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Nerve Growth Factor Increases Choline Acetyltransferase Activity in Developing Basal Forebrain Neurons

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Nerve growth factor (NGF) is a neuronotrophic protein. Its effects on developing peripheral sensory and sympathetic neurons have been extensively characterized, but it is not clear whether NGF plays a role during the development of central nervous system neurons. To address this point, we examined the effect of NGF on the activity of neurotransmitter enzymes in several brain regions. Intracerebroventricular injections of highly purified mouse NGF had a marked effect on the activity of choline acetyltransferase (ChAT), a selective marker of cholinergic neurons. NGF elicited prominent increases in ChAT activity in the basal forebrain of neonatal rats, including the septum and a region which contains neurons of the nucleus basalis and substantia innominata. NGF also increased ChAT activity in the hippocampus and neocortex, terminal regions for the fibers of basal forebrain cholinergic neurons. In analogy with the response of developing peripheral neurons, the NGF effect was shown to be selective for basal forebrain cholinergic cells and to be dose-dependent. Furthermore, septal neurons closely resembled sympathetic neurons in the time course of their response to NGF. These observations suggest that endogenous NGF does play a role in the development of basal forebrain cholinergic neurons.

INTRODUCTION

Diffusible trophic substances may play a key role in the differentiation of the nervous system. Among these, nerve growth factor (NGF) is a well-characterized protein with effects on dorsal root sensory and sympathetic neurons of the peripheral nervous system (PNS) that have been extensively investigated. Its actions on developing neurons include enhanced viability and accelerated morphological and biochemical differentiation[16,50]. Moreover, administration of NGF to developing animals prevents the degeneration of sensory[15] and sympathetic neurons[20] that normally occurs during this period. Conversely, administration of NGF antibodies at appropriate times during development causes destruction of sensory and sympathetic neurons[16,23,41,50] an effect produced by antibody-mediated sequestration of endogenous NGF[13]. The ability of NGF injections to prevent naturally occurring neuronal degeneration and of its antibodies seemingly to enhance this process, indicates that endogenous NGF plays a key role in shaping the developing PNS. Moreover, studies of NGF effects on its responsive peripheral neurons have provided a model for investigating trophic relationships in other neuronal populations and for understanding essential aspects of neural development.

Recent studies have suggested that cholinergic neurons of the basal forebrain also respond to NGF. These neurons are considered to be important for memory and other cognitive processes and loss of these cells has been documented in patient's with Alzheimer's disease[5,51]. Several observations suggest that NGF is an endogenous trophic factor for these

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neurons. First, mammalian brain contains messenger RNA encoding NGF[27,49] and material which reacts with NGF antibodies[8,27,29]. Interestingly, rather high levels of both have been found in hippocampus and neocortex[27,49], the target areas for the fibers of cholinergic neurons located in the septum and nucleus basalis, respectively[35]. Second, Schwab and co-workers demonstrated that neuronal cell bodies in the basal forebrain cholinergic complex were selectively labeled after retrograde transport of iodinated NGF preparations from neocortex and hippocampus[46,47]. This result suggests that the fibers of basal forebrain neurons are like those of responsive peripheral neurons in their ability to bind NGF and transport it retrogradely[50]. Finally, the activity of choline acetyltransferase (ChAT; acetyl-CoA: choline-O-acetyltransferase, EC 2.3.1.6), an enzyme selectively localized in cholinergic neurons which catalyzes the synthesis of acetylcholine[32], was increased in the basal forebrain of neonatal rats treated with NGF[12]. Neurotransmitter synthesizing enzymes in peripheral sympathetic neurons respond to NGF in similar fashion[16].

In spite of these observations, the role of endogenous NGF as a trophic factor for basal forebrain cholinergic neurons remains uncertain, largely because of the apparent inability of NGF antibodies to alter the survival of these neurons in developing animals[12]. It was unclear why NGF preparations demonstrated activity while NGF antibodies had no effect. However, one interpretation is that NGF, itself, was inactive on CNS cholinergic neurons and that the NGF samples used in these studies were contaminated with another active agent.

