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NEUROTROPHINS AND NEUROTROPHIN SIGNAL TRANSDUCTION IN CHOLINERGIC NEURONS OF THE MOUSE FOREBRAIN

by Nicole L. Ward

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy and Neurobiology

at

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DALHOUSIE UNIVERSITY

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ABSTRACT

Nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4) are neurotrophins and bind to the p75 NGF receptor ($p75^{NGFR}$). Previously our laboratory suggested that $p75^{NGFR}$ induced apoptosis in a subpopulation of cholinergic forebrain neurons during postnatal development. Using transgenic mice and a detailed and quantitative approach, we attempted to identify *in vivo* i. the putative $p75^{NGFR}$ "neurocidal", or death-inducing ligand that induced apoptotic cell death of these neurons; ii. the intracellular signaling pathways activated by $p75^{NGFR}$ during developmental apoptotic cell death and following injury in cholinergic forebrain neurons; and iii. the role of $p75^{NGFR}$ in the potential atrophy or maintenance of cholinergic medial septum neurons during aging.

We determined that BDNF and NT-4 were not "neurocidal" in the developing cholinergic medial septum by analyzing transgenic mice lacking BDNF or NT-4. Between P6 and P15 in control mice, the number of choline acetyltransferase (ChAT)-immunopositive neurons in the medial septum and neostriatum increased, not decreased as previously suggested. BDNF-deficient (-/-) mice had fewer and smaller ChAT-positive neurons, and ~3 times more TUNEL-labeled cells in the medial septum compared to control littermates, and had reduced cholinergic hippocampal innervation. NT-4 -/- mice did not differ from control mice, suggesting that BDNF, but not NT-4 was necessary during postnatal development.

$p75^{NGFR}$ can signal *in vitro* through activation of the c-Jun N-terminal kinase (JNK) pathway and nuclear translocation of NF_KB. Activation of JNK can lead to apoptosis in some cells. We investigated these activities in cholinergic forebrain neurons of control and $p75^{NGFR}$ -/- mice during development and following fimbria fornix transection. Different patterns of staining for JNK cascade proteins were observed during development and following injury with similar patterns observed in both control and $p75^{NGFR}$ -/- mice. No differences in NF_KB or TUNEL labeling were found. Thus, $p75^{NGFR}$ does not appear to have a critical role in this activation, nor does this activation necessarily lead to cell death.

Discrepancies with data sets in our laboratory caused us to re-analyze the fate of the cholinergic forebrain neurons in $p75^{NGFR}$ -/- and control mice during development. We confirmed the increase in the number of ChAT-positive neurons in the forebrain during postnatal development and determined that $p75^{NGFR}$ -/- mice have more ChAT-positive neurons than control mice at P6 only. Thus, cholinergic medial septum and neostriatum neurons do not die during the postnatal time period and $p75^{NGFR}$ does not mediate their apoptosis.

We also investigated whether the absence of $p75^{NGFR}$ would affect the cholinergic septohippocampal system during aging in mice. In young, middle aged, and aged control mice, the total number and size of ChAT-positive medial septum neurons and the cholinergic hippocampal innervation did not differ, consistent with the idea that $p75^{NGFR}$ does not play a role in the maintenance of the septohippocampal system during aging.

Thus, BDNF is necessary for normal postnatal maturation of cholinergic forebrain neurons, and $p75^{NGFR}$ does not signal via the JNK cascade, activation of NF_KB, or induce apoptotic cell death in these neurons. Lastly, $p75^{NGFR}$ does not appear to have a critical role in maintaining the cholinergic cell number or size during senescence.

LIST OF ABBREVIATIONS AND SYMBOLS

°C	degree (s) Celsius
-/-	deficient
µg	microgram
µl	microliter
µm	micrometer
AC	anterior commissure
AChE	acetylcholinesterase
AD	Alzheimer's disease
ALS	amyotropic lateral sclerosis
ANOVA	analyses of variance
APTEX	3-aminopropyltriethoxy-silane
ASM	acidic sphingomyelinase
BDNF	brain derived neurotrophic factor
bp	base pairs
CC	corpus callosum
cer	ceramide
CHAIN	Change in Heteroreceptor Association Induced by Neurotrophins
ChAT	choline acetyltransferase
c-Junp	phosphorylated c-Jun
CNS	central nervous system
CTX	cortex
DAB	diaminobenzidene
DAG	diacylglycerol
DIC	differential interference contrast
DNA	deoxyribonucleic acid

dNTP	deoxynucleoside 5'-triphosphate
DRG	dorsal root ganglion
E	embryonic
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
HDB	horizontal limb of the diagonal band of the nucleus of Broca
IgG	immunoglobulin G
ION	isthmo optic nucleus
IP_3	inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
JNKK	c-Jun N-terminal kinase kinase
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
LNGRF	p75 low affinity NGF receptor
LS	lateral septum
LTD	long term depression
LTP	long term potentiation
LV	lateral ventricle
M	Molar
MAPK	mitogen activated protein kinase
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonuleic acid

MS	medial septum
n	number of animals
NF_KB	nuclear factor kappa B
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NIH	National Institutes for Health
NRIF	neurotrophin receptor interacting factor
NSM	neutral sphingomyelinase
NTR	neurotrophin receptor
NT-3	neurotrophin-3
NT-4	neurotrophin-4/5
P	postnatal
p	probability
$p75^{NGFR}$	p75 nerve growth factor receptor
PCR	polymerase chain reaction
PI-3K	phospatidylinositol – 3 kinase
PIP_2	phosphatidylinsoitol 3,4 bisphosphate
PKC	protein kinase C
PLC	phospholipase-C
r	correlation coefficient
SAPK	stress activated protein kinase
SCG	superior cervical ganglion
SDS	sodium dodecyl sulfate
SEK1p	phosphorylated SEK1
SEM	standard error of the mean
Ser	serine
SH3	src homology 3

SNT	suc 1-associated neurotrophic factor target
SOS	son of sevenless
SRE	serum response element
SRF	serum response factor
SSC	standard sodium citrate
TBS	Tris buffered saline
TdT	terminal deoxynucleotidyl transferase
TNF	tumor necrosis factor
TNFR	tunor necrosis factor receptor
Trk	tropomyosin receptor kinase
TUNEL	TdT-mediated dUTP-biotin nick end labeling
vAChT	vesicular acetylcholine transporter
VDB	vertical limb of the diagonal band of the nucleus of Broca

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CHAPTER 1:

GENERAL INTRODUCTION

The discovery and history of NGF and neurotrophins

The development of the nervous system is dependent on stages of cell proliferation, formation of synaptic connections, maturation, differentiation and cell death. During gestation, an abundance of neurons are born and form connections. However, smaller numbers of these connections are actually maintained into adulthood, due to naturally occurring programmed cell death which reduces the number of neurons to adult levels (Hamburger and Oppenheim, 1982; Oppenheim, 1991).

In an attempt to better understand the determination of cell fate and the role of interactions between newly formed neurons and their connections, it was determined that changes in the size of a nervous system target corresponded to changes in the size of the cell center (nucleus or ganglion). In the late 1940s, Hamburger and Levi-Montalcini (1949) demonstrated that removal of a limb bud resulted in hypoplasia (defective development and reduced number of cells in a tissue) of the developing neural center. Conversely, they showed that transplanting an extra limb produced hyperplasia (an abnormal increase in the number of cells in a tissue). In addition, they found that cell death occurred normally during development and that following limb bud destruction the neural centers normally supplying the destroyed limb began to degenerate (Hamburger and Levi-Montalcini, 1949).

The work of Hamburger and Levi-Montalcini (1949) identified the target as having a critical role in determining neuronal fate and size of the nucleus (i.e., the number of neurons). This is significant as during normal development, neurons die at the time their processes enter the target (Oppenheim, 1991). Subsequent studies (Levi-Montalcini and Hamburger, 1951, 1953) identified a soluble diffusible agent produced by muscle-like neoplastic cells that acted on sympathetic and sensory neurons. This agent was named nerve growth factor (NGF). NGF is the prototypic member of the neurotrophin family, a family of homodimer polypeptides that promote survival, growth and differentiation of selected neurons in the peripheral and central nervous systems (PNS and CNS,

respectively; Bothwell, 1995; Maness et al., 1994; Yuen et al., 1996).

In the last 50 years, our understanding of the actions of NGF on the survival, differentiation, growth and maintenance of neurons has been confirmed and has extended to include the "neurotrophic hypothesis". This hypothesis states that once a developing neuron has grown its process into its target, it competes with other developing neurons of the same type for a limited supply of trophic (nutrition) support produced by the target. In a Darwinian manner, those neurons which are successful at competing for neurotrophin survive, those that are not, die (Barde, 1989; Lucidi-Phillipi and Gage, 1993; Yuen et al., 1996). To be consistent with this theory, neurotrophins need to be diffusible, able to act on specific cell surface receptors (localized to the processes of innervating structures), and be present at concentrations lower than that necessary to maintain the viability of the innervating neurons. NGF meets all of these requirements.

For over two decades, NGF offered the only basis for the concept that trophic molecules were necessary for neuronal survival. Work done by Stanley Cohen (1960) provided evidence that NGF was critical for cell survival during normal development of the peripheral sympathetic nervous system, such that anti-NGF antibodies injected into newborn rodents specifically destroyed this region. Studies of the PNS, specifically the peripheral sympathetic neurons and sympathetic ganglia, have offered the most insight into the functions of NGF. Moreover, the "neurotrophic model" can be extended into the central nervous system (CNS), where target neurons synthesize trophic support for their afferent neurons. In the early 1980s it was suggested and demonstrated that NGF has an important role in the developing, adult and injured CNS. Evidence was found supporting this in the cholinergic basal forebrain, where neurons send their axons to the hippocampus and neocortex, where NGF is produced (Korsching et al., 1985; Shelton and Reichardt, 1986). The cholinergic neurons in the basal forebrain were also able to retrogradely transport NGF (Schwab et al., 1979; Seiler and Schwab, 1984), suggesting that these neurons were utilizing NGF. During development, the activity of the

acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT), and the number and size of neurons was increased or reduced following enhancement or inhibition of NGF levels (Auburger et al., 1987; Gahwiler et al., 1987; Johnston et al., 1987; Large et al., 1986; Mobley et al., 1986). Moreover, exogenous NGF could lead to cell hypertrophy in the adult CNS (Garofalo et al., 1992), and after undergoing an injury, prevent cell shrinkage and loss of ChAT activity (Hefti, 1986; Williams et al., 1989).

The Neurotrophin family

In addition to NGF, mammalian members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4). In general, these neurotrophic factors act on distinct, but overlapping populations of neurons to regulate neurotransmitter synthesis, promote cell survival and cause neurite outgrowth (Ip and Yancopoulos, 1996; Lewin and Barde, 1996). Many of these neurotrophic actions are not only critical for the normal development and maintenance of the nervous system, but also play a regenerative or protective role in the nervous system's ability to respond to injury (Ip and Yancopoulos, 1994).

Members of the neurotrophin family bind to two different types of transmembrane receptors: the high-affinity tyrosine kinase receptors (Trk, pronounced track: tropomyosin receptor kinase), and the low-affinity p75 neurotrophin receptor ($p75^{NGFR}$).

The Trk family of neurotrophin receptors

The identification of the mechanism by which the neurotrophins exert their biological activity did not occur until the early 1990s. Originally, the neurotrophin receptor was identified as the product of the trk oncogene, a chimeric oncoprotein found in a human colon carcinoma (Martin-Zanca et al., 1986a, 1986b, 1989). However in 1991, the physiological role of the Trk tyrosine kinase was identified as being the signaling receptor for NGF (Hempstead et al., 1991; Kaplan et al., 1991a, b). Subsequent studies

revealed that the Trk tyrosine kinase was a member of a small family of highly related receptors that included TrkB (Middlemas et al., 1991) and TrkC (Lamballe et al., 1991). Under physiological conditions, these Trk receptors exhibit overlapping specificities for their respective neurotrophins: NGF mediates its effects via TrkA, BDNF and NT-4 activate the TrkB receptor, and NT-3 binds preferentially to TrkC (Glass & Yancopoulos, 1993). Whereas the interaction of NGF, BDNF, and NT-4 with their cognate receptor is highly specific, NT-3 is also capable of activating TrkA (Cordon-Cardo et al., 1991) and TrkB (Soppet et al., 1991) (Fig. 1.1).

The Trk proteins possess a tripartite structure consisting of an extracellular domain for ligand recognition, a single transmembrane domain, and a cytoplasmic domain which contains a tyrosine kinase for initiating the signaling cascade (Schneider & Schwegel, 1991). A second class of alternatively spliced TrkB (2 truncated isoforms) and TrkC (3 truncated isoforms) receptors also exists, containing the same extracellular and transmembrane domains as their full-length receptor, but having a short cytoplasmic domain instead of a catalytic kinase domain (Middlemas et al., 1991; Tsoulfas et al., 1993). Several functions of the truncated form have been proposed including inhibition of full-length TrkB signaling by forming functionally inactive receptor-ligand heterodimers and the sequestering of BDNF and NT-4 to reduce their local availability (Eide et al., 1996; Fryer et al., 1997). Alternatively, these truncated Trks may be capable of signaling on their own, although this has yet to be determined.

Neurotrophin signal transduction by the Trk receptors

Signal transduction is initiated when the neurotrophin homodimer binds the receptor causing a ligand-induced conformational change in the extracellular domain resulting in receptor homodimerization. Receptor dimerization is believed to be necessary for high-affinity neurotrophin binding and resultant trophic activity. It is currently thought that contact between the two cytoplasmic domains induces a conformational change that

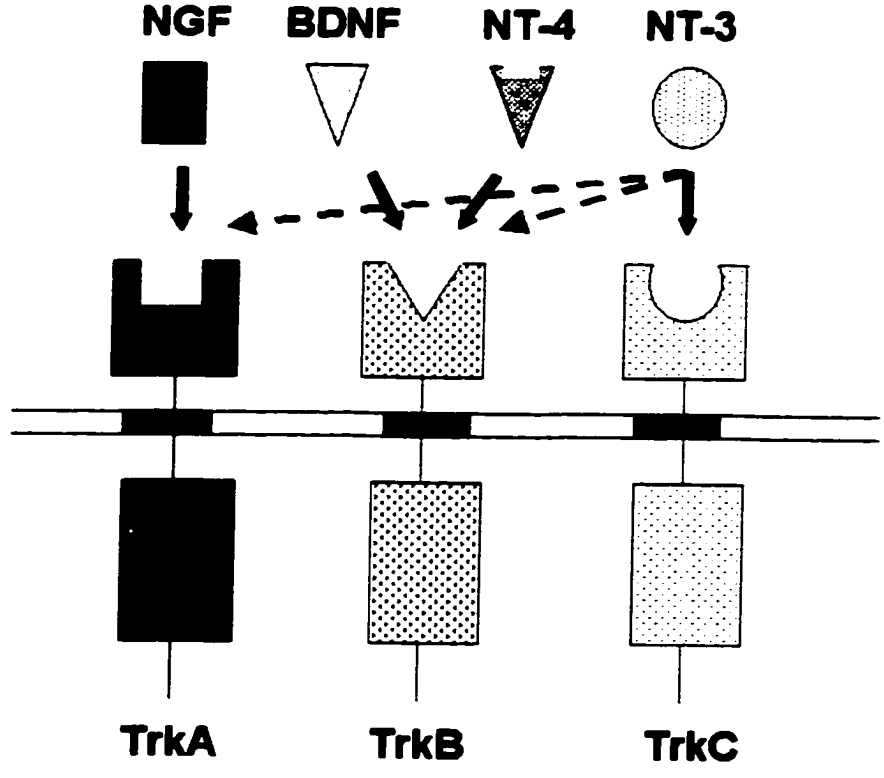


Figure 1.1. A schematic representation of the ligand / receptor relationship between the neurotrophin family of ligands and the Trk family of tyrosine kinase receptors. Thick arrows indicate primary ligand / receptor interactions, thin dashed arrows indicate secondary interactions. (Adapted from Barbacid, 1994).

stimulates catalytic activity leading to mutual transphosphorylation between the two receptor molecules in the dimer (Van der Geer et al., 1994). The tyrosine-phosphorylated Trk acts as a recruitment structure for a variety of intracellular adapter proteins and enzymes that ultimately propagate the ligand signal (Knipper et al., 1993; Stephens, 1994; Vetter et al., 1991). The main focus in the following discussion will be on a downstream target of Trk activity, specifically the Ras pathway, which can interact with $p75^{NGFR}$-induced signaling events (discussed later).

As schematically illustrated in Fig 1.2, following Trk phosphorylation, the phosphorylated intracellular tyrosines act as binding sites for Shc, a linker protein that binds to tyrosine residues on phosphorylated Trk and Grb2. Grb2 interacts via its SH3 (src homology 3) domains, with the proline rich regions of SOS (son of sevenless), a GDP/GTP exchange protein. SOS mediates the exchange of GDP and GTP binding to the membrane bound protein Ras. Ras is inactive when GDP is bound to it and becomes activated when GDP is exchanged for GTP via SOS. Upon activation, Ras is capable of binding to Raf (which has MAP KKK activity, mitogen activating protein kinase kinase kinase). Ras binding to Raf serves to localize Raf, which is normally cytoplasmic, to the membrane. Activated Raf catalyzes the phosphorylation of MAPKK (MEK) at two serines (217 and 221). MAPKK in turn is a dual specificity kinase that catalyzes the phosphorylation of MAPK on two threonine residues (183 and 185). Activated MAPK translocates to the nucleus and catalyzes the phosphorylation of the transcription factor Elk on multiple serines. It has been proposed that ability of Elk to bind to the SRE (serum response element) and form a ternary complex with the SRF (serum response factor) might be enhanced with the phosphorylation of Elk. Elk phosphorylation then activates transcription (for reviews see Bonni and Greenberg, 1997; Heumann, 1994; Kaplan and Stephens, 1994; and Segal and Greenberg, 1996).

Additional downstream targets of activated Trk (also seen in Fig. 1.2) include activation of phospholipase-C gamma (PLCγ), phosphatidylinositol – 3 kinase (PI-3K),

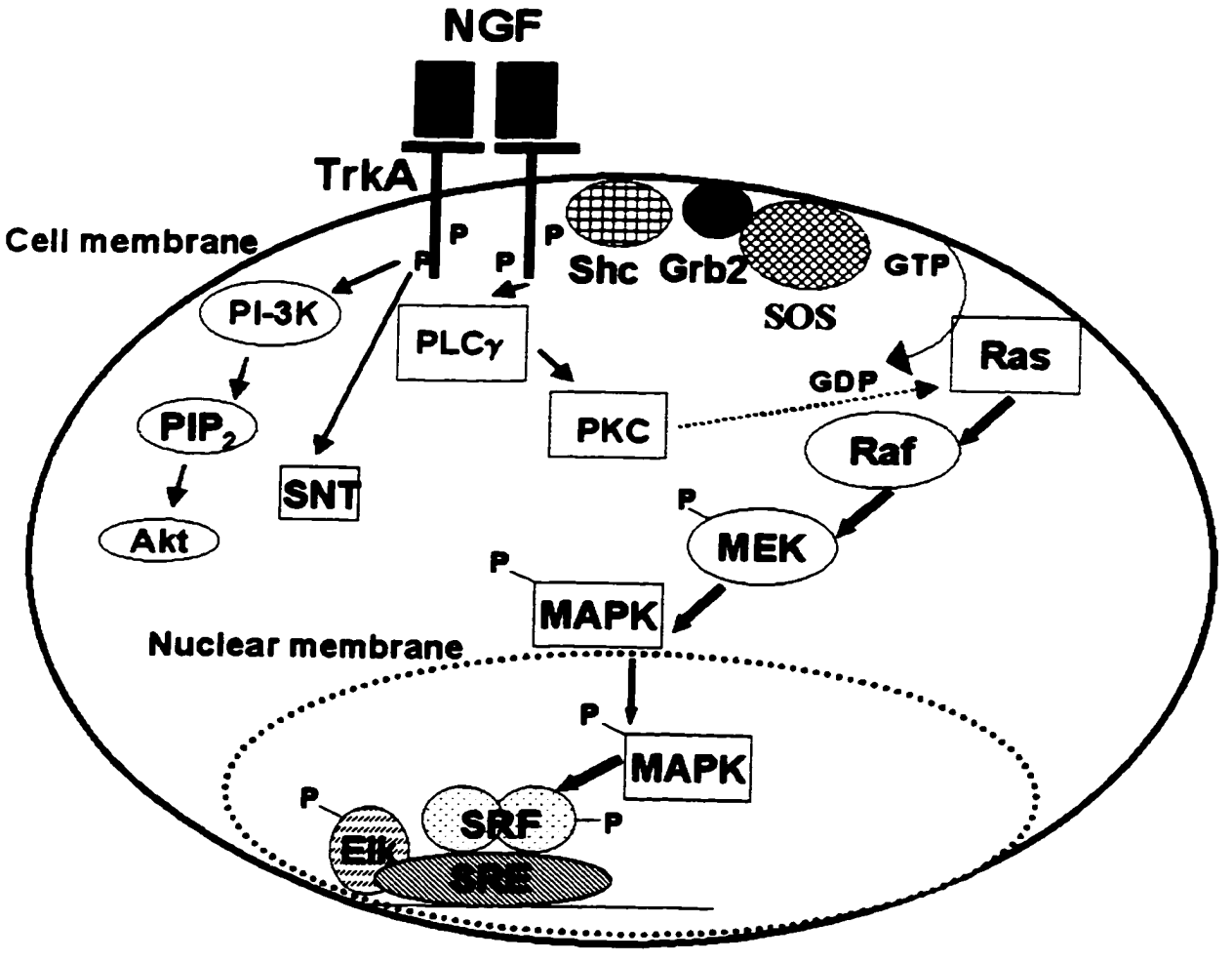


Figure 1.2. A schematic representation of the Ras - MAP kinase pathway activated by neurotrophins as described in the text (including abbreviations). P = phosphorylated (Adapted from Segal and Greenberg, 1996).

and SNT (suc 1-associated neurotrophic factor target). Following Trk autophosphorylation, PLCγ is activated and it can in turn activate Ras via PKC. In addition, PLCγ can also catalytically cleave phosphatidylcholine from the cell membrane leading to the formation of the second messenger diacylglycerol (DAG), and inositol-bisphosphate (IP_2). DAG itself, can activate protein kinase-C (PKC), and IP_2 can lead to increased intracellular calcium levels, all of which lead to cell activation (for review see Bonni and Greenberg, 1997). Similarly, TrkA can also activate PI-3K, although the actual association of the two is still being debated. Activation of PI-3K results in the formation the phospholipid second messenger phosphatidylinsoitol 3,4 bisphosphate (PIP_2) which can then activate Akt, a serine/threonine kinase, (Dudek et al., 1997) which can mediate cell survival, and inhibit apoptosis through its interactions with Bcl-2 (for review see Bonni and Greenberg, 1997). Lastly, Trk also appears to be able to signal through a protein called SNT. Although not well characterized, evidence suggests a role for this signaling protein in elongation and maintenance of neurites and cell survival (Peng et al., 1995).

The $p75^{NGFR}$ neurotrophin receptor

The second neurotrophin receptor, the p75 nerve growth factor receptor ($p75^{NGFR}$), was identified in the late 1980s before Trks were discovered (Chao et al., 1986; Johnson et al., 1986). The term p75 nerve growth factor is misleading as subsequent studies showed that all neurotrophins bind to this molecule, not just NGF (Rodriguez-Tebar et al., 1990, 1993), thus it can also be referred to as the p75 neurotrophin receptor ($p75^{NTR}$). In addition, it has been called the low-affinity NGF receptor (LNGFR). This too is misleading as recent studies have shown that $p75^{NGFR}$ is capable of forming high-affinity binding sites (Ross et al., 1998).

Following sequence analyses, $p75^{NGFR}$ was found to contain a cysteine-rich amino terminal, indicative of an extracellular ligand-binding domain, a short hydrophobic

segment similar to that of other transmembrane receptor sequences, and a carboxy terminus possessing numerous basic acids suggesting an intracellular domain (Johnson et al., 1986). However, absent from the intracellular domain was any indication of a known signal transduction mechanism, i.e., a serine/threonine specific kinase, tyrosine specific kinase, or GTP-binding protein. In fact, it was originally determined that $p75^{NGFR}$ was insufficient for high-affinity binding and NGF responsiveness (Chao et al., 1986; Radeke et al., 1987). The finding that the Trk receptors served as high-affinity binding sites for their respective neurotrophin ligand only cast further question to the relevance of $p75^{NGFR}$ in neurotrophin functioning. However, shortly thereafter, related receptors were characterized, including two tumor necrosis factor receptors (TNFR1 and TNFR2), CD40, and Fas (for review see Chao, 1994). These receptors had similarly small cytoplasmic domains and could transduce a potent signal (Beutler and van Huffel, 1994). Little similarity exists between the TNF and neurotrophin ligands. Nonetheless, their respective receptors share a similar elongated structure displaying an alignment of intracellular amino acid sequences, called the "death domain". This finding helped lead to the discovery that $p75^{NGFR}$ has two very different roles in neurotrophin functioning: one that is Trk-dependent and another that is Trk-independent.

Both the high-affinity Trk receptors and the low-affinity $p75^{NGFR}$ are capable of homo- and heterodimerization; however, the role of $p75^{NGFR}$ in the formation of the high-affinity receptor and the resultant trophic activity is still being debated (Barbacid, 1993). Numerous studies have shown that high-affinity binding of neurotrophins requires the co-expression of $p75^{NGFR}$ and currently, three models describing Trk-$p75^{NGFR}$ interactions have been proposed. Figure 1.3 summarizes the four proposed models.

In the first model (Fig. 1.3A), functional high-affinity neurotrophin receptors exist as heterodimeric complexes of at least two subunits: $p75^{NGFR}$ and Trk (A, B, or C). One of two events are proposed: first, that NGF binds rapidly to $p75^{NGFR}$, increasing the local concentration of NGF for TrkA, and possibly even transfers the neurotrophin to the Trk

Figure 1.3. A summary of proposed models for Trk-p75^{NGFR} interactions.
Different mechanisms have been proposed accounting for p75^{NGFR} and Trk family member interactions. Representations of the models proposed by Chao and Hempstead (1995) (A); Bothwell (1995) (B), and Ross et al. (1998) (C). ● represents NGF.

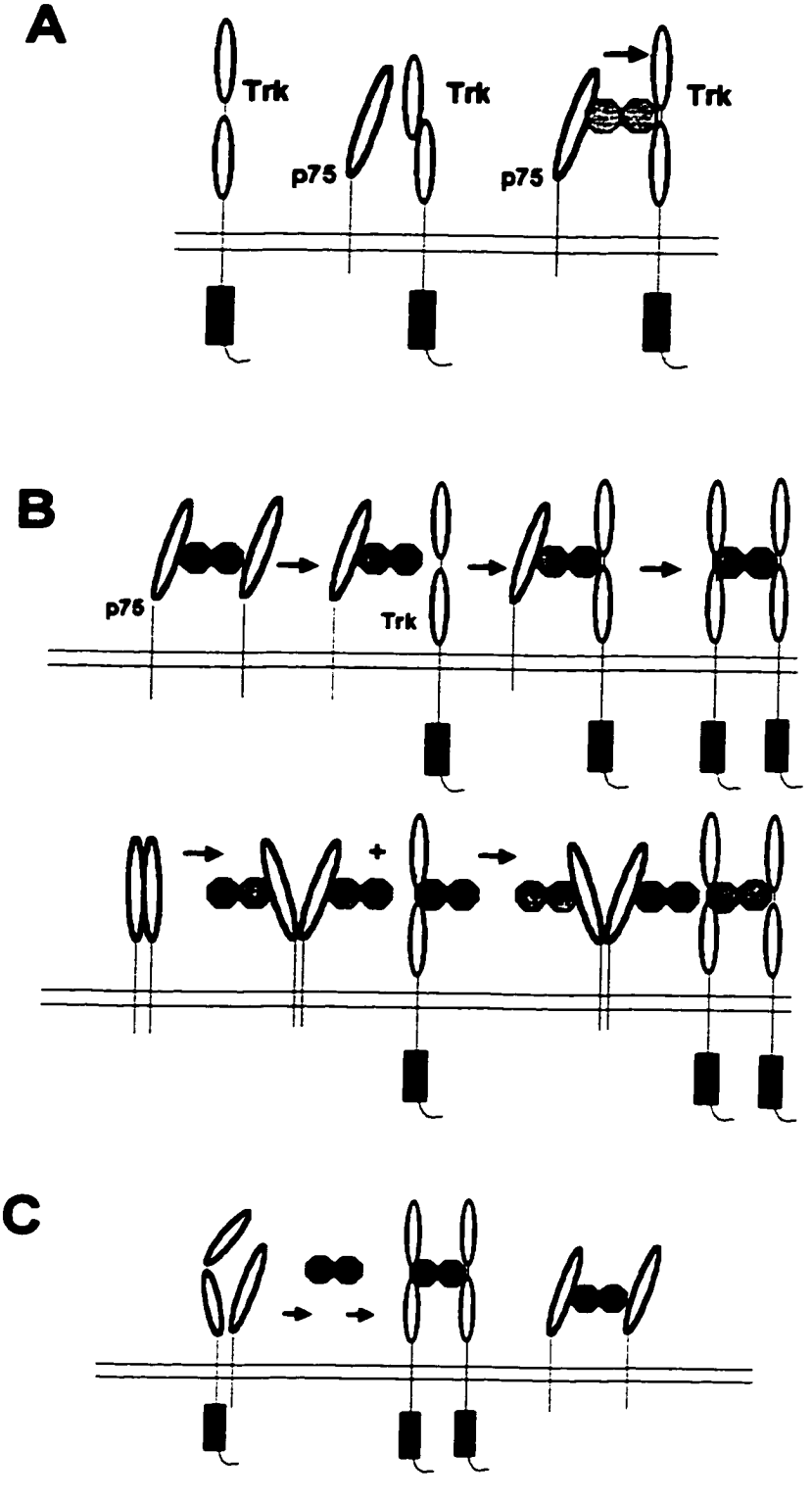


Figure 1.3

receptor; second that $p75^{NGFR}$ and TrkA do not interact directly. Rather, in the absence of ligand Trk and $p75^{NGFR}$ directly interact. In this model, $p75^{NGFR}$ changes the conformation of the Trk receptor, producing a high-affinity binding site (for reviews Chao, 1992; Chao, 1994; Chao and Hempstead, 1995). In contrast, a number of studies have indicated that $p75^{NGFR}$ is not necessary for signaling (Glass & Yancopoulos, 1993). For instance, isolated Trk receptors in the absence of $p75^{NGFR}$ can mediate functional response to physiologically relevant concentrations of the neurotrophins (Ip et al., 1993; Kaplan et al., 1991). Similarly, biochemical interactions of TrkA and $p75^{NGFR}$ have been difficult to demonstrate, although under certain conditions they can be co-immunoprecipitated (Huber and Chao, 1995; Ross et al., 1996).

The second model (Fig. 1.3B) incorporates the asymmetry of the NGF molecule. NGF has regions specific for TrkA and $p75^{NGFR}$ binding, largely independent of each other. The Trk binding sites are localized to one face of the NGF dimer, allowing for the binding of two TrkA molecules following NGF dimerization. The $p75^{NGFR}$ binding sites are localized to the top of the dimer, partly overlapping with TrkA. Models for interactions then suggest that NGF binding to $p75^{NGFR}$ or TrkA monomers cause the formation of $p75^{NGFR}$-TrkA receptor heterodimers, which then act as intermediates in the formation of Trk homodimers. Alternatively, $p75^{NGFR}$ may exist primarily as a dimer, followed by a conformational change following binding to two dimeric molecules of NGF. The conformationally-altered $p75^{NGFR}$ dimer could then bind to TrkA and induce TrkA homodimers (Bothwell, 1995). The third, and most recently proposed model (Fig. 1.3C) has been called the CHAIN model (for Change in Heteroreceptor Association Induced by Neurotrophins). This model proposes that TrkA and $p75^{NGFR}$ physically interact in the absence, but not the presence of NGF (Ross et al., 1998). This interaction is mediated via the intracellular regions of both receptors and inhibits the formation of homodimers (either TrkA or $p75^{NGFR}$). Following NGF binding, the TrkA:$p75^{NGFR}$ heterodimer undergoes a conformational change leading to the favoring of homodimer

receptor interactions (Ross et al., 1998).

Whereas Trks alone appear to mediate many responses to the neurotrophins, evidence also suggests that $p75^{NGFR}$ can significantly alter Trk activity (for reviews see Chao, 1994 and Greene and Kaplan, 1995). There are at least three distinct effects of $p75^{NGFR}$ on Trk functioning. The first involves ligand-binding specificity. In PC12 and sympathetic neuron cell cultures NGF binds to TrkA when both $p75^{NGFR}$ and TrkA are present. If $p75^{NGFR}$ is absent, TrkA can also bind to and mediate responses to NT-3, which normally binds to TrkC or under some conditions to TrkB (Benedetti et al., 1993; Lee et al., 1994). Similarly, $p75^{NGFR}$ appears to convey specificity for BDNF and NT-4 binding to TrkB. Site-directed mutagenesis studies have revealed a role for $p75^{NGFR}$ in regulating biological responsiveness to NT-4, such that NT-4 but not BDNF requires $p75^{NGFR}$ for efficient signaling through TrkB (Ibanez, 1996; Ryden et al., 1995)

The second effect suggests that $p75^{NGFR}$ can enhance the proportion of TrkA receptors able to bind to their cognate ligand with high affinity. When $p75^{NGFR}$ and TrkA are co-expressed in the proper ratio (10:1), a substantial number of high-affinity sites are observed. Furthermore, when $p75^{NGFR}$ undergoes a deletion mutation, these high-affinity binding sites are eliminated (Battleman et al., 1993; Hempstead et al., 1990). The requirement for a ratio of $p75^{NGFR}$ to TrkA receptors implies that a stoichiometric relationship may exist in order for neurotrophin binding and signaling to successfully occur.

Finally, the third Trk-dependent effect of $p75^{NGFR}$ is to enhance TrkA:NGF -induced Trk tyrosine kinase activity. Verdi et al. (1994) showed that overexpressing $p75^{NGFR}$ in MAH sympathoprogenitor cell lines enhanced the biological and signaling activity of Trk. Similarly, Barker and Shooter (1994) found that inhibiting NGF binding to $p75^{NGFR}$ in PC12 cells also reduced NGF-mediated Trk autophosphorylation. Therefore, an important role for $p75^{NGFR}$ may be to provide more specificity to the interactions of neurotrophins with members of the Trk family. Thus, the preceding evidence suggests

that when $p75^{NGFR}$ is co-expressed, the Trk family members may respond much more selectively and effectively.

Trk-dependent functioning notwithstanding, $p75^{NGFR}$ now also appears to be capable of triggering cellular responses without the participation of Trk receptors. For example, in the CNS of $p75^{NGFR}$-deficient (-/-) mice, an ~85-90% reduction in the transport of ^{125}I-labelled NGF and BDNF was found in the cholinergic neurons of the medial septum following injection into the hippocampal formation (Hagg et al., 1996).

$p75^{NGFR}$ appears to promote Schwann cell migration in developing or regenerating peripheral nerves. These nerves express high levels of both NGF and $p75^{NGFR}$ in the absence of TrkA (Anton et al., 1994). These authors report increased Schwann cell migration in denervated sciatic nerve compared to normal nerves, and could inhibit this effect following treatment with antibodies to NGF or $p75^{NGFR}$ (Anton et al., 1994). Similarly, following NGF administration to a human melanoma cell line expressing $p75^{NGFR}$ in the absence of TrkA, a dose-dependent enhancement of penetration of the extracellular matrix was found. The absence of TrkA, and consequent lack of p75-TrkA heterodimers or TrkA-TrkA homodimers, suggests a signaling role for $p75^{NGFR}$ (Herrmann et. al., 1993). Lastly, mRNA induction of the neural adhesion molecule NILE/L1 following NGF exposure in PC12 cells (that contain $p75^{NGFR}$ and TrkA) has been found and does not require TrkA (Itoh et al., 1995). These Trk-independent functions imply the existence of a $p75^{NGFR}$-specific signal.

Signal transduction linked to $p75^{NGFR}$

As a member of the TNF receptor family, $p75^{NGFR}$ has been found to have an extracellular domain homologous to that of TNF. It now also appears that the intracellular portions of TNF receptor 1 (TNFR1), Fas, and $p75^{NGFR}$ all contain a short segment of similarity referred to as the "death domain" (Feinstein et al., 1995). The cellular responses to activation of TNFR1 and Fas includes activation of gene

transcription via nuclear factor κ B ($NF_\kappa B$), sphingomyelin hydrolysis resulting in the generation of ceramide, and the regulation of cell survival/apoptosis. The structural similarities of $p75^{NGFR}$ to the TNF receptor family raise the possibility that similar signaling functions and pathways might exist.

The first clear evidence for $p75^{NGFR}$ signaling came from work by Dobrowsky et al. (1994). These authors showed that binding of any neurotrophin to $p75^{NGFR}$ in T9 glioma cells or NIH3T3 cells activated sphingomyelinase resulting in increased ceramide production. Recently, these observations have been extended to primary oligodendrocyte cultures, where following treatment with NGF, but not BDNF or NT-3, a long lasting release of ceramide and the activation of c-jun amino terminal kinase (JNK; also called stress-activated protein kinase; SAPK) were observed (Cassacia-Bonnefil et al., 1996). The $p75^{NGFR}$ has also been found to activate $NF_\kappa B$ in rat Schwann cells following binding of NGF (Carter et al., 1996). Whereas the signaling of the $p75^{NGFR}$ is not clearly known or understood at this time, the preceding evidence has led to a great deal of 'hinting' that the $p75^{NGFR}$ signaling pathway may mimic the TNF signaling pathway, which is much more clearly understood (Carter et al., 1996; Dobrowsky et al., 1994; Majdan et al., 1997; 1995; Xia et al., 1995). For TNFR I and II and Fas, receptor interacting proteins such as TRAD, TRAF, RIP, and FLICE and FADD directly link the death domain of the membrane receptor to the intracellular proteins and caspases whose activation ultimately can lead to apoptotic death (for review see Baker and Reddy, 1996). Thus far only one interacting factor has been identified and named neurotrophin receptor interacting factor (NRIF) (Casademunt et al., 1998). This protein has been found to interact with the intracellular portion of the $p75^{NGFR}$ following NGF binding. NRIF then causes the activation and nuclear translocation of $NF_\kappa B$. Little evidence other than that presented in the published abstract has been reported, although a manuscript is currently in preparation (Elisabeth Casademunt, personal communication). Currently, no other interacting factors homologous to the Fas and TNFR interacting proteins have been

identified for $p75^{NGFR}$. Figure 1.4 outlines a proposed TNFR pathway (Testi, 1996). It is expected that similarities between TNFR2 and $p75^{NGFR}$ signaling pathways will be found.

Newly discovered neurotrophin functions

The classic role for members of the neurotrophin family dates back to the era in which NGF was first discovered (Levi-Montalcini and Hamburger, 1951, 1953) and includes the promotion of cell survival, growth, differentiation, and sprouting. These events most likely occur in response to signaling through Trk:Trk and/or Trk:p75 interactions. Now, newly discovered, less classical roles for neurotrophins have been found.

In vitro investigations studying PNS sensory neurons have shown that neurotrophins can regulate neuronal phenotype without corresponding changes in cell number (Lewin et al., 1992; Ritter et al., 1991). In addition, *in vitro* work aimed at elucidating neurotrophin physiology has revealed a mitogenic role for Trk C and NT-3 on newly migrating neural crest cells (Kalcheim et al., 1992; Pinco et al., 1993), and increases in the number of neurons in cultured dorsal root ganglions (Wright et al., 1992). Moreover, it is now known that neurotrophins can specifically act at promoting survival or neurite extension, even through the same receptor. For example, in NGF-dependent sympathetic neurons, NT-3 can mediate neuritogenesis but not cell survival as effectively as NGF following binding to TrkA. In these same cells, NT-3 that activates TrkC was unable to support cell survival or neuritogenesis, suggesting that NGF and NT-3 differentially regulate the TrkA receptor (Belliveau et al., 1997).

Classically, NGF was thought to affect synaptic connectivity by influencing growth of neuronal processes, thus leading to its name. However, NGF has also been shown to influence cell body size, dendritic complexity (for review see Lewin and Barde, 1996),

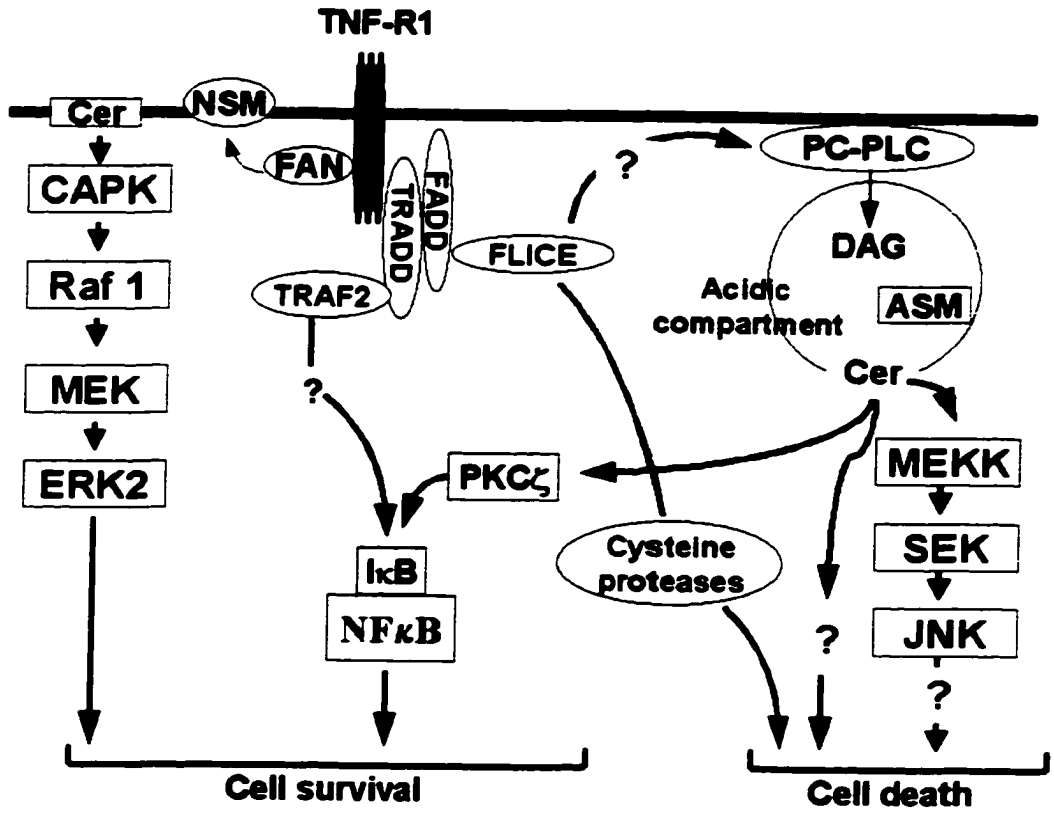


Figure 1.4. A schematic representation of the TNFR1 ceramide-dependent pathway proposed as the signaling pathway for $p75^{NTR}$. NSM, neutral sphingomyelinase; ASM, acidic sphingomyelinase; PC-PLC, phosphatidylcholine-specific phospholipase C; DAG, diacylglycerol; Cer, ceramide. It is not known whether $p75^{NTR}$ has the same receptor interaction factors as TNFR1 (FAN, TRADD, FADD, FLICE, TRAF2). (Adapted from Testi, 1996).

and the number and size of synaptic contacts in the brain (Garofalo et al., 1992).

