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Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation

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The levels of nerve growth factor (NGF) and its mRNA in the rat central nervous system were determined by two-site enzyme immunoassay and quantitative Northern blots, respectively. Relatively high NGF levels (0.4–1.4 ng NGF/g wet weight) were found both in the regions innervated by the magnocellular cholinergic neurons of the basal forebrain (hippocampus, olfactory bulb, neocortex) and in the regions containing the cell bodies of these neurons (septum, nucleus of the diagonal band of Broca, nucleus basalis of Meynert). Comparatively low, but significant NGF levels (0.07-0.21 ng NGF/g wet weight) were found in various other brain regions. $mRNA^{NGF}$ was found in the hippocampus and cortex but not in the septum. This suggests that magnocellular cholinergic neurons of the basal forebrain are supplied with NGF *via* retrograde axonal transport from their fields of innervation. These results, taken together with those of previous studies showing that these neurons are responsive to NGF, support the concept that NGF acts as trophic factor for magnocellular cholinergic neurons.

Key words: enzyme immunoassay/hippocampus/Northern blot/retrograde axonal transport/septum

Introduction

In the peripheral nervous system, nerve growth factor (NGF) acts as a survival and maintenance factor for sympathetic and sensory neurons derived from the neural crest (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). In contrast, it is not yet established whether NGF exerts a similar function in the central nervous system. It has been demonstrated that, unlike peripheral noradrenergic neurons, the catecholaminergic neurons of the central nervous system are not responsive to NGF (Konkol *et al.*, 1978; Schwab *et al.*, 1979; Olson *et al.*, 1979; Dreyfus *et al.*, 1980). However, several lines of evidence suggest a possible function of NGF for magnocellular cholinergic neurons located in the basal forebrain nuclei that give rise to a diffuse projection pattern extending throughout the cerebral cortex (Godfrey *et al.*, 1980; Bigl *et al.*, 1982; McKinney *et al.*, 1983; Fibiger, 1982; Wainer *et al.*, 1984; Cuello and Sofroniew, 1984). NGF is selectively taken up by cholinergic nerve terminals in the neocortex and hippocampus and transported retrogradely to the basal nuclei of the forebrain (Schwab *et al.*, 1979; Seiler and Schwab, 1984), indicating the presence of NGF receptors on these neurons. Lesion of the cholinergic input to the hippocampus leads to the ingrowth of peripheral sympathetic axons into the hippocampus (Loy and Moore, 1977) suggesting that these may be attracted by increased NGF levels in the hippocampus. Further, NGF increases the content of choline acetyltransferase (responsible for synthesis of the transmitter acetylcholine) *in vivo* in septum, hippocampus and cortex of newborn rats and *in vitro* in aggregation cultures of embryonic forebrain and in dissociated cell cultures (Gnahn *et al.*, 1983; Honegger and Lenoir, 1982; Hefti *et al.*, 1985). However, antibodies to NGF neither impaired the survival nor decreased the basal choline acetyltransferase content of central cholinergic neurons (Honegger and Lenoir, 1982; Gnahn *et al.*, 1983; Hefti *et al.*, 1985). Thus it remained unclear whether or not the responsiveness of cholinergic neurons to exogenous NGF reflects a physiological function of NGF in the central nervous system. An essential step towards the resolution of this issue is to determine if NGF is indeed present *in vivo* in the central nervous system.

Therefore we used a sensitive enzyme immunoassay for NGF (Korsching and Thoenen, 1983) and quantitative Northern blot analysis for $mRNA^{NGF}$ (Heumann *et al.*, 1984) to determine the NGF content of various rat brain regions and to examine possible correlations with the magnocellular cholinergic system.

