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Trophic Factor Effects on Cholinergic Innervation in the Cerebral Cortex of the Adult Rat Brain

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Abstract

The cholinergic pathway ascending from the nucleus basalis magnocellularis (NBM) to the cortex has been implicated in several important higher brain functions such as learning and memory. Following infarction of the frontoparietal cortical area in the rat, a retrograde atrophy of cholinergic cell bodies and fiber networks occurs in the basalocortical cholinergic system. We have observed that neuronal atrophy in the NBM induced by this lesion can be prevented by intracerebroventricular administration of exogenous nerve growth factor (NGF) or the monosialoganglioside GM_1. In addition, these agents can upregulate levels of cortical choline acetyltransferase (ChAT) activity in the remaining cortex adjacent to the lesion site. Furthermore, an enhancement in cortical high-affinity ^3H-choline uptake and a sustained in vivo release of cortical acetylcholine (ACh) after K^+ stimulation are also observed after the application of neurotrophic agents. Moreover, these biochemical changes in the cortex are accompanied by an anatomical remodeling of cortical ChAT-immunoreactive fibers and their synaptic boutons.

Index Entries: Nerve growth factor (NGF); nerve growth factor receptor (NGFr); monosialoganglioside (GM_1); nucleus basalis magnocellularis (NBM); cholinergic synapses; cortex; microdialysis; acetylcholine (ACh) release; high-affinity choline uptake.

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Introduction

Neurotrophic factors play a fundamental role in the development of the peripheral (PNS) and central nervous systems (CNS). It has been recently demonstrated that in addition to their developmental functions, endogenously produced or exogenously applied neurotrophic agents have pronounced effects in the adult fully differentiated CNS. NGF originally discovered by Rita Levi-Montalcini and coworkers (Levi-Montalcini and Angeletti, 1968), remains the prototype for these responses in the PNS and CNS, and the considerable list of putative neurotrophic agents is continuously growing (for review, *see* Hefti et al., 1989; Maisonpierre et al., 1990).

As discussed below, some of the most dramatic examples of growth factor effects in the adult CNS have been provided by NGF in the injured cholinergic system. Noncholinergic neuronal populations also respond to growth factors. For example, it has been shown recently that ciliary neurotrophic factor (CNTF) can rescue axotomized spinal motoneurons (Oppenheim et al., 1991) and that brain-derived neurotrophic factor (BDNF) can protect embryonic dopaminergic neurons of the *substantia nigra* from the cytotoxic effects of methyl-phenyl-tetrahydro-pyridine (MPTP) (Hyman et al., 1991). NGF cannot substitute for BDNF or CNTF in these cases. However, studies involving the exogenous administration of trophic factors suggest that different sets of CNS neurons, perhaps independent of the type of transmitter(s) produced by them, are susceptible to the trophic actions of endogenously generated factors, thereby favoring the idea that trophic factors might play a role in reparative attempts of the adult CNS. Interestingly, other agents that are not *bona fide* neurotrophic factors, such as gangliosides, also can protect CNS neurons from injury. Gangliosides have been shown to exert a variety of putative trophic effects on neuronal cells both in vitro and in vivo (for review, *see* Skaper et al., 1985; Cuello, 1990).

In this article, we discuss the actions of NGF and the monosialoganglioside GM_1 on the structure and function of the cholinergic presynaptic terminal network in the injured neocortex of the adult rat. We are particularly pleased to be making a contribution in memory of Eduardo P. De Robertis, a most inspiring forerunner and teacher of A. C. Cuello, a member of our group. De Robertis had a particular interest in cholinergic neurotransmission and greatly contributed to the concept of synaptic vesicle storage and release of neurotransmitters (for review of his early work, *see* De Robertis, 1964). We would like to think that he would have been delighted to see the progress made in this field, including the discovery of the remarkable plasticity of central cholinergic neurons.