Similarly, other neurotrophins, specifically BDNF, have also been shown to be involved in enhancing synaptic development, function, and synaptic plasticity (for reviews see Lu and Figurov, 1997; McAllister et al., 1999). Recent studies have shown that neurotrophins potentiate synaptic transmission at the neuromuscular junction (Lohof et al., 1993; Wang and Poo, 1997) and in the brain (Croll et al. 1994; Lessmann et al., 1994). Neurotrophins are also involved in the development of synapses in the visual system (Riddle et al., 1995; 1997) and play important roles in long-term potentiation (LTP), a cellular model for learning and memory (Figurov et al., 1996; Kang et al., 1997). In addition, neurotrophins play a particularly specific and important role in axonal branching and control of dendritic complexity *in vitro* and *in vivo* (reviewed in McAllister et al., 1999). For example, in the CNS, neurons within each cortical layer respond differently to BDNF and NT-3, with each neurotrophin having distinct and opposite effects on the length and complexity of both basal and apical dendrites (McAllister et al., 1995). These results provide functional evidence for neurotrophin-dependent expression of cell phenotype, cell survival, mitogenesis, dendritic outgrowth, and synaptic efficacy.

In addition to cell culture and organotypic slice work, models of neuronal injury and neurotrophin manipulation *in vivo* have also offered tools for studying and understanding the functional role of neurotrophins. For example, antibodies and anti-sense treatment against neurotrophins and their receptors has enabled the further understanding of the role of neurotrophins in synaptic activity (Liou and Fu, 1997) and neurotransmitter release (Sala et al., 1998). Similarly, studies involving neurotrophin inhibition have led to enhanced knowledge of seizure development and the associated changes in synaptic organization (Adams et al., 1997; Ferencz et al., 1997; Holtzman and Lowenstein, 1995), neurotrophin-mediated calcium regulation (Montcouquiol et al., 1997), LTP (Dragunow et al., 1997) and long term depression (LTD; Ruberti et al., 1997), in addition to the role

of neurotrophins in learning and memory (Gutierrez et al., 1997). Moreover, further knowledge has also been garnered by studying neurotrophins during injury of the spinal cord (McTigue et al., 1998; Oudega and Hagg, 1996) the nigro-striatal pathway (Hagg, 1998), and the fimbria fornix septohippocampal system (Hagg et al., 1989; Hagg et al., 1990; Hagg and Varon, 1993), to name a few.

With the advent of genetic engineering, scientists have been able to generate and study the effects of removal of both the Trk and individual neurotrophin genes. Mice with a Trk deletion have significant cell loss, loss of target innervation (Trk -/- Fagan et al., 1997; Smeyne et al., 1994; Trk B -/- Klein et al., 1993; Alcanatara et al., 1997; Trk C -/- Klein et al., 1994), and ultimately, early postnatal mortality. Similar results are found in NGF -/- (Chen et al., 1997), BDNF -/- (Ernfors et al., 1994) and NT-3 -/- (Wilkinson et al., 1996) animals. The early postnatal mortality of homozygous knockout animals demonstrates the critical role for these protein ligands and receptors, such that following the genetic removal normal PNS and CNS system development is disrupted leading to death.

The cholinergic septohippocampal system and its development

The cholinergic septohippocampal system is a system of the CNS in which neurotrophin effects have been widely studied (for reviews see Henderson, 1996; Rylett and Williams, 1994). These cholinergic neurons express both $p75^{NGFR}$ and TrkA receptors (Holtzman et al., 1992; Gibbs and Pfaff, 1994; Hefti et al., 1986; Lee et al., 1998; Sobreviela et al., 1994), and respond to NGF (Lapchak et al., 1993; Li et al., 1995; Mobley et al., 1986). NGF is synthesized in hippocampal neurons (Ayer-LeLievre, 1988; Korsching et al., 1985), taken up by terminals of septohippocampal cholinergic neurons and then retrogradely transported (via the fimbria-fornix pathway) back to the cell bodies found in the medial septum-diagonal band complex where it has been localized (Conner and Varon, 1992; Johnson et al., 1987; Lauterborn et al., 1991; Seiler

and Schwab, 1984). NGF then is able to stimulate the expression of the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT; Holtzman et al., 1992; Vantini et al., 1989). One model that is commonly used to study neurotrophin effects on this system involves transection of the fimbria-fornix. This manipulation results in axotomy of the cholinergic neurons and causes many cholinergic neurons to lose their ability to synthesize and express ChAT (Sofroniew et al., 1990, 1993), an easily measured marker. Following administration of exogenous trophic factors (NGF or BDNF), ChAT immunoreactivity is restored in many of the cholinergic cells axotomized by fimbria-fornix transection (Hagg et al., 1988; Knusel et al., 1992; Morse et al., 1993; Widmer et al., 1993). The medial septum-hippocampal circuit offers a useful model system to study the role of neurotrophin-mediated events following trophic manipulation.

The development of the cholinergic septo-hippocampal system is best understood in terms of the development (neurogenesis and differentiation) of the cholinergic neurons, the growth of their axons towards their targets in the molecular layer of the dentate gyrus, and the development and maturation of the ChAT and AChE enzymes.

In the rat, neurogenesis of the cholinergic neurons in the basal forebrain begins as early as embryonic day (E) 12-13.5 (Armstrong et al, 1987; Brady et al., 1989; Semba and Fibiger, 1988), with reports suggesting that these neurons are the first to become postmitotic in the septum (Semba, 1992). However, in the medial septum cholinergic neurons become post-mitotic with a peak at E15 and E16 in the rat (Semba and Fibiger, 1988). In the mouse, cholinergic neurons in the medial septum and diagonal band are generated around E5 (Schambra et al., 1989). In the rat, ChAT-immunoreactive neurons develop in a caudal to rostral direction beginning at the dorsal tip of the vertical limb of the diagonal band of the nucleus of Broca (VDB), appearing between E14-18 (Semba and Fibiger, 1988). These immature neurons arise from the germinal zone located in the medial wall of the lateral ventricle, just ventral to the developing corpus callosum, and then migrate medio-ventrally to their final destination. In the mouse, the cholinergic

neurons of the VDB and the horizontal limb of the diagonal band (HDB) appear between E14-17 (Schambra et al., 1989). These neurons originate from the germinal zone of the anterior horn of the lateral ventricle and migrate either ventrally (HDB), or medially (VDB), depending on dorsal location. In the mouse, by E17-18 cholinergic septal neurogenesis is complete, and cells become post-mitotic (Schambra et al., 1989). Similar embryonic events occur in rats; however, the time during gestation differs slightly, as the gestation period for mice is 19 days and for rats is 21-22 days (Armstrong et al., 1987; Brady et al., 1989).

Postnatally, cholinergic basal forebrain neurons continue to mature through increases in size. Most of the information on postnatal maturation comes from studies in the rat, and similar to neurogenesis, maturation appears to proceed in a caudal to rostral fashion (Koh and Loy, 1989). The increases in size and dendritic complexity are transient and are observed until the third postnatal week, followed by cell shrinkage and simplification of dendritic structure to levels similar to those observed in adults (Gould et al., 1989, 1990; Sofroniew et al., 1987).

The development and maturation of the fiber innervation of the septohippocampal and hippocamposeptal projections are interdependent, and follow a similar time-pattern to that observed in the neurogenesis and maturation of the medial septal neurons in both rat and mouse. The hippocamposeptal projections develop first from postmitotic-nonpyramidal neurons, reaching the septal region by E16 in rat and E15 in mouse (Linke et al., 1995; Super and Soriano, 1994). These early-formed hippocamposeptal fibers may act as guidance clues for the outgrowing septal fibers (Linke and Frötscher, 1993), as the septohippocampal fibers develop later and only reach the hippocampus after the hippocampal axons reach their target, at E17-18 in rats and at E17 in mice (Super and Soriano, 1994). Septohippocampal fibers finally reach the dentate gyrus by E19 in mice, and by E20 in rats, with very little change occurring until P3 (Linke and Frötscher, 1993; Super and Soriano, 1994). Fiber innervation then continues to increase in density and

arborization until adult patterns are established by P14 – P21 in the rat (reviewed in Semba, 1992). A similar chronological progression exists for cholinergic innervation of the remaining hippocampus, with an adult-like pattern appearing by the third postnatal week.

The developmental and maturation of the expression of ChAT, the rate limiting synthesizing enzyme, and a frequently used marker to identify these neurons, mimics this pattern. ChAT immunoreactivity has been observed as early as E12 in rats and E13.5 in mice at very low levels (Schambra et al., 1987). From this developmental period, enzyme activity continues to increase peaking around P30 for both mice and rats in the medial septum, and decreasing to adult levels by 5 months (Thal et al., 1991; Virgili et al., 1991).

AChE-positive fibers have been reported emerging from the septum by E20 in rat and by E18-19 in mouse, extending towards the hippocampus through the fimbria by P0. AChE activity, like that of ChAT, is extremely low embryonically and early into postnatal development. In fact, in both rat and mouse AChE staining in the hippocampus does not become readily visible until 2 – 4 days after birth (Matthews et al., 1974) at which time AChE-positive fibers are consistently seen in the rostral parts of the dentate gyrus, hippocampus, and subiculum. By P5 – P11, stained fibers can be observed throughout the rostro-caudal aspects of the hippocampus, and between P14 – P21, AChE enzyme activity and staining pattern, reach adult levels (Milner et al., 1983; Thal et al., 1991; Virgili et al., 1991).

Transgenic technology has been used effectively for the *in vivo* determination of the role of specific neurotrophins and their receptors in the development of the cholinergic septohippocampal system. Analyses of medial septum cholinergic cell numbers, cell size, and innervation of the hippocampus in NGF- and TrkA- deficient mice have revealed the importance of these proteins. In these animals, fewer and smaller ChAT-positive medial septum neurons, and reduced cholinergic innervation of the hippocampus

(Chen et al., 1997; Crowley et al., 1994; Fagan et al., 1997) were observed, consistent with the classic neurotrophin theory that these proteins are critical for regulating the normal developmental processes.

Newly emerging hypotheses for neurotrophins and their receptors

With the discovery of Trk-independent $p75^{NGFR}$ functions, and the identification of its independent signaling ability, recent evidence is pointing towards a novel role for neurotrophins and their receptors.

The signaling similarities of $p75^{NGFR}$ to TNFR1 whose activation often leads to apoptotic events led to the very 'unclassical' prediction that perhaps $p75^{NGFR}$ could also induce apoptosis. One of the first indications that $p75^{NGFR}$ could initiate an apoptotic signal was seen in certain $p75^{NGFR}$-expressing neuroblastoma cell lines following NGF withdrawal (Rabizadeh et al., 1993). These findings led to the prediction that $p75^{NGFR}$ induced cell death in a ligand-independent manner. Shortly thereafter, Barrett and Bartlett (1994) showed that cultured post-natal dorsal root ganglion (DRG) cells treated with anti-sense oligonucleotides against $p75^{NGFR}$ survived longer than control DRG cells in the absence of neurotrophin. Similar results were obtained using the same approach in PC12 cells (Barrett and Georgiou, 1996)

One of the first indications that $p75^{NGFR}$ could initiate an apoptotic signal *in vivo* was observed in 1994. von Bartheld et al. (1994) found that BDNF retrogradely transported back to chick isthmo-optic nuclei (ION; which express $p75^{NGFR}$) and promoted the survival of these neurons while NGF increased the number of dying cells. Interestingly, these results were interpreted to suggest that NGF decreased endogenous BDNF binding to TrkB by blocking the $p75^{NGFR}$ co-receptor, thereby resulting in cell death of some BDNF-dependent neurons. It was only in their 1996 article that the authors showed that there was no TrkA localized in the ION, while full length TrkB was present, suggesting that NGF binding to $p75^{NGFR}$ in cells expressing $p75^{NGFR}$ and not TrkA, might induce

apoptosis. Recently, two groups have reported direct evidence for mediation of cell death by $p75^{NGFR}$ upon NGF binding. Frade et al. (1996; also Frade and Barde, 1998) showed that early retinal cells expressing $p75^{NGFR}$, and not TrkA, undergo cell death that could be prevented by application of anti-NGF, anti-$p75^{NGFR}$ antibodies, or a $p75^{NGFR}$-inhibiting peptide (dc28-36). Furthermore, Cassacia-Bonnefil (1996) showed NGF-induced death via $p75^{NGFR}$ in rat oligodendrocytes not expressing TrkA. Moreover, these authors showed that cell death was correlated to increased ceramide production and JNK activation. More recently, Majdan et al. (1997) have shown that overexpression of the intracellular domain of the $p75^{NGFR}$ receptor leads to profound reductions in numbers of sympathetic and peripheral sensory neurons as well as cell loss in the neocortex where normally little or no $p75^{NGFR}$ is expressed. This same group has also demonstrated that when sympathetic neuron survival is maintained with low quantities of NGF or KCl, activation of $p75^{NGFR}$ with BDNF causes neuronal cell death. These results were confirmed in BDNF -/- mice where sympathetic neuron numbers were found to be increased relative to BDNF +/+ littermate controls (Bamji et al., 1998). These results suggest that endogenous neurotrophins may act as "neurocidal" or death-inducing ligands following binding to $p75^{NGFR}$.

Van der Zee and colleagues have recently shown that during development of the basal forebrain, ~25% of the cholinergic cells in the medial septum of the basal forebrain undergo programmed cell death between postnatal (P) day 6 and 15, and that these cells do not express TrkA. Furthermore, this programmed cell death was prevented following the systemic administration of a $p75^{NGFR}$-inhibiting peptide (Van der Zee et al., 1996). Other groups have now shown that $p75^{NGFR}$ also negatively regulates cholinergic neuronal phenotype of the basal forebrain cholinergic neurons, including cell size, target innervation, and neurotransmitter synthesis (Yeo et al., 1997).

These results imply that the presence of Trk may abolish the apoptotic ability of $p75^{NGFR}$. Evidence suggests that Trk signaling may block or inhibit $p75^{NGFR}$ signaling.

Xia et al. (1995) found that NGF-withdrawal in PC12 cells caused a sustained activation of JNK and a corresponding inhibition of MAPKs. This dominant-interfering theory regarding various components of the JNK and MAPK signaling pathways is supported by the preceding studies, mentioned above (Frade et al., 1996; Frade and Barde, 1998; Van der Zee et al., 1996) in which $p75^{NGFR}$ exerted its death-inducing role only when TrkA was absent. In fact, this may generalize to any Trk signaling pathway, although at present there is no evidence suggesting this. Nevertheless, the blocking effect of TrkA on $p75^{NGFR}$ enhances the knowledge regarding our predicted signaling pathways and their interactions with each other as depicted in Figure 1.5. Recently, the specific effects of competition between TrkA and $p75^{NGFR}$ were examined in oligodendrocytes (Yoon et al., 1998). The authors report that activation of TrkA negated $p75^{NGFR}$ – initiated cell death and correlated to MAPK activation and suppression of JNK activity initiated by $p75^{NGFR}$. Nuclear translocation of NF_KB was unaffected. These results further support the suggestion that TrkA can simultaneously activate survival responses and suppress death signaling. Thus, the end result may be a series of checks and balances between signaling events. The selective interplay between tyrosine kinases and cytokine receptors may provide a means for predicting cell fate, such that the more dominant signaling pathway may be capable of canceling or blocking the effect of the less weighted one, resulting in cell survival or cell death.

Rationale

In the last four years, the understanding of the function and role of the $p75^{NGFR}$ has changed from one of neglect, due to the belief it could not transduce a signal, to one of rapidly growing interest and importance. The discovery that $p75^{NGFR}$ could activate $NF_\kappa B$ (Carter et al., 1996), initiate ceramide formation via sphingomyelinase activation (Dobrowsky et al., 1994, 1995; for review see Carter and Dobrowsky, 1998), and mediate apoptosis (for review see Carter and Lewin, 1997) has sustained this interest.

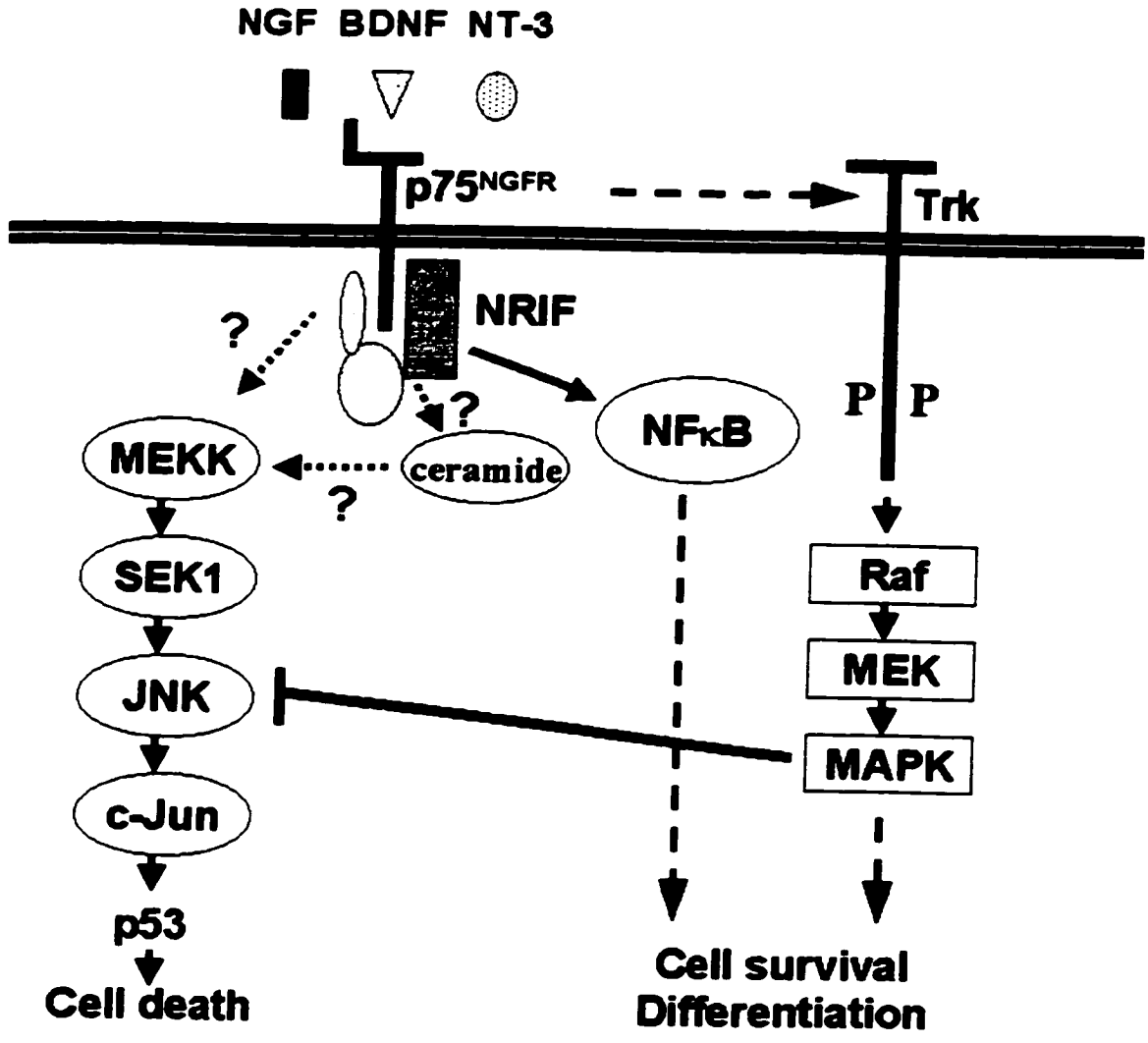


Figure 1.5. A schematic representation of the predicted neurotrophin receptor signaling pathways and their possible interactions.

Nonetheless, the factors underlying the induction and modulation of $p75^{NGFR}$-mediated apoptosis are still unclear. Currently, two theories exist regarding ligand binding to $p75^{NGFR}$; one that suggests that it is necessary, the second that it is not. Neurotrophin effects via $p75^{NGFR}$ most likely are cell type-, environment-, and development-specific. Currently, in the basal forebrain the need for $p75^{NGFR}$-ligand interactions for the initiation of apoptosis is unknown.

In the basal forebrain, in addition to other forebrain brain regions expressing $p75^{NGFR}$, the $p75^{NGFR}$-specific signaling pathways and their interactions with Trk signaling events have not been studied. Proposed pathways have been suggested based on the similarities between $p75^{NGFR}$ and TNFR1 but these have only been tested in culture systems, and have yet to be identified in *in vivo* systems.

Lastly, cholinergic neurons of the forebrain have been shown to shrink and have decreased retrograde transport in aged animals. In addition, these neurons have been shown to degenerate during Alzheimer's Disease (AD). The role of $p75^{NGFR}$ is not well understood in aging-related events or in AD.

Objectives

1. To identify *in vivo* the putative $p75^{NGFR}$ ligand that induces apoptotic cell death of a subpopulation of cholinergic forebrain neurons.

2. To identify *in vivo* the intracellular signaling pathways activated by $p75^{NGFR}$ during developmental apoptotic cell death and following injury in cholinergic forebrain neurons.

3. To determine *in vivo* the role of $p75^{NGFR}$ in the degeneration of cholinergic medial septum neurons during aging.

We studied the cholinergic forebrain in an attempt to meet these objectives as this region provides a model system in which to study the *in vivo* interactions of TrkA and $p75^{NGFR}$. Understanding the death-mediating role and signaling pathways of $p75^{NGFR}$ will provide basic information regarding p75 receptor-mediated events. Moreover, it may provide insight into new strategies for the treatment of cell death in the forebrain in AD and Down's syndrome where these cholinergic neurons degenerate and die.

CHAPTER 2:

BDNF IS NEEDED FOR POSTNATAL MATURATION OF CHOLINERGIC BASAL FOREBRAIN NEURONS IN VIVO

The results presented in the following chapter have been submitted for publication to the journal *Experimental Neurology*.

INTRODUCTION

The neurotrophins are a family of neurotrophic factors which promote cell survival, neurite outgrowth, phenotypic maturation and synaptic functioning (Ip and Yancopoulos, 1996; Lewin and Barde, 1996). Mammalian neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4). Under physiological conditions NGF mediates its survival-promoting effects via TrkA, BDNF and NT-4 preferentially activate TrkB, and NT-3 preferentially activates TrkC (Bothwell, 1995). All neurotrophins can bind to the p75 NGF receptor ($p75^{NGFR}$) which plays a role in neurotrophin transport, ligand-binding specificity, Trk functioning (Bothwell, 1995; Chao and Hempstead, 1995) and under certain conditions, induction of apoptosis (Carter and Lewin, 1997; Chao et al., 1998).

Basal forebrain cholinergic neurons project their axons throughout the hippocampal formation and neocortex and are important for learning and memory (Everitt and Robbins, 1997). In the medial septum of the basal forebrain of developing and adult mammals, including mice, cholinergic neurons express TrkA and $p75^{NGFR}$ (Lee et al., 1998; Sobreviela et al., 1994). NGF is known for its role in maintaining cell body size and ChAT (Mobley et al., 1986; Vantini et al., 1989) and its ability to prevent or reverse degeneration in adult and aging mammals (Hagg et al., 1989b; Williams et al., 1986). In fact, heterozygous NGF- or TrkA-deficient mice have fewer and smaller ChAT-positive medial septum neurons (Chen et al., 1997; Fagan et al., 1997).

Basal forebrain cholinergic neurons also express the full-length transducing TrkB receptor for BDNF and NT-4 during development and in adulthood (Fryer et al., 1996; Masana et al., 1993; Yan et al., 1997). Cultured embryonic and early postnatal cholinergic neurons respond to BDNF with increased survival and increased levels of ChAT and AChE (Alderson et al., 1990; Nonomura and Hatanaka, 1992). In adult rats, treatment with BDNF can prevent axotomy-induced degeneration and loss of ChAT (Knusel et al., 1992; Koliatsos et al., 1994; Morse et al., 1993). BDNF mRNA and protein levels increase during the first two postnatal weeks peaking between P19-24

(Forster et al., 1993; Timmusk et al., 1994), with levels of full-length Trk B decreasing after P7 to adult levels (Altar et al., 1994; Masana et al., 1993). This expression pattern parallels the critical period for postnatal maturation of the cholinergic medial septal neurons (Mobley et al., 1986). Therefore, it is conceivable that BDNF plays a significant role during development and further maintenance of the basal forebrain cholinergic neurons.

In direct contrast to the classic neurotrophin hypothesis, recent evidence suggests that neurotrophins can also initiate apoptosis during development (Bamji et al., 1998; Cassaccia-Bonnefil et al., 1996; Frade et al., 1996; Frade and Barde, 1998; Van der Zee et al., 1996). In the medial septum of the basal forebrain of developing control mice, ~25% of the cholinergic neurons reportedly do not express TrkA and die between postnatal day (P) 6 and 15 (Van der Zee et al., 1996). Similar findings were reported in the developing neostriatum (Van der Zee and Hagg, 1998). This loss was not observed in $p75^{NGFR}$ - deficient mice or in control mice injected with a $p75^{NGFR}$ - interfering peptide (dc28-36; Van der Zee et al., 1996). This raised the possibility that an endogenous ligand may induce cholinergic cell death through binding to $p75^{NGFR}$ in the absence of Trk receptors (see also Yoon et al., 1998). In fact, endogenous NGF reportedly induces $p75^{NGFR}$ - mediated neuronal apoptosis in developing chick retina, since the cell loss can be prevented by antibodies against NGF or $p75^{NGFR}$, or by dc28-36 peptide (Frade et al., 1996; Frade and Barde, 1998). Moreover, activation of $p75^{NGFR}$ with BDNF causes death of sympathetic neurons *in vitro* and BDNF - deficient mice have more peripheral sympathetic neurons than control littermates (Bamji et al., 1998). Thus, endogenous neurotrophins can act as death-inducing or "neurocidal" ligands. It follows that elimination of the neurocidal neurotrophin through genetic deletion, such as found in neurotrophin-deficient mice, could result in an increased number of certain neuronal populations that survive into adulthood.

In homozygous NGF, BDNF, NT-3, NT-4, and Trk (A, B, or C) - deficient mice, the

basal forebrain has been described previously but not in sufficient quantitative detail to reveal differential survival or loss of a subpopulation of cholinergic neurons (Alcantara et al., 1997; Chen et al., 1997; Crowley et al., 1994; Ernfors et al., 1994; Klein et al., 1993; Klein et al., 1994; Liu et al., 1994; Smeyne et al., 1994; Wilkinson et al., 1996). One of the problems of such analyses is the fact that very few homozygous deficient mice survive after the second postnatal week. Heterozygous NGF - or TrkA - deficient mice have reduced numbers of ChAT - positive neurons in the medial septum (Chen et al., 1997; Fagan et al., 1997), suggesting that NGF is needed for their development and is probably not neurocidal in this system. Here, we analyzed BDNF, NT-3, and NT-4 deficient mice using a detailed quantitative approach to investigate the potential role of neurotrophins during postnatal development and maturation of the cholinergic neurons of the medial septum and neostriatum of the mouse forebrain.

MATERIALS AND METHODS

Animals and genotyping. All animal procedures were approved by the Animal Care Committee of Dalhousie University and conformed to Canadian Council on Animal Care guidelines. Deep anesthesia was achieved by intraperitoneal injection of 6.5 mg/kg sodium pentobarbitol. Breeding pairs of mice (inbred mixed Balb/c-B6-129/Sv background) heterozygous (+/-) for a null mutation in the BDNF or NT-3 genes or homozygous (-/-) for a deletion of the NT-4 gene were purchased from Jackson Laboratory (Bar Harbor, ME). The genotype of offspring from the BDNF +/- and NT-3 +/- mice was determined using DNA extracted from tail clippings obtained from anesthetized animals (Walsh et al., 1991) and a polymerase chain reaction (PCR) protocol supplied by Jackson Laboratories (www.jax.org). Oligonucleotide primers were used with the following sequences:

BDNF: 1. 5' - GGG AAC TTC CTG ACT AGG GG - 3'
2. 5' - ATG AAA GAA GTA AAC GTC CAC - 3'
3. 5' - CCA GCA GAA AGA GTA GAG GAG - 3'

NT-3 1. 5' - CCT GGC TTC TTT ACA TCT CG - 3'

2. 5' - TGG AGG ATT ATG TGG GCA AC - 3'

3. 5' - GGG AAC TTC CTG ACT AGG GG - 3'

PCR analyses were performed using Pharmacia Biotech rTaq and buffer (Quebec, Canada) under the following conditions: 0.4 μm oligonucleotide (as indicated); 200 μm dNTPs; 1.5 mM $MgCl_2$ as supplied in buffer; 0.25 units rTaq polymerase; denature 1 minute (min) at 94 °C, followed by 30 cycles of: denature 30 seconds at 94.0 °C; anneal 1 min at 50.0°C (BDNF), 53.0 °C (NT-3); and extend 1 min at 72.0 °C. The PCR reaction products were separated electrophoretically on a 1.2% agarose gel. PCR products generated using BDNF primers 1 and 2 revealed a single band of ~340 basepairs (bp) (targeted deficient allele), and those generated with primers 2 and 3 a band of ~275 bp (wildtype allele). Similar analyses using NT-3 primers 1 and 3 revealed a single band of 350 bp (targeted deficient allele), and those generated with primers 1 and 2 revealed a single band of 250 bp (wildtype allele). For both neurotrophin genes, heterozygous animals yielded both amplification products.

Histological procedures. At P6, P15 or adulthood (2-3 months), anesthetized littermates were weighed and then perfused transcardially with cold (4°C; 2 - 15 ml) phosphate buffered saline, followed by cold 4% paraformaldehyde in 0.1M phosphate buffer (4°C; 4 - 30 ml). The brains were post-fixed for 24 hours in 4% paraformaldehyde and cryoprotected in a 30% sucrose / phosphate buffer solution for 24 hours. The brains were then weighed and 30μm thick coronal sections through the septum and hippocampal formation were cut with a freezing microtome. To visualize the cholinergic neurons, every third section through the entire septal nucleus and vertical limb of the diagonal band was processed for immunocytochemistry using an affinity-purified polyclonal goat antibody against ChAT (Ab144P, Chemicon, Temecula, CA). In short, free floating tissue sections were incubated sequentially with 3% rabbit serum in Tris buffered saline (TBS) containing 0.3% Triton X-100 for thirty minutes, primary antibody Ab144P at a 1:4000 dilution in 1% rabbit serum TBS-triton for 16 hours (P15 and adult tissue) or 36 hours (P6 tissue) at 4°C, biotinylated rabbit anti-goat IgG (1:300; Vector laboratories;

Burlingame, California) in TBS for ninety minutes, and avidin-biotin-peroxidase complex (1:600; ABC Elite kit, Vector Laboratories) in TBS for one hour. Immunoreactive products were revealed with a diaminobenzidine (DAB) reaction which was intensified with 0.67% ammonium nickel sulfate. In between steps, the sections were washed 3 x 10 minutes in TBS. Adjacent sections throughout the septum of P15 BDNF transgenic mice were processed for $p75^{NGFR}$ immunostaining using the polyclonal anti - REX antibody (generous gift of Dr. Louis Reichardt, UCSF) at 1:30.000 following the same protocol adjusted for rabbit primary antibodies. All stained sections were mounted on gelatin-coated glass slides, dehydrated, and coverslipped in Permount. Two identical sections (spaced 180 μm) in the medial septum of every animal were stained with cresyl violet. A series of three sections spanning 720 μm in the dorso-rostral hippocampal formation of P6 and P15 BDNF animals were mounted on gelatin-coated slides and processed for acetylcholinesterase or AChE (Hedreen et al., 1985) to visualize cholinergic axons in the hippocampus. Promethazine (0.2mM, Sigma) was used as an inhibitor of non-specific esterases. Adjacent sections were stained with polyclonal anti-REX and polyclonal goat anti-vesicular acetylcholine transporter (vAChT; Ab1578, 1:20,000, Chemicon, Temecula, CA) as additional methods of visualizing cholinergic innervation of the hippocampus.

TUNEL labeling to detect DNA fragmentation. Adjacent sections to those used in the histological analyses were mounted on 2% APTEX (3-aminopropyltriethoxy-silane; cat #A-3648; Sigma Chemicals, St. Louis, MO) -coated slides. Four sections spanning 540μm of the medial portion of the medial septum (every sixth section between 240 - 780μm rostral to the anterior commissure) were processed for TUNEL using the Oncor ApopTag *In situ* Apoptosis Detection kit - peroxidase (Cat #S7100-Kit Ed.1.5; Oncor Inc, Gaithersburg, MD). For a positive control, separate tissue was incubated with serially diluted RQ1 RNAse free DNAse I (activity = 1 unit / μl; Promega, Madison, WI) in DNAse buffer (1M Tris HCl with 6mM MgCl2) for one hour at 37° C before the first step of the TUNEL protocol. Experimental tissue was incubated under the same conditions with buffer, and all tissue was thoroughly washed in 2xSSC before beginning

the TUNEL kit protocol. As a negative control, separate tissue underwent the complete protocol, but were incubated with a solution containing distilled water instead of TdT enzyme. Following staining, slides were dehydrated and coverslipped in Permount.

Quantitative analysis. In each mouse, every third section through the entire rostro-caudal extent of the medial septum was used to determine the number of ChAT-positive neurons. To ensure consistency between animals, section number 0 (zero) was identified as the most rostral section through the anterior commissure decussation. In adjacent sections of P15 BDNF mice the number of $p75^{NGFR}$ -positive neurons was also determined. Neuronal cell body profiles larger than 9 μm in longest diameter were counted on one half of the medial septum, whose ventral border was defined by an imaginary line through the arms of the anterior commissure. The total number of cholinergic medial septum neurons in one hemisphere was calculated according to the formula: Total number = 3 x (counted profiles x section thickness / (mean cell body diameter + section thickness)) (Abercrombie, 1946). The mean diameter was determined by measuring the longest diameter of 25 randomly chosen cholinergic (ChAT and $p75^{NGFR}$ stained) neurons per animal. Neostriatal cholinergic neurons were counted in one hemisphere in identical sections using the same protocol as used in the medial septum. Potential size changes of non-cholinergic medial septum neurons were estimated in the following manner: subtracting the sum of the cell body diameters of 8 cholinergic neurons (8 x mean diameter) from the sum of cell body diameters of 50 cresyl violet stained neurons (cells larger than 9 μm to exclude glia) and dividing the result by 42. This is based on the findings that ~17 % (i.e., ~8/50) of medial septum neurons are cholinergic (Hagg et al 1992; Williams et al., 1986). Volumes (mm^3) of the septum and vertical limb of the diagonal band (bilateral) and the volumes occupied by cholinergic neurons in these regions (Fig. 9) were determined in every sixth section of all P6 and P15 BDNF transgenic mice, using an interactive image analysis system. For each of the AChE-, $p75^{NGFR}$ -, and vAChT - stained sections through the hippocampal formation, a grid was placed over the left dorsal blade of the molecular layer of the dentate gyrus. The number of stained fibers intersecting one of three distinct lines of the grid (spaced ~60

μm) were counted in the molecular layer in each of the sections and presented as mean number of fibers per section.

All data collection was completed blind to the animal genotype. Between group (i.e., genotype, age) analyses were performed using the Mann-Whitney U test and statistical significance was defined as $p < 0.05$. For BDNF transgenic animals at P6, P15, and adult, n = (+/+) 4, 6, 8; (+/-) 6, 10, 7, (-/-) 4, 4, 0, respectively. Adult BDNF -/- mice were not analyzed as few survived beyond two weeks. For NT-3 transgenic animals at P6 and P15 n = (+/+) 5, 5; (+/-) 5, 9; (-/-) not viable, respectively. For NT-4 (-/-) transgenic animals at P15 n = 5. Each group (excluding NT-4 -/-) contained mice from at least two different litters.

RESULTS

BDNF -/- mice have fewer ChAT-positive forebrain neurons than their control littermates

Sections through the medial septum revealed that BDNF -/- mice had fewer ChAT-positive neurons than their +/+ and +/- littermates at P6 as well as at P15 (Fig. 2.1). Moreover, the cholinergic neurons of the BDNF -/- mice had a reduced ChAT-immunostaining intensity. At P6, the total number of ChAT-positive neurons on one side of the medial septum of BDNF +/+ (321 ±11; SEM; n = 4) and +/- (324 ± 17; n=6) mice was not significantly different and was ~23% higher than the number of ChAT-positive neurons in BDNF -/- mice (263 ±24 $p < 0.05$; n = 4; Fig. 2.2). At P15, ~45% more ChAT-positive neurons were detectable in BDNF +/+ mice (467 ±22; $p < 0.01$; n = 6) than at P6. The number of neurons at P15 in BDNF +/- (392 ±38; n = 10) or BDNF -/- mice (312 ±28; n=4) was not significantly higher than at P6. At P15, BDNF +/+ mice had ~33% ($p < 0.01$) more ChAT-positive neurons than their -/- littermates but the number was not significantly different from that of the BDNF +/- littermates. The number of neurons in adult BDNF +/+ and +/- mice (n = 8 and 7, respectively) were similar to that observed at P15 (Fig. 2.2).

Figure 2.1. BDNF -/- mice have fewer cholinergic medial septum neurons and reduced ChAT levels.

ChAT-immunostained coronal sections through the medial septum of P6 BDNF +/+ (A), +/- (B), and -/- (C) and P15 BDNF +/+ (D) and +/- (E) and -/- (F) mice. Note that ChAT-immunostaining of the individual neurons is less intense in BDNF -/- mice. Arrowheads indicate the midline of the medial septum. Magnification bar = 100 μm. Insets are 10x higher magnifications of individual neurons in the corresponding mice.

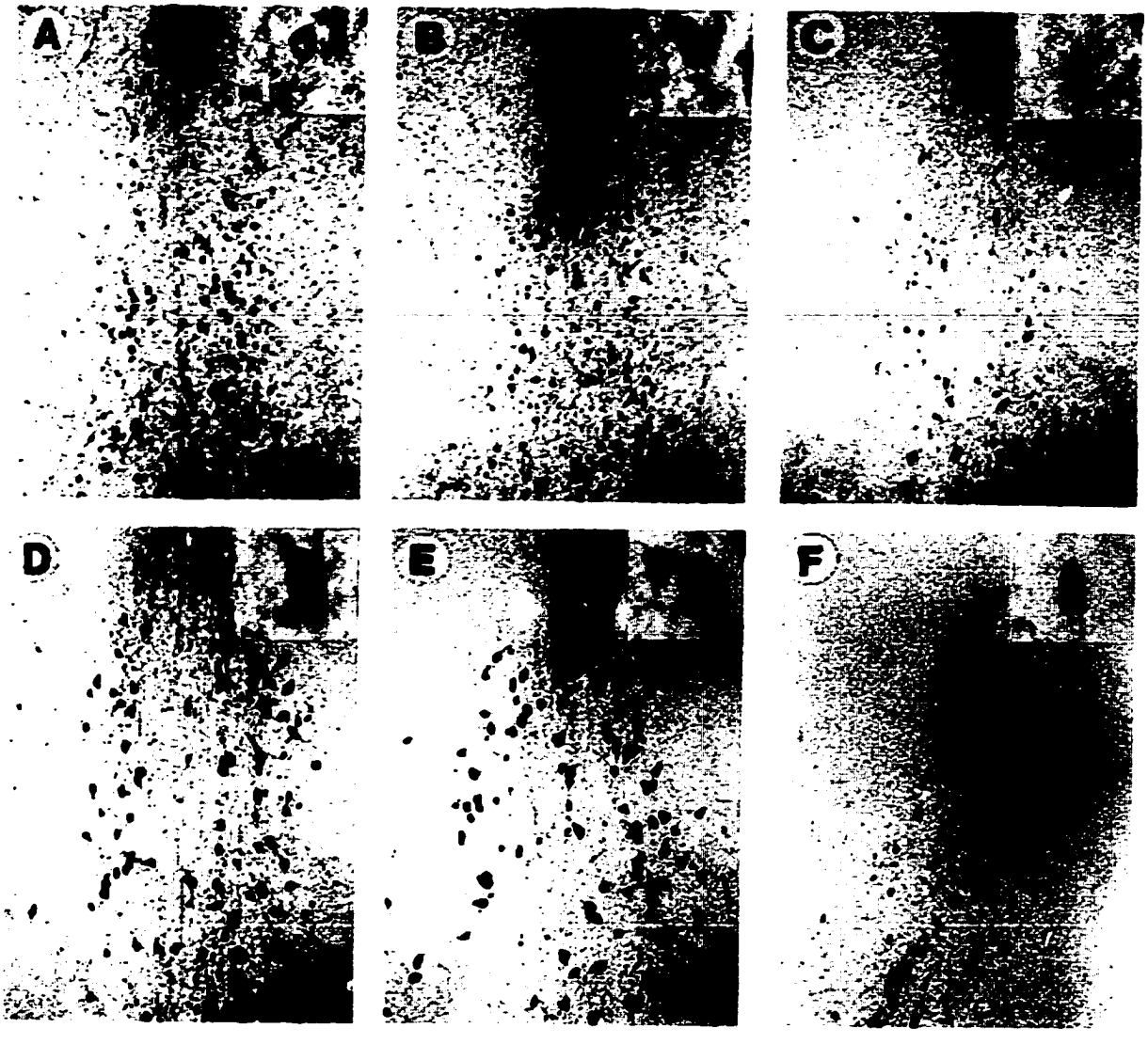


Figure 2.1

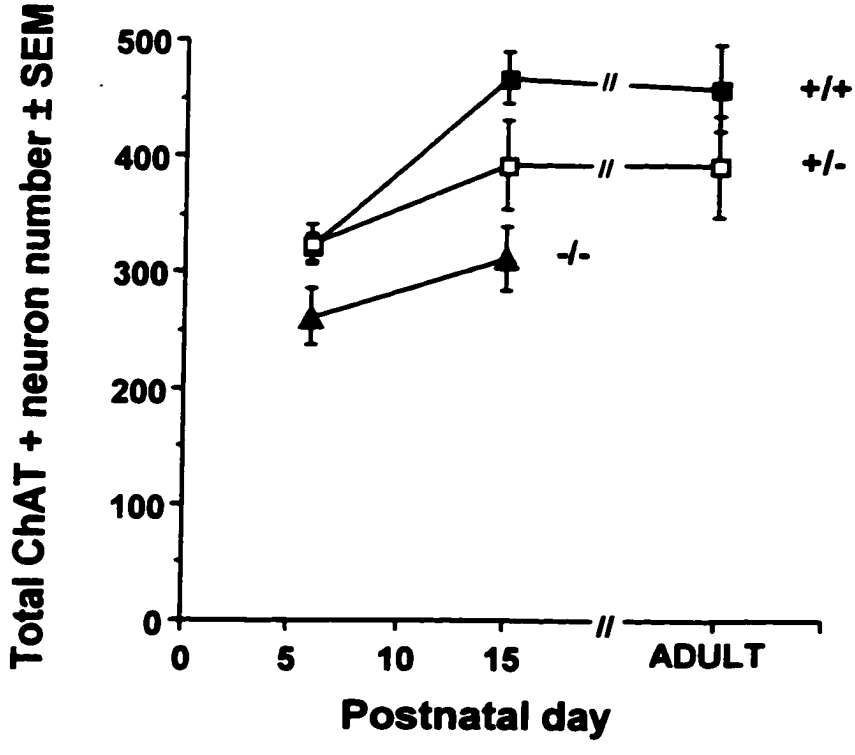


Figure 2.2. The total number of cholinergic medial septum neurons is lower in BDNF -/- mice.

