Dynamic Expression of Neurotrophin Receptors during Sensory Neuron Genesis and Differentiation

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To identify potential functions for neurotrophins during sensory neuron genesis and differentiation, we determined the temporal and spatial protein expression patterns of neurotrophin receptors throughout the process of sensory neurogenesis in the dorsal root ganglia (DRG). We show that neurotrophin receptors are expressed early, being first detected on subsets of migrating neural crest cells, and that trkC is among the earliest markers of neural lineage specification. In the immature DRG, we find that both trkC and p75[NTR] are expressed on subsets of dividing progenitor cells *in vivo.* Furthermore, our data directly reveal distinct patterns of trk receptor expression by individual sensory neurons from the time of their inception with all early arising cells initially being trkC[+], some subsets of whom also coexpress either trkA or trkB or both. As sensory neurons innervate their targets and establish their mature identities, the spectrum of trk receptors expressed by individual neurons is altered. The stereotyped trk receptor expression profiles identified here may potentially correspond to distinct lineages of sensory neurons. These data, in conjunction with other studies, argue for multiple functions for neurotrophins during the process of sensory neuron differentiation, including effects on both neural crest and DRG mitotically active progenitor cells, in addition to possibly influencing the establishment of sensory neuron identity. © 2000 Academic Press

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INTRODUCTION

The sensory neurons of the dorsal root ganglia (DRG) are an extremely heterogeneous cell population. They are distinguished by several characteristics, including sensory modality, neurotransmitter content, site of central and peripheral target innervation, morphology, and growth factor dependencies (Scott, 1992). An outstanding question then, is how each distinctive identity is established. DRG neurons are the descendants of neural crest cells (Horstadius, 1950; Weston, 1963; LeDourain, 1986; Bronner-Fraser and Fraser, 1988; and see Sharma *et al.,* 1995) and once postmitotic, a majority will undergo target-regulated programmed cell death (Hamburger and Levi-Montalcini, 1949; Pannesi, 1974; Carr and Simpson, 1978). A thorough analysis of the intervening events between these two milestones is critical if we are to identify the mechanisms regulating sensory neuron identity. Recent work has demonstrated that in the absence of two bHLH genes, *ngn1* and *ngn2,* DRG fail to form, and furthermore each gene regulates a distinct wave of neurogenesis in the DRG, consistent with clonal analyses and morphological birth-dating studies (Carr and Simpson, 1978; Frank and Sanes, 1991; Ma *et al.,* 1999).

During the period of target-regulated cell death, a hallmark feature of DRG neurons is their differential response to members of the neurotrophin family of growth factors (Lindsay, 1996). Pain and temperature afferents depend on nerve growth factor (NGF) yet proprioceptors require neurotrophin-3 (NT-3) for survival (summarized in Farinas and Reichardt, 1996, and Snider and Silos Santiago, 1996; Oakley *et al.,* 1995, 1997). The receptors which mediate these activities include members of the trk family of receptor tyrosine kinases and the structurally distinct p75[NTR] receptor (Bothwell, 1995; Dechant and Barde, 1997). Intriguingly, the onset of neuronal expression of neurotrophin receptors can precede the period of target innervation

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by several days (Mu *et al.,* 1993; Lefcort *et al.,* 1996) and studies conducted over the past few years have implicated activities for neurotrophins in key early events in sensory neurogenesis which occur *prior* to target innervation, including the proliferation and differentiation of neural crest cells and of precursor cells in the DRG (Kalcheim *et al.,* 1992; Pinco *et al.,* 1993; Kahane and Kalcheim, 1994; Davies, 1994; Henion *et al.,* 1995; Farinas *et al.,* 1996; Elshamy and Ernfors, 1996; Sieber-Blum and Zhang, 1999). For example, blockade of trkC or its ligand NT-3 prior to the onset of target-regulated cell death results in a loss of ca. 25% of the cells in the nascent DRG (Gaese *et al.,* 1994; Lefcort *et al.,* 1996). To interpret these data, a thorough identification of the cell types expressing neurotrophin receptors during each of these early critical events is required—from neural crest migration through the genesis and differentiation of nascent DRG neurons—and comprises one of the major aims of this study.

The second aim of our study was to determine how the discrete patterns of trk receptor expression observed in the mature DRG develop. That is, do all nascent DRG neurons express solely one particular trk receptor from their inception or rather is the profile of trk receptors expressed by an individual sensory neuron altered as the cell matures. Such information is required in order to dissect the relative roles of environmental signals vs intrinsic determinants of sensory neuron identity.

Our results reveal (1) discrete, stereotyped receptor patterns on identifiable cell types, including expression on a subset of neural crest cells and mitotically active DRG progenitor cells, and (2) dynamic trk receptor expression profiles in that the spectrum of trk receptors expressed by an individual neuron can switch within hours of neurogenesis, coincident with axonal outgrowth. Furthermore, our data are consistent with the existence of distinct lineages of sensory neurons which can be distinguished by their repertoire of expressed trk receptors. These data provide a conceptual framework with which to interpret the multitude of activities attributed to neurotrophins by identifying the intermediate cell types and developmental events which could be influenced by neurotrophins during the genesis and differentiation of sensory neurons.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chicken embryos were obtained from Truslow Farms (Chestertown, MD) and placed in a rocking incubator at 37°C (Kuhl, Flemington, NJ). Eggs were windowed and embryos staged according to Hamburger and Hamilton (1951).

Immunocytochemistry

Embryos were fixed in 4% paraformaldehyde/PBS from 3 h to overnight (depending on the age) at 4°C. Embryos were rinsed in PBS, cryoprotected in 30% sucrose/PBS overnight, embedded in OCT (Miles, Elkhart, IN), and cryosectioned at 7–10 μm. Slides were rehydrated in PBS for 20 min and placed in blocking buffer (10% normal goat serum, 1% glycine, 3% BSA, 0.4% Triton X-100 in 30 mM Tris, 150 mM NaCl) for 1 h followed by overnight incubation in 1° antibody at 4°C. Slides were rinsed in blocking buffer and incubated in either biotinylated secondary antibodies followed by a 1-h incubation in fluorescein DCS avidin (Vector Laboratories, Burlingame, CA) or fluorophore-conjugated secondary antibodies for 1 h (The Jackson Laboratory, Bar Harbor, ME). Slides were mounted in Prolong Antifade (Molecular Probes, Eugene, OR) and examined on a Nikon FXA microscope. To visualize chromatin, sections were incubated for 15 min in DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; 0.3 μg/ml; Molecular Probes). Mitotic nuclei could be readily identified using DAPI labeling on the basis of chromatin morphology, particularly during metaphase through telophase stages. Two additional approaches used to identify dividing cells included (1) labeling with the anti-phosphohistone H3 antibody (Upstate Biotechnology, NY; gift from Drs. Olivia Bermingham-McDonogh and Edwin Rubel, University of Washington). The H3 histone is phosphorylated only during the G2–M phase of the cell cycle (Hendzel *et al.,* 1997). (2) Eggs were injected with BrdU (50 μg/egg) onto the chorioallantoic membrane, 1–2 h before removal and fixation of embryos, and then sectioned and immunolabeled with an anti-BrdU monoclonal antibody (Novocastra; Vector Laboratories). To determine whether BrdU$^+$ or phosphorylated H3$^+$ dividing cells expressed neurotrophin receptors, sections were double labeled with one of the anti-trk receptor antibodies or the anti-p75 antibody, CHEX.

Primary antibodies included rabbit polyclonal antibodies to trkA, B, and C receptors (produced as described in Lefcort *et al.,* 1996) and the monoclonal antibodies listed below.

