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The Role of Brain-Derived Neurotrophic Factor (BDNF) in Development and Survival in the Mammalian Nervous System

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ABSTRACT:

The nerve growth factor family of neurotrophins play multiple roles in both the developing and mature mammalian nervous systems. During development, neurotrophins are typically thought to act as retrogradely-derived signals that mediate the survival of innervating neurons. However, recent studies have highlighted different mechanisms of delivery and multiple functions for certain neurotrophins, extending their scope of biological activity beyond that suggested by the neurotrophic hypothesis. This thesis highlights two novel roles for the neurotrophin, brain-derived neurotrophic factor (BDNF), in the mammalian nervous system, and the mechanisms by which these events are mediated.

First, we show that BDNF is anterogradely transported in central noradrenergic neurons to regulate the development of target neuron populations such as the neocortex, and to mediate the survival of injured motor neurons. This provides the first evidence of a functional role for anterogradely-derived BDNF during the survival and development of target neuron populations. Two transgenic mouse constructs were used to determine the role of anterogradely-derived BDNF; the previously characterized dopamine β-hydroxylase (DBH):BDNF transgenic mice, and Tα1:nlacZ transgenic mice which have been extensively characterized within the scope of this thesis.

Second, we show that BDNF mediates apoptosis via the p75NTR in neurons lacking TrkB. Indeed, BDNF-mediated p75NTR activation results in increased sympathetic neuron death *in vitro*, and ablation of BDNF and p75 *in vivo*, (BDNF-/- or p75-/- mice), results in an increased number of SCG neurons following the period of naturally occurring cell death. Together, these data indicate that p75NTR can mediate apoptosis, and that this mechanism is essential for naturally-occurring cell death.

We also show that the tumor suppressor protein, p53, is essential during naturally-occurring sympathetic neuron death, and that p53 protein levels are increased following both BDNF-mediated activation of p75, and NGF withdrawal. Moreover, we postulate that both the p75-mediated and NGF withdrawal-induced upregulation of p53 is mediated by the MEKK-JNK pathway. The p53 tumor suppressor protein has been shown to play an apoptotic role in several cell types, including in neurons exposed to external stressors, such as excitotoxicity and DNA damage. This is the first time p53 has been demonstrated to play an apoptotic role during the development of the mammalian nervous system.

RÉSUMÉ:

Les neurotrophines de la famille de "nerve growth factor (NGF)" sont des polypeptides très importants jouant des rôles multiples dans le développement et le maintient du système nerveux mammifière. Au cours du développement, les neurotrophines agissent de manière rétrograde et médient la survie des neurones qui inervent la cible. Cependant, diverses rècentes études ont démontré que certaines neurotrophines pouvaient être transportées des mécanismes diffèrents et possèder des fonctions plus vastes que celles suggérées par l'hypothèse classique des neurotrophines.

Cette thèse doctorale met eu évidence deux nouveaux rôles de "brain-derived neurotrophic factor (BDNF)", un membre de la famille des neurotrophines, ainsi que les mécanismes par lesquels cette neurotrophine médie ces fonctions.

Premièrement, nous demonstrons que BDNF est transporté de manière antérograde dans les neurones noradrènergiques, dans le but de règuler le developpement de populations de neurones cibles, telles les neurones du neocortex. De plus BDNF permet la survie de neurones moteurs blessées. Ce sont les premières évidences démontrant que BDNF transporté de manière antérograde joue un rôle dans la survie et le développement de neurones-cibles.
Deux lignées de souris transgéniques ont été utilisées pour ces études. Une lignée exprimant le transgène dopamine β-hydroxylase (DBH):BDNF ainsi qu'une lignée exprimant le transgène Tα1:nlacZ ont été caractérisées en detail dans cette thèse.

Deuxièmement, on démontre que BDNF cause l'apoptose, en activant le récepteur membraine p75, de neurones n'exprimant pas le recepteur TrkB. De plus, l'activation de p75 par BDNF cause une augmentation de l'apoptose des neurones sympathiques en culture. L'absence de BDNF et de p75 *in vivo* (souris knockout BDNF-/-, p75-/-) rèsulte en une augmentation du nombre de neurones du ganglion cervicale supérièur. Ces résultats indiquent que p75 médie l'apoptose et que ce mécanisme est nécessaire à l'apoptose naturelle qui a lieu lors du développement.

Finalement, nous démontrons que le suppresseur de tumeurs p53 est nécessaire dans le mécanisme d'apoptose naturelle des neurones sympathiques. On note une augmentation de la quantité de p53 lorsque p75 est activé par BDNF ou lorsque les neurones sympathiques sont deprivées de NGF. De plus, nous potulons que le mécanisme par lequel p53 augmente, implique les proteines MEKK et JNK. C'est la première fois que p53 est impliqué dans l'apoptose lors du développement du système nerveux.

ACKNOWLEDGEMENTS:

I would like to express my extreme gratitude to my supervisor, Dr. Freda Miller, for her guidance and support throughout the past 5 years. Not only has she provided me with a stimulating research environment, but has also been a friend whose scientific and personal advice has proved to be invaluable over the years.

I would also like to thank my thesis committee, Dr. Philip Barker, Dr. Dusica Maysinger and Dr. David Kaplan, for their excellent guidance. I would especially like to thank Dr. David Kaplan for his unending help with the preparation of this thesis, his tutalage in the signaling field, and his wonderful Margharita mixes.

Undoubtably, my greatest discovery in the Miller lab has been my husband, Dave, who went from being my labmate to friend to fiancée in a matter of days. Thanks for all your support, your strength, your love, and help. It has truly been wonderful to go through this period side-by-side with you.

Much of the work in this thesis has been performed in collaboration with many members of this laboratory, and I would like to thank you all for the commraderie, the excellent teamwork and the wonderful scientific discussions.

To the entire Miller laboratory, past and present, I am deeply grateful to you for making the past five years so remarkable. I have learned from you all (namely): Dan, Wendong, Xiu Ming, Audrey, Rahul, Ruth, Hiba, Carrie, Andrew G., Andrew V., Christine, Marta, Irene, Raquel, Judi, Jean, Jim, Jasi, Jose, Doron and Dave – that's a *lot* of gratitude.

To my family, both here and abroad, thanks for your unyielding support. A special thanks to my parents, Xerxes and Thrity, and to my brother, Zubin, who have celebrated each exciting result with me, and who have survived my crash courses in neurobiology.

This research was made possible through studentships provided by the Rick Hansen Man in Motion Foundation and the Medical Reseach Council of Canada. In addition, I would like to thank the Network for the Centres of Excellence for the supplemental studenship which has enabled me to attend several scientific meetings, and the Standard Life Foundation which supported me while I wrote this thesis through the Standard Life Dissertation Fellowship.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Apaf-1	apoptotic protease activating factor 1
BAE cells	bovine aortic endothelial cells
BDNF	brain-derived neurotrophic factor
BH1/2	Bcl-2 homology 1/2
CaM kinase	Ca^{2+}/calmodulin-dependent protein kinase
caspase	cysteine-containing aspartate-specific protease
CED	cell death abnormal
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DBH	dopamine β-hydroxylase
DR3	death receptor 3
DRG	dorsal root ganglia
EGF	epidermal growth factor
EPSP	excitatory postsynaptic potential
Erk	extracellular signal-related kinase
ES cells	embryonic stem cells
FADD	Fas-associated death domain protein
FGF	fibroblast growth factor
FLICE	FADD-like ICE
GABA	γ-aminobutyric acid
GAP-43	growth-associated protein-43
GCKR	germinal center kinase related
GDNF	glial cell line-derived neurotrophic factor
HGF	hepatocyte growth factor
ICE	interleukin-1-converting enzyme
IGF	insulin-like growth factor
IL-1	interleukin 1
IMAN	lateral magnocellular nucleus of the anterior neostriatum
JNK	c-jun amino-terminal kinase
LC	locus coeruleus
LIF	leukaemia inhibitory factor
LTP	long-term potentiation
MACH	MORT1-associated CED-3 homolog
MAH	myc-infected, adrenal-derived, HNK-1^+
MAP	microtubule associated protein
MCF7	human breast carcinoma
MEK1	MAP kinase kinase 1
MEKK1	MAP kinase kinase kinase 1
MKK4	JNK kinase
MORT1	mediator of receptor-induced toxicity
N-CAM	neural cell-adhesion molecule
NF-1	neurofibromin-1

NFκB	nuclear factor κB
NGF	nerve growth factor
NT-3,4/5,6	neurotrophin –3,4/5,6
p75NTR	p75 neurotrophin receptor
PC12	pheochromocytoma cell line
PH	pleckstrin homology
PI3-kinase	phosphoinositide 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC-γ	phospholipase C-γ
PNS	peripheral nervous system
PTB domain	phosphotyrosine binding domain
PtdIns	phosphoinositides
RA	robust nucleus of the archstriatum
Rb	retinoblastoma
RIP	receptor interacting protein
ROS	reactive oxygen species
SA	sympathoadrenal
SAPK	stress-activated protein kinase
SCG	superior cervical ganglia
SEK	SAPK kinase
SH2	Src homology 2
SHP	SH2-containing tyrosine phosphatase
SIF	small immunofluorescent
SOD1	superoxide dismutase 1
SNc	substantia nigra pars compacta
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain protein
TRAF	TNFR-associated factor 1
Trk	tropomyosin-related kinase
U937 cells	human monoblastic leukaemia cells
VIP	vasoactive intestinal peptide

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CONTRIBUTION TO PAPERS

Bamji,S.X., and Miller,F.D. (1996) Comparison of the Expression of a Tα1:nlacZ Transgene and Tα1 α-Tubulin mRNA in the Mature Central Nervous System. *J.Comp.Neurol.* 374:52-69.

My contribution to this paper includes the complete characterization of the pattern of Tα1:nlacZ transgene expression in the brain of two transgenic mouse lines. *In situ* labeling of Tα1 mRNA in the rat brain was performed by a former summer student in the laboratory, however I analyzed the pattern of Tα1 mRNA expression in the rat brain, and made a detailed comparison of this expression with the expression pattern of the Tα1:nlacZ transgene. Tα1:nlacZ transgenic mice were previously generated in Dr. Freda Miller's laboratory.

Bamji,S.X.*, Fawcett,J.P.*, Causing,C.G., Aloyz,R., Ase,A.R, Reader,T.A., McLean,J.H., and Miller,F.D. (1998) Functional Evidence that BDNF is an Anterograde Neuronal Trophic Factor in the CNS. *J. Neurosci.* 18:2808-2821.

My contribution to this paper includes all the functional evidence that BDNF is anterogradely transported in the CNS. This includes the analysis of the cortex in DBH:BDNF transgenic mice and control mice (Fig.3.5), and facial axotomy studies (Fig.3.6). For the analysis of cortical size in BDNF overexpressors and control mice, I perfused the animals, sectioned and stained all brains, measured the width of the cortex in two regions of the brain, and counted all cortical neurons in a delineated region of the brain. For the facial axotomy studies, I performed all the surgeries on neonatal and adult DBH:BDNF transgenic mice and control mice, perfused all animals, and sectioned and stained the brains. I then counted the number of surviving neurons in the manipulated mice and measured the area of the neurons. Furthermore, I designed and performed a number of *in vivo* studies which contributed to our understanding of the role of BDNF in cortical development, however, these experiments were not included in this paper.

BDNF and dopamine-β-hydroxylase (DBH) double labeling (Figs.3.1 and 3.2) was performed by J.P. Fawcett. BDNF and DBH immunolabeling in the locus coeruleus, and DBH immunolabeling in the hippocampus (Fig.3.3) was performed by C.G. Causing ,and all biochemistry (Fig 3.4., panel in Fig.3.6) was performed by R.Aloyz. Monoamine distribution

analysis (Tables 3.1, 3.2) was performed by A.R. Ase in Dr.T.A. Reader's laboratory. DBH:BDNF transgenic mice were maintained by animal technicians and C.G. Causing.

Bamji,S.X.*, Majdan,M.*, Pozniak,C.D., Belliveau,D.J., Aloyz,R., Kohn,J., Causing,C.G., and Miller,F.D. (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally-occurring sympathetic neuron death. ***J.Cell Biol.*** **140:911-923.**

This paper represents a highly collaborative effort, and I have contributed to virtually every aspect of this study along with my co-authors. My major contribution to this paper involves the survival studies reported in figure 4.2 and figure 4.4a, however, my specific contribution to each figure is outlined below.

Figure 4.1: An enormous number of primary sympathetic neuron cultures were required for all *in vitro* experiments. My specific contribution to figure 1 was the culturing of SCG neurons along with D.J. Belliveau. D.J. Belliveau performed all the washouts and induced the cells with the appropriate neurotrophins for figures 4.1a-d. I performed all the washouts and induced the cells with the appropriate neurotrophins for figures 4.1e,f. After the cells were induced with the various neurotrophins, R.Aloyz performed Western blot analysis on the lysates. Thus, my contribution to figure 4.1 includes half of the culturing of all SCG neurons, and all the washouts and inductions for figure 4.1e,f.

Figure 4.2: I predominantly performed survival studies involving the addition of BDNF to limiting concentrations of NGF (Fig.4.2B,C) or KCL (D,E), however D.J. Belliveau also aided in these studies including helping in the culturing of sympathetic neurons, and induction of the neurons with the different neurotrophins. For figures 4.2b-e, I performed half of the cultures with D.J. Belliveau and performed 80% of the washouts, inductions with the various neurotrophins, and MTT survival assays. The comparison between sympathetic neuron survival mediated by different concentrations of NGF and NT-4 (Fig.4.2A) were predominantly performed by D.J. Belliveau, however, I cultured half of the SCG neurons required for this experiment.

Figure 4.3: TUNEL labeling experiments shown in figure 4.3 were performed entirely by J.Kohn.

Figure 4.4: I contributed largely to survival assays performed with the p75 antibody, REX, seen in figure 4.4a. C. Pozniak performed half the culturing required for this study, however I performed all washouts on the cells, all the inductions with various neurotrophins

and antibodies, and performed the MTT survival studies. Survival experiments shown in figure 4.4b were performed by M.Majdan.

Figures 4.5, 4.6: Analysis of sympathetic neurons in BDNF-/- and p75-/- mice shown in figures 4.5 and 4.6, and in table 4.1 were performed by M.Majdan, C.Pozniak and myself. M.Majdan counted SCG neurons from p75-/- mice as represented by figures 4.6d-f and the last column in table 4.1. C.Pozniak counted SCG neurons from BDNF-/- mice as shown in figure 4.6b,c and the last column in table 4.1. M.Majdan, C.Pozniak and myself contributed equally to estimated BDNF-/- and p75-/- SCG neuron counts as shown in table 4.1.

Bamji,S.X.*, Aloyz, R.S.*, Pozniak,C.D.*, Toma,J.G., Atwal,J., Kaplan,D.R., and Miller,F.D. (1998) P53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *J.Cell Biol.*143:1691-1703.

This manuscript is also the result of a highly collaborative effort, and my contribution to each figure listed below:

Figure 5.1: I generated and maintained all primary symapthetic neuron cultures required for this figure in conjunction with a technician. R.Aloyz performed all NGF washouts and biochemistry on these samples.

Figure 5.2: I performed the survival assays depicted in figure 5.2a,b in conjunction with C.D.Pozniak. This includes generating neuron cultures, viral infections, washouts and MTT survival assays. Figures 5.2c-e were constructed by J.G.Toma.

Figure 5.3: Neuronal cultures, viral infections, and NGF washouts for this figure were entirely completed by myself. R.Aloyz then performed Western blot analysis on the lysates. Figures 5.3d and e were performed by J.G. Toma and R.Aloyz.

Figure 5.4: This figure was largely constructed by myself. After the cells were induced with the various neurotrophins, R.Aloyz performed Western blot analysis on the lysates.

Figure 5.5: This figure was performed by R.Aloyz and J.G. Toma.

Figure 5.6: Neuronal counts in p53-/- and wildtype SCGs at two timepoints, and TUNEL labeling in p53-/- and wildtype SCGs were performed in conjunction with C.D. Pozniak. C.D.Pozniak and I contributed equally to this work.

* These authors contributed equally to this work

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1. **Bamji S.X.***, Aloyz,R.*, Pozniak,C.D.*, Kaplan,D.R., and Miller,F.D. P53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. **(1998)** *J.Cell Biol.*, 143:1691-1703.

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2. **S.X. Bamji***, M. Majdan*, C.D. Pozniak, D.J. Belliveau, R. Aloyz, J.Kohn, C.G. Causing and F.D. Miller. **(1997)** The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally-occurring sympathetic neuron death. *Soc. Neurosci. Abst.* 23:339.

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10. Evidence that anterograde secretion of BDNF affects the development and survival of target neurons in the central nervous system. **(1996)** *Fourth International Conference on Nerve Growth Factor and Related Molecules.* East Sussex, England.

RATIONALE:

The nerve growth factor family of neurotrophins plays multiple roles in both the developing and mature mammalian nervous systems. The neurotrophin, BDNF, has specifically been shown to mediate a variety of biological effects from events surrounding neurogenesis, to neuronal survival and modulation of synaptic plasticity. The present studies were undertaken to further our understanding of the role of BDNF in both the CNS and PNS. We have concentrated on two areas of BDNF biology; the transport of BDNF within a neuron, and the effect of BDNF on neurons expressing the p75NTR, but lacking the TrkB receptor. Together, we believe these studies will provide additional information on how BDNF mediates the complex interplay that takes place during the sculpting of the mammalian nervous system, and specifically, how BDNF may be transported to effect the survival and death of neurons during programmed cell death.

OBJECTIVES OF THE THESIS:

There are four main objectives of this thesis. The first objective was to further characterize a model system that could be used as a useful "tool" to examine perturbations in neural development in transgenic mice, such as the dopamine β-hydroxylase (DBH):BDNF trangenic mice, and to rapidly identify neuronal populations that are responsive to extrinsic cues, such as BDNF, that regulate the growth of mature neurons *in vivo*.

The second objective was to examine the role of BDNF in the CNS, and specifically, to examine the functional role of anterogradely-derived BDNF. We focussed on the anterograde transport of BDNF in noradrenergic neurons of the locus coeruleus, a neuronal population known to express BDNF and to exert trophic control of a wide variety of target populations.

The third objective was to examine the role of BDNF on neuronal populations that express the p75NTR but lack the TrkB receptor. We focussed on sympathetic neurons of the rat SCG as a biologically relevant model system, as these neurons are exposed to BDNF at their targets, but do not express TrkB. In addition to furthering our understanding of the role of BDNF on neurons lacking the TrkB receptor, this study provided a model system to examine

the role of the p75 neurotrophin receptor. Indeed, as neurons are exposed to many neurotrophins derived from a variety of sources such as targets, afferent innervation, and glial cells, it is important to examine the effects of these neurotrophins on neurons lacking their cognate Trk receptors, and the contribution of the p75 receptor in mediating these events.

The final objective of this thesis was to examine the signaling pathways activated upon binding of BDNF to the p75NTR. As BDNF-mediated p75NTR activation was shown to be a necessary component of developmental sympathetic neuron death, this study encompassed signaling pathways activated both upon p75 activation and NGF withdrawal, two events known to occur during programmed cell death in sympathetic neurons.

CHAPTER 1: LITERATURE REVIEW

The aim of this chapter is to review the current understanding of the roles of neurotrophins, in particular nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), in the development of the vertebrate nervous system with emphasis on the role of neurotrophins in mediating neuronal survival and death. First, the nerve growth factor family of neurotrophins and their receptors are introduced, focussing on the role of neurotrophins in the development of the sympathetic nervous system. Second, the regulation of survival by neurotrophins is reviewed including the role of innervating neurons in mediating target development and survival, and the molecular mechanisms by which neurotrophins are thought to confer neuronal protection. Finally, the molecular events leading to neuron death, as well as the role of neurotrophins in this process are examined.

I. The Nerve Growth Factor (NGF) Family Neurotrophins:

The lifetime of a neuron, from a proliferative neuroblast to a mature, differentiated neuron may be divided into three major phases. Initially, in the proliferative phase, a neuroblast divides at a rate which appears to be a function of its position in the developing nervous system. Terminal mitosis marks the "birth date" of a neuron, which is shortly followed by a phase of neurite outgrowth, elicited by the expression of a number of neuronal growth-associated genes. Following the successful negotiation of an axonal pathway involving growth regulation and guidance cues, specific target-cell recognition triggers growth cone collapse and synaptogenesis, marking the beginning of the final phase of neuronal development, neuronal maturation.

Not every neuroblast, however, will realize its potential to differentiate and mature. During the development of the nervous system, many of the immature neurons of the CNS and PNS are eliminated in a self-directed process of cell death termed "apoptosis". This extensive process of cell death is developmentally regulated and occurs amidst the flurry of cell growth taking place in the developing nervous system.

The nerve growth factor family of neurotrophic factors constitutes a class of protein molecules that has been shown to exert a multitude of effects during neuronal development and maturation. Neurotrophin-mediated signals are thought to promote neuronal survival and

impinge on all three phases of neuronal development as well as the transitional events that separate them. Much of the conceptual framework surrounding the suspected function of neurotrophic factors has emerged from nearly fifty years of study of the prototypical neurotrophic factor, nerve growth factor (NGF).

Since the initial discovery of NGF by Rita Levi-Montalcini and Viktor Hamburger (reviewed in Levi-Montalcini and Angeletti,1968), a whole family of neurotrophins have been identified. At present, five neurotrophins have been described: nerve growth factor (NGF) (reviewed in Perez-Polo et al.,1972; Bradshaw et al.,1984), brain-derived neurotrophic factor (BDNF) (Barde et al.,1982; Leibrock et al.,1989), neurotrophin-3 (NT-3) (Ernfors et al.,1990; Hohn et al.,1990; Kaisho et al., 1990; Maisonpierre et al.,1990; Rosenthal et al.,1990), neurotrophin-4/5 (NT-4/5) (Berkemeier et al.,1991; Hallböök et al.,1991; Ip et al.,1992), and neurotrophin-6 (NT-6) (Gotz et al.,1994) which has thus far only been found in the teleost fish, *Xiphophorus maculatus*. The members of this family share 50-60% amino acid homology, and each have been shown to enhance the survival of specific neurons *in vivo* and/or *in vitro*. In addition to neurotrophins, other families of growth factors may also participate in aspects of neuronal development and survival. These include: insulin-like growth factors (IGFs) (reviewed in Gorio et al.,1998; D'Ercole et al.,1996), epidermal growth factor (EGF) (reviewed in Hicks et al.,1998), fibroblast growth factor (FGF) (reviewed in Hicks et al.,1998; Grothe and Wewetzer,1996), transforming growth factor (TGF) (reviewed in Sieber-Blum and Zhang,1997; Unsicker and Krieglstein,1996), ciliary neurotrophic factor (CNTF) (Kuzis and Eckenstein,1996; Horton et al.,1996) and glial cell line-derived neurotrophic factor (GDNF) (reviewed in Bjorklund et al.,1997).

Early investigations into the binding properties of NGF on responsive peripheral neurons revealed that these cells express two receptor populations (Sutter et al.,1979). NGF bound to some sites with higher affinity and slow dissociation rates, and to other sites with low affinity and fast dissociation rates (for review see Bothwell,1995). The first NGF receptor to be molecularly characterized and cloned was the p75 receptor, initially called the low affinity NGF receptor. At the time that p75 was cloned, this receptor defined a new family of proteins now known to include proteins such as CD40, Fas and the tumor necrosis factor α (TNFα) receptors, that are predominantly expressed by immune cells (Chao,1994). Although the precise function of the p75 receptor remained elusive for many years, it soon became clear that

all neurotrophins are able to bind to the p75 receptor, now commonly known as the p75 neurotrophin receptor (p75NTR).

The second NGF receptor to be discovered was shown to be a previously identified proto-oncogene, a transmembrane tyrosine kinase termed Trk (tropomyosin-related kinase) which is an essential component for the high affinity binding properties of NGF (Kaplan et al.,1991a,b; Klein et al.,1991). This soon led to the rapid identification of a family of Trk related proteins now known to constitute the signal transducing element of cellular responses to NGF, BDNF, NT-3 and NT-4/5. The Trk tyrosine kinase family of receptors consists of three genes *trkA*, *trkB* and *trkC*, all of which exhibit binding specificity for the different neurotrophins. The TrkA receptor represents the preferred receptor for NGF, but can also be activated, to a lesser degree, by NT-3 (Belliveau et al.,1997) and NT-4/5 (Bamji et al.,1998). The TrkB receptor is unique within the Trk family in that it can be activated equally well by two ligands, BDNF (Glass et al.,1991) and NT-4/5 (Klein et al.,1992). TrkB can also be activated with lower efficiency by NT-3, at least when expressed in fibroblasts (Glass et al.,1991). The TrkC receptor is the least promiscuous Trk receptor and binds only to NT-3 (Lamballe et al.,1991).

Our current concepts of neurotrophin function stem primarily from four types of experimental approaches. First, studies of the spatiotemporal pattern of expression of the neurotrophins and their receptors have provided important clues regarding the site and nature of neurotrophin function. Second, primary neuronal cultures have been used to rigorously characterize the biological activities of the neurotrophins. The biological activity of the neurotrophins have also been examined *in vivo*, in the attempt of furthering our understanding of the physiological roles of neurotrophins. Finally, gene targetting of the neurotrophins and their cognate receptors has been used to confirm some of the functions of the neurotrophins that were predicted by the other three experimental approaches, however, this approach has cast doubt on other functions. In addition to their functions during the development of the nervous system, the neurotrophins can promote the recovery of injured neurons in the adult nervous system (Yuen and Mobley,1996). These observations have generated interest in the theraputic potential of neurotrophins in neurodegenerative diseases. An overview of the biological activities of the neurotrophins, which have been previously reviewed extensively, (Snider,1994; Lewin and Barde,1996; Ip and Yancopolous,1996) will be presented in the following sections.

(i) Nerve Growth Factor (NGF):

Cellular targets whose survival depends on NGF have been well characterized and can be classified under three main categories: (i) neural crest derived cells, including sympathetic neurons, paraganglia cells, chromaffin cells (including the neoplastic PC12 cells), and neural crest-derived sensory neurons, (ii) cholinergic neurons of the central nervous system (CNS), including cells of the corpus striatum, basal forebrain, septum and the nucleus of the diagonal band of Broca, and (iii) nonneuronal cells including mast cells and other cells of the immune system (reviewed in Thoenen and Barde,1980; Levi-Montalcini,1987).

(a) The Role of NGF in Survival - Neurotrophic Hypothesis:

According to the neurotrophic theory of neuron survival, the type and density of neurons that will ultimately innervate a target are determined by the expression of specific factors within the target (reviewed in Oppenheim,1989). In this classical theory of neurotrophic support, limiting amounts of neurotrophic factors are synthesized in target cells of the responsive neuron, secreted in a soluble form into the extracellular space, taken up by receptors on the responsive neuron, and then retrogradely transported to the soma of the neurotrophin-dependent cell. Neurons innervating a target compete with one another for the limiting target-derived neurotrophin. Neurons that are successfully able to acquire sufficient amounts of this limiting neurotrophin, survive, whereas cells unable to sequester sufficient neurotrophin, die. Thus, the neurotrophic hypothesis provides an explanation of how specific neural systems are able to match the number of neurons to the size and requirements of the target field.

The basis for this theory comes from a number of studies, largely performed in avian and mammalian sensory and sympathetic ganglion cells that depend on NGF for survival. First, it was demonstrated that NGF is produced in the targets of these NGF-dependent populations and that the amount of NGF synthesized in the target fields of NGF-dependent neurons is proportional to the number of innervating neurons (Korshing and Thoenen,1983; Heumann et al.,1984; Shelton and Reichardt,1984). Second, neurons that innervate NGF-expressing target fields express the TrkA and p75 receptors. Furthermore, developmental time course studies have shown that the expression of NGF in the targets of NGF-dependent sensory

(Davies et al.,1987) and sympathetic (Korshing and Thoenen,1988) neurons, as well as the expression of NGF receptors in these neurons (Wyatt and Davies,1993), begin immediately prior to the time of target innervation. Thus, prior to the time of target innervation, when neither NGF nor its receptor is expressed, the survival of the innervating neurons is not dependent upon NGF. Third, studies have demonstrated that after uptake by sensory and sympathetic fibres from their target fields, NGF is retrogradely transported from the target to the cell soma by fast axonal transport where it exerts its survival promoting effects (Hendry et al.,1974a,b; Korsching and Thoenen,1983). Indeed, the pattern of retrograde axonal transport of target-derived NGF correlates with its trophic actions. For example, NGF is retrogradely transported by neurons of the dorsal root ganglion (DRG) (Stoeckel et al.,1975), and sympathetic neurons of the SCG (Hendry et al.,1974a,b), and in the central nervous system, NGF is retrogradely transported from the dorsal hippocampus to the NGF-dependent neurons of the medial septal nucleus and the vertical limb of the diagonal band of Broca (DiStefano et al.,1992). Finally, in the absence of target-derived NGF, innervating neurons die. This was first demonstrated *in vivo* by experiments where denervation of target tissue, or administration of NGF antibodies to neonatal rats, resulted in the elimination of the paravertebral sympathetic chain (Levi-Montalcini et al.,1969). In contrast, systemic treatment of neonatal mice with exogenous NGF causes marked hypertrophy of the peripheral sympathetic system (Angeletti et al.,1971; Aloe et al.,1975). This hypertrophy is also observed when neurons are made to innervate larger target territories (Hollyday and Hamburger,1976; Hollyday et al.,1977).

Studies have demonstrated that biologically active ^{125}I-labelled BDNF and NT-3 can also be retrogradely transported in a wide variety of neuronal cell types that depend on these trophic factors for survival in both the peripheral and central nervous systems, extending the generality of the neurotrophic hypothesis to other neurotrophins (DiStefano et al.,1992). The neurotrophic hypothesis has, however, been broadened in recent years by the demonstration that multiple neurotrophic factors regulate the survival of certain populations of neurons. For example, some sensory neurons depend on several different neurotrophic factors that may act either concurrently or sequentially during target field innervation (reviewed in Phillips and Arminini,1996; Davies,1997). In addition, there are aspects of neurotrophin action that do not conform to the classical neurotrophic hypothesis. Indeed, some populations of sensory neurons depend on particular neurotrophins before significant neuronal death takes place, raising the

possibility that the supply of these neurotrophins is not limiting at this stage of development (Buchman and Davies,1993). There is also evidence that neurotrophins can act in an autocrine or paracrine manner, and can also be anterogradely transported. However, despite the growing wealth of information on the multiple roles and modes of action of neurotrophic factors, the neurotrophic hypothesis best explains how neuronal target fields in the developing peripheral nervous system regulate their innervation density (reviewed by Davies,1996).

(b) Pattern of NGF Expression in the Mamalian Nervous System:

It has been clearly demonstrated in the PNS that both NGF mRNA and protein are synthesized in the target fields of NGF-sensitive neurons at the time of arrival of the first nerve fibres (Davies et al.,1987). In contrast to the periphery, where NGF is synthesized in non-neuronal mesenchyme and epithelial cells (Bandtlow et al.,1987; Davies et al.,1987), the target-derived action in the brain appears to involve neuron-neuron interactions. Here, NGF may either be retrogradely derived from other neurons to support survival, or may support survival in an autocrine manner. For example, the response of striatal cholinergic interneurons to NGF (Mobley et al.,1989; Vantini et al.,1989) implies a retrograde mode of action for NGF which is also considered to be local as the targets of these interneurons are contained within the corpus striatum. Similarly, the synthesis of NGF (and BDNF) in pyramidal and granule cells of the hippocampus (Ernfors et al.,1990) agrees with the proposed role of NGF as a retrograde message for their afferents, the basal forebrain cholinergic neurons. Co-expression of NGF and TrkA on individual neurons of the central nervous system, namely hippocampal neurons (Ernfors et al.,1990; Kokaia et al.,1993), and cerebellar granule cells (Muller et al.,1997) also suggest that support of these cells could be provided through autocrine mechanisms.

The source of NGF within the developing and adult nervous system is not solely confined to the targets of responsive neurons, however. For example, NGF is developmentally expressed in Schwann cells that ensheath the axons of peripheral nerves (Heumann et al.,1987). NGF is also expressed in glial cells throughout the developing brain, suggesting that CNS glia exhibit a generalized capacity to express the NGF gene (Lu et al.,1991). Indeed, NGF has been shown to be developmentally expressed in all three major types of glial cells in the brain including oligodendrocytes, microglia and astrocytes (Lu et al.,1991). NGF levels in glial cells of the PNS and CNS are highest during development, decrease in adult animals, and are then

reinduced in the mature nervous system following injury (Heumann et al.,1987; Lu et al.,1991). As glial cells actively proliferate both during development and after neuronal injury, it has been suggested that actively growing glia in the developing or injured brain regulate neuronal growth through the elaboration of NGF (Heumann et al.,1987; Lu et al.,1991).

(c) NGF Loss-of-Function (Knockout), and Gain-of-Function Mice:

The introduction of molecular genetics to the field of neurotrophins, through the use of germline-targetted ("knockout") mice, enabled the unique opportunity to determine the *in vivo* activities of neurotrophins and their receptors. Mice null for the NGF gene, NGF knockout (NGF-/-) mice, are born alive but show reduced growth and survival, with a maximum life span of 4 weeks (Crowley et al.,1994). Analysis of mice null for the NGF gene revealed a virtual loss of sympathetic ganglia by ten days of life. In addition, NGF-/- mice lack the small-sized, TrkA expressing sensory neurons within the dorsal root and trigeminal ganglia that are believed to respond to pain and temperature stimuli. This confirms previous *in vitro* and *in vivo* results demonstrating the critical dependence of these specific neuronal populations on target-derived NGF for survival that can not be compensated for by other related neurotrophins.

Despite early reports that NGF-/- mice display no gross structural defects in the CNS (Crowley et al.,1994), more subtle changes in behaviour and the pattern of innervation have been reported upon closer examination (Chen et al.,1997). Indeed, recent examinations of the brain of heterozygous mutant mice have revealed a decrease in the size and number of cholinergic septal cells, and a decrease in the choiinergic innervation of the hippocampus with concommitant deficits in memory acquisition and retention (Chen et al.,1997).

In general, mice expressing a null mutation for either the neurotrophins or their receptors have produced surprisingly few gross CNS defects. It has been suggested that the apparent lack of impressive findings in the CNS, as compared with the PNS of these mice, may reflect a fundamental difference in the regulation of cell survival in the CNS (Davies,1994). Alternatively, it has been suggested that the CNS may possess redundant neurotrophic pathways as evidenced by the coexpression of different Trk receptors on specific neuronal populations (Davies,1994). Furthermore, there is a constant concern that compensatory effects may mask the effects of the mutation, thereby downplaying the importance of the gene.

In addition to knockout studies, two studies have examined the effects of overexpressing NGF in the developing mouse. To examine the effects of increased target-derived NGF on the sympathetic nervous system, the superior cervical ganglion (SCG) was characterized in transgenic mice overexpressing NGF in keratinized epthelium by the K14 keratin promoter (Davis et al.,1996). As expected, increased NGF expression in targets of sympathetic neurons prior to programmed cell death resulted in an increased number of SCG neurons, as well as a dramatic hypertrophy of sympathetic neurons, which correlated with an increased innervation of the skin (Davis et al.,1996).

Overexpression of NGF in sympathetic neurons themselves as in dopamine β-hydroxylase (DBH):NGF transgenic mice, results in an increased number of sympathetic fibres and an enlargement of the sympathetic trunk and nerves growing to the peripheral target (Hoyle et al.,1993). These effects are thought to occur through autocrine regulation. Although there is an increased number of sympathetic axons reaching peripheral tissues, terminal sympathetic innervation within tissues is decreased in DBH:NGF transgenic mice. This may be explained by the lack of competition for limiting amounts of neurotrophin at the target due to the local acquisition of NGF. It is also possible that the appropriate levels of terminal TrkA activation is required for normal sympathetic innervation of target tissues. As these mice overexpress NGF prior to the period of naturally-occurring cell death, it would be interesting to see whether autocrinely-derived NGF can decrease apoptosis, and thereby increase the number of neurons in the SCG.

(d) The Role of NGF in Neuronal Differentiation: Neurogenesis

The expression of NGF mRNA in target and glial cells relatively late during the development of the nervous system (~E15) may explain why NGF does not typically play a role during early neuronal differentiation and cell cycle exit. During the development of the sympathetic nervous system, for example, NGF does not appear to affect the differentiation of sympathoadrenal progenitor cells to sympathetic neuroblasts, nor does it affect the survival of neuroblasts. In this situation, neuroblasts have been defined as dividing cells that possess a variety of neuronal characteristics, including catecholamines (Cohen,1974), tyrosine hydroxylase (Rothman et al.,1980), neurofilament protein (DiCicco-Bloom and Black,1988), and neuritic processes (DiCicco-Bloom et al.,1990). These proliferating, neuron-like cells have

also been termed precursor cells (Anderson and Axel,1986), and immature neurons (Ernsburger et al.,1989). Similarly, the immortalized MAH cell line, which is derived from sympathoadrenal progenitor cells, is unresponsive to NGF by criteria of neurite outgrowth, mitotic rate, and survival (Anderson and Axel,1986).

Although NGF does not influence neuronal development at the stage where a neuroblast differentiates into a neuron, NGF has been shown to exert an effect on the phenotype of specific differentiated cells, resulting in the transdifferentiation of cells into a sympathetic phenotype. For example, evidence for a close developmental relationship between chromaffin cells and sympathetic neurons (described later) initially came from observations that transplantation of postnatal adrenal medulary tissue into the anterior chamber of the eye produced an outgrowth of neuritic processes (Olson,1970), and that neurite outgrowth can be induced from dissociated postnatal chromaffin cells by NGF *in vitro* (Unsicker et al.,1978). In addition, exposing the adrenal medulla to exogenous NGF *in vivo*, results in the differentiation of a majority of cells into cells exhibiting a sympathetic phenotype, which includes the expression of functional cholinergic synapses (Ogawa et al.,1984).

NGF has been shown to promote the differentiation of PC12 cells, a clonal cell line derived from a rat medullary tumor, from a chromaffin-like to a sympathetic neuron-like phenotype. Upon addition of NGF to cycling PC12 cells, these cells cease proliferating (Greene and Tischler,1976), increase their ability to synthesize neurotransmitters characteristic of sympathetic neurons (Schubert et al.,1977), and become more electrically excitable (Dichter et al.,1997). The ability of NGF to inhibit proliferation in these cells is due to the expression of TrkA receptors on PC12 cells. The ability of undifferentiated PC12 cells to differentiate into cells with many of the phenotypic properties of sympathetic neurons upon activation of the TrkA receptor, has resulted in the widespread use of PC12 cells as an *in vitro* model for studying neuronal differentiation.

(e) Role of NGF in Neuronal Differentiation: Neurite Outgrowth and Gene Expression

The first suggestion that changes in gene expression are important to the biological actions of NGF came from studies on NGF-induced neurite outgrowth in PC12 cells. It was initially observed that upon addition of NGF to PC12 cells, a characteristic lag time of about 48 hours occurred before neurite outgrowth was observed (Greene and Rukenstein,1981). Further

analysis revealed that inhibition of RNA and protein synthesis during this lag time resulted in a block of neurite outgrowth (Greene and Rukenstein,1981). Proteins whose synthesis are regulated by NGF to promote the outgrowth of neurites from differentiated PC12 cells include, microtubule associated proteins (MAPs) (Black et al,1986), tau proteins, tubulins, including α- and β- tubulin (Drubin et al.,1985), the growth-associated protein GAP-43 (Skene,1989), and the neural cell-adhesion molecule N-CAM (Prentice et al.,1987).

It was subsequently demonstrated that in the developing nervous system, NGF is able to specifically upregulate the expression of a subset of genes important in growth and differentiation, including those encoding the p75 neurotrophin receptor (Miller et al.,1991;1994), Tα1 α-tubulin (Mathew and Miller,1990;1993), and tyrosine hydroxylase (Miller et al.,1991). In the sympathetic nervous system the magnitude of these increases in gene expression was shown to occur in a graded fashion with respect to NGF both *in vivo* (Miller et al.,1994) and *in vitro* (Ma et al.,1992). Interestingly, the concentration-dependent changes in gene expression occurred from 10-200ng/ml NGF. It is now believed that the first response to be elicited is neuronal survival, which occurs at the low end of the NGF curve (1-10ng/ml NGF). Once the survival threshold is reached, neurite outgrowth and gene expression are regulated in a graded fashion as a function of receptor activation. This extended concentration curve is biologically reasonable; during the period of target innervation, neurons need to sequester a small amount of NGF to ensure their survival, but are thereafter required to innervate an increasingly larger target area during the growth of the animal, necessitating graded regulation of neuronal growth and gene expression (reviewed in Miller,1994).

Interestingly, NGF differentially regulates the expression of its two receptors, resulting in a net increase in the expression of the p75NTR, but not the TrkA receptor. It has become increasingly apparent that NGF's ability to alter the ratio of p75 to TrkA receptors may be extremely important during the development of the nervous system as discussed later (reviewed in Miller,1994).

Neuronal responses to NGF are also spatially regulated, resulting in a robust increase in gene expression when NGF is applied to cell bodies and proximal axons, and a weaker response when NGF is applied to distal axons and neuronal terminals (Toma et al.,1997). This is consistent with the recent finding that TrkA expression is differentially distributed in neurites

and cell bodies of sympathetic neurons; TrkA expression being approximately 4-fold greater in cell bodies compared to neurites (Kohn et al.,1997).

In a similar tissue culture system designed to selectively expose different parts of the neuron to NGF, the promotion of growth by NGF was shown to be largely confined to distal neurite regions directly exposed to extracellular NGF (Campenot,1977;1982). This work demonstrates that the local growth of neurites is primarily controlled by local exposure to NGF, and cannot be robustly induced by NGF application to cell bodies and proximal neurites, supporting the hypothesis that target NGF levels regulate sympathetic innervation *in vivo* (Campenot,1977;1982). Thus, NGF not only increases neurite outgrowth and gene expression, but does so in a concentration-dependent, spatially-regulated fashion which depends on the region of the neuron exposed to NGF.

(ii) Brain-Derived Neurotrophic Factor (BDNF):

(a) The Role of BDNF in Survival:

Despite the high similarity between NGF and BDNF as indicated by their strictly conserved cysteine residues (Barde et al.,1982; Leibrock et al.,1989), they are dissimilar enough to exhibit different neuronal specificities. This is due to the differential distribution of TrkA and TrkB receptors on different neuronal populations, with NGF exerting its effects on TrkA expressing cells, BDNF exerting its effects on TrkB expressing cells, and both exerting their effects on cells expressing both TrkA and TrkB. For example, although cholinergic neurons of the basal forebrain nuclei are able to respond to both BDNF and NGF (Alderson, et al.,1990), only BDNF is able to promote the survival of TrkB expressing embryonic retinal ganglion cells (Johnson, et al.,1986), mesencephalic dopaminergic cells (Hyman, et al.,1991), as well as placode- and non-neural crest-derived sensory neurons, including the proprioceptive mesencephalic trigeminal neurons (Davies et al.,1986). These types of neurons do not express the TrkA receptor and are unable to respond to NGF at physiological concentrations (reviewed by Korching ,1993). Thus, overlapping but distinct neuronal populations respond to the various neurotrophins. Interestingly, BDNF can promote the survival of cultured motor neurons during development (Oppenheim et al.,1992), and can rescue a percentage of motor neurons from axotomy-induced death in neonatal rats (Sendtner et al.,1992; Yan et al.,1992).

BDNF is able to support the survival of innervating neurons in the classic, retrograde manner similar to that seen with NGF. Indeed, spinal motor neurons can bind and retrogradely transport [^{125}I] -BDNF and [^{125}I] -NT-3 administered into sciatic nerve (DiStefano et al.,1992). Furthermore, BDNF is expressed in the targets of BDNF-responsive neurons including the tectum, which is innervated by BDNF-dependent retinal ganglion cells (Ma, et al.,1998), in muscle targets of motor neurons (Koliatsos et al.,1993), and in hippocampal neurons that are innervated by neurons of the medial septal nucleus and the vertical limb of the diagonal band of Broca (DiStefano et al.,1992). This pattern is observed throughout the CNS and PNS.

Expression of BDNF mRNA in subsets of DRG neurons (Ernfors et al.,1988; Schecterson and Bothwell,1992), raised the possibility that sensory neurons could obtain BDNF by autocrine or paracrine routes operating in the ganglion. Indeed, work using antisense BDNF oligonucleotides in single cell cultures of adult DRG neurons has demonstrated the operation of a BDNF autocrine loop in a subset of these neurons, which is required for their survival (Acheson et al.,1995). BDNF is also believed to act in a paracrine manner in the DRG, and rigorous analysis of the expression of BDNF in different subsets of neurons has shown that the highest levels of BDNF mRNA are expressed in NGF-dependent cutaneous sensory neurons, which can mediate the survival of BDNF-dependent sensory populations in coculture (Robinson et al.,1996). BDNF mRNA expression was observed at lower levels in BDNF-dependent cutaneous sensory neurons, and was undetectable in BDNF-dependent proprioceptive neurons (Robinson et al.,1996). This work provides a clear example of how neurons may depend on neurotrophins for survival, yet obtain these from sources other than their targets.

(b) The Role of BDNF in Neuronal Differentiation: Neurogenesis

In addition to BDNF's survival-promoting activity on postmitotic motor neurons (Oppenheim et al.,1992; Sendtner et al.,1992; Yan et al.,1992; McKay et al.,1996), recent data suggests that BDNF is also able to stimulate the proliferation and/or differentiation of motor neuron precursors (Jungbluth et al.,1997). In this study, Jungbluth et al.(1997) report that motor neuron progenitors express the TrkB receptor, and that treatment of these cells with BDNF leads to a significant increase in the number of motor neurons. This increase in the number of motor neurons is not thought to be due to an increase in the survival of differentiated

motor neurons, as the ablation of BDNF-expressing cells results in a decreased number of motor neurons, without a concomitant increase in the number of apoptotic cells. Instead, it is believed that BDNF can either act as a mitogen for motor neuron precursors or influence the adoption of a motor neuron fate by TrkB expressing precursor cells. Further studies need to be performed to distinguish between these two possibilities. Interestingly, the only BDNF expression detectable at this stage is by a subset of ventrally-projecting, interneurons in the dorsal neural tube. The authors have therefore speculated that BDNF produced by dorsal interneurons stimulates proliferation and/or differentiation of motor neuron precursors after anterograde axonal transport and release in proximity to the TrkB-expressing motor neuron progenitors.

BDNF has also been shown to enhance the differentiation of stem cell-derived neuronal precursors (Ahmed et al.,1995). In these *in vitro* experiments, application of BDNF to EGF-generated neuronal precursors derived from embryonic and adult striatum, resulted in a marked enhancement in neurite outgrowth, as well as the rapid differentiation of these precursors into neurons that express GABA and substance P. Once again, BDNF did not appear to act as a survival factor for neuronal precursors.

Finally, BDNF has been implicated in mediating differentiation of the neural retina (Cohen-Cory and Fraser,1994) and the retinal pigment epithelium (Liu et al.,1997) both of which arise from a single layer of neuroectoderm. BDNF is believed to mediate this differentiation in an autocrine/paracrine manner, as BDNF and its receptor, TrkB, are coexpressed in these cells. Indeed, when a dominant negative mutant of the TrkB receptor was expressed in developing embryos, there was a severe arrest of retinal pigment epithelium differentiation as evidenced by persistence of nestin- and Notch-positive neuroblasts (Liu et al.,1997).

Thus, BDNF not only mediates the survival of TrkB positive neurons in a target-derived fashion, but is also crucial to the early development of the developing nervous system by regulating the differentiation and/or proliferation of neuroblasts in an autocrine/paracrine or anterograde fashion. Indeed, in this regard it appears that the actions of BDNF during early neural development are clearly distinct from those of NGF, which does not appear to be responsible for mediating the transition from neuroblast to neuron in either the PNS or CNS.

(c) BDNF Loss-of-Function (Knockout) Mice:

BDNF and its receptor, TrkB, are widely expressed in the embryonic, postnatal, and adult central and peripheral nervous systems (Hofer et al.,1990; Schecterson and Bothwell,1992; Merlio et al.,1992) and much work has been performed to examine the role of BDNF in the developing and adult nervous systems. These studies have been complimented *in vivo* through the generation of BDNF knockout (BDNF-/-) mice (Jones et al.,1994; Ernfors et al.,1994) and TrkB-/- mice (Klein et al.,1993). BDNF homozygote mutant mice exhibit a substantial reduction in the number of cranial and spinal sensory neurons, including the trigeminal, geniculate, vestibular, petrosal-nodose and dorsal root ganglia, confirming its survival-promoting function in these neurons.

Although *in vitro* studies have shown that BDNF, NT-4/5 and NT-3 can promote the survival of purified rat motorneurons (Henderson et al.,1993), and BDNF can support the survival of axotomized facial motor neurons (Koliatsos et al.,1993), motor neurons appear normal in BDNF-/- mice, and BDNF/NT-4/5 double knockout mice (Ernfors et al.,1994, Jones et al.,1994; Conover et al.,1995; Liu et al.,1995). This may be due to compensatory effects commonly observed in null mutant mice. Indeed, as facial motor neurons also express the TrkC receptor, BDNF/ NT-4/5/ NT-3 triple mutant mice may be necessary to observe a decrease in facial motor neuron survival. Interestingly, there is a decrease in the survival of axotomized facial motor neurons in adult BDNF+/- mice, indicating that BDNF is crucial for the survival of injured motor neurons, though it does not appear to be essential for survival during the period of naturally-occurring cell death (Fawcett et al.,1998).

Interestingly, it has been demonstrated that there is an increased number of neurons in the superior cervical ganglia (SCG) of BDNF-/- mice (Bamji et al.,1998). SCG neurons do not express the BDNF receptor, TrkB, and a full account of how BDNF plays a negative role on the survival of sympathetic neurons is described in chapters IV and V.

In addition to its role in mediating the survival of neurons, BDNF has been shown to affect the innervation density of BDNF expressing target tissues. For example, although TrkB-expressing preganglionic neurons normally innervate BDNF-expressing sympathetic neurons of the SCG, this innervation density is significantly decreased in BDNF-/- mice (Causing et al.,1997). The dramatic reduction in innervation density in BDNF-/- mice cannot fully be explained by the decrease in survival of preganglionic neurons. Thus, it has been postulated

that BDNF can regulate the innervation density of BDNF-expressing neuron populations independent of its survival effects. As an interesting adjunct, a recent study has reported a reduction in axonal arborization and synaptic density of hippocampal afferents in TrkB-/- and TrkC-/- mice (Martinez et al.,1998).

In general, BDNF-/- mice display no gross structural abnormalities in the CNS. It has been noted, however, that many neurons exhibit an altered expression of neuropeptide Y and calcium-binding proteins, suggesting that they do not function normally and that BDNF regulates the phenotype of these neurons (Jones et al.,1994).

BDNF-/- mice have also revealed a role for BDNF in mediating long-term potentiation. This is further discussed in in section I(e) below.

(d) Role of BDNF in Neuronal Differentiation: Neurite Outgrowth and Gene Expression

Recently, two studies have shown that BDNF is able to regulate the expression of growth-associated genes in the CNS, both in intact cells and in injured neurons (Fournier et al.,1997; Kobayashi et al.,1997). Kobayashi et al. (1997) show that BDNF can induce an increase in Tα1 α-tubulin expression in both injured and intact rubrospinal neurons, whereas the induction of GAP-43 mRNA appears to require additional signals associated with axotomy. The stimulation of GAP-43 and Tα1 α-tubulin expression by application of BDNF was shown to correlate with an increase in the number of rubrospinal neurons regenerating into peripheral nerve implants, thus implicating a role for BDNF in mediating axon outgrowth and gene induction. In contrast to BDNF's affect on rubrospinal neurons, BDNF was able to stimulate the expression of GAP-43, but not Tα1 α-tubulin in axotomized retinal ganglion cells (Fournier et al.,1997). Thus, regulation of regeneration-associated genes by BDNF is context-dependent and distinct in different neuronal systems.

(e) Neurotrophins and Neuronal Plasticity:

Although neurotrophins are traditionally thought to be signaling proteins essential for neuronal survival and differentiation, a series of recent studies suggests a novel role of neurotrophins in synaptic transmission and plasticity (reviewed in Lo,1995; Thoenen,1995; Bonhoeffer,1996; Lu and Figurov,1997). For example, BDNF and NT-3 have been shown to rapidly enhance synaptic transmission at the developing neuromuscular junction *in vitro* (Lohof

et al.,1993). Further analyses indicate that this effect is a result of enhanced transmitter release, most likely caused by increased calcium concentrations at the nerve terminal (Stoop and Poo,1995,1996). NT-3 also has been shown to have a long-term effect on the maturation of neuromuscular synapses (Wang et al.,1995; Liou et al.,1997). Moreover, the expression of NT-3 in postsynaptic muscle cells is regulated by nerve cell innervation and membrane depolarization, and it is postulated that this activity-dependent expression of NT-3 may serve as a retrograde signal for modulating synaptic efficacy and/or stabilizing the neuromuscular synapses (Xie et al.,1997).

In the CNS, neurotrophins appear to have both long-term and acute effects on synaptic function and plasticity. Studies on long-term effects have focussed primarily on the visual system, highlighting the ability of neurotrophins to prevent the formation of occular dominance columns (Maffei et al.,1992; Cabelli et al.,1995; Riddle et al.,1995). The acute effects of neurotrophins on neuronal activity and synaptic transmission have been observed in cultured hippocampal neurons and in slices by a number of laboratories. For example, Knipper et al.,(1994) reported that NGF and BDNF enhance high K^+-induced release of acetylcholine from hippocampal synaptosomes. Next, Kang and Schuman (1995) showed that BDNF and NT-3 rapidly enhance basal excitatory synaptic transmission in CA1 synapses of adult hippocampal slices, and that this effect requires protein synthesis in the dendrites and axons (Kang and Schuman,1996). In contrast, other reports have demonstrated that BDNF does not affect basal excitatory synaptic transmission, but rapidly reduces inhibitory postsynaptic current (IPSCs) at CA1 synapses in hippocampal slices (Tanaka et al.,1997).

Studies in BDNF-/- mice have also revealed a role for BDNF in mediating long-term potentiation (LTP). In BDNF-/- mice, there was a severe impairment of hippocampal LTP (Korte et al.,1995) which could be rescued by either recombinant BDNF (Patterson et al.,1996) or infection with BDNF-containing adenovirus (Korte et al.,1996), suggesting that the absence of BDNF, rather than cumulative developmental defects, is responsible for the impairment of LTP. In addition, application of BDNF promotes LTP induction in neonatal hippocampal slices, where endogenous BDNF levels are low (Figurov et al.,1996).

BDNF has recently been shown to act presynaptically to modulate synaptic transmission and plasticity by regulating neurotransmitter release (Gottschalk et al.,1998). This is not to say that BDNF does not act on postsynaptic neurons to modulate LTP. For example,

BDNF may regulate ion channel properties, allowing for faster repolarization of postsynaptic membranes. Indeed, BDNF has been shown to enhance the expression of K^+ channels that are critical in membrane repolarization (Du et al.,1997). Another possibility is that BDNF induces morphological changes in postsynaptic dendrites and/or spines. In slices derived from neonatal ferret visual cortex, long-term treatment with BDNF elicited a dramatic change in dendritic arborizations (McAllister et al.,1995,1996). BDNF has also been shown to modulate post-synaptic NMDA channels directly via mechanisms not involving TrkB receptors (Jarvis et al.,1997). Thus, in addition to their actions in mediating survival and differentiation, neurotrophins clearly play a role in modulating neuronal plasticity and synapse formation.

iii) The Role of Neurotrophins in the Development of the Sympathetic Nervous System:

A central problem in developmental neurobiology is understanding the cellular and molecular mechanisms that generate the diversity of cell types found in the nervous system. One system that has been investigated in detail is the sympathoadrenal (SA) lineage (see Fig.1.1 for timeline for the development of the SCG). This lineage derives from neural crest cells that migrate ventrally from the top of the neural tube to either the dorsal aorta, where they aggregate and differentiate to form sympathetic neurons, or to the region of the adrenal gland, where they differentiate to form chromaffin cells (reviewed in Anderson,1997).

Bipotential sympathoadrenal progenitor cells have been shown to exist *in vivo*, and individual cells coexpressing neuron-specific and chromaffin-specific antigenic markers have been observed in early (E12.5) sympathetic ganglia primordia (Anderson et al.,1991; Carnahan and Patterson,1991a). These cells have been isolated from E14.5 rat adrenal glands (Anderson and Axel,1986; Anderson 1988) using the monoclonal antibody HNK-1 (Abo and Balch,1981), and from sympathetic ganglia (Carnahan and Patterson,1991b) using novel monoclonal antibodies generated by an immunosuppression technique (Carnahan and Patterson,1991b). Further analysis of these purified cell populations *in vitro* (Anderson and Axel,1986; Carnahan and Patterson,1991a) has confirmed that many SA progenitor cells are bipotential and able to develop into either chromaffin cells or sympathetic neurons depending upon the culture conditions.

To further examine how bipotential SA progenitor cells differentiate into committed sympathetic neuroblasts and chromaffin cells, an SA progenitor cell line, called MAH (Myc-

infected, Adrenal-derived, HNK-1+) cells, were produced by retrovirally introducing the avian v-myc oncogene into SA progenitors (Birren and Anderson,1990). It was shown that neither primary SA progenitors nor MAH cells respond to NGF by criteria of neurite outgrowth, survival, or induction of neuron-specific genes (Birren and Anderson,1990). This proved interesting as NGF has the ability to trigger neurite outgrowth from postnatal chromaffin cells, small immunofluorecsent (SIF) cells and PC12 cells, initially suggesting that NGF is an important determinant of neuronal fate in the SA lineage. This lack of NGF-responsiveness is thought to be due to the lack of expression of NGF receptors, as NGF has been shown to induce a maturation and survival response in MAH cell expressing NGF receptors (Verdi et al.,1994). Instead, the mitogen fibroblast growth factor (FGF) was shown to induce differentiation of both primary SA progenitor cells and MAH cells, suggestting that FGF is responsible for promoting neuronal differentiation of bipotential, embryonic SA progenitors, to committed sympathoblasts (Birren and Anderson,1990). These sympathoblasts have been observed in mammalian and avian sympathetic ganglia as early as E13 (Anderson et al.,1991; Carnahan and Patterson,1991a).

FGF also stimulates the proliferation and survival of immature sympathetic neuroblasts. Other factors that also maintain the survival of these cells include depolarization, insulin, insulin-like growth factor-1 (IGF-1), and vasoactive intestinal peptide (VIP) (for review see Rohrer,1990), as well as hepatocyte growth factor (HGF) (Maina et al.,1998). Interestingly, although mature sympathetic neurons express the HGF receptor, Met, and can respond to HGF with increased neurite outgrowth, HGF is unable to suport the survival of these neurons (Maina et al.,1998, Yang et al.,1998).

The survival of sympathetic neurons is independent of NGF before their axons reach the sources of NGF in the periphery (Davies et al.,1987; Korching and Thoenen,1988; Fagan et al.,1996). Thus, the question remained of how differentiating neurons acquire their responsiveness to, and ultimately their trophic dependence upon, NGF.

There has been some controversy in this field regarding the maintenance of sympathetic neuroblasts and early sympathetic neurons by neurotrophins. Early studies suggested that sympathetic neuroblasts initially depend on NT-3 for survival via activation of the TrkC receptor, and that these neurons subsequently lose their responsiveness to NT-3 and become

dependent on target-derived NGF, and TrkA activation, for survival. However, several recent observations have made it clear that such a straight-forward interpretation may not be justified.

First, administration of anti-NT-3 antibodies to postnatal rats *in vivo* results in the death of up to 80% of SCG neurons, suggesting that even mature sympathetic neurons are dependent on endogenous NT-3, as well as NGF, for survival (Zhou and Rush, 1995). Although experiments have confirmed the existence of an NT-3-dependent, mature sympathetic neuron population, the high loss of mature sympathetic neurons in experiments performed by Zhou and Rush (1995) may be explained by cross-reactivity of the NT-3 antibody with NGF. This is highly plausible as more than 50% of sympathetic neurons stay alive *in vitro* in the absence of NT-3 -producing glial cells, and unless sympathetic neurons themselves produce NT-3, this is the only source of the neurotrophin in culture.

Second, analysis of NT-3 knockout and wildtype SCGs have cast a doubt on the proposed role of NT-3 in promoting the survival of sympathetic neuroblasts. Indeed, there appeared to be no apparent difference in the number of SCG neurons in wildtype and NT-3 knockout mice at E15.5, although at E17.5 and later stages, NT-3 knockout mice exhibited a smaller number of SCG neurons than control mice (reviewed in Davies,1997). Indeed, there is a 55% decrease in the number of SCG neurons in mature NT-3-/- mice compared to wildtype mice (Ernfors et al.,1994).

Finally, although *trkC* mRNA is highly expressed in early sympathetic ganglia, there is no significant difference in the number of SCG neurons in TrkC-/- mice compared to wildtype mice (Fagan et al.,1996), suggesting that NT-3 signaling via the TrkC receptor is not required for the genesis or survival of early sympathetic neurons *in vivo*, nor for the survival effects of NT-3 on mature neurons. Indeed, NT-3's ability to activate the TrkA receptor on SCG neurons, and to mediate limited neuronal survival, point to the possibility that NT-3 may be exerting its survival effects via the TrkA receptor (Belliveau et al.,1997). This is corroborated by the fact that both TrkA-/- and NT-3-/- mice display decreased survival of SCG neurons at similar timepoints, beginning at E15.5 (reviewed in Davies,1997). Together, these data indicate that NT-3 is not required for the survival of sympathetic neuroblasts and early sympathetic neurons, but mediate the survival of sympathetic neurons at later stages via the TrkA receptor.

Although sympathetic neurons are not dependent on NGF for survival before reaching their target tissues, they express TrkA receptors indicating that they are responsive to NGF (Wyatt and Davies,1993). In fact, there is strong evidence that the differing spatial and temporal patterns of NGF expression within target tissues, exerts a key role in establishing and maintaining appropriate connections. Indeed, the ability of supplemental NGF to dramatically increase the density of innervating neurons argues strongly that targets are able to regulate the type and density of innervating neurons through the synthesis and release of limiting quantities of NGF (Bjerre et al.,1975).

Sympathetic neurons undergo programmed cell death over a wide developmental window between E17 and P15 (Wright et al.,1983). The binding of target-derived NGF to TrkA receptors activates a variety of signal transduction pathways that lead to the survival of mature, NGF-responsive sympathetic neurons. The role of neurotrophins in survival and apoptosis of sympathetic neurons and the molecular mechanisms by which these actions are mediated will be extensively reviewed in a later section.

In addition to its role in mediating survival, the targets of sympathetic neurons have also been shown to exert an effect on the phenotype of the sympathetic neuron with respect to neurotransmitter phenotype. Elegant studies have shown that sympathetic neurons innervating the footpad, change their neurotransmitter phenotype from adrenergic to cholinergic, and that factors released from the target are responsible for this phenotypic switch. This has been extensively reviewed by Story C. Landis (1996) and will not be further discussed here.

The role of TrkA and p75 in mediating survival and death of sympathetic neurons is one of the key foci of this thesis. For this reason, the temporal pattern of expression of these receptors in the SCG is outlined here and in figure 1.1. At E13, the SCG contains proliferating cells that express many features of differentiated neurons (reviewed in Anderson,1997). These immature neurons or sympathoblasts survive in culture without NGF and NGF does not induce c-fos expression. Low levels of p75 and trkA mRNAs are expressed at this stage *in vivo*. In E14 cultures, NGF induces *c-fos* expression in 10-15% of the neurons and mediates the survival of a similar number of neurons (Deckworth and Johnson,1993). The proportion of neurons responding to NGF increased with age, reaching 90% in E18 cultures. The *in vivo* level of *trkA* mRNA increased markedly from E14 onward, but, in contrast to sensory neurons (in which p75 and trkA mRNA levels increase in parallel) (Wyatt and Davies,1993), the

increases in *trkA* mRNA and p75 mRNA are out of step with one another in the developing SCG. The level of *trkA* mRNA initially increases much more rapidly than that of p75 mRNA, so that by E17 the level of *trkA* mRNA is almost 7-fold higher than that of p75 mRNA. However, after E17, the level of p75 mRNA increases rapidly and approaches that of *trkA* mRNA postnatally. According to Wyatt and Davies (1995), the level of p75 mRNA never exceeds the level of *trkA* mRNA, whereas Verdi and Anderson (1994) claim that the level of p75 mRNA is tenfold greater than that of *trkA* mRNA by P1. Furthermore, in E14 sympathetic neuron cultures, the level of *trkA* mRNA increases in the absence of neurotrophins or KCl. In contrast, the level of p75 mRNA in E14 cultures is enhanced by NGF but is unaffected by KCL (Wyatt and Davies,1995).

Finally, it is important to note the distribution of TrkA and p75 in cultured sympathetic neurons. The TrkA receptor is preferentially enhanced in sympathetic neuron cell bodies relative to neurites, and it is estimated that there is approximately 3-4 fold more TrkA in the cell soma (Kohn et al.,1997). Conversely, the distribution of the p75NTR is relatively uniform in cell bodies and neurites, with approximately 1.5-1.8 fold more p75NTR expression in cell bodies (Kohn et al.,1997).

iv) The Tyrosine Kinase Family of Neurotrophin Receptors (Trk Receptors):

(a) Structure of Trk Receptors:

To understand the mechanism by which neurotrophins exert their effects, it is necessary to examine the properties of neurotrophin receptors. The TrkA receptor tyrosine kinase was initially isolated as an oncogenic protein found in human colonic carcinoma (Martin-Zanca et al.,1986:1989), and it was not until two years later that TrkA was described as the principle receptor for NGF (Kaplan et al.,1991a,b; Klein et al.,1991). The TrkA receptor appears to be both necessary and sufficient to mediate the biological effects of NGF. Indeed, PC12 cells that lack functional TrkA (nnr5 cells) are unable to differentiate in response to NGF, whereas transfection of the TrkA receptor into these cells restores their ability to differentiate in the presence of NGF (Loeb et al.,1991). Subsequent studies have shown that the related Trk tyrosine kinases, TrkB and C, are responsible for mediating the biological effects of the other members of the neurotrophin family.

All of the full-length membrane tyrosine kinase receptors share common structural features that include: an extracellular domain containing a signal peptide and a region for neurotrophin binding, a single transmembrane domain, and a cytoplasmic region that encompasses the tyrosine kinase catalytic domain (Berkemeier et al.,1991; Squinto et al,,1991; Klein et al.,1991,1992). Two isoforms of the TrkA protein have been described, one differing from the other by a 6-amino acid insert into the extracellular domain (Barker et al.,1993; Horigome et al.,1993). When tested in fibroblasts, the 6 amino acid insert did not appear to affect the receptor's ligand binding specificity or its ability to transduce functional signals in response to NGF (Barker et al.,1993). In rats and humans, only the longer isoform is expressed in neuronal cells at appreciable levels, whereas the shorter isoform, the form of TrkA originally cloned, is expressed in non-neuronal cells (Barker et al.,1993; Horigome et al.,1993). PC12 cells have been shown to express both TrkA isoforms (reviewed in Barbacid,1994). It would be of great interest to know whether the two TrkA isoforms play different functional roles, or whether these two receptor isoforms have differential abilities to interact with the p75 receptor.

The *trkB* gene is more complicated than that of *trkA*. Indeed, in addition to the full length receptor (TrkB.FL), alternative splicing of the *trkB* gene results in the generation of two TrkB isoforms that lack the tyrosine kinase domain (TrkB.T1 and TrkB.T2) (Klein et al.,1990; Middlemas et al.,1991), and one splice variant with mutations in the extracellular region of the TrkB receptor (Strohmaier et al.,1996). The two truncated TrkB isoforms that lack the intracellular tyrosine kinase domains posses the same extracellular and transmembrane domains as the full length receptor, and have been shown to bind to both BDNF and NT-4 (Klein et al.,1990; Middlemas et al.,1991). Although little is known about truncated TrkB receptor function, a number of hypotheses have emerged from examining the expression pattern of these receptors. A number of studies have shown that both TrkB.T1 and TrkB.T2 are expressed in neurons and glia in both the PNS (Armanini et al.,1995; Ninkina et al.,1996) and CNS (Armanini et al.,1995; Fryer et al.,1996), and are predominantly expressed at the time of axon arrival at targets (Escandon et al.,1993,1994; Allendoerfer et al.,1994; Armanini et al.,1995) and in the mature nervous system (Armanini et al.,1995; Fryer et al.,1996). The ability of truncated TrkB isoforms to reduce the ability of TrkB.FL to induce particular BDNF-dependent events (Ninkina et al.,1996; Fryer et al.,1997), and their ability to rapidly bind to and internalize BDNF, has led to the hypothesis that these truncated TrkB isoforms act as

dominant-negative elements in cells expressing the full length TrkB receptor. Indeed, it has been suggested that truncated receptors may act as "sponges" to soak up excess ligand and act as a barrier to diffusion (Biffo et al.,1995; Fryer et al.,1997). As BDNF is highly expressed in the CNS, and as BDNF-responsive populations are separated by short distances in the brain, the development of a system that prevents the rapid diffusion of BDNF appears plausible. Thus, these receptors may act to restrict widespread BDNF availability, and to allow for the independent development of BDNF-responsive systems. Another model suggests that the truncated TrkB receptors may be able to signal independently (Baxter et al.,1997). This has been demonstrated by the ability of BDNF to increase the rate of acidic metabolite release from cells expressing TrkB.T1 and TrkB.T2, a common physiological consequence of many signaling pathways. These issues may be resolved *in vivo* through the generation of specific truncated TrkB knockout mice.

In addition to truncated TrkB receptors, a TrkB splice variant lacking a region of the extracellular domain encoded by exon 9, has been identified (Strohmaier et al.,1996). Although the full length TrkB receptor can bind to NT-3 and NT-4/5 in addition to BDNF, this splice variant was unable to bind to either NT-3 or NT-4/5. Previous studies have shown that BDNF and NT-4/5 do not have identical effects when tested on neurons, though both seem to be recognized equally well in a TrkB-expressing fibroblast cell line (Ip et al.,1992; Klein et al.,1992; Dechant et al.,1993). For example, BDNF supports the survival of more chick sensory neurons than NT-4/5, and the binding of radiolabelled BDNF to neuronal receptors cannot be fully prevented by NT-4/5 (Dechant et al.,1993). It is possible that this enigma may now be explained by the identification of this new splice variant that differentially binds BDNF and NT-4/5.

Finally, a larger isoform of molecular weight 195 kD has recently been observed in cortical tissue. It is believed that this isoform is specifically enriched in dendrites as there is preferential phosphorylation of this isoform in response to activity-dependent secretion of BDNF from the axons of adrenergic and noradrenergic cells (R. Aloyz, D.R. Kaplan and F.D. Miller, unpublished data).

There are also a number of splice variants of the *trkC* gene, resulting in the expression of eight TrkC receptor isoforms. Four of these isoforms lack the intracellular tyrosine kinase domain (Tsoulfas et al.,1993; Valenzuela et al.,1993) and thus resemble the previously defined

truncated variants of TrkB; three other forms contain variable-sized inserts within their kinase domains (Lamballe et al.,1991,1993; Tsoulfas et al.,1993; Valenzuela et al.,1993). Transcripts encoding all forms of TrkC can be detected throughout the nervous system and display substantial overlap as well as mutually exclusive distribution patterns with transcripts for TrkB. The function of these numerous TrkC receptor isoforms are still unknown, however it is possible that they are responsible for tailoring the responsiveness of the cell to NT-3. Indeed, although forms of TrkC containing insertions in the kinase domain can be phosphorylated in response to NT-3, they do not mediate proliferation in fibroblasts or neuronal differentiation in PC12 cells, and have been shown to possess ten-fold less kinase activity than wildtype TrkC in PC12 cells (Tsoulfas et al.,1993; Valenzuela et al.,1993).

(b) Trk Loss-of-Function (Knockout) Mice:

A great deal of information concerning the importance of the Trk receptors *in vivo*, has come from genetic studies involving the generation of transgenic mice deficient for a particular Trk receptor. In some instances, these studies have highlighted the importance of these receptor tyrosine kinases in mediating the development and survival of neuronal populations. In other instances, however, the effects of deleting these receptors have been surprisingly mild, especially in the central nervous system, suggesting that other molecules are able to compensate for the deletion.

TrkA knockout mice (TrkA-/-) display a number of sensory abnormalities including deficiencies in nociception and thermoception, whereas motor function is preserved (Smeyne et al.,1994). Neuroanatomical evaluation of these mice have revealed extensive neuronal loss in trigeminal, dorsal root and sympathetic ganglia, thus demonstrating the dependence of these cells on TrkA signaling to mediate survival. In contrast, examination of the cholinergic neurons of the medial septum which have been shown to respond to NGF, displayed no significant neuronal loss, but showed a severe decrease in the number of cholinergic fibres projecting from these nerve cells to the hippocampus (Fagan et al.,1997). This decrease in the cholinergic innervation of the hippocampus is similar to that seen in NGF+/- mice (Chen et al.,1997). Thus, the effects of knocking out the TrkA receptor largely correlates with the effects seen in NGF knockout mice. It is important to note, however, that TrkA-/- mice display more severe effects than NGF-/- mice. For example, 70% of DRG neurons in the L4

and L5 lumbar ganglia are lost in NGF-/- mice (Crowley et al.,1994), whereas over 80% of these neurons are lost in TrkA-/- mice (Smeyne et al.,1994). This discrepancy highlights the ability of other neurotrophins such as NT-3 to mediate their actions via the TrkA receptor.