Presented is the total number of ChAT-positive neurons (±SEM) on one side of the medial septum area of BDNF-deficient (-/-) mice and their littermate controls (+/+ and +/-) at P6, P15 and adulthood.

Sections through the neostriatum revealed that BDNF -/- mice had fewer ChAT-positive neurons than their +/+ littermates at P6 and at P15 in addition to a reduction in ChAT-immunostaining intensity (Fig. 2.3). At P6, the total number of ChAT-positive neurons in the regions analyzed was ~24% higher in BDNF +/+ mice than in their BDNF -/- littermates (2001 ±63 vs. 1530 ±207; $p < 0.05$). Between P6 and P15 the total number of ChAT-positive cells increased ~54% in both BDNF +/+ (2001±63 vs. 3090 ±117) and BDNF -/- mice (1530 ±207 vs. 2370 ±144), and the ~24% difference between BDNF +/+ and -/- mice was maintained ($p< 0.05$).

Rostro-caudal distribution of cholinergic neurons does not change in BDNF -/- mice

Distribution graphs of the number of ChAT-positive neurons throughout the rostro-caudal extent of the septal nucleus demonstrated that at P6, the rostro-caudal length of the "cholinergic" septum (all sections containing ChAT-positive neurons) was similar in all BDNF mice (Fig. 2.4, +/- mice not shown). More ChAT-positive neurons were found along the midportion of the rostro-caudal extent of the medial septum in BDNF +/+ mice than in the BDNF -/- littermates. At P15, BDNF +/+ and +/- mice had significantly greater rostro-caudal septal lengths than at P6 (1.04 ±0.04 vs. 0.56 ± 0.06 mm (+/+); 0.92 ± 0.03 vs. 0.57 ± 0.02 mm (+/-)). The length of the septal region in BDNF -/- mice did not increase significantly between P6 and P15 (0.56 ±0.07 vs. 0.81 ±0.1 mm). The distribution graphs also revealed that the smaller number of neurons observed in BDNF -/- mice at P15 was present throughout the nucleus (Fig. 2.4).

$p75^{NGFR}$ - immunostaining confirms that BDNF -/- mice have fewer cholinergic neurons

Because the lower number of ChAT-positive neurons observed in BDNF -/- littermates could represent a reduced level of ChAT expression, sections were immunostained for $p75^{NGFR}$, an independent marker of cholinergic neurons in the basal forebrain (Hagg et al., 1992). At P15, the total number of ChAT-positive neurons did not significantly differ from the number of $p75^{NGFR}$-positive neurons in BDNF +/+ (467 ±22

Figure 2.3. BDNF -/- mice have fewer and smaller cholinergic neurons in the developing striatum

ChAT-immunostained coronal sections through the striatum of one hemisphere of P6 BDNF +/+ (A), P6 BDNF -/- (B), P15 BDNF +/+ (C) and P15 BDNF -/- (D) mice show that BDNF -/- mice have fewer and smaller cholinergic neurons, in addition to having less ChAT. Magnification bar = 100 μm. Insets are 10x higher magnifications of individual neurons in the corresponding mice. Left side of the photomicrographs is medial.

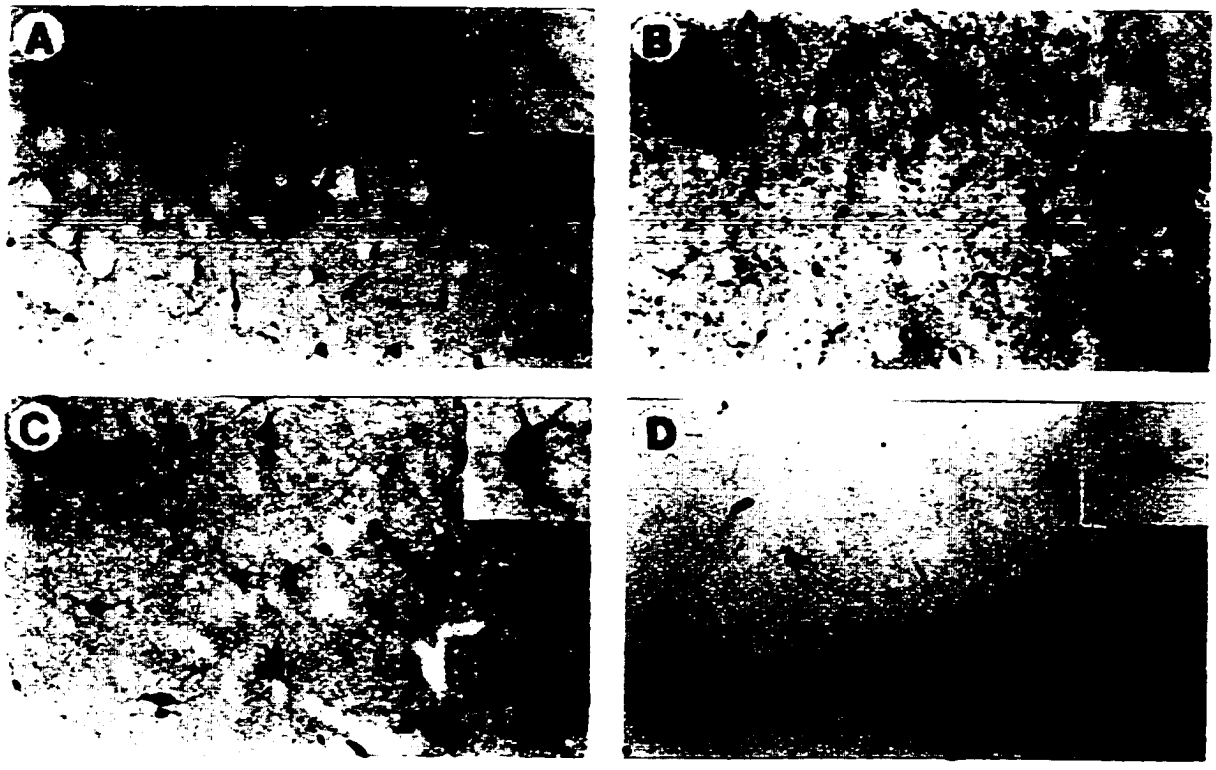


Figure 2.3

Figure 2.4. BDNF -/- mice have fewer cholinergic neurons throughout the septum and the distribution does not change from P6-P15.

The numbers of ChAT- positive neurons (±SEM) in coronal sections through one side of the medial septum show that P15 BDNF -/- mice have fewer cholinergic neurons than their wild-type control littermates throughout the rostro-caudal extent of the nucleus at P15 (closed squares) but not at P6 (open squares). Section number 0 is the most rostral section through the anterior commissure decussation. Also note that at P15, BDNF -/- mice have fewer ChAT-positive neurons in the rostral portion of the nucleus compared to BDNF +/+ mice.

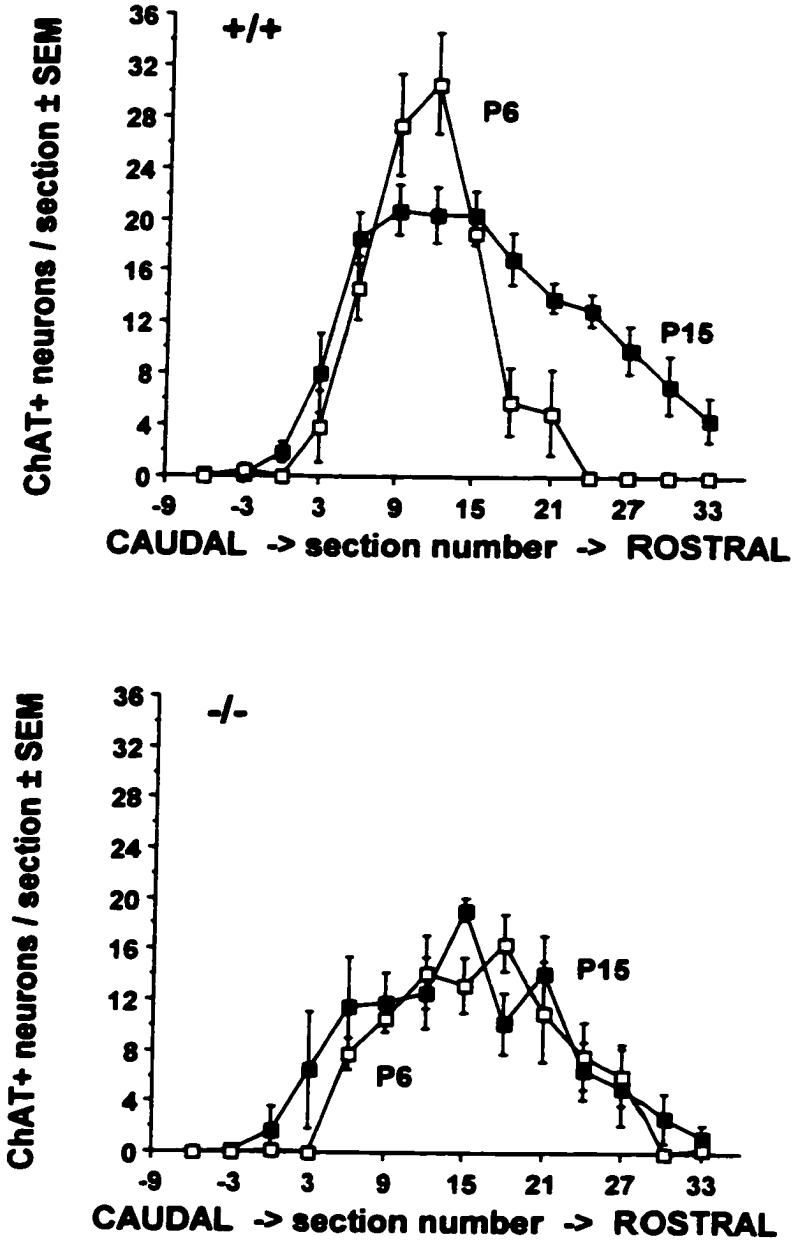

+/+
P6
P15
ChAT+ neurons / section ± SEM
36
32
28
24
20
16
12
8
4
0
-9
-3
3
9
15
21
27
33
CAUDAL -> section number -> ROSTRAL
-/-
P15
P6
ChAT+ neurons / section ± SEM
CAUDAL -> section number -> ROSTRAL

Figure 2.4

vs. 391 ±27) and BDNF +/- mice (392 ±38 vs. 405 ±23). In BDNF -/- mice, the number of ChAT-positive neurons was greater than the number of $p75^{NGFR}$-positive neurons (312 ± 28 vs. 246 ± 14; $p < 0.05$). Distribution graphs of the number of $p75^{NGFR}$-positive neurons confirmed that the number of cholinergic neurons in P15 BDNF -/- mice was lower throughout the rostro-caudal extent of the septal nucleus (not shown).

BDNF -/- mice have more TUNEL-labeled forebrain cells than their BDNF +/+ littermates

To investigate whether the lower number of cholinergic neurons in BDNF -/- mice could be explained by increased cell death, sections through the medial septum were processed for TUNEL, a marker of DNA fragmentation and a characteristic of apoptosis. At P6, BDNF -/- mice had ~3 times more TUNEL-positive cells in the medial septum than their control (+/+) littermates (16.5 ± 4.1 vs. 5.7 ± 0.7; $p < 0.05$; Fig. 2.5). Between P6 and P15, the number of TUNEL-positive cells significantly decreased for BDNF -/- mice (10.5 ± 2.6; $p < 0.05$) but did not change for BDNF +/+ littermates (5.0 ± 1.3) littermates ($p = 0.11$).

In the striatum, the number of TUNEL-positive profiles was higher (but not significantly) at P6 (46 ±13 (+/+); 64 ±17 (-/-)) than at P15 (26 ±4 (+/+); 24 ±3 (-/-)), with no differences observed between BDNF +/+ and -/- mice.

Cholinergic neurons in BDNF -/- mice do not show a normal developmental size increase

At P6, the cell body diameters of the cholinergic medial septum neurons was not significantly different between any of the mice (~15.0 μm; Figs. 2.1 and 2.6). Between P6 and P15, the cell body diameter increased ~45% and ~38% ($p < 0.001$) in BDNF +/+ and +/- mice, respectively, but not in BDNF -/- mice (Figs. 2.1 and 2.6). At P15, the cell diameters were significantly greater in BDNF +/+ and BDNF +/- mice (21.6 ± 0.5 μm or 25% and 20.3 ± 0.4 μm or 20%, respectively; $p < 0.002$) than in BDNF -/- mice. The

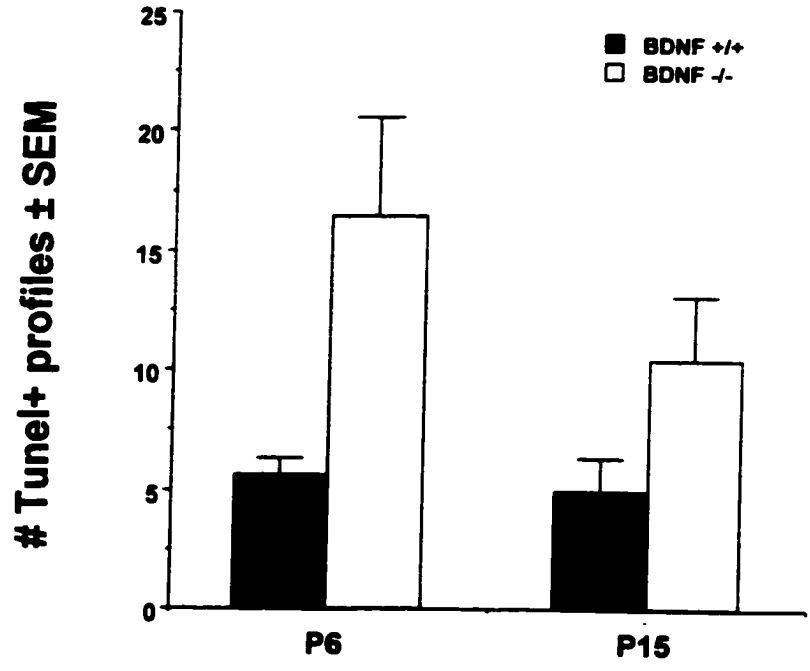


Figure 2.5. BDNF -/- mice have increased DNA fragmentation in the medial septum at P6.

Presented is the total number of TUNEL-positive cells (±SEM) in four sections of one side of the medial septum at P6 and P15 for BDNF +/+ and -/- mice. At P6, BDNF -/- mice have significantly more TUNEL-positive cells than their BDNF +/+ littermates.

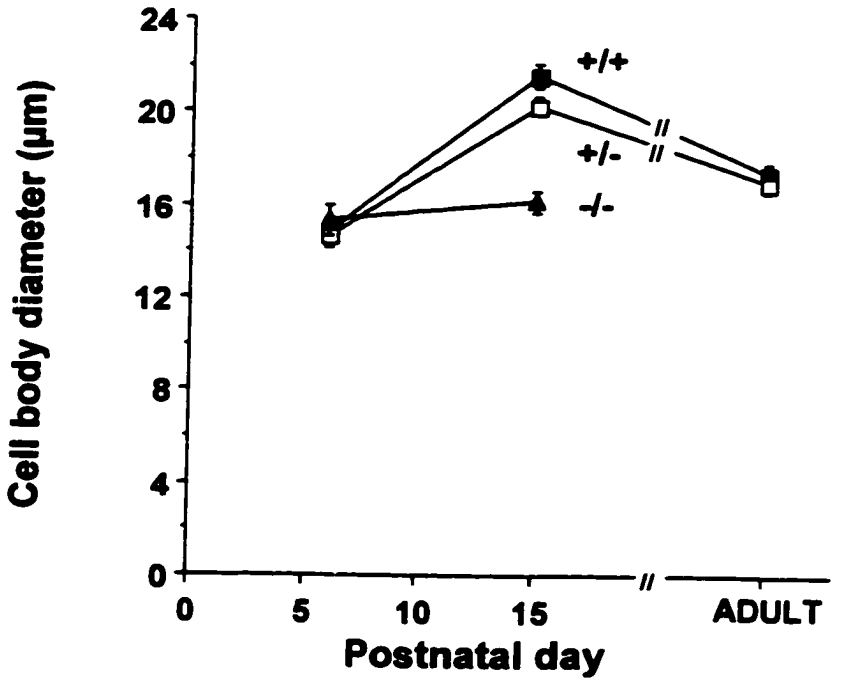


Figure 2.6. Cholinergic septal neurons of BDNF -/- mice do not show developmental hypertrophy.

Presented are the cell body diameters (±SEM) of ChAT-positive medial septum neurons in BDNF -/- (triangles) mice and their littermate controls (+/+, filled squares; +/- open squares) at P6, P15, and adulthood. In BDNF +/+ and +/- mice, the size of the cholinergic neurons increased between P6 and P15 and then decreased to adult values. In the BDNF -/- mice, the diameter remained unchanged between P6 and P15.

diameter of the cholinergic cell bodies in the medial septum decreased significantly between P15 and adulthood ($p < 0.001$) in BDNF +/+ and BDNF +/- mice. BDNF -/- adult mice were not analyzed as few survive longer than 2-3 weeks. To confirm that BDNF -/- mice had smaller cell diameters than BDNF +/+ mice at P15, and not simply decreased ChAT levels (which could be reflected in smaller cell diameters), we measured cell diameters in tissue stained against the membrane receptor $p75^{NGFR}$. BDNF +/+ mice had significantly larger $p75^{NGFR}$-positive cholinergic medial septal neurons (21.1 ± 0.7) than their BDNF -/- littermates (16.5 ± 0.3; $p < 0.001$). Moreover, the size of the $p75^{NGFR}$-positive cells did not differ from the ChAT-positive cell diameters.

To determine whether the lack of postnatal size increase was specific to the population of cholinergic medial septum neurons, the average cell body diameter of the non-cholinergic neuron population was estimated. At P15, the diameter of non-cholinergic neurons in BDNF -/- mice (16.8 ± 0.6 μm) was not significantly different from that in BDNF +/+ littermates (15.3 ± 0.3 μm).

At P6, the cell body diameters of the cholinergic neurons in the neostriatum were not significantly different between any of the mice (~14.5 μm; Fig. 2.3). Between P6 and P15, the cell body diameter of the striatal cholinergic neurons increased ~42% ($p < 0.001$) in BDNF +/+ mice (from 14.6 ±0.7 to 20.7 ±0.6 μm) and ~12% ($p < 0.01$) in BDNF -/- mice (from 14.4 ±0.2 to 16.1 ±0.4 μm). At P15, the cell diameters were significantly larger in BDNF +/+ mice (~29%; $p < 0.001$) than in BDNF -/- mice.

Cholinergic hippocampal innervation develops with reduced AChE in BDNF -/- mice

To evaluate whether BDNF had an effect on the development of the septohippocampal pathway, the cholinergic innervation of the dentate gyrus was analyzed with AChE, a widely used marker for cholinergic fibers. At P6, the number of AChE-positive fibers observed in the molecular layer of the dentate gyrus was not significantly different between any of the mice (~12-14 fibers in 3 measured fields; Fig. 2.7). Between P6 and P15, the number of AChE-positive fibers increased significantly in BDNF +/+ (71 ± 2) and BDNF +/- (63 ± 4; not shown) mice ($p < 0.01$) with no differences between the

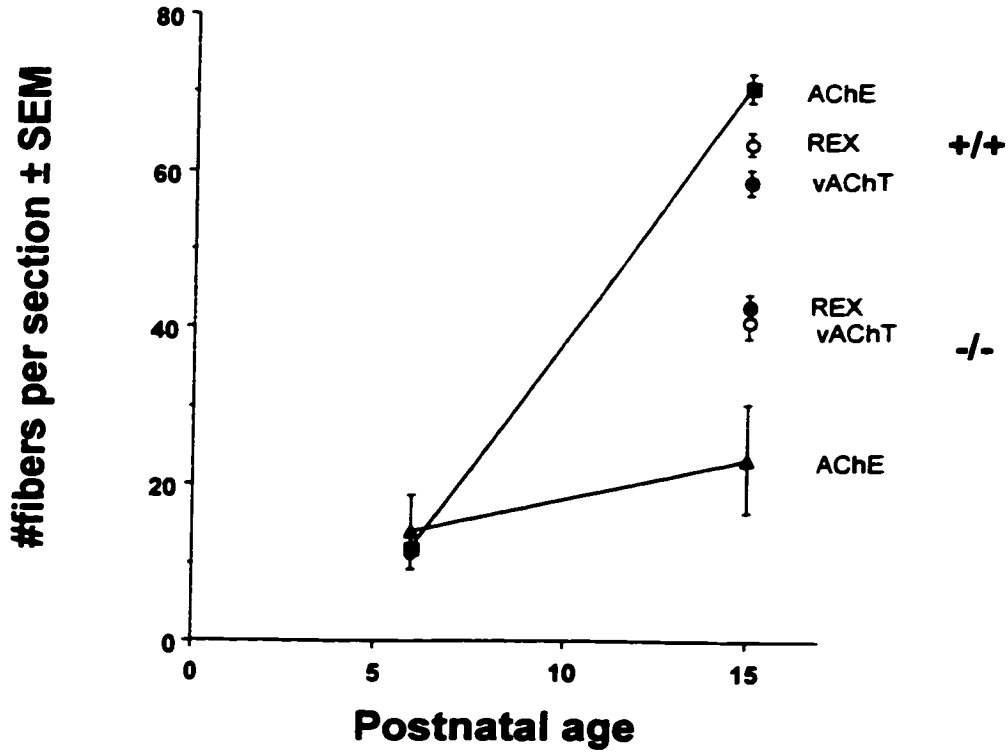


Figure 2.7. Cholinergic hippocampal innervation is reduced in BDNF -/- mice.
The number of AChE-positive cholinergic fibers in the molecular layer of the dorsal blade of the left dentate gyrus increased between P6 and P15 in littermate controls (+/+, filled squares) but not in BDNF -/- (filled triangles) mice. Also presented are the number of $p75^{NGFR}$- (open circles) or vAChT-immunostained (closed circles) fibers in BDNF +/+ and BDNF -/- mice, revealing that the cholinergic axons had a reduced level of AChE. Values represent the mean sum of 3 measurements per section made over three sections through the hippocampal formation of each animal.

two types of mice. In BDNF -/- littermates the number of AChE-positive fibers did not change significantly between P6 and P15 (23 ± 2; Fig. 2.7), resulting in the appearance of a much less extensive cholinergic innervation of the dentate gyrus (Fig. 2.8). The thickness of the molecular layer did not differ between any of the mice at P6 or at P15. At P15, other hippocampal regions (e.g., CA1, CA2, and CA3) also appeared to have a reduced number of AChE-positive fibers in BDNF -/- mice. Various regions of the neocortex of P15 BDNF -/- mice also had a lower density of AChE-positive fibers and a reduced number of AChE-positive neuronal cell bodies (not shown).

Because the reduced number of AChE-positive fibers could represent a reduction in the phenotypic marker AChE, adjacent sections of P15 mice were immunostained for $p75^{NGFR}$ or vAChT, two additional cholinergic markers. Staining intensity was similar for $p75^{NGFR}$ and AChE, such that clearly defined fibers were observed (Fig. 2.8). Despite the fact that the vAChT-staining was lighter (Fig. 2.8), which made counting more difficult, the number was similar to the number of $p75^{NGFR}$-positive fibers. At P15, BDNF +/+ mice had ~35% more counted $p75^{NGFR}$-positive fibers and ~27% more counted vAChT-stained fibers than their BDNF -/- littermates ($p75^{NGFR}$: 64 ±1 vs. 41 ±2, $p < 0.05$; vAChT: 59 ±2 vs. 43 ±2, $p < 0.05$; Fig.2.7). An estimate of the actual number of fibers in three dimensions, suggests that BDNF -/- mice have ~50-60% fewer cholinergic ($p75^{NGFR}$- or vAChT-positive) fibers in the dentate gyrus. The numbers of $p75^{NGFR}$- or vAChT-positive fibers were lower in BDNF +/+ mice and greater in BDNF -/- mice when these were compared to the number of AChE-positive fibers ($p < 0.02$ and 0.05, respectively).

Basal forebrain regions are smaller in BDNF -/- mice at P15 but not at P6

To assess whether BDNF had more general effects on basal forebrain development, the volumes of the septum (lateral and medial), diagonal band region, and the sub-regions occupied by the cholinergic neurons were measured (Fig. 2.9). At P6, the volumes of these four regions did not differ between BDNF -/- mice and their +/+ or +/- littermates (Fig. 2.9; diagonal band not shown). Between P6 and P15, the volumes of all four

Figure 2.8. Cholinergic hippocampal innervation is reduced in P15 BDNF -/- mice. AChE-stained (A, B, C, D) coronal sections through the hippocampal formation at P15 suggest that the density of cholinergic innervation in the dentate gyrus molecular layer of BDNF -/- mice (B and D) is greatly reduced compared to their BDNF +/+ (A and C) littermates. In sections stained for $p75^{NGFR}$ the difference between BDNF +/+ (E) and BDNF -/- (F) was obvious but less pronounced than with AChE. Staining for vAChT revealed similar differences between BDNF +/+ (G) and BDNF -/- (H) mice. Note the similar staining intensities of AChE- and $p75^{NGFR}$- stained sections of control mice (C and E). Magnification bar for A and B = 200 μm and for C through H = 25 μm. Arrowheads delineate the border of the dentate gyrus and the hippocampal fissure. Asterisks indicate the granule cell layer of the dentate gyrus.

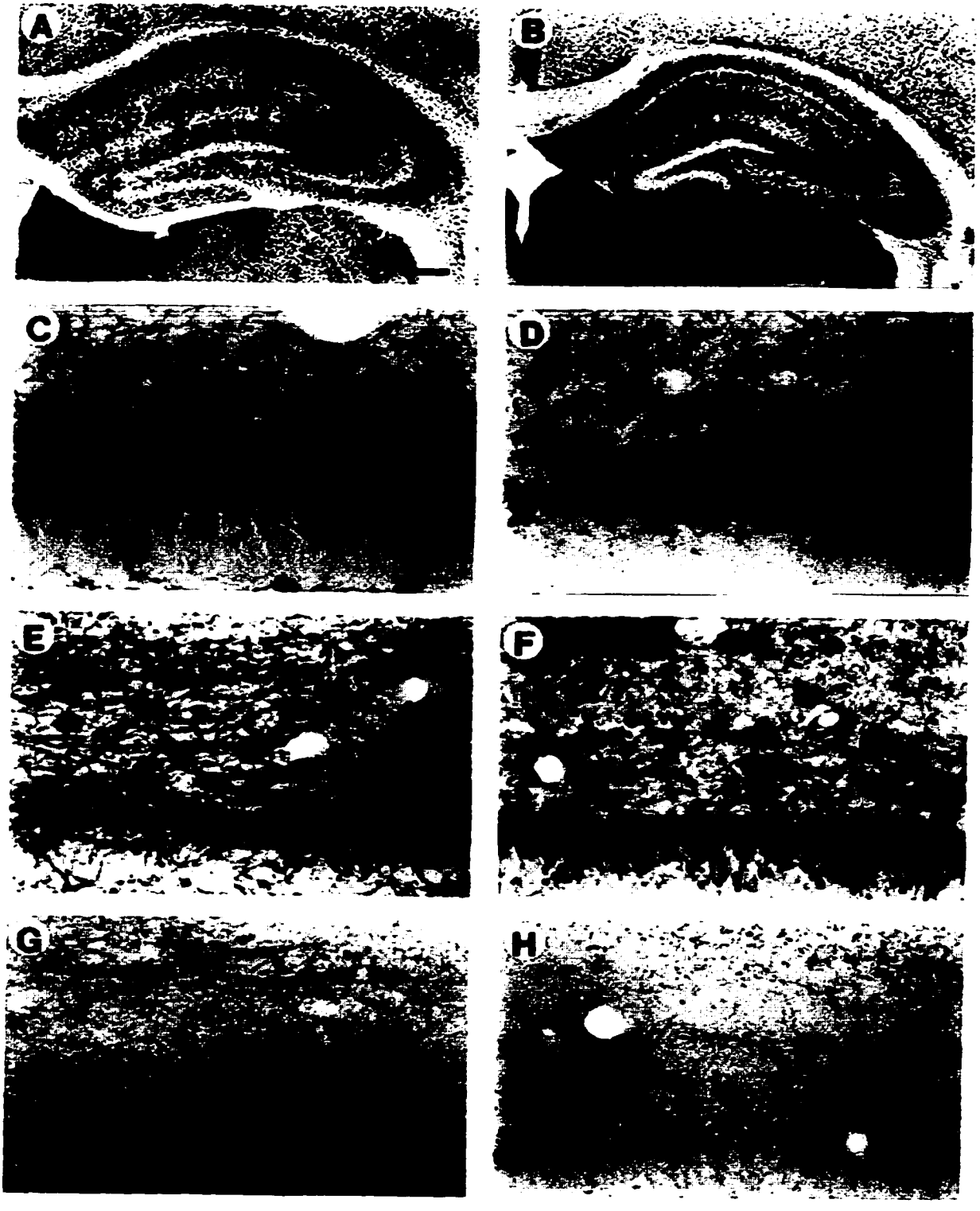
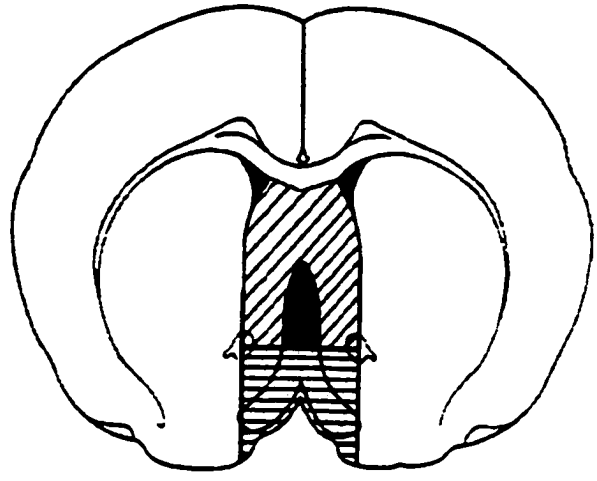
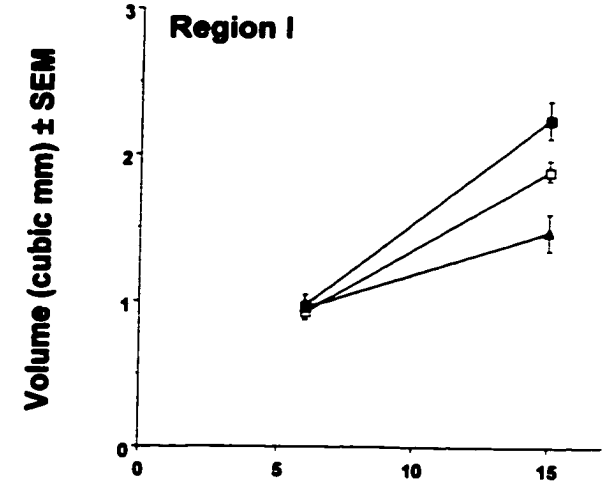


Figure 2.8

Figure 2.9. Basal forebrain development is reduced in BDNF -/- mice after P6. Illustration of the basal forebrain regions for which volumes were determined. Region I (diagonal-hatching) represents the entire septum, Region II (gray shading) is occupied by the cholinergic neurons of the medial septum, Region III (horizontal hatching) represents the diagonal band area, and Region IV (stippled) is occupied by the cholinergic cells of the vertical limb of the diagonal band nucleus. As shown in the graphs, the volume of Regions I and II was the same at P6 in BDNF -/- (triangles) mice as in their littermate controls (+/+, filled squares; +/- open squares) mice, and lower in region I at P15. Note the different ordinate scale for Regions I and II.




Region I
Volume (cubic mm) ± SEM
3
2
1
0
0
5
10
15

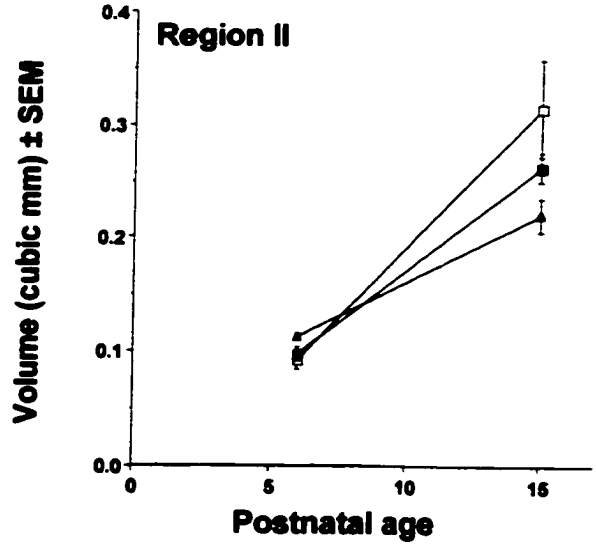

Region II
Volume (cubic mm) ± SEM
0.4
0.3
0.2
0.1
0.0
0
5
10
15
Postnatal age

Figure 2.9

regions increased significantly ($p < 0.05$) in all mice (Fig. 2.9), reflective of the continued postnatal development of this region. At P15, the volume of the septum and diagonal band region of BDNF -/- mice was significantly smaller than that of their littermates ($p < 0.01$). However, at P15, the volume of the cholinergic cell region in the septum (Region II, the medial septum area in which the number of cholinergic neurons was determined) was not significantly different between BDNF -/- mice and their littermates. The volume of the cholinergic vertical limb region (Region IV) was smaller in BDNF -/- mice ($p < 0.02$; not shown).

Body weight positively correlates with cholinergic neuron and fiber numbers at P15 but not at P6

To assess potential influences of processes that regulate body and brain weight on the development of the basal forebrain, correlation analyses for values in individual mice were performed. At P6, the average brain weights of BDNF +/+, +/- and -/- mice did not differ (230 ±14 mg, 204 ± 3 mg, and 289 ± 29 mg), and body weights did not differ (3.25 ± 0.25 g, 3.0 ± 0.26 g, and 3.34 ± 0.14 g). At P6, no correlation was observed between body or brain weight and the number of ChAT-positive neurons, AChE-positive fibers in the dentate gyrus, or basal forebrain volumes (not shown). At P15, brain weights of BDNF +/+, +/- and -/- mice did not differ (606 ± 33 mg, 585 ± 51 mg, and 497 ± 94 mg) but BDNF -/- mice had a lower body weight (~64% and 55%, respectively) than the littermates (+/+, 6.98 ± 014 g; +/-, 5.55 ± 0.52 g; -/-, 2.50 ± 0.75g; $p < 0.05$). Body weight correlated positively with the number of ChAT-positive medial septum neurons (Figure 2.10A; $r = 0.520$; $p < 0.02$), the number of AChE-positive fibers (Fig 2.10B; $r = 0.894$; $p < 0.001$), the number of $p75^{NGFR}$-positive fibers ($r = 0.896$; $p < 0.005$), and the number of vAChT-positive fibers ($r = 0.937$; $p < 0.001$). Body weight also correlated positively with the volume of the septum/vertical limb of the diagonal band (Regions I plus III; Figure 2.10C; $r = 0.723$; $p < 0.001$) and the volume of the cholinergic cell regions (Regions II plus IV; $r = 0.619$; $p < 0.005$; not shown). At P15, brain weight did not correlate with the number of cholinergic neurons in the medial septum (Fig. 2.10D),

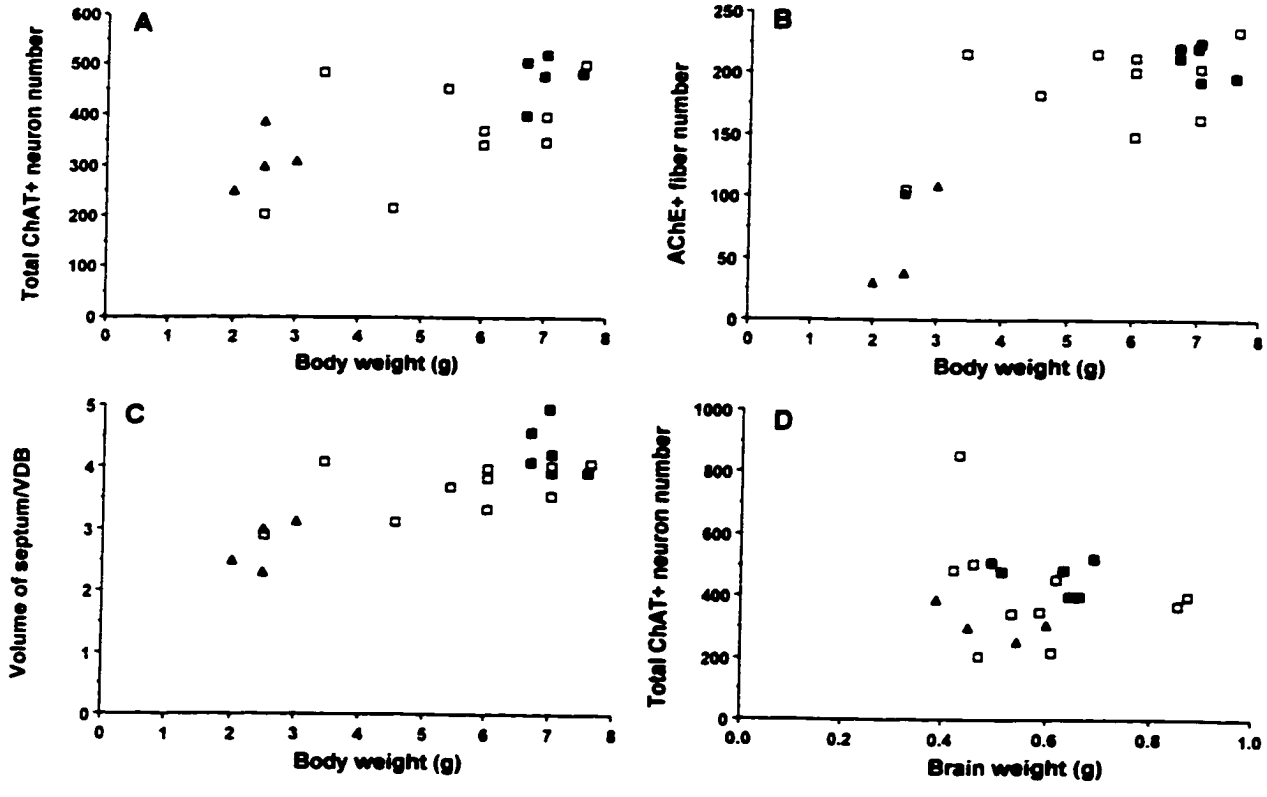


Figure 2.10. Body weight but not brain weight is correlated with basal forebrain measures of cholinergic neurons.

A) Plots of the number of ChAT-positive medial septum neurons in individual mice against the body weight of those mice at P15 reveal a positive correlation ($p < 0.02$). BDNF -/- mice are indicated by triangles, BDNF +/- by open squares and BDNF +/+ by solid squares. Note that low body weight is also accompanied by low numbers of neurons in some BDNF +/- mice. Body weight also correlated with B) the number of AChE-positive fibers in the molecular layer of the dentate gyrus ($p < 0.001$) and C) the volume of the septum plus diagonal band regions ($p < 0.001$). D) Brain weight did not correlate with the number of ChAT-positive medial septum neurons (shown here) or with the other measures (not shown).

the density of cholinergic hippocampal innervation, or the basal forebrain volumes.

NT-3 +/- mice have the same number but smaller cholinergic neurons than control mice

At P6, the number of ChAT-positive neurons in the medial septum of NT-3 +/+ and +/- mice (n = 5 each) were similar to each other and to BDNF +/+ control mice (n = 4) (Table 2.1). The number of ChAT-positive neurons was higher at P15 than at P6 and the same for NT-3 +/+ and +/- littermates (at P15 n = 5 and 9, respectively; Table 2.1). No NT-3-/- pups survived beyond P1 (viability of NT-3 -/- mice = 0 / 24 littermates).

The average cell body diameter of the cholinergic medial septum neurons increased ($p < 0.001$) between P6 and P15 in both NT-3 +/+ and +/- mice (Table 2.1). The cell body diameters of NT-3+/- mice were not significantly different from controls (NT-3+/+ or BDNF +/+) at P6 but were significantly lower at P15 ($p < 0.05$; $p < 0.001$, respectively).

NT-4-/- mice have the same number but smaller cholinergic neurons than control mice

At P15, the total number of ChAT-positive neurons in the medial septum of NT-4-/- mice (n = 5) was not different from that in control mice (BDNF +/+, NT-3+/+; Table 1). The cell body diameter of the cholinergic neurons in NT-4-/- mice was ~11% less ($p < 0.001$) than that of control mice (Table 2.1).

Table 2.1 Number and diameter of cholinergic neurons in NT-3 +/-, NT-4 -/-, and control mice

	Number of cholinergic neurons ± SEM			
	NT-3		NT-4	BDNF
	+/+	+/-	-/-	+/+
P6	288 ± 27	258 ± 27	ND	321 ± 21
P15	477 ± 21	426 ± 24	507 ± 12	467 ± 21
	Cell body diameter ± SEM			
P6	13.4 ± 0.5	14.7 ± 0.4	ND	14.9 ± 0.5
P15	20.3 ± 0.7	18.4 ± 0.4*	18.3 ± 0.3*	21.6 ± 0.5

ND = not determined

* significantly different from controls

DISCUSSION

This study provides evidence that BDNF is needed for the maturation of the septohippocampal cholinergic system in the forebrain. Our detailed quantitative results suggest that BDNF is necessary during postnatal development for i) survival of cholinergic neurons and/or increases in the amount of the cholinergic markers, ChAT and $p75^{NGFR}$; ii) increases in the size of cholinergic medial septum neurons; and iii) expansion and maturation of the cholinergic projections to the hippocampal formation.

