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Neurotrophin-3 and trk receptors in the developing mouse trigeminal ganglion

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NEUROTROPHIN-3 AND TRK RECEPTORS IN THE DEVELOPING MOUSE TRIGEMINAL GANGLION

by

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DISSERTATION

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Neurotrophin-3 and trk receptors in the developing mouse trigeminal ganglion

George A. Wilkinson

Abstract

The neurotrophins are a family of secreted polypeptides required for neuronal survival and other aspects of nervous system development. Their principal survival actions are mediated by binding to the trk family of receptor tyrosine kinases. Gene inactivation experiments have shown that mice lacking neurotrophins or trk receptors manifest specific nervous system deficits. Mice lacking neurotrophin-3 (NT-3) are born with severe neuronal deficits, with the trigeminal ganglion lacking 65% of the normal number of neurons. Because of the many potential roles for NT-3 in the developing nervous system, we undertook a detailed comparison of the development of the trigeminal ganglion in wild-type mice and in mice lacking NT-3.

The neuronal deficit in the trigeminal ganglion of mice lacking NT-3 emerges between embryonic stages (E)10.5 and E13.5. From E10.5-E12.5, neuronal numbers were reduced relative to wild-type in association with increased apoptotic death but without change in numbers or proliferation of precursors. From E12.5-E13.5, neuronal numbers in mutants decreased rapidly, accompanied by elevated apoptosis and changes in precursor populations. Therefore the trigeminal neuronal deficit in mice lacking NT-3 is predominantly due to apoptotic elimination of neurons.

Using a genetic reporter construct, it was determined that from E11.5 to E13.5 NT-3 is expressed in peripheral targets of the trigeminal ganglion but not within the ganglion nor in the CNS targets of these neurons.

Stages E11.5-E13.5 in the wild-type trigeminal ganglion are associated with extensive changes in trk receptor expression in vivo and neurotrophin responsiveness of trigeminal neurons in vitro. To examine neuronal expression of trk receptors, we raised antibodies against rat trkB and rat trkC. In wild-type mice from E11.5-E13.5, expression of each trk receptor is restricted to neurons in the trigeminal ganglion, and numbers of neurons expressing trkC or trkB decreased whereas numbers expressing trkA increased. In mice lacking NT-3 at E11.5, the numbers of neurons expressing trkC and numbers expressing trkB were severely reduced whereas numbers expressing trkA were largely unaffected.

William C. Mobley
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Chapter I. INTRODUCTION

During the development of the vertebrate nervous system, many more neurons are produced during development than persist in the adult. In many parts of the nervous system, more than half of the neurons initially generated are eliminated by cell death (reviewed in Oppenheim, 1991). This naturally occurring cell death is observed in a wide variety of species, neuronal populations and regions of the nervous system. Neurons which are destined to die during this process undergo a characteristic sequence of morphological changes such as such as condensation of chromatin, nuclear fragmentation, and breakdown into membrane-bound fragments which are phagocytosed by neighboring cells (Wyllie et al., 1980) These changes are part of the cell death process called apoptosis and are distinct from the morphological signs of necrosis due to conditions such as injury or disease. It is now understood that the morphological hallmarks of apoptotic death are correlated with activation of a highly conserved molecular "death program." (e.g. Zou et al., 1997; reviewed in Deshmukh and Johnson, 1997) Thus, the massive cell death observed in the developing nervous system seems to reflect a regulated physiological process.

A seminal investigation into the development of the chick dorsal root ganglia (Hamburger and Levi-Montalcini, 1949) showed that the extent of naturally occurring neuronal cell death varies

considerably from ganglion to ganglion. Cervical and thoracic dorsal root ganglia, which innervate small peripheral territories, showed a great degree of naturally occurring cell loss, whereas lumbar dorsal root ganglia, which innervate large peripheral territories in the hind limb, showed much less cell death. The degree of naturally occurring cell death could be dramatically increased by removing the normal target field of the lumbar ganglia, i.e., by extirpating the limb bud. Subsequent experiments have shown that the survival of neurons in many systems depends upon interactions with their target tissue. Ablation of target tissue exacerbates the normally occurring neuronal death, and augmenting target tissue via transplantation preserves neurons which normally would be eliminated. (reviewed in Purves and Lichtman, 1986; Oppenheim, 1991) Moreover, decreasing the number of neurons which innervate a target results in improved survival of the remaining neurons (Pilar et al., 1980); while increasing the numbers of neurons increases the degree of subsequent neuronal death (O'Leary and Cowan, 1984; reviewed in Purves and Lichtman, 1986). These observations led to the proposal that neuronal apoptosis during development functions to match the number of surviving neurons to the target tissue.

The selective evolutionary advantage which is gained through this phenomenon is not well understood. It is thought that most

vertebrate cell types require signals from other cells to avoid programmed cell death (reviewed in Jacobson et al., 1997) This interdependence of cells may be a strategy employed by complex organisms to eliminate misplaced or malfunctioning cells and insure correct numbers of particular cell types (see Raff, 1992). In this respect, target-dependent neuronal death may help to remove incorrect axonal connections (reviewed in Purves and Lichtman, 1986). Alternatively, overproduction of neurons may provide a sort of safety factor insuring an adequate size match between two tissues which grow at different rates over different developmental stages (Oppenheim, 1991).

Nerve Growth Factor and the neurotrophic hypothesis

How might matching between innervating neurons and target tissues be accomplished? Targets are thought to control the surviving numbers of innervating neurons by producing limiting amounts of some factor which is necessary for the survival of neurons, thus engendering a competition among afferents for the factor. Early experiments identified Nerve Growth Factor (NGF), a small dimeric secreted polypeptide, as a target-produced factor which played this role in the maintenance of sympathetic and spinal sensory ganglia. (reviewed in Levi-Montalcini, 1987; Purves and Lichtman, 1986) The

extensive characterization of the biology and cellular mechanisms of NGF actions on developing neurons led to the formulation of a series of predictions about the properties expected of all target derived survival factors (Purves and Lichtman, 1986). This list of properties, which collectively are known as the neurotrophic hypothesis, are intended to distinguish between a genuine target-derived survival factor and metabolic or nutritive components of the environment (Purves and Lichtman, 1986; Barde 1988; Davies, 1988):

(1) The factor should be produced by the target in limiting amounts.

(2) Innervating neurons should express specific receptors for the factor.

(3) Axon terminals should be able to take up the factor at the level of the target.

(4) Without access to this factor the innervating neurons die in excess during the naturally occurring period of cell death.

(5) Experimental addition of exogenous factor will spare neurons that would die in normal animals.

Subsequent work has resulted in the identification of many potent survival factors for neurons, including a family of proteins

homologous to NGF (see below), and members of the FGF, TGF-ß, and cytokine families. All show restricted patterns of expression and promote the survival of different, though overlapping, neuronal populations. GDNF, a distantly related member of the TGF-ß family, can support sensory and autonomic neurons (Buj-Bello et al., 1995), dopaminergic midbrain neurons (Lin et al., 1993) and motor neurons (Henderson et al., 1994, Yan et al., 1995). CNTF and LIF, which are related to cytokines such as IL-6, act on many different classes of neurons including motor neurons (Ernsberger et al., 1989; Murphy et al., 1993; Barbin et al., 1994).

Very few neuronal survival factors have been subjected to analyses addressing all of the above criteria for a neurotrophic factor. Specifically, levels of expression of survival factors are seldom quantified in target tissues, and experiments in which exogenous factor is added to prevent cell death are less common than deprivation experiments based on genetic approaches (see below). However, the neurotrophic hypothesis still serves as a useful initial framework for considering the participation of candidate molecules in target dependent cell death.

The neurotrophin family

NGF is now recognized as the prototypical member of a homologous family of proteins, called the neurotrophins. Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) , and NT-4/5 have been shown to act potently to support the survival of sensory neurons and other populations (reviewed in Lewin and Barde, 1996) These more recently identified members of the neurotrophin family have proved to support distinct, but partially overlapping neuronal populations in a target-derived manner reminiscent of the demonstrated role of NGF (BDNF: placodally derived sensory neurons, Davies et al., 1986, Robinson et al., 1996; NT-3: muscle spindle afferents, Hory-Lee et al., 1993, Kucera et al., 1995). However, studies of their distribution and pharmacological actions has also implicated the newer neurotrophins in biological roles which fall outside the neurotrophic hypothesis (see Korschung, 1993; Lewin and Barde, 1996). The specific roles of the neurotrophins in the development of the nervous system remains an area of active investigation.

Trk receptors and $p75^{NTR}$

The neurotrophins bind to two types of transmembrane glycoproteins, the trk family of receptor tyrosine kinases and

$p75^{NTR}$, of the cytokine receptor superfamily which includes type I and II TNF receptors (reviewed in Carter and Lewin, 1997). Whereas all the neurotrophins bind to $p75^{NTR}$ with comparable affinity, binding to the trks shows a greater degree of specificity. NGF binds to trkA; BDNF and NT-4/5 bind to trkB; and NT-3 binds principally to trkC but can also activate trkA and trkB (reviewed in Bothwell, 1995). This specificity of binding, combined with in vitro studies indicating that $p75^{NTR}$ signaling is dispensable for neuronal survival and differentiation (Weskamp and Reichardt, 1991; Ibañez et al., 1992) has led to the conclusion that the major survival-promoting actions of neurotrophins are mediated by the trk receptors. The overall role of the $p75^{NTR}$ receptor is still uncertain (reviewed in Carter and Lewin, 1997).

Binding of neurotrophins to the trk receptors causes receptor dimerization and leads to activation of the intrinsic tyrosine kinase activity. The activated kinase of each receptor molecule in the dimer first phosphorylates the other receptor subunit (Kaplan et al., 1991 a, b), which increases the catalytic activity of the kinase (Mitra, 1991). The receptor molecules then become autophosphorylated on multiple sites. Some of these phosphorylation sites serve as recognition sites for adapter proteins and enzymes which become activated and

transduce the ligand binding event into an intracellular signal (reviewed in Segal and Greenberg, 1996). These proteins include phospholipase Cγ (PLCγ1; Vetter et al., 1991; Obermeier et al., 1993a; Stephens et al., 1994), PI-3 kinase (Ohmichi et al.. 1992; Obermeier et al., 1993b) and the adapter protein Shc (Obermeier et al., 1993b; Stephens et al., 1994). PI-3 activation appears to be necessary for the survival effects of NGF binding to trkA (Yao and Cooper, 1995), while the Ras-MAP kinase pathway initiated through PLCγ1 and Shc participates in differentiation responses (reviewed in Kaplan and Stephens, 1994).

Splice variants of trk receptors

Each of the trk genes encodes a number of differentially spliced variants with different expression patterns and signaling properties. In addition to the canonical trkA receptor, a trkA variant has been identified which lacks a six amino acid stretch near the transmembrane region (Barker et al., 1993). The two trkA isoforms have distinct distributions, and the variant containing the six amino acids shows enhanced responsiveness to NT-3 (Clary and Reichardt, 1994). Two truncated trkB variants have been described which lack a tyrosine kinase domain. (Klein et al,. 1991; Middlemas et al., 1991);

These may be capable of some sort of intrinsic signaling, (Baxter et al., 1997). They are also postulated to perform non-signaling roles, depending on their specific cellular context, such as sequestering or concentrating ligand (Klein et al., 1991; Middlemas et al., 1991). Truncated trkB receptors expressed in neurons which also express full-length trkB could interfere with signaling in response to BDNF or NT-4 binding, by forming inactive heterodimers with full-length trkB in response to ligand binding. (Eide et al., 1996; Ninkina et al., 1996).

Analysis of trkC cDNA clones has led to an even more complicated picture, with four kinase-containing isoforms and four additional isoforms which lack a kinase domain. Three of the kinase containing forms contain inserts of 14, 25, or 39 amino acids in the tyrosine kinase domain (Valenzuela et al., 1993; Tsoulfas et al., 1993; Lamballe et al., 1993). These kinase insert variants show reduced activation of the MAP kinase pathway relative to the canonical kinase isoform (Guiton et al., 1995; Tsoulfas et al., 1996). Four trkC receptors lacking a tyrosine kinase domain have been identified. The contribution to neuronal survival, if any, of these variants is not clear.

The existence of so many isoforms of trk receptors, many of which are predicted to have minimal or even negative influences on the ability of neurons to survive in the presence of neurotrophins,

complicates efforts to delineate populations responsive to neurotrophins. Most reagents used to study the distribution of trkA, trkB, and trkC do not distinguish among the various isoforms of these receptors. However, failure to express any trk or $p75^{NTR}$ can rule out the ability of a cell to respond to neurotrophins.

Mutant mice as a means for testing functions of neurotrophins

Recently developed techniques allow the generation of mutant mouse strains with targeted mutations ablating the products of single preselected genes (reviewed in van der Neut, 1997). In order to disrupt expression of a gene product, the relevant genomic sequence can be replaced with any other DNA sequence, allowing the insertion of DNA encoding a foreign protein into the exon encoding the original. This not only results in inactivation of the original gene but, with judicious choice of the replacement sequence, results in expression of an easily identified foreign marker in its place.

These molecular genetic deletion experiments (or "knockouts") have proven to be a powerful approach to investigating the roles of neurotrophins and the trk receptors in the development of the mouse. (reviewed in Reichardt and Fariñas, 1997) The elimination of a single gene product allows testing of the essential survival

functions of that protein without the complex environmental changes associated with tissue deletion or toxicity issues associated with antibody-mediated depletion experiments. In the case of the newly described neurotrophins, analyses of mice with targeted null mutations have allowed rapid insights into their function comparable to years of earlier work on NGF.

The trigeminal ganglion as a model system

The trigeminal ganglion is a frequently used model system to evaluate the roles of neurotrophins in the developing nervous system. This ganglion supplies sensory innervation to the oral cavity and skin of the face. The early development and relatively large size of the mouse trigeminal ganglion has allowed these neurons to be cultured and their responsiveness to neurotrophins evaluated at various stages in development. Cultures of embryonic trigeminal ganglion neurons show some survival in response to each of the four neurotrophins (Paul and Davies, 1995; Buchman and Davies, 1993) and each of the trks and $p75^{NTR}$ are expressed in the ganglion in vivo (Arumae et al, 1993; Ibañez et al, 1993; Ernfors et al., 1992). The peripheral targets of trigeminal ganglion neurons express each of the neurotrophins in differing temporal and spatial patterns (Arumae et al., 1993; Ibañez et al, 1993). Finally, the principal target

tissue of the mouse trigeminal ganglion, the whisker pad, is heavily innervated by an elaborate and highly stereotyped array of sensory endings (Rice et al., 1993) which allows analyses of the consequences of genetic deletions upon specific subclasses of sensory endings (Rice et al., 1997; Fundin et al., 1997).

The development of the trigeminal ganglion in the mouse has been characterized in extensive detail (Figure 1). The first neurons are born around embryonic stage (E) 9.5. Neurogenesis takes place from this point until about E13.5, with a peak in the mouse around E11-E12 (Altman and Bayer, 1993). The first axons contact the peripheral target at E10.5, and target innervation proceeds until around E15.5 (reviewed in Davies, 1988). Starting around E13.5, neuronal numbers in the trigeminal ganglion decrease in a period of naturally occurring cell death in which 30-50% of neurons are eliminated (Davies and Lumsden, 1984).

Elegant work by the Davies lab and others has demonstrated the remarkable developmental synchrony in the trigeminal system between NGF synthesis in the periphery, NGF responsiveness in trigeminal neurons, and the innervation of the periphery by trigeminal neurons. NGF is manufactured in the cutaneous target tissues of the trigeminal in amounts which vary in proportion to final innervation density (Davies et al., 1987; Harper and Davies, 1990).

Synthesis of NGF is correlated with the arrival of the first innervating trigeminal axons at E10.5, increases rapidly up to E13.5, and then shows a gradual decline correlated with the reduction of neuronal numbers during the period of naturally occurring cell death (Davies, 1987). Expression of trkA and $p75^{NTR}$ by neurons, and the in vitro survival requirement of trigeminal neurons for NGF, also increases dramatically at stages E12-E13, just prior to the onset of the period of naturally occurring cell death. (Wyatt and Davies, 1992; Buchman and Davies, 1993) Finally, disruption of the genes encoding NGF (Crowley et al., 1994) or trkA (Smeyne et al., 1994) results in loss of about 70% of trigeminal neurons, underscoring the importance of endogenous, target-derived NGF in the survival of trigeminal ganglion neurons.

In light of the importance of NGF as a neurotrophic factor for trigeminal neurons, it is somewhat suprising that mice lacking NT-3 also develop severe deficits in the trigeminal ganglion, with 65% of the neurons missing at birth (Fariñas et al., 1994; Ernfors et al., 1994). Thus, the lack of NT-3 leads to losses of neurons which also require another neurotrophin. We wanted to know the basis for the trigeminal deficit seen in mice lacking NT-3. Since NT-3 and its principal receptor, trkC, were known to be widely expressed in early

embryos, we considered possible developmental roles of NT-3 that would result in a deficit of this size.

Potential roles of NT-3 in the developing trigeminal ganglion

Neurotrophin-3 has been proposed to play a number of roles in the developing nervous system. For example, migrating crest cells express trkC, the NT-3 receptor (Tessarollo et al., 1992), and in vitro experiments show that NT-3 can influence survival and proliferation of these cells (Kalcheim et al., 1992). Also, NT-3 can act as a survival factor for neuroblasts in vitro (Birren et al., 1993; diCicco-Bloom et al., 1993) and has been proposed to act as such in vivo (ElShamy and Ernfors, 1996). If these very early proposed actions of NT-3 constituted its principal role in the development of the trigeminal ganglion, one would expect to see early defects in the ganglion of NT-3 knockout mice, with neuronal and nonneuronal trigeminal cells equally affected, and defects in proliferation of progenitor cells.

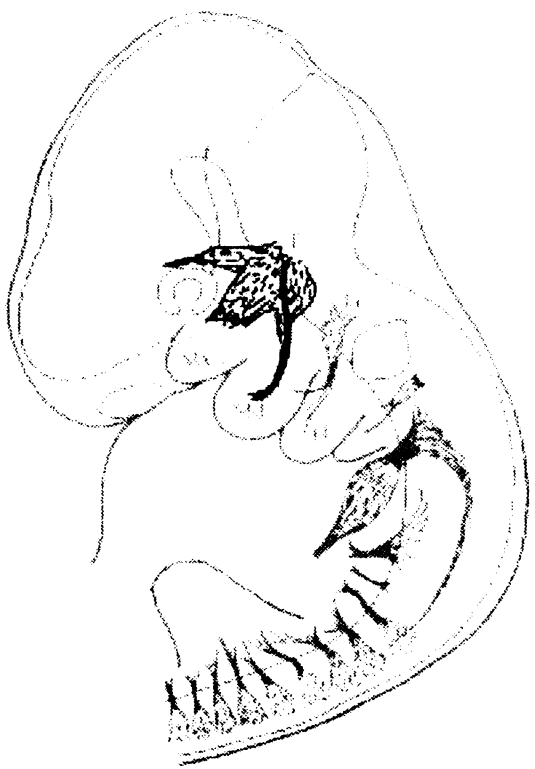
NT-3 has also been shown to have effects on neurons themselves. In vitro, NT-3 accelerates the differentiation of spinal sensory neurons from progenitor cells (Wright et al., 1992; Henion et al., 1995). NT-3 has been shown to promote survival of embryonic trigeminal cells in vitro, some of which later become dependent on

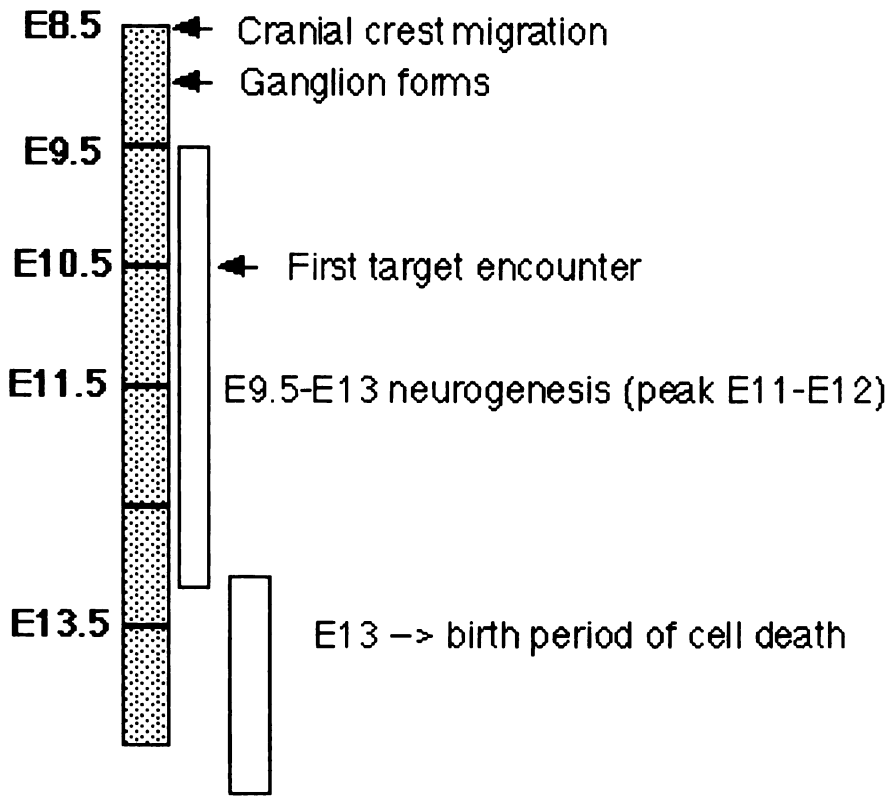
other neurotrophins (Buchman and Davies, 1993) Finally, NT-3 has been shown to support 1a afferents in a classical neurotrophic fashion, that is as a limiting target derived substance. (Hory-Lee et al., 1993; Kucera et al., 1995). Although the trigeminal ganglion lacks 1a afferents, it is possible that another class of ending would also depend on NT-3 in a similar manner. Each of these later proposed actions, should they represent a major function of NT-3 in the development of the ganglion, would result in mutant embryos with deficits in neurons but not in precursor cells. A role in differentiation could show up as a deficit in the earliest neuronal numbers, i.e. E9.5-E10.5; whereas a transitional or trophic requirement for NT-3 may not be seen until later stages. Any of these effects would be accompanied by elevated apoptotic death.

In order to evaluate these potential roles of NT-3 in the development of the trigeminal ganglion, we undertook an extensive comparison of the development of this ganglion in wild-type mice and NT-3 knockout mice. In order to distinguish among effects on precursors and effects on neurons, we analyzed numbers of neurons and precursor cells at a variety of embryonic stages. We also compared the proliferation of precursor cells and the rates of apoptosis in the trigeminal ganglion in wild-type and mutant

embryos. We used a foreign lacZ construct which was inserted into the NT-3 locus to visualize sources of NT-3 during the development of the trigeminal ganglion. Finally, we used antibodies against the trk receptors to evaluate the dynamics of trk-expressing populations in wild-type and NT-3 mutant mice.

Figure 1. Development of the trigeminal ganglion in wild-type mice. Cranial crest migration occurs over stages E8-E8.5 (Trainor and Tam, 1995) and the ganglion is first discernible in histological sections at stage E9.0 (Theiler, 1989). Neurogenesis commences around stage E9.5, and continues until about stage E13 (Davies and Lumsden, 1983) with a peak around stages E11-E12 (Altman and Bayer, 1983). The first axons contact their peripheral targets at stage E10. 5 (Davies and Lumsden, 1983) and is this process is thought to be completed at stage E15 (Davies, 1988). The period of naturally occurring cell death begins around stage E13.5 and continues until birth at 20 days of gestation. (Davies and Lumsden 1983).