Results

Specificity of the detection method for NGF

In general the NGF determination was performed with the monoclonal antibody 27/21 (Korsching and Thoenen, 1983). To exclude the possibility that this antibody detects a different molecule in brain, that merely shares one epitope with NGF, we also performed the enzyme immunoassay with affinity-purified

Table I. NGF levels in rat central nervous system

Tissue		ng NGF/g wet weight
Hippocampus	Regions innervated by magnocellular cholinergic neurons[a,b]	1.41 ± 0.08
Olfactory bulb		0.40 ± 0.04
Neocortex		0.53 ± 0.08
Septum	Regions containing the cell bodies of magnocellular cholinergic neurons[a,b]	0.51 ± 0.06
Diagnonal band of Broca		0.71 ± 0.11
Nucleuc basalis of Meynert		0.37 ± 0.04
Corpus striatum	Regions with other types of cholinergic neurons[a]	0.08 ± 0.01
Retina		0.07 ± 0.01
Hindbrain + Medulla oblongata		0.07 ± 0.02
Hypothalamus		0.16 ± 0.02
Cerebellum	Regions not containing cholinergic neurons[a]	0.21 ± 0.03
Optic tectum		0.07 ± 0.01

Values given are means ± SEM of at least four independent experiments each involving quadruplicate determinations. The NGF content is corrected for the recovery of added mouse 2.5S NGF (60–90% at an exogenous NGF concentration of 0.125 ng/ml) and expressed as equivalents of mouse 2.5S NGF.

[a]Fibiger, 1982; Wainer *et al.*, 1984, Cuello and Sofroniew, 1984.

[b]Lehmann *et al.*, 1980; Godfrey *et al.*, 1980; Bigl *et al.*, 1982; McKinney *et al.*, 1983.