Generation of the TrkB-/- mutant mouse, in which the full-length receptor is not expressed but the truncated form is, results in the survival of mutant mice to birth, though most die within the first postnatal week (Klein et al.,1993). The cause of death soon after birth is thought to be due to their inability to feed. This may be the result of a severe depletion of neurons in the nodose and petrosal ganglia, which normally function to relay visceral sensory information from the gastrointestinal and cardiopulmonary systems. The trigeminal ganglia of these mice also show a depletion of one-half of the normal number of neurons and the DRG neurons are decreased in number by almost one-third. Interestingly, neuronal expression of the extracellular domain of TrkB is clearly present in some DRG neurons in TrkB-/- mice, suggesting that some neurons which express TrkB are not dependent on TrkB signaling for survival (reviewed in Snider and Silos-Santiago,1996). Presumably, these are neurons in which TrkB is colocalized with other Trks. It is probably this redundancy of receptor expression (with TrkC for example [Barbacid,1994]) which accounts for the lack of any gross structural defects in the CNS of TrkB-/- mice as well.

TrkC-/- mice begin to display uncoordinated movements soon after birth, suggestive of an abnormality in proprioception (Klein et al.,1994). Indeed, histological examination has confirmed the absence of Ia proprioceptive muscle afferent projections to spinal motorneurons in these mice. Furthermore, there is a decrease in the number of large myelinated axons in the dorsal root and posterior columns of the spinal cord, indicating a need for NT-3-mediated TrkC signaling in the survival of these neurons. Interestingly, the loss of DRG neurons is more extensive in NT-3 null mutants compared to TrkC null mutants, indicating that NT-3 may support additional classes of neurons at early developmental stages, possibly via TrkA (Farinas et al.,1994).

(v) The p75 Neurotrophin Receptor

Thus far, we have discussed the biological roles of the neurotrophins in specific neuronal populations during development, and the role of Trk receptors in mediating these effects. Although the p75NTR was the first neurotrophin receptor to be identified, the

discovery of the Trk family of receptor tyrosine kinases as the cognate receptor for the neurotrophins left the p75 NTR with a seemingly diminished role, especially as it was clear that binding to the p75 receptor could not account for the survival functions exhibited by the neurotrophins. However, since then it has been clearly demonstrated that p75 plays a very important role in mediating the survival and death of developing neurons both by regulating signaling events mediated by the Trk receptor, and by signaling itself.

In addition to its potential role in neurons, p75 may serve a variety of functions in non-neuronal cells as evidenced by its widespread distribution throughout non-neuronal tissues including the muscle, maxillary pad, kidney, spleen, thymus and lung (Lomen-Hoerth and Shooter, 1995; Wheeler et al., 1998).

(a) The Relationship Between p75 and Trk Receptors:

Many of the *in vitro* investigations regarding the function of p75, and, in particular, the relationship between the Trk and p75 receptors, have been carried out using NGF in PC12 cells. It has been postulated that the p75NTR plays an important role as a coreceptor for the Trks. This is particularly thought to be true for TrkA receptors which typically coexpress with p75 on NGF-responsive cells, in contrast to cells expressing TrkB or TrkC which may not necessarily express p75 (Schechterson and Bothwell, 1992; Verge et al., 1992). Indeed, several groups, in particular that of Moses Chao and his colleagues, have demonstrated that both TrkA and p75 receptors are required to produce high affinity binding sites for NGF (Chao, 1994). NGF has been previously shown to bind to p75 with low affinity (Kd 10^{-9}M) (Klein et al., 1991; Jing et al., 1992), and although there has been some debate regarding the binding of NGF to the TrkA receptor, it is generally believed that most of the binding sites for Trk family receptors are of a low-affinity nature (Kaplan et al., 1991a; Klein et al., 1991; Hempstead et al., 1991; Jing et al., 1992; Meakin and Shooter, 1992; Battleman et al., 1993). Coexpression of both TrkA and p75 in PC12 cells leads to the formation of a limited number of high-affinity NGF binding sites (Kd 10^{-12}M) (Hempstead et al., 1990; Battleman et al., 1993; Mahadeo et al., 1994), however these high affinity binding sites are only observed when the ratio of p75:TrkA receptors is high (Jing et al., 1992). It is important to note that this high affinity binding is not required for NGF to mediate its biological effects (Weskamp and Reichardt, 1991).

Formation of high-affinity binding sites on PC12 cells are generally believed to be mediated by p75-TrkA heteromers (Hempstead et al.,1991; Mahadeo et al.,1994), and recent evidence indicates that p75 and TrkA can interact either directly or via an intermediate protein. For example, Trk and p75 receptors have been shown to coimmunoprecipitate in PC12 cell lines (Ross et al.,1998) and 293 cells (P.A.Barker, personnal communication). Furthermore, TrkA and p75 have recently been shown to copatch in both the absence and presence of NGF in SF9 insect cells (Ross et al.,1996). This ability of p75 to copatch with TrkA was shown to be specific as p75 did not copatch with other tyrosine kinase receptors and was not dependent on tyrosine kinase activity. Copatching of p75 and TrkA receptors have also been observed on sympathetic neurons (Kohn et al.,1997).

The possibility that both p75 and TrkA participate in high-affinity binding by NGF implies that co-expression of these two receptors may lead to a greater sensitivity to neurotrophins (Davies et al.,1993; Verdi et al.,1994; Scheibe and Wagner,1992). Indeed, a number of studies have revealed a facilitating role for p75 in Trk function. For example, sympathoadrenal cells (Mah cells) expressing only TrkA, extended neurites and survived in the presence of NGF, but when coexpressed with p75, showed an 8-fold higher tyrosine phosphorylation of TrkA (Verdi et al.,1994). In addition, a 4-fold higher concentration of NGF is necessary to elicit a survival response in cultured trigeminal sensory neurons in p75-/- mice (Davies et al.,1993). Finally, there is a significant decrease in the number of sensory neurons in p75-/- mice (Lee et al.,1992).

This appears to be contradictory to the plethora of recent studies which have indicated that p75 plays a role in mediating neuronal death (see section I v(d)). Indeed, sympathetic neurons of the SCG have been shown to be more sensitive to limiting concentrations of NGF in p75-/- mice, and less NGF was required to mediate their survival (Bamji et al.,1998). A reexamination of prior work may also support the idea that the p75NTR acts as a negative regulator of survival upon binding to NGF. For example, there is a trend towards an increase in the number of surviving sensory neurons, and an increase in the number of neurite bearing cells, upon addition of the p75NTR functional antibodies, REX and CHEX (Weskamp and Reichardt,1991). These same antibodies were shown to decrease the number of high affinity sites in PC12 cells (Weskamp and Reichardt,1991).

In yet another study, the p75 antibody, MC192, and BDNF were used to disrupt the formation of high affinity binding sites between p75 and TrkA (Barker and Shooter,1994). In this study, treatment of PC12 cells with these ligands resulted in lowered TrkA tyrosine phosphorylation and decreased early gene induction. Although this decrease in tyrosine phosphorylation of the Trk receptor was initially interpreted to display a disruption in high affinity binding of NGF, it has recently been demonstrated by the same group that BDNF-induced activation of the p75 receptor in PC12 cells can decrease TrkA tyrosine phosphorylation via independent signaling events (MacPhee and Barker,1997). In addition, the p75 antibody, MC192, is generally accepted to behave as an agonist for the p75 receptor, as MC192 decreases the survival of neonatal sympathetic neurons both *in vivo* (Johnson et al.,1989), and *in vitro* (M.Majdan and F.D. Miller, unpublished data) in a similar manner to that seen upon p75NTR activation. This may explain the ability of MC192 to activate the p75 receptor in a similar manner as BDNF, and the ability of both these ligands to decrease TrkA tyrosine phosphorylation in PC12 cells.

Finally, higher concentrations of mutant NGF that bind TrkA but not p75 were required to promote the survival of sensory neurons (Horton et al.,1997). Although intriguing, it is impossible to overlook the distinct possibility that the mutant NGF may not activate TrkA at the same dose as wildtype NGF. This needs to be clarified to definitively state that p75 enhances the actions of NGF. This is not to say that p75 is unable to regulate high affinity binding, and unable to enhance NGF's biological actions at specific stages in sensory neuron development. A model of how p75 may play a dual role during different stages of sensory neuron development is outlined in chapter VI. Thus, although it appears that p75 can interact with TrkA to generate high affinity binding sites, it is becoming increasingly apparent that the p75NTR plays a negative role in mediating the survival of many primary neuronal populations.

In light of the fact that alterations in the ratio of p75 and TrkA receptors can effect NGF binding, and considering the differences in p75 to TrkA ratios in PC12 cells and many neuronal populations, as well as variations in p75:TrkA ratios at different stages of development, it is entirely possible that the ability to form high affinity binding varies between neuronal populations and at different developmental stages within the same neuronal population. Thus, the contextual framework is very important during the examination of p75 and TrkA interactions and their biological effects.

One of the most important differences between neural cells and PC12 cells is the absence of a p75-mediated death loop in PC12 cells. Indeed, BDNF is not able to elicit a death response in PC12 cells, nor is NGF able to mediate death in PC12nnr cells. Thus, the biological responses of cells upon TrkA and p75 activation not only depends on the ratio of TrkA to p75, but also on the cellular context.

The p75 receptor is also believed to play a role in ligand discrimination by the Trk receptors, at least in a PC12 cell context (Benedetti et al.,1993). In primary neurons, however, it is possible to reinterpret p75's role in ligand discrimination by the negative survival effect mediated by the p75 receptor. For example, sympathetic neurons from p75 -/- mice are more sensitive to NT-3 than sympathetic neurons from wildtype mice (Lee et al.,1994). Although this was initially interpreted to indicate that a cell's response to a particular neurotrophin may be more selective in the presence of p75 (Chao,1994), an alternative explanation is that p75 has a dampening effect on Trk function in response to ligands that bind weaker than NGF to TrkA, and stronger than NGF to p75. Indeed, NT-3's ability to promote greater survival in sympathetic neurons from p75-/- mice may be interpreted as either the lack of a p75 activated signal "dampening" the survival signals of TrkA, or due to the lack of a direct apoptotic signal via p75 (this idea is further discussed in section v(c), (d) of this chapter). Similarly, the ability of NT-3 to activate TrkA in the PC12 cell line is enhanced when the binding of NT-3 to p75 is prevented by function-perturbing antibodies, or when p75 expression is very low (Clary and Reichardt,1994; Benedetti et al.,1993).

(b) p75 Loss-of-Function (Knockout) and Gain-of-Function Mice:

To elucidate the role of p75 in neural development, p75 knockout mice carrying a null mutation in the neurotrophin binding region (exon 3) of the p75 gene, were generated (Lee et al.,1992). Mutant mice were shown to be viable and exhibit a dramatic decrease in cutaneous sensory innervation, which is associated with decreased heat sensitivity and the development of skin ulcerations. Thus, the overall phenotype of p75 null mice, including the partial loss of NGF-dependent sensory neurons, was thought to be a milder version of the previously-described NGF null mutant mice.

Although the initial report of the p75NTR knockout phenotype appears to support the originally-reported function of p75 as a positive regulator of TrkA activity, more recent studies

have uncovered other defects in the p75NTR knockout mice which are not consistent with this hypothesis. In particular, mutant animals display tissue-specific deficits in sympathetic innervation, resulting in normal innervation of the iris and the sweat glands in some footpads, but the selective depletion of sympathetic innervation in other sweat glands and the complete lack of innervation to the pineal gland (Lee et al.,1994). Furthermore, it has recently been reported that the absence of p75NTR can be neuroprotective during development (Van der Zee et al.,1996; Yeo et al.,1997; Bamji et al.,1998). Indeed, studies have revealed an increase in the number of cholinergic forebrain neurons in p75-/- mice or in wildtype mice injected with a peptide that blocks NGF binding to p75 (Van der Zee et al.,1996; Yeo et al.,1997), with a coincident increase in cholinergic innervation of the hippocampus (Yeo et al.,1997). Similarly, there is an increase in the number of sympathetic neurons in the SCG of p75-/- mice following the period of programmed cell death, which is consistent with an increased sensitivity of cultured p75-/- sympathetic neurons to NGF (Bamji et al.,1998; futher discussed in Chapter IV). Finally, there is an increased survival of motor neurons following injury in p75-/- mice, as compared to wildtype mice (Ferri et al.,1998).

To further elucidate the role of the p75NTR in development, transgenic mice which express the intracellular domain of the p75NTR in peripheral and central neurons (p75ICD mice) were generated (Majdan et al.,1997). These animals demonstrated a marked decrease in the number of sympathetic and peripheral TrkA-expressing sensory neurons, as well as cell loss in the neocortex, where there is normally little or no p75NTR expression. In addition, biochemical analysis suggested that these effects were not attributable to a p75-dependent reduction in TrkA activation. Instead these results suggest that the intracellular domain of p75 may act as a constitutive activator of signaling cascades that regulate apoptosis.

Interestingly, there was a decreased survival of axotomized facial motor neurons in adult p75ICD mice as compared to wildtype mice (Majdan et al.,1997). This, together with evidence suggesting that the loss of p75 leads to the protection of injured motor neurons (Ferri et al.,1998), suggests that p75 may also play a negative role in TrkB responsive neurons following injury. As the number of motor neurons in p75-/- mice and p75ICD mice appear to be similar to that of wildtype mice, p75 may not play a crucial role during programmed cell death in BDNF-responsive populations.

As an interesting adjunct, a number of naturally-occurring, developmentally-regulated p75 splice variants containing the transmembrane and intracellular domain have been recently identified (A.Vaillant and F.D. Miller, unpublished data; Y.A.Barde, personal communication). These p75NTR splice variants are still present in p75-/- mice that were developed by targeting exon 3 in the p75 gene, known to encode the neurotrophin binding region of the extracellular domain. In light of the fact that the p75 intracellular domain may play a role in the constitutive signaling of apoptosis, it may be necessary to reevaluate some of the work done in p75-/- mice. This puts yet another interesting spin on the interpretation of the role of the enigmatic p75 receptor.

(c) p75 and Signaling:

Clues to signaling pathways through which p75 may mediate its actions have come from the analysis of the structurally related tumor necrosis factor family of receptors (TNF receptors). These include TNFR1, TNFRII, the Fas antigen (also referred to as Apo1), CD40, CD30, CD27, DR3, and the lymphoxin β receptor (for reviews see Gruss,1996; Wallach et al.,1997). These receptors are characterized by an extracellular domain containing four cysteine-rich repeats, however, the intracellular domains are substantially divergent and lack any catalytic domain that could provide clues about their mechanism of action. The exception to this is a short segment of homology in Fas and TNFRI referred to as the "death domain" since mutations in this domain inhibit the activation of apoptosis by these receptors (Brakebusch et al.,1992). Recently, regions weakly homologous to the death domain have been observed in other family members including p75 although thus far the function of this domain in the p75 receptor remains unknown (Feinstein et al.,1995). Thus far, the TNF receptor family has been shown to regulate both cell survival and apoptosis via a number of interacting proteins and signaling pathways, including increased production of the lipid messenger ceramide via increased sphingomyelinase activity, activation of the JNK pathway, and activation of gene transcription via nuclear factor κB (NFκB).

The first clear evidence for p75 signaling came from studies by Dobrowsky et al. (1994,1995), who found that binding of neurotrophins to p75 in T9 glioma cells, NIH-3T3 and PC12 cells activated sphingomyelinase, and subsequently, ceramide production, in the absence of their preferred Trk receptor. Interestingly, in PC12 cells that express both the TrkA and p75

receptors, NGF was unable to induce sphingomyelin hydrolysis until TrkA tyrosine kinase activity was inhibited with K252a (Dobrowsky,1995). These data support the idea that the p75NTR serves as a common signaling receptor for the neurotrophins through induction of sphingomyelin hydrolysis, and that crosstalk exists between Trk and p75NTR-dependent pathways such that TrkA signaling may suppress signaling via the p75NTR. In addition, activation of the p75 receptor with NGF in rat oligodendrocytes resulted in an increase in ceramide and c-jun amino-terminal kinase (JNK) activity, whereas the neurotrophins BDNF and NT-3 were not able to mediate these effects (Casaccia-Bonefil et al.,1996). Finally, our lab has demonstrated that p75 can mediate increased c-jun phosphorylation and p53 expression in rat sympathetic neurons following BDNF application (Bamji et al.,1998; Aloyz et al.,1998: see Chapters IV,V).

Concurrent with increases in ceramide levels and JNK activity, activation of the p75 receptor resulted in increased death of cultured oligodendrocytes (Casaccia-Bonefil et al.,1996). As rat oligodendrocytes do not express the TrkA receptor, but do express low levels of TrkB and TrkC, one can conclude that p75 can only effectively signal and mediate death upon binding of a neurotrophin when its cognate Trk receptor is not present, or as Dobrowsky (1994) has shown, when the activity of the cognate Trk receptor is blocked. Alternatively, it is possible that differential binding of the different neurotrophins to the p75NTR may cause p75 to interact with different intracellular signaling proteins, with only NGF stimulating the p75-mediated apoptotic pathway (for review see Chao et al.,1998). Surprisingly, addition of NGF or TNFα to human oligodendrocyes expressing p75 but not TrkA did not result in either death or JNK activation, but did mediate the translocation of NFκB translocation to the nucleus (Ladiwala et al.,1998). This discrepancy can be explained by differences in cellular context and experimental methodology.

The idea that Trk signaling can suppress p75-mediated events was further confirmed in a recent study by Yoon et al. (1998), who show that NGF does not increase JNK activity and cell death in cultured rat oligodendrocytes that were manipulated to express the TrkA receptor. This TrkA-mediated rescue involved not only activation of survival signals, but also a simultaneous supression of a death signal mediated by p75.

P75-mediated NFκB activation and translocation to the nucleus has been demonstrated following NGF application in Schwann cells (Carter et al.,1996) and human oligodendrocytes

that do not express the TrkA receptor (Ladiwala et al.,1998). Interestingly, this p75-mediated event was specifically induced by NGF, and neither BDNF nor NT-3 were able to produce a signal (Carter et al.,1996). Together, these data suggest that neurotrophin binding to the p75NTR results in the activation of at least three signal transduction molecules, NFκB, c-jun, and ceramide. In section III *ii*(b), I will discuss our laboratory's identification of the first signaling pathway required for p75-mediated neuronal cell death consisting of MEKK, JNK, p53 and BAX.

(d) p75NTR and Apoptosis:

The similarity of p75 receptor signaling in certain cell types to signaling in the TNFR family, whose activation often leads to apoptotic events, led to the notion that the p75NTR can also initiate apoptosis. One of the first indications that p75NTR may be responsible for mediating neuronal cell death was suggested by Rabizadeh et al. (1993), who observed that expression of p75NTR induced neural cell death in a constitutive manner. In this study, transfection of the p75NTR in immortalized cerebellar neuronal cell lines resulted in rapid apoptosis which could be rescued by application of NGF or p75 agonist antibodies. Although this finding was interpreted to indicate that p75NTR mediates a ligand-independent, Trk-independent, apoptotic effect that can be inhibited by ligand binding, more recent evidence suggests that p75 can mediate apoptosis in a ligand-dependent fashion that can be modulated by Trk signaling.

The apoptotic function of the p75 receptor has also been demonstrated in cells of the chick isthmo-optic nucleus, which expresses the p75 but not the TrkA receptor. Here, exogenous application of NGF increased the number of dying cells (von Bartheld et al.,1994). Although NGF was initially thought to promote death by decreasing binding of endogenous BDNF to TrkB by blocking the p75 "co-receptor", another interpretation is that exogenous NGF specifically activates the p75NTR, thus increasing the activation of apoptotic pathways.

More direct evidence for ligand-activated mediation of cell death by p75 has been reported by four groups in addition to our own. First, Frade et al. (1996) have shown that early retinal cells expressing p75NTR, but not TrkA, undergo cell death that can be prevented by application of antibodies to NGF, thereby demonstrating naturally-occurring NGF-mediated apoptosis during chick development via p75NTR. Recently, Frade and Barde (1998) have

extended these studies and demonstrate that microglia-derived NGF is responsible for naturally-occurring cell death in the developing retina. Second, Casaccia-Bonefil et al. (1996), have shown that NGF is able to induce apoptosis via activation of the p75 receptor in cultured rat oligodendrocytes that do not express TrkA. Third, a p75NTR function-blocking antibody was able to mimic the actions of exogenous NGF in cerebellar granule cells, resulting in increased survival and Bcl-2 expression (Muller et al.,1997). Finally, NGF was shown to mediate death of embryonic trigeminal neurons lacking the TrkA receptor in a p75-mediated manner (Davey and Davies,1998). Interestingly, NGF-mediated p75 activation was only able to induce apoptosis when these neurons were maintained with CNTF, and not when these neurons were maintained with BDNF, indicating that the activation of different survival signaling pathways in trigeminal neurons influences their susceptibility to p75-mediated apoptosis (Davey and Davies,1998).

It has been proposed that p75 can also mediate apoptosis in the absence of direct ligand activation, and that this can be blocked by activation of Trk receptors. For example, Barrett and Bartlett (1994) have shown that treatment of cultured postnatal mouse DRG neurons with antisense oligonucleotides to p75NTR results in an increase in the amount of survival in the absence of neurotrophin signaling. Similarly, overexpression of the intracellular domain of the p75NTR in p75NTR ICD-expressing transgenic mice resulted in a decrease in the number of small, unmyelinated DRG neurons, and sympathetic neurons of the SCG (Majdan et al.,1997). Although expression of the intracellular domain of p75 is able to mediate neuronal apoptosis (Majdan et al.,1997), it would be interesting to see whether full-length p75 normally signals in a ligand-independent fashion or whether neurotrophins produced by these neuronal populations themselves can mediate p75NTR activation in an autocrine/paracrine fashion.

Although some aspects of the signaling pathways downstream of p75 have been elucidated recently, mechanisms of receptor activation and proximal signaling events are unknown. Analysis of the p75NTR "death domain" has revealed that this domain is unable to self associate (Liepinsh et al.,1997), nor is it able to associate with other death domains of the same subtype (Varfolomeev et al.,1996). Thus, the mechanism of activation of p75 appears to be different from that of Fas and TNFR1, in which the direct association between intracellular death domains is crucial for signaling after ligand binding (Boldin et al.,1996; Muzio et al.,1996). Interestingly, NRIF, the only protein isolated so far that is capable of specifically

interacting with the p75ICD, at least in non-neuronal cells, does not seem to contain a death domain (Carter et al.,1996b).

II. Regulation of Survival By Neurotrophins:

i) Afferent regulation of Neuronal Survival:

Programmed cell death is a major event during the development of the mammalian nervous system, and emphasis has systematically been placed on the roles of either the peripheral targets or central postsynaptic neurons in the control of neuronal survival. However, it is also widely known that afferent input to targets can regulate the survival of target neurons. The role of afferent supply in regulating the survival of target neurons has been typically studied *in vivo* by either examining the effects of deafferentiation or hyperinnervation on target neuron survival.

For example, the assessment of cell death in the isthmo-optic nucleus of the chick following tectal lesions of various sizes, suggests that complete deafferentiation might lead to total cell loss in the nucleus (Clarke,1985). Similarly, in the lumbar spinal cord of the chick, the early removal of either dorsal root or supraspinal afferents increases motor neuron death during the period of naturally-occurring cell death (Hamburger,1975; Okado and Oppenheim,1984).

The hypothesis that afferent supply can regulate the survival of target cells is further strengthened by experiments in which enlargement of the afferent supply leads to the rescue of neurons in the target populations. For example, following retrograde degeneration of the dorsal lateral geniculate nucleus, there is increased sprouting of the retinal ganglion cells into the nucleus of the optic tract and superior colliculus. These hyperinnervated areas had increased numbers of neurons when compared with control rats (Cunningham et al.,1979). Thus, it is generally believed that both the targets and the afferent supply interact in the control of developmental cell death at the level of single cells, ensuring the proper matching of neuron number trans-synaptically.

Various hypotheses have been raised to explain the afferent control of neuronal survival. These mechanisms include activity-dependent processes triggered by neurotransmitters and neuromodulators, and regulation by trophic factors. Indeed, depolarization enhances the survival of several neuronal populations *in vitro* (Bennet and White,1981; Larmet et al.,1992; Lampe et al.,1995), and the specific blockade of postsynaptic

receptors increases neuron loss during the period of naturally-occurring death (Meriney et al.,1987; Maderdrut et al.,1988). These studies suggest that postsynaptic membrane depolarization, triggered by neurotransmitters, may be involved in the control of neuronal survival by active afferents.

Recently, however, it has become increasingly apparent that neurotrophins can be anterogradely transported and released at the site of the target. Indeed, recent studies have proposed that these anterogradely-derived neurotrophic factors may be released like neurotransmitters and peptides in an activity-dependent, regulated manner to mediate the survival and development of target neurons.

The first indication that neurotrophins could be anterogradely transported along axons and released into the postsynaptic cleft was the observation that exogenous NT-3 and BDNF can be anterogradely transported by retinal ganglion cells to their target, the optic tectum (von Bartheld et al.,1996). By examining the pattern of distribution of one of the neurotrophins, BDNF, it soon became evident that endogenous neurotrophins are also transported in the anterograde direction in both the CNS and PNS. For example, immunohistochemical evidence has suggested that BDNF can be anterogradely transported along sensory neurons of the dorsal root ganglia and released in the dorsal horn (Zhou and Rush,1996; Michael et al.1997). The anterograde transport of neurotrophins in the brain has classically been demonstrated by the use of highly specific antibodies, which have identified the presence of BDNF and NT-3 in nerve terminals of brain and PNS populations that do not contain the corresponding mRNA. Indeed, in the brain, BDNF protein, but not its mRNA, was found in the neostriatum (Radka et al.,1996; Altar et al.,1997), neocortex, mossy fibres of the hippocampal granule neurons, medial habenula, central amygdala, lateral septum and spinal cord (Conner et al.,1997; Smith, et al.,1997; Yan et al.,1997). Conversely, neurons that project to these areas are rich in BDNF mRNA.

In addition to being anterogradely transported along neurons of the CNS and PNS, recent studies have suggested that the neurotrophins, BDNF and NT-3 may be secreted in a regulated fashion. Goodman and colleagues (1996) have demonstrated that BDNF co-localizes with the secretory granule marker, chromogranin A, and is released from hippocampal dendrites by a regulated pathway dependent on extracellular calcium. In addition, BDNF immunoreactivity is concentrated over dense-core vesicles in BDNF-immunoreactive dorsal

root ganglia cells (Michael et al.,1997). This observation is further corroborated by recent biochemical studies demonstrating that BDNF is enriched in a vesicular fraction of synaptosomes, its distribution being similar to that of synaptotagmin, which is associated with synaptic vesicles and large dense-core vesicles (Fawcett et al.,1997). The location of BDNF in synaptic vesicles suggests that anterogradely-transported BDNF is released in a regulated, activity-dependent, fashion.

The first *in vivo* demonstration implicating anterogradely-transported neurotrophins in the regulation of neuronal survival came from work done in zebra finches by Johnson and collegues (1997). In this study, removal of presynaptic input from the lateral magnocellular nucleus of the anterior neostriatum (IMAN) to the robust nucleus of the archistriatum (RA), caused massive RA neuron death. This death could be rescued following BDNF, NT-3 or NT4/5 (but not NGF) infusions. Moreover, IMAN neurons express both BDNF and NT-3 and transport these neurotrophins in the anterograde direction. Together, this correlative evidence suggested that anterogradely-transported neurotrophins may be released into the RA to mediate target neuron survival.

BDNF has also been shown to anterogradely regulate survival of CNS neurons in the mammalian brain. In a recent paper by Altar and collegues (1997), inhibition of axonal transport or deafferation depleted the expression of BDNF in nerve terminals in brain areas such as the striatum that lack BDNF mRNA. Concomittant with a decrease in BDNF protein in nerve terminals, there was a decrease in the number of striatal neurons containing the calcium-binding protein, parvalbumin. It is unclear whether decreases in the number of parvalbumin-containing cells in the striatum reflect a decrease in the survival of these neurons, or a decrease in the level of parvalbumin expression.

Finally, endogenous BDNF has been reported to be anterogradely transported in central noradrenergic neurons, regulating the survival and differentiation of target neurons (Fawcett et al.,1998; Chapter III).

Interestingly, although BDNF, NT-3 and NT4/5 have all been shown to be anterogradely transported in the CNS and PNS, there is no evidence that NGF is transported along axons and released at target sites. This observation could mark one of the key differences between the regulation of survival mediated by NGF and the other neurotrophins.

(ii) Target-Derived Regulation of Neuronal Survival - NGF/TrkA Signaling:

The application of a neurotrophin to a neuron which expresses the appropriate receptors may influence survival, growth, differentiation, proliferation, and, in some cases, even death. Therefore, elucidation of molecular mechanisms responsible for the diverse array of responses to neurotrophic factors is fundamental to an understanding of the neurobiology of neurotrophin action. For this reason, the following discussion outlines our current understanding of neurotrophin signal transduction, which is the sequence of molecular events that characterizes a cell's transmission of growth factor signals from the receptor at the cell's surface to the cytoplasm and nucleus.

Much of the work in the field of neurotrophin signal transduction has been done *in vitro* on PC12 cells, where extensive studies have been performed to investigate the activation of signaling cascades upon activation of the TrkA receptor by its ligand, NGF (for review see Kaplan and Stephens,1994). The first event in neurotrophin signaling is the homodimerization of TrkA receptors, leading to the activation of the intrinsic tyrosine kinase activity of TrkA (Jing et al.,1992), with one TrkA subunit catalyzing the phosphorylation of the other subunit in *trans* (Kaplan et al.,1991a,b; Klein et al.,1991). There are five tyrosine phosphorylation sites on the TrkA receptor, including three that reside within the kinase domain, and two that lie outside this domain (Middlemas et al.,1994; Stephens et al.,1994). Trk tyrosine phosphorylation reaches its maximum within 5 to 10 minutes of NGF binding and is then attenuated over a period of several hours (Kaplan et al.,1991b; Segal et al.,1996), most likely by dephosphorylation and internalization of the receptor (Hempstead et al.,1992; Jing et al.,1992; Loeb and Greene,1993). The tyrosine phosphorylated sites of the TrkA receptor serve both as recognition or docking sites for specific intracellular proteins containing either a Src homology 2 (SH2) domain, or a phosphotyrosine binding (PTB) domain (reviewed in Pawson and Scott,1997), and to regulate receptor kinase activity and transphosphorylation (Cunningham et al.,1996; reviewed in Greene and Kaplan,1995).

Previous work using Trk mutants that are defective in either associating with, or stimulating the activities of, intracellular signaling proteins has revealed several signal transduction pathways that are used to mediate a variety of biological effects. Proteins which directly bind to the TrkA receptor to mediate its biological effects include the adaptor proteins Shc and its neural-specific isoform N-shc (Nakamura et al.,1996), phospholipase C-γ (PLC-γ),

the phosphotyrosine phosphatase SHP-1 (SH2-containing tyrosine phosphatase-1), (reviewed in Greene and Kaplan,1995), FRS2 (Kouhara et al.,1997) and two novel phospho Trk interacting proteins, SH2-B and rAPS (Qian et al.,1998).

Propagation of the TrkA signal within PC12 cells and neurons is mediated by both Ras-dependent and Ras-independent signaling pathways (reviewed in Kaplan and Miller,1998). Although the identity of TrkA effectors that mediate Ras-dependent and Ras-independent TrkA signaling in neurons remains largely unknown, Shc has been shown to mediate NGF induced Ras-dependent signaling in PC12 cells (Stephens et al.,1994), and FRS2, SH2-B and rAPS have been shown to mediate NGF induced Ras-dependent signaling in 293 cells (Qian et al.,1998). The most well-studied pathway of Ras activation involves the Shc-Grb2-SOS-Ras pathway. Following its association with TrkA, Shc becomes phosphorylated, thereby enabling it to interact with the SH2 domain containing protein, Grb2. Grb2 then associates with the Ras GTP exchange factor, SOS, via two SH3 domains, translocating SOS to the plasma membrane where it activates Ras (reviewed in Kaplan and Stephens,1994; Greene and Kaplan,1995; Segal and Greenberg,1996; Kaplan and Miller,1997; Vojtek and Der,1998).

(a) Ras Activation and Neuronal Survival:

Once activated, Ras stimulates the activity of several downstream activators, the best characterized of which is the serine-threonine kinase, Raf, which lies upstream of the MEK1/2-MAPK pathway (for reviews see Kaplan and Stephens,1994; Greene and Kaplan,1995; Segal and Greenberg,1996; Kaplan and Miller,1997; Vojtek and Ders,1998; see Figs.1.2, 1.3). A number of Ras effectors in addition to Raf have been reported including PI 3-kinase (Kodari et al.,1994; Rodriguez-Viciana et al.,1994) which plays an important role in neuronal survival, growth and morphology (Dudek et al.,1997; Lavie et al.,1997; Philpott et al.,1997; Crowder and Freeman,1998; Polakiewicz et al.,1998), RalGDS, a guanine nucleotide exchange factor which indirectly activates the small GTPases Cdc42 and Rac, and MEKK, a serine-threonine kinase which activates the JNK signaling pathway (reviewed in Vojtek and Ders,1998).

Although the direct activation of many effector molecules by Ras has not been examined in neuronal cells, Cdc42, Rac (Bazenet et al.,1998) and MEKK (Aloyz et al.,1998; Eilers et al.,1998) have been shown to be involved in stimulating cell death pathways in sympathetic neurons. However, despite Ras's ability to activate both positive and negative

survival pathways in non-neuronal cells, Ras activity has been shown to be essential for a large portion of rat sympathetic neuron survival (Nobes and Tolkovsky,1995; Vogel et al.,1995; Nobes et al.,1996; Markus et al.,1997; Mazzioni et al.,1998). Indeed, knockout mice lacking the GTPase neurofibromin-1 (NF-1), a negative regulator of Ras activity, exhibit an increased number of sympathetic neurons *in vivo*, and an increased survival of cultured sympathetic neurons in the absence of neurotrophin (Vogel et al.,1995). Conversely, transgenic mice which constitutively expressed Ras, exhibit growths resembling neuroblastoma, a tumor of the sympathetic chain (Sweetner et al.,1997). Furthermore, the ability of NGF, CNTF, LIF and cAMP to mediate rat sympathetic neuron survival has been shown to be critically dependent on functional Ras protein (Nobes and Tolkovsky,1995; Markus et al.,1997). In these studies, neutralizing antibodies to Ras inhibited survival mediated by these different survival factors (Nobes and Tolkovsky,1995), whereas constitutively active Ras was found to be sufficient to rescue NGF-deprived SCG neurons (Nobes et al.,1996). Our laboratory has recently confirmed these results, and show that constitutively activated Ras (RasV12) supports, and dominant-negative Ras suppresses, approximately half of the survival responses of rat sympathetic neurons (Mazzoni et al.,1998). The fact that only half of NGF's survival effects are mediated via Ras, points to the presence of multiple survival pathways in sympathetic neurons. These Ras-independent survival pathways may involve PI 3-kinase-Akt, SNT, and/or PLCγ.

This is not to say that all primary neurons require Ras activity for survival. Indeed, the role of Ras in neurotrophin-regulated cell survival and differentiation is believed to vary depending on cell type. For example, embryonic chick sensory neurons (Borasio et al.,1993) and sympathetic neurons from the chick superior cervical ganglia (Markus et al.,1997) were shown to require Ras for survival, whereas embryonic chick sympathetic neurons derived from the more caudally located lumbosacral chain ganglia did not respond to activated Ras (Markus et al.,1997). This would suggest that primary neurons derived from different lineages and species differ in their requirement for Ras in NGF-mediated survival pathways.

How does Ras mediate survival in rat sympathetic neurons? Studies performed in the Miller and Kaplan laboratories suggest that Ras mediates its actions through both the PI 3-kinase-Akt and Raf-MEK-MAPK signaling pathways, with the PI 3-kinase-Akt pathway mediating a slightly higher proportion of Ras's survival effects (Mazzoni et al.,1998; see Figs.1.2, 1.3). In addition to activating downstream survival pathways, Ras has also recently

been shown to mediate survival by suppressing apoptotic signaling pathways (Mazzoni et al.,1998; Fig.1.3). Indeed, expression of constitutively activated Ras (RasV12) suppresses c-jun phosphorylation, and increases in p53 and Bax levels, in the absence of NGF (Mazzoni et al.,1998). Conversely, expression of dominant-negative Ras in sympathetic neurons leads to increased c-jun phosphorylation and increased p53 and Bax levels even in the presence of NGF (Mazzoni et al.,1998). The following sections highlight the roles of the PI 3-kinase-Akt and Ras-Raf-MEK-MAPK pathways in sympathetic neuron survival.

(b) The PI 3 kinase/ PKB/Akt Signaling Pathway:

PI 3-kinase is activated by NGF and other neurotrophic factors in neuronal cells in either a Ras-dependent, or independent fashion (reviewed in Kaplan and Stephens, 1994). Ras-independent PI 3-kinase activation is believed to be mediated via the recruitment of PI 3-kinase to the plasma membrane by the effector proteins Shc-Grb2-Gab-1 (J.Korhonen and D.R.Kaplan, unpublished data; see Fig.1.2; 1.3). This enzyme phosphorylates phosphoinositides (PIs), leading to the generation of the phospholipid second messengers, phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P3], phosphatidylinositol (3,4)-diphosphate [PtdIns(3,4)P2] and PtdIns(3)P (for a review see Kapeller and Cantley,1994).

The serine-threonine kinase Akt/PKB has been shown to be a downstream effector of PI 3-kinase (Franke et al.,1995; see Fig.1.2, 1.3). Akt/PKB contains a pleckstrin homology (PH) domain that is essential for its activation in response to growth factors (Franke et al.,1995; Andjelkovic et al.,1996) and in response to PI 3-kinase activation (Klippel et al.,1996; Franke et al.,1997; Marte et al.,1997). Indeed, it is believed that this PH domain binds directly to the phosphoinositides [PtdIns(3,4,5)P3] and [PtdIns(3,4)P2] which are produced as a result of PI 3-kinase activation (Franke et al.,1997), thereby recruiting Akt/PKB to the plasma membrane and stimulating its activity. Full activation of Akt/PKB requires phosphorylation at two major sites, Thr308 in the kinase domain, and Ser473 in the carboxy domain (Alessi et al.,1997). Support for the idea that Akt/PKB activation occurs downstream of PI 3-kinase comes from the demonstration that i) constitutively active forms of PI 3-kinase results in stimulation of Akt/PKB (Klippel et al.,1996; Franke et al.,1997; Marte et al.,1997), ii) Akt/PKB overexpression prevents neuronal apoptosis in the absence of PI 3-kinase activity (Dudek et al.,1997; Crowder and Freeman,1998), and iii) dominant-negative forms of Akt/PKB interfere

with IGF-1 and NGF-mediated survival in spite of PI 3-kinase activation (Dudek et al.,1997; Crowder and Freeman,1998).

PI-3 kinase activity has been shown to be both sufficient and necessary for the survival of NGF-dependent rat sympathetic neurons (Crowder and Freeman,1998), and for insulin growth factor-1 (IGF-1)-mediated survival of cultured cerebellar granule cells (D'Mello et al.,1997; Dudek et al.,1997). Indeed, expression of constitutively active PI 3-kinase mediated over 75% of sympathetic neuron survival in the absence of NGF (Crowder and Freeman,1998). The necessity of PI 3-kinase in mediating NGF's survival response has recently been confirmed by the Kaplan and Miller laboratories who show that expression of dominant-negative Akt, or treatment with the PI 3-kinase inhibitor, LY294002, inhibit over 60% of NGF-mediated sympathetic neuron survival (N.Marsh, J.Atwal, F.D.Miller and D.R.Kaplan, unpublished data). This would indicate that the PI 3-kinase-Akt pathway is the major survival pathway activated by NGF in rat symapthetic neurons. The discrepancy between sympathetic neuron death induced by PI 3-kinase inhibition and NGF withdrawal indicate the presence of additional factors which are responsible for NGF-mediated sympathetic neuron survival, albeit to a lesser degree than PI 3-kinase. In contrast, Philpott and collegues (1997) argue that PI 3-kinase is sufficient, but not necessary for sympathetic neuron survival. One plausible reason for this discrepancy includes the inability to express sufficient levels of dominant negative PI 3-kinase by microinjection. This does not, however, explain why the PI 3-kinase inhibitor, LY294002, failed to decrease the survival of sympathetic neurons in this study.

Although the PI 3-kinase-Akt pathway is belived to be the major survival pathway in sympathetic neurons, PI 3-kinase is also able to activate other downstream effectors which may account for some of the survival mediated by PI 3-kinase (Shepherd et al.,1998). Indeed, inhibition of PI 3-kinase activity results in increased symapthetic neuron death compared to dominant-negative Akt (N.Marsh, J.Atwal, F.D.Miller and D.R.Kaplan, unpublished data). Thus, Ras-dependent and Ras-independent activation of PI 3-kinase mediates the majority of NGF-induced survival in sympathetic neurons, and the majority of PI 3-kinase's effects are mediated by Akt.

The mechanism by which Akt functions to promote survival is just recently beginning to be understood. It was initially postulated that activation of the PI-3 kinase-Akt signaling pathway culminates in the phosphorylation of the BCL-2 family member BAD, thereby

suppressing apoptosis and promoting survival (Datta et al.,1997). In the absence of phosphorylation, BAD is thought to induce cell death by forming heterodimers with BCL-X_L resulting in the concommitant generation of BAX homodimers. This model, known as the "rheostat model" is further discussed in section III of this chapter. It must be noted, however, that Akt has only been shown to phosphorylate BAD in myeloid precursor cells (Datta et al.,1997), and that BAD is not phosphorylated in most neuronal cells, indicating that this is not the major mechanism by which Akt mediates neuronal survival.

More recently, Akt has been shown to induce phosphorylation of caspase-9, which prevents the cytochrome c-induced proteolytic processing of this caspase (Cardone et al.,1998; Fig.1.3), thus preventing the caspase cascade that typically culminates in cell death. As caspase-9 has been shown to be an important caspase during neuronal apoptosis (Hakem et al.,1998; Kuida et al.,1998), it is conceivable that Akt mediates neuronal survival via the phosphorylation of this caspase.

Preliminary evidence from the Miller laboratory suggests that expression of dominant-negative Akt in sympathetic neurons results in decreased survival, increased c-jun phosphorylation and increased p53 levels in the presence of NGF (R.Aloyz, D.R.Kaplan, and F.D.Miller, unpublished results; Fig.1.3). Together, this suggests that Akt may impinge on multiple apoptotic signaling proteins to inhibit cell death both upstream and downstream of Bax and Bad, the relative contribution of which may depend on cellular context.

In accordance with the idea that multiple cross-talk occurs between survival and death signaling pathways, Akt activity has been shown to be suppressed by C2-ceramide (Summers et al.,1998; Zhou et al.,1998; Zundel and Giaccia,1998; Fig.1.3). Although it has been shown that ceramide acts upstream of Akt (Zhou et al.,1998; Zundel and Giaccia,1998), it is unclear whether this regulation occurs at the level of PI 3-kinase due to conflicting reports (Zhou et al.,1998; Zundel and Giaccia,1998).

(c) The Ras/ MapKinase Signaling Pathway

Ras and its downstream effectors Raf, MEK and MAP kinase have been shown to be necessary and sufficient for NGF-mediated neuritogenesis, but not survival of PC12 cells (Greene and Kaplan, 1995, Kaplan and Miller,1997). As Ras activation correlates with sustained MEK and MAPK activities in neurons (Rosen et al.,1994), it was postulated that this

pathway may play a role in neurotrophin-mediated survival and neuritogenesis. However, several studies have clearly demonstrated that activation of the Raf/MEK/MAP kinase pathway is insufficient for many of the responses of immortalized hippocampal neurons and primary neurons to neurotrophic factors (reviewed in Kaplan and Miller,1997). First, although prolonged activation of Raf-1 induced differentiation in immortalized hippocampal neurons, prolonged activation of MEK and MAPK was insufficient to elicit this response (Kuo et al.,1996). Second, sustained activation of Ras and MAPK was insufficient to mediate the survival of primary hippocampal pyramidal neurons (Marsh and Palfrey,1996). Third, persistent stimulation of the Ras/MAPK pathway was insufficient to mediate neuritogenesis (Vaillancourt et al.,1995) or increases in calcium channel currents (Pollack and Rane,1996) in PC12 cells. Fourth, inhibition of MEK activity with the MEK inhibitor, PD98059, failed to block NGF-dependent survival of rat sympathetic neurons (Creedon et al.,1996; Virdee and Tolkovsky,1996; Vaillant et al.,1998), and NGF-mediated neurite outgrowth from chick sensory and sympathetic neurons (Klinz et al.,1996). Interestingly, although inhibition of MEK activity with the MEK inhibitor, PD98059, failed to block NGF-dependent survival of rat sympathetic neurons, PD98059 was able to significantly inhibit survival mediated by RasV12 (Mazzoni et al.,1998). Together, one can conclude that: 1) Ras plays a role in mediating neurotrophin-mediated survival of most neuronal populations including sympathetic neurons of the rat SCG, 2) approximately 50% of survival mediated by NGF in sympathetic neurons occurs in a Ras-dependent manner, 3) Ras mediates survival via the PI 3-kinase and Ras-Raf-MEK1/2-MAPK pathways, 4) Ras-dependent and Ras-independent activation of PI 3-kinase accounts for the majority of NGF-induced sympathetic neuron survival, and 5) the overall contribution of the Ras-Raf-MEK1/2-MAPK signaling pathway in NGF-mediated sympathetic neuron survival is minor.

(d) Depolarization-Mediated Survival:

Although neurotrophic factors are the major determinants of neuronal survival during the development of the vertebrate nervous system, other factors also make important contributions. Electrical activity is thought to be one of these factors (Franklin and Johnson,1992; Schmidt and Kater, 1995). This is suggested by experiments showing that removal of afferent input or pharmacological blockade of electrical activity or

neurotransmission causes the death of some types of developing neurons (Lipton, 1986; Maderdrut et al., 1988; Ruitjer et al., 1991; Catsicas et al., 1992; Galli-Resta et al., 1993). Similarly, *in vitro* experiments have demonstrated that maintenance of developing neurons in cell culture medium containing elevated concentrations of K^+ greatly enhances the survival of these neurons (for review see Franklin and Johnson, 1992). This increased extracellular K^+ concentration is believed to enhance survival by causing a chronic depolarization of cells that may promote survival by mimicking the effects of naturally-occurring electrical activity. Franklin et al. (1995) have shown that sustained depolarization of neonatal SCG neurons with K^+ leads to increased survival by inducing the influx of Ca^{2+} through voltage-gated L-type Ca^{2+} channels as withdrawal of extracellular Ca^{2+}, or blockage of L-type Ca^{2+} channels abolishes the K^+-mediated survival (see Fig. 1.2). Increases in intracellular Ca^{2+} levels can activate Ca^{2+}-sensitive enzymes such as protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Indeed, calmodulin antagonists block the survival of depolarized rat sympathetic neurons (Franklin et al., 1995; Vaillant et al., 1998) and chick spinal cord motorneurons (Soler et al., 1998) at concentrations that do not affect the survival of cells maintained in NGF, indicating that calmodulin is involved in calcium-mediated survival of neurons.

Previous studies have shown that an increase in intracellular Ca^{2+} is able to activate Ras in PC12 cells (Rosen et al., 1994; for review see Finkbeiner and Greenberg, 1996; Fig. 1.2). As mentioned previously, NGF-mediated Ras activation leads to the activation of MAPK via Raf and MEK1. Interestingly, the 44 kDa MAPK protein is phosphorylated upon K^+-mediated depolarization of sympathetic neurons, leading to the intriguing idea of a common survival pathway in both NGF-mediated and K^+-mediated survival. Although inhibition of MAPK in depolarized cerebellar granule cells did not affect K^+-mediated survival of these neurons (Miller et al., 1997), our laboratory has recently shown a significant reduction in K^+-mediated sympathetic neuron survival upon application of the MEK1 inhibitor, PD98059 (Vaillant et al., 1998).

Activation of the PI-3 kinase pathway may be stimulated by increases in intracellular Ca^{2+} levels potentially via a Ras-dependent mechanism (see Fig. 1.2). Although inhibition of PI-3 kinase activity has been shown to block depolarization-mediated survival of cerebellar granule cells (Miller et al., 1997), there is still some contention as to the role of PI-3 kinase in

mediating depolarization-induced survival. Indeed, Soler et al. (1998) have recently demonstrated that PI-3 kinase is not activated upon depolarization of chick spinal motor neurons, and that inhibition of PI-3 kinase does not suppress the survival-promoting effect of K^+ treatment. Similar to that seen in cerebellar granule cells, our laboratory has recently shown that PI 3-kinase activity is essential for K^+-mediated sympathetic neuron survival (Vaillant et al.,1998). Indeed, blocking PI 3-kinase activity with the PI 3-kinase inhibitor, LY294002, decreased K^+-mediated survival, albeit to a lesser degree that PD98059 (Vaillant et al.,1998). This result suggests that although both the Ras-MAPK and the PI 3-kinase-Akt pathways play a role in sympathetic neuron survival, the Ras-MAPK pathway plays a greater role in K^+-mediated survival, whereas the PI 3-kinase-Akt pathway plays a greater role in NGF-mediated survival.

III. Neurotrophic Factors and Cell Death:

i) Apoptosis:

The molecular mechanisms underlying cell death are not well understood. Based upon morphological criteria, several different modes of cell death have been defined (Schweichel and Merker,1973; Beaulaton and Lockshin,1982; Clarke,1990) for which different underlying molecular mechanisms are believed to exist. Among the different types of cell death, the mode of apoptosis (Kerr et al.,1972) has received much attention since many cell types exhibit this mode of death during development and adulthood. At early stages of apoptosis, morphologically visible cytoplasmic changes are subtle. There is a progressive loss of cell volume, but mitochondria and other organelles remain physically intact for an extended period. Among the principle criteria that characterize cell death as apoptotic are the condensation of nuclear chromatin (Kerr et al.,1972), activation of endonucleases and the early fragmentation of DNA (for reviews see Bursch et al.,1990; Deckworth and Johnson,1993; Vaux,1993). Transient blebbing of the plasma membrane occurs after which all cytoplasmic organelles start to degenerate, become compartmentalized into membrane-bound apoptotic bodies and are then engulfed by phagocytic cells.

(a) Apoptosis and the Development of the Nervous System:

The development of most regions of the vertebrate nervous system, including sensory, motor and autonomic neurons of both the peripheral nervous system (PNS) and CNS, includes a distinct phase of neuronal degeneration during which a substantial proportion of the neurons initially generated die (Oppenheim, 1991). This degeneration primarily adjusts the size of the neuronal population to the size or the functional needs of its projection field. However, in this process, it also eliminates any neurons whose axons have grown to the wrong target (Clarke and Cowan, 1976; O'Leary and Cowan, 1982).

This type of death, known as programmed cell death or naturally-occurring cell death, occurs via an apoptotic mechanism, and it is generally believed that programmed cell death is due to insufficient target-derived trophic support (reviewed by Oppenheim, 1991; Deckworth and Johnson, 1993). For example, in most systems undergoing programmed cell death, it has been possible to show that all, or at least the great majority of the axons, have reached the target field before the onset of cell death (Clarke and Cowan, 1976; Oppenheim and Chu-Wang, 1977). Furthermore, studies have shown that injection of NGF antibodies into newborn rats increased the death of sympathetic neurons (Levi-Montalcini et al., 1969; Angeletti et al., 1971; Wright et al., 1983), and that these cells die by a process of apoptosis. In addition, NGF-deprived embryonic sympathetic neurons *in vitro* die by apoptosis (Martin et al., 1988).

Several *in vitro* (Martin et al., 1988; Scott and Davies, 1990; Edwards et al., 1991) and *in vivo* (Oppenheim, 1991) studies have shown that neuronal death is prevented by inhibitors of protein and RNA synthesis, suggesting that, in most cases, apoptotic death of neurons involves *de novo* protein synthesis.

(b) Temporal Analysis of Events Associated with NGF Withdrawal-Induced Death of Sympathetic Neurons:

The most well-examined system of trophic factor deprivation-induced apoptosis is based on the role of NGF in maintaining sympathetic neurons during development (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979) and in the adult (Gorin and Johnson, 1980). The current understanding of the temporal events associated with NGF withdrawal-induced death has been analyzed by Deckworth and Johnson (1993), and is summarized here and in figure 1.4.

Studies demonstrate that morphological changes in cultured sympathetic neurons occur approximately 19 hours after NGF deprivation. These changes include irregularly shaped cellular outlines, the expression of small vacuoles, and a shift in the nucleus to one side of the soma. After 36 hours, 95% of the neurons are atrophied and half the neurons have died, whereas 48 hours after NGF withdrawal, 95% of the neurons are dead (Deckworth and Johnson, 1993).

A neuron that has been deprived of its neurotrophin will eventually lose its ability to respond to reapplication of its physiological trophic factor with long-term survival. Upon loss of this ability, the neuron is committed to die. All sympathetic neurons can be rescued by readdition of NGF up to 12 hours after trophic factor deprivation (Deckworth and Johnson, 1993). After 22 hours, half of the neurons are committed to die, and after 48 hours, >95% of all sympathetic neurons are committed to die (Deckworth and Johnson, 1993). The time course of rescue with NGF precedes the time course of neuronal death by approximately 5 hours, indicating that there is a period in the life of a neuron during which the degenerative processes has become irreversible but has not yet caused the death of the neuron (Deckworth and Johnson, 1993).

The atrophy of the neuronal soma indicates a reduction of cell body mass that may be caused at least in part, by a decrease of total neuronal protein caused by a decrease in the levels of protein synthesis and RNA synthesis. In fact, within 12 hours after onset of NGF deprivation, glucose uptake, protein synthesis, and RNA synthesis fall dramatically, followed by a moderate decrease in mitochondrial function (Deckworth and Johnson, 1993).

Interestingly, a comparison of the rescue of NGF-deprived sympathetic neurons with either NGF alone or NGF plus the protein synthesis inhibitor, cyclohexamide, reveals no difference in the number of rescued cells (Deckworth and Johnson, 1993). This suggests that protein synthesis is not required for NGF to protect NGF-deprived neurons from cell death. One could also hypothesize that NGF's role in mediating cell survival is to suppress apoptotic pathways.

Fragmentation of neuronal DNA can be observed in NGF-deprived sympathetic neurons, approximately 5 hours before cell death, and concurrent with commitment to die (Deckworth and Johnson, 1993). The temporal and pharmacological characteristics of DNA fragmentation is consistent with DNA fragmentation being part of the mechanism that commits

the neuron to die. Thus, trophic factor deprivation-induced death occurs by apoptosis and is an example of programmed cell death.

(c) Cell-Cycle -Associated Genes and Apoptosis:

It has been suggested that apoptosis could result from a situation where cell proliferation is initiated, but cannot be successfully completed. For example, the *c-myc* oncogene, which is a potent activator of proliferation, can induce apoptosis under conditions of restrained cell proliferation such as serum deprivation (Evan et al.,1992). Indeed, cells undergoing apoptosis have been shown to display some characteristics of mitotic cells, such as chromatin condensation, breakdown of the nuclear envelope, rounding up of the cell, and some cytoskeletal changes. On the basis of these observations, it has been proposed that in postmitotic cells such as neurons, apoptosis may represent an abortive attempt by cells to re-enter the cell cycle (Batistatou et al.,1993; Heintz,1993; Rubin et al.,1993). Supporting this concept is the fact that trophic factor withdrawal-induced death of PC12 cells and sympathetic neurons can be inhibited by treatment with pharmacological blockers of the G1/S phase of the cell cycle (Farinelli and Greene,1996). Cyclin D1, which is involved in the progression of the cell cycle, is also induced in NGF-deprived sympathetic neurons (Freeman et al.,1994). However, the inability of antisense cyclin D1 to decrease the susceptibility of sympathetic neurons to NGF deprivation, raises the possibility that cyclin D1 induction may not be necessary for apoptosis (Greenlund et al.,1995).

Another gene known to be involved in the control of neuronal cell cycle progression, is the retinoblastoma (*Rb*) gene. Mice lacking the *Rb* gene display abnormal and massive neuron loss during development and die prenatally (Lee et al.,1992; Slack et al.,1998). Interestingly, in cortical precursor cells, pRb was determined to be essential for cells to exit the cell cycle, but not required for the induction of neuronal genes such as Tα1 α-tubulin (Slack et al.,1998). Functional ablation of the pRb family in cortical progenitor cells *in vitro* by expression of a mutant adenovirus E1A protein, resulted in cortical progenitor death in cells expressing neuronal genes, presumably as a consequence of "mixed signals" deriving from their inability to undergo terminal mitosis (Slack et al.,1998). Ablation of pRb in postmitotic cortical neurons did not effect neuronal survival (Slack et al.,1998). Together, these data raise the possibility that neuronal apoptosis is inextricably linked to the cell cycle, and may arise from "mixed

signals" to proliferate while expressing neuronal markers that typically indicate terminal mitosis. Indeed, it would be interesting to see whether cortical precursor cells lacking pRb undergo apoptosis following the supression of neuronal gene induction.

The tumor suppressor protein, p53, is another cell-cycle-associated molecule that has been implicated in the induction of apoptosis. For instance, the expression of p53, which arrests the cell cycle at the G1/S border in response to DNA damage, is increased during apoptosis in some paradigms. A complete discussion of the role of p53 in mediating apoptosis is outlined in section ii (c) below.

(ii) Molecular Mechanisms Underlying Apoptosis:

(a) The Tumor Necrosis Factor (TNF) Family of Receptors and Apoptosis:

It has been postulated that apoptosis is a default pathway, activated automatically in the absence of incoming survival signals from the cell's environment. However, it has also been demonstrated that, in certain circumstances, cells can be stimulated to undergo apoptosis by activation of cell surface receptors. For example, activation of the tumor necrosis factor 1 receptor (TNFR1) (Tartaglia et al.,1991), Fas (also known as CD95 and Apo-1) (Itoh et al.,1991), and the recently identified DR3 (death receptor-3) (Chinnaiyan et al.,1996; Kitson et al.,1996) initiates apoptosis in certain cells. These three receptors are all members of the TNF receptor superfamily, and share a region of similarity in their intracellular domains with the Drosophila cell death protein, reaper (Itoh and Nagata,1993). This region of similarity is termed the death domain and has been shown to be necessary for the apoptotic functions of the TNFR1 (Tartaglia et al.,1993) and Fas (Behrmann et al.,1994) receptors.

Interestingly, the p75NTR, which shares structural homology with the TNF family of receptors, also contains a region in its intracellular domain that is homologous to the death domain (Liepinsh et al.,1997). It is still unclear, however, whether this region is essential for p75 mediated apoptosis. As TNFR1, Fas, DR3 and p75 can mediate apoptosis, and as all four receptors contain homologies in their death domains it is important to review signaling pathways mediated by the TNF family of receptors as it may shed light on signaling events mediated by the p75NTR. Although p75NTR, TNFR1, Fas, and DR3 all mediate apoptosis and contain cytoplasmic death domains, most other family members do not contain this death

domain and function in stimulation of immune responses rather than the induction of apoptosis (Smith et al.,1994).

A major advance in understanding the early events in TNF signaling was the identification of proteins that become recruited to TNF receptors following ligand-induced trimerization of these receptors (Rothe et al.,1994,1995; Hsu et al.,1995). Being protein-protein interaction domains, the death domains of TNFR1 and Fas recruit other death domain containing proteins, TRADD (TNFR1-associated death domain protein), FADD/MORT1 (Fas-associated death domain protein/ mediator of receptor-induced toxicity), and RIP (receptor interacting protein) to the trimerized receptors (Hsu et al.,1995,1996a,1996b; Chinnaiyan et al.,1995; Boldin et al.,1996; Stanger et al.,1995). Some of these directly interact with the death domain of the receptor, and others interact indirectly via the death domain of other adaptor proteins (see Figs.1.5, 1.6).

TRADD, one of the first TNFR1-associated proteins identified, has been shown to mediate both apoptosis and NFκB activation upon recruitment to TNFR1 (Hsu et al.,1995). Furthermore, overexpression of either full-length TRADD or the TRADD death domain is sufficient to modulate TRADD self-association, NFκB activation, and apoptosis (Hsu et al.,1995). It is interesting that TRADD can mediate both apoptosis and NFκB activation, as NFκB clearly plays an anti-apoptotic role in TNFR1 signaling (Liu et al.,1996). Furthermore, TRADD-induced apoptosis, but not NFκB activation, can be inhibited by CrmA, a specific inhibitor of the ICE family that is encoded in the cowpox virus genome (Hsu et al.,1995). These results suggest that TRADD may mediate two diverse signaling pathways of life and death and that the apoptotic pathway involves ICE-like proteases.

Despite the fact that the Fas receptor also contains a death domain and can initiate apoptosis, the death domain of Fas is unable to associate with TRADD (Baker and Reddy,1996; see Fig.1.6). This result, in conjuction with others, suggests a divergence in TNFR1 and Fas mediated apoptotic pathways.

FADD/MORT1 appears to be highly selective in its association with Fas via death domain interactions (Fig.1.6), however, a only a weak direct interaction between FADD and TNFR1 has been detected (Chinnaiyan et al.,1995). FADD's interaction with TNFR1 is typically thought to be mediated indirectly though TRADD via death domain interactions (Fig.1.5). Analogous to TRADD, overexpression of FADD/MORT1 can induce apoptosis

which is inhibitable by CrmA (Chinnaiyan et al.,1995), lending support to the idea that Fas and TNFR1-mediated apoptosis involves ICE or a related protease. Interestingly, in contrast to TRADD, overexpression of the FADD/MORT1 death-domain alone cannot induce apoptosis, and mutagenesis studies have shown that the "death effector domain" of FADD/MORT1, which is in the amino terminus, is crucial for FADD's apoptotic function. This suggests that the critical apoptotic part of TRADD is its death domain, which mediates the interaction between FADD and TNFR1, whereas the critical function of FADD/MORT1 may be to recruit death-inducing factors to the receptor via it's "death effector domain" (Fig.1.5). Indeed, it has recently been shown that FADD's death effector N-terminal domain recruits the ICE-like protease MACH (MORT1-associated CED-3 homolog)/FLICE (FADD-like ICE) to activated receptor complexes (Boldin et al.,1996; Muzioet al.,1996). MACH/FLICE, also known as caspase-8, associates with the receptor through caspase-8's amino terminal death effector domain (Boldin et al.,1996; Muzioet al.,1996; Fig.,1.6). Overexpression of caspase-8 induces apoptosis which can be blocked by inhibitors specific for the ICE family. It has been suggested that cleavage of caspase-8 may mediate the downstream effects of apoptosis.

RIP is yet another death domain-containing protein shown to interact with the cytoplasmic domain of Fas, however, in addition to its death domain, RIP contains a tyrosine kinase domain at its amino terminus (Stanger et al.,1995). RIP-induced apoptosis requires the death domain, but not the kinase domain of RIP. This protein has recently been shown to interact with TRADD through death domain interactions, and it is postulated that TRADD acts as an adapter protein to recruit RIP to TNFR1 (Hsu et al.,1996b; Ting et al.,1996). Overexpression of RIP induces NFκB activation (Hsu et al.,1996b), and cell lines lacking RIP were found to be defective in the activation of NFκB in response to TNF (Ting et al.,1996). However, mutant Jurkat cells lacking the RIP protein were fully susceptible to Fas-mediated apoptosis, suggesting that RIP plays an essential role in mediating NFκB activation by TNF, but is dispensible for Fas-induced apoptosis (Ting et al.,1996).

The only two components of the TNFR1 complex that can activate JNK upon transient expression are RIP and TRAF2 (TNFR-associated factor 2) (Fig.1.5). While TRAF2 does not mediate apoptosis (Hsu et al.,1996a), RIP does (Stanger et al.,1995; Hsu et al.,1996b), and thus it was postulated that RIP may mediate TNF-induced apoptosis via activation of the JNK pathway. However, a recent study indicates that JNK activation by RIP is not responsible for

RIP's apoptotic effects (Liu et al.,1996). In this study, RIP mutants that lacked the death domain and were incapable of mediating apoptosis, were fully competent in mediating JNK activation. Furthermore, expression of the RIP death domain which was sufficient to induce apoptosis (Stanger et al.,1995; Hsu et al.,1996b) did not activate JNK (Liu et al.,1996). Thus, they concluded that although RIP is capable of inducing both apoptosis and JNK activation, the two responses can be separated and involve different functional domains of this protein. Further evidence that JNK activation and TNFR1-mediated apoptosis are separate events is provided by the findings that mutant FADD lacking the death domain blocked TNF-induced apoptosis, but not JNK activation, while wild type FADD induced apoptosis but not JNK activation (Liu et al.,1996). Although this study appears to clearly demonstrate that in human breast carcinoma (MCF7) cells, JNK is not involved in TNF-mediated apoptosis, it is not hard to envisage a scenario in which the JNK pathway does indeed lead to apoptosis, but with a slower timecourse. The rapid apoptosis mediated by activation of FADD and the ICE-like protease pathway (4-6 hours; Clement and Stamenkovic,1994) could easily mask a slower, JNK-mediated apoptotic event. Indeed, TNF treatment has been shown to lead to increased ceramide levels, activation of the JNK pathway, and apoptosis in a number of other cell lines (described later).

Despite the focus on the TNFR1 and Fas receptors and their associated proteins, the TRAF1 (TNFR-associated factor 1) and TRAF2 (TNFR-associated factor 2) proteins which interact with the cytoplasmic domain of TNFR2, were actually the first TNFR superfamily associated proteins to be found (Rothe et al.,1994). TNFR2 has been associated with TNF's ability to stimulate cell proliferation and activation of NFκB (Tartaglia et al.,1991). TRAF1 and TRAF2 can form heterodimers via interactions between their TRAF domains, and the interaction of TRAF1 with TNFR2 appears to be mediated through TRAF2.

TRAF2 interacts not only with TNFR2, but also with TNFR1 via TRADD (Hsu et al.,1996a) (Fig.1.5). TRAF2 is believed to mediate the activation of both NFκB and JNK. Whether TRAF2 is necessary for NFκB activation is still unclear as TRAF2 mutants lacking the RING finger inhibit TNF-induced NFκB activation (Hsu et al.,1996a), but dominant negative forms of TRAF2 are unable to abrogate the activation of NFκB (Lee et al.,1997). The *in vivo* expression of a dominant negative TRAF2 protein demonstrated the need for TRAF2 in TNF-mediated activation of JNK (Lee et al.,1997). Recently, a TNF-responsive

serine/threonine protein kinase termed germinal center kinase related (GCKR) protein has been identified and shown to act downstream of TRAF2 to activate the JNK pathway, but not the NFκB pathway (Shi and Kehrl,1997; Fig.1.5). It is commonly believed that TRADD is a bifurcating point in the TNFR1 signaling pathway that interacts with TRAF2 to mediate NFκB and JNK activation and with FADD to induce apoptosis via the ICE-like proteases (Hsu et al.,1996a).

Another mechanism by which TNFR1 and Fas are believed to mediate its actions is by the production of the second messenger, ceramide (Kolesnick and Golde,1994) (Figs.1.5, 1.6). Occupancy of either TNFR1 or Fas was reported to activate sphingomyelinases, phospholipases that catalyze ceramide production from sphingomyelin (Weigmann et al.,1994; Gulbins et al.,1995). When applied exogenously, ceramide can cause apoptosis (Obeid et al.,1993) and NFκB (Weigmann et al.,1994) and JNK activation (Verheij et al.,1996). Indeed, JNK activation was proposed to mediate apoptosis in response to either TNF or exogenous ceramide via a c-jun-dependent mechanism (Verheij et al.,1996). Here, expression of a dominant negative c-jun mutant inhibited TNF and ceramide-induced death (Verheij et al.,1996). In addition, sphingosine-1-phosphate, a metabolite of ceramide, has been shown to counteract the ceramide-induced activation of JNK (Cuvillier et al.,1996), resulting in increased ERK activation (Wu et al.,1995). This points to a potential cross-talk between these two pathways.

Many researchers interested in unraveling the signaling pathways mediated by p75 activation have turned to signaling events mediated by the TNF family of receptors as a starting point in their endeavors. Indeed, TNFR1, Fas and p75 can all mediate apoptotic events, increase intracellular ceramide levels, enhance NFκB translocation to the nucleus and activate the JNK pathway. However, the upstream events in TNF receptors and p75 do not appear to be similar.

TNF has been shown to rapidly induce apoptosis via the ceramide-JNK-c-jun pathway in U937 human monoblastic leukaemia cells and bovine aortic endothelial (BAE) cells (approximately 6 hours following TNF application) (Verheij et al.,1996). Conversely, p75NTR activation is able to increase ceramide levels, activate the JNK pathway, and mediate cell death with apparently different timecourses. In oligodendrocytes, cell death was observed 4-6 hours after activation of the p75 receptor with NGF (Casaccia-Bonnefil et al.,1996). This is similar to the rapid timecourse seen upon application of TNF to U937 and BAE cells (Verheij

et al.,1996). Conversely, in sympathetic neurons, c-jun phosphorylation occurs 8-12 hours after p75 activation, and neuronal apoptosis is observed 36-48 hours following activation of this receptor (Bamji et al.,1998). This is similar to the timecourse of c-jun phosphorylation and cell death following NGF withdrawal in sympathetic neurons (Estus et al.,1994; Ham et al.,1995). The disparity between the temporal events following activation of the p75 receptor in oligodendrocytes and sympathetic neurons may be attributed to differences in cell type. Another explanation is that in sympathetic neurons, other signaling events (ie. TrkA signaling) may be impinging on the p75-mediated signal cascade.

(b) The JNK Signaling Pathway and Apoptosis:

Extracellular stimuli, such as tumor necrosis factor-α (TNF-α) or interleukin 1 (IL-1), activate JNK through a cascade in which MAP kinase kinase kinase 1 (MEKK1) phosphorylates and activates a Thr-Tyr protein kinase, SEK1 (also termed MKK4 or JNKK), which then phosphorylates and activates JNK (Lange-Carter et al.,1993; Minden et al.,1994; Derijard et al.,1995; Lin et al.,1995). Activation of JNK results in the phosphorylation of c-jun at serine residues 63 and 73 and increases its potential to activate transcription by translocating to the nucleus (Hibi et al.,1993; Derijard et al.,1994; Kallunki et al.,1994; Kyriakis et al.,1994; Sluss et al.,1994).

The MEKK1-SEK1-JNK pathway has been shown to play a role in NGF withdrawal-induced death of PC12 cells, and sympathetic neurons (Xia et al.,1995; Eilers et al.,1998). Our laboratory has recently shown that p53 lies downstream of this apoptotic pathway and is both sufficient (Slack et al.,1996) and necessary for sympathetic neuron apoptosis following NGF withdrawal and p75 activation (Aloyz et al.,1998; Chapter V). Indeed, withdrawal of NGF (Estus et al.,1994; Ham et al.,1995) and activation of p75 (Bamji et al.,1998) resulted in increased JNK activity, and increased p53 and Bax levels (Aloyz et al.,1998; Chapter V). Moreover, expression of constitutively active MEKK1 resulted in increased JNK activity (Eilers et al.,1998), and increased p53 and Bax levels, in the presence of NGF (Aloyz et al.,1998; Chapter V). Interestingly, although a dominant negative mutant of SEK1 (SEK*AL*) was able to inhibit cell death mediated by constitutively active MEKK1, SEK*AL* did not prevent increases in c-jun expression, JNK activity or cell death after NGF withdrawal (Eilers et al.1998). Thus, although the MEKK1-SEK1-JNK pathway is sufficient for neuronal cell

death, multiple pathways may impinge downstream of SEK1 to mediate sympathetic neuron death.

In accordance with this, the small GTP-binding protein, Cdc42, and its downstream effector, Rac1, has been shown to be required for NGF withdrawal-induced neuronal death (Bazenet et al.,1998). Indeed, overexpression of these Rho-like GTPases increased c-jun phosphorylation and death in the presence of NGF, whereas dominant negative mutants blocked these effects. Although Cdc42 and Rac1 clearly act upstream of JNK, the point at which this modulation occurs remains to be elucidated. It would be interesting to see whether Cdc42 - Rac1 act upstream of the MEKK1-SEK1-JNK pathway, or whether multiple pathways do, indeed, impinge on this pathway downstream of MEKK1.

Studies involving the microinjection of neutralizing antibodies, or the expression of dominant-negative mutants, have suggested a critical role for c-jun in NGF withdrawal-induced death of cultured sympathetic neurons (Estus et al.,1994; Ham et al.,1995). These studies demonstrate that c-jun is both sufficient and necessary for sympathetic neuron death. Although we had initially postulated that c-jun lies downstream of JNK and uptream of p53 in the apoptotic pathway, more recently, JNK has been shown to directly interact with and stabilize the p53 protein (Fuchs et al.,1998). Recently, JNK and p53 has been shown to coimmunoprecipitate in sympathetic neurons (R.Aloyz, D.R.Kaplan, F.D.Miller, unpublished results), and we predict that JNK may directly stabilize p53 to mediate neuronal apoptosis. Thus, the role of c-jun in sympathetic neuron death is now in question.

The events that occur upstream of MEKK1 in sympathetic neuron death are still unknown, however one potential candidate in p75-mediated cell death is ceramide. Indeed, p75NTR activation is able to increase ceramide levels, activate the JNK pathway, and mediate cell death in non-neuronal cells (Casaccia-Bonnefil et al.,1996). Moreover, ceramide has been shown to act upstream of JNK to mediate its apoptotic effects (Verheij et al.,1996; Fig.1.3), although the point at which this modulation occurs remains to be elucidated. In addition to mediating cell death via activation of the JNK pathway (Verheij et al.,1996), ceramide has recently been shown to inhibit Akt activity in a number of cell types including motor neurons (Summers et al.,1998; Zhou et al.,1998; Zundel and Giaccia,1998; Fig.1.3). Thus, ceramide may act upstream of the JNK pathway to mediate p75NTR-induced cell death at least in non-neuronal cells, and potentially in neurons via suppression of Akt activity.

