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Neuronal responses to brain injury: effects of nerve growth factor and acidic fibroblast growth factor on morphological changes and gene expression

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Dedicated to:

The 21st century author(s) of "Treatment and Cure of Cancer" and to my lovely and brave sister Maria Gorett Cavalcante de Figueiredo Silveira.

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CONTRIBUTIONS OF CO-AUTHORS

STATEMENT OF CONTRIBUTION

All ideas which resulted in the experimental work of this thesis were either mine (chapter VI, appendices A and B), my supervisor's (chapters II and III) or from both of us (chapters IV and V). I was responsible for the initial preparation of all manuscripts. With exception of the manuscript of chapter II, all the manuscripts were mainly the result of my own work in the organization and interpretation of data. With exception of processing of total RNA for PCR and the cortical tissue for measurement of NGF protein, development of hybridized probes in *in situ* hybridization experiments, measurement of ChAT activity as well as image analysis described in chapter II, all other experiments were mainly processed by myself.

Dr. Malgorzata Skup was the first author of the manuscript entitled "Intraventricular application of BDNF and NT-3 failed to protect NBM cholinergic neurons" (Chapter 2). Dr. Skup's principal contributions were the image analysis of the collected data and the manuscript's final editing. She also shared the responsibility of performing surgeries and preparing tissues for immunocytochemistry.

Dr. Pedro Piccardo was the second author of the manuscript entitled "Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in spatial memory task following cortical devascularization" (Chapter 3). Dr. Piccardo's main contribution was his assistance with the preparation of septal cell culture.

Dr. Dusica Maysinger was the third author of the manuscript entitled "Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in spatial memory task following cortical devascularization" (Chapter 3). Dr. Maysinger's contribution was the measurement of cortical choline acetyltransferase enzymatic assays.

Dr. Paul Clarke was the fourth author of the manuscript entitled "Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in spatial memory task following cortical devascularization" (Chapter 3). Dr. Clarke's contribution was the supervision of the behavioral experiments' blind observer, Ms. Laura Fernandes.

Dr. Kathleen Plüss was the second author of the manuscript entitled "Neocortical NGF and its mRNA are induced *in vivo* by acidic FGF" (Chapter 4). Under the supervision of Dr. Uwe Otten, the fourth author, Dr. Plüss processed the total RNA for PCR and the cortical tissue for the measurement of NGF protein.

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Mrs. Annie Bedard was the third author of the manuscript entitled "Differential expression of $p75^{NGFR}$ and GAP-43 genes in nucleus basalis, thalamus and adjacent cortex following neocortical infarction and NGF treatment" (Chapter 5). Mrs. Bedard's contribution was the labelling of the oligonucleotide, the preparation of solutions, and the development of hybridized probes.

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Dr. Dusica Maysinger was the fifth author of the manuscript entitled "Effects of perinatal hypo- and hyperthyroidism on the levels of nerve growth factor and its low-affinity receptor in cerebellum" (Appendix A). Dr. Maysinger's contribution was the measurement of the second section of the NGF concentration in cerebellum.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
aCSF	Artificial cerebrospinal fluid
$ACTH^{1-24}$	Proopiocortin-derived peptide
AD	Alzheimer's disease
aFGF	Acidic fibroblast growth factor
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CaM	Calmodulin
ChAT	Choline acetyltransferase
CNS	Central nervous system
DAB	Diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
dp-GAP-43	Dephosphorylated GAP-43
E	Embryonic day
ELISA	Enzyme linked immunoassay
FG	FluoroGold
FGF	Fibroblast (or heparin-binding) growth factor
FGFR	FGF receptor
GAP-43	Growth-associated phosphoprotein-43
GFAP	Glial fibrillary acidic protein
HAChU	High-affinity choline uptake
HRP	Horseradish peroxidase
HSPG	Heparan sulphate proteoglycans
IgG	Immunoglobulin G
IP_3	Inositol-1-4-5-triphosphate
IR	Immunoreactivity
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCID	Microcomputer imaging device
MS/VB	Medial septum / ventral limb of the diagonal band
NBM	Nucleus basalis magnocellularis
nbMt	Nucleus basalis of Meynert
NGF	Nerve growth factor
NT-3	Neurotrophin-3
NTF	Neurotrophic factors
p-GAP-43	Phosphorylated GAP-43
$p75^{NGFR}$	low-affinity NGF receptor
PAP	Peroxidase-antiperoxidase
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PBS-T	PBS plus 0.1% Triton X-100
PIP	Phosphatidylinositol-4-phosphate
PIP_2	Phosphatidylinositol-4-5 biphosphate
PKC	Protein kinase C

PLP	Myelin Proteolipid protein
PNS	Peripheral nervous system
PO	Postnatal day 0
PTU	Propylthiouracil
TR	Thyroid hormone receptor
trk	Tropomyosin receptor kinase
trkA	High-affinity tyrosine kinase receptor for NGF
trkB	High-affinity tyrosine kinase receptor for BDNF
trkC	High-affinity tyrosine kinase receptor for NT-3
PCR	Polymerase chain reaction

ABSTRACT

NEURONAL RESPONSES TO BRAIN INJURY: EFFECTS OF NERVE GROWTH FACTOR AND ACIDIC FIBROBLAST GROWTH FACTOR ON MORPHOLOGICAL CHANGES AND GENE EXPRESSION

The major goal in the work of this thesis was to identify neurotrophic factors (NTFs) capable of rescuing atrophied nucleus basalis magnocellularis (NBM) neurons. Emphasis was placed on the atrophy caused by surgical neocortical infarction, and its recovery, defined by the size of choline acetyltransferase (ChAT)-immunopositive neurons. The selection of pharmacological agents used in this study was based either on their similarity to nerve growth factor, which provides an efficient protection to NBM cholinergic neurons, or to basic fibroblast growth factor (bFGF), which has been shown to prevent cell death of septal cholinergic neurons. Two neurotrophins, brain-derived neurotrophic factor and neurotrophin-3, failed to rescue NBM cholinergic neurons, while a similar dose of acidic fibroblast growth factor (aFGF) was able to rescue them. Notably, lesioned NBM neurons treated with aFGF displayed somata size, as well as neuritic processes (dendrites and axons) and ChAT activity in the NBM similar to sham-operated animals. NBM cholinergic degeneration was consistent with the observed memory deficits, which was also prevented by aFGF treatment. The possibility of a direct effect of aFGF in NBM neurons remains obscure. However, our *in vivo* demonstration of aFGF-induced increase of NGF and its mRNA, in the neocortex of adult animals, entirely supports an indirect mechanism mediating aFGF effects. As endogenous NGF seemed to be responsible for these aFGF effects, we decided to further investigate other properties of NGF in these neurons. One goal was to analyze the effects of neocortical infarction and NGF treatment on low-affinity NGF receptor ($p75^{NGFR}$) and growth-associated phosphoprotein-43 (GAP-43) mRNAs in NBM neurons. The increase of endogenous NGF in lesioned animals was consistent with the transient increase in the expression of $p75^{NGFR}$ mRNA, which was most pronounced after the exogenous application of NGF. Although GAP-43 plays an important role in neuronal regeneration and responded to cortical lesion with enhanced expression in NBM and pyramidal neurons, its expression was unaffected by NGF treatment. On the seventh post-lesion day, at the time when GAP-43 mRNA expression was increased in NBM and pyramidal neurons, which were displaying normal cell size, GAP-43 expression was

dramatically reduced in thalamic neurons which were undergoing an accentuated cell death process. Atrophy of NBM cholinergic neurons, as a consequence of thyroid hormone deprivation during and after brain development, has been described in neonatal and young adults. Adult animals displaying such atrophy did not reveal alteration in the mRNA expression of $p75^{NGFR}$ and trkA, the high-affinity NGF receptor. These neurons became further atrophic following cortical infarction but not when they were surgically lesioned and treated with NGF. However, NGF did not increase the size of post-hypothyroid neurons with cortical lesion to that of sham-operated or NGF-treated and lesioned euthyroid animals. The neuronal size acquired three weeks after NGF treatment and cortical devascularization is approximately the size we found when post-hypothyroid and euthyroid animals were 60 days old (i.e., 15 days before lesion and NGF treatment). This suggests that NGF is not capable of reversing neuronal phenotype in post-hypothyroid rats to that of normally differentiated animals. Results from this and other studies indicate that a cascade of events (e.g., increase of inflammatory cytokines, immediate-early genes, FGFs, etc.) are activated in response to brain injury, leading to an increase of NGF and other putative neurotrophic factors. These newly produced neurotrophic factors may induce some neuronal changes but they do not reach a sufficient level to promote neuronal regeneration of NBM neurons.

RÉSUMÉ

RÉPONSES NEURONALES À UNE LÉSION CÉRÉBRALE: LES EFFETS DU FACTEUR DE CROISSANCE NEURONAL ET DU FACTEUR DE CROISSANCE ACIDE DES FIBROBLASTES SUR LES CHANGEMENTS MORPHOLOGIQUES ET L'EXPRESSION DE CERTAINS GÈNES

L'objectif premier de cette thèse était d'identifier les facteurs neurotrophiques (FNTs) capables de sauvegarder les neurones atrophiés du noyau basal magnocellulaire (NBM). Nous nous sommes concentré sur l'atrophie neuronale causée par un infarctus néocortical induit chirurgicalement et sur la récupération qui s'en suit. Nous avons quantifié l'atrophie à partir de la taille des neurones immunopositifs à la choline acétyltransférase (ChAT). Nous avons choisi d'utiliser certains agents pharmacologiques sur la base de leur ressemblance avec le facteur de croissance neuronal (FCN), qui procure une protection efficace des neurones cholinergiques du NBM, et de leur ressemblance avec le facteur de croissance basique des fibroblastes (FCFb), qui prévient la mort des neurones cholinergiques du septum. Deux neurotrophines, le facteur neurotrophique dérivé du cerveau et la neurotrophine-3 n'ont pas permis de sauvegarder les neurones cholinergiques du NBM. Cependant, une dose équivalente du facteur de croissance acide des fibroblastes (FCFa) a permis de sauvegarder ces neurones. Notamment, chez des animaux lésés, mais traités avec du FCFa, le NBM était comparable à celui d'individus contrôles quant à la taille des corps cellulaire et des protubérances cellulaires (dendrites et axones) et quant à l'activité de la ChAT. La dégénérescence des cellules cholinergiques du NBM concorde avec la présence de déficits de mémoire. Or, le traitement au FCFa aide également à prévenir le développement de tels déficits. La question demeure de savoir si le FCFa affecte directement ou non les neurones du NBM. Nous avons démontré, *in vivo*, une augmentation de FCN de même que de son ARNm dans le néocortex d'individus adultes suite à l'administration de FCFa. Cette observation concorde entièrement avec l'idée d'un mécanisme d'action indirecte du FCFa sur les neurones du NBM. Comme le FCN endogène semblait être responsable de ces effets, nous avons décidé d'étudier plus en profondeur les propriétés du FCN chez ces neurones. Nous avons donc analysé les effets d'un infarctus nécortical et d'un traitement au FCN sur les niveaux de récepteurs à faible affinité du FCN ($p75^{RFCN}$) et les niveaux d'ARNm de la phosphoprotéine-43 associée à la croissance (PAC-43) chez les

neurones du NBM. L'augmentation de FCN endogène chez les animaux lésés concordait avec une augmentation passagère de l'expression d'ARNm du $p75^{RFCN}$. Cette augmentation étaient maximale après une administration de FCN exogène. Bien que la PAC-43 joue un rôle important au niveau de la régénération neuronale, et bien que son niveau d'expression ait augmenté chez les neurones pyramidaux et du NBM suite à une lésion corticale, sont expression demeura inchangée suite à un traitement au FCN. Sept jours après la lésion, alors que l'expression du PAC-43 avaient augmenté dans les neurones pyramidaux et du NBM, elle était dramatiquement réduite dans le thalamus où l'on retrouvait un processus accéléré de mort cellulaire. On a déjà décrit une atrophie des neurones cholinergiques du NBM suite à une privation d'hormone thyroïdienne au cours du développement cérébral chez le jeune et l'adulte. Dans notre cas, chez des animaux adultes qui montraient une telle atrophie neuronale, nous n'avons pas trouvé de changement de niveau d'expression d'ARNm du $p75^{RFCN}$, ni du récepteurs au FCN à haute affinité, le trkA. Nous avons observé une atrophie encore plus prononcée de ces neurones suite à un infarctus cortical, sauf lors d'un traitement au FCN. Cependant, un traitement au FCN suite à une lésion corticale n'a pas eu pour conséquence d'augmenter la taille des neurones des rats *posthypothyroïdiens* à un degré comparable à celle des neurones des *euthyroïdiens*. La taille des neurones atteinte après trois semaines de traitement au FCN suite à une dévascularisation corticale était approximativement la même que chez les animaux *posthypothyroïdiens* et *euthyroïdiens* 15 jours avant la lésion (âge: 60 jours). Cela suggère que le FCN n'est pas capable de restaurer le phénotype normal chez les rats *posthypothyroïdiens*. Nos résultats, ainsi que ceux d'autres études, indiquent qu'une cascade d'événements (par ex.: augmentation de cytokines inflammatoires, gènes prompts-immédiats, FCFs, etc.) est déclenchée suite à une lésion cérébrale, entraînant une augmentation des niveaux de FCN et d'autres facteurs neurotrophiques potentiels. Ces facteurs trophiques nouvellement produits induisent probablement certains changements neuronaux, mais demeurent insuffisants pour promouvoir la régénération des neurones du NBM.

CHAPTER I

INTRODUCTION

The last decade has seen many new concepts emerge in the neurosciences aided, in part, by the development of reliable analytical techniques such that our understanding of the consequences of brain lesions and the potential for repair within the central nervous system (CNS) has greatly expanded. The main goal of this thesis was to advance our understanding of the mechanisms governing maintenance of phenotypic characteristics of nucleus basalis magnocellularis (NBM) cholinergic neurons. Molecular biology, immunocytochemistry, neurochemistry and behavioral sciences were all employed to perform the work of this thesis. The results will be described in the five chapters that follow and which include five papers, in addition to information provided by two other publications in the appendix section (published, in press or submitted). The spectra of activities of three neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) as well as acidic fibroblast growth factor (aFGF) were investigated for their ability to prevent degeneration of NBM cholinergic neurons. Other functions ascribed to NGF and aFGF, such as their capacity to induce important genes in the basalo-cortical cholinergic system, were also investigated. Another aspect of this thesis was to verify the consequences of thyroid hormone deprivation on the development of cholinergic NBM neurons and to investigate their possible recovery with NGF.

Data directly related to the objectives of the present work are reviewed in this section. The distribution of forebrain cholinergic neurons, particularly neurons of the NBM, their sites of innervation and the lesion model used in this work are reviewed in section I.1. Section I.2 reviews reports pertaining to neurotrophins and their receptors. The trophic action of aFGF and other aFGF-related compounds, as well as their expression in response to brain injury are all reviewed in section I.3. The specificity of neurotrophic factors in rescuing a given neuron is reviewed in section I.4. Section I.5 outlines some aspects of the regulation and role of growth-associated protein-43 (GAP-43) in the context of neuronal injury and neurotrophic therapy. Section I.6 seeks to integrate information regarding the postulated interaction between thyroid hormones and NGF on the development of basal forebrain cholinergic neurons. These widely different

approaches have yielded convergent results that illustrate the interaction and limitations of NGF as a specific trophic factor for NBM cholinergic neurons. Section I.7 presents the overall framework including the statement of the problem and objectives.

I.1. Forebrain cholinergic neurons

The anatomy of cholinergic pathways projecting from the basal forebrain and of cholinergic interneurons within striatum, islands of Calleja and neocortex has been delineated only in the last two decades (Fig. I.1). Mapping of these cholinergic pathways was initially defined using a histochemical technique for detection of acetylcholinesterase (AChE) (Shute and Lewis, 1961; 1963; Lewis and Shute, 1967; Butcher and Bilezikjian, 1975; Butcher, 1978; 1983). Immunocytochemical studies employing monoclonal antibodies against a reliable cholinergic marker, choline acetyltransferase (ChAT), in combination with retrograde tracing have subsequently defined these systems more accurately (Sofroniew et al., 1982; Eckenstein and Sofroniew, 1983; Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1983; Wainer et al., 1984; Woolf et al., 1986). The designation Ch1-Ch4 was proposed for rat and primate brains (Mesulam et al., 1983a; 1983b) (see below).

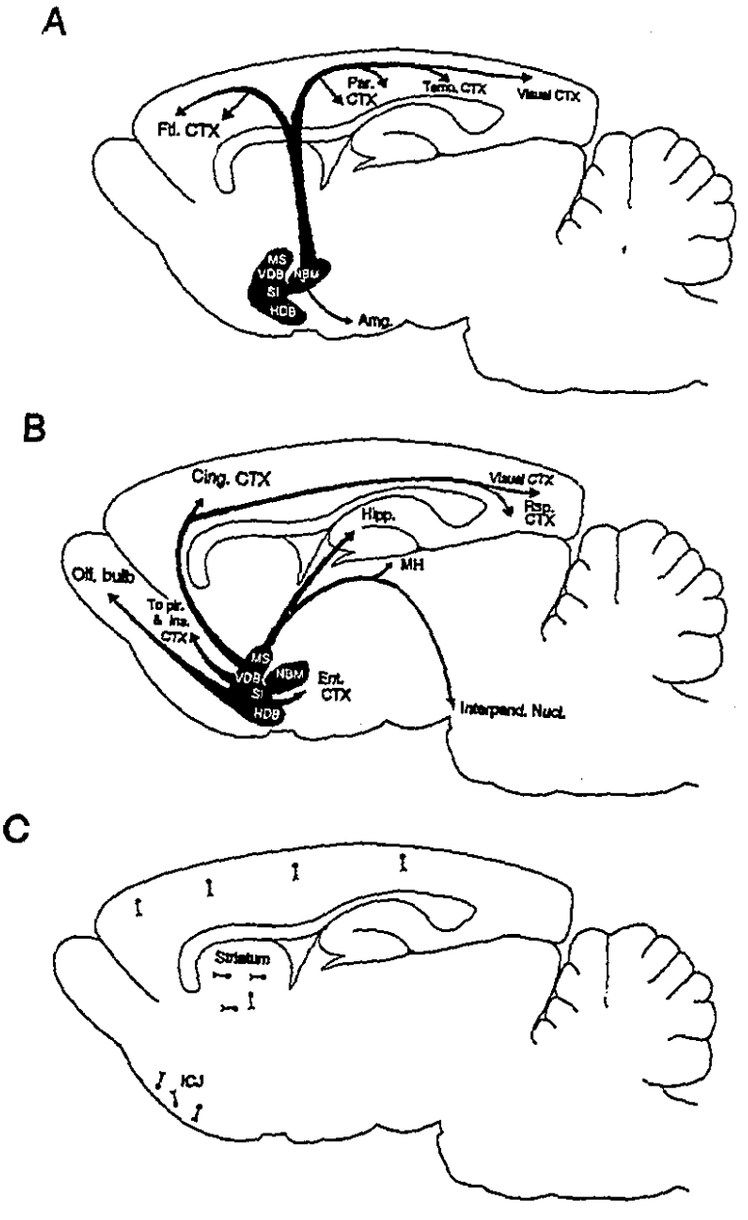
The major source of cholinergic innervation to the hippocampus and cortex arises from a continuum of cholinergic neurons situated in the basal forebrain. This region includes neurons of the medial septal nucleus (MS or Ch1) which project to the hippocampus, the nuclei of the vertical limb of the diagonal band of Broca (VDB, or Ch2) which project to the hippocampus, the nuclei of the horizontal limb of the diagonal band of Broca (HDB, or Ch3) which project to the olfactory bulb as well as to medial and (mostly) limbic cortex, and the nucleus basalis magnocellularis (NBM or Ch4; the equivalent in primates was originally referred to as the nucleus basalis of Meynert or substantia innominata before its cholinergic nature was known). This group projects mostly to neocortex but also to amygdala and limbic cortex (Mesulam et al., 1983a; 1983b; Rye et al., 1984; Bigl et al., 1982; Woolf and Butcher, 1982). Evidence suggesting cortical targets for cholinergic neurons originating in the nucleus basalis

(Kodama, 1929; Das, 1971; Divac, 1975; Kievet and Kuypers, 1975; Lehmann et al., 1980; McKinney et al., 1983; Lewis and Shute, 1967; Krnjevic and Silver, 1965; Wenk et al., 1980; Coyle et al., 1978; Gorin and Johnson, 1979; Vaughn et al., 1981) was confirmed by studies which combined ChAT and AChE immunocytochemistry with histochemical tracers (Bigl et al., 1982; Woolf et al., 1983; 1986). Bigl and colleagues (1982) used fluorescent tracer histology in combination with AChE analysis to demonstrate the cholinergic projections from basal forebrain. In this study, these investigators described that the frontal cortex receives a larger number of fibers from the nucleus basalis. They also found evidence, which has been confirmed more recently (Price and Stern, 1983; Saper, 1984; Carey and Rieck, 1987; Luiten et al., 1987), for a rostrocaudal topography for the basalo-cortical projections: the anterior, the mid and the posterior portions from the NBM project predominantly to the frontal, parietal and occipital cortical lobes, respectively. In this thesis, the designation NBM will refer to the rodent equivalent which includes the group of large cholinergic neurons situated in the innermost portion of the globus pallidus and encroaching on the capsula interna (Cuello and Sofroniew, 1984; Sofroniew et al., 1987). We can regard these neurons as equivalent. However, some discrepancies exist in the literature, most likely as a result of lack of familiarity with the comparative neuroanatomy of rodents and primates.

The contribution of intrinsic neurons to cortical cholinergic innervation is a matter of controversy (Eckenstein and Thoenen, 1983; Houser et al., 1983; Levey et al., 1984). These neurons are probably absent in primates, including man, but they might account for as much as 30% of the cortical cholinergic innervation in rodents (Johnston et al., 1981). The presence of these neurons in the cortex of rat was confirmed by one recent *in situ* hybridization study employing a radiolabelled probe complementary to ChAT mRNA (Lauterborn et al., 1993), but not by another study using digoxigenin-labelled RNA probes and *in situ* hybridization histochemistry to examine ChAT mRNA (Oh et al., 1992).

Patterns of ChAT-immunoreactive fibers found in the rat cerebral cortex were described in detail (Lysakovski et al., 1989). These investigators distinguished eight

Figure I.1 Drawings modified from (Woolf, 1991) illustrating the pathways of cholinergic neurons from the NBM (A) and the remaining basal forebrain neurons (B), as well as the location of the cholinergic interneurons (C). For the purpose of this thesis, illustrative projections from basal forebrain cholinergic neurons were subdivided into these two groups shown in A and B. Abbreviations: Cing. CTX, cingulate cortex; Ent. CTX, entorhinal cortex; Ftl CTX, frontal cortex; HDB, horizontal limb of the diagonal band of Broca; ICJ, islands of Calleja; Ins. CTX, insular cortex; Interpend. Nucl., interpendular nucleus; NBM, nucleus basalis magnocellularis; MH, medial habenula; MS, medial septal nucleus; Olf. bulb, olfactory bulb; Par. CTX, parietal cortex; Pir. CTX, piriform cortex; Rsp. CTX, retrosplenial cortex; SI, substantia innominata; VDB, vertical limb of the diagonal band of Broca.


A
Par. CTX
Temp. CTX
Visual CTX
Frl. CTX
MS
VDB
NBM
SI
HDB
Amg.
B
Cing. CTX
Visual CTX
Rsp. CTX
Hipp.
MH
Olf. bulb
To pir. & ins. CTX
MS
VDB
NBM
SI
HDB
Ent. CTX
Interpend. Nucl.
C
Striatum
ICJ

neocortical or isocortical patterns and five allocortical patterns (composed of three or four layers, including piriform and entorhinal areas) which correlated with functional differences between the respective cortical regions as follows. Motor/frontal cortex: ChAT-immunoreactive fibers showed a homogeneous pattern in motor area four, which is agranular and contained large pyramidal cells in layer V. These cholinergic fibers formed a dense tangentially oriented band in layer I and had moderate, homogeneous distribution in the remaining layers. However, this pattern of ChAT immunoreactivity does not correlate with the laminae which are believed to be of primary importance for information processing in output layers V and VI. Parietal cortex (primary sensory, sensorimotor, secondary sensory and association cortices): the pattern of ChAT-immunopositive fibers reflected the diverse neuronal distribution in this area of cortex. This cortical region also includes a mixed motor-sensory function (characterized by a granular layer IV and broad layers V and VI with large pyramidal cells in layers V). According to the study of Lysakovski et al. (1989), the largest portion of the parietal cortex exhibits a "primary sensory pattern" of ChAT innervation which appears to concentrate in the thalamocortical input areas and is characterized by very dense fibers at the border between layers IV and V, moderately dense fibers in layers I and V and least dense fibers in layers II, III and VI. Temporal cortex: showed a primary sensory pattern in which ChAT immunoreactive fiber density was greatest in layers I and V, lowest in layer VI and moderate in layers II-III. Occipital (visual) cortex: had dense tangential fibers in layer I in primary and secondary visual areas and somewhat less dense ChAT-immunoreactive fibers between the border of layers IV and V, but only in primary sensory cortex. The functional correlates to these anatomical findings will require further associative analysis using physiological and pharmacological approaches.

One of the experimental lesion models which has been most widely used to investigate the effects of neurotrophic factors on basal cholinergic neurons is fimbria-fornix transection. This axotomy model (Daitz and Powell, 1954) interrupts projections from the medial septum and vertical limb of the diagonal band of Broca to the hippocampus (Fig. I.1, A). The result of fimbria-fornix transection is total retrograde

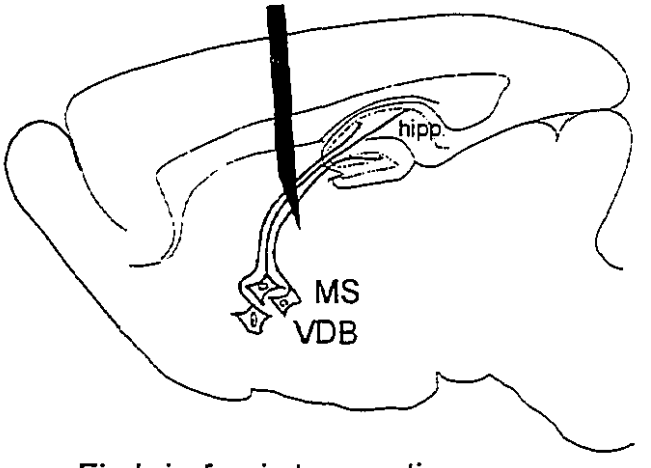
degeneration with profound atrophy and neuronal cell death. A second surgical lesion model, which is used in this work to study cholinergic neurons of the basalo-cortical pathway, is "the target plus cholinergic terminal network removal" model. Cortical devascularization (or cortical ablation) results in partial anterograde degeneration and moderate cell shrinkage in the NBM, while cell number remain unchanged (Figure I.2, B) (Sofroniew et al., 1983; 1987). This model also leads to a marked depletion of ChAT activity in the microdissected NBM. The devascularizing lesion is performed by removing a flap of bone (1.0 x 1.0 cm) with coordinates 3 mm anterior to Bregma extending to 7 mm posterior to Bregma; the medial limit is approximately 2 mm from the midline and extends laterally 10 mm. After cutting the underlying dura, all vessels and the pia mater are gently rubbed away with sterile saline-soaked cotton swabs. The areas affected include complete atrophy of parietal 1 and forelimb, and part of frontal 1, hindlimb and parietal 2 regions (Zilles, 1985). The resulting decorticated region approximately fits the size of the devascularized area (ie., 1.0 x 1.0 cm). Most of the atrophic neurons are found in the midportion of the NBM (Garofalo et al., 1992; 1993; Garofalo and Cuello, 1990; 1993;1994), within an area delineated by Bregma -1.20 mm to -1.50 mm [coordinates according to Paxinos atlas (Paxinos and Watson, 1986)]. As a consequence of this infarction, which affects parietal cortex, degeneration occurs mainly in the midportion of NBM, as previously described (Price and Stern, 1983; Saper, 1984; Carey and Rieck, 1987; Luiten et al., 1987) (see above).

I.2. Neurotrophins and their receptors in rat forebrain

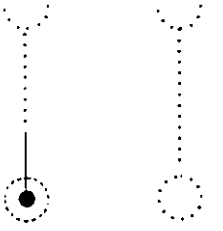
In the last years we have seen a phenomenal advance in the discovery and functional characterization of neurotrophins. NGF remains the prototypic neurotrophic factor and was the first demonstrated to be required for normal development (Levi-Montalcini and Angeletti, 1963; Cohen, 1960; Johnson et al., 1983). NGF was originally purified from mouse submandibular gland (Angeletti and Bradshaw, 1971), where it occurs as a complex having a molecular weight of approximately 140kD and a sedimentation coefficient of 7S (Varon et al., 1968). This 7S complex contains three

Figure I.2. The septal-hippocampal (A) and basalo-cortical (B) lesion models of basal cholinergic neurons.(A) Axotomy model: cholinergic axons projecting from medial septum (MS) and ventral limb of the diagonal band of Broca (VDB) are partially or totally sectioned (fimbria-fornix transection) resulting in total anterograde degeneration and cell death illustrated by dotted drawings. (B) Target plus terminal removal model: the surgical lesion consists of partial cortical devascularization (see text for details) which results in partial anterograde degeneration and moderate shrinkage of NBM cholinergic neurons. Drawings modified from previous report (Cuello, 1993).

A AXOTOMY MODEL

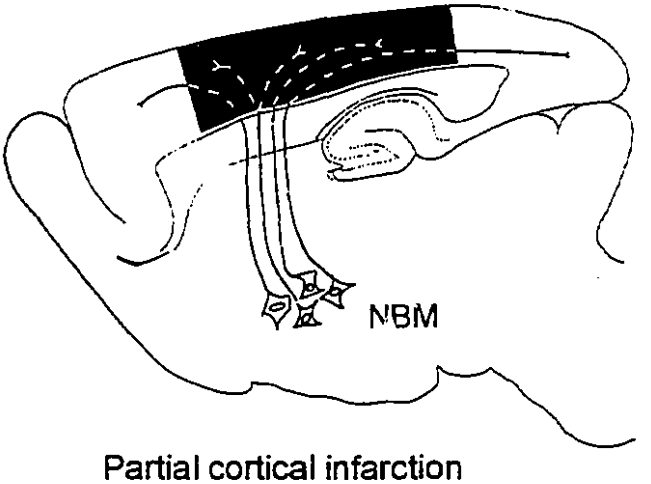


Fimbria-fornix transection

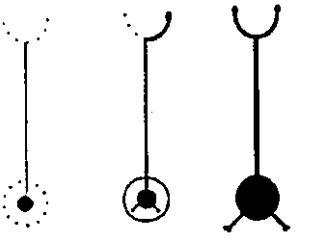


Atrophy / Cell death

B TARGET PLUS TERMINAL REMOVAL MODEL



Partial cortical infarction



Moderate cell shrinkage

subunits, α, β and γ, with the stoichiometry $\alpha_2\beta\gamma_2$ [for review see (Thoenen and Barde, 1980; Greene and Shooter, 1980)]. The biological activity of NGF is mediated only by the β subunit which has often been referred to simply as "NGF". This active form of NGF, when first isolated and sequenced, was known as 2.5S (30kD) NGF (Bocchini and Angeletti, 1969). NGF is synthesized as a 305 amino acid long prepo-NGF precursor (Scott et al., 1983) which undergoes proteolytic cleavage at dibasic amino acid residues to yield a 118 amino acid mature NGF protein (Greene et al., 1969; Greene and Shooter, 1980; Thoenen and Barde, 1980). Structural analysis of β- NGF reveals that the monomer forms a flat surface and that the dimer, which is the active form of the NGF molecule (Varon and Shooter, 1970), forms by the association of two roughly parallel identical 118 amino acid subunits (McDonald et al., 1991). The nucleotide sequence coding for β-NGF has recently been determined in mouse (Scott et al., 1983), human (Ullrich et al., 1983), rat (Whittemore et al., 1988), and chicken (Ebendal et al., 1986). Cloning of these genes allowed the deduction of the sequence of the mature NGF in several other species (Ebendal, 1992).

The mouse β-NGF gene consists of five exons and four introns comprising more than 43kb which, by alternative RNA splicing, generates four different transcripts (Selby et al., 1987). Other members of the neurotrophin family identified by sequence homology are BDNF (Barde et al., 1982; Leibrock et al., 1989), NT-3 (Maisonpierre et al., 1990b; Hohn et al., 1990; Rosenthal et al., 1990), NT-4 (Hallböök et al., 1991; Ip et al., 1992) and NT-5 (Berkemeier et al., 1991). Six cysteine residues, which form three central disulfide bonds that maintain biological activity, are strictly conserved among these five molecules. Sedimentation equilibrium showed that BDNF, NT-3, and NGF exist as strongly associated dimers in phosphate-buffered saline (PBS), pH 7.1 (Narhi et al., 1993). Although these neurotrophins share considerable sequence similarities, it is known that, at least for NGF, BDNF and NT-3, distribution and sites of biological activity are different (Ernfors et al., 1990a; Ibáñez et al., 1991).

NGF binds with low affinity (Kd approximately 10^{-9} M) and high affinity (Kd approximately 10^{-11} M) to separate sites (Sutter et al., 1979; Meakin and Shooter, 1991).

The low affinity site is a 75-80 kD receptor glycoprotein (Johnson et al., 1986; Radeke et al., 1987) referred as $p75^{NGFR}$. $p75^{NGFR}$ has an extracellular domain consisting of four cysteine repeats which bind neurotrophin, one transmembrane domain, and a relatively short cytoplasmic domain (Johnson et al., 1986; Radeke et al., 1987; Welcher et al., 1991; Baldwin et al., 1992). The second binding site belongs to the family of tyrosine kinase receptors: trkA is the high-affinity receptor for NGF, encoded by the $p140^{trk}$ gene (Kaplan et al., 1991b; Klein et al., 1991a; Meakin and Shooter, 1991), while trkB and trkC are the high-affinity receptors for BDNF and NT-3, respectively (Klein et al., 1989; 1991b; Lamballe et al., 1991). It is still debatable what role the $p75^{NGFR}$ receptor plays since it binds all neurotrophins with equivalent affinity (Rodríguez-Tébar et al., 1990; Bothwell, 1991; Squinto et al., 1991). The suggested designation, p75, seems more appropriate to its promiscuous binding; however, since this affinity for multiple ligands was not clear when this work began, the designation $p75^{NGFR}$ will be used for the purpose of this thesis. Previous studies have indicated that biological responses to NGF require interaction with the high-affinity receptor (Herrup and Shooter, 1973; Sutter et al., 1979) which may involve binding to $p140^{trk}$ exclusively (Ibáñez et al., 1992), although $p140^{trk}$ also binds this factor with low affinity (Kaplan et al., 1991a; 1991b). Other studies have suggested that high-affinity binding requires expression of both proteins, $p75^{NGFR}$ and $p140^{trk}$ (Green and Greene, 1986; Hempstead et al., 1991). A conceptual framework has emerged in which NGF responses may be mediated by an heterodimer ($p75^{NGFR}$ + $p140^{trk}$) or simply by a $p140^{trk}$ homodimer (Ragsdale and Woodgett, 1991). However, it is of interest that other studies support the importance of $p75^{NGFR}$ in biological responses to NGF including results obtained using homozygous animals lacking $p75^{NGFR}$ (Lee et al., 1992), gene transfer experiments (Pleasure et al., 1990; Berg et al., 1991; Matsushima and Bogenmann, 1990), antisense oligonucleotides against $p75^{NGFR}$ (Wright et al., 1992) and mutant or chimeric forms of $p75^{NGFR}$ (Hempstead et al., 1990; Yan et al., 1991). Little information is available on the cooperative involvement of $p75^{NGFR}$ and $p140^{trk}$ in activating intracellular signalling pathways of NGF; nonetheless, multiple lines

of evidence suggest that NGF activity depends upon the expression of both neurotrophin receptor types.

In accordance with the conventional neurotrophic hypothesis which predicts expression of neurotrophins in target tissues (Purves, 1986; Oppenheim, 1991), data have so far confirmed that the expression of NGF is limited to cell populations that receive innervation by NGF-responsive neurons (Korsching and Thoenen, 1983; Heumann et al., 1984; Shelton and Reicherdt, 1986; Bandtlow et al., 1987; Davies et al., 1987; Wheeler and Bothwell, 1992; Schecterson and Bothwell, 1992). In the forebrain, it was possible to establish a firm correlation between levels of both $p75^{NGFR}$ protein and mRNA (Taniuchi et al., 1986; Hefti et al., 1986; Kordower et al., 1988; Pioro and Cuello, 1990a; 1990b; Cavicchioli et al., 1989; Higgins et al., 1989; Vazquez and Ebendal, 1991) and $p140^{trk}$ mRNA (Holtzman et al., 1992; Merlio et al., 1992; Venero et al., 1994) in the cell bodies of basal forebrain cholinergic neurons and the levels of NGF mRNA in their targets, the hippocampus and cerebral cortex (Korsching et al., 1985; Shelton and Reicherdt, 1986; Whittemore et al., 1986; Goedert et al., 1986). Forebrain neurons expressing trkB or trkC are widely distributed while $p140^{trk}$ expression is highly restricted (Klein et al., 1989; 1990; Lamballe et al., 1991). The highest levels of $p140^{trk}$ mRNA in the forebrain are consonant with previous binding studies which used iodinated murine or recombinant human NGF to demonstrate high-affinity sites in basal forebrain and caudate putamen (Richardson et al., 1986; Altar et al., 1991). ^{125}I-labelled BDNF and NT-3 are retrogradely transported by distinct populations of CNS neurons located mainly in the hippocampus (DiStefano et al., 1992). BDNF binding was detected in medial septal and vertical diagonal band areas after injection of ^{125}I-labelled BDNF into the dorsolateral septum but not when it was injected intracerebroventricularly (Morse et al., 1993). In binding assays using brain sections, ^{125}I-labelled NT-3 did not bind basal forebrain neurons (Altar et al., 1993). Distribution of these neurotrophins and their receptors in septohippocampal and basalo-cortical regions are shown in Table I.1. These data highlight the necessity of interpreting the role of these neurotrophins within the context of the cellular environment where they, and their receptors, are preferentially

expressed. There is 50% homology among the extracellular domains of the trk receptors and approximately the same degree of homology among the neurotrophins; however, restricted coupling has been observed *in vitro*. NGF binds to trkA only, although trkA also binds NT-3 and NT-4/NT-5. With the exception of NGF, all neurotrophins bind to trkB receptors, while trkC binds NT-3 only. It is not yet clear whether all of these interactions occur *in vivo* [for review see (Chao, 1992b; Chao, 1992a; Korsching, 1993)].

One interesting feature of the trkB gene is that it gives rise to two noncatalytic forms of trkB, termed $p95^{trk}$, which lack the cytoplasmic domain (Middlemas et al., 1991) but retain the extracellular domains of the full-length receptor.This truncated receptor is abundantly expressed in ventricular ependymal cells and choroid plexus (Klein et al., 1990) where it has been suggested to act as a scavenger receptor capable of binding high levels of neurotrophins without participating in signalling. This notion is in agreement with the accumulation of exogenous BDNF circumscribing the cerebral ventricles (ipsilateral to the lesion) (Morse et al., 1993).

I.3. - Fibroblast growth factors

A vast number of studies relating to the biology of the seven members of the family of fibroblast growth factors (FGF) has been produced since the first identification (Armelin, 1973) of an unknown substance in pituitary extracts which induced mitogenesis of fibroblasts. This FGF, which later proved to be basic FGF (Gospodarowicz, 1975) and the six other structurally homologous peptides, also called heparin-binding growth factors, are expressed in a wide variety of tissues. Acidic FGF (aFGF, acidic isoelectric point) and basic FGF (bFGF, basic isoelectric point) are also called FGF-1 and FGF-2, respectively. This nomenclature, whereby the FGFs are numbered consecutively from FGF-1 to FGF-7, has alleviated the confusion created by different names, but the FGF designation is still not appropriate because it implies that the actions of these proteins are specific for fibroblasts. For convenience, however, the designations aFGF and bFGF will be used in this thesis. Data generated over the past 20

Table I.1. Semi-quantitative representation of approximate mRNA levels of neurotrophins and their receptors in rat brain.

A. Basalo-cortical pathway

CHOLINERGIC PROJECTION ORIGIN		CHOLINERGIC TARGET REGION	
RECEPTOR	**NBM**	**NEOCORTEX**	**NEUROTROPHIN**
$p75^{NGFR}$	+ + + +	-	
	-/+	+/+ +	NGF
trkA	+ + +	-	
	-	+/+ +	BDNF
trkB	-/+ ?	+ +	
	-	+	NT-3
trkC	-	+/+ +	

B. Septo-hippocampal pathway

CHOLINERGIC PROJECTION ORIGIN		CHOLINERGIC TARGET REGION	
RECEPTOR	**MS/DB**	**HIPPOCAMPUS**	**NEUROTROPHIN**
$p75^{NGFR}$	+ + + +	-	
	-/+	+ + +	NGF
trkA	+ + +	-	
	-	+ + + +	BDNF
trkB	+#	+ + +	
	-	+ + +	NT-3
trkC	-*	+ + + +	

Abbreviations: MS/DB, medial septum and diagonal band (vertical and horizontal limbs); NBM, nucleus basalis magnocellularis; NT, neurotrophin. The approximate mRNA levels are indicated by high (+ + + +), moderate (+ +/+ + +), low (+/+ +) and very low or undetected (-/+). For reviews see (Korsching et al., 1985; Goedert et al., 1986; Whittemore et al., 1986; Shelton and Reicherdt, 1986; Phillips et al., 1990; Maisonpierre et al., 1990a; Ernfors et al., 1990b; Vazquez and Ebendal, 1991; Squinto et al., 1991; Ceccatelli et al., 1991; Holtzman et al., 1992; Morse et al., 1993; Merlio et al., 1992; Schecterson and Bothwell, 1992; Merlio et al., 1993; Lapchak et al., 1993; Ceccatelli et al., 1991; Heckers et al., 1994; Gibbs and Pfaff, 1994; Venero et al., 1994). The co-localization of abundant levels of neurotrophins and their high affinity receptors (indicated by shading) suggests the possibility of a local autocrine effect. # trkB protein reported in a binding study, injecting BDNF (12 μg/day) into the dorsolateral septum, for 14 days (Morse et al., 1993). * Data from binding studies with ^{125}I-labelled NT-3 in brain sections (Altar et al., 1993).

years have mainly focused on the ability of FGFs to affect cell proliferation, differentiation and angiogenesis [for general review see (Gospodarowicz, 1991; Basilico and Moscatelli, 1992)]. Table I.2 summarizes these properties of FGFs. FGFs bind to two classes of specific cell surface FGF receptors (FGFR). One of these receptors appears to be heparan sulphate proteoglycans (Jeanny et al., 1987; Kiefer et al., 1990). Recent observations suggest that, apart from serving as a major stable reservoir for FGFs, cell surface proteoglycans are an essential component of the FGF signalling pathway. The second class of receptor corresponding to the high-affinity receptors has been cloned and identified as a member of the tyrosine kinase receptor family. These FGFRs possess extracellular immunoglobulin-like domains and intracellular split tyrosine kinase domains. Four such receptors have been identified: FGFR-1 (flg), FGFR-2 (bek), FGFR-3, and FGFR-4 (Kornbluth et al., 1988; Dionne et al., 1990; Ruta et al., 1989; Lee et al., 1989; Pasquale, 1990; Keegan et al., 1991; Partanen et al., 1991). Four membrane-bound isoforms and four secreted isoforms of FGFRs have been described [for review:(Givol and Yayon, 1992)]. FGFR-1 and FGFR-2 both bind aFGF and bFGF with high affinity having a K_d in the range of 100-500 pM (Dionne et al., 1990). FGFR-3 preferentially binds aFGF and shows approximately 20-fold lower affinity for bFGF (Ornitz and Leder, 1992). FGFR-4 binds aFGF with a similar affinity to that of FGFR-1 (Kd 200-600 pM). It was reported (Partanen et al., 1991) that FGFR-4 bound aFGF but not bFGF; however, more recent studies have found that this receptor binds aFGF, bFGF, FGF-4 and FGF-6, but not FGF-7 (Ron et al., 1993; Vainikka et al., 1993). The strong affinity of all members of the FGF family for the glycosaminoglycan heparin led to the discovery of cell surface heparan sulphate proteoglycans (HSPG) (Shing et al., 1984; Burgess and Maciag, 1989). In contrast to the binding of NGF to its receptors, the binding of FGFs to their high affinity receptors is dependent on the interaction between the FGF and HSPG. Attempts to map the heparin binding domains of aFGF and bFGF revealed at least two sites, one of which is common to both FGFs and corresponds to one of the FGFR binding domains. Modification of this site (by deletion or methylation of lysine residues) resulted in a reduction in its affinity for both heparin and FGFR as well

Table I.2. Classification and general properties of fibroblast growth factors

	FGF-1	FGF-2	FGF-3	FGF-4	FGF-5	FGF-6	FGF-7
Other names	aFGF	bFGF	int-2	hst/K-FGF /ks			KGF
Number of amino acids #	155	155, 196, 201, 210	239, 271	206	267	?	194
N-terminal hydrophobic leader sequence	Does not exist	Does not exist	Release not efficient	Secreted	Secreted	Secreted	Secreted
Suggested high affinity receptors	R-1 (Flg) R-2 (Bek) R-3 (Cek) R-4	R-1 R-2 R-4		R-1 R-2			R-2
Avid heparin binding	Yes	Yes	No	Yes	Yes	Yes	Yes
Oncogenic potential	Very limited	Very limited	High*	High**	High***	?	?
Expression	Ubiquitous (higher in brain)	Ubiquitous (higher in brain)	During development (brain)	During development	During development (and in adult brain)	?	?

Most of the data are for human FGFs (Gospodarowicz et al., 1986; Gospodarowicz, 1991; Otto et al., 1989; Basilico and Moscatelli, 1992; Thomas, 1987; Kornbluth et al., 1988; Dionne et al., 1990; Ruta et al., 1989; Lee et al., 1989; Pasquale, 1990; Keegan et al., 1991; Partanen et al., 1991; Ron et al., 1993; Vainikka et al., 1993; Ornitz and Leder, 1992). The functional redundancy (seven distinct FGFs with high cross-reactivity and 4 FGFRs known) to date raises the question of specificity of FGFs. The diversity of FGFRs is explained by the gene structure of FGFR. The human FGFR-1 gene consists of 19 exons, which indicate the possibility for the expression of many variant forms of FGFR-1. Therefore, the above FGFs and FGFRs may, in fact, represent an oversimplified list. Abbreviations: int-2, refers to the second gene identified as a very frequent site of integration of the mouse mammary tumor virus; hst; human stomach tumor/K-FGF, Kaposi's FGF/ ks, Kaposi's sarcoma gene; KGF, keratinocyte growth factor. Fibroblast growth factor receptor cDNAs received other names according to their sources: R1 (FGFR-1), is equivalent to the mammalian flg and the chicken cek1 transcripts; R2 (FGFR-2), is the equivalent to the human bek or K-Sam proteins; R3 (FGFR-3) is equivalent to the murine flg-2 and chicken cek2; R4 (FGFR-4) has no other name. # = Primary human translation product; * = virally-activated expression has been implicated in the formation of mouse mammary tumours; ** = discovered in human stomach cancers and Kaposi's sarcoma;. *** = originally identified as an oncogene product.

as a decrease in biological potency [for a review see (Basilico and Moscatelli, 1992)]. Heparin affinity for FGFs ranges from 0.6 M for FGF-7 to 1.6 M for bFGF; aFGF occupies an intermediary position. In the absence of heparin, rapid loss of FGF activity occurs due to inactivation or degradation by proteases (Rosengart et al., 1988a; Salsela et al., 1988; Gospodarowicz and Cheng, 1986; Sommer and Rifkin, 1989; Thomas et al., 1991). These studies have shown that heparin binding to acidic and basic FGF prolongs their biological half lives. Interestingly, it was reported that heparin dependence of aFGF was differentially regulated in the PNS and CNS, with aFGF being much less heparin dependent in subcortical tissue (Stock et al., 1992). Also from the same laboratory, it was shown that the mitogenic effect of aFGF on fibroblasts was strongly potentiated by heparin while that of bFGF was only slightly potentiated (Eckenstein et al., 1991a). Since aFGF and bFGF are not secreted by the endoplasmic reticulum-Golgi pathway, an alternate mechanism, one which could be activated as a consequence of tissue damage, has been postulated for their release (Burgess and Maciag, 1989). Subsequently, the FGFs released near the damaged extracellular tissue would be bound to exposed heparan sulfate-containing subendothelial basement membrane and extracellular matrix, effectively concentrating and storing these proteins at sites of injury (Baird and Ling, 1987; Rosengart et al., 1988b).

FGFs are found in high levels in the brain (Gospodarowicz et al., 1987; Burgess and Maciag, 1989) where the aFGF and bFGF seem to be the most abundant mitogenic factors (Thomas, 1987). Although little is known about the precise role of these two FGFs in the brain, evidence to date indicates that aFGF, bFGF and, probably, FGF-5 (Lindholm et al., 1994) have the potential to be important neurotrophic factors for future therapeutic neurology. Thus, this review focuses mainly on data pertaining to the neurotrophic effects of FGF, namely neuronal differentiation, growth, and survival. In PC12 cells, aFGF and bFGF induced neurite outgrowth in a manner similar to that caused by NGF (Togari et al., 1983; 1985; Rydel and Greene, 1987; Schubert et al., 1987; Wagner and D'Amore, 1986). Treatment with aFGF or bFGF promoted the survival and differentiation of primary cell cultures from various regions of the rat brain

(Morrison et al., 1986; Unsicker et al., 1992; Walicke, 1988; Walicke and Baird, 1991). Thus suggesting that aFGF and bFGF may be of importance for the development and maintenance of some populations of neurons including those from cerebral cortex (Morrison et al., 1986), hippocampus (Walicke, 1988), and septal area (Grothe et al., 1989). Identification of the sites where FGFs and their receptors are localized in the brain, provides further insight into the role of FGF for each of these neuronal populations (Table I.3). Very different distribution patterns of aFGF, bFGF and their receptors in the rat forebrain are revealed by measurements of the respective protein and mRNA levels, as well as by retrograde labelling of iodinated-aFGF and -bFGF of specific neuronal populations in naive and lesioned animals. The predominant localization of bFGF to astrocytes contrasts to the preferential neuronal localization of aFGF. Furthermore, the populations of forebrain neurons with moderate levels of aFGF are located in the NBM, caudate-putamen, globus pallidum; aFGF is only weakly expressed in frontal cortex. Neurons expressing moderate levels of bFGF are found in the hippocampus while low levels appear in the cingulate cortex. The moderate levels of iodinated-bFGF binding to the thalamus suggested that bFGF bound to FGFR-3, which is predominantly expressed in thalamus; however, this binding did not occur following aFGF injection, which was unexpected because FGFR-3 was shown to have higher affinity for aFGF than for bFGF (see above). It is interesting that aFGF, bFGF, FGFR-1 and FGFR-2 are upregulated following neuronal injury. The expression of other FGFs and receptors after injury has not been tested. This upregulation, observed predominantly in reactive astrocytes, led investigators to explore the functional role of these putative neurotrophic factors in the process of neuronal regeneration. These data, in conjunction with the first experiments *in vivo* to show bFGF prevented loss of cholinergic neurons after fimbria-fornix transection (Anderson et al., 1988; Otto et al., 1989; Gómez-Pinilla et al., 1992b), were further strong arguments for a specific role for bFGF as a neurotrophic factor. However, the apparent failure to detect FGFS in neurons of the medial septum and ventral limb of the diagonal band of Broca is in agreement with an indirect effect,

Table I.3. Relative expression of aFGF, bFGF and FGF receptors in naive and lesioned rat forebrain.

	aFGF	bFGF	FGFR-1	FGFR-2	FGFR-3	FGFR-4	^{125}I-aFGF binding	^{125}I-bFGF binding
NAIVE RAT	(Prot.)	(Prot.)	(mRNA)	(mRNA)	(mRNA)	(mRNA)		
Strong			Hipp.	Corpus callosum	-	Medial habenular nucleus	-	S.N./ L.hyp./ S. mm.n/
Moderate	NBM/ Hypoth./ Caud./Put. Pall./F.ctx Claust.	Ubiquitous in astrocytes & some neurons: S.hip.n., C. ctx., CA2	Olf. bulb Ctx (L. V) C. ctx/ Ent. ctx.	(abundant in oligoden-drocytes)	Thal., Hipp., Cortex	-	Lateral hypothala-mic area	Pyr.c.hipp /thalamic nuclei/ Subiculum
Weak	Thal.		Ctx (I, V)		-	-	-	P. ctx/ C.ctx
AFTER LESION								
Protein and/or mRNA	Increased in gran. cells of DG **	Increased in reactive astrocytes at CA1,Caud Put.,T.ctx, C.cal.,CA4 * Increased in gran. cells of DG ** Increased in hipp. #	Strong and prolonged increase in astrocytes of CA1 * Increased in pyr cells of hipp. and gran.cells of DG **	Slight and transient increase in astrocytes (mainly in CA1) *	?	?	?	?

Abbreviations: CA1,CA2, CA3, CA4, subfields of hippocampus; Caud., caudate; Claust., claustrum; C.ctx, cingulate cortex; Ctx (I,V), cortical layers I and V; Ent. ctx, entorhinal cortex; F. ctx, frontal cortex; gran. cells of DG, granular cells of dentate gyrus; Hipp., hippocampus; Hypoth., hypothalamus; L. hyp. area, lateral hypothalamus area; P. ctx, parietal cortex; Pall., pallidum; Pyr. cells of hipp., pyramidal cells of hippocampus; S.hip.n.,septohippocampal nucleus; S. mm. n., supramammillary nucleus; S.N., substantia nigra; Thal., thalamus. The increase in protein and mRNA was identified close to wounds and areas of cell death, in lesion models, following transient forebrain ischemia *; after fimbria-fornix transection or entorhinal lesion # and following kainate-induced seizures **. For reviews:(Eckenstein et al., 1991; Wanaka et al., 1990; Kiyota et al., 1991; Ferguson and Johnson, Jr., 1991; Gómez-Pinilla et al., 1992a; Takami et al., 1992; Eckenstein et al., 1991b; Stock et al., 1992; Woodward et al., 1992; Takami et al., 1993; Bugra et al., 1994; Itoh et al., 1994).

that aFGF and bFGF are perhaps mediated by glial cells as initially proposed (Barde, 1987). In support of this notion, *in vitro* studies demonstrated potent stimulators of NGF release from astrocytes of newborn rats and mice (Yoshida and Gage, 1991; Ono et al., 1991; Yoshida and Gage, 1992). A central question that applies to the family of neurotrophins, particularly NGF, is that neuronal cell survival is dependent upon limiting amounts of these neurotrophic factors in the targets of these neurons. For NGF, there is a clear correlation between the amounts of NGF receptor in basal cholinergic neurons and the expression of target-derived NGF (Table I.1). In the case of aFGF and bFGF, to date, several studies failed to find any correlation between the supply of these FGFs and their binding to high-affinity receptors in surviving cholinergic neurons (Table I.3). More experiments will be necessary to elucidate whether the neurotrophic effects of FGFs on neurons are indeed direct or indirect.

I.4. - Neurotrophic factors used to promote repair of basal forebrain cholinergic neurons

The limited restorative capacity of fully differentiated CNS neurons has often frustrated the analysis of basic mechanisms involved in this process. Until some years ago, restorative neurology was viewed as an impossible task. However, the discovery of neurotrophic factors and receptors, as well as advances in understanding the cellular regulatory processes underlying neuronal injury, cell death and neuronal repair, provide some optimism that the potential exists for neurotrophic factors to therapeutically influence neurons of human adult brains. A major goal of studies on naturally occurring or induced atrophy and cell death of CNS neurons has been to identify specific and efficient neurotrophic factors capable of rescuing these neurons. In selecting models for neuronal injury and repair, the sympathetic, sensory, and basal forebrain cholinergic neurons merit attention because of their sensitivity to NGF extracted in abundance from mouse salivary glands. As heterogeneity of the neurotrophic factors was discovered, the idea that they may perform different functions in different target organs according receptor expression was advanced. With progress in genetic and molecular biology techniques, rapid access to large amounts of recombinant neurotrophic factors allowed

their use in several neuronal lesion models. The effects mediated by interaction of exogenous neurotrophic factors with sensitive neurons and non-neuronal cells include local trophic interactions, transfer of information through retrograde messenger mode and anterograde trophic signalling, as well as autocrine signalling [for a review see (Korsching, 1993)]. While this work does not dispute the merit of other neuronal types being studied for regenerative purposes, the focus of this work is to specifically address the repair of basal forebrain cholinergic neurons, particularly of the NBM, whose axonal terminals and cortical targets have been injured by devascularization. The NBM is the rodent equivalent of the nucleus basalis of Meynert (nbMt) which has has been implicated in disorders of cholinergic transmission such as dementia (Bartus et al., 1985).

Extensive cell loss and neurofibrillary tangle formation in the nbMt (Saper et al., 1985; Whitehouse et al., 1982), loss of choline acetyltransferase activity in nbMt and cortex (Bowen and Smith, 1976; Davies and Maloney, 1976; Perry et al., 1977; Rossor et al., 1982; Henke and Lang, 1983; Sims et al., 1983) and marked reduction of muscarinic (type 2) and nicotinic receptors in cortex and hippocampus (Rinne et al., 1984; Mash et al., 1985; Flynn and Mash, 1986; Nordberg and Winblad, 1986; Perry et al., 1986; Whitehouse et al., 1986; Araujo et al., 1988; Svensson et al., 1992) are consistently implicated in Alzheimer's Disease (AD) neuropathology. Some animal models of cholinergic hypofunction display AD-related symptoms such as memory, attention, or cognitive deficits (Lippa et al., 1980; Sherman et al., 1981; Bartus, 1981; 1982; 1987). Pharmacological agents used to enhance cholinergic function reversed behavioral deficits in these animal models [for review see (Smith, 1988)], but failed to do so in AD patients. One possible reason for this apparent discrepancy is that the cholinergic hypofunction in AD is usually superimposed on an advanced aging process, where the abnormal neuronal cytoarchitecture does not respond to conventional cholinergic agonists. Alternate therapeutic strategies, using cholinergic agonists and/or drugs that induce release of acetylcholine by acting on non-cholinergic receptors, could be beneficial in the treatment of some AD patients (Quirion et al., 1990). Furthermore, other non-cholinergic systems appear to be defective; AD may be a multisystem disorder

with the primary insult originating in the cerebral cortex (Cuello and Sofroniew, 1984; Mesulam, 1986; Price, 1986) with a cholinergic involvement secondary to such cortical pathology. Agents which are specific trophic factors for basal forebrain cholinergic neurons are presently being seriously considered for treatment of AD patients. The first report on intracranial infusion of NGF to an AD patient described a 25% increase in [^{11}C]nicotine binding in frontal and temporal cortical areas, as visualized by positron emission tomography, and this was correlated with a transient performance increment in delayed word recognition (Seiger et al., 1993).

Three distinct neuronal types are responsive to NGF: sensory, sympathetic, and selective populations of central cholinergic neurons (Thoenen et al., 1987). The limited number of neurons supported by NGF has led to speculation that additional neurotrophic factors play a role and several experimental approaches have been used to test whether other neurotrophins can rescue central neurons. The specificities of some neurotrophic factors in rescuing these neurons are reviewed in Table I.4 which highlights the responsiveness of central cholinergic neurons in comparison with other neuronal cell types. These data indicate that more than one neurotrophic factor may exert an effect (e.g., neuronal survival, neuronal differentiation or increased neurite growth) on a given neuron and vice versa, several types of neurons may be protected by one neurotrophic factor. This apparent lack of specificity was recently examined (Korsching, 1993). In her review, Korsching proposed a modification in the concept of neurotrophic factor, claiming that a high degree of specificity can be achieved by detailed regulation of the spatial and temporal expression for all modifications associated with neurotrophic factors, their receptors, and intracellular signalling components. A group of at least four neurotrophic factors and the putative trophic factor monosialoganglioside (GM1) were reported to protect basal forebrain cholinergic cells from induced degeneration. Among central cholinergic neurons, those located between the substantia nigra and locus ceruleus (pedunculopontine tegmental nucleus and the dorsolateral tegmental nucleus)(Woolf and Butcher, 1986; Green and Greene, 1986; Rye et al., 1987) seem to not respond to NGF.

Table I.4. Specificities of NTFs in rescuing cholinergic and other neurons from degeneration

NTFs	LESION MODEL	REGENERATIVE RESPONSE	
		CENTRAL CHOLINERGIC NEURON	OTHER NEURONAL TYPES
NGF	FF transection	Prevents MS/VDB neuronal loss or ChAT activity (Hefti et al., 1984; Hefti, 1986; Williams et al., 1986; Kromer, 1987; Montero and Hefti, 1988; Gage et al., 1988)	
	FF transection (primates)	Prevents MS/VDB neuronal loss (Tuszynski et al., 1990; Koliatsos et al., 1990; Tuszynski et al., 1991; Koliatsos et al., 1991)	
	Cort. devasc.	Prevention of NBM neuronal atrophy (Cuello et al., 1989)	
	Cort. devasc. (primates)	Prevention of NBM neuronal atrophy (Liberini et al., 1993)	
	Spinal cord injury		Partial recovery of corticospinal tract (Fernandez et al., 1993)
	Atrophy (aged rats)	Partial recovery of atrophy (basal forebrain and striatum)(Fisher et al., 1987)	
	Sympathetic and sensory nerve lesion		Reversed natural or induced cell death (Thoenen and Barde, 1980; Levi-Montalcini, 1982; Thoenen et al., 1987)
	Sciatic nerve transection		Prevention of neuronal loss in lumbar sensory ganglia (Rich et al., 1987)
	Sciatic nerve transection		Partially restores sensory neuron volume(Verge et al., 1989)
BDNF	Dopaminergic neurons *in vitro*		Improved survival (Hyman et al., 1991)
	MPTP lesion *in vitro*		Protected dopaminergic neurons (Hyman et al., 1991)
	Striatal lesion		No protection of fetal dopaminergic neurons *in vitro* (Knüsel et al., 1992)
	Sciatic nerve transection		Prevented death of motoneurons in newborn rats (Yan et al., 1992)
	Facial nerve transection		Prevented death of motoneurons in newborn rats (Sendtner et al., 1992; Koliatsos et al., 1993)

	Avian motoneuron deafferentation		Rescued avian motoneurons from naturally occurring or induced cell death (Oppenheim et al., 1992)
	FF transection	Partial recovery of MS/VDB neuronal loss (Morse et al., 1993; Knüsel et al., 1992; Widmer et al., 1993)	
	Spinal cord lesion		Failed sprouting of sectioned corticospinal tract (Schnell et al., 1994)
	Axotomy at cervical and thoracic levels		Prevented degeneration of rubrospinal motoneurons (Tetzlaff et al., 1994; Tetzlaff et al., 1993)
NT-3	Corticospinal transection		Sprouting of corticospinal tract (Schnell et al., 1994)
bFGF	FF transection	Prevented MS/VDB neuronal loss (Anderson et al., 1988; Otto et al., 1989; Otto et al., 1989)	
	Cortical infarction		Prevention of thalamic degeneration (Yamada et al., 1991)
	MPTP lesion		Partial recovery of dopaminergic neurons (Chadi et al., 1993)
CNTF	Motoneuron axotomy		Prevent degeneration of motoneurons (Sendtner et al., 1990; Oppenheim et al., 1991)
	FF transection	Prevented cell death and atrophy of MS/VDB neurons (Hagg et al., 1992)	Prevented cell death and atrophy of unknown septal neurons (Hagg et al., 1992)
	Nigrostriatal transection		Prevented degeneration of dopaminergic neurons (Hagg and Varon, 1993)
GDNF	Midbrain cultures		Enhanced survival of dopaminergic neurons (Lin et al., 1993)
NGF,BDNF,NT-3,NT-4, NT-5,bFGF,CNTF and LIF	DRG models		Exerted some effect on survival and differentiation of sensory neurons(Lindsay, 1988; Diamond et al., 1992; Ruit et al., 1992; Leibrock et al., 1989; Carter and Krause, 1990; Rosenthal et al., 1990; Hallböök et al., 1991; Berkemeier et al., 1991; Lin et al., 1990; Murphy et al., 1991; Eckenstein et al., 1990)

Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF,ciliary neurotrophic factor; Cort. devasc., cortical devascularization; DRG, dorsal root ganglions; GDNF, glial cell line-derived neurotrophic factor; FF, fimbria-fornix transection; LIF, leukemia inhibitory factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS/VDB, medial septum and ventral limb of the diagonal band of Broca; NBM, nucleus basalis magnocellularis; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; NT-5, neurotrophin-5; NTFs, neurotrophic factors.

NGF failed to induce changes in ChAT activity in these neurons *in vitro* (Knusel and Hefti, 1988) and in developing rat brain (Gnahn et al., 1983; Mobley et al., 1985). NGF binding sites in radioautographic studies (Richardson et al., 1986; Raivich and Kreutzberg, 1987), $p75^{NGFR}$ and $p140^{trk}$ mRNAs (Holtzman et al., 1992) and $p75^{NGFR}$-like immunoreactivity (Pioro and Cuello, 1990b) are highly co-localized with cholinergic markers. There is considerable overlap in nuclei containing both $p75^{NGFR}$-like and ChAT immunoreactivity in the brain stem of colchicine-treated animals (Pioro and Cuello, 1990a). However, this last correlation suggests that the neurotrophic factor for these neurons could be any of the neurotrophins described so far. Furthermore, taking into account the preferential distribution of endogenous NGF and its mRNA in the hippocampus and cortex in contrast to the relatively low levels in target areas of pontine cholinergic neurons (i.e.,thalamus, septum, hypothalamus and caudate-putamen) (Korsching et al., 1985; Shelton and Reichardt, 1986; Whittemore and Seiger, 1987), it is unlikely that NGF is a specific agent for cholinergic pontine projections.

I.5. - Expression, regulation and putative role of growth-associated phosphoprotein-43 (GAP-43) in neuronal outgrowth

Studies reported over the past eighteen years provide converging evidence for a specific role of the polypeptide GAP-43 (also called B-50, F1, P57 or pp46) in neuronal morphogenesis. This protein, originally called B-50, was identified as a synaptosomal plasma membrane protein (Zwiers et al., 1978; 1979; 1980). The critical involvement of GAP-43 seems to occur (i) during the period of neurite outgrowth, particularly in the motile tips (growth cones) of elongating neurites during neuronal development and regeneration (Skene and Willard, 1981a; 1981b; 1981c; Benowitz and Lewis, 1983; Redshaw and Bisby, 1984; Benowitz and Routtenberg, 1987; Skene, 1989) and (ii) during the establishment of neuronal polarity when one of the neuronal processes elongates to become the axon (Dotti et al., 1988; Goslin et al., 1990b). Goslin et al. (1990b), using immunofluorescence microscopy to investigate the localization of GAP-43, reported that it is selectively distributed to the axonal domain in developing neurons and

is absent from dendrites and their growth cones. This selective GAP-43 distribution, occurring coincidentally with the acquisition of axonal characteristics, strongly implied that this protein has a role in neuronal outgrowth. However, PC12 cells with markedly decreased levels of GAP-43 were still able to initiate extension (Baetge and Hammang, 1991) implying that GAP-43 is involved in more advanced stages of neurite outgrowth, perhaps in growth cone function and/or the operation of the presynaptic terminal. GAP-43 is synthesized as a soluble protein, transported through rapid axonal transport in a vesicle-associated form and associated with neuronal membranes in less than 20 min (Skene and Willard, 1981a; 1981b; Skene and Virag, 1989). More mature neurons exhibited less restricted GAP-43 localization with more homogeneous distribution throughout the entire axon (Goslin et al., 1990b). However, this observation *in vitro* is not in line with experiments *in vivo* showing that, during maturation, GAP-43 disappears from axons within fiber tracts but persists in some regions enriched in axon terminals and synapses (Gispen et al., 1985; Oestreicher and Gispen, 1986).

The GAP-43 gene was cloned in rat (Basi et al., 1987; Nielander et al., 1987; Rosenthal et al., 1987; Grabczyk et al., 1990; Nedivi et al., 1992) and in human (Nielander et al., 1993). The gene consists of three exons and two large introns: exon 1 codes for the N-terminal 10 amino acid domain involved in GAP-43 binding to membranes; exon 2 codes for a large central protein domain containing several functional and structural units (sites for protein kinase C-mediated phosphorylation and calmodulin binding) (Alexander et al., 1988; Labate and Skene, 1989; Liu et al., 1991), and exon 3 encodes a portion of the C-terminal protein domain that may interact with elements of the submembranous cytoskeleton (Nedivi et al., 1992).