Number of ChAT-positive medial septum neurons increases between P6 and P15 in control mice

Previous work had suggested that the number of cholinergic medial septum neurons decreases between P6 and P15 and that this was due to apoptosis of a subpopulation of cholinergic neurons (Van der Zee et al., 1996). Here, we observed an increase in the total number of ChAT-positive neurons between P6 and P15 in control mice, confirming recent findings by others (Fagan et al., 1997). This apparent discrepancy can be explained in part by variances in the methodology of data collection. Here, the entire nucleus was analyzed, whereas previous work presented primarily the midportion of the nucleus where decreases in neuron numbers occur between P6 and P15 (Fig. 2.4). The postnatal increase in the total number of cholinergic neurons was again confirmed in our most recent study of 129/Sv and Balb/c mice, the same strains used by Van der Zee et al. (1996, 1998; Chapter 4, this thesis). The total number of cholinergic medial septum neurons in adults that we observed here corresponds to that reported by others (Chen et al., 1997; Fagan et al., 1998; Yeo et al., 1997) who used unbiased stereological methods. This increase in total number of ChAT-positive neurons most likely reflects an increase in detectability resulting from developmental increases in ChAT expression, in addition to increases in neuron size (>9 μm diameter criterium used), both of which are known to occur between P7 and P25 (Gould et al., 1991; Mobley et al., 1986; Semba, 1992).

BDNF is needed for postnatal development of medial septum and neostriatal cholinergic neurons

BDNF -/- mice had 23% fewer cholinergic medial septum neurons and less intense ChAT-immunostaining at P6 than their littermates (+/+ and +/-). This difference was even greater at P15 because the normal increase in the number of ChAT-positive neurons was not observed in BDNF -/- mice. The lesser expression of ChAT in the BDNF -/- mice may have led to an underestimation of the actual number of cholinergic neurons. At P15, the number of ChAT-positive neurons in all groups was similar to the number of neurons immunoreactive for $p75^{NGFR}$, an additional marker for these cholinergic neurons. However, it is possible that $p75^{NGFR}$ expression was also decreased in BDNF -/- mice as BDNF can increase the number of $p75^{NGFR}$-positive neurons in vitro (Alderson et al., 1990). At P6, the number of TUNEL-positive cells, a marker of DNA fragmentation and apoptotic cell death, was ~3 times greater in BDNF -/- than in BDNF +/+ mice. This is consistent with the possibility that BDNF is necessary for survival of a third of the cholinergic medial septum neurons. It is also conceivable that non-cholinergic neurons are dying during this critical period, and further study will be required to resolve this issue. However, in the adult medial septum, the effects of BDNF appear to be specific for the cholinergic neurons. The size of the non-cholinergic neurons, which include the GABAergic septohippocampal neurons, appeared to be similar in BDNF -/- and BDNF +/+ mice at P15. Moreover, BDNF is selectively transported by the cholinergic neurons (DiStefano et al., 1992; Hagg et al., 1996) and injured GABAergic neurons do not respond to BDNF (Koliatsos et al., 1994). Thus, BDNF appears at least to be required for normal postnatal maturation of these neurons, including increases in ChAT and $p75^{NGFR}$. We were unable to resolve whether more cholinergic neurons would have disappeared after P15 as few BDNF -/- mice live longer.

In the neostriatum, BDNF -/- mice had ~24% fewer cholinergic interneurons than BDNF +/+ control mice at P6 and P15, and less intense ChAT-immunostaining. At P6 and P15 the number of TUNEL-positive cells in BDNF -/- mice was similar as in BDNF +/+ mice. This suggests that if cell death is responsible for the lower number of cholinergic neurons in the neostriatum of BDNF -/- mice, it occurs before P6, consistent

with the earlier development of this nucleus as compared to the medial septum (Gould et al., 1991; Semba, 1992).

Previous reports concluded that the cholinergic basal forebrain neurons were not affected in BDNF -/- or TrkB-/- mice (Alcantara et al., 1997; Conover et al., 1995; Ernfors et al., 1994; Jones et al., 1994). However, those studies lacked the detailed quantitative approach that is needed to reveal the loss of a sub-population of neurons. An important role for BDNF during development was conceivable since BDNF promotes the survival of embryonic and early postnatal cholinergic neurons in vitro (Alderson et al., 1990; Nonomura and Hatanaka, 1992). Moreover, adult levels of full-length transducing TrkB mRNA and protein in the basal forebrain are reached by birth (Fryer et al., 1996; Masana et al., 1993; Yan et al., 1997). Detailed analyses of the developing cerebellum revealed differences, previously not identified, indicating the need for BDNF for normal development and survival of granule cells, growth of Purkinje cell dendrites, and formation of horizontal layers and foliation patterns to occur (Schwartz et al., 1997).

BDNF is needed for size increases of cholinergic neurons

In BDNF +/+ and BDNF +/- control mice, as also reported for rats (Gould et al., 1991), the size of the cholinergic neurons increased to a maximum around P15 and decreased again to adult values. This raises the possibility that the cell size is related to the extent of cholinergic innervation which develops over the first two postnatal weeks, and the subsequent pruning of cholinergic dendrites directly following (Gould et al., 1989; Koh and Loy, 1989). At P6, the size of the cholinergic neurons was the same in BDNF -/- mice as in their littermates, but normal developmental size increase did not occur despite the increase in the extent of their hippocampal innervation. Thus, mechanisms other than those related to process volume apparently are also involved in regulating cell body size. Alternatively, the reduced size of the cholinergic neurons in P15 BDNF -/- mice could also reflect a degenerative process, similar to that seen after axotomy in the adult mammals (Hagg et al., 1989a).

In the neostriatum, the cholinergic neurons of BDNF -/- mice were of similar size at P6 as those in control mice but did not undergo as extensive an increase in size between

P6 and P15 as those of control mice. This suggests that BDNF plays a role in determining cholinergic cell body size also in the neostriatum.

Expansion and maturation of postnatal hippocampal cholinergic innervation is dependent on BDNF

In normal rats, the number of cholinergic fibers that reach the hippocampal formation increases between E20 and P3, and the adult pattern of cholinergic innervation is reached between P14 and P21 (Linke and Frötscher, 1994; Semba 1992). The current and previous (Hohmann and Ebner, 1985) results suggest that this time-line is similar in normal mice.

At P6, the cholinergic innervation patterns and density were the same in BDNF -/- mice as in their littermates, suggesting that BDNF is not necessary for the initial outgrowth of the cholinergic axons. At P15, the number of $p75^{NGFR}$- or vAChT-positive cholinergic fibers in the dentate gyrus of BDNF -/- mice was reduced by ~50-60% compared to their littermates. Because the number of cholinergic neurons was reduced by only ~33%, this suggests that the postnatal expansion of the cholinergic innervation is dependent on BDNF. TrkB also appears to be essential for normal axonal branching, and normal development of synaptic contacts of hippocampal afferents (Martinez et al., 1998). The levels of AChE were disproportionally reduced in BDNF -/- mice compared to the levels of $p75^{NGFR}$ and vAChT. Therefore, BDNF appears to play a role in the maturation of the transmitter-metabolizing pathway (AChE) as it does in the maturation of the transmitter-synthesizing pathway (reduced ChAT in cell bodies of BDNF -/- mice).

Levels of BDNF mRNA in the hippocampal formation are low over the first postnatal week and increase from P7 to peak at adult levels between P19 and P24 (Maisonpierre et al., 1990; Timmusk et al., 1994). Cholinergic transmission reportedly increases BDNF expression in the hippocampal formation (da Penha Berzaghi et al., 1993). Therefore, it is possible that increases in the levels of hippocampal BDNF and development of the cholinergic innervation and phenotypic maturation are interdependent. Such a relationship may also exist at the level of the synapse as BDNF potentiates highly active

synapses and is involved in synaptic plasticity in the developing hippocampus (Gottschalk et al., 1998).

Peripheral deficits may affect development after P6

BDNF -/- mice have severe peripheral sensory and sympathetic deficits, leading to stunted growth and death of the animals by postnatal week 2-3 (Conover et al., 1995; Ernfors et al., 1994; Jones et al., 1994). Such general events, including nutritional deficits, could play a role in brain development and counter our interpretation that BDNF is specifically needed for the development of cholinergic neurons. However, at P6, when the number and immunostaining intensity of ChAT-positive neurons was reduced in BDNF -/- mice, the weights of the body and brain were not significantly different between any of the mice. Thus, the lack of BDNF in the brain and not peripheral events are likely responsible for the cholinergic deficits seen in P6 BDNF -/- mice.

Between P6 and P15, BDNF -/- mice did not gain body weight as much as their littermates. The gain in brain weight was normal in BDNF -/- mice, suggesting that most regions of the brain develop normally. In direct contrast to this normal weight gain, cholinergic deficits in the septohippocampal system were observed in P15 BDNF -/- mice, in addition to volumes of septal and diagonal band regions being reduced. At P15, the individual body weights (but not brain weights) correlated positively with several of these basal forebrain measures, including the number of ChAT-positive neurons. These findings would be consistent with the interesting possibility that nutrients, or other influences from the periphery, have a selective influence on basal forebrain development after P6.

Other neurotrophins and development of cholinergic basal forebrain neurons

Not all cholinergic neurons disappeared in BDNF -/- mice, raising the possibility that other neurotrophic factors also play a role in their development. Like BDNF, NT-4 activates TrkB and can support cholinergic forebrain neurons *in vitro* and *in vivo* (Alderson et al., 1996; Friedman et al., 1993; Nonner et al., 1996). Preliminary results in our laboratory suggest that NT-4 -/- mice have a normal number of ChAT-positive

medial septum neurons, suggesting that NT-4 is not critical for the development of these neurons (Table 2.1). One explanation for the lack of clear effects of NT-4 deficits is that NT-4 is found in much smaller amounts than BDNF (Timmusk et al., 1993), i.e., that BDNF can compensate for NT-4 deficits, while NT-4 cannot completely compensate for BDNF deficits. This is suggested by the fact that double BDNF/NT-4 deficient mice exhibit more severe sensory neuronal losses in the nodose-petrosal complex (Conover et al., 1995). Our finding that the cholinergic basal forebrain neurons are smaller in P15 NT-4 -/- mice than controls suggests that NT-4 plays a role in the development of this system.

Because none of the homozygous NT-3 deficient mice survived past P1, we cannot exclude the possibility that NT-3 plays a role in the death or survival of the cholinergic basal forebrain neurons. Heterozygous NT-3 deficient mice had smaller cholinergic neurons which suggests that NT-3 plays a supportive role during postnatal development of these neurons. This is conceivable since levels of hippocampal NT-3 mRNA and protein are high during the first week of postnatal life and decrease to lower (but not negligible) levels in adults (Maisonpierre et al., 1990).

NGF is clearly important for the development of cholinergic basal forebrain neurons. Heterozygous NGF-deficient adult mice have significantly fewer and smaller medial septum cholinergic neurons (Chen et al., 1997). NGF also reportedly affects cholinergic hippocampal innervation (Chen et al., 1997; Crowley et al. 1994), regulates the size of these cholinergic neurons during development (Li et al., 1995) and is regulated by cholinergic transmission (da Penha Berzaghi et al., 1993).

In summary, our data suggest that BDNF and NT-4 are not neurocidal ligands for $p75^{NGFR}$ in the cholinergic basal forebrain. Rather, our results indicate that BDNF is essential for normal development of these neurons and their innervation of the hippocampal formation. Thus, the development of cholinergic neurons and their projections appears to be dependent on an intricate interdependent regulation mechanism involving at least BDNF and NGF.

CHAPTER 3:

SEK1/MKK4, C-JUN, AND NF_KB ARE DIFFERENTIALLY ACTIVATED IN FOREBRAIN NEURONS DURING DEVELOPMENT AND INJURY IN BOTH CONTROL AND $p75^{NGFR}$-DEFICIENT MICE

The results presented in the following chapter have been submitted for publication to the journal *European Journal of Neuroscience*.

INTRODUCTION

$p75^{NGFR}$ is a member of the tumor necrosis factor receptor (TNFR) superfamily having sequence similarities in the intracellular domain to TNFR I, TNFR II, Fas/Apo-1, and CD40 (Chao and Hempstead, 1995). This intracellular domain can interact with downstream signaling molecules required for mediating apoptosis (Baker and Reddy, 1996). Specifically, this region can lead to formation of the lipid second messenger ceramide, which in turn can activate MEKK, leading to SEK1/MKK4/JNKK phosphorylation, which results in activation of JNK/SAPK, leading to phosphorylation of c-Jun on serine (ser) residues 63 and 73 (Minden and Karin, 1997). The presence of this domain in $p75^{NGFR}$ led to speculation that $p75^{NGFR}$ may also signal via similar intracellular signaling pathways (reviewed in Carter and Lewin, 1997; Dechant and Barde, 1997).

Recent in vitro evidence supports this idea. In various cell types, neurotrophin binding to $p75^{NGFR}$ mediates increased sphingomyelin hydrolysis leading to increased ceramide levels (Dobrowsky et al., 1994, 1995). Similarly, following NGF binding, increases in ceramide levels and JNK activity were observed in $p75^{NGFR}$-expressing oligodendrocytes (Casaccia-Bonnefil et al., 1996). In addition, $p75^{NGFR}$ reportedly can activate the nuclear translocation of NF_KB (Carter et al., 1996). However, little evidence exists regarding $p75^{NGFR}$-mediated signaling events *in vivo*.

The biological consequences of the proposed $p75^{NGFR}$ signaling pathways are not straightforward. Ceramide increases correlate to JNK activation and perhaps cell death, however ceramide can also activate mitogenic downstream signaling cascades in certain cells (Testi, 1996). JNK activation can occur independent of cell death and c-Jun activation can lead to either cell death or several trophic responses (Herdegen et al., 1997; Lenczowski et al., 1997; Virdee et al., 1997). Similarly, the nuclear translocation of NF_KB has been linked to cell survival and cell death (O'Neill and Kaltschmidt, 1997).

Basal forebrain cholinergic neurons offer a model system for studying *in vivo* $p75^{NGFR}$ signaling. In the medial septum of the basal forebrain of developing and adult mammals, including mice, cholinergic neurons express $p75^{NGFR}$ (Li et al. 1995; Sobreviela et al., 1994). Cholinergic neurons of the neostriatum express $p75^{NGFR}$ until

the end of the first postnatal week (Koh and Loy, 1989).

Here, we used immunohistochemistry in an attempt to determine the role of $p75^{NGFR}$ in activating members of the JNK cascade (phosphorylation of SEK1, c-Jun63, and c-Jun 73) and in inducing nuclear translocation of the p50 sub-unit of NF_KB *in vivo*. JNK-activation was not evaluated because available antibodies do not work in immunohistochemistry. We analyzed the forebrain of mice during postnatal development when cholinergic neurons in particular undergo changes including sprouting and maturation (Semba, 1992) and several forebrain regions show signs of apoptosis (TUNEL labeling). We also analyzed the septohippocampal cholinergic neurons of adult mice after a unilateral fimbria fornix transection which induces c-Jun expression (Haas et al., 1996) and degenerative changes (Armstrong et al., 1987; Hagg et al., 1989). We have recently determined that the cholinergic forebrain neurons of $p75^{NGFR}$ -/- mice do not undergo less cell death than two control strains (Ward and Hagg, 1999a, Chapter 4, this thesis) contrary to a previous report (Van der Zee et al., 1996). Nonetheless, the $p75^{NGFR}$ -/- mice provide an opportunity to assess the role of $p75^{NGFR}$ in JNK pathway and NF_KB signaling *in vivo*.

MATERIALS AND METHODS

Animals. All animal procedures were approved by the Animal Care Committee of Dalhousie University and conformed to Canadian Council on Animal Care guidelines. Deep anesthesia was achieved by intraperitoneal injection of 6.5 mg/kg sodium pentobarbital. Breeding pairs of mice homozygous (-/-) for a deletion of the $p75^{NGFR}$ gene (Lee et al., 1992) and their DNA controls (129/Sv, Balb/c) were purchased from Jackson Laboratory (Bar Harbor, ME).

During development, uninjured mice were analyzed at postnatal (P) day 6, 8, 10, and 15: n = 6, 6, 4, 6 for 129/Sv mice; n = 5, 6, 5, 5 for Balb/c mice; and n = 6, 6, 5, 6 for $p75^{NGFR}$ -/- mice, respectively. Each group contained mice from at least two different litters.

Fimbria-fornix transection. Adult (at least 12 weeks old) male control and $p75^{NGFR}$ -/- mice were anesthetized and placed in a Kopf stereotaxic apparatus and received a

unilateral aspirative transection of the right fimbria fornix (Armstrong et al., 1987; Hagg et al., 1988). This procedure interrupts the projections from the cholinergic and GABAergic neurons in the ipsilateral medial septum and vertical limb of the diagonal band nucleus to the ipsilateral hippocampal formation. The mice were analyzed 1, 3, 7 or 14 days after the lesion, with n = 5, 3, 2, 3, for 129/Sv mice; n = 5, 3, 2, 3, for Balb/c mice and n = 3, 3, 3, 3, for $p75^{NGFR}$ -/- mice, respectively.

Immunohistological procedures. At the specified postnatal or post-lesion times, mice were anesthetized, weighed, and then perfused transcardially with cold (4°C; 2 - 15 ml for P6 - adult) phosphate buffered saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (4°C; 4 - 30 ml for P6 – adult). The brains were post-fixed for 24 hours in 4% paraformaldehyde and cryoprotected in a 30% sucrose / phosphate buffer solution for 24 hours. The brains were then weighed and 30 μm thick coronal sections through the septum were cut with a freezing microtome. To visualize activity of the JNK signaling cascade in developing and lesioned forebrain neurons, standardized sections in the midportion along the rostro-caudal axis of the septum (see "Quantitative analyses") were processed for immunocytochemistry using affinity-purified polyclonal rabbit antibodies against phosphorylated-SEK1 (SEK1p, 1:1,000 dilution; cat #9151; New England Biolabs; Beverly, MA), serine 63-phosphorylated c-Jun (c-Jun63p) (1:3,000 dilution; cat #9261), serine 73-phosphorylated c-Jun (c-Jun73p) (1:10,000 dilution; cat #9164), or phosphorylation state-independent c-Jun (1:3000 dilution; cat #9162). In short, free floating tissue sections were incubated sequentially with 3% goat serum in Tris buffered saline (TBS) containing 0.3% Triton X-100 for thirty minutes, primary antibody at the indicated dilution in 1% goat serum TBS-Triton for 16 hours at 4°C, biotinylated goat anti-rabbit IgG (1:300; Vector laboratories; Burlingame, CA) in TBS for ninety minutes, and avidin-biotin-peroxidase complex (1:600; ABC Elite kit, Vector Laboratories) in TBS for one hour. Immunoreactive products were revealed with a diaminobenzidine (DAB) reaction that was intensified with 0.67% ammonium nickel sulfate. In between steps, the sections were washed 3 x 10 minutes in TBS. In the lesioned mouse brains, an additional series of sections was double-labeled for co-localization of choline acetyltransferase (ChAT) and either c-Jun63p or c-Jun73p. To

reveal the c-Juns, the same protocol as first described was used with biotinylated donkey anti-rabbit IgG (1:500; Chemicon; Temecula, CA) as the secondary antibody. Immediately following the DAB-nickel (black) reaction, the sections were processed using an affinity-purified polyclonal goat antibody against ChAT (Ab144P, Chemicon, Temecula, CA) using the same protocol adjusted for goat primary antibodies and DAB reaction product without Nickel (brown). To visualize nuclear translocation of NF_KB in developing and lesioned mice, sections adjacent to those used for visualization of the JNK cascade members, were double-labeled for co-localization of ChAT and the p50 subunit of NF_KB (Ab392; Dr. W. Greene; Gladstone Institute, UCSF, CA; Doerre et al., 1993; Walker et al., 1992), using a similar protocol as described above. Other sections were double-labeled for NF_KB-p50 and ChAT, or SEK1p and ChAT, using immunofluorescence secondary antibodies conjugated to Cy2 (NF_KB-p50; SEK1p) and Cy3 (ChAT) at 1:500 using a protocol adjusted appropriately for immunofluorescence. DAB-stained sections were mounted on gelatin-coated glass slides, dehydrated, and coverslipped in Permount. Immunofluorescence-stained sections were mounted on gelatin-coated slides and were not coverslipped.

Western Blotting. To ensure specificity of the SEK1p and c-Junp antibodies, we performed Western analyses of forebrain tissue from P15 mice and from adult mice that had received a fimbria-fornix transection 7 days earlier. Anesthetized mice were perfused transcardially with cold (4°C; 2 - 15 ml) phosphate buffered saline in order to clear blood from the brain. On ice the forebrain, from the olfactory bulbs to the most rostral portion of the fimbria fornix, was dissected and frozen fresh on dry ice. Frozen tissue was homogenized in 1 ml of 0.32 M sucrose in 100 mM phosphate buffer, and 25 mg of sample protein was placed in sample buffer containing 5% β-mercaptoethanol and 10% sodium dodecyl sulfate (SDS). Proteins were then electrophoretically separated in a 10% SDS-polyacrylamide gel and transferred to Immobilon PVDF membranes (Millipore, Toronto, Canada) using a previously described method (Towbin et al., 1979). Membranes were blocked for two hours in blotto (0.1% Tween 20 with 5% skim milk powder in TBS) followed by three washes in TBS-Tween 20, and an overnight incubation at 4°C in the appropriate primary antibody (SEK1p, c-Jun73p, c-Jun63p) or SEK1

phosphorylation-state independent antibody (#06-621-MN; Upstate Biotechnology; New York) at 1:1000 in TBS-Tween 20 with 5% bovine serum albumin (Fraction V, cat #A-8022; Sigma Chemicals, St. Louis, MO). On day 2, membranes were washed 3 x 15 minutes in TBS-Tween 20 followed by a one hour incubation in blotto containing a peroxidase-conjugated goat antibody raised against rabbit IgG (1:5000; Vector Laboratories Inc. Burlingame, CA). After 3 x 15 minute washes in PBS-Tween 20, the antigen-antibody complexes were visualized with the ECL detection system (Amersham; Oakville, Canada).

TUNEL Labeling. A separate group of P8 Balb/c and $p75^{NGFR}$ -/- mice were sacrificed and had their tissue fixed as described in the immunohistological procedures section. The brains were cryoprotected using a 15% sucrose / phosphate buffer solution over 24 hours and 10 μm thick coronal brain sections were sectioned using a cryostat and mounted on 2% APTEX (3-aminopropyltriethoxy-silane; cat #A-3648; Sigma Chemicals, St. Louis, MO) coated slides. Six equidistant sections throughout the septum (see "Quantitative analyses") of each mouse were processed for TUNEL (to detect DNA fragmentation characteristic of apoptosis) using the Intergen ApopTag *In situ* Apoptosis Detection kit - peroxidase (Cat #S7100-Kit Ed.1.5; Intergen, Purchase, NY). For a positive control, separate tissue was incubated with serially diluted RQ1 RNAse free DNAse I (activity = 1 unit / μl; Promega, Madison, WI) in DNAse buffer (1 M Tris HCl with 6 mM $MgCl_2$) for one hour at 37°C before the first step of the TUNEL protocol. All experimental tissue was incubated at the same time under the same conditions with buffer, and all tissue was thoroughly washed in 2xSSC before beginning the TUNEL kit protocol. As a negative control, separate tissue underwent the complete protocol, but was incubated with a solution containing distilled water instead of TdT enzyme. Following staining, sections were dehydrated and coverslipped in Permount.

Quantitative analyses. In each developing mouse brain, two sections per immunostain, spanning 360 μm (~36% of the septum) in the midportion along the rostro-caudal axis of the medial septum were used to determine the number of SEK1p, c-Jun63p, c-Jun73p, and double-labeled NF_KB-p50 and ChAT immunoreactive neurons. In each lesioned adult mouse brain, three sections spanning 360 μm were used to determine

the number of SEK1p-positive and c-Jun73p-positive cells, and neurons double-labeled for ChAT and c-Jun63p, c-Jun73p, or nuclear NF_KB-p50, respectively, on the lesioned and non-lesioned (internal control) side. To ensure consistency between animals, section number 0 (zero) was identified as the most rostral section through the anterior commissure decussation. Cell and nuclear profiles were counted with a 400x magnification. For cells with immunoreactive cytoplasm, only neuronal cell body profiles larger than 9 μm in longest diameter were counted. Positively stained cells were counted in the left hemisphere in all sections for the following additional forebrain regions: the lateral septum, the vertical limb of the diagonal band of the nucleus of Broca (VDB), the horizontal limb of the diagonal band of the nucleus of Broca (HDB), the neostriatum (caudate-putamen), the nucleus accumbens, and a 30 μm wide region surrounding the vertical portion of the lateral ventricles (medial, lateral). Individual TUNEL-positive profiles were counted under Nomarski filter conditions at 400x magnification, as no counterstain was used and background staining was very low.

All data collection was blind to the animal strain and age. All data was analyzed using a two-tailed Student's t-test.

RESULTS

Western analyses confirm specificity of antibodies

Since the SEK1p, c-Jun63p, and c-Jun73p antibodies had not been tested *in vivo*, we tested these antibodies using forebrain tissue from P15 mice and adult mice with a 7-day old fimbria fornix transection in Western blot analyses. In tissue from lesioned mice, each of the c-Junp antibodies (ser 63 and 73) revealed bands corresponding to the expected ~46 kDa migratory position of c-Jun (Figs. 3.1A and 3.1B; Kyriakis et al., 1994). In tissue from P15 mice the SEK1p antibody revealed a major band corresponding to the expected molecular weight (~46 kDa) of SEK1 (Fig. 3.1C; Sanchez et al., 1994). Forebrain tissue taken from non-lesioned adult control mice did not show corresponding bands for any of the phospho-specific antibodies and was used as a negative control. The antibody against NF_KB-p50 (AB392) was made against the N-terminal peptide of KBF-1, the p50 homodimer, and has been characterized previously (Clemens et al., 1997; Doerre

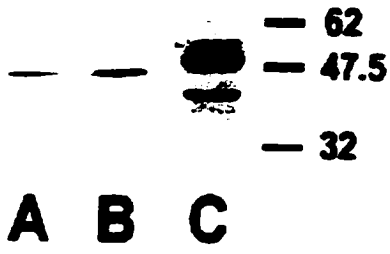


Figure 3. 1. SEK1p, c-Jun63p, and c-Jun73p antibodies are specific.
Western immunoblot of total protein extracted from the forebrain region of mice 7 days following fimbria fornix transection demonstrated a single band at ~46 kDa immuno-reactive for c-Jun63p (A) and for c-Jun73p (B). In forebrain tissue from P15 mice the major SEK1p immuno-reactive band was observed at ~46 kDa (C).

et al., 1993; Walker et al., 1992).

Development

SEK1 is activated during development but does not predict activation of c-Jun on serine residues 63 or 73

In control mice (Balb/c and 129/Sv) robust SEK1p immunoreactivity in neurons of the medial septum was observed at every developmental age analyzed up to P15 (Fig. 3.2B and 3.3A) but essentially no positive cell bodies were detectable in adulthood. During development, the maximum mean number of SEK1p - positive neurons in the medial septum was similar to that of the cholinergic neurons in one strain of control mice (Balb/c; Fig. 3.3). In the other control strain (129/Sv), the maximum number of SEK1p – positive neurons in the medial septum was higher at P8 than Balb/c and $p75^{NGFR}$ -/- mice. Double-labeled sections revealed that some of the neurons contained SEK1p and ChAT, but some ChAT-positive neurons did not have SEK1p, and some SEK1p-positive neurons were not ChAT-positive (not shown). In the lateral neostriatum SEK1p immunoreactivity in neurons was observed at every developmental age analyzed increasing from P6 and reaching a maximum at P15 (Fig. 3.3D). Double-labeled sections from P15 mice, revealed that essentially every ChAT-positive striatal neuron was also SEK1p immunoreactive (Fig. 3.4). No positive cell bodies stained for SEK1p were detected in the adult neostriatum. SEK1p immunostaining of neurons appeared throughout the developing forebrain and was also observed in neurons of the cortex (Fig. 3.5A and 3.5B), the VDB, and the HDB (refer to Fig. 3.12). SEK1p-immunostaining of fiber processes was seen in the developmental tissue in the cortex, the striatum, and the septum. In adults, the fiber staining was less distinct, with a more homogenous appearance of the neuropil. In the cortex of both developmental and adult mice, fiber staining was localized to the apical dendrites of essentially all layers of neurons with fibers coursing perpendicular to the brain surface (Fig. 3.5). Thin fibers were also observed running parallel to the brain surface in the first cortical layer. No SEK1p or c-Junp was observed, at any age, in white matter. Western analyses on adult control forebrain tissue demonstrated the presence of a SEK1 positive band (phosphorylation-

Figure 3. 2. Medial septum neurons have SEK1p but little c-Junp during development. Compared to the number of ChAT-positive neurons in the developing (P8) mouse medial septum (A) similar numbers of neurons have immunostaining for SEK1p (B) but few neurons contain c-Jun63p (not shown) and c-Jun73p (C). The medial septum of such mice contains many neurons with c-Jun (phosphorylation state-independent; D). Magnification bar = 50 μm. Arrowhead indicates mid-line of the septum. Insert in C is a 5-fold magnification of C.

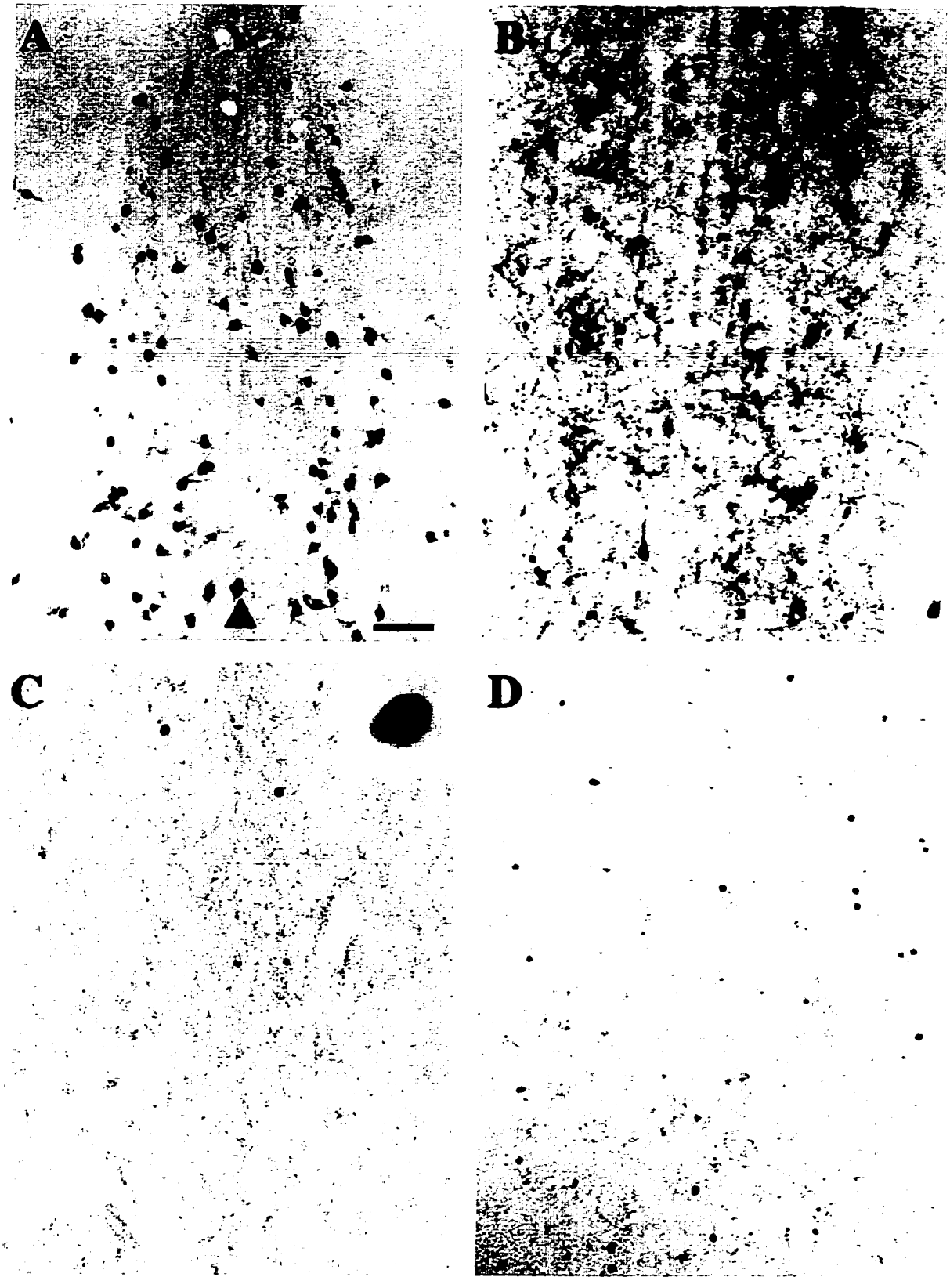


Figure 3.2

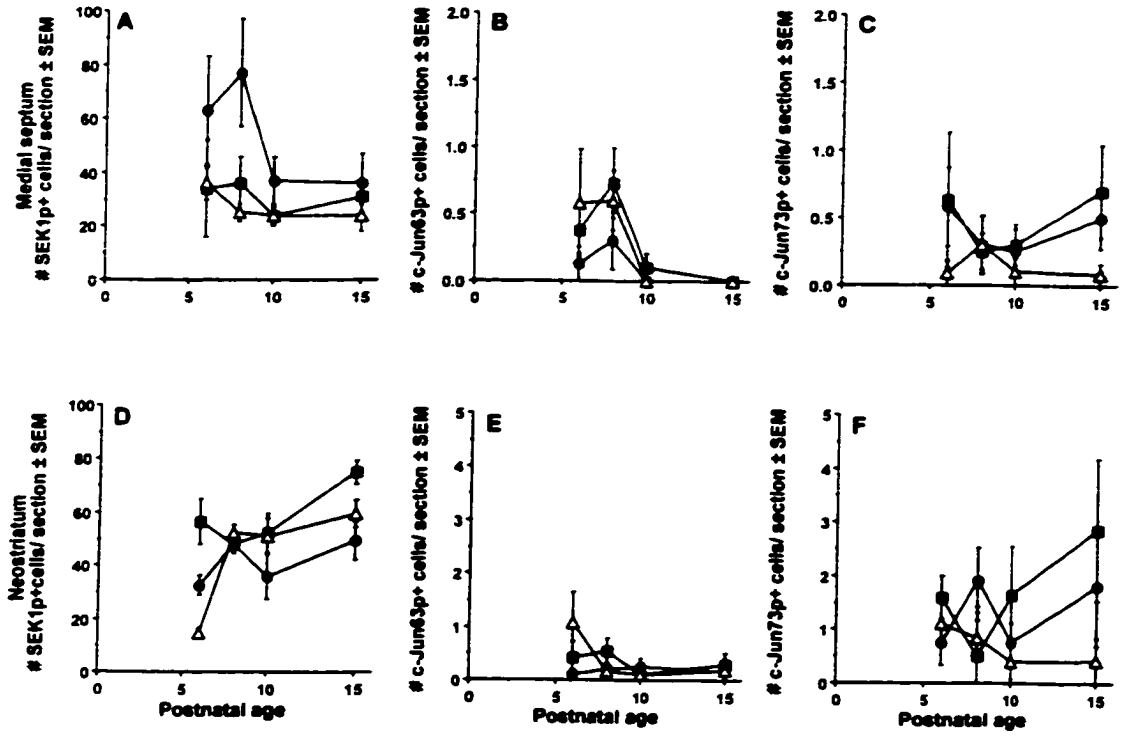


Figure 3. 3. In the forebrain during postnatal development the activation of SEK1 and c-Jun differs and overall is unaffected by $p75^{NGFR}$.

SEK1p in the medial septum of control mice (circles = 129/Sv; squares = Balb/c) was observed at every developmental age analyzed (A) and did not predict levels of c-Jun63p (B) or c-Jun73p (C). Note the different ordinate scales. Overall $p75^{NGFR}$ -/- mice (triangles) did not differ from control mice. In the lateral striatum of control mice, the number of SEK1p-positive neurons increased between P6 and P15 (D), and only a few cells immuno-positive for c-Jun63p (E) and c-Jun73p (F) were observed. Only at P6 were differences observed for SEK1p between the $p75^{NGFR}$ -/- mice and both controls in the lateral striatum. At adulthood SEK1p and c-Junp values were zero.

Figure 3. 4. SEK1p and ChAT are co-localized in the developing neostriatum. SEK1p immunoreactivity is observed at P15 in the developing neostriatum (A). Double labeling for ChAT (B) and SEK1p (C) reveal that almost all ChAT-immunopositive neurons are also SEK1p-immunopositive. Arrow indicates a neuron that is only labeled for SEK1p. Magnification bar in A is 100 μm and 25 μm in B and C. CTX = cortex, CC = corpus callosum

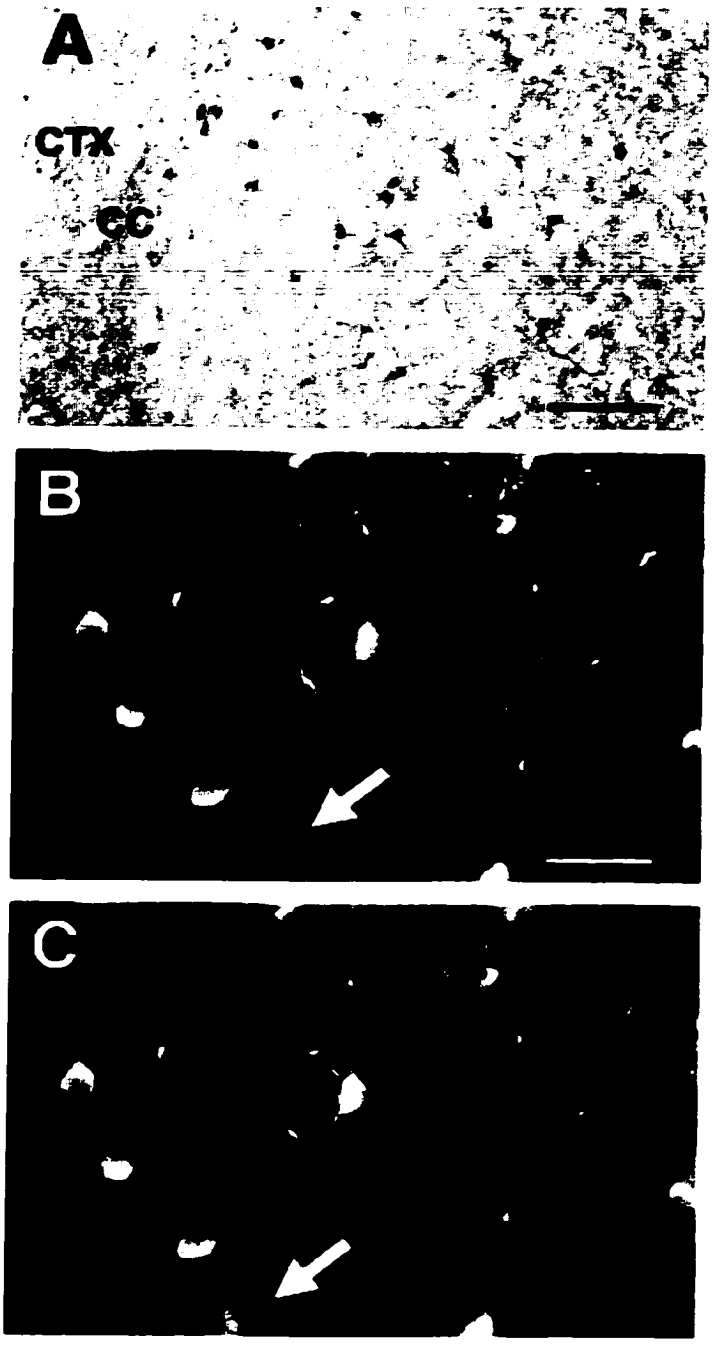


Figure 3.4

Figure 3. 5. SEK1p-positive fibers are seen during developmental through adulthood, while SEK1p-positive cells are found only during development.

In the parietal cortex of developing (P15, A and C) and adult (B and D) mice, SEK1p fibers are observed coursing perpendicular to the brain surface. SEK1p-positive cell bodies are only observed in the developmental tissue, where fiber staining appears to be more intense. Magnification bar in A is 50 μm for A and B and 25 μm for C and D.

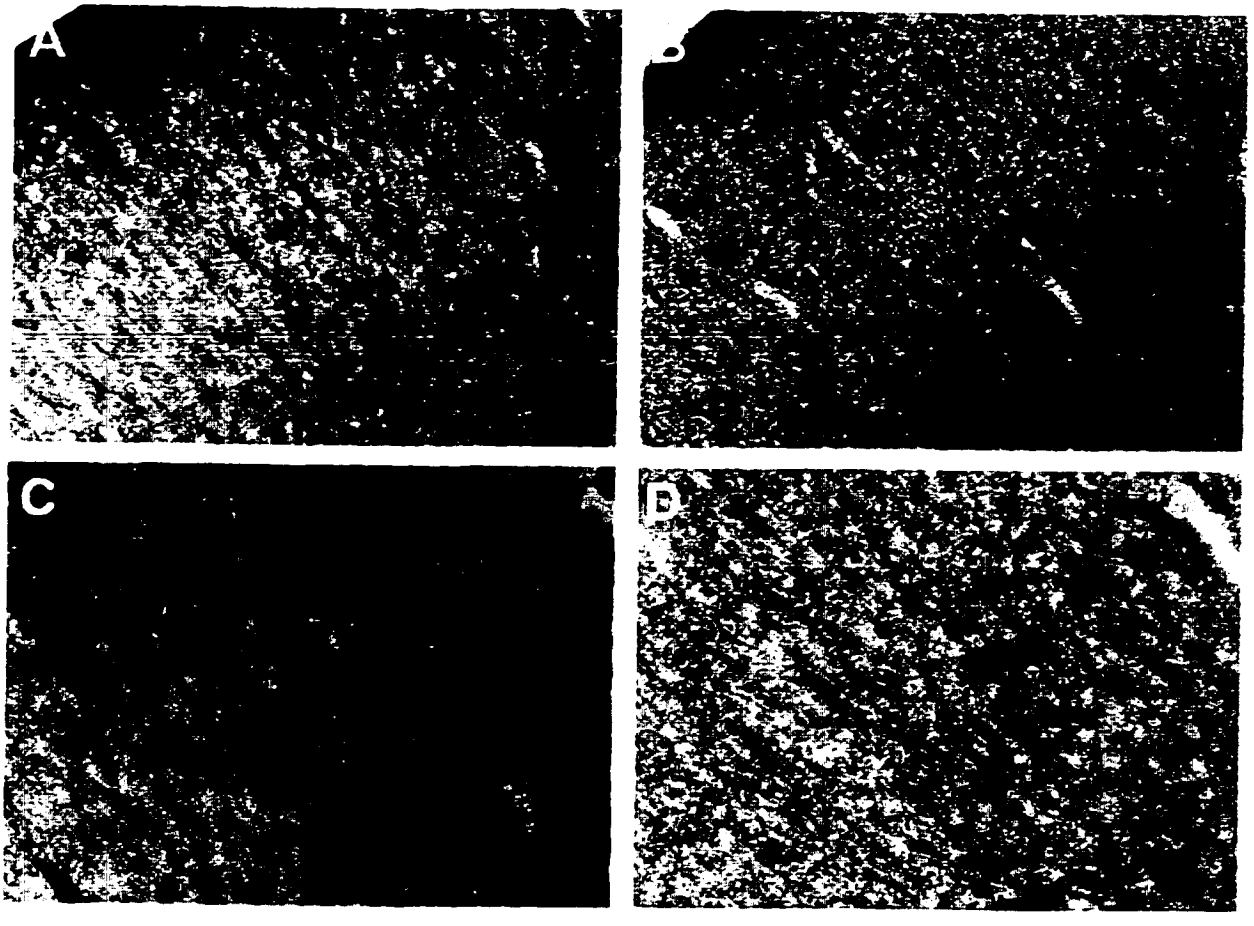


Figure 3.5

state independent; not shown), but the absence of a clear SEK1p band (not shown).