NGF Receptor Sites in Cholinergic Neurons

Nerve growth factor receptors (NGFr) are presumably synthesized in the neuronal soma and anterogradely transported to nerve terminals; NGFr present in the cholinergic terminal network of the neocortex would bind specifically with NGF, and then the complex is retrogradely transported to the cell soma (Seiler and Schwab, 1984; Taniuchi et al., 1986). The molecular events of NGF's subsequent biological action on the cell remains an issue of intensive investigation. Recently, the high-affinity NGFr has been proposed to comprise two proteins: one, the low-affinity NGFr ($p75^{NGFR}$) and the other, a tyrosine kinase ($p140^{Prototrk}$) encoded by the *trk* proto-oncogene (Kaplan et al., 1991; Hempstead et al., 1991). The monoclonal antibody (MAb) 192-IgG, originally reported by Chandler et al. in 1984 (*see also* Taniuchi and Johnson, 1985), is thought to recognize the low-affinity form of the receptor, although there is some evidence that it may also recognize the high-affinity form *(see below)*. Several laboratories, including our own, have used this antibody to demonstrate the cellular and subcellular localization of these binding sites (Batchelor et al., 1989; Dawbarn et al., 1988; Eckenstein, 1988; Gomez-Pinilla et al., 1987; Kiss et al., 1988; Koh and Loy, 1989; Pioro and Cuello, 1990; Pioro et al., 1988, 1990; Schweitzer, 1987; Sofroniew et al., 1988; Springer et al., 1987; Yan

and Johnson, 1988). Using light microscopy, we have demonstrated that these sites are closely associated with some CNS cholinergic systems and other noncholinergic sites. Figure 1 illustrates this point.

Since cholinergic neurons of the NBM as well as those of the medial septum are particularly responsive to NGF, it was interesting to investigate the subcellular localization of NGFr-immunoreactive (NGFr-IR) sites in this important forebrain neuronal group. Our ultrastructural studies (Pioro et al., 1990) revealed that immunoperoxidase complexes are present in the rough endoplasmatic reticulum (RER), providing unequivocal evidence for local biosynthesis (Fig. 2). Visualization of NGFr-IR in the RER indicates that the epitope recognized by 192-IgG appears very early in the biosynthesis of the receptor. Some of the aggregates of immunoreactive vesicles may represent secondary lysosomes where NGFr is catabolized, whereas others, e.g., in dendrites, may be packaged NGFr's traveling to or from the cell membrane. The patches of more intense NGFr-IR along the cell membrane may be the result of receptor clustering that usually occurs in coated pits prior to internalization (for review, *see* Brodsky, 1988; Goldstein et al., 1979). The fact that 192-IgG recognizes a site that does not interfere with the binding of NGF at least in vitro, permits the possibility of NGF being complexed with the immunolabeled receptor. Our ultrastructural evidence of NGFr internalization indicates that 192-IgG recognizes an epitope that is also present in the high-affinity form of the receptor because low-affinity NGFr apparently is not internalized.

Morphological, Biochemical, and Functional Plasticity of Cholinergic Neurons

Anatomical and Biochemical Changes

Following infarction of the frontoparietal cortical area in the rat, retrograde atrophy of cholinergic cell bodies and fiber networks occurs in the basalocortical cholinergic system. Immunocytochemical analysis revealed that ChAT-immunoreactive cells are still visible in the NBM even 120 d after the cortical devascularizing lesion, but are in a shrunken state with retracted dendritic profiles (Sofroniew et al., 1983; Stephens et al., 1985). This lesion (Fig. 3) involves extensive disruption of the pia mater, resulting in a cortical infarct and gradual atrophy of ascending NBM cholinergic fibers, thus differing from the axotomy technique used for the septal-hippocampal pathway. It has been used conveniently to investigate secondary retrograde changes affecting NBM cholinergic neurons when deprived of neuronal targets and their terminal networks.

Several studies have provided evidence for the protective and preventative effects of putative trophic factors at biochemical and morphological levels in this model (Cuello et al., 1989; Cuello, 1990; Maysinger et al., 1990a). More specifically, administration of the monosialoganglioside GM_1, NGF, or a combination of both administered intracerebroventricularly shortly after lesion prevents the reduction in ChAT enzymatic activity and cholinergic cell shrinkage in the NBM. Even more striking effects were observed in the remaining ipsilateral cortex adjacent to the lesion site where an increase above control values in ChAT enzymatic activity was noted following administration of trophic factors. This suggests that either hyperactivity of the remaining cortical cholinergic terminals occurs as a compensatory mechanism or possibly, that presynaptic terminals are altered morphologically. Both subthreshold and effective concentrations of the ganglioside GM_1, when applied in combination with NGF, have supramaximal effects on ChAT activity (Cuello et al., 1989; Garofalo and Cuello, 1990). Cortical ChAT activity largely represents the biosynthetic activity of cholinergic synaptic boutons of fibers ascending from the basal forebrain. Indeed, Garofalo and colleagues (1990) demonstrated, using electron and light microscopic quantitative techniques, that an increase in both cortical ChAT-immunolabeled varicosities and fiber density occurs in lesioned animals treated with GM_1 and NGF simultaneously (Fig. 4a,b).