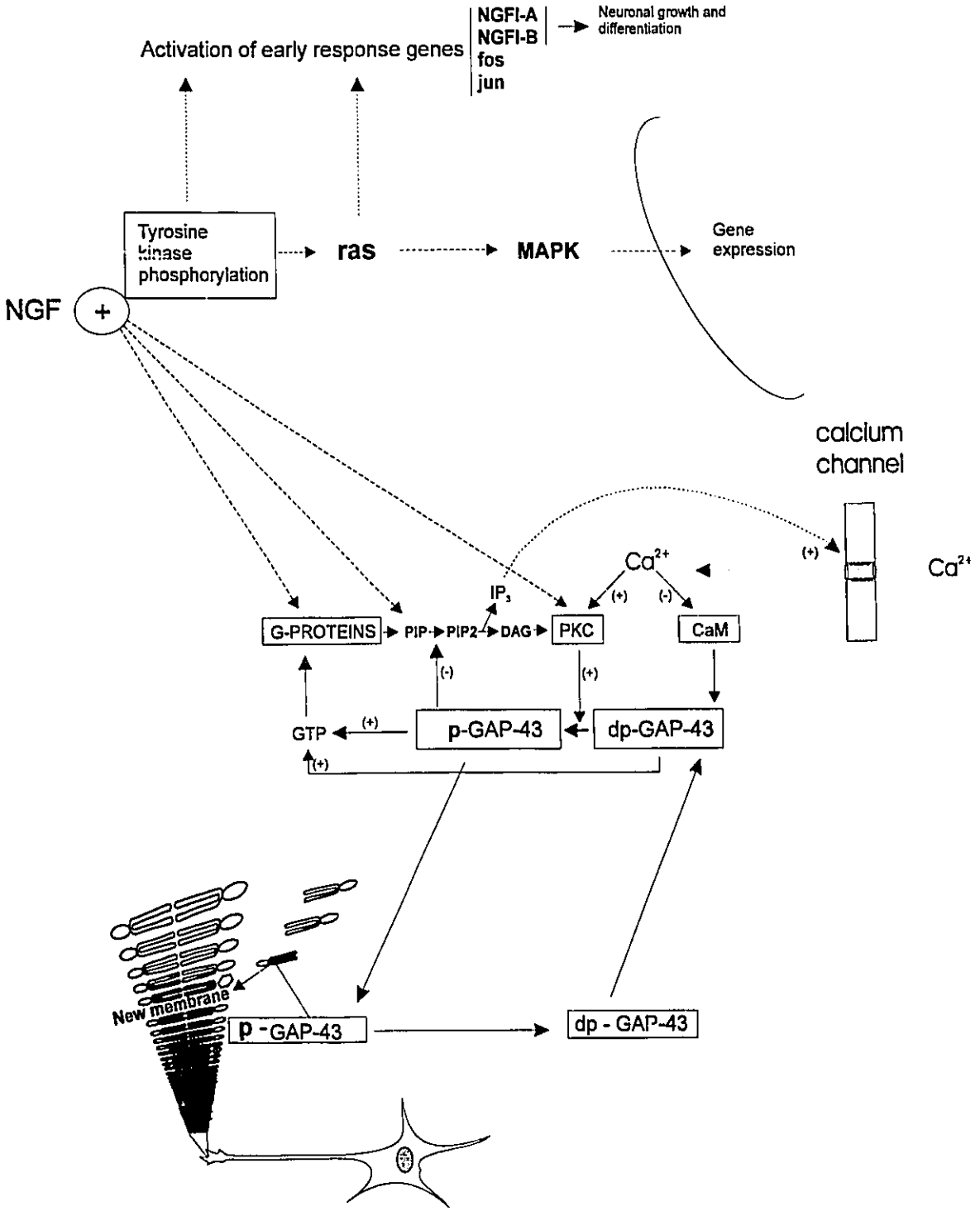
It is interesting to note that NGF induces phosphorylation of GAP-43 (Meiri and Burdick, 1991), probably via protein kinase C (Hama et al., 1986; Meiri et al., 1991). It remains to be defined if this effect is critically important for neuronal outgrowth during development or neuronal regeneration. Experiments *in vitro* suggest that NGF signalling pathways may be interconnected with a GAP-43 phosphorylation cycle (Coggins and Zwiers, 1991). With a view to defining the roles of NGF and GAP-43 and

the link between these proteins, a hypothetical diagram integrating available information about their potential second messengers is depicted in Fig. I.3. GAP-43 stimulates the binding of GTP to the α subunit of the G-protein (Strittmatter et al., 1990; 1993) which is found in high concentration in neuronal growth cone membranes (Simkowitz et al., 1989). GAP-43 was termed an unusual G-protein regulator because it is an intracellular protein (Van Hooff et al., 1989), whereas G-proteins generally are activated by transmembrane receptor-extracellular ligand complexes (Gilman, 1987). GAP-43 stimulation of $G\alpha_o$ and $G\alpha_i$ is also remarkable because it is independent of the $\beta\gamma$ subunits, is insensitive to pertussis toxin, and does not require phospholipid insertion (Strittmatter et al., 1991). The evidence that NGF and other neurotrophins also stimulate G-proteins is based on the similarity between the intracellular domain of $p75^{NGFR}$ and mastoparan, a peptide which stimulates $G\alpha_i$ and $G\alpha_o$ GTPase activity (Feinstein and Larhammer, 1990). Interestingly, during development, motion of nerve growth cones ceases on contact with particular targets. Collapse of growth cones in culture is induced by mastoporan and blocked by pertussis toxin, suggesting that growth cone collapse is mediated by G-proteins (Igarashi et al., 1993). In PC12 cells, GAP-43 was mainly associated with lysosomal structures, including multivesicular bodies, secondary lysosomas and Golgi apparatus while GAP-43 was undetected in plasma membrane. After 48 h of NGF application, GAP-43 was predominantly located in the plasma membrane surrounding sprouting microvilli, lamellipodia and filopodia (Van Hooff et al., 1989). It is appealing to propose that GAP-43 phosphorylation induced by NGF is one step leading to GAP-43 binding to the membrane (see lower part of Fig. I.3). Certain proopiocortin-derived peptides, particularly $ACTH^{1-24}$, have consensus sequences similar to the GAP-43 N-terminus, which includes the binding domain of calmodulin (CaM) and the protein kinase C ($serine^{41}$) phosphorylation site [for a review (Coggins and Zwiers, 1991)]. The fact that $ACTH^{1-24}$ is able to inhibit phosphorylation of GAP-43 *in vitro*, could be used as a tool to further explore whether the phosphorylation induced by NGF is required to initiate neuronal morphogenesis. GAP-43 is also phosphorylated by casein kinase-II (CK-II) at a distinct phosphorylation site, tentatively ascribed to either $serine^{145}$

or serine[192] (Pisano et al., 1988). It has been demonstrated *in vitro* that GAP-43 is not a substrate for other kinases such as cyclic AMP-dependent protein kinases and calcium-calmodulin-dependent kinases (I and II)(Aloyo et al., 1983; 1988), although these enzymes and others (e.g., protein kinase A and C, mitogen-activated protein (MAP2) kinase and protein kinase N) are upregulated by NGF [for reviews see (Levi and Alemà, 1991; Halegoua et al., 1991)]. These data suggest that NGF exerts its effects by multiple routes and that the protein kinase C/GAP-43 pathway is one mechanism which, while not exclusive to NGF, may still be under its influence. In support with this idea, high basal levels of GAP-43 mRNA correlated with the presence of high-affinity NGF binding sites in rat sensory neurons (Verge et al., 1990). Direct evidence that GAP-43 plays an important role in neuronal regeneration (Skene, 1989) was provided by experiments correlating depletion of GAP-43 by antisense oligonucleotides (Jap Tjoen San et al., 1992; Aigner and Caroni, 1993) or inhibition with antibodies (Shea et al., 1991), with inhibition of neurite outgrowth. Attempts have been made to relate lesion-induced changes in GAP-43 expression to functional neurochemical mechanisms mediating neuroplasticity (Coggins and Zwiers, 1991; Bisby and Tetzlaff, 1992). GAP-43 was shown to be more specifically associated with the growth of new membranes (Goslin et al., 1988; 1990a). This is in agreement with the dramatic decrease in GAP-43 concentrations following synaptogenesis and the recapitulation of this event during nerve regeneration (Benowitz et al., 1988; Van der Zee et al., 1989; Meiri and Gordon-Weeks, 1990). GAP-43 is also expressed in non-neuronal cells (Plantinga et al., 1993), refuting its prior designation as a neuron-specific phosphoprotein (Coggins and Zwiers, 1991). However, this phosphoprotein seems to be regulated in a very special way in neurons. High concentrations of GAP-43 have been correlated with retention of the ability to reshape their terminal arbors by neurons of the associative cortex (Benowitz et al., 1988), olfactory bulbs (Verhaagen et al., 1989; 1990; 1993), and enteric nervous system (Sharkey et al., 1990), among others. By contrast, neurons which failed to regenerate after injury also failed to express GAP-43 (Skene and Willard, 1981b; Skene, 1989; 1992; Doster et al., 1991; Tetzlaff et al., 1991). The limited capacity of the CNS to

Figure I.3. A hypothetical diagram showing NGF signalling leading to neuronal growth, differentiation, neuronal regeneration and cell proliferation. Cellular ras proteins function as regulated GDP/GTP switches that control transmission of information from tyrosine kinases activated by NGF (Ng and Shooter, 1993; Khosravi-Far and Der, 1994). This pathway is partially shown in this diagram. Mitogen-activated protein kinase (MAPK) has been implicated as a critical downstream component of ras signal transduction leading to cell proliferation. Activation and phosphorylation of trkA also results in activation of early response genes: *fos*, *Jun*, NGFI-A (Krox24 or zif268), NGFI-B/nur77, Egr [for reviews see (Levi and Alemà, 1991; Halegoua et al., 1991)]. The role of GAP-43 in signal transduction was reviewed by Coggins and Zwiers (1991). For the interconnections from activated NGF receptor upstream to the downstream G-proteins/PIP/PKC cycle and to other pathways not shown in this diagram (e.g. activation of Na^+/K^+ ATPase, upregulation of Ca^{2+}/calmodulin protein kinase II, as well as activation of pKA and pKN protein kinases) (Feinstein and Larhammer, 1990; Levi and Alemà, 1991; Halegoua et al., 1991). For details on the effect of NGF-induced phosphorylation of GAP-43 which may lead to new membrane formation (lower part of the diagram) see (Van Hooff et al., 1989; Goslin et al., 1988; 1990a). Abbreviations: CaM, calmodulin; DAG, diacylglycerol; dp-GAP-43, dephosphorylated GAP-43; p-GAP-43, phosphorylated GAP-43; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4-5-biphosphate; IP_3, inositol-1-4-5-triphosphate; PKC, protein kinase C.

Hypothetical NGF and GAP-43 signal transduction pathways



regenerate has been attributed to the limited amounts of specific trophic factors and to the presence of inhibitors of growth cones (Luo et al., 1993; Schwab et al., 1993; Caroni and Schwab, 1993; Bandtlow et al., 1993). More recently, evidence was presented that an injured central neuron is able to over-express GAP-43 and regenerate in response to a neurotrophin (BDNF) (Tetzlaff et al., 1993; 1994). This study was performed in rubrospinal motoneurons, in which GAP-43 overexpression induced by the lesion depended upon the distance from the axotomy site to the cell body: GAP-43 mRNA increased when the axotomy was performed at cervical but not at thoracic level (Tetzlaff et al., 1990). This is in line with the regeneration of rubrospinal neurons into cervical but not thoracic transplants of peripheral nerve (Richardson et al., 1984). Similarly, the expression of GAP-43 protein occurred in retinal ganglion cells when the injury was within 3 mm of the eye but not for axotomies at a distance of 6 mm (Doster et al., 1991). This distance-dependent regulation of the GAP-43 expression was not observed in lesioned PNS neurons (Tsui et al., 1991; Schreyer and Skene, 1993), suggesting that distinct environmental or intracellular signals are regulating the transcription of the GAP-43 gene. It was hypothesized that signal molecules act as repressors or stimulators of GAP-43 expression (Bisby, 1984); however, there is no evidence of such physiological molecules operating in the PNS or CNS.

I.6. - Thyroid hormones in CNS development

It is well known that thyroid hormones play a crucial role in the growth and differentiation of the brain. It is not clear what severity of thyroid deficiency is tolerated during pregnancy before there is fetal brain damage. Monitoring for clinical features suggestive of thyroid deficiency and measuring hormonal levels in pregnant women, in conjunction with follow up mental status examinations of the offspring of at-risk pregnancies and the use of replacement therapy, when necessary, have decreased the number of cretinic (mentally retarded) children. However, the prevalence of thyroid deficiency in pregnant women is still high (Kein et al., 1991). Thyroid hormone deprivation is very often caused by iodine deficiency, which leads to endemic cretinism.

The clinical picture of endemic cretinism depends upon the length and severity of the pre- and, mainly, postnatal thyroid hormone deficiency. When the thyroid deficiency is transmitted vertically from mother to fetus, usually around the time of the midtrimester, the result is neurological, intellectual and audiometric deficits. If this hormonal deficiency is prolonged after birth, the clinical picture is more severe. In this case, the children are typically dwarfed, sexually immature and exhibit marked clinical features of myxedema, in addition to signs of neurological damage [for an updated review see (Boyages and Halpern, 1993)]. Two frequently used animal models of thyroid hormone deficiency are: (1) a chemically-induced model, in which blockage of thyroid hormone synthesis is achieved with the administration of propylthiouracil (PTU), which also inhibits the peripheral conversion of thyroxine (T4, less potent) to triiodothyronine (T3, more potent) and (2) the surgical model where animals undergo partial or total thyroidectomy with or without administration of a lower concentration of PTU. Permanent brain damage occurs in hypothyroid rat pups and humans unless replacement therapy with thyroid hormone is started immediately after birth (Rabié and Legrand, 1973; Legrand, 1967; Klein et al., 1972). A genetic model, mutant hypothyroid mice, have also been employed (Beamer et al., 1981) but, for unknown reasons, these mice have not exhibited the abnormalities (Sugisaki et al., 1991) previously described in rats with chemically-induced hypothyroidism (Nicholson and Altman, 1972). Using these models, extensive biochemical and morphological abnormalities have been characterized in thyroid hormone-deficient rats [for reviews see (Dussault and Ruel, 1987; Legrand, 1979; 1983)].

Nuclear proteins have been identified as thyroid hormone receptor (TR) alpha and beta, which are products of (c-erbA-related) separate genes (Weinberger et al., 1986; Benbrook et al., 1988; Hodin et al., 1989; Green and Chambon, 1988; Murray et al., 1988; Sap et al., 1986; Forrest et al., 1990). Each gene gives rise to three isoforms of which TR alpha-1, TR beta-1 and TR beta-2 bind T3 and mediate the action of thyroid hormone (Sherer et al., 1993; Lechan et al., 1993; Hodin et al., 1990). These receptors behave as ligand-activated transcription factors through binding specific enhancer

elements referred to as hormone response elements in target genes (Tsai et al., 1988; Glass et al., 1987). The supposition that the developing brain, more than the mature brain, is an important target of thyroid hormone action is based on the fact that the maximal rate of thyroxine secretion by the rat thyroid gland occurs before the pre-weaning period (Vigouroux, 1976), coincident with the highest expression of biologically active TR mRNAs in the brain (Forrest et al., 1991; Sherer et al., 1993; Wills et al., 1991). TR expression correlates in turn with very high T3 binding in most of the brain regions studied (Schwartz and Oppenheimer, 1978; Perez-Castillo et al., 1985). During development, thyroid hormone has been shown to be required in neuronal and glial processes including cell migration, myelination and synaptogenesis (Legrand, 1984). The growth factor inducible gene NGFI-A is an immediate-early response gene activated by signals that lead to growth and differentiation (Milbrandt, 1987; Christy et al., 1988; Lemàire et al., 1988). Developing rat brain, but not adult animals, during thyroid hormone deprivation showed a marked decrease in NGFI-A mRNA which increased within 1 h of T3 treatment (Pipaon et al., 1992). It is interesting that NGFI-A is also induced by NGF (Milbrandt, 1987; Watson and Milbrandt, 1990). Such convergence of activity is in agreement with the hypothesis that thyroid hormones interact with NGF to promote the growth and differentiation of developing basal forebrain cholinergic and other neurons (Hayashi and Patel, 1987; Patel et al., 1988; Legrand and Clos, 1991; Clos and Legrand, 1990). It is worth noting that thyroid hormone activity in the brain of pre-weaning rats is coincident with the sharp development of forebrain cholinergic neurons (Coyle and Yamamura, 1976; Gould et al., 1991; Singh and McGeer, 1977). In support of the hypothesis that central cholinergic neurons respond to thyroid hormone, TR was found to be localized in ChAT-positive CNS neurons in culture (Garza et al., 1990) and, indeed, atrophic somata and abnormal processes of basal cholinergic neurons are seen in animals with thyroid hormone deprivation from birth (Gould and Butcher, 1989). Central cholinergic neurons which appear to be most sensitive to neonatal hypothyroidism are the same neurons which are responsive to NGF (Oh et al., 1991). It is unclear whether the reduced TR alpha receptor mRNA observed in target areas of

septohippocampal cholinergic neurons of Alzheimer's disease patients (Sutherland et al., 1992) represent a possible etiologic risk factor, are a consequence of the disease or are just a coincidence. Permanent abnormalities caused by hypothyroidism and thyroid hormone therapy are other possible risk factors in the development of Alzheimer's disease that remain to be further investigated (Butcher and Woolf, 1989; Woolf and Butcher, 1990). Hypothyroid-induced abnormalities in cerebellar neurons have been widely studied but will not be discussed here [for review see (Rabié and Legrand, 1973; Nicholson and Altman, 1972)].

Numerous phenotypic alterations have been described in myelin sheaths of differentiated oligodendrocytes as a consequence of thyroid hormone deprivation in early life, including reduced amounts of myelin-associated glycoprotein, myelin basic protein, cholesterol, cerebrosides, sulfatides, glycolipids, sulfolipids and gangliosides (Flynn et al., 1977; Bhat et al., 1979; 1981; Balazs et al., 1969; Shanker et al., 1987; Walravens and Chase, 1969; Rodriguez-Peña et al., 1993). Decreased enzymatic activity (Bhat et al., 1979), increased mRNA instability (Rodriguez-Peña et al., 1993) and changes in the rate of transcription of myelin basic protein (Farsetti et al., 1991) have been demonstrated in oligodendrocytes from hypothyroid animals. The patterns of expression of myelin-associated glycoprotein and its mRNA was studied in neonatal rats (Rodriguez-Peña et al., 1993). These investigators reported that more caudal brain regions which myelinate last (i.e., cerebellum, hypothalamus and mesencephalon) are more affected by hypothyroidism than rostral areas which myelinate earlier (i.e., hippocampus, striatum and cerebral cortex). The transient dependency of myelin genes on thyroid hormone during myelination of the developing brain has been attributed to the transitory overexpression of TR in oligodendrocytes of neonatal animals (Rodriguez-Peña et al., 1993; Mellström et al., 1991; Yusta et al., 1988; Hubank et al., 1990).

I.7. Statement of the problem

A comprehensive experimental approach by which we understand and test the function of neurotrophic factors in regeneration is based largely on work conducted in

animals with surgical neuronal lesions. Ever since NGF was discovered to be a potent factor for rescuing basal forebrain cholinergic neurons, it has seemed natural to hypothesize that two other structurally similar neurotrophins, BDNF and NT-3, could also be capable of protecting NBM cholinergic neurons. Obvious differences in the expression of trkA, trkB and trkC receptors in basal forebrain cholinergic neurons (see above) would suggest that BDNF and NT-3 are probably ineffective in promoting regeneration of NBM cholinergic neurons; however, this notion is contradicted by a recent finding that BDNF is at least able to partially prevent degeneration of septal neurons following fimbria-fornix transection. A preliminary objective was to test doses of BDNF and NT-3 which are equivalent to previously employed saturating doses of NGF using the same paradigm. Intracerebroventricular administration of these neurotrophins was initiated immediately before partial unilateral cortical devascularization. Assessment of neurotrophic efficiency was based on analysis of the post-lesion size, as well as the density of neuritic processes (dendrites and axons) of NBM cholinergic neurons identified by ChAT immunocytochemistry **(Chapter II)**.

The same procedure was carried out to investigate the therapeutic profile of aFGF, a neurotrophic factor with strong potential to rescue NBM cholinergic neurons. The intriguing features of this NTF are its ability to elicit very diverse effects in neuronal and non-neuronal cells and the fact that another member of the same family, bFGF, has been shown to prevent septal cholinergic cell death despite the current lack of evidence that these neurons express FGF receptors. Therefore, the neurotrophic properties of aFGF were studied *in vitro* using the cortical infarction model by analysing changes in the size of NBM cholinergic neurons and density of their neuritic processes (dendrites and axons) within the NBM, ChAT activity and morphology of astrocytes (*in vivo* and *in vitro*), and performance of treated-animals in water maze tasks **(Chapter III)**.

Another property of aFGF was described *in vitro*, whereby astrocytes derived from newborn brain explants released NGF in response to aFGF or bFGF treatments. This effect had not yet been described *in vivo* using mature animals. Thus, my next

objective was to address this question by producing cortical infarction in animals and assessing NGF protein and mRNA levels in the remaining cerebral cortex **(Chapter IV)**.

Much has been learned about the physiological and pharmacological properties of NGF in basal forebrain neurons. To the present, the response of the low-affinity and high-affinity NGF receptors to NBM neurons injury and NGF treatment have not been described. There is evidence that cortical injury may induce an increase in NGF expression but not to levels sufficient to rescue NBM cholinergic neurons. One of the objectives in the next chapter was to determine whether lesioned animals with injury-induced or exogenously exhibiting increased levels of NGF would display changes in the expression of $p75^{NGFR}$ mRNA in NBM neurons **(Chapter V)**. The participation of NGF in eliciting neuronal growth, preventing degeneration and inducing sprouting of peripheral and central neurons encouraged me to investigate changes in the mRNA expression of a putative marker of regeneration, growth GAP-43, employing the same *in vivo* conditions. Analysis of GAP-43 expression in NBM neurons was compared to the expression in other neurons that are not sensitive to NGF **(Chapter V)**. The distribution of these three mRNAs was investigated using *in situ* hybridization procedures.

The role of NGF in the repair of NBM cholinergic neurons is becoming well characterized. However, atrophied NBM cholinergic neurons in mature animals deprived of thyroid hormone during development were not yet evaluated with respect to NGF responsiveness. The capability of NGF to reestablish normal neuronal size or to prevent further damage caused by cortical infarction, produced in these animals at maturity, was assessed by morphometric analysis of ChAT-immunoreactive NBM neurons at two adult ages. Furthermore, expression of $p75^{NGFR}$ and $p140^{trk}$ was determined in mature animals by *in situ* hybridization in order to investigate whether thyroid hormone deprivation during development significantly affected mRNA levels of these receptors in NBM neurons **(Chapter VI)**. Of added interest in this study, parallel experiments in immature thyroid deficient animals (Appendices A and B) revealed several alterations, including in the expression of $p75^{NGFR}$ and its mRNA in pups.

REFERENCES

Aigner L, Caroni P (1993) Depletion of 43-kD growth-associated protein in primary neurons leads to diminished formation and spreading of growth cones. J Cell Biol 123:417-429.

Alexander KA, Wakim BT, Doyle GS, Walsh KA, Storm DR (1988) Identification and characterization of the calmodulin binding domain of neuromodulin, a neurospecific calmodulin-binding protein. J Biol Chem 263:7544-7549.

Aloyo VJ, Zwiers H, Gispen WH (1983) Phosphorilation of B-50 protein by calcium-activated phospholipid dependent protein kinase and B-50 protein kinase. J Neurochem 41:649-653.

Aloyo VJ, Zwiers H, De Graan PNE, Gispen WH (1988) Phosphorilation of the neuronal protein kinase C substrate B-50: in vitro assay conditions alter sensitivity to ACTH. Neurochem Res 13:343-348.

Altar CA, Burton LE, Bennett GL, Dugich-Djordjevic M (1991) Recombinant human nerve growth factor is biologically active and labels novel high-affinity binding sites in rat brain. Proc Natl Acad Sci USA 88:281-285.

Altar CA, Criden MR, Lindsay RM, DiStefano PS (1993) Characterization and topography of high-affinity ^{125}I-neurotrophin-3 binding to mammalian brain. J Neurosci 13:733-743.

Anderson KJ, Dam D, Lee S, Cotman CW (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332:360-362.

Angeletti RH, Bradshaw RA (1971) Nerve growth factor from mouse submaxillary gland: Amino acid sequence. Proc Natl Acad Sci USA 68:2417-2420.

Araujo DM, Lapchak PA, Robitaille Y, Gauthier S, Quirion R (1988) Differential alterations of various cholinergic markers in cortical and subcortical regions of human brain in Alzheimer's disease. J Neurochem 50:1914-1923.

Armelin HA (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci USA 70:2002-2006.

Baetge EE, Hammang JP (1991) Neurite outgrowth in PC12 cells deficient in GAP-43. Neuron 6:21-30.

Baird A, Ling N (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: Implications for a role of heparinase-like enzymes in the neovascular response. Biochem Biophys Res Commun 142:428-435.

Balazs R, Brooksbank BWL, Davison AN, Eayrs JT, Wilson DA (1969) The effects of neonatal thyroidectomy on myelination in the rat brain. Brain Res 15:219-232.

Baldwin AN, Bitler CM, Welcher AA, Shooter EM (1992) Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor ($p75^{NGFR}$). J Biol Chem 267:8352-8359.

Bandtlow CE, Heumann R, Schwab ME, Thoenen H (1987) Cellular localization of nerve growth factor synthesis by in situ hybridization. EMBO J 6:891-899.

Bandtlow CE, Schmidt MF, Hassinger TD, Schwab ME, Kater SB (1993) Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science 259:80-83.

Barde Y-A, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:857-866.

Barde Y-A (1987) What, if anything, is a neurotrophic factor ?. Trends Neurosci 11:343-346.

Bartus RT (1981) Physostigmine and recent memory: Effects in young and aged non-human primates. Science 206:1087-1089.

Bartus RT, Dean RL, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408-417.

Bartus RT, Dean RL, Pontecorvo MJ, Flicker C (1985) Integration of animal and human reserch from clinical to preclinical perspectives. In: Memory dysfunctions (Olton D, Gamzu E, Corkin S eds), pp 332-358. New York: New York Academy of Sciences.

Bartus RT, Dean RL, Flicker C (1987) Cholinergic psychopharmacology: An integration of human and animal research on memory. In: Psychopharmacology: The third generation of progress (Meltzer HT ed), pp 219-232. New York: Raven Press.

Basi GS, Jacobson RD, Virág I, Schilling J, Skene JHP (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell 49:785-791.

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115-165.

Beamer WG, Eicher EM, Maltais LG, Southard JL (1981) Inherited congenital goiter in mice. Science 212:61-63.

Benbrook D, Lernhardt E, Pfahl M (1988) A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature 333:669-672.

Benowitz LI, Apostolides PJ, Perrone-Bizzozero N, Finklestein SP, Zwiers H (1988) Anatomical distribution of the growth associated protein GAP-43/B-50 in the adult rat brain. J Neurosci 8:339-352.

Benowitz LI, Lewis LR (1983) Increased transport of 44,000- to 49,000-dalton acidic proteins during regeneration of the goldfish optic nerve: A two-dimensional gel analysis. J Neurosci 3:2153-2163.

Benowitz LI, Routtenberg A (1987) A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism and synaptic plasticity. Trends Neurosci 10:527-532.

Berg MM, Sternberg DW, Hempstead BL, Chao MV (1991) The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. Proc Natl Acad Sci USA 88:7106-7110.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A (1991) Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. Neuron 7:857-866.

Bhat NR, Sarlieve LL, Subba Rao G, Pieringer RA (1979) Investigations on myelination in vitro: regulation by thyroid hormone in cultures of dissociated cells from embryonic mice. J Biol Chem 254:9342-9344.

Bhat NR, Subba Rao G, Pieringer RA (1981) Investigations on myelination in vitro: regulation of sulpholipid synthesis by thyroid hormone in cultures of dissociated brain cells from embryonic mice. J Biol Chem 256:1167-1171.

Bigl V, Woolf NJ, Butcher LL (1982) Cholinergic projections fron the basal forebrain to frontal, parietal, temporal, occipital and cingulate cortices: a combined fluorescent tracer and acetylcholinesterase analysis. Brain Res Bull 8:727-749.

Bisby MA (1984) Retrograde axonal transport and nerve regeneration. In: Advances in Neurochemistry: VI. Axonal transport (ed), pp 45-67. New York: Plenum.

Bisby MA, Tetzlaff W (1992) Changes in cytoskeletal protein synthesis following axon injury and during axon regeneration. Mol Neurobiol 6:107-123.

Bocchini V, Angeletti PU (1969) The nerve growth factor: Purification as a 30,000-molecular weight protein. Proc Natl Acad Sci USA 64:787-794.

Bothwell M (1991) Keeping track of neurotrophin receptors. Cell 65:915-918.

Bowen DM, Smith CD (1976) Neurotransmitter related enzymes and indices of hypoxia in senile dementia and other abiotrophies. Brain 99:459-496.

Boyages SC, Halpern JP (1993) Endemic cretinism: toward a unfying hypothesis. Thyroid 3:59-69.

Bugra K, Pollard H, Charton G, Moreau J, Ben-Ari Y, Khrestchatisky M (1994) aFGF, bFGF and flg mRNAs show distinct patterns of induction in the hippocampus following kainate-induced seizures. Eur J Neurosci 6:58-66.

Burgess WH, Maciag T (1989) Heparin binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 58:575-606.

Butcher LL (1978) Recent advances in histochemical techniques for the study of central cholinergic mechanisms. In: Cholinergic Mechanisms and Psychopharmacology (Jenden DJ ed), pp 93-124. New York: Plenum Press.

Butcher LL (1983) Acetylcholinesterase histochemistry. In: Handbook of Chemical Neuroanatomy (ed), pp 1-49. Amsteradam: Elsevier.

Butcher LL, Bilezikjian L (1975) Acetylcholinesterase-containing nuerons in the nostriatum and substantia nigra revealed after puntate intracerebral injection of di-isopropylfluorphosphate. Eur J Pharmacol 34:115-125.

Butcher LL, Woolf NJ (1989) Neurotrophic agents may exacerbate the pathologic cascade of Alzheimer's disease. Neurobiol Aging 10:557-570.

Carey RG, Rieck RW (1987) Topographic projections to the visual cortex from the basal forebrain in the rat. Brain Res 424:205-215.

Caroni P, Schwab ME (1993) Oligodendrocyte- and myelin-associated inhibitors of neurite growth in the adult nervous system. Adv Neurol 61:175-179.

Carter MS, Krause JE (1990) Structure, expression, and some regulatory mechanisms of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K, and neuropeptide gamma. J Neurosci 10:2203-2214.

Cavicchioli L, Flanigan TP, Vantini G, Fusco M, Polato P, Toffano G, Walsh FS, Leon A (1989) NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. Eur J Neurosci 1:258-262.

Ceccatelli S, Ernfors P, Villar MJ, Persson H, Hökfelt T (1991) Expanded distribution of mRNA for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the rat brain after colchicine treatment. Proc Natl Acad Sci USA 88:10352-10356.

Chadi G, Moller A, Rosén L, Janson AM, Agnati LA, Goldstein M, Ögren S-O, Pettersson RF, Fuxe K (1993) Protective actions of human recombinant basic fibroblast growth factor on MPTP-lesioned nigrostriatal dopamine neurons after intraventricular infusion. Exp Brain Res 97:145-158.

Chao MV (1992a) Neurotrophin receptors: A window into neuronal differentiation. Neuron 9:583-593.

Chao MV (1992b) Growth factor signaling: where is the specificity. Cell 68:995-997.

Christy BA, Lau LF, Nathans D (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci USA 85:7857-7861.

Clos J, Legrand C (1990) An interaction between thyroid hormone and nerve growth factor promotes the development of hippocampus, olfactory bulbs and cerebellum. A comparative biochemical study. Growth Factors 3:205-220.

Coggins PJ, Zwiers H (1991) B-50 (GAP-43): Biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. J Neurochem 56:1095-1106.

Cohen S (1960) Purification and metabolic effects of a nerve growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. Proc Natl Acad Sci USA 46:302-311.

Coyle JT, Molliver ME, Kuhar MJ (1978) In situ injection of kainic acid, a new method for selectively lesioning neuronal cell bodies while sparring axons of passage. J Comp Neurol 180:301-324.

Coyle JT, Yamamura HI (1976) Neurochemical aspects of the ontogenesis of cholinergic neurons in the rat brain. Brain Res 118:429-440.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

Cuello AC (1993) Trophic responses of forebrain cholinergic neurons: a discussion. Prog Brain Res 98:265-277.

Cuello AC, Sofroniew MV (1984) The anatomy of CNS cholinergic neurons. Trends Neurosci 7:74-78.

Daitz HM, Powell TPS (1954) Studies on the connections of the fornix system. J Neurol Neurosurg ,Psych 17:75-82.

Das GD (1971) Projections of the interstitial nerve cells of the globus pallidus: a study of retrograde changes following cortical ablation.. Z Anat Entwicklungsgesch 133:135-160.

Davies AM, Bandtlow C, Heumann R, Korsching S, Rohrer H, Thoenen H (1987) Timing and site of nerve growth factor synthesis in developping skin in relation to innervation and expression of the receptor. Nature 326:353-358.

Davies P, Maloney A (1976) A selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2:1403

Diamond J, Foerster A, Holmes M, Coughlin M (1992) Sensory nerves in adult rats regenerate and restore sensory function to the skin independently of endogenous NGF. J Neurosci 12:1467-1476.

Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burguess WH, Jaye M, Schlessinger J (1990) Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J 9:2685-2692.

DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ (1992) The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983-993.

Divac I (1975) Magnocellular nuclei of the basal forebrain project to neocortex, brain stem and olfactory bulb. Review of some functional correlates.. Brain Res 93:385-398.

Doster SK, Lozano AM, Aguayo AJ, Willard MB (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6:635-647.

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8:1454-1468.

Dussault JH, Ruel J (1987) Thyroid hormones and brain development. Ann Rev Physiol 49:321-334.

Ebendal T, Larhammar D, Persson H (1986) Structure and expression of chicken β nerve growth factor gene. EMBO J 5:1483-1487.

Ebendal T (1992) Function and evolution in the NGF family and its receptors. J Neurosci Res 32:461-470.

Eckenstein F, Shipley GD, Nishi R (1991a) Acidic and basic fibroblast growth factors in the nervous system: Distribution and differential alteration of levels after injury of central versus peripheral nerve. J Neurosci 11:412-419.

Eckenstein F, Woodward WR, Nishi R (1991b) Differential localization and possible functions of aFGF and bFGF in the central and peripheral nervous systems. Ann NY Acad Sci 638:348-360.

Eckenstein F, Sofroniew MW (1983) Identification of central neurons containing both choline acetyltransferase and acetylcholinesterase and of central neurons containing only acetylcholinesterase. J Neurosci 3:2286-2291.

Eckenstein F, Thoenen H (1983) Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. Neurosci Lett 36:211-215.

Eckenstein FP, Esch F, Holbert T, Blacher RW, Nishi R (1990) Purification and characterization of a trophic factor for embryonic peripheral neurons: Comparison with fibroblast growth factors. Neuron 4:623-631.

Eckenstein FP, Shipley GD, Nishi R (1991) Acidic and basic fibroblast growth factors in the nervous system: Distribution and differential alteration of levels after injury of central versus peripheral nerve. J Neurosci 11:412-419.

Ernfors P, Ibáñez CF, Ebendal T, Olson L, Persson H (1990a) Molecular cloning and neurotrophic activities of a protein with structural similarities to β-nerve growth factor: developmental and topographic expression in the brain. Proc Natl Acad Sci USA 87:5454-5458.

Ernfors P, Wetmore C, Olson L, Persson H (1990b) Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron 5:511-526.

Farsetti A, Mitsuashi T, Desvergne B, Robbins J, Nikodem V (1991) Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. J Biol Chem 266:23226-23232.

Feinstein DL, Larhammer D (1990) Identification of a conserved protein motif in a group of growth factor receptors. FEBS Lett 272:7-11.

Ferguson IA, Johnson EM, Jr. (1991) Fibroblast growth factor receptor-bearing neurons in the CNS: Identification by receptor-mediated retrograde transport. J Comp Neurol 313:693-706.

Fernandez E, Pallini R, Lauretti L, Mercanti D, Serra A, Calissano P (1993) Spinal cord transection in adult rats: Effects of local infusion of nerve growth factor on the corticospinal tract axons. Neurosurgery 33:889-893.

Fisher W, Wictorin K, Bjorklund A, Williams LR, Varon S, Gage FH (1987) Ameriolation of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329:65-68.

Flynn DD, Mash DC (1986) Characterization of l[^{3}H]nicotine binding in human cerebral cortex: comparison between Alzheimer's disease and the normal. J Neurochem 47:1948-1986.

Flynn TJ, Pieringer DS, Pieringer RA (1977) Effect of altered thyroid function on galactosyl diacylglycerol metabolism in myelinating rat brain. J Biol Chem 252:5864-5870.

Forrest D, Sjoberg M, Vennstrom B (1990) Contrasting developmental and tissue-specific expression of alpha and beta thyroid hormone receptor genes. EMBO J 9:1519-1528.

Forrest D, Hallbook F, Persson H, Vennstrom B (1991) Distinct functions for thyroid homone receptors alpha and beta in brain development indicated by differential expression of receptor genes. EMBO J 10:269-275.

Gage FH, Armstrong DM, Williams LR, Varon S (1988) Morphologic response of axotomized septal neurons to nerve growth factor. J Comp Neurol 269:147-155.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1992) Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. Proc Natl Acad Sci USA 89:2639-2643.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1993) Potentiation of nerve growth factor-induced alterations in cholinergic fibre length and presynaptic terminal size in cortex of lesioned rats by the monosialoganglioside GM1. Neuroscience 57:21-40.

Garofalo L, Cuello AC (1990) Nerve growth factor and the monosialoganglioside GM1 modulate cholinergic markers and affect behavior of decorticated adult rats. Eur J Pharmacol 183:934-935.

Garofalo L, Cuello AC (1993) Nerve growth factor and the monosialoganglioside GM1: Analogous and different in vivo effects on biochemical, morphological and behavioral parameters of adult cortically leisoned rats. Exp Neurol (In Press)

Garofalo L, Cuello AC (1994) Pharmacological characterization of nerve growth factor and/or monosialoganglioside GM1 effects on cholinergic markers in the adult lesioned brain. (submitted)

Garza R, Puymirat J, Dussault JH (1990) Immunocytochemical localization of thyroid hormone nuclear receptors in cultured acetylcholinesterase-positive neurons: a correlation between the presence of thyroid hormone nuclear receptors and L-tri-iodothyronine morphological effects. Neuroscience 36:473-482.

Gibbs RB, Pfaff DW (1994) In situ hybridization detection of trkA mRNA in brain: Distribution, colocalization with $p75^{NGFR}$ and up-regulation by nerve growth factor. J Comp Neurol 341:324-339.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615-649.

Gispen WH, Leunissen JLM, Oestreicher AB, Verkleij AJ, Zwiers H (1985) Presynaptic localization of B-50 phosphoprotein: The (ACTH)-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. Brain Res 328:381-385.

Givol D, Yayon A (1992) Complexity of FGF receptors: Genetic basis for structural diversity and functional specificity. FASEB J 6:3362-3369.

Glass CK, Franco R, Weinberger C, Albert VR, Evans RM, Rosenfeld MG (1987) A c-erb-A binding site in rat growth hormone gene mediates trans-activation by thyroid hormone. Nature 329:738-741.

Gnahn H, Hefti F, Heuman R, Schwab ME, Thoenen H (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain?. Dev Brain Res 9:45-52.

Goedert M, Fine A, Hunt SP, Ullrich A (1986) Nerve growth factor mRNA in peripheral and central rat tissues and in the human nervous system: lesion effects in the rat brain and levels in Alzheimer's Disease. Mol Brain Res 1:85-92.

Gorin PD, Johnson EM (1979) Experimental autoimmune model of nerve growth factor deprivation: effects of developing peripheral sympathetic and sensory neurons. Proc Natl Acad Sci USA 76:5382-5386.

Goslin K, Schreyer DJ, Skene JHP, Banker G (1988) Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. Nature 336:672-674.

Goslin K, Schreyer DJ, Skene JHP, Banker G (1990a) Changes in the distribution of GAP-43 during the development of neuronal polarity. J Neurosci 10:588-602.

Goslin K, Schreyer DJ, Skene JHP, Banker G (1990b) J Neurosci 10:588-602.

Gospodarowicz D (1975) Purification of a fibroblast growth factor from bovine pituitary. J Biol Chem 250:2515-2520.

Gospodarowicz D, Neufeld G, Schweigerer L (1986) Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Different 19:1-17.

Gospodarowicz D, Ferrara N, Aschweigerer L, Neufeld G (1987) Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 8:95-114.

Gospodarowicz D (1991) Biological activities of fibroblast growth factors. Ann NY Acad Sci 638:1-8.

Gospodarowicz D, Cheng J (1986) Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475-484.

Gould E, Woolf NJ, Butcher LL (1991) Postnatal development of cholinergic neurons in the rat: I. Forebrain. Brain Res Bull 27:767-789.

Gould E, Butcher LL (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347-3358.

Gómez-Pinilla F, Lee JW, Cotman CW (1992a) Basic FGF in adult rat brain: Cellular distribution and response to entorhinal lesion and fimbria-fornix transection. J Neurosci 12:345-355.

Gómez-Pinilla F, Lee JW-K, Cotman CW (1992b) Basic FGF in adult rat brain: Cellular distribution and response to entorhinal lesion and fimbria-fornix transection. J Neurosci 12:345-355.

Grabczyk E, Zuber MX, Federoff HJ, Ng S-C, Pack A, Fishman MC (1990) Cloning and characterization of the rat gene encoding GAP-43. Eur J Neurosci 2:822-827.

Green S, Chambon P (1988) Nuclear receptors enhance our understanding of transcription regulation. Trends Gen 4:309-314.

Green SH, Greene LA (1986) A single M_r=103,000 ^{125}I-β-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. J Biol Chem 261:15316-15326.

Greene LA, Shooter EM, Varon S (1969) Subunit interaction and enzymatic activity of mouse 7S nerve growth factor. Biochemistry 8:3735-3741.

Greene LA, Shooter EM (1980) The nerve growth factor: Biochemistry, synthesis and mechanism of action. Annu Rev Neurosci 3:353-402.

Grothe C, Otto D, Unsicker K (1989) Basic fibroblast growth factor promotes *in vitro* survival and cholinergic development of rat septal neurons: Comparison with the effects of nerve growth factor. Neuroscience 31:649-661.

Hagg T, Quon D, Higaki J, Varon S (1992) Ciliary neurotrophic factor prevents neuronal degeneration and promotes low affinity NGF receptor expression in the adult rat CNS. Neuron 8:145-158.

Hagg T, Varon S (1993) Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons *in vivo*. Proc Natl Acad Sci USA 90:6315-6319.

Halegoua S, Armstrong RC, Kremer NE (1991) Dissecting the mode of action of a neuronal growth factor. Curr Top Microbiol Immunol 165:119-169.

Hallböök F, Ibáñez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Hama T, Huang P, Guroff G (1986) Protein kinase C as a component of nerve growth factor sensitive phosphorilation system in PC12 cells. Proc Natl Acad Sci USA 83:2352-2357.

Hayashi M, Patel AJ (1987) An interaction between thyroid hormone and nerve growth factor in the regulation of choline acetyltransferase activity in neuronal cultures, derived from the septal-diagonal dand region of the embryonic rat brain.. Dev Brain Res 36:109-120.

Heckers S, Ohtake T, Wiley R, Lappi DA, Geula C, Mesulam M (1994) Complete and selective denervation of rat neocortex and hippocampus but not amygdala by an immunotoxin against the p75 NGF receptor. J Neurosci 14:1271-1289.

Hefti F, Dravid A, Hartikka J (1984) Chronic intraventricular injections of nerve growth factor elevate hippocampal choline acetyltransferase activity in adult rats with partial septo-hippocampal lesions. Brain Res 293:305-311.

Hefti F (1986) Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 6:2155-2162.

Hefti F, Hartikka J, Salvaterra PM, Weiner WJ, Mash DC (1986) Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neurosci Lett 69:37-41.

Hempstead BL, Patil N, Thiel B, Chao MV (1990) Deletion of Cytoplasmic Sequences of the Nerve Growth Factor Receptor Leads to Loss of High Affinity Ligand Binding. J Biol Chem 265:9595-9598.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low affinity NGF receptor. Nature 350:678-683.

Henke H, Lang W (1983) Cholinergic enzymes in neocortex, hippocampus and basal forebrain of non-neurological and senile dementia of Alzheimer-type patients. Brain Res 267:218-291.

Herrup K, Shooter EM (1973) Properties of the β-nerve growth factor receptor of the avian dorsal root ganglia. Proc Natl Acad Sci USA 70:3884-3888.

Heumann R, Korsching S, Scott J, Thoenen H (1984) Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. EMBO J 3:3183-3189.

Higgins GA, Koh S, Chen KS, Gage FH (1989) NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3:247-256.

Hodin RA, Lazar MA, Wintman BI, Darling DS, Koenig RJ, Larsen PR, Moore DD, Chin WW (1989) Identification of a thyroid hormone receptor that is pituitary-specific. Science 244:76-79.

Hodin RA, Lazar MA, Chin WW (1990) Differential and tissue-specific regulation of the multiple rat c-erbA messenger RNA species by thyroid hormone. J Clin Invest 85:101-105.

Hohn A, Leibrock J, Bailey K, Barde Y-A (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 344:339-341.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen C-K, Valletta JS, Zhou J, Long JB, Mobley WC (1992) $p140^{trk}$ mRNA marks NGF-responsive forebrain neurons: Evidence that *trk* gene expression is induced by NGF. Neuron 9:465-478.

Houser CR, Crawford GD, Barber RP, Salvaterra PM, Vaughn JE (1983) Organization and morphological characteristics of cholinergic neurons: an immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Res 266:97-119.

Hubank M, Sinha AK, Gullo D, Ekins RP (1990) Nuclear triiodothyronine (T3) binding in neonatal rat brain suggests a direct glial requirement for T3 during development. J Endocrinol 126:409-415.

Hyman C, Hofer M, Barde Y-A, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230-232.

Ibáñez CF, Ebendal T, Persson H (1991) Chimeric molecules with multiple neurotrophic activities reveal structural elements determining the specificities of NGF and BDNF. EMBO J 10:2105-2110.

Ibáñez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. Cell 69:329-341.

Igarashi M, Strittmatter SM, Vartanian T, Fishman MC (1993) Mediation by G proteins of signals that cause collapse of growth cones. Science 259:77-79.

Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R, III, Squinto SP, Persson H, Yancopoulos GD (1992) Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 89:3060-3064.

Itoh N, Yazaki N, Tagashira S, Miyake A, Ozaki K, Minami M, Satoh M, Ohta M, Kawasaki T (1994) Rat FGF receptor-4 mRNA in the brain is expressed preferentially in the medial habenular nucleus. Mol Brain Res 21:344-348.

Jap Tjoen San ERA, Schmidt-Michels M, Oestreicher A, Gispen WH, Schotman P (1992) Inhibition of nerve growth factor-induced B-50/GAP-43 expression by antisense oligomers interferes with neurite outgrowth of PC12 cells. Biochem Biophys Res Commun 187:839-846.

Jeanny J-C, Fayein N, Moenner M, Chevallier B, Barritault DJ, Courtois Y (1987) Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membrane. Exp Cell Res 171:63-75.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M (1986) Expression and structure of the human NGF receptor. Cell 47:545-554.

Johnson EM, Osborne PA, Rydel RE, Schimidt RE, Pearson J (1983) Characterization of the effects of autoimmune nerve growth factor depreivation in the developing guinea pig. Neuroscience 8:631-642.

Johnston MV, McKinney M, Coyle JT (1981) Neocortical cholinergic innervation: a description of extrinsic and intrinsic components in the rat. Exp Brain Res 43:159-172.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a) The *trk* proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 252:554-558.

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine activity of the *trk* proto-oncogene product induced by NGF. Nature 350:158-160.

Keegan K, Johnson DE, Williams LT, Hayman MJ (1991) Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. Proc Natl Acad Sci USA 88:1095-1099.

Kein RZ, Haddow JE, Fein JD, Brown RS, Hermos RJ, Pulkkinen A, Mitchell ML (1991) Prevalence of thyroid deficiency in pregnant women. Clin Endocr 35:41-46.

Khosravi-Far R, Der CJ (1994) The ras signal transduction pathway. Cancer Metas Rev 13:67-89.

Kiefer MC, Stephans JC, Crawford K, Okino K, Barr PJ (1990) Ligand affinity cloning and structure of a heparin sulphate proteoglycan receptor for fibroblast growth factor. Proc Natl Acad Sci USA 87:6985-6989.

Kievet J, Kuypers HGJM (1975) Subcortical afferents to the frontal lobe in the rhesus monkey studied by means of retrograde hroseradish peroxidase transport. Brain Res 85:261-266.

Kiyota Y, Takami K, Iwane M, Shino A, Miyamoto M, Tsukuda R, Nagaoka A (1991) Increase in basic fibroblast growth factor-like immunoreactivity in rat brain after forebrain ischemia. Brain Res 545:322-328.

Klein AH, Meltzer S, Kennedy FN (1972) Improved prognosis in congenital hypothyroidism treated before age 3 months. J Pediatr 81:912-915.

Klein R, Parada LF, Coulier F, Barbacid M (1989) trkB, a novel tyrosine kinase receptor expressed during mouse neural development. EMBO J 8:3701-3709.

Klein R, Conway D, Parada LF, Barbacid M (1990) The trkB tyrosine kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-656.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991a) The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klein R, Nanduri V, Jing S, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991b) The *trk*B tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395-403.

Knusel B, Hefti F (1988) 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.

Knüsel B, Beck KD, Winslow JW, Rosenthal A, Burton LE, Widmer HR, Nickolics K, Hefti F (1992) Brain-derived neurotrophic factor administration protects basal forebrain cholinergic but not nigral dopaminergic neurons from degnerative changes after axotomy in the adult rat brain. J Neurosci 12(11):4391-4402.

Kodama S (1929) Pathalogisch-anatomische Untersuchungen mit Bezug auf die sogennanten Basalganglien und ihre Adnexe. Neurol Psychiat Abn (Shweiz Arch neurol Psychiol) 8:1-206.

Koliatsos VE, Nauta HJW, Clatterbuck RE, Holtzman DM, Mobley WC (1990) Mouse nerve growth factor prevents degeneration of axotomized basal forebrain cholinergic neurons in the monkey. J Neurosci 10:3801-3813.

Koliatsos VE, Clatterbuck RE, Nauta HJW, Knüsel B, Burton LE, Hefti FF, Mobley WC, Price DL (1991) Human nerve growth factor prevents degeneration of basal forebrain cholinergic neurons in primates. Ann Neurol 30:831-840.

Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL (1993) Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. Neuron 10:359-367.

Kordower JH, Bartus RT, Bothwell M, Schatteman G, Gash DM (1988) Nerve growth factor receptor immunoreactivity in the nonhuman primate (*Cebus apella*): distribution, morphology, and colocalization with cholinergic enzymes. J Comp Neurol 277:465-486.

Kornbluth S, Paulson KE, Hanfusa H (1988) Novel tyrosine kinase identified by phosphotyrosine antibody screening of cDNA libraries. Mol Cell Biol 8:5541-5544.

Korsching S, Auburger G, Heumann R, Scott J, Thoenen H (1985) 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.

Korsching S (1993) The neurotrophic factor concept: A reexamination. J Neurosci 13:2739-2748.

Korsching S, Thoenen H (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation.. Proc Natl Acad Sci USA 80:3513-3516.

Krnjevic K, Silver A (1965) A histochemical study of cholinergic fibers in the cerebral cortex. J Anat 99:711-759.

Kromer LF (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235:214-216.

Labate ME, Skene JHP (1989) Selective conservation of GAP-43 structure in vertebrate evolution. Neuron 3:299-310.

Lamballe F, Klein R, Barbacid M (1991) *Trk*C, a new member of the *trk* family of protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Lapchak PA, Araujo DM, Hefti F (1993) BDNF and trkB mRNA expression in the rat hippocampus following entorhinal cortex lesions. NeuroReport 4:191-194.

Lauterborn JC, Isackson PJ, Montalvo R, Gall CM (1993) In situ hybridization localization of choline acetyltransferase mRNA in adult rat brain and spinal cord. Mol Brain Res 17:59-69.

Lechan RM, Qi Y, Berrodin TJ, Davis KD, Schwartz HL, Strait KA, Oppenheimer JH, Lazar MA (1993) Immunocytochemical delineation of thyroid hormone receptor beta 2-like immunoreactivity in the rat central nervous system. Endocrinology 132:2461-2469.

Lee K-F, Huber J, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Target mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral nervous system. Cell 69:737-749.

Lee PL, Johnson DE, Cousens LS, Fried VA, Williams LT (1989) Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 245:57-60.

Legrand C, Clos J (1991) Biochemical, immunocytochemical and morphological evidence for an interaction between thyroid hormone and nerve growth factor in the developing cerebellum of normal and hypothyroid rats. Dev Neurosci 13:382-396.

Legrand J (1967) Variations, as a function of age, of the response of the cerebellum to the morphogenetic action of the thyroid in rats. Arch d'Anat Microsc Morphol Exp 56:291-307.

Legrand J (1979) Morphogenetic action of thyroid hormones. Trends Neurosci 2:234-236.

Legrand J (1983) Hormones thyroïdiennes et maturation du système nerveux. J Physiol (Paris) 78:603-652.

Legrand J (1984) Effects of thyroid hormones on central nervous system. In: Neurobehavioral Teratology (Yanay J ed), pp 331-363. Amsterdam: Elsevier.

Lehmann J, Nagy JJ, Atmadja S, Fibiger HC (1980) The nucleus basalis magnocellularis: the origin of a cholinergic projection to the neocortex of the rat. Neuroscience 5:1161-1174.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde Y-A (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149-152.

Lemàire P, Revelant O, Bravo R, Charnay P (1988) Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci USA 85:4691-4695.

Levey AI, Armstrong DM, Atweh SF, Terry RD, Wainer BH (1983) Monoclonal antibodies to choline acetyltransferase: production, specificity and immunohistochemistry. J Neurosci 3:1-9.

Levey AI, Wainer BH, Rye DB, Mufson EJ, Mesulam M-M (1984) Choline acetyltransferase-immunoreactive neurons intrinsic to rodent cortex and distinction from acetylcholinesterase-positive neurons. Neuroscience 13:341-353.

Levi A, Alemà S (1991) The mechanism of action of nerve growth factor. Annu Rev Pharmacol Toxicol 31:205-228.

Levi-Montalcini R (1982) Developmental neurobiology and the natural history of nerve growth factor. Annu Rev Neurosci 5:341-362.

Levi-Montalcini R, Angeletti PU (1963) Essential role of the nerve growth factor on the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. Dev Biol 7:653-659.

Lewis PR, Shute CCD (1967) The cholinergic limbic system: Projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supraoptic crest. Brain 90:521-540.

Liberini P, Pioro EP, Maysinger D, Ervin FR, Cuello AC (1993) Long-term protective effects of human recombinant nerve growth factor and monosialoganglioside GM1 treatment on primate nucleus basalis cholinergic neurons after cortical infarction. Neuroscience 53:625-637.

Lin L-FH, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130-1132.

Lin LF, Armes LG, Sommer A, Smith DJ, Collins F (1990) Isolation and characterization of ciliary neurotrophic factor from rabbit sciatic nerves. J Biol Chem 265:8942-8947.

Lindholm D, Hartikka J, Da Penha Berzaghi M, Castrén E, Tzimagiorgis G, Hughes RA, Thoenen H (1994) Fibroblast growth factor-5 promotes differentiation of cultured rat septal cholinergic and Raphe serotonergic neurons: Comparison with the effects of neurotrophins. Eur J Neurosci 6:244-252.

Lindsay RM (1988) The role of neurotrophic growth factors in development, maintenance, and regeneration of sensory neurons. In: The making of the nervous system (Parnavelas JG, Stern CD, Stirling RV eds), pp 148-165. Oxford UP: Oxford.

Lippa AS, Pelham RW, Beer B, Critchett DJ, Dean RL, Bartus RT (1980) Brain cholinergic dysfunction and memory in aged rats. Neurobiol Aging 1:13-19.

Liu Y, Chapman ER, Storm DR (1991) Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. Neuron 6:411-420.

Luiten PGM, Gaykema RPA, Traber J, Spencer DG (1987) Cortical projection patterns of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin. Brain Res 413:229-250.

Luo Y, Raible D, Raper A (1993) Collapsin: a protein in brain that induces the collapse and paralysis of growth cones. Cell 75:217-227.

Lysakovski A, Wainer BH, Bruce G, Hersh LB (1989) An atlas of the regional and laminar distribution of choline acetyltransferase immunoreactivity in rat cerebral cortex. Neuroscience 28:291-336.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Weigand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990a) NT-3, BDNF and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. Neuron 5:501-509.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990b) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Mash DC, Flynn DD, Potter LT (1985) Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 228:1115-1117.

Matsushima H, Bogenmann E (1990) Nerve growth factor (NGF) induces neuronal differentiation in neuroblastoma cells transfected with the NGF receptor cDNA. Mol Cell Biol 10:5015-5020.

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundel TL (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature 354:411-414.

McKinney M, Coyle JT, Hedreen JC (1983) Topographic analysis of the innervation of the rat cortex and hippocampus by the forebrain cholinergic system.. J Comp Neurol 217:103-121.

Meakin SO, Shooter EM (1991) Molecular investigations on the high-affinity nerve growth factor receptor. Neuron 6:153-163.

Meiri KF, Bickerstaff LE, Schwob JE (1991) Monoclonal antibodies show that kinase C phosphorilation of GAP-43 during axonogenesis is both spatially and temporally restricted in vivo. J Cell Biol 112:991-1005.

Meiri KF, Burdick D (1991) Nerve growth factor stimulation of GAP-43 phosphorylation of intact isolated growth cones. J Neurosci 11:3155-3164.

Meiri KF, Gordon-Weeks PR (1990) GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. J Neurosci 10:256-266.

Mellström B, Naranjo JR, Santos A, Gonzalez AM, Bernal J (1991) Independent expression of the alpha and beta c-erbA genes in developing rat brain. Mol Endocr 5:1339-1350.

Merlio J-P, Ernfors P, Jaber M, Persson H (1992) Molecular cloning of rat *trk*C and distribution of cells expressing messenger RNAs for members of the *trk* family in the rat central nervous system. Neuroscience 51:513-532.

Merlio J-P, Ernfors P, Kokaia Z, Middlemas DS, Bengzon J, Kokaia M, Smith M-L, Siesjö BK, Hunter T, Lindvall O, Persson H (1993) Increased production of the TrkB protein tyrosine kinase receptor after brain insults. Neuron 10:151-164.

Mesulam M-M, Mufson EJ, Levey AI, Wainer BH (1983a) Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. J Comp Neurol 214:170-197.

Mesulam M-M, Mufson EJ, Wainer BH, Levey AI (1983b) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience 10:1185-1201.

Mesulam MM (1986) Alzheimer plaques and cortical cholinergic innervation - Matters Arising. Neuroscience 17(1):275-276.

Middlemas DS, Lindberg RA, Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two trncated receptors. Mol Cell Biol 11:143-153.

Milbrandt J (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797-799.

Mobley WC, Rutkowski JL, Tennekoon GI, Buchanana K, Johnson MW (1985) Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. Science 229:284-287.

Montero CN, Hefti F (1988) Rescue of lesioned septal cholinergic neurons by nerve growth factor: specificity and requirement for chronic treatment. J Neurosci 8:2986-2999.

Morrison RS, Sharma A, De Vellis J, Bradshaw RA (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary cultures. Proc Natl Acad Sci USA 83:7537-7541.

Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, Miller J, DiStefano PS, Altar CA, Lindsay RM, Alderson RF (1993) Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J Neurosci 13:4146-4156.

Murphy M, Reid K, Hilton DJ, Bartlett PF (1991) Generation of sensory neurons is stimulated by leukemia inhibitory factor. Proc Natl Acad Sci USA 88:3498-3501.

Murray MB, Zilz ND, McCreary NL, MacDonald MJ, Towle HC (1988) Isolation and charaterization of rat cDNA clones for two distinct thyroid hormone receptors. J Biol Chem 263:12770-12777.

Narhi LO, Rosenfeld R, Talvenheimo J, Prestrelski SJ, Arakawa T, Lary JW, Kolvenbach CG, Hecht R, Boone T, Miller JA (1993) Comparison of the biophysical characteristics of human brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor. J Biol Chem 268:13309-13317.

Nedivi E, Basi GS, Virag I, Skene JHP (1992) A neural-specific GAP-43 core promoter located between unsual DNA elements that interact to regulate its activity. J Neurosci 12:691-704.

Ng NF, Shooter ES (1993) Activation of p21ras by nerve growth factor in embryonic sensory neurons and PC12 cells. J Biol Chem 268:25329-25333.

Nicholson JL, Altman J (1972) The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. II. Synaptogenesis in the molecular layer. Brain Res 44:25-36.

Nielander HB, Schrama LH, Van Rozen AJ, Kasperaitis M, Oestreicher AB, De Graan PNE, Gispen WH, Schotman P (1987) Primary structure of the neuron-specific phosphoprotein B-50 is identical to growth-associated protein GAP-43. Neurosci Res Commun 1:163-172.

Nielander HB, De groen PC, Eggen BJL, Schrama LH, Gispen WH, Schotman P (1993) Structure of the human gene for the neural phosphoprotein B-50 (GAP-43) Mol Brain Res 19:293-302.

Nordberg A, Winblad B (1986) Reduced numbers of [^{3}H]nicotine and [^{3}H]acetylcholine binding sites in frontal cortex of Alzheimer brains. Neurosci Lett 72:115-121.

Oestreicher AB, Gispen WH (1986) Comparison of the immunocytochemical distribution of the phosphoprotein B-50 in the cerebellum and hippocampus of mature and adult rat brain. Brain Res 375:267-279.

Oh JD, Butcher LL, Woolf NJ (1991) Thyroid hormone modulates the development of cholinergic terminal fields in the rat forebrain: Relation to nerve growth factor receptor. Dev Brain Res 53:133-142.

Oh JD, Woolf NJ, Roghani A, Edwards RH, Butcher LL (1992) Cholinergic neurons in the rat central nervous system demonstrated by *in situ* hybridization of choline acetyltransferase mRNA. Neuroscience 47:807-822.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453-501.

Oppenheim RW, Prevette D, Qin-Wei Y, Collins F, MacDonald J (1991) Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. Science 251:1616-1618.

Oppenheim RW, Qin-Wei Y, Prevette D, Yan Q (1992) Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755-757.

Ornitz D, Leder P (1992) Ligand specificity and heparin dependence of fibroblast growth factor receptors 1 and 3. J Biol Chem 267:16305-16311.

Otto D, Frotscher M, Unsicker K (1989) Basic fibroblast growth factor and nerve growth factor administered in gelfoam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22:83-91.

Otto G, Frotsher M, Unsicker K (1989) Basic fibroblast growth factor and nerve growth factor administered in gel foam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22:83-91.

Partanen J, Mäkelä TP, Eerola E, Korhonen J, Hirvonen H, Claesson-Welsh L, Alitalo K (1991) FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. EMBO J 10:1347-1354.

Pasquale EB (1990) A distinctive family of embryonic protein-tyrosine kinase receptors. Proc Natl Acad Sci USA 87:5812-5816.

Patel AJ, Hayaschi M, Hunt A (1988) Role of thyroid hormone and nerve growth factor in the development of choline acetyltransferase and other cell-specific marker enzymes in the basal forebrain of the rat. J Neurochem 50:803-811.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Perez-Castillo A, Bernal J, Ferreiro B, Pans T (1985) The early ontogenesis of thyroid hormone receptor in the rat fetus. Endocrinology 117:2457-2461.

Perry EK, Perry RH, Blessed G, Tomlinson BE (1977) Necropsy evidence of central cholinergic deficits in senile dementia. Lancet 1:189

Perry EK, Perry RH, Smith CJ, Purohit D, Bonham J, Dick DJ, Candy JM, Edwardson JA, Fairbairn A (1986) Cholinergic receptors in cognitive disorders. Can J Neurol Sci 13:521-527.

Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread Expression of BDNF But Not NT3 by Target Areas of Basal Forebrain Cholinergic Neurons. Science 250:290-294.

Pioro EP, Cuello AC (1990a) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system--II. Brainstem, cerebellum and spinal cord. Neuroscience 34:89-110.

Pioro EP, Cuello AC (1990b) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system: Effect of colchicine and correlation with the cholinergic system-I.Forebrain. Neuroscience 24:57-87.

Pipaon C, Santos A, Perez-Castillo A (1992) Thyroid hormone up-regulates NGFI-A gene expression in rat brain during development. J Biol Chem 267:21-23.

Pisano MR, Hegazy MG, Reimann EM, Dokas LA (1988) Phosphorilation of protein B-50 (GAP-43) from adult rat brain cortex by casein kinase II. Biochem Biophys Res Commun 155:1207-1212.

Plantinga LC, Verhaagen J, Gispen WH (1993) The expression of B-50/GAP-43 in Schwann cells. Ann NY Acad Sci 679:412-417.

Pleasure SJ, Reddy UR, Venkatakrishnan G, Roy AK, Chen J, Ross AH, Trojanowski JQ, Pleasure DE, Lee VMY (1990) Introduction of nerve growth factor (NGF) receptors into a medulloblastoma cell line results in expression of high- and low-affinity NGF receptors but not NGF-mediated differentiation. Proc Natl Acad Sci USA 87:8496-8500.

Price DL (1986) New perspectives on Alzheimer's disease. Annu Rev Neurosci 9:489-512.

Price JL, Stern R (1983) Individual cells in the nucleus basalis-diagonal band complex have restricted axonal projections to the cerebral cortex in the rat. Brain Res 269:352-356.

Purves D (1986) The trophic theory of neural connections. Trends Neurosci 9:486-489.

Quirion R, Aubert I, Robitaille Y, Gauthier S, Araujo DM, Chabot JG (1990) Neurochemical deficits in pathological brain aging: specificity and possible relevance for treatment strategies. Clin Neuropharmacol 13:S73-S80.

Rabié A, Legrand J (1973) Effects of thyroid hormone and undernourishment on the amount of synaptosomal fraction in the cerebellum of the young rat. Brain Res 61:267-278.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Ragsdale C, Woodgett J (1991) trking neurotrophic receptors. Nature 350:660-661.

Raivich G, Kreutzberg GW (1987) The localisation and distribution of high affinity β-nerve growth factor binding sites in the central nervous sytem of the adult rat. A light microscopic autoradiographic study using [^{125}I] β-nerve growth factor. Neuroscience 20:23-36.

Redshaw JD, Bisby MA (1984) Proteins of fast axonal transport in the regenerating hypoglossal nerve of the rat. Can J Physiol Pharmacol 62:1387-1393.

Rich KM, Luszczinski JR, Osborne PA, Johnson EM, Jr. (1987) Nerve growth factor protects adult sensory neurons from cell death and atrophy caused by nerve injury. J Neurocytol 16:261-268.

Richardson PM, Issa VM, Aguayo AJ (1984) Regeneration of long spinal axons in the rat. J Neurocytol 13:165-182.

Richardson PM, Verge Issa VMK, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 6:2312-2321.

Rinne J, Rinne JK, Laasko K, Paljarvi L, Rinne UK (1984) Reduction in muscarinic receptor binding in limbic areas of Alzheimer brain. J Neurol Neurosurg Psychiatry 47:651-653.

Rodriguez-Peña A, Ibarrola N, Iñiguez MA, Muñoz A, Bernal J (1993) Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. J Clin Invest 91:812-818.

Rodríguez-Tébar A, Dechant G, Barde Y-A (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Ron D, Reich R, Chedid M, Lengel C, Cohen OE, Chan AM-L, Neufeld G, Miki T, Tronick SR (1993) Fibroblast growth factor receptor 4 is a high affinity receptor for both acidic and basic fibroblast growth factor but not for keratinocyte growth factor. J Biol Chem 268:5388-5394.

Rosengart TK, Johnson WV, Friesel R, Clark R, Maciag T (1988a) Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. Biochem Biophys Res Commun 152:432-440.

Rosengart TK, Kupferschmidt J, Ferrans VJ, Casscells W, Maciag T, Clark RE (1988b) Heparin-binding growth factor-I (endothelial cell growth factor) binds to endothelium *in vivo*. J Vasc Surg 7:311-317.

Rosenthal A, Chan SY, Henzel W, Haskell C, Kuang W-J, Chen E, Wilcox JN, Ullrich A, Goeddel DV, Routtenberg A (1987) Primary structure and mRNA localization of protein F1, a growth-related protein kinase C substrate associated with synaptic plasticity. EMBO J 6:3641-3646.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4:767-773.

Rossor MN, Svendsen C, Hunt S, Mountjoy CG, Roth M, Iversen LL (1982) The substantia innominata in Alzheimer's disease: A histochemical and Biochemical analysis of cholinergic marker enzymes.. Neuroscience Letters 28:217-222.

Ruit KG, Elliott JL, Osborne PA, Yan Q, Snider WD (1992) Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron 8:573-587.

Ruta M, Burgess WH, Givol D, Epstein JI, Neiger N, Kaplow JM, Crumley G, Dionne CA, Jaye M, Schlessinger J (1989) Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the fms-like gene (flg). Proc Natl Acad Sci USA 86:8722-8726.

Rydel RE, Greene LA (1987) Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation. J Neurosci 7:3639-33653.

Rye DB, Wainer BH, Mesulam M-M, Mufson EJ, Saper CB (1984) Cortical projections arising from the basal forebrain: a study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase. Neuroscience 13:627-643.

Rye DB, Saper CB, Lee HJ, Wainer BH (1987) Pendunculopontine tegmental nucleus of the rat: Cytoarchitecture, cytochemistry and some extrapyramidal connections of the mesopontine tegmentum. J Comp Neurol 259:483-528.

Salsela O, Moscatelli D, Sommer A, Rifkin DB (1988) Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol 107:743-751.

Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B (1986) The c-erb-A protein is a high-affinity receptor for thyroid hormone. Nature 324:635-640.

Saper C, German D, White C (1985) Neuronal pathology in the nucleus basalis and associated cell groups in senile dementia of the Alzheimer type: possible role in cell loss. Neurology 35:1089-1095.

Saper CB (1984) Organization of cerebral cortical afferent systems in the rat. II. magnocellular basal nucleus. J Comp Neurol 222:313-342.

Schecterson LC, Bothwell M (1992) Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9:449-463.

Schnell L, Schneider R, Kolbeck R, Barde Y-A, Schwab ME (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367:170-173.

Schreyer DJ, Skene JHP (1993) Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. J Neurobiol 7:959-970.

Schubert D, Ling N, Baird A (1987) Multiple influences of heparin-binding growth factor on neuronal development. J Cell Biol 104:635-643.

Schwab ME, Kapfhammer JP, Bandtlow CE (1993) Inhibitors of neurite growth. Annu Rev Neurosci 16:565-595.

Schwartz HL, Oppenheimer JH (1978) Ontogenesis of 3,5,3'-triiodothyronine receptors in neonate rat brain: dissociation between receptor concentration and stimulation of oxygen consumption by 3,5,3'-triiodothyronine. Endocrinology 103:943-948.

Scott J, Selby M, Urdea M, Quiroga M, Bell GI, Rutter WJ (1983) Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. Nature 302:538-540.

Seiger Å, Nordberg A, Von Holst H, Bäckman L, Ebendal T, Alafuzoff I, Amberla K, Hartvig P, Herlitz A, Lilja A, Lundqvist H, Långström B, Meyerson B, Persson A, Viitanen M, Winblad B, Olson L (1993) Intracranial infusion of purified nerve growth factor to an Alzheimer patient: The first attempt of a possible future treatment strategy. Behav Brain Res 57:255-261.

Selby MJ, Edwards R, Sharp F, Rutter WJ (1987) Mouse nerve growth factor gene: structure and expression. Mol Cell Biol 7:3057-3064.

Sendtner M, Kreutzberg GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345:440-441.

Sendtner M, Holtmann B, Kolbeck R, Thoenen H, Barde Y-A (1992) Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature 360:757-759.

Shanker GA, Campagnoni AT, Pieringer RA (1987) Investigations on myelinogenesis in vitro: developmental expression of myelin basic protein mRNA and its regulation by thyroid hormone in primary cell cultures from embryonic mice. J Neurosci Res 17:220-224.

Sharkey KA, Coggins PJ, Tetzlaff W, Zwiers H, Bisby MA, Davison JS (1990) Distribution of growth-associated protein, B-50/(GAP-43) in the mammalian enteric nervous system. Neurosci 38:13-20.

Shea TB, Perrone-Bizzore ML, Benowitz LI (1991) Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neritogenesis. J Neurosci 11:1685-1690.

Shelton DL, Reichardt LF (1986) Studies on the expression of the B-NGF gene in the CNS: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci USA 83:2714-2718.

Shelton DL, Reicherdt LF (1986) Studies on the expression of β-nerve growth factor gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for sveral distinct populations of neurons. Proc Natl Acad Sci USA 83:2714-2718.

Sherer TT, Sylvester SR, Bull RJ (1993) Differential expression of the c-erbA mRNAs in the developing cerebellum and cerebral cortex of the rat. Biol Neonate 63:26-34.

Sherman KA, Kuster JE, Dean RL, Bartus RT, Friedman E (1981) Presynaptic cholinergic mechanisms in brain of aged rats with memory impairments. Neurobiol Aging 2:99-104.

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296-1299.

Shute CCD, Lewis PR (1961) Cholinergic nervous pathways in the forebrain. Nature 189:332-333.

Shute CCD, Lewis PR (1963) Cholinesterase-containing systems of the brain of the rat. Nature 199:1160-1164.

Simkowitz P, Ellis L, Pfenninger KH (1989) Membrane proteins of the nerve growth cone and their developmental regulation. J Neurosci 9:1004-1017.

Sims NR, Bowen DM, Allen SJ, Smith CCT, Thomas D, Davison AN (1983) Presynaptic cholinergic dysfunction in patients with Alzheimer's disease. J Neurochem 40:503-509.

Singh VK, McGeer EG (1977) Choline acetyltransferase in developing rat brain and spinal cord. Brain Res 127:159-163.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Skene JHP (1992) Retrograde pathways controlling expression of a major growth cone component in the adult CNS. In: The nerve growth cone (Letourneau PC, Kater SB, Macagno ER eds), pp 463-475. New York: Raven Press.

Skene JHP, Virag I (1989) Post-translational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein. J Cell Biol 108:613-625.

Skene JHP, Willard M (1981a) Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J Cell Biol 89:86-96.

Skene JHP, Willard M (1981b) Axonally transported proteins associated with growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96-103.

Skene JHP, Willard M (1981c) Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion cell axons. J Neurosci 1:419-426.

Smith G (1988) Animal models of Alzheimer's disease: experimental cholinergic denervation. Brain Res Rev 13:103-118.

Sofroniew MV, Eckenstein F, Thoenen H, Cuello AC (1982) Topography of choline acetyltransferase-containing neurons in the forebrain of the rat. Neurosci Lett 33:7-12.

Sofroniew MV, Pearson RC, Eckenstein F, Cuello AC, Powell TP (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res 289:370-374.

Sofroniew MV, Pearson RCA, Powell TPS (1987) The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. Brain Res 411:310-331.

Sommer A, Rifkin DB (1989) Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. J Cell Physiol 138:215-220.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, DiStefano PS, Yancopoulos GD (1991) *trk*B encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Stock A, Kuzis K, Woodward WR, Nishi R, Eckenstein FP (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12:4688-4700.

Strittmatter SM, Valenzuela D, Kennedy TE, Neer EJ, Fishman MC (1990) G_o is a major growth cone protein subject to regulation by GAP-43. Nature 344:836-841.

Strittmatter SM, Valenzuela D, Sudo Y, Linder ME, Fishman MC (1991) An intracellular guanine nucleotide release protein for Go. GAP-43 stimulates isolate alpha subunits by a novel mechanism. J Biol Chem 266:22465-22471.

Strittmatter SM, Cannon SC, Ross EM, Higashijima T, Fishman MC (1993) GAP-43 augments G protein-coupled receptor transduction in xenopus laevis oocytes. Proc Natl Acad Sci USA 90:5327-5331.

Sugisaki T, Noguchi T, Beamer WG, Kosak LP (1991) Genetic hypothyroid mice: normal cerebellar morphology but altered glycerol-3-phosphate dehydrogenase in bergmann glia. J Neurosci 11:2614-2621.

Sutherland MK, Wong L, Somerville MJ, Handley P, Yoong L, Bergeron C, McLachlan DR (1992) Reduction of thyroid receptor c-erb A alpha mRNA levels in the hippocampus of Alzheimer as compared to Huntington brain. Neurobiol Aging 13:301-312.

Sutter A, Riopelle LF, Harris-Harrik RM, Shooter EM (1979) Nerve growth factor receptors: characterization of two distinct classes of binding sites on chick embryo sensory gnglia cells. J Biol Chem 254:5972-5982.

Svensson A-L, Alafuzoff I, Nordberg A (1992) Characterization of muscarinic receptor subtypes in Alzheimer and control brain cortices by selective muscarinic antagonists. Brain Res 596:142-148.

Takami K, Iwane M, Kiyota Y, Miyamoto M, Tsukuda R, Shiosaka S (1992) Increase of basic fibroblast growth factor immunoreactivity and its mRNA level in rat brain following transient forebrain ischemia. Exp Brain Res 90:1-10.

Takami K, Kiyota Y, Miyamoto M, Tsukuda R, Igaraschi K, Shino A, Wanaka A, Shiosaka S, Tohyama M (1993) Upregulation of fibroblast growth factor-receptor messenger RNA expression in rat brain following transient forebrain ischemia. Exp Brain Res 97:185-194.

Taniuchi M, Schweitzer JB, Johnson EM, Jr. (1986) Nerve growth factor receptor molecules in rat brain. Proc Natl Acad Sci USA 83:1950-1954.