At all ages that were evaluated, only 1-3 medial septum neurons per section had nuclei that were positive for c-Jun63p or c-Jun73p (Fig. 3.2C, Figs. 3.3B and 3.3C). Similarly, in the lateral neostriatum, only a few c-Jun63p or c-Jun73p positive cells could be detected (Figs. 3.3E and 3.3F). Very few, if any, c-Junp immunostained nuclei were detected in the entire forebrain. Correlation analyses for each age between SEK1p, c-Jun63p, and c-Jun73p failed to reveal any significant interactions in the medial septum, lateral neostriatum, or any of the other forebrain regions that were analyzed. During these postnatal times, the medial septum (Fig. 3.2D), lateral striatum, and forebrain cortices contained many neurons with nuclei immunostained for c-Jun (phosphorylation-state independent).

$p75^{NGFR}$ -/- mice do not differ from control mice in their ability to activate SEK1 or c-Jun during postnatal development

Overall, during postnatal development no differences in numbers of neurons immunostained for SEK1p, c-Jun63p or c-Jun73p were observed between the two control strains and $p75^{NGFR}$ -/- mice in the medial septum or the lateral striatum (Fig. 3.3A-F). The distribution and neuronal morphology was similar for both control and $p75^{NGFR}$ -/- mice. Phosphorylation of SEK1 and c-Jun was the same in $p75^{NGFR}$ -/- as in control mice in all regions of developing postnatal forebrain that we analyzed (data not shown). By adulthood, immunostaining of cell bodies for SEK1p and c-Junp was negligible in both control and $p75^{NGFR}$ -/- mice.

NF_KB is localized to nuclei of cholinergic neurons during development in both control and $p75^{NGFR}$ -/- mice

NF_KB-p50 immunoreactivity was observed in the nucleus of most neurons in the forebrain at every developmental age analyzed (Fig. 3.6A), and remained high into adulthood. To assess the activation (or nuclear translocation) of NF_KB in cholinergic neurons of the medial septum and lateral neostriatum during development, we looked at co-immunolocalization of the p50 subunit of NF_KB in the nucleus of ChAT-positive

Figure 3. 6. NF_KB is localized to most neurons of the medial septum.
NF_KB-p50 immunostaining is seen in almost every cell of the developing medial septum (A) and is localized in the nucleus of ChAT-positive stained neurons (arrows in B). Magnification bar = 50 μm in A and 10 μm in B. Arrowheads indicate mid-line of the medial septum. Arrow is A and B indicate the same neuron.

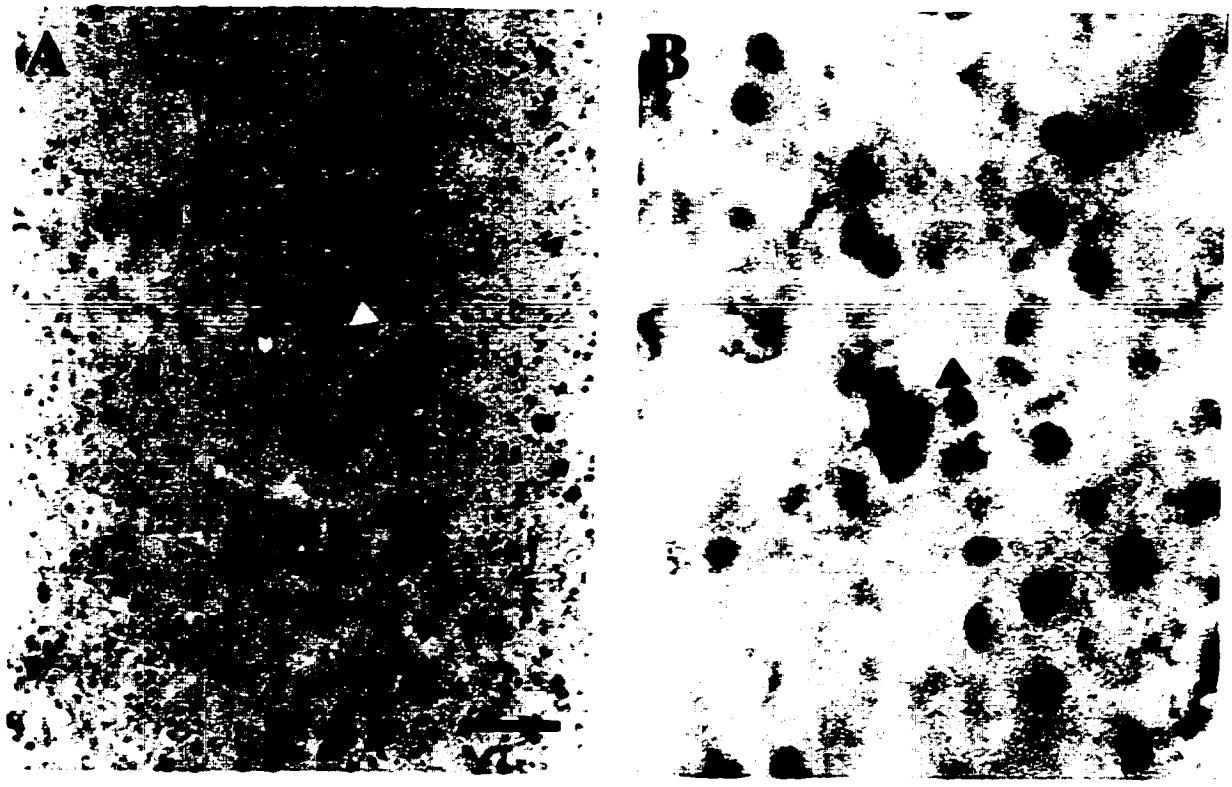


Figure 3.6

stained cells in two strains (Balb/c and 129/Sv) of control mice (Fig. 3.6B). The number of cholinergic neurons with nuclear NF_KB-p50 did not significantly differ between Balb/c and 129/Sv mice and these controls were therefore grouped for all further analyses. In the developing medial septum and neostriatum of control mice the number of NF_KB-p50-positive nuclei in ChAT-positive neurons increased with age peaking at P15 (Figs. 3.7A and 3.7D). During this same time period, the number of detectable ChAT-positive neurons also increased in both regions (Figs. 3.7B and 3.7E). Therefore, during development the average percentage of ChAT-positive neurons in the medial septum and lateral neostriatum with nuclear NF_KB-p50 immunostaining ranged between ~70-85% (Figs. 3.7C and 3.7F).

Between P6 and P10, $p75^{NGFR}$ -/- mice had more NF_KB-p50 positive nuclei in ChAT-positive neurons (Fig. 3.7A). However, since the number of detectable ChAT-positive medial septum neurons was greater in $p75^{NGFR}$ -/- mice at these times (Fig. 3.7B), the percentage of ChAT-positive neurons with NF_KB-p50 positive nuclei was similar in control and $p75^{NGFR}$ -/- mice (Fig. 3.7C). In the lateral neostriatum, the number of detectable ChAT-positive neurons with NF_KB-p50 positive nuclei increased during development in concert with the number of ChAT-positive neurons in both $p75^{NGFR}$ -/- and control mice (Figs. 3.7D and 3.7E), thus the percent of cholinergic neurons expressing NF_KB-p50 remained equal (Fig. 3.7F).

Following axotomy in the adult

Immunostaining in the medial septum for SEK1p remains low but staining for c-Junp increases after fimbria fornix transection

To assess the activation of the JNK signaling cascade following injury, SEK1p and c-Junp immunostaining was analyzed in adult mice 1, 3, 7, or 14 days following a unilateral fimbria fornix transection. Within the medial septum, the staining pattern of SEK1p did not obviously change on either the lesioned or nonlesioned side (Fig. 3.8B). On the lesioned side, at most ~1-2 cells per section stained positive throughout the entire medial septal region analyzed. No SEK1p immunoreactivity was observed in cell bodies at the site of the lesion; i.e. in the fimbria fornix. Light SEK1p immunoreactivity in fibers was

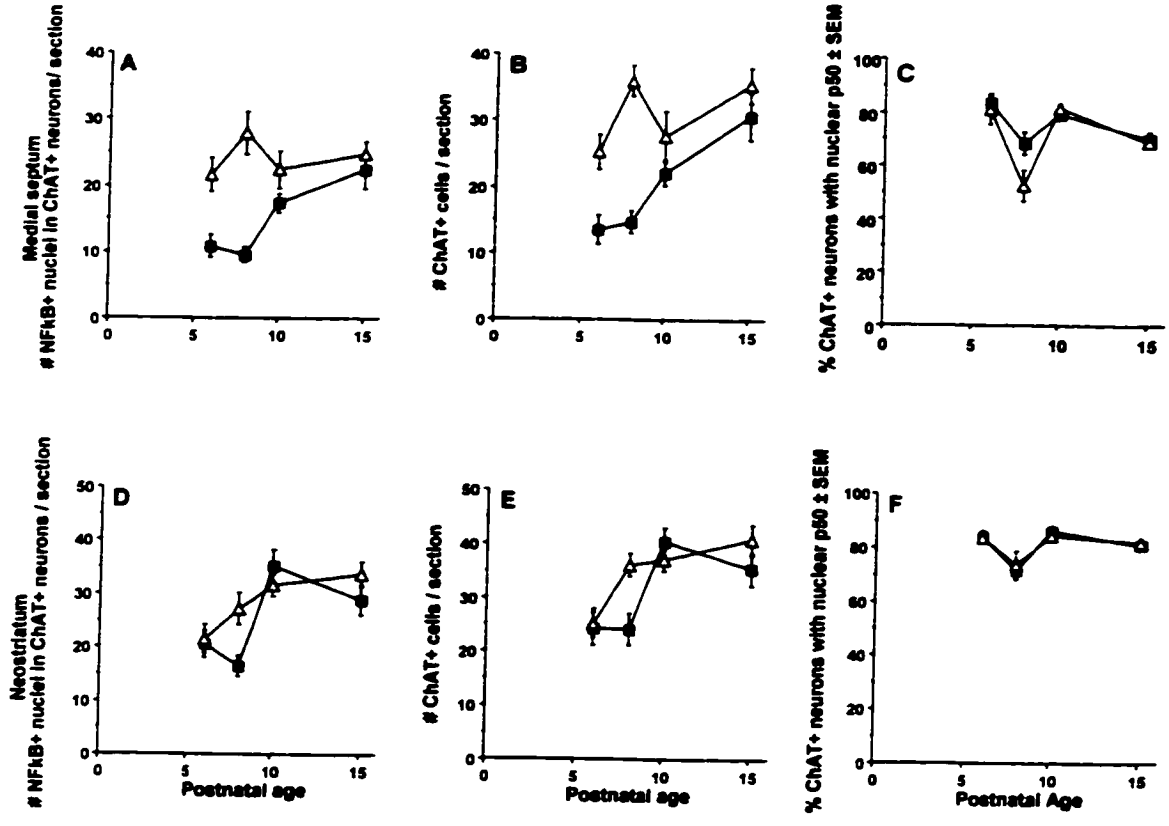


Figure 3. 7. Nuclear translocation of NF_KB-p50 is unaffected by $p75^{NGFR}$ in developing cholinergic forebrain neurons.

A) The number of cholinergic neurons with nuclear NF_KB-p50 immunostaining increases during postnatal development in control (squares), but not in $p75^{NGFR}$ -/- mice (triangles). B) In concert, the number of ChAT-stained neurons during development demonstrates that $p75^{NGFR}$ -/- mice have significantly more ChAT-positive neurons at P6, P8, and P10 than control mice, but by P15 no differences are found. C) When expressed as a percentage of ChAT-positive neurons with nuclear NF_KB-p50, no major differences between $p75^{NGFR}$ -/- and control mice are observed. In the lateral striatum, the number of ChAT-positive neurons with nuclear NF_KB-p50 immunostaining (D), number of ChAT-positive neurons (E), and the percentage of cholinergic neurons with NF_KB-p50 (F) were similar between $p75^{NGFR}$ - /- and control mice at all ages.

Figure 3. 8. Medial septum cells contain c-Junp immunostaining but essentially no SEK1p 7 days following fimbria fornix transection.

On the side ipsilateral (left) to the fimbria fornix transection, fewer neurons contain ChAT immunostaining (A). Very few cells (arrow) stained for SEK1p on the lesioned side, although fiber staining for SEK1p was observed on both the lesioned and nonlesioned sides (B). In comparison to ChAT (A), many more neurons contained c-Jun63p (not shown) and c-Jun73p (C). The presence of $NF_{K}B$ in large cells appears to decrease after the lesion (D). Magnification bar = 50 μm. Arrowhead indicates mid-line of medial septum.

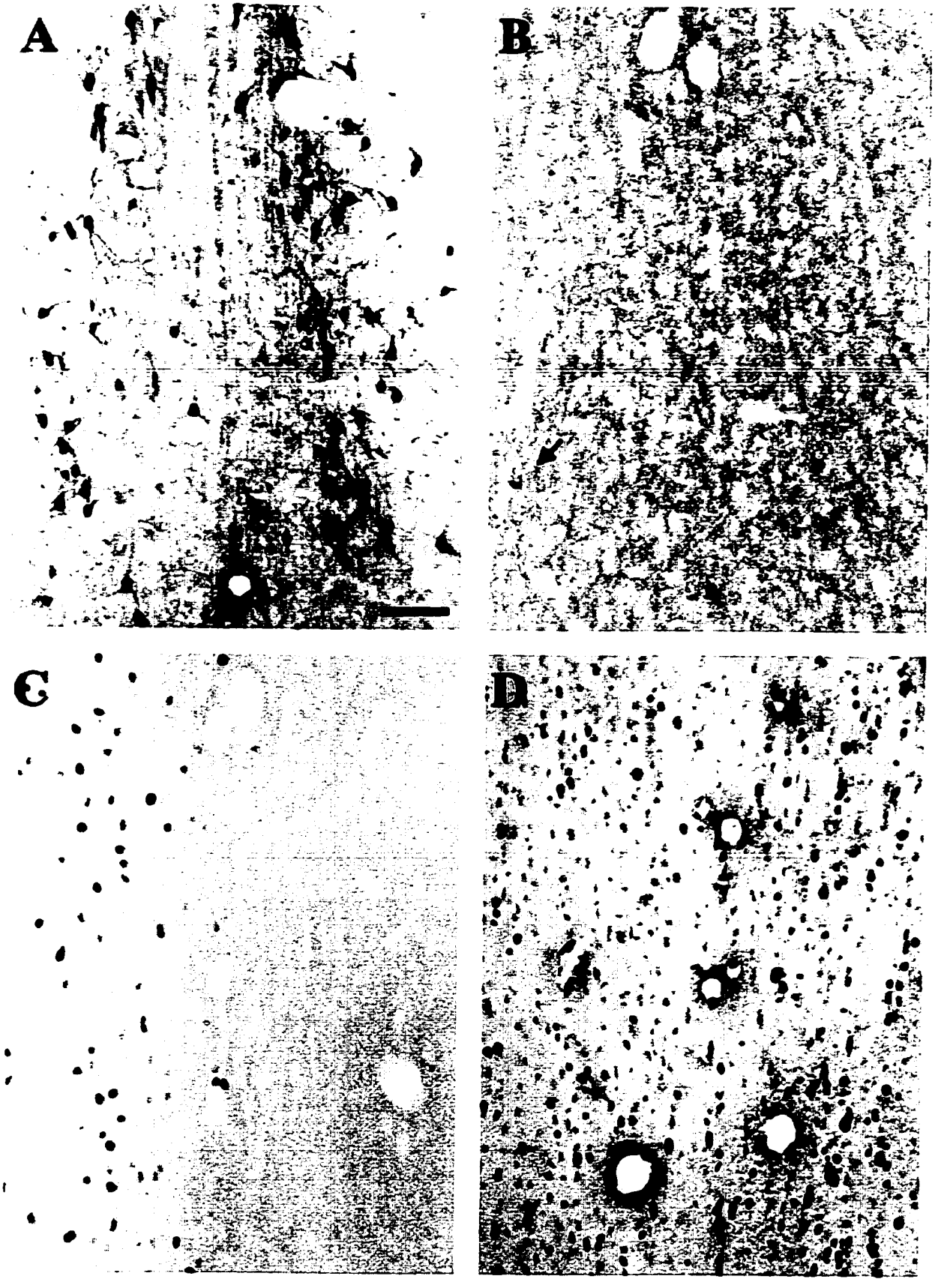


Figure 3.8

the same on the lesioned and nonlesioned side of the medial septum (Fig. 3.8B).

Robust immunostaining for c-Jun63p and c-Jun73p (Fig. 3.8C) was observed in numerous nuclei of neurons in the ipsilateral medial septum after fimbria fornix transection. Overall, the number of cells (per section) immunoreactive for c-Jun63p did not significantly differ from the number of cells immunoreactive for c-Jun73p, but was consistently lower at every time point measured (Figs. 3.9A and 3.9B). The number of nuclei with immunostaining for c-Jun63p and c-Jun73p increased with time after the lesion, starting as early as 1 day post-axotomy and reaching a maximum of 100 and 135 positive nuclei, respectively, by day 7 (Figs. 3.9A and 3.9B). By day 14 following the lesion, overall c-Junp levels were lower again and the number of c-Jun63p-positive nuclei was close to normal, negligible levels while the number of c-Jun73p-positive nuclei was approximately 40% of maximum. On the non-lesioned side, essentially no c-Junp positive nuclei were observed. Correlation analyses between the number of SEK1p neurons and c-Jun63p- or c-Jun73p-positive nuclei per mouse failed to reveal any significant interactions.

SEK1 and c-Jun are similarly activated after axotomy in p75^{NGFR} -/- and control mice

Following fimbria fornix transection, the number of neurons with immunostaining for SEK1p (0-1; not shown), c-Jun63p, and c-Jun73p (Fig. 3.9) was largely similar in control and p75^{NGFR} -/- mice on the lesioned side of the medial septum.

The total number of c-Junp-positive medial septum neurons was greater than the number of ChAT-positive neurons. Since this could obscure a potential difference between p75^{NGFR} -/- and control mice, we assessed the co-localization of c-Junp and ChAT in lesioned neurons. At 7 days after axotomy, p75^{NGFR} -/- mice had as many ChAT-positive neurons with c-Jun immunostaining as control mice (Fig. 3.10). In both strains the number of c-Jun73p containing ChAT-positive neurons was greater than the number with c-Jun63p ($p < 0.001$; Fig. 3.10).

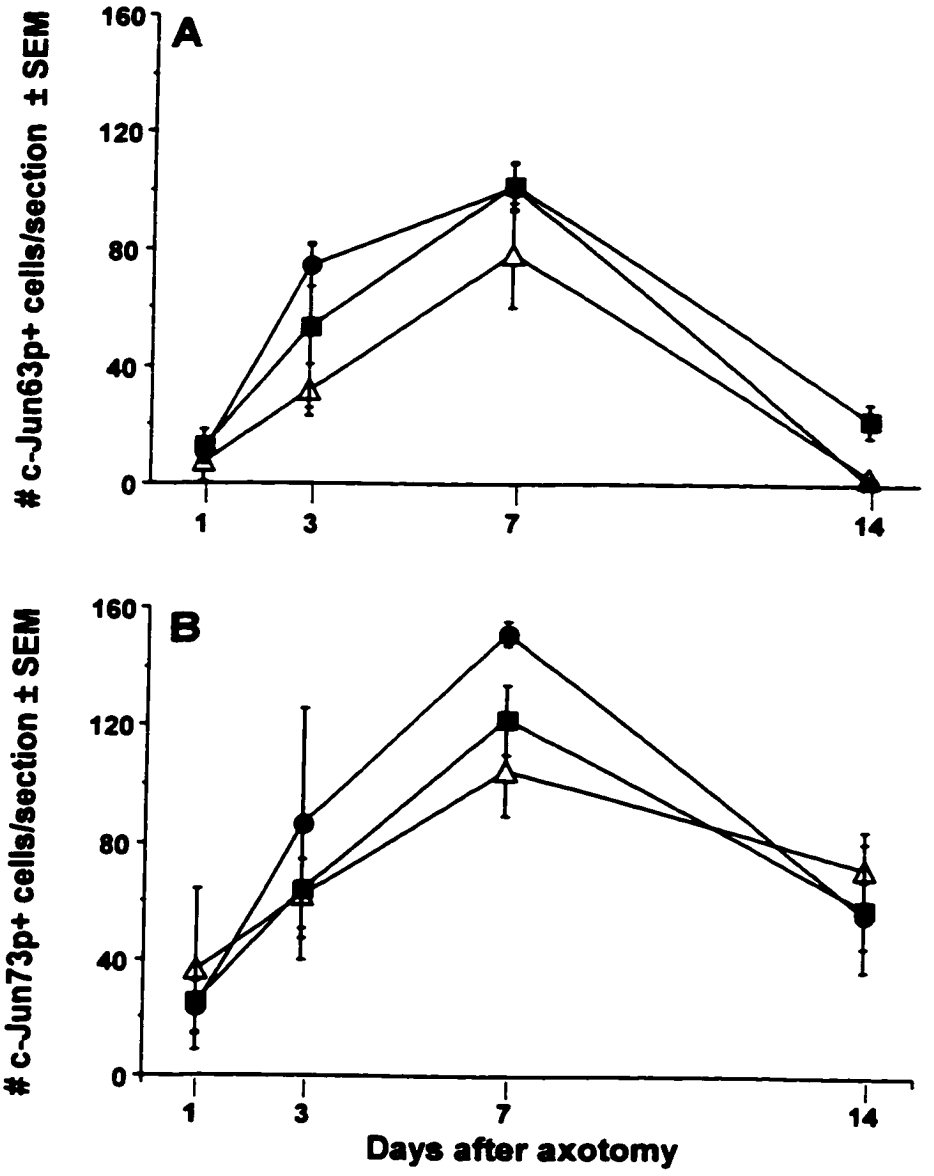


Figure 3. 9. c-Junp increases following fimbria fornix transection in the septum of control and $p75^{NGFR}$ -/- mice.

Following fimbria fornix transection in control mice (circles = 129/Sv; squares = Balb/c), the number of neurons with nuclei immunoreactive for c-Jun63p (A) and c-Jun73p (B) increased from day 1 through day 7. By day 14 postlesion, c-Jun63p levels were significantly lower than c-Jun73p levels ($p < 0.02$), which are ~60% lower than at 7 days. At any time after the lesion, the number of neurons with nuclear c-Junp in control and $p75^{NGFR}$ -/- mice (triangles) was not significantly different.

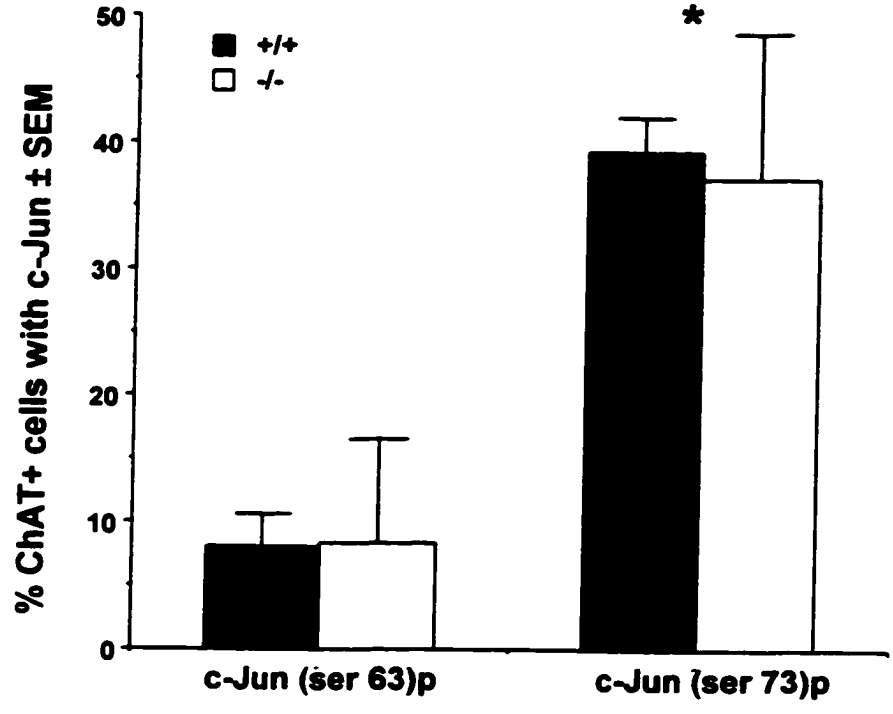


Figure 3. 10. c-Jun63 and c-Jun73 activation is similar in axotomized cholinergic medial septal neurons of $p75^{NGFR}$ -/- and control mice.

Seven days after axotomy, the same number of cholinergic neurons have immunostaining for c-Jun73p and c-Jun63p in control and $p75^{NGFR}$ -/- mice. Note that the number of ChAT-positive neurons with c-Jun73p is ~30% more than that with c-Jun63p (* = $p < 0.001$).

Nuclear NF_KB staining is reduced in concert with ChAT in axotomized medial septum cholinergic neurons in control and $p75^{NGFR}$ -/- mice.

Potential changes in the activation of NF_KB-p50 in cholinergic neurons following injury, was assessed in immunostained medial septum sections from adult animals 3, 7, or 14 days following unilateral fimbria fornix transection. NF_KB-p50 immunoreactivity was observed in the nucleus of most cells in the forebrain on both the lesioned and nonlesioned side (Fig. 3.8D). On the side ipsilateral to the fimbria fornix transection, the number of NF_KB-p50 immunoreactive nuclei in ChAT-positive (cholinergic) neurons decreased to ~30% at 3 days after the injury and remained the same at later times (Fig. 3.11A). The number of ChAT-positive neurons also decreased with time after axotomy (Fig. 3.11B). Between day 3 and day 14 following fimbria fornix transection, the number of double-labeled NF_KB-p50 in ChAT-positive neurons decreased by 2 (Fig. 3.11A) and the number of single-labeled ChAT-positive neurons decreased by 5 (Fig. 3.11B). This difference results in an apparent increase in percentage from 60% to 90% in control mice (Fig. 3.11C).

In $p75^{NGFR}$ -/- mice the number of ChAT-positive and ChAT-positive neurons with NF_KB-p50 nuclear staining was similar to that in control mice on the lesioned side of the medial septum at all post-lesion times (Figs. 3.11A and 3.11B). Following the calculation to percentage of cholinergic neurons expressing NF_KB-p50, at 14 days post-axotomy, $p75^{NGFR}$ -/- mice had ~27% fewer ChAT-positive neurons with nuclear NF_KB-p50 (Fig. 3.11C; $p < 0.02$). This difference reflects the fact that at 14 days post-lesion, the $p75^{NGFR}$ -/- mice had a few (3 cells/section) more ChAT-positive neurons per section than control mice but the same number of NF_KB-p50 containing ChAT-positive neurons.

In vivo signaling and cell death

Activation of SEK1 or c-Jun during postnatal development does not always correspond with DNA fragmentation

We compared the presence of TUNEL-positive labeling with SEK1p or c-Junp-positive staining in cells of various forebrain regions of P6 and P8 mice. The HDB had high numbers of both SEK1p immunostained neurons (~14 cells/section) and TUNEL-positive profiles (~12/section; Figs. 3.12A and 3.12E). SEK1p and TUNEL-staining both

Figure 3. 11. Nuclear NF_KB-p50 is reduced in concert with ChAT after axotomy in control and $p75^{NGFR}$ -/- mice.

At 3, 7, and 14 days following fimbria fornix transection, control (squares) as well as $p75^{NGFR}$ -/- mice (triangles) have similarly reduced numbers of NFKB-p50-containing ChAT- positive neurons (A) and ChAT-positive neurons (B) in the lesioned medial septum. The percentage of ChAT-positive neurons with nuclear NF_KB-p50 reveals an increase with time after injury for control but not $p75^{NGFR}$ -/- mice (C).

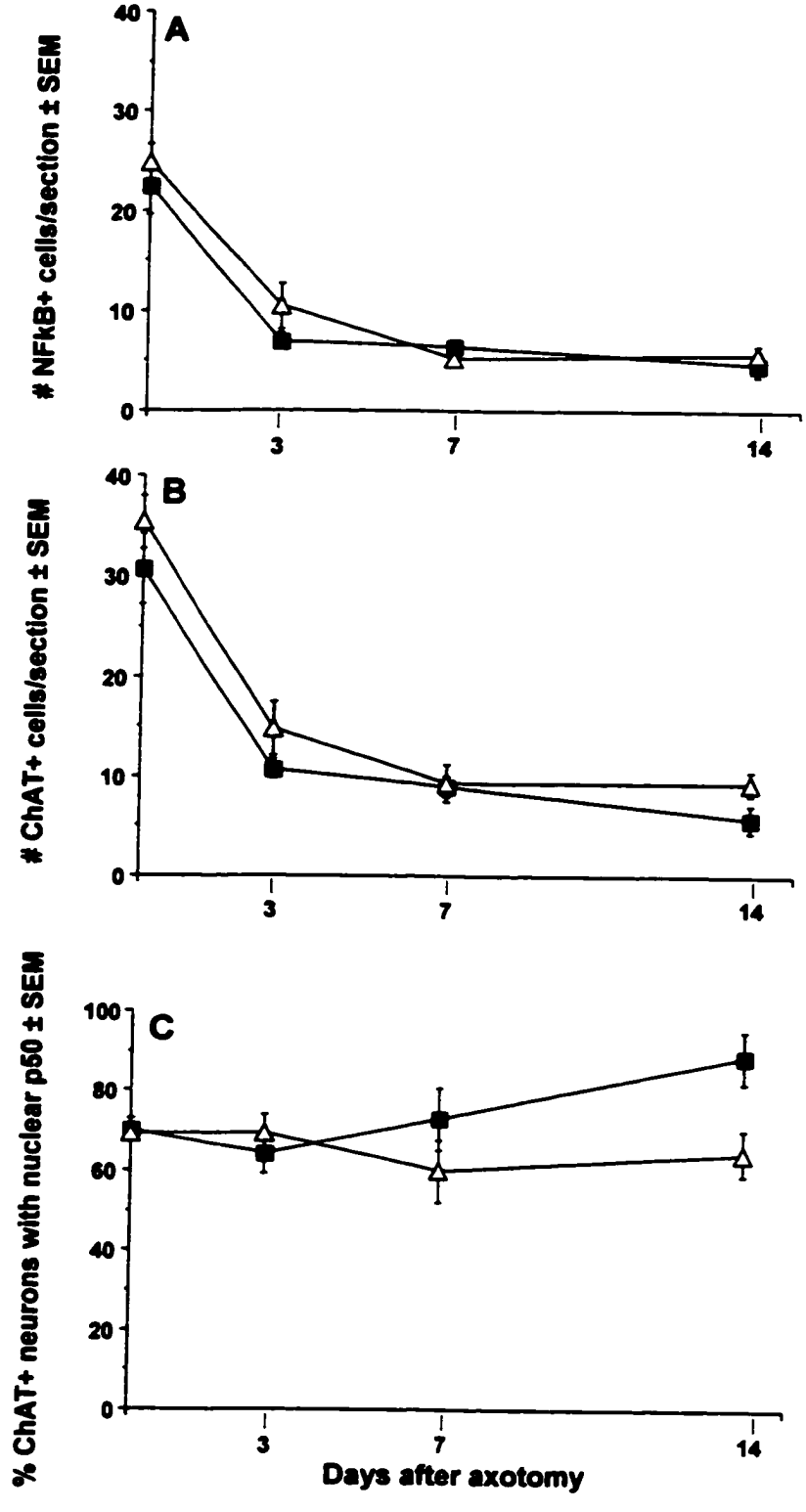

A
NFkB+ cells/section ± SEM
40
30
20
10
0
3
7
14
B
ChAT+ cells/section ± SEM
40
30
20
10
0
3
7
14
C
% ChAT+ neurons with nuclear p50 ± SEM
100
80
60
40
20
0
3
7
14
Days after axotomy

Figure 3. 11

Figure 3. 12. SEK1 or c-Jun activity does not necessarily predict DNA fragmentation during postnatal development.

In the horizontal limb of the diagonal band of the nucleus of Broca (HDB) many neurons are immunostained for SEK1p (A), many cell have TUNEL labeling (E), but none are immunostained for c-Junp (I). In the medial septum (MS; B), large numbers of SEK1p-positive neurons are observed with very few TUNEL-positive (F) or c-Junp-positive cells (J). The nucleus accumbens (region surrounding the anterior commissure, AC) contained no SEK1p stained neurons (C), many TUNEL positive cells (G), and a few c-Junp-positive nuclei (K). In the lateral septum (LS), no SEK1p stained neurons (D), no TUNEL positive profiles (H) and very few c-Junp-positive nuclei (L) are observed. LV = lateral ventricle. Arrows indicate single cells. Magnification bar = 50 μm and insert (in A and E) = 10 μm.

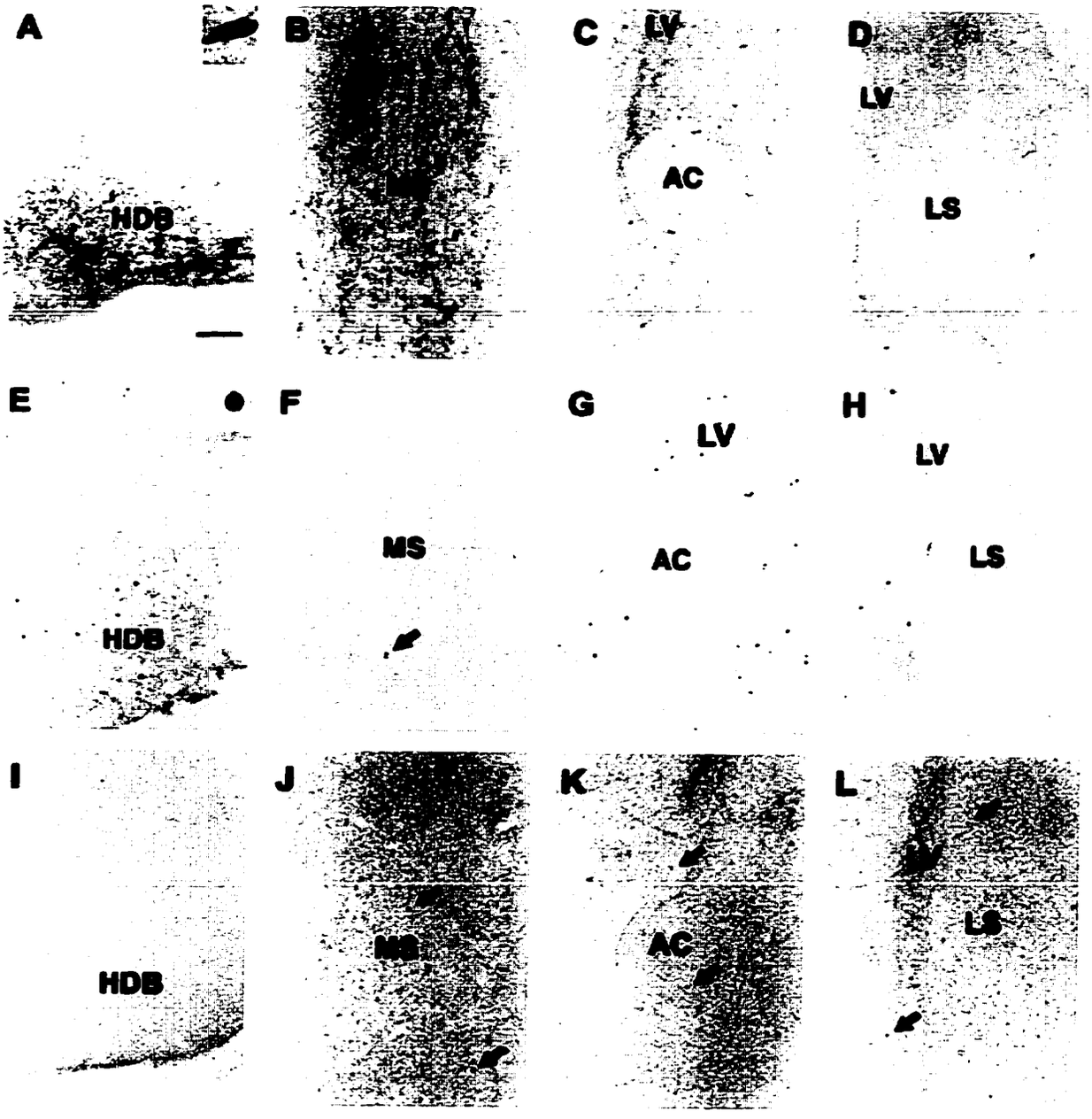


Figure 3.12

appeared to be low in the developing lateral septum (~0-1 and ~0-3 cells/section, respectively; Figs. 3.12D and 3.12H). In contrast to these regions where SEK1p and TUNEL immunostaining appeared to correspond, other regions of the developing forebrain showed a polarity between the two. The medial septum, like the HDB, showed high numbers of SEK1p immunoreactive cells (~30 cells/section), but had low numbers of TUNEL-positive profiles (~1-2 cells/section; Figs. 3.12B and 3.12F). In contrast, the nucleus accumbens had very few neurons stained for SEK1p (~0-1 cells/section), but had a high number of cells with TUNEL staining (~20-30 cells/section; Figs. 3.12C and 3.12G). High numbers of TUNEL-positive profiles were also observed. In the absence of SEK1p staining in the regions surrounding the lateral ventricles, specifically the dorsal regions (~18-24 cells/section; not shown) and those bordering the medial and lateral sides (5-10 cells/section; Fig. 3.12H). A similar lack of predictability was found for c-Junp and TUNEL, such that in all examined regions, low levels of either c-Jun63p or c-Jun73p were observed (0 – 3 positively-stained nuclei/section/region; Fig. 3.12I-L). Thus, the number of SEK1p or c-Junp positive cells was not consistently high or low compared to the number of TUNEL-positive cells in the various regions.

DISCUSSION

Recent *in vitro* evidence has suggested that $p75^{NGFR}$ can activate the JNK and NF_KB signaling pathways which have been linked to trophic or stress and degenerative responses (Dechant and Barde, 1997; Ip and Davis, 1998; O'Neill and Kaltschmidt, 1997). The present study took advantage of antibodies that recognize the activated forms of these signaling proteins. We determined in tissue sections of mice, whether forebrain neurons that normally express $p75^{NGFR}$ (predominantly cholinergic) would have different activation patterns in control and $p75^{NGFR}$ -/- mice. Our results provide *in vivo* evidence that i) SEK1 activation is prevalent mainly during development and that c-Jun (ser 63 and 73) activation is predominantly seen in injured forebrain neurons of adults; ii) NF_KB-p50 is localized to nuclei of most forebrain neurons including the medial septal and neostriatal cholinergic neurons during these periods; iii) these activation patterns are the same in $p75^{NGFR}$ -/- mice, suggesting that $p75^{NGFR}$ does not regulate the activity of these signaling proteins; and iv) that activation of these proteins can occur independent of DNA fragmentation.

SEK1 and c-Jun activities are regulated differently during development and after injury

During postnatal development robust SEK1p immunostaining was detectable throughout the forebrain in both cholinergic and non-cholinergic neurons and their fibers. This is consistent with recent findings (Lee et al., 1999) that SEK1 mRNA and protein is present in the embryonic, developing and adult forebrain. In adults, very light staining of neuropil and some fibers was seen in the tissue, confirming reports that SEK1 protein is expressed in the cytoplasm and dendrites of neurons (Lee et al., 1999). Western analyses on non-lesioned adult tissue revealed a SEK1- but not a SEK1p-positive band (adult results not shown), suggesting low levels of SEK1 activation. Thus, higher levels of SEK1 expression (Lee et al., 1999) and activation may play a role during postnatal maturation of certain nuclei of the central nervous system. Sprouting and innervation of target regions in the cholinergic forebrain, are all known to occur during postnatal development (Linke and Frotscher, 1993; Semba, 1992). In the developing medial

septum. SEK1p was seen in ChAT-positive and ChAT-negative neurons, while in the neostriatum SEK1p was localized almost exclusively to the cholinergic interneurons. The difference between these two types of "cholinergic" regions could reflect the slightly earlier maturation of the cholinergic neurons of the neostriatum (Gould et al. 1991; Semba, 1992).

In contrast to the clear presence of SEK1p, very few cells throughout the developing forebrain contained c-Jun63p and c-Jun73p during development despite the clear presence of c-Jun. This suggests that other phosphorylation sites on c-Jun (Karin et al., 1997; Smeal et al., 1992) may be phosphorylated during postnatal development, and that such activation is related to growth-associated events during postnatal development of the forebrain. Thus, in none of the forebrain regions did levels of SEK1p immunostaining correspond to or appear to predict to the phosphorylation of c-Jun (ser63 or 73). This was unexpected since previous reports have shown that JNK binds to the c-Jun transactivation domain and phosphorylates it on ser-63 and ser-73 (Derijard et al., 1994; Minden et al., 1994). This suggests that alternate downstream signaling targets of the JNK pathway could be affected, such as p38 (Minden and Karin, 1997). In addition, an alternate upstream activator of c-Jun (and JNK) such as MKK7 (Tournier et al., 1997), could explain the apparent discrepancy between the presence of SEK1p and absence of c-Junp. It is also possible that an, as yet unknown, substrate of SEK1p is involved.

In contrast to the developmental results, following fimbria fornix transection in adult mice, the levels of neuronal SEK1p remained low, and levels of c-Junp increased in many neurons, revealing again a disjunction between SEK1 and c-Jun activation. The maximum number of neurons with c-Jun73p immunostaining detected at 7 days post-lesion was consistent with the idea that only the axotomized cholinergic and GABAergic septohippocampal neurons contained activated c-Jun, confirming reports by others (Butterworth and Dragunow, 1996; Haas et al., 1996). Immunostaining for c-Jun63p was observed in fewer medial septum neurons at all times after the lesion. In addition, a greater proportion of injured cholinergic neurons contained c-Jun73p than c-Jun63p. These differences may represent an earlier and longer activation of c-Jun73p, or possibly a different role for activation of c-Jun63p, perhaps more in degeneration, as previously

suggested (Eilers et al., 1998; Watson et al., 1998). These results also suggest that signaling proteins other than SEK1, such as MKK7, may be involved in activating upstream substrates of c-Jun resulting in phosphorylation on these two residues. Other pathways could include those activated through Ras or Raf (Radziwill et al., 1995; Westwick et al., 1994) or the ERK-type of MAPKs (Leppa et al., 1998).

NF_KB is found in most cells and is associated with ChAT in the developing and injured forebrain

During development, in adulthood, and even after injury, NF_KB was observed in most neurons of the forebrain. During development and following axotomy, approximately 70-80% of the ChAT-positive cholinergic neurons contained nuclear NF_KB-p50. This may suggest that NF_KB activation is somehow associated with the regulation of ChAT expression. On the other hand, these results may also be interpreted such that constitutive levels of nuclear NF_KB are present in most neurons (Kaltschmidt et al., 1994), and the changes observed simply represent the counting procedure which is dependent on increasing or decreasing levels of detectable ChAT. Whichever the case may be, the widespread presence of nuclear NF_KB in normal and injured neurons suggests that activated NF_KB in the brain participates in normal and critical functions.