Indeed, contaminants are present in most NGF preparations and bioactivities due to the presence of other molecules have been incorrectly attributed to NGF (for review see ref. 14). To determine whether NGF or a contaminant was responsible for the activity on CNS cholinergic neurons, we prepared highly purified mouse NGF. Herein, we report that these preparations elicited prominent increases in ChAT activity in the basal forebrain of neonatal rats, indicating that NGF is active on CNS cholinergic neurons. NGF effects were manifested in regions containing the cell bodies and fibers of basal forebrain cholinergic neurons. In analogy with the receptor-mediated activities demonstrated for PNS neurons[16,50], the NGF effect was shown to be selective for cholinergic neurons and to be dose-dependent. Furthermore, the time course of the NGF effect closely resembled that for the response of developing peripheral sympathetic neurons. These results suggest that NGF does play a role in the development of basal forebrain cholinergic neurons.

MATERIALS AND METHODS

NGF isolation and characterization

Submandibular glands of adult male Swiss–Webster mice were dissected or obtained commercially (Pel-Freez). NGF was isolated by a modification of a previously described method[37]. NGF molecules were eluted from the second carboxymethyl cellulose column (CM-52, Whatman; column dimensions 2.5 × 17 cm; flow rate = 60 ml/h) with a sodium chloride gradient prepared by mixing equal (150 ml) volumes of 50 mM Tris-HCl, pH 9.0, with the same buffer containing 0.4 M sodium chloride in a linear gradient mixer (Pharmacia, GM-1).

Preparative electrofocusing was conducted in an electrofocusing column (LKB 8100 Ampholine electrofocusing column, 110 ml). Equal (1.4 ml) volumes of pH 7–9 and pH 9–11 ampholytes (LKB Ampholines) were added. To 51.9 ml of a 5% sucrose solution in water were added 2.1 ml of the ampholyte mixture; the remainder was added to 53.3 ml of 50% sucrose. Individual 4.6 ml gradient fractions were prepared to span sucrose concentrations of 5–50%. NGF samples submitted to electrofocusing were lyophilized, redissolved in a small volume of 0.2% acetic acid and incorporated into one or two midgradient fractions. The cathode solution was 240 mM sodium hydroxide in 60% sucrose. The anode solution was 1% phosphoric acid. Focusing was conducted at 600 V for 18–24 h at 4 °C. Absorbance at 280 nm and pH (4 °C) were determined on 1.5 ml fractions.

Gel filtration of NGF samples was conducted according to a published method[9]. Electrofocused NGF samples (1–2 mg) were lyophilized and redissolved in 400 μl of 2 N acetic acid. After addition of 100 μl of 50% sucrose, the sample was carefully layered on a Sephadex G-75 (Pharmacia) column (0.8 × 120 cm) equilibrated in 2 N acetic acid at 4 °C. The column had been previously calibrated with marker proteins that included bovine carbonic anhydrase, soybean

trypsin inhibitor, and bovine pancreatic RNAse A (Sigma). Column flow rate was 7.5 ml/h and 1.0 ml fractions were collected.

For reverse-phase HPLC, NGF was loaded onto a μBONDAPAK C18 column (Waters Associates) (19 mm × 15 cm; flow rate = 9 ml/min) in 0.1% trifluoroacetic acid (TFA) (Fisher) and eluted with a complex gradient of acetonitrile (Fisher-HPLC grade) in 0.1% TFA. Fractions were diluted with 2 vols. of cold 0.2% acetic acid and dialyzed against the same at 4 °C. Samples were then lyophilized, redissolved in a small volume of 0.2% acetic acid, centrifuged to remove any particulate matter, and the concentration was determined by best-fit of amino acid analysis data (Beckman 6300) with the published sequence[3].

NGF samples were submitted to analysis in isoelectric focusing (IEF) polyacrylamide tube gels as described[38]. The procedure of Laemmli[28] was used for sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE) in 15% polyacrylamide slab gels. After fixing in 20% trichloroacetic acid, the gels were stained[37]. Gels were scanned and peaks were quantitated[38]. Biological activity in vitro was measured by an established method[43] in which neurite outgrowth from dissociated dorsal root ganglion neurons was scored. Readings were made 24–72 h after plating.

Renin activity was determined as described[38]. The activity of the submandibular gland homogenate under these conditions was 1014 nmol angiotensin I formed/mg protein/h. Prior trypsinization of the homogenate[22] did not increase renin activity.