Tetzlaff W, Tsui BJ, Balfour JK (1990) Rubrospinal neurons increase GAP-43 and tubulin mRNA after cervical but not after thoracic axotomy. Soc Neurosci Abstr 16:338

Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11:2528-2544.

Tetzlaff W, Kobayashi NR, Bedard AM (1993) BDNF prevents atrophy of rubrospinal neurons after axotomy. Soc Neurosci Abstr 19:1104

Tetzlaff W, Kobayashi NR, Giehl KMG, Tsui BJ, Cassar SL, Bedard AM (1994) Response of rubrospinal and corticospinal neurons to injury and neurotrophins. (submitted)

Thoenen H, Bandtlow C, Heumann R (1987) The physiological function of nerve growth factor in the central nervous system: Comparison with the periphery. Rev Physiol Biochem Pharmacol 109:145-178.

Thoenen H, Barde Y-A (1980) Physiology of nerve growth factor. Physiol Rev 60:1284-1335.

Thomas KA (1987) Fibroblast growth factors. FASEB J 1:434-440.

Thomas KA, Ortega S, Soderman D, Schaeffer M-T, DiSalvo J, Gimenez-Gallego G, Linemeyer D, Kelly L, Menke J (1991) Structural modifications of acidic fibroblast growth factor alter activity, stability, and heparin dependence. Ann NY Acad Sci 638:9-17.

Togari A, Baker D, Dickens G, Guroff G (1983) The neurite promoting effect of fibroblast growth factor on PC12 cells. Biochem Biophys Res Commun 114:1189-1193.

Togari A, Dickens G, Kuzuya H, Guroff G (1985) The effect of fibroblast growth factor on PC12 cells. J Neurosci 5:307-316.

Tsai SY, Carlstedt-Duke J, Weigel NL, Dahlman K, Gustafsson JA, Tsai MJ, O'Malley BW (1988) Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell 55:361-369.

Tsui BJ, Cassar SL, Tetzlaff W (1991) Changes in mRNA levels for GAP-43, tubulin and neurofilament-M in rat spinal motoneurons after proximal versus distal axotomy. Soc Neurosci Abstr 17(1):47(Abstract)

Tuszynski MH, Sang UH, Amaral DG, Gage FH (1990) Nerve growth factor infusion in the primate brain reduces lesion-induced neural degeneration. J Neurosci 10:3604-3614.

Tuszynski MH, Sang UH, Yoshida K, Gage FH (1991) Recombinant human nerve growth factor infusions prevent cholinergic neuronal degeneration in the adult primate brain. Ann Neurol 30:625-636.

Ullrich A, Gray A, Berman C, Dull TJ (1983) Human β-nerve growth factor gene sequence highly homologous to that of mouse. Nature 303:821-825.

Unsicker K, Reichert-Preibsch H, Wewetzer K (1992) Stimulation of neuron survival by basic FGF and CNTF is a direct effect and not mediated by non-neuronal cells: Evidence from single cell cultures. Dev Brain Res 65:285-288.

Vainikka S, Partanen J, Bellosta P, Coulier F, Basilico C, Jaye M, Alitalo K (1993) Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. EMBO J 11:4273-4280.

Van der Zee CEEM, Nielander HB, Vos JP, Lopes da Silva S, Verhaagen J, Oestreicher AB, Schrama LH, Schotman P, Gispen WH (1989) Expression of growth-associated protein B50(GAP-43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. J Neurosci 9:3506-3512.

Van Hooff CO, Holthuis JC, Oestreicher AB, Boonstra J, De Graan PN, Gispen WH (1989) Nerve growth factor-induced changes in the intracellular localization of the protein kinase substrate B-50 in pheochromocytoma PC12 cells. J Cell Biol 108:1115-1125.

Varon S, Nomura J, Shooter EM (1968) Reversible dissociation of the mouse nerve growth factor protein into different subunits. Biochemistry 7:1296-1303.

Varon S, Shooter EM (1970) The nerve growth factor proteins of the mouse submaxillary gland. In: Biochemistry of brain and behavior (Bowman RE, Datta SP eds), pp 41-64. Ney York: Plenum.

Vaughn JE, Barber RP, Ribak CE, Houser CR (1981) Methods for the immunocytochemical localization of proteins and peptides involved in neurotransmission. In: Current trends in morphological techniques, vol. III (Johnson JE ed), pp 33-70. Florida: CRC Press.

Vazquez ME, Ebendal T (1991) Messenger RNAs for *trk* and the low affinity NGF receptor in basal forebrain. NeuroReport 2:593-596.

Venero JL, Knüsel B, Beck KD, Hefti F (1994) Epression 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.

Verge VMK, Riopelle RJ, Richardson PM (1989) Nerve growth factor receptors on normal and injured sensory neurons. J Neurosci 9:914-922.

Verge VMK, Tetzlaff W, Richardson PM, Bisby MA (1990) Correlation between GAP-43 and nerve growth factor receptors in rat sensory neurons. J Neurosci 10:926-934.

Verhaagen J, Oestreicher AB, Gispen WH, Margolis FL (1989) The expression of the growth associated protein B-50/GAP-43 in the olfactory system of the neonatal and adult rats. J Neurosci 9:683-691.

Verhaagen J, Oestreicher AB, Grillo M, Khew-Goodall YS, Gispen WH (1990) Neuroplasticity in the olfactory system: differential effects of central and peripheral lesions of the primary pathway on the expression of B-50/GAP-43 and the olfactory marker protein. J Neurosci Res 26:31-44.

Verhaagen J, Zhang Y, Hamers FP, Gispen WH (1993) Elevated expression of B-50 (GAP-43)-mRNA in a subpopulation of olfactory bulb mitral cells following axotomy. J Neurosci 35:162-169.

Vigouroux E (1976) Dynamic study of post-natal thyroid function in the rat. Acta Endocrinol (Copenh) 83:752-762.

Wagner JA, D'Amore PA (1986) Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. J Cell Biol 103:1363-1367.

Wainer BH, Levey AI, Mufson EJ, Mesulam MM (1984) Cholinergic systems in mammalian brain identified with antibodies against choline acetyltransferase. Neurochem Int 6:163-182.

Walicke PA (1988) Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J Neurosci 8:2618-2627.

Walicke PA, Baird A (1991) Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. J Neurosci 11:2249-2258.

Walravens P, Chase HP (1969) Influence of thyroid hormone on formation of myelin lipids. J Neurochem 16:1477-1484.

Wanaka A, Johnson EM, Jr., Milbrandt J (1990) Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. Neuron 5:267-281.

Watson MA, Milbrandt J (1990) Expression of the nerve growth factor-regulated NGFI-A and NGFI-B genes in the developing rat. Development 110:173-183.

Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM (1986) The c-erb-A gene encodes a thyroid hormone receptor. Nature 324:641-646.

Welcher AA, Bitler CM, Radeke MJ, Shooter EM (1991) Nerve growth factor binding domain of the nerve growth factor receptor. Proc Natl Acad Sci USA 88:159-163.

Wenk H, Bigl V, Meyer V (1980) Cholinergic projections from magnocellular nuclei of the basal forebrain to cortical areas in rats. Brain Res Rev 2:295-316.

Wheeler EF, Bothwell M (1992) Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. J Neurosci 12:930-945.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, DeLong MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237-1239.

Whitehouse PJ, Martino AM, Antuono PG, Lowenstein PR, Coyle JT, Price DL, Kellar KJ (1986) Nicotinic acetylcholine binding sites in Alzheimer's disease. Brain Res 371:146-151.

Whittemore SR, Ebendal T, Lärkfors L, Olson L, Seiger Å, Strömberg I, Persson H (1986) Developmental and regional expression of β nerve growth factor messenger RNA and protein in the rat central nervous system. Proc Natl Acad Sci USA 83:817-821.

Whittemore SR, Friedman PL, Larhammar D, Persson H, Gonzalez-Carvajal M, Holets VR (1988) Rat β-nerve growth factor sequence and site of synthesis in the adult hippocampus. J Neurosci Res 20:403-410.

Whittemore SR, Seiger Å (1987) The expression, localization and functional significance of β-nerve growth factor in the central nervous system. Brain Res Rev 12:439-464.

Widmer HR, Knüsel B, Hefti F (1993) BDNF protection of basal forebrain cholinergic neurons after axotomy: complete protection of p75NGFR-positive cells. NeuroReport 4:363-366.

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A, Gage FH (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc Natl Acad Sci USA 83:9231-9235.

Wills KN, Zhang XK, Pfahl M (1991) Coordinate expression of functionally distinct thyroid hormone receptor alpha isoforms during neonatal brain development. Molec Endocr 5:1109-1119.

Woodward WR, Nishi R, Meshul CK, Williams TE, Coulombe M, Eckenstein FP (1992) Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. J Neurosci 12:142-152.

Woolf NJ, Eckenstein F, Butcher LL (1983) Cholinergic projections from the basal forebrain to the frontal cortex: a combined fluorescent tracer and immunohistochemical analysis. Neurosci Lett 40:93-98.

Woolf NJ, Hernit MC, Butcher L (1986) Cholinergic and non-cholinergic projections from the rat basal forebrain revealed by combined choline acetyltransferase and Phaseolus vulgaris leucoagglutinin immunohistochemistry. Neurosci Lett 66:281-286.(Abstract)

Woolf NJ (1991) Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 37:475-524.

Woolf NJ, Butcher LL (1982) Cholinergic projections to the basolateral amygdala: a combined Evans Blue and acetylcholinesterase analysis. Brain Res Bull 8:751-763.

Woolf NJ, Butcher LL (1986) Cholinergic systems in the rat brain: III. Projections from the pontomesencephalic tegmentum to the thalamus, tectum, basal ganglia and basal forebrain. Brain Res Bull 16:603-637.

Woolf NJ, Butcher LL (1990) Dysdifferentiation of structurally plastic neurons initiates the pathologic cascade of Alzheimer's disease: towards a unifying hypothesis. In: Brain cholinergic systems (Steriade M, Biesold D eds), pp 387-438. New York: Oxford University Press.

Wright EM, Vogel KS, Davies AM (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9:139-150.

Yamada K, Kinoshita A, Kohmura E, Sakaguchi T, Taguchi J, Kataoka K, Hayakawa T (1991) Basic fibroblast growth factor prevents thalamic degeneration after cortical infarction. J Cereb Blood Flow Metab 11:472-478.

Yan H, Schlessinger J, Chao MV (1991) Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. Science 252:561-563.

Yan Q, Elliott J, Snider WD (1992) Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature 360:753-755.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

Yusta B, Besnard F, Ortiz-Caro J, Pascual A, Aranda A, Sarlieve L (1988) Evidence of the presence of nuclear 3,5,3'-triodothyronine receptors in secondary cultures of pure rat oligodendrocytes. Endocrinology 122:2278-2284.

Zilles K (1985) A stereotaxic atlas. Berlin: Springer-Verlag.

Zwiers H, Wiegant VM, Schotman P, Gispen WH (1978) ACTH-induced inhibition of endogenous rat brain protein phosphorilation in vitro: structure activity. Neurochem Res 3:455-463.

Zwiers H, Tonnaer J, Wiegant VM, Schotman P, Gispen WH (1979) ACTH-sensitive protein kinase from rat brain membranes. J Neurochem 33:247-256.

Zwiers H, Schotman P, Gispen WH (1980) Purification and some characteristcs of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. J Neurochem 34:1689-1699.

CHAPTER II

INTRAVENTRICULAR APPLICATION OF BDNF AND NT-3 FAILED TO PROTECT NBM CHOLINERGIC NEURONS

Malgorzata H. Skup, Bonald C. Figueiredo and A. Claudio Cuello

ABSTRACT

Quantitative analysis of choline acetyltransferase (ChAT) immunoreactive neurons was used to evaluate the protective potential of brain-derived neurotrophic factor (BDNF) or NT-3 against retrograde changes in nucleus basalis magnocellularis (NBM) cholinergic neurons after unilateral partial devascularization of the rat neocortex. A daily dose of 12 μg, proven to be effective for NGF and aFGF in the same experimental paradigm, was administered intracerebroventricularly by minipump infusion for a one-week period. Thirty days after lesioning, neuronal shrinkage and loss of neuritic processes were not prevented by treatment. The results indicate that BDNF and NT-3 are not as effective as NGF and aFGF in protection of NBM cholinergic neurons against lesion-induced changes in adult rat brain.

INTRODUCTION

Brain derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989) and Neurotrophin-3 (NT-3) (Maisonpierre et al., 1990) are homologs of the best characterized neurotrophin - nerve growth factor (NGF) (Levi-Montalcini, 1987). Extensive studies on the role of this prototypic trophic molecule in the adult central nervous system (CNS) have been carried out in recent years. Thus, the distribution of this protein and the pattern of its mRNA expression match the topography of target fields for brain cholinergic pathways. Furthermore, forebrain cholinergic neurons prominently display both low- and high-affinity binding sites to NGF (Chao, 1992), observations which are consistent with its postulated trophic and reparative effects on these neurons (Hefti et al., 1989; Cuello, 1993). More recently new peptides closely related to NGF have been described, namely BDNF, NT-3 and NT-4/5. Despite high structural homology with NGF and similar affinity to the low-affinity NGF receptor ($p75^{NGFR}$) (Chao, 1992), BDNF and NT-3 seemingly operate via different high-affinity neurotrophin receptors. Thus, BDNF acts predominantly on trkB (Chao, 1992) while NT-3 acts preferentially on trkC (Chao, 1992). Distinct spatial patterns of expression of NGF, BDNF and NT-3 mRNAs have been reported. BDNF mRNA has the most widespread

distribution and is very conspicuous in many areas of the cortex as well as in septum, amygdala, and the ependymal lining of the lateral ventricles (Phillips et al., 1990; Wetmore et al., 1990). The distribution of BDNF and its receptors (Phillips et al., 1990; Wetmore et al., 1990; Merlio et al., 1992; Feinstein et al., 1993) suggests that this peptide might exert widespread actions within the CNS, in contrast to the apparently more restricted actions of the NGF and NT-3 molecules. The partial overlap of expression patterns of trkB and trkC and their ligands imply a possible auto- or paracrine function rather than the target-dependent action of NGF. The differential biological role of these neurotrophins in the adult CNS remain to be established. After some brain insults, BDNF and NGF appear to be similarly regulated (Lindvall et al., 1992). However, these two neurotrophins respond differently to selective deafferentation of the hippocampus (Lapchak et al., 1993). *In vitro* NGF and, less potently, BDNF promote survival of septal cholinergic cells, up-regulate the expression of $p75^{NGFR}$ (Alderson et al., 1990), and elevate choline acetyltransferase (ChAT) activity of developing septal cholinergic neurons (Knüsel et al., 1991). Recent *in vivo* morphological studies revealed some protection from atrophy of axotomized septal cholinergic neurons with BDNF treatment (Knüsel et al., 1992; Morse et al., 1993). In this study, we aimed to characterize the effects of exogenously administered BDNF and NT-3 on the basalo-cortical pathway of the forebrain cholinergic system. This system is highly vulnerable to lesions of the target tissue and nerve terminal network. Cortical devascularization causes well-characterized retrograde changes in the (NBM), where the actions of these two novel neurotrophins can be accurately assessed in relation to the prototypical actions of NGF.

MATERIALS AND METHODS

Surgical procedure and administration of neurotrophic factors:

Twenty-four adult male Wistar rats (Charles River Breeding Laboratories, Quebec, 300-325 g) were used. To prevent infection, all the animals were pretreated with Tribrissen (24% solution, 0.02 ml/100 g body weight, s.c.) 0.5-1 h before surgery. Under Equithesin anesthesia (4.0 ml/kg, i.p.), 18 animals were subjected to unilateral

devascularizing cortical lesions, essentially as previously described (Figueiredo et al., 1993). Briefly, after removal of a 1 cm^2 flap of bone, extending longitudinally from 3 mm anterior to Bregma to 1 mm anterior to Lambda, and perpendicularly 1 cm downwards starting 1 mm from the midline, dura mater was cut out and all the exposed pia arachnoid vasculature was disrupted and removed with the use of a fine surgical needle. The lesion caused a gradual atrophy followed by a complete loss of the underlying cortex (Fig. II.1b, d, f) that encompassed portions of the frontal 1, forelimb, hindlimb and parietal 1 and 2 cortical regions, leaving intact cingulate (anteriorly, Fig. II.1a-b, c-d) and retrosplenial (posteriorly, Fig. II.1e-f), frontal 2, insular, perirhinal and entorhinal areas. Animals in which histological verification of the extent of the lesion revealed damage of the subcallosal structures were eliminated from the study. Six animals which had undergone sham operations consisting of bone removal only served as controls. Rats were implanted, immediately prior to devascularization, with permanent stainless steel cannulae (23 gauge) into the right lateral ventricle [coordinates from Bregma (Paxinos and Watson, 1986): AP, -0.8; L, 1.4; V, 3.5]. The cannulae were connected to subcutaneous Methylene blue (0.01%, BDH Chemicals)-filled osmotic minipumps (Alzet 2001) via coiled polyethylene tubing (Intramedic, PE-60). This tubing was filled with either vehicle [artificial cerebrospinal fluid (aCSF) supplemented with 0.1% bovine serum albumin (BSA, Boehringer-Mannheim)] or with BDNF or NT-3 solutions (Regeneron Pharmaceuticals, USA). Recombinant human BDNF and NT-3, prepared from conditioned media of Chinese hamster ovary cells (Altar et al., 1992), were formulated in phosphate-buffered saline (PBS), pH 7.3, at a concentration of 1 mg/ml. The bioactivity of both proteins was confirmed in bioassay in cultures of dorsal root and nodose ganglia explants or dissociated neurons. Prior to loading, frozen samples were thawed at 37°C and diluted 1:1 in 2x aCSF plus BSA to a final neurotrophin concentration of 0.5mg/ml. After the seven day continuous infusion (12μg/24μl/day), all rats were anaesthetized and the minipumps and connecting tubing were removed. Total infusion volume was determined to be 178$\pm$10 μl per rat in the BDNF-treated group, and

160±12 μl per rat in the NT-3-treated group, corresponding to an average total dose of 12.7±0.7 μg and 11.4±0.9 μg respectively.

Immunocytochemistry

Thirty days after the lesion (i.e. 23 days after the end of neurotrophin or vehicle administration) animals were anaesthetized with Equithesin (4.5 ml/kg), and perfused through the ascending aorta as previously described (Figueiredo et al., 1993). The brains were removed, postfixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, for 2h, and kept in 10% sucrose-0.1M PB at 4°C overnight followed by infiltration in 30% sucrose-PB the next day. Subsequently, 50μm coronal sections were cut on a freezing sledge microtome (Baldwin, Inc., Cambridge, U.K.) and collected in PBS, pH 7.4. The free-floating sections were washed in PBS plus 0.1% Triton X-100 (PBS-T), treated with hydrogen peroxide (0.3%) for 20 min to reduce unspecific staining, washed repeatedly in PBS-T, and incubated at 4°C overnight in anti-ChAT monoclonal antibody (Boehringer Mannheim, 1:25 dilution in PBS-T). The immunocytochemical reaction was developed by the PAP technique with the use of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen. All steps following incubation with primary antibody were carried out at room temperature using PBS-T for washes and reagent dilutions. In brief, after washings (2x 15 min), sections were incubated for 1 h in a rabbit anti-rat IgG (1:50, prepared in our laboratory). Afterwards, sections were washed (2x 15 min) and incubated for 90 min in the rat peroxidase-antiperoxidase complex (mouse antiperoxidase 1:30, Medicorp, Canada, HRP type VI, Sigma, 5 μg/ml). After rinsing (3x 15 min), sections were incubated in 0.06% solution of DAB for 10 min, and then for an additional 10 min in the same solution containing 0.01% hydrogen peroxide. The reaction was stopped by removal of the solution and rinsing. Following three rinses (3x 15 min), sections were mounted on chrom-alum subbed slides, dried, dehydrated in ascending concentrations of ethanol, cleared in xylene and cover-slipped with Entallan (BDH Inc., Quebec, Canada).

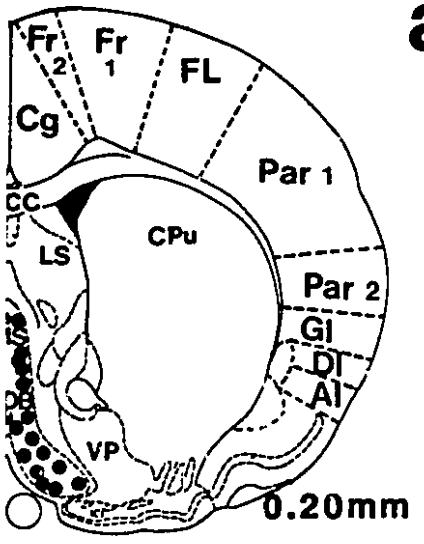
Light microscopic measurements

A total of three - six ChAT-immunostained brain sections from the intermediate portion (coordinates from Bregma -1.20 to -1.50, see Fig. II.1) of the NBM were analyzed per animal. Two NBM fields - dorsal and medioventral - in one plane of focus were investigated. The cells were visualized with an Olympus BH-2 microscope and the image (magn. 200x) was transferred via an MTICCD 72 camera to an image analysis system - Microcomputer Imaging Device (MCID, Brock University, St. Catharines, Ontario, Canada) for quantitative analysis of the area of the ChAT-immunopositive NBM neurons. The mean cross-sectional area of the cell bodies was calculated using a program with a fully automated sampling tool. Differences in the mean cross-sectional area of ChAT-immunoreactive cell bodies between experimental groups were estimated by ANOVA followed by Newman-Keuls' test. The level of statistical significance was set at $P<0.05$.

RESULTS

Thirty days after unilateral cortical devascularization, there was a significant shrinkage of ChAT-immunopositive neuronal cell bodies and a decrease in the density of neurites in the NBM ipsilateral to the lesion. The cholinergic neurites corresponded largely to dendrite profiles and occasionally to axonal profiles. As illustrated by the light microscopic micrographs (Fig. II.2), the changes in neuronal morphology caused by injury were observed both in the dorsal (the upper fields of Fig. II.2a-b) and ventral (the bottom fields of Fig. II.2a-b) parts of the intermediate regions of the NBM (mid-basalis). Quantitative analysis of the material revealed that reduction of the cross-sectional area of the ChAT-immunoreactive cholinergic neurons occurred with the same intensity both in its dorsal and medioventral parts (Fig. II.3), confirming our previous results with this model (Cuello, 1993; Figueiredo et al., 1993). The lateroventral part of the mid-basalis was much less affected while the anterior and posterior NBM regions were not affected by the cortical injury applied in these experiments. One-week's treatment of lesioned rats

Figure II.1. Schematic representation of rat brain coronal sections (a, c, e) at different levels from Bregma and matching brain slices (b, d, f) showing localization and extent of the cortical lesion one month after cortical devascularization.


a
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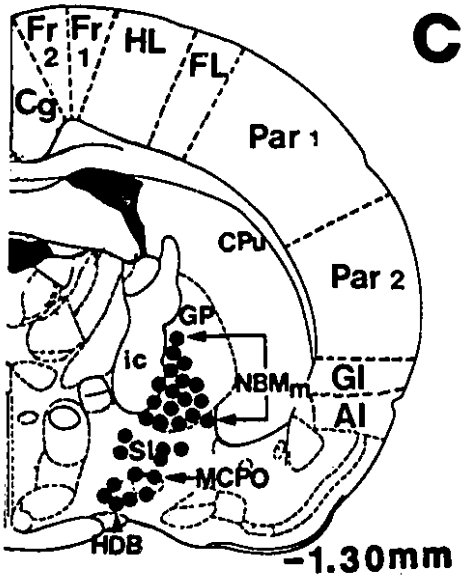
b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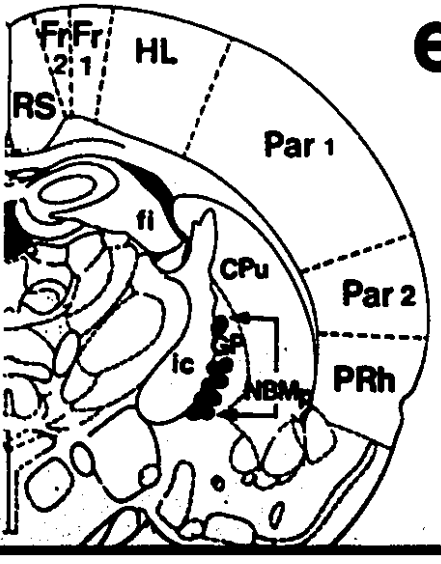
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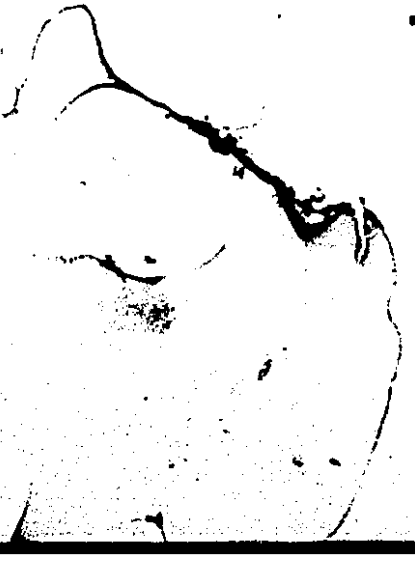
f

FIG. II.2. Microscopic appearance of ChAT-immunoreactive neurons in the intermediate nucleus basalis magnocellularis: sham operated (panel a), lesioned vehicle-treated (panel b), lesioned BDNF-treated (panel c), lesioned NT-3-treated (panel d) rats. Areas framed in a - d shown at higher magnification in a', b', c'', and d'', respectively. High-power photomicrographs show cell shrinkage and the loss of neuritic processes. Scale bar in low magnification photomicrographs = 125 μm, in high magnification photomicrographs = 20 μm.

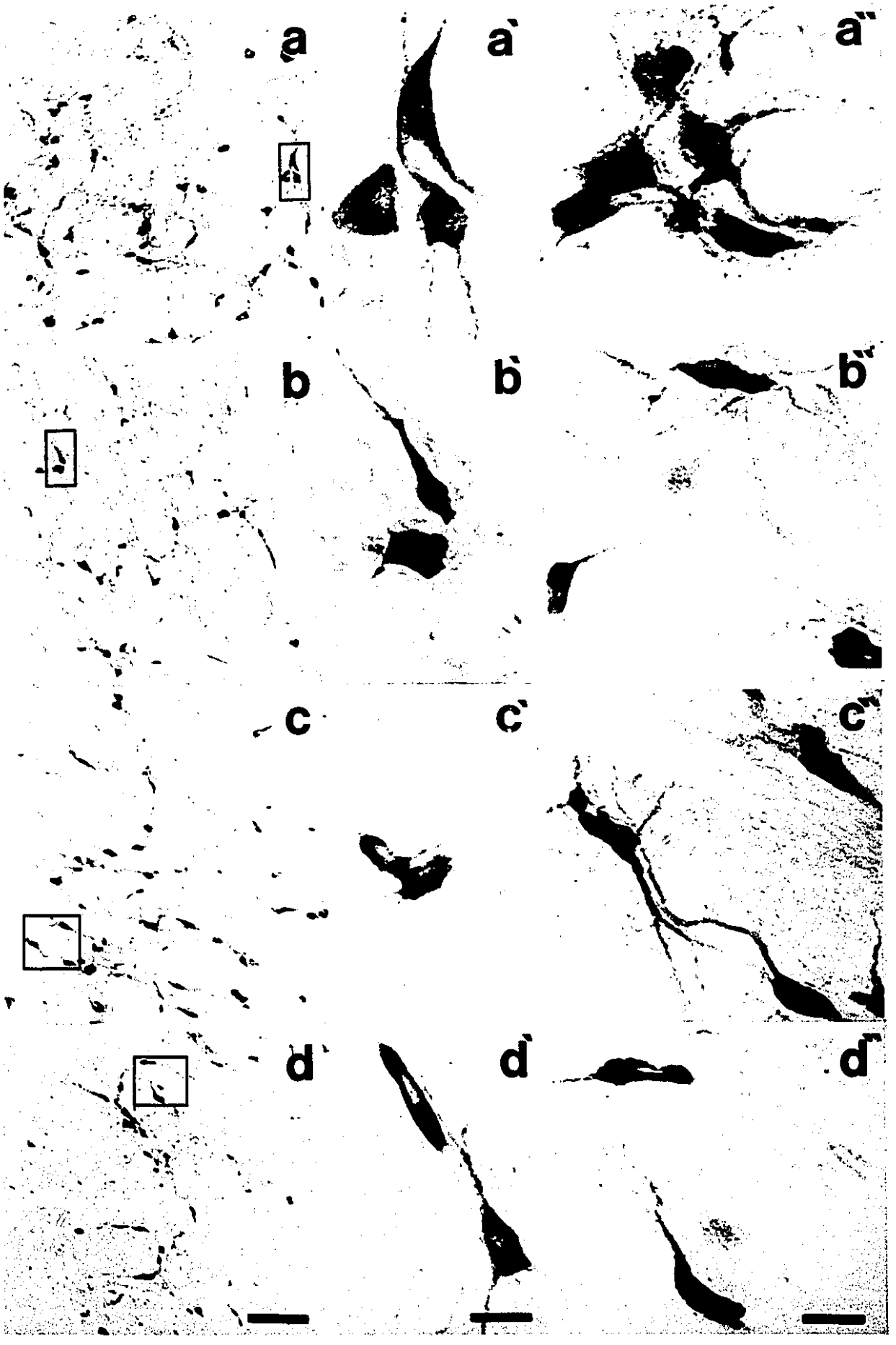
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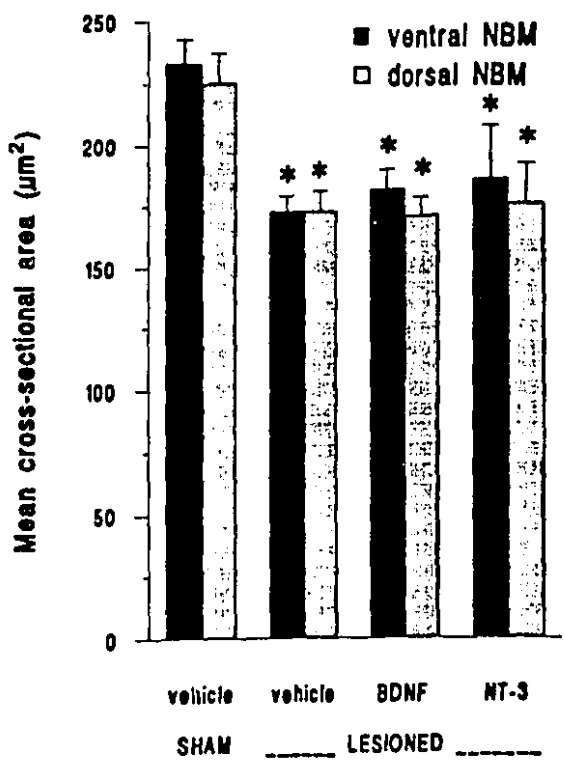
FIG. II.3. Quantitative evaluation of the effect of the cortical lesion and neurotrophin treatment on the cell size of ChAT-immunoreactive neurons in the intermediate NBM ipsilateral to the injury. Bars represent means $\pm$ S.E.M. of the cross-sectional area of randomly selected ChAT-immunoreactive neurons. Number of animals in sham operated group = 6, in other operated groups = 4. $^{*}P<0.05$ compared to sham operated group (ANOVA, followed by *post-hoc* Newman-Keuls' test).


250
200
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0
Mean cross-sectional area (μm²)
ventral NBM
dorsal NBM
vehicle
vehicle
BDNF
NT-3
SHAM
LESIONED

with BDNF or NT-3 at a daily dose of 12μg did not reduce atrophy of ChAT-immunopositive neurons (Fig. II.3). Neuronal cell bodies remained shrunken, and no restoration of neurite networks was observed (Fig. II.2, panels c and d, respectively).

DISCUSSION

In the search for effective and selective factors in the experimental therapeutics of degenerative neuronal processes in the CNS, we investigated the biological activity of BDNF and NT-3 in a model of retrograde degeneration of cholinergic neurons of the NBM after partial cortical devascularization. Obliteration of superficial pial vessels supplying the selected area of the cerebral cortex causes a gradual infarction resulting in cortical necrosis which affects the terminal network of NBM cholinergic neurons (Cuello, 1993). Our previous studies demonstrated that the intracerebroventricular infusion of NGF (Cuello, 1993) or aFGF (Figueiredo et al., 1993) to the lesioned animals provides protection of NBM cholinergic cell bodies and their neuritic network when observed one month after lesioning and three weeks after cessation of treatment. Here we demonstrate that BDNF and NT-3, when administered in the same experimental paradigm at doses previously found to be effective for NGF or aFGF, failed to prevent forebrain NBM cholinergic neurons from undergoing cell body shrinkage and retraction of neuritic networks in the NBM.

Lack of effect of either of the neurotrophins used in this study should be considered in terms of responsiveness of NBM cholinergic neurons to these factors, but also in terms of dose requirements and parenchymal penetration. Lack of data on the post-lesion regulation of trkB and trkC mRNA expression in NBM cholinergic neurons restricts the possibility to confidently address the issue of expected cellular responsiveness. Although both trkB and trkC mRNA expression are detectable in the NBM region, their levels appear to be much lower than that of trkA (Merlio et al., 1992). Evidence for the presence of functional BDNF receptors on NBM cholinergic cells comes from studies demonstrating trkB immunopositivity (Feinstein et al., 1993) and BDNF transport to the NBM after intracortical or amygdalar administration of that

factor (Kroin et al., 1993). However, it has only been shown recently that intraventricular BDNF (Morse et al., 1993) administration results in very limited diffusion from the ventricles into the adjacent neural tissue, thus, seriously limiting its availability to the NBM neurons.

The absence of a demonstrable effect of BDNF on cholinergic NBM neurons reported in the present study is comparable to the apparent lack of BDNF-mediated effects on lesion size or cholinergic cell sparing in the striatum following excitotoxic injury (Frim et al., 1993). Continuous intrastriatal BDNF administration in doses equivalent to those applied by us also failed to modify striatal ChAT activity and high-affinity choline uptake (HAChU) (Altar et al., 1992). Cell culture studies on embryonic basal forebrain neurons revealed their responsiveness to porcine and rhBDNF (Alderson et al., 1990; Knüsel et al., 1991; 1992), but showed that rhBDNF is 90 times less potent than NGF in affecting cell survival and elevation of ChAT activity (Knüsel et al., 1992); NT-3 was ineffective in that assay (Knüsel et al., 1991). Up to the present, there are no available *in vivo* studies in the cholinergic system reporting NT-3 effectiveness in neural repair. Although data obtained in cell culture studies can be roughly extrapolated to *in vivo* systems, they imply much lower potency of BDNF and NT-3 than of NGF on cholinergic cells. In the present study we used a dose (12 μg/day) of BDNF and NT-3 equal to the maximum dose of NGF and aFGF applied in previous studies (Garofalo et al., 1992, 1994; Cuello, 1993; Figueiredo et al., 1993). This dose was 120 times higher than the NGF ED50 established for the maintenance of control NBM ChAT activity in devascularized rats and supramaximal to the NGF dose causing full protection of NBM cholinergic neurons from retrograde shrinkage (Garofalo et al., 1992, 1994; Cuello, 1993). Furthermore, our choice of infusion concentration (0.5 mg/ml) allowed us to overcome technical restrictions of limited release of BDNF and NT-3 from minipumps (Altar et al., 1992), reported when lower concentrations were tested. In effect, this level of exogenously applied neurotrophin was 2.7 times higher than the BDNF dose previously shown to protect axotomized septal neurons from degeneration after a three-week administration period (Knüsel et al., 1992; Widmer et al., 1993). The

latter treatment resulted in the protection of a restricted number of cholinergic neurons which contrasts to the more complete response to NGF. Because the response of ChAT-immunopositive cells was weaker than $p75^{NGFR}$ immunoreactive cells, the authors speculated that BDNF up-regulates $p75^{NGFR}$ mRNA but not ChAT mRNA in septal cholinergic neurons. While long-lasting treatment (Knüsel et al., 1992; Widmer et al., 1993) may be postulated to cause the $p75^{NGFR}$ upregulation, in the present study no BDNF or NT-3 effect on $p75^{NGFR}$-immunoreactive neurons was observed after three weeks from cessation of treatment (not shown). The reported BDNF-induced rescue of part of the septal cholinergic neurons from axotomy-caused cell loss is not paralleled by its effects on biochemical parameters of functional recovery (Lapchak et al., 1993; Hefti et al., 1993). BDNF treatment did not affect the diminished ChAT activity and HAChU levels in synaptosomal preparations from the septal area ipsilateral to the lesion (Hefti et al., 1993), neither did it attenuate post-lesion decrease of ACh synaptosomal content (Hefti et al., 1993), and ACh release from hippocampal slices (Lapchak et al., 1993). Although these findings suggest a lower potency of BDNF in comparison to NGF, further studies applying other administration routes and doses of novel neurotrophins may disclose their effectiveness for protection of NBM cholinergic neurons after injury.

CONCLUSION

This study has shown that the continuous intraventricular administration of BDNF or NT-3 is apparently non-efficacious in the protection or recovery of cholinergic neurons of the NBM undergoing retrograde degeneration after partial cortical devascularization. The differential *in vivo* responses of basal cholinergic neurons following intraventricular delivery of various neurotrophins may be partly due to their significantly different ability to penetrate brain parenchyma and by individual responsiveness of particular neuronal cell populations. Our negative data, together with recently described findings indicating that BDNF offers less protection to injured cholinergic neurons than NGF, point to the latter as the neurotrophin of choice when attempting to counteract degenerative changes in basal forebrain cholinergic neurons.

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REFERENCES

Alderson RF, Alterman AL, Barde Y-A, Lindsay RM (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron 5:297-306.

Altar CA, Boylan CB, Jackson C, Hershenson S, Miller J, Wiegand SJ, Lindsay RM, Hyman C (1992) Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover *in vivo*. Proc Natl Acad Sci USA 89:11347-11351.

Barde Y-A. Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:549-553.

Chao MV (1992) Neurotrophin receptors: A window into neuronal differentiation. Neuron 9:583-593.

Cuello AC (1993) Trophic responses of forebrain cholinergic neurons: a discussion. Prog Brain Res 98:265-277.

Feinstein SC, Matheson CR, Radeke MJ, Welcher AA, Blumberg D, Talvenheimo J, Yan Q (1993) Immunocytochemical localization of *trk*A and *trk*B in rat central nervous system. Soc Neurosci Abstr 19:249(Abstract)

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Frim DM, Uhler TA, Short MP, Ezzedine ZD, Klagsbrun M, Breakefield XO, Isacson O (1993) Effects of biologically delivered NGF, BDNF and bFGF on striatal excitotoxic lesions. NeuroReport 4:367-370.

Garofalo, L., Ribeiro-da-Silva, A., and Cuello, A.C. (1992) Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. Proc.Natl.Acad.Sci.USA 89:2639-2643, 1992.

Garofalo, L. and Cuello, A.C. Nerve growth factor and the monosialoganglioside GM1: Analogous and different *in vivo* effects on biochemical, morphological, and behavioral parameters of adult cortically lesioned rats. *Exp.Neurol.* 125:195-217, 1994.

Hefti F, Hartikka J, Knusel B (1989) Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. Neurobiol Aging 10:515-533.

Hefti F, Knusel B, Lapchak PA (1993) Protective effects of NGF and BDNF on basal forebrain cholinergic neurons in adult rats with partial fimbrial transections. Prog Brain Res 98:257-263.

Knüsel B, Winslow JW, Rosenthal A, Burton LE, Seid DP, Nikolics K, Hefti F (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci USA 88:961-965.

Knüsel B, Beck KD, Winslow JW, Rosenthal A, Burton LE, Widmer HR, Nickolics K, Hefti F (1992) Brain-derived neurotrophic factor administration protects basal forebrain cholinergic but not nigral dopaminergic neurons from degnerative changes after axotomy in the adult rat brain. J Neurosci 12(11):4391-4402.

Kroin JS, Sobreviela T, Penn RD, Mufson EJ (1993) Retrograde transport of rHBDNF following cortical, amygdaloid, hippocampal and striatal infusion in rat. Soc Neurosci Abstr 19:260(Abstract)

Lapchak PA, Araujo DM, Hefti F (1993) Cholinergic regulation of hippocampal brain-derived neurotrophic factor mRNA expression: Evidence from lesion and chronic cholinergic drug treatment studies. Neuroscience 52:575-585.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde Y-A (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149-152.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237:1154-1162.

Lindvall O, Ernfors P, Bengzon J, Kokaia Z, Smith M-L, Siesjö BK, Persson H (1992) Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. Proc Natl Acad Sci USA 89:648-652.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Merlio J-P, Ernfors P, Jaber M, Persson H (1992) Molecular cloning of rat *trk*C and distribution of cells expressing messenger RNAs for members of the *trk* family in the rat central nervous system. Neuroscience 51:513-532.

Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, Miller J, DiStefano PS, Altar CA, Lindsay RM, Alderson RF (1993) Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J Neurosci 13:4146-4156.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread Expression of BDNF But Not NT3 by Target Areas of Basal Forebrain Cholinergic Neurons. Science 250:290-294.

Wetmore C, Ernfors P, Persson H, Olson L (1990) Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by *in situ* hybridization. Exp Neurol 109:141-152.

Widmer HR, Knüsel B, Hefti F (1993) BDNF protection of basal forebrain cholinergic neurons after axotomy: complete protection of p75NGFR-positive cells. NeuroReport 4:363-366.

One attempt was made to provide recovery of NBM neurons following intraventricular administration of BDNF and NT-3 separately. As shown in the previous chapter, this approach failed and is in agreement with a recent report showing that intraparenchymal treatment with BDNF did not rescue NBM neurons following ibotenic acid infusion (Dekker et al., 1994). These results were based on assessement of low-affinity NGF receptor ($p75^{NGFR}$)-immunoreactive neurons, a basal forebrain cholinergic marker, that was reported to be more sensitive than choline acetyltransferase (ChAT) to the *in vivo* effects of BDNF (Knüsel et al., 1992; Widmer et al., 1993; Morse et al., 1993). The failure to protect NBM cholinergic neurons [data from our study (Skup et al., 1994) and from others (Dekker et al., 1994)] is not in line with the protection of neurons from MS/VB (Knüsel et al., 1992; Widmer et al., 1993; Morse et al., 1993). There is no evidence for a differential expression of high-affinity receptor for BDNF (trkB) between NBM, and, medial septum and vertical limb of diagonal band of Broca (MS/VB). One remote possibility for these conflicting results, could be that BDNF may prevent cell death of MS/VB cholinergic neurons without being able to maintain neuronal cell size in the NBM. Although further studies, using higher doses of BDNF, would be necessary to clarify these differences between MS/VB and NBM, it was decided to concentrate our efforts in a thorough understanding on the effects of acidic fibroblast growth factor (aFGF). This FGF shares about 55% amino acid sequence identities (Gimenez-Gallego et al., 1985; Esch et al., 1985) with basic FGF which has been shown to rescue MS/VB neurons (Anderson et al., 1988; Otto et al., 1989). Furthermore, both FGFs have been shown to produce similar effects in *vitro* and in non-neuronal cells (see section 1.3. in Chapter I). Thus, data shown in the next chapter indicate that aFGF effects in NBM are similar to those of bFGF in MS/VB neurons. A coverage of the aFGF effects indicating protection of NBM neurons, as well as an improvement in the performance of a memory task, suggest that this neurotrophic agent offers great potential in the therapy of clinical problems of patients displaying an equivalent degenerative process.

REFERENCES

Anderson KJ, Dam D, Lee S, Cotman CW (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332:360-362.

Dekker AJ, Fagan AM, Gage FH, Thal LJ (1994) Effects of brain-derived neurotrophic factor and nerve growth factor on remaining neurons in the lesioned nucleus basalis magnocellularis. Brain Res 639:149-155.

Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Böhlen P, Guillemin R (1985) Primary structure of bovine basic fibroblast growth factor (FGF) and comparison with the amino terminal sequence of bovine brain acidic FGF. Proc Natl Acad Sci U S A 82:6507-6511.

Gimenez-Gallego G, Rodkey J, Bennet C, Rios-Candelore M, DiSalvo J, Thomas KA (1985) Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. Science 230:1385-1388.

Knüsel B, Beck KD, Winslow JW, Rosenthal A, Burton LE, Widmer HR, Nickolics K, Hefti F (1992) Brain-derived neurotrophic factor administration protects basal forebrain cholinergic but not nigral dopaminergic neurons from degnerative changes after axotomy in the adult rat brain. J Neurosci 12(11):4391-4402.

Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, Miller J, DiStefano PS, Altar CA, Lindsay RM, Alderson RF (1993) Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J Neurosci 13:4146-4156.

Otto D, Frotscher M, Unsicker K (1989) Basic fibroblast growth factor and nerve growth factor administered in gelfoam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22:83-91.

Skup M, Figueiredo BC, Cuello AC (1994) Intraventricularly applied BDNF and NT-3 failed to protect NBM cholinergic neurons. Neuroreport (in press) 5(8):

Widmer HR, Knüsel B, Hefti F (1993) BDNF protection of basal forebrain cholinergic neurons after axotomy: complete protection of p75NGFR-positive cells. NeuroReport 4:363-366.

CHAPTER III

EFFECTS OF ACIDIC FIBROBLAST GROWTH FACTOR ON CHOLINERGIC NEURONS OF NUCLEUS BASALIS MAGNOCELLULARIS AND IN A SPATIAL MEMORY TASK FOLLOWING CORTICAL DEVASCULARIZATION

B.C. Figueiredo, P. Piccardo, D. Maysinger, P.B.S. Clarke and A.C. Cuello

ABSTRACT

The ability of acidic fibroblast growth factor (aFGF) to elicit a trophic response in the nervous system of the rat was tested *in vitro* and *in vivo*. Treatment of cultured septal cells with aFGF resulted in an elongation of glial processes as assessed by immunostaining for glial fibrillary acidic protein. Increased choline acetyltransferase (ChAT) was also observed. The responses to aFGF *in vivo* were studied in rats trained in a spatial memory task using the Morris water maze. Randomly selected animals were subjected to unilateral cortical devascularization. This lesion results in partial unilateral infarction of the neocortex and in retrograde degeneration of the nucleus basalis magnocellularis (NBM). Animals were tested post-lesion for memory retention and were then sacrificed for morphological studies. Intracerebroventricular administration of aFGF (0.6 μg/h for seven days starting at surgery) prevented the lesion-induced impairment in this test and reduced the NBM cholinergic degeneration, as assessed by morphometric ChAT-like immunoreactivity and radioenzymatic assay for ChAT activity. The preservation of the phenotype of injured cholinergic neurons of the NBM by aFGF was indicated by the maintenance of the cross-sectional area of cell bodies and mean length of neuritic processes one month after surgery. The effect of aFGF in non-cholinergic cells remains to be investigated.

It is suggested that aFGF may alleviate the lesion-induced deficit in the memory retention task by preventing disruption of functional connections between NBM and intact cortical areas. aFGF may act together with other trophic factors such as NGF to modulate the development and/or plasticity of cholinergic central nervous system (CNS) neurons. These observations are consistent with the idea that aFGF may ameliorate NBM-dependent memory impairments that occur in degenerative diseases.

INTRODUCTION

The FGF (or heparin-binding) family refers to an expanding list of structurally homologous peptides which occur in a wide variety of peripheral and CNS tissues

(Basilico and Moscatelli, 1992). Several lines of evidence suggest that FGFs are involved in cell proliferation, differentiation, maintenance in angiogenesis, vascular repair, and chemotaxis in many regions of the CNS (Baird and Klagsbrun, 1991; Baird and Ling, 1987; Bjornsson et al., 1991; Burgess and Maciag, 1989; Gospodarowicz et al., 1986; Lobb, 1988; Rifkin and Moscatelli, 1989; Walicke, 1988). High levels of FGFs have been found in the brain (Burgess and Maciag, 1989; Gospodarowicz et al., 1987). The topographical distribution of FGF targets in rat brain has recently been elucidated through the study of receptor-mediated retrograde transport of aFGF and basic FGF (bFGF) (Ferguson et al., 1991), the best-studied and most abundant member of the FGF family of trophins (Thomas, 1987). Neither aFGF nor bFGF accumulate in basal forebrain cholinergic neurons (Ferguson et al., 1991). However, high levels of endogenous aFGF have been found in specific subcortical neuronal populations, including magnocellular neurons in the septal area and nucleus basalis (Stock et al., 1992). These findings suggest that endogenous aFGF may play a role in neuronal regeneration after injury or during development. Evidence for a role of FGFs in the repair of cholinergic neurons comes from studies showing that bFGF may rescue septal cholinergic neurons following fimbria-fornix lesions (Anderson et al., 1988; Gómez-Pinilla et al., 1992; Otto et al., 1989). That FGFs might have a direct (Knusel et al., 1990) and/or an indirect neurotrophic effect mediated via glia cells is supported by experiments showing that aFGF and bFGF increase NGF concentration in astrocyte cell cultures (Ono et al., 1991; Yoshida and Gage, 1991; 1992) and in developing hippocampus (Spranger et al., 1990). Furthermore, it has been shown that levels of aFGF, aFGF mRNA, and bFGF increase following CNS injury and that infusion of aFGF or bFGF after CNS insult prevents degeneration of spinal cord neurons (Sweetnam et al., 1991).

In patients with Alzheimer's disease taken to autopsy, histopathological changes in the brain are associated with reduced cerebral cortical ChAT activity and acetylcholine levels (Bowen and Smith, 1976; Davies and Maloney, 1976). These chemical alterations appear to reflect degenerative changes occurring in the cortical cholinergic projection neurons of the NBM (Whitehouse et al., 1982). The functional deficits characteristic of

Alzheimer's disease have been linked, at least in part, to this cholinergic loss. These observations have promoted a great deal of interest in the neurobiology of NBM which is the principal source of extrinsic cholinergic input to the neocortex (Fibiger, 1982; Luiten et al., 1987; Saper, 1984; Sofroniew et al., 1987; Woolf, 1991). Shrinkage of NBM cholinergic neurons was described one to several months after cortical devascularization (Sofroniew et al., 1983, 1987). However, it is unclear whether these atrophic neurons may still become hypertrophic after NGF. NGF has been shown to promote hippocampal reinnervation of lesioned cholinergic septal neurons (Hagg et al., 1990; Hefti, 1986; Kromer, 1987; Williams et al., 1986) to rescue injured cholinergic neurons of the NBM using an animal model of infarction (Cuello et al., 1989), or injury caused by injection of neurotoxin in the NBM (Haroutunian et al., 1989). In addition to NGF, bFGF affects the survival and transmitter metabolism of cultured embryonic rat septal neurons (Grothe et al., 1989; Knusel et al., 1990).

In the present experiments, we have assessed the therapeutic potential of aFGF in an animal model of retrograde degeneration of cholinergic neurons of the NBM following a partial devascularizing lesion of the cerebral cortex. We have investigated the effects of aFGF in the cell shrinkage of NBM cholinergic neurons which follows cortical devascularization and we have examined whether aFGF treatment of lesioned rats could modify their performance in a memory paradigm using the Morris water maze.

MATERIALS AND METHODS

Animals

Male Wistar rats (300-325g) and pregnant Sprague-Dawley rats (embryonic day 16-17) were purchased from Charles River (Quebec) and housed individually with a 12 h light/dark schedule at a temperature of 20-22^{0}C. They were given free access to food and water.

In vivo experiments

Three groups of animals were used: (i) sham-operated animals treated with vehicle only; (ii) devascularized rats treated with vehicle; and (iii) devascularized rats treated with aFGF (generously provided by Dr. K. A. Thomas, Merck-Sharp-Dohme) for seven days. Male rats were anaesthetized with Equithesin (2.5 ml/kg, i.p.) and placed in a stereotaxic frame. A stainless steel cannula (23 gauge) was permanently implanted into the right lateral ventricle [coordinates from Bregma: (Paxinos and Watson, 1986) AP, -0.8; L, 1.4; V, 3.5]. Osmotic minipumps (Alzet 2001, 1μl/h, seven days) were loaded with dye (0.01% Methylene blue, BDH Chemicals) and pretested before being connected to the cannulae through coiled polyethylene tubing (Intramedic, PE-60). aFGF (12 μg/24 μl per day) or vehicle [phosphate-buffered saline/artificial cerebrospinal fluid (PBS/CSF) 1:1 and 0.1% bovine serum albumin] was loaded into the tubing with a small amount of mineral oil added to the minipump end to prevent mixing with the dye released from the minipump.

Cortical devascularization

The animal model used in this work involves the gradual process of infarction resulting from the obliteration of superficial pial vessels supplying the selected cortical area. The cortical necrosis engulfs the terminal network of NBM cholinergic neuritic processes which project to the neocortex (Fibiger, 1982; Luiten et al., 1987; Saper, 1984; Sofroniew et al., 1987; Woolf, 1991). The outcome of this lesion is a gradual retrograde degeneration and eventual shrinkage of NBM cholinergic neuron cell bodies and neuritic processes (Sofroniew et al., 1983, 1987), as opposed to the transection of the fimbria-fornix which results in an apparent cell loss (Hefti, 1986; Kromer, 1987). Animals were trained in a Morris maze task and were then subjected to left-side devascularizing cortical lesions, as previously described (Sofroniew et al., 1983, 1987; Stephens et al., 1985). All animals were allowed to survive one month after surgery. In brief, the devascularizing lesion was performed by removing a flap of bone and underlying dura and gently rubbing away all vessels and most of the pia mater with sterile, saline-soaked cotton swabs. Control animals received a sham operation consisting

of craniotomy only. Minipumps were implanted during this surgery. Seven days after the lesion or sham operation, the animals were anaesthetized and the pumps and tubings were removed. The animals survived for a period of one month. Memory retention in all animals was then evaluated for four days and two days following the behavioral tests, animals were sacrificed. However, only animals showing well-defined lesions of the cortex (including parietal 1, forelimb, and part of frontal 1, hindlimb and parietal 2 regions (Zilles, 1985) without invasion of underlying subcallosal brain regions) were considered for further analysis. For immunocytochemical studies, 18 animals were sacrificed under Equithesin anaesthesia (2.5 ml/kg) by transcardiac perfusion with fixative as previously described (Pioro and Cuello, 1990).

Choline acetyltransferase activity determination

ChAT enzymatic activity was determined in tissue from microdissected areas according to Fonnum's procedure (Fonnum, 1975), and proteins were measured according to the procedure described by Bradford (Bradford, 1976). Six to eight animals from each group were used for biochemical studies. They were decapitated and the remaining cortex (cingulate 1 and 2, frontal 2 and part of frontal 1 and hindlimb regions) and NBM were microdissected from fresh tissue slices as previously described (Stephens et al., 1985).

Performance in the water maze

The behavioral effects of these devascularizing lesions were investigated using a previously reported procedure (Elliott et al., 1989) originally described by Morris (Morris, 1984). The test chamber consisted of a pool (diameter 140 cm; height 42 cm) containing water made blue by the addition of powdered non-toxic paint and containing a submerged blue platform. Rats were placed into the pool at different start points; the platform was kept in the same place throughout all trials. Starting randomly from four locations (north, east, south or west), close to the wall of the pool, all animals were able to learn to locate the hidden platform within 20 sec after four days of training (four

trials/day with intervals of 15 min) before surgery or treatment with drugs. Animals then underwent surgery one day after the final four-day training sessions and were retested starting 25 days later. The swimming path for each rat during each trial (total of four trials per day) was registered on a map of the pool by an observer who was blind to the experimental condition. In addition, the time required to reach platform was measured.

In vitro experiments

In preparation for *in vivo* studies, we studied septal cell cultures treated with different concentrations of aFGF. The septal area was dissected from the brains of 16 - 17 day-old embryonic rats as described (Dunnet et al., 1993). Dissociated septal cells (a mixed neuronal-glial cell population) were prepared (Cuello et al., 1989) and resuspended in culture medium to a concentration of 0.7 X 10^6 (Bowen and Smith, 1976) viable cells/well. Cells were grown in 24 multiwell plates, at 37 °C in 5% CO_2, on glass coverslips coated with 100 μg/ml poly-L-lysine. aFGF was diluted to a stock solution of 1 μg/ml in culture medium, aliquoted and stored at -20°C. Septal cultures received no treatment for the first 24 h, aFGF (25 or 50 ng/ml) was then added to three cultures and three untreated cultures served as controls. As a positive control, additional septal cultures were treated with 25 ng/ml NGF (Prince laboratories). Treated cultures were fully fed four times with NGF or aFGF-enriched medium on days one, three, six, and eight. The cells were grown for a total of nine days and then prepared for immunocytochemistry or determination of ChAT activity. A total of six controls and six independent cultures incubated with each concentration were studied in two independent experiments.

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Free-floating sections

After a 20 min washout with 10% glucose in phosphate buffer solution (PBS), the brains were removed and the appropriate area was blocked and stored in 30% sucrose-phosphate buffer at 4°C for two to four days. Coronal sections (50 μm thick) were

obtained by using a freezing sledge microtome. The sections were washed (15 min) in PBS plus 0.1% Triton X-100 (PBS-T). Sections were pretreated with hydrogen peroxide (0.3 %) to reduce background staining and were further processed free-floating. After three washes in PBS-T (10 min per wash), sections were incubated with the antibodies anti-glial fibrillary acidic protein (GFAP) (antiserum dilution 1/200, DAKO) and rat ChAT monoclonal antibody (Boehringer) for 18 h at 4°C in PBS-T solution. All subsequent steps were carried out at room temperature using PBS-T for washes, dilution of antibodies and 3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. After washing (2x10 min), sections were incubated for 2 h either in rabbit anti-rat serum (1/50; prepared in our laboratory) for anti-ChAT or in an undiluted solution of the McC8 bispecific antibody (Kenigsberg et al., 1990) (Medicorp, Canada) which recognizes rabbit IgG (anti-GFAP) and horseradish peroxidase (HRP) simultaneously. Sections were then rinsed three times in PBS-T and were further incubated for 2 h either in monoclonal rat peroxidase-antiperoxidase (Cuello et al., 1984) (1/30; Medicorp, Canada) for anti-ChAT or in a solution of HRP type VI (Sigma, 5 μg/ml in PBS-T) for GFAP immunostaining. After washing (4x10 min), sections were incubated in a 0.06% solution of DAB for 15 min and a subsequent 10 min in the same solution containing 0.01% hydrogen peroxide. After a final washing, sections were mounted on subbed slides, dehydrated, cleared and coverslipped.

Cell culture

The septal cells were washed (3x10 min) in 0.01M PBS, fixed with 2% paraformaldehyde and 15% saturated picric acid mixture in 0.1 M phosphate buffer (pH 7.2) for 30 min, and left to dry for 20 min at room temperature. To reduce background, the cells were treated with 0.3% hydrogen peroxide in PBS for 10 min, then washed in PBS (3x5 min). All antibody incubation steps, intervening washes, and DAB reaction were performed at room temperature using PBS + 10% horse serum as the solvent. The polyclonal antibody anti-GFAP was incubated with the cells for 2 h. After washing the wells (2x10 min), these cells were incubated for 1 h with the second antibody, a

monoclonal bi-specific (Kenigsberg et al., 1990) (MCC8 cell line, Medicorp, Canada). After the septal cells were rinsed, they were incubated for 1 h in a solution consisting only of HRP. All subsequent steps were performed as described for free-floating immunostaing.

Morphometric analysis

Randomly selected animals were used for immunocytochemistry image analysis investigations of ChAT-immunoreactive neurons of the NBM. A total of six brains/group were analyzed. To assess the mean cross-sectional area and total length of neuritic processes in the brain, sections stained for ChAT from control, lesioned non-treated, and aFGF-treated animals were analyzed with a computerized image analyzer (Quantimet 920; Cambridge Instruments). For these studies, the area containing the middle portion of NBM (approximately Bregma -1.30 to -1.80 mm according to Paxinos and Watson (Paxinos and Watson, 1986)) was analyzed. Morphometric data is expressed as mean cross-sectional area of cell-bodies or mean length of neuritic processes $\pm$ standard error of the mean (S.E.M.). The image analysis used to detect and quantify immunoreactive neuritic processes (axons and dendrites) employed a skeletonization routine which measures total area covered by neuritic processes as described by Mize et al (1988). These measurements were obtained from ChAT-immunoreactive neurons of the ventral and dorsal mid-portions of the NBM one month after surgery. These parameters were chosen in order to determine whether treatment with aFGF would prevent the shrinkage of these cholinergic cells in the NBM ipsilateral to the cortical lesion. Most of the devascularizing lesions result in a well-defined atrophy of the parietal 1, forelimb, part of frontal, hindlimb and parietal 2 regions.

Statistical analysis

Differences in average time to locate the platform in the Morris water maze, the mean cross-sectional area of cell bodies, and the mean length of neuritic processes of ChAT-immunoreactive neurons or ChAT activity among groups were compared with

ANOVA followed by Newman-Keuls' *post hoc* test. The level of statistical significance was set at $P<0.05$.

RESULTS

Performance in water maze task

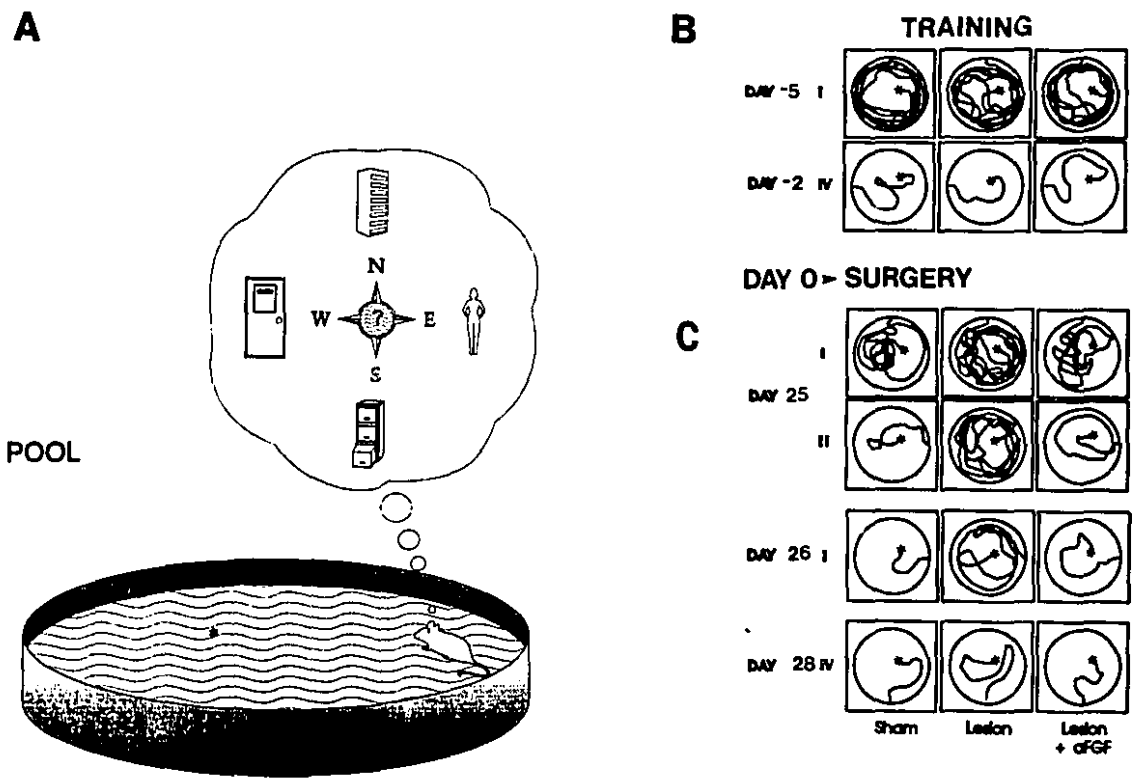
Before surgery, all animals were able to learn to locate the hidden platform (Fig. III.1A) after four days of training (Fig. III.1B). Cortical devascularization produced severe impairment in task performance 25 days post-lesion. A typical swimming pattern of lesioned vehicle-treated animals, shown in Fig. III.1C, illustrates the significant impairment in performance of the task in comparison with sham-operated rats. In contrast to the vehicle-treated animals, lesioned rats treated with aFGF were not impaired (Table III.1). There was a great deal of variation in the time spent by the lesioned rats treated with vehicle only to locate the platform. However, the time spent was significantly longer ($P<0.05$) only in the first trial (Table III.1) when compared with the performance of lesioned animals treated with aFGF 12 μg/rat/ per day. The protection afforded by aFGF treatment was observed on the first day but not on the following three days (Table III.1). Lesioned vehicle-treated animals were still able to relearn the same task (Table III.1).

Immunocytochemistry

Neuronal changes observed in decorticated vehicle-treated animals consisted mainly of cholinergic cell shrinkage, and loss of neuritic processes (Fig. III.2B,E). The NBM, contralateral to the lesioned cortex in vehicle-treated animals, showed longer multipolar neurons than the ipsilateral side one month after surgery. As illustrated by the light-microscopic micrographs shown in Fig. III.2C and F, aFGF infused for seven days in lesioned animals that survived a further 23 days, reduced the shrinkage of ChAT-immunoreactive neurons and maintained the level of staining for ChAT immunoreactivity compared with vehicle-treated rats (Fig. III.2B, E). As determined by the cross-sectional

Figure III.1

Morris water maze: (A) a pool (diameter 140 cm; height 42 cm) containing water made blue by the addition of powdered non-toxic paint and containing a submerged blue platform (shown by asterisks in A, B and C). Rats were placed into the pool at different start points; the platform was kept in the same place throughout all trials, so that the spatial location of "reference points" (such as illustrated by the door shown in the cartoon) are not altered. Starting randomly from four locations (north, east, south or west), close to the wall of the pool, all animals were able to learn to locate the hidden platform within 20 sec after four days of training (four trials/day with intervals of 15 min) before surgery or treatment with drugs. Typical swimming patterns of: (B) all groups of animals prior to any surgical manipulation and drug treatments [starting five days before ("-5") surgery]; and (C) all groups 24 days after surgical manipulation and 17 days following respective drug treatments. The platform was kept in the same place (located close to the center of the pool, as shown by the asterisks) throughout all trials. Numerals I-IV refer to the trials from day -5 to day -2 (- = before surgery) or from day 25 to day 28 (after surgery). Note disorderly swimming pattern in animals lesioned and treated with vehicle at day -5 and at the first trial of day 26. However, animals treated with vehicle were able to relearn and perform this task as well as control animals.


A
N
W
E
S
POOL
B
TRAINING
DAY -5 I
DAY -2 IV
DAY 0 ► SURGERY
C
I
DAY 25
II
DAY 26 I
DAY 28 IV
Sham
Lesion
Lesion + aFGF

area of cell bodies and length of the neuritic processes (Table III.2), neurons of the NBM ipsilateral to the lesioned cortex in aFGF-treated rats showed a substantial reduction of the degenerative changes compared with lesioned vehicle-treated rats. The detected cholinergic neuritic processes were consistently less abundant in areas with prevalent shrunken cell bodies, found mostly in the mid-portion of the NBM of vehicle-treated rats.

Choline acetyltransferase activity

The decrease in ChAT activity was statistically significant in the NBM ipsilateral to the lesion in vehicle-treated animals. In contrast, such a reduction in ChAT activity was not observed in aFGF-treated or sham-operated rats by 30 days (Fig. III.3). There

Table III.1. Effects of acidic fibroblast growth factor in water maze behavior

	Average time (sec) to locate platform		
Water maze	Control (sham)	Lesion + vehicle	Lesion + aFGF
On first day			
Trial I	38.4 ± 10.2	118.9 ± 38.1*	27.1 ± 9.6
Trial II	12.5 ± 5.4	48.0 ± 22.1	20.2 ± 7.6
Trial III	10.9 ± 4.3	29.6 ± 12.7	12.0 ± 7.0
Trial IV	22.8 ± 15.	41.6 ± 18.3	18.2 ± 12.0
Over all four trials			
Day 1	18.0 ± 4.8	62.0 ± 17.3*	15.4 ± 3.8
Day 2	8.1 ± 0.9	28.3 ± 12.8	14.0 ± 2.0
Day 3	6.1 ± 0.8	23.8 ± 6.3	11.4 ± 2.7
Day 4	11.6 ± 6.2	14.3 ± 1.8	10.5 ± 3.8

Animals underwent surgery one day after the final training sessions and were retested commencing 24 days later. Each value is the mean ± S.E.M. of eight animals per group. The difference is statistically significant (* $P<0.05$, ANOVA and Newman-Keuls' test) from sham-operated or lesioned aFGF-treated groups.

Table III.2. Morphometric analysis of *in vivo* acidic fibroblast growth factor effects in reducing retrograde degeneration of choline acetyltransferase-immunoreactive neurons of nucleus basalis magnocellularis

ChAT-immunopositive neurons	Control (sham)	Lesion + vehicle	Lesion + aFGF
Mean cross-sectional area (μm^2)			
Ventral NBM	234.5 ± 11.7	163.9 ± 21.3*	227.4 ± 11.8
Dorsal NBM	227.5 ± 13.2	157.8 ± 20.1*	214.3 ± 17.3
Mean length of neuritic processes (arbitrary units)			
Ventral NBM	42.0 ± 3.3	25.0 ± 6.1*	40.0 ± 3.8
Dorsal NBM	48.5 ± 5.9	21.2 ± 5.9*	44.0 ± 7.4

Animals survived one month after cortical devascularization. Each value is the mean ± S.E.M. of six animals per group. The difference is statistically significant (* $P<0.05$, ANOVA and Newman-Keuls' test) from sham-operated or lesioned aFGF-treated groups.

Table III.3. Effects of partial unilateral cortical devascularization and acidic fibroblast growth factor on choline acetyltransferase activity

Brain region	ChAT activity (nmol of acetylcholine/mg protein per h)		
	Control (sham)	Lesion + vehicle	Lesion + aFGF
NBM			
Contralateral to the decortication	109.8 ± 12.2	110.9 ± 10.8	93.0 ± 10.0
Ipsilateral to the decortication	99.2.± 6.6	58.1 ± 6.3*	87.2 ± 10.2
Cortex			
Contralateral to the decortication	27.6 ± 1.6	27.8 ± 2.6	27.0 ± 1.6
Ipsilateral to the decortication	29.4 ± 1.5	29.0 ± 0.9	27.3 ± 1.3

Animals survived one month after cortical devascularization. Microdissected cortical tissues are the remaining adjacent cortex, which includes cingulate, frontal 2 and part of frontal 1 and hindlimb cortices. Mean values ± S.E.M. of six to eight-animals per group are shown. The difference is statistically significant (* $P<0.05$, ANOVA and Newman-Keuls' test) from sham-operated or lesioned aFGF-treated groups.

Table III.4. Effects of acidic fibroblast growth factor and nerve growth factor on choline acetyltransferase activity in septal cultures

Septal culture treatment	ChAT activity (CPM)
Control	5000 ± 470
aFGF (25 ng/ml per well)	10250 ± 580*
aFGF (50 ng/ml per well)	10850 ± 400*
NGF (25 ng/ml per well)	12500 ± 950*

Dissociated fetal rat septal cells were grown for one week in the presence of aFGF, NGF or in their absence. Data were pooled from three experiments and are expressed as mean ± S.E.M. The absolute value of ChAT activity in control cultures was 4.8 nmol of acetylcholine/h per well. The difference is statistically significant (* $P<0.05$, ANOVA and Newman-Keuls' test) from control groups.

Figure III.2

***In vivo* effects of aFGF on protection of NBM cholinergic neurons.** Immunostaining of NBM neurons using anti-ChAT monoclonal antibody. ChAT-immunoreactive neurons in the mid-portion of the NBM, ipsilateral to the cortical lesion are shown in: (i) sham-operated rat (A,D); (ii) lesioned animals (B,E); (iii) aFGF-treated lesioned rats (C,F). Observe in high magnification (E) that the cell shrinkage is predominantly due to cytoplasmic atrophy which correlate with a scant neurite length. Significant protection of the NBM cholinergic cells occurred in animals that received aFGF, as can be observed by the preservation of cell bodies and their processes (Fig. III.2C,F), in contrast to the atrophy and decreased fiber length in ChAT-immunopositive neurons of lesioned vehicle-treated rats (B,E).