E8.5
Cranial crest migration
Ganglion forms
E9.5
E10.5
First target encounter
E11.5
E9.5-E13 neurogenesis (peak E11-E12)
E13.5
E13 –> birth period of cell death

Chapter II: Neurotrophin-3 is a survival factor in vivo for embryonic mouse trigeminal neurons

(Wilkinson et al., J. Neurosci 16:7661-7669, 1996)

ABSTRACT

Mice lacking neurotrophin-3 (NT-3) have previously been shown to be born with severe sensory deficits. This study characterizes the developmental course of this deficit in the trigeminal sensory ganglion, which in NT-3 homozygous mutants contains only 35% of the normal number of neurons at birth. At embryonic day 10.5 (E10.5), normal numbers of neurons, as assessed by expression of neurofilament protein, and of total cells are present in the ganglia of mutant homozygotes. During the next three days (E10.5-13.5) virtually all of the deficit develops, after which mutant animals retain only approximately 30% the normal number of neurons. Quantification of neuronal and neuronal precursor numbers in normal and mutant animals reveals that neurons are specifically depleted in the absence of NT-3. A deficiency in precursor proliferation is only seen after most of the neuronal deficit has developed. Numbers of apoptotic cells in the ganglia of mutant animals are elevated during this same interval, indicating that the neuronal deficit is caused, in large part, by increased cell death of embryonic neurons.

To determine sources of NT-3 in the trigeminal system, we examined the expression pattern of β-galactosidase in mice in which

lacZ has replaced the NT-3 coding exon. E10.5-11.5 embryos exhibit intense reporter expression throughout the mesenchyme and epithelia of the first branchial arch. β-galactosidase expression in E13.5 embryos is largely confined to the oral epithelium and to the mesenchyme underlying the skin. Throughout the E10.5-13.5 interval, the trigeminal ganglion and its targets in the central nervous system do not express reporter activity.

We conclude that NT-3 acts principally as a peripherally-derived survival factor for early trigeminal neurons.

INTRODUCTION

The neurotrophins are a family of related proteins, including NGF, BDNF, NT-3, and NT-4/5, required for the survival of many classes of neurons (reviewed in Korsching, 1993). Their major actions are mediated by a family of receptor tyrosine kinases named trkA, B, and C; each neurotrophin also interacts with another receptor, p75NTR (reviewed in Bothwell, 1995). Examinations of mice with targeted mutations in the genes encoding neurotrophins or their receptors have revealed specific deficiencies at birth associated with each mutation (reviewed in Fariñas & Reichardt, 1996). Characterization of neuronal losses at birth in the dorsal root

ganglion has shown that animals lacking NT-3 exhibit complete elimination of proprioceptive neurons (Fariñas et al., 1994; Ernfors et al., 1994; Tesarollo et al., 1994; Tojo et al., 1995) but they additionally suffer severe losses in sensory neuron populations including neurons which are dependent on other neurotrophins postnatally (Fariñas et al., 1994). This suggests that NT-3 is required during embryogenesis for multiple populations of sensory neurons, which is consistent with the widespread expression of NT-3 and its major receptor, trkC, during embryogenesis (e.g. Ernfors et al., 1992; Tesarollo et al., 1993; Lamballe et al., 1994).

Of particular interest is the trigeminal ganglion, where a lack of NT-3 causes a reduction in the number of neurons at birth of more than 60% relative to wild-type animals. The trigeminal ganglion primarily supplies sensory innervation to derivatives of the first branchial arch including facial skin and the oral cavity. Interestingly, this ganglion does not contain proprioceptive neurons in wild-type animals. Thus the deficits observed in animals lacking NT-3 must involve other neuronal classes. It is not known, however, how the trigeminal ganglion deficit is generated during the development of these animals. The development of this ganglion and its innervation patterns have been intensely studied as a model system for characterizing the roles of neurotrophins (reviewed in Davies, 1994).

The temporal and spatial patterns of neurotrophin and neurotrophin receptor expression have been mapped in great detail and correlated with stages of development within the ganglion and in innervation of target fields (Arumae et al., 1993; Buchman and Davies, 1993; reviewed in Davies, 1994).

Previous observations have revealed several actions of NT-3 which might contribute to the development of the trigeminal ganglion in vivo and account for aspects of the deficit observed in NT-3 mutant homozygotes. NT-3 has been shown to accelerate the differentiation of spinal sensory neurons from progenitor cells (Wright et al., 1992). NT-3 has been shown to promote survival of embryonic trigeminal neurons in vitro, some of which later become dependent upon other neurotrophins (Buchman and Davies, 1993). NT-3 can act as a survival factor for neuroblasts in vitro (Birren et al., 1993; diCicco-Bloom et al., 1993; Karavanov et al., 1995) and some evidence indicates that it may also do so in vivo (e.g. ElShamy et al., 1995; ElShamy and Ernfors, 1996). Finally, studies have shown that NT-3 application in vitro increases the proliferation of sensory neuron precursors (Memberg and Hall, 1995).

In the present work, we evaluate the possible roles of NT-3 by examining the details of development of the trigeminal ganglion in normal and NT-3-deficient mice. We find that the neuronal

deficiency in animals lacking NT-3 appears during a comparatively short period of development which coincides with the peak of neurogenesis and axonal innervation of targets. We show that the neuronal deficit is associated with an abnormally high frequency of apoptosis. Examination of NT-3 expression indicates that NT-3 is derived from sources in the surrounding mesenchyme and target fields. The results indicate that the deficit reflects the loss of neurons dependent upon obtaining this factor from peripheral sources.

Materials and Methods

Mice with a targeted mutation in the NT-3 gene in which the coding region of the lacZ gene replaces the coding exon for NT-3 (Fariñas et al., 1994) were obtained from our colony, and bred out over the C57/Bl6 background. Animals were genotyped by DNA blot analysis as described (Fariñas et al., 1994).

Females in estrus were paired with males overnight and examined for vaginal plugs the following morning. For the purposes of staging embryos, pregnant females were regarded as having conceived at midnight. Some litters were additionally staged using Theiler (1989). Dams were sacrificed by cervical dislocation at noon and the embryos dissected out and placed immediately into Carnoy's

fixative (60% Ethanol, 30% Chloroform, 10% Acetic Acid). Embryos were dehydrated, embedded in paraffin, sectioned at 7 microns on a rotary microtome, mounted in series and stained with cresyl violet.

For immunohistochemisty sections were rehydrated through a graded series of alcohols. Endogenous peroxidases were quenched using 10 mM Tris pH 7.5, 150 mM NaCl (TBS) containing 10% methanol and 3% hydrogen peroxide. Sections were rinsed in TBS, then blocked in TBS containing 10% normal goat serum, 0.1% Triton X-100 (Sigma, St. Louis) 1% glycine, and 2-3% BSA (Sigma). Primary antibodies were added, in blocking solution, as follows: Rabbit anti-Neurofilament 150-kD subunit (Chemicon, Temecula, CA; 1:2000); Anti-BrdU (Novocastra, Newcastle upon Tyne; 1:100) (see below). Immunoreactivity was detected using the appropriate biotinylated secondary antibody and biotin/avidin/biotin peroxidase reagents from the Vectastain detection kit (Vector Labs, Burlingame, CA), following the manufacturer's instructions.

Counts of neurons and cells

The trigeminal ganglion was mapped in paraffin series from three mutant and three wild-type animals for each stage analyzed. Every fifth section through the ganglion was photographed at high magnification, and positive profiles containing nucleoli were counted

in the resulting montage. No correction was made in the counts for split nucleoli. The numbers of trigeminal precursors in embryos up to stage E13.5 was calculated as the average number of Nissl profiles minus the average number of neurofilament positive profiles. (Satellite cells are not born until after these stages; see Altman and Bayer, 1982.)

Counts of pyknotic profiles

The density of pyknotic profiles was measured in Nissl-stained paraffin sections through the trigeminal of five wild type and five mutant embryos at E11.5 and E13.5. Widely spaced (by at least 30 microns) sections representing 8-10% of the total cell number were photographed as above, and the number of pyknotic profiles was divided by the total number of cells within the sections. Care was taken to exclude red blood cells (Coggeshall et al., 1994).

Analysis of BrdU incorporation

Pregnant dams were injected intraperitoneally with 5-bromo-2-deoxy-uridine (BrdU; Sigma) (50 mg/kg body weight) two hours before sacrifice. The embryos were dissected, embedded, sectioned and mounted as above.

Before staining with anti-BrdU antibody, sections were treated with 2 N HCl in 0.05 M phosphate buffered saline pH 7.2 (PBS) at 37°C for 20 min.; neutralized in 0.1 M borate buffer pH 8.5 for 5 min.; washed once in TBS; and then treated with peroxidase/methanol and stained following the protocol for immunohistochemistry described above.

LacZ staining of whole mounts and sections

Embryos up to age E13.5 were fixed for 1-2 hours in ice-cold 2% paraformaldehyde in PBS pH7.3. They were then either stained immediately for lacZ activity as whole mounts or frozen and cryosectioned at 10-30 microns for staining of sections.

Sections or whole mounts were placed into XGal staining solution (PBS pH7.3 containing 2mM $MgCl_2$, 0.02% NP-40 (Sigma), 0.01% Sodium deoxycholate, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, and 1 mg/ml X-Gal (Boehringer, Indianapolis, IN). Specimens were developed overnight with shaking at 37°C, washed extensively with PBS pH 7.3, and postfixed in 4% paraformaldehyde in PBS pH 7.3. Control material never showed color development under these conditions. Sections were then either immunostained for neurofilament (as above) or coverslipped with glycerol and stored.

In pilot experiments, the expression pattern of lacZ was compared in sections through the heads of heterozygous and homozygous mutant animals. No difference was found in the tissue distribution of lacZ product between animals of the different genotypes. Whole mounts were therefore performed on heterozygotes, while sections of homozygotes and heterozygotes were studied.

Comparison of Maxillary and Ophthalmic Branches of the Trigeminal Ganglion

7 micron paraffin sections cut in the coronal plane through the heads of E13.5 embryos were immunostained for NF-150 as above. The areas of the ophthalmic and maxillary branches of the trigeminal nerve were measured at the level of the posterior margin of the optic chiasm using the Neurolucida computerized tracing system (Microbrightfield, Inc., Colchester, VT).

Results

The NT-3 deficit emerges early in trigeminal development

In order to analyze the time-course of the development of the trigeminal defect in animals lacking NT-3, we counted the numbers of neurons and of all cells in the trigeminal ganglion of mutant and

wild type embryos at several stages of development. Neurons in E10.5, E11.5, and E13.5 embryos were counted as immunoreactive profiles in series stained for neurofilament 150kD protein (NF-150), a ubiquitous early neuronal marker (Cochard and Paulin, 1984) (illustrated in Figures 1 A,B). Neurons in E15.5 and P0 animals were counted according to morphological criteria in Nissl stained material. Total cells were counted in Nissl stained material at E10.5, E11.5, and E13.5.

Mutant embryos at E10.5 showed no detectable difference in neuronal number when compared with wild-type embryos (Figure 2; Table 1). Over the next days of development, during which most trigeminal neurons are born and extend axons to their peripheral targets (Davies and Lumsden, 1984), significant and increasing deficits in neuronal number were seen in mutant ganglia. At E11.5, mutants have only 60% as many neurofilament positive profiles as their wild-type counterparts. By E13.5, mutant animals have only 30% of the normal number of neurons. The number of neurons in mutant ganglia, and the deficit, remain roughly constant in size from E13.5 until birth. The trigeminal ganglia in heterozygous animals contained 52,210 $\pm$ 6821 neurons at E13.5, which is not significantly different from wild-type animals at this stage (wild-type embryos contain 48,755 $\pm$ 3943 neurons at E13.5). This suggests that NT-3 is

not present in limiting amounts with respect to the survival of these neurons at this stage.

To quantitate the numbers of precursor cells between E11.5 and E13.5 in normal and homozygous mutant embryos, we estimated the numbers of this population by subtracting neuronal numbers from the total numbers of cells present in the ganglion. (The major class of nonneuronal cells in the adult ganglion, the satellite cells, are not born until after these stages; see Altman and Bayer, 1982; see also Discussion.) At E10.5 and E11.5 (Figure 2; Table 1), the number of trigeminal precursor cells is similar in mutants and wild-type embryos. An approximately 30% reduction in precursor numbers is seen in E13.5 mutant animals, although this difference is not statistically significant. This deficit occurs later in development compared to the defect in neurons, which is already substantial at E11.5 and is essentially complete at E13.5. Therefore the absence of NT-3 affects neurons earlier and more severely than precursor cells. Neurons consequently represent a smaller fraction of all cells compared to wild type at both stages (Compare Figs. 1 A,B; Table 1).

Cell death is elevated in NT-3 mutants

The trigeminal ganglia of E10.5 mutant embryos contain normal numbers of cells and neurons. The subsequent deficit could

therefore be due to a failure of precursors to proliferate or due to an increase in cell death. In order to investigate the latter possibility, we measured the density of pyknotic profiles at E11.5 and E13.5 (Table 2). We found substantial numbers of pyknotic profiles in all stages and genotypes during the interval over which the deficit is occurring (not shown). We found a significant, approximately 2-fold increase in density of pyknotic profiles in E11.5 mutants relative to wild type and a highly significant, 2.5-fold increase at E13.5 (see Figure 1 C,D). Thus the emergence of the neuronal deficit correlates with an abnormally high rate of cell death in mutant animals.

To examine possible effects of the NT-3 deficiency on precursor proliferation, we also determined the number of cells that incorporate BrdU at different stages in normal and homozygous mutant animals (Table 2; Figure 2 E,F). At E11.5, there is no significant difference in numbers of BrdU positive cells between wild type and mutant embryos. At E13.5, we observed a reduction in total numbers of BrdU positive profiles in homozygous mutants; when this was normalized to the independently measured numbers of precursor cells, we found a highly significant, approximately 2-fold reduction in the intrinsic rate of proliferation. This indicates a change of precursor populations subsequent to elimination of neurons. Since neurogenesis is almost complete in mice by this stage (see Altman

and Bayer, 1982), these changes seem unlikely to substantially reduce the final neuronal number, but may reduce glial numbers.

NT-3 is expressed throughout the periphery of the trigeminal system

We took advantage of the β-galactosidase reporter construct inserted into the NT-3 locus to analyze the expression of NT-3 in the early trigeminal system by staining whole mounts of embryos using lacZ histochemistry (Figure 3). While our results provide greater resolution than published in situ hybridization studies (Arumae et al., 1993; reviewed in Davies, 1994), they are consistent with the patterns of expression found in those studies. We additionally stained some embryos using TuJ1 (Easter et al., 1993), a monoclonal antibody which recognizes the neuron-specific β3 isoform of tubulin, to reveal axonal projections (Figure 3B). At E9.5, when the first trigeminal neurons are born and begin to extend neurites (Davies and Lumsden, 1984; Easter et al., 1993), lacZ expression is already detectable in the first branchial arch (*arrow*, Figure 3A), the presumptive target of most trigeminal neurons. The midline of the roof of the mesencephalon is also intensely stained. By E10.5 (Figure 3B), the reporter expression has greatly intensified and spread throughout the developing mandibular and maxillary processes. The

olfactory pit (not shown) and eye-cup are stained to intermediate intensity. TuJ1 counterstaining reveals that the anterior margin of the trigeminal ganglion is surrounded by NT-3 expressing cells. At E11.5 (Figure 3C), the expression pattern changes, with more intense expression toward the distal half of the maxillary (*Mx* in Figure 3C) and mandibular processes. This trend is continued in E13.5 embryos, in which expression in the maxillary territory is most intense in the mystacial pad (*MP* in Fig. 3D) and distinctly less in the surrounding regions. The nostrils, ears and mesenchyme surrounding the eyeball remain intensely stained. This pattern of staining remains similar for several days after E13.5 (not shown; see also Tojo et al., 1995).

In order to examine the trigeminal system in greater detail, we also stained cryostat sections from embryos with one or two copies of the gene replacement for lacZ activity, alone or in conjunction with NF-150 immunohistochemistry (Figure 4). In E11.5 animals (Fig. 4 A,B), nearly every cell in the distal ends of the maxillary and mandibular processes, including the presumptive epidermis (*ep* in Figure 4A), was stained. Lineage tracing studies (Trainor and Tam, 1995, and references therein) have shown that these structures are populated by cells of three different developmental origins which occupy segregated final locations within the arch. The epithelial lining is derived from ectoderm, while the subadjacent mesenchyme

is derived from cells of neural crest origin. The deep mesenchymal (*mch* in Figure 4A) interior is derived from paraxial mesoderm. The staining seen throughout the depth of these structures at this stage indicates that cells of all three lineages express NT-3 at this time. Thus, axons approaching their cutaneous targets are bathed in NT-3 along their entire trajectory (Figure 4B). We found staining in other trigeminal targets, including the corneal ectoderm, the nostrils and the oral epithelia (not shown). In E12.5 animals (Figure 4C), expression appears to be reduced in the presumptive epidermis, while remaining intense throughout the mesenchyme. By E13.5 (Figure 4 E,F) reporter expression is no longer detectable in ectodermally derived targets such as the epidermis, instead being confined to mesenchyme adjacent to the epidermis (Figure 4E), hair follicles (Figure 4F) and the eyeball (not shown). Thus NT-3 is transiently and intensely expressed in trigeminal target tissues from E11.5 to E13.5, which correlates with the developmental interval over which trigeminal neurons are lost in mutants.

In sections from embryos of stages E11.5 through E13.5, the vast majority of trigeminal cells are negative for reporter expression (illustrated in Fig. 4D). While others have shown expression of NT-3 in a small number of sensory neurons at later stages as assessed by a similar lacZ reporter (Tojo et al., 1996), we find no evidence for

neuronal expression at the earlier stages during which the deficits in neuronal number are emerging in the trigeminal ganglia of animals lacking NT-3. We do find a very small number of positive cells, none of them neurons, within the ganglion at these stages (*arrows* in Figure 4D). In agreement with other reports (Buchman and Davies, 1993; Arumae et al., 1993), we find no evidence of NT-3 expression in the central nervous system targets of the trigeminal ganglion neurons (not shown).

The mystacial pad of the snout, which is the most densely innervated cutaneous target in the adult mouse, is derived during development from the first branchial arch (indicated by an *arrow* in Figure 3 A,C,D). This structure expresses very high levels of NT-3 during the developmental interval over which trigeminal neurons are abnormally lost in mice lacking NT-3 (Buchman and Davies, 1993; and present results). We wondered whether NT-3 might be especially important within the first branchial arch for the maintenance of immature trigeminal neurons. If so, then the neurons innervating this tissue would be more affected by the absence of NT-3 than neurons supplying regions which express less NT-3. In order to test this idea, we compared the effect of the NT-3 mutation on the cross-sectional area of the ophthalmic nerve, which supplies areas outside the first branchial arch which express much lower levels of

NT-3 (Buchman and Davies, 1993; see Figure 3), and the maxillary nerve, which supplies the maxillary process of the first branchial arch including the presumptive mystacial pad.

We measured the cross-sectional area of the ophthalmic and maxillary nerves in paraffin sections cut in the coronal plane through E13.5 embryos, in wild-type, heterozygous, and mutant embryos. (Figure 5; Table 3) We found that neither nerve trunk was significantly reduced in area in heterozygous animals. However, the ophthalmic nerve in homozygous mutants was reduced by 60% in cross-sectional area, while the maxillary nerve was reduced by 50% (Table 3). This result suggests that neurons supplying targets outside the first branchial arch are depleted to a similar extent to neurons supplying targets within the arch. Thus the intense expression of NT-3 in the first branchial arch likely does not correspond to a differential requirement by trigeminal neurons innervating those territories for NT-3.

Discussion

Results presented in this paper show that the deficiency in neuronal numbers seen in the trigeminal ganglion in animals lacking NT-3 emerges rapidly over a short interval in the development of

the animal. This period, between stages E10.5-E13.5, is characterized in homozygous mutants by a progressive depletion of neurons from the pool of all trigeminal cells relative to wild-type embryos. During these stages, apoptotic cell death is elevated in the trigeminal ganglion of mutants relative to wild type; whereas neither the incorporation of BrdU into proliferating trigeminal cells, nor the numbers of trigeminal precursor cells, is reduced in mutants until after the birth of most neurons. Our lacZ based assay for NT-3 expression shows that NT-3 is produced throughout the periphery of the trigeminal system, but not within the ganglion nor the CNS targets of ganglion neurons. We conclude that the neuronal defect seen in the trigeminal ganglion of mutant mice is due to the abnormal cell death of neurons. Thus NT-3 acts in wild type mice as a peripherally-derived survival factor for early trigeminal ganglion neurons.

Neurons and precursors in the developing trigeminal ganglion

NT-3 has been proposed to perform a variety of actions in the developing nervous system (for review see Korsching, 1993). In order to evaluate these potential roles it was necessary for us to track the dynamics of both the neuronal pools and the precursor pools that give rise to them. As discussed below, we believe our

measurements, centering around the expression of neurofilament-150 by trigeminal neurons, can yield an accurate assessment of the relative dynamics of these two populations in the early ganglion.

We chose expression of neurofilament-150, a member of the family of neuron-specific middle molecular mass intermediate filament proteins, as our benchmark for identifying trigeminal neurons. During embryogenesis these proteins are expressed by all sensory neurons, with the onset of expression at the cellular level concomitant with initial axonogenesis by recently born sensory neurons (Cochard and Paulin, 1984). Thus, while our counts based on NF-150 may omit some cells in the earliest stages of commitment to neuronal fate, it unambiguously identifies all neurons from an early stage.

Our observations indicate that the temporal expression of NF-150 by early trigeminal ganglion neurons is not disrupted in mice lacking NT-3. We find normal numbers of NF-150 expressing cells in E10.5 mutants, indicating the appropriate temporal expression of this antigen in the absence of NT-3 by the earliest trigeminal neurons. Additionally, our counts of total cell numbers at E11.5 and E13.5 (Table 1) suggest that the deficiencies seen in neurofilament counts of mutant embryos at these stages reflect the absence of cells rather than delay or failure of neurons to express NF-150 in mutant

animals. If the absence of NT-3 were to cause a delay in the expression of NF-150, one might expect to see increased numbers of neurofilament-negative cells in mutant animals relative to wild-type animals. Instead, we find that the difference between wild-type and mutant embryos in total cell numbers is equal to or greater than the difference in neuronal counts at both E11.5 and E13.5. Thus, we conclude that the neurons are actually absent in mutants, rather than being present and failing to express NF-150. Finally, consistent with this conclusion, the development of the peripheral projections of trigeminal neurons in material stained for NF-150 appears to be temporally appropriate in mutant embryos (data not shown). Thus NF-150 appears to mark an equivalent population of neurons in both wild-type and mutant embryos.

We selected the term "precursor" to designate members of the population (or populations) of proliferating cells in the trigeminal ganglion of mid-gestation embryos which give rise to the mature cell types found in the adult ganglion (i.e., principally neurons and satellite cells). Neurogenesis studies indicate that neurons are born from this pool over stages E9.5 to E13.5, with a peak over stages E11-E12 (see Altman and Bayer, 1982). We believe that the quantification of the number of trigeminal ganglion cells which do not express neurofilament indeed provides a good estimate of the numbers of

precursors at the stages examined for the purposes of this study. Cell cycle studies in early sensory neurons show that essentially all neurofilament negative cells can be labeled in E11.5 animals by repeated pulses of BrdU over a 9 hour period (unpublished observations), indicating that all of the neurofilament-negative cells counted in this study are indeed proliferating. By considering the numbers and proliferative properties of this pool at stage E11.5, we can appraise whether neurons are being born in normal numbers in mutant animals (see below).

NT-3 directly affects the neuronal population

In interpreting our results, we find no evidence for an effect of this mutation on the earliest events of gangliogenesis, since the E10.5 ganglion in mutant animals contains normal numbers of trigeminal cells and neurons. However, after this initial stage, we find a rapid depletion of the number of neurons in mutants relative to wild type. This decrease, seen in the absence of a detectable effect on the precursor population during neurogenesis, suggests that NT-3 acts directly on the neuronal population. Moreover, the increase in cell death seen at these stages strongly suggests that the loss of neurons is due to neuronal apoptosis.