Animal treatments and enzyme assays

Pregnant Sprague–Dawley rats were individually housed and kept on a 12-h daylight schedule. The birth of litters was registered every 12 h and the day of birth was recorded as postnatal day 1. NGF samples were injected intracerebroventricularly (i.c.v.)[38]. Control animals received an equal amount of cytochrome *c*, a molecule physiochemically similar to NGF but which lacks trophic activity, or the vehicle alone. Unless otherwise stated, injections were given on postnatal days 2, 4, 6 and 8, and animals were sacrificed on day 12. Angiotensins I and II (Boehringer-Mannheim) were administered in the same fashion. Injections were well tolerated.

Animals were killed by decapitation. The brains were quickly removed, sectioned sagittally, and dissected on a chilled glass plate. Dissected regions included: septum, hippocampus, nucleus basalis/substantia innominata (NB/SI), frontolateral neocortex, corpus striatum, ventral midbrain/substantia nigra, and dorsal pons. The NB/SI was dissected as follows: a knife cut linked the root of the ventricle behind the septum to the base of the brain by passing through the anterior commissure; another knife cut was placed parallel to the first and 1.5 mm caudal to it; with fine, curved forceps a 2 mm thick tissue piece was removed which extended from the base of the brain 1.5 mm dorsally. Histological examination showed that neurons of the nucleus basalis, substantia innominata and horizontal limb nucleus of the diagonal band were included (unpublished observations). The ventral one-third of the midbrain is herein referred to as ventral midbrain/substantia nigra. Assays of choline acetyltransferase, tyrosine hydroxylase and glutamate decarboxylase were those used by Johnston et al.[24]. Enzyme activity was expressed in relation to the concentration of soluble protein[31]. In pups receiving 30 μg doses of NGF, but not controls, the rate of increase of body weight and length was reduced. At 12 days of age, body weight was 75–85% of normal. No significant change was seen in brain weight or in brain protein per tissue weight. NGF-treated pups were normally active and there was no gross change in brain morphology.

RESULTS

Purification of NGF

Mouse NGF prepared by some protocols contains significant amounts of renin activity[6,17]. NGF isolated by ion exchange chromatography contained only 2 parts in 10^5 of the renin activity in the submandibular gland homogenate. Purity by IEF gel and SDS-PAGE analysis was ≥94% and ≥92%, respectively. Bioactivity in vitro was present at NGF concentrations shown to be effective in prior studies[43].

For studies to determine that NGF, and not a contaminant, was responsible for the effect on ChAT activity, two additional purification schemes were used: one in which molecules were isolated by charge and size characteristics and another in which separation was achieved by reverse-phase HPLC. In the