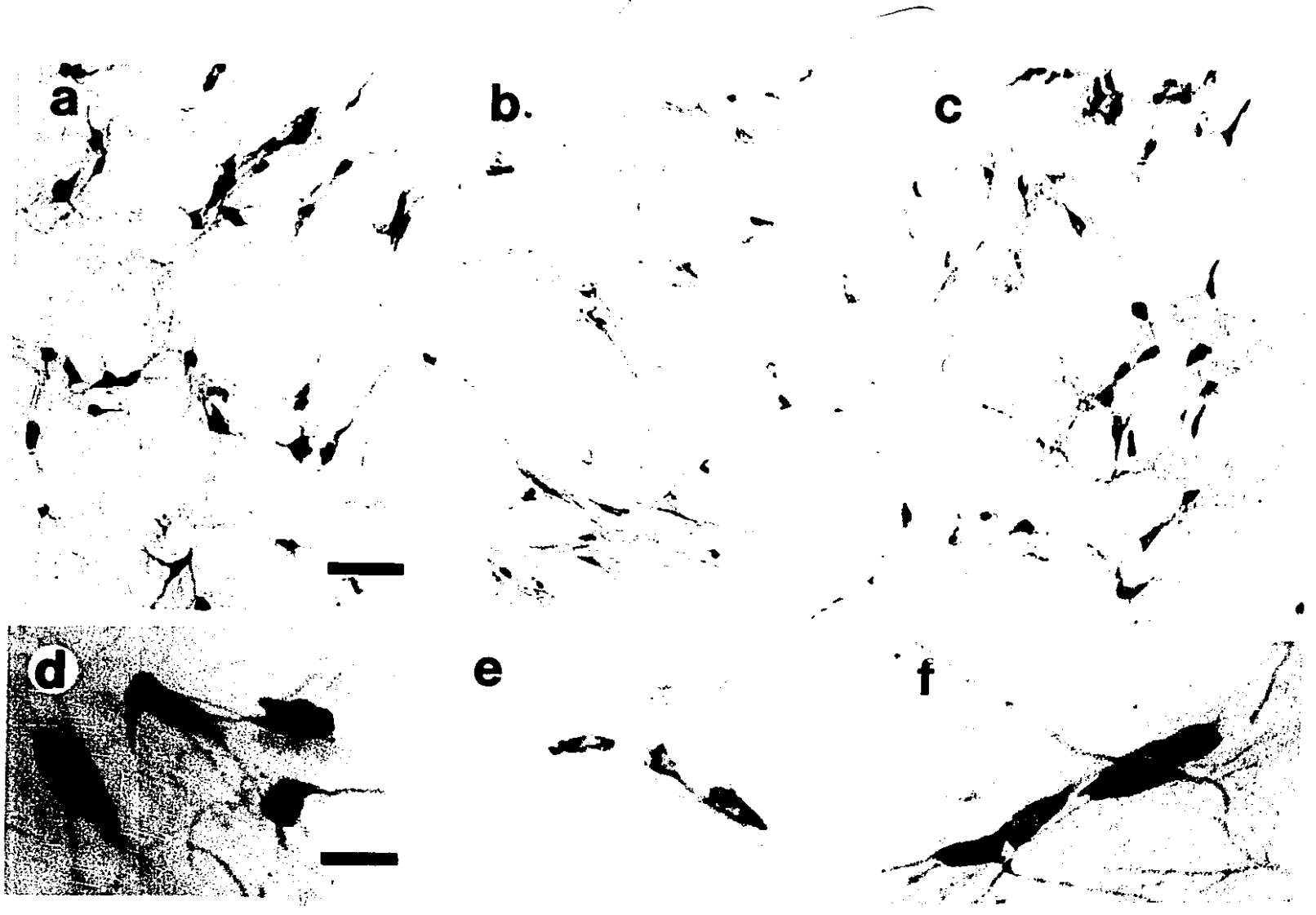
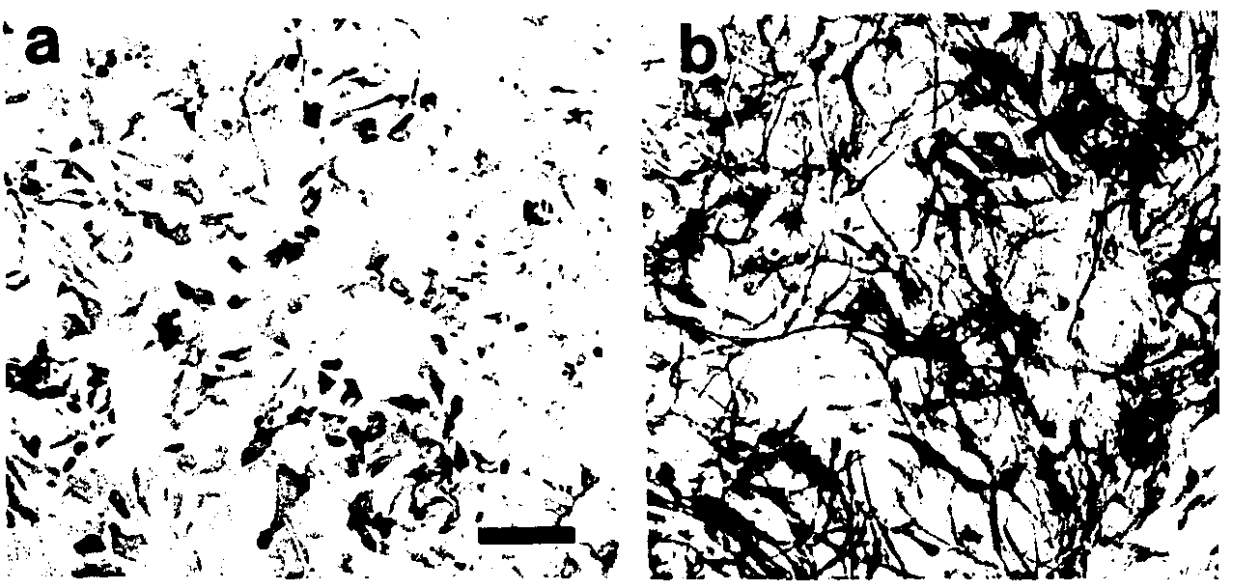

a
b.
c
d
e
f

Figure III.3

Effects of aFGF *in vitro* on immunoreactivity for GFAP. Detection of GFAP-immunoreactive cells in septal cell culture. Note the more prominent GFAP immunoreactivity and the increased mean neurite length in aFGF-treated cells (50 ng/ml) shown in B, in contrast to untreated cells shown in A. Scale bar in a = 50 μm, and also applies to B.


a
b

was no significant difference in ChAT activity in the remaining adjacent cortex among any of the experimental groups or comparing the remaining cortex, ipsilateral to the lesion, with the correspondent contralateral cortical area (Table III.3).

In vitro studies

Cultures treated with 0.05 μg/ml aFGF showed enlarged astrocytic processes and increased GFAP immunoreactivity (Fig. III.3B). ChAT activity increased more than 100% after a nine-day exposure to aFGF (25 or 50 ng/ml). Similar increases in ChAT activity were observed with 25 ng/ml NGF (Table III.4). Cultures treated with 25 ng/ml NGF in combination with 50 ng/ml aFGF did not show increased ChAT activity over that detected when either trophic factor was administrated alone (data not shown).

DISCUSSION

In contrast to the established effects of bFGF in preventing death of basal forebrain cholinergic cells (Anderson et al., 1988; Gómez-Pinilla et al., 1992; Otto et al., 1989), the properties of aFGF are not as well characterized. This is due, in part, to the heparin dependence of aFGF which limits the use of this growth factor. In this study, we have presented biochemical, morphological, and functional evidence which indicates that short-term continuous infusion of aFGF, without heparin, starting immediately after injury can save injured forebrain cholinergic cells from shrinkage.

The mechanism underlying this effect is not known. However, we hypothesize that the preservation of functional connections between the NBM and the remaining cortical areas is a possible explanation for the effects of aFGF improving the retention of learnt spatial localization tasks in decorticated rats. However, it is also possible that other non-cholinergic cells in the NBM or cortex may be affected by aFGF treatment and may also be responsible for reducing the deficits in water maze efficacy induced by the lesion. Although direct evidence to support such a hypothesis is lacking, our immunolocalization and morphometry data show that aFGF preserved the size of the cell body and the processes of cholinergic neurons in the NBM to the same extent as NGF

or the ganglioside GM1 have shown in previous studies (Cuello et al., 1986, 1989; Elliott et al., 1989).

In accordance with the preservation of ChAT immunoreactivity, aFGF prevented the decrease in ChAT activity in the NBM ipsilateral to the cortical lesion. However, aFGF did not increase baseline ChAT activity above control levels in the remaining ipsilateral cortex, in contrast to what has been observed following administration of an equimolar dose of NGF (Cuello et al., 1989) or GM1 (5 mg/kg per day) (Elliott et al., 1989). This suggests that the mechanisms of action of aFGF may differ from those of NGF and GM1 or that a higher dose of aFGF might be required to attain the same upregulation in ChAT activity.

It is possible that glial cells play a role in the efficacious preservation of cholinergic cells by aFGF. Although the increase in ChAT activity in bFGF-treated septal cell culture may not require glial cells (Knusel et al., 1990), glial cells respond to aFGF and bFGF with increased release of NGF *in vitro* (Ono et al., 1991; Yoshida and Gage, 1991; 1992), and *in vivo* (Spranger et al., 1990) which may result in further stimulation of septal cells by NGF. NGF is known to provide direct support to cholinergic neurons (Cuello et al., 1989; Hagg et al., 1990; Haroutunian et al., 1989; Hefti, 1986; Kromer, 1987; Williams et al., 1986) and to participate in injury-induced synaptic plasticity (Garofalo et al., 1992) in the cerebral cortex. The apparent absence of aFGF receptors in forebrain cholinergic cells (Ferguson et al., 1991) is consistent with an indirect action of aFGF in these cells via NGF. However, bFGF receptors may be found in forebrain cholinergic cells as suggested by a direct cholinergic response to bFGF in *in vitro* study (Knusel et al., 1990). In the present study, we found no significant difference in GFAP immunoreactivity between aFGF-treated and vehicle-treated rats after the lesion (data not shown). However, aFGF-treated septal cultures revealed hypertrophic astrocytes and an increase in ChAT activity similar to the changes observed in NGF-treated cultures. The absence of *in vivo* aFGF effect on GFAP immunoreactivity could be due to a lack of sensitivity of GFAP immunostaining.

Therefore, further studies using western and northern blot analysis may be helpful in detecting changes in GFAP protein and its mRNA.

Demonstration of trophic effects *in vitro* with aFGF suggests that a variety of trophic factors may act together to modulate the development and/or plasticity of the CNS. The effect of aFGF following axotomy of septal cholinergic neurons has not yet been described and its specific function in the CNS is not well understood. While our data would indicate that *exogenously* applied aFGF promotes preservation of cholinergic neurons, it is as yet unclear what role large amounts of *endogenous* aFGF in the NBM (Stock et al., 1992) may play as part of a general reaction to cortical injury.

In the present study, a lower dose of aFGF (6.0 μg/day) was ineffective in preventing shrinkage of cholinergic cells in the NBM of lesioned rats. The lack of effect at this dosage could be due to loss of FGF activity secondary to rapid inactivation (Damon et al., 1993; Gospodarowicz and Cheng, 1986; Müller et al., 1989; Thomas et al., 1991) and degradation by proteases (Rosengart et al., 1988a; Salsela et al., 1988; Sommer and Rifkin, 1989). Heparin has been shown to bind to acidic and basic FGF prolonging their biological half-lives. If heparin were to be similarly effective in protecting aFGF in our *in vivo* model, perhaps 6 μg/day or less of aFGF in combination with heparin would be sufficient to obtain the same results found with 12 μg/day of aFGF alone. This is in agreement with a recent study showing that aFGF bioactivity was much more dependent on heparin in sciatic nerve than in subcortical telencephalon (Stock et al., 1992). It is interesting to note that the absence of heparin (Thomas et al., 1991) reduces the duration of the total biological activity of aFGF to less than 2 h. This suggests that most of the aFGF administered to the rats in our study was probably rapidly lost. However, aFGF was delivered by constant infusion (0.5 μg/h, seven days), thus minimizing the loss in relation to bolus dose administration. The beneficial effect of aFGF we observed in the absence of heparin suggests that retention of learnt spatial localization could be due to an indirect effect mediated via endogenous release of NGF. This is supported by the preservation of ChAT activity and the normal cytoarchitecture of NBM cholinergic cells similar to that produced by exogenously applied NGF (Cuello

et al., 1989; Haroutunian et al., 1989; Mandel et al., 1989). The recent finding of intense immunoreactivity for aFGF in NBM neurons (Stock et al., 1992) could be interpreted as evidence for the interaction of *endogenous* aFGF with cells producing target-derived NGF from neocortex. However, it is not yet clear if the aFGF produced in NBM is localized in cholinergic neurons. It is also unclear how aFGF interacts with target cells as it lacks a signal sequence for secretion. If the effects of aFGF observed in the present study are the consequence of direct action of aFGF on NBM cholinergic neurons, we should consider the possibility of an autocrine effect in NBM neurons similar to that described for bFGF in cultured endothelial cells (Sakaguchi et al., 1988; Sato and Rifkin, 1988).

CONCLUSION

It appears that the efficacy of 0.5 μg/h aFGF for seven days might be due in part to portioning of the protein onto the exposed heparan sulfate-containing subendothelial basement membrane and extracellular matrix, effectively concentrating aFGF at sites of injury (Bjornsson et al., 1991; Rosengart et al., 1988a; 1988b). Basic FGF has been reported to prevent death of septal cholinergic cells following fimbria-fornix lesion (Anderson et al., 1988; Gómez-Pinilla et al., 1992; Otto et al., 1989) and this effect occurred at much lower doses than the doses of aFGF used in the present study. These reports suggest that bFGF may be considerably more potent than aFGF in the CNS. Basic FGF appears to be less dependent on heparin than does aFGF in such models [for review see Burgess and Maciag, (1989); Eckenstein et al., (1991)]. In contrast, it has been reported that aFGF was more efficacious than bFGF in releasing NGF from tissue cultures (Yoshida and Gage, 1991). Based on previously reported work and the present observations, we speculate that a number of trophic substances interact following insult to the CNS and that aFGF, or a closely related trophic factor, may be involved in mediating the response to injury, and that the exogenous application of some of these agents can facilitate or achieve repair of specific sets of CNS neurons.

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REFERENCES

Anderson KJ, Dam D, Lee S, Cotman CW (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332:360-362.

Baird A, Klagsbrun M (1991) The fibroblast growth factor family: An overview. Ann NY Acad Sci 638:xi-xii.

Baird A, Ling N (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: Implications for a role of heparinase-like enzymes in the neovascular response. Biochem Biophys Res Commun 142:428-435.

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115-165.

Bjornsson TD, Dryjski M, Tluczek J, Mennie R, Ronan J, Mellin TN, Thomas KA (1991) Acidic fibroblast growth factor prmotes vascular repair. Proc Natl Acad Sci USA 88:8651-8655.

Bowen DM, Smith CD (1976) Neurotransmitter related enzymes and indices of hypoxia in senile dementia and other abiotrophies. Brain 99:459-496.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principal of dye binding. Ann Biochem 72:248-254.

Burgess WH, Maciag T (1989) Heparin binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 58:575-606.

Cuello AC, Milstein C, Wright B, Bramwell S, Priestley JV, Jarvis J (1984) Development and application of a monoclonal rat peroxidase-antiperoxidase (PAP) immunocytochemical reagent. Histochemistry 80:257-261.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

Damon DH, Lobb RR, D'Amore PA, Wagner JA (1993) Heparin potentiates the action of acidic fibroblast growth factor by prolonging its biological half-life. J Cell Physiol

Davies P, Maloney AJF (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2 (8000):1403

Dunnet SB, Wishaw IQ, Bunch ST, Fine A (1993) Acetylcholine-rich neuronal grafts in the forebrain of rats: Effects of environmental enrichment, neonatal noradrenaline depletion, host transplantation site and regional source of embryonic donor cells on graft size and acetylcholinesterase-positive fiber outgrowth. Brain Res

Eckenstein FP, Shipley GD, Nishi R (1991) Acidic and basic fibroblast growth factors in the nervous system: Distribution and differential alteration of levels after injury of central versus peripheral nerve. J Neurosci 11:412-419.

Elliott PJ, Garofalo L, Cuello AC (1989) Limited neocortical devascularizing lesions causing deficits in memory retention and choline acetyltransferase activity--effects of the monosialoganglioside GM1. Neuroscience 31:63-76.

Ferguson IA, Schweitzer JB, Bartlett PF, Johnson EM, Jr. (1991) Receptor-mediated retrograde transport in CNS neurons after intraventricular administration of NGF and growth factors. J Comp Neurol 313:680-692.

Fibiger HC (1982) The organization and some projections of cholinergic neurons of the mammalian forebrain. Brain Res Rev 4:327-388.

Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. J Neurochem 24:407-409.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1992) Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. Proc Natl Acad Sci USA 89:2639-2643.

Gospodarowicz D, Neufeld G, Schweigerer L (1986) Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Different 19:1-17.

Gospodarowicz D, Ferrara N, Aschweigerer L, Neufeld G (1987) Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 8:95-114.

Gospodarowicz D, Cheng J (1986) Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475-484.

Gómez-Pinilla F, Lee JW-K, Cotman CW (1992) Basic FGF in adult rat brain: Cellular distribution and response to entorhinal lesion and fimbria-fornix transection. J Neurosci 12:345-355.

Grothe C, Otto D, Unsicker K (1989) Basic fibroblast growth factor promotes *in vitro* survival and cholinergic development of rat septal neurons: Comparison with the effects of nerve growth factor. Neuroscience 31:649-661.

Hagg T, Vahlsing HL, Manthorpe M, Varon S (1990) Nerve growth factor infusion into the denervated adult rat hippocampal formation promotes its cholinergic reinnervation. J Neurosci 10:3087-3092.

Haroutunian V, Kanof PD, Davis KL (1989) Attenuation of nucleus basalis of Meynert lesion induced cholinergic deficits by nerve growth factor. Brain Res 487:200-203.

Hefti F (1986) Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 6:2155-2162.

Kenigsberg RL, Semenenko FM, Cuello AC (1990) Development of a bi-specific monoclonal antibody for simultaneous detection of rabbit IgG and horseradish peroxidase: use as a general reagent in immunocytochemistry and enzyme-linked immunosorbent assay. J Histochem Cytochem 38:191-198.

Knusel B, Michel PP, Schwaber JS, Hefti F (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development *in vitro* by nerve growth factor, basic fibroblast growth factor, epidermal growh factor, insulin and the insulin-like growth factors I and II. J Neurosci 10:558-570.

Kromer LF (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235:214-216.

Lobb RR (1988) Clinical applications of heparin-binding growth factors. Eur J Clin Invest 18:321-336.

Luiten PGM, Gaykema RPA, Traber J, Spencer DG (1987) Cortical projection patterns of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin. Brain Res 413:229-250.

Mandel RJ, Gage FH, Thal LJ (1989) Spatial learning in rats: Correlation with cortical choline acetyltransferase and improvement with NGF following NBM damage. Exp Neurol 104:208-217.

Mize RR, Holdefer RN, Nabors LB (1988) Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis. J Neurosci Methods 26:1-24.

Morris R (1984) Developments of a water maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47-60.

Müller SN, Thomas KA, DiSalvo J, Levine EM (1989) Stabilization by heparin of acidic fibroblast growth factor mitogenicity for human endothelial cells *in vitro*. J Cell Physiol 140:439-448.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Otto D, Frotscher M, Unsicker K (1989) Basic fibroblast growth factor and nerve growth factor administered in gelfoam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22:83-91.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Pioro EP, Cuello AC (1990) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system: Effect of colchicine and correlation with the cholinergic system-I.Forebrain. Neuroscience 24:57-87.

Rifkin DB, Moscatelli D (1989) Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol 109:1-6.

Rosengart TK, Johnson WV, Friesel R, Clark R, Maciag T (1988a) Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. Biochem Biophys Res Commun 152:432-440.

Rosengart TK, Kupferschmidt J, Ferrans VJ, Casscells W, Maciag T, Clark RE (1988b) Heparin-binding growth factor-I (endothelial cell growth factor) binds to endothelium *in vivo*. J Vasc Surg 7:311-317.

Sakaguchi M, Kajio T, Kawahara K, Kato K (1988) Antibodies against basic fibroblast factor inhibit the autocrine growth of pulmonary artery endothelial cells. FEBS Lett 233:163-166.

Salsela O, Moscatelli D, Sommer A, Rifkin DB (1988) Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol 107:743-751.

Saper CB (1984) Organization of cerebral cortical afferent systems in the rat. II. magnocellular basal nucleus. J Comp Neurol 222:313-342.

Sato Y, Rifkin DB (1988) Autocrine activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J Cell Biol 107:1199-1205.

Sofroniew, M.V., Pearson, R.C., Eckenstein, F., Cuello, A.C., and Powell, T.P. (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res. 289:370-374.

Sofroniew MV, Pearson RCA, Powell TPS (1987) The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. Brain Res 411:310-331.

Sommer A, Rifkin DB (1989) Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. J Cell Physiol 138:215-220.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Nahernoe M, Thoenen H (1990) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system - comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. Eur J Neurosci 2:69-76.

Stephens PH, Cuello AC, Sofroniew MV, Pearson RC, Tagari P (1985) Effect of unilateral decortication on choline acetyltransferase activity in the nucleus basalis and other areas of the rat brain. J Neurochem 45:1021-1026.

Stock A, Kuzis K, Woodward WR, Nishi R, Eckenstein FP (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12:4688-4700.

Sweetnam PM, Sanon HR, White LA, Brass BJ, Jaye M, Whittemore SR (1991) Differential effects of acidic and basic fibroblast growth factors on spinal cord cholinergic, GABAergic, and glutamatergic neurons. J Neurochem 57:237-249.

Thomas KA (1987) Fibroblast growth factors. FASEB J 1:434-440.

Thomas KA, Ortega S, Soderman D, Schaeffer M-T, DiSalvo J, Gimenez-Gallego G, Linemeyer D, Kelly L, Menke J (1991) Structural modifications of acidic fibroblast growth factor alter activity, stability, and heparin dependence. Ann NY Acad Sci 638:9-17.

Walicke PA (1988) Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J Neurosci 8:2618-2627.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, DeLong MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237-1239.

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A, Gage FH (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc Natl Acad Sci USA 83:9231-9235.

Woolf NJ (1991) Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 37:475-524.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

Zilles K (1985) A stereotaxic atlas. Berlin: Springer-Verlag.

The knowledge gained in the previous chapter points to another valuable feature of aFGF, as a neurotrophic factor capable of rescuing NBM cholinergic neurons. We have described morphological and behavioral changes elicited by intracerebroventricular administration of aFGF. However, studies on the distribution of the four high-affinity FGF receptors in the rat brain (see Table I.3 in Chapter I), ruled out a direct action of aFGF in NBM neurons. These data and the detailed examples of a large number of FGF effects present in non-neuronal cells, particularly in astroglia (Ono et al., 1991; Walicke and Baird, 1991; Schubert, 1992; Araujo and Cotman, 1992; Basilico and Moscatelli, 1992; Figueiredo et al., 1993), suggest that aFGF and bFGF indirectly protected NBM neurons. Trophic factors released from astrocytes in response to application of aFGF could be involved in this mechanism (Barde, 1989). A description of both aFGF and bFGF effects in astrocytes *in vitro* and in developing hippocampus (Spranger et al., 1990; Ono et al., 1991; Yoshida and Gage, 1991; Yoshida and Gage, 1992) revealed that NGF is one of the neurotrophic factors released by astrocytes. Therefore, it was necessary to investigate whether this interesting feature of aFGF could be demonstrated in target regions of the cholinergic NBM neurons of adult animals. Since reactive astrocytes (Herrera and Cuello, 1992) and increased NGF levels (Gasser et al., 1986; Weskamp et al., 1986) occur following brain injury, our next chapter focuses on the *in vivo* regulation of NGF levels induced by cortical devascularization and treatment with aFGF. A large body of evidence indicates that a cascade of signalling and cell-recognition molecules are activated in response to brain injury. Inflammatory cytokines (Woodroofe et al., 1991) and other molecules, such as c-fos protooncogene (Dragunow and Robertson, 1988), play an important role as starting events in this cascade. However, Chapter IV is aimed primarily at changes in NGF levels which seems to be a later event.

REFERENCES

Araujo DM, Cotman CW (1992) Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J Neurosci 12:1668-16678.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115-165.

Dragunow M, Robertson HA (1988) Brain injury induces c-fos protein (s) in nerve and glial-like cells in adult mammalian brain. Brain Res 455:295-299.

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Gasser UE, Weskamp G, Otten U, Dravid AR (1986) Time course of the elevation of nerve growth factor (NGF) content in the hippocampus and septum following lesions of the septohippocampal pathways in rats. Brain Res 376:351-356.

Herrera DG, Cuello AC (1992) Glial fibrillary acidic protein immunoreactivity following cortical devascularizing lesion. Neuroscience 49:781-791.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Schubert D (1992) Synergistic interactions between transforming growth factor beta and fibroblast growth factor regulate Schwann cell mitosis. J Neurobiol 23:143-148.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Nahernoe M, Thoenen H (1990) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system - comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. Eur J Neurosci 2:69-76.

Walicke PA, Baird A (1991) Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. J Neurosci 11:2249-2258.

Weskamp G, Gasser UE, Dravid AR, Otten U (1986) Fimbria fornix-lesion increases nerve growth factor content in adult rat septum and hippocampus. Neurosci Lett 70:121-126.

Woodroofe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ (1991) Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J Neuroimunol 33:227-236.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

CHAPTER IV

ACIDIC FGF INDUCES NGF AND ITS mRNA IN NEOCORTEX OF ADULT RATS

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ABSTRACT

Recently we reported that human recombinant acidic fibroblast growth factor (aFGF) is capable of preventing degeneration of nucleus basalis magnocellularis neurons *in vivo* and inducing growth of astrocytes *in vitro*. In the present study, the effects of aFGF on the concentration of nerve growth factor (NGF) and its messenger RNA were investigated in rat cerebral cortex following unilateral cortical infarction. Lesioned animals exhibited a significant increase ($P < 0.05$) of NGF in the remaining cortex ipsilateral to the lesion. After combining cortical lesion with intracerebroventricular application of aFGF (12 μg/day for 7 days), we observed an 8-fold increase in the NGF concentration and a marked increase in the level of steady state NGF mRNA relative to controls ipsilaterally, and a less pronounced aFGF effect in the contralateral cerebral cortex. These results support the hypothesis that the neurotrophic effects previously shown for aFGF and basic FGF (bFGF) in neurotrophin-sensitive neurons is mediated by inducing increased production of NGF within the injured central nervous system (CNS) of adult animals.

INTRODUCTION

The fact that NGF shares similar therapeutical properties with aFGF and bFGF in rescuing basal cholinergic neurons has led several investigators to suggest a physiological interaction between these neurotrophic factors (Spranger et al., 1990; Yoshida and Gage, 1991). Protection of these cholinergic neurons by exogenous NGF is in agreement with the abundance of NGF mRNA in the hippocampus and cerebral cortex which is matched by expression of low- and high-affinity NGF receptors in afferent basal forebrain cholinergic neurons [for reviews see (Hefti et al., 1989; Barde, 1989; Cuello, 1993; Altar et al., 1991a; Raivich and Kreutzberg, 1987; Richardson et al., 1986; Merlio et al., 1992; Feinstein et al., 1993; Altar et al., 1991b)]. In contrast, among the best known FGFs, an inverse distribution of aFGF and FGF receptors (FGFRs) has been described: moderate levels of aFGF were found in basal forebrain cholinergic neurons (Stock et al., 1992) while the high-affinity FGFR-1 (fgl), and to a lesser extent FGFR-2

(bek), are expressed at a higher level in the hippocampus and the cerebral cortex (Wanaka et al., 1990; Asai et al., 1993; Dionne et al., 1990). It is worth noting that aFGF is expressed almost exclusively in specific neuronal populations (Eckenstein et al., 1991; Stock et al., 1992; Nobuyuki et al., 1994; Elde et al., 1991; Janet et al., 1987; Tourbach et al., 1991; Wilcox and Unnerstall, 1992) while bFGF is associated with both neurons and astrocytes (Grothe et al., 1991; Woodward et al., 1992; Kato et al., 1992) and predominates in astrocytes following brain injury (Finklestein et al., 1988; Kiyota et al., 1991; Takami et al., 1992; Eckenstein et al., 1991; Frautschy et al., 1991; Gómez-Pinilla et al., 1992). FGFR-1 is predominantly found in neurons (Wanaka et al., 1990; Asai et al., 1993; Takami et al., 1993) while FGFR-2 is more abundantly expressed in oligodendrocytes and astrocytes (Asai et al., 1993; Takami et al., 1993). However, Takami and colleagues (1993) reported a strong and prolonged increase of FGFR-1 in contrast to a weak and transient increase of FGFR-2 in astrocytes following transient forebrain ischemia. Also recently van der Wal and collegues (1994) described a strong, seizure-associated induction of FGFR-1 protein expression in the hippocampal and cortical astrocytes, followed only by its appearance in neuron-like cells spread throughout the hippocampus and cerebral cortex (Van der Wal et al., 1994). The increase in NGF brain levels occurring after lesions (Gasser et al., 1986; Korsching et al., 1986; Weskamp et al., 1986a; Weskamp et al., 1986b) is considered to be partly due to its accumulation in the target structures to the injured projections that normally transport retrogradely NGF. Additionally, an important source of the increased NGF levels can be glial cells as proved by the studies showing increased NGF-immunoreactivity in astrocytes of medial septum and hippocampus after brain injury (Bakhit et al., 1991; Oderfeld-Nowak et al., 1992). Basic FGF was shown to increase the synthesis of NGF and its mRNA in developing hippocampus (Spranger et al., 1990). However, regulation of NGF levels by aFGF in target sites of the basalo-cortical cholinergic systems has not yet been reported in studies conducted in adult animals with brain injury. The present study was designed to investigate whether the levels of the NGF protein and its mRNA are altered in the adult CNS *in vivo* following intracerebroventricular administration of aFGF in rats immediately before partial unilateral cortical infarction.

MATERIALS AND METHODS

Animals

In this study three groups of male Wistar rats (300-325 g) were used: 1) control, naive rats; 2) devascularized rats treated with vehicle for seven days, and 3) devascularized rats treated with aFGF for seven days. Groups consisted initially of 6 animals each. After verification of the lesion extent and a volume of the solutions pumped to the cerebral ventricle, brains of 6 naive, 5 lesioned vehicle-treated, and 4 lesioned aFGF-treated rats were chosen for assays.

Surgery

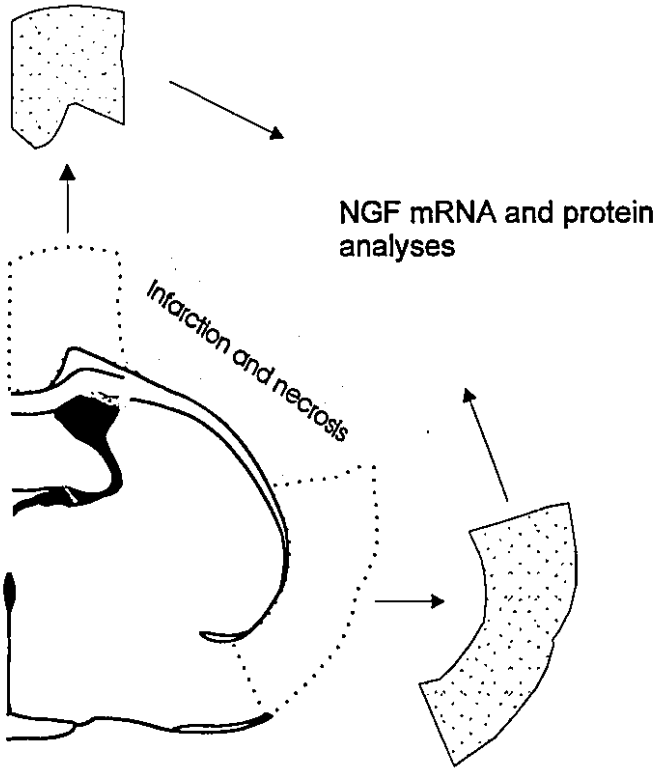
Rats were anaesthetized with Equithesin (2.5 ml/kg, i.p.) and placed in a stereotaxic frame. A stainless steel cannula (23 gauge) was permanently implanted into the right lateral ventricle [coordinates from Bregma (Paxinos and Watson, 1986): AP, -0.8; L, 1.4; V, 3.5]. Osmotic minipumps (Alzet 2001, 1 μl/h, seven days) were loaded with dye (0.01% Methylene blue, BDH Chemicals) and pretested before being connected to the cannulae by coiled polyethylene tubing (Intramedic, PE-60). Human recombinant acidic FGF (Merck, Rahway, New Jersey, USA), 100μg in a total volume of 200μl of vehicle or the same volume of vehicle only [artificial cerebro-spinal fluid, containing 0.1% bovine serum albumin and heparin (200 μg/ml)] was loaded into the tubing with a small amount of mineral oil added to the minipump end to prevent mixing with the dye released from the minipump. Animals were subjected to left-side devascularizing lesions as described previously (Sofroniew et al., 1983; Stephens et al., 1985). In brief, the devascularizing lesion was performed by removing a flap of bone and underlying dura and gently rubbing away all vessels and the pia mater with sterile saline-soaked cotton swabs. Control animals did not receive any operation. The cortical regions analysed were sampled as illustrated schematically in Fig. IV.1 and included entire cerebral cortex around the lesion placement affecting parietal and part of frontal cortices. Strictly matched samples from the contralateral cortices were taken as well, which did not include equivalent lesioned cortex.

Reverse transcription and PCR

Total RNA was extracted using acid guanidinium-thiocyanate-phenol-2-mercaptoethanol

Figure IV.1.

Schematic coronal brain section (lesioned hemisphere) at the level of the most extensive damage to the infarction area. The areas demarcated with dots represent the samples of the cortical tissue dissected out and pooled with the analogous regions taken at the level of the frontal and occipital lobes (not shown) for determination of NGF protein levels and its mRNA expression. Strictly matched areas from the contralateral hemispheres were subsequently microdissected for NGF protein and mRNA assays, performed in a separate set of experiments.


NGF mRNA and protein analyses
Infarction and necrosis

(RNAzol B), according to the manufacturer's instructions (CINNA/BIOTECH Laboratories). The RNA was re-suspended in RNAse-free water and the concentration calculated from optical density. The determination of NGF mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) was performed according to the procedure recently reported by Scully and Otten (Scully and Otten, 1993). In brief, 1 μg of total RNA was denatured at 90 °C for 5 min and reversed-transcribed at 37 °C for 1 h. Five μl of this reaction mixture was used for amplification by PCR. Sense and anti-sense NGF-specific primers are 5'-CCAAGGACGCAGCTTTCTAT-3' and 5'-CTCCGGTGAGTCCTGTTGAA-3', respectively. Equivalent amounts of cDNA were amplified in parallel with S12 ribosomal protein specific primers to control for random variations in amplification efficiency. Twenty cycles for NGFmRNA and 18 cycles for S12 mRNA were performed. Negative controls were reverse-transcribed either without RNA or without reverse transcriptase, while positive controls were reversed-transcribed from murine fibroblast mRNA or amplified from plasmids containing cDNA of the NGF gene. Samples were electrophoresed through a 1.2% agarose gel and blotted in 0.4 N NaOH onto nylon membranes (Boehringer Mannheim). Two cDNA probes specific for the NGF or S12 bands produced by RT-PCR were labelled using digoxigenin (DIG system), and the signal detected according to the manufacturer's instructions (Boehringer, Mannheim). KodaK X-Omat AR films were exposed and quantified using a Bio-Rad Model 620 densitometer. The NGF mRNA levels were expressed as absorbance and normalized to the corresponding S12 signal. The assays of NGF mRNA concentrations in the samples of the ipsi- and contralateral cortices were performed in separate sets of experiments.

Enzyme-linked immunoassay (ELISA) for NGF

NGF-like protein determinations were performed using a sensitive two-site immunosorbent assay (Weskamp and Otten, 1987). NGF concentrations were measured in the remainder of the cerebral cortical tissue circumscribing the decorticated area and the corresponding cortices contralateral to the lesion (Fig. IV.1). Cortices were dissected, weighed and stored at -80 °C. The tissue was homogenized in 750 μl phosphate-buffered saline containing 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 0.5% bovine serum albumin (BSA), and 0.1% of aprotinin pH 7.4 and centrifuged for 3 min at room

temperature. One hundred μl aliquots were placed in 96-well polystyrene plate immunocoated with polyclonal goat anti-NGF antibody or with a non-immune goat serum. Determinations were performed in duplicate and corrected for the background. The absolute values were expressed as equivalents of mouse 2.5 S NGF and changes in NGF concentration were expressed as % control.

Statistical analysis

Differences between two groups of values for NGF or its mRNA were analyzed using unpaired, two-tailed Student't test with Bonferroni's adjustments.

RESULTS

The decorticated area, seven days after surgery, corresponded approximately to the size of the partial cortical devascularization (1 x 1 cm^2). Digoxigenin labeled-cDNA blots representing NGF mRNA in cortical tissue of three animal groups and those of ribosomal protein specific primers of S12 controls are shown in Figure IV.2. Densitometric measurements of the blots shown in figure 2 revealed values ranging from 141 to 362% of control. These values denoted a statistically significant increase in the level of steady state NGF mRNA in ipsilateral cortical tissues from aFGF-treated rats bearing unilateral cortical infarction (n=4), in relation to lesioned vehicle-treated or non-operated control (naive) rats respectively (n=5) (Figure IV.3B). The increase in the level of NGF mRNA in the contralateral cortex was less pronounced and averaged 160 % of control levels (Fig. IV.3B). The mean NGF mRNA levels in both cerebral hemispheres of vehicle-treated and lesioned animals without trophic factor were not statistically different from respective controls. Microdissected ipsilateral cortices of 5 vehicle-treated and lesioned animals exhibited a moderate increase ($P < 0.05$) in concentrations of NGF protein in comparison to controls (n=6) (Fig. IV.3A). There was no effect of the lesion on the NGF protein concentration in the contralateral cortex. Infusion of aFGF for seven days resulted in an approximately eight-fold increase of NGF protein concentration in the ipsilateral remaining cortex in relation to controls. NGF protein levels were approximately 4 times higher than in vehicle-treated lesioned animals (Fig. IV.3A). Acidic FGF also elicited a statistically significant

Figure IV.2.

Effect of lesion and aFGF treatment on expression of NGF mRNA in the cerebral cortex on the ipsilateral side of the lesion, seven days after surgery. Rats were treated with aFGF continuously infused to the lateral ventricle ipsilaterally to the lesion placement (12μg/day) and were sacrificed on the last day of aFGF administration. Total cortical RNA (1 μg) was assayed by reverse transcriptase-polymerase chain reaction (RT-PCR) as described in the text. The RT-PCR products were subjected to agarose gel electrophoresis, blotted onto nylon membranes, and hybridized to DIG-labelled specific probes for NGF (402 bp) and S12 (368 bp). Lane 1 = lesioned, vehicle-treated animals; lane 2 = naive animals; lane 3 = lesioned, aFGF-treated animals; lane 4 = negative (no RNA in the RT-reaction); lane 5 = positive control (unlabeled NGF cDNA-probe).


1 2 3 4 5
NGF ▶
– 402 bp
S12 ▶
– 368 bp

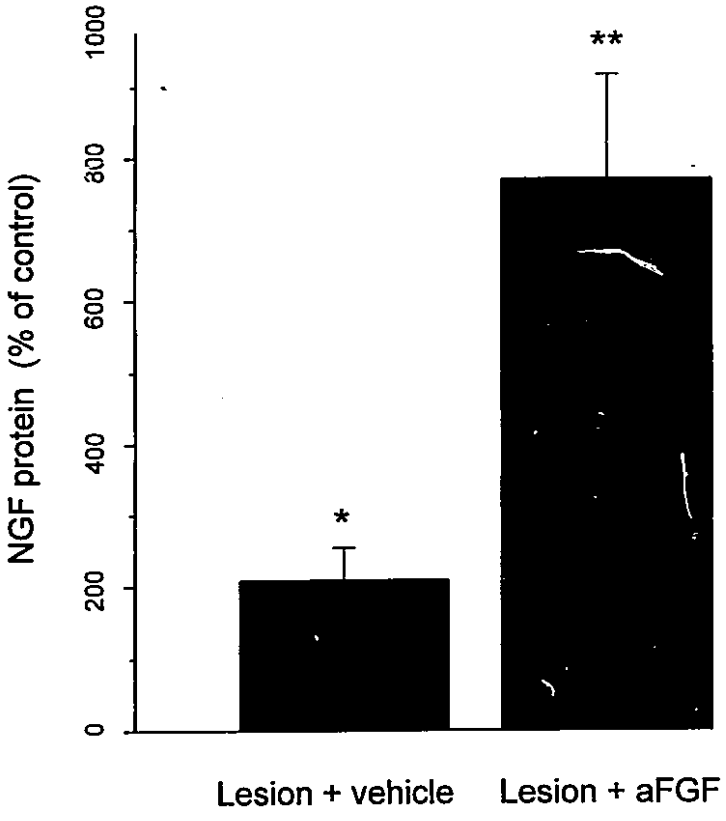
Figure IV.3.

Effect of lesion and aFGF treatment on expression of NGF in the cerebral cortex seven days after surgery. Rats were continuosly infused with aFGF (12μg/day) to the lateral ventricle ipsilateral to the lesion placement and were killed on the last day of aFGF administration. Column 1, naive animals; column 2, vehicle-treated and lesioned animals; column 3, aFGF-treated and lesioned animals.

A: NGF protein concentration in cerebral cortices ipsi- and contralateral to the lesion. NGF protein is expressed as pg/100 mg wet cortical tissues $\pm$ S.E.M. In aFGF-treated animals NGF protein levels in cerebral cortices show a significant increase ipsi- and contralateral to the lesion. *$P<0.05$ compared to naive animals; **$P<0.01$ compared to naive or lesioned animals.

B: NGF mRNA concentration in cerebral cortices ipsi- and contralateral to the lesion. Densitometric evaluation of the NGF mRNA signals were normalized to the corresponding S12 signal and expressed as absorbance $\pm$ S.E.M. *$P<0.05$ compared to naive or lesioned animals; **$P<0.01$ compared to naive or lesioned animals.

Figure IV.3A


NGF protein (% of control)
0
200
400
600
800
1000
*
**
Lesion + vehicle
Lesion + aFGF

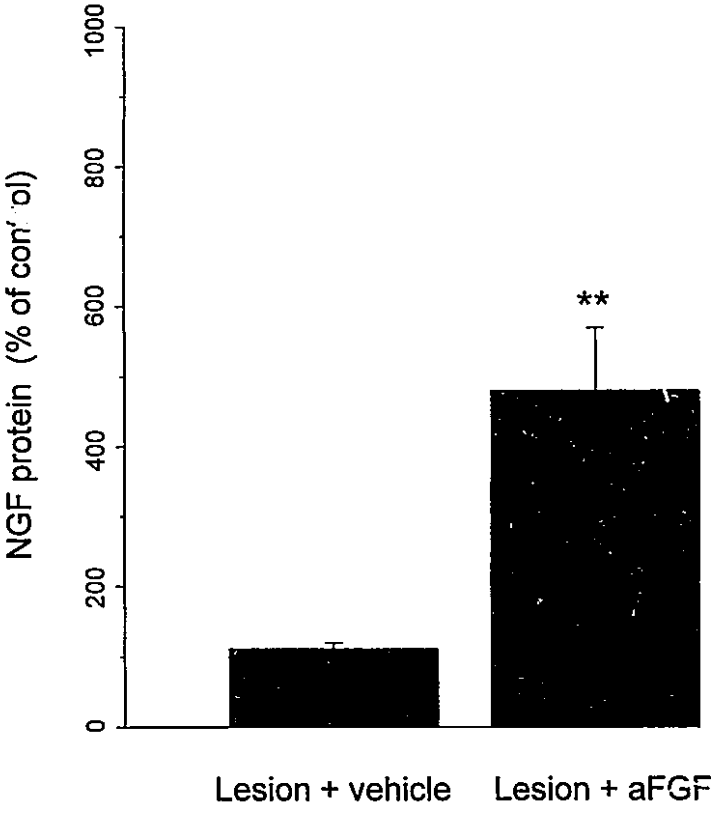
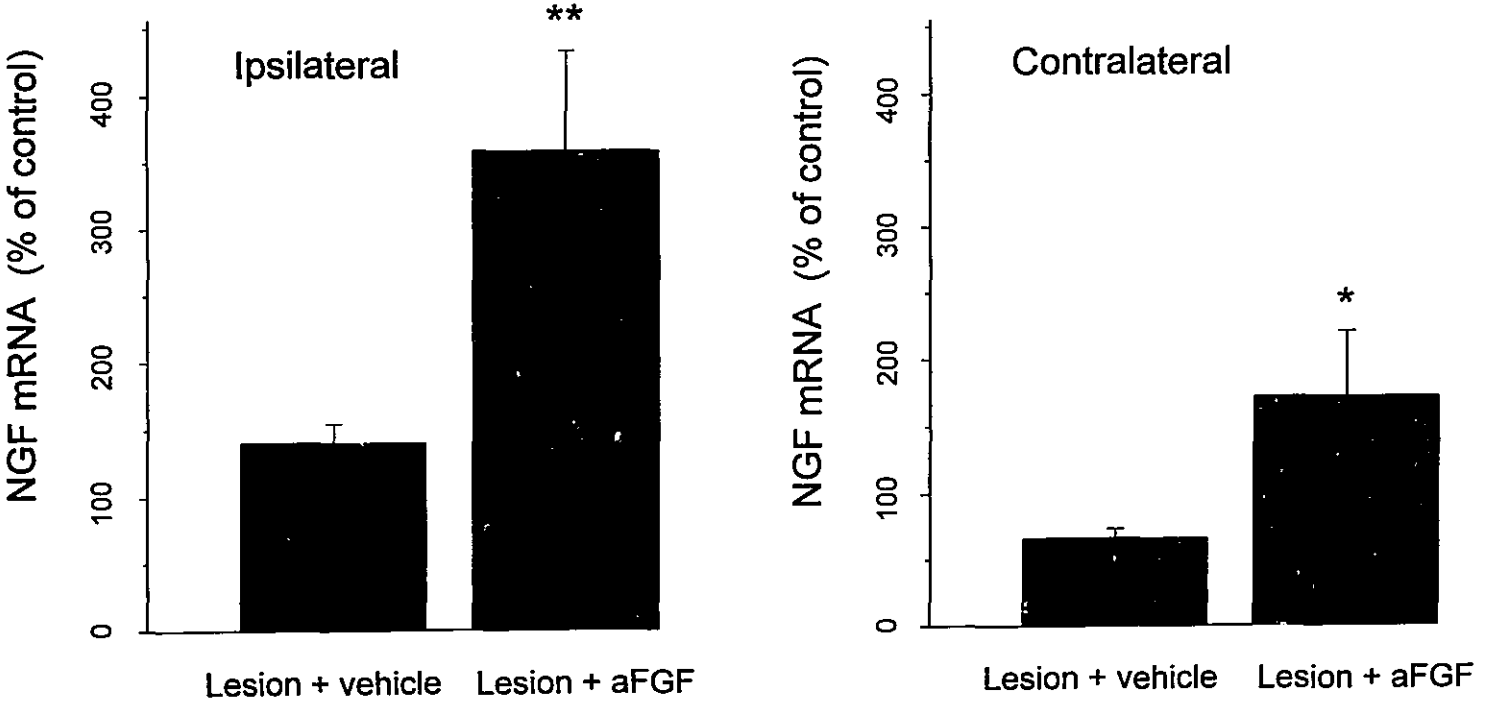

NGF protein (% of con' ol)
0
200
400
600
800
1000
**
Lesion + vehicle
Lesion + aFGF

Figure IV.3B


Ipsilateral
**
NGF mRNA (% of control)
0
100
200
300
400
Lesion + vehicle
Lesion + aFGF
Contralateral
*
NGF mRNA (% of control)
0
100
200
300
400
Lesion + vehicle
Lesion + aFGF

increase in the cortical NGF protein concentration contralateral to the devascularizing lesion.

DISCUSSION

The finding that bFGF can prevent cell death of basal forebrain cholinergic neurons after fimbria-fornix transection has provoked great interest in the therapeutic potential of this agent (Anderson et al., 1988). More recent studies from our own group proved that aFGF treatment (12 μg/day for seven days) in a paradigm similar to the one in the present study leads to a protection of basalo-cortical cholinergic neurons and, furthermore, to the preservation of spatial memory in rats bearing cortical ischemic lesions (Figueiredo et al., 1993). It is unclear how these effects are achieved as there is no evidence for the presence of FGFRs in basal forebrain cholinergic neurons. In the reported study (Figueiredo et al., 1993), astrocyte responsiveness to aFGF was also identified in mixed-septal cell cultures which exhibited intense glial fibrillary acidic protein-immunoreactive processes after aFGF treatment (Figueiredo et al., 1993). It is debatable whether aFGF and bFGF interact directly with basal cholinergic neurons. Comprehensive binding and *in situ* hybridization studies have failed to detect FGF binding sites and corresponding FGF receptor mRNAs (Ferguson and Johnson, Jr. 1991; Wanaka et al., 1990) in the medial septum and nucleus basalis magnocellularis. These results would suggest that the neurotrophic effects of FGFs in these neurons are indirect. It has been documented that aFGF or bFGF can directly stimulate newborn brain astrocytes to produce NGF *in vitro* (Spranger et al., 1990; Ono et al., 1991; Yoshida and Gage, 1991; Yoshida and Gage, 1992). The present study provides the first direct *in vivo* evidence for an aFGF mediated increase in NGF and its mRNA in adult animals. The evidence of increased NGF-immunoreactivity in basal forebrain astrocytes after hippocampal lesion (Bakhit et al., 1991), septal lesion (Oderfeld-Nowak et al., 1992), as well as the dramatic reactive gliosis in the cerebral cortex (Herrera and Cuello, 1992), and the increase of bFGF in astrocytes as a consequence of brain injury (Eckenstein et al., 1991; Frautschy et al., 1991; Gómez-Pinilla et al., 1992; Finklestein et al., 1988; Kiyota et al., 1991; Takami et al., 1992), all indicate that reactive astrocytes may be activated by their own FGFs in a cascade of postinjury phenomena leading to possible autocrine or paracrine effects which

might promote NGF synthesis. Furthermore, the overexpression of FGFR-1 and FGFR-2 in astrocytes in response to brain lesion (Takami et al., 1993) may increase the sensitivity of astrocytes to aFGF. This concept is supported by our results showing an eight-fold increase of NGF concentration relative to controls after aFGF treatment as well as a four-fold increment relative to lesioned animals treated with vehicle. The fact that FGFR-1 and FGFR-2 are abundantly expressed in the cerebral cortex suggest that aFGF could be released from afferent basolocortical cholinergic neurons (Stock et al., 1992), probably by a mechanism other than that requiring the N-terminal hydrophobic leader sequence. Alternatively, following the concept of Eckenstein and coworkers (Eckenstein et al., 1991) that FGFs may act as injury-related molecules, aFGF that is quiescent and unreleasable in a physiological situation could trigger a trophic cascade after being released from damaged cellular stores. This could also lead to increased synthesis of cortical NGF from neurons or from astrocytes. Interestingly, our results showed an increase of the cortical NGF caused by the lesion only, thus supporting the possibility that some of the NGF detected ipsilaterally 7 days after surgery may be due to its accumulation resulting from impairment of its retrograde transport to the nucleus basalis magnocellularis neurons. However an evident stimulation of NGFmRNA expression and NGF levels by aFGF points to the NGF enhanced synthesis in the cortical tissue. Direct evidence for aFGF and bFGF stimulation of NGF synthesis at the transcriptional level has been provided by complete inhibition of the aFGF-induced secretion of NGF by cultured astrocytes and fibroblasts, after blockade of transcription with cycloheximide and actinomycin (Yoshida and Gage, 1991). The differential sites of expression of aFGF, bFGF and their receptors may be helpful in disclosing the physiological role of FGFs. The fact that FGFR-1 and FGFR-2 bind aFGF and bFGF (Dionne et al., 1990), that FGFR-3 binds aFGF preferentially (for review: (Basilico and Moscatelli, 1992)), and that FGFR-4 is not expressed in the cerebral cortex (Itoh et al., 1994), suggests that aFGF may be as or more efficient than bFGF in inducing synthesis of NGF in the rat cerebral cortex.

CONCLUSIONS

These *in vivo* experiments demonstrate that aFGF is capable of increasing the concentration of NGF and the level of steady state NGF mRNA in rat cerebral cortex after

cortical injury, supporting the concept that the protective effects of aFGF against neuronal degeneration in injured CNS of adult rats are caused by increased production of NGF.

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REFERENCES

Altar CA, Burton LE, Bennett GL, Dugich-Djordjevic M (1991a) Recombinant human nerve growth factor is biologically active and labels novel high-affinity binding sites in rat brain. Proc Natl Acad Sci USA 88:281-285.

Altar CA, Dugich M, Armanini M, Bakhit C (1991b) Medial-lateral gradient of neostriatal NGF receptors relationship to cholinergic neurons and NGF-like immunoreactivity. J Neurosci 11:828-836.(Abstract)

Anderson KJ, Dam D, Lee S, Cotman CW (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332:360-362.

Asai T, Wanaka A, Kato H, Masana Y, Seo M, Tohyama M (1993) Differential expression of two members of FGF receptor gene family, FGFR-1 and FGFR-2 mRNA, in the adult rat central nervous system. Mol Brain Res 17:174-178.

Bakhit C, Armanini M, Bennett GL, Wong W-LT, Hansen SE, Taylor R (1991) Increase in glial-derived nerve growth factor following destruction of hippocampal neurons. Brain Res 560:76-83.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115-165.

Cuello AC (1993) Trophic responses of forebrain cholinergic neurons: a discussion. Prog Brain Res 98:265-277.

Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burguess WH, Jaye M, Schlessinger J (1990) Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J 9:2685-2692.

Eckenstein FP, Shipley GD, Nishi R (1991) Acidic and basic fibroblast growth factors in the nervous system: Distribution and differential alteration of levels after injury of central versus peripheral nerve. J Neurosci 11:412-419.

Elde R, Cao Y, Cintra A, Brelje TC, Pelto-Huikko M, Juntilla T, Fuxe K, Petterson RF, Hökfelt T (1991)

Prominent expression of acidic fibroblast growth factor in motor and sensory neurons. Neuron 7:349-364.(Abstract)

Feinstein SC, Matheson CR, Radeke MJ, Welcher AA, Blumberg D, Talvenheimo J, Yan Q (1993) Immunocytochemical localization of *trk*A and *trk*B in rat central nervous system. Soc Neurosci Abstr 19:249

Ferguson IA, Johnson EM, Jr. (1991) Fibroblast growth factor receptor-bearing neurons in the CNS: Identification by receptor-mediated retrograde transport. J Comp Neurol 313:693-706.

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Finklestein SP, Apostolides PJ, Caday CG, Prosser J, Philips MF, Klagsbrun M (1988) Increased basic fibroblast growth factor receptor (bFGF) immunoreactivity at the site of local brain wounds. Brain Res 460:253-259.

Frautschy SA, Walicke PA, Baird A (1991) Localization of basic fibroblast growth factor and its mRNA after CNS injury. Brain Res 553:291-299.

Gasser UE, Weskamp G, Otten U, Dravid AR (1986) Time course of the elevation of nerve growth factor (NGF) content in the hippocampus and septum following lesions of the septohippocampal pathways in rats. Brain Res 376:351-356.

Gómez-Pinilla F, Lee JW-K, Cotman CW (1992) Basic FGF in adult rat brain: Cellular distribution and response to entorhinal lesion and fimbria-fornix transection. J Neurosci 12:345-355.

Grothe C, Zachmann K, Unsicker K (1991) Basic FGF-like immunoreactivity in the developing and adult rat brainstem. J Comp Neurol 305:328-336.

Hefti F, Hartikka J, Knusel B (1989) Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. Neurobiol Aging 10:515-533.

Herrera DG, Cuello AC (1992) Glial fibrillary acidic protein immunoreactivity following cortical devascularizing lesion. Neuroscience 49:781-791.

Itoh N, Yazaki N, Tagashira S, Miyake A, Ozaki K, Minami M, Satoh M, Ohta M, Kawasaki T (1994) Rat FGF receptor-4 mRNA in the brain is expressed preferentially in the medial habenular nucleus. Mol Brain Res 21:344-348.

Janet T, Miehe M, Pettmann B, Labourdette G, Sensenbrenner M (1987) Ultrastructural localization of fibroblast growth factor in neurons of rat brain. Neurosci Lett 80:153-157.(Abstract)

Kato H, Wanaka A, Tohyama M (1992) Co-localization of basic fibroblast growth factor-like immunoreactivity and its receptor mRNA in the rat spinal cord and the dorsal root ganglion. Brain Res 576:351-354.

Kiyota Y, Takami K, Iwane M, Shino A, Miyamoto M, Tsukuda R, Nagaoka A (1991) Increase in basic fibroblast growth factor-like immunoreactivity in rat brain after forebrain ischemia. Brain Res 545:322-328.

Korsching S, Heumann S, Thoenen H, Hefti F (1986) Cholinergic denervation of the hippocampus by fimbria lesion leads to a transient accumulation of NGF without change in NGF mRNA content. Neurosci Lett 66:175-180.

Merlio J-P, Ernfors P, Jaber M, Persson H (1992) Molecular cloning of rat *trk*C and distribution of cells expressing messenger RNAs for members of the *trk* family in the rat central nervous system. Neuroscience 51:513-532.(Abstract)

Nobuyuki I, Yazaki N, Tagashira S, Miyake A, Ozaki K, Minami M, Satoh M, Ohta M, Kawasaki T (1994) Rat FGF receptor-4 mRNA in the brain is expressed preferentially in the medial habenular nucleus. Mol Brain Res 21:344-348.

Oderfeld-Nowak B, Bacia A, Gradrowska M, Fusco M, Vantini G, Leon A, Aloe L (1992) In vivo activated brain astrocytes may produce and secrete nerve growth factor-like molecules. Neurochem Int 21:455-461.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Raivich G, Kreutzberg GW (1987) The localisation and distribution of high affinity β-nerve growth factor binding sites in the central nervous sytem of the adult rat. A light microscopic autoradiographic study using [^{125}I] β-nerve

growth factor. Neuroscience 20:23-36.

Richardson PM, Verge Issa VMK, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 6:2312-2321.

Scully JL, Otten U (1993) Glucocorticoid modulation of neurotrophin expression in immortalized mouse hippocampal neurons. Neurosci Letts 155:11-14.

Sofroniew MV, Pearson RC, Eckenstein F, Cuello AC, Powell TP (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res 289:370-374.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Näher-Noé M, Thoenen H (1990) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. Eur J Neurosci 2:69-76.

Stephens PH, Cuello AC, Sofroniew MV, Pearson RC, Tagari P (1985) Effect of unilateral decortication on choline acetyltransferase activity in the nucleus basalis and other areas of the rat brain. J Neurochem 45:1021-1026.

Stock A, Kuzis K, Woodward WR, Nishi R, Eckenstein FP (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12:4688-4700.

Takami K, Iwane M, Kiyota Y, Miyamoto M, Tsukuda R, Shiosaka S (1992) Increase of basic fibroblast growth factor immunoreactivity and its mRNA level in rat brain following transient forebrain ischemia. Exp Brain Res 90:1-10.

Takami K, Kiyota Y, Miyamoto M, Tsukuda R, Igaraschi K, Shino A, Wanaka A, Shiosaka S, Tohyama M (1993) Upregulation of fibroblast growth factor-receptor messenger RNA expression in rat brain following transient forebrain ischemia. Exp Brain Res 97:185-194.

Tourbach A, Oliver L, Jeanny JC, Gumpel M (1991) Acidic fibroblast growth factor (aFGF) is expressed in the neuronal and glial spinal cord cells of adult mice. J Neurosci Res 29:560-568.(Abstract)

Van der Wal EA, Gómez-Pinilla F, Cotman CW (1994) Seizure-associated induction of basic fibroblast growth factor and its receptor in the rat brain. Neuroscience 60:311-323.(Abstract)

Wanaka A, Johnson EM, Jr., Milbrandt J (1990) Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. Neuron 5:267-281.

Weskamp G, Gasser UE, Dravid AR, Otten U (1986a) Fimbria fornix-lesion increases nerve growth factor content in adult rat septum and hippocampus. Neurosci Lett 70:121-126.

Weskamp G, Lorez HP, Keller HH, Otten U (1986b) Cholinergic but not monoaminergic denervation increases nerve growth factor content in the adult rat hippocampus and cerebral cortex. Naunyn Schmiedebergs Arch Pharmacol 334:346-351.

Weskamp G, Otten U (1987) An enzyme-linked immunoassay for nerve growth factor (NGF): a tool for studying regulatory mechanisms involved in NGF production in brain and peripheral tissues. J Neurochem 48:1779-1786.

Wilcox BJ, Unnerstall JR (1992) Expression of acidic fibroblast growth factor mRNA in the developing and adult rat brain. Neuron 12:142-152.(Abstract)

Woodward WR, Nishi R, Meshul CK, Williams TE, Coulombe M, Eckenstein FP (1992) Nuclear and cytoplasmic localization of basic fibroblast growth factor in astrocytes and CA2 hippocampal neurons. J Neurosci 12:142-152.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

Results shown in the preceding chapter was the first demonstration in brain of adult animals that exogenous application of aFGF induces substantial increase in cortical NGF, providing a key to the mechanism of how aFGF prevents degeneration of NBM cholinergic neurons. While still limited by technical difficulties, we were unable to characterize the cell type(s) responsible for such increase in NGF protein following lesion and aFGF treatment. The cells expressing this neurotrophin is likely to be astroglial which express receptors to FGF receptors (see Table 3 in Chapter I) and that respond to aFGF and bFGF *in vitro*, with increased release of NGF (Spranger et al., 1990; Ono et al., 1991; Yoshida and Gage, 1991; Yoshida and Gage, 1992). It has been described that NGF protein in unlesioned brain is mainly expressed in neurons (Conner et al., 1992; Conner and Varon, 1992). However, on closer examination of the immunoreactivities for glial fibrillary acidic protein and NGF protein, following brain injury, they were mainly co-localized and enhanced in astrocytes (Bakhit et al., 1991). Therefore, our current understanding of these events led us to reiterate the proposition from previous investigators (Spranger et al., 1990; Barde, 1989), that NGF is released from astrocytes following stimulation by aFGF and bFGF. This seems to be the only mechanism available to explain the protection of basal forebrain neurons by FGFs. This conclusion reinforces the role of NGF as a specific neurotrophic factor for these neurons and stimulates further studies on NGF effects in these cholinergic neurons. Therefore, search for changes in the expression of proteins involved in signalling NGF effects has attracted our attention to analyze changes in the mRNAs of two proteins. The regulation of $p75^{NGFR}$, known to be upregulated by NGF (Higgins et al., 1989), and one putative marker of regeneration and sprouting, such as growth-associated protein-43 (GAP-43) which is upregulated by NGF in PC12 cells (Mooradian, 1990), were analyzed in response to cortical infarction and NGF treatment. Next chapter presents the first analysis of these NBM mRNAs at different post-lesion days. This study also addresses changes in GAP-43 mRNA in neurons that are not responsive to NGF, one approach that allows better evaluation of the NGF effects.

REFERENCES

Bakhit C, Armanini M, Bennett GL, Wong W-LT, Hansen SE, Taylor R (1991) Increase in glial-derived nerve growth factor following destruction of hippocampal neurons. Brain Res 560:76-83.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Conner JM, Muir D, Varon S, Hagg T, Manthorpe M (1992) The localization of nerve growth factor-like immunoreactivity in the adult rat basal forebrain and hippocampal formation. J Comp Neurol 319:454-462.

Conner JM, Varon S (1992) Distribution of nerve growth factor-like immunoreactive neurons in the adult rat brain following colchicine treatment. J Comp Neurol 326:347-362.

Higgins GA, Koh S, Chen KS, Gage FH (1989) NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3:247-256.

Mooradian AD (1990) Metabolic fuel and amino acid transport into the brain in experimental hypothyroidism. Acta Endocrinol (Copenh) 122(2):156-162.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Nahernoe M, Thoenen H (1990) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system - comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. Eur J Neurosci 2:69-76.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

CHAPTER V

DIFFERENTIAL EXPRESSION OF $p75^{NGFR}$ AND GAP-43 GENES IN NUCLEUS BASALIS, THALAMUS AND ADJACENT CORTEX FOLLOWING NEOCORTICAL INFARCTION AND NGF TREATMENT

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ABSTRACT

A loss of target-derived neurotrophic factors is hypothesized to be one of the major determinants of central nervous system (CNS) neuronal degeneration vulnerability. In order to obtain further insight into early neuronal responses to injury, lesion-induced alterations in the expression of low-affinity nerve growth factor (NGF) receptors and growth-associated phosphoprotein-43 (GAP-43) genes in nucleus basalis magnocellularis (NBM), thalamic and neocortical neurons were studied. For this purpose, unilateral cortical devascularization operations were conducted on adult rats. They received, i.c.v. via minipump, either vehicle or NGF (12 μg/day) and were sacrificed at 1, 3, 7 and 15 days post-lesion. *In situ* hybridization studies, using ^{35}S-labelled oligonucleotide probes for p75^{NGFR} and GAP-43 mRNAs revealed that these genes were differentially regulated following the lesion. In the NBM, p75^{NGFR} mRNA initially increased on day 3 and decreased on days 7 and 15 post-lesion. GAP-43 mRNA levels were significantly increased in the NBM on post-lesion days 3 and 7. Moreover, in contrast to p75^{NGFR}, GAP-43 mRNA levels were moderately increased in pyramidal neurons located in the remaining adjacent cortex at all time points days of all decorticated animals. In the lateral and ventroposterior nuclei of the thalamus, GAP-43 mRNA was slightly increased on day 3 and was dramatically decreased, significantly below unlesioned controls, on post-lesion days 7 and 15. NGF application prolonged the transient increase of p75^{NGFR} mRNA noted in lesioned animals treated with vehicle. However, only NGF-treated animals had higher p75NGFR mRNA levels in postlesion days 1 and 7, indicating that at these timepoints lesion alone does not cause significant change in this expression. In addition, NGF-induced increase in p75^{NGFR} mRNA in the NBM contralateral to the lesion suggested an effect independent of the lesion. NGF treatment did not affect the expression of GAP-43 in any of the areas studied. Three cell types, NBM, pyramidal and thalamic neurons, were probably affected in different ways by the devascularization with respect to lesion extent. Consequently, the remaining number of synaptic contacts in each of these brain areas is very likely different which may lead to a differential regulation of GAP-43 mRNA. Furthermore, our results are in line with the concept that GAP-43 is regulated by target-derived factors other than NGF and that the increase in

$p75^{NGFR}$ mRNA in lesioned animals may occur as a consequence of increased neurotrophin activity.

INTRODUCTION

NGF, the prototype for a family of neurotrophins (Thoenen, 1991; Ebendal, 1992), prevents cell death of lesioned cholinergic septal neurons (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Hagg et al., 1990) and rescues NBM cholinergic neurons injured by cortical devascularization (Cuello et al., 1989) or neurotoxins (Haroutunian et al., 1989). Critical reports providing evidence for a trophic response to NGF by cholinergic basal forebrain neurons (Honegger and Lenoir, 1982; Gnahn et al., 1983; Mobley et al., 1985) were supported by a subsequent study describing high affinity NGF binding to rat brain sections (Richardson et al., 1986). The NBM is responsible for the majority of the extrinsic cholinergic input to the neocortex (Fibiger, 1982; Saper, 1984; Luiten et al., 1987; Sofroniew et al., 1987; Woolf, 1991) which is the most abundant source of NGF in the basalo-cortical pathway (Korsching et al., 1985; Goedert et al., 1986; Large et al., 1986; Shelton and Reichardt, 1986; Whittemore et al., 1986). The mechanism by which NGF initiates signaling involves binding to and activation of a tyrosine kinase receptor ($p140^{trk}$) (Martin-Zanca et al., 1986; Hempstead et al., 1991; Kaplan et al., 1991a; 1991b; Klein et al., 1991a) which is a transducing receptor similar to those of other growth factors (Ullrich and Schlessinger, 1990; Cordon-Cardo et al., 1991; Glass et al., 1991; Klein et al., 1991b; Lamballe et al., 1991; Squinto et al., 1991; Soppet et al., 1991; Schlessinger and Ullrich, 1992). NGF also binds to (transmembrane glycoprotein) a low-affinity receptor ($p75^{NGFR}$) (Johnson et al., 1986; Radeke et al., 1987) which plays an important role in developing sensory neurons (Lee et al., 1992). However, it continues to remain unclear as to whether there are any $p75^{NGFR}$-dependent effects of NGF in the brain.

Important features of NGF action include increases of mRNA levels in CNS neurons for $p140^{trk}$ (Holtzman et al., 1992; Venero et al., 1994; Gibbs and Pfaff, 1994),

$p75^{NGFR}$ (Cavicchioli et al., 1989; Gage et al., 1989; Higgins et al., 1989; Holtzman et al., 1992) and choline acetyltransferase (ChAT) (Higgins et al., 1989; Venero et al., 1994), as well as increases of $p75^{NGFR}$ mRNA levels in neurons of the peripheral nervous system (PNS) (Lindsay et al., 1990; Miller et al., 1991; Verge et al., 1992). These data further support the possibility that the NBM-derived cholinergic neurons projecting to neocortex (Fibiger, 1982; Saper, 1984; Luiten et al., 1987; Sofroniew et al., 1987; Woolf, 1991), which undergo retrograde degeneration after neocortical devascularization (Stephens et al., 1985; Sofroniew et al., 1983, 1987), may alter the levels of $p75^{NGFR}$ mRNAs in the NBM. Although increased neurotrophic activity in the cortex adjacent to injury site has been shown (Nieto-Sampedro et al., 1983), after an extensive frontoparietal ischemic decortication, a significant loss of target-derived NGF may be expected to alter gene expression in NBM neurons. In agreement with this hypothesis, previous studies have shown that without interventive treatment with exogenous NGF, axotomized septo-hippocampal (Hefti, 1986; Williams et al., 1986) and injured cholinergic NBM neurons (Cuello et al., 1989; Garofalo et al., 1992) degenerate.

We are interested in studying the mechanism that underlies retrograde degeneration of cholinergic NBM neurons. Therefore, we examined the expression of $p75^{NGFR}$ gene in the NBM following a lesion which deletes targets of NBM cholinergic neurons and compromise several of their cortical terminals. Also included in this study, an assessment of GAP-43 mRNA, a putative marker of neuronal plasticity (Benowitz and Routtenberg, 1987; Skene, 1989; Benowitz and Perrone-Bizzozero, 1991; Coggins and Zwiers, 1991; Gispen et al., 1991; Neve et al., 1991; Bisby and Tetzlaff, 1992). Evidence which suggests that GAP-43 plays an important role during growth of developing neurons (Skene and Willard, 1981; Jacobson et al., 1986; Meiri et al., 1986; Goslin et al., 1988; McGuire et al., 1988; De la Monte et al., 1989; Figueiredo et al., 1993a) as well as during regeneration of axotomized peripheral neurons (Tetzlaff et al., 1989; 1991; Van der Zee et al., 1989) is accumulating. Moreover, a correlation has been shown between the restorative capacity of the CNS (Richardson et al., 1984) and the ability to express GAP-43 in damaged CNS neurons (Tetzlaff et al., 1991; Theriault et al., 1992).

The ischemic lesion of the neocortex used for the present study allowed an *in vivo* analysis of mRNA levels in two cortico-petal systems, one from the NBM and the other from the thalamus, as well as in an cortico-fugal neuronal population. An increase of GAP-43 protein and its mRNA has been shown to occur in other CNS regions as a result of peripheral or central injury (Dunn-Meynell et al., 1992; Levin and Dunn-Meynell, 1993), and in association with synaptic plasticity involved in long-term potentiation (Nelson and Routtenberg, 1985). Unilateral vibrissectomy induced the message for GAP-43 earlier and more intensely in the ipsilateral trigeminal nucleus than in the contralateral rat cortex (lamina IV) but did not alter the expression in the ventrobasal thalamus (Levin and Dunn-Meynell, 1993). Thus, induction of GAP-43 mRNA is associated with intrinsic properties found in some neurons as a result of neuronal insult. The main objective of this investigation was to determine whether the vulnerability of NBM neurons to degeneration following cortical devascularization may or may not be linked to changes in the expression of $p75^{NGFR}$ and GAP-43 genes.

MATERIAL AND METHODS

Animal and tissue preparation

Three groups of animals were used: 1) Sham-operated animals treated with vehicle only; 2) cortically devascularized rats treated with vehicle, and 3) cortically devascularized rats treated with NGF for 1, 3 and 7 days. Animals survived 1, 3, 7 and 15 days after the lesion. Animals surviving 15 days received NGF for only 7 days. Adult male Wistar rats (300-325g) were anesthestized with Equithesin (2.5 ml/kg, i.p.) and placed in a stereotaxic frame. A stainless steel cannula (23 gauge) was permanently implanted into the right lateral ventricle [coordinates from Bregma (Paxinos and Watson, 1986): AP, -0.8; L, 1.4; V, 3.5]. Osmotic minipumps (Alzet 2001, 1μl/hr, seven days) were loaded with dye (0.01% Methylene blue, BDH Chemicals) and pretested before being connected to the cannulae through coiled polyethylene tubing (Intramedic, PE-60). NGF 12 μg/24 μl/day or vehicle (artificial cerebro-spinal fluid and 0.1% bovine serum albumin) was loaded into the tubing with a small amount of mineral oil added to the

minipump end to prevent mixing with the dye released from the minipump. Animals were subjected to left-side cortical devascularizing lesions as described previously (Stephens et al., 1985; Sofroniew et al., 1987). In brief, the devascularizing lesion was performed by removing a flap of bone and underlying dura and gently rubbing away all vessels and the pia mater with sterile saline-soaked cotton swabs. Control animals received a sham operation consisting of a craniotomy only. The decorticated areas included parietal 1, forelimb, and part of frontal 1, hindlimb and parietal regions (Zilles, 1985). The resulting decorticated region approximately fit the coordinates of the devascularized area (1.0 x 1.0 cm). A typical cortical lesion at the level of mid-portion of NBM is shown in Fig. V.1. Four rats were sacrificed under Equithesin anesthesia at each time-point, 1, 3, 7 and 15 days after the operation. For these studies, the mid-portion of the NBM [approximately Bregma -1.20 mm to -1.50 mm according to Paxinos atlas (Paxinos and Watson, 1986)] was analyzed. The region was chosen based on previous studies which indicated that neurons in this region of the NBM project predominantly to the devascularized parietal cortex. This was further confirmed by the present study using 1.5% FluoroGold (FG) injected, between parietal cortical layers V and III (2 μl in each of four sites, see Fig. 2A).