Where is the NT-3 required by trigeminal neurons produced? The expression pattern of our lacZ reporter gene indicates that at E11.5, NT-3 is available along the entire trajectory of maxillary axons including final target regions. We do not observe staining within the ganglion but it is possible that NT-3 is available within the ganglion, via diffusion from nearby mesenchymal cells (Figure 3B; Figure 4D). The onset of the deficit in the trigeminal ganglion occurs between ages E10.5 and E11.5, a time span during which the first trigeminal axons are beginning to reach their targets (Figures 4B and 5B; see also Davies and Lumsden, 1984). This suggests that the earliest trigeminal neurons may not require NT-3 for survival until their axons have reached the vicinity of their targets. Alternatively, the onset of the deficit in mutants might reflect a requirement for NT-3 by later born neurons. One prediction of this alternative idea is that the earliest neurons would be relatively unaffected by the loss of NT-3. This could be determined via a detailed neurogenesis study comparing wild type to mutant animals.

Developmental studies of trigeminal neurons in vitro have demonstrated a switchover in the neurotrophin dependence of early trigeminal neurons (Buchman and Davies, 1993). Cultures of early trigeminal neurons can survive in the presence of NT-3, BDNF, or NT-4, whereas most neurons from cultures of later trigeminal ganglia

survive only in the presence of NGF. The switchover is observed in cultures from E11 through E13 mice, which corresponds well to the stages at which we see neuronal losses in vivo in mice lacking NT-3. This switchover further corresponds to a period of rapid increase in NGF expression in the presumptive epidermis at the times (stages E12-E13) that we see a relative decrease in the expression of NT-3 in that tissue (Davies et al., 1987; discussed in Davies, 1994). This raises the intriguing possibility that the survival of trigeminal neurons depends on obtaining NT-3 and NGF in sequence from the same tissue. Since the size of the trigeminal ganglion deficit is very large in animals lacking NT-3 and in animals lacking NGF, at least some trigeminal neurons must require both factors for survival (discussed in Fariñas and Reichardt, 1996). While NGF and NT-3 utilize trkA and trkC, respectively, as their primary receptors, trigeminal neurons in this situation might in fact respond to both factors via the identical receptor, namely, trkA. TrkA is expressed very early by most trigeminal neurons (Arumae et al., 1993; and unpublished observations), and both NT-3 and NGF are capable of signaling through this receptor (Clary and Reichardt, 1994; Davies et al., 1995). However, trigeminal neurons in cultures from stage E11-E13 animals do not survive in identical numbers in response to NT-3 and NGF (Buchman and Davies, 1993), suggesting complexities in the ligand-

receptor system in the trigeminal ganglion. Both differential splicing of trkA and its coexpression with $p75^{NTR}$ have been shown to modulate the efficacy of trkA activation by NT-3 (Clary and Reichardt, 1994). Either mechanism might explain the changes in relative sensitivity of trigeminal neurons to NT-3 compared to NGF seen over this interval. Evaluation of this hypothesis would require additional study of the relationship between the extension of peripheral trigeminal axons in vivo and the expression of functional NT-3 receptors. In any event, the fact that the phenotype in vivo of mice lacking NT-3 results in incomplete elimination of trigeminal neurons suggests that other survival factors could be supporting these neurons at this stage.

Late disruption in precursor populations

It seems likely that lack of NT-3 does not primarily affect the generation of neurons by precursor cells during the interval over which the neuronal deficit is emerging. Our quantification of precursor numbers (Table 1; Figure 2) indicates that these cells are present in normal numbers in mutant mice at E11.5, and that most of these cells survive throughout the interval of the deficit in the absence of NT-3. Consequently, NT-3 does not appear to be an essential survival factor for the majority of precursors in vivo. In

addition, the numbers of trigeminal cells incorporating BrdU is not diminished in mutant animals at this stage (Table 2). Thus, precursor cells are not significantly diminished in numbers nor slowed in proliferation by the absence of NT-3 at E11.5, when neurogenesis is at its maximum (see Altman and Bayer, 1982). This suggests that neurons are being generated in normal numbers at this stage. Therefore, the simplest inference from these findings is that the neuronal defect seen in the trigeminal ganglion of E11.5 mutants does not arise primarily from a diminished generation of neurons by precursors. Instead, the progressive decrease of neurons as percentage of all cells in mutants, relative to wild-type embryos, indicates that the lack of NT-3 directly affects neuronal numbers via the neurons themselves.

While not seen in earlier embryos, we do observe a change in the intrinsic rate of proliferation of precursors at E13.5. (Table 2). Since this change occurs after the onset of the neuronal deficiencies, which are already seen in E11.5 animals, we cannot determine if they represent direct or indirect effects of the absence of NT-3. By E13.5 almost all neurons have been born, and the changes seen in E13.5 animals in themselves could reflect decreased generation of satellite cells (see Altman and Bayer, 1982). Consistent with this hypothesis, NT-3 has been shown to act as a mitogen for oligodendrocyte

precursors (Barres et al., 1994). Therefore the proliferative changes seen at E13.5 probably do not contribute substantially to the deficit in neuronal numbers.

Our results provide strong evidence that neurons are the population most affected by the absence of NT-3 in the developing trigeminal ganglion. Our quantification of neuronal and non-neuronal pools clearly shows that the former pool is specifically depleted in animals lacking NT-3 in the absence of effects on precursor populations. Following the initial review of this paper, another study (ElShamy and Ernfors, 1996b) was published that argues that precursors, not neurons, are the cells primarily affected in the absence of NT-3. They observe colabelling of BrdU-label with some TUNEL positive cells and observe an increased proportion, versus wild type, of TUNEL-positive cells colabelled with anti -nestin, a marker for neural progenitors.

The two sets of observations might be reconciled if the neurons which die in the trigeminal ganglion of NT-3 knockout mice at E11.5 have commmited to do so shortly after their final mitosis. A five to six hour BrdU labelling protocol was used in their experiments, and other markers for progenitor cells have been shown to persist in newborn neurons (e.g. Cochard and Paulin, 1984, Memberg and Hall, 1995a). However, in our analysis of development of the deficiency in

the dorsal root ganglia of NT-3 mutant mice, we also have evidence indicating that there may be methodological problems associated with the non-standard BrdU labelling protocol used by these authors (Fariñas et al., submitted).

In conclusion, our results indicate that lack of NT-3 results in abnormal elimination of trigeminal neurons early in development, with a subsequent disruption of precursor populations, possibly as an indirect effect of the loss of neurons. Thus NT-3 acts as a peripherally-derived survival factor for these neurons in the wild-type mouse. Further understanding of the cellular events associated with the neuronal requirement for NT-3 will require additional investigation of the relationship between the defects seen in knockout mice and the expression of functional NT-3 receptors by trigeminal neurons.

Figure 1. Summary of the trigeminal phenotype in embryos lacking NT-3. For each pair of photographs, representative sections from wild-type (**A,C,E**) and mutant (**B,D,F**) animals are compared. These observations are quantitated in Tables 1 and 2. **A,B.** E13.5 material stained for neurofilament-150 and counterstained for cresyl violet. Neurons are depleted relative to overall trigeminal populations. **C,D.** E13.5 material stained for cresyl violet. The density of pyknotic profiles is greatly elevated in mutants. *Arrowheads* indicate red blood cells. **E,F.** E11.5 material stained for BrdU incorporation by proliferating cells. Proliferation is unchanged at this stage. Scale bar, 100 microns.

Figure 2. Numbers of trigeminal neurons (squares) and precursor cells (diamonds) in wild type (closed symbols) and NT3- (open symbols) animals during development. Neurons in E10.5, E11.5, and E13.5 animals were counted as profiles immunopositive for neurofilament 150 kD protein. Precursors for those stages were calculated as total (Nissl) cells minus neurons. E15.5 and P0 neurons were identified on the basis of morphology in Nissl material. The means +/- S.D. of counts from three separate animals are shown for each point plotted.

Figure 3. Changes in expression of a lacZ reporter construct inserted into the NT-3 locus. Whole mounts of heterozygous animals. (See text). **A**. E9.5. Staining is observed in the first branchial arch (*arrow*) and the anterior (*a*) and posterior (*p*) neuropores of the mesencephalon. **B**. E10.5, counterstained for TuJ1 to show axonal projections. The lacZ reaction was underdeveloped to allow visualization of axons. . **C**. E11.5 embryo. Expression of NT-3 is strongest at the distal half of the maxillary process (*Mx*). **D**. E13.5 embryo. Reporter expression in the maxillary territory is strongest in the mystacial pad (*MP*) and distinctly less elsewhere.

Figure 4. Changes in lacZ reporter expression during development. (**E**,**F**).**A**. Maxillary process of an E11.5 embryo homozygous mutant. Intense reporter expression is seen in the presumptive epidermis (*ep*) and in the superficial (derived from neural crest) and deep (derived from mesoderm) mesenchyme (*mch*). **B**. E11.5, lower magnification of the maxillary process, immunostained for NF-150 to reveal axons. Axons approaching their cutaneous targets encounter NT-3 producing cells along their entire trajectory. Homozygous mutant. **C**. Maxillary process of an E12.5 homozygous mutant embryo. Intense expression persists in the mesenchyme, but the skin staining begins to weaken. **D**. Vicinity of

the trigeminal in an E12.5 homozygous mutant embryo. NT-3 is expressed by mesenchyme surrounding the anterior edge of the trigeminal, and by a few nonneuronal cells (*arrows*) within the ganglion. **E, F**. Mystacial pad of an E13.5 heterozygote sectioned at 20 microns. Expression is confined to the mesenchyme underlying the skin (**E**) and surrounding whisker follicles (**F**). Scale bar for each image is 50 microns.

Figure 5. Comparison of the peripheral branches of the trigeminal nerve in wild-type and mutant mice. **A.** Schematic drawing of an E13.5 embryo (adapted from Theiler, 1989) indicating the plane of section used for the camera lucida analysis. The thin opthalamic branch (dorsal) and the thick maxillary branch (middle) were compared in wild type, heterozygous, and mutant mice. The mandibular branch (ventral) travels obliquely to this plane and was not analyzed. *O.N.* designates the optic nerve. **B.** Camera lucida drawing showing the opthalmic and maxillary branches of the trigeminal nerve in a wild-type embryo. The opthalmic nerve is hatched; the maxillary fascicles are cross-hatched. *OX* indicates the optic chiasm. **C.** Camera lucida drawing of the same complex in a mutant animal. The opthalmic (hatched) and maxillary (cross-

hatched) branches of the ganglion are both depleted (see Table 3). Scale bars: 100 microns.

Table 1. Subpopulations in the trigeminal ganglion in wild type and NT-3 deficient mice.

	E10.5			E11.5			E13.5		
	Wild Type	Mutant		Wild Type	Mutant		Wild Type	Mutant	
Neurons	6093 ± 2065	5375 ± 1738	88%	25545 ± 3562	14743 ± 5513•	58%	48755 ± 3943	15217 ± 1023•••	31%
Total Cells	24733 ± 1389	25542 ± 8151	100%	61228 ± 10914	46270 ± 13104	77%	82027 ± 12216	39192 ± 6604••	48%
Precursors	18640 ± 2488	20167 ± 8334	100%	36833 ± 11480	31527 ± 14216	88%	33272 ± 12836	23975 ± 6682	72%
% Neurons	25	21		42	32		59	39	

Percentages following entries for mutant animals show the mutant population as a percentage of wild type. **Neurons** is reported as the mean +/- standard deviation (SD) of counts of cells immunopositive for NF-150 in three animals for each group. **Total Cells** is reported as the mean +/- SD of counts of Nissl profiles in three animals for each group. **Precursors** is calculated as Neurons minus Total Cells for each group. Uncertainty is calcluated via propagation of errors. **%Neurons** is calculated as the percentage of neurons relative to total cells.

• $p < 0.05$ (Two-tailed student's t-test)

•• $p < 0.01$

••• $p < 0.001$

Table 2. Proliferation and pyknosis in the trigeminal ganglion of wild type and NT-3 deficient mice.

	E11.5		E13.5	
	Wild Type	Mutant	Wild Type	Mutant
$BrdU^+$	10878 +/- 2548	9047 +/- 368	4528 +/- 381	1820 +/- 281**
% $BrdU^+$	30.4 +/- 0.7	28.7 +/- 1.1	13.6 +/- 1.1	7.6 +/- 1.1**
% Pyknotic	3.2 +/- 1.5	6.1 +/- 2.5*	3.0 +/- 0.5	8.2 +/- 2.5**

$BrdU^+$ is reported as the mean +/- SD of counts of cells immunopositive for BrdU incorporation in 3 animals in each group. % $BrdU^+$ is calculated as the mean percentage +/- SD of BrdU positive cells relative to numbers of precursors. % Pyknotic is reported as the mean +/- SD of the percentage of pyknotic profiles relative to total cells for five animals in each group.

* $p<0.05$, One-tailed Student's t-test

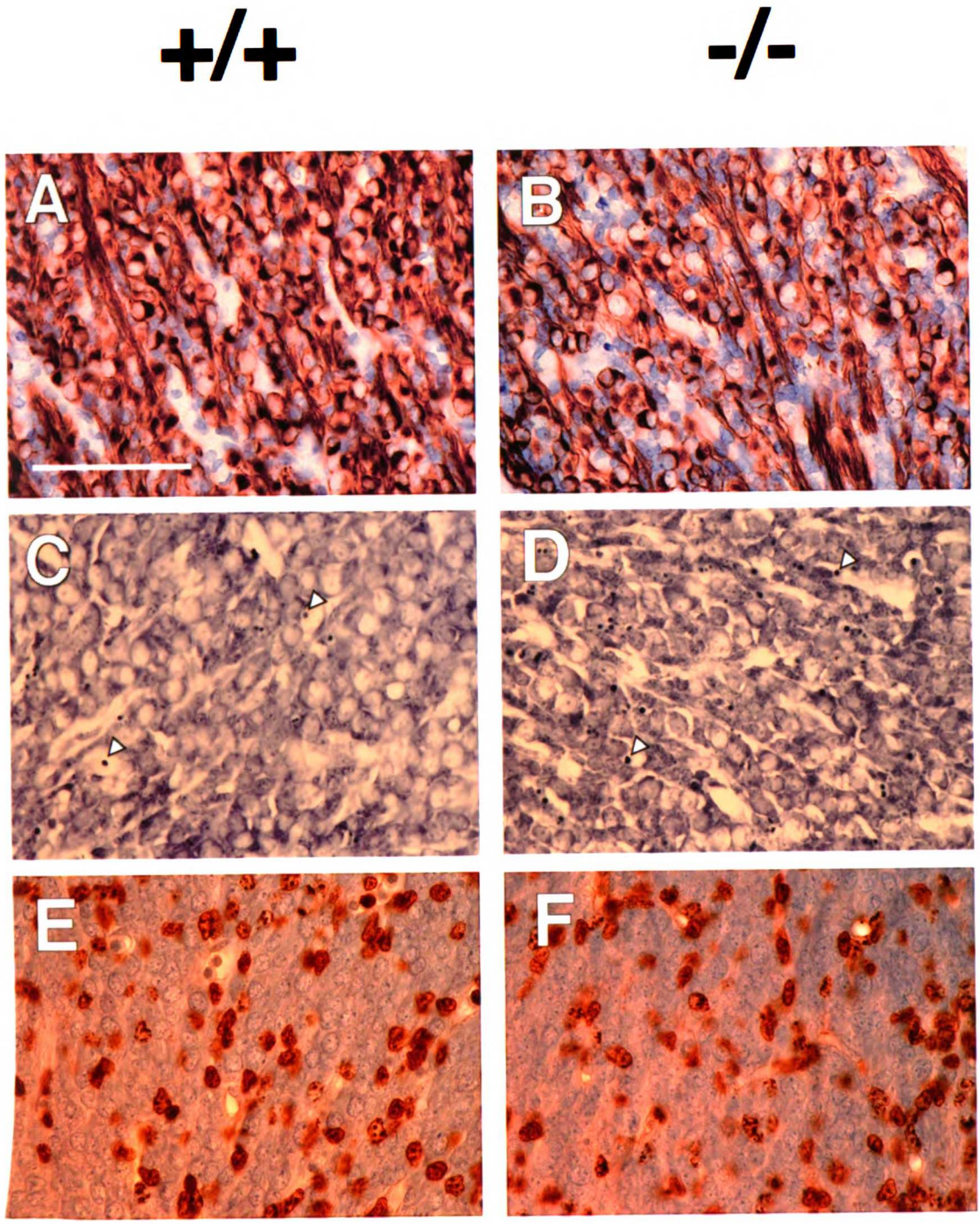
** $p< 0.01$

Table 3. Cross-sectional area (in μm^2)of the maxillary and opthalmic branches of the trigeminal in wild type, heterozygous or homozygous mutant mice.

	+/+	+/-	-/-
Maxillary	8486 +/- 227	8564 +/- 1630	4650 +/- 1604**
Opthalmic	1958 +/- 223	1761 +/- 190	766 +/- 220**

Each entry represents the mean +/- SD of the measurements on three animals in E13.5 animals. Plane of section is indicated in figure 5A.

** $p < 0.01$, One-tailed Student's t-test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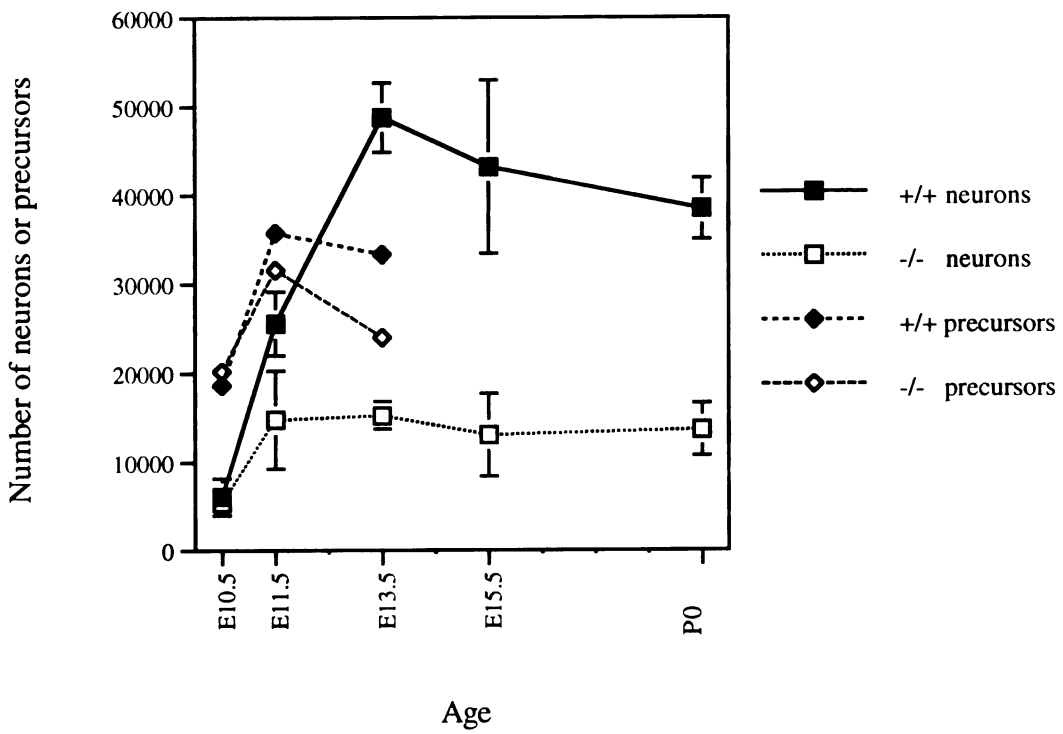
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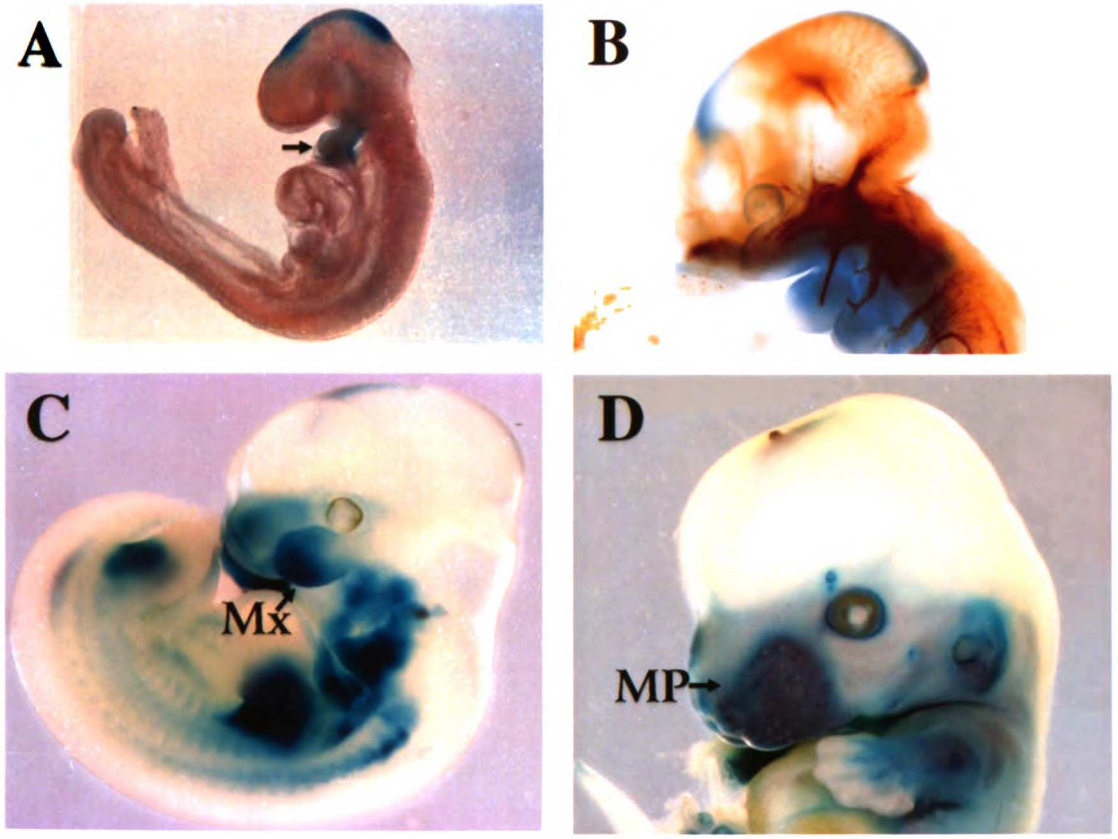
50000
40000
30000
20000
10000
0
E10.5
E11.5
E13.5
E15.5
P0
Age
+/+ neurons
-/- neurons
+/+ precursors
-/- precursors


A
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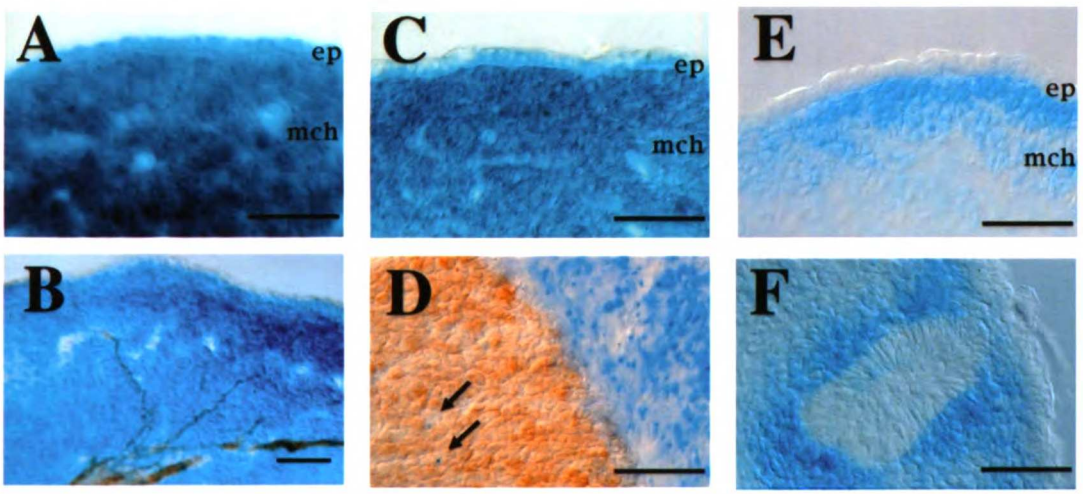
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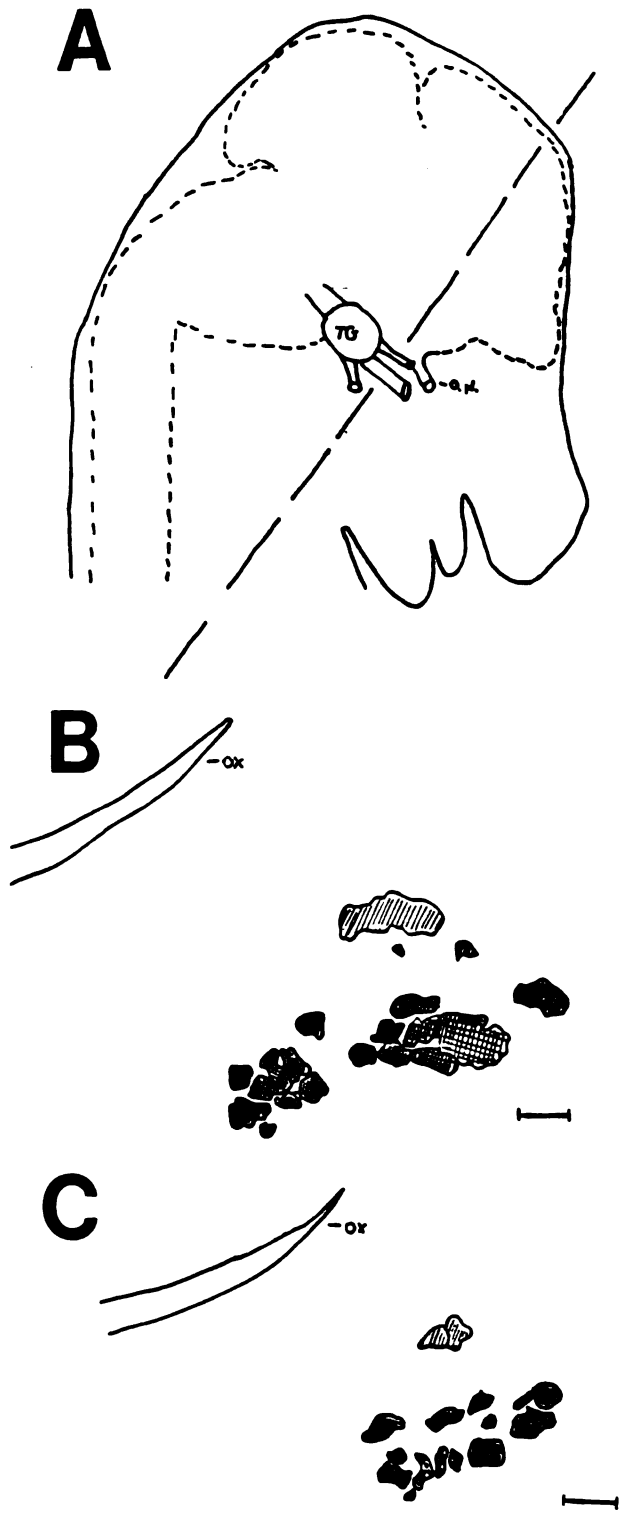
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Chapter III.