Evidence that ceramide is involved in the p75NTR-mediated pathway in sympathetic neurons derives from its ability to inhibit neuritogenesis (de Chaves et al.,1997,1998), similar to that seen upon p75 activation (Kohn et al.,1998). Interestingly, addition of ceramide to sympathetic neurons has been shown to prevent apoptosis following NGF deprivation (Ito and Horigome,1995). Thus, the role of ceramide in NGF withdrawal-induced death and p75NTR-mediated death in sympathetic neurons is still unclear.

In accordance with the idea that multiple cross-talk occurs between survival and death pathways, the MEKK1-SEK1-JNK pathway has recently been shown to be suppressed via Akt (N.Marsh, J.Atwal, F.D.Miller, and D.R.Kaplan, unpublished results). Indeed, expression of dominant-negative Akt results in increased c-jun phosphorylation and increased p53 levels in the presense of NGF. This result implies that Akt acts upstream of JNK to suppress apoptosis, however, as SEK*AL* is unable to prevent neuronal apoptosis in the absence of NGF, it is difficult to postulate which signaling molecule(s) is directly suppressed by Akt.

(c) The Role of p53 in Neuronal Apoptosis:

The p53 tumor supressor gene is the most frequently mutated gene in human tumors (Vogelstein,1990; Hollstein et al.,1991), and reintroduction of p53 into transformed cells can induce either growth arrest (El-Deiry et al.,1993; Xiong et al.,1993) or apoptosis (Yonish-Rouach et al.,1991). As a tumor supressor, p53's primary role is to mediate cell cycle arrest after DNA damage. P53-mediated growth arrest is believed to be dependent on transcriptional activation of a number of proteins that aid exit from the cell cycle including the cyclin kinase inhibitor, p21 (WAF-1) (El-Deiry et al.,1993; Picksley and Lane,1994). Human p53 is a protein of 393 amino acids and can be divided structurally and functionally into four domains. The N-terminus contains an acidic transcription-activation domain that interacts with the transcriptional machinery of the cell and regulates gene expression (Levine,1997). The next region is the sequence-specific DNA-binding domain that is frequently mutated in cancer cells (Kern et al.,1991; Oren,1997). The carboxyl terminus of p53 can function as an autonomous domain capable of binding non-specifically to different forms of DNA, including damaged DNA (Wang et al.1993; Lee et al.,1995; Reed et al.,1995). P53 binds to DNA as a tetramer, and the region which is responsible for p53 tetramerization, the oligomerization domain, lies within the carboxyl terminus of p53 (Lee et al.,1994; Clore et al.,1995, Jeffrey et al.,1995).

Normally, endogenous p53 levels are relatively low in the cell due to its rapid degradation (Levine,1997). P53 protein levels then increase in response to cellular stress, potentially due to changes in conformation or phosphorylation that prevent its rapid degradation.

The consensus is that p53 can induce growth arrest or apoptosis depending on the physiological circumstances or cell type. For example, p53 accumulates and directs apoptosis in response to DNA damage in skin (Ziegler et al.,1994), thymocytes (Clarke et al.,1993; Lowe et al.,1993), and intestinal epithelium (Clarke et al.,1994; Merritt et al.,1994; for review see Oppenheim,1991), but does not appear to be essential for all aspects of apoptosis in normal murine development (Donehower et al.,1992; Armstrong et al.,1995).

Most recently, p53 has been implicated in playing a role in neuronal apoptosis (reviewed in Hughes et al.,1997). For example, it has been demonstrated *in vivo* that p53 mRNA and protein levels are induced in neurons after focal ischemia and prior to neuronal death (Li et al.,1994). The increased expression of p53 mRNA in the injured brain in addition to p53 protein is interesting as in many other cell types and tissues, p53 protein induction usually occurs via post-translational stabilization (Chernov et al.,1998; Fuchs et al.,1998; Thomas and White,1998; reviewed in Blagosklonny,1997), not transcription. Wood and Youle (1995) have also demonstrated an effect of p53 gene deletion on cerebellar granule cell apoptosis *in vivo*. Cerebellar granule cells typically undergo apoptosis in response to genotoxic agents such as γ-irradiation, however, in p53-/- mice, γ-irradiation failed to induce cerebellar granule cell apoptosis, further implicating p53 in injury-induced neuronal apoptosis.

Similarly, *in vitro* studies have demonstrated that following kainic acid treatment there is a dramatic increase in p53 levels prior to neuronal apoptosis (Sakhi et al.,1994), whereas neurons cultured from p53-/- mice do not die in response to kainic acid treatment (Morrison et al.,1996; Xiang et al.,1996). Furthermore, when cerebellar granule cells were treated with γ-irradiation, or other DNA damaging agents, wildtype neurons expressing p53 underwent massive cell death, while p53-/- neurons were resistant to injury (Enokido et al.,1996).

The question of whether p53 is sufficient to mediate neuronal apoptosis has been directly addressed by overexpressing wildtype p53 in cultured sympathetic (Slack et al.,1996) and hippocampal neurons (Jordan et al.,1997) using adenovirus-mediated transfection of the p53 gene. In both these cell types, expression of p53 was sufficient to induce apoptosis. Overexpression of the cyclin-dependent kinase inhibitor, p21 (WAF1), did not induce apoptosis

in hippocampal cells, however, indicating that p53's role in neuronal death is distinct from its actions relating to cell cycle arrest (Jordan et al.,1997).

Thus far it has been shown that p53 is necessary to mediate neuronal death in response to certain types of injury and DNA damage, and that p53 is sufficient to induce neuronal death. We have recently examined the role of p53 in mediating neurotrophin withdrawal-induced death, and programmed cell death, and show that p53 is necessary to mediate cell death in the absence of trophic factors (see Chapter V). This initially appears contradictory to previous work demonstrating that cultured neurons from p53-deficient transgenic mice undergo apoptosis in the absence of neurotrophins (Donehower et al.,1992; Davies and Rosenthal,1994). However, our results do not dispute the fact that p53-/- neurons undergo cell death, but indicate that cell death is decreased in p53-/- mice. Similarly, although the number of SCG neurons appear unaltered in p53-/- mice compared to wildtype mice when plated in culture (Sadoul et al.,1996), upon more rigorous examination, our laboratory has demonstrated that p53 does indeed play a role in naturally-occurring cell death, and have shown increased neuronal numbers in the SCG of p53-/- mice during the period of programmed cell death (see Chapter V).

Recently, two groups have independently identified a structural homolog of p53, p73 (Jost et al.,1997; Kaghad et al.,1997). Two human isoforms of p73 have been cloned that differ at their C-termini: p73α (636 aa) and a shorter form, p73β (499 aa) that occur via alternative splicing. Further investigation of the protein sequence revealed considerable homology between p53 and p73. Structurally, p73 shares 29% homology with p53 in the transcriptional activation domain, 63% homology in the DNA binding domain, and 38% homology in the oligomerization domain (Kaghad et al.,1997). Yeast two hybrid assays have revealed that p73α forms only weak homophilic interactions and slightly stronger interactions with p73β. Conversely, p73β forms strong homophilic interactions and weak interactions with both p73α and p53. Although p73 shares structural similarities with p53, and may form interactions with p53, the role p73 may play in neurons is largely unknown (Kaghad et al.,1997). In addition to the structural similarities between p53 and p73, they also share common functional characteristics. For example, p73 α and β are able to induce the p21/WAF1 protein in neuroblastoma cell lines (Kaghad et al.,1997) and osteosarcoma cell lines (Jost et al.,1997) resulting in growth arrest. In addition p73 α and β are able to cause apoptosis in baby hamster

kidney cells. Despite its ability to induce p21/WAF1, p73 is not responsive to DNA damage, suggesting that p53 and p73 do not have completely redundant functions in cells. Interestingly, p73 has recently been observed in rat SCGs and brain (C.Pozniak and F.D.Miller, unpublished results), implying a biological function for p73 in the nervous system.

More recently, a third member of the p53-related family, p51, has been identified using degenerate PCR (Osada et al.,1998). There are two splice variants of the p51 gene, p51A and p51B, the former encoding a 448-amino-acid protein with molecular weight of 50.9 kDa, and the latter encoding a 641-amino-acid protein with a molecular weight of 71.9kDa. Comparison of the amino acid sequence revealed that p51 is more similar to p73β than p53 in the transcription activation domain, DNA binding domain, and oligomerization domain. P51 is able to induce growth suppression and apoptosis when transfected into p53-deficient cells, and is able to induce p21/WAF-1 through p53 regulatory elements. In addition, some human epidermal tumors were shown to have mutations in p51.

The consequence of having three p53 family genes has yet to be demonstrated. One possibility is that the simultaneous expression of multiple p53 family genes in a single cell may increase the chances of acquiring mutations, producing mutant p53-like proteins which may interfere with normal p53 function in a dominant-negative manner (Kern et al.,1992). Another possibility is that these proteins may subsume similar roles in cell-cycle arrest and apoptosis, but in response to different cellular stressors such as DNA damage, which upregulates p53 levels but not p73. Yet another possibility is that that the differential expression of the p53 family genes results in the ability of p53 to assume diferent roles and differentially activate specific genes in different cell types. Although much work is needed to elucidate the functions of the various p53-like proteins, it is clear that the expression of these proteins adds another dimension to the biological roles of p53.

How p53 directs growth arrest and apoptosis has been the focus of attention of many laboratories. Most evidence suggests that p53 functions as a transcription factor as p53 contains sequence-specific DNA-binding activity and can activate or repress transcription. Although p53-dependent transcription is clearly responsible for the promotion of growth arrest (El-Deiry et al.,1993; Xiong et al.,1993), the role of p53-dependent transcription in apoptosis has been more controversial. It has been postulated that p53 may mediate apoptosis by activating the transcription of death genes such as *bax* (Miyashita and Reed,1995) or repressing

the transcription of survival genes such as *bcl-2* (Tilly et al.,1995). Indeed, in p53-/- mice, *bax* expression is depressed and *bcl-2* expression is elevated relative to wild-type controls (Miyashita et al.,1994). *Bax* expression is upregulated by wild-type p53 expression but not by expression of the transcriptionally defective p53 mutant, which is in agreement with the *bax* transcriptional activation model for p53-dependent apoptosis (Han et al.,1996). Furthermore, ectopic *bax* expression induces apoptosis even when p53 is constitutively in the mutant conformation, suggesting that Bax alone is sufficient to induce apoptosis and lies downstream of p53 (Han et al.,1996).

Bax has also recently been shown to be necessary for neuronal apoptosis. For exampie, Bax has been shown to be required for neuronal death after NGF withdrawal and during sympathetic neuron development (Deckworth et al.,1996). Similarly, there is a widespread elimination of naturally-occurring neuronal death in *Bax*-deficient mice (White et al.,1998). Furthermore, Xiang et al (1998) demonstrate that deletion of the *Bax* gene results in neuronal protection following injury and p53 activation, indicating that Bax is both suffiecient and necessary for p53-mediated neuronal apoptosis. In sympathetic neurons, the induction of apoptosis by neurotrophin withdrawal requires transcriptional/translational activity (Martin et al.,1988) lending further support to the intriguing idea that p53 plays a role in mediating trophic factor withdrawal-induced death and programmed cell death, by transcriptionally upregulating *bax* expression.

The Rheostat model of neuronal apoptosis states that the ratio of apoptotic to anti-apoptotic proteins of the Bcl-2 family controls cell survival (further described in section d). According to this model, either the upregulation of Bax or down-regulation of Bcl-2 would be sufficient to induce apoptosis. Thus, p53 may mediate apoptosis by transcriptionally increasing the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members.

In contrast to the suggestion that p53 induces apoptosis via the transcriptional upregulation of *bax*, other evidence suggests that p53 can induce apoptosis in the presence of inhibitors of transcription and protein synthesis, indicating that regulation of transcription may not be the sole mechanism of apoptotic induction (Caelles et al.,1994). Indeed, although some studies have shown that transcriptionally defective mutant p53 is unable to induce apoptosis (Sabbatini et al.,1995; Yonish-Rouach et al.,1996), other evidence suggests that mutant p53 proteins can, indeed, induce apoptosis in a transcriptionally-independent manner (Bissonnette

et al.,1997) . Thus, although it appears that p53 mediates neuronal apoptosis through a transcription-dependent manner, based on the inability of neurons to undergo apoptosis in the presence of transcription/translation inhibitors (Deckworth and Johnson,1993), and based on the nuclear localization of p53 in injured or dying neurons (Wood and Youle,1995; Hughes et al.,1996), the dependence on transcriptional events in p53-mediated apoptosis in other cell types may vary.

Recently, yet another model for p53-induced apoptosis has been put forth, based on the observation that many of the genes that are transcriptionally regulated by p53 encode proteins that can generate or respond to oxidative stress (Polyak et al.,1997). According to this model, p53-mediated apoptosis could result through a three step process: 1) the transcriptional induction of redox-related genes, 2) the formation of reactive oxygen species, and 3) the oxidative degradation of mitochondrial components, culminating in cell death (Polyak et al.,1997).

It has been known for a long time that production of reactive oxygen species (ROS) causes cell damage, and several lines of evidence support a causal role for ROS in neuronal apoptosis as well (reviewed in Slater et al.,1995). For example, exogenously provided oxidants that increase the production of ROS, induce apoptosis in neuronal cell lines (Estevez et al.,1995). In addition, neuronal apoptosis induced by a variety of stimuli, including serum and trophic factor deprivation (Ferrari et al.,1995, Satoh et al.,1996), K^+ deprivation (Schultz et al.,1996), glutamate-mediated excitotoxicity (Bonfocco et al.,1995), and ischaemia (Linnik et al.,1993), is accompanied by increased production of ROS. Treatment of these neurons with ROS scavengers, antioxidants, and ROS-metabolizing enzymes can protect against apoptosis in many cases (Bonfocco et al.,1995; Ferrari et al.,1995; Satoh et al.,1996).

A key enzyme in the metabolism of ROS is copper-zinc superoxide dismutase (SOD1). In PC12 cells, antisense oligonucleotides against SOD1 inhibit cell survival, supporting a neuroprotective role for this enzyme (Troy and Shelanski,1994). However, in cultured sympathetic neurons, microinjection of SOD1 delays, but does not completely prevent NGF withdrawal-induced apoptosis (Greenlund et al.,1995). Moreover, readdition of NGF rescues sympathetic neurons from death even after full blown increases in ROS production (Greenlund et al.,1995). Similarly, in serum-deprived PC12 cells and embryonic cortical neurons, survival factors prevent apoptosis without affecting the increase in ROS levels (Enokido et al.,1992;

Hinshaw et al.,1993). Taken together, these results suggest that elevated ROS levels are in themselves not fatal, and can be compensated for by other trophic factors. Thus, although p53 may play a role in transcriptionally activating genes which encode proteins that generate or respond to oxidative stress (Polyak et al.,1997), this may not be the primary mechanism by which p53 mediates apoptosis, especially in neurons.

P53 is typically thought to play a role in mediating cell death in response to DNA damage and cellular excitotoxicity, but p53 also plays a role in mediating TNF-induced death. TNF has been shown to induce p53 expression in C6 rat glioma cells during apoptosis, and this apoptotic effect was partially inhibited by actinomycin D and cyclohexamide, suggesting that in this system, TNF-induced death is dependent upon new RNA and protein synthesis (Yin et al.,1995). In addition, the loss of p53 function resulted in the inability of TNF to kill MCF7 human breast carcinoma cells, indicating that p53 is the main mediator of TNF-induced death in this system (Cai et al.,1997).

Consistent with the idea that TNF-mediated cell death may involve the JNK pathway, it has recently been reported that MEKK1/JNK signaling is able to stabilize and activate p53 (Fuchs et al.,1998). JNK has been shown to associate with (Adler et al.1997), and phosphorylate (Lees-Miller et al.,1992, Wang and Eckhart,1992; Milne et al.,1995) p53, thereby decreasing p53 ubiquitination, and increasing its half-life (Fuchs et al.,1998). MEKK1/JNK signaling not only increases p53 stability, but also increases transcriptional activation, and potentiates the ability of p53 to initiate programmed cell death (Fuchs et al.,1998). Recently, JNK and p53 have also been shown to associate in sympathetic neurons of the rat SCG (R.Aloyz, F.D.Miller and D.R.Kaplan, unpublished results).

Taken together, these data suggest a model for the mechanism of p53-induced apoptosis. In this model, p53 mRNA and protein levels are increased in response to DNA damage, excitotoxicity, trophic factor withdrawal and TNF induction. P53 then translocates to the nucleus where it transcriptionally upregulates Bax production, and potentially downregulates the production of the anti-apoptotic Bcl-2 protein. This shift in the balance between Bax and Bcl-2, enhances the production of Bax/Bax homodimers, and decreases Bax/Bcl-2 heterodimers, leading to apoptosis.

(d) The Role of the Ced/Bcl-2 Family in Apoptosis:

Several proteins have been implicated in modulating the death process following stimulation of an apoptotic cascade. Considerable understanding of the genetic features of developmental programmed cell death has been derived from studies of the nematode *Caenorhabditis elegans*. Genetic studies have determined that two *C. elegans* genes, *ced-3* and *ced-4* (Ellis and Horvitz, 1986) are required for apoptosis. While these genes activate a cellular death program, another gene, *ced-9*, inhibits apoptosis and mutations of this gene results in the death of many cells that would normally survive (Hengartner et al., 1992).

Interest in these *C.elegans* genes has been greatly increased following the discovery of homologous mammalian genes. The best described of these are those of the Bcl-2 family. Bcl-2 shares marked sequence homology with the *C.elegans* death-inhibitory protein ced-9 (reviewed in Hengartner and Horvitz, 1994) and just as ced-9 acts to inhibit apoptotic death in the nematode, Bcl-2 opposes mammalian cell death (reviewed in White, 1996). Bcl-2 is the founding member of an expanding family of proteins whose principle homology is clustered within three conserved motifs called the Bcl-2 homology 1 (BH1), homology 2 (BH2) and homology 3 (BH3) that are required for the regulation of apoptosis and protein-protein interactions (reviewed in Williams and Smith, 1993). Currently, the Bcl-2 family includes Bax, Bcl-X_L, Bcl- X_S, Bad, Bak, Mcl-1, and A1 (reviewed in White, 1996). Whereas Bcl-2, Bcl- X_L, and Mcl-1 are anti-apoptotic proteins, Bcl-X_S, Bax, Bak and Bad promote apoptosis.

Evidence that Bcl-2 can regulate the survival of developing neurons initially came from studies in which an expression vector containing *bcl-2* cDNA was microinjected into cultured neurons. Overexpression of Bcl-2 prevented the death of cultured sympathetic (Garcia et al., 1992) and NGF dependent sensory neurons (Allsopp et al., 1993) upon NGF withdrawal, and rescued proprioceptive neurons deprived of either BDNF or NT-3 (Allsopp et al., 1993). Interestingly, overexpression of Bcl-2 rescues proprioceptive neurons that are supported initially by BDNF, but does not rescue proprioceptive neurons initially supported by CNTF, indicating that the choice of cell death pathways can be influenced by the neurotrophic factors to which they have been exposed (Allsopp et al., 1993). Transgenic mice that overexpress *bcl-2* in neurons from the neuron-specific enolase promoter display hypertrophy of the brain, and have increased numbers of neurons in the facial nucleus and retinal ganglion cell layer (Martinou et al., 1994). As overexpression of Bcl-2 can rescue neurotrophin-deprived neurons

in vitro, and can prevent naturally-occurring cell death *in vivo*, it is somewhat surprising that the nervous system of Bcl-2-/- mice appear to behave normally during development (Nakayama et al.,1993; Veis et al.,1993). A somewhat delayed degeneration of neurons was, however, observed in motor neurons, sympathetic and sensory neurons, well after the period of naturally-occurring cell death, indicating that Bcl-2 may be crucial for the maintenance of specific populations of neurons (Michaelidis et al.,1996). It is possible that a more rigorous analysis of neuron numbers may be necessary to reveal developmental neuronal deficits, and/or Bcl-2-related proteins may compensate for the loss of Bcl-2. In contrast, deletion of the apoptotic Bax protein in Bax-/- mice results in the rescue of neonatal sympathetic neurons deprived of NGF, and axotomized facial motorneurons (Deckworth et al.,1996).

How does the Bcl-2 family act to regulate cell survival and death? One model that has been proposed by Korsmeyer and colleagues is the Rheostat model (Oltvai et al.,1993; reviewed in Oltvai and Korsmeyer,1994). This model is based on the idea that members of the Bcl-2 family can form functional homodimers, or heterodimerize with other selected members of this family. The most examined pair of interacting proteins is Bcl-2 and Bax. Mammalian cells expressing heterodimers of Bcl-2 and Bax exert death-inhibitory action, whereas Bax-Bax homodimers facilitate death (Yin et al.,1994). Mutations within the BH1 or BH2 regions of Bcl-2 that disrupt Bcl-2's ability to heterodimerize with Bax, but not to form Bcl-2 homodimers, result in the inability of Bcl-2 to counter apoptosis, demonstrating that Bcl-2 must heterodimerize with Bax to function (Yin et al.,1994).

The Bcl-2 related protein, Bad, also forms heterodimers with Bcl-2 and Bcl-X_L and can counteract the death-inhibitory effect of Bcl- X_L, (Yang et al.,1995). Thus, the overall theme that emerges is that apoptotic and anti-apoptotic proteins interact with one another and the outcome that prevails depends on the ratio of the death promoter to the death suppressor. Although it is believed that the death promoter/death suppressor ratio is inherent to the cell, external factors can alter this balance and push the cell towards apoptosis or survival. Indeed, in some forms of cell death, p53 has been shown to increase the ratio of Bax to Bcl-2, thereby increasing the ratio of pro-apoptotic Bax/Bax homodimers to anti-apoptotic Bcl-2/Bax heterodimers.

(e) Caspases, Endonucleases and Apoptosis:

The cloning of *ced-3* has revealed that its product is similar to the mammalian cysteine protease interleukin-1β-converting enzyme (ICE) (Yuan et al.,1993), providing further evidence of evolutionary conservation of apoptotic pathways between *C. elegans* and vertebrates. Residues essential for substrate recognition and catalysis are conserved between Ced-3 and ICE (Walker et al.,1994). Overexpression of Ced-3 or ICE in mammalian cells induces apoptosis that is inhibitable by Bcl-2 (Miura et al.,1993). Interestingly, the cowpox *crmA* gene product, which binds to and inhibits ICE (Ray et al.,1992; Komiyama et al.,1994) inhibits apoptosis induced by NGF deprivation of neuronal cells (Gagliardini et al.,1994), and by the TNFα and Fas antigens (Enari et al.,1995; Los et al.,1995; Tewari and Dixit,1995). This provides strong evidence for the role of cysteine proteases in mediating multiple apoptotic pathways. Other ICE-related genes including nedd-2/ich-1, TX/Ich-2/ICErel-II, ICErel-III, Mch-2, and CPP32, have been identified whose products similarly induce apoptosis, indicating the existence of a mutigene family encoding proteases that are involved in apoptosis (reviewed in Gorman et al.,1998). These enzymes are named caspases (for cysteine-containing aspartate-specific proteases) because they all contain the amino acid cysteine in their active sites, and clip their protein targets next to the amino acid aspartate. Among these targets are the caspases themselves, which start out as inactive proteins called zymogens that need to be cut to be fully active. This suggests that activation of a caspase could produce a cascade of proteolysis with active caspases cutting and activating more caspases. There has been a number of studies that show that these enzymes are indeed turned on this way, in a sequence of cutting that begins with initiator caspases and end with endonucleases that cause fragmentation of DNA and apoptosis (Enari et al.,1998).

The question of how the first initiatior caspase gets turned on has been recently examined, and a number of groups have shown that some initiator caspase zymogens can cut and activate each other when they are in close proximity, even though they have only at most one-fiftieth of the protein cutting ability of active enzymes (Muzio et al.,1998; Yang et al.,1998). This finding could explain how the Fas receptor is able to rapidly mediate apoptosis. Fas has previously been shown to draw caspase-8 zymogen molecules together (Muzio et al.,1998), and this proximity may allow precursors to activate one another.

Apoptosis is not always triggered by cell surface receptors however, and recently a mechanism for inducing caspase activation in response to growth factor deprivation, DNA damage, oxidizing agents, and excitotoxicity has been proposed. This mechanism involves a role for the mitochondrial protein, cytochrome c, as a trigger to activate caspases.

Cytochrome c has been shown to bind to a newly discovered protein called apoptotic protease activating factor-1 (Apaf-1) (Li et al.,1997). This binding allows Apaf-1 to bind to and activate caspase-9, which in turn activates one of the executioner caspases, caspase-3 (Li et al.,1997). Although it is not known how Apaf-1 activates caspase-9, it has been hypothesized that it acts as the Fas receptor appears to, by bringing together multiple zymogens that can activate one another (Li et al.,1997). Once the executioner caspases are activated, they cut other proteins and caspases, and a caspase cascade begins.

In the absence of caspase-9 (caspase-9-/- mice), there is a marked decrease in apoptosis during brain development (Kuida et al.,1998; Hakem et al.,1998). Furthermore, although cytochrome c is translocated to the cytosol of caspase-9-/- ES cells after UV irradiation (Hakem et al.,1998), the cytochrome c-mediated cleavage of caspase-3 is absent and can be restored only upon addition of *in vitro*-translated caspase-9 (Kuida et al.,1998). Together these new data further demonstrate that caspase-9 acts downstream from cytochrome c, but is a critical upstream activator of the caspase cascade *in vivo*.

For a long time the link between caspases and apoptotic cell death was not clear, however, recently, Shigekazu Nagata and colleagues (1998) have demonstrated that one of the caspase's targets is a DNA-cleaving enzyme known as an endonuclease (Enari et al.,1998). When the caspase target is clipped, it frees the endonuclease to enter the nucleus and begin cutting DNA. This is the first time that caspases have been linked to endonucleases. Caspases also act to cut gelsolin, a protein that normally binds to actin filaments and help give a cell its shape (Kothakota et al.,1997). After losing shape, cells undergoing apoptosis normally break into membrane-encased apoptotic bodies that are engulfed by scavenger cells.

In both worm and mammalian cells, the antiapoptotic members of the Bcl-2 family act upstream of the excecutioner caspases and prevent their protelytic processing into active killers. One of the proposed mechanisms for this involves the inhibition of cytochrome c release from the inner mitochondrial membrane by the anti-apoptotic members of the Bcl-2 family. Indeed, both Bcl-2 (Kluck et al.,1997) and Bcl- X_L (Kharbanda et al.,1997) have been shown to block

the accumulation of cytochrome c. As Bcl-2 and Bcl-X_L have channel-forming activity, it was proposed that they may participate in regulating the permeability of mitochondria (reviewed in Reed,1997). During apoptosis, large (approximately 2.9 nm) channels open in the inner membrane of the mitochondrion (Bernardi et al.,1994; Zoratti and Szabo,1995) which may contribute to the generation of oxygen free radicals, dumping of stored Ca2+ into the cytosol, and the release of mitochondrial proteins (Petit et al.,1996) including cytochrome c. Overexpression of Bcl-2 and Bcl-X_L inhibits the opening of these "megapores" (Susin et al.,1996) whereas Bax overexpression induces it (Xiang et al.,1996; Antonsson et al.,1997). It is still unclear, however, whether this phenomena is the cause of cytochrome c release, or an effect of the apoptotic program.

More recently, Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, has been identified and has been shown to mediate cytochrome c release from mitochondria in response to activation of the TNF and Fas cell surface death receptors (Li et al.,1998; Luo et al.,1998). Bid normally exists in the cytosol as an inactive precursor that becomes activated upon cleavage by caspase-8, the caspase activated by cell surface receptors such as Fas and TNFR1. After cleavage, the COOH fragment of Bid translocates to the mitochondria where it is sufficient to induce the release of cytochrome c (Li et al.,1998; Luo et al.,1998). It is possible that Bid could form a complex with a pro-apoptotic Bcl-2 family member to trigger cytochrome c release. Alternatively, Bid may inactivate an anti-apoptotic member of the Bcl-2 family that plays an active role in preventing cytochrome c release. Interestingly, Bid initiates the release of cytochrome c without evoking gross mitochondrial swelling and changes in permeability (Luo et al.,1998).

It must be noted here, that caspase-8 can initiate two pathways leading to the activation of downstream caspases. Caspase-8 can directly activate downstream caspases like caspase-3, caspase-6, and caspase-7, by directly cleaving them (Muzio et al.,1997), or can indirectly activate caspases via cleavage of Bid, and the subsequent release of cytochrome c. The latter pathway may be regulated by Bcl-2 or Bcl- X_L, while a caspase-8 inhibitor like CrmA blocks both pathways. The contributions of these two pathways to Fas-induced death may vary between cell types, presumably due to different levels of activated caspase-8 and its downstream substrates in a particular cell type (Scaffidi et al.,1998).

Recently, it has been shown that blocking cytochrome c activity within intact sympathetic neurons inhibits NGF withdrawal-induced apoptosis (Neame et al.,1998). In this study, cytochrome c was reported to be redistributed from the mitochondria to the cytoplasm following NGF withdrawal. However, although microinjection of SCG neurons with an antibody to cytochrome c blocks NGF withdrawal-induced apoptosis, microinjection of cytochrome c does not increase apoptosis in either the presence of absence of NGF. This data suggests that cytochrome c is an intrinsic, but not limiting component of the neuronal apoptotic pathway.

Together, this provides a model for the mediation of apoptosis in neuronal cells (Fig.1.7). Here, external stimuli, including factors that mediate DNA damage and excitotoxicity, activation of Fas or TNFR1 receptors, or trophic factor withdrawal lead to the upregulation of p53 mRNA and/or protein synthesis by undefined signal transduction pathways. P53 then translocates to the nucleus where it functions to transcriptionally upregulate pro-apoptotic genes such as Bax, and downregulate anti-apoptotic genes such as Bcl-2. This alters the ratio of pro-apoptotic/anti-apoptotic proteins and there is an increase in the level of Bax/Bax homodimers and a decrease in Bax/Bcl-2 heterodimers. This shift induces the release of cytochrome c from the inner mitochondrial membrane and enables its accumulation in the cytosol. Cytochrome c, potentially via binding to Apaf-1, activates caspase-9, which in turn cuts and activates caspase-3, thereby activating a caspase cascade. These caspases can cleave and activate endonucleases, which translocate to the nucleus to chop DNA, one of the hallmarks of apoptosis.

Timeline for the Development of Sympathetic Neurons of the Superior Cervical Ganglia (SCG)

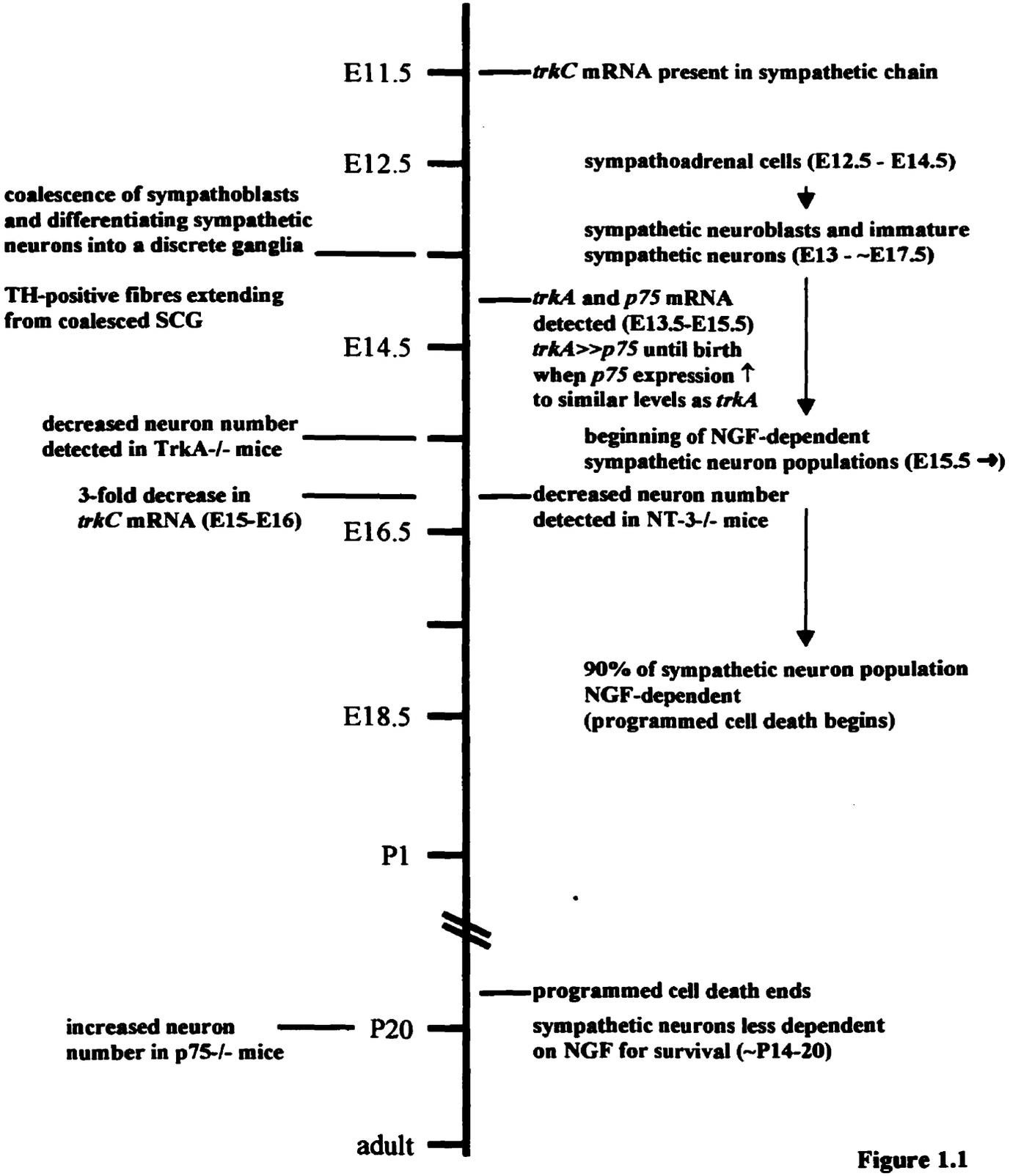

Figure 1.1

Model of the Signaling Pathways Involved in TrkA-Mediated and Depolarization-Induced Sympathetic Neuron Survival

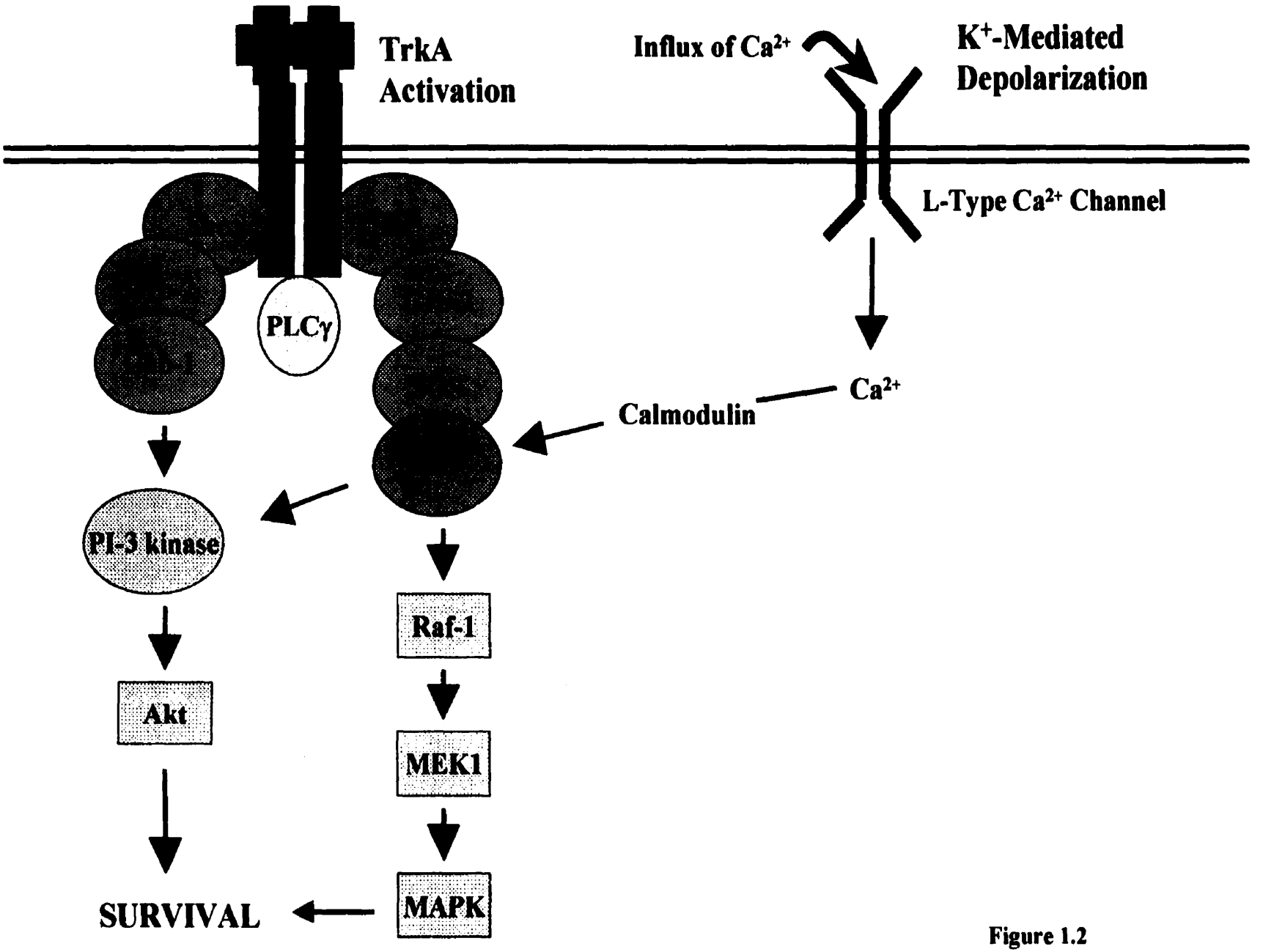

Figure 1.2

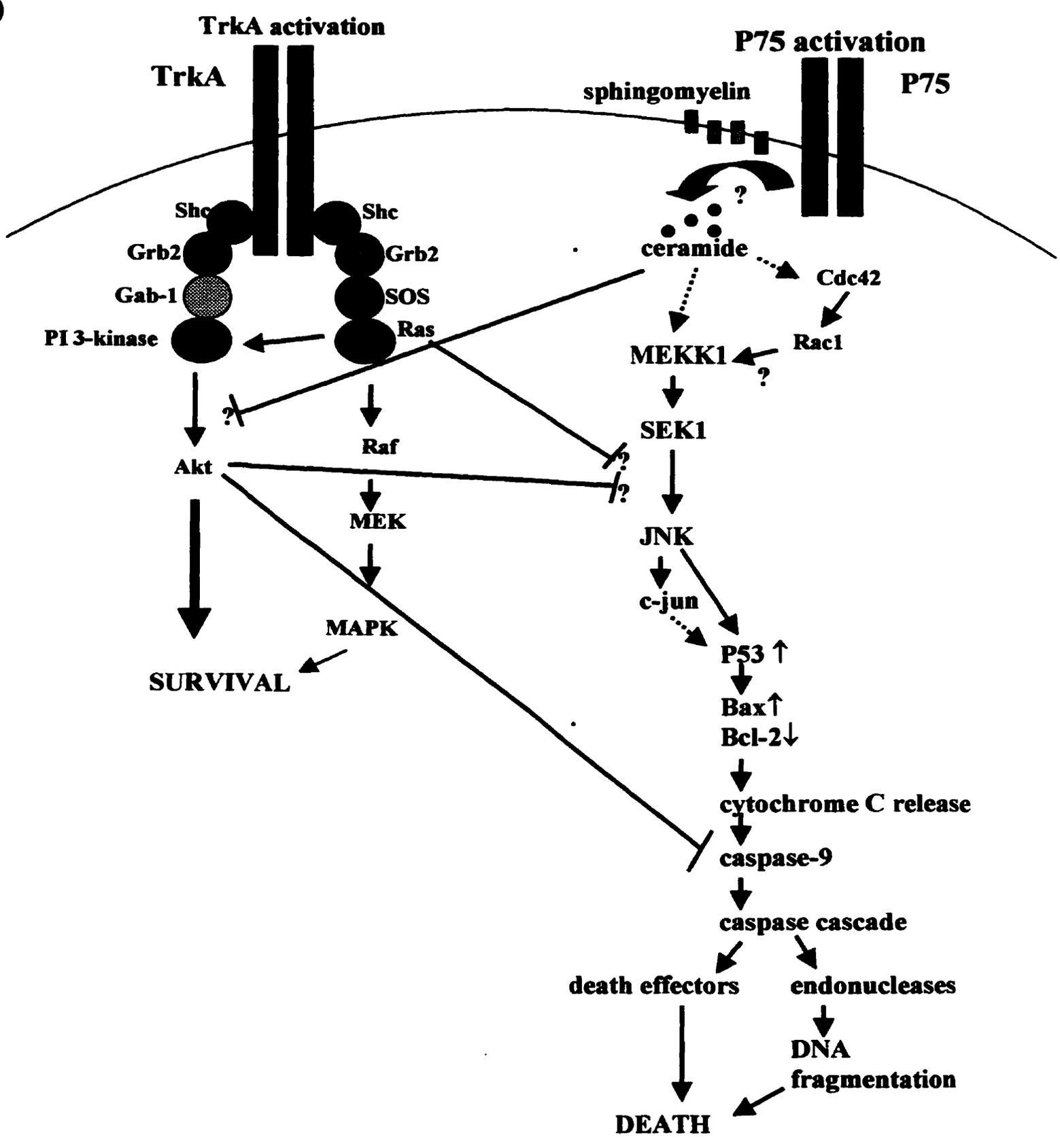
TrkA activation
TrkA
P75 activation
P75
sphingomyelin
Shc
Shc
Grb2
Grb2
Gab-1
SOS
PI 3-kinase
Ras
ceramide
Cdc42
Rac1
MEKK1
SEK1
Raf
Akt
MEK
JNK
c-jun
MAPK
P53 ↑
SURVIVAL
Bax↑
Bcl-2↓
cytochrome C release
caspase-9
caspase cascade
death effectors
endonucleases
DNA
fragmentation
DEATH

Figure 1.3

Timeline of Events Involved in NGF Withdrawal-Induced and p75NTR-Mediated Sympathetic Neuron Death

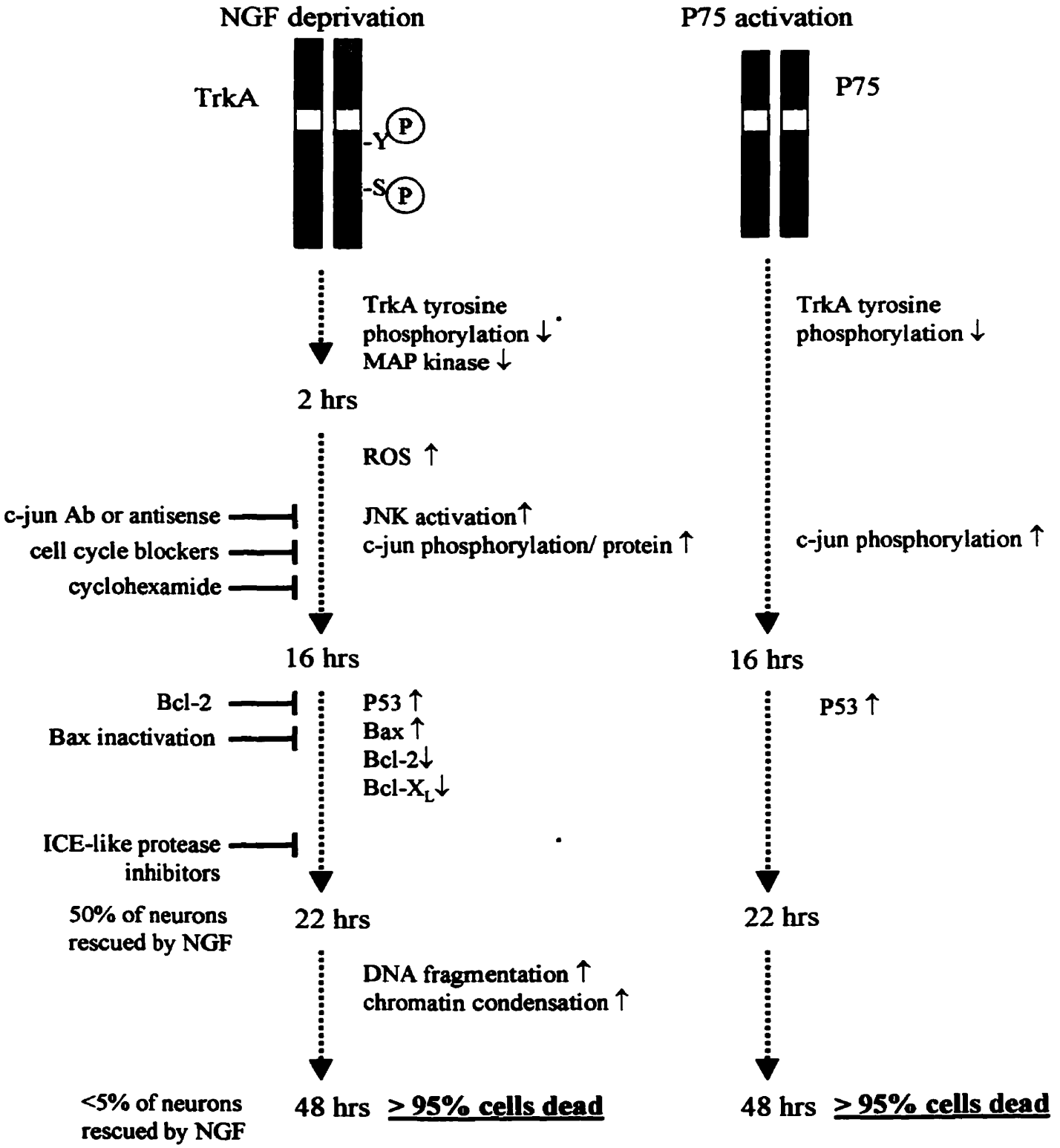

Figure 1.4

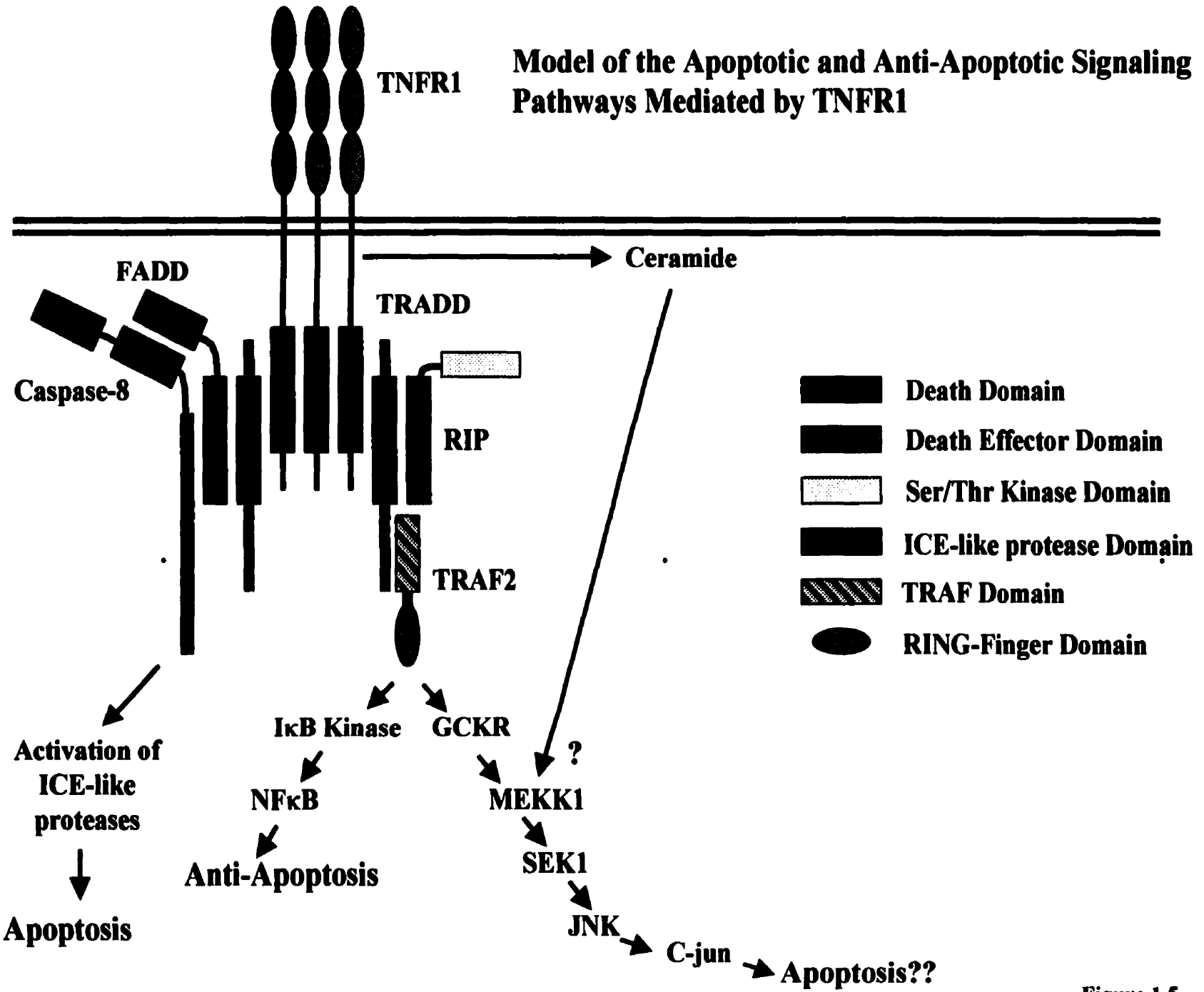
Model of the Apoptotic and Anti-Apoptotic Signaling Pathways Mediated by TNFR1
TNFR1
Ceramide
FADD
TRADD
Caspase-8
RIP
TRAF2
Death Domain
Death Effector Domain
Ser/Thr Kinase Domain
ICE-like protease Domain
TRAF Domain
RING-Finger Domain
Activation of ICE-like proteases
Apoptosis
IκB Kinase
GCKR
NFκB
Anti-Apoptosis
?
MEKK1
SEK1
JNK
C-jun
Apoptosis??

Figure 1.5

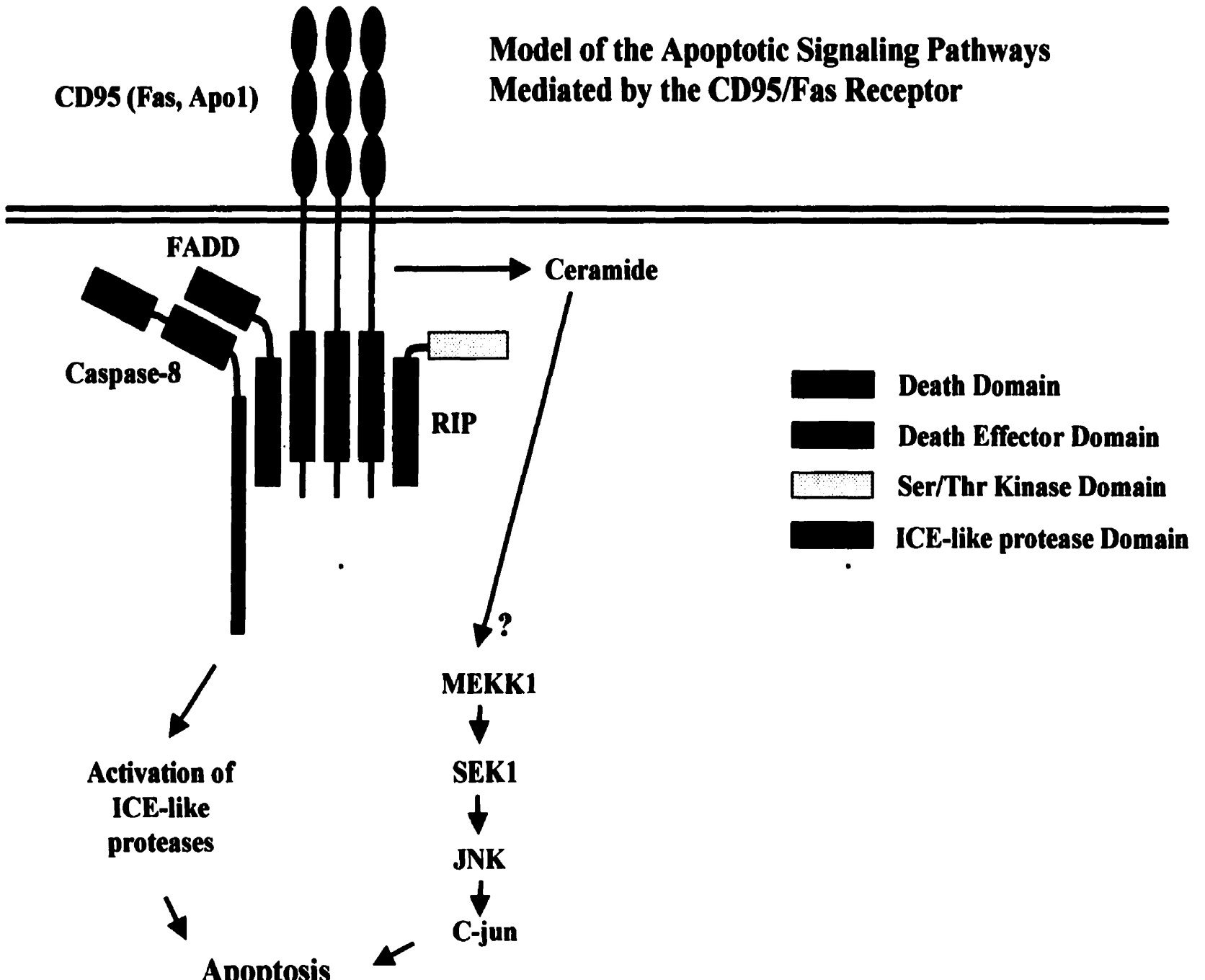
Model of the Apoptotic Signaling Pathways
Mediated by the CD95/Fas Receptor
CD95 (Fas, Apo1)
FADD
Caspase-8
RIP
Ceramide
?
MEKK1
SEK1
JNK
C-jun
Activation of
ICE-like
proteases
Apoptosis
Death Domain
Death Effector Domain
Ser/Thr Kinase Domain
ICE-like protease Domain

Figure 1.6

Model of the Signaling Pathway Involved in Sympathetic Neuron Death Following NGF Deprivation and p75NTR Activation

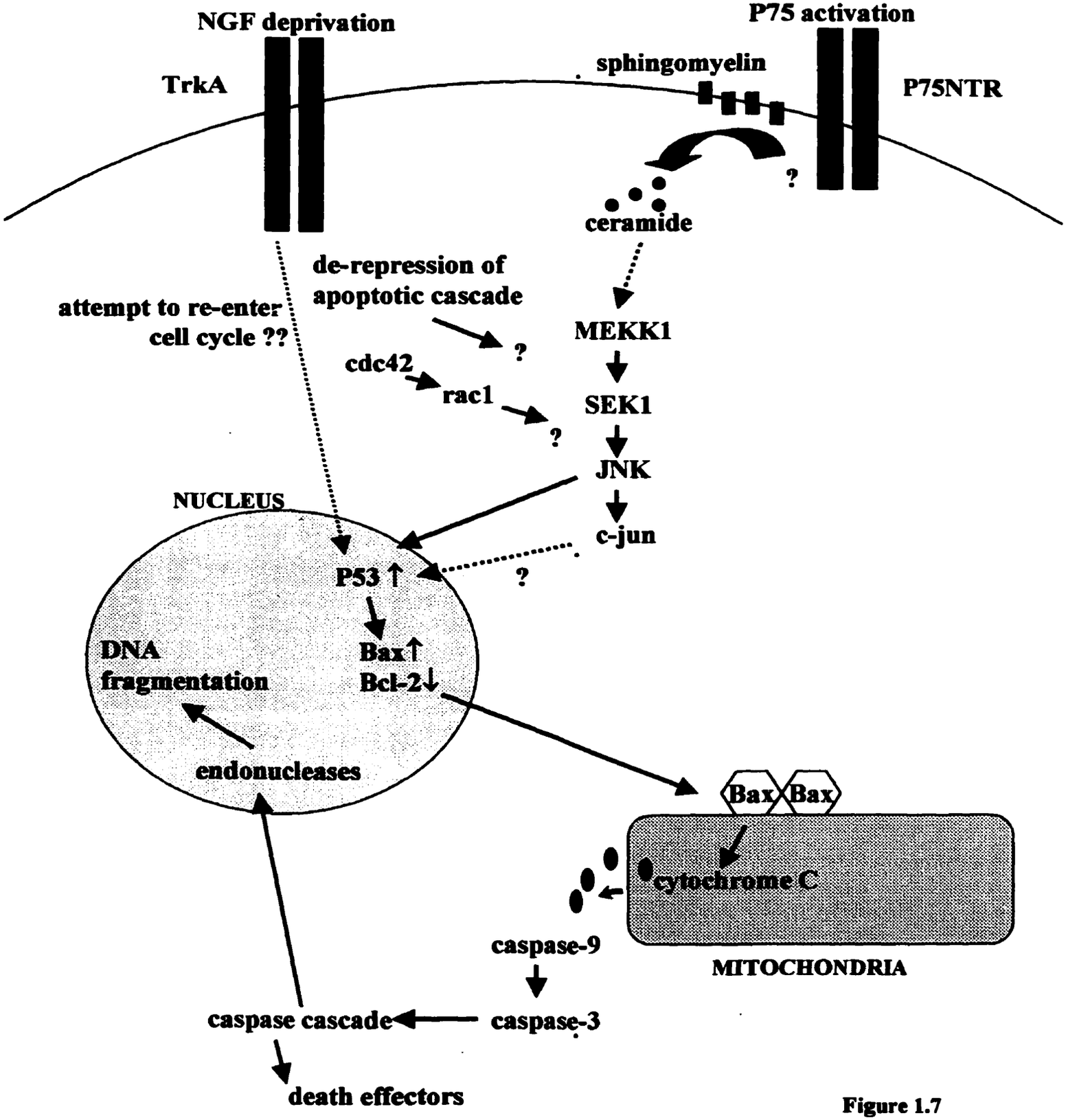

Figure 1.7

V. Figure Legends:

Figure 1.1: Timeline for the development of sympathetic neurons of the superior cervical ganglia (SCG).

Figure 1.2: Model of the signaling pathways involved in TrkA-mediated and depolarization-induced sympathetic neuron survival.

Figure 1.3: Model of the signaling pathways involved in TrkA-mediated survival, and p75NTR-mediated sympathetic neuron death. Question marks: (red) has not been shown in symapthetic neurons, (blue) not known whether effect mediated at this point. Dashed arrows: connection between effector molecules unknown.

Figure 1.4: Timeline of events involved in NGF withdrawal-induced and p75NTR-mediated sympathetic neuron death.

Figure 1.5: Model of the apoptotic and anti-apoptotic signaling pathways mediated by the TNF receptor 1. Question mark: not known whether effect mediated at this point.

Figure 1.6: Model of the apoptotic signaling pathways mediated by the CD95/ Fas receptor. Question mark: not known whether effect mediated at this point.

Figure 1.7: Model of the signaling pathways involved in sympathetic neuron death following NGF deprivation and p75NTR activation. Question marks: (red) has not been shown in symapthetic neurons, (blue) not known whether effect mediated at this point. Dashed arrows: connection between effector molecules unknown.

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CHAPTER II: COMPARISON OF THE EXPRESSION OF A Tα1:nLACZ TRANSGENE AND Tα1 α-TUBULIN mRNA IN THAT MATURE CENTRAL NERVOUS SYSTEM

I. Preface

The aim of this chapter is to further characterize a model system that has been used to analyze the molecular genetic mechanisms underlying neuronal development and growth. This model system has focussed on the regulation of the Tα1 α-tubulin gene. The Tα1 gene is expressed at high levels during the growth of developing and mature neurons, and in transgenic mice, 1.1kb of 5' flanking sequence from the Tα1 gene is sufficient to target gene expression to early developing neurons (Gloster et al.,1994), and to regulate levels of expression as a function of neuronal growth (Gloster et al.,1994; Wu et al.,1994). In the mature, uninjured nervous system, expression of both the endogenous Tα1 gene and the Tα1:nlacZ transgene is reduced. Moreover, consistent with the idea that transcription of this gene is associated with ongoing structural growth, preliminary studies have suggested that expression of the endogenous Tα1 gene is not homogeneous in different populations of mature neurons. To examine which subsets of neurons express Tα1 mRNA and to determine to what degree this pattern of expression correlates with growth capability, we examined the pattern of Tα1 mRNA expression in the mature brain. To examine whether 1.1 kb of the Tα1 promoter could faithfully drive the expression of a marker gene in an appropriate pattern, we compared the pattern of Tα1:nlacZ transgene expression and Tα1 mRNA expression in the mature brain.

Upon confirming the ability of the 1.1 kb Tα1 promoter to confer an appropriate pattern of gene expression in the mature, uninjured nervous system, the Tα1:nlacZ transgene will provide a useful "tool" to examine perturbations in neural development in transgenic mice, and to define extrinsic cues that regulate the growth of mature neurons in vivo ***(examples of this are shown in Chapter III).***

II. Abstract:

We have previously demonstrated that one member of the α-tubulin multigene family, termed Tα1 in rats, is a panneuronal gene that is regulated as a function of neuronal growth and regeneration. Moreover, 1.1 kb of 5' upstream region from this gene is sufficient to direct expression of a marker gene to growing neurons in transgenic mice. In this paper, we have characterized the distribution of the Tα1:nlacZ transgene in the mature central nervous system of two lines of transgenic mice, and have compared its expression to that of the endogenous Tα1 α-tubulin mRNA. These results demonstrate that the pattern of expression of the Tα1:nlacZ transgene is similar to that of Tα1 mRNA, with a few notable differences. Furthermore, expression of the transgene and the mRNA within the mature brain is panneuronal and, in many cases, is highest in those populations of neurons that show some capacity for morphological growth. These results, together with our previous studies on mature regenerating neurons (Gloster et al., 1994; Wu et al., 1994) suggest that the Tα1:nlacZ transgene will provide a useful marker of growth-associated gene expression in the mature nervous system.

III. Abbreviations:

3	oculomotor nucleus
6	abducens nucleus
7	facial nucleus
ACo	anterior cortical amygdaloid nucleus
AHi	amygdalohippocampal area
Amb	ambiguus nucleus
Amy	amygdala
AO	anterior olfactory nucleus
AOB	accessory olfactory bulb
BL	basolateral amygdaloid nucleus
BM	basomedial amygdaloid nucleus
CA1	field CA1 of Ammon's horn
CA3	field CA3 of Ammon's horn
CG	central gray
CPu	caudate putamen
Cx	cerebral cortex
DB	diagonal band of Broca
DM	dorsomedial hypothalamic nucleus
DR	dorsal raphe
Ent	entorhinal cortex
GrC	granule cells
Gl	glomerular layer
Hi	hippocampus
IC	inferior colliculus
ICj	islands of Calleja
LC	locus coeruleus
LS	lateral septal nucleus
LSO	lateral superior olive
MG	medial geniculate nucleus
MHb	medial habenula
Mi	mitral cells of the olfactory bulb
MS	medial septum
Pa	paraventricular hypothalamic nucleus
PB	parabrachial nucleus
PBG	parabigeminal nucleus
PDTg	posterodorsal tegmental nucleus
Pir	piriform cortex
Pk	cerebellar Purkinje cellc
PLCo	posterolateral cortical amygdaloid nucleus
PMCo	posteromedial cortical amygdaloid nucleus
PV	paraventricular thalamic nucleus
R	red nucleus
SC	superior colliculus
SCh	suprachiasmatic nucleus

SG supragenicular nucleus
SNc substantia nigra pars compacta
SO supraoptic nucleus
Sp5 spinal trigeminal nucleus
TT tenia tecta
Ve vestibular nucleus
VMH ventromedial hypothalamic nucleus
VMHDM ventromedial hypothalamic nucleus (dorsomedial)
VMHVL ventromedial hypothalamic nucleus (ventrolateral)
ZI zona incerta

IV. Introduction:

Microtubules, which are assembled from α- and ß-tubulins, comprise the major cytoskeletal component of growing neurites (Daniels, 1972), thereby playing an essential structural role during neuronal growth as well as providing the substrate for axonal transport (Vale et al., 1985). In mammals, at least six different α-tubulin genes (Villasante et al., 1986) and five different ß-tubulin genes (Wang et al., 1986) are expressed in neural and non-neural tissues at various times during development. We have previously demonstrated that, of two α-tubulin genes known to be expressed in the embryonic nervous system of the rat, one, termed Tα1, is abundantly expressed in developing neurons during morphological growth, while a second, termed T26, is constitutively expressed in neurons and non-neuronal cells (Miller et al., 1987). It is likely that the Tα1 isotype, which is homologous to the human bα1, and mouse Mα1 isotypes (Lemischka et al., 1981; Cowan et al., 1983; Lewis et al., 1985), is incorporated into the majority of neuronal microtubules during development, since Tα1 mRNA comprises 1-2% of the total mRNA, and >95% of the total α-tubulin mRNA in the embryonic nervous system (Miller et al., 1987).

Expression of Tα1 α-tubulin mRNA is also correlated with the growth of mature neurons. Following axotomy of motor (Miller et al., 1989), and sympathetic (Mathew and Miller, 1990) neurons, Tα1 mRNA increases rapidly, and then decreases to control levels following target reinnervation. If regeneration is unsuccessful (Miller et al., 1989), as with CNS neurons (Tetzlaff et al., 1991), Tα1 mRNA levels remain elevated. These increases appear to be due, to a great extent, to loss of repressive homeostatic signals (Mathew and Miller, 1993; Wu et al., 1993) that are likely target derived (Wu et al., 1994). Tα1 α-tubulin mRNA levels are also increased during collateral sprouting of adult sympathetic neurons (Mathew and Miller, 1990), and in dentate granule cells of the hippocampus in response to elevated excitatory input (Causing et al., 1995). The increase in mature sympathetic neurons and, potentially in granule cells, is presumably in response to the increased availability of neurotrophins, since exogenous neurotrophins can increase Tα1 α-tubulin mRNA both *in vivo* (Mathew and Miller, 1990; Miller et al., 1994), and in culture (Ma et al., 1992; Krivko and Miller, 1994). Thus, expression of Tα1 mRNA is high during developmental growth, is downregulated as a function of neuronal maturation, and is then increased in response to extrinsic cues like growth factors that regulate the growth of mature neurons.

In an attempt to elucidate the molecular genetic mechanisms that allow neurons to couple gene expression to morphological growth, we have identified the promoter region for the Tα1 α-tubulin gene (Gloster et al., 1994). In transgenic mice, 1.1 kb of the 5' upstream region of this gene is sufficient to regulate expression of a ß-galactosidase marker gene as a function of neuronal growth: expression of the transgene is induced as soon as neurons are born, is maintained at high levels during neuronal development, and is subsequently reinduced when neurons regenerate or sprout (Gloster et al., 1994; Wu et al., 1994). In the mature, uninjured nervous system, expression of the transgene is reduced, and, consistent with the idea that transcription of this gene is associated with ongoing structural growth, expression of the transgene is not homogeneous in mature neurons. Given that process outgrowth is ongoing at a low level in normal animals (Purves et al., 1986, 1987; Lichtman et al., 1987; Harris and Purves, 1989), and that nerve terminal sprouting may be a normal physiological mode by which some neurons change connectivity (Chang and Greenough, 1984), we felt it would be of interest to determine which subsets of adult neurons express Tα1 mRNA and/or the Tα1:nlacZ transgene, and to determine to what degree these patterns of expression were correlated with growth capability.

In the present paper, histochemical staining with X-gal was used to visualize the pattern of expression of the Tα1:nlacZ transgene in the adult mouse brain, and this was directly compared to the pattern of expression of Tα1 α-tubulin mRNA in the adult rat brain, as determined by *in situ* hybridization. These data demonstrate a strong correlation between the pattern of expression of the Tα1:nlacZ transgene and the endogenous Tα1 mRNA, and indicate that, even in the mature nervous system, transcription of this gene may be associated with morphological growth and/or structural plasticity.

V. Materials and Methods:

Animals: Adult Sprague Dawley rats (2 to 3 months of age) were used to map the endogenous Tα1 α-tubulin mRNA using *in situ* hybridization, whereas Tα1:nlacZ transgenic CD1 mice (of no less than 2 months of age) were used to map the expression of the Tα1:nlacZ transgene using X-gal staining. Rats were chosen for the *in situ* hybridization analysis because the promoter used in the transgenic construct was derived from the rat. Two lines of Tα1:nlacZ transgenic mice were used for the present study: 1) a higher expressing line, K6 and 2) a lower expressing line, Q54, both of which have been previously described (Gloster *et.al.*,1994). Three rats were analyzed for the *in situ* hybridization studies, and five mice from each of the two transgenic lines for mapping transgene expression.

***In Situ* Hybridization Studies:** Adult rats were deeply anaesthetized with sodium pentobarbital (35 mg/kg), and perfused transcardially with 4% paraformaldehyde in 0.1M NaH_2PO_2 (pH 7.3). Brains were then removed and postfixed in the same paraformaldehyde fix overnight. Following cryoprotection through graded sucrose solutions (12%, 16% and 18%), the brains were frozen and serially sectioned at 10 μm intervals onto chrom-alum-subbed slides. Antisense ^{35}S-RNA probes specific for Tα1 α-tubulin mRNA were generated and hybridized to these sections as described (Miller et.al.,1987,1989). Hybridized slides were air dried and apposed to Kodak XRP film for 12 hours to 3 days to obtain X-ray images. The slides were subsequently dipped in Kodak NTB-2 emulsion, exposed for 2-7 days, developed, and analyzed using dark-field microscopy. For quantitative evaluation, only brain sections from the same *in situ* hybridization run, which were exposed for identical periods of time, were utilized. Alternate sections were stained with cresyl violet to facilitate mapping.

LacZ Staining Studies: Expression of the Tα1:nlacZ transgene was characterized in two lines of transgenic mice, K6 and Q54, that express the transgene at different levels. To perform this analysis, mature transgenic brains were assessed by histochemical staining with X-gal, a reagent that leads to deposition of blue crystals in cells with an active E. coli ß-galactosidase. This analysis was done on either entire mouse brains, or on 1-2 mm thick coronal brain slices. Such thick coronal slices were then sectioned on a cryostat for analysis at the resolution of single stained cells. In these transgenic animals, the ß-galactosidase marker gene has been

engineered to include a nuclear localization signal, so that only the nuclei of positive cells will stain.

Adult Tα1:nlacz transgenic mice were deeply anaesthetized with sodium pentobarbital (35 mg/kg), perfused transcardially with 4% paraformaldehyde in phosphate buffer, and the brains removed and postfixed in paraformaldehyde for 20-30 minutes. The brains were then dissected, and sliced into sections 1-2mm thick on a mouse brain matrix (Activational Systems Inc.). They were then rinsed three times, for 30 minutes each, with a wash containing 0.1M NaH_2PO_2 (pH 7.3), 2mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 as previously described (Gloster et.al.,1994). The staining reaction was performed by incubating the tissue at 37°C in a reaction mix containing all the components of the rinse buffer with the addition of 1 mg/ml X-gal, 5mM $K_3Fe(CN)_6$, and 5mM $K_4Fe(CN)_6$ (at pH 7.3-7.6). For the Q54 line, staining was done for 12-24 hours, and for the K6 line for 30-90 minutes. Under these conditions, no background staining of control, nontransgenic brain slices was observed, even at timepoints of 12-24 hours. Brain slices to be used for sectioning were cryoprotected in graded sucrose solutions (12%, 16%, 18%) as previously described (Miller et.al.,1987), and sectioned on the cryostat. For quantitative evaluation, comparisons were made only within a given brain, ensuring that all brain slices were treated identically, and stained with X-gal for the same period of time.

Semi-Quantitative Data Analysis: To determine the pattern of expression of endogenous Tα1 α-tubulin mRNA, film and emulsion autoradiographs were prepared from coronal sections of CD1 rat brains hybridized to probes specific for Tα1 mRNA. Identification of brain structures and qualitative assessments of hybridization intensity using a 0 to 3+ scale were made using the Rat Brain Atlas of Paxinos and Watson (1986).

"+" : hybridization levels low but above background

"++" : moderate hybridization levels

"+++": high relative hybridization levels

The pattern of expression, and relative levels of transgene activity in the transgenic K6 and Q54 mouse lines were determined using X-gal staining. Qualitative assessments of staining were made using a 0 to 3+ scale, with comparisons being made only between brain slices derived from the same animal, and stained for the same period of time. Since X-gal

staining represents an all-or-none phenomenon, with blue staining appearing beyond a threshold level, individual brains of a given line were stained for differing periods of time, so that the scale represents the rapidity with which the staining appears in a given nucleus or region relative to other structures in the same brain. Within a given line, the relative intensity of staining, given this index, was consistent from brain to brain, as follows:

"+" : low levels of staining in a fixed time period

"++" : moderate levels of staining in a fixed time period

"+++": high levels of staining in a fixed time period

VI. Results:

X-gal staining of the entire brain (Fig.2.1) revealed that the overall pattern of transgene expression was similar between the two lines, but that there were also regions of significant differences. Dorsal (Fig.2.1A,B) and ventral (Fig.2.1C,D) perspectives of the entire stained mouse brain demonstrate some of the most obvious differences in expression pattern. In line K6, the transgene is expressed throughout the cortex, although there is significant variation in both the levels and pattern of staining from region to region (Fig. 2.1B). In contrast, in line Q54, the transgene is expressed in clusters of cortical neurons, with more clusters rostrally than caudally (Figs. 2.1A, 2.1E). This punctate pattern of staining on the surface of the cortex represents columns of stained cortical cells, as seen in coronal sections (Figs. 2.2A-G, 2.3A).

