). In the present study, DEX elevated NGF levels in the septum, which is the site of origin of cholinergic neurons, and in the hippocampus and cortex, which are brain regions containing the terminal projections of these same neurons (35). Peak elevations in NGF mRNA preceded increased NGF protein levels, in each region tested, by 4–6 hr after DEX administration. This relatively short time period between maximal increases in mRNA and in protein indicates that the increased NGF is likely synthesized locally within each region, including the septum. Retrograde transport of NGF protein to specific regions is unlikely to play a role in the measured elevations, because this process takes up to 24 hr to occur (36). The presence of NGF and its induction within the septum are also of potential therapeutic significance, because the cholinergic neurons that degenerate in Alzheimer's disease originate in this region (6).

NGF administered ICV elicited a Trk phosphorylation response *in vivo* that was identical to the response observed in PC12 cells and in primary cultures of septal neurons (22, 28). Although the anti-Trk antiserum recognizes all three isoforms of Trk, the phosphorylated Trk in these studies was likely TrkA, because NGF has high affinity and selectivity for this receptor subtype (21, 22). Increases in Trk phosphorylation were not apparent in the hippocampus after ICV injection, although NGF-responsive cholinergic neurons project to this region. At the time points used in these experiments, ICV

administered NGF accumulates in the septal region and penetrates significantly less into the hippocampus (37), which may account for the lack of Trk phosphorylation observed. Nevertheless, these data demonstrate that NGF elicits the same initial biochemical events in the adult rat septum as it does in PC12 cells and primary cultured septum. Importantly, these results indicate that elevated levels of Trk phosphorylation can be used as an index of biologically active NGF and for assessment of increased NGF activity in the CNS.

To test whether DEX elevated NGF levels sufficiently to mediate NGF signaling, Trk phosphorylation levels were assessed in brain regions and at a dose at which NGF levels were increased. DEX evoked a time-dependent increase in Trk phosphorylation that paralleled the time-dependent elevation in NGF protein (Fig. 6). There was an absence of increased Trk phosphorylation at time points at which NGF levels were at base-line values. Similarly to the effects of exogenously administered NGF, the increased Trk phosphorylation occurred exclusively within the septum. Lack of Trk phosphorylation in the hippocampus may be due to the relatively low level of NGF protein induction in the hippocampus, compared with the septum. Nevertheless, the septum displayed the highest levels of induced NGF protein and simultaneous elevations in Trk phosphorylation, indicating that these events are causally related. We can conclude from these data that the DEX-induced NGF within the septal region is biologically active and that an NGF-inducing compound can initiate NGF signaling in the CNS. In addition, these data demonstrating DEX up-regulation of NGF mRNA, NGF protein, and Trk phosphorylation, all within the septum, provide additional evidence that the induced levels of this neurotrophic factor were synthesized and had local effects within this region.

Because the Trk antibody used in these studies recognizes all Trk forms, it is not apparent whether other neurotrophins regulate measured Trk phosphorylation levels. DEX-down-regulated levels of BDNF mRNA and neurotrophin-3 levels have been reported to be minimally affected by DEX (17), suggesting that these related neurotrophins are probably not activating their respective receptors. In addition, the increased Trk phosphorylation is not likely due to increased levels of the Trk protein itself, because 1) DEX does not affect Trk mRNA levels in a neuronal precursor cell line (38) and 2) we are measuring the phosphorylated form, which is generated only through activation by an agonist, such as NGF (21, 22).

In the present study, we demonstrated that systemic administration of DEX elevates NGF mRNA, which leads to an increase in NGF protein that, in turn, is sufficient to evoke an NGF-mediated biological response. These biochemical sequelae occur within the septum, a brain region in which NGF-responsive cholinergic neurons degenerate in Alzheimer's disease. Although DEX itself may not be a suitable pharmacological agent for this use, because it has inherent neurotoxic potential with chronic usage (39), these studies demonstrated that, in theory, increased NGF biological activity can be achieved with a single injection of an NGF-inducing compound, and they are supportive of an effort for developing these types of compounds for the treatment of neurodegenerative disorders.

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