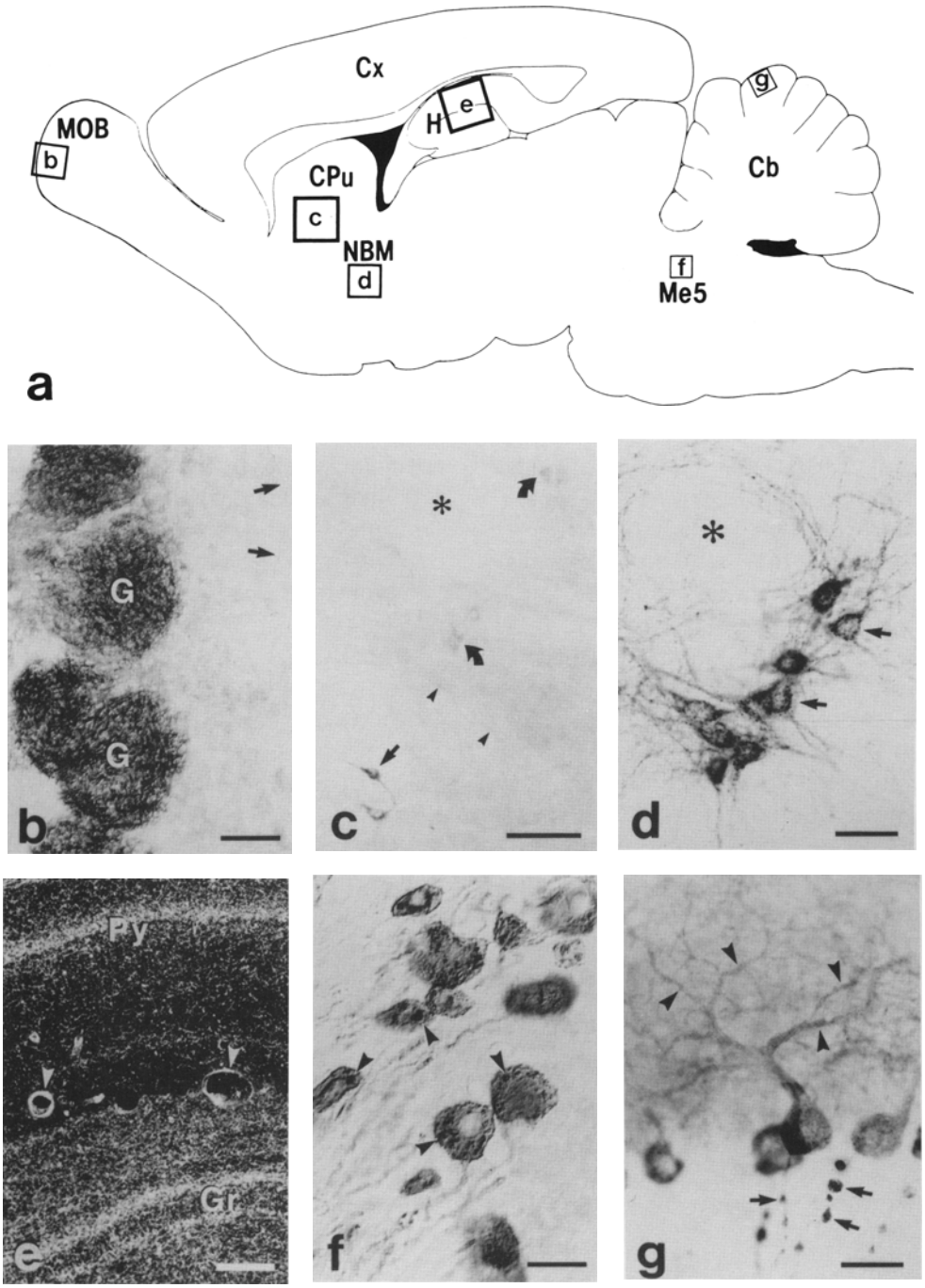


Fig. 1. Location and LM appearance of NGFr-IR neurons and fibers in the rat CNS. **A:** Schematic representation of sagittally sectioned rat brain, indicating sites of photomicrographs of NGFr-IR neurons and fibers (b–g). **B:** Low-magnification appearance of dense NGFr-IR fiber plexes in the glomerular layer of the main olfactory bulb. G = glomerulus (arrow points to a long-stained fiber within the external plexiform layer). **C:** High-magnification view of scattered NGFr-positive neurons in the caudate putamen (cp); asterisk indicates bundles of unstained myelinated fibers with individual NGFr-IR area (arrowhead). Arrows show NGFr-IR neurons in ventral caudate putamen and more faintly stained ones in the dorsal cp. **D:** Aggregates of NGFr-containing magnocellular neurons of the NBM (arrows); asterisks indicate fibers of the internal capsule. **E:** Darkfield micrograph of NGF-IR fiber plexes in the hippocampal complex. **f:** Densely stained unipolar neurons of the mesencephalic trigeminal nucleus containing prominent aggregates of NGFr reaction products in perikarya (arrowheads). **g:** High-magnification view of sagittally sectioned cerebellum from a colchicine-treated animal. Arrowheads indicate immunoreactive dendrites of the Purkinje neurons, and arrows point to proximal axons. (Cuello et al., 1990, with permission.)