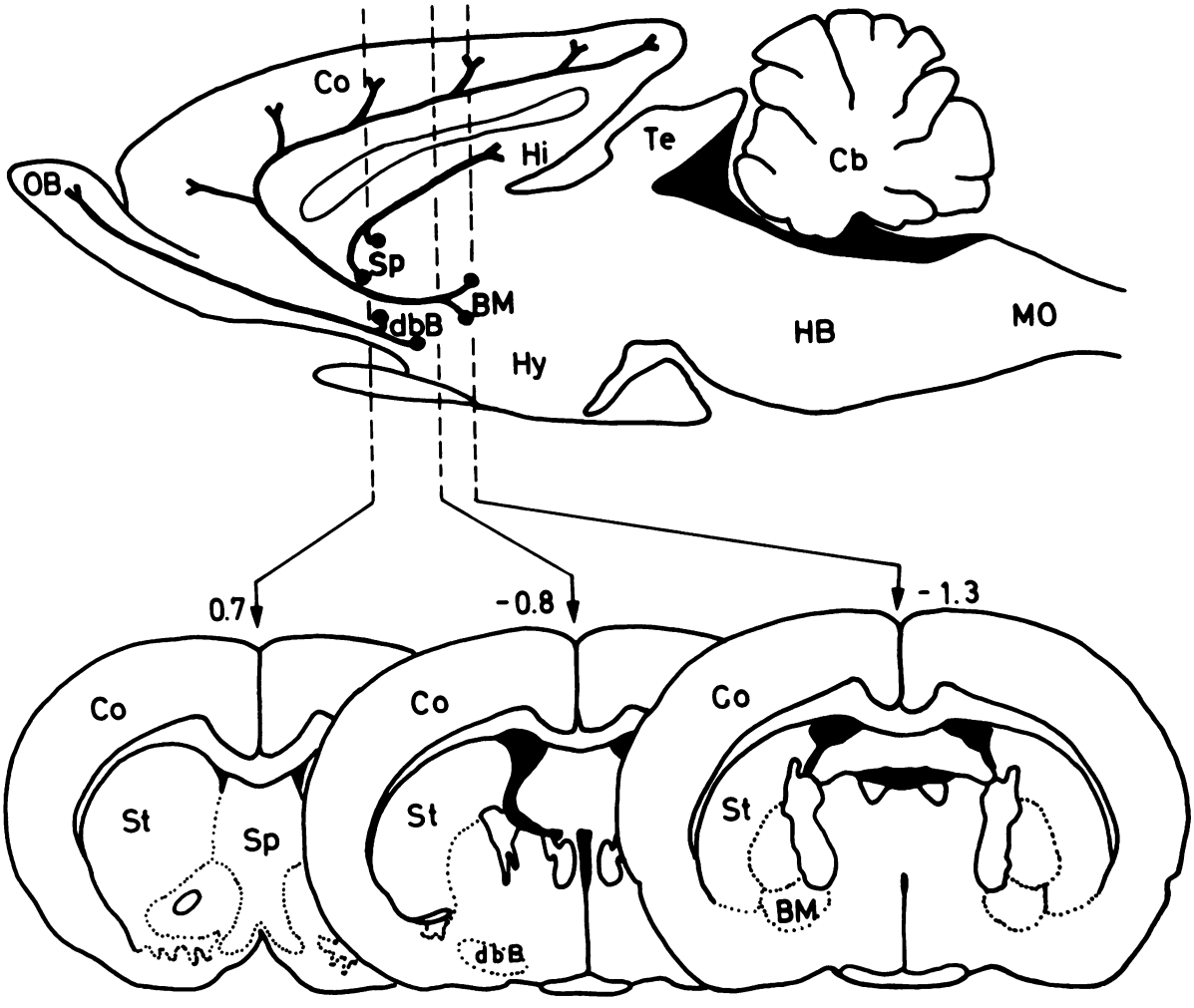

Fig. 1. Schematic representation of the rat brain. Bold lines in the sagittal section (figure top) represent the pathways of the magnocellular cholinergic system originating from the basal forebrain nuclei (modified from Cuello and Sofroniew, 1984). Ventricular space is shown in black. Dashed lines in the transverse sections (after Paxinos and Watson, 1982) indicate the dissected regions. The numbers refer to the distances in mm from the bregma point. BM, nucleus basalis of Meynert; Cb, cerebellum; Co, neocortex; DB, diagonal band of Broca; HB, hindbrain; Hi, hippocampus; Hy, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; Sp, septum; St, corpus striatum; Te, optic tectum.

polyclonal sheep anti-NGF antibodies for determination of the hippocampal NGF content. The value obtained (1.38 ± 0.25 ng NGF/g wet weight) was in good agreement with the result obtained using monoclonal antibodies (Table I), indicating that the monoclonal antibody 27/21 indeed detects NGF in brain tissue.

Influence of dissection time on NGF levels

Since the time needed for dissection varied considerably for the different brain regions, we investigated whether the NGF levels were stable during prolonged dissection times. Pairs of hippocampi were dissected rapidly (<5 min). One hippocampus was frozen immediately on dry ice, whereas the other one was left at room temperature in a wet chamber for 15 – 180 min before freezing. This was several-fold longer than the maximal dissection times needed. No significant decrease in NGF content was observed during that time period (i.e., <10% decrease after 3 h with a confidence coefficient of 0.95). The poly(A)$^+$ mRNA preparations were shown to be stable for at least 2 h at room temperature (Figure 2).

Influence of sympathetic denervation on brain NGF levels

The synthesis of NGF in target tissues of peripheral sympathetic neurons is well established (cf. Thoenen and Barde, 1980; Korsching and Thoenen, 1983; Heumann *et al.*, 1984; Shelton and Reichardt, 1984; Korsching and Thoenen, 1985a). To exclude the possibility that substantial amounts of NGF measured in brain are due to the presence of sympathetically innervated vascular tissue within the brain, we performed a unilateral excision of the superior cervical ganglion (SCG). The sympathetic innervation of the cerebral vasculature originates exclusively from the SCG and does not cross the midline (Edvinsson, 1975). The excision of the SCG should lead to an NGF accumulation in the target tissues it innervates, since removal of NGF by retrograde axonal transport is prevented. This effect has in fact been demonstrated in peripheral target tissues after destruction of sympathetic nerve terminals by 6-OH-dopamine (Korsching and Thoenen, 1985a). Indeed, 3 days after SCG excision, the NGF

Table II. NGF levels within the hippocampus

Region	ng NGF/g wet weight[a]
Dentate gyrus	2.0 ± 0.3
CA3+4	2.0 ± 0.3
CA1+2	0.8 ± 0.2

[a]Values are means ± SEM of three independent determinations. Method as described in the legend of Table I.