Expression of trk receptors and roles of NT-3 in vivo in the developing mouse trigeminal ganglion

ABSTRACT

Animals lacking neurotrophin-3 (NT-3) are born with severe sensory deficits, with the trigeminal ganglion missing 70% of the normal number of neurons. Our previous study of the development of the trigeminal ganglion in NT-3 knockout mice showed that the neuronal deficit in this ganglion emerges between embryonic stages (E)11.5 and E13.5 due to abnormal elimination of neurons. This interval corresponds in wild-type animals to rapid changes in the distribution of trk receptor mRNAs in the ganglion. In order to understand the neuronal distribution of trk receptors in the developing trigeminal ganglion, we raised antisera against rat trkB, called RTB, and rat trkC, called RTC. We used these new antisera and RTA, an antiserum against trkA to examine expression patterns of the trk receptors in the trigeminal ganglion of wild-type embryos and NT-3 mutant embryos between stages E11.5 and E13.5.

At all stages examined, the expression of trks within the trigeminal ganglion was confined to neurons. The extent of expression of each of the trks changes rapidly in wild-type embryos from E11.5-E135. RTC labels 93% of neurons at E11.5 and only ~10% of neurons at E13.5. RTB labels~40% of neurons at E11.5 and ~10% of neurons at E13.5. RTA labels 50% of neurons at stage E11.5 and

essentially 100% of neurons at E12.5. At E11.5, trkC is expressed by many neurons which also express trkA or trkB. At E13.5, trkC is not coexpressed with trkA or trkB.

The absence of NT-3 has disparate consequences for trigeminal neurons expressing trkA, trkB, or trkC. Neurons expressing trkC and neurons expressing trkB are severely reduced in E11.5 embryos. By contrast, the numbers and rate of increase of RTA immunopositive neurons in mutants is relatively unaffected from E11.5-E12.5.

We propose a model for the expression of trk receptors and actions of NT-3 in the E11.5 trigeminal ganglion. TrkC is transiently expressed by all neurons prior to definitive expression of a single trk. NT-3 acts on this early pool by influencing expression of trkC. NT-3 is specifically required for the survival of all definitive trkC expressing neurons, and for many definitive trkB expressing neurons. TrkA expressing populations are relatively unaffected by the absence of NT-3. In this model, the deficits seen in animals lacking NT-3 are more severe than that seen in animals lacking trkC in part because of a functional interaction in vivo of NT-3 with trkB.

INTRODUCTION

The neurotrophins are a family of related polypeptides required for the survival of developing neurons and other aspects of

nervous system development. Their principal effects on neurons are exerted through binding to the trk family of receptor tyrosine kinases. NGF binds to trkA; BDNF and NT-4 bind to trkB; and NT-3 binds principally to trkC but has been shown in vitro to bind to trkA and trkB (reviewed in Bothwell, 1995) Each neurotrophin additionally binds to another protein, called $p75^{NTR}$, but the contribution of this interaction to the survival of developing neurons is still incompletely understood (Lee et al., 1993; Stucky and Koltzenburg, 1997).

Knockout experiments in mice have underscored the importance of the neurotrophins and the trk receptors in the developing nervous system. Elimination of the function of any of the neurotrophins or the trk receptors results in mice with specific nervous system deficiencies (reviewed in Reichardt and Fariñas, 1997). For example, animals lacking NT-3 are born with severe sensory deficits and die shortly after birth. Of particular interest, the trigeminal ganglion in NT-3 knockout animals lacks about 70% of the wild-type numbers of neurons. This deficit includes many classes of neurons, including some which depend on other neurotrophins postnatally (Fariñas et al., 1994).

In a previous study of the neuronal deficit in the trigeminal ganglion of NT-3 knockout mice (Wilkinson et al., 1996), we

determined that the deficit in neuronal numbers in this ganglion occurred during a restricted developmental interval due to apoptotic elimination of neurons. This interval, between stages E10.5-E13.5, is associated in wild-type mice with substantial developmental changes in the expression of the trkA, trkB and trkC receptors in the trigeminal ganglion. In situ studies (Arumae et al., 1993; Ernfors et al., 1992) have shown that trkB and trkC are widely expressed in the ganglion at E11.5 but show restricted expression by E13.5. In contrast, they found that trkA is expressed by increasing proportions of trigeminal neurons over the same period. Furthermore, in vitro data (Buchman and Davies, 1993; Paul and Davies, 1995) show that cultured trigeminal neurons obtained from E11-E14 embryos progressively change in their ability to respond to added neurotrophins. Cultures of E11 trigeminal neurons exhibit 100% survival in the presence of added NT-3, BDNF or NT-4/5, but almost no survival response to added NGF. Cultured trigeminal neurons from later staged embryos show an apparent switchover in neurotrophin responsiveness, so that cultured E14 trigeminal neurons exhibit 100% survival in response to NGF with only small numbers surviving in response to BDNF, NT-4/5, or NT-3 (Buchman and Davies, 1993; Paul and Davies, 1995). However, it is not clear how the changes in neurotrophin responsiveness observed in vitro

might relate to the survival requirement for NT-3 in the more complex environment encountered by these neurons in vivo. Furthermore, the extent of neuronal expression of the trkB and trkC has been unknown.

To address these questions, we have raised polyclonal antisera against the extracellular domains of rat trkB and rat trkC. These antisera are expected to recognize all isoforms of rodent trkB (reviewed in Barbacid, 1994; Ninkina et al., 1997) and trkC (reviewed in Barbacid, 1994). These affinity purified antisera along with a previously generated antiserum recognizing rat trkA (RTA; Clary et al., 1994) have allowed us to obtain an overview of the expression of trkA, trkB, and trkC in vivo by embryonic trigeminal cells in wild-type and NT-3 knockout animals.

Using these tools, we have determined that the expression of each of the trks in the trigeminal ganglion is confined to neurons at all stages examined. Secondly, the pattern of neuronal trkC and trkA expression is highly dynamic during this interval. The percentages of neurons expressing trkC decreases dramatically from E11.5 to E13.5, accompanied by an increase in trkA expressing neurons. Finally the lack of NT-3 in vivo has distinct consequences on the dynamics of populations expressing trkA, trkB, and trkC during the stages examined.

METHODS

Generation of cDNAs encoding the extracellular domain of rat trkB and rat trkC

cDNA segments encoding the extracellular domain of rat trkB (Rtb-ex) or the extracellular domain of rat trkC (Rtc-ex) were obtained using PCR to amplify that portion of the cDNA from a full-length cDNA clones. The oligonucleotides used to amplify also introduced sequences specifying a 6xHistidine motif followed by a myc tag to the 3' terminus of each construct. The olignucleotides sequences used for trkB were: (N-terminal) CCC GAA TTC GCC ACC ATG TCG CCC TGG CCG AGG TGG CAT TGG and (C-terminal) GTT GCT GAC CAA ACC AAT CGG GAG CAT CAC CAT CAC CAT CAC GGC GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG TAG CTC GAG AAG G . The oligonucleotide sequences used for trkC were: (N-terminal) CCC ACT AGT GCC ACC ATG GAT GTC TCT CTT TGC CCA and (C-terminal) CCT TCT CGA GCT ACA GGT CCT CCT CGG AGA TCA GCT TCT GCT CGC CGT GAT GGT GAT GGT GAT GGT CTT CCT CTG GTT TGT GGG TCA G. Amplified PCR fragments were cloned into the vector pCR3 using the TA cloning kit (Invitrogen), which was used as the expression vector. The cloning junctions were sequenced and multiple clones were

screened in transient cos cell transfections assays for appropriate expression.

Purification of Rtb-ex and Rtc-ex from cos cell supernatants

Constructs were transfected using lipofectamine into large scale cultures of cos cells. Cells were then transferred into expression medium (DME, 1% Nutridoma-HU; pen/strep). Supernatants were harvested at 48 and 96 hours, sterile filtered, and diluted with the following: PMSF (to 100 ug/ml); NaCl (to final concentration 400 mM); Tris pH 8.0 (to final 20 mM); leupeptin (to 10 ug/ml) and pepstatin (5 ug/ml).

The supernatant was passed over a wheat germ lectin column. After washing with 20 mM Tris-Cl pH 8.0, 300 mM NaCl, lectin-binding proteins eluted using 500 mM N-Acetyl Glucosamine (Sigma) in 300mM NaCl, 20 mM Tris-Cl 8.0. The following was added to eluate fractions: PMSF (100 ug/ml); Na-Imidazole (10 mM pH 8.0); Beta-mercaptoethanol (10mM) ; glycerol (30% final concentration); Tween-20 (to 0.1%).

The wheat germ lectin eluate was batch-adsorbed to NTA Nickel beads (Qiagen) for 3 hr at 4 °C. After binding, the beads were washed in wash buffer (10mM Tris-Cl pH 8.0; 300 mM NaCl; 0.1% Tween-20 (Sigma)). Protein was batch eluted using 10mM Tris-Cl pH

8.0; 100 mM NaCl; 500 mM Imidazole (pH 8.0). Eluate was concentrated on a Centricon-30 (Amicon) and dialyzed extensively versus TBS.

Purified RTB-ex was used to immunize a rabbit and purified RTC-ex was used to immunize a goat. Initial injections in both cases were 150 ug. Subsequent boosts were 100 ug of purified protein each.

Affinity purification of RTB and RTC

Purified Rtb-ex and Rtc-ex (a generous gift of Allan Bates and Bill Mobley, UCSF) obtained from a Picchia pastoralis expression system (pPIC9K expression vector; Invitrogen) were coupled to cyanogen bromide CNBR4MB beads (Pharmacia) following the manufacturer's instructions. Coupled beads were incubated in 10mM Tris pH 7.5 150 mM NaCl (TBS) overnight to block unreacted groups and then used as an affinity matrix.

Partially purified RTB or RTC immunoglobulin (ammonium sulfate 35% saturated cut; dialyzed into TBS) was bound to affinity matrix using batch incubation. Beads were washed twice in 10 mM Tris-Cl pH 7.5 , 300 mM NaCl, once in TBS and then loaded onto a mini-column. Immunoglobulin was eluted using 150mM glycine-Cl

pH 2.3 and immediately neutralized by adding Tris-Cl pH 8.0 to a final concentration of 100 mM. Eluates were pooled and then dialyzed versus TBS. Na-Azide was added to .05% and samples were stored at 4 °C. The affinity matrix was immediately returned to neutral pH by washing in TBS.

Western blot analysis of the specificities of RTB and RTC

Cos cells were transiently transfected with expression constructs directing expression of full-length rat trkA, trkB, or trkC. 5 ug of each full-length construct cloned into the pCR3 expression vector (Invitrogen) was mixed with 40 microliters of lipofectamine (GIBCO) to transfect 100 mM dishes. After 48 hours, cells were lysed in 1 ml RIPA buffer containing PMSF (100 ug/ml) and leupeptin (1 ug/ml). 1/100 volume of each lysate was analyzed by Western blot using affinity purified RTB (1 ug/ml), affinity purified RTC (1 ug/ml), or RTA (1 ug/ml).

Immunohistochemistry

Mice with a targeted mutation in the NT-3 gene were obtained from our colony on a C57B/6 background. Additional wild-type mice

were obtained by crossing C57B/6 mice. Animals were genotyped by Southern blot as described.

Females in estrus were paired with males overnight and examined for vaginal plugs the following morning. For the purposes of staging embryos, pregnant females were regarded as having conceived at midnight. Some embryos were also staged using the criteria of Theiler (1989). Dams were killed by cervical dislocation at noon and the embryos rapidly dissected out and placed into Carnoy's fixative (10% glacial acetic acid; 30% chloroform; 60% ethanol). Embryos were dehydrated, embedded in paraffin, sectioned at 7 microns on a rotary microtome, and mounted in series.

For immunohistochemistry, sections were rehydrated through a series of alcohols. Endogenous peroxidases were quenched using 10 mM Tris pH 7.5, 150 mM NaCl (TBS) containing 10% methanol and 3% hydrogen peroxide. Sections were rinsed in TBS then blocked using TBS containing 0.4% Triton X-100 (Sigma); 1% glycine, 3% BSA (Fraction V; Sigma) and 10% normal serum from the host species of the secondary antibody to be used (see below). Primary antibodies were added in blocking solution as follows: rabbit anti-neurofilament medium molecular weight (NF-M) subunit (Chemicon, 1:200); rabbit anti-trkA (Clary et al., 1994, 1:500); affinity purified

rabbit anti-trkB (5 ug/ml); affinity purified goat anti-trkC (5 ug/ml); mouse anti-NF-M (Sigma; 1:100).

For detection of single antigens, immunoreactivity was detected using biotinylated goat anti-rabbit antibody (Vector; 1:300) or biotinylated rabbit anti-goat (Vector; 1:300) and biotin-avidin-biotin peroxidase reagents from the Vectastain detection kit (Vector) following the manufacturer's instructions.

For immunofluoresecence detection of two antigens, trkC immunoreactivity was detected using biotinylated horse anti-goat antibody (vector; 1:300) followed by Texas Red Avidin (Zymed; 1:1000). Rabbit NF-M and trkB immunoreactivity were detected using donkey anti-rabbit antibody (Jackson, 1:300) coupled to either FITC or Cy3. Mouse NF-M immunoreactivity was detected using goat anti-rat antibody (Cappel, 1:100) coupled to either FITC or Texas Red. Fluorescent samples were photographed at high magnification, with fluorescence from each channel exposed on a separate negative. Exposures were digitally superimposed using Photoshop 3.05 software (Adobe Systems).

Counts of immunopositive profiles

The trigeminal ganglion was mapped in paraffin series for at least two animals for each genotype and antigen at each stage

examined. In most cases, every sixth section through the ganglion was photographed at high magnification and immunopositive profiles containing nucleoli were counted in the resultant montages. No correction for split nucleoli was made to the counts.

In the following instances of low numbers of positive profiles, immunoreactive neurons were counted directly at the microscope using a graticule: E11 mutant trkC; E12 wild-type and mutant trkB; E13 wild-type trkB and trkC and mutant trkB.

Counts of double label experiments

The following pairs of antigens were subjected to semi-quantitative analysis: At E11.5, trkB and neurofilament; trkC and neurofilament. At E12.5, trkB and trkC. At E13.5, trkA and trkC.

Double label photomicrographs were assembled as above from regions of the trigeminal ganglion which contained many profiles immunopositive for each antigen. Prints of the resultant superposition were counted by hand for profiles expressing one or two antigens.

RESULTS

Generation of antisera specific for the extracellular domain of rat trkB and rat trkC

Expression cassettes directing the secreted expression of the extracellular domain of rat trkB (Rtb-ex) or the extracellular domain of rat trkC (Rtc-ex) were transfected on a large scale into cos cells and the exogenous products were purified from the supernatants. (see Methods). Polyclonal antisera were raised using these purified extracellular domains, rabbit for Rtb-ex and goat for Rtc-ex. These antisera, called RTB and RTC, respectively, were then affinity purified against additional Rtb-ex or Rtc-ex obtained using a Picchia pastoralis expression system.

Results in figure 1 document the specificity of these sera in immunoblot analysis. Western blots of lysates of cos cells transiently transfected with full-length rat trkA, trkB, and trkC, or of untransfected cos cells, were probed with RTA, RTB or RTC. RTA (left panel) recognized only lysates of trkA transfected cells. RTB (middle panel) recognized only lysates of trkB transfected cells, and RTC (right panel) recognized only lysates of trkC transfected cells.

Immunohistochemical analysis of parasagittal sections through E14.5 embryos (Figure 2) confirmed that the distributions of cells immunoreactive for RTB and RTC closely match the distributions of cells expressing trkB mRNA (Klein et al., 1989, 1990; Middlemas et al., 1991) and trkC mRNA(Tessarollo et al., 1993; Lamballe et al., 1994). RTB (Figure 2B) recognizes subsets of neurons within the

trigeminal ganglion (TG) and dorsal root ganglia (DRG), and reacts intensely with the entire spinal cord. In addition, this antibody recognizes many nonneuronal structures including the mesenchyme surrounding trigeminal and dorsal root ganglia and mesenchymal structures in the snout and abdomen. Importantly, this antibody recognizes the presumptive ependyma and choroid plexus (cp), structures which express trkB, but not trkC or trkA (reviewed in Barbacid et al., 1994). RTC (5 ug/ml; Figure 2C) recognizes a subset of neurons within the trigeminal ganglion (TG), dorsal root ganglia (DRG) and the superior cervical ganglion (scg). RTC immunoreactivity is widespread within the central nervous system but especially intense in the 1a afferents of the spinal cord (1a) and the presumptive dentate gyrus (dg).

For comparison, we include a section immunostained with RTA (Figure 1B) to show the distribution of trkA. RTA recognizes many neurons within the trigeminal ganglion(TG) and dorsal root ganglia (DRG). In addition RTA recognizes the superior cervical ganglion (scg) and processes in the superficial layers of the dorsal spinal cord. We conclude that the three antisera specifically recognize their respective antigens in immunohistochemical assays.

Distribution of trkA in the E11.5-E12.5 wild-type trigeminal ganglion

In the trigeminal ganglion of E11.5-E12.5 embryos (Figure 3A,B,D), RTA recognizes a large number of neurons. Quantification of numbers of RTA immunoreactive profiles (Table I; Figure 4A) shows that the fraction of trigeminal neurons expressing trkA increases greatly during this period. At E11.5, 50% of neurons are immunopositive for RTA. By E12.5, the proportion approaches 100% of all neurons. We did not evaluate stage E13.5, since in vitro data on the NGF responsiveness of trigeminal neurons (Buchman and Davies, 1993) and in situ data (Ernfors et al., 1992; Arumae et al., 1993) already indicate that almost all trigeminal neurons express trkA at this stage.

Distribution of trkB in the E11.5-E13.5 wild-type trigeminal ganglion

Sections containing the trigeminal ganglion of E11.5-E13.5 embryos stained with RTB reveal rapid changes in the pattern of expression of this antigen (Figure 5A,C,E,F). At E11.5 (Figure 5A,E), RTB labels widespread profiles distributed with greater frequency toward the anterior of the ganglion. Axons of trigeminal neurons are intensely labeled along their entire length (Figure 5A). In E13.5

embryos (Figure 5C,F), RTB immunoreactivity within the trigeminal ganglion has become confined to a much smaller proportion of neuronal and vascular profiles. The spatial skew of labeled neurons persists, but is less obvious in this figure because of the decreased prominence of labeled neurons relative to the many unlabeled cells and labeled vascular profiles which are distributed without spatial bias. At this stage, the mesenchyme surrounding the trigeminal ganglion is strongly immunoreactive.

Double labeling experiments using RTB and a monoclonal antibody against medium molecular weight subunit of neurofilament (NF-M; Figure 6A-C) shows that RTB immunoreactivity within the E11.5 ganglion is restricted to neurons. RTB immunopositive profiles (red) almost always coincide with NF-M immunopositivity (green). The mesenchymal sheath of the ganglion (red arrow) is immunoreactive for RTB but negative for neurofilament. Conversely, the neurofilament-positive motor root of the trigeminal (green arrow) is not immunoreactive for trkB. A count of 2 double label experiments (see methods) showed that in sections stained for both RTB and neurofilament, 98 profiles were double-labeled; 48 were labeled only with neurofilament; and 6 were labeled with trkB but not with neurofilament. Thus, about 94% of RTB immunoreactive cells

are neuronal, indicating that trkB is expressed by at most a small subset of precursor cells within the trigeminal ganglion at this time.

Quantification of the numbers of RTB immunoreactive profiles in the E11.5-E13.5 trigeminal ganglion (Table I; Figure 4A) indicates that the number of neurons expressing trkB decreases by about twofold during this period, even as the total number of neurons within the ganglion is increasing rapidly. E11.5 embryos contain ~10,000 RTB immunopositive neurons, whereas by E13.5 there are only ~4,600.

Distribution of trkC in the E11.5-E13.5 wild-type trigeminal ganglion

Staining experiments in E11.5-E13.5 embryos stained with RTC (Figure 5 B,D,G,H) show dramatic developmental changes in the expression of this antigen in the trigeminal ganglion. RTC immunoreactive cells are very abundant within the E11.5 trigeminal ganglion (Figure 5B,G). As is seen with RTB, central and peripheral axonal projections are intensely labeled by RTC. At stage E13.5 (Figure 5D,H), the extent of labeling has decreased dramatically so that by RTC immunoreactive profiles represent only a restricted set of trigeminal neurons.

Double labeling studies using RTC in combination with a polyclonal antiserum against NF-M (Figure 6D-F) shows that trkC expression is also restricted to neurons at stage E11.5. The very abundant RTC labeled profiles (red) almost always collocalize with neurofilament (green). A count of 2 double label experiments identified in 124 double-labeled profiles, 18 profiles labeled only with neurofilament, and 6 profiles labeled only with trkC. Thus 95% of trkC positive profiles are neurofilament positive.

Quantification of numbers of RTC immunoreactive profiles (Table I; Figure 4A) shows that 93% of neurons express trkC at stage E11.5. In E12.5 embryos, this percentage has decreased, with RTC immunopositive cells representing 37% of all neurons. In E13.5 embryos the numbers of RTC immunopositive neurons has decreased to about 10% of all neurons, a result consistent with previous measures of NT-3 survival responsiveness of E13 trigeminal ganglion neurons (Buchman and Davies, 1993) and quantification of the percentage of trkC expressing neuronal profiles in the juvenile rat thoracic DRG (Wright and Snider, 1995).