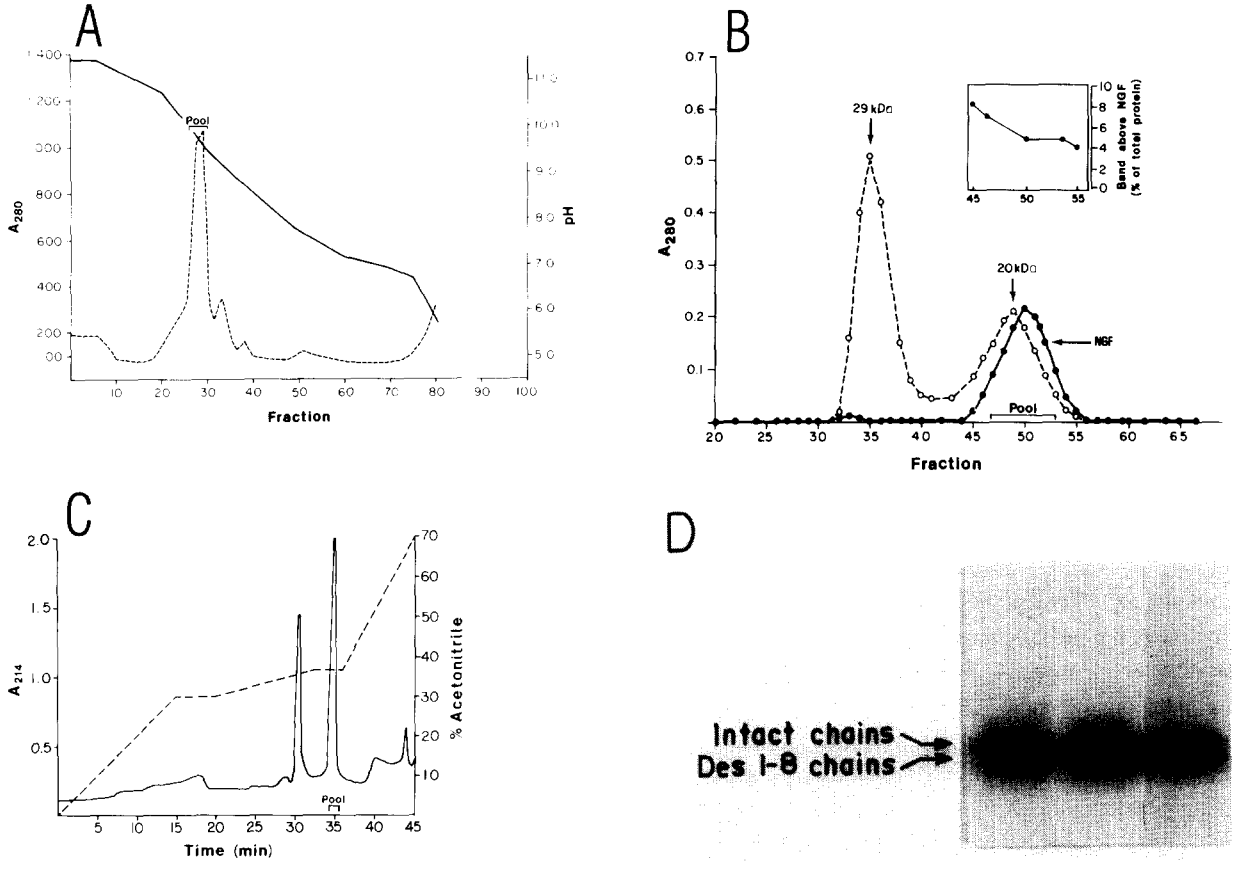

Fig. 1. Purification of NGF. A: electrofocusing column preparation of NGF. Ampholyte-containing sucrose gradient fractions were prepared as described in Materials and Methods. Lyophilized NGF (5.0 mg) was included in one or two midgradient fractions. The sample was focused at 600 V for 22 h at 4 °C. Fractions (1.5 ml) were collected and absorbance at 280 nm (dashed line) and pH (at 4 °C; solid line) were determined. Material was pooled as indicated. Recovery of NGF in the pool was 45%. B: gel filtration of NGF. A column of Sephadex G-75 was prepared and calibrated as described. Markers indicate the elution positions of bovine carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20 kDa) determined during calibration (open circles). Electrofocused NGF (1.5 mg) was applied and eluted and absorbance at 280 nm was determined on 1.0 ml fractions (closed circles). Material was pooled as indicated. Recovery was 50%. The inset shows the amount of the band above NGF on SDS-PAGE, as percentage of total protein, versus fraction number. C: reverse-phase HPLC of NGF. NGF (100 μg) was loaded on a μBONDAPAK C18 column (19 mm × 15 cm; flow rate = 9 ml/min) in 0.1% trifluoroacetic acid and eluted with a complex gradient of 0.1% trifluoroacetic acid in acetonitrile. Material was pooled as shown. D: SDS-PAGE analysis of different NGF samples. The gels are oriented such that bands of lower apparent molecular weight are nearer the bottom. Left panel: NGF (25 μg) prepared by ion exchange chromatography. Middle: NGF (30 μg) further processed by electrofocusing and gel filtration chromatography. Note, in each preparation, the presence of intact chains and those in which enzymatic cleavage has removed the amino-terminal eight amino acids[37]. Also note approximately equal amounts of a band at a higher apparent molecular weight than NGF. Right: NGF (50 μg) prepared by reverse-phase HPLC. The contaminant above NGF has been removed.

first, NGF prepared by ion exchange chromatography was submitted to preparative electrofocusing. In these experiments a single major peak was present and minor peaks were partially resolved (Fig. 1A). IEF gel analysis indicated that the two minor peaks located at pH values immediately below the main peak consisted largely of NGF species which lacked the carboxy-terminal arginine residue of one or both chains of the dimer[39]. On IEF gels the pooled fractions (Fig. 1A) consisted predominantly of NGF chains intact at the C-terminus, so-called β_1-NGF[39]. This material had the same bioactivity in vitro as the starting material.