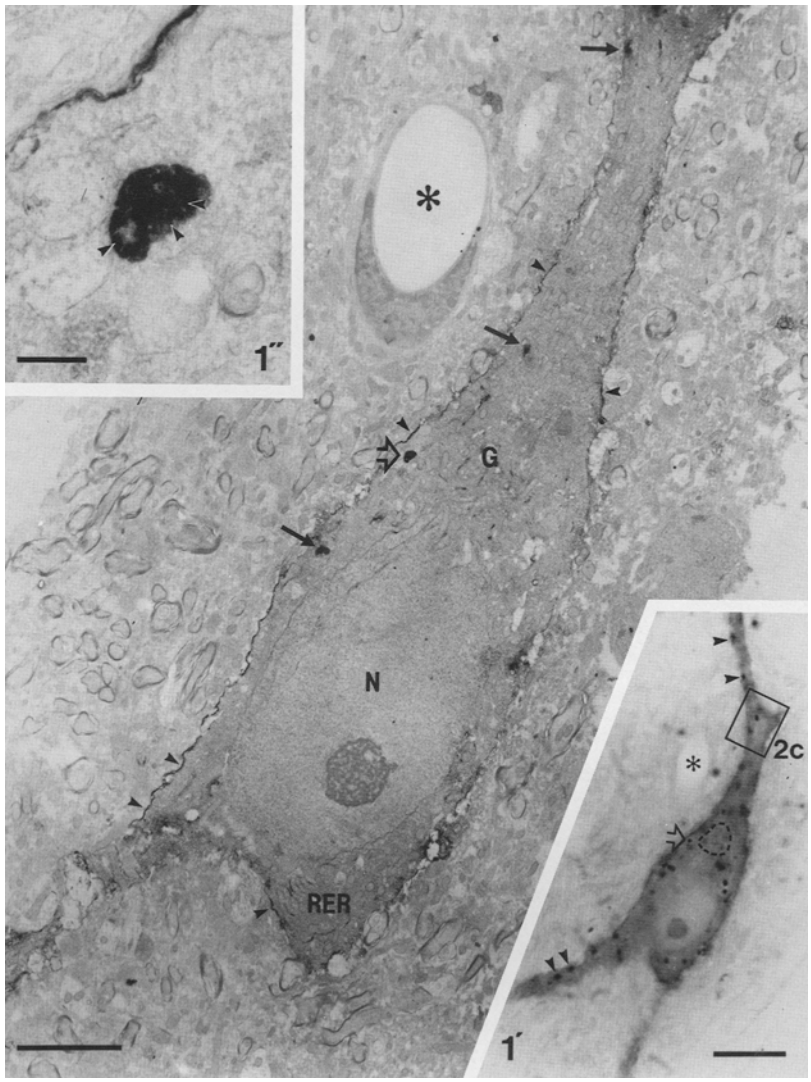


Fig. 2. Low-magnification EM of an NGFr-IR neuron in the NBM of an adult rat brain. Inset I shows the LM appearance of this neuron in an Epon-embedded semithin section. Asterisks indicate the lumen of the same blood vessel. Prominent NGFr-IR is present in a patchy distribution along the cell membrane (arrowheads). Segments of RER and Golgi bodies (G) also contain reaction product. No specific NGFr-IR is seen in the nucleus (N). 2C indicates distal primary dendrites. 1" high-magnification of NGFr-IR granule. Scale bar for low-magnification EM = 4 μm. Inset: 1' = 10 μm. Inset 1" = 0.3 μm. (Cuello et al., 1990, with permission).

