Dynamic expression of trk receptors during sensory neuron differentiation
by Jason Thomas Rifkin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University
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Abstract:
Studies over the past several years have provided evidence for the functions of neurotrophins during development of the nervous system prior to their well characterized role in target-mediated cell death. To define the potential functions of neurotrophins during this early period of development we looked at the spatial and temporal expression patterns of neurotrophin receptors throughout the formation of motorneurons, within the ventral horn of the spinal cord, and sensory neurons of the dorsal root ganglion (DRG) in an avian model. To this end we have used a highly specific panel of antibodies to the avian trkA, trkB, and trkC receptors and p75 and compared their expression to that of known neuronal cellular markers. We have found that p75 is the first of the neurotrophin receptors to be expressed (at stage 14), followed by TrkC (stage 15/16), then trkA (stage 18), and finally trkB (stage 22). At stage 18 trkA is expressed only on a subpopulation of trkC+ cells, and trkA+ and trkC+ cells show a reduction of the HNK-1 carbohydrate moiety. At stage 24, trkC is expressed by the vast majority of cells within the developing DRG, while by E6 (and beyond) trkA becomes the most prominently expressed trk receptor in the DRG. At ES, trkB and trkC are expressed primarily by cells in the VL region of the DRG while trkA predominates in the DM region of the DRG. Early in development, trk receptors are expressed in the distal regions of peripheral axon afferents. Both trkC (at stage 24) and p75 (at stage 18, 24) receptors are expressed by mitotically active cells. Finally, at E6, BEN is now coexpressed with trkA and is no longer coexpressed with trkC; while, at E13, NF (which was expressed throughout the DRG) is now localized to the VL region of the DRG. In conclusion, our data point to two additional potential functions for neurotrophins during the development of the dorsal root ganglion: regulating proliferation of sensory neuroblasts and influencing patterns of axon outgrowth during target innervation.

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A thesis submitted in partial fulfillment
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Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

December 1996

N378
R44749

APPROVAL

of a thesis submitted by

Jason Thomas Rifkin

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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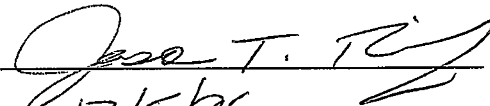
Signature 

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"For the real amazement, if you wish to be amazed, is this process. You start out as a single cell derived from the coupling of a sperm and an egg; this divides in two, then four, then eight, and so on, and at a certain stage there emerges a single cell which has as all its progeny the human brain. The mere existence of such a cell should be one of the great astonishments of the earth. People ought to be walking around all day, all through their waking hours calling to each other in endless wonderment, talking of nothing except that cell."

Lewis Thomas

ACKNOWLEDGEMENTS

I would like to thank the members of my committee for all of their support and advice and encouragement throughout my graduate program. I would especially like to thank my Major Advisor, Frances Lefcort, for her support and friendship, and for enhancing my ability to think for myself.

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ABSTRACT

Studies over the past several years have provided evidence for the functions of neurotrophins during development of the nervous system prior to their well characterized role in target-mediated cell death. To define the potential functions of neurotrophins during this early period of development we looked at the spatial and temporal expression patterns of neurotrophin receptors throughout the formation of motorneurons, within the ventral horn of the spinal cord, and sensory neurons of the dorsal root ganglion (DRG) in an avian model. To this end we have used a highly specific panel of antibodies to the avian trkA, trkB, and trkC receptors and p75 and compared their expression to that of known neuronal cellular markers. We have found that p75 is the first of the neurotrophin receptors to be expressed (at stage 14), followed by TrkC (stage 15/16), then trkA (stage 18), and finally trkB (stage 22). At stage 18 trkA is expressed only on a subpopulation of trkC^{+} cells, and trkA^{+} and trkC^{+} cells show a reduction of the HNK-1 carbohydrate moiety. At stage 24, trkC is expressed by the vast majority of cells within the developing DRG, while by E6 (and beyond) trkA becomes the most prominently expressed trk receptor in the DRG. At E8, trkB and trkC are expressed primarily by cells in the VL region of the DRG while trkA predominates in the DM region of the DRG. Early in development, trk receptors are expressed in the distal regions of peripheral axon afferents. Both trkC (at stage 24) and p75 (at stage 18, 24) receptors are expressed by mitotically active cells. Finally, at E6, BEN is now coexpressed with trkA and is no longer coexpressed with trkC; while, at E13, NF (which was expressed throughout the DRG) is now localized to the VL region of the DRG. In conclusion, our data point to two additional potential functions for neurotrophins during the development of the dorsal root ganglion: regulating proliferation of sensory neuroblasts and influencing patterns of axon outgrowth during target innervation.