Raising RTC in goat allowed us to directly compare the staining pattern of this antiserum that of RTB or RTA using double labeling. Double labeling using RTC and RTB (Figure 7) reveals that at stage E11.5 many profiles are RTC/RTB double positive (Figures 7A-C),

consistent with the widespread neuronal distribution of each of these antigens as described above. Many neurons are immunoreactive for RTC and not RTB, reflecting the greater abundance in the E11.5 trigeminal ganglion of neurons expressing trkC. Conversely, a subpopulation of neurons clustered near the anterior pole of the ganglion are immunoreactive for RTB, but not RTC. These neurons which stain with RTB but not RTC are distributed along the entire anterior edge of the trigeminal ganglion (Figure 7D) and in the most ventral portion of the dorsal root ganglia (Figure 7E). In E12.5 and E13.5 embryos, RTB labels a distinct set of neurons from RTC throughout the ganglion (Figure 7F-G). Counts from 2 double label experiments with E12.5 embryos showed that 57 profiles were positive for RTC, 31 profiles were positive for RTB, and none were labeled with both RTB and RTC.

Double staining with RTC and RTA (Figure 8) shows extensive overlap in the E11.5 trigeminal ganglion (Figure 8A-C). Different neurons display different relative levels of RTC and RTA immunoreactivity, ranging from strong RTC immunoreactivity and no detectable RTA immunoreactivity (red profiles in Figure 8C) to weak RTC immunoreactivity and strong RTA immunoreactivity. By E13.5, these two antibodies label distinct populations (Figure 8D-F). Counts of two double label experiments in E13.5 embryos showed that 94

profiles were positive for RTC, 172 were positive for RTA, and only 6 were double labeled.

Effect of loss of NT-3 on expression of trks by embryonic trigeminal neurons

In a previous study (Wilkinson et al., 1996) we determined that the deficit in neuronal numbers in the trigeminal ganglion of animals lacking NT-3 emerged after stage E10.5 and was complete by stage E13.5. In order to evaluate the effects of this mutation on the survival of neurons expressing different trk receptors, we used the RTB and RTC antisera to compare series through the trigeminal ganglion of wild-type animals and animals homozygous null for NT-3.

Quantification of the numbers of neurons in the E12.5 mutant trigeminal ganglion using NF-M as a marker for neurons (Table I) shows an increase in neuronal numbers at this stage compared to the E11.5 ganglion. Neuronal numbers decline again in E13.5 embryos, to levels seen at birth (Wilkinson et al., 1996).

E11.5 wild-type embryos show RTC staining on nearly all trigeminal ganglion neurons (Figure 9E). By contrast, in E11.5 embryos homozygous for the NT-3 knockout, the numbers of RTC immunopositive profiles have been severely reduced. (Figure 9B,F,H) Furthermore, RTC immunoreactive debris associated with pyknotic

profiles is seen throughout the ganglion (Figures 9F,H). By E12.5, RTC immunoreactive profiles have been eliminated from the mutant ganglion (Figure 9G), although axons travelling through the trigeminal ganglion from the trigeminal mesencephalic nucleus remain immunopositive (lower right of Figure 9G). Quantification of RTC immunopositive profiles in E11.5 mutant embryos (Table I) shows that only 9% of neurons are immunopositive for trkC, compared with 93% in wild-type embryos, indicating that many neurons do not express detectable levels of trkC. In addition, the widespread incidence of RTC immunopositive debris in these mutants (arrows, Figure 9H) indicates increased apoptotic elimination of neurons expressing trkC.

E11.5 wild-type embryos show widespread trigeminal expression of trkB. In mutant embryos, RTB immunopositive cells are visibly less frequent in the trigeminal ganglion at this stage (Figure 9A; compare to Figure 5A). However, the remaining RTB positive neurons show apparently normal morphology (Figure 9D) and stain with normal intensity, compared to those seen in wild-type embryos (Figure 9C). There is no obvious change in the anterior spatial bias of immunoreactive profiles from that observed in wild-type animals. (Compare Figures 9A, 5A).

Comparison of numbers of RTB immunoreactive profiles (Table 1; Figure 4C) in animals lacking NT-3 with wild-type embryos shows that mutant ganglia contain 60% fewer RTB immunoreactive neurons at stage E11.5. The presence of RTB immunoreactive pyknotic debris (data not shown) suggests that this deficit reflects at least in part an increased apoptotic elimination of trkB expressing neurons. The deficit in RTB immunopositive neurons in embryos lacking NT-3 becomes smaller at stages E12.5-E13.5 (Table I; Figure 4C) as the numbers of neurons expressing trkB in wild-type embryos decrease whereas the numbers in mutant animals remain essentially the same.

We also compared RTA immunoreactivity in the E11.5-E12.5 trigeminal of wild-type and mutant embryos. Qualitative examination of the staining patterns of the trigeminal of mutant embryos reveals apparently normal frequency and morphology of RTA labeled neurons at E12.5 (Figure 3B,C, D, E). Quantification (Table I; Figure 4B) reveals significantly diminished numbers of trkA expressing neurons at stages E11.5 and E12.5. However, the number of RTA labeled neurons represents an unchanged proportion of all neurons, and increases at a comparable rate, in mutants and wild-type embryos (Table I, Figure 4C). Taken together, these observations indicate that the trkA expressing population is not as

strongly affected at these stages in animals lacking NT-3 as the trkB and trkC expressing populations.

DISCUSSION

We have generated antisera against the extracellular domain of rat trkB and rat trkC. Western blot analysis combined with immunohistochemical experiments allow us to conclude that RTB and RTC specifically recognize rat trkB and rat trkC.

Our double labeling experiments comparing RTB and RTC with the distribution of NF-M staining show that trkB and trkC are almost entirely restricted to neurons throughout these stages. We find approximately 95% of immunoreactive profiles are positively associated with neurofilament immunoreactivity, indicating that these receptors are primarily expressed by neurons in the early trigeminal ganglion. This finding contradicts a general expectation (reviewed in Lewin and Barde, 1996; see also Chapter IV) that neuronal precursors would express trkC. Studies of the distribution of trkC expressing cells in the developing chick DRG (Lefcort et al., 1996; Henion et al., 1995) indicate that only a small proportion of neural crest cells migrating into the DRG express trkC. Preliminary data from our lab (I. Fariñas, unpublished observations) suggests

that trkC is expressed by migrating neural crest cells which do not contribute to sensory ganglia. We are now investigating the distribution of this antigen in the trigeminal ganglion of E9.5-E10.5 embryos.

Changes in trk expression in the E11.5-E13.5 trigeminal ganglion.

The distribution of trkA, trkB, and trkC changes rapidly in the trigeminal ganglion from E11.5-E13.5 (Table I; Figure 3A). TrkC and trkB are widely expressed in the E11.5 trigeminal ganglion of wild-type embryos and are much less numerous in E13.5 animals. The decrease in numbers of trkC positive neurons (open circles, Figure 3A) appears to be the result of transient expression of trkC by embryonic neurons.

The decrease in the total number of trkB expressing neurons from E11.5-E13.5 in wild-type embryos (open diamonds, Figure 3A) could reflect naturally occurring cell death within this population. Alternatively, trkB could be transiently expressed by some trigeminal neurons in a manner similar to that seen for trkC. Data from trkB knockout mice (Piñon et al., 1996) show that indicates that these stages represent the onset of a survival requirement for trkB signaling by trigeminal neurons. TrkB knockout mice show elevated

pyknosis in the trigeminal ganglion at stages E11-E13, with a peak at stage E12 (Piñon et al., 1996). This peak correlates well with the developmental stages in which trkB expressing neurons stop coexpressing trkC in the wild-type ganglion (Figure 6). Thus, we favor the interpretation that the decrease in neurons expressing trkB in the E11.5-E13.5 wild-type ganglion represents a period of naturally occurring cell death associated with a requirement by these neurons for signaling though trkB.

Whereas trkB and trkC expressing populations decrease in absolute numbers and as a percentage of all trigeminal neurons, numbers of trkA expressing neurons increase rapidly from E11.5 to E12.5. Neurons expressing trkA represent about 50% of all trigeminal neurons at stage E11.5 and at stage E12.5 represent essentially all trigeminal neurons.

The E11.5 ganglion contains many neurons which express both trkB and trkC, and in addition many neurons express both trkC and trkA (Figures 6,7). The degree of colocalization decreases very rapidly over the next 48 hours, so that by E12.5 trkC and trkB, and by E13.5 trkC and trkA, are expressed by nonoverlapping populations. Given the rapid decline in overall trkC immunopositive profiles during these stages, we interpret these changes as transient expression of trkC by populations which later express trkB or trkA

exclusive of trkC. Although we have not determined the potential coexpression of trkB and trkA, the small fraction of all trigeminal neurons which express trkB at E12.5 and E13.5 means that the majority of trkA positive cells do not express trkB at those stages. Conversely, because trkA expressing neurons are only moderately affected by the absence of NT-3 at stage E11.5, whereas numbers of trkB expressing neurons decrease greatly, we favor the idea that most trkB expressing neurons do not express trkA at this stage. However, a recent study (Moshnyakov et al., 1996) using single-cell RT-PCR to detect trkA, trkB and trkC mRNA in the cytoplasm of individual cultured embryonic trigeminal neurons did detect individual neurons which express all three trk receptors. About 2/3 of E12 and about 1/2 of E17 rat trigeminal neurons examined by those authors expressed all three trk receptors. However, these percentages should be interpreted with caution, since the results of others (Arumae et al., 1993; Ernfors et al., 1992; Klein et al., 1991; Tesarollo et al., 1993) indicate that trkC and trkB are expressed by only a small subset of E15 mouse or E17 rat trigeminal neurons.

Model of dynamics of expression of trkA, trkB and trkC in the E11.5 trigeminal ganglion

The observation of rapid changes in distribution of trk receptors in the E11.5-E13.5 trigeminal ganglion suggests a model (Figure 10) in which most newborn trigeminal neurons transiently express trkC at stage E11.5 before definitively expressing only a single trk. This process is manifested by the frequent coexpression at E11.5 of trkC with trkB, and trkC with trkA, followed by separate expression of trkC compared to trkB, and trkC compared to trkA, at later stages.

Although this model is based on observations of the E11.5 trigeminal ganglion, a similar developmental sequence may occur for trigeminal neurons born at E12.5. The number of trkC immunoreactive profiles at E12.5 is approximately the same as the number of neurons born over the previous 24 hours (See Table 1). Since neurogenesis ceases at E13 (Altman and Bayer, 1982), the number of trkC expressing neurons at stage E13.5 may represent the number of definitive trkC expressing neurons..

At the cellular level, the transition is completed after a neuron has extended an axon well into the periphery, as evidenced by the intense trkC immunoreactivity seen on the trigeminal nerves at E11 (Figure 3C) and on the endings of trigeminal axons approaching their targets (Frank Rice, personal communication).

Neurons of the definitive trkB population are hypothesized to stop coexpressing trkC sooner than many cells of the definitive trkA population. The segregation of trkB and trkC expressing populations appears to be essentially complete at E12.5. Some profiles immunoreactive for RTB, but not RTC are already detected in their biased spatial location at E10.5 (data not shown).

Changes in expression of trkA, trkB and trkC in the trigeminal ganglion of NT-3 knockout mice

The lack of NT-3 has disparate consequences for the populations which express trkA, trkB, or trkC in the E11.5 trigeminal ganglion. Neurons expressing trkA are relatively unaffected and neurons expressing trkB are severely depleted. Neurons expressing trkC are severely depleted and expression of trkC by surviving neurons may also be affected. We use our model of the dynamics of wild-type trk receptor expression to propose multiple actions of NT-3 in the trigeminal ganglion at stage E11.5.

Neurons expressing trkA are diminished in number, but represent an unchanged percentage of all neurons in the absence of NT-3

In stages E11.5 and E12.5, absolute numbers of trkA expressing neurons are significantly decreased in embryos lacking NT-3 (Table 1). However, this population represents an essentially normal fraction of all neurons at E11.5 and E12.5. Thus, NT-3 is not specifically required for the survival of trkA expressing neurons at stage E11.5.

Depletion of trkB expressing neurons in the absence of NT-3

E11.5 embryos lacking NT-3 show greatly decreased numbers of neurons immunopositive for trkB relative to wild-type (Table I, Figure 3C). This could reflect abnormal cell death of trkB expressing neurons or failure of neurons to express trkB. The simplest interpretation of this observation is that it reflects an exacerbation or acceleration of the process which causes numbers of trkB expressing neurons to decrease in the wild-type trigeminal ganglion. Thus, we favor the idea that this decrease relative to wild type embryos reflects elevated cell death of trkB expressing neurons in animals lacking NT-3. This interpretation will be considered in more detail in chapter IV.

We observed a strong spatial skew in the distribution of trkB expressing neurons in the wild-type E11.5 trigeminal ganglion. Furthermore, we noted that the most anterior trkB expressing

neurons did not coexpress trkC. In terms of our model of transient expression of trkC, the most anterior trkB expressing neurons are more developmentally advanced relative to neurons which coexpress trkC.

We do not notice an obvious change in the spatial skew of trkB expressing profiles in the absence of NT-3. This presents a problem for a strict "treadmill" model in which the survival requirement for NT-3 occurs at the same developmental stage for all trkB expressing neurons. In this model, in animals lacking NT-3 the most developmentally advanced neurons at the anterior of the ganglion should be eliminated first. However, this expectation may not be fulfilled if the most mature neurons are able to bypass the requirement for NT-3 by obtaining other trophic factors. At least some neurons express trkB without trkC in the E10.5 ganglion (data not shown), at a stage when presumptive epidermis, which expresses BDNF and NT-4 at these stages, (Arumae et al., 1993) is considerably closer to the ganglion.

These issues might be resolved by careful quantification of the position and numbers of trkB and trkC coexpressing profiles in wild type tissue compared to surviving populations in the mutant. The most direct test, a colocalization experiment in mutant tissue, is not feasible because of massive disruptions in RTC immunoreactivity in

the mutant background (see below). Perhaps careful birthdating experiments in mutant tissue could distinguish the survival of recently generated neurons from neurons which are more developmentally advanced.

Severe disruptions of trkC expression in animals lacking NT-3

Mutant embryos at stage E11.5 show severely weakened RTC staining and widespread RTC immunoreactive debris. The E11.5 phenotype may represent a developmentally recent onset of these changes, since lumbar dorsal root ganglia in mutant embryos have a more wild-type appearance at this stage (data not shown). In the E12.5 ganglion, trkC immunoreactive neurons have been eliminated. In the context of the model, we interpret these findings as reflecting a specific survival requirement for NT-3 by definitive trkC expressing neurons.

TrkC is expressed by 93% of all neurons in the E11.5 wild-type trigeminal ganglion and only 10% of neurons in embryos lacking NT-3. This indicates that many surviving neurons at E11.5 do not express detectable levels of trkC in the absence of NT-3. In this regard, it is interesting to note that levels of trkC kinase mRNA (standardized to GAPDH mRNA) are depleted by about 50% in the

E11.5 mutant trigeminal ganglion relative to wild-type (E. Huang et al., submitted). Thus, NT-3 may affect the levels of expression of trkC by embryonic trigeminal neurons. This possibility should be explored quantitatively at the protein level.

An essentially normal percentage of trigeminal ganglion neurons expresses detectable levels of trkA in the absence of NT-3 at E11.5, (see below), suggesting that NT-3 specifically influences trkC expression.

NT-3 has at least three roles in the developing trigeminal ganglion.

When considering the disparate effects of the absence of NT-3 on trkA, trkB, and trkC expressing populations, we propose three actions of NT-3 in the E11.5 trigeminal. NT-3 affects the survival of the definitive trkB pool and is essential for the survival of the definitive trkC pool. NT-3 may also increases the expression of trkC by E11.5 trigeminal neurons.

The effect of the absence of NT-3 in the mouse trigeminal ganglion is more severe than that seen in animals lacking trkC (Fariñas et al., 1994; L. Tessarollo, personal communication). TrkC knockout animals are lacking 20-25% of their trigeminal neurons (Piñon et al., 1996; L. Tessarollo, personal communication) whereas

animals lacking NT-3 are lacking 65% of their trigeminal neurons at birth (Fariñas et al., 1994). The severe depletion of the trkB and trkC expressing populations and the relatively unchanged trkA expressing population seen in NT-3 mutants suggest that NT-3 interacts functionally with trkB in the E11.5 trigeminal ganglion. Later events, to be discussed in Chapter IV, also contribute to the final size of the trigeminal deficit in animals lacking NT-3.

Multiple isoforms of trkB and trkC

The amino acid sequence of the extracellular domain is common all known variants of trkB (Ninkina et al., 1997; reviewed by Barbacid, 1994) and trkC(reviewed by Barbacid, 1994). It is expected that RTB and RTC will recognize all isoforms of these receptors. Throughout this paper we refer to the aggregate distribution of all isoforms of the trkB receptor as trkB, and similarly with trkC.

In principle, we do not know whether the immunoreactivity studied in this paper represents forms of trkB or trkC capable of mediating neurotrophin survival signals. However, evidence from other sources suggests that the RTB immunostaining seen in this study represents functional trkB receptor. Cultures of E11.5 trigeminal neurons show robust survival responses to BDNF, a

specific trkB ligand, indicating the presence of functional trkB on those neurons (Buchman and Davies, 1993). Furthermore, a study of the relative abundance of full-length and truncated variants of trkB in the trigeminal ganglion (Ninkina and Davies, 1996) shows that mRNA encoding the full-length form is the most abundant isoform in the trigeminal ganglion at the stages considered here. That study may represent an underestimate of the prominence of the full-length form on trigeminal neurons, since nearby structures expressing truncated trkB such as the mesenchyme (Figure 2A; Klein et al., 1993) may have been included in their samples.

Much less is known about the distribution of trkC isoforms in the trigeminal at this stage. However, the widespread impact of the absence of NT-3 on E11.5 trigeminal neurons (Figure 8A) indicates the presence of NT-3 receptors on those neurons capable of influencing aspects of trkC expression. Although trkA or trkB together could fulfill this role, the simplest interpretation of the data is that these neurons express a signaling isoform of trkC.

Conclusion

The isolation of antibodies specific for rat trkB and rat trkC has allowed us to dissect the dynamics of trk expression in the mouse trigeminal ganglion at the stages when neurons are being abnormally

lost in animals which lack NT-3. These studies have led to some suprising insights, including the prominent role played by trkC in the E11.5 ganglion and the rapidity with which neurons can alter surface expression of trk receptors and hence their sensitivity to environmental neurotrophins. It will be of great interest to distinguish the roles of specific isoforms, especially of trkC, in the development of sensory populations.

Figure 1: RTB is specific for rat trkB and RTC is specific for rat trkC in Western blot analysis. Cos cells were transiently transfected with full length rat trkA, trkB or trkC, or untransfected. After 48 hr, lysates were subjected to Western blot analysis with RTA (1 ug/ml; left panel), affinity purified RTB (1 ug/ml; center panel), or affinity purified RTC (1 ug/ml; right panel). RTA reacts only with lysates of cos cells transfected with rat trkA. RTB reacts only with lysates of cos cells transfected with rat trkB and RTC reacts only with lysates of cos cells transfected with rat trkC.

Figure 2. Immunohistochemical analysis of parasagittal sections through an E14.5 embryo. RTA (A) stains many neurons of the trigeminal ganglion (TG) and dorsal root ganglia (DRG) and reacts intensely with the superficial layers of the spinal cord. The superior cervical ganglion (scg) is also labeled. RTB (B) stains

small numbers of neurons in the trigeminal ganglion and DRGs; throughout the spinal cord; and nonneuronal structures such as the mesenchyme surrounding the trigeminal ganglion and DRGs. RTB also recognizes the choroid plexus (cp, arrow) and presumptive ependyma, structures which are known to express trkB but not trkA or trkC. RTC (C) stains small numbers of neurons in the TG and DRGs and stains the spinal cord. The superior cervical ganglion is also labeled. Within the central nervous system, RTC labels 1a afferents in the spinal cord (1a) and the presumptive dentate gyrus (dg). Scale bar, 1 mm.

Figure 3. RTA immunoreactivity in the trigeminal ganglion of E11.5-E12.5 wild-type and NT-3$^{-/-}$ embryos. A. RTA staining in the E11.5 wild-type trigeminal ganglion. **B-C.** RTA staining in the wild-type (**B**) and mutant (**C**) E12.5 trigeminal ganglion. Scale bar (A-C) = 50 microns. **D-E.** RTA staining in the wild-type (**D**) and mutant (**E**) trigeminal ganglion. RTA labels numerous profiles throughout the trigeminal ganglion in animals of both genotypes. Scale bar for D-E= 200 microns.

Figure 4. Quantification of numbers of trk-expressing neurons in the mouse trigeminal ganglion. A. Total neurons

and neurons expressing trkA, trkB, and trkC in the wild-type trigeminal ganglion. Neurons (filled squares) were counted as profiles immunoreactive for NF-M. Neurons expressing trkA (open diamonds), trkB (open circles), or trkC (open triangles) were counted as profiles immunoreactive for RTA, RTB, and RTC, respectively. **B.** TrkA expressing neurons in the trigeminal ganglion of wild-type embryos (filled squares) and embryos lacking NT-3 (open squares). **C.** TrkB expressing neurons in the trigeminal ganglion of wild-type embryos (filled squares) and embryos lacking NT-3 (open squares). The mean ± S.D. of counts from two separate animals. are shown for each point plotted.

Figure 5. Rapid changes in the pattern of trkB and trkC expression in the E11.5-E13.5 trigeminal ganglion. **A.** RTB recognizes numerous cells in the E11.5 trigeminal ganglion. These cells occur with greater frequency toward the anterior of the ganglion (to the right in this image). Peripheral and central axonal projections are intensely labeled. **B.** RTC recognizes widespread profiles throughout the E11.5 trigeminal ganglion. Scale bar for A- B, 500 microns. **C.** In E13.5 embryos, RTB recognizes a much smaller proportion of neurons as well as vascular profiles within the ganglion. The mesenchyme surrounding the ganglion is also intensely

labeled. The spatial skew of immunoreactive profiles seen in E11.5 embryos persists, but appears to be less pronounced. **D.** At E13.5, RTC recognizes only a small fraction of trigeminal neurons. . Note the differences in staining pattern between RTB and RTC where the trigeminal root enters the hindbrain. Scale bar for C-D, 200 microns. **E-H.** High magnification of RTB staining at E11.5 (**E**) and E13.5 (**F**) and RTC staining at E11.5 (**G**) and E13.5 trigeminal ganglion (**H**). Scale bar for E-H, 50 microns.

Figure 6. TrkB and trkC are primarily expressed by neurons in the E11.5 trigeminal ganglion. A-C. RTB (red) and NF-160 (green) double labeling of E11.5 trigeminal ganglion. RTB label is confined to neurofilament positive profiles, as seen in the double label image (**C**). The mesenchymal sheath surrounding the ganglion (red arrow) is RTB but negative for neurofilament, and the motor root of the trigeminal (green arrow) is neurofilament positive and RTB negative. **D-F**. RTC (red) and NF-150 (green) double labeling of E11.5 trigeminal ganglion. RTC label is confined to neuronal profiles. Scale bar for all images = 50 microns.

Figure 7. RTB and RTC double labeling in the E11.5-E13.5 trigeminal ganglion. A-C. RTB (red) and RTC (green) double

labeling near the exit point of the maxillary nerve (outside this field to the top right) from the anterior pole of the E11.5 trigeminal ganglion. Many neurons are labeled with RTC but not RTB. A population clustered toward the anterior pole of the ganglion is labeled with RTB but not RTC. **D-E.** This pattern is seen elsewhere along the anterior edge of the trigeminal ganglion and in dorsal root ganglia. The top of the micrograph corresponds to anterior in **D**. and ventral in **E**. **F-G**. In the E12.5 trigeminal ganglion (**F**) and 13.5 trigeminal ganglion (**G**), RTB and RTC label distinct populations. Scale bar for all images = 50 microns.

Figure 8. RTA and RTC double labelling in the E11.5-E13.5 trigeminal ganglion. **A-C**. E11 trigeminal ganglion. RTC (red) and RTA (green) label extensively colocalized neuronal populations, although some profiles are positive for each single label. **D-F**. At E13.5, RTC (red) and RTA (green) label distinct populations. Scale bar for all images = 50 microns.