Antibody	Cells labeled
HNK-1	Neural crest
NF-M	Neurons, some progenitors (Bennett *et al.,* 1987; Bennett, 1987)
Tuj-1	Neurons, some progenitors (Memberg and Hall, 1995)
Ben	Neurons
Hu	Neurons, progenitors

Ben and HNK-1 were obtained from the Developmental Hybridoma Bank (University of Iowa); the antibody to NF-M which recognizes both the phosphorylated and the nonphosphorylated 160- to 180-kDa neurofilament subunits was obtained from Drs. V. Lee (University of Pennsylvania) and D. Lurie (University of Montana); Tuj-1 was obtained from Dr. A. Frankfurter (University of Virginia); a monoclonal antibody supernatant against Hu was obtained from Drs. P. Henion and J. Weston (University of Oregon) (Marusich *et al.,* 1993); CHEX, a polyclonal antibody to p75$^{\text{NTR}}$, was obtained from Dr. L. F. Reichardt (UCSF) (Weskamp and Reichardt, 1991). To corroborate the immunolabeling patterns obtained with the CHEX antibody, we stained several sections with two monoclonal antibodies to chick p75$^{\text{NTR}}$, Nos. 7902 and 7412 (gift from Dr. M. Bothwell, University of Washington). For Tables 1 and 3, all quantification of trk subpopulations was conducted on sections from three embryos for each time point. Embryos were sectioned transversely through the trunk (8–10 μm) and each slide contained sections from throughout the trunk axis. Thus adjacent sections on any individual slide were from axial levels at least 100–150 μm apart. Cell counts were made from sections that were double labeled with two primary antibodies and fluorescently tagged

TABLE 1

Characterization of TrkC$^+$ and TrkA$^+$ Neural Crest Cells (E2.75/St. 18)

mAb	% TrkC$^+$	% TrkA$^+$
NF	97	23
Tuj-1	97 $\pm$ 1	27 $\pm$ 7
Ben	98 $\pm$ 2	21 $\pm$ 1
Hu	86 $\pm$ 2	23 $\pm$ 5

Note. Sections from three embryos (St. 18/St. 19) were double labeled with antibodies to either trkC or trkA and to NF, β3 tubulin, Ben, or Hu, and of the cells that expressed one of these neural markers, the percentage that also coexpressed trkC or trkA was determined. In each embryo, ca. 40–70 cells were counted and the numbers represent the mean % of double-labeled cells + SEM, except for neurofilament staining in which just one embryo was labeled.

secondary antibodies. All trk$^+$ cells with an entire cross section and nucleus contained within the section were counted. Given the considerable distance between sections, there was no chance of double counting individual cells. All cells counted were either neurons or neural progenitors since none of the trk antibodies used labels glial cells. For Fig. 1 and Table 1, confirmation that the cells were among the migrating neural crest was achieved by staining adjacent sections with the HNK-1 antibody. Table 3 is to be understood as follows: of the trkX-positive cells, the percentage that coexpressed trkY is listed separately from the percentage that coexpressed trkZ; this categorization is repeated for each of the trk subpopulations. On a given section, only two of the three trk subpopulations could be analyzed so each pairing is presented separately. To determine absolute numbers of trk$^+$ cells within the DRG at embryonic day 4.5 (E4.5), embryos were serially sectioned (7 μm) and stained with one of the three trk primary antibodies followed by secondary antibody linked to HRP and counterstained with Nissl. The number of trk$^+$ cells in every fourth section was determined in the wing innervating DRG ganglion 14 and the percentage of trk$^+$ cells per ganglion determined in one or two embryos as described in Lefcort *et al.* (1996).

Immunolabeling of Two Trk Receptor Family Members in the Same Section

All three anti-trk receptor antibodies were made in rabbit. To be able to detect two different trk family members on the same section, we exploited the considerable signal amplification provided by the TSA indirect procedure (NEN, Boston, MA) system and devised a double labeling protocol based on Michael *et al.* (1997); all reagents were used at the kit-recommended concentrations and buffers. Briefly, the first primary antibody was added at 0.1 μg/ml overnight, followed by a 1-h incubation in biotinylated secondary antibody, 30-min incubation in streptavidin–HRP, 10-min incubation in biotinyl tyramide, 30-min incubation in fluorescein DCS avidin (1:600; Vector). Sections were then incubated with the second primary antibody overnight at 4°C, followed by standard amplification with a Cy3-tagged secondary antibody (1:2000; The Jackson Laboratory). This method avoids cross-reactivity of the second secondary antibody with the first primary antibody
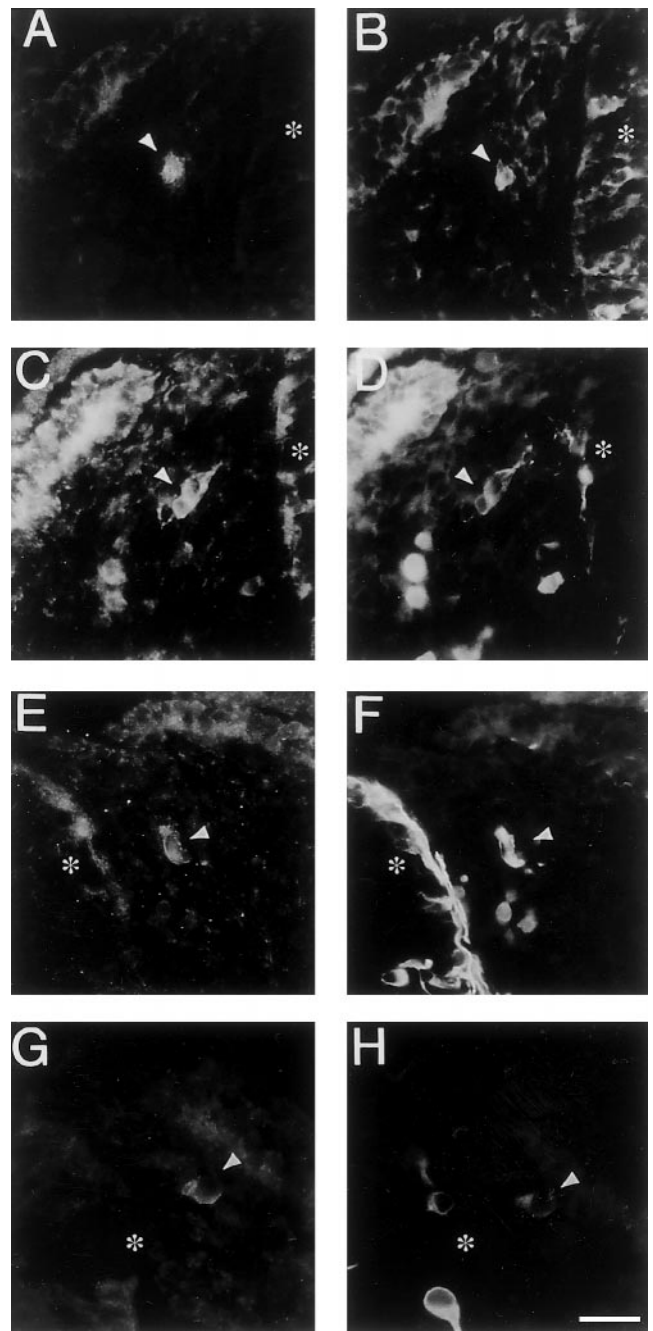
FIG. 1. TrkC is expressed by a discrete subset of neurogenic neural crest cells. Transverse sections through E2.25/St. 15 embryos immunolabeled with the anti-trkC IgG (CTC IgG; A, C, E, G) and with the Hu (B), Ben (D), NF 160- to 180-kDa (F), or Tuj-1 (H) IgGs. Arrowheads point to a neural crest cell which coexpresses trkC with one of the other identified markers. The indicated cell(s) is the only trkC$^+$ cell among a stream of migrating neural crest cells in these sections. Asterisk (*) marks the neural tube. Bar, 25 μm.
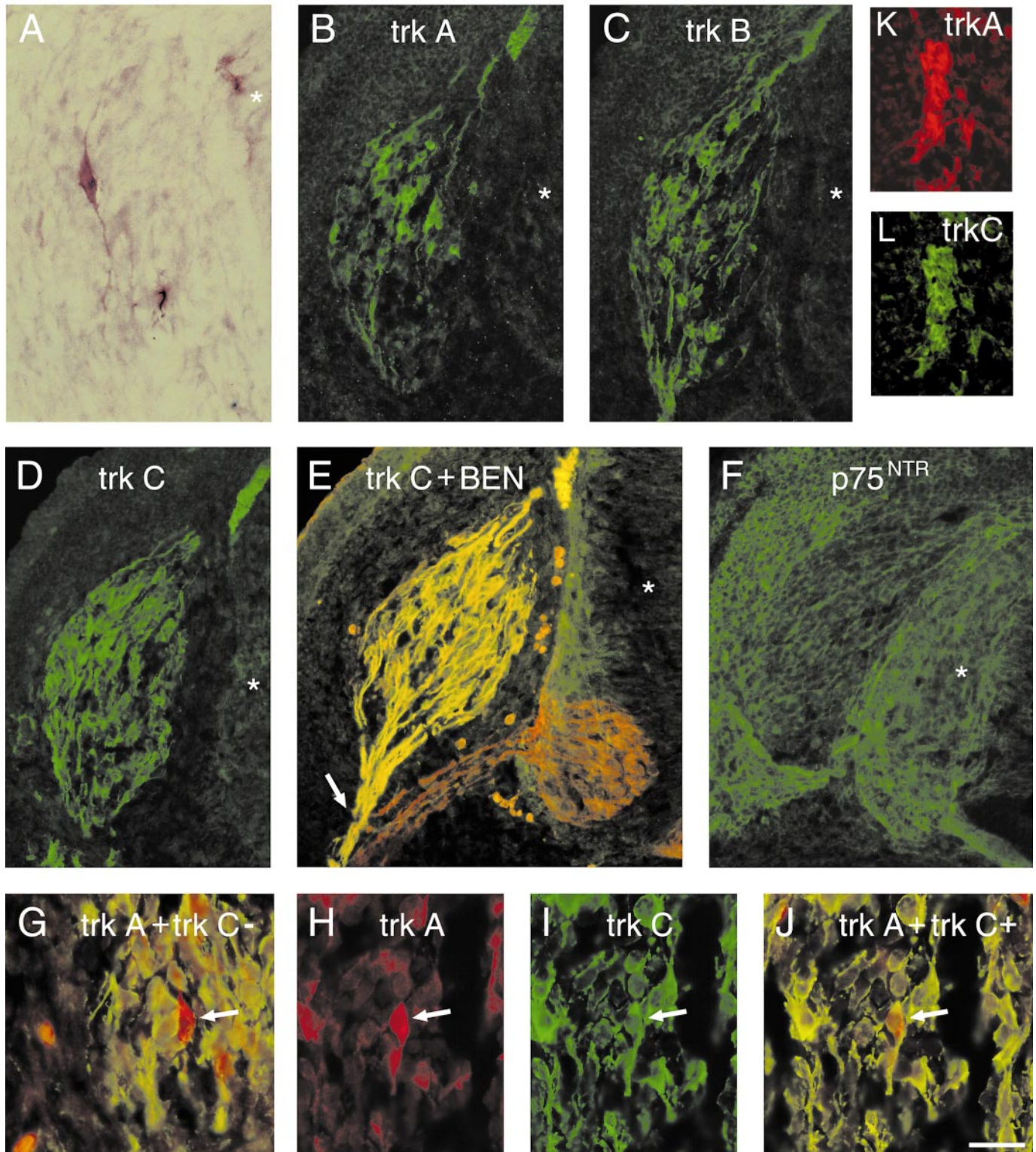
FIG. 2. Neurotrophin receptor expression in the nascent, immature DRG. Transverse sections through embryos were stained with anti-neurotrophin receptor antibodies. (A) E3/HH St. 19 embryonic section labeled with the anti-trkA IgG (CTA IgG) reveals a single trkA+ cell situated lateral to the neural tube. Within 1.5 days, the nascent DRG has formed and trkA expression is observed on ca. 20% of the cells in the DRG (B). At this same age, E4–4.5/HH St. 24–25, ca. 47% of the cells express trkB (C) while about 63% of the cells express trkC

