NERVE GROWTH FACTOR AND/OR MONOSIALOGANGLIOSIDE GM1-INDUCED NEUROPLASTICITY IN BRAIN OF DECORTICATED ADULT AND AGED RATS

by

Lorella Garofalo

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Nerve growth factor- and/or ganglioside GM1-induced plasticity in vivo

Lorella Garofalo
Department of Pharmacology and Therapeutics PhD

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ABSTRACT

The ability of two putative trophic agents, nerve growth factor (NGF) and the monosialoganglioside GM1, to induce neurochemical, morphological and behavioral recovery following injury to the adult rat basalo-cortical cholinergic pathway was studied. Treatment of unilaterally decorticated rats with these agents was shown to: attenuate deficits in cholinergic markers of the nucleus basalis magnocellularis (NBM), prevent shrinkage of choline acetyltransferase (ChAT)- and $p75^{NGFR}$-immunoreactive (IR) NBM neurons, and stimulate cortical ChAT activity and high affinity choline uptake, in a dose-dependent manner with equal efficacies but different potencies. Quantitative light and electron microscopic studies, assisted by image analysis, showed that GM1 or NGF treatment also similarly attenuated lesion-induced deficits in cortical ChAT-IR fiber length. By contrast, NGF, but not GM1, treatment caused significant synaptic remodelling in the remaining cortex of adult lesioned rats; this was reflected by increases in ChAT-IR varicosity number, presynaptic terminal size, and in the number of boutons with synaptic contacts. GM1 treatment only attenuated such lesion-induced deficits. Exogenous GM1 was also shown to potentiate NGF-induced effects on basalo-cortical cholinergic markers and on cortical synaptic remodelling, but did not affect the affinity or number of NGF binding sites in brain membranes isolated from lesioned animals. This suggests that GM1 probably affects an alternative step of the NGF signal transduction cascade to potentiate NGF effects. Moreover, NGF or GM1 treatment were also shown to: distinctly regulate striatal cholinergic markers, differ with respect to the delay possible in treatment time onset for effective protection from retrograde degeneration, and diversely affect the behaviour of these animals in passive avoidance and Morris water maze tasks. In aged rats (>20 months), NGF and/or GM1 treatment were also shown to effectively prevent decortication-induced retrograde degeneration of NBM

cholinergic neurons and to stimulate cortical cholinergic markers. However, in contrast to that in young adult rats, chronic (30 days) NGF and/or GM1 treatment was necessary to produce these effects.

The work of this thesis has thus provided evidence that the injured adult rat basalo-cortical cholinergic pathway can exhibit substantial neurochemical and morphological plasticity in response to NGF and/or GM1 treatment. In particular, it has been shown that these agents cause significant alterations in cholinergic innervation.

Lorella Garofalo
Département de Pharmacologie et Thérapeutique Ph.D.

LE FACTEUR DE CROISSANCE NEURONALE ET LE MONOSIALOGANGLIOSIDE GM1 PROVOQUENT UNE PLASTICITE NEURONALE DANS LE CERVEAU DE RATS DECORTIQUES ADULTES ET AGES

RESUME

Nous avons étudié la capacité de deux agents trophiques, le facteur de croissance neuronal (NGF) et le monosialoganglioside GM1, à provoquer une plasticité neurochimique, morphologique et comportementale suite à une lésion du système cholinergique basalocortical chez le rat adulte. Nous avons observé que le traitement de rats décortiqués unilatéralement avec ces agents a) atténue les déficits des marqueurs cholinergiques du noyau basal magnocellulaire (NBM), b) prévient le rétrécissement des neurones du NBM immunoréactifs (IR) à la choline acétyltransférase (ChAT) et au récepteur p75 du NGF et c) stimule, avec une efficacité égale mais une puissance relative différente, l'activité corticale de la ChAT et le système de captation à haute affinité de la choline. Des études quantitatives en microscopie optique et électronique ont montré que le traitement au GM1 ou au NGF atténue de façon similaire les déficits des projections ChAT-IR provoqués par la lésion. Toutefois, seulement le traitement au NGF induit un remodelage synaptique du cortex résiduel chez le rat lésé; ceci se reflétant par des augmentations a) du nombre de varicosités ChAT-IR, b) de la surface des terminaisons présynaptiques et c) du nombre de boutons avec contacts synaptiques. Le traitemnet au GM1 ne fait qu'atténuer les déficits provoqués par la lésion.

Nous avons aussi démontré que le GM1 augmente les effets du NGF sur les marqueurs cholinergiques de la projection NBM-cortex et sur le remodelage synaptique du cortex, sans toutefois affecter l'affinité ou le nombre de sites de liasion au NGF dans des membranes de cerveaux d'animaux lésés. Ceci suggère que le GM1 affecte probablement une étape post-réceptorielle dans la cascade de la transduction du signal induit par le NGF. Le NGF et le GM1 affectent aussi de façon différentielle les marqueurs cholinergiques striataux. En effect,

ils diffèrent en ce qui concerne le délai maximum possible avant l'initiation pour une protection efficace contre la dégénérescence rétrograde et quant au comportement des animaux lésés dans des tests d'apprentissage ("passive avoidance" et "Morris water maze"). Enfin, chez les rats âgés (>20 mois), nous avons observé que le traitement au NGF et/ou GM1 prévient la dégénérescence rétrograde induit par la décortication des neurones cholinergiques du NBM et stimule les marqueurs cholinergiques corticaux. Toutefois, contrairement aux jeunes adultes, un traitement chronique (30 jours) au NGF et/ou au GM1 est nécessaire pour induire une telle protection chez l'animal âgeé.

Les travaux présentés dans cette thèse ont donc permis de mettre en évidence que l'innervation basalo-cortical-cholinergique du rat adulte lésé répond par une plasticité neurochimique et morphologique à un traitement au NGF et/ou GM1. Plus spécifiquement, il a été démontré que ces agents causent des changements significatifs de l'innervation cholinergique corticale.

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ABBREVIATIONS USED IN THESIS

AcCoA	Acetyl Coenzyme A
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine Triphosphate
ATPase	Adenosine 5'-Triphosphate
BSA	Bovine serum albumin
Cer	Ceramide
ChAT	Choline Acetyltransferase
Ch	Choline
CMP	Cytidine Monophosphate
c.s.f.	cerebrospinal fluid
CTX	Neocortex
EDTA	Ethylenediamine Tetraacetic Acid
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
GAD	Glutamic Acid Decaroboxylase
Gal	Galactose
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcNAc	N-acetylglucosamine
GM1	monosialoganglioside GM1
gr	gram
HACU	High-Affinity Choline Uptake
HDB	Horizontal Limb of the Diagonal Band of Broca
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethansulfonic Acid
HIPP	Hippocampus
HRP	Horseradish Peroxidase
5-HT	Serotonin
i.c.v.	intracerebroventricular
i.m.	intramuscular

^{125}I-NGF	Iodinated Nerve Growth Factor
i.p.	intraperitoneal
IR	immunoreactive
l	liter
mg	milligram
ml	milliliter
NA	Noradrenaline
NAN	N-acetylneuraminic Acid (sialic acid)
NBM	Nucleus Basalis Magnocellularis
nbM	Nucleus Basalis of Meynert
NGF	Nerve Growth Factor
PAP	Peroxidase Anti-Peroxidase
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
SEPT	Medial Septum
PBS + T	Phosphate Buffered Saline containing 0.2% Triton
UDP	Uridine Diphosphate
μg	microgram
VDB	Vertical Limb of the Diagonal Band of Broca
wt	weight

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INTRODUCTION

The work of this thesis assessed whether two putative trophic agents, the monosialoganglioside GM1 and nerve growth factor (NGF), could induce substantial recovery and neuroplasticity in the adult and aged rat brain. Particular emphasis was placed on examining the effects of these agents on the nucleus basalis magnocellularis (NBM) and cortex following retrograde degeneration of the basalo-cortical cholinergic pathway. The introduction to this thesis, therefore, reviews the phenomenon of retrograde degeneration (section 1.1) and provides an overview of early work which challenged the dogma that the adult central nervous system (CNS) is immutable (section 1.2). The neurochemistry and neuroanatomy of brain cholinergic systems are subsequently reviewed in section 1.3. The last two sections of the introduction provide comprehensive reviews of gangliosides (section 1.4) and NGF (section 1.5). Also included in this section is the statement of the problem (section 1.6).

1.1 RETROGRADE DEGENERATION IN THE CENTRAL NERVOUS SYSTEM

Separation of a nerve cell body from its axon results in the degeneration of the distal severed axon [Wallarian degeneration, (Waller, 1852)], in addition to degeneration of the cell body and its remaining connected proximal axon (retrograde degeneration). The process of retrograde degeneration in the peripheral (PNS) and central nervous systems has often been studied comparatively with the aim of obtaining some understanding of the basis of neuronal regenerative activity. The series of morphological changes exhibited by the neuronal perikarya following retrograde degeneration were termed *primäre Reizung* (primary irritation) by Nissl (1894), "*réaction à distance*" by Marinesco (1898) and *the axonal reaction* by Meyer (1901). The original description of the retrograde degenerative process is attributed to Nissl (1892) who reported that after (24 hours) axotomy of rabbit facial motoneurons the "chromatinkörper" or chromatin bodies begin to disperse and disintegrate. These chromatin bodies, also called cytoplasmic basophilic material, are now referred to as Nissl substance and can be visualized using basic dyes such as Toluidine or Methylene blue as well as Cresyl violet. Additional changes described by Nissl were that during the few days after axonal injury, the cell body became swollen, the nucleus was displaced from its usual central

position towards the cell membrane, and basophilic material disappeared from the cytoplasm. The term *chromatolysis* has also been used to describe these events (Marinesco, 1896). Such alterations are considered as prototypic responses of perikarya to axonal injury. Subsequently, unless the neuron recovered, the soma was observed to shrink and die. Further studies have demonstrated that other cellular responses to axonal injury can include alterations in: RNA metabolism, axonal transport, mitochondria, microtubules, neurofilaments, lysosomes and rough endoplasmic reticulum [for review see: (Liberman, 1971; Grafstein, 1973; Spencer et al.1985)]. However, the original perikaryal changes noted by Nissl, as well as the aforementioned features are not always noted following axonal injury and greatly depend upon injury type, the proximity of the cell body to the injury, the species and age of the subject, in addition to the type and location of the neuron involved (Liberman, 1971; Grafstein, 1973). These factors also appear to play a role in determining whether or not neurons succumb to the injury. It has been proposed that retrograde degeneration could in part result from the loss of target derived trophic factors (see section 1.5). However, investigators have yet to identify consistent characteristics of the retrograde degenerative process which might be responsible for the failure of certain neurons to recover from axonal damage.

Neurons of the adult mammalian CNS are more likely to degenerate following injury than are those of the PNS. For example, in brain, rapid degeneration of specific sensory relay neurons has been shown to occur in the thalamus following cortical lesions (Peacock and Combs, 1965; Chow and Dewson, 1966; Barron and Doolin, 1968; Matthews, 1973; Barron, 1983). In addition, an early study by Kodama (1929) reported that cortical damage in humans results in the retrograde degeneration of neurons in the nucleus basalis. Similarly, experimental models involving surgical lesions of the cortex have also shown that an apparent loss of large neurons from the basal nucleus occurs in the rabbit (Das, 1971), as well as in human and monkey brains (Pearson et al.1983); such a deficit is comparable to the neuronal degeneration noted in the nucleus basalis of Meynert (nbM) in post-mortem brains afflicted with Alzheimer's disease (Whitehouse et al.1982). Moreover, cortical ablation in rats, induced by a devascularizing lesion, has been reported to cause prototypic signs of retrograde neuronal degeneration (Sofroniew et al.1983). In particular, it was shown that following short post-operative times, cholinergic neurons in the nucleus basalis appear swollen and have

eccentric nuclei, while at subsequent times the perikarya are notably shrunken. This experimental model was used for the studies presented in this thesis which tested whether retrograde degeneration of nucleus basalis cholinergic neurons in adult and aged animals could be prevented pharmacologically. It is demonstrated that the timely administration of neurotrophic agents such as the monosialoganglioside GM1 and/or nerve growth factor (NGF) may be beneficial in this regard (see section 3.2).

1.2 PLASTICITY IN THE CENTRAL NERVOUS SYSTEM

In contrast to the adult PNS, the CNS appears to have lost the capacity for axonal regeneration with higher evolution. Over the years, a large part of the research effort in the neurosciences has been directed towards developing experimental approaches to overcome this limitation. The basis of our understanding of nerve regeneration can be attributed to the work of Ramón y Cajal. His classic studies, published in 1913, on the degeneration and regeneration of the nervous system have served as the foundation of several present investigations. Ramón y Cajal's experiments, as did those of others at that time, centered for the most part on examining the regenerative capacity of long coarse myelinated systems and, particularly, the descending connections of the spinal cord. He noted that following transection of the spinal cord in mammals, nerve fibers begin to regrow but that this process is aborted after a few post-operative weeks (Ramón y Cajal, 1928). This led him to state that "*...everything may die. Nothing may be regenerated*" thus, reinforcing the pessimistic view held by many scientists at that time with respect to CNS regeneration. However, Ramón y Cajal proposed that central neurons did not lack the capacity for regrowth but rather that this "*... derives from external conditions, the presence or absence of auxiliary factors that are indispensable to the regenerative process...*". Moreover, he suggested that "*.... if experimental neurology is to some day supply artificially the deficiencies in question, it must accomplish these two objects: it must give to sprouts, by means of adequate alimentation, a vigourous capacity for growth; and, place in front of the disoriented nerve cones and in the thickness of the tracts of the white matter and neuronic foci, specific orienting substances.*" (Ramón y Cajal, 1928). Ramón y Cajal's suggestions have since been heeded by numerous

investigators but it has taken until nearly the present day for the dogma of central nervous system "hard-wiring" to be overcome.

Systematic research of CNS regeneration did not occur for some time after Ramón y Cajal's reports. Early studies which involved placing substances with regenerative ability, such as sciatic nerve tissue, into the brain or spinal cord were hampered by a lack of appropriate histological techniques and were levied heavy criticism. In 1940, however, Sugar and Gerard (1940) reinvestigated the regenerative ability of the young adult mammalian spinal cord. In their study, the spinal cords of 3 to 5-week-old rats were transected between levels T5 and T13, which caused 2-3 mm gaps. They noted, confirming Cajal's observations, that sprouting was abortive. However, Sugar and Gerard were able to show improved fiber outgrowth in some cases and especially in instances where the gap was filled with degenerated sciatic nerve or muscle tissue, what they considered "*a proper orienting substance*". Similar studies were also pursued by other laboratories [(Freeman et al.1949; Windle and Chambers, 1950a; Windle and Chambers, 1950b; Freeman, 1952; Windle, 1956), for review see: (Windle, 1956; Clemente, 1964)] who reported analogous degrees of regenerative success in experimental animals. The serendipitous findings of Windle and Chambers (1950a) however, had the greatest impact because the injury-induced glial scar was highlighted as a possible reason why the CNS was non-conducive for growth. Furthermore, their study represents the first attempt to pharmacologically induce CNS regeneration. In original experiments designed to determine the site of action of bacterial pyrogenic agents, Windle and Chambers noted that in dogs with spinal injuries "pyromen", a preparation from a *Pseudomonas* species, reduced scar tissue and allowed fiber outgrowth to persist for months and in some cases years [(Windle and Chambers, 1950a; Windle and Chambers, 1950b); see (Windle, 1956) for review]. Although little recovery of posture or locomotion occurred in these animals, Windle and his colleagues were able to record nerve impulses across the transected nerve segment up to a distance of 30 mm. Similar studies were subsequently done to assess the growth of peripheral nerve in brain. In one such study, the right temporal branch of the facial nerve in cats was inserted into the right temporal lobe of the cerebrum to a depth of 3-4 mm. Cats were then treated, every second day for 28 days, with pyromen R, a complex bacterial polysaccharide from *Pseudomonas* species IV. It was noted that the glial barrier was inhibited and that growth occurred. Similar results were obtained by these

authors using adrenocorticotrophic hormone (ACTH) (Clemente and Windle, 1954). However, these reports, and those of others at that time, which indicated that the CNS can be manipulated to allow growth, were regarded sceptically and were overshadowed by literature advocating that the adult CNS lacked regenerative ability.

Advances in neurosurgical, histochemical and transplantation techniques have since allowed the definitive demonstration that substantial regrowth of transected central axons can occur in the presence of a peripheral nerve bridge [for review see: (Aguayo et al.1982)]. More recently, it has been shown that axonal growth occurs in the injured CNS of rats treated with an antibody directed against myelin associated proteins (Schnell and Schwab, 1990). As well, studies have combined the use of peripheral grafts or matrices with infusion of growth promoting agents such as NGF to demonstrate that axotomized central fibers can regrow over long distances and can reinnervate their denervated target in the adult brain (Hagg et al.1990; Kawaja et al.1992).

1.2.1 COLLATERAL SPROUTING

In late 1940s the phenomenon of collateral sprouting, which is regarded as a fundamental concept in neurobiology, was introduced by Edds (1953). While working with peripheral systems, Edds noted that following transection of nerve fibers, which innervate muscle, remaining intact fibers sprout and can restore function to the denervated tissue. Similar observations of functional and anatomical collateral sprouting were made for other systems, such as sensory fibers which innervate the skin (Weddel et al.1941; Diamond et al.1976; Diamond et al.1992). The work of Liu and Chambers (1958) extended this concept to the CNS and revitalized interest in CNS regeneration. At that time, these investigators demonstrated, albeit by indirect methods, that following denervation of the spinal cord, induced by transecting the dorsal roots, remaining intact fibers sprout within the cord. That this could also occur in brain was comprehensively shown by Geoffery Raisman using electron microscopic techniques (Raisman, 1969; Raisman and Field, 1973). Raisman's studies focused on examining the innervation of the rat septal nucleus following injury. The septum, which is part of the basal forebrain nuclear complex receives input mainly from the fimbria-fornix and the medial forebrain bundle (reviewed in section 1.2.4). The innervation

from the medial forebrain bundle was shown to terminate on septal perikarya while that from the fimbria-fornix on dendritic processes. Raisman noted that if either of these pathways were transected, remaining fibers sprouted and formed synaptic contacts with the area which lost its synaptic input (Raisman, 1969). Sprouting was noted to begin within one week and was complete after approximately one month. Collateral sprouting has now been shown to occur, using a variety of techniques, in a number of brain areas including, the thalamus (Wall and Egger, 1971), the red nucleus (Nakamura et al.1974), the hippocampus (dentate gyrus) (Lynch et al.1972b; Lynch et al.1973; Matthews et al.1976a; Matthews et al.1976b) as well as other brain regions (Goodman and Horel, 1967; Moore et al.1971; Stenevi et al.1972). Extensive studies have assessed collateral sprouting using the rat hippocampal formation as an experimental model. Such studies have previously been substantially reviewed (Björklund and Stenevi, 1979; Cotman et al.1981; Gage and Björklund, 1986; Steward, 1986) and thus, will be mentioned here in brief. Cotman, Steward and Lynch (1973; 1974) were the first to demonstrate that collateral sprouting occurs within the dentate gyrus of the hippocampal formation. The dentate gyrus receives innervation predominantly from the entorhinal cortex, the contralateral hippocampus and the septum. This innervation exhibits a characteristic laminar pattern (see section 1.3.3a). Following lesions of the entorhinal cortex, a sprouting into denervated regions is elicited from surviving afferents. This has been illustrated using acetylcholinesterase (AChE) immunohistochemistry [(Lynch et al.1972a; Nadler et al.1977); for review see: (Cotman and Nadler, 1978)] as well as by tract tracing techniques (Stanfield and Cowan, 1982). Additional studies further examined sprouting responses of various chemically identified transmitter systems within the denervated hippocampus and noted that both homotypic (from same system) and heterotypic (from different system) sprouting occurred (Gage and Björklund, 1986). It was suggested that homotypic collateral sprouting in the CNS may result in functional recovery (Azmitia et al.1978; Gage et al.1983) and the term *compensatory collateral sprouting* was proposed for this phenomenon. Azmitia and coworkers (1978) demonstrated that following unilateral lesions of the cingulum bundle, regrowth of 5-hydroxytryptamine fibers into the hippocampus coincided with improvements in an asymmetrical behavioral response in rats. These studies were extended by others who examined if such a correlation also existed for more complex (cognitive) behaviors (Gage and Björklund, 1986). Rats with bilateral lesions of the supracallosal striae in the cingulate gyrus

showed improved performance in a forced-choice T-maze alternation task which paralleled the recovery of neurochemical markers for hippocampal noradrenaline, serotonin and acetylcholine (Gage and Björklund, 1986). Post-lesion increases in these markers were previously shown to match the appearance of collateral sprouting (Gage et al.1983). In addition, improvements in performance of rats with aspirative lesions of the medial cingulate cortex and supracallosal stria in a spatial memory based task (Morris water maze) were also found to correlate with recovery of choline acetyltransferase (ChAT) activity as well as noradrenaline (NA) and serotonin (5-HT) uptake in the hippocampus. Compensatory collateral sprouting and behavioral recovery were thus suggested to be causally linked. However, unequivocal evider. for this has yet to be provided.

1.2.2 TERMINAL SPROUTING AND REACTIVE SYNAPTOGENESIS

Sprouting is not always accompanied by synapse formation. However, the formation of synaptic contacts between the proliferating presynaptic process and the denervated post-synaptic cell has also been shown to occur following CNS injury, and is referred to as "*reactive synaptogenesis*" (Raisman and Field, 1973; Cotman and Nadler, 1978). This phenomenon, as mentioned in section 1.2.1, was first observed by Raisman (1969). Based on his electron microscopic studies of the septum, Raisman also proposed that particular stages underlie this event. He reported that first, the degenerated presynaptic element is removed by reactive astrocytes. Subsequently, the astrocytic process is displaced by the incoming axon terminal and then the synaptic differentiation is formed (Raisman, 1985). The molecular mechanisms which can regulate synaptogenesis remain to be elucidated. Quantitative electron microscopic techniques have further been used to study reactive synaptogenesis in the dentate gyrus, where synapse replacement following denervation also occurs (Matthews et al.1976a; Lee et al.1977; McWilliams and Lynch, 1978; McWilliams and Lynch, 1979; Hoff et al.1982a; Hoff et al.1982b). Moreover, the relationship between terminal proliferation and reactive synaptogenesis has been investigated (Steward and Vinsant, 1983). It was shown that at early post-lesion stages reactive synaptogenesis in the dentate gyrus, which was induced by entorhinal cortex lesions, derives predominatly from terminal proliferation rather than multiple synapse formation by individual terminals [for

review see (Steward, 1986)].

1.2.3 POSSIBLE INVOLVEMENT OF GROWTH FACTORS IN CNS COLLATERAL SPROUTING, TERMINAL SPROUTING OR SYNAPTOGENESIS

That growth factors can mediate collateral sprouting or lesion induced synaptogenesis was suspected long before the presence of such agents in the mammalian brain was confirmed. Early studies demonstrated that extracts from brain tissue at a lesion site were neuritogenic when applied to *in vitro* systems (Nieto-Sampedro et al.1983). Such trophic responses could be detected in extracts taken from either mechanically, chemically or ischemically damaged developing, mature or aged brains. In addition, increases in the levels of trophic activity were noted to be dependent upon post-lesion time and the age of the animal. In younger animals, maximal levels of trophic activity were observed earlier and were higher than those noted in older rats (Needles et al.1985). It was suspected that this could account for the diminished recovery exhibited by the aged CNS when compared to the young. It has now been shown that several neurotrophins are present in brain (reviewed in section 1.5.9) and that their levels are indeed increased following injury (see section 1.5.1). NGF has been shown to exert neurotrophic effects upon central cholinergic neurons (reviewed in section 1.5.5). Attempts to assess the role of endogenous NGF in phenomena such as collateral sprouting and/or reactive synaptogenesis have been hampered by technical constraints such as the lack of adequate antibody penetration into CNS tissues (discussed in section 1.5.3). The work of this thesis (section 3.2) provides the first direct evidence, using quantitative light and electron microscopic immunocytochemical techniques, that exogenous NGF can induce substantial synaptic remodelling in the remaining cortex of decorticated adult rats. NGF treatment is shown to cause substantial growth of cortical cholinergic fibers, to increase varicosity number and the size of presynaptic terminals. Moreover, the number of varicosities which were synaptic was also increased by NGF. It is further reported that the monosialoganglioside GM1 can potentiate these NGF-induced effects (section 3.2).

1.3 ACETYLCHOLINE IN THE CENTRAL NERVOUS SYSTEM

Work by Otto Loewi (1921) first gave credence to the idea that neurotransmission occurred via chemical rather than electrical means. His landmark work which involved the collection of perfusates following stimulation of a frog heart and their subsequent application to a second heart preparation, led him to postulate the existence of a chemical which he named "Vagusstoff". This substance was later identified as acetylcholine (ACh) (Loewi and Navratil, 1926), a choline ester which was shown to be present in peripheral mammalian tissues. That ACh might also act as a neurotransmitter in brain was inferred by studies in the 1930s which demonstrated that ACh was released from parasympathetic, preganglionic and motor neuron fibers (Feldberg and Krayer, 1933; Feldberg and Gaddum, 1934; Dale et al.1936; Feldberg, 1945). Its presence in brain was first reported by Chang and Gaddum (1933) and later by Dale [for review see: (Dale, 1965)]. Despite the fact that ACh has been recognized as a brain neurotransmitter for over fifty years, much still needs to be learned with respect to its regulation, release and function.

1.3.1 SYNTHESIS AND RELEASE OF ACETYLCHOLINE

Nachmanson and Machado (1943) first discovered, in soluble extracts of cholinergic tissues, the enzyme responsible for the synthesis of ACh. This enzyme, known as choline acetyltransferase (ChAT; EC 2.3.1.6), is a globular protein with an approximate molecular mass of 68,000 (Rossier, 1976; Eckenstein and Thoenen, 1982; Crawford et al.1982; Bruce et al.1985) although, other estimates quoted are higher [approx 73,000: (Hersh et al.1984; Cozzari and Hartman, 1983)]. In neuronal tissues, the distribution of ChAT correlates highly with that of ACh. Thus, measures of ChAT activity are often used to determine the density of cholinergic innervation in various tissues. Acetylcholine is synthesized by ChAT which transfers the acetyl group of acetyl-Coenzyme A (AcCoA) to choline (Ch) thus, forming an ester bond between acetate and Ch (AcCoA + Ch $\rightleftharpoons$ ACh + CoA). The predominant reaction proceeds sequentially with AcCoA first binding to the enzyme followed by Ch. After synthesis, ACh dissociates prior to CoA (White and Cavallito, 1970; White and Wu, 1973; Ryan and McClure, 1980). The reaction is reversible but its equilibrium is shifted to the

right. Michaelis-Menten constants (Km) of 18- 47 μM for AcCoA and 0.4-1.0 mM for Ch have been reported for mammalian brain ChAT (White and Wu, 1973; Malthe-Sorenssen et al.1978). In addition, it was noted that Ch can be competitively inhibited by ACh and non-competitively inhibited by CoA. By contrast, AcCoA can be non-competitively inhibited by ACh and competitively inhibited by CoA.

ChAT is synthesized in the cell body and is transported, by slow axonal transport [(Saunders et al.1973; Heiwall et al.1979); for review see: (Dahlström, 1983)] to neuronal processes. It is found in both axons and dendrites (Kasa et al.1970; Houser et al.1983), but experiments involving subcellular fractionation of brain tissue indicate that the highest proportion of ChAT is located in isolated nerve endings known as synaptosomes (Hebb and Whittaker, 1958; Whittaker and Sheridan, 1965; Tucek, 1967). In the nerve terminal, ChAT is mostly localized in the cytoplasm (Fonnum, 1967; Fonnum, 1968). However, other forms of ChAT exist as has been shown by studies employing differential solubilization techniques to brain tissue (Benishin and Carroll, 1983). In addition to the largest form of ChAT, which is soluble and cytoplasmic, membrane bound forms of the enzyme have also been identified in brain, ganglia and Torpedo electric organ. One of these ChAT forms is ionically associated with the membrane and is released following exposure to high salt solutions, while the second membrane bound form requires detergent to be extracted (Benishin and Carroll, 1983; Eder-Colli and Amato, 1985; Eder-Colli et al.1986). These three forms of ChAT appear to share similar physico-chemical properties such as molecular mass, isoelectric points and antigenic sites (Badamchian and Carroll, 1985; Peng et al.1986). Moreover, no difference is noted with regard to their affinities for choline and AcCoA (Benishin and Carroll, 1983; Eder-Colli and Amato, 1985; Eder-Colli et al.1986). These forms differ, however, with respect to pH optima, product inhibition, heat stability and substrate specificity (Smith and Carroll, 1980; Benishin and Carroll, 1983; Eder-Colli and Amato, 1985; Eder-Colli et al.1986). In addition, they can be differentially stimulated by veratridine or potassium (Carroll, 1987) although, no evidence yet exists to suggest distinct roles of the various ChAT forms in the synthesis of ACh. Recent work appears to indicate a lack of involvement of membrane bound ChAT in regulating basal ACh synthesis (Schmidt and Rylett, 1992), but its role under stimulating conditions has yet to be examined. Biochemical and structural analysis of rat brain ChAT has been limited because of the small amounts of

this enzyme in this tissue. However, ChAT has now been cloned and sequenced from various species (Itoh et al.1986; Berrard et al.1987; Brice et al.1989; Toussaint et al.1992). Several potential phosphorylation sites have been identified on the enzyme but the biological significance of this remains to be determined. Therefore, how the transcription of the ChAT gene or the activity of the enzyme are regulated remains to be to be established.

ChAT is generally not thought to play a regulatory role in the biosynthesis of ACh since it is present in kinetic excess [for review see: (Tucek, 1988)]. The synthesis of ACh in nerve terminals is generally thought to depend upon the provision of Ch. Choline can be obtained from blood plasma, intracellular hydrolysis of choline phospholipids (from blood or synthesized directly by brain glia and neurons) or from extracellular hydrolysis of ACh released at cholinergic synapses [for review see: (Ansell and Spanner, 1982)]. Under physiological conditions, most of the Ch used for ACh synthesis is obtained from the extracellular space. Choline is taken up by the nerve terminal by high and low-affinity proteins located in the membrane [reviewed by: (Yamamura and Snyder, 1973; Kuhar and Murrin, 1978; Jope, 1979; Murrin, 1980; Tucek, 1985)]. The high affinity carrier is both sodium and chloride dependent and is highly sensitive to hemicholinium-3 while the low affinity carrier is sodium independent and has a lower sensitivity to hemicholinium-3 [(Suszkiw and Pilar, 1976), for review see: (Ducis, 1988)]. The other substrate for ACh synthesis, AcCoA, is produced in mammalian brain intramitochondrially from glucose and pyruvate (Tucek, 1988). How AcCoA reaches ChAT which is located outside the mitochondrion is not fully understood. It has been proposed that it may be transformed into another substance, perhaps citrate, which could act as an acetyl carrier across the membrane, and once having crossed it would be converted back to AcCoA (Greville, 1969). Alternatively, AcCoA is thought to be supplied by acetylcarnitine (Dolezal and Tucek, 1981; Tzagoloff, 1982) or to be obtained by direct passage through Ca^{2+} induced hydrophilic pores (or channels) in the inner mitochondrial membrane (Benjamin et al.1983).

Therefore, in cholinergic neurons AcCoA is obtained from glucose, and choline is retrieved from the extracellular space as starting materials for ACh synthesis. ChAT then synthesizes ACh which is either retained within the cytosol or subsequently stored in vesicles. That ACh was not homogenously distributed in brain tissue was originally noted by Hebb and Whittaker (1958), who identified "free" and "bound" pools of ACh. More extensive studies

on ACh compartmentalization were done using the cat superior cervical ganglion as a model, where 4 pools of ACh were identified (Birks and McIntosh, 1961). These include: a "stationary pool", not releasable from the ganglia, and which comprises approximately 15% of total ganglionic ACh; a "readily releasable" pool; a "less readily releasable" pool and a "surplus pool" (Birks and McIntosh, 1961; Collier and Katz, 1971).

Both basal and stimulated release of ACh occurs. Basal (or spontaneous) ACh release, which occurs at rest, does not require extracellular calcium and is thought to derive from cytoplasmic rather than vesicular stores (Boksa and Collier, 1980; Carroll, 1983). By contrast, the stimulated release of ACh is thought to occur in a quantal fashion, from vesicles, by the process of exocytosis and is known to require extracellular calcium and the inhibition of Na^+/K^+ ATPase (Meyer and Cooper, 1981). Acetylcholine content is maintained at constant concentrations within the nerve terminal (Collier and MacIntosh, 1969). How ACh synthesis is regulated to adapt to the rate of ACh release has been the subject of much research. Factors which appear to play a role in regulating ACh synthesis include [reviewed by: (Tucek, 1988)]: (1) alterations in the content of ACh in the nerve terminal; (2) choline uptake (ie: the activation of the high-affinity choline carrier); (3) post-stimulation hyperpolarization; (4) calcium effects on AcCoA supply; (5) metabolic changes in the availability of choline or AcCoA; and (6) ChAT activity.

Once released, ACh interacts with either nicotinic or muscarinic receptors on cholinoceptive cells. Nicotinic and muscarinic presynaptic receptors also exist which can modulate release of other neurotransmitters or can act as autoreceptors to regulate ACh release itself (DeBelleroche and Gardiner, 1982). The action of ACh is terminated by cholinesterases which hydrolyze its ester bond to release the products, choline and acetate. Acetylcholinesterase [AChE (EC 3.1.1.7)] [for review see (Brimijoin, 1983)] is found in substantial amounts in all cholinergic neurons. However, it is also found in glia and other neurons. Moreover, ACh is not its only substrate since the purified enzyme also hydrolyzes a number of peptides, including substance P (Chubb et al.1980). Thus, AChE is found to be more widely distributed than ACh.

1.3.2 NEUROANATOMY OF CHOLINERGIC PATHWAYS IN BRAIN

Advances in the characterization of brain cholinergic systems have paralleled improvements in the development of neuroanatomical techniques for their identification. Ideally, visualizing ACh itself would best serve to identify cholinergic neurons or pathways in brain. However, no such technique exists. Despite some reports of monoclonal antibodies for ACh, (Geffard et al.1985) studies demonstrating their usefulness for immunocytochemical purposes in mammals have yet to arise. The first opportunity to identify cholinergic neurons was provided by the development of a histochemical technique for AChE by Koelle and Friedenwald (1949). Using this method, the production of a brown precipitate, occurring within brain tissue after its incubation in acetylthiocholine and coppersulfide, was taken to indicate the presence of hydrolysis by AChE hence, indirectly detecting the enzyme. The resolution of this technique was later improved by Karnovsky and Roots (1964) who added ferricyanide to yield a finer precipitate. Employing this method in combination with surgical lesions, Shute and Lewis (Shute and Lewis, 1961; Shute and Lewis, 1963; Lewis et al.1967a; Shute and Lewis, 1967; Lewis and Shute, 1967) were able to map cholinergic pathways in the rat brain using the rationale that interruption of axons should cause a buildup of AChE in the cell body, where it is synthesized, and result in a decrease of its content at terminal fields. However, the fact that AChE can hydrolyze substrates other than ACh (see section 1.3.1) and is also found in non-cholinergic neurons (see below) cast doubt on the reliability of these findings. The staining method for AChE was considerably improved (Butcher and Bilezikjian, 1975; Butcher, 1978; Butcher, 1983) by the addition of a step requiring the pretreatment of animals, a few hours prior to processing the brain for AChE histochemistry, with di-isopropylphosphofluoride (DFP), an irreversible AChE antagonist. This procedure allowed enhanced detection of AChE positive cells and was based on the assumption that *de novo* synthesis of AChE in cholinergic neurons occurred faster and in greater amounts than in non-cholinergic neurons (Butcher, 1983). This technique was employed quite successfully and is still in current use today, but is only reliable for certain brain areas (Fibiger, 1982; Eckenstein and Sofroniew, 1983; Levey et al.1983b; Wainer et al.1984). More dependable approaches to detect cholinergic neurons emerged in the late 1970s and early 1980s when antibodies became available which were specifically raised against ChAT [for review see:

(Wainer et al.1984)]. Comparative studies of ChAT and AChE immunohistochemistry showed that in some brain areas correspondence between ChAT and AChE did not occur. For example, dopaminergic neurons within the substantia nigra which were shown to be AChE positive (Butcher et al.1975) were ChAT negative. Thus, ChAT is considered a more reliable marker of cholinergic neurons and was used for the neuroanatomical studies undertaken for this thesis. However, this procedure is also not without faults (Landis, 1985) and greatly depends upon the sensitivity and specificity of the antibody used. Several well characterized ChAT antibodies are now available (Levey et al.1983a; Hedreen et al.1983; Houser et al.1985; Eckenstein et al.1981) including the one used for this thesis (Eckenstein and Thoenen, 1982).

Many ChAT immunoreactive (IR) neurons have been identified in brain by several research groups (Butcher and Woolf, 1984; Butcher and Bilezikjian, 1975; Sofroniew et al.1982; Armstrong et al.1983; Wainer et al.1984). These include neurons of the basal forebrain complex [which comprise the: medial septal nucleus, nuclei of the vertical as well as horizontal limb of the diagonal band of Broca (VDB, HDB) and nucleus basalis (nucleus basalis of Meynert or substantia innominata in humans and primates)], striatum, nucleus accumbens, motor nuclei, parabrachial system, reticular formation and others. Since the work of this dissertation focused predominantly upon the basal forebrain complex and striatum only the efferent and afferent projections of cholinergic neurons within these groups will be reviewed. The following references include reviews of other cholinergic systems (Kasa, 1986; Woolf, 1991). Moreover, since the rat was used as an experimental model emphasis will be placed on their description in this species. Major cholinergic pathways in the rat brain are shown in Figure 1.1.

1.3.3 NEURONS OF THE BASAL FOREBRAIN

Initially identified by Meynert (1872), after whom it is named, the distinct aggregation of large neurons within the human substantia innominata were characterized by Kölliker (1896) as the basal nucleus (*Meynertsches Basalganglion*). Similar neurons are noted in primates, cats and rodents, however, variations in their anatomical localization have contributed to the confusion which pervades the literature with respect to nomenclature of this cell

population(s). Brockhaus (1942), using Nissl staining, originally classified the basal nuclear complex as comprising three nuclear subdivisions consisting of: (1) neurons within the medial septum and diagonal band of Broca, (2) nuclear groups within the tuberculum olfactorium and (3) neurons of the nucleus basalis of Meynert (nbM). Such subdivisions are still considered appropriate although they have been somewhat redefined (Fibiger, 1982; Mesulam et al.1983a; Mesulam et al.1983b; Woolf et al.1984). It is now clear that the majority of neurons within the basal forebrain nuclear complex are cholinergic. Advances in methodologies allowing such chemical identification, in addition to progress in tract tracing techniques permitting connectivity patterns to be defined, have accentuated the need to reassess basal forebrain nomenclature. Studies by Mesulam and colleagues initially conducted using primate brains (Mesulam et al.1983a) and later extended to rat (Mesulam et al.1983b) suggest a subdivision of the basal forebrain nuclear complex into 4 major sectors: Ch1 (representing cholinergic neurons in the septum which project to the hippocampus), Ch2 (representing cholinergic neurons in the VDB which project to the hippocampus), Ch3 (representing cholinergic neurons in the HDB which project to the olfactory bulb as well as to medial and mostly limbic cortex), Ch4 (representing cholinergic neurons in the magnocellular preoptic field, substantia innominata and nucleus basalis which project to limbic cortex, amygdala/limbic cortex and remaining cerebral cortex, respectively). However, such designations for the rat have been met with some resistance especially by investigators who prefer to view the basal forebrain as a continuum (Schwaber et al.1987). Moreover, it remains debatable, for example, whether magnocellular preoptic neurons are components of the HDB or nucleus basalis. A particular disorder exists within the literature with regard to which neurons comprise the nucleus basalis. Investigators have often used this term to include neurons of the substantia innominata or to represent all cholinergic neurons of the basal forebrain nuclear complex excepting those of the diagonal band and medial septum. For the purpose of this thesis, the division of the basal forebrain complex adopted was essentially that described by Mesulam except that the traditional rather than the Ch nomenclature was employed. Furthermore, the term nucleus basalis magnocellularis (NBM) was used to refer to the group of large cholinergic neurons encroaching the internal capsule and situated medial to and within the globus pallidus. The basal forebrain subdivisions adopted for this thesis are illustrated in Figure 1.2.

1.3.3a Cholinergic projection from the septum and VDB to hippocampus

The hippocampal formation consists of four major subdivisions which are all interconnected by substantial association and commissural pathways [for review see: (Swanson et al.1987)]. The dentate gyrus and Ammon's horn (comprising CA1, CA2 and CA3 regions) are usually considered as the hippocampal region while the subicular and entorhinal subdivisions are usually referred to as the retrohippocampal region. A comprehensive review of the interconnections and trisynaptic circuitry of the hippocampal formation has been provided by Swanson and coworkers (1987).

That a projection exists from the septum to hippocampus was suggested by Diatz and Powell (1954) who showed that retrograde degeneration of septal neurons occurs following extensive hippocampal lesions. The combined lesion/AChE immunohistochemical studies of Lewis and Shute (1967b) provided the first indication that this projection was cholinergic. Their work was supported by the neurochemical findings of Oderfeld-Nowak (1974) who reported that electrolytic lesions of various portions of the septum/VDB result in deficits of hippocampal ChAT and AChE activities at various post-lesion times. Subsequent evidence for the existence of a septo-hippocampal pathway was provided by tract tracing studies. Meibach and Sigel (1977) demonstrated that injection of horse radish peroxidase (HRP) into the hippocampus resulted in the retrograde labelling of septal/VDB neurons. Similar studies using alternative tracers as well as work which combined the use of tract tracing with ChAT or AChE immunocytochemistry have further confirmed that cholinergic neurons of the medial septum and VDB project to the hippocampus (Alonso and Köhler, 1984; Rye et al.1984; Woolf et al.1984; Amaral and Kurz, 1985; Gaykema et al.1990). The cingulate, entorhinal, perirhinal, subicular and retrospenial cortices have also been shown to receive cholinergic input from septum/VDB neurons [(Woolf et al.1984; Woolf et al.1986); for review see: (Swanson et al.1987)]. Moreover, portions of the rat visual cortex are also innervated by VDB cholinergic neurons although a similar projection does not appear in the cat or primate (Mesulam et al.1983a; Irle and Markowitsch, 1984). A clear depiction of the topographic organization of the cholinergic septal/VDB projection to the hippocampus has also been provided. Four pathways (3 dorsal, 1 ventral) have been identified [for review see: (Swanson et al.1987)]. The dorsal pathways arise from cholinergic neurons in the medial septum and

dorsal VDB and proceed either: (1) through the dorsal fornix to innervate the dorsal hippocampus, (2) through the fimbria, to diffusly innervate the hippocampus, or (3) through the supracallosal striae to innervate more caudal portions of the hippocampal formation. The fourth pathway emerges from the more ventral VDB cholinergic neurons whose fibers enter the ventral temporal hippocampus via the ansa lenticularis. The fimbria-fornix pathway has been reported to contribute 60% to hippocampal innervation while the supracallosal striae and ansa lenticularis pathways appear to contribute 30% and 10%, respectively (Storm Mathisen and Blackstad, 1964; Gage et al.1983). ChAT and AChE immunocytochemical studies have shown that cholinergic fibers are present throughout the hippocampal formation but that a particularly dense innervation is noted in the hilus of the dentate gyrus (Swanson et al.1987). Moreover, studies at the electron microscopic level have identified hippocampal pyramidal and agranular neurons as cholinoceptive cells (Frotscher and Léránth, 1985).

Medial septum/VDB ChAT positive neurons are the most anteriorly located cells of the cholinergic basal forebrain complex (see Figure 1.2). Of the total neurons in the septum it was shown using HRP-WGA (horse radish peroxidase conjugated wheat germ agglutinin) tract tracing combined with ChAT immunocytochemistry that 30-35% of the cells in the medial septum and 45-55% in the VDB projecting to the hippocampus are cholinergic (Wainer et al.1985). A population of GABAergic cells in the septum which project to the hippocampus has also been identified and reported to comprise 30% of the neurons in the septal-diagonal-band area (Köhler et al.1984; Zaborsky et al.1986). Cholinergic neurons in the septum have been shown to receive cholinergic input from the ventral tegmental area and catecholaminergic input from the locus coeruleus [for review see: (Swanson et al.1987). In addition, pathways from the HDB, medial habenula and paraventricular hypothalamic nuclei to the septum have also been noted [for review see: (Swanson et al.1987)]. A pathway from the hippocampus to the septum, which innervates GABAergic septal neurons, has also been identified (Zaborsky et al.1986).

The hippocampus, in addition to receiving afferents from the septum/VDB, also receives input from the entorhinal cortex. The entorhinal input is thought to be glutamatergic and projects through the perforant pathway to reach the dentate gyrus. Moreover, a projection from the contralateral entorhinal cortex has also been noted the crossed temporodentate projection (Goldwitz et al.1975); for review see (Swanson et al.1987) . These afferent

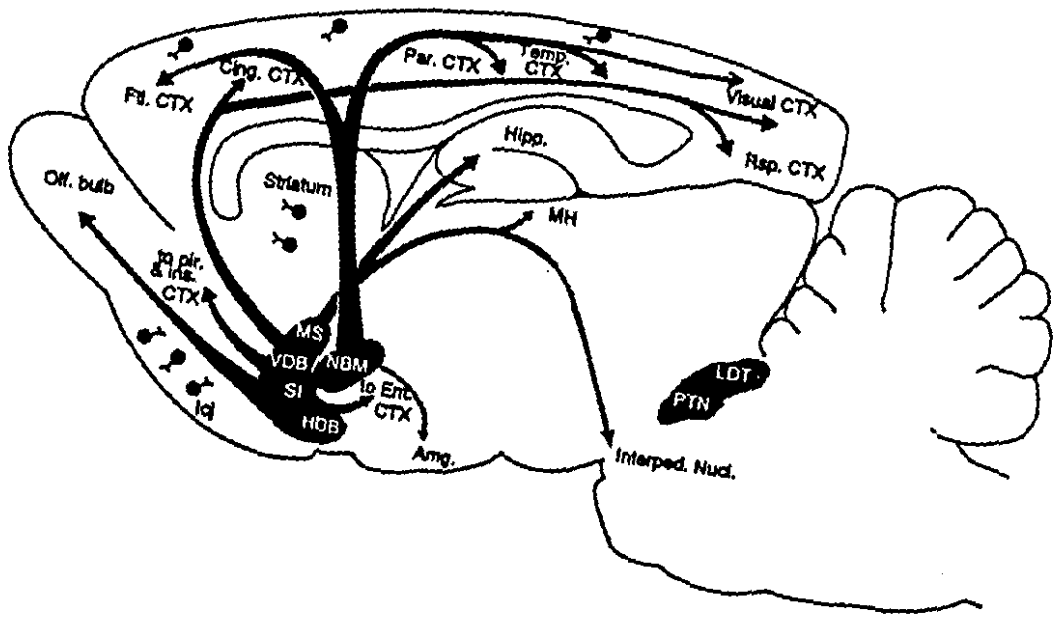
projections innervate the hippocampus in a characteristic laminar fashion. For example, the ipsilateral entorhinal input terminates in the outer two-thirds of the molecular layer of the dentate gyrus while the crossed temporodentate projection terminates in the inner one third of the molecular layer. The cholinergic projection from the medial septum/VDB, as mentioned above, predominatly innervates the hilar region of the dentate gyrus. The presence of intrinsic ChAT-IR hippocampal neurons has also been reported. Such neurons are found scattered throughout all layers of the dentate gyrus, Ammon's horn and the subiculum (Houser et al.1983; Levey et al.1984; Frotscher and Léránth, 1985; Matthews et al.1987), although their numbers are low. Muscarinic and nicotinic receptors have also been detected in the hippocampus by ligand autoradiography but their distribution does not completely parallel that of the cholinergic innervation (Clarke et al.1985; Cortés and Palacios, 1986; Levey et al.1991).

1.3.3b CHOLINERGIC PROJECTION FROM THE HDB TO THE OLFACTORY BULB

The HDB is situated below the anterior commissure and lateral to ChAT positive neurons of the VDB (see Figure 1.2). Rostral cholinergic neurons of the HDB have been shown to project predominantly to the olfactory bulb (Woolf et al.1984; Záborsky et al.1986; Gaykema et al.1990). More medial ChAT positive cells within the HDB also have projections to the cingulate, retrosplenial, entorhinal, perirhinal cortices and interpeduncular nucleus (Woolf et al.1984; Woolf and Butcher, 1985). Moreover, the basolateral amygdala has also been reported to receive cholinergic input from the HDB (Woolf and Butcher, 1982; Woolf et al.1984). In turn, the anterior olfactory nucleus, basolateral amygdala, orbitofrontal, cingulate, entorhinal, insular and piriform cortices project to the HDB [(Woolf and Butcher, 1986); for review see (Woolf, 1991)]. Additional afferents have been reported to come from the ventral tegmental area, dorsal and median raphe, pedunculopontine and lateral dorsal tegmental nuclei (Woolf, 1991).

Figure 1.1 Illustration of the distribution of cholinergic pathways in the rat brain. Parasagittal rat brain sections are shown depicting the projections of (A) basal forebrain cholinergic neurons and (B) pontomesencephalic neurons. Abbreviations: Cing.CTX, cingulate cortex; CNN, cranial nerve nuclei; DCN, deep cerebellar nuclei; DR, dorsal raphe; 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; Lateral. Hypot., lateral hypothalamus; LC, locus ceruleus; LDT, lateral dorsal tegmental nucleus; MBN, nucleus basalis magnocellularis; MH, medial Habenula, MRN, medullary reticular nuclei; MS, medial septal nucleus; Olf. bulb, olfactory bulb; Par. CTX, parietal cortex; Pir.CTX, piriform cortex; PRN, pontine reticular nuclei; PTN, pedunculopontine tegmental nucleus; Rp. CTX, retrosplenial cortex; SI, substantia innominata, SN, substantia nigra; Thal., thalamus; VDB, vertical limb of the diagonal band of Broca; VN, vestibular nuclei. Drawings are a modification of that presented in (Woolf, 1991).

A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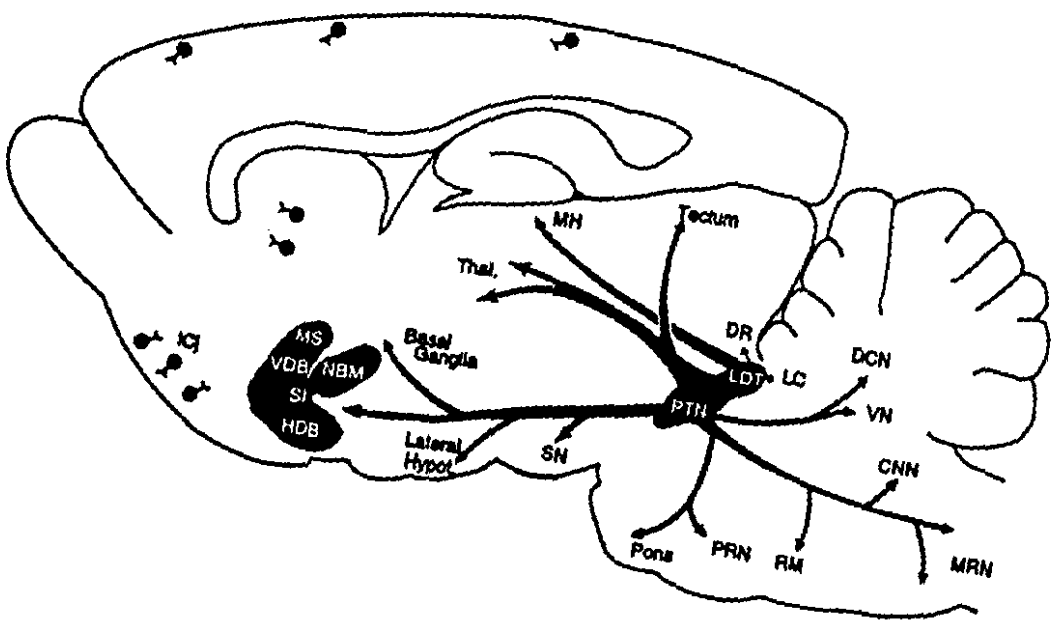
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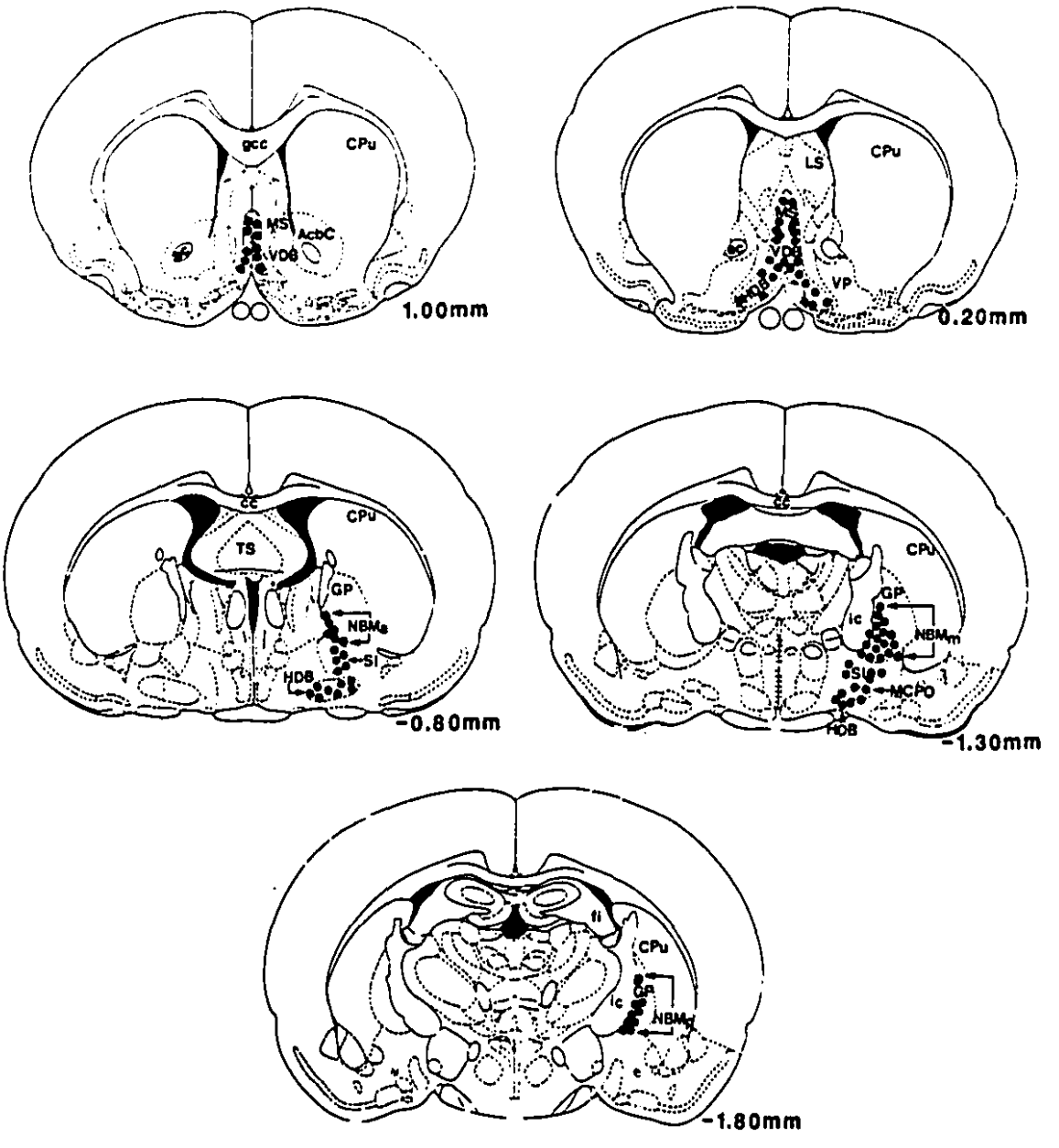


Figure 1.2 Illustration of the topography of the basal forebrain cholinergic system at various rostrocaudal levels. Black circles indicate the distribution of cholinergic neurons. Distance from Bregma is indicated on the lower right hand side of each figure. Abbreviations: ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen; fi, fimbria; gcc, genu of the corpus callosum; GP, globus pallidus; HDB, horizontal limb of the diagonal band of Broca, LS, lateral septum; MBNa,m,p, nucleus basalis magnocellularis anterior, mid and posterior parts; MCPO, medial central preoptic nucleus; SI, substantia innominata; MS, medial septum; VDB, vertical limb of the diagonal band of Broca, VP, ventral pallidum.

1.3.3c CHOLINERGIC PROJECTION FROM THE NBM TO NEOCORTEX

The observation by Kodama (1929) and later by Das (1971) that degeneration occurs in the nucleus basalis following damage to the cortex provided the first indication for the existence of a basalo-cortical pathway. Support for their suggestion was provided by subsequent studies which showed that neurons of the nucleus basalis, in various mammalian species, were retrogradely labelled following injections of tracers into the cortex (Divac, 1975; Kievet and Kuypers, 1975; Lehmann et al.1980; McKinney et al.1983). That this projection in the rat was cholinergic was suggested by the studies of Lewis and Shute (1967) using AChE histochemical techniques. However, an earlier study by Krnjevic and Silver (1965) had also proposed a basalo-cortical cholinergic pathway for the cat brain. Lesions of the rat NBM have been reported to cause marked decreases in cortical ChAT and AChE activities, as well as reduced AChE staining in cortical areas extending from the medial limbic cortex to the rhinal fissure (Wenk et al.1980). Similar studies by Coyle (1978) and Johnson (1979; 1981) proposed that basal forebrain neurons contributed up to 70% of the cholinergic innervation to the fronto-parietal cortex. Definitive confirmation of an ascending cholinergic innervation from the nucleus basalis to cortex was provided by studies which employed ChAT or AChE immunocytochemistry combined with tracers (Bigl et al.1982; Woolf et al.1983; Rye et al.1984; Woolf et al.1986). Although these latter studies significantly advanced our understanding of NBM projections to cortex, their topography has yet to be firmly established. The discrepancies which exist between various reports can be accounted for by differences in methodologies and delineations of the nucleus basalis employed (Lehmann et al.1980; Bigl et al.1982; Woolf et al.1983; McKinney et al.1983; Mesulam et al.1983b; Price and Stern, 1983; Rye et al.1984; Saper, 1984; Woolf et al.1986; Carey and Rieck, 1987). Cholinergic fibers ascending from the NBM have been shown to traverse the caudate and continue within the corpus callosum prior to turning into the cortex (Eckenstein et al.1988). The consensus appears to be that projections from the NBM to cortex are arranged in an anterior to posterior fashion. That is, cholinergic neurons in the anterior portion of the rat NBM project predominantly to the frontal cortex while those of the mid and posterior NBM project, respectively, predominantly to parietal and occipital cortices. The main

recipient of NBM cholinergic fibers, however, is the frontal cortex (Bigl et al.1982). A detailed analysis of the cortical projection patterns of the NBM has been provided by Luiten and coworkers (1987). This study examined the projections over the entire cortical mantle following injections of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) into numerous portions of the rat NBM. In addition to providing further support for an anterior-posterior topographic arrangement for NBM projections, this study also reported that a ventrolateral to dorsomedial pattern exists. That is, the anterior NBM sends more fibers to medial cortical regions while the posterior NBM sends more fibers to lateral cortical regions.

The collateralization of cholinergic fibers from the NBM within cortex remains debatable. Investigators who have addressed this issue have, for the most part, infused different fluorescent tracers into specific neocortical areas and have assessed the number of NBM double-labelled cells. Using such a technique, Bigl and coworkers (1982) reported that AChE positive neurons of the basal forebrain projecting to frontal cortex area 10 and those projecting to parietal area 2 and temporal area 4 do not provide afferents to other cortical regions. However, a few basal forebrain neurons (approximately 3%) appeared to project to both cingulate area 29 and occipital area 29. McKinney and coworkers (1983) reported that several neurons in the NBM were double labelled after separate injections of Fast Blue and Nuclear Yellow into frontal and parietal cortices. By contrast, Price and Stern (1983), using the same technique showed that cortical projections of individual cells in the nucleus basalis are restricted to an area less that 1-1.5 mm in diameter. Thus, evidence obtained so far favours that individual NBM cholinergic neurons in rat have a restricted distribution in cortex. It should be noted, however, that individual cholinergic fibers appear to branch extensively within specific cortical regions and to innervate several layers within that area (Eckenstein et al.1988). This has also been suggested by Luiten and coworkers (1987) based on tract tracing studies.

Approximately 90% of the neurons within the NBM which project to cortex are believed to be cholinergic (Mesulam et al.1983b). In addition to a projection to cortex, NBM cholinergic neurons have also been shown to project to the amygdala, although these cells are distinct from those which project to cortex (Carlsen et al.1985). Afferents to the NBM come from the intralaminar thalamus, zona incerta, ventral tegmental area, dorsal and median

raphe, pedunculopontine and tegmental nuclei [for review see: (Woolf, 1991)]. It has also been shown that the substantia nigra accounts for the majority of the tyrosine hydroxylase (TH) immunoreactive network in the NBM (Martinez-Murillo et al.1988) and that this dopaminergic input appears to be presynaptic to NBM cholinergic neurons. A more detailed study, which used HRP-WGA tract tracing combined with immunocytochemistry, revealed that following injections of this tracer into the NBM, 25% of TH and 10% of serotonin positive neurons were labelled in the mesencephalic region (Jones and Cuello, 1989). Moreover, less than one 1% of cholinergic neurons in the pedunculopontine and lateral tegmental nuclei were labelled (Jones and Cuello, 1989).

1.3.4 DISTRIBUTION OF THE CHOLINERGIC INNERVATION IN THE RAT NEOCORTEX

That ACh is a neurotransmitter in the mammalian cerebral cortex has been established for over 30 years [for review see: (Krnjevic, 1985)]. This was initially based on the presence of ChAT or AChE activities, as well as ACh content in cortex (Feldberg, 1933; Hebb and Silver, 1956; Hebb et al.1963). Subsequently, ACh release was detected from cortex (Mitchell, 1963; Dudar, 1977; Florian et al.1987; Maysinger et al.1988) and cortical responses to ACh were recorded using electrophysiological techniques (Krnjevic and Phillis, 1963). Various cortical subdivisions have been identified based on cytoarchitectonic studies. Over the years, however, improvements in methodologies have prompted investigators to suggest alternative cortical maps to the one originally proposed by Brodmann, which was designed primarily for primates (Brodmann, 1909). The terminology for the subdivisions of the cortex employed for this thesis follows that suggested by Zilles (1985). The rat neocortex (also known as isocortex) is subdivided into 4 major sectors namely, frontal, parietal, temporal and occipital cortices, which themselves have been further subdivided. As described above, the majority of the cholinergic innervation to the rat neocortex is extrinsic and originates predominantly from the NBM. However, an intrinsic cholinergic innervation was also proposed for the rat cortex since basal forebrain lesions failed to suppress all cortical cholinergic activity (Johnston et al.1979; Lehmann et al.1980; Johnston et al.1981). Although AChE positive cortical neurons were not detected in early studies (Butcher and Woolf, 1984),

subsequent reports which used ChAT immunocytochemistry revealed the presence of ChAT-IR neurons in the rat cortex [(Sofroniew et al.1982; Houser et al.1983; Eckenstein and Baughman, 1983; Levey et al.1984; Lysakowski et al.1989); this thesis]. Additional work showed that these neurons colocalize VIP (Eckenstein and Baughman, 1983). However, because AChE-IR cortical neurons are not observed and ChAT-IR neurons not detected in the cat or primate cortex, some investigators have questioned their existence in the rat. Recently, a study has shown that the rat cortex lacks ChAT mRNA (Oh et al.1991), although this finding could be attributed to a lack of sensitivity of the in situ hybridization technique employed and thus, requires confirmation. Consensus at present is that the cortical cholinergic innervation in the rat derives from intrinsic (accounting for approximately 30%) and extrinsic (accounting for approximately 70%) sources. ChAT-IR neurons observed in the rat cortex are found throughout cortical layers II-VI but are most concentrated in layers II and III. These neurons are small, bipolar and have vertically oriented dendrites (Houser et al.1985).

The distribution of cholinergic fibers in cortex has been examined by several research groups (Eckenstein and Baughman, 1983; Houser et al.1985), but a most comprehensive study was recently accomplished by Lysakowski and coworkers (1989) who identified 13 different patterns of ChAT-IR fiber distribution throughout the entire rat cerebral cortex. Four different fiber network patterns were noted in the neocortex. These were classified as: motor/frontal, primary sensory, secondary sensory and association patterns. The motor/frontal pattern is noted within frontal cortex areas 1 and 2 (Fr1, Fr2) as well as in the dorsal agranular and insular cortices (DAG). The ChAT-IR fiber distribution in these regions is dense and tangentially oriented in cortical layer I and is homogenous throughout layers II-VI. The primary sensory pattern is observed in the hindlimb (HL) and forelimb (FL), parietal 1 (Par1), gustatory cortex (Gu), temporal cortex area 1 (Te1), occipital cortex area 1 binocular (Oc1B), occipital cortex area 1 monocular (Oc1M) and granular cortical regions. In these areas, a dense pattern of ChAT-IR fibers is present in cortical layer I and V as well as in lower layer IV, while layers II, III and VI show a lighter fiber density. The secondary sensory pattern is exhibited by parietal cortex area 2 (Par2), occipital cortex area 2 lateral part (Oc2L), occipital cortex 2 medio-medial part (Oc2MM), occipital cortex 2 medio-lateral part (Oc2ML) and temporal cortex area 2 (Te2). Within these regions, a dense staining of

ChAT-IR fibers is noted in layer I, a moderate staining is noted in layers II and III, while lighter staining occurs in layers V and VI. The association pattern is exhibited by some portions of the parietal cortex area 1 (Par1) and occipital cortex area 1 lateral part (Oc2L). These show a uniform ChAT-IR fiber distribution across all layers. It has also been established that a large proportion of cholinergic fibers ascending from the NBM terminate in cortical layer V of the fronto/parietal region (Eckenstein and Baughman, 1983; Luiten et al.1987).

The cortex has also been shown to contain muscarinic and nicotinic receptors by radioligand binding or autoradiographic techniques (Clarke et al.1985; Cortés and Palacios, 1986; Swann and Hewitt, 1988; Vige and Briley, 1989; Wang et al.1989). In the rat brain, nicotinic receptors (defined by [^{3}H] nicotine binding) were predominantly noted in layers III and IV in all cortical regions except the retrosplenial cortex, where nicotine binding was prominent in layer I (Clarke et al.1985). [^{3}H] quinuclidinyl benzilate (QNB) binding revealed the presence of muscarinic receptors throughout layers I and III and another dense band in lower cortical layers (Kuhar and Yamamura, 1976). More recently, in situ hybridization as well as immunocytochemical techniques have allowed the detection of various subtypes of muscarinic receptors (Levey et al.1991). This has revealed a differential distribution of m_1, m_2 and m_4 subtypes of muscarinic receptors in the rodent cortex. Regionally, m_1 immunoreactivity was more abundant in fronto-parietal cortical areas rather than in the retrosplenial cortex. The converse was true for m_2 distribution. Within cortical layers, m_1 immunoreactivity was most dense in layers II/III and V, while m_2 immunoreactivity was densest in layer IV and in the border of layers V/VI. Immunoreactivity for m_4 was found to be considerably less dense than that of the other receptor subtypes and was localized in layers II/III. A patchy m_4 immunoreactivity was also detected in layer IV. These results were found to be consistent with the distribution of the mRNA for these subtypes (Buckley et al.1988; Weiner and Brann, 1989).

Few studies have comprehensively examined the ultrastructural features of the rat cortical cholinergic innervation. Previous work at the electron microscopic level identified ChAT-IR synaptic vesicle filled profiles throughout the rat cortex (Houser et al.1985). The ChAT positive synapses observed were mainly of the symmetric type and were noted to be predominantly associated with medium sized dendritic shafts of unknown origin. ChAT-IR

synaptic contacts were also seen associated with apical and probably basilar dendrites of pyramidal neurons, as well as with ChAT negative non-pyramidal neurons (Houser et al.1985).

1.3.5 STRIATAL CHOLINERGIC INTERNEURONS

Because of their similar architecture, the caudate and putamen in the rat are considered to be one complex known as the striatum (or neostriatum). Neurochemical studies have indicated that the striatum contains high levels of ChAT, high-affinity choline uptake and ACh, as well as muscarinic and nicotinic receptors [(Hebb and Silver, 1956; Kobayashi et al.1975; Clarke et al.1984); for review see: (Gerfen, 1992)]. Attempts to denervate this brain region failed to cause cholinergic deficits (Butcher and Butcher, 1974). Similarly, studies involving the injection of fluorescent tracers into striatal efferent targets failed to retrogradely label striatal neurons. Such studies suggested that the cholinergic population in this brain region was intrinsic. Indeed, ChAT positive striatal interneurons have been identified (Woolf and Butcher, 1981; Armstrong et al.1983; Houser et al.1983; Wainer et al.1984). These neurons have been shown to be large and aspiny, to be distributed throughout the striatum and to innervate immediate striatal regions [(Woolf and Butcher, 1981; Bolam et al.1984a; Bolam et al.1984b); for review see: (Graybiel and Ragsdale, 1983)]. ChAT-IR striatal interneurons have been found to be presynaptic to striatal GABAergic neurons which project to the substantia nigra [for review see: (Gerfen, 1992)].

Afferents to the rat striatum arise from neocortical regions, in particular, from cortical layer V pyramidal cells; a projection which appears to be glutamatergic (Spencer, 1976). Moreover, the substantia nigra and locus coeruleus provide catecholaminergic afferents to ChAT positive striatal cells (Chang, 1988; Pickel and Chan, 1990). A small number of cholinergic neurons of pedunculopontine and laterodorsal tegmental nuclei also project to striatum (Woolf and Butcher, 1986).

1.3.6 FUNCTIONAL ROLE OF CHOLINERGIC PATHWAYS IN BRAIN

Brain cholinergic systems have been implicated in many complex and diverse functions such as: sleep, wakefulness, arousal, attention, aggression, locomotor behavior as well as learning and memory [for review see: (Hannin, 1983)]. Cholinergic involvement in learning and memory has received particular attention predominantly because Alzheimer's disease (AD), which is characterized by a progressive memory loss, is associated with a reduction of presynaptic cholinergic markers (Bowen and Smith, 1976; Davies and Maloney, 1976) and degeneration of neurons in the nucleus basalis of Meynert (Whitehouse et al.1982; Pearson et al.1983). This latter observation suggested that the basal forebrain cholinergic system was especially involved in these higher functions. However, early psychopharmacological research with animals had previously provided clues that cholinergic activity could affect learning and memory. In particular, studies by Herz (1960) as well as Myers and Domino (1964) showed that blocking cholinergic function in the CNS can affect recent memory. Moreover, cognitive deficits associated with aging were suggested to be due to decreased cholinergic function since drugs which blocked cholinergic activity produced a dementia-like syndrome and memory loss in young human subjects (Drachman and Leavitt, 1974). Subsequent studies, using either rats or primates, further demonstrated that cholinergic antagonists, such as scopolamine, disrupt behavior in learning and memory-based tasks [for review see: (Bartus et al.1987)]. Moreover, young rats given anticholinergic drugs were shown to develop memory disorders comparable to those noted in some aged animals (Bartus et al.1982). As mentioned above, the finding that neurons in the nucleus basalis of Meynert degenerated in AD prompted investigators to assess the role of the basalo-cortical cholinergic system in learning and memory. This has primarily been done by lesioning the rat NBM, the counterpart of the nucleus basalis of Meynert, and to a lesser degree, areas of the neocortex. Anterograde damage to the NBM caused by either electrolytic lesions or infusions of neurotoxic substances have been shown to impair either the acquisition and/or retention of several memory-based tasks [for review see: (Dekker et al.1991a; Fibiger, 1991)]. Similarly, damage to portions of the neocortex also affect performance in such tasks (Sutherland et al.1982; Kolb et al.1983; DiMattia and Kesner, 1988; Bermudez-Rattoni et al.1991). It has

been reported that these deficits can be reversed by treating such animals with cholinomimetic agents [for review see: (Bartus et al.1987; Hagan and Morris, 1988; Fibiger, 1991; Dekker et al.1991a)]. However, it has also been proposed that behavioral impairments noted following NBM lesions may arise from non-specific damage occurring to areas in the vicinity of the NBM and perhaps could be attributed to disruption of NBM projections to the amygdala (Page et al.1991). In addition, alterations in neurochemicals other than acetylcholine are also thought to play a role (Dunnett et al.1991; Page et al.1991; Wenk et al.1989). Thus the exact involvement of the NBM-to-cortex cholinergic pathway in mnemonic processes remains to be fully established.

1.3.7 NEURODEGENERATIVE DISEASES ASSOCIATED WITH ALTERED CHOLINERGIC FUNCTION

As mentioned above, the most well-known neurodegenerative disease associated with cholinergic dysfunction is Alzheimer's disease (AD). However, the cholinergic deficit in AD represents a small part of the changes which are noted in this disorder. The pathology of AD is complex and consists predominantly of a marked accumulation of neurofibrillary tangles (bundles of paired helical filaments) in neuronal cell bodies and the presence of senile plaques in the cortical neuropile. The plaques are surrounded by dystrophic neurites, activated microglia and fibrillary astrocytes and contain a central deposit of extracellular amyloid fibrils (the core). Beta-amyloid deposition is considered a prime suspect in the initiation of the degenerative alterations occurring in AD. Consequently, the amyloid-ß-protein and its precursor protein, ß-APP, (Kang et al.1987) have been the subject of much research. However, how the pathogenesis of AD develops remains unknown. An excellent review of the molecular pathology of AD has recently been provided by Selkoe (1991). In addition to the neuronal loss or shrinkage which is noted in the nucleus basalis of brains with AD, apparent cell losses are also observed in the locus coeruleus, hippocampus, amygdala, cortex as well as other brain areas. Consequently, although acetylcholine is the neurotransmitter most consistently shown to be decreased, norepinephrine, serotonin, dopamine and somatostatin have also been reported to be diminished.

Degeneration of cholinergic neurons of the basal forebrain and diminished cholinergic markers have also been shown to occur in Down's syndrome (Mann et al.1984), Korsakoff's disease (Arendt et al.1983), progressive supranuclear palsy (Tagliavini et al.1984) and dementia pugilistica (Uhl et al.1982). Patients with such pathological disorders have also been noted to exhibit cognitive impairments. Decreases in basal forebrain cholinergic neurons, in addition to the characteristic dopaminergic cell loss in the substantia nigra, have also been noted in brains from patients afflicted with Parkinson's disease with associated dementia. Cholinergic function is also altered in non-dementia related diseases such as amyotrophic lateral sclerosis which is characterized by the loss of both upper (brain) and lower (spinal cord) motoneurons.

1.4 GANGLIOSIDES

The discovery of gangliosides, which are sialic acid containing glycosphingolipids, basically stemmed from two areas of research, namely interest in the chemical composition of the brain and in the lipids which accumulate in particular human disorders. The work of Johann Ludwig Wilhelm Thudichum, a London surgeon-chemist, in 1874-1876 provided the basis for the identification of sphingolipids and the eventual recognition of gangliosides. His contribution to this field, however, was only fully recognized after his death. An interesting account of the skepticism which his work initially received along with a detailed outline of the history of shingolipid research have been reported by Hakomori (1983). Thudichum correctly identified cerebroside, a sugar containing lipid, as well as shingosine, the most charactersitic component of glycosphingolipids. In addition, "protagon", a glycoside named as such by Leibrech (1865) and believed to be an essential chemical component of brain, was determined to be a mixture of cerebroside and sphingomyelin by Thudichum. Significant progress towards the identification of present day gangliosides was also provided by the work of Gunnar Blix who reported the presence of a compound which was widely distributed in various glycoproteins and which he named "sialic acid" (Blix, 1938). The definitive description of the composition of gangliosides is attributed to Ernest Klenk who in 1935 described the large accummulation of a compound he named "Substanz X" in the brain of a patient who died of Tay-Sachs' disease (Klenk, 1935). The methanolyzate of "Substanz X", was shown by Klenk to exhibit similar chemical properties as the compound reported by Blix, thus indicating the presence of sialic acid. Subsequent work with bovine brain and further isolation of this material led Klenk to propose the name "ganglioside" for this group of glycolipids (Klenk, 1942), because he believed that they were concentrated in the "Ganglienzellen" (neurons) of the gray matter. Despite the difficulties associated with the separation and purification of gangliosides at that time, Klenk isolated ganglioside preparations free of contaminating phopholipids and cerebrosides. Based on his analysis, Klenk proposed that gangliosides consisted of fatty acid, shingosine, hexose, galactosamine and sialic acid.

That gangliosides are heterogeneous substances was first proposed by Lars Svennerholm

(1954). However, it was not until 1963 that the definitive structure of four mammalian brain gangliosides, now known as GM1, GD1a, GD1b and GT1b (nomenclature according to Svennerholm, see below), was reported by Kuhn and Wiegandt. These investigators also established that ganglio-N-tetraose (Galß1-3GalNAcß1-4Galß1-4Glc-Cer) comprised the ganglioside core and clearly demonstrated that the position of substitution by sialosyl residues was to the middle galactosyl and to the terminal galactosyl residue of this core structure (Kuhn and Wiegandt, 1963). Advances in isolation and purification procedures have since allowed the discovery of over 60 ganglioside structures which have been found in both neural and extraneuronal tissues (Ledeen, 1983).

1.4.1 PHYSICO-CHEMICAL FEATURES OF GANGLIOSIDES

Gangliosides are located in the outer leaflet of the plasma membrane of all vertebrate cells but are particularly concentrated in brain (Ledeen et al.1976; Ledeen, 1985). They are held within the plasma membrane by virtue of their lipidic ceramide portion, while the oligosaccharide chains of gangliosides protrude from the cell surface (Figure 1.3). With few exceptions, all gangliosides can be classified into a series according to the sequence and type of chemical bonds in their oligosaccharide structure (Table 1.1). The most current and widely used nomenclature for gangliosides was established by Svennerholm (1980). According to his simplified scheme, sialic acid residues of the ganglioside are designated as M, D, T, Q and P indicating the presence of mono, di, tri, tetra or poly sialosyl residues, respectively. The rest of the molecule is assigned a number according to the length of the carbohydrate chain with increments above one indicating shorter chain lengths. The oligosaccharide portion of gangliosides consist of a sequence of neutral carbohydrates (glucose, galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine) to which one or more sialic acid residues are linked via an α-ketosidic bond. The presence of sialic acid is the feature which characterizes gangliosides within the glycolipid family. It is also a generic term for derivatives of neuramininc acid of which the major form found in most brains is N-acetylneuraminic acid (NAN). Brains of some species also contain minor amounts of N-glycolylneuraminic acid but this form, along with NAN, is widely distributed in extraneural tissues (Ledeen, 1985). Sialic acid is thought to play an important role in ganglioside functions since its carboxy group can

dissociate at physiological pHs thus, conferring to gangliosides their characteristic negative charge. Such a property of this molecule also renders gangliosides highly hydrophilic.

TABLE 1.1: CLASSIFICATION OF GANGLIOSIDES

SERIES	EXAMPLE	OLIGOSACCHARIDE STRUCTURE
GALA	GM4	NANα2-3GAlß1-1'Cer
HEMATO	GM3	NANα2-3Galß1-4Glcß1-1'Cer
NEOLACTO	LM1 OR SPG	NANα2-3Galß1-4GlcNAcß1-3Galß1-4Glcß1-1'Cer
GLOBO		NANα2-3Galß1-3GalNAcß1-3Galα1-4Galß1-4Glcß1-1'Cer
GANGLIO	GM1	Galß1-3GalNacß1-4Galß1-4Glcß1-1'Cer 3 α | 2 NAN

Adapted from (Yu, 1984). Abbreviations: Cer, ceramide; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; NAN, n-acetylneuraminic acid.

The lipidic portion or ceramide of gangliosides is composed of fatty acid, stearate being the major form in brain gangliosides, joined by an amide bond to sphingosine or a similar long chain base. This portion of the molecule is highly hydrophobic due to the presence of the 2 hydrocarbon tails. The vast heterogeneity of gangliosides has been attributed to differences in the composition of both the oligosaccharide and ceramide portions. Due to their chemical structure, gangliosides show strong amphiphilic properties that dictate their behavior in solution. In contrast to phospholipids, in water, gangliosides form micelles rather than vesicles or bilayers due to their large hydrophilic sugar headgroups. Concentrations and temperatures at which gangliosides can form micelles range widely between 10^{-9}-10^{-2} M and 0-100^{0} C, respectively, depending upon the ganglioside species and saturation of its hydrocarbon tail (Corti et al.1987).

The monosialoganglioside GM1 has perhaps been the most studied ganglioside to date because of its putative neuronotrophic and neuritogenic properties (see section 1.4.7). As implied by its nomenclature, it contains one sialic acid group linked to an inner galactose residue (Figure 1.4) which confers its negative charge. GM1 has an approximate molecular weight of 1545 but in solution can form large micelles containing between 100-300 molecules with an estimated molecular weight of 500,000 daltons (Bach et al.1982). One mole of GM1 has been reported to bind 22-30 moles of water (Bach et al.1982). Several semisynthetic derivatives of GM1 have been made (see Figure 1.5) and these include: AGF2, an internal ester of GM1; LIGA4 and LIGA20, compounds in which the fatty acid tail at the 2-amino position is substituted by acetyl or dichloroacetyl groups, respectively; GM1-OH and GM1-CH3, derivatives in which the negative charge of sialic acid is removed via substitution of its carboxy group with a primary alcohol (GM1-OH) or by creating a methylester (GM1-CH3). Several other derivatives also exist (Alidino et al.1984; Manev et al.1989; Lipartiti et al.1992). AGF2 undergoes slow hydrolysis in serum to give rise to the original GM1 compound but has a longer plasma half-life (AGF2: 180 $\pm$ 4 minutes; GM1: 145 $\pm$ 13 minutes) and a wider volume of distribution (AGF2: 105 $\pm$ 3.5 ml/kg, GM1: 60 $\pm$ 2.3 ml/kg) (Alidino et al.1984). AGF2 mimics the effects of GM1 but is more potent in some instances (Alidino et al.1984). The LIGA compounds have been assesed *in vitro* and also appear to be more active than GM1 (Manev et al.1989; Lipartiti et al.1992). GM1-OH and GM1-CH3 have also been tested *in vitro* and were neuritogenic in 3-cell culture systems (Neuro-2A, PC12 and dorsal root ganglia cells) (Cannella et al.1990).

1.4.2 BIOSYNTHESIS OF GANGLIOSIDES

Sphingosine, a long-chain aliphatic amine, is the building block of shingolipids and it is formed from palmitoyl-CoA and l-serine by a series of reactions involving NADPH, pyridoxyl phosphate and FAD (Lehninger, 1975). To form N-acylsphingosine or ceramide (the hydrophobic lipid chain of gangliosides), the amino group of sphingosine is acylated by a long chain fatty acyl-CoA in a reaction involving the microsomal enzyme sphingosine acyltransferase. Subsequently, the cerebrosides are formed from ceramide and UDP-D-glucose or UDP-D-galactose. Much of what is presently known about ganglioside

biosynthesis has been established by *in vitro* studies and can be attributed to the early work of Rosemann and coworkers [(Kaufman et al.1968; Arce et al.1971; Roseman, 1970; Maccioni et al.1971; Maccioni et al.1978; Fishman et al.1972; Basu et al.1973); for review: (Fishman and Brady, 1976)]. Following the formation of cerebroside, the biosynthesis of gangliosides proceeds in a step-wise manner through the sequential addition of individual sugar and sialic acid groups to the growing glycolipid (Figure 1.6). The process is mediated by membrane bound glycosyltransferases which reside on the luminal side of the Golgi cisternae. Carrier proteins are believed to serve as transporters for the sugar nucleotides which are synthesized in the cytosol and cannot penetrate the Golgi membrane where the synthesizing enzymes are located [for review see: (Yasuf et al.1984)]. As can be seen (Figure 1.6), the product of a transferase reaction becomes the specific acceptor substrate for the next enzyme. Lactosylceramide (Cer-Glc-Gal) appears to be the common precursor for all gangliosides of the ganglio series, while GM3 is a central branching point where the biosynthetic route splits towards 2 different series of gangliosides (a and b). N-acetylgalactosaminyltransferase (GM2 synthetase) which converts GM3 to GM2 is thought to have a key regulatory role in ganglioside biosynthesis since only tissues or cells with this enzyme have more complex gangliosides (Fishman and Brady, 1976). Although studies by Roseman and colleagues made significant progress towards the elucidation of ganglioside biosynthesis, the concept of precursor product relationship may not hold *in vivo* since labelling studies have yet to demonstrate significant levels of precursors (Arce et al.1971; Maccioni et al.1971; Caputto et al.1976). Rather, it has been suggested that separate enzyme systems exist which give rise to two distinct ganglioside pools, a small one of transient precursors and a large pool of end products (Caputto et al.1976; Miller-Podraza et al.1982). Ganglioside biosynthesis, *in vivo*, may therefore be more complex than proposed.

The site of ganglioside synthesis is the golgi apparatus in the cell perikaryon. This has definitively been shown by studies using the chick optic system where the cell body was separated from nerve endings (Landa et al.1979). It was shown that the limited synthesis which occurs at nerve endings most likely arises due to the action of membrane-bound degradative enzymes (sialidases), a process known as "ganglioside trimming" (Preti et al.1980).

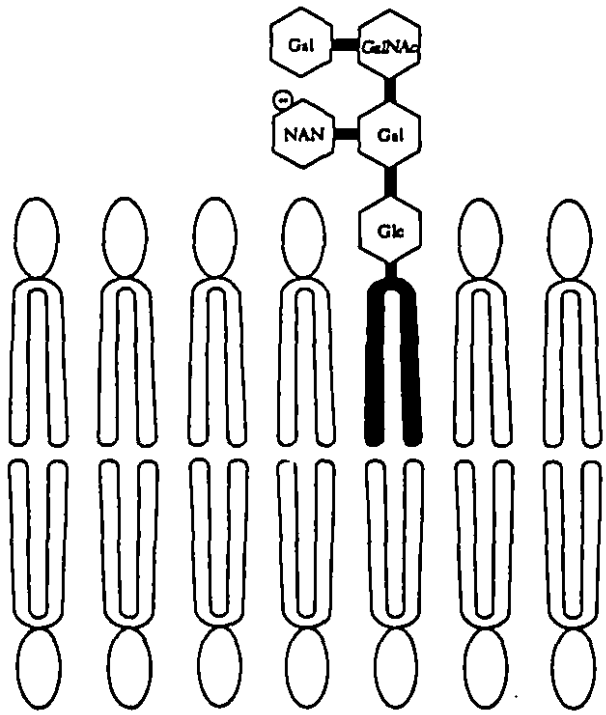


Figure 1.3 Localization of gangliosides in plasma membrane. Gangliosides are held within the outer leaflet of the plasma membrane lipid bilayer via their hydrophobic ceramide moieties. The oligosaccharide portions of the ganglioside molecule protrude from the cell surface. Ganglioside shown is GM1. Abbreviations: Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; NAN, N-acetylneuraminic acid.

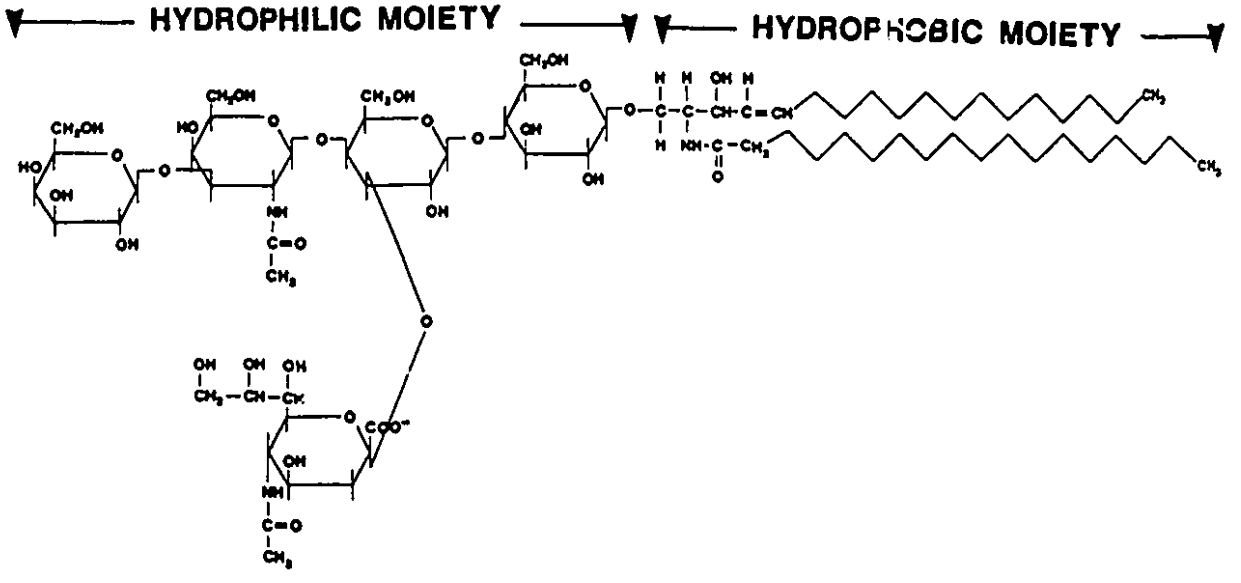


Figure 1.4 Chemical structure of the monosialoganglioside GM1.