A similarly striking difference in transgene expression between the two lines was noted in the cerebellum (Fig. 2.1A,B) and in the islands of Calleja (Fig. 2.1C,D). In both structures, neurons expressed the transgene in line K6, but did not in any significant numbers in line Q54. In the cerebellum of line K6, the transgene was expressed in most, if not all Purkinje cells (Fig. 2.1B, 2.H). In contrast, in line Q54, staining of Purkinje cells was, in most cases, undetectable (Fig. 2.1A), with the exception of the occasional positive cell that was observed on cryostat sections (Fig. 2.4K). Similarly, the islands of Calleja were robustly stained in line K6 (Fig.1D,F), with little or no staining in line Q54 (Fig. 2.1C).

A more detailed analysis of transgene expression was performed by X-gal staining of 1-2 mm thick coronal brain sections (Figs. 2.2, 2.3). Qualitative assessments of X-gal staining were made between structures in a given transgenic line using a 0 to 3 + scale, and are summarized in Table 2.1.

Expression of the Tα1:nlacZ transgene was observed, at varying levels, in specific neuronal populations within the olfactory system, cerebral cortex, basal forebrain, hippocampal formation, amygdala, hypothalamus, thalamus, midbrain, pons, medulla, and cerebellum. Analysis of expression of the endogenous Tα1 α-tubulin mRNA by *in situ* hybridization revealed that, with a few exceptions, the pattern of expression of the endogenous mRNA was very similar to that observed for the transgene. These results, for both the transgene and the endogenous mRNA, are summarized in Table 2.1, and will be presented here region by region.

Olfactory system: Analysis of 1-2 mm coronal slices revealed that the relative pattern of transgene expression was similar in the olfactory bulbs of lines K6 and Q54. The large mitral cells of both the main and accessory olfactory bulbs, exhibited high relative levels of staining (Figs. 2.2A,B, 2.3C). The juxtaglomerular cells of the main olfactory bulb also expressed the transgene in both lines, albeit at somewhat lower levels, while transgene expression was detected in only the occasional granule cell. These patterns were confirmed on cryostat sections of the olfactory bulb (Fig. 2.4H-I). The anterior olfactory nucleus also stained moderately in both transgenic lines (Fig. 2.2B).

As observed for the transgene in both lines, *in situ* hybridization revealed that the mitral cells expressed the highest levels of Tα1 mRNA, with the neurons of the juxtaglomerular region of the main olfactory bulb expressing somewhat lower mRNA levels (Fig. 2.4G). The anterior olfactory nucleus also expressed Tα1 mRNA at moderate to high levels (Fig. 2.5A). One discrepancy in the pattern of mRNA versus transgene expression was, however, observed with regards to the granule cells; Tα1 mRNA was moderately expressed in these neurons (Fig. 2.4G), whereas transgene expression was very low (Fig. 2.4H-I).

Cerebral Cortex: As previously noted on the dorsal aspect of the stained whole brain (Fig. 2.1A,B,E), the pattern of expression of the transgene differed in the cerebral cortex of K6 versus Q54 mice. In the 2.K6 transgenic mouse line, the marker gene was expressed in distinct laminae (Figs. 2.2C-H, 3B). Transgene expression was highest in layer V and in layers II/III, although the transgene was also expressed in other populations of cortical neurons. Moreover, relative levels of transgene expression differed from region to region. Expression of the transgene in neurons of the piriform cortex was amongst the highest in the entire brain (Fig. 2.2D-G). The transgene was also expressed at high levels in the parietal cortex, with neurons in other regions of the cortex expressing significantly lower levels. In all regions, however, the distinctive variation in laminar distribution was maintained.

In contrast, in the Q54 transgenic mouse line, a columnar pattern of Tα1:nlacZ transgene expression was observed in the cerebral cortex (Figs. 2.2A-G, 2.3A). These columns included labelled, blue-stained cells that extended from the corpus callosum to the pia. The intensity of staining in a given column of cells was not uniform; superimposed on the columnar staining pattern was the same relative pattern of laminar distribution noted in the K6 cortex,

with cells of layers V and II/III staining the most intensely. These columns were not consistently distributed about the midline, although in many cases, pairs of columns were observed in approximately the same location relative to the midline (For example, see Fig. 2.1E). The size of individual columns was similar, as noted either on the whole brain (Fig. 2.1E), or on coronal slices (Fig. 2.3A), but the pattern of columns was distinctive from animal to animal. However, in all animals, the greatest density of columns occurred in the rostral half of the cortex (Fig. 2.1A).

In addition to these columns, neurons of the piriform cortex were intensely stained in sections from the Q54 line, as was observed for the K6 line, and represented one of the highest levels of transgene expression in these brains (Fig. 2.2D-E). However, the number of positive neurons in the Q54 piriform cortex was significantly lower than in the K6 piriform cortex, as observed for the rest of the cerebral cortex.

Expression of the endogenous Tα1 mRNA was similar to the pattern observed for the Tα1:nlacZ transgene in line K6, with expression throughout the cortex and some enrichment in certain laminae (Fig. 2.5A-F). The laminar distribution was, however, much less obvious for the mRNA than for the transgene. Also similar to transgene expression was the very high level of mRNA expression in the piriform cortex (Fig. 2.5B-D).

Basal Forebrain and Basal Ganglia: The pattern of transgene expression was generally similar for both lines in the basal forebrain region. The transgene was expressed at the highest relative levels in neurons of the tenia tecta in both lines. The medial septal nucleus and the vertical and horizontal limbs of the diagonal band of Broca exhibited moderate relative expression of ß-galactosidase (Fig. 2.2C,D). In contrast, neurons of the lateral septal nucleus expressed the transgene, but at lower levels, (Fig. 2.2D) and only a very small subset of neurons expressed the transgene in the caudate/putamen and globus pallidus (Fig. 2.2C,D). One obvious discrepancy between the two lines was in the Islands of Calleja. As noted on the stained ventral surface of the whole brain (Fig. 2.1C,D,F), the large neurons of the Islands of Calleja stained strongly in the K6 transgenic mouse line, but did not stain in the Q54 line (Fig. 2.2C).

A similar pattern of expression was observed for the endogenous Tα1 mRNA. Highest levels of mRNA expression were detected in the tenia tecta (Fig. 2.5B), with moderate levels of

expression in the medial septal nucleus and the horizontal and vertical limbs of the diagonal band of Broca. Tα1 mRNA was expressed at low levels in the lateral septal nucleus, and hybridization approached background levels in the basal ganglia. Tα1 mRNA levels were also low in the neurons of the Islands of Calleja (Fig. 2.5B), in agreement with the pattern observed in Q54, but not K6.

Hippocampal Formation: In the hippocampus, pyramidal neurons of Ammon's horn, hilar interneurons, and dentate granule cells all stained positively for ß-galactosidase, albeit with significant variations in relative levels of expression. In line K6, pyramidal neurons of fields CA1 and CA2, and granule cells of the dentate gyrus all expressed high relative levels of ß-galactosidase (Figs. 2.2E-H, 2.3D). Cryostat sections revealed that hilar interneurons also expressed the transgene, although at somewhat lower levels, and that, surprisingly, pyramidal neurons of the CA3 field expressed very low relative levels of ß-galactosidase, with most of the expression within this region of the hipocampus likely due to expression in scattered interneurons (Fig. 2.4C).

In line Q54, transgene expression was particularly obvious in the granule cells of the dentate gyrus (Fig. 2.2E-G), but scattered positive cells were present throughout the remainder of the hippocampal formation (Fig. 2.4B). Again, as observed for the neocortex, the number of cells expressing the transgene in any region of the hippocampal formation was significantly lower than in line K6 (Fig. 2.4B,C).

Expression of Tα1 α-tubulin mRNA was high in the hippocampus, and was relatively homogeneous in the dentate granule cells, in the hilar neurons, and in the pyramidal cells of regions CA1, CA2 and CA3, as detected both on film autoradiographs (Fig. 2.5C-E), and emulsion-dipped sections (Fig. 2.4A). The lack of transgene staining in area CA3 in line K6 (Fig. 2.4C) therefore represents a marked discrepancy with expression of the endogenous Tα1 mRNA.

Amygdala: As seen on whole brain (Fig. 2.1C,D,G) and confirmed on coronal slices (Figs. 2.2E-H, 2.3G), the amygdaloid complex is one of the most darkly stained regions of the brain in both lines of transgenic mice. The areas of the amygdala which stained most intensely for the transgene included the anterior cortical amygdaloid area (Fig. 2.2E), the

basolateral/basomedial amygdaloid nucleus (Figs. 2.2F,G, 2.3G), the amygdalohippocampal area, and the amygdalopiriform transition zone (Fig. 2.2F-H). This pattern of transgene expression is very similar to the pattern of endogenous Tα1 mRNA expression, with high expression in the anterior cortical amygdaloid area (Fig. 2.5C), the basolateral and basomedial amygdaloid nucleus (Fig. 2.5D), the amygdalohippocampal area (Fig. 2.5D), and the amygdalopiriform transition zone.

Hypothalamus: In the hypothalamus, transgene expression was again very similar for the two lines, with the expression pattern differing only in terms of the number of stained neurons, as previously discussed for the neocortex and the hippocampal formation. Staining was high in the suprachiasmatic nucleus (Fig. 2.2E), as well as in the ventromedial hypothalamic nucleus (dorsomedial) (Fig. 2.2F, 2.3E), paraventricular hypothalamic nucleus, supramammillary and medial mammillary nucleus (Fig. 2.2G), and tuberomammillary nucleus. The dorsomedial hypothalamic nucleus (Figs. 2.2F, 2.3E), and the ventromedial hypothalamic nucleus (ventrolateral) (Figs. 2.2F, 2.3E), were moderately stained. Moderate to low staining was further observed throughout the remainder of the hypothalamus as shown in table 2.1.

The pattern of endogenous mRNA expression in the hypothalamus was similar to that of the transgene, with high levels in the suprachiasmatic nucleus (Fig. 2.5C), paraventricular hypothalamic nucleus (Fig. 2.5C), and the tuberomammillary nucleus (Fig. 2.5D). As observed for the transgene, there was moderate to low expression of the endogenous mRNA throughout the remainder of the hypothalamus. One outstanding discrepancy between the pattern of expression of the transgene and the endogenous mRNA was observed in the supraoptic nucleus. The endogenous Tα1 mRNA was highly expressed in this area (Fig. 2.5C), whereas the supraoptic nucleus did not stain for the transgene in either transgenic line (Fig. 2.2E).

Thalamus and Subthalamus: The Tα1:nlacZ transgene was expressed throughout the thalamus, with a pattern of expression that was similar in both lines. The highest relative level of staining was observed in neurons of the medial habenula, as detected on coronal slices (Fig. 2.2E-G) and confirmed on cryostat sections (Fig. 2.4E-F). Other nuclei which highly expressed the transgene included the medial geniculate nucleus (Fig. 2.2G,H), the suprageniculate nucleus (Fig. 2.2G,H), the ventral lateral geniculate nucleus (Fig. 2.2G), and the zona incerta (Fig.

2.2F). Moderate to lower levels of neuronal staining were observed throughout the remainder of the thalamus and subthalamus.

Like the transgene, the highest relative level of expression of Tα1 mRNA was in neurons of the medial habenula, as observed on film autoradiographs (Fig. 2.5C,D), and on emulsion-dipped sections (Fig. 2.4D). High levels of Tα1 mRNA expression were also observed in the suprageniculate nucleus (Fig. 2.5E), whereas the medial geniculate nucleus (Fig. 2.5E) and ventral lateral geniculate nucleus express the endogenous mRNA more moderately. Moderate to low relative levels of expression were observed in the remainder of the thalamus.

Midbrain: Within the midbrain, the Tα1:nlacZ transgene was expressed at the highest levels in the substantia nigra, the superior colliculus, and the oculomotor nucleus. The staining in the substantia nigra was not, however, homogenous. Neurons of the dopamine rich pars compacta expressed the transgene robustly, while neurons of the pars reticulata and pars lateralis expressed the transgene only at very low levels (Fig. 2.2G). This pattern of staining was observed in both lines of mice. The superior colliculus also stained relatively robustly in both lines, although it, too, did not stain homogenously (Fig. 2.2H,I). Within the superior colliculus, the optic nerve layer stained the most robustly, with the other layers expressing the transgene at lower levels. The oculomotor nucleus stained relatively highly in both the Q54 and K6 transgenic lines (Fig. 2.2H,I). A number of additional midbrain structures expressed the transgene at somewhat lower relative levels, including the midbrain central gray (Fig. 2.2H), the red nucleus, the Edinger-Westphal nucleus, and the nucleus of Darkschewitsch. In all of these cases, the pattern of expression was similar in both lines, although the number of positive neurons in each structure was always lower in line Q54.

Expression of the endogenous Tα1 mRNA mirrored that of the transgene throughout the midbrain, with the exception of the superior colliculus. Although clearly expressed, the relative level of expression of the endogenous mRNA in this area was much lower than that of the transgene (Fig. 2.5E,F). However, within the superior colliculus, the pattern of hybridization was similar: Tα1 mRNA was not expressed homogeneously, but was expressed at higher levels in the optic nerve layer.

Pons/Medulla: Expression of the transgene was very high in certain nuclei of the pons and medulla. In particular, neurons of the locus coeruleus were one of the most quickly and intensely staining structures in the brains of both transgenic lines (Fig. 2.2K). Other highly staining nuclei in the pons and medulla included the abducens nucleus (Fig. 2.2L), the superior olive (Fig. 2.2K), the parabigeminal nucleus (Fig. 2.2I), the parabrachial nucleus (Fig. 2.2K), ambiguus nucleus, the inferior olive, and the lateral, medial, spinal and superior vestibular nuclei (Fig.2L). Lower relative levels of transgene expression were observed in the remainder of the nuclei, as shown in table 2.1. A number of differences in transgene expression between the two lines were also observed in the pons and hindbrain. Specifically, in the dorsal and median raphe (Fig. 2.2I), as well as the inferior colliculus (Fig. 2.2J), relative transgene expression in the Q54 line was significantly lower than in the K6 transgenic line.

Levels of transgene expression were not comparable in functionally similar neurons of the pons and medulla. For example, some motor neurons were highly stained for the transgene (abducens nucleus, ambiguus nucleus), some were moderately stained (facial nucleus, hypoglossal nucleus, oculomotor nucleus and trochlear nucleus), and others were minimally stained (trigeminal motor nucleus). Similarly, sensory neurons also expressed the transgene at different levels with high levels in the spinal trigeminal nucleus, moderate levels in the mesencephalic trigeminal nucleus, and low levels in the pontine trigeminal nucleus.

Like the transgene, high Tα1 mRNA expression was observed in the locus coeruleus (Fig. 2.5G), abducens nucleus, parabigeminal nucleus (Fig. 2.5F), posterodorsal tegmental nucleus (Fig. 2.5G), trochlear nucleus, ambiguus nucleus (Fig. 2.5H), facial motor nucleus, hypoglossal nucleus, and the lateral vestibular nucleus. The expression of Tα1 mRNA in the dorsal raphe and the median raphe was high and resembled levels of transgene expression in the K6 transgenic line. One notable difference in the pattern of expression of the transgene and Tα1 mRNA was observed in the parabrachial nucleus. Whereas the transgene was highly expressed in the parabrachial nucleus in both transgenic lines, endogenous Tα1 mRNA was poorly expressed in this area (Fig. 2.5G).

Cerebellar Cortex: As seen from the dorsal view of the entire brain (Fig. 2.1A), and confirmed on the 2mm thick sections (Figs. 2.2K,L, 2.3H), virtually all of the Purkinje cells in line K6 expressed the transgene at moderate levels, whereas those in line Q54 did not. Closer

examination of cryostat sections confirmed this pattern of expression for the K6 line (Fig. 2.4L), and also demonstrated the occasional transgene-positive Purkinje cell in line Q54 (Fig. 2.4K). This therefore appears to be an extreme example of the phenomena of only a subset of neurons in any given structure expressing the transgene in line Q54 relative to line K6. The transgene was not, however, detectably expressed in granule cells of line Q54, while only the occasional very lightly-stained nucleus was detected in the granule cell layer of line K6 (Fig. 2.4K,L). This is in contrast to the endogenous Tα1 mRNA, which was expressed in both Purkinje cells and granule cells of the cerebellum, with higher mRNA levels in the Purkinje cells (Figs. 2.4J, 2.5G,H).

VII. Discussion:

We have recently demonstrated that, in transgenic mice, 1.1 kb of the 5' flanking region of the Tα1 α-tubulin gene is sufficient to regulate expression of a lacZ marker gene in a neuron-specific, temporally appropriate fashion (Gloster et al., 1994). In this paper, we extend those results, by mapping expression of the transgene in the adult central nervous system of two different lines of transgenic mice, and by comparing the pattern of transgene expression with that of the endogenous Tα1 α-tubulin mRNA. These results indicate that expression of the Tα1:nlacZ transgene is similar to that of Tα1 mRNA. Moreover, expression of both the transgene and the mRNA within the mature brain is panneuronal and, in many cases, is highest in those populations of neurons that show some capacity for morphological growth. These results, together with experiments demonstrating increased expression of the transgene in regenerating facial (Gloster et al., 1994) and sympathetic (Wu et al., 1994) neurons indicate that the Tα1 transgene will provide a useful marker of growth-associated gene expression in the mature nervous system.

Tα1 α-tubulin mRNA distribution in the mature CNS

The Tα1 α-tubulin gene is one member of the α-tubulin multigene family that is apparently specialized for high levels of expression during the growth of both developing and mature neurons (Miller et al., 1987; Miller et al., 1989; Mathew and Miller, 1990). Within the mature nervous system, the induction of Tα1 mRNA is perhaps more accurately viewed as a reflection of the potential for growth, as opposed to growth itself, since Tα1 mRNA increases even in situations where functional growth does not occur as, for example, in injured rubrospinal neurons (Tetzlaff et al.,1991). Furthermore, in cases where growth is limited, as, for example, in some types of neuronal sprouting (Mathew and Miller, 1993; Steward, 1995), Tα1 mRNA levels do not increase, presumably because basal levels of synthesis of cytoskeletal components are sufficient.

The data presented here demonstrate that, in the unperturbed adult brain, some neurons maintain higher relative levels of expression of Tα1 mRNA. Interestingly, Tα1 α-tubulin mRNA and GAP-43 mRNA, which encodes a phosphoprotein that is also enriched during neuronal growth (Skene, 1984; Basi et al., 1987; Karns et al., 1987; Neve et al., 1987), and

which is essential for growth-cone function *in vivo* (Strittmatter et.al.,1995), appear to be elevated in at least some of the same populations of mature neurons. As demonstrated here for Tα1 α-tubulin mRNA, GAP-43 mRNA is expressed at increased levels in olfactory bulb mitral cells and in the hippocampal formation (De la Monte et al., 1989; Kruger et.al.,1993), as well as in the dorsal raphe nucleus, the substantia nigra pars compacta, and the locus coeruleus (Bendotti et al., 1991). Although this data is correlative, colocalization of two mRNAs important to neuronal growth in adult neurons is an intriguing observation.

It remains to be determined whether populations of CNS neurons that express higher levels of Tα1 α-tubulin mRNA share biological features. A number of different explanations, none of which are mutually exclusive, can be invoked to account for the observed differential distribution. First, it may be that some neurons undergo constant structural remodelling, thereby requiring higher basal levels of synthesis of growth-associated proteins. In the peripheral nervous system, where such remodelling has been directly visualized (Purves et.al.,1986), dramatically different rates of terminal remodelling have been observed for corneal sensory neurons (Harris and Purves,1989) versus motoneurons (Lichtman et.al.,1987), indicating that not all populations of neurons are equally dynamic. If such a principle generalizes to the CNS, then it may well be that Tα1 mRNA is higher in those neurons that display a higher rate of synaptic or dendritic remodelling. For example, the olfactory sensory neurons that innervate the dendrites of the olfactory bulb mitral cells are continuously lost and replaced (Graziadei et al., 1980). Higher levels of Tα1 mRNA in the mitral cells might therefore reflect ongoing structural remodelling in response to these new inputs. Support for this hypothesis derives from our studies demonstrating that Tα1 α-tubulin mRNA increases during the sprouting of both sympathetic (Mathew and Miller,1993) and hippocampal (Causing et al.,1996) neurons.

Second, increased basal levels of expression of Tα1 α-tubulin mRNA may correlate with growth potential. For example, intact neurons of the entorhinal cortex (Lin et.al.,1992; Steward,1992) and the hippocampus proper (Scheff et.al.,1988; Steward,1992; Leanza et.al.,1993) are capable of significant sprouting and regrowth during lesion-induced reactive synaptogenesis, while neurons of the locus coeruleus sprout for months following damage (Pickel et al., 1974). All of these populations of neurons express Tα1 mRNA at high relative levels. Finally, expression of Tα1 mRNA may be higher in large neurons that maintain large

axons and/or extensive dendritic arbors. For example, the large layer V pyramidal cells of the motor cortex, which project their axons to the spinal cord, express elevated levels of Tα1 mRNA relative to other cortical neurons. However, many large neurons that maintain extensive arborizations, such as the cerebellar Purkinje cells, do not express particularly high levels of Tα1 mRNA, indicating that although size may be a factor, there is no simple correlation between neuronal size and Tα1 mRNA expression.

Tα1:nlacZ transgene expression in the mature CNS

Mapping of the expression of the Tα1:nlacZ transgene in two different lines of transgenic mice revealed that, in the majority of structures, there was a good correspondence between transgene expression in the two lines. There were, however, a number of differences, with the most striking being a difference in the number of neurons that expressed the transgene. In line Q54, only a subset of neurons in any given structure were transgene-positive, whereas in line K6, the majority of neurons stained with X-gal. This phenomenon, which is termed partial penetrance, is not uncommon for transgene expression in the nervous system. For example, the dopamine ß-hydroxylase promoter, which targets gene expression to noradrenergic neurons, drives marker gene expression in anywhere from 5 to 95% of sympathetic neurons (Mercer et.al.,1991). This variation in positive cell number which, in the case of the Tα1:nlacZ transgene, differs from line to line, but not within lines, is likely due to the genomic insertion site of the transgene (Al-Shawi et.al.,1990; Pravtcheva et.al.,1994).

Regardless of the underlying molecular mechanism(s), the partial penetrance observed in line Q54 produced an interesting pattern of transgene expression in the neocortex. In particular, the transgene-positive cells in line Q54 are organized in columns that span the width of the neocortex. The simplest interpretation of this neuroanatomical organization is that the potential for transgene expression is limited to a small population of progenitor cells within the ventricular zones, and that the transgene-positive neurons that derive from these progenitors preferentially migrate laterally within a relatively restricted region. Support for this hypothesis derives from the fact that these columns of transgene-expressing cells are observed as early as embryonic day 15, and thus, must arise during neurogenesis (S. Bamji, A. Gloster, and F. Miller, unpublished observations). These observations therefore support previous studies indicating that the neuronal progeny of any given cortical lineage are largely limited to lateral

migration, thereby forming radial units (Rakic,1988). These data do not, however, address the possibility that a small subset of cortical neurons migrate long distances tangentially (Austin and Cepko,1990; O'Rourke et.al.,1992), as suggested by a number of recent studies (for review see Herrup and Silver,1994).

In contrast to Q54, in line K6 the transgene is apparently expressed throughout the cortex. However, there are marked variations both in laminar distribution, and in rostral/caudal distribution. In general, in line K6, transgene expression is highest either in cortical neurons with large projection axons (such as the large pyramidal cells of layer V) or in those populations of neurons thought to be most structurally "plastic", such as cortical neurons of the piriform cortex. Whether or not sensory input would modulate this pattern of expression remains to be determined.

With one exception, the other differences between lines K6 and Q54, as described here, involve the absence of transgene expression in line Q54 relative to line K6. For example, in line Q54, the transgene is not detectably expressed in the islands of Calleja or in the vast majority of Purkinje cells of the cerebellum. However, the fact that the occasional Purkinje cell in line Q54 is transgene-positive suggests that these are simply extreme cases of partial penetrance, with only a very few neurons in these structures expressing the transgene, as opposed to an absolute absence of expression. The one exception to this generalization involves pyramidal cells of the hippocampus. In line Q54, scattered positive cells are observed throughout the entirety of the pyramidal cell layers, whereas, in line K6, pyramidal neurons of the CA3 region do not apparently express the transgene. This difference is particularly striking given that the endogenous Tα1 mRNA is homogeneously expressed in pyramidal cells. It is difficult to rationalize this difference except on the basis of a somewhat unusual position effect where expression in CA3 pyramidal neurons is selectively repressed in line K6.

Other than the aforementioned differences, there is good correspondence between the pattern of transgene expression in these two lines. There is an equally good correlation between expression of the Tα1:nlacZ transgene and the endogenous Tα1 mRNA. Again, however, a number of diffences were documented, with the most striking being in granule cells. The endogenous Tα1 mRNA is expressed at relatively robust levels in granule cell populations within the olfactory bulb, the hippocampus, and the cerebellum. In marked contrast, the Tα1:nlacZ transgene is expressed at low levels in hippocampal granule cells, and is not

expressed at all in the other granule cell populations in line Q54, with only the occasional positive cell in line K6. A similar difference was noted in the supraoptic nucleus where the endogenous mRNA is expressed at relatively high levels, but transgene expression is low or undetectable.

In contrast, in a number of structures, expression of the transgene is higher than expression of the endogenous mRNA. Specifically, transgene expression is high in the superior colliculus in both lines, and the islands of Calleja stain intensely in line K6. Expression of Tα1 mRNA is low in both of these regions. Thus, in a few structures, the endogenous mRNA is highly expressed and the transgene is not, whereas in a few others, transgene expression is high and the endogenous mRNA is not.

A number of explanations could explain these discrepancies. First, some of this variability may be due to the natural variability between rats and mice although, in both cases, it is the rat Tα1 promoter that is being assayed.

Second, cis elements that are important for gene expression in subsets of neurons may be absent from the 1.1 kb promoter fragment used in these studies. These elements could be located either upstream and/or downstream from our promoter fragment, and might regulate transcriptional levels positively, as in the case of the supraoptic nucleus, or negatively, as in the case of the superior colliculus. Precedent for separable cis-elements targetting gene expression to subpopulations of neurons has previously been obtained with other neuron-specific promoters (Vandaele et al.,1991).

Third, some of these differences could be attributed to position effects. The expression of genes introduced into mice by embryo microinjection is known to be dependent on the integration site of the transgene (Jaenisch et al., 1981; Palmiter and Brinster, 1986). Direct evidence for position effects has been provided by the cloning of transgenes and their reintroduction into mouse embryos; in such studies, integration of a transgene at a novel site correlated with alterations in its expression pattern (Harbers et al., 1981; Al-Shawi et al., 1990). Such differences can likely be attributed to integration of the transgene close to the regulatory elements of other genes, which then inappropriately influence transgene expression. Such a phenomenon provides the basis for "enhancer-trap" screening for novel genetic control elements (Korn et.al.,1992).

Finally, at least some of these differences can be explained by the fact that the endogenous Tα1 α-tubulin mRNA is subject to posttranscriptional regulation, whereas the ß-galactosidase mRNA generated from the transgene is not. Previous work indicates that the cellular tubulin monomer:polymer ratio directly regulates tubulin mRNA levels posttranscriptionally (Ben-Ze'ev et al., 1979; Cleveland et al., 1983; Caron et al., 1985; Gay et al., 1987; Cleveland et al., 1988). Specifically, an increase in the pool of tubulin monomers causes degradation of tubulin mRNA as it is being translated. We have recently demonstrated that Tα1 α-tubulin mRNA stability is regulated by a similar posttranscriptional mechanism in cultured sympathetic neurons (J. Toma and F. Miller, unpublished observations). Thus, the Tα1 mRNA levels that we assess using *in situ* hybridization are a reflection of both transcriptional and posttranscriptional mechanisms. In contrast, ß-galactosidase levels in the Tα1:nlacZ mice will reflect only transcriptional activity from the Tα1 promoter. This difference could explain the aforementioned discrepancies. In neurons where transgene expression is higher than the endogenous mRNA, as in the superior colliculus, posttranscriptional degradation may mask a higher relative rate of transcription. Conversely, in neurons where the endogenous mRNA is apparently higher, such as, for example, in granule cells, the endogenous Tα1 mRNA pool might be stabilized by rapid utilization of the tubulin monomer pool for ongoing structural remodelling. We have recently obtained evidence for such posttranscriptional "fine tuning" during the regeneration of sympathetic neurons (Wu et al., 1994).

The data presented here also suggest that expression of the Tα1:nlacZ transgene could be used as a sensitive assay for environmental cues that regulate the growth of mature neurons. Tα1 α-tubulin mRNA is itself responsive to growth factors (Ma et.al.,1992), and the promoter region for this gene includes a large number of consensus sequence elements for extrinsic cues such as steroid hormones, and some members of the cytokine family (Gloster et al., 1994). In fact, we have recently crossed the line K6 Tα1:nlacZ mice with transgenic mice expressing brain-derived neurotrophic factor (BDNF) from the dopamine-ß-hydroxylase promoter. Interestingly, expression of the Tα1:nlacZ transgene is increased in at least some populations of BDNF-responsive neurons within the mature brain of these animals (S. Bamji, C. Causing, and F. Miller, unpublished observations), suggesting that this marker may allow us to map growth-factor positive neurons *in vivo*.

In summary, we have characterized expression of a Tα1:nlacZ transgene in the nervous system of two lines of adult transgenic mice and have directly compared these patterns of expression with that of the endogenous Tα1 α-tubulin mRNA. The results show a good correlation between expression of the transgene and the endogenous mRNA, and demonstrate that, in many cases, the highest levels of expression of both are detected in neurons that show some capacity for morphological growth. These results, together with our studies on mature regenerating neurons (Gloster et al., 1994; Wu et al., 1994) suggest that the Tα1:nlacZ transgene will provide a useful marker of growth-associated gene expression in the mature nervous system.

Table 2.1: Summary of the Regional Distribution of Tα1 α-Tubulin mRNA in the Adult Rat Brain, and Tα1:nlacZ Expression in the Adult Q54 and K6 Transgenic Mouse Brains

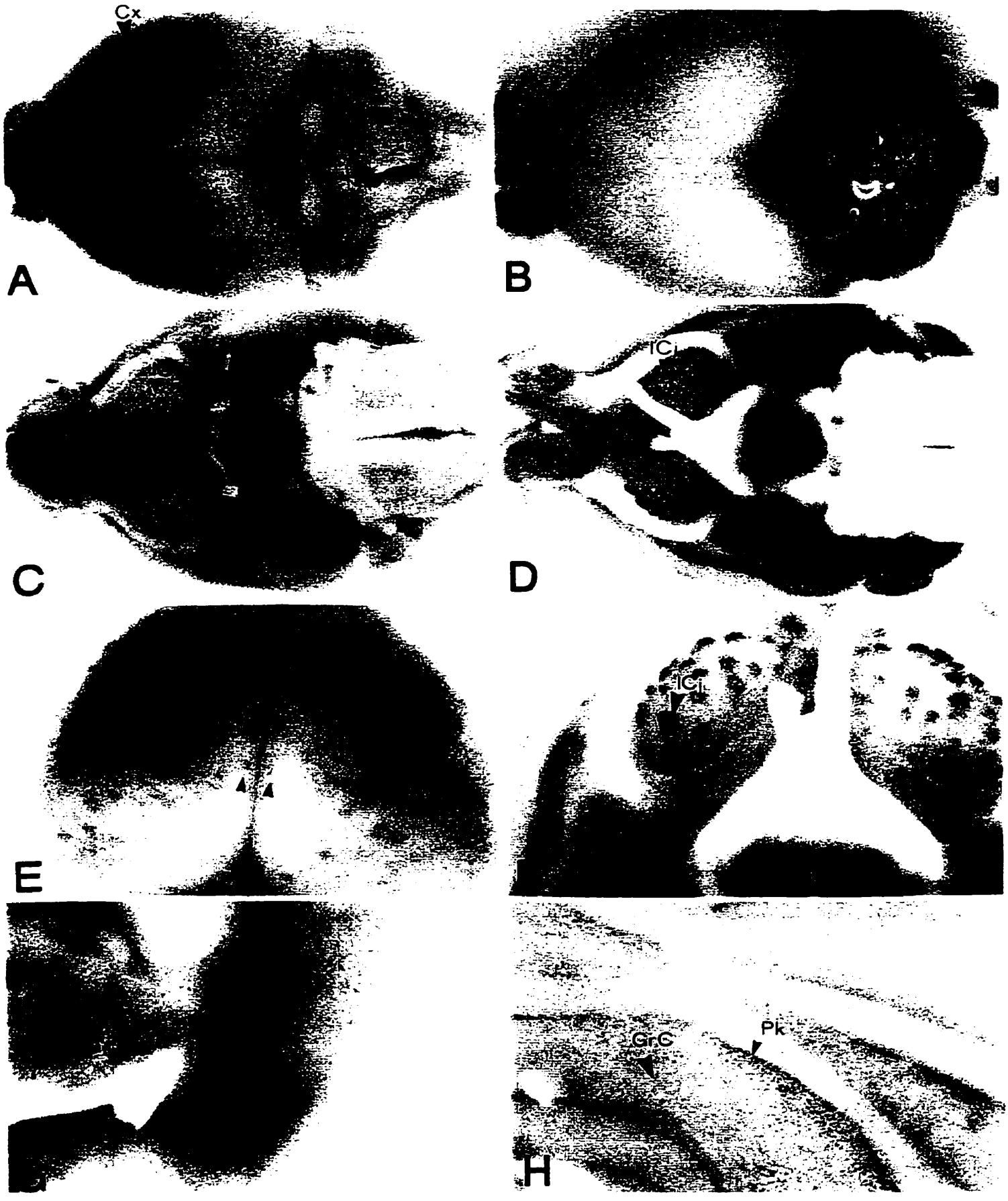
Brain Region	Endogenous mRNA	Q54 Line	K6 Line
Olfactory System			
accessory olfactory bulb: mitral cell layer (Mi)	+++	+++	+++
anterior olfactory nucleus (AOD, AOL, AOE, AOM, AOP)	++	++	++
main olfactory bulb:			
mitral cell layer (Mi)	+++	+++	+++
internal granular layer (IGr)	++	+	+
glomerular layer (Gl)	++	++	++
Cerebral Cortex			
cingulate (Cg)	+	+	+
claustrum (Cl)	+	+	+
dorsal endopiriform cortex (DEn)	+	+	+
entorhinal (Ent)	+	+	+
frontal (Fr)	+	+	+
infralimbic (IL)	+	+	+
parietal (Par)	+	++/+++	++
piriform (Pir)	+++	+++	+++
Basal Ganglia			
caudate/putamen (CPu)	+	+	+
globus pallidus (GP)	+	+	+

Basal Forebrain Region			
bed nucleus of the stria terminalis (BST)	+	+	+
Islands of Calleja (ICj)	+	+	+++
lateral septal nucleus, dorsal (LSD)	-	-	++
lateral septal nucleus, intermediate (LDI)	+	+	+
medial septal nucleus (MS)	++	++	++
nucleus of the diagonal band, horizontal limb (HDB)	++	++	++
nucleus of the diagonal band, vertical limb (VDB)	++	++	++
Hippocampal Formation			
CA1	+++	++	+++
CA2	+++	++	+++
CA3	+++	++	+
dentate gyrus (DG)	+++	+++	+++
hilus of the dentate gyrus (Hil)	+++	+++	+++
tenia tecta (TT)	+++	+++	+++
Amygdala			
amygdalohippocampal area ((AHi)	+++	+++	+++
amygdalopiriform transition zone (APir)	+++	+++	+++
anterior cortical amygdaloid nucleus (ACo)	+++	++	+++
basomedial (BM) and basolateral (BL) amygdaloid nucleus	+++	+++	+++
central lateral amygdaloid nucleus (CeL)	+	+	+
medial anterior amygdaloid nucleus (MeA)	+	+	+
posteromedial (PMCo)/ posterolateral (PLCo) cortical amygdaloid nucleus	+++	+++	+++

Hypothalamus			
arcuate (Arc)	++	+	++
dorsomedial hypothalamic nucleus (DMC)	++	+++	+++
lateral hypothalamic area (LH)	+	+	+
lateral mammilary nuclei (LM)	++	++	++
medial mammilary nucleus (MM)	+	+++	+++
medial preoptic nucleus (MnPO)	++	++	++
paraventricular hypothalamic nucleus, magnocellular (PaMP) and parvocellular (PaLM)	+++	+++	+++
subincertal nucleus (SubI)	++	++	++
supramammilary nucleus (SuM)	++	+++	+++
supraoptic nucleus (SO)	+++	-	-
tuberal magnocellular nucleus (TMC)	+++	+++	+++
tuberomammillary nucleus (TM)	+++	+++	+++
ventromedial hypothalamic nuclei (VMH)	++	++	++
Thalamus			
central medial nucleus (CM)	++	+	++
centrolateral nucleus (CL)	+	+	+
medial habenular nucleus (MHb)	+++	+++	+++
medial geniculate (MG)	++	+++	+++
paracentral nucleus (PC)	+	+	+
parafascicular nucleus (PF)	+	+	++
paraventricular nucleus (PV)	++	++	++
reuniens nucleus (Re)	++	+	+
rhomboid nucleus (Rh)	++	+	+
supragenicuiate nucleus (SG)	+++	+++	+++
ventral lateral geniculate (VLG)	++	+++	+++
ventromedial thalamic nucleus (VM)	+	++	++

Subthalamus			
subthalamic nucleus (STh)	++	+	+
zona incerta (ZI)	++	+++	+++
Midbrain			
Edinger-Westphal nucleus (EW)	++	++	++
interfascicular nucleus (IF)	+	++	++
midbrain central gray (CG)	++	++	++
nucleus of Darkschewitsch (Dk)	++	++	++
occulomotor nucleus (3)	++	+++	++
red nucleus (R)	++	+	++
substantia nigra, pars compacta (SNC)	+++	+++	+++
substantia nigra,pars reticulata (SNR)	+	+	+
superior colliculus (SC)	+	++	+++
Pons			
A5 norepinephrine cells	++	++	+++
abducens nucleus (6)	+++	+++	+++
dorsal raphe (DR)	+++	+	+++
inferior colliculus (IC)	+	+	+++
lateral superior olive (LSO)	+	+	++
laterodorsal tegmental nucleus (LDTg)	++	++	++
locus coeruleus (LC)	+++	+++	+++
median raphe (MnR)	+++	+	+++
mesencephalic trigeminal nucleus (Me5)	++	++	++
motor trigeminal nucleus (Mo5)	++	+	+
parabigeminal nucleus (PBG)	+++	++	+++
parabrachial nucleus, medial (MPB) and lateral (LPB)	+	+++	+++
pontine nuclei (Pn)	++	+	+

posterodorsal tegmental nucleus (PDTg)	+++	+	+++
principal sensory trigeminal nucleus (Pr5)	+	+	+
nucleus of the spinal trigeminal tract (Sp5)	++	++	+++
trochlear nucleus (4)	+++	+++	++
Medulla			
ambiguus nucleus (Amb)	+++	+++	+++
facial motor nucleus (7)	+++	++	++
gigantocellular reticular nucleus (Gi)	+	+	+
hypoglossal nucleus (12)	+++	++	++
inferior olive (IOD)	++	+++	+++
lateral vestibular nucleus (LVe)	+++	+++	+++
medial vestibular nucleus (MVe)	++	+++	+++
spinal vestibular nucleus (SPVe)	++	+++	+++
superior vestibular nucleus (SuVe)	++	+++	+++
Cerebellum			
granule cell layer	++	-	-
Purkinje cell layer	++	+	++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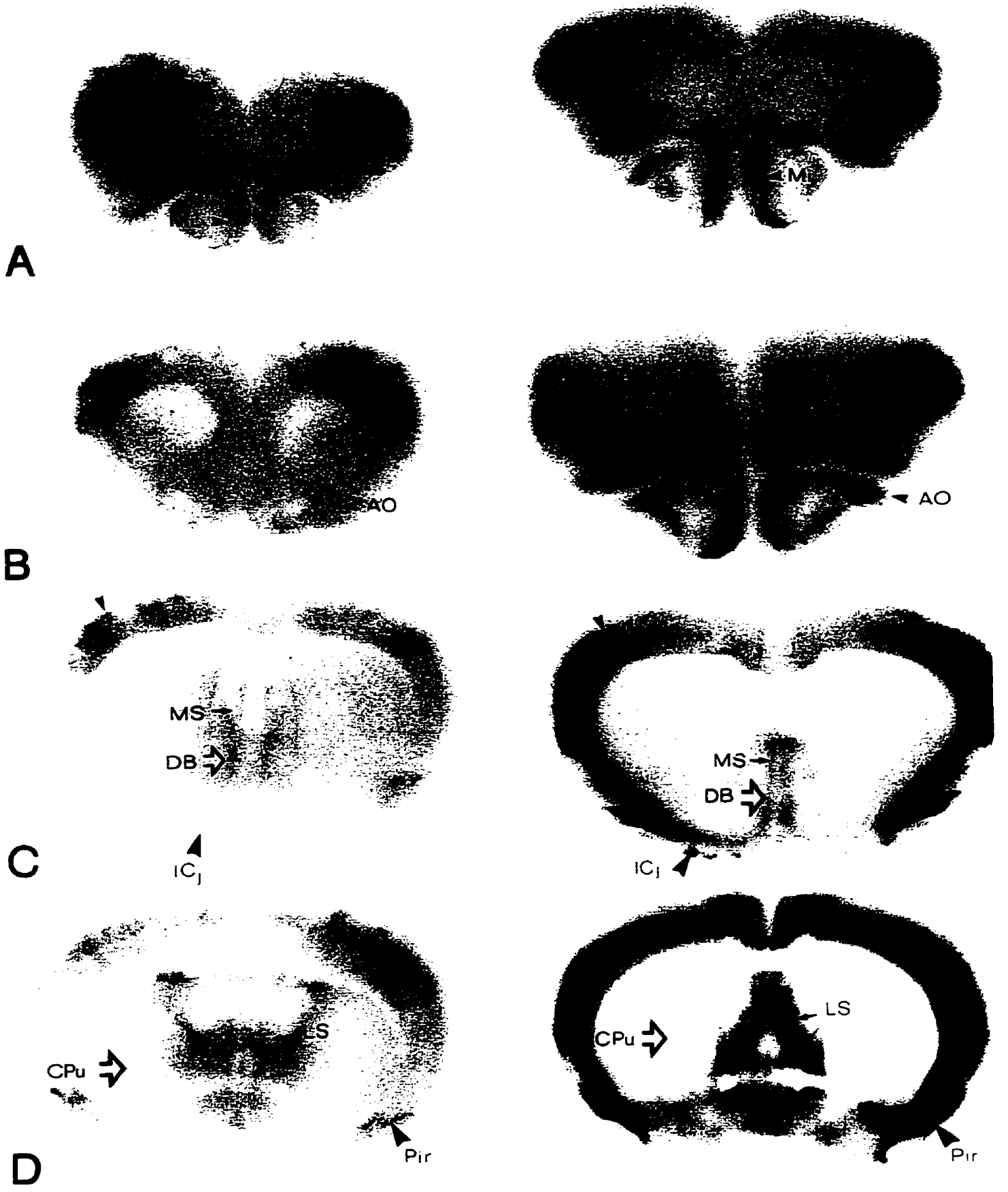
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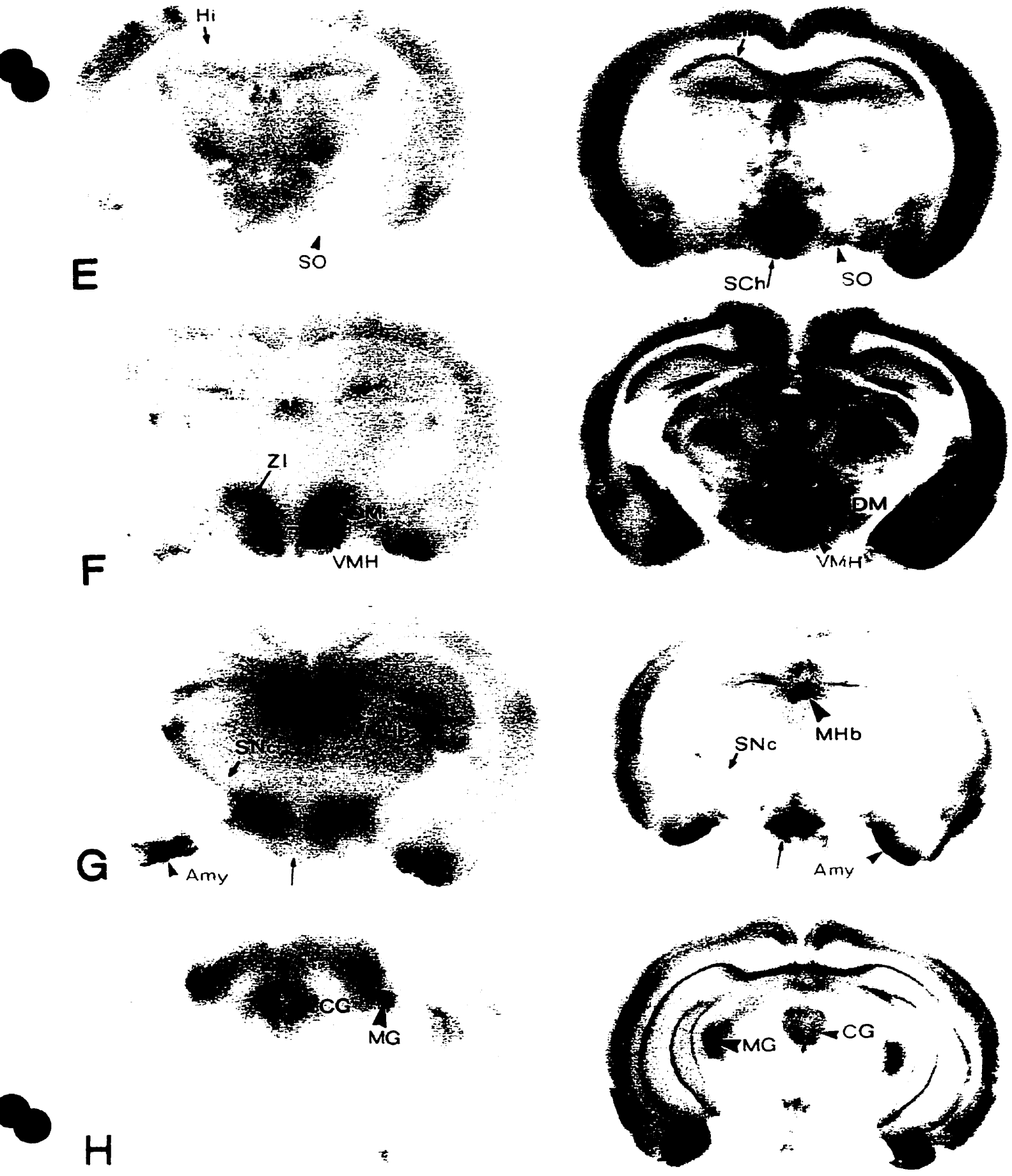
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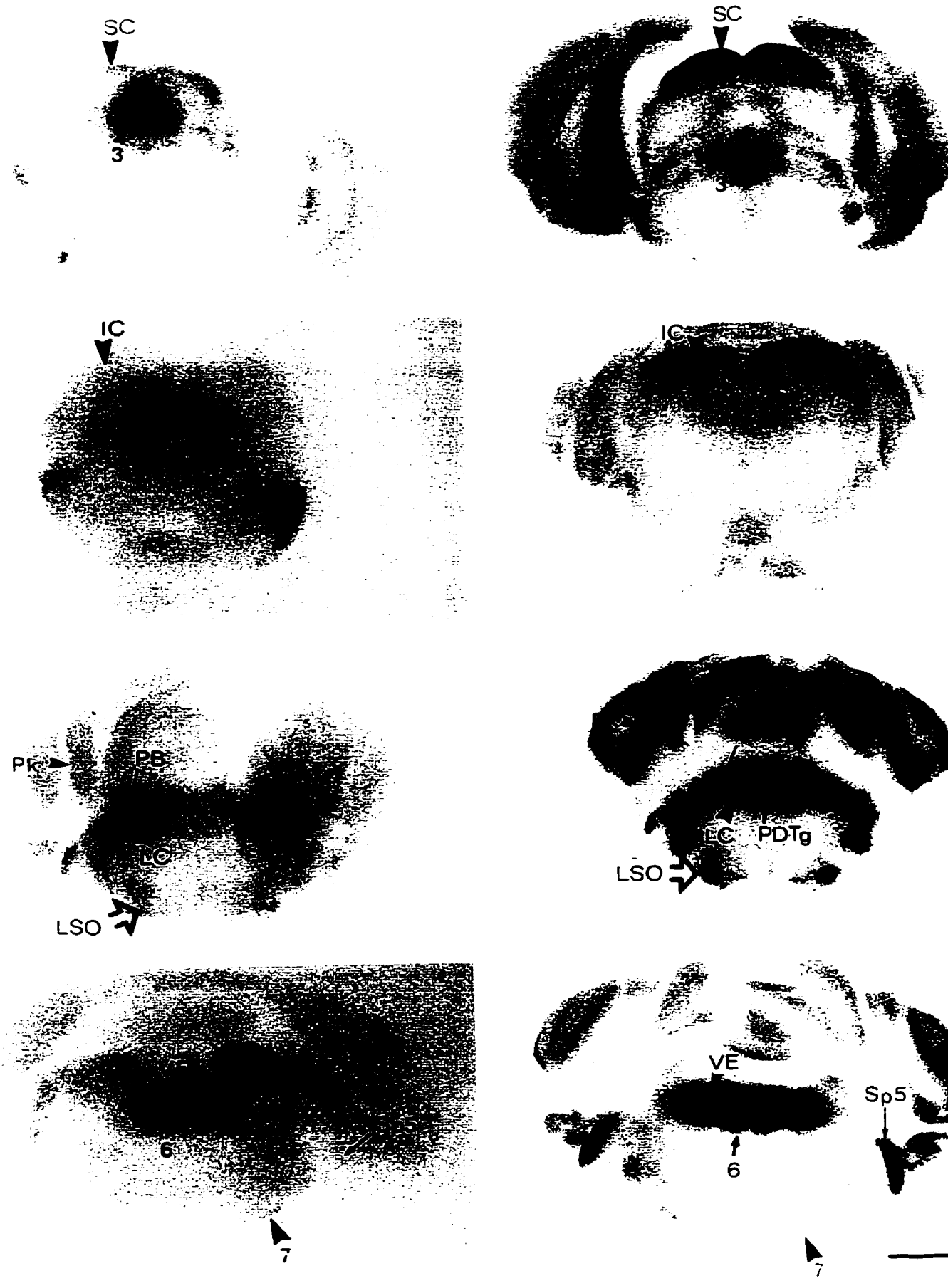
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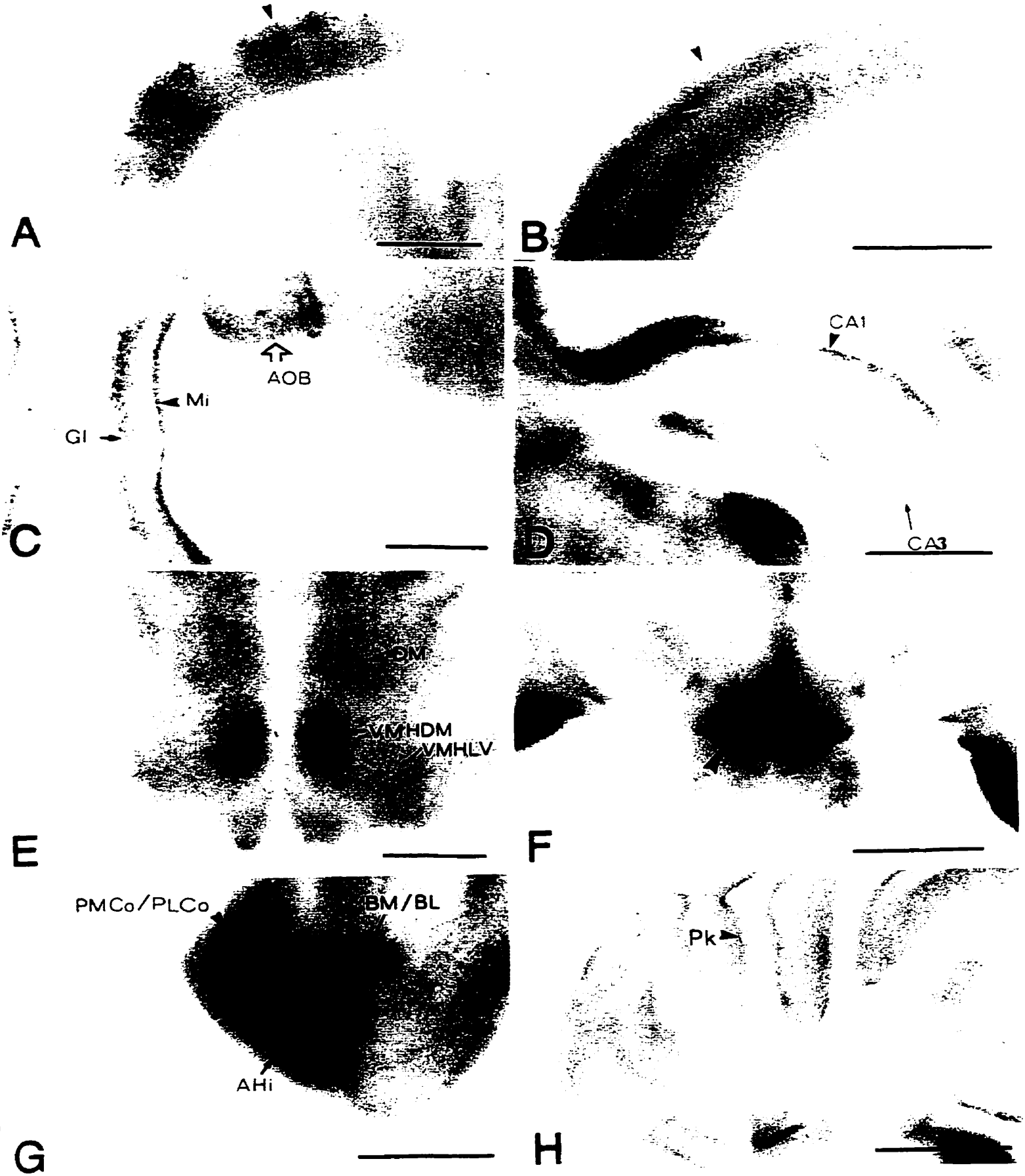
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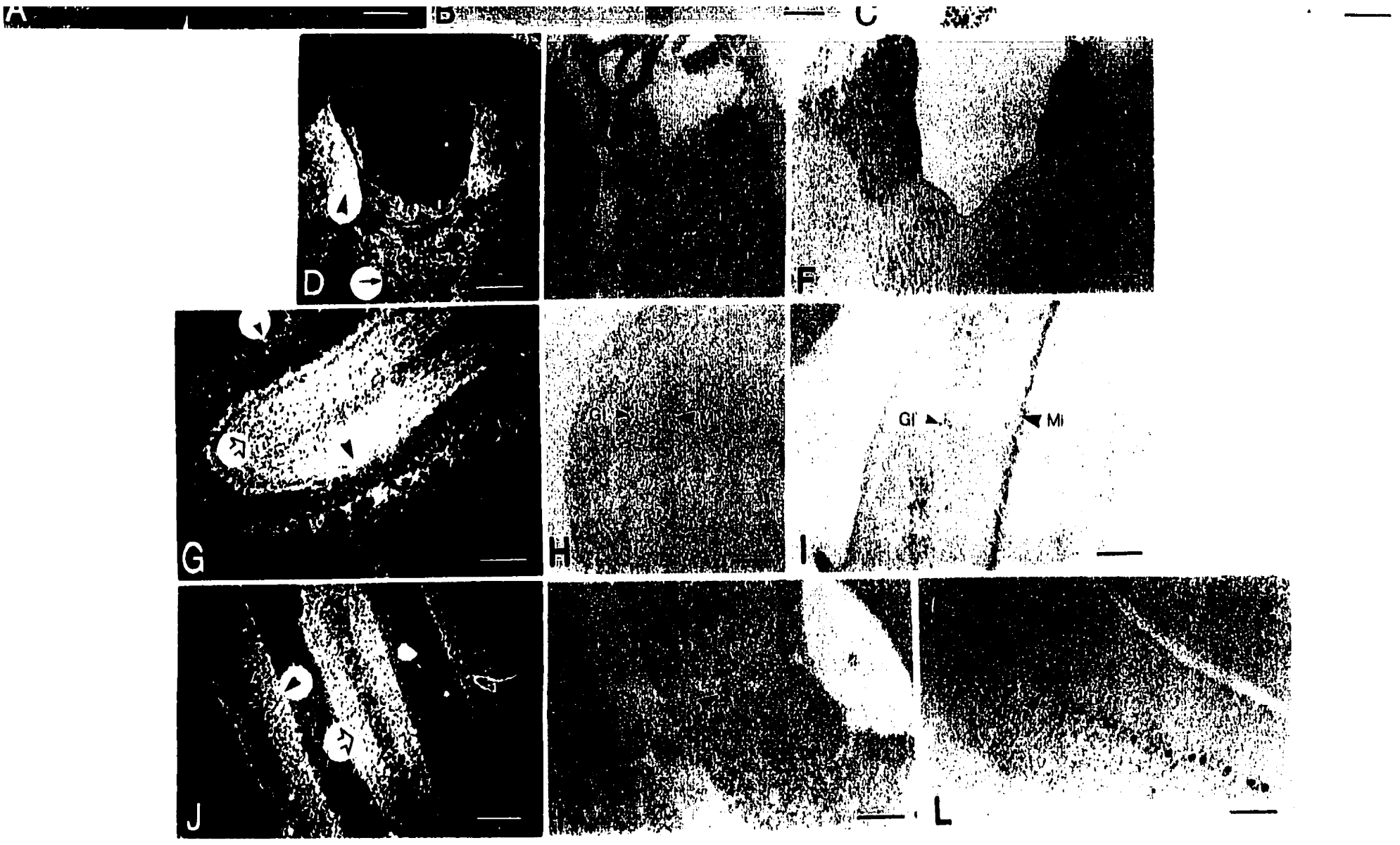
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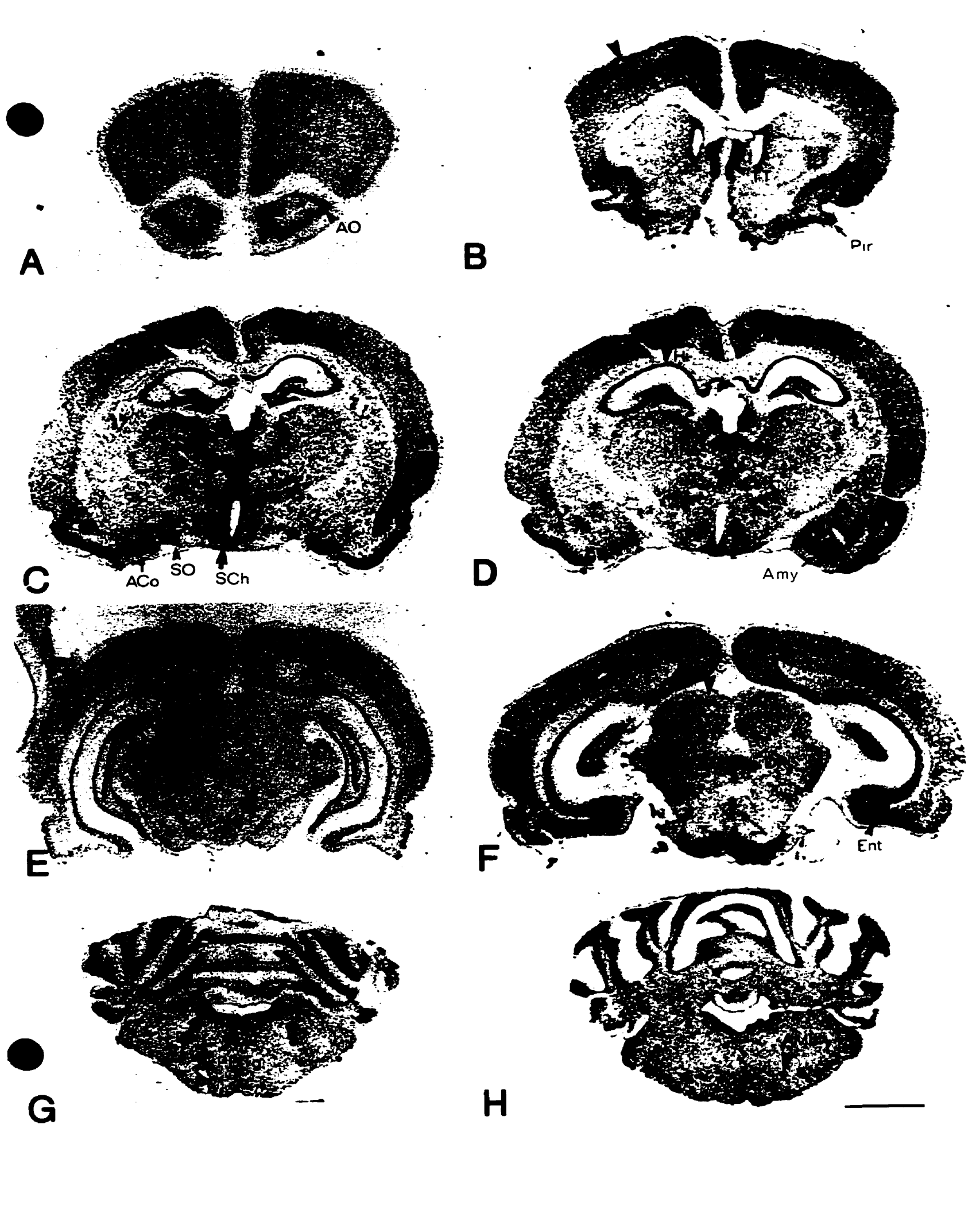
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IX. Figure Legends:

Figure 2.1: ß-galactosidase reporter gene expression in mature Tα1:nlacZ transgenic mice. Pictures A,C,E, and G represent staining in line Q54, whereas pictures B,D,F and H represent staining in the K6 transgenic line. (A) Dorsal view of mature mouse brain from line Q54 showing columnar pattern of cortical staining (large arrowhead) and lack of staining in cerebellar Purkinje cells (small arrowhead). Scale bar = 2.5 mm. (B) Dorsal view of mature mouse brain from line K6 showing staining in the cerebellar Purkinje cells (arrowhead). Scale bar = 2.5 mm. (C) Ventral view of mature mouse brain from line Q54 showing dark staining in the amygdala (large arrowhead), and lack of staining in the Islands of Calleja (small arrowhead). Scale bar = 2.5 mm. (D) Ventral view of mature mouse brain from line K6 showing dark staining in the amygdala (large arrowhead), and staining in the Islands of Calleja (small arrowhead). Scale bar = 2.5 mm. (E) Close-up of Q54 cortex showing a symmetrical pattern of staining in some columns (small arrowhead). Scale bar = 1.25 mm. (F) Close-up of islands of Calleja in line K6 (arrowhead). Scale bar = 0.83 mm (G) Close-up of amygdala in line Q54. (H) Cerebellar Purkinje cells (small arrowhead) and granule cells (large arrowhead). Scale bar = 0.83 mm.

Figure 2.2: ß-galactosidase reporter gene expression in mature Tα1:nlacZ transgenic mice. 1-2 mm thick coronal slices were stained using X-gal and photographed. Brain slices shown on the left represent the Q54 transgenic mouse line, whereas brain slices shown on the right represent the K6 transgenic line. Comparable brain sections are shown in each row to facilitate comparison. (A) Mitral cells (arrowhead). (B) Anterior olfactory nucleus (arrowhead). (C) Comparison between the columnar and laminar pattern of cortical staining (small arrowhead), Islands of Calleja (large arrowhead), medial septum (arrow), and diagonal band of Broca (open arrow). (D) Caudate putamen (open arrow), lateral septal nucleus (small arrowhead), piriform cortex (large arrowhead). (E) Hippocampus (short arrow), suprachiasmatic nucleus (long arrow), supraoptic nucleus (arrowhead). (F) Zona incerta (long arrow), dorsomedial hypothalamic nucleus (short arrow), ventromedial hypothalamic nucleus (arrowhead). (G) Medial habenula (large arrowhead), amygdala (small arrowhead), mammillary bodies (long arrow), substantia nigra pars compacta (short arrow). (H) Central gray (small arrowhead), medial geniculate nucleus (large arrowhead). (I) Superior colliculus (large arrowhead),

oculomotor nucleus (small arrowhead). (J) Inferior colliculus (arrowhead). (K) Purkinje cell layer (small arrowhead), locus coeruleus (large arrowhead), parabrachial nucleus (long arrow), posterodorsal tegmental nucleus (short arrow), superior olive (open arrow). (L) Vestibular nucleus (small arrowhead), facial nucleus (large arrowhead), abducens (short arrow), spinal trigeminal nucleus (long arrow). Scale bar = 2.5mm.

Figure 2.3: ß-galactosidase reporter gene expression in mature Tα1:nlacZ transgenic mice. 2mm thick coronal slices were stained using X-gal. Pictures B-H represent the K6 transgenic mouse line. (A) Columnar pattern of transgene expression in the Q54 cortex (arrowhead). Scale bar = 1.25mm. (B) Laminar pattern of transgene expression in K6 cortex (arrowhead). Scale bar = 1.25mm. (C) Glomerular cell layer of the olfactory bulb (arrow), mitral cell layer (arrowhead), accessory olfactory bulb (open arrow). Scale bar = 0.83mm. (D) Hippocampus with the arrows denoting the CA1 (arrowhead) and CA3 (arrow) fields. Scale bar = 0.83mm. (E) Dorsomedial hypothalamic nucleus (arrowhead), ventromedial hypothalamic nucleus - dorsomedial (short arrow), ventromedial hypothalamic nucleus - ventrolateral (long arrow). Scale bar = 0.83mm. (F) Mammilary bodies (arrowhead). Scale = 1.25mm. (G) Amygdalohippocampal area (short arrow), basolateral/basomedial amygdaloid nucleus (long arrow), posterolateral/ posteromedial cortical amygdaloid nucleus (arrowhead). Scale bar = 0.83mm. (H) Purkinje cell layer of the cerebellum (arrowhead). Scale bar = 1.25mm.

Figure 2.4: Comparison of Tα1 mRNA expression and Tα1:nlacZ transgene expression in the brain of adult CD1 rats and transgenic mice, respectively. A,D,G, and J represent sections of the mature rat brain probed for Tα1 α-tubulin mRNA, dipped in photographic emulsion, developed and photographed using darkfield microscopy. B,E,H, and K represent ß-galactosidase staining in the Q54 transgenic line and C,F,I and L represent staining in the K6 transgenic line. (A-C) Hippocampus, with the arrows denoting the pyramidal cell layers CA1 (large arrowhead) and CA3 (arrow), and the border between CA1 and CA3 (open arrow). Scale bar (A) = 100μm. Scale bar (B,C) = 200μm. (D-F) Medial habenula (arrowhead), and paraventricular thalamic nucleus (arrow). Scale bar (D,F) = 200μm. Scale bar (E) = 125μm. (G-I) Olfactory bulb, showing the mitral cell layer (large arrowhead), glomerular cell layer (small arrowhead), and granule cells (open arrow). Scale bar (G) = 100μm. Scale bar (H,I) =

200μm. (J-L) Purkinje cell layer (arrowhead), and granule cells (open arrow). Scale bar (J) = 100μm. Scale bar (K,L) = 125μm.

Figure 2.5: Film autoradiographs of coronal sections from adult rat brain illustrating regional variations in hybridization intensity of the probe specific for Tα1 α-tubulin mRNA. Regions of greater hybridization intensity correspond to higher levels of mRNA and appear darker in these prints. (A) Anterior olfactory nucleus (arrowhead). (B) Laminar pattern of cortical Tα1 mRNA expression (large arrowhead), tenia tecta (small arrowhead), piriform cortex (arrow). (C) Paraventricular hypothalamic nucleus (arrowhead), supraoptic nucleus (small arrowhead), suprachiasmatic nucleus (large arrow), anterior cortical amygdaloid nucleus (small arrow). (D) Hippocampus (large arrowhead), medial habenula (large arrow), amygdala (small arrow). (E) Supragenículate nucleus (arrow), substantia nigra pars compacta (large arrowhead), red nucleus (small arrowhead). (F) Superior colliculus (large arrowhead), entorhinal cortex (small arrowhead), dorsal raphe (arrow), parabigeminal nucleus (open arrow). (G) Parabrachial nucleus (open arrow), locus coeruleus (arrowhead) posterodorsal tegmental nucleus (arrow). (H) Ambiguus nucleus (arrowhead). Scale bar = 2.5mm.

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CHAPTER III: FUNCTIONAL EVIDENCE THAT BDNF IS AN ANTEROGRADE NEURONAL TROPHIC FACTOR IN THE CENTRAL NERVOUS SYSTEM

I. Preface:

The aim of this chapter is to examine the role of BDNF in the developing central nervous system. Initially, our strategy was to examine the role of BDNF in the CNS by overexpressing BDNF in adrenergic and noradrenergic neurons from the dopamine-β-hydroxylase (DBH) promoter which has previously been shown to confer gene expression specifically in these neurons (Mercer et al.1991). To examine the effects of overexpressing BDNF, we crossed the DBH:BDNF transgenic mice to the K6 line of Tα1:nlacZ transgenic mice described in Chapter II. Here, the Tα1:nlacZ transgene was used as an in vivo ***reporter for neurotrophin-responsiveness and neuronal patterning in the brain following gene manipulation. Somewhat surprisingly, analysis of these mice revealed that Tα1:nlacZ expression was increased in BDNF-responsive neurons that are innervated by adrenergic and/or noradrenergic fibers (see Fig.3a). These studies have provided evidence that BDNF may be anterogradely transported and released onto target neurons in the CNS, and have allowed us to "map" BDNF-responsive neurons in the mature CNS. It remains unclear whether the increase in Tα1:nlacZ expression in the targets of BDNF overexpressing neurons is due to an increase in the level of Tα1:nlacZ transgene expression within neurons, or whether there are a larger number of neurons now expressing the transgene. This is because in addition to being a qualitative reporter of BDNF-responsiveness, the Tα1:nlacZ transgene is a quantitative marker of neurons. In spite of this shortcoming, the Tα1:nlacZ reporter gene provides crucial evidence that one of the roles of BDNF in the brain includes its anterograde transport and release into the vicinity of the target.***

In addition to the observation that Tα1:nlacZ transgene expression is upregulated in the targets of BDNF-overexpressing adrenergic and noradrenergic neurons, there are perturbations in the pattern of Tα1:nlacZ expression in the cortex of BDNF-overexpressing mice compared to control mice (Fig.3b). As discussed in Chapter II, the K6 line of Tα1:nlacZ transgenic mice exhibits an even, laminar pattern of transgene expression in the

cortex. The perturbations and disruptions in the laminar pattern of transgene expression in BDNF-overexpressing mice ranged from mild to severe as shown in figure 3b, and approximately 30-35% of BDNF-overexpressors exhibited these cortical perturbations. This provides evidence that anterogradely transported BDNF can be secreted onto targets, resulting in functional consequences for target neuron morphology.

Although additional studies were needed to confirm that endogenous BDNF is anterogradely transported, and that anterogradely-derived BDNF can regulate the survival and differentiation of target neurons, the use of the Tα1:nlacZ transgene as a "tool" to rapidly determine subtle alterations in the development of the brain after genetic manipulation, was highly successful. The following section furthers the examination of BDNF as an anterogradely-derived trophic factor in the brain.

II. Abstract:

In this report, we have tested the hypothesis that brain-derived neurotrophic factor (BDNF) is an anterograde neurotrophic factor in the central nervous system, focussing on central noradrenergic neurons that synthesize BDNF. Double-label immunocytochemistry for BDNF and dopamine-ß-hydroxylase (DBH), a marker for noradrenergic neurons, demonstrated that BDNF is partially localized to noradrenergic nerve fibers and terminals in the adult rat brain. To test the functional importance of this anterograde BDNF, we analyzed transgenic mice carrying a dopamine ß-hydroxylase:BDNF (DBH:BDNF) minigene. Increased synthesis of BDNF in noradrenergic neurons of DBH:BDNF mice caused elevated TrkB activation throughout postnatal life in the neocortex, a noradrenergic target region. This afferently-regulated increase in TrkB receptor activity led to long-lasting alterations in cortical morphology. To determine whether noradrenergic neuron-expressed BDNF also anterogradely regulated neuronal survival, we examined a second noradrenergic target, neonatal facial motoneurons. One week following axotomy, 72% of facial motoneurons were lost in control animals, while only 30-35% were lost in DBH:BDNF transgenic mice. Altogether, these results indicate that BDNF is anterogradely transported to fibers and terminals of noradrenergic neurons, that anterogradely-secreted BDNF causes activation of TrkB in target regions, and that this secretion has functional consequences for target neuron survival and differentiation. This presynaptic secretion of BDNF may provide a cellular mechanism for modulating neural circuitry, either in the developing or mature nervous system.

III. Introduction:

The neurotrophic factor hypothesis postulates that trophic factors produced and released by target neurons regulate the survival and differentiation of their innervating neurons (reviewed in Oppenheim, 1991). Developing neurons may, however, be equally dependent for survival and differentiation upon afferent neuronal input (reviewed in Linden, 1994). For example, deafferentation can increase the number of neurons lost during the period of naturally-occurring cell death (Sohol and Narayahan, 1975; Clarke, 1985; Furber et al., 1987; Linden and Pinon, 1987) and hyperinnervation can increase survival of target neurons (Cunningham et al., 1979). These findings suggest the existence of anterogradely-released trophic factors that are functionally similar to retrogradely-acting growth factors like nerve growth factor (reviewed in Levi-Montalcini, 1987).