because the latter is used at a very low concentration and is relatively inaccessible to the second secondary antibody. In each experiment, a lack of nonspecific cross-reactivity was ensured by the inclusion of sections from E8 embryos in which there is a clear spatial segregation in the trkA-, trkB-, and trkC-positive cell populations in the DRG (see Fig. 5) and by determining whether any aberrant staining patterns were observed; for example, of the three receptors, only trkC is expressed on a subset of spinal cord interneurons, trkA and trkC are expressed on sympathetic neurons at E3.5–E4.5, and trkB is the only trk receptor expressed on cells surrounding the perimeter of the neural tube at E3.5–E4.5.

RT-PCR

mRNA from E4.5 DRG was isolated using MicroPoly(A)Pure (Ambion, Inc) and treated with RNase-free DNase I (Promega) for 30 min at 37°C. The pelleted and resuspended mRNA was then subjected to RT-PCR using the one step Access RT-PCR system (Promega) with specific primers for the kinase-containing trkC isoform (forward TTCGAGTACATGAAGCATGGGG, reverse GCCTTGGGTAATGCAACTCAATG) or the truncated, kinase-deleted isoform of trkC (forward ATGTTGAATCCAATAAGC-CTCCC, reverse CATCAGAACAGGGACTTTTAAGG). Primer controls lacking mRNA were also included (data not shown). In addition to being DNase treated, controls were included in which the reverse transcriptase was omitted from the PCR reaction (lanes 2 and 6, Fig. 3).

RESULTS

Several major events comprise the generation and differentiation of sensory neurons in the DRG: neural crest cells migrate, proliferate, and coalesce lateral to the neural tube to begin forming the DRG; progenitor cells within the nascent DRG then proliferate to generate the neurons and glia of the DRG; and following their birth, postmitotic neurons establish their mature identities with the projection of axons to stereotyped targets in the CNS and periphery (Scott, 1992). To elucidate the function(s) of neurotrophins during sensory neurogenesis and differentiation, we investigated the repertoire of neurotrophin receptors expressed on defined cells during each of the following events: (1) migration of neural crest cells (E2–3/HH St. 14–19), (2) the peak of neurogenesis within the developing DRG (E4–4.5/HH St. 24–25), and (3) E6–E13, the period of target-mediated cell death. Part of this work has appeared in abstract form (Rifkin *et al.,* 1996).

Neurotrophin Receptor Expression during Early Sensory Neurogenesis

Neural crest migration. Along the chick trunk axis, the neural crest emanates from the neural tube beginning about E2/HH St. 12 with the cessation of neural crest migration by about E3/HH St. 20 (Tosney, 1978; Loring and Erickson, 1987; Lalliel and Bronner-Fraser, 1988; see also Sharma *et al.,* 1995). Given the rostral–caudal developmental gradient, to determine the onset of trk receptor expression we focused on one axial level, the brachial region, where neural crest migration begins ca. E2/HH St. 13 (Tosney, 1978). At E2.25 no immunoreactivity for any of the trk receptors was detected, although p75^{NTR} was broadly and strongly expressed on the majority of migrating neural crest as demonstrated by coexpression with the HNK-1 neural crest marker (data not shown; Nordlander *et al.,* 1993; Schachner *et al.,* 1995) in addition to expression on somites but not in the overlying embryonic ectoderm. This broad expression of p75^{NTR} protein at this age is consistent with the previously described *in situ* hybridization data for p75^{NTR} mRNA expression (Heuer *et al.,* 1990) and with descriptions of p75^{NTR} protein expression on neural crest cells *in vitro* (Bernd, 1985; Stemple and Anderson, 1992; Hapner *et al.,* 1998).

The first trk receptor to be expressed in the neural crest is trkC, which appears in the brachial region at approximately E2.5/HH St. 15–16 on a small subpopulation of migrating crest cells on the ventral lateral pathway in close proximity to the neural tube. At this age, neither trkA nor trkB is expressed on migrating neural crest cells (data not shown). At E2.75/St. 18, we determined that 6% (35/563) of migrating HNK-1$^+$ neural crest cells were trkC$^+$.

To determine the identity of the trkC$^+$/HNK-1$^+$ cells, we double labeled sections with anti-trkC IgG and several monoclonal antibodies which identify discrete cell types. These antibodies include (a) Hu, which recognizes a member of the Elav family of RNA binding proteins and has been previously observed on a subset of dividing sensory neural progenitors in addition to nascent DRG neurons (Marusich *et al.,* 1993). (b) Ben (also referred to as DMI/GRASP; SC1), which recognizes a transiently expressed cell adhesion molecule present on the cell surface of several different groups of peripherally projecting neurons (Pourquie *et al.,* 1990; Burns *et al.,* 1991; Tanaka *et al.,* 1991; Chedotal *et al.,* 1995) and is known to be expressed by postmitotic neurons.

(D). (E) Double labeling at E4–4.5 with the neuronal marker Ben (red) and the CTC IgG (green) reveals that the vast majority of the neurons express trkC (yellow). Arrow points to trkC$^+$ nascent projection axons. (F) At E4–4.5 all cells in the nascent DRG express p75^{NTR}. (G–J) Sections through E4–4.5 double labeled with the CTC and CTA IgGs reveals that while the majority of trkA$^+$ cells at this age coexpress trkC (H–J, same field, arrow points to trkA$^+$/trkC$^+$ yellow cell), 10% of the trkA$^+$ cells do not coexpress trkC (G, arrow points to trkA$^+$/trkC$^-$ cell which is red. TrkC-only cells are green, trkA-only cells are red, and cells expressing both trkA and trkC are yellow. Asterisk (*) marks the spinal cord (A–F). Bar: A, 25 μm; B–F, 50 μm; G–J, 80 μm.