$p75^{NGFR}$ does not alter SEK1, c-Jun, or NF_KB activities in mouse forebrain

No obvious differences in phosphorylation of SEK1 or c-Jun in the developing, adult or injured forebrain were found between control and $p75^{NGFR}$ -/- mice suggesting that $p75^{NGFR}$ does not play a major role in activating these two members of the JNK signaling cascade. This does not exclude the possibility that in the forebrain another receptor such as TNFR activates SEK1, JNK, c-Jun, and NF_KB (Testi, 1996). Consistent with our results, others have reported a lack of JNK activation by $p75^{NGFR}$ in cultured adult human oligodendrocytes (Ladiwala et al., 1998). However, in cultured rat oligodendrocytes and mouse sympathetic neurons $p75^{NGFR}$-dependent activation of JNK and c-Jun, respectively has been observed (Bamji et al., 1998; Casaccia-Bonnefil et al., 1996).

Following NGF binding, $p75^{NGFR}$ reportedly can induce translocation of NF_KB to the

nucleus in cultured Schwann cells and fibroblasts (transfected with $p75^{NGFR}$; Carter et al., 1996). Our comparison between control and $p75^{NGFR}$ -/- mice suggests that $p75^{NGFR}$ does not play a major or specific role in inducing nuclear translocation of NF_KB-p50 *in vivo* during development or following injury. In fact, essentially all neurons had NF_KB immunostaining in their nucleus consistent with previous work demonstrating constitutive activation of NF_KB in neurons *in vivo* (Kaltschmidt et al., 1994). However, even in identified cholinergic neurons, we did not detect obvious differences between $p75^{NGFR}$ -/- and control mice. The apparent difference observed in Figure 3. 11C reflects the observation that the injured septum of $p75^{NGFR}$ -/- mice had 3 more detectable ChAT-positive neurons per section than control mice.

An alternative interpretation for the lack of observed differences in SEK1, c-Jun and NF_KB activation between control and these $p75^{NGFR}$ -/- mice is suggested by the fact that they all have a natural splice variant of $p75^{NGFR}$ (Dechant and Barde, 1997). This $p75^{NGFR}$ variant lacks the neurotrophin-binding domain but consists of the first cysteine-rich repeat of its extracellular domain, the transmembrane domain, and the intracellular domain. The intracellular domain of $p75^{NGFR}$ might be able to signal as its over-expression in transgenic mice leads to activation of JNK and NF_KB, and the death of neurons normally and not normally expressing $p75^{NGFR}$ (Majdan et al., 1997). Therefore, the lack of a difference in signaling between $p75^{NGFR}$ -/- and control mice that we observed may reflect the presence of the intracellular $p75^{NGFR}$ domain of the splice variant. If so, this would be consistent with findings by others that $p75^{NGFR}$ can signal without neurotrophin binding (Barrett and Bartlett, 1994; Barrett and Georgiou, 1996; Rabizadeh et al., 1993).

SEK1 and c-Jun do not predict or induce cell death

Some work has suggested that activation of the JNK pathway and/or c-Jun can result in apoptotic cell death (Bamji et al., 1998; Casaccia-Bonnefil et al., 1996; Estus et al., 1995; Ham et al., 1995; Majdan et al., 1997;). Here, SEK1p and c-Junp were observed in neurons of developing mice (P6 or P8) in most regions of the forebrain but did not show a clear predictive relationship with TUNEL-staining (an indicator of DNA fragmentation,

a characteristic of apoptosis). For instance, in the medial septum many neurons contained SEK1p, but few contained c-Junp or TUNEL labeling. In contrast, the nucleus accumbens contained many TUNEL-positive cells but very few SEK1p or c-Junp labeled neurons. Other regions (HDB) contained large numbers of SEK1p and TUNEL-positive and no c-Junp-positive cells. Following fimbria fornix transection in adult mice, the number of ChAT-positive neurons decreased, and c-Jun activation increased. Such axotomized neurons do not appear to die since ChAT-staining can be recovered in most of them with NGF treatment started long after the lesion (Hagg et al., 1988, 1989). Moreover, others have failed to find TUNEL labeling in the septum after fimbria fornix transection (Butterworth and Dragunow, 1996; Haas et al., 1996, but see Koliatsos et al., 1994). Together with others (Cuvillier et al., 1996; Liu et al., 1996; Verheij et al., 1996; Virdee et al., 1997), our study provides further evidence that members of the JNK pathway, including c-Jun63 and 73, can signal independent of cell death (Ip and Davis, 1998).

Apoptosis can occur in the presence of a dominant-negative form of SEK1, suggesting that SEK1 may be necessary for cell survival (Lenczowski et al., 1997). Our findings, and those of others (Lee et al., 1999; Yang et al., 1997) suggest that SEK1 activity is robust and critical for normal postnatal development. Moreover, the developing forebrain of JNK-/- mice shows increased caspase activation and apoptotic cell death, suggesting that JNK activation is necessary for normal development of this brain region (Kuan et al., 1999). In fact, activation of the JNK cascade in neurons of mice reportedly increases ~3-15 fold following normal exploratory behaviors (Xu et al., 1997), providing further evidence that JNK cascade activation can be involved in normal physiological function.

In summary, these results demonstrate that: SEK1 and c-Jun activities are differently regulated during development and following axotomy in adults, and that activation of these two members of the JNK signaling cascade and NF_KB are not different between control and $p75^{NGFR}$ -/- mice, and can occur in the absence of cell death. We are currently unable to resolve whether the observed lack of effect is due to the presence of a

functional intracellular domain of $p75^{NGFR}$ in these mice.

CHAPTER 4:

$p75^{NGFR}$ AND CHOLINERGIC NEURONS OF THE DEVELOPING FOREBRAIN: A RE-EXAMINATION

The results presented in the following chapter have been accepted for publication in the journal *Developmental Brain Research.*

INTRODUCTION

The family of neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4). Traditionally neurotrophins have been recognized for promoting cell survival, neurite outgrowth, and phenotypic maturation (Ip and Yancopoulos, 1996; Lewin and Barde, 1996) through the activation of specific high-affinity transmembrane tyrosine kinase (Trk) receptors (Bothwell, 1995). NGF binds to and activates TrkA, BDNF and NT-4 activate TrkB, and NT-3 activates TrkC. All neurotrophins bind with equal affinity to another receptor, $p75^{NGFR}$, which is functionally involved in mediating retrograde transport, providing ligand binding specificity, and enhancing Trk functioning (Bibel et al., 1999; Chao and Hempstead, 1995; Curtis et al., 1995).

Several reports now provide evidence suggesting an apoptosis-inducing role for $p75^{NGFR}$ in various developing cell types (Carter and Lewin, 1997; Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Majdan et al., 1997). NGF binding to $p75^{NGFR}$ induced neuronal apoptosis in chick retina in the absence of TrkA, and such cell death was prevented following treatment with antibodies against NGF, $p75^{NGFR}$ or a $p75^{NGFR}$-interfering peptide (dc28-36) (Frade and Barde, 1998; Frade et al., 1996). Similarly, BDNF activation of $p75^{NGFR}$ caused cultured sympathetic neurons to die, and in BDNF-deficient mice, more peripheral sympathetic neurons were observed than in control littermates (Bamji et al., 1999). The interactions and balance between the signaling cascades of TrkA and $p75^{NGFR}$ may predict the final fate of the cell (Kaplan and Miller, 1997; Yoon et al., 1998), such that $p75^{NGFR}$ is able to induce cell death in the absence of a Trk-initiated signaling cascade (Xia et al., 1995).

The cholinergic neurons of the developing and adult medial septum contain TrkA and $p75^{NGFR}$ (Koh and Loy, 1989; Li et al., 1995; Sobreviela et al., 1994). The cholinergic interneurons of the neostriatum contain TrkA, and until the end of the first postnatal week also $p75^{NGFR}$. These regions provide a model system in which to study the *in vivo*

interactions of TrkA and $p75^{NGFR}$. The use of transgenic mice containing a genetic deletion for each of these receptors has allowed for further detailed study of the role of TrkA and $p75^{NGFR}$ in cholinergic cell survival, and target innervation of the septo-hippocampal and striatal systems during development and in adulthood. In control mice, the number of detectable cholinergic neurons in the medial septum, neostriatum, and the cholinergic innervation of the hippocampal formation, increases with age, reaching mature levels between P14 and P21 (Fagan et al., 1997; Gould et al., 1991; Linke and Frötscher, 1993; Semba, 1992). TrkA-deficient mice had significantly reduced numbers of cholinergic neurons in the medial septum and neostriatum, reportedly caused by enhanced cell death, and reduced cholinergic innervation of the hippocampal formation (Fagan et al., 1997).

The reports about the effects of deleting $p75^{NGFR}$ have been conflicting. Our laboratory and others have suggested that $p75^{NGFR}$ -/- mice have more (Hagg et al., 1997; Van der Zee et al., 1996; Yeo et al., 1997), and yet others have suggested they have fewer (Peterson et al., 1997; 1999) cholinergic medial septal neurons than control mice. In the neostriatum, $p75^{NGFR}$ -/- mice may have more cholinergic neurons (Van der Zee and Hagg, 1998), although others have not observed this (Peterson et al., 1999; Yeo et al., 1997).

Inconsistencies between results describing the normal postnatal developmental process for medial septal cholinergic neurons have been reported, i.e., decreasing cholinergic cell numbers due to death (Van der Zee et al., 1996) versus increasing cell numbers (Fagan et al., 1997; Ward and Hagg, 1999b, Chapter 2, this thesis). The various discrepancies lead us to re-examine the cholinergic septo-hippocampal system and the neostriatum during postnatal development and in adulthood in new sets of two DNA control strains (129/Sv and Balb/c) and in $p75^{NGFR}$ -/- mice.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Dalhousie University Animal Care Committee and conformed to Canadian Council on Animal Care guidelines. Deep anesthesia was achieved by intraperitoneal injection of 6.5 mg/kg sodium pentobarbitol. Breeding pairs of mice homozygous (-/-) for the $p75^{NGFR}$ gene (Lee et al., 1992) (Jackson Laboratory number, JR2124) and their DNA controls (129/Sv, JR2448; Balb/c, JR0651) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Histological procedures. At P6, P15 or adulthood (3-4 months), anesthetized littermates were perfused transcardially with cold (4°C; 2 - 15 ml) phosphate buffered saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (4°C; 4 - 30 ml). Brains were post-fixed for 24 hours in 4% paraformaldehyde and cryoprotected in a 30% sucrose / phosphate buffer solution for 24 hours before sectioning. Brains were weighed, marked on the left hemisphere, and 30 μm thick coronal sections through the septum were cut with a freezing microtome. To visualize the cholinergic neural cell bodies, every third section through the entire septal nucleus and the nucleus of the vertical limb of the diagonal band of Broca (VDB) was processed for immunocytochemistry using an affinity-purified polyclonal goat antibody against ChAT (Ab144P, Chemicon, Temecula, CA). In short, free-floating tissue sections were incubated sequentially with 3% rabbit serum in Tris buffered saline (TBS) containing 0.3% Triton X-100 for thirty minutes, primary antibody Ab144P at a 1:4000 dilution in 1% rabbit serum TBS-triton for 16 hours (P15 and adult tissue) or 36 hours (P6 tissue) at 4°C, biotinylated rabbit anti-goat IgG (1:300; Vector laboratories; Burlingame, California) in TBS for ninety minutes, and avidin-biotin-peroxidase complex (1:600; ABC Elite kit, Vector Laboratories) in TBS for one hour. Immunoreactive products were revealed with a diaminobenzidine reaction in the presence of 0.67% ammonium nickel sulfate for intensification. In between steps, the sections were washed 3 x 10 minutes in TBS. All stained sections were mounted on gelatin-coated glass slides, dehydrated, and

coverslipped in Permount.

To visualize cholinergic axons in the hippocampal formation, a series of four sections spanning on average ~700 μm in the dorso-rostral part of the hippocampal formation of P6, P15, and adult mice were mounted on gelatin-coated slides and processed for acetylcholinesterase (AChE; Hedreen et al., 1985). Adaptations to the original protocol were made such that tissue was incubated twice for thirty minutes in freshly mixed enzyme solution and Promethazine (0.2 mM, Sigma) was used as an inhibitor of non-specific esterases. All tissue was stained at one time in an effort to exclude any differences in staining intensity as a variable.

TUNEL Labeling. A separate group of P8 Balb/c and $p75^{NGFR}$ -/- (n = 4 each) mice were perfused and their brains post-fixed as described above. The brains were cryoprotected in 15% sucrose / phosphate buffer over 24 hours and 10 μm thick coronal sections through the septal areas were cut using a cryostat and mounted on 2% APTEX (3-aminopropyltriethoxy-silane; cat #A-3648; Sigma Chemicals, St. Louis, MO) -coated slides. In addition, unstained archived tissue stored at –70 °C, from the same mice used by Van der Zee et al.(Van der Zee and Hagg, 1998; Van der Zee et al., 1996) also were used. Six sections in each animal were processed for TUNEL using the Oncor ApopTag *In situ* Apoptosis Detection kit - peroxidase (Cat #S7100-Kit Ed.1.5; Intergen, Purchase, NY). For a positive control, separate tissue was incubated with serially diluted RQ1 RNAse free DNAse I (activity = 1 unit / μl; Promega, Madison, WI) in DNAse buffer (1 M Tris HCl with 6 mM $MgCl_2$) for one hour at 37 °C before the first step of the TUNEL protocol. Experimental tissue was incubated under the same conditions with buffer, and all tissue was thoroughly washed in 2x SSC before beginning the TUNEL kit protocol. As a negative control, separate tissue underwent the complete protocol, but was incubated with a solution containing distilled water instead of TdT enzyme. Following staining, slides were dehydrated and coverslipped in Permount.

Quantitative analysis. In each mouse, every third section through the entire rostro-caudal extent of the medial septum (total = 14 sections) was used to determine the number of ChAT-positive neurons. To ensure consistency between animals, section number 0 (zero) was identified as the most rostral section through the decussation of the anterior commissure. Neuronal cell body profiles larger than 9 μm in longest diameter were counted on the left side of the medial septum or VDB, with the border between the two regions defined by an imaginary line through the arms of the anterior commissure. The ventral boundary of the VDB was defined by an imaginary horizontal line through the ventral surface in the midline of the brain. The lateral boundaries of the VDB were defined using vertical lines through the borders of either the nucleus accumbens or the Islands of Calleja. In the same sections, neuronal cell bodies of the neostriatum were counted in one hemisphere (representing ~50% of neostriatal volume). The total number of cholinergic neurons in each region was calculated according to the formula: Total number = 3 x (counted profiles x section thickness / (mean cell body diameter + section thickness); Abercrombie, 1946). This value was then used to estimate the total number in both hemispheres. The mean diameter was determined by measuring the longest diameter of 25 randomly chosen ChAT-stained cholinergic neurons per animal, in each respective region.

For each of the AChE-stained sections through the hippocampal formation (4 per animal), three lines were placed over the left dorsal blade of the outer molecular layer of the dentate gyrus. The number of stained fibers intersecting each of the three distinct lines (spaced ~60 μm apart) were counted in each of the sections and were represented as total number of fibers per section. At P6, the length of each line used was ~72 μm, to account for the shorter width of molecular layer. At P15 and adulthood, the length of lines used was 120 μm. The lines covered the dorsal portion of the outer molecular layer only, as to exclude the measurement of the inner molecular layer, whose anatomical boundaries are difficult to identify. The number of fibers transecting the three lines was

then divided by the total length of the three lines to provide a density measure of the number of fibers per millimeter.

For TUNEL-stained tissue, as no counterstain was used, and background staining was very low, a DIC (differential interference contrast) filter was used for counting individual TUNEL-positive profiles. In order to ensure consistency across all animals, the most caudal section was 240 μm rostral to the anterior commissure, and then every sixth section rostral to this point, spanning a total distance of ~310 μm (6 sections) was stained and counted. This region represents the mid-portion of the medial septum where most of the cholinergic neurons are found. TUNEL-positive cell profiles were counted in the left hemisphere in every section.

All data collection was completed blind to the animal genotype. Between group analyses were performed with ANOVA and post-hoc student Newman-Keuls analysis using Statistica software (Statsoft; Tulsa, OK) with an alpha of 0.05. For 129/Sv control mice at P6: n = 6; at P15: n = 9; and at adulthood: n = 6. For Balb/c control mice at P6: n = 7; at P15: n = 9; and at adulthood: n = 6. For $p75^{NGFR}$ -/- mice at P6: n = 7; at P15: n = 6; and at adulthood: n = 6. For each strain, mice used represent different litters and different breeding parents.

RESULTS

$p75^{NGFR}$ -/- mice have more detectable basal forebrain ChAT-positive neurons than control mice at P6 but not later

Analyses of the overall effects revealed that the number of ChAT-positive neurons in the medial septum differed significantly between mouse strains ($p < 0.0001$, ANOVA, $F(2, 53) = 19.45$) and with age ($p < 0.004$, ANOVA, $F(2, 53) = 6.02$). Post-hoc analyses of the main effect of strain indicated that $p75^{NGFR}$ -/- and 129/Sv mice had more ChAT-positive medial septum neurons than Balb/c mice ($p < 0.004$ and 0.02 respectively). Post-hoc analyses of the main effect of age indicated that P15 and adult mice had more

ChAT-positive neurons than at P6, with no differences between the two ($p < 0.0002$ and 0.0001 respectively).

Analyses of the simple effects in control mice were consistent with the overall effects. Between P6 and P15, the number of ChAT-positive neurons increased in the medial septum in 129/Sv and Balb/c mice ~64% and ~62%, respectively (Figs. 4.1 and 4.2A; $p < 0.0002$). Between P15 and adulthood, the number of ChAT-positive medial septum neurons did not change, and remained significantly higher than at P6 ($p < 0.0001$).

Analyses of the simple effects in $p75^{NGFR}$ -/- mice revealed that the number of ChAT-positive neurons in the medial septum remained constant at P6, P15 and adulthood. At P6, $p75^{NGFR}$ -/- mice had ~31% and ~56% more ChAT-positive medial septum neurons than 129/Sv and Balb/c mice, respectively (Figs. 4.1 and 4.2A; $p < 0.026$ and 0.005, respectively; $p75^{NGFR}$ -/- 969 ± 99; 129/Sv 738 ± 20, Balb/c 623 ± 48). At P15, $p75^{NGFR}$ -/- and 129/Sv mice had similar numbers of ChAT-positive medial septum neurons, and both had ~15% more ChAT-positive neurons than Balb/c mice (Figs. 4.1 and 4.2A; $p < 0.053$ and 0.039, respectively; $p75^{NGFR}$ -/- 1073 ± 41; 129/Sv, 1093 ± 34, Balb/c, 930 ± 60). When 129/Sv and Balb/c control mice were separated using date of birth and breeding parents, overlap between strains was observed for the number of ChAT-positive neurons at P6 and P15 for some of the litters. Thus, although the ANOVA suggests a difference between the two control strains, the overlap between certain litters suggests that this may not be the case. By adulthood, the $p75^{NGFR}$ -/-, 129/Sv and Balb/c mice had similar numbers of ChAT-immunoreactive neurons in the medial septum ($p75^{NGFR}$ -/- 1122 ± 67;129/Sv 1187 ± 107, Balb/c 1061 ± 124).

Overall, the number of ChAT-positive neurons in the VDB differed with mouse strain ($p < 0.02$, ANOVA, $F(2, 46) = 12.45$) and age ($p < 0.02$, ANOVA, $F(2, 46) = 4.29$). A significant interaction was found between mouse strain and age, suggesting that both strain and age of the mice contributed to the differences observed, and suggesting that these two factors are not independent ($p < 0.014$, ANOVA, $F(2, 46) = 3.19$). Across all

Figure 4.1. $p75^{NGFR}$ -/- mice have more detectable cholinergic medial septum neurons than control mice during development only.

ChAT-immunostained coronal sections through the medial septum of P6, P15, and adult Balb/c (A, B, C), 129/Sv (D, E, F), and p75-deficient (-/-) (G, H, I) mice. Note that at P6, p75 -/- mice (G) have more ChAT-positive neurons than both Balb/c (A) and 129/Sv (D) mice and that by adulthood all mice appear to have similar numbers of ChAT-positive neurons (C, F, I). Arrowheads indicate the midline of the medial septum. Arrows indicate the position of the anterior commissure (not shown) used to define the boundary between the medial septum and VDB. Magnification bar = 200 μm.

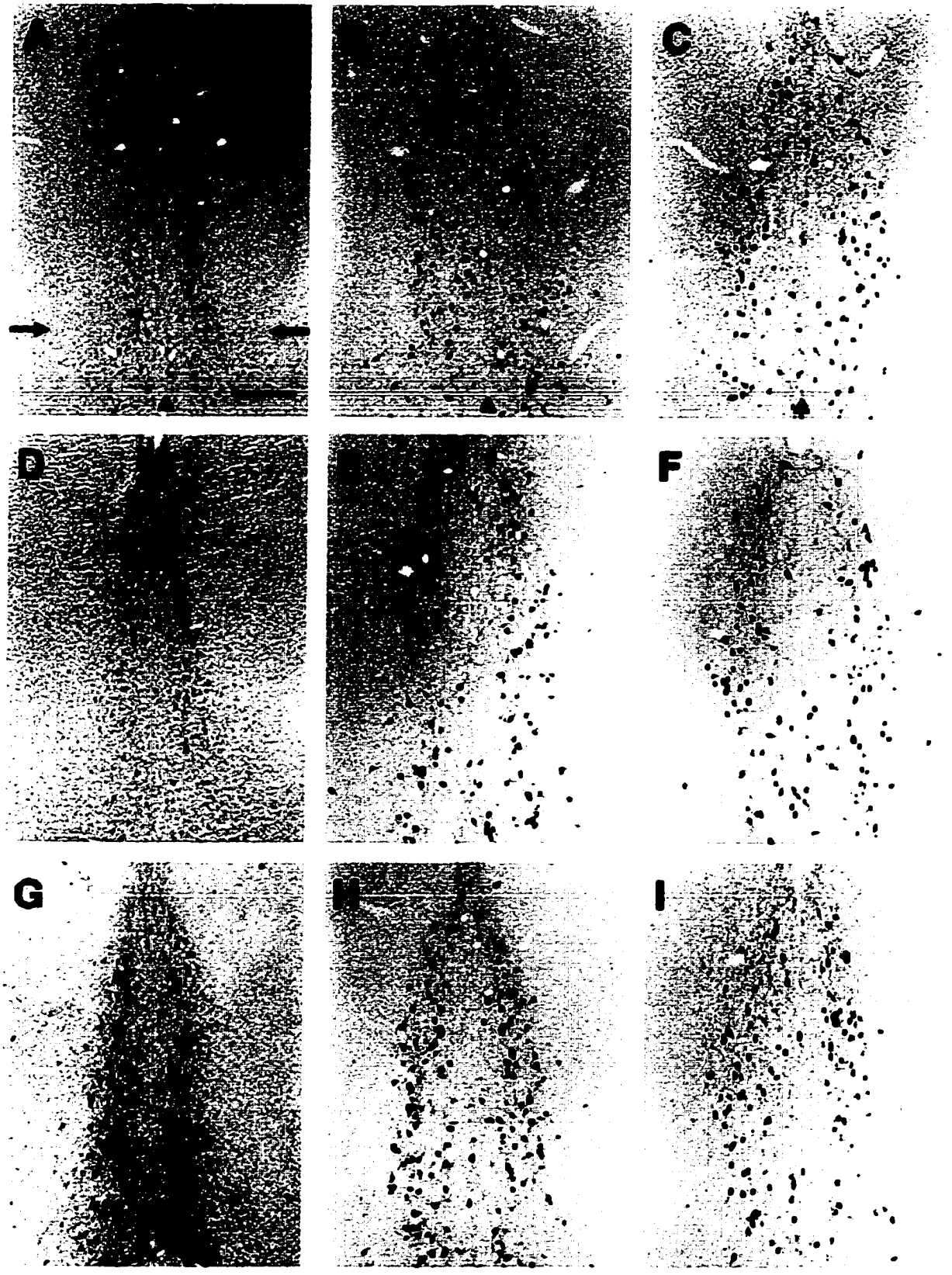


Figure 4.1

Figure 4.2. The total estimated number of basal forebrain cholinergic neurons is greater in $p75^{NGFR}$ -/- mice at P6 only.

Presented is the total estimated number of ChAT-positive neurons (±SEM) in both hemispheres of the medial septum area (A) the VDB (B) and the neostriatum (C) of $p75^{NGFR}$ -/- mice (triangles) and their DNA controls (129/Sv, filled squares; Balb/c, open squares) at P6, P15 and adulthood.

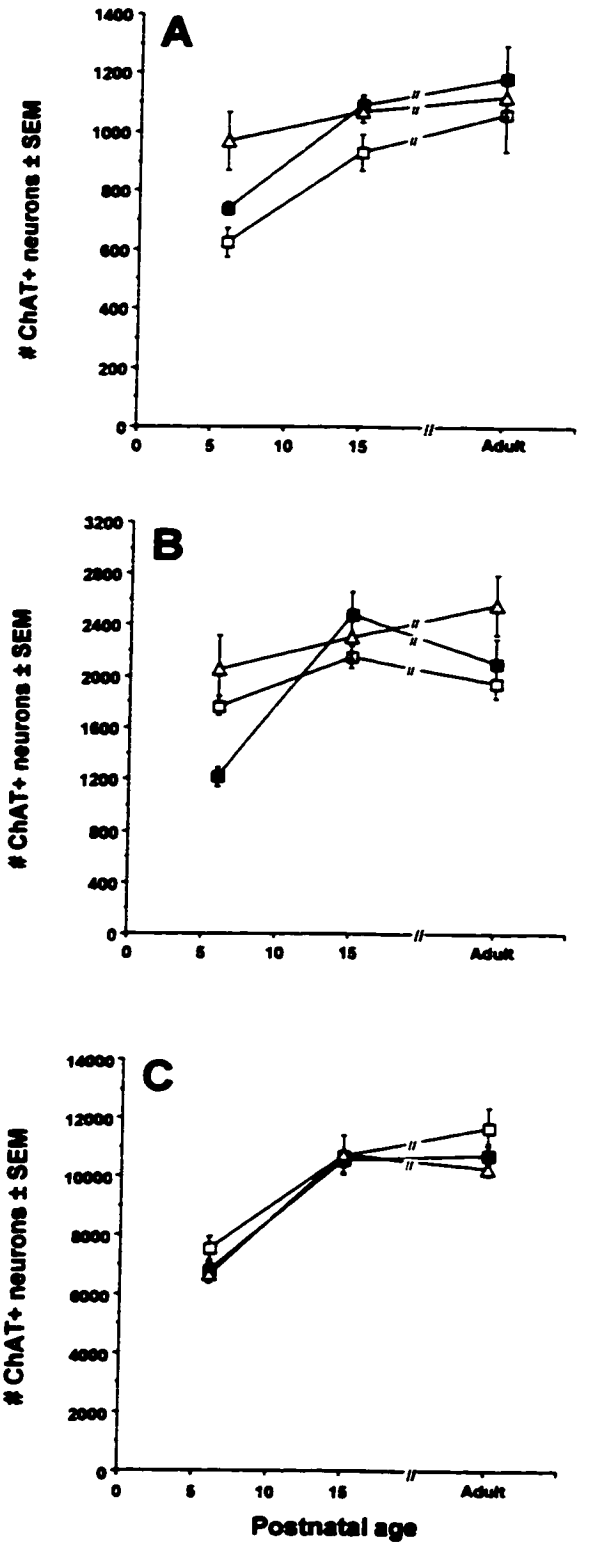

A
ChAT+ neurons ± SEM
1400
1200
1000
800
600
400
200
0
0
5
10
15
Adult
B
ChAT+ neurons ± SEM
3200
2800
2400
2000
1600
1200
800
400
0
0
5
10
15
Adult
C
ChAT+ neurons ± SEM
14000
12000
10000
8000
6000
4000
2000
0
0
5
10
15
Adult
Postnatal age

Figure 4.2

ages $p75^{NGFR}$ -/- mice had more ChAT-positive VDB neurons than Balb/c and 129/Sv mice (Fig. 4.2B; $p < 0.024$ and 0.02 respectively). Across all mouse strains, P15 and adult mice had significantly more ChAT-positive VDB neurons than mice at P6 ($p < 0.0003$ and 0.0003, respectively). No differences were observed between P15 and adult mice, or between Balb/c and 129/Sv mice.

Analyses of the simple effects revealed inconsistencies with the overall effects such that Balb/c and $p75^{NGFR}$ -/- mice had significantly more ChAT-positive VDB neurons at P6 than 129/Sv mice ($p < 0.029$ and 0.005, respectively, $p75^{NGFR}$ -/- 2057 ± 262; 129/Sv 1218 ± 74, Balb/c 1768 ± 71; Fig. 4.2B). Moreover, in Balb/c and $p75^{NGFR}$-deficient mice, the number of ChAT-positive VDB neurons did not change significantly with age, whereas the number of ChAT-positive neurons in the VDB of 129/Sv mice was significantly greater at P15 and adulthood than at P6 ($p < 0.0001$; at P15, $p75^{NGFR}$ -/- 2310 ± 175; 129/Sv 2485 ± 170, Balb/c 2152 ± 85; at adulthood, $p75^{NGFR}$ -/- 2560 ± 237; 129/Sv 2100 ± 194, Balb/c 1951 ± 117).

The anterior commissure was used as a landmark to define the border between the medial septum and VDB. To account for potential differences between the strains in the position of the anterior commissure, we added the numbers of ChAT-positive neurons in the medial septum and VDB (data not shown). Overall, the number of ChAT-positive neurons in the combined medial septum-VDB region differed with mouse strain ($p < 0.001$, ANOVA, $F(2, 48) = 7.64$) and age ($p < 0.0001$, ANOVA, $F(2, 48) = 32.49$). Across all ages $p75^{NGFR}$ -/- mice had more ChAT-positive neurons than Balb/c and 129/Sv mice ($p < 0.0008$ and 0.01 respectively), and Balb/c and 129/Sv mice did not differ. This finding is consistent with the idea that the two control strains do not differ and the differences observed in the individual regions may reflect differences in the dorso-ventral position of the anterior commissure within each strain.

Analyses of the simple effects revealed that at P6 only, $p75^{NGFR}$ -/- mice had significantly more ChAT-positive total medial septum-VDB neurons than both 129/Sv

and Balb/c mice ($p < 0.008$ and 0.016, respectively, $p75^{NGFR}$ -/- 4029 ± 461; 129/Sv 2693 ± 92, Balb/c 3014 ± 124). In P15 and adult 129/Sv and Balb/c mice, significantly more ChAT-positive VDB neurons were found than at P6 ($p < 0.0001$; at P15, $p75^{NGFR}$ -/- 4400 ± 156; 129/Sv 4670 ± 227, Balb/c 3973 ± 163; at adulthood, $p75^{NGFR}$ -/- 4804 ± 324; 129/Sv 4474 ± 260, Balb/c 3748 ± 631). In $p75^{NGFR}$ -/- mice only, the number of ChAT-positive medial septum-VDB neurons did not change significantly with age.

$p75^{NGFR}$ -/- and control mice have similar numbers of neostriatal cholinergic neurons

At P6, no differences in the total number of ChAT-positive neostriatal neurons were observed between $p75^{NGFR}$ -/- (6675 ± 305), 129/Sv (6804 ± 296), and Balb/c mice (7026 ± 593). Between P6 and P15, the total number of ChAT-positive neostriatal neurons increased ~56% in all three strains of mice (Fig. 4.2C; $p < 0.001$). At P15, again no differences were observed between the three mouse strains ($p75^{NGFR}$ -/- 10723 ± 632; 129/Sv 10731 ± 368; and Balb/c 10704 ± 662). Between P15 and adulthood, no changes in ChAT-positive cell numbers occurred, and the number of ChAT-positive neurons in adults ($p75^{NGFR}$ -/- 10257 ± 236; 129/Sv 10726 ± 401; Balb/c mice 11682 ± 670) remained significantly higher than at P6 ($p < 0.001$) (Fig. 4.2C). Similar results were found when only the lateral aspect of the developing and adult neostriatum were examined (data not shown).

$p75^{NGFR}$ -/- mice have larger cholinergic neurons in the medial septum but not the neostriatum

The diameter of cholinergic neuronal cell bodies in the medial septum changed with age ($p < 0.0001$, ANOVA, $F(2, 53) = 101.70$), such that between P6 and P15 they increased by ~26% ($p < 0.001$), and then decreased by ~10% between P15 and adulthood ($p < 0.001$; Fig. 4.3A). The diameter of these neurons was different between the mouse strains ($p < 0.0001$, ANOVA, $F(2, 53) = 19.28$). At P6, $p75^{NGFR}$ -/- mice had

significantly larger cholinergic neurons than 129/Sv ($p < 0.0048$) and Balb/c mice ($p < 0.047$), and no difference in diameter was found between the two control strains (Fig. 4.3A). At P15, no differences in diameter were observed between $p75^{NGFR}$ -/- and both control strains. At adulthood the neurons of $p75^{NGFR}$ -/- mice again had significantly greater diameters (~17% and ~16%, respectively) than that of 129/Sv ($p < 0.009$) and Balb/c mice ($p < 0.0005$). Adult 129/Sv mice had larger (~6%) neurons than the Balb/c mice ($p < 0.048$).

In the VDB, the diameter of cholinergic neurons was different with age ($p < 0.0001$, ANOVA, $F(2, 49) = 64.89$). Between P6 and P15 the size of the VDB cholinergic neurons increased ~22% ($p < 0.0001$; Fig. 4.3B). Between P15 and adulthood, cholinergic neurons remained the same size ($p = 0.054$), and remained significantly larger (~18%) than at P6 ($p < 0.0001$). Overall, no differences between the different mouse strains were observed. However, analyses of the simple effects revealed at P6 only, that Balb/c and $p75^{NGFR}$ -/- mice had significantly larger (~14% greater diameter each) cholinergic neurons than 129/Sv mice (Fig. 4.3B; $p < 0.0006$).

In the neostriatum, the diameter of cholinergic neurons increased ~24% ($p < 0.0001$) between P6 and P15 and then decreased ~4% between P15 and adulthood ($p < 0.01$; Fig. 4.3C). In contrast to the medial septum, no differences in cell size were observed between any of the three strains of mice, at any age.

$p75^{NGFR}$ -/- and control mice have similar numbers of TUNEL-positive profiles in the developing postnatal forebrain

A previous study had reported that control mice have more TUNEL-positive profiles in the septum and neostriatum than $p75^{NGFR}$ -/- mice during development, with the largest difference observed at P8 (Van der Zee and Hagg, 1998; Van der Zee et al., 1996). In the current study P8 Balb/c and $p75^{NGFR}$ -/- mice had similar, low numbers of TUNEL-positive cells in the medial septum (Fig. 4.4A). TUNEL-positive profiles were observed

Figure 4.3. $p75^{NGFR}$ -/- mice have larger medial septum cholinergic neurons. Presented are the cell body diameters (μm ± SEM) of ChAT-positive medial septum (A), VDB (B), and neostriatum (C) neurons in $p75^{NGFR}$ -/- (triangles) mice and their DNA controls (129/Sv, filled squares; Balb/c open squares) at P6, P15, and adulthood. $P75^{NGFR}$ -/- mice had larger ChAT-positive neurons in the medial septum, but not in the VDB or neostriatum. In all mice the diameter of the cholinergic neurons in the medial septum, VDB, and neostriatum increased between P6 and P15. In control, but not $p75^{NGFR}$ -/- mice, the diameter of medial septum neurons decreased again to adult values.

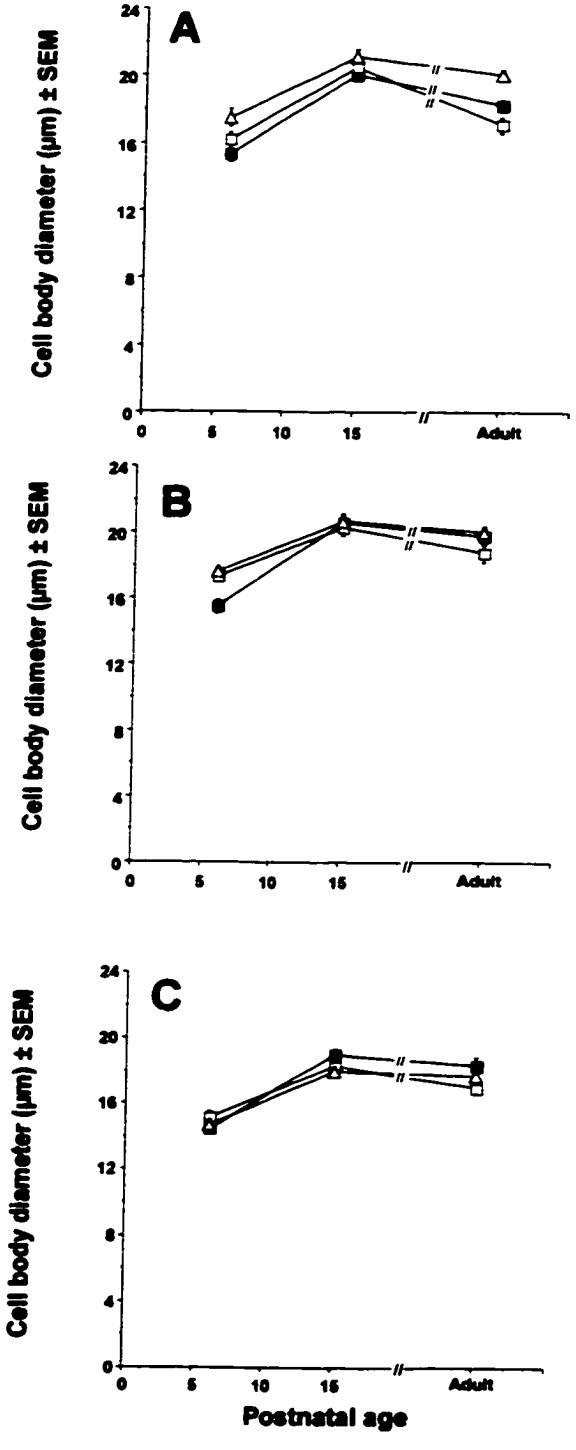

A
B
C
Cell body diameter (µm) ± SEM
Cell body diameter (µm) ± SEM
Cell body diameter (µm) ± SEM
0
4
8
12
16
20
24
0
5
10
15
Adult
Postnatal age

Figure 4.3

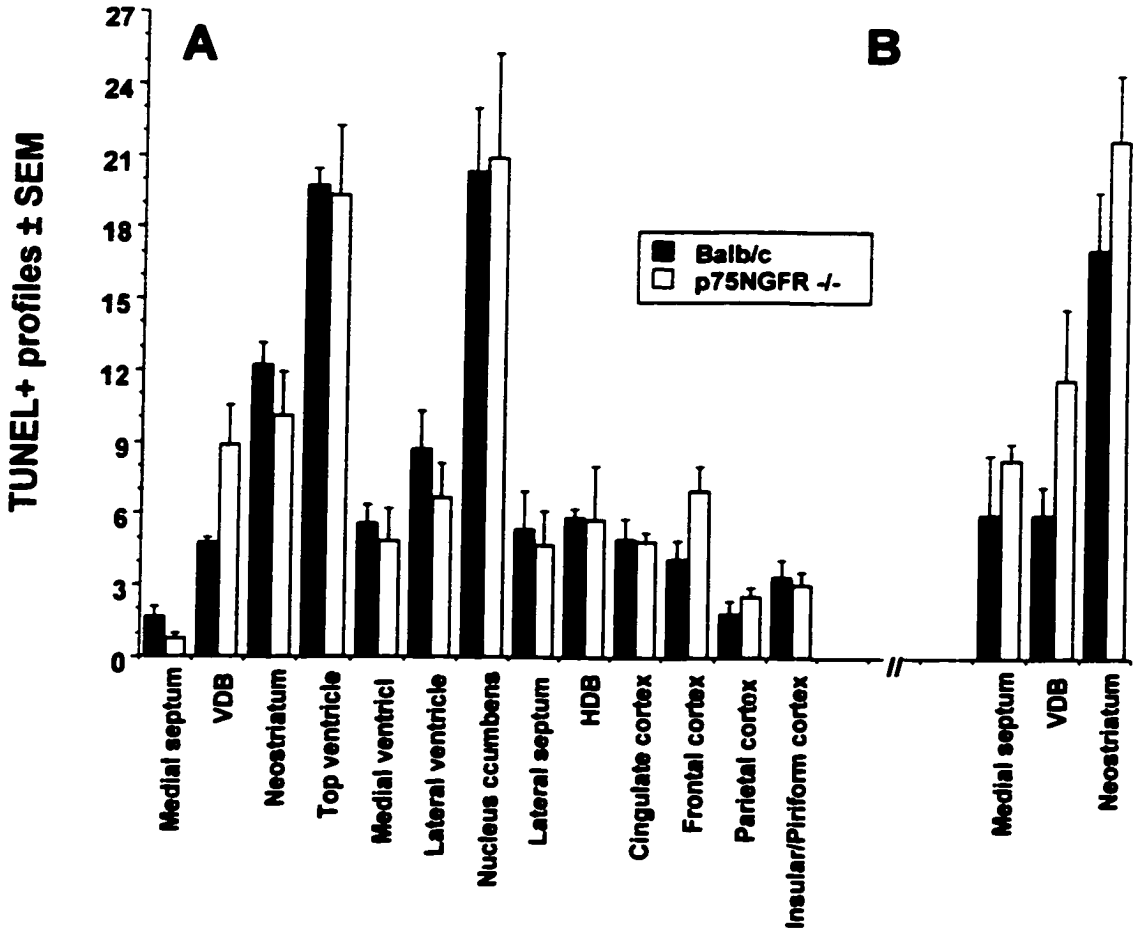


Figure 4.4. At P8, $p75^{NGFR}$ -/- mice have a similar number of cells with signs of apoptosis (DNA fragmentation) as Balb/c mice.

Presented is the total number of TUNEL-positive cells (±SEM) in six sections of one side of a variety of forebrain regions from the current mice (A) and from archived tissue of the Van der Zee (1996, 1998) studies (B) at P8. In most regions (except the VDB), $p75^{NGFR}$ -/- mice had the same number of TUNEL-positive cells as Balb/c control mice (A, $p < 0.01$; B, $p < 0.05$).

in all regions adjacent to the lateral ventricles, in the nucleus accumbens, the neostriatum, the lateral septum, the vertical (VDB) and horizontal (HDB) limbs of the diagonal band of the nucleus of Broca, and all the forebrain cortices (Fig. 4.4A). In only one forebrain region, the VDB, were differences between Balb/c and $p75^{NGFR}$ -/- mice observed, such that the $p75^{NGFR}$ -/- mice had more TUNEL-positive cells ($p < 0.01$).