Functional Changes

Although trophic factor administration to rats with either cortical or NBM lesions can improve biochemical and morphological parameters, a necessary question to address is whether these changes correlate with relevant physiological functions. Marked morphological changes induced by NGF and GM_1 are accompanied by an increase in high-affinity choline uptake (HACU) over control values in isolated cortical synaptosomes from 30-d postlesion rats. The increments produced by each factor separately (22 and 30%, respectively) were inferior to those noted after combined GM_1 and NGF treatment

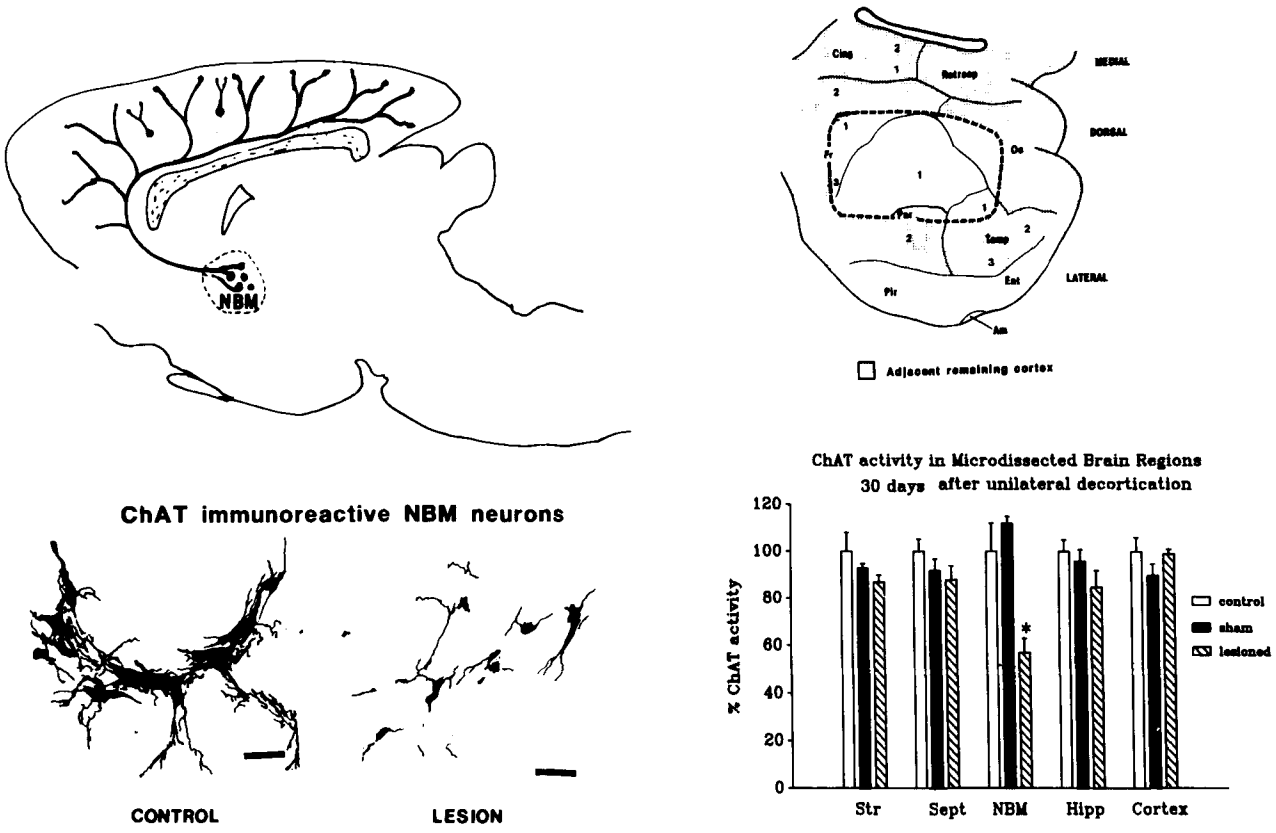


Fig. 3. Schematic representation of the cortical lesion. To left: Sagittal rat brain section illustrating the NBM-to-cortex cholinergic pathway and cortical cholinergic interneurons. Top right: The unshaded area within the dashed rectangle represents the brain regions affected by the lesion. These include frontal (fr) 1, and 3, parietal (par) 1, and portions of parietal 2 and occipital (oc) neocortex. The shaded areas indicate brain regions unaffected by the cortical infarction. Cing: cingulate cortex; Retrosp, retrosplenial cortex; Temp, temporal cortex; Pir, piriform cortex; Ent, entorhinal cortex; and Am, amygdaloid body. Bottom left: Schematic appearance of ChAT-IR neurons in the NBM of control unoperated (right) and lesioned animals (left) 30 d postsurgery. Scale bar: 50 μm. Bottom right: Effect of the cortical lesion on ChAT activity in various brain areas. Str, striatum; sept, septum; NBM, nucleus basalis magnocellularis; and hipp, hippocampus. $^{*}p < 0.01$ from control or sham NBM; ANOVA and posthoc Newman-Keul's test.