One neuronal population that may play an afferent trophic role during development is brainstem noradrenergic neurons, which innervate a large number of CNS structures, including the cerebral cortex (Jones and Moore, 1977; reviewed in Foote et al., 1983). These central noradrenergic neurons are born from E10 to E13 (Lauder and Bloom, 1974), and project axons into targets such as the neocortex while cortical neurons are still undergoing birth, migration, and differentiation (Altman and Bayer, 1990; Bayer and Altman, 1990). A trophic role for these early afferents is supported by lesion studies that demonstrate that loss of noradrenergic afferents during development alters both the number and morphology of cortical neurons (Maeda et al., 1974; Felten et al., 1982).

The nature of the signal responsible for the trophic effects of noradrenergic afferents is still unknown. However, locus coeruleus neurons make brain-derived neurotrophic factor (BDNF) (Castren et al., 1995; Conner et al., 1997), a member of the neurotrophin family (Barde et al., 1982; Leibrock et al., 1989) known to have effects upon many CNS populations, including the developing cortex (Ghosh et al., 1994; Nawa et al., 1994; McAllister et al., 1995; 1996; Cabelli et al., 1995). Moreover, recent evidence indicates that neurotrophins, including BDNF, can be anterogradely transported in neurons. Specifically, i) exogenous neurotrophins can be anterogradely transported in the developing chick central nervous system (von Bartheld et al., 1996), ii) BDNF is present in axons and, potentially, terminals of peripheral (Zhou and Rush, 1996; Michael et al., 1997), and central neurons (Fawcett et al.,1997; Conner et al.,1997), iii) in the CNS, BDNF is localized to vesicles in presynaptic terminals (Fawcett et al.,1997), and iv)

neurotrophins can be synthesized and released in an activity-dependent fashion (reviewed in Thoenen, 1995). Finally, TrkB receptors have recently been localized to neuronal dendrites in both the hippocampus and cortex (Fryer et al., 1996; Yan et al., 1997a).

On the basis of these recent findings, we hypothesized that BDNF may be a trophic signal derived from noradrenergic afferents. In this paper, we have tested this hypothesis and demonstrate that BDNF is localized to axons and terminals of noradrenergic neurons, that increased BDNF secretion within the cortex from noradrenergic afferents can lead to long-lasting changes in cortical organization, and that increased secretion of BDNF onto developing target neurons can modify neuronal survival itself.

IV: Materials and Methods:

Animals and Surgical Procedures

The DBH:BDNF mice used in these studies, lines D481 and D498, have been previously described (Causing et al., 1997), and were bred and genotyped as described. As controls for these transgenic mice, we used either littermates or animals of the same genetic background that were null for the DBH:BDNF transgene. Mice heterozygous for a targeted mutation in the BDNF gene (Ernfors et al., 1994) were obtained from Jackson Labs (Bar Harbor) and maintained, bred and genotyped as described previously (Causing et al., 1997).

For the TrkB and BDNF biochemical analysis, adult or 1 week old DBH:BDNF, BDNF +/- or appropriate wildtype controls were sacrificed by rapid decapitation. For immunocytochemistry, animals were sacrificed with sodium pentobarbital (65mg/kg) then transcardially perfused with 4% paraformaldehyde or 4% paraformaldehyde/15% picric acid in phosphate buffer (PB, pH 7.4). Brains were then removed and postfixed in 4% paraformaldehyde or 4% paraformaldehyde/15% picric acid in PB for 30 min at 4EC and subsequently cleared of picric acid with phosphate-buffered saline (PBS, pH 7.4). Alternatively, rats were transcardially perfused with 37EC heparinized-PBS followed by 2% parformaldehyde in 0.1M PB, pH 7.3, and were then postfixed for 2 hours at 4°C. All sections were cryoprotected in graded sucrose solutions and sectioned on the cryostat prior to immunocytochemical analysis.

For the facial motoneuron studies, postnatal day 5 wildtype or transgenic animals were anaesthetized by inhalation with metofane to affect. Alternatively, adult female BDNF heterozygote mice ranging in age from 2.5 to 3 months were aneasthetized using sodium pentobarbital (35mg/kg). Following complete anaesthetization (in accordance with an animal care protocol meeting the standards of the Canadian Council on Animal Care) the facial nerve was transected unilaterally at the stylomastoid foramen. One week following nerve transection, young or adult animals were perfused with phosphate buffered saline (pH 7.4) and 4% parformaldehyde in 0.1M NaH_2PO_4, following which the brains were removed and postfixed overnight in the same fixative at 4°C. Brains were then cryoprotected overnight in 30% sucrose, and sectioned on a cryostat.

Histological and Morphometric Analysis

For morphometric analysis of the neocortex, 16 μm sections were stained using cresyl violet, and neuronal counts were performed in 528 μm wide strips extending from corpus callosum to pia, in two separate regions of the forebrain.

For the analysis of facial motoneuron size and number, 16 μm serial coronal sections were collected throughout the extent of the facial motor nuclei, and stained with cresyl violet. For size determination, neuronal profiles containing a nucleus with a distinct nucleolus were displayed on a video screen, and the cellular cross-sectional areas were measured using a computer-based image analysis system (Biocom, France). For neuronal numbers, all neurons containing a nucleus in the facial nuclei were counted on every fifth serial, 16 μm section using the same image analysis system, which prevents double measurements of profiles. The numbers obtained using this approach are not corrected for split nucleoli. Results were expressed as mean values ± the standard error of the mean, and were tested for significance using the one-tailed Student's t-test.

Antibodies, Immunoprecipitations and Western Blot Analysis

The following, previously-described antibodies were used for the biochemical studies described here; anti-TrkBin, which specifically recognizes the intracellular domain of TrkB (Fryer et al., 1996), anti-TrkBout, which specifically recognizes the extracellular domain of full-length and truncated TrkB (Knusel et al., 1994), anti-panTrk 203, which recognizes the intracellular domain of all Trk family receptors (Hempstead et al., 1992), anti-BDNF, which recognizes mature BDNF (Santa Cruz) (Causing et al., 1997; Fawcett et al., 1997), and anti-phosphotyrosine 4G10 (UBI).

For biochemical analysis of TrkB, different regions of 1 week old and adult mouse brains were dissected out, homogenized and lysed in Tris buffered saline (TBS) lysis buffer (Knusel et.al.,1994) containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 0.2 μg/ml leupeptin, and 1.5 mM sodium vanadate. Total Trk protein was immunoprecipitated using 3 μl of anti-panTrk 203. The immunoprecipitates were collected with Protein A-sepharose (Pharmacia) for 1.5 hours at 4°C followed by centrifugation. Immunoprecipitates were then washed three times with cold lysis buffer, boiled in sample buffer (2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.05% bromophenol blue) for 5 minutes, and electrophoresed on 7.5% SDS-polyacrylamide minigels.

After electrophoresis, proteins were transferred to 0.2 μm nitrocellulose for 1 hour at 0.5 amps, and the membranes washed 2 x 10 minutes in TBS. For all antibodies except anti-phosphotyrosine, for which membranes were blocked in 2% BSA (Sigma Chemical Co), membranes were blocked in 5% non-fat dry milk, in TBS for 1.5 hours. Membranes were then washed 2 x 10 minutes in TBS, and the primary antibodies were used overnight at 4EC at dilutions of 1:10,000 for antiphosphotyrosine 4G10 (UBI), 1:2000 for anti-panTrk 203 and 1:5000 for anti-TrkBout, and 1:2000 for anti-TrkBin. Secondary antibodies were incubated for 1.5 hours at room temperature, and were used at dilutions of 1:10,000 for a goat-anti-mouse horse radish peroxidase (HRP) antibody and 1:10,000 for a goat-anti-rabbit HRP antibody (Boehringer Mannheim Biochemicals). Detection was carried out using enhanced chemiluminescence (Amersham) and XAR X-ray film (Kodak). Results were quantitated by image analysis, and statistical significance determined using Student's t-test.

For biochemical analysis of BDNF, brain lysates were boiled in sample buffer, and 20-100 μg of protein was separated on a 15% SDS-polyacrylamide minigel, as previously described (Causing et al., 1997; Fawcett et al., 1997). Following transfer, the nitrocellulose was blotted with an antibody specific to BDNF (Santa Cruz) at a 1:5000 dilution, using the protocols described above. As controls, we used human recombinant BDNF (obtained from Amgen), and an extract of PC12 cells infected with vaccinia virus encoding BDNF (Causing et al., 1997).

Immunocytochemistry

Two different BDNF antibodies were used for the immunocytochemical studies (the Santa Cruz antibody that was used for Western blots does not work for immunocytochemistry). One, an anti-peptide antibody, was used for detection of BDNF synthesis in the DBH:BDNF transgenic mice, and has previously been shown to work for immunocytochemistry (Patterson et al., 1996; W. Friedman and D. Kaplan, unpublished data). A second, more sensitive BDNF antibody (RAB) was used for the BDNF/DBH double-labelling studies; this antibody has previously been characterized (Yan et al., 1997) and demostrated to detect BDNF in nerve fibers and terminals (Conner et al., 1997; Fawcett et al., 1997; Yan et al., 1997b), and was the kind gift of Dr. A. Welcher (Amgen). A commercially available antibody (EugeneTech) was used for detecting dopamine-ß-hydroxylase immunocytochemically. For fluorescence immunocytochemical detection of BDNF and DBH in locus coeruleus neurons, 14 μm-thick cryosections on slides were

post-fixed with 4% paraformaldehyde in 0.1M phosphate buffer for 2 min at room temperature and washed 2 X 10 min in 1M HEPES-buffered saline (HBS, pH 7.4). Sections were permeabilized with HBS + 0.2% Triton X-100, blocked with 3% serum for 45 min at room temperature, and incubated with the primary antibody in HBS +0.2% Triton X-100 + 3% serum overnight at 4°C at a dilution of 1:500 for anti-BDNF and 1:600 for anti-DBH. Slides were then washed 3 X 15 min in HBS. Binding of the primary antibody was visualized with a rhodamine-conjugated secondary antibody (goat anti-rabbit IgG at a 1:200 dilution; Jackson). Slides were then washed, mounted with a 10% glycerol, 90% PBS solution, and coverslipped. In all cases, control and transgenic tissue were prepared at the same time, and analyzed on the same slides.

For immunoperoxidase detection of DBH, the primary antibody was utilized as above (1:600 dilution; Eugene Tech), with the exception that DBH was visualized using a biotin-conjugated secondary antibody (goat anti-rabbit dilution 1:200; Vector Laboratories), and PBS (pH 7.4) was used as a buffer. Slides were then washed as before, incubated with the ABC complex (Vector Laboratories) for 1 hr at room temperature, and then with DAB for approximately 5 minutes, or until desired staining was reached. In all cases control and transgenic tissue were prepared at the same time and analyzed on the same slides.

For the double-label analysis of BDNF and DBH, 10 μm thick sections were blocked in PB (pH 7.4) containing 10% normal goal serum, and 0.2% Triton X-100 for 1 hour. BDNF (AMGEN) and DBH antibodies were then added to the blocking solution at dilutions of 1:5000 and 1:1000, respectively for 24 hours at 4°C. Sections were washed three times in the blocking solution, and then incubated 1 hour in blocking solution containing an anti-rabbit CY3-conjugated antibody to detect anti-BDNF, and an anti-mouse CY2-conjugated antibody to detect anti-DBH, both used at a dilution of 1:1000. Sections were then washed in PBS, and mounted in 50% glycerol in PBS containing 2% DABCO. Sections were analyzed and photographed on a Zeiss Axioscope, using filters that were designed for double-label analysis using these two fluorochromes.

Quantitation of the relative level of immunoreactivity for BDNF in locus coeruleus neurons from DBH:BDNF versus control animals was carried out using image analysis. Five different pairs of line D481 versus control animals were analyzed. Specifically, transgenic and control neurons were analyzed by immunofluorescence at the same time (and in many cases, on the same slide), and were photographed using the same exposure and developing times. These

photographic images were scanned, and the relative fluorescence intensity was measured and expressed as mean optical density per neuron. The background level of fluorescence, measured lateral to the locus coeruleus neuron cluster, was subtracted from the values obtained for BDNF-positive cells. Statistical significance was determined using Student's t-test.

Measurement of Tissue Monoamines

Animals were sacrificed by swift decapitation with a guillotine, their brains quickly removed and frozen at -80°C. The brains were later placed on a cold plate to thaw, and series of 1.0-1.5 mm thick sections (usually 4-5) were cut. The brains from six wild-type and six transgenic mice were dissected over the cold plate under microscopic observation, as previously described (Reader and Grondin, 1987; Reader et al., 1989), and the following eight regions were taken for the monoamine assays: frontal and entorhinal-piriform cortices, neostriatum (caudate-putamen), thalamus, hypothalamus, hippocampus, cerebellum and brainstem. The discrete tissue samples (10-20 mg wet weight) were placed in tubes already containing 1-2 ml of cold monochloroacetic acid 0.1N with 2.15 mM Na_2EDTA, disrupted in a glass homogenizer with a Teflon pestle, and the homogenates centrifuged at 39,000 x g for 45 min at 4°C. The pellets were dissolved overnight in 0.5 ml of 1 N NaOH for protein determinations (Lowry et al., 1951) and the supernatants assayed by high-performance liquid chromatography (HPLC) with electrochemical detection, following well established procedures (Lakhdar-Ghazal et al., 1986; Sauve and Reader, 1988). Briefly, the supernatants were filtered through 0.45 µm pores (GS, Millipore, Bedford, MA) and injected into the 3 µm particle-size chromatographic column (100.0 x 4.1 mm; Adsorbosphere Catecholamine; Alltech Associates Inc., Deerfield, IL). The isocratic mobile phase was 0.1 N monochloroacetic acid ajusted to pH 3.3 with 1 N NaOH and containing 800 mg/l of Na_2EDTA, 300 mg of sodium octyl sulfate and 10% of HPLC-grade methanol. The flow was set at 0.6 ml/min, the temperature of the column was kept at 37EC, and the electrochemical detector (Model M-400, Princeton Applied Research, Princeton, NJ) set at a gain of 50 nA, full scale for neostriatum and brainstem and at 20 nA full scale for the remaining regions. The eluted compounds were oxidized with a glassy carbon electrode at a potential of 0.68 V relative to the Ag/AgCl reference electrode. The peaks generated by the compounds were recorded, and their surfaces integrated with a Hewlett Packard 3392A integrator. For every chromatographic run, external standards containing 1.25 ng of each of the authentic monoamines were injected to quantify peak area as well as retention times.

Both parameters showed very good reproducibility, with a coefficient of variation never exceeding 5%. Although the method allows for the separation of catecholamines, indoleamines and their major metabolites (Reader and Grondin, 1987), only the tissue levels of noradrenaline (NA), dopamine (DA), 3,4-dihyroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methyoxytyramine (3-MT), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) are reported here, in nanograms per milligram of protein.

V. Results:

BDNF and Dopamine-ß-Hydroxylase are Partially Colocalized in Fibers and Nerve Terminals in the Adult Brain

To determine whether BDNF, which is synthesized by central noradrenergic neurons (Costren et al., 1995; Conner et al., 1997), is anterogradely-transported into noradrenergic axons and nerve terminals, we performed double-label immunocytochemical analysis for BDNF and dopamine-B-hydroxylase (DBH), a marker for noradrenergic and adrenergic neurons and fibers (Figs. 3.1 and 3.2) using a specific BDNF antibody (RAB) from AMGEN which has previously been characterized (Conner et al., 1997; Fawcett et al., 1997; Yan et al., 1997b) and a commercially available monoclonal (DBH) antibody. We chose to focus upon the brainstem for these studies, since both BDNF- and DBH-positive fibers are abundant in this brain region (Conner et al., 1997; Moore and Card, 1984).

This analysis revealed that, although many DBH-positive fibers were not immunoreactive for BDNF, and many BDNF-positive fibers were not immunoreactive for DBH, these two proteins were highly colocalized in certain brainstem structures (Fig. 3.1). As previously reported (Conner et al., 1997), noradrenergic neuron cell bodies were largely negative for BDNF-immunoreactivity, although faint immunostaining could occasionally be seen in the soma of DBH-positive noradrenergic neurons (Fig. 3.1A-C). However, BDNF-immunoreactivity was detected in the network of DBH-positive processes that surround noradrenergic cell groups, such as those of the ventrolateral reticular group (Fig. 3. 1A-C). In these locations, DBH-positive fibers were also BDNF-positive and, in some cases, BDNF immunoreactivity was detected in DBH-positive processes as they exited the noradrenergic cell soma (Fig. 3.1A-C). At higher magnification, this colocalization could be seen to occur in single noradrenergic fibers (Fig. 3. 2A-C). These data, together with previous in situ hybridization data (Castren et al., 1995), indicate that BDNF is synthesized by noradrenergic neurons, and is transported from the cell soma into noradrenergic processes.

BDNF was also colocalized to DBH-positive fibers rostral and lateral to noradrenergic cell groups. In particular, BDNF was colocalized with DBH-positive fibers, presumably axons, in the rostro-ventrolateral peri-olivary region (Figs. 3.1D-F). Higher-resolution analysis (Fig. 3.2D-F) revealed that, in some cases, this colocalization occurred in fibers coursing through the tissue section (Fig. 3.2D-F), while in others the colocalized immunoreactivity was punctate in nature,

reflecting either noradrenergic terminals and/or axons cut in cross-section (Fig. 3.2D-F, arrow heads). Both DBH and BDNF teminal staining was also seen in the raphi pallidus, a terminal field of noradrenergic innervation (data not shown). Thus, BDNF can be immunocytochemically detected in noradrenergic axons and nerve terminals.

DBH:BDNF Mice Overexpress BDNF in Central Noradrenergic Neurons

These data indicate that BDNF is anterogradely transported by noradrenergic afferents, and suggest that it may be secreted from noradrenergic terminals, thereby regulating the development and/or maintenance of their target neurons. To test this hypothesis, we took advantage of transgenic mice that overexpress BDNF in noradrenergic neurons from the dopamine-ß-hydroxylase promoter (DBH:BDNF mice) (Causing et al.,1997). We have previously demonstrated that in two lines of DBH:BDNF mice, D498 and D481, BDNF synthesis is increased approximately 2 to 4-fold in sympathetic noradrenergic neurons, with no ectopic expression and/or secretion of BDNF into the circulation. To determine whether BDNF is also overexpressed in central noradrenergic neurons, as predicted (Hoyle et al., 1994), we examined the locus coeruleus. Immunocytochemical analysis using a BDNF-specific antibody (Patterson et al., 1996; W. Friedman and D. Kaplan, unpublished data) demonstrated BDNF-immunoreactivity detectable in a few neuronal cell bodies within the control locus coeruleus (Fig. 3.3A). In contrast, BDNF-immunoreactivity was evident in most locus coeruleus neuron bodies in DBH:BDNF animals from lines D481 (Fig. 3.3B) and D498 (data not shown). Image analysis of the fluorescence intensity per locus coeruleus neuron revealed that BDNF-immunoreactivity was reproducibly increased in four different pairs of control versus line D481 DBH:BDNF animals (5.52±0.29 versus 11.19±0.38, n = 52 and 78, $p<0.0001$; 11.27±0.57 versus 16.09±0.72, n = 41 and 90, $p<0.0001$; 9.57±0.35 versus 17.04±0.87, n = 141 and 71, $p<0.0001$; 9.88±1.78 versus 21.57±1.61, n = 15 and 43, $p<0.0002$; in all four pairs, the data represents the mean fluorescence intensity ± the standard error, and n represents the number of neurons analyzed). Thus, the DBH:BDNF transgene was expressed in both central and peripheral (Causing et al., 1997) noradrenergic neurons.

To confirm this conclusion, we also quantitated BDNF in the brains of control versus DBH:BDNF mice by Western blot analysis. We initially examined the brains of 1 week old mice, after the onset of expression from the dopamine-ß-hydroxylase promoter (Kapur et al., 1991), at a

time when endogenous BDNF levels are low (Maisonpierre et al., 1990). Analysis of Western blots revealed that brainstem BDNF levels were increased approximately 1.8-fold (n = 5 each, $p<0.003$) in 1 week-old transgenic animals of line D498 relative to their control littermates (Fig. 3.4C, top panels). BDNF levels were similarly increased approximately 2.4-fold (n = 5 each, $p<0.0007$) in the brainstem of adult animals of line D498 (Fig. 3.4C, bottom panels) and D481 (data not shown). In contrast, BDNF levels were unchanged in the cortex of adult line D481 animals (data not shown) or in the neonatal or adult cortex of line D498 DBH:BDNF mice (n = 5 each, $p>0.05$) (Fig. 3.4A,B, bottom panels), as previously reported in the spinal cord (Causing et al., 1997). Both the cortex and spinal cord are locations where there are no resident noradrenergic or adrenergic neurons. Together with our previous findings (Causing et al., 1997), this analysis indicates that BDNF synthesis is increased in developing and mature noradrenergic neurons of DBH:BDNF mice, and that this overexpression is confined to regions of the nervous system predicted by previous analysis of the 1.6 kb DBH promoter (Hoyle et al., 1994).

Increased BDNF Synthesis by Noradrenergic Neurons Anterogradely Regulates Cortical Development

If BDNF synthesized by noradrenergic neurons is anterogradely transported and secreted onto target neurons, then we would predict, in the DBH:BDNF mice, an increase in the baseline level of TrkB autophosphorylation in targets such as the neocortex. To test this prediction, we isolated cortical tissue from neonatal and adult mice, immunoprecipitated the lysates with anti-panTrk 203, and then analysed the immunoprecipitates by Western blot analysis with anti-phosphotyrosine. We reprobed the blots for total TrkB receptor levels using anti-TrkBout, and normalized the level of increase in Trk autophosphorylation relative to total TrkB levels. This analysis indicated that levels of autophosphorylation of a 145 kD Trk band were specifically increased 2.8-fold (n = 8 each, $p<0.003$) in the cortex of 1 week-old DBH:BDNF versus control animals (Fig. 3.4B, top panels), although cortical BDNF levels were unchanged (Fig. 3.4B, bottom panels). Similarly, in the adult DBH:BDNF cortex, the level of autophosphorylation of the 145 kD Trk band was increased approximately 2.5-fold relative to controls (n = 8 each, $p<0.05$) (Fig. 3.4A, top panels). In the neonatal cortex, the increased Trk autophosphorylation was limited to this 145 kD Trk band (Fig. 3.4B), while in adult animals, we also observed increased autophosphorylation of a 190 kD Trk-immunoreactive band (Fig. 3.4A). This phosphorylated Trk

band likely corresponds to a novel, 190 kD TrkB that has been previously reported in both the peripheral (Bhattacharyya et al., 1997) and central (Roback et al., 1995) nervous systems (R. Aloyz, D. Kaplan, and F.D. Miller, unpublished observations). Thus, increased BDNF expression within brainstem noradrenergic neurons led to increased Trk receptor autophosphorylation in the neocortex, a noradrenergic target region.

To determine whether the observed increase in TrkB receptor activation in the neocortex modified cortical development and/or maintenance, coronal sections of control versus DBH:BDNF brains were stained with cresyl violet, and the gross morphology of the neocortex compared. Comparisons were made at two levels as shown in Fig. 3.5A. At the anterior level, the depth of the cortex from the corpus callosum to the pia was consistently smaller in animals of line D498 than in controls (Fig. 3.5B, top panels). In contrast, cortical depth was similar in controls versus DBH:BDNF mice at the more posterior level (Fig. 3.5B, bottom panels). This observation was quantitated by determining the number of neurons in 528 μm wide strips of cortex in control versus transgenic animals. At the anterior level, there were 2269.0 +/- 41.45 cells per 528 μm wide strip of transgenic cortex, compared to 2505 +/- 21.55 in controls, a statistically significant decrease of 10% ($p<0.002$, $n = 5$ each). At the posterior level, there were 1860.38 +/- 54.44 cells in DBH:BDNF mice, compared to 1953.7 +/- 68.1 cells in control mice, numbers that were statistically similar ($p>0.05$); $n = 5$ each). Consistent with these observations, noradrenergic innervation is most dense in the rostral neocortex (Schlumpf et al., 1980). Thus, increased anterograde secretion of BDNF from noradrenergic neurons affects cortical development and/or maintenance, most probably through activation of endogenous TrkB receptors.

Increased Synthesis of BDNF by Noradrenergic Neurons Rescue Neonatal Facial Motoneurons from Axotomy-Induced Death

Our cortical results support the hypothesis that anterogradely-secreted BDNF regulates the development and/or maintenance of noradrenergic target neurons. To further test this hypothesis, we turned to neonatal facial motoneurons because i) motoneurons receive noradrenergic innervation (Pickel et al., 1974; McBride and Sutin, 1976), and ii) neonatal facial motoneurons, which normally die in response to facial nerve axotomy, can be rescued by application of exogenous BDNF (Sendtner et al., 1993; Koliatsos et al., 1993). We predicted that neonatal facial

motoneurons would be rescued from axotomy-induced death in DBH:BDNF mice because of increased BDNF supplied by their noradrenergic input.

To perform these experiments, we initially characterized facial motoneuron size and number in DBH:BDNF mice. Image analysis of serial, cresyl violet-stained coronal sections from the brainstem of P12 control versus DBH:BDNF mice revealed that facial neuron numbers were unchanged by expression of BDNF in noradrenergic neurons (Control, 2987±514; D481, 3248±200; D498, 2838±471; n = 3 animals each; $p>0.3$ for all comparisons). In contrast, the average cross-sectional area of facial motoneurons was increased approximately 35% and 25%, respectively, in lines D481 and D498 relative to control animals (Fig. 3.6A) (Control, 256.8±9.2 μm^2; D481, 384.8±8.5 μm^2; D498, 333.8±15.4 μm^2; n = 4 animals each for D498 and control, and 3 for D481; $p<0.002$ for both comparisons).

We next determined whether increased BDNF from noradrenergic neurons could rescue the death of axotomized, neonatal facial motoneurons, as can exogenous BDNF (Sendtner et al., 1993; Koliatsos et al., 1993). For these experiments, the facial nerve was unilaterally transected at postnatal day 5, and 1 week later the number of facial motoneurons in the contralateral control versus ipsilateral transected facial nuclei determined. Specifically, serial 16 μm coronal sections were collected throughout the entirety of the facial motor nuclei, and the number of motoneurons determined in every fifth cresyl violet-stained section (Fig. 3.6B). In control littermates, only 28% of axotomized neonatal facial motoneurons survived relative to their uninjured counterparts (control, uninjured neuron number, 2579±546; control axotomized neuron number, 631±75; n = 4 each). In contrast, in animals of lines D481 and D498, 66% and 69% of facial motoneurons, respectively, remained in the axotomized versus uninjured facial nuclei (line D481 uninjured neuron number, 3248±200; line D481 injured neuron number, 2133±159; line D498 uninjured neuron number, 2521±460; line D498 injured neuron number, 1721±277; n = 3 for D481 and n = 4 for D498). This increase in facial motoneuron survival in the DBH:BDNF mice was highly significant relative to control animals ($p<0.006$ for D498; $p<0.009$ for D481), and is similar to the level of rescue previously reported for exogenous BDNF (Sendtner et al., 1993; Koliatsos et al., 1993).

Decreased Synthesis of BDNF in BDNF+/- Mice Leads to Increased Death of Axotomized Adult Facial Motoneurons

Our data indicate that alterations in BDNF levels directly regulate the survival of injured motoneurons. To test this hypothesis further, we examined BDNF+/- mice, in which one BDNF allele has been mutated by targeted recombination. To confirm that BDNF levels are decreased in the brains of BDNF+/- mice relative to controls, we quantitated BDNF by Western blot analysis. This analysis revealed that BDNF levels were decreased approximately two-fold in regions throughout the adult BDNF+/- brain (Fig. 3.6D), as previously reported using ELISAs (Altar et al., 1997). To determine whether this decrease in endogenous BDNF levels affected survival of injured motoneurons, the facial nerve was unilaterally transected in adult BDNF+/- mice or their BDNF+/+ littermates and one week later, the number of surviving facial motoneurons was determined by morphological analysis (Fig. 3.6C). This analysis revealed that, in BDNF+/+ littermates, 87 $\pm$1.6% of transected facial motoneurons remained relative to the control, untransected motoneurons, as previously observed in control mice (Majdan et al., 1997). In contrast, in BDNF+/- mice, only 71 $\pm$4% of transected facial motoneurons remained, relative to the untransected facial motoneurons in the same animals (Fig. 3.6C), a statistically significant decrease of 18% ($p<0.005$, n=3 animals each). These results, together with those obtained from the DBH:BDNF mice support the hypothesis that the survival of injured facial motoneurons is at least partially dependent upon BDNF, and that relatively small alterations in BDNF levels can tightly regulate the survival of injured BDNF-responsive neurons.

Noradrenergic Innervation is Not Altered in DBH:BDNF Mice

Although our results indicate that anterogradely transported BDNF regulates neuronal development and maintenance by activation of the TrkB receptor, noradrenaline has been previously proposed to have trophic effects (Maeda et al., 1974; Felten et al., 1982; Foote et al., 1983), and it is formally possible that some of the observed biological effects in the DBH:BDNF mice are due to increased catecholaminergic innervation. To address this possibility, we examined morphological and neurochemical measures of noradrenergic innervation density. Initially, we compared the level of immunostaining for dopamine-ß-hydroxylase, which is specific to noradrenergic and adrenergic neurons, in the brains of control versus DBH:BDNF mice (Fig. 3.3C-F). As predicted, anti-DBH robustly stained the noradrenergic neurons of the locus

coeruleus, with no significant qualitative different between control (Fig. 3.3C) and D481 (Fig. 3.3D) or D498 (data not shown) animals. To determine whether the level of noradrenergic target innervation was altered, we examined the hippocampus, which has a well-defined pattern of noradrenergic innervation (Swanson and Hartman, 1975; Loy et al., 1980; Moudy et al., 1993). In control (Fig. 3.3E), D481 (Fig. 3.3F), and D498 (data not shown) animals, the pattern and density of DBH-positive innervation was similar. Thus, the pattern of noradrenergic innervation was not apparently altered in DBH:BDNF mice.

To assess quantitatively catecholaminergic innervation, tissue levels were measured in discrete CNS regions, using HPLC with electrochemical detection. This method allowed for the measurement of noradrenaline (NA) and dopamine (DA), of the major metabolites of DA, namely DOPAC, HVA and 3-MT, as well as of the indoleamines serotonin (5-HT) and 5-HIAA (Tables 3.1-3.2). In all regions examined, the levels of noradrenaline were similar in control versus transgenic animals ($p>0.05$), confirming that noradrenergic innervation was not altered in these animals. In frontal cortex (Fro) the main catecholamine was NA, and although levels of this catecholamine were unaltered, this was the only CNS region where endogenous DA levels were found to be somewhat higher in transgenic mice (Table 3.1). The entorhinal-piriform (EnPi) cortex had similar levels of NA and DA, as expected, and there were no differences detected. In hippocampus (Hipp) DA and its metabolites were not detected in the majority of samples; the main catecholamine was NA which was unaltered. The main catecholamine in the neostriatum (NS; caudate-putamen) was DA, and there were also high levels of its metabolites DOPAC, HVA and 3-MT; this was the only region in which significant levels of 3-MT were detected and measured in all samples. The indoleamines 5-HT and 5-HIAA were present in all these regions, with values ranging from 3 to 5 ng/mg protein, but there were no significant differences between control and transgenic mice, suggesting a normal 5-HT innervation of these forebrain areas. In the thalamus (Thal) of both control and transgenic mice there were moderate levels of NA, but also of DA and its metabolites DOPAC and HVA (Table 3.2) and somewhat higher (5-7 ng/mg protein) levels of 5-HT and 5-HIAA. Interestingly, in the hypothalamus (Hypo) the tissue levels of DA were lower in the transgenic mice relative to controls. In spite of this reduction in endogenous DA, the levels of its metabolites DOPAC and HVA were unchanged, suggesting an increased turnover rate. The levels of the indoleamines 5-HT and 5-HIAA were similar in control and transgenic mice. In the brainstem (BS) the high contents of NA (4-5 ng/mg protein), 5-HT (8

ng/mg protein) and 5-HIAA (8-9 ng/mg protein) reflect the fact that this region contains the nuclei of origin of noradrenergic and serotonergic projections. There were no differences between control and transgenic mice in levels of NA, DA, DOPAC, HVA, or the indoleamines in this region. Finally, in the cerebellum (CB) only moderate to low levels of NA, 5-HT and 5-HIAA were measured, and the tissue contents were the same in control and transgenic mice.

VI. Discussion:

Evidence indicates that neuronal survival and differentiation is dependent upon the appropriate establishment of afferent inputs (reviewed in Linden, 1994). In this paper, we have tested the hypothesis that BDNF functions as an anterograde trophic factor for central noradrenergic neurons. The results reported here support our hypothesis and lead to three major conclusions. First, our data indicate that BDNF is synthesized by noradrenergic neurons and is anterogradely transported into noradrenergic axons and nerve terminal fields. Second, we demonstrate that overexpression of BDNF in noradrenergic neurons of DBH:BDNF mice has measurable anterograde effects on the cortex, leading to increased activation of cortical TrkB receptors, and differences in cortical morphology. Third, our studies demonstrating rescue of axotomized neonatal facial motoneurons in DBH:BDNF mice indicate that increased BDNF from noradrenergic afferents can directly regulate neuronal survival. Together, these data indicate that anterogradely-secreted BDNF can modulate neuronal survival and differentiation, and suggest that the effects of noradrenergic afferents on target neuron development may be at least partially mediated by BDNF.

Previous studies have demonstrated that BDNF is present in axons and nerve terminals in the mature central nervous system (Conner et al., 1997; Fawcett et al., 1997; Yan et al., 1997), and that TrkB, the preferred receptor for BDNF, is present on the dendrites of many central neurons (Fryer et al., 1996). Together, these data led us to hypothesize that BDNF might be an anterograde neurotrophic factor within the central nervous system, and that it might mediate some of the previously-described effects of afferent innervation on target neuron development. To test this hypothesis, we focussed upon central noradrenergic neurons which synthesize BDNF mRNA (Castren et al., 1995), and which have long been thought to play an organizational role in development of targets such as the neocortex (Maeda et al., 1974; Felton et al., 1982). The double-labelling data presented here indicate that BDNF may well be an anterograde trophic factor for these neurons; BDNF is localized to processes of noradrenergic neurons as they exit the cell soma, in noradrenergic axons in regions lateral to the cells themselves, and in noradrenergic terminal fields. This colocalization data, together with previous studies demonstrating synthesis of BDNF mRNA by noradrenergic neurons (Castren et al., 1995; Conner et al., 1997), strongly supports the hypothesis that BDNF is a candidate anterograde neurotrophic factor for this class of neurons.

One noradrenergic target that might well be regulated by BDNF secreted from noradrenergic afferents is the neocortex. Previous studies indicate that a chemical lesion of developing noradrenergic neurons perturbs cortical development (Maeda et al., 1974; Felton et al., 1982), indicating a key regulatory role for early-arriving afferents. Moreover, both BDNF and NT-4 regulate cortical development. Specifically, exogenous application of BDNF prevents the formation of ocular dominance columns (Cabelli et al., 1995) and regulates pyramidal neuron dendritic growth (McAllister et al., 1995), while exogenous NT-4 causes neuronal heterotopias, possibly by causing excess neurons to migrate into the developing marginal zone (Brunstrom et al., 1997). Moreover, endogenous BDNF is necessary for differentiation of cortical interneurons (Jones et al., 1994), and is thought to play an autocrine/paracrine role in cortical neuron survival (Ghosh et al., 1994). These effects presumably occur through activation of TrkB (Klein et al., 1991; 1992), which is present at high levels during corticogenesis (Knusel et al., 1994; Escandon et al., 1994), and which is expressed on cortical neurons throughout the developing and mature cortex (Cabelli et al., 1995; Fryer et al., 1996; Yan et al., 1997b).

Our studies with the DBH:BDNF mice support the hypothesis that noradrenergic afferents are likely to provide one endogenous source of BDNF for the developing cortex. Previous work demonstrates that the 1.6 kb DBH promoter used in these studies is highly specific for noradrenergic and adrenergic cells, and is not ectopically expressed (Hoyle et al., 1994). Our data support this conclusion. The two lines of DBH:BDNF mice described here express increased BDNF in sympathetic neurons of the SCG and in noradrenergic neurons of the locus coeruleus, with no apparent increase in BDNF in the spinal cord or cortex (Causing et al., 1997; data shown here). Moreover, the effects described here cannot be ascribed to increased systemic BDNF, since i) BDNF is undetectable in the circulation of these animals (Causing et al., 1997), and ii) sensory neurons of the dorsal root ganglia, which hypertrophy in response to BDNF, are unaffected in DBH:BDNF mice (C. Causing and F. Miller, unpublished data). In addition, we have demonstrated that the pattern and levels of noradrenergic innervation in the CNS (data shown here) and PNS (Causing et al., 1997) of these mice are apparently normal. Thus, the cortical effects we observe are best explained by increased secretion of BDNF from noradrenergic and, potentially, adrenergic afferents.

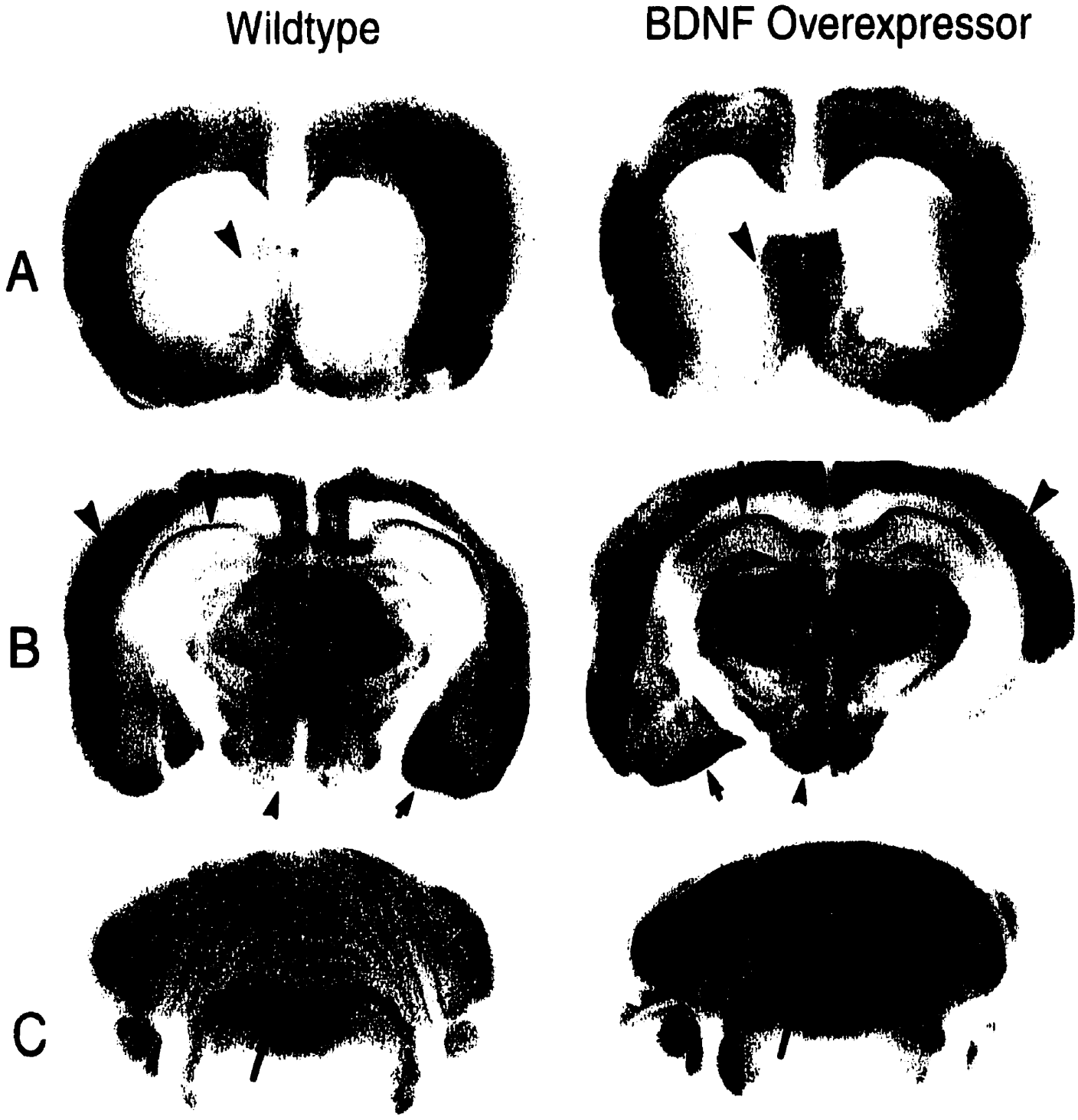
Interestingly, our data indicate that there is an increase in cortical Trk autophosphorylation without an apparent increase in cortical BDNF levels. The lack of a detectable increase in cortical

BDNF is relatively easy to explain; it is likely that the amount of BDNF derived from noradrenergic afferents is small relative to the total amount of BDNF in the cortex, and therefore, even a 2 to 4-fold increase in the amount of BDNF from this source is masked by endogenous cortical BDNF. Why, then, is there a two to three-fold increase in the basal level of Trk autophosphorylation? We propose that much of the BDNF present in cortical neurons and/or their afferents is packaged in secretory vesicles (Fawcett et al., 1997), and is therefore unavailable to neuronal TrkB receptors until it is released by neuronal activity, much like a neuropeptide. In contrast, although the amount of BDNF delivered by noradrenergic afferents is low relative to the total amount of BDNF in the cortex, it is likely that this BDNF is being routinely secreted, since noradrenergic neurons are constitutively active during waking hours (Harley, 1991). Thus, we propose that the amount of TrkB receptor activation in any given region of the cortex is not only a function of the amount of BDNF present in that region, but also of the amount of BDNF secretion that occurs in response to neuronal activity. Support for this model derives from our recent findings that pharmacological activation of cortical afferent systems leads to rapid and dramatic increases in cortical TrkB autophosphorylation, presumably as a consequence of activity-dependent BDNF release (R. Aloyz, J. Fawcett, and F.D. Miller, unpublished data).

What are the cortical perturbations observed in the DBH:BDNF mice in response to this increased TrkB receptor activation? In rats, noradrenergic neurons are born from E10 to E13 (Lauder and Bloom, 1974), and their afferents reach the developing cortex by E16 (Schlumpf et al., 1980), a timepoint when cortical neurons are actively being born, migrating and differentiating (Altman and Bayer, 1990; Bayer and Altman, 1990). These afferents project through and branch into the marginal zone (Levitt and Moore, 1979), which is essential for the appropriate formation of cortical layers (Caviness, 1982), and which is one site of perturbed cortical development in response to NT-4 (Brunstrom et al., 1997). The DBH promoter is turned on in central noradrenergic neurons shortly after they are born (Hoyle et al., 1994), and our results confirm the neonatal expression of the DBH:BDNF transgene. The resultant increase in cortical Trk receptor activation throughout postnatal development presumably causes the decrease in neuronal number observed in the anterior DBH:BDNF cortex. This phenotype could result from any number of underlying mechanisms, including premature neurogenesis, inappropriate migration, and/or alterations in neuronal survival; our data do not discriminate amongst these possibilities.

Although the cellular mechanisms underlying the cortical phenotype reported here are unclear, the facial motoneuron studies indicate that afferent BDNF can directly regulate neuronal survival. Facial motoneurons in the DBH:BDNF mice are not rescued from developmental death, likely because they develop early relative to their noradrenergic innervation (Bayer and Altman, 1994). However, in neonates, when we know the DBH:BDNF transgene is on, increased BDNF from noradrenergic neurons causes facial motoneuron hypertrophy, and rescues these neurons from axotomy-induced death. Whether endogenous afferent BDNF plays a similar role is unclear. However, our data with the BDNF+/- mice demonstrate that endogenous BDNF is essential for the survival of these injured neurons, although these studies do not directly address the source of this endogenous BDNF. Moreover, these latter studies demonstrate that relatively small alterations in endogenous BDNF (approximately two-fold) are sufficient to determine motoneuron survival following injury. Thus, these two studies together strongly support the idea that alterations in the amount of BDNF secreted from noradrenergic afferents could play a role in regulating the survival of developing or injured target neurons.

In summary, our immunocytochemical data indicate that BDNF is present in the axons and terminals of at least some noradrenergic neurons, and our studies with the DBH:BDNF mice indicate that such an afferent source of BDNF can affect the survival, differentiation and maintenance of target neurons. We suggest that the previously-documented trophic effects of noradrenergic afferents on cortical development are at least partially mediated by BDNF. Moreover, we suggest that activity-dependent presynaptic neurotrophin secretion and subsequent activation of postsynaptic Trk receptors may provide a cellular mechanism for modulating neural circuitry either during development and/or in the mature nervous system.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BDNF Overexpressor
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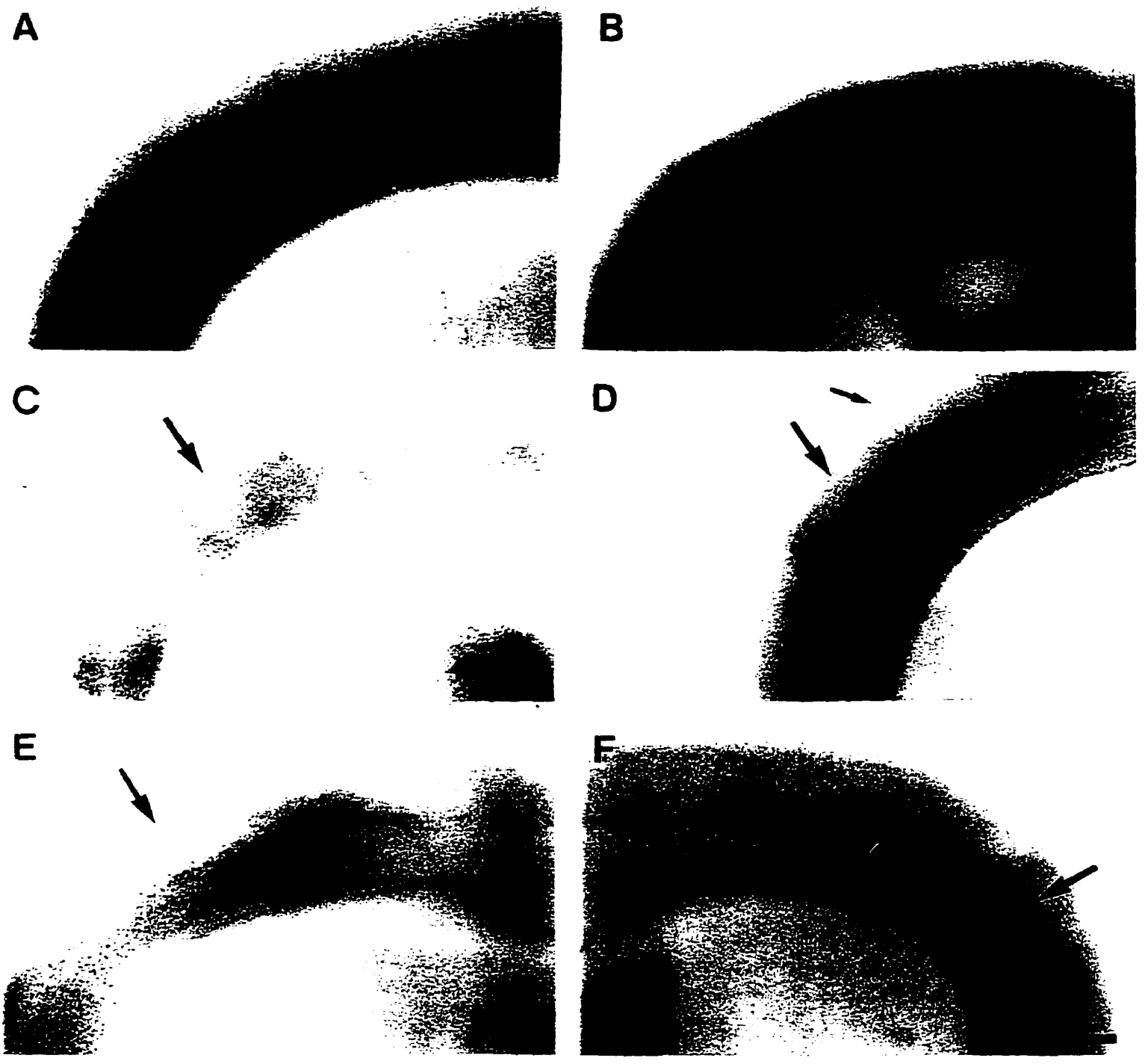
B
C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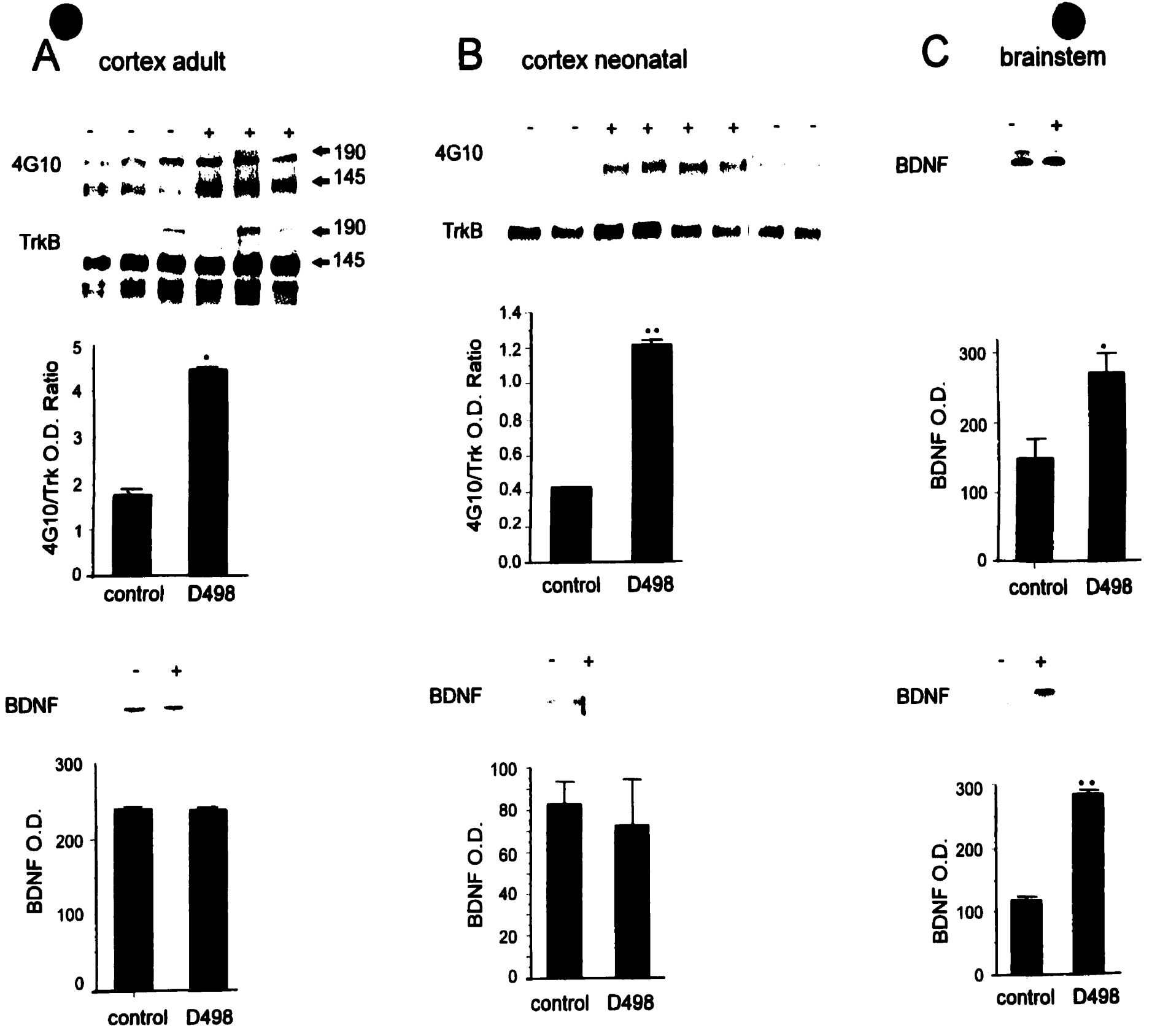
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control
D498
B
cortex neonatal
4G10
TrkB
4G10/Trk O.D. Ratio
control
D498
BDNF
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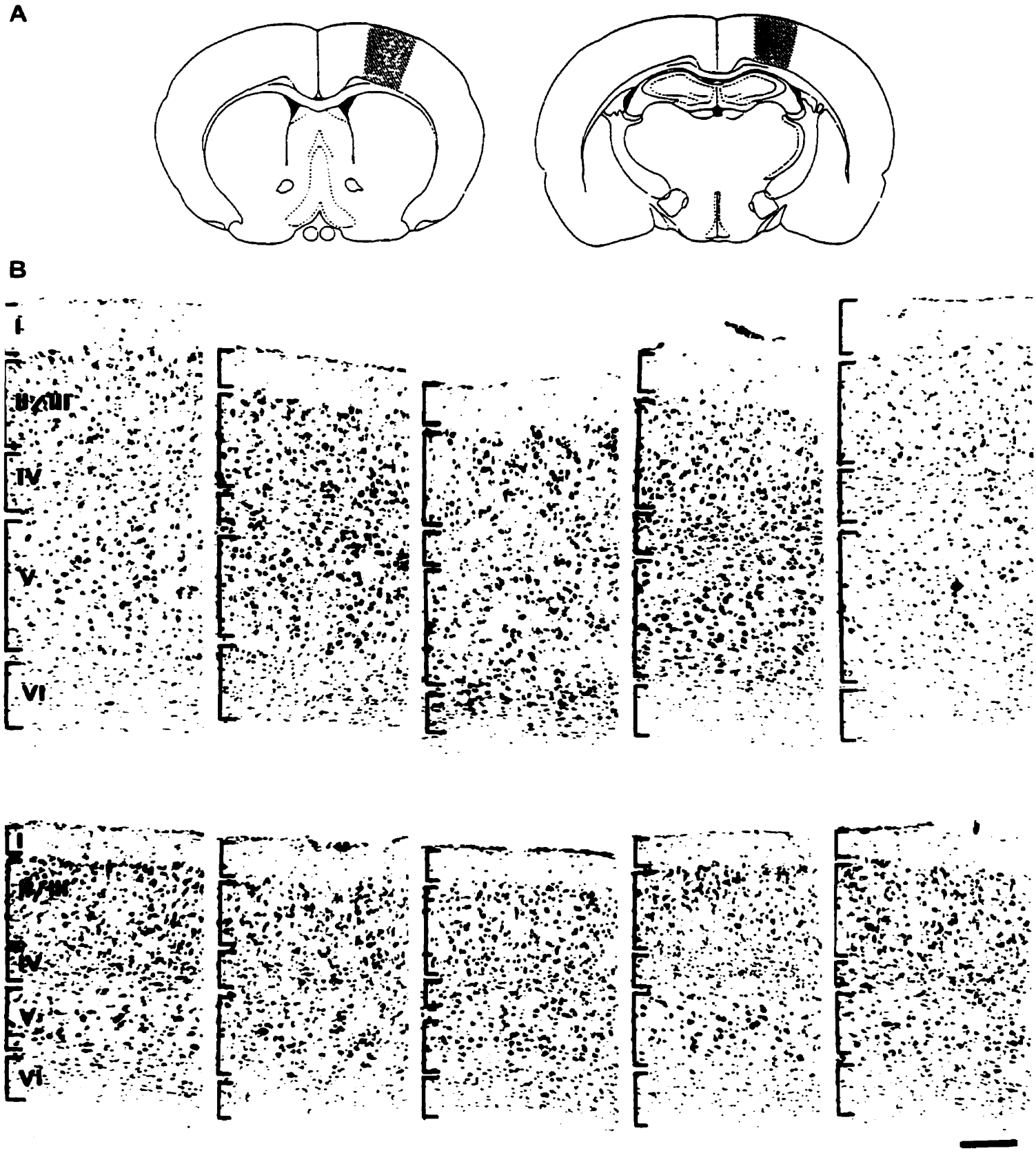
control
D498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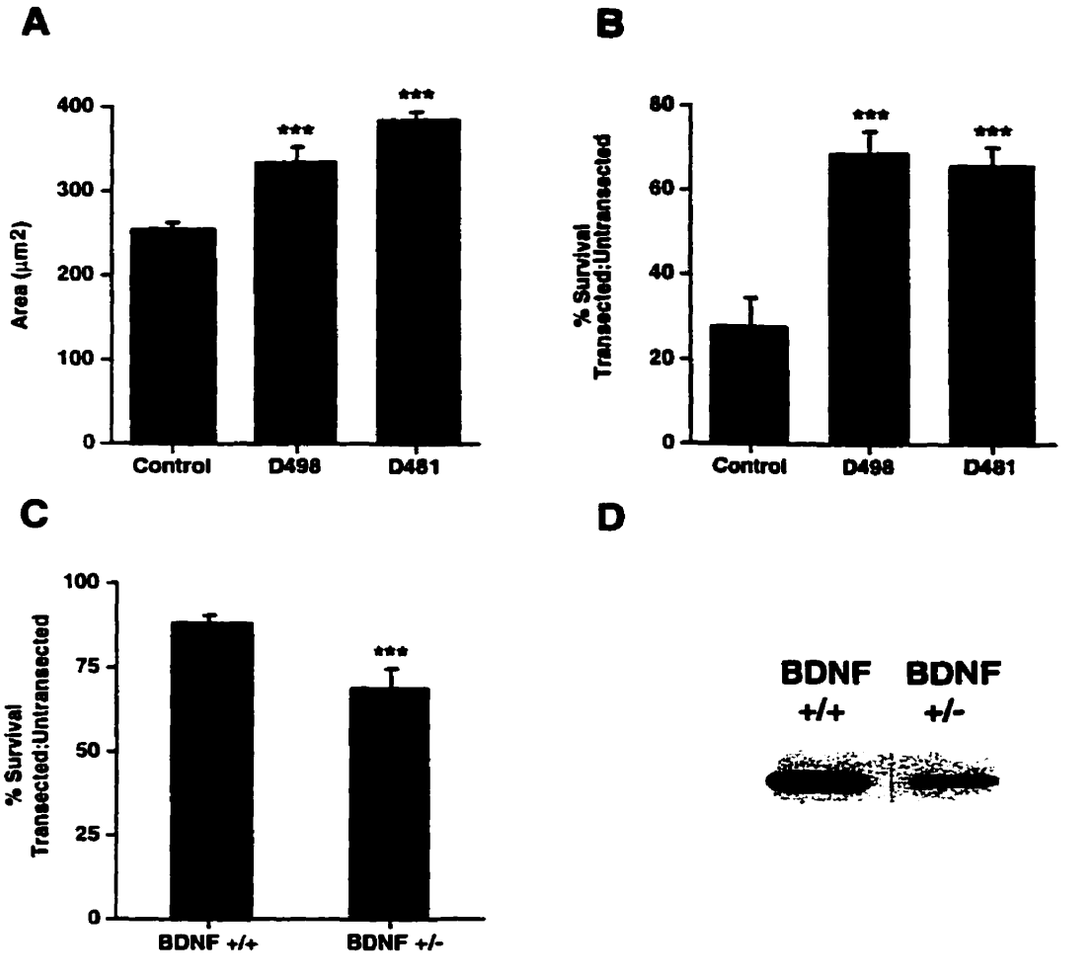
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% Survival Transected:Untransected
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***
Control
D498
D481
C
100
75
50
25
0
% Survival Transected:Untransected
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BDNF +/+
BDNF +/-
D
BDNF +/+
BDNF +/-

Table 1. Monoamines and metabolites in frontal (Fro) and entorhinal-piriform (EnPi) cortices, hippocampus (Hipp), and neostriatum (NS) of control and transgenic mice

Compound		Fro	EnPi	Hipp	NS
NA	Control	1.12 ± 0.16	0.90 ± 0.23	2.85 ± 0.91	4.77 ± 0.92
	Transgenic	1.72 ± 0.31	0.91 ± 0.18	3.63 ± 0.72	3.46 ± 0.94
DA	Control	0.16 ± 0.05	1.16 ± 0.40	n.d.	58.26 ± 3.13
	Transgenic	0.34 ± 0.06*	0.77 ± 0.16	n.d.	64.50 ± 9.29
DOPAC	Control	0.22 ± 0.05	0.75 ± 0.34	n.d.	19.09 ± 4.42
	Transgenic	0.30 ± 0.04	0.72 ± 0.22	n.d.	16.72 ± 2.51
HVA	Control	n.d.	n.d.	n.d.	5.13 ± 0.98
	Transgenic	n.d.	n.d.	n.d.	5.74 ± 1.02
3-MT	Control	n.d.	n.d.	n.d.	4.42 ± 0.46
	Transgenic	n.d.	n.d.	n.d.	5.46 ± 0.97
5-HT	Control	4.02 ± 1.31	5.25 ± 1.18	4.75 ± 1.18	3.25 ± 0.74
	Transgenic	4.71 ± 0.69	4.53 ± 1.65	7.03 ± 1.57	3.73 ± 0.74
5-HIAA	Control	2.90 ± 0.80	3.38 ± 0.80	5.70 ± 1.29	4.32 ± 0.74
	Transgenic	2.25 ± 0.33	3.04 ± 0.79	6.90 ± 1.18	4.77 ± 1.19

The values are the means ± SEM (n = 6) in nanograms per milligram of protein. The compounds are NA, noradrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylalanine; HVA, homovanillic acid; 3-MT, 3-methoxytyramine; 5-HT, serotonin; and 5-HIAA, 5-hydroxyindole-3-acetic acid. n.d., Not determined. Statistical comparisons were made by Student's t test.

*$p < 0.05$.

Table 2. Monoamines and metabolites in thalamus (Thal), hypothalamus (Hypo), brainstem (BS), and cerebellum (CB) of control and transgenic mice

Compound		Thal	Hypo	BS	CB
NA	Control	3.74 ± 0.80	13.03 ± 1.35	5.51 ± 1.08	1.38 ± 0.18
	Transgenic	3.56 ± 0.70	8.15 ± 1.76	4.69 ± 0.77	1.10 ± 0.45
DA	Control	2.30 ± 0.74	2.13 ± 0.45	1.05 ± 0.18	n.d.
	Transgenic	1.73 ± 0.61	0.99 ± 0.11*	0.96 ± 0.21	n.d.
DOPAC	Control	2.23 ± 0.54	6.72 ± 0.53	0.99 ± 0.16	n.d.
	Transgenic	2.89 ± 0.75	6.39 ± 1.64	0.86 ± 0.17	n.d.
HVA	Control	2.50 ± 0.75	2.82 ± 1.11	1.01 ± 0.18	n.d.
	Transgenic	3.40 ± 2.51	3.23 ± 0.72	1.23 ± 0.27	n.d.
5-HT	Control	5.08 ± 0.63	6.61 ± 1.33	7.97 ± 1.09	1.83 ± 0.43
	Transgenic	5.85 ± 1.03	7.76 ± 1.98	8.42 ± 0.97	1.89 ± 0.55
5-HIAA	Control	7.61 ± 0.42	8.12 ± 1.66	9.48 ± 1.20	1.59 ± 0.28
	Transgenic	6.99 ± 1.25	7.37 ± 1.41	8.64 ± 1.31	1.95 ± 0.27

The values are the means ± SEM (n = 6) in nanograms per milligram of protein. The compounds are NA, noradrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylalanine; HVA, homovanillic acid; 5-HT, serotonin; and 5-HIAA, 5-hydroxyindole-3-acetic acid. n.d., Not determined. Statistical comparisons were made by Student's t test.

*$p < 0.05$.

VIII. Figure Legends:

Figure 3.a: Comparison of β-galactosidase reporter gene expression in adult Tα1:nlacZ transgenic and wildtype mice– 2mm thick coronal slices stained with X-gal. (A) basal forebrain (arrowhead), (B) hippocampus (long arrow), amygdala (short arrow), neocortex (large arrowhead), hypothalamus (small arrowhead), (C) locus coeruleus (long arrow), cerebellar Purkinje cells (short arrow).

Figure 3.b: β-galactosidase reporter gene expression in adult Tα1:nlacZx DBH:BDNF transgenic mice – 2mm thick coronal brain slices stained with X-gal. (A) Wildtype cortex, (B-F) Cortex of DBH:BDNF transgenic mice. Arrows represent perturbations in X-gal staining in DBH:BDNF cortex. Scale bar = 0.15mm.

Figure 3.1. Colocalization of BDNF and dopamine-ß-hydroxylase in fibers and nerve terminals in the adult rat brain. Fluorescence photomicrographs of the rat brainstem double-labelled with antibodies to BDNF (A,D; visualized using a CY3-labelled secondary antibody), and DBH (B,E; visualized using a CY2-labelled secondary antibody). Panels (C,F) are photographic double-exposures; in these panels, yellow indicates regions of double-labelling. (A,B,C) The region of noradrenergic innervation rostral to the ventrolateral reticular region. Noradrenergic neuron cell bodies are DBH-positive (asterix), but are largely negative for BDNF, with some cells exhibiting faint BDNF-immunoreactivity (large arrow head). However, the processes of these noradrenergic neurons are largely positive for both BDNF and BDNF (arrows). (D,E,F) The ventrolateral peri-olivary region is immunoreactive for both the BDNF antibody (D) and DBH antibody (E) with punctate staining which may represent terminals or cut fibers. Space bars = 50 μm.

Figure 3.2. BDNF and DBH are colocalized in axons in the brainstem. Fluorescence photomicrographs of brainstem sections double-labelled for BDNF and DBH as described in Fig. 3.1. (A-C) BDNF and DBH are colocalized in fine processes in the rostral ventrolateral reticular region (arrows). (D-F) BDNF and DBH are colocalized in fibers (arrows) in the ventrolateral peri-olivary region. The occasional single double-labelled punctate structure can be visualized in this region (arrow head). Space bars = 25 μm.

Figure 3.3. (A,B) BDNF expression is increased in the locus coeruleus of DBH:BDNF mice. Fluorescence photomicrographs at the level of the locus coeruleus in control (A) and line D481 DBH:BDNF (B) animals that were immunostained with an antibody specific to BDNF. Note that in control locus coeruleus, the level of immunostaining is not above background, while in the DBH:BDNF locus coeruleus, there are many BDNF-immunoreactive cell bodies (arrows). **(C-F) The level and pattern of dopamine-ß-hydroxylase immunostaining is not altered in DBH:BDNF mice.** (C,D) Fluorescence micrographs at the level of the locus coeruleus in control (C) and line D481 DBH:BDNF (D) animals that were immunostained with an antibody specific to dopamine-ß-hydroxylase, which recognizes noradrenergic and adrenergic neurons and fibres. The pattern and level of DBH immunostaining are similar in both cases. (E,F) Darkfield micrographs of coronal sections of the hippocampus from control (E) and line D481 DBH:BDNF (F) brains that have been immunostained with anti-DBH, and visualized with peroxidase. Both photographs derive from a similar level of the hippocampus, and the dentate granule cell (DGc) and pyramidal cell (Py) layers are marked. Note that, in darkfield, the DBH-positive nerve fibers appear bright silver/yellow (arrows), and there there are no apparent differences in the pattern or density of DBH-immunoreactive fibers in transgenic versus control animals. Size bar = 100 µm.

Figure 3.4. (A,B) Endogenous levels of TrkB autophosphorylation are increased in the cortex of DBH:BDNF mice, while BDNF levels are unchanged. (Top panels) Cortical lysates from individual adult (A) and 1 week old (B) control (-) and line D498 DBH:BDNF (+) animals were immunoprecipitated with anti-panTrk, and then analyzed by Western blots with antiphosphotyrosine (4G10). To ensure that the observed increases reflected an increase in the activation of TrkB, the blots were reprobed with anti-TrkBout (TrkB). Image analysis quantitation was used to normalize the level of autophosphorylation of the 145 kD TrkB band relative to levels of TrkB protein. The normalized data (shown in the graphs, with an n of at least 3 individual animals in each case) were analyzed statistically for significance using a Students t-test. *$p<0.05$, **$p<0.005$. 145 and 190 indicate the size of the two TrkB isoforms. (Bottom panels) Western blot analysis of BDNF protein in the cortex of individual adult (A) and 1 week old (B) control (-) and line D498 DBH:BDNF (+) mice. The graphs represent image analysis quantification of the data obtained on the same Western blot of three individual control and transgenic animals, with the

optical density (O.D.) being arbitrary numbers. **(C) BDNF levels are increased in the brainstem of DBH:BDNF mice.** Western blot analysis of BDNF protein in the brainstem of individual 1 week old (top panels) and adult (bottom panels) control (-) versus line D498 DBH:BDNF (+) mice. The graphs represent image analysis quantification of the data obtained on the same Western blot of three individual control and transgenic animals, with the optical density (O.D.) being arbitrary numbers. Statistical analysis of these data demonstrate that BDNF is significantly increased in the brainstem of adult and neonatal DBH:BDNF animals. *$p<0.05$, **$p<0.005$.

Figure 3.5: Overexpression of BDNF in noradrenergic neurons leads to decreased neuronal numbers in the anterior neocortex. (A) Schematic drawing showing the rostral and caudal levels that were analyzed for morphology of the neocortex. (B) Photomicrographs of coronal Nissl-stained sections of the neocortex of control and DBH:BDNF transgenic mice. Right and leftmost panels are from control animals, whereas the three innermost panels are all from line D498 DBH:BDNF animals. The top set represents the rostral level of the neocortex indicated in (A) and the bottom set represents the caudal level. Brackets define approximate boundaries of the different cortical layers indicated in the leftmost panel. Size bar = 70 μm.

Figure 3.6: **(A) Facial motorneurons are hypertrophied in neonatal DBH:BDNF transgenic mice.** The average cross-sectional area of P12 facial motoneurons were determined by image analysis of cresyl violet-stained coronal sections of the appropriate brainstem level of DBH:BDNF mice of lines D498 and D481 versus their control littermates. In both lines of DBH:BDNF mice, facial motoneurons were significantly hypertrophied. ***$p<0.005$. **(B) Neonatal facial motoneurons are rescued from axotomy-induced death in DBH:BDNF transgenic animals.** The facial nerve of 5 day old control and DBH:BDNF animals were unilaterally transected and, seven days later, serial 16 μm sections were collected throughout the entirety of the facial nuclei. The number of facial motoneurons was then determined in the contralateral, control versus ipsilateral, transected facial nuclei by image analysis quantification of the number of facial motoneurons in every fifth section. The results of this analysis are presented as a ratio of the number of neurons in the transected versus untransected nuclei. The mean number of facial motoneurons within each group is presented within the text. Note that in control littermates, only 28% of facial motoneurons remain 1 week following a facial nerve transection, while in lines

D498 and D481, 69% and 66% of facial motoneurons remain, respectively. ***$p<0.005$, n = at least 3 animals in each group. **(C) Survival of transected facial motoneurons is reduced in adult BDNF+/- mice compared to their BDNF+/+ littermates.** Methods similar to those described in (B) were used with the exception that unilateral facial nerve transections were performed on 3 month old BDNF+/- versus BDNF+/+ animals. The results of this analysis are presented as a ratio of the number of neurons in the transected versus untransected nuclei. Note that in BDNF+/+ littermates, 87% of motoneurons remain 1 week following a facial nerve transection, while in BDNF+/- mice, only 71% of facial motoneurons remain. ***$p<0.005$, n = 3 animals in each group. **(D) BDNF levels are decreased in the BDNF+/- brain.** Western blots of equal amounts of protein from the cortex of BDNF+/- versus BDNF+/+ mice were probed with an antibody to BDNF. A similar decrease was observed in other regions of the BDNF+/- brain (data not shown).

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CHAPTER IV: THE P75 NEUROTROPHIN RECEPTOR MEDIATES NEURONAL APOPTOSIS AND IS ESSENTIAL FOR NATURALLY-OCCURRING SYMPATHETIC NEURON DEATH

I. Preface:

The "neurotrophic hypothesis" states that target-derived trophic factors mediate the survival of innervating neurons. However, recent studies have highlighted different mechanisms of delivery and multiple functions for certain neurotrophins, extending their scope of biological activity beyond that suggested by the neurotrophic hypothesis. Indeed, accumulating evidence suggesting that BDNF, as well as NT-3 and NT-4/5, can be anterogradely transported in the CNS have demonstrated a novel means by which neurotrophins regulate the development of the mammalian nervous system. One example of this, and the first examination of the functional relevance of anterogradely-derived BDNF on survival and the development of target neurons in the mammalian nervous system, has been discussed in chapter III.

Until recently, the effects of target-derived, or anterogradely-derived neurotrophins have been believed to be mediated through appropriate Trk receptors, while the effects of neurotrophin binding to the p75 neurotrophin receptor (NTR) have remained elusive. More recently, however, studies have demonstrated that neurotrophin binding to the p75NTR results in the activation of various signal transduction pathways. In certain cellular contexts, the result of p75 activation appears to be the induction of apoptosis, implicating the neurotrophins in the control of cell death in addition to survival. For this reason, we hypothesized that neurotrophins could play a novel role during the development of the nervous system and mediate apoptosis via the p75NTR. As the examination of this hypothesis threatened to be very complicated in the central nervous system, we turned to the sympathetic neurons of the peripheral nervous system as a model to test our hypothesis.

The aim of this chapter is to examine the role of target-derived, and autocrine/paracrine-derived BDNF on neurons that do not express the cognate BDNF receptor, TrkB, but do express the p75 neurotrophin receptor. The model system used to examine this question is the sympathetic neurons of the superior cervical ganglia (SCG).