In situ hybridization

In situ hybridization was performed as previously described (Schalling et al., 1988) with some modifications (Verge et al., 1992). In brief, brains were removed from unperfused animals, decapitated under deep Equithesin anesthesia, and were frozen in dry ice. *In situ* hybridization was performed on tissue sections of lesioned animals mounted adjacent to sections of control animals, taken at the same NBM

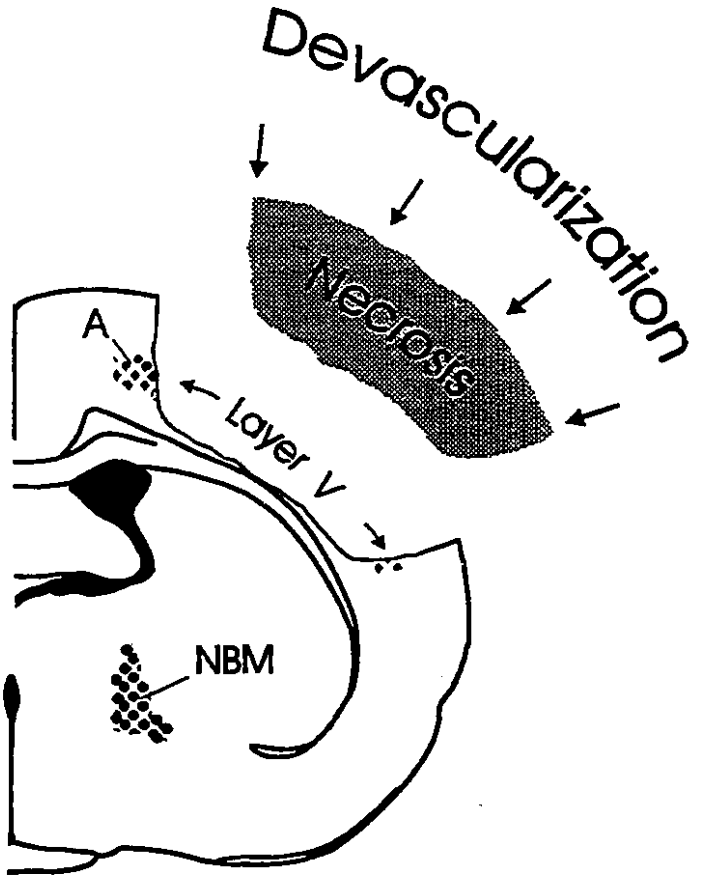


Figure V.1. Schematic illustration of the extent of a typical lesion in a brain section located at the level of the mid NBM. The cortical devascularization produced a well defined frontoparietal necrosis without direct involvement of subcortical structures. Enhanced expression of GAP-43 mRNA was detected in cortical layer V (shaded) of the remaining cortices adjacent to the lesion, lateral to the cingulate and located mainly in the hind- and forelimb cortical regions (site "A"). The measurements of silver grains (pixel) in cells were done in sections taken at the level shown in the diagram up to more posterior regions (anterior-posterior -1.40 to -2.80 from bregma, Paxinos atlas (Paxinos and Watson, 1986)). Thus, sampling neurons located in the mid-portion of the NBM.

level. Coronal sections (10 μm thick) were cut using a cryostat, were thaw-mounted onto Superfrost Plus slides (Fisher), and were stored at -70°C. Tissue sections were processed for *in situ* hybridization of $p75^{NGFR}$ and GAP-43 mRNAs using ^{35}S-labelled oligonucleotide probes. A 50-mer DNA oligonucleotide, 5'-GCATCGGTAGTAGCAGAGCCATCTCCCTCCTTCTTCTCCACACCATCAGC AA-3', was used. It is complementary to the rat GAP-43 mRNA which corresponds to the counterpart of base pairs 220-270 of the rat GAP-43 coding region (Basi et al., 1987; Nielander et al., 1987). This part is downstream of the coding region for the conserved calmodulin domain. For $p75^{NGFR}$ mRNA, a 50-mer antisense DNA oligonucleotide 5'-ACAAGGCCCACGACCACAGCAGCCAAGATGGAGAATAGACAGGAATGAG -3', was prepared complementary to the cDNA encoding the highly conserved transmembrane segment of $p75^{NGFR}$ (Ernfors et al., 1989). Sections were taken from the freezer and dried for 10 min at room temperature, followed by fixation for 20 min in cold (10-12°C) 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After washing (4x5min) in phosphate-buffered saline (PBS), sections were dehydrated through 60%, 80%, 95%, and 99% ethanol, deproteinized with chloroform for 2 min and subsequently rinsed with 99% and 95% ethanol for 1 min each. Subsequently, the sections were dried for 20 min at room temperature and incubated for 18 h at 42°C in a hybridization buffer (100 μl/slide) containing 50% deionized formamide, 4x SSC, 10% dextran sulfate, 0.02 M phosphate buffer, 50 gm/100 ml salmon sperm DNA, 200 mM dithiothreitol and 106 cpm of [α-35S]dATP-labelled probe/100 μl/slide (terminal transferase labelling according to Ausubel et al., (1987). Following the hybridization process, the sections were washed with 4x SSC for 20 min at room temperature, followed by washout in 2x SSC at 50°C (1 x 20 min), 1x SSC at 50°C (4x 20 min), 1x SSC at 55°C (1x 20 min), 0.5x SSC at 45°C (1 x 20 min) and 0.25x SSC at 45°C 1x 20 min). The sections were then rinsed in double distilled water for 1 min at 30°C and then four times (5 min each) at room temperature, and were dried at room temperature. The sections were dipped in NTB2 emulsion (diluted 1:1 with distilled water) and exposed in the dark for five to eight days for GAP-43 and $p75^{NGFR}$ hybridized probes. Following development, the sections were counterstained with Methylene blue plus fuchsin, were dehydrated, and coverslipped.

Immunocytochemistry

The polyclonal anti-glial fibrillary acidic protein (anti-GFAP, Dako) was incubated for 12 h at 4°C with 10 μm-, cryostat cut, free-floating coronal brain sections taken at the level of the thalamus. All subsequent antibody incubation steps, intervening washes and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) reactions, were performed at room temperature using PBS (pH 7.4) as the solvent. After washing (2x15 min), the sections were incubated for 2 h with the second antibody, a monoclonal bi-specific (Kenigsberg et al., 1990) (MCC8 cell line, anti-peroxidase and anti-rabbit IgG, Medicorp, Canada). Subsequently, the sections were incubated for 1 h with horseradish peroxidase (HRP; Sigma, Type IV) only. The sections were washed with PBS (4x15 min) and then developed with 0.06% DAB containing 0.01% hydrogen peroxide for 10 min followed by a washing in PBS. After washing in PBS, the sections were mounted on subbed slides (in gelatin) and left to dry before dehydration and coverslip-mounting. After dehydration in a number of alcoholic solutions and immersion in xylene, the slides were subsequently coverslipped with a mounting media (Entellan).

Quantification of silver grains

The number of silver grains per area of cytoplasm was quantified using the M1 version of an MCID Image analysis system (Microcomputer Imaging Device; Imaging Research Inc., St. Catharine, Ont., Canada). Measurements were performed using light microscopy with an oil immersion objective connected to an image analysis system. Measurements were performed in NBM, cortical pyramidal and thalamic neurons neurons located ipsi- and contralateral to the cortical devascularization. The number of silver grains on lesioned side was compared with that of the contralateral side in the same group and with the side ipsilateral to the sham-operation in the control vehicle-treated animals and of NGF-treated lesioned animals. For each brain section, the background measured from corpus callosum areas contralateral to the lesion was subtracted from signal obtained for each probe. Labeled cells used for analysis exhibited optimal distribution of grains in the cytoplasm. All cells with silver grains above background values were included in the quantification. Only cells with evident cytoplasm and nucleus

were included in our analysis. Since we could not distinguish between lesioned and unlesioned neurons, it is likely that non-injured cells adjacent to the injured cells were included in the quantification.

Statistical analysis

Differences in the number of grains among the groups were compared using ANOVA followed by a post-hoc test (Newman-Keuls'). The level of statistical significance was set at $p < 0.05$.

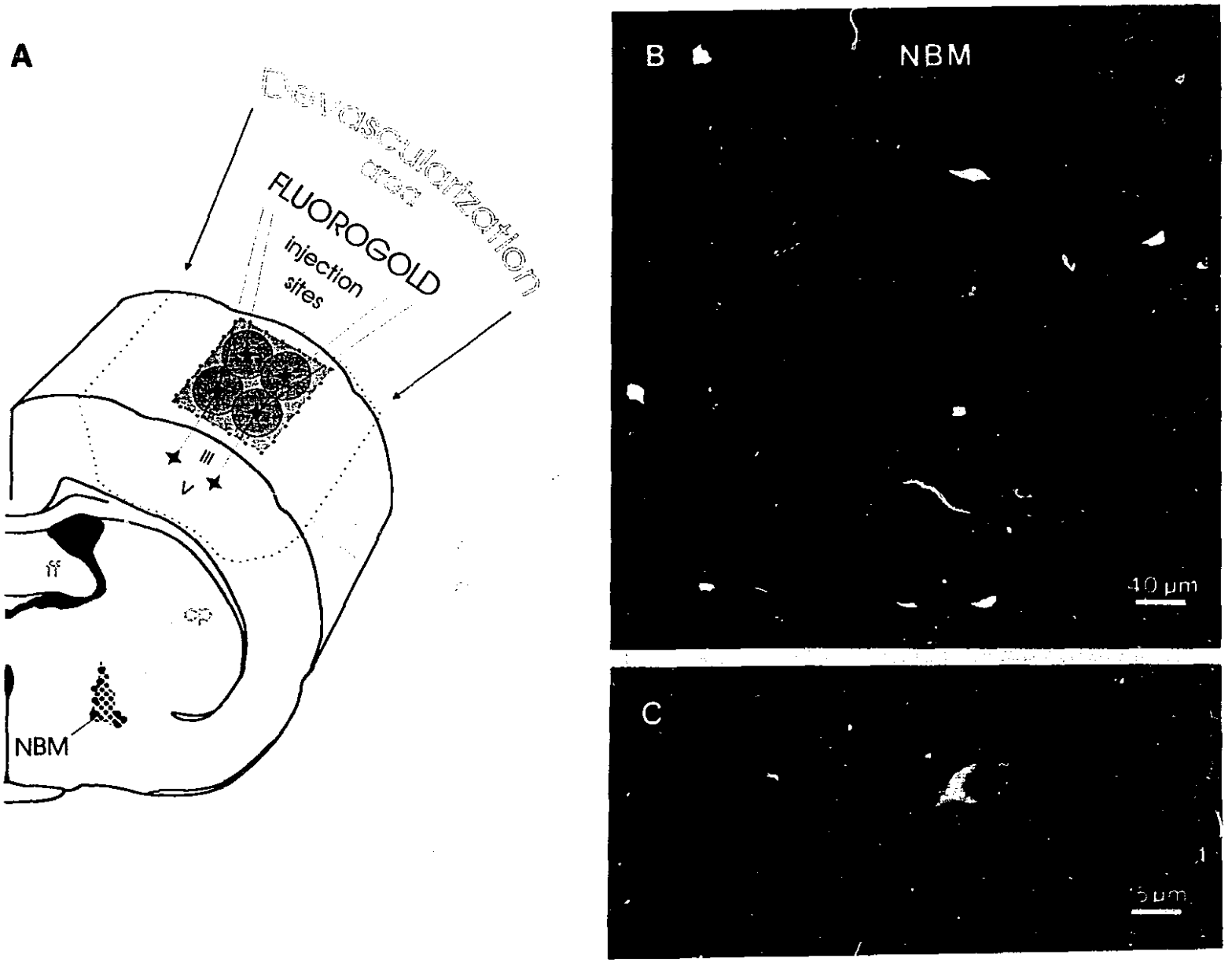
RESULTS

Cholinergic basalo-cortical pathway

In this study our quantitative analysis was restricted to neurons in the mid-NBM. Although cholinergic neurons in the NBM are distributed in a diffused maner, previous studies by others (Bigl et al., 1982; Luiten et al., 1987; Eckenstein et al., 1988) and from our own group (Cuello et al., 1989; Figueiredo et al., 1993b) indicate that the parietal cortex receives innervation predominantly from neurons in the mid portion of this nucleus. This was confirmed by the present report which examined the retrograde transport of FluoroGold (FG). As shown in Fig. V.2A, 1.5% FG in saline (4x 2 μl) was injected between cortical layers III and V covering a central area (parietal) corresponding to approximately 40% of the devascularized region. Using this procedure, a large number of neuronal cell bodies located in the

Figure V.2

Retrograde labelling of NBM cholinergic cell bodies with fluoroGold (FG). A. Illustration of the FG injection sites located in the parietal cortex: four injections of 2 μl of 1.5% FG in saline were done between cortical layers V and III as shown in the diagram. B. Photomicrograph shows labelled cells taken from the mid-basalis (level shown as in A). C. FG-labelled cell taken at high magnification.


A
Devascularization area
FLUOROGOLD
injection sites
III
V
ff
cp
NBM
B
NBM
10 µm
C
5 µm

mid portion of the NBM were labelled (Fig. V.2B). Fig. V.2C shows in high power detail the labelling with very low FG-background level. This number of FG-labelled cells noted was slightly less than the number of ChAT-immunostained cells generally observed. These findings suggest that we probably include a small number of unlesioned neurons which were not projecting to the selected site of the cortical lesion. However, it must be taken into account that the area corresponding to the cortical lesion is larger than the cortical area that received FG.

Cross-sectional area

Quantification of the cross-sectional area ($\mu m^2 \pm$ SEM) of NBM cells ipsilateral to the lesion, containing positive signal for $p75^{NGFR}$ above background values, revealed a mean cross-sectional area in sham-operated animals of 216 $\pm$ 18. However, different values were obtained between 254 $\pm$ 17, measured on 3rd post-lesion day of lesioned NGF-treated animals, and 203 $\pm$ 23 of the 15th post-lesion day of vehicle-treated animals. This was the only statistical difference among all measured cross-sectional areas. The cross-sectional area on the 7th post-lesion day of NGF-treated animals ipsilateral to the lesion (242 $\pm$ 31), exhibited higher variability among the five animals than the other experimental groups.

$p75^{NGFR}$ mRNA levels

The expression of $p75^{NGFR}$ mRNA was similarly analysed at 4 post-lesion days. At 1 or 3 days postlesion, we observed an increase in the level of $p75^{NGFR}$ mRNA in several cells of the mid-NBM of lesioned vehicle-treated animals (Fig. V.3). Although the histogram displaying all measurements at 1 and 3 post-lesion days reveals a moderate elevation of $p75^{NGFR}$ hybridization signal in some NBM neurons ipsilateral to the lesion, the mean number of pixels obtained from all cells indicate only a modest, but statisticaly significant, increase of $p75^{NGFR}$ mRNA levels only on post-lesion day 3 (Table V.1). On day 7 and 15 post-lesion the level of $p75^{NGFR}$ mRNA in many NBM cells of lesioned vehicle-treated animals was decreased below levels detected in sham-operated

vehicle-treated rats. This decrease, however, was not significantly reduced relative to sham-operated animals. The decrease in signal for $p75^{NGFR}$ mRNA at day 15 in lesioned animals was significantly different to that observed on day 1. Interestingly, after NGF application, $p75^{NGFR}$ mRNA levels rose in NBM lesioned neurons at 1, 3 and 7 days post-lesion (Fig. V.3-N1,-N3,-N7) to reach maximal levels at 7 days post-lesion, an increase that was statistically significant during the time period that NGF was applied. By 15 days post-lesion, the number of pixels ipsilateral to the lesion (i.e. 8 days after cessation of treatment) was slightly higher but not statistically different from the sham-operated control. Grain countings/μm^2 $\pm$ SEM (for $p75^{NGFR}$ hybridized probes) in the NBM contralateral to the lesion in NGF-treated animals at postlesion day 3 (11.82 ± 0.64, $n=4$) and 7 (13.83 ± 0.42, $n=3$) were increased ($p<0.05$) relative to sham-operated controls (please see Table V.1). These results indicated that NGF also increased levels of its low affinity receptor mRNA in unlesioned NBM neurons. However, in NBM neurons contralateral to the lesion in vehicle-treated rats $p75^{NGFR}$ mRNA levels were similar to those of sham-operated animals at all post-lesion days. $p75^{NGFR}$ mRNA was not detected in the thalamus or neocortex. Analysis of other brain areas which express $p75^{NGFR}$ mRNA were not included in this study. arrows point to single cells. Scale bar in C7=20 μm and applies to all bright-field photomicrographs. Scale bar in N15=90 μm and apllies all dark-field photomicrographs.

GAP-43 mRNA levels

GAP-43 mRNA levels, in contrast to $p75^{NGFR}$, changed following cortical devascularization in all three brain areas studied: NBM, thalamus and neocortex, where it was differentially regulated after surgical lesion. In the NBM, the highest level of GAP-43 mRNA was detected in lesioned animals 7 days after the surgery, as shown in bright and dark field photomicrographs in Figure V.4. The mean number of pixels per NBM cell cross-sectional area (μm^2) ipsilateral to the lesion were significantly elevated

Figure V.3

Expression of $p75^{NGFR}$ mRNA in NBM

Dark- and bright-field photomicrographs of silver grains detected in the mid-portion of the nucleus basalis. Sections from sham-operated control (C), lesion + vehicle (V) and lesion + NGF (N) treated animals are shown at their respective 1, 3, 7 and 15 postlesion days as indicated by the subscripted number. The density of silver grains/cell was higher on V1 and V3 and declined on V7 and V15 compared with respective controls. Note the more intense increase of $p75^{NGFR}$ mRNA in N3 and N7. In N15, a time point 8 days after interruption of NGF-treatment, labelling of $p75^{NGFR}$ mRNA detected was higher than on V15 but not different from C15. Black and white

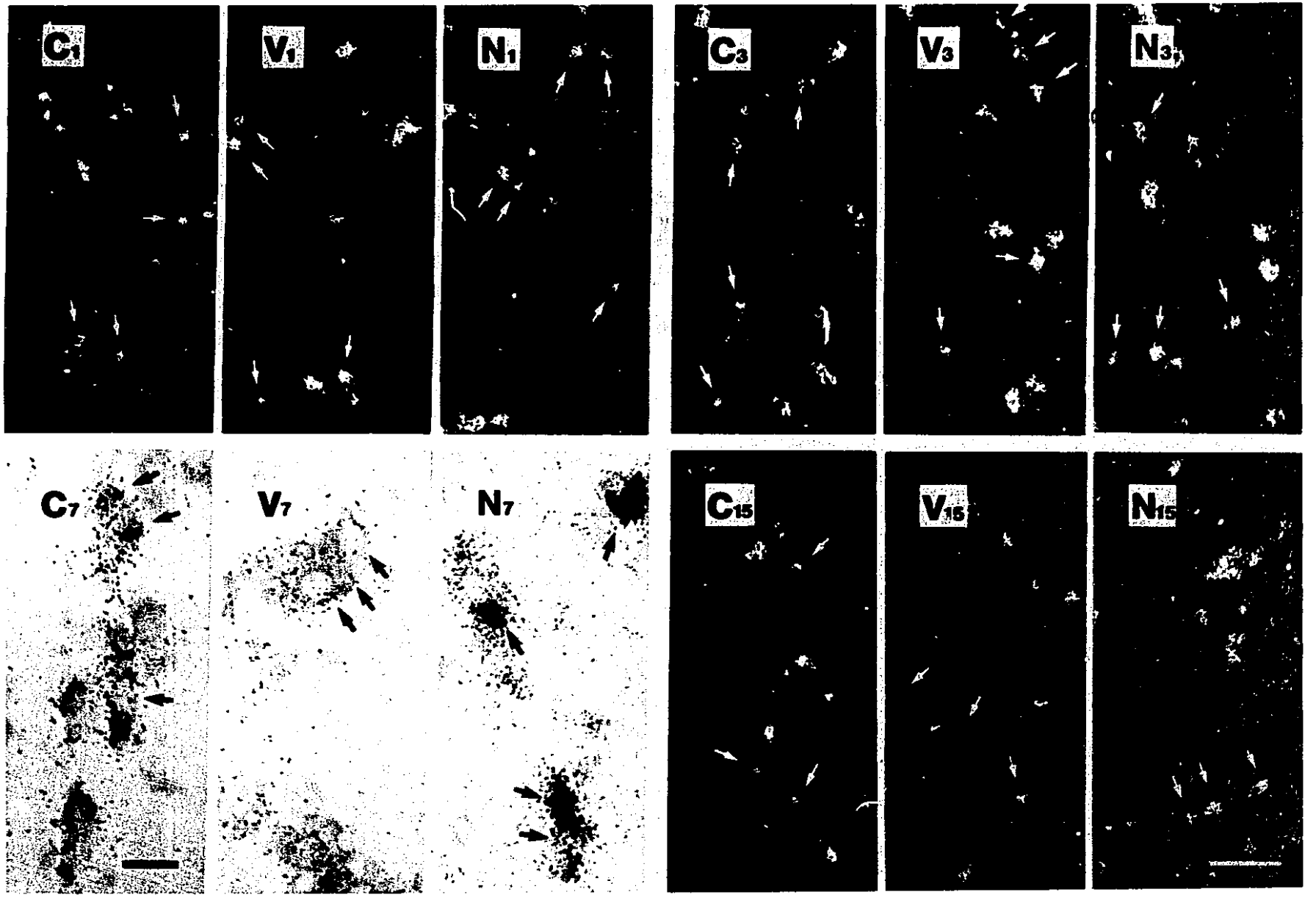

C1
V1
N1
C3
V3
N3
C7
V7
N7
C15
V15
N15

Table V.1 $p75^{NGFR}$ mRNA levels in nucleus basalis ipsilateral to the lesion

	Mean number of silver grains (pixels)/ cross-sectional cell area (μm^2)		
Post-lesion day	Control	Lesion + Vehicle	Lesion + NGF
1	8.70 ± 0.75	10.91 ± 0.50	12.11 ± 0.62*
3	7.13 ± 0.48	11.42 ± 1.43*	13.29 ± 1.29*
7	8.62 ± 0.39	7.26 ± 0.78$^{\Delta}$	14.25 ± 0.76*$^{\Delta}$
15	8.53 ± 0.55	6.24 ± 0.42$^{\Delta}$	9.98 ± 0.59$^{\Delta}$

Values represent mean ± S.E.M., n=4-5 animals/group. * $p < 0.05$ from control ; $^{\Delta}$ $p < 0.05$ from day 1 values within the same group; $^{\Delta}$ $p < 0.05$ from lesion + vehicle (ANOVA followed by post-hoc Newman-Keuls' test).

on days 3 and 7 after devascularization (Table V.2). GAP-43 mRNA levels were reduced on post-lesion day 15 and were not statistically different from sham-operated animals nor GAP-43 mRNA was detected in lesioned animals 7 days after the surgery, as shown in bright and dark field photomicrographs in Figure V.4. The mean number of pixels per NBM cell cross-sectional area (μm^2) ipsilateral to the lesion were significantly elevated on days 3 and 7 after devascularization (Table V.2). GAP-43 mRNA levels were reduced on post-lesion day 15 and were not statistically different from sham-operated animals nor from levels detected in the contralateral NBM. In the neocortex, GAP-43mRNA was detected (Fig. V.5) and quantitated in layer V. Changes in Gap-43 mRNA in layer V were noted in pyramidal cells located lateral to the cingulate region and medial to the decorticated area (site "A", shown in Fig. V.1) including parts of hind- and fore-limb cortical regions. GAP-43 mRNA levels were increased in pyramidal cells at day 1 post-lesion (Fig. V.5, compare C1 with V1 and N1). These levels remained elevated on all post-lesion days studied (1, 3, 7 and 15) and reached their highest level on post-lesion day 3 (Table V.3). In individual pyramidal cells GAP-43 mRNA was increased up to 6-fold as compared with controls. In the thalamus, GAP-43 mRNA increase in cells of the ipsilateral lateral nucleus was noted on post-lesion day 1 animals. (data not shown). However, this mRNA value (5.25 ± 1.02) was not statistically different from that obtained for sham-operated animals (3.2 ± 0.81). After day 3, GAP-43 mRNA levels in thalamic neurons decreased progressively below levels detected in sham-operated animals (Fig. V.6). This decrease was more accentuated (a 38% reduction in large cell number) in the lateral thalamic nucleus of vehicle-treated animals as compared with the contralateral side ($P<0.05$, student's t test) and was parallel to the increase of picnotic

Figure V.4

Expression of GAP-43 mRNA in NBM

Dark- and bright-field photomicrographs taken from animals that survived seven days after the lesion. Sections from sham-operated control (C), lesion + vehicle (V) and lesion + NGF (N) treated animals are shown. Note the increased hybridization signal in lesioned groups, V7 and N7. Clusters of silver grains from overlapping cells are seen in V7 (bright-field) and N7 (dark-field) photomicrographs. Small solid arrows (black and white) point to single cells while open arrows (black and white) point to clusters of 2 or more cells. Scale bar in N7 (bright-field)=20 μm and also applies to C7 and V7 bright-field micrographs. Scale bar in N7(dark-field)=40 μm and also apllies to C7 and V7 dark-field micrographs.

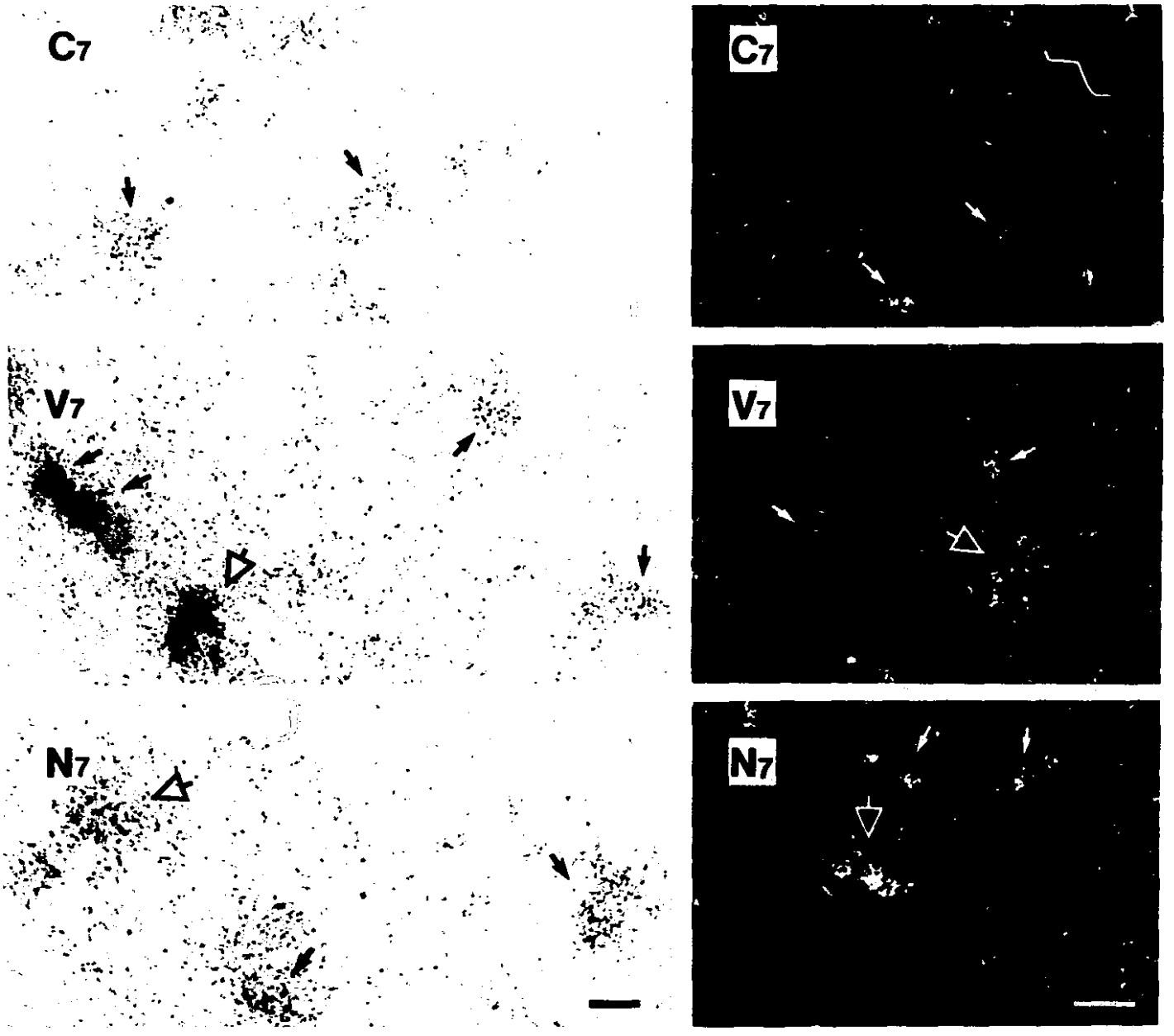

C7
V7
N7
C7
V7
N7

Table V.2 GAP-43 mRNA levels in nucleus basal isipsilateral to the lesion

	Mean number of silver grains (pixels)/ cross-sectional cell area (μm^2)		
Post-lesion day	Control	Lesion + Vehicle	Lesion + NGF
1	4.33 ± 0.28	4.37 ± 0.19	3.92 ± 0.28
3	5.55 ± 0.50	7.40 ± 0.36*▲	6.84 ± 0.64*▲
7	3.77 ± 0.30	11.54 ± 0.86*▲	9.68 ± 0.57*▲
15	4.41 ± 0.18	5.30 ± 0.34	4.21 ± 0.26

Values represent mean ± S.E.M., n=4-5 animals/group. * $p < 0.05$ from control ; ▲ $p < 0.05$ from day 1 values within the same group; △ $p < 0.05$ from lesion + vehicle (ANOVA followed by post-hoc Newman-Keuls' test).

Figure V.5

Expresion of GAP-43 mRNA in neocortex

The distribution of silver grains reflecting GAP-43 mRNA expression is shown in dark-field photomicrographs for cortical layers III, IV, V and VI, as shown in C1, which applies to all dark-field panels. In bright-field photomicrographs, C3 and V3, cortical layer V is shown at the center of the micrograph. Sections from sham-operated control (C), lesion + vehicle and lesion + NGF (N) treated animals are shown. Subscripted numbers correspond to post-lesion days. Expression of GAP-43 mRNA in N3, N7 and N15 were not shown as they did not differ from their to respective vehicle-treated groups (V3, V7 and V15). Note that the density of silver grains in all lesioned groups, V and N, are higher in layer V: the labelling is already elevated in V1 and N1. A much higher signal for GAP-43 mRNA is observed in V3 and N3. This signal declined in V7 and V15 (N7 and N15, not shown), but it remained higher than in control preparations. Scale bar in V15=90 μm and applies to all dark-field photomicrographs. Scale bar in C3=25 μm and also applies to V3 (bright-field).

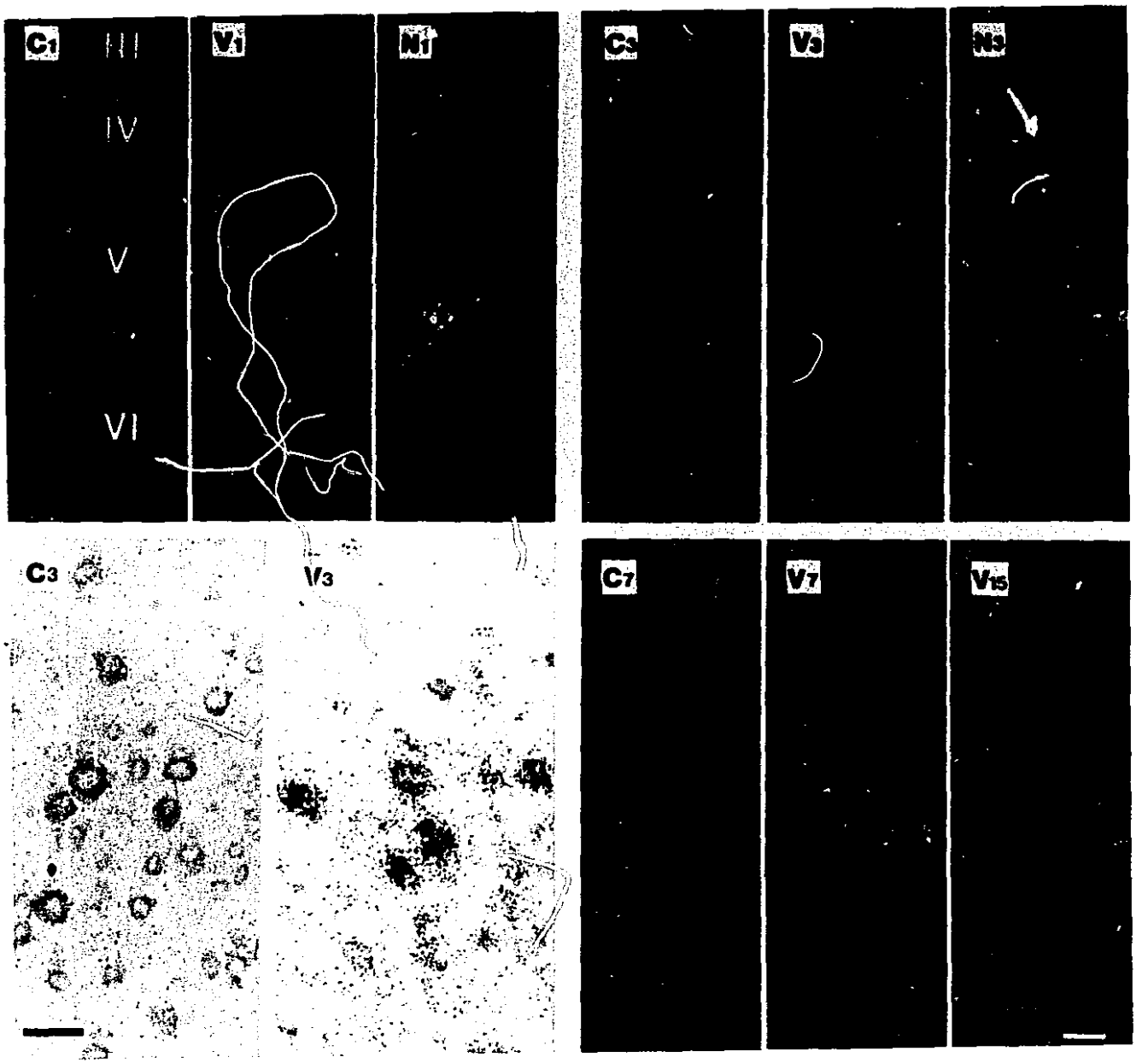

C1
III
IV
V
VI
V1
N1
C3
V3
C5
V5
N5
C7
V7
V15

Table V.3 GAP-43 mRNA levels in pyramidal cells ipsilateral to the lesion

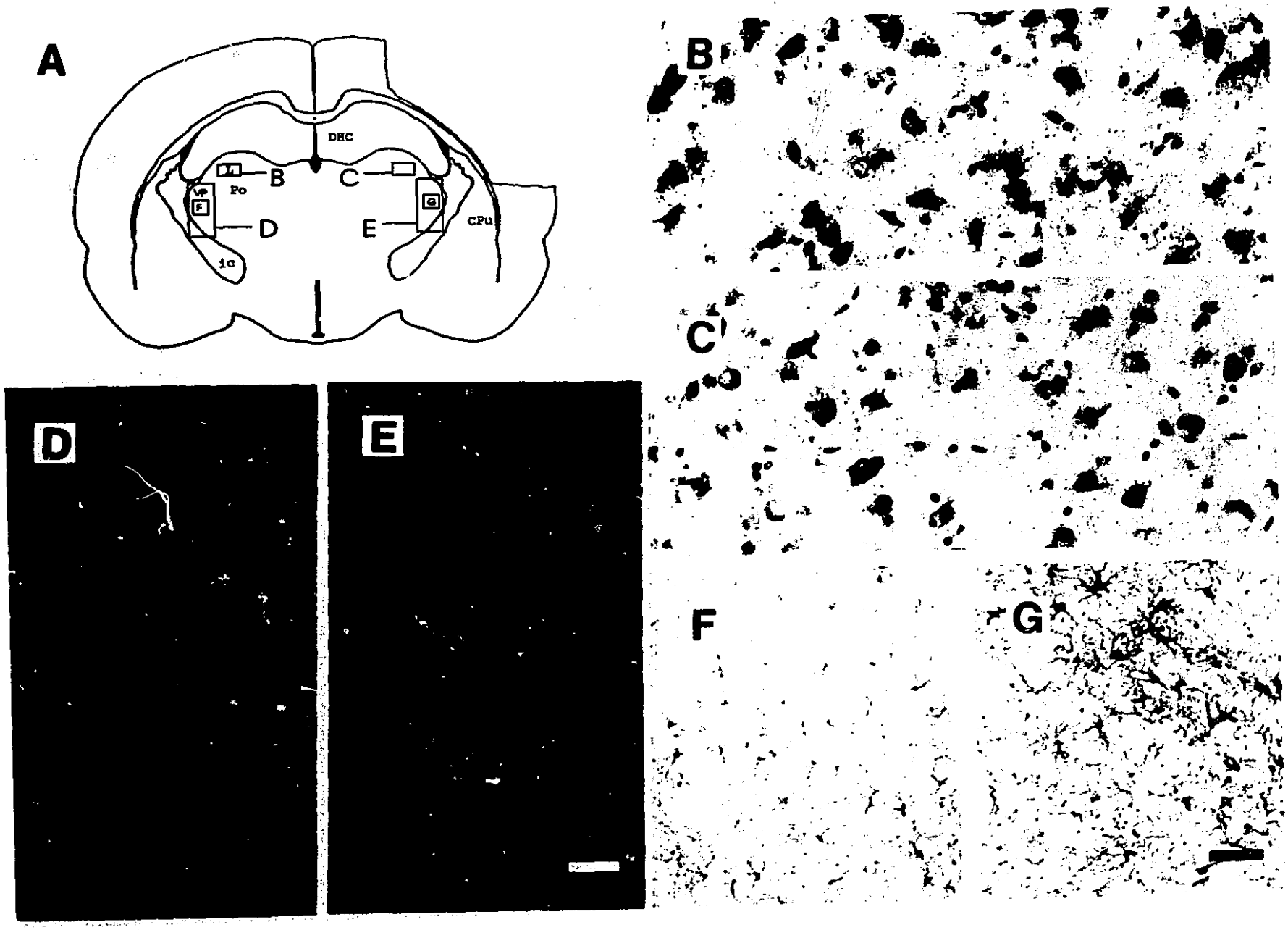
	Mean number of silver grains (pixels)/ cross-sectional cell area (μm^2)		
Post-lesion day	Control	Lesion + Vehicle	Lesion + NGF
1	3.45 ± 0.42	7.30 ± 0.30*	6.26 ± 0.31*
3	2.87 ± 0.27	10.81 ± 0.33*	10.44 ± 0.6*
7	3.49 ± 0.23	8.77 ± 0.82*	8.69 ± 0.28*
15	3.19 ± 0.40	4.53 ± 0.33*▲	4.76 ± 0.43*▲

Values represent mean ± S.E.M., n=4-5 animals/group. * $p < 0.05$ from control ; ▲ $p < 0.05$ from day 1 values within the same group; ▲ $p < 0.05$ from lesion + vehicle (ANOVA followed by post-hoc Newman-Keuls' test).

Figure V.6

Expression of GAP-43 mRNA in thalamus

Distribution of silver grains in lateral (L) and ventroposterior (VP) nuclei of the thalamus, ipsi- and contralateral to the lesion, are shown according to the diagram shown in A: Large capital letters B, C, D, E and G refer to respective photomicrographs taken from animals that survived 7 days after lesion. B, bright-field photomicrograph taken from the lateral nucleus contralateral to the lesion: Note the homogeneous population of large cells (counterstained with methylene blue), each presenting a higher number of silver grains than an the ipsilateral side (C). Observe in C that the number of large cells is diminished, and other cells, atrophic and with picnotic nuclei, are seen in higher number. This decrease in GAP-43 mRNA seen in C is better visualized in E under darkfield illumination (ventroposterior nucleus ipsilateral to the lesion) in contrast with that normal pattern of distribution seen in D. At this 7th post-lesion day, immunoreactivity in 10 μm-thick sections, revealed reactive astrocytes ipsilateral to the lesion (G), in contrast to the contralateral pattern noted in the unlesioned ventroposterior nucleus (F). Scale bar in E=120 μm and applies to D. Scale bar in G=40 μm and also applies to B, C and F.


A
DHC
B
C
Po
VP
F
D
E
G
CPu
ic
B
C
D
E
F
G

cells (Fig. V.6C) as well as astrocytic reaction detected by glial-fibrillary acidic protein immunoreactivity in the lateral and ventroposterior thalamic nuclei (Fig. V.6G). GAP-43 mRNA levels were not affected by exogenous application of NGF in any of these brain regions.

DISCUSSION

In the present study, attempts were made to correlate NGF treatment with expression of the mRNAs for the low-affinity NGF receptor and for GAP-43, a protein implicated in neuronal growth and regeneration. Continuous infusion of NGF for a period of seven days has been shown to rescue NBM cholinergic neurons from degeneration caused by cortical infarction. Treatment with this neurotrophin prevented neuronal atrophy, the difference in cell size between treated and untreated animals becoming apparent one month post-lesion (Cuello et al., 1989). Prior to this time point, atrophy of NBM cholinergic neurons in untreated animals is not easily distinguished.

$p75^{NGFR}$ mRNAs in the nucleus basalis

A principal finding of the present study is that devascularizing lesions of the cerebral cortex cause changes in the expression of two mRNAs investigated, $p75^{NGFR}$ and GAP-43 in NBM cells. Alterations of these mRNAs in the cholinergic basalo-cortical pathway may be an important aspect of cellular responsiveness to injury and may allow us to predict corresponding changes in protein production by these neurons. Changes in the expression of both low and high affinity NGF receptors presumably involves a number of factors induced by neuronal injury. It is conceivable that these changes reflect the loss of tissue in the target tissue which is generally assumed to be the principal source of NGF to NBM neurons (Korsching et al., 1985; Goedert et al., 1986; Shelton and Reichardt, 1986; Whittemore et al., 1986; Todd and Spike, 1993). Cortical devascularization elicited increases in $p75^{NGFR}$ mRNA which were statistically greater at three days post-lesion, declining seven days after the lesion. This transient increase could be secondary to other agents (e.g. expression of endogenous growth factors) which

may be similarly elevated during the early post-lesion days. This tempting conclusion is supported by reports that a transitory increase in NGF occurs in the NBM following neocortical suction ablation (Lorez et al., 1988) in the remaining cerebral cortex following cortical infarction (see chapter IV) and in the septum following fimbria-fornix transection (Gasser et al., 1986; Weskamp et al., 1986) and furthermore, that blockage of axonal transport with vincristine prevents an increase in $p75^{NGFR}$ mRNA following transection of the rat hypoglossal nerve (Hayes et al., 1992).

This insult-induced increase in NGF and its low affinity receptor in the basalo-cortical pathway may be part of a protective cascade of events serving to repair or retard damage to cholinergic neurons. As noted above, these neurons become atrophic at one month post-lesion if not untreated with neurotrophic agents (Stephens et al., 1985; Sofroniew et al., 1983;1987). In the axotomy lesion model (fimbria-fornix transection), $p75^{NGFR}$ mRNA was shown to be decreased in the medial septum but was increased in neurons that did not degenerate (horizontal limb of the diagonal band of broca) (Gibbs et al., 1992). Exogenous NGF increased $p75^{NGFR}$ mRNA levels in the NBM ipsi- and contralateral to the lesion, confirming previous studies conducted in other neuronal cell types (Cavicchioli et al., 1989; Gage et al., 1989; Higgins et al., 1989; Holtzman et al., 1992; Verge et al., 1992). Another more recent study reported that NGF selectively increases the ratio of $p75^{NGFR}$ to $p140^{trk}$ receptors on mature sympathetic neurons (Miller et al., 1994).

GAP-43 mRNA in NBM, neocortex and thalamus

Our rationale for investigating GAP-43 mRNA in the cortical devascularization lesion model was that NGF was shown to induce sprouting of cholinergic fibers and hypertrophy of their boutons in the remaining neocortex (Garofalo et al., 1992; 1993). Several lines of evidence suggest that GAP-43 plays an important role in neuronal regeneration [for review see (Skene, 1989)]. Consonant with this notion, depletion of GAP-43 using antisense oligonucleotides (Jap Tjoen San et al., 1992; Aigner and Caroni, 1993) or functional inhibition with antibodies (Shea et al., 1991) reduced neurite outgrowth. Attempts have been made to relate post-lesion changes in GAP-43 to

functional neurochemical mechanisms linked to neuroplasticity (Cogginc and Zwiers, 1991; Bisby and Tetzlaff, 1992). However, it is unclear how GAP-43 is involved in the brain regions affected by these decortication experiments and whether NGF is involved in its regulation. The transient increase of GAP-43 mRNA in large NBM cells ipsilateral to the lesion implies that ischemic-induced degeneration of the target, coupled with retrograde degeneration of the basalo-cortical pathway, provides an adequate stimulus for GAP-43 expression in projecting neurons. However, it is unclear whether this increase in GAP-43 mRNA, which is probably occurring in cholinergic neurons, plays a role in the transient hypertrophy of these neurons after cortical infarction (Sofroniew et al., 1987). In contrast to the rapid cell death seen in the thalamus (Matthews, 1973), NBM cholinergic neurons do not die as a result of this lesion but become atrophic in the first month after lesioning (Sofroniew et al., 1983; 1987). NGF infused for seven days after cortical devascularization (Cuello et al., 1989) or after lesions with neurotoxins (Haroutunian et al., 1989), is able to prevent degeneration in the NBM and induce sprouting of cholinergic terminals (Garofalo et al., 1992; 1993). Based on the present results, GAP-43 mRNA expression is not regulated by NGF as was reported for the NGF-induced expression of GAP-43 in PC12 cells (Mooradian, 1990). Other levels of regulation by NGF may exist, however, including induction of phosphorylation of the GAP-43 protein (Meiri and Burdick, 1991). Regulation of GAP-43 by a target-derived factor is supported by the observation in sciatic nerve that interruption of axonal transport with vinblastine or colchicine increased the expression of GAP-43 in the dorsal root ganglion (DRG) (Basi and Skene, 1988; Woolf et al., 1990; Smith and Skene, 1993). These data imply that derepression of GAP-43 is involved rather than induction of a positive signal coming from the injury site or the proximal stump. This concept of repression of GAP-43 expression is further supported by the observation that colchicine, when applied proximal to a nerve cut, fails to prevent a GAP-43 increase (Smith and Skene, 1993). NGF is unlikely to be the candidate in the DRG as it did not reverse the increase in GAP-43 after axotomy despite the fact that there is a good correlation between which DRG neurons express high affinity NGF receptors and their baseline level of GAP-43 (Verge et al., 1990).

A difference in the regulation of GAP-43 mRNA levels in PNS versus CNS in response to injury has been noted: the lesion-to-cell body distance seems not to influence GAP-43 expression in the PNS (Tsui et al., 1991; Schreyer and Skene, 1993), but it may be critically important for retinal ganglion cells (Doster et al., 1991) for the rubrospinal system (Tetzlaff et al., 1991) and for corticospinal neurons (Tetzlaff and Giehl, 1991). Thalamic and NBM neurons are located approximately 3 to 5 mm from the large decorticated region in our model, a distance which is similar to the maximal lesion distance capable of enhancing GAP-43 in retinal ganglion cells (Doster et al., 1991) but shorter than the distance which increases GAP-43 in rubrospinal neurons (Tetzlaff et al., 1991). The slight increase of GAP-43 mRNA in some neurons on the third post-lesion day in the thalamus, although not statistically significant, occurred in conjunction with rapid degeneration in these nuclei suggesting that other post-lesion time-points must be critically important and should be investigated. It is clear from our experiments, and others, that the degenerative process in the thalamus (Matthews, 1973) is more accentuated and faster than in the NBM (Garofalo et al., 1992; 1993). Thalamic neurons exhibited a statistically significant (56%) decrease in GAP-43 mRNA at seven days post-lesion (data not shown). This decrease was noted in thalamic cells of normal size, in partially atrophic neurons, and in cells with a pycnotic appearance. This condition may explain why GAP-43 is increased in the NBM and decreased in the thalamus on the seventh post-lesion day. The occurrence of degenerative changes in the thalamus coincides with astrocytic hyperactivity (detected by GFAP immunoreactivity) as has been shown in other studies (Ross and Ebner, 1990; Herrera and Cuello, 1992), and which was four-six fold higher than in the NBM. It is unclear whether or not this difference is linked to any transient cellular processes involved in regeneration. Cortical infarction may also damage pyramidal neurons in the remaining cortical tissue adjacent to the lesion where we observed an increase in GAP-43 mRNA at all post-lesion days. It is interesting that pyramidal cells exhibited the highest levels of GAP-43 mRNA and that this trend persisted for a minimum of 15 days. However, mRNA analysis in combination with tracing studies would be necessary to characterize the lesion occurring in these neurons. To this end, Giehl and colleagues (1994, in preparation) have performed

experiments to elucidate this problem using an axotomy lesion model. Cortical ischemia may induce an inflammatory reaction and local accumulation of cytokines and other factors that may be important for regulation of GAP-43 expression; this cascade was shown to be associated with GAP-43 induction and axonal regeneration in DRG cells (Lu and Richardson, 1991; 1993). However, the fact that only pyramidal neurons in lamina V are seemingly affected by the devascularizing lesion would suggest that the upregulation of GAP-43 mRNA may be mainly due to interruption of the cortico-cortical fibres.

CONCLUSION

Differential regulation of $p75^{NGFR}$ and GAP-43 mRNAs after partial and unilateral cortical infarction was noted in three neuronal populations: NBM, pyramidal and thalamic neurons. The mRNA expression in each of these groups of neurons was differentially affected. These changes are attributed to the extent at which the corresponding neurons are compromised by the cortical lesion. In this experimental model, the presence of high levels of NGF within the CNS is capable of inducing the rapid upregulation of the mRNA for the $p75^{NGFR}$ in the ipsilateral NBM. Some grain countings in NBM contralateral to the lesion revealed similar NGF effects in $p75^{NGFR}$ mRNA. This indicated that NGF effects is bilateral, i.e., independent of the lesion. In the NBM of lesioned animals, GAP-43 mRNA was increased between three and seven days post-lesion while, in pyramidal cells, its induction was noticed between 1 and 15 days post-lesion and it was of higher magnitude indicating that, in these neurons, GAP-43 mRNA expression is differentially regulated by the cortical lesion. In thalamus, there was initially a slight increase followed rapidly with a decrease in GAP-43 mRNA accompanying degeneration and death of these neurons evident from the seventh post-lesion day.

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REFERENCES

Aigner L, Caroni P (1993) Depletion of 43-kD growth-associated protein in primary neurons leads to diminished formation and spreading of growth cones. J Cell Biol 123:417-429.

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K (1987) Current protocols in molecular biology. New York: John Wiley and Sons.

Basi GS, Jacobson RD, Virág I, Schilling J, Skene JHP (1987) Primary structure and trnscriptional regulation of GAP-43, a protein associated with nerve growth. Cell 49:785-791.

Basi GS, Skene JHP (1988) Regulation of GAP-43 gene expression during axonal regeneration in sensory neurons. Soc Neurosci Abstr 14:408.

Benowitz LI, Perrone-Bizzozero NI (1991) The relationship of GAP-43 to the development and plasticity of synaptic connections. Ann NY Acad Sci 627:58-74.

Benowitz LI, Routtenberg A (1987) A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism and synaptic plasticity. Trends Neurosci 10:527-532.

Bigl V, Woolf NJ, Butcher LL (1982) Cholinergic projections fron the basal forebrain to frontal, parietal, temporal, occipital and cingulate cortices: a combined fluorescent tracer and acetylcholinesterase analysis. Brain Res Bull 8:727-749.

Bisby MA, Tetzlaff W (1992) Changes in cytoskeletal protein synthesis following axon injury and during axon regeneration. Mol Neurobiol 6:107-123.

Cavicchioli L, Flanigan TP, Vantini G, Fusco M, Polato P, Toffano G, Walsh FS, Leon A (1989) NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. Eur J Neurosci 1:258-262.
Coggins PJ, Zwiers H (1991) B-50 (GAP-43): Biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. J Neurochem 56:1095-1106.

Cordon-Cardo C, Tapley P, Jing S, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, Barbacid M (1991) The *trk* tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-183.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

De la Monte SM, Federoff HJ, Ng S-C, Crabczyk E, Fishman MC (1989) GAP-43 gene expression during development: persistence in a distinctive set of neurones in the mature central nervous system. Dev Brain Res 46:161-168.

Doster SK, Lozano AM, Aguayo AJ, Willard MB (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6:635-647.

Dunn-Meynell AA, Benowitz LI, Levin BE (1992) Vibrissectomy induced changes in GAP-43 immunoreactivity in the adult rat barrel cortex. J Comp Neurol 315:160-170.

Ebendal T (1992) Function and evolution in the NGF family and its receptors. J Neurosci Res 32:461-470.

Eckenstein FP, Baughman RW, Quinn J (1988) An anatomical study of cholinergic innervation in rat cerebral cortex. Neuroscience 25(2):457-474.

Ernfors P, Henschen A, Olson L, Persson H (1989) Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2:1605-1613.

Fibiger HC (1982) The organization and some projections of cholinergic neurons of the mammalian forebrain. Brain Res Rev 4:327-388.

Figueiredo BC, Almazan G, Ma Y, Tetzlaff W, Miller FD, Cuello AC (1993a) Gene expression in the developing cerebellum during perinatal hypo- and hyperthyroidism. Mol Brain Res 17:258-268.

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993b) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Gage FH, Batchelor P, Chen KS, Chin D, Higgins GA, Koh S, Deputy S, Rosenberg MB, Fischer W, Björklund A (1989) NGF receptor reexpression and NGF-mediated cholinergic neuronal hypertrophy in the damaged adult neostriatum. Neuron 2:1177-1184.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1992) Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. Proc Natl Acad Sci USA 89:2639-2643.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1993) Potentiation of nerve growth factor-induced alterations in cholinergic fibre length and presynaptic terminal size in cortex of lesioned rats by the monosialoganglioside GM1. Neuroscience 57:21-40.

Gasser UE, Weskamp G, Otten U, Dravid AR (1986) Time course of the elevation of nerve growth factor (NGF) content in the hippocampus and septum following lesions of the septohippocampal pathways in rats. Brain Res 376:351-356.

Gibbs RB, Chao MV, Pfaff DW (1992) Effects of fimbria fornix and angular bundle transection on expression of the p75NGFR mRNA by cells in the medial septum and diagonal band of broca: correlations with cell survival, synaptic reorganization and sprouting. Mol Brain Res 11:207-219.

Gibbs RB, Pfaff DW (1994) In situ hybridization detection of trkA mRNA in brain: Distribution, colocalization with $p75^{NGFR}$ and up-regulation by nerve growth factor. J Comp Neurol 341:324-339.

Gispen WH, Nielander HB, De Graan PNE, Oestreicher AB, Schrama LH, Schotman P (1991) Role of the growth-associated protein B-50/GAP-43 in neuronal plasticity. Mol Neurobiol 5:61-85.

Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, Goldfarb M, Yancopoulos GD (1991) TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 66:405-413.

Gnahn H, Hefti F, Heuman R, Schwab ME, Thoenen H (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain?. Dev Brain Res 9:45-52.

Goedert M, Fine A, Hunt SP, Ullrich A (1986) Nerve growth factor mRNA in peripheral and central rat tissues and in the human nervous system: lesion effects in the rat brain and levels in Alzheimer's Disease. Mol Brain Res 1:85-92.

Goslin K, Schreyer DJ, Skene JHP, Banker G (1988) Changes in the distribution of GAP-43 during the development of neuronal polarity. J Neurosci 10:588-602.

Hagg T, Vahlsing HL, Manthorpe M, Varon S (1990) Nerve growth factor infusion into the denervated adult rat hippocampal formation promotes its cholinergic reinnervation. J Neurosci 10:3087-3092.

Haroutunian V, Kanof PD, Davis KL (1989) Attenuation of nucleus basalis of Meynert lesion induced cholinergic deficits by nerve growth factor. Brain Res 487:200-203.

Hayes RC, Wiley RG, Amstrong DM (1992) Induction of nerve growth factor receptor (p75NGFr) mRNA within hypoglossal motoneurons following axonal injury. Mol Brain Res 15:291-297.

Hefti F (1986) Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 6:2155-2162.

Hempstead BL, Martin-Zanca D, Kapian DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low affinity NGF receptor. Nature 350:678-683.

Herrera DG, Cuello AC (1992) Glial fibrillary acidic protein immunoreactivity following cortical devascularizing lesion. Neuroscience 49:781-791.

Higgins GA, Koh S, Chen KS, Gage FH (1989) NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3:247-256.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen C-K, Valletta JS, Zhou J, Long JB, Mobley WC (1992) p140^{trk} mRNA marks NGF-responsive forebrain neurons: Evidence that *trk* gene expression is induced by NGF. Neuron 9:465-478.

Honegger P, Lenoir D (1982) Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. Dev Brain Res 3:229-238.

Jacobson RD, Virag I, Skene JHP (1986) A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. J Neurosci 6:1843-1855.

Jap Tjoen San ERA, Schmidt-Michels M, Oestreicher A, Gispen WH, Schotman P (1992) Inhibition of nerve growth factor-induced B-50/GAP-43 expression by antisense oligomers interferes with neurite outgrowth of PC12 cells. Biochem Biophys Res Commun 187:839-846.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M (1986) Expression and structure of the human NGF receptor. Cell 47:545-554.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a) The *trk* proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 252:554-558.

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine activity of the *trk* proto-oncogene product induced by NGF. Nature 350:158-160.

Kenigsberg RL, Semenenko FM, Cuello AC (1990) Development of a bi-specific monoclonal antibody for simultaneous detection of rabbit IgG and horseradish peroxidase: use as a general reagent in immunocytochemistry and enzyme-linked immunosorbent assay. J Histochem Cytochem 38:191-198.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991a) The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klein R, Nanduri V, Jing S, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991b) The *trk*B tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395-403.

Korsching S, Auburger G, Heumann R, Scott J, Thoenen H (1985) 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.

Kromer LF (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235:214-216.

Lamballe F, Klein R, Barbacid M (1991) *Trk*C, a new member of the *trk* family of protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Large TH, Bodary SC, Clegg DO, Weskamp G, Otten U, Reichard LF (1986) Nerve growth factor gene expression in the developing rat brain. Science 234:352-355.

Lee K-F, Huber J, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Target mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral nervous system. Cell 69:737-749.

Levin BE, Dunn-Meynell A (1993) Regulation of growth-associated protein 43 (GAP-43) messenger RNA associated with plastic change in the adult rat barrel receptor complex. Mol Brain Res 18:59-70.

Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H, Lindholm D (1990) Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. Eur J Neurosci 2:389-396.

Lorez H, vonFrankenber M, Weskamp G, Otten U (1988) Effect of bilateral decortication on nerve growth factor content in nucleus basalis and neostriatum of adult rat brain. Brain Res 454:355-360.

Lu X, Richardson PM (1991) Inflamation near the nerve cell body enhances axonal regeneration. J Neurosci 11:972-978.

Lu X, Richardson PM (1993) Induction by macrophages of regeneration associated molecules in sensory neurons. Soc Neurosci Abstr 19:451.

Luiten PGM, Gaykema RPA, Traber J, Spencer DG (1987) Cortical projection patterns of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin. Brain Res 413:229-250.

Martin-Zanca D, Hughes SH, Barbacid M (1986) A human oncogene formed by the fusion of truncated tropomyosin and protein kinase sequences. Nature 319:743-748.

Matthews S (1973) Death of the central neuron: An electron microscopic study of thalamic retrograde degneration following cortical ablation. J Neurocytol 2:265-288.

McGuire CB, Snipes GJ, Norden JJ (1988) Light-microscopic immunolocalization of the growth and plasticity-associated GAP-43 in the developing rat brain. Dev Brain Res 41:277-291.

Meiri KF, Pfenninger KH, Willard MB (1986) Growth-associated protein, GAP-43, a polypeptide that is induced when neurons exrends axons, is a component of growth cones and corresponds to pp46, a major polypeptide of subcellular fraction enriched in growth cones. Proc Natl Acad Sci USA 83:3537-3541.

Meiri KF, Burdick D (1991) Nerve growth factor stimulation of GAP-43 phosphorylation of intact isolated growth cones. J Neurosci 11:3155-3164.

Miller FD, Mathew TC, Toma JG (1991) Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. J Cell Biol 112:303-312.

Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994) Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. Dev Biol 161:206-217.

Mobley WC, Rutkowski JL, Tennekoon GI, Buchanana K, Johnson MW (1985) Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. Science 229:284-287.

Mooradian AD (1990) Metabolic fuel and amino acid transport into the brain in experimental hypothyroidism. Acta Endocrinol (Copenh) 122(2):156-162.

Nelson R, Routtenberg A (1985) Characterization of protein F1 (47 kDa, 4.5 pI): A kinase C substrate directly related to neural plasticity. Exp Neurol 89:213-244.

Neve RL, Ivins KJ, Benowitz LI, During MJ, Geller AI (1991) Molecular analysis of the function of the neuronal growth-associated protein GAP-43 by genetic intervention. Mol Neurobiol 5:131-141.

Nielander HB, Schrama LH, Van Rozen AJ, Kasperaitis M, Oestreicher AB, De Graan PNE, Gispen WH, Schotman P (1987) Primary structure of the neuron-specific phosphoprotein B-50 is identical to growth-associated protein GAP-43. Neurosci Res Commun 1:163-172.

Nieto-Sampedro M, Manthorpe M, Barbin G, Varon S, Cotman C (1983) Injury-induced neuronotrophic activity in adult rat brain: correlation with survival of delayed implants in the wound cavity. J Neurosci 3:2219-2229.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.
Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Richardson PM, Issa VM, Aguayo AJ (1984) Regeneration of long spinal axons in the rat. J Neurocytol 13:165-182.

Richardson PM, Verge Issa VMK, Riopelle RJ (1986) Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 6:2312-2321.

Ross DT, Ebner FF (1990) Thalamic retrograde degeneration following cortical injury: an excitotoxic process. Neuroscience 35:525-550.

Saper CB (1984) Organization of cerebral cortical afferent systems in the rat. II. magnocellular basal nucleus. J Comp Neurol 222:313-342.

Schalling M, Dagerlind Å, Brené S, Hallman H, Djurfeldt M, Persson H, Terenius L, Goldstein M, Schlesinger D, Hökfelt T (1988) Coexistence and gene expression of phenylethanolamine N-methyltransferase, tyrosine hydroxylase and neuropeptide tyrosine in the rat and bovine adrenal gland: Effects of reserpine. Proc Natl Acad Sci USA 85:8306-8310.

Schlessinger J, Ullrich A (1992) Growth factor signaling by receptor tyrosine kynases. J Neurosci 9:383-391.

Schreyer DJ, Skene JHP (1993) Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. J Neurobiol 7:959-970.

Shea TB, Perrone-Bizzore ML, Benowitz LI (1991) Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neritogenesis. J Neurosci 11:1685-1690.

Shelton DL, Reichardt LF (1986) Studies on the expression of the B-NGF gene in the CNS: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci USA 83:2714-2718.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Skene JHP, Willard M (1981) Axonally transported proteins associated with growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96-103.

Smith DS, Skene JHP (1993) Axotomy-induced transcription is a prerequisite for regenerative axon elongation but not for sprouting. Soc Neurosci Abstr 19:679.

Sofroniew MV, Pearson RC, Eckenstein F, Cuello AC, Powell TP (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res 289:370-374.

Sofroniew MV, Pearson RCA, Powell TPS (1987) The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. Brain Res 411:310-331.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, Nikolics K, Parada LF (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the *trk*B tyrosine kinase receptor. Cell 65:895-903.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM, DiStefano PS, Yancopoulos GD (1991) *trk*B encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Stephens PH, Cuello AC, Sofroniew MV, Pearson RC, Tagari P (1985) Effect of unilateral decortication on choline acetyltransferase activity in the nucleus basalis and other areas of the rat brain. J Neurochem 45:1021-1026.

Tetzlaff W, Zwiers H, Lederis K, Cassar L, Bisby MA (1989) Axonal transport and localisation of B50/GAP-43-like immunoreactivity in regenerating sciatic and facial nerves of the rat. J Neurosci 9:1303-1313.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11:2528-2544.

Tetzlaff W, Giehl KMG (1991) Axotomized corticospinal neurons increase GAP-43 mRNA after sub-cortical lesions but not after pyramidal lesions. Abstr IBRO Third World Congr Neurosci 3:29(Abstract).

Theriault E, Tetzlaff W, Tator CH (1992) Elevated gene expression in the red nucleus after spinal cord compression injury. NeuroReport 3:559-562.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165-170.

Todd AJ, Spike RC (1993) The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog Neurobiol 41:609-638.

Tsui BJ, Cassar SL, Tetzlaff W (1991) Changes in mRNA levels for GAP-43, tubulin and neurofilament-M in rat spinal motoneurons after proximal versus distal axotomy. Soc Neurosci Abstr 17(1):47(Abstract).

Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

Van der Zee CEEM, Nielander HB, Vos JP, Lopes da Silva S, Verhaagen J, Oestreicher AB, Schrama LH, Schotman P, Gispen WH (1989) Expression of growth-associated protein B50(GAP-43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. J Neurosci 9:3506-3512.

Venero JL, Knüsel B, Beck KD, Hefti F (1994) Epression 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.

Verge VMK, Tetzlaff W, Bisby MA, Richardson PM (1990) Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons. J Neurosci 10:2018-2025.

Verge VMK, Merlio J-P, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hökfelt T, Richardson PM (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: Responses to injury and infusion of NGF. J Neurosci 12:4011-4022.
Weskamp G, Gasser UE, Dravid AR, Otten U (1986) Fimbria fornix-lesion increases nerve growth factor content in adult rat septum and hippocampus. Neurosci Lett 70:121-126.

Whittemore SR, Ebendal T, Lärkfors L, Olson L, Seiger Å, Strömberg I, Persson H (1986) Developmental and regional expression of β nerve growth factor messenger RNA and protein in the rat central nervous system. Proc Natl Acad Sci USA 83:817-821.

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A, Gage FH (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc Natl Acad Sci USA 83:9231-9235.

Woolf CJ, Reynolds ML, Molander C, OBrien C, Lindsay RM, Benowitz LI (1990) The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. Neuroscience 34:465-478.

Woolf NJ (1991) Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 37:475-524.

Zilles K (1985) A stereotaxic atlas. Berlin: Springer-Verlag.

As outlined in the previous chapter, expression of $p75^{NGFR}$ mRNA in NBM neurons of animals with cortical infarction may reflect increases or fluctuations in neocortical NGF levels. Reduction of $p75^{NGFR}$ mRNA to levels below controls, occurring two weeks after the lesion, was prevented by NGF treatment for seven days. Such treatment prevented atrophy of cholinergic neurons examined one month after the lesion (Cuello et al., 1989; Garofalo and Cuello, 1990; 1993; Garofalo et al., 1993). Therefore, atrophy of NBM cholinergic neurons, associated with decreased gene expression of $p75^{NGFR}$ in these neurons are consequences of reduced levels of target-derived NGF. This is in line with the notion that target-derived NGF regulate the structural and chemical phenotype of basal forebrain cholinergic neurons (Sofroniew et al., 1993). Some of the features of human cretinism are reproducible in rodents. One such feature is the atrophy of NBM cholinergic neurons described in young and 50 day old rats (Gould and Butcher, 1989). Moreover, deficiency of NGF was suggested to occur in hypothyroidism (Walker et al., 1979). Therefore, we decided to investigate whether post-hypothyroid adult animals (with physiological levels of thyroid hormones after brain development with thyroid hormone deprivation) would display levels of low- and high-affinity NGF receptor mRNAs in the NBM which correlated with the alteration in the size of NBM cholinergic neurons. Secondly, it was of interest to investigate whether these NBM neurons of post-hypothyroid animals would maintain their size following cortical infarction and NGF treatment.

REFERENCES

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

Garofalo L, Ribeiro-da-Silva A, Cuello AC (1993) Potentiation of nerve growth factor-induced alterations in cholinergic fibre length and presynaptic terminal size in cortex of lesioned rats by the monosialoganglioside GM1. Neuroscience 57:21-40.

Garofalo L, Cuello AC (1990) Nerve growth factor and the monosialoganglioside GM1 modulate cholinergic markers and affect behavior of decorticated adult rats. Eur J Pharmacol 183:934-935.

Garofalo L, Cuello AC (1993) Nerve growth factor and the monosialoganglioside GM1: Analogous and different in vivo effects on biochemical, morphological and behavioral parameters of adult cortically leisoned rats. Exp Neurol (In Press)

Gould E, Butcher LL (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347-3358.

Sofroniew MV, Cooper JD, Svendsen CN, Crossman P, Ip NY, Lindsay RM, Zafra F, Lindholm D (1993) Atrophy but not death of adult septal cholinergic neurons after ablation of target capacity to produce mRNAs for NGF, BDNF, and NT3. J Neurosci 13:5263-5276.

Walker P, Weichsel ME, Fisher DA, Guo SM (1979) Thyroxine increases nerve growth factor concentration in adult mouse brain. Science 204:427-429.

CHAPTER VI

EXPRESSION OF $p75^{NGFR}$ AND $gp140^{trk}$ mRNAs AND ChAT IMMUNOREACTIVITY IN NUCLEUS BASALIS NEURONS OF POSTHYPOTHYROID RATS: NGF PREVENTS FURTHER ATROPHY BUT DOES NOT RESTORE CELL SIZE FOLLOWING CORTICAL INFARCTION

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ABSTRACT

We have tested the hypotheses that trophic factor therapy can reestablish the cellular characteristics of nucleus basalis magnocellularis (NBM) neurons in posthypothyroid rats and whether NGF treatment can prevent further atrophy of these neurons following cortical infarction. Animals which developed while deprived of thyroid hormone were used to assess effects on cell surface area, expression of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs and therapeutic responses to NGF by NBM cholinergic neurons in adulthood. Rats were rendered hypothyroid by the addition of propylthiouracil (PTU) to their diet beginning on embryonic day 19 (E19) until the age of one month. At this time, both the pups and their dams continued to receive 0.05% PTU in their diet and were thyroidectomized. At 60 days, PTU treatment was interrupted and thyroxine levels were restored to normal by daily subcutaneous administration of physiological levels of thyroxine. Morphometric analysis identified atrophied NBM cholinergic cells at two ages, days 75 and 105, identified by *in situ* hybridization for $p75^{NGFR}$ and $gp140^{trk}$ mRNAs in Methylene blue stained cells (day 75) and choline acetyltransferase (ChAT) immunostaining (day 105). The mean number of silver grains (pixels) per μm^2 (mean $\pm$ S.E.M.) of cell body cross-sectional area for $p75^{NGFR}$ mRNA in the NBM of euthyroid rats was 3.43 ± 0.89, which was not statistically different from posthypothyroid animals (4.02 ± 1.07). A similar finding was noted for $p140^{trk}$ mRNA: Mean number of grains in the euthyroid group was 5.54 ± 0.96 and was not statistically different from the posthypothyroid group (6.32 ± 1.45). NGF treatment in adulthood (between days 75 and 82) did not restore cell surface area from early thyroid deprivation. However, NGF prevented further atrophy of NBM neurons following cortical devascularization inflicted in adulthood. These data show that in the posthypothyroid rat, as in the euthyroid rat, NGF can replace the loss of presumed target derived trophic support and that posthypothyroid NBM neurons which express similar amounts of mRNA for NGF receptors are responsive to NGF. Moreover, these findings indicate that the cellular atrophy of NBM neurons during and after thyroid hormone depletion is probably not due to a loss of trophic support or a failure to express NGF receptors.

INTRODUCTION

Nerve growth factor (NGF) is the best characterized member of the neurotrophin family. Low- and high-affinity receptors for NGF have been described. However, some controversy still exists as to whether the low-affinity neurotrophin binding protein p75 plays a crucial role in the formation of the high-affinity NGF receptor complex (Klein et al., 1991; Ibáñez et al., 1992), or whether the high-affinity receptor is formed by dimers of the tyrosine kinase receptor ($gp140^{trk}$) (Hempstead et al., 1991; Kaplan et al., 1991).

In brain NGF, mRNA is expressed mainly in regions innervated by cholinergic neurons of the basal forebrain (Korsching et al., 1985; Shelton and Reichardt, 1986; Whittemore et al., 1986; Goedert et al., 1986). This notable coincidence, along with the demonstration of NGF retrograde transport by these neurons (Seiler and Schwab, 1984), provide a rationale for the use of NGF to restore the morphological, biochemical, and functional status of cholinergic nerve cells. Indeed, NGF has proven to be the most specific neurotrophic factor tested so far for basal forebrain cholinergic neurons (Honegger and Lenoir, 1982; Gnahn et al., 1983; Mobley et al., 1985; Thoenen, 1991; Ebendal, 1992; Hefti, 1986; Hagg et al., 1990; Kromer, 1987; Williams et al., 1986; Cuello et al., 1989; Haroutunian et al., 1989; Cuello, 1993). This notion is further supported by the expression of $p75^{NGFR}$ and $p14^{trk}$ mRNA on these neurons (Holtzman et al., 1992). It has suggested that NGF may be required for normal development of basal cholinergic neurons (Thoenen et al., 1987; Whittemore and Seiger, 1987). Conversely, during early postnatal development, the activity-dependent regulation of NGF and brain-derived neurotrophic factor (BDNF) expression is also influenced by the cholinergic system (Da Penha Berzaghi et al., 1993).