(c) An antibody which recognizes the 160- to 180-kDa neurofilament subunit. The particular neurofilament antibody we used (see Materials and Methods) has been shown previously to recognize both differentiated neurons and a subset of proliferating neuronal progenitor cells (Bennett, 1987; Bennett *et al.,* 1987). (d) The Tuj-1 antibody which recognizes the β3 neural-specific isoform of tubulin has also been shown to be expressed by both differentiated peripheral neurons in addition to a subset of dividing sympathoblasts and sensory progenitors (Memberg and Hall, 1995). As shown in Table 1 (HH St. 18) and Fig. 1 (HH St. 15/16), the majority of the trkC$^+$ cells within the migrating neural crest were NF$^+$ (38/39), Tuj-1$^+$ (136/140), Ben$^+$ (115/117), and Hu$^+$ (122/142). Therefore the trkC receptor is coexpressed with markers of both mitotically active neuronal progenitor cells and postmitotic neurons, indicating that these trkC$^+$ cells represent either a neural progenitor subpopulation within the migrating neural crest cell and/or nascent postmitotic neurons which will contribute to the DRG (see Discussion and Henion *et al.,* 1995).

By E2.75/HH St. 18, a subset of cells among the migrating crest lateral to the neural tube begins to coexpress trkA (Fig. 2A). While all of the trkA$^+$ cells coexpressed NF, β3 tubulin, Ben, and Hu, Table 1 shows that this trkA$^+$ subpopulation comprises only about 20–25% of the total cells which express these marker proteins at this age. These data are in stark contrast to the trkC expression pattern observed at this age and would suggest that the trkA$^+$ subpopulation represents a distinct subset not only of the neuronal population but also most likely of the trkC$^+$ subpopulation, since close to 100% of neuronal markers were coexpressed in cells that were trkC$^+$ (except for Hu). Immunocytochemical background difficulties prohibited a direct analysis of coexpression of trkC and trkA at HH St. 18.

Formation of the DRG. By E3/HH St. 20, neural crest migration wanes in the brachial region (see also Sharma *et al.,* 1995), and an aggregation and coalescence of neural crest cells occurs lateral to the neural tube such that nascent DRG become evident (Teillet *et al.,* 1987; Lalliel and Bronner-Fraser, 1988). Between E3/HH St. 19–20 and E4.5/HH St. 24–25 a dramatic change in trk receptor expression occurs in the nascent DRG. While at E3 only an occasional trkA$^+$ cell is observed (Fig. 2A), within 1.5 days, ca. 23% of the nascent cells in the DRG are trkA$^+$ (Fig. 2B). Given their morphology and coexpression of neural markers Ben and NF (data not shown), these cells appear to be nascent, immature, spindle-shaped bipolar neurons. No trkA$^+$ dividing cells were observed, suggesting that trkA is not expressed by proliferating progenitors at this age. The temporal window for the change in trkA expression within the nascent DRG is quite narrow in that at E3.5–4/HH St. 23–24 only about 15% of the cells are trkA$^+$, while 12–24 h later, the percentage of trkA$^+$ cells has increased to about 30%. A similar rapid and dramatic increase in trkA expression in the nascent DRG is also observed in the mouse (Farinas *et al.,* 1998). At E3.5–4/HH St. 23–24, while only a
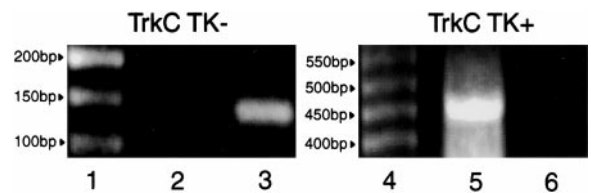
FIG. 3. Both kinase-positive and kinase-negative isoforms of trkC are expressed during the peak of neurogenesis. RT-PCR from DNase-treated mRNA isolated from E4.5 DRG and amplified with primers which would anneal to kinase-minus isoforms (TrkC TK$^-$; lanes 2 and 3) or to isoforms containing the kinase domain (TrkC TK$^+$; lanes 5 and 6). Lanes 1 and 4 contain molecular weight markers. In lanes 2 and 6, the reverse transcriptase was omitted from the PCR and no product obtained.

minority of DRG neurons are trkA$^+$, the sympathetic neurons in the primary chain next to the dorsal aorta are very intensely trkA immunopositive (Fig. 2K). Thus widespread, intense trkA expression in the sympathetic ganglia precedes that in the DRG. TrkB expression begins at approximately E3/HH St. 22 and by E4.5 about 47% of the nascent DRG cells are trkB$^+$ (Fig. 2C). At E4.5, of the three trk receptors, trkC is the most ubiquitously expressed (Fig. 2D; ca. 63% of all cells; Lefcort *et al.,* 1996) and is coexpressed with all of the markers analyzed, including NF$^+$, Hu$^+$, Tuj-1$^+$, and Ben$^+$, and therefore is present on the majority of the DRG neurons as illustrated in Fig. 2E. Previous work from Bernd and colleagues has shown that over 90% of the Hu$^+$ cells in the nascent DRG contain mRNA for trkC (Zhang *et al.,* 1994). In addition to being trkA$^+$, cells in the primary chain of sympathetic ganglia also strongly coexpress trkC (Fig. 2L) although the intensity of trkC signal decreases between E3.5 and E4.5 (data not shown). The remaining 30–35% of the cells in the DRG which do not express any of the trk receptors nor any neural markers are located in regions of the DRG known to contain primarily mitotically active progenitor cells (e.g., dorsomedial pole and along the DRG perimeter; F. Lefcort, unpublished observations). All cells, both neurons and progenitor cells, are p75^{NTR+} at E4.5 (Fig. 2F).

Multiple isoforms of trkC have been identified in the chick and mammal (Tsoulfas *et al.,* 1993; Valenzuela *et al.,* 1993; Garner and Large, 1994; Menn *et al.,* 1998). Since different trkC isoforms can mediate discrete activities (Garner and Large, 1994; Hapner *et al.,* 1998), to identify the range of potential functions for the strong and widely expressed trkC in the immature DRG, we determined which isoforms were expressed during the peak period of neurogenesis (E4.5) by RT-PCR (Fig. 3). Transcripts for both tyrosine kinase-positive (TK$^+$) and -negative (TK$^-$) isoforms were identified consistently in the immature DRG. The isoform which lacks the middle third of the tyrosine kinase domain (Garner and Large, 1994) and the kinase insert-containing isoform (Garner and Large, 1994) were observed often (data not shown). Since the TK$^+$ and TK$^-$ have distinct

TABLE 2

Neurotrophin Receptor Expression on Proliferating Cells in the DRG (E4.5)

Mitotically active	Neurotrophin receptor[+]	%
	TrkA[+]	
118	0	0
	TrkB[+]	
85	0	0
	TrkC[+]	
122	24	20
	(prophase-14)	
	(metaphase-5)	
	(anaphase/telophase-5)	
	p75[NTR+]	
97	96	99

Note. Sections from four embryos (E4.5) were labeled with antibodies to trkA, trkB, trkC, or p75[NTR] and incubated in the nuclear stain DAPI (see Materials and Methods). Dividing cells in the interior region of the DRG were identified based on chromatin morphology and scored with respect to whether they were immunopositive for one of the neurotrophin receptors.

and overlapping activities, expression of both isoforms would facilitate multiple responses to NT-3, including cellular survival, proliferation, and differentiation.

Neurotrophin Receptor Expression on Mitotically Active DRG Progenitor Cells

E4.5–E5 falls within the peak period of neurogenesis in the nascent DRG (Carr and Simpson, 1978). Thus, an important question to be addressed is whether mitotically active cells express neurotrophin receptors during DRG formation.

Sections from E4.5/HH St. 24–25 embryos were analyzed for the presence of mitotically active cells (Table 2). By E4.5, all mitotically active cells in all regions of the DRG were p75[NTR+] (Table 2). There are three "zones" of mitotic activity in the DRG at this age: the dorsal medial pole, a layer that is one to two cell diameters thick encompassing the perimeter of the entire DRG, and an intermingled subpopulation of dividing cells in the interior region of the DRG (Lefcort, unpublished observation). To look for trk receptor expression on dividing cells, we restricted our analysis to the interior region of the DRG, the only region of the three described in which trk receptors are expressed (see Fig. 2). We saw no dividing cells which were trkB[+] or trkA[+] (0/85 and 0/118, respectively) in this region. However, strikingly, 20% (24/122) of the dividing cells in the interior region of the DRG were trkC[+]. As shown in Fig. 4 and summarized in Table 2, trkC^{+} dividing cells were observed in all phases of mitosis as defined by chromatin

morphology, including prophase, metaphase (Fig. 4C), and telophase (Fig. 4D). Other methods used to identify dividing cells included immunolabeling BrdU-injected embryos (Fig. 4A; 2/10 BrdU[+]trkC^{+} cells observed) and labeling sections with an antibody to phosphorylated histone H3, which is phosphorylated only in the G2–M phase of the cell cycle (Fig. 4B; Hendzel *et al.,* 1997). These are the first reported direct observations of trk receptor expression on dividing cells *in vivo* and suggest that neurotrophins could potentially influence the survival, proliferation, or fate of dividing progenitor cells in the nascent DRG via activation of trkC receptors and/or p75[NTR]. Although not quantified at this age, many trkC^{+} dividing cells were also observed at E3.5/HH St. 22/23.