Electrofocused NGF was applied to a Sephadex G-75 column. A single, broad major peak was eluted at an apparent molecular weight of about 18 kDa (Fig. 1B). This result agreed with prior observations of the migration of NGF under these conditions[9] and sug-

gested that the NGF dimer (26.5 kDa)[3] interacted with the column and was thereby retarded in its migration relative to the marker proteins. Pooled material (Fig. 1B) was fully active in vitro and was devoid of renin activity. Purity was ≥97.5% on IEF gels and ≥94% by SDS-PAGE.

Consistently present on SDS-PAGE in spite of the additional purification was a single band, or occasionally a closely spaced doublet, which migrated at an apparent molecular weight about 0.7 kDa heavier than did the NGF monomer (Fig. 1D). This material constituted about 4% of total protein whether the NGF samples were prepared by ion exchange chromatography alone or were further processed by electrofocusing and gel filtration. Interestingly, the co-purified material was present in small amounts through the NGF peak (Fig. 1B inset), suggesting interaction of this molecule with NGF. In order to remove this material, NGF prepared by ion exchange chromatography was submitted to reverse-phase HPLC (Fig. 1C). The minor peak at 29 min contained the impurity. The second major peak (34 min) was devoid of the heavier material and contained NGF with purity ≥98.5%. HPLC-purified NGF had full bioactivity in vitro. Further characterization of individual HPLC fractions will be given elsewhere.

Highly purified NGF increases basal forebrain ChAT activity

There was concern that an impurity in the NGF preparation, and not NGF, was responsible for the previously demonstrated effect on basal forebrain cholinergic neurons[12,47]. Renin activity is present in mouse NGF samples prepared by the protocols used for earlier studies[6,17]. Experiments with bovine NGF, which is virtually devoid of renin activity[17], indicated that this contaminant was unlikely to be responsible for the observed increases in ChAT activity[12]. In agreement with these observations, ICV injections of angiotensin I and II in the same amount (30 μg) and schedule used for NGF failed to alter ChAT activity (data not shown).

To exclude contaminants, NGF samples were submitted to two additional isolation schemes. In the first of these, electrofocusing was followed by gel filtration. A 2–4% increase in purity was observed. This material was equipotent to NGF prepared by ion exchange chromatography in the biological assay in vitro. In experiments in vivo, these NGF preparations were compared for their effect on ChAT activity in septum. Repeated 10 μg doses were given. The increases in ChAT activity were indistinguishable (ChAT activity mean ± S.E.M. as percent control; ion-exchange NGF = 172 ± 10; further purified NGF = 155 ± 15; n = 4 each group). NGF prepared by reverse-phase HPLC was devoid of the only impurity consistently present in SDS-PAGE of samples submitted to electrofocusing and gel filtration. The bioactivity in vivo of HPLC-purified NGF was also indistinguishable from that of the starting material (animals decapitated day 10; ChAT activity mean ± S.E.M. as percent control; ion-exchange NGF = 369 ± 9; HPLC NGF = 320 ± 9; n = 4 for each group).

NGF selectively affects basal forebrain cholinergic neurons

Injections of NGF prepared by ion-exchange chromatography produced significant increases in ChAT activity in regions containing cholinergic cell bodies and their fibers (Table I). The greatest effects were seen in the former. In septum and NB/SI, ChAT activity levels were more than doubled. The increases recorded for hippocampus and neocortex were more modest (50–60%) but still statistically significant. The principal source of ChAT activity in hippocampus and neocortex is the cholinergic neurons of the basal forebrain[5,24,34,35] and increases in ChAT activity are almost certainly due to the action of NGF on these cells; however, the possibility that NGF also had an effect on cholinergic interneurons cannot be excluded (also see ref. 38). The NGF effect appeared to be centrally mediated in that ChAT activity was unchanged when NGF was given in the same dose and schedule but by subcutaneous injection.

