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Trophic Effect of Exogenous Nerve Growth Factor on Rat Striatal Cholinergic Neurons: Comparison between Intraparenchymal and Intraventricular Administration

JOSE L. VENERO, FRANZ HEFTI, and BEAT KNUSEL

Andrus Gerontology Center, Division of Neurogerontology, University of Southern California, Los Angeles, California 90089-0191

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SUMMARY

Penetration into the brain is an important consideration in the pharmacological use of neurotrophic factors for the treatment of brain neurodegeneration, e.g., in Alzheimer's disease. Furthermore, intracerebroventricular treatment with nerve growth factor (NGF) has been found to induce side effects, including aberrant sympathetic sprouting and weight loss. Such findings suggest that direct intraparenchymal application of minimal amounts of trophic factors might be therapeutically desirable. We compared the effectiveness of intrastriatal and intracerebroventricular administrations of NGF on striatal cholinergic neurons in adult rats. Daily intrastriatal administration for 1 week of ≥50 ng of NGF resulted in an increase in mRNA levels for choline acetyltransferase (ChAT) in striatal cholinergic cells to ~2-fold over control. A daily intraventricular dose of 4.5 μg of NGF was required for a similar response. Both 5 and 50 ng of NGF/day failed to induce an effect on transmembrane protein tyrosine kinase *trk*A mRNA levels, but injections of 750 or 1500 ng/day of NGF up-regulated *trk*A mRNA expression to ~2-fold of control. NGF delivered intracerebroventricularly failed to induce an observable change in striatal *trk*A mRNA, even at a dosage of 4.5 μg of NGF/day. These quantitative differences in NGF actions were reflected at the level of NGF receptors. Using Western blotting procedures, we found pronounced tyrosine phosphorylation of Trk-type proteins 2 hr after intrastriatal injection of 50 ng of NGF. Maximal responses were seen with either 150 or 750 ng of NGF. For maximal activation of Trks by intraventricular NGF injection, 4.5 μg of NGF was required. Taken together, our results strongly favor intraparenchymal injections or infusions of NGF, and possibly other trophic factors, for therapeutical applications to maximize the effects on the targeted neuronal populations and to minimize undesirable side effects.

NGF is the first discovered and best characterized member of a small family of four closely related proteins known as the neurotrophins (for a review, see Ref. 1). The other neurotrophins are BDNF, NT-3, and NT-4/5, discovered independently in slightly differing forms in amphibians and mammals (1). Neurotrophins bind and activate a group of transmembrane protein tyrosine kinases, commonly referred to as Trks. Despite a certain degree of ambiguity in the relationships between the individual neurotrophins and the different Trks, NGF is recognized to be the predominant ligand for TrkA, BDNF and NT-4/5 are mainly ligands for TrkB, and NT-3 is the ligand for TrkC (for a review, see Ref. 2). However, NT-3 also can activate TrkB, and both NT-3 and NT-4/5 are capable of interacting with TrkA in cell lines overexpressing this receptor (1). Binding of the neurotrophins to Trks initiates a signaling cascade with tyrosine phosphorylation of the receptor and of several intracellular substrates (3). These effects can be observed within <5 min (4). All neurotrophins also bind to the so-called low affinity neurotrophin receptor (p75NTR) with very similar affinity (5). The function of this membrane-spanning protein is unknown, and no signal transduction mechanisms have been identified. It has been suggested that p75NTR might be involved in the formation of functional high affinity NGF receptors in brain and PC12 cells (6–8). Furthermore, p75NTR has been implicated in mediating neuronal death (9).

Trophic actions of NGF within the central nervous system are remarkably confined to the cholinergic population of the basal forebrain and the striatum and a small number of other discrete cell groups (10). Other cholinergic cell populations of the brain, e.g., pontine cholinergic neurons are not affected by NGF (11). However, recently several discrete noncholinergic populations of neurons have been described in mammals as also expressing *trk*A mRNA, in some cases in conjunction with mRNA for p75NTR (10, 12, 13). NGF has been

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ABBREVIATIONS: Trk, tyrosine receptor kinase protein; *trk*, tyrosine receptor kinase gene; BDNF, brain-derived neurotrophic factor; bp, basepair(s); ChAT, choline acetyltransferase; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; p75NTR, low affinity neurotrophin receptor; SSC, standard saline citrate

shown to prevent cell death of the basal forebrain cholinergic neurons after axotomy of their hippocampal projection by fimbria/fornix transection (14). *In situ* hybridization studies have established not only the presence of *trk*A mRNA in the basal forebrain but also its colocalization with mRNA for ChAT and p75NTR in this brain area (15, 16). The striatal cholinergic interneurons represent a second major population of NGF responsive cells in the brain (17, 18). NGF protein (19) and mRNA (20) are present in the striatum. However, although *trk*A mRNA and protein are found in both the developing and adult striata, p75NTR mRNA and protein are detectable only in the developing striatal cholinergic interneurons (21–24). Intraventricular injections of NGF increase different phenotypic markers of adult striatal cholinergic neurons, like ChAT activity (18) and ChAT mRNA and *trk*A mRNA expression (15, 16), and induce hypertrophy of these neurons (21). Moreover, intrastriatal NGF administration protects striatal cholinergic neurons from degeneration after quinolinic acid injection into the striatum in an animal model of Huntington's disease (25, 26). Together, these observations suggest a prominent role of TrkA in mediating NGF responses in striatal cholinergic neurons.

Intracerebral injections of neurotrophic factors have been proposed as treatment for several neurodegenerative diseases (for a review, see Ref. 27). Due to the proteinous nature of the neurotrophins and other neurotrophic factors and to the presence of binding proteins in brain cells for some of these factors, penetration of the injected trophic protein into the brain parenchyma is an important consideration in any attempt at trophic factor treatment. For example, it has been shown that penetration rates for the closely related neurotrophins NGF and BDNF vary considerably (28). For the neurotrophin BDNF, it has also been found that direct injection into the septal parenchyma more effectively rescues medial septal cholinergic neurons after axotomy than do intraventricular injections (4, 29). Furthermore, intracerebroventricular treatment with NGF has been found to result in aberrant sympathetic neurite sprouting (30) along with reduced weight gain and food intake (31, 32). These findings have been corroborated in clinical trials, where NGF was found to induce undesirable side effects of hyperalgesia and weight loss (33). Observations directly indicating a central effect of BDNF on appetite control have also been reported (34). Taken together, these findings suggest that direct intraparenchymal injections of minimal amounts of trophic factors are therapeutically more suitable than intracerebroventricular application of larger doses. To directly compare the effectiveness of intraventricular and intraparenchymal trophic factor treatment, we injected various doses of NGF into the striatum or into the lateral ventricle of adult rats. The close anatomic relationship of the striatum to the lateral ventricle and the scattered distribution of the cholinergic neurons in this structure provide an ideal model for such a comparison at the cellular level. ChAT and *trk*A mRNA levels in individual neurons were analyzed by *in situ* hybridization. To test for effects of the NGF treatment on Trk-type NGF receptors, we further assessed NGF-induced Trk tyrosine phosphorylation by using immunoprecipitation and Western blotting.