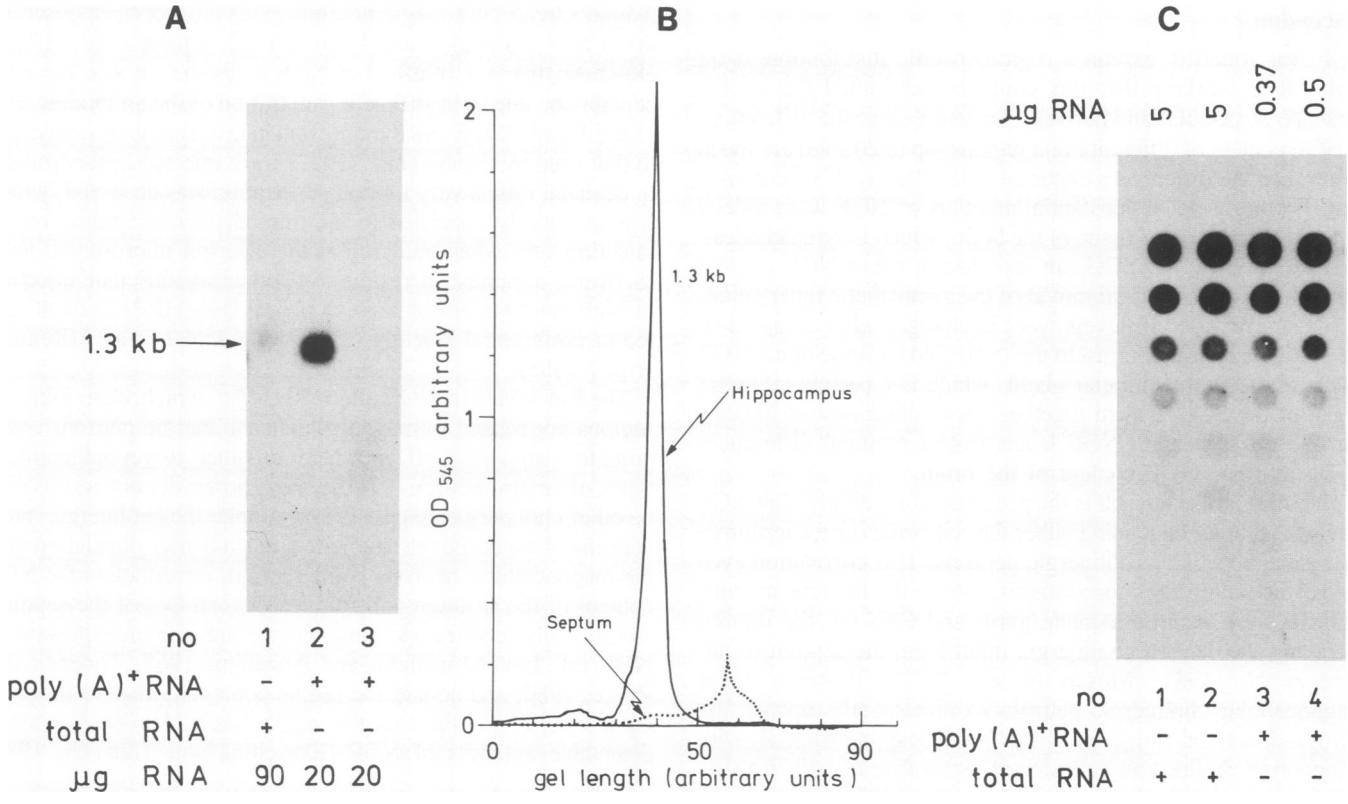

Fig. 2. mRNA^{NGF} in the septo-hippocampal system: Northern blot and poly(A)$^+$ quantification. (**a**) Poly(A)$^+$ RNA preparations of hippocampus (**lane 2**) and septum (**lane 3**) were subjected to quantitative Northern blot analysis as described in Materials and methods. **Lane 1**: total RNA of hippocampus. (**b**) Densitometric analysis of (**a**), **lanes 2** and **3**. (**c**) Dot blot hybridisation of the poly(A)$^+$ and total RNA preparations used in (**a**) using ^{32}P-labeled oligo(dT) as a probe according to Heumann *et al.* (1984). Two-fold dilution steps beginning with the indicated amounts of RNA are shown. The identical intensities of the hippocampal (**lane 3**) and septal (**lane 4**) poly(A)$^+$ RNA preparations demonstrate their identical purity. The poly(A)$^+$ content of total RNA preparations from hippocampus dissected within 7 min (**lane 1**) is undistinguishable from that of hippocampus left for 2 h at room temperature (**lane 2**), indicating a lack of degradation of poly(A)$^+$ mRNA during that time period.

content of the submandibular gland, a target organ of the SCG, increased 14-fold above the contralateral control levels. In contrast, in the same animals, the NGF levels of hippocampus, cerebellum and corpus striatum were unchanged ($p < 0.05$), indicating that NGF both in regions with high and low NGF levels does not originate from vascular (mesodermal) tissue, but from the central nervous system parenchyma.

Regional differences of NGF levels in brain

Hippocampus, olfactory bulb, neocortex, septum, diagonal band of Broca and nucleus basalis of Meynert (Figure 1) contained between 0.4 and 1.4 ng NGF/g wet weight (Table I). Considerably lower amounts of NGF (0.07 – 0.21 ng NGF/g wet weight) were found in several other brain regions, i.e., cerebellum, hindbrain + medulla oblongata, optic tectum, hypothalamus, corpus striatum and retina (Figure 1, Table I). Neocortex and hippocampus were analysed in further detail. Since no difference was found between NGF levels of parietal, occipital, frontal, temporal and cingulate cortex ($p < 0.05$), only the value for the whole cortex is given (Table I). No differences in the NGF content of hippocampal subregions were found when analysed in three segments along the dorsal-ventral axis ($p < 0.05$). However, subdivision of the hippocampus according to the functional regions [dentate gyrus, CA1 to CA4 (Isaacson and Pribram, 1975)] revealed a 2- to 3-fold higher NGF content in dentate gyrus and CA3+4 region as compared with CA1+2 (Table II).