BDNF is expressed in both the targets of these sympathetic neurons, and in sympathetic neurons themselves. Thus, this model system provides a biologically relevant system in which to examine the in vivo ***effect of BDNF on neurons that do not express the cognate TrkB receptor. In addition to its biological relevance, this homogeneous population of neurons provides an ideal system to examine the effects of p75 receptor activation and the mechanism by which these effects are mediated.***

II. Abstract:

To determine whether the p75 neurotrophin receptor (p75NTR) plays a role in naturally-occurring neuronal death, we examined neonatal sympathetic neurons that express both the TrkA tyrosine kinase receptor and p75NTR. When sympathetic neuron survival is maintained with low quantities of NGF or KCl, the neurotrophin BDNF, which does not activate Trk receptors on sympathetic neurons, causes neuronal apoptosis and increased phosphorylation of c-jun. Function-blocking antibody studies indicate that this apoptosis is due to BDNF-mediated activation of p75NTR. To determine the physiological relevance of these culture findings, we examined sympathetic neurons in BDNF-/- and p75NTR-/- mice. In BDNF-/- mice, sympathetic neuron number is increased relative to BDNF+/+ littermates, and in p75NTR-/- mice, the normal period of sympathetic neuron death does not occur, with neuronal attrition occurring later in life. This deficit in apoptosis is intrinsic to sympathetic neurons, since cultured p75NTR-/- neurons die more slowly than do their wild-type counterparts. Together, these data indicate that p75NTR can signal to mediate apoptosis, and that this mechanism is essential for naturally-occurring sympathetic neuron death.

III. Introduction:

The p75 neurotrophin receptor (p75NTR) (Johnson et al., 1986; Radeke et al., 1987) is the first-discovered member of a family of receptors, including fas and TNFR1, that have been shown to mediate cellular differentiation and apoptosis (reviewed in Chao, 1994). p75NTR can interact with all of the mammalian members of the neurotrophin family (reviewed in Levi-Montalcini, 1987; Barde, 1989; Snider, 1994), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), with approximately equivalent affinities (Rodriguez-Tebar et al., 1990; 1992). In contrast, the other family of neurotrophin receptors, the Trk tyrosine kinases (reviewed in Barbacid, 1994), display a specificity for binding individual neurotrophins; NGF and NT-3 for TrkA (Cordon-Cardo et al., 1991; Klein et al., 1991; Kaplan et al., 1991a; 1991b), BDNF and NT-4 for TrkB (Soppet et al., 1991), and NT-3 for TrkC (Lamballe et al., 1993; Tsoulfas et al., 1993). While much is known about the signaling and biological roles of the Trks in neurons *in vivo* and *in vitro*, the functional role of p75NTR in neurons has remained elusive.

p75NTR was originally reported to function as a positive regulator of TrkA activity in a number of neural cell lines (Barker et al., 1994; Ip et al., 1993; Benedetti et al., 1993; Verdi et al., 1994). The initial report of the p75NTR knockout mouse apparently supported this hypothesis, since these mice exhibited a loss of nociceptive and thermosensitive sensory neurons (Lee et al., 1992), phenotypes observed in a more severe form in the NGF (Crowley et al., 1994) and TrkA knockout mice (Smeyne et al., 1994). However, other defects have been observed in the p75NTR knockout mice that are more difficult to explain on the basis of p75NTR positively regulating TrkA, such as a selective loss of sympathetic innervation without an apparent loss of sympathetic neurons (Lee et al., 1992; 1994) and an increase in the number of basal forebrain cholinergic neurons (van der Zee et al., 1996) with a coincident increase in cholinergic innervation of the hippocampus (Yeo et al., 1997).

More recent evidence indicates that, in addition to modulating Trk function, p75NTR signals on its own. Specifically, in neural cell lines and glial cells, neurotrophin binding to p75NTR stimulates the generation of ceramide (Dobrowsky et al, 1994; 1995; Casaccia-Bonnefil et al., 1996), activation of NFkB and translocation of this protein to the nucleus (Carter et al., 1996), and enhancement of Jun kinase (JNK) activity (Casaccia-Bonnefil et al., 1996). Such signalling likely mediates the effects of NGF on Schwann cells, which do not express TrkA, and

which respond to NGF with migration (Anton et al., 1994). Similarly, NGF-mediated activation of p75NTR signalling in cultured oligodendrocytes leads to apoptosis (Casaccia-Bonnefil et al., 1996).

In spite of this progress in our understanding of p75NTR function in cell lines and glial cells, the functional role of this receptor in neurons remains elusive. However, a number of recent studies indicate that p75NTR may play a role in regulating neuronal survival. First, Rabizadeh et al. (1993) reported that p75NTR decreased survival of a neural tumor cell line in a ligand-independent fashion, while Barrett and Bartlett (1994) reported that functional ablation of p75NTR enhanced survival of cultured postnatal sensory neurons. However, neither of these reports determined whether p75NTR mediated these responses on its own, or by modulating Trk activity. Second, addition of NGF *in vivo* to neurons of the developing chick isthmo-optic nucleus, which express p75NTR but not TrkA, led to cell death by an undefined mechanism (von Bartheld et al., 1994). Third, addition of anti-NGF antibodies to the developing chick retina led to decreased death of cells that express p75NTR but not TrkA (Frade et al., 1996). In this latter study, unlike that with the chick isthmo-optic nucleus neurons, addition of excess NGF had no effect. Finally, expression of the intracellular domain of p75NTR as a transgene in mouse neurons resulted in apoptosis of selected populations of peripheral and central neurons, indicating the cell-death inducing potential of p75NTR (Majdan et al., 1997). However, while these studies implicate p75NTR in neuronal apoptosis, the mechanism whereby this occurs, and its physiological roles remain unclear.

We have addressed these issues by investigating the role of p75NTR in postnatal rat sympathetic neurons during the developmental period of naturally-occurring cell death. These neurons express relatively high levels of TrkA and p75NTR, low levels of functional TrkC (Belliveau et al., 1997), and have an absolute requirement for NGF for survival (Levi-Montalcini et al., 1960a; 1960b). We have recently demonstrated that TrkA activation is necessary for sympathetic neuron survival, and that NGF and NT-3 are both capable of eliciting TrkA activation on these neurons (Belliveau et al., 1997). However, surprisingly, while NT-3 is as effective as NGF in mediating neuritogenesis, it is 2- to 4-fold less effective at mediating neuronal survival at equivalent levels of TrkA activation. Since NT-3 is much less efficient at activating TrkA than NGF (Cordon-Cardo et al., 1991; Belliveau et al., 1997) and approximately equivalent at binding p75NTR (Rodriguez-Tebar et al., 1992), we hypothesized that this difference in survival was a

consequence of a balance between differential activation of TrkA versus p75NTR on sympathetic neurons. In studies reported here, we have tested this hypothesis. Our data indicate that p75NTR can signal to mediate neuronal apoptosis, and that this mechanism is essential for naturally-occurring sympathetic neuron cell death.

IV. Materials and Methods:

Primary Neuronal Cultures

Mass cultures of pure sympathetic neurons from the superior cervical ganglion (SCG) of postnatal day 1 rats were prepared as previously described (Ma et al., 1992). Neurons were plated on rat tail collagen coated tissue culture dishes; 4 well plates (Falcon) for morphological measurements, 6 well plates for biochemistry, and 48 well plates for MTT assays. Low density SCG cultures were used for all of the survival assays; for the MTT assays, neurons were used at a density of 1,500-2,000 neurons per well of a 48 well plate. Similar neuronal densities were used for the TUNEL assays, but the neurons were plated on poly-d-lysine and laminin. For biochemistry, approximately 100,000 neurons were plated per well of a 6 well dish. For all experiments, neurons were initially cultured for 5 days in the presence of 10 ng/ml or 50 ng/ml NGF. At the end of this 5 day selection, neurons were washed several times in neurotrophin-free media prior to addition of the new neurotrophin- or KCL-containing media.

NGF for these experiments was purified from mouse salivary glands (CedarLane, Hornby, ON). Purified recombinant NT4 was obtained from Genentech (South San Francisco, CA). Two sources of recombinant human BDNF (Amgen,Thousand Oaks, CA; Preprotech, Rocky Hill, NJ) were used for these experiments to ensure that the results were not due to unsuspected toxicity associated with one of the sources. Both preparations of BDNF caused TrkB autophosphorylation on TrkB-expressing NIH-3T3 cells (D. Kaplan, unpublished data). Moreover, these sources of BDNF have been used on primary cultures of cortical progenitor cells, cortical neurons, and hippocampal neurons, with no evidence of cytotoxicity (data not shown). The p75NTR function-blocking antibody REX (Weskamp et al., 1991) (the kind gift of Dr. L. Reichardt, U.C.S.F.) is directed against the extracellular domain of the receptor, and was used as an antiserum at a dilution of 1:150.

Mouse sympathetic neurons were cultured by a modification of the method used for rat neurons. Specifically, neurons were dissected and triturated as for rat neurons (Ma et al., 1992), except that neurons were dissociated in the presence of media instead of saline solution. Neurons were then plated on collagen-coated culture dishes in Ultraculture media (BioWhittaker) containing 2mM glutamine, 100 units Penicillin/ml, 100 μg streptomycin/ml, 3% fetal bovine serum (Life Technologies), and 50 ng/ml NGF. Three days following plating, the neurons were fed with the same media containing 0.5% cytosine arabinoside.

TUNEL and Survival Assays

TUNEL assays were performed as previously described (Slack et al., 1996). For survival assays, analysis was performed 48 hours later using non-radioactive cell proliferation (MTT) assays (Celltitre 96, Promega). Twenty microliters of the MTT reagent as added to each well and left for 90 min. followed by the addition of 200 µl of solubilization solution to lyse the cells. Each condition was repeated in quadruplicate. In each assay, baseline (0% survival) was considered to be 0 ng/ml NGF, and 10 ng/ml NGF was considered to be 100% survival. All other conditions were related to these values. For the REX experiment, neurons were washed with neurotrophin-free media for 3 hours, and were then preincubated in media + KCl + REX for 2 hours, at which point the BDNF was added for 48 hours.

Survival assays for p75NTR-/- versus p75NTR+/+ neurons were performed by counting randomly-selected fields of cultured neurons. Specifically, after 5 days in culture, neurons were washed with four changes of neurotrophin-free media for a total of 2.5 hours, and then maintained in media with no added neurotrophins. The number of phase-bright neurons with neurites in randomly-selected, 5.3 mm^2 fields was counted immediately following the switch to NGF-free media, and was recounted in the same fields at 24 hour intervals for 5 days. In each individual experiment, each condition was repeated in triplicate. As controls for the p75NTR-/- neurons, neurons were cultured from mice of either C129 or CD1 backgrounds. Results for neurons from either background were similar, and were therefore combined. In three of the five experiments analyzed, p75NTR-/- and p75NTR+/+ neurons were cultured at the same time in the same 48 well plates to eliminate potential variability.

Immunoprecipitations and Western Blot Analysis

For Trk assays, sympathetic neurons were lysed in Tris buffered saline (TBS) lysis buffer (Knusel et al., 1994) containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 0.2 µg/ml leupeptin, 5 mM Phenanthroline, and 1.5 mM sodium vanadate. 1 ml of cold TBS was added to one or two wells of a 6 well dish, and cells were collected, resuspended in 100 µl lysis buffer, and rocked for 20 min at 4° C. Each sample was vortexed for 10 sec, and cleared by centrifugation. The lysates were normalized for protein concentration using a BCA Protein Assay Reagent (Pierce, Rockford,

III). Total Trk protein was immunoprecipitated using 3 μl of anti-panTrk 203 (the kind gift of Dr. David Kaplan), and TrkA using 2 μl of anti-TrkA RTA (the kind gift of Dr. L. Reichardt). The immunoprecipitates were collected with Protein A-sepharose (Pharmacia) for 1.5 hours at 4° C followed by centrifugation.

For Western blot analysis, precipitates were washed three times with cold lysis buffer, boiled in sample buffer (2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.05% bromophenol blue) for 5 min, and electrophoresed on 8.0% SDS-polyacrylamide minigels. After electrophoresis, proteins were transferred to 0.2 μm nitrocellulose for 1.0 hours at 0.5 Amps, and the membrane washed 2 x 10 min in TBS. For all antibodies except anti-phosphotyrosine, for which membranes were blocked in 2% BSA (Sigma Chemical Co), membranes were blocked in 5% non-fat dry milk, in TBS for 2.5 hours. Membranes were then washed 2 x 10 min in TBS, and the primary antibodies were used overnight at 4°C at dilutions of 1:5000 for antiphosphotyrosine 4G10 (UBI), and 1:1000 or 1:2000 for anti-panTrk 203. Secondary antibodies were incubated for 1.5 hours at room temperature, and were used at dilutions of 1:10,000 or 1:20,000 for a goat-anti-mouse horse radish peroxidase (HRP) antibody (Boehringer Mannheim Biochemicals; used for anti-phosphotyrosine), 1:10,000 for a goat anti-rabbit HRP antibody (Boehringer Mannheim Biochemicals; used for anti-TrkA) and 1:2000 for Protein A-HRP (Sigma Chemical Co; used for anti-panTrk). Detection was carried out using enhanced chemiluminescence (Amersham) and XAR X-ray film (Kodak). Results were quantitated by image analysis.

For biochemical analysis of c-jun, the collected cells were incubated 20 minutes at 4°C in lysis buffer plus 0.5% SDS. After protein determination, 50 μg of protein was boiled in sample buffer, and separated on a 10% acrylamide gel, transferred, and Western blotted with antibodies specific to c-jun at a 1:1000 dilution, using the protocols described above. For the c-jun experiments, two different antibodies were used, anti-c-jun J3192 (Transduction Labs), and anti-c-jun SC-822 (Santa Cruz). Both antibodies produced similar results on Western blots.

Analysis of BDNF -/- and p75NTR -/- Mice

Mice heterozygous for a targetted mutation in the BDNF gene (Ernfors et al., 1994) and homozygous for a targetted mutation in the p75NTR gene (Lee et al., 1992) were obtained from Jackson Labs (Bar Harbor). The BDNF -/- mice were maintained in a C129/BalbC background. The p75NTR-/- mice were originally generated in a C129/BalbC background (Lee et al., 1992),

were crossed back into a C129 background prior to purchase from Jackson Labs, and were then maintained as homozygotes. Progeny from BDNF heterozygote crosses were screened for the mutant allele(s) using PCR. To amplify the wildtype allele, two PCR oligonucleotide primers were used (5'-ATGAAAGAAGTAAACGTCCAC-3' and 5'-CCAGCAGAAAGAGTAGAGGAG-3') to generate a 275 nucleotide product. To amplify the mutant allele, two oligonucleotide primers specific to this allele were used (5'-GGAACTTCCTGACTAGGGG-3' and 5'-ATGAAAGAAGTAAACGTCCAC-3') to generate a 340 nucleotide product.

For morphometric analysis, the SCG were removed and immersion-fixed in 4% paraformaldehyde in phosphate buffer (PB) for 1 hour to overnight at 4° C. Ganglia were cryoprotected in graded sucrose solutions, 7 μm thick sections were serially cut on a cryostat, and every section was collected and thaw-mounted onto chrom-alum subbed slides. Slides were stained with cresyl violet and morphometric analyses were performed using a computer-based image analysis system (Biocom, France). Neuronal sizes were determined by randomly measuring the cross-sectional area of neurons containing a distinct nucleolus. Neuronal numbers were determined by counting all neuronal profiles with nucleoli on every third section for the BDNF -/- mice, and every fourth section for the p75NTR -/- mice, as per Coggeshall (1984). This sampling frequency (every 21 and 28 μm, respectively) ensures that neurons are not double-counted, since the average neuronal diameter does not exceed 21 μm in any of the groups examined (see Results section). This method does not correct for split nucleoli. As a second approach, neuronal numbers were estimated using measurements of total SCG volume and neuronal density. To determine ganglion volume, the combined cross-sectional area of every third (BDNF-/-) or every fourth (p75NTR-/-) serial section was measured. This cross-sectional area was normalized for total number of sections, and multiplied by 7 μm (the section thickness) to obtain the total SCG volume. To measure mean neuronal density, the number of neurons in a randomly-applied sampling window of 175 μm x 175 μm was counted. For the BDNF-/- and BDNF+/+ animals, the sampling window was 182 μm x 182 μm, and the mean neuronal densities were then normalized to a 175 μm x 175 μm sampling window (a normalization factor of 1.08). To estimate neuronal number from these two parameters, we calculated SCG volume x (mean neuron number/214,375 μm^3), where 214,375 μm^3 is the sampling window volume (175 μm x 175 μm x 7 μm). This calculation was performed either i) on an individual ganglion basis or ii) using

the mean volume and the mean pooled neuronal density obtained from at least 3 individual animals. Since the numbers obtained were similar in both cases, only the latter calculation is presented in the results. Statistical results were expressed as the mean ± the standard error of the mean and were tested for significance by a one-tailed Student's t-test.

V. Results:

Differential Neurotrophin Binding to TrkA versus p75NTR Correlates with Sympathetic Neuron Survival versus Death

To determine whether differences in neurotrophin binding to TrkA versus p75NTR regulate neuronal survival, as suggested by our previous results (Belliveau et al., 1997), we compared NGF and NT-4, both of which bind only TrkA and p75NTR on sympathetic neurons. For these studies, postnatal day 1 sympathetic neurons were first selected for 5 days in 10 ng/ml NGF, washed free of NGF, exposed to varying concentrations of NGF and NT-4, and analyzed for TrkA activation relative to neuronal survival. For the biochemical measurements, neurons were exposed to these two neurotrophins for ten minutes, cell lysates were immunoprecipitated with anti-TrkA (RTA; Clary et al., 1994), and the precipitated TrkA was analyzed for autophosphorylation on Western blots using anti-phosphotyrosine. This biochemical analysis revealed that NT-4 was similar to NT-3 in its ability to activate TrkA on sympathetic neurons (Fig. 4.1A; data not shown). NT-4 was, however, approximately 10 to 20-fold less efficient at activating TrkA than NGF (Fig. 4.1A), with 2.5 ng/ml NGF being equivalent to approximately 50 ng/ml NT-4.

To compare the survival promoting activity of these two TrkA/p75 ligands, we then cultured the NGF-dependent neurons in 0 to 10 ng/ml NGF or 10 to 100 ng/ml NT-4 for 2 days (Fig. 4.2A). MTT assays revealed that 2.5 and 5.0 ng/ml NGF supported approximately 55% and 85% sympathetic neuron survival, respectively. In contrast, 50 and 100 ng/ml NT-4, which lead to approximately equal levels of TrkA activation (Fig. 4.1A) supported only approximately 10% and 25% neuronal survival, respectively. Thus, when compared to NGF, NT-4 was approximately 40-fold less potent at mediating neuronal survival, and approximately 5-fold less efficient when normalized for similar levels of TrkA autophosphorylation (Figs. 4.1A, 4.2A). Since NT-4 binds far less well to TrkA than NGF, and more efficiently to p75NTR (Rodriguez-Tebar et al., 1992), we hypothesized that, at low levels of TrkA activity, coincident activation of p75NTR might "override" the TrkA survival signal.

To test this hypothesis, we cultured sympathetic neurons in limiting amounts of NGF with and without the presence of a p75NTR ligand, BDNF. We chose the neurotrophin BDNF, which binds to p75NTR (Rodriguez-Tebar et al., 1990), since BDNF does not bind to the two Trk receptors present on neonatal sympathetic neurons, TrkA and TrkC (Lamballe et al., 1991). We

first confirmed biochemically that BDNF is selective for p75NTR on these neurons, by examining short- and long-term total Trk autophosphorylation. For the short-term experiments, neonatal sympathetic neurons were grown for 5 days in 10 ng/ml NGF, and were then exposed to BDNF for 10 minutes. Cellular lysates were immunoprecipitated with an antibody that recognizes all members of the Trk receptor family (anti-panTrk 203; Hempstead et al., 1992), and the precipitated Trk examined for BDNF-induced tyrosine autophosphorylation by probing Western blots with anti-phosphotyrosine. These studies demonstrated that concentrations of BDNF ranging from 30 to 100 ng/ml (Fig. 4.1B) did not lead to short-term activation of Trk receptors on sympathetic neurons.

For the long-term experiments, sympathetic neurons were selected for 5 days in 10 ng/ml NGF, and then were switched for two days either to 10 ng/ml NGF plus 100 ng/ml BDNF or to 10 ng/ml NGF plus 100 ng/ml NGF; 10 ng/ml NGF was required in these experiments to support neuronal survival. Two days later, the Trk proteins were analyzed for BDNF or NGF-induced tyrosine phosphorylation (Fig. 4.1C). These studies revealed that, as previously reported (Belliveau et al., 1997), 110 ng/ml NGF led to higher levels of long-term Trk autophosphorylation than did 10 ng/ml NGF. However, Trk autophosphorylation in 10 ng/ml NGF plus 100 ng/ml BDNF was unchanged relative to 10 ng/ml NGF alone. Thus, as predicted, BDNF does not activate either TrkA or TrkC on sympathetic neurons, and represents a p75NTR-selective ligand for these experiments.

To test the hypothesis that at low levels of TrkA activity, coincident activation of p75NTR could "override" the TrkA survival signal, we cultured sympathetic neurons in 50 ng/ml NGF for 5 days, and then switched them into 2.5 ng/ml NGF for two days, with or without BDNF. 50 ng/ml NGF was used for the initial selection since we have previously demonstrated (Belliveau et al., 1997) that p75NTR levels are significantly higher in sympathetic neurons cultured in 50 ng/ml NGF versus 10 ng/ml NGF, whereas TrkA levels remain constant. 2.5 ng/ml NGF was chosen for the survival assay since this concentration of NGF is limiting for TrkA activation and neuronal survival under our assay conditions (Fig. 4.2A). MTT assays demonstrated that 2.5 ng/ml NGF alone mediated survival of 50-55% of sympathetic neurons, and that the addition of 100 or 200 ng/ml BDNF reduced this survival by 43% and 58%, respectively (Fig. 4.2B). In contrast, BDNF had less of an effect when TrkA activation was increased; addition of 100 ng/ml BDNF in the presence of 5 ng/ml NGF reduced survival by 25% (Fig. 4.2C), and addition of BDNF to 10 ng/ml

NGF (which supports 100% survival) had no effect (Fig. 4.2C). Thus, BDNF reduces TrkA-mediated neuronal survival, but only when the survival signal is suboptimal.

BDNF-Mediated Activation of p75NTR Causes Sympathetic Neuron Apoptosis in the Absence of TrkA Activation

One explanation for these data is that BDNF causes activation of p75NTR, and that a p75NTR-mediated apoptotic signalling cascade can override survival signals mediated by low levels of TrkA activation. An alternative explanation is that BDNF competes with low levels of NGF for binding to p75NTR, thereby affecting NGF binding to TrkA (Barker et al., 1994). To differentiate these two possibilities, we performed survival assays using KCl instead of NGF to maintain sympathetic neuron survival. We first confirmed, as previously reported (Franklin et al., 1995), that KCl maintained sympathetic neuron survival without activating Trk by measuring Trk autophosphorylation in neurons exposed to KCl for 10 minutes or 24 hours in the absence of neurotrophin (Fig. 4.1D). Specifically, after an initial selection for 5 days in NGF, neurons were washed free of NGF, and then were exposed to 25 or 50 mM KCl for 10 minutes or 24 hours. Cellular lysates were immunoprecipitated with anti-panTrk, and the precipitates were analyzed by Western blot analysis with anti-phosphotyrosine (Fig. 4.1D). These experiments confirmed that, as previously reported (Franklin et al., 1995), KCl did not lead to an increase in autophosphorylation of Trk either in the short- or long-term.

We then performed survival assays with KCl as we had done for NGF and BDNF; sympathetic neurons selected in 50 ng/ml NGF for 5 days were switched to KCl with or without BDNF for two days. These assays were performed under two conditions. In one case, we used 25 mM KCl, which mediates maximal sympathetic neuron survival (130% relative to 10 ng/ml NGF), and in the second, we used 12.5 mM KCl, which is suboptimal, mediating approximately 80-85% survival (Fig. 4.2D). MTT assays revealed that the addition of 100 ng/ml BDNF to 12.5 or 25 mM KCl decreased neuronal survival significantly in both cases (Fig. 4.2D). To determine the concentration range of this BDNF-mediated killing, we performed similar experiments using 12.5 mM KCl with the addition of 30 to 200 ng/ml BDNF. MTT assays revealed a concentration-dependent decrease in neuronal survival commencing at 30 ng/ml BDNF (Fig. 4.2E). Thus, BDNF was sufficient to "override" survival signals mediated by KCl in a concentration-dependent fashion.

To demonstrate that the lack of neuronal survival detected using MTT assays corresponded to neuronal apoptosis, we monitored the neurons morphologically, and by TUNEL assays. Sympathetic neurons were first selected in 50 ng/ml NGF, were washed free of neurotrophin, and then were switched to 12.5 or 25 mM KCl with or without BDNF. As controls, neurons were switched to 10 ng/ml NGF or were maintained without NGF. Eighteen hours following the switch, neurons maintained in 10 ng/ml NGF or 12.5 mM KCl exhibited no TUNEL-positive nuclei, while neurons withdrawn from NGF or maintained in 12.5 mM KCl + 100 ng/ml BDNF showed many TUNEL-positive nuclei (Fig. 4.3A-D). Similar results were obtained with neurons maintained in 25 mM KCl (data not shown). By 48 hours, morphological examination revealed that all neurons maintained in 10 ng/ml NGF were alive (Fig. 4.3E), while in 12.5 mM KCl, many neurons were alive, but some were also showing morphological signs of cell death (Fig. 4.3F), consistent with the MTT survival assays (Fig. 4.2D). In contrast, no neurons were apparently alive after 48 hours of culture in 0 NGF or in 12.5 mM KCl plus 100 ng/ml BDNF (Fig. 4.3G,H).

We also performed similar TUNEL assays on sympathetic neurons maintained in suboptimal concentrations of NGF and coincidently exposed to BDNF. Specifically, sympathetic neurons were cultured for 5 days in 50 ng/ml NGF, and switched to 5 ng/ml NGF plus or minus 100 ng/ml BDNF. TUNEL-labelling was performed 24 hours following this switch; this analysis demonstrated a statistically-significant increase in the number of TUNEL-labelled cells in the cultures exposed to BDNF ($p<0.03$; data not shown).

Together, these data suggest that BDNF leads to p75NTR activation, and that this activation causes neuronal apoptosis. To directly test this hypothesis, we used the function-blocking p75NTR antibody, REX (Weskamp et al., 1991; Cassaccia-Bonnefil et al., 1996). Specifically, neurons were cultured in 50 ng/ml NGF for 5 days, and were then switched to 25 mM KCl with or without the addition of 100 ng/ml BDNF and/or REX. Two days later, MTT assays were used to monitor neuronal survival. These experiments (Fig. 4.4A) demonstrated that REX was able to inhibit the BDNF-mediated apoptosis of sympathetic neurons, indicating that p75NTR mediates most, if not all, of the apoptotic effects of BDNF.

BDNF Leads to Increased Phosphorylation of c-Jun

Previous work has demonstrated that increased expression and phosphorylation of c-jun is necessary for sympathetic neuron apoptosis caused by NGF withdrawal (Ham et al., 1995; Estus et

al., 1994). Moreover, p75NTR has been demonstrated to activate the kinases that phosphorylate c-jun, the jun kinase (JNK) family, in cultured oligodendrocytes (Casaccia-Bonnefil et al., 1996). We therefore examined c-jun in sympathetic neurons induced to undergo apoptosis by p75NTR activation. To perform these experiments, neurons were selected for 5 days in 50 ng/ml NGF, and then were switched to 12.5 or 25 mM KCl plus or minus BDNF. 18 hours following this switch, cellular lysates were analyzed by probing Western blots with an antibody to c-jun. As controls, neurons were maintained in 10 ng/ml NGF, or were withdrawn from NGF.

These experiments demonstrated that, as previously reported (Ham et al., 1995), NGF withdrawal led to a shift in c-jun to a larger size, indicative of phosphorylation (Fig. 4.1E). A similar shift in c-jun size was detected in neurons cultured in either 12.5 (Fig. 4.1E) or 25 (Fig. 4.1F) mM KCl plus 100 or 200 ng/ml BDNF, relative to KCl alone. Thus, BDNF induces c-jun phosphorylation in sympathetic neurons coincident with neuronal apoptosis, suggesting that one of the signal transduction pathways leading to neuronal apoptosis in response to NGF withdrawal is similar to that induced by p75NTR activation.

Sympathetic Neuron Number is Increased and Size Decreased in BDNF -/- Mice

These *in vitro* experiments indicate that, when neonatal sympathetic neurons are exposed to a suboptimal survival signal, activation of p75NTR by BDNF leads to neuronal apoptosis. If a similar cellular mechanism occurred *in vivo* during the period of naturally-occurring cell death, then one would predict that in the BDNF -/- mice (Ernfors et al., 1994; Jones et al., 1994), there would be an increase in the number of sympathetic neurons. To test this prediction, we analyzed the number of sympathetic neurons of the superior cervical ganglion at postnatal day 15, immediately following the period of target competition and cell death (Hendry and Campbell, 1976; Wright et al., 1983). Specifically, SCG from BDNF +/+ (Fig. 4.5A) and -/- (Fig. 4.5B) littermates were serially sectioned at 7 μm, and the number of neurons with an identifiable nucleolus were counted on every third section. This analysis demonstrated a statistically-significant increase of 36% in the relative number of sympathetic neurons in BDNF -/- mice relative to their BDNF +/+ littermates (BDNF +/+: 15,690±617, BDNF -/-: 21,318±1627, $p = 0.009$; $n = 6$ animals each) (Fig. 4.5B, Table 4.1).

To confirm the increase in number of sympathetic neurons in the absence of BDNF, we estimated neuronal number using a second approach. Specifically, we first determined the volume

of the SCG by measuring the total cross-sectional area of every third section throughout the entirety of individual ganglia, and then normalizing for sampling frequency and section thickness (Table 1). We then determined the mean number of neurons in a randomly-selected sampling volume of 214,375 $\square m^3$ from 3 animals of each genotype (Table 4.1). This latter analysis demonstrated that neuronal density was significantly higher in the SCG of BDNF-/- animals versus their BDNF +/+ littermates ($p<0.0001$; Table 4.1, Fig. 4.5A,B). The volume and density measurements were then used to estimate the total number of neurons within the SCG as 14,358±437 in the BDNF+/+ animals and 17,150±328 in the BDNF-/- animals (Table 4.1), a statistically-significant increase ($p = 0.003$). These numbers are statistically similar to those derived by direct counting ($p>0.05$), and support the conclusion that there are increased numbers of sympathetic neurons in the BDNF-/- animals.

Finally, we determined the size of sympathetic neurons from the BDNF+/+ and BDNF-/- animals. This morphological analysis revealed a statistically-significant decrease in sympathetic neuron cross-sectional area (Fig. 4.5A,B; 4.6A) of approximately 1.6-fold in the absence of BDNF (BDNF+/+: 222.24±6.50 μm^2, BDNF-/-: 142.49±9.21 μm^2, $p = 0.002$; $n = 3$ animals each), a finding that is consistent with increased numbers of neurons competing for limiting amounts of NGF. Thus, these findings indicate that BDNF *in vivo* may well mediate sympathetic neuron apoptosis, as we have documented *in vitro*.

Sympathetic Neuron Death is Developmentally-Delayed in p75NTR-/- Mice

If, as our results from the BDNF -/- mice suggest, ligand-mediated activation of p75NTR is essential for naturally-occurring sympathetic neuron death, then this process should also be perturbed in the absence of p75NTR. To test this prediction, we examined sympathetic neuron numbers in the p75NTR-/- mice during the normal period of developmental death, which occurs during the first few postnatal weeks in rats (Wright et al., 1983; Hendry and Campbell, 1976; Hendry, 1977). Since the p75NTR-/- mice analyzed in this study have been bred into a C129 background and are maintained as homozygotes, we made two types of comparisons; an internal comparison from birth to adulthood in the p75NTR-/- mice, and a comparison of p75NTR-/- mice with control animals of the same C129 background.

For this analysis, we collected serial 7 μm thick sections of the SCG, and counted the total number of neurons containing a nucleolus in every fourth section (Fig. 4.5C-E). Analysis of

control C129 mice revealed that, as previously reported in rats, naturally-occurring sympathetic neuron death in the SCG occurred between birth and P23. Sympathetic neuron number at P1 was 25,328±927, at P4 was 23,196±568, at P23 was 13,415±1682 (a number similar to that observed in the BDNF +/+ animals at P15 [Table 4.1]), and by adulthood was 14,667±799 (Figs. 4.6D,F, Table 4.1). These counts demonstrated a 42% decrease in number of SCG neurons between birth and P23, after which numbers remained constant ($p = .0003$, P4 versus P23; $p>0.05$, P23 versus adult; n = 5 P1s, 3 P4s, 5 P23s, and 5 adults). In contrast, in p75NTR-/- mice, the neuron number at P4 was 32,468±2362, at P23 was 40,275±1368, and by adulthood was 22,254±1404 (Figs. 6E,F, Table 4.1). Thus, in the absence of p75NTR, sympathetic neuron number did not decrease between birth and P23, as seen in controls, but instead increased by 24% between birth and P23, and then decreased 45% between P23 and adulthood ($p = 0.02$, P4 versus P23; $p = 0.006$, P23 versus adult; n = 4 P4s, 5 P23s, and 5 adults). This developmental profile of sympathetic neuron number in the p75NTR-/- mice is very similar to that observed when neonatal rats are given NGF systemically from P6 to P21 (Hendry and Campbell, 1976); naturally-occurring sympathetic neuron death is eliminated, and the number of sympathetic neurons in the SCG increases 20% between P6 and P21, an increase attributed to the rescue of sympathetic neurons that are born postnatally (Hendry, 1977).

To obtain an independent estimate of sympathetic neuron number over this developmental interval, we measured SCG volumes and neuronal density as we had for the BDNF-/- mice (Table 4.1). Analysis of control C129 mice revealed that SCG volume increased from birth to P23 and then remained relatively constant into adulthood (Table 4.1), as previously observed in rats (Hendry and Campbell, 1976). In contrast, in the p75NTR -/- mice, SCG volume increased from P4 to P23, but then decreased between P23 and adulthood. Moreover, p75NTR-/- SCG volume at P23 was 1.7-fold larger than SCG volume in the corresponding C129 animals ($p<0.05$). Similar differences were noted in neuronal density. In the control C129 animals, neuronal density decreased from P4 to P23 ($p<0.05$, Table 4.1), and then remained constant into adulthood. In contrast, in the p75 NTR -/- SCG, neuronal density was not significantly altered between P4 and P23 ($p = 0.27$), but decreased between P23 and adulthood ($p<0.05$, Table 4.1).

The neuronal numbers estimated from the measurements of ganglia volume and neuronal density confirmed the conclusions obtained from counting neuronal profiles (Table 4.1). In the control C129 SCG, estimated neuron number decreased between P4 and P23 ($p = 0.013$), and then

remained constant, while in the p75 NTR -/- SCG, estimated neuron number increased from P4 to P23, and then decreased from P23 to adulthood ($p = 0.030$, Table 4.1). Moreover, the absolute numbers obtained using this estimation were statistically similar to those obtained by direct counting (in all cases, $p>0.05$, Table 4.1). Thus, in the absence of p75NTR, normal developmental death of sympathetic neurons does not occur, but is instead replaced by a delayed period of neuronal loss between P23 and adulthood.

Since rat sympathetic neurons increase in size over this developmental interval, we also determined neuronal size. As reported in rats (Hendry and Campbell, 1976), neuron size increased gradually from birth to adulthood in control C129 animals (Figs. 4.5, 4.6B); the average cross-sectional area at P1 to P4 was 114.86±6.24 μm^2, at P23 was 240.73±16.43 μm^2, and in adults was 317.06±26.60 μm^2 (Fig. 4.6C). These measurements correspond to diameters of 12.1 μm, 17.5 μm, and 20.1 μm respectively, numbers very similar to those previously reported in rats (Hendry and Campbell, 1976). Sympathetic neurons from p75NTR-/- mice were similar in size to controls at P4 (cross-sectional area, 121.46±6.44 μm^2; diameter, 12.4 μm; $p = .25$, $n = 3$ animals each) and in adulthood (cross-sectional area, 318.72±5.17 μm^2; diameter, 20.1 μm; $p = .47$, $n = 3$ animals each) (Figs. 4.5, 4.6B). In contrast, at P23, p75NTR-/- sympathetic neurons were significantly smaller than their control counterparts (cross-sectional area, 167.47±11.15 μm^2; diameter, 14.6 μm; $p = .011$, $n = 3$) (Figs. 4.5, 4.6B), consistent with increased neurons in the p75NTR-/- mice competing for limiting amounts of trophic support.

Cultured p75NTR-/- Sympathetic Neurons Die More Slowly Following NGF Withdrawal

The delay in sympathetic neuron death in the p75NTR-/- mice could be due to alterations either intrinsic or extrinsic to the neurons themselves. To differentiate between these two possibilities, we cultured p75NTR-/- sympathetic neurons and examined their rate of cell death following NGF withdrawal. Specifically, sympathetic neurons of the postnatal day 1 mouse SCG were cultured for 4 or 5 days in 50 ng/ml NGF, were washed to remove the NGF, and were then maintained in 0 or 10 ng/ml NGF-containing media for 5 days. Fields of neurons were counted immediately upon NGF withdrawal, and at 24 hour intervals subsequent to the withdrawal.

This analysis (Fig. 4.4B) revealed p75NTR-/- sympathetic neurons died significantly more slowly than their wildtype counterparts in the absence of NGF, indicating that the delay in neuronal loss observed *in vivo* was intrinsic to the neurons themselves. Specifically, 48 hours

following NGF withdrawal, 81.6 ± 2.9% p75NTR-/- neurons plated at day 0 were still alive, compared to 55.6 ± 4.8% of control neurons ($p < 0.005$, $n = 5$ each). At 72 hours, 72.7 ± 3.5% of p75NTR-/- neurons were alive versus 39.8 ± 3.9% of controls ($p < 0.0001$, $n = 5$), while at 96 hours, 63.9 ± 5.8% of p75NTR-/- neurons were still alive, as opposed to 30.14 ± 2.9% of controls ($p<0.005$, $n=5$). By 120 hours, the last timepoint examined, 59.8 ±6.4% of p75NTR-/- neurons were still alive, compared to only 19.6 ± 2.3% of control neurons ($p<0.0005$, $n=5$). Moreover, at this final timepoint, all of the control neurons were clearly dying, with fragmented neurites, while the p75NTR-/- neurons were clearly still healthy with neurites intact.

Thus, the lack of p75NTR in sympathetic neurons themselves leads to a significant delay in apoptosis of these neurons following NGF withdrawal, results that are consistent with the *in vivo* delay in naturally-occurring cell death.

VI. Discussion:

Data presented in this paper demonstrate that BDNF-mediated activation of p75NTR is both necessary and sufficient for the developmental apoptosis of sympathetic neurons competing for limiting amounts of survival factors. Specifically, these experiments indicate that, in culture, BDNF-mediated activation of p75NTR is sufficient to "override" suboptimal survival signals, whether they derive from TrkA activation or chronic depolarization, thereby leading to sympathetic neuron apoptosis. Coincident with this apoptosis, BDNF leads to phosphorylation of the immediate early gene product, c-jun, a biochemical event that is necessary for sympathetic neuron apoptosis following NGF withdrawal (Ham et al., 1995; Estus et al., 1994), presumably via activation of p75NTR signaling. The *in vivo* relevance of these culture experiments is indicated by two findings. First, in BDNF -/- mice (Ernfors et al., 1994; Jones et al., 1994), sympathetic neuron number is increased relative to BDNF +/+ littermates, an effect consistent with the lack of activation of p75NTR by BDNF. This is the first time such an increase in neuronal number has been reported in neurotrophin knockout mice. Second, naturally-occurring sympathetic neuron death does not occur normally in p75NTR -/- animals, but there is instead delayed neuronal loss between P23 and adulthood. This delay in neuronal loss is intrinsic to the neurons themselves, since p75NTR-/- sympathetic neurons display a similar delayed death in culture. Thus, although sympathetic neuron death can occur without p75NTR, this receptor is required for developmental apoptosis to occur rapidly and appropriately.

What is the biological rationale for having two neurotrophin receptors, one of which, TrkA, mediates survival, and one of which, p75NTR, mediates apoptosis? We propose that p75NTR provides a molecular mechanism for ensuring rapid and active apoptosis when a neuron is unsuccessful in competing for adequate amounts of the appropriate neurotrophin. If a sympathetic neuron reaches the appropriate target and sequesters NGF, TrkA is robustly activated, and any coincident activation of p75NTR is insufficient to override this survival signal. Conversely, if a neuron is late-arriving and/or reaches an inappropriate target, then TrkA would be only weakly induced as a consequence of the lack of NGF, and p75NTR would be robustly activated by neurotrophins such as BDNF. Our data indicate that the net outcome of such a scenario would be the rapid apoptotic elimination of that neuron. Such a perturbation in the normal period of naturally-occurring cell death could at least partially explain the perturbations in innervation observed previously in the p75NTR-/- mice; there is cholinergic hyperinnervation of

the hippocampus in these mice (Yeo et al., 1997), and sympathetic innervation is perturbed, with targets such as the iris being normally innervated and targets such as the pineal gland completely lacking sympathetic innervation (Lee et al., 1994). Data presented here indicates that this perturbed sympathetic innervation is not due to a deficit in neuronal number, but is instead presumably due to the failure of p75-dependent mechanisms that are essential for matching neurons to their target organs during the period of naturally-occurring cell death.

This model does not imply that p75NTR activation is the only means by which sympathetic neurons die in the absence of TrkA activation. In fact, our data demonstrating loss of sympathetic neurons between P23 and adulthood, and the death (albeit slow) of cultured p75NTR-/- sympathetic neurons argue that inadequate levels of TrkA activation do ultimately lead to the death of sympathetic neurons even in the absence of p75NTR. Instead, we propose that p75NTR activation represents a mechanism whereby exposure to an "inappropriate" neurotrophin is sufficient to cause a rapid apoptotic death even in the presence of low levels of TrkA activation. Such a mechanism would ensure that neurotrophins like NT-3 and NT-4, which weakly activate TrkA (as shown here) would not maintain sympathetic neuron survival and circumvent the absolute requirement for NGF, thereby leading to inappropriate target innervation. In fact, our data indicate that BDNF is not the only apoptotic p75NTR ligand *in vivo*; after naturally-occurring cell death, there are only approximately 36% more sympathetic neurons in the BDNF-/- mice, but at least twice as many in the p75NTR-/- mice, implying that other ligands, potentially NT-3 and/or NT-4, act through p75NTR during developmental sympathetic neuron death.

If ligand-mediated activation of p75NTR is required for it to play an essential role in sympathetic neuron apoptosis, then why is there a delay in death of cultured p75NTR-/- versus control neurons in the absence of exogenous neurotrophins? We have previously demonstrated (Causing et al., 1997) that BDNF is synthesized in the rodent SCG *in vivo*. Moreover, cultured sympathetic neurons synthesize BDNF, and BDNF can be detected in media conditioned by sympathetic neurons (C.C.G. Causing, R. Aloyz, and F. Miller, unpublished data). On the basis of these findings, we propose that autocrine BDNF may play a role in cultured sympathetic neuron apoptosis following NGF withdrawal, a hypothesis we are currently testing. Does sympathetic neuron-derived BDNF play a similar role *in vivo*? Although our data demonstrate an increased number of sympathetic neurons in BDNF-/- mice, it is unclear whether the BDNF that is critical for this effect derives from sympathetic neurons themselves and/or from sympathetic neuron

targets. In this regard, we have previously demonstrated that BDNF made by sympathetic neurons *in vivo* regulates their level of preganglionic input (Causing et al., 1997). One could therefore speculate that BDNF made by sympathetic neurons might serve both to eliminate those sympathetic neurons that did not innervate an appropriate target (and thereby receive adequate TrkA activation), and to ensure adequate preganglionic input for those neurons that did successfully compete for target territory.

The developmental profile of sympathetic neuron number observed in the absence of p75 NTR is very similar to that observed when neonates are supplied with exogenous NGF, lending support to the notion that the absence of p75 NTR rescues sympathetic neurons from the normal period of naturally-occurring cell death. When NGF is given systemically to rats from P6 to P21, not only are the neurons that normally die rescued, but, as observed in the p75 NTR -/- mice, there is an increase of approximately 20% in the number of neurons (Hendry and Campbell, 1976). This increase is presumably due to the fact that, during the early period of naturally-occurring cell death, sympathetic neuroblasts continue to proliferate; however, these later-born neurons are preferentially susceptible to programmed cell death, and the increase in number of neurons born is masked by the neuronal death that occurs at the same time (Hendry, 1977). It is therefore likely that the increased number of sympathetic neurons observed at P23 in p75NTR-/- mice is due to the same phenomenon.

What are the biochemical mechanisms that allow p75NTR activation to override a TrkA or KCl-mediated survival signal? Our results demonstrate that BDNF stimulated a hyperphosphorylation of c-jun concomitant with the appearance of apoptosis. Hyperphosphorylation of c-jun has been previously noted in postnatal rat sympathetic neurons deprived of NGF (Ham et al., 1995). Serum and NGF withdrawal from PC12 cells also leads to sustained activation of JNK, the kinase that phosphorylates c-jun (Xia et al., 1995). The necessity of c-jun phosphorylation in these apoptotic responses was suggested by three types of experiments; (1) expression of dominant-inhibitory c-jun in rat sympathetic neurons protected against apoptosis caused by NGF deprivation (Ham et al., 1995), (2) microinjection of sympathetic neurons with antibodies to c-jun inhibited cell death induced by NGF deprivation (Estus et al., 1995), and (3) expression in PC12 cells of dominant-inhibitory MEKK1, which inhibits the activity of JNK, reduced apoptosis, while expression of activated MEKK1, which indirectly activates JNK, increased apoptosis (Xia et al., 1995). The observation that BDNF also stimulates

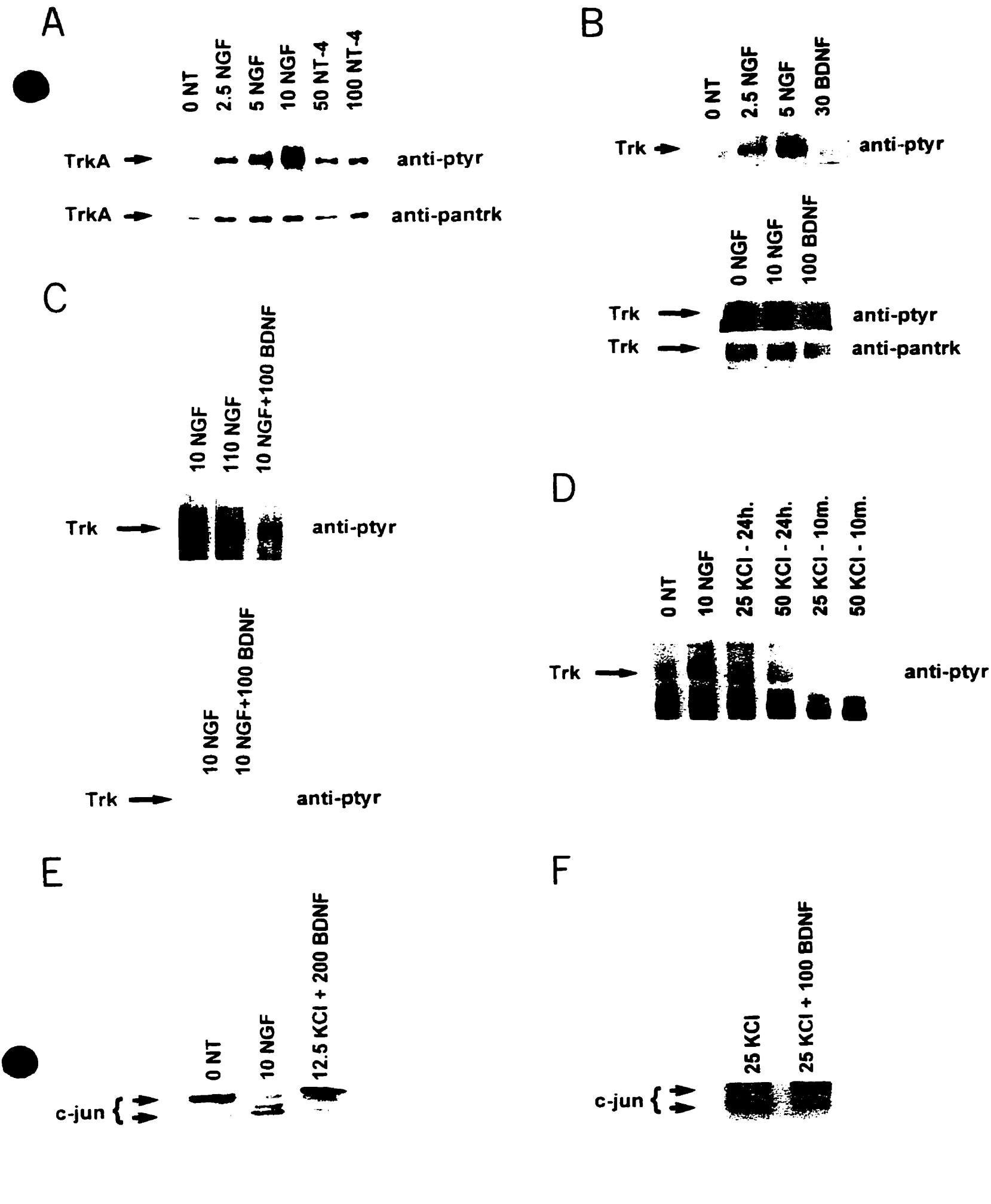
c-jun phosphorylation suggests that the MEKK1-MKK4-JNK pathway may function in apoptosis induced by both NGF deprivation and by activation of p75NTR. However, we do not know whether the components of the JNK pathway are necessary or sufficient for p75NTR-mediated cell death. p75NTR activates several other signaling proteins, including NF-kB and perhaps ceramide-activated kinases, which have been hypothesized to function in the induction of apoptosis (Casaccia-Bonnefil et al., 1996). The role of each of the p75NTR-stimulated signaling proteins in apoptosis awaits experiments in which the activities of these molecules are selectively blocked in BDNF-treated sympathetic neurons.

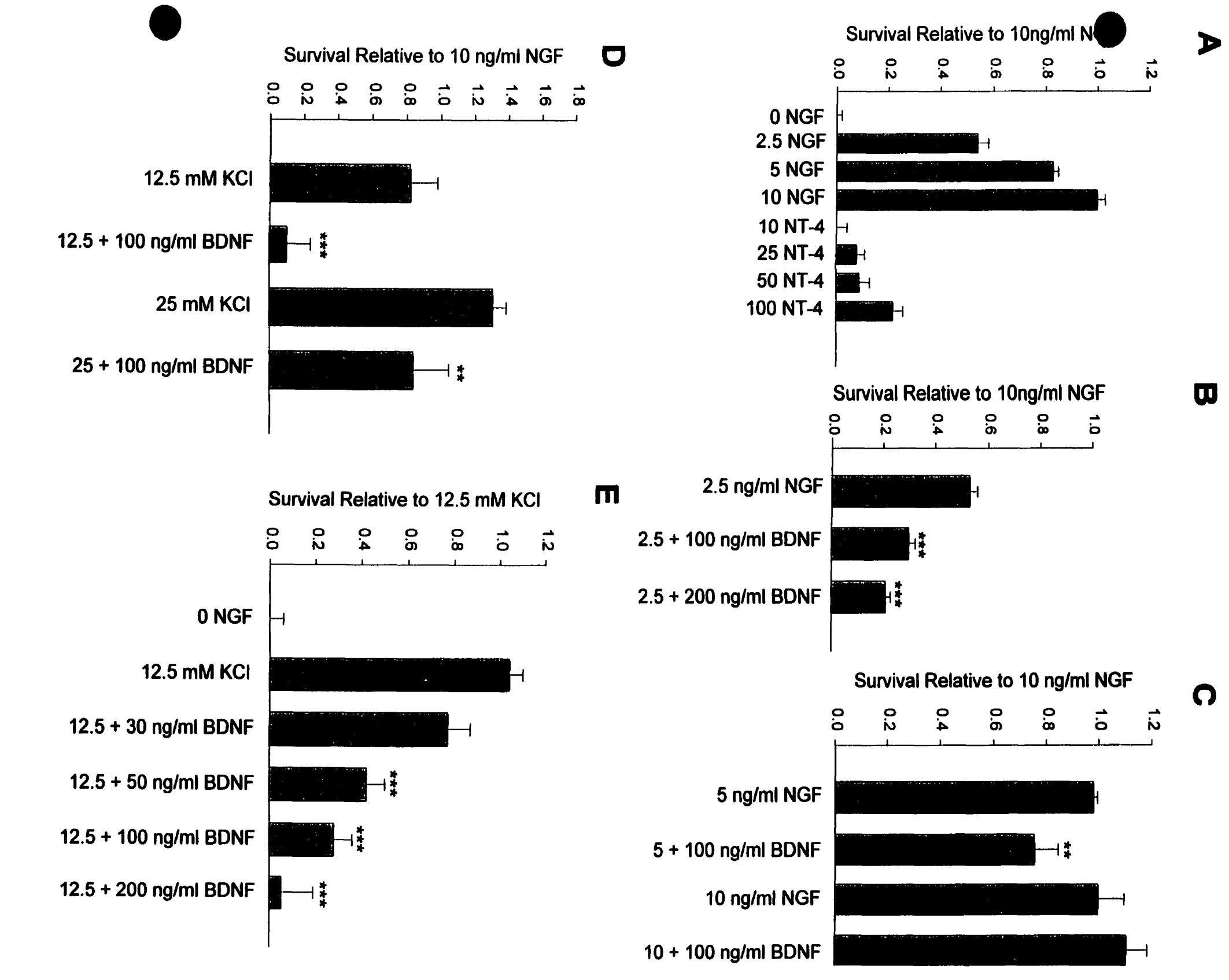
p75NTR activation could also antagonize a suboptimal TrkA survival signal by a direct action on the TrkA receptor itself. Such a mechanism might involve alterations in p75NTR:TrkA interactions to modify a high-affinity signaling complex (Hempstead et al., 1991; Ross et al., 1995; Wolf et al., 1995) and/or a direct effect of p75NTR signaling on the TrkA receptor involving, for example, serine/threonine phosphorylation by ceramide-activated kinases (MacPhee et al., 1997). However, our data indicate that even if p75NTR activation directly regulates TrkA function, there must be further downstream actions of p75NTR that lead to apoptosis. This conclusion derives from our finding that BDNF-mediated activation of p75NTR led to apoptosis in the absence of TrkA activation, when neuronal survival was maintained by chronic depolarization. It is also likely that cross-talk between the p75NTR and TrkA is bidirectional. In this regard, Dobrowsky et al. (1994) have demonstrated that a p75NTR-mediated ceramide increase was antagonized by coincident activation of TrkA. Thus, one of the mechanisms whereby TrkA may support neuronal survival is by silencing a neurotrophin-mediated p75NTR death signal.

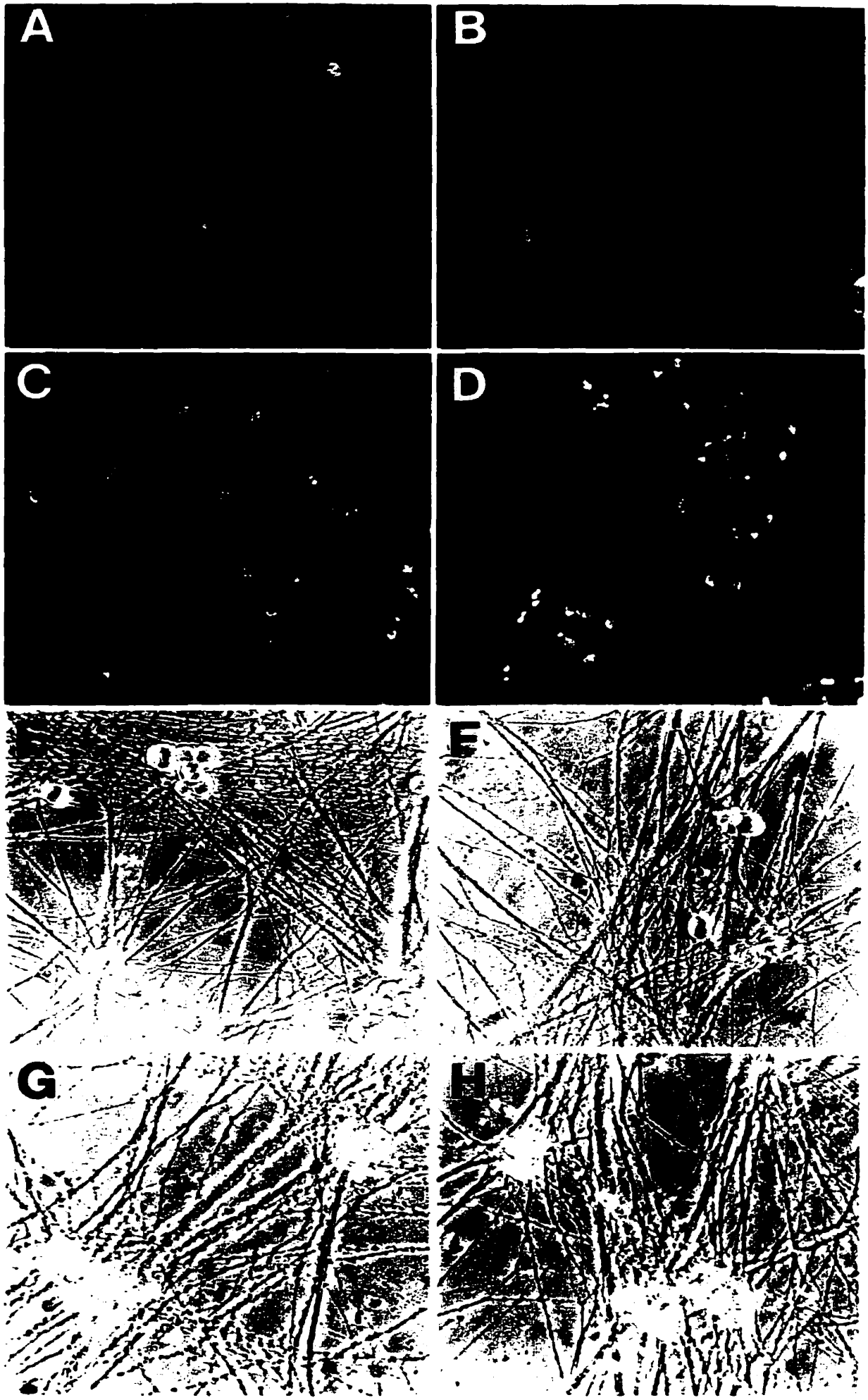
The finding of an increased number of sympathetic neurons in the BDNF -/- mice was predicted by our culture findings, but was unexpected in light of previous studies on this growth factor. The neurotrophins, including BDNF, are traditionally thought to regulate neuronal survival positively via the Trk family of tyrosine kinase receptors (reviewed in Snider, 1994). Here, BDNF antagonizes NGF or KCl-induced survival signals through the activation of p75NTR. Such a mechanism, whereby the precise cohort of neurotrophins to which a neuron is exposed determines life versus death at the time of target innervation provides a level of sophistication that was previously unsuspected. However, this additional level of complexity provides a mechanism whereby neurons can recognize not only whether they are exposed to the "right" neurotrophin, but

whether or not they are seeing the "wrong" neurotrophin. It is possible that such functional antagonism between Trk and p75NTR provides not only a mechanism for determining neuronal survival, but also a mechanism for elimination of axonal collaterals that innervate the inappropriate target.

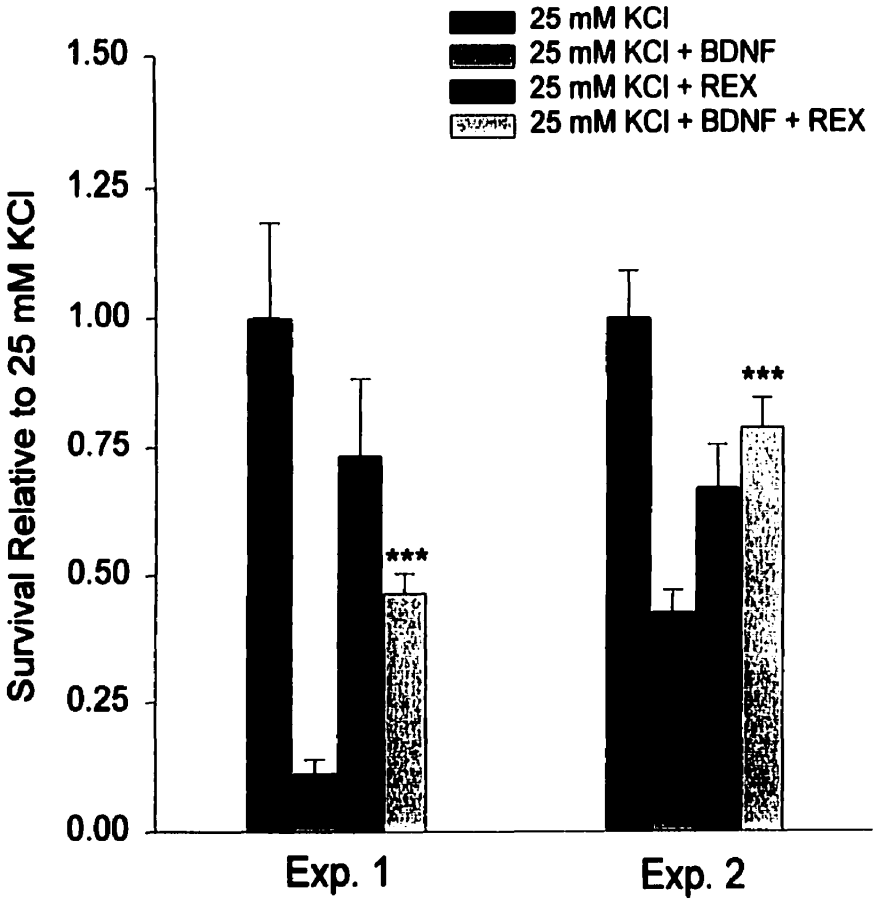
Finally, although our findings indicate that p75NTR can signal to mediate sympathetic neuron apoptosis during the period of naturally-occurring cell death, they do not imply that p75NTR-mediated signaling inevitably results in neuronal apoptosis. It is more likely that, like other members of this family, the outcome of p75NTR-mediated signal transduction cascades is a function of cellular context. For example, depending upon the cellular environment, p75NTR regulates cell migration (Anton et al., 1994), gene expression (Itoh et al., 1994), and can positively modulate TrkA signaling (Verdi et al., 1994; Barker et al., 1994). However, our findings indicate that during the period of naturally occurring sympathetic neuron death, the apoptotic actions of p75NTR are essential for the establishment of appropriate neuron:target interactions.


A
0 NT
2.5 NGF
5 NGF
10 NGF
50 NT-4
100 NT-4
TrkA
anti-ptyr
TrkA
anti-pantrk
B
0 NT
2.5 NGF
5 NGF
30 BDNF
Trk
anti-ptyr
0 NGF
10 NGF
100 BDNF
Trk
anti-ptyr
Trk
anti-pantrk
C
10 NGF
110 NGF
10 NGF+100 BDNF
Trk
anti-ptyr
10 NGF
10 NGF+100 BDNF
Trk
anti-ptyr
D
0 NT
10 NGF
25 KCl - 24h.
50 KCl - 24h.
25 KCl - 10m.
50 KCl - 10m.
Trk
anti-ptyr
E
0 NT
10 NGF
12.5 KCl + 200 BDNF
c-jun
F
25 KCl
25 KCl + 100 BDNF
c-jun

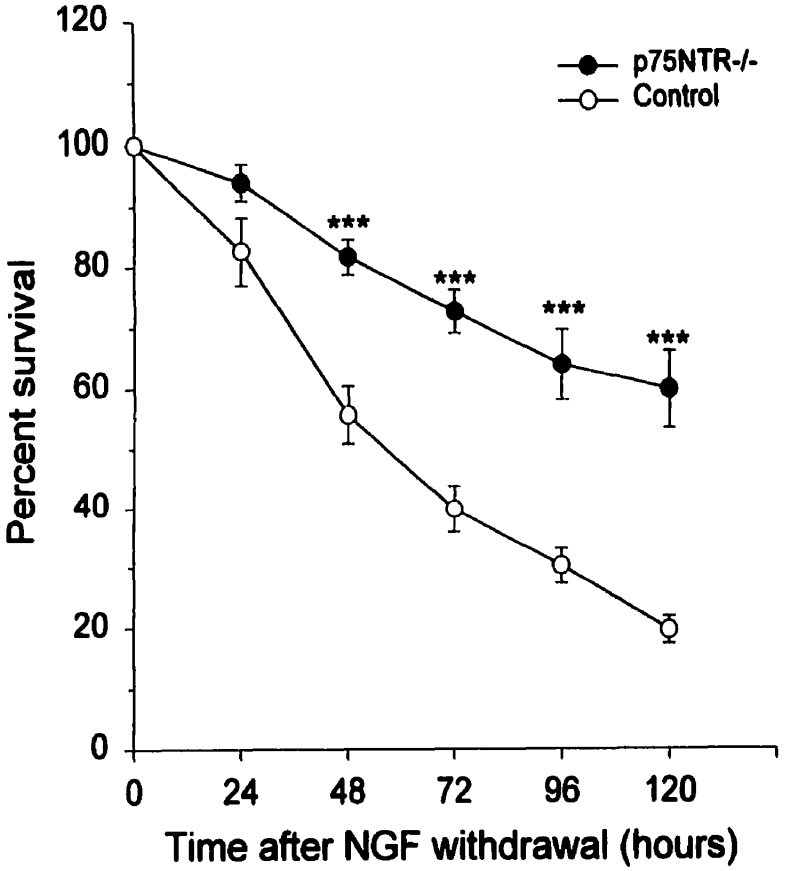

A
Survival Relative to 10ng/ml N
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0 NGF
2.5 NGF
5 NGF
10 NGF
10 NT-4
25 NT-4
50 NT-4
100 NT-4
B
Survival Relative to 10ng/ml NGF
0.0
0.2
0.4
0.6
0.8
1.0
2.5 ng/ml NGF
2.5 + 100 ng/ml BDNF
2.5 + 200 ng/ml BDNF
***
***
C
Survival Relative to 10 ng/ml NGF
0.0
0.2
0.4
0.6
0.8
1.0
1.2
5 ng/ml NGF
5 + 100 ng/ml BDNF
10 ng/ml NGF
10 + 100 ng/ml BDNF
**
D
Survival Relative to 10 ng/ml NGF
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
12.5 mM KCl
12.5 + 100 ng/ml BDNF
25 mM KCl
25 + 100 ng/ml BDNF
***
**
E
Survival Relative to 12.5 mM KCl
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0 NGF
12.5 mM KCl
12.5 + 30 ng/ml BDNF
12.5 + 50 ng/ml BDNF
12.5 + 100 ng/ml BDNF
12.5 + 200 ng/ml BDNF
***
***
***

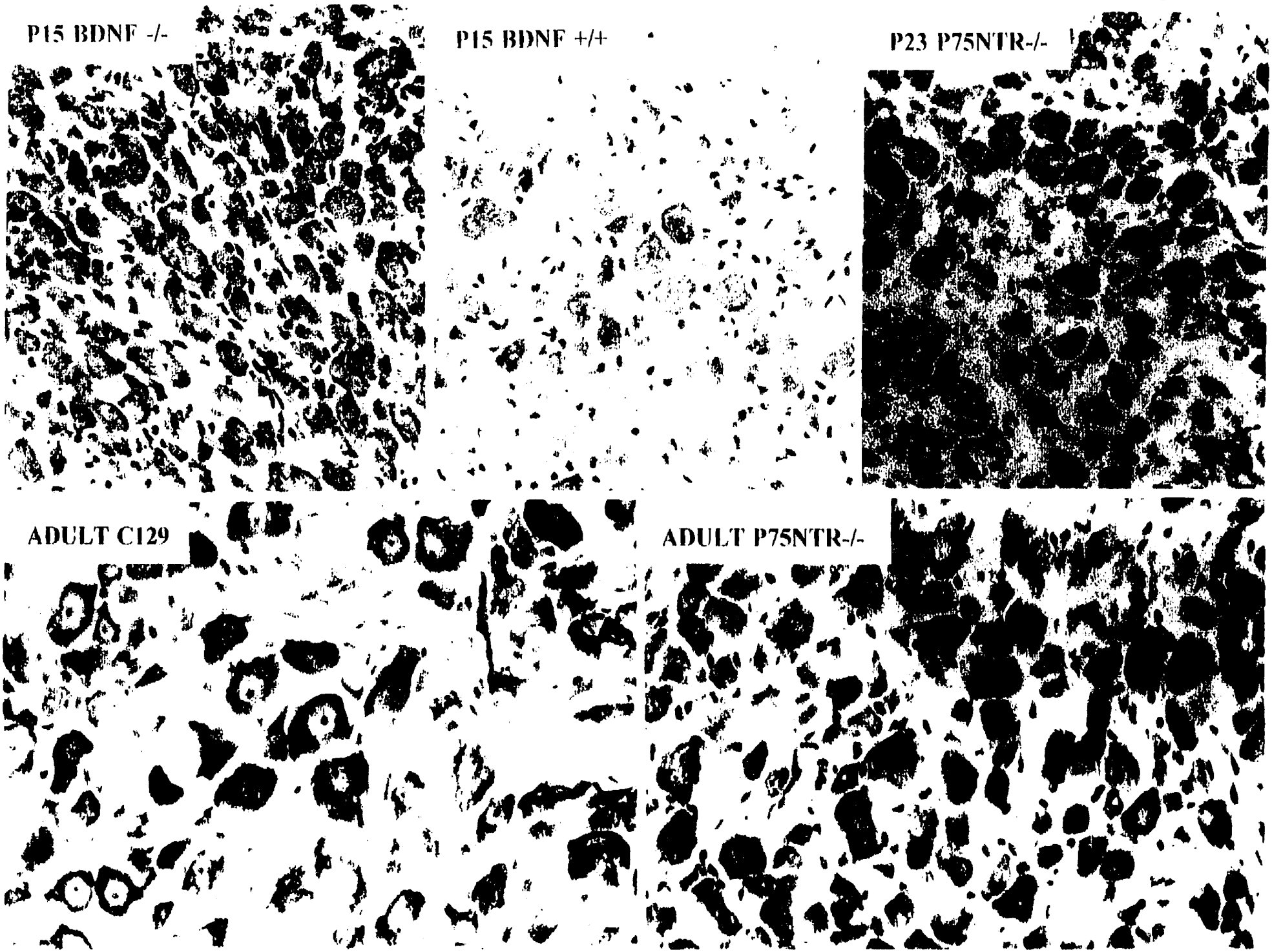

A
B
C
D
E
G
H

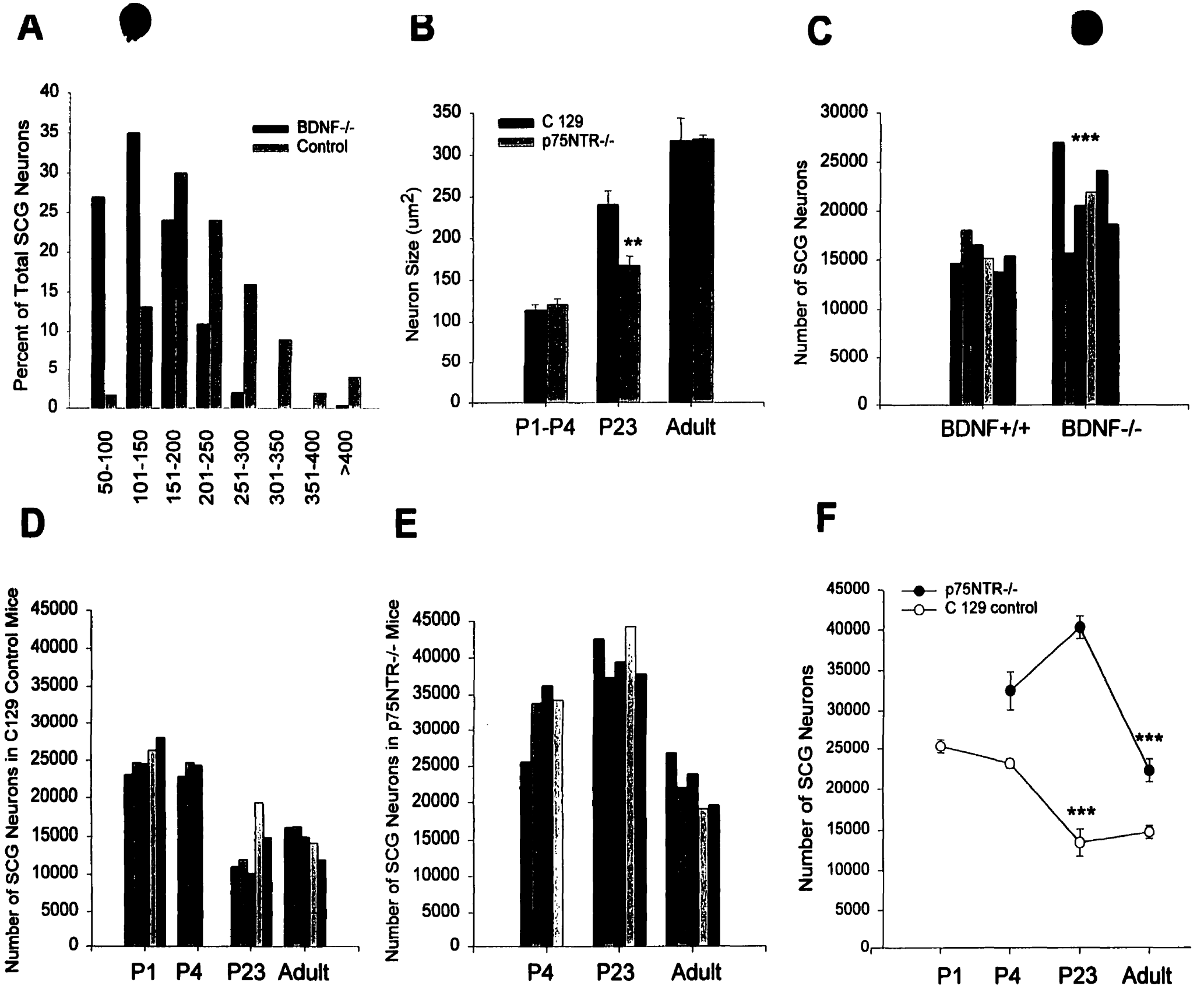
A


25 mM KCl
25 mM KCl + BDNF
25 mM KCl + REX
25 mM KCl + BDNF + REX
Survival Relative to 25 mM KCl
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
***
Exp. 1
Exp. 2

B


p75NTR-/-
Control
Percent survival
120
100
80
60
40
20
0
***
***
***
***
0
24
48
72
96
120
Time after NGF withdrawal (hours)


P15 BDNF -/-
P15 BDNF +/+
P23 P75NTR-/-
ADULT C129
ADULT P75NTR-/-


A
Percent of Total SCG Neurons
BDNF-/-
Control
50-100
101-150
151-200
201-250
251-300
301-350
351-400
>400
B
Neuron Size (um2)
C 129
p75NTR-/-
**
P1-P4
P23
Adult
C
Number of SCG Neurons
***
BDNF+/+
BDNF-/-
D
Number of SCG Neurons in C129 Control Mice
P1
P4
P23
Adult
E
Number of SCG Neurons in p75NTR-/- Mice
P4
P23
Adult
F
p75NTR-/-
C 129 control
Number of SCG Neurons
***
***
P1
P4
P23
Adult

Group	SCG volume (mm^3)	Neuron number per 214 375μm^3	Total estimated neuron number	Total counted neuron number
C129 P1	.106 ± .008	56 ± 7.2	27 698 ± 3846	25 328 ± 927
C129 P4	.121 ± .001	40 ± 0.3	22 577 ± 171	23 196 ± 568
C129 P23	.120 ± .012	32 ± 2.2	17 912 ± 1355	13 415 ± 1683
C129 adult	.121 ± .009	30 ± 1.5	16 933 ± 906	14 667 ± 799
p75NTR-/- P4	.170 ± .021	39 ± 2.9	30 927 ± 2576	32 468 ± 2362
p75NTR-/- P23	.202 ± .028	44 ± 6.7	41 460 ± 7197	40 275 ± 1368
p75NTR-/- adult	.172 ± .005	26 ± 3.9	20 860 ± 3223	22 254 ± 1404
BDNF+/+ P15	.102 ± .014	30 ± 0.8	14 358 ± 437	15 690 ± 617
BDNF-/- P15	.083 ± .004	44 ± 0.8	17 150 ± 328	21 318 ± 1627

VIII. Figure Legends:

Figure 4.1. (A) NT-4 activates TrkA on sympathetic neurons. Sympathetic neurons were selected in 10 ng/ml NGF for 5 days, washed free of neurotrophin, and then exposed to various concentrations of NGF (from 2.5 to 10 ng/ml) or NT-4 (50 and 100 ng/ml) for ten minutes. Cellular lysates were immunoprecipitated with an antibody specific to TrkA, and probed first with anti-phosphotyrosine (anti-ptyr) to visualize TrkA autophosphorylation, and then reprobed with anti-panTrk to monitor total TrkA levels (anti-pantrk). All samples were normalized for equal amounts of protein. **(B,C) BDNF does not activate Trk on sympathetic neurons.** (B) To examine short-term Trk autophosphorylation, sympathetic neurons were washed free of NGF and exposed to 2.5, 5, or 10 ng/ml NGF or to 30 or 100 ng/ml BDNF for 10 minutes. Total Trk protein was immunoprecipitated with anti-panTrk, and Trk autophosphorylation analysed by blotting with anti-phosphotyrosine (anti-ptyr). In the bottom panel, the same blot was reprobed with panTrk antibody 203 (anti-pantrk) to ensure that similar amounts of total Trk protein were present in all lanes. (C) To examine long-term Trk autophosphorylation, sympathetic neurons were selected in 10 ng/ml NGF, and then switched to 10 ng/ml NGF (10 NGF), 110 ng/ml NGF (110 NGF), or 10 ng/ml NGF + 100 ng/ml BDNF (10 NGF + 100 BDNF). Analysis of Trk autophosphorylation was performed as in (B). In all cases, samples were normalized for equal amounts of protein, and blots were reprobed for total Trk protein levels. **(D) KCl treatment does not lead to Trk activation on sympathetic neurons.** Sympathetic neurons were washed free of neurotrophin, and switched to 10 ng/ml NGF for 10 minutes, or to 25 or 50 mM KCl for 10 minutes or 24 hours. Trk autophosphorylation was analyzed as in (B). **(E,F) BDNF-mediated activation of p75 leads to increased phosphorylation of c-jun.** Sympathetic neurons were selected in 50 ng/ml NGF, washed free of neurotrophin, and then switched to 10 ng/ml NGF, or 12.5 or 25 mM KCl plus or minus BDNF. Eighteen hours later, cellular lysates were analyzed for c-jun on Western blots. (E) As previously reported (Ham et al., 1995), the size of c-jun increases in response to NGF withdrawal, a shift indicative of increased phosphorylation. 200 ng/ml BDNF caused a similar size shift in the presence of 12.5 mM KCl. (F) This size shift was specific to BDNF and not to KCl, as shown here with 25 mM KCl plus or minus 100 ng/ml BDNF.