INTRODUCTION

Development of the nervous system is a complex, intricate process involving cell proliferation, migration, differentiation, survival, and synapse formation (Chao, 1992). Subsequent to gastrulation, neurulation ensues and involves the changing of the shape of the ectodermal cells within the dorsal portion of the developing embryo, which will form the neural plate (Jacobson, 1981; Smith et al., 1991). The cells of the neural plate begin to invaginate, while the adjacent neural folds begin to rise and finally fuse converting the neural plate into the neural tube (Erickson et al., 1983; Nichols, 1981). Many theories have been proposed as to the mechanisms underlying the initiation of the shape changes that result in the neural groove, and subsequent neural tube. These include: pushing and pulling forces (His, 1874); adhesion interactions (Edelman, 1984); and structural changes in the size and shape of the neural epithelial cells (Trinkhaus, 1969). The neural tube gives rise to the central nervous system (CNS) including the spinal cord and all of the spinal cord's various cell types. This occurs via the differentiation of neuroepithelial cells within the wall of the neural tube giving rise to the various populations of neurons and supportive cells in the spinal cord (Gilbert, 1994).

Neurons generally develop in a ventral to dorsal gradient within the spinal cord, with the motorneurons developing first and projecting their axons into the periphery (Lumsden et al., 1991; Placzek et al., 1991). In the chick, the first motorneurons appear (leave the mitotic cycle) at stage (St.) 15, with all of the population being born by St. 23 (Hollyday et al., 1977). By approximately St. 29 one half of the motorneuron population has died due to programmed cell death (Hamburger, 1975; Oppenheim et al., 1978; Lanser et al., 1984). Considerable research has implicated the involvement of the notochord and floor plate in the development of the motorneurons through cell-cell contacts and extrinsic factors (Van Straten et al., 1985; Smith et al., 1989). Placzek et al., 1991, found that following removal of the notochord early in development, the floor plate did not form and motorneurons failed to properly develop. Placzek et al., 1991 also discovered that adding an additional notochord would lead to the formation of an another floor plate within the spinal cord. The extrinsic factor mediating these ventral patterning effects is most likely sonic hedgehog (Ericson et al., 1995).

The peripheral nervous system develops from migrating neural crest cells which give rise to the DRG, sympathetic and parasympathetic ganglia, and supportive cells (Lallier et al., 1988; Vogel et al., 1992; Marusich et al., 1991; Weston, 1991; Serbedzija et al., 1990). The neural crest cells form

between the ectoderm and the dorsal region of the recently closed neural tube, and their migration occurs in a rostral to caudal gradient (Holmdahl, 1928). There are two streams of neural crest cell migration: (1) a dorsolateral route which will give rise to the melanocytes and has been studied in relation to pigmentation patterns (Rawles, 1948); and (2) a ventrolateral route which travels lateral to the neural tube and both medial to and through the developing somites (Weston, 1963; Bronner-Fraser, 1986). This second migratory pathway travels only along the anterior portion of the adjacent somite (Bronner-Fraiser, 1986). This stream of cells will give rise to the autonomic ganglia, glial cells, adrenomedullary cells, and of most interest in this study, to the cells of the DRG. The mechanisms involved in this migration are not completely understood, but it is believed that the extracellular matrix through which the neural crest cells migrate is of utmost importance (Pratt et al., 1975; Toole, 1976; Derby, 1978; Rovasio et al., 1983).