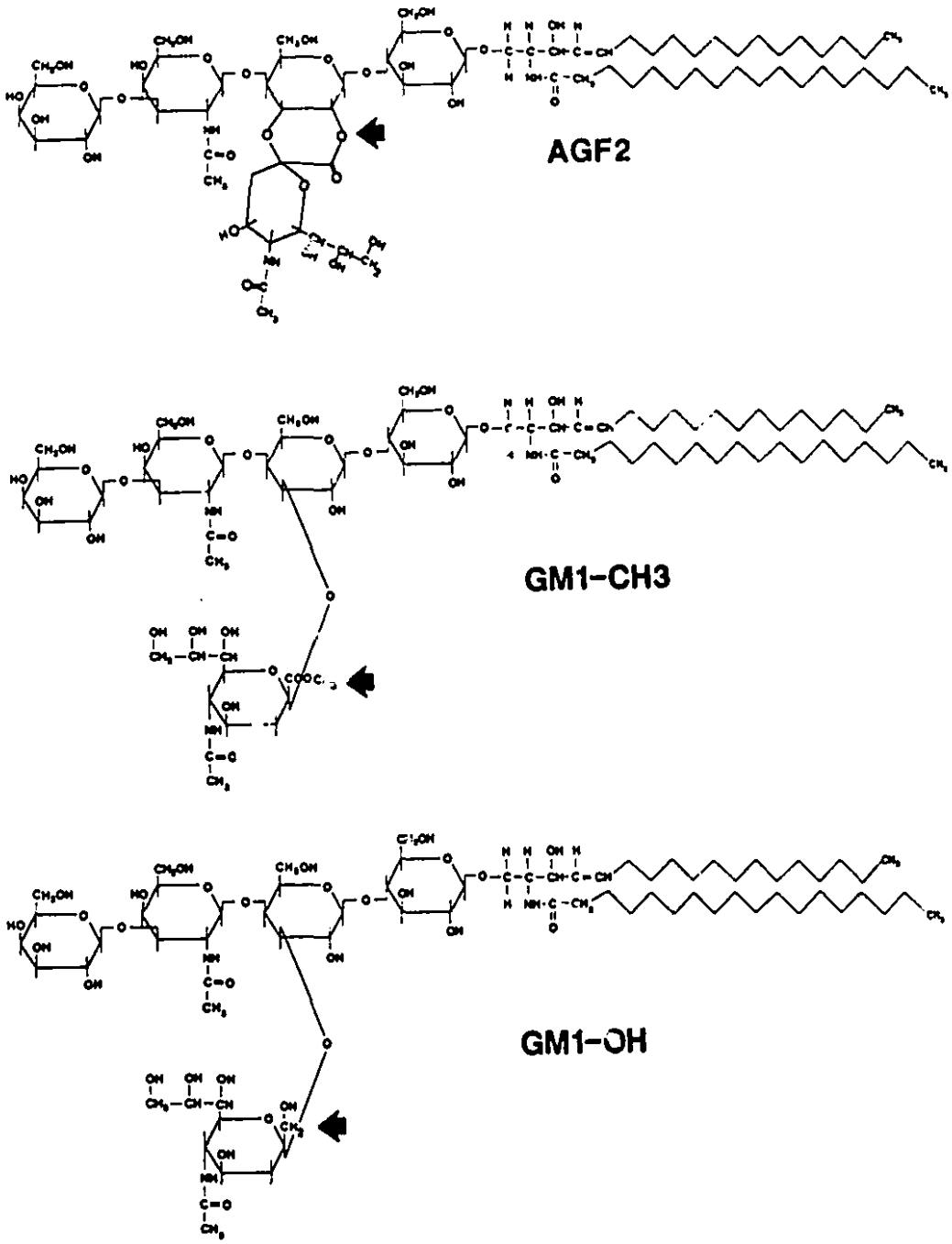
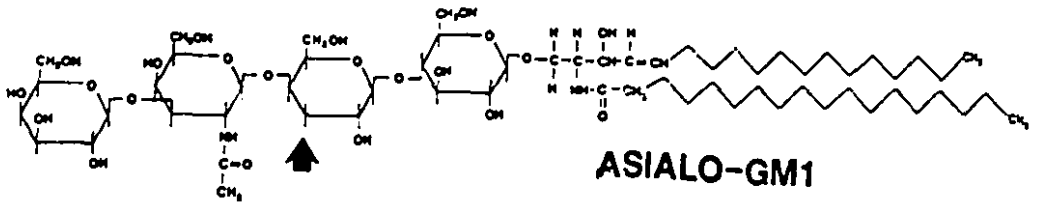
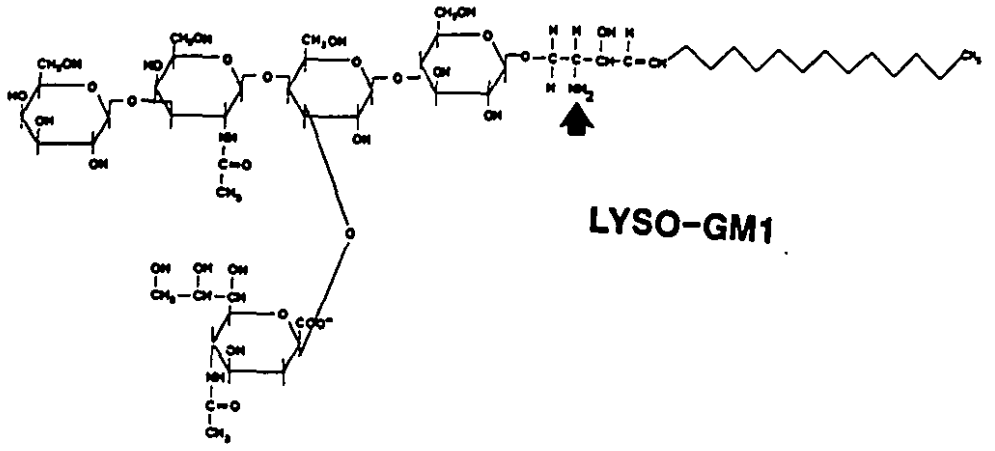


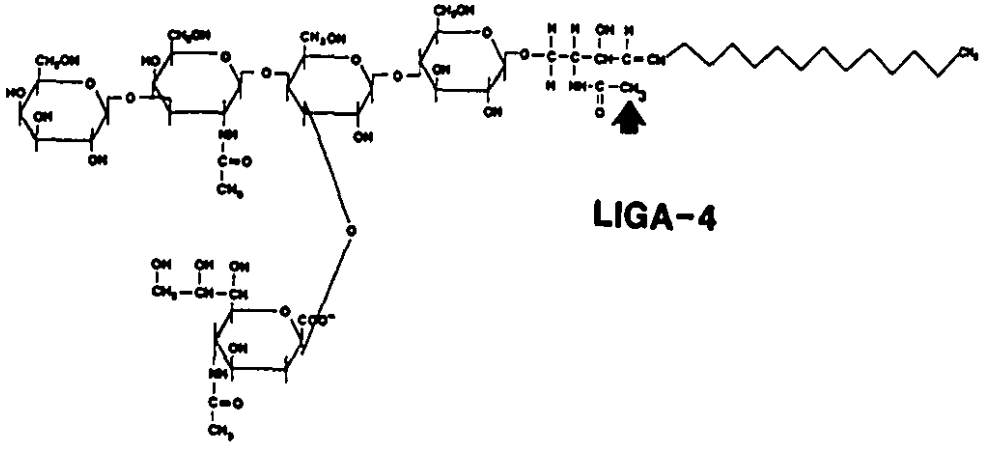
Figure 1.5 Chemical structures of some derivatives of the monosialoganglioside GM1. Arrow indicates sites of modification from the parent GM1 molecule.



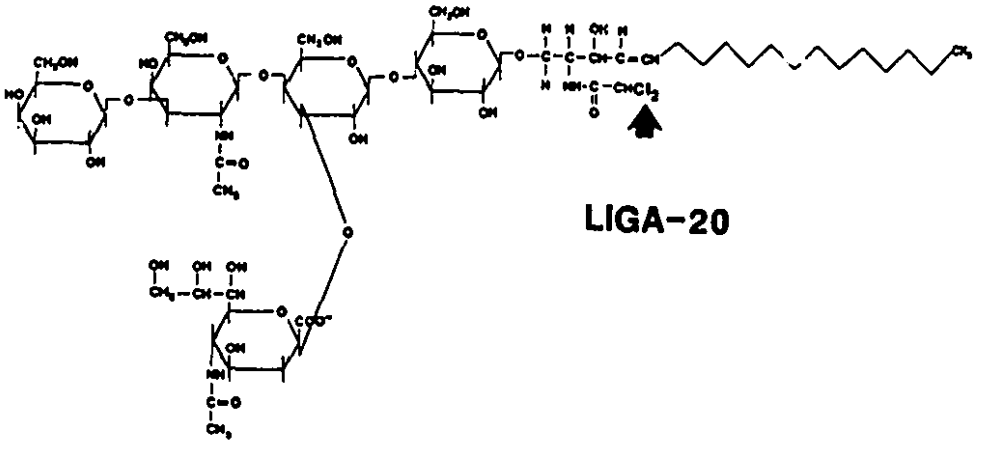
ASIALO-GM1



LYSO-GM1



LIGA-4



LIGA-20

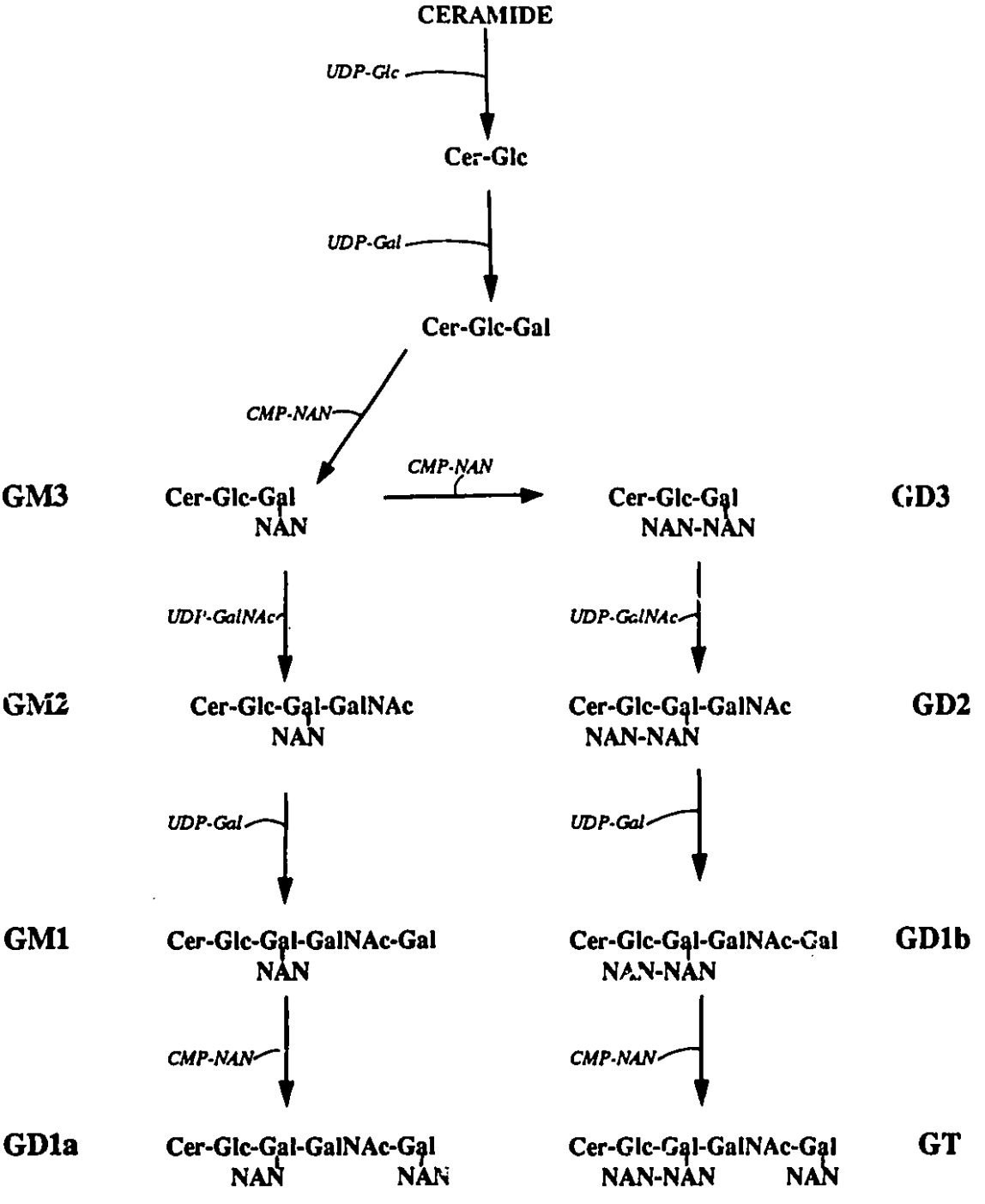
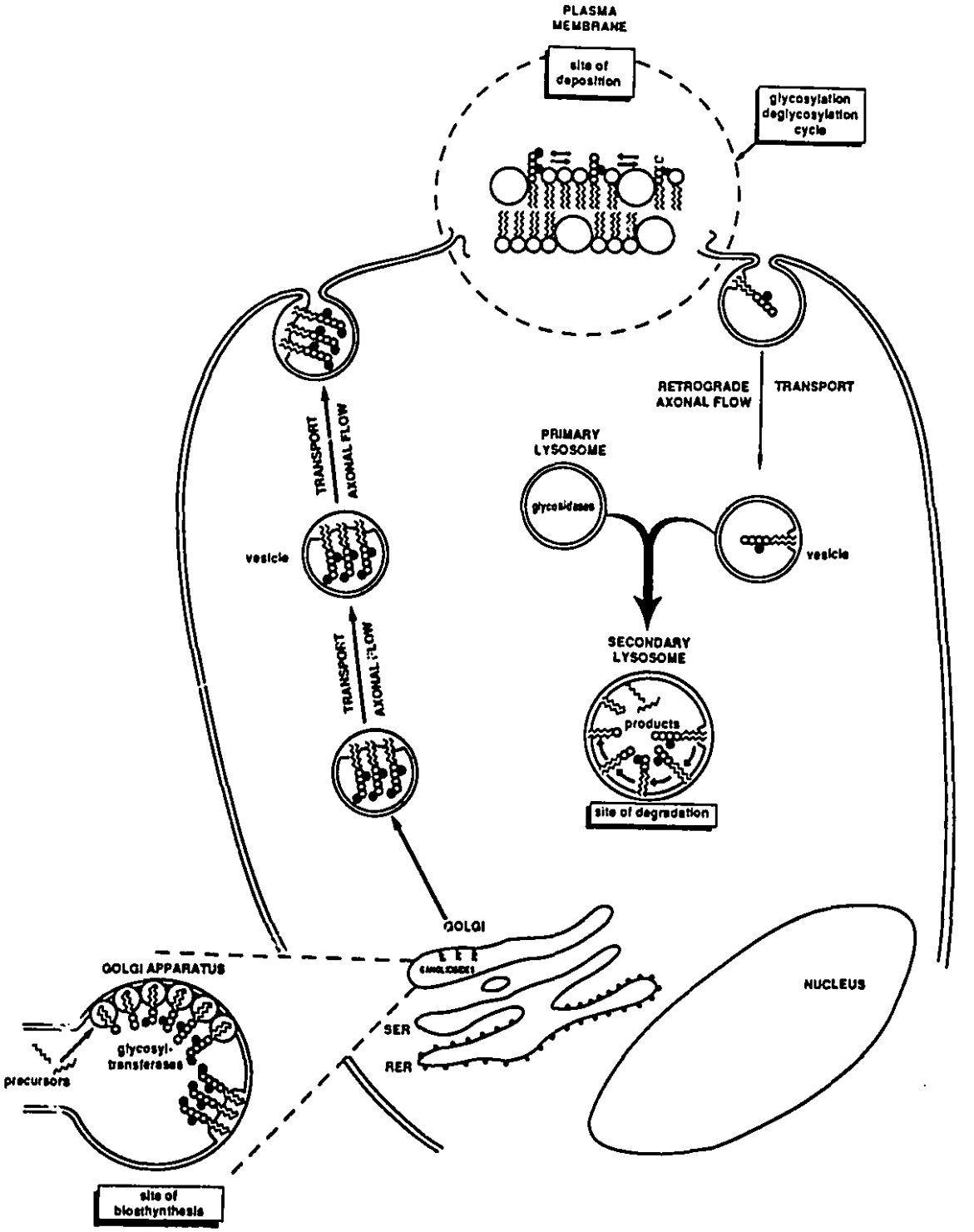


Figure 1.6 Outline of ganglioside biosynthetic pathway (adapted from Fishman, P.H. and Brady R.O., 1973). Abbreviations: CER, ceramide; Gal, galactose: GalNAc, N-acetylgalactosamine; Glc, glucose; NAN, N-acetylneuraminic acid.

How gangliosides are transported to the plasma membrane, where they reside, following synthesis remains unclear. Axonal transport of gangliosides was first reported in the goldfish optic nerve by Forman and Ledeen (1972). Work in the rat brain subsequently showed that newly synthesized gangliosides are membrane bound in a manner unavailable to degradative enzymes (Landa et al.1981). It has been suggested that at the sites of the golgi apparatus a process takes place to form vesicles which carry gangliosides. In these vesicles, gangliosides would be associated with the membrane and located asymmetrically on the inner surface (Figure 1.7). Fusion of the transport-vesicle with the plasma membrane would then automatically expose the ganglioside on the outer surface of the membrane. The details of this proposed mechanism have yet to be elucidated. Transport of gangliosides from the cell body to the plasma membrane is a fast process having been reported to be approximately 300-400 mm/day in both the PNS and CNS (Miller-Podraza and Fishman, 1982; Aquino et al.1985; Aquino et al.1987).

Figure 1.7 Illustration of major compartments of ganglioside metabolism. The biosynthesis of gangliosides is catalyzed by membrane-bound transferases in the lumen of the endoplasmic reticulum and Golgi stacks. The membrane-bound gangliosides are then transported to the plasma membrane where some degradation of gangliosides can occur via membrane-bound sialidases. Gangliosides are catalyzed predominantly in lysosomal compartments. Drawing modified from that shown in (Tettamanti, 1984; Tettamanti et al.1987).


PLASMA MEMBRANE
site of deposition
glycosylation deglycosylation cycle
RETROGRADE AXONAL FLOW
TRANSPORT
PRIMARY LYSOSOME
glycosidases
vesicle
TRANSPORT
AXONAL FLOW
vesicle
TRANSPORT
AXONAL FLOW
SECONDARY LYSOSOME
products
site of degradation
GOLGI
GOLGI APPARATUS
glycosyl-transferases
precursors
SER
RER
NUCLEUS
site of biosynthesis

1.4.3 REGULATION OF GANGLIOSIDE SYNTHESIS

The enzymes thought to play a key role in the regulation of ganglioside biosynthesis are: GM3 synthetase (CMP-sialic acid: LacCer sialosyltransferase), GM2 synthetase (GM3:UDP-GalNAc N-acetylgalactosaminyltransferase) and GD3 synthetase (GM3: CMP NeuAc sialosyl-transferase) which produce respectively, GM3, GM2 and GD3. The synthesis of gangliosides can be regulated by affecting their synthesizing enzymes either at the transcription or post-transcription level. The former is inferred from changes noted in ganglioside levels during development or oncogenic transformation, instances when alterations in gene expression are known to occur. Support for this suggestion was provided by work demonstrating that the ganglioside content of cells can be altered following transfection of oncogene containing DNA. One such report demonstrated that transfection of the human adenovirus type 12 early gene, EIA, altered ganglioside synthesis in a rat fibroblast cell line (3Y1) (Sanai and Nagai, 1989). Moreover, transfection with the myc, src, H-ras or fes oncogenes also altered ganglioside expression in cells (Nakaishi et al.1988; Nagai et al.1986). With respect to post-translational modifications, phosphorylation has been proposed to regulate ganglioside-synthesizing enzymes. Phosphotyrosine residues have been identified on the liver CMP-sialic-lactosylceramide sialyltransferase enzyme by Western blot analysis (Sweeley, 1991). Furthermore, agents known to affect protein kinase C and to alter phosphorylation such as butyric acid, retinoic acid and phorbol esters have also been shown to cause significant elevations in GM3 synthetase as well as in GM2 synthetase (Fishman et al.1974; Macher et al.1978; Burczak et al.1983). Moreover, addition of cyclic AMP to rat brain microsomes has also been reported to increase ganglioside synthesis (Scheideler and Dawson, 1986). Alternatively, agents which decrease cyclic AMP levels inhibit ganglioside synthesizing enzymes (Dawson et al.1980; McLawhon et al.1981). The phosphorylation sites and the mechanism by which enzyme activity is regulated remain to be fully elucidated. Feedback regulation as a mechanism of controlling ganglioside biosynthesis has also been suggested. Gangliosides GT1b and GD1a have been shown to be potent inhibitors of GM2 synthetase in microsomes of chick retina *in vitro* (Nores and Caputto, 1984), but the contribution of this mode of regulation to *in vivo* biosynthesis remains unknown. Alterations in ganglioside synthesis have also been reported following changes in metabolic state. For

example, a greater incorporation of precursor label occurs in gangliosides of chicks exposed to light compared to those kept in dark (Caputto et al.1982) and in young rather than aged rats [for review see: (Ando, 1983)]. As well, anaesthesia, hypoxia and brain lesions can also cause alterations in ganglioside synthesis [(Domanska-Janik, 1988); for review see (Ando, 1983)].

1.4.4 GANGLIOSIDE TURNOVER AND DEGRADATION

Various turnover rates have been reported for endogenous gangliosides (20-30 days). Discrepancies between studies are most likely due to differences in the type of labelled perscursors employed and systems examined. For example, the turnover rate for GM1 in myelin has been reported to be higher than that of GM1 in rat whole brain (Suzuki, 1970). In addition, rates are thought to be overestimated because reutilization and recycling of labelled sugars can occur which is not easily accounted for. The turnover rate for sialic acid, which is thought to be an important functional group of the ganglioside molecule has been reported to be 6-8 days in rats (Ando, 1983).

The fate of exogenously administered gangliosides has been studied mostly by using GM1 labelled with tritium ([^{3}H]) on its terminal galactose ([^{3}H]Gal-GM1) or shingosine ([^{3}H]Sph-GM1) portion of the molecule. Gangliosides administered intravenously have been shown to bind serum albumin rendering it less susceptible to the actions of plasma hydrolases (Irwin and Irwin, 1982). The half life of GM1 in blood has been reported to range between 2. 75 and 3.8 hours (Barkai and Di Cesare, 1975). [^{3}H]Gal-GM1 administered by various routes (intramuscular, i.m.; subcutaneous, s.c.; intravenous, i.v.) to mice was shown to incorporate at particularly high concentrations in liver (Orlando et al.1979). Much of the labelled GM1 was recovered bound tightly and stably to the plasma membrane. At peak times, the non-volatile radioactivity recovered in brain was approximately 1/30 th that of liver. Similar results were obtained in studies which employed rats (Masco and Seifert, 1988; Ghidoni et al.1986). When injected intracerebroventricularly (i.c.v.), [^{3}H]Gal-GM1 was shown to be rapidly incorporated into membranes and did not appear to be catabolized until after 96 hours (Masco and Seifert, 1988). Using [^{3}H]Sph-GM1 injected intracisternally, Ghidoni and

coworkers (1989) also noted stable incorporation of GM1 into brain plasma membrane with no extensive break down prior to 96 hours. However, at later times both degradative and biosynthetic compounds were identified in rat brain tissue. Degradative products noted were GM2, GM1 lacotsyl, glycosyl and ceramide. The biosynthetic products noted were GD1a, GD1b and sphingomyelin.

All brain cell types contain the appropriate enzymes necessary for ganglioside catabolism although their levels may differ. For example, concentrations of ß-galactosidase, which initiates the catabolism of GM1, have been found to be greater in neuronal cell bodies than in astroglia and oligodendroglial fractions of adult rabbit and bovine brain (Freysz et al.1979). In accordance with what is known about the metabolism of other membrane-bound compounds it has been suggested that gangliosides are internalized, along with a patch of the plasma membrane to give rise to vesicles. These vesicles then fuse with primary lysosomes where degradation of gangliosides takes place. The degradation of gangliosides proceeds in a sequential manner and in reverse order to its biosynthesis [for review see: (Ando, 1983; Fishman and Brady, 1976; Ledeen, 1989)]. Most of the degradative enzymes are indeed lysosomal as evidenced by their sedimentation coefficients, enhanced activities in the presence of detergents and acidic pH optima (Ledeen, 1989). An exception to this are the sialidases which can be found both within and outside lysosomal structures (Schengrund and Rosenberg, 1970; Tettamanti et al.1972; Schengrund et al.1976; Schengrund et al.1979). Some degradation of gangliosides is thought to occur nonlysosomally. Sialic acid residues of multisialated gangliosides can be removed by sialidases located in plasma (Schengrund et al.1976; Schengrund et al.1979) or synaptic (Schengrund and Rosenberg, 1970; Tettamanti et al.1972) membranes. The importance of lysosomal degradation, however, has been made apparent by inborn lysosomal disorders where the absence of ß-galactosidase and ß-hexosaminidase enzymes in these compartments result in the intralysosmal accumulation of GM1 or GM2 leading to pathological consequences (see section 1.4.9).

Catabolism of GM1 begins with the action of ß-galactosidase which converts it into GM2. Two distinct genetic forms of this enzyme exist in mammalian brain, one which hydrolyzes GM1 and the other GalCer (Suzuki et al.1980). GM2 is then converted to GM3 by N-acetyl ß hexosaminidase. This enzyme exists as two isoforms, A and B, consisting of respectively, a heterodimer of α and ß subunits and a homodimer of 2 ß subunits (Sandhoff et al.1989).

Only hexosaminidase A can degrade GM2. Activator proteins are also required by ß-galactosidase and hexosaminidase enzymes for ganglioside degradation. These activator proteins, also located within the lysosome, appear to function by removing single gangliosides from the lysosomal membrane and presenting them to the hydrolytic enzyme in a 1:1 complex (Conzelmann et al.1982). *In vitro* the "GM2 activator" protein has been shown to be specific for hexosaminidase A which hydrolyzes GM2. The other, known as "sulfatide activator (SAP-1)", activates ß-galactosidase which catabolizes GM1 (Furst et al.1986; Ledeen, 1989). GM1 and GM2 are thought to be resistant to most sialidases due to steric hindrance caused from an "oxygen cage" surrounding the ketosidic linkage of NAN (Schauer et al.1980; Harris and Thornton, 1978). By contrast, GM3 can be catabolized by sialidases since no such steric barrier exists in this molecule. Subsequent degradation, after GM3, involves the enzyme glucosyl-ceramide ß-glucosidase which cleaves the final carbohydrate, GLc. Ceramide is then hydrolyzed to fatty acid and shingosine which are then also further degraded (Lehninger, 1975).

1.4.5 GANGLIOSIDE EXPRESSION DURING DEVELOPMENT

Different patterns as well as increases in gangliosides can be noted during development in both the rat and human cerebral cortex (Vanier et al.1971). In the rat brain, ganglioside composition and biosynthesis is altered between embryonic day 14 (E14) and birth. In addition, a major increase in ganglioside concentration occurs between birth and post-natal day 17 (Yavin and Yavin, 1979; Irwin and Irwin, 1979; Irwin et al.1980; Hilbig et al.1982; Irwin and Irwin, 1982). At E14, GM3 and GD3 are the predominant species in rat brain. By E16 gangliosides of the "b" series such as GD1b, GT1b and GQ1b increase in content while after E18 the "a" series gangliosides such as GM1, GD1a and GT1b appear. At this time point, GM3 and GD3 decrease. In humans, a major increase in ganglioside concentration occurs between the the 15th week of gestation and 6 months of post-natal life (Suzuki, 1965; Rahmann, 1980). In frontal cortex GD1a, which is predominant in newborn brain subsequently decreases with age. This was also noted for GM1. By contrast, GD1b and GT1b both increase with age, reach adult levels at 30 years and are maintained up to 90 years (Ando, 1983). In both species, such changes in ganglioside composition have been

shown to correlate with developmental events such as cell proliferation, migration, myelination and with the formation of synaptic contacts. In brief, it appears that during times of neuronal and glial multiplication GD3 and GM3 predominate (Yates, 1986). The content of GD3 decreases after neuroblast mitosis ceases, at which time highly sialyated complex gangliosides begin to accumulate (Rösner, 1980; Hilbig et al.1982; Rösner, 1982). Gangliosides of the "a" pathway such as GM1 and GD1a as well as those of the "b" pathway such as GD1b and GT1b increase substantially during periods of synaptogenesis and neuronal growth (Rösner, 1980; Willinger and Schachner, 1980). Levels of GD3 are known to increase just before the onset of myelination when oligodendroglia proliferation occurs. During myelination, GM1 and GM4 accumulate. Further changes in the ganglioside composition of myelin also occurs with age (Suzuki, 1967; Yu and Yen, 1975), as evidenced by the progressive increases in the proportion of GM4 and GM1. Although the role of gangliosides in myelin is not known they are thought important for intermolecular interactions with myelin basic protein (Cochran et al.1982).

1.4.6 LOCALIZATION OF GANGLIOSIDES IN THE ADULT NERVOUS SYSTEM

A particulary high concentration as well as diversity and complexity of gangliosides exist in brain as compared to other extraneural tissues or organs [for review see: (Ledeen, 1983; Ledeen, 1989)]. Although early studies indicated that gray matter had a higher concentration of gangliosides than white matter, which suggested a preferential neuronal localization, astroglia and oligodendroglia have now been shown to contain substantial amounts of gangliosides (Ledeen, 1989). On a molar basis, 4-6% of total neuronal phosholipid content consists of gangliosides. They are thus only minor components of the membrane. Differences in ganglioside concentrations between tissues have been reported. Their distribution in some areas of the rat brain is shown in Table 1.2.

TABLE 1.2: GANGLIOSIDE COMPOSITION OF ADULT RAT BRAIN

BRAIN AREA	PERCENT OF TOTAL LIPID BOUND SIALIC ACID							
	GM1	GM2	GM3	GM4	GD1a	GD1b	GT1b	GQ1b
CORTEX	14.1	3.2	3.2	-	32.1	13.2	22.7	5.1
STRIATUM	17.5	1.8	3.5	-	40.6	13.4	14.8	2.6
HYPOTHALAMUS	13.6	1.9	5.5	-	27.1	16.6	20.9	5.1
PONS-MEDULLA	13.0	0.3	5.1	2.0	16.3	19.2	24.7	3.1

Adapted from (Ando, 1983)

1.4.7 GANGLIOSIDE EFFECTS

The hypothesis that gangliosides could regulate growth and differentiation derived in large part from the observations of Purpura and Suzuki who demonstrated that ganglioside storage diseases were characterized by an ectopic neurite outgrowth (Purpura and Suzuki, 1976; Purpura, 1978). In addition, studies showing alterations of these agents during development as well as oncogenic transformation lent further support to the notion of their participation in growth processes. The function of gangliosides as neuritogenic or neuronotrophic agents both *in vitro* and *in vivo* are discussed in sections 1.4.7a and 1.4.7b. In addition, a function for gangliosides as "receptors" or receptor modulators is described in section 1.4.8.

1.4.7a IN VITRO EFFECTS OF GANGLIOSIDES

Gangliosides have been shown to have both neuritogenic (ie: affecting neuronal process

number, length or branching) and neurotrophic (ie: regulating neuronal survival and maintenance) activity *in vitro*. In 1977, Obata and collaborators noted that synapse formation, reflected by measures of end plate potentials, in co-cultures of chick embryo spinal cord and pectoral muscle could be stimulated by 0.25 mM GM1 (Obata, 1977). Subsequent to this study, other reports examined the effects of gangliosides in a number of cell culture systems. The most commonly used were Neuro-2A, a neuroblastoma clonal cell line, and dorsal root ganglia primary cell cultures. Extensive neuronal sprouting of Neuro-2A cells was noted after a 40-hour incubation in media supplemented with 250 μg/ml of a ganglioside mixture isolated from bovine brain (Roisen et al.1981). This sprouting response was seen as early as 6 hours after the ganglioside mixture was added. Moreover, an increase in spine-like projections were noted by scanning electron microscopy. Dorsal root ganglia cells also showed increases in process number when exposed to ganglioside concentrations of 75 μg/ml and 750 μg/ml (Roisen et al.1981). Furthermore, the ganglioside mixture used also caused an elevation in the levels of ornithine decarboxylase (ODC); the rate limiting enzyme in polyamine biosynthesis, which is often used as an indicator of metabolic activity or growth. A mixture of gangliosides composed of GM1, GD1a, GD1b and GT1b at a concentration of 125 μg/ml was also found to increase neurite formation in Neuro-2A cells (Dimpfel et al.1981).

Investigations using individual ganglioside species showed that 100 μg/ml of GM1 alone could increase process length of S20Y neuroblastoma cells (Carine and Schengrund, 1984). Neurite outgrowth and the formation of apparently mature synaptic contacts were also induced by GM1 in Neuro 2A cells (Spoerri, 1983). These effects of GM1 on Neuro-2A cells were shown to correlate with its stable insertion into the neuronal plasma membrane (Facci et al.1984). However, some skepticism arose with regard to ganglioside-induced effects *in vitro*, because of a report which indicated that ganglioside-induced mitogenesis of B103 and B104 cells (cell lines derived from chemically induced rat brain tumors) was due to peptide contaminants rather than the gangliosides themselves (Morgan et al.1983). This issue was resolved by Byrne and collaborators (1983) who employed extensive chromatographic and proteolytic procedures to remove possible contaminants from the ganglioside sample, and who demonstrated that the neuritogenic activity of gangliosides in Neuro-2A cells failed to be diminished. Furthermore, 11 different ganglioside species were examined in this cell culture system and were all shown to be highly active, with the

exception of GM4. Thus, in addition to confirming the neuritogenic properties of gangliosides, this study also indicated an apparent lack of specificity of Neuro 2A cells for individual ganglioside species. This lack of specificity, however, does not hold for all cell types since S2OY murine neuroblastoma cells exhibit neuritogenesis in the presence of GM1 but not GD1a (Carine and Schengrund, 1984), while human neuroblastoma GOTO and NB-1 cells appear responsive only to GQ1b (Nakajima et al.1986). Interestingly, these cells which show structural specificity for gangliosides require concentrations one magnitude lower than that needed by others. Cells with such specificity requirements have been classified either as: "N"-type cells, which exhibit narrow ganglioside specificity and require low (nM range) optimal ganglioside concentrations; or "M"-type cells, which exhibit broad ganglioside specificity and require high (μM range) optimal ganglioside concentrations (Tsuji et al.1988). It has been suggested that cellular mechanisms which mediate ganglioside responses may differ depending on cell type or animal species.

Primary neuronal culture systems in which GM1 has been shown to promote neurite outgrowth include: embryonic day 8 (E8) and E15 chick dorsal root ganglia (Roisen et al.1981; Leon et al.1984a; Doherty et al.1985; Skaper and Varon, 1985; Skaper et al.1985; Cannella et al.1988), chick E8 forebrain (Skaper et al.1985), guinea pig spinal root ganglia (Hauw et al.1981), E8 ciliary chick ganglia (Skaper and Varon, 1985; Skaper et al.1985), E11 sympathetic ganglia (Skaper and Varon, 1985), rat E18 hippocampus (Skaper et al.1985), E8 cerebral cortex (Skaper et al.1985) and E18 striatum (Skaper et al.1985). Mouse mesencephalic (Leon et al.1988), rat cerebellar (Manev et al.1989) and rat septal cells (Hartikka and Hefti, 1988; Cuello et al.1989) are also responsive to gangliosides. In mesencephalic cells, the addition of exogenous GM1 to the culture medium was shown to increase ^{3}H-dopamine uptake in a time- and dose-dependent manner as well as to enhance the long-term survival of these cells (Leon et al.1988). Such events were again found to correlate with the stable insertion of GM1 into the plasma membrane. The addition of a-sialoGM1, sialic acid or the oligosaccharide portion of GM1 to the culture media failed to elicit responses indicating that the entire molecule is necessary for biological activity. In cerebellar cultures, GM1 was shown to inhibit delayed neuronal death induced by glutamate (Manev et al.1989). Liga 4 and Liga 20, derivatives of GM1, produced similar effects but proved more potent than the parent molecule (Manev et al.1989). In cultures of basal forebrain

cholinergic neurons GM1 stimulated ChAT activity (Hefti et al.1985a; Cuello et al.1989), but did not alter survival or neurite outgrowth of these cells (Hefti et al.1985a). By contrast, enhanced survival as well as differentiation was induced by gangliosides in B104 cells (Morgan and Siefert, 1979). In addition, an increase in cell number, as well as body area, primary neurite length and the sprouting of secondary processes were noted in ganglioside-treated primary cultures of embryonic day 8 chick cerebrum (Massarelli et al.1985).

Inhibitory effects of gangliosides have also been reported. Treatment of rat basal forebrain cultures with a mixture of ganglioside species, GM1 alone or GD1a alone, has been shown to attenuate astrocytic proliferation and to increase the number of process-bearing astrocytes (Hefti et al.1985a). Ganglioside inhibition of cell proliferation has also been reported for 3T3 cells, BHK cells, human oral epidermoid carcinoma cells (KB) and human ovarian epidermoid carcinoma cells (A431) (Bremer et al.1984b; Bremer et al.1986). In addition, GM1 has been shown to prevent or reverse the dibutyryl cyclic AMP (dBcAMP) and forskolin-induced conversion of astroglial cells from flat to a stellate morphology (Skaper et al.1986).

A particularly interesting aspect of ganglioside effects especially illustrated by *in vitro* studies is that a relationship may exist between gangliosides and neuronotrophic agents. It is known that particular cell populations depend upon specific trophic molecules for survival. A clear example of this situation is the dependence which sensory and sympathetic neurons have, during development, for nerve growth factor (NGF) (reviewed in section 1.5.2). In most instances, gangliosides have been unable to substitute for the neurotrophic factors that particular neurons require (Leon et al.1984a). However, GM1 has been reported to prevent neuronal death of dorsal root ganglia cells following NGF withdrawl and to enhance the actions of such agents (Leon et al.1984b). For example, NGF-induced neurite outgrowth is potentiated by GM1 in E8 dorsal root ganglia and E11 sympathetic ganglia as well as in other primary neuronal cultures (Leon et al.1984b; Doherty et al.1985; Skaper et al.1985). In addition, antibodies to GM1 can block the neuritogenesis induced in chick embryo sensory ganglia by NGF (Schwartz and Spirman, 1982) or conditioned media (Spoerri et al.1988). Perhaps the best example, however, that exogenous GM1 can potentiate NGF-induced effects has been provided by work using a pheochromocytoma cell line (PC12). PC12 cells are derived from a chromaffin cell tumor and do not require NGF for survival (Greene and

Tischler, 1976). These cells stop dividing however, and differentiate, adopting a neuronal phenotype, in the presence of NGF. This is exemplified by morphological changes such as the extention of neuritic processes (Greene and Tischler, 1976). GM1 alone does not promote neurite extension in PC12 cells but it does potentiate this phenomenon in cells pretreated with NGF or when co-administered with NGF (Ferrari et al.1983; Katoh-Semba et al.1984). It had thus been suggested that gangliosides facilitate rather than initiate the execution of a growth or differention program (Leon et al.1984b; Skaper et al.1985). This effect of GM1 is not limited to peripheral or fetal neurons. Treatment of rat septal cell cultures with NGF in combination with GM1 increases ChAT activity significantly above that induced by each agent alone (Cuello et al.1989). Moreover, GM1 also enhances NGF effects on adult mouse sympathetic ganglia (Spoerri, 1986). The actions of trophic agents other than NGF have also been shown to be potentiated by GM1. Indeed, the effects of ciliary neurotrotphic factor (CNTF) and the "NGF unlike" trophic actions of cell-conditioned medium on parasympathetic neurons and dorsal root ganglia are also enhanced by GM1 (Skaper et al.1985). A further notable observation from *in vitro* studies is that gangliosides appeared to have optimal effects especially when culture conditions (eg: serum, substratum, growth factors) were manipulated to moderately restrict neuronal survival or neurite extension. This led to the proposal that a balance between inhibitory and permissive conditions is required in order for gangliosides to be effective (Skaper et al.1985).

1.4.7b IN VIVO EFFECTS OF GANGLIOSIDES

1.4.7b$_1$ Peripheral nervous system

Gangliosides have been shown to enhance regrowth and to restore function to sympathetic, motor and sensory peripheral neurons following injury. The first demonstration of such ganglioside effects was provided by Ceccarelli and coworkers in 1976 who showed that ganglioside treatment could enhance the reinnervation of the cat nictating membrane. Recovery was noted following either pre- or post-ganglionic denervation indicating that gangliosides lacked specificity for neuronal phenotype (cholinergic; adrenergic) (Ceccarelli et al.1976b). In addition, in a separate study these authors showed that treatment of rats with

a ganglioside bovine brain mixture could enhance the functional reinnervation of the denervated rat extensor digitorum longus (EDL) gastrocnemius muscle following sciatic nerve crush (Ceccarelli et al.1976a). This work was extended by Gorio and coworkers who reported that the daily intramuscular administration of 50 mg/kg of a bovine brain mixture of gangliosides accelerated the sprouting rate and speed of synapse formation during EDL muscle reinnervation (Gorio et al.1980). This was assessed using electrophysiological and morphological techniques at the light and electron microscopic level. In another model, which examined ganglioside effects on peripheral nerve, rat soleus muscle were partly denervated and ganglioside treatment was shown to increase the sprouting capacity of motor neurons by 50% (Gorio et al.1983). Further to an effect of gangliosides on peripheral nerve sprouting, local infusions of a ganglioside mixture (Cronassial®: 21% GM1, 40% GD1a, 16% GD1b, 10% GT1b) were shown to enhance axonal regeneration of sciatic nerves (Sparrow and Grafstein, 1982). Several other studies have also reported that enhanced regeneration of peripheral nerves occurs with ganglioside treatment [for review see: (Gorio et al.1985)]. Significant increases in the number of regenerating fibers as well as in axonal and myelin area have been demonstrated using quantitative methods (Mengs and Stotzem, 1987).

Additional studies of ganglioside effects on peripheral nerve regeneration were conducted using a model of diabetic neuropathy. Mutant diabetic mice C57BL/Ks(db/db) have been shown to develop a symmetrical neuropathy. Sensory and motor neurons of these animals show deficits in conduction velocity and axonal atrophy (Gorio et al.1981; Norido et al.1982). When these mice were treated with gangliosides an improvement in nerve conduction velocity as well as axonal morphometry occurred, and normal neurological function appeared to be restored (Norido et al.1984). Interestingly, gangliosides proved ineffective if treatment was initiated between 80-150 days of mouse life when high doses of insulin can improve neuropathy. At later stages, when mice cease to respond to insulin (150-280 days), an effect of gangliosides was noted. This observation was particularly interesting since it suggested that the state of the neuron or its environment could influence ganglioside actions. Thus, a balance between inhibitory or permissive circumstances, as suggested by *in vitro* studies (see section 1.4.7a), could also be required for *in vivo* ganglioside effectiveness.

The neurotoxic effects of 6 hydroxy-dopamine (6OH-DA) on noradrenergic terminals in both neonatal and adult mice irides were also attenuated by ganglioside treatment (Jonsson

et al.1984). Moreover, GM1 was also shown to be a neurotrophic agent for peripheral sensory systems since it could diminish the capsacin-induced depletion of substance P in the superficial layers of the spinal cord (Gorio et al.1986). Since both sympathetic and sensory peripheral neurons were previously shown to be dependent on NGF (see section 1.5.2) it was proposed that GM1 may exert its neuroprotective effects by interacting with such an endogenous trophic agent (Jonsson et al.1984). Support for this idea has recently been provided by a study which examined the effects of NGF in newborn rats subjected to vinblastine sympathectomy. It was shown that the NGF-induced recovery of noradrenaline (NA) content in these rats was potentiated by concurrent GM1 treatment (Vantini et al.1988).

1.4.7b$_2$ Central nervous system

Exogenous gangliosides were first reported to have a neuroprotective effect in brain by Wojick who demonstrated that daily i.m. injections of 50 mg/kg of a ganglioside mixture (GM1, GD1a, GD1b, GT1b) could enhance recovery of hippocampal ChAT and AChE activities following septal lesions (Wojcik et al.1982). Since these effects were noted only after 18 post-lesion days it was suggested that gangliosides could hasten hippocampal collateral sprouting. However, no direct evidence to support this proposal was provided. Subsequent work tested the effects of GM1 alone in animals in which the nigro-striatal dopaminergic pathway was transected. These studies firstly indicated that gangliosides also lacked specificity for neuronal phenotype in the CNS, as was the case *in vitro* and for the PNS. Using this rat nigro-striatal hemitransection lesion model it was shown that daily intraperitoneal (i.p.) administration of GM1 (30 mg/kg, for 30 days) attenuated decreases in striatal and substantia nigral tyrosine hydroxylase (TH) Vmax (Toffano et al.1983; Toffano et al.1984c). Moreover, immunohistochemical studies using fluorescent-linked TH antibodies showed an increased fluorescence in the striatum of GM1-treated animals when compared to their vehicle treated counterparts or controls (Toffano et al.1984c). It was suggested that this reflected a sprouting of dopaminergic striatal axons. Additional work with this lesion model, employing quantitative methods, showed that 10 mg/kg of GM1, given i.p. for 56 days, also prevented neuronal degeneration of dopaminergic nigral neurons occurring after

hemitransection (Agnati et al.1983c). Furthermore, the length and density of nigral neuronal dendrites were restored (Agnati et al.1983c). The enhanced immunofluoresence noted in the striatum was attributed to either augmented levels of TH in spared axons or was suggested to arise from collateral sprouting, but no distinction between these possibilities was made. Similar neuroprotective effects were obtained with the GM1 derivative AGF2, albeit at lower doses; this was suggested to reflect the longer half-life of this compound (Alidino et al.1984). Treatment of nigro-striatal hemitransected rats with GM1 was also shown to counteract the lesion-induced dopaminergic receptor supersensitivity occurring in striatum, as well as to attenuate apomorphine-induced rotational behavior (Agnati et al.1983b). Such behavioral improvements following nigro-striatal hemitransection lesions were confirmed by others (Sabel et al.1985). In addition, GM1 was also shown to improve rodent behavior in spatial reversal tasks following bilateral radiofrequency lesions of the caudate nucleus (Sabel et al.1984). This suggested that GM1 could restore normal function to the injured nigro-striatal pathway.

In addition to improving recovery of dopaminergic pathways following surgical lesions, GM1 was also shown to be effective following neurotoxic lesions of the CNS. Infusions of 6OH-DA into the rat neocortex reduced cortical NA levels by 80-90% and significantly decreased NA immunofluorescence. These lesion-induced deficits were prevented by GM1 treatment when initiated 3 days prior to toxin infusion and continued until the animals were sacrificed (Kojima et al.1984). It was not possible to establish from this study, whether the recovery of NA occurred due to blockade of the toxin effect, enhanced levels of NA or regeneration of injured fibers. By contrast, GM1 (30mg/kg/day) given i.p., beginning immediately post-lesion and continued for 24 days, did not attenuate deficits in striatal TH activity induced by 6OH-DA lesions of the substantia nigra (Toffano et al.1984a). It was suggested that this could be due to insufficient penetration of the GM1 molecule since this lesion did not disrupt the blood brain barrier to the extent the hemitransection lesion did. An alternative explanation provided was that 6OH-DA blocked the effects of endogenous trophic agents which GM1 would potentiate to enhance recovery. However, their results could also be interpreted as reflecting a dependence of GM1 effectiveness on the lesion extent. Such a dependence was demonstrated for the septo-hippocampal pathway (Gradkowska et al.1986). In this latter model, it was shown that although GM1 treatment (30 mg/kg/day, i.m. for 6

or 21 days) could significantly augment hippocampal ChAT, AChE and 5HT activities following modest lesions of the fimbria-fornix, only a small recovery was noted in animals with extensive lesions of this pathway.

More recently, GM1 has been shown to enhance recovery of dopaminergic pathways following neurotoxic lesions induced by MPTP (Hadjiconstantinou and Neff, 1988). Mice which received 30 mg/kg/day of MPTP for 7 days showed a 50% decrease in striatal DA levels. The monosialoganglioside GM1 (30mg/kg/day, i.p. for 23 days) was shown to partially restore normal striatal DA levels and to fully restore the levels of its metabolite, dihydroxyphenyl acetic acid (DOPAC). However, the lesion-induced deficit in striatal synaptosomal DA uptake was not significantly improved in MPTP-lesioned rats which received GM1 treatment, suggesting a lack of recovery of striatal dopaminergic fibers. A subsequent immunocytochemical study showed that MPTP decreased dopaminergic cell density in the substantia nigra and that remaining cells were significantly reduced in size (Hadjiconstantinou et al.1989). In this case, only the deficit in dopaminergic cell size was attenuated by the GM1 treatment. The beneficial effects of GM1 treatment following MPTP lesions on striatal dopamine and DOPAC levels were also demonstrated by Schneider and coworkers (1989). However, these authors showed that the MPTP lesion-induced deficit in striatal TH-IR fiber density could be ameliorated by GM1 treatment. In contrast to the work of Hadjiconstantinou and colleagues (1989), the MPTP lesion in this study did not cause neuronal loss in the substantia nigra. Thus, differences in lesion extent could account for discrepancies with regard to the degree of striatal dopaminergic fiber recovery induced by the ganglioside. GM1 treatment has also been shown to significantly attenuate Parkinsonian-like behavioral symptoms in MPTP-treated primates, to enhance the levels of striatal dopamine and its metabolites, as well as to increase striatal TH-IR fiber density in the primate brain (Schneider et al.1992).

The *in vivo* effects of gangliosides on the injured basalo-cortical cholinergic pathway had also been studied, although not systematically prior to the undertaking of this thesis. As previously discussed (section 1.3.6 and 1.3.7), the counterpart of this pathway in humans was shown to be significantly affected in Alzheimer's disease as reflected by the loss (Whitehouse et al.1982) or skrinkage (Pearson et al.1983) of neurons in the nucleus basalis of Meynert (nbM). Deficits in cortical cholinergic presynaptic markers have also been documented for

this disease (Bowen and Smith, 1976; Davies and Maloney, 1976). Over the years, there has been some debate as to whether these deficits reflect a primary injury to the nbM or result secondarily as a consequence of degenerative changes originally occuring in cortex [see for example: (Mesulam, 1986; Arendt and Bigl, 1986)]. Animal models attempting to mimic the cholinergic dysfunction in Alzheimer's disease have for the most part induced anterograde damage to the NBM via electrolytic or neurotoxic lesions [for review see: (Dekker et al.1991a; Fibiger, 1991)]. Unilateral electrolytic lesions of the NBM have been shown to cause deficits in ChAT activity and high-affinity choline uptake in cortex (Casamenti et al.1985). The daily intraperitoneal administration of GM1 (30mg/kg/day), beginning immediately post-lesion, was shown to facilitate recovery of cortical high-affinity choline uptake and ChAT activity (Pedata et al.1984; Casamenti et al.1985), but effects on NBM cholinergic neurons were not examined. The impaired performance exhibited by these animals in an active avoidance task was also shown to be attenuated by the GM1 treatment (Casamenti et al.1985). Whether exogenous GM1 could prevent retrograde degeneration of NBM cholinergic neurons was first examined by Cuello and colleagues (1986) using a decortication lesion model. Unilateral devascularizing cortical lesions have been shown to cause significant decreases in ChAT activity in the NBM (Stephens et al.1985) and shrinkage of ChAT-IR NBM neurons (Sofroniew et al.1983); deficits which were shown to be maximal at 30 days post-lesion. Treatment with GM1 (30 mg/kg/day, i.p.), initiated immediately after lesioning and continued until the 30th post-lesion day, maintained ChAT-IR neuronal size and ChAT activity in the NBM (Cuello et al.1986; Stephens et al.1987). Moreover, this treatment increased ChAT activity, above control levels, in the remaining ipsilateral cortex adjacent to the lesion site (Stephens et al.1987). The work of this thesis confirms and extends these findings. It is shown that GM1 effects on brain cholinergic markers are centrally mediated since doses which were ineffective when given i.p. proved to be neuroprotective when given i.c.v., via minipump (see section 3.1.1, 3.1.2). As well, it is shown that short-term (7 days) continuous i.c.v. infusion of GM1 in decorticated rats results in the long-term attenuation and increase, respectively, of cholinergic deficits in the NBM and cortical cholinergic presynaptic markers (section 3.1). These effects were found to be dependent upon the dose and delay of treatment time onset employed for GM1 (see section 3.1.10). Moreover, comprehensive quantitative studies, which assessed effects within specific NBM regions, illustrate that the

size and fiber network of ChAT-IR neurons most severely affected by the cortical lesion are fully protected from retrograde degeneration by GM1 treatment (section 3.2.1). The degree of neuroprotection accorded by GM1 was found to be equivalent to that produced by NGF (section 3.2.1). However, these agents are shown to differentially affect ultrastructural features of the cortical cholinergic innervation (section 3.3.2) and the performance of lesioned rats in memory-based tasks (section 3.3). It is also demonstrated that exogenous GM1 can increase NGF efficacy in stimulating ChAT and high-affinity choline uptake in the NBM and remaining cortex of cortically lesioned adult rats (section 3.1.5). The work of this thesis further shows that exogenous GM1 also attenuates neurochemical and neuroanatomical deficits in lesioned aged rats (section 3.4). Furthermore, a GM1-induced potentiation of NGF effects on cholinergic markers in the basalo-cortical cholinergic system is also noted for these aged decorticated animals (section 3.5). Related findings noted in other animal models are commented on in the discussion (section 4).

1.4.8 GANGLIOSIDES AS POSSIBLE "RECEPTORS" OR RECEPTOR MODULATORS

The notion that gangliosides could function as "receptors" was first suggested by work which demonstrated that these agents could bind and inactivate cholera toxin (enterotoxin from *Vibrio cholerae*) with high affinity and specificity (van Heyningen, 1984). The monosialoganglioside GM1 was shown to be particularly effective in this respect (Holmgren et al.1974; Hollenberg et al.1974). Toxin concentrations of 10^{-9} M were shown to be inhibited by 50% with 10^{-8} M GM1. Furthermore, the ability of transformed cells, *in vitro*, to bind cholera toxin was shown to correlate with ganglioside content (Cuatrecasas, 1973). This correlation was particularly high with respect to GM1 content. In addition, radiolabeled exogenous GM1 was shown to be taken up by the cell and to confer toxin sensitivity. The monosialoganglioside GM1 has now been shown to bind the B subunit of cholera toxin (Fishman and Brady, 1976). Studies which reinforced the notion that GM1 could serve as a "receptor" for cholera toxin were conducted using a line of ganglioside deficient cells (NCTC 2071A). These cells can be grown in a serum-free medium and are unresponsive to cholera toxin. After exposure to nanomolar concentrations of GM1, these cells have been shown to take up the ganglioside, bind the toxin and respond by accumulating cyclic AMP

(Moss et al.1976; Fishman et al.1976; Fishman, 1986). Other gangliosides which can also be taken up by these cells do not confer toxin sensitivity. Similar effects were noted using C6 glioma cells (Fishman et al.1980; Miller-Podraza et al.1982). Although GM1 appears to fulfil the requirements of a receptor (ie: bind ligand with high affinity, specificity and exert biological action), the involvement of GM1 in numerous other functions precludes its acceptance as such. Moreover, because epithelial cells of the intestine, which are the initial targets of cholera toxin, lack GM1 (Morita et al.1980) the biological relevance of a GM1-cholera toxin interaction and the role of GM1 in the pathogenesis of cholera toxin remain ill defined. Gangliosides have also been proposed as "receptors" for other toxins such as: tetanus toxin (van Heyningen, 1984) and *E. coli* enterotoxin (Holmgen et al.1982), but evidence for this is particularly weak. As well, the physiological importance of these observations is dubious since under physiological conditions binding of gangliosides to these toxins is reduced (Critchley et al.1986). Studies have also implicated gangliosides as "receptors" for viruses, in particular Sandai virus (Markwell et al.1981). These reports were again based on work in which ganglioside deficient cell lines were shown to become virus sensitive following addition of either GQ1b, GT1b or GD1a. A role for gangliosides as components of glycopeptide hormone receptors such as thyrotropin (TSH) has also been proposed (Kohn, 1978; Lacetti et al.1983). However, as in studies conducted with tetanus toxin, conditions were used to optimize binding of the hormone. Under more physiological conditions gangliosides inhibit low and not high-affinity binding of the hormone, which is linked to biological activity (Pekonen, 1980).

A role for gangliosides as receptor modulators has been proposed by several studies. Neurotransmitter receptors such as the serotonin $5HT_1$ receptor in NCB-20 cells have been shown to be modulated by gangliosides (Barry-Kravis and Dawson, 1985). In particular, GQ1b has been shown to couple the receptor G protein and the adenylate cyclase complex. Incubation of these cells with exogenous gangliosides increased serotonin receptor affinity 10 fold and also reduced EC50 values for serotonin-stimulated cyclic AMP production. The most effective ganglioside was GQ1b, but GM1 and GM2 were also shown to increase serotonin receptor affinity and potency. Interestingly, cell adhesion receptors have also been shown to be modulated by gangliosides (Kleinman et al.1979). This could explain ganglioside effects on cell attachment or adhesion. GD1a or GT1b have been shown to inhibit fibronectin

mediated cell attachment to a collagen coated substratum as well as fibronectin-collagen complexes (Kleinman et al.1979). Antibodies directed against GD2 and GD3 have been reported to inhibit attachment of human melanoma cells on a number of extracellular matrix proteins including: fibronectin, vitronectin, collagen, laminin as well as on peptides containing a sequence (arg-gly-asp) common to adhesive proteins (Cheresh et al.1986). Cheresh and coworkers have further demonstrated in biochemical, immunocytochemical and functional studies that GD2 forms calcium-dependent complexes with the vitronectin glycoprotein receptor and augments its fuction (Cheresh et al.1987).

In vitro studies have also shown that gangliosides can modulate growth factor receptors (Bremer and Hakomori, 1983; Hanai et al.1987). As described in section 1.4.7a gangliosides can bimodally regulate cell growth (ie: inhibit or stimulate) and can induce neuritogenesis. The receptors for epidermal growth factor (EGF) and platelet derived growth factor (PDGF), agents which exert mitogenic effects, can be regulated by gangliosides. Epidermal growth factor stimulated receptor phosphorylation in membrane preparations of both KB and A431 human epidermoid carcinoma cell lines has been shown to be inhibited by GM3, but binding of EGF to its receptor was not attenuated (Bremer et al.1986). It has been proposed that gangliosides allosterically regulate tyrosine phosphorylation of the EGF receptor by binding to specific receptor sites. In swiss 3T3 fibroblast cells, GM3, and to a lesser degree GM1, altered PDGF receptor affinity, but not number, and reduced phosphorylation of the PDGF receptor (Bremer et al.1984b). By contrast, in PC12 cells NGF receptor affinity or receptor number were unaffected following exposure to 10^{-6} or 10^{-5} M GM1 (Ferrari et al.1983). The work of this thesis shows that the *in vivo* administration of GM1 to cortically devascularized rats does not apparently affect NGF receptor binding or number in the ipsilateral remaining cortex and striatum of these animals (section 3.4). However, modulation of receptor binding and affinity following the *in vivo* administration of exogenous GM1 has been shown for several neurotransmitter systems (Agnati et al.1983a; Agnati et al.1983b; Hollman and Seifert, 1986; Hollman and Seifert, 1988).

1.4.9 ALTERATIONS OF GANGLIOSIDES IN DISEASE STATES

As previously mentioned gangliosides were discovered, in part, due to interest in identifying the lipids which accumulate in certain diseases of the nervous system. Many neuronal storage disorders are now known to be characterized by an abnormal accumulation of gangliosides. The first clinical observations reported of neuronal storage disease were made by Tay (1881) and a detailed description was first provided by Sachs (1887). Initially, the disorder, now known as Tay-Sachs disease, was classified by Sachs as Amaurotic family idiocy (Sachs, 1903). This was based on the fact that patients were blind as well as mentally retarded and that the disease prevailed in particular families. Cortical pyramidal neurons are severely affected in this disease as evidenced by marked swelling of cell bodies and dendrites, eccentrically placed nuclei and degeneration. It is now known that this results due to an accumulation of gangliosides arising from specific defects in ganglioside degradative enzymes stemming from genetic abnormalities. These genetic abnormalities in man can be classified according to the enzyme affected. Some of these are outlined in Table 1.3. Tay-Sachs is recognized as the prototype of all ganglioside storage diseases. It is caused by an almost total lack of ß-N-acetylhexosaminidase A activity (Okada and O'Brien, 1969; Sandhoff, 1969) which appears to be soley responsible for the degradation of GM2 ganglioside *in vivo*. Hexosaminidase B activity is either normal or increased. Increases in GM2 can reach as high as 100 times normal levels. A juvenile form of this disorder exists where the deficiency of hexosaminidase is partial rather than complete, hence leading to a slower clinical course and milder pathological and compositional abnormalities (Suzuki et al.1970; Suzuki and Suzuki, 1970). Genetic abnormalities in ß-galactosidase give rise to GM1 gangliosidosis.

TABLE 1.3: GENETIC DISORDERS CAUSED BY PRIMARY ABNORMALITIES IN GANGLIOSIDE METABOLISM

DISEASE	DEFECTIVE CATABOLIC ENZYME	DEFECTIVE ACTIVATOR
GM2 GANGLIOSIDOSIS	ß-N-ACETYLHEXOSAMINIDASE	-
TAY-SACHS	ß-N-ACETYLHEXOSAMINIDASE A, complete defect	-
JUVENILE GM2-GANGLIOSIDOSIS	ß-N-ACETYLHEXOSAMINIDASE A, partial defect	-
GM1 GANGLIOSIDOSIS	ß-GALACTOSIDASE	-
MUCOLIPIDOSIS IV	SIALIDASE DEFECT	-
GM2 GANGLIOSIDOSIS A-B VARIANT	-	HEXOSAMINIDASE-GM2 ACTIVATOR

Adapted from (Suzuki, 1984).

These disorders are not restricted to humans since several animal species (cats, dogs, cattle, swine) also have genetic disorders of ganglioside metabolism and show similar genetic, clinical and pathological features (Baker et al.1976; Suzuki, 1985). These animals provide particularly useful research models for the development and testing of enzyme replacement strategies using DNA recombinant technology.

In contrast to neuronal storage disorders where excesses in ganglioisdes mediate the disease process, deficiencies in gangliosides have been noted in diseases such as multiple sclerosis. This disease is characterized by focal lesions in myelin arising from unknown sources. Investigations of ganglioside content in brain or spinal cord of these patients illustrated that GM4 a myelin specific ganglioside, is completely lost as are GM1 and GM2, while GD3 and GM3 are remarkably increased (Yu et al.1974). Abnormalities in gangliosides are also noted in amyotrophic lateral sclerosis, a disease characterized by muscle atrophy secondary to degeneration of motor neurons. In this disease, GM2 and GD3 are increased while GD1b, GT1b, GQ1b are all decreased (Rapport, 1981). Interestingly, immunological activity against

gangliosides has been detected in serum samples from patients with the above diseases, as well as in patients afflicted with Alzheimer's disease (Arnon et al.1980; Indo et al.1984; Chapman et al.1988). However, the pathological significance of these findings remains unclear.

1.4.10 POSSIBLE MECHANISMS UNDERLYING GANGLIOSIDE EFFECTS

The diversity of ganglioside effects have complicated attempts to elucidate the possible molecular mechanisms involved in their actions. Indeed, since gangliosides can activate or inhibit cell proliferation, can induce differentiation as well as promote regeneration of peripheral nerves and recovery following CNS injury, it is likely that they activate a number of signal transduction pathways. Several investigators have examined the possibility that the neuroprotective effects of gangliosides occur as a consequence of their interaction with the cell membrane and proteins held therein. Because gangliosides have been shown to incorporate into neuronal membranes, it has been proposed that this may help maintain membrane integrity following injury (Toffano et al.1980). The neuroprotective effects of gangliosides have also been attributed to a regulation of ion permeability. It is known that injury can cause significant alterations in ion balance (Choi, 1990). In particular, ischemic brain injury has been shown to decrease the activity of Na^+/K^+ ATPase and to increase calcium influx (Karpiak et al.1991). The latter effect, *in vitro*, has been shown to correlate with neuronal death (Choi, 1990). Gangliosides have been reported to interact with calcium (Rahmann, 1992) and to modulate calcium influx in cell cultures (Wu et al.1990; Wu and Ledeen, 1991). In addition, isolated brain membranes treated with GM1 showed increased Na^+/K^+ ATPase activity, which correlated with their insertion into the neuronal membrane (Leon et al.1981). *In vivo*, GM1 treatment has also been shown to attenuate deficits in Na^+/K^+ ATPase activity and calcium influx in animals with ischemic brain injuries (Li et al.1986; Karpiak et al.1991).

Increases in the levels of excitatory amino acids are also thought to contribute to brain injury (Rothman and Olney, 1987; Choi, 1988). In particular, the excessive stimulation of glutamate receptors has been reported to cause significant increases in intracellular calcium concentrations and to induce neuronal death (Choi, 1990; Manev et al.1990). As well,

glutamate has been shown to promote the translocation of protein kinase C (pKC) from the cytosol to membranes of cerebellar cells in culture (Vaccarino et al.1987). A similar translocation of pKC has been shown to occur in fetal rat brain following complete obstruction of the maternal blood flow (Magal et al.1990) and in the adult brain following ischemia (Kochlar et al.1989; Onodera et al.1989; Olah et al.1990). Treatment with GM1 has been shown to block the activation and translocation of pKC which is evoked by glutamate in cultured cells (Vaccarino et al.1987), and to prevent ischemia-induced down regulation and translocation of pKC to membranes *in vivo* (Magal et al.1990). Moreover, *in vitro* studies have shown that gangliosides can attenuate the glutamate induced protracted increase in intracellular calcium which is correlated with neuronal death (DeErausquin et al.1990). Therefore, gangliosides could exert their neuroprotective effects by attenuating these acute events induced by injury.

Much work has been directed towards examining phosphorylation as a possible signal transduction mechanism for gangliosides. Indeed, gangliosides have been shown to stimulate the phosphorylation of a number of proteins (Goldenring et al.1985) and to modulate the activity of several protein kinases including: pKC (Kreutter et al.1987), Ca^{2+}-ectokinase (Tsuji et al.1985), Ca^{2+} dependent protein kinase (Goldenring et al.1985), and Ca^{2+}/calmodulin dependent protein kinase (Cimino et al.1987). The physiological significance of ganglioside-induced alterations of protein kinases is not well defined but since such kinases have been implicated in cell proliferation and differentiation it is possible that they form part of the ganglioside signal transduction cascade which produce these effects.

Gangliosides could also exert their neurotrophic or neuritogenic effects indirectly by altering the phosphorylation state of growth factor receptors [(Bremer et al.1986; Bremer and Hakomori, 1982); also see section 1.4.8]. Some interesting studies in this regard examined the effects of the alkaloid-like compound K252a in PC12 cells. K252a was originally described as an inhibitor of pKC and cyclic nucleotide dependent kinases (Kase et al.1987). It is now recognized as a specific inhibitor of NGF-induced biological responses such as neurite outgrowth. The high-affinity NGF receptor, $p140^{trk}$, has intrinsic tyrosine kinase activity (reviewed in section 1.5.6) which can be inhibited by K252a (Berg et al.1992). Interestingly, GM1 can prevent K252a inhibition of NGF responses in PC12 cells (Ferrari et al.1992).

1.5 NERVE GROWTH FACTOR

The high order and topographic arrangement, characteristic of the adult central nervous system, arises from a series of events during development which include cell proliferation, migration, differentiation and formation of synaptic contacts, in addition to pruning of particular neuronal populations and synapses. The understanding of these events as well as notions on neuronal survival and regeneration were greatly enhanced by the discovery of nerve growth factor (NGF). The identification of NGF originated from studies directed towards examining the effects of chick embryo limb ablation on sensory and motor neurons, with the aim of distinguishing whether neuronal loss occurred due to degeneration or a lack of differentiation. While working in Victor Hamburger's laboratory, Rita Levi-Montalcini astutely concluded that the reason mouse tumor could substitute for a removed limb and maintain spinal cord and sensory ganglia neurons in the chick embryo, as demonstrated by Bueker (1948), was due to a soluble factor which the tumor released. Indeed, Levi-Montalcini showed that Bueker's tumor (mouse sarcoma 180) secreted a soluble factor that stimulated the survival and growth of sympathetic and sensory ganglion cells (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953). This work gave support to the "neurotrophic hypothesis" which suggested that neurons are dependent upon a target-derived growth factor for their survival. A bioassay for "nerve growth promoting factor" was subsequently devised by Levi-Montalcini and the substance responsible, NGF, was purified by Stanley Cohen (Levi-Montalcini et al.1954; Cohen et al.1954); work for which they were awarded the 1986 Nobel prize in Medicine.

1.5.1 STRUCTURE, SYNTHESIS AND REGULATION OF NGF

The use of snake venom in initial attempts to purify NGF surprisingly indicated that the venom itself contained nerve growth promoting activity (Cohen and Levi-Montalcini, 1956). This prompted a search for rich sources of NGF which led to the discovery that the homologue of the snake venom gland in male mice, the submaxillary gland, was such a source. This finding facilitated the purification of the NGF protein. The biologically active molecule of NGF, ß-NGF, is present in a complex consisting of 3 different subunits, $\alpha_2\beta\gamma_2$

[for review: (Thoenen and Barde, 1980; Greene and Shooter, 1980; Darling et al.1983)]. This complex also known as 7S NGF, based on its sedimentaion coefficient, has a molecular weight of 140,000 daltons (Varon et al.1968) and contains two zinc ions which stabilize the complex (Pattison and Dunn, 1975; Bothwell and Shooter, 1977), but the individual subunits can dissociate at pHs below 5.0 and above 8.0 (Varon et al.1968). The γ subunit is an esteropeptidase which is responsible for the proteolytic activity of 7S NGF (Greene et al.1969). It belongs to the kallikrein family of trypsin-like serine proteases, has a molecular weight of 26,000, a pI of 5.2-5.8 and is glycosylated (Varon and Shooter, 1970). Up to six forms (γ^1-γ^6) of this subunit have been identified, all of which have similar esterase activities (Server and Shooter, 1977). Its function is not clear but it is believed to be involved in the proteolytic processing of the ß-NGF precursor. It has been shown, for example, that the γ subunit can cleave the bond between two arginine residues in the C-terminal amino acid sequence of ß-NGF which consists of Arg-Arg-Gly (Berger and Shooter, 1977; Bothwell and Shooter, 1978). The α subunit has a high homology (80%) to the γ subunit, suggesting that it is also a glandular kallikrein. Its pI has been reported as 4.1-4.6 (Varon and Shooter, 1970), but it has been shown to lack enzymatic activity. Four forms of the α subunit have been identified which do not show differential binding to ß-NGF. The function of the α subunit remains largely unknown but it is thought to protect ß-NGF from proteolytic degradation and to regulate its biological activity. ß-NGF is the subunit responsible for the growth promoting activity of NGF (Frazier et al.1973a; Stach and Shooter, 1974). It is first synthesized as a large precursor (prepro-NGF) (Scott et al.1983), but the steps involved in the processing of this precursor and the identification of intermediates have yet to be firmly established. ß-NGF exists as a noncovalently linked dimer and is composed of 2 identical chains comprised of 118 amino acids (Varon and Shooter, 1970). ß-NGF may lose an arginine at the N terminus and eight amino acids from its C terminus during isolation and still retains full biological activity (Bocchini and Angeletti, 1969). This form was the first to be isolated and sequenced and is known as 2.5S NGF. The amino acid sequence of ß-NGF is known (Angeletti and Bradshaw, 1971). In addition, the crystal structure of the murine ß-NGF dimer has been determined (McDonald and Blundell, 1991). This study revealed that each subunit consists of 3 antiparallel pairs of ß strands which together from a flat surface. Six cysteine residues have been identified which form 3 central disulfide bonds. Furthermore,

4 loop regions exist. These contain variable residues which distinguish NGF from other related molecules and are thought to confer receptor specificity (McDonald and Blundell, 1991). A clustering of positively charged side chains comprising residues Asp 30-Lys 34 are believed to provide complementary interaction with the acidic low-affinity NGF receptor (McDonald and Blundell, 1991). Each 118 amino acid flat subunit is held together by hydrophobic interactions to form the NGF dimer. The NGF dimer is very stable; a dissociation equilibrium constant less than 10^{-13} has been reported (Bothwell and Shooter, 1978) along with a molecular weight and pI of 26,500 and 9.3, respectively (Bocchini and Angeletti, 1969). The biological activity of ß-NGF depends on its conformation. Cleavage of the 3 disulfide bonds result in the complete loss of biological activity (Greene and Shooter, 1980; Thoenen and Barde, 1980). Moreover, site-directed mutagenesis studies have shown that modification of the Val 21 residue notably reduced receptor binding and biological activity (Ebendal, 1992). Modification of Arg 99 and Arg 102 or Trp 21 similarly resulted in the loss of biological activity (Cohen et al.1980; Ibáñez et al.1990). It has also been noted that Trp 20 may be important for stability of the protein (Frazier et al.1973b; Cohen et al.1980).

The NGF gene has now been cloned in many species including chick, mouse, rat, cattle and man [for review: (Ebendal, 1992)]. These have been shown to have 70-90% sequence homology. The mouse ß-NGF gene covers more than 43 kb and consists of 5 exons separated by 4 introns (Selby et al.1987). It is present as a single copy in both mouse and human genomes where it is localized to chromosome 3 and 1, respectively (Francke et al.1983; Zabel et al.1985). Promotor regions for the rat and mouse NGF have been identified (Zheng and Heinrich, 1988). There are 2 major (A and B) and 2 minor (C and D) transcripts for ß-NGF in mouse which differ due to alternative RNA splicing (Selby et al.1987). Transcript A encodes a large precursor which is primarily found in the submaxillary gland and placenta of mouse, while transcript B encodes a shorter precursor and is found in other tissues (Selby et al.1987). These have the same NGF coding sequence at their 3' end but differ at the 5' end. The A and C transcripts are similar but the C form has an independent promotor upstream from A. The D transcript is similar to B but it has an intron spliced out indicating that it may be a partially processed transcript. The two minor forms (C and D) comprise only 1% of NGF mRNA in some tissues but in others such as cortex can comprise 5-10% of the

total NGF mRNA (Selby et al.1987). The purpose and function of these different transcripts is not clearly understood.

NGF can be regulated at the level of transcription by androgens. Levels of NGF in the submaxillary gland are higher in male than in female mice (Isackson et al.1987). In addition, NGF levels have been shown to be increased in testosterone-treated females and to be decreased in castrated male mice (Ishii and Shooter, 1975). Similar results are not obtained for other species or tissues. Why this effect is particular to the mouse and submaxillary gland remains to be determined. The developmental regulation of NGF has been shown both in the PNS and CNS [(Ernfors et al.1988; Ernfors et al.1989; Ernfors et al.1990a; Friedman et al.1991b); for review: (Thoenen et al.1987)]. In the adult, NGF levels are altered following injury. For example in the PNS, sciatic nerve lesion results in an increase in NGF mRNA (Heumann et al.1987a; Heumann et al.1987b). By contrast, in the CNS, hippocampal NGF mRNA levels are unaffected following lesions of the septo-hippocampal pathway (Korsching et al.1986), however, an increase in NGF protein levels is noted (Grasser et al.1986; Korsching et al.1986). This has been suggested to reflect an accumulation of the protein due to an interruption in its retrograde transport. NGF mRNA is increased in brain, however, following electrical activity or by excitatory amino acids. In particular, kindling seizures have been shown to increase NGF mRNA in the hippocampus (Bengzon et al.1992). Similar increases have been noted following hypoglycemia, transient ischemia and the application of glutamate (Thoenen et al.1991; Lindvall et al.1992). Moreover, interleukin 1 ß has also been shown to increase the mRNA for NGF in hippocampal and rat sciatic nerve cultures (Friedman et al.1992; Lindholm et al.1987). It is thought that some of these increases are mediated by immediate early genes such as c-fos (Hengerer et al.1990).

1.5.2 NGF INVOLVEMENT IN DEVELOPMENT, SURVIVAL AND DIFFERENTIATION OF PERIPHERAL NEURONS

The growth promoting effects of purified NGF were first demonstrated using an *in vitro* bioassay system consisting of embryonic day 8-10 chick dorsal root ganglia. The addition of NGF resulted in an outgrowth of fibers creating a "halo effect" which surrounded the ganglia (Levi-Montalcini and Angeletti, 1963). Exposure to NGF antiserum was shown to block this

phenomenon (Levi-Montalcini, 1964). The dependence of these neurons on NGF for survival during particular developmental periods was evident from studies which showed that administration of NGF antibodies to developing rodents resulted in the death of a great majority of sympathetic and sensory neurons (Levi-Montalcini and Booker, 1960), while treatment of newborn rats with exogenous NGF caused a hypertrophy of sympathetic ganglia. In culture, explanted superior cervical ganglia and sympathetic neurons require NGF for survival, and withdrawl of NGF after short-term culture leads to cell death [(Levi-Montalcini and Angeletti, 1963); for review: (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980)]. In addition, naturally occuring cell death of sympathetic (Oppenheim et al.1982) 1982) and sensory ganglia (Hamburger et al.1981) was shown to be prevented by NGF treatment. However, only neural crest-derived sensory ganglia are affected by NGF. In the lumbar dorsal root ganglia, calcitonin gene related peptide (cGRP) and substance P, but not somatostatin, immunoreactive neurons exhibit high-affinity binding to NGF (Verge et al.1989). The role of NGF as a retrograde trophic factor was reinforced by studies which demonstrated that death of sympathetic neurons, as a consequence of 6-OH DA or vinblastine treatment are attenuated by NGF. Moreover, studies also indicated that NGF can be taken up by sympathetic and sensory fibers and be retrogradely transported to the cell body (Korsching and Thoenen, 1983a). In addition, NGF protein and its mRNA were detected in target areas of sympathetic and sensory neurons (Korsching and Thoenen, 1983b). The role of NGF as a target-derived trophic factor was made particularly evident by the work of Campenot (1977) who showed, using a chambered culture system, that NGF applied at terminal areas could support survival of sympathetic neurons. It was also demonstrated by this study that the direction of neurite outgrowth could be shifted towards areas enriched with NGF. Nevertheless, the role of NGF as a chemotactic agent is not particularly evident during development, but a close correlation exists between the density of fiber innervation of NGF responsive neurons and levels of NGF, as well as its mRNA, in target sites. One system where the timing of innervation and the appearance of NGF has been well established is the whisker pad of the mouse. The whisker pad receives its innervation from sensory neurons in the trigeminal ganglia. NGF mRNA in the whisker pad is only noted upon the arrival of the first fibers from the trigeminal ganglion (Davies et al.1987). Levels of NGF protein are detected half a day after the appearance of its mRNA. Subsequently, NGF protein levels are

reduced in the whisker pad, while mRNA levels stabilize. This is thought to reflect the retrograde flow of NGF.

The involvement of NGF in differentiation has been shown by both *in vivo* and *in vitro* studies. Sympathetic ganglia appear to lose their dependence on NGF for survival with increasing age (Thoenen and Barde, 1980; Coughlin and Collins, 1985). In the rat, after the third post-natal week, antibodies against NGF fail to cause death of sympathetic ganglia (Rosenfeld et al.1983). However, NGF is needed for normal neuronal function such as the maintenance of TH, dopamine ß-hydroxylase (DBH) and neuronal size (Thoenen and Barde, 1980). Exogenous NGF has also been shown to regulate the expression and content of cGRP and substance P levels in adult sensory neurons, TH activity in the superior cervical ganglia and to increase dendritic arborization [(Lindsay and Harmar, 1989) for review see: (Thoenen and Barde, 1980; Johnson et al.1986)]. In early post-natal periods, NGF treatment increases synapse number in superior cervical ganglia (Purves et al.1988). Moreover, exogenous NGF can attenuate the retraction of synapses which occurs following axon transection, while antibodies against NGF cause the loss of synapes from mature ganglion cells (Purves and Nja, 1976; Njå and Purves, 1978). In mature sensory neurons exogenous NGF has also been shown to regulate axonal caliber, neurofilament content and nuclear localization following sciatic nerve transection (Gold et al.1991). The differentiative properties of NGF are particularly exemplified by studies using PC12 cells. These cells proliferate in serum containing media and, in the absence of NGF, resemble chromaffin cells with respect to their ability to synthesize and secrete catecholamines. In response to NGF these cells cease dividing and extend neuritic processes (Greene and Tischler, 1976). Moreover, increases in ChAT and AChE activities are also noted in these cells following incubation with NGF (Edgar and Thoenen, 1978; Greene and Rukenstein, 1981; Heumann et al.1984).

1.5.3 NGF AS A TROPHIC AGENT FOR CENTRAL CHOLINERGIC NEURONS

On the basis of studies in the PNS, initial work conducted with regard to NGF in the CNS was biased towards catecholaminergic systems. Indeed, an initial report by Schwab and coworkers (1979) was entitled *"Nerve growth factor (NGF) in the rat CNS: Absence of*

specific retrograde axonal transport and tyrosine hydroxylase induction in locus coeruleus and substantia nigra" despite the fact that specific retrograde transport of ^{125}I NGF was noted in the basal forebrain. Neurons of the basal forebrain were believed, at that time, to be cholinergic based on AChE immunohistochemistry. A response of cholinergic neurons to NGF was first demonstrated *in vitro* using aggregate cultures of cholinergic telencephalic neurons (Honegger and Lenoir, 1982). Addition of NGF to these cultures was shown to increase ChAT activity in a dose-dependent manner. The greatest dose employed, 30 ng/ml, caused a 209% increase in ChAT activity. Subsequently, it was shown that injections of NGF to newborn rats stimulated septal and hippocampal (Gnahn et al.1983) as well as striatal (Mobley et al.1985) ChAT activity. Confirmation that this effect was indeed due to NGF and not possible purification contaminants, such as renin, was provided by Mobley and coworkers (1986). It was later shown that NGF can be retrogradely transported from the cortex to the basal forebrain (Seiler and Schwab, 1984a). Moreover, the presence of NGF and its mRNA in brain were shown to correlate with cholinergic innervation (Korsching et al.1985). These observations indicated that NGF could be a trophic factor for CNS cholinergic neurons.

However, in contrast to the PNS where a role for NGF in the maintenance of cholinergic neuronal survival during development is well established, such a role of NGF for cholinergic CNS neurons is ambiguous. In culture, exogenous NGF appeared not to affect neuronal survival or fiber outgrowth of embryonic septal cholinergic neurons (Hefti et al.1985b), but it was shown to augment ChAT activity (Hefti et al.1985b; Cuello et al.1989). However, in cultures of cholinergic basal forebrain neurons from 13-day-old post-natal rats, NGF was shown to increase cell survival (Hatanaka et al.1988). As well, addition of NGF to these cultured cells was shown to increase the high potassium-induced stimulation of ACh release (Takei et al.1989). Subsequent reports showed that the effects of NGF on cholinergic neurons in culture were dependent upon plating density and the presence of glia (Hartikka and Hefti, 1988). At low plating density anti-NGF antibodies decreased survival of embryonic day 17 septal cholinergic neurons, but NGF was not required for survival if neurons were plated at high density (Hartikka and Hefti, 1988). More recently, it was shown that cultured septal neurons exposed to NGF for short periods of time die if NGF is withdrawn from the media (Svendsen et al.1991). However, neurons which were kept for longer periods of time in culture prior to NGF withdrawal survived.

In vivo, as previously discussed, antibodies against NGF led to the death of peripheral sympathetic neurons in newborn rats. However, the intracerebroventricular injection of polyclonal antibodies to NGF from birth until the 7th post-natal day failed to affect ChAT activity levels in the rat cortex, hippocampus and septum (Gnahn et al.1983). That this could be due to a lack of adequate penetration of NGF antibodies in CNS tissue was suggested based on work which showed that injections of NGF antibodies into the deafferented hippocampus prevents the ingrowth of peripheral sympathetic fibers only within a 1 mm radius of the injection site (Springer and Loy, 1985). More recently, Vantini and coworkers (1989) have shown that i.c.v. injections of anti-NGF IgG and Fab fragments into newborn rats cause transient decreases in septal, hippocampal, cortical and striatal ChAT activity, but no evidence of an effect on neuronal survival was provided. Thus, whether NGF indeed regulates cholinergic neuronal survival during development awaits confirmation and improved procedures to neutralize endogenous NGF *in vivo*. However, a role for NGF in neurochemical and neuroanatomical recovery following injury to CNS cholinergic pathways has been established (see section 1.4)

1.5.4 DISTRIBUTION OF NGF AND ITS mRNA IN BRAIN

1.5.4a DEVELOPMENTAL EXPRESSION

The appearance of NGF and its mRNA in brain matches the development of cholinergic innervation. In the rat whole brain, NGF mRNA was initially observed on post-natal day 1 (Whittemore et al.1986). However, more recent studies have demonstrated the presence of NGF mRNA as early as embryonic day 13 (Maisonpierre et al.1990a). Half maximal values for NGF mRNA were shown to be reached at 1 to 2 post-natal weeks, while adult levels were achieved by the third post-natal week (Whittemore et al.1986). NGF protein was noted in rat whole brain on embryonic day 16, and was shown to peak by the third post-natal week and to subsequently decline to adult levels (Whittemore et al.1986). Studies which have assessed the regional distribution of NGF and its mRNA in the developing rat brain have shown that NGF mRNA expression is highest in hippocampus and cortex, but high amounts were also detected in the olfactory bulb (Maisonpierre et al.1990a).

1.5.4b EXPRESSION IN THE ADULT BRAIN

The high correlation of NGF and its mRNA with cholinergic innervation was actually first noted in the adult brain (Korsching et al.1985). High levels of both the NGF protein and its mRNA were detected in target areas of basal forebrain cholinergic neurons, such as the hippocampus and cortex. By contrast, NGF mRNA was very low in the septum and striatum. Approximate amounts of 1.4 ng of NGF per mg (wet weight) of tissue were reported for the hippocampus. In the neocortex and NBM, NGF levels of 0.5 ng/mg wet weight and 0.37 ng/mg wet weight of tissue, respectively were detected. The low levels of NGF mRNA detected in the basal forebrain supports the notion that NGF in the NBM derives from the cortex via retrograde transport (Seiler and Schwab, 1984b). No difference in the levels of NGF protein and its mRNA were noted among cortical regions examined (parietal, occipital, frontal, temporal, cingulate), which correlates with the widespread distribution of cholinergic innervation in cortex (see section 1.3.4). Differences were noted, however, in subdivisons of the hippocampus. In this brain area, NGF levels were 2 to 3 times higher in hippocampal regions with dense cholinergic innervation such as the dentate gyrus, CA3 and CA4 areas when compared to CA1 and CA2 regions. The NGF gene has been localized to neurons in the hippocampus and cortex (Ayer-LeLievre et al.1988), but glia are also known to express NGF (Lu et al.1991; Furukawa et al.1986) and can apparently increase its production when stimulated chemically or subsequent to lesions (Yoshida and Gage, 1991; Yoshida and Gage, 1992).

1.5.5 NGF AS A NEUROTROPHIC AGENT FOR ADULT CNS CHOLINERGIC NEURONS

Several investigations have now provided evidence that NGF can serve as a neuroprotective agent for injured adult CNS cholinergic neurons. Initial studies reported that exogenous NGF could attenuate deficits in septal and hippocampal ChAT activity following partial transection of the fimbria-fornix in rats (Hefti et al.1984). This was achieved by injecting 10 μg of 2.5S NGF, i.c.v., 2 times per week for a total treatment time of 4 weeks.

The increases noted in ipsilateral hippocampal ChAT activity ranged from 20 to 60%, depending on the hippocampal region assessed, while septal ChAT activity was increased 60% above that of untreated lesioned rats. In this experimental paradigm, the increase in hippocampal ChAT activity was attributed to activation of remaining fibers and not sprouting since AChE immunoreactivity in the hippocampus was not altered by the NGF treatment. It was later reported that this NGF treatment also attenuated the apparent 50% loss of AChE-IR septal neurons induced by the aforementioned lesion (Hefti, 1986). At the same time, Williams and coworkers (1986) showed that continuous (17 day) infusion of 7S NGF (1 μg/week), administered i.c.v. via minipump, prevented retrograde degeneration of AChE-IR septal neurons and furthermore, also appeared to increase AChE-IR fiber density in the caudal dorsal lateral septum of adult rats with lesions of the fimbria-fornix and supracallosal striae. Subsequently, Kromer (1987) demonstrated that continuous infusion of 2.5S NGF (3 μg/day for 14 days) could prevent the apparent loss of ChAT-IR septal neurons even after bilateral fimbria-fornix lesions. The possibility that fimbria-fornix transection caused a down regulation of ChAT immunoreactivity in septal neurons, which was interpreted as neuronal loss, was proposed by Hagg and coworkers (1988). Their work showed that delayed administration of NGF to rats with transections of the fimbria-fornix resulted in the reappearance of ChAT-IR neurons. Initiating NGF treatment up to one month after lesioning was shown to rescue approximately 50% of the septal neurons which were apparently lost. Since neurons in the adult brain are post-mitotic, it was suggested that NGF caused an upregulation of the ChAT protein rather than promoted neuronal survival.

The neuroprotective effects of NGF following retrograde degeneration of NBM cholinergic neurons are demonstrated by the work of this thesis. It is shown that a short-term (7 day) continuous treatment, i.c.v. via minipump, with 2.5S NGF can attenuate decreases in NBM ChAT activity, ChAT-IR neuronal size and fiber length which are noted 30 days after decortication (section 3.2). This provided the first evidence that exogenous NGF can promote long-term recovery of central cholinergic neurons. Moreover, exogenous NGF was found to augment both ChAT activity and high-affinity choline uptake in the remaining ipsilateral cortex adjacent to the lesion site. These effects were also noted to persist after cessation of drug adminstration and depended upon the dose and delay in the treatment time onset of NGF (see section 3.1). The anatomical correlates of this NGF-induced neuroplasticity in target

sites of basal forebrain cholinergic neurons are also shown by the work of this thesis (section 3.2). Quantitative light and electron microscopic studies using ChAT immunocytochemistry demonstrate that exogenous NGF can cause substantial synaptic remodelling in the remaining cortex of lesioned rats. Moreover, it is shown that such NGF-induced *in vivo* effects can be potentiated by exogenous GM1 (section 3.1).

Exogenous NGF has also been shown to attenuate cognitive deficits in normal aged animals (Fischer et al.1987; Fischer et al.1991). The performance of aged rats in a spatial memory task (Morris water maze) was shown to be significantly improved by NGF treatment. Moreover, exogenous NGF reversed the neuronal atrophy in the basal forebrain and striatum of these behaviorally impaired rats. Nerve growth factor treatment can also ameliorate deficits in cholinergic markers noted in normal aged rats (Williams, 1991b). The work of this thesis shows that NGF treatment can attenuate lesion-induced deficits in NBM ChAT activity and cell size, as well as stimulates cortical high-affinity choline uptake in decorticated aged animals (section 3.4).

Additional related studies exploring the neuroprotective effects of NGF in adult or aged animals following brain injury, which were published while the experimental work of this thesis was in progress, are commented upon in the discussion (section 4.1).

1.5.6 NGF MECHANISM OF ACTION

Early studies showed that the effects of NGF are initiated by its interaction with specific receptors. Initial characterization of NGF receptors was done using chick embryonic sympathetic (Costrini et al.1979; Massague et al.1981) and sensory (Sutter et al.1979) neurons. These reports identified two NGF receptor populations which exhibited either high (Kd 10^{-11})- or low (Kd 10^{-9})-affinity binding for NGF. Subsequent work which explored the differences in these two receptor populations was largely conducted using PC12 cells. Cross-linking studies estimated molecular weights of 140,000 for the high affinity and 80,000 daltons for the low-affinity receptor (Massague et al.1981; Hosang and Shooter, 1985). The low-affinity receptor was susequently cloned in several species, including the chick, rat as well as human and was shown to be quite homologous (Johnson et al.1986; Radeke et

al.1987). It is now referred to as $p75^{NGFR}$ (or $p75^{LNGFR}$). This low-affinity receptor is a highly glycosylated single peptide chain which contains approximately 400 amino acid residues, a single membrane spanning domain and a larger extracellular than intracellular portion. The extracellular domain contains 4 cysteine rich regions which are conserved in the chick, rat as well as human, and which contain the binding domain for NGF (Large, 1989; Baldwin et al.1992; Welcher et al.1991; Yan and Chao, 1991). Its mechanism of signal transduction is not known but G proteins are thought involved because of the presence of a domain in its intracellular portion which resembles mastoparan, a peptide which stimulates $G\alpha_i$ and $G\alpha_0$ GTPase activity (Feinstein and Larhammer, 1990). The low-affinity receptor also binds other neurotrophins [Brain Derived Neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4 (NT-4) and Neurotrophin-5 (NT-5), see section 1.5.8] with the same kd (10^{-9} M) but different dissociation and association rates have been noted (Rodriguez-Tébar et al.1990; Squinto et al.1991; Rodríguez-Tébar et al.1992).