Since not all brain regions were analysed for NGF content, the average value for whole brain was also determined (0.27 ± 0.07 ng NGF/g wet weight). This excludes the possibility that a particular rich source for NGF has been overlooked.

Table III. mRNA^{NGF} levels in the rat central nervous system

Tissue	mRNA^{NGF} [fg/µg poly(A)$^+$] ± SEM	(*n*)
Hippocampus	530 ± 60	(3)
Neocortex	350 ± 70	(3)
Septum	< 80	(3)
Corpus striatum	~ 40[a]	(2)
Cerebellum	~ 80[a]	(2)

[a]NGF-specific bands could only be determined after prolonged exposure times (7 days) of the films.

mRNA^{NGF} content of different brain regions

The mRNA^{NGF} levels were determined by quantitative Northern blot analysis (Heumann *et al.*, 1984). Hippocampus (Figure 2, Table III) and cortex (Table III) contained about as much mRNA^{NGF} as peripheral tissues which are densely innervated by the sympathetic nervous system (see Heumann *et al.*, 1984). In contrast, mRNA^{NGF} levels were >5-fold lower in corpus striatum and cerebellum (Table III). The mRNA^{NGF} levels thus reflect the NGF protein levels (high mRNA^{NGF} levels in regions with high NGF content and very low mRNA^{NGF} in regions with low NGF content), with the exception of regions containing the cell bodies of magnocellular cholinergic neurons (e.g., septum), where mRNA^{NGF} levels were below the detection limit (Figure 2a,b, Table III).

Discussion

NGF was found to exhibit a region-specific distribution in rat brain. It is unlikely that this could be accounted for by the presence of vascular and perivascular tissue since the differences in NGF content of different brain regions (up to 20-fold) are much larger than the differences expected in the density of vascularisation. Furthermore, if substantial amounts of NGF were indeed present in the vascular tissue of the brain, which is sympathetically innervated by the SCG, an increase in brain NGF content would be expected after removal of the sympathetic innervation. However, brain NGF levels were unchanged after removal of the SCG, in distinct contrast to the >10-fold increase of the NGF levels of the submandibular gland, which is a peripheral target organ of the SCG. Taken together, these observations indicate that the differences in NGF levels reflect regional differences in the intrinsic NGF content of the brain.