Materials and Methods

Administration of NGF. Female Wistar rats (180–200 g; Charles River, Wilmington, MA) were used for this study. Recombinant human NGF was produced and characterized as described (4). The rats were anesthetized with 75 mg/kg Nembutal and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) to conform with the brain atlas of Paxinos and Watson (35). A cannula system from Plastics One (Roanoke, VA) was used for NGF injections. A metal guiding cannula (22 gauge) with threaded plastic casing was permanently attached to the skull with miniature screws and dental cement. For intrastriatal injections, the intracerebral end of the guiding cannula was placed 0.5 mm caudal, 2.5 mm lateral, and 6.5 mm ventral to bregma. For intraventricular NGF administration, the coordinates were 2.5 mm posterior, 1.9 mm lateral, and 4.0 mm below bregma. Between injections, the guiding cannula was closed with a solid dummy cannula. At the time of injections, the dummy cannula was replaced temporarily by a small-bore (28 gauge) metal cannula protruding 0.5 mm from the internal end of the guiding cannula. This cannula was connected by flexible tubing to a Hamilton syringe containing recombinant human NGF or cytochrome *c* serving as a control protein. The proteins were dissolved in 0.01 M Na-succinate and 0.14 M NaCl with 15 mg/ml gentamicin, and 1 or 2.5 μl of the solutions was injected over a time period of 90 sec for intrastriatal and intraventricular injections, respectively. The first injection of growth factor was given at the day of surgery, and animals were killed after they had received daily injections for 1 week. Four different doses of NGF were tested for daily intrastriatal injections: 5, 50, 750, and 1500 ng/day. For daily intraventricular injections, NGF was tested at a dosage of 4500 ng/day. Control animals received daily injections of 1500 ng cytochrome *c* into the striatum or 4.5 μg cytochrome *c* into the ventricle for 1 week.

Preparation of riboprobes. Both ChAT and *trk*A cDNA probes were transcribed in the presence of [^{35}S]UTP (1300 Ci/mmol; New England Nuclear, Boston, MA). ChAT sense and antisense riboprobes were generated from pChAT.SX2, a plasmid containing a 208-bp segment of rat ChAT genomic sequence covering 132 bp of exonic sequence (kindly provided by Dr. Nozomu Mori, University of Southern California, Los Angeles, CA; see Ref. 36). For riboprobe recognizing *trk*A mRNA, a pGEM-3 vector (Promega, Madison, WI) containing a 464-bp coding region of the extracellular domain of the mouse trkA sequence was kindly provided by Drs. Dionisio Martin Zanca and Luis F. Parada (NCI, Frederick, MD) (see Ref. 37).

***In situ* hybridization histochemistry.** One week after the cannula implantation, the animals were decapitated while under light Nembutal anesthesia, and the brains were removed inmediately and frozen in isopentane at −15°. *In situ* hybridization on brain sections was carried out following a modification of a procedure described in detail elsewhere (38). Thaw-mounted 10-μm sections were postfixed for 30 min in 4% paraformaldehyde, followed by three 10-min washes in phosphate-buffered saline, pH 7.4. Sections were treated for 1 min in 0.1 M triethanolamine, followed by 10 min in acetic anhydride/0.1 M triethanolamine to decrease nonspecific binding. After a 1-min wash in 2× SSC, sections were dehydrated in a series of increasing concentrations of ethanol and then air-dried. The sections were hybridized for 3 hr at 50° with the corresponding [^{35}S]cRNA, rinsed in 4× SSC/20 mM dithiothreitol, and then rinsed in 4× SSC alone. Sections were subjected to 30 min of RNase digestion at 37° (20 g/ml RNase A in 0.5 M NaCl, 0.01 M Tris·HCl, 0.001 M EDTA, pH 8.0), washed for 2 hr in 2× SSC at 25° followed by 0.1× SSC at 60° for 1 hr, dehydrated in a series of ethanol, air-dried, and either exposed to film or processed for emulsion autoradiography.

***In situ* hybridization data analysis.** Silver grains over individual *trk*A or ChAT mRNA-expressing neurons in 10-μm-thick sections were quantified with the use of a Computer Enhanced Video Densitometer. After dry film autoradiography, slides were dipped in Amersham LT1 emulsion (diluted 1:1 with water) and exposed in the dark at 4°. Slides were then developed in D-19 (Kodak) at 15° for 2.5 min, fixed for 4 min in fixer (Kodak), and counterstained with Cresyl Violet. Labeling was considered specific when grain accumulation over individual cells with a large nucleus exceeded five times the background value. At a 100× magnification, the area with high grain

density over an individual cell body was delineated, and the number of grains within this field was counted. Because of the high levels of labeling, the typical disposition of the clusters over the cell body, and the high magnification, the delineation was unequivocal. Only well-separated cells were selected for quantification. Representative sections corresponding to plates 14 and 15 of the rat brain atlas of Paxinos and Watson (35) from four animals for each treatment were analyzed. A minimum of 100 cells were measured for each treatment.

Analysis of Trk tyrosine autophosphorylation. Rats were provided with cannulas as in the experiments involving *in situ* hybridization for ChAT or *trk*A mRNA. After a recovery period of 5 days, the rats were treated with a single injection of NGF directly into the striatum or into the lateral ventricle. Two hours after this intrastriatal or intraventricular NGF injection, the rats were decapitated while under light Nembutal anesthesia, and the striatum was quickly dissected on ice. Striata from injected and from uninjected control sides were collected separately from two animals each and pooled into ice-cold Krebs' buffer. Further processing of the tissue was as described in detail previously (4). Briefly, the striatal tissue was chopped with a tissue chopper (McIlwain; Brinkmann Instruments, Westburg, NY) in two directions at 200-μm thickness and transferred into gassed (5% CO_2/95% O_2) Krebs' solution at 37°. Two washes with fresh gassed Krebs' at 15-min intervals were performed; after another 15 min, four samples were prepared from each pooled tissue and placed into 1.5-ml Eppendorf tubes on a heating block at 37°. Half of the samples were incubated acutely for 4 min with NGF to determine maximal possible NGF response in the samples. The tissue was then lysed in 0.5 ml lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 500 μM orthovanadate), and the lysate was frozen at −70° until further assay by Western blotting. Western blotting was performed as described (4). Trk family proteins were immunoprecipitated with rabbit anti-pan Trk 203 (provided by Dr. D. Kaplan, NCI, Frederick, MD). Precipitates were collected with protein A/Sepharose and washed three times with lysis buffer and once with water. The precipitates were then boiled in sample buffer (2% sodium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol, and 0.25% bromophenol blue) for 5 min and electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide minigels before transfer to nitrocellulose. Protein blots were probed overnight at 4° with the monoclonal antiptyr antibody 4G10 (UBI) in Tris-buffered saline with 0.2% Tween-20. Blots were analyzed using an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Statistical analysis. Results are expressed as mean ± standard error. Grain count analysis of cells expressing either ChAT or *trk*A mRNA were assessed by one-way analysis of variance followed by Scheffé's test.