The high-affinity NGF receptor has now been identified as $p140^{trk}$ (Trk, *trk*A or $gp140^{prototrk}$), a tyrosine kinase receptor which is encoded by the trk proto-oncogene (Klein et al.1991; Kaplan et al.1991a; Hempstead et al.1991; Kaplan et al.1991b). Similar type receptors exist for the other neurotrophins (see section 1.5.8). For an outline of the studies which led to the identification of $p140^{trk}$ as the high-affinity NGF receptor see: (Baranga, 1991; Meakin and Shooter, 1992). $p140^{trk}$ is also a heavily glycosylated peptide with a single membrane spanning domain but it is larger (160,000 daltons) than $p75^{NGFR}$ and its cytoplasmic domain contains tyrosine kinase activity. In this respect, $p140^{trk}$ resembles other receptors such as EGF, FGF, insulin and PDGF [for review see: (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992)]. That $p140^{trk}$ indeed mediates the actions of NGF was suggested by its presence in NGF responsive neurons such as dorsal root ganglia, PC12 and neuroblastoma cells. In addition, it has been shown that a mutant PC12 cell line which was non responsive to NGF exhibited neurite outgrowth and cell survival after transfection with trk cDNA and addition of NGF (Loeb et al.1991). An issue of current controversy is what constitutes the high-affinity NGF binding site. There is evidence that $p140^{trk}$ alone can serve as the NGF high-affinity receptor (Weskamp and Reichardt, 1991; Ibáñez et al.1992) while other studies indicate that both $p75^{NGFR}$ and $p140^{trk}$ are needed (Hempstead et al.1989; Yan et al.1991). A recent report presents a strong case that $p140^{trk}$ homodimers constitute the high

affinity binding site and are necessary for NGF binding as well as biological activity (Jing et al.1992). However, this study, as have others, used heterologous cultured cell lines and whether these results can be extrapolated to neurons is unclear. A recent study has employed herpes simplex virus (HSV-1) vectors to successfully transfect $p75^{NGFR}$ into primary cortical neuronal cultures (Battleman et al.1993). Future work employing this technology could perhaps allow the nature of the high-affinity binding site in neurons to be assessed.

Subsequent to binding of NGF to its receptor and activation of tyrosine kinase, the signal transduction cascade which follows to give rise to the neurotrophic or neuritogenic actions of NGF remains to be fully elucidated. Early events elicited by NGF include tyrosine phosphorylation, membrane ruffling and the activation of early response genes such as: Egr, NGFI-A, Krox24, zif268, *fos* and *Jun* family members, NGFI-B/nur77, ß actin and ornithine decarboxylase [(Cremins et al.1986; Mutoh and Gurpff, 1989; Hatanaka et al.1978; Wu et al.1989; Boonstra et al.1983); for review see: (Levi and Alemà, 1991; Halegoua et al.1991)]. An important candidate for the NGF signal transduction pathway specific for differentiation appears to be $p21^{ras}$. Studies have shown that microinjections of oncogenic ras protein in PC12 cells elicits neurite outgrowth (Bar-Sagai and Feramisco, 1985). In addition, anti-$p21^{ras}$ antibodies have been shown to block NGF-induced neurite extension in PC12 cells (Hagag et al.1986). NGF has also been shown to activate Na^+/K^+ ATPase, to modulate calcium and sodium influx, to upregulate Ca^{2+}/calmodulin protein kinase II, phosphoinositol turnover as well as the following protein kinases: pKA, pKC, MAP2 pK and pKN [for review see: (Levi and Alemà, 1991; Halegoua et al.1991)]. Which of these events or combination of events are responsible for the neuroprotective or regenerative effects of NGF in the CNS remains to be determined.

1.5.7 LOCALIZATION AND REGULATION OF NGF RECEPTORS IN BRAIN

Early binding studies had shown that specific binding of NGF occurred in homogenates of chick or rat brain, which exhibited a single low-affinity binding site (Frazier et al.1974a; Frazier et al.1974b). Cross linking studies subsequently showed that two NGF receptor proteins existed in brain, which were similar to those observed in PC12 cells and sympathetic ganglia (Taniuchi et al.1986b). Additional binding studies showed high-affinity NGF binding

to membranes from the striatum of adult rats (Richardson et al.1986). NGF binding sites in rat brain were then mapped using autoradigraphy. It was demonstrated that high-affinity binding of ^{125}I-NGF occurred predominantly in the basal forebrain nuclear complex, which comprises the medial septum, VDB, HDB and NBM (Richardson et al.1986; Ravich and Kreutzberg, 1987). The distribution of NGF high-affinity binding sites was shown to correlate with that of cholinergic neurons (Richardson et al.1986; Ravich and Kreutzberg, 1987). High affinity binding was also noted in the hippocampus and striatum but not in cortex. Further work which investigated the distribution of NGF receptors in brain used the monoclonal antibody 192-IgG (Mab 192) which recognizes the low-affinity NGF receptor, $p75^{NGFR}$ (Chandler et al.1984). With this technique receptors were also noted in the basal forebrain and were found to parallel the distribution of ChAT-IR neurons in this area [(Kiss et al.1988; Dawbarn et al.1988; Pioro and Cuello, 1990), this thesis section 3.2]. However, Mab 192 immunoreactive neurons were not noted in striatum. In situ hybridization studies examining the distribution of $p75^{NGFR}$ mRNA showed similar results (Gibbs et al.1989). A systematic study exploring the distribution of $p140^{trk}$ in the rat brain has yet to be undertaken. One study shows the presence of $p140^{trk}$ cDNA in the medial septum and VDB, but little to no $p140^{trk}$ expression was detected in cortex (Vazquez and Ebendal, 1991). Expression, or lack thereof, of $p140^{trk}$ in other brain areas was not shown. A more recent study shows $p140^{trk}$ expression in the rat NBM but again little to no expression was noted in cortex (Merlio et al.1993). Similarly, little to no expression was seen in hippocampus. These investigators also demonstrated that expression of $p140^{trk}$ mRNA was not altered after kindling, cerebral ischemia or hypoglycemia. This was in contrast to the elevated levels of Trk B (high-affinity receptor for BDNF see section 1.5.8) mRNA which were noted in the dentate gyrus following these stimuli. $p140^{trk}$ expression has been shown to be upregulated in response to NGF in PC12 cells (Meakin and Shooter, 1992), and in normal brain (Holtzman et al.1992). In sensory neurons expression of $p140^{trk}$ mRNA decreases along with that for $p75^{NGFR}$ following injury and was shown to be restored by NGF treatment (Verge et al.1992). $p75^{NGFR}$ is upregulated by NGF in PC12 cells, as well as following NGF administration or brain injury *in vivo* (Gage et al.1989; Higgins et al.1989; Cavicchioli et al.1989). In particular, $p75^{NGFR}$ is detected in the injured striatum, using Mab 192 which shows little to no immunoreactivity in intact striata [(Gage et al.1989); this thesis section

3.3). In the periphery, Schwann cells and fibroblasts distal to the sciatic nerve lesion show increases in $p75^{NGFR}$ (Taniuchi et al.1986a; Taniuchi et al.1988; Johnson et al.1988). Similarly, motoneurons also increase $p75^{NGFR}$ after injury (Ernfors et al.1989). NGF receptor levels have been shown to be upregulated by basic fibroblast growth factor (b-FGF) (Doherty et al.1988) and retinoic acid (Haskell et al.1987) in particular cell lines. Moreover, a down regulation of NGF binding or $p75^{NGFR}$ mRNA has been observed, *in vitro*, following exposure of cells to dexamethasone or testosterone (Tocco et al.1988; Persson et al.1990). Since the role of $p75^{NGFR}$ is not clear, the significance of such changes remain to be determined.

1.5.8 OTHER NGF RELATED MOLECULES

More recently, several other trophic agents related to NGF have been detected in the mammalian CNS. These are now collectively called "neurotrophins". Following NGF, brain dervived neurotrophic factor (BDNF) was the first such molecule to be identified. Initially purified by Barde and coworkers (1982), BDNF was subsequently cloned (Leibrock et al.1989) and was shown to have a high homology to NGF. This feature greatly facilitated the cloning, using the PCR technique, of other factors such as Neurotrophin-3 (NT-3) (Maisonpierre et al.1990b; Maisonpierre et al.1991; Rosenthal et al.1990; Hohn et al.1990), Neurotrophin-4 (NT-4) (Hallböök et al.1991) and Neurotrophin-5 (NT-5) (Berkemeier et al.1991). NT-5 however, has been proposed to be a species paralog of NT-4 (Ip et al.1992). These neurotrophins are differentially expressed in the CNS during development and their tissue distribution also differs in adulthood (Ernfors et al.1990a; Wetmore et al.1990; Ernfors et al.1990b; Maisonpierre et al.1991; Maisonpierre et al.1990a; Friedman et al.1991a; Phillips et al.1990). Moreover, these factors affect different cell populations. For example, NGF but not BDNF enhances survival and neurite outgrowth of sympathetic neurons. By contrast, NT-3 and BDNF, but not NGF can promote survival of nodose sensory neurons (Barde, 1989). As well, BDNF, but not NGF, protects cultured nigral dopaminergic neurons from the neurotoxic effects of MPP^+ (Hyman et al.1991). In basal forebrain cultures BDNF has been shown to be less potent than NGF in elevating levels of ChAT activity (Knüsel et al.1991). As previously mentioned, BDNF and NT-3 have also been shown to bind $p75^{NGFR}$.

Moreover, their high-affinity receptors have intrinsic tyrosine kinase activity and belong to the trk family. They have been identified as Trk B for BDNF and Trk C for NT-3 [see (Chao, 1992), for review].

1.6 STATEMENT OF THE PROBLEM

When this thesis was initiated the extent to which exogenous trophic agents, such as GM1 or NGF, could facilitate recovery of injured cholinergic pathways in the adult or aged rat brain had yet to be determined. In particular, comprehensive studies examining whether these agents modulate cholinergic presynaptic markers were lacking. Moreover, no direct evidence had been provided which showed that either of these agents could induce growth of injured cholinergic fibers or significantly alter cholinergic innervation. With respect to GM1, it had been shown that continued treatment, given i.p., with this agent could attenuate deficits in cholinergic markers in the septo-hippocampal pathway following fimbria-fornix transection (Gradkowska et al.1986) and in cortex following neurotoxic NBM lesions (Casamenti et al.1985). Moreover, it was reported that chronic i.p. administration of GM1 could prevent the atrophy of ChAT-IR neurons in the NBM following decortication (Cuello et al.1986). At that time, it was also shown that NGF and specific NGF binding sites were present in the adult brain, and that their distribution correlated highly with that of cholinergic innervation (Korsching et al.1985; Richardson et al.1986). The possibility that NGF could prevent retrograde degeneration of NBM cholinergic neurons had yet to be demonstrated. Studies which were done prior to the undertaking of this thesis focused on assessing NGF effects in hippocampus and septum of fimbria-fornix lesioned rats, an axotomy lesion model, where NGF was shown to prevent deficits in cholinergic markers (Hefti et al.1984) as well as the apparent loss of AChE-IR neurons of the medial septum (Williams et al.1986; Hefti, 1986).

The ultimate goal of this thesis was to determine whether cholinergic neurons of the adult rat NBM exhibit substantial neurochemical plasticity, morphological recovery and fiber growth in response to GM1 or NGF treatment. Moreover, since there was some evidence based on *in vitro* studies that the effects of NGF could be enhanced by GM1 (Ferrari et al.1983), whether NGF-induced effects on adult CNS cholinergic neurons could also be augmented by this agent *in vivo* was tested. The animal model used for these studies involved unilateral devascularizing cortical lesions. This injury causes cortical atrophy and retrograde degeneration of NBM cholinergic neurons, deficits which are also noted in AD brains. Four main objectives were pursued in the work of this thesis. First, the ability of NGF or GM1 treatment to attenuate decortication-induced deficits in NBM ChAT activity, and to modulate

cortical ChAT activity and HACU were compared. The ability of GM1 to augment NGF-induced effects was also determined. Particular emphasis was placed on examining effects on the basalo-cortical cholinergic pathway, but other brain areas were also studied. The dose requirements and time constraints for these effects were established. Second, the neuroanatomical correlates of the NGF and/or GM1 induced neurochemical plasticity were investigated. Quantitaitive light immunocytochemical studies, assisted by image analysis, were undertaken to determine whether these agents could induce long-term recovery of NBM neuronal morphology, an event which could suggest that neurons reestablish post-synaptic contacts. For this purpose the effects of the lesion, NGF and/or GM1 treatment on both ChAT and $p75^{NGFR}$ immunoreactivity, as markers of NBM cholinergic neurons, were determined. Moreover, electron microscopic quantitative techniques were employed to assess whether fiber outgrowth and synaptic remodelling occurred in the remaining NBM target area as a result of these treatments. Third, the behavioral consequences of NGF and/or GM1 treatment were examined in order to compare the functional effects of these agents. Fourth, the response of the aged decorticated rat brain to these agents was studied. The results of experiments pertaining to each objective are presented in the results section of this thesis in the order described above. Thus, neurochemical studies are presented in section 3.1; neuroanatomical studies are presented in section 3.2, behavioral studies in section 3.3, and effects of NGF and/or GM1 in aged unilaterally decorticated rats in section 3.4.

MATERIALS AND METHODS

2.1 *SURGICAL PROCEDURES*

2.1.1 Animals

Male Wistar rats, purchased from Charles River Breeding Laboratories (St. Constant, Québec), weighing either between: 300-350 g (Adult) or 750-950 g (Aged) prior to each experiment were used. The animals were kept in the McGill McIntyre Animals Center where they were housed, two per cage, in a temperature-controlled room (22^{0}C) on a 12 hour light/dark cycle. Rats were permitted free access to food and water.

2.1.2 Unilateral devascularizing cortical lesion

The cortical devascularizing lesion which was used for the studies of this thesis has previously been described, albeit briefly, in the following reports (Sofroniew et al., 1983; Stephens et al., 1985). Since the original method was somewhat modified a detailed description of the procedure is provided here.

Throughout the surgical procedure care was taken to ensure aseptic conditions. Surgical instruments were autoclaved prior to use and were kept in 70% ethanol during surgery. Animals were anaesthetized with 3ml/kg Equithesin (see section 2.7.1 for composition and preparation), given intraperitoneally (i.p.). The heads of the animals were shaved using an Oster Golden A5 animal clipper and the skin was wiped with an iodine solution. The animals were then placed in a stereotaxic apparatus (David Kopf Instruments; California). A midline incision was made into the skin which exposed the dorsal surface of the rat skull. The fascia and left temporalis muscle were retracted to also expose the left lateral skull surface. The edge of a large portion of the left skull bone, extending [coordinates from Bregma (Paxinos and Watson, 1986): For young adult rats: Anterior: 3.2 mm, Posterior: 6.2 mm, Laterally from the midline: 1.0 mm and extending ventrally to the squamosal bone; For aged rats: Anterior: 3.6 mm, Posterior: 7.0 mm, Laterally from the midline: 1.0 mm and extending ventrally to the squamosal bone] was drilled through, using a dental drill (Foredom electric Company, CT, USA) without disrupting the underlying dura. Cool sterile phosphate buffered saline (PBS), pH 7.4, was dripped onto the skull surface, while drilling, to prevent

overheating. The exposed dura was carefully cut horizontally across its most ventral aspect and vertically along both sides, and was carefully drawn back to facilitate later replacement. The exposed pia arachnoid vasculature was then disrupted using a fine surgical needle. Following haemostasis the dura was replaced and the temporal muscle with attached fascia was used to cover the lesion site. The skin was subsequently sutured using Supramid white surgical thread, and Nifulidone (Sanofi, Canada), a nitroflurazone (0.2%) ointment was applied to the wound. This lesion results in a gradual atrophy and complete loss, unilaterally, of the frontal 1 & 3, parietal 1 and portions of the frontal 2, parietal 2 and occipital areas of the rat neocortex. Figure 2.1 illustrates the extent of a typical lesion 30 days post-surgery. Sham operated animals had an equivalent amount of skull bone removed, but the dura was left intact and the cortex was not lesioned.

2.1.3 Drug Treatment

Prior to cortical lesioning, two stainless steel sterile screws (LoMat, Montréal, Canada) were inserted into the right (ie: contralateral to the eventual lesion site) skull bone. One was placed approximately 4 mm anterior to bregma, while the other approximately 5 mm posterior to bregma. A small round hole was drilled through the right skull bone at the following coordinates from Bregma (Paxinos and Watson, 1986): For young adult rats: Anterior/Posterior: -0.8 mm; Lateral: 1.3 mm; Ventral: 3.5 mm; For aged rats: Anterior/Posterior: -1.4 mm; Lateral: 1.8 mm; Ventral: 3.5 mm. A stainless steel (23 gauge) sterile cannula was then stereotaxically guided into the lateral ventricle using the coordinates mentioned. The cannula was secured to the skull bone and to the screws using dental cement (Minit-Weld, Teledyne-Gets, Illinois).

In initial studies, the Alzet osmotic minipumps were filled directly with the drug solution. However, with this method it was difficult to measure the amount of drug, if any, left over and thus, whether animals received different total infusion volumes was difficult to assess. To circumvent this problem a modification of the procedure described by Vahlsing and coworkers (1989) was employed. Coiled polyethelyene tubing (Intramedic PE-60, Clay Adams, New Jersey) was prepared by wrapping 30 cm of the tubing around a steel tube (Diameter: 4 mm) and heating it in a low-temperature oven for approximately 5 minutes. The

coiled tubing was kept overnight in 70% ethanol under UV radiation in a sterile culture hood. The osmotic minipumps were filled, under a sterile culture hood, with a dye solution (0.1% methylene blue, BDH) (Vahlsing et al.1989) which was prefiltered through 0.22 μm Millipore filters. Small lengths of uncoiled sterile tubing (PE-60) were connected to the pumps and they were placed in sterile vials or tubes. The tubes or vials contained sterile PBS, which covered the body of the pump. The pumps were kept overnight in a warm room, at 37°C prior to use. This was done to ensure that the pumps were functional, and that they pumped at the same rate prior to inserting them into the animals. Before filling with the drug solution, the coiled tubing was shaken free of ethanol and dried with sterile gauze. The tubing was filled using a hypodermic needle (23 gauge), with either: vehicle [PBS in initial studies and subsequently artificial cerebral spinal fluid (c.s.f.) (see section 2.7.2 for composition) + 0.1% bovine serum albumin (BSA)], NGF, GM1 or both these agents in combination (NGF/GM1) at various doses. The tubing was subsequently filled with an air pocket and a small amount of mineral oil (Nujol). The ends, containing mineral oil, were connected to Alzet 2001 (7 day delivery), 2002 (15 day lifespan) or 2ML1 (30 day lifespan) minipumps which were pretested to confirm drug deliver rates of 1, 0.5, and 2.5 μl/hr, respectively. The other end of the coiled tubing was connected to the permanent cannulae. After appropriate times the pumps and tubing were removed from anaesthetized rats and total infusion volume was determined for each animal to confirm dosages of NGF and/or GM1 received. The pumps were very accurate; S.E.M. for total infusion volume was always less than 10 μl per group. The 2ML1 pumps were only used in aged rats because these pumps are large in size. For adult rats which received treatments for 30 days, 2002 pumps were used and were replaced.

2.2 PROCEDURES FOR NEUROCHEMICAL STUDIES

2.2.1 Preparation of fresh rat brain slices

For neurochemical studies rats were sacrificed after appropriate post-lesion times by decapitation. The brain was quickly removed, placed on ice and was taken to a cold room (4°C) where brain slices were prepared. This procedure has previously been described in

some detail (Cuello and Carson, 1983) and will thus be briefly reported here. The ventral surface of the brain was wiped free of blood and was adhered to the base of a McIlwain tissue chopper using a cyanoacrylate adhesive (Loctite, Super Bonder 495). Coronal (300-350 μm) brain sections were cut with the machine set on manual control. Each slice which was cut was detached from the blade by gently injecting PBS, pH 7.4 with a hypodermic needle (23 gauge). The brain slice was lifted off the blade and chopper base with the needle, and was placed in cold PBS, pH 7.4. The slice was quickly collected onto a glass slide, and was kept on a bed of ice prior to microdissection of brain areas of interest. Such fresh brain slices can be seen in Figure 2.1.

2.2.2 Microdissection of brain areas from fresh tissue slices

Microdissection of various brain areas were performed with the aid of a Wild microscope (Heerbrugg, Switzerland) equipped with a cold stage. The slides containing the brain slices were placed on the cold stage and were viewed by transillumination. The extent of the lesion in each section was recorded for every animal. Specific brain areas were distinguished based on the contrast provided by alterations in myelin content; areas containing more myelin appeared darker (see Figure 2.1). With the exception of the NBM and HDB all other areas studied could be distinguished in this way. Dissections of the NBM and HDB were done based on photographs of the distribution of ChAT-IR neurons in those nuclei. To ensure reproducibility from animal to animal particular brain regions (e.g.: anterior commissure, internal capsule, globus pallidus, caudate putamen) were used as landmarks and dissection boundaries were strictly followed from animal to animal.

2.2.3 Tissue preparation for determination of ChAT or GAD activity

In instances where only ChAT or GAD activity were measured, the microdissected tissue (septum, VDB, HDB, striatum, NBM, hippocampus, cortex) was immediately frozen at -70^{0}C. Samples were then thawed and were hand homogenized in 10 volumes of 10 mM sodium EDTA buffer pH 7.4 containing 0.5% triton X-100. In some cases choline uptake was also assessed for the cortex, hippocampus and striatum. In this instance, microdissected

tissue were placed in an Eppendorf tube containing 5 volumes (5ml/g tissue) of ice-cold 0.32M sucrose in 5 mM HEPES buffer pH 7.4. The tissue were gently and briefly (2 up and down strokes, manually) homogenized using a loose fitting Teflon pestle. A portion (100 μl) of the homogenized sample was removed, was placed in an Eppendorf tube kept on dry ice, and was stored at -70^{0}C for later analysis of ChAT activity. Once thawed, sodium EDTA and triton X-100 were added to the sample at a final concentration of 10mM and 0.5%, respectively, and the sample was subsequently rehomogenized by hand prior to processing for ChAT activity. ChAT activity in control tissue prepared via either procedure did not differ.

2.2.4 Tissue preparation for determination of choline uptake

Microdissected cortices, hippocampi and striati were collected and were placed on ice. The tissues were weighed and P_2 synaptosomal fractions were immediately prepared and processed for choline uptake. Crude P_2 synaptosomes were isolated essentially as previously described (Gray and Whittaker, 1962) and will be described here in brief: in instances where choline uptake alone was assessed, tissues were suspended in 20 volumes of ice cold 0.32 M sucrose in 5mM HEPES buffer, pH 7.4, and were homogenized (10 up and down strokes at 840 rpm) in a glass tube (0.25mm wall clearance) with a Teflon pestle. In cases where ChAT or GAD activity were also measured, the tissue was previously briefly and mildly homogenized in 5 volumes of 0.32M sucrose buffered with 5 mM HEPES, pH 7.4 (see section 2.2.1). To this homogenate, 15 more volumes (original wet weight) of sucrose buffer were added and this suspension was further homogenized (10 up and down strokes at 840 rpm) in a glass tube (0.25mm wall clearance) with a Teflon pestle. The homogenate was then centrifuged at 1,000 *g* for 10 min. The resulting supernatant was recentrifuged at 12,000 *g* for 15 min to obtain a crude synaptosomal pellet. The pellet was resuspended in 20 volumes 0.32M sucrose buffered with 5 mM HEPES buffer, pH 7.4, and was used to assess choline uptake. Choline uptake of synaptosomes from control animals prepared using either procedure did not differ.

Figure 2.1 Representative fresh coronal brain slices showing the anterior to posterior extent of the unilateral devascularizing cortical lesion from a subject sacrificed 30 days post-lesion. 300-350 μm thick coronal brain sections were obtained using a McIlwain tissue chopper. The extent of the lesion was examined under a Wild (Heerbrugg, Switzerland) microscope and was recorded for every subject. The lesion resulted in a complete atrophy of the frontal 1 & 3, parietal 1 and portions of the frontal 2, parietal 2 and occipital neocortical regions. The distance from bregma is shown in millimeters (mm) in the lower right hand side of each figure (Paxinos G. and Watson, *The Rat Brain in Stereotaxic Coordinates*, 2nd Edition, 1986).

3.20mm

-1.40mm

1.20mm

-3.30mm

-0.92mm

-4.80mm

2.2.5 Tissue preparation for choline uptake kinetics

For studies examining the kinetics of choline uptake, synaptosomes were obtained from cortices and striati of control unoperated and cortically lesioned rats which received, i.c.v. via an Alzet 2001 osmotic minipump, either: vehicle (artificial c.s.f. + 0.1% BSA) or maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day) for 7 days. Animals were sacrificed 30 days post-lesion and P_2 synaptosomal fractions were isolated from microdissected brain tissue as described above; the first step being that tissues were immediately suspended in 20 volumes of ice cold sucrose buffer prior to homogenization (10 up and down strokes at 840 rpm) and centrifugation (see section 2.2.4). The resulting synaptosomal pellet was resuspended in 20 volumes (w/v) of ice-cold 0.32M sucrose buffered with 5 mM HEPES, pH 7.4, and was used in the assay. It was necessary to pool tissue from 3 animals per group in order to have sufficient synaptosomal fractions to test the various choline concentrations.

2.2.6 Isolation of "membrane bound" and "soluble" forms of ChAT

The activities of "membrane bound" and "soluble" forms of ChAT were assessed in the cortex and striatum of decorticated rats which received either: vehicle (artificial c.s.f. + 0.1% BSA) or maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), i.c.v. via an Alzet 2001 osmotic minipump for 7 days. The animals were sacrificed at 30 days post-lesion. "Soluble" and "membrane" bound forms of ChAT were isolated using a modification of the procedure described by Benishen and Carroll (1983). P_2 synaptosomal fractions were obtained as described (section 2.2.4). The synaptosomal pellet was homogenized in 6 volumes dd H_2O and centrifuged at 100,000 g for 1 hour. The crude vesicular pellet obtained was resuspended in 3 volumes of 100 mM sodium phosphate buffer, pH 7.4, was homogenized, and centrifuged at 100,000 g for 30 minutes. The supernatants obtained from these two centrifugations were pooled and taken as the "soluble" fraction. The membrane pellet was resuspended in 100 mM sodium phosphate buffer, pH 7.4, containing 0.5% triton X-100 and was used as the "membrane bound" pool.

2.2.7 Preparation of brain tissue for NGF receptor binding studies

Membranes from cortices of control unoperated, lesion vehicle or GM1 (1.5 mg/day, 7 days) and/or NGF (12 μg/day, 7 days) treated rats sacrificed at the indicated post-lesion times were prepared as previously described (Banerjee et al.1973). Briefly, tissue from 3 animals per group were pooled and were suspended in 10 volumes (w/v) of ice-cold Krebs-Tris buffer (Composition in mM: 143 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 9.8 Dextrose; 25 $NaHCO_3$; 15.5 Tris-HCl), pH 7.4, containing 0.1% bacitracin (Sigma) and 5 X 10^{-5}M leupeptin (Sigma) as protease inhibitors. The tissue was homogenized and then centrifuged for 10 minutes at 1000 g. The pellet was discarded and the supernatant was centrifuged at 7710 g for 10 minutes. The resulting supernatant was then centrifuged at 100,000 g for 60 minutes. The pellet from this centrifugation was resuspended in Krebs-Tris buffer pH 7.4 containing cytochrome C (1mg/ml, Sigma), 0.1 % bacitracin (Sigma) and 5 X 10^{-5}M leupeptin (Sigma) and was used for the binding assay.

2.3 NEUROCHEMICAL ASSAYS

2.3.1 ChAT activity

ChAT activity was determined according to the method of Fonnum (1975). With this radioenzymatic method radiolabeled acetylcholine, which is formed in the assay, is extracted directly into a scintillation mixture leaving the radiolabeled acetyl CoA precursor in the aqueous phase.

All of the steps described were carried out with the Eppendorf tubes, containing samples, kept on a bed of wet ice. Five μl of an incubation medium consisting of: 300 mM NaCl (Fisher), 20 mM Na_2EDTA (Fisher), 50 mM $NaH_2PO_4.2H_2O$ (Fisher), 8 mM choline chloride (Sigma), 0.1 mM eserine sulphate (Sigma) pH 7.4, and ^{14}C-Acetyl CoA (1 nM/5 μl; Specific activity: 60 mCi/mMol; Amersham, Canada Ltd.) were added to 2 μl of the microdissected tissue homogenates. The tubes were transferred to a shaking water bath at 37^{0}C for 15 minutes. The reaction was stopped by returning the tubes to the wet ice and

quickly adding 1.0 ml of ice cold phosphate buffer (finishing buffer: 10 mM $Na_2HPO_4.2H_2O/NaH_2PO_4$, pH 7.4). The samples were then transferred, using a disposable plastic pipet, to scintillation vials. The tubes were washed twice with 1 ml of finishing buffer. The washings were also transferred to the scintillation vials. Two ml of a freshly prepared ion-exchange medium [tetraphenylboron (5 gr/1000 ml, Sigma) in acetonitrile (Fisher)] was added to the scintillation vials. This was followed by 10 ml of toluene scintillant containing PPO (5 gr/l, Sigma) and POPOP (0.1 gr/l, Sigma). The vials were capped, inverted gently and were allowed to stand 60 minutes before determination of radioactivity by a Beckman LS5801 liquid scintillation counter. Blanks which were included in the assay procedure contained homogenizing buffer in place of tissue homogenate. Standards consisted of: 5 μl of the incubation medium which contained 1 nM ^{14}C-Acetyl CoA (60 mCi/mMol); 5 ml ethoxyethanol (ethylene glycol monoethyl ether, Fisher) and 10 ml scintillation fluid.

2.3.2 GAD activity

GAD activity was assessed radiometrically by measuring the amount of liberated $^{14}CO_2$ from ^{14}C glutamate using a modification of procedures described by (Albers and Brady, 1959; Atterwill et al.1981; Legay et al.1987) All steps were carried out with the tubes placed on a bed of wet ice, under a fume hood.

A reaction mixture was prepared on the day of the assay which contained: 50 μl of ^{14}C-glutamic acid (Specific activity: 50 μCi/ml, Amersham, Canada Ltd); 25 μl K-glutamate (Sigma) (0.05 M); 50 μl phosphate buffer (10 mM $Na_2HPO_4.2H_2O/NaH_2PO_4$, pH 7.4); and 15 μl of pyridoxal phosphate (1mg/ml; Sigma), as well as 10 μl of dithiothreitol (11.4 mg/ml; Sigma). Tissue homogenates (prepared as described in section 2.2.3) were centrifuged at high speed for 5 minutes in a Beckman microfuge. Five μl aliquots of the supernatant were placed in 1.8 ml Elkay Eppendorf tubes. Five μl of the reaction mixture was added to each tube. Whatman filter paper (Standard No. 1), previously cut into circles (9 mm diameter) and to which 15 μl of hyamine hydroxide (1M methylbenzathonium hydroxide in methanol, Sigma) was quickly added, were immediately placed in each tube such that the filter rested, snugly, just above the reaction mixture. The tubes were quickly and tightly closed, and were placed in a water bath, at 37^{0}C for 2 hours. After this

incubation, the tubes were placed on ice and the filters were quickly removed and were placed in scintillation vials containing 10 ml Toluene based scintillation fluid (Fisher, containing 0.1 g/l POPOP (Sigma) and 5.0 g/l PPO (Sigma).

2.3.3 [^{3}H] Choline uptake

For choline uptake, 100 μl duplicate synapotosomal preparations were preincubated at 37^{0}C for 5 minutes in 800 μl Kreb's-buffer containing either sodium (composition in mM: 118 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 9.8 dextrose; 25 $NaHCO_3$; 5 HEPES) or in the absence of sodium (same buffer composition except that NaCl and $NaHCO_3$ were replaced by 252mM sucrose and 15.8 mM Tris-phosphate buffer). Following this preincubation, 100 μl of appropriate Krebs buffer containing [^{3}H] choline ([methyl ^{3}H] choline chloride; Amersham, 82 Ci/mmol) was added. Final concentration in the incubation mixture was 0.50μM [^{3}H] choline (0.1 μCi/ml). Uptake was terminated, after a 4 minute incubation at 37^{0}C, by the addition of 4 ml ice-cold Kreb's buffer to each tube. Parallel incubations were carried out at 4^{0}C to account for non specific binding and diffusion. Samples were then immediately filtered under reduced pressure through Whatman GF/B filters which were presoaked for 15 minutes in 0.05% polyethyleneamine (Sigma). Filters were then washed 3 times with 4 ml ice-cold Krebs buffer. [^{3}H] choline uptake was determined by measuring the radioactivity retained by the filters; filters were allowed to stand overnight in 5.0 ml Scintiverse (Fisher) before determination of radioactivity by liquid scintillation spectrometry. Uptake data refers to the difference between uptake at 37^{0}C and 4^{0}C.

In instances where tissue amounts were low, sample portions were reduced by half and the assay proceeded exactly as described above. That is, 50 μl duplicate synaptosomal preparations were preincubated at 37^{0}C for 5 minutes in 400 μl Kreb's-buffer containing either sodium or in the absence of sodium. Following this preincubation, 50 μl of appropriate Krebs buffer containing [^{3}H] choline ([methyl ^{3}H] choline chloride; Amersham, 82 Ci/mmol) was added. Final concentration in the incubation mixture was 0.50μM [^{3}H] choline (0.1 μCi/ml). Uptake was terminated, after a 4 minute incubation at 37^{0}C, by the addition of 2 ml ice-cold Kreb's buffer to each tube. Samples were filtered as described above and the

filters were washed 3 times with 2 ml ice-cold Krebs buffer.

2.3.3a Choline uptake kinetics

Triplicate 50 μl synaptosomal fractions of cortices and striati were preincubated in 400 μl of Krebs buffer for 5 minutes at 37^{0}C. Following this preincubation, 50 μl of [^{3}H] choline ([methyl ^{3}H] choline chloride; Amersham, 82 Ci/mmol) at concentrations ranging between 0.1 μM-1μM, to assess high affinity, or 2 μM-100 μM, to assess low affinity, choline uptake were added to respective tubes. The uptake assay was continued as described above (section 2.33).

2.3.4 ^{125}I-NGF receptor binding assay

Membrane aliquots (1.2-1.6 mg/ml; see section 2.2.5 for preparation) were incubated, at room temperature for 2 hours, with concentrations of ^{125}I-NGF (see section 2.3.8 for preparation and specific activity) ranging from 0.01-20 nM with or without a 400 fold excess of non-radiolabelled NGF. Total incubation volume was 0.1 ml. The bound ligand was separated from free using a previously described centrifugation procedure (Vale and Shooter, 1985). Briefly, the binding mixture was layered over 0.2 ml of 10% (w/v) sucrose dissolved in PBS contained in 0.5 ml microfuge tubes (Evergreen Scientific, California). Tubes were centrifuged for 60 seconds in a microfuge and were then quickly frozen in liquid nitrogen. The tips containing the membrane pellet were subsequently cut and radioactivity was determined with a gamma counter (Intertechnique). Specific binding (total minus that in presence of non-radiolabelled NGF) ranged between 25 and 70%.

2.3.5 Determination of protein content

Tisuue protein content was determined according to the method of Bradford (1976). The dye binding reagent used was either purchased from Bio-Rad or was prepared as follows: 50 mg Coomassie Brilliant Blue G-250 were dissolved in 23.8 ml absolute ethanol and was stirred for 30 minutes. Concentrated orthophosphoric acid (Fisher), 50 ml, was added and

the mixture was left to stir for an additional 30 minutes. Prior to use, the Bio-RAD concentrate was diluted 1/5 or the prepared concentrate was diluted (1/6) and were filtered through Whatman (Standard No. 1) paper. Of this diluted concentrate , 2.5 ml were added to 5 μl of tissue homogenate diluted in 95 μl of double distilled water (dd H_2O). The samples were vortexed and left to stand for 15 minutes. Absorbance of the samples were read, before 1 hour, at 595 nm using a Pye Unicam PU8600 UV/VIS spectrophotometer. Bovine serum albumin (BSA) (5-100 μg/ 5 μl) was used as a standard.

2.4 Isolation and Purification of 2.5S NGF

2.5S NGF was isolated from submaxillary glands taken from CD1 male mice (retired breeders, 25-30g) and was purified using a modification of the procedure described by Bocchini and Angeletti (1969).

2.4.1 Preparation of gland homogenate

Submaxillary glands were dissected taking care to remove as much connective tissue as possible. The glands were collected on ice and were kept frozen at least overnight at -70^{0}C. The frozen glands were allowed to thaw and were placed in a cold blender containing cold double distilled water (dd H_2O) (3ml/gr of gland) and were homogenized at high speed for 2 minutes with brief interruptions every 30 seconds. The resulting homogenate was centrifuged for 30 minutes at 13,000 rpm using a Sorvall centrifuge. The supernatant was subsequently decanted and was filtered through gauze. The precipitate was discarded. To the filtered supernatant an amount of water was added equal to one-half its original volume. Subsequently, streptomycin sulfate (Boehringer Mannheim) at a final concentration of 56 mg per gram of gland was then added. The streptomycin sulfate was originally dissolved in 0.1M Tris-HCl buffer, pH 7.5 at a concentration of 1 gram streptomycin sulfate in 10 ml of the Tris buffer. The diluted gland supernatant was placed in a beaker on ice in a cold room (4^{0}C) and while it stirred the prepared streptomycin solution was added slowly such that the operation lasted 5 minutes. The solution was left stirring for 40 minutes and was subsequently centrifuged for 30 minutes at 13,000 rpm (Sorvall). The supernatant was

removed and lyophilized, and the precipitate was discarded.

2.4.2 Fractionation

The first column used for fractionation of the gland extract was a Sephadex G-100 (fine grade, Pharmacia) column (dimensions: 2.5 cm X 110 cm). The column was kept in a cold room at 4^0C. The Sephadex was obtained in dry powder form and was allowed to swell in 20 ml/gr of dd H_2O, under vacum, for 72 hours at room temperature. The column was carefully packed according to the specifications of Pharmacia (see: Gel Filtration Theory and practice, by Pharmacia, pgs 48-49) ensuring that no air bubbles were retained. The column was equilibrated with 50 mM Tris-HCl buffer containing 5 X 10^{-4}M EDTA, the flow rate was adjusted to 20 ml/hr and 5 ml fractions were collected. Prior to initiating fractionation of the gland extract, the void volume and homogeneity of the column bed were verified by applying a sample of Blue Dextran 2000 (2 mg/ml; Sigma) onto the column. Moreover, molecular weight standards (Hemoglobin: 64,500 mw (Sigma); Ribonuclease A: 13,000 mw; Chymotrypsinogen A: 25,000 mw; Ovalbumin: 43,000 mw; Blue Dextran 2000: 2,000,000; Pharmacia) were also put through the column to establish a molecular weight distribution profile. After these steps, 4-5 column volumes of the eluent (50 mM Tris-HCl buffer containing 5 X 10^{-4}M EDTA) were passed before application of the actual sample.

The lyophilized gland supernatant (section 2.3.6a) was dissolved in cold dd H_2O (1-1.5 mg/gr of glands) and was centrifuged at 15,000 rpm (Sorvall) for 25 minutes. The precipitate was discarded and the supernatant was applied to the pre-equilibrated Sephadex G-100 (Pharmacia) column (Flow rate: 20 ml/hr; Fraction size collected: 5 ml). The fractions were collected and were read at 280 nm. The pooled fractions collected (see Figure 2.2 A; 1st G-100 pool) were diluted with 50 mM Na Acetate buffer pH 5.0 until the optical density was between 1.0- 1.5. This sample was then dialyzed overnight against 50 mM Na Acetate buffer pH 5.0 at 4^0C. The dialysate was then centrifuged (30 minutes, 13,000 rpm) and the supernatant collected was applied to a Whatman CM-52 (Carboxymethyl cellulose, Pre-swollen, microgranular) cation-exchange column.

The column was prepared as follows: The CM52 was added to 0.1M Na Acetate buffer pH 5.0 (20ml/ gr CM52) in a vacum flask. The slurry was gently swirled and the pH was

checked and adjusted to 5.0. Once the slurry settled the supernatant was discarded and the slurry was redispersed in 0.1M Na Acetate buffer pH 5.0 (4 ml/gr CM 52). After the slurry once again settled, the supernatant was discarded and 10 ml/gr of a 50 mM Na Acetate buffer pH 5.0 was added. The slurry was warmed in a water bath (60-70°C) and was placed under vacum for 10 minutes. The column (dimensions: 1.5 cm X 25 cm) was carefully packed with the cooled gel, in a cold room (4°C), and 50 mM Na Acetate buffer pH 5.0 was run through the column at a high flow rate (100 ml/hr) until the column bed height was constant (usually 2-3 hrs). The flow rate was subsequently reduced to 40 ml/hr.

After absorption of the sample, the column was washed with 50 mM Na Acetate buffer, pH 5.0 at the same flow rate (40 ml/hr) for 1-2 hours. Subsequently, a 5 chambered parabolic gradient was applied consisting of:

1st chamber: 250 ml 50 mM Na Acetate pH 5.0
2nd chamber: 250 ml 50 mM Na Acetate pH 5.0 + 100 mM NaCl
3rd chamber: 250 ml 50 mM Na Acetate pH 5.0 + 100 mM NaCl
4th chamber: 250 ml 50 mM Na Acetate pH 5.0 + 600 mM NaCl
5th chamber: 250 ml 50 mM Na Acetate pH 5.0 + 800 mM NaCl

3 ml fractions were collected and the optical density of the collected fractions was read at 280 nm. NGF is contained in the last peak emerging from the column. The pooled fractions of this last peak (CM-52 pool, see Figure 2.2 B) was dialyzed against water (2-3 changes) overnight at 4°C for no more than 24 hours. The dialysate was lyophilized and was resuspended in 50 mM Ammonium Acetate buffer pH 4.2. The sample was applied onto a 2nd Sephadex G-100 column (Dimensions: 1.5 cm X 100 cm), which was prepared and verified for packing homogeneity and molecular weight distribution as described previously. This column however, was equilibrated with 50 mM ammonium acetate buffer, pH 4.2 (15 ml/hr). The fractions collected (2 ml) were read at 280 nm. 2.5S NGF is contained in the last peak which emerges from this fractionation (see Figure 2.2 C). Middle fractions comprising the last peak were pooled (2nd G-100 pool) and were dialyzed against water (3 changes) for 24 hours maximum. The dialysate was collected and lyophilized. The lyophilized sample was dissolved in dd H_2O (2-5 ml) and was centrifuged at 13,000 rpm (Sorvall) for 30 minutes. The supernatant was collected and optical density read at 280 nm. The volume was adjusted to obtain a concentration of NGF at 2mg/ml. The solution was

filtered using a 0.22 μm millipore filter (low protein retainer) and was aliquoted into autoclaved vials. The samples were lyophilized and kept at -20°C.

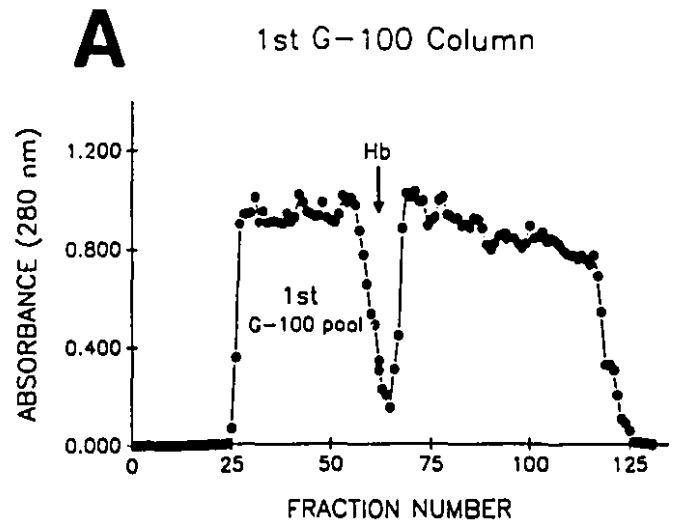
If the sample was shown not to be pure by gel electrophoresis (see 2.3.6c), a second CM-52 column was prepared and the sample was run again in the cation-exchange column, and/or fractionation through the second G-100 column was repeated.

2.4.3 Verification of 2.5S NGF purity

The purity of the 2.5S NGF isolated was tested using one dimensional sodium dodecylsulfate-polyacrylamide (SDS) gradient gel electrophoresis. The separating gel consisted of 7.5-15% acrylamide, 0.3% bis-acrylamide, 8% glycerol, 0.37 M Tris-HCl, pH 8.8. Polymerization was achieved with 0.03% ammonium persulfate (APS) and 0.1% TEMED. The stacking gel consisted of: 4% acrylamide, 0.8% bis-acrylamide, 0.12 M Tris-HCl, pH 6.8, 0.1% SDS, polymerized with 0.1% APS and 0.05% TEMED. The tank buffer consisted of: 25mM Tris-HCl, 200mM glycine and 2mM SDS, pH 8.3. Two micrograms of purified NGF were dissolved in 10 μl of sample buffer consisting of (V/V): 25% 0.5M Tris-HCl, pH 6.8; 20% Glycerol, 4% SDS, 10% 2-ß-mercaptoethanol and 0.005% (w/v) bromophenol blue and was boiled for 5 minutes. Molecular weight standards (Bio-Rad) were diluted (1/20) in the same buffer and were also boiled. Ten to 20 μl of the samples were applied per well. Electrophoresis was performed using a Bio-Rad PROTEAN™ II apparatus, which included a cooling system. Samples were run through the stacking gel at 15 mA and through the separating gel at 25 mA.

The gel was subsequently stained with a solution containing: 0.1% Coomassie Brilliant Blue 250 in 40 % methanol/ 10% acetic acid. The gel was destained with 40% methanol/10% acetic acid for 2-3 hours followed by 10% methanol/ 10% acetic acid (several changes) until background was clear and was photographed (Figure 2.3).

Figure 2.2 Purification of 2.5S NGF from the submaxillary gland of male mice. Representative graphs from one purification experiment are shown. (A) Gel filtration of a mouse gland extract on a Sephadex G-100 column equilibrated with 50 mM Tris-HCl buffer containing 5 X 10^{-4}M EDTA. Thirty-five ml of gland extract were loaded. Fraction volume collected was 5 ml. Hb indicates fraction number where the hemoglobin molecular weight (64,500) standard eluted. (B) Chromatography of the 1st G-100 pool on a CM-52 column equilibrated with 50 mM Na Acetate buffer pH 5.0 and eluted with a parabolic salt gradient (0 to 800 mM NaCl). (C) Gel filtration of the 1st CM-52 pool on a second G-100 column equilibrated with 50 mM ammonium acetate buffer pH 4.2. Two ml fractions were collected.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1st G−100 Column
Hb
1st
G−100 pool
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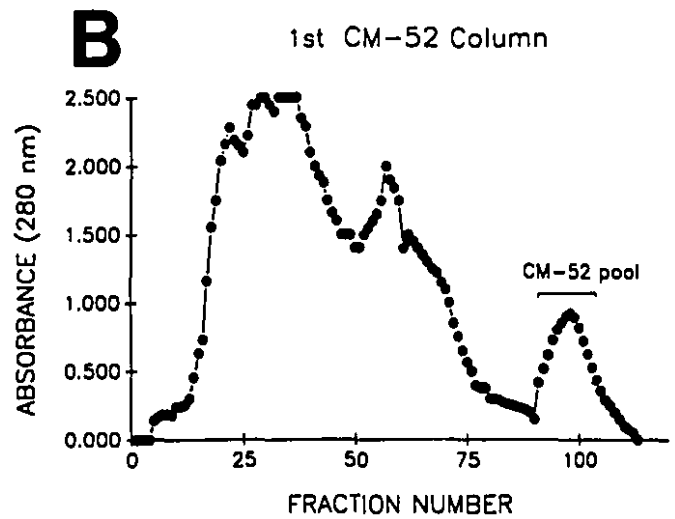
0.800
0.400
0.000
0
25
50
75
100
125
FRACTION NUMBER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CM−52 pool
ABSORBANCE (280 nm)
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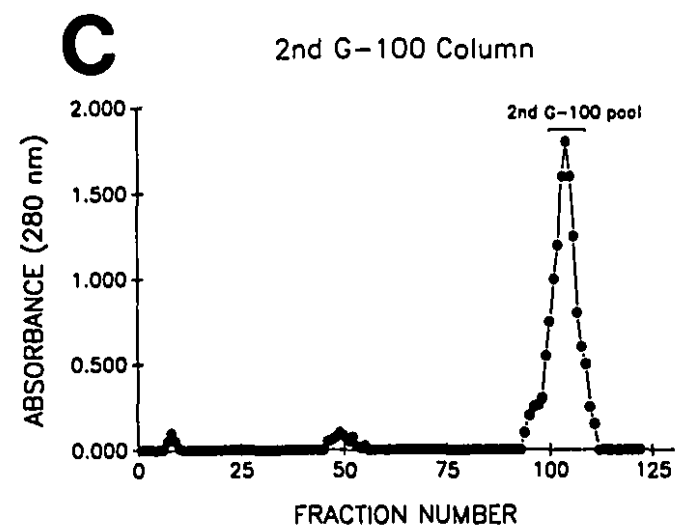
2.000
1.500
1.000
0.500
0.000
0
25
50
75
100
FRACTION NUMBER


C
2nd G−100 Column
2nd G−100 pool
ABSORBANCE (280 nm)
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1.500
1.000
0.500
0.000
0
25
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75
100
125
FRACTION NUMBER

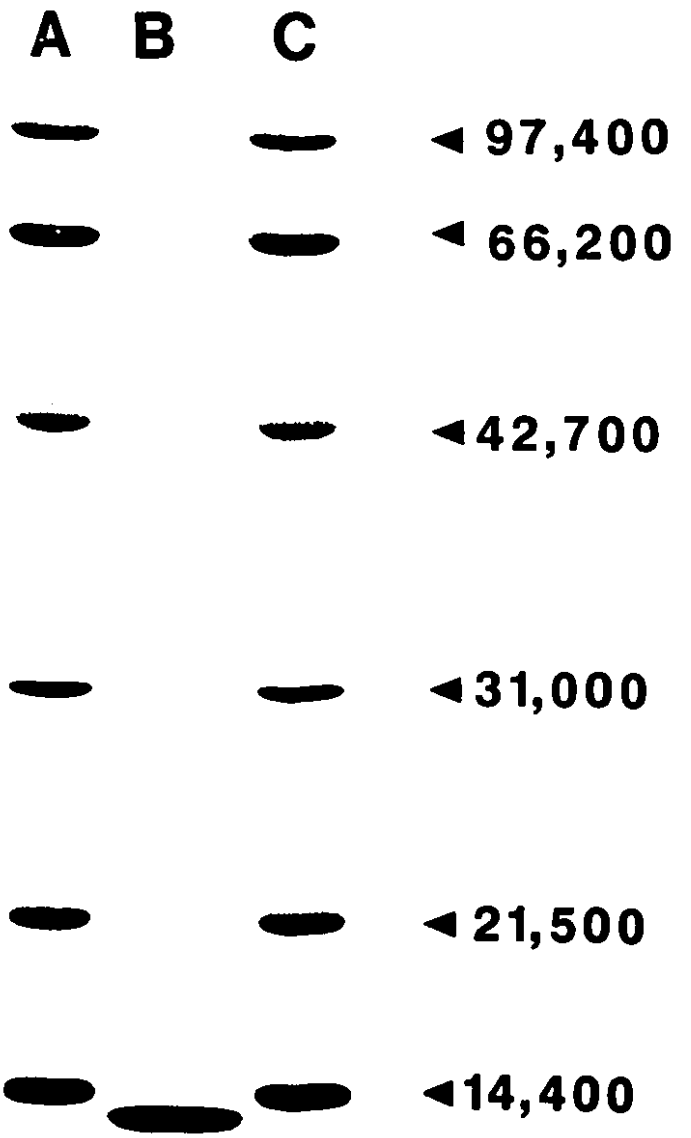


Figure 2.3 One dimensional SDS gel electrophoresis of purified 2.5S NGF. Lanes A and C: molecular weight standards; lane B: purified 2.5S NGF.

2.4.4 Biological activity of 2.5S NGF

The biological activity of the purified 2.5S NGF was tested by assessing neurite extension induced by the purified sample in pheochromocytoma cells (PC12) cells as previously described (Greene and Tischler, 1976) (see Figure 2.4). The cells were obtained from American Type Culture Collection (Rockville, Massachussets) and were plated on collagen (from rat tail) coated coverslips in 24 multiwell plates (1.3×10^5 cells per well). The culture media consisted of: DMEM (Gibco), 10% horse serum (Gibco), 5% fetal bovine serum (Gibco) and 1% KNS (Kanamycin/ Neomycin/ Streptomycin; Gibco). The purified NGF was dissolved in medium at final concentrations of 0.01 to 1 nM and was added to the cells. Media was changed every second day and neurite extension was assessed at 8 days. The purified peptide showed an EC_{50} ranging from 1-to-4 X 10^{-10}M.

2.4.5 Iodination of 2.5S NGF:

The entire iodination procedure was conducted, with the samples under a fume hood, in a room especially designated for such purposes. Purified 2.5S NGF was iodinated using IODO-GEN™ iodination reagent (Pierce, Illinois USA). An Eppindorf tube was coated with a 0.1% (w/v) solution of IODO-GEN™ dissolved in chloroform. The dissolved IODO-GEN™ reagent was thoroughly dried under a stream of nitrogen at room temperature and the reaction vessel was washed once with phosphate buffered saline (PBS), pH 7.4. To the coated tube a 0.2% (w/v) solution of 2.5S NGF dissolved in PBS was added followed by 0.4 mCi of ^{125}I (Amersham IMS.30). The reaction was allowed to proceed for 10 minutes at room temperature with occasional gentle mixing. The sample was subsequently removed from the reaction vessel, and 10 μl of the sample (pre-column sample) was set aside for calculation of specific activity (see below). The rest of the sample was applied to a Sephadex G-25 column (fine grade, Pharmacia) in order to separate ^{125}I-NGF from unreacted ^{125}I. The reaction vessel was washed with 100 μl of 0.5M NaI (Sigma) which was also transferred to the column after the original sample was absorbed. The sample was eluted with elution buffer, consisting of: 0.01M sodium acetate buffer pH 4.0, containing 0.1% BSA and 150 mM NaCl. A 10 ml disposable pipette was used for the column. The top orange border of

the pipet was broken and the bottom was stuffed with cotton. The Sephadex beads were swollen in PBS containing 0.1% BSA (5ml/mg). The column was packed and then was washed with 20 ml of elution buffer, prior to applying the reaction vessel sample. Fractions of 1 ml were collected. From each fraction 10 μl was taken and transferred to scintillation vials for determination of radioactivity with a gamma counter. The labelled NGF peak was pooled and a 10 μl sample was taken (post-column sample) to determine specific activity.

To determine specific activity of the ^{125}I-NGF, 990 μl of elution buffer was added to the 10 μl pre-column and post-column samples set aside. Ten μl aliquots of each sample were then transferred to Eppindorf tubes. The samples were diluted by adding 490 μl of elution buffer, were mixed and 500 μl of a 20% TCA (Trichloroacetate, Sigma) solution was added. The samples were incubated on a bed of wet ice for 20 minutes. They were then centrifuged in a microfuge for 10 minutes at the highest speed. The radioactivity in the supernatant and the pellet from each tube was subsequently determined using a gamma counter. Specific activity of the ^{125}I-NGF obtained ranged from 950-2000 cpm/fmole.

Biological activity of ^{125}I-NGF was tested by assessing neurite extension of PC12 cells. A loss in biological activity of 10-30% of ^{125}I-NGF as compared to non-radiolabelled 2.5S NGF was noted.

2.5 Monosialoganglioside GM1

The monosialoganglioside GM1 was generously provided by Fidia spa in lyophilized form. GM1 was isolated from bovine brain and was more than 99% pure.

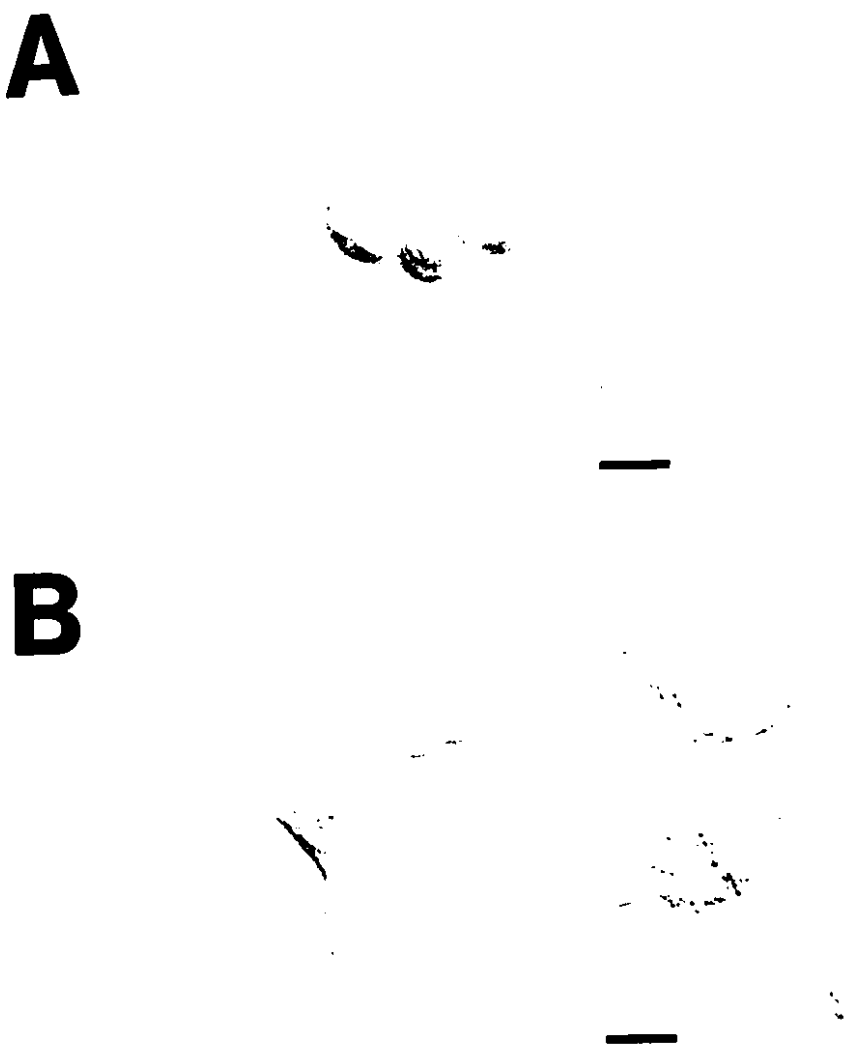


Figure 2.4 Photomicrographs showing the biological activity of the purified 2.5S NGF. PC12 cells were grown in the (A) absence and (B) presence of 0.1 nM of purified 2.5S NGF. After 8 days the coverslips on which the cells were grown were removed from the multiwell plates and were placed, face down, on glass slides containing 50% gelatin (diluted in PBS). Cells were viewed using Nomarski optics. Scale bar= 10 μm.

2.6 *IMMUNOCYTOCHEMICAL PROCEDURES*

2.6.1 Rat perfusion

All rats were fixed by vascular perfusion. The rats were firstly anaesthetized with 3ml/kg of Equithesin (see section 2.7.1 for composition), given i.p. and were subsequently affixed, using masking tape, to a perfusion table. The left or right jugular vein, immediately above the clavicle, was then exposed and injected with (sequentially), 0.1 ml Heparin (10 USP units/ml, Sigma) and 1 ml of freshly prepared 1% sodium nitrite (Sigma). These agents serve, respectively, to prevent blood clotting and to provoke vasodilation of small caliber blood vessels. After 1 to 2 minutes the thorax was opened and the heart exposed. The tip of the heart was then cut and a cannula was quickly inserted through the left ventricle into the aorta. The aorta was clamped to prevent backflow. The right atrium was subsequently cut and a vascular rinse (for composition see section 2.5.3) was allowed to flow at maximum rate for approximately 1 minute. Perfusion with the fixative (see section 2.4.2 and 2.4.3) was then initiated.

2.6.2 Fixation for light microscopic immunocytochemical studies

For light microscopic studies the fixative consisted of 500 ml of a mixture containing 3% paraformaldehyde (BDH), 0.1% glutaraldehyde (Sigma), 15 % (v/v) saturated picric acid (BDH) and 0.1M sodium phosphate buffer (PB), pH 7.4 at room temperature (for buffer composition and fixative preparation see sections 2.7.3 and 2.7.4, respectively). The perfusion was initiated with a high flow rate for the first five minutes. The flow rate was subsequently reduced so that total perfusion time with the 500 ml of fixative was 20 minutes. A perfusion with 250 ml of 0.5% hydrogen peroxide in PBS lasting for 30 minutes followed. This step serves to eliminate remaining free aldehyde groups from the fixative, which reduces background staining and increases specific staining. Subsequently, 500 ml of 10% sucrose in 0.1 M PB were perfused into the animal for 30 minutes. Brains were subsequently removed from animals and were placed in 30% sucrose buffered with 0.1M PB, pH 7.4 and were kept overnight at 4°C. The purpose of the perfusion with, and overnight infiltration in

sucrose is to remove all traces of fixative from the tissue and to cryoprotect it.

2.6.3 Fixation for electron microscopic immunocytochemical studies

For electron microscopic studies the rats were fixed, for 30 minutes, with 500 ml of a 3% paraformaldehyde, 0.1% glutaraldehyde, 15% (v/v) saturated picric acid and 0.1M PB, pH 7.4 mixture at room temperature (for buffer composition and fixative preparation see sections 2.7.3 and 2.7.4, respectively). This was followed by perfusion with another 500 ml of the same fixative, but devoid of Glutaraldehyde, for another 30 minutes. The rats were subsequently perfused with 10% sucrose in 0.1M PB, pH 7.4 for 20 minutes. The brains were removed, relevant areas were blocked and stored in 30% sucrose in 0.1M PB, pH 7.4 overnight at 4°C.

2.6.4 Processing of tissue for light microscopy immunocytochemistry

2.6.4a Tissue sectioning

For light microscopy 50 μm-thick coronal brain sections were cut using a freezing sledge microtome (Reichert). The sections were collected free-floating, in PBS, in either 12 (10-12 sections/well) or 24 (6-8 sections/well) multiwell tissue culture plates. Alternative sections were usually collected for ChAT, $p75^{NGFR}$ and Nissl staining. The sections were washed once with PBS, and subsequently with PBS containing 0.2% Triton X-100 (PBS+T) prior to incubation with the primary antibodies.

2.6.4b ChAT immunocytochemistry

The ChAT rat monoclonal antibody used for the studies of this thesis was developed and characterized by Eckenstein and Thoenen, 1982 (Boehringer Mannheim). The cell line was generously provided by Dr. H. Thoenen and the antibody was recloned in our laboratory. The spent cell supernatant was used diluted 1/100 in PBS+T. Sections were incubated in the primary antibody overnight at 4°C, in multiwell plates kept on an undulating tray. Subsequent

steps were carried out at room temperature. The antibody solution was removed using a plastic transfer pipet and the sections were washed twice (15 minutes each) with PBS+T. A link antibody (rabbit anti-rat IgG; Sigma), diluted 1/50, was then added to the sections, and they were incubated for 60 minutes on a shaker tray. Afterwards, sections were washed twice (15 minutes each) with PBS+T. The sections were then incubated in a peroxidase anti-peroxidase (PAP) complex for 60 minutes. The anti-peroxidase antibody was a rat monoclonal [(Cuello et al.1984); Medicorp, Canada) and was used diluted 1/30. Horseradish peroxidase (HRP, SigmaType VI) was added to the diluted anti-peroxidase antibody solution, at a final concentration of 5 μg/ml, at least 1 hour prior to its addition to the sections. Following incubation in the PAP complex, the sections were washed twice (15 minutes each time) with PBS+T. Subsequent steps were carried out under a fume hood. The sections were incubated in 0.06% DAB (3,3'-diaminobenzidine tetrahydrochloride solution; Sigma; see section 2.7.5 for preparation and precautions necessary), dissolved in PBS+T for 10 minutes. Hydrogen peroxide (H_2O_2; A&C Co.) was then added to the sections, in the DAB solution, to a final concentration of 0.01% (30% stock solution diluted in water). The samples were carefully agitated and the reaction was allowed to proceed for 4-8 minutes. The intensity of the immunostaining was verified using a stereomicroscope. The reaction was stopped by carefully removing the incubation medium and replacing it with PBS+T. The sections were then washed three times (15 minutes per wash) with PBS+T. If the sections were not immediately mounted onto gelatin subbed slides (see section 2.5.8 for preparation) they were kept overnight at 4°C in PBS.

2.6.4c $p75^{NGFR}$ immunocytochemistry

To detect the low affinity NGF receptor ($p75^{NGFR}$) the rat monoclonal antibody Mab 192, previously characterized by Chandler and Johnson, 1984 was used. Brain sections were incubated in undiluted spent supernatant from 192 IgG hybridoma cells, grown in our laboratory, which were generously provided by Eugene Johnson Jr. The steps in the immunocytochemical procedure followed those as described for ChAT immunostaining except that the link antibody used was a rabbit anti-mouse IgG (diluted 1/50; Sigma), and the anti-peroxidase antibody used was a mouse monoclonal [diluted 1/30; (Semenenko et al.1985);

Medicorp Canada].

2.6.4.d Nissl staining

Nissl substance was detected using cresyl violet (Sigma) staining. After brain sections were cut using a freezing sledge microtome and were collected in PBS, they were washed once (15 min) with PBS and were subsequently immediately mounted onto gelatin subbed glass slides. The sections were left to air dry overnight at room temperature and were subsequently dehydrated through ascending alcohols as follows: slides were sequentially incubated for 2 minutes in, absolute ethanol; 95% ethanol containing 6 drops concentrated acetic acid per 200 ml; 80% ethanol; 70% ethanol; distilled water containing 6 drops concentrated acetic acid per 200 ml. The slides were then incubated in a 0.1% cresyl violet (Sigma, see section 2.5.7 for preparation) solution for 2-3 minutes. The slides were transferred to distilled water, containing 6 drops concentrated acetic acid per 200 ml, for approximately 15 seconds. Subsequently, the slides were placed in 70% ethanol and then in 80% ethanol for 30 seconds each time. The sections were differentiated in a 95% ethanol solution, containing 6 drops of concentrated acetic acid per 200 ml, until the Nissl substance and cell nuclei appeared dark purple on a clear background. The sections were rinsed twice in absolute ethanol (30 seconds each), were cleared in xylene, and mounted (see section 2.6.4e)

2.6.4e Mounting medium used for light microscopy

After the immunostained sections were collected onto subbed glass slides they were allowed to dry overnight at room temperature prior to dehydrating in ascending alcohols. This was done by sequentially incubating the slides, for 5 minutes at a time, in: 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, 100% ethanol and 100% ethanol. The slides were then cleared in xylene (10-15 minutes) and were cover slipped (Baxter cover slips, 24 x 50 mm; CanLab) using Entellan (E.Merck, Germany).

2.6.5 Processing of tissue for electron microscopy immunocytochemistry

2.6.5a Tissue sectioning

For electron microscopy, the blocked brain regions (approximate coordinates from Bregma (Paxinos and Watson, 1986): -1.10 to -1.50 mm), which were kept overnight stored in 30% sucrose, were then frozen in liquid nitrogen, transferred to 0.1M PB, pH 7.4 and thawed at room temperature. The cortical area of interest was trimmed and 50 μm thick coronal sections were cut using a Vibratome containing 0.1M PB, pH 7.4 at 4^{0}C. Sections were collected in 6 multiwell plates containing 0.1M PB, pH 7.4 and were kept on ice.

2.6.5b ChAT immunostaining for electron microscopy

After collecting the Vibratome cut sections, they were incubated for 30 minutes in 1% sodium borohydride (dissolved in PBS; Sigma) and were washed repeatedly with PBS (at least 5 changes over a 1 hour period). The sections were then incubated overnight at 4^{0}C in the anti-ChAT antibody (diluted 1/100 in PBS). Subsequent steps were carried out at room temperature. Sections were washed twice, 15 minutes each time, with PBS and were incubated in the link antibody (rabbit anti-rat IgG, diluted 1/50 in PBS; Sigma) for 1 hour. Afterwards, the sections were washed twice, 15 minutes each, with PBS. They were then incubated for 2 hours in a rat monoclonal anti-horseradish peroxidase (HRP) antibody [(Cuello et al.1984); Medicorp Canada], were washed twice (15 minutes each) with PBS, and were incubated for another 2 hours in HRP (5μg/ml in PBS, Sigma type VI). The sections were then washed three times (15 minutes each) with PBS prior to proceeding with the DAB reaction.

A metal intensified DAB reaction was used (Adams, 1981). The DAB (0.05%) was dissolved in PBS and cobalt chloride (final concentration 0.025%) and nickel ammonium sulfate (final concentration 0.02%) were added (see section 2.7.5 for preparation and precautions). The sections were incubated, in this mixture, for 15 minutes at room temperature. H_2O_2 was subsequently added at a final concentration of 0.01% (30% stock

solution diluted with water). The samples were carefully agitated and the reaction was allowed to proceed for 4-8 minutes. The intensity of the immunostaining was verified using a stereomicroscope. The reaction was stopped by carefully removing the incubation medium and replacing it with PBS. The sections were washed twice (15 minutes each) with PBS and once with 0.1M PB, pH 7.4. Afterwards, the sections were rinsed in PBS and were osmicated in 1% osmium tetroxide (Meca Lab Ltd, Montréal, Québec) in 0.1M PB (see section 2.7.6 for preparation) for 1-2 hours at 4°C. The osmium was removed with a disposable pasteur pipet and was discarded in corn oil. The sections were then washed twice with 0.1M PB and were subsequently dehydrated by sequentially incubating them in the following solutions: 50% ethanol for 5 min; 70% ethanol for 5 min; 90% ethanol for 5 min; 95% ethanol for 5 min; 100% ethanol for 10 min; 100% ethanol for 10 min; propylene oxide (Meca Lab Ltd, Montréal, Québec) for 10 min; a 1:1 epon (Meca Lab Ltd., Montréal, Québec)/propylene oxide mixture, for 2 hours or overnight; a 2:1 epon/propylene oxide mixture for 2 hours; pure epon for 2 hours. The sections were then flat embedded with plastic cover slips on an acetate sheet, which was affixed with masking tape to an oven rack. They were kept in the oven at 55°C for 24 hours. The epon embedded and coverslipped sections were carefully detached from the acetate sheet and were examined with a light microscope. Relevant cortical regions were trimmed with a blade and were reembeded, with the plastic coverslip facing down, on the base of plastic conic capsules whose tops had been cut out. The capsule was filled with epon and kept in an oven at 60°C for 48 hours. Cortical layer V was subsequently trimmed. Ultrathin sections (70 nm) were obtained using an Ultracut E Ultramicrotome (Reichert-Jung, Austria) equipped with a diamond knife (Diatome Ltd, Switzerland) and were collected on Formvar-coated one slot grids. The samples were observed non counterstained with a Philips 410 electron microscope.

2.7 SOLUTIONS FOR NEUROCHEMICAL AND NEUROANATOMICAL STUDIES

2.7.1 Equithesin anaesthesia

Equithesin contained 10 mg/ml of sodium pentobarbital (BDH) and 40 mg/ml of chloral hydrate (Fisher). It was prepared as follows: 17g of chloral hydrate were dissolved in 171

ml of propylene glycol (Sigma) and were mixed for several hours (Solution A); 3.9 g sodium pentobarbital were dissolved in 46 ml of 90% ethanol (Solution B); 8.5 g magnesium sulfate (Fisher) were dissolved in 183 ml ddH_2O (Solution C). Solutions A and B were combined and mixed, and solution C was subsequently added. The mixture was stored, in a dark glass bottle, at 4°C and was used within 1 month.

2.7.2 Rat artificial cerebral spinal fluid

The composition of the c.s.f. used was: 128.6 mM NaCl; 2.6 mM KCl; 2mM $MgCl_2$; 1.4 mM $CaCl_2$; 25 mM $NaHCO_3$; 5mM glucose and 1.3 mM $NaH_2PO_4.H_2O$, pH 7.4.

2.7.3 Buffers

Sodium phosphate (Sörensen's) buffer (PB), pH 7.4 ,(0.2M PB), was prepared as follows: 19 ml of a stock solution (A) consisting of 0.2M NaH_2PO_4 was mixed with 81 ml of a second stock solution (B) containing 0.2M Na_2HPO_4. Solution A was used to adjust the pH to 7.4.

Phosphate buffered saline (PBS), pH 7.4 consisted of: 50 ml/l of 0.2M PB, 8.8 g/l NaCl and 0.2 g/l KCl. The pH was adjusted to 7.4 using 1N NaOH. For PBS+T, 2ml/l of Triton X-100 (Fisher) were added to the PBS buffer.

Vascular rinse contained: 50 ml/l 0.2M PB, 8 g/l NaCl, 0.25 g/l KCl and 0.50 g/l $NaHCO_3$. The pH was adjusted to 7.4 using 1N NaOH.

2.7.4 Fixatives

An 8% paraformaldehyde solution was used as a stock solution for preparation of the fixatives. This 8% paraformaldehyde stock was prepared as follows: 680 ml of dd H_2O in an Erlenmeyer flask were heated, on a hot plate, to 90°C, under a fume hood. The flask was removed from the hot plate and 80 g of paraformaldehyde (BDH) were added to the heated water and the solution was mixed. Once the paraformaldehyde was dissolved, drops of 1N

NaOH (usually 10-16) were added to the solution, while it stirred, until the solution cleared. The solution was cooled in a bed of wet ice and was filtered through 0.22 μm Millipore filters, under vacum. Usually, 4 liters of this solution were prepared, which were kept in a dark bottle at 4^0C and used within 1-2 weeks.

The saturated picric acid solution was prepared as follows: 25 g of picric acid were added to 1 liter dd H_2O in a bottle covered with aluminum foil. The solution was left to mix overnight at room temperature and was stored at 4^0C. Prior to use, the solution was filtered through Whatman (Standard No 1) filter paper.

The paraformaldehyde/glutaraldehyde/picric acid mixture used for light and electron microscopy fixation contained: 174 ml 8% paraformaldehyde, 2ml of a 25% glutaraldehyde solution, 74 ml of filtered saturated picric acid and 250 ml 0.2M PB. These components were mixed immediately prior to use. When glutaraldehyde was omitted, 252 ml of 0.2 M PB were added instead. When used for electron microscopy the solution, after mixing, was filtered.

2.7.5 Preparation of metal-intensified DAB solution

DAB is a possible carcinogen and was thus, handled while wearing disposable gloves and a mask. Aluminum paper was used to weigh this compound in order to prevent dispersion of the powder. All solutions remaining, materials and containers exposed to DAB were neutralized by immersing them in a 20% bleach solution. The metal-intensified DAB solution was prepared as follows: 10 mg of DAB were dissolved in 20 ml PBS. While the solution stirred, 0.5 ml of 1% cobalt chloride was slowly added, dropwise. Subsequently, 0.4 ml of a 1% nickel ammonium sulfate solution was similarly added.

2.7.6 Preparation of the osmium solution

Gloves and safety glasses were worn while preparing the osmium solution. All reagents were mixed under a fume hood. Osmium ampoules which contained 2ml of 4% osmium tetroxide were mixed with 4 ml of 0.2M PB, pH 7.4 and 2 ml ddH_2O. The solution was kept refrigerated until use.

2.7.7 Cresyl violet stain

Cresyl Violet (0.5 g) was added to 25 ml of dd H_2O. This solution was left to stir overnight. Sodium acetate (0.1 g) was added to 200 ml dd H_2O and was mixed with the cresyl violet solution. Glacial acetic acid (1.5 ml) was subsequently added followed by 275 ml dd H_2O. The solution was heated to 60°C and left to cool at room temperature for 2 hours. The mixture was stirred, the pH adjusted to 4, and was subsequently filtered through Whatman (Standard No. 1) paper. The solution was kept in a glass bottle, at room temperature, and was re-used several times.

2.7.8 Preparation of gelatin subbed slides

Gelatin (5 g/l, Sigma) and chromic potassium sulfate (0.5 g/l; Chrome Alum, Sigma) were added to water, which was heated to 60°C while the mixture stirred. After the compounds were completely dissolved clean glass slides (Fisher, Superfrost 25 x 75 mm) were dipped in the solution. The slides were dried overnight in a warm room at 37°C.

2.8 LIGHT MICROSCOPIC QUANTIFICATION

2.8.1 NBM cell and fiber measurements

Six sections from the anterior [approximate coordinates from Bregma (Paxinos and Watson, 1986): -0.70 to -1.00 mm), 6 sections from the posterior [approximate coordinates from Bregma (Paxinos and Watson, 1986): -1.60 to -1.80 mm) and 10 sections from the mid [approximate coordinates from Bregma: -1.00 to -1.55 mm] portion of the rat NBM were used for quantification. Alternate sections at each level were used to quantify ChAT or $p75^{NGFR}$-IR neurons and fibers. Thus, for each staining 3 sections from anterior and posterior, and 5 sections from the mid-NBM of each rat were quantified. To visualize ChAT or $p75^{NGFR}$ immunoreactive (IR) NBM neurons and neuritic processes a Polyvar (Reichert) microscope equipped with a 10X plan-apochromatic objective, a 12.5X projection lens and an automated stage was used. A video camera was employed to capture the image into the

image analysis system (Quantimet 920, Cambridge Instruments) [see Figure 2.5]. All neurons in one plane of focus and in a total of 2 fields (425μm X 550μm) per section for anterior and posterior, and 4 fields (425μm X 550μm) per section for the mid basalis were measured (see Figure 2.6). The Quantimet 920 program used for the measurements is provided in appendix A.

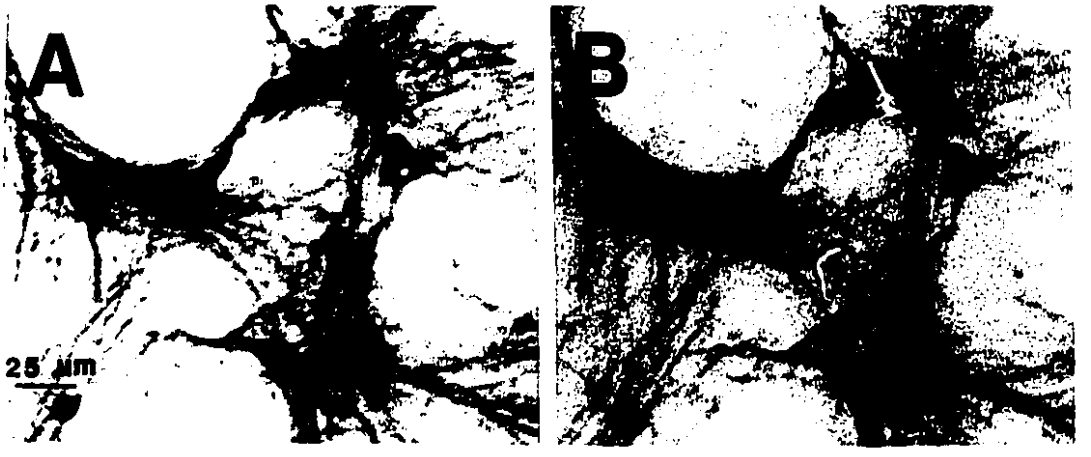
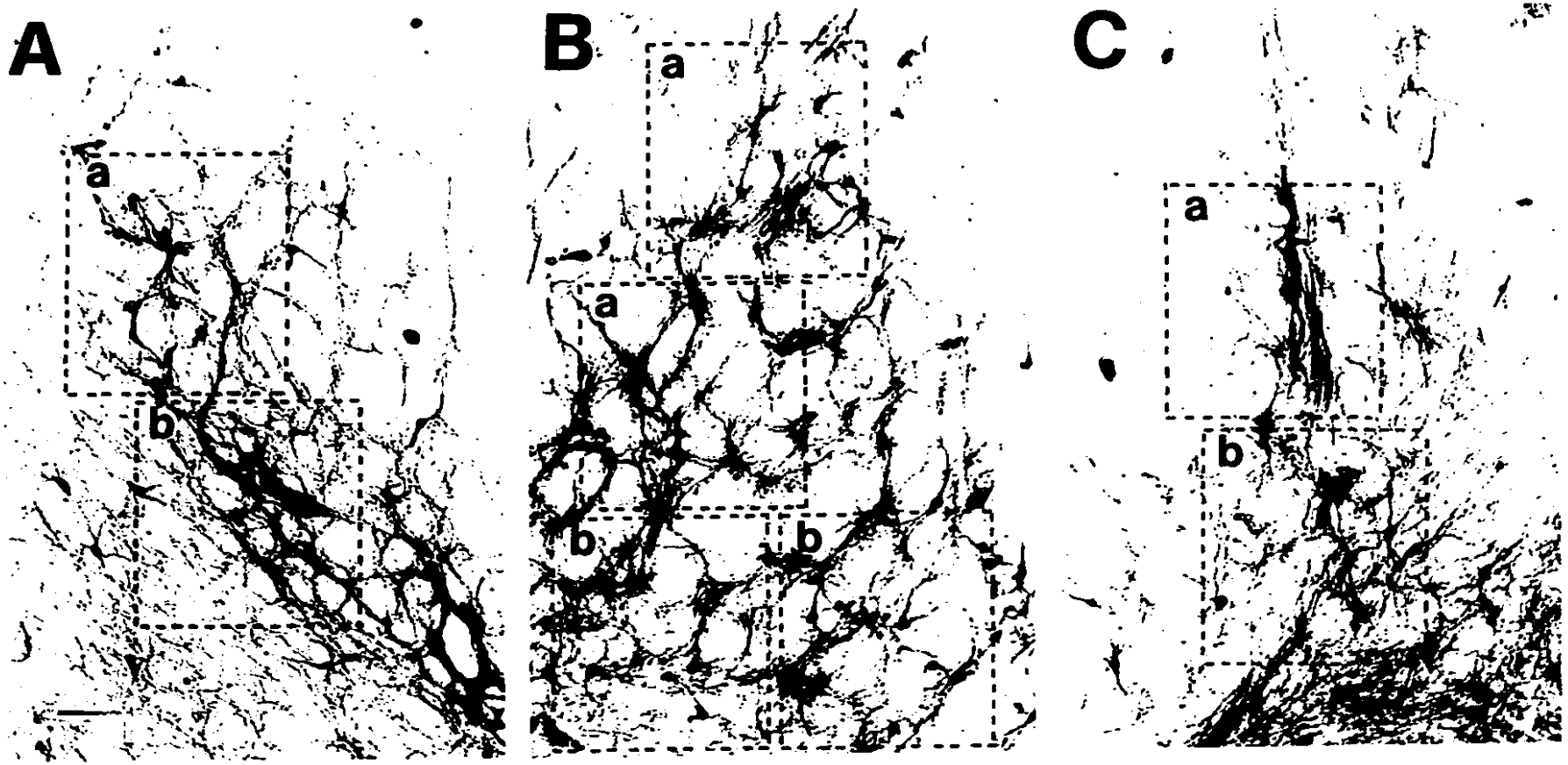


Figure 2.5 ChAT-IR neurons in the NBM as detected by the image analysis system (Quantimet 920). (A) Grey Image and (B) Grey plus binary image. Images of ChAT-IR neurons were transferred onto the image analysis (Quantimet 920) screen using a Polyvar microscope equipped with a 10x objective lens and a 12.5x projection lens connected to a Plumbicon camera. Cells were photographed directly from the image analysis screen using a Nikon camera. Scale bar as indicated in the photo applies to both panels.

Figure 2.6 Illustration of the subdivisions of the NBM used for quantification. A differential distribution of ChAT-IR neurons can be noted throughout the (A) anterior, (B) mid and (C) posterior portions of the NBM. Dashed boxes are representative of the areas quantified. (a) dorsal, (b) ventral, scale bar = 125 μm for all panels.


A
a
b
B
a
a
b
b
C
a
b

2.8.2 *Cortical fiber measurements*

ChAT-IR cortical fibers were quantified using the Polyvar microscope equipped with an automated stage, as well as a 40X plan apochromatic objective and a 12.5X projection lens, connected to the image analysis system. Fibers in all cortical layers adjacent to the lesion site, specifically, within cortical area 4 (Lysakowski et al., 1989) at the mid-basalis level were measured (see Figure 2.7). Five 50 μm thick coronal brain sections [coordinates from Bregma (Paxinos and Watson, 1986): -1.20 to -1.40 mm) from each animal were used for quantification. To ensure that equivalent cortical regions were quantified in all groups, measurements were always initiated at a fixed distance (100 μm) from the mid-point of the cingulum in each section. A total of two focal planes, in each of 12 adjacent fields (105μm X 135 μm), were used to sample cortical layers I through VI within that region (see Figure 2.7). Labelled fibers were measured by the automated image analysis system which employed a segmented fiber analysis program, involving skeletonization (Mize et al.1988), to perform the quantification. With this method, detected fibers are extracted from background and reduced to a single pixel in width (see Figure 2.8). The total number of pixels quantified per area therefore, represents total fiber length (ie: total area occupied by fibers) (Mize et al., 1988). Some of the measurements were performed by an operator who was blinded to the treatment groups. The computer program used for such measurements is provided in appendix A.

2.8.3 *Cortical varicosity measurements*

To quantify the number of cortical ChAT-IR varicosities the Polyvar microscope equipped with an automated stage, as well as a 100X oil immersion plan apochromatic objective and a 12.5X projection lens connected to the image analysis system, was used. Two focal planes in each of twenty-four automatically scanned fields (40μm X 51μm), covering cortical layers I through VI, were counted in the same five brain sections used for the fiber measurements for each rat. All measurements were performed by an operator who was blinded to the treatment groups. See Appendix A for computer program.

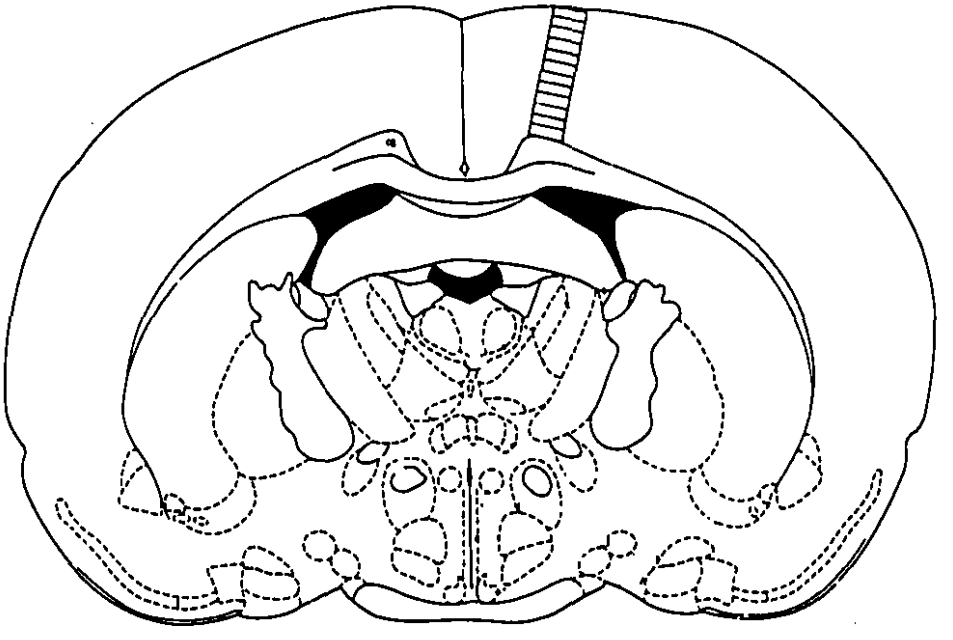


Figure 2.7 Illustration of the cortical area in which ChAT-IR fibers were quantified. ChAT-IR fibers were quantified, using an image analysis system (Quantimet 920), in five sections taken at the level of the mid-basalis from each animal. Twelve fields sampled cortical layers I through VI within the cortical region. See methods for additional details

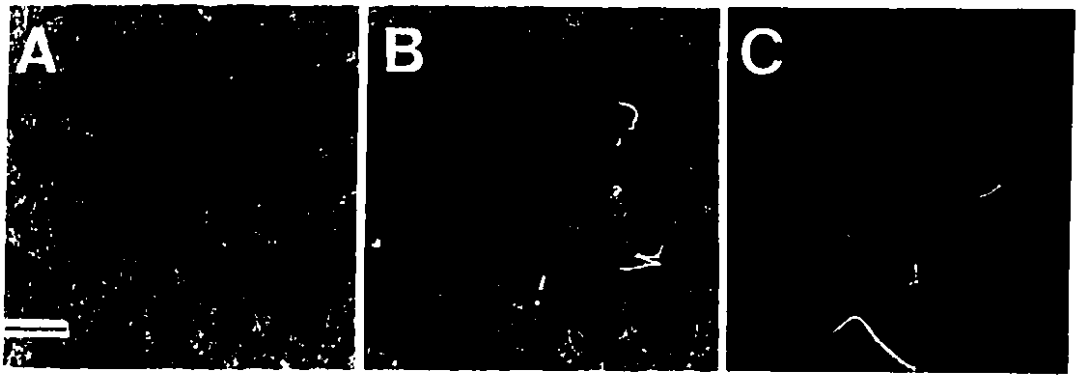


Figure 2.8 Detection of ChAT-IR cortical fibers by image analysis. (A) Grey image, (B) grey plus binary image and (C) Binary image. ChAT-IR fiber images were transferred onto the image analysis (Quantimet 920) screen using a Polyvar microscope equipped with a 40x oil objective lens and a 12.5x projection lens connected to a Plumbicon camera. Fibers were automatically detected using a program involving skeletonization (Mize et al., 1988). With this method, after detection from background, binary images are reduced to one pixel widths. Scale bar= 10 μm for all panels.

2.9 *ELECTRON MICROSCOPIC QUANTITATIVE ANALYSIS*

2.9.1 Measurement of bouton morphometric features

Layer V was selected from cortical tissue processed for EM immunocytochemistry as described above. Ultrathin sections were obtained, collected on Formvar-coated one slot grids and observed non counterstained with a Philips 410 electron microscope equipped with a goniometer stage. The first 26 ChAT immunoreactive varicosities observed in two grids from separate blocks of each animal (n=4 rats/group) were recorded directly onto videotape using a Sony U-matic videocassette recorder (V0-5600). The tapes were replayed using an identical videocassette recorder and the images were transferred to the image analysis system. The immunostained varicosities were automatically detected (see Figure 2.9) and measured with the aid of an operator blinded to the treatment groups. Morphometric features of the ChAT-IR boutons quantified included: mean cross sectional area, perimeter, length, breadth and shape factor ($p^2/4\pi a$). Identification of synaptic differentiations was facilitated by employing the goniometer stage of the electron microscope which allowed rotation and tilting of the grids. A total of 104 varicosities were quantified per group. See appendix A for program used.

2.9.2 Three dimensional serial reconstruction

Serial sections (12-30 per bouton; approximately, 70 nm in thickness each) were also obtained from another 3-4 boutons detected at random from each group. Electron micrographs of each profile and a video camera were used to transfer the images into the image analysis system. The captured image was digitized and the immunostained profiles were automatically detected. After erosion and subtraction an outline was generated. Areas of synaptic contact previously confirmed under the EM and marked on the micrograph were then detected and automatically combined with the generated outline to produce one image forming part of the serial reconstruction. Subsequent profiles were similarly processed. Outlined boutons were stored, displaced by fixed distances and stacked (Blackstad, 1981), thus, reconstructing a three dimensional image (see Figure 2.9). See appendix A for program.