(66%) (Garofalo and Cuello, 1989). These results are illustrated in Fig. 5, and the findings complement those of DiPatre and collaborators (1989), who showed that treatment with NGF and GM_1 improved cortical choline uptake following NBM lesions.

Further evidence that the morphological and biochemical changes occurring in the remaining ipsilateral cortex of animals treated with GM_1 and NGF may be functionally significant is provided by data from in vivo neurotransmitter release studies in fully awake and free-moving animals. Cortical ACh release can be considered indicative of the activity of cholinergic fibers ascending to the cortex. The release of ACh is affected by changes in physiological states and also by a variety of selective drug treatments (Pepeu, 1983).

Cortical ACh output can be modulated either via presynaptic receptors located on cholinergic nerve endings or by altering cholinergic neuronal activity in the NBM. An example of the latter case was provided by Casamenti and coworkers (1986), who by using the cortical cup technique, demonstrated that electrical stimulation of the NBM results in an ipsilateral increase of ACh release from the frontoparietal areas of the cor-

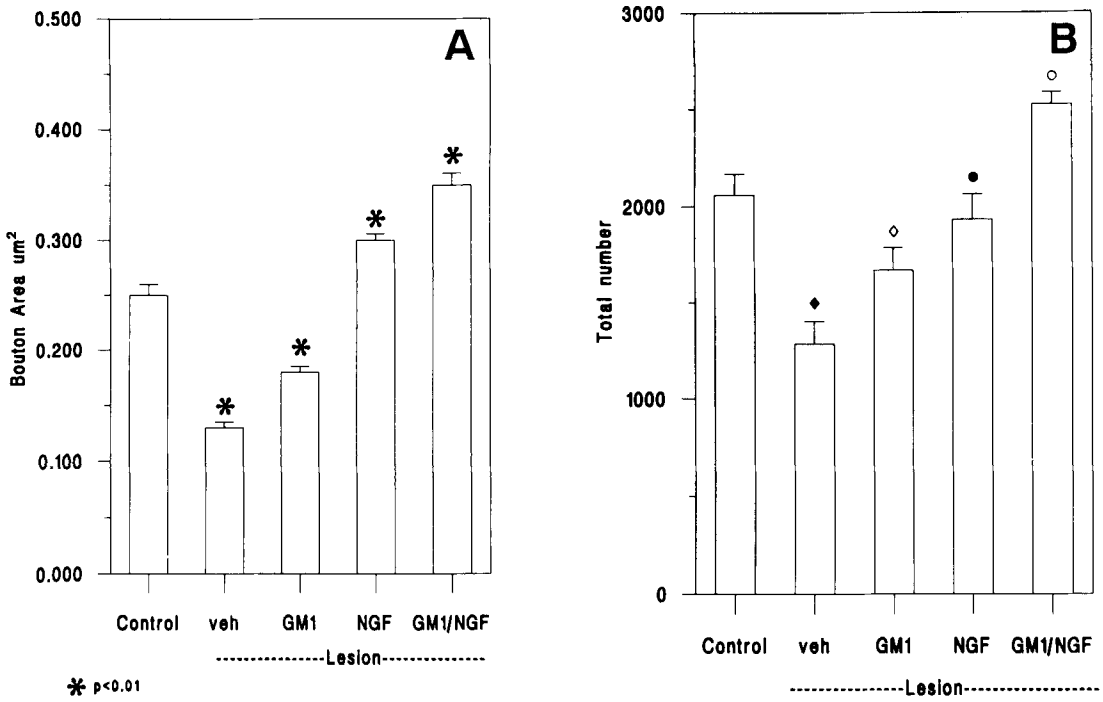


Fig. 4. Morphometric features of cortical cholinergic terminals. **A:** ChAT-IR bouton surface area (expressed in μm^2) in cortical layer V of nonlesioned control animals (control) and 30 d postlesion vehicle (veh: artificial CSF + 0.1% BSA), GM_1 (5 mg/d), NGF (12 μg/d), or GM_1/NGF-treated rats. Animals received vehicle or these factors intracerebroventricularly, via minipump, for 7 d beginning immediately postlesion. They were then sacrificed 30 d postlesion (i.e., 23 d after termination of drug administration), and the brains were processed for EM immunocytochemistry. Ultrathin brain sections were obtained, collected on Formvar-coated one slot grids, and observed noncounterstained with a Philips 410 EM. ChAT-IR boutons observed in two grids from separate blocks of each animal were recorded onto video tape and then transferred to an image analysis system (Quantimet 920) where they were automatically detected and measured with the aid of an operator blinded to the treatment groups. Eighty boutons were quantified per group $^*p < 0.01$ from control, ANOVA, and posthoc Newman-Keul's test. **B:** Cortical ChAT-IR fiber number. Four 50 μm thick