Figure 9. Effect of the lack of NT-3 on RTB and RTC immunoreactivity in the E11.5 trigeminal ganglion. **A.** RTB immunoreactivity in the E11.5 trigeminal ganglion of a homozygous mutant. Fewer profiles are labeled than in wild-type embryos

(compare Figure 2A; see also Table 1). The spatial skew of RTB labeled profiles seen in wild-type embryos is also present in mutant embryos. **B.** RTC immunoreactivity in the E11.5 trigeminal ganglion of a homozygous mutant. Immunoreactivity is grossly disrupted over the entire ganglion. **C,D.** High magnification photomicrographs of RTB immunoreactivity in the E11.5 trigeminal ganglion near the exit point of the maxillary nerve of a wild-type (**C**) and a mutant (**D**) embryo. Neurons in mutant embryos appear to be stained with equal intensity as in wild-type embryos. **E,F.** High magnification photomicrographs of RTC immunoreactivity in the E11.5 ganglion of a wild-type (**E**) and a mutant (**F**) embryo. In the mutant embryo, few cells with abnormal morphology remain RTC immunopositive. Other cells stain very weakly. Cellular debris associated with pyknotic profiles is also immunoreactive for RTC.

Figure 10. Model of dynamic of trk expression and the roles of NT-3 in the E11.5 wild-type trigeminal ganglion. This schematic diagram interprets the expression patterns of trkA, trkB, and trkC, and the coexpression of trkB/trkC and trkA/trkC, observed in the wild type trigeminal ganglion, with special focus on stage E11.5. All newborn neurons express trkC, and can be further classified as those which express trkA and trkC (about 50%), those

which express trkB and trkC (40%) and those which only express trkC. At later stages, neurons which definitively express trkA or trkB do not coexpress trkC. At the cellular level, this transition is completed after the neuron has extended an axon well into the periphery toward final target tissues. NT-3 is essential for the survival of the definitive trkC population, supports survival of definitive trkB neurons, and may also influence the levels of trkC expressed by early neurons.

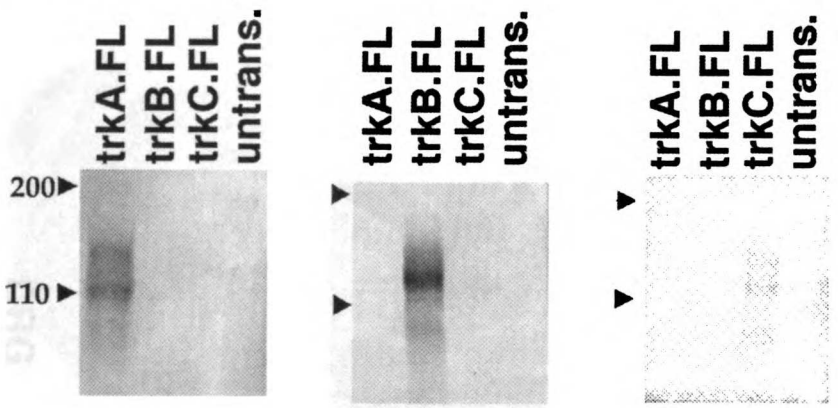
Table I. Quantification of numbers of trigeminal ganglion neurons immunoreactive for RTA, RTB, and RTC, stages E11.5-E13.5, in wild-type and mutant animals. Counts are reported as the mean ± S.D. of at least two measurements for each group. Percentages show each mutant measurement as a percentage of the corresponding wild-type measurement.

E11.5	Wild-type	Mutant	% of wt
Neurofilament[a]	25545 ± 3562	14743 ± 5513	58%
RTA	13299 ± 1243	9309 ± 1005	70%
RTB	9903 ± 666	3725 ± 182**	38%
RTC	23837 ± 1453	1328 ± 237***	6%
E12.5			
Neurofilament	42170 ± 960	31353 ± 2181*	74%
RTA	42093 ± 1413	29415 ± 1862*	70%
RTB	6266 ± 1005	3552 ± 509*	57%
RTC	15758 4650	(0)	(0)
E13.5			
Neurofilament[a]	48755 ± 3943	15217 ± 1023***	31%
RTB	4608 ± 280	3318 ± 229*	72%
RTC	5548 ± 392	(0)	(0)

* p<.05, Student's two-tailed T-test
**p< .01
*** p<.001

[a] Neurofilament counts of wild-type and mutant E11.5 and E13.5 are from Wilkinson et al., 1996.

Figure III-1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trkB.FL
trkC.FL
untrans.
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110
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Blot:
RTA
RTB
RTC

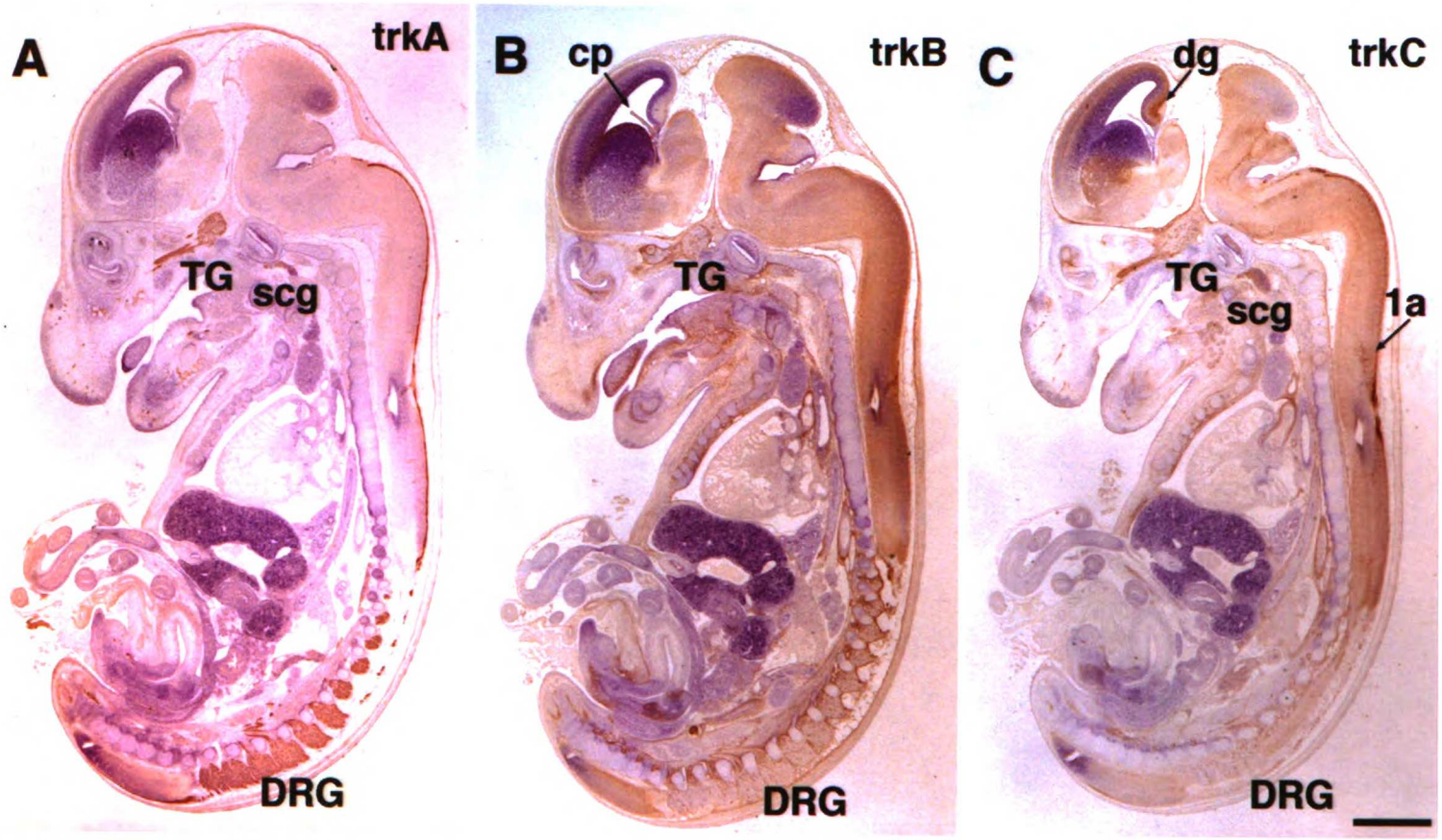
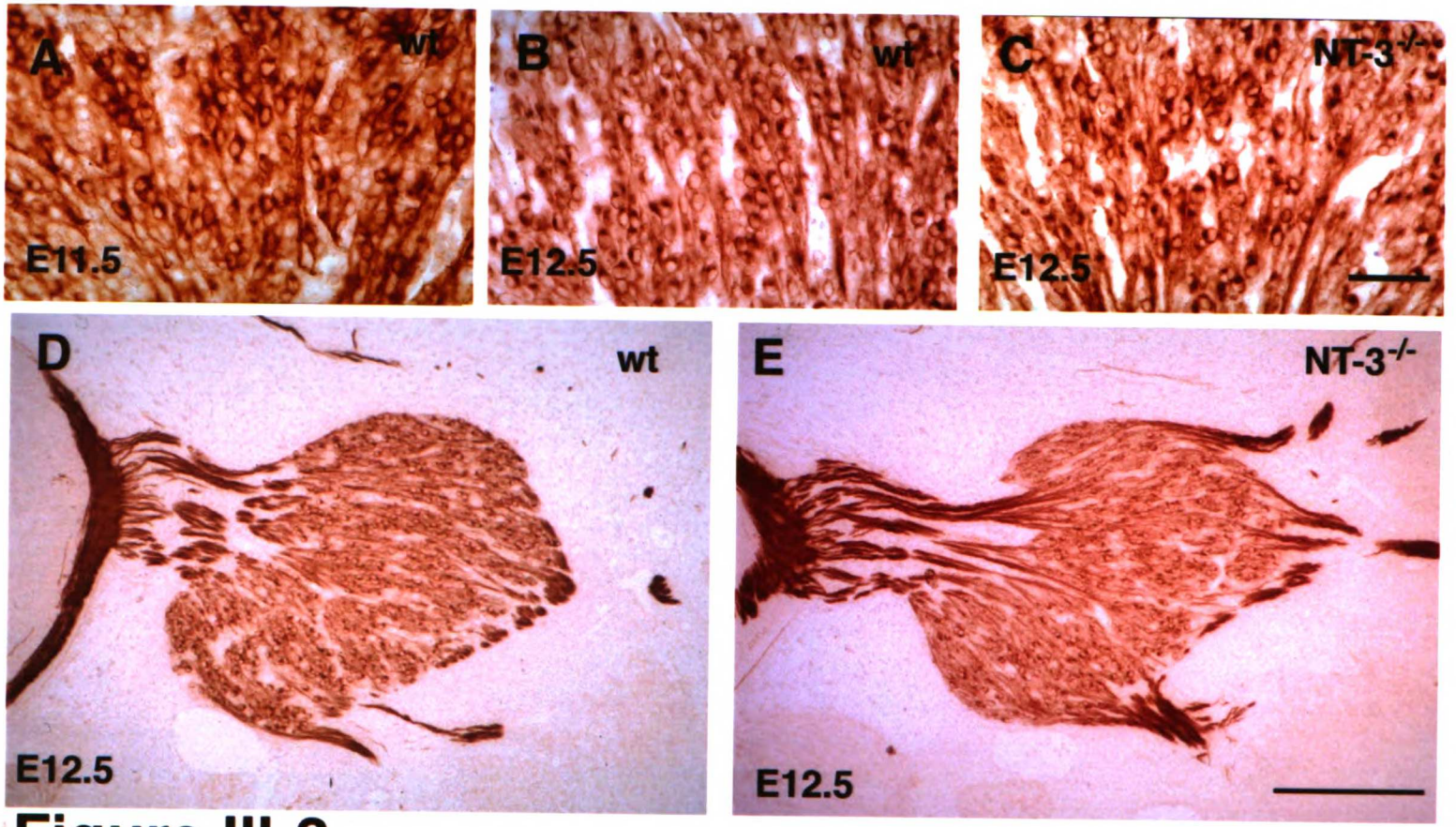


Figure III-2



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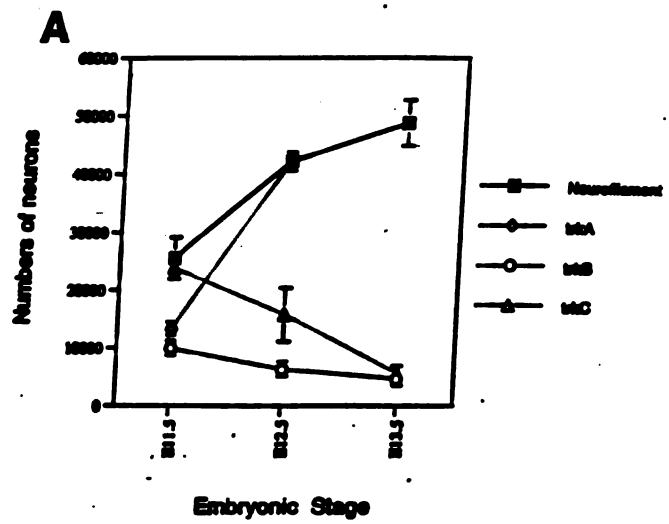
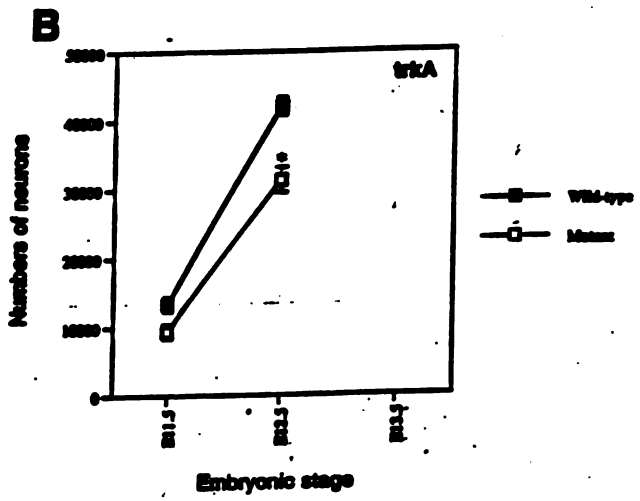

A
Numbers of neurons
Embryonic Stage
E11.5
E12.5
E13.5


B
trkA
Numbers of neurons
Embryonic stage
E11.5
E12.5
E13.5

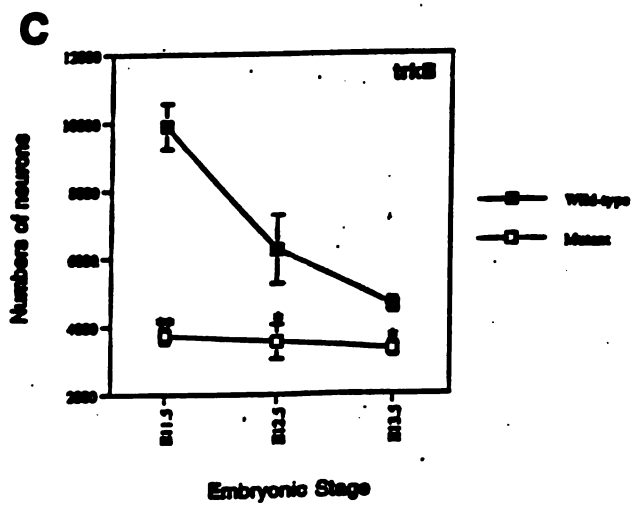
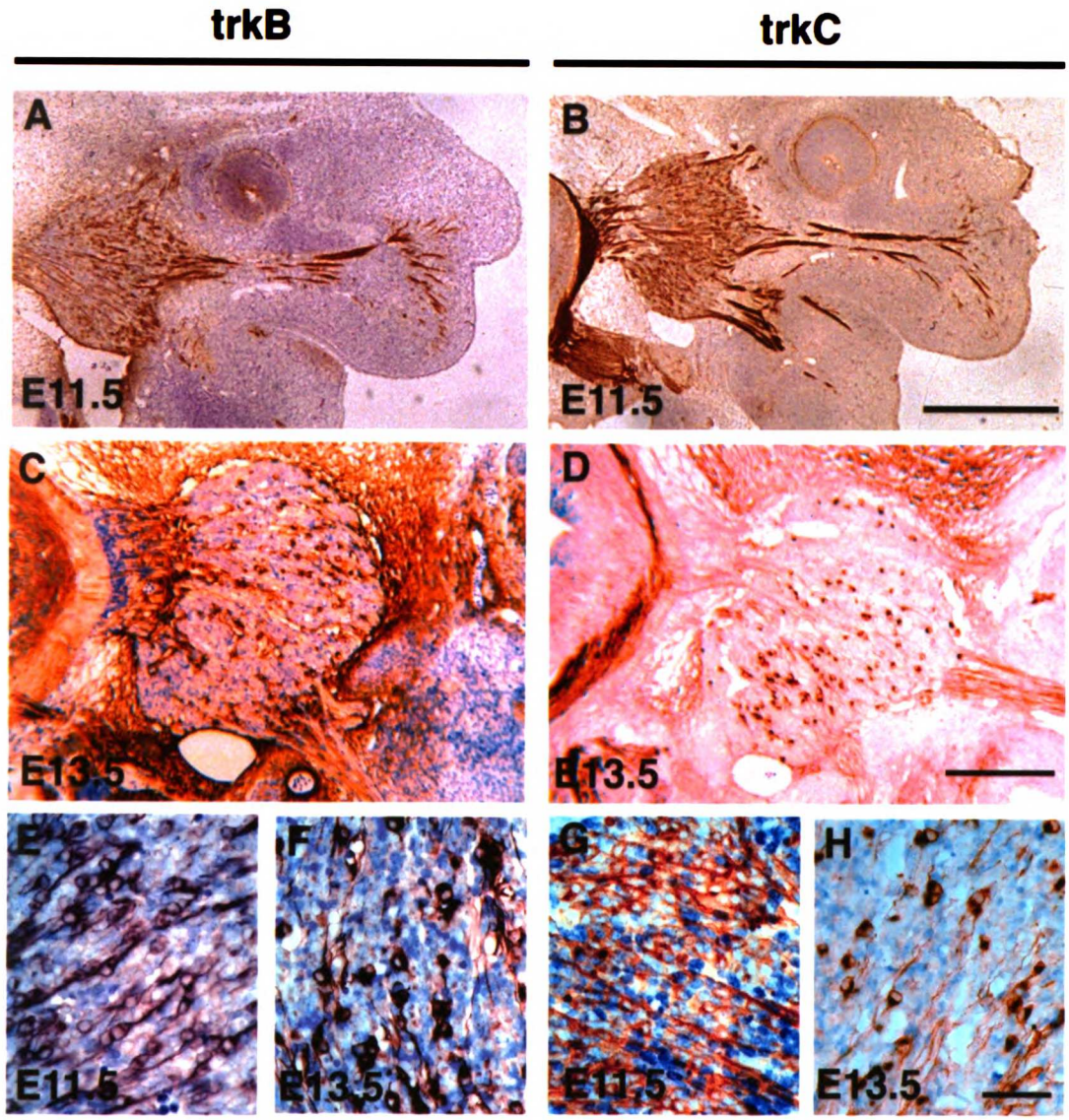