The full extent and range of pathological circumstances in which NGF can elicit protection of central cholinergic neurons is not entirely known. In these studies, we investigated whether NGF can reverse the phenotypic changes occurring in the NBM after developmental thyroid hormone deprivation (Gould and Butcher, 1989). A large number of phenotypic alterations in the CNS caused by a deficiency in thyroid hormone

during development have been described (Legrand, 1983; Dussault and Ruel, 1987; Porterfield and Hendrich, 1993). These alterations include varicosities (spheroidal swellings) along the proximal axon in cholinergic nucleus basalis neurons (NBM)(Gould and Butcher, 1989) similar to those described in Purkinje neurons (Figueiredo et al., 1993a). The developmental significance of thyroid hormones for neurite outgrowth (Nicholson and Altman, 1972; Legrand, 1979; Rami et al., 1986: Gould and Butcher, 1989) may be attributable, in great part, to reduced amounts of cytoskeletal proteins in hypothyroid rats (Chaudhury et al., 1985; Faivre et al., 1983; Lakshmanan et al., 1981; Nunez, 1985). Among the consequences described were stunted dendritic trees of cholinergic NBM neurons that persisted into adulthood (Gould and Butcher, 1989). These alterations may result in permanent impairment of neuronal connectivity, thus, leading to neuronal dysfunction. Several other studies indicate that ChAT activity in the CNS is sensitive to alterations of thyroid hormone levels (Honegger and Lenoir, 1980; Atterwill et al., 1984; Hefti et al., 1986; Hayashi and Patel, 1987; Patel et al., 1988; Valcana, 1971; Kalaria and Prince, 1985; Gould and Butcher, 1989; Oh et al., 1991). A persistent decrease in ChAT activity was reported to be selective to subcortical cholinergic neurons (Patel et al., 1987; Kalaria and Prince, 1985) but in another report, despite persistent blockage of thyroid hormone synthesis (Gould and Butcher, 1989), ChAT immunoreactivity was shown to be recovered in the NBM of hypothyroid animals. It is not clear which process, if any, can be regulated by an interaction between thyroid hormone and NGF in basal forebrain cholinergic neurons (Hayashi and Patel, 1987; Patel et al., 1988) or in other brain areas (Legrand and Clos, 1991; Clos and Legrand, 1990). Previous studies from our laboratory (Figueiredo et al., 1993a; 1993b, see appendixes A & B) have shown that in hypothyroid pups, in addition to a delayed expression of $p75^{NGFR}$, this protein accumulates in neurons such as Purkinje cells, probably due to reduced axonal transport. We undertook the present study to assess the effects of prolonged thyroid hormone deprivation on NBM cholinergic cell neuronal size and on the expression of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs. Secondly, we determined, in these animals, the extent to which NBM neurons are affected by cortical lesions and

intracerebroventricular NGF infusions. A cortical infarction model of retrograde degeneration of NBM neurons (Sofroniew et al., 1983) was used for this purpose.

MATERIALS AND METHODS

Experimental animals

Wistar pups were rendered hypothyroid by giving dams a 0.4% propylthiouracil (PTU) enriched diet beginning on E19 (Kalaria and Prince, 1985). On the day of birth, six male pups were assigned to each mother. Offsprings of dams that had not regularly ingested their PTU-treated food from the second day of treatment were removed from this study. The amount of ingested food was controlled daily, and did not differ from that of controls, from birth until pups were 30 days old. When the pups reached 30 days of age, they and their dams were fed a 0.05% PTU enriched diet and the pups were submitted to thyroidectomy. These animals continued to receive PTU to eliminate possible hormone secretion from any remaining thyroid tissue and were left with the dam until 60 days of age. As a consequence of the hypothyroidism and the surgery, the mean body weight of these rats was 84 ± 11 g (mean ± S.D., n=26) when they were 60 days old. This represented 29.4% of the mean body weight of sham-operated control (285 ± 19 g; n=28). After reaching 60 days of age, these animals were allowed to recover by withdrawing PTU from their diet and restoring physiological levels of thyroid hormones by daily subcutaneous injections of L-thyroxine 5 ng/g of body weight until they were 75- or 105-days old. The injected dose corresponded approximately to the amount of hormone normally secreted by the gland (Vigouroux, 1976). This group of previously hypothyroid animals that received thyroxine replacement therapy from the second post-birth month are referred to as "posthypothyroid" rats. To reduce the number of animals removed due to low food consumption, PTU was first well-mixed with 6% sucrose before it was added to the appropriate food. After 15 days without PTU ingestion, at the age of 75 days, the mean body weight of these previously PTU-treated rats was 124 ± 13 g (n=21) in contrast with 367 ± 16 g of controls.

***In situ* hybridization**

In situ hybridization was performed as previously described (Schalling et al., 1988) with some modifications (Verge et al., 1992). Briefly, brains were removed from unperfused 75 day old male rats (without cortical lesion), posthypothyroids and controls, decapitated under deep anaesthesia with Equithesin, and frozen in dry ice. The *in situ* hybridization was performed with tissue sections of experimental animals adjacent to those of control animals, taken at the same NBM level. Coronal sections (10 μm thick) were cut using a cryostat, were thaw-mounted onto Superfrost Plus slides (Fisher), and kept at -70°C. Tissue sections were processed for the *in situ* hybridization of $gp140^{trk}$ and $p75^{NGFR}$ mRNAs using ^{35}S-labelled oligonucleotide probes. For $p75^{NGFR}$ mRNA, a 50-mer antisense DNA oligonucleotide,
5'-ACAAGGCCCACGACCACAGCAGCCAAGATGGAGAATAGACAGGAATGAG -3', was prepared complementary to the cDNA encoding the highly conserved transmembrane segment of $p75^{NGFR}$ (Ernfors et al., 1989). The DNA oligonucleotide antisense $gp140^{trk}$ probe used in this study was
5'-AAGGTTGAACTCAAAAGGGTTGTCCATGAAGGCAGCCATGATGGAGGC-3', previously used by Verge et al. (1992), which is the counterpart of base pairs 1198-1245 in the rat $p140^{trk}$ sequence (Meakin et al., 1992). Sections were taken from the freezer and dried for 10 min at room temperature, followed by fixation in cold (10-12°C) 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. After washing (4x5min) in phosphate-buffered saline (PBS), sections were dehydrated through 60%, 80%, 95% and 99% ethanol, deproteinized with chloroform for 2 min and subsequently rinsed with 99% and 95% ethanol, 1 min each. The sections were then dried for 20 min at room temperature and incubated for 18 h at 42°C in a hybridization buffer (100 μl/slide) containing 50% deionized formamide, 4x SSC, 10% dextran sulfate, 0.02 M phosphate buffer, 50 mg/100 ml salmon sperm DNA, 200 mM dithiothreitol, and 10^6cpm of [α-35S]dATP-labelled probe (terminal transferase labelling according to Ausubel et al., 1987). After hybridization, the sections were washed with 4 x SSC for 20 min at room temperature and were followed with a washout in 2 x SSC at 50°C (1 x 20 min),

1 x SSC at 50°C (4 x 20 min), 1 x SSC at 55°C (1 x 20 min), 0.5 x SSC at 45°C (1 x 20 min), and 0.25 x SSC at 45°C 91 x 20 min). The sections were then rinsed in double distilled water for 1 min at 30°C and at room temperature (4 x 5 min), and dried at room temperature. The sections were dipped in NTB2 (Kodak) emulsion (diluted 1:1 with distilled water) and exposed in the dark for five - eight days for $p75^{NGFR}$ and 12-14 days for $gp140^{trk}$ probe. Following development, the sections were counterstained with Methylene blue and fuchsin dehydrated and cover-slipped.

Cortical devascularization

Three groups of 75 day old posthypothyroid animals and three groups of naive control animals were operated as follows: 1) sham-operated animals treated with vehicle only, 2) devascularized rats treated with vehicle, and 3) devascularized rats treated with NGF for seven days. Male rats were anaesthetized with Equithesin (2.5 ml/kg, i.p.) and placed into a stereotaxic frame. A stainless steel cannulae (23 gauge) was directed into the right lateral ventricle [coordinates from Bregma (Paxinos and Watson, 1986): AP, -0.8; L, 1.4; V, 3.5] through a small burr hole in the skull and was affixed to the bone skull with dental cement. Osmotic minipumps (Alzet 2001, 1μl/hr, seven days) were loaded with dye (0.01% Methylene blue, BDH chemicals) and pretested before being connected to the cannulae through coiled polyethylene tubing (Intramedic, PE-60). NGF 12 μg/24 μl/day or vehicle (phosphate-buffered-saline/artificial CSF 1:1 and 0.1% bovine serum albumin) were loaded into the tubing with a small amount of mineral oil added to the minipump end to prevent mixing with the dye.

Animals were subjected to left-side devascularizing cortical lesions, as previously described (Sofroniew et al., 1983; Stephens et al., 1985). In brief, the devascularizing lesion was performed by removing a flap of bone and underlying dura and gently rubbing away all vessels and most of the pia mater with sterile, saline-soaked cotton swabs. Control animals received a sham operation consisting of a craniotomy only. Minipumps were implanted during this surgery. Seven days, after the lesion or sham operation, the animals were anesthestized and the pumps and tubings were removed. One month after

the lesion, the animals were sacrificed by transcardial perfusion under Equithesin anesthesia.

Immunocytochemistry

Rats were deeply anesthestized with Equithesin (0.40 ml/100g i.p.) and briefly perfused through the ascending aorta with a phosphate buffer (PB; pH 7.4) vascular rinse. Subsequently, a 20 min fixation with a 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid mixture in 0.1M phosphate buffer (pH 7.2) was immediately followed by a 20 min perfusion with 0.5% hydrogen peroxide in PBS. A final 20 min washout with a 10% glucose-PB solution was followed by removal and storage of brains in 30% sucrose-PB at 4°C for 72-120 h. Forty μm-thick coronal sections were cut on a freezing sledge microtome.

Free-floating sections were incubated overnight at 4°C in a monoclonal antibody anti-ChAT (Boehringer) diluted 1/25. All subsequent steps were carried out at room temperature using PBS containing 0.2% Triton X-100 (pH 7.4) for washes, dilution of antibodies and 3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. After washing, the sections were incubated for 2 h with the second antibody, a rabbit anti-rat serum (1/50; prepared in our laboratory). Subsequently, the sections were incubated with horseradish peroxidase (HRP; Sigma, Type IV) and a monoclonal rat anti-peroxidase (Cuello et al., 1984) (Medicorp, Canada) to develop ChAT immunoreactivity. After washing for 15 min (4x), sections were incubated in a 0.06% solution of DAB for 10 min and a subsequent 10 min in the same solution containing hydrogen peroxide (0.01%, final concentration). After washing, sections were mounted on subbed slides, dehydrated, cleared, and coverslipped.

MORPHOMETRIC ANALYSIS

Quantification of silver grains

The number of silver grains per area of cytoplasm was quantified using the M1 version of an MCID Image analysis system (Microcomputer Imaging Device; Imaging Research Inc., St. Catharine, Ont., Canada). Measurements were performed using light microscopy with an oil immersion objective connected to an image analysis system. Measurements were performed in ventral and dorsal NBM neurons. For each brain section, the background measured from corpus callosum areas was subtracted from signal obtained for each probe. Labeled cells used for analysis exhibited optimal distribution of grains in the cytoplasm. All cells with silver grains above background values were included in the quantification. Only cells with evident cytoplasm and nucleus were included in our analysis.

Immunocytochemistry image analysis

To assess the mean cross-sectional area of NBM neurons, sections stained for ChAT from control, vehicle-treated, as well as lesioned NGF-treated 105 day old animals (one month after cortical lesion) were analyzed. For these studies, the area containing the mid-portion of the NBM [approximately Bregma -1.30 mm to -1.80 mm according to the atlas of Paxinos and Watson (1986)] was analyzed using the M1 system. A total of six brains/group were analyzed. ChAT-immunoreactive neurons in the ventral and dorsal part of the mid-NBM were examined with respect to their mean cross-sectional area one month after surgery. This parameter was chosen in order to determine whether posthypothyroid animals could respond to NGF-treatment following cortical infarction. Morphometric data are expressed as mean cross-sectional area and mean fiber length $\pm$ the standard error of the mean (S.E.M.).

Statistical analysis

Statistical significance of the number of grains (pixel) per μm^2 of the cell body cross-sectional area and differences in the cross-sectional area of Methylene blue stained cells (75 day old animals) were assessed using Student't test. Differences in cross-sectional area of ChAT-immunopositive neurons among groups were compared with

ANOVA followed by a *post hoc* Newman-Keuls' test. The level of statistical significance was set at $P<0.05$.

RESULTS

In situ hybridization

We examined the levels of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs in the NBM of 75 day old non-devascularized posthypothyroid rats in order to compare them with the therapeutic profile of NGF in such animals. In another group of 75 day old animals, the effect of the lesion and NGF treatment was analyzed one month later (see below). Therefore, we assessed levels of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs before cortical lesion and NGF-treatment in order to define whether the possible failure of NGF to rescue NBM cholinergic neurons in posthypothyroid rats could be related to alterations of these receptors. The hybridization signal for both oligonucleotides, $p75^{NGFR}$ and $gp140^{trk}$, appeared to be over neuronal perikarya only. Our measurements of silver grains were conducted in the mid-portion of the NBM. This area has been reported to predominantly project to the parietal cortex by several studies in addition to our own (Bigl et al., 1982; Luiten et al., 1987; Eckenstein et al., 1988; Cuello et al., 1989; Figueiredo et al., 1993c). Fig. VI.1A shows bright-field photomicrographs exhibiting the hybridization signal for the $p75^{NGFR}$ probe in euthyroid animals which is very similar to the signal seen in posthypothyroid animals (Fig. VI.1B). The mean number of silver grains (pixels) per μm^2 of cell body cross-sectional area (number of grains/μm^2 $\pm$ S.E.M.) (Table VI.1) for $p75^{NGFR}$ in euthyroid rats (n=5) was 3.43 $\pm$ 0.89, which was not statistically different from posthypothyroid animals (n=5), 4.02 $\pm$ 1.07. Similar findings were noted for $p140^{trk}$ (Fig. VI.1C,D): the expression of this mRNA in euthyroid rats (Fig. VI.1C), is similar to that in the posthypothyroid animal (Fig. VI.1D). The mean number of grains (pixel/μm^2) in the euthyroid group was 5.54 $\pm$ 0.96, which was not statistically different from that of the posthypothyroid group, 6.32 $\pm$ 1.45. However, in 75 day old posthypothyroid rats, the mean cross-sectional area of NBM cells, visualized by Methylene blue staining, with positive label for $p75^{NGFR}$ or $gp140^{trk}$ was statistically

smaller ($P < 0.05$) 173 ± 15 (μm^2 ± S.E.M.) than in euthyroid animals, 234 ± 11 (μm^2 ± S.E.M.). The cross-sectional area of NBM neurons contralateral to the lesion side of NGF-treated posthypothyroid rats was not statistically different from sham-operated posthypothyroid animals.

Morphometric analysis of ChAT-immunopositive neurons

The immunocytochemical investigation of ChAT-immunoreactive sites in the NBM neurons from euthyroid and posthypothyroid animals confirmed an atrophy of these cholinergic cells, as previously reported (Sofroniew et al., 1983; 1987). The morphological characteristics of these ChAT immunoreactive neurons in sham-operated (105 day old), euthyroid and posthypothyroid animals, is shown in Figs. VI.2A,B and 2C,D, respectively. The measurement of the cross-sectional area of ChAT positive neurons in the sham-operated animals (105-day old) confirmed the differences described for Methylene blue stained cells in 75 day old animals. The mean cross-sectional area of ChAT-positive neurons of posthypothyroid animals (n=5) was 181 ± 17, which was significantly reduced as compared with controls (n=5), 237 ± 14 (Table VI.2).

Unilateral cortical infarction resulted in the expected retrograde atrophy of the NBM ChAT-immunopositive neurons in euthyroid rats treated with vehicle (See Fig. VI.3A,B). Intense neuronal shrinkage and retraction of neurites can be observed in Fig. VI.3B. Also, predictably, NGF application to devascularized rats for seven days (12 μg/24 μl/day) prevented the atrophy of these cholinergic neurons (Fig. VI.3C,D). Posthypothyroid rats, with a reduced cross-sectional area of NBM cholinergic neurons, became further atrophic after cortical devascularization (Fig. VI.4A,B) and also responded to NGF therapy which prevented their atrophy. Numerical data of cell body cross-sectional areas following cortical lesion are shown in Table VI.2.

Figure VI.1

Bright-field photomicrographs show coronal sections taken at the level of the mid-NBM. Neurons in the ventral NBM are shown which display hybridization signal for $p75^{NGFR}$ (A and B) and $p140^{trk}$ mRNAs (C and D). A and C were from control euthyroid, while B and D were from posthypothyroid 75 day old rats. Note that the number of silver grains, comparing individual cells, is similar between A and B as well as between C and D. At ventral (shown photomicrographs) and dorsal NBM (not shown), cell surface area from some isolated cells positive for $p75^{NGFR}$ or $p140^{trk}$ mRNAs exhibited smaller size in posthypothyroid animals (B and D). Scale bar in D=50 μm and also applies to A,B and C .

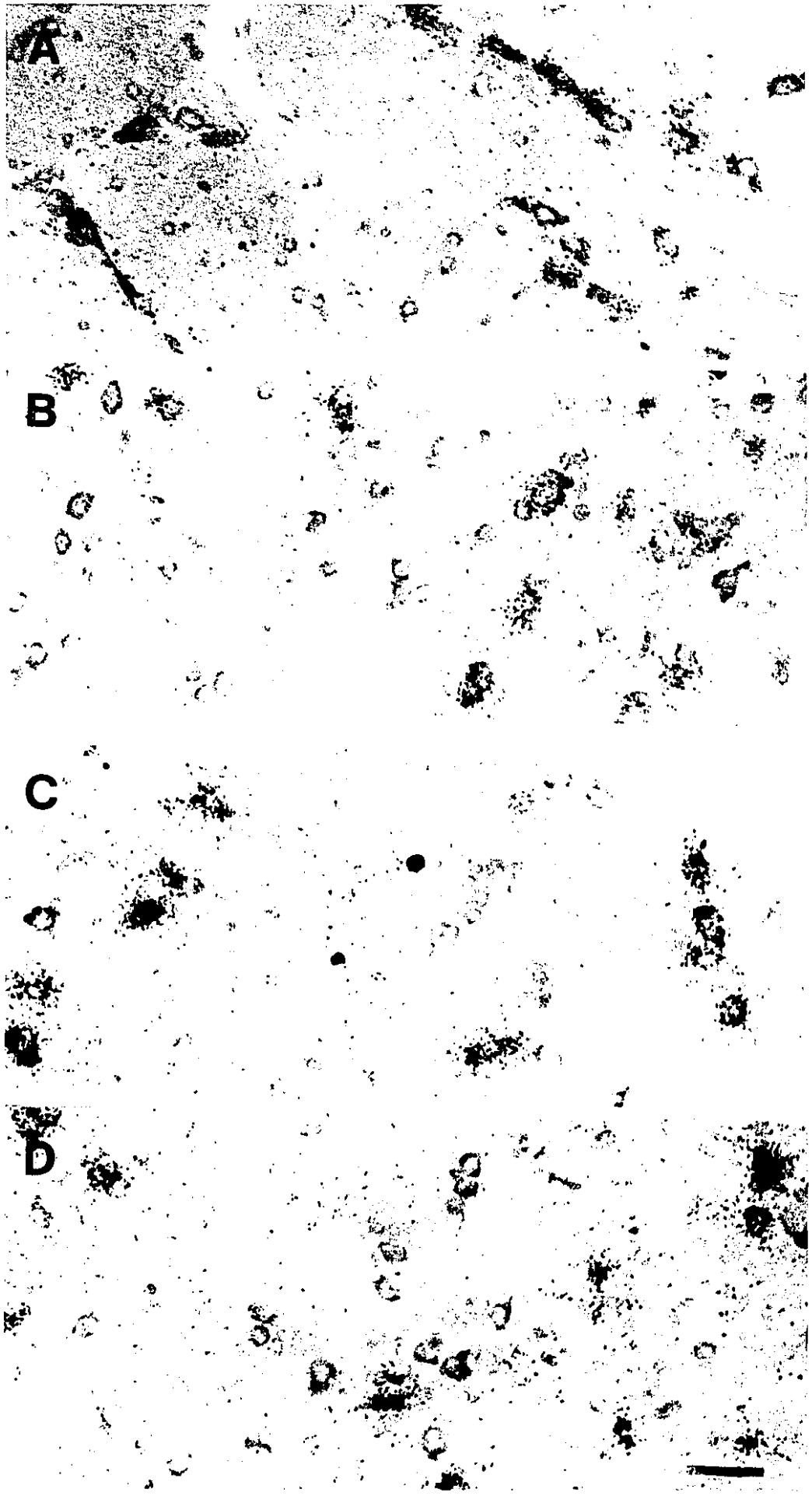
A
B
C
D

Table VI.1
Effects of thyroid hormone deprivation on mid-NBM neurons of 75 day old rats

	Number of grains (pixels)/μm^2 of cell surface		Cell surface
Groups	$p75^{NGFR}$ mRNA	$p140^{trk}$ mRNA	(μm^2)
Euthyroid	3.43 ± 0.89	5.54 ± 0.96	234 ± 11
Posthypothyroid	4.02 ± 1.07	6.32 ± 1.45	173 ± 15*

The cross-sectional area was measured in methylene blue-stained cells which were expressing grains above background levels. Values represent the mean of five animals/group (± S.E.M.). * $P<0.05$ versus euthyroid (Student' t test).

Figure VI.2

Photomicrographs of coronal sections taken at the level of the mid-NBM. ChAT-immunoreactive neurons in the dorsal mid-NBM of sham-operated 105 day old animals. (A and B) Sections from euthyroid rats: framed area in A corresponds to the high magnification photo shown in B. Smaller cell surface area is clearly seen in posthypothyroid rats, at low (C) and high power (D). Scale bar in C=60 μm and also applies to A. Scale bar in C, same for A, 60 μm. Scale bar in D=30 μm and also applies to B.

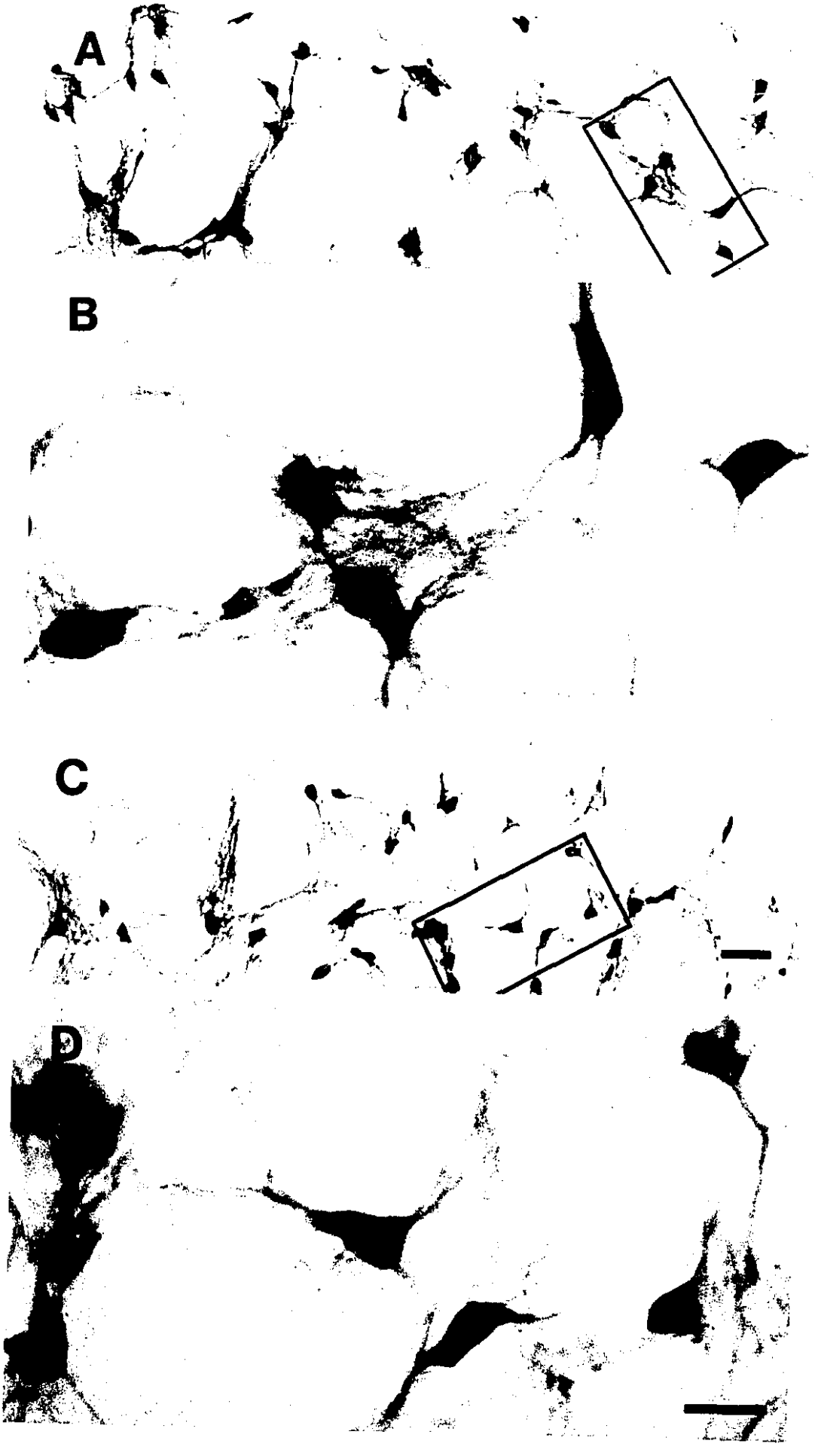
A
B
C
D

Figure VI.3

Photomicrographs of coronal sections from lesioned-animals at the level of mid-NBM. ChAT-immunoreactive neurons in the dorsal mid-NBM ipsilateral to the lesion, in euthyroid 105-day-old animals, one month after cortical devascularization (A and B). Sections from lesioned animals treated with vehicle: Framed area in A corresponds to the high power photo shown in B. NGF infusion for seven days confirmed recovery of the cell cross-sectional area ipsilateral to the lesion, as is clearly noted in photomicrographs C and D in contrast to the vehicle-treated animals. Photo in D corresponds to the framed area seen in C. Scale bar in C=60 μm and also applies A. Scale bar in B=30 μm and also applies to D.


A
B
C
D

Figure VI.4

Photomicrographs of coronal sections from lesioned-animals at the level of mid-NBM. ChAT-immunoreactive neurons in the dorsal mid-NBM, ipsilateral to the lesion, in posthypothyroid 105 day old animals one month after cortical devascularization, (A and B). Sections from lesioned animals treated with vehicle: Framed area in A corresponds to the high power photo shown in B. The regenerative capacity of cholinergic neurons in response to NGF infusion is clearly noted in photomicrographs C and D in contrast to the vehicle-treated animals. Photo in D corresponds to the framed area seen in C. Scale bar in A=60 μm and also applies to C. Scale bar in B=30 μm and also applies to D.

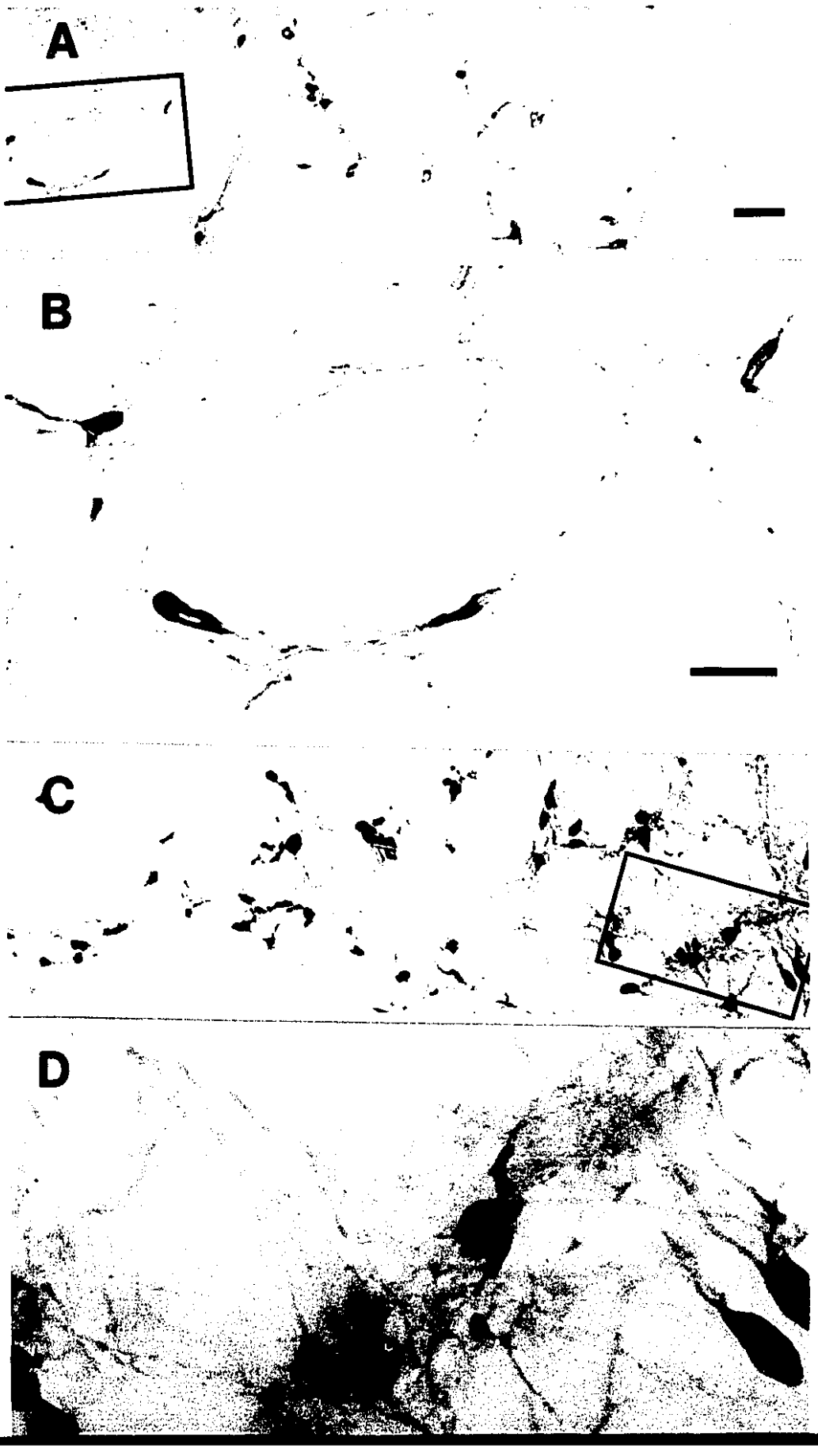
A
B
C
D

Table VI.2

Cell cross-sectional area of ChAT-immunopositive mid-NBM neurons in 105 day old rats

	Euthyroid	Posthypothyroid
Groups	$\mu m^2 \pm$ SEM	$\mu m^2 \pm$ SEM
Ipsilateral NBM		
Sham-operated + vehicle	237 ± 14	181 ± 17▲
Lesioned + vehicle	166 ± 21*	131 ± 23*
Lesioned + NGF	211 ± 17	174 ± 15▲
Contralateral NBM		
Lesioned + NGF	224 ± 12	190 ± 09**

Values represent the mean of five animals/group (± S.E.M.). * $p < 0.05$ versus sham-operated or NGF-treated rats with equivalent thyroid status. ▲ $p < 0.05$ versus respective euthyroid group. ** $p < 0.05$ versus respective euthyroid group. (ANOVA followed by *post hoc* Newman-Keuls' test).

Devascularization reduced cross-sectional area of posthypothyroid animals to 131 ± 23 (n=5), which was prevented by NGF treatment, 174 ± 15 (n=4). The size of the NBM cholinergic neurons ipsilateral to the lesion of NGF-treated and lesioned animals were not statistically different from ipsilateral sham-operated euthyroid animals, Thus, confirming previous results (Cuello et. al., 1989). Similar experimental group (NGF + lesion) in posthypothyroid animals was also not statistically different from the sham-operated group. In both euthyroid and posthypothyroid animals NGF treatment did not change significantly the cross-sectional area of contralateral NBM cholinergic neurons in lesioned rats as compared with sham-operated rats (Table VI.2). The size of NBM ChAT-positive neurons contralateral to the lesion in NGF-treated euthyroid rats was still larger ($p < 0.05$) than the same cells in posthypothyroid rats. These results indicated that while NGF restored the size of NBM cholinergic neurons ipsilateral to the cortical lesion to that of sham-operated posthypothyroid, its action was insufficient to bring the cells to a size equivalent to that of euthyroid rats (Table VI.2).

DISCUSSION

Atrophied cholinergic neurons in posthypothyroid rats were identified at two ages, 75 and 105 days, using two different markers, Methylene blue staining of cells containing substantial levels of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs, and ChAT immunoreactivity. These findings were observed after reestablishing physiological levels of thyroid hormones. The noted persistent atrophy of NBM cholinergic neurons confirms a previous study (Gould and Butcher, 1989) and is consistent with the irreversible effects observed in a wide variety of brain neuronal cell types (Legrand, 1983; Dussault and Ruel, 1987; Porterfield and Hendrich, 1993; Rabié et al., 1979). An exception is the rat olfactory epithelium which exhibits a profound ability to recover from prolonged thyroid hormone deprivation from birth (Paternostro and Meisami, 1993). The NBM cholinergic cells of posthypothyroid adult rats, ipsi- and contralateral the cortical infarction, failed to revert to their normal (euthyroid) size in response to NGF. Thus, once the adult morphology

of CNS neurons is acquired, trophic factors might have a modest effect on phenotypic expression. This effect is apparently not sufficient to re-structure these neurons to their normal size. However, cholinergic neuronal recovery in hypothyroid rats could occur if growth factor therapy were implemented before the critical period of brain development. A central question concerns NGF responsiveness in atrophic NBM cholinergic cells. An unaltered expression of $p75^{NGFR}$ and $gp140^{trk}$ mRNAs in the NBM of posthypothyroid animals suggest normal post-receptor NGF signalling. This is supported by the observation that NGF restored the size of NBM cholinergic neurons to that of sham-operated posthypothyroid but not to that of control euthyroid rats. The finding that NGF prevented decreases in cell cross-sectional area induced by cortical infarction is not in accordance with the notion of deficient axonal transport of NGF-NGF receptor complexes in posthypothyroid animals, as previously suggested for the cerebellum of hypothyroid neonates (Figueiredo et al., 1993a). The overall action of thyroid hormone on developing cholinergic cells and the cerebellum was proposed to involve an interaction with NGF (Hayashi and Patel, 1987; Patel et al., 1988; Legrand and Clos, 1991). Susceptibility of some brain cholinergic neurons to thyroid hormone deprivation was suggested to be selectively related to the sensitivity of these neurons to NGF (Oh et al., 1991). These reports are compatible with thyroxine-induced increases of NGF levels in the adult mouse brain (Walker et al., 1979). This increase in NGF level was observed in cerebellar neuroblasts from 5 day old rats *in vitro* (Charrasse et al., 1992) and in cerebellum of thyroxine-treated newborn rats but not in 15 or 30 day old rats (Figueiredo et al., 1993b). It would be of particular interest, in this regard, to investigate whether this decrease in cholinergic neuronal size as described above, could be prevented by concomitant NGF treatment during early development. It is likely that a reduction in neuronal size results primarily not only from a failure of a direct influence exerted by thyroid hormone during neuronal growth but also from decrements in many other thyroid hormone-dependent or independent macromolecules, including NGF, which are involved in cell growth and differentiation. The growth factor inducible gene NGFI-A is an immediate-early response gene induced by signals that lead to growth and differentiation (Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988). Pipaon and

colleagues (1991; 1992) described that developing rat brain, but not adult animals with thyroid hormone deprivation, had marked decrease of NGFI-A while triiodothyronine treatment increased NGFI-A within 1 h. Interestingly, NGFI-A was also shown to be induced by NGF (Milbrandt, 1987; Watson and Milbrandt, 1990). Thus, a deficiency of NGFI-A in thyroid hormone deprived pups may have contributed to the decreased NBM cholinergic cell size. Furthermore, it is conceivable that a disturbed metabolism caused by thyroid hormone deficiency also leads to deficits in particular brain nutritional elements (Mooradian, 1990). It appears that the consequences of thyroid hormone deprivation from birth involve decrements in many specific and unspecific factors. NGF treatment did not affect the size of unlesioned cholinergic neurons (contralateral NBM in animals with cortical devascularizing lesion) in both euthyroid and posthypothyroid rats suggesting that under our experimental conditions NGF does not induce permanent changes in fully mature animals. results from this and other studies indicate that for successful recovery of cholinergic neurons appropriate levels of NGF must be available following a trauma but, it is unknown whether earlier NGF treatment could prevent the deleterious and, so far, permanent effects of neonatal hypothyroidism. In a previous study, NGF treatment of rat pups significantly increased forebrain ChAT activity in euthyroid but not in PTU-treated pups (Patel et al., 1988). These findings could suggest that NGF per se may not induce other phenotypic changes, such as size of cholinergic cells in thyroid hormone deprived pups. Collectively, results from this study and from others, to date, would indicate that thyroid hormone replacement therapy initiated before the critical neonatal period of development could prevent the apparent permanent and irreversible effect of thyroid deficiency in CNS cholinergic neurns.

CONCLUSION

In this study we have found that NGF was unable to restore cholinergic phenotype in neurons of the NBM which developed abnormally due to early thyroid hormone deprivation. However, this neurotrophin was efficacious in protecting the same cells from further atrophy induced by unilateral cortical infarction. This positive biological

response to NGF is in accordance with the unchanged mRNA levels of low and high-affinity NGF receptors in posthypothyroid animals.

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REFERENCES

Atterwill CK, Kingsbury A, Nicholls J, Prince A (1984) Development of markers for cholinergic neurones in re-aggregate cultures of foetal rat whole brain in serum-containing and serum-free media: Effects of triiodothyronine (T3). Br J Pharmacol 83:89-102.

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K (1987) Current protocols in molecular biology. New York: John Wiley and Sons.

Bigl V, Woolf NJ, Butcher LL (1982) Cholinergic projections fron the basal forebrain to frontal, parietal, temporal, occipital and cingulate cortices: a combined fluorescent tracer and acetylcholinesterase analysis. Brain Res Bull 8:727-749.

Charrasse S, Jehan F, Confort C, Brachet P, Clos J (1992) Thyroid hormone promotes transient nerve growth factor synthesis in rat cerebellar neuroblasts. Dev Neurosci 14:282-289.

Chaudhury S, Chartterjee D, Sarkar PK (1985) Induction of brain tubulin by triiodothyronine: dual effect of the hormone on the synthesis and turnover of the protein. Brain Res 339:191-194.

Christy BA, Lau LF, Nathans D (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci USA 85:7857-7861.

Clos J, Legrand C (1990) An interaction between thyroid hormone and nerve growth factor promotes the development of hippocampus, olfactory bulbs and cerebellum. A comparative biochemical study. Growth Factors 3:205-220.

Cuello AC, Milstein C, Wright B, Bramwell S, Priestley JV, Jarvis J (1984) Development and application of a monoclonal rat peroxidase-antiperoxidase (PAP) immunocytochemical reagent. Histochemistry 80:257-261.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

Cuello AC (1993) Trophic respcnses of forebrain cholinergic neurons: a discussion. Prog Brain Res 98:265-277.

Da Penha Berzaghi M, Cooper J, Castrén E, Zafra F, Sofroniew M, Thoenen H, Lindholm D (1993) Cholinergic regulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) but not neurotrophin-3 (NT-3) mRNA levels in the developing rat hippocampus. J Neurosci 13:3818-3826.

Dussault JH, Ruel J (1987) Thyroid hormones and brain development. Ann Rev Physiol 49:321-334.

Ebendal T (1992) Function and evolution in the NGF family and its receptors. J Neurosci Res 32:461-470.

Eckenstein FP, Baughman RW, Quinn J (1988) An anatomical study of cholinergic innervation in rat cerebral cortex. Neuroscience 25(2):457-474.

Ernfors P, Henschen A, Olson L, Persson H (1989) Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2:1605-1613.

Faivre C, Legrand C, Rabié A (1983) Effects of thyroid deficiency and corrective effects of thyroxine on microtubules and mitochondria in cerebellar purkinje cell dendrites of developing rats. Dev Brain Res 8:21-30.

Figueiredo BC, Almazan G, Ma Y, Tetzlaff W, Miller FD, Cuello AC (1993a) Gene expression in the developing cerebellum during perinatal hypo- and hyperthyroidism. Mol Brain Res 17:258-268.

Figueiredo BC, Otten U, Strauss S, Volk B, Maysinger D (1993b) Effects of perinatal hypo- and hyperthyroidism on the levels of nerve growth factor and its low-affinity receptor in cerebellum. Dev Brain Res 72:237-244.

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993c) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Gnahn H, Hefti F, Heuman R, Schwab ME, Thoenen H (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain?. Dev Brain Res 9:45-52.

Goedert M, Fine A, Hunt SP, Ullrich A (1986) Nerve growth factor mRNA in peripheral and central rat tissues and in the human nervous system: lesion effects in the rat brain and levels in Alzheimer's Disease. Mol Brain Res 1:85-92.

Gould E, Butcher LL (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347-3358.
Hagg T, Vahlsing HL, Manthorpe M, Varon S (1990) Nerve growth factor infusion into the denervated adult rat hippocampal formation promotes its cholinergic reinnervation. J Neurosci 10:3087-3092.

Haroutunian V, Kanof PD, Davis KL (1989) Attenuation of nucleus basalis of Meynert lesion induced cholinergic deficits by nerve growth factor. Brain Res 487:200-203.

Hayashi M, Patel AJ (1987) An interaction between thyroid hormone and nerve growth factor in the regulation of choline acetyltransferase activity in neuronal cultures, derived from the septal-diagonal dand region of the embryonic rat brain.. Dev Brain Res 36:109-120.

Hefti F (1986) Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J Neurosci 6:2155-2162.

Hefti F, Hartikka J, Bolger MB (1986) Effect of thyroid hormone analogs on the activity of choline acetyltransferase in cultures of dissociated septal cells.. Brain Res 375:413-416.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature 350:678-683.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen C-K, Valletta JS, Zhou J, Long JB, Mobley WC (1992) $p140^{trk}$ mRNA marks NGF-responsive forebrain neurons: Evidence that *trk* gene expression is induced by NGF. Neuron 9:465-478.

Honegger P, Lenoir D (1980) Triiodothyronine enhancement of neuronal differentiation in aggregating fetal brain cells cultured in a chemically defined medium. Brain Res 199:425-434.

Honegger P, Lenoir D (1982) Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. Dev Brain Res 3:229-238.

Ibáñez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low-affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. Cell 69:329-341.

Kalaria RN, Prince AK (1985) Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neurons markers and DNA concentrations in the rat corpus striatum. Int J Dev Neurosci 3:655-666.

Kaplan DR, Martin-Zanca D, Parada LF (1991) Tyrosine phosphorylation and tyrosine activity of the *trk* proto-oncogene product induced by NGF. Nature 350:158-160.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991) The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Korsching S, Auburger G, Heumann R, Scott J, Thoenen H (1985) 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.

Kromer LF (1987) Nerve growth factor treatment after brain injury prevents neuronal death. Science 235:214-216.

Lakshmanan J, Mansfield H, Weichsel ME, Hoath S (1981) Neonatal hypothyroidism - A biochemical disorder of α-tubulin metabolism. Biochem Biophys Res Commun 100:1587-1596.

Legrand C, Clos J (1991) Biochemical, immunocytochemical and morphological evidence for an interaction between thyroid hormone and nerve growth factor in the developing cerebellum of normal and hypothyroid rats. Dev Neurosci 13:382-396.

Legrand J (1979) Morphogenetic action of thyroid hormones. Trends Neurosci 2:234-236.

Legrand J (1983) Hormones thyroïdiennes et maturation du système nerveux. J Physiol (Paris) 78:603-652.

Lemàire P, Revelant O, Bravo R, Charnay P (1988) Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci USA 85:4691-4695.

Luiten PGM, Gaykema RPA, Traber J, Spencer DG (1987) Cortical projection patterns of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin. Brain Res 413:229-250.

Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM (1992) The rat *trk* protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci USA 89:2374-2378.

Milbrandt J (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797-799.

Mobley WC, Rutkowski JL, Tennekoon GI, Buchanana K, Johnson MW (1985) Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. Science 229:284-287.

Mooradian AD (1990) Metabolic fuel and amino acid transport into the brain in experimental hypothyroidism. Acta Endocrinol (Copenh) 122(2):156-162.

Nicholson JL, Altman J (1972) The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. II. Synaptogenesis in the molecular layer. Brain Res 44:25-36.

Nunez J (1985) Microtubules and brain development: the effects of thyroid hormones. Neurochem Int 32:959-968.

Oh JD, Butcher LL, Woolf NJ (1991) Thyroid hormone modulates the development of cholinergic terminal fields in the rat forebrain: Relation to nerve growth factor receptor. Dev Brain Res 53:133-142.

Patel AJ, Hayaschi M, Hunt A (1987) Selective persistent reduction in choline acetyltransferase activity in basal forebrain of the rat after thyroid deficiency during early life. Brain Res 422:182-185.

Patel AJ, Hayaschi M, Hunt A (1988) Role of thyroid hormone and nerve growth factor in the development of choline acetyltransferase and other cell-specific marker enzymes in the basal forebrain of the rat. J Neurochem 50:803-811.

Paternostro MA, Meisami E (1993) Developmental plasticity of the rat olfactory receptor sheet as shown by complete recovery of surface area and cell number from extensive early hypothyroid growth retardation. Dev Brain Res 76:151-161.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Pipaon C, Santos A, Perez-Castillo A (1992) Thyroid hormone up-regulates NGFI-A gene expression in rat brain during development. J Biol Chem 267:21-23.

Porterfield SP, Hendrich CE (1993) The role of thyroid hormones in prenatal and neonatal neurological development. Current perspectives. Endocr Rev 14:94-106.
Rabié A, Patel AJ, Legrand J (1979) Effect of thyroid deficiency on the growth of the hippocampus in the rat: a combined biochemical and morphological study. Dev Neurosci 2:183-194.

Rami A, Patel AJ, Rabié A (1986) Thyroid hormone and development of the rat hippocampus: morphological alterations in granule and pyramidal cells. Neuroscience 19:1217-1226.

Schalling M, Dagerlind Å, Brené S, Hallman H, Djurfeldt M, Persson H, Terenius L, Goldstein M, Schlesinger D, Hökfelt T (1988) Coexistence and gene expression of phenylethanolamine

N-methyltransferase, tyrosine hydroxylase and neuropeptide tyrosine in the rat and bovine adrenal gland: Effects of reserpine. Proc Natl Acad Sci USA 85:8306-8310.

Seiler M, Schwab ME (1984) Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. Brain Res 300:34-39.

Shelton DL, Reichardt LF (1986) Studies on the expression of the B-NGF gene in the CNS: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. Proc Natl Acad Sci USA 83:2714-2718.

Sofroniew MV, Pearson RC, Eckenstein F, Cuello AC, Powell TP (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res 289:370-374.

Sofroniew MV, Pearson RCA, Powell TPS (1987) The cholinergic nuclei of the basal forebrain of the rat: normal structure, development and experimentally induced degeneration. Brain Res 411:310-331.

Stephens PH, Cuello AC, Sofroniew MV, Pearson RC, Tagari P (1985) Effect of unilateral decortication on choline acetyltransferase activity in the nucleus basalis and other areas of the rat brain. J Neurochem 45:1021-1026.

Thoenen H, Bandtlow C, Heumann R (1987) The physiological function of nerve growth factor in the central nervous system: Comparison with the periphery. Rev Physiol Biochem Pharmacol 109:145-178.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165-170.

Valcana T (1971) Effects of neonatal hypothyroidism on the development of acetylcholinesterase and choline acetyltransferase activities in rat brain. In: Influences of Hormones on the Nervous System (Ford DH ed), pp 174-184. Basel: S.Karger.

Verge VMK, Merlio J-P, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hökfelt T, Richardson PM (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: Responses to injury and infusion of NGF. J Neurosci 12:4011-4022.

Vigouroux E (1976) Dynamic study of post-natal thyroid function in the rat. Acta Endocrinol (Copenh) 83:752-762.

Walker P, Weichsel ME, Fisher DA, Guo SM (1979) Thyroxine increases nerve growth factor concentration in adult mouse brain. Science 204:427-429.

Watson MA, Milbrandt J (1990) Expression of the nerve growth factor-regulated NGFI-A and NGFI-B genes in the developing rat. Development 110:173-183.

Whittemore SR, Ebendal T, Lärkfors L, Olson L, Seiger Å, Strömberg I, Persson H (1986) Developmental and regional expression of β nerve growth factor messenger RNA and protein in the rat central nervous system. Proc Natl Acad Sci USA 83:817-821.

Whittemore SR, Seiger Å (1987) The expression, localization and functional significance of β-nerve growth factor in the central nervous system. Brain Res Rev 12:439-464.

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A, Gage FH (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc Natl Acad Sci USA 83:9231-9235.

CHAPTER VII

GENERAL DISCUSSION

An initial goal of this thesis, was to assess whether brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) elicit protection of nucleus basalis magnocellularis (NBM) cholinergic neurons, as occurs with NGF treatment (Cuello et al., 1989; Garofalo and Cuello, 1990; 1994a; 1994b; Haroutunian et al., 1989). The therapeutic properties of these two neurotrophins were examined with respect to their ability to prevent atrophy of these neurons. The results presented in Chapter II (Skup et al., 1993; 1994), show that these two neurotrophins do not elicit protection of NBM cholinergic neurons after injection into the lateral cerebral ventricle for seven days. The injected amounts of BDNF and NT-3 were approximately equimolar to the saturating dose (12 μg/day) reported for NGF to rescue these neurons (Garofalo and Cuello, 1994b). The lack of response to such moderate amounts of BDNF is in agreement with a recent study which demonstrated that intraparenchymal treatment with BDNF did not rescue NBM neurons following ibotenic acid infusion (Dekker et al., 1994). Our results are also in accordance with a report showing that this agent at the same dosage and administration route, fails to protect septal cholinergic neurons following fimbria-fornix transection (as revealed by ChAT immunocytochemistry) even after 14 days of treatment (Morse et al., 1993). These findings are in agreement with the very low to undetectable levels of trkB mRNA in basal forebrain neurons (see Table I.1 in Chapter I), as well as with the lack of BDNF binding to basal forebrain neurons after intracerebroventricular injection of iodinated BDNF at 12 μg/day, for 14 days (Morse et al., 1993). In radioautographic receptor binding studies, ^{125}I-NT-3 did not bind to basal forebrain neurons (Altar et al., 1993). The presence of very high levels of truncated trkB mRNA (Klein et al., 1990), together with the accumulation of exogenous BDNF circumscribing the cerebral ventricles (ipsilateral to the lesion) (Morse et al., 1993), suggested limited penetration into the subjacent neural parenchyma. However, when BDNF (12 μg/day, 14 days) was injected into the dorsolateral septum it accumulated in a discrete number of neurons in the medial septal nucleus and vertical limb of the diagonal band of Broca (MS/VB) (located approximately 2-3 mm ventral to the injection site) and was shown to rescue septal neurons following fimbria-fornix transection (Morse et al., 1993). There was no record in this binding study (Morse et al., 1993) that other basal forebrain

cholinergic neurons, in particular the NBM, accumulated BDNF after injection into the dorsolateral septum. This suggests that the ventricular ependymal cell layer and subjacent regions are not the only limiting factors affecting distribution and specific binding of BDNF to basal forebrain cholinergic neurons. In contrast with these and our own results, previous studies from another laboratory reported that intracerebroventricular infusion of lower amounts of BDNF (4.5 μg/day, for 20 days) increased by 100% (Knüsel et al., 1992) and by 55% (Widmer et al., 1993) the percentage of visible ChAT-positive neurons in the MS/VB, ipsilateral to the fimbria-fornix transected side, compared with the number of cells in cytochrome C-treated and lesioned animals. Immunocytochemistry to $p75^{NGFR}$ revealed either similar (Knusel et al., 1992) or a larger number of neurons in the MS/VB, ipsilateral to the fimbria-fornix transected side of BDNF-treated animals (Morse et al., 1993; Widmer et al., 1993) as compared with the number of ChAT positive neurons. These studies suggest that intracerebroventricular administration of BDNF rescues these cholinergic neurons. It was argued that discrepancies between results with ChAT and $p75^{NGFR}$ immunoreactivities could be due to a loss of ChAT expression occurring before $p75^{NGFR}$ expression (Widmer et al., 1993). However, another explanation for these conflicting results between ChAT and $p75^{NGFR}$ immunoreactivities could be attributed to the injection site used in these studies (Knüsel et al., 1992; Widmer et al., 1993). Injections were not only intracerebroventricular, but also intrahippocampal (as suggested by the location of the injection cannula) distal to the site of the axotomy. Results from studies with BDNF-treated septal cell cultures (25 ng/ml/day) treated for 12 days (Alderson et al., 1990) revealed potent increases in ChAT activity (260%) and in the number of $p75^{NGFR}$-immunopositive neurons (100%). Differences obtained in *in vivo* experiments with single BDNF (4.5 μg/day) injections (Knüsel et al., 1992; Widmer et al., 1993) versus continuous infusion via minipump (12 μg/day) (Skup et al., 1993; Morse et al., 1993) could suggest a loss of BDNF activity during chronic administration by minipump. However, there was negligible loss in the biological activity of either NGF or BDNF recovered from minipumps (Altar et al., 1992). Provided that the only way for an efficient BDNF-treatment is through intraparenchymal injection, it is unlikely that BDNF could be considered for the

treatment of injured basal forebrain cholinergic neurons when NGF seems more potent and efficient by the intracerebroventricular route. However, BDNF has been shown to be a potent neurotrophic factor for other CNS neurons (see Table I.4 in chapter I).

The second objective of this thesis was to test, for the first time, whether aFGF could rescue NBM cholinergic neurons in the same animal model. The interest in this NTF emerged from two lines of evidence: (1) the report of a potent effect of bFGF (6ng/day), in preventing atrophy and cell death of medial septal cholinergic neurons caused by fimbria-fornix transection (Anderson et al., 1988); and (2) that bFGF, in experiments conducted *in vitro* and in neonatal animals (Spranger et al., 1990a), and aFGF, in *in vitro* studies (Yoshida and Gage, 1991; Ono et al., 1991), induced an increase in NGF levels. These data provided important information allowing us to predict whether aFGF could be efficacious in our lesion model. However, evidence derived from experiments using peripheral tissues, indicating that aFGF stability and bioactivity depended upon high concentrations of heparin (Damon et al., 1993; Gospodarowicz and Cheng, 1986; Müller et al., 1989; Thomas et al., 1991; Rosengart et al., 1988), were initially discouraging because the use of heparin could not be considered due to an increased risk of bleeding in devascularized animals. After a successful pilot experiment involving the injection of several aFGF doses, treatments at 12 μg/day, in the absence of heparin, proved to be efficacious in preventing atrophy of NBM cholinergic neurons. This daily amount of aFGF caused a considerable decrease in animal body weight during treatment time. The weight loss was more accentuated than that noted for vehicle-treated decorticated animals and is in agreement with a previous study (Hanai et al., 1989). aFGF also preserved ChAT-immunoreactive neuritic processes (dendrites and axons) in the NBM ipsilateral to the cortical infarction, which is in line with the noted preservation of ChAT activity in this area and its increased activity in mixed-septal cell cultures. The fact that heparin was not used in these experiments (Figueiredo et al., 1992) suggested that the final amount of aFGF required to exert these effects was small. However, it later became clear that aFGF in CNS tissue is much less heparin-dependent than in peripheral nerves (Stock et al., 1992). The well known pleiotropic effects of the FGF family have been widely described [for a review see (Basilico and Moscatelli, 1992)]. Among these

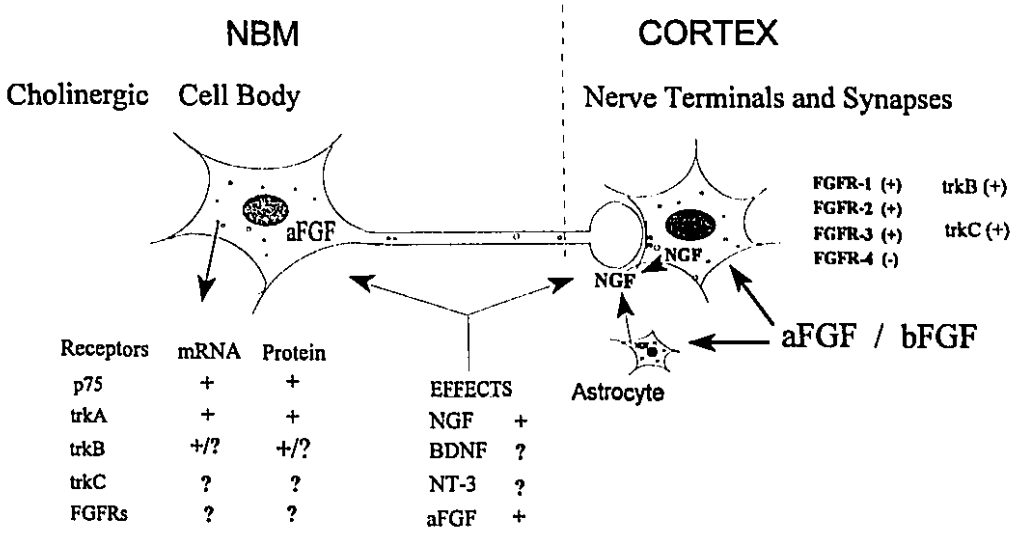


Figure VII.1. Schematic diagram of the basolo-cortical pathway indicating possible target sites for neurotrophic factors. The sites of expression of neurotrophic factor receptors are shown for NBM cholinergic neurons and neocortex (see Tables 1.1 and 1.3). The observations from this study confirmed that NGF rescues NBM cholinergic neurons. This protective effect was also observed after aFGF-treatment but not following BDNF or NT-3 treatment. According to the literature (see chapter 1), some endogenous sources of FGFs are: NBM cholinergic neurons (aFGF) and astrocytes (aFGF and bFGF). All FGF receptors (FGFRs), with the exception of FGFR-4, are expressed in the neocortex. Results from this work and that of others suggest that aFGF may rescue NBM cholinergic neurons indirectly, by increasing the synthesis of NGF from astrocytes and neurons. Detection of receptor protein or mRNA is indicated by (+), or a (-) when undetected.

effects is that aFGF can interact with non-neuronal cells as well. In our study, aFGF was able to induce a robust growth in glial cells, as detected by glial fibrillary acidic protein (GFAP)-immunocytochemistry of mixed glial-septal cell cultures. Figure VII.1. is a schematic diagram which summarizes the distribution of some target sites of neurotrophic factors, their effects on NBM cholinergic neurons, and a possible mechanism for the effects of aFGF on NBM cholinergic neurons. It has previously been described that animals with cortical infarctions exhibit memory deficits (Elliott et al., 1989). This lesion model was used to investigate whether the phenotypic changes induced by aFGF in NBM cholinergic neurons could also correspond to an improvement of memory deficits.

Some of the possible functional consequences of the infarction inflicted on the rat cerebral cortex may not be attributable to disruption of the cholinergic basalo-cortical pathway but could also be due to injury of other cortico-fugal or cortico-petal pathways, as well as due to the loss of intrinsic cortical circuits. It is clear that this NBM lesion model and all others reporting lesions of cholinergic basal forebrain neurons [for reviews see Refs. (Voytko et al., 1994; Bartus et al., 1985; Price, 1986)] do not mimic all the cognitive deficits noted in ageing and Alzheimer's disease (AD). However, studies using these models have revealed much about cholinergic function in learning and memory processes. While we find it important to acknowledge the pitfalls of the cortical devascularization model, and that there are points of overlap between behavioral deficits caused by unilateral NBM cholinergic hypoactivity and possible hypofunction of other neurotransmitter systems, restoration of the behavioral deficit with NGF treatment (Garofalo and Cuello, 1990; 1994a) is in agreement with evidence linking cholinergic failure to learning and memory deficits in this lesion model. This study also supports previous work (Fischer et al., 1987; 1991) showing amelioration of cholinergic neuronal atrophy and spatial memory impairment in aged animals by NGF treatment. Such work is in agreement with the notion that cholinergic transmission is one of the major systems involved in the pathology of AD. However, it has been suggested that the cholinergic hypothesis represents an oversimplified concept of the biological basis of AD, which clearly has a multi-transmitter, multi-neuronal system pathology. The therapeutic potential of cholinergic agonists is of limited value for the treatment of patients with AD

(Fibiger, 1991; Dunnet and Fibiger, 1993). The hypothesis favouring the occurrence of retrograde degeneration in AD, involving degeneration of the cerebral cortex and hippocampus (Cuello and Sofroniew, 1984; German et al., 1987), partially supports the validity of the cortical infarction model which causes retrograde degeneration of the basalo-cortical system. In this condition, the performance of animals in the water maze test exhibited statistically significant impairment in the first trial after surgery, suggesting a deficit in recalling the behavioral task which was learned one month before (occurring in the last five days of training immediately before cortical lesion). This memory deficit was prevented by aFGF treatment [Chapter III, (Figueiredo et al., 1993c)]. The experimental paradigm utilized in the behavioral tests described here was designed to analyze performance in recalling a previous learned task (memory), but it was clearly observed that the untreated animals were able to find and climb the platform as controls did in the last two days of the four-day training period (one month after lesion). Results of recent experiments from our laboratory (in preparation), designed to assess acquisition of memory, revealed that animals with cortical infarction exhibit impairments in the learning process. Moreover, results from studies with human volunteers treated with diazepam and scopolamine (Ghoneim and Mewaldt, 1975) and with monkeys treated with scopolamine (Aigner et al., 1991) suggest that acetylcholine affects the acquisition of new memories to a greater degree than the recall of previously learnt ones.

At the same time as our first communication which showed that aFGF improves impairments in the recall of a previously learnt behavioral task (Figueiredo et al., 1992), other studies reported similar findings obtained using mice and, in addition, provided further insights for a physiological role of endogenous aFGF (released from ependymal cells) in memory facilitation (Oomura et al., 1993; 1992). These investigators described that 2 h after food-intake, intraperitoneal or intracerebroventricular infusions of glucose, concentrations of aFGF increased 1000 times in cerebrospinal fluid (CSF); a finding indicating that aFGF is secreted, despite contradictory arguments (see Table I.2 in Chapter I). The evidence that secreted aFGF is responsible for facilitation of memory (increased retention in a passive-avoidance test and reduced time to find a submerged platform in a water maze test) was indicated by inhibition of this effect following

intracerebroventricar injection of an anti-aFGF antibody (Oomura et al., 1993). In experiments using radiofrequency-induced lesions of basal forebrain neurons in mice, which result in severe impairment in the acquisition but not in the retention process of a passive avoidance test, basic FGF improved acquisition but did not affect ChAT activity (Ishihara et al., 1992). Furthermore, aFGF was shown to enhance long-term potentiation (LTP), induced by a tetanus of 20 pulses at 60 Hz, in the dentate gyrus (Hisajima et al., 1992). The involvement of cholinergic transmission in learning and memory has been associated with enhanced LTP (Blitzer et al., 1990; Burgard and Sarvey, 1990) and enhanced activity of inhibitory interneurons (McCormick and Prince, 1986; Pitler and Alger, 1992). This coordinated inhibitory modulation has been proposed to lead to efficient memory storage (Hasselmo and Bower, 1993). However, memory storage mechanisms are probably dependent on multi-transmitter systems.

More recently, toxicological and immunological studies conducted in rats, mice and volunteers treated with bovine bFGF, failed to find adverse side-effects caused by bFGF (Aguilar et al., 1993). These authors reported the presence of radioactivity in cerebral capillaries and in the brain parenchyma after 5 and 25 min of an intravenous injection of ^{131}I-bFGF, respectively. The fact that FGF receptors are expressed in endothelial cells (Schweigerer et al., 1993), led these authors to propose a receptor-mediated bFGF transport, similar to that described for transferrin (Fishman et al., 1987), or to the carrier-mediated transport of vasopressin across the blood-brain-barrier (Banks et al., 1987). The main findings from these studies was the observation that intramuscular administration of bovine bFGF (once or twice a month, over 7-12 months) to children who were mentally retarded due to perinatal hypoxia, significantly improved the mental development of these children (Aguilar et al., 1993). The only adverse effect of bFGF treatment reported, was that 10% of the children treated exhibited a slight hyperactivity and irritation during the first two or three applications. These findings are in accord with our observations and, to my knowledge, this is the first report on the use of FGF for human brain therapy. Although the physiological and clinical significances of these findings with bFGF provoke a number of investigations in patients with cerebral trauma and degenerative diseases, it is important to realise the risk of serious side

effects, especially with regard to continuous stimulation of glial cells. Furthermore, use of human recombinant acidic or basic FGF would reduce the risk of antibody synthesis and other effects which are more likely to occur with bovine FGF.

To further elucidate the effects of aFGF in cholinergic neurons and glial cells (Chapter III), we then verified whether aFGF could induce increases of NGF and its mRNA in the neocortex of adult animals (Chapter IV), an effect that was shown in hippocampus of neonatal animals treated with bFGF (Spranger et al., 1990b). Our results demonstrate, firstly, that cortical infarction alone induces NGF, a finding that confirmed previous studies using different types of brain injury (Gasser et al., 1986; Korsching et al., 1986; Weskamp et al., 1986a; 1986b; Bakhit et al., 1991). The idea that NGF was being produced mainly by reactive glial cells following cortical infarction (Herrera and Cuello, 1992) was indicated by studies showing the co-localization of increased immunoreactivity to both NGF and GFAP in astrocytes following injury (Bakhit et al., 1991; Oderfeld-Nowak et al., 1992). Inducers of NGF synthesis also include FGFs, which probably produce this effect by activating NGF synthesis mainly in astrocytes. These arguments were suggested by results showing that aFGF and/or bFGF increase the release of NGF from astrocyte cell cultures (Spranger et al., 1990a; Wanaka et al., 1990; Ono et al., 1991; Yoshida and Gage, 1992). The first *in vivo* evidence that aFGF can induce an increase of NGF and its mRNA in cerebral cortex of adult animals was provided by our study (Chapter IV). This effect occurs in various brain areas and at different ages, concurring with previous reports showing that bFGF elicited an increase in NGF in the hippocampus of developing rats (Spranger et al., 1990b). It has been suggested that in situations where inflammatory agents are elevated, such as in cortical infarction, these agents may also lead to increased levels of NGF. Interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), platelet derived growth factor (PDGF), lipopolysaccharide and other agents have been shown to augment levels of NGF and its mRNA in brain and in non-neuronal cells *in vitro* (Lindholm et al., 1987; 1988; Spranger et al., 1990a; Friedman et al., 1990; Steiner et al., 1991; Yoshida and Gage, 1992). Amongst them, IL-1β and FGFs appear to cause a greater increase in the synthesis of NGF than do the other cytokines. Interestingly, their regional distribution in brain

reveals a high degree of co-localization for IL-1β and NGF (Bandtlow et al., 1990). The same conclusion can be reached with respect to the sites of expression of FGFs and NGF (see Tables I.1 and I.3). Brain macrophages or microglia react to injuries or chronic diseases by proliferating and expressing cytokines; these can be down-regulated by corticosteroids (Ganter et al., 1992). Interleukin-1β induction of NGF mRNA was shown to involve activation of prostaglandin signal transduction, as well as c-fos and c-jun (Friedman et al., 1992; 1990; Hartung et al., 1989). Figure VII.2 shows a simplified mechanism of the possible involvement of injury-induced cytokines in neurotrophin effects. The graphs on the right-side of the diagram are a hypothetical illustration of the activation of a cascade of trophic factors. In summary, following cortical injury an increase in cytokines is proposed (Woodroofe et al., 1991; Ganter et al., 1992) which in turn could induce the release of FGFs from astroglia and neurons (Araujo and Cotman, 1992). According with our observations, these cytokines and FGFs may then cause increased levels of endogenous NGF. However, such an increase in endogenous NGF may not reach the threshold necessary to promote recovery of NBM cholinergic neurons, which may thus become atrophic if left untreated (Sofroniew et al., 1983). The evidence of increased immunoreactivity to NGF in basal forebrain astrocytes, as well as to bFGF in astrocytes consequent to brain injury (Finklestein et al., 1988; Kiyota et al., 1991; Takami et al., 1992), support our findings on the *in vivo* effect of aFGF on the levels of NGF and its mRNA in rat cerebral cortex (Chapter IV). These results are also in agreement with previous studies in developing hippocampus (Spranger et al., 1990a), and *in vitro* (Yoshida and Gage, 1991). In the latter study, it was demonstrated that pharmacological blockade of transcription by cycloheximide and actinomycin completely inhibited the aFGF-induced secretion of NGF by cultured astrocytes and fibroblasts. These observations suggest that FGF regulates NGF synthesis at the level of transcription. It is worth noting that while bFGF has been more widely studied to rescue basal forebrain cholinergic and other neurons, experiments *in vitro* with cultured brain-derived cells have failed to show that bFGF is more efficient than aFGF in inducing synthesis of NGF. It is conceivable that this induction of NGF synthesis is, so far, the only mechanism in favour of an indirect action of FGFs on basal forebrain

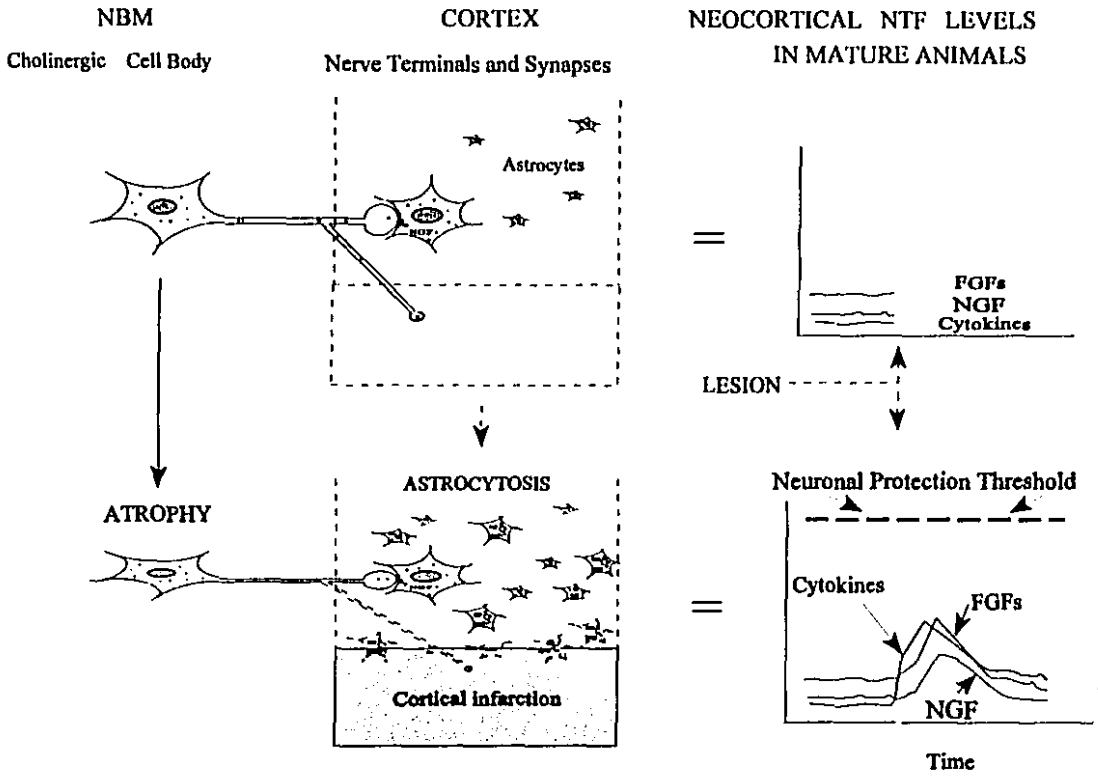


Figure VII.2. Following partial unilateral cortical lesion (devascularization) and cortical infarction, the inflammatory process initiates a cascade of events. The inflammatory cytokines, such as interleukin-1 whose action may be mediated by prostaglandins, c-fos and c-jun (Hartung et al., 1989; Friedman et al., 1990;1992), may increase the synthesis of FGFs, NGF, other neurotrophic factors (NTFs) or neurotransmitter. FGFs synthesised by astrocytes and probably neurons may be released following injury of these cells or by another mechanism. This release of FGF may subsequently initiate another event, such as the induction of NGF synthesis in astrocytes (autocrine effect) or neurons. It is speculated that the final levels of NGF and other neurotrophic factors are insufficient to prevent atrophy of NBM cholinergic neurons, but may be sufficient to activate other events, such as the upregulation of $p75^{NGFR}$ gene expression. Although the mechanisms involved are poorly understood and perhaps not exactly as described in this schematic diagram, the represented here are events were based upon a number of in vitro and in vivo studies (see text).

neurons, as initially proposed (Barde, 1989).