To assess the selectivity of the NGF effect, the activities of two other neurotransmitter enzymes were measured in NGF-treated animals. Tyrosine hydroxylase activity serves as a marker for catecholaminergic neurons[4], and glutamate decarboxylase activity is contained in neurons that use γ-aminobutyric acid as neurotransmitters[33]. NGF injections were not associated with significant changes in the activities of either of these neurotransmitter enzymes (Table I, footnote).

TABLE I

The effect of NGF treatment on the activity of ChAT and other neurotransmitter enzymes in several neonatal rat brain regions*

Animals received i.c.v. injections of 30 μg of NGF, the same dose of cytochrome *c* or injection vehicle alone (controls) on postnatal days 2, 4, 6 and 8 and were decapitated on day 12. Brains were processed as indicated. There was no significant difference in ChAT activity between controls which received cytochrome *c* and those which received injection vehicle alone. Preincubation of the ChAT assay mixture with from 1 to 30 μg of NGF had no effect on enzyme activity. Values represent mean ± S.E.M. Number of animals is listed in parentheses.

Brain region	*ChAT activity (nmol acetylcholine/h/mg protein)*		*% Change*
	Control	*NGF Treatment*	
Septum	57 ± 4 (10)	173 ± 15 (8)***	204
Hippocampus	31 ± 4 (10)	47 ± 3 (7)**	52
NB/SI	25 ± 2 (19)	62 ± 6 (10)***	148
Neocortex	26 ± 2 (10)	42 ± 3 (8)***	62

* Data for the activities of tyrosine hydroxylase and glutamate decarboxylase are listed as follows: region, control value (animal number), NGF-treatment value (animal number). Values are mean ± S.E.M. In no case did NGF treatment produce a significant difference. For tyrosine hydroxylase activity (pmol/h/mg protein): septum, 864 ± 100 (4), 1142 ± 270 (4); hippocampus, 31 ± 3 (9), 36 ± 12 (8); neocortex 52 ± 8 (8), 67 ± 17 (8); ventral midbrain/substantia nigra, 1997 ± 290 (5), 2236 ± 342 (4); dorsal pons, 685 ± 89 (4), 886 ± 140 (4). For glutamate decarboxylase activity (nmol/h/mg protein): septum, 14.7 ± 2 (5), 18.6 ± 1 (4); neocortex, 6.9 ± 0.7 (5), 7.3 ± 0.7 (5); corpus striatum, 9.5 ± 1.4 (5), 10.5 ± 0.9 (5).

** Students *t*-test $P < 0.01$.

*** $P < 0.001$.

Dose–response characteristics of the NGF effect

The in vivo dose–response of NGF and ChAT activity was determined for basal forebrain cholinergic neurons and for regions which receive their fibers. NGF was given i.c.v. in the schedule used for earlier experiments. In the septum and NB/SI even 1-μg doses of NGF produced significant increases in ChAT activity with higher doses producing a greater effect (Fig. 2). Doses of 30 μg elevated ChAT activity 200% in the septum and 140% in the NB/SI. In the neocortex, ChAT activity increases were detected at 3 μg with maximal increases of 80% occurring at the 10 μg dose. A dose–response relationship was not observed for hippocampus; increases in ChAT activity were recorded at the lowest dose and were little changed at higher doses.

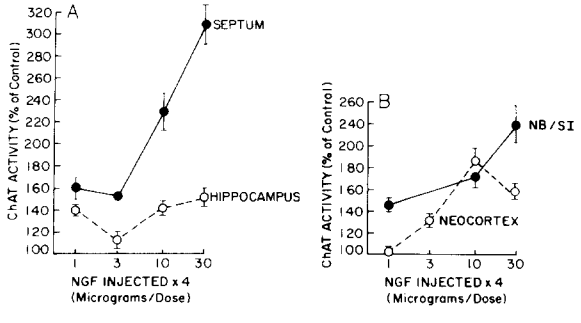

Fig. 2. Dose–response relationship for NGF and ChAT activity. The indicated dose of NGF was injected via the schedule given in Table I. Control animals received the same dose of cytochrome *c* or injection vehicle alone. Animals were decapitated at postnatal day 12 and tissues were processed as described. A and B depict ChAT activity responses in different brain regions. Values are mean ± S.E.M. For the 1, 3 and 10 μg doses, n = 3. For the 30 μg dose the n ranged from 7 to 10.