Figure 4.2. (A) NT-4 is 5-fold less efficient than NGF at supporting sympathetic neuron survival at equivalent levels of TrkA autophosphorylation. A comparison of sympathetic

neuron survival in response to 2.5-10 ng/ml NGF versus 10-100 ng/ml NT-4, as monitored using MTT assays that measure mitochondrial function and cell survival. Neonatal sympathetic neurons were cultured in 10 ng/ml NGF for 5 days in 48 well dishes, washed free of neurotrophin-containing medium, and then incubated for 2 days in various concentrations of NGF or NT-4, as indicated on the x-axis. Each point represents the values pooled from 3 independent sets of survival assays, each of which was performed in quadruplicate. In these assays, absolute values are normalized so that the value obtained with 0 neurotrophin is 0% survival, while that obtained with 10 ng/ml NGF is considered 100% survival. Error bars represent the standard error. **(B-D) BDNF decreases sympathetic neuron survival in the presence of limiting quantities of NGF (B,C) or KCl (D).** Neurons were selected in 50 ng/ml NGF for 5 days, and then switched to 2.5 ng/ml NGF, 5 ng/ml NGF, 10 ng/ml NGF, 12.5 mM KCl or 25 mM KCL, with or without 100 or 200 ng/ml BDNF for 2 days. Survival was monitored by MTT assays. Results are expressed relative to those obtained with 10 ng/ml NGF, and represent the mean ± standard error. Panels B and D include the combined data from 3 independent experiments performed in quadruplicate. Panel C represents the data from one representative experiment, performed in quadruplicate (**$p<0.05$, ***$p<0.005$ relative to NGF (B,C) or KCl (D) alone). **(E) BDNF decreases sympathetic neuron survival in a concentration-dependent fashion.** Neurons were selected in 50 ng/ml NGF for 5 days, and then switched to 12.5 mM KCl with or without concentrations of BDNF ranging from 30 to 200 ng/ml for 2 days. Survival was monitored by MTT assays. Results are normalized relative to those obtained with 12.5 mM KCl alone, and represent the mean ± the standard error from the combined data of 4 independent experiments (***$p<0.005$ relative to 12.5 mM KCl alone).

Figure 4.3. Sympathetic neurons undergo apoptosis in response to BDNF. (A-D) Fluorescence photomicrographs of sympathetic neurons analyzed by TUNEL-labelling. Neurons were selected in 50 ng/ml NGF for 5 days, washed free of neurotrophin, and then switched to 12.5 mM KCl with (D) or without (B) 100 ng/ml BDNF for 18 hours. As controls, sister cultures were maintained in 50 ng/ml NGF (A) or withdrawn from NGF (C). Eighteen hours later, neurons were analyzed for apoptosis using TUNEL-labelling for fragmented nuclear DNA. (E-H) Phase-contrast photomicrographs of cultured sympathetic neurons. Neurons were cultured as above, and then switched to 12.5 mM KCl with (H) or without (F) 100 ng/ml BDNF for 48 hours. As

controls, sister cultures were maintained in 50 ng/ml NGF (E) or withdrawn from NGF (G). Size bar = 74μm.

Figure 4.4. (A) The p75NTR antibody, REX, inhibits the BDNF-mediated apoptosis of sympathetic neurons. A comparison of sympathetic neuron survival, as monitored using MTT assays, in response to 25 mM KCl ± BDNF with and without the presence of the function-blocking anti-p75NTR, REX. Sympathetic neurons were cultured in 50 ng/ml NGF for 5 days, washed free of neurotrophin-containing medium, and then incubated for 2 days in 25 mM KCl or 25 mM KCl + 100 ng/ml BDNF with or without REX. Results of two representative experiments, each of which was performed in triplicate, are shown. In all cases, results represent the mean ± standard error, and are normalized so that the survival mediated by 25 mM KCl alone is 100%. REX by itself had no significant effect ($p>0.05$) on sympathetic neuron survival as mediated by 25 mM KCl. (***$p< 0.004$ for the comparison between 25 mM KCl + REX and 25 mM KCL + 100 ng/ml BDNF + REX). **(B) Death of cultured p75NTR-/- neurons is delayed following NGF withdrawal.** Timecourse of survival of cultured p75NTR-/- and p75NTR+/+ (control) neurons following NGF withdrawal. Representative fields of neurons were counted immediately upon NGF withdrawal, and again every 24 hours. Results are normalized so that the number of neurons at the time of NGF withdrawal is 100%. Results represent the mean ± the standard error of the combined data from 5 individual experiments each for the p75NTR-/- and control neurons; every individual experiment was performed in triplicate. (***$p<0.002$ for the comparison between p75NTR-/- and control neurons at each timepoint).

Figure 4.5. Morphology of sympathetic neurons of the superior cervical ganglion in BDNF -/- and p75NTR -/- mice. Photomicrographs of cresyl violet-stained cross-sections of the SCG from (A) P15 BDNF-/- mice, (B) P15 BDNF+/+ littermates, (C) P23 p75NTR-/- mice, (D) adult C129 mice, and (E) adult p75NTR-/- mice. Size bar = 38.5 μm.

Figure 4.6. At P15 to P23, sympathetic neuron number is increased in the SCG of BDNF -/- and p75NTR -/- mice. (A) Cross-sectional area of P15 BDNF-/- neurons relative to control neurons from animals of the same background, plotted as a size distribution histogram, in bins of 50 μm^2. Note that the entire population of BDNF-/- sympathetic neurons is shifted to a smaller

size relative to BDNF+/+ (Control) neurons of the same age. (B) Mean cross-sectional areas of sympathetic neurons of the SCG in p75NTR-/- mice at various developmental timepoints relative to control C129 mice. Note that from P1 to P4, and in adulthood, the size of sympathetic neurons is similar in the presence or absence of p75NTR, but that at P23, neurons lacking p75NTR are significantly smaller. Neuron size is expressed as cross-sectional area in um^2, and error bars represent the standard error (**$p<0.05$). (C) The number of sympathetic neurons of the SCG is increased in P15 BDNF-/- animals relative to their control littermates, as determined by counting. The number of neurons in each of 6 control BDNF+/+ and BDNF -/- animals are shown; each bar represents the number of neurons in the SCG of one animal of the indicated genotype. The means of these two groups are significantly different (***$p<0.01$). The means ± the standard errors are summarized in Table 1 as "Total counted neuron number". (D) Sympathetic neuron number in the SCG of control C129 mice at developmental timepoints encompassing the period of naturally-occurring sympathetic neuron death. The number of neurons counted in the SCG at P1, P4, P23, and adulthood are shown; each bar represents the number of neurons in the SCG of one C129 animal of the indicated age. Five animals were analyzed at P1, 3 at P4, 5 at P23, and 5 at adulthood. The means ± standard errors of these groups are plotted in panel (F). (E) Sympathetic neuron number in the SCG of p75NTR-/- mice at developmental timepoints encompassing the period of naturally-occurring sympathetic neuron death. The number of neurons counted in the SCG at P4, P23, and adulthood are shown; each bar represents the number of neurons counted in the SCG of one p75NTR-/- animal of the indicated age. Four animals were analyzed at P4, 5 at P23, and 5 adults. The means ± standard errors of these groups are plotted in panel (F). (F) Comparison of the timecourse of naturally-occurring sympathetic neuron death in the SCG of p75NTR-/- versus control (C129 control) animals. The points represent the mean ± standard error of the counts shown in panels (D) and (E). There is a highly significant decrease in neuronal number from P4 to P23 in the control C129 mice, and from P23 to adulthood in the p75NTR-/- mice (***$p<0.007$). The means for all of these groups are summarized in Table 1 as "Total counted neuron number".

TABLE 4.1. Morphometric Analysis of the SCG from BDNF-/- and p75NTR-/- Mice. Data are presented for the SCG from two different groups of experimental versus control animals; P15 BDNF+/+ and BDNF-/- littermates in a BalbC/C129 background, and p75NTR-/- mice in a C129

background versus control C129 animals. "SCG volume" and "Neuron number per 214,375 μm^3" are expressed as the mean ± SE, and were measured as described in the Experimental Procedures (n = 3 animals for each group). The "Total estimated neuron number" is calculated as mean SCG volume x (mean neuron number/214,375 μm^3). The "Total counted neuron number" represents the mean ± SE of total SCG neurons determined by counting neurons with a nucleolus in every third (BDNF-/- and BDNF+/+) or fourth (C129 and p75NTR-/-) section, as described in the Experimental Procedures. The data obtained from each individual animal is illustrated in Fig. 6. Note that the total estimated neuron numbers and the total counted neuron numbers are statistically similar ($p>0.05$ in all cases).

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CHAPTER V: P53 IS ESSENTIAL FOR DEVELOPMENTAL NEURON DEATH AS REGULATED BY THE TRKA AND P75 NEUROTROPHIN RECEPTORS

I. Preface:

The previous chapter focuses on the ability of neurotrophins to mediate apoptosis by binding to the p75NTR. Although p75 has been previously shown to mediate the translocation of NFκB from the cytoplasm to the nucleus, to increase JNK activity and to increase ceramide levels, the signal transduction pathway through which p75 mediates its apoptotic effect remained unknown. In chapter IV, it was demonstrated that the binding of BDNF to the p75 receptor leads to the phosphorylation of c-jun, a signaling molecule downsteam of the MEKK-SEK-JNK pathway. C-jun is also phosphorylated in sympathetic neurons upon withdrawal of NGF, leading us to hypothesize that there is a convergence in apoptotic signaling pathways between trophic factor withdrawal-induced death and p75-mediated death. To test this hypothesis we have focussed on the p53 protein which has recently been implicated in the induction of neuronal death, and which is believed to lie downstream of signal transduction pathways due to its function as a transcription factor and its relatively late activation in response to cellular stress.

The aim of this chapter is to establish the role of the p53 protein in mediating NGF withdrawal-induced apoptosis and p75-mediated apoptosis, and to investigate the potential upstream members of this signal transduction pathway. We have once again utilized sympathetic neurons of the rat SCG to test our hypothesis, and have proposed a biologically relevant model for the role of p53 in programmed cell death of SCG neurons. This model is in accordance with, and compliments the proposed model for the role of p75 in mediating programmed cell death and is further discussed in this chapter.

II. Abstract:

Naturally-occurring sympathetic neuron death is the result of two apoptotic signalling events: one normally suppressed by NGF/TrkA survival signals, and a second activated by the p75 neurotrophin receptor. Here we demonstrate that the p53 tumor suppressor protein, likely as induced by the MEKK-JNK pathway, is an essential component of both of these apoptotic signalling cascades. In cultured neonatal sympathetic neurons, p53 protein levels are elevated in response to both NGF withdrawal and p75NTR activation. NGF withdrawal also results in elevation of a known p53 target, the apoptotic protein Bax. Functional ablation of p53 using the adenovirus E1B55K protein inhibits neuronal apoptosis as induced by either NGF withdrawal or p75 activation. Direct stimulation of the MEKK-JNK pathway using activated MEKK1 has similar effects; p53 and Bax are increased and the subsequent neuronal apoptosis can be rescued by E1B55K. Expression of p53 in sympathetic neurons indicates that p53 functions downstream of JNK and upstream of Bax. Finally, when p53 levels are reduced or absent in p53+/- or p53-/- mice, naturally-occurring sympathetic neuron death is inhibited. Thus, p53 is an essential common component of two receptor-mediated signal transduction cascades that converge on the MEKK-JNK pathway to regulate the developmental death of sympathetic neurons.

III. Introduction:

Naturally-occurring neuronal death is an essential component of neural development in which up to 50% of any given neuronal population is lost as a mechanism for establishing appropriate neuron:target cell connections (reviewed in Oppenheim, 1991). Sympathetic neurons of the peripheral nervous system, which require nerve growth factor (NGF) for their survival (reviewed in Levi-Montalcini, 1987), have been a prototype for analysis of the mechanisms regulating this event. During the first two postnatal weeks, sympathetic neurons compete for limiting amounts of target-derived NGF, which binds to and activates TrkA tyrosine kinase receptors (Kaplan et al., 1991a; 1991b; Klein et al., 1991) on neuronal teminals, thereby mediating a retrograde neuronal survival signal (Campenot et al., 1982; Senger et al., 1997; Riccio et al., 1997). Traditionally, it has been thought that the absence of such a TrkA retrograde signal was sufficient to cause rapid neuronal apoptosis during developmental death. However, we have recently shown that the lack of such a NGF/TrkA-mediated survival signal is, of itself, insufficient for appropriate sympathetic neuron death. Instead, a second neurotrophin receptor, p75NTR (Johnson et al., 1986; Radeke et al., 1987), is required for this process; in the absence of p75NTR, sympathetic neuron apoptosis is delayed both in culture and in vivo (Bamji et al., 1998). Moreover, ligand-mediated activation of p75NTR is sufficient to cause sympathetic neuron apoptosis (Bamji et al., 1998). Thus, appropriate developmental sympathetic neuron death occurs when p75NTR is activated coincident with suboptimal NGF/TrkA survival signals (reviewed in Miller and Kaplan, 1998). This convergent regulation of neuronal apoptosis provides a mechanism whereby sympathetic neurons are able to recognize not only whether they are receiving adequate amounts of NGF, but also whether or not they are exposed to "inappropriate" neurotrophins, potentially as a function of late or inappropriate target innervation.

The intracellular mechanisms responsible for transducing these receptor-mediated apoptotic cascades in sympathetic neurons remain ill-defined, although activation of the JNK pathway occurs following both p75NTR activation (Bamji et al., 1998; Casaccia-Bonnefil et al., 1996) and NGF withdrawal (Ham et al., 1995; Estus et al., 1994) and, in the case of NGF withdrawal, is a necessary early event in the apoptotic pathway. Moreover, Bax is essential for sympathetic neuron apoptosis both in culture and during naturally-occurring death in vivo (Deckwerth et al., 1996; Easton et al., 1997). One protein that is known to regulate transcription of Bax (Miyashita and Reed, 1995) and that has been implicated in cellular apoptosis is the p53

tumor suppressor protein (reviewed in Hainut, 1995; Levine, 1997; Jacks and Weinberg, 1996). However, although p53 has been implicated in neuronal death in response to DNA damage or cellular stress (Sakhi et al., 1994; Li et al., 1994; Morrison et al., 1996; Xiang et al., 1998; Wood and Youle, 1995), this protein has not previously been thought to play a role in naturally-occurring cell death nor has it been shown to act downstream of death receptor activation or of the MEKK-JNK pathway.

In this regard, we have previously demonstrated that increased expression of p53 was sufficient to cause sympathetic neuron apoptosis in the presence of NGF (Slack et al., 1996). On the basis of this observation, together with the fact that Bax is essential for naturally-occurring sympathetic neuron death (Deckwerth et al., 1996; Easton et al., 1997), we hypothesized that p53 was an essential component of the signalling pathways causing sympathetic neuron apoptosis either in response to NGF withdrawal and/or to p75NTR activation. In this paper, we test this hypothesis, and demonstrate that both p75NTR activation and NGF withdrawal cause increased expression of p53, likely as a consequence of activation of the MEKK-JNK (Yan et al., 1994; Derijard et al., 1994) pathway, and that this convergent regulation of p53 is essential for normal naturally-occurring sympathetic neuron death.

IV. Materials and Methods:

Primary Neuronal Cultures

Mass cultures of pure sympathetic neurons from the superior cervical ganglion (SCG) of postnatal day 1 rats were prepared as previously described (Belliveau et al., 1997; Bamji et al., 1998). Neurons were plated on rat tail collagen coated tissue culture dishes; 6 well plates for biochemistry, and 48 well plates for survival assays. Low density SCG cultures were used for all of the survival assays; for the survival assays in NGF withdrawal and p75 activation experiments, neurons were used at densities of 4000-6000 and 2000-4000 neurons per well of a 48 well plate, respectively. For biochemistry, approximately 40,000-50,000 neurons were plated per well of a 6 well dish. For all experiments not involving viral infection, neurons were initially cultured for 5 days in the presence of 50 ng/ml NGF. At the end of this 5 day selection, neurons were washed several times in neurotrophin-free media prior to addition of the new neurotrophin- or KCL-containing media.

NGF for these experiments was purified from mouse salivary glands (CedarLane, Hornby, ON). Two sources of recombinant human BDNF (Amgen,Thousand Oaks, CA; Preprotech, Rocky Hill, NJ) were used for these experiments; similar results were obtained with both, as discussed previously (Bamji et al., 1998).

Virus Infections and Survival Assays

Six different recombinant adenoviruses were used for these experiments. Two, those expressing E1B55K, the E1B55K mutant A262 (Yew et al., 1990), and p53 (Slack et al., 1996) have been previously constructed and described. We also constructed adenoviruses (Massie et al., 1995) expressing activated MEKK1 (Eilers et al., 1998), and Bcl-xl (Boise et al., 1993) in the Ad5 backbone (Bett et al., 1994), which drives expression from the CMV promoter. As a control for these viruses, we used a recombinant adenovirus in the same Ad5 backbone expressing E. coli ß-galactosidase, as we have previously described (Slack et al., 1996; the kind gift of Dr. Frank Graham, McMaster University, Hamilton, Ontario).

All recombinant adenoviruses were purified on CsCl gradients, as we have previously described (Slack et al., 1996). Infectious titer was determined by plaque assay on 293 cells (Graham and Prevec, 1991).

For the experiments involving E1B55K rescue from NGF withdrawal, neurons were cultured for 3-4 days in 50 ng/ml NGF, and then were infected overnight with various mois of recombinant adenovirus in serum-free media containing 50 ng/ml NGF. The next day, the virus was removed, and the cells were fed with fresh media containing 10 ng/ml NGF. The following day, cells were washed free of NGF with 3 hour-long washes in serum-free, NGF-free media, and then were switched to media with or without 10 ng/ml NGF. Two days later, neuronal survival was assessed. For the p75 activation experiments, the protocol was similar with the exception that, after the washes to remove NGF, neurons were switched to media containing 50 mM KCl with or without 100 ng/ml BDNF for two days.

For the experiments with the activated MEKK1 or p53 adenoviruses, neurons were plated at a density of 10,000 to 12,000 neurons/well in 48 well plates (Falcon) with 20 ng/ml NGF. On the fifth day of culture, medium was removed, the virus was added in a volume of 200 ul DMEM media (Gibco) containing 2 mM glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin (all from BioWhittaker), and 10% fetal bovine serum. After 16-18 hours of infection, the virus-containing media was removed and was replaced with Ultraculture media containing 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 ng/ml NGF. Cultures were maintained for 48 hours, washed four times for one hour each with growth factor-free media, and then switched into the same media containing various concentrations of NGF.

Survival assays were performed as previously described (Slack et al., 1996; Bamji et al., 1998) using non-radioactive cell proliferation (MTT) assays (CellTitre 96, Promega). 50 µl of the MTT reagent as added to each well and left for 90 min. followed by the addition of 100 µl of solubilization solution to lyse the cells. Each condition was repeated in triplicate. In each assay, baseline (0% survival) was considered to be 0 ng/ml NGF, and 10 ng/ml NGF was considered to be 100% survival, unless stated otherwise. All other conditions were related to these values.

Western Blot Analysis

For biochemistry, sympathetic neurons were lysed in Tris buffered saline (TBS) lysis buffer (Knusel et al., 1994) containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 0.2 µg/ml leupeptin, 1.5 mM sodium vanadate, and 0.1% SDS. Cells were collected in cold PBS by gentle scraping to detach them from the collagen substratum, were washed three times with the same

buffer, and then were resuspended in 50 to 100 µl of lysis buffer, following by rocking for 10 min at 4E C. After a 10 minute centrifugation, the lysates were normalized for protein concentration using a BCA Protein Assay Reagent (Pierce, Rockford, Ill). Equal amounts of protein (10 to 50 µg) were then boiled in sample buffer for 5 minutes and separated by SDS-PAGE. After electrophoresis, proteins were transferred to 0.2 µm nitrocellulose for 1.5 hours at 0.6 Amps, and the membrane was washed 3 times with TBS. For all antibodies, the membranes were blocked in 3% non-fat-milk in TBST (blotto) for 1.5 hours at room temperature. The membranes were then incubated overnight at 4E C with the primary antibodies in blotto: anti-p53 (Oncogene Neurosciences), anti-p53 (Novacastra), anti-p21 (Transduction Laboratories), anti-p27 (Transduction Laboratories), anti-Bad (Transduction Laboratories), anti-Bcl2 (Transduction Laboratories), anti-Bclxl (Santa Cruz), anti-Bax (Santa Cruz), anti-E1B55K antibody 2A6, anti-human c-myc (PharMingen), anti- phosphothr183/tyr184JNK (New England Biolabs; Derijard et al., 1994), anti-phosphoser473Akt (New England Biolabs), anti-phosphothr183/tyr185Erk (Promega), anti-TrkA (RTA; the kind gift of Dr. L. Reichardt) (Weskamp and Reichardt, 1991), anti-tyrosine hydroxylase (Chemicon Laboratories), or anti-tubulin (Oncogene Neurosciences). After incubation with the primary antibodies, membranes were washed 4 times with TBST over 40 minutes, and incubated with the secondary antibody for 1.5 hours at room temperature. The secondary antibodies (goat-anti-mouse or goat-anti-rabbit HRP from Boehringer Mannheim Biochemicals) were used at 1/10,000 dilution. After 3 washes with TBST, detection was carried out using the ECL chemiluminescence reagent from Amersham and XAR X-ray film from Kodak.

Analysis of p53 -/- Mice

Mice heterozygous for a targetted mutation in the p53 gene (Donehower et al., 1992) were obtained from GenPharm International (Mountainview, CA). p53-/- mice were maintained in a C57Bl/6 background, as homozygotes or heterozygotes. Progeny from p53-/- x p53+/- or from p53 heterozygote crosses were screened for the mutant allele(s) using PCR. To amplify the mutant allele, PCR was conducted for 35 cycles (94°C for 30 sec, 55°C for 60 sec, 72°C for 90 sec) using the following oligonucleotides: GTGGGAGGGACAAAAGTTCGAGGCC for the 5' end, and TTTACGGAGCCCTGGCGCTCGATGT for the 3' end. This results in a 200 nucleotide fragment in mutant mice and no product in wildtype mice. To amplify the wildtype allele, the same oligonucleotide was used as the 5' primer and ATGGGAGGCTGCCAGTCCTAACCC was

used as the 3' primer. This results in amplification of a 600 nucleotide fragment in heterozygote and wildtype mice, and no product in the p53 -/- mice.

For morphometric analysis, the SCG were removed and immersion-fixed in 4% paraformaldehyde in phosphate buffer (PB) for 1 hour to overnight at 4°C. Ganglia were cryoprotected in graded sucrose solutions, 7 μm thick sections were serially cut on a cryostat, and every section was collected and thaw-mounted onto chrom-alum subbed slides. Slides were stained with cresyl violet and morphometric analyses were performed using a computer-based image analysis system (Biocom, France). Neuronal numbers were determined by counting all neuronal profiles with nucleoli on every third section, as per Coggeshall (1984). This sampling frequency (every 21 μm) ensures that neurons are not double-counted, since the average neuronal diameter does not exceed 21 μm in any of the groups examined (Bamji et al., 1998). This method does not correct for split nucleoli. Statistical results were expressed as the mean ± the standard error of the mean and were tested for significance by a one-tailed Student's t-test.

For TUNEL analysis, SCGs were dissected from p53+/- and p53+/+ littermates at postnatal day 7. Ganglia were fixed for 30 minutes in 4% paraformaldehyde, cryoprotected in graded sucrose solutions, and 7 um thick sections cut on a cryostat. Every third section was collected and thaw-mounted onto chrom-alum subbed slides and TUNEL staining immediately performed using the Boehringer-Mannheim in situ cell death detection kit. The number of TUNEL-positive cells on every third section was determined by fluorescence microscopy, and these numbers were used to determine the total number of TUNEL-positive cells per ganglia. Results were tested for significance by a one-tailed Student's t-test.

V. Results:

p53 and Bax are Elevated Following NGF Withdrawal from Sympathetic Neurons

We have previously demonstrated that an increase in p53 levels is sufficient to cause sympathetic neuron apoptosis in the presence of NGF (Slack et al., 1996). To determine whether endogenous p53 protein levels were ever similarly elevated during sympathetic neuron apoptosis, we cultured sympathetic neurons from neonatal animals, a time when developmental death of these neurons is ongoing. Initially, we examined p53 during sympathetic neuron apoptosis induced by NGF withdrawal; this apoptosis is relatively slow, taking approximately 48 hours, and is transcription-dependent (Deckwerth and Johnson, 1993; reviewed in Johnson and Deckwerth, 1993). Neurons were cultured for 5 days in the presence of 50 ng/ml NGF, NGF was withdrawn, and then the cellular levels of p53 were quantitated using Western blots with anti-p53 at various timepoints post-withdrawal (Fig. 5.1A). This analysis revealed that p53 levels were elevated approximately 3-fold by 16 hours following NGF withdrawal (Fig. 5.1A). Elevation of p53 protein was first observed at 12 hours (data not shown), and was maintained until at least 36 hours post NGF-withdrawal (Fig. 5.1A). Interestingly, this timecourse corresponds to the "commitment point" following which NGF-withdrawn sympathetic neurons cannot be rescued from apoptotic death (Johnson and Deckwerth, 1993). Thus, NGF withdrawal leads to an increase in p53 levels that correlates with the timecourse of commitment to an apoptotic death.

To determine whether this increase in p53 had functional consequences, we examined the expression of a well-characterized p53 transcriptional target, the cyclin-dependent kinase p21 (El-Diery et al., 1993). Western blot analysis of equal amounts of protein from sympathetic neurons at various timepoints following NGF withdrawal revealed that p21 levels were increased 2-fold by 16 hours following NGF withdrawal, and that this increase was maintained for at least 36 hours (Fig. 5.1B). This increase was not specific to p21, since levels of p27, a second cyclin-dependent kinase (Toyoshima and Hunter, 1994), were also increased approximately 2-fold at 24 and 36 hours following NGF withdrawal (Fig. 5.1C).

We next investigated the levels of Bax, another transcriptional target of p53 (Miyashita and Reed, 1995) that is known to be essential for naturally-occurring sympathetic neuron death (Deckwerth et al., 1996; Easton et al., 1997). Western blot analysis revealed that, like p53, Bax levels increased approximately 2-fold following NGF withdrawal (Fig. 5.1F). This increase was first observed at 16 hours, and levels were maximal by 24 hours (Fig. 5.1F). Interestingly,

Western blot analysis revealed that a second proapoptotic member of this family, Bad (Yang et al., 1995), was also increased 3-fold following NGF withdrawal (Fig. 5.1D). Like Bax, this increase in Bad commenced at approximately 16 hours post-withdrawal, the committment point for apoptosis. In contrast to Bad and Bax, Western blot analysis for the anti-apoptotic protein Bcl-2 (Hockenberry et al., 1990) revealed that levels of this protein were relatively constant over this timecourse, decreasing slightly at 24 hours and later. A similar decrease was observed for another prosurvival member of this family, Bcl-xl (Boise et al., 1993), which was decreased by 36 hours following NGF withdrawal (Fig. 5.1G). Thus, these data indicate i) that two transcriptional targets of p53, p21 and Bax, are increased following NGF withdrawal, and ii) that the balance of proapoptotic to prosurvival members of the Bcl2 family is significantly shifted following removal of NGF.

We also examined signalling proteins that are activated in response to TrkA receptor activation, including the ERKs (Virdee and Tolkovsky, 1995; Creedon et al., 1996) and Akt, a serine/threonine kinase thought to play a key role in promoting NGF-dependent sympathetic neuron survival (Crowder and Freeman, 1998). Western blot analysis with an antibody specific to the phosphorylated form of the serine/threonine kinase Akt revealed that, 12 hours following NGF withdrawal, phosphorylation of Akt was greatly reduced (Fig. 5.1H). Western blot analysis with an antibody specific to the phosphorylated form of the ERKs revealed a similar decrease in the phosphorylation of these proteins following NGF withdrawal (Fig. 5.1H). Thus, NGF withdrawal leads to a decrease in signalling via these TrkA-regulated prosurvival pathways coincident with induction of potentially proaptotic pathways.

Elevated p53 is Necessary for Sympathetic Neuron Apoptosis in Response to NGF Withdrawal

To determine whether this increase in p53 protein levels was an essential component of the apoptotic cascade that follows NGF withdrawal, we took advantage of the adenoviral E1B55K protein, which functionally ablates p53 (Yew et al., 1992). Specifically, neonatal sympathetic neurons were cultured for 3-4 days in 50 ng/ml NGF, and then were infected with one of two recombinant, replication-defective adenoviruses; one of these adenoviruses expressed E1B55K while the second expressed a mutant E1B55K protein (A262) that is defective in its ability to bind and ablate p53 (Yew et al., 1990). As a further control, neurons were infected with a similar moi

of recombinant adenovirus expressing ß-galactosidase (Slack et al., 1996). Two days following viral infection, neurons were washed free of NGF and two days later, neuronal survival was measured using MTT assays (Fig. 5.2A). These experiments demonstrated that E1B55K, but not the E1B55K mutant A262 or ß-galactosidase, was able to rescue sympathetic neurons from apoptosis. In 5 independent experiments, E1B55K rescued 49% (100 moi) and 63% (500 moi) of the neurons relative to those kept alive in 10 ng/ml NGF, increases which were highly significant ($P<0.005$ in both cases). In none of these experiments did either the A262 or ß-galactosidase virus have a significant effect on neuronal survival (Fig. 5.2A).

To ensure that the effects of E1B55K were being mediated via p53, we examined levels of p53 in NGF-withdrawn sympathetic neurons following viral infection; the vector used not only ablates p53 through the actions of E1B55K, but also targets p53 for degradation through the adenoviral E4orf6 product (Teodoro and Branton, 1997; Yew and Berk, 1992; Querido et al., 1997). For these experiments, sympathetic neurons were initially selected in 50 ng/ml NGF for 3 days, were infected with the adenoviruses expressing E1B55K or the mutant E1B55K, and two days later were withdrawn from NGF. As a control, neurons were not infected or were infected with the ß-galactosidase virus. Analysis of p53 protein levels 36 hours following this treatment revealed that levels of p53 protein were similarly elevated in NGF-withdrawn neurons that were uninfected, or that were expressing either ß-galactosidase or the mutant E1B55K (Fig. 5.3A). In contrast, p53 protein levels were significantly reduced in the neurons expressing E1B55K, as predicted. Reprobing of the same blot with an antibody specific for the cytoskeletal protein tubulin confirmed that equal amounts of protein were present in all of the samples (Fig. 5.3B). Moreover, the differential effect of E1B55K versus the A262 mutant was not due to a difference in levels of expression of these two proteins, since the A262 protein was expressed at higher levels than E1B55K (Fig. 5.3C). These experiments therefore indicate that elevated p53 protein levels are essential for NGF-withdrawal induced apoptosis of sympathetic neurons.

p53 is Downstream of p75NTR and is Essential for p75NTR-Mediated Neuronal Apoptosis

We have previously demonstrated that the immediate early protein, c-jun, is hyperphosphorylated in sympathetic neurons following p75NTR activation (Bamji et al., 1998) as it is following NGF withdrawal (Ham et al., 1995; Estus et al., 1994). To determine whether p53 elevation was also a common downstream component of these two apoptotic signalling cascades,

we examined p53 levels in sympathetic neurons in conditions where p75NTR activation leads to apoptosis. Specifically, for these experiments, sympathetic neurons were cultured for 5 days in 50 ng/ml NGF and then were switched to KCl, which maintains sympathetic neuron survival in the absence of TrkA activation (Franklin et al., 1995). We then added the neurotrophin BDNF, which selectively binds p75NTR but not Trk receptors on sympathetic neurons (Bamji et al., 1998), and determined cellular p53 levels (Fig. 5.4A). As seen with NGF withdrawal, p53 levels were increased at 24 and 36 hours in neurons maintained in KCl plus BDNF relative to those in KCl alone. Reprobing of the same blot with an antibody to tyrosine hydroxylase revealed that equal amounts of protein were present in all of the samples (Fig. 5.4B). This increase was already apparent at 12 hours, a timepoint when p53 levels were only first starting to increase following NGF withdrawal (data not shown). Thus, p53 elevation is downstream of both p75NTR activation and NGF withdrawal.

To determine whether this increased p53 was essential for p75NTR-mediated apoptosis, we performed rescue experiments using the E1B55K adenovirus. Specifically, neurons were cultured in 50 ng/ml NGF for 3-4 days, were infected overnight with recombinant adenovirus, and two days later were switched to media containing 50 mM KCl plus or minus 100 ng/ml BDNF. Neuronal survival was then measured after 48 hours using MTT assays (Fig. 5.2B). As we have previously reported (Bamji et al., 1998), the addition of BDNF to 50 mM KCl caused a decrease in sympathetic neuron survival of approximately 54% (Fig. 5.2B). Expression of the mutant E1B55K had no significant effect on this decrease (P=0.11). In contrast, expression of E1B55K rescued 100% of the p75NTR-driven apoptosis (P=0.012) (Fig. 2B), a rescue similar to that observed for NGF-withdrawal induced apoptosis. Thus, p53 elevation was an essential component of the apoptotic cascades induced in cultured sympathetic neurons by both NGF withdrawal and p75NTR activation.

p53 is Downstream of the MEKK-JNK Pathway, and is Essential for MEKK-Mediated Neuronal Apoptosis

These results indicated that the apoptotic cascades originating from NGF withdrawal and p75NTR activation share two common components; hyperphosphorylation of c-jun and p53 elevation. On the basis of these findings, we hypothesized that p53 was downstream of the MEKK-JNK pathway, which leads to c-jun hyperphosphorylation (Yan et al., 1994; Derijard et

al., 1994). To test this hypothesis, we generated a recombinant adenovirus expressing an activated form of MEKK1 which has previously been shown to cause sympathetic neuron apoptosis and c-jun hyperphosphorylation (Eilers et al., 1998). We first confirmed that this virus expressed the recombinant myc epitope-tagged MEKK1 protein, and that it was capable of activating the MEKK1 downstream target, JNK (Yan et al., 1994), in sympathetic neurons. Specifically, sympathetic neurons were grown in 20 ng/ml NGF for 4 days, were infected with 20 moi of recombinant virus expressing activated MEKK1 or ß-galactosidase, and then were analyzed two days later for expression of the myc-tagged MEKK1 on Western blots with anti-myc (Fig. 5.5A). Analysis of equal amounts of protein from ß-galactosidase versus MEKK1-infected sympathetic neurons revealed the presence of a myc-immunoreactive protein of the appropriate size, 35 kD, in the MEKK1-infected neurons (Fig. 5.5A). To confirm that virally-expressed MEKK1 was capable of activating JNK, we performed similar experiments and then analyzed the lysates for phosphorylation of JNK using a phospho-JNK antibody (Fig. 5.5B). In this experiment, we compared MEKK1-infected sympathetic neurons to sympathetic neurons withdrawn from NGF, where JNK is known to be activated (Eilers et al., 1998). Western blot analysis revealed that the activated MEKK1 adenovirus caused phosphorylation of JNK to the same level as did NGF withdrawal, relative to neurons maintained in 10 ng/ml NGF alone (Fig. 5.5B).

We next determined whether adenovirus-mediated expression of activated MEKK1 was sufficient to cause sympathetic neuron apoptosis, as was previously reported with microinjection (Eilers et al., 1998). Specifically, neurons were selected in 20 ng/ml NGF for 5 days, infected with various concentrations of recombinant virus expressing activated MEKK1 or ß-galactosidase, maintained in 20 ng/ml NGF for a further 4 days, and then assayed for neuronal survival. Results from four separate experiments revealed that activated MEKK1 led to neuronal apoptosis; at 20, 50, or 100 moi, sympathetic neuron survival was decreased to 52%, 54%, and 44%, respectively, relative to neurons infected with the same concentration of ß-galactosidase virus (Fig. 5.2C). Similar results were obtained with neurons maintained in 10 ng/ml and 50 ng/ml NGF (data not shown).

We next determined whether activation of the MEKK-JNK pathway caused elevation of p53 levels, as we had hypothesized. Specifically, sympathetic neurons were maintained in 20 ng/ml NGF for 4 days, were infected with 20 or 50 moi of the activated MEKK1 adenovirus, and were then maintained in 10 ng/ml NGF for a further 2 days prior to analysis. To ensure that viral

infection itself did not induce p53, we infected sister cultures with the ß-galactosidase adenovirus. Western blot analysis of equal amounts of protein revealed that p53 levels were increased when sympathetic neurons were infected with 20 or 50 moi of MEKK1 adenovirus, and that the magnitude of this increase was similar to that observed following NGF withdrawal for 48 hours (Fig. 5.5C). In contrast, no increase in p53 levels was seen upon infection with similar mois of the ß-galactosidase adenovirus (data not shown), consistent with the observation that this latter virus had little or no effect on neuronal survival (Fig. 5.2C). Thus, activation of the MEKK-JNK pathway led to increased levels of p53 in the presence of NGF.

To determine whether p53 was an essential component of the MEKK1-induced apoptotic signalling cascade, we asked whether the E1B55K adenovirus could rescue the apoptotic effects of activated MEKK1. To perform these experiments, sympathetic neurons were cultured for 5 days in 20 ng/ml NGF, and were infected with 50 moi of the MEKK1 virus plus or minus 500 moi of the E1B55K virus. As a baseline for this study, we first determined whether coinfection with E1B55K reduced the p53 levels induced by activated MEKK1 alone (Fig. 5.3D). Western blot analysis of sympathetic neurons infected for two days revealed that p53 protein levels were reduced in neurons expressing E1B55K plus activated MEKK1 relative to those expressing MEKK1 alone (Fig. 5.3D). Reprobing of the same blot with an antibody specific for TrkA confirmed that equal amounts of protein were present in all of the samples (Fig. 5.3E).

To determine whether this reduction in p53 levels mediated by E1B55K rescued sympathetic neurons from apoptosis induced by activated MEKK1, we performed similar experiments and, four days following infection, MTT assays were performed. As negative controls in this experiment, we used adenoviruses expressing the mutant A262 protein or ß-galactosidase. Results of three separate experiments indicated that the E1B55K adenovirus was able to significantly rescue MEKK1-induced neuronal apoptosis (Fig. 5.2D) relative to MEKK1 alone, or relative to MEKK1 plus the ß-galactosidase or A262 adenovirus ($p<0.005$ for E1B55K in all cases) (Fig. 5.2D). Thus, elevation of p53 protein levels is necessary for sympathetic neuron apoptosis following activation of the MEKK-JNK pathway.

One potential downstream candidate for the apoptotic effects of p53 in sympathetic neurons following MEKK-JNK pathway activation is the proapoptotic protein Bax. To determine whether Bax levels were increased in response to the MEKK1 adenovirus as they were following NGF withdrawal (Fig. 5.1F), we selected sympathetic neurons for 4 days in 20 ng/ml NGF,

infected them with 50 moi of the MEKK1 adenovirus, switched them into 10 ng/ml NGF and two days later performed Western blots with anti-Bax. This analysis revealed that Bax levels were increased approximately 2-fold in sympathetic neurons expressing activated MEKK1 (Fig. 5.5D), an increase similar in magnitude to that seen following NGF withdrawal (Fig. 5.1F).

Previous studies have demonstrated that Bax is necessary for sympathetic neuron apoptosis following NGF withdrawal (Deckwerth et al., 1996). To determine whether proapoptotic proteins like Bax are also necessary for sympathetic neuron apoptosis following activation of the MEKK-JNK pathway, we generated a recombinant adenovirus expressing a prosurvival member of this pathway, Bcl-xl (Boise et al., 1993). Using this adenovirus, we then asked whether altering the balance between prosurvival versus proapoptotic members of this family could rescue MEKK1-induced apoptosis. Specifically, sympathetic neurons were cultured for 5 days, and were infected with 50 moi of the MEKK1 adenovirus plus or minus various concentrations of Bcl-xl virus. For comparison, neurons were infected with 50 moi of MEKK1 virus plus 500 moi E1B55K or A262. Neurons were then maintained in 20ng/ml NGF for 4 additional days prior to measuring survival using MTT assays. This analysis revealed that the Bcl-xl adenovirus was able to rescue the apoptotic effects of activated MEKK1 at 20 (data not shown) or 50 moi (Fig. 5.2E). A similar rescue was observed with the E1B55K virus (Fig. 5.2E), while no rescue was observed with the A262 virus (Fig. 5.2E). Similar results were obtained when the MEKK1 adenovirus was used at 100 moi, although the magnitude of the rescue effect with lower concentrations of Bcl-xl was decreased (data not shown).

p53 is Upstream of Bax and Downstream of JNK

These experiments demonstrated that JNK, p53 and Bax are all downstream of activated MEKK, and that elevated p53 is essential for MEKK-induced apoptosis. To determine whether p53 is downstream of JNK and upstream of Bax, as we had hypothesized, we took advantage of a recombinant adenovirus expressing human p53 (previously described in Slack et al., 1996). Specifically, sympathetic neurons were cultured for 4 days in 20 ng/ml NGF, were infected with 20 moi of p53-expressing adenovirus and 48 hours later, cell lysates were analyzed by Western blots with anti-p53, anti-phosphoJNK or anti-Bax. This analysis revealed that the p53 adenovirus increased expression of p53 to levels similar to those observed following NGF withdrawal (Fig. 5.5G), but that this elevated expression of p53 had no effect on JNK phosphorylation in

sympathetic neurons maintained in 20 ng/ml NGF (Fig. 5.5E). In contrast, infection with the p53 virus caused an increase in the levels of Bax protein (Fig. 5.5F) that was similar in magnitude to that observed following NGF withdrawal (Fig. 5.1F) or following infection with the activated MEKK1 adenovirus (Fig. 5.5D). Together with the previous experiments with activated MEKK1, these experiments indicate that MEKK and JNK are upstream of p53, while Bax is downstream (Fig. 5.7).

p53 is Essential for Naturally-Occurring Sympathetic Neuron Death In Vivo

Our previous work indicates that naturally-occurring sympathetic neuron death is a result both of suboptimal activation of the TrkA receptor and of coincident activation of p75NTR (Bamji et al., 1998). Since, in culture, both of these types of neuronal apoptosis require p53, we hypothesized that p53 would also be essential for sympathetic neuron death in vivo. To test this hypothesis, we examined the superior cervical ganglia (SCG) of transgenic mice in which the p53 gene was deleted by homologous recombination (Donehower et al., 1992). In control mice, the SCG contains approximately 20000 - 25000 neurons at birth, depending on the genetic background. Over the ensuing two weeks approximately half of these neurons are lost so that by P15, control SCGs contain approximately 13,000 neurons (Bamji et al., 1998). We therefore chose to analyze the SCG from p53+/+, p53+/- and p53-/- mice at two timepoints; postnatal days 1 and 15, timepoints immediately preceding and following the normal period of naturally-occurring cell death (Fig. 5.6A,D).

Analysis of the SCG at postnatal day 1 revealed that sympathetic neuron numbers prior to the period of naturally-occurring death were similar regardless of the presence or absence of p53 (Fig. 5.6D). The SCG of p53+/+ animals contained 23,976 $\pm$ 2764 neurons (n=3), a number similar to mice of other genetic backgrounds (Bamji et al., 1998), while those from p53+/- and p53-/- animals contained 20,537 $\pm$2514 (n=5) and 19016 $\pm$ 2675 (n=3), respectively (Fig. 5.6D). All of these numbers were statistically similar ($P>0.1$ for all comparisons). However, analysis of the SCG from animals of these same genotypes at P15 (Fig. 5.6A,D), following the period of naturally-occurring death, revealed significant differences. In control p53+/+ animals the P15 SCG contained 13,163 $\pm$ 875 neurons (n=5) (Fig. 5.6D). In contrast, the SCG of p53 +/- animals contained 20,352 $\pm$ 944 neurons (n=6), and that of p53-/- mice contained 20,600 $\pm$ 1709 neurons

(n=3), statistically significant increases of 55% and 56%, respectively (P<0.005 in both cases). A comparison within the same genotype from P1 to P15 revealed that while the p53+/+ ganglia lost approximately 45% of its sympathetic neurons over this timeframe (P<0.05) (Fig. 5.6D), there was no significant loss of sympathetic neurons in either the p53+/- or p53-/- SCG over the same period (Fig. 5.6D) (P>0.3).

To confirm that this difference in neuron number in the SCG during the period of naturally-occurring cell death reflected a deficit in apoptosis in the p53+/- and p53-/- mice, and not due to an increase in the proliferation of neuronal progenitors that occurs for a short period postnatally in the SCG (Hendry, 1977), we analyzed the total number of apoptotic cells in the SCG at postnatal day 7, when sympathetic neuron apoptosis is ongoing. To perform this analysis, SCG from p53+/- and p53+/+ littermates were sectioned, and every third section was analyzed by in situ TUNEL-labelling. This analysis revealed that TUNEL-positive cells were detected in the ganglia of both p53+/- and p53+/+ animals (Fig. 5.6B), but that the total number of apoptotic nuclei was significantly decreased in the p53+/- ganglia (p53+/- SCG, mean = 661 $\pm$19, n=5; p53+/+ SCG, mean = 1025 $\pm$ 155, n=4; p = 0.016) (Fig. 5.6C). Thus, the magnitude of sympathetic neuron apoptosis is decreased when p53 levels are decreased in vivo.

VI. Discussion:

In this study, we have examined the role of the p53 tumor suppressor in naturally-occurring sympathetic neuron death. Our findings support an essential role for p53 in this process and, more specifically, support the following conclusions. First, following NGF withdrawal of neonatal sympathetic neurons, p53 levels are increased with a timecourse that is consistent with it playing a role in the "commitment" to apoptosis. The magnitude of this increase is similar to that required to induce sympathetic neuron apoptosis using adenovirus-mediated transduction of p53 (Slack et al., 1996). Second, levels of two p53 transcriptional targets, p21 and Bax, are also increased; the latter of these two, Bax, has already been shown to be essential for sympathetic neuron apoptosis following NGF withdrawal (Deckwerth et al., 1996). Third, p53 levels are also increased when sympathetic neuron apoptosis is induced by p75NTR activation, with a similar timecourse to that observed following NGF withdrawal. Fourth, a decrease in levels of p53 mediated by the E1B55K protein is sufficient to inhibit sympathetic neuron apoptosis induced either by NGF withdrawal or by p75NTR activation, indicating that increased p53 levels are both necessary and sufficient (Slack et al., 1996) for sympathetic neuron apoptosis. Fifth, NGF withdrawal and p75NTR activation may induce apoptosis via a pathway involving MEKK-JNK-p53-Bax (Fig. 5.7) since i) expression of activated MEKK1, which is sufficient to cause sympathetic neuron apoptosis, leads to elevated levels of p53 and Bax, ii) expression of p53 leads to elevated Bax levels, but does not affect JNK activation, and iii) elevated p53 is essential for MEKK-induced apoptosis. Finally, the physiological relevance of these observations is indicated by our findings that, when p53 is reduced or absent, naturally-occurring sympathetic neuron death is inhibited in vivo. Thus, our data indicate that p53 is a common, essential target during developmental sympathetic neuron death that is downstream of the MEKK-JNK pathway and that may well integrate apoptotic signals deriving both from p75NTR activation and from a lack of NGF/TrkA receptor activation.

The p53 tumor suppressor protein encodes a transcriptional regulator that functions to control cell proliferation and apoptosis in a cell context-dependent fashion (reviewed in Levine, 1997; Jacks and Weinberg, 1998). The precise mechanism by which p53 mediates apoptosis is not well understood, but it is believed to proceed by a number of mechanisms including direct transactivation, transcriptional repression, and direct involvement in DNA cleavage (Caelles et al., 1994; Elledge and Lee, 1995; Lane, 1993; Miyashita and Reed, 1995). Within the nervous

system, p53 is upregulated in neurons following a number of traumatic insults, including kainic acid and ischemia (Sakhi et al., 1994; Li et al., 1994; Wood and Youle, 1995), and is necessary for excitotoxicity-induced apoptosis of hippocampal and cortical neurons (Morrison et al., 1996; Xiang et al., 1998). However, p53 has not been thought to be involved in naturally-occurring neuronal cell death, nor has it been thought to be an essential downstream effector of death receptor activation. In particular, a number of previous studies have failed to implicate p53 in sympathetic neuron death. In one study, Martinou et al. (1995) demonstrated that microinjection of the adenoviral protein E1B19K, but not E1B55K, rescued sympathetic neurons from apoptosis due to NGF withdrawal. The inability to rescue sympathetic neuron apoptosis with microinjected E1B55K as opposed to adenovirally-expressed E1B55K is likely due to the lower levels of expression obtained using microinjection. In this regard, we have ourselves compared the rescue of sympathetic neurons using adenovirally-expressed E1B19K versus E1B55K, and E1B19K was able to rescue sympathetic neurons at titers approximately 10-fold lower than those needed for E1B55K (L. Pacquet, F. Miller, and D. Kaplan, unpublished data). In a second set of studies, cultured p53-/- sympathetic neurons died following NGF withdrawal, leading to the conclusion that p53 played no role in developmental sympathetic neuron death (Sadoul et al., 1996; Davies and Rosenthal, 1994). The studies presented here demonstrate that neurons with lowered p53 levels still die in vivo, although the magnitude of this death is significantly lower than in controls; these results do not necessarily contradict the finding that p53-/- neurons die in culture following NGF withdrawal. Moreover, results might also differ in response to acute inhibition of p53 (as mediated via E1B55K) versus a chronic loss of p53 (as in p53-/- mice). For example, the developmental absence of p53 may well cause compensatory upregulation of parallel apoptotic pathways. Precedent for such compensation derives from our previous studies with another tumor suppressor protein, pRb. In Rb-/- mice, cortical neurons are unable to differentiate appropriately and, as a consequence, undergo neuronal apoptosis in vivo (Slack et al., 1998). However, when the same Rb-/- cortical progenitors are cultured and undergo the transition to postmitotic neurons in vitro, they differentiate normally and do not undergo apoptosis (Slack et al., 1998); this difference is explained by a selective upregulation of p107 and p130, two other Rb family members, in culture but not in vivo (Ruth Slack, personal communication). A similar explanation could be invoked for cultured p53-/- neurons; sympathetic neurons express p73 (C. Pozniak and F.

Miller, unpublished data), an apoptotic p53 family member (Kaghad et al., 1997; Jost et al., 1997) that might well compensate for the lack of p53 in a mutant p53 background.

What is the signal transduction pathway that derives either from p75NTR activation or from a lack of activation of TrkA and that leads to increased p53? Our data implicate the MEKK-JNK pathway (Yan et al., 1994; Derijard et al., 1994) (Fig. 7). This pathway is activated in sympathetic neurons in response to either NGF withdrawal (Ham et al., 1995; Eilers et al., 1998) or p75NTR activation (Bamji et al., 1998), and the resultant hyperphosphorylation of c-jun is necessary for NGF-withdrawal induced sympathetic neuron death (Ham et al., 1995; Estus et al., 1994). The data presented here indicate that activation of the MEKK-JNK pathway causes sympathetic neuron death via a p53-dependent mechanism, thereby providing a potential direct link from p75 to p53. Moreover, our finding that the stress-induced (Herdegen et al., 1997) MEKK-JNK pathway acts via p53 in neurons may explain why excitotoxicity-induced death of CNS neurons can be inhibited by deletion of either the JNK3 (Yang et al., 1997) or p53 genes (Morrison et al., 1996; Xiang et al., 1998).

Are there other pathways that cause elevated p53 following NGF withdrawal or p75NTR activation? A second candidate upstream pathway involves deregulation of the cell cycle; in cycling cells, p53 is viewed as a "fail-safe" mechanism that causes cellular apoptosis when proliferative mechanisms are deregulated (reviewed in Levine, 1997; Jacks and Weinberg, 1996). Such cell cycle deregulation has been proposed to occur in sympathetic neurons following NGF withdrawal (Park et al., 1997; Freeman et al., 1994; Rubin et al., 1993). It is therefore possible that NGF withdrawal causes two different signals, MEKK-JNK activation and cell cycle deregulation, that together converge on p53. Thus, p53 may function as a sensor to induce neuronal apoptosis when the sum of these upstream signals reaches a critical level.

What are the downstream events that are responsible for p53-mediated neuronal apoptosis? One potential downstream mechanism involves the protein Bax, which is known to be essential for sympathetic neuron apoptosis (Deckwerth et al., 1996), and for p53-dependent death of cortical neurons (Xiang et al., 1998). Data presented here indicate that, following the commitment of sympathetic neurons to apoptosis, the balance of proapoptotic versus prosurvival members of the bcl-2 family is considerably shifted to the proapoptotic, and that one of the changes contributing to this shift is an increase in Bax protein levels. Moreover, we demonstrate that increased expression of p53 alone is sufficient to cause increased Bax protein levels in sympathetic neurons. Since Bax

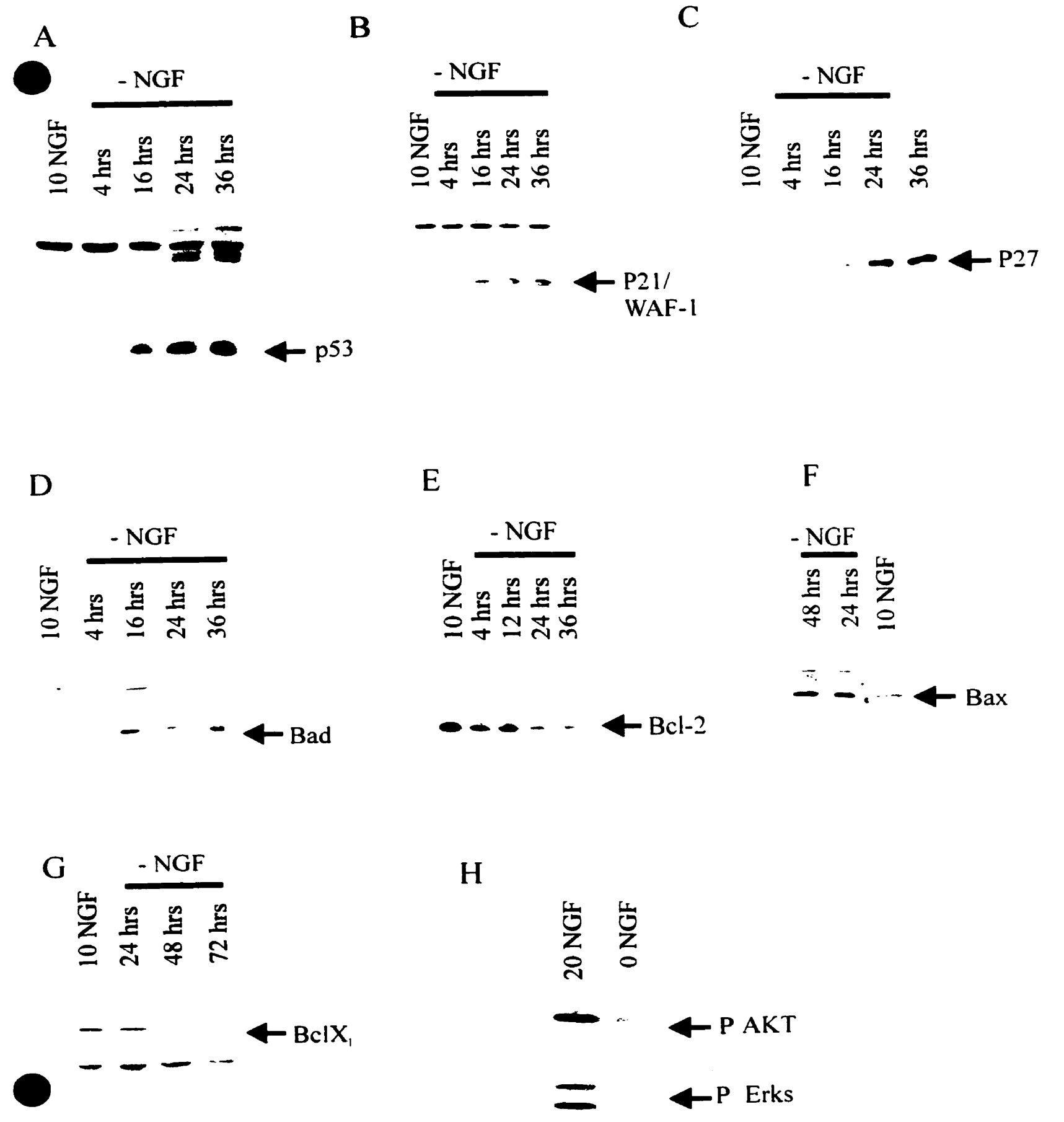
is a direct transcriptional target of p53 (Miyashita and Reed, 1995), we propose that elevated p53 protein levels cause increased p53-dependent transcription of Bax, and that this is sufficient to tip the balance towards neuronal apoptosis. In support of this hypothesis, exogenous p53 is unable to mediate apoptosis of Bax-/- cortical neurons (Xiang et al., 1998), Bax-/- sympathetic neurons do not undergo apoptosis in response to NGF withdrawal (Deckwerth et al., 1996), and increased expression of Bcl2 or Bcl-xl (Garcia et al., 1992; Gonzalez-Garcia et al., 1995) in sympathetic neurons rescues NGF withdrawal induced cell death.

The in vivo data presented here support a number of additional conclusions. First, our studies demonstrate that the postnatal period of naturally-occurring sympathetic neuron death is inhibited, but not completely eliminated when p53 levels are lowered, indicating that p53 is essential for normal apoptosis in the SCG, but also suggesting that other, potentially parallel death pathways are important. Such pathways may involve other p53 family members such as p73 (Kaghad et al., 1997; Jost et al., 1997) or p51 (Osada et al., 1998; Trink et al., 1998). Second, our data showing decreased apoptosis in the p53+/- SCG support the idea that it is not the presence or absence of p53 that determines apoptosis, but it is instead the levels of p53 that are important. Precedent for such a gene dosage effect has been documented with regard to p53 and tumorigenesis (Harvey et al., 1993; Donehower et al., 1992). Third, our in vivo data demonstrate that, although p53 may be essential embryonically for regulating the proliferation and survival of sympathetic neuroblasts (Vogel and Parada, 1998), that by birth any resultant deficits are not obvious, since neuron numbers in the newborn p53+/+ versus p53-/- SCG are not significantly different.

What is the biological rationale for p53 acting as a "threshold" to neuronal apoptosis? During the period of naturally-occurring death, sympathetic neurons compete for limiting amounts of trophic support to ultimately ensure an appropriate target innervation density. The net result of this competition is a loss of approximately 40-50% of sympathetic neurons over the first two weeks of postnatal life. Our previous data indicate that both TrkA and p75NTR are required for this process (Bamji et al., 1998). Moreover, these same studies demonstrate that robust activation of TrkA antagonizes p75NTR-derived apoptotic signals and that, conversely, p75NTR can override TrkA-derived survival signals. On the basis of the current study, we propose that p53 is a convergent downstream target that "sums" opposing signals deriving from TrkA versus p75NTR activation, and that apoptosis is triggered when the balance of "death" signals deriving from

p75NTR is greater than the "survival" signals deriving from TrkA. In cellular terms, this ensures that all neurons that are incapable of sequestering sufficient target-derived NGF are rapidly and efficiently eliminated from competition.

One final issue derives from the emerging similarities between sympathetic neuron apoptosis as induced by NGF withdrawal and p75NTR activation. In both cases, the MEKK-JNK pathway is induced, and in both cases, p53 is required for apoptosis. In the case of p75, while this receptor has been demonstrated to signal via a number of pathways, including ceramide production (Dobrowsky et al., 1994), JNK activation (Casaccia-Bonnefil et al., 1996), c-jun hyperphosphorylation (Bamji et al., 1998) and NFkB activation (Carter et al., 1996), the results presented here are the first to identify a required component, p53, of the p75NTR apoptotic pathway. Given the similarities between NGF withdrawal and p75NTR activation, we propose that NGF-withdrawal-induced apoptosis may be, to a large extent, a p75NTR-mediated process. In particular, our previous work (Bamji et al., 1998) indicates that NGF-withdrawal induced apoptosis of sympathetic neurons is greatly delayed in the absence of p75NTR. We propose that, during naturally-occurring sympathetic neuron death, p75NTR may well provide the basis for a constitutive apoptotic signal in sympathetic neurons via one of two different mechanisms. First, there may be an autocrine p75NTR-dependent apoptotic loop; sympathetic neurons synthesize BDNF (Causing et al., 1997) that they process and secrete (C.G. Causing, R. Aloyz, and F.D. Miller, unpublished observations). Since BDNF can bind to p75NTR to cause sympathetic neuron apoptosis (Bamji et al., 1998), and since BDNF is required in vivo for a portion of appropriate naturally-occurring sympathetic neuron death (Bamji et al., 1998), then this may indicate the presence of an ongoing autocrine BDNF:p75NTR death signal. A second possibility is that p75NTR functions to mediate a constitutive death signal in the absence of ligand, as previously suggested (Rabizadeh et al., 1993). However, it is clear that addition of exogenous BDNF is essential for a maximal p75NTR-dependent apoptotic signal (Bamji et al., 1998), indicating that even if a constitutive death mechanism(s) is in place, activation of p75NTR by exogenous, potentially target-derived, neurotrophins is likely to play a key role. In either case, exposure to an appropriate neurotrophin, in this case NGF, would lead to TrkA activation, thereby providing a mechanism for overriding p75 and p53-mediated apoptosis.


