# THE GENERATION OF PERIPHERAL NEURON DIVERSITY FROM MAMMALIAN PROGENITOR CELLS IN VITRO

Thesis by

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To my parents, Gary and Janice Greenwood,

and my sisters, Erica and Amanda

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## Abstract

The vertebrate peripheral nervous system is comprised of many different kinds of neurons that develop from a common progenitor pool, the neural crest. Factors that control the specification of most neuronal subtypes are not well understood. The studies presented in this thesis describe how and when sensory precursors become different from those of the autonomic lineage, and how subtype diversity is generated within the sensory neuron class.

The development of peripheral neurons was assayed in vitro using rat neural tube explant cultures as a source of neural crest, and antibody staining for lineage-specific transcription factors to identify neuronal subtypes. A chemically defined medium supported the differentiation of sensory but not autonomic neurons in these explants. When cultures were challenged with the extrinsic factor BMP2 to induce autonomic neurons, the division and differentiation of sensory precursors was unperturbed. Therefore, the neural crest contains a population of dividing precursors that are operationally determined to the sensory neuron fate.

The majority of sensory neurons that differentiated in defined medium displayed characteristics of the muscle afferent subtype including dependence on the neurotrophins BDNF and NT-3, expression of the marker ER81, and lack of expression of trkA. In contrast, the addition of serum or BMP2 induced the development of many neurons that expressed the cutaneous afferent marker, trkA. Neither serum nor BMP2 is sufficient to induce trkA in post-migratory, differentiated neurons; instead, these factors induce the neural tube to produce additional sensory precursors, some of which differentiate into cutaneous afferents. We also observed that the neurotrophins BDNF and NT-3, which could directly regulate ER81 expression in differentiated neurons, did not prevent the development of trkA expressing neurons. This implies that serum/BMP2 have early

affects on the specification of sensory subtypes while neurotrophins regulate acquisition of particular subtype characteristics.

In addition to their ability to promote the development of the cutaneous afferent sensory subtype, serum/BMP2 induced patterning in neural tube explants. In the presence of these factors, the location of sensory and autonomic neurons in the neural crest outgrowth of the explant resembled the arrangement of these neuronal subtypes along the dorsoventral axis in vivo.

# Table of Contents

# List of Tables and Illustrations

# Chapter 1

# Introduction

## The specification of sensory neuron fates in the vertebrate peripheral nervous system

Specification is the process by which cells choose developmental pathways and become different from each other. A single cell generates the vast diversity of cell types found in a mature organism through many consecutive specification events . Significant progress has been made in identifying the molecules that act as intrinsic and extrinsic cues in the specification of particular cell fates. However, such cues do not have the same effects on all progenitor cells because the potential of a cell is limited by its developmental history. Therefore, when studying the specification of a cell type it is necessary to identify the precursors that can and cannot respond in a predicted manner to particular cues. In this way, one is able determine how the timing and nature of specification events are coordinated to achieve the precise patterning of cell types in the embryo.

One system in which the study of cell fate specification is particularly appropriate is in the development of the peripheral nervous system (PNS) of vertebrate embryos. The neuronal types in the PNS are highly diverse but derive largely from a common progenitor pool: the neural crest. The neural crest consists of precursors that delaminate from the neural tube and then migrate through the embryo before reaching their sites of differentiation (Hörstadius, 1950; Le Douarin, 1982). A longstanding question about the development of this system is centered around the origin of heterogeneity among different types of neurons (reviewed in Le Douarin, 1982; Le Douarin, 1986; Anderson, 1989; Sieber-Blum, 1990; Marusich and Weston, 1991; Anderson, 1993; Bronner-Fraser, 1993; Le Douarin et al., 1994; Anderson, 1997; Groves and Bronner-Fraser, 1999). Specifically, how much of the heterogeneity is due to inherent early biases among the neural crest, and how much is due to the differential response of multipotent cells to environmental signals present at sites of differentiation? The goal of my thesis is to address this question in the context of the specification of sensory neurons, one particular class of cells in the PNS. The work presented in Chapters 2 and 3 describes how the specification of sensory neurons and the regulation of some characteristics of sensory

subtype are affected by different environmental conditions in vitro. These data emphasize the existence of heterogeneity among neural crest precursors that contribute to sensory neurons. The intention of this chapter is to put this work in context. The first section provides a brief overview of neuronal diversity in the PNS. It is followed by a discussion that reviews the evidence pertaining to how and when sensory neuron fates become specified during development.

## Overview of neuronal subtypes in the PNS

The PNS contains two main classes of neurons (Fig. 1): sensory (somatic) and autonomic (Kandel et al., 1991). The general function of sensory neurons is to transduce stimuli from the external environment into signals that are relayed to the central nervous system (CNS). Sensory neurons are found in cranial ganglia, which serve sensory functions in the head and neck, and dorsal root ganglia (DRGs) that occur in a metameric pattern within the vertebral column adjacent to the spinal cord, and which carry somatic information from the rest of the body. Many subtypes of sensory neurons can be found within each DRG. Conversely, the three classes of autonomic neurons, sympathetic, parasympathetic, and enteric, are generally found in separate ganglia at various locations in the body. Autonomic neurons regulate homeostatic processes such as heart rate, body temperature, and digestion among others. Despite the heterogeneity within each class, sensory and autonomic neurons can be distinguished from each other based on location, function, and, more recently, gene expression.

Significant effort has been put into the molecular characterization of sensory and autonomic neurons and has led to the identification of combinations of markers that can identify subsets of neurons with reasonable certainty. The ability to distinguish between