The most striking result is the presence of relatively high NGF levels in all areas containing either the cell bodies or the terminals of the magnocellular cholinergic neurons. This correlation even holds true within the hippocampus, where the highest amounts of NGF were found in dentate gyrus and CA3+4, the regions receiving the densest cholinergic input from the septum (Crutcher *et al.*, 1981; Mosko *et al.*, 1973). Precisely these magnocellular cholinergic neurons exhibit specific uptake and retrograde axonal transport of NGF and respond to NGF with induction of choline acetyltransferase (Schwab *et al.*, 1979; Seiler and Schwab, 1984; Honegger and Lenoir, 1982; Gnahn *et al.*, 1983; Hefti *et al.*, 1985). The correlation of NGF content with the presence of either magnocellular cholinergic nerve terminals or cell bodies is consistent with the concept that NGF is synthesized in target regions of the magnocellular cholinergic neurons, is taken up by their terminals, and is transported retrogradely to their cell bodies. Further support for this interpretation is derived from the exclusive presence of $mRNA^{NGF}$ in the target regions of magnocellular cholinergic neurons (hippocampus, cortex), but not in the region containing the cell bodies of these neurons (septum). These results indicate that NGF in the septum orginates from retrograde axonal transport rather than from local synthesis, representing a situation analogous to that of the peripheral nervous system, where the predominant NGF supply for sympathetic neurons originates from their target organs, and is not synthesized in the ganglia (Heumann *et al.*, 1984; Korsching and Thoenen, 1985a; cf. Levi-Montalcini *et al.*, 1975).

The NGF and also the $mRNA^{NGF}$ levels found in areas innervated by the magnocellular cholinergic neurons are in the range of those sympathetically innervated peripheral organs (Korsching and Thoenen, 1983; Heumann *et al.*, 1984). However, the NGF levels in the regions containing the cell bodies of these neurons are much lower than those of the sympathetic ganglia. One possible explanation is that, unlike in sympathetic ganglia, the magnocellular cholinergic neurons are only a small subpopulation in the brain regions containing their cell bodies (cf. Eckenstein and Sofroniew, 1983).

The results of both the NGF and $mRNA^{NGF}$ determinations taken together with earlier observations regarding retrograde axonal transport and enzyme induction by NGF (Schwab *et al.*, 1979; Seiler and Schwab, 1984; Gnahn *et al.*, 1983) point to a possible endogenous function of NGF for magnocellular cholinergic neurons of the basal forebrain. In analogy to the peripheral nervous system, where NGF antibodies impair function and/or survival of NGF-responsive neurons, it would then be expected that antibodies to NGF impair the function of magnocellular cholinergic neurons. This has not been observed up to now (Honegger and Lenoir, 1982; Gnahn *et al.*, 1983). However, the lack of effect of NGF antibodies *in vivo* can conceivably be due to insufficient penetration of the antibodies. For example, when IgG-HRP monoconjugates in amounts equivalent to that used by Gnahn *et al.* (1983) were injected intracortically in neonatal rats, a very limited penetration was observed, which was essentially restricted to the immediate surrounding of the injection site, when analysed at the electron microscopic level (Schwab, unpublished results). Also, intraventricular injection of high amounts of horseradish peroxidase (HRP) in adult cats led to preferential labeling of the perivascular spaces (Rennels *et al.*, 1985).

The low levels of NGF and $mRNA^{NGF}$ which were found in regions not related to magnocellular cholinergic neurons could point to a supportive effect of NGF on other, as yet unidentified, types of central neurons. These may, however, not necessarily be other cholinergic neurons; for example, the cholinergic interneurons of the corpus striatum are a much denser population than the magnocellular neurons in the septum, nevertheless the NGF content of the striatum is 6-fold lower than that of the septum. Additionally, cholinergic motoneurons are also unresponsive to NGF (cf. Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980) and neither the region containing their cell bodies (spinal cord) nor their target region (skeletal muscle) contain detectable amounts of NGF (Korsching and Thoenen, 1983, 1985b).