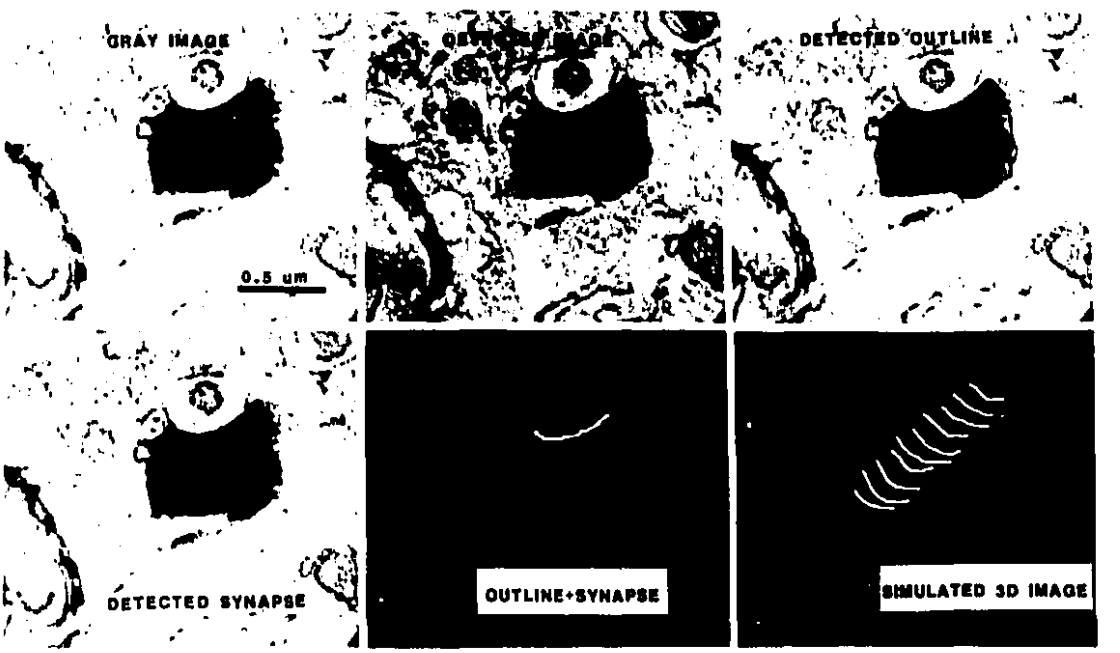


Figure 2.9 Three dimensional serial reconstruction of cortical ChAT-IR varicosities in layer V of rat neocortex. Panels from left to right, beginning at the top, show: Grey image; Grey plus binary image; Grey image plus the binary image of the bouton outline; Grey image plus binary image of the detected synapse; Binary images of the bouton and synapse; Binary image of the reconstructed bouton. Scale bar = 0.5 μm and applies to all panels except lower right one.

2.10 Behavioral Testing

The animals were trained prior to any surgical manipulations in two memory based tasks, passive avoidance and the Morris water maze. Previous studies have shown that animals with these cortical lesions show performance deficits in these two tasks (Elliott et al.1989). The behavioral performance of the animals in both tasks was scored by an investigator who was blinded to the treatment groups.

2.10.1 Passive Avoidance

The passive avoidance test apparatus (84 x 30 x 30 cm) was divided into two equal halves, one painted white, the other black. Access to either side was permitted through an opening (10 x 10 cm) which could be closed with a trap door. The white side had a clear persplex top and was illuminated, while the black side was covered with a black painted top. The floors of both sides comprised of steel bars (8 mm in diameter, spaced 10 mm apart); those on the dark side of the box were connected by a scrambling device (Lafayette Inst. Co., Lafayette IN), to a shock generator (Lafayette No. 58020).

Prior to surgery all rats were habituated for three days to the passive avoidance box. The first two days, rats had free access to either the light or dark compartments of the box. On the third day the time taken for the rats to enter the dark side was recorded. Once the rat entered the dark compartment, the trap door was closed and 15s later the rat received a 0.45mA x 1s scrambled footshock. The subject remained in the dark compartment for an additional 15s before it was returned to its cage. All rats were trained (15 min trial interval) to remain in the light compartment, until each reached a 300s latency criterion. Retention of the learnt response was tested 24 hours later. Only rats with similar levels of performance were selected for the study.

Thirty days post-surgery (ie: 2 weeks after the end of drug infusions) animals were retested in this behavioral task. Latency to enter the dark side of the passive avoidance box was recorded on two consecutive days (Test days 1 & 2). Animals which reentered the box on Test day 1 were reshocked and were retested 24 hours later (Test day 2).

2.10.2 Water Maze

Prior to surgery, rats were also trained in a Morris water maze task. The water maze testing apparatus used was originally described by Morris (1984). For experiments of this thesis it consisted of a white plastic pool (diameter: 140 cm; height 42 cm) placed in the center of a test room. The pool was filled to a depth of 30-35 cm with water in which non-toxic powder tempera dark blue paint (Weber Costello, Mississauga Ontario) had been dissolved. A circular transparent platform (10 cm in diameter) covered with a plastic mesh (Type S74616-F; Sargent-Welch) was placed in one part of the pool such that it was covered with 1 cm of water and was not visible. Trials were initiated from previously designated and fixed parts of the pool (North, South, East, West). At the start of each trial the rat was placed in the water with its face positioned towards the side of the pool. The time taken for the rat to locate the hidden platform and the route which it travelled were recorded for each subject on every trial. The training paradigm consisted of one block of four trials, spaced 15 minutes apart, per day for four consecutive days. Start positions within each block varied between trials. Once the rat located the platform, it was allowed 30s to acquire spatial information from external cues in the room, i.e. shelves, cupboards, stools, investigators. A 180s cutoff point was used in this study. If during the first few trials the rat failed to locate the platform in that time the subject was placed on it and left for 30s. Rats which showed equal levels of acquisition (ie: similar escape latency times and ability to locate the platform using a direct route in the last set of trials) in this task were selected for this study.

Thirty days post-lesion (ie: 2 weeks after end of drug infusions) the animals were retested using the same experimental paradigm as described for training. Following reacquisition of the task the rats were retested once more 2 weeks later in one block of four trials.

These rats were subsequently tested in a cued version of this water task. In this study the platform was moved to a new location and was elevated 3 cm above the surface of the water thus making it visible. The same testing paradigm as described above was used. This allowed indirect assessment as to whether motivation, altered swim speeds, or motor problems were affecting performance.

RESULTS

3.1 *NEUROCHEMICAL CORRELATES OF UNILATERAL DEVASCULARIZING CORTICAL LESIONS AND TROPHIC FACTOR TREATMENT*

Initial studies of this thesis confirmed (Stephens et al., 1985) that a unilateral devascularizing cortical lesion affecting mainly the frontal/parietal adult rat cortex causes significant decreases (40-50%) in ChAT activity in the ipsilateral NBM at 30 days post-lesion, and does not affect the activity of this enzyme in the contralateral NBM (Figure 3.1). Other ipsilateral or contralateral subcortical brain areas such as the septum, striatum and hippocampus show no change in this cholinergic marker (Figure 3.1). In addition, ChAT activity in the remaining ipsilateral cortex adjacent to the lesion site, and in its contralateral counterpart, also remains at control levels (Figure 3.1).

3.1.1 *Effect of short-term versus long-term and intraperitoneal versus intracerebroventricular treatment with the monosialoganglioside GM1 on NBM and cortical ChAT activity*

Previous work had shown that treatment of decorticated rats with daily intraperitoneal (i.p.) injections of GM1 at 30 mg/kg, beginning immediately post-lesion and continuing for 30 days, could attenuate the lesion-induced deficit in NBM ChAT activity and could stimulate ipsilateral cortical ChAT activity above control levels (Stephens et al., 1987). In order to further examine this apparent neuroprotective effect of GM1, subsequent studies undertaken for this thesis assessed (1) whether a short-term i.p. treatment could similarly prevent deficits in NBM ChAT activity and (2) if these effects could be attributed to gangliosides acting upon the CNS. For these studies adult rats were unilaterally decorticated and received, immediately post-lesion, either vehicle, phosphate buffered saline (PBS), or daily i.p. injections of GM1 (30 mg/kg) for 7 or 30 days. Other decorticated animals had permanent cannulae implanted into their right lateral ventricle through which GM1 (1.5 mg/day) was continuously infused for 7 days by a subcutaneously placed Alzet 2001 osmotic minipump. For comparison, GM1 at this lower dose of 1.5 mg/day was also administered i.p. daily for 7 days. A group of

sham operated rats were similarly treated. For the sham operations, an amount of skull bone was removed which was equivalent in size to that taken from lesioned subjects, but the dura was left intact and the cortex of these animals was not lesioned. As shown by Figure 3.2, ChAT activity in the ipsilateral NBM or cortex of sham operated animals was not altered by any of the treatments. Lesioned rats which received vehicle either i.p. or intracerebroventricularly (i.c.v.) had significant decreases in NBM ChAT activity at 30 days post-lesion. By contrast, NBM ChAT activity in lesioned rats which received, beginning immediately post-lesion, daily i.p. injections of 30 mg/kg/day of GM1 for either 7 or 30 days was not significantly different from control unoperated or sham operated animals. In addition, both these treatment paradigms induced an increase, above control values, in ChAT activity of the remaining ipsilateral cortex. Moreover, the lower dose (1.5 mg/day) given i.c.v. also had a protective effect but, was ineffective when given i.p.. These results demonstrate that short-term treatment with GM1 can provoke long-term recovery or activation of cholinergic markers following brain injury and furthermore, suggest that these effects of GM1 are centrally mediated. Moreover, that GM1 treatment did not affect ChAT activity in sham operated animals indicates that injury creates a permissive environment for GM1 effects. This is in line with the proposal, based on in vitro observations, that gangliosides are effective in particular circumstances such as, for example, when a balance between permissive and inhibitory conditions exists (Skaper and Varon, 1985).

3.1.2 Dose dependent effects of exogenous NGF or GM1 on ChAT activity

Reports indicating that NGF, a well known trophic factor for peripheral sympathetic and sensory neurons, was present in the adult mammalian brain (Korsching et al, 1985) prompted us to investigate whether NGF could also serve as a neuroprotective agent for injured adult NBM cholinergic neurons and in particular, to compare and contrast its effects with those produced by GM1 treatment. For this purpose, experiments were undertaken to establish dose response curves for the i.c.v. effects of exogenous NGF and GM1 on brain cholinergic markers.

Adult rats were unilaterally decorticated and received, i.c.v. via minipump for 7 days,

vehicle [artificial cerebrospinal fluid (c.s.f.) + 0.1% BSA], GM1 or NGF at various doses. The rats were subsequently sacrificed at 30 days post-lesion. Lesioned rats which received vehicle showed decreases in ChAT activity in the ipsilateral NBM of approximately 50% (Figure 3.3B, Figure 3.4B). The activity of this enzyme, in lesioned rats, was not affected in the contralateral NBM nor in other ipsilateral or contralateral brain areas examined (striatum, septum, hippocampus, cortex) (Figure 3.5, Table 3.1). Treatment with NGF or GM1 beginning immediately post-lesion and continuing for 7 days prevented, in a dose dependent manner, this lesion induced deficit (Figure 3.5B, 3.6B). In addition, these agents caused an increase, above control levels, in ipsilateral cortical ChAT activity (Fig 3.5A, 3.6A). The ED50 for NGF to produce these effects was 0.1 μg/day for the NBM and 1 μg/day for stimulation of cortical ChAT activity. By contrast, the dose required for exogenous GM1 to similarly attenuate NBM or to stimulate cortical ChAT activity was much greater (ED50 for NBM: 375 μg/day; ED50 for Cortex: 675 μg/day). These short term (7 days) treatments with NGF or GM1 did not affect ChAT activity in the contralateral NBM or cortex, nor in the septum or hippocampus (Table 3.1). Increases in ChAT activity were observed however, in both the ipsilateral and contralateral striati of lesioned animals which received NGF (Figure 3.5A, 3.5B). The ED50 for the NGF-induced stimulation of ipsilateral striatal ChAT activity was 1 μg/day while lower doses (ED50: 0.5 μg/day) increased striatal ChAT activity contralateral to the lesion, that is, on the side of the NGF infusion. However, these ED50 values for striatum may be incorrect since a clear plateau for the striatal dose-reponse curve was not noted, and higher doses were not tested.

These results show that exogenous GM1 or NGF are equally efficacious in preventing cholinergic deficits in the ipsilateral NBM and in augmenting ChAT activity in the remaining ipsilateral cortex of decorticated animals. However, NGF is more potent than GM1 in this respect. Furthermore, it appears that striatal cholinergic interneurons are particularly sensitive to NGF since ChAT activity in other similarly nonlesioned brain regions (ipsilateral and contralateral septum and hippocampus; contralateral NBM and cortex) do not show NGF-induced alterations in ChAT activity.

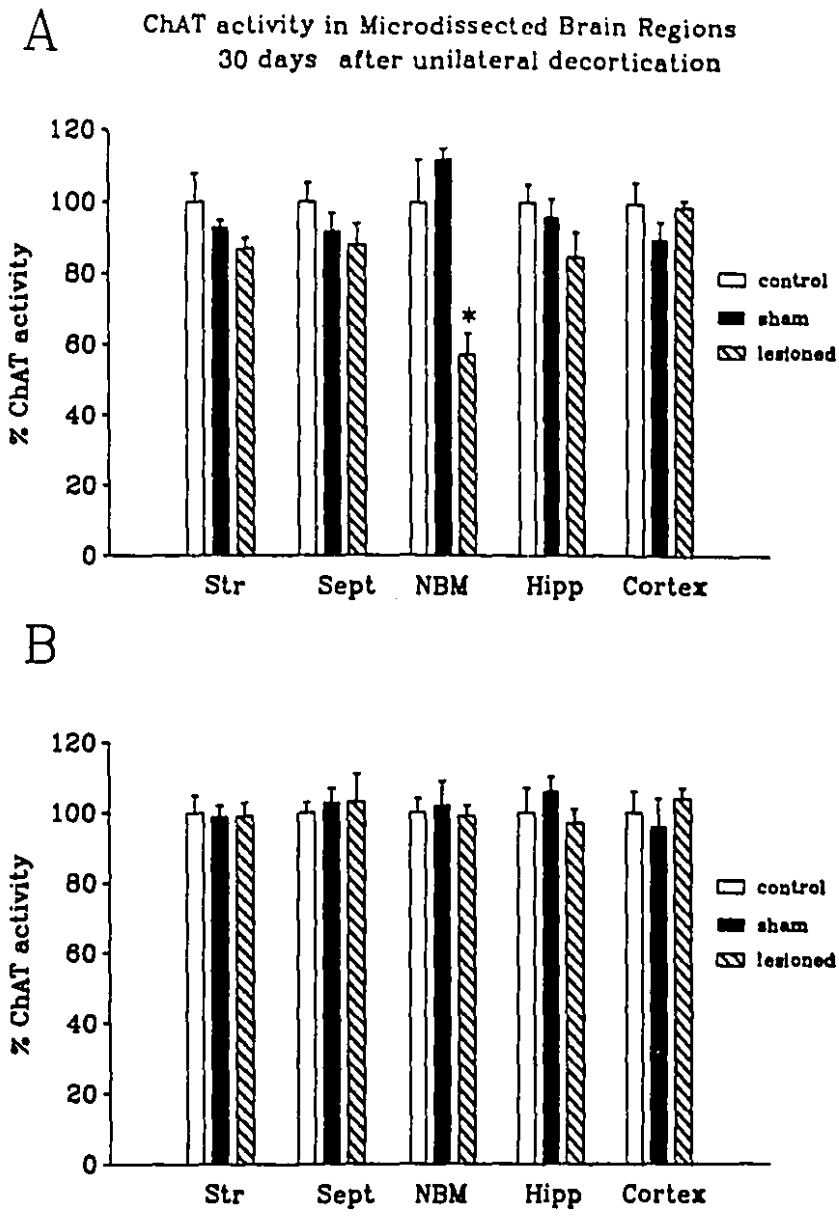


Figure 3.1 Effect of unilateral decortication on ChAT activity in (A) ipsilateral and (B) contralateral cortical and subcortical brain regions of adult male Wistar rats at 30 days post-lesion. Animals (n= 6/group) were cortically lesioned as described in methods. Sham operated animals had an equivalent amount of skull bone, as their lesioned counterparts, removed but, the dura was left intact. Unoperated animals served as controls. ChAT activity and protein content in microdissected brain areas were determined using the methods of Fonnum (1975) and Bradford (1976), respectively. Values are expressed as percent control. ChAT activity (nMol ACh/mg protein/hr) in control animals were for the: ipsilateral NBM: 63.56 ± 1.66; contralateral NBM: 61.82 ± 1.75; ipsilateral septum (Sept): 55.60 ± 1.55; contralateral septum (Sept): 51.32 ± 2.66; ipsilateral hippocampus (Hipp): 63.87 ± 0.99; contralateral hippocampus (Hipp); 61.56 ± 0.88; ipsilateral cortex: 38.66 ± 0.75; contralateral cortex: 40.21 ± 0.65; ipsilateral striatum (Str): 126.55 ± 2.03, contralateral striatum (Str): 125.77 ± 1.62. Error bars represent S.E.M., *$p < 0.05$ from respective control values, ANOVA, post-hoc Newman-Keuls' test.

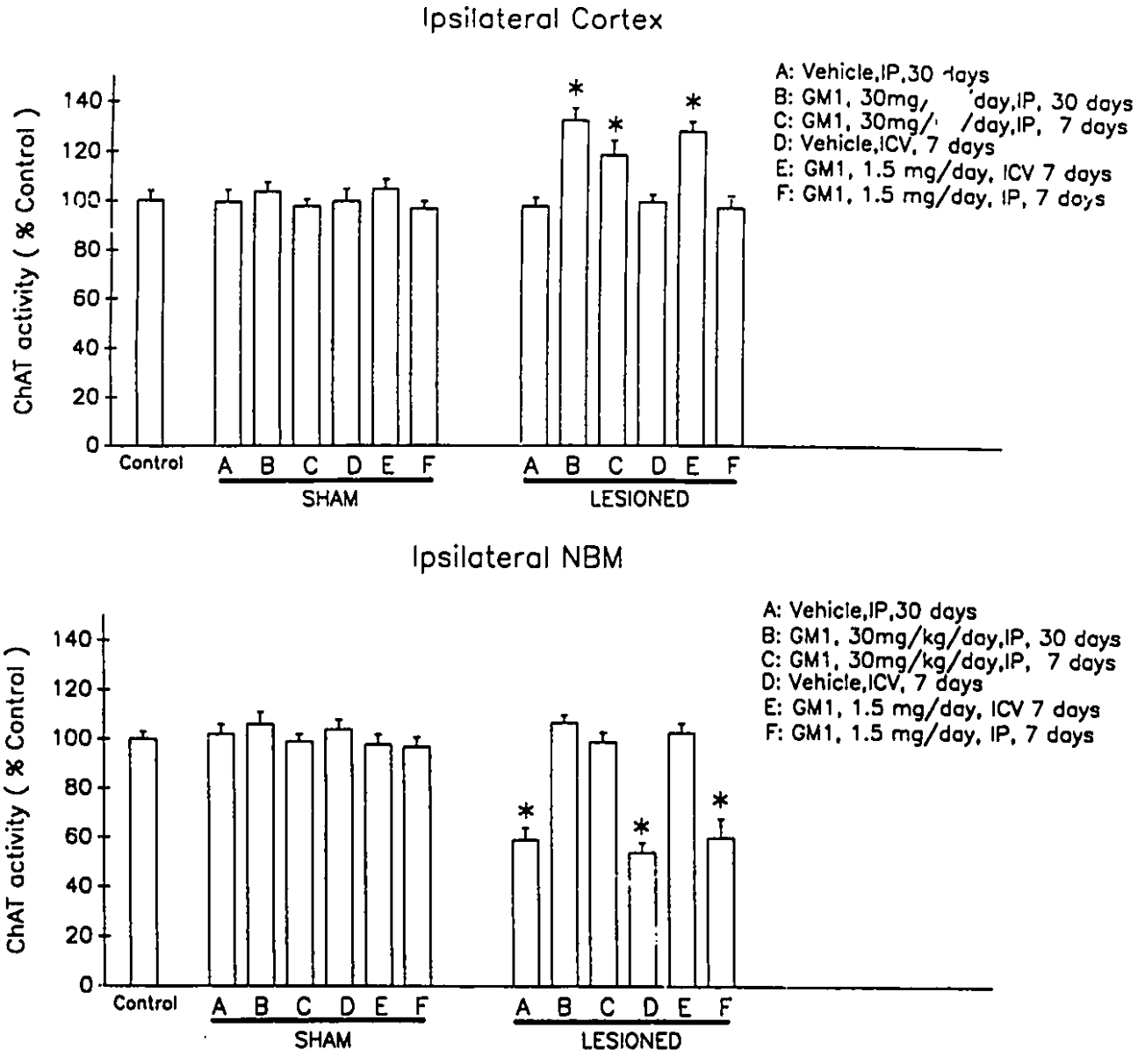


Figure 3.2 Effect of short-term (7 days) versus chronic (30 days) and intraperitoneal (IP) versus intracerebroventricular (ICV) GM1 treatment of adult unilaterally decorticated rats on NBM and cortical ChAT activity. Decorticated rats received, beginning immediately post-lesion, daily IP injections of GM1 at doses of 30 mg/kg/day for 7 or 30 days, while another group of lesioned rats received GM1 at doses of 1.5 mg/day either IP or ICV (continuously, via minipump) for 7 days. Sham operated animals were similarly treated. All animals were sacrificed at 30 days post-lesion. ChAT activity and protein content in microdissected brain areas were determined using the methods of Fonnum (1975) and Bradford (1976), respectively. Values are expressed as percent control. ChAT activity (nMol ACh/mg protein/hr) in control animals was for the: ipsilateral NBM: 58.56 ± 1.66; contralateral NBM: 55.82 ± 1.95; ipsilateral cortex: 35.60 ± 0.75; contralateral cortex: 33.32 ± 1.66. Error bars represent S.E.M., n=6-9 animals/group. *$p < 0.05$ from respective control value, ANOVA, post-hoc Newman-Keuls' test.

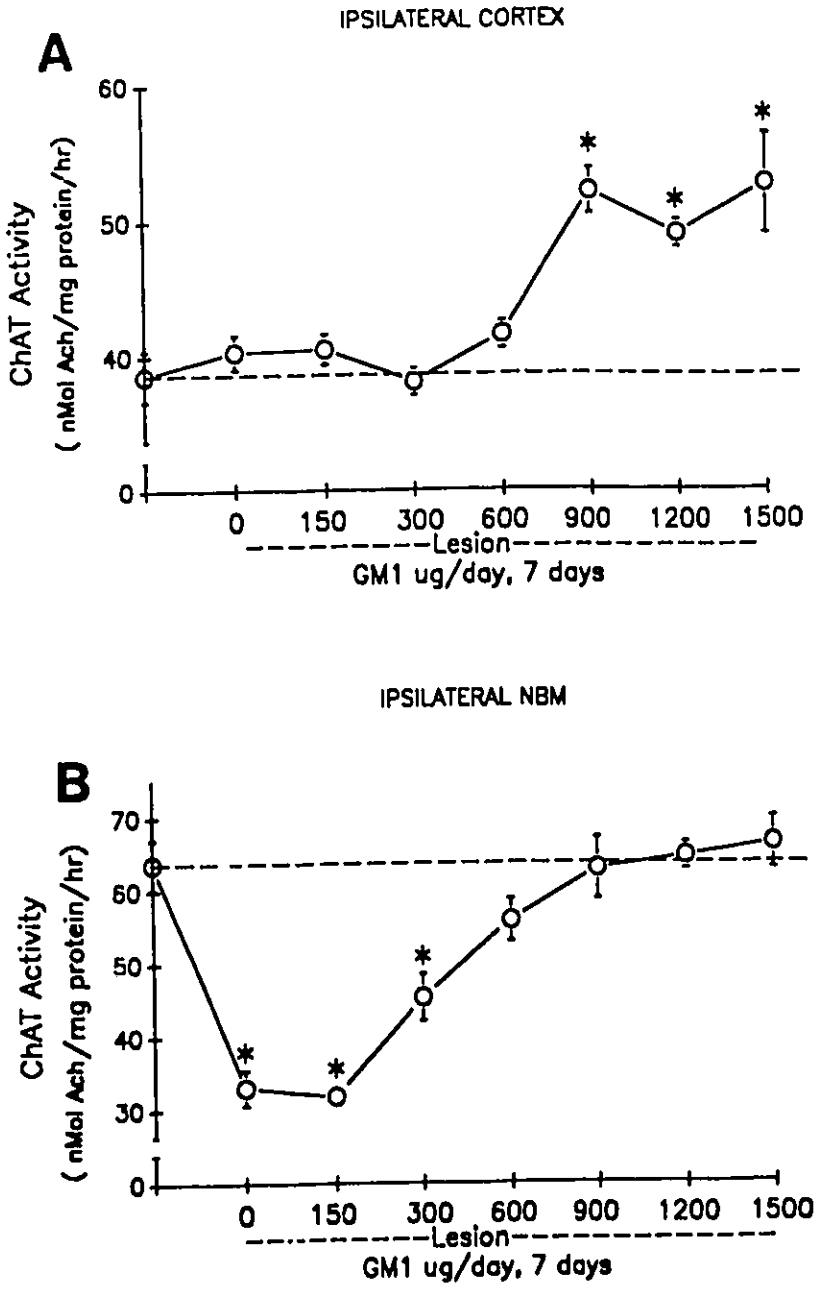


Figure 3.3 Dose dependent effects of GM1 treatment on ipsilateral (A) Cortical and (B) NBM ChAT activity of unilaterally decorticated rats. Animals were cortically lesioned as described in methods and received, i.c.v via minipump, immediately post-lesion either vehicle (artificial c.s.f. + 0.1 % BSA) [0] or GM1 at dosages of 150 to 1500 μg/day for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatment) and ChAT activity was determined in microdissected NBM and cortices as described by Fonnum (1975). Protein content was assessed using the method described by Bradford (1976). n= 6 animals/group. Error bars represent S.E.M.. *$p < 0.05$ from control, ANOVA, post-hoc Newman-Keuls'. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

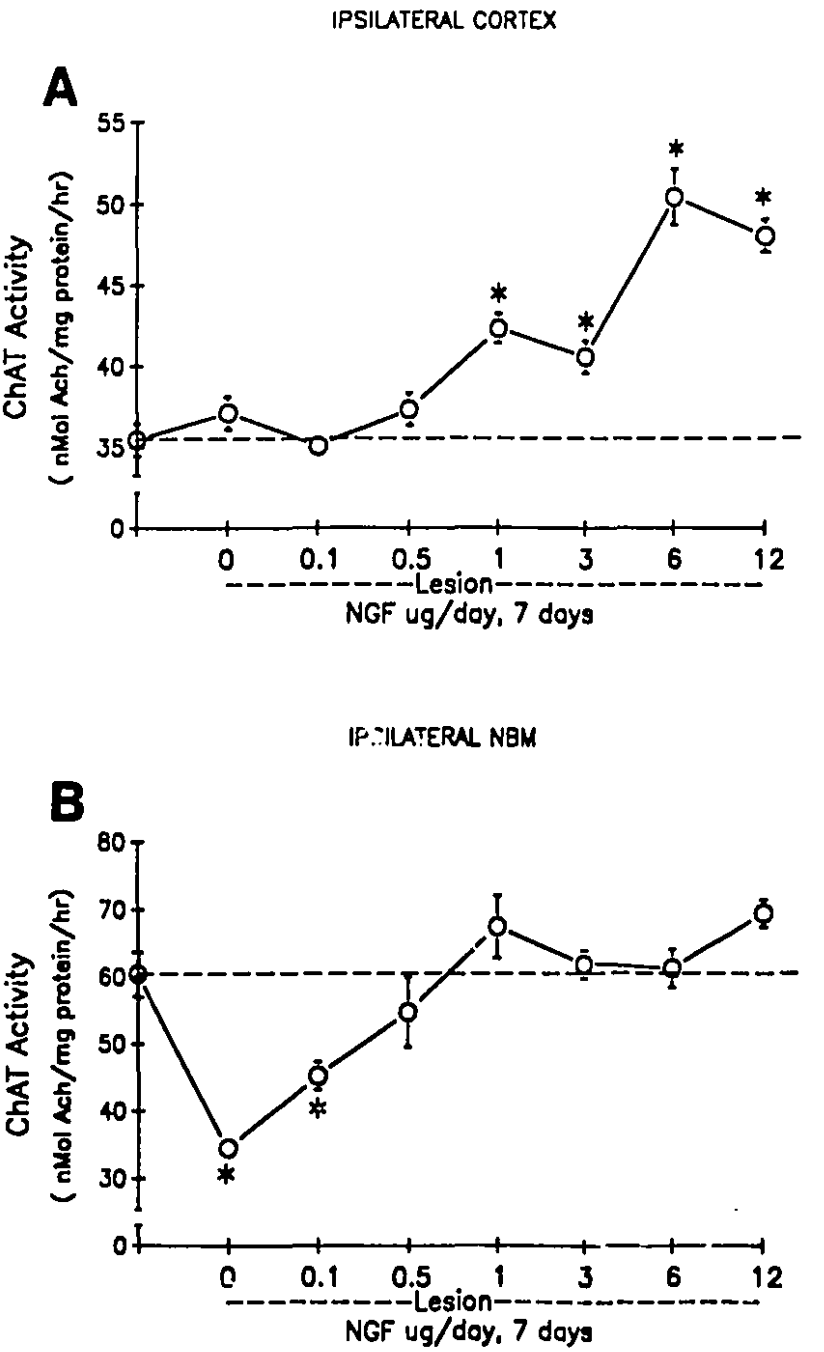


Figure 3.4 Dose dependent effects of NGF treatment on ipsilateral (A) Cortical and (B) NBM ChAT activity of unilaterally decorticated rats. Animals were cortically lesioned as described in methods and received, i.c.v. via minipump, immediately post-lesion either vehicle [0], consisting of artificial c.s.f. + 0.1 % BSA, or NGF at dosages of 0.1 to 12 μg/day for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatment) and ChAT activity was determined in microdissected NBM and cortices according to the method of Fonnum (1975). Protein content was assessed as described by Bradford (1976). n= 6 animals/group. Error bars represent S.E.M.. *$p < 0.05$ from control, ANOVA, post-hoc Newman-Keuls' test. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

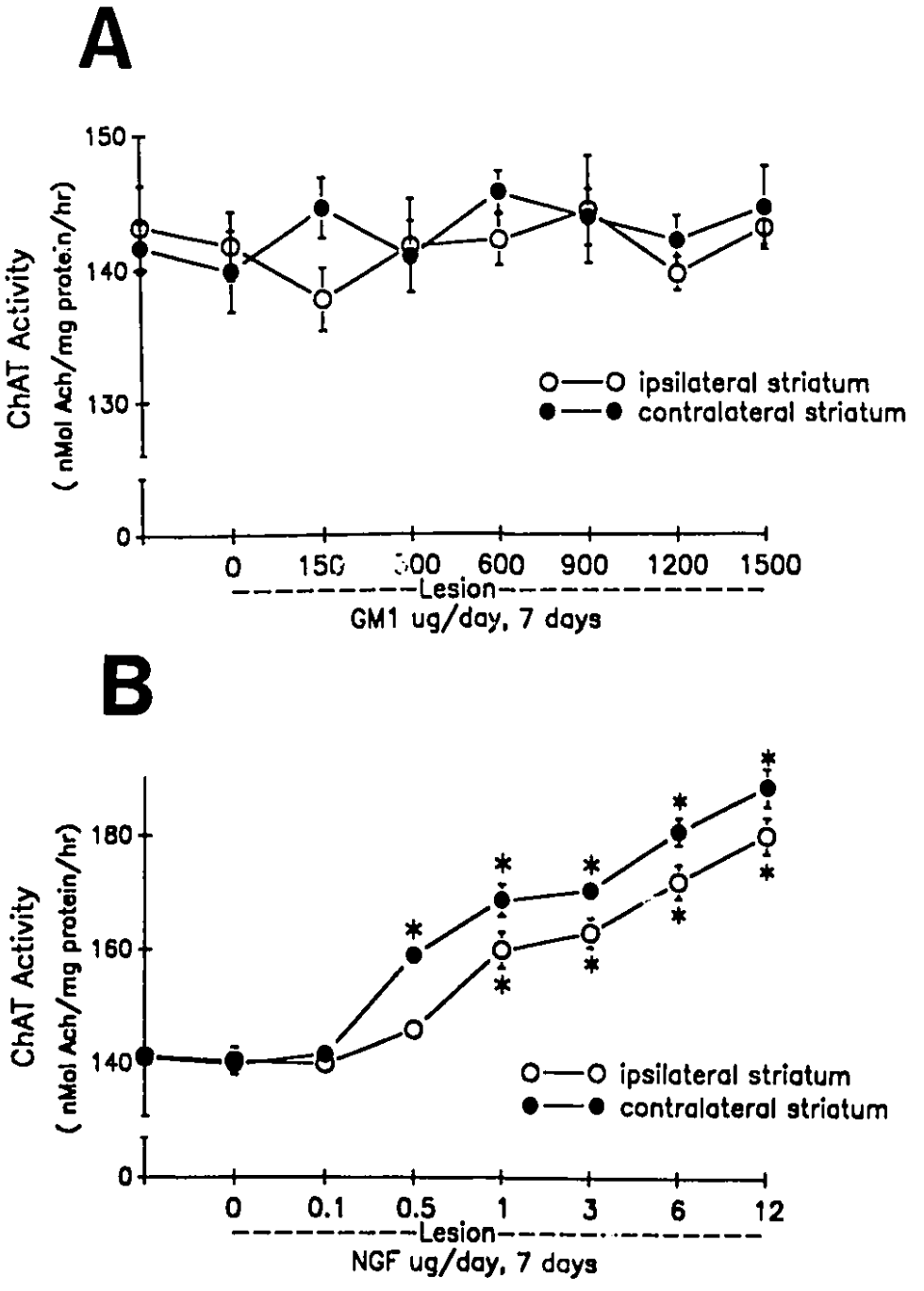


Figure 3.5 Dose dependent effects of (A) GM1 or (B) NGF treatment on ipsilateral and contralateral striatal ChAT activity of unilaterally decorticated rats. Animals were cortically lesioned as described in methods and received, i.c.v. via minipump, immediately post-lesion either vehicle [0] (artificial c.s.f. + 0.1 % BSA), GM1 at dosages of 150 to 1500 μg/day or NGF at dosages of 0.1 to 12 μg/day for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatment) and ChAT activity was determined in the microdissected tissue according to the method of Fonnum (1975). Protein content was assessed as described by Bradford (1976). n= 6 animals/group. Error bars represent S.E.M.. *$p<0.05$ from control, ANOVA, post-hoc Newman-Keuls' test. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

TABLE 3.1 **DOSE DEPENDENT EFFECTS OF GM1 OR NGF ON ChAT ACTIVITY**

BRAIN AREA	Lesion + GM1 (μg/day)							
	Control	0	150	300	600	900	1200	1500
Contralateral NBM	59.32±1.61	58.28±2.22	57.84±1.61	57.82±1.30	61.68±1.31	61.22±1.45	58.11±1.25	59.35±1.36
Contralateral Cortex	39.33±0.71	39.17±1.76	37.57±0.86	38.64±2.10	38.67±0.85	38.50±0.78	39.18±0.97	39.47±0.97
Ipsilateral Septum	51.21±1.09	53.21±1.52	51.77±0.88	50.43±1.16	51.97±1.69	50.51±1.05	53.32±2.14	53.28±1.68
Contralateral Septum	53.67±1.76	50.42±1.34	52.78±1.55	54.32±2.09	50.64±0.98	53.45±1.12	50.76±1.69	52.25±1.09
Ipsilateral Hippocampus	60.00±0.86	59.49±2.37	59.77±1.39	59.70±1.51	62.39±2.83	63.30±3.43	63.48±1.42	63.14±3.14
Contralateral Hippocampus	62.76±1.67	61.36±1.76	60.28±2.10	63.89±1.77	60.96±1.15	64.79±2.98	64.31±2.25	65.54±2.51

BRAIN AREA	Lesion + NGF (μg/day)							
	Control	0	0.1	0.5	1	3	6	12
Contralateral NBM	60.19±1.41	58.81±1.07	59.10±0.93	59.93±1.31	61.22±1.67	61.10±1.02	62.84±2.11	62.83±2.33
Contralateral Cortex	38.87±0.88	38.81±0.76	39.15±0.95	38.84±1.35	37.96±1.58	38.46±0.98	38.74±1.07	40.23±1.62
Ipsilateral Septum	51.95±1.70	51.44±1.35	51.83±1.81	51.63±1.32	50.44±1.36	51.65±2.47	53.83±1.99	52.97±2.29
Contralateral Septum	49.86±2.25	53.67±2.06	50.63±1.67	50.98±0.98	53.62±1.67	54.51±1.33	51.90±1.89	54.43±2.56
Ipsilateral Hippocampus	61.78±3.81	59.78±2.69	58.97±2.39	61.16±1.14	62.86±2.78	61.30±2.00	61.94±2.06	64.03±2.72
Contralateral Hippocampus	58.63±2.76	62.98±1.97	63.67±2.05	64.55±2.90	65.77±3.35	64.99±2.25	63.87±1.99	65.77±3.02

Adult male Wistar rats were unilaterally decorticated and immediately received, i.c.v. via minipump, various amounts of GM1 or NGF for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after end of drug administration). Microdissected brain areas were assayed for ChAT activity (nMol Ach/mg protein/hr) as described by Fonnum (1975). Protein content was measured according to the method of Bradford (1976). Data were analyzed using ANOVA and post-hoc Newman-Keuls test ($p<0.05$) on the group means. No significant changes were noted among groups. n= 6 animals/group.

3.1.3 Dose dependent effects of exogenous NGF or GM1 treatment on cortical choline uptake of lesioned animals

Although ChAT is regarded as a good cholinergic marker, the uptake of choline is considered to be the rate limiting step for the synthesis of ACh and thus, is thought to more adequately reflect the functional activity of the cholinergic terminal (reviewed in section 1.3.1). Therefore, the effects of NGF or GM1 treatment on high affinity choline uptake (HACU) were examined. HACU was assayed using synaptosomes prepared from the ipsilateral or contralateral cortices, hippocampi or striati of control unoperated or 30 day post-lesion rats. Lesioned rats had received, beginning immediately after surgery, either vehicle or various concentrations of GM1 or NGF, i.c.v. via an Alzet 2001 osmotic minipump for 7 days. As shown by Figure 3.6, NGF or GM1 treatment caused a dose dependent stimulation of ipsilateral cortical HACU in lesioned rats. ED50 values were, respectively, 1.5 μg/day and 700 μg/day which are close to what was noted for stimulation of cortical ChAT activity. As observed for ChAT activity, these short-term treatments with GM1 or NGF did not affect hippocampal HACU nor HACU in the contralateral cortex (Table 3.2). However, striatal HACU was stimulated in a dose dependent manner by NGF but not GM1 treatment (Figure 3.7). The ED50 for NGF stimulation of striatal HACU was greater than that necessary to augment ChAT activity (ED50 ipsilateral striatum: 3 μg/day). In addition, as was noted for ChAT activity, stimulation of HACU in the contralateral striatum (on infusion side) occurred with lower amounts of NGF (ED50 1.5 μg/day).

3.1.4 In vitro effects of exogenous NGF or GM1 on cortical and striatal choline uptake

The in vitro effects of exogenous NGF or GM1 on cortical and striatal HACU were also examined. Synaptosomes prepared from the ipsilateral cortex or striatum of lesioned rats were preincubated for 5 or 15 minutes with various concentrations of GM1 or NGF, or with vehicle (Krebs buffer + 0.1% Bacitracin and 5 X 10^{-5} M Leupeptin). In vitro, neither NGF nor GM1, at the doses tested, altered cortical or striatal HACU (Figure 3.8). This suggests that these agents do not directly affect the choline transporter.

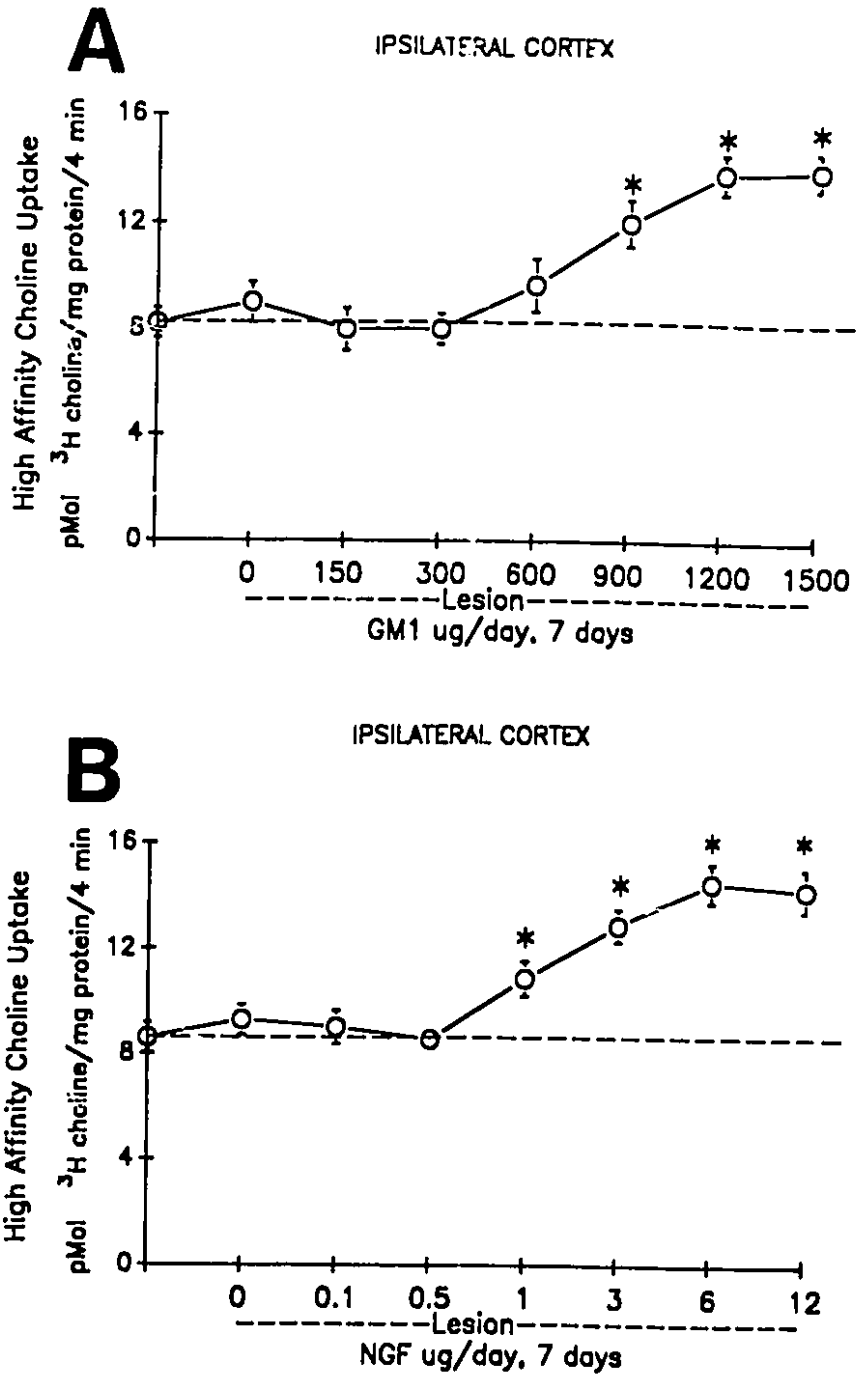


Figure. 3.6 Dose dependent effects of (A) GM1 or (B) NGF on high affinity choline uptake in the ipsilateral cortex of decorticated rats. Animals were cortically lesioned as described in methods and received, i.c.v. via minipump, immediately post-lesion either vehicle [0] (artificial c.s.f. + 0.1 % BSA), GM1 at dosages of 150 to 1500 μg/day or NGF at dosages of 0.1 to 12 μg/day for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatment) and HACU of cortical synaptosomes was determined as described in methods. Protein content was assessed using the method of Bradford (1976). n= 6 animals/group. Values represent difference between mean uptake at 37° and 0° C ± S.E.M.. *$p<0.05$ from control, ANOVA, post-hoc Newman-Keuls' test. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

TABLE 3.2 HIGH AFFINITY [^{3}H] CHOLINE UPTAKE OF HIPPOCAMPUS AND CONTRALATERAL CORTEX FROM DECORTICATED RATS TREATED WITH NGF OR GM1

GROUP	pMol [^{3}H] choline/mg protein/4 min		
	Contralateral Cortex	Ipsilateral Hippocampus	Contralateral Hippocampus
Control			
Lesion + Vehicle	7.7 ± 1.2	11.5 ± 0.6	10.6 ± 0.8
Lesion + GM1, 150 μg/day	8.8 ± 0.9	10.8 ± 1.2	11.4 ± 0.6
Lesion + GM1, 300 μg/day	8.2 ± 0.8	11.8 ± 0.9	11.7 ± 0.6
Lesion + GM1, 600 μg/day	8.4 ± 0.6	11.5 ± 1.4	10.5 ± 0.9
Lesion + GM1, 900 μg/day	8.8 ± 1.2	11.6 ± 1.8	11.3 ± 1.0
Lesion + GM1, 1200 μg/day	8.4 ± 0.9	10.9 ± 1.2	11.8 ± 0.9
Lesion + GM1, 1500 μg/day	8.9 ± 0.8	11.1 ± 0.5	10.7 ± 0.8
Control	9.1 ± 0.6	11.6 ± 1.2	10.8 ± 0.9
Lesion + Vehicle	8.6 ± 0.8	11.2 ± 1.0	11.5 ± 0.9
Lesion + NGF, 0.1 μg/day	9.2 ± 1.4	10.9 ± 1.0	11.7 ± 0.7
Lesion + NGF, 0.5 μg/day	8.6 ± 0.8	10.9 ± 1.0	11.6 ± 1.0
Lesion + NGF, 1 μg/day	9.4 ± 0.9	11.3 ± 1.2	11.1 ± 0.8
Lesion + NGF, 3 μg/day	8.6 ± 0.8	10.6 ± 1.1	10.9 ± 1.0
Lesion + NGF, 6 μg/day	9.2 ± 0.8	11.6 ± 0.7	11.7 ± 0.8
Lesion + NGF, 12 μg/day	8.5 ± 0.6	11.9 ± 1.2	10.9 ± 1.0

Adult male Wistar rats were unilaterally decorticated and immediately received, ICV via minipump, various doses of GM1 or NGF for 7 days. Rats were sacrificed at 30 days post-lesion (ie: 23 days after end of drug treatment). High affinity choline uptake, assayed as described in methods, of synaptosomes from the ipsilateral and contralateral hippocampus as well as the contralateral cortex are shown. Data were analyzed using ANOVA which indicated no significant differences between groups. Numbers represent difference between mean uptake at 37^0 and 0^0 C ± S.E.M. n= 6 animals/group.

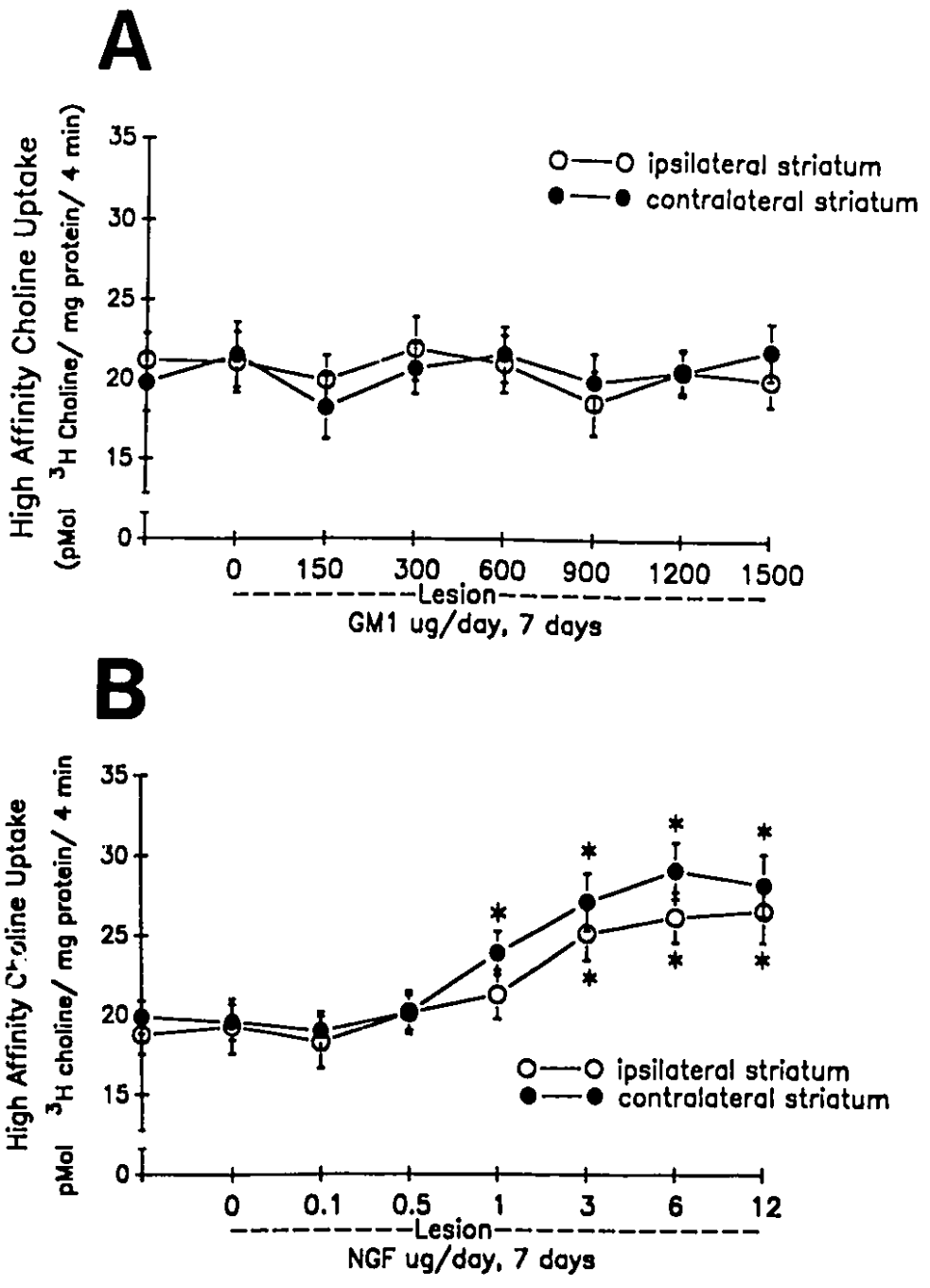


Figure 3.7 Dose dependent effects of (A) GM1 or (B) NGF on high affinity choline uptake of the ipsilateral and contralateral striati of decorticated rats. Animals were cortically lesioned as described in methods and received, i.c.v. via minipump immediately post-lesion, either vehicle [0] (artificial c.s.f. + 0.1 % BSA), GM1 at dosages of 150 to 1500 μg/day or NGF at dosages of 0.1 to 12 μg/day for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatment) and HACU of cortical synaptosomes was determined as described in methods. Protein content was assessed using the method of Bradford (1976). n= 6 animals/group. Numbers represent difference between mean uptake at 37° and 0° C ± S.E.M. *$p<0.05$ from control, ANOVA, post-hoc Newman-Keuls' test. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

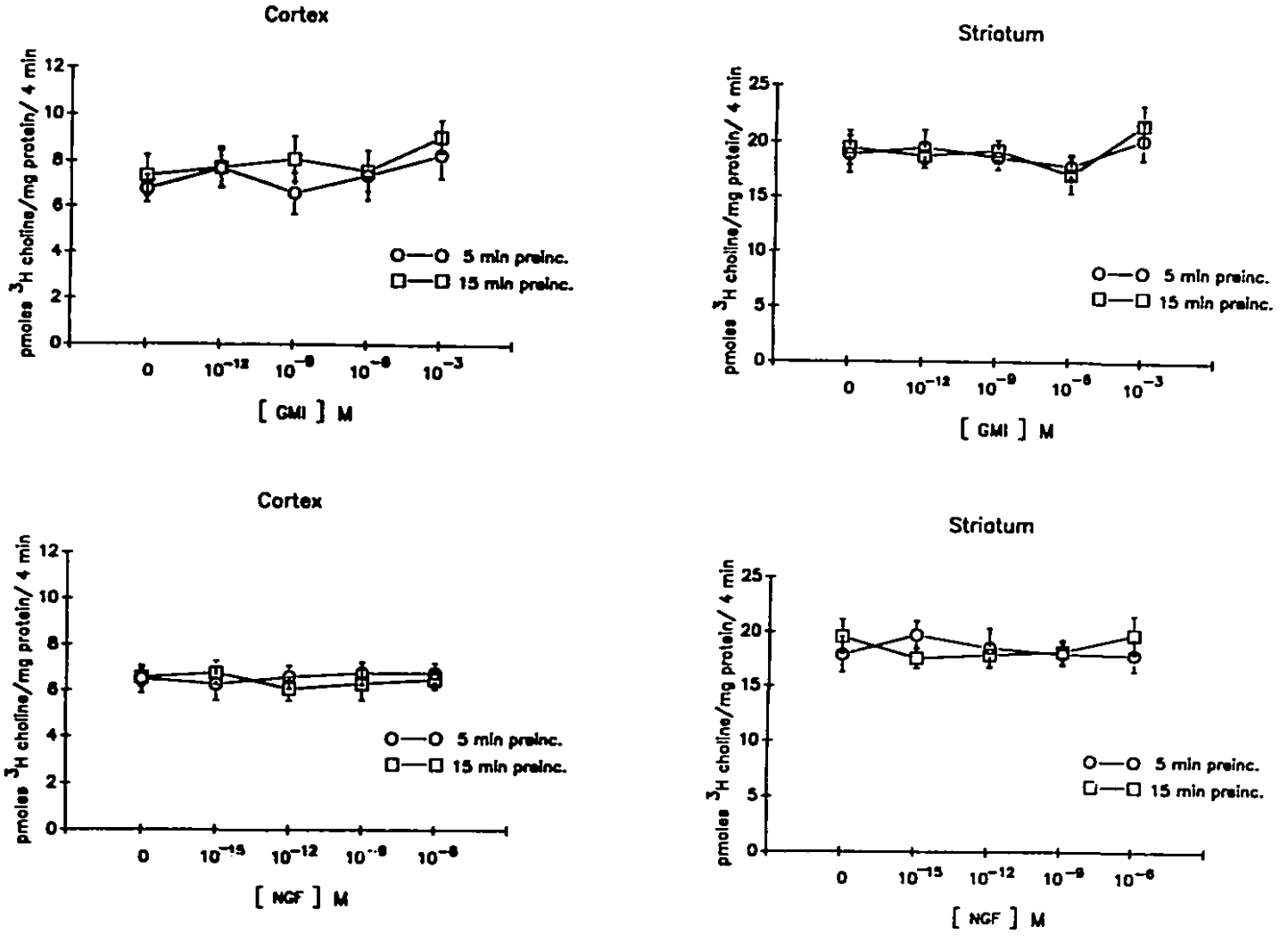


Figure 3.8 In vitro effects of NGF or GM1 on high affinity choline uptake in cortical (A-B) or striatal (C-D) synaptosomes. Synaptosomes from lesioned animals were preincubated for 5 or 15 minutes with vehicle [0], NGF [10^{-6} to 10^{-15} M] or GM1 [10^{-3} to 10^{-12} M] prior to the addition of 0.5 μM [^{3}H] choline. See methods for assay details. Values represent difference between mean uptake at 37° and 0°C of 5-6 separate experiments. Error bars indicate S.E.M.. ANOVA, with post-hoc Newman-Keuls' test on the group means confirmed that there was no significant difference among groups.

3.1.5 Potentiation of NGF effects on ChAT activity and HACU by GM1

As described in the introduction (1.4.10) the molecular mechanisms through which GM1 induces its neuritogenic or neurotrophic effects in vitro or in vivo have yet to be clarified. In vitro studies indicated however, that GM1 could potentiate the actions of neurotrophic agents such as NGF (Ferrari et al., 1983; Leon et al., 1984). In view of evidence which showed that NGF was present in brain (Korsching et al., 1985) and that, following injury, neurotrophic activity in brain extracts increased (Neito-Sampedro et al., 1983), we investigated whether GM1 could potentiate NGF-induced effects in vivo. For this purpose, the effect of GM1 on the NGF dose response curve was studied. Cortically lesioned rats were treated with various concentrations of NGF in combination with a dose of GM1 (150 μg/day) which when given alone did not affect ChAT activity or HACU. Figure 3.9 shows the dose response curve for NGF effects on cortical (Figure 3.9A) and NBM (Figure 3.9B) ChAT activity when co-administered with GM1. To facilitate comparison, the dose response curves for NGF alone (data from Figure 3.4 expressed as percent control) were also plotted. As can be seen, GM1 does not affect potency but does potentiate the maximal efficacy of NGF. ChAT activity in the NBM and cortex of lesioned rats which received both NGF and GM1 was potentiated at each effective NGF dose. Similarly, this dose of GM1 was able to further augment NGF effects on cortical HACU (Figure 3.10). Moreover, if lesioned rats were treated with maximal effective doses of both GM1 (1.5 mg/day, 7 days) and NGF (12 μg/day, 7 days), NBM and cortical ChAT activity at 30 days post-lesion were augmented significantly above that induced by each agent alone (Table 3.3). Similar results were noted for cortical HACU (Figure 3.13). These findings showed, for the first time, that GM1 can potentiate NGF-induced effects on cholinergic markers in the adult CNS in vivo.

In contrast to the NBM and cortex, co-administration of GM1 did not potentiate NGF effects on ChAT activity and HACU in the ipsilateral or contralateral striatum (Figure 3.11).

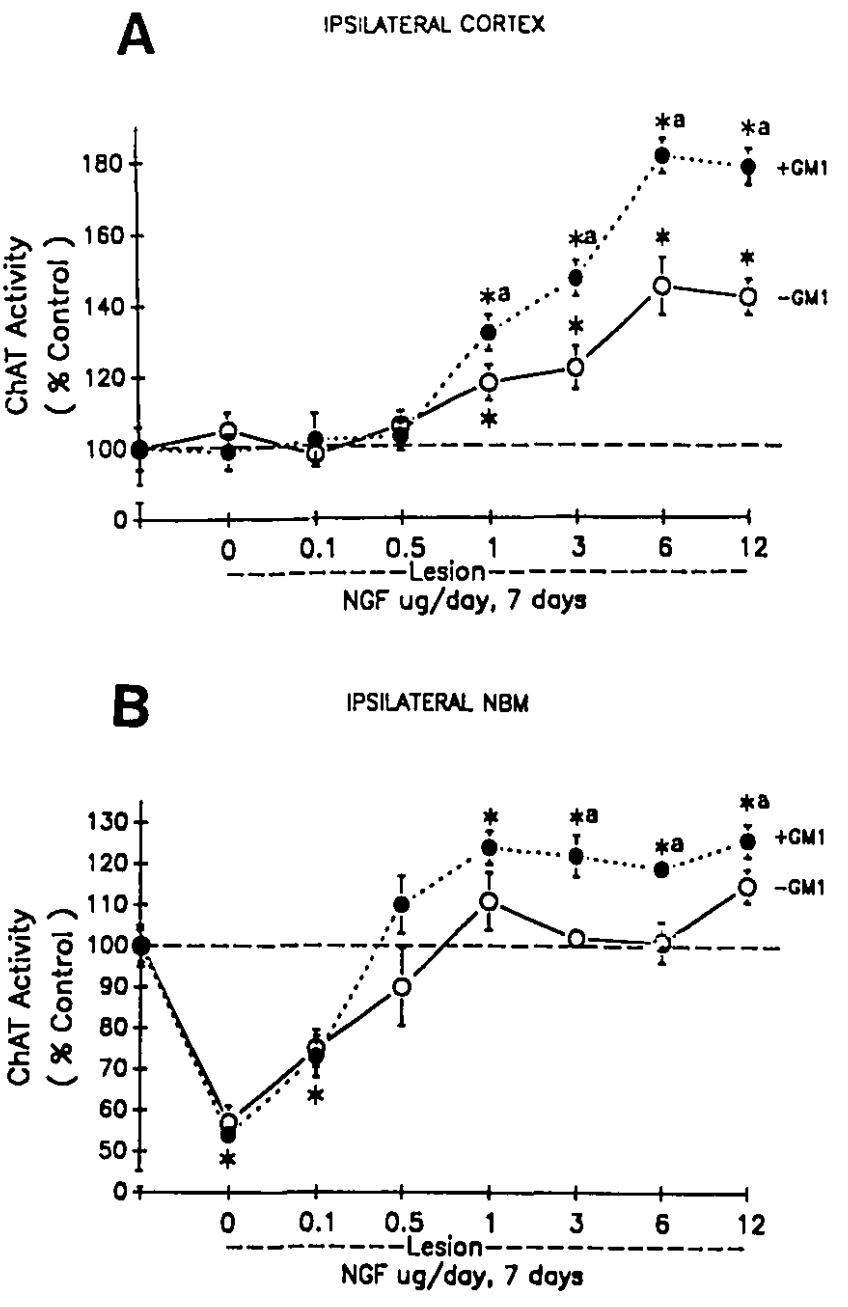


Figure 3.9 Potentiation of NGF-induced effects on (A) cortical and (B) NBM ChAT activity by GM1. Animals were decorticated and received, i.c.v. via minipump, various amounts of NGF in combination with an ineffective dose of GM1 (150 μg/day), beginning immediately post-lesion and continuing for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatments). To facilitate comparison, the dose response curve for NGF treatment alone (from Figure 3.4) is also plotted with the values expressed as percent control. Control values for ChAT activity in the NGF+GM1 experiment were: for the NBM, 58.56 $\pm$ 1.95 nMol ACh/mg protein/hr and for the cortex, 34.53 $\pm$ 0.75 nMol ACh/mg protein/hr. ChAT activity for control animals in the NGF alone treated groups were: for the NBM, 60.44 $\pm$ 3.11 nMol ACh/mg protein/hr and for the cortex, 35.47 $\pm$ 0.80 nMol ACh/mg protein/hr. ChAT activity and protein content were determined according to the methods of Fonnum (1976) and Bradford (1976), respectively. Error bars indicate S.E.M.. *$p<0.05$ from control values, $^{a}p<0.05$ from lesion NGF alone treated groups, ANOVA post-hoc Newman-Keuls' test. n= 6 animals/group. Point on y-axis represents control value. Note that the x-axis is not drawn to scale.

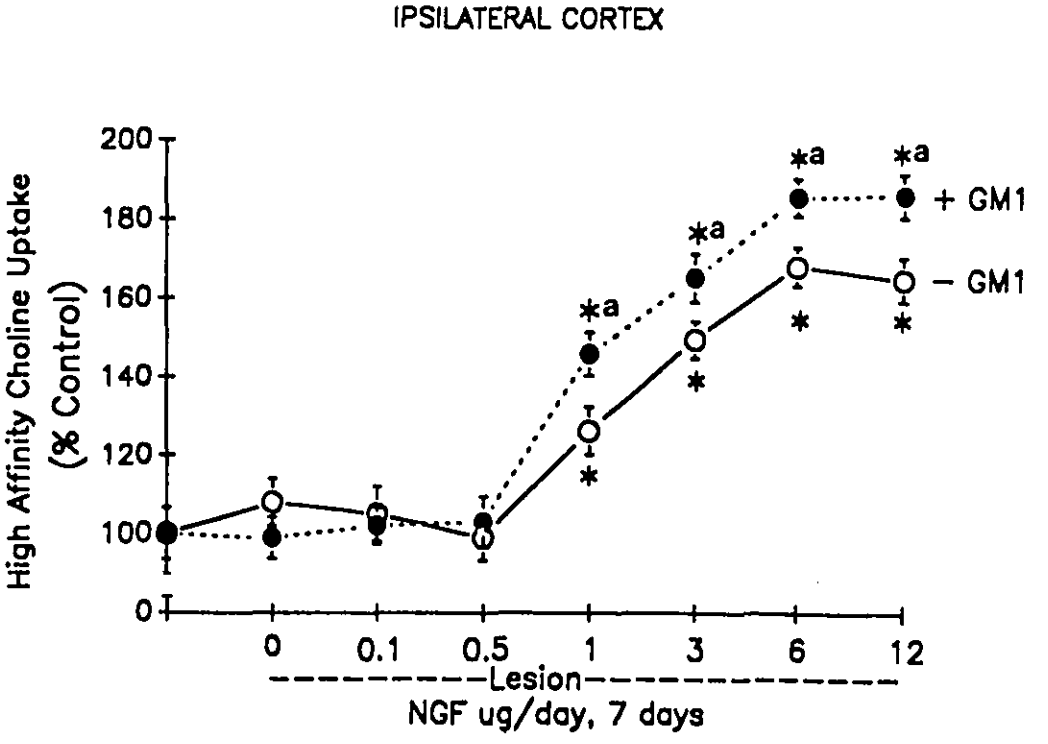


Figure 3.10 Potentiation of NGF-induced effects on cortical high affinity choline uptake by GM1. Animals were decorticated and received, i.c.v. via minipump, various amounts of NGF in combination with an ineffective dose of GM1 (150 μg/day) beginning immediately post-lesion and continuing for 7 days. Rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatments). To facilitate comparison, the dose response curve for NGF treatment alone (from Figure 3.6B) is also plotted with the values expressed as percent control. HACU of cortical synaptosomes was determined as described in methods. Protein content was measured according to Bradford (1976). The control value for HACU in the NGF+GM1 experiment was: 8.73 $\pm$ 0.61 pMol [^{3}H] choline/mg protein/4 min and in the NGF alone treated groups was: 8.62 $\pm$ 0.56 pMol [^{3}H] choline/mg protein/4 min. Error bars indicate S.E.M.. *$p<0.05$ from control values, $^a p<0.05$ from lesion NGF alone treated groups, ANOVA post-hoc Newman-Keuls' tests. n= 6 animals/group. Point on y-axis represents control value. Note that x-axis is not drawn to scale.

TABLE 3.3 EFFECT OF MAXIMAL GM1 AND NGF DOSES ON NBM AND CORTICAL ChAT ACTIVITY

		IPSILATERAL NBM		*IPSILATERAL CORTEX*	
GROUP	n	ChAT ACTIVITY	% CONTROL	ChAT ACTIVITY	% CONTROL
Control	6	57.67 ± 3.86	-	35.81 ± 2.39	-
Lesion + Vehicle	6	31.16 ± 3.17	54*	35.85 ± 1.74	100
Lesion + GM1	5	61.94 ± 6.55	107	50.70 ± 2.44	142*
Lesion + NGF	5	50.94 ± 3.75	88	47.63 ± 3.12	132*
Lesion + NGF/GM1	5	69.41 ± 1.06	120*§	84.82 ± 10.42	237*§

Adult male Wistar rats were unilaterally decorticated and immediately received GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days, i.c.v. via minipump. ChAT activity in brain tissues was assessed at 30 days post-lesion. Values for ChAT activity are mean ± S.E.M. and are expressed as nMol ACh/mg protein/hr. n= number of rats per group. *$p<0.05$ from Control, § $p<0.05$ from lesion + GM1 or lesion + NGF treated groups. ANOVA, post-hoc Newman-Keuls' test.

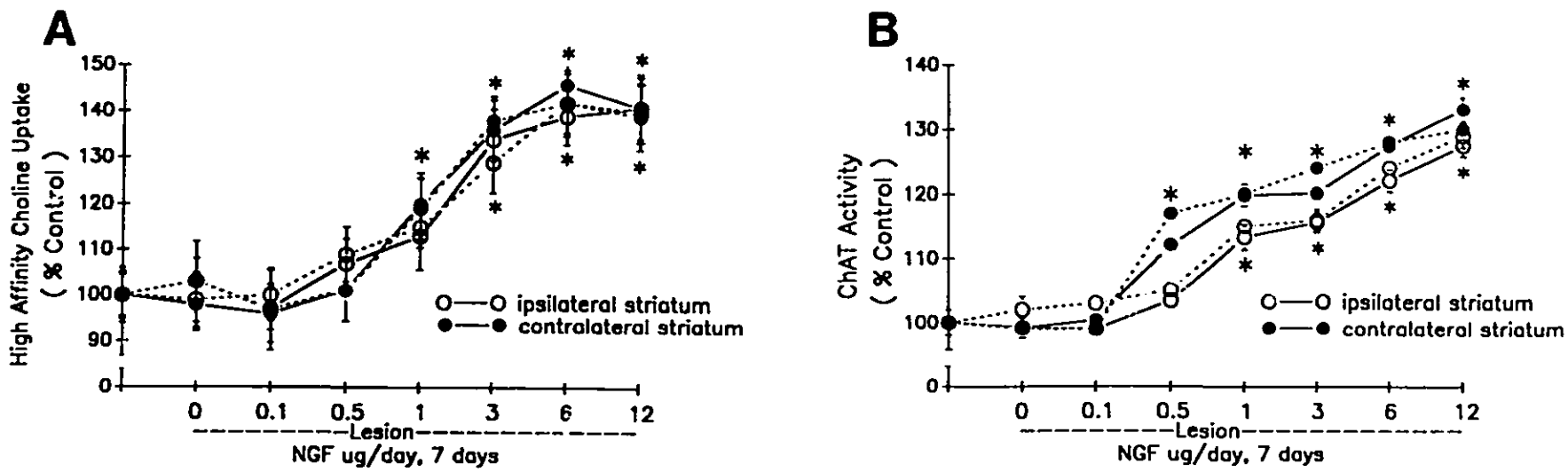


Figure 3.11 Lack of potentiation of NGF effects on striatal (A) high affinity choline uptake and (B) ChAT activity by GM1. Adult rats were decorticated and received, i.c.v. via minipump, various amounts of NGF in combination with an ineffective dose of GM1 (150 μg/day) beginning immediately post-lesion and continuing for 7 days. Animals were sacrificed 30 days post-lesion (ie: 23 days after the end of drug treatments). To facilitate comparison, the dose response curves for NGF treatment alone (solid lines) (data from Figures 3.5B and 3.7B) are also plotted with the values expressed as percent control. HACU of striatal synaptosomes was determined as described in methods. ChAT activity and protein content were determined according to the methods of Fonnum (1975) and Bradford (1976), respectively. Control values for striatal HACU in the NGF+GM1 experiment were: ipsilateral: 20.52 ± 1.25 pMol [^{3}H] choline/mg protein/4 min; contralateral: 21.20 ± 1.43 pMol [^{3}H] choline/mg protein/4 min. For the NGF alone treated groups, control values were: ipsilateral: 18.76 ± 1.21 pMol [^{3}H] choline/mg protein/4 min; contralateral: 19.85 ± 1.03 pMol [^{3}H] choline/mg protein/4 min. Control values for ChAT activity were: for the NGF+GM1 treated groups, ipsilateral: 138.92 ± 1.25 nMol ACh/mg protein/hr; contralateral: 140.54 ± 1.15 nMol ACh/mg protein/hr. For the NGF alone treated groups, control values were: ipsilateral: 141.16 ± 0.98 nMol ACh/mg protein/hr; contralateral: 140.98 ± 1.24 nMol ACh/mg protein/hr. Error bars indicate S.E.M.. *$p<0.05$ from control values, ANOVA post-hoc Newman-Keuls tests. n= 6 animals/group. Point on y-axis represents control value. Note that x-axis is not drawn to scale.

3.1.6 Effects of exogenous NGF and/or GM1 on "soluble" or "membrane bound" forms of ChAT

Extraction of ChAT from brain has revealed that multiple forms of this enzyme exist (reviewed in section 1.3.1). The largest form appears to be soluble, but ChAT associated with membrane fractions has also been shown. The distinct involvement of these various forms of ChAT in ACh synthesis is not yet clear but it is thought that the membrane bound form by virtue of its proximity to the choline transporter, may play a particular role in ACh synthesis. To examine whether NGF or GM1 treatment differentially altered "membrane bound" or "soluble" forms of ChAT, lesioned animals were treated beginning immediately post-lesion, i.c.v. via minipump, with maximal doses of GM1 (1.5 mg/day) and/or NGF(12 μg/day) for 7 days and were sacrificed at 30 days post-lesion. In control unoperated animals both the cortex and striatum showed higher levels of "soluble" ChAT as compared to the "membrane bound" form (Table 3.4). This is in line with what was previously reported for the rat (Benishen and Carroll, 1983). The cortical lesions did not cause disparate alterations of either enzyme forms in these brain areas (Table 3.4). Exogenous GM1 treatment stimulated cortical "soluble" ChAT activity by 37% and similarly the "membrane bound" form by 35%. Comparable increases in "soluble" (40% above control) and "membrane bound" forms (38% above control) were also noted in lesioned rats which received NGF treatment (Table 3.4). Furthermore, although co-treatment of decorticated animals with both NGF and GM1 further augmented the activity of both ChAT forms, the percent increase for both were again comparable (Table 3.4).

"Soluble" and "membrane" bound forms of ChAT in the striatum were also not differentially affected by the lesion or GM1 treatment. As well, lesioned rats which received NGF or NGF/GM1 treatment showed equivalent increases in both forms of striatal ChAT (Table 3.4).

3.1.8 NGF and/or GM1 effects on cortical choline uptake kinetics

The effects of NGF and/or GM1 treatment on choline uptake were further studied by assessing whether these agents could distinctly alter its kinetic constants. For this purpose,

cortical or striatal synaptosomes from control, lesion vehicle or lesioned animals which received, i.c.v. via minipump, maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day) for 7 days were incubated with concentrations of [^{3}H] choline ranging from 0.1-1 μM, to assess high affinity, and 2-100 μM, to examine low affinity uptake. K_m and V_{max} values were calculated from double reciprocal plots of velocity versus substrate concentration according to Lineweaver-Burk (1934). V_{max} values for choline uptake, in unoperated rats, which presumably reflect the number of uptake sites, were lower in cortex as compared to the striatum (Table 3.5). This is in line with what has previously been reported for the rat brain (Yamamura and Snyder, 1973). No significant differences were noted for HACU K_m values of these brain areas, but greater Km values for low affinity choline uptake were noted for the striatum as compared to cortex. Thirty days post-lesion vehicle treated rats showed no alterations in K_m or V_{max} values in the cortex or striatum when compared to their control counterparts (Table 3.5). Short-term (7 days) treatment of cortically lesioned rats with either GM1 or NGF caused an increase in V_{max} but did not affect the K_m of high affinity cortical choline uptake (Figure 3.12A-B, Table 3.5). Lesioned animals which received both NGF and GM1 showed increases in cortical HACU V_{max} which were significantly greater than that induced by each agent alone (Table 3.5). Effects on the kinetic parameters of the low affinity transporter were less marked. Although a trend towards an increase in V_{max} was noted, this failed to reach significance in any of the treatment cases. Kinetic constants of striatal choline uptake were not affected by the GM1 treatment (Figure 3.12 C-D, Table 3.5). However, NGF treatment caused a significant increase in V_{max} but did not affect the K_m of striatal HACU. Decorticated animals which received both NGF and GM1 showed no further alterations in striatal high or low affinity choline uptake kinetic constants (Figure 3.12 C-D, Table 3.5).

3.1.9 Time dependence of NGF and/or GM1 effects on ChAT activity and HACU

The time course of the lesion induced changes and effects of trophic agents were also studied in these brain areas. For this purpose, adult animals were unilaterally decorticated and received either vehicle or maximal doses of NGF (12 μg/day) or GM1 (1.5 mg/day) for

a period of 7 days. Animals were sacrificed at either 1, 5, 15 or 30 days post-lesion. An increase in ChAT activity was noted in the ipsilateral NBM at 1 day post-lesion and a time dependent decrease in ChAT activity subsequently occurred (Figure 3.13C). GM1 and/or NGF treatment attenuated the 1 day post-lesion increase in NBM ChAT activity as well as the decreases observed at later times. ChAT activity in the ipsilateral NBM of lesioned rats which received both NGF and GM1 was significantly increased above control levels, and that induced by each agent alone, at both 15 and 30 days post-lesion.

A time dependent stimulation of cortical ChAT activity and cortical HACU was also noted. As was observed for the NBM, ChAT activity in the cortex was also found to be significantly increased above control values on post-lesion day 1 (Figure 3.13A). By contrast, cortical HACU in 1 day post-lesion rats was not significantly different from control values. At subsequent post-lesion times cortical ChAT activity and HACU in vehicle treated animals did not vary from control values (Figure 3.13A, 3.13B). Cortical ChAT activity was stimulated, significantly above control values, at both 15 and 30 days post-lesion in GM1 treated rats. Increases in HACU at these time points also occurred but, were significant only at 30 days post-lesion in GM1 treated lesioned animals. The time dependent effects of NGF treatment on cortical ChAT activity and HACU matched what was observed for GM1 treated lesioned rats (Figure 3.13A, 3.13B). Co-treatment of lesioned rats with both NGF and GM1 did not expedite the increase of basalo-cortical cholinergic markers. However, a significantly greater induction of cortical ChAT activity and cortical HACU was noted at these post-lesion times when compared to that occurring in lesioned rats which received either agent alone (Figure 3.13).

In striata from decorticated rats, ChAT activity and HACU remained at control levels at all post-lesion times analyzed (Figures 3.14A and 3.14B). Lesioned rats which received GM1 treatment showed no change in the activity of ChAT or HACU at any time examined. By contrast, an increase in striatal ChAT activity was noted by post-lesion day 5 in NGF and NGF/GM1 treated lesioned animals (Figure 3.14B). HACU was similarly affected (Figure 3.14A).

TABLE 3.4 EFFECTS OF NGF AND/OR GM1 TREATMENT ON THE ACTIVITY OF "SOLUBLE" OR "MEMBRANE BOUND" FORMS OF ChAT IN THE DECORTICATED RAT BRAIN

BRAIN AREA and GROUP	ChAT ACTIVITY (nMol ACh/mg protein/hr)	
	"SOLUBLE"	"MEMBRANE BOUND"
CORTEX		
Control	22.2 ± 0.8	7.4 ± 0.5
Lesion + Vehicle	20.6 ± 1.0	7.7 ± 0.6
Lesion + GM1	30.5 ± 1.2*	10.0 ± 0.5*
Lesion + NGF	31.1 ± 0.9*	10.3 ± 0.7*
Lesion + NGF/GM1	46.4 ± 1.1*a	14.6 ± 1.1*a
STRIATUM		
Control	74.9 ± 1.6	21.3 ± 1.6
Lesion + Vehicle	72.9 ± 1.1	24.4 ± 1.2
Lesion + GM1	72.1 ± 1.9	20.7 ± 1.0
Lesion + NGF	104.9 ± 2.6*	32.1 ± 2.1*
Lesion + NGF/GM1	101.3 ± 2.3*	32.7 ± 2.0*

Decorticated animals received immediately post-lesion either vehicle (artificial c.s.f. + 0.1% BSA) or maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), i.c.v. via minipump, for 7 days. The rats were sacrificed 30 days post-lesion (ie: 23 days after the end of drug administration). "Soluble" and "membrane bound" associated ChAT activity were extracted from microdissected ipsilateral cortical and striatal tissues as described in methods. ChAT activity and protein content were determined according to the methods of Fonnum (1975) and Bradford (1976), respectively. Values are mean ± S.E.M. from 3 separate experiments. *$p < 0.05$ from control, $^{a}p < 0.05$ from lesion + GM1 and Lesion + NGF treated groups, ANOVA post-hoc Newman-Keuls' test.

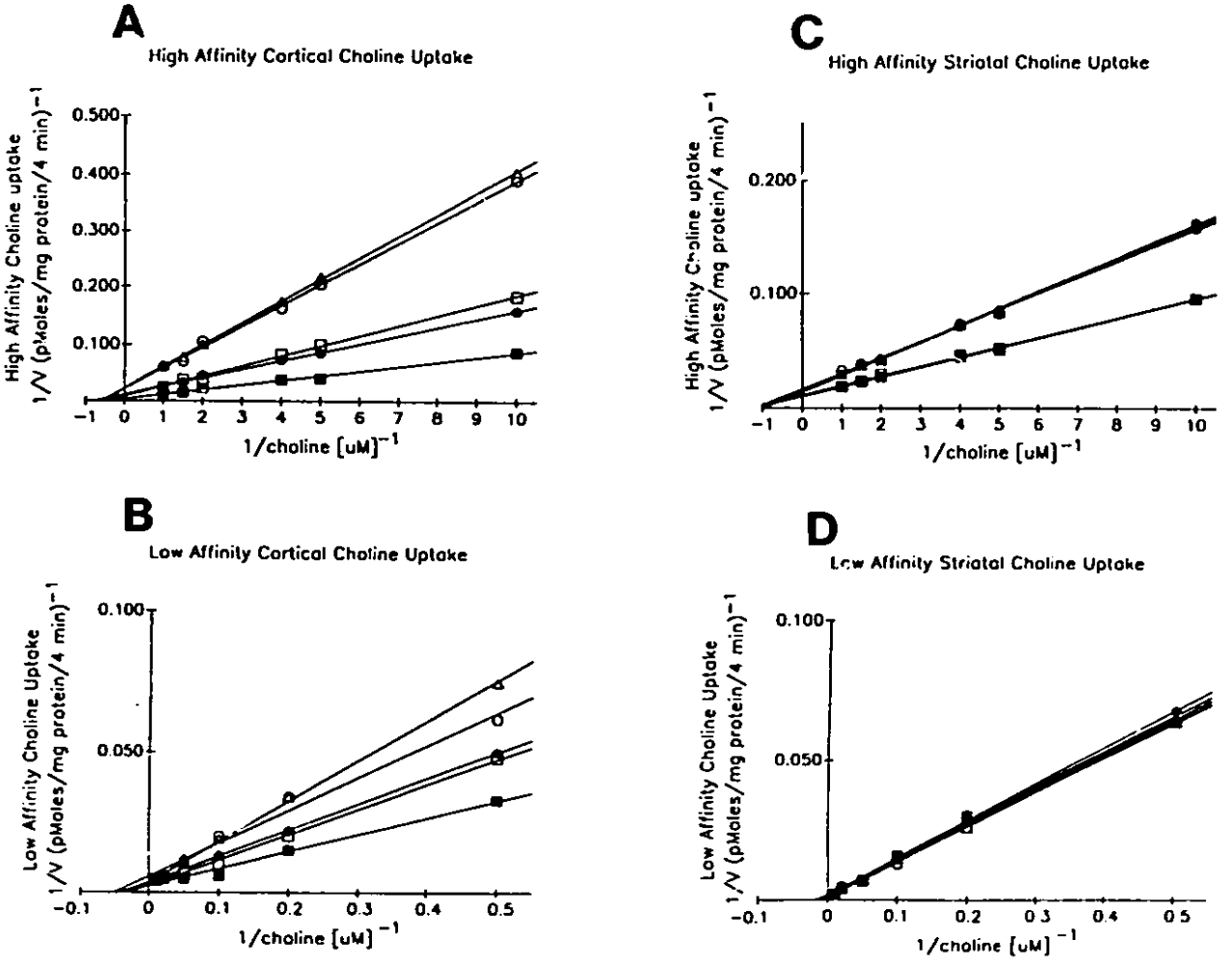


Figure 3.12 Lineweaver-Burk plots of the kinetics of [^{3}H] choline uptake by cortical (A-B) and striatal (C-D) synaptosomes from control (○—○), lesioned vehicle (△—△), lesioned GM1 (□—□) lesioned NGF (●—●) and lesioned NGF+GM1 treated groups (■—■). Decorticated rats received vehicle or maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), i.c.v. via minipump, beginning immediately post lesion and continuing for 7 days. Animals were sacrificed 30 days post-lesion. [^{3}H] Choline concentrations were varied from 0.1 -to- 1 μM and 2 -to- 100 μM to assess high and low affinity uptake, respectively. Representative plots taken from one experiment are shown. See Table 3.5 for kinetic constants.

GROUP and BRAIN AREA	EFFECT OF DECORTICATION, GM1 AND/OR NGF TREATMENT ON KINETIC CONSTANTS OF [^{3}H] CHOLINE UPTAKE IN CORTICAL AND STRIATAL SYNAPTOSOMES			
	HIGH AFFINITY		LOW AFFINITY	
CORTEX	K_m (μM)	V_{max} (pMol/mg protein/4 min)	K_m (μM)	V_{max} (pMol/mg protein/4 min)
Control	1.91 ± 0.15	44.5 ± 1.6	32.8 ± 4.9	311 ± 56
Lesion + Vehicle	1.83 ± 0.20	46.0 ± 3.1	33.4 ± 4.9	305 ± 39
Lesion + GM1	1.92 ± 0.37	80.4 ± 4.4*	27.6 ± 1.6	283 ± 18
Lesion + NGF	2.04 ± 0.25	87.6 ± 10.0*	28.3 ± 3.1	364 ± 22
Lesion + NGF/GM1	1.60 ± 0.23	130.6 ± 11.2*a	32.0 ± 5.7	412 ± 36
STRIATUM				
Control	1.26 ± 0.09	73.0 ± 1.8	79.0 ± 11.6	669 ± 74
Lesion + Vehicle	1.30 ± 0.22	77.0 ± 7.8	67.1 ± 9.8	583 ± 94
Lesion + GM1	1.26 ± 0.18	75.0 ± 6.4	94.0 ± 20.7	778 ± 168
Lesion + NGF	1.38 ± 0.02	124.9 ± 2.9*	102.8 ± 17.4	737 ± 169
Lesion + NGF/GM1	1.34 ± 0.08	122.3 ± 8.1*	84.6 ± 18.5	851 ± 140

Table 3.5: Animals were decorticated as described in methods and received either vehicle (artificial c.s.f. + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days, i.c.v. via minipump, beginning immediately post-lesion. Rats were sacrificed at 30 days post-lesion (ie: 23 days after the end of drug administration). Synaptosomes isolated from the ipsilateral cortex or striatum of these animals were incubated with [^{3}H] choline concentrations ranging from 0.1 μM -to- 1 μM or 2 μM -to- 100 μM, to assess high and low affinity uptake, respectively. K_m and V_{max} values were obtained from Lineweaver-Burk plots and represent mean ± S.E.M. of 3-4 separate experiments. *$p<0.05$ from control, $^a p<0.05$ from lesioned GM1 and lesioned NGF treated groups, ANOVA, post-hoc Newman-Keuls test.

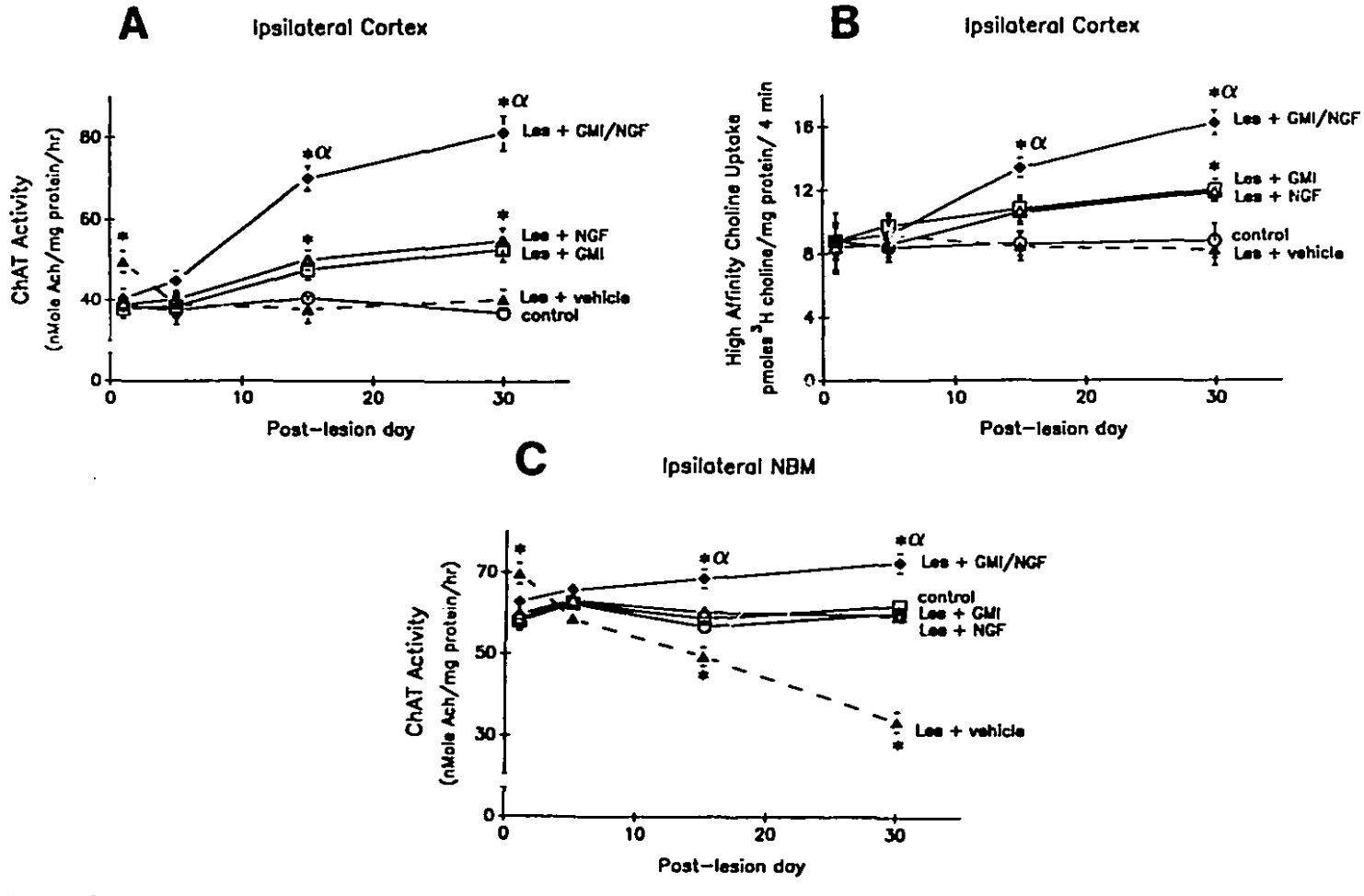


Figure 3.13 Time dependent effects of NGF and/or GM1 treatment on (A) ChAT activity and (B) high affinity choline uptake in the ipsilateral cortex and, (C) ChAT activity in the ipsilateral NBM of lesioned rats. Decorticated rats received maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), i.c.v. via minipump, beginning immediately post-lesion and continuing for 7 days. Animals were sacrificed at 1, 5, 15 or 30 days post-lesion. High affinity choline uptake was determined as described in methods, ChAT activity and protein content were assayed according to Fonnum (1975) and Bradford (1976), respectively. n= 5-9 animals/group. Error bars represent S.E.M.. *$p<0.05$ from control, α $p<0.05$ from lesion NGF and lesion GM1 treated animals, ANOVA, post-hoc Newman-Keuls test.