In the experimental decortication model used for this thesis, two portions of the neocortex exhibit dramatic inflammatory reactions after the lesion: one part undergoes a rapid and progressive inflammation which leads to total necrosis beneath the devascularized cortical surface, while the second part, corresponding to the remaining cortex, undergoes a transient inflammatory reaction with dramatic astrocytosis (Herrera and Cuello, 1992). The concentrations of NGF and inflammatory cytokines may increase and decrease very rapidly in the first case, while in the remaining cortex, these events may be prolonged and degeneration may not occur. Thus, different concentrations of NGF, cytokines or other trophic factors, which could exist in these two parts of the cortex could differently alter the phenotype (neuronal cell size, ChAT activity and gene expression) of NBM neurons innervating these two cortical areas. A third possible explanation, reflecting an intermediary situation, may exist: one group of NBM neurons may be partially lesioned because they innervate different cortical regions, infarcted and non-infarcted areas (see illustration in Figure VII.2). Although we were unable to detect differences with respect to the extent of lesion incurred per neuron, our observations on the expression of the low-affinity NGF receptor mRNA in the NBM revealed differences in this expression, sometimes between two closely situated neurons. The data described in all chapters (II-VI) of this thesis were exclusively recorded from mid-NBM neurons, therefore, one would expect that most were either partially or totally affected by the lesion. A combination of immunocytochemistry with different tracers injected into distinct neocortical areas could be employed in future studies to identify neurons which were partially or completely lesioned or which were unlesioned following cortical infarction.

The increased NGF concentration observed in the remaining cortex on the seventh post-lesion day (the only time point investigated) may not be sufficient to rescue cholinergic neurons from atrophy, but it may somehow help "to postpone" the atrophy observed one month after lesion (Chapters II, III, VI). The level of $p75^{NGFR}$ mRNA in the ipsilateral NBM was increased on the third day but returned to normal levels on the seventh post-lesion day (Chapter V). If such changes in $p75^{NGFR}$ mRNA expression is a reflection of NGF cortical levels, it would suggest that on the third post-lesion day, NGF

concentrations in the remaining neocortex may be significantly higher than in sham-operated animals. However, the expression of $p75^{NGFR}$ mRNA observed in the NBM on seventh post-lesion day was not statistically different from sham-operated animals. This finding does not correlate with the increased concentration of NGF noted in the remaining cortex at that time. This would suggest that either the NGF levels achieved are insufficient to induce $p75^{NGFR}$, or that neurons in the mid-NBM are not fully stimulated by the NGF concentrations present in the remaining cortex. Decreased levels of $p75^{NGFR}$ mRNA noted in cholinergic NBM neurons (15 days after lesion) of vehicle-treated animals may be a consequence of reduced lesion-induced access of these neurons to target-derived NGF. This is in agreement with the decrease in ChAT activity, atrophy, and reduced density of neuritic processes in the NBM occurring one month after cortical lesion. In our studies the NBM cells of animals treated with exogenous NGF showed an increased expression of $p75^{NGFR}$, confirming previous results using comparable protocols (Cavicchioli et al., 1989; Gage et al., 1989; Higgins et al., 1989; Holtzman et al., 1992; Verge et al., 1992). One week after interruption of NGF treatment, the levels of $p75^{NGFR}$ mRNA in the NBM were not statistically different from sham-operated animals, but were statistically higher than in vehicle-treated animals.

The studies described in this thesis revealed that the expression of GAP-43 mRNA in the CNS was related to the lesion status of neurons rather than to the degree of NGF stimulation. The profile of GAP-43 mRNA expression in two NGF-insensitive neurons, cortical pyramidal cells and thalamic neurons, were compared with the observed pattern of GAP-43 mRNA in NGF-responsive NBM cholinergic neurons. Partial unilateral infarction of the cerebral cortex elicited a transient and statistically significant increase in GAP-43 mRNA in NBM cholinergic neurons. When the cortical lesion was combined with NGF treatment, no statistically significant changes occurred in the levels of GAP-43 mRNA when compared with lesioned vehicle treated animals. However, it is possible that in neurons where NGF may play an important role during development or regeneration, such as basal forebrain cholinergic neurons, NGF might affect GAP-43 activity via a mechanism independent of transcription (e.g., phosphorylation; see below). The effect of bFGF on the expression of GAP-43 was not investigated for this thesis. However,

recent *in vitro* experiments show that bFGF increases the level of GAP-43 mRNA in glial progenitor cells (Deloulme et al., 1993). Given that with present methodology FGF receptors are undetectable in the NBM of adult animals, the possibility that aFGF and/or bFGF can directly increase GAP-43 levels in NBM cholinergic cells seems very remote.

The fact that NGF can influence post-translational regulation of GAP-43 has become increasingly evident (see Fig. I.3 in Chapter I). One mechanism by which NGF can modulate GAP-43 is via phosphorylation. Perhaps the best documented evidence of this was work showing that NGF activates protein kinase C (PKC) which can phosphorylate GAP-43 (Meiri and Burdick, 1991). However, since a large number of other substances can similarly activate PKC, the issue of specificity with respect to involvement of PKC in regeneration mechanisms remains unclear. Neuronal differentiation has also been shown to be influenced by other second messenger systems involving *ras*, whose activation (formation of a *ras*.GTP complex) by NGF in PC12 cells (Nakafuku et al., 1992) or in embryonic sensory neurons (Ng and Shooter, 1993), seems to be regulated by tyrosine kinases rather than by PKC. Since PKC appears to require diacylglycerol and Ca^{2+} to phosphorylate GAP-43 (Aloyo et al., 1983; 1988; Alexander et al., 1987; Apel et al., 1990) this implies that NGF effects on PKC is mediated through these two activators. The functional role of a PKC-dependent phosphorylation site (serine^{41}) on GAP-43 is unclear, but it seems to facilitate binding of GAP-43 to the membrane, a step which could lead to the generation of new membranes, filopodial formation and/or cell spreading (Van Hooff et al., 1989; Strittmatter et al., 1994) (see Fig. I.3 in Chapter I). The finding that dephosphorylated GAP-43 can be bound to calmodulin as long as intracellular free Ca^{2+} remains at very low levels (Rasmussen, 1983; Carafoli, 1987), indicates that Ca^{2+} also plays an important role in the post-translational regulation of GAP-43. Incoming Ca^{2+} can bind calmodulin leaving GAP-43 free to be phosphorylated. These dynamic changes occurring on the N-terminal phosphorylation site of GAP-43 appear to be totally independent of the mechanisms which control the second site of its phosphorylation on serine^{145} or serine^{192} [for review see (Coggins and Zwiers, 1991)].

Pyramidal neurons exhibited a strong, rapid, and prolonged increase of GAP-43 mRNA as a consequence of the ischemic cortical lesion which indicated a possible role for GAP-43 in these cells. These neurons are the most abundant neurons in the cerebral cortex. The cells showing a statistically significant increase in GAP-43 mRNA were located very close to the lesion site. In order to understand the possible types of lesions in pyramidal cells which led to increases in GAP-43 mRNA, it is necessary to review the anatomic characteristics of these cells. Although the name of this cell is derived from its shape, a large body of anatomical data has shown that these pyramidal cells are not only heterogeneous in their morphology and projection sites, but also in their physiological and chemical characteristics [for review see (Thomson and Deuchars, 1994)]. Pyramidal cells convey processed information leaving the cortex via their long axons to reach other cortical or subcortical areas. These neurons exhibit a complex dendritic arborization which consists of (1) basal dendrites close to the cell body, (2) apical dendrites directed towards more superficial cortical layers and (3) terminal dendritic tulft directed to the cortical surface, and the descending axons which emit axonal collaterals. In the devascularization lesion model, we did not characterize which part of the remaining pyramidal neurons were lesioned. Further tracing studies would be necessary for such characterization. We speculate that the axonal collaterals and/or the descending axons are most likely affected by the adjacent infarcted cortical region.

Thalamocortical connections, which relay information from specific sensory thalamic nuclei to temporal, parietal, and occipital cortices, where the bulk of thalamocortical fibers terminate preferentially in layer IV (Jacobson and Trojanowski, 1975), are largely affected by manipulation of these cortical regions. Cell death of thalamic neurons, as a consequence of cortical ablation and retrograde degeneration (Matthews, 1973), also occurred rapidly following cortical devascularization. This cell death was progressive and by the seventh day after cortical lesion, a variable degree of cell atrophy as well as the presence of pycnotic nuclei in lateral, posterior, and ventroposterior thalamic nuclei were noted. On the first and third post-lesion days, some neurons revealed increased levels of GAP-43 mRNA. However, this increase was not statistically significant when mean grain counts were considered for all neurons in the

thalamus and indicated that a more intense and statistically significant increase of GAP-43 mRNA levels might occur between the first and the third post-lesion days. It could be inferred from these observations that thalamic neurons exhibit a tendency to increase GAP-43 mRNA, for short periods, at an intermediary time-point between the fast upregulation occurring in pyramidal neurons and the more retarded increase occurring in NBM neurons on the third post-lesion day. Fig. VII.3 illustrates the time-courses of the varying GAP-43 mRNA levels exhibited by these different neuronal populations (NBM, thalamic, pyramidal) which probably arise as a function of the distance between the neuronal somata to the lesion site. This is in line with what was described for other CNS neurons with respect to GAP-43 mRNA expression in response to injury (Doster et al., 1991; Tetzlaff et al., 1991; Tetzlaff and Giehl, 1991). It is speculated that the noted differences in duration of GAP-43 mRNA expression may be a consequence of multiple factors such as the extent of the lesion affecting each group of neurons, the number of remaining synaptic contacts, the intrinsic cellular properties linked to degeneration and neuronal repair, as well as the influence of external factors such as inflammatory cytokines (Lu and Richardson, 1991; 1993). Further studies are necessary to elucidate the physiological significance of this differential regulation of GAP-43 mRNA levels in these three neuronal populations. Much evidence exists which indicates that GAP-43 plays an important role in neuronal regeneration [for review see (Skene, 1989; Jap Tjoen San et al., 1992; Aigner and Caroni, 1993; Shea et al., 1991)]. NGF treatment, however, did not affect the expression of GAP-43 mRNA in any of these three neuronal populations, especially not in NGF-responsive NBM neurons. A distinct pattern of GAP-43 mRNA expression (such as in amplitude or duration) which could suggest its association with atrophy or regeneration of NBM neurons, was not detected when untreated animals were compared with those which received NGF. However, as explained above, this neurotrophin may play an important role in the regenerative processes by stimulating the phosphorylation of the N-terminal site of GAP-43, via activation of protein kinase C and/or other second messengers (see diagram in Fig. I.3).

A concerted action of thyroid hormones and neurotrophic factors appears to be important for setting appropriate conditions for neuronal growth during development. Our

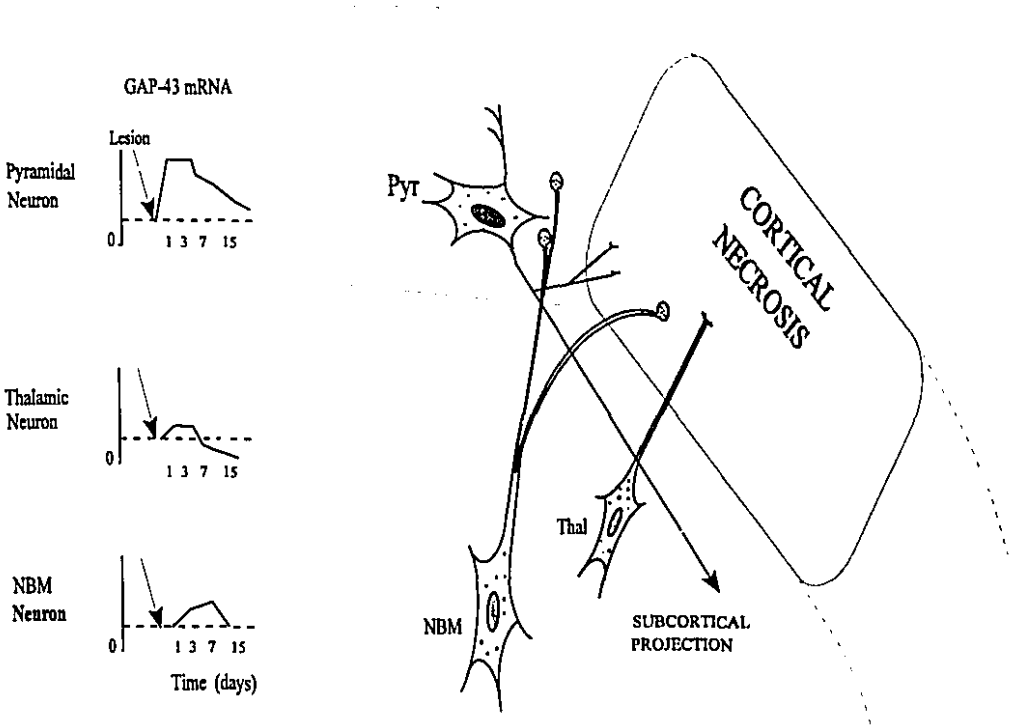


Figure VII.3. An interpretation for the differential regulation of GAP-43 mRNA after unilateral partial cortical devascularization. The time-courses and amplitudes of GAP-43 mRNA shown on the left side of this schematic diagram correspond approximately to the measured number of grains (pixels) per cell area after several post-lesion days. It can be hypothesized that the distance between the lesion site and the cell somata, the extention of the lesion affecting each neuron, the number of remaining synaptic contacts and other cellular and extracellular factors (inflammatory cytokines) influence the expression of GAP-43 mRNA. Pyramidal cells are located less than 1 mm from the lesion site but it is not clear which part of the neuron was more likely lesioned. In this diagram a lesion of the axonal collaterals is shown, however, pyramidal basal dendrites and the projecting subcortical fibers may also be affected. The observation that the total number of thalamic neurons in the lateral, posterior and ventroposterior nuclei, ipsilateral to the lesion were reduced, and that a large number of remaining neurons were atrophic on post-lesion day 7, indicated that a large number of neurons were undergoing a process of cell death. This situation may explain, in part, the decrease in GAP-43 mRNA levels noted since the third post-lesion day. Atrophy and presumably cell death were noted to a much lesser extent in pyramidal neurons as compared with thalamic neurons. Abbreviations: Pyr, pyramidal neuron; Thal, thalamic neuron; NBM, nucleus basalis magnocellularis.

understanding of thyroid hormone effects in the developing brain has been advanced by recent developments in the characterization and distribution of thyroid hormone receptors in brain. The apparent determining factor for such a dramatic change, from an absolute thyroid hormone dependency controlling brain development to an almost global lack of effect of thyroid hormone imbalance in adult animals, seems to be drastic decrease with age of thyroid hormone receptors in the brain (Rodriguez-Peña et al., 1993; Mellström et al., 1991; Yusta et al., 1988; Hubank et al., 1990).

With respect to the main thrust of this work, our findings on hypothyroidism-induced atrophy of NBM cholinergic neurons in mature animals, after an interval of normal levels of thyroid hormones (posthypothyroid animals) revealed: (1) unaltered expression of low- and high-affinity NGF receptor mRNAs, (2) no effect of unilateral cortical devascularization on the size of NBM cholinergic neurons treated with NGF, and (3) the failure of NGF in restoring the size of NBM cholinergic neurons to that of mature euthyroid animals.

The levels of $p75^{NGFR}$ and $p140^{trk}$ mRNAs, expressed as the number of grains (pixels) per cell area, were not statistically different between posthypothyroid and mature euthyroid animals. An oversimplified way of presenting these results would be to express the number of grains (pixels) per cell, disregarding the cell area. Use of this approach would have produced opposite data and incorrect interpretation of the results between posthypothyroid and euthyroid rats. The invariably serious neurological consequences of thyroid hormone deprivation during brain development of children led to the preconceived view that the consequences of thyroid deprivation during development are unaltered with increasing age. Although the mental retardation exhibited by most patients would support this assumption, experimental studies conducted at the cellular level have shown that several changes observed in thyroid hormone deprived developing animals are not similarly seen in adult animals. Some of these differences are illustrated in our studies. For instance, in the developing cerebellum of hypo- and hyperthyroid pups, the mRNA levels of the low-affinity NGF receptor, GAP-43, Tα1 α-tubulin, and two myelin specific proteins (proteolipid protein and myelin basic protein) [(Figueiredo et al., 1993a), Appendix A] were altered in the first two weeks after birth, but these

differences gradually disappeared. Although protein levels of low- and high-affinity NGF receptors in the NBM of these animals were not determined, it could still be inferred that whatever these differences may be, if any, between levels in posthypothyroid versus normal animals, this did not prevent NGF from protecting NBM cholinergic neurons. In a parallel study of these animals with cortical lesion which received NGF-treatment for seven days, the final mean cell size of these NBM neurons of posthypothyroid animals, ipsi- and contralateral to the lesion, remained statistically different from euthyroid animals. This persistent atrophy of NBM neurons in decorticated rats treated with NGF between days 15 and 22, subsequent to 45 days of thyroid hormone replacement therapy, is in agreement with the proposal that susceptibility to thyroid hormone manipulations may involve sensitivity to NGF, especially in basal forebrain neurons (Kalaria and Prince, 1985; Patel et al., 1987; Oh et al., 1991). However, other neuronal abnormalities, perhaps in proximal axons, as described for Purkinje cells of the cerebellum (Appendix A) could also play a role. Although Purkinje cells in neonatal rats express high levels of low-affinity NGF receptor, it seems that these neurons are particularly sensitive to neurotrophin-3, which was shown to be regulated by thyroid hormones (Lindholm et al., 1993). Moreover, we were unable to detect $p140^{trk}$ mRNA in the cerebellum of adult animals using *in situ* hybridization techniques (unpublished results). Whether abnormalities in Purkinje cells of hypothyroid pups correlated with decreases in NGF concentration in cerebellum [(Figueiredo et al., 1993b), Appendix B], as suggested by other studies (Walker et al., 1979; Gibbs et al., 1992) was also investigated. We observed that hypothyroidism did not affect the levels of NGF, but that thyroxine induced increases in NGF concentration, only on postnatal day two. Furthermore, we did not find statistically significant changes in the levels of NGF and its mRNA in hippocampus and cortex, nor in NGF mRNA of the developing cerebellum of young and adult rats with hypo- or hyperthyroidism (unpublished results). However, it is possible that the effects of thyroxine on NGF levels observed in the cerebellum on postnatal day two is due to an indirect effect on the differentiation of astroglia (Aizenman and Vellis, 1987). Whether, thyroid hormone deficiency affects astroglia resulting in abnormal development of basal forebrain neurons has yet to be tested. However, NGF

effects in basal forebrain cholinergic neurons *in vitro* were shown to be enhanced by astroglia (Hartikka and Hefti, 1988). It remains controversial whether thyroxine-induced increases in NGF levels also occurs in the brain of adult rats, or if this effect is restricted only to mice.

Atrophied NBM cholinergic neurons caused by hypothyroidism were previously noted in younger rats (50 days old), and have been described as a feature of aging in several species and genotypes, including humans with normal neuropathology or Alzheimer's disease (AD) [for a review see (Finch, 1993)]. Abnormalities in cholinergic neurons arising as a consequence of thyroid hormone therapy has been proposed as a risk factor for the development of senile dementia of the AD type (Butcher and Woolf, 1989; Woolf and Butcher, 1990). The somewhat similar neurological symptoms displayed by hypothyroid patients and those with AD has stimulated interesting investigations. One study demonstrated that subclinical hypothyroidism associated with autoimmune thyroiditis is more commonly found in patients with Down Syndrome or AD (Percy et al., 1990). Decreased thyroid hormone receptor (c-erb A alpha) mRNA levels were noted in patients with AD but not in Huntington controls (Sutherland et al., 1992) which would support the idea that normal levels of thyroid hormone would reduce the risk of AD (Lejeune, 1991).

SUMMARY

A survey of putative neurotrophic factors for basal forebrain cholinergic neurons, closely related to two factors of recognized therapeutical properties (i.e., either NGF or bFGF), led us to test three human recombinant peptides: BDNF, NT-3 and aFGF. These growth factors have three-dimensional molecular structures similar to either NGF or bFGF and, therefore, could have related biological functions. An essential step in understanding the function of these trophic factors for NBM cholinergic neurons was provided by preliminary results demonstrating their ability to partially or completely prevent atrophy of these neurons. Our data indicated that besides NGF, only aFGF was likely to play a protective role for these neurons. Further experiments with FGF revealed other biological

features, such as maintenance of ChAT activity in the lesioned NBM, or increase of this activity in a mixed-septal cell culture, promotion of glial cell growth *in vitro*, as well as improvement in rodent memory deficits. Acidic FGF elicited increases in the levels of NGF and its mRNA in the neocortex of adult animals, suggesting that this effect is currently the only explanation for its indirect trophic action on NBM neurons. As NGF came into play as the endogenous mediator of aFGF, our next series of experiments provided new insights into the regulatory mechanisms involving this neurotrophin. During NGF treatment, low-affinity NGF receptor gene expression was upregulated, and after NGF withdrawal $p75^{NGFR}$ mRNA levels did not decrease in lesioned NBM neurons. These observations are in line with the absence of NBM cholinergic neuronal atrophy resulting one month after lesion. By contrast, NGF treatment did not affect expression of the GAP-43 gene, however, cortical infarction induced a differential regulation of this gene in three neuronal populations. On the seventh post-lesion day, GAP-43 mRNA levels were elevated in the NBM, decreased in thalamic neurons, which were undergoing a process of cell death, and, moderately elevated in pyramidal neurons. Posthypothyroid rats, an equivalent animal model of the human cretinism, exhibited atrophy of NBM neurons at all ages examined, but displayed normal levels of low- and high-affinity NGF receptors in these neurons, and responded to NGF treatment by failing to further atrophy after neocortical infarction. It is worth considering that these data and the results obtained by others (as discussed in this thesis) would support the possible application of NGF/aFGF in clinical trials aimed to prevent atrophy of cholinergic neurons following brain injury (e.g. stroke).

REFERENCES

Aguilar LC, Islas A, Rosique P, Hernandez B, Portillo E, Herrera JM, Cortes R, Cruz S, Alfaro F, Martin R, Cantu JM (1993) Psychometric analysis in children with mental retardation due to perinatal hypoxia treated with fibroblast growth factor (FGF) and showing improvement in mental development. J Intel Disab Res 37:507-520.

Aigner L, Caroni P (1993) Depletion of 43-kD growth-associated protein in primary neurons leads to diminished formation and spreading of growth cones. J Cell Biol 123:417-429.

Aigner TG, Walker DL, Mishkin M (1991) Comparison of the effects of scopolamine administered before and after acquisition in a test of visual recognition memory in monkeys. Behav Neural Biol 55:61-67.

Aizenman Y, Vellis J (1987) Synergistic action of thyroid hormone, insulin and hydrocortisone on astrocyte differentiation. Brain Res 414:301-308.

Alderson RF, Alterman AL, Barde Y-A, Lindsay RM (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron 5:297-306.

Alexander KA, Cimler BM, Meier KE, Storm DR (1987) Regulation of calmodulin binding to P-57. J Biol Chem 262:6108-6113.

Aloyo VJ, Zwiers H, Gispen WH (1983) Phosphorilation of B-50 protein by calcium-activated phospholipid dependent protein kinase and B-50 protein kinase. J Neurochem 41:649-653.

Aloyo VJ, Zwiers H, De Graan PNE, Gispen WH (1988) Phosphorilation of the neuronal protein kinase C substrate B-50: in vitro assay conditions alter sensitivity to ACTH. Neurochem Res 13:343-348.

Altar CA, Boylan CB, Jackson C, Hershenson S, Miller J, Wiegand SJ, Lindsay RM, Hyman C (1992) Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover *in vivo*. Proc Natl Acad Sci USA 89:11347-11351.

Altar CA, Criden MR, Lindsay RM, DiStefano PS (1993) Characterization and topography of high-affinity ^{125}I-neurotrophin-3 binding to mammalian brain. J Neurosci 13:733-743.

Anderson KJ, Dam D, Lee S, Cotman CW (1988) Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332:360-362.

Apel ED, Byford MF, Au D, Walsh KA, Storm DR (1990) Identification of the protein kinase C phosphorilation site in neuromodulin. Biochemistry 29:2330-2335.

Araujo DM, Cotman CW (1992) Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. J Neurosci 12:1668-16678.

Bakhit C, Armanini M, Bennett GL, Wong W-LT, Hansen SE, Taylor R (1991) Increase in glial-derived nerve growth factor following destruction of hippocampal neurons. Brain Res 560:76-83.

Bandtlow CE, Meyer M, Lindholm D, Spranger H, Heumann R, Thoenen H (1990) Regional and cellular codistribution of Interleukin 1β and nerve growth factor mRNA in the adult rat brain: Possible relationship to the regulation of nerve growth factor synthesis. J Cell Biol 111:1701-1711.

Banks WA, Kastin AJ, Horvath A, Machals EA (1987) Carrier-mediated transport of vasopressin across the blood-brain barrier of the mouse. J Neurosci Res 18:326-332.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Bartus RT, Dean RL, Pontecorvo MJ, Flicker C (1985) Integration of animal and human reserch from clinical to preclinical perspectives. In: Memory dysfunctions (Olton D, Gamzu E, Corkin S eds), pp 332-358. New York: New York Academy of Sciences.

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115-165.

Blitzer RD, Gil O, Landau EM (1990) Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. Neurosci Letts 119:207-210.

Burgard EC, Sarvey JM (1990) Muscarinic receptor activation facilitates the induction of long-term potentiation (LTP) in the rat dentate gyrus. Neurosci Letts 116:34-39.

Butcher LL, Woolf NJ (1989) Neurotrophic agents may exacerbate the pathologic cascade of Alzheimer's disease. Neurobiol Aging 10:557-570.

Carafoli E (1987) Intracellular calcium homeostasis. Ann Rev Biochem 4869:139-38.

Cavicchioli L, Flanigan TP, Vantini G, Fusco M, Polato P, Toffano G, Walsh FS, Leon A (1989) NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. Eur J Neurosci 1:258-262.

Coggins PJ, Zwiers H (1991) B-50 (GAP-43): Biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. J Neurochem 56:1095-1106.

Cuello AC, Garofalo L, Kenigsberg RL, Maysinger D (1989) Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons. Proc Natl Acad Sci USA 86:2056-2060.

Cuello AC, Sofroniew MV (1984) The anatomy of CNS cholinergic neurons. Trends Neurosci 7:74-78.

Damon DH, Lobb RR, D'Amore PA, Wagner JA (1993) Heparin potentiates the action of acidic fibroblast growth factor by prolonging its biological half-life. J Cell Physiol

Dekker AJ, Fagan AM, Gage FH, Thal LJ (1994) Effects of brain-derived neurotrophic factor and nerve growth factor on remaining neurons in the lesioned nucleus basalis magnocellularis. Brain Res 639:149-155.

Deloulme JC, Laenge P, Janet T, Sesenbrenner M, Baudier J (1993) Expression of neuromodulin (GAP-43) and its regulation by basic fibroblast growth factor during the differentiation of 0-2A progenitor OFF1s. J Neurosci Res 36:147-162.

Doster SK, Lozano AM, Aguayo AJ, Willard MB (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6:635-647.

Dunnet SB, Fibiger HC (1993) Role of forebrain cholinergic systems in learning and memory: relevance to the cognitive deficits of aging and Alzheimer's dementia. Prog Brain Res 98:413-420.

Elliott PJ, Garofalo L, Cuello AC (1989) Limited neocortical devascularizing lesions causing deficits in memory retention and choline acetyltransferase activity--effects of the monosialoganglioside GM1. Neuroscience 31:63-76.

Fibiger HC (1991) Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. Trends Neurosci 14:220-223.

Figueiredo BC, Maysinger D, Piccardo P, Clarke PBS, Cuello AC (1992) Effects of acidic fibroblast growth factor in cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Cholinergic Neurotransmission: Function and Disfunction 51(Abstract)

Figueiredo BC, Almazan G, Ma Y, Tetzlaff W, Miller FD, Cuello AC (1993a) Gene expression in the developing cerebellum during perinatal hypo- and hyperthyroidism. Mol Brain Res 17:258-268.

Figueiredo BC, Otten U, Strauss S, Volk B, Maysinger D (1993b) Effects of perinatal hypo- and hyperthyroidism on the levels of nerve growth factor and its low-affinity receptor in cerebellum. Dev Brain Res 72:237-244.

Figueiredo BC, Piccardo P, Maysinger D, Clarke PBS, Cuello AC (1993c) Effects of acidic fibroblast growth factor on cholinergic neurons of nucleus basalis magnocellularis and in a spatial memory task following cortical devascularization. Neuroscience 56:955-963.

Finch CE (1993) Neuron atrophy during aging: programmed or sporadic ?. Trends Neurosci 16 (3):104-110.

Finklestein SP, Apostolides PJ, Caday CG, Prosser J, Philips MF, Klagsbrun M (1988) Increased basic fibroblast growth factor receptor (bFGF) immunoreactivity at the site of local brain wounds.

Brain Res 460:253-259.

Fischer W, Wictorin K, Björklund A, Williams L, Varon S, Gage FH (1987) Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329:65-68.

Fischer W, Björklund A, Chen K, Gage FH (1991) NGF improves spatial memory in aged rodents as a function of age. J Neurosci 11:1889-1906.

Fishman JB, Rubin JB, Handrahan JV, Connor JR, Fine RE (1987) Receptor-mediated transcytosis of transferrin across the blood-brain barrier. J Neurosci Res 18:299-304.

Friedman WJ, Larkfors L, Ayer-LeLievre C, Ebendal T, Olson L, Persson H (1990) Regulation of beta-nerve growth factor expression by inflammatory mediators in hippocampal cultures. J Neurosci Res 27:374-382.

Friedman WJ, Altiok N, Fredholwm BB (1992) Mechanism of nerve growth factor mRNA regulation by interleukin-1β in hippocampal cultures: Role of second messengers. J Neurosci Res 33:37-46.

Gage FH, Batchelor P, Chen KS, Chin D, Higgins GA, Koh S, Deputy S, Rosenberg MB, Fischer W, Björklund A (1989) NGF receptor reexpression and NGF-mediated cholinergic neuronal hypertrophy in the damaged adult neostriatum. Neuron 2:1177-1184.

Ganter S, Northoff H, Mannel D, Gebicke-Harter PJ (1992) Growth control of cultured microglia. J Neurosci Res 33:218-230.

Garofalo L, Cuello AC (1990) Nerve growth factor and the monosialoganglioside GM1 modulate cholinergic markers and affect behavior of decorticated adult rats. Eur J Pharmacol 183:934-935.

Garofalo L, Cuello AC (1994a) Nerve growth factor and the monosialoganglioside GM1: Analogous and different in vivo effects on biochemical, morphological and behavioral parameters of adult cortically leisoned rats. Exp Neurol 125:195-217.

Garofalo L, Cuello AC (1994b) Pharmacological characterization of nerve growth factor and/or monosialoganglioside GM1 effects on cholinergic markers in the adult lesioned brain. (submitted)

Gasser UE, Weskamp G, Otten U, Dravid AR (1986) Time course of the elevation of nerve growth factor (NGF) content in the hippocampus and septum following lesions of the septohippocampal pathways in rats. Brain Res 376:351-356.

German DC, White CL, III, Sparkman DR (1987) Alzheimer's disease: neurofibrillary tangles in nuclei that project to the cerebral cortex. Neuroscience 21:305-312.

Ghoneim MM, Mewaldt SP (1975) Effects of diazepam and scopolamine on storage, retrieval and organizational processes in memory. Psychopharmacologia 44:257-262.

Gibbs RB, Chao MV, Pfaff DW (1992) Effects of fimbria fornix and angular bundle transection on expression of the p75NGFR mRNA by cells in the medial septum and diagonal band of broca: correlations with cell survival, synaptic reorganization and sprouting. Mol Brain Res 11:207-219.

Gospodarowicz D, Cheng J (1986) Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475-484.

Hanai K, Oomura Y, Kay Y, Nishikawa K, Shimizu N, Morita H, Plata-Salaman CR (1989) Central action of acidic fibroblast growth factor in feeding regulation. Am J Physiol 256:R217-R223.

Haroutunian V, Kanof PD, Davis KL (1989) Attenuation of nucleus basalis of Meynert lesion induced cholinergic deficits by nerve growth factor. Brain Res 487:200-203.

Hartikka J, Hefti F (1988) Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. J Neurosci 8(8):2976-2985.

Hartung HP, Schafer B, Heininger K, Toyka KV (1989) Recombinant interleukin-1 beta stimulates eicosanoid production in rat primary culture astrocytes. Brain Res 489:113-119.

Hasselmo ME, Bower JM (1993) Acetylcholine and memory. Trends Neurosci 16 (6):218-222.

Herrera DG, Cuello AC (1992) Glial fibrillary acidic protein immunoreactivity following cortical devascularizing lesion. Neuroscience 49:781-791.

Higgins GA, Koh S, Chen KS, Gage FH (1989) NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. Neuron 3:247-256.

Hisajima H, Saito H, Abe K, Nishiyama N (1992) Effects of acidic fibroblast growth factor on hippocampal long-term potentiation in fasted rats. J Neurosci Res 31:549-553.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen C-K, Valletta JS, Zhou J, Long JB, Mobley WC (1992) p140^{trk} mRNA marks NGF-responsive forebrain neurons: Evidence that *trk* gene expression is induced by NGF. Neuron 9:465-478.

Hubank M, Sinha AK, Gullo D, Ekins RP (1990) Nuclear triiodothyronine (T3) binding in neonatal rat brain suggests a direct glial requirement for T3 during development. J Endocrinol 126:409-415.

Ishihara A, Saito H, Nishiyama N (1992) Basic fibroblast growth factor ameliorates learning deficits in basal forebrain-lesioned mice. Japanese J Pharmacol 59:7-13.

Jacobson S, Trojanowski JQ (1975) Corticothalamic neurons and thalamocortical terminal fields: an investigation in rat using horseradish peroxidase and autoradiography. Brain Res 85:385-401.

Jap Tjoen San ERA, Schmidt-Michels M, Oestreicher A, Gispen WH, Schotman P (1992) Inhibition of nerve growth factor-induced B-50/GAP-43 expression by antisense oligomers interferes with neurite outgrowth of PC12 cells. Biochem Biophys Res Commun 187:839-846.

Kalaria RN, Prince AK (1985) Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neurons markers and DNA concentrations in the rat corpus striatum. Int J Dev Neurosci 3:655-666.

Kiyota Y, Takami K, Iwane M, Shino A, Miyamoto M, Tsukuda R, Nagaoka A (1991) Increase in basic fibroblast growth factor-like immunoreactivity in rat brain after forebrain ischemia. Brain Res 545:322-328.

Klein R, Conway D, Parada LF, Barbacid M (1990) The trkB tyrosine kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-656.

Knüsel B, Beck KD, Winslow JW, Rosenthal A, Burton LE, Widmer HR, Nickolics K, Hefti F (1992) Brain-derived neurotrophic factor administration protects basal forebrain cholinergic but not nigral dopaminergic neurons from degnerative changes after axotomy in the adult rat brain. J Neurosci 12(11):4391-4402.

Korsching S, Heumann S, Thoenen H, Hefti F (1986) Cholinergic denervation of the hippocampus by fimbria lesion leads to a transient accumulation of NGF without change in NGF mRNA content. Neurosci Lett 66:175-180.

Lejeune J (1991) Pathogenie de la debilite de l'intelligence dans la trisomy 21. Annales de Genetique 34:55-64.

Lindholm D, Heumann R, Meyer M, Thoenen H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. Nature 330:658-659.

Lindholm D, Heumann R, Hengerer B, Thoenen H (1988) Interleukin 1 increases stability and transcription of mRNA encoding nerve growth factor in cultured rat fibroblasts. J Biol Chem 263:16348-16351.

Lindholm D, Castrén E, Tsoulfas P, Kolbel R, Berzaghi MP, Leingärtner A, Heisenberg CP, Tesarollo L, Parada LP, Thoenen H (1993) Neurotrophin-3 induced by tri-iodothyronine in cerebellar granule cells promotes Purkinje cell differendiation. J Cell Biol 122:443-450.

Lu X, Richardson PM (1991) Inflamation near the nerve cell body enhances axonal regeneration. J Neurosci 11:972-978.

Lu X, Richardson PM (1993) Induction by macrophages of regeneration associated molecules in sensory neurons. Soc Neurosci Abstr 19:451

Matthews MA (1973) Death of the central neuron: an electron microscopic study of thalamic retrograde degeneration following cortical ablation. J Neurocytol 2:265-288.

McCormick DA, Prince DA (1986) Mechanisms of action of acetylcholine in the guinea-pig cerebral cortex *in vitro*. J Physiol 375:169-194.

Meiri KF, Burdick D (1991) Nerve growth factor stimulation of GAP-43 phosphorylation of intact isolated growth cones. J Neurosci 11:3155-3164.

Mellström B, Naranjo JR, Santos A, Gonzalez AM, Bernal J (1991) Independent expression of the alpha and beta c-erbA genes in developing rat brain. Mol Endocr 5:1339-1350.

Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, Miller J, DiStefano PS, Altar CA, Lindsay RM, Alderson RF (1993) Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J Neurosci 13:4146-4156.

Müller SN, Thomas KA, DiSalvo J, Levine EM (1989) Stabilization by heparin of acidic fibroblast growth factor mitogenicity for human endothelial cells *in vitro*. J Cell Physiol 140:439-448.

Nakafuku M, Satoh T, Kaziro Y (1992) Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active Ras.GTP complex in rat pheochromocytoma PC12 cells. J Biol Chem 267:19448-19454.

Ng NF, Shooter ES (1993) Activation of p21ras by nerve growth factor in embryonic sensory neurons and PC12 cells. J Biol Chem 268:25329-25333.

Oderfeld-Nowak B, Bacia A, Gradrowska M, Fusco M, Vantini G, Leon A, Aloe L (1992) In vivo activated brain astrocytes may produce and secrete nerve growth factor-like molecules. Neurochem Int 21:455-461.

Oh JD, Butcher LL, Woolf NJ (1991) Thyroid hormone modulates the development of cholinergic terminal fields in the rat forebrain: Relation to nerve growth factor receptor. Dev Brain Res 53:133-142.

Ono T, Saito H, Kishimoto T, Okumoto T, Miyamoto K (1991) Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes. Neurosci Lett 126:18-20.

Oomura Y, Sasaki K, Suzuki K, Muto T, Li AJ, Ogita Z, Hanai K, Tooyama I, Kimura H, Yanaihara N (1992) A new brain glucosensor and its physiological significance. Amer J Clin Res 55:278S-282S.

Oomura Y, Sasaki K, Li AJ (1993) Memory facilitation induced by food intake. Physiol Behav 54:493-498.

Patel AJ, Hayaschi M, Hunt A (1987) Selective persistent reduction in choline acetyltransferase activity in basal forebrain of the rat after thyroid deficiency during early life. Brain Res 422:182-185.

Percy ME, Dalton AJ, Markovic VD, Crapper McLachlan DR, Gera GE, Hummel JT, Rusk AC, Somerville MJ, Andrews DF, Walfish PG (1990) Autoimmune thyroiditis associated with mild "subclinical"hypothyroidism in adult with Down syndrome: a comparison of patients with and without manifestation of Alzheimer's disease. Am J Med Genetics 36:148-154.

Pitler TA, Alger BE (1992) Cholinergic excitation of GABAergic interneurons in the rat hippocampal slice. J Physiol 450:127-141.

Price DL (1986) New perspectives on Alzheimer's disease. Annu Rev Neurosci 9:489-512.

Rasmussen H (1983) Cellular calcium metabolism. Ann Intern Med 98:809-816.

Rodriguez-Peña A, Ibarrola N, Iñiguez MA, Muñoz A, Bernal J (1993) Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. J Clin Invest 91:812-818.

Rosengart TK, Johnson WV, Friesel R, Clark R, Maciag T (1988) Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. Biochem Biophys Res Commun 152:432-440.

Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fidder JC, Gospodarowicz D (1993) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325:257-259.

Shea TB, Perrone-Bizzore ML, Benowitz LI (1991) Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neritogenesis. J Neurosci 11:1685-1690.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Skup M, Figueiredo BC, Albert P, Cuello AC (1993) BDNF and NT-3 do not prevent cholinergic neurons of the nucleus basalis magnocellularis from atrophy caused by cortical devascularization. Soc Neurosci Abstr 19:657

Skup M, Figueiredo BC, Cuello AC (1994) Intraventricularly applied BDNF and NT-3 failed to protect NBM cholinergic neurons. Neuroreport (in press) 5(8):

Sofroniew MV, Pearson RC, Eckenstein F, Cuello AC, Powell TP (1983) Retrograde changes in cholinergic neurons in the basal forebrain of the rat following cortical damage. Brain Res 289:370-374.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Nahernoe M, Thoenen H (1990a) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system - comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and in vivo. Eur J Neurosci 2:69-76.

Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Näher-Noé M, Thoenen H (1990b) Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: Comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and *in vivo*. Eur J Neurosci 2:69-76.

Steiner P, Pfeilschifter J, Boeckh C, Radeke H, Otten U (1991) Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor synthesis in rat mesangial cells. Am J Physiol 261(5):F792-F798.

Stock A, Kuzis K, Woodward WR, Nishi R, Eckenstein FP (1992) Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12:4688-4700.

Strittmatter SM, Valenzuela D, Fishman MC (1994) An amino-terminal domain of the growth-associated protein GAP-43 mediates its effects on filopodial formation and cell spreading. J Cell Sci 107:195-204.

Sutherland MK, Wong L, Somerville MJ, Handley P, Yoong L, Bergeron C, McLachlan DR (1992) Reduction of thyroid receptor c-erb A alpha mRNA levels in the hippocampus of Alzheimer as compared to Huntington brain. Neurobiol Aging 13:301-312.

Takami K, Iwane M, Kiyota Y, Miyamoto M, Tsukuda R, Shiosaka S (1992) Increase of basic fibroblast growth factor immunoreactivity and its mRNA level in rat brain following transient forebrain ischemia. Exp Brain Res 90:1-10.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11:2528-2544.

Tetzlaff W, Giehl KMG (1991) Axotomized corticospinal neurons increase GAP-43 mRNA after sub-cortical lesions but not after pyramidal lesions. Abstr IBRO Third World Congr Neurosci 3:29(Abstract)

Thomas KA, Ortega S, Soderman D, Schaeffer M-T, DiSalvo J, Gimenez-Gallego G, Linemeyer D, Kelly L, Menke J (1991) Structural modifications of acidic fibroblast growth factor alter activity, stability, and heparin dependence. Ann NY Acad Sci 638:9-17.

Thomson AM, Deuchars J (1994) Temporal and spartial properties of local circuits in neocortex. Trends Neurosci 17:119-130.

Van Hooff CO, Holthuis JC, Oestreicher AB, Boonstra J, De Graan PN, Gispen WH (1989) Nerve growth factor-induced changes in the intracellular localization of the protein kinase substrate B-50 in pheochromocytoma PC12 cells. J Cell Biol 108:1115-1125.

Verge VMK, Merlio J-P, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hökfelt T, Richardson PM (1992) Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: Responses to injury and infusion of NGF. J Neurosci 12:4011-4022.

Voytko ML, Olton DS, Richardson RT, Gorman LK, Tobin JR, Price DL (1994) Basal forebrain lesions in monkeys disrupt attention but not learning and memory. J Neurosci 14:167-186.

Walker P, Weichsel ME, Fisher DA, Guo SM (1979) Thyroxine increases nerve growth factor concentration in adult mouse brain. Science 204:427-429.

Wanaka A, Johnson EM, Jr., Milbrandt J (1990) Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. Neuron 5:267-281.

Weskamp G, Gasser UE, Dravid AR, Otten U (1986a) Fimbria fornix-lesion increases nerve growth factor content in adult rat septum and hippocampus. Neurosci Lett 70:121-126.

Weskamp G, Lorez HP, Keller HH, Otten U (1986b) Cholinergic but not monoaminergic denervation increases nerve growth factor content in the adult rat hippocampus and cerebral cortex. Naunyn Schmiedebergs Arch Pharmacol 334:346-351.

Widmer HR, Knüsel B, Hefti F (1993) BDNF protection of basal forebrain cholinergic neurons after axotomy: complete protection of p75NGFR-positive cells. NeuroReport 4:363-366.

Woodroofe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ (1991) Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J Neuroimunol 33:227-236.

Woolf NJ, Butcher LL (1990) Dysdifferentiation of structurally plastic neurons initiates the pathologic cascade of Alzheimer's disease: towards a unifying hypothesis. In: Brain cholinergic systems (Steriade M, Biesold D eds), pp 387-438. New York: Oxford University Press.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Res 538:118-126.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14-25.

Yusta B, Besnard F, Ortiz-Caro J, Pascual A, Aranda A, Sarlieve L (1988) Evidence of the presence of nuclear 3,5,3'-triodothyronine receptors in secondary cultures of pure rat oligodendrocytes. Endocrinology 122:2278-2284.

ORIGINAL CONTRIBUTIONS ARISING FROM THE WORK OF THIS THESIS

1. Two members of the neurotrophin family, BDNF and NT-3, were shown not to be efficacious in rescuing NBM cholinergic neurons in contrast to NGF, the prototype member of the family.

2. These studies have shown that despite the lack of FGF receptors, aFGF treatment of animals with partial unilateral cortical infarction prevented neuronal atrophy and the decrease in ChAT-immunoreactive neurites (dendrites and axons), as well as the depletion in ChAT activity in the nucleus basalis magnocellularis (NBM), ipsilateral to the lesion.

3. The experiments described in this thesis demonstrated for the first time that aFGF is capable of recovering memory deficits (impairment in recalling the location of a submerged platform in a pool).

4. We have demonstrated that unilateral partial neocortical infarction of adult rats elicited, per se, an increase in NGF protein in the remaining neocortex, ipsilateral to the lesion. This increase was even higher after aFGF treatment. The fact that these animals have also shown a moderate increase of NGF mRNA levels indicated de novo synthesis of NGF synthesis in aFGF-treated animals.

5. It was found that neocortical infarction induced an early increase in the levels of low-affinity NGF receptor ($p75^{NGFR}$) mRNA in the NBM which decreased below control levels in the following post-lesion days examined, 7 and 15. In addition, NGF treatment caused further increase on post-lesion day 3, and restored levels of $p75^{NGFR}$ mRNA on post-lesion day 15.

6. We described for the first time that the levels of GAP-43 mRNA are unaffected by NGF treatment but they are transiently increased in the NBM after cortical

infarction. This upregulation occurred at the same period that decreased expression of this gene was observed in thalamic neurons, which were undergoing accentuated degeneration and neuronal cell death. These observations are consistent with the idea that neuronal injury may enhance expression of the GAP-43 gene, however, we suggest that in the case of neuronal injury leading to cell death, decreased expression of the GAP-43 precedes cell death.

7. We demonstrated that atrophied NBM neurons of posthypothyroid animals express normal mRNA levels of low- and high-affinity NGF receptors which are apparently responsive to trophic therapy as these abnormally developed neurons were rescued by NGF after unilateral partial neocortical infarction.

APPENDIX A

GENE EXPRESSION IN THE DEVELOPING CEREBELLUM DURING PERINATAL HYPO- AND HYPERTHYROIDISM

B. C. Figueiredo, G. Almazan, Y. Ma, W. Tetzlaff, F. D. Miller and A. C. Cuello

ABSTRACT

The intensity of $p75^{NGFR}$ receptor-like immunoreactivity and the mRNAs encoding $p75^{NGFR}$, Tα1 α-tubulin, GAP-43, and the myelin proteins MBP and PLP were measured in the developing cerebellum to study the effects of perinatal thyroid hormone inbalance in rats. Results compared to age-matched controls provide *in vivo* evidence for differential gene regulation by thyroid hormone in the developing cerebellum. We found that $p75^{NGFR}$ immunoreactivity was strikingly elevated in hypothyroid rats, where as $p75^{NGFR}$ mRNA content remained only twice as high as that of control levels on post-natal day 15 (P15). When $p75^{NGFR}$ receptor immunoreactivity was still elevated in hypothyroid rats, Purkinje cells exhibited proximal axonal varicosities, axonal twisting and differences in axonal caliber. The mRNAs encoding proteins involved with neurite growth-promoting elements, Tα1 α-tubulin and GAP-43, were also increased in hypothyroidism, possibly reflecting a neuronal response to a deficiency in, or damage to, cerebellar neurons, or a general delay in their down regulation. Similar increases were not observed for the myelin specific genes. MBP and PLP mRNAs were first detected on P2 of hyperthyroid rats, and they increased with age. Hypo- or hyperthyroidism did not affect the initial onset of MBP and PLP expression, however, hyperthyroidism increased levels of PLP and MBP mRNAs between P2 and P10. By contrast, the most consistent decrease in MBP and PLP mRNAs in rats with thyroid hormone deficiency was observed only on P10. At later times (P15 and P30), the two mRNA levels were similar to controls in all groups. These results are consistent with a role for thyroid hormone in the earlier stages of cerebellar myelination. Hypothyroidism led to specific increases in Tα1 α-tubulin and GAP-43 mRNAs, and in the immunoreactivity and mRNA levels of p^{75NGFR} receptor - all changes that may play a role in the observed abnormal neuronal outgrowth.

INTRODUCTION

Cerebellar development extends well into postnatal life, a time during which this system is exposed to substantial environmental influences. Several studies have shown that an inbalance in thyroid hormone levels is extremely detrimental to cerebellar

development. The primary objective of such studies has been to determine the thyroid requirement for efficient cerebellar histogenesis (Clos and Legrand, 1973; Legrand, 1967; 1983; Nicholson and Altman, 1972a). Other studies have reported alterations in the timing and level of proteins and neurotransmitters [for review see (Dussault and Ruel, 1987)], behavioral responses (Klein et al., 1972; Legrand, 1984; Raiti and Newns, 1971) and gene expression (Aniello et al., 1991a; 1991b; Muñoz et al., 1991). Developmentally-regulated expression of central nervous system (CNS) genes appears to be accounted for by their mRNA accumulation during specific developmental events such as neuronal differentiation, neurite outgrowth, synaptogenesis and myelination. One such gene is the low-affinity nerve growth factor receptor ($p75^{NGFR}$) which undergoes a 10 to more than 100-fold decrease in its mRNA in certain CNS regions following early postnatal brain development (Buck et al., 1988; Ernfors et al., 1988; Large, 1989; Maisonpierre et al., 1990).

The timing of the transient increase on the expression of the $p75^{NGFR}$ mRNA in the cerebellum (Buck et al., 1988; Maisonpierre et al., 1990) coincides with the developmental onset of Purkinje cell growth, proliferation of cerebellar microneurons (Altman, 1972; Altman and Bayer, 1978; Legrand and Clos, 1991) and the beginning of cerebellar myelination (Reynolds and Wilkin, 1988). As cerebellar development proceeds, $p75^{NGFR}$ decreases; myelin specific proteins, however, such as myelin basic protein (MBP) and myelin proteolipid protein (PLP), the two principal protein components of myelin, increase. Physiological concentrations of thyroid hormones are required for normal myelin synthesis [for review see (Dussault and Ruel, 1987)]. MBP and PLP constitutes most of the total protein of myelin and they are more efficiently synthesized *in vitro* in the presence of thyroid hormone (Almazan et al., 1985; Campagnoni et al., 1987). Thus, thyroid hormones seem to directly affect the levels of expression of MBP and PLP, probably at the transcriptional level, as suggested by mRNA determinations *in vitro* (Shanker et al., 1987) and mRNA determination in total brain samples (Muñoz et al., 1991). We are interested in assessing cerebellar MBP and PLP mRNAs because, through their expression, we can indirectly estimate the maturation states of oligodendroglial cells. To-date, no study has shown MBP and PLP mRNA

expression in the cerebellum during several postnatal periods of hypo- and hyperthyroidism.

As a result of their effect on neurite outgrowth during early stages of development, thyroid hormones affect numerous brain functions. Thyroid hormone deficiency reduced the size of granule cell axons and the dendritic tree of Purkinje cells, leading to the establishment of disturbances in axodendritic synaptic densities (Hajós et al., 1973; Legrand, 1967; Nicholson and Altman, 1972a). It has been suggested that these abnormalities are secondary to several deficits in the synthesis and assembly of cytoskeleton proteins, such as tubulin (Chaudhury et al., 1985; Chaudhury and Sarkar, 1983; Faivre et al., 1983; Gonzales and Geel, 1978; Lakshmanan et al., 1981; Nunez, 1985), microtubule-associated protein (Fellous et al., 1979; Francon et al., 1977) and filamentous actin (Faivre Sarrailh and Rabié, 1988; Siegrist Kaiser et al., 1990). A regulatory role has also been suggested for thyroid hormone at the transcriptional level of tau (Aniello et al., 1991a) and tubulin isotype genes (Aniello et al., 1991b) in the developing cerebellum although not in the cerebral hemispheres. However, the marked abnormalities in cytoskeletal processes have been described in the cerebellum (Legrand, 1979; Nicholson and Altman, 1972b) and other brain regions (Gould and Butcher, 1989; Rami et al., 1986) of thyroid-deficient rats, which indicate more complex levels of thyroid hormone regulation. The abnormalities in neurite outgrowth result in impaired connectivity among neurons and may lead to permanent defective neuronal function if thyroid hormone deficiency is prolonged throughout the period of cerebellar development.

The deleterious effects of perinatal thyroid imbalance on synaptic connections may also be linked to elements which appear to play important roles in neurite extension, such as the developmentally-regulated Tα1 α-tubulin (Miller et al., 1989) and the growth-associated protein GAP-43 expression (Skene et al., 1986). GAP-43 is specifically enriched in the membrane skeleton of growth cones, where it apparently controls outgrowth (Skene, 1989; Skene et al., 1986). We have included immunocytochemistry for $p75^{NGFR}$ in an attempt to correlate morphological and biochemical events, as well as to compare the immunoreactivity (IR) of $p75^{NGFR}$ with $p75^{NGFR}$ mRNA levels. Although

$p75^{NGFR}$ is found in very high levels during critical phases of the cerebellar cortex development where this receptor may play an important role, so far, its function remains unknown. The present study has compared the changes in $p75^{NGFR}$ IR and its mRNA with the morphological and behavioral abnormalities found in hypothyroid rats without assigning a specific role to $p75^{NGFR}$. The effects of excessive doses of thyroxine and thyroid deficiency in the expression of the mRNAs encoding Tα1 α-tubulin, GAP-43, $p75^{NGFR}$, PLP and MBP were studied during several stages of the cerebellar development. Our data show a disproportion between $p75^{NGFR}$-like IR in band-like areas of the cerebellum and the total cerebellar level of $p75^{NGFR}$ mRNA. The abnormal pattern of Purkinje axons is also compared to the absolute increase in tubulins and GAP-43 gene expression.

MATERIALS AND METHODS

Animals and tissue processing

Wistar pups were made hypothyroid by providing some of the dams with a 0.4% propylthiouracil (PTU) enriched diet beginning on embryonic day 19 (E19) (Kalaria and Prince, 1985). The begining of the gestation was carefully verified and the day of birth was considered to be day 0. On the day of birth, six male pups were assigned to each mother. Offsprings of dams that had not ingested their PTU-treated food regularly from the second day of treatment were removed from the experiments. The amount of ingested food was controlled daily, and ranged from approximately 30 to 75 g/day/dam (as in control), from birth until their pups became 30 days old. To greatly reduce the number of animals removed because of low food consumption, 6% sucrose was added to PTU before mixing with the appropriate food. Other male pups, six per dam, were subcutaneously injected daily, either with physiological saline or with 0.3 μg thyroxine/g body weight from the day of birth (P0). Neonatal naive pups were also included in the control group. The animals were decapitated (on P2, P6, P10, P15 or P30) and their cerebelli were kept frozen at -70°C until RNA extraction. For purposes of immunocytochemistry, the pups were sacrificed transcardially by perfusion on P17. All

hypothyroid pups remained with their dams until the day of sacrifice and the rest of the animals were weaned at the end of the third postnatal week.

Immunocytochemistry

The processing of cerebellar tissue for immunocytochemistry was essentially the same as that described earlier (Pioro and Cuello, 1990b) except that the pups were perfused with smaller volumes of fixative. Rats were deeply anesthetized with Equithesin (0.40 ml/100 g i.p.) and perfused briefly through the ascending aorta with phosphate buffer (PB; pH 7.4) vascular rinse. Subsequently, a 20 min fixation with a 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid mixture in 0.1 M PB (pH 7.2) was immediately followed by a 20 min perfusion with 0.5% hydrogen peroxide in phosphate-buffered saline (PBS). A final 20 min washout with a 10% glucose-PB solution was followed by removal and storage of cerebelli in 30% sucrose-PB at 4°C for 72-120 h. Fifty-μm-thick coronal sections were cut on a freezing sledge microtome.

Free-floating sections were incubated overnight at 4°C in supernatant from 192-IgG hybridoma cells (generously provided by E. M. Johnson) with 10% horse serum or with a non-specific mouse IgG. Characteristics of the 192-IgG anti-$p75^{NGFR}$ have been previously outlined in detail (Chandler et al., 1984; Taniuchi and Johnson, Jr., 1985). All subsequent steps were carried out at room temperature using PBS containing 0.2% Triton X-100 (pH 7.4) for washes, dilution of antibodies plus horse serum, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. After washing, sections were incubated for 2 h in rabbit anti-mouse IgG (1/50) and then washed again. Subsequently, the sections were incubated for 1 h in monoclonal mouse peroxidase-antiperoxidase (Semenenko et al., 1985) (PAP; 1/30; Medicorp, Canada). After washing for 5 min (4x), sections were incubated in a 0.06% solution of DAB for 10 min, and a subsequent 8 min in the same solution containing 0.01% hydrogen peroxide (final concentration). After washing, sections were mounted on subbed slides, dehydrated, cleared and coverslipped.

cDNA clones and probe preparation

The cDNA probe for MBP mRNA was prepared using the 1.4 kb *Hin*dIII-EcoRI insert from pMBPSP64 (Mentaberry et al., 1986) containing a partial cDNA from rat 14KDa MBP. The cDNA probe for PLP mRNA was prepared using a partial rat cDNA (Gardinier et al., 1986). GAP-43 cDNA probe was generated from the 1.1 kb cDNA clone, kindly provided by Dr. Pate Skene (Basi et al., 1987). The MBP, PLP and GAP-43 probes were prepared by random oligonucleotide priming. Probes for Tα1 α-tubulin mRNA were prepared as described (Miller et al., 1987). For studies, a subcloned 310 nucleotide *Eco* RI/Bam HI fragment containing nucleotides 400-710 of the rat cDNA (Radeke et al., 1987) was used to generate riboprobes (Miller et al., 1991).

Preparation of RNA and RNA blotting

Total RNA was extracted from five (P2), three (P6), two (P10) pooled, or individual cerebelli (P15 and P30) in 4 M guanidium thiocyanate and purified by ultracentrifugation through 5.7 M CsCl. RNA was solubilized and denatured in 50% formaldehyde, 6% formamide and 20 mM MOPS buffer at 65°C for 1 h. RNA (10 μg/lane) was then loaded onto a 1.5% agarose/formaldehyde gel, transferred and immobilized onto a nylon membrane (Hybond-N, Amersham). Eight blots (n=3 experiments) were hybridized overnight at 42°C in 50% formamide, 0.8 M NaCl, 0.5% SDS, 50 mM sodium phosphate (pH 6.5) and 10^6 cpm/ml of ^{32}P-labeled probes. Blots were washed in 0.1 X SSC, 0.1% SDS at 45-50°C and exposed to Kodak XAR-5 film with an intensifier screen. The signals were quantified by densitometry using an LKB laser densitometer. To detect significant differences between groups for different probes, the Newman-Keuls' test was used. Values given represent means ± S.E.M. of three experiments. Criterion for significance was $P<0.05$. The migration of 18S and 28S RNAs were compared in blots stained with Methylene blue.

RESULTS

General signs of thyroid imbalance

The physical and behavioral effects of perinatal hypothyroidism were mainly opposite to those of hyperthyroidism (Gould and Butcher, 1989). However, there were a few exceptions, such as adult body, cerebral and cerebellar sizes and weights [for review see ref. (Nicholson and Altman, 1972a)], which were reduced in both experimental groups (more reduced in hypothyroidism) as compared to age-matched controls. In normal pups, coordinated creeping was observed around P9-11, fully opened eyes between P11 and P13, and exploratory behavior started more evidently around P13-16. In hyperthyroid animals, these signs were observed three to five days earlier and were usually accompanied by motor hyperactivity and fine tremor. However, in hypothyroid neonates, the coordinated creeping, opening of the eyes and the innate exploratory behavior were retarded 7 to 12 days. Accentuated motor deficit persisted in hypothyroid animals up to the oldest age examined, i.e. 30 days after interruption of PTU treatment (P30). The motor abnormalities observed in hypothyroid rats included uncoordinated gait and frequent and sudden involuntary leaps, which were observed after P40 and usually at the onset of or during progression. Most of the time, however, the hypothyroid animals were generally hypoactive and the hyperthyroid rats more hyperactive than age-matched controls.

Immunocytochemistry

Purkinje cells, the molecular layer and the external granular layer of hypothyroid rats expressed increased $p75^{NGFR}$-like IR on P17 (Fig. 1C). In age-matched controls, the same cell groups expressed lower IR (Fig. 1A), which however, was greater than in thyroxine-treated pups (Fig. 1B). In all experimental and control neonates, the most intense $p75^{NGFR}$-like IR was detected in the external granular layer and cortical areas of posterior lobules with band-like distribution. Purkinje cells of hypothyroid rats were characterized by striking differences in axonal caliber and abnormalities in their skeletal processes, such as twisting and varicosities (spheroidal swelling) in proximal axon (Fig. 1D,E).

Northern blot analysis

Figure 1

Micrographs showing $p75^{NGFR}$-like IR in coronal sections through lobule 8 (A and B) or lobules 8 and 9 (C-E) of developing cerebelli of P17. A: low magnification of cerebellar cortex of normal rats revealing light-to-moderate $p75^{NGFR}$-like IR in the molecular layer (ML), with slightly stronger appearance of band-like immunoreactivity; Bar in A - C= 63 μm. B: light $p75^{NGFR}$-like IR in the molecular layer of hyperthyroid rats. C: strong $p75^{NGFR}$-like IR in all layers of the cerebellar cortex of hypothyroid rats, with striking immunoreactivity in the external granular layer (EGL), followed by the molecular and Purkinje cell layers in bands. D, E: high magnification of the Purkinje cell (P) projecting axons through the internal granular layer (IGL). Observe in D, different axonal calibers (open arrows) and twisting appearance of Purkinje cell axon (small solid arrows): Bar in D and E=10 μm. E represents a high magnification of framed area in C showing varicosity (spheroidal swelling) in proximal axon and different calibers of axons.

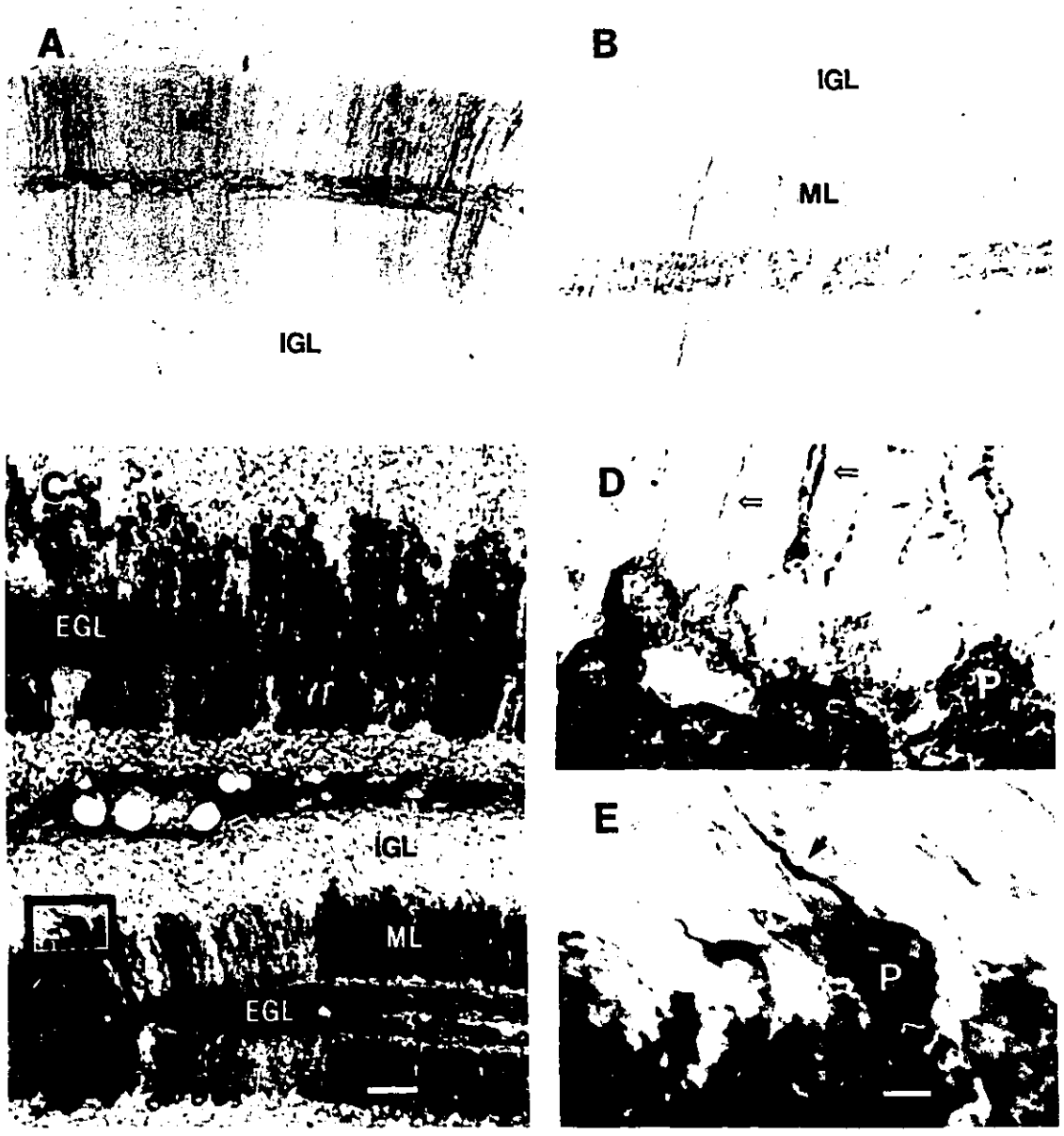
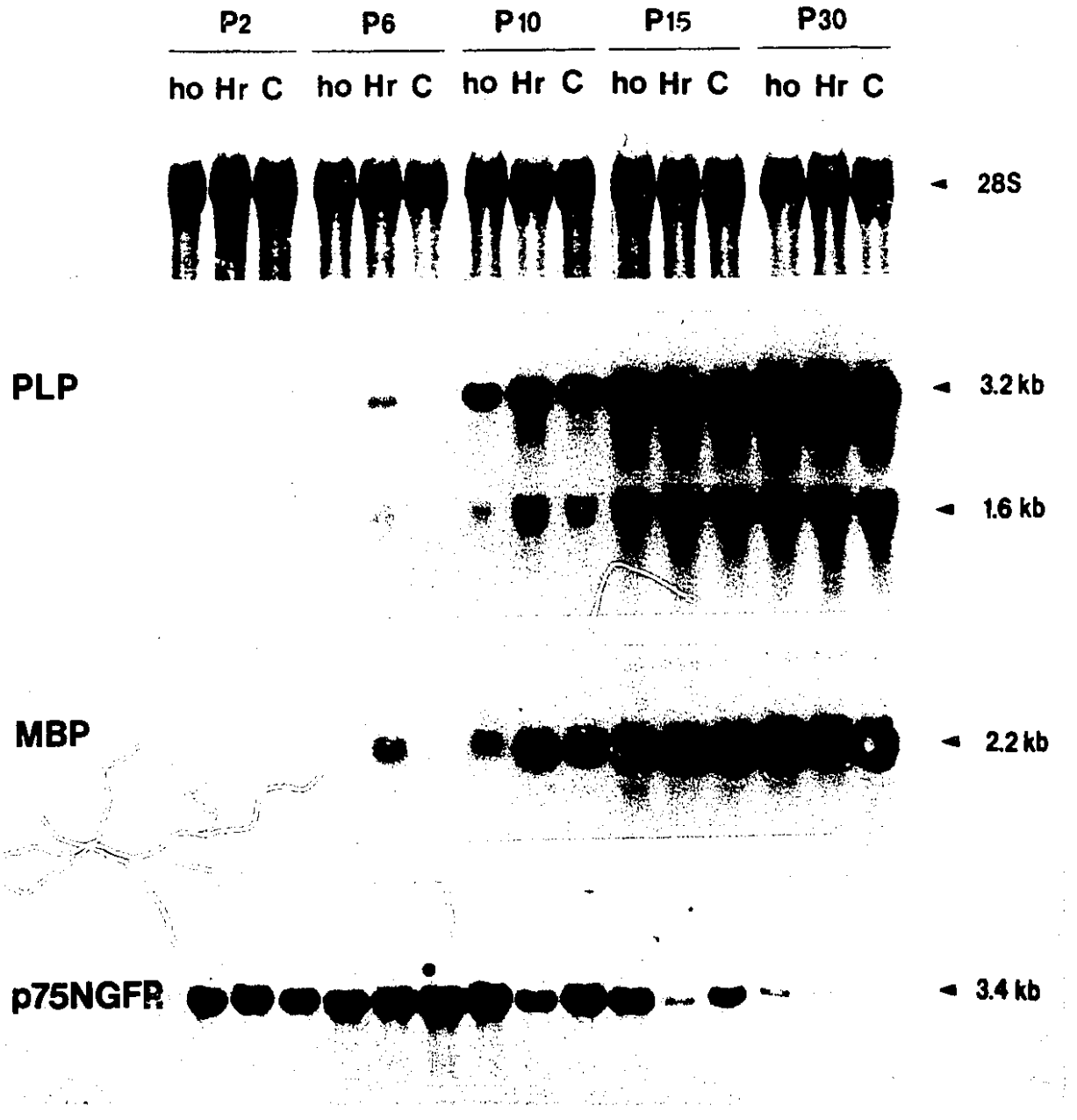

A
IGL
B
IGL
ML
C
EGL
IGL
ML
EGL
D
P
E
P

Figure 2

Time-courses of PLP, MBP and $p75^{NGFR}$ mRNAs in developing cerebellum. Ten μm of total RNA per lane were used for each developmental time-point. On top are blots (migration of 28S RNA) stained with Methylene blue. Some signals not shown above were detected only in longer exposure of radioautograms such as: (1) for MBP and PLP at P2 of hyperthyroid rats and the signals of hypothyroid and controls rats on P6; (2) the signals for $p75^{NGFR}$ at P30 in eu- and hyperthyroid rats. Transcript sizes indicated in kilobases at right. Top: ho, hypothyroid; Hr, hyperthyroid; C, control (euthyroid); P, postnatal day.

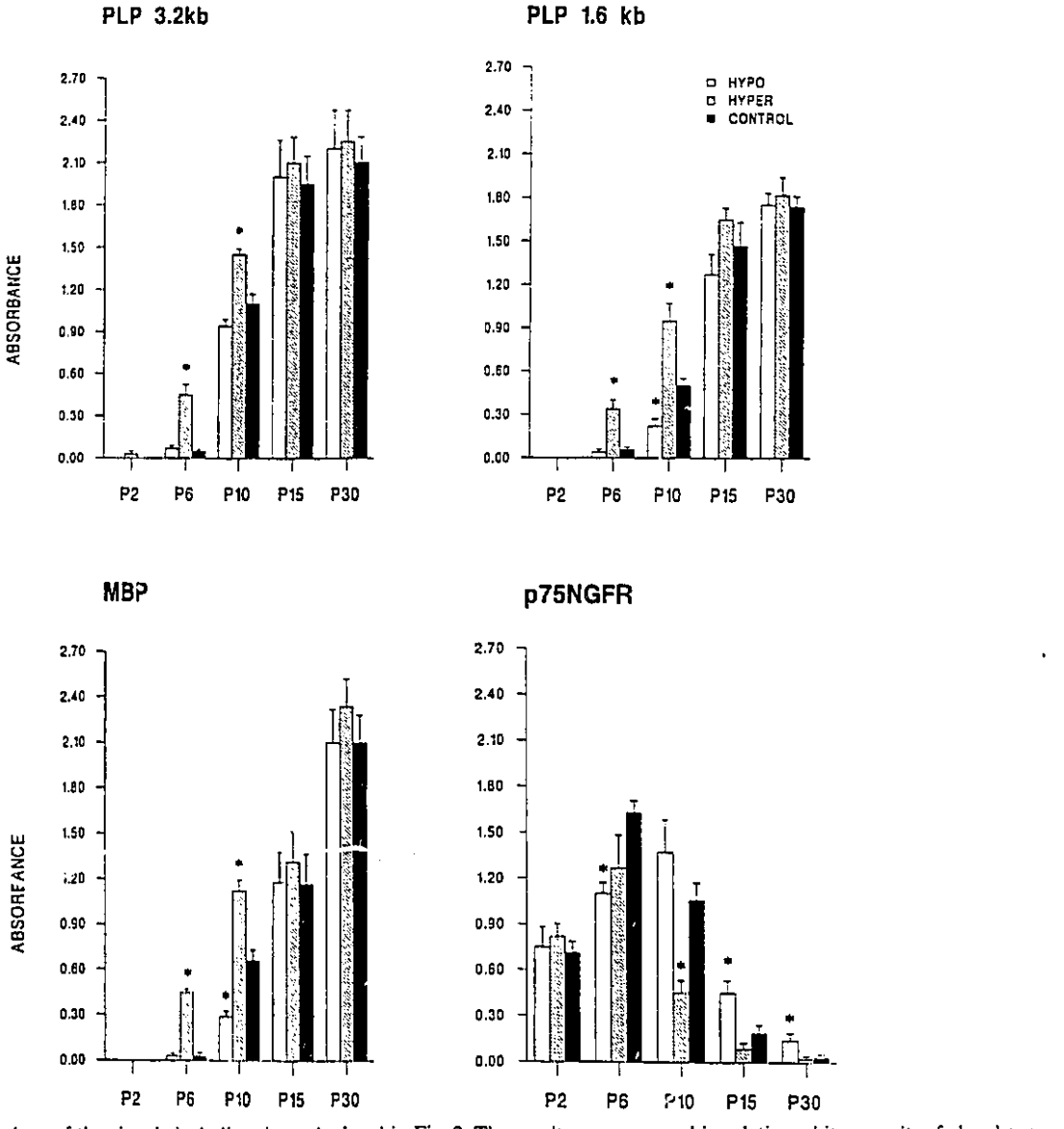

P2
P6
P10
P15
P30
ho Hr C ho Hr C ho Hr C ho Hr C ho Hr C
28S
PLP
3.2 kb
1.6 kb
MBP
2.2 kb
p75NGFR
3.4 kb

Northern blot analysis of total RNA indicated that the 3.2 and 1.6 kb PLP and the 2.2 kb MBP mRNA transcripts increased progressively in the developing cerebellum. MBP and PLP turned on simultaneously during the first week of postnatal life and their expression stabilized at maximal levels two - three weeks later. PLP and MBP mRNAs were first expressed at low levels on P2 in thyroxine-treated rats, but in age-matched controls they were detected upon longer exposures of Northern blots (Fig. 2). Between P6 and P10 there was a rapid rise in the levels of expression of both mRNAs in thyroxine-treated pups, as compared to control and hypothyroid rats. In hyperthyroid rats at P6, the levels of MBP and PLP mRNAs, clearly observed in longer exposed blots, were four to six-fold higher than in age-matched controls (Fig. 3); however, this difference disappeared on P15 and P30 when both mRNAs in experimental animals stabilized to control levels. Unexpectedly, thyroid deficiency produced no significant changes in the levels of expression of PLP 3.2 kb mRNA at any of the times studied. The MBP and the 1.6 kb PLP mRNA levels were significantly lower than control only at P10 (Figs. 2 and 3). The blots shown in Figs. 2 and 4A were slightly over-exposed in order to show the relative intensity of mRNA levels in all time points examined. However, for quantification and comparison, other blots with less exposure were included.