Results

Expression of ChAT and *trk*A mRNA in the striatum. *In situ* hybridization of brains from control animals and from NGF-treated animals with antisense riboprobes for ChAT mRNA resulted in specific labeling of a subpopulation of striatal neurons scattered throughout the striatum (Fig. 1, A, C, and E), as observed previously (16). In parallel hybridization experiments with a riboprobe for *trk*A mRNA, the distribution of the labeled cells was very similar to the distribution of the striatal cholinergic interneurons (Fig. 1, B, D, and F), confirming earlier results using *in situ* hybridization and immunocytochemistry (15, 16, 23). Immunolabeling experiments have been used to show colocalization of TrkA with the cholinergic marker enzyme ChAT (22). Compared with the untreated control side, daily intrastriatal injections for 1 week of 1.5 μg control protein (cytochrome *c*) resulted in a modest up-regulation of ChAT mRNA expression in individual striatal cholinergic cell bodies (Fig. 1A). No up-regulation of *trk*A mRNA was seen with this treatment (Fig. 1B), and no effects on ChAT or *trk*A mRNA levels were observed with intraventricular injection of a higher dosage of 4.5 μg cytochrome *c*/day (data not shown; Ref. 16). Treatment of the animals with 750 ng of NGF/day intrastriatally (Fig. 1, C and D) resulted in clearly higher levels of mRNA expression for ChAT and *trk*A in the striatum on the injected brain side, although 4.5 μg of NGF/day administered intraventricularly only resulted in an increase of the mRNA for ChAT but not for TrkA (Fig. 1, E and F).

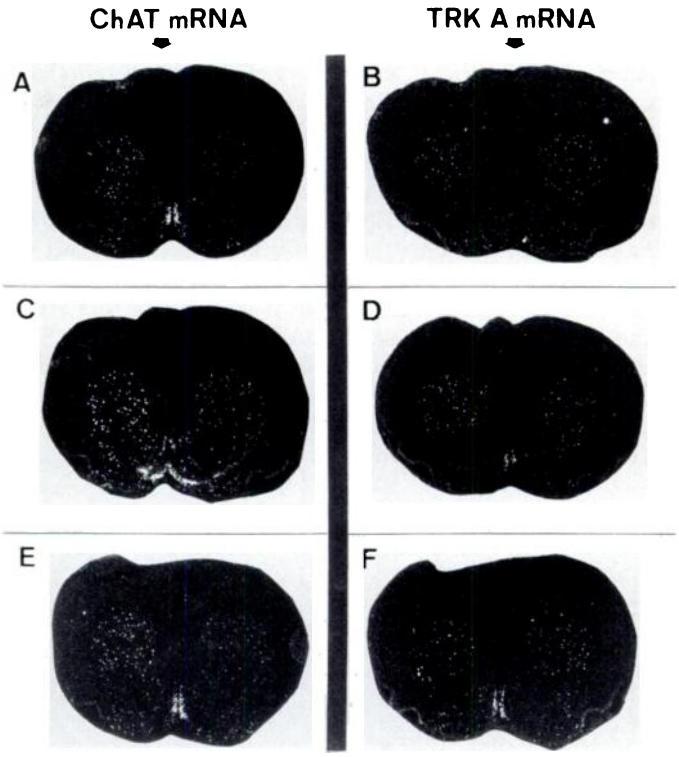

Fig. 1. Cells expressing ChAT or *trk*A mRNA in rat forebrain after injection of cytochrome *c* or NGF. Rats were treated for 1 week with control protein (cytochrome *c*) or NGF by daily injection directly into the striatum or into the lateral ventricle. One day after the last injection, the brains were processed by *in situ* hybridization for ChAT (A, C, and E) and *trk*A mRNA (B, D, and F). Experimental conditions were unilateral intrastriatal injection of cytochrome *c* (A and B), unilateral intrastriatal injection of 750 ng of NGF (C and D), and unilateral intraventricular injection of 4.5 μg of NGF (E and F). *Left*, injected side. Intrastriatal delivery of NGF is more effective in inducing both ChAT mRNA over striatal cholinergic cells than is intraventricular administration, even at the higher dosage used. *trk*A mRNA is increased only after direct intrastriatal delivery of the trophic factor. Note the modest induction of ChAT mRNA levels but not *trk*A mRNA levels after intrastriatal injection of cytochrome *c*.

Dose-response analysis for ChAT mRNA induction in the striatum. To further analyze the injection-induced changes in ChAT mRNA levels, we treated rats daily for 1 week with cytochrome *c* or various amounts of NGF and performed cell count analysis of the hybridization signal. In a previous study (16), we showed that after daily intraventricular administration of NGF for 1 week in fimbria transected animals, there was a medial-to-lateral gradient decrease in the NGF-induced up-regulation of ChAT expression in the striatum ipsilateral to the side of the administration. We therefore performed cell count analysis only in the striatal

area proximal to the ventricular walls within 1 mm from the ventricle (medial striatum). As observed previously (Fig. 1A), intrastriatal treatment with 1.5 μg of cytochrome *c* induced an increase in the labeling for ChAT (Fig. 2, A and B). Quantitative analysis showed this increase to be 166% over the contralateral untreated control side (Fig. 3). Daily intrastriatal injection of the lowest dosage of NGF (5 ng/day) failed to induce ChAT mRNA expression significantly above the level observed with the cytochrome *c* control injections (177% of control values) (Figs. 2C and 3). However, daily intrastriatal injections of 50 ng of NGF resulted in a statistically significant increase of ChAT mRNA levels over individual cells to 267% of the uninjected contralateral side (Figs. 2D and 3). Similar effects were seen with intrastriatal injections of 750 and 1500 ng/day of NGF, which increased ChAT mRNA expression to 356% and 367% of control values, respectively (Figs. 2, E and F, and 3). Although this result suggests maximal NGF stimulation at 750 ng/day of injected protein, we cannot rule out that our failure to detect a higher effect on ChAT mRNA expression with the higher dosage of 1.5 μg/day might be due to saturation of the photographic emulsion used to detect the signal of the radiolabeled probe. However, direct measurement of tyrosine phosphorylation of Trk-type NGF receptors after intrastriatal NGF injections (see below) confirmed full activation of these receptors at ≥150 ng of injected NGF. Unlike our earlier observations with intraventricular injections mentioned above, we did not notice a gradient of labeling intensity away from the injection site of the intrastriatal injections. This might be due to a potentially more even distribution of NGF throughout the tissue and possibly slower clearance from the brain with intraparenchymal application compared with intraventricular injections.