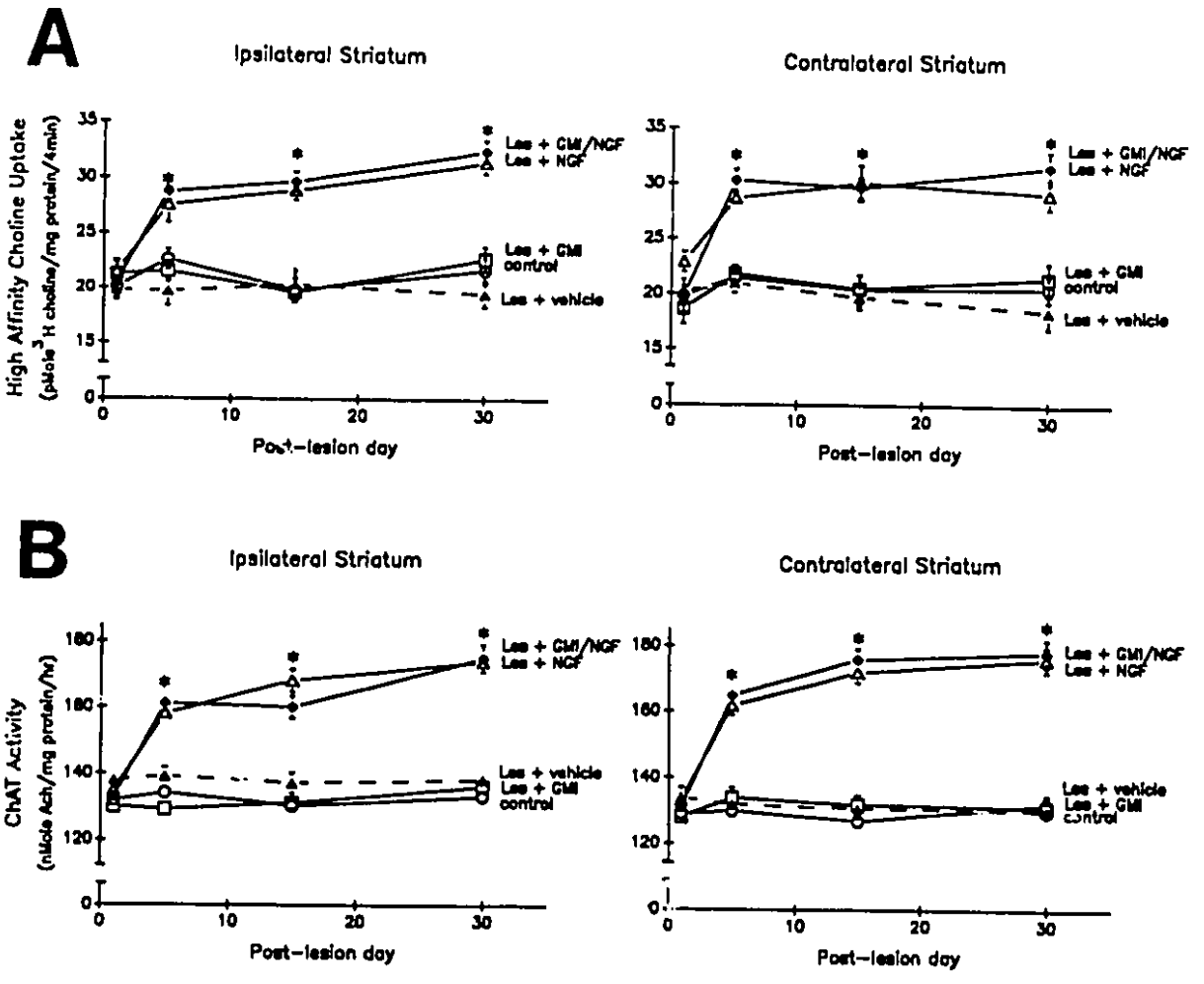


Figure 3.14 Time dependent effects of NGF and/or GM1 treatment on striatal (A) high affinity [^{3}H] choline uptake and (B) ChAT activity. Decorticated rats received maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), i.c.v. via minipump, beginning immediately post-lesion and continuing for 7 days. Animals were sacrificed at 1, 5, 15 or 30 days post-lesion. High affinity choline uptake was determined as described in methods, ChAT activity and protein content were assayed according to Fonnum (1975) and Bradford (1976), respectively. n= 5-9 animals/group. Error bars represent S.E.M.. *$p < 0.05$ from control, ANOVA, post-hoc Newman-Keuls' test.

3.1.9 Lack of effect of NGF and/or GM1 treatment on GAD activity in decorticated rats

To assess the possible effects of the lesion or trophic factor treatment on GABAergic function, GAD activity was measured in ipsilateral and contralateral microdissected brain samples from 1, 5, 15 and 30 days post-lesion animals, which received immediately after decortication, i.c.v. via minipump, either vehicle or maximal doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day) for 7 days. GAD activity was not altered by the lesion in any of the brain areas studied at these post-lesion times (Table 3.6). Moreover, NGF and/or GM1 treatment failed to significantly alter the activity of GAD in the brain areas examined (Table 3.6).

3.1.10 Necessity for early administration of GM1, but not NGF, to attenuate deficits in NBM ChAT activity

The effects of short-term (7 days) versus chronic (30 days) administration of maximal doses of NGF (1 μg/day) and/or GM1 (900 μg/day) on NBM and cortical ChAT activity and cortical HACU at 30 days post-lesion were also examined. As shown by Figures 3.15 A-D and 3.16 A-D a 7 day or 30 day treatment with GM1 or NGF, when initiated immediately post-lesion, maintained NBM ChAT activity and augmented cortical ChAT activity to an equal degree. The percent increase in ChAT activity was for the NBM: *7 and 30 day treatment with GM1, respectively*, 105 ± 5; 107 ± 6, *7 and 30 day treatment with NGF, respectively*: 102 ± 5; 110 ± 5, and for cortex: *7 and 30 day treatment with GM1, respectively*: 133 ± 6; 139 ± 6; *7 and 30 day treatment with NGF, respectively*: 136 ± 7; 139 ± 8. Differences were noted however, in the treatment time onset necessary to induce these effects. If a short term treatment paradigm was employed the onset of GM1 administration could be delayed no more than 1 day for full preservation of NBM ChAT activity at 30 days post-lesion (Figure 3.15B). A partial protective effect resulted if this treatment was delayed for 2 days (Figure 3.15B). By contrast, with a continuous treatment paradigm, lesion-induced deficits in NBM ChAT activity were partially attenuated by GM1 after a 4 day delay in treatment time onset (Figure 3.15D). On the other hand, NGF treatment could be delayed up to 6 (short-term treatment) or 14 (chronic treatment) days post-

lesion and still prevent the decrease in NBM ChAT activity noted on post-lesion day 30 (Figure 3.16B, 3.16D). However, a 14 day delay in NGF treatment onset failed to stimulate ChAT activity in the remaining cortex (Figure 3.16A). Initiating NGF treatment at 30 days post-lesion and continuing treatment for 2 weeks did not attenuate lesion induced deficits in NBM ChAT activity nor was the activity of this enzyme stimulated in the remaining ipsilateral cortex. However, if treatment was continued for 4 weeks a partial recovery in NBM ChAT activity occurred. Alterations in cortical HACU following these various treatment paradigms followed what was noted for cortical ChAT activity (Table 3.7 A-B).

3.1.11 Lack of effect of GM1 treatment on NGF binding to cortical or striatal membranes

To investigate whether the in vivo administration of GM1 could affect the density or affinity of cortical or striatal NGF binding sites, animals were cortically lesioned and immediately received, i.c.v. via minipump, vehicle or maximal (1.5 mg/day) doses of GM1 for 7 days. Animals were sacrificed at 1, 5, 15 and 30 days post-lesion and specific binding of ^{125}I-NGF to cortical and striatal membranes was determined at these post-lesion times. Scatchard analysis of the binding data from cortical membranes of control unoperated animals, done using computer assisted nonlinear regression (EBDA-LIGAND programs, McPherson, 1985), revealed one NGF binding site with a Kd of 5.0 $\pm$ 0.3 nMole and Bmax of 85 $\pm$ 4 fmole/mg protein (Table 3.7). By contrast, Scatchard analysis of ^{125}I-NGF saturation binding data for striatal membranes showed a curvilinear plot. The data was best fit, using the LIGAND program, to a two site model. Kd values of 0.13 $\pm$ 0.01 and 23 $\pm$ 3 nMol, and Bmax values of 1.7 $\pm$ 0.2 and 67 $\pm$ 7 fmol/mg protein were obtained for striata from unoperated control rats. Neither the Kd nor the Bmax of NGF binding to cortical or striatal membranes were altered, at any of the post-lesion times examined, in lesioned vehicle or GM1 treated rats (Figure 3.17, Table 3.8; Figure 3.18, Table 3.9).

TABLE 3.6 **GAD ACTIVITY (nMole $^{14}CO_2$/mg protein/hr)**

	NBM	SEPTUM	STRIATUM	HIPPOCAMPUS	CORTEX
POST-LESION DAY 1					
Control	220 ± 22	200 ± 16	454 ± 20	140 ± 11	280 ± 15
Lesion + Vehicle	235 ± 18	208 ± 13	472 ± 19	143 ± 10	295 ± 12
Lesion + GM1	226 ± 24	211 ± 15	449 ± 16	140 ± 15	276 ± 10
Lesion + NGF	228 ± 24	216 ± 11	458 ± 22	143 ± 14	282 ± 20
Lesion + NGF/GM1	219 ± 19	217 ± 17	459 ± 21	139 ± 11	285 ± 18
POST-LESION DAY 5					
Control	210 ± 28	198 ± 10	449 ± 10	144 ± 8	275 ± 8
Lesion + Vehicle	225 ± 15	207 ± 9	476 ± 20	148 ± 7	298 ± 9
Lesion + GM1	218 ± 14	215 ± 11	452 ± 15	150 ± 10	290 ± 10
Lesion + NGF	220 ± 12	212 ± 7	456 ± 14	143 ± 9	290 ± 10
Lesion + NGF/GM1	222 ± 11	215 ± 11	455 ± 13	146 ± 8	291 ± 7
POST-LESION DAY 15					
Control	215 ± 18	203 ± 7	452 ± 19	139 ± 7	283 ± 11
Lesion + Vehicle	224 ± 20	212 ± 9	486 ± 16	142 ± 6	296 ± 6
Lesion + GM1	220 ± 10	209 ± 9	461 ± 12	146 ± 10	277 ± 8
Lesion + NGF	225 ± 15	214 ± 10	459 ± 14	138 ± 9	277 ± 8
Lesion + NGF/GM1	227 ± 13	222 ± 11	458 ± 21	144 ± 5	282 ± 6
POST-LESION DAY 30					
Control	219 ± 18	213 ± 11	455 ± 17	145 ± 5	274 ± 12
Lesion + Vehicle	226 ± 15	207 ± 7	488 ± 12	143 ± 6	280 ± 9
Lesion + GM1	229 ± 13	209 ± 8	472 ± 11	139 ± 10	275 ± 7
Lesion + NGF	215 ± 21	198 ± 10	479 ± 9	148 ± 9	272 ± 6
Lesion + NGF/GM1	229 ± 16	214 ± 8	474 ± 10	147 ± 7	284 ± 8

Animals were decorticated as described in methods and received either vehicle (artificial c.s.f. + 0.1% BSA) or GM1 (1.5 mg/day) and/or NGF (12 μg/day), i.c.v. via minipump, beginning immediately post-lesion and continuing for 7 days. Rats were sacrificed at the post-lesion times indicated. Microdissected brain areas were assayed for GAD activity essentially as described by Atterwill and coworkers (1981). Protein content was assessed using the method described by Bradford (1976). Values shown are for ipsilateral brain areas. Contralateral brain areas were similarly unaffected. ANOVA and post-hoc Newman-Keuls' test on the group means showed no significant differences between groups.

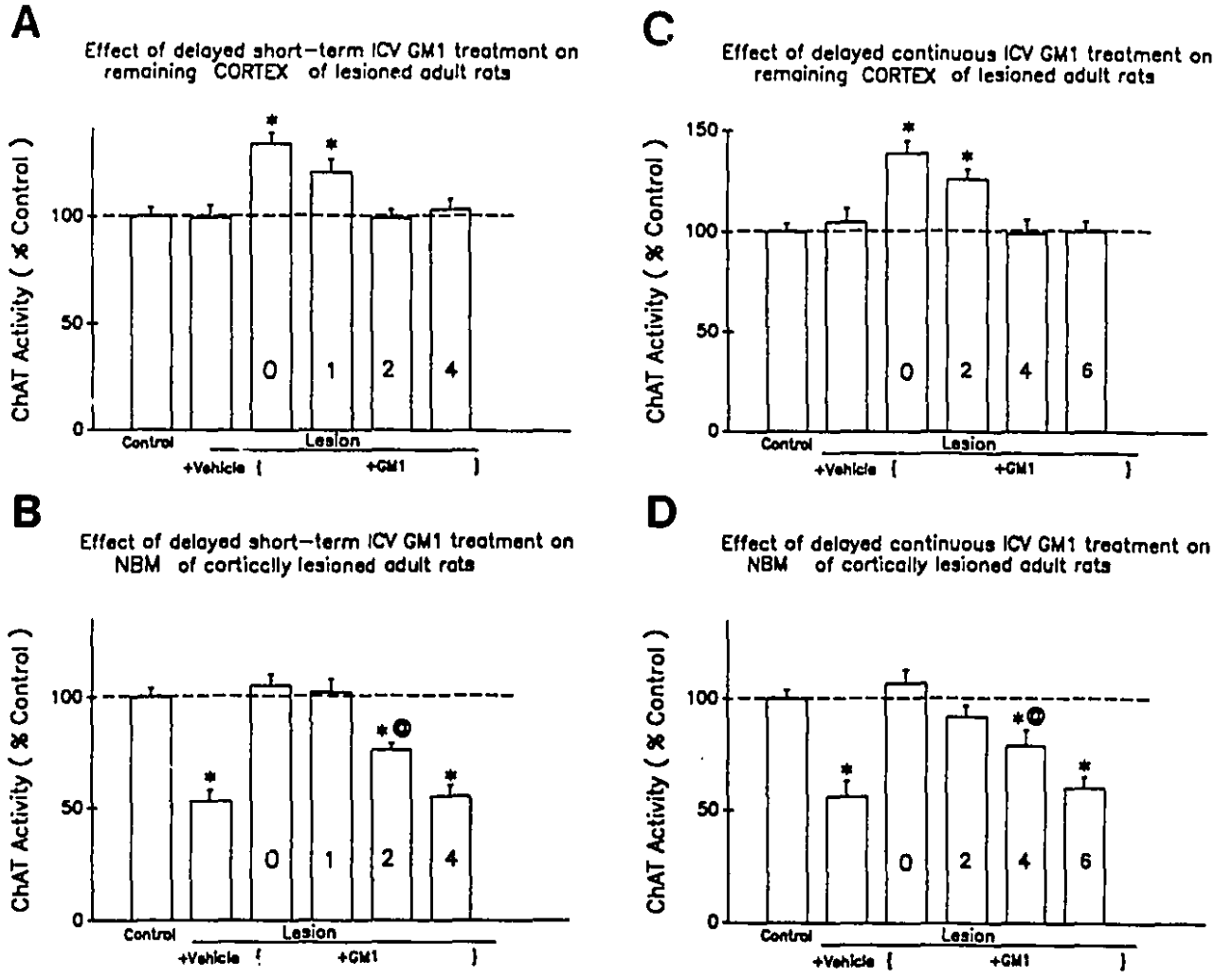


Figure 3.15 Effect of short-term (A-B) or chronic (C-D) GM1 treatment of decorticated rats. Animals were decorticated and received a short-term (7 days) or chronic (30 days) treatment with maximal doses of GM1 (900 μg/day), i.c.v. via minipump, beginning immediately (0) post-lesion or after a 1, 2, 4 or 6 day delay as indicated within bar graphs. *$p<0.05$ from control, @$p<0.05$ from lesion vehicle treated group, ANOVA post-hoc Newman-Keuls test. n= 5-9 animals/group. Control values of ChAT activity were (expressed as mean nMol ACh/ mg protein/hr $\pm$ S.E.M.), for experiment (A-B) NBM: 59.32 $\pm$ 1.55, cortex: 37.17 $\pm$ 0.98 and for experiment (C-D) NBM: 57.97 $\pm$ 1.55, cortex: 40.10 $\pm$ 1.11.

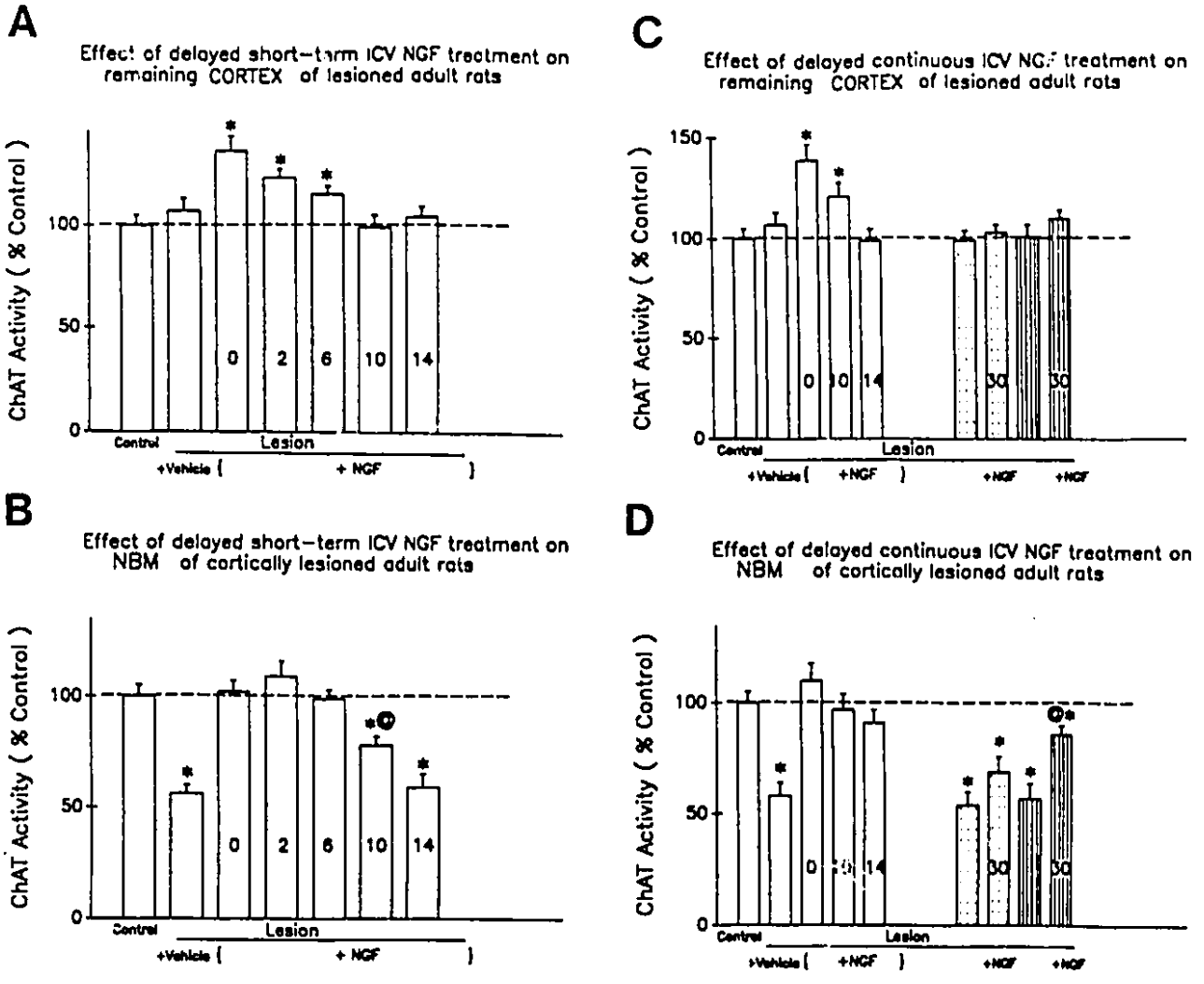


Figure 3.16 Effect of short-term (A-B) or chronic (C-D) NGF treatment on NBM and cortical ChAT activity of lesioned adult rats. Animals were decorticated and received short-term (7 days) or chronic (30 days) treatment with maximal doses of NGF (1 μg/day), i.c.v. via minipump, beginning immediately (0) post-lesion or after a 2, 6, 10 or 14 day delay as indicated within bar graphs. Dotted bar graphs represent decorticated animals treated with vehicle or NGF for 2 weeks after a 30 day delay in treatment onset. Bar graphs with vertical lines represent decorticated animals which received vehicle or NGF treatment for 30 days following a 30 day delay in treatment time onset. *$p < 0.05$ from control, @$p < 0.05$ from respective lesion vehicle treated group, n= 5-9 animals/group. ANOVA post-hoc Newman-Keuls test. Control values of ChAT activity were (expressed as mean nMol ACh/mg protein/hr $\pm$ S.E.M.), for experiment (A-B) NBM: 56.99 $\pm$ 2.01, cortex: 36.32 $\pm$ 1.13 and, for experiment (C-D), NBM: 59.68 $\pm$ 1.21, cortex: 38.89 $\pm$ 0.87.

Table 3.7 EFFECT OF DELAYED SHORT-TERM OR CHRONIC GM1 OR NGF TREATMENT ON HIGH AFFINITY [^{3}H] CHOLINE UPTAKE OF THE REMAINING CORTEX OF LESIONED RATS

A

Control	100 ± 6	100 ± 7
Lesioned + Vehicle	99 ± 5	105 ± 7
	7 days of GM1 treatment	*30 days of GM1 treatment*
Lesioned + 0 day delay	138 ± 6*	143 ± 5*
Lesioned + 1 day delay	127 ± 4*	-
Lesioned + 2 day delay	103 ± 5	131 ± 5*
Lesioned + 4 day delay	104 ± 8	99 ± 6
Lesioned + 6 day delay	-	101 ± 5

B

Control	100 ± 10	100 ± 8
Lesioned + Vehicle	105 ± 8	98 ± 5
	7 days of NGF treatment	*30 days of NGF treatment*
Lesioned + 0 day delay	144 ± 6*	149 ± 8*
Lesioned + 2 day delay	131 ± 4*	-
Lesioned + 6 day delay	115 ± 4	-
Lesioned + 10 day delay	106 ± 7	129 ± 5*
Lesioned + 14 day delay	99 ± 5	103 ± 4

C

Lesioned + Vehicle	99 ± 5	101 ± 6
	14 days of NGF treatment	*30 days of NGF treatment*
Lesioned + 30 day delay	107 ± 6	113 ± 5

Animals were decorticated as described in methods and received either vehicle, (A) GM1 (900 μg/day) or (B and C) NGF (1 μg/day) for 7 or 30 days, i.c.v. via minipump, beginning immediately post-lesion or after the delays indicated. Rats were sacrificed on post-lesion day 30. Values represent high affinity uptake of [^{3}H] choline, expressed as percent control, of synaptosomes isolated from the remaining ipsilateral cortex. See methods for assay details. n= 5-9 animals/group. *$p<0.05$ from control, ANOVA post-hoc Newman-Keuls test. Control values for: 7 days of GM1 treatment: 8.53 ± 0.63 pMoles/^{3}H choline/mg protein/4 min; 30 day of GM1 treatment: 8.17 ± 0.55 pMol/^{3}H choline/mg protein/4 min; for 7 days of NGF treatment: 8.49 ± 0.85 pMol/^{3}H choline/mg protein/4min; for 30 days of NGF treatment: 8.22 ± 0.66 pMol/^{3}H choline/mg protein/4min.

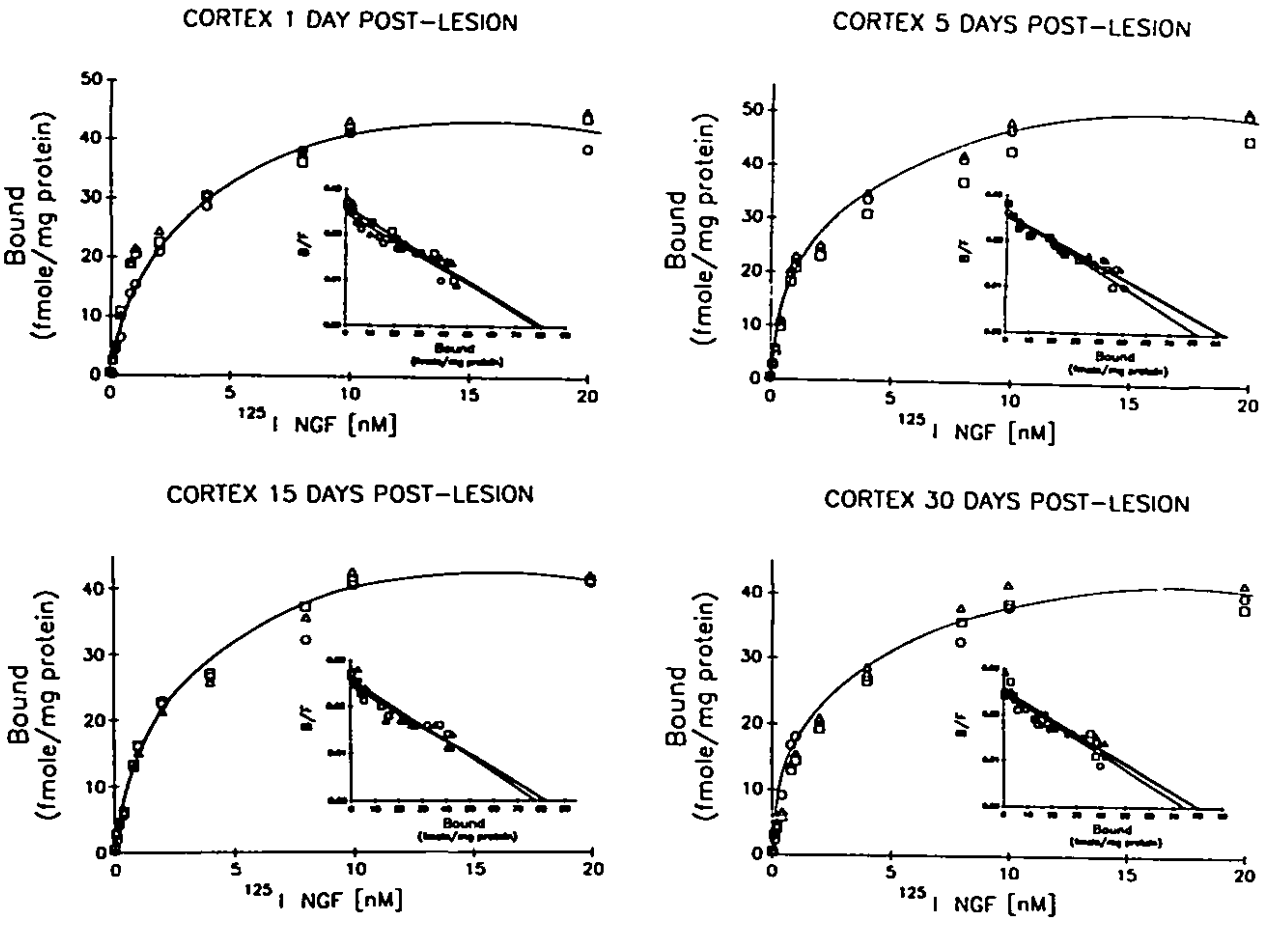


Figure 3.17 Specific binding of ^{125}I-NGF in the presence of excess cold NGF to cortical membranes from control (○—○), lesion vehicle (△—△) and lesion GM1 treated rats (□—□). GM1 (1.5 mg/day) was administered i.c.v., via minipump, beginning immediately post-lesion and lasted for 7 days. NGF binding to cortical membranes was examined at 1, 5, 15 and 30 days post-lesion. See methods for details of the binding assay. Binding data were analyzed using EBDA followed by LIGAND binding programs (MacPherson, 1985). Inset: Scatchard plots of the same data showing Kd and Bmax values similar to those obtained by nonlinear analysis. Binding data presented for each post-lesion time point are from a representative experiment. Binding parameters from several experiments are summarized in Table 3.8.

TABLE 3.8 EFFECT OF GM1 TREATMENT ON NGF RECEPTOR BINDING IN CORTEX

	Kd nM	Bmax (fmol/mg protein)
Control	5.0 ± 0.3	85 ± 4
1 day post-lesion		
Lesion + Vehicle	5.3 ± 0.6	85 ± 12
Lesion + GM1	5.2 ± 0.6	84 ± 8
5 days post-lesion		
Lesion + Vehicle	5.9 ± 0.8	87 ± 6
Lesion + GM1	5.8 ± 0.7	87 ± 7
15 days post-lesion		
Lesion + Vehicle	4.5 ± 0.6	77 ± 3
Lesion + GM1	4.9 ± 1.2	91 ± 7
30 days post-lesion		
Lesion + Vehicle	4.6 ± 0.3	80 ± 4
Lesion + GM1	5.0 ± 2.4	87 ± 3

Isolated cortical membranes from unoperated or decorticated rats which received, i.c.v. via minipump beginning immediately post-lesion, either: vehicle (artificial c.s.f. + 0.1% BSA) or GM1 (1.5 mg/day) for 7 days were incubated with concentrations of ^{125}I-NGF ranging from 0.01 nM to 20 nM in the presence or absence of excess cold NGF. Kd and Bmax values were obtained from Scatchard analysis of specific binding using computer assisted nonlinear regression (EBDA-LIGAND programs, McPherson 1985). ANOVA followed by a post-hoc Newman-Keuls test on the group means showed no significant differences. Numbers represent mean ± S.E.M. from 3-4 experiments per lesion time point.

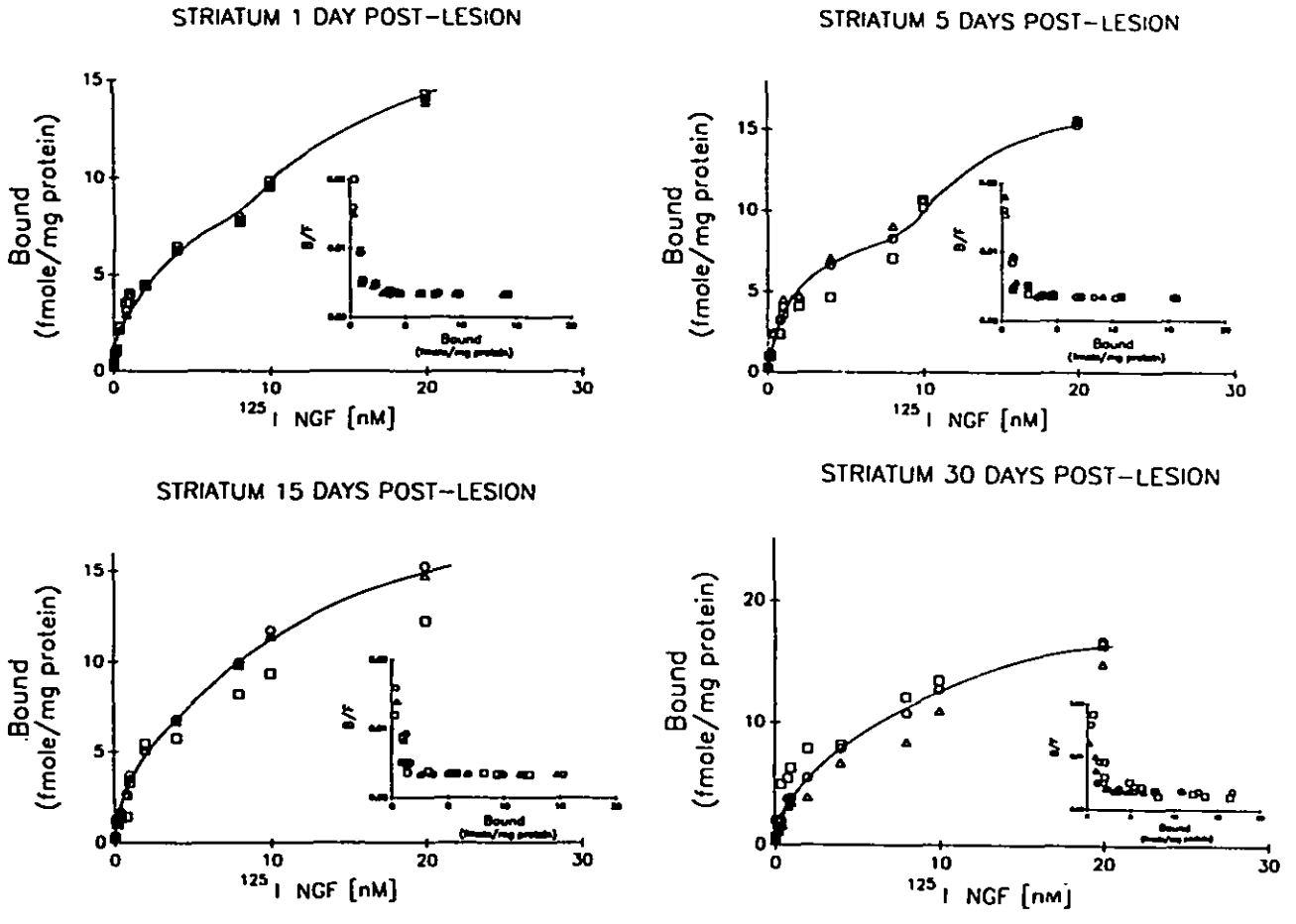


Figure 3.18 Specific binding of ^{125}I-NGF in the presence of excess cold NGF to striatal membranes from control (○—○), lesion vehicle (△——△) and lesion GM1 treated rats (□—□). GM1 (1.5 mg/day) was administered i.c.v., via minipump, beginning immediately post-lesion and lasted for 7 days. NGF binding to striatal membranes was examined at 1, 5, 15 and 30 days post-lesion. See methods for details of the binding assay. Binding data were analyzed using EBDA followed by LIGAND binding programs (MacPherson, 1985). Inset: Scatchard plots of the same data. Binding data presented for each post-lesion time point are from a representative experiment. Binding parameters from several experiments are summarized in Table 3.9.

TABLE 3.9 EFFECT OF GM1 TREATMENT ON NGF RECEPTOR BINDING IN STRIATUM

	Kd (nM)		Bmax (fmol/mg protein)	
	High affinity	Low affinity	High affinity	Low affinity
Control	0.13 ± 0.01	23 ± 3	1.7 ± 0.2	67 ± 7
1 day post-lesion				
Lesion + Vehicle	0.09 ± 0.01	19 ± 3	1.8 ± 0.3	69 ± 14
Lesion + GM1	0.11 ± 0.02	21 ± 2	1.8 ± 0.2	69 ± 11
5 days post-lesion				
Lesion + Vehicle	0.11 ± 0.02	19 ± 3	2.2 ± 0.4	70 ± 4
Lesion + GM1	0.09 ± 0.02	22 ± 5	2.0 ± 0.3	74 ± 9
15 days post-lesion				
Lesion + Vehicle	0.18 ± 0.03	20 ± 3	2.3 ± 0.5	66 ± 8
Lesion + GM1	0.13 ± 0.03	23 ± 5	1.8 ± 0.2	66 ± 6
30 days post-lesion				
Lesion + Vehicle	0.16 ± 0.04	22 ± 5	2.1 ± 0.3	63 ± 6
Lesion + GM1	0.12 ± 0.02	21 ± 4	2.2 ± 0.2	76 ± 7

Isolated striatal membranes from unoperated or decorticated rats which received, i.c.v. via minipump beginning immediately post-lesion, either: vehicle (artificial c.s.f. + 0.1% BSA) or GM1 (1.5 mg/day) for 7 days were incubated with concentrations of ^{125}I-NGF ranging from 0.01 nM to 20 nM in the presence or absence of excess cold NGF. Kd and Bmax values were obtained from Scatchard analysis of specific binding using computer assisted nonlinear regression (EBDA-LIGAND programs, McPherson 1985). ANOVA followed by a post-hoc Newman-Keuls test on the group means showed no significant differences. Numbers represent mean ± S.E.M. from 3-4 experiments per lesion time point.

3.2 *NEUROANATOMICAL CORRELATES OF UNILATERAL DEVASCULARIZING CORTICAL LESIONS AND TROPHIC FACTOR TREATMENT*

The effects of the lesion, GM1 and/or NGF treatment on the morphology of cholinergic neurons in the NBM were assessed quantitatively with the assistance of an image analysis system. Moreover, the anatomical correlates of the trophic factor-induced neurochemical plasticity in cortex of lesioned animals were examined using both light and electron microscopic techniques coupled to image analysis.

3.2.1 *Effects of the cortical lesion GM1 and/or NGF treatment on NBM ChAT and $p75^{NGFR}$-like immunoreactive neurons and neurites*

ChAT-IR neurons in the NBM have previously been shown to undergo prototypic signs of retrograde degeneration following unilateral decortication (Sofroniew et al., 1983). In particular, it was noted that at 7 days post-lesion cholinergic neurons of the NBM show an increase in perikaryal size as well as exhibit eccentrically placed nuclei. At subsequent time points (30 days), these neurons were shown to be shrunken but, no significant cell loss was detected. Moreover, it was demonstrated that this shrinkage of NBM ChAT-IR neurons was prevented in decorticated animals which received, beginning immediately post-lesion, daily i.p. injections of GM1 (30 mg/kg/day) for thirty days (Cuello et al., 1986).

The work of this thesis extended these studies and examined (1) whether a short-term i.c.v. treatment with GM1 could equally prevent retrograde degeneration of NBM cholinergic neurons, (2) if NGF treatment could similarly affect NBM cholinergic neuronal morphology, (3) whether concurrent treatment with both NGF and GM1 could elicit enhanced recovery and (4) the effects of the lesion and/or trophic factor treatment on NBM cholinergic neurite length. Moreover, the effects of the lesion GM1 and/or NGF treatment on the expression of NGF receptor-like immunoreactivity in the NBM was also examined for the first time. For this, the monoclonal antibody 192-IgG (Mab 192) (Chandler et al., 1984), which presumably recognizes the low affinity NGF receptor ($p75^{NGFR}$), was used.

Because cholinergic neurons are diffusely organized within the NBM, in order to more

accurately assess the effects of GM1 and/or NGF treatment, image analysis was employed and ChAT as well as $p75^{NGFR}$-IR cell size and number, in addition to neuritic processes were quantified. The neuritic processes measured included the dendritic components of NBM neurons and to a lesser extent cholinergic fibers ascending from the brainstem reticular formation (Jones and Cuello, 1989). For quantitative purposes the NBM was divided into anterior, mid and posterior portions each of which were further subdivided into dorsal and mid portions as described in methods. Adult male Wistar rats were unilaterally cortically lesioned and received, beginning immediately post-lesion either, vehicle (artificial CSF + 0.1% BSA), or maximal doses of GM1 (1.5 mg/day) and/or NGF (12 μg/day), i.c.v. via minipump for 7 days. The animals were sacrificed at 30 days post-lesion by vascular perfusion and the brains were processed for ChAT or $p75^{NGFR}$ immunocytochemistry for light microscopic analysis. No significant decrease in the number of ChAT or $p75^{NGFR}$-IR neurons was noted in any of the NBM subdivisions on post-lesion day 30 (Table 3.10). However, a significant shrinkage of ChAT-IR neurons was noted predominantly in the mid portion of the ipsilateral NBM (Figure 3.19; Figure 3.20; Table 3.11). Cholinergic neurons in the anterior and posterior NBM were also reduced in size but not significantly from control values. In addition, a significant decrease in the mean length of ChAT-IR neurites occurred in NBM subdivisions of lesioned vehicle treated rats. As was noted for cholinergic cell size, the greatest deficit in mean neurite length occurred in the mid portion of the NBM (Dorsal: 44% decreases; Ventral: 42% decrease) (Table 3.11). In anterior or posterior NBM regions, small decreases were evident, but were not significant. Since the unilateral devascularizing cortical lesion causes a degeneration of mainly fronto/parietal cortical areas, these results support previous work, which employed anterograde tract tracing techniques, to show that the cholinergic projection from the NBM to cortex follows an anterior to posterior distribution pattern (Luiten et al., 1987). Similar lesion-induced changes were noted for $p75^{NGFR}$-IR NBM neurons (Figure 3.21; Table 3.12). That ChAT and $p75^{NGFR}$ immunoreactivity are highly correlated in the NBM was confirmed while the work of this thesis was in progress (Kiss et al., 1988; Dawbarn et al., 1988; Pioro and Cuello, 1990) and is further supported by the quantitative results presented here. Short-term (7 days) NGF and/or GM1 treatment prevented the lesion-induced decrease in cholinergic cell size as well as preserved cholinergic neuritic processes in the mid-NBM, where the detrimental effects of the lesion were most evident

(Figure 3.19; Figure 3.20; Table 3.11). Although a trend towards an increase in cell size and neurites were observed in NGF/GM1 treated rats, particularly in the mid-NBM, these were not significantly different from control values (Table 3.12). In addition, the small decreases in ChAT and $p75^{NGFR}$-IR cell size detected in the anterior and posterior NBM were no longer evident in lesioned NGF and/or GM1 treated rats. Unlesioned animals which also received either NGF and/or GM1 showed no change in cholinergic cell size or neuritic processes when compared to unoperated animals (Table 3.12). Moreover, the cholinergic neurons in the contralateral NBM were similarly unaffected by the lesion and treatments (Table 3.13).

These studies demonstrate that cholinergic neurons in select portions of the NBM are differentially affected by the unilateral devascularizing cortical lesion and that GM1 and/or NGF treatment can equally prevent retrograde degeneration of NBM cholinergic neurons.

TABLE 3.10: EFFECT OF CORTICAL DEVASCULARIZATION ON ChAT AND $p75^{NGFR}$-IR CELL DENSITY

	ChAT-IR		$p75^{NGFR}$	
	Control	Lesion + Vehicle	Control	Lesion + Vehicle
ANTERIOR NBM	41.2 ± 8.0	47.8 ± 8.0	41.7 ± 6.0	38.5 ± 3.0
MID-DORSAL NBM	46.3 ± 6.0	56.0 ± 3.0	40.7 ± 6.0	46.6 ± 3.0
MID-VENTRAL NBM	49.6 ± 6.0	57.4 ± 3.0	55.3 ± 2.0	63.4 ± 10
POSTERIOR NBM	52.9 ± 9.5	58.5 ± 4.0	49.4 ± 4.5	61.3 ± 9.0

Values represent mean Abercrombie corrected number of ChAT or $p75^{NGFR}$-IR neurons per field. Neurons were quantified in two fields of each subdivision of the ipsilateral rat NBM, 30 days post-lesion, using an image analysis system (see methods for details), n= 4 animals/group.

Figure 3.19 ChAT-IR neurons in the mid portion of the rat NBM in (A) control unoperated, (B) lesion vehicle, (C) lesion GM1, (D) lesion NGF and (E) lesion NGF+ GM1 treated rats. Scale bar= 200 for all panels

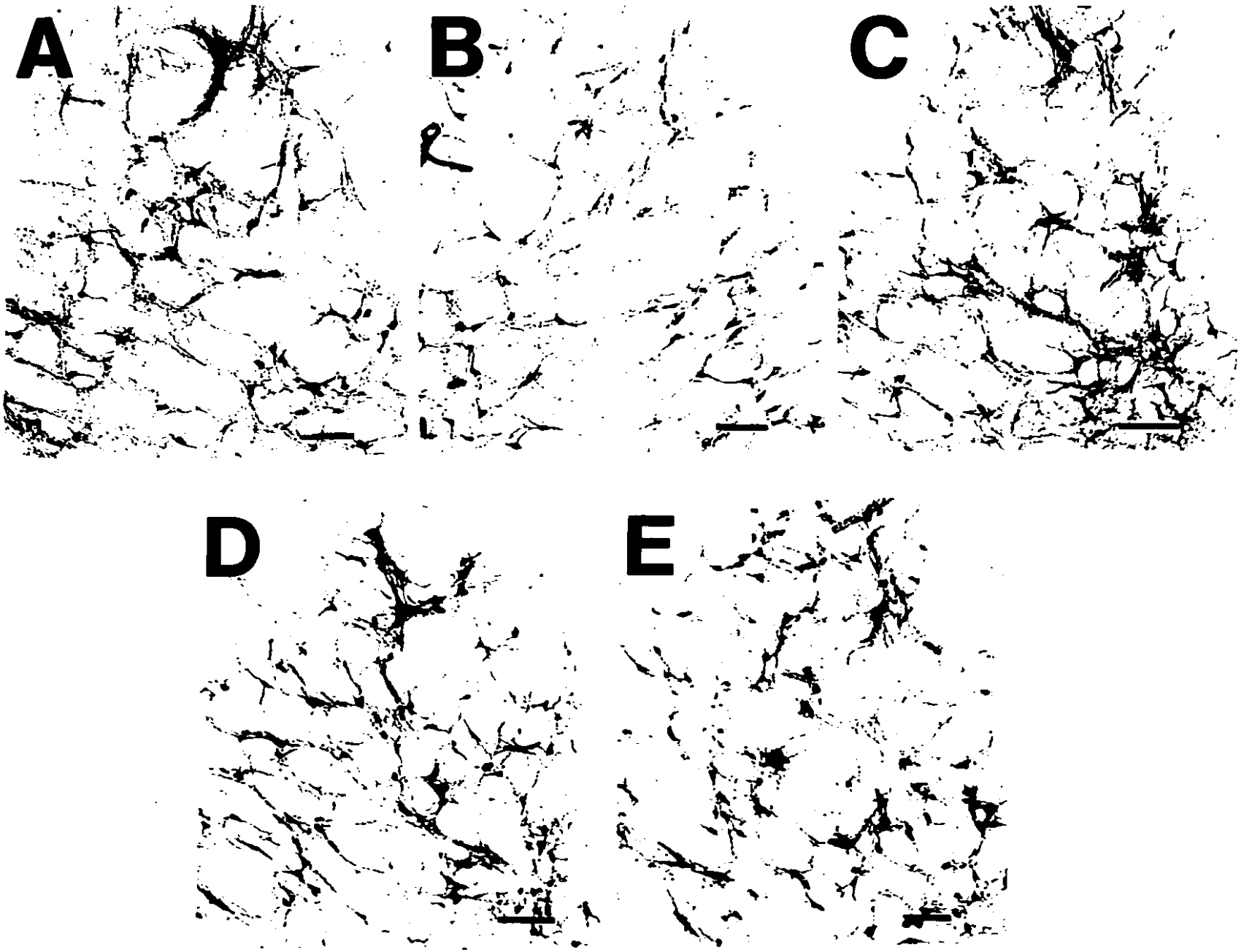
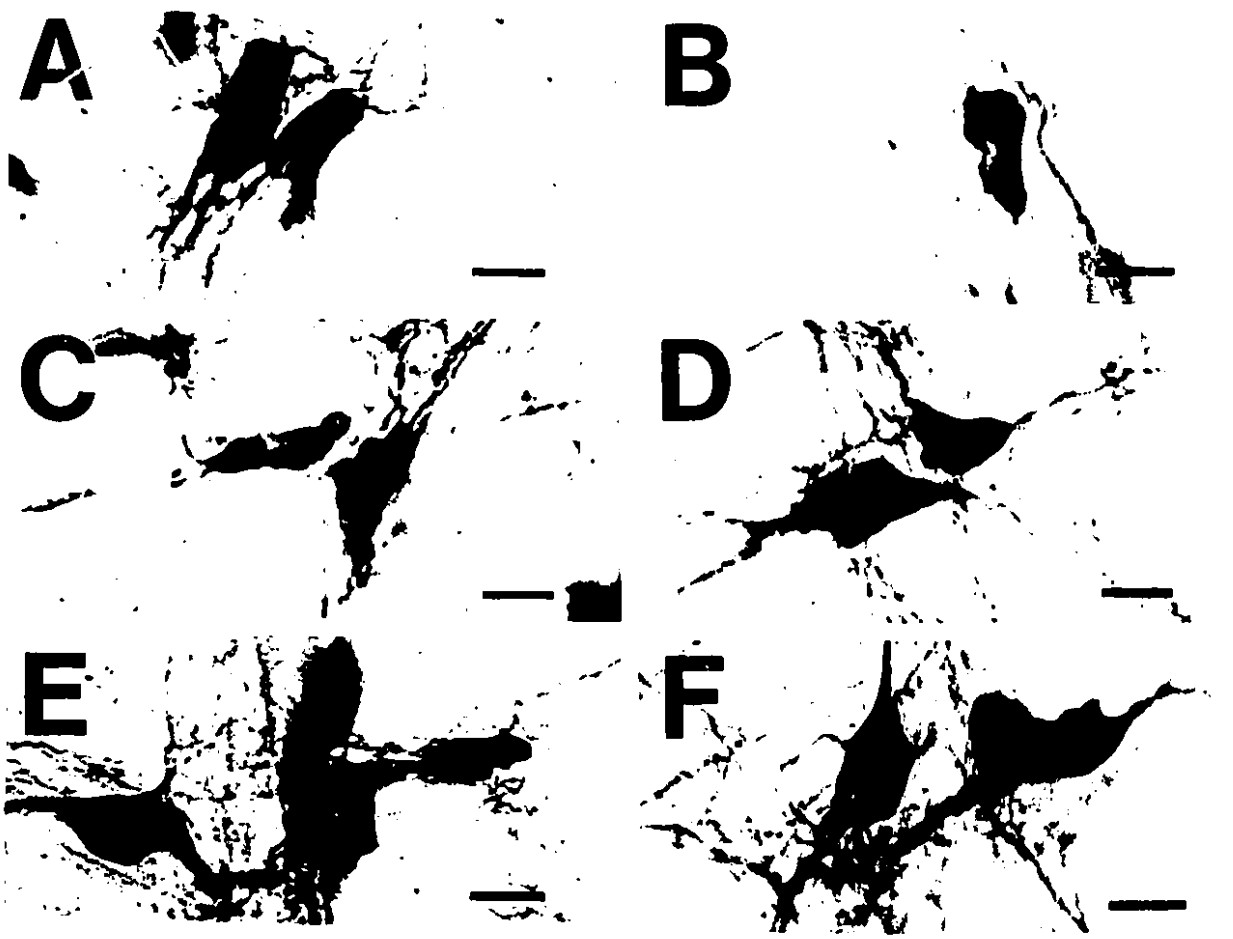

A
B
C
D
E

Figure 3.20 ChAT-IR neurons shown at high magnification from the (A) dorsal mid-NBM of control unoperated, (B) dorsal mid-NBM and (C) ventral mid-NBM of a 30 days post-lesion vehicle treated rat, and dorsal mid-NBM of a 30 days post-lesion (D) GM1, (E) NGF and (F) NGF+GM1 treated lesioned rat. Lesioned rats were treated beginning immediately post-lesion, i.c.v. via minipump, for 7 days. Scale bar= 20μm.


A
B
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D
E
F

Figure 3.21 $p75^{NGFR}$-IR neurons in the mid-NBM of (A) dorsal mid-NBM of control unoperated, (B) dorsal mid-NBM and (C) ventral mid-NBM of a 30 days post-lesion vehicle treated rat, and dorsal mid-NBM of a 30 days post-lesion (D) GM1, (E) NGF and (F) NGF+GM1 treated lesioned rat. Lesioned rats were treated beginning immediately post-lesion, i.c.v. via minipump, for 7 days. Scale bar= 40μm

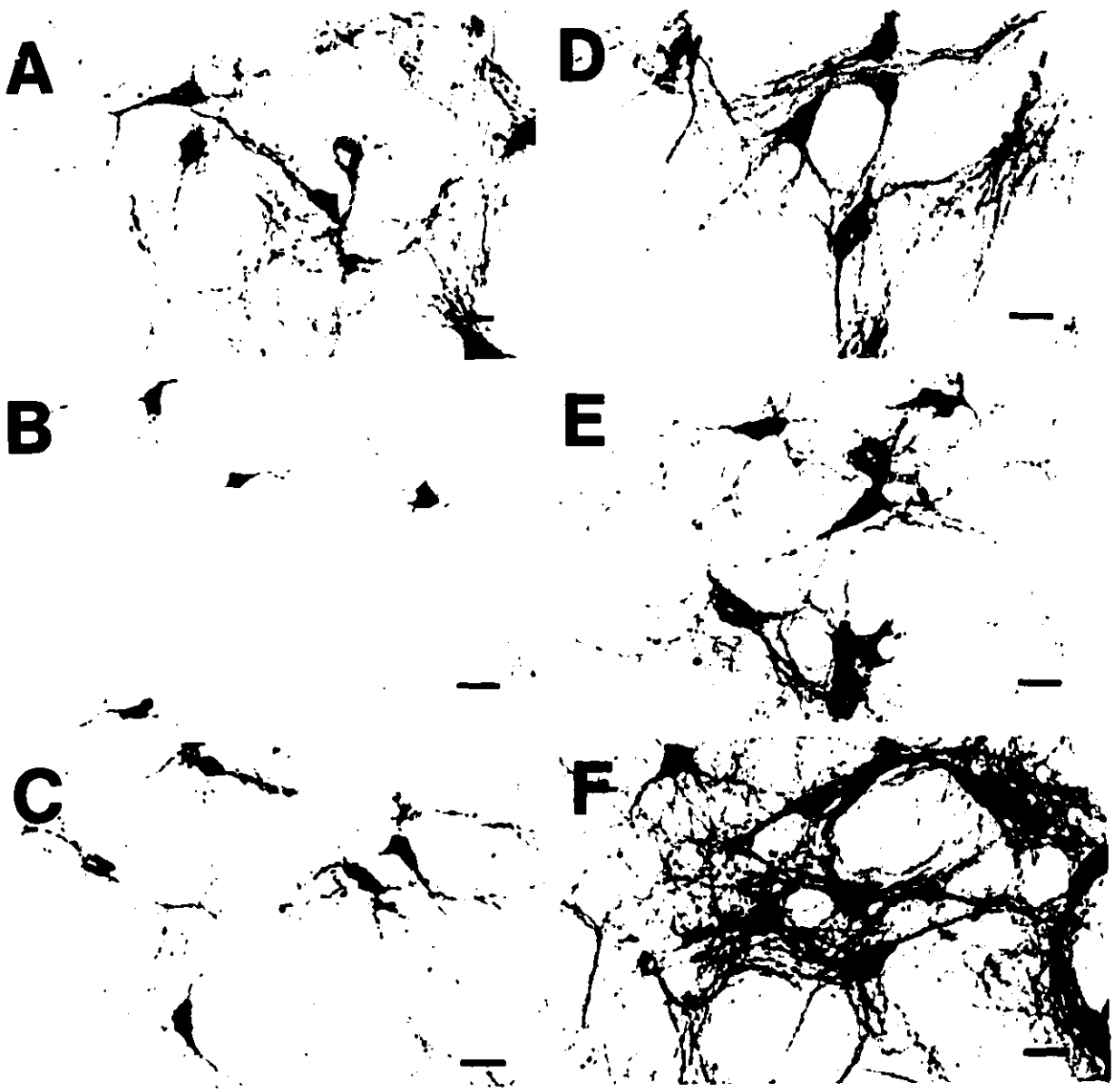

A
D
B
E
C
F

TABLE 3.11: EFFECT OF CORTICAL DEVASCULARIZATION, NGF AND/OR GM1 TREATMENT ON ChAT-IR CELL BODIES AND NEURITIC PROCESSES IN THE IPSILATERAL NBM

PERIKARYAL MEAN CROSS SECTIONAL AREA (μm^2)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
Control	241 ± 13	254 ± 12	260 ± 10	243 ± 14
Control + GM1	245 ± 9	247 ± 12	269 ± 18	253 ± 14
Control + NGF	242 ± 7	268 ± 9	255 ± 14	255 ± 22
Control + NGF/GM1	256 ± 10	270 ± 9	272 ± 8	252 ± 15
Lesion + Vehicle	213 ± 9	166 ± 8*	170 ± 11*	214 ± 12
Lesion + GM1	256 ± 13	243 ± 10	237 ± 14	245 ± 9
Lesion + NGF	248 ± 8	251 ± 9	256 ± 11	245 ± 11
Lesion + NGF/GM1	252 ± 10	278 ± 13	270 ± 9	249 ± 8

MEAN NEURITE LENGTH (mm)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
Control	54.0 ± 14.0	54.9 ± 13.6	58.9 ± 14.3	52.9 ± 9.9
Control + GM1	53.0 ± 9.0	54.7 ± 4.0	53.6 ± 4.0	53.6 ± 5.0
Control + NGF	53.0 ± 6.0	54.1 ± 10.0	56.1 ± 6.2	56.1 ± 6.0
Control + NGF/GM1	52.3 ± 10.0	51.0 ± 12.0	52.5 ± 4.5	52.5 ± 5.0
Lesion + Vehicle	46.3 ± 9.9	31.1 ± 10.1*	33.9 ± 9.6*	41.3 ± 9.7
Lesion + GM1	52.4 ± 8.7	50.9 ± 11.1	61.42 ± 10.2	51.3 ± 8.2
Lesion + NGF	59.7 ± 8.8	52.5 ± 8.6	59.0 ± 9.7	56.1 ± 5.9
Lesion + NGF/GM1	62.6 ± 5.6	69.5 ± 10.5	73.9 ± 10.2	68.2 ± 9.9

Animals were unilaterally decorticated and treated i.c.v. with either vehicle, GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days. Another group of animals were not lesioned (control) but also received GM1 and/or NGF. Rats (n=4/group) were sacrificed thirty days post lesion (ie: 23 days after the end of drug infusions) and the brains were processed for ChAT immunocytochemistry (see methods for details). Three sections from the anterior and posterior and 5 sections from the mid basalis area were used for quantification with an image analysis system (Quantimet 920). Numbers represent mean cross sectional area of ChAT-IR neurons or mean length of ChAT-IR neurites ± S.E.M. in the ipsilateral NBM. *$p<0.05$ from control values, ANOVA, post-hoc Tukey test.

TABLE 3.12: EFFECT OF CORTICAL DEVASCULARIZATION, NGF AND/OR GM1 TREATMENT ON $p75^{NGFR}$-IR CELL BODIES AND NEURITIC PROCESSES IN THE IPSILATERAL NBM

PERIKARYAL MEAN CROSS SECTIONAL AREA (μm^2)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
Control	229 ± 8	244 ± 10	250 ± 9	236 ± 8
Control + GM1	233 ± 6	246 ± 9	251 ± 8	242 ± 10
Control + NGF	231 ± 5	248 ± 10	245 ± 11	255 ± 22
Control + NGF/GM1	236 ± 7	252 ± 8	261 ± 10	254 ± 10
Lesion + Vehicle	195 ± 10	162 ± 10*	175 ± 9*	203 ± 14
Lesion + GM1	236 ± 11	239 ± 9	247 ± 6	238 ± 8
Lesion + NGF	237 ± 8	242 ± 6	250 ± 10	242 ± 10
Lesion + NGF/GM1	249 ± 10	260 ± 11	261 ± 9	248 ± 10

MEAN NEURITE LENGTH (mm)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
Control	46.5 ± 11.0	50.7 ± 8.6	52.3 ± 9.4	46.6 ± 10.2
Control + GM1	48.7 ± 9.0	52.0 ± 9.0	51.6 ± 10.0	47.3 ± 9.8
Control + NGF	48.6 ± 10.0	53.6 ± 9.0	54.6 ± 8.4	48.7 ± 7.5
Control + NGF/GM1	50.2 ± 10.3	55.6 ± 10.5	57.7 ± 6.9	48.5 ± 8.0
Lesion + Vehicle	38.5 ± 8.8	29.4 ± 8.8*	31.3 ± 8.2*	38.6 ± 7.5
Lesion + GM1	47.6 ± 7.6	51.6 ± 8.0	55.5 ± 9.9	47.7 ± 10.0
Lesion + NGF	49.3 ± 9.0	55.5 ± 9.1	54.9 ± 10.1	49.9 ± 8.5
Lesion + NGF/GM1	54.5 ± 7.0	63.6 ± 12.5	64.9 ± 11.2	58.9 ± 10.2

Animals were unilaterally decorticated and treated i.c.v. with either vehicle, GM1 (1.5 mg/day) and/or NGF (12 μg/day). Another group of animals were not lesioned (control) but also received GM1 and/or NGF. Rats (n=4/group) were sacrificed thirty days post lesion (ie: 23 days after the end of drug infusions) and the brains were processed for $p75^{NGFR}$ immunocytochemistry (see methods for details). Three sections from the anterior and posterior and 5 sections from the mid basalis area were used for quantification with an image analysis system (Quantimet 920). Numbers represent mean cross sectional area of $p75^{NGFR}$-IR neurons or mean length of $p75^{NGFR}$-IR neurites ± S.E.M. in the ipsilateral NBM. *$p<0.05$ from control values, ANOVA, post-hoc Tukey test.

Table 3.13: EFFECT OF CORTICAL DEVASCULARIZATION, NGF AND/OR GM1 TREATMENT ON ChAT AND $p75^{NGFR}$-IR CELL BODIES AND NEURITIC PROCESSES IN THE CONTRALATERAL NBM

PERIKARYAL MEAN CROSS SECTIONAL AREA (μm^2)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
ChAT-IR				
Control	239 ± 11	244 ± 8	255 ± 7	238 ± 11
Lesion + Vehicle	244 ± 8	246 ± 7	248 ± 9	244 ± 9
Lesion + GM1	246 ± 10	252 ± 5	255 ± 9	245 ± 8
Lesion + NGF	255 ± 9	255 ± 10	261 ± 10	250 ± 5
Lesion + NGF/GM1	255 ± 8	258 ± 9	255 ± 9	248 ± 10
$p75^{NGFR}$				
Control	240 ± 9	245 ± 10	247 ± 11	233 ± 12
Lesion + Vehicle	233 ± 11	237 ± 11	250 ± 8	241 ± 10
Lesion + GM1	245 ± 10	244 ± 9	248 ± 7	247 ± 8
Lesion + NGF	248 ± 8	252 ± 12	254 ± 8	253 ± 6
Lesion + NGF/GM1	251 ± 10	258 ± 11	262 ± 11	255 ± 8

MEAN NEURITE LENGTH (mm)

Group	Anterior NBM	Dorsal Mid NBM	Ventral Mid NBM	Posterior NBM
ChAT-IR				
Control	49.0 ± 11.0	60.3 ± 11.5	66.9 ± 13.5	55.6 ± 7.5
Lesion + Vehicle	51.0 ± 10.5	57.5 ± 6.0	55.5 ± 8.0	57.6 ± 9.0
Lesion + GM1	55.0 ± 8.6	58.9 ± 10.4	67.8 ± 9.5	60.4 ± 10.0
Lesion + NGF	54.8 ± 10.7	62.6 ± 10.0	66.8 ± 7.5	64.8 ± 6.0
Lesion + NGF/GM1	56.9 ± 9.5	66.6 ± 9.5	69.5 ± 10.3	66.4 ± 7.5
$p75^{NGFR}$				
Control	44.6 ± 8.0	52.5 ± 10.0	47.5 ± 7.5	44.8 ± 9.5
Lesion + Vehicle	49.0 ± 10.0	49.7 ± 6.5	44.6 ± 11.0	52.5 ± 7.0
Lesion + GM1	53.5 ± 7.5	46.8 ± 5.5	58.5 ± 9.0	46.6 ± 8.0
Lesion + NGF	55.6 ± 6.5	58.7 ± 6.5	58.5 ± 7.5	56.1 ± 10.5
Lesion + NGF/GM1	55.8 ± 5.5	60.5 ± 9.0	59.7 ± 6.0	60.5 ± 9.0

Animals were unilaterally decorticated and treated i.c.v. with either vehicle, GM1 (1.5 mg/day) and/or NGF (12 μg/day). Another group of animals were not lesioned (control) but also received GM1 and/or NGF. Rats (n=4/group) were sacrificed thirty days post lesion (ie: 23 days after the end of drug infusions) and the brains were processed for ChAT or $p75^{NGFR}$ immunocytochemistry (see methods for details). Three sections from the anterior and posterior and 5 sections from the mid basalis area were used for quantification with an image analysis system (Quantimet 920). Numbers represent mean cross sectional area of ChAT or $p75^{NGFR}$-IR neurons or mean length of ChAT or $p75^{NGFR}$-IR neurites ± S.E.M. in the ipsilateral NBM. ANOVA followed by a post-hoc Tukey test showed no significant differences among groups.

3.2.2 *NGF or GM1 treatment preserves, while NGF/GM1 treatment augments, ChAT-IR fiber length in cortex*

To assess the anatomical correlates of exogenous GM1 and/or NGF induced increases in cortical cholinergic activity (shown in section 3.1), the effects of these agents on the cortical ChAT-IR fiber network were studied. As for the neurochemical study, adult rats were unilaterally decorticated and received immediately post-lesion, i.c.v. via minipump, either vehicle or maximal doses of GM1 (1.5 mg/day) and/or NGF (12 μg/day) for a period of 7 days. Animals were sacrificed at 30 days post-lesion (ie: 23 days after the end of drug treatment) and were processed for ChAT immunocytochemistry at the light microscopic level. The area of neocortex assessed was taken at the level of the mid basalis [1.20 -to- 1.40 mm posterior to bregma, (Paxinos and Watson, 1986)], 100 μm from the mid-point of the cingulum. This corresponds to cortical area 4 as described by Lysakowski and collaborators (1989). ChAT-IR fibers observed in this area in control unoperated rats were fine and appeared uniformly distributed across all layers (Figure 3.22), excepting in cortical layer I where fibers appeared tangentially oriented as previously described (Houser et al., 1985). A deficit in the cortical ChAT-IR fiber network was noted throughout all 6 layers of this cortical region in lesion vehicle treated animals (Table 3.14, Figure 3.22). Decreases in ChAT-IR fiber length ranged from 31% to 50%; the greater losses occurring in layers II, IV, and V. ChAT-IR interneurons observed within this area of cortex did not appear affected by the lesion. Immediate treatment with either GM1 or NGF prevented the decrease in cholinergic fiber length, in all cortical layers (Table 3.14 Figure 3.22 C,D). However, when administered together these agents caused a significant increase in the cortical cholinergic innervation (Table 3.14; Figure 3.22E). Unlesioned animals which also received GM1, NGF or NGF/GM1 treatment showed no change in their cortical fiber network when compared to their vehicle treated counterparts (Table 3.14).

3.2.3 *GM1 and/or NGF effects on ChAT-IR cortical axonal varicosities*

ChAT-IR cortical varicosities were observed to be unevenly distributed along cholinergic fibers and were decreased in number in the remaining cortex of lesion vehicle treated animals

(Figures 3.23A, 3.23B, Table 3.15). These decreases ranged from 26 to 39% between cortical layers. Whereas exogenous GM1 prevented this loss, NGF treatment instead augmented varicosity number above control levels (Figures 3.23A, 3.23B, Table 3.15). Lesioned rats which received both NGF and GM1 showed an increase in axonal varicosity number which was significantly greater than that obtained in control or lesion NGF treated rats (Table 3.15). GM1 and/or NGF did not affect varicosity number in unlesioned animals.

3.2.4 GM1 treatment does not affect cortical ChAT-IR presynaptic terminal size but does potentiate NGF induced effects

The ultrastructural effects of GM1 and/or NGF treatment on cholinergic presynaptic terminals were studied using electron microscopy. Decorticated rats received, i.c.v. via minipump, either vehicle or maximal doses of GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days and were sacrificed at 30 days post-lesion by transcardial perfusion with fixation buffer. Layer V from cortical tissue processed for ChAT immunocytochemistry for electron microscopy was chosen for study since it is known to be a major terminal area of ascending NBM fibers (Luiten et al., 1987; Eckenstein et al., 1988). A random sample (n= 104 boutons/group) of ChAT-IR profiles in cortical layer V of unoperated control animals had a mean cross sectional area, as quantified by image analysis, of $0.223 \pm 0.013\ \mu m^2$. ChAT-IR boutons in this region contained a moderate concentration of vesicles and although their post-synaptic targets were not comprehensively studied, the boutons were mostly found apposed to non immunoreactive dendritic profiles most likely arising from either pyramidal or nonpyramidal cells as previously shown (Houser et al., 1985). Occasionally, cholinergic boutons were also observed in contact with nerve cell bodies. Synaptic differentiations noted were almost invariably of the symmetric type. In lesion vehicle treated animals no obvious ultrastructural changes were evident excepting a remarkable difference in the size of the cholinergic boutons (Figure 3.24). Quantification confirmed that a significant shrinkage occurred in boutons of lesion vehicle treated rats (Figure 3.27). As well, these boutons showed a high density of synaptic vesicles and were usually intensely immunoreactive suggesting that increases in cholinergic activity may occur following injury perhaps as a compensatory mechanism. This could account for our previous observations showing control

levels of ChAT activity in this remaining cortical area of lesion vehicle treated rat (section 3.1). Exogenous GM1 treatment did not significantly modify this noted lesion-induced shrinkage (Figure 3.25, Figure 3.27). Thus, although GM1 treatment can maintain the cholinergic fiber network in lesioned rats, at the dosages and treatment times employed here, it does not significantly alter the ultrastructural changes characteristic of lesioned animals. By contrast, NGF treatment of cortically devascularized rats significantly increased, above control values, the mean cross sectional area, breadth, length, perimeter and shape factor of cholinergic boutons in this cortical region (Figure, 3.25; Figure 3.27). In decorticated animals which received NGF co-administered with GM1, a further augmentation in bouton size was observed (Figure 3.26). As well, an increase in the irregularity of the bouton shape was noted and confirmed quantitatively by the shape factor measurement. In unlesioned rats, ChAT-IR bouton size was not altered by NGF or NGF/GM1 treatment (Figure 3.27).

3.2.5 Effects of lesion, NGF and/or GM1 treatment on synapse number

The number of boutons with synaptic contacts in cortical layer V were also quantified. All boutons were examined using the goniometer stage of the electron microscope which facilitated identification of synapses. In control unoperated rats approximately 38% of ChAT-IR boutons within this area were found to have synaptic contacts. In lesioned vehicle treated animals the number of cholinergic boutons with synaptic contacts was decreased to 22%, whereas, lesioned animals which received GM1 treatment had control number of synapses (Figure 3.28). This suggests that gangliosides can perhaps facilitate the maintenance of contact between a cholinergic terminal and its post synaptic target following injury. By contrast, NGF treatment of lesioned rats caused a significant increase in synaptic number. In these cases NGF treatment nearly doubled synapse number in this area (Figure 3.28). This suggests that in addition to synaptic remodelling NGF may also play a role in synaptogenesis. Lesioned rats which received both NGF and GM1 showed no further increase in synapse number (Figure 3.27). As well, unoperated rats which received NGF and/or GM1 treatment showed no alteration in synapse number (Figure 3.28). No obvious changes in post-synaptic targets were detected among the groups. Moreover, the morphology of synaptic specializations in lesioned treated animals was identical to control.

Figure 3.22 Effect of lesion, GM1 and/or NGF treatment on the ChAT-IR fiber network in area 4 (Lysakowski et al., 1989) of the rat cortex at the level of the mid basalis (approximately 1.30 mm posterior to bregma). Dark field photomicrographs of (A) control, (B) lesion vehicle, (C) lesion GM1, (D) lesion NGF and (E) lesion NGF+GM1 treated rats. Animals were unilaterally decorticated and treated immediately, ICV, with either vehicle, GM1 and/or NGF as described in methods. Thirty days post-lesion (ie: 23 days after the end of drug treatment) the rats (n=4/group) were sacrificed and the brains processed for ChAT immunocytochemistry for light microscopic analysis (see methods for details). Note the reduced ChAT-IR fiber network in all cortical layers in lesion vehicle treated rats (B) particularly, in layers IV, V and VI. C and D show that the ChAT-IR fiber network is maintained by either GM1 or NGF treatment, respectively while it is enhanced, above control levels, in animals which received both NGF and GM1 (E). Scale bar in (A) = 100 μm and applies to all panels.

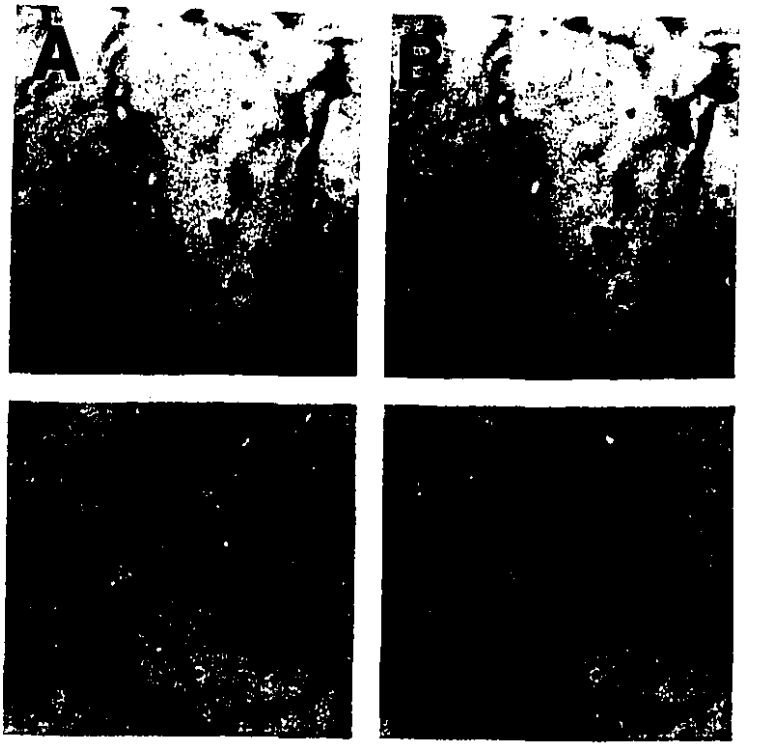


Figure 3.23A ChAT-IR varicosities in layer V of cortical area 4 (Lysakowski et al., 1989) at the level of the mid basalis (approximately 1.30 mm posterior to bregma) in rat. Grey (A and C) and Grey plus binary images (B and D) of ChAT-IR varicosities in (A & B) control, and (C & D) lesion vehicle treated rats. Scale bar= 10 μm

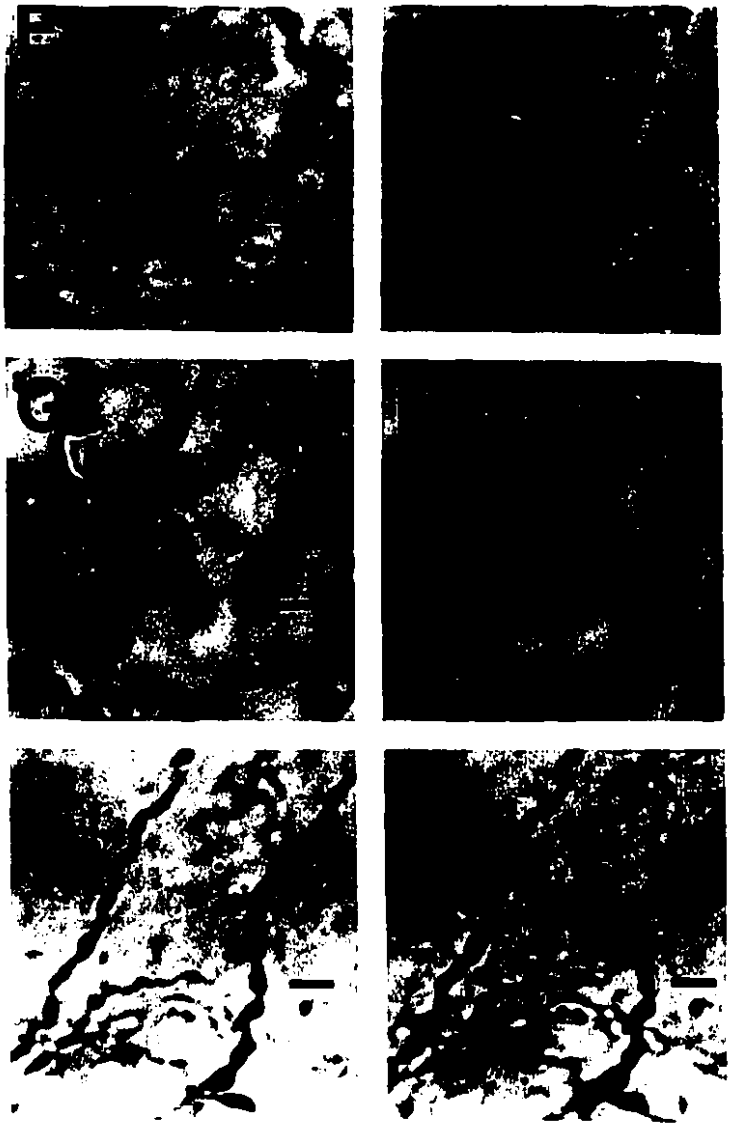


Figure 3.23B ChAT-IR varicosities in layer V of cortical area 4 (Lysakowski et al., 1989) at the level of the mid basalis (approximately 1.30 mm posterior to bregma) in rat. Grey (E,G and I) and Grey plus binary images (F,H and J) of ChAT-IR varicosities in (E & F) lesion GM1, (G & H) lesion NGF, and (I & J) lesion NGF/GM1 treated rats. Scale bar= 10 μm

TABLE 3.14: CORTICAL ChAT IMMUNOREACTIVE FIBER LENGTH (μm)

Cortical Layers	CONTROL			
	UNTREATED	+GM1	+NGF	+NGF/GM1
I	6641 ± 343	7084 ± 1030	5185 ± 766	6753 ± 353
II	7889 ± 532	7938 ± 1177	8268 ± 1140	8122 ± 871
III	6788 ± 652	6958 ± 554	7560 ± 1149	7515 ± 879
IV	10480 ± 630	9126 ± 805	9218 ± 828	8840 ± 610
V	20282 ± 712	17609 ± 1084	17473 ± 924	17407 ± 978
VI	16027 ± 1099	14824 ± 607	14270 ± 1485	12836 ± 1553
Cortical Layers	LESIONED			
	+VEHICLE	+GM1	+NGF	+NGF/GM1
I	4577 ± 801	6197 ± 968	8053 ± 977	10786 ± 732 *§
II	4207 ± 477 *	8365 ± 524	8610 ± 985	12434 ± 1235*§
III	4412 ± 451 *	6888 ± 590	8861 ± 969	12085 ± 551 *§
IV	5226 ± 798 *	9079 ± 847	12923 ± 1038	16905 ± 1113*§
V	11110 ± 927 *	18973 ± 1028	20827 ± 1354	28412 ± 1164*§
VI	10221 ± 772 *	14660 ± 1363	16833 ± 734	22500 ± 1549*§

Lesioned rats received either vehicle, NGF (12 μg/day) and/or GM1 (1.5 mg/day) i.c.v. as described in methods. A group of nonlesioned animals (control) also received NGF and/or GM1. Rats (n=4/group) were sacrificed thirty days post lesion (ie: 23 days after the end of drug infusions) and the brains were processed for ChAT immunocytochemistry. ChAT-IR fiber length in cortical layers I-VI were quantified, by an operator blinded to the treatment groups, using an Image analysis system (Quantimet (920) (see methods for details). Numbers represent mean fiber length (μm) ± S.E.M. *$p<0.05$ from control, § $p<0.05$ from lesion NGF or GM1 treated rats, ANOVA, post-hoc Newman-Keuls test.

TABLE 3.15: CORTICAL ChAT IMMUNOREACTIVE VARICOSITY NUMBER

Cortical Layers	CONTROL			
	UNTREATED	+GM1	+NGF	+NGF/GM1
I	886 ± 69	997 ± 25	900 ± 80	992 ± 74
II	1007 ± 37	1083 ± 63	920 ± 36	992 ± 74
III	964 ± 61	1088 ± 31	871 ± 59	1098 ± 129
IV	1874 ± 74	2122 ± 83	1468 ± 206	2060 ± 77
V	3144 ± 103	3240 ± 62	2941 ± 121	3334 ± 11
VI	2174 ± 161	2004 ± 301	2282 ± 138	2281 ± 129
Cortical Layers	LESIONED			
	+VEHICLE	+GM1	+NGF	+NGF/GM1
I	613 ± 69 *	967 ± 58	1327 ± 84 *	1620 ± 70 *§
II	618 ± 34 *	996 ± 83	1383 ± 80 *	1638 ± 95 *§
III	615 ± 61 *	947 ± 40	1219 ± 52 *	1504 ± 60 *§
IV	1201 ± 100 *	1815 ± 67	2322 ± 97 *	2818 ± 115*§
V	2271 ± 128 *	3316 ± 334	4300 ± 178 *	4986 ± 406 *
VI	1614 ± 165 *	2286 ± 175	3048 ± 65 *	3675 ± 216*§

Lesioned rats received either vehicle, NGF (12 μg/day) and/or GM1 (1.5 mg/day) i.c.v. as described in methods. A group of nonlesioned animals (control) also received NGF and/or GM1. Rats (n=4/group) were sacrificed thirty days post lesion (ie: 23 days after the end of drug infusions) and the brains were processed for ChAT immunocytochemistry. Values represent mean number of cortical ChAT-IR varicosities ± S.E.M. quantified, by an operator blinded to the treatment groups, using an Image analysis system (Quantimet (920) (see methods for details). *$p < 0.05$ from control, §$p < 0.05$ from lesion NGF or GM1 treated rats, ANOVA, post-hoc Newman-Keuls test.

Figure 3.24 Electron micrographs of ChAT-IR boutons from layer V of rat cortex at the level of the mid-basalis (between 1.20 and 1.40 mm posterior to Bregma). Three representative cholinergic presynaptic terminal profiles observed in ultrathin sections from control unoperated (A-C) and lesion vehicle (D-F) treated animals are shown. Arrows indicate synapses which were confirmed in adjacent sections. Scale bar in (A) = 0.5 μm and applies to all panels.

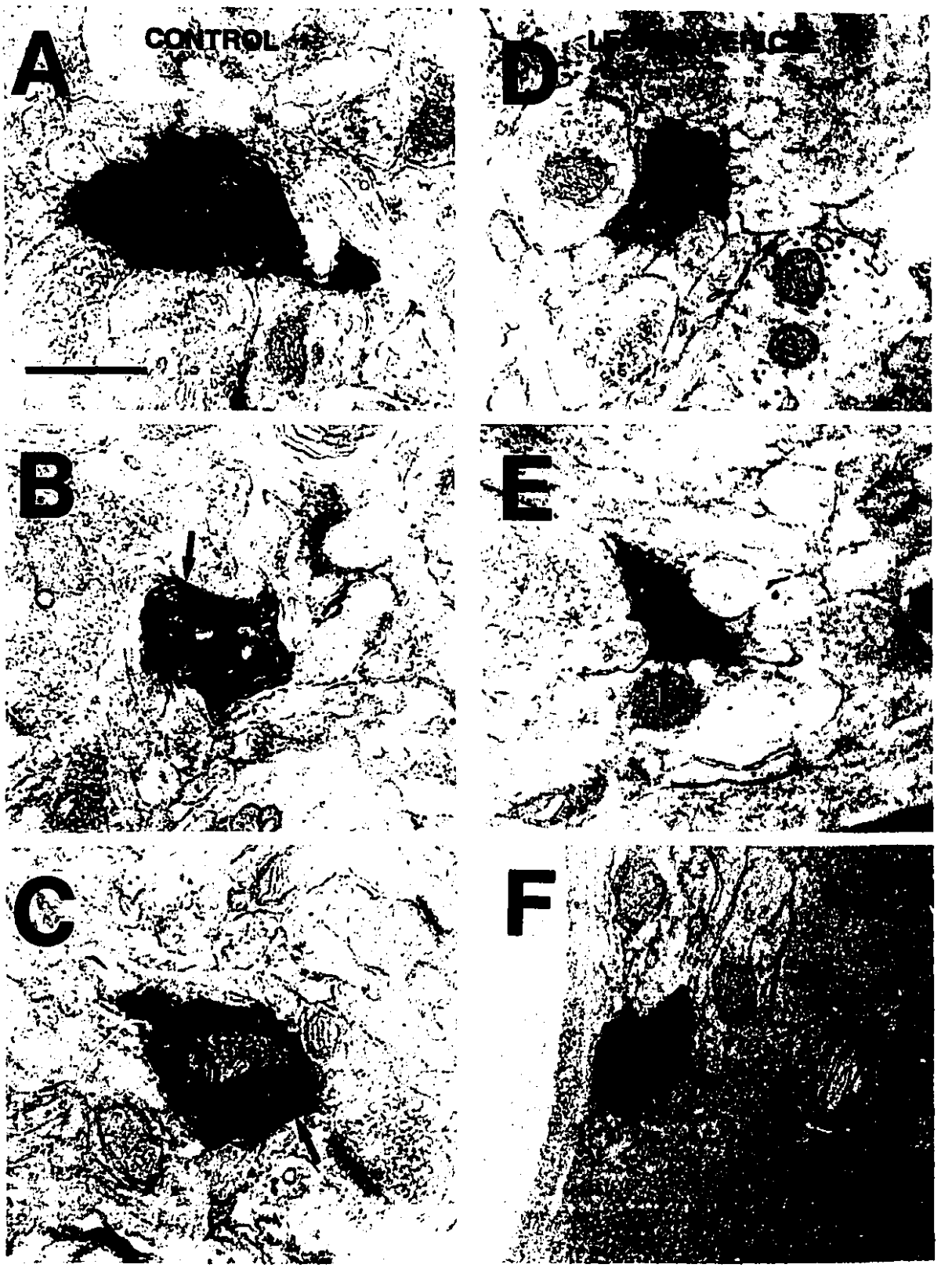

A
CONTROL
D
B
E
C
F

Figure 3.25 Electron micrographs of ChAT-IR boutons from layer V of rat cortex at the level of the mid-basalis (between 1.20 and 1.40 mm posterior to Bregma). Three representative cholinergic presynaptic terminal profiles observed in ultrathin sections from lesion GM1 (A-C) and lesion NGF (D-F) treated animals are shown. Arrows indicate synapses which were confirmed in adjacent sections. Scale bar in (A) = 0.5 μm and applies to all panels.

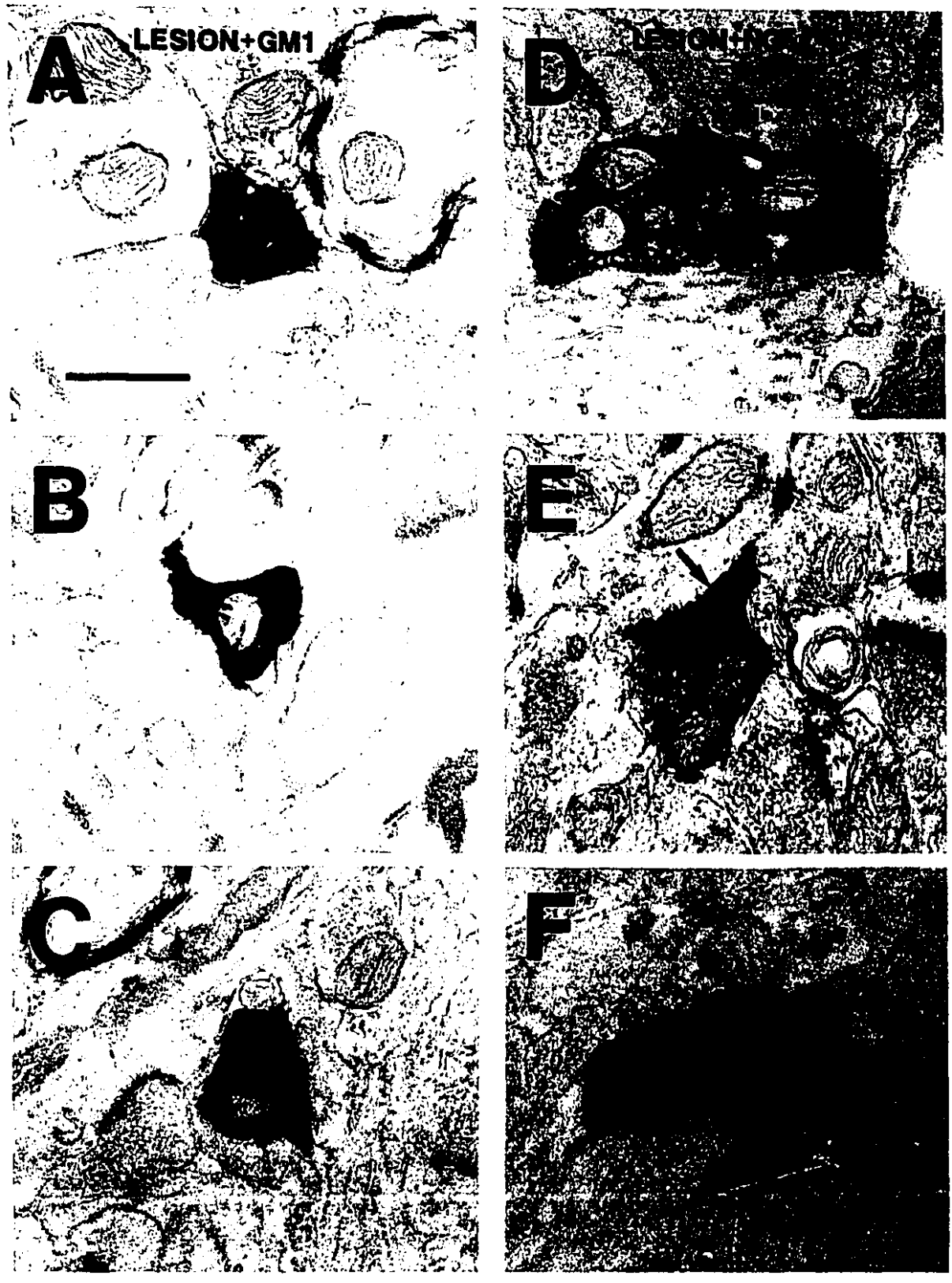

A
LESION+GM1
B
C
D
E
F

Figure 3.26 Electron micrographs of ChAT-IR boutons from layer V of rat cortex at the level of the mid-basalis (between 1.20 and 1.40 mm posterior to Bregma). Three representative cholinergic presynaptic terminal profiles observed in ultrathin sections from lesion NGF+GM1 (A-C) treated animals are shown. Arrows indicate synapses which were confirmed in adjacent sections. Scale bar in (A) = 0.5 μm and applies to all panels.