Archived unstained tissue sections from P8 Balb/c and $p75^{NGFR}$ -/- mice for which differences in TUNEL labeling had been previously reported (Van der Zee and Hagg, 1998; Van der Zee et al., 1996) were stained for TUNEL. These sections revealed no differences between the Balb/c and $p75^{NGFR}$ -/- mice in the medial septum, or the entire neostriatum. Moreover, they confirmed the current data indicating $p75^{NGFR}$ -/- mice have more TUNEL-positive cells than Balb/c mice in the VDB (Fig. 4.4B; $p < 0.05$). Differences in the numbers of TUNEL-positive cells obtained for control mice between the archived and new tissue may reflect differences in the TUNEL kits used.

$p75^{NGFR}$ -/- mice have a similar number of AChE-positive fibers in the molecular layer of the dentate gyrus as control mice

To evaluate whether $p75^{NGFR}$ had an effect on the development of the septo-hippocampal pathway, the cholinergic innervation of the dentate gyrus was analyzed with histochemical staining for AChE, a widely used marker for cholinergic fibers.

The density of AChE-positive fibers estimated in the outer molecular layer of the dentate gyrus was different between the mouse strains ($p < 0.0004$, ANOVA, $F(2, 42) = 16.92$). Across all ages $p75^{NGFR}$ -/- and 129/Sv mice had more AChE-positive fibers than Balb/c mice ($p < 0.0001$ and 0.001 respectively). The density of AChE-positive fibers also changed with age ($p < 0.0001$, ANOVA, $F(2, 42) = 334.61$). Between P6 and P15, the density of AChE-positive fibers increased ~ 4 fold in all three strains of mice (Figs. 4.5 and 4.6; $p < 0.0001$). Between P15 and adulthood, the density of AChE-positive fibers

Figure 4.5. Adult $p75^{NGFR}$ -/- mice appear to have a similar densities of cholinergic innervation of the dentate gyrus.

AChE-stained coronal sections through the dentate gyrus of the hippocampal formation at P6 (A and B) P15 (C and D) and adulthood (E and F). At P6 and P15 the density of cholinergic innervation in the dentate gyrus molecular layer is greater in $p75^{NGFR}$ -/- (B and D) than in Balb/c mice (A and C). By adulthood, cholinergic innervation is similar for Balb/c (E) and $p75^{NGFR}$ -/- mice (F). Magnification bar = 50 μm. The asterisk indicates the granule cell layer of the dentate gyrus.

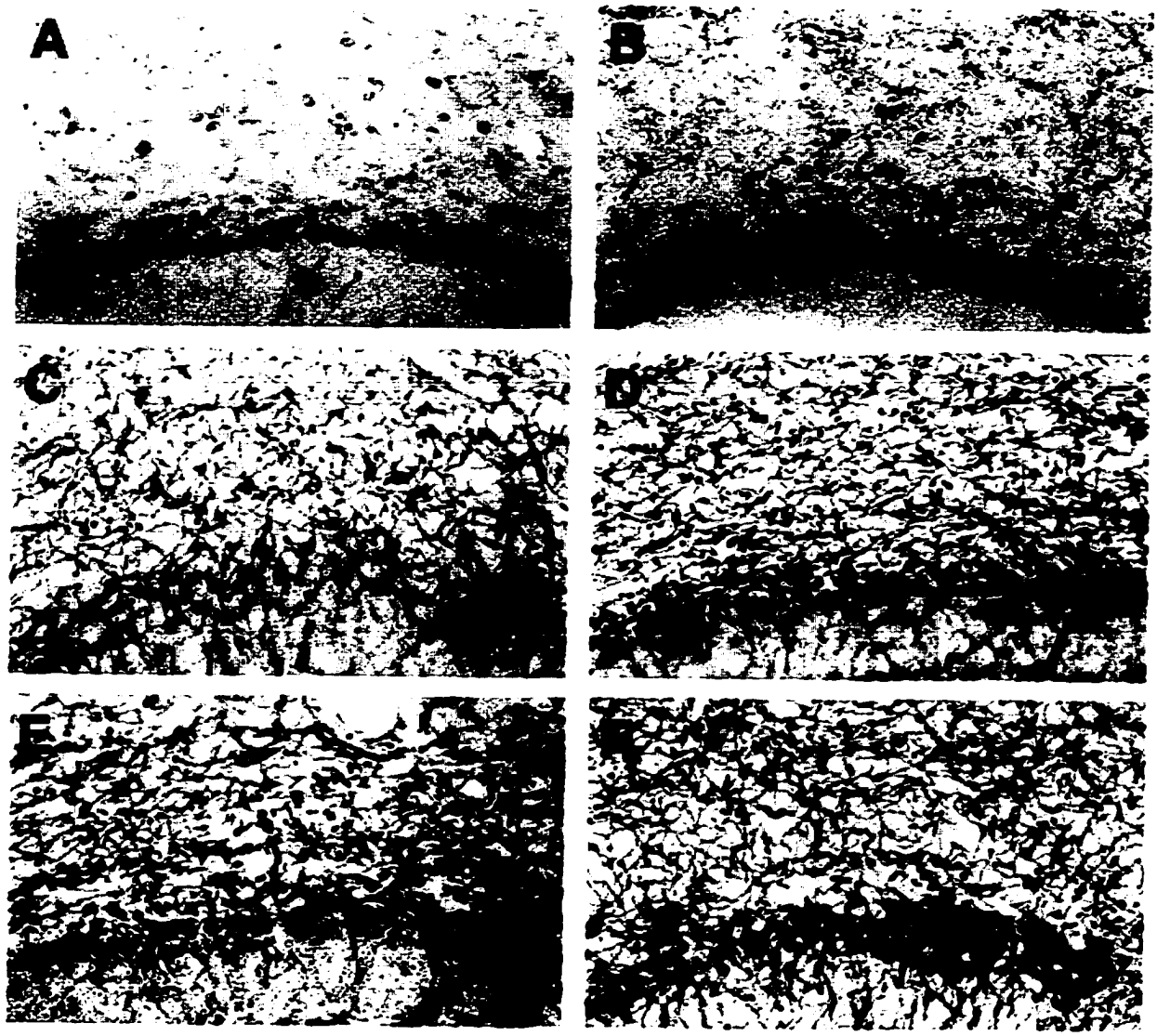


Figure 4.5

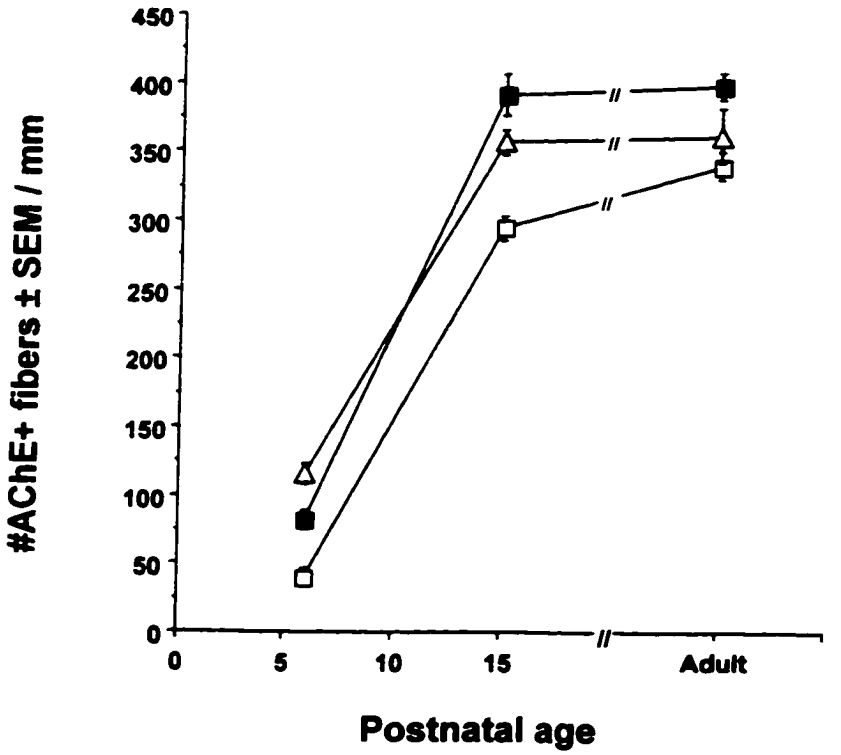


Figure 4.6. The density of AChE-positive fibers per millimeter in the outer molecular layer of the dentate gyrus is similar in $p75^{NGFR}$ -/- and control mice.

The density of AChE-positive cholinergic fibers intersecting three perpendicularly placed lines over the molecular layer of the dorsal blade of the left dentate gyrus increased between P6 and P15 in all mice. Across all ages, $p75^{NGFR}$ -/- (triangles) mice have a similar number of AChE-positive fibers as one of the control strains (129/Sv, closed squares; Balb/c, open squares). Values represent the mean sum of 3 measurements per section over four sections through the hippocampal formation of each animal divided by the total length of the linear probe.

did not change, and remained significantly higher than at P6 ($p < 0.001$). By adulthood, all mice had similar densities of AChE-positive fibers (~339-405 fibers/mm; Figs. 4.5 and 4.6). The width of the entire molecular layer of adults was similar in all three strains ($p75^{NGFR}$ -/- 158 ± 5μm; 129/Sv 158 ± 3μm; and Balb/c 160 ± 9μm).

Analyses of the simple effects showed that at P6, $p75^{NGFR}$ -/- mice had more dense AChE-innervation of the outer molecular layer than 129/Sv and Balb/c mice ($p < 0.03$ and $p < 0.004$, respectively). By P15, $p75^{NGFR}$ -/- and 129/Sv mice had a similarly dense innervation, and more dense than Balb/c mice ($p < 0.02$ and $p < 0.004$, respectively). By adulthood, all mice had a similar density of AChE-positive fibers of the outer molecular layer ($p = 0.08$, not significantly different).

DISCUSSION

We detected inconsistencies between results in our laboratory describing the normal development of forebrain cholinergic neurons and the role of $p75^{NGFR}$ in this process. This lead to our thorough re-examination of the cholinergic septo-hippocampal system and the neostriatum during postnatal development and in adulthood in two control strains and $p75^{NGFR}$ -/- mice. In contrast to previous reports from our laboratory, our current results suggest that i) the number of ChAT-positive forebrain neurons increases, not decreases during normal postnatal maturation; ii) the absence of $p75^{NGFR}$ does not affect cell numbers in the adult mouse forebrain; and iii) $p75^{NGFR}$ does not induce apoptotic cell death in a subpopulation of cholinergic neurons. Moreover, in contrast to others, we find that iv) the absence of $p75^{NGFR}$ does not affect the density of cholinergic innervation of the hippocampal formation. Finally, v) the absence of $p75^{NGFR}$ apparently causes increases of ChAT and cell body size of septal cholinergic neurons.

Number of ChAT-positive medial septum, VDB, and neostriatum neurons increases between P6 and P15 in control mice

Previous work had suggested that the number of cholinergic medial septum and lateral neostriatum neurons decreases between P6 and P15 and that this was due to apoptosis of a subpopulation of cholinergic neurons (Van der Zee and Hagg, 1998; Van der Zee et al., 1996). In contrast, we observed an increase in the total number of ChAT-positive neurons in the medial septum and rostral neostriatum in control mice, confirming recent findings by ourselves and others (Fagan et al., 1997; Ward and Hagg, 1999b, Chapter 2, this thesis). This increase most likely reflects the increased detectability of these cells, due to developmental increases in ChAT expression, in addition to increases in neuron cell body size (>9 μm diameter criterium used), both of which occur between P7 and P25 (Gould et al., 1991; Mobley et al., 1986; Semba, 1992).

By P15, the number of cholinergic neurons had reached their adult values. The total number of neurons on both sides of the medial septum of the adult control mice (~1000-

1200) was in the range of that reported by others (~1700, Yeo et al., 1997; ~1200, Fagan et al., 1998; and Dr. J. Long, ~1600, personal communication) who used unbiased stereological quantification methods.

One possible explanation for the reported developmental decrease in the number of cholinergic neurons (Van der Zee and Hagg, 1998; Van der Zee et al., 1996) is that the data was not collected in a sufficiently systematic and comprehensive manner.

Absence of $p75^{NGFR}$ does not affect cell numbers in the adult medial septum, VDB, or neostriatum

We report here that the number of ChAT-positive neurons in the medial septum, VDB, or the neostriatum in adult $p75^{NGFR}$ -deficient mice did not differ from that in control mice. This is contrary to previous reports where $p75^{NGFR}$ -/- mice had more (Hagg et al., 1997; Van der Zee and Hagg, 1998; Van der Zee et al., 1996; Yeo et al., 1997) or fewer (Peterson et al., 1997, 1999) ChAT-positive neurons in these regions. A comparison between laboratory groups, and within our own laboratory revealed significant differences in methodology, strain of controls, and breeding strategy used (Hagg et al., 1997; Peterson et al., 1997, 1999; Van der Zee and Hagg, 1998; Van der Zee et al., 1996; Yeo et al., 1997).

Within our laboratory, we have now examined the entire rostro-caudal extent of the medial septal nucleus whereas previously (Hagg et al., 1997; Van der Zee and Hagg, 1998; Van der Zee et al., 1996), only subsections of the medial septum were examined.

In the previous study, the $p75^{NGFR}$ -/- mice purchased from The Jackson Laboratory were compared to predominantly Balb/c control mice and some F2 generation C57BL/6J/Black29 control (not Balb/c and 129/Sv mice as claimed). Here, we used $p75^{NGFR}$ -/- mice that had been inbred by us an additional minimum of 6 generations, and compared them to the two strains considered to be the DNA controls (129/Sv, Balb/c). Maintaining a null-mutant transgenic line by inbreeding into itself can lead to the generation of new substrains of mice (Banbury Conference on genetic background in mice, 1997). Thus, across multiple generations of breeding changes in gene expression could conceivably compensate for the loss of function induced by removal of $p75^{NGFR}$.

Therefore, the less stringent application of quantification criteria, including anatomical boundaries and a potential drift in the $p75^{NGFR}$ -/- line could explain discrepancies within our lab.

Comparison between our results and the work presented by Yeo and colleagues (1997; reporting ~50% greater number of cholinergic medial septum neurons in $p75^{NGFR}$ -/- mice) suggests that differences in the generation of $p75^{NGFR}$ -/- mice could also account for some of the discrepancies. The $p75^{NGFR}$ -/- mice obtained from Jackson were generated on a mixed background strain of Balb/c/129/Sv. Yeo and colleagues (1997), back-bred the commercial (Jackson) mice into Balb/c mice to generate heterozygote F1 progeny, mated these, and analysed the F2 offspring. This may represent an area in which differences may have occurred.

Comparison between our results and the work published by Peterson and colleagues (1997, 1999; reporting a ~37% lower number of cholinergic medial septum neurons in $p75^{NGFR}$ -/- mice) again suggest that the method of $p75^{NGFR}$ -/- generation may contribute to the lack of consistent results between various laboratories. These authors used the parental heterozygote strain (Balb/c/129/Sv) which had been inbred more than 25 generations, and then crossed these mice to compare their offspring. Therefore, these animals may represent a different $p75^{NGFR}$ -/- substrain. The values presented by Peterson et al. for the estimated number of cholinergic neurons in the medial septum of control mice were ~5-10 fold greater than that by others (Fagan et al., 1996; 1998; Yeo et al., 1997), all using an optical disector technique. This suggests that there may be problems with Peterson's quantification methods.

The development and generation of another line of $p75^{NGFR}$ -/- mice with particular attention to breeding and maintainence of the genetic line may help to resolve the issue of the role $p75^{NGFR}$ in development and functioning of the cholinergic neurons of the medial septum (see comments below).

$p75^{NGFR}$ does not induce death in a subpopulation of cholinergic neurons

Our current results, in contrast to previous reports (Van der Zee and Hagg, 1998; Van der Zee et al., 1996), suggest that the number of ChAT-positive neurons increases during

postnatal development and is not different in adult control and $p75^{NGFR}$ -/- mice. Consistent with this observation was the lack of an increased number of TUNEL-positive cells in Balb/c mice compared to $p75^{NGFR}$ -/- mice at P8. Thus it appears that $p75^{NGFR}$ does not induce apoptotic cell death in a subpopulation of developing cholinergic medial septum or neostriatum neurons. In fact, newly processed sections of the identical animals used in the earlier studies also failed to reveal differences in TUNEL-labeling between $p75^{NGFR}$ -/- and control mice. The discrepancy between the current results and previously reported results may reflect the lack of strict application of criteria for anatomical boundaries, or perhaps limited control of staining conditions. Similar to the medial septum and neostriatum, $p75^{NGFR}$ -/- mice did not differ from control mice for TUNEL staining in other forebrain regions, including the subependymal regions of the ventricles, the nucleus accumbens, the lateral septum, the HDB or any of the cortical regions of the forebrain. This is not to say $p75^{NGFR}$ does not induce cell death in some neurons of the nervous system in vivo. It has been demonstrated that $p75^{NGFR}$ can induce death in peripheral sympathetic neurons following BDNF binding (Bamji et al., 1998), and in developing chick retinal cells following NGF binding (Frade and Barde, 1998; Frade et al., 1996).

An intriguing possibility is that the recently described natural splice variant of $p75^{NGFR}$, which lacks the extracellular neurotrophin binding domain in control and these $p75^{NGFR}$ -/- mice (Dechant and Barde, 1997), contributes to cell death equally in these mice. The intracellular domain of $p75^{NGFR}$ might be able to signal on its own, as its over-expression in transgenic mice leads to activation of JNK and NF_KB, and the death of neurons normally and not normally expressing $p75^{NGFR}$ (Majdan et al., 1997). Such signaling ability by an alternative $p75^{NGFR}$ is also suggested by our recent observation that MKK4/SEK1, c-Jun, and NF_KB activation during development or following injury is similar in these $p75^{NGFR}$ -/- mice and controls (Ward and Hagg, 1999c, Chapter 3, this thesis). Thus, the $p75^{NGFR}$ splice variant may induce apoptotic death in the developing cholinergic forebrain. Development and thorough analyses of a new transgenic mouse with a complete null mutation will be important to resolve this issue.

Interestingly, the developing $p75^{NGFR}$ -/- mice had more TUNEL-positive nuclei in the VDB than Balb/c mice. However, no differences in the number of ChAT-positive neurons were observed between these mice, suggesting that a cell population other than the cholinergic neurons in the VDB of $p75^{NGFR}$ -/- mice are affected.

Absence of $p75^{NGFR}$ does not obviously affect development of cholinergic hippocampal innervation

In normal rats, the number of cholinergic fibers that reach the hippocampal formation increases between embryonic day 20 and P3, and the adult density and pattern of cholinergic innervation is reached between P14 and P21 (Linke and Frötscher, 1993; Semba, 1992). The current and previous (Hohmann and Ebner, 1985; Ward and Hagg, 1999c, Chapter 2, this thesis) results suggest that this time-line is similar in normal mice. Moreover, the general developmental process also appears similar for $p75^{NGFR}$ -/- mice. By P15 $p75^{NGFR}$ -/- mice had reached adult levels of cholinergic innervation and did not differ from the 129/Sv control strain of mice. This is apparent contrast to a previous report (Yeo et al., 1997) which provided qualitative evidence for in increase in ChAT-immunostaining in the molecular layer of the dentate gyrus of $p75^{NGFR}$ -/- mice and quantitative evidence for increase numbers of ChAT-immunoreactive fibers in the CA1 region of the hippocampus.

Beyond the limitations of the AChE staining technique, including variation in staining quality, further discrepancy may arise from the method of data collection. Most groups, including ourselves, report fiber data as a density measure (e.g., number of fibers intersecting lines) and differences in where lines are positioned could lead to differences in results. Intrinsic difficulties exist when attempting to quantify fiber innervation of a structure, including the use of density measures, and lack of three-dimensional analyses. In order to resolve the issue of fiber-innervation the design and use of an appropriate stereological method for coronal tissue sections will be necessary.

$p75^{NGFR}$ -/- mice have more detectable ChAT in the developing medial septum and VDB and more detectable AChE in the developing dentate gyrus

At P6 the number of ChAT-positive neurons in the medial septum and VDB of $p75^{NGFR}$ -/- mice was greater than that of both Balb/c and 129/Sv mice. Consistent with this finding, was the observation that at P6 $p75^{NGFR}$ -/- mice had more AChE-positive fibers than both Balb/c and 129/Sv mice. This suggests the absence of $p75^{NGFR}$ allows the cholinergic neurons of the medial septum and their projecting fibers to express ChAT and AChE enzymes earlier and is consistent with the findings that $p75^{NGFR}$ -/- mice have increased ChAT enzyme levels in the adult medial septum (Yeo et al., 1997). However, in that study, the adult $p75^{NGFR}$ -/- mice had more cholinergic neurons in contrast to the present mice. Thus it remains to be seen whether increased ChAT staining is equivalent to increased ChAT in development.

In the developing neostriatum, where $p75^{NGFR}$ is expressed only until the first postnatal week, the number of ChAT-positive neurons was similar in $p75^{NGFR}$ -/- and control mice at any age, consistent with the finding that $p75^{NGFR}$ -/- mice have a normal number of ChAT-positive neurons and ChAT enzyme levels in the adult striatum (Yeo et al., 1997). The lack of an observed effect of $p75^{NGFR}$ in the young postnatal neostriatum may reflect the earlier maturation of this nucleus, the lack of projections outside of the nucleus (cholinergic neurons in the neostriatum are interneurons) or possibly the low levels of $p75^{NGFR}$.

The mechanism behind $p75^{NGFR}$-regulated ChAT expression is currently unknown. However, it is conceivable that $p75^{NGFR}$ may be involved in the activation of downstream signaling molecules that interact with ChAT synthesis and/or degradation, possibly via c-Jun whose AP-1 binding site has been localized to the cloned rat ChAT gene (Hahn et al., 1992).

$p75^{NGFR}$ -/- mice have larger cholinergic neurons in the medial septum but not the neostriatum

In control and $p75^{NGFR}$-deficient mice, consistent with that reported for rats (Gould et al., 1991), the size of the cholinergic medial septum neurons increased to a maximum around P15 and then decreased to adult values. These findings are consistent with the

possibility that cell body size relates to the total volume of their processes, which increase with cholinergic innervation over the first two postnatal weeks, and then is followed by the subsequent pruning of cholinergic dendrites (Gould et al., 1989; Koh and Loy, 1989).

In $p75^{NGFR}$ -/- mice, the neurons were larger at P6, consistent with the idea that $p75^{NGFR}$ may contribute to the regulation of the maturation rate of this nucleus. Adult cholinergic medial septum neurons of $p75^{NGFR}$ -/- mice were also larger than both 129/Sv and Balb/c mice as reported before (Yeo et al., 1997). No differences in size of neostriatal cholinergic neurons between strains were observed confirming a previous report (Yeo et al., 1997).

Conclusions

In summary, these results suggest that $p75^{NGFR}$ does not affect the number of cholinergic neurons in the adult forebrain, induce apoptotic cell death in a subpopulation of cholinergic neurons, or affect the cholinergic innervation of the adult hippocampal formation. $p75^{NGFR}$ appears to regulate ChAT and AChE expression and neuron size during development of the cholinergic septo-hippocampal system.

The discrepancies between various groups regarding the number of cholinergic neurons in adult $p75^{NGFR}$ -/- mice appears to arise from differences in breeding and data collection. Thus, breeding and maintainance of mouse colonies, choice of control animals, and proper use of anatomical quantification methods are important if data is to be replicable and interpreted similarly between different studies. Lastly, development of a new strain of mice with the complete removal of $p75^{NGFR}$ and appropriate controls, combined with the proper use of stereological anatomical quantification is expected to facilitate the elucidation of the role of $p75^{NGFR}$ in the developing and adult cholinergic septo-hippocampal system.

CHAPTER 5:

CHOLINERGIC MEDIAL SEPTUM NEURONS DO NOT DEGENERATE IN AGED CONTROL OR $p75^{NGFR}$ -/- MICE

The results presented in the following chapter have been submitted for publication to the journal *Neurobiology of Aging.*

INTRODUCTION

The neurotrophins are a family of growth factors which promote cell survival, neurite outgrowth, phenotypic maturation and synaptic functioning (Ip and Yancopoulos, 1995; Lewin and Barde, 1996). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4) are all members of the neurotrophin family. NGF mediates its survival-promoting effects via the transmembrane tyrosine kinase receptor TrkA, BDNF and NT-4 via TrkB, and NT-3 via TrkC (Bothwell, 1995). All neurotrophins bind to another receptor, p75 NGF receptor ($p75^{NGFR}$). The role of $p75^{NGFR}$ includes assisting in neurotrophin transport, enhancing ligand binding specificity, and increasing Trk functioning (Bibel et al., 1999; Bothwell, 1995; Chao and Hempstead, 1995) and under certain conditions, induction of apoptosis (Carter and Lewin, 1997; Chao et al., 1998).

Basal forebrain cholinergic neurons offer a model system for studying the *in vivo* role of neurotrophins and their receptors. In the medial septum of the basal forebrain of adult mammals, including mice, cholinergic neurons express TrkA and $p75^{NGFR}$ (Koh and Loy, 1989; Li et al. 1995; Sobreviela et al., 1994). In the medial septum, NGF apparently maintains cell body size and choline acetyltransferase (ChAT) levels (Mobley et al., 1986; Vantini et al., 1989), and can prevent or reverse degenerative changes caused by axotomy and aging (Fischer et al., 1987; Hagg et al., 1989; Hefti 1986; Williams et al., 1986). These effects appear to be regulated through TrkA since transgenic mice with a genetic deletion of NGF (+/-) or TrkA (-/-) have fewer and smaller ChAT-positive medial septum (Chen et al., 1997; Fagan et al., 1997). The medial septum of adult $p75^{NGFR}$ -/- mice has similar numbers of ChAT-positive neurons as control mice (Ward and Hagg, 1999a, see Chapter 4, this thesis; also see Yeo et al., 1997). These $p75^{NGFR}$ -/- mice have larger cell bodies, and possibly increased levels of ChAT (Ward and Hagg, 1999a, see Chapter 4, this thesis; Yeo et al., 1997), which is unexpected since their transport of NGF

is greatly reduced (Hagg et al., 1996). It is possible that the expected effects of no $p75^{NGFR}$ are only revealed during aging.

During aging of rodents, basal forebrain cholinergic neurons reportedly may be lost (Altavista et al., 1990; Armstrong et al., 1993; Fischer et al., 1991; Gilad et al., 1987) and can atrophy (Backman et al., 1996; Cooper et al., 1994; De Lacalle et al., 1996; Fagan et al., 1998; Fischer et al., 1987; Mesulam et al., 1987), a process that can be reversed with NGF treatment (Backman et al., 1996; Fischer et al., 1987; 1991; Gustilo et al., 1999; Martinez-Serrano et al., 1995). Aged animals also have reduced levels of ChAT and AChE activities (Baxter et al., 1999; Springer et al., 1987), and reduced expression of TrkA mRNA (Cooper et al., 1994). Moreover, NGF transport by the basal forebrain cholinergic neurons is reduced in aged rats (Cooper et al., 1994; De Lacalle et al., 1996; Koh and Loy, 1988), and possibly in Alzheimer's disease (Higgins and Mufson, 1989) where these neurons die.

Decreases in performance of learning- and memory-related behavioral tests have been reported in aged rodents (Aubert et al., 1995; Backman et al., 1996; Fischer et al., 1987, 1991; Lee et al., 1994; Sugaya et al., 1998). Basal forebrain cholinergic neurons project their axons throughout the hippocampal formation and neocortex and are important for learning and memory (Bigl et al., 1982; Everitt and Robbins, 1997).

In an attempt to determine whether $p75^{NGFR}$ plays a role in the normal aging process, here we investigated whether the cholinergic septohippocampal system would be differentially affected in young (6-8 months), middle aged (12-18 months), and aged (18 - 23 months) $p75^{NGFR}$ -/- mice.

MATERIALS AND METHODs

Animals. Breeding pairs of mice homozygous (-/-) for a deletion of the $p75^{NGFR}$ gene (Lee et al., 1992) and one of their DNA controls (129/Sv) were purchased from The Jackson Laboratory (Bar Harbor, ME). The 129/Sv strain more closely resembles the

$p75^{NGFR}$ -/- line in terms of the numbers of cholinergic neurons and density of cholinergic hippocampal innervation than the Balb/c DNA control strain (Ward and Hagg, 1999a, Chapter 4, this thesis). Sixty-four male and female offspring from these breeding pairs were sacrificed as either young (6-8 months of age), middle aged (12 – 18 months of age, average 14 months), or aged (18 - 23 months of age, average 20 months). All animal protocols were approved by the Dalhousie University Animal Care Committee and conformed to Canadian Council on Animal Care guidelines. Anesthesia was achieved by intraperitoneal injection of 6.5 mg/kg sodium pentobarbitol.

Tissue Processing, Immunohistochemistry and Histochemistry. At "young", "middle aged", or "aged" time points, anesthetized mice were perfused transcardially with cold (4°C; 15 ml) phosphate buffered saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (4°C; 30 ml). Following 24 hours post-fixation in 4% paraformaldehyde, the brains were cryoprotected in a 30% sucrose / phosphate buffer solution for 24 hours in preparation for sectioning. Brains were marked on the left hemisphere and 30 µm thick coronal sections through the septum and hippocampal formation were cut with a freezing microtome. Immunohistochemistry was performed in order to visualize the cholinergic neural cell bodies. Every third section through the entire septal nucleus (total number of sections = 14) was processed for immunohistochemistry using an affinity-purified polyclonal goat antibody against ChAT (Ab144P, Chemicon, Temecula, CA). Free-floating tissue sections were incubated sequentially with 3% rabbit serum in Tris buffered saline (TBS) containing 0.3% Triton X-100 for thirty minutes, primary antibody Ab144P at a 1:4000 dilution in 1% rabbit serum TBS-triton for 16 hours at 4°C, biotinylated rabbit anti-goat IgG (1:300; Vector laboratories; Burlingame, California) in TBS for ninety minutes, and avidin-biotin-peroxidase complex (1:600; ABC Elite kit, Vector Laboratories) in TBS for one hour. Immunoreactive products were revealed with a diaminobenzidine reaction in the presence of 0.67% ammonium nickel sulfate for intensification. In between steps, the sections

were washed 3 x 10 minutes in TBS. All stained sections were mounted on gelatin-coated glass slides, dehydrated, and coverslipped in Permount.

Cholinergic axons in the hippocampal formation were visualized by AChE staining in a series of four sections spanning ~700 μm in the dorso-rostral part of the hippocampal formation. Hippocampal tissues from all mice were mounted on gelatin-coated slides and all slides were processed with random strain and age assignment to one of three batches for AChE histochemistry (Hedreen et al., 1985). Adaptations to the original protocol were made such that tissue was incubated twice for thirty minutes in freshly prepared enzyme solution and Promethazine (0.2 mM, Sigma) was used as an inhibitor of non-specific esterases.

Quantification. In each mouse, every third section through the entire rostro-caudal extent of the medial septum (total = 14 sections) was used to determine the number of ChAT-positive neurons. To ensure consistency between animals, section number 0 (zero) was identified as the most rostral section through the decussation of the anterior commissure. Neuronal cell body profiles larger than 9 μm in longest diameter were counted on the left side of the medial septum, with the ventral border defined by an imaginary line through the arms of the anterior commissure. The total number of cholinergic neurons in each region was estimated according to the formula: Total number = 3 x (counted profiles x section thickness / (mean cell body diameter + section thickness)) (Abercrombie, 1946). This value was then used to estimate the total number in both hemispheres. The mean diameter was determined by measuring the longest diameter of 25 randomly chosen ChAT-stained cholinergic neurons per animal. Digitized images of the sections that were used to determine the cell body diameter were used to determine the cross sectional area of ChAT-positive neuronal cell bodies larger than 9 μm in diameter (NIH Image, Wayne Rasband, NIH, USA, http://rsb.info.gov/nih-image/).

For each of the AChE-stained sections through the hippocampal formation (4 per animal), three lines (~120 μm each) were placed over the left dorsal blade of the outer

molecular layer of the dentate gyrus. The number of stained fibers intersecting each of these three distinct lines (spaced ~60 μm) were counted in each of the sections and represented as a density measure, i.e., total number of counted fibers per section / total length of the three lines (0.36 mm). The width of the entire molecular layer was measured in the same sections.

All tissue processing and data collection was completed blind to the animal age and genotype. Between group analyses were performed with ANOVA and post-hoc Student Newman-Keuls analysis using Statistica software (Statsoft; Tulsa, OK) with an alpha of 0.05. For young, middle aged, and aged 129/Sv control mice: n = 11, 8, 15, respectively and for young, middle aged, and aged $p75^{NGFR}$-/- mice: n = 9, 9, 12.

RESULTS

Young, middle aged, and aged $p75^{NGFR}$ -/- and control mice have similar numbers of medial septum cholinergic neurons

The appearance of the cholinergic neurons of the medial septum was similar between control and $p75^{NGFR}$ -/- mice at young, middle aged, and aged time-points (Fig. 5.1). ChAT-positive neurons were visible at all three time-points, and did not appear to change in either the intensity of ChAT staining or the number of ChAT-positive neurons.

The ANOVA revealed overall effects for the number of ChAT-positive neurons in the medial septum differed significantly between mouse strains ($p < 0.02$, ANOVA, $F(1, 58) = 6.72$) but not with age. Post-hoc analyses of the main effect of strain (i.e., no age group separation) indicated that 129/Sv mice had more ChAT-positive medial septum neurons than $p75^{NGFR}$ -/- mice ($p < 0.011$).

However, in contrast to the overall effects, which indicated a difference between control and $p75^{NGFR}$ -/- mice, analyses of the simple effects of strain failed to reveal any differences. Control and $p75^{NGFR}$ -/- mice did not differ in the number of cholinergic neurons in the young ($p = 0.37$; 129/Sv 1357 ± 49; $p75^{NGFR}$ -/- 1277 ± 70), middle aged

Figure 5.1. Young and aged 129/Sv and $p75^{NGFR}$-/- mice have similar numbers of cholinergic medial septum neurons in the forebrain.

ChAT-immunostained coronal sections through the medial septum of 129/Sv mice (A and B) do not differ from $p75^{NGFR}$ -/- mice (C and D) at young (A and C) and aged (B and D) time-points. Arrowheads indicate the midline of the medial septum. Arrows indicate the position of the anterior commissure (not shown) used to define the ventral boundary of the medial septum. Magnification bar = 200 μm.

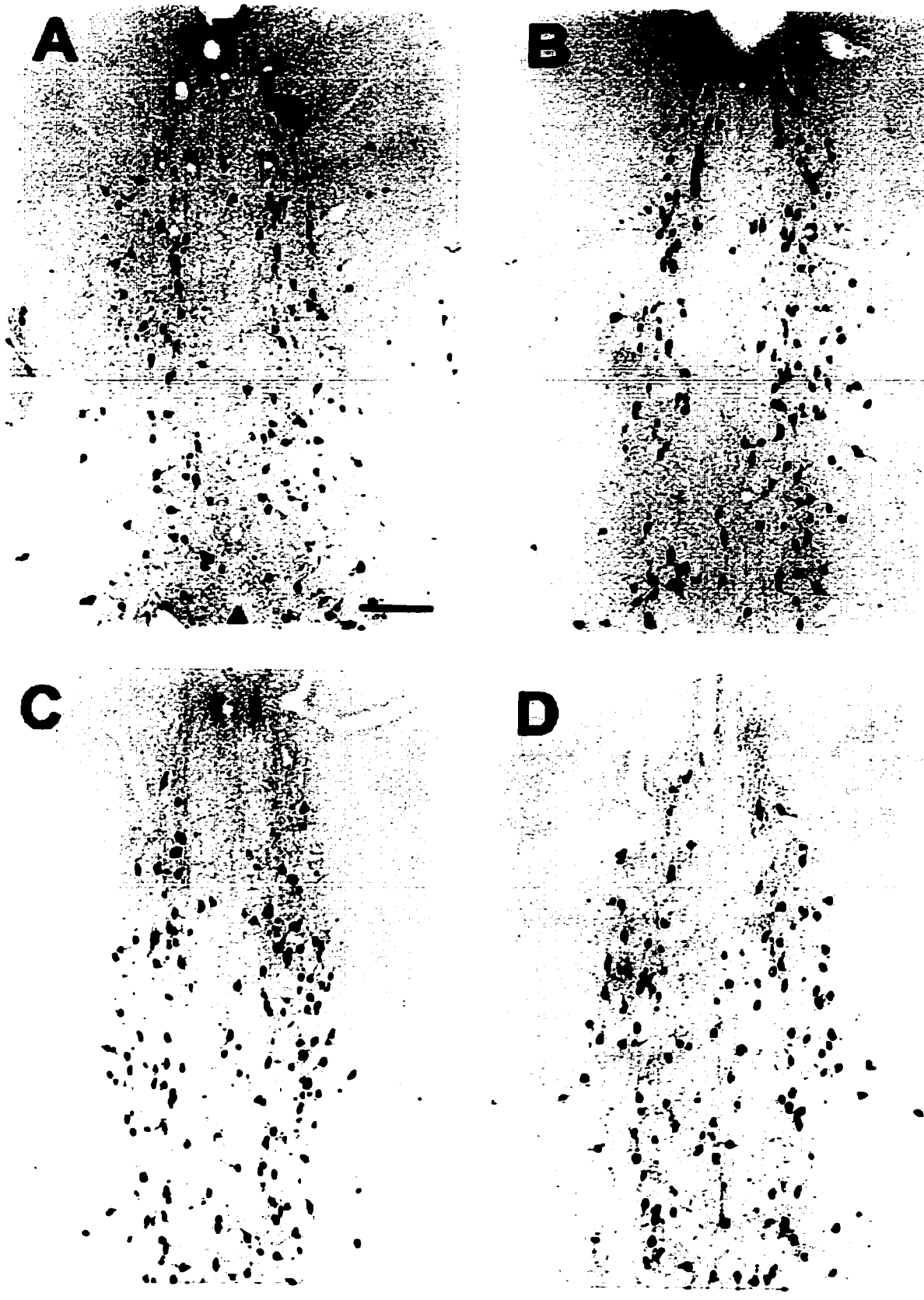


Figure 5.1

($p = 0.06$; 129/Sv 1327 ± 100; $p75^{NGFR}$ -/- 1141 ± 71), or aged groups ($p = 0.15$; 129/Sv 1345 ± 54; $p75^{NGFR}$ -/-1180 ± 92) (Fig. 5.2A). Analyses of the simple effects of age were consistent with the overall effects, such that no differences in the number of cholinergic neurons were observed in control or $p75^{NGFR}$ -/- mice between young, middle aged, and aged groups (Figs. 5.1 and 5.2). The variability in the number of ChAT-positive neurons did not appear to change in control 129/Sv mice. However, the distribution for the $p75^{NGFR}$ -/- animals suggested a sub-group may exist, such that some mice had lower numbers of ChAT-positive neurons than the lowest control mice (Fig. 5.2A). The number of ChAT-positive neurons was similar for male and female mice (129/Sv, 1381 ± 36 vs 1277 ± 73, $p75^{NGFR}$ -/-, 1212 ± 50 vs 1199 ± 98, respectively) and the $p75^{NGFR}$ -/- "sub-group" contained both male and female mice.

Cholinergic cell body size does not change with age in both $p75^{NGFR}$ -/- and control mice

Analyses of the overall effects revealed that the diameter of the cholinergic cell bodies differed significantly between mouse strains ($p < 0.003$, ANOVA, $F(1, 58) = 9.66$) but not with age. Post-hoc analyses of the strain difference suggested that overall, $p75^{NGFR}$ -/- mice have larger cholinergic cell bodies than 129/Sv mice ($p < 0.0026$).

Analyses of the simple effects of strain revealed differences only at the aged time-point, such that the cholinergic neurons of $p75^{NGFR}$ -/- mice had an ~12% greater diameter (19.7 ± 0.4 μm) than those of 129/Sv mice (17.6 ± 0.3 μm) (Fig. 5.2B). No differences were observed between the two strains of mice at the young (129/Sv 18.1 ± 0.5 μm; $p75^{NGFR}$ -/- 19.0 ± 0.6 μm) or middle aged (129/Sv 17.9 ± 0.5 μm; $p75^{NGFR}$ -/- 18.4 ± 0.6μm) time-points. Consistent with the overall effects, the cell body diameter of cholinergic neurons in 129/Sv and $p75^{NGFR}$ -/- mice did not change between young, middle aged, or aged time-points (Fig. 5.2B).

Figure 5.2. The number of cholinergic neurons in the medial septum remains constant and neurons do not atrophy during aging in 129/Sv and $p75^{NGFR}$ -/- mice.

Presented is the total number of ChAT-positive neurons in both hemispheres of the medial septum area (A), the average cholinergic cell body diameter (B) and cross-sectional area (C) for each $p75^{NGFR}$ -/- (open triangles) and 129/Sv (closed triangles) mouse at young, middle aged, and aged time-points. Note that a possible sub-group of $p75^{NGFR}$ -/- mice may exist (A), having fewer neurons than the lowest control value.

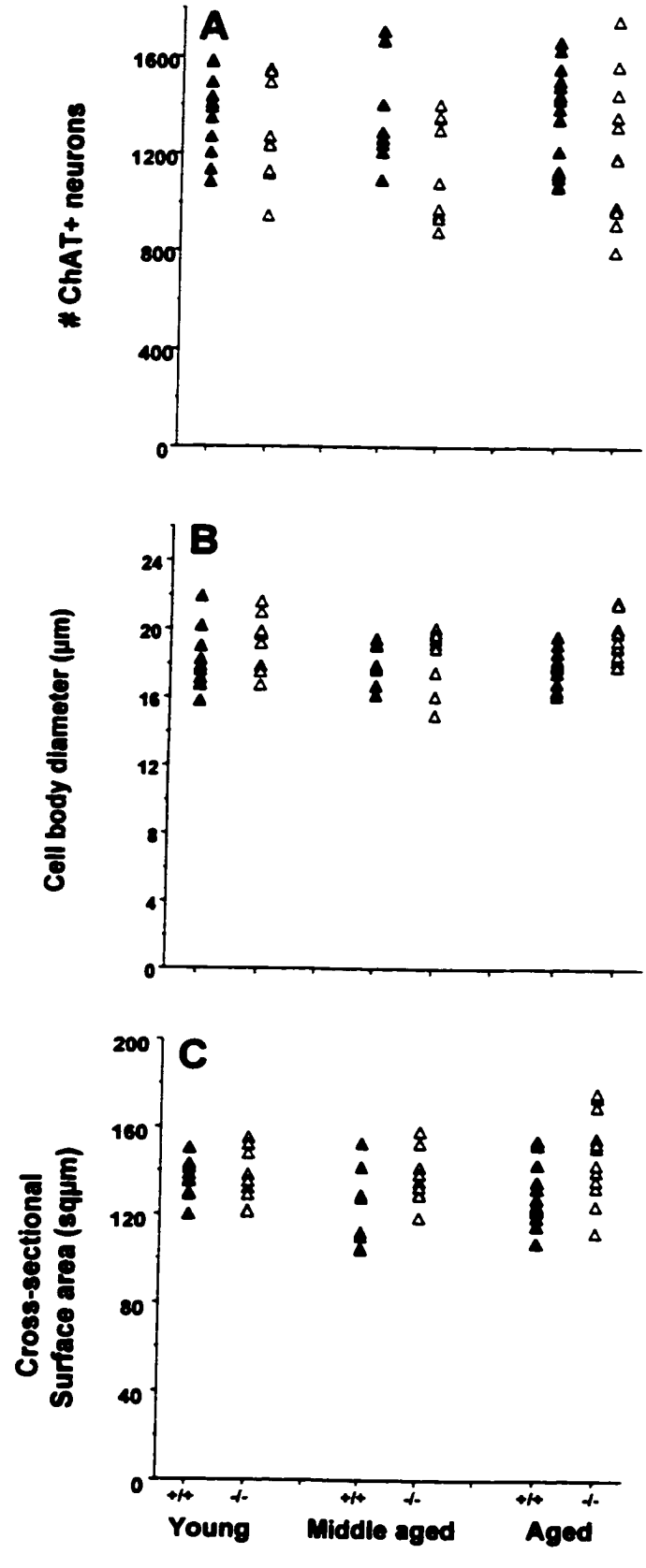

A
ChAT+ neurons
1600
1200
800
400
0
B
Cell body diameter (µm)
24
20
16
12
8
4
0
C
Cross-sectional Surface area (sqµm)
200
160
120
80
40
0
+/+
-/-
+/+
-/-
+/+
-/-
Young
Middle aged
Aged

Figure 5.2

Measurements of cross-sectional area (Fig. 5.2C) confirmed the cell body diameter results. Across all ages, $p75^{NGFR}$ -/- mice had larger cholinergic cross-sectional areas than 129/Sv mice ($P < 0.0029$; $F(1, 58) = 9.71$). Again, no changes in size were found with age.