Death in the Nascent DRG

As previously described, neurogenesis in the DRG occurs between E4.5 and E7. Yet, apoptotic figures are observed as early as E4.5 in the nascent DRG (Hamburger and Levi Montalcini, 1949; Carr and Simpson, 1978). Carr and Simpson described apoptotic cells which had incorporated [3H]thymidine within 2 h of fixation. Thus birth and death happen simultaneously in the nascent DRG. In fact we saw several examples of dividing cells sitting adjacent to dying cells (data not shown). To determine the identity of the apoptotic cells in the nascent DRG, we ascertained the trk receptor expressed on apoptotic cells in sections through DRG of E4.5 embryos. We counted only cells in which we could unambiguously determine that the cell was in fact trk immunopositive and considered only apoptotic figures in the interior region of the ganglion, where trk^{+} cells are located. Forty percent of apoptotic cells were trkC[+] (8/20) while 21% (7/34) were trkB[+]. We found no trkA^{+} apoptotic cells at this age. These data are consistent with the time course of cell differentiation in the DRG—the larger diameter neurons are born and differentiate prior to the smaller diameter neurons (Hamburger and Levi Montalcini, 1949; Carr and Simpson, 1978).

Trk Receptor Patterns Are Altered as Neurons Differentiate

After E4.5, neuronal differentiation ensues with axons projecting toward their cutaneous, muscle, and visceral targets followed shortly by target innervation (Scott, 1992; Honig *et al.,* 1998). It is from this time point onward (>E4.5) that the expression pattern of trk receptors within the ganglion is so dramatically altered. By E6 the trk-positive subpopulations begin to segregate into the spatial patterns that will persist in the mature animal. This spatial segregation becomes so distinct that by E7.5 the majority of DRG neurons express only one trk family member (Fig. 5). TrkA is expressed on the dorsal medial 2/3 of the DRG (Fig. 5), while trkB and trkC are expressed primarily by the large-diameter neurons which are restricted to the ventral lateral regions of the DRG (Fig. 5). A similar expression
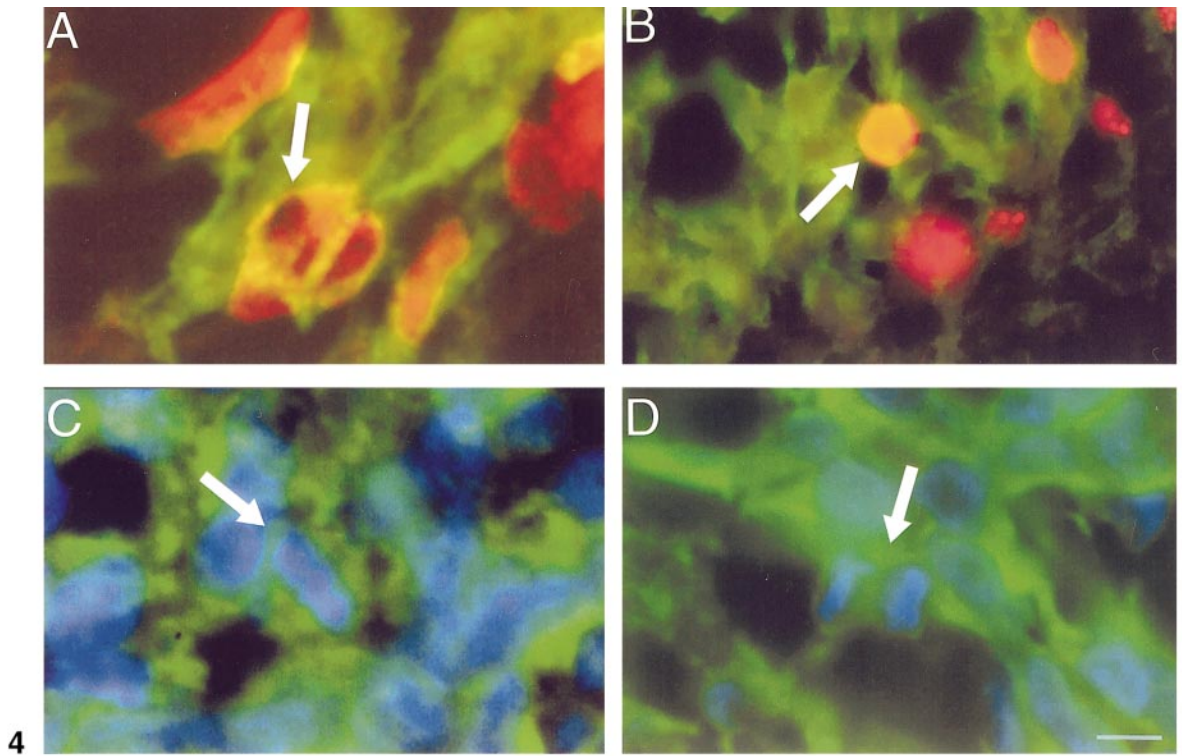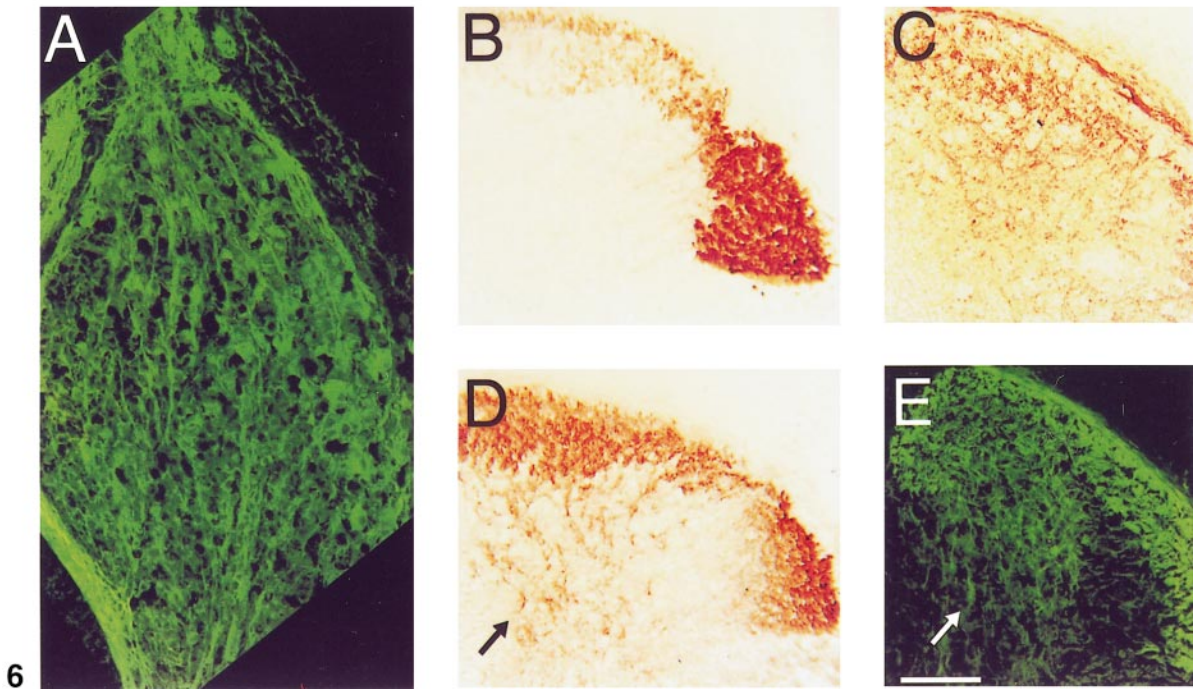
FIG. 4. A subpopulation of mitotically active DRG progenitor cells express trkC. Transverse sections through embryos at E4–4.5 double labeled with the CTC IgG (green) and (A, red) antibody to BrdU, (B, red) antibody to phosphorylated histone H3, or (C and D) the chromatin stain DAPI. Orange cells (marked by arrow) in A and B indicate BrdU$^+$/trkC$^+$ cell (A) or a trkC$^+$ cell in the G2–M phase of the cell cycle (B). In A, three BrdU$^+$ cells are observed together in a cluster and arrow points to one of them, which is also trkC$^+$. (C) Arrow indicates a trkC$^+$ cell in metaphase; (D) arrow indicates a trkC$^+$ cell in anaphase. Bar: B, 20 μm; A, C, and D, 30 μm.