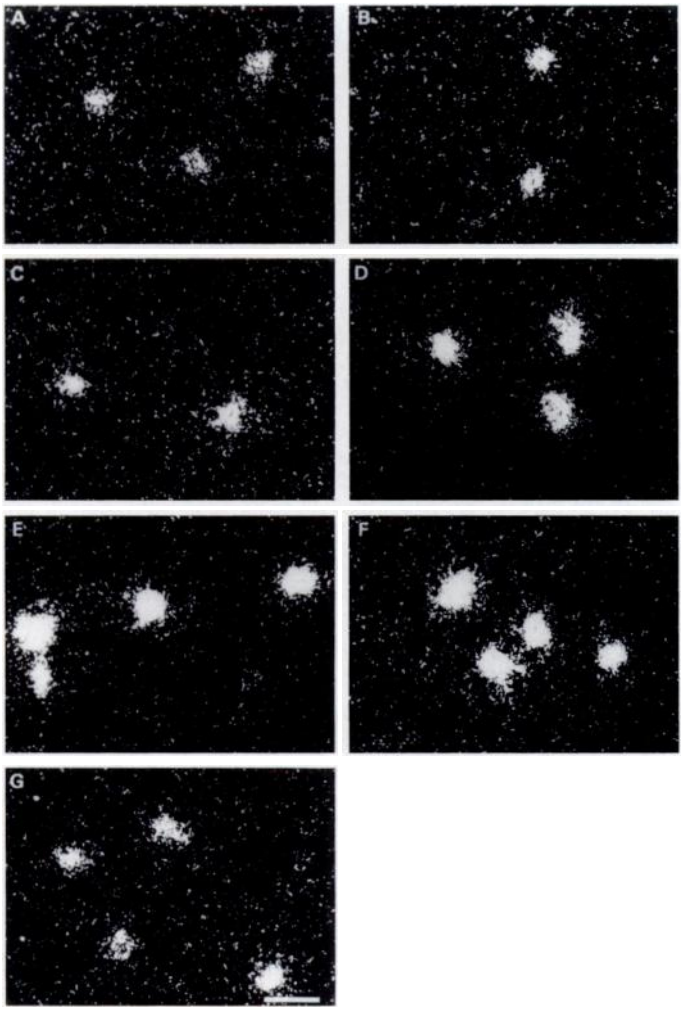

Fig. 2. Dose-response experiment for ChAT mRNA induction after intrastriatal NGF treatment and comparison with intraventricular application. Animals were treated as detailed in legend to Fig. 1. B–G, High magnification dark-field photographs of striatum ipsilateral to the injection. A, Striatum contralateral to cytochrome *c*-injection (1.5 μg). B, Striatum ipsilateral to cytochrome *c*-injection (1.5 μg). Intrastriatal NGF doses in 1 μl volume were C, 5 ng; D, 50 ng; E, 750 ng; and F, 1.5 μg. G, Striatum ipsilateral to injections from an animals receiving 4.5 μg of NGF in 2.5 μl volume daily into the lateral ventricle. Note the intense induction of ChAT mRNA expression after intrastriatal NGF injections of either 750 or 1500 ng. Less induction is observed with the higher dose of 4.5 μg of NGF administered intraventricularly. *Scale bar*, 100 μm.

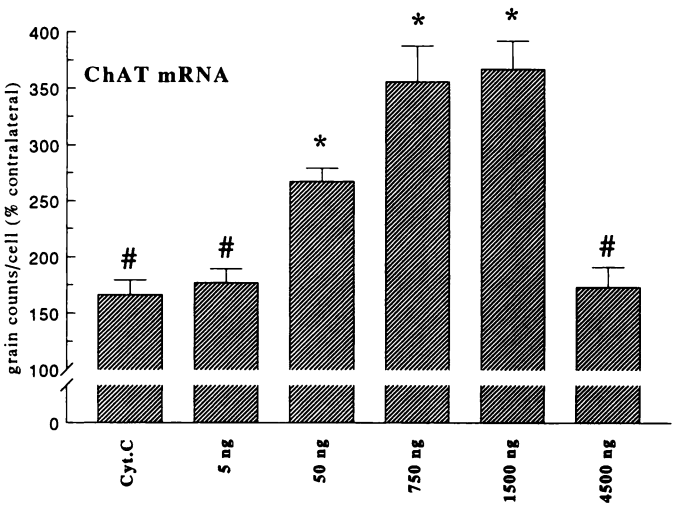

Fig. 3. Quantification of the number of grains per neuron for the experiment detailed in legend to Fig. 2. Results are expressed as percentage of values measured in the contralateral striatum of cytochrome *c* (*Cyt.C*)-injected animals. Intrastriatal injections: cytochrome *c* (1.5 μg) and NGF at 5, 50, 750, and 1500 ng/day. Intraventricular injections: NGF at 4.5 μg/day. Results were analyzed by analysis of variance followed by Scheffé's test. *, $p < 0.05$ for comparison with the ipsilateral side of cytochrome *c*-injected animals. #$p < 0.05$ for comparison with the contralateral side of cytochrome *c*-injected animals. Maximal increases are seen with 750 ng and 1.5 μg of intrastriatal NGF; 5 ng of intrastriatal NGF did not increase ChAT mRNA levels beyond cytochrome *c*-induced levels. Only a moderate increase beyond respective control was seen with 4.5 μg of intraventricular NGF.

Our results, which demonstrate maximal effect of NGF injections directly into the striatal parenchyma at a daily NGF dose of ≥750 ng, were then compared with the effects of injections into the lateral ventricle. A dosage of 4.5 μg/day of NGF was injected directly into the ventricular space. This treatment resulted in an increase of ChAT mRNA levels on the injected side to 173% of the uninjected contralateral control side (Figs. 1E, 2G, and 3). Because daily intraventricular injections of cytochrome *c* during 1 week did not result in changes of ChAT mRNA in the striatum (data not shown; Ref. 16), we conclude that the effect of 4.5 μg of intraventricular NGF on striatal ChAT mRNA corresponds approximately to the effect of 50 ng of intrastriatal NGF.

Dose-response analysis for *trk*A mRNA induction in the striatum. We performed cell count analysis on sections

hybridized with a probe specific for *trk*A for the same striatal area (medial striatum) as selected for measuring ChAT mRNA expression in individual neurons. In contrast to our observation of ChAT mRNA expression, intrastriatal injections of the control protein cytochrome *c* did not affect cellular *trk*A mRNA levels in the striatum (Figs. 1B, 4B, and 5). Also, higher doses of NGF were required for an effect on *trk*A expression than for ChAT mRNA induction. Intrastriatal NGF at a dosage of 5 or 50 ng/day failed to induce any change in *trk*A mRNA levels (Figs. 4, C and D, and 5). Injections of the higher daily dosages of NGF of 750 ng/day or 1.5 μg/day, however, strongly up-regulated *trk*A mRNA expression to 222% or 229% of control levels, respectively (Figs. 4, E and F, and 5). Similar to the effects on ChAT mRNA, this result of intrastriatal NGF injections clearly differed from the results of intraventricular NGF injections. NGF, delivered intracerebroventricularly, even at a dosage of 4.5 μg/day, failed to induce any observable change in the cellular level of *trk*A mRNA on the injected side compared with the uninjected control side (Figs. 1F, 4G, and 5).