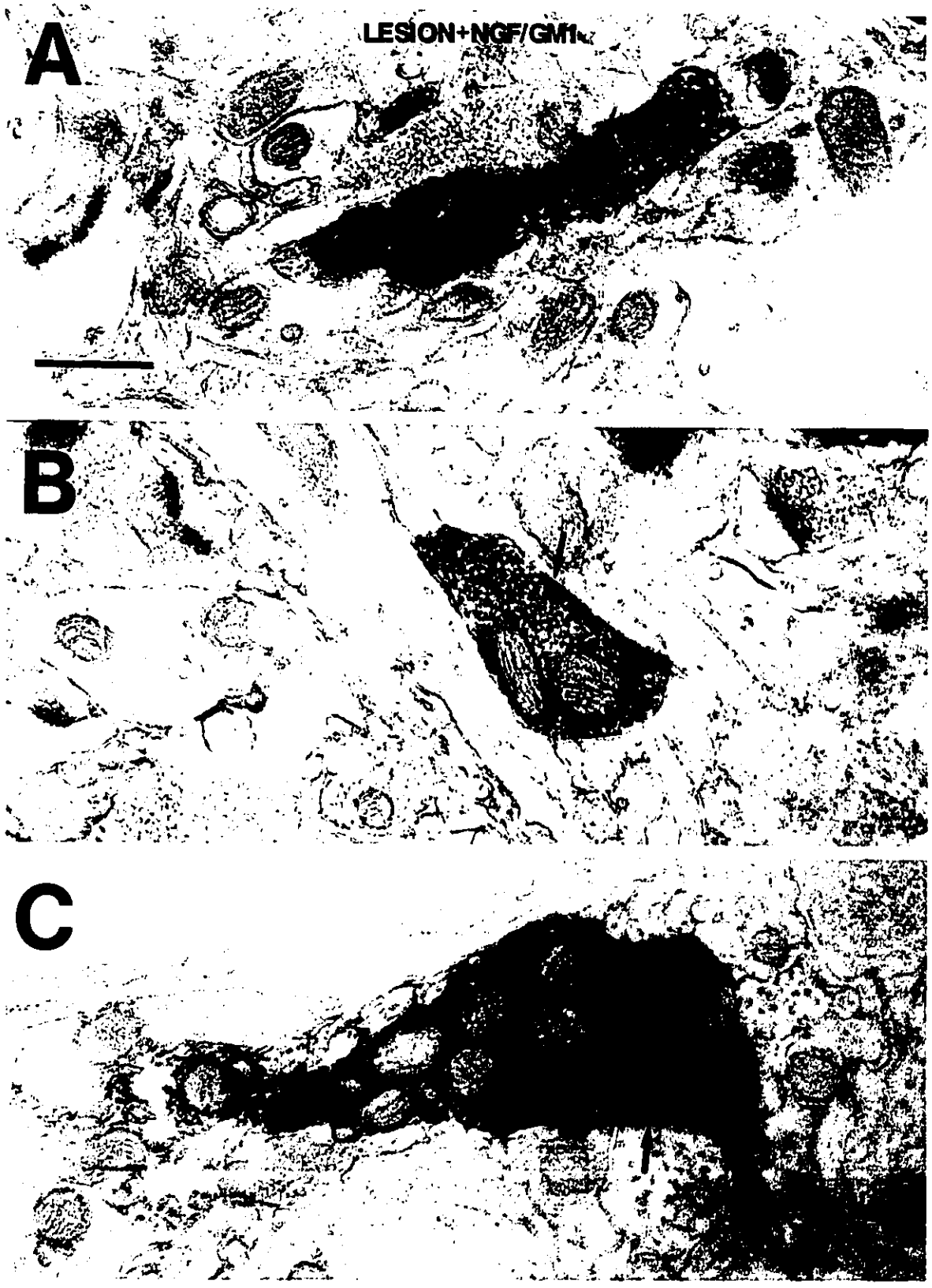
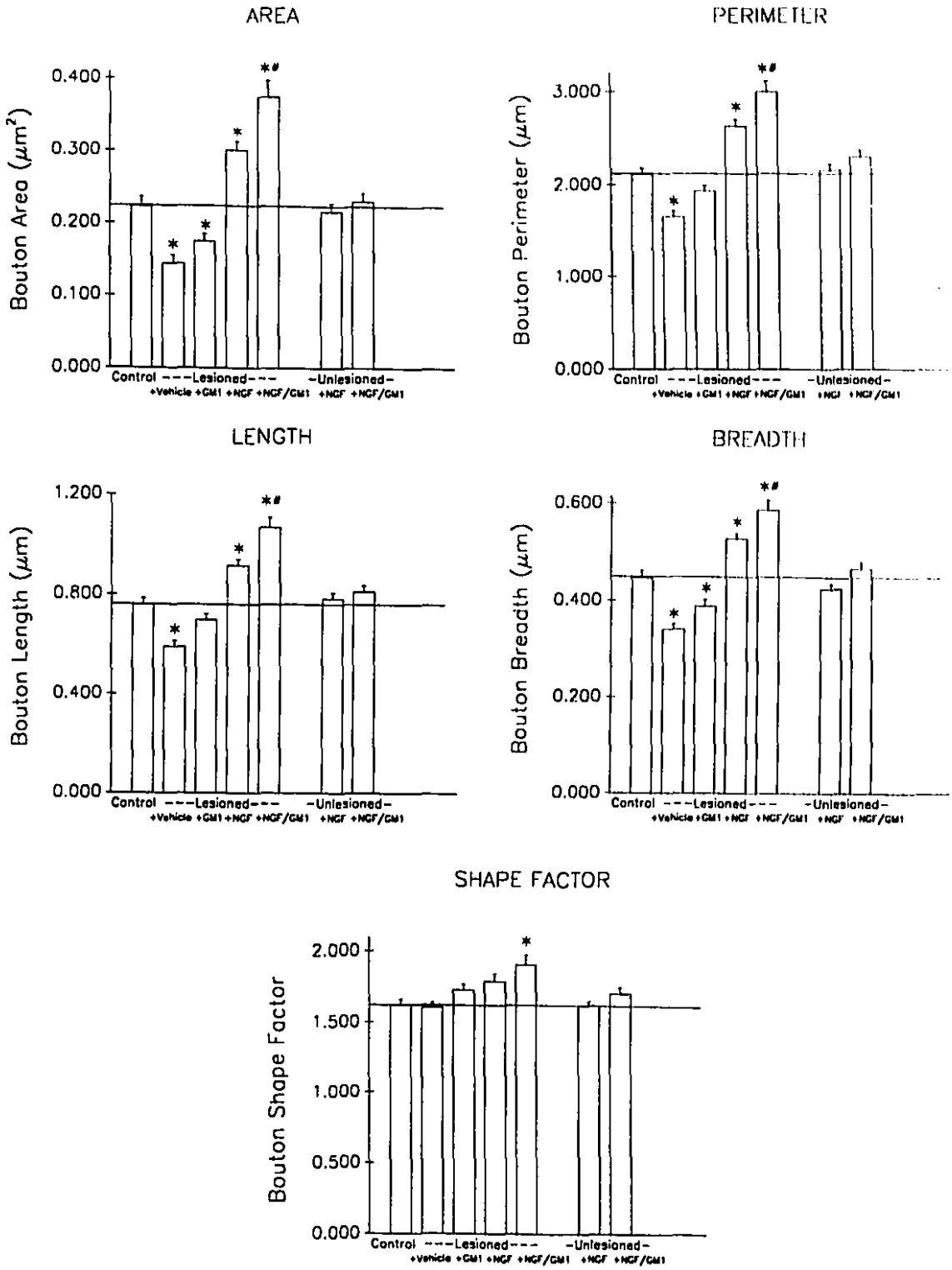

A
LESION+NGF/GM1
B
C

Figure 3.27 Quantitative morphometric analysis of cortical cholinergic boutons in layer V of rat cortex. Animals were unilaterally decorticated and received either vehicle, GM1 and/or NGF as described in methods. A group of unlesioned rats also received NGF alone or NGF+GM1 treatment. Animals (n=4/group) were sacrificed 30 days post-lesion (ie: 23 days after end of drug treatment) and the brains were processed for ChAT immunocytochemistry for EM analysis. The area, perimeter, length, breadth and shape factor of ChAT-IR boutons were quantified using an image analysis system (Quantimet 920) by an operator blinded to the treatment groups (see methods for details). *$p < 0.01$ from control, #$p < 0.01$ from lesion NGF treated group, ANOVA, post-hoc Tukey test. Error bars represent S.E.M.


AREA
Bouton Area (μm^2)
0.400
0.300
0.200
0.100
0.000
Control
Lesioned
Unlesioned
+Vehicle +GM1 +NGF +NGF/GM1 +NGF +NGF/GM1
PERIMETER
Bouton Perimeter (μm)
3.000
2.000
1.000
0.000
LENGTH
Bouton Length (μm)
1.200
0.800
0.400
0.000
BREADTH
Bouton Breadth (μm)
0.600
0.400
0.200
0.000
SHAPE FACTOR
Bouton Shape Factor
2.000
1.500
1.000
0.500
0.000

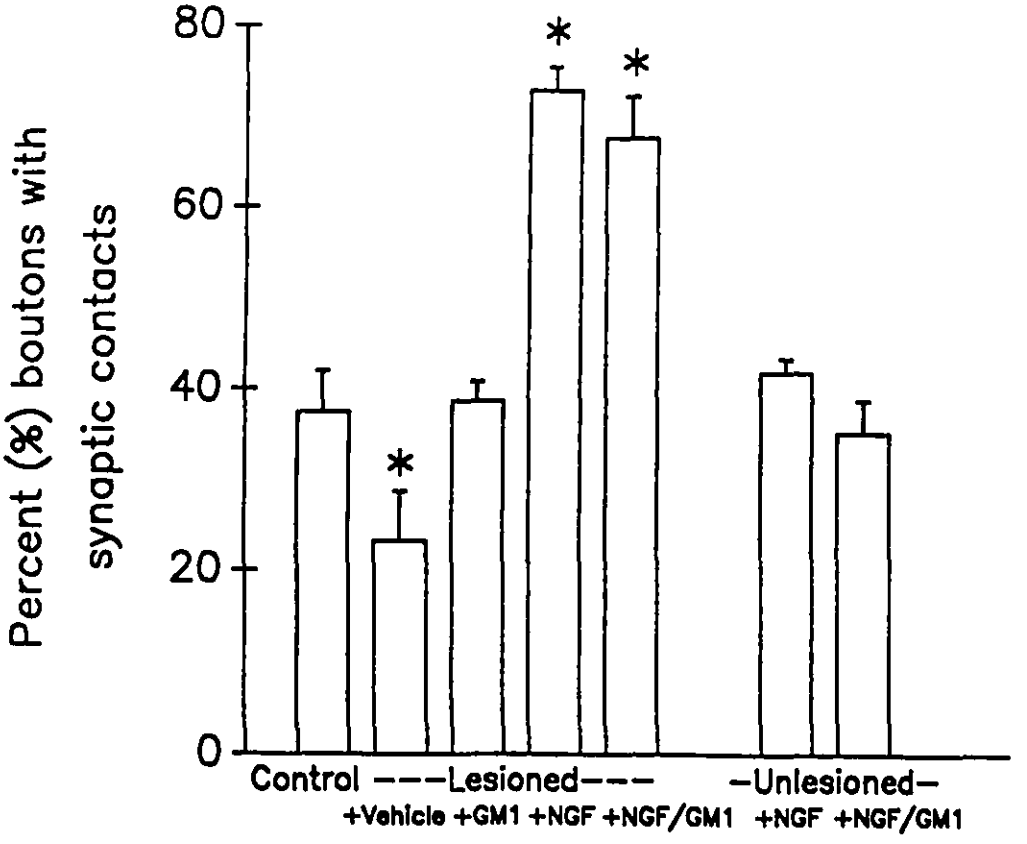


Figure 3.28 Percentage of ChAT-IR varicosity profiles with a visible synaptic contact. *$p < 0.05$ ANOVA post-hoc Tukey test.

3.2.6 Effect of lesion, NGF and/or GM1 treatment on bouton volume and synaptic area

To further examine the effects of lesion and factor administration on structural parameters of ChAT-IR boutons, serial reconstruction of these elements was undertaken. An additional three to four cholinergic boutons, selected at random, were reconstructed per group with the aid of an image analysis system. The mean volume of ChAT-IR boutons in layer V of unoperated animals was found to be $0.170 \pm 0.042\ \mu m^3$. By contrast, bouton volume of lesion vehicle treated rats was significantly reduced and the entire presynaptic terminal appeared considerably shrunken (Figures 3.29; 3.32). Reconstructed boutons of lesioned GM1 treated rats were found to be of intermediate size falling between those of control and lesion vehicle treated animals, suggesting that GM1 may be partially effective in maintaining cortical cholinergic presynaptic terminal size (Figures 3.30; 3.32). In addition, these boutons also had a high density of synaptic vesicles and were usually intensely immunoreactive, which could account for our previous observations showing augmented ChAT activity in this remaining cortical area following GM1 treatment (section 3.1). NGF treatment, on the other hand, caused a significant increase in bouton volume above control levels (Figures 3.30; 3.32). Each individual profile which formed part of the reconstruction was significantly larger than those observed in control animals. A similar occurrence was noted for lesioned NGF/GM1 treated rats where the increase in bouton size was even more apparent (Figures 3.31; 3.32). As well, the area of the bouton which was synaptic appeared enhanced in NGF or NGF/GM1 treated rats. Measures of synaptic area revealed a trend towards an increase in this parameter, as well, when compared to control values (Control: $0.00072 \pm 0.00035\ \mu m^2$; Lesion NGF: $0.00094 \pm 0.00060\ \mu m^2$; Lesion NGF/GM1: $0.00157 \pm 0.00050\ \mu m^2$).

Figure 3.29 Serial reconstruction of ChAT-IR boutons in layer V of rat cortex. Reconstructed boutons as generated with the assistance of the image analysis system are shown for control unoperated (A) and lesion vehicle (C) treated rats. Highlighted areas in the reconstructed bouton represent regions of synaptic contact. Right hand side of panels A and C shows the image when rotated 180°. EM micrographs of ChAT-IR profiles, from the middle portion of each reconstructed varicosity (indicated by white arrows in A and C) are shown for control (B) and lesion vehicle (D) treated animals. Black arrow in B indicates a synapse. Scale bar= 0.50 μm for all panels.

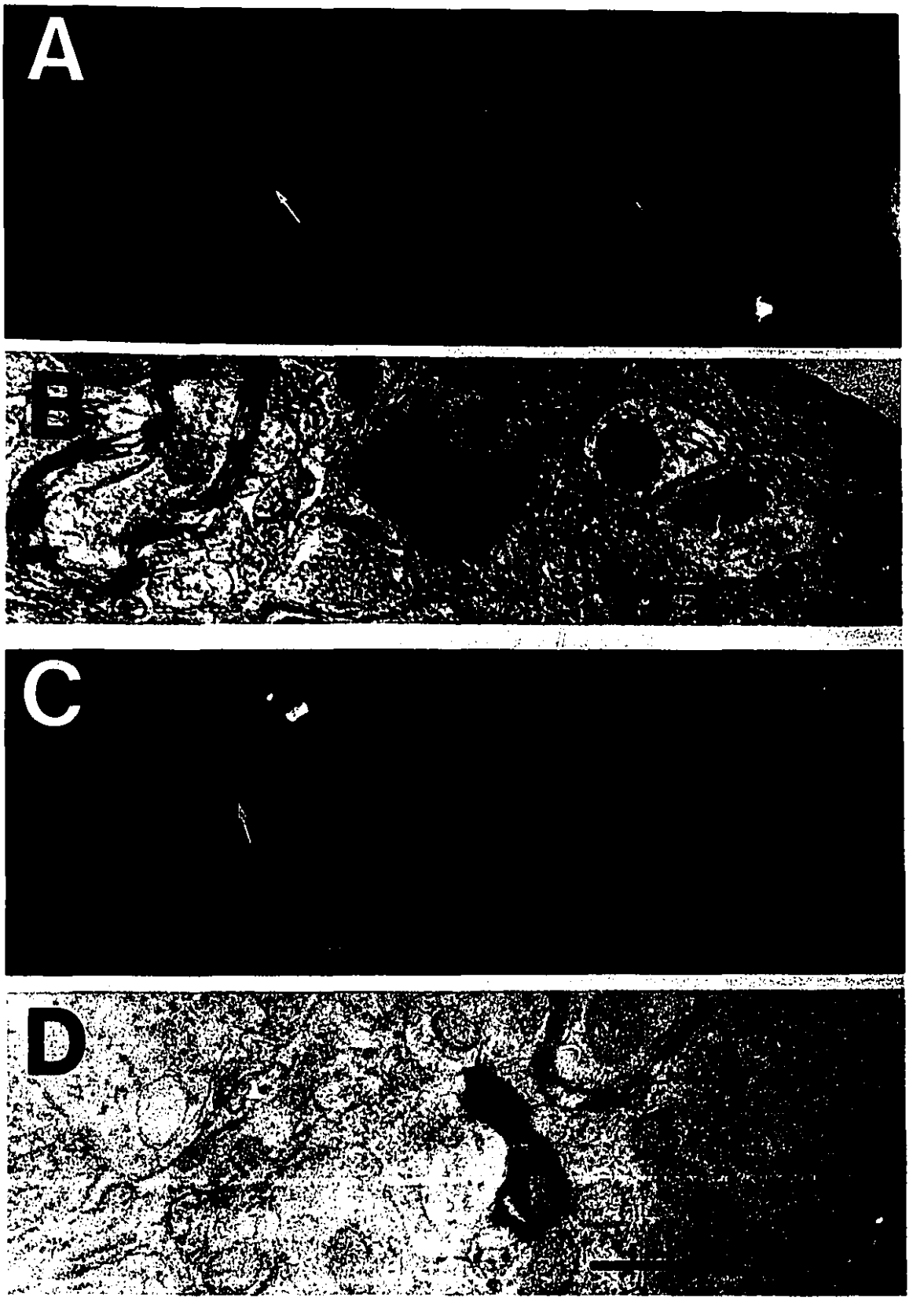

A
B
C
D

Figure 3.30 Serial reconstruction of ChAT-IR boutons in layer V of rat cortex. Reconstructed boutons as generated with the assistance of the image analysis system are shown for lesion GM1 (A) and lesion NGF (C) treated rats. Highlighted areas in the reconstructed bouton represent regions of synaptic contact. Right hand side of panels A and C shows the image when rotated 180^0. EM micrographs of ChAT-IR profiles, from the middle portion of each reconstructed varicosity (indicated by white arrows in A and C) are shown for control (B) and lesion vehicle (D) treated animals. Scale bar = 0.50 μm for all panels.

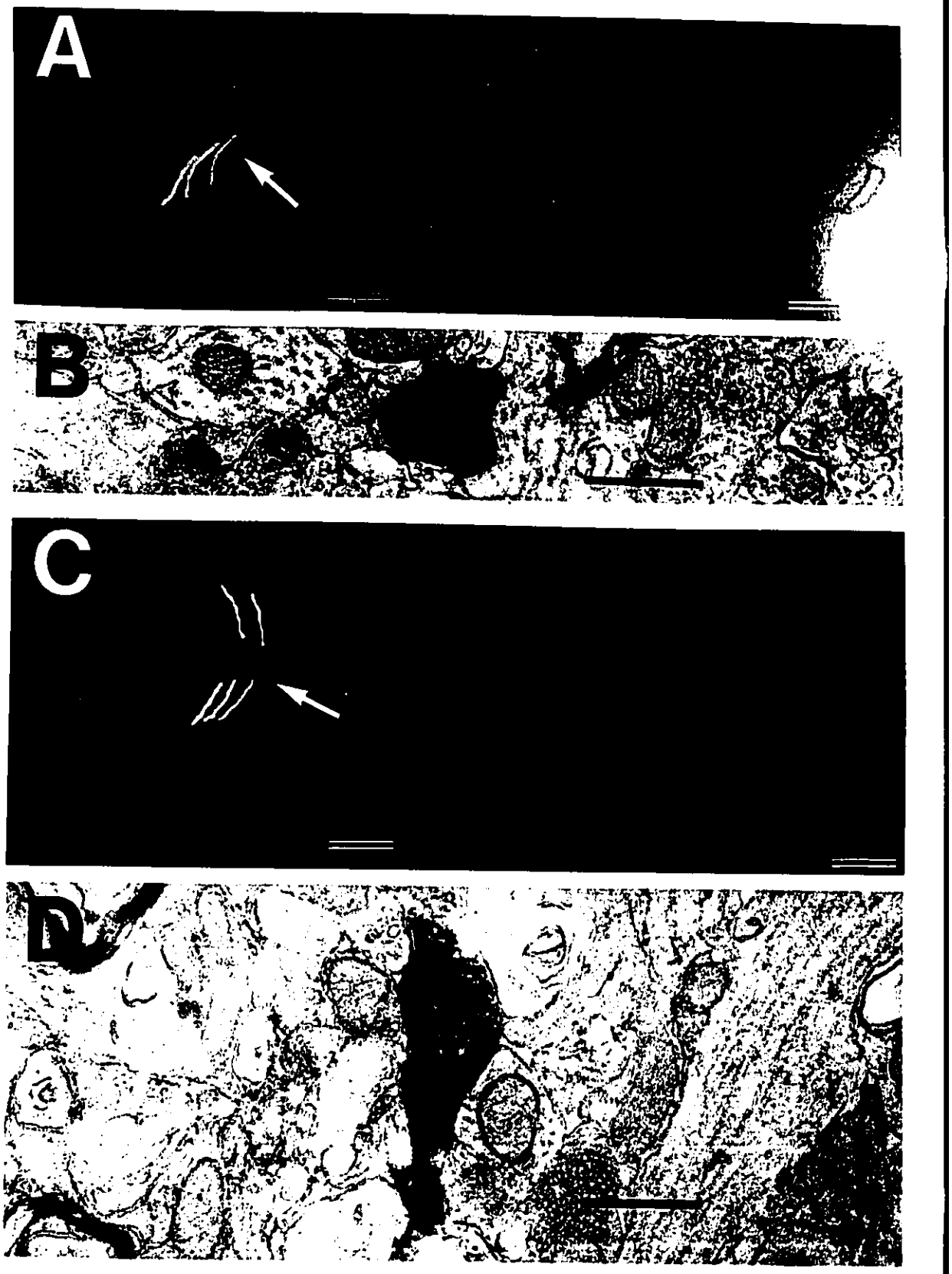
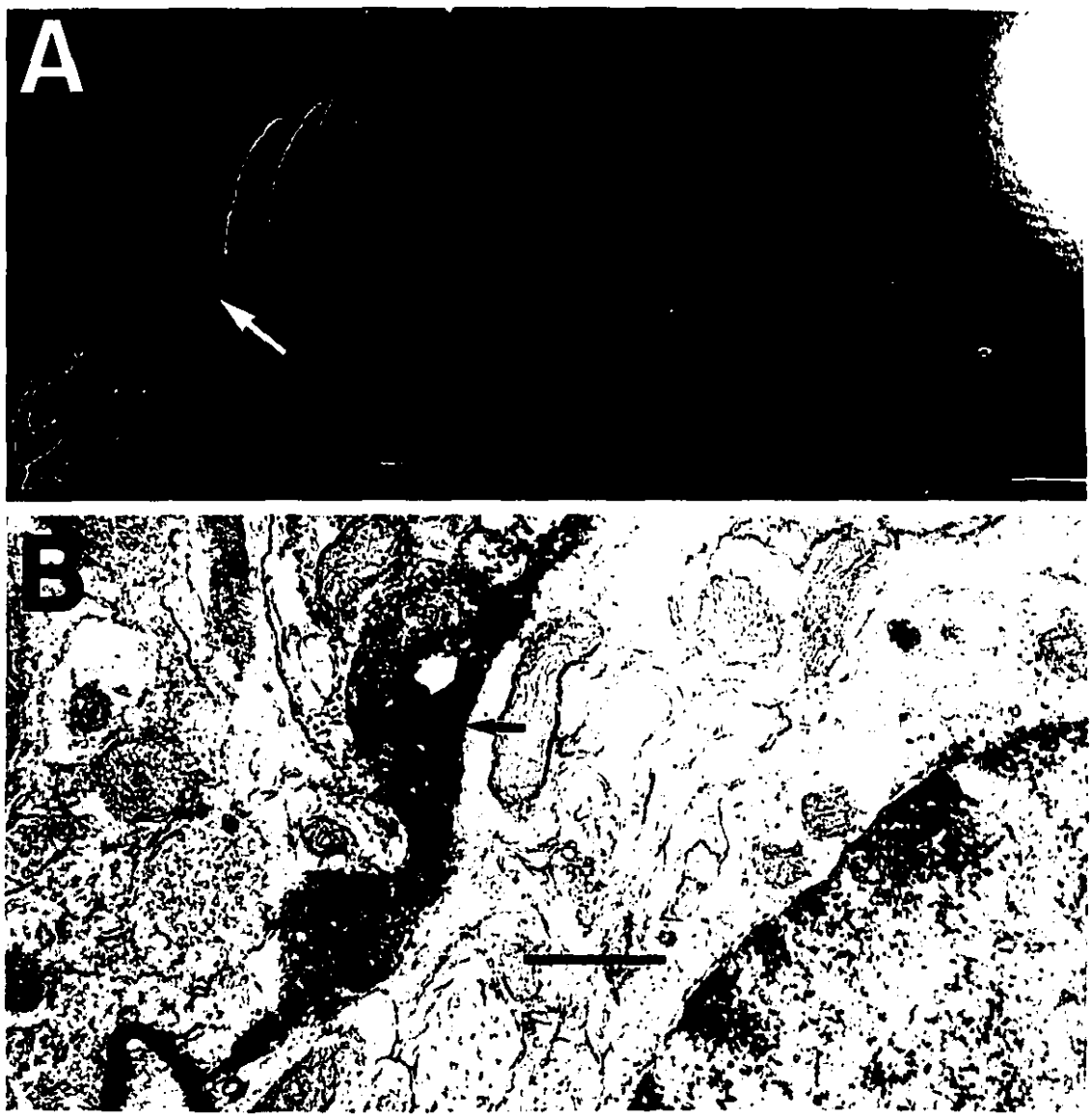

A
B
C
D

Figure 3.31 Serial reconstruction of a ChAT-IR bouton in layer V of rat cortex. A reconstructed bouton as generated with the assistance of the image analysis system is shown for a lesion NGF/GM1 (A) treated animal. Highlighted areas in the reconstructed bouton represent regions of synaptic contact. At the right hand side of panel A, the image is shown when rotated 180^0. An EM micrograph of a ChAT-IR profile, from the middle portion of the reconstructed varicosity (indicated by a white arrow in A) is shown in panel B. Black arrow in B points to synapse. Scale bar= 0.50 μm for both panels.


A
B

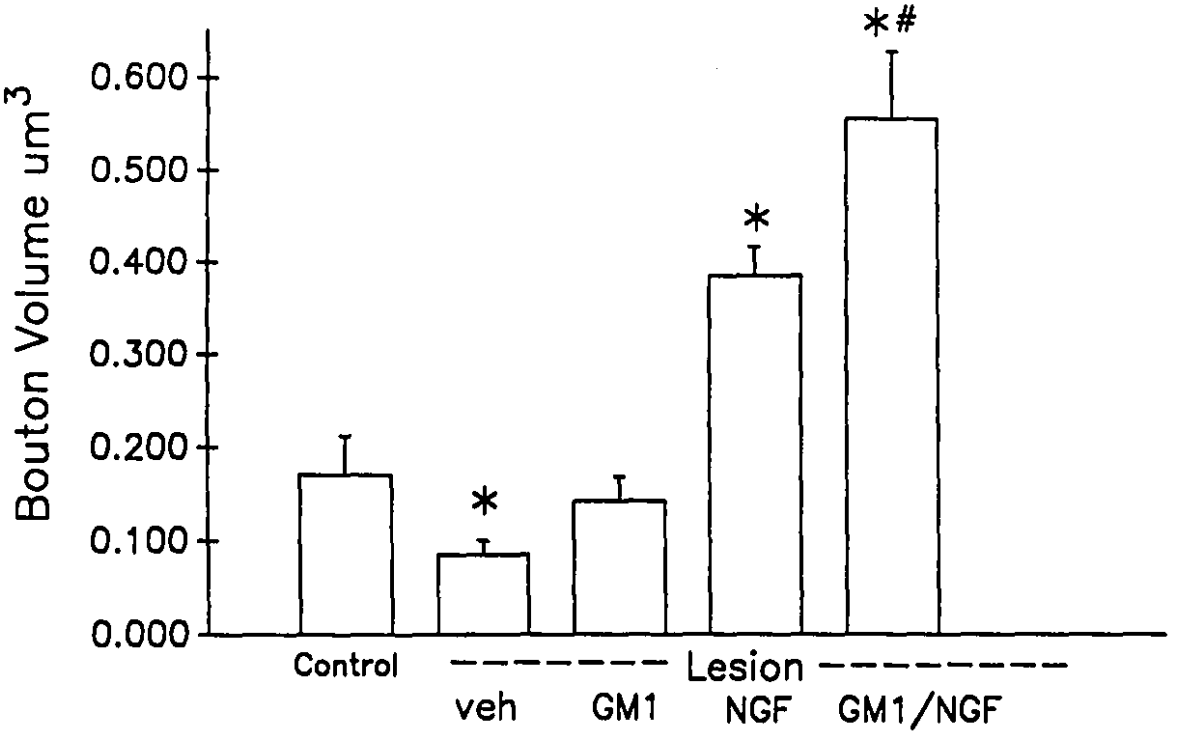


Figure 3.32 Volume of ChAT-IR boutons in layer V of rat cortex as determined from the serial reconstruction. *P<0.05 from control, #p<0.05 from lesion NGF treated group, ANOVA, post-hoc Newman-Keuls' test.

3.3 *BEHAVIORAL CORRELATES OF UNILATERAL DEVASCULARZING CORTICAL LESIONS AND TROPHIC FACTOR TREATMENT*

The behavioral effects of the cortical lesion, GM1 and/or NGF treatment were also assessed in adult rats. For this purpose, adult male Wistar rats were pretrained in two behavioral tasks, passive avoidance and the Morris water maze. Following acquisition of the tasks, the rats were unilaterally decorticated and received, i.c.v. via minipump, either vehicle, GM1 (750 μg/day) and/or NGF (6 μg/day) for 14 days and were subsequently retested in the tasks at 30 days post-lesion (ie: 16 days after end of drug treatment). Animals were sacrificed following behavioral studies at 52 days post-lesion. The neurochemical and immunocytochemical effects of the lesion and/or trophic factor administration in several brain areas were also assessed at this extended post-lesion time point.

3.3.1 *Effect of the cortical lesion, GM1 and/or NGF treatment on rat body weight*

All vehicle treated cortically lesioned rats showed a significant decrease in body weight 10 and 30 days post-lesion but recovered normal body weight by the 52 nd post-lesion day (Figure 3.33). By contrast, lesioned rats which received GM1 treatment recovered this lesion-induced initial weight loss more rapidly (Figure 3.33). Rats which were treated with either NGF or NGF/GM1 showed the slowest weight recovery, although by the 52 nd post-lesion day their body weight did not differ significantly from control animals (Figure 3.32). No other physiological differences were apparent between the control and lesioned groups. In a previous study (Elliott et al., 1989) such decorticated animals were tested for olfactory, auditory or sensory-related deficits but, other than exhibiting a small but significant increase in overnight locomotor activity and an increase in foot faults on a horizontal ladder these animals did not differ from control unoperated animals.

3.3.2 *Alterations in passive avoidance behavior by decortication, GM1 and/or NGF treatment*

Prior to surgery all rats were trained in a passive avoidance task. On the first training trial,

after a 3 day habituation to the box, all rats entered the dark compartment of the box within 20 seconds. When retested, 15 minutes or 24 hours after having received a footshock, none of the animals failed to stay on the illuminated side of the box. The animals were subsequently retested in the task thirty days post lesion on two consecutive days [Test day 1 (Figure 3.34A) and Test day 2 (Figure 3.34B)]. Animals which entered the dark side of the box on test day 1 were reshocked. Analysis of variance revealed significant differences among the groups ($F(4, 84) = 13.33$, $p<0.01$) and trial days ($F(1,84) = 23.29$, $p<0.01$). The interaction between group and trial day was also significant ($F(4, 84) = 3.75$, $p<0.05$). Newman-Keuls comparison ($p<0.05$) of the latency time means showed that lesion vehicle and lesion GM1 treated rats have retention deficits in this task and that exogenous GM1 treatment facilitated task reacquisition. By contrast, NGF and NGF/GM1 treated animals did not differ in performance when compared to unoperated control animals (Figure 3.34).

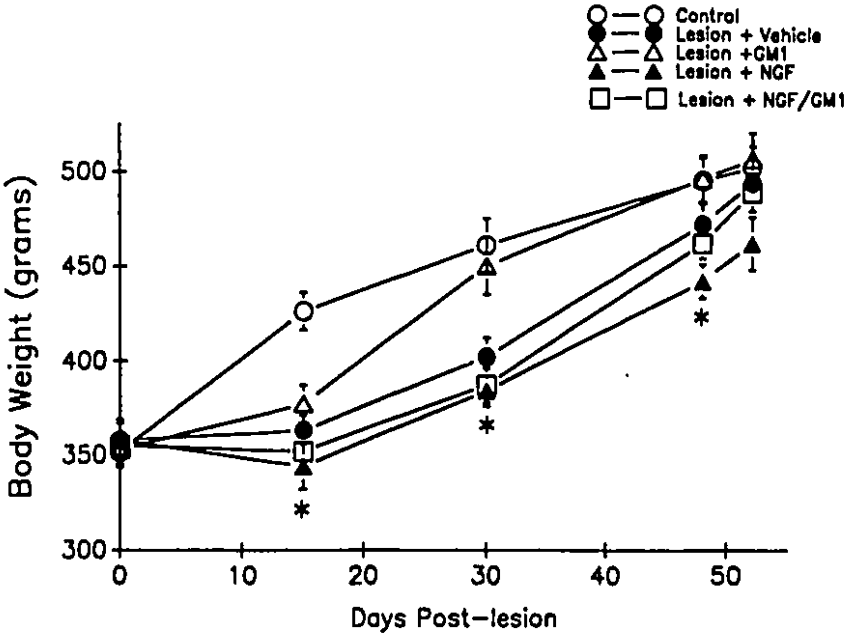


Figure 3.33 Mean rat body weight for animals in each group at various post-lesion and treatment times. *$p<0.05$ from control on day 14 for all lesioned groups. On day 30, *$p<0.05$ from control for all lesioned groups excepting Lesion + GM1. On day 42, only Lesion+NGF treated animals were significantly (*$p<0.05$) different from control. ANOVA, post-hoc Newman-Keuls' test.

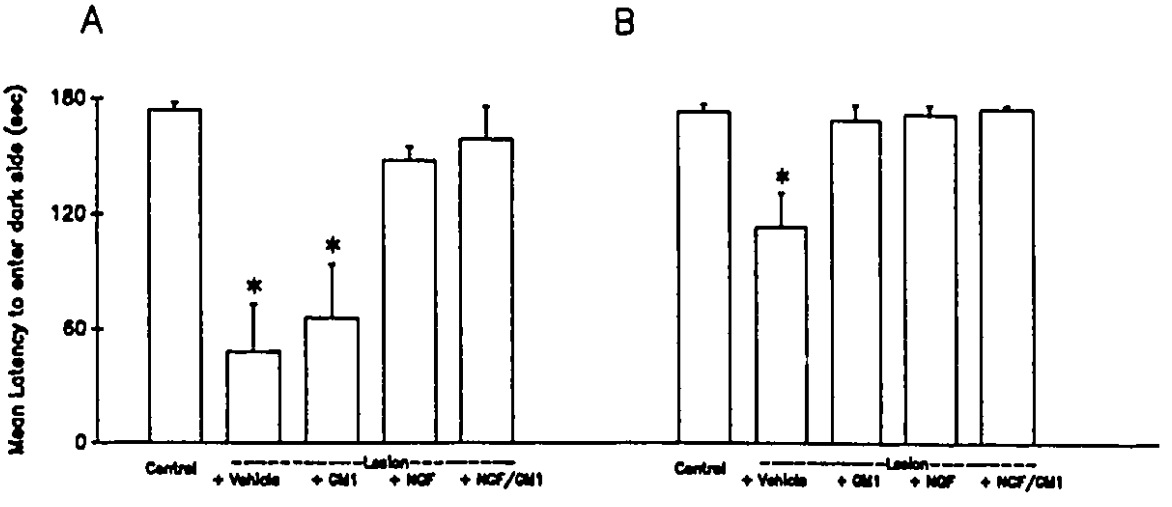


Figure 3.34 Passive avoidance (A) retention and (B) reacquisition. Unoperated rats were trained in the task and after acquisition were either left unoperated or were lesioned and treated immediately with vehicle, NGF (6 μg/day) and/or GM1 (750 μg/day) for 2 weeks as described. Animals were retested in the passive avoidance task on days 30 (A= Test day 1) and 31 (B= Test day 2) post-lesion. *$p < 0.05$, ANOVA post-hoc Newman-Keuls test.

3.3.3 Attenuation of cortical lesion induced performance deficits in the Morris water Maze by GM1 and/or NGF treatment

Unoperated rats quickly learned the water maze task. By the 2nd training day most rats could locate the hidden platform within 20-30 seconds (Figure 3.35A). By the fourth trial on day four of training only two rats failed to use a direct route to find the platform and were therefore not used in this study. When retested after thirty days, analysis of variance showed significant effects of group ($F(4,168)=45.90$, $p=0.001$), trial days ($F(3,168)=85.77$, $p=0.001$) and group by trial day interaction ($F(12,168)=18.56$, $p=0.001$). Newman-Keuls post-hoc comparisons of the group means, on the 30th post-lesion day, demonstrated that both lesion vehicle and lesion GM1 treated animals showed significantly ($p<0.05$) longer latencies to find the platform than all other groups. By contrast, escape latency times for NGF or NGF/GM1 treated lesioned animals did not differ significantly from control animals (Figure 3.35B). Although the latency to escape the water was similar for lesion vehicle or GM1 treated rats on the first day of testing, analysis of the swim profiles showed that the lesion vehicle treated animals spent more time circling the perimeter of the pool than did rats from other groups (Figure 3.36). On post-lesion day 31, only lesion vehicle treated rats showed significantly ($p<0.05$) longer escape latency times when compared to control rats. GM1 treated lesioned rats quickly reacquired the task whereas lesioned rats which received vehicle required more training trials and showed a more random search strategy (Figure 3.36). Two weeks following reacquisition of the task (post-lesion day 47) mean escape latency times did not differ significantly among the experimental groups (ANOVA, $F(4,42)=34.30$, $p=0.56$) (Figure 3.35). However, lesion vehicle treated rats still failed to use a direct route to locate the hidden platform. When the platform was moved to another quadrant of the pool and was elevated above the surface of the water, lesioned rats which received vehicle had the shortest escape latency times in the initial trials (Figure 3.37). Animals in the other groups, in the initial trials, spent more time searching for the platform in its previous location before heading towards the visible platform.

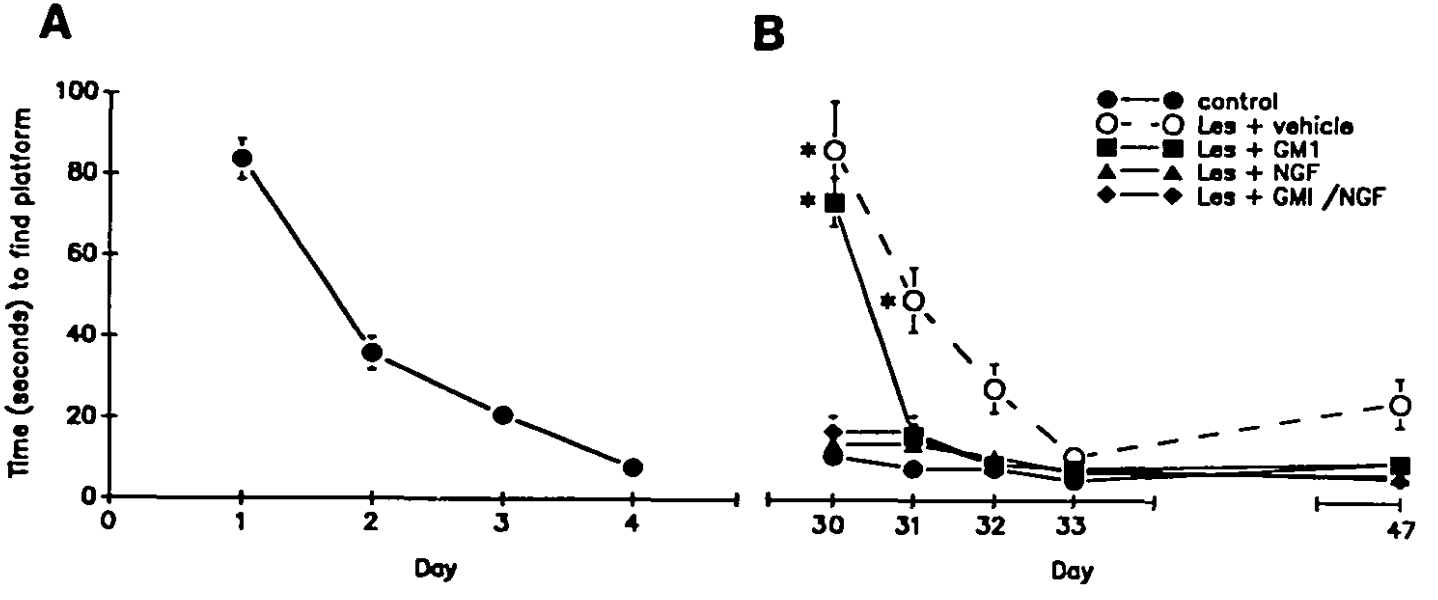


Figure 3.35 (A) Pre-operative and (B) post-operative mean escape latency times for rats tested in the Morris water maze with a hidden platform. Unoperated animals were trained to find the platform as described in methods. After acquisition animals were lesioned and treated, i.c.v. via minipump for 14 days, with either vehicle, NGF (6 μg/day) and/or GM1 (750 μg/day) as described. All animals were retested in the task beginning 30 days post-lesion (ie: 2 weeks after the end of drug treatment) for 4 consecutive days. After reacquisition all animals were retested once more 2 weeks later (post-lesion day 47). *$p < 0.05$ ANOVA, post-hoc Newman-Keul's test.

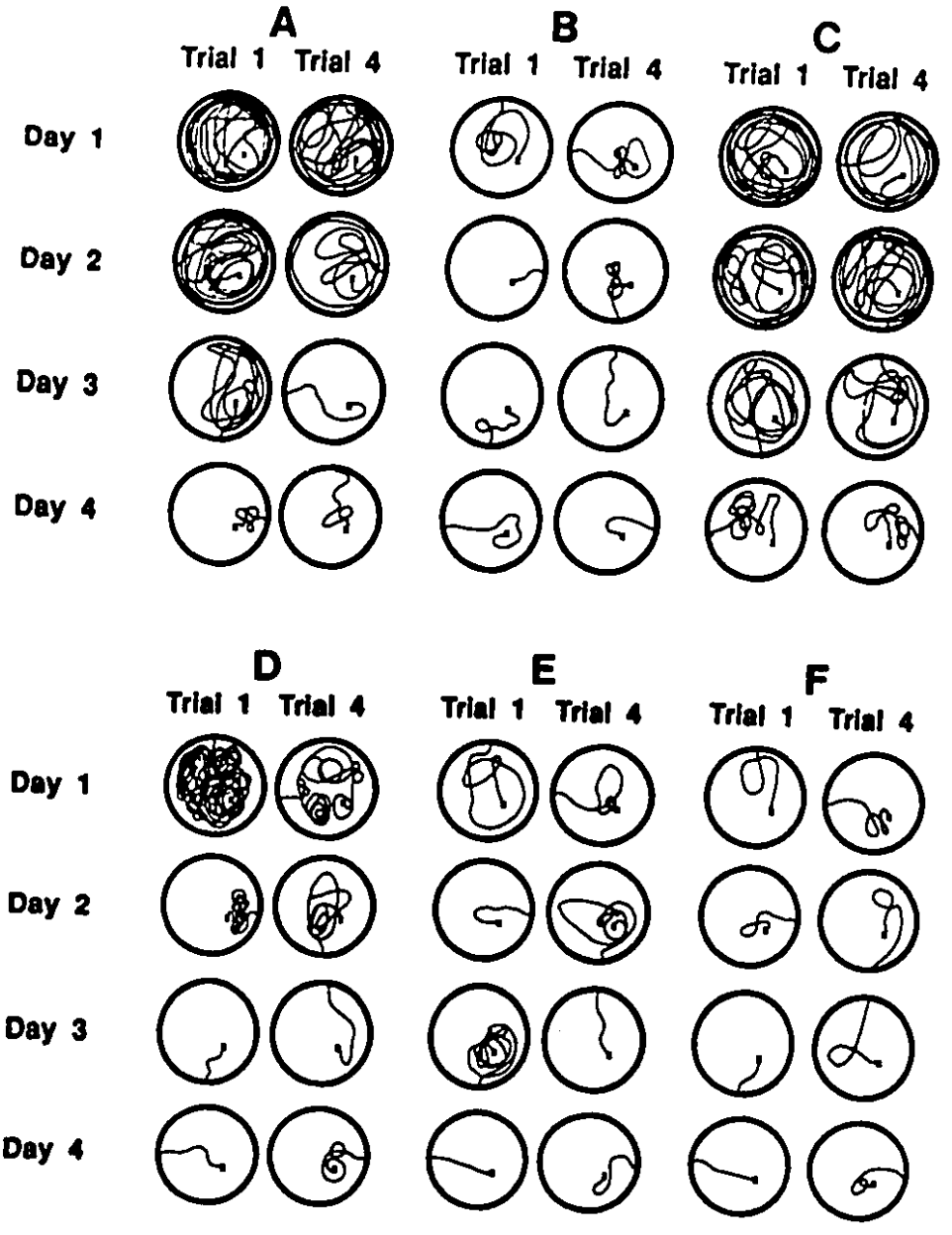


Figure 3.36 (A) Typical swim patterns of pre-operative rats on trials 1 and 4 of each test day. After acquisition animals were lesioned and treated, i.c.v. via minipump for 14 days, with either vehicle, NGF (6 μg/day) and/or GM1 (750 μg/day) as described. Swim patterns of (B) unoperated rats, (C) lesion vehicle, (D) lesion GM1, (E) lesion NGF and (F) Lesion NGF+GM1 treated rats on trails 1 and 4 of days 30 (Day 1), 31 (Day 2), 32 (Day 3) and 33 (Day 4) post-lesion. Swim patterns shown are from a rat, within each group, whose escape latency times most closely matched that of the mean.

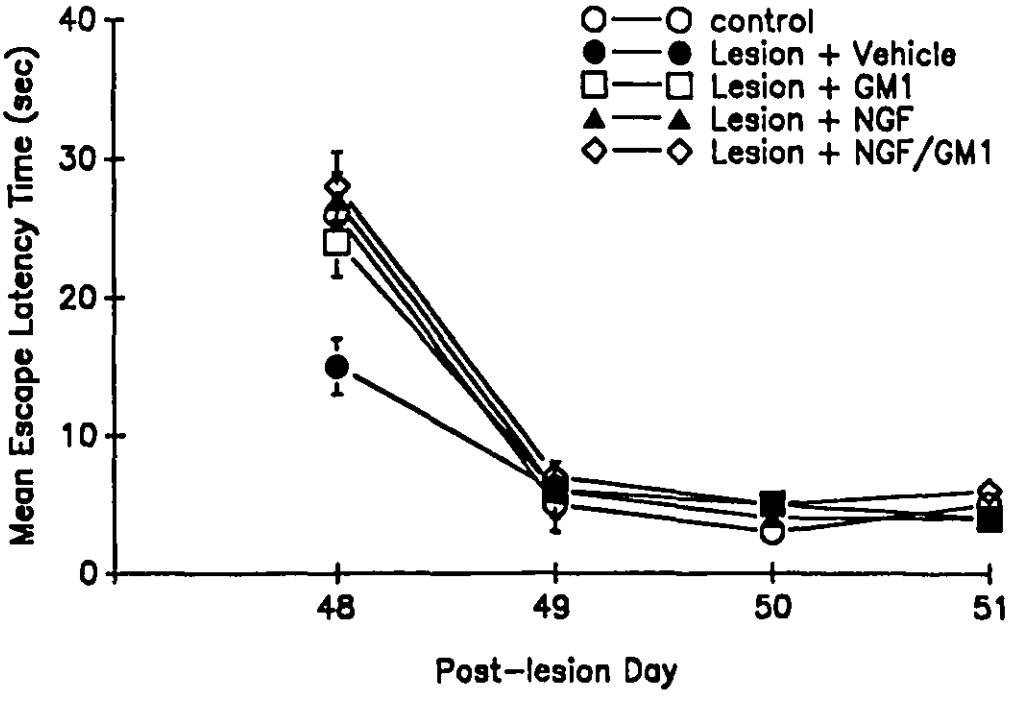


Figure 3.37 Mean escape latency times for all groups in the Morris water Maze after the platform was moved to a new location and was made visible.

3.3.4 Assessment of lesion extent

Rats were sacrificed, following the end of behavioral studies, by decapitation or by aldehyde fixation for neurochemical and immunocytochemical analysis, respectively. Coronal brain sections obtained from each animal were analyzed under the microscope prior to microdissection or quantification to assess and record the lesion extent. Rats used for the data analysis had a complete loss of the frontal 1 & 2 and parietal 1 as well as a partial loss of the frontal 3, parietal 2 and occipital neocortical regions. The corpus callosum was spared in the more anterior regions but was however, in some animals, thinner or absent in more posterior areas. Several animals were discarded from the study since their lesions did not conform to criteria. These included : one lesion vehicle (LV1), one lesioned GM1 (LG3) and one lesioned NGF/GM1 (LN/G5) treated rat who along with loss of the corpus callosum also had small damage to the dorso-lateral striatum; one lesion vehicle (LV5) treated animal with a misplaced cannula which damaged the septum and who had enlarged ventricles; one lesion NGF (LN8) and one vehicle (LV2) treated rat with slight thinning of the dorsal hippocampus. Interestingly, whereas LV1 performed poorly in the water maze (excessive circling and required the most trials to acquire the task), LG3 and LN/G5 despite the additional striatal damage did not differ in water maze performance from other GM1 or NGF/GM1 treated lesioned rats. LV5 showed signs of hyperactivity and attempted to jump off the platform in the water maze several times. This animal was also the quickest to enter the dark side of the passive avoidance box. LN8 and LV2 did not differ in their performance from other animals within their respective groups but were also not used in the data analysis.

3.3.5 Alterations in cholinergic and GABAergic markers

3.3.5a ChAT activity

Fifty-two days following unilateral decortication, ChAT activity in the ipsilateral NBM of vehicle treated animals was significantly decreased. However, the deficit in ChAT activity was not greater than that noted on post-lesion day 30 following such a lesion (section 3.1). ChAT activity in the contralateral NBM did not differ from control values (Table 3.16). The

remaining ipsilateral cortex adjacent to the lesion site had control levels of ChAT activity as did the contralateral equivalent cortical area (Table 3.16). These data extend the previous findings of this thesis with respect to the time course of NBM cholinergic deficits following decortication. NGF or GM1 treatment given for 2 weeks intracerebroventricularly, beginning immediately following surgery, maintained ChAT activity in the ipsilateral NBM of lesioned rats up to 38 days following termination of treatment. Furthermore, a significant increase in ChAT activity, above control levels, was noted in the remaining ipsilateral cortex (Table 1). Rats which received both NGF and GM1 showed a potentiation in both NBM and cortical ChAT activity which was significantly greater than that observed in animals which received either factor alone (Table 3.16). At this post-lesion time point ChAT activity was not altered in other ipsilateral or contralateral subcortical brain regions such as the striatum (Table 1), septum (control: 52.65 $\pm$ 1.67, Lesion+Vehicle: 50.96 $\pm$ 0.88; ipsilateral), vertical limb of the diagonal band (VDB) (control: 48.33 $\pm$ 1.02, Lesion + Vehicle: 49.46 $\pm$ 0.78; ipsilateral), horizontal limb of the diagonal band (HDB) (Table 1), or hippocampus (Table 1). ChAT activity in subcortical brain areas not invaded by the lesion was not altered by GM1 or NGF treatment. An exception to this however, was the striatum where ChAT activity was augmented, significantly above control values, by both NGF or NGF/GM1 treatment (Table 3.16). In contrast to the ipsilateral NBM and cortex, no potentiation of striatal ChAT activity was observed in animals which received both NGF and GM1. Furthermore, a trend towards increased ChAT activity was noted in the ventral hippocampus of NGF and NGF/GM1 treated lesioned rats. However, only ChAT activity in the contralateral ventral hippocampus of NGF/GM1 treated animals was significantly greater than control values (Table 3.16).

3.35b Choline uptake

In parallel with the ChAT activity results, choline uptake was also unaffected in the remaining ipsilateral cortex adjacent to the lesion site and in the contralateral equivalent area of cortically lesioned vehicle treated rats (Table 3.17). Lesioned animals which received GM1 or NGF showed increases, above control values, in both sodium dependent and sodium independent cortical choline uptake (Table 3.17). Animals treated with both NGF and GM1

showed an increase in cortical choline uptake which was significantly greater than that induced by either factor alone (Table 3.17). Striatal choline uptake was also unaffected by the lesion or by GM1 treatment. However, NGF treatment appeared to increase striatal choline uptake (Table 3.17), although not significantly above control values. This suggests that NGF effects on striatal choline uptake may be transient. Moreover, in agreement with the ChAT activity results, no potentiation of striatal choline uptake was noted when NGF was given in combination with GM1. Hippocampal choline uptake was not affected by the lesion or by the various treatments.

3.35c GAD activity

To assess possible alterations occurring in GABAergic function at this lesion time point, GAD activity (nMole $^{14}CO_2$ released/mg protein/hour) was measured in subcortical and cortical brain areas of these lesioned animals. GAD activity, examined 52 days post-lesion, in unilaterally cortically devascularized rats was not affected by the lesion or treatments in the ipsilateral or contralateral NBM, cortex, striatum, HDB, dorsal or ventral hippocampus (Table 3.18). Moreover, GAD activity in the septum (control: 207 ± 21; Lesion vehicle: 192 ± 23; Lesion + GM1: 215 ± 11 ; Lesion + NGF: 206 ± 14; and Lesion + NGF/GM1: 221 ± 13) or VDB (control: 195 ± 21; Lesion+ vehicle: 176 ± 16; Lesion + GM1: 186 ± 14; Lesion + NGF: 196 ± 19; Lesion NGF/GM1: 188 ± 12) was similarly unaffected by the lesion or treatments.

Table 3.16: Effect of GM1 and/or NGF on ChAT activity in 52 day post-lesion adult unilaterally decorticated rats.

	IPSILATERAL	CONTRALATERAL
NBM		
Control	67.51 ± 1.75	71.81 ± 0.91
Lesion + Vehicle	35.66 ± 0.94*	68.18 ± 2.32
Lesion + GM1	66.68 ± 1.85	65.63 ± 0.68
Lesion + NGF	66.22 ± 4.81	63.35 ± 2.22
Lesion + NGF/GM1	93.45 ± 3.00*§	63.09 ± 2.73
CORTEX		
Control	45.34 ± 0.75	43.70 ± 1.25
Lesion + Vehicle	47.39 ± 1.19	42.80 ± 0.77
Lesion + GM1	59.03 ± 0.93*	43.90 ± 0.85
Lesion + NGF	58.54 ± 1.72*	42.60 ± 1.31
Lesion + NGF/GM1	89.10 ± 2.32*§	44.20 ± 2.92
STRIATUM		
Control	100.91 ± 2.87	101.33 ± 1.19
Lesion + Vehicle	101.61 ± 3.40	104.04 ± 2.42
Lesion + GM1	106.81 ± 1.29	99.85 ± 1.02
Lesion + NGF	127.56 ± 1.92*	137.16 ± 2.20*
Lesion + NGF/GM1	132.52 ± 1.52*	139.05 ± 2.05*
HDB		
Control	56.08 ± 1.76	55.35 ± 1.52
Lesion + Vehicle	52.98 ± 2.51	54.46 ± 2.42
Lesion + GM1	56.11 ± 1.61	53.85 ± 2.51
Lesion + NGF	54.85 ± 1.18	54.59 ± 1.00
Lesion + NGF/GM1	54.76 ± 1.58	55.14 ± 1.57
DORSAL HIPPOCAMPUS		
Control	43.47 ± 2.87	43.92 ± 1.19
Lesion + Vehicle	46.38 ± 3.40	47.35 ± 2.42
Lesion + GM1	45.65 ± 1.29	45.96 ± 1.02
Lesion + NGF	42.86 ± 1.92	50.14 ± 2.20
Lesion + NGF/GM1	47.87 ± 1.52	43.12 ± 2.05
VENTRAL HIPPOCAMPUS		
Control	56.31 ± 0.48	56.48 ± 0.96
Lesion + Vehicle	58.88 ± 0.42	55.24 ± 1.40
Lesion + GM1	56.53 ± 1.28	55.43 ± 2.01
Lesion + NGF	61.68 ± 2.45	63.76 ± 2.71
Lesion + NGF/GM1	62.72 ± 1.96	67.43 ± 2.54♦

Numbers represent mean ± S.E.M. nMole ACh/mg protein/hr. n= 6 animals/group, see methods for details. * $p<0.01$ from Control group, § $p<0.01$ from Lesion+GM1 and Lesion+NGF group, ♦ $p<0.05$ from Control group, ANOVA post-hoc Tukey test.

Table 3.17: Effect of GM1 and/or NGF on choline uptake in unilaterally decorticated rats.

	Sodium Dependent		Sodium Independent	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
CORTEX				
Control	8.42 ± 0.53	7.94 ± 0.51	2.15 ± 0.28	2.04 ± 0.18
Lesion + Vehicle	8.48 ± 0.54	8.15 ± 0.54	2.35 ± 0.28	2.35 ± 0.26
Lesion + GM1	14.04 ± 1.01*	8.55 ± 0.44	3.90 ± 0.17*	2.73 ± 0.17
Lesion + NGF	12.90 ± 0.41*	9.45 ± 1.31	3.64 ± 0.26*	2.15 ± 0.33
Lesion + NGF/GM1	20.88 ± 1.00*§	8.06 ± 0.30	4.83 ± 1.07*	2.33 ± 0.20
STRIATUM				
Control	20.10 ± 1.44	21.60 ± 3.84	5.40 ± 0.76	5.20 ± 1.05
Lesion + Vehicle	17.50 ± 2.47	18.46 ± 2.23	4.56 ± 0.61	4.42 ± 0.53
Lesion + GM1	18.92 ± 1.40	20.16 ± 1.23	4.87 ± 0.42	6.16 ± 1.07
Lesion + NGF	25.38 ± 1.40	29.56 ± 3.29	7.44 ± 1.27	7.16 ± 1.20
Lesion + NGF/GM1	28.35 ± 3.39	29.19 ± 1.71	7.90 ± 0.83	7.98 ± 1.53

Animals were lesioned and received, beginning immediately post-lesion, NGF (6μg/day) and/or GM1 (750 μg/day) i.c.v. via an Alzet 2002 osmotic minipump for 2 weeks. Rats were sacrificed 52 days post-lesion. Numbers represent mean ± S.E.M. pmoles ^{3}H choline/mg protein/4 min. n = 6 animals/group, see materials and methods for details, *$p<0.01$ from Control group, §$p<0.01$ from Lesion + GM1 and Lesion + NGF groups, ANOVA post-hoc Tukey test.

Table 3.16: Effect of GM1 and/or NGF on GAD activity in 52 day post-lesion adult unilaterally decorticated rats.

	IPSILATERAL	CONTRALATERAL
NBM		
Control	216 ± 30	225 ± 15
Lesion + Vehicle	230 ± 17	215 ± 22
Lesion + GM1	220 ± 14	233 ± 21
Lesion + NGF	221 ± 11	215 ± 14
Lesion + NGF/GM1	225 ± 12	223 ± 13
CORTEX		
Control	279 ± 17	285 ± 20
Lesion + Vehicle	273 ± 10	274 ± 16
Lesion + GM1	285 ± 12	277 ± 14
Lesion + NGF	281 ± 11	290 ± 11
Lesion + NGF/GM1	275 ± 12	293 ± 15
STRIATUM		
Control	458 ± 14	455 ± 16
Lesion + Vehicle	488 ± 27	444 ± 18
Lesion + GM1	466 ± 12	459 ± 12
Lesion + NGF	450 ± 11	460 ± 19
Lesion + NGF/GM1	452 ± 18	461 ± 21
HDB		
Control	198 ± 20	201 ± 18
Lesion + Vehicle	211 ± 22	199 ± 12
Lesion + GM1	212 ± 16	215 ± 13
Lesion + NGF	215 ± 13	221 ± 12
Lesion + NGF/GM1	199 ± 15	207 ± 19
DORSAL HIPPOCAMPUS		
Control	112 ± 5	110 ± 10
Lesion + Vehicle	119 ± 11	115 ± 8
Lesion + GM1	115 ± 9	109 ± 12
Lesion + NGF	120 ± 19	107 ± 11
Lesion + NGF/GM1	117 ± 17	114 ± 10
VENTRAL HIPPOCAMPUS		
Control	191 ± 21	195 ± 17
Lesion + Vehicle	170 ± 23	185 ± 18
Lesion + GM1	175 ± 15	176 ± 20
Lesion + NGF	182 ± 13	177 ± 11
Lesion + NGF/GM1	176 ± 19	199 ± 14

Numbers represent mean ± S.E.M. nMole $^{14}CO_2$ released/mg protein/hr. n= 6 animals/group, see methods for details. ANOVA, with post-hoc Tukey test confirmed no significant differences among groups.

3.3.6 Immunocytochemical light microscopic analysis

3.36a Nissl

Cresyl Violet staining showed that no apparent neuronal loss occurs in the rat NBM, up to 52 days following extensive unilateral cortical lesions (Figure 3.38 A-B). Cell number in other subcortical brain areas such as the septum, VDB, HDB, striatum or hippocampus also appeared unaffected. However, some neuronal loss and an increase in non-neuronal cell density were detected in the ventrolateral nucleus of the dorsal thalamus (Figure 3.38 C-F). This was also evident in Nissl stained material from GM1 and/or NGF treated lesioned rats.

3.36b ChAT IMMUNOREACTIVITY

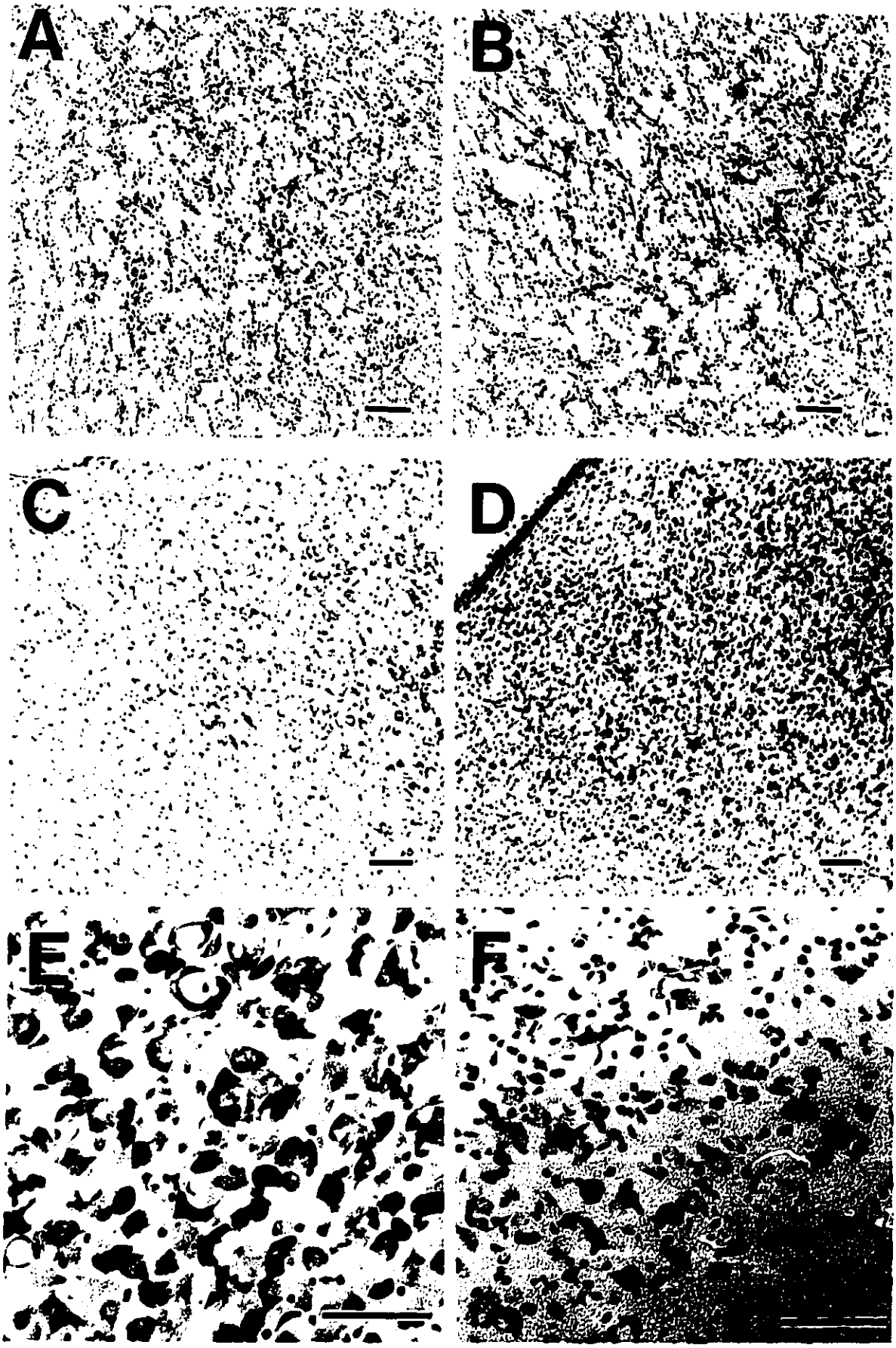
Quantitative light microscopic analysis of NBM ChAT-IR and $p75^{NGFR}$-IR neurons and neurites was also undertaken for this lesion time point. As previously described, for quantitative purposes, the NBM was divided into anterior, mid and posterior portions each of which were further subdivided into dorsal and ventral areas. This study shows that cholinergic neurons in the NBM can persist in a shrunken state at least up to 52 days after decortication (Table 3.19, Figure 3.39). The majority of affected neurons were again located in the dorsal mid basalis area where cell shrinkage approached 45% in animals of the present study. Cholinergic neurons in the ventral mid and anterior basalis are more modestly affected as reflected by decreases in mean cross sectional areas of 29 and 24% respectively. However, these areas are more significantly affected by the lesion at this time point when compared with effects at 30 days post-lesion (~12% decrease in cell size; Table 3.11). ChAT-IR neurons in the posterior NBM were not significantly affected by the lesion (Table 3.20). A decrease in NBM ChAT immunoreactivity was also noted in the ipsilateral mid dorsal NBM of vehicle treated lesioned rats. Moreover, 52 days after cortical lesioning, cholinergic neurite length in the NBM is also diminished (Table 3.20). Significant decreases in ChAT-IR neuritic length occurred only in the ipsilateral dorsal mid NBM (Figure 3.39, Table 3.20). However, the quantification results show a trend towards decreased ChAT-IR neuritic length in the ventral mid NBM as well. In agreement with the observations for Nissl substance,

cholinergic cell density (ie: number of ChAT-IR neurons per area) was unaltered throughout the NBM of lesion vehicle treated rats (Table 3.19). Continuous GM1 (750 μg/day) or NGF (6 μg/day) administration to lesioned rats for 2 weeks beginning immediately post-lesion preserves the size (Table 3.20A) of cholinergic neurons and maintains the ChAT-IR neuritic network (Table 3.20B) in the NBM up to 38 days after termination of treatment. This effect was noted in all NBM areas affected by the lesion (anterior, dorsal and ventral mid NBM). In lesioned rats which received both NGF and GM1 mean cross sectional area of NBM ChAT-IR neurons and neuritic length were similarly preserved in these regions. Although not significantly different from control values, a trend towards an increase in the ChAT-IR neurite network in the NBM, particularly in the ventral mid NBM, was noted with NGF/GM1 treatment (Table 3.20B).

In the remaining neocortex, the ChAT-IR fiber network of lesion vehicle treated rats appeared significantly decreased when compared to control animals (Figure 3.40). By contrast, that in lesioned rats which received GM1 or NGF treatment did not seem to differ from control rats. However, animals which received both GM1 and NGF tended to have a richer network of ChAT-IR fibers (Figure 3.40).

Cholinergic neurons or fibers in the septum, VDB, HDB and hippocampus appeared unaltered by the lesion or treatments. As well, ChAT-IR fiber density in the amygdala was also apparently unaffected by the cortical lesion or treatments. In the striatum, few hypertrophic ChAT-IR neurons were noted in NGF or NGF/GM1 treated lesioned rats (Figure 3.41). These neurons, were restricted to striatal areas either beneath the lesion site or adjacent to the lateral ventricles. In addition, when compared with control, lesion vehicle and lesion GM1 treated rats, lesioned rats which received NGF or NGF/GM1, tended to show ChAT-IR fibers in striatal areas adjacent to the lateral ventricles. (Figure 3.41F).

Figure 3.38 Staining for Nissl substance in the (A-B) NBM of (A) control unoperated and (B) lesion vehicle treated rats and in the (C-D) ventrolateral nucleus of the dorsal thalamus of (C) control unoperated and (D) lesion vehicle treated animals. Higher magnification of the dorsal thalamus is shown in E and F for control and lesion vehicle treated rats, respectively. Scale bars: A and B = 125 μm; C and D = 100 μm: E and F = 50 μm.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B
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E
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Figure 3.39 ChAT-IR neurons in the mid portion of the NBM of (A) control unoperated, (B) lesion vehicle, (C) lesion GM1, (D) lesion NGF and (E) lesion NGF+GM1 treated rats. Neurons in the dorsal and ventral portions of the nucleus, as indicated by the arrows, are shown at higher magnification in respectively, (a) and (a_1) for control unoperated, (b) and (b_1) for lesion vehicle, (c) and (c_1) for lesion GM1, (d) and (d_1) for lesion NGF and (e) and (e_1) for lesion NGF+GM1 treated animals. Scale bar = 200 μm for A-E and 25 μm for a/a_1-e/e_1.

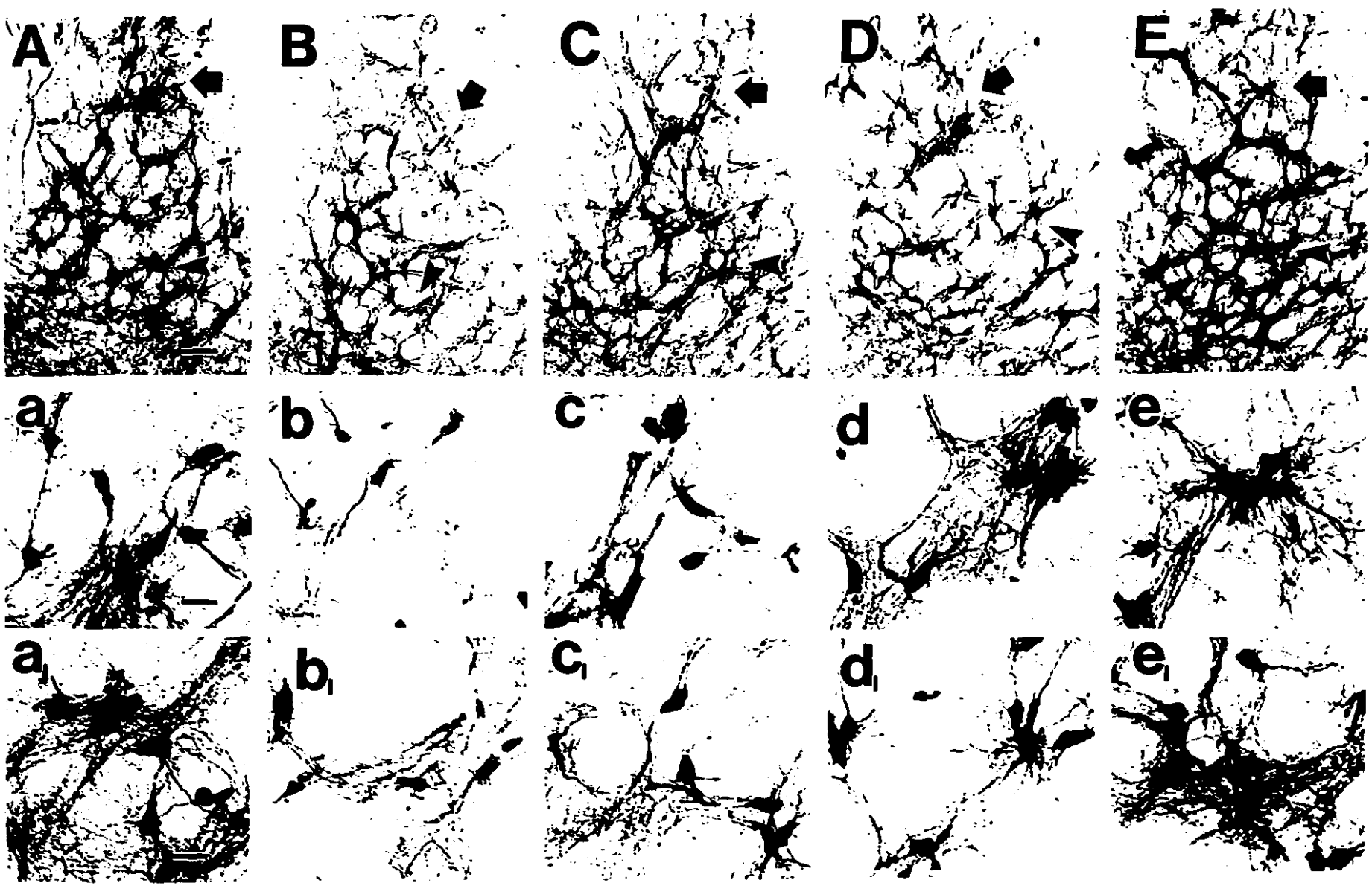
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Figure 3.40 Dark field photomicrographs of ChAT-IR fibers in layer V of (A) control unoperated, (B) lesion vehicle, (C) lesion GM1, (D) lesion NGF and (E) lesion NGF+GM1 treated rats. Animals were sacrificed after behavioral studies (ie: 52 days post-lesion or 38 days after end of drug administration) and the brains were processed for ChAT immunocytochemistry as described in methods. Scale bar = 45 μm for all panels.

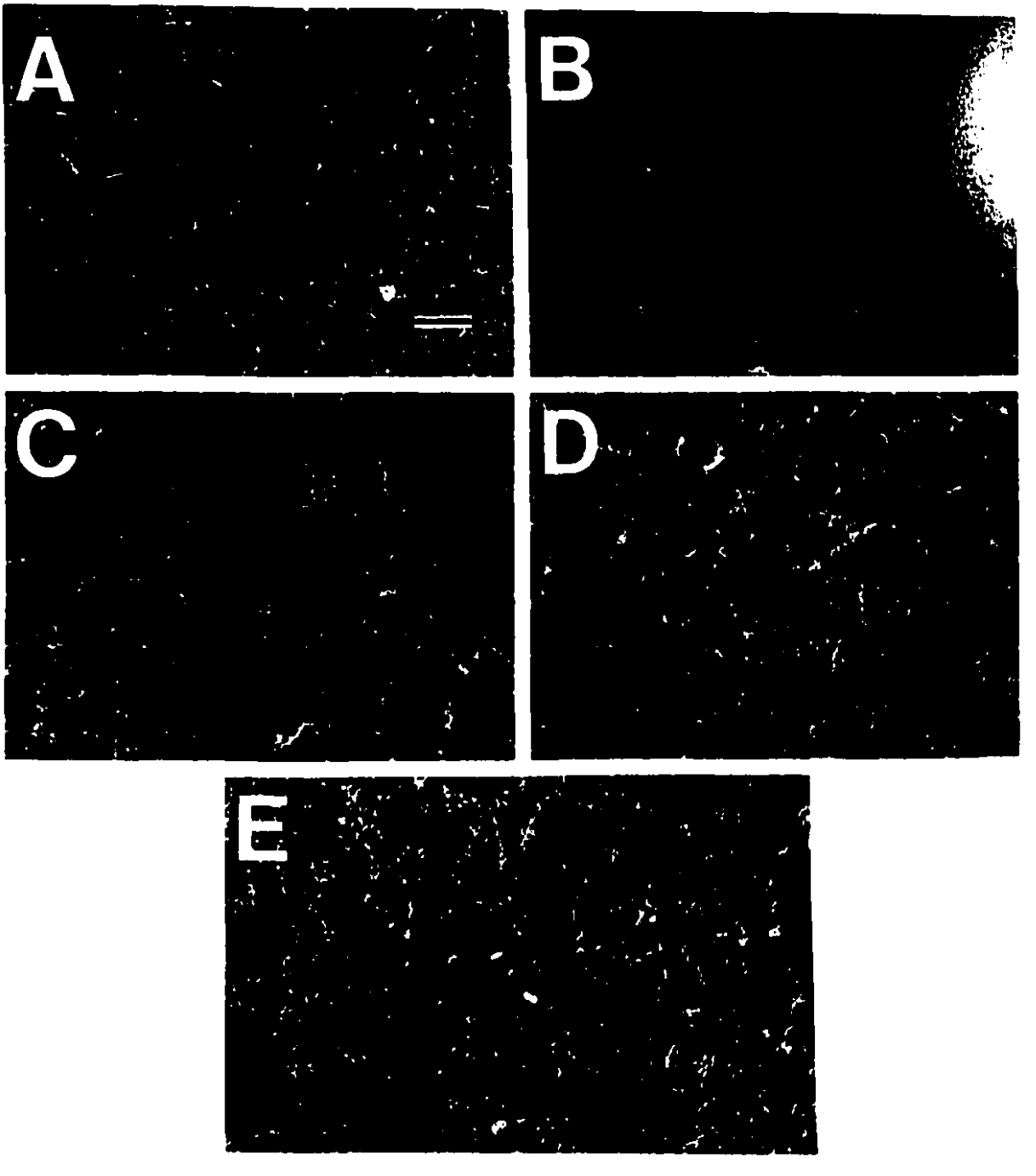
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Table 3.19 ChAT and p75^{NGFR}-IR cell density in discrete regions of the rat NBM

A ANTERIOR NBM

GROUP	ChAT-IR Neurons		NGFr-IR Neurons	
	ipsilateral	contralateral	ipsilateral	contralateral
Control	30 ± 2	27 ± 5	38 ± 9	39 ± 4
L+Veh	33 ± 3	36 ± 2	38 ± 9	33 ± 5
L+GM1	36 ± 2	32 ± 5	38 ± 7	39 ± 10
L+NGF	36 ± 3	36 ± 2	37 ± 6	32 ± 7
L+N/G	33 ± 4	34 ± 5	30 ± 9	34 ± 9

B POSTERIOR NBM

GROUP	ChAT-IR Neurons		NGFr-IR Neurons	
	ipsilateral	contralateral	ipsilateral	contralateral
Control	32 ± 3	33 ± 2	34 ± 7	32 ± 4
L+Veh	29 ± 2	33 ± 3	35 ± 4	38 ± 5
L+GM1	32 ± 3	30 ± 3	33 ± 9	32 ± 11
L+NGF	34 ± 3	35 ± 3	34 ± 5	31 ± 7
L+N/G	33 ± 4	37 ± 2	30 ± 6	35 ± 6

C DORSAL MID NBM

GROUP	ChAT-IR Neurons		NGFr-IR Neurons	
	ipsilateral	contralateral	ipsilateral	contralateral
Control	33 ± 2	38 ± 4	34 ± 6	31 ± 3
L+Veh	31 ± 2	36 ± 4	38 ± 7	36 ± 3
L+GM1	31 ± 2	31 ± 2	36 ± 8	33 ± 4
L+NGF	33 ± 2	31 ± 4	35 ± 6	37 ± 5
L+N/G	35 ± 3	36 ± 4	32 ± 7	34 ± 9

D VENTRAL MID NBM

GROUP	ChAT-IR Neurons		NGFr-IR Neurons	
	ipsilateral	contralateral	ipsilateral	contralateral
Control	43 ± 3	46 ± 4	40 ± 6	33 ± 9
L+Veh	41 ± 2	42 ± 4	42 ± 14	39 ± 9
L+GM1	41 ± 2	39 ± 2	45 ± 4	45 ± 10
L+NGF	41 ± 2	43 ± 3	36 ± 6	36 ± 7
L+N/G	49 ± 3	51 ± 5	36 ± 9	41 ± 11

Cortically lesioned animals (L) were immediately administered vehicle (Veh), GM1 (750μg/day), NGF (6μg/day) or both agents in combination (N/G). Animals n=3-4/group were sacrificed 52 days post-lesion (5 weeks following the end of drug infusions) and were processed for ChAT or p75^{NGFR} immunocytochemistry. See methods for details. Values represent Abercrombie corrected number of ChAT or p75^{NGFR}-IR neurons per field in each region of the rat NBM (see methods for details).

TABLE 3.20

(A) Mean Cross Sectional area (μm^2) of NBM ChAT immunoreactive neurons

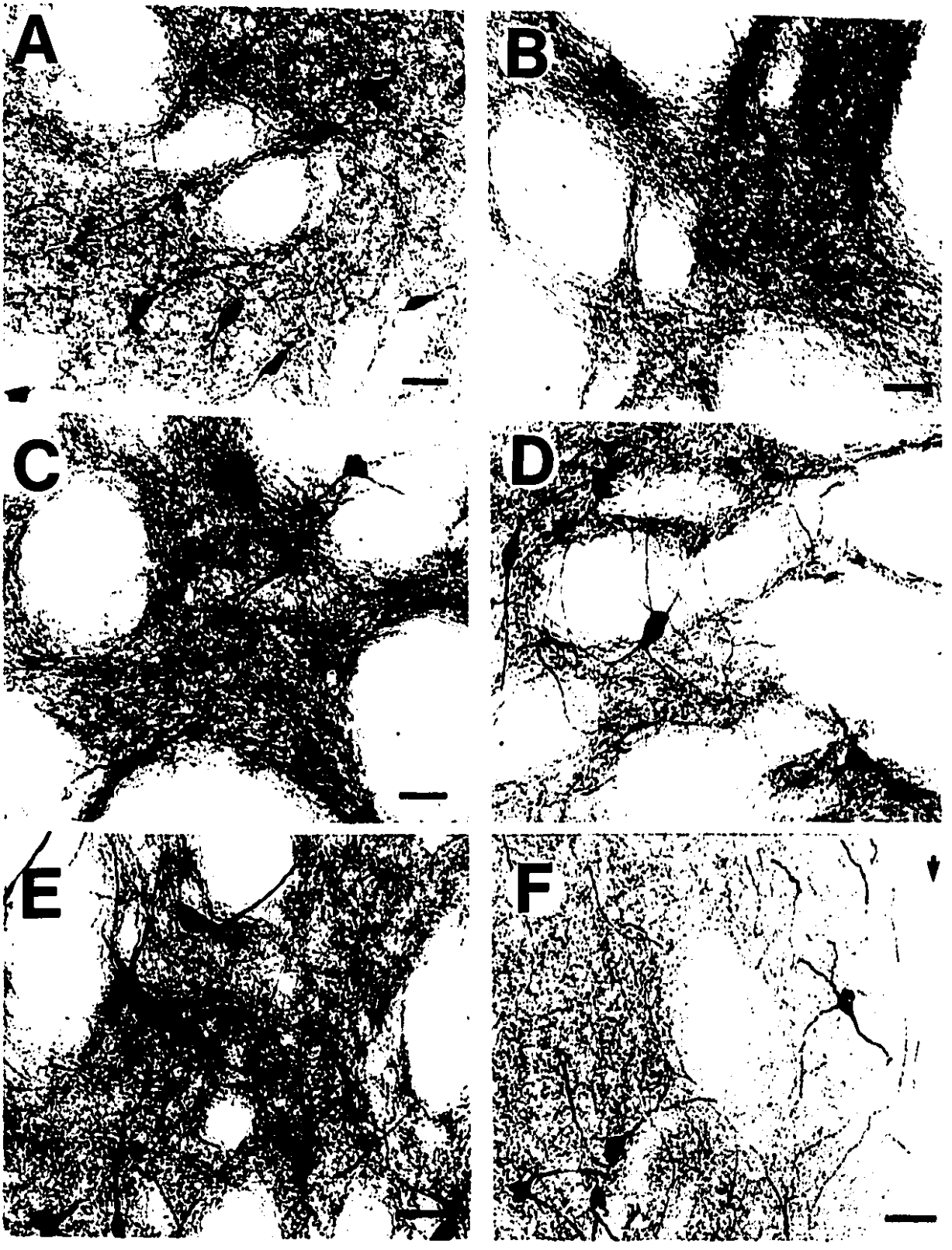
GROUP	ANTERIOR NBM		DORSAL MID NBM		VENTRAL MID NBM		POSTERIOR NBM	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
Control	244 ± 10	247 ± 12	260 ± 8	260 ± 5	266 ± 6	264 ± 7	253 ± 6	247 ± 14
L+Veh	186 ± 16 *	235 ± 9	147 ± 5 *	265 ± 5	189 ± 6 *	265 ± 8	214 ± 11	239 ± 12
L+GM1	242 ± 9	232 ± 11	253 ± 7	250 ± 10	259 ± 7	242 ± 9	248 ± 10	248 ± 8
L+NGF	243 ± 14	244 ± 11	274 ± 10	276 ± 11	271 ± 7	273 ± 10	269 ± 8	269 ± 16
L+N/G	243 ± 14	244 ± 11	282 ± 10	276 ± 11	281 ± 7	258 ± 8	281 ± 11	260 ± 13

(B) Mean Length (mm) of NBM ChAT immunoreactive Neurites per area

GROUP	ANTERIOR NBM		DORSAL MID NBM		VENTRAL MID NBM		POSTERIOR NBM	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
Control	58 ± 5	58 ± 10	57 ± 4	60 ± 5	78 ± 6	82 ± 4	51 ± 6	54 ± 5
L+Veh	47 ± 10	62 ± 4	30 ± 2 *	52 ± 4	59 ± 4	75 ± 4	50 ± 8	54 ± 3
L+GM1	64 ± 7	71 ± 8	56 ± 3	63 ± 5	72 ± 4	74 ± 3	62 ± 4	60 ± 4
L+NGF	69 ± 11	63 ± 8	69 ± 4	67 ± 4	88 ± 3	80 ± 3	65 ± 5	70 ± 6
L+N/G	76 ± 8	70 ± 9	73 ± 7	77 ± 9	100 ± 13	110 ± 13	71 ± 7	69 ± 4

Cortically lesioned animals (L) were immediately administered, i.c.v. via minipump, vehicle (Veh), GM1 (750μg/day), NGF (6μg/day) or both agents in combination (N/G). Animals n= 3-4/group were sacrificed 52 days post-lesion (5 weeks following the end of drug infusions) and were processed for ChAT immunocytochemistry. See methods for details. Numbers represent (A) mean cross sectional area ± S.E.M. of NBM ChAT-IR neurons and (B) mean length ± S.E.M. of NBM ChAT-IR neurites quantified by image analysis as described in methods. * $p<0.05$ from respective control groups, ANOVA, post-hoc Tukey test.

Figure 3.41 ChAT-IR neurons in the striatum of (A) control unoperated, (B) lesion vehicle, (C) lesion GM1, (D) lesion NGF and (E) lesion NGF/GM1 treated rats. Animals were sacrificed after behavioral studies (ie: 52 days post-lesion or 38 days after end of drug administration) and the brains were processed for ChAT immunocytochemistry as described in methods. In (F) the striatal area adjacent to the lateral ventricle (indicated by arrow) is shown for an NGF treated lesioned rat. Scale bar= 50 μm for all panels.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3.3.6c p75^{NGFR} IMMUNOREACTIVITY

The effect of the cortical lesion and various treatments on the expression of nerve growth factor receptor-like immunoreactivity in the NBM and other brain areas was also examined at 52 days post-lesion using the monoclonal antibody 192 IgG (MAb 192) (Chandler et al., 1984). p75^{NGFR}-IR cell density in control animals was not significantly different from ChAT-IR cell density per area (Table 3.19) throughout the NBM. In the ipsilateral dorsal mid NBM of lesioned animals which received vehicle, p75^{NGFR}-IR neurons were shrunken (Table 3.21A) and a decreased p75^{NGFR} immunoreactive neurite network (Table 3.21B) was also noted in this area in agreement with that observed for ChAT immunoreactivity. The mean cross sectional area of p75^{NGFR}-IR neurons in the anterior NBM was also diminished in lesioned vehicle treated rats but no significant alterations were noted in the posterior NBM. p75^{NGFR}-IR neurite length was not affected by the lesion in these two subdivisions of the NBM. GM1, NGF, or NGF/GM1 treatment maintained the morphological appearance of NGF receptor positive neurons and their neuritic network in all NBM areas affected by the lesion (Table 3.21A). As well, p75^{NGFR}-IR neurons in the septum, VDB, HDB appeared unaffected by the lesion or treatments. Few p75^{NGFR} positive neurons were observed in the striatum of control animals and these were restricted to its ventrolateral quadrant (Figure 3.42 A). Lesion vehicle or GM1 treated rats showed similar p75^{NGFR} immunoreactivity in striatum when compared to unoperated control animals. By contrast, a more intense immunoreaction to MAb 192 was apparent in the ventrolateral striati of lesion NGF or NGF/GM1 treated rats (Figure 3.42B). Extensive p75^{NGFR}-IR neuritic processes were also noted (Figure 3.42C). Some light p75^{NGFR}-IR neurons and fibers were also observed in striatal areas adjacent to the ventricles of NGF and NGF/GM1 treated lesioned animals. This suggested that NGF can upregulate p75^{NGFR} expression. While this work was in progress a report appeared also indicating that NGF infusion to the striatum increases p75^{NGFR} immunostaining (Gage et al., 1989).