C
trkB
Numbers of neurons
Embryonic Stage
E11.5
E12.5
E13.5

Figure III-5



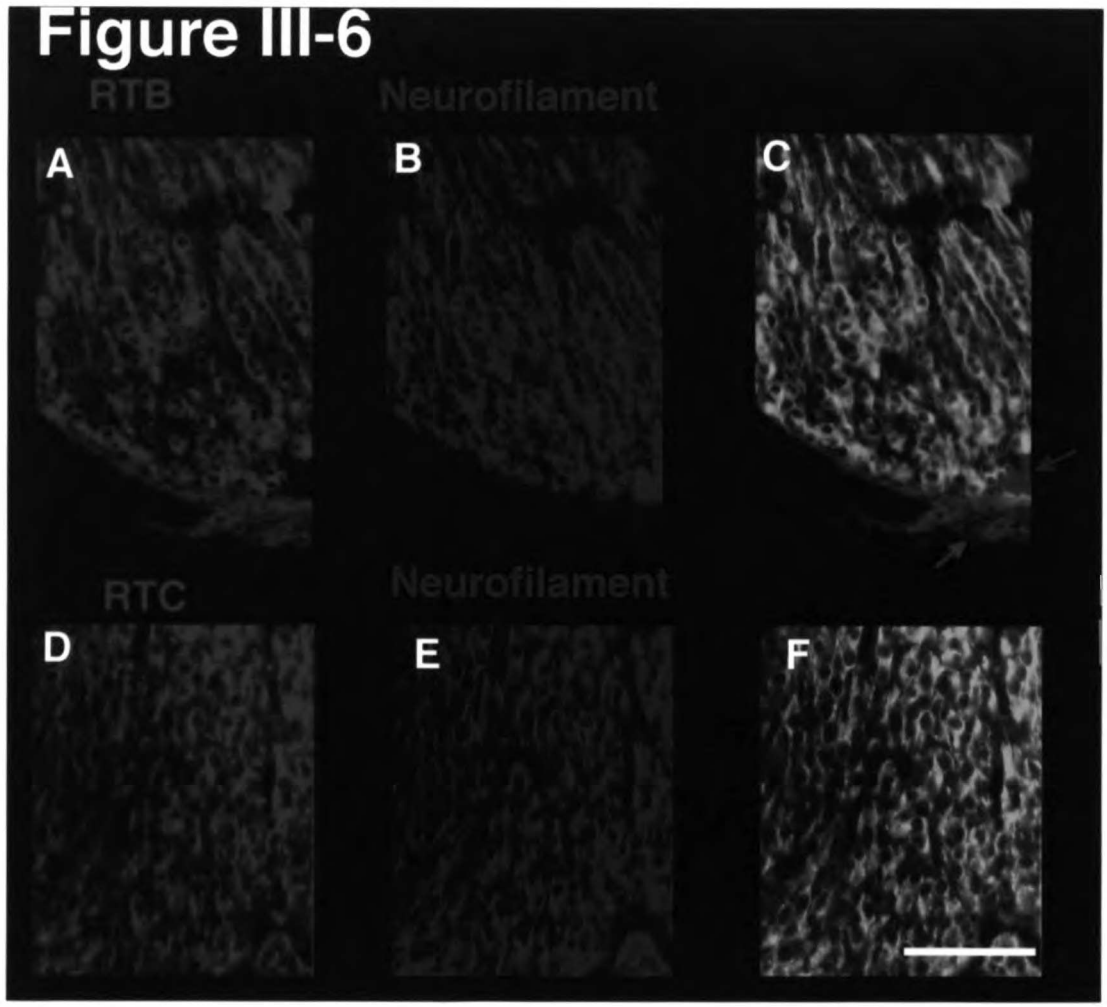


Figure III-6
RTB
Neurofilament
A
B
C
RTC
Neurofilament
D
E
F

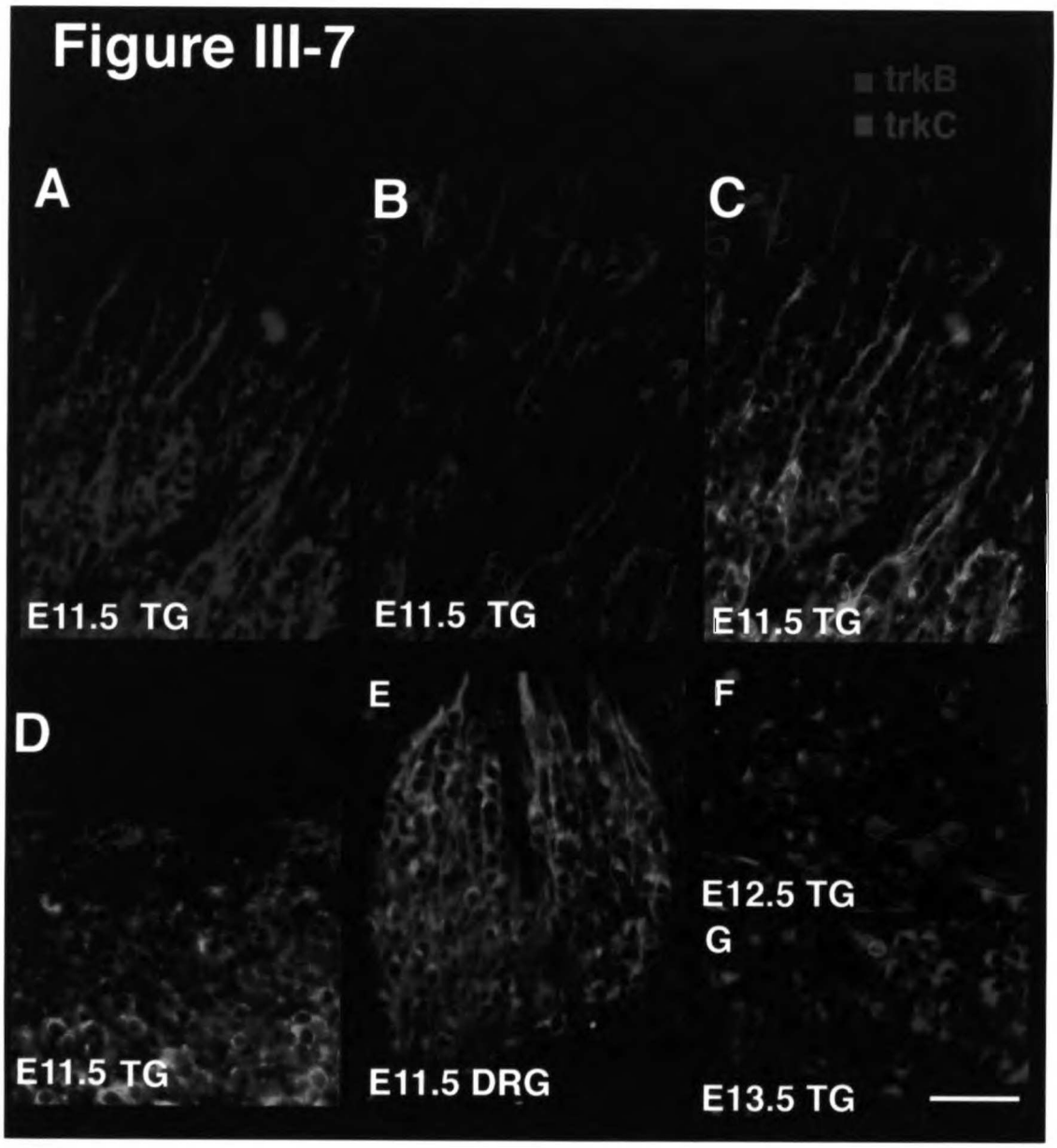
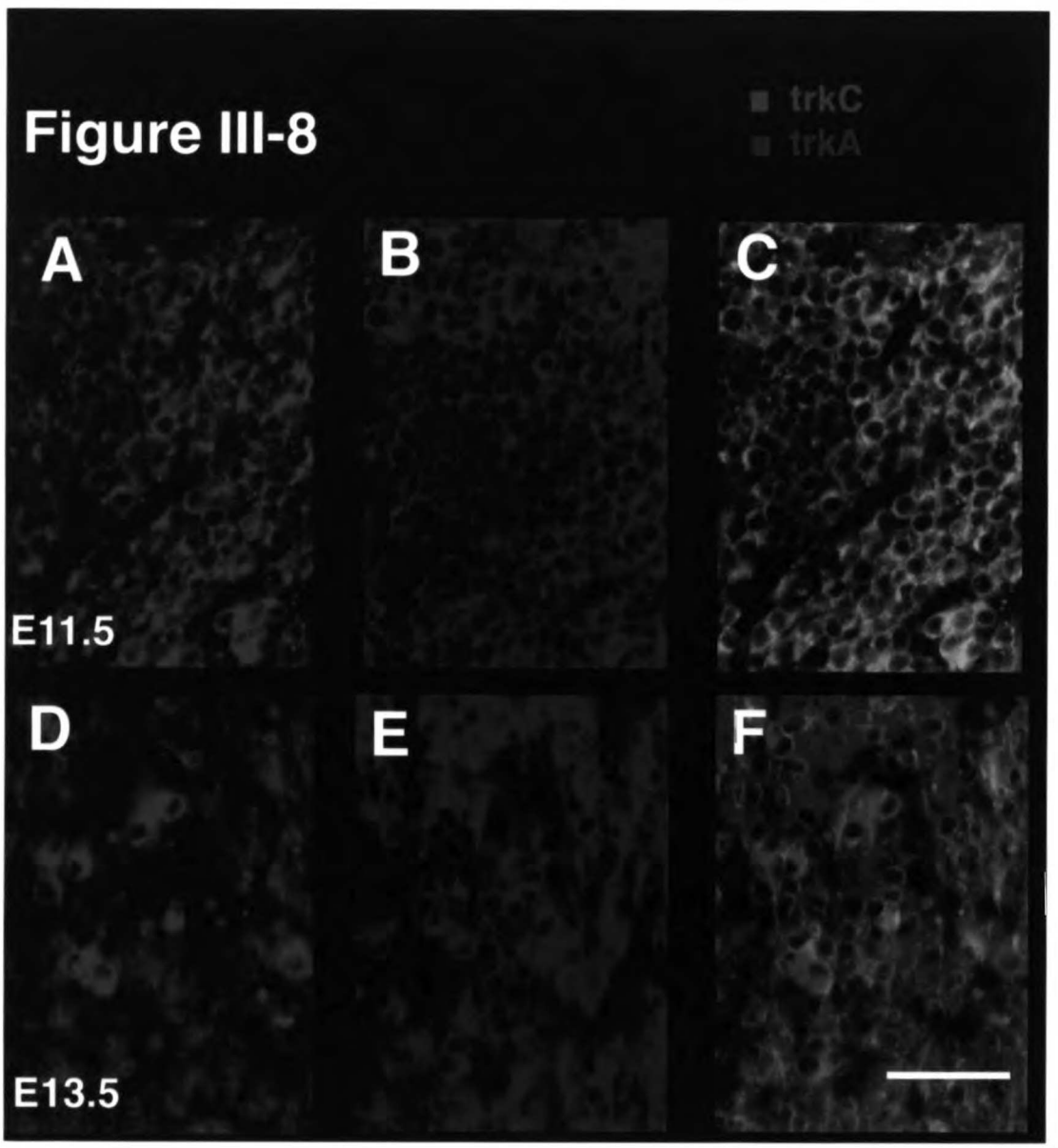


Figure III-7
trkB
trkC
A
B
C
E11.5 TG
E11.5 TG
E11.5 TG
D
E
F
E11.5 TG
E11.5 DRG
E12.5 TG
G
E13.5 TG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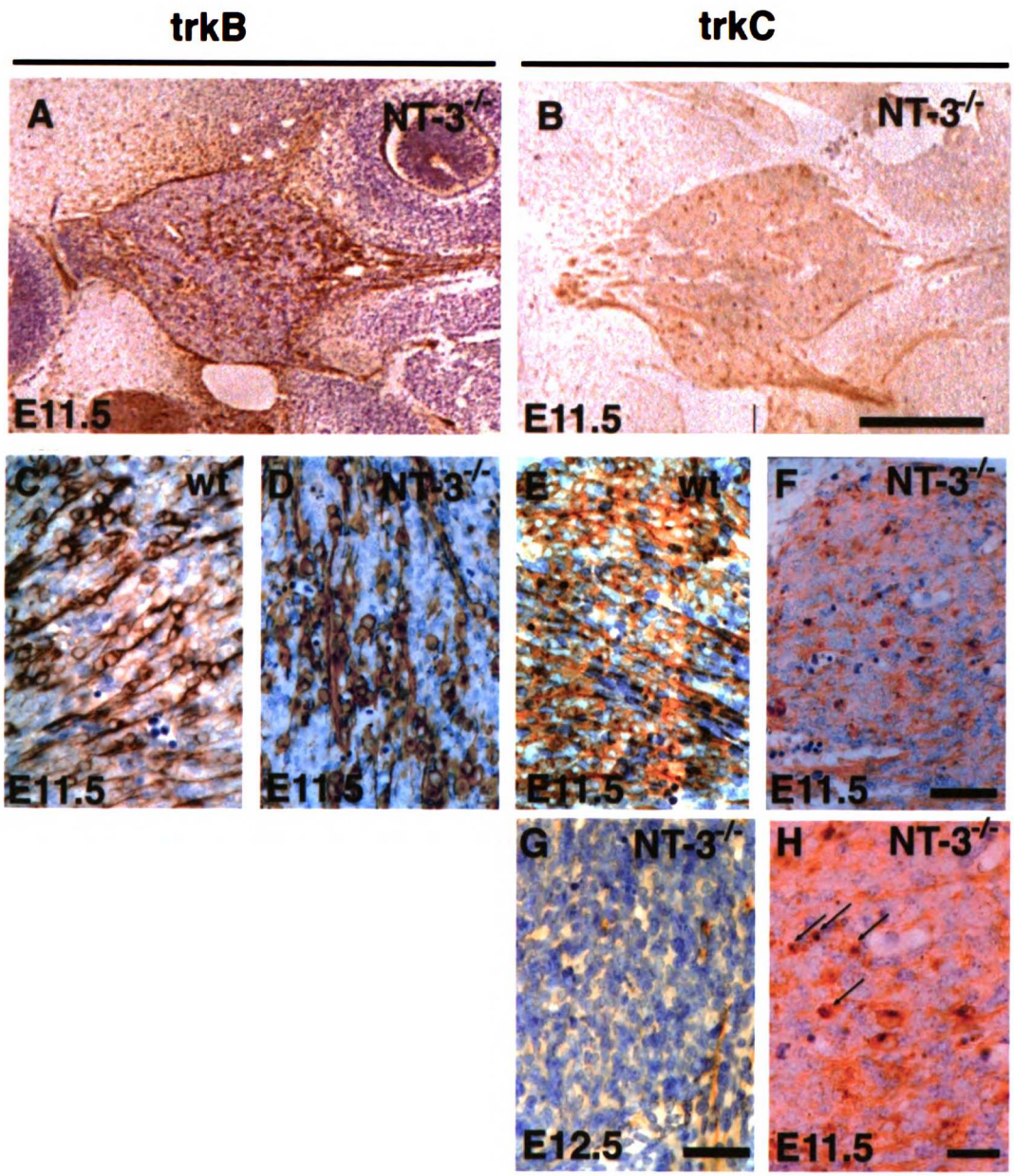
trkC
trkA
A
B
C
E11.5
D
E
F
E13.5

Figure III-9


trkB
trkC
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NT-3-/-
E11.5
B
NT-3-/-
E11.5
C
wt
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D
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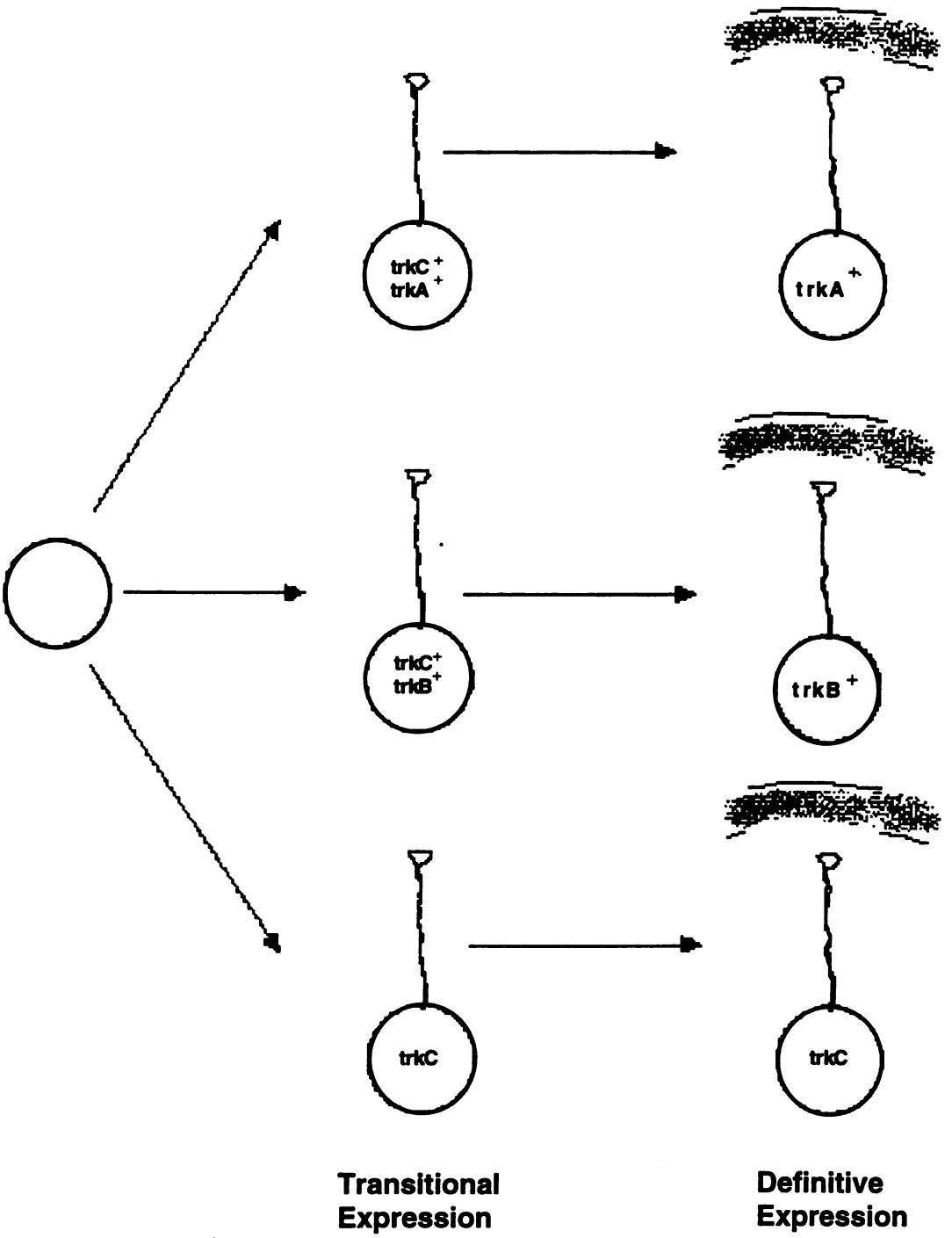
E11.5
E
wt
E11.5
F
NT-3-/-
E11.5
G
NT-3-/-
E12.5
H
NT-3-/-
E11.5


trkC+ trkA+
trkA+
trkC+ trkB+
trkB+
trkC
trkC
Transitional Expression
Definitive Expression

CHAPTER IV: DISCUSSION

Experiments presented in this thesis compare the development of the trigeminal ganglion in wild-type and NT-3 mutant mice. Counts of numbers of neurons in embryos at stages E10.5 -P0 revealed that the neuronal deficit in mice lacking NT-3 emerges between stages E10.5-E13.5. We therefore closely examined stages E11.5-E13.5, measuring numbers of precursor cells; rates of proliferation and apoptosis; sources of NT-3 in the trigeminal system; and expression of trkA, trkB, and trkC.

Analyses of wild-type mice revealed rapid changes in the extent of expression of trkA, B, and C in wild-type embryos at stages E11.5-E13.5. Quantification of numbers of neurons expressing trkA, trkB and trkC in the trigeminal ganglion yielded a model, presented in Chapter 3, in which all trigeminal neurons transiently express trkC prior to definitive expression of a single trk.

This model could be tested by a "pulse chase" experiment, in which BrdU is injected into dams at 11. 5 days p.c at varying intervals before sacrifice and recovery of embryos. In embryos obtained at short intervals after injection, BrdU incorporation should be detected primarily in precursor cells, predicted to not express any trk receptor. In embryos obtained after longer intervals, BrdU incorporation would be detected in newborn trigeminal neurons, which are predicted to coexpress trkC. Finally, at intervals up to 24

hr. , BrdU incorporation would be detected in older trigeminal neurons, which are predicted to express trkB without trkC.

Parallel analysis of the development of the trigeminal ganglion in mice lacking NT-3 shows that neurons are abnormally eliminated in these animals via apoptosis during stages E11.5-E13.5, initially without changes in the numbers or proliferation of precursor cells. Quantification of the trkA, trkB and trkC expressing neurons in the NT-3 knockout embryos at these stages shows disparate effects of the mutation on each subpopulation. At the onset of the neuronal deficit in E11.5 mutants, trkC and trkB expressing populations are severely depleted whereas trkA expressing populations are relatively unaffected by the mutation. In E11.5 mutant embryos, trkC immunoreactivity is associated with only 10% of surviving neurons whereas in E11.5 wild-type embryos, trkC is expressed by 93% of neurons. This suggests that NT-3 may additionally act to increase the levels of expression of trkC in these neurons.

Roles of NT-3 in the trigeminal ganglion

Our results indicate that NT-3 is necessary for the survival of embryonic trigeminal neurons. We find depletion of trkC and trkB expressing neurons at stage E11.5, before most trigeminal ganglion

neurons have encountered their targets. Very similar actions of NT-3 have been proposed to account for the phenotype of the dorsal root ganglia (DRGs) of these mice (Fariñas et al., 1996). This proposed role is also consistent with results obtained in avian DRGs. A strong decrease in embryonic neuronal survival in the DRGs has been observed in vivo in 3 day old quail embryos treated with antibodies against NT-3 (Gaese et al., 1994) or in 3.5-7 day old chick embryos treated with antibodies against trkC (Lefcort et al., 1996).

Other work indicates a specific later role for NT-3 in supporting the survival of muscle sensory neurons. Retrogradely labeled muscle sensory neurons survive in vitro in approximately two-fold greater numbers in response to added NT-3 compared to added BDNF or NGF (Hory-Lee et al., 1993). Furthermore, injection of a function-blocking antibody against NT-3 in vivo in developing chick at stages E6-E9 interferes with survival of large diameter muscle afferents (Oakley et al., 1995). NT-3 knockout mice lack 1a afferents at birth, as ascertained by examination of central projections (Enrfors et al., 1994; Fariñas et al., 1994) and the sensory innervation of muscle (Ernfors et al., 1994). Muscle spindle afferents can be selectively rescued, in mice carrying a null mutation in the NT-3 gene, by transgenic expression of NT-3 under a myogenin promoter, resulting in muscle-specific expression of NT-3 (Wright et al., 1997).

Finally, work from other laboratories indicates that there may be some effect of NT-3 on the final morphology of peripheral sensory endings. Specifically, NT-3 may favor Merkel cell-type contacts by peripheral fibers. Transgenic mice overexpressing NT-3 under a keratin promoter show enlargement of skin touch domes and increased numbers of associated Merkel cells (Albers et al., 1996). Conversely, mice lacking NT-3 show postnatal elimination of Merkel contacts (Airaksinen et al., 1995).

Our conclusions contradict published analyses of NT-3 knockout mice (ElShamy and Ernfors, 1996a,b) which conclude that the neuronal deficits in the trigeminal ganglion and DRGs in mutants arises principally through abnormal death of precursor cells. ElShamy and Ernfors based their conclusions on the following arguments: (1)They observe reduced numbers of cells which had incorporated BrdU in the developing DRG and trigeminal ganglion of mutant animals; (2) They observe double labeling of cells which had incorporated BrdU with the TUNEL marker for an early apoptotic phenotype; (3)They observed colocalization of TUNEL staining and nestin, a marker for preneuronal cells (ElShamy and Ernfors, 1996 a,b).

In our studies, we have concluded that the trigeminal deficit in NT-3 knockout mice arises from abnormal elimination of neurons, in the absence of effects on precursor cells. Our results directly contradict the results of ElShamy and Ernfors (1996 a,b) on each of the particulars cited by them: (1) We find no decrease in incorporation of BrdU by dividing trigeminal cells (Wilkinson et al., 1996); (2) at lower doses of BrdU injection cells which have incorporated BrdU are not labeled by the TUNEL method (Fariñas et al., 1996). This may indicate a toxicity problem with the BrdU injection protocol used by ElShamy and Ernfors (1996a,b). (3) TUNEL colocalization with neurofilament is frequently observed (Fariñas et al., 1996).

Our further analyses strengthened our interpretation that neurons, not precursor cells, are dying abnormally in the NT-3 knockout animals. Our counts of neurons and precursor cells showed no deficits in either measurement in E10.5 mutants and exclusively a neuronal deficit in E11.5 mutants. This is not consistent with abnormal losses of uncommitted progenitor cells, which would result in early losses of both neurons and precursors.

The strongest in vitro evidence for an action of NT-3 on proliferating cells has been obtained from cultures of sympathetic precursors (Birren et al., 1993; diCicco-Bloom et al., 1993; Verdi and

Anderson, 1994). ElShamy et al. (1996) had initially suggested that NT-3 acted principally on precursor cells in the sympathetic sytem. It is worth pointing out that subsequent analyses of the sympathetic system (Fagan et al., 1996; Wyatt et al., 1997), have concluded that NT-3 appears to act exclusively on postmitotic neurons in vivo

Our recent finding that the trkA, trkB and trkC are confined to neurons at stages E11.5-E13.5 strongly favors our model of a direct action of NT-3 upon embryonic trigeminal neurons. Therefore, the observed colocalization of trkC with neurofilament in the E11.5 trigeminal ganglion should be examined rigorously (see below).

FUTURE DIRECTIONS

In this section, I will speculate on the potential significance of the findings of this thesis in relation to major concepts of the field, and to indicate some future directions of study.

Expression of trkC by wild-type embryonic trigeminal neurons

Our results show that almost all neurons are immunoreactive for trkC at stage E11.5. The percentage of immunoreactive neurons rapidly decreases so that by E13.5 they represent about 10% of all neuronal profiles. This trend is consistent with reports of the

survival of cultured E11.5 and E13.5 trigeminal neurons in response to NT-3 in vitro (Buchman and Davies, 1993).

The more suprising finding is that trkC in vivo is primarily associated with neurofilament immunopositivity. By implication, trkC is expressed by at most a small subset of precursor cells. NT-3 and trkC are expressed as early as E7 in developing mouse embryos. Additionally, in situ experiments have detected trkC on migrating trunk crest cells (Tessarollo et al., 1993) and, in chick, on the placodal progenitors of the trigeminal ganglion (Williams et al., 1995). These data, along with reports of actions of NT-3 on sympathetic neuroblasts in vitro (Birren et al., 1993; diCicco-Bloom et al., 1993; Verdi and Anderson, 1994) have led to a general expectation in the field that trkC would be expressed on preneuronal cells (e.g. Lewin and Barde, 1996; but see Davies, 1994). None of these reports reflect specifically on the stages of trigeminal development studied here. Furthermore, studies of the development of chick DRGs has found that only a minor subpopulation of migrating neural crest cells in the forming DRG expresses trkC (Henion et al., 1995; Lefcort et al., 1996). This leaves open the possibility that trkC protein, which has been shown by experiments in this thesis to be subject to rapid redistribution, is transiently expressed by early trigeminal

progenitors and removed before stage E11.5. In any event, this suprising finding should be examined closely in the future.

This observation can be confirmed and extended by immunohistochemical approaches comparing hallmarks of precursor cells (BrdU incorporation or nestin or PCNA immunoreactivity) with trkC immunoreactivity at stage E11.5 as well as E9.5-E10.5 embryos. Alternatively, DAPI colabeling at these stages could give a feeling for the density of all cells in instances where expression appears to be restricted to neurons. The population of neurons within a field could then be related to the much more numerous precursors. In the case of the trigeminal ganglion, special attention should be paid to potential trkC expression by the trigeminal placode.

TrkC immunoreactivity in the ganglion and NT-3 expression in the periphery show a remarkable developmental synchrony over stages E11.5-E13.5. TrkC is transiently expressed by all trigeminal neurons at stage E11.5, including neurons which also express trkB or trkA. This suggests that most trigeminal neurons could be sensitive to NT-3 at this stages. Also at E11.5, NT-3 is synthesized by cells along the entire peripheral route taken by extending trigeminal axons, raising the possibility of a direct in vivo action of NT-3 on trigeminal neurons at this stage. In support of this idea, there is evidence from mutant embryos that NT-3 influences the levels of

trkC expressed by these neurons in vivo, since only 10% of surviving neurons express detectable levels of trkC in E11.5 NT-3 mutants. By E13.5, NT-3 expression in the periphery has become restricted to dermal mesenchyme, and in the trigeminal ganglion the extent of trkC immunoreactivity has decreased greatly. In particular, trkB and trkA expressing neurons no longer express trkC.