Septal cholinergic neurons undergo major developmental changes during the early postnatal period. This is reflected in the approximately 6-fold increase in ChAT activity in hippocampus that occurs during the first two postnatal weeks[40]. The experimental schedule used above extended from the first postnatal week well into the second and the response of septal neurons to NGF was thus observed over a rather broad span in development. In order to determine whether the response of septal cholinergic neurons to NGF was present during both the first and second postnatal weeks, a single dose (30 μg) was given on either postnatal day 2 or 8. The animals were killed 2 days after injection and ChAT activity was determined. Pronounced and comparable increases were detected in both instances (mean ± S.E.M.; inject day 2 and assay day 4, control = 34 ± 5 nmol acetylcholine formed/h/mg protein (n = 2), NGF = 75 ± 6 (n = 3), 120% increase, $P < 0.05$; inject day 8 and assay day 10, control = 45 ± 3 (n = 2); NGF = 78 ± 7 (n = 3), 73% increase, $P < 0.02$). Sensitivity to NGF

administration was thus clearly present throughout this period. Interestingly, in a previous study the response of basal forebrain cholinergic neurons in intact mature animals was considerably less than for neonates[12].

Time course of the response to NGF

The ability to evoke ChAT activity increases with a single injection allowed examination of the time course of the NGF effect. After an initial lag period of approximately 24 h, i.c.v. administered NGF produced a rise in ChAT activity which was sustained for at least 48 h (Fig. 3). Activity returned to control levels by postnatal day 10. This pattern closely resembled that for the response of developing sympathetic neurons to systemically administered NGF[25]. Moreover, in both instances, repeated doses were required for NGF effects to be maintained for more than a few days. These findings suggest that similar processes may attend the action of NGF in these neuronal systems.

DISCUSSION

Cholinergic neurons of the basal forebrain appear to play a key role in memory and other cognitive processes[5,10]. In the studies reported here we have examined the effect of NGF administration during the development of these neurons. No precise timetable for the development of septal neurons has been established, but it appears that neurite outgrowth reaches the hippocampal formation by fetal day 20 and that by postnatal day 14 septal fibers have assumed the highly segregated pattern of the adult[36]. Neurochemical evidence indicates that most cholinergic synapses form during the second and third postnatal weeks[48]; cholinergically mediated electrical activity in hippocampus appears on postnatal day 10, gradually assuming the adult pattern over the next 2 weeks[30]. Our NGF studies were conducted during the time that the neurites of septal neurons were maturing and beginning to establish functional connections with their target cells. We found that i.c.v. injections of NGF elicited prominent increases in the activity of ChAT in the septum and in the hippocampus which receives fibers from septal cholinergic cell bodies. By dissection we showed that the NGF effect was also registered in the NB/SI, which contains another population of basal forebrain cholinergic cell bodies, and in the neocortex, which receives NB/SI cholinergic fibers.

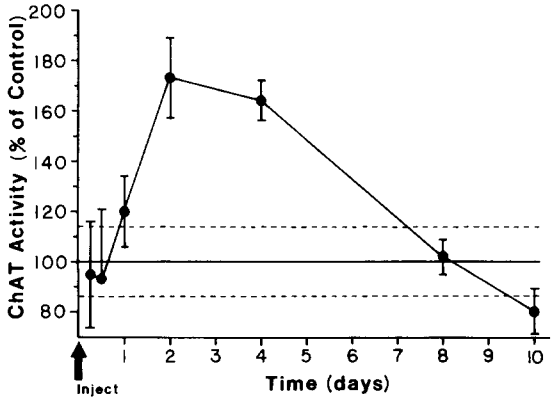

Fig. 3. Time course of response of septal neurons to NGF. A single (30 μg) dose of NGF was administered on postnatal day 2. Animals were decapitated at intervals following injection, as indicated, and septum was dissected and assayed for ChAT activity. Controls received injection vehicle. Values represent mean ± S.E.M. as percent control. n = 4 for both treatments at all times except that n = 3 for NGF at 48 h. Dashed lines represent the average S.E.M. for ChAT determinations in control animals.