TABLE 3.21

(A) Mean Cross Sectional area (μm^2) of NBM NGFr immunoreactive neurons

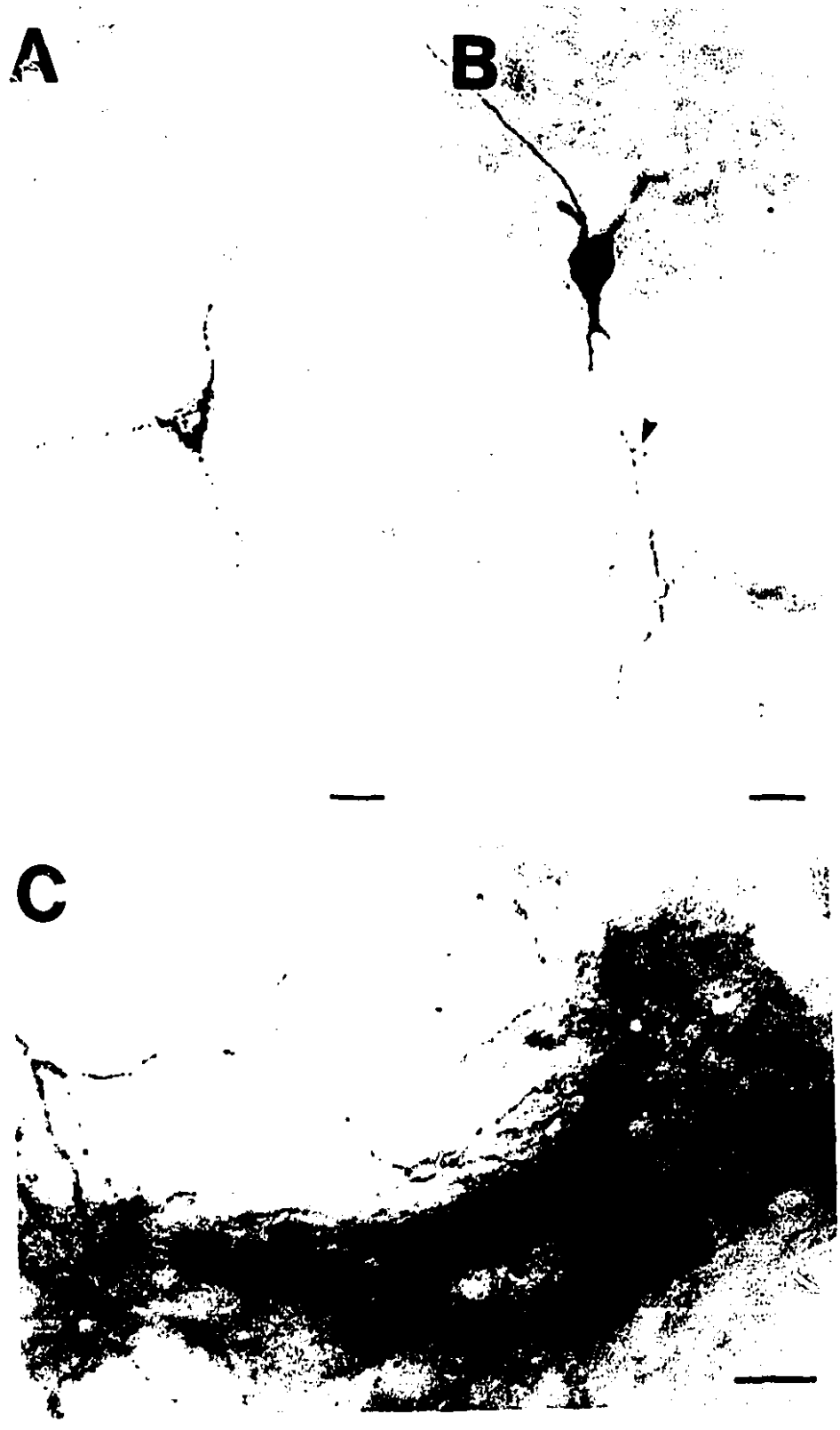
GROUP	ANTERIOR NBM		DORSAL MID NBM		VENTRAL MID NBM		POSTERIOR NBM	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
Control	226 ± 8	225 ± 8	258 ± 14	241 ± 10	255 ± 15	259 ± 8	236 ± 14	231 ± 12
L+Veh	180 ± 15 *	239 ± 10	167 ± 5 *	232 ± 6	179 ± 11 *	261 ± 10	192 ± 5	233 ± 16
L+GM1	234 ± 16	229 ± 11	254 ± 4	253 ± 13	268 ± 8	261 ± 12	252 ± 6	256 ± 15
L+NGF	247 ± 14	225 ± 8	248 ± 4	245 ± 8	253 ± 10	250 ± 4	248 ± 17	246 ± 9
L+N/G	267 ± 21	235 ± 11	283 ± 16	245 ± 15	281 ± 15	257 ± 8	251 ± 17	241 ± 10

(B) Mean Length (mm) of NBM NGFr immunoreactive Neurites per area

GROUP	ANTERIOR NBM		DORSAL MID NBM		VENTRAL MID NBM		POSTERIOR NBM	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
Control	55 ± 6	69 ± 14	49 ± 4	50 ± 4	61 ± 6	56 ± 5	51 ± 5	52 ± 6
L+Veh	43 ± 14	56 ± 6	27 ± 8 *	53 ± 7	42 ± 14	53 ± 7	44 ± 11	42 ± 5
L+GM1	59 ± 10	47 ± 8	61 ± 15	53 ± 5	62 ± 14	55 ± 6	59 ± 13	49 ± 6
L+NGF	50 ± 7	55 ± 14	55 ± 6	63 ± 8	68 ± 13	72 ± 10	52 ± 2	57 ± 10
L+N/G	50 ± 6	60 ± 8	53 ± 10	54 ± 10	60 ± 6	64 ± 8	54 ± 8	59 ± 4

Cortically lesioned animals (L) were immediately administered, i.c.v. via minipump, vehicle (Veh), GM1 (750μg/day), NGF (6μg/day) or both agents in combination (N/G). Animals n=3-4/group were sacrificed 52 days post-lesion (5 weeks following the end of drug infusions) and were processed for NGFr immunocytochemistry. See methods for details. Numbers represent (A) mean cross sectional area ± S.E.M. of NBM $p75^{NGFR}$-IR neurons and (B) mean length ± S.E.M. of NBM $p75^{NGFR}$-IR neurites quantified by image analysis as described in methods. * $p<0.05$ from respective control groups, ANOVA, post-hoc Tukey test.

Figure 3.42 $p75^{NGFR}$-IR neurons in the striatum of (A) control unoperated and (B) lesion NGF treated animals as detected using the Mab 192 IgG. Note the increase in perikaryal and neuritic immunoreactivity in lesioned rats which received NGF. Arrowhead in (B) indicates area shown at higher magnification in (C). Scale bar = 20 μm for all panels.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3.4 *EFFECTS OF DECORTICATION NGF AND/OR GM1 TREATMENT ON THE AGED RAT BRAIN*

In early experiments with aged rats, retired breeders from Charles River breeding laboratories (St. Constant, Québec) were used. These male Wistar rats ranged between 9-12 months in age. In initial neurochemical and immunocytochemical studies it was noted that deficits in basal forebrain cholinergic activity and neuronal morphology were inconsistent in these animals. That is, while some of these aged rats had diminished levels of brain cholinergic markers and an apparent reduction in the number of NBM cholinergic neurons others were indistinguishable from young adult rats. Since older animals were unavailable at that time, for subsequent studies, rats were kept housed in the McGill McIntyre animal center to age further. Male Wistar rats between 20-26 months of age (weighing 850-970 grams) showed more consistent deficits in basal forebrain cholinergic markers when compared with young adults (Figure 3.43) and were therefore, used for the studies presented here. These animals however, were problematic since at this age approximately 40-50% of the animals died of natural causes. As well, they did not tolerate the surgical procedures well and often had respiratory problems, infections, skin lesions and tumors (both peripheral and central). Animals with such health problems were not included in the studies therefore, total number of animals per group available for analysis was ususally low and few experiments were possible (n= 3-5).

3.4.1 *Short-term treatment with GM1 and/or NGF fails to attenuate cholinergic deficits induced by a unilateral cortical devascularizing lesion*

As occurs in adult rats, unilateral devascularizing cortical lesions in aged animals cause decreases in NBM ChAT activity at 30 days post-lesion (Figure 3.44). The decrease in the activity of this enzyme was also in the range of 40-50% as obtained for young adult rats (section 3.1). However, in contrast to adult rats, short-term (7 days) treatment, i.c.v. via minipump, of aged decorticated rats with doses of NGF (12 μg/day) and/or GM1 (1.5 mg/day), which were maximally effective in young adult rats did not attenuate this deficit in the NBM. In addition, cortical ChAT activity was not stimulated above control values as

occurred in young adult decorticated rats. High affinity choline uptake in cortex, hippocampus and striatum were also unaffected by the lesion and the drug treatments (Figure 3.44). Other brain areas examined, which were unaffected by the lesion with respect to ChAT activity, also showed no alterations in the activity of this enzyme following drug treatments (Figure 3.45).

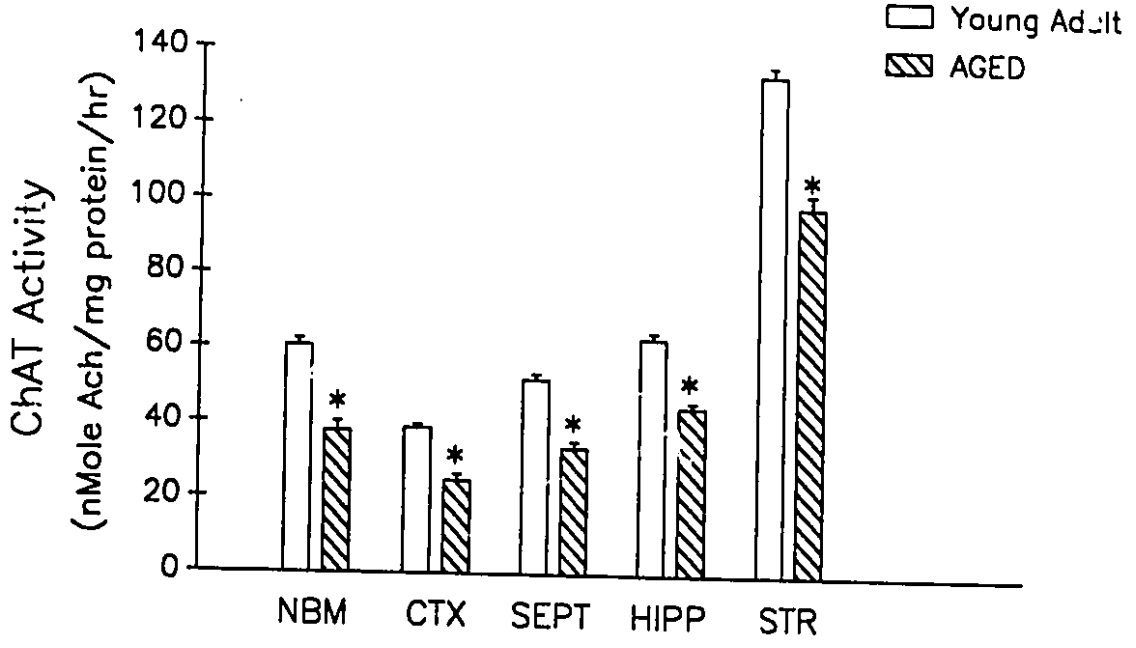

Figure 3.43 Comparison of ChAT activity in various brain areas of young adult (300-350 gr., ~ 4months old) and aged male Wistar rats (850-970 gr., >20 months old). * $p<0.05$ from young adult counterpart, Student t test. n = 6 animals/group. Abbreviations: CTX, cortex; SEPT, septum; HIPP, hippocampus; STR, striatum.

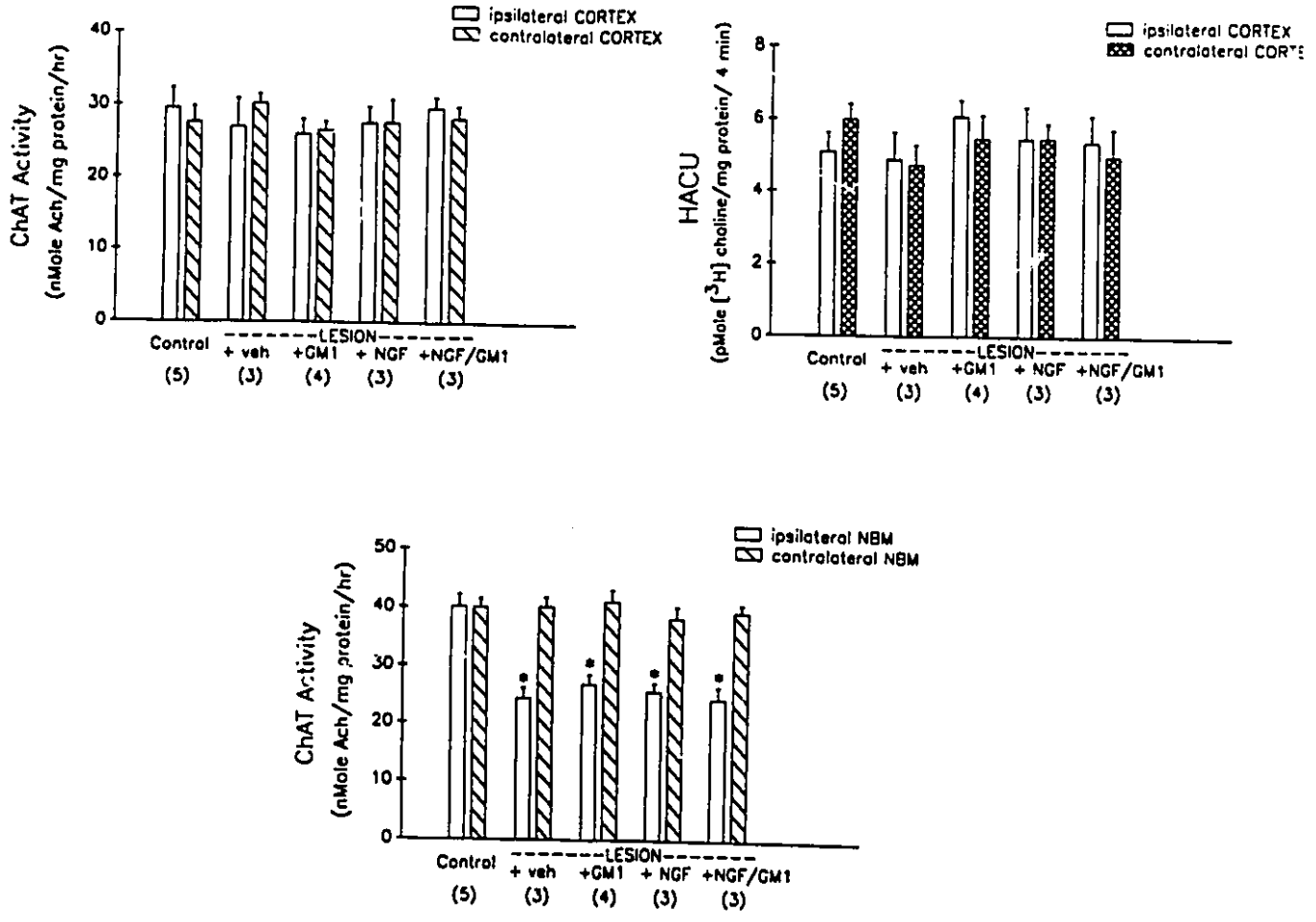

Figure 3.44 Effects of unilateral decortication, GM1 and/or NGF short-term treatment on the NBM and cortex of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in the microdissected NBM and remaining cortex according to the procedure described by Fonnum (1975). Cortical HACU was measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p < 0.05$ from control, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

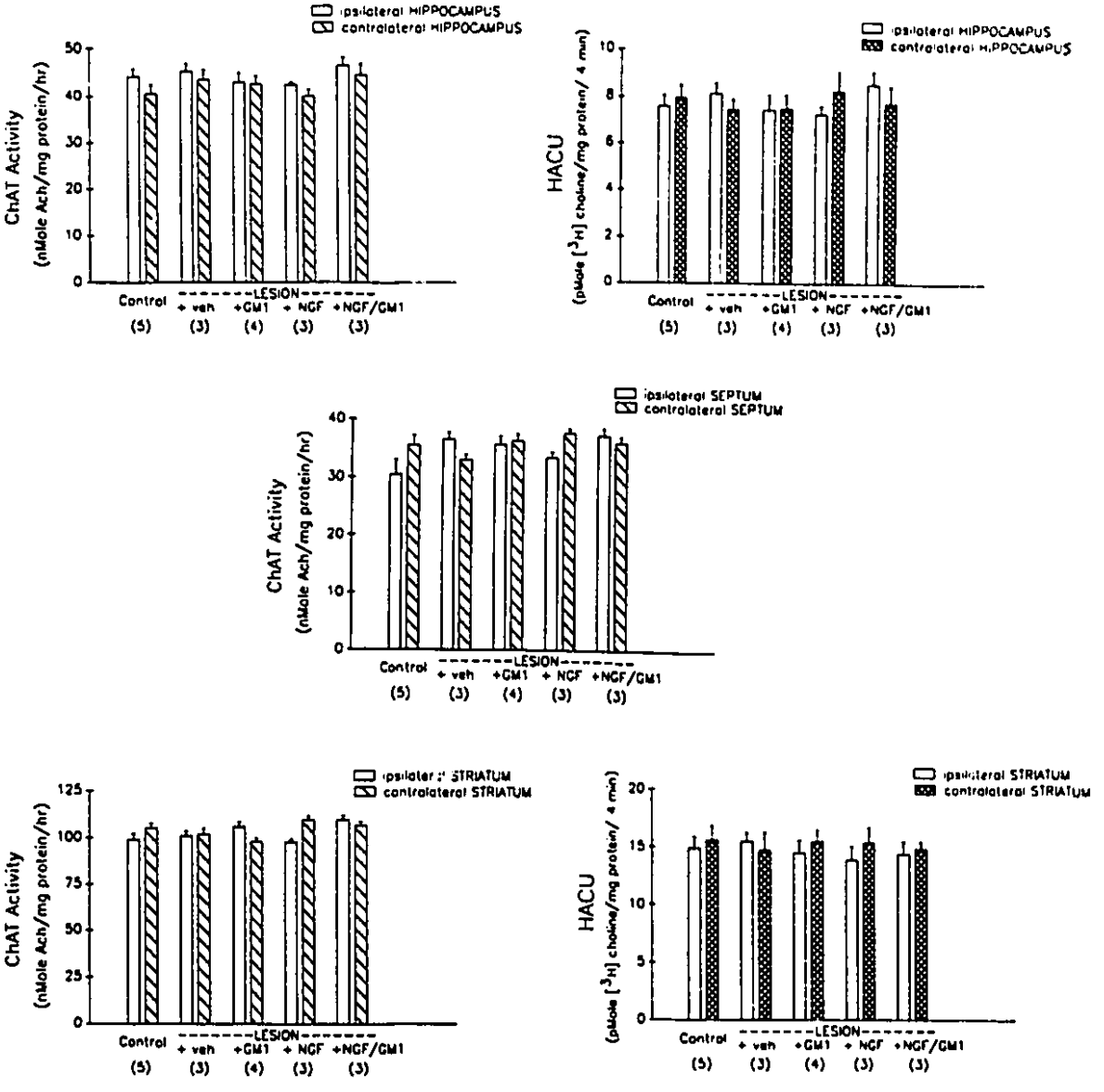

Figure 3.45 Effects of unilateral decortication, GM1 and/or NGF short-term treatment on the striatum, septum and hippocampus of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 7 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in the microdissected striatum, septum and hippocampus according to the procedure described by Fonnum (1975). Striatal and hippocampal HACU were measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., $^{*}p<0.05$ from control, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

3.4.2 Requirement for chronic treatment to attenuate cholinergic deficits in decorticated aged rats

In subsequent studies whether cholinergic deficits in the NBM could be prevented by chronic administration of these factors was tested. Aged rats were cortically lesioned and received, i.c.v. via minipump beginning immediately post-lesion either vehicle (artificial c.s.f + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 1 month. Lesioned animals which received either GM1 or NGF treatment had ChAT activity values in the ipsilateral NBM which were maintained at control levels (Figure 3.46). Moreover, ChAT activity was significantly augmented above control values in the ipsilateral remaining cortex. In animals which received both NGF and GM1, ChAT activity was significantly increased in both the NBM and cortex above that obtained by each agent alone (Figure 3.46). Similar observations were noted for cortical HACU (Figure 3.46). As was the case for young adult rats, ChAT activity in the striatum was also significantly increased by NGF but not GM1 treatment (Figure 3.47). NGF treatment also caused a significant increase in septal and hippocampal ChAT activity of aged lesioned rats (Figure 3.47). By contrast, GM1 treatment did not affect ChAT activity in the septum, hippocampus or striatum. Lesioned animals which received both NGF and GM1 treatment showed a trend towards an increase in septal ChAT activity, as well as hippocampal ChAT activity and HACU above that noted in lesioned aged rats which received only NGF treatment, however this increase was not significant.

Lower doses of these agents, which were still maximally effective in young adult rats (NGF: 0.5 μg/day; GM1 600 μg/day) were only partially effective in attenuating cholinergic deficits when given for 1 month to decorticated aged animals (Figure 3.48). This dose of NGF did however, increase ChAT activity in the striatum, but not in septum and hippocampus (Figure 3.49). If treatment time with these lower doses was extended to 2 months a clear protection of NBM ChAT activity and stimulation of cortical ChAT activity as well as high affinity choline uptake were noted (Figure 3.50). Moreover, stimulation of ChAT activity was noted in the septum, hippocampus and striatum of NGF treated lesioned animals (Figure 3.51). HACU was also augmented for the latter two brain areas. GM1 treatment alone at this extended dosage did not affect cholinergic markers in brain areas other than the ipsilateral NBM and remaining cortex but did potentiate NGF effects on ChAT

activity in the basal forebrain (Figures 3.50; 3.51). However, striatal and hippocampal HACU were not further increased in lesioned aged rats which received both NGF and GM1. It should also be noted that at the 60 days post-lesion time point ChAT activity in the ipsilateral NBM of aged rats which received vehicle was not further decreased, in lesion vehicle treated rats when compared with 30 days post-lesion rats.

3.4.3 Chronic NGF and/or GM1 treatment prevent retrograde degeneration of NBM cholinergic neurons

Comprehensive quantitative analysis of NBM cholinergic neuronal morphology in experimental aged rats was not possible for all treatment paradigms because of the low number (n= 2-3) of rats per group available for study. This was mainly due to the fact that many of the aged rats did not survive anaesthesia prior to perfusion, and that several of the aged brains processed showed poor immunocytochemical quality, which rendered samples inadequate for computerized image analysis. A sufficient number (n= 3) of animals were obtained, for immunocytochemical analysis, only for the 2 month treatment paradigm.

In addition to decreased NBM ChAT activity the number of ChAT-IR neurons in the mid-portion of the NBM of aged (>20 months) unoperated animals appeared significantly reduced as compared to that noted in young adult rats (*Abercrombie corrected number per field*: control aged: 26 $\pm$ 7, n=5; control young adult: 48 $\pm$ 5, n= 3). Moreover, quantitative analysis of ChAT-IR neurons in this brain area revealed that they were considerably diminished in size when compared to their young adult counterparts (control aged: 186 $\pm$ 11 μm^2, n =5; control young adult: 249 $\pm$ 8 μm^2, n=3).

In contrast to their younger counterparts, aged rats which were unilaterally decorticated and received vehicle showed an apparent loss of ChAT-IR cell density in the mid-NBM at 60 days post-lesion (Figure 3.52, Table 3.22). Moreover, the mean cross sectional area of NBM ChAT-IR neurons and the NBM ChAT-IR fiber network in lesioned vehicle treated aged rats were also decreased (Table 3.22). By contrast, rats which received, beginning immediately post-lesion, GM1 (600 μg/day) or NGF (0.5 μg/day), i.c.v. via minipump for 2 months showed an increase in ChAT-IR cell density in the mid-NBM (Figure 3.52; Table 3.22). These treatments also preserved ChAT-IR neuronal size and the fiber network in the

mid-NBM of lesioned aged rats (Figure 3.53; Table 3.22). Moreover, a trend towards an increase in NBM cholinergic cell size, but not fiber length, was apparent in lesioned aged rats which received concurrent NGF and GM1 treatment at the above doses (Table 3.22).

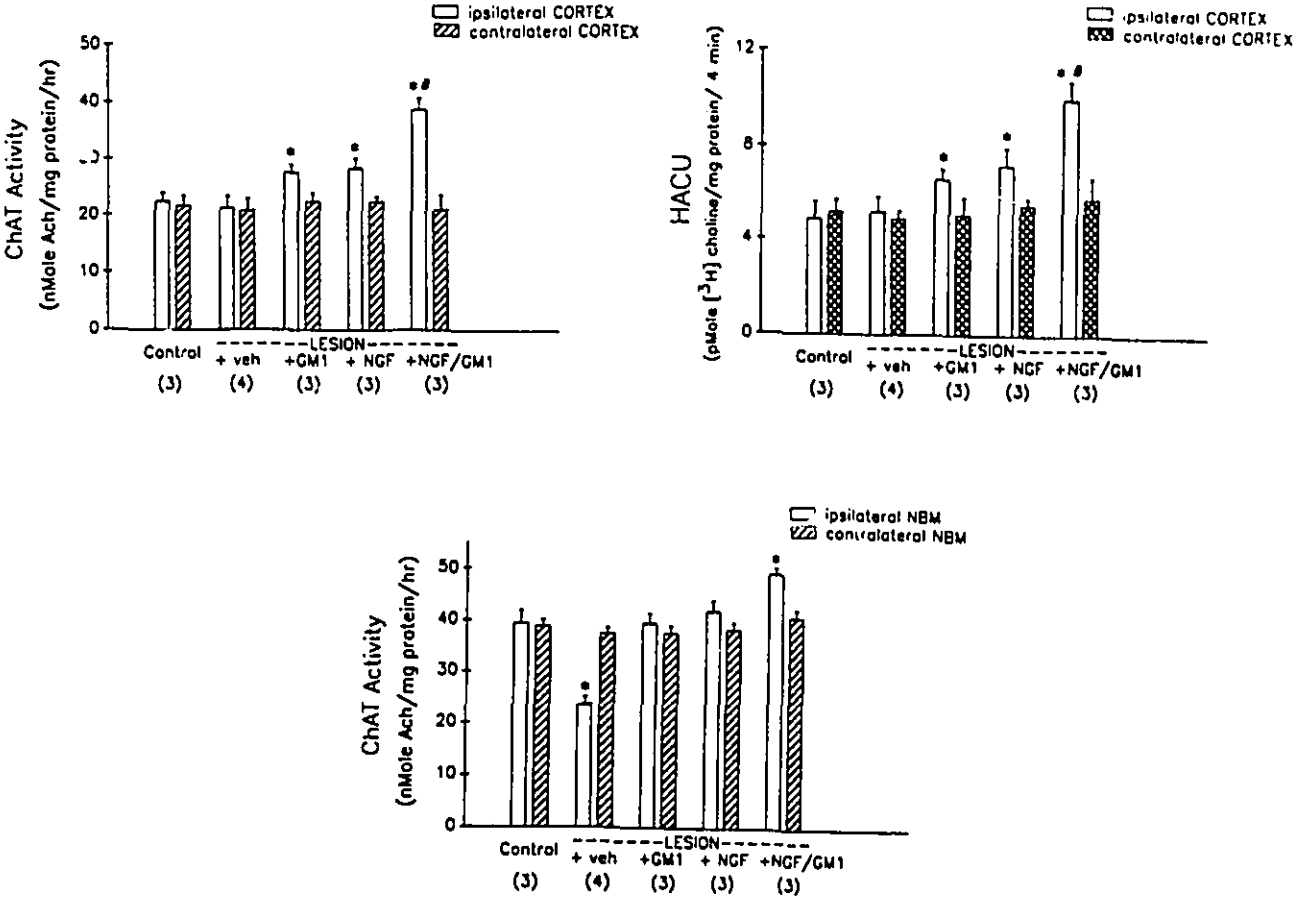

Figure 3.46 Effects of unilateral decortication, GM1 and/or NGF chronic (1 month) treatment on the NBM and cortex of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 29 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in microdissected NBM and cortex according to the procedure described by Fonnum (1975). Cortical HACU was measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p < 0.05$ from control, #$p < 0.05$ from lesion NGF treated group, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

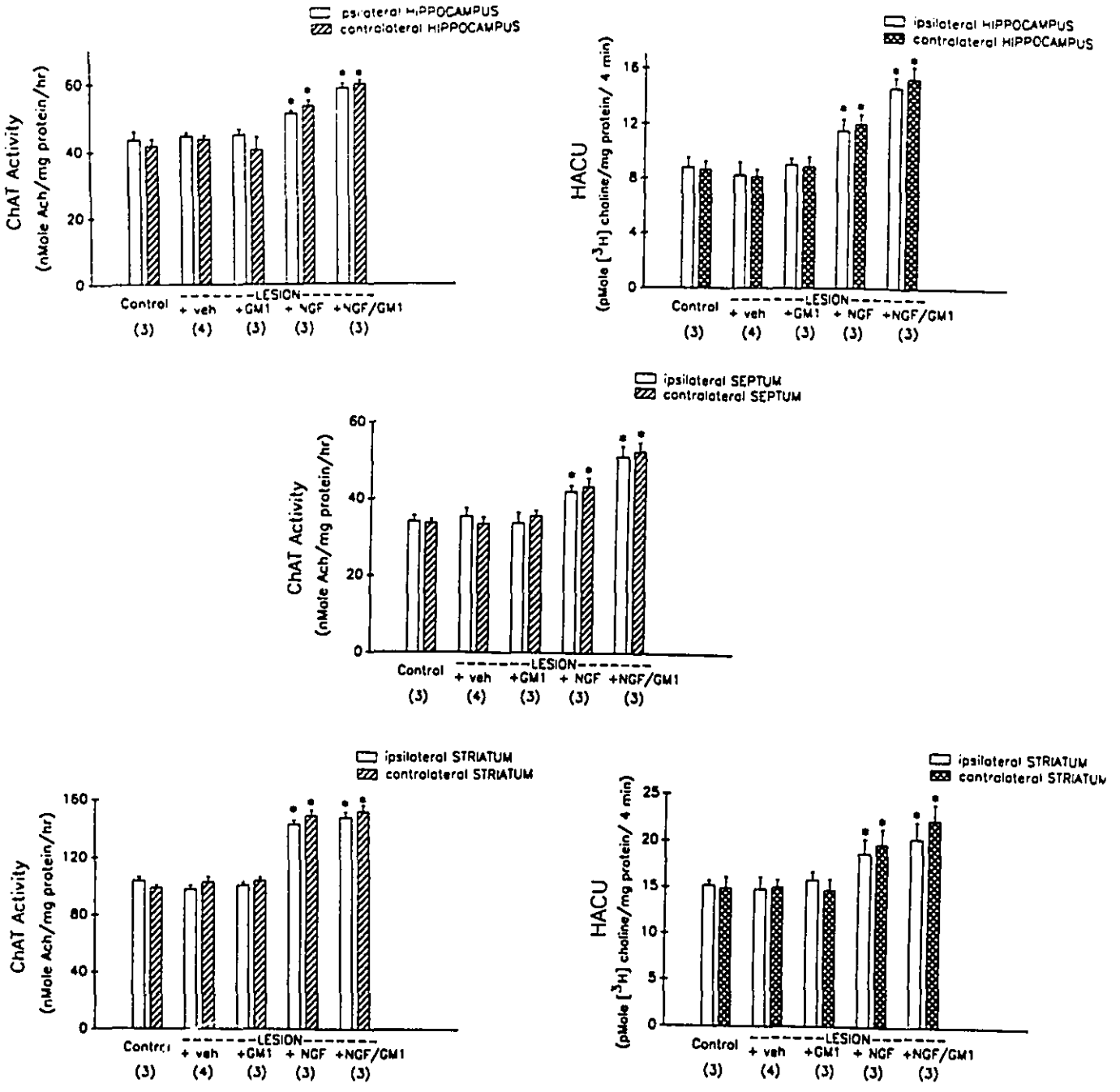

Figure 3.47 Effects of unilateral decortication, GM1 and/or NGF chronic (1 month) treatment on the striatum, septum and hippocampus of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (1.5 mg/day) and/or NGF (12 μg/day) for 29 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in microdissected striatum, septum and hippocampus according to the procedure described by Fonnum (1975). Cortical HACU was measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p < 0.05$ from control, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

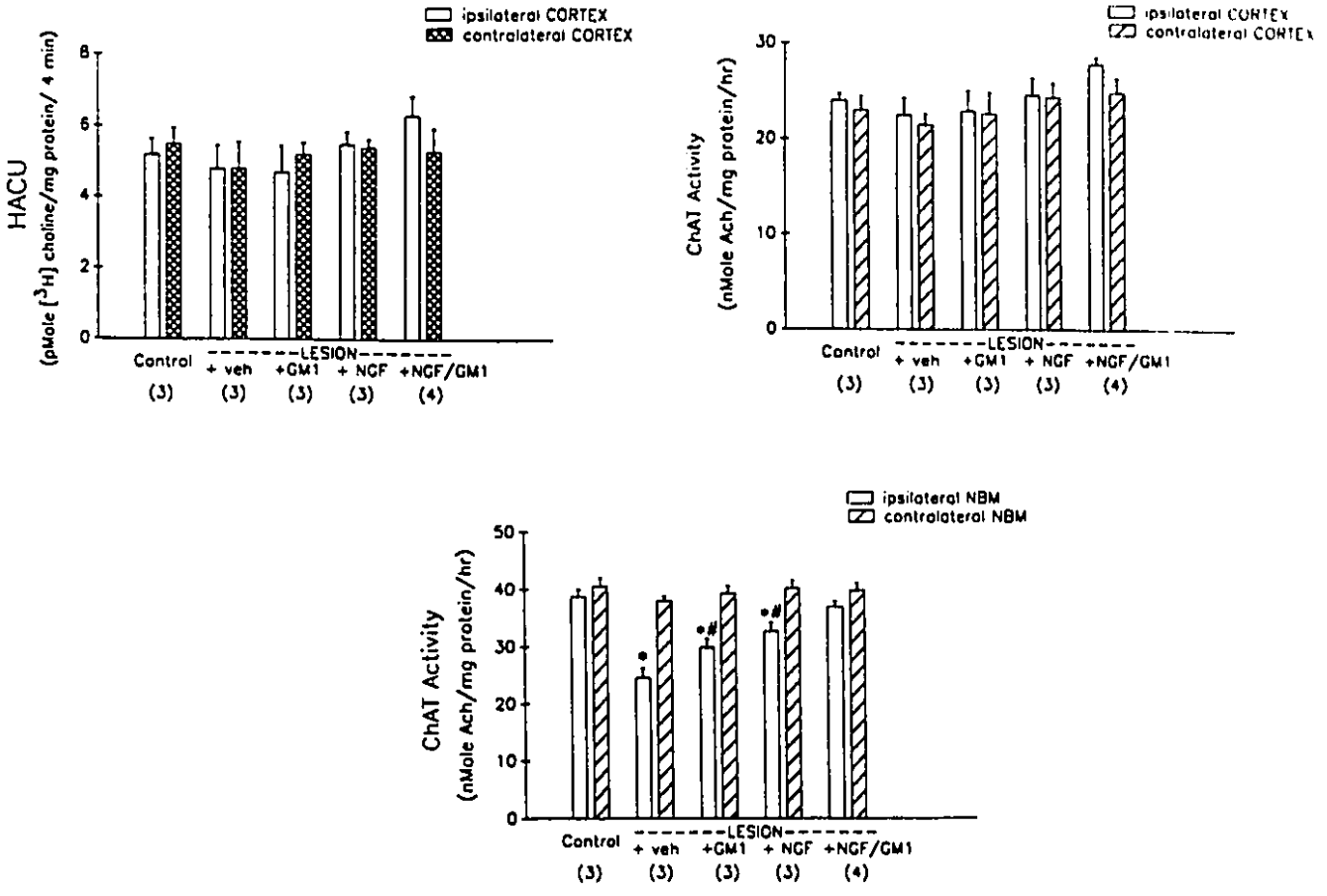

Figure 3.48 Effects of unilateral decortication, GM1 and/or NGF chronic 1 month treatment on the NBM and cortex of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 29 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in microdissected tissue according to the procedure described by Fonnum (1975). Cortical HACU was measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p<0.05$ from control, #$p<0.05$ from lesion vehicle treated group, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

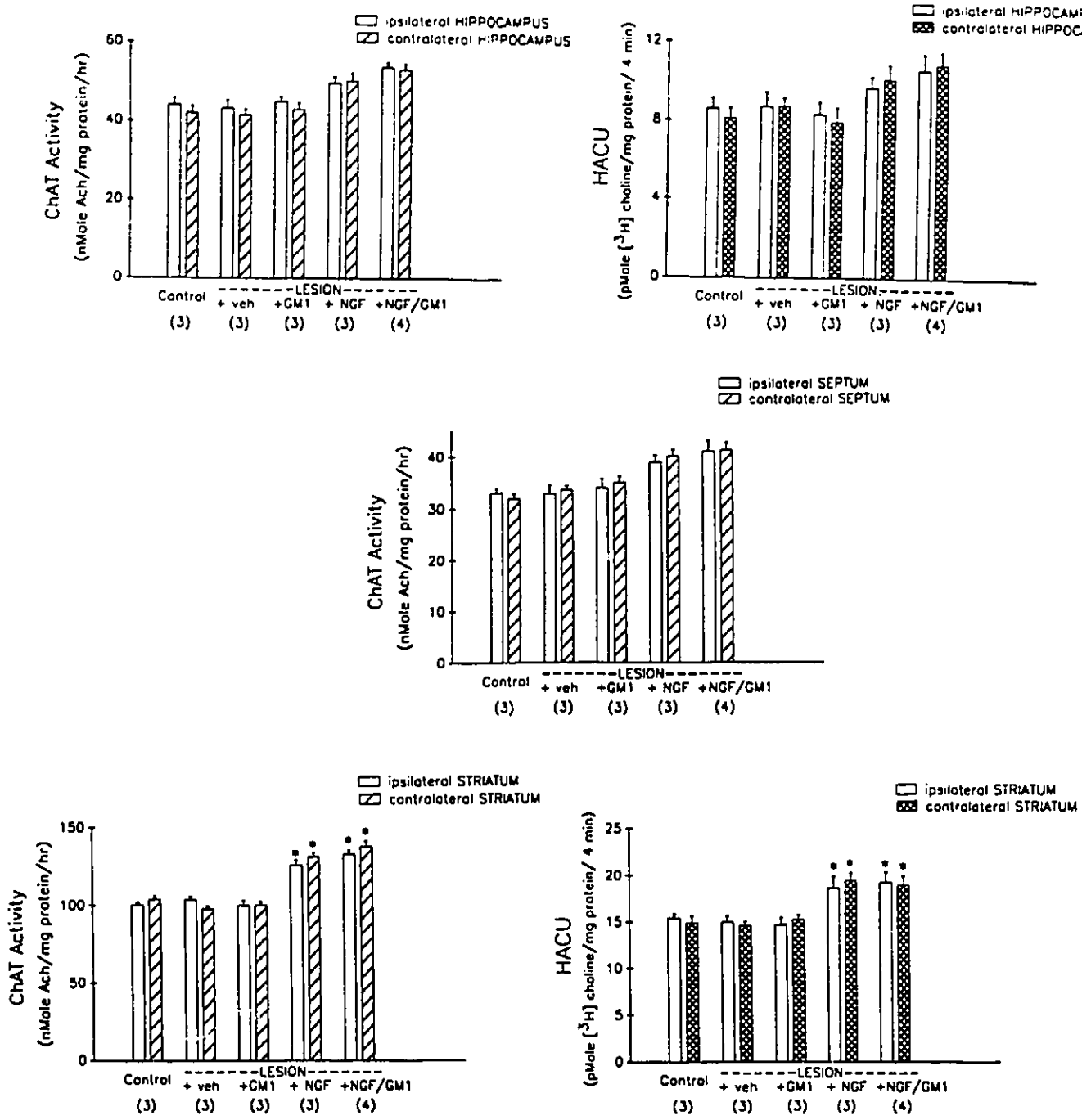

Figure 3.49 Effects of unilateral decortication, GM1 and/or NGF chronic (1 month) treatment on the striatum, septum and hippocampus of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 29 days. Animals were sacrificed on post-lesion day 30 and ChAT activity was measured in microdissected striatum, septum and hippocampus according to the procedure described by Fonnum (1975). Striatal and hippocampal HACU were measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p<0.05$ from control, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

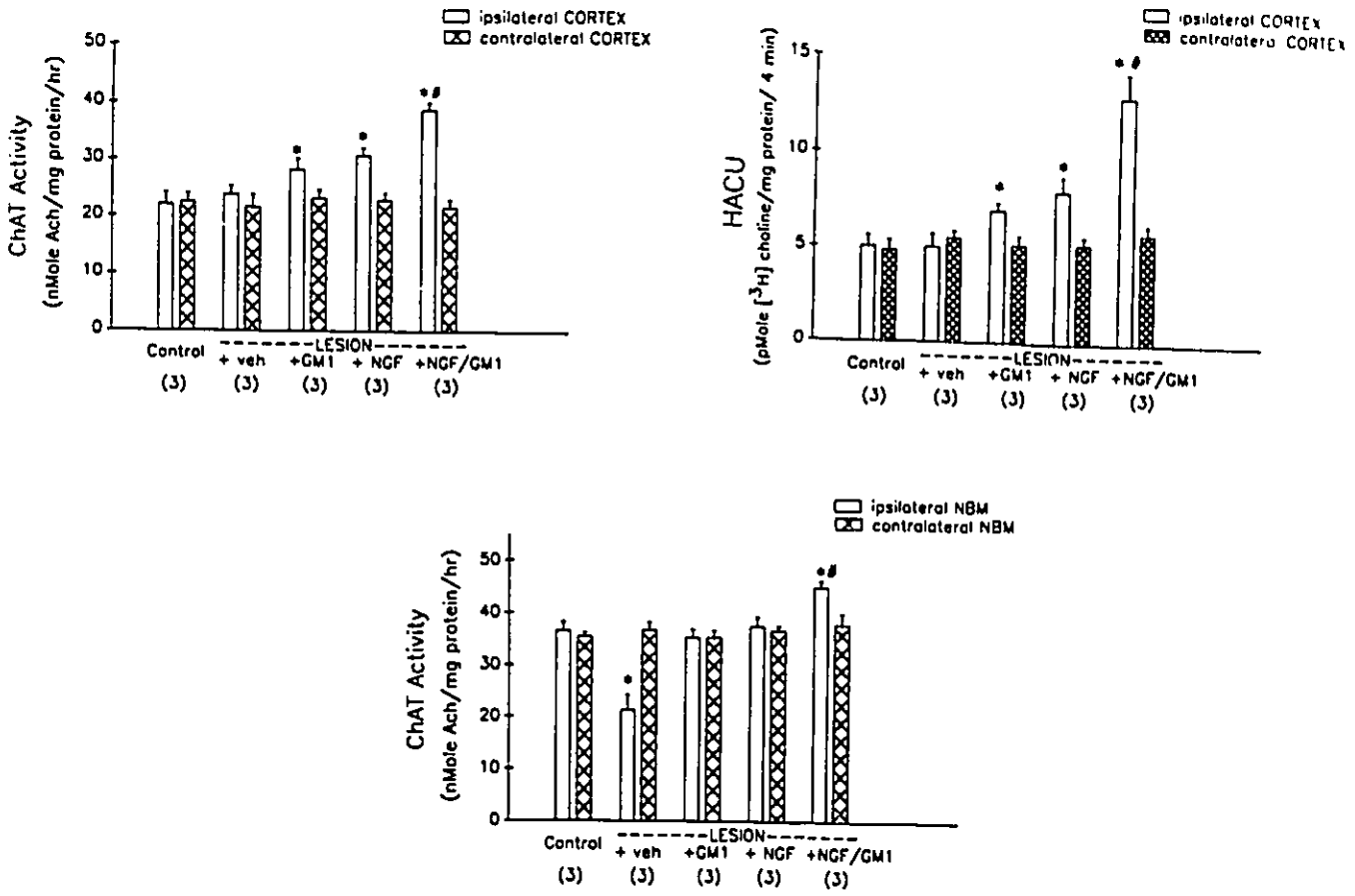

Figure 3.50 Effects of unilateral decortication, GM1 and/or NGF chronic (2 months) treatment on the NBM and cortex of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 59 days. Animals were sacrificed on post-lesion day 60 and ChAT activity was measured in microdissected tissue according to the procedure described by Fonnum (1975). Cortical HACU was measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p < 0.05$ from control, #$p < 0.05$ from lesion NGF treated group, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

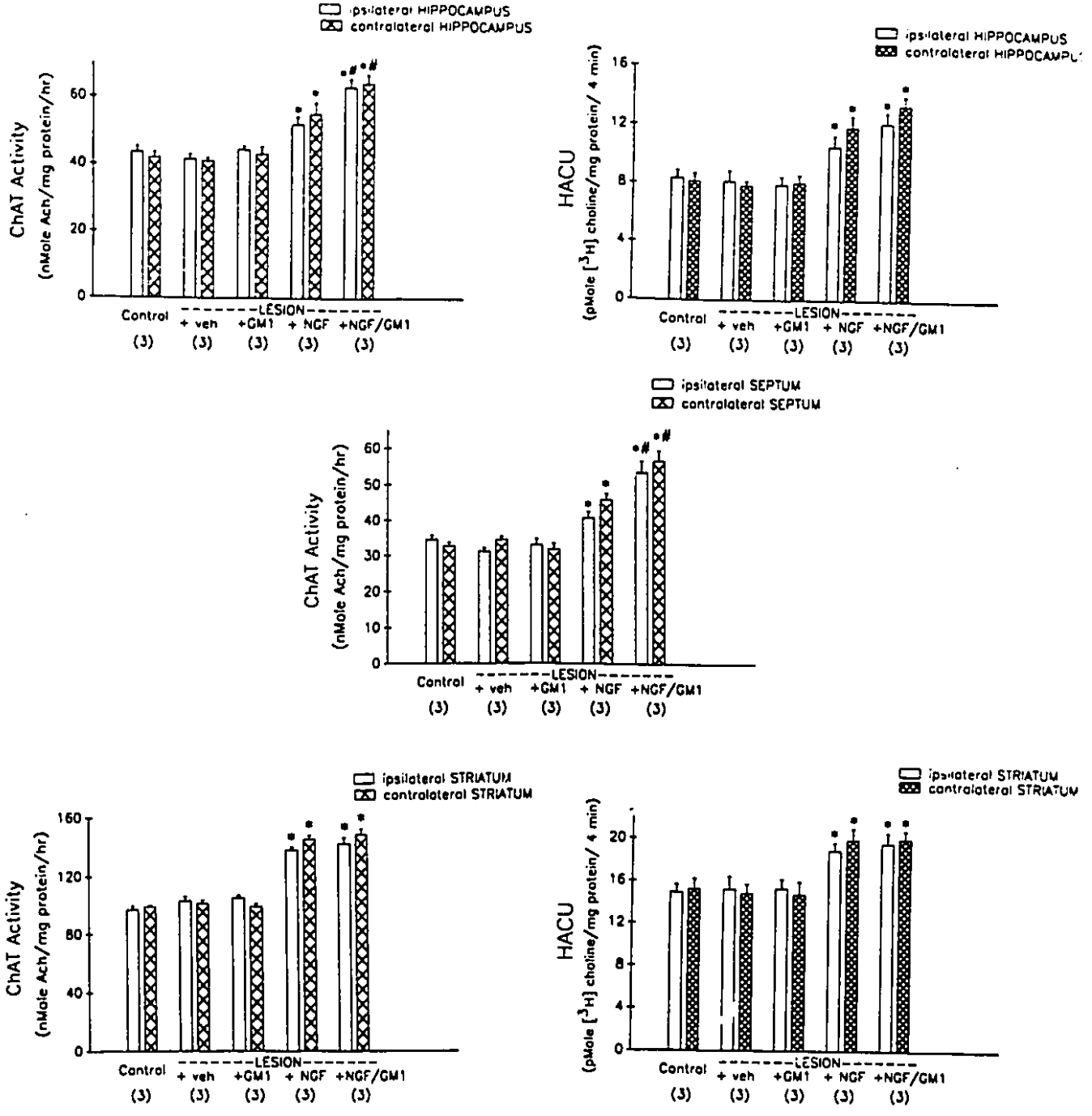

Figure 3.51 Effects of unilateral decortication, GM1 and/or NGF chronic (2 months) treatment on the striatum, septum and hippocampus of aged lesioned rats. Animals were unilaterally decorticated and received, i.c.v. via minipump, beginning immediately post-lesion either: vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 59 days. Animals were sacrificed on post-lesion day 60 and ChAT activity was measured in microdissected striatum, septum and hippocampus according to the procedure described by Fonnum (1975). Striatal and hippocampal HACU were measured as described in methods. Protein content was determined according to the method of Bradford (1976). Error bars represent S.E.M., *$p<0.05$ from control, #$p<0.05$ from lesion NGF treated group, ANOVA, post-hoc Newman-Keuls test. Numbers below each bar graph indicate animals per experimental group.

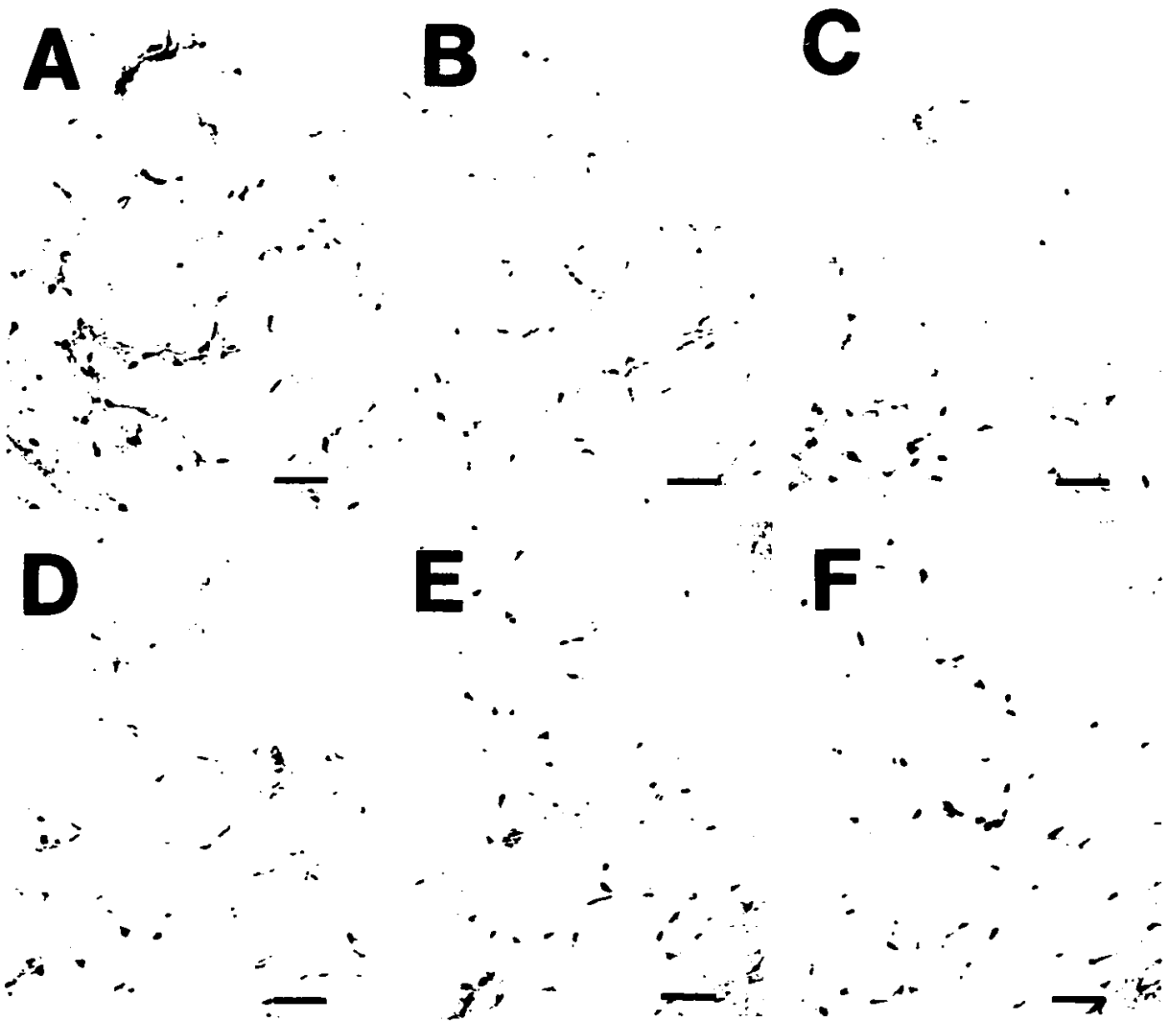
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Figure 3.52 ChAT-IR neurons in the mid portion of the NBM in a young 4 month old unoperated rat (A), and an unoperated aged rat (B). (C-D) ChAT-R neurons from the mid-NBM of a decorticated aged rat, at 60 days post-lesion, which received (C) vehicle, (D) GM1, (E) NGF and (F) NGF in combination with GM1. Aged animals were unilaterally decorticated as described in methods and received i.c.v. via minipump, beginning immediately post-lesion either, vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 59 days. Animals were sacrificed at 60 days post-lesion by transcardial perfusion and the brains were processed for ChAT immunocytochemistry as described in methods. Scale bar= 100 μm.

Figure 3.53 High magnification of ChAT-IR neurons in the aged (> 20 months) rat NBM. ChAT-IR neurons in the (A) dorsal-mid NBM of a control unoperated aged rat, (B) dorsal-mid and (C) ventral mid-NBM of a 60 days post-lesion aged rat which received vehicle. (D-F) ChAT-IR neurons in the dorsal-mid NBM of a 60 days post-lesion aged rat which received (D) GM1, (E) NGF or (F) NGF/GM1 treatment. Aged animals were unilaterally decorticated as described in methods and received i.c.v. via minipump, beginning immediately post-lesion either, vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day) and/or NGF (0.5 μg/day) for 59 days. Animals were sacrificed at 60 days post-lesion by transcardial perfusion and the brains were processed for ChAT immunocytochemistry as described in methods. Scale bar= 40 μm.


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Table 3.22

Effect of lesion NGF and/or GM1 treatment on ChAT-IR neurons and fibers in the NBM of aged rats			
GROUPS	Cell number (number/area)	Cell cross sectional area (μm^2)	Fiber length (mm/area)
Control	28 ± 6	184 ± 8	38 ± 9
Lesion + Vehicle	13 ± 7*	151 ± 6*	20 ± 7*
Lesion + GM1	29 ± 8	182 ± 9	37 ± 6
Lesion + NGF	30 ± 9	188 ± 8	40 ± 5
Lesion + NGF/GM1	31 ± 7	199 ± 6	41 ± 6

Aged (>20 months) male Wistar rats were cortically leisoned and were immediately given, i.c.v. via minipump, vehicle (artificial c.s.f. + 0.1% BSA), GM1 (600 μg/day), NGF (0.5 μg/day) or both agents in combination (NGF/GM1) for 59 days. Animals (n= 3/group) were sacrificed on post-lesion day 60 and were processed for ChAT immunocytochemistry. Cell number, size and fiber length were quantified using an image analysis system as descibed in methods. Cell number represents the Abercrombie corrected values. *$p<0.05$ from respective control value, ANOVA post-hoc Newman-Keul's test.

DISCUSSION

4.0 GENERAL DISCUSSION

Establishing adequate pharmacological treatments for disorders associated with brain cholinergic dysfunction remains an important goal for neuroscientists. So far, therapeutic strategies have consisted of administering cholinergic agonists or anticholinesterase drugs. However, this has resulted in limited success because such therapies require the presence of viable and functional neurons which are often absent in the diseased brain. This thesis examined whether two agents, NGF and GM1 could regulate cholinergic markers, prevent retrograde neuronal degeneration and induce recovery following injury to the basalo-cortical cholinergic pathway in the rat brain. In addition, whether GM1 could potentiate NGF-induced effects in the CNS *in vivo* was studied.

4.1 Devascularizing cortical lesions in the rat: a model for retrograde degeneration of NBM cholinergic neurons

Most *in vivo* studies which have assessed responses of basal forebrain cholinergic systems to injury have, for the most part, relied heavily upon experimental paradigms involving axon transection of the septo-hippocampal pathway (Hefti, 1986; Kromer, 1987; Williams et al.1986; Hagg et al.1988), or when studying the basalo-cortical system, neurotoxic anterograde NBM lesions (DiPatre et al.1989; Mandel et al.1989b; Haroutunian et al.1989; Dekker et al.1992). By contrast, for the work of this thesis a unilateral devascularizing lesion was used to produce an extensive ischemic cortical infarction in the rat. This lesion causes a gradual loss of the affected cortex and retrograde degeneration of NBM cholinergic neurons [(Sofroniew et al.1983), present study]. Its advantage over other models of retrograde degeneration in current use (e.g. axotomy) is that it likely more closely simulates potential pathophysiological events of CNS trauma and degeneration. The damage induced by cortical devascularization could arise for example following severe head trauma or stroke, whereas axotomies of brain pathways are not encountered beyond experimental situations. Moreover, in contrast to axotomy lesion models such as fimbria-fornix transections the extent of neuronal injury induced by cortical devascularization can be easily detected using ChAT immunocytochemistry. This is due to the fact that a loss of cholinergic neuronal phenotype

does not occur in the NBM of decorticated adult rats. A particular disadvantage of the fimbria-fornix lesion model is that up to 80% of septal neurons are undetected by ChAT or AChE immunocytochemistry in such lesioned rats (see for example Hagg et al. 1989a). This makes it difficult to ascertain the exact extent of atrophy versus neuronal loss by such techniques (further discussed section 4.4). Nevertheless, some limitations of the cortical devascularization lesion model should be discussed. One drawback, which however also applies to other animal models involving surgical interventions, is that some care was required to ensure lesion reproducibility from animal to animal. As described in methods, the decortication procedure used in this thesis was standardized by establishing stereotaxic landmarks to ensure that equivalent cortical areas were lesioned in each case. Nevertheless, this precaution was not fool proof. Animals sometimes showed variations in cortical vascularization and in post-lesion bleeding, factors which could not be controlled for. Thus, it was necessary to prepare supplementary animals to ensure that an adequate number of samples were available for analysis. A particular weakness of the cortical devascularization lesion model, when compared with septo-hippocampal lesion models, is that in contrast to the hippocampus, where projections from the medial septum and their topographic arrangement are well established, less is known about the organization and topography of NBM projections to cortex. This problem was circumvented by undertaking a painstaking comprehensive quantitative analysis, assisted by a computerized imaging system, of all layers within a specific cortical area and of neurons throughout the entire NBM. In addition, although decortication specifically produces retrograde degeneration of NBM cholinergic neurons, other subcortical areas which also project to, or receive input from, the cortex are most probably affected. These include regions such as: the raphe nucleus, the locus coeruleus and the thalamus. Consequently, decortication can be expected to also produce deficits in other neurotransmitter systems like dopamine and serotonin. Neurochemical or neuroanatomical changes induced by the devascularizing lesion and the effects of trophic agents in these pathways were not addressed by this thesis. Rather, the work of this dissertation focused primarily on examining effects upon the basalo-cortical cholinergic system.

4.2 Lesion induced cholinergic deficits in the NBM

Unilateral devascularizing lesions of the rat cortex cause cholinergic deficits, as indicated by significant decreases in ChAT activity, in the ipsilateral NBM and not in other basal forebrain areas. This correlates with previous reports which have shown, using neuroanatomical techniques, that the major target region for NBM cholinergic neurons is the cortex (Fibiger, 1982; Bigl et al.1982; Saper, 1984; Luiten et al.1987). Decorticated animals showed time-dependent decreases in NBM ChAT activity. These deficits were first found to be significant at day 15 post-lesion, thus suggesting that a gradual degeneration of NBM cholinergic neurons occurs. These findings correlate with previous work which showed that shrinkage of NBM ChAT-IR neurons is first notable 14 days after decortication (Sofroniew et al.1983). The augmented NBM and cortical ChAT activity observed at post-lesion day 1 could be attributed to compensatory responses of the neurons to the trauma or alternatively, to a transient stimulation of the basalo-cortical pathway by neurotoxic substances released upon injury (Nilsson et al.1990). Such neurotoxic substances include excitatory amino acids like glutamate or aspartate which could alter ion flux. That cortical HACU was not similarly affected on post-lesion day 1 suggests that this activation does not result in significant changes in the synthesis of ACh or synaptic activity of cholinergic neurons. At 30 days post-lesion NBM ChAT activity was significantly decreased by 40-50%. Similar decreases in the activity of this enzyme were noted at 52 days post-lesion. Detailed quantitative analysis of NBM ChAT and $p75^{NGFR}$-IR cell number and size at these post-lesion times confirmed that cortical devascularization does not cause a significant loss of NBM cholinergic neurons rather, ChAT-IR neurons atrophy as evidenced by decreases in their mean cross sectional cell areas (Sofroniew et al.1987). The work of this thesis has also shown that cholinergic neurons in the dorsal mid portion rather than in the anterior or posterior subdivisions of the NBM are more deeply affected by this lesion, and furthermore that a significant decrease in the NBM ChAT-IR fiber network occurs. Moreover, a correlative shrinkage of ChAT and $p75^{NGFR}$-IR neurons in each NBM subdivision was shown. These results concur with work which used *phaseolus vulgaris* leucoagglutinin as an anterograde tract tracer to confirm that NBM projections to cortex maintain an anterior to posterior topographic arrangement (Luiten et al.1987), and with studies published while the work for this thesis was in progress,

revealing that ChAT and $p75^{NGFR}$ immunoreactivity are colocalized in the basal forebrain (Batchelor et al.1989). The lack of significant NBM ChAT-IR cell death observed following decortication suggests that NBM cholinergic neurons somehow retain some means of trophic support following substantial loss of their target site. This could be achieved if these neurons have sustaining collaterals in unaffected cortical areas, if they project to other subcortical regions or if they do not depend exclusively upon their terminal target for survival. The latter possibility has recently been investigated by Sofroniew and colleagues (1990) with respect to septal cholinergic neurons. These authors showed that whereas transection of the fimbria-fornix causes a loss of ChAT-IR septal neurons, complete ablation of the hippocampus induced by the neurotoxin NMDA fails to do so. Since septal cholinergic neurons project almost exclusively to the hippocampus (Amaral and Kurz, 1985) it was suggested that these neurons do not completely depend upon target derived growth factors for survival. However, an alternative interpretation of their results is that injured neurons left with substantial portions of their axons intact can still obtain some trophic support from local sources, while axotomized neurons cannot. In particular, a source of trophic agents may be provided by glia (Yoshida and Gage, 1991; Lu et al.1991) which are known to proliferate following brain damage (Mathewson and Berry, 1985). Therefore, levels of endogenous factors, which are known to increase following injury (Nieto-Sampedro et al.1983; Lorez et al.1988) could be insufficient to prevent neuronal death following axotomy, but may sustain injured neurons whose axons remain substantially intact. It is well established that the extent of neuronal death is related to the distance of the injury from the cell body and that the severity of the lesion can affect the degree of neuronal recovery possible (Lieberman, 1971; Grafstein, 1973; Sofroniew and Isacson, 1988). The lesion used for the studies of this thesis injured the most terminal part of the axon, and thus neurons may remain viable because they can still take up, transport and respond to endogenous trophic agents produced locally. This is likely to be the case since projections of NBM cholinergic neurons to subcortical areas (Carlsen et al.1985) and the degree of their collateralization in the cortex appear minimal (Price and Stern, 1983). This feature may be species specific since in primates individual nbM cholinergic neurons appear to project to widespread cortical areas (Mesulam et al.1986). Although NBM cholinergic neurons in adult decorticated animals persist in a shrunken state, at least as shown here up to 52 days post-lesion, their terminal target area and, perhaps trophic agents held

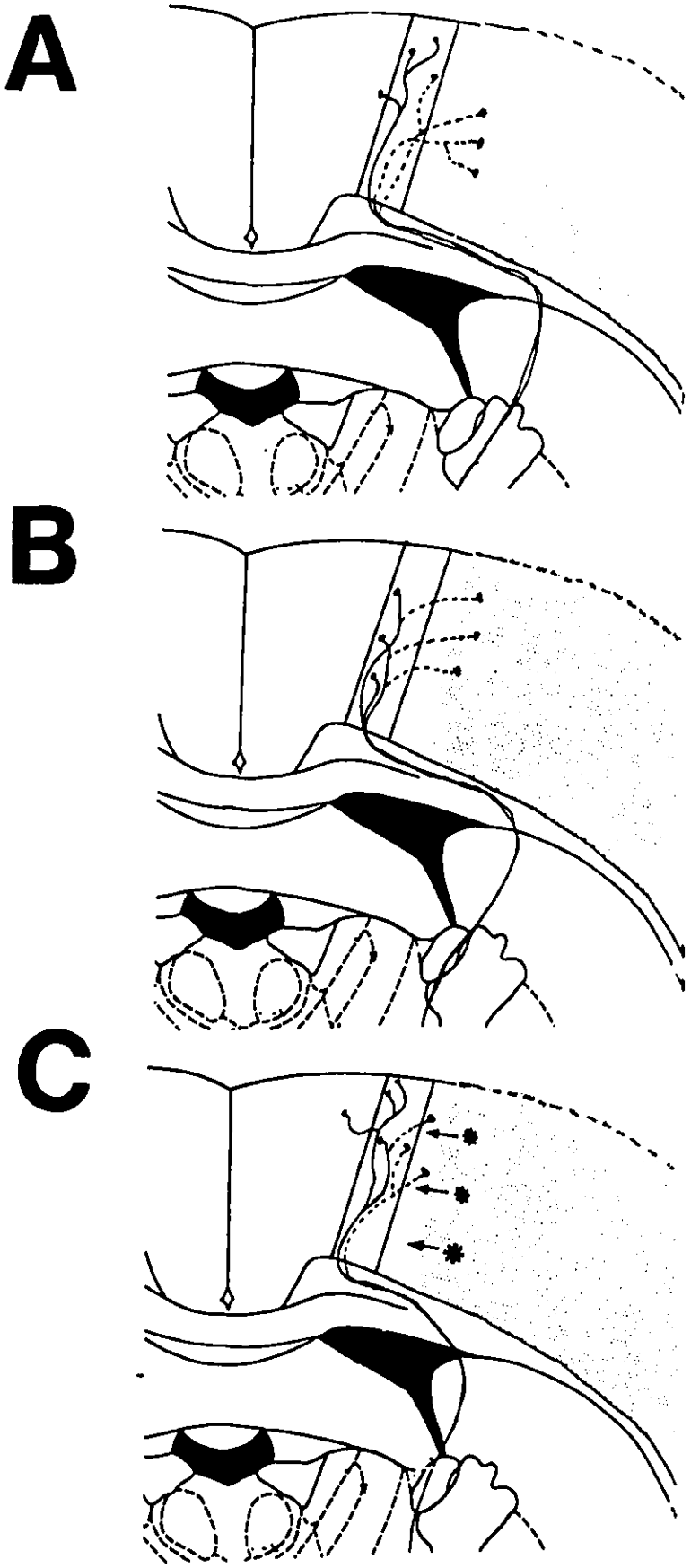
therein, are necessary for the maintenance of neuronal morphology as well as normal levels of neurochemical markers.

4.3 Effect of unilateral decortication on the cortical cholinergic innervation

The remaining ipsilateral cortex adjacent to the lesion site showed no change in ChAT activity or HACU at 5, 15, 30 or 52 days post-lesion when compared to its contralateral counterpart or to cortices of unoperated animals. Such observations could indicate that this remaining cortical area receives input from NBM neurons unaffected by the lesion or that fibers do degenerate, but remaining ones within that region are activated to maintain ChAT activity and HACU at control levels. The latter may be the case since a decrease in the ChAT-IR fiber network was noted in the ipsilateral remaining cortex of lesioned vehicle treated rats, while cortical fibers which remained appeared intensely ChAT-IR (section 3.2.3). The majority of cholinergic fibers in the rat cortex are known to derive from the basal forebrain (Lehmann et al.1980; Fibiger, 1982; Mesulam et al.1983b). Numerous studies have examined the topography of cortical projections from these neurons (reviewed in section 1.3.3c) however, consensus as to their precise morphological organization has yet to be reached. Ascending fibers from individual NBM cholinergic neurons have been reported to be confined within cortical areas no greater than 1.5 mm (Price and Stern, 1983). However, cortical cholinergic fibers have been shown to branch substantially and to innervate several cortical layers (Eckenstein et al.1988). Thus, the lesion-induced loss of ChAT-IR fibers noted in the remaining cortex adjacent to the lesion site of vehicle treated lesioned rats could indicate that: (1) a degeneration of fibers which originate from neurons projecting to the lesioned area, but which traverse through the remaining intact cortex, occurs; (2) collaterals, in the remaining cortex, from injured neurons are retracted or also degenerate; or (3) the loss of fibers arises as a consequence of secondary damage occurring due to the proximity of the cortical area quantified to the lesion site. Of these three possibilities, which are illustrated in Figure 4.1, the latter is less likely to occur since intrinsic ChAT-IR neurons and their fibers in the cortical area examined did not appear to be altered by the lesion. In addition, although all cortical layers are known to receive input from basal forebrain cholinergic neurons a more intense immunostaining has been reported for layer II, lower

layer IV and upper layer V (Houser et al.1985; Luiten et al.1987; Eckenstein et al.1988; Lysakowski et al.1989) areas which appeared more significantly affected by the lesion. However, these observations provide only indirect support that decreases in cortical ChAT-IR fibers occur due to atrophy of NBM cholinergic neurons. Direct evidence for this could be obtained, in future studies, by using anterograde tracers such as *phaseolus vulgaris* leugoagglutinin (PHL-A) to establish the origin of the cortical ChAT-IR fibers under investigation.

Figure 4.1 Schematic representation of the possible effects of unilateral decortication on cholinergic fibers in the remaining ipsilateral cortex. Few fibers are shown for clarity. Shaded areas indicate cortical region affected by the lesion. Dashed lines indicate degenerated fibers. (A) Anterograde degeneration of cholinergic fibers which innervate lesioned cortical areas, but which traverse nonlesioned regions is shown; (B) Illustrates possible degeneration of collaterals from ChAT-IR cortical fibers; (C) Shows that the noted fiber loss could arise as a consequence of the diffusion of neurotoxic substances from nearby injured areas.


A
B
C

4.4 NGF and GM1 prevent retrograde degeneration of cholinergic neurons in the rat NBM

The work of this thesis has shown that continuous i.c.v infusion of either GM1 or NGF to lesioned rats can attenuate deficits in NBM ChAT activity (section 3.1). This could be attributed to the prevention of neuronal atrophy accorded by these factors. Indeed, it was confirmed, using quantitative light microscopic immunocytochemical techniques, that treatment of decorticated rats with GM1 or NGF attenuates NBM ChAT-IR cell shrinkage and the loss of the ChAT-IR fiber network in this area (section 3.2). However, increases in the *de novo* synthesis of ChAT or alterations in its turnover could also contribute to the neurochemical recovery noted. Although the former has yet to be unequivocally demonstrated, increases in ChAT mRNA following NGF treatment have been reported using in-situ hybridization techniques (Higgins et al.1989). How ChAT expression is regulated remains to be established. A recent study has shown that the gene for ChAT contains several AP1 sites (Ibáñez and Persson, 1992). AP1 sites refer to a sequence, -TGACTCA-, which was first identified as a phorbol ester inducible promotor element and the binding site for a transcription factor activity termed AP1 (Sheng and Greenberg, 1990). The product of two immediate early genes, c-fos and c-jun, interact with each other to form a heterodimeric transcription factor complex which can bind AP1 sites and potentially stimulate transcription (Sheng and Greenberg, 1990). Since NGF has been shown to upregulate c-fos, both *in vitro* and *in vivo* (Midbrant, 1986; Sharp et al.1989), NGF could conceivably regulate ChAT expression via activation of this transcription factor. Whether GM1 treatment also affects ChAT mRNA levels remains to be determined. However, previous work has shown, in fimbria-fornix lesioned rats, that GM1 can increase ChAT Vmax, suggesting increases in protein number (Skup et al.1987). Upregulation of ChAT expression rather than prevention of neuronal death has been proposed to account for the apparent preservation of cholinergic septal cell number observed following fimbria-fornix transection. In contrast to cortical devascularization, which caused no significant loss of NBM cholinergic neurons up to 52 days post-lesion, decreases in the number of septal ChAT-IR neurons were noted at approximately 2 weeks after fimbria-fornix transection. Several investigators have now shown that exogenous NGF can prevent this apparent neuronal degeneration (Hefti, 1986; Williams

et al.1986; Kromer, 1987). However, the reduced number of septal cholinergic neurons noted after axotomy has been suggested to be due to a decrease in the detection of markers such as ChAT, AChE and $p75^{NGFR}$ rather than neuronal death. This was based on studies which showed that when the onset of NGF treatment was delayed (2 weeks-to-months) for fimbria-fornix lesioned rats, a reappearance of ChAT-IR septal neurons occurred (Hagg et al.1988). Since neurons in the adult rat brain are post-mitotic, it was suggested that fimbria-fornix transection caused down regulation of ChAT expression rather than cell death. This could occur because the injured cell diverts its energy towards the synthesis of proteins necessary for the maintenance of its structure and repair (Spencer et al.1985). Whether neuronal death subsequently arises appears to depend upon a number of factors, which were previously mentioned in the introduction to this thesis (section 1.1). More recently, Fischer and Björklund (1991) have shown, using the fluorescent retrograde tracer fluorogold to prelabel septal neurons, that fimbria-fornix transection in adult rats causes down regulation of marker enzymes such as AChE and $p75^{NGFR}$ as well as cell atrophy (exemplified by neuronal shrinkage) and actual cell death. Moreover, it was reported that treatment with NGF could prevent all three events. The work of this thesis has comprehensively shown, with the aid of an image analysis system, that NGF or GM1 treatment can equally prevent shrinkage of NBM cholinergic neurons and can also preserve the NBM ChAT-IR fiber network in adult decorticated rats. In addition, it was demonstrated that these agents confer long-term recovery to injured NBM cholinergic neurons. A 7 or 14 day treatment with NGF or GM1 maintained neuronal morphology and neurite length in various subdivisions of the NBM up to 3 or 5 weeks after termination of treatment, respectively. This neuroprotection was particularly evident in the dorsal mid-portion of the NBM where neurons were most severely affected by the decortication. Long-term preservation of NBM neuronal morphology suggests that NGF or GM1 treatment could stabilize neurons after injury and stimulate the reestablishment of contact with post-synaptic targets. The latter could account for the absence of a long-term dependence of injured NBM cholinergic neurons on exogenous trophic agents. Our finding that short-term treatment with NGF induces synaptogenesis in cortex and that GM1 can maintain innervation supports this proposal (see section 4.7). By contrast, Montero and Hefti (1988) showed that a chronic administration of NGF is needed to prevent the apparent septal cholinergic cell loss which occurs following fimbria-fornix transection. Differences between

our findings and theirs can be attributed to several methodological factors. Firstly, different dosages and mode of administration were employed for NGF. For the neuroanatomical experiments reported in this thesis, murine 2.5S NGF was administered i.c.v., at 12 or 6 μg/day, continuously via minipump for 7 or 14 days, respectively and then animals were left untreated for an additional 3 or 5 weeks, respectively. In the study of Montero and Hefti, murine 2.5S NGF at 5 μg/day was injected into the cerebroventricular space either: (1) once daily on the first and third day after fimbria-fornix transection, and subsequently lesioned rats were left untreated for 4 weeks; or (2) once every second day, for 4 weeks and the animals were then left untreated for another 4 weeks. In their report, both these paradigms were shown to be ineffective in preventing septal ChAT-IR neuronal loss after fimbria-fornix transection. Whether a continuous short-term treatment with NGF could result in the long-term survival of septal neurons after fimbria-fornix transection remains to be investigated. It is more likely however, that the divergent lesion models employed in these studies account for the noted differences. As previously discussed, in the fimbria-fornix transection model an axotomy is produced which quickly severs the neuron from its terminal target and depending upon the proximity of the transection to the cell body, various degrees of neuronal death ensue (Sofroniew and Isacson, 1988). By contrast, the cortical devascularizing lesion affects only the target region. Thus, the injury is restricted to the most terminal segment of the axon and a gradual degeneration leading mostly to cell shrinkage occurs. Therefore, the ultimate response of the neuron to such injuries and the potential endogenous agents (ex: glia, interleukins, macrophages, other neurotrophins) which are upregulated after these traumas (Mathewson and Berry, 1985; Nieto-Sampedro et al.1983; Lorez et al.1988) could differ and perhaps influence the degree of neuroplasticity possible. Such events may be pivotal in defining the outcome of exogenous trophic factor effects. In addition, since cortical devascularization appears to produce less severe neuronal damage, most likely because a sizeable portion of the axon is left intact, it is possible that the injured neuron is more amenable to the effects of putative trophic agents. This may indeed be the case since NGF has been shown to stimulate axotomized septal cholinergic neurons to regenerate when a peripheral nerve or collagen matrix are implanted to provide a bridge (Hagg et al.1990; Kawaja et al.1992). In particular, a study by Kawaja and coworkers (1992) has demonstrated that these regenerated fibers can reestablish contact with the hippocampus. This is in line

with our observation that NGF treatment can provoke synaptogenesis in the injured adult mammalian brain (see section 3.2).

The dependence of NGF effects on lesion type and extent was clearly demonstrated by the study of Williams and coworkers (1989) who assessed NGF effects on septal/VDB ChAT activity following various lesions to the septo-hippocampal pathway. It was shown that treatment with exogenous NGF did not affect ChAT activity in the medial septum/VDB of animals which received aspirative lesions of the cingulate cortex and supracallosal striae, but that modest increases occurred in animals which received partial knife transections of the fimbria-fornix. Moreover, the activity of ChAT was substantially augmented, by NGF treatment, in the septum/VDB of rats subjected to complete aspirative transection of the fimbria-fornix. The effect of GM1 on septo-hippocampal cholinergic markers has also been shown to similarly depend upon the extent of denervation induced by the lesion (Gradkowska et al.1986). However, for GM1, this effect was not restricted to cholinergic systems. The magnitude of the GM1-induced attenuation of deficits in hippocampal 5-HT after fimbria-fornix transection also appeared to depend upon the degree of denervation. In addition, the extent of the GM1-induced recovery of striatal dopamine and its metabolites in MPTP treated mice was also influenced by the degree of damage induced (Hadjiconstantinou and Neff, 1988; Schneider and Yuwiler, 1989). A dependence of the neuroprotective effects of GM1 on lesion extent could also account for discrepancies between studies reporting the need for chronic treatment (Hadjiconstantinou and Neff, 1988), or lack thereof [this thesis, (Schneider and Yuwiler, 1989)] with this agent, and for reports indicating that GM1 does not promote recovery following particular brain injuries (Toffano et al.1984b; Stephens et al.1988; Walsh et al.1989). That the effects of putative neurotrophic agents such as NGF or GM1 can vary according to lesion extent and type of trauma incurred suggests that the activities of these factors may depend upon neuronal state or other substances released after injury (discussed further in section 4.9). Such findings are important in view of the fact that trophic factor administration is being considered for the treatment of certain human neurodegenerative diseases [(Hefti et al.1989), also discussed in section 4.14].

4.5 Effect of NGF or GM1 on cortical cholinergic innervation

In the remaining cortex adjacent to the lesion site NGF or GM1 treatment caused a dose dependent augmentation in ChAT activity and HACU, above control levels. These effects could be attributed to an increase in ChAT or HACU protein number in remaining terminals. Alternatively, these findings could be interpreted as indicative that sprouting of cortical cholinergic fibers occurred. That the increase in these neurochemical markers in NGF or GM1 treated decorticated rats was significant at approximately 15 days after initiation of treatment would be consistent with the occurrence of fiber growth. However, activation of mechanisms conferring NGF or GM1 sensitivity following injury, which lead to ChAT upregulation and transport could also be time dependent. NGF or GM1 induced sprouting in the CNS has now been inferred from several studies (Gradkowska et al.1986; Rosenberg et al.1988; Gage et al.1988). Most of these reports however, have interpreted increases in ChAT or AChE immunoreactivity as sprouting. This is problematic, and at best, provides only indirect evidence for sprouting since it can be argued that NGF or GM1 treatment could increase the expression of ChAT or AChE proteins in fibers which were previously below the detection limit of the immunocytochemical procedure. The work of this thesis has provided direct evidence, using quantitative electron microscopic techniques, that NGF treatment causes terminal sprouting in the injured adult CNS. By contrast, GM1 treatment at the dosages and duration employed for these studies did not induce this phenomenon. Although either GM1 or NGF treatment maintained the cholinergic fiber network in the remaining cortex of lesioned animals at control levels, only NGF treatment significantly increased ChAT-IR varicosity number and presynaptic terminal size. This was somewhat surprising since the neurochemical data failed to show a distinction between GM1 and NGF effects. In particular, the time course and extent of the supranormal increases in cortical ChAT activity and cortical HACU induced by NGF or GM1 in lesioned rats were equivalent. In addition, both agents caused an increase in HACU Vmax without affecting its Km. NGF or GM1 treatment also similarly increased "soluble" or "membrane bound" forms of ChAT activity. Based on our electron microscopic studies it appears that supranormal increases in cortical cholinergic markers in NGF treated lesioned rats can be attributed to both sprouting and increases in the number of ChAT proteins or HACU sites per terminal. By contrast, for

GM1, only the latter phenomenon appears applicable.

As mentioned in the introduction to this thesis, it is thought that "membrane bound" ChAT could play a particular role in ACh synthesis because of its proximity to the choline carrier. Since gangliosides incorporate into neuronal membranes, whether GM1 treatment preferentially activated "membrane bound" ChAT was of particular interest to test. "Membrane bound" and "soluble" ChAT forms in cortex and striatum were shown not to be differentially affected by GM1 and/or NGF treatment. That these factors increase both "membrane bound" and "soluble" ChAT activity to the same extent could indicate that in addition to possibly augmenting ChAT protein number exogenous NGF or GM1 also maintain normal distributions of ChAT pools.

The increase induced by GM1 or NGF treatment in cortical HACU Vmax of lesioned rats further suggests that these agents augment the activity of cholinergic neurons *in vivo*. Increases in cortical HACU Vmax could indicate that NGF or GM1 treatment affect the turnover or augment the number of choline uptake sites. Factors which have been implicated in the modulation of the choline transporter include: arachidonic acid, phospholipase A2, calcium and calmodulin (Boksa et al.1988; Yamada et al.1988; Yamada et al.1991b; Salterelli et al.1990), as well as phosphorylation (Breer and Knipper,1990; Chaterjee and Bhatnagar,1990; Knipper et al.1992). However, how the choline transporter is regulated physiologically remains to be clarified. Thus, the possible mechanisms through which NGF or GM1 could affect choline transport also remain to be determined. GM1 or NGF treatment have also been reported to attenuate transient decreases in cortical HACU or ChAT activity following electrolytic or neurotoxic lesions of the NBM (Casamenti et al.1985; DiPatre et al.1989; Haroutunian et al.1986; Haroutunian et al.1989). As well, it was shown using an anterograde NBM lesion model that GM1 treatment can attenuate deficits in cortical ACh release (Florian et al.1987). Similar findings of enhanced cortical ACh release were noted, using *in vivo* microdialysis, in NBM lesioned rats which received NGF treatment (Dekker et al.1991b). The effects of NGF or GM1 treatment on ACh release in the cortical devascularization lesion model used for this thesis were investigated by another member of our laboratory. It was demonstrated, using the microdialysis technique, that decorticated rats which received NGF, i.c.v. via minipump for 7 days, had increased basal cortical ACh release, *in vivo*, at 30 days post-lesion (Maysinger et al.1992a). Moreover, a trend towards

an increase in the levels of potassium stimulated ACh release was noted. By contrast, GM1 treatment did not significantly alter basal Ach release but did significantly increase potassium-stimulated ACh release measured in a portion of remaining cortex (Maysinger et al.1988). These results support the neurochemical and ultrastructural findings of this thesis which indicate that cortical cholinergic innervation is augmented by these agents. In adult rats with full transections of the fimbria-fornix continuous treatment, via minipump, with murine NGF has also been reported to increase hippocampal HACU (Williams and Rylett, 1990). Furthermore, using a partial fimbria-fornix transection lesion model, it has been shown that injections of recombinant human NGF, into the cerebroventricular space every second day for three weeks, can increase ACh synthesis as well as its spontaneous and evoked release in hippocampal slices from adult lesioned rats (Lapchak and Hefti, 1991). These alterations were attributed to an activation of remaining uninjured fibers since in another study similar NGF treatment did not appear to stimulate significant hippocampal AChE fiber growth (Junard et al.1990). Such studies showing that NGF or GM1 treatment can enhance neurotransmitter release confirm that these agents alter the presynaptic function of cortical or hippocampal cholinergic fibers. Investigations assessing whether NGF or GM1 treatment affect muscarinic or nicotinic receptors in the cortical devascularization lesion model have yet to be completed. In the fimbria-fornix lesion model it has recently been shown that chronic NGF treatment augments the density of both M1 and M2 receptors in hippocampus (Lapchak et al.1993). Therefore, NGF at least can alter both pre- and post-synaptic cholinergic markers.

The ED50s for NGF or GM1 to affect cholinergic markers in decorticated rats differed substantially. During the course of this thesis a study was published which examined the dose requirements for NGF to attenuate cholinergic deficits in the septum following fimbria-fornix transection, which reported an ED50 for NGF of 0.12 μg/day (Williams et al.1989). This is similar to that noted here for NBM ChAT activity (0.1 μg/day). However, in the present study it was further shown that higher doses of NGF are required to stimulate cortical ChAT and HACU above control levels (ED50: 1 and 1.5 μg/day, respectively). This suggests that greater amounts of NGF are required to activate mechanisms necessary for the transport of ChAT or HACU proteins from cell bodies in the NBM, where they are synthesized, to terminals in cortex. Alternatively, higher doses may be necessary to elicit mechanisms which

induce sprouting. Dosages of GM1 needed to stimulate cortical ChAT activity or HACU were also higher than those required to stimulate NBM ChAT activity. Dose response curves for GM1 effects in other models of cholinergic brain injury are unavailable for comparison. The difference in dosage required for NGF or GM1 to attenuate neurochemical deficits or to stimulate cholinergic markers was approximately 3000 fold. However, what amount of each compound is actually taken up by NBM neurons has not been established. Using autoradiography techniques, it was shown that uninjured basal forebrain neurons accumulate only a small portion of total radiolabeled NGF injected i.c.v. (Ferguson et al.1991). Similar studies have yet to be done with GM1, but as described in section 1.3.4 of the introduction to this thesis ^{3}H-GM1 injected i.c.v. readily accumulates throughout the brain and is subsequently metabolized (Masco and Seifert, 1988). Whether a metabolite of GM1 mediates its neuroprotective effects *in vivo*, remains unknown. *In vitro*, GM1 enhances neuronal survival of mesencephalic cells (Leon et al., 1988). By contrast, neither a-sialo GM1, sialic acid alone nor the oligosaccharide portion of the GM1 molecule produce similar effects. However, the ceramide portion of GM1 can be further metabolized to shingosine, which is thought to mediate some of the intracellular effects of GM1 (Tettamanti and Riboni, 1993).

In contrast to GM1, NGF interacts with specific receptors (Greene and Shooter, 1980), which could account for the low amounts of NGF, as compared to GM1, needed for *in vivo* neuroprotection. Basal forebrain cholinergic neurons have been shown to express both low and high affinity receptors for NGF (Kiss et al.1988; Dawbarn et al.1988; Pioro and Cuello, 1990; Vazquez and Ebendal, 1991; Merlio et al.1993). Moreover, NGF has been shown to be retrogradely transported from target areas such as hippocampus and cortex to basal forebrain cholinergic neurons (Seiler and Schwab, 1984b). The high affinity NGF receptor, p140^{trk}, has intrinsic tyrosine kinase activity (Kaplan et al.1991a; Klein et al.1991) and appears responsible for NGF signal transduction while the role of the low affinity NGF receptor is not yet clear. As reviewed in the introduction to this thesis the molecular mechanisms underlying the neurotrophic or neuritogenic effects of NGF have yet to be fully elucidated. Most studies in this regard have been conducted using *in vitro* systems involving cell lines, and little information is available on how the neuroprotective effects of these agents are mediated, *in vivo*, in adult mammalian neurons. If one extrapolates from what has been reported for *in vitro* NGF effects, it is possible to speculate that multiple mechanisms

also contribute to its *in vivo* effects. For example, as is the case *in vitro*, both transcription dependent and transcription independent mechanisms could mediate NGF-induced neuroprotection of cholinergic neurons or the induction of fiber outgrowth *in vivo*. Firstly, since NGF has been shown to regulate Na^+/K^+ ATPase and calcium flux it is possible that NGF treatment could attenuate early imbalances in Na^+ or Ca^{2+} induced by the lesion (reviewed section 1.5.6). Moreover, since $p140^{trk}$ mRNA has been localized in the adult brain NGF-induced tyrosine phosphorylation could activate subsequent kinases or genes leading to the synthesis of specific proteins involved in the maintenance of neuronal morphology or neurite outgrowth. Indeed growth associated proteins such as neurofilaments, tubulin, GAP-43 and others have been shown to be activated by NGF [(Dickson et al.1986; Meiri and Burdick, 1991), for review see (Halegoua et al.1991)].

In contrast to NGF, GM1 does not interact with specific receptors, lacks specificity for neuronal phenotype in the CNS, and the molecular mechanisms for the neuroprotection it accords are more nebulous. Interestingly, however, GM1 appears to share several of the above mentioned functions associated with NGF. For example, GM1 has been shown to: regulate membrane enzymes such as Na^+/K^+ ATPase (Karpiak et al.1991; Li et al.1986); modulate calcium influx or intracellular calcium levels (Wu et al.1990; Wu and Ledeen, 1991; DeErausquin et al.1990); and to activate the synthesis of proteins necessary for growth (Rybak et al.1983). In addition, GM1 has been shown to block the neurotoxic effects of agents like glutamate (Skaper et al.1991; Leon et al.1990) and to potentiate the actions of neurotrophic factors *in vitro* and *in vivo* [this thesis, (Ferrari et al.1983; Katoh-Semba et al.1984; Leon et al.1984b; Vantini et al.1988; DiPatre et al.1989). Increases in glutamate and neurotrophic activity as well as destabilization of calcium levels and Na^+/K^+ ATPase are events known to occur soon after injury (Choi, 1990). It has been shown that following cerebral ischemia in rats disturbances in these components can be attenuated by GM1 treatment (Li et al.1986; Karpiak et al.1991). Such observations are consistent with the early onset treatment time required for GM1 to prevent retrograde degeneration of cholinergic NBM neurons after cortical devascularization. The work of this thesis has shown that short-term treatment with GM1 cannot be delayed more than 2 days in order to significantly attenuate NBM ChAT activity while continuous GM1 treatment cannot be delayed more than 4 days to prevent decreases in NBM ChAT activity. This indicates that GM1 predominantly

acts as a protective rather than restorative agent following CNS injury. Similar findings were observed after surgical or neurotoxic lesions of dopaminergic pathways (Toffano et al.1984c; Hadjiconstantinou and Neff, 1988). Thus, GM1 treatment appears to block acute effects of brain damage or to facilitate an intrinsic neuroprotective program activated by the injured cell soon after trauma. These results support *in vitro* studies which indicate that GM1 facilitates rather than initiates trophic programs (Skaper and Varon, 1985). By contrast, NGF seems to have some restorative ability since delaying the onset of treatment in decorticated animals by 14 days still prevented deficits in NBM ChAT activity following decortication. Whether such delays in initiating GM1 or NGF treatment following cortical devascularization can also restore neuronal morphology was not investigated. As previously discussed, in fimbria-fornix lesioned rats which received delayed NGF treatment a reappearance of ChAT-IR septal neurons occurred (Hagg et al.1988). Although maximal recovery was noted if NGF treatment was initiated within 3-to-7 days after lesioning, approximately 50% of ChAT-IR neurons apparently lost after fimbria-fornix transection were rescued even if NGF treatment was delayed by 96 days (Hagg et al.1989a). Moreover, normal cell size was restored even if NGF treatment was delayed by 2 weeks. As previously mentioned, a study which examined the fate of prelabelled medial septal neurons following fimbria-fornix transection provided direct evidence that delayed NGF treatment can reverse septal cholinergic neuronal shrinkage (Fischer and Björklund, 1991). In the delayed treatment paradigms used for experiments of this thesis cortical ChAT activity or HACU were not augmented above control levels in NGF or GM1 treated decorticated rats. This suggests that early administration is necessary to provoke sprouting of cortical cholinergic fibers or to activate the necessary mechanisms to transport ChAT or HACU proteins to cortex.