Analyses of the simple effects of strain revealed differences in cross-sectional area between 129/Sv and $p75^{NGFR}$ -/- mice at the aged time-point only ($p < 0.009$; Fig. 5.2C). The cholinergic neuronal cell bodies of $p75^{NGFR}$ -/- mice had an ~14% larger cross-sectional area (147 ± 7 μm^2) than those of 129/Sv mice ($p < 0.0026$; 129 ± 4 μm^2). At the middle-aged time-point, $p75^{NGFR}$ -/- mice (138 ± 4 μm^2) appeared to have larger cross-sectional areas than 129/Sv mice (123 ± 6 μm^2), however the p-value did not reach significance ($p < 0.065$). No differences were observed at the young time-point (129/Sv 135 ± 3 μm^2; $p75^{NGFR}$ -/- 137 ± 4 μm^2).

For both sets of measures, cell body diameter and cross-sectional area, no sub-groupings were evident, suggesting that all mice aged similarly. In addition, no sex differences were apparent (cell body diameter, 129/Sv, (♂) 17.9 ± 0.3 μm vs (♀) 18.0 ± 0.5 μm, $p75^{NGFR}$ -/-, (♂) 19.0 ± 0.3 μm vs (♀) 19.0 ± 0.8 μm; cross-sectional cell area, 129/Sv (♂) 130 ± 3 μm^2 vs (♀) 127 ± 3 μm^2, $p75^{NGFR}$-/- (♂) 141 ± 4 μm^2 vs (♀) 142 ± 4 μm^2).

Cholinergic innervation of the outer molecular layer of the dentate gyrus is similar in young, middle aged, and aged $p75^{NGFR}$ -/- and control mice

The density of cholinergic fibers in the molecular layer of the dentate gyrus (Fig. 5.3), and the overall size of the hippocampal formation (not shown) appeared similar for control and $p75^{NGFR}$ -/- mice at young, middle aged, and aged time-points. In some mice, AChE staining appeared denser in the inner molecular layer as compared to the outer molecular layer of the dentate gyrus, but was not limited to a specific age or strain.

Figure 5.3. Young and aged $p75^{NGFR}$ -/- and 129/Sv mice have similar densities of cholinergic innervation of the dentate gyrus.

AChE-stained coronal sections through the dentate gyrus of the hippocampal formation at young (A and C) and aged (B and D) time-points. Cholinergic innervation is similar for 129/Sv (A and B) and $p75^{NGFR}$ -/- mice (C and D). Magnification bar = 50 μm. The asterisk indicates the granule cell layer of the dentate gyrus.

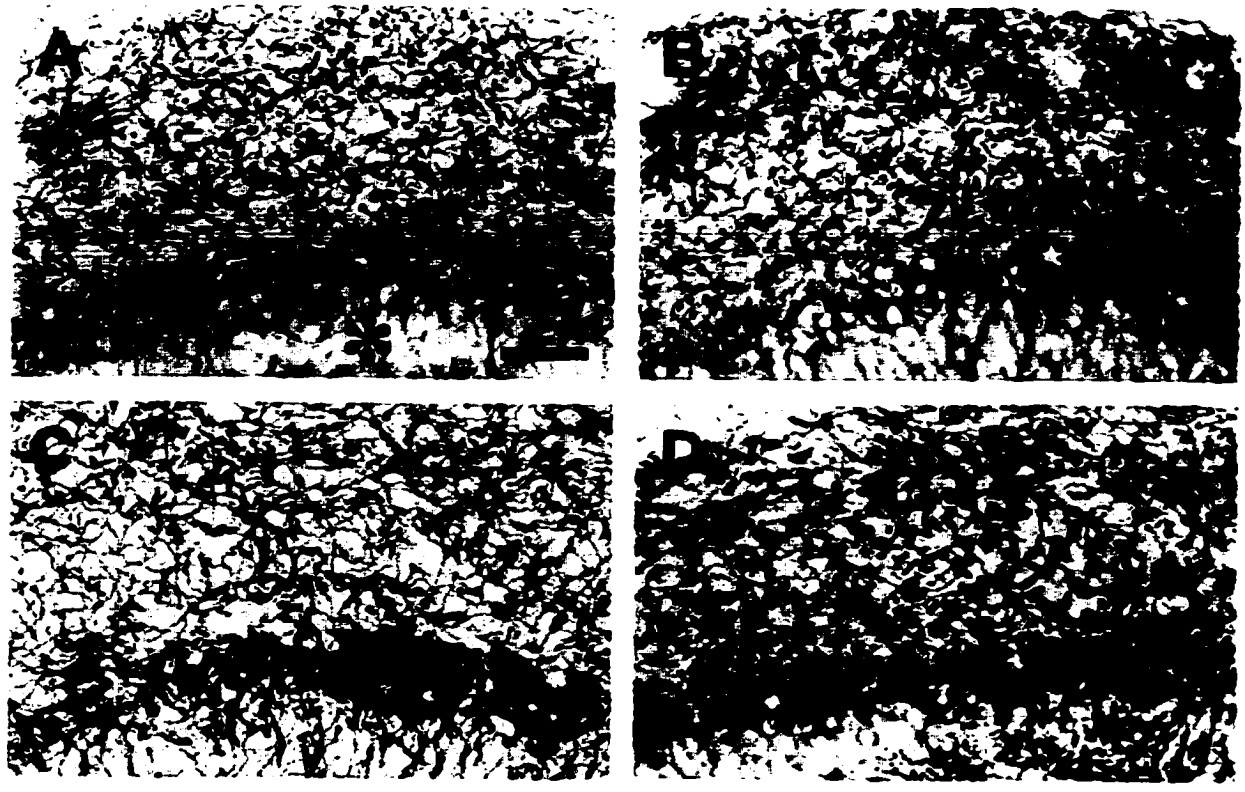


Figure 5.3

The density of AChE-positive fibers in the outer molecular layer of the dentate gyrus was not different between 129/Sv and $p75^{NGFR}$-/- mice, or between young (129/Sv 347 ± 8 fibers per mm; $p75^{NGFR}$ -/- 334 ± 8), middle aged (129/Sv 339 ± 10; $p75^{NGFR}$ -/- 354 ± 13), and aged mice (129/Sv 338 ± 2; $p75^{NGFR}$ -/- 350 ± 8) (Fig. 5.4A). To account for possible changes in the width of the entire molecular layer associated with aging or absence of $p75^{NGFR}$, we measured the width (μm) of this region in the same sections as were used to determine the fiber density. No differences were observed between 129/Sv and $p75^{NGFR}$-/- mice nor between young (160 ± 2 μm; 156 ± 3 μm), middle aged (157 ± 3 μm; 160 ± 2 μm), or aged (157 ± 1 μm; 158 ± 3 μm) mice (Fig. 4B). Again, no differences were observed between male and female mice (density, 129/Sv, (♂) 339 ± 7 fibers per mm vs (♀) 345 ± 15, $p75^{NGFR}$ -/-, (♂) 342 ± 10 vs (♀) 358 ± 16; width, 129/Sv (♂) 158 ± 2 μm vs (♀) 159 ± 3 μm, $p75^{NGFR}$-/- (♂) 158 ± 2 μm vs (♀) 157 ± 3 μm).

Figure 5.4. The density of AChE-positive fibers per millimeter in the outer molecular layer of the dentate gyrus is similar in $p75^{NGFR}$ -/- and control mice at all three time-points.

A) The density of AChE-positive cholinergic fibers intersecting three perpendicularly placed lines over the outer molecular layer of the dorsal blade of the left dentate gyrus was similar for 129/Sv (closed triangles) and $p75^{NGFR}$ -/- mice (open triangles) at young, middle aged, and aged time-points. Values represent the mean sum of 3 measurements per section over four sections through the hippocampal formation of each animal divided by the total length of the linear probe. B) No differences were observed for the width of the entire molecular layer of the dentate gyrus between 129/Sv (closed triangles) and $p75^{NGFR}$ -/- mice in the same tissue sections. Note that similar values may overlap and appear as a single point.

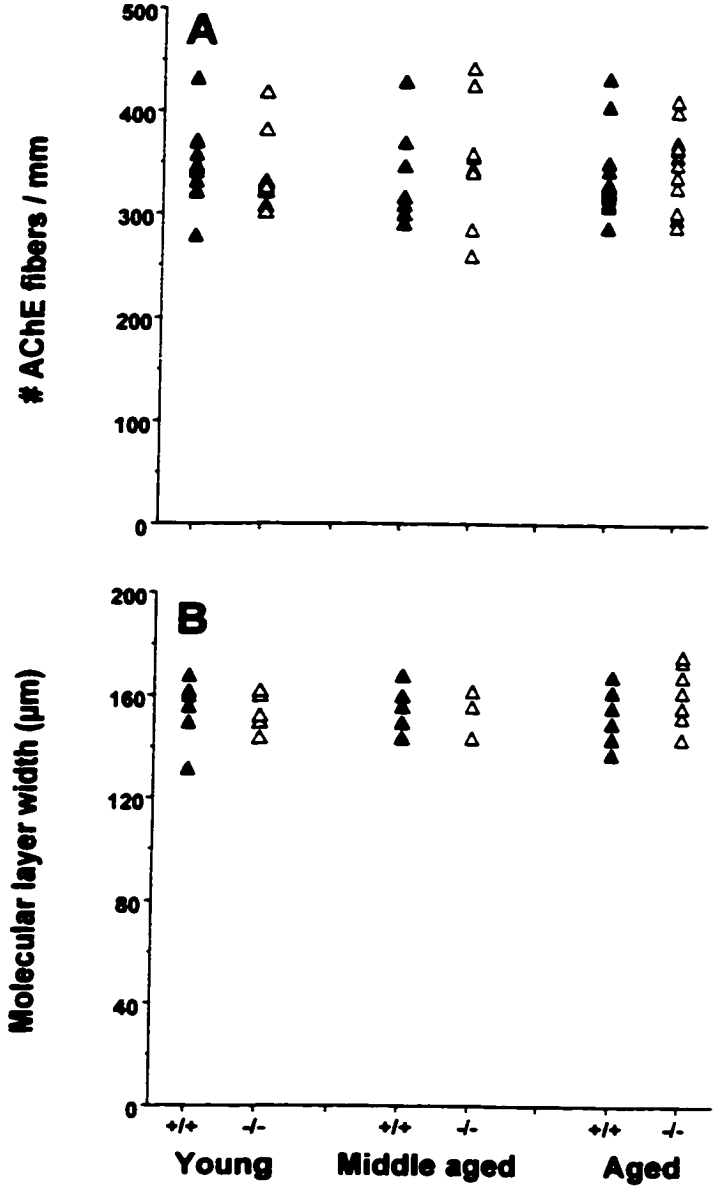

A
AChE fibers / mm
500
400
300
200
100
0
B
Molecular layer width (µm)
200
160
120
80
40
0
+/+
-/-
+/+
-/-
+/+
-/-
Young
Middle aged
Aged

Figure 5.4

DISCUSSION

The present study provides evidence that absence of $p75^{NGFR}$ does not affect the normal aging process of the cholinergic septohippocampal system. In both control and $p75^{NGFR}$ -/- mice: i) the number of ChAT-positive neurons remains constant; ii) cholinergic neuronal cell bodies do not atrophy; and iii) cholinergic innervation of the outer molecular layer of the dentate gyrus remains unchanged.

Medial septum cholinergic neuron numbers do not change in aging control or $p75^{NGFR}$ -/- mice

We report here that the number of ChAT-positive neurons in the medial septum of young, middle aged, and aged $p75^{NGFR}$ -/- and 129/Sv mice does not change with age. This suggests that cholinergic neuron loss or reduction of ChAT below detectable levels does not occur during the normal process of aging in rodents. Finding no change in cell numbers conflicts with previous reports suggesting that cholinergic cell loss was a natural component of the aging process in rats (Altavista et al., 1990; Armstrong et al., 1993; Fischer et al., 1991; Gilad et al., 1987) but confirms other reports in rats (Backman et al., 1996; Cooper et al., 1994; Gustilo et al., 1999; De Lacalle et al., 1996; Lee et al., 1994). The discrepancy among results could reflect differences in techniques or in rodent strains. For example, previous work in rats showing a decrease in cholinergic neurons made use of diisofluorophosphate to detect cholinergic neurons. However, this method does not distinguish between age-related decreases in AChE enzyme levels and actual cholinergic cell loss (Altavista et al., 1990; Fischer et al., 1987; Gilad et al., 1987). However, this explanation is not supported by reports in which ChAT was used as a marker that also indicate cell loss (Armstrong et al., 1993; Fischer et al., 1991). An alternative explanation is the strain of rat used. No change in cell numbers has been consistently reported in Sprague-Dawley (Cooper et al., 1994; De Lacalle et al., 1996; Lee et al., 1994; but see Fischer et al., 1991) and Fischer 344 rats (Backman et al., 1996; Gustilo et al., 1999). In

contrast, cell loss has been reported for Wistar and Brown-Norway strains (Gilad et al., 1987; Altavista et al., 1990).

The number of cholinergic neurons in the medial septum of normal control 129/Sv mice in the present study (~1100-1300) is in the range of that reported by others (~1700 Yeo et al., 1997; ~1200 Fagan et al., 1998; and Dr. J. Long, ~1600, personal communication), who used unbiased stereological quantification methods, and confirms the findings of others for aged CD1 and C57Bl/6 mice (Fagan et al., 1998; Hornberger et al., 1985; Mesulam et al., 1987).

In our studies (Ward and Hagg, 1999a, see Chapter 4, this thesis), we found similar numbers of cholinergic medial septum neurons in $p75^{NGFR}$ -/- and 129/Sv mice. Interestingly, two previous reports that addressed the same question came to opposite conclusions. Thus, Yeo et al. (1997) suggested that $p75^{NGFR}$ -/- mice have more and Peterson et al., (1999) suggested that $p75^{NGFR}$ -/- mice have fewer ChAT-positive neurons. Factors such as differences in methodology, background strain of controls, and breeding strategy may explain these differences (Ward and Hagg, 1999a, see Chapter 4, this thesis).

Endogenous NGF has been shown to be important for the expression of ChAT (Vantini et al., 1989; Mobley et al., 1986). In addition, $p75^{NGFR}$ is necessary for high affinity binding to TrkA signaling (Chao and Hempstead, 1995) and for efficient neurotrophin transport from the hippocampal formation to the cholinergic medial septum neurons (Hagg et al., 1996). Thus, it was somewhat unexpected that in $p75^{NGFR}$ -/- mice the neurons of the medial septum maintain their ChAT expression with age, with the exception of a small sub-population of $p75^{NGFR}$-/- mice that had fewer neurons. Both NGF and TrkA-deficient mice also have ChAT-positive basal forebrain neurons (Chen et al., 1997; Fagan et al., 1997). Thus it is possible that an alternative means of trophic support may compensate in $p75^{NGFR}$ -/-, NGF -/-, and TrkA -/- transgenic mice, such as the utilization of other growth factors.

Another explanation for no age-related changes in $p75^{NGFR}$ -/- mice is the finding that these mice have increased levels of ChAT as young adults (Yeo et al., 1997). In other words, even if age-related reductions in ChAT were greater in $p75^{NGFR}$ -/- mice, this might not be detectable using immunostaining procedures. These caveats related to the nature of transgenic mice, therefore, leave open the possibility of reduced NGF transport in Alzheimer's disease (Mufson et al., 1989; Higgins and Mufson, 1989; Mufson et al., 1995) where cholinergic basal forebrain neurons have reduced levels of ChAT and do atrophy.

Cholinergic neurons do not atrophy with age in control or $p75^{NGFR}$ -/- mice

In both 129/Sv and $p75^{NGFR}$ -/- mice, cell body size as measured by diameter and cross-sectional area did not change with age. This is in contrast to previous reports, which suggested that cell body atrophy occurs with age in C57/Bl6 (Fagan et al., 1998) and CD1 mice (Mesulam et al., 1987). The difference in results is probably not due to the age of the mice, as the present study analyzed mice of similar age, i.e., ~24-25 months of age to those by Fagan et al. (1998) and Mesulam et al. (1987). Moreover, Hornberger and colleagues (1985) analyzed both cell body diameter and surface area in exceptionally old C57Bl/6NNIA mice at 53 months of age, and did not find cell atrophy. In the present study, it remains possible that in the 129/Sv mouse strain, cell body atrophy could occur in very aged animals. We did not analyze mice older than ~23 months of age, by which time they become frail. The cell area measures (~130 μm^2) of the present study are in the same range of what others report for young control mice (~130 μm^2 Mesulam et al., 1987; ~125 μm^2 Yeo et al., 1997, ~150 μm^2 Fagan et al., 1997). Thus it is unlikely that methodological considerations account for the differences among studies. Rather, the differences may be due to strain differences. We used 129/Sv mice while others have used CD-1 (Mesulam et al., 1987), C57Bl/6 mice (Fagan et al., 1998, but see Hornberger et al., 1985). In a recent study, no atrophy was found for the

cholinergic medial septum neurons in aged Fischer 344 rats (Gustilo et al., 1999), which is consistent with previous reports in cognitively impaired Sprague Dawley rats (Fischer et al., 1991; Lee et al., 1994). However, others have reported atrophy in similar and different strains (Altavista et al., 1990; Backman et al., 1996; Cooper et al., 1994; Fischer et al., 1987; De Lacalle et al., 1996). Thus, strain differences may not account for all the discrepancy.

Lastly, no age-related morphological changes in the current control mice is consistent with no change in learning- and memory-related behaviours (see Stanford et al., 1998)

$p75^{NGFR}$ -/- mice have larger neurons than control mice

The cell body size of the ChAT-positive neurons of $p75^{NGFR}$-/- mice was significantly larger than that of 129/Sv mice only in the aged group. We (Ward and Hagg, 1999a, see Chapter 4, this thesis) and others (Yeo et al., 1997) have reported that young adult $p75^{NGFR}$ -/- mice have larger cholinergic medial septum neurons than control mice. In the latter experiments, young mice were examined ~4 – 5 months of age, whereas in the current studies we used mice between 6-8 months for the "young" time-point. During normal development, a transient increase in cell body size is observed between P6 and P15 followed by a decrease to adult levels (Gould et al., 1991). In $p75^{NGFR}$ -/- mice, this decrease to adult levels at ~3-5 months is less than in control mice (Ward and Hagg, 1999a; see Chapter 4, this thesis). Therefore, it is conceivable that $p75^{NGFR}$ -/- mice may mature more slowly, reaching their "adult" sizes at 6 – 8 months.

Cholinergic hippocampal innervation remains constant during aging and in the absence of $p75^{NGFR}$

Cholinergic medial septum neurons project their axons to the hippocampal formation and, therefore changes in the cholinergic innervation might also be expected to be reflected in aging animals if cholinergic neurons differ with age. In our study, the density

of AChE-positive fibers in the outer molecular layer of the dentate gyrus and the width of the molecular layer remained unchanged through young, middle aged, and aged time-points. This is consistent with the lack of atrophy of the cholinergic medial septum neurons and the idea that cholinergic cell body size may relate to the total volume of their processes (Koh and Loy, 1989). On the other hand, others (Fagan et al., 1998) have reported an increase in cholinergic fiber density in normal aging mice, despite atrophy of the medial septum cholinergic neurons. A possible explanation for the apparent discrepancy between our studies and that of Fagan and colleagues (1998) may be due to technical factors: Fagan and colleagues (1998) used a cycloid probe designed for use in "vertical" sections (sections in which the sectioning plane has been randomized; Gokhale, 1990) in coronal sections, and no corrections for tissue shrinkage appear to have been used. It should be noted that currently available techniques for quantification of a structure in a single (e.g., coronal) plane, including the use of density measures, do not consider the three dimensional nature of such structures. The necessity of analyzing neural structures, in particular fibers, in a variety of randomly sectioned planes is impractical, as this makes identification of anatomical structures difficult. A new method able to measure the total length of fibers in standard "anatomical" sections is currently under development (Mouton et al., 1999).

The cholinergic fiber density of $p75^{NGFR}$-/- mice did not differ from control mice. A previous report (Yeo et al., 1997), has provided qualitative evidence that young adult $p75^{NGFR}$ -/- mice have an increase in ChAT-immunostaining in the molecular layer of the dentate gyrus and quantitative evidence for an increase in the number of ChAT-immunoreactive fibers in the CA1 region of the hippocampus. However, these authors also report an increase in the number of ChAT-positive medial septum neurons.

The density of cholinergic fibers in the dentate gyrus of $p75^{NGFR}$-/- mice did not change during aging, nor did it change in control mice. Thus, the lack of $p75^{NGFR}$ does not appear to make neurons and axons more vulnerable to the aging process. As

discussed above, alternative strategies for trophic support may develop to compensate for the reduced efficiency of neurotrophin binding and transport following genetic disruption of $p75^{NGFR}$. On the other hand, neurotrophins may not be involved in maintenance of axonal projections during adulthood and aging.

In summary, the results of the present investigation show that the number and size of cholinergic neurons in the medial septum and their innervation of the hippocampal formation do not change during aging, and that the absence of $p75^{NGFR}$ does not have an effect on these morphological parameters.

CHAPTER 6:

GENERAL DISCUSSION

Summary of the work

The current body of work was initiated to identify the ligand that binds to $p75^{NGFR}$ and induces apoptotic cell death of a sub-population of developing cholinergic forebrain neurons, and then to identify the intracellular signaling pathways involved in this death. In addition, the *in vivo* role of $p75^{NGFR}$ during the degeneration of cholinergic medial septum neurons during aging was to be determined.

In an attempt to determine the putative $p75^{NGFR}$-neurocidal ligand, possibly a neurotrophin, for the developing cholinergic forebrain neurons, we determined that these neurons did not die through developmentally-regulated apoptosis, and that the number of ChAT-positive neurons in the medial septum and neostriatum actually increased postnatally in BDNF +/+ mice. The cholinergic neurons of the basal forebrain, the cholinergic septohippocampal system in particular, of BDNF -/- mice did not increase in number or size beyond P6, suggesting that BDNF was necessary for normal development of these regions. This is consistent with the expected survival promoting effects of neurotrophic factors. Concurrent with these experiments, we examined the activities of two specific signal transduction pathways in cholinergic cell populations of the developing and injured forebrain in control and $p75^{NGFR}$-deficient mice. Previous *in vitro* and *in vivo* work had shown that $p75^{NGFR}$ could transduce an intracellular signal through phosphorylation of JNK, c-Jun, and p53, and could induce nuclear translocation of the transcription factor $NF_{\kappa}B$ (Aloyz et al., 1998; Bamji et al., 1998; Carter et al., 1996; Cassacia-Bonnefil et al., 1996; Dobrowsky et al., 1994; Frade et al., 1996; Majdan et al., 1997). In spite of this, we found no differences in the phosphorylation activities of SEK1 (JNKK/ MKK4), c-Jun, or the nuclear translocation of $NF_{\kappa}B$ between control and $p75^{NGFR}$-/- mice. We also observed no differences in TUNEL labeling (as an indicator of apoptosis) at P8, suggesting that $p75^{NGFR}$ did not induce cell death in a sub-population of cholinergic medial septum neurons, and that in the absence of cell death, $p75^{NGFR}$ did not signal through activation of SEK1 or c-Jun. However, we did observe differences in

activation and predicted patterns of SEK1 and c-Jun between developing and injured neurons, suggesting a different role for those signaling molecules. Because we could not replicate previous reports within our own laboratory (Van der Zee et al., 1996; Hagg et al., 1997; Van der Zee and Hagg, 1998), we re-examined the role of $p75^{NGFR}$ during postnatal development. Previously, it was reported that the number of ChAT-positive medial septum and neostriatum neurons of control mice decreased postnatally, and was due to $p75^{NGFR}$-mediated apoptotic cell death. The study of the brains of new groups of $p75^{NGFR}$ -/- mice revealed that $p75^{NGFR}$ did not induce cell death in a sub-population of cholinergic neurons in the forebrain. Rather, we found that the total number of ChAT-positive neurons in the adult $p75^{NGFR}$ -/- mouse was similar to that of control mice. Interestingly, the present results suggest that $p75^{NGFR}$ may, in fact, negatively regulate cell size, ChAT and AChE activities in the cholinergic septohippocampal system at P6. We also examined the role of $p75^{NGFR}$ during aging. Evidence suggests that during aging, cholinergic neurons can atrophy, TrkA mRNA levels decrease, and NGF transport is reduced (Cooper et al., 1994; Fagan et al., 1998; Fischer et al., 1987; Mesulam et al., 1987). However, we found no changes in cholinergic septohippocampal anatomy related to aging for either control 129/Sv or $p75^{NGFR}$ -/- mice, suggesting that $p75^{NGFR}$ or neurotrophin transport does not play significant roles in the cholinergic septohippocampal system during aging. Alternatively, it may be that $p75^{NGFR}$ -/- mice have compensatory mechanisms, including support by growth factors other than neurotrophins.

BDNF-induced neural protection in the cholinergic forebrain

We demonstrated that BDNF plays a significant role in maintaining the phenotype and possibly the survival of the cholinergic neurons of the both the medial septum and neostriatum. Historically, NGF has been the most studied neurotrophin in the cholinergic forebrain neurons but both NGF and BDNF appear to be necessary for the normal development of the cholinergic medial septum and neostriatum. The trophic effects of

BDNF on motor and midbrain neurons has been shown (for reviews see Lindsay, 1994; Mufson et al., 1999; Sendtner et al., 1996). Moreover, the therapeutic potential of BDNF for preventing cell loss associated with ALS and Parkinson's disease has been tested in animal models of these pathologies (Gimenez y Ribotta et al., 1997; Hagg, 1998; Kaal et al., 1997; Son et al., 1999), and in clinical trials (currently in progress). In Alzheimer's disease (AD), in which cholinergic neurons of the forebrain shrink and are lost, retrograde transport of neurotrophins may be reduced (Higgins and Mufson, 1989). These cholinergic neurons express TrkB and are BDNF responsive. In addition, BDNF and TrkB levels decrease in aged rodents (Croll et al., 1998; Katoh et al., 1998) and in AD brains (Connor et al., 1997), suggesting that loss of BDNF trophic support contributes to the pathology. Recent studies in animals (Pan et al., 1998; Wu and Partridge, 1999) have shown that BDNF delivery to the CNS can now be accomplished via intravenous injection, offering therapeutic strategies for treating cholinergic-related neurodegenerative disease without the use of craniotomy and cannulation of the lateral ventricle. Thus, therapeutic treatment with BDNF may help prevent or delay cholinergic cell death in AD.

In the present studies, the cholinergic medial septal neurons in BDNF -/- mice did not increase in size or number postnatally, compared to control littermates, and the density of cholinergic fibers innervating the hippocampal formation did not increase, which is not surprising considering the number of projecting cholinergic neurons in the medial septum of BDNF -/- mice did not increase postnatally. Nonetheless, the extent of the reduced cholinergic innervation suggests that the reduction is likely due to more than just the reduced number of projection neurons. It is plausible that BDNF -/- mice also have reduced sprouting and synapse development, contributing to the reduction in cholinergic fiber innervation in the dentate gyrus. Consistent with this suggestion is the finding that BDNF is involved in synapse development, sprouting, and synaptic and dendritic plasticity (Causing et al., 1997; Gottschalk et al., 1998). Although we did not evaluate

any behavioral deficits associated with these neuroanatomical deficiencies, it may be that the spatial and cognitive performance of these mice is compromised. The region of the brain implicated in spatial and cognitive tasks, specifically learning and memory, is the hippocampal formation. BDNF is expressed in the hippocampus and BDNF mRNA levels increase following Morris water maze training in regions directly associated with spatial memory processing (Kesslak et al., 1998). Therefore, exogenous BDNF may offer strategies not only for prevention of cell loss associated with neurodegeneration, but also for enhancement of spatial cognition.

Intracellular signaling

We studied the activation of a proposed specific $p75^{NGFR}$ mediated intracellular signaling pathway in an attempt to understand the downstream mechanisms underlying $p75^{NGFR}$-induced apoptosis. Contrary to previous *in vitro* findings (for review see Frade and Barde, 1998; Kaplan, 1998; Kaplan and Miller, 1997), we showed that $p75^{NGFR}$ does not differentially activate members of the JNK cascade in either developing or injured mouse cholinergic medial septum neurons. Although we considered several reasons for this finding, the most likely explanation is that these proteins are activated by $p75^{NGFR}$ (only) in models of cell death, but not in the absence of cell death. We determined that the system we analyzed lacked $p75^{NGFR}$-induced cell death. In cell populations in which $p75^{NGFR}$-mediated apoptosis occurs, i.e., sympathetic neurons of the SCG (Aloyz et al., 1998; Bamji et al., 1998) and neurons in the developing retina (Frade et al., 1996; Frade and Barde, 1998), JNK, c-Jun, and p53 are activated, and the prevention of their activation can prevent apoptosis. Our results suggest that $p75^{NGFR}$ does not signal via these proteins in the absence of cell death.

In our *in vivo* study of the $p75^{NGFR}$-mediated signaling cascades, we determined that SEK1 has a critical role during postnatal maturation and development, but not following injury. In addition, SEK1 activity does not necessarily predict downstream c-Jun

phosphorylation, consistent with the idea that alternative downstream proteins, such as p38, exist. C-Jun activity also appears to be able to signal in the absence of SEK1 activity. This difference between signaling proteins suggests a complexity to these signal transduction pathways that remains to be determined. Recent progress in the field of signal transduction has shown that *in vitro* events are oversimplified. Our results indicate the complexities of working in *in vivo* systems. *In vitro* models using cell lines and purified cells are not complicated by the existence of multiple cell types and cell interactions. More interesting, and representative of a normal context, organ and endocrine interactions, multiple-system communication and environmental stress all affect intra- and inter-cellular communication in the whole animal. This may explain why well-characterized *in vitro* events often cannot be replicated in the whole animal. Further complexity in signal transduction is introduced with specific context, such as during development, senescence, or injury.

One intracellular protein can act upstream or downstream from other proteins in multiple signaling cascades. Similarly, specific cascades can be activated by multiple receptors. Moreover, there are numerous points at which activation or inhibition of these signaling cascades can occur. Thus, the ability to identify a specific intracellular signaling cascade, activated by a specific transmembrane receptor, localized to a specific population of nervous system cells, may be difficult to achieve. This shouldn't however, prevent the study of this hypothesis, as the relevance and importance of understanding the signaling mechanism behind a biological effect, is of extreme importance.

Ultimately, understanding cell communication will provide knowledge and insight across multiple disciplines, including that of immunology, pathology, cell biology, and neurobiology. Comprehension of the molecular mechanisms underlying intra- and intercellular communication in normal biology is intended to provide increased insight into the causes of pathology. Understanding a cell's natural ability to develop, establish itself, and thrive is important for understanding why a cell degenerates or dies. The

understanding of the signaling process provides information and insight into cell behaviour, and may eventually be used as biological markers. Instead of waiting for post-mortem results, changes in signaling protein and transcription factor levels in CSF, plasma, and tissue samples may offer additional and alternative methods of diagnosis for specific diseases.

Role of $p75^{NGFR}$ in the cholinergic forebrain during early postnatal development, following injury in adults, and during senescence

The roles of $p75^{NGFR}$ include one that is Trk-dependent, i.e., increasing receptor affinity for a specific neurotrophin; and another that is Trk-independent, i.e., inducing apoptotic cell death (Bredesen and Rabizadeh, 1997). The cholinergic neurons in the mouse medial septum are a useful model system in which to study these, as they contain Trk (A, B, and C) and $p75^{NGFR}$ (Altar et al., 1994; Escandon et al., 1994; Fryer et al., 1996; Gibbs and Pfaff, 1994; Hefti et al., 1986; Holtzman et a., 1992; Lee et al., 1998; Sobreviela et al., 1994; Nonomura et al., 1995). We studied the cell fate of these neurons during postnatal development, following injury in adult mice, and during senescence in the presence and absence of $p75^{NGFR}$.

Previous reports (Van der Zee et al., 1996; Hagg et al., 1997; Van der Zee and Hagg, 1998) reported $p75^{NGFR}$-induced cell death in a subpopulation of cholinergic medial septum and neostriatal neurons. We found that $p75^{NGFR}$ did not induce (apoptotic) cell death in the developing cholinergic medial septum or neostriatum. Our findings suggest that during postnatal development, $p75^{NGFR}$ can negatively regulate the immunohistological detectability of medial septum cholinergic neurons and possibly cell size. In contrast to control mice which reached adult cholinergic medial septum neuron values at P15, $p75^{NGFR}$ -/- mice reached adult levels at P6. No differences in the total number of cholinergic medial septum neurons were found between adult control and $p75^{NGFR}$ -/-mice. Conflicting findings to these have been reported, with more (Yeo et al.,

1997) and fewer (Peterson et al., 1997, 1999) neurons reported for $p75^{NGFR}$ -/- mice. However, these strains of $p75^{NGFR}$ -/- mice were generated and maintained differently than the mice we used. Resolution of the role of $p75^{NGFR}$ in cholinergic forebrain neurons in normal young adults may become clear following the generation of new mice and re-analysis of the region. If $p75^{NGFR}$ is involved in regulating ChAT enzyme levels, as reported by Yeo and colleagues (1997), the expectation would be to see larger neurons and increased ChAT activity in $p75^{NGFR}$ -/- mice. Conversely, if fewer and smaller neurons were observed, this would be consistent with the idea that $p75^{NGFR}$ is necessary for cell development and maturation.

$p75^{NGFR}$ may cause cell death of a subpopulation of neurons in the medial septum. For this to occur, $p75^{NGFR}$ would need to be localized to neurons in the absence of Trk (A, B or C). Interactions between downstream intracellular proteins activated by $p75^{NGFR}$ (JNK) and Trk (MAPK) result in the MAPK inhibition of JNK phosphorylation, resulting in either a trophic or mitogenic response (Xia et al., 1995). In order for $p75^{NGFR}$ to induce cell death through this specific pathway (which has repeatedly been shown to be involved in $p75^{NGFR}$-mediated apoptosis), $p75^{NGFR}$ must exist in the absence of a MAPK activating receptor (such as Trk). Because cholinergic forebrain neurons can express multiple Trk receptors, a neuron may not express TrkA but alternatively may express TrkB or TrkC (Altar et al., 1994; Escandon et al., 1994; Fryer et al., 1996; Nonomura et al., 1995). Thus, very few neurons express $p75^{NGFR}$ in the absence of a Trk receptor, making it unlikely that a $p75^{NGFR}$-mediated death signal and consequent cell death could occur in neurons expressing both high and low affinity neurotrophin receptors. $p75^{NGFR}$-mediated cell death (in the developing retina and SCG; Aloyz et al., 1998; Bamji et al., 1997; Frade and Barde, 1998; Frade et al., 1996) occurs during the period of naturally occurring programmed cell death (Burek and Oppenheim, 1996). This suggests that the presence of $p75^{NGFR}$, in the absence of Trk, controls the development of these specific cell populations. The cholinergic neurons of the medial

septum do not represent a region of the CNS known to undergo developmental programmed cell death (Semba, 1992), providing further support for the finding that $p75^{NGFR}$ does not mediate apoptotic cell death in a subpopulation of cholinergic forebrain neurons.

Consistent with this was our observation that SEK1, c-Jun, and NF_KB signaling was not different between control and $p75^{NGFR}$ -/- mice during development and following injury. In fact, other than increased cell body size in the young (3-5 month old) $p75^{NGFR}$ -/- mice, no apparent differences were detected between adult animals, including their response to fimbria fornix transection. These findings support the idea that $p75^{NGFR}$ may have little to do with maintenance of the cholinergic medial septum neurons. Nonetheless, this does not rule out the possibility that another gene, perhaps encoding a growth factor, is upregulated in response to the deletion of $p75^{NGFR}$, or that homologues to $p75^{NGFR}$ exist and can compensate for $p75^{NGFR}$ absence.

The similar patterns of immunostaining in $p75^{NGFR}$ -/- and control mice for phosphorylated SEK1, c-Jun, and nuclear NF_KB during development and following an injury in adulthood may reflect the presence of a recently described functional splice variant of $p75^{NGFR}$ (Dechant and Barde, 1997). This splice variant consists of the first cysteine-rich repeat of the extracellular domain, the transmembrane domain, and the intracellular domain of $p75^{NGFR}$. This splice variant is expressed in the current $p75^{NGFR}$ -/- mice (Dechant and Barde, 1997), and may be able to transduce a signal on its own. Transgenic mice overexpressing the intracellular portion of $p75^{NGFR}$ have increased activation of JNK and NF_KB, and increased death of neurons normally and not normally expressing $p75^{NGFR}$ (Majdan et al., 1997). In addition, $p75^{NGFR}$ can signal without neurotrophin binding (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Barrett and Georgiou, 1996). Therefore, until mice are generated with the complete removal of the $p75^{NGFR}$ gene, including all splice variants, or an alternate approach is taken, such as using blocking antibodies, peptides, or oligonucleotides, it will be difficult to determine

whether $p75^{NGFR}$ has a trophic, apoptotic, or no significant role at all in the developing and injured cholinergic neurons in the forebrain.

In addition to the study of $p75^{NGFR}$ in the developing and injured cholinergic septohippocampal system, we also studied $p75^{NGFR}$-influences in this region in senescent mice. Previous reports suggested that in aged mice, cholinergic medial septum neurons atrophy (Fagan et al., 1998; Fischer et al., 1987; Mesulam et al., 1987), and have reduced NGF transport, two events in which $p75^{NGFR}$ is involved. In this thesis no atrophy of the cholinergic medial septum neurons and no decreases in cholinergic innervation of the dentate gyrus were found in young and aged control and $p75^{NGFR}$ -/- mice. These findings confirm our previous results that showed no difference in the number of cholinergic medial septum neurons in control and $p75^{NGFR}$ -/- mice in young adulthood (~3 months) and in adults undergoing fimbria fornix transection. These results conflict with the proposed Trk-dependent role of $p75^{NGFR}$, i.e., that $p75^{NGFR}$ enhances high-affinity binding of neurotrophins to their cognate Trk receptor. Our findings suggest that Trk receptors can maintain efficient neurotrophin binding and signal transduction in the absence of $p75^{NGFR}$ during injury and in aging. Alternatively, other growth factors or cytokines may compensate for $p75^{NGFR}$ withdrawal and the consequent change in trophic support.

Methodological considerations

During the current body of work, limitations and problems with currently existing methodology became evident. Transgenic technology has provided the scientific community a better understanding of the role of specific proteins in development, pathology, and physiological functioning. This has been accomplished by removing, adding, or altering the efficacy of a gene, and analyzing changes in structure, anatomy, morphology, or functioning. However, limitations exist when mice are not maintained on the same embryonic stem cell line or parental strain of mice. For example, three different

$p75^{NGFR}$ -/- mouse (sub) strains appear to exist. The mice we used were from The Jackson Laboratory and maintained by breeding homozygous knockout mice. The mice used by Peterson et al. (1997; 1999) were from the original stock generated in the laboratory of Dr. R. Jaenisch (Lee et al., 1992) and the colony was maintained by breeding heterozygote mice. A third group (Yeo et al., 1997) purchased homozygous deficient mice from The Jackson Laboratory, and back-bred these into Balb/c controls, analyzing the F2 offspring of heterozygote breeding couples. The lack of standardized techniques for breeding and maintaining a transgenic colony of mice made comparison of results across research groups difficult.

In addition to the inherent problems of breeding and maintaining transgenic lines of mice, further complications arise due to limitations in standards for collecting, counting, and measuring anatomical structures. It was hoped that with the advent of stereology these issues would be resolved (for review see West, 1999). This has not yet been the case. Different laboratories have reported different values for control mice (Peterson et al., 1997; 1999 vs. Yeo et al., 1997, Fagan et al., 1996; 1998), and the same laboratory has reported different numbers for identical mice (Peterson et al., 1997 vs. Peterson et al., 1999). In addition, one lab has reported very different numbers for different control mice (Fagan et al., 1996 vs. Fagan et al., 1998). This may reflect technique, training, or an understanding of the proper use and interpretation of the stereological technique. Until these issues are resolved, it will continue to be difficult to compare quantitative data across laboratories.

Future directions

The further study and application of BDNF as a therapeutic tool for treatment and prevention of neurodegenerative disease will be necessary to determine if BDNF can be used effectively to treat AD and other diseases affecting the cholinergic septohippocampal systems. In addition, there is still a need for more basic research into

cell communication. Clearly, there is strong potential for knowledge based on the signal transduction field to offer alternative therapeutic strategies and lead to the development of new diagnostic assays. These will further enhance the understanding of the normal developmental and aging processes, in addition to the mechanisms underlying pathological events.

Understanding the role of $p75^{NGFR}$ in the developing, injured, and senescent cholinergic forebrain continues to be controversial. The generation of new $p75^{NGFR}$ -/- transgenic mice seems to be an important step. In addition, it is imperative that $p75^{NGFR}$ -/- mice be bred and maintained with attention to physiological and genetic controls and have their anatomy analyzed by individuals skilled in application and interpretation of stereological techniques. For researchers interested in the role of $p75^{NGFR}$ in the cholinergic forebrain and $p75^{NGFR}$-mediated signaling in the medial septum, the effect of deleting the entire gene, including that which encodes the splice variant will be of great interest. In particular, the comparison of signaling in the different strains of $p75^{NGFR}$ -/- mice will offer insightful information regarding the potential role and function of the $p75^{NGFR}$ splice variant. In fact, pharmacological intervention of the signaling ability of this variant or portions of the intracellular domain of $p75^{NGFR}$ may provide additional therapeutic targets.

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