Many different intrinsic and extrinsic factors play a role in this migration and subsequent formation of the peripheral nervous system. These include various cell adhesion molecules and cell surface receptors. There are also a multitude of exogenous ligands acting via receptor proteins to activate intracellular signaling cascades, which will lead to changes in the expression patterns of various gene products. These include numerous trophic factors which

act on populations of cells in the process of development and promote survival, proliferation, or differentiation of these cells. In the development of the nervous system these trophic factors are collectively referred to as neurotropic factors. Throughout the development of the central and peripheral nervous systems these neurotrophins and their associated receptors play an important role, and a role that appears to expand as more research in this area is undertaken.

The first of the neurotrophins to be discovered was Nerve Growth Factor (NGF), discovered by Rita Levi-Montalcini and Victor Hamburger in association with Stanley Cohen in the early 1950's (Levi-Montalcini et al., 1956; Levi-Montalcini et al., 1960). It was found that mouse sarcoma tissue transplanted into a chick embryo promoted outgrowth of sympathetic and sensory neurons into the sarcoma tissue. This growth was also induced by an extract of snake venom. A 44kD protein responsible for this "growth promoting effect" was termed nerve growth factor, and so began the fascination with neurotrophins in the development of the nervous system. Brain derived neurotrophic factor (BDNF) was subsequently discovered by Barde and his group in 1982 by purifying the protein through homogenization of thousands of swine brains. It was found that there existed a considerable sequence homology (approximately 50%) between NGF and BDNF (Hohn et al., 1990; Jones et al., 1990; Maisopierre et al., 1990;

Rosenthal et al., 1990), and this fact was used to identify a third neurotrophin. Hohn et al., 1990; Jones et al., 1990; Maisopierre et al., 1990; and Rosenthal et al., 1990 all utilized degenerate oligonucleotides (from homologous regions of the two previously discovered neurotrophins) in PCR reactions using rat or human genomic DNA to discover a novel neurotrophin called Neurotrophin-3 (NT-3). The three previously mentioned neurotrophins will be the major focus, in this thesis, because of their prominent effect on cell populations within the developing nervous system.

The neurotrophins are exogenous ligands and must therefore act at a receptor on or within the cell to exert their effects. Previous data had identified a low-affinity receptor for NGF, p75, which does not contain a tyrosine kinase domain (Johnson et al., 1986: Radeke et al., 1987).It was discovered that NGF bound with a high affinity to an unknown receptor, which was thought to be p75. Since p75 binds NGF with a low affinity it was therefore realized that there must also be another receptor binding NGF (Weskamp et al., 1991). It was theorized that the tyrosine protein kinase superfamily could contain a likely candidate for the receptor (Barbacid et al., 1991), given that a large number of the genes within this superfamily act as growth factor receptors (Yarden et al., 1988; Ullrich et al., 1990). Since the trks fall into this tyrosine protein kinase superfamily they were considered a candidate for this role. The trk

receptors are a family of proto-oncogenes (Barbacid et al., 1991), and were originally detected in 3T3 cells (Martin-Zanca et al., 1986). They derive their name from a rearrangement of nonmuscle tropomyosin fused to the cytoplasmic and transmembrane domain of a kinase receptor (therefore trk - tropomyosin receptor kinase). Trk receptor proteins display a C-2 type extracellular domain similar to that of an immunoglobulin (Willimas et al., 1987), which makes them members of the immunoglobulin superfamily. Intracellularly they contain a tyrosine kinase domain, and upon binding ligand (neurotrophin) (Klein et al., 1991) the trk receptor dimerizes forming a homodimer complex (Eide et al., 1996) which leads to the autophosphorylation of the tyrosine kinase domain. This event initiates an intracellular signaling cascade that directly or indirectly acts on transcriptional events within the cell (Jing et al., 1992; Fantl et al., 1993).