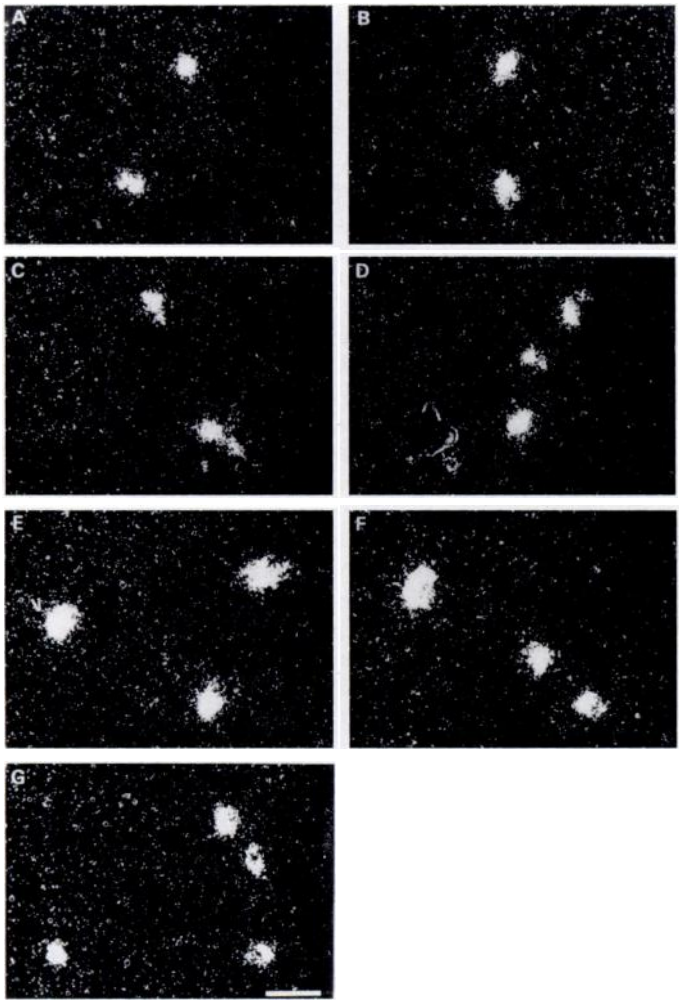

Fig. 4. Dose-response experiment for *trk*A mRNA induction after intrastriatal NGF treatment and comparison with intraventricular application. Sections adjacent to those used for Fig. 2 were hybridized with a probe specific for *trk*A. B–G, High magnification dark-field photographs of striatum ipsilateral to the injection. A, Striatum contralateral to cytochrome *c* injection (1.5 μg). B, Striatum ipsilateral to cytochrome *c* injection (1.5 μg). Intrastriatal NGF doses in 1 μl volume were C, 5 ng; D, 50 ng; E, 750 ng; and F, 1.5 μg. G, Striatum ipsilateral to injections from an animals receiving 4.5 μg of NGF in 2.5 μl volume daily into the lateral ventricle. *Scale bar*, 100 μm.

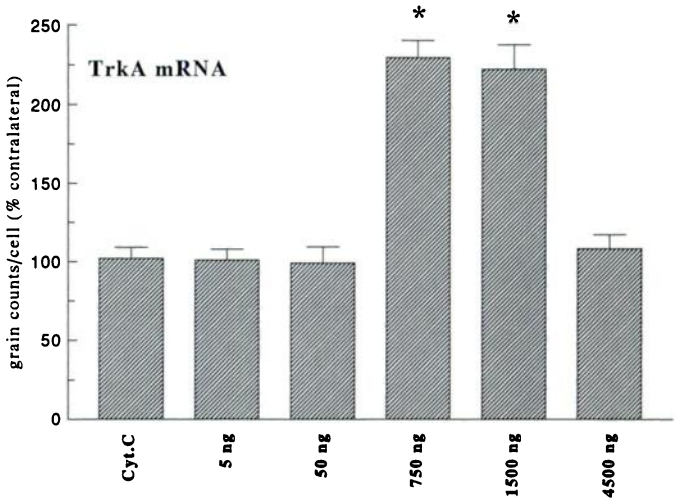

Fig. 5. Quantification of the number of grains per neuron for the experiment detailed in legend to Fig. 4. Results are expressed as percentage of values measured in the contralateral striatum. Intrastriatal injections: cytochrome *c* (*Cyt.C*) (1.5 μg) and NGF doses of 5, 50, 750, and 1500 ng. Intraventricular injections: NGF at 4.5 μg. Results were analyzed by analysis of variance followed by Scheffé's test. *, $p < 0.05$ for comparison with the ipsilateral side of cytochrome *c*-injected animals. Only intrastriatal NGF injections at a dosage of 750 ng/day or 1.5 μg/day (not intraventricular injections of the higher dose of 4.5 μg of NGF) were effective in increasing striatal cellular levels of *trk*A mRNA.

Induction of Trk tyrosine phosphorylation by intrastriatal and intraventricular NGF injections. Our *in situ* hybridization results demonstrated more effective stimulation of NGF-responsive striatal neurons with injection of the trophic factor directly into the striatal parenchyma than into the lateral ventricle, despite the close proximity of the striatum to the ventricular space. To test whether these quantitative differences in NGF actions are reflected at the level of the NGF receptors, we assessed Trk-type receptor activation by measuring Trk tyrosine autophosphorylation using a recently developed procedure (4). Rats received a single injection of NGF directly into the striatum or into the lateral ventricle, using the same cannula system as for the repeated injections in the experiments described above. Two hours after the injection, the animals were killed, the striatum was quickly dissected, and microslices were prepared. Pairs of samples were prepared from the slices. One sample of each pair was lysed without further NGF treatment to assess tyrosine phosphorylation status of the tissue after the *in vivo* treatment. The other sample was incubated under physiological conditions with NGF to test for the maximal response capacity of the same tissue. Trk-type proteins were then immunoprecipitated and analyzed for phosphotyrosine content by immunoblotting (4). Tyrosine phosphorylation of Trk-type receptors in brain tissue slices after neurotrophin stimulation was previously found to correlate with tyrosine phosphorylation of several secondary signal transduction proteins, suggesting stimulation of the kinase activity of the Trk-type receptors (4). Using this assay, we found persistent tyrosine phosphorylation on Trks 2 hr after injections of 50, 150, or 750 ng of NGF directly into the striatum (Fig. 6A). Comparison with the phosphotyrosine signal in samples acutely incubated with NGF during the assay demonstrated that maximal possible stimulation of Trk-type NGF receptors

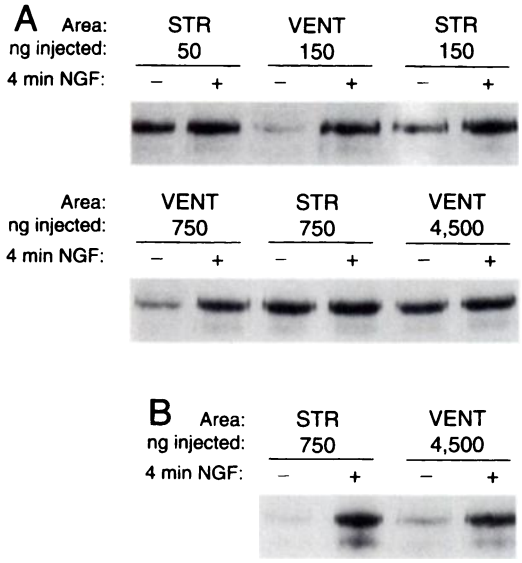

Fig. 6. Direct intrastriatal NGF injections are more effective in inducing Trk tyrosine autophosphorylation than intraventricular injections. Adult rats were provided with guiding cannulas directed at the striatum (*STR*) or at the lateral ventricle (*VENT*), respectively. The animals were allowed to recover from stereotactic surgery for 5 days and then were injected with the amounts of NGF indicated in the figure in a total volume of 1 μl. Two hours after the injection, microslices were prepared from injected and contralateral control striata and maintained for 45 min at 37° in CO_2/O_2 gassed Krebs' buffer with two buffer changes. Aliquots of the slices were then prepared, and some of the aliquots were incubated for 4 min at 37° with a maximal active concentration of NGF (100 ng/ml). After incubation, the tissue was lysed and immunoprecipitated with anti-pan Trk serum. Trk proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-ptyr as described in Materials and Methods. Pronounced Trk tyrosine autophosphorylation after intrastriatal injections is observed with 50 or 150 ng of injected NGF and is maximum with 750 ng. In contrast, 4.5 μg of NGF is required for maximal response with intraventricular injections. A, Samples prepared from injected side of the brain. B, Samples prepared from the uninjected (contralateral) side.