pattern is also evident at later stages of development (E10; Oakley *et al.,* 1997). While this spatial segregation pattern is not observed in the mouse or rat, it is conserved in the human DRG (Shelton *et al.,* 1995). The trkB and trkC subpopulations in the ventral lateral region were entirely distinct and nonoverlapping (Figs. 5D and 5E). When double labeling with the trkA antibody, we never saw large-diameter neurons in the ventral lateral regions which expressed trkA (Figs. 5A–5C and 5E). However, we did find subpopulations of cells in the dorsal medial pole which were either trkA$^+$/trkC$^+$ or trkA$^+$/trkB$^+$ (Figs. 5A, 5B, and 5F). These data combined would suggest that at least a subset of these small-diameter neurons in the dorsal medial pole of the DRG could coexpress all three trk family members. Retrograde studies have revealed a population of trkC$^+$ cutaneous afferents (Oakley *et al.,* 1997). Although the trkB$^+$ dorsomedially located cells persist at E10, the trkC$^+$ dorsomedially located cells do not (Oakley *et al.,* 1997). As at the younger ages examined, p75^{NTR} is expressed by apparently all of the neurons in the DRG at E7.5 (Fig. 6A).

By determining trk receptor expression profiles on the central projections of DRG neurons, the lamination pattern of functionally discrete subclasses of sensory neuron can be identified in the spinal cord (Figs. 6B–6E). At E9, trkA central afferents terminate primarily in Rexed Laminae II and possibly in I and III; trkB$^+$ afferents terminate primarily in II and III and possibly I; trkC$^+$ afferents terminate in II, but mainly in III in addition to the trkC$^+$ projections to motor neurons in the ventral spinal cord (Oakley *et al.,* 1997; Dr. Joel C. Glover, personal communication; Eide and Glover, 1997). p75^{NTR} continues to be expressed on all central afferents at this age (Fig. 6E).

### Dynamic Switching of Trk Receptor Expression in the Nascent DRG

How do these mature patterns of trk receptor expression arise? Is this pattern established at the time of neuronal inception, or does the trk receptor repertoire expressed by an individual neuron change as a cell differentiates, contacts central and peripheral targets, and establishes its mature identity? *In vitro* studies have provided evidence that trigeminal neurons switch their class of trk receptor as they mature (Pinon *et al.,* 1996; Davies, 1997). To directly determine whether DRG sensory neurons coexpress or change expression of the different trk family members during their ontogenesis, we developed a protocol to double label sections with two different anti-trk receptor antibod-

ies (see Materials and Methods). We followed trk receptor expression patterns at three ages, (1) E3.5/HH St. 22–23, just after the coalescence of neural crest cells and the beginning of neuronal differentiation; (2) E4.5/HH St. 25, 1 day later, which is well into the period of neurogenesis; and (3) E8, during the period of target-mediated programmed cell death. We found that initially, while the majority of immature neurons express trkC at both E3.5 and E4.5 (Fig. 2), a subset of those cells at both ages also coexpresses either trkA or trkB or both trkA and trkB (Table 3). Interestingly, all trkA$^+$ or trkB$^+$ neurons coexpress trkC at E3.5 (Table 3; Figs. 2H–2J). In fact, in addition to coexpressing trkC, the majority of trkA$^+$ cells also coexpress trkB ($64 \pm 6\%$) and the majority of the trkB$^+$ cells also coexpress trkA ($80 \pm 5\%$). However, that pattern is altered within 24 h for by E4.5; not only are trkA$^+$/trkC$^-$ (Fig. 2G) and trkB$^+$/trkC$^-$ cells now present but also only 7% of the trkB$^+$ cells coexpress trkA and only 36% of the trkA$^+$ cells coexpress trkB. These data could be explained by (1) two distinct temporal waves of genesis of trkA$^+$ cells and trkB$^+$ cells, with the early (E3.5) ones coexpressing two or three trk receptors followed by subpopulations born later ($\geq$E4.5) that express solely either trkA or trkB, and/or (2) a subset of the trkA$^+$ and trkB$^+$ subpopulations which originally did coexpress trkC having within 1 day ceased expressing trkC and one of the two other trk receptors. Evidence pointing to (1) includes the observation that the absolute number of trkB$^+$ cells in the ganglion increases dramatically between those two time points (data not shown but compare the number of trkB$^+$ cells counted at E3.5 and E4.5). It is within the following 24 h (by E6) that the majority of cells in the DRG are trkA$^+$ and the relative percentages of trkB$^+$ and trkC$^+$ cells decrease (see Fig. 5), suggesting that the birth of each trk subpopulation is staggered with the trkC$^+$ subpopulation born first, some of whom coexpress one or both of the other trk receptors, followed by the birth of a large population of trkB$^+$ cells (most of whom initially also coexpress trkC), with the bulk of the ultimate trkA$^+$ subpopulation born last.

DISCUSSION

The goal of this study was to identify the events comprising early sensory neurogenesis and differentiation which could be modulated by neurotrophins. We show that based on receptor protein expression profiles, neurotrophins could potentially influence neural crest differentiation; the survival, proliferation, and/or differentiation of DRG mitoti-

FIG. 6. Neurotrophin receptor expression in mature DRG and on afferent projections to the spinal cord. (A) p75^{NTR} is expressed on essentially all the neurons in the DRG. (B) TrkA$^+$ afferents terminate predominantly in Rexed Laminae II and possibly somewhat in I and III in the E9 spinal cord. (C) TrkB$^+$ afferents terminate primarily in II and III and possibly in I. (D) TrkC$^+$ afferents terminate predominantly in laminae II and III in addition to more ventral laminae in the cord (arrow). (E) p75^{NTR+} afferents terminate in all of the above-mentioned laminae. Bar: A, 55 μm; B–E, 45 μm.
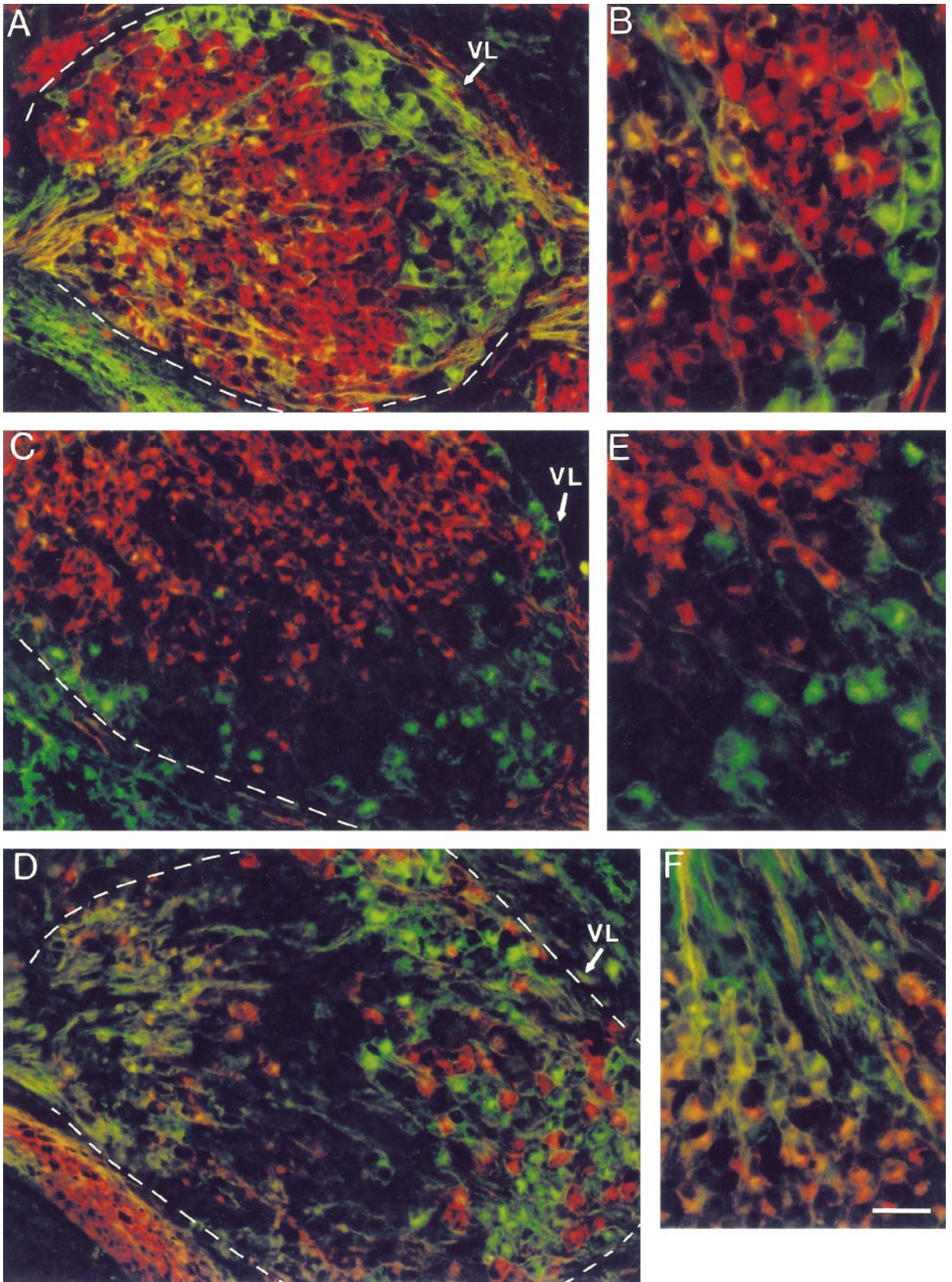
TABLE 3