In accord with investigations in vivo and in vitro of the effect of other NGF preparations[12,19,21], these data clearly document an effect on basal forebrain cholinergic neurons. However, to establish that NGF itself was responsible, highly purified NGF was produced in each of two substantially different chromatographic protocols. We noted that these NGF preparations retained full bioactivity in vivo and in vitro. These data indicate that CNS cholinergic neurons do respond to NGF.

The effect of NGF was selective for cholinergic neurons in that no change was produced in the other neurotransmitter enzyme markers examined. NGF did not affect catecholaminergic neurons, which also send their fibers over long distances to the neocortex and hippocampus[4] or neurons intrinsic to neocortex, which use γ-aminobutyric acid as neurotransmitter[33]. Interestingly, however, NGF has been shown to act on another population of cholinergic neurons in the CNS which differ in their morphology and physiology from those in the basal forebrain. Cholinergic interneurons of the corpus striatum respond to NGF with dose-dependent increases in ChAT activity[38]. Selectivity of NGF for CNS cholinergic neurons has also been suggested in morphological studies. Radiola-

beled NGF was selectively transported from injection sites in neocortex and hippocampus to neurons of the basal forebrain cholinergic complex[46,47]. Selectivity of NGF action in the CNS is likely to be due to the presence of NGF-specific receptors on the cell bodies and fibers of cholinergic neurons. Consistent with this suggestion, recent data indicate that high-affinity NGF receptors are present in the basal forebrain and hippocampus[44]. Furthermore, the dose-related nature of ChAT activity increases suggests that this effect is mediated by NGF receptors. Further studies will be required to characterize in detail the regional distribution of NGF receptors in brain and to show whether they are selectively localized to cholinergic neurons.

The ontogenesis of ChAT activity in hippocampus may serve as an index of cholinergic synapse formation[40,48]. The specific activity of ChAT in the rat dentata gyrus increases rapidly during the latter portion of the second and the third postnatal weeks[40] and it is during this period that most synapses are formed in the molecular layer[7]. The effect of NGF on ChAT activity in hippocampus suggests that NGF may be accelerating the rate of cholinergic synapse formation. Alternatively, only the pace of neurochemical differentiation may be increased. In either event, NGF treatment may significantly alter developing cholinergic neurites and their interaction with target cells in the hippocampus and neocortex.

NGF treatment increased ChAT activity either by increasing the number of ChAT molecules or by increasing the activity of preformed molecules. Increased de novo synthesis of ChAT is suggested by the 24–48 h period which intervened between NGF injection and the first increase in ChAT activity in the septum. New synthesis of tyrosine hydroxylase molecules[18] may occur during a similar lag period in developing sympathetic neurons[25]. In order to elucidate the mechanism of the NGF effect on ChAT activity it will be necessary to determine the specific activity of ChAT molecules in NGF-treated and control animals. Recent experience with immunoprecipitation of ChAT molecules suggests that such determinations will be possible[45].

The physiological significance of NGF that is endogenous to brain is not yet clear. Changes in the number of NGF receptors in chick forebrain during embryonic life[11,52] suggest that endogenous NGF may be important to CNS neurons during development. Furthermore, the concentration of NGF contained within mouse brain synaptosomes appears to be developmentally regulated, with highest levels present during early postnatal life[29]. Interestingly, the concentration of NGF in neonatal brain (approx. 10^{-9} to 10^{-10} M)[29] is comparable to the ED_{50} concentration of NGF for ChAT activity increases in septal neurons in vitro[19]. Nevertheless it is not known whether NGF is necessary for the viability of developing CNS cholinergic neurons, as it is for responsive neurons in the PNS. A prior study provided no evidence that NGF antibodies had an effect on ChAT activity[12]; however, antibodies may not have adequately penetrated brain tissue in these experiments[27]. In view of our observations and of others reviewed herein, a full evaluation of the effect of NGF antibodies is in order. To effect penetration of NGF antibodies, advantage may be taken of the fact that the blood–brain barrier is permeable in utero[1,2]. In preliminary experiments, ChAT activity was decreased by approximately 50% in the basal forebrain of rats injected with NGF-antibodies in utero[42]. Additional studies in vivo may well provide conclusive evidence for a role of NGF in the growth and maturation of basal forebrain cholinergic neurons.

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the investigator(s) adhered to the 'Guide for the Care and Use of Laboratory Animals', as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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