4.6 GM1 potentiates NGF-induced increases in NBM and cortical ChAT activity and cortical HACU

The work of this thesis demonstrated that GM1 can increase the effects of exogenous NGF on cholinergic makers following brain injury in adult rats (section 3.1). A subsequent study reported a similar effect using a lesion model involving anterograde damage to the NBM;

exogenous GM1 was shown to potentiate the NGF-induced attenuation of deficits in cortical ChAT activity and HACU following unilateral electrolytic NBM lesions (DiPatre et al.1989). This phenomenon is not restricted to CNS cholinergic systems since it has also been shown that GM1 can potentiate the neuroprotective effects of NGF on NA levels following vinblastine induced sympathectomy in newborn rats (Vantini et al.1988). These findings suggest that the neuroprotective effects of GM1, *in vivo*, may in part involve the potentiation of endogenous trophic agents. Although this notion is also supported by *in vitro* studies (reviewed in introduction section 1.4.7a), the aforementioned results still only provide indirect evidence that the *in vivo* neuroprotective effects of GM1 on CNS neurons are mediated by such interactions. Attempts to provide direct evidence for this were hampered by technical constraints. Ideally, one would have tested whether the *in vivo* neurotrophic effects of GM1 treatment could be inhibited by blocking endogenous NGF activity. However adequate methods to block endogenous NGF activity are still unavailable. The use of antibodies in this regard is problematic since specific antibodies to NGF have not been obtained by many research groups, and often show poor penetration in adult CNS tissues (Springer and Loy, 1985). Nevertheless, in addition to the *in vivo* and *in vitro* evidence showing that GM1 can potentiate exogenous NGF effects, circumstantial evidence also exits to suggest an interaction between GM1 and endogenous trophic agents *in vivo*. For example, as shown by the work of this thesis and that of others (Oderfeld-Nowak et al.1984), exogenous GM1, given either i.p. or i.c.v, has no effect on cholinergic markers of unoperated or unlesioned adult rats. Since levels of endogenous trophic agents are low in the adult uninjured brain, but are significantly increased following injury it is possible that the effects of GM1 are dependent upon the presence of appropriate levels of endogenous trophic agents.

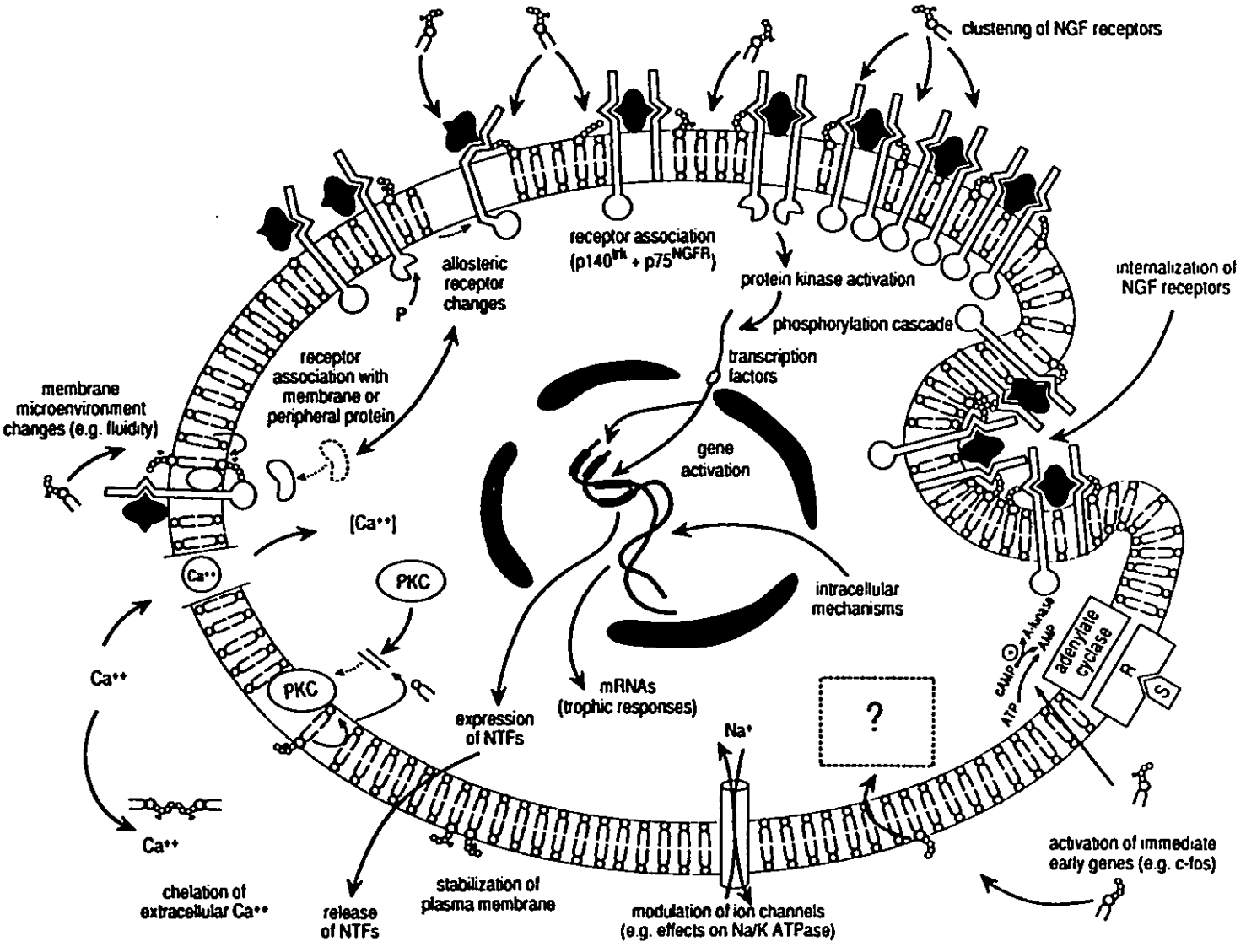
The experiments presented in this thesis show that GM1 increases the efficacy but not the potency of NGF in modulating cholinergic markers (section 3.1.5). This suggests that GM1 does not sensitize cholinergic neurons to exogenous NGF but rather that GM1 may share some aspect of the NGF signal transduction cascade. The interaction between NGF and GM1 appeared to be synergistic rather than additive since ineffective doses of GM1 were able to potentiate the effects induced by maximal NGF doses. Since exogenous GM1 has been shown to incorporate into the plasma membrane (Toffano et al.1980), an interaction at the NGF

receptor level was suspected. Precedent for such a possibility existed since gangliosides have been shown to modulate PDGF and EGF receptors (Bremer et al.1984a; Bremer et al.1986). NGF binds to both low and high affinity receptors. It remains controversial whether in particular neurons $p140^{trk}$ alone or $p140^{trk}$ complexed with $p75^{NGFR}$ mediate the functional activity of NGF (Berg et al.1991; Bothwell, 1991; Hempstead et al.1991; Jing et al.1992). Previous studies using autoradiography techniques showed an apparent lack of NGF high affinity binding in the adult rat cortex (Richardson et al.1986; Ravich and Kreutzberg, 1987). In addition, in situ hybridization studies have so far failed to detect significant levels of $p140^{trk}$ mRNA in cortex (Vazquez and Ebendal, 1991). By contrast, other investigators who used isolated membrane preparations, distinguished both high (35 pM) and low (20 nM) affinity NGF binding sites in adult rat cortex (Alberch et al.1991a; Alberch et al.1991b). Analysis of NGF binding to cortical membranes shown by the work of this thesis (section 3.1.11) revealed one binding site with a kd of 5 nM. However, Scatchard analysis indicated that saturation was not achieved. Thus, it is possible that two sites may exist. Studies using higher concentrations of the ligand were not pursued due to the limited amounts of ^{125}I-NGF available. Discrepancies among binding studies for NGF could be attributed to a number of factors, such as differences in: the sensitivity of detection techniques, preparation of tissues, assay conditions, ligand purity or ligand labelling. A difference in the latter, in particular, was noted between our study and that by Albrech and coworkers (1991b). Specific activity for the ^{125}I-NGF used in this thesis ranged from 950-2000 cpm/fmole while that in their study was 1950-3500 cpm/fmole.

NGF receptor binding to cortical membranes isolated from decorticated rats which received vehicle did not differ from controls at any of the post-lesion times examined. Moreover, GM1 treatment did not affect the Kd nor the Bmax of NGF binding to cortical membranes (section 3.1.11). These results however, do not completely exclude that GM1 can enhance NGF signal transduction by affecting its receptor. NGF receptor activation involves phosphorylation, a process which occurs rapidly. GM1 has previously been shown to affect phosphorylation of PDGF and EGF receptors *in vitro* (Bremer et al.1984a; Bremer et al.1986). Thus, it is possible that GM1 may modulate NGF receptor phosphorylation leading to increased functional activity. In PC12 cells, GM1 potentiates NGF induced neurite outgrowth (Ferrari et al.1983). This effect of NGF has been shown to be blocked by K-252a,

a general protein kinase inhibitor. Moreover, it has recently been shown that K-252a blocks tyrosine kinase activity and NGF-mediated autophosphorylation of $p140^{trk}$ (Berg et al.1992; Tapley et al.1992). It is thought that this may occur because K-252a competes with ATP binding to the kinase catalytic domain (Kase et al.1987). Interestingly, GM1 has been shown to prevent the K-252a-induced inhibition of NGF-induced neurite outgrowth (Ferrari et al.1992). This is thought not to occur because GM1 blocks K-252a entry into the cell since other glycolipids are inactive (Ferrari et al.1992). Speculatively, it could therefore be proposed that GM1, or perhaps a GM1 metabolite, interacts with $p140^{trk}$ such that a conformational change occurs in the receptor to favour ATP binding to the kinase catalytic domain. Alternatively, GM1 may by pass K-252a receptor inhibition to activate steps in the NGF signal transduction cascade, down stream from the receptor, which induce neurite outgrowth. These hypotheses could easily be tested using cell culture systems. However, whether the results obtained could also apply to adult neurons *in vivo* would be difficult to confirm. A scheme of the cellular mechanisms activated by NGF or GM1 and possible sites of interaction are shown in Figure 4.2.

Figure 4.2 Illustration of the possible molecular mechanisms modulated by NGF or GM1 and potential sites of interaction for these two agents.  = NGF;  = ganglioside;  = phospholipid;  = $p140^{trk}$;  = $p75^{NGFR}$


clustering of NGF receptors
receptor association
(p140trk + p75NGFR)
allosteric
receptor
changes
P
protein kinase activation
phosphorylation cascade
transcription
factors
internalization of
NGF receptors
receptor
association with
membrane or
peripheral protein
membrane
microenvironment
changes (e.g. fluidity)
gene
activation
[Ca++]
Ca++
PKC
PKC
intracellular
mechanisms
Ca++
mRNAs
(trophic responses)
expression
of NTFs
Na+
?
cAMP
ATP
AMP
adenylate
cyclase
R
S
Ca++
chelation of
extracellular Ca++
release
of NTFs
stabilization of
plasma membrane
modulation of ion channels
(e.g. effects on Na/K ATPase)
activation of immediate
early genes (e.g. c-fos)

4.7 GM1 potentiates NGF induced alterations in cortical cholinergic presynaptic terminals

As previously described, treatment with NGF or GM1 maintained the length of cortical cholinergic fibers at control levels. By contrast, decorticated rats which received both GM1 and NGF showed significant increases in the cortical ChAT-IR fiber network, above control levels. This indicates that these agents help preserve and could induce neural connectivity following injury. The possibility that the increased fiber network is due to augmented levels of ChAT protein allowing for improved immunocytochemical detection of the fibers cannot be completely excluded. However, this is unlikely as remaining fibers in lesion vehicle treated rats had a tendency to appear more intensely immunostained when compared to NGF alone or NGF/GM1 treated lesioned animals. Moreover, that a growth of cholinergic fibers indeed occurred is supported by our ultrastructural quantitative studies. The noted terminal hypertrophy detected at the electron microscopic level and the increase in the number of axonal varicosities indicate that NGF and, especially NGF/GM1 treatment cause a significant hyperinnervation of this remaining cortical region. Although our methodology did not determine the contribution of intrinsic ChAT-IR neurons to the increased fiber network, it is expected to be minor since the majority of cortical cholinergic fibers have been shown to derive from the NBM (Eckenstein et al.1988). In addition, intrinsic ChAT-IR neurons have not been shown to express, so far, mRNAs for either $p140^{trk}$ or $p75^{NGFR}$ and thus, may not respond to NGF (Vazquez and Ebendal, 1991). Moreover, a recent study has shown that NGF treatment does not affect the size of cortical ChAT-IR interneurons in NBM lesioned rats (Dekker and Thal, 1993). Whether our observations represent a regeneration of injured fibers or a sprouting response from collateral fibers is not clear. Enlarged terminals have previously been noted in both regenerating or sprouting axons (Hwang et al.1986; Steward et al.1988). Therefore, the NGF and NGF/GM1-induced hypertrophy of cortical presynaptic terminals noted in our investigation could be attributed to either events. An illustration of these possible changes is shown in Figure 4.3. Based on the magnitude of the alterations in the fiber network it is favoured that a sprouting of cortical cholinergic fibers occurred.

Lesion-induced collateral sprouting is a phenomena which has been extensively studied in the hippocampal formation [for review see: (Cotman et al.1981; Steward, 1986; Gage and

Björklund, 1986)]. Following destruction of a major source of extrinsic innervation from the entorhinal cortex, lesion induced growth occurs in the dentate gyrus (Steward et al.1974). As well, an increase in synapses has also been shown in this area (Steward et al.1988). The time course for these events varies depending on the lesion induced. After entorhinal cortex lesions, the first signs of reactive growth are noted at 4-5 days, while maximal sprouting, assessed using AChE immunohistochemistry, has been reported to be reached at 12-15 days post-lesion. Reactive synaptogenesis in the lesioned hippocampus has been shown to occur between 9-to-30 days post-lesion [for review see: (Cotman, 1985)]. It was speculated for some time that these events involved growth factors. Indeed, it was subsequently shown that following injury brain tissue extracts exhibited growth promoting activity when tested *in vitro* (Nieto-Sampedro et al.1983). Such increases were first noted at 5 days post-lesion and were maximal by 10 days post-lesion, at which time neurotrophic activity induced by these brain extracts was 4 times higher than that of basal levels. It has recently been shown that antibodies to NGF can block entorhinal lesion-induced collateral sprouting of AChE fibers in the hippocampus (Van der Zee et al.1992). However, the antibody used was not specific for NGF thus, it remains unclear whether endogenous NGF or another neurotrophin directly or indirectly mediates this phenomenon. As mentioned in the introduction to this thesis (section 1.5.11), BDNF, NT-3 and NT-4/NT-5 are neurotrophins which are also present in target areas of basal forebrain cholinergic neurons. BDNF has been shown to be partially effective, and less potent, in preventing the apparent loss of septal ChAT-IR neurons following fimbria-fornix transection (Knüsel et al.1992).

The area of remaining ipsilateral cortex quantified in the 30 days post-lesion vehicle treated rats used for this thesis showed a decrease in the cholinergic fiber network, indicating that no lesion-induced collateral sprouting of cortical cholinergic fibers occurs. Moreover, although it was not quantified, a decrease in the fiber network was still apparent in such lesioned rats at 52 days post-lesion. Whether collateral sprouting occurs, in the remaining cortex of decorticated rats, at subsequent post-lesion times is not known. A lack of cortical cholinergic fiber sprouting, assessed using AChE immunocytochemistry, was also reported for 3 month post-NBM lesioned ferrets (Henderson, 1991). The results of this thesis have shown that NGF or GM1 treatment can attenuate lesion-induced deficits in cortical cholinergic fiber length and that these agents can also induce synaptic remodelling. The

possible molecular mechanisms which underlie such events were not established, but it is likely that synaptic remodelling necessitates the participation of other agents in addition to growth factors. Movement of axonal growth cones has been shown to be influenced by molecules in the extracellular matrix and on cell surfaces (Skene, 1989; Rathjen et al.1992). These molecules include agents such as: laminin, neural cell adhesion molecules (NCAM), fibronectin, heparan sulfate proteoglycan in addition to other growth associated molecules, which are probably concomitantly expressed during neuronal repair such as: the α-1 Tubulin (α-1T) isoform, GAP-43 and others [for review see: (Lander, 1989; Skene, 1989)]. GAP-43 is expressed in brain (Benowitz et al.1988; Jacobson et al.1986) and is thought involved in synaptic plasticity [(Skene, 1989); for review see: (Masliah et al.1991a)]. In addition, a laminin-like antigen has been detected in neurons of the adult rat brain (Hagg et al.1989c). The expression of this laminin-like antigen in septal neurons was shown to decrease after fimbria-fornix transection, and its levels to be restored by NGF treatment (Hagg et al.1989c). Gangliosides themselves have been reported to have cell adhesion properties or to modulate the effects of cell adhesion molecules (Baker, 1988; Cheresh et al.1987), such effects of GM1 could facilitate the maintenance of normal levels of synapses in the remaining cortex of cortically devascularized animals. However, whether these extracellular matrix molecules or other growth associated proteins are upregulated in the remaining cortex of NGF and/or GM1 treated decorticated rats remains to be shown. Moreover, the full extent of the synaptic remodelling induced by NGF or NGF/GM1 treatment in the adult injured brain remains to be explored. It would be of interest to establish, in future studies, whether the noted fiber outgrowth and terminal hypertrophy is restricted to the cortical layer and region examined for this thesis, or if it also occurs in other cortical areas. In particular, the possibility that NGF-induced synaptic remodelling is correlated with the distance from the injury site would be of interest to assess. An important issue which also remains to be resolved is whether appropriate connections are being formed. In NGF or NGF/GM1 treated lesioned rats, where the incidence of synaptic contacts was increased 95% above control levels, the morphological features of the synapses were identical to those observed in controls. However, whether their post-synaptic targets were correct could not be determined by the methods used in this thesis. In future studies, double labelling immunocytochemistry combined with electron microscopy should be used to establish whether NGF and/or GM1 treatment induce aberrant connections.

In related work, it has been shown that NGF treatment can significantly augment dendritic arborization in layer V of the aged rat cortex. This indicates that significant changes can also be induced by this neurotrophin at the post synaptic level (Mervis et al.1991).

Exogenous GM1 administered concurrently with NGF potentiated NGF-induced increases in fiber length, varicosity number and presynaptic terminal size. However, NGF-induced increases in synaptic number were not altered by GM1 co-treatment, which indicates that not all NGF effects are modulated by GM1.

4.8 Differential effects of NGF and GM1 on the striatum

Striatal ChAT activity or HACU were unaffected by decortication at 1, 5, 15 and 30 days post-lesion. The innervation of the normal striatum is complex, and its circuitry in rat has yet to be fully established at the ultrastructural level. The striatum is known to receive input from glutamatergic cortical neurons, located predominantly in layer V, in widespread cortical areas [(Gerfen and Sawchenko, 1984); for review see: (Gerfen, 1992)]. This input is thought to be presynaptic to striatal medium spiny neurons which use GABA as a principal transmitter, and which innervate dopaminergic neurons in the substantia nigra. Substantia nigra dopaminergic neurons, in turn, project to the striatum and innervate cholinergic interneurons (reviewed in introduction 1.3.5). That decortication does not appear to affect the function of striatal cholinergic interneurons reflects that these interneurons do not receive direct input from the cortex, and suggests that striatal cholinergic function recovers rapidly from the loss of cortical-striatal innervation. Previous studies have also shown that ChAT activity, choline uptake or ACh content are not altered, up to 4 weeks following frontal cortex ablation in the rat (Hassler et al.1982). However, the lack of effect of cortical lesions on striatal cholinergic markers could also be explained by the fact that the cortical glutamatergic input to striatum has a differential distribution. That is, glutamatergic neurons in neocortical areas appear to primarily innervate the dorsal striatum while those in allocortical areas innervate the ventral striatum [for review see: (Gerfen, 1992)], since cholinergic markers were measured in homogenates from the whole striatum it is possible that small alterations in their levels were masked.

In contrast to the basalo-cortical pathway, dissimilar neurochemical effects of NGF and

GM1 treatment were noted in both the ipsilateral and contralateral striatum. Striatal ChAT activity and HACU, which were unaffected by the lesion, were augmented by NGF but not GM1 treatment. This increase was noted as early as 5 days post-lesion. In addition, an apparent hypertrophy of striatal cholinergic neurons was noted in NGF treated rats (section 3.3), thus supporting other studies indicating that striatal cholinergic interneurons are particularly responsive to exogenous NGF (Mobley et al.1985; Hagg et al.1989b; Gage et al.1989). This has been attributed to the low levels of endogenous NGF normally present in this area and the abundance of high affinity NGF binding sites localized in this region (Richardson et al.1986; Ravich and Kreutzberg, 1987; Altar et al.1991). Interestingly, in contrast to the basal forebrain, little or no immunoreactivity to the monoclonal antibody Mab 192, thought to recognize $p75^{LNGFR}$, is noted in the adult uninjured striatum (Gage et al.1989; Hagg et al.1989b; Pioro and Cuello, 1990). However, increases in the expression of this low-affinity receptor are noted following injury or NGF infusion [present study, (Gage et al.1989)]. The apparent inability of GM1 to potentiate NGF-induced increases in striatal ChAT activity or HACU in our decorticated animals could be attributed to receptor differences. On the other hand, this could indicate that injury activates particular mechanisms or releases agents which GM1 affects to indirectly potentiate the actions of NGF (further discussed below in section 4.9).

In contrast to the data obtained for cortex, NGF binding to striatal membranes best fit a two site model exhibiting both high and low affinity binding. Although this agrees with previous reports examining NGF binding to striatum either using autoradiography or membrane binding techniques, the Kd and Bmax values obtained were significantly different than those reported (Richardson et al.1986). This discrepancy, as previously discussed, could be attributed to differences in experimental procedures.

4.9 Effects of NGF and/or GM1 on nonlesioned brain areas

The results presented in this thesis show that NGF or GM1 treatment do not increase cholinergic markers in brain areas, except the striatum, which were not directly affected by the lesion. These include regions such as the septum, VDB, HDB, hippocampus, contralateral NBM or contralateral cortex. Moreover, NGF or NGF/GM1 treatment did not alter the

cortical cholinergic innervation in unlesioned animals. It could therefore be suggested that the normal adult CNS has mechanisms which can deal with excessive amounts of trophic factors or that injury creates a permissive environment for NGF or GM1 effects. Possibly, brain damage could lead to the suppression of inhibitory agents or alternatively, the activation of substances which facilitate the actions of, or through which exogenous trophic agents could act to indirectly affect cholinergic innervation. Although little is known with regard to the first possibility, it has been shown that interleukins, excitatory amino acids, and c-fos, which are all upregulated following injury can modulate NGF (Hengerer et al.1990; Zafra et al.1991; Thoenen et al.1991; Zafra et al.1990). Moreover, glia which proliferate after brain injury, have been proposed to secrete NGF itself or agents which can modulate NGF (Furukawa et al.1986; Yoshida and Gage, 1991; Yoshida and Gage, 1992). Indeed, interleukin-3, b-FGF, and a-FGF have been shown to exert neurotrophic effects upon basal forebrain cholinergic neurons *in vivo* (Kamegai et al.1990; Anderson et al.1988; Figueiredo et al.1992). It is possible therefore, that the *in vivo* effects of NGF or GM1 could be facilitated by such agents.

Some studies have shown however, that the intact septo-hippocampal pathway or NBM of adult rats may also respond to exogenous NGF by increasing ChAT activity (Fusco et al.1989) or cell size (Fischer and Björklund, 1991). NGF itself has been shown to upregulate $p75^{NGFR}$ immunoreactivity and its mRNA (Cavicchioli et al.1989). Moreover, $p140^{trk}$ mRNA can also be upregulated by exogenous NGF in the adult brain (Holtzman et al.1992). The effects of NGF on ChAT activity in the normal septo-hippocampal pathway of adult rats appears transient since the activity of this enzyme returns to control levels after NGF treatment is stopped (Fusco et al.1989). Discrepancies with respect to the effects, or lack thereof, of NGF in normal rats have been attributed to differences in rat sex or strain and in dosage, biological activity, duration or method of NGF treatment employed (Williams et al.1989). Effects of NGF in brain regions other that the striatum or ipsilateral NBM and cortex were perhaps not noted in the present study because a short-term NGF treatment was used. In decorticated animals which received NGF, i.c.v. via minipump for 14 days a trend towards an increase in ChAT activity in the ventral hippocampus was noted at 52 days post-lesion. Thus, it is possible that with higher doses of NGF and/or GM1 or extended treatment times cholinergic markers could be augmented, and synaptic remodelling could be induced

in normal adult male Wistar rats.

4.9 NGF and/or GM1 treatment fails to affect GAD activity in the adult brain

The majority of studies with NGF have focused on the cholinergic system. However, GABAergic neurons are present in the basal forebrain and have been shown to express high affinity NGF binding *in vitro* (Dreyfus et al.1989). A GABAergic projection from the septum to hippocampus in the rat has been demonstrated (Köhler et al.1984). However, the apparent loss in GAD immunopositive septal neurons, following fimbria-fornix transection, was shown not to be prevented by NGF treatment (Montero and Hefti, 1988). In the rat, NBM GABAergic neurons appear to be intrinsic in contrast to what is noted for cats or monkeys (Fisher et al.1988). Since GABA transmission is also thought to play a role in learning and memory (Dudchenko and Scuter, 1991) and in view of the fact that GM1 has previously been shown to exert effects on injured non cholinergic pathways [reviewed in section 1.4.7b_2], GAD activity, a marker of GABAergic function, was also measured in the experimental animals used for the work of this thesis. GAD activity was found to be unaltered, at various post-lesion times, in the NBM, striatum and all other brain areas examined in decorticated rats. This is in agreement with previous studies which showed that NBM GAD activity was not affected by excitotoxic cortical lesions (Lehmann et al.1980), and that striatal GAD activity remains at control levels after decortication (Hassler et al.1982). The results obtained for striatum were surprising because striatal GABAergic neurons have been reported to be directly innervated by glutamateric fibers from cortex. As previously discussed (section 1.3.5) however, since the input from neocortex appears to be concentrated in the dorsal striatum changes in GAD activity, measured in the whole striatum, could be masked. NGF and/or GM1 treatment did not alter GAD activity in any ipsilateral or contralateral brain area examined. This provides further support for studies which propose that NGF specifically affects cholinergic systems in the CNS and that GM1 does not affect intact pathways.

4.10 NGF or GM1 treatment differentially affect behaviour of cortically lesioned rats

In order to assess and compare the functional consequences of GM1 and/or NGF treatment in this lesion model the behaviour of these animals was studied in two tasks, passive avoidance and the Morris Water maze. These two tasks have been extensively used in NBM-cortex lesion paradigms. The passive avoidance task is a rather crude test for learning and memory, but because of its simplicity is frequently used for experiments involving rodents. The task involves that the rat learn and recall to avoid an aversive stimulus (footshock) upon entering a dark compartment of a shuttle box. Rats therefore are required to suppress an instinctive behavior, to seek the dark, by sitting "passively" in the illuminated side of a shuttle box. By contrast, the Morris water maze is a task which can test both spatial and non-spatial learning and memory in rodents. Originally, it was developed to test a rat's ability to learn, recall and go to a place in space delineated only by extramaze cues (Morris, 1984). In the task, rats are required to locate a platform submerged below the surface of the water, and fixed at a particular position in a circular pool. This task has some advantages over other tests of spatial memory, such as the T-maze or radial-arm maze, in that acquisition of the task is quite rapid, thus obviating the need for extensive pretraining and allowing the handling of large groups of animals. In addition, no food reward is involved, thus, dismissing the need to starve the animals and of odor trails. Moreover, several variations of the task can be used. For example, "place learning" (spatial test) can be examined by assessing the rat's ability to locate a hidden platform (submerged below that pool water); "cue learning" (non-spatial test) can be studied by assessing the ability of the rat to learn to swim to a visible platform. This non-spatial test, in particular, is often used to provide indirect evidence that rats do not have sensory, motor or motivational disturbances which could influence performance. Additional variations of the Morris water maze task have also been adopted (McNamara and Skelton, 1993).

Lesions of the cortex have previously been shown to disrupt rodent behavior in learning and memory based tasks. For example, neurotoxic lesions of the insular or frontal cortices have been reported to disrupt passive avoidance retention (Bermudez-Rattoni et al.1991; Fukuchi et al.1987). As well, an intact cortex has been shown to be important for proper spatial navigation in the rat. Kolb and colleagues (1983) employing the suction ablation

cortical lesioning technique demonstrated that damage to the medial frontal, orbital frontal and cingulate cortices produce significant impairments in water maze place learning while injury to parietal areas resulted in only minor deficits. However, in a study where a larger extent of the rat parietal cortex was lesioned it was shown that this area also contributes significantly, perhaps to a greater degree than the hippocampus, to spatial memory (DiMattia and Kesner, 1988). The unilateral cortical lesion employed for this thesis also caused deficits, albeit mild, in spatial navigation and passive avoidance retention and reacquisition. That performance deficits were noted in unilaterally cortically lesioned rats could perhaps be attributed to the less stringent training paradigms which were employed in this investigation. Mandel and coworkers (1989a) have shown that training schedule greatly influences the degree of behavioural deficits exhibited, by NBM lesioned rats, in the Morris water maze task. Such a finding may be related to the extent of memory consolidation permitted by the training paradigm which could thus alter task difficulty, and allow the manifestation of minor deficits.

GM1 or NGF treatment distinctly affected performance of lesioned animals. In particular, NGF or NGF/GM1 treatment abolished retention deficits while GM1 treatment instead facilitated the reacquisition of both tasks. With respect to the cholinergic basalo-cortical system, NGF or GM1 treatment produce similar neurochemical effects and have, so far, only been shown to differentially affect the cortical cholinergic network as assessed by EM immunocytochemistry (section 3.2). Based on these results, it is tempting to ascribe the observed differences in behaviour to alterations in cholinergic innervation of the remaining frontal cortex. The remaining basalo-cortical pathway has previously been proposed to play a role in behavioral recovery following NBM lesions (Haroutunian et al.1990). For example, rats with basal forebrain lesions have been shown to exhibit retention deficits in a passive avoidance task, which can be attenuated by the administration of physostigmine. In rats which received both NBM and frontal cortex lesions physostigmine fails to improve passive avoidance retention. Although this evidence is indirect, it suggests that the behavioral recovery induced by physostigmine is mediated by the remaining NBM innervation of the frontal cortex. However, interpretation of the behavioural results of this thesis are complicated by several factors. Firstly, as with all behavioral studies involving brain lesions it is difficult to fully attribute deficits and their reversal by drugs to alterations in specific

pathways or learning and memory. In particular, because such lesions invariably also cause sensory, motor or motivational disturbances. Despite numerous investigations [for review see: (Fibiger, 1991; Dekker et al.1991a)] which have shown that NBM lesions cause behavioral deficits in passive avoidance and Morris water maze tasks which can be attenuated by cholinergic drugs, fetal tissue transplants to cortex, NGF or GM1 treatment, work by Page and coworkers (1991) suggests that the NBM-to cortex cholinergic pathway may have little influence on rodent mnemonic processes as assessed by these tasks. Instead these NBM lesion-induced behavioural deficits have been suggested to reflect disturbances in fear or motivation arising either from non specific damage to nuclei in the vicinity of the NBM or disruption of NBM projections to the amygdala (Dunnett et al.1991; Page et al.1991). Moreover, the involvement of alterations in neurochemicals other than acetylcholine (ex: neurotensin, GABA) has also been suggested (Wenk et al.1989). In animals of the present study, a large portion of the neocortex is lost and a significant decrease in ChAT activity occurs in the NBM. These animals do not appear to exhibit any major physiological differences when compared to their unlesioned counterparts. In a previous study, similarly decorticated rats were tested for disturbances in various sensorimotor related behaviours, and other than an increase in overnight locomotor activity and footfaults in a ladder walking task, lesioned animals did not differ from their control counterparts (Elliott et al.1989). However, two important behaviours, fear and motivation, which could also alter performance in passive avoidance and Morris water maze tasks were not examined for decorticated animals. Although other subcortical brain areas appeared undisturbed by the devascularizing cortical lesion, the amygdala and in particular the striatum and thalamus were affected as exemplified by the presence of gliosis in these brain areas at various post-lesion times (Herrera and Cuello, 1992). Thus, it is possible that the observed deficits noted in our animals, particularly in the passive avoidance task, are due to alterations in fear or motivation. In fact, decorticated animals which received vehicle, in contrast to control or lesion GM1 treated rats, were reported to defecate less in the passive avoidance box (Elliott et al.1989). This could reflect reduced fear.

Similarly, alterations in non-mnemonic processes could also account for the noted performance deficits exhibited by lesioned rats in the Morris water maze. Results of the cue studies undertaken in this report together with previous work showing that cortically

devascularized rats have no significant motor deficits (Elliott et al.1989) provide indirect evidence that motivation, altered swim speeds or swimming ability are not significantly altered in these animals. However, it was noted that lesioned rats tend to spend more time swimming along the edge of the pool in initial trials. Unilateral cortical lesions are known to induce contralateral sensory neglect (Cowey and Bozek, 1974). This, or perhaps alterations in visual attentional function could have affected task performance. A further factor to consider is that the cortex also receives catecholaminergic and serotoninergic inputs from the locus coeruleus and raphe nucleus, respectively (Moore and Bloom, 1979; Moore et al.1978). The contribution of these pathways to the observed behaviors remains to be addressed as does the possibility that the diverse behavioural effects induced by GM1 and NGF treatment arise as a consequence of their distinct neurochemical effects on the striatum and hippocampus. Alterations in function of the latter in particular, is known to affect rodent performance in passive avoidance and Morris water maze tasks (Morris et al.1982).

Lesioned animals which received both NGF and GM1 performed no differently, in both tasks, than did those which received NGF treatment alone. The tests employed in the present study perhaps lacked sufficient difficulty to allow a distinction. Nevertheless, it is of interest to note that NGF/GM1 treatment did not adversely affect rodent behavior despite the excessive augmentation in basalo-cortical cholinergic markers induced by their co-treatment.

4.11 Effect of decortication and trophic factor treatment on the thalamus

In animals used for behavioral studies, the effect of the lesion on subcortical brain areas, other than the NBM, in lesioned vehicle or lesioned trophic factor treated rats was also examined. ChAT or $p75^{NGFR}$-IR neurons in the medial septum, VDB, HDB did not appear to differ, in morphology, from their respective controls. However, a neuronal loss and an increase in non-neuronal cells were detected by Nissl staining in the ventrolateral nucleus of the dorsal thalamus. The latter finding most likely reflects lesion-induced gliosis (Herrera and Cuello, 1992). Whether a more sustained administration of NGF or GM1 could effectively prevent thalamic retrograde degeneration following decortication or whether these thalamic neurons respond to other neurotrophins remains to be determined. Recently, BDNF and NT-3, but not NGF, have been shown to be retrogradely transported to the thalamus, following

intrahippocampal injection (DiStefano et al.1992). In addition, treatment with b-FGF has also been r ·ported to prevent retrograde degeneration of thalamic neurons after cortical infarction (Yamada et al.1991a). Thus, perhaps BDNF, NT-3, or b-FGF, rather than NGF, may subserve some function in the thalamus.

4.12 Implications of trophic factor induced synaptic remodelling in the adult brain

A major finding of this thesis is that cholinergic presynaptic terminals in brain injured adult rats can be manipulated by exogenous agents. In particular, direct evidence for an involvement of NGF and GM1 in CNS synaptic remodelling has been provided. Sprouting and synaptic remodelling have been suggested to serve to enhance functional recovery in the CNS (Gage and Björklund, 1986). In NGF or NGF/GM1 treated lesioned rats both terminal hypertrophy and an increase in synaptic contacts were noted. While the significance of the latter is implicit, since it is accepted that information is transmitted through synapses, the finding that terminal hypertrophy occurs may also have important implications for function. The results of this thesis show that approximately 38% of ChAT-IR varicosities in cortical layer V have synaptic differentiations. This is in contrast to the less than 5% which is noted for 5-HT or noradrenaline innervation in the rat cortex (Beaudet and Descarries, 1978) and would favour that cholinergic transmission in cortex is predominantly "wired". However, this has yet to be comprehensively proved and thus, the possibility exists that non synaptic arrangements could also exist for cholinergic systems in particular cortical areas. Non synaptic innervation has been proposed to serve as an efficient way to produce generalized effects without having to contact every cell (Descarries et al.1991). This type of innervation could also account for the mismatch of receptors which exists in brain. The significance of a low number of fixed synapses has been suggested to reflect that these structures are dynamic, allowing for the movement and reshaping of varicosities along the parent fiber according to stimulation (Descarries et al.1991). In fact, dynamic events such as learning and memory are thought to involve alterations in synapses (Greenough, 1988). Long term potentiation (LTP), which is considered a good model for learning and memory, has been shown to involve changes in both pre- and post-synaptic sites (Van Herreveld and Fifkova,

1975; Applegate et al.1987). Moreover, animals exposed to new environments show changes in synaptic shape, dendritic number and spines (Greenough et al.1985). The presence of enlarged varicosities could therefore also be considerably significant with respect to function. Thus, in addition to the potential use of NGF and GM1 to facilitate the reestablishment of disrupted neural connections and to promote functional repair of the adult mammalian brain, their identification as agents which can affect synapses provides a powerful tool with which molecular mechanisms underlying synaptic plasticity can be further studied.

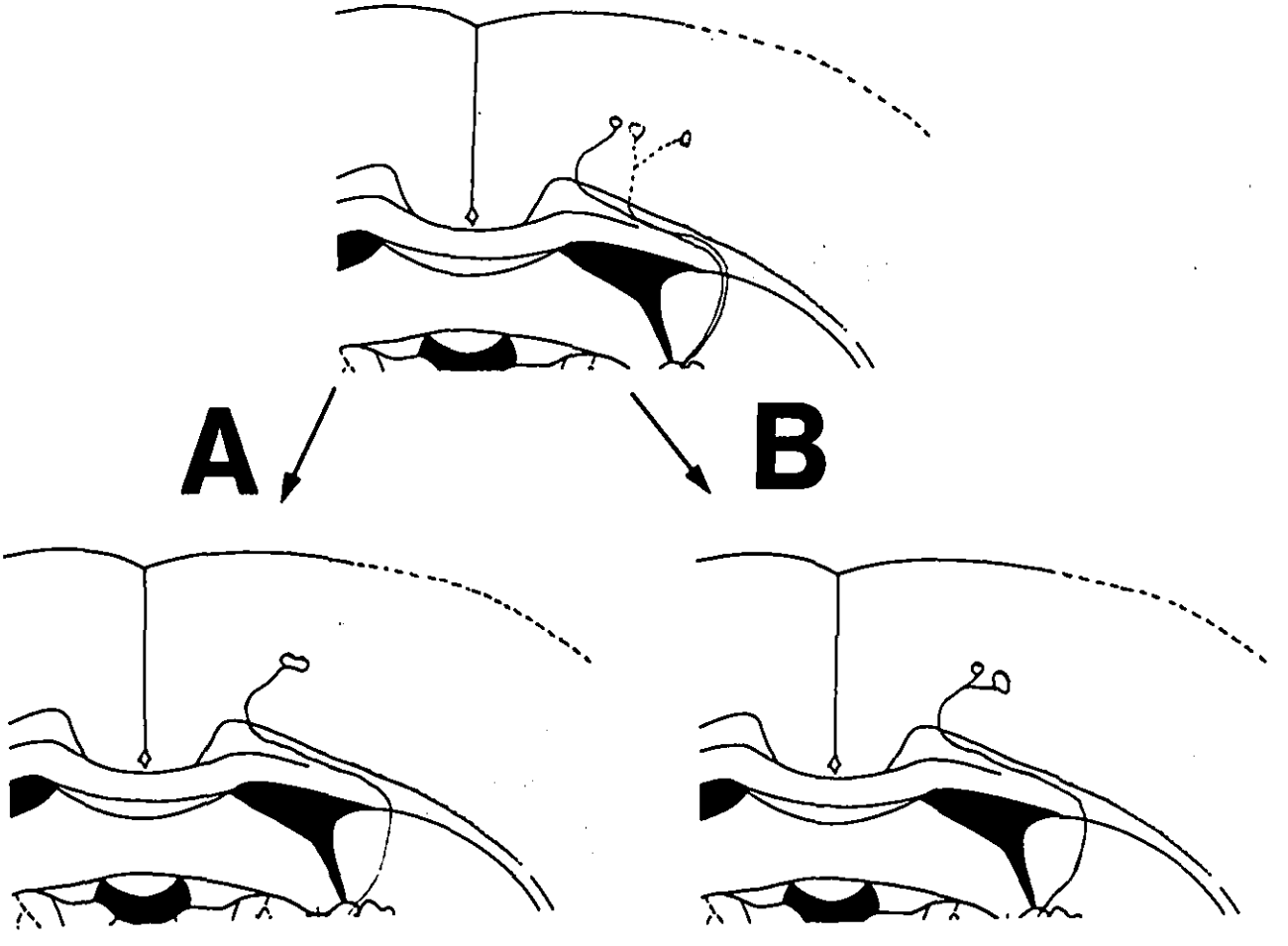


Figure 4.3 Potential alterations induced by NGF or NGF/GM1 treatment on cortical cholinergic presynaptic terminals. Increases in bouton size could be attributed to (A) an outgrowth of existing terminals to occupy vacated synaptic sites or (B) sprouting of an uninjured or injured fiber.

4.13 Cholinergic deficits in the basal forebrain of aged rats and effects of decortication

Although it is generally accepted that cognitive deficits associated with aging are due to impaired cholinergic function, particularly of the basal forebrain, several studies have failed to show significant alterations in cholinergic markers in the aged rodent brain [for review see: (Decker, 1987)]. Such discrepancies have been attributed to differences in animal strain or sex, or in assay procedures for cholinergic neurochemical markers. For example, AChE-positive cells in the medial septum, VDB, striatum and NBM of aged behaviorally impaired female Sprague-Dawley rats have been reported to be show an age-related decrease in neuronal size (Fischer et al.1987). In another study, behaviorally impaired aged (22-24 months) female Sprague-Dawley rats were shown to have minor deficits in ChAT activity in the striatum and brainstem, but the activity of this enzyme was not found to be different in cortex, hippocampus and septum when compared with young rats (Hellweg et al.1990). In contrast, aged (22-24 months) male Sprague-Dawley rats showed significant decreases in striatal ChAT activity and ACh content, as well as HACU in the striatum, frontal cortex and hippocampus (Hadjiconstantinou et al.1992). In aged male Fischer 344 rats significant decreases in ChAT activity were noted in cortex and striatum but not in hippocampus (Michalek et al.1989), while other investigators using this same rat strain reported age-related losses of hippocampal ChAT activity (Sherman et al.1981). In addition, 24 month old male Fisher 344 rats have been shown to have significant decreases in ChAT activity in the medial septum/VDB and striatum but not in NBM when compared to their younger counterparts. In particular, in this rat strain, a loss of medial septum/VDB ChAT activity was observed only in rats older than 19 months, while a striatal deficit was noted in rats older than 7 months (Williams, 1991b).

In the male Wistar rats used for the studies of this thesis significant and reliable decreases in cholinergic presynaptic markers were noted in rats older than 20 months of age. When compared with their young counterparts such animals showed significant decreases in ChAT activity in the septum, hippocampus, NBM, cortex as well as striatum. Cortical, hippocampal and striatal HACU were also decreased as compared to young rats. Moreover, an apparent loss of cholinergic neurons was also evident in the NBM of these aged rats. Decortication of aged rats caused significant decreases in ChAT activity in the ipsilateral NBM but did not

affect the activity of this enzyme in other brain areas. Moreover, ChAT activity and HACU in the remaining ipsilateral cortex adjacent to the lesion site was not altered by the lesion. These effects of the devascularizing cortical lesion on cholinergic markers are therefore similar to what was observed for young adult rats. However, in contrast to younger animals, decorticated aged rats showed an apparent decrease in ChAT and $p75^{NGFR}$-IR neurons in the ipsilateral NBM. It is possible that this reflects a down regulation of ChAT and $p75^{NGFR}$ expression because some of these neurons reappeared following treatment with NGF and/or GM1 (section 3.4; also see 4.14). The inability of aged rats to maintain cholinergic phenotype in the NBM after decortication is in contrast to what occurs in young adult decorticated rats. This could be attributed to the fact that in aged rats, in contrast to young animals, lower levels of endogenous trophic agents are upregulated following brain damage (Needles et al.1985). These reduced amounts may be insufficient to maintain the expression of ChAT and $p75^{NGFR}$ after injury.

4.14 Necessity for chronic treatment with NGF and/or GM1 to attenuate decortication-induced cholinergic deficits in aged rats

When this thesis was initiated whether NGF or GM1 could attenuate cholinergic deficits associated with aging in rats was not known. However, during the course of this thesis several studies were published which showed that NGF treatment could increase the levels of basal forebrain cholinergic presynaptic markers (Williams, 1991b), as well as reverse basal forebrain and striatal cholinergic neuronal atrophy and improve behavior of normal aged rats (Fischer et al.1987; Fischer et al.1991). Increases in brain cholinergic markers, in normal aged rats, were also shown to be induced by GM1 treatment (Hadjiconstantinou et al.1992). The work of this thesis has shown that NGF and/or GM1 treatment can attenuate cholinergic deficits which occur in the aged rat NBM after brain injury. In contrast to young adult rats however, it was found that short-term (7 days) treatment with NGF and/or GM1, which were maximally effective in young adult rats, did not prevent decreases in NBM ChAT activity of aged animals at 30 days post-lesion. However, lesioned aged rats which received continuous infusions of these agents for thirty days showed control levels of NBM ChAT activity as well as a stimulation of ChAT activity and HACU in the remaining cortex. In

addition, NBM ChAT-IR cell density and morphology were restored. Therefore, NGF or GM1 treatment can also attenuate lesion-induced cholinergic deficits in the aged decorticated rat brain. Thus, in contrast to young adult rats, short-term treatment with NGF or GM1 is insufficient to render injured NBM cholinergic neurons of aged rats independent from exogenous sources of trophic factors. This could indicate that these short-term treatments do not stimulate aged injured cholinergic neurons to reestablish post-synaptic contacts. Whether these neurons loose dependence on exogenous sources of trophic agents after chronic treatment was not tested. Interestingly, a study has shown that the density of AChE positive fibers in the lateral septum of fimbria-fornix lesioned aged rats was increased after a 2 month treatment, but not a 15 day treatment, with NGF (15 μg/day, injected twice weekly) (Yunshao et al.1992). NGF has also been shown to attenuate deficits in septal ChAT activity which occur in the medial septum following fimbria-fornix transection in aged rats (Yunshao et al.1991), and to prevent the apparent loss of $p75^{NGFR}$-IR septal neurons in such lesioned aged animals (Montero and Hefti, 1989).

In aged cortically devascularized rats which received chronic treatment with both NGF and GM1 cholinergic markers were augmented in the ipsilateral NBM and cortex significantly above that induced by each agent alone. Thus, suggesting that gangliosides can potentiate NGF-induced effects in the aged rat brain as well. Long-term treatment with NGF, but not GM1, also augmented ChAT activity and HACU, above control levels, in areas unaffected by the lesion such as, the septum, the hippocampus, and the striatum. It was shown, while the work of this thesis was in progress, that sensitivity of brain areas, such as the medial septum/VDB and striatum, to NGF increases with age in male Fischer 344 rats (Williams, 1991b). This may also be the case for male Wistar rats, although it was not systematically investigated by this thesis.

The increased cholinergic markers in the cortex of decorticated rats which received NGF and/or GM1 suggest that these treatments augment the synthesis and transport of ChAT and HACU proteins in the basalo-cortical pathway or that sprouting of cholinergic terminals also occurs in the aged brain. It would be of particular interest to confirm this latter possibility at the ultrastructural level and to investigate whether synaptic remodelling, as well as appropriate connections, are induced by these agents in the aged cortex.

4.14 Potential use of trophic agents for the treatment of human brain injury or disease

The ultimate goal of studies examining neuroplasticity of the rat central nervous system is to develop treatments for human brain injury or neurodegenerative diseases. The findings that GM1, and in particular NGF, stimulate cholinergic markers as well as prevent neuronal atrophy, provided some hope that such agents could be used to treat human disorders where brain cholinergic function is impaired, such as Alzheimer's disease (AD). The observation that NGF receptors were present in human brain (Hefti et al.1986; Mufson et al.1989; Allen et al.1989; Kerwin et al.1990; Kerwin et al.1991; Mrzljak and Goldman-Rakic, 1993) reinforced that notion. The rodent models used to explore NGF or GM1 effects on cholinergic systems have now also been extended to primates. As occurs in rats, lesions of the fimbria-fornix in monkeys have been shown to cause an apparent loss of cholinergic neurons in the septum which can be prevented by NGF treatment (Tuszynski et al.1990; Tuszynski et al.1991; Koliatsos et al.1991). In addition, the cortical devascularization lesion model has been reproduced in primates, and NGF and/or GM1 treatment have been shown preserve nbM ChAT-IR cell size up to 6 months after a short-term (3 weeks) treatment (Pioro et al.1993; Liberini et al.1993). In contrast to NGF, GM1 has previously been used clinically. In fact Cronassial, which comprises a mixture of gangliosides (21% GM1, 40% GD1a, 16% GD1b, 10% GT1b) has been in use, in some countries (Italy, Spain, Brazil) for over 15 years, predominantly for the treatment of post-operative or diabetic polyneuropathy. Surprisingly, despite its extensive use in humans, few well controlled double-blind experiments with sufficient patients have been published which demonstrate the effectiveness of GM1 treatment for these purposes in humans [for review see: (Massaroti, 1986)]. However, many reports claim that intramuscular injections of Cronassial can significantly improve, as assessed both clinically and electrophysiologically, peripheral nerve function (Massaroti, 1986; Bradley et al.1988). Studies have also shown that gangliosides may be beneficial for the treatment of certain human CNS injuries. GM1 was shown to significantly improve recovery following brain injury induced by stroke (Bassi et al.1984; Argentino et al.1989). Moreover, a randomized placebo-controlled study has been published showing positive effects of GM1 following human spinal cord injury (Geisler et al.1991). However, although studies in humans are in progress, neither GM1 nor NGF treatment has yet been

reported to be beneficial for the treatment of AD. Initially, it was proposed that a deficit in a trophic factor(s) could contribute to the neuronal loss or shrinkage noted in the basal forebrain in AD (Appel, 1981). However, there is some indication that this may not be so, since normal levels of NGF mRNA have been noted in AD brains (Goedert et al.1986). Several investigators have cautioned against the use of NGF treatment for humans (Butcher and Woolf, 1989; Saffran, 1992). Practical reasons opposing its use are that extensive surgical procedures are required since NGF needs to be administered i.c.v., and that infusion devices could also be exacting to install, refill or could cause infection. Attempts to circumvent this problem have included the development of genetically modified cells to provide a continuous source of NGF (Rosenberg et al.1988), the successful microencapsulation of NGF (Maysinger et al.1992b) or the development of methods to facilitate its passage through the blood brain barrier (Friden et al.1993). The former two however, still necessitate surgery, while the beneficial effects of the latter have yet to be shown. The successful synthesis of NGF analogues which could be administered via less invasive routes and still be active have yet to be reported . However, based on studies in rodents, it appears that the effects of NGF, or GM1, may vary depending upon the extent of damage incurred. Since none of the lesion models used so far for the study trophic factor effects are representative of AD it is difficult to predict whether agents such as NGF or GM1 would ultimately be beneficial. Moreover, animal studies have shown that the success of such trophic agents in improving cholinergic function and reversing neuronal atrophy declines after extended post-lesion times. This would restrict the use of agents such as NGF, or GM1, for AD since diagnosis of this disease is usually made only after the brain has sustained significant damage. In addition, NGF treatment may provoke alterations which may be detrimental such as: weight loss (Williams, 1991a), hyperinnervation of cerebral blood vessels (Isaacson et al.1990) and possibly aberrant sprouting. The latter in particular has been reported to contribute to the pathogenesis of AD (Masliah et al.1991c) and potentially other neurological disorders (Sutula et al.1988). Furthermore, with respect to AD, NGF has been shown to upregulate ß-amyloid (Mobley et al.1988) which is thought to be a causal agent for this disorder. Nevertheless, so far, few reports showing deleterious effects of NGF and/or GM1 on cognitive function have surfaced. Rather, studies indicate that these agents improve rodent performance in memory based tasks following injury or aging [(Fischer et al.1987;

Elliott et al.1989; Fischer et al.1991; Dekker et al.1992), this thesis]. Moreover, cognitive deficits in AD have been attributed to a loss of synapses (Masliah et al.1991d; Masliah et al.1991b). Thus, the findings of this thesis which show that exogenous NGF, in particular when co-administered with GM1, causes significant synaptic remodelling in the injured brain are particularly relevant. Future studies should assess whether similar synaptic remodelling is induced by NGF and/or GM1 treatment in primate brain, and furthermore establish whether the behaviour of these animals is altered.

4.15 Summary

The work of this thesis has shown that the adult mammalian brain is capable of considerable neuroplasticity in response to trophic agents. It has been demonstrated that cholinergic markers in the decorticated adult rat brain can be similarly and distinctly regulated by NGF and GM1. Furthermore, the demonstration of an interaction between these agents suggests that GM1 may share some part of the NGF signal transduction pathway, *in vivo*, indicating that it could serve as a pharmacological tool not only to help elucidate the molecular mechanisms underlying NGF effects but also to potentially increase trophic factor efficacy in disorders where their actions are found to be restricted. Moreover, direct evidence has been provided, which demonstrates that terminal sprouting and synaptogenesis can be induced, in the adult mammalian brain, by these agents.

The discovery that several other neurotrophins (BDNF, NT-3, NT-4/NT-5) in addition to NGF and other growth factors (e.g.: b-FGF, a-FGF, CNTF) are also present in brain provide abundant tools with which plasticity processes in the adult or aged brain could be further studied. It remains a challenge to identify exactly how such agents can possibly individually or collectively regulate neuronal repair or reinnervation in the CNS. Such future studies could help further our understanding of normal function in the CNS or of how to treat neurodegenerative diseases.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The data presented are original observations and appear in the following manuscripts which have been published or are under consideration for publication:

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

Garofalo, L., Elliott, P.J. and Cuello A.C. Behavioral response of rats with cortical lesions to cholinomimetics. *Physiology and Behavior* **52**: 971-977, 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. Neurology, in Press*

Garofalo L., Ribeiro-da-Silva A. and Cuello A.C, Potentiation of nerve growth factor induced alterations in cholinergic fiber length and presynaptic terminal size by the monosialoganglioside GM1, *Neuroscience, in Press*

Garofalo L. and Cuello A.C. Characterization of nerve growth factor and/or monosialoganglioside GM1 effects on cholinergic markers in the adult lesioned rat brain. Submitted

Garofalo L. and Cuello A.C. Necessity of long-term treatment for recovery of cholinergic markers and neurons in the injured brain of aged rats. In preparation.

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

Elliott P. J., Garofalo L. and Cuello A.C., Limited neocortical devascularizing lesions causing deficits in memory retention and choline acetyltransferase activity--effects of the monosialoganglioside GM1. *Neuroscience* **31**: 63-76, 1989.

Stephens,P.H., Tagari, P., Garofalo, L., Maysinger, D., Piotte, M. and Cuello A.C., Neural plasticity of basal forebrain cholinergic neurons: Effects of gangliosides. *Neurosci. Lett.* **80**: 80-84, 1987

The work of this thesis has provided the following contributions to original knowledge:

1. By comparing various routes of administration (i.p. versus i.c.v.), evidence has been provided to support the notion that GM1 exerts its neuroprotective effects by acting directly in the CNS.

2. The effects of exogenous NGF were assessed for the first time in a lesion model causing retrograde degeneration of NBM cholinergic neurons in the rat. The dose-dependent effects of NGF were compared to those produced by GM1 treatment. It has been shown that short-term treatment (7days) with either of these agents can attenuate deficits in NBM ChAT activity, noted at 30 days post-lesion. Moreover, NGF or GM1 treatment stimulate cortical ChAT activity and HACU in a dose-dependent manner. The ED50 for NGF-induced attenuation of decreases in NBM ChAT activity was 0.1 μg/day, while that for GM1 was 375 μg/day. Higher doses were shown to be needed to stimulate cortical ChAT activity and HACU (ED50: NGF: 1 and 1.5 μg/day, respectively; GM1: 600 and 700 μg/day, respectively). Furthermore, the effects of NGF or GM1 treatment on NBM or cortical ChAT activity and HACU were shown to be time-dependent.

3. The size and distribution of ChAT and $p75^{NGFR}$-IR neurons were quantitatively compared throughout various subdivisions of the NBM in control and decorticated rats and were found to correlate. The work of this thesis is the first to show that short-term treatment (7 days) with NGF or GM1 can induce a long-term protection of NBM ChAT and $p75^{NGFR}$-IR neuronal morphology and their fiber length following injury. A lack of long-term dependence of injured NBM cholinergic neurons on exogenous trophic agents suggests that NGF or GM1 could induce these neurons to reestablish contact with post-synaptic targets.

4. It was demonstrated that the effects of NGF and/or GM1 were restricted to specific CNS regions in decorticated adult rats. ChAT activity in the septum, hippocampus or contralateral NBM or cortex, and HACU in the contralateral cortex or in hippocampus of decorticated animals were unaffected by NGF and/or GM1 treatment.

5. It has been shown that NGF and GM1 exert distinct effects on both the ipsilateral and contralateral striatum. In particular, it was demonstrated that a 7 day i.c.v. treatment with various GM1 doses caused no alterations in striatal ChAT activity or HACU, while NGF treatment stimulated these cholinergic markers in striatum in a dose-dependent manner.

6. The work of this thesis provided the first evidence that co-treatment with GM1 potentiates *in vivo* NGF-induced effects on NBM ChAT activity and cortical ChAT activity of decorticated rats. Moreover, the effects of NGF on cortical HACU were also shown to be potentiated by GM1. The monosialoganglioside GM1 was also shown to augment NGF efficacy, but not to alter NGF potency. It is suggested that GM1 may share some part of the NGF signal transduction cascade which mediates its neuroprotective effects *in vivo*.

7. The activities of membrane bound or soluble forms of ChAT in the remaining ipsilateral cortex or striatum were shown not to be differentially affected by decortication. Moreover, NGF or GM1 treatment were shown not to distinctly affect the activities of membrane bound or soluble ChAT.

8. This thesis provides the first evidence that decortication does not alter the kinetic parameters of HACU in the remaining cortex or striatum. Moreover, NGF and/or GM1 treatment were shown to increase cortical HACU Vmax and not to affect Km. This suggests that these agents could augment the number of HACU sites.

9. It was also demonstrated that in contrast to the basal forebrain, NGF effects on ChAT activity and HACU in striatum were not potentiated by GM1 co-treatment. It is suggested that this could be accounted for by the apparent difference in NGF receptors in striatum versus basal forebrain. As shown by others and also by the work of this thesis, the striatum, in contrast to the basal forebrain, does not express abundant $p75^{NGFR}$ immunoreactivity.

10. Specific binding of NGF to cortical or striatal membranes, isolated from decorticated rats at various post-lesion times, were shown not to differ from that of unoperated rats. Furthermore exogenous GM1 treatment did not alter NGF binding to cortex or striatum of

lesioned animals.

11. Decortication was shown to cause a significant decrease in the ChAT-IR fiber network in the remaining cortex of adult rats. This decrease was attenuated by GM1 or NGF treatment. Moreover, exogenous NGF, but not GM1, was shown to significantly augment, above control levels, the number and size of cortical ChAT-IR axonal varicosities in these animals. In addition, it was demonstrated that GM1 treatment could prevent significant decreases in the number of ChAT-IR varicosities with synaptic contacts in cortical layer V of lesioned rats. Furthermore, NGF treatment was shown to increase synapse number significantly above control levels. This work provided the first direct evidence that GM1 can facilitate the maintenance of synaptic contacts following injury, and that NGF treatment causes terminal sprouting as well as synaptogenesis in the adult injured mammalian brain.

12. The NGF-induced synaptic remodelling noted in cortex was shown to be potentiated by GM1, thus, demonstrating that the growth promoting effects of NGF can also be modulated by GM1 *in vivo*.

13. Rats with unilateral devascularizing cortical lesions were shown to exhibit retention and reacquisition deficits in passive avoidance and Morris water maze tasks which were distinctly affected by GM1 and NGF treatment. GM1 treatment was shown to improve reacquisition of both tasks, while NGF and NGF/GM1 treatment instead abolished retention deficits. This demonstrated that NGF or GM1 treatment can induce distinct functional effects.

14. It has been shown that NGF and/or GM1 treatment can also effectively prevent decortication-induced decreases in NBM ChAT activity in aged male Wistar rats. In addition, these treatments also stimulate cortical ChAT activity and HACU in these animals. However, in contrast to young adult rats, aged lesioned rats required chronic treatment. This could reflect the decreased plasticity associated with aging, but indicates that NGF and/or GM1 can promote some recovery of function in the injured aged brain.

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APPENDIX A

A IMAGE ANALYSIS PROGRAMS

A.1 Cell and fiber measurements in the NBM

```
Cambridge Instruments Quantimet 920  Quips/MX: V05.01MX USER: GUILLERMO
Routine: BCEL2    DATE:      RUN: 0        SPECIMEN: 0

          Pause Message
            SET UP FIRST FIELD OF FIRST NBM SECTION
            WITH X10 1K OBJECTIVE AND X1.25 TURRET
            YOU CAN MEASURE UP TO 16 SECTIONS
            WITH 2 FIELDS PER SECTION

            WHEN OPTICAL CONDITIONS ARE OK
            SELECT CONTINUE AND ENTER SECTION
            THICKNESS IN MICRONS!!!!!!!!!!!!
Scanner   (No. 1 Plumbicon  AUTO-BRIGHTNESS  SENS=  1.02  Pause)
Thickness  := 50.
Input THICKNESS
For FIELD
FLAG1   :=        0.
FLAG2   :=        0.
FLAG3   :=        0.
For LOOPCOUNT  = 1  to   2
Image ACCEPTED  (Binary,Res=1,Horiz=1,Vert=1)
Binary Ouput goes to ACCEPTED
Clear Binary Output
Image FIBRES   (Binary,Res=1,Horiz=1,Vert=1)
Binary Output goes to FIBRES
Clear Binary Output
Image CELLS   (Binary,Res=1,Horiz=1,Vert=1)
BInary Output goes to CELLS
Clear Binary Output
CALL STANDARD
Calibrate User Specified  (Cal Value  =  1, picturepoint per pixel)
Image Frame is Rectangle  (X: 50,  Y: 50,    W: 800,   H: 600,  )
If FIELDNUM  >  1. then
Pause Message
          SET UP NEXT FIELD
Scanner     (No. 1 Plumbicon   AUTOBRIGHTNESS SENS=  1.02 Pause )
Else
Endif
Pause Message
          SET DETECTOR FOR FIBRES
Detect 2D  (Darker than 56  and Lighter than  20 PAUSE )
Amend     (Close by 1 )
Amend     (SKELETON - Sub mode = Peel Ends )
Amend     (SKELETON - Sub mode = None )
Image Frame is Standard Image Frame
Live Frame is Rectangle ( X:  45, Y:  45,   W:  810,   H:  625,  )
Measure Feature       AREA     X.FCP     Y.FCP
          with limits   10.  <= AREA     <   100000.
          into array FEATURE  ( of 600 features and   3 parameters )
If FLAG1  <  1. then
TOTFIB   := Field sum of FEATURE  AREA * 0.00065500
AVFIB                :=  TOTFIB/ FEATURE VALID.OBJ
TOTELEMEN          := FEATURE VALID.OBJ
Else
```

```
STOTFIB := Field sum of FEATURE   AREA * 0.00065500
SAVFIB  := STOTFIB / FEATURE VALID.OBJ
STOTELEM        := FEATURE VALID.OBJ
Endif
Accept FEATURE AREA from 10. to      100000.
Binary Output goes to FIBRES
Transfer Accepted Features to Binary Output
Binary Output goes to DETECTED
Clear Binary Output
Calibrate Microscope            (Cal Value = 0.6800 microns    per pixel)
                                (Optics  = Transmitted)
                                (Objective = x 10.0, Proj. Lens  = x 12.5)
Pause Message
        SET DETECTOR FOR CELL BODIES
Detect     (Darker than 84 and Lighter than  20 PAUSE)
Amend    (Close by  5)
Measure Feature      AREA     X.FCP     Y.FCP
        with limits          49.8 <=AREA      < 214512
        into array FEATURE1 ( of 50 features   and 3 parameters)
Accept FEATURE1  AREA from           50. to      10000.
Binary Output goes to CELLS
Transfer Accepted Features into Binary Output
Binary Input A is CELLS
Binary Input B is CELLS
Measure feature      AREA     X.FCP     Y.FCP
        into array FEATURE1 ( of 200 features and   3 parameters)
Pause Message
        EDIT IMAGE IF NEEDED
Edit (pause)
Binary Input A is FIBRES
Binary Input B is CELLS
Selected Display                (Binary A) (Binary B) (grey) (Frame)
Pause Message
        CELLS AND FIBRES
        PHOTOGRAPH OR 'CONTINUE'
Colour Setup (Pause)-Load Binary B of LUT GREY
        with colour (R  0, G  0,B  190)
Measure feature                 AREA     X.FCP     Y.FCP              ROUNDNESS
        into array FEATURES2 ( of 50 features and   10 parameters)
If  FEATURE2 VALID.OBJ > 0. then
CL.FRAREA       :=      0.20600
If FLAG1 < 1. then
AREASUM         := Field sum of FEATURE2  AREA
MEANAREA        := AREASUM / FEATURE2 OBJ.COUNT
ROUNDSUM        := ROUNDSUM /FEATURE2 VALID.OBJ
LENEQCIRC       := 2. * ( (MEANAREA / PI ) * EXP ( - 2. ) )
CELDENS         := FEATURE2 VALID.OBJ / CL.FRAREA
CORDENS         := ( ( FEATURE2 VALID.OBJ * THICKNESS ) / (LENEQCIRC + THICKNESS )          ) )    /
CL.FRAREA
Else
SAREASUM        := Field sum of FEATURE2  AREA
SMEANAREA       := SAREASUM / FEATURE2 VALID.OBJ
SROUNDSUM       := Field sum of FEATURE2  ROUNDNESS
SMEANROUN       := SROUNDSUM / FEATURE 2 VALID.OBJ
SLENEQCIR       := 2. * ( (SMEANAREA / PI ) * EXP ( - 2. ) )
SCELDENS        := FEATURE2 VALID.OBJ / CL.FRAREA
SCORDENS        := ( ( FEATURE2 VALID.OBJ * THICKNESS ) / (SLENEQCIR + THICKNESS )          ) )    /
CL.FRAREA
Endif
FAREASUM        := (MEANAREA  + SMEANAREA ) / 2.
FMEANROUN       := (MEANROUN + SMEANROUN ) / 2.
FCELDENSM       := ( CELDENS + SCELDENS ) / 2.
```

```
FCORDENS        := ( CORDENS + SCORDENS ) / 2 .
FELEMENDN       := ( ( TOTELEMEN + STOTELEMN ) / 2. ) / CL.FRAREA
FTOTFIBDN       := ( ( TOTFIB + STOTFIB ) / 2. ) / CL.FRAREA
FAVFIBLN        := ( AVFIB + SAVFIB ) / 2.
RTOFDNCDN       := FTOTFIBDN / FCELDENS
RTOFDNCOR       := FTOTFIBDN / FCORDENS

If FLAG1 < 1. then
Pause Message
0
        SET FLAG 1 AND
        'CONTINUE' TO
        MEASURE SECOND FIELD

        OR SET FLAG 20
        AND 'CONTINUE' TO QUIT
Define FLAG1 on Pause SFK 1 with text
        <SET FLAG FOR SEC> <OND >
Define FLAG2 on Pause SFK 2 with text
        <SET TO FINISH>
Pause
Else
LOOPCOUNT       := 2.
Else
Emdif
Else
LOOPCOUNT       := LOOPCOUNT - 1.
Pause Message
0
        NO CELLS WERE MEASURED
        FOR THIS FIELD
        SELECT 'CONTINUE' AND TRY AGAIN
Disable FLAG1
Disable FLAG2
Pause
Endif
Next
Distribute FMANREA (Units SQUM    ) vs FIELDNUM
        into AREA from   1.00 to    16.00, differential
Distribute FMEANROUN (Units UNITS    ) vs FIELDNUM
        into SHAPE from   1.00 to    16.00, differential
Distribute FCELDENS (Units CELLS/SQMM) vs FIELDNUM
        into CELDENS from   1.00 to    16.00, differential
Distribute FCORDENS (Units CELLS/SQMM    ) vs FIELDNUM
        into CORDENS from   1.00 to    16.00, differential
Distribute FTOTFIBDN (Units MM/SQMM    ) vs FIELDNUM
        into FIBDENS from   1.00 to    16.00, differential
Distribute FELEMENDN (Units NO/SQMM    ) vs FIELDNUM
        into ELDENS from   1.00 to    16.00, differential
Distribute FAVFIBLN (Units MM     ) vs FIELDNUM
        into AVFIB from   1.00 to    16.00, differential
Distribute RTOFDNCDN (Units MM/CELL    ) vs FIELDNUM
        into RATIO from   1.00 to    16.00, differential
Distribute RTOFDNCOR (Units MM/CORCEL    ) vs FIELDNUM
        into CORATIO from   1.00 to    16.00, differential
Disable FLAG1
Disable FLAG2
If  FIELDNUM  > 15. then
Pause Message
        YOU HAVE QUANTIFIED
        16 SECTIONS NOW
        PLEASE MOVE TO NEXT ANIMAL
```

```
        BY SELECTING 'FINISH'
Else
Pause Message
        SELECT 'CONTINUE' TO
        MEASURE NEXT SECTION
X
        ELSE 'FINISH TO END
Endif

Print       " TOTAL ELEM / AREA "
Print       TOTELEMEN / CL.FRAREA
Print       STOTELEMN / CL.FRAREA
Print       " TOTAL  FIB / AREA"
Print       TOTFIB / CL.FRAREA
Print       STOTFIB / CL.FRAREA
Print       "  AVG FIB"
Print       AVFIB
Print       SAVFIB
Print       "  AREA    MEAN AREA"
Print       AREA SUM  , MEANAREA
Print       SAREASUM,  SMEANAREA
Print       " ROUNDNESS  MEAN ROUNDNESS"
Print       ROUNDSUM  ,  MEANROUND
Print       SROUNDSUM,  SMEASNROUN
Print       "  CEL  DENS   COR  DENS"
Print       CELDENS,  CORDENS
Print       SCELDENS ,  SCORDENS
Print       "—" , FIELDNUM

Pause
Next FIELD
FLAG1    := 0.
FLAG2    := 0.
Pause Message
        SET FLAG1 FOR HISTO DISPLAY
        SET FLAG2 FOR HISTO PRINT
Define FLAG1 on Pause SFK 1 with text
        <SET FOR  HISTO   ><DISPLAY >
Define FLAG2 on Pause SFK 2 with text
        <SET FOR HISTO  ><PRINT>
Pause
If FLAG2 > 0. then
Print Distribution ( AREA, differential, bar chart, scale=    0.000)
Print Distribution ( SHAPE, differential, bar chart, scale=    0.000)
Print Distribution ( CELDENS, differential, bar chart, scale=    0.000)
Print Distribution ( CORDENS, differential, bar chart, scale=    0.000)
Print Distribution ( FIBDENS, differential, bar chart, scale=    0.000)
Print Distribution ( ELDENS, differential, bar chart, scale=    0.000)
Print Distribution ( AVFIB, differential, bar chart, scale=    0.000)
Print Distribution ( RATIO, differential, bar chart, scale=    0.000)
Print Distribution ( CORATIO, differential, bar chart, scale=    0.000)
Else
Endif
If  FLAG1 > 0. then
Display Histogram AREA   (LIN)  differential PAUSE
Display Histogram SHAPE   (LIN)  differential PAUSE
Display Histogram CELDENS   (LIN)  differential PAUSE
Display Histogram CORDENS   (LIN)  differential PAUSE
Display Histogram FIBDENS   (LIN)  differential PAUSE
Display Histogram ELDENS   (LIN)  differential PAUSE
Display Histogram AVFIB   (LIN)  differential PAUSE
Display Histogram RATIO   (LIN)  differential PAUSE
```

```
Display Histogram CORATIO  (LIN)  differential PAUSE
Else
Endif
END OF PROGRAM
```

A.2 Cortical fiber measurements

```
Cambridge Instruments Quantimet 920  Quips/MX: V05.01MX USER: GUILLERMO
Routine: CORFB DATE:        RUN: 0  SPECIMEN: 0

FLAG3    := 3.
Scanner   (No. 1 Plumbicon AUTO-BRIGHTNESS   SENS= 1.00 PAUSE
Enter specimen identity
RRLLS    := 0.
Input RRLLS
For FIELD
SID      := 0.
Input SID
Pause Message
FOR SUBSEQUENT FIELDS
CLICK ONCE TO REDUCE0
THE STAGE Y VALUE BY 120 MICRONS
NOW 'CONTINUE'
Pause
CALL STANDARD
For LOOPCOUNT =  1 TO    2
TOTFIB
Binary Input A is DETECTED
Binary Input B is DETECTED
Binary Output goes to  DETECTED
Clear Binary Output
Pause Message
SELECT FOCAL PLANE USING FOCUS CONTROL
Pause
Pause Message
SET DETECTOR FOR FIBRES
Detect 2D          (Darker than  56 Lighter than  20 PAUSE)
Amend    (DILATE by  2- Vertically)
Amend    (ERODE by  2- Horizontally)
Amend    (DILATE by  2- Vertically
Amend    (SKELETON - Sub mode = None)
Amend    (SKELETON - Sub mode = Peel Ends)
Calibrate User Specified (Cal Value = 1, picturepoint per pixel)
Image ACCEPTED  (Binary, Res=1,Horiz=1,Vert=1,)
Measure FEATURE  AREA    X.FCP    Y.FCP
          with limits          10        <= AREA          < 2000
          into array FEATURE ( of 500 features and  4 parameters)
Accept FEATURE AREA from 10. to      10000.
Binary Output goes to ACCEPTED
Clear Binary Output
Transfer Accepted features to Binary Output
Binary Output A is ACCEPTED
Binary Output B is ACCEPTED
Binary Output goes to FIBRES
Binary Input A is FIBRES
Image Transfer from A <OR> B to Binary Output
```

```
Binary Input B is FIBRES
Selected Display      (Binary A) (grey) (Frame)
Pause Message
SUMMED FIBRES FROM FOCAL PLANES DISPLAYED
Colour Transfer (Pause) - LUT GREY, Full resolution,
        Text On, Window (150,104)
Pause Message
SELECT 'CONTINUE' FOR ANOTHER FOCAL PLANE
ELSE 'FINISH' TO MOVE TO NEXT FIELD
Pause
Next
Amend    (DILATE by  2- Vertically)
Amend    (SKELETON - Sub mode = None)
Amend    (SKELETON - Sub mode = Peel Ends)
Measure FEATURE  AREA     X.FCP     Y.FCP
        with limits          10        <= AREA          < 2000
        into array FEATURE ( of 500 features and   4 parameters)
FEATURE1  CALC  ;= AREA * 0.00016300
TOTFIB   ; Field sum of FEATURE1  AREA
FTCNT    ;= FEATURE1 OBJ.COUNT
FIBDENS          ;= TOTFIB/0.016100
SMPLMN           ;= (TOTFIB/FEATURE1 VALID.OBJ) * 0.00016300
SMPLVR  ;= (YOTFIB^2. - FEATURE1 VALID.OBJ * SMPLMN^ 2.)/FEATURE1
        VALID.OBJ
LGSD     ;=  0.50000 * LN (SMPLVR)
SMPLSD   ;= EXP (LGSD)
Distribution of COUNT v CALC (Units MM      )
        from FEATURE1 IN HISTO1 FROM 0.00100 TO 0.0250
        in 64 bins (LIN)
Open output (HSTXXX) with XXX defined by SID
File #0 RRLLS, SID, STAGEY
File #2 FTCNT
File Distribution (HISTO1)
Close Output
Display Histogram HISTO1 (LIN) differential
Print "FT1)C", FEATURE1 OBJ.COUNT, "   ", " FT1VO", FEATURE! VALID>OBJ
Print "TOTFIB", TOTFIB
Print "RRLLS", RRLLS, "    ", "SID", SID, "   ", STAGEY", STAGEY
Print "SMPLMN", SMPLMN, "    ", "SMPLVR", SMPLVR
Print "   "
Pause Message
0
        TOTFIB IS TOTAL FIBER LENGTH
        FIBDENS IS LENGTH OF FIBER/MM2
        STAGE Y IS DISTANCE FROM DURA
        IN MICRONS
Pause
Next FIELD
Print Distribution (HISTO1, differential, bar chart, scale =     0.00)
END OF PROGRAM
```

A.3 Cortical Varicosity measurements

```
Cambridge Instruments Quantimet 920  Quips/MX: VO5.01MX USER: GUILLERMO
Routine: DNUMXX DATE:     RUN: 1  SPECIMEN: 0

FLAG3   := 3.
```

```
Scanner   (No. 1 Plumbicon AUTO-BRIGHTNESS   SENS= 1.00 PAUSE
Enter specimen identity
RRLLS    := 0.
Input RRLLS
For FIELD
SID       := 0.
Input SID
Calibrate User Specified (Cal Value = 1, picturepoint per pixel)
CALL STANDARD
For LOOPCOUNT  = 1 to     2
Binary Input A is DETECTED
Binary Input B is DETECTED
Binary Output goes to DETECTED
Clear Binary Output
Pause Message
SELECT FOCAL PLANE
USING FOCUS CONTROL
Pause
Pause Message
SET DETECTOR FOR VARICOSITIES
Pause
Detect     (Darker than 170 and Lighter than 20 Pause)
Amend     (Close by            1)
Edit (pause)
Image ACCEPTED  (Binary, Res=1,Horiz=1,Vert=1,)
Measure FEATURE  AREA     X.FCP     Y.FCP
          with limits           20          <= AREA          < 175.
          into array FEATURE ( of 500 features and  3 parameters)
If LOOPCOUNT < 2. then
SUMVAR           ;= Field sum of FEATURE     AREA
NUMVAR           := FEATURE VALID.OBJ
MEANVAR          := SUMVAR/NUMVAR
Else

SSUMVAR          ;= Field Sum of FEATURE     AREA
SNUMVAR          ; FEATURE VALID.OBJ
SMEANVAR         ; SSUMVAR/SNUMVAR
End if
TOTAVAREA        ;= (MEANVAR + SMEANVAR)/2
TONUM  ;= NUMVAR + SNUMVAR
Accept FEATURE AREA from
Binary Output goes ACCEPTED
Clear Binary Output
Transfer Accepted Features to Binary Output
Binary Input A is ACCEPTED
Binary Input B is ACCEPTED
Selected Display      (Binary A) (Binary B) (Grey) (Frame)
Pause
Clear Binary Output
Next
Print      "*******************************"
Print      "FIELD NUMBER =", FIELDNUM
Print      "RRLLS   =", RRLLS
Print      "SID                =", SID
Print      "MEAN AREA    =", (MEANVAR +SMEANVAR)/2
Print      "FEATURE NUMBER =", TONUM
Print      "*******************************"
Pause Message
CLICK Y STAGE TO NEXT FIELD
SELECT 'CONTINUE'
TO MOVE TO NEXT FIELD
ELSE 'FINISH'
```

```
Pause
Next FIELD
END OF PROGRAM
```

A.4 Measurements of cortical boutons

```
Cambridge Instruments Quantimet 920 Quips/MX; V05.01MX USER:
ROUTINE: CFEM DATE:                RUN: 1 SPECIMEN:

CALL STANDARD
FLAG3   := 3.
Enter specimen identity
Pause Message
        SET UP CALIBRATION IMAGE
Pause
CCTV Transfer
Calibrate User specified (Cal Value = 0.0067766 microns per pixel)
                          (          PAUSE   )
Colour Transfer- LUT GREY, FULL resolution
        Text On, Window (150,102)
For FIELD
Pause Message
0
        SET UP FIELD
PAUSE
CCTV Transfer
Pause Message
        SET DETECTOR LEVEL TO <
                FILL TERMINAL
Detect          (Darker than 61 PAUSE)
PAUSE Message
        ERODE BY ONE TO REMOVE NOISE
        THEN DILATE & ERODE TO FILL HOLES
Amend   (ERODE by       0 PAUSE)
PAUSE Message
        USE ACCEPT & COVER TO FINISH IMAGE
Edit (pause)
Measure feature     AREA            PERIMETER       X.FCP   Y.FCP
                                LENGTH BREADTH          ROUNDNESS
        with limits     0.00961 <= AREA             <  18.4
        into array FEATURE ( of 200 features and        7 parameters)
Print FEATURE, AREA, PERIMETER, LENGTH, BREADTH, ROUNDNESS
Distribution of COUNT v AREA
        from feature in GLOM from   0. to 5.000
        in  5 bins (LIN)
Distribution of COUNT v PERIMETER (Units MICRONS)
        from FEATURE in GLOMPERIN from   0. to 30.00
        in 5 bins (LIN)
Distribution of COUNT v LENGTH (Units MICRONS)
        from FEATURE in GLOMLNGTH from   0. to 5.000
        in 5 bins (LIN)
Distribution of COUNT v BREADTH (Units MICRONS)
        from FEATURE in GLOMBRED from    0 to 5.000
        in 5 bins (LIN)
Distribution of COUNT v ROUNDNESS (Units MICRONS)
        from FEATURE in GLOMRND from    0 to 10.00
        in 5 bins (LIN)
Pause Message
```

```
PREPARE NEXT FIELD
OR FINISH TO QUIT
Pause
Next FIELD
Print Distribution (GLOM, differential, bar chart, scale=        0.00)
Print Distribution (GLOMPERIM, differential, bar chart, scale=        0.00)
Print Distribution (GLOMLNGTH, differential, bar chart, scale=        0.00)
Print Distribution (GLOMBRED, differential, bar chart, scale=  0.00)
Print Distribution (GLOMRND, differential, bar chart, scale=  0.00)
END OF PROGRAM
```

A.5 Bouton three dimensional reconstruction

```
Cambridge Instruments Quantimet 920 Quips/MX; V05.01MX USER: GUILLERMO
ROUTINE: 3D3      DATE:                RUN: 0   SPECIMEN: 0

Live Frame is Rectangle (X: 353, Y : 87, W: 235, H: 612, )
Scanner   (No. 2 Plumbicon AUTO-BRIGHTNESS  SENS= 1.67
Image DETECTED (Binary,Res=1,Horiz=1,Vert=1,)
Image WORK (Binary,Res=1,Horiz=1,Vert=1,)
Image INK (Binary,Res=1,Horiz=1,Vert=1,)
Image EDITED (Binary,Res=1,Horiz=1,Vert=1,)
Image AMENDED (Binary,Res=1,Horiz=1,Vert=1,)
Image 3D (Binary,Res=1,Horiz=1,Vert=1,)
Image SY (Binary,Res=1,Horiz=1,Vert=1,)
Image SYTEMP (Binary,Res=1,Horiz=1,Vert=1,)
Image GRID (Binary,Res=1,Horiz=1,Vert=1,)
Image FILL (Binary,Res=1,Horiz=1,Vert=1,)
Binary Input A is DETECTED
Binary Input B is DETECTED
Binary Output goes to 3D
Clear Binary Output
Binary Output goes to GRID
Clear Binary Output
Binary Output goes to DETECTED
Clear Binary Output
Binary Output goes to SY
Clear Binary Output
Binary Output goes to SYTEMP
Clear Binary Output
Live Frame is Standard Live Frame
SHIFTX  :=      16.
SHIFTY  :=      0.
SIZEX           :=      200.
SIZEY           :=      100.
STARTX  :=      0.
STARTY  :=      0.
SECTIONS        :=      2.
DISTANCE        :=      0.070000
TOTVOL  :=      0.
TEMPVOL         :=      0.
TOTSURF         :=      0.
TEMPSURF        :=      0.
TEMPSY  :=      0.
SYTOTAREA       :=      0.
```

```
CALL GRID
Pause Message
0
        SET UP LARGEST SECTION
        THEN 'CONTINUE'
Colour Transfer- LUT GREY,Full resolution,
        Text On,Window (130,105)
Scanner          (No. 2 Plumbicon AUTO-BRIGHTNESS SENS= 1.67 PAUSE )
Selected Display          (Binary A) (Grey) (Frame)
Pause Message
0
        ESTIMATE SIZE IN X AND Y *
        DIMENSIONS (GRID 100X100)2

        SELECT 'CONTINUE' TO ENTER
        SIZE AND NUMBER OF SECTIONS
Pause
Input SIZEX
Input SIZEY
Input SECTIONS
Input DISTANCE
Image Frame is Standard Image Frame
SHIFTX  :=      8.
STARTY  :=      680. - SIZEY
SHIFTY  := STARTY
Binary Output goes to DETECTED
Clear Binary Output
Binary Input A is DETECTED
Binary Input B is DETECTED
Colour Transfer - LUT GREY < Full resolution,
        Full frame
Define FLAG1 on Pause SFK 1 with text
        <SET FOR DETECTOR>
Define FLAG2 on Pause SFK 2 with text
        <SET FOR OUTLINE > <ONLY>
Disable FLAG3
Pause Message

        SET FLAG1 TO USE DETECTOR
        THEN 'CONTINUE'

        OR

        SET FLAG 2 FOR OUTLINE ONLY
        AND 'CONTINUE'
Pause
Calibrate User Specified (Cal Value = 0.005000 microns   per pixel)
For FIELD
If  (FIELDNUM - 1.) < SECTIONS then
IF FIEDLNUM > 1. then
SHIFTX  := SHIFTX + ( (872. - SIZEX )/ (SECTIONS - 1.))
SHIFTY  := SHIFTY - ( STARTY / (SECTIONS - 1.))
Else
SHIFTX  := 8.
Endif
Image Frame is Rectangle (X:SHIFTX   , Y:SHIFTY   , W:SIZEX   , H:SIZEY   , )
Binary Input A is DETECTED
Binary Input B is DETECTED
Clear Binary Output
If FLAG > 0. then
Disable FLAG1
Disable FLAG2
```

```
Pause Message
        CENTER SECTION IN FRAME
        THEN CONTINUE
Pause
Pause Message
        SET DETECTOR FOR SECTION OUTLINE
Detect 2D  (Darker than  44 Pause )
Pause Message

        EDIT IF NECESSARY
        THEN CONTINUE
Amend    (OPEN by    2 PAUSE )
Edit (pause)
Else
Scanner   (No. 2 Plumbicon AUTO-BRIGHTNESS   SENS= 1.67 )
Selected Display      (Grey)(Frame)
Pause Message

        CENTRE SECTION IN FRAME
        THEN OUTLINE WITH 'DRAW'
Edit (pause)
Amend    (DILATE by   2  PAUSE )
Endif
If FLAG2 > 0. then
Binary Input B is DETECTED
Binary Output goes to DETECTED
Image Transfer from Binary B <FILL HOLES> to Binary Output
Else
Endif
Measure feature                AREA              PERIMETER       X.FCP    Y.FCP
                               LENGTH
        into array FEATURE ( of 200 features and   5 parameters )
SECTAREA        := Field sum of FEATURE    AREA
SECTPERIN       := Field sum of FEATURE    PERIMETER
TOTVOL := ( SECTAREA * DISTANCE ) + TEMPVOL
TEMPVOL         :=  TOTVOL
TOTSURF         := (SECTPERIM * DISTANCE ) + TEMPSURF
TEMPSURF        := TOTSURF
Binary Output goes to AMENDED
Clear Binary Output
Amend    (ERODE by   3)
Binary Input A is DETECTED
Binary Input B is AMENDED
Binary Output goes to EDITED
Image Transfer from A <AND NOT> B to Binary Output

Binary Input A is EDITED
Binary Input B is EDITED
Binary Output goes to DETECTED
Pause Message
        'EDIT' IMAGE TO ACCEPT SYNAPSES
Pause
Edit (pause)
Binary Input A is DETECTED
Binary Input B is DETECTED
Amend           (SKELETON-Sub mode = None)
Measure feature                AREA    X.FCP    Y.FCP
        into array FEATURE1 ( of   10 features and  5 parameters )
Amend           (DILATE by 1 )
SYSAREA         := Field sum of FEATURE1   AREA
SYTOTAREA       :=  (SYAREA * DISTANCE ) + TEMPSY
TEMPSY :=  SYTOTAREA
```

```
Binary Input A is DETCETED
Binary Input B is SYTEMP
Binary Output goes to SYTEMP
Image Transfer from A <OR> B to Binary Output
Image Frame is Standard Image Frame
Binary Input A is 3D
Binary Input B is 3D
Binary Output goes to FILL
Clear Binary Output
Image Transfer from Binary B <FILL HOLES> to Binary Output
Binary Input A is EDITED
Binary Input B id FILL
Binary Output goes to EDITED
Image Transfer from A <AND NOT> B to Binary Output

TEMPORARY PATCH
If FIELDNUM > 1. then
Binary Input A is SYTEMP
Binary Input B is FILL
Binary Output goes to SYTEMP
Image Transfer from A <AND NOT> B to Binary Output
Else
Endif

Binary Input A is SYTEMP
Binary Input B is SY
Binary Output goes to SY
Image Transfer from A <OR> B to Binary Output

Binary Input A is EDITED
Binary Input B is 3D
Binary Output goes to 3D
Image Transfer from A <OR> B to Binary Output
Else
Binary Input A is 3D
Binary Output B is SY
Selected Display    (Binary A)(Binary B) (Frame)
Disable FLAG1
Disable FLAG2
Colour Setup (Pause) - Load Binary A of LUT GREY
        with colour (R 255, G 255, B 255)
Pause Message
SELECT 'FINISH'
Pause
Endif
Next FIELD
Print       "********************"
Print       "TOTAL FEATURE VOLUME =" , TOTVOL
Print       "TOTAL FEATURE SURFACE AREA =" , TOTSURF
Print       "TOTAL SYNAPSES AREA =" , SYTOTAREA
END OF PROGRAM


to DETECTED
Pause Message
        'EDIT' IMAGE TO ACCEPT SYNAPSES
Pause
Edit (pause)
Binary Input A is DETECTED
Binary Input B is DETECTED
Amend               (SKELETON-Sub mode = None)
Measure feature                 AREA    X.FCP    Y.FCP
```

```
        into array FEATURE1 ( of  10 features and  5 parameters )
Amend           (DILATE by 1 )
SYSAREA         := Field sum of FEATURE1  AREA
SYTOTAREA       := (SYAREA * DISTANCE ) + TEMPSY
TEMPSY := SYTOTAREA
Binary Input A is DETCETED
Binary Input B is SYTEMP
Binary Output goes to SYTEMP
Image Transfer from A <OR> B to Binary Output
Image Frame is Standard Image Frame
Binary Input A is 3D
Binary Input B is 3D
Binary Output goes to FILL
Clear Binary Output
Image Transfer from Binary B <FILL HOLES> to Binary Output
Binary Input A is EDITED
Binary Input B id FILL
Binary Output goes to EDITED
Image Transfer from A <AND NOT> B to Binary Output

TEMPORARY PATCH
If FIELDNUM > 1. then
Binary Input A is SYTEMP
Binary Input B is FILL
Binary Output goes to SYTEMP
Image Transfer from A <AND NOT> B to Binary Output
Else
Endif

Binary Input A is SYTEMP
Binary Input B is SY
Binary Output goes to SY
Image Transfer from A <OR> B to Binary Output

Binary Input A is EDITED
Binary Input B is 3D
Binary Output goes to 3D
Image Transfer from A <OR> B to Binary Output
Else
Binary Input A is 3D
Binary Output B is SY
Selected Display    (Binary A)(Binary B) (Frame)
Disable FLAG1
Disable FLAG2
Colour Setup (Pause) - Load Binary A of LUT GREY
        with colour (R 255, G 255, B 255)
Pause Message
SELECT 'FINISH'
Pause
Endif
Next FIELD
Print       "********************"
Print       "TOTAL FEATURE VOLUME =" , TOTVOL
Print       "TOTAL FEATURE SURFACE AREA =" , TOTSURF
Print       "TOTAL SYNAPSES AREA =" , SYTOTAREA
END OF PROGRAM
```