A
- NGF
10 NGF
4 hrs
16 hrs
24 hrs
36 hrs
p53
B
- NGF
10 NGF
4 hrs
16 hrs
24 hrs
36 hrs
P21/
WAF-1
C
- NGF
10 NGF
4 hrs
16 hrs
24 hrs
36 hrs
P27
D
- NGF
10 NGF
4 hrs
16 hrs
24 hrs
36 hrs
Bad
E
- NGF
10 NGF
4 hrs
12 hrs
24 hrs
36 hrs
Bcl-2
F
- NGF
48 hrs
24 hrs
10 NGF
Bax
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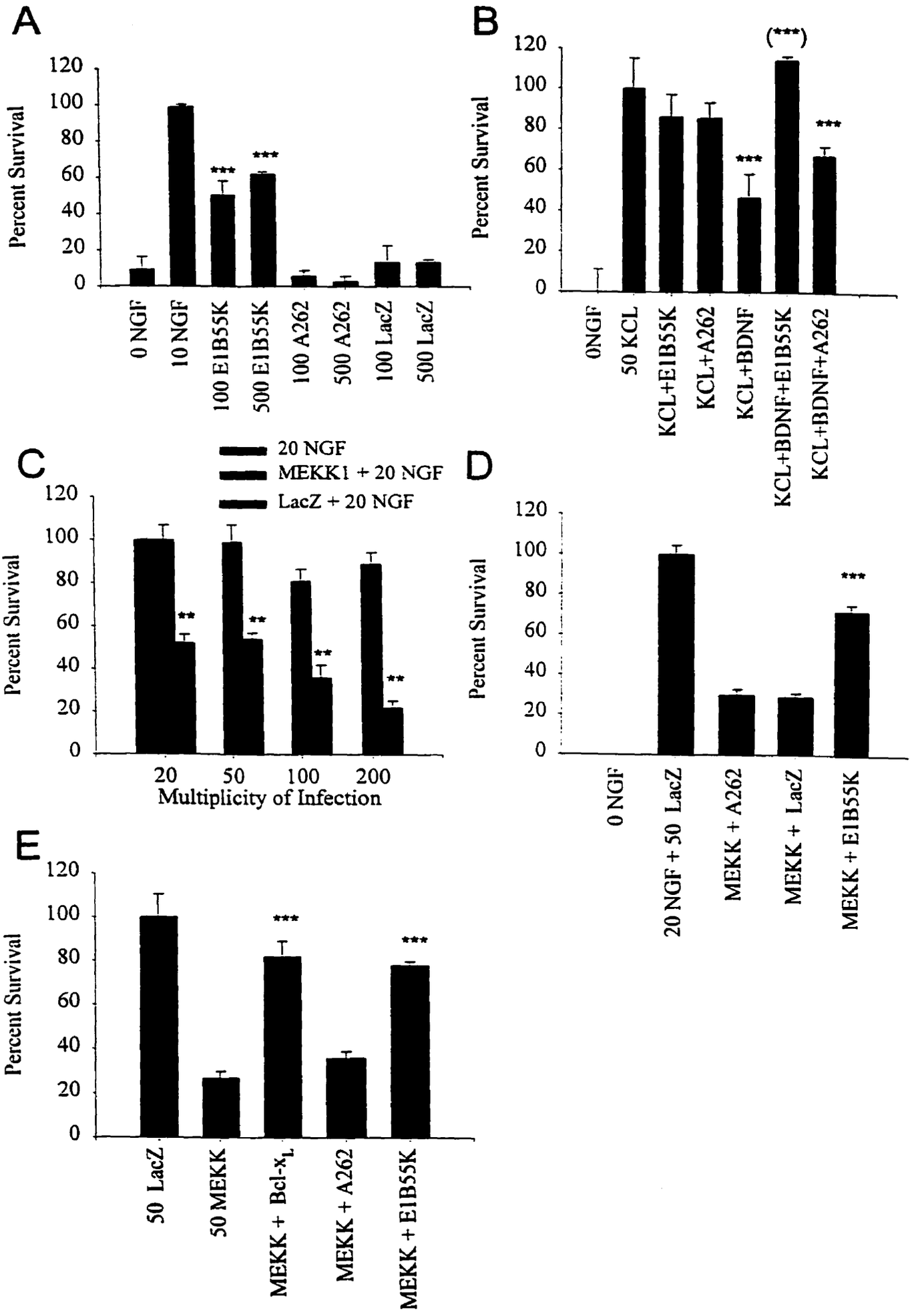
10 NGF
24 hrs
48 hrs
72 hrs
BclX$_L$
H
20 NGF
0 NGF
P AKT
P Erks


A
Percent Survival
120
100
80
60
40
20
0
***
***
0 NGF
10 NGF
100 E1B55K
500 E1B55K
100 A262
500 A262
100 LacZ
500 LacZ
B
Percent Survival
(***)
***
***
0NGF
50 KCL
KCL+E1B55K
KCL+A262
KCL+BDNF
KCL+BDNF+E1B55K
KCL+BDNF+A262
C
20 NGF
MEKK1 + 20 NGF
LacZ + 20 NGF
Percent Survival
**
**
**
**
20
50
100
200
Multiplicity of Infection
D
Percent Survival
***
0 NGF
20 NGF + 50 LacZ
MEKK + A262
MEKK + LacZ
MEKK + E1B55K
E
Percent Survival
***
***
50 LacZ
50 MEKK
MEKK + Bcl-x$_L$
MEKK + A262
MEKK + E1B55K

A

0 NGF
10 NGF
A262
E1B55K

p53

B

0 NGF
10 NGF
A262
E1B55K

Tubulin

C

A262
E1B55K

E1B55K

D

20 NGF
MEKK/E1B55K
MEKK

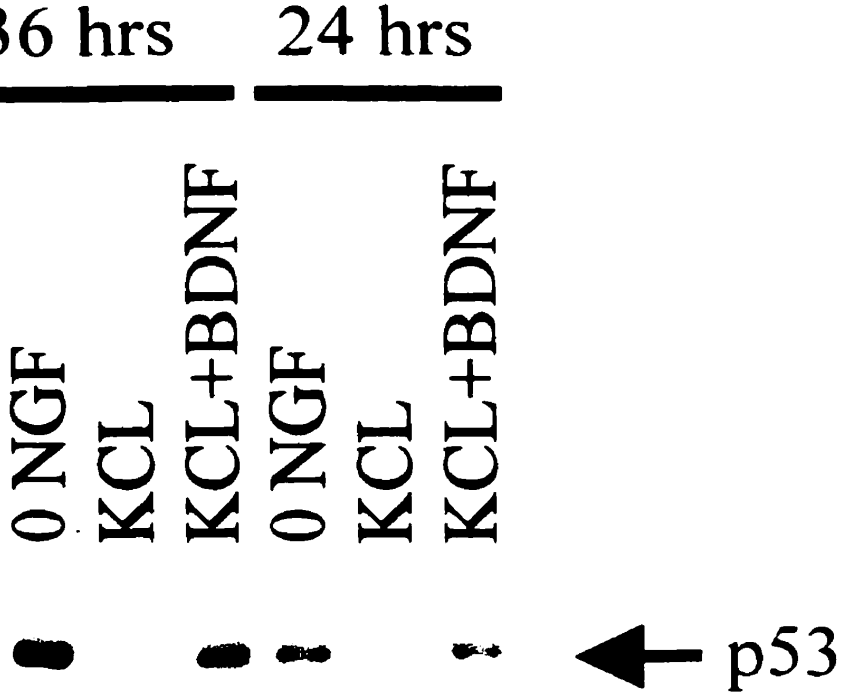
p53

E

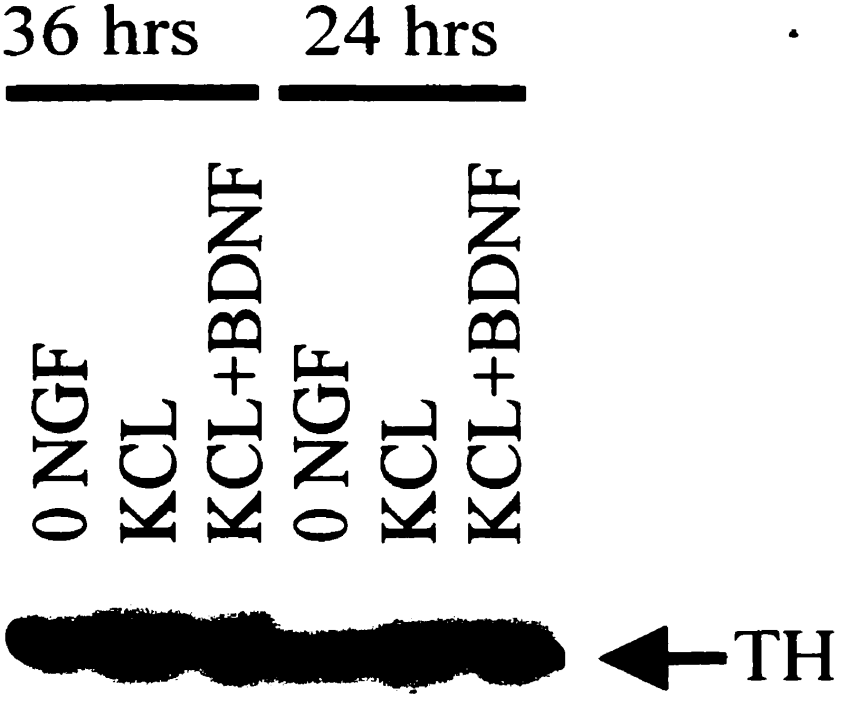
20 NGF
MEKK/E1B55K
MEKK

Trk A

A

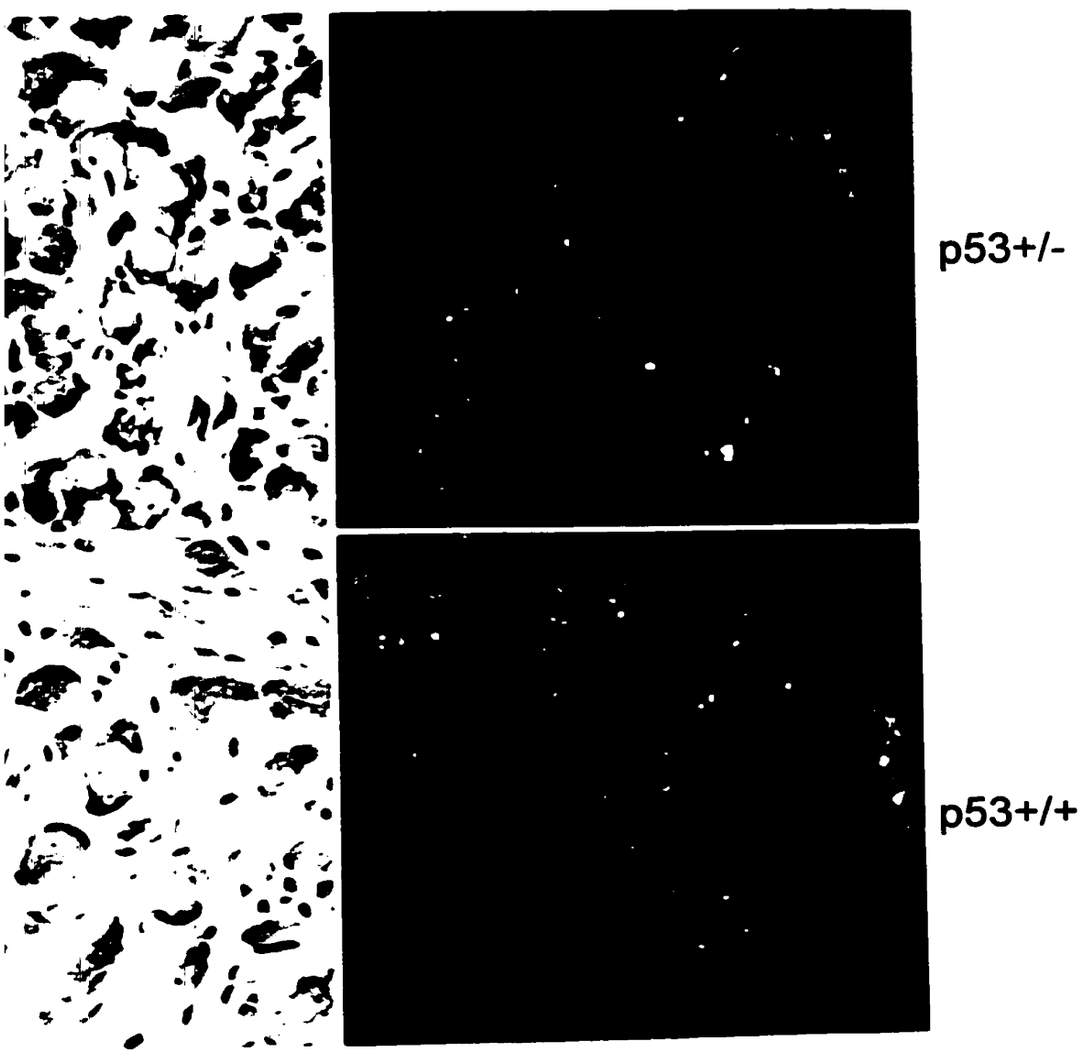

36 hrs
24 hrs
0 NGF
KCL
KCL+BDNF
0 NGF
KCL
KCL+BDNF
p53

B

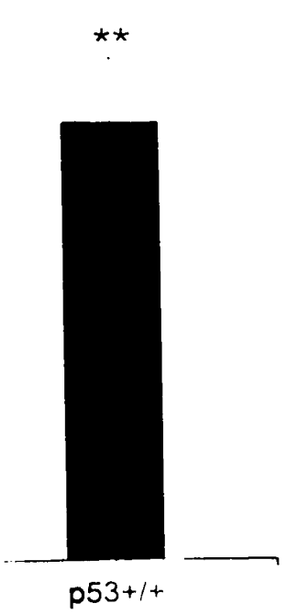

36 hrs
24 hrs
0 NGF
KCL
KCL+BDNF
0 NGF
KCL
KCL+BDNF
TH

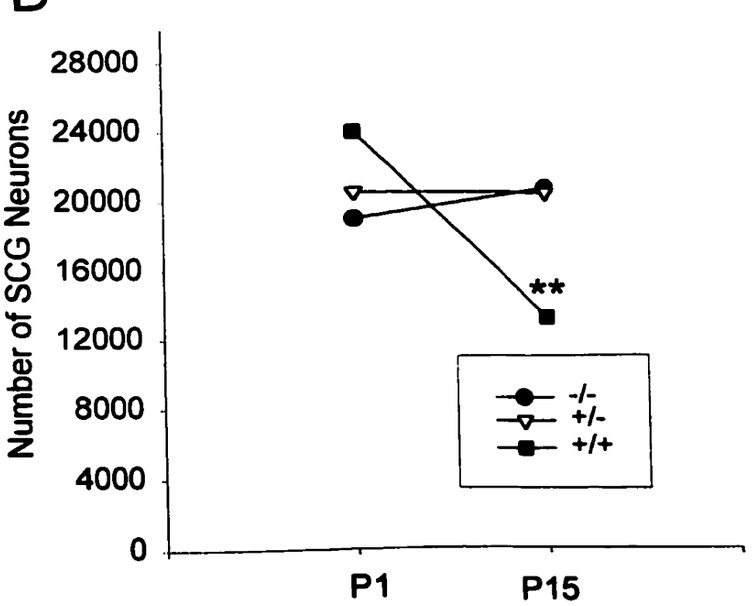
A
control
20 moi MEKK
MEKK
B
0 NGF
10 NGF
48 hrs
50 moi MEKK
Phospho-JNK
C
0 NGF
10 NGF
20 moi MEKK
p53
D
10 NGF
50 moi MEKK
BAX
E
20 NGF
20 moi p53
Phospho-JNK
F
20 NGF
20 moi p53
BAX
G
20 NGF
20 moi p53
50 moi MEKK
0 NGF
p53

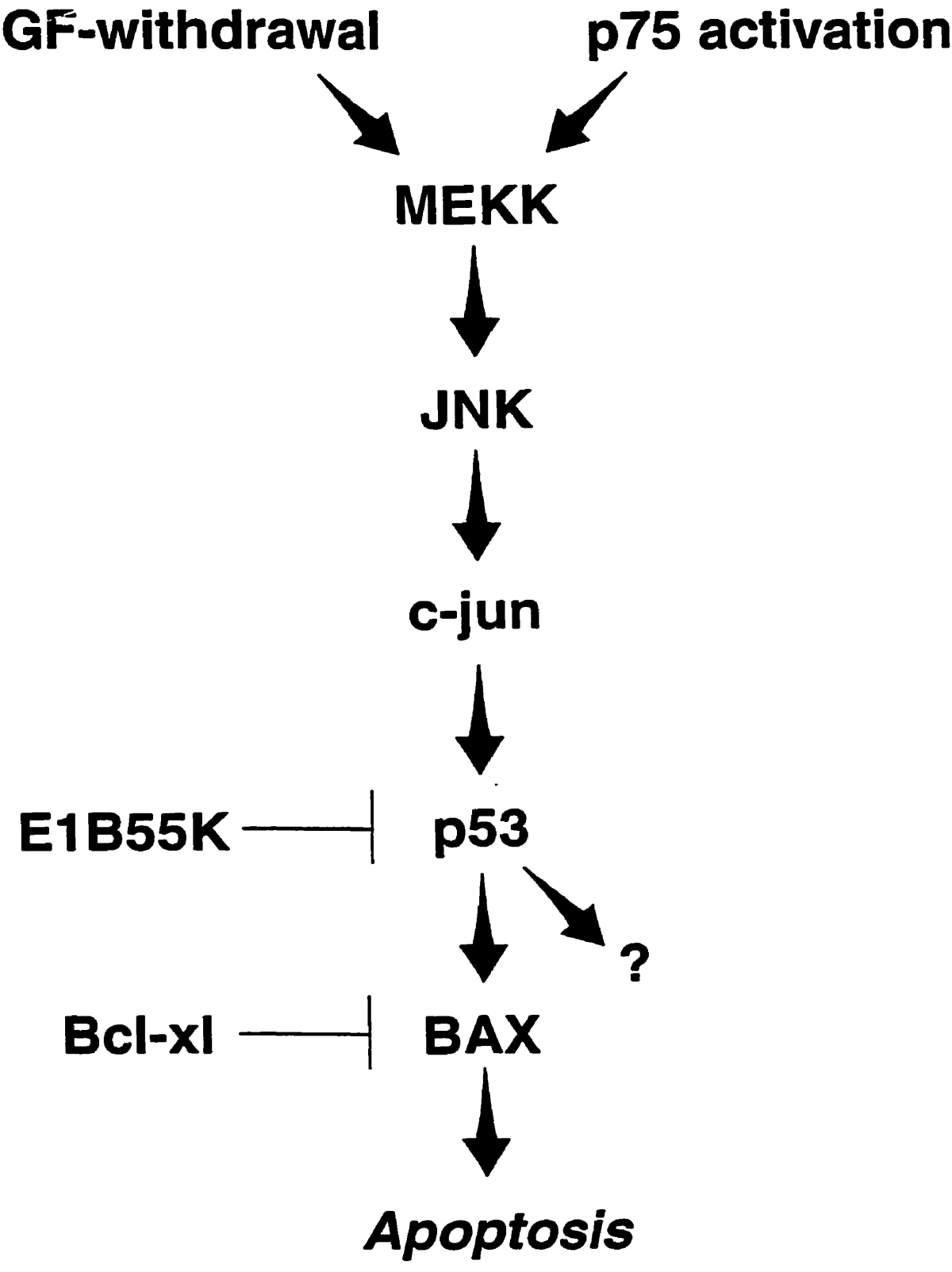
B


p53+/-
p53+/+

D


**
p53+/+


Number of SCG Neurons
28000
24000
20000
16000
12000
8000
4000
0
**
-/-
+/-
+/+
P1
P15


NGF-withdrawal
p75 activation
MEKK
JNK
c-jun
E1B55K
p53
?
Bcl-xl
BAX
Apoptosis

VIII. Figure Legends:

Figure 5.1. p53 and its transcriptional targets, p21 and Bax, are increased during NGF-withdrawal induced apoptosis of sympathetic neurons. (A-G) Western blot analysis of equal amounts of protein derived from sympathetic neurons either maintained in 10 ng/ml NGF (NGF), or at various timepoints from 4 to 48 hours following withdrawal of NGF (-NGF).Western blots were probed with antibodies specific to p53 (A), p21 (B), p27 (C), Bad (D), Bcl-2 (E), Bax (F), or Bcl-xl (G). Note that p53, p21, p27, Bad and Bax all increase following NGF withdrawal, while Bcl-2 and Bcl-xl decrease. (H) Western blot analysis of equal amounts of protein derived from sympathetic neurons either maintained in 20 ng/ml NGF, or withdrawn from NGF for 12 hours (0 NGF). Western blots were probed with antibodies specific to phosphorylated forms of Akt (P AKT) or ERKS (P Erks). Note that phosphorylation of these TrkA targets was significantly decreased following NGF withdrawal.

Figure 5.2. E1B55K-mediated inhibition of p53 rescues sympathetic neuron apoptosis as induced by NGF withdrawal (A), p75NTR activation (B), and activated MEKK (C-E). (A) p53 and NGF withdrawal-induced apoptosis. Sympathetic neurons were infected in parallel with recombinant adenovirus expressing E1B55K, the mutant E1B55K A262, or ß-galactosidase (LacZ) at titers of 100 or 500 moi and NGF was withdrawn from the media two days later. After 48 hours, cell survival was measured by MTT assays. 0 NGF represents uninfected sympathetic neurons that were also withdrawn from NGF, while 10 NGF represents uninfected sympathetic neurons maintained in the presence of 10 ng/ml NGF for the course of the experiment. Results are those obtained in a representative experiment performed in triplicate, and represent the mean +/- the standard error. Similar results were obtained in five separate experiments. *** indicates those values that are significantly different from the 0 NGF control ($P<0.005$). Note that only those neurons transduced with E1B55K were rescued from NGF withdrawal-induced apoptosis. (B) p53 and p75-induced apoptosis. Sympathetic neurons were infected with recombinant adenovirus expressing E1B55K or the mutant E1B55K A262 at a titer of 500 moi and two days later were switched into 50 mM KCl (KCL+E1B55K and KCL+A262) or 50 mM KCl plus 100 ng/ml BDNF (KCL+BDNF+E1B55K and KCL+BDNF+A262). As controls, uninfected neurons were switched into 50 mM KCl (50 KCL), into 50 mM KCl plus 100 ng/ml BDNF (KCL+BDNF), or were withdrawn from NGF (0NGF). After 48 hours, cell survival was measured using MTT

assays. Results are those obtained in a representative experiment performed in triplicate, and represent the mean +/- standard error. Similar results were obtained in 4 separate experiments. *** indicates those values that are significantly different from 50 mM KCl alone, and (***) indicates those values amongst the points that received BDNF that are significantly different from KCL+BDNF ($P<0.005$). Note that, as previously reported (Bamji et al., 1998), BDNF reduces neuronal survival in the presence of KCl, and that E1B55K but not A262 is able to completely rescue this BDNF-mediated apoptosis. (C) MEKK1-induced apoptosis. Sympathetic neurons were infected with recombinant adenovirus expressing activated MEKK1 or ß-galactosidase (LacZ) at concentrations of 20 to 200 moi, and were maintained in 20 ng/ml NGF for 4 days following infection. As controls, uninfected neurons were maintained for the entire experiment in 20 ng/ml NGF. Cell survival was measured using MTT assays, and results are those obtained in a representative experiment performed in triplicate, and represent the mean +/- standard error. Similar results were obtained in 4 separate experiments. ** indicates those values that are significantly different between MEKK1 and the ß-galactosidase control at a given moi ($P<0.005$). Note that activated MEKK1 decreases sympathetic neuron survival in a concentration-dependent fashion. (D) p53 and MEKK1-induced apoptosis. Sympathetic neurons were infected with 50 moi of MEKK1 plus 500 moi of E1B55K (MEKK + E1B55K), A262 (MEKK + A262) or ß-galactosidase (MEKK + LacZ), were maintained in 20 ng/ml NGF for four days following infection, and survival was then measured using MTT assays. As further controls, neurons were infected with 50 moi ß-galactosidase virus (20 NGF + 50 LacZ) for the same timeperiod, or were withdrawn from NGF for the final two days. Results are those obtained in a representative experiment performed in triplicate, and represent the mean +/- standard error. Results are normalized so that 0 NGF is 0% survival, and 20 ng/ml NGF plus 50 moi ß-galactosidase (20 NGF + 50 LacZ) is 100% survival. Similar results were obtained in 3 separate experiments. *** indicates that E1B55K significantly rescues MEKK1-induced killing of sympathetic neurons ($P<0.005$). (E) p53 in JNK-Bax cell death pathway. Sympathetic neurons were infected with 50 moi of the activated MEKK1 adenovirus (50 MEKK) plus or minus 50 moi Bcl-xl (MEKK + Bcl-xl), 500 moi E1B55K (MEKK + E1B55K) or 500 moi A262 (MEKK + A262) adenoviruses. As a control, neurons were infected with 50 moi ß-galactosidase adenovirus (50 LacZ). Four days following the initial infection, during which time neurons were maintained in 10 ng/ml NGF, survival was measured using MTT assays. Results are those obtained in a representative

experiment performed in triplicate, and represent the mean +/- standard error. Results are normalized so that 0 NGF is 0%, and 10 ng/ml NGF plus 50 moi ß-galactosidase adenovirus is 100% survival. *** indicates those values that are significantly different from MEKK alone ($P<0.005$).

Figure 5.3. E1B55K reduces p53 levels in sympathetic neurons withdrawn from NGF. (A) Western blot analysis for p53 in sympathetic neurons that were withdrawn from NGF for 36 hours (0 NGF), that were maintained in 10 ng/ml NGF (10 NGF), or that were infected with 500 moi of recombinant adenovirus expressing A262 or E1B55K, and two days later were withdrawn from NGF for 24 hours. Equivalent amounts of protein from lysed neurons were electrophoresed, transferred to nitrocellulose filters, and p53 protein levels were assessed using anti-p53. Note that p53 levels increase following NGF withdrawal, and that expression of E1B55K reverses this increase, while expression of A262 has no effect. (B) The same blot as in panel (A) reprobed for the cytoskeletal protein tubulin. (C) Western blot analysis for E1B55K using anti-E1B55K in equal amounts of protein derived from sympathetic neurons infected for 36 hours with adenoviruses expressing E1B55K or the E1B55K mutant A262. The antibody used for these studies recognizes both the wildtype and mutant proteins. (D) Western blot analysis for p53 in sympathetic neurons that were maintained in 20 ng/ml NGF, and that were infected with 50 MOI of recombinant adenovirus expressing activated MEKK1 (MEKK) with or without 200 MOI of E1B55K for 36 hours. Equivalent amouonts of protein from lysed neurons were electrophoresed, transferred to nitrocellulose filters, and p53 protein levels were assessed using anti-p53. (E) The same blot as in panel (D) reprobed for TrkA.

Figure 5.4. p53 levels increase during apoptosis of sympathetic neurons as induced by BDNF-mediated activation of p75NTR. (A) Western blot analysis for p53 in equal amounts of protein derived from sympathetic neurons that were cultured in 50 ng/ml NGF for 4 days, and then were washed free of NGF and switched into 50 mM KCl (KCL), 50 mM KCl plus 100 ng/ml BDNF (KCL+BDNF), or media containing no NGF or KCl (0 NGF) for 24 or 36 hours. Note that p53 levels in the neurons treated with KCl and BDNF are similar to those in 0 NGF, and are greater than those maintained in KCl alone. (B) The same blot as in panel (A) reprobed for the

neurotransmitter enzyme, tyrosine hydroxylase (TH) to demonstrate that equal amounts of protein were present in each of the lanes.

Figure 5.5. (A-D) p53 and Bax protein levels increase following activation of the MEKK-JNK pathway in sympathetic neurons. (A) Western blot analysis for c-myc in equal amounts of protein derived from sympathetic neurons infected with 20 moi myc-tagged MEKK1 (20 moi MEKK) or ß-galactosidase (control) adenovirus for 48 hours. In both cases, neurons were maintained in 20 ng/ml NGF for the entirety of the experiment. (B) Western blot analysis for phospho-JNK in equal amounts of protein derived from sympathetic neurons that were infected with 50 moi MEKK1 adenovirus and maintained for 48 hours (50 moi MEKK), or from uninfected sister cultures that were either maintained in 10 ng/ml NGF (10 NGF), or that were withdrawn from NGF for 48 hours (0 NGF). Note that the level of phospho-JNK immunoreactivity is similar in neurons withdrawn from NGF or transduced with activated MEKK1. (C) Western blot analysis for p53 in equal amounts of protein derived from sympathetic neurons that were infected with 20 moi MEKK1 adenovirus and maintained in 20 ng/ml NGF for 48 hours (20 moi MEKK), or from uninfected sister cultures that were maintained in 10 ng/ml NGF (10 NGF), or that were withdrawn from NGF for 48 hours (0 NGF). Note that p53 protein levels are increased by activated MEKK1 as they are by NGF withdrawal. (D) Western blot analysis for Bax in equal amounts of protein derived from sympathetic neurons that were infected with 50 moi MEKK1 adenovirus and maintained in 20 ng/ml NGF for 48 hours (50 moi MEKK), or from uninfected sister cultures that were maintained in 10 ng/ml NGF for the same timeperiod. **(E,F) Increased expression of p53 in sympathetic neurons causes increased Bax protein, but does not affect phosphorylation of JNK.** (E) Western blot analysis for phospho-JNK in equal amounts of protein derived from sympathetic neurons infected with 20 moi MEKK1 adenovirus and maintained in 20 ng/ml NGF for 48 hours, or from uninfected sister cultures that were maintained in 20 ng/ml for the same timeperiod. (F) Western blot analysis for Bax in equal amounts of protein derived from sympathetic neurons infected with 20 moi MEKK1 adenovirus and maintained in 20 ng/ml NGF for 48 hours, or from uninfected sister cultures maintained in 20 ng/ml NGF for the same timeperiod. (G) Western blot analysis for p53 in equal amounts of protein derived from sympathetic neurons maintained in 20 ng/ml NGF and infected with 20 moi p53

adenovirus or 50 moi activated MEKK adenovirus for 30 hours. As a control, neurons were withdrawn from NGF (0 NGF) for 30 hours.

Figure 5.6. The number of sympathetic neurons in the superior cervical ganglia is increased when p53 levels are decreased in vivo. (A) Photomicrographs of cresyl-violet stained sections through the SCG of wildtype (p53+/+) versus p53+/- mice of the same genetic background at postnatal day 15, when naturally-occurring sympathetic neuron death is complete. (B) Fluorescent photomicrographs of in situ TUNEL labelling in the SCG of p53+/+ versus p53+/- mice of the same genetic background at postnatal day 7, when developmental sympathetic neuron death is ongoing. (C) The number of TUNEL-positive cells in the SCG of P7 mice that are heterozygous (p53+/-) or homozygous (p53-/-) for a p53 mutation created by homologous recombination (Donehower et al., 1992). Results are expressed as the mean ∀ standard error of the total number of TUNEL-positive cells per SCG (n=5 for p53+/- and n=4 for p53+/+). ** indicates that these two values are significantly different p=0.016. (D) The number of neurons in the SCG of P1 or P15 mice that are either p53+/+ (control), or heterozygous (p53+/-) or homozygous (p53-/-) for the p53 mutant allele. Results are expressed as the mean ∀ standard error (n=3 for P1 p53+/+, 5 for P1 p53+/-, 3 for P1 p53-/-, 5 for P15 p53+/+, 6 for P15 p53+/- and 3 for P15 p53-/-). ** indicates values significantly different from p53+/+ SCG of the same age ($P<0.05$). Note that while there is a statistically significant loss of 45% of the neurons in the p53+/+ SCG over this timeperiod (**, $P<0.05$), there is no significant loss of neurons in either the p53+/- or p53-/- SCG ($P>0.3$).

Figure 5.7. Model of sympathetic neuron apoptosis induced by NGF withdrawal or p75NTR activation. Both of these apoptotic stimuli increase p53 and Bax protein levels, and require elevated p53 levels to efficiently promote cell death. Neuronal death induced by activated MEKK1 also increases p53 and Bax protein levels, and requires elevated levels of p53 protein. NGF withdrawal-mediated sympathetic neuron death increases JNK and c-jun phosphorylation and activity, and requires c-jun (Ham et al., 1995; Estus et al., 1994). p75NTR activation in sympathetic neurons also leads to hyperphosphorylation of c-jun, presumably via JNK (Bamji et al., 1998). Bax is also essential for sympathetic neuron apoptosis in vivo and in vitro (Deckwerth et al., 1996; Easton et al., 1997). The precise MEKK or JNK family member in these pathways is not known, and other intermediate proteins, such as SEK1 (Sanchez et al., 1994) are likely

involved as intermediate steps in this hypothetical cascade. Moreover, although this model shows JNK signalling to p53 via c-jun, recent work indicates that JNK can directly interact with and phosphorylate p53 (Hu et al., 1997). p53 may induce death by increasing Bax protein or activity, or via other p53 target proteins (Polyak et al., 1997).

IX. Acknowledgements:

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CHAPTER 6: GENERAL DISCUSSION

I. Overview of the Role of Neurotrophins in the Development of the Vertebrate Nervous System:

Neurotrophins exert multiple effects on the developing and mature nervous system. In addition to their well-defined role in promoting and maintaining the survival of differentiated neurons, these proteins affect neuron precursor cell proliferation and differentiation, regulate neurotransmitter and neuropeptide synthesis, and influence neuronal morphology and synaptic plasticity throughout life (reviewed in Gu,1995; Hallböök et al.,1995; Sendtner,1995). A brief account of the roles neurotrophins play during neuronal development is described in this section, which is aimed at integrating the findings of the previous chapters and setting them in a general developmental framework.

One of the earliest roles for neurotrophins during neuronal development is the induction of terminal mitosis in proliferating neuroblasts. Interestingly, this ability is not exhibited by all neurotrophins, and thus far, only BDNF and NT-3 have been shown to affect the "birth date", or terminal mitosis, of a neuron (Cohen-Cory and Fraser,1994; Ahmed et al.,1995; Liu et al.,1997; Jungbluth et al.,1997). The inability of NGF to elicit a similar effect on dividing neuroblasts is probably due to the lack of expression of its cognate receptor, TrkA, in these cells (Verdi et al.,1994). Indeed, the timecourse of expression of the neurotrophins and their cognate Trk receptors indicates that BDNF and NT-3 play crucial roles during early neuronal development, whereas NGF exerts its effects at later timepoints. How are BDNF and NT-3 targeted to dividing neuroblasts? It has recently been shown that BDNF is secreted in an autocrine/ paracrine manner by dividing neuronal precursors, to affect the differentiation of these cells (Cohen-Cory and Fraser,1994; Liu et al.,1997). This represents a local mechanism of control whereby timing of differentiation within populations is regulated by that population itself. It is crucial however to coordinate the development of the nervous system, and it is highly likely that distant neuronal populations are also able to impinge on newly developing systems, to coordinate their development. How does this occur? We hypothesize that established neuronal populations might temporally regulate differentiation within newly developing target populations, via the anterograde secretion of neurotrophins. Indeed, correlative data has suggested that BDNF is anterogradely transported by early developing

interneurons to mediate the differentiation of motor neuron progenitors (Jungbluth et al.,1997). Our results demonstrate that BDNF is anterogradely transported by noradrenergic neurons in the developing CNS, and we postulate that this early secretion of BDNF in the vicinity of TrkB-expressing cortical neuroblasts may regulate the differentiation of these cells. These effects may be direct, or indirect via the regulation of neurotrophin expression in target neurons, and is further discussed below.

The survival of neurons immediately following exit from the cell cycle, and prior to target innervation, is generally believed to be neurotrophin-independent. *In vitro* studies of different populations of cranial sensory neurons have shown that the initial duration of neurotrophin independence is controlled by an intrinsic timing mechanism and correlates with the distance an axon needs to cover prior to target innervation (Vogel and Davies, 1991). For example, vestibulocochlear neurons have close targets and survive for only a short duration in the absence of neurotrophins, whereas nodose neurons have distant targets and are neurotrophin-independent for a longer duration (Vogel and Davies, 1991). This is not to say that neurotrophins do not exert any effects at this developmental stage. Indeed, sympathetic neurons already express their TrkA and p75 receptors at this point, and we hypothesize that their differential activation may play a role during the formation of neuronal connections (discussed below). Moreover, early sympathetic neurons express the TrkC receptor, which may modulate other neuronal functions, although it is not required for survival (Fagan et al.,1996).

According to the classical neurotrophin hypothesis, innervating neurons become dependent on limiting quantities of target-derived neurotrophin for survival, in a mechanism that allows a target to dictate its innervation density. However upon closer examination, neuronal development at this stage appears more complicated. Indeed, multiple neurotrophic factors from multiple sources have been shown to act concurrently and sequentially during target field innervation in several neuronal systems, and have been shown to exert both positive and negative effects on neuronal survival. The final outcome after exposure to multiple neurotrophins depends on the receptor repertoire expressed by the neuron as well as their spatio-temporal pattern of expression. What is the biological significance of having multiple neurotrophic factors acting concurrently or sequentially on neuronal populations? First, let us

examine how multiple neurotrophic factors can act *concurrently* on a neuronal population with a set receptor repertoire, to sculpt the development of that population.

The rapid elimination of excess neurons that are unable to sequester appropriate levels of target neurotrophins during the time of innervation, is generally referred to as programmed cell death. Our laboratory has recently shown that the absence of the appropriate neurotrophin alone is insufficient to drive rapid neuronal apoptosis, which further requires the activation of the p75 receptor by other neurotrophins (Bamji et al.,1998). We hypothesise that it is the precise cohort of neurotrophins to which a neuron is exposed that determines cell survival or death (see Fig.6.1), and postulate that these neurotrophins can be derived retrogradely from target cells, anterogradely from afferent neurons, locally in an autocrine/ paracrine manner, or from glial cells. Such a mechanism, whereby neurotrophins act positively and negatively to affect neuronal survival, reveals a level of sophistication that was previously unpredicted by the neurotrophin hypothesis, and may play an essential role in the matching of neurons with their targets throughout the nervous system.

To ensure appropriate target innervation and overall circuit fidelity, it is also important to have a mechanism that ensures the elimination of axon collaterals that have innervated inappropriate targets. Recent evidence has suggested that neurotrophins can inhibit the growth and elongation of axons via the p75NTR in the absence of their cognate Trk receptors (Kimpinski et al.,1998). We now propose that this provides a system whereby inappropriate target innervation is determined by the neurotrophin repertoire expressed in targets and the receptor repertoire on innervating neurons, and that this inappropriate innervation is actively eliminated by the action of one or more of these neurotrophins on the p75NTR (Fig.6.1). Thus, we propose that during the time of innervation multiple neurotrophic factors act to ensure appropriate target innervation. Although it has yet to be determined whether innappropriate target innervation takes place in the absence of the p75NTR, ectopic expression of NGF in glial cells of transgenic mice resulted in aberrant sympathetic innervation to these cells within the cerebellum (Walsh and Kawaja,1998). This aberrant sympathetic innervation was increased when NGF was ectopically expressed in
p75 -/- mice (Walsh and Kawaja,1998).

Following the successful innervation of a target, sympathetic neurons are faced with innervating an increasingly large area as the target increases in size. In order to respond to

increases in target area and, therefore, increases in NGF levels, a neuron must be able to respond to large variations of NGF in a graded fashion. We propose that the negative modulation of TrkA tyrosine kinase signaling by p75 plays an integral role in enabling the neuron to respond to a large range of NGF concentrations. Moreover, increases in terminally-derived NGF upregulate the ratio of p75 to TrkA, suggesting NGF may widen its own concentration response range via a modulatory feedback loop that regulates neuronal responses to NGF itself. Indeed, the NGF-induced increase in the ratio of p75 to TrkA could serve to ensure that the growth response would occur over a much broader concentration range than predicted by the number of TrkA receptors alone (reviewed in Miller, 1994)

These are situations whereby neurotrophins acting concurrently on a given neuronal population can exert very different and indeed, opposing effects, on the cell via the Trk and p75 receptors. This is not to say that neurotrophins always exert opposing effects on cells. Indeed, given the receptor repertoire of the cell, multiple neurotrophins may act synergistically to mediate the survival of the cell as in the case of BDNF and NT-3 on early trigeminal neurons, and, NT-3 and NGF on mature sympathetic neurons. What is the biological significance of neurotrophin redundancy? In fact, although neurotrophins may act redundantly to mediate the survival of a neuron, it is still not clear whether these factors mediate exactly the same biological effects, or whether they mediate subtly different influences not detectable by examination of gross effects like survival and death. Indeed, it is likely that as the mechanisms by which neurotrophins mediate their effects are studied in greater detail, differences between the intracellular signaling events and corresponding biological effects that are mediated by the different neurotrophins will become apparent.

Neurotrophins may also exert distinct effects at different timepoints during the development of a neuron. In this sense, there are changes in the functions of neurotrophins, and changes in the particular neurotrophins serving these functions, throughout the development and maturation of different neuronal lineages. For example, following the period of neurotrophin-independency, trigeminal sensory neurons becomes transiently responsive to both BDNF and NT-3, which mediate a synergistic effect on survival (Davies et al., 1986). Following a brief period during which a neuron is responsive to BDNF, NT-3 and NGF, the neuron loses its responsiveness to BDNF and NT-3, and becomes dependent on NGF for survival. Although sympathetic neurons were believed to undergo a similar switch in

neurotrophin responsiveness, from NT-3-dependence to NGF-dependence, such a straightforward change in neurotrophin dependency is not as evident. Despite the fact that sympathetic neurons are not generally believed to require TrkC signaling for survival (Fagan et al.,1996), the high expression of TrkC at early timepoints during sympathetic neuron development may affect subtle changes on the role of NT-3 on sympathetic neurons. It is still unclear what these roles may be.

The recent realization that neurotrophins can also be anterogradely transported in the CNS and PNS makes it important to examine the effects of innervating neurons and their neurotrophin repertoire, on the survival of target populations. Indeed, our lab has recently shown that overexpression of BDNF in afferent noradrenergic neurons, can mediate the survival of injured facial motor neurons (Fawcett et al.,1998). Such a scheme represents an afferent-supplied adjunct to retrograde signaling that characterizes the neurotrophins.

Recent studies have made it clear that the role of BDNF and NT-3 in the CNS extends beyond that of differentiation and trophic support. Indeed, BDNF and NT-3 (but not NGF) produce rapid, neuromodulatory effects in a variety of adult systems, and can augment neuronal firing rates, neurotransmitter release, metabolism and the phenotypic expression of enzymes in cholinergic, dopaminergic, serotinergic or peptidergic neurons (reviewed in Lu and Figurov,1997). In the CNS, the rapid effects of BDNF are best described for the long-term potentiation of hippocampal synaptic responses (reviewed in Lu and Figurov,1997). This role for BDNF has implications for learning and memory, and the failure of cognitive abilities in dementia including Parkinson's and Alzeimer's disease.

II. Anterograde Transport of BDNF in the CNS:

(i) Regulation of BDNF Secretion:

The anterograde transport of neurotrophins in the brain has classically been demonstrated by the use of highly specific antibodies, which have identified the presence of BDNF and NT-3 in nerve terminals of brain and PNS populations that do not contain the corresponding mRNA (Radka et al.,.1996; Altar et al.,1997; Conner et al.,1997; Smith et al.,1997;Yan et al.,1997). We have furthered these studies and have demonstrated a functional role for anterogradely-secreted BDNF in mediating the development and survival of target neurons. How is this release of trophic factor regulated? Several studies have suggested that neurons of the CNS are

able to target neurotrophins to the regulated secretory pathway for release at presynaptic nerve terminals (reviewed in Altar and DiStefano,1998). This activity-dependent release of anterogradely transported neurotrophins is in keeping with the proposed neurotransmitter-like role for neurotrophins in the CNS. CNS neurons contain both constititive and regulatory release pathways, the latter being used to package neurotransmitters within synaptic vesicles and neuropeptides within large dense-core vesicles (reviewed in Kelly,1991). Indeed, BDNF immunoreactivity in the rat brain is enriched in synaptosomal fractions, its distribution resembling that of synaptotagmin, a protein associated with synaptic vesicles and large dense-core vesicles at nerve terminals (Fawcett et al.,1997). Furthermore, endogenous BDNF in cortex (Grisbeck et al.,1995) and [^{125}I]-BDNF preloaded into a synaptosomal fraction of cortex and hippocampus (Murray-Rust et al.,1993) can be released following depolarization with potassium and glutamate, similar to that seen with neuropeptides.

Noradrenergic neurons of the locus coeruleus exhibit slow spontaneous activity that fluctuates systematically with spontaneous changes in the vigilance of the animals (Foote et al.,1980). Although we hypothesize that BDNF secretion from noradrenergic LC neurons varies with changes in its discharge activity, this has yet to be examined. This could provide a potential molecular mechanism for coupling alterations in activity in neural circuits with morphological and functional changes in those systems.

(ii) Role of Anterogradely-Derived BDNF in the Mammalian CNS:

What is the role of anterogradely transported BDNF in noradrenergic neurons? The early, prenatal formation of noradrenergic projections to the forebrain has led to the proposition that these axons exert a trophic and regulatory influence during the ontogeny of the cerebral cortex (Olson and Seiger,1972). Although it was previously believed that this trophic influence was largely due to norepinephrine release, the perturbations we have observed in the cortex of DBH:BDNF transgenic mice, indicate that this trophic factor may also be crucial in the regulation of cortical development by noradrenergic afferents. Indeed, the perturbations in the neocortex as demonstrated by the pattern of Tα1:nlacZ expression in Tα1:nlacZxDBH:BDNF transgenic mice, and in nissl stained sections of DBH:BDNF mice, are not attributable to indirect effects such as changes in the number of noradrenergic neurons of the locus coeruleus, changes in the pattern of noradrenergic innervation, nor to changes in catecholamine levels in

the cortex. (Fawcett et al.,1998). This phenotype may be achieved through a number of different mechanisms, including premature neurogenesis, inappropriate migration, and alterations in neuronal survival. Indeed, there is precedent that increased BDNF in the vicinity of the developing neocortex can exert all three effects. However, we hypothesize that the cortical phenotype of DBH:BDNF transgenic mice, is primarily due to the premature expression of BDNF in afferent noradrenergic axons, and the consequent premature "push" that dividing neuroblasts receive towards untimely differentiation. First, there is a decrease in the size of the neocortex and in number of cortical neurons in DBH:BDNF transgenic mice. Early differentiation of cortical neuroblasts may deplete the precursor pool and severely decrease the number of total cortical neurons, resulting in a reduction in cortical width. Interestingly, the number of neurons and width of the neocortex is most severely affected at the anterior-most part of the neocortex, one of the first areas innervated by LC neurons, and one of the last cortical areas to reach maturity. Together, these two occurrences could provide the widest window of opportunity for LC-derived BDNF to influence cortical precursors, and may explain the more severe depletion of cortical neurons in the anterior neocortex. Secondly, when heterozygote DBH:BDNF transgenic mice were crossed to Tα1:nlacZ mice, β-galactosidase-expressing blue cells were observed at earlier timepoints in the cortex of the BDNF overexpressing progeny compared to wildtype littermates following X-gal staining (data not shown). As the Tα1:nlacZ transgene is turned on in neuronal cells following exit from the cell cycle (Gloster et al.,1998), the early expression of this transgene in BDNF overexpressing mice (at E13), indicates premature differentiation of cortical precursors.

During cortical development, cells in the ventricular zone exit the cell cycle and migrate to their appropriate position in the cortical molecular layer, its position depending on the birth date of the cell (reviewed in Bayer and Altman,1991). There is some indication that changes in the neurotrophin level and indeed, alterations in the noradrenergic innervation of the cortex, may affect the migration of cells. For example, cortical heterotopias (groups of ectopic cells) were observed in the molecular layer of mice injected with NT-4, and to a lesser degree, BDNF (Brunstrom et al.,1997). In addition, ectopic neurons were observed in brains treated with 6-hydroxydopamine (6-OHDA) (Lidov and Molliver,1982). This data does not differentiate between effects on neuronal migration and proliferation, nor does it unequivocally tie loss of innervation effects to loss of neurotrophins. Further experiments are required to elucidate the

mechanism of BDNF action on the developing cortex. However, as the time of cell cycle exit may affect the survival and/or the position a neuron is destined to migrate to, it will be very hard to distinguish between differentiation and migration effects on cortical progenitors and neurons, respectively.

Although the use of transgenic mice has proved to be extremely important in uncovering the biological functions of the neurotrophins *in vivo*, a cautionary note must be added against interpreting these effects to be direct, as opposed to being secondary effects upon manipulation of an entire system. Several factors suggest that the effects we observed on cortical development were directly mediated by anterogradely-derived BDNF from noradrenergic afferents. First, TrkB activity is increased in BDNF overexpressing mice, and secondly, there are spatial changes in the severity of the perturbations that correlate with noradrenergic inputs. However, it may be that in such a coordinated system, other factors may influence cortical phenotype and may themselves be influenced by the overexpression of BDNF in LC neurons. For example, increases in the level of BDNF in noradrenergic neurons, may have an effect on other neurotrophins expressed by these neurons, and may result in decreased NT-3 expression in neurons of the LC. Indeed, neurotrophin compensation has previously been thought to account for the lack of gross morphological effects in the CNS of many neurotrophin knockout mice, and may be evolutionarily essential to dampen the effect of alterations in one system, that could severely perturb the function of many networks in the brain.

BDNF also plays an important role in regulating the expression of neuropeptides in the brain as evidenced by the decreased expression of neuropeptide Y (NPY), parvalbumin, and calbindin in BDNF-/- mice (Jones et al.,1994). Recently, our lab has shown that calbindin expression is increased in the septum of DBH:BDNF transgenic mice, and that treatment of wildtype mice with 6-OHDA results in a decreased expression of this neuropeptide (Fawcett et al.,1998). It is therefore conceivable that alterations in the growth and survival of neuronal populations in the brain are due to secondary effects of BDNF on fluctuations in neuropeptide levels which may then directly influence survival and differentiation, possibly via its ability to modulate neuronal activity. While the precise mechanisms remain to be elucidated, these findings expand potential influences of neurotrophins into more dynamic aspects of brain function than previously envisioned.

We have also demonstrated that anterogradely-derived BDNF can mediate the survival of target neurons. This is very important in examining the effects of neurotrophins in neurodegenerative diseases. For example, noradrenergic neurons of the locus coeruleus are severely affected in patients with Alzeimer's and Parkinson's disease with a trend for greater neuronal loss in patients with concurrent dementia (Zweig, et al.,1993). Neurons of the locus coeruleus innervate both the substantia nigra pars compacta (SNc), and the pyramidal cells of the hippocampus. One of the etiologies of Parkinson's disease is the severe depletion of neurons within the SNc (Quik et al.,1979), which depend on BDNF for survival (Hofer et al.,1991). Although it is tempting to speculate that decreased exposure to BDNF results in the death of midbrain dopaminergic neurons, this population does not appear to be dependent on BDNF for survival as evidenced by studies in BDNF-/- mice (Ernfors et al.,1994).

BDNF has been implicated in regulating synaptic plasticity and long-term potentiation in the hippocampus, a mechanism believed to be associated with memory formation and learning (reviewed in Cain,1998; Frey and Morris,1998). Indeed, decreased expression of BDNF in BDNF-/- mice resulted in decreased formation of LTP, which was rescued by exogenous applications of BDNF (Korte et al.,1995,1996). This perturbation in hippocampal function and LTP formation may be relevant to the dementia seen in Alzeimer's patients and Parkinson's patients with severe LC neuron loss. Thus, anterogradely-derived BDNF from noradrenergic afferents may play a role in hippocampal LTP and memory formation.

(iii) Model for the Role LC-Derived BDNF as a Mediator of "State of Arousal" Effects on Brain Development:

Together, we suggest that BDNF may be anterogradely transported to effect the development, the phenotype and the survival of target neurons. What is the biological significance of anterogradely-derived BDNF? It is important to consider that the level of BDNF synthesis (Ernfors,1991; Lindefors et al.,1992) and release (Griesbeck et al.,1995; Androutsellis-Theotokis et al.,1996) is increased by activity induced either by pharmacological or direct stimulation. Together with the biological effects of anterogradely-derived BDNF, this activity-dependent modulation of BDNF synthesis and release, can be used to propose a model for encoding long-lasting changes in the development and function in the CNS as a result of changes in the environment and the general state of arousal of the animal.

Neurons of the locus coeruleus generally act as postsynaptic integrators of external and internal inputs, regulating the state of arousal of the animal by altering the functional activity of target neurons (reviewed in Foote et al.,1983). We propose that during development, neurons of the LC establish a baseline level of activity that is dependent on environmental inputs during specific developmental windows. Lowering this baseline level of activity (for example, in a stimulus-deprived environment), may lower the synthesis and release of growth factors such as BDNF (reviewed in Lindholm et al.,1994). Conversely, in high stimulus environments, the baseline level of LC neuron activity is high, and the synthesis and release of BDNF is concommitantly increased (reviewed in Lindholm et al.,1994). Alterations in the level of BDNF being secreted into target areas, as determined by the level of activity in those circuits, may play an essential role in regulating the development and survival of targets during critical developmental widows.

In support of this idea, alterations in neurotrophin levels (McAllister, et al.,1997) and noradrenergic innervation (Maeda et al.,1974) has been shown to affect the growth and morphology of cortical dendrites, and alterations in BDNF levels have been shown to affect the survival of differentiated cortical neurons (Ghosh et al.,1994), and synaptogenesis (Braun et al.,1996). Thus, in a deprived environment, decreased secretion of BDNF from neurons of the LC, may result in decreased survival, growth and synapse formation. These effects will be long-lasting, even if the level of environmental stimulation returns to normal following the critical developmental window. This model, although speculative, provides a novel framework for thinking about the molecular mechanisms underlying psychopathology.

Within the field of psychology, it is generally believed that the strength of memory formation is dependent on the state of arousal of the animal and its concentration on a given task or stimulus (reviewed in Barkley,1997). Here, we propose a model whereby LC-derived BDNF enhances memory formation via its activity-dependent release at the hippocampus. During the state of increased vigilance, there is an increase in the spontaneous activity of LC neurons, resulting in a potential concommitant increase in BDNF levels being secreted to target areas. This increase in BDNF levels at the hippocampus results in enhanced LTP formation. Conversely, during a state of decreased vigilance, less BDNF is secreted to targets in an activity-dependent manner, decreasing the chances of an LTP event and a decreased chance of memory consolidation.

Thus, we propose that the LC-derived, activity-dependent anterograde secretion of BDNF, mediates a number of biological effects throughout the lifetime of an animal and may explain the differences in animals reared in environmentally-rich conditions versus animals reared in a deprived environment.

III. The Role of the p75 Neurotrophin Receptor in the Development of Sympathetic Neurons of the SCG:

The p75 neurotrophin receptor plays a multifaceted role during the development of the mammalian nervous system. Indeed, analysis of p75 null mutant mice and supporting *in vitro* data have revealed potential functions of p75 in target innervation and neuronal survival and death. The following sections outline potential models of how p75 may act to negatively regulate neuron growth and survival, and how these negative roles reconcile with its ability to form high affinity binding sites for NGF.

(i) Role of p75 in the Regulation of Axon Outgrowth and the Appropriate Innervation of Targets:

Evidence suggests that the total number of axonal branches extending from a given neuron tends to be conserved, and may be regulated by the limiting production of factors made in the neuronal cell body which are needed by the axons for their growth and survival (reviewed in Smalheiser and Crain,1984). This process, termed "sibling neurite bias", is one in which excess or inappropriate synaptic contacts are "pruned down" to stabilize a limited number of axon terminals, usually those possessing an optimal specificity of contacts and accruement of trophic factors (reviewed in Smalheiser and Crain,1984). We propose that the p75NTR plays an integral role during target innervation and may regulate the extension or retraction of axon collaterals via its local modulation of the TrkA receptor.

Evidence for a role of p75 in target innervation is derived from p75-/- mice, which exhibit a lack of sympathetic innervation to the pineal gland and reduced innervation to specific footpads (Lee et al.,1994). We propose that functional antagonism of the p75 and TrkA receptors acts to "fine-tune" the innervation of targets in a similar mechanism to that involved in regulation of neuronal survival.

Expression of *trkA* and p75 mRNA are first detected in sympathetic neurons at E13.5, just prior to the time when these neurons innervate proximal targets (Wyatt and Davies,1995). One of the first potential roles of the p75NTR during sympathetic neuron innervation may be to slow down axon growth as the axon arrives at the target, via its activation by target-derived neurotrophins. Indeed, a recent study suggests that the p75 second messenger, ceramide, acts in distal axons, but not cell bodies, to negatively regulate neurite growth (Posse de Chaves et al.,1997). Moreover, specific activation of p75 in sympathetic neurons decreases neurite branching, an effect reversible using antibodies to the p75 receptor (Kohn et al.,1998). We speculate that NGF-responsive neurons regulate neuritic growth via cross-talk between TrkA and p75NTR signaling pathways. A mechanism can be envisioned in which the secretion of target-derived NGF into the proximal environment just prior to axon arrival favors growth, and potentially affects neuronal pathfinding to direct axons to the target. As axons get closer to the target, target secretion of other neurotrophins such as BDNF, which may be expressed at lower levels or may not be able to diffuse as far into the proximal environment, may activate the p75 receptor to inhibit local growth via ceramide.

One model of neuronal pathfinding suggests that growth-promoting and growth-inhibiting signals act in concert at the growth cone where they are translated into a directed growth response (reviewed in Tessier-Lavigne and Goodman,1996). The local generation of ceramide may be one inhibitory signal regulating neuronal growth and pathfinding. Although activation of the p75 receptor has been shown to mediate apoptosis in sympathetic neurons, it is possible that at this developmental stage, activation of p75 cannot mediate apoptosis due to the immaturity of the apoptotic signaling cascade and the potential absence of crucial apoptotic signaling molecules.

Following target innervation, axon collaterals from the same neuron compete with one another for trophic factors which will not only determine neuronal survival, but also determine whether an axon collateral becomes stabilized or resorbed. This competition among growing sibling neurites could provide an additional mechanism by which elongation and survival of a neurite can be affected not only by its local environment, but by signals acting on its siblings growing in distant regions. In this way, small differences in target cues acting upon different axon collaterals can be amplified (reviewed in Smalheiser and Crain,1984). Therefore, "sibling bias" may contribute to the establishment of specific neural connections, as well as to the loss

of inappropriate ones. We propose that the maintenance of axon-target contact is regulated by the level of TrkA signaling occurring at a specific collateral. Indeed, one can imagine a scenario in which a neuron extends two collaterals that innervate different targets. If one of those collaterals arrives early at a target and is able to sequester high levels of NGF, then the robust TrkA signaling would retrogradely mediate the survival of that neuron, and would locally promote growth of that axon. Activation of p75 by target-derived growth factors would be integrated, but would not override this signal. If the second collateral arrives at a target that has already been innervated or arrives at an inappropriate target, the low level of available NGF would result in weak TrkA activation, and ligand-activated p75 would act locally to attenuate the growth of that specific axonal branch. The net result would be the retraction of one axon collateral and the maintenance of the other (see Fig.6.1).

Interestingly, the level of sympathetic innervation to the pineal gland is increased in BDNF-/- mice, and cannot be explained by the increase in sympathetic neuron number alone. The pineal gland normally produces BDNF (Kohn et al.,1998), and we propose that in the absence of BDNF-mediated p75 activation, local signals for retraction are absent, resulting in a persistence of sympathetic innervation. Thus, p75 may play a similar negative modulatory role in neurite outgrowth of TrkB-expressing, BDNF-responsive neurons.

Somewhat counter-intuitively, there is a lack of sympathetic innervation to the pineal gland, and reduced innervation of specific sweatpads in p75-/- mice (Lee et al.,1994). The lack of specific target innervation in p75-/- mice can be explained in two ways. First, strong survival and growth signals mediated by robust TrkA activation in the absence of p75, may result in the persistent innervation of early targets which sequester the majority of growth proteins for axon arborization, thus preventing the formation or growth of axon collaterals to late targets such as the pineal gland. Evidence for this comes from transgenic mice which overexpress NGF in keratinized skin (K14:NGF mice) (Guidry et al.,1998). Here, examination of sympathetic innervation to the footpad revealed an increased innervation of the dermis, and a concommitant decrease in the innervation of sweat glands and blood vessels (Guidry et al.,1998). Thus, the robust activation of TrkA in one target area may result in differential sympathetic innervation.

Another example of this can be seen in DBH:NGF mice, which express NGF in sympathetic neurons themselves (Hoyle et al.,1993). There is an overall decrease in the innervation density of sympathetic neurons in these mice, supporting the idea that in the

presence of strong local survival signals, neurites are not required to "seek" additional sources of neurotrophin. This would account for the decreased sympathetic innervation pattern. It would be of great interest to see whether there is a higher incidence of inappropriate target innervation in these mice.

Another explanation for the apparent lack of sympathetic innervation to the pineal gland in p75-/- mice may be the inability of these axons to pathfind to this specific target area. It is well known that axons grow along previously established axonal routes to arrive at target areas. It is possible that p75 aids in neuronal pathfinding in early neurons in the absence of prior axonal routes. As the pineal gland is not innervated by either sensory or motor neurons, it is possible that sympathetic neuites are required to pathfind to the pineal gland independently. In the absence of p75, this axon pathfinding may be severely compromised, resulting in the absence of pineal innervation.

Although it is relatively easy to conceptualize how TrkA and p75 may act locally to initiate a growth or retraction signal in specific axons, it is harder to imagine how a neuron integrates signals from all neurites, and interprets the spatial arrangement of Trk signaling such that it facilitates growth or maintenance of one collateral, while causing another to retract. Indeed, this has yet to be examined.

(ii) The Role of p75 in Neuronal Survival and Death:

a) Biological Consequences:

Once a neuron becomes dependent on NGF for survival and competes for limiting amounts of target-derived NGF during the period of naturally-occurring cell death, the role of the p75 receptor shifts to rapidly eliminate neurons that are unable to sequester adequate amounts of NGF. We postulate that the rapid pruning of these neurons makes biological sense, to allow for the "best" neurons to innervate the target. We believe that this is a result of functional antagonism between the TrkA and p75 signaling pathways, briefly described below. Our model of how the p75 receptor regulates neuronal survival and death has previously been described in chapters IV and V and reviewed in Miller and Kaplan (1998), and will not be further discussed here.

b) Signaling Events Mediated by the p75 Neurotrophin Receptor:

Apoptosis serves to control cell numbers during development and throughout life. It is important during the normal development of the nervous system and is a feature of neurodegenerative diseases (reviewed in Gelbard et al.,1997). Therefore, understanding the signaling pathways regulating apoptosis in neurons is of particular interest. Sympathetic neuronal apoptosis is known to occur upon withdrawal of NGF *in vitro* and *in vivo*, and the inability to sequester sufficient NGF during development is believed to be one of the hallmarks of cells undergoing programmed cell death. We have recently shown that sympathetic neurons undergo apoptosis upon selective activation of the p75NTR with BDNF (Bamji et al.,1998). We have also attempted to unravel the potential signaling cascade by which p75 mediates its apoptotic function (Aloyz et al.,1998). In the following section, the potential signaling pathways leading to sympathetic neuron death will be discussed, focussing on NGF withdrawal-induced death and p75NTR-mediated death.

We propose that NGF withdrawal-induced death and p75NTR activation-induced death, may be mediated by the same apoptotic signaling pathway. Indeed, given the similarities between NGF withdrawal and p75NTR activation, we propose that NGF withdrawal-induced apoptosis may be, to a large extent, a p75NTR-mediated process. That is to say, in the absence of sufficient TrkA signaling, we predict that target-derived and aurtocrine/paracrine-derived neurotrophic factors impinge on the p75NTR and rapidly induce apoptosis. This will be discussed focussing on the temporal events of sympathetic neuron death after these two events, as well as the timecourse of activation of apoptotic signaling molecules.

The first evidence that NGF withdrawal-induced death and p75NTR activation-induced death are similar in sympathetic neurons derives from the timecourse of neuronal death following NGF withdrawal (Deckworth and Johnson,1993) or p75 activation (Bamji et al.,1998), which occur approximately 48 hours following experimental manipulation. This timecourse of cell death is different from that observed in rat oligodendrocytes where p75 activation led to apoptosis in 4 hours (Casaccia-Bonefil et al.,1996).

Second, we have shown that NGF withdrawal-induced apoptosis is greatly delayed in p75NTR null mutant mice *in vitro*, and that programmed cell death is delayed *in vivo* (Bamji et al.,1998). This indicates that the absence of TrkA signaling is insufficient for rapid apoptosis, which requires activation of the p75NTR-mediated apoptotic signaling cascade. This is the

best biological evidence showing that NGF withdrawal-induced death is, to a large extent, a p75-mediated process. The neurotrophin binding region of the extracellular domain was targetted for disruption in p75-/- mice, however these mice have recently been shown to express splice variants containing the intracellular domain of p75 (A.Vaillant, D.R.Kaplan, F.D. Miller, unpublished results; Y.A. Barde, personnal communication). Although there is an absence of ligand-activated p75 signaling in p75 -/- mice, which may account for the decrease in cell death seen *in vivo* and *in vitro*, it is possible that the intracellular region of p75 may function to mediate a constitutive death signal in the absence of ligand as previously suggested (Majdan et al.,1997). This constitutive signal may account for the delayed cell death seen in p75 knockout mice. It would therefore be very interesting to see whether there is a complete rescue of NGF-deprived neurons in the absence of the entire p75 receptor.

Extensive work by a number of laboratories has provided information on the temporal activation of apoptotic proteins following NGF withdrawal, and the timepoints after which blocking these proteins is no longer able to rescue cells from death (see Fig.1.4). Although such an extensive examination of the timecourse of events has not been examined after p75 activation, evidence shows that c-jun becomes hyperphosphorylated (Estus et al.,1994; Ham et al.,1995; Bamji et al.,1998) and p53 levels increase (Aloyz et al.,1998) with similar timecourses following NGF deprivation and p75 activation (Fig.1.4). Thus similarities in the timecourse of activation of key apoptotic factors provides the third piece of evidence that the signaling cascade activated by p75 and NGF deprivation is, by and large, similar.

How does ligand-dependent and/or ligand-independent p75 signaling lead to apoptosis? One possibility is that p75NTR activation antagonizes suboptimal TrkA survival signals by a direct effect on the TrkA receptor as shown in PC12 cells by MacPhee and Barker (1997). Although intriguing, activation of the p75 receptor with BDNF in sympathetic neurons (Bamji et al.,1998), or constititive p75 activation in Tα1:p75ICD transgenic mice (Majdan et al.,1997), does not effect tyrosine phosphorylation of the TrkA receptor. P75 may, however, mediate apoptosis by inhibiting signaling pathways *downstream* of the TrkA pathway as previously demonstrated (Yoon et al.,1998). P75 is also able to directly signal apoptosis independently of TrkA, as demonstrated by its ability to mediate apoptosis when neuronal survival is maintained by chronic depolarization (Bamji et al.,1998).

What are the players in the p75-mediated signaling cascade? Although the complete mechanism by which apoptosis is triggered by p75NTR is unknown, sustained elevation of ceramide levels by activation of the sphingomyelin pathway may be involved (Casaccia-Bonnefil et al.,1996; Frago et al.,1998; reviewed in Dobrowsky and Carter,1998). Indeed, ceramide has been shown to increase JNK levels and mediate cell death (Verheij et al.,1996). JNK can be activated by SEK1, which is in turn, activated by MEKK1 (Lange-Carter et al.,1993; Minden et al.,1994; Derijard et al.,1995; Lin et al.,1995). Although it appears that ceramide lies upstream from JNK activation, it is unclear whether it lies upstream of MEKK1 (as shown on Figs. 1.3, 1.7), or whether it activates JNK through a parallel pathway. In addition to activating the JNK pathway, ceramide may mediate neuronal apoptosis by inhibiting Akt activity (Summers et al.,1998; Zhou et al.,1998; Zundel and Giaccia,1998).

One of the targets of JNK is c-jun, and increased phosphorylation of c-jun is also observed upon activation of p75 (Bamji et al.,1998). Although c-jun has been shown to be required for sympathetic neuron death (Estus et al.,1994; Ham et al.,1995) and was thought to lie upstream of p53 in the sympathetic neuron death pathway, more recent evidence suggests that JNK may be directly responsible for increasead p53 levels (Fuchs et al.,1998; R.Aloyz, D.R.Kaplan, and F.D.Miller,unpublished results).

P53, in turn may promote apoptosis by influencing the transcription of *bcl-2* and *bax* genes (reviewed in Evan and Littlewood,1998). Mechanistically, the shift toward the increase in Bax/Bax homodimer formation permits the release of cytochrome C from the mitochondria (reviewed in Evan and Littlewood,1998). Blocking cytochrome C has recently been shown to rescue NGF-deprived sympathetic neurons (Neame et al.,1998), suggesting that this mechanism of apoptosis can be generalized to sympathetic neurons. In addition, the p75 receptor family member, TNFR1, has recently also been shown to mediate caspase activation via cytochrome C release in certain cell types (Luo et al.,1998; Srinivasan et al.,1998). The release of mitochondrial cytochrome C causes the activation of caspases, leading to structural alterations in the cell and culminating in cell death (reviewed in Evan and Littlewood,1998). Caspase-9 knockout mice have recently been generated and have shown that neuronal death is perturbed in the absence of this apoptotic protein (Hakem et al.,1998; Kuida et al.,1998).

It is likely that cross-talk between p75NTR and TrkA is bidirectional, and that one of the mechanisms whereby TrkA supports neuronal survival is by silencing a neurotrophin-

mediated death signal (Yoon et al.,1998). This may occur at may levels and is now thought to include suppression of caspase-9 by Akt (Cardone et al.,1998; Fig.1.3), suppression of the JNK pathway by Akt (R.Aloyz, D.R.Kaplan, and F.D.Miller, unpublished data), and supression of the JNK pathway by Ras (Mazzoni et al.,1998).

c) Differential Roles of p75 During Development: Analysis of the Role of p75 in Migration and High Affinity Binding:

Thus far we have explained how the p75 neurotrophin receptor may negatively regulate TrkA signaling and/or directly mediate apoptosis. Although this is clearly an important role for the p75NTR, one cannot discount the fact that certain neuronal populations are compromised in the absence of p75. Indeed, in p75-/- mice, there is a significant decrease in the number of large and small diameter sensory neurons in the dorsal root ganglion (Stucky and Koltzenburg,1997). Here we discuss two possible mechanisms by which p75NTR expression can positively influence sensory neuron development; via its role in the migration of sensory neuron precursors, and via its ability to positively regulate the survival of sensory neurons at specific developmental stages through the formation of high affinity NGF receptors. This section is therefore aimed at uncovering potential alternative roles for the p75 receptor during the development of the mammalian nervous system, and providing a hypothesis of the role of high affinity binding *in vivo*, using NGF-dependent sensory neurons as a model system.

P75NTR activation has been shown to cause enhanced cellular migration in Schwann cells (Anton et al.,1994), epidermal Langerhans cells (Wang et al.,1997), and in tumor cells (Iwamoto et al,1996). In addition, activation of the p75 receptor has been shown to induce the gene for the neural cell adhesion molecule, NILE/L1, in PC12 cells (Itoh et al.,1995), and enhance extracellular matrix penetration of human melanoma cells (Hermann et al.,1993). Thus, one hypothesis of why there is a decrease in the number of sensory neurons in p75-/- mice may be due to perturbations in the ability of sensory neuron precursor cells to migrate to distant sites where they coalesce to form sensory ganglia.

The decrease in the number of sensory neurons in p75-/- mice was initially believed to reflect the ability of p75 to act as a "co-receptor" for TrkA. This was supported by primary culture experiments using embryonic sensory neurons from E18 p75-/- mice, which indicated that nearly four-fold more NGF was required to maintain the survival of p75-/- neurons at this

stage compared to wildtype cultures (Lee et al.,1994). In addition, expression of p75 antisense oligonucleotides resulted in decreased sensory neuron survival at early embryonic timepoints (Barrett and Bartlett,1994). Together, these two results suggest that the decrease in sensory neuron number in p75-/- mice may be due to decreased survival of *early* sensory neurons which require p75 for survival.

Interestingly, expression of p75 antisense oligonucleotides resulted in increased survival in E19-P2 sensory neurons (Barrett and Bartlett,1994). Indeed, accumulating evidence suggests that p75 acts as a negative mediator of survival and growth in late embryonic and postnatal sensory neurons. When sensory neurons from postnatal mice were sorted into high- and low-p75 populations, low p75 cells were found to exhibit enhanced survival in the absence of neurotrophins, while cells with high p75 levels had reduced survival, compared to the overall population (Barrett et al.,1998). In addition, reducing p75 levels using antisense oligonucleotides prevented the loss of axotomized sensory neurons in the dorasl root ganglia of newborn mice (Cheema et al.,1996). Finally, activation of the p75NTR in cultured P1 sensory neurons had a negative effect on axon outgrowth similar to that seen in sympathetic neurons (Kimpinski et al.,1998).

How can the differential ability of p75 to mediate neuron survival and death be explained? Thus far the mechanism(s) responsible for this profound switch is unknown and further research is required to distinguish between multiple putative scenarios. One potential explanation as to why embryonic sensory neurons and sympathetic neurons differ in their response to p75 activation during programmed cell death may be their differential ability to form high affinity NGF binding sites.

It has long been known that p75 and TrkA receptors can form high affinity binding sites for NGF, which is dependent on the relative ratios of p75 and TrkA. Indeed, high affinity binding has been observed in cell lines displaying a high p75 to TrkA ratio (Hempstead et al.,1990; Battleman et al.,1993; Mahadeo et al.,1994), but not when p75 to TrkA levels are lower (Jing et al.,1992). The developmental expression of p75 and *trkA* mRNA appear to be very similar in sensory neurons, and increase in parallel with the acquisition of the NGF survival response at E12 (Wyatt and Davies,1993). In contrast, the increases in *trkA* and p75 mRNA are out of step with one another in the developing SCG (Wyatt and Davies,1995). In these neurons, the level of *trkA* mRNA initially increases much more rapidly than that of p75

mRNA, so that near the onset of programmed cell death (E17), the level of *trkA* mRNA is almost seven-fold higher than that of p75 (Wyatt and Davies,1995). The level of p75 mRNA has been shown to increase from E17 onward, but is still three-fold lower at birth and two-fold lower at P4 (Wyatt and Davies,1995). The level of *trkA* and p75 mRNA at later dates is unknown. These differences in the developmental profile of p75 and *trkA* mRNA expression in sympathetic and sensory neurons, may provide a plausible explanation for the differential role of p75 during the development of these two populations.

One can imagine a scenario whereby the high expression of p75 in early embryonic sensory neurons leads to the formation of high affinity binding sites for NGF via p75-TrkA interactions, as well as activates a p75-mediated death pathway. If p75's contribution to high affinity binding, and thus enhanced NGF responses, is greater than its ability to signal apoptosis, then the net effect of the p75NTR on these neurons would be as a positive regulator of survival. The loss of p75, such as in p75 null mutant mice, would therefore result in a net decrease in the level of TrkA signaling in response to a limited level of NGF that can no longer bind to TrkA with high affinity. These high affinity binding sites have been observed in chick sensory neurons (Sutter et al.,1979).

Although high affinity NGF binding has not been examined in sympathetic neurons, it is plausible that the relatively low p75:TrkA ratio in these neurons results in the absence of, or fewer high affinity NGF binding sites. Here, p75 expression leads to activation of the death pathway, and the net effect of the p75NTR on these neurons would be as a negative regulator of survival. This could explain why there is an increase in the number of NGF-responsive basal cholinergic (Van der Zee et al.,1996; Yeo et al.,1997) and sympathetic (Bamji et al.,1998) neurons in p75 null mutant mice. As the expression pattern of *trkA* and p75 mRNA is largely unknown in late embryonic and postnatal sensory neurons, it is difficult to say whether the negative effect of p75 at this developmental stage reflects differences in the ability to form high affinity NGF binding sites. Together, it is possible to propose a model whereby differences in p75:TrkA ratios in different neuronal populations or at different stages within a population, determines the net effect of p75 activation in mediating survival or death.

One cannot discount the possibility that p75 may exert differential effects on neuronal populations due to differences in the expression pattern of naturally-occurring p75 splice variants. Indeed, although the function of the various splice variants are still largely unknown,

these factors may mediate a constitutive death signal or may exhibit a dominant-negative role for high affinity binding or apoptotic signaling.

Thus far the p75 neurotrophin receptor has only been observed to exert deleterious effects on TrkA-expressing neurons that depend on NGF for survival. It is of great interest to determine whether the p75 receptor subsumes a similar role in BDNF-, NT-3- and NT-4-dependent populations. Analysis of p75-/- mice (Lee et al.,1994; Stucky and Koltzenburg,1997) and Tα1:p75ICD transgenic mice (Majdan et al.,1997) would indicate that p75 exerts a minimal effect on TrkB- and TrkC-expressing neurons, at least during the period of naturally-occurring cell death. Indeed, TrkB- and TrkC-expressing cells do not appear to be grossly compromised in p75-/- mice (Lee et al.,1994; Stucky and Koltzenburg,1997) and Tα1:p75ICD transgenic mice (Majdan et al.,1997), although expression of p75ICD does appear to have a negative effect in the neocortex (Majdan et al.,1997). There are a number of plausible explanations for this. It is possible that TrkB and TrkC mediate their survival effects through different signaling pathways that cannot be negatively modulated by p75 activation. Indeed, preliminary evidence suggests that the Ras-MAPK pathway plays a much more important role in TrkB-mediated survival than TrkA, which mediates survival primarily through the PI 3-kinase-Akt pathway (J.Atwal, D.R.Kaplan). Another explanation may be that the p75NTR is typically coexpressed with TrkA in NGF-responsive neurons, in contrast to cells expressing TrkB or TrkC which may not necessarily express p75 (Schechterson and Bothwell,1992; Verge et al.,1992). This does not, however, explain why these neurons aren't compromised in Tα1:p75ICD transgenic mice which express the p75ICD in all neurons (Majdan et al.,1997). Thus, it is impossible to determine whether p75 is unable to modulate survival and death in specific neuronal subtypes that are dependent on specific neurotrophins, or whether this receptor is predominantly absent in these populations.

Evidence that p75 may play a negative role in survival of BDNF-responsive neurons comes from studies which have examined the survival of axotomized motor neurons in p75-/- mice (Ferri et al.,1998) and in Tα1:p75ICD transgenic mice (Majda et al.,1997). Indeed, the loss of p75 lead to the protection of injured motor neurons (Ferri et al.,1998), and overexpression of p75 in Tα1:p75ICD mice resulted in decreased survival of axotomized facial motor neurons compared to wildtype mice (Majdan et al.,1997).

IV. Future Directions and Clinical Perspectives:

The pace of research into the mechanisms by which neurotrophins mediate their functions has rapidly accelerated over the past few years, leading to an increased understanding of the delivery of neurotrophins to responsive populations, and the intracellular biochemical pathways that propagate neurotrophin signals from the plasma membrane to the nucleus. Together, these studies will provide further insight into the roles of neurotrophins in degenerative disorders of the nervous system as well as into the pathogenesis of these disorders. Indeed, an understanding of how neurotrophins are transported to responsive neuronal populations either in the anterograde or retrograde direction, will provide further information regarding the role of neurotrophins in the etiology of disease or in the occurrence of secondary symptoms following degeneration of a specific population of neurons. For example, degeneration of a particular population of neurons may effect other populations via its inability to provide an adequate source of anterogradely-derived trophic support. Furthermore, characterization of the neurotrophin activated apoptotic signaling mechanisms via the p75 and Trk receptors, should provide insights into the pathogenesis of neurodegenerative disorders. An understanding of the regulation of the p75 receptor, its splice variants, and the members of the apoptotic signaling pathway may provide an understanding of why specific neuronal populations are more susceptible to neuronal degeneration.

In addition to learning about the role of positive and negative neurotrophin signaling pathways in the normal development of the nervous system, characterization of the neurotrophin-activated signaling mechanisms could provide a basis for the potential development of novel strategies in the treatment of degenerative disorders of the nervous system. Controlled clinical trials of neurotrophins in diseases such as amyotrophic lateral sclerosis and diabetic neuropathy have revealed that administration of neurotrophins is often accompanied by adverse effects. An important goal for understanding the mechanisms that enhance neuronal survival and death will provide the rationale for the development of theraputic agents that specifically regulate the survival and death pathways to mediate the appropriate survival effects.

VI. References

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