Coexpression Pattern of Trk Receptors during Sensory Neurogenesis

	E3.5 (St. 22/23)	E4–4.5 (St. 24/25)
TrkA$^+$ cells		
TrkA$^+$/trkB$^+$	64 ± 6% (351)	36 ± 5% (173)
TrkA$^+$/trkC$^+$	100% (397)	90 ± 6% (259)
TrkB$^+$ cells		
TrkB$^+$/trkA$^+$	80 ± 5% (312)	7 ± 1% (885)
TrkB$^+$/trkC$^+$	100% (300)	93 ± 1% (482)
TrkC$^+$ cells		
TrkC$^+$/trkA$^+$	36 ± 3% (1096)	9 ± 2% (2410)
TrkC$^+$/trkB$^+$	23 ± 3% (1003)	36 ± 7% (1194)

Note. Sections from three or four embryos for each stage were double labeled with two of the anti-trk receptor antibodies (see Materials and Methods) and the percentage of cells unambiguously immunopositive for two receptors was determined. Total number of cells counted for each coexpression pair is identified within the parentheses.

cally active progenitor cells; and guidance of sensory axons, in addition to their well-characterized role in promoting neuronal survival (summarized in Fig. 7).

TrkC Expression and Function during Early Sensory Neurogenesis

We have identified a discrete subset (6%) of trkC$^+$ cells among the migrating neural crest as early as E2.5/HH St. 15–16. These cells were always located immediately laterally to the neural tube; neural crest cells which migrate along this medial pathway are those that give rise to the neural and glial lineages rather than to melanocytes (Weston, 1998). Our data suggest that these cells are neurogenic as they coexpress neurofilament and Ben and, by E2.75, β3-tubulin. Morphologically these trkC$^+$ cells appear as both nondifferentiated asymmetric neural crest cells and polarized, nascent neural precursor cells (Wright *et al.,* 1992; Lefcort *et al.,* 1996). The question then is whether these cells are nascent postmitotic cells in an early stage of neural differentiation or, rather, a discrete class of neural progenitor cell.

What is the function of this early trkC expression? NT-3 is expressed in the neural tube (Pinco *et al.,* 1993) and surrounding mesenchyme (Farinas *et al.,* 1996; Hallbook et

al., 1993) during this early time period and would thus be theoretically available to activate trkC receptors on migrating neural crest cells. Henion *et al.* (1995) have shown that *in vitro,* clusters of neurogenic neural crest cells express trkC mRNA and additional *in vitro* studies have shown that NT-3 can cause both the proliferation and the neural differentiation of neural crest cells (Kalcheim *et al.,* 1992; Pinco *et al.,* 1993; Chalazonitis *et al.,* 1994; Henion *et al.,* 1995). Furthermore, Hapner *et al.* (1998) have shown that ectopic overexpression and activation of trkC in neural crest cells *in vitro* can promote both their proliferation (via the TK$^+$ isoform) and neural differentiation (via either the TK$^+$ or the TK$^-$ isoform). In mice, trkC mRNA and protein have been detected in migrating neural crest cells (Tessarollo *et al.,* 1993; Donovan *et al.,* 1996) but not in a second study (Farinas *et al.,* 1998). In mice in which the NT-3 gene has been deleted, there is a superficially normal complement of neural crest cells; however, if a small percentage of trkC$^+$ neural crest cells exist in mice comparable to that in chick, additional studies would be required to detect a potentially subtle deficit in neural crest cell number or composition (Farinas *et al.,* 1996, and see Tessarollo *et al.,* 1997). We also find that p75^{NTR} is expressed on the vast majority of migrating neural crest cells, including dividing neural crest cells, *in vivo.* p75^{NTR} may function to transduce some of the neurotrophin activities identified on neural crest cells *in vitro* (Wright *et al.,* 1992; Verdi *et al.,* 1994; Seiber-Blum and Zhang, 1999) and/or perhaps influence the migration of neural crest cells, since p75^{NTR} has been shown to enhance Schwann cell migration *in vitro* (Anton *et al.,* 1995). Antibody blockade of p75^{NTR} *in vitro* abrogates the ability of NT-3 to induce neural differentiation of neural crest cells via the truncated, TK$^-$ isoform of trkC (Hapner *et al.,* 1998).

During the peak period of neurogenesis in the DRG, E4.5, 20% of the mitotically active cells in the interior region of the DRG are trkC$^+$. Do these cells represent a discrete subclass of DRG progenitor or rather a "snapshot" of a dynamic expression of trkC expression, on all progenitor cells? No trkB$^+$ nor trkA$^+$ dividing progenitor cells were observed. Given the extensive coexpression of trkC receptors with trkA or trkB at this age, these trkC$^+$ progenitor cells must belong to the subpopulation of DRG cells that express solely trkC. In contrast, all dividing progenitor cells in all regions of the DRG expressed p75^{NTR}. A similar analysis in the mouse DRG did not detect trkC$^+$ progenitor cells (Farinas *et al.,* 1998). This may reflect a species

FIG. 5. Coexpression of trk family members by individual DRG neurons in mature DRG. E8 DRG labeled with (A, B) anti-trkA IgG (red) and anti-trkB IgG (green). Cells coexpressing both receptors are located in the dorsal medial (DM) pole and appear yellow. (B) Same section as in (A) but under higher magnification. (C, E, F) E8 DRG section labeled with anti-trkA IgG (red) and anti-trkC IgG (green). Cells coexpressing trkA and trkC are yellow (F) and are localized to the DM pole of the ganglion (not visible in C and E). (D) E8 DRG section double labeled with both anti-trkB IgG (red) and anti-trkC IgG (green). Cells expressing both receptors are localized at the DM pole and appear yellow. Dashed line demarcates border of ganglion; VL, ventrolateral region of the DRG. Bar: A, C, D, 45 μm; B, E, F, 25 μm.
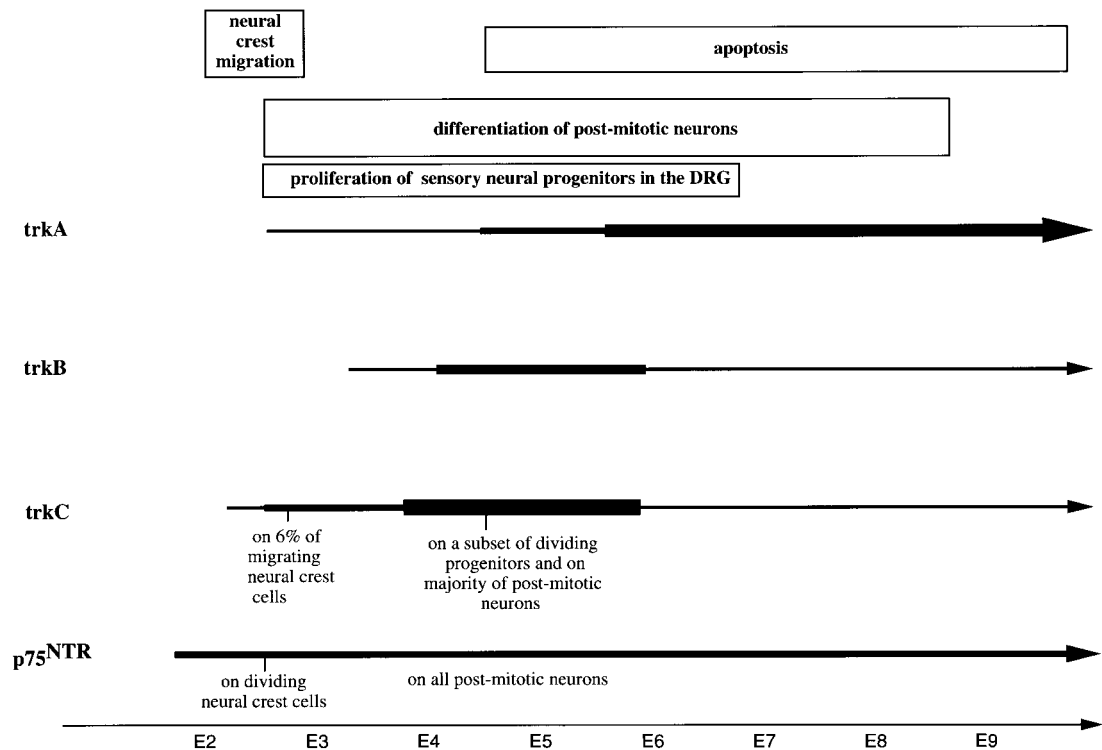
FIG. 7. Summary timeline of sensory neurogenesis in the avian DRG. References include Carr and Simpson (1978) and Tosney (1978).