coronal brain sections at the level of the midbasalis were used for fiber quantification. ChAT-IR profiles were counted with the aid of an image analysis system (Quantimet 920) in all layers of cortex adjacent to the lesion site. veh = vehicle; GM_1, NGF, or GM_1/NGF (*see* **A** for details). ◆, $p < 0.01$ from control, lesion + NGF, and lesion + GM_1/NGF-treated groups; $p < 0.05$ from GM_1-treated group. ◊, $p < 0.01$ from control, and $p < 0.05$ from lesion + vehicle-treated group. ●, $p < 0.01$ from lesion + vehicle, and lesion + GM_1/NGF-treated groups. ○, $p < 0.01$ from control, lesion + vehicle, lesion + GM_1, and lesion + NGF-treated groups.

tex. This increase in ACh output (approx 40% on average) was followed by activation of the electrocorticogram. In a series of studies, we used in vivo microdialysis (Ungerstedt, 1984; Benveniste, 1989) to study the effects of cortical devascularizing lesions and subsequent treatment with putative neurotrophic agents on basal and stimulated ACh release. A microdialysis probe was implanted diagonally into the anterior portion of the left sensorimotor cortex at a 40° angle. A continuous flow of artificial cerebrospinal fluid (CSF) was delivered (2 μL/min); fractions were collected at 20-min intervals and concentrations of ACh were determined using HPLC (Maysinger et al., 1988). Under basal conditions, extracellular cortical ACh concentrations were in the 29–31 n*M* range in intact, saline-treated controls, and only insignificantly lower in lesioned animals, microdialyzed 30 d following lesion (Fig. 6). Interestingly, the putative trophic agent GM_1, administered either locally via biodegradable microspheres or centrally (i.e., intracerebroventricularly) via osmotic minipumps (Alzet 2001), produced an outflow of ACh

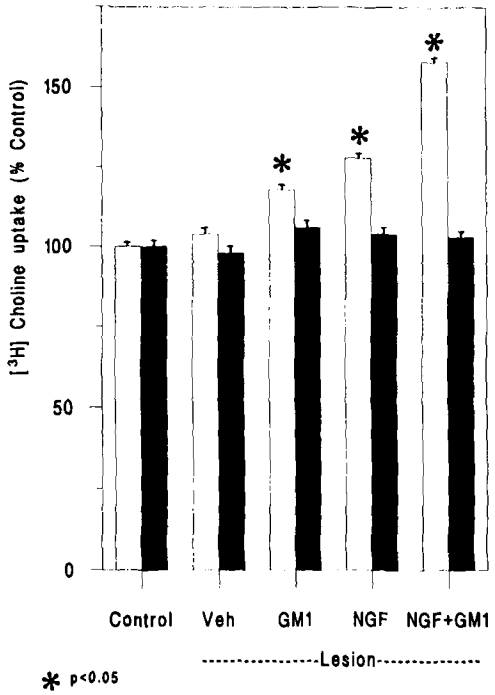
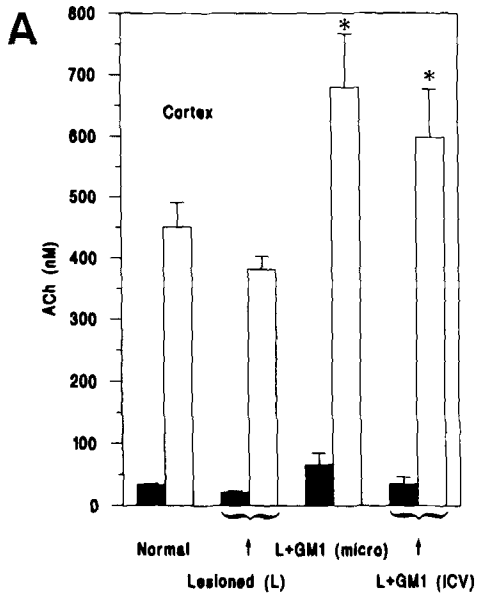


Fig. 5. High-affinity choline uptake (HACU) in the ipsilateral remaining cortex of adult rats, following unilateral cortical devascularizing lesion. HACU was determined in synaptosomal preparations (Simon et al., 1976) in four groups of animals: Veh = lesioned receiving (icv) vehicle alone; NGF (12 μg/d, 7 d); GM_1 (5 mg/d, 7 d), or both agents in combination (NGF + GM_1) at the above doses, intracerebroventricularly via minipump. $^*p < 0.05$ difference from the control nonlesioned animals. □, Ipsilateral CTX; ■, Contra-lateral CTX.