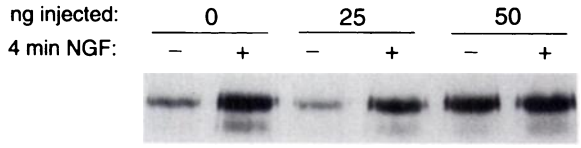

Fig. 7. Fifty nanograms of NGF are required in intraparenchymal injections directly into the striatum to induce pronounced NGF receptor tyrosine phosphorylation. Animals were treated as detailed in legend to Fig. 6, but only intrastriatal injections were performed. Indicated nanogram amounts of NGF were injected in 1 μl total volume.

due to the *in vivo* treatment was achieved with injections of 150 and 750 ng of the trophic protein (Fig. 6A). Intraventricular injection of NGF was also effective in inducing Trk-type protein tyrosine phosphorylation (Fig. 6A). However, similar to the effects of NGF injections on induction of cellular mRNAs, much higher doses were required. Only at the highest dose of 4.5 μg did intracerebroventricularly applied NGF elicit significant Trk tyrosine phosphorylation (Fig. 6A). This dose is ~30-fold higher than the dose required for maximal stimulation using intrastriatal injection of NGF. Contralateral to the injection site, no significant activation of Trk-type NGF receptors was observed with either intrastriatal or intracerebroventricular injection, demonstrating the rather local effect of NGF application (Fig. 6B). In a separate experiment, we determined the minimal required dose of intrastriatal NGF to elicit a clear response of NGF receptor tyrosine phosphorylation. As shown in Fig. 7, compared with the constitutive level of Trk tyrosine phosphorylation no change was seen 2 hr after intrastriatal treatment with 25 ng of NGF, although 50 ng elicited a clear response.

Discussion

To compare the effectiveness of intraparenchymal and intraventricular neurotrophic factor treatment in the brain, NGF was injected into the striatum or into the lateral ventricle, and cellular and molecular effects of these treatments were measured in the striatum. To assess the neurotrophic effect in the NGF-responsive cholinergic striatal interneurons, we used *in situ* hybridization for ChAT and *trk*A mRNA and Western blotting for phosphotyrosine on Trk-type NGF receptors. Neurotrophin-induced tyrosine phosphorylation on Trks has previously been shown to correlate with tyrosine phosphorylation of several second messenger proteins in brain tissue, indicating activation of the Trk-type receptors (4). Our findings can be summarized as follows: (i) daily intrastriatal administration of control protein, cytochrome *c*, resulted in a modest but significant up-regulation of ChAT mRNA expression but not *trk*A mRNA expression in striatal cholinergic neurons. (ii) NGF, when applied to the striatum, induced a dose-dependent increase in ChAT mRNA levels in individual striatal cholinergic cell bodies. In our experiments, the threshold value for NGF to induce ChAT mRNA expression was as low as 50 ng/day, and the lowest dose to induce maximal effect was 750 ng/day. (iii) *trk*A mRNA-containing cells also responded to intrastriatal NGF injections with an increase in *trk*A mRNA. However, the dose requirements for *trk*A mRNA induction were clearly higher than for ChAT mRNA induction. (iv) Dose requirements for inducing tyrosine phosphorylation of Trk-type NGF receptors by intrastriatal or intraventricular NGF injections were very similar to the requirements for induction of ChAT mRNA. (v) Intrastriatal NGF delivery was significantly more efficient than intraventricular NGF administration in up-regulating cellular marker mRNAs of the striatal cholinergic neurons and in inducing Trk autophosphorylation.

The striatum in the rat is in close proximity to the lateral wall of the lateral ventricle, which was used for the intraventricular injections. The much higher dose requirements for NGF effects in the striatum with intraventricular NGF administration than with intrastriatal NGF injections suggest that trophic factor deposited into the ventricular space diffuses into adjacent brain tissue only to a small degree. Most of the protein is probably quickly cleared from the brain or reaches ineffectively low concentrations in any given brain area. This is demonstrated by the fact that no significant signal was seen on the contralateral side after intracerebroventricular injection of the factor. Our findings with the Trk tyrosine phosphorylation assay indicate that even the initial concentration of NGF in the striatum probably never reaches levels comparable to the concentration achieved with direct intraparenchymal injections. This finding is significant with regard to the potential use of neurotrophic factor therapy in neurodegeneration. For example, it is well established that basal forebrain cholinergic neurons are a major central nervous system target for NGF (for a review, see Ref. 27). These neurons, which are implicated in learning and memory, are

severely affected in Alzheimer's disease (39). In animal experiments, NGF has been shown to prevent the death of septal/diagonal band cholinergic neurons after axotomy by fimbria/fornix transection, to restore ChAT activity to control levels after lesions of the nucleus basalis, and to improve the behavioral performance of aged impaired animals (for a review, see Ref. 27). In most of the described studies, NGF was administered intraventricularly in relatively large doses (microgram amounts). At these doses, NGF treatment is known to induce remarkable side effects, e.g., promotion of aberrant sympathetic neurite sprouting (30) along with reduced weight gain and food intake (31, 32). Similar findings have been observed in limited clinical trials, where NGF has been found to induce hyperalgesia and weight loss (33). Our findings now indicate that the same desired effects but fewer side effects might be achieved by more precisely targeted intraparenchymal infusion of smaller amounts of the trophic factor. Although we did not specifically quantify the effects of NGF injections on basal forebrain cholinergic neurons in the present study, we did not notice up-regulation of ChAT or *trk*A mRNA in the medial septum with intrastriatal injections of NGF (data not shown). For intraventricular injections, Holtzman *et al.* (15) reported increases of ChAT and *trk*A mRNAs to 200% and 170% of control, respectively, after intraventricular treatment for 7 days with 30 μg of NGF/day. We found similar results in fimbria/fornix-lesioned rats with daily intraventricular NGF injections of 4.5 μg (16). These increases are very similar to the effects observed in striatum after intraventricular NGF injections in the present study but less than those observed after intrastriatal injections. Similarly, at the level of Trk tyrosine phosphorylation, high doses of intraventricular NGF are required to elicit a response of Trk-type receptors in the medial septum/basal forebrain.[1] Taken together, these observations suggest a very similar NGF response in basal forebrain as in striatal cholinergic neurons.