Klein et al., 1991 determined that NGF bound to the human proto-oncogene trk (to be later deemed "trkA", a 140 kD glycoprotein) by cross linking NGF to trk, and through immunoprecipitation experiments. This binding of trk receptor to NGF ligand induced rapid phosphorylation of the trk intracellular kinase domain (Klein et al., 1991; Kaplan et al., 1991). Trk B was subsequently isolated by molecular characterization of trk-related transcripts. It was found to be a glycoprotein of 145 kD with a 57% homology to the

extracellular domain of trk A and 88% homology with the intracellular kinase domain (Klein et al., 1989). It was later determined that BDNF, and to a lesser extent NT-3, bound with high affinity to trk B (Squinto et al., 1991). Lamballe, et. al., (1991) identified a third member of this family, trk C, as a 145 kD glycoprotein and showed that trk C specifically bound NT-3 and not NGF or BDNF. These receptor tyrosine kinases are believed to have the ability to promote growth and proliferation, or to halt growth and promote differentiation depending on the receptor, its ligand, and the type of cell in which it is expressed (Schlessinger et al., 1992).

For a long period of time it was believed that the sole role of neurotrophin/trk receptor interactions was one that promoted survival during a period of target-mediated cell death (Purves, 1988). During the formation of the nervous system many potential neurons die in a competitive struggle to gain access to a limited supply of survival promoting neurotrophins which are produced by the target tissue (Hamburger et al., 1981; Oppenheim, 1991). Those cells which receive these nourishing ligands will survive and set up the neural network, while those that do not will die through the mechanism of apoptosis (Hamburger et al., 1982). Apoptosis is a neat and orderly process which results in the condensation of the cells chromatin followed immediately by phagocytosis by neighboring cells (McConkey et al., 1992).

Various genes (depending on the cell type involved) are activated or repressed and this leads to cell death. It is termed programmed cell death because these genes are systematically turned on or off by endogenous or exogenous cues resulting in survival or death.

Recent evidence points to the involvement of neurotrophins and their receptors in the development of the nervous system prior to target mediated cell death (Davies, 1994). Migrating neural crest cells express trk C mRNA and will proliferate in response to NT-3 in vitro (Kalcheim et al., 1992). Moreover, NT-3 has been shown to accelerate differentiation of newly born spinal sensory neurons (Wright et al., 1992), as well as induce the proliferation of precursor populations (DiCicco-Bloom et al., 1993; Pinco et al., 1993). Most strikingly, the number of DRG neurons is dramatically reduced in NT-3 knockout mice (Farinas et al., 1996). Farinas et al., 1996 (in press) have shown that an increase in apoptotic figures and a depletion of the neuroblast pool leads to this reduction of 60-70% of DRG neurons compared to controls.

The DRG develops from the ventral-lateral migratory pathway of the neural crest cells (Raible et al., 1995), differentiating into sensory neurons, as well as supportive cells (Figure 1). During development, the DRG will grow in size and cell number and the sensory neuron cell bodies will send afferent extensions into the spinal cord and to their

peripheral targets, where they will synapse onto their appropriate targets. Finally, target-mediated cell death occurs, pruning the number of sensory neurons in the DRG to the level that will persist in the adult.