In normal rats, levels of $p75^{NGFR}$ mRNA increased from the earliest point examined, P2 to P6, declined on P10 and decreased rapidly during the next five days (Figs. 2 and 3). On P30, $p75^{NGFR}$ mRNA in control and hyperthyroid rats was expressed at barely detectable levels. In hyperthyroid animals, levels of $p75^{NGFR}$ mRNA declined much more dramatically from P6 to P10, and were barely detectable at P15 and P30. By contrast, in animals with thyroid hormone deficiency, $p75^{NGFR}$ mRNA level did not decrease from P6 to P10, and only first decreased from P10 to P15.

Subsequently, we compared the Tα1 α-tubulin and GAP-43 mRNA levels with the morphological features found in Purkinje cell axons of hypothyroid rats. In control animals, the time-courses of expression of GAP-43 and Tα1 α-tubulin mRNAs were similar; levels were highest at P2, decreasing progressively thereafter to P30, the oldest age examined (Fig. 4A,B). In hypothyroid animals, Tα1 and GAP-43 mRNAs were

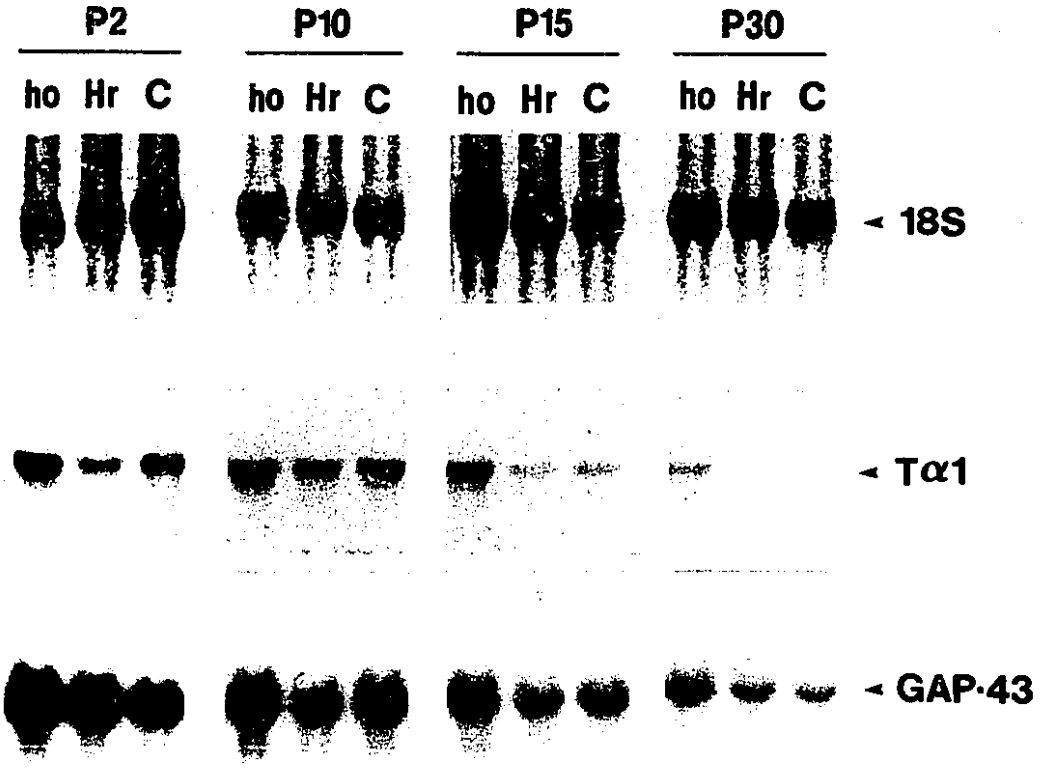
Fig. 3

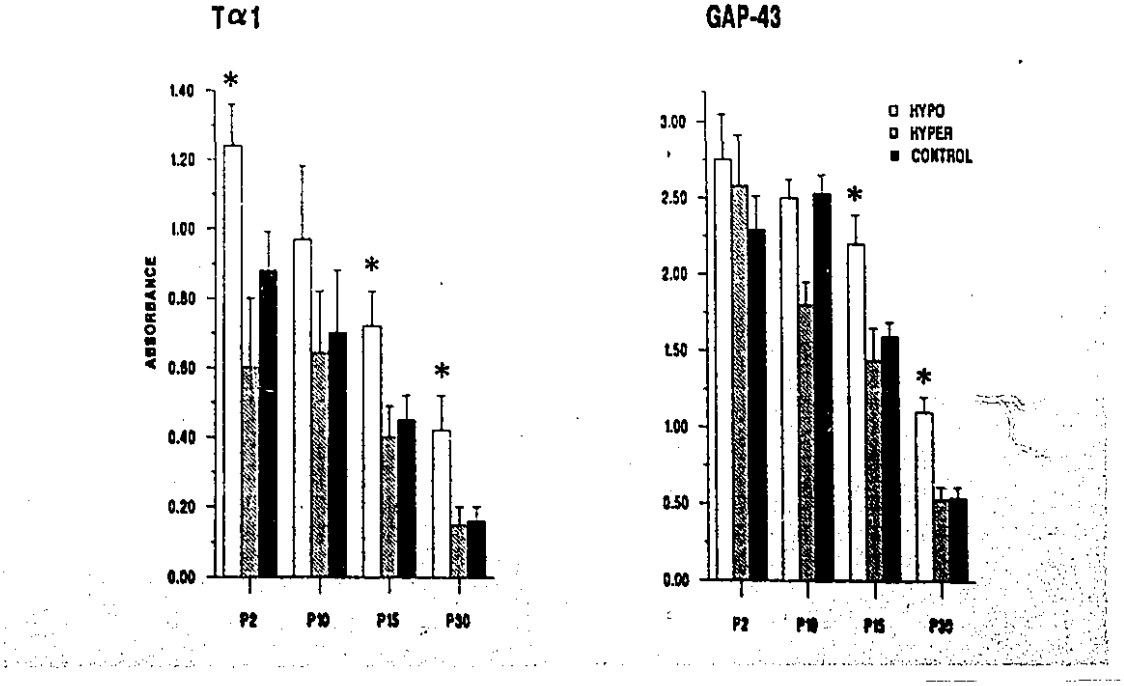


Densitrometric values of the signals including those depicted in Fig. 2. The results are expressed in relative arbitrary units of absorbance. Bars represent the means ± S.E.M. of multiple exposures of three RNA preparations. * $P < 0.05$, ANOVA followed by post hoc Newman–Keuls' test compared to age-matched controls. HYPO, hypothyroid rats; HYPER, hyperthyroid rats; CONTROL, euthyroid rats; In the *x*-axis, P, postnatal day.

Figure 4 A,B:

Time-courses of tubulin Tα1, α-tubulin, and GAP-43 mRNAs in developing cerebellum. A: 10 μm of total RNA per lane was used for each time-point. Top, blots (migration of 18S RNA) stained with Methylene blue. Positive signals for Tα1 mRNA on P30 of hyperthyroid and control animals required longer exposure of radioautograms. ho, hypothyroid rats; Hr, hyperthyroid rats; C, euthyroid animals; P, postnatal day. B: quantitation of the signals including those depicted in A. The results are expressed in relative arbitrary units of absorbance. Bars represent the mean ± S.E.M. of multiple exposures of three RNA preparations. * $P<0.05$, ANOVA followed by *pos hoc* Newman-Keuls' test compared to age-matched controls. HYPO, hypothyroid rats; HYPER, hyperthyroid rats; CONTROL, euthyroid rats. In the *X*-axis, P, postnatal day.


P2
P10
P15
P30
ho Hr C
ho Hr C
ho Hr C
ho Hr C
18S
Tα1
GAP-43


Tα1
GAP-43
ABSORBANCE
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
3.00
2.50
2.00
1.50
1.00
0.50
0.00
P2
P10
P15
P30
HYPO
HYPER
CONTROL
*

significantly increased at P15 and P30 relative to aged-matched controls. In thyroxine-treated rats, the mRNA levels were significantly lower at P2 (Tα1) and P10 (GAP-43) in relation to aged-matched controls.

DISCUSSION

We have examined some of the features of cerebellar development that are dramatically affected by thyroid hormone imbalance and have found some evidence of compensatory responses by the neurons. In particular, we studied the effects of hypo- and hyper-thyroidism in expression of $p75^{NGFR}$, Tα1 and GAP-43 mRNAs because of their involvement in differentiation and growth. For comparison, we demonstrated that thyroid hormone can exert some control on oligodendrocyte gene expression (e.g. MBP and PLP) during the early period of cerebellar myelinogenesis.

Our data on $p75^{NGFR}$ mRNA time-course in control rats show a distribution similar to the findings in previous studies (Buck et al., 1988; Maisonpierre et al., 1990; Wanaka and Johnson, Jr., 1990). We expected to find a high $p75^{NGFR}$ mRNA content in hypothyroid animals based upon the dramatic increase in the $p75^{NGFR}$-like IR found between P13 and P18 (Fig. 1 shows data from P17) as compared to age-matched controls. However, on P15 we observed only double the amount of $p75^{NGFR}$ mRNA found in age-matched controls. A reduced $p75^{NGFR}$ IR shown on P10 in hypothyroid rats (Legrand and Clos, 1991), compared to our data on higher $p75^{NGFR}$ IR seven days later, indicated a retarded increase in $p75^{NGFR}$ on the developing cerebellum. The 192-IgG antibody appears to recognize $p75^{NGFR}$ (Green and Greene, 1986; Taniuchi and Johnson, Jr., 1985), the low-affinity receptor of the NGF superfamily, which binds to brain-derived neurotrophic factor (BDNF) (Rodríguez-Tébar et al., 1990) or neurotrophin-3 (Rodríguez-Tébar et al., 1992); therefore, wherever we cite "$p75^{NGFR}$" immunoreactivity we are actually referring to "$p75^{NGFR}$-like" immunoreactivity. In addition, other reasons for the apparent discrepancy between the levels of $p75^{NGFR}$ mRNA and the $p75^{NGFR}$ IR in hypothyroid rats in comparison to age-matched controls (P17), may be due to the

following two hypotheses: (i) thyroid hormone deficiency may reduce the degradation rate of $p75^{NGFR}$ for the same reasons as for other proteins, i.e., decreased enzyme activity [for review see ref. (Dussault and Ruel, 1987)]; or (ii) perhaps $p75^{NGFR}$ accumulated in neurons, such as Purkinje cells, as a result of reduced anterograde transport due to modification in the content or assembly of cytoskeleton proteins (Chaudhury et al., 1985; Chaudhury and Sarkar, 1983; Faivre et al., 1983; Faivre Sarrailh and Rabié, 1988; Fellous et al., 1979; Francon et al., 1977; Gonzales and Geel, 1978; Lakshmanan et al., 1981; Nunez, 1985; Siegrist Kaiser et al., 1990), which are necessary for the axonal transport of $p75^{NGFR}$, as demonstrated by colchicine treatment (Pioro and Cuello, 1990a; 1988; Pioro et al., 1991). The retardation in the disappearance of the external granular layer during thyroid hormone deficiency (Nicholson and Altman, 1972a) and during treatment with antibody anti-$p75^{NGFR}$ (Legrand and Clos, 1991) is in agreement with the prolonged $p75^{NGFR}$ IR observed in this layer. In contrast, the faint $p75^{NGFR}$ IR found in hyperthyroid rats (Fig. 1B) was also expected and indicated early termination of cell proliferation in the external granular layer, accompanied by early disappearance of the germinative layer (Nicholson and Altman, 1972a). At the very least, these data demonstrate that this synchronized shift in the expression of $p75^{NGFR}$, which corresponds to a common low-affinity receptor in the growing list of the NGF family of neurotrophins (Thoenen, 1991), correlated with the period of cell proliferation and maturation of cerebellar cortical neurons. This correlation reinforces the possibility of an important physiological role for a neurotrophin, acting via $p75^{NGFR}$, for the developing cerebellar cortex; however, other experimental approaches are necessary to test this hypothesis.

The developmental significance of thyroid hormones for neurite outgrowth has been demonstrated in different brain tissues (Gould and Butcher, 1989; Legrand, 1979; Nicholson and Altman, 1972b; Rami et al., 1986). In this study, we have extended these observations by demonstrating that the perturbation of Purkinje cells axonal outgrowth occurs simultaneously with the over-expression of genes involved with neurite outgrowth. To illustrate the alteration of neurite outgrowth induced by perinatal hypothyroidism, we have described defective entities in Purkinje cells, the only efferent neurons of the cerebellar cortex. This study has focused only on Purkinje cells because of the simplicity

of their projecting fibers. In hypothyroid rats at P17, these fibers are easily visualized in $p75^{NGFR}$- IR band-like areas of the cerebellar lobes. The morphological features noted include varicosities (spheroidal swelling) along proximal axons, very similar to those observed in nucleus basalis neurons (Gould and Butcher, 1989), twisting as well as striking changes in the caliber of Purkinje cell axons (Figs. 1D, E). These alterations, together with the reduced arborization of dendritic spines of Purkinje cells and with the already defective contacts of granule cells, between afferent mossy fibers and Purkinje cells [for review see ref. (Legrand, 1979)], result in permanent impairment of neuronal connectivity, contributing to the generation of motor abnormalities of hypothyroid rats. All of these changes in neurite outgrowth may be largely attributable to reduced amounts of cytoskeletal proteins in hypothyroid rats (Chaudhury et al., 1985; Chaudhury and Sarkar, 1983; Faivre et al., 1983; Faivre Sarrailh and Rabié, 1988; Fellous et al., 1979; Francon et al., 1977; Gonzales and Geel, 1978; Lakshmanan et al., 1981; Nunez, 1985; Siegrist Kaiser et al., 1990) and to a reduction in Tα1 and GAP-43 proteins. The increase in the expression of the developmentally-regulated Tα1 gene, at all ages in hypothyroid rats examined in this study and in a recent report (Aniello et al., 1991b), may reflect primarily: (i) an intrinsic neuronal response to a reduced level of Tα1 protein, (ii) an impairment of or abnormal neuronal growth, and/or (iii) a delayed down-regulation of Tα1.

Surprisingly, the timing and content of Tα1 in thyroxine-treated animals did not differ significantly from controls, which indicates that Tα1 expression is not affected by accelerated cerebellar development during thyroxine treatment. Similarly, the increased amount of GAP-43 mRNA at P15 and P30 in hypothyroid rats may be a compensatory reaction of cerebellar neurons to the perturbed GAP-43 function in growth cones (Alexander et al., 1987; Liu and Storm, 1990; Skene et al., 1986) and/or a reaction to regenerate any neuronal damage (Benowitz and Lewis, 1983; Tetzlaff et al., 1991). The earlier increase of Tα1 in relation to a later increase in GAP-43 mRNA suggests either that an already superabundant production of GAP-43 mRNA may occur in naive animals, or that the Tα1 gene is more sensitive to the deleterious effects of hypothyroidism. On the basis of a large body of evidence on the inefficient growth of cerebellar neurons

deprived of thyroid hormones, it was reasonable to expect increased amounts of Tα1 and GAP-43 mRNAs in the developing cerebellum of hypothyroid rats. The inverse relationship between the levels of mRNA and its protein was also previously described for actin mRNA (Faivre Sarrailh et al., 1990), and the higher levels of tau, α1-, ß2- and β4-tubulin mRNAs in the cerebellum of hypothyroid rats (Aniello et al., 1991a; 1991b) certainly correlate with low amounts of their proteins. However, since other tubulin isotype mRNAs were not affected in the same way (Aniello et al., 1991b), the authors of this study have concluded that these genes are regulated differently by thyroid hormone.

We were interested in observing the time-course of MBP and PLP gene expression during the critical phase of the cerebellar development, without assigning a role to any particular MBP or PLP isoform. Our results show that in thyroxine-treated animals, the MBP and PLP mRNAs in the developing cerebellum increase several-fold when examined at an early age (P6-P10). By contrast, thyroid hormone deficiency reduced both mRNA levels only at P10 as compared to age-matched controls. These results raise the question of when and at what level of myelin protein synthesis is thyroid hormone required to maintain normal levels of MBP and PLP *in vivo*. Our results show that at older ages, P15 and P30, hypo- or hyperthyroidism did not affect MBP or PLP mRNA levels. These MBP data correlate partially, at early postnatal ages, with a recent study by Farsetti and coworkers (Farsetti et al., 1991). In contrast, their study shows that hypothyroid animals have lower levels of MBP mRNA at all developmental ages examined. They used total brain mRNA (including mRNA from areas in very different developmental stages) in a RNAse protection assay in mice or slot-blot analysis in rat, and a cDNA clone which did not include exon 2 or part of exon 7 of the MBP gene. It is known that the isoforms with exon 2 (17 and 21.5 kDa) predominate in younger animals and the other isoforms without exon 2 (14 and 18.5 kDa) predominate in older animals (Barbarese et al., 1978). Another reason for this apparent discrepancy is that they have normalized their densitometric values of MBP mRNA to that of β-actin mRNA, which in the developing cerebellum is much higher in hypothyroid rats after P10 (Faivre Sarrailh et al., 1990). This differential tissue gene regulation (i.e. total brain

versus developing cerebellum) by thyroid hormone may occur during the first month of postnatal life, as recently demonstrated for some mRNAs encoding tubulin isotypes (Aniello et al., 1991b). For instance, these investigators reported that hypothyroidism caused a slight increase of ß2-tubulin in the cerebellum, whereas it is decreased in cerebral hemispheres. The same differential regulation has been reported for tau mRNA, which was slightly changed in the cerebral hemispheres of hypothyroid rats, whereas the level of tau mRNA in the cerebellum was maintained at a higher level than in the euthyroid controls (Aniello et al., 1991a). The reduced level of PLP mRNA in total brain tissue induced by thyroid hormone deprivation during brain maturation cannot be widespread to all brain areas, as was suggested in a recent study (Muñoz et al., 1991). One important finding which has emerged from another study has been that differential thyroid hormone receptor mRNAs were reported in the developing cerebellum (Forrest et al., 1991), suggesting different functions for the thyroid hormone in the developing cerebellum. The situation is quite different in mature rats, in which thyroid hormone excess or deficiency does not affect MBP gene expression in total brain (Farsetti et al., 1991), a finding which is in agreement with our data on late cerebellar development. There is no information available on the effects of thyroid hormones on cerebellar myelin protein mRNAs *in vivo*. Much of our knowledge is derived from primary cell cultures from cerebral hemispheres, which generally have shown that thyroid hormone regulation of myelin protein synthesis occurs mainly at the level of transcription and translation (Almazan et al., 1985; Amur et al., 1984; Bhat et al., 1981; Campagnoni et al., 1987; Matthieu et al., 1987; Shanker et al., 1984; 1987).

We conclude that thyroid hormone regulation of cerebellar myelinogenesis *in vivo,* in addition to its effect on mRNA levels, may be more crucial at the post-transcriptional level. The neuronal mRNA levels of $p75^{NGFR}$, Tα1 and GAP-43, are directly or indirectly (e.g. mediated by neuronal damage) regulated by thyroid hormone. Therefore, thyroid hormone appears to play an important role in the neuronal maturation of the developing cerebellum, since the thyroid deficiency causes neuronal abnormalities and alters different neuronal markers.

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REFERENCES

Alexander KA, Cimler BM, Meier KE, Storm DR (1987) Regulation of calmodulin binding to P-57. J Biol Chem 262:6108-6113.

Almazan G, Honegger P, Matthieu J-M (1985) Triiodothyronine stimulation of oligodendroglial differentiation and myelination. A developmental study. Dev Neurosci 7:45-54.

Altman J (1972) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 145:399-463.

Altman J, Bayer SA (1978) Prenatal development of the cerebellar system in the rat. I. Cytogenesis of the deep nuclei and the cortex of the cerebellum. J Comp Neurol 179:23-48.

Amur SG, Shanker G, Pieringer RA (1984) Regulation of myelin basic protein (arginine) methyltransferase by thyroid hormone in myelinogenic cultures of cells dissociated from embryonic mouse brain. J Neurochem 43:494-498.

Aniello F, Couchie D, Bridoux AM, Gripois D, Nunez J (1991a) Splicing of juvenile and adult tau mRNA variants is regulated by thyroid hormone. Proc Natl Acad Sci U S A 88:4035-4039.

Aniello F, Couchie D, Gripois D, Nunez J (1991b) Regulation of five tubulin isotypes by thyroid hormone during brain development. J Neurochem 57:1781-1786.

Barbarese E, Carson JH, Braun PE (1978) Accumulation of the four myelin basic proteins in mouse brain during development. J Neurochem 31:779-782.

Basi GS, Jacobson RD, Virág I, Schilling J, Skene JHP (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell 49:785-791.

Benowitz LI, Lewis LR (1983) Increased transport of 44,000- to 49,000-dalton acidic proteins during regeneration of the goldfish optic nerve: A two-dimensional gel analysis. J Neurosci 3:2153-2163.

Bhat NR, Subba Rao G, Pieringer RA (1981) Investigations on myelination in vitro: regulation of sulpholipid synthesis by thyroid hormone in cultures of dissociated brain cells from embryonic mice. J Biol Chem 256:1167-1171.

Buck CR, Martinez HJ, Chao MV, Black IB (1988) Differential expression of the nerve growth factor receptor gene in multiple brain areas. Dev Brain Res 44:259-268.

Campagnoni AT, Hunkeler MJ, Moskaitis JE (1987) Translational regulation of myelin basic protein synthesis. J Neurosci Res 17:102-110.

Chandler CE, Parsons LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 259:6882-6889.

Chaudhury S, Chartterjee D, Sarkar PK (1985) Induction of brain tubulin by triiodothyronine: dual effect of the hormone on the synthesis and turnover of the protein. Brain Res 339:191-194.

Chaudhury S, Sarkar PK (1983) Stimulation of tubulin synthesis by thyroid hormone in the developing rat brain. Biochim Biophys Acta 763:93-98.

Clos J, Legrand J (1973) Effects of thyroid deficiency on the different cell populations of the cerebellum in the young rat. Brain Res 63:450-455.

Dussault JH, Ruel J (1987) Thyroid hormones and brain development. Ann Rev Physiol 49:321-334.

Ernfors P, Hallböök F, Ebendal T, Shooter EM, Radeke MJ, Misko TP, Persson H (1988) Developmental and regional expression of β nerve growth factor mRNA in the chick and rat. Neuron 1:983-996.

Faivre Sarrailh C, Ferraz C, Liautard JP, Rabie A (1990) Effect of thyroid deficiency on actin mRNA content in the developing rat cerebellum. Int J Dev Neurosci 8:99-106.

Faivre Sarrailh C, Rabié A (1988) A lower proportion of filamentous to monomeric actin in the developing cerebellum of thyroid-deficient rats. Brain Res 469:293-297.

Faivre C, Legrand C, Rabié A (1983) Effects of thyroid deficiency and corrective effects of thyroxine on microtubules and mitochondria in cerebellar purkinje cell dendrites of developing rats. Dev Brain Res 8:21-30.

Farsetti A, Mitsuashi T, Desvergne B, Robbins J, Nikodem V (1991) Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. J Biol Chem 266:23226-23232.

Fellous A, Lennon AM, Francon J, Nunez J (1979) Thyroid hormones and neurotubule assembly in vitro during brain development. Eur J Biochem 101:365-376.

Forrest D, Hallbook F, Persson H, Vennstrom B (1991) Distinct functions for thyroid homone receptors alpha and beta in brain development indicated by differential expression of receptor genes. EMBO J 10:269-275.

Francon J, Fellous A, Lennon AM, Nunez J (1977) Is thyroxine a regulatory signal for neurotubule assembly during brain development?. Nature 266:188-190.

Gardinier MV, Macklin WB, Diniak AJ, Deininger PL (1986) Characterization of myelin proteolipid mRNAs in normal and jimpy mice. Mol Cell Biol 6:3755-3762.

Gonzales LW, Geel SE (1978) Quantitation and characterization of brain tubulin (colchicine-binding activity) in developing hypothyroid rats. J Neurochem 30:237-245.

Gould E, Butcher LL (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347-3358.

Green SH, Greene LA (1986) A single M_r=103,000 ^{125}I-β-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. J Biol Chem 261:15316-15326.

Hajós F, Patel AJ, Balázs R (1973) Effects of thyroid deficiency on the synaptic organization of the cerebellar cortex. Brain Res 50:387-401.

Kalaria RN, Prince AK (1985) Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neurons markers and DNA concentrations in the rat corpus striatum. Int J Dev Neurosci 3:655-666.

Klein AH, Meltzer S, Kennedy FN (1972) Improved prognosis in congenital hypothyroidism treated before age 3 months. J Pediatr 81:912-915.

Lakshmanan J, Mansfield H, Weichsel ME, Hoath S (1981) Neonatal hypothyroidism - A biochemical disorder of α-tubulin metabolism. Biochem Biophys Res Commun 100:1587-1596.

Large TH (1989) Structure and developmental expression of nerve growth factor receptor in the chicken nervous system. Neuron 2:1123-1134.

Legrand C, Clos J (1991) Biochemical, immunocytochemical and morphological evidence for an interaction between thyroid hormone and nerve growth factor in the developing cerebellum of normal and hypothyroid rats. Dev Neurosci 13:382-396.

Legrand J (1967) Analyse de l'action morphologique des hormones thyroidienes sur le cervelet du jeune rat. Arch Anat Microsc Morphol Exp 56:205-244.

Legrand J (1979) Morphogenetic action of thyroid hormones. Trends Neurosci 2:234-236.

Legrand J (1983) Hormones thyroïdiennes et maturation du système nerveux. J Physiol (Paris) 78:603-652.

Legrand J (1984) Effects of thyroid hormones on central nervous system. In: Neurobehavioral Teratology (Yanay J ed), pp 331-363. Amsterdam: Elsevier.

Liu YC, Storm DR (1990) Regulation of free calmodulin levels by neuromodulin: neuron growth and regeneration. Trends Pharmacol Sci 11:107-111.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Weigand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990) NT-3, BDNF and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. Neuron 5:501-509.

Matthieu JM, Roch J, Eng L, Honegger P (1987) Triiodothyronine controls myelin protein gene expression in aggregating brain cell cultures. Ann Endocrinol 48:164-169.

Mentaberry A, Adesnik M, Atchison M, Norgard EM, Alvarez F, Sabatini DD, Colman DR (1986) Small basic proteins of myelin from central and peripheral nervous systems are encoded by the same gene. Proc Natl Acad Sci U S A 83:1111-1114.

Miller FD, Naus CCG, Durand M, Bloom FE, Milner RJ (1987) Isotypes of α-tubulin are differentially regulated during neuronal maturation. J Cell Biol 105:3065-3073.

Miller FD, Tetzlaff W, Bisby MA, Fawcett JW, Milner RJ (1989) Rapid induction of the major embryonic alpha-tubulin mRNA, T alpha 1, during nerve regeneration in adult rats. J Neurosci 9:1452-1463.

Miller FD, Mathew TC, Toma JG (1991) Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. J Cell Biol 112:303-312.

Muñoz A, Rodriguez Pena A, Perez Castillo A, Ferreiro B, Sutcliffe JG, Bernal J (1991) Effects of neonatal hypothyroidism on rat brain gene expression. Mol Endocrinol 5:273-280.

Nicholson JL, Altman J (1972a) The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. I. Cell proliferation and differentiation. Brain Res 44:13-23.

Nicholson JL, Altman J (1972b) The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. II. Synaptogenesis in the molecular layer. Brain Res 44:25-36.

Nunez J (1985) Microtubules and brain development: the effects of thyroid hormones. Neurochem Int 32:959-968.

Pioro EP, Ribeiro-da-Silva A, Cuello AC (1991) Similarities in the ultrastructural distribution of nerve growth factor receptor-like immunoreactivity in cerebellar Purkinje cells of the postnatal and colchicine-treated adult rat. J Comp Neurol 305:189-200.

Pioro EP, Cuello AC (1988) Purkinje cells of adult rat cerebellum express nerve growth factor receptor immunoreactivity: light microscopic observations. Brain Res 455:182-186.

Pioro EP, Cuello AC (1990a) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system--II. Brainstem, cerebellum and spinal cord. Neuroscience 34:89-110.

Pioro EP, Cuello AC (1990b) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system: Effect of colchicine and correlation with the cholinergic system-I.Forebrain. Neuroscience 24:57-87.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Raiti S, Newns GH (1971) Cretinism: early diagnosis and its relation to mental prognosis. Arch Dis Child 46:692-694.

Rami A, Patel AJ, Rabié A (1986) Thyroid hormone and development of the rat hippocampus: morphological alterations in granule and pyramidal cells. Neuroscience 19:1217-1226.

Reynolds R, Wilkin GP (1988) Development of macroglial cells in rat cerebellum II. An in situ immunohistochemical study of oligodendroglial lineage from precursor to mature myelinating cell. 102 Development:409-425.

Rodríguez-Tébar A, Dechant G, Barde Y-A (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rodríguez-Tébar A, Dechant G, Götz R, Barde Y-A (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J 11:917-922.

Semenenko FM, Bramwell S, Sidebottom E, Cuello AC (1985) Development of a mouse antiperoxidase secreting hybridoma for use in the production of a mouse PAP complex for immunocytochemistry and as a parent cell line in the development of hybrid hybridomas. Histochemistry 83:405-408.

Shanker G, Rao GS, Pieringer RA (1984) Investigations on myelinogenesis in vitro: regulation of 5'-nucleotidase activity by thyroid hormone in cultures of dissociated cells from embryonic mouse brain. J Neurosci Res 11:263-270.

Shanker GA, Campagnoni AT, Pieringer RA (1987) Investigations on myelinogenesis in vitro: developmental expression of myelin basic protein mRNA and its regulation by thyroid hormone in primary cell cultures from embryonic mice. J Neurosci Res 17:220-224.

Siegrist Kaiser CA, Juge Aubry C, Tranter MP, Ekenbarger DM, Leonard JL (1990) Thyroxine-dependent modulation of actin polymerization in cultured astrocytes. A novel, extranuclear action of thyroid hormone. J Biol Chem 265:5296-5302.

Skene JHP, Jacobson RD, Snipes GJ, McGuire CB, Norden JJ, Freeman JA (1986) A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. Science 233:783-786.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127-156.

Taniuchi M, Johnson EM, Jr. (1985) Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the nerve growth factor receptor. J Cell Biol 101:1100-1106.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA (1991) Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11:2528-2544.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165-170.

Wanaka A, Johnson EM, Jr. (1990) Developmental study of nerve growth factor receptor mRNA expression in the postnatal rat cerebellum. Dev Brain Res 55:288-292.

APPENDIX B

EFFECTS OF PERINATAL HYPO- AND HYPERTHYROIDISM ON THE LEVELS OF NERVE GROWTH FACTOR AND ITS LOW-AFFINITY RECEPTOR IN CEREBELLUM

B.C. Figueiredo, U. Otten, S. Strauss, B. Volk and D. Maysinger

ABSTRACT

Deficits or excesses of thyroid hormones during critical periods of mammalian cerebellar development can lead to profound biochemical and morphological abnormalities in this system. The goal of this study was to investigate the effects of perinatal hypo- and hyperthyroidism on the ontogeny of nerve growth factor (NGF) and its low-affinity receptor ($p75^{NGFR}$) in the rat cerebellum. The concentration of NGF and of $p75^{NGFR}$ immunoreactivity (IR) were measured, several days after birth, in cerebelli of rats which had received propylthiouracil (PTU) or thyroxine. NGF concentration was markedly enhanced only on postnatal day two (P2) in hyperthyroid rats, whereas in hypothyroid (PTU-treated) rats, NGF values were similar to age-matched controls. These observations suggest that thyroid hormone affects NGF synthesis during early periods of cerebellar development. In Purkinje cells of control animals, $p75^{NGFR}$ IR peaked at P10. In hypothyroid rats, the expression of $p75^{NGFR}$ was retarded, peaking at P15, whereas in hyperthyroid rats it was advanced, peaking at P8. The increased $p75^{NGFR}$ IR found in Purkinje cell bodies and the delayed disappearance of $p75^{NGFR}$ IR from the external granular layer of hypothyroid rats suggest different roles for thyroid hormone in the developing cerebellum. We conclude that $p75^{NGFR}$ and NGF are independently regulated by thyroid hormone during critical periods of cerebellar development. The effect of thyroid hormone deficiency in $p75^{NGFR}$ content in Purkinje cells may involve complex mechanisms such as impaired efficiency of axonal transport.

INTRODUCTION

The cerebellar cortex is a structure with well-defined afferent and efferent connections, in which neuron development is largely postnatal. These features facilitate the study of drug effects on Purkinje cell differentiation and on proliferation and maturation of cerebellar microneurons after birth (Altman, 1972). Imbalances in thyroid hormone levels during critical periods of cerebellar development have significant affects on the proliferation and differentiation of cerebellar neurons (Clos and Legrand, 1973; Legrand, 1983; Nicholson and Altman, 1972; Chaudhury et al., 1985). These studies

have shown alterations in the number and size of cerebellar neurons and their axonal density. The length, density and extent of dendritic branching of these cerebellar neurons are also changed. Reduced neurite outgrowth in the nervous system of hypothyroid animals has been attributed, in part, to impairments in actin filament formation (Faivre Sarrailh and Rabié, 1988) and in microtubule assembly (Faivre et al., 1983; Nunez, 1985). A neuronal form of thyroid hormone receptor has been identified (Forrest et al., 1991; Mitsuashi et al., 1988; Thompson et al., 1987), thus supporting the notion that thyroid hormones act directly on the brain.

The presence of the low-affinity nerve growth factor receptor ($p75^{NGFR}$) (Pioro and Cuello, 1988; Pioro et al., 1991; Rodríguez-Tébar et al., 1990) and its mRNA (Buck et al., 1988; Wanaka and Johnson, Jr., 1990) in the cerebellum indicates that NGF may play an important role in cerebellar development. Preliminary studies proposed that thyroid hormones induce the synthesis of NGF (Walker et al., 1979; 1981), potentiate the effect of NGF (Hayashi and Patel, 1987; Patel et al., 1988), or may somehow regulate the NGF gene (Dracopoli et al., 1988). A higher expression of neurotrophin-3 (NT-3) than of NGF during early postnatal development of the cerebellum (Legrand and Clos, 1991) also suggests a role for NT-3 in the differentiation or establishment of early connections of cerebellar neurons. The well known over-expression of $p75^{NGFR}$ and its probable involvement in the maturation and differentiation of developing cerebellar cortical neurons constitutes an excellent developmental system to study its regulation by thyroid hormone.

In this study, neonatal rats were made either hypo- or hyperthyroid in order to evaluate changes in NGF levels and in the expression of $p75^{NGFR}$ in the developing cerebellum. These parameters were chosen to test the hypothesis of whether thyroid hormone can induce the synthesis of NGF and $p75^{NGFR}$. In order to establish the extent of these changes within an approximate one month postnatal period, we quantified $p75^{NGFR}$ IR and measured concentrations of NGF in the cerebellum of euthyroid, hypothyroid, and hyperthyroid rats at different neonatal ages. These data provide evidence consistent with an involvement of thyroid hormones in the early regulation of NGF and $p75^{NGFR}$ expression in both retarded and accelerated cerebellar development.

MATERIALS AND METHODS

Animals and drug administration

Pregnant Wistar rats were fed a diet containing 0.4% propylthiouracil (PTU) beginning on embryonic day 19 (E19) as described by Kalaria and Prince (Kalaria and Prince, 1985) in order to render their pups hypothyroid. The PTU was mixed with 6% sucrose to ensure food consumption was equivalent to that of control dams. Food intake was monitored daily and the pups from dams that had not ingested their PTU-containing food regularly since the second day of treatment were not used for the study. On the day of birth (P0) other male pups were pooled and randomly assigned to produce litters of 6 pups per dam. From P0 until the day of sacrifice, these pups were injected subcutaneously each day with wither physiological saline (control group) or 0.3 μm/gm body weight thyroxine (T4) (hyperthyroid group). Neonatal naive pups were also included in the control group. All hypothyroid pups remained with their dams until the day of perfusion or decapitation, but control and hyperthyroid animals surviving more than three weeks were weaned at the end of the third postnatal week. Pups were sacrificed by perfusion for immunocytochemistry processing from P7 to P30. Determination of NGF levels in cerebellar tissue was done in treated and control animals on P2, P15 and P30 (n=6 to 10 per group).

Immunocytochemistry

The processing of cerebella for immunocytochemistry was essentially as previously described (Pioro and Cuello, 1990), except that pups younger than P30 were perfused with smaller volumes of fixative, according to their ages and body weights. Rats were deeply anaesthetized with Equithesin (0.35 ml/100 g i.p.) and perfused briefly through the ascending aorta with a phosphate buffer (PB; pH 7.4) vascular rinse. Subsequently, a 20 min fixation with a 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid mixture in 0.1 M phosphate buffer (pH 7.2) was immediately followed by a 20 min perfusion with 0.5% hydrogen peroxide in phosphate-buffered saline (PBS). A final 20 min washout with a 10% glucose-PB solution was followed by

removal and storage of cerebella in 30% sucrose-PB at 4°C for 72-120 h. Coronal sections (50 μm thick) were cut on a freezing sledge microtome.

Free-floating sections were incubated overnight at 4°C in supernatant from 192-IgG hybridoma cells (generously provided by E. M. Johnson) and with 10% horse serum or with a non-specific mouse IgG. Characterization of the 192-IgG anti-$p75^{NGFR}$ have previously been outlined in detail (Chandler et al., 1984; Taniuchi and Johnson, Jr., 1985). Because other neurotrophins also appear to bind to $p75^{NGFR}$ (see Discussion) $p75^{NGFR}$-IR should be taken to represent $p75^{NGFR}$ like-IR. All subsequent steps were carried out at room temperature, and PBS containing 0.2% Triton X-100 (pH 7.4) was used for washes, dilution of antibodies + horse serum, as well as for the 3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. After washing, sections were incubated for 2 h in rabbit anti-mouse IgG (1/50) and then washed again. Subsequently, the sections were incubated for 1 h in monoclonal mouse peroxidase-antiperoxidase (Semenenko et al., 1985) (PAP; 1/30; Medicorp, Canada). After washing for 5 min (4x), sections were incubated in a 0.06% solution of DAB for 10 min and a subsequent 8 min in the same solution containing 0.01% hydrogen peroxide (final concentration). After washing, sections were mounted on subbed slides, dehydrated, cleared and coverslipped. The relative optical density measurements of $p75^{NGFR}$-IR in Purkinje cell bodies were obtained from multiple sections using the Quantimet 920 (Cambridge Instruments, UK) image analysis system. In control animals, cerebellar sections quantified were from days 8 to 11. In hyperthyroid and hypothyroid rats they were from days 7-10 and 14-17, respectively. These age-points were chosen because above or below these periods proper detection of $p75^{NGFR}$ IR was not possible either because Purkinje cells were not well defined or the immunoreactivity was too low. The $p75^{NGFR}$ staining intensity was well reproduced in three different immunocytochemical experiments. All data (Figs. 3 and 4) were analyzed using ANOVA and the Newman-Keuls' *post hoc* test, with a level of significance of $P<0.05$.

Enzyme linked immunoassay (ELISA) for NGF

NGF concentrations were measured in cerebellar tissue of euthyroid (n = 6), hyperthyroid (n = 6) and hypothyroid (n = 6) animals at days P2, P15 and P30. Cerebella of all experimental groups were removed on ice at postnatal days 2, 15 and 30. Each cerebellum was sonicated in 750 μl of sample buffer (PBS/0.05% Tween/0.5% bovine serum albumin) containing enzyme inhibitors (0.1 M phenylmethylsulfonyl-fluoride, 0.1 benzethonium chloride and 0.1% aprotinin). Sonicated tissue was centrifuged (12,000 rpm, at 4°C) for 5 min and supernatant aliquots (100 μl) were used for NGF immunoassay (ELISA). A two-site enzyme-linked immunoassay (ELISA) was used to measure NGF content (Weskamp and Otten, 1987). Briefly, polystyrene 96 well microtiter immunoplates (Nunc, Copenhagen, Denmark) were coated with polyclonal goat anti-NGF anti-bodies or with non-immune goat serum. All incubations were performed in a moist chamber and, after each incubation step, the plates were washed four times with phosphate-buffered saline (PBS/0.05% Tween 20). After an 8 h coating step at 4°C, nonspecific binding was blocked with PBS/0.05% Tween 20 containing 1% fetal calf serum for 2 h at 20°C. One hundred μl samples to be tested for NGF were added to each well and were incubated for 12 h at 4°C. Each plate contained a complete standard curve ranging from 0 to 100 pg/ml of NGF. Six μg of monoclonal antibody against NGF (clone 23c4 or clone 30) per well was added and incubated for 9 h at 4°C. Biotinylated goat immunoglobulins (1:8000) (Zymed, USA) were incubated overnight at 4°C and the procedure was continued by adding peroxidase-conjugated streptavidin (Zymed, USA) for 3 h at 4°C. Immune sandwiches were detected by the addition of *o*-phenylenediamine (4mg/plate) 4 in a freshly prepared solution containing 5 μl of 30% H_2O_2 in 10 ml substrate buffer. The reaction proceeded for 7-10 min at 20°C and was stopped with H_2SO_4. Optical density was measured at 490 nm using a Dynatech MR 700 microplate reader. Absorbance of samples and standards was corrected for nonspecific binding (i.e. the absorbance in wells coated with normal goat serum). The NGF content in the samples was determined in relation to a NGF standard curve. Data were not corrected for recovery of NGF from the samples, which was routinely 70-90% and were accepted only when values were greater than two standard deviations above the blank. Using these criteria, the limit of sensitivity of the NGF ELISA averaged 1 pg per assay.

RESULTS

Physical and behavioral signs of hypo- and hyperthyroidism

The physical and behavioral effects of perinatal hypothyroidism were, for the most part, similar to those previously described (Gould and Butcher, 1989). The adult body, cerebral and cerebellar sizes and weights (Nicholson and Altman, 1972) were reduced in both experimental groups as compared to age-matched controls. In normal pups, coordinated creeping was observed around P9-11, fully opened eyes between P11 and P13 and exploratory behavior was more evident around P13-16. In hyperthyroid animals, these signs were observed three-five days earlier and were usually accompanied by motor hyperactivity and fine tremor. However, in hypothyroid neonates, the coordinated creeping, opening of the eyes and the innate exploratory behavior were retarded 7-12 days. Accentuated motor deficits persisted in hypothyroid animals up to the oldest age examined, i.e. 30 days after interruption of PTU treatment (P30).

$p75^{NGFR}$ immunocytochemistry

The distribution of $p75^{NGFR}$-IR, as revealed by the monoclonal antibody 192-IgG, was studied in selected cerebellar regions of crus 1 ansiform lobule and simple lobule (Fig. 1) (Paxinos and Watson, 1986). Light microscopic examination of $p75^{NGFR}$ positive neurons revealed maximal density of immunoreactive material in the external granular layer, mostly distributed in the proliferative zone, in all experimental groups. The quantification was not processed at the same age-points for all three groups because the period of minimal detectable $p75^{NGFR}$ IR in individual Purkinje cell bodies occurred at three different periods (Fig. 3). In control animals, increased intensity of $p75^{NGFR}$ immunostaining, synchronous with the growth of Purkinje cells, was observed during the first 10 postnatal days. In Purkinje cells of hypothyroid rats, the most intense expression of $p75^{NGFR}$ immunoreactivity was retarded until (P15) (Fig. 2a) whereas the highest intensity was found on P10 in control animals (Fig. 2b). In hyperthyroid pups, the

Figure 1

Coronal sections through the crus 1 ansiform lobule and simple lobule of cerebellum of 15-day-old (a,b,c) and 30-day-old (d,e,f) rats. Developmental changes in $p75^{NGFR}$ IR can be observed in hypothyroid (a,d), euthyroid (b,e) and hyperthyroid rats (c,f) in Purkinje cell layer (arrows), molecular layer (ml), internal granular layer (igl) and external granular layer (egl). Very intense $p75^{NGFR}$ IR is observed in Purkinje cells and the external granular layer of hypothyroid rats in contrast to age-matched controls and hyperthyroid rats. Hyperthyroid rats show much less $p75^{NGFR}$ IR than controls. Bar = 63 μm.

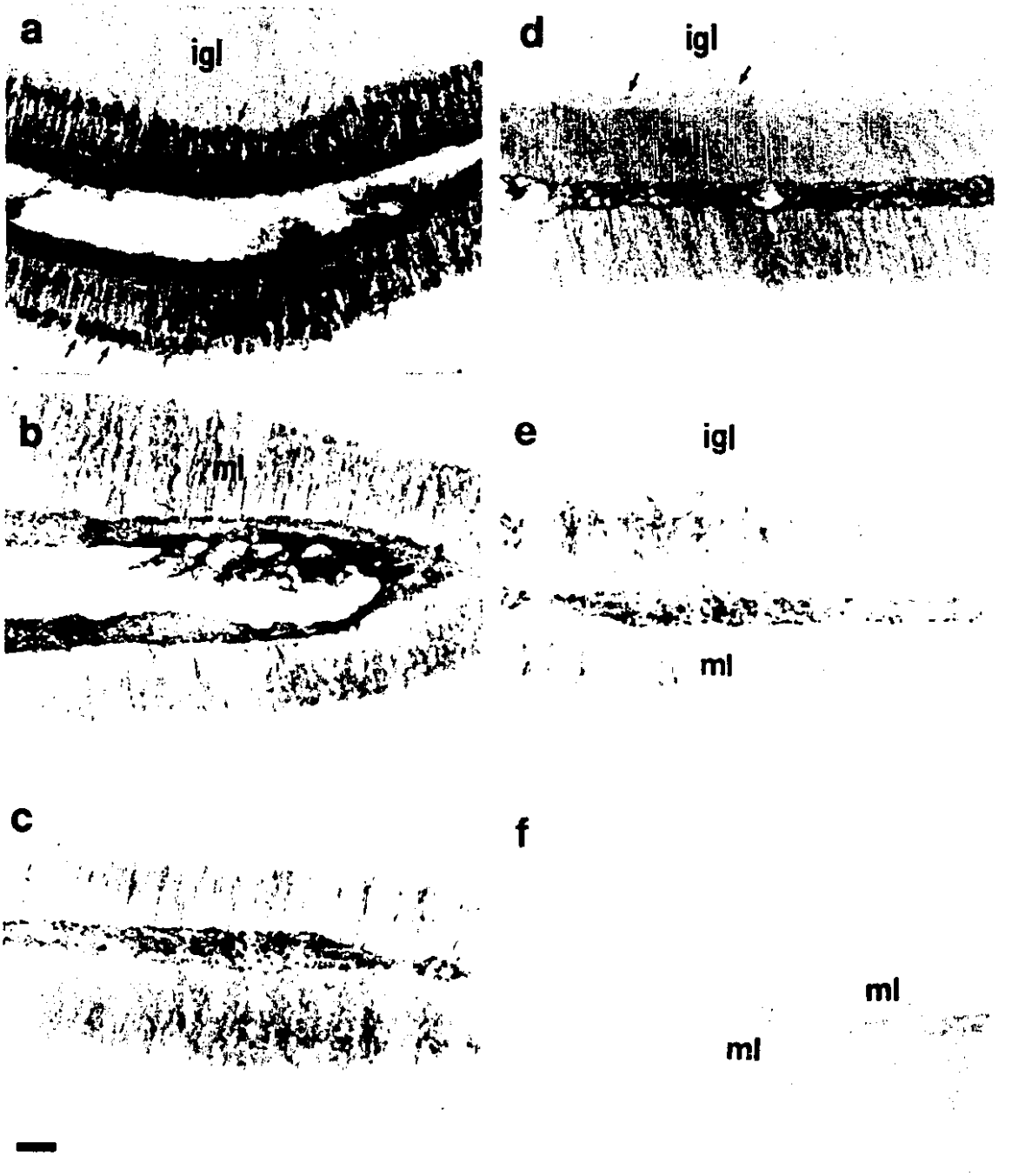
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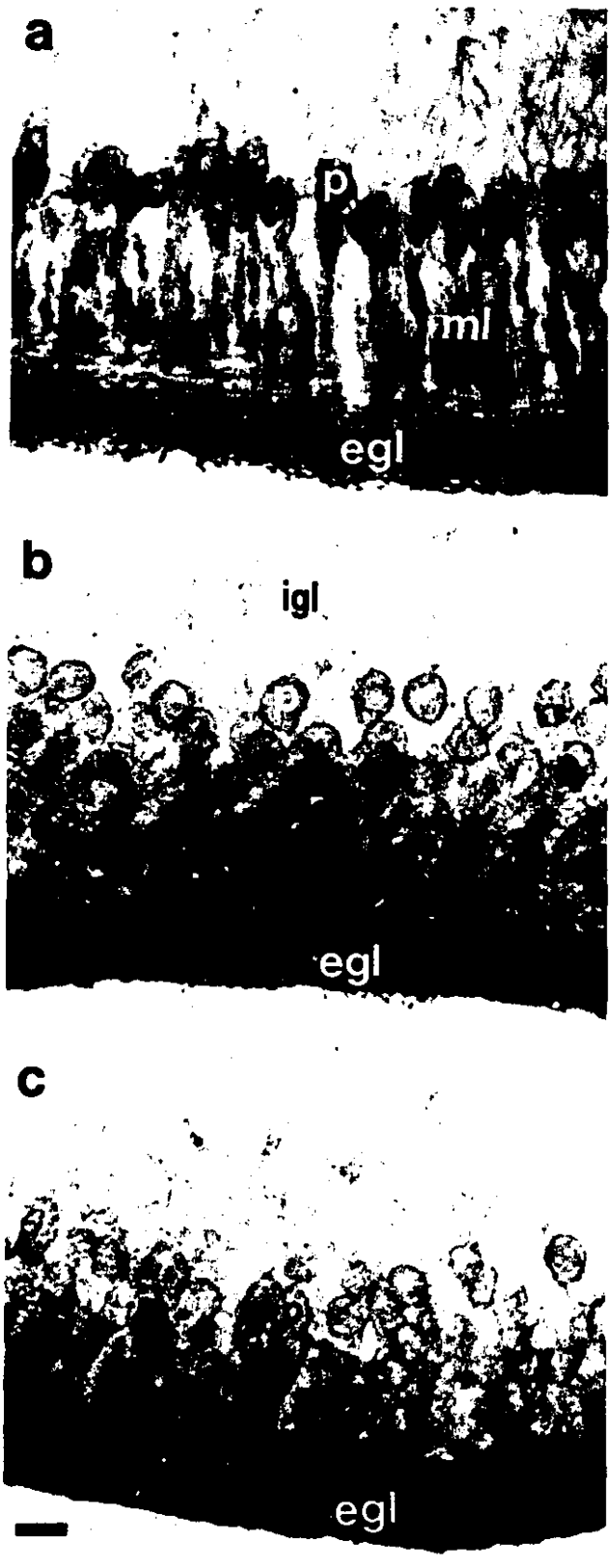
Figure 2

Coronal sections showing the cerebellar cortex through the crus 1 ansiform lobule. The photomicrographs were taken on the day with maximal $p75^{NGFR}$ IR in Purkinje cells in hypothyroid (a = PD15), control (b = PD10) and hyperthyroid (c = PD8) rats. Note the marked increase in immunoreactivity in Purkinje cells of hypothyroid rats (Fig. 2a). Abbreviations: Purkinje cell (p); molecular layer (ml); internal granular layer (igl) and external granular layer (egl). Bar = 20 μm.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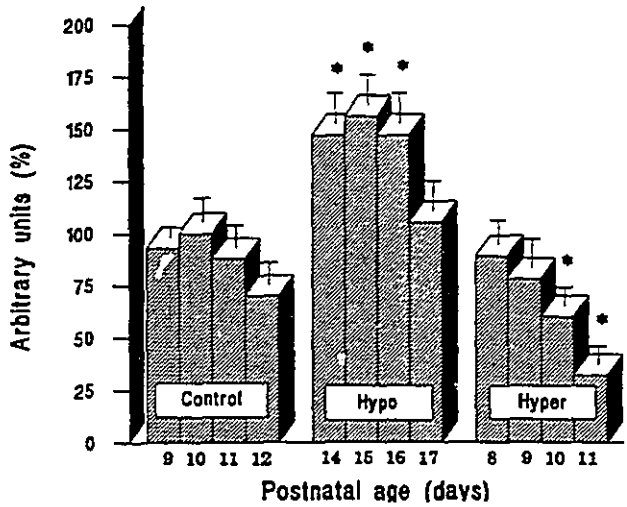

Fig. 3. Relative optical density of p75NGFR IR (in % of arbitrary units) in Purkinje cell bodies (partially shown in Fig. 2). In each group, the 4 consecutive ages represent maximal immunostaining found in comparison to other ages in the same group. Bars refer to means ($n = 6$–8) ±S.E.M. * $P < 0.05$ vs. maximal control value (i.e. at P10) (ANOVA, Newman–Keuls' post-hoc test).

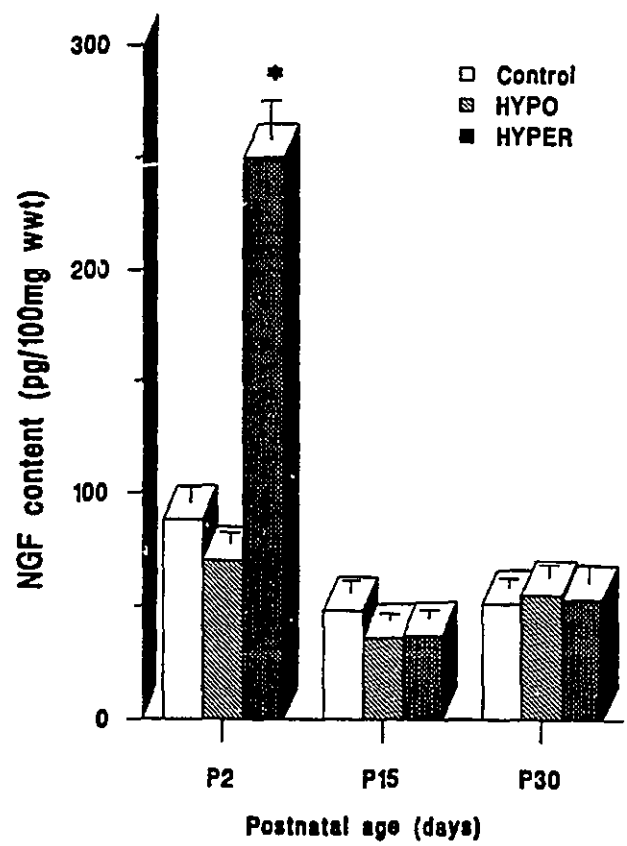

Fig. 4. NGF content in developing cerebellum of control ($n = 6$–10/group), thyroid hormone deficient (hypo, $n = 6$–10/group) and hyperthyroid rats (hyper, $n = 6$–12/group). Bars refer to means± S.E.M. * $P < 0.05$ vs. age-matched control (ANOVA, Newman–Keuls' post hoc test).

maximal density of $p75^{NGFR}$ IR in Purkinje cells was found on P8 (Fig. 2c). The maximal immunoreactivity (arbitrary units, Fig. 3) of the $p75^{NGFR}$ immunoreactive material determined in Purkinje cell bodies of hyperthyroid pups (shown in Fig. 2a) was significantly higher than that found in respective controls (Fig. 2b) or in hyperthyroid rats (Fig. 2c). $p75^{NGFR}$ immunoreactivity in naive animals was not significantly different from saline-treated controls.

As the Purkinje cells became more differentiated (after P10), the immunoreactivity decreased (Fig. 1, compare a, b, c corresponding to P15 with d,e,f at P30). $p75^{NGFR}$ IR was noticeably higher in the external granular layer and Purkinje cells of hypothyroid animals (Fig. 1a,d) compared to age-matched controls (Fig. 1b,e) and hyperthyroid rats (Fig. 1c,f). A sparse pattern of $p75^{NGFR}$ staining was observed in the molecular layer of hypothyroid neonates on P15 (Fig. 1a), but was observed in younger control animals only. Occasional Purkinje cells with positive immunoreactive cytoplasms were seen dispersed over the cortex of the oldest examined hypothyroid rats (P30, Fig. 1d) and the intensity of immunostaining in the cortex was more pronounced than in age-matched control rats (Fig. 1e). On the other hand, the pattern of $p75^{NGFR}$ IR in hyperthyroid animals on P30 (Fig. 1f) was similar to that of normal mature rats.

NGF concentrations

NGF concentrations were measured in euthyroid, hypo- and hyperthyroid rats at postnatal days 2, 15 and 30. There was a significant increase in NGF content in hyperthyroid rats only on P2 in comparison with age-matched controls (Fig. 4). Levels in naive pups did not differ from their saline-treated counterparts. The treatment with PTU did not significantly affect the NGF content in the cerebellum at any of the three time points examined.

DISCUSSION

The present study demonstrates that prolonged thyroid deficiency in rodents can delay the time-course as well as accentuate the intensity of $p75^{NGFR}$ IR in cerebellar Purkinje cells during early maturation. A rise in $p75^{NGFR}$ IR in Purkinje cells correlates

with the retarded maturation of the cerebellar cortex in hypothyroid rats (Altman, 1972; Nicholson and Altman, 1972; Chaudhury et al., 1985; Rabié et al., 1979), and thus, could represent part of a more general neuronal reaction which occurs in the developing cerebellum following insult. If this were the case, then $p75^{NGFR}$ could be activated by one or more neurotrophins some how implicated in trophic responses in developing Purkinje or other cerebellar cell types. NGF, and other neurotrophins such as BDNF, NT-3 and neurotrophin-4 (NT-4) can bind with similar affinities to the $p75^{NGFR}$ (Hallböök et al., 1991; Rodríguez-Tébar et al., 1990; 1992), suggesting that any of these neurotrophins can interact with $p75^{NGFR}$ during postnatal cerebellar development. Therefore, rat 192-IgG antibody appears to recognize a common neurotrophin receptor, $p75^{NGFR}$ (Ernfors et al., 1990; Green and Greene, 1986; Rodríguez-Tébar et al., 1990; 1992; Taniuchi and Johnson, Jr., 1985), and is described in this paper as representing $p75^{NGFR}$-like IR. Our results on the time-course of $p75^{NGFR}$-like IR in the developing cerebellum of normal rats correlate with previous reports of $p75^{NGFR}$ mRNA (Buck et al., 1988; Walker et al., 1979) levels and/or high affinity NGF-binding sites (Cohen-Cory et al., 1989).

The increased $p75^{NGFR}$ IR seen in hypothyroid rats was anatomically selective. IR in Purkinje cells in hypothyroid rats was increased relative to control. IR in the external granular layer was similar for experimental control rats. This latter cerebellar region was richest in $p75^{NGFR}$ IR during the first two postnatal weeks in control and experimental animals. The retarded appearance and increase amount of $p75^{NGFR}$ immunoreactive material found in hypothyroid rats may also involve alterations at the post-transcriptional level since results of $p75^{NGFR}$ mRNA in these animals [reported elsewhere (Figueiredo et al., 1993)] indicate a retarded expression but no increase in level relative to control. Thus, the changes in $p75^{NGFR}$ IR in hypothyroid rats could not simply be attributed to an active neuronal response resulting in increased $p75^{NGFR}$ synthesis. These phenomena may also represent passive accumulation of the receptor in the Purkinje cell bodies possibly due to an impairment in the axonal transport system. Consistent with this hypothesis there is evidence (Faivre et al., 1983; 1985; Francon et al., 1977; Nunez, 1985) that specific modifications occur in cytoskeletal components of

neurons, including Purkinje cells, in hypothyroid rats. In addition, $p75^{NGFR}$ immunostaining in mature rats treated with an inhibitor of axonal transport (colchicine) resembled the pattern observed in the developing cerebellum on day P12 (Pioro et al., 1991) and in rats of the present study.

Previous investigators (Legrand and Clos, 1991) showed a decrease in $p75^{NGFR}$ IR in the cerebellar cortex of hypothyroid rats on P10, also seen by us, but failed to show a retarded increase in this receptor because they did not examine effects at further age-points. The sparse pattern of $p75^{NGFR}$ immunostaining in the molecular layer of hypothyroid pups may suggest retardation in Purkinje cell dendritic growth and branching as well as spine formation (Legrand, 1979) and/or a decrease in synapse density in the molecular layer (Chaudhury et al., 1985; Vincent et al., 1982). Thyroid hormone deficiency did not increase $p75^{NGFR}$ IR in the external granular layer; however, there was a retarded disappearance of $p75^{NGFR}$ IR in this layer in hypothyroid rats. Such a finding was also reported in a recent study using antibody anti-$p75^{NGFR}$ (Legrand and Clos, 1991). These results suggest that thyroid hormone and neurotrophins, acting via $p75^{NGFR}$, may play an important role in the maturation of the external granular layer.

Thyroxine treatment brought forward the $p75^{NGFR}$ IR peak in Purkinje cells by only two days. This suggests considerable autonomy of $p75^{NGFR}$ regulation in Purkinje cells during the early phase of thyroxine treatment. The rapid decrease in $p75^{NGFR}$ IR observed in the external granular layer between the second and the third week of thyroxine treatment was in accordance with the previously described early termination of cell proliferation and disappearance of the external granular layer (Chaudhury et al., 1985). The pale immunostaining in the cerebellar cortex observed on P30 of thyroxine-treated rats resembled that found in adult normal animals in the present study (data not shown) and in a previous report (Pioro and Cuello, 1988). This similarity indicates a rapid down regulation of $p75^{NGFR}$ in thyroxine-treated rats. In contrast to our observations in the developing cerebellum, the distribution of $p75^{NGFR}$ IR in the developing forebrain following PTU-treatment decreased $p75^{NGFR}$ IR, while no alteration was detected for $p75^{NGFR}$ IR in thyroxine-treated pups (Oh et al., 1991). These discrepancies in the effects of postnatal hypo- and hyperthyroidism may be related to

different sensitivities of regionally selective neurons and may indicate that $p75^{NGFR}$ is regulated differentially by neuronal and environmental factors. Mutation of the gene encoding $p75^{NGFR}$ (Lee et al., 1992) suggested that $p75^{NGFR}$ has an important role in the development and function of sensory neurons of mice. However, these investigators have not yet extended their studies on possible morphological or functional alterations in the central nervous system of these mutated mice.

NGF levels in control animals at P15 were reduced 50% from those at P2, but they were not statistically different from those at P30 (Fig. 4). This would suggest a physiological role for NGF during early cerebellar development. Why PTU did not show an effect on NGF content, as opposed to the stimulatory effect by thyroxine on P2, could be due to an incomplete blockage of thyroid hormone synthesis by PTU or to the residual effect of maternal thyroid hormone transferred to the fetus late in gestation (Mitsuashi et al., 1988; Obregón et al., 1984). Consistent with the absence of thyroid hormone effect on NGF mRNA levels (Muñoz et al., 1991), our findings also suggest that thyroid hormone is not required for NGF synthesis at P15 and P30. Preliminary results (from this laboratory) also indicate absence of a thyroid hormone effect on NGF mRNA at P2. This indicates that the increase in NGF levels at P2 probably involve post-translational mechanisms which take place at an earlier postnatal developmental stage of cerebellar neurons. The absence of significant changes in NGF content in hypo- and hyperthyroid rats in comparison with age-matched controls on P15 and P30 does not agree with previous studies in mice (Walker et al., 1979; Walker et al., 1981). Such a discrepancy could be due to species difference and/or reliability of the NGF assay used in those studies (Walker et al., 1979; 1981). Although NGF gene expression in salivary glands of mice (Black et al., 1992) seems to be regulated by the thyroid hormones, we cannot at present extrapolate these findings to a different neural system such the rat cerebellum.

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REFERENCES

Altman J (1972) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 145:399-463.

Black MA, Lefebvre F, Pope L, Lefebvre YA, Walker P (1992) Thyroid hormone and androgen regulation of nerve growth factor gene expression in the mouse submandibular gland. Mol Cell Endocrinol 84:145-154.

Buck CR, Martinez HJ, Chao MV, Black IB (1988) Differential expression of the nerve growth factor receptor gene in multiple brain areas. Dev Brain Res 44:259-268.

Chandler CE, Parsons LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 259:6882-6889.

Chaudhury S, Chatterjee D, Sarkar PK (1985) Induction of brain tubulin by triiodothyronine: dual effect of the hormone on the synthesis and turnover of the protein. Brain Res 339:191-194.

Clos J, Legrand J (1973) Effects of thyroid deficiency on the different cell populations of the cerebellum in the young rat. Brain Res 63:450-455.

Cohen-Cory S, Dreyfus CF, Black IB (1989) Expression of high- and low-affinity nerve growth factor receptors by Purkinje cells in the developing rat cerebellum. Exp Neurol 105:104-109.

Dracopoli NC, Rose E, Whitfield GK, Guidon PT, Jr., Bale SJ, Chance PA, Kourides IA, Housman DE (1988) Two thyroid hormone regulated genes, the beta-subunits of nerve growth factor (NGFB) and thyroid stimulating hormone (TSHB), are located less than 310 kb apart in both human and mouse genomes. Genomics 3:161-167.

Ernfors P, Ibáñez CF, Ebendal T, Olson L, Persson H (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to β-nerve growth factor: developmental and topographic expression in the brain. Proc Natl Acad Sci USA 87:5454-5458.

Faivre Sarrailh C, Rabié A (1988) A lower proportion of filamentous to monomeric actin in the developing cerebellum of thyroid-deficient rats. Brain Res 469:293-297.

Faivre C, Legrand C, Rabié A (1983) Effects of thyroid deficiency and corrective effects of thyroxine on microtubules and mitochondria in cerebellar purkinje cell dendrites of developing rats. Dev Brain Res 8:21-30.

Faivre C, Legrand C, Rabié A (1985) The microtubular apparatus of cerebellar Purkinje cell dendrites during postnatal development of the rat: the density and cold-stability of microtubules increase with age and are sensitive to thyroid hormone deficiency. Int J Dev Neurosci 3:559-565.

Figueiredo BC, Almazan G, Ma Y, Tetzlaff W, Miller FD, Cuello AC (1993) Gene expression in the developing cerebellum during perinatal hypo- and hyperthyroidism. Mol Brain Res 17:258-268.

Forrest D, Hallbook F, Persson H, Vennstrom B (1991) Distinct functions for thyroid homone receptors alpha and beta in brain development indicated by differential expression of receptor genes. EMBO J 10:269-275.

Francon J, Fellous A, Lennon AM, Nunez J (1977) Is thyroxine a regulatory signal for neurotubule assembly during brain development?. Nature 266:188-190.

Gould E, Butcher LL (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347-3358.

Green SH, Greene LA (1986) A single M_r=103,000 ^{125}I-β-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. J Biol Chem 261:15316-15326.

Hallböök F, Ibáñez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Hayashi M, Patel AJ (1987) An interaction between thyroid hormone and nerve growth factor in the regulation of choline acetyltransferase activity in neuronal cultures, derived from the septal-diagonal dand region of the embryonic rat brain. Dev Brain Res 36:109-120.

Kalaria RN, Prince AK (1985) Effects of thyroid deficiency on the development of cholinergic, GABA, dopaminergic and glutamate neurons markers and DNA concentrations in the rat corpus striatum. Int J Dev Neurosci 3:655-666.

Lee K-F, Huber J, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Target mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral nervous system. Cell 69:737-749.

Legrand C, Clos J (1991) Biochemical, immunocytochemical and morphological evidence for an interaction between thyroid hormone and nerve growth factor in the developing cerebellum of normal and hypothyroid rats. Dev Neurosci 13:382-396.

Legrand J (1979) Morphogenetic action of thyroid hormones. Trends Neurosci 2:234-236.

Legrand J (1983) Hormones thyroïdiennes et maturation du système nerveux. J Physiol (Paris) 78:603-652.

Mitsuashi T, Tennyson GE, Nikodem VM (1988) Alternative splicing generates messages encoding rat c-erbA proteins that do not bind thyroid hormone. Proc Natl Acad Sci U S A 85:5804-5808.

Muñoz A, Rodriguez Pena A, Perez Castillo A, Ferreiro B, Sutcliffe JG, Bernal J (1991) Effects of neonatal hypothyroidism on rat brain gene expression. Mol Endocrinol 5:273-280.

Nicholson JL, Altman J (1972) The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. I. Cell proliferation and differentiation. Brain Res 44:13-23.

Nunez J (1985) Microtubules and brain development: the effects of thyroid hormones. Neurochem Int 32:959-968.

Obregón MJ, Mallol J, Pastor R, Morrealle de Escobar G, Escobar del Rey F (1984) L-thyroxine and 3,5,3'-triiodo-L-thyronine in rat embryos before onset of fetal thyroid function. Endocrinology 114:305-307.

Oh JD, Butcher LL, Woolf NJ (1991) Thyroid hormone modulates the development of cholinergic terminal fields in the rat forebrain: Relation to nerve growth factor receptor. Dev Brain Res 53:133-142.

Patel AJ, Hayashi M, Hunt A (1988) Role of thyroid hormone and nerve growth factor in the development of choline acetyltransferase and other cell-specific marker enzymes in the basal forebrain of the rat. J Neurochem 50:803-811.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Pioro EP, Ribeiro-da-Silva A, Cuello AC (1991) Similarities in the ultrastructural distribution of nerve growth factor receptor-like immunoreactivity in cerebellar Purkinje cells of the postnatal and colchicine-treated adult rat. J Comp Neurol 305:189-200.

Pioro EP, Cuello AC (1988) Purkinje cells of adult rat cerebellum express nerve growth factor receptor immunoreactivity: light microscopic observations. Brain Res 455:182-186.

Pioro EP, Cuello AC (1990) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system: Effect of colchicine and correlation with the cholinergic system-I.Forebrain. Neuroscience 24:57-87.

Rabié A, Faivre C, Clavel MC, Legrand J (1979) Sequential effects of thyroxine on the developing cerebellum of rats made hypothyroid by propylthiouracil. Brain Res 161:469-479.

Rodríguez-Tébar A, Dechant G, Barde Y-A (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487-492.

Rodríguez-Tébar A, Dechant G, Götz R, Barde Y-A (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J 11:917-922.

Semenenko FM, Bramwell S, Sidebottom E, Cuello AC (1985) Development of a mouse antiperoxidase secreting hybridoma for use in the production of a mouse PAP complex for immunocytochemistry and as a parent cell line in the development of hybrid hybridomas. Histochemistry 83:405-408.

Taniuchi M, Johnson EM, Jr. (1985) Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the nerve growth factor receptor. J Cell Biol 101:1100-1106.

Thompson CC, Weinberger C, Lebo R, Evans RM (1987) Identification of a novel thyroid hormone receptor expressed in the mammalian central nervous system. Science 237:1610-1614.

Vincent J, Legrand C, Rabie A, Legrand J (1982) Effects of thyroid hormone on synaptogenesis in the molecular layer of the developing rat cerebellum. J Physiol (Paris) 78:729-738.

Walker P, Weichsel ME, Fisher DA, Guo SM (1979) Thyroxine increases nerve growth factor concentration in adult mouse brain. Science 204:427-429.

Walker P, Weil ML, Weichsel ME, Jr., Fisher DA (1981) Effect of thyroxine on nerve growth factor concentration in neonatal mouse brain. Life Sci 28:1777-1787.

Wanaka A, Johnson EM, Jr. (1990) Developmental study of nerve growth factor receptor mRNA expression in the postnatal rat cerebellum. Dev Brain Res 55:288-292.

Weskamp G, Otten U (1987) An enzyme-linked immunoassay for nerve growth factor (NGF): a tool for studying regulatory mechanisms involved in NGF production in brain and peripheral tissues. J Neurochem 48:1779-1786.

APPENDIX C

AUTHORIZATION TO REPRINT