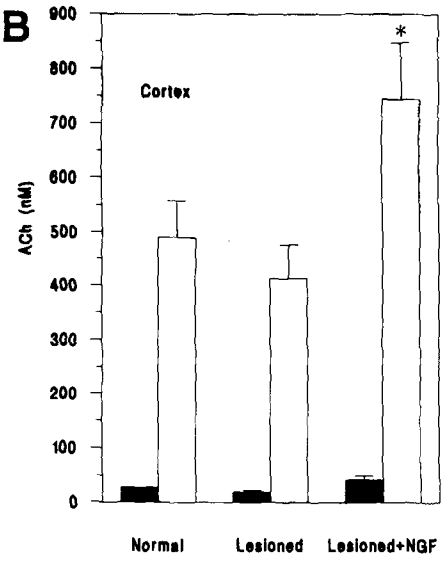


Fig. 6. Release of ACh in vivo. **A:** Extracellular concentrations of ACh, as determined by HPLC from the rat cortex in nonlesioned (normal) and lesioned animals treated with GM_1 [icv and microencapsulated (micro)]. $^*p < 0.05$ difference from the lesioned, vehicle-treated, and nonlesioned control animals (ANOVA and posthoc Newman Keul's test). **B:** Extracellular concentrations of ACh in vivo from the rat cortex in nonlesioned (normal) and lesioned, NGF-treated animals. Black bars = basal conditions (artificial CSF + 10 μ*M* neostigmine). Open bars = stimulated conditions (artificial csf + 10 μ*M* neostigmine + 100 μ*M* KCl). $^*p < 0.05$ difference from the lesioned, vehicle-treated, and nonlesioned animals. ■, Basal; □, Stimulated.

from the adjacent cortex of lesioned animals that was greater than that observed in intact control animals and that was sustained for a prolonged period of time (Fig. 6A) (Maysinger et al., 1990a).

Similarly, NGF administered into the lateral ventricle for 1 wk augmented extracellular ACh above control levels, following both stimulating and nonstimulating conditions (Fig. 6B) (Maysinger et al., 1990b). The decrease in ACh release observed in cortically lesioned vehicle-treated rats might represent an impairment of intrinsic and extrinsic cortical cholinergic systems. Intrinsic cholinergic neurons are spread throughout several layers of the cortex and account for approx 30% of total cortical ACh (Johnston et al., 1981). However, we interpret the

decrease in ACh in cortices of lesioned animals to be, for the most part, the result of an impairment of the extrinsic cholinergic innervation to the cortex. Mainly because the remaining ipsilateral cortex received no direct insult, ChAT-positive neurons localized in the NBM ipsilateral to the lesion side are atrophied and biochemical cholinergic deficits are also detected in the microdissected NBM. The fact that GM_1 or NGF treatment enhanced cortical ACh output concurs with the finding that there are NGFr sites in ascending cholinergic fibers in the rat neocortex (Pioro and Cuello, 1990) and that GM_1 can facilitate trophic factor-mediated effects (Vantini et al., 1988; Cuello et al., 1989).

Conclusions

Our experimental data on the cortical lesion model presented here, along with results obtained in several other laboratories using the septohippocampal transection paradigm (Hefti et al., 1986; Williams et al., 1986; Kromer, 1987), demonstrate that exogenous NGF can affect the morphology of cholinergic cell bodies. We are now providing the first, direct morphological evidence that NGF can significantly alter the size of presynaptic cortical cholinergic terminals and suggest the occurrence of significant changes in the number of ChAT-immunoreactive boutons and synapses. These changes correlate with improved cholinergic function substantiated by the increase in HACU and in vivo ACh release noted in these animals. Although the precise mechanism(s) of action of GM_1 and NGF remains unknown, the ability of these agents to alleviate biochemical and morphological cholinergic deficits following brain injury and to induce presynaptic remodeling of cortical cholinergic elements lends support to the view that functional recovery in the CNS of adult animals might be achieved through prolonged and controlled administration of neurotrophic agents. This notion is strengthened by behavioral studies that show an improvement in the performance of trophic factor-treated cortically lesioned rats (Garofalo and Cuello, 1990; Elliot et al., 1989). In summary, the ability of NGF to mediate synaptic plasticity in the adult and fully differentiated CNS demonstrates that neuronal connections can be potentially induced and exogenously manipulated, thus opening new avenues for regeneration studies involving the mature CNS.

Acknowledgments

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