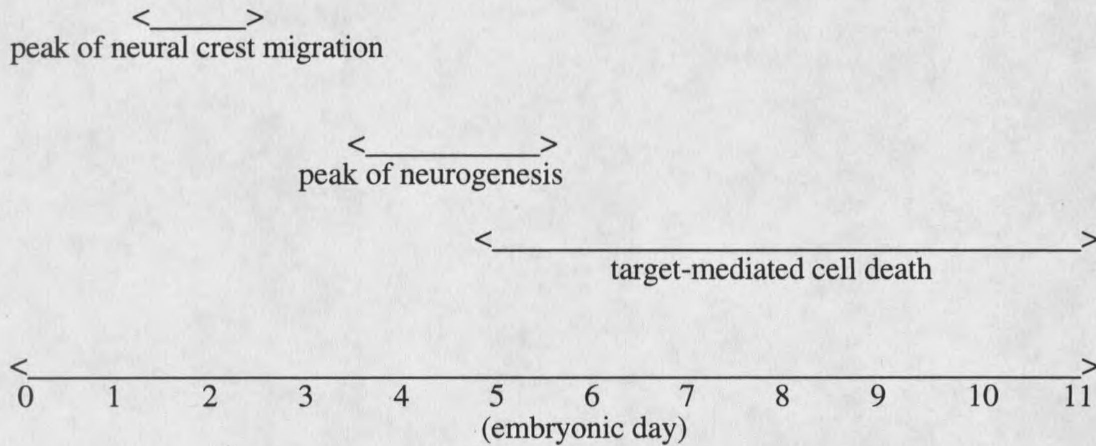

Figure 1 Critical periods in the development of the sensory neurons of the DRG (Carr, 1978; Lallier, 1988).

EXPERIMENTAL OBJECTIVES AND RATIONALE

The goal of this study was to understand the role trk receptors play in development of the nervous system. To accomplish this task we have looked at single cell resolution of trk receptor proteins utilizing immunofluorescense to determine their temporal and spatial expression patterns. Previous studies have utilized in situ hybridization to determine the expression patterns of trk receptors, and a more definitive study determining protein expression needed to be performed. The highly specific antibodies utilized in this study provide a far greater amount of cellular resolution than in situ hybridization. We have focused our attention on the DRG and the interaction between neurotrophins and their respective trk receptors because neurotrophins are known to promote survival of neurons within the developing DRG (Davies et al., 1985; Lindsay et al., 1985; Davies et al., 1986; Farinas et al., 1996; Lefcort et al., 1996; Oakley et al., 1995). We examined several ages that correspond to: (1) migration of the neural crest cells to the region of DRG formation (Marusich et al., 1991), (Vogel et al., 1992) (st. 14-19), (2) the peak of neurogenesis within the developing DRG (st. 22-25) (Lallier et al., 1988), and (3) E6-E13, the period of target mediated cell death (Purves, 1986)(Figure 1). To examine this question we used a highly specific panel of antibodies to the

trk receptors (Lefcort et al., 1996), as well as antibodies to well known antigenic determinants including: BEN, recognizing a cell-adhesion molecule present on sensory and motorneuron populations (Pouquie et al., 1990); Tuji, recognizing a neuronal specific B-tubulin subunit (Memberg et al., 1994); NF, recognizing the 160 kD neurofilament subunit (Bennett et al., 1984); HNK-1, recognizing a carbohydrate moiety associated with several cell-adhesion molecules (Nordlander et al., 1993; Schachner et al., 1995); Hu, recognizing a vertebrate RNA binding protein (Marusich et al., 1993); and p75, recognizing the low affinity growth factor receptor (Weskamp et al., 1991). Use of these known antigenic determinants allowed observations of the expression of trk receptors in relation to events occurring throughout development of the peripheral nervous system. For instance, HNK-1 is present on the majority (but not all) of the migrating neural crest cells. NF, Tuji, and Hu will stain neuronal cells which may still be mitotically active; while BEN is believed to stain mature neurons within the DRG and ventral horn of the spinal cord. To identify mitotically active cells we labeled sections with 4',6-diamidino-2-phenylindole (DAPI) or injected BromodeoxyUridine (BrdU) and stained with an anti-BrdU antibody (Yoshida et al., 1987; Waid et al., 1995).

In addition, it was of interest to determine the expression patterns of trk receptors within the developing

motorneuron populations. This is critical because it could help us to understand why the motorneurons develop where they do and how their axonal extensions find their targets. This was accomplished by viewing sections at various axial levels early on in development. The axial level is of importance due to an anterior to posterior gradient of development over time (Hollyday et al., 1977).

METHODS

Embryos

Fertilized White Leghorn chicken embryos were obtained from Truslow Farms (Chestertown, Maryland) and incubated at 37° C in a rocking incubator (Kuhl, Flemington, NJ). Eggs were then windowed and embryos staged at various timepoints according to Hamburger and Hamilton, 1951. Staging could generally be determined by the development of the limb buds, but counting the somites was necessary for the youngest of the embryos. This is accomplished by injecting India Ink underneath the embryo to produce a background contrast suitable for determining the number of somites present.