Injection of the control protein cytochrome *c* directly into the striatum resulted in up-regulation of ChAT mRNA. The reason for this effect is not clear. No increase in cholinergic properties after treatment with this protein is seen in primary cultures of embryonic rat brain containing cholinergic neurons of the basal forebrain or the striatum.[2] It seems unlikely that developmental changes in the cholinergic neurons might be responsible for this difference. Rather, it seems possible that lesions of the striatum might result in the endogenous production and release of trophic factor(s), possibly including NGF. Reactive astrocytes, a common observation along the trajectory of intracerebral injections, show increased synthesis of mRNA for NGF (40). A low level of constitutive activation of Trk-type NGF receptors, presumably due to the presence of endogenous NGF, can indeed be detected in our Western blots (Figs. 6 and 7).

Our results showed up-regulation of ChAT mRNA occurred at lower doses of injected NGF than up-regulation of *trk*A mRNA. This result confirms a conclusion derived from earlier cell culture data that trophic treatment does not necessarily affect equally all biochemical and molecular parameters of a responsive cell but might influence only rather specific cellular processes (41). It has been speculated that the low affinity neurotrophin receptor protein p75NTR might be involved in regulating NGF responses, particularly at low concentrations of the ligand (6, 42, 43). It is conceivable that interactions of some but not all of the proteins involved in NGF signal transduction with the activated NGF receptor in responsive cells (3) might be regulated by p75NTR. Considering the absence of detectable levels of p75NTR mRNA and protein in the striatum, such a mechanism seems unlikely to explain our observed differences with ChAT and *trk*A mRNA. Although lesions have been shown to result in an up-regulation of p75NTR in the adult striatum (21), the similarity of our results with intrastriatal and intraventricular injections that do not result in striatal lesioning and, presumably, up-regulation of p75NTR adds further evidence that p75NTR might not be responsible for the observed differences.

Our results not only have relevance for NGF and the cholinergic neurons of the basal forebrain but also particularly have implications for possible neurotrophic factor therapy in Huntington's disease, an affliction characterized by severe striatal neurodegeneration, mainly of the GABAergic neurons (for a review, see Ref. 44). We have previously demonstrated the ability of intrastriatally delivered NGF to prevent the reduction in the number of cells expressing either ChAT mRNA or *trk*A mRNA, caused by a single intrastriatal injection of quinolinic acid, in an animal model of Huntington's disease (26). Whether NGF administration, possibly combined with BDNF or NT-4/5, which have been shown to stimulate striatal GABAergic neurons (45–47), is useful for recovering some of the neurotransmitter systems affected by this fatal disease awaits clarification. Another approach to treatment in this disease might be the use of a genetically engineered neurotrophin with multiple activities, as recently proposed by different researchers (48–50). Our current results strongly suggest, probably also for such altered neurotrophins, that intraparenchymal rather than intraventricular injections or infusions of the trophic factor(s) might be useful to maximize the effects on the targeted neuronal populations and to minimize undesirable side effects.

[1] B. Knusel, unpublished observations.
[2] B. Knusel and F. Hefti, unpublished observations.

References

1. Bothwell, M. Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* **18**:223–253 (1995).
2. Barbacid, M. The Trk family of neurotrophin receptors. *J. Neurobiol.* **25**:1386–1403 (1994).
3. Kaplan, D. R., and R. M. Stephens. Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.* **25**:1404–1417 (1994).
4. Knusel, B., S. Rabin, H. R. Widmer, F. Hefti, and D. R. Kaplan. Neurotrophin-induced trk receptor phosphorylation and cholinergic neuron response in primary cultures of embryonic rat brain neurons. *Neuroreport* **3**:885–888 (1992).
5. Rodriguez Tebar, A., G. Dechant, R. Gotz, and Y. A. Barde. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* **11**:917–922 (1992).
6. Hempstead, B. L., D. Martin Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao. High-affinity NGF binding requires coexpression of the Trk proto-oncogene and the low-affinity NGF receptor. *Nature (Lond.)* **350**:678–683 (1991).
7. Kahle, P., P. A. Barker, E. M. Shooter, and C. Hertel. p75 nerve growth factor receptor modulates p140trkA kinase activity, but not ligand internalization, in PC12 cells. *J. Neurosci. Res.* **38**:599–606 (1994).
8. Barker, P. A., and E. Shooter. Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* **13**:203–215 (1994).
9. Rabizadeh, S., J. Oh, L. T. Zhong, J. Yang, C. M. Bitler, L. L. Butcher, and D. E. Bredesen. Induction of apoptosis by the low-affinity NGF receptor. *Science (Washington D. C.)* **261**:345–348 (1993).
10. Holtzman, D. M., J. Kilbridge, Y. Li, E. T. Cunningham, Jr., N. J. Lenn, D. O. Clary, L. F. Reichardt, and W. C. Mobley. TrkA expression in the CNS:

evidence for the existence of several novel NGF-responsive CNS neurons. *J. Neurosci.* **15**:1567–1576 (1995).
11. Knusel, B., and F. Hefti. Development of cholinergic pedunculopontine neurons *in vitro*: comparison with cholinergic septal cells and response to nerve growth factor, ciliary neuronotrophic factor, and retinoic acid. *J. Neurosci. Res.* **21**:365–375 (1988).
12. Venero, J. L., and F. Hefti. TrkA NGF receptor expression by non-cholinergic thalamic neurons. *Neuroreport* **4**:959–962 (1993).
13. Gibbs, R. B., and D. W. Pfaff. *In situ* hybridization detection of *trkA* mRNA in brain: distribution, colocalization with p75NGFR and up-regulation by nerve growth factor. *J. Comp. Neurol.* **341**:324–339 (1994).
14. Hefti, F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* **6**:2155–2162 (1986).
15. Holtzman, D. M., Y. Li, L. F. Parada, S. Kinsman, C. K. Chen, J. S. Valletta, J. Zhou, J. B. Long, and W. C. Mobley. p140trk mRNA marks NGF-responsive forebrain neurons: evidence that trk gene expression is induced by NGF. *Neuron* **9**:465–478 (1992).
16. Venero, J. L., B. Knusel, K. D. Beck, and F. Hefti. Expression of neurotrophin and trk receptor genes in adult rats with fimbria transections: effect of intraventricular nerve growth factor and brain-derived neurotrophic factor administration. *Neuroscience* **59**:797–815 (1994).
17. Mobley, W. C., J. L. Rutkowski, G. I. Tennekoon, K. Buchanan, and M. V. Johnston. Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science (Washington D. C.)* **229**:284–287 (1985).
18. Johnston, M. V., J. L. Rutkowski, B. H. Wainer, J. B. Long, and W. C. Mobley. NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochem. Res.* **12**:985–994 (1987).
19. Korsching, S., G. Auburger, R. Heumann, J. Scott, and H. Thoenen. Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* **4**:1389–1393 (1985).
20. Shelton, D. L., and L. F. Reichardt. Studies on the expression of the *beta* nerve growth factor (NGF) gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several neuronal populations. *Proc. Natl. Acad. Sci. USA* **83**:2714–2718 (1986).
21. Gage, F. H., P. Batchelor, K. S. Chen, D. Chin, G. A. Higgins, S. Koh, S. Deputy, M. B. Rosenberg, W. Fischer, and A. Bjorklund. NGF receptor reexpression and NGF-mediated cholinergic neuronal hypertrophy in the damaged adult neostriatum. *Neuron* **2**:1177–1184 (1989).
22. Sobreviela, T., D. O. Clary, L. F. Reichardt, M.M. Brandabur, J.H. Kordower, and E.J. Mufson. TrkA-immunoreactive profiles in the central nervous system: colocalization with neurons containing p75 nerve growth factor receptor, choline acetyltransferase, and serotonin. *J. Comp. Neurol.* **350**:587–611 (1994).
23. Steininger, T. L., B. H. Wainer, R. Klein, M. Barbacid, and H. C. Palfrey. High-affinity nerve growth factor receptor (Trk) immunoreactivity is localized in cholinergic neurons of the basal forebrain and striatum in the adult rat brain. *Brain Res.* **612**:330–335 (1993).
24. Ringstedt, T., H. Lagercrantz, and H. Persson. Expression of members of the trk family in the developing postnatal rat brain. *Brain Res. Dev. Brain Res.* **72**:119–131 (1993).
25. Davies, S. W., and K. Beardsall. Nerve growth factor selectively prevents excitotoxin induced degeneration of striatal cholinergic neurones. *Neurosci. Lett.* **140**:161–164 (1992).
26. Venero, J. L., K. D. Beck, and F. Hefti. Intrastriatal infusion of nerve growth factor after quinolinic acid prevents reduction of cellular expression of choline acetyltransferase messenger RNA and trkA messenger RNA, but not glutamate decarboxylase messenger RNA. *Neuroscience* **61**:257–268 (1994).
27. Hefti, F. Neurotrophic factor therapy for nervous system degenerative diseases. *J. Neurobiol.* **25**:1418–1435 (1994).
28. Yan, Q., C. Matheson, J. Sun, M. J. Radeke, S. C. Feinstein, and J. A. Miller. Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression. *Exp. Neurol.* **127**:23–36 (1994).
29. Morse, J. K., S. J. Wiegand, K. Anderson, Y. You, N. Cai, J. Carnahan, J. Miller, P. S. DiStefano, C. A. Altar, and R. M. Lindsay. Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons after fimbria transection. *J. Neurosci.* **13**:4146–4156 (1993).
30. Isaacson, L. G., and K. A. Crutcher. The duration of sprouted cerebrovascular axons after intracranial infusion of nerve growth factor. *Exp. Neurol.* **131**:174–179 (1995).
31. Williams, L. R. Hypophagia is induced by intracerebroventricular administration of nerve growth factor. *Exp. Neurol.* **113**:31–37 (1991).
32. Lapchak, P. A., and F. Hefti. BDNF and NGF treatment in lesioned rats: effects on cholinergic function and weight gain. *Neuroreport* **3**:405–408 (1992).
33. Petty, B. G., D. R. Cornblath, B. T. Adornato, V. Chaudhry, C. Flexner, M. Wachsman, D. Sinicropi, L. E. Burton, and S. J. Peroutka. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. *Ann. Neurol* **36**:244–246 (1994).
34. Pelleymounter, M. A., M. J. Cullen, and C. L. Wellman. Characteristics of BDNF-induced weight loss. *Exp. Neurol.* **131**:229–238 (1995).
35. Paxinos G., and Watson C. *The Rat Brain in Stereotaxic Coordinates*. Academic Press, London (1986).
36. Mori, N., Y. Tajima, H. Sakaguchi, D. J. Vandenbergh, H. Nawa, and P. M. Salvaterra. Partial cloning of the rat choline acetyltransferase gene and *in situ* localization of its transcripts in the cell body of cholinergic neurons in the brain stem and spinal cord. *Brain Res. Mol. Brain Res.* **17**:101–111 (1993).
37. Martin Zanca, D., M. Barbacid, and L. F. Parada. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* **4**:683–694 (1990).
38. Pasinetti, G. M., D. G. Morgan, S. A. Johnson, S. P. Lerner, M. A. Myers, J. Poirier, and C. E. Finch. Combined *in situ* hybridization and immunocytochemistry in the assay of pharmacological effects on tyrosine hydroxylase mRNA concentration. *Pharmacol. Res.* **21**:299–311 (1989).
39. Whitehouse, P. J., D. L. Price, A. W. Clark, J. T. Coyle, and M. R. DeLong. Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann. Neurol.* **10**:122–126 (1981).
40. Arendt, T., M. K. Bruckner, T. Krell, S. Pagliusi, L. Kruska, and R. Heumann. Degeneration of rat cholinergic basal forebrain neurons and reactive changes in nerve growth factor expression after chronic neurotoxic injury. II. Reactive expression of the nerve growth factor gene in astrocytes. *Neuroscience* **65**:647–659 (1995).
41. Beck, K. D., B. Knusel, and F. Hefti. The nature of the trophic action of brain-derived neurotrophic factor, des(1–3)-insulin-like growth factor-1, and basic fibroblast growth factor on mesencephalic dopaminergic neurons developing in culture. *Neuroscience* **52**:855–866 (1993).
42. Davies, A. M., K. F. Lee, and R. Jaenisch. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* **11**:565–574 (1993).
43. Verdi, J. M., S. J. Birren, C. F. Ibanez, H. Persson, D. R. Kaplan, M. Benedetti, M. V. Chao, and D. Anderson. p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* **12**:733–745 (1994).
44. Purdon, S. E., E. Mohr, V. Ilivitsky, and B. D. Jones. Huntington's disease: pathogenesis, diagnosis and treatment. *J. Psychiatry Neurosci.* **19**:359–367 (1994).
45. Mizuno, K., J. Carnahan, and H. Nawa. Brain-derived neurotrophic factor promotes differentiation of striatal GABAergic neurons. *Dev. Biol.* **165**:243–256 (1994).
46. Widmer, H. R., and F. Hefti. Stimulation of GABAergic neuron differentiation by NT-4/5 in cultures of rat cerebral cortex. *Brain Res. Dev. Brain Res.* **80**:279–284 (1994).
47. Ventimiglia, R., P. E. Mather, B. E. Jones, and R. M. Lindsay. The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons *in vitro*. *Eur. J. Neurosci.* **7**:213–222 (1995).
48. Urfer, R., P. Tsoulfas, D. Soppet, E. Escandon, L. F. Parada, and L. G. Presta. The binding epitopes of neurotrophin-3 to its receptors trkC and gp75 and the design of a multifunctional human neurotrophin. *EMBO J.* **13**:5896–5909 (1994).
49. Ilag, L. L., R. Curtis, D. Glass, H. Funakoshi, N. J. Tobkes, T. E. Ryan, A. Acheson, R. M. Lindsay, H. Persson, and G. D. Yancopoulos. Pan-neurotrophin 1: a genetically engineered neurotrophic factor displaying multiple specificities in peripheral neurons in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **92**:607–611 (1995).
50. Friedman, W. J., I. B. Black, H. Persson, and C. F. Ibanez. Synergistic trophic actions on rat basal forebrain neurons revealed by a synthetic NGF/BDNF chimeric molecule. *Eur. J. Neurosci.* **7**:656–662 (1995).

Send reprint requests to: Dr. Beat Knusel, University of Southern California, Andrus Gerontology Center, 3715 McClintock Avenue, Los Angeles, CA 90089-0191.