In conclusion, our results substantiate the role of NGF as endogenous trophic factor for magnocellular cholinergic neurons of the central nervous system. These observations are of potential clinical relevance owing to the correlation of the Alzheimer form of presenile dementia with atrophy of magnocellular cholinergic neurons (Whitehouse *et al.*, 1982; Pearson *et al.*, 1983).

Materials and methods

Dissection technique

Wistar rats, weighing 150–200 g, were decapitated and the brain was removed. Hippocampus, neocortex, olfactory bulb, septum, hypothalamus, cerebellum, optic tectum, hindbrain and medulla oblongata were dissected from the whole brain. Transverse slices of the hippocampus were subdivided in dentate gyrus, CA3+4 and CA1+2 (Isaacson and Pribram, 1975) along the visible intersection lines with the aid of a stereo dissection microscope. The region of the diagonal band of Broca (including the nucleus of the diagonal band of Broca), the corpus striatum and the nucleus basalis region were dissected from frontal slices (cuts at −1.3 mm, −0.8 mm and 0.7 mm from the bregma point, Figure 1). The vertical limb of the diagonal band of Broca was included in the septal region. Only the central part of the nucleus basalis region was dissected, since the borders of this area are not well defined in the rat (Lehmann *et al.*, 1980). Dissected tissues were frozen on dry ice and stored at −70°C.

Unilateral sympathetic denervation of the brain

Wistar rats were ether-anaesthetized and the left SCG was excised. The success of the surgical procedure was evaluated by the nearly complete ptosis at the operated side. Three days after surgery, when denervation of the cerebral arteries was complete (Kobayashi *et al.*, 1983), submandibular gland, hippocampus, corpus striatum and cerebellum from the operated and unoperated side were removed and analysed for NGF content.

Determination of NGF content

NGF levels were determined by a previously described two-site enzyme immunoassay with a detection limit of 5 pg NGF/ml corresponding to 0.01 fmol NGF per assay (Korsching and Thoenen, 1983). The test uses the monoclonal anti-NGF antibody 27/21 and affinity-purified polyclonal sheep-anti-NGF antibodies (Korsching and Thoenen, 1983; Stöckel *et al.*, 1976).

Determination of $mRNA^{NGF}$

$mRNA^{NGF}$ was measured using quantitative Northern blot analysis as described recently (Heumann *et al.*, 1984). Briefly, RNA was prepared according to Melera and Rusch (1973), poly(A)$^+$ RNA was isolated with oligo(dT)-cellulose (Aviv and Leder, 1972), digested with DNase I to remove residual DNA (Tullis

and Rubin, 1980), glyoxylated and electrophoresed as described by Thomas (1980). The purity of the poly(A)$^+$ preparation was checked by dot blot hybridisation with ^{32}P-labeled oligo(dT) (Figure 2c) according to Heumann *et al.* (1984). After transfer to nitrocellulose the blots were hybridized for 16–20 h at 65°C in 50% formamide with a single-stranded ^{32}P-labeled RNA-probe (6 x 10^8 d.p.m./μg) derived from the large *PstI* insert of a NGF cDNA clone (Scott *et al.*, 1983). This insert was recloned in the plasmid SP64 (Melton *et al.*, 1984) whose SP6 promoter allows asymmetric transcription of the DNA template. This results in labeled RNA complementary to the mRNA^{NGF} (RNA^{NGF+}). The specificity of this hybridisation method had been shown previously by the absence of hybridisation when RNA-probes of opposite polarity (RNA^{NGF-}) were used (Heumann *et al.*, 1984). Tissue mRNA^{NGF} contents were determined by densitometric scanning of the autoradiograms and expressed as equivalents of *in vitro* synthesized unlabelled RNA^{NGF-}. This standard was included in each gel in several known concentrations.

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