Fixation/Tissue Sectioning

Whole embryos were fixed in 4% paraformaldehyde/ Phosphate-Buffered Saline (PBS) from 8 hours to overnight (depending on the age). This fixing procedure crosslinks the proteins within the tissue and therefore keeps the embryo in a "preserved" state for future examination. Embryos were then rinsed in PBS and cryoprotected in 30% sucrose/PBS overnight. This helps protect the tissue from temperature extremes that occur in the freezing process and future

cryosectioning. They were then placed in 1:1 OCT:PBS for several hours, followed by straight OCT (Miles, Elkhart, IN). The embryos were then oriented in a plastic block containing OCT and flash frozen in an ethanol/dry ice bath. Tissue used for BrdU immunocytochemistry was fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid). The Tissues were then cryosectioned at 10 um on a Frigocut 2800 (Reichert-Jung) and sections were placed on gelatin subbed slides. Slides were subbed in chromium/gelatin solution (300ml de-ionized (dI) H_20, 2.5g Knox gelatin, and 0.5g chromium potassium sulfate), to ensure minimal loss of sectioned material. For motorneuron studies the axial level of the embryos was determined by the presence of limbs and the size of the ventral horn. Generally, in the DRG studies, brachial sections were obtained for the sake of consistency.

Fluorescense Immunocytochemistry

Tissue was initially rehydrated in PBS for 20 minutes and then placed in blocking buffer (10% normal goat serum, 1% glycine, 3% BSA, 0.4% Triton X-100 in Tris-Buffered Solution (TBS)) for one hour. This buffer contains a detergent to expose antigenic sites within the cell membrane, as well as large globular proteins to block any non-specific binding of antibodies. Slides were initially treated with an Avidin/ Biotin blocking kit (Vector Laboratories, Burlingame, CA)

prior to incubation with primary antibodies overnight at 4 C (approximately 1ug/ml), until it was found that the Avidin/Biotin blocking step was unnecessary. Following incubation in primary antibodies, slides were rinsed in block 4 times at 10 minutes/rinse and then incubated in either biotinylated or fluorescent conjugated secondary antibodies for 1 hour. Sections exposed to biotinylated secondary antibodies were then rinsed 4 times at 10 minutes/rinse in block and incubated in fluorescent conjugated avidin for 1 hour. This was followed by 4 more 10 minute rinses in block and finally slides were cover slipped utilizing a Prolong Anti-Fade Kit (Molecular Probes) to minimize loss of fluorophore signal over time.

Antibodies

Rabbit polyclonal antibodies to the trkA, B, and C receptors were produced as described in Lefcort, et al., 1996. These were used at approximately 1ug/ml. To amplify the signals of these antibodies biotinylated goat-anti rabbit H&L IgG (Vector Laboratories, Burlingame, CA) was used as second antibody at 1:450. For the polyclonal fluorescence signal, Fluorescein Avidin DCS/Cell Sorting Grade or Streptavidin-Cy3 (Vector Laboratories, Burlingame, CA) was used at 1:1000.

For double labelling experiments appropriate mouse monoclonal antibodies were used in association with the trk receptor antibodies. Supernatant containing BEN, an antibody which specifically recognizes an adhesion molecule expressed by post-mitotic neurons, was used at 1:50 (Developmental Hybridoma Bank, U. of Iowa). HNK-1 supernatant, which recognizes a carbohydrate moiety associated with many cell adhesion molecules, was used at 1:10 (Developmental Hybridoma Bank, U. of Iowa). NFM supernatant, which recognizes the phosphorylated and non-phosphorylated form of the 160 kD neurofilament protein, was used at 1:100 (Virginia Lee, University of Pennsylvania). Tuji, recognizing a neuronal specific ß-tubulin isoform, ascites fluid was used at 1:5000 (A. Frankfurter, U. of Virginia). A monoclonal antibody supernatant against Hu, a vertebrate neuron specific family of RNA binding proteins, was used at 1:100 (J. Weston, University of Oregon). Also, an anti-BrdU antibody (Novocastra Laboratories) was used at 1:500. Fluorescent conjugated secondary antibodies were used to visualize these antigens. Finally, the nuclear stain, DAPI, was applied to visualize individual nuclei.