difference; given the different evolutionary pressures on the development of their PNS, these species may have adopted diverse functions for trkC. Alternatively, given the multitude of trkC receptor isoforms identified (Valenzuela *et al.,* 1993; Tsoulfas *et al.,* 1993; Lamballe *et al.,* 1994; Garner and Large, 1994), the immunolabeling patterns may reflect differences in the repertoire of trkC isoforms expressed by mouse and chick DRG progenitor cells. In both studies, the antibodies used would not have distinguished the class of receptor isoform immunolabeled.

What roles, if any, might trkC serve in mitotically active DRG progenitor cells? Both Elshamy and Ernfors (1996; Elshamy *et al.,* 1998) and Farinas *et al.* (1996, 1998) have described an alteration in behavior of precursor cells in mice with targeted deletion of the NT-3 gene: either their death or their premature exit from the cell cycle. Recently we have shown that direct activation of trkC, either with NT-3 or with the trkC-activating antibody, CTC IgG (Lefcort *et al.,* 1996), promotes the proliferation and possibly survival of DRG progenitor cells *in vitro* (Hapner and Lefcort, in preparation) and we and others have previously shown that antibody blockade of trkC or its ligand, NT-3, prior to target-regulated programmed cell death reduces DRG neuronal cell number significantly (Gaese *et al.,* 1994; Lefcort *et al.,* 1996). Goldstein and colleagues have shown that early applications of exogenous NGF induce the proliferation of progenitor cells in Froriep's ganglion (Geffen

and Goldstein, 1996). Thus, NT-3 could play a key role in the formation of DRG by regulating the behavior of mitotically active DRG progenitor cells (and see Ockel *et al.,* 1996). Our data would suggest that this activity(ies) must be mediated by trkC and/or p75^{NTR}, because these are the only two neurotrophin receptors expressed on progenitor cells. The fact that transcripts for kinase-containing and truncated isoforms were identified in the immature E4.5 DRG would support a multitude of activities for NT-3, including survival, proliferation, and neuronal differentiation (Hapner *et al.,* 1998; Garner and Large, 1994).

In situ hybridization studies have also demonstrated expression of trk mRNAs at E4.5 in the DRG (Kahane and Kalcheim, 1994; Williams *et al.,* 1993; Schropel *et al.,* 1995; Zhang *et al.,* 1994; Williams and Ebendal, 1995), and the temporal expression patterns are in general concordant with our protein data albeit with a few differences. For example, Yao *et al.* (1994) find transcripts for trkB in neural crest cells by RT-PCR and two other reports do not find trkA transcripts until E5–6 (Williams and Ebendal, 1995; Zhang *et al.,* 1994) although Schropel *et al.* (1995) find trkA transcripts in the DRG at E4.5, in agreement with our protein studies.

As noted previously at this age (E4.5) for trkC (Lefcort *et al.,* 1996), all three trk receptors and p75^{NTR} were detected along the entire lengths of the nascent projecting axons, both their central and their peripheral projections (see Figs.

2B–2E). Furthermore, this axonal expression completely overlapped with that of Ben, which has been previously shown to be expressed on projecting growth cones (Chetdotal *et al.,* 1995). These data are consistent with a potential role for neurotrophins in the guidance or support of nascent axons as they project toward their targets.

Role of Neurotrophins in Establishment of Cell Identity

Our data raise a number of key issues concerning the specification of cell identity in the DRG. We find that from their inception, distinct classes of sensory neurons exist as defined by their profile of trk receptor expression: one class expresses only trkC and includes cells that are mitotically active, one class coexpresses trkC and trkA, while another coexpresses trkC and trkB and a fourth class coexpresses all three trk receptors. However, as the vast majority of these cells mature, they cease expression of trkC and ultimately express only either trkA or trkB. Recent work by Anderson and colleagues (Ma *et al.,* 1999; Anderson, 1999) has demonstrated the existence of two waves of neuronal differentiation in the DRG, each regulated by discrete genes, *ngn1* and *ngn2,* with the former responsible for generation of the majority of the trkA$^+$ neurons and a minority of the trkB and trkC subpopulations, while the latter is required for the generation of the majority of the trkC$^+$ and trkB$^+$ neurons. We show here that prior to E4.5, all trkA$^+$ and trkB$^+$ neurons coexpress trkC, but that after E4.5, subsets of neurons appear which express solely trkA or trkB; these later arising populations may derive from the *ngn1* precursor cells which generate the second wave of neurons in the DRG. In addition, our data would support the existence of dynamic switching of trk receptor expression as neurons differentiate, which has been shown to occur in the trigeminal ganglion (Davies, 1997, and see Huang *et al.,* 1999), consistent with a model of different neurotrophin activities being required during the sequential stages of neural differentiation (see Liebl *et al.,* 1997). Interestingly, we identified a subpopulation of cells in the mature DRG which express more than one trk receptor (Fig. 5). While the functional modalities subserved by cells expressing a single trk family member have been fairly well characterized (Oakley *et al.,* 1995, 1997; Snider and Silos-Santiago, 1996), the functional identities of the small-diameter multi-trk receptor-expressing cells in the dorsomedial pole are unknown. Possibly, they are immature cells generated toward the end of neurogenesis which will later differentiate into 1 of the 20 known subclasses of DRG neuron (Scott, 1992). Alternatively, they may comprise a functionally distinct subpopulation of DRG neuron; for example, it has been shown in the adult rat that trkA$^+$/trkB$^+$ neurons comprise a significant proportion of the visceral afferents (McMahon *et al.,* 1994) and in mice in which both the trkB and the trkC genes have been deleted, a subpopulation of cells which innervate the intermediate zone of the spinal cord, an area innervated by myelinated mechanoreceptors, is lost (Silos-Santiago *et al.,* 1997).

Given that trk receptor expression has been shown to correlate with specific sensory neuronal modalities, the question remains as to how causal a role, if any, a particular pattern of trk receptor expression might play in influencing the ultimate functional identity of an individual sensory neuron. That is, does a particular pattern of trk receptor expression bias or predispose the future differentiation pattern or function of a sensory neuron? Or rather, have their final functional identities already been predetermined and reflected by divergent patterns of trk receptor expression on nascent neurons? In the latter case, then, this early trk receptor expression can serve as a predictive marker for some of the ultimately distinctive functional classes of sensory neurons. We show here that differences in the particular pattern of trk receptor expression arise early— several days prior to target innervation, suggesting the existence of intrinsically different neuronal subpopulations due to inherited cues or intercellular signals within the DRG (Ma *et al.,* 1999; Perez *et al.,* 1999). However, these distinctive trk receptor profiles are altered as sensory neurons extend axons and innervate their targets, implicating extrinsic, environmental cues in ultimately influencing sensory neuron identity, as has been shown for regulation of ETS gene expression and by work of Frank and colleagues (Frank and Westerfield, 1982; Smith and Frank, 1987; Shah *et al.,* 1994; Mendell, 1996; Lewin, 1996; Oakley *et al.,* 1997; Wright *et al.,* 1997; Lin *et al.,* 1998; Chen and Frank, 1999). The identification of both these intrinsic and extrinsic signals will be required to elucidate the mechanisms regulating the phenotypic specification of sensory neurons.

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