This synchrony seems to be reflected in an early requirement for NT-3 by trkB and trkC expressing neurons. Definitive trkC expressing neurons are eliminated and trkB neurons are severely depleted at stages when trkC and NT-3 are both widely expressed in the trigeminal system. However, NT-3 signaling through trkC appears to be neither necessary nor sufficient for survival of trkA expressing neurons. Mice which do not express any form of trkC lack 21% of trigeminal neurons at birth (Tessarollo et al., Soc. Neuro Abs. 1996), indicating that most trkA expressing trigeminal neurons can survive without trkC signaling. Furthermore, the population which expresses trkA along with trkC survive in only slightly reduced numbers at stages E11.5-E12.5 in embryos lacking NT-3. (present study). Finally, embryos lacking NGF show a neuronal deficit in the trigeminal at stage E11.5 (unpublished observations), a time of extensive coexpression of trkC by trkA populations, suggesting NT-3 mediated

signaling through trkC is not sufficient for the survival of those neurons.

A first step toward understanding the function of widespread trkC and NT-3 expression with respect to the trkA expressing population would be to determine which isoforms of trkC are expressed in the E11.5 trigeminal ganglion. Messenger RNA analyses of the entire wild-type ganglion could enumerate the isoforms of trkC which are expressed at stage E11.5, and antibodies specific for individual variants, such as have been developed for variants of trkB (e.g. Fryer et al., 1996) could determine which isoforms of trkC are expressed by trkA expressing neurons. The genetic data raise the possibility that trkA expressing neurons are expressing isoforms of trkC which are unable to mediate a survival response to NT-3.

trkB expression in the wild-type trigeminal ganglion

The total number of trkB expressing neurons in the wild type ganglion decreases from 9900 neurons at stage E11.5 to 4600 neurons at stage E13.5. This could be due to naturally occurring cell death or transient expression of trkB by some of these neurons. Based on the correlation of decreasing neuronal numbers in the wild-type trigeminal ganglion with the onset of trkB signaling dependence as seen in trkB knockout mice (Piñon et al., 1996), we favor the idea that the decrease in numbers reflects the onset of naturally occurring

cell death. This interpretation that could be directly verified by showing colocalization of trkB and the TUNEL marker for apoptosis. In the case that some neurons transiently express trkB, they would almost certainly later express trkA, since trkA is expressed by essentially all neurons at E12.5. An experimental prediction of this hypothesis would be coexpression of trkB and trkA by individual trigeminal neurons.

TrkB expressing neurons are observed most frequently in the anterior of the E11.5 trigeminal ganglion. The prominent spatial skew of trkB expressing neurons within the ganglion seems to persist in later embryos and in the absence of NT-3. The early appearance of this phenomenon raises the possibility that it reflects initial expression of trkB by a spatially biased population. The trkB expressing population need not be of separate developmental origin (e.g. derived from the trigeminal placode) because the dorsal root ganglion, a purely crest-derived population, manifests a comparably biased early distribution of trkB positive neurons.

If the initial expression of trkB by neurons is indeed influenced by spatially biased factors, the most attractive possibility at present is an inductive signal, possibly soluble, originating in peripheral tissue. Specifically, we can argue against an inhibitory signal generated within the trigeminal ganglion and a soluble inhibitory

signal generated by the central nervous system. If the initial expression of trkB were inhibited by some factor released by trigeminal neurons, one would expect a decline in the proportion of trkB expressing neurons as overall numbers of trigeminal neurons increased. Also, the initial expression of trkB would be favored in neurons at the margins of the ganglion, where levels of the inhibitory factor would be least. These predictions are consistent with what is observed. However, the posterior margin of the ganglion should be equally favored, which is not observed.

There is indirect reason to believe that the anterior bias in trkB expressing neurons seen in the trigeminal ganglion is not the result of a factor inhibitory to the initial expression of trkB released by the central nervous system. Dorsal root ganglia (DRGs) show a biased distribution of trkB expressing neurons toward their ventral margin. Because the spinal cord is medial to, rather than dorsal to, the DRGs, an inhibitory factor released from the CNS would be expected to favor initial expression of trkB by lateral DRG neurons rather than ventral neurons.

If the initial expression of trkB is in fact influenced by soluble factors, some critical agent may be present in the medium used for low-density trigeminal neuron cultures by Buchman and Davies (1993) and Paul and Davies (1995). These authors report 150%

survival of E11 trigeminal neurons (normalized to initially plated neurons) cultured in a defined medium in the presence of added BDNF. By contrast, culture in the presence of added NGF yielded only 20% survival. We observe roughly equal numbers of trkA and trkB expressing neurons in vivo in the E11.5 trigeminal ganglion. The seven-fold in vitro increase of neurons seen in the presence of BDNF relative to that seen in the presence of NGF may therefore reflect a selection for expression of trkB by neurons born in vitro. It would be of interest to compare the extent of expression of trkA, trkB, and trkC by trigeminal neurons cultured in this defined system. This may allow identification of factors which can influence the expression of trkB by early trigeminal neurons.

Switchover in neurotrophin dependence in the wild-type trigeminal ganglion

A study of changes in neurotrophin responsiveness in developing trigeminal neurons (Buchman and Davies, 1993) has demonstrated that cultures of trigeminal neurons taken from E10 embryos exhibit robust survival responses to added BDNF, NT-4, or NT-3 but almost no response to added NGF. Cultures obtained from older embryos show a progressive changeover, so that almost 100% of E14 trigeminal neurons survive in response to added NGF but only

about 10% survive in the presence of BDNF, NT-4, or NT-3. These and other observations (Paul and Davies, 1995; Piñon et al, 1996) have led the authors to propose that individual trigeminal neurons undergo a switch in neurotrophin dependence, depending on trkB signaling prior to requiring trkA signaling (Davies, 1997).

Our results show that the in vivo basis for the switchover seen in these cultures is that a much smaller percentage of neurons express trkB or trkC in the E13.5 trigeminal ganglion relative to E11.5. Conversely, trkA expressing neurons comprise about 50% of all neurons at E11.5 and essentially 100% of all neurons at E13.5. Thus, a diminished survival of E13.5 trigeminal neurons to added BDNF relative to E11.5 neurons likely reflects the decrease in the percentages of neurons which express trkB. It is not necessary to invoke a dominant inhibitory effect exerted by truncated trkB receptors (Ninkina et al., 1996; Davies, 1997) to account for this change.

Moreover, the available evidence suggests that individual trigeminal neurons do not switch expression from trkB to trkA as a general phenomenon. The absolute numbers of trkB immunopositive neurons at stages E12.5 are not consistent with a role for trkB expressing neurons as a precursor state for neurons destined to express trkA. Between stages E11.5-E12.5, some 15,000 new neurons

are born in the trigeminal ganglion, and essentially all of these express trkA at E12.5, thus doubling the number of trkA expressing neurons. Nevertheless the number of trkB immunopositive profiles actually decreases over these stages. Secondly, the spatially biased position occupied by trkB expressing neurons in the trigeminal ganglion does not correlate well with the even distribution seen of trkA positive neurons. Finally, at E11.5, a stage at which trkB expressing neurons are potentially numerous enough to serve as the source of trkA expressing neurons, the trkA and trkB expressing populations show distinct behaviors in the absence of NT-3. This would not be expected if the trkA populations seen at E11.5 in NT-3 knockouts had passed through an obligatory trkB dependent phase.

Instead, our observations suggest that the trkB and trkA expressing populations are largely independent in vivo. The observation of elevated cell death in trkB kinase knockout embryos at stages E11-E12 followed by elevated cell death in trkA knockout embryos at stages E13-E15 (Piñon et al., 1996) is most consistent with separate periods of naturally occurring neuronal death for these two populations. In this light, it may be interesting to evaluate the status of the trkB expressing population with respect to peripheral and central target encounter during its naturally occurring period of cell death. The NGF sensitive population has been demonstrated to

undergo a period of naturally occurring cell death after most axons have encountered the peripheral sources of NGF (see introduction). By contrast, the best studied trkC dependent population, the 1a afferents, are eliminated by the absence of NT-3 prior to final target encounter (Kucera et al., 1995).

The most direct verification of these issues is to examine the potential coexpression of trkB and trkA by individual trigeminal neurons in vivo and in vitro.

The phenotype of NT-3 knockout embryos

Counts of neurons in the trigeminal ganglion of embryos lacking NT-3 rise from approximately 15,000 neurons at E11.5 to 31,000 neurons at E12.5. The number of neurons then decreases to 15,000 neurons at E13.5, after which the number of neurons remains roughly constant up to birth. These changes are reminiscent of a burst of neurogenesis at the expense of precursors seen in the DRGs of E12.5 NT-3 knockout embryos (Fariñas et al., 1996). A similar phenomenon has also been observed at these stages in the DRGs of NGF knockout animals (Fariñas et al., unpublished observations). This may represent a perturbation of the controls of neurogenesis in both mutant backgrounds. Because of this possibility, and in light of the small changes seen in proliferation and precursor numbers seen in

the trigeminal ganglion of NT-3 knockout embryos at stages E13.5 (Wilkinson et al., 1996), we interpret results from E12.5 and older embryos with caution.

trkC expression in NT-3 knockout embryos

NT-3 appears to be necessary for the survival of trigeminal neurons which definitively express trkC, which represent about 10% of all neurons in the E13.5 ganglion. At least some of these neurons are already dying by E11.5, and the elimination appears to be complete by E12.5 (see also Tessarollo et al., 1994).

Analysis of the innervation pattern of the intervibrissal skin of the mystacial pad in NT-3 knockouts or trkC knockouts (Rice et al., 1997) has identified a class of large caliber, CGRP/Substance P/Galanin immunopositive, free nerve ending which is eliminated prenatally in both of these mutant backgrounds. This sensory ending class may correspond to the trkC immunopositive neurons which are eliminated in the E13.5 trigeminal ganglion in NT-3 knockout animals. It would be of interest to study the formation of this class of ending with respect to trkC immunoreactivity. The relationship of this type of ending to supplies of NT-3 could be evaluated in mice carrying the lacZ reporter knock-in to the NT-3 locus.

trkB expression in embryos lacking NT-3

Counts of neurons expressing trkB in the E11.5 trigeminal ganglion of embryos lacking NT-3 show a severe depletion of this population relative to wild type. This could be due to an increase in cell death among neurons expressing trkB or failure of neurons to express trkB. We favor the interpretation that this represents increased cell death of trkB expressing neurons at E11.5, immediately prior to their naturally occurring period of cell death. The coexpression of trkC by these neurons at this stage may reflect a survival requirement for NT-3 these neurons. This interpretation could be verified directly by double label experiments with trkB and TUNEL.

If trkB expressing neurons survive but fail to express trkB in the absence of NT-3, then our data indicate that they do not convert to expressing another trk receptor, for instance trkA. TrkA expressing neurons are present in unchanged proportion relative to all neurons in E11.5 embryos lacking NT-3. Thus, abnormal conversion of trkB expressing neurons to expressing trkA would have to be accompanied by elevated cell death among trkA expressing neurons. Alternatively, neurons which do not express trkB in NT-3 knockouts might not express trkA. However, in E11.5

embryos lacking NT-3, the numbers of neurons expressing trkA (9000 neurons) added to the number expressing trkB (3700 neurons) accounts for 85% of the total number of neurons (14,700). The postulated presence of 6000 neurons which express neither trkB nor trkA in mutant animals would imply large numbers of neurons which express both trkB and trkA. This seems unlikely, given the very different numerical consequences for trkB and trkA expressing populations of the absence of NT-3. An experimental prediction of this model is that trkB expressing neurons would also express trkA in E11.5 NT-3 knockout embryos. Also, a developmental study of numbers of trkB expressing neurons in wild type and mutant embryos at stages E10.5 and possibly earlier could support the possibility of failure of neurons to express trkB in the absence of NT-3.

At stages E12.5-E13.5, numbers of trkB immunoreactive neurons in mutant embryos converge with wild-type counts, as wild type numbers decrease while numbers remain constant in the mutant. The observed convergence of wild-type and mutant numbers of trkB immunopositive profiles at E13.5 emphasizes the importance of developmental studies in evaluating the consequences of genetic mutations. The earliest disruptions in mutant embryos, i.e.

the ones presumed to result most directly from the absence of the gene product of interest, may be masked by subsequent phenomena.

With specific regard to trkB, this observation indicates that the trigeminal phenotype of BDNF and NT-4 knockout animals should be reevaluated with reference to these early stages despite the absence of a numerical neuronal deficit in these genetic backgrounds at stage P0 (Conover et al., 1994; Liu et al., 1994). The small size of the trkB expressing population in the trigeminal ganglion makes the anti-trkB antibody a valuable tool.

trkA expression in embryos lacking NT-3.

Numbers of trkA immunopositive neurons in the trigeminal ganglia of E11.5 and E12.5 embryos lacking NT-3 are 70% of numbers seen in the wild type ganglia at the same time. Although this difference represents a comparatively mild deficit compared to the consequences of this mutation for numbers of trkC and trkB immunopositive neurons at the same stages, it is nevertheless statistically significant at E12.5. Since trkA positive neurons make up the same proportion of all neurons in wild-type and NT-3 knockouts at these stages, these decreased absolute numbers of trkA expressing neurons may reflect a NT-3 sensitive neuronal state prior to definitive expression of trkA. If this pool survives in diminished

numbers in the absence of NT-3, then definitive trkA expressing neurons would be generated in normal proportion from this reduced pool.

The neurons lost during the decrease in neuronal numbers from E12.5 to E13.5 in the trigeminal ganglion of NT-3 knockout embryos are predicted to be predominantly of the population which expresses trkA, since this population makes up essentially 100% of neurons present in the E12.5 ganglion. Loss of these neurons could be due to a survival requirement for NT-3 signaling through trkA. NT-3 is capable of signaling through trkA in E14 trigeminal neurons (Davies et al., 1995) In this case, the requirement for NT-3 would be expressed relatively late in the development of those neurons, since the E11.5-E12.5 trigeminal ganglion in NT-3 knockout embryos has only slightly reduced numbers of trkA expressing neurons. The finding that NGF knockout embryos show a deficit at E11.5 (unpublished observations) indicates that the cellular requirement of trkA expressing neurons for NT-3 and NGF would be almost simultaneous.

An alternative interpretation is that the loss of neurons from the trigeminal ganglion in NT-3 knockout embryos between stages E12.5-E13.5 reflects a general decrease in the competence of these neurons to survive related to the postulated secondary pathologies in

E13.5 mutant embryos described above. There are several lines of experiments which could clarify the later trigeminal phenotype of NT-3 knockout animals. First, a calculation of in vivo precursor numbers in the E12.5 mutant trigeminal will test whether the increase in neuronal numbers seen at in the trigeminal of NT-3 knockouts at this stage is accompanied by a strong decrease in precursor numbers, as is seen in the DRGs of NT-3 knockout embryos at E12.5. Secondly, in vitro assays could test the competence of E12 trigeminal neurons to survive in the presence of added NT-3 or added NGF.

Placodal contribution to the mouse trigeminal ganglion

The trigeminal ganglion is derived during development from placodal as well as neural crest cells. In chick, the contributions of these two populations in the developing trigeminal ganglion are readily distinguishable. Placodally derived neurons are born first and occupy the anterior rim of the ganglion, forming an anterolateral "cap" (D'Amico-Martel and Noden, 1980; Noden, 1980). Thus the dorsomedial (DM) lobe of the chick trigeminal, which contributes most of the source neurons of the ophthalmic nerve, is enriched in crest derived populations, whereas the anterolateral (AL) lobe, which primarily contains source neurons of the maxillary and mandibular

nerves, is comprised of neruons of neural crest and placodal origin. Intriguingly, neurons cultured from the two lobes show distinct neurotrophin responsiveness. Chick DM neurons respond well to NGF, while chick VL neurons are supported by BDNF (reviewed in Davies, 1988).

The situation in mouse is not as clear. The mouse trigeminal ganglion does not develop in two distinct lobes, as seen in the chick, and the relative size of the placodal and the neural crest derived populations in the adult trigeminal ganglion is uncertain (reviewed in Altman and Bayer, 1983). Futhermore, cultures of anterior and posterior mouse trigeminal ganglion do not show different responsiveness to NGF (Davies and Lumsden, 1983).

We wondered whether the severe defects in neuronal numbers seen in animals lacking NT-3 might reveal a dual developmental origin in the mouse trigeminal ganglion. For example, in chick, DM trigeminal neurons undergo a switch in neurotrophin dependence whereas AL trigeminal neurons do not (Buj-Bello et al., 1995). Given our initial assessment of NT-3 as a transitional survival factor, we hypothesized that the neural crest derived portion of the mouse trigeminal would require NT-3 while the placodal portion would not. We therefore compared the effects of the absence of NT-3 relative to wild-type on the cross-sectional area of the mouse ophthalmic nerve,

which in the chick is supplied exclusively by neural crest derived neurons, to the effects on the cross-sectional area of the mouse maxillary nerve, which should be supplied by neurons of placodal as well as neurons of neural crest origin. We hypothesized that the ophthalmic nerve would be more severely diminished than the maxillary nerve in the absence of NT-3. However, our measurements showed that both branches of the trigeminal nerve were apparently equally diminished in the absence of NT-3 (Chapter II, Figure 5, and accompanying text).

The question of dual origins of the trigeminal ganglion arose again when we observed the anterior distribution of trkB expressing neurons. Their distribution is reminiscent of the distribution of BDNF-responsive placodally derived neurons seen in the chick trigeminal. However, this skewed distribution is mirrored in the purely neural crest derived DRGs.

Thus, in our studies, we have not encountered differences in the trigeminal neurons which would distinguish placodal from crest derived populations. Our results are most consistent with a mouse trigeminal ganglion derived principally from crest cells, since the deficit in the trigeminal ganglion in the absence of NT-3 resembles

that of the DRGs in extent, in developmental timing, and in mechanistic details.

There is reason to believe that cells of placodal origin contribute some neurons to the mouse trigeminal ganglion. Neuregulin knockout embryos (Meyer and Birchmeier, 1995) and ErbB2 knockout embryos (Lee et al., 1995) show severe disruptions of cranial ganglia derived from neural crest but not in placodally derived ganglia. The trigeminal ganglia in these embryos are severely reduced but do show a residual neuronal population, which Meyer and Lee (1995) propose is placodally derived. The number of these neurons in the trigeminal ganglia of these mutants was not reported. Interestingly, the trigeminal ganglion of neuregulin or ErbB2 knockout embryos lacks a mandibular nerve. This is somewhat suprising in light of the proposal that the surviving trigeminal neurons in these knockouts derive from the trigeminal placode. From the studies in chick it would be predicted that placodal neurons would contribute heavily to the mandibular branch of the trigeminal nerve. Perhaps some other aspect of the deficiencies in these mice results in abnormal peripheral connectivity. Alternatively, the placodal contribution to the mouse trigeminal ganglion may occupy an unexpected topological location within the ganglion. It should be

noted that all three trigeminal nerve branches are present in NT-3 knockout animals.

Neurotrophins in the E11.5 trigeminal ganglion

Our observations of the development of the trigeminal ganglion in NT-3 knockout mice are consistent with a model (Figure IV-1) in which NT-3 performs 3 different roles in the E11.5 trigeminal system. (1) NT-3 is available along the trajectory taken by axons of trigeminal neurons to the periphery. NT-3 is necessary for the survival of definitive trkC expressing neurons and supports the survival of trkB expressing neurons, probably before they reach their final targets. (2) In the absence of NT-3, far fewer surviving neurons express detectable levels of trkC, which suggests that NT-3 regulates the levels of expression of trkC. (3) Other workers have provided evidence that NT-3 acts as a target-derived trophic factor for muscle sensory neurons and possibly other classes of sensory neurons.

NT-3 appears to be unique among the four neurotrophins in that it is synthesized by mesenchymal cells along the entire trajectory taken by trigeminal neurons toward the periphery. NT-4, BDNF and NGF are primarily expressed in the final targets of trigeminal neurons. NT-4 and BDNF have been shown by in situ to be

confined in the periphery to the presumptive epidermis and dermal mesenchyme, respectively (Arumae et al., 1992). BDNF is also synthesized in the hindbrain, a major central nervous system target of trigeminal ganglion neurons. (Buchman and Davies, 1992). NGF has been shown by in situ and by two-site immunoassay to be synthesized only by the presumptive epidermis (Davies et al., 1987; reviewed in Davies, 1987). The distribution of NT-3 combined with the widespread early expression of trkC by embryonic trigeminal allows NT-3 to act uniquely in vivo as the "early neurotrophin" by supporting the survival of classes of sensory neurons, which later depend on other neurotrophins.

CONCLUSION

Our analysis of the development of the trigeminal ganglion in wild-type and NT-3 knockout mice has revealed unexpected simplicity in this ganglion. In spite of the many different actions of NT-3 on neurons and neuronal precursors observed in vitro, we can explain the onset of the deficit seen in the trigeminal ganglion at stage E11.5 almost entirely in terms of failure of embryonic trigeminal neurons to survive. In later stages embryos, the neuronal deficit appears to reflect complex changes the the trigeminal

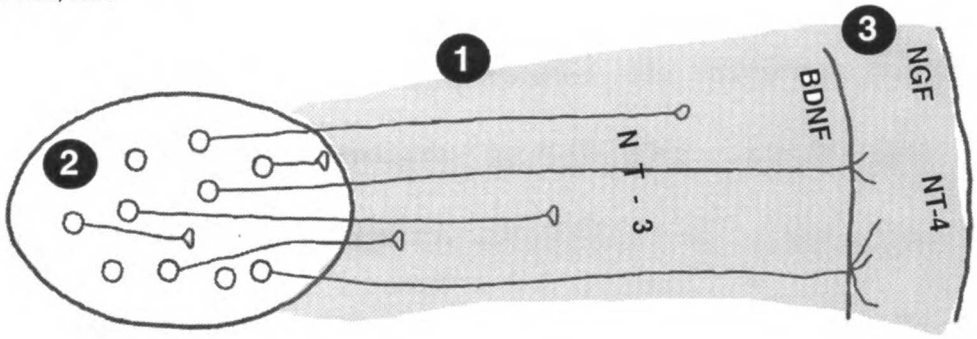
ganglion, including changes in precursor cell behavior. (Fariñas et al., 1996; and present results).

Using a combination of genetic manipulations and molecular markers for neuronal subpopulations has allowed a substantial clarification of the consequences of the absence of NT-3 on the neurons of the trigeminal ganglion. This approach should be broadly applicable in understanding the effects of null mutations in other systems in the future.

Figure IV-1. Trk receptors and neurotrophins in the E11.5 trigeminal system. Shown in this schematic diagram are the results of our studies on the distribution of NT-3 and trk receptors and the in situ and protein studies of others (Arumae et al., 1992; Buchman and Davies, 1993; Enrfors et al., 1992) on the other neurotrophins. The hindbrain (not shown), which contains CNS targets of trigeminal ganglion neurons expresses BDNF (Buchman and Davies, 1993). Sites of NGF synthesis have been localized by in situ methods and 2-site immunoassay (reviewed by Davies, 1987). BDNF and NT-4 have been localized in the periphery by in situ methods (Ernfors et al., 1992; Arumae et al., 1992). NT-3 has been localized using in situ (Arumae et al., 1993) and using a lacZ reporter construct (Wilkinson et al., 1996). Within this environment, NT-3 plays multiple roles in the support of trigeminal neurons, including many other neurons which later rely on other neurotrophins.

ROLES OF NT-3 IN THE E11.5 TRIGEMINAL SYSTEM

Pinon et al., 1996
Arumae et al., 1993
Buchman and Davies, 1993
Ernfors et al., 1992



GANGLION

trkC (all neurons)
trkB (neurons)
trkA (neurons)

TARGET TISSUE

BDNF, NT-4 by in situ
NT-3 by lacZ

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