BrdU Injections

Embryos were injected, in ovo, with 10ul of a 6.6mg/ml solution of Bromodeoxyuridine (BrdU) in sterile Tyrodes

buffer (composed of CaCl, $MgCl_2$, KCl, NaCl, $NaPO_4$, and D-glucose), on the chorioallantoic membrane. This was generally accomplished by producing a small tear in the allantoic membrane, followed by application of the BrdU with a micropipette. BrdU is a thymidine analogue incorporated into the DNA of cells during DNA replication (S phase) and can be visualized using monoclonal antibodies to it (Gratzner, 1982).

BrdU Immunocytochemistry

BrdU staining was accomplished using the above mentioned immunocytochemical procedures, but the tissue was initially pretreated with a denaturing agent to expose the recently incorporated BrdU. This was accomplished by exposing the tissue to either 2N HCl for 30 minutes (Yoshida et al., 1987), or to 0.07M NaOH for 2 minutes (Waid et al., 1995). Both treatments are followed by numerous rinses in PBS. We found that the HCl pretreatment worked well with the BrdU stain, but somehow destroyed the antigenic epitope of the trk receptors and therefore inhibited the binding of our trk antibodies. On the other hand, the NaOH did not disrupt the binding sites of our antibodies, but destroyed the internal portion of the tissue being stained. This destruction almost always resulted in the loss of the tissue in the area of DRG

formation and was therefore unsatisfactory. Postfixing the tissue in 1% paraformaldehyde for 2 minutes prior to the denaturing treatments (personal communication with David Waid, U. of Minnesota), only partially overcame this problem.

Results

Trk Expression on Developing Spinal Motor Neurons

It was of interest to examine the expression patterns of the trk receptors within the ventral horn of the spinal cord, where motorneurons will develop and reside, throughout development of the avian embryo. Thus both the stage of development and the axial level of the embryo were experimental variables. We looked at time points in early development prior to the onset of target-mediated cell death while members of Ronald Oppenheim's laboratory (Bowman-Gray Medical School, Wake Forest University) examined the later stages. We chose three stages: E2.75, E4.5, and E6.

Motorneuron populations were identified using a monoclonal antibody (BEN) to a cellular adhesion glycoprotein present on motorneurons (Pourquie et al., 1990). By double labelling with different fluorescent secondary antibodies to the trk and BEN primary antibodies we could determine if the trk receptors were indeed present on the motorneuron population.

At E 2.75 (Stage 18) there was no trk expression within the ventral horn of the spinal cord at any axial level. By E4.5 (Stage 25) faint trkB staining was observed within all axial levels of the ventral horn, but this could not be

clearly distinguished from background. At E6 the pattern of trk expression became more interesting with trkB present only at the lumbar level, and only over the medial column of the ventral horn (Figure 2c). There was a distinct lack of staining over the lateral column of the ventral horn. TrkC appeared to be present on the motorneuron population throughout all axial levels at E6 (Figure 2a), yet showed the same selective pattern of expression at the lumbar level where it is distinctively not expressed in the lateral column of the ventral spinal cord. (Figure 2b). The lack of either trkB or trkC expression in the lateral column of the ventral horn was more apparent in horseradish-peroxidase staining experiments performed by Frances Lefcort.

Sensory Neurogenesis

Stage 14

The earliest timepoint examined in these experiments was Stage 14, corresponding to day 2.25 of development (E2.25). Here we noticed no trk receptor expression, but did observe the widespread expression of the p75 receptor (Figure 3a). Of our panel of monoclonal antibodies, only HNK-1 showed expression in the region of the migrating neural crest (Figure 3b). p75 expression was present on the majority of the tissue, including the HNK-1 positive cells and therefore within the migrating neural crest.

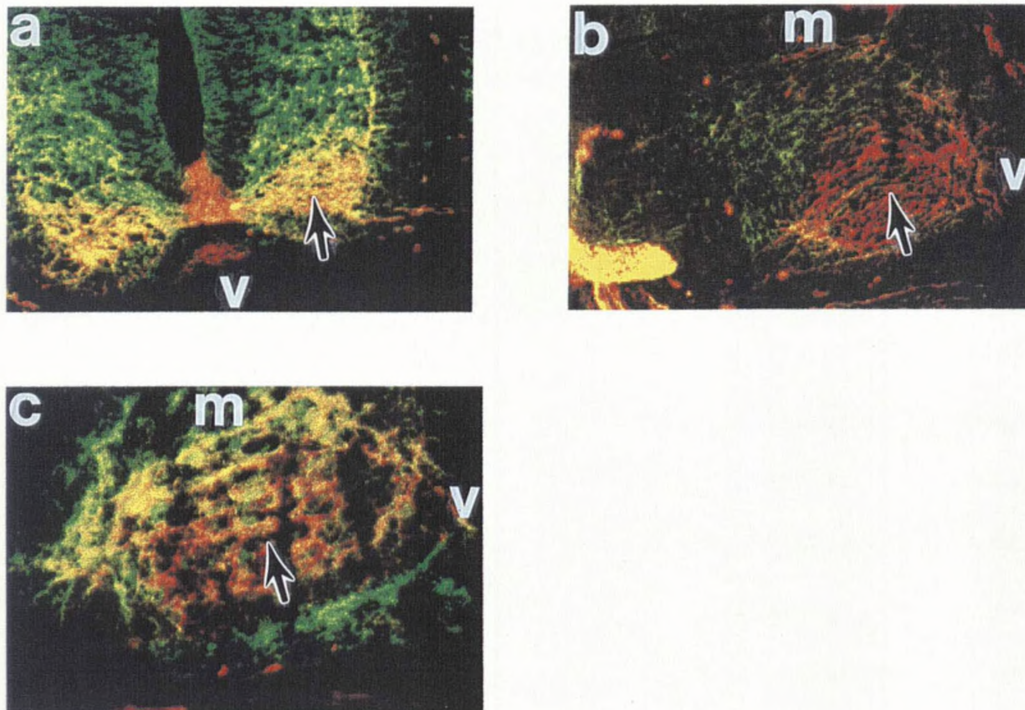

Figure 2 TrkC expression patterns in the ventral horn of the E6 embryo. (a) TrkC (green) and BEN (red) coexpress in the cervical ventral horn producing a yellow color during double exposure photography (arrow). (b) Absence of trkC and trkB (c) staining observed over the lateral motor column of the lumbar ventral horn (arrow). (v = ventral, m = medial, l= lateral column of ventral horn).

Figure 3 Early expression of p75 within the stream of migrating neural crest cells, stage 14. (a) Migrating streams of neural crest identified with HNK-1. (b) P75 positive cells seen within the neural crest, as well as the surrounding tissue. (v = ventral, s = spinal cord, so = somite).

Stage 15/16

At approximately E2.5/Stage 15-16 days we noticed the first evidence of trk expression within the migrating neural crest. TrkC is expressed on only a very small population of cells, and is coexpressed with Hu (Figure 4a,b) BEN (Figure 4c,d) and NF (Figure 4e,f). NF, the antibody we used, labels the 160kD neurofilament protein which is believed to be present in differentiated as well as proliferating neuronal progenitor cells (Bennett et al., 1984; Bennett et al., 1985). BEN is a transiently expressed cell adhesion molecule present on the cell surface of several different groups of peripherally projecting neurons (Pourquie et al., 1990), and is believed to be present on post-mitotic neuronal cells. Hu is a family of vertebrate RNA binding proteins that are found in mitotically active cells within the developing DRG (Marusich et al., 1993). Therefore the trkC receptor coexpresses with antigens recognizing both mitotically active neuronal progenitor cells and post-mitotic neurons.

We also noticed that there was no Tuji staining in the periphery and trkC positive cells were Tuji negative (Fig. 4g,h). There was no trk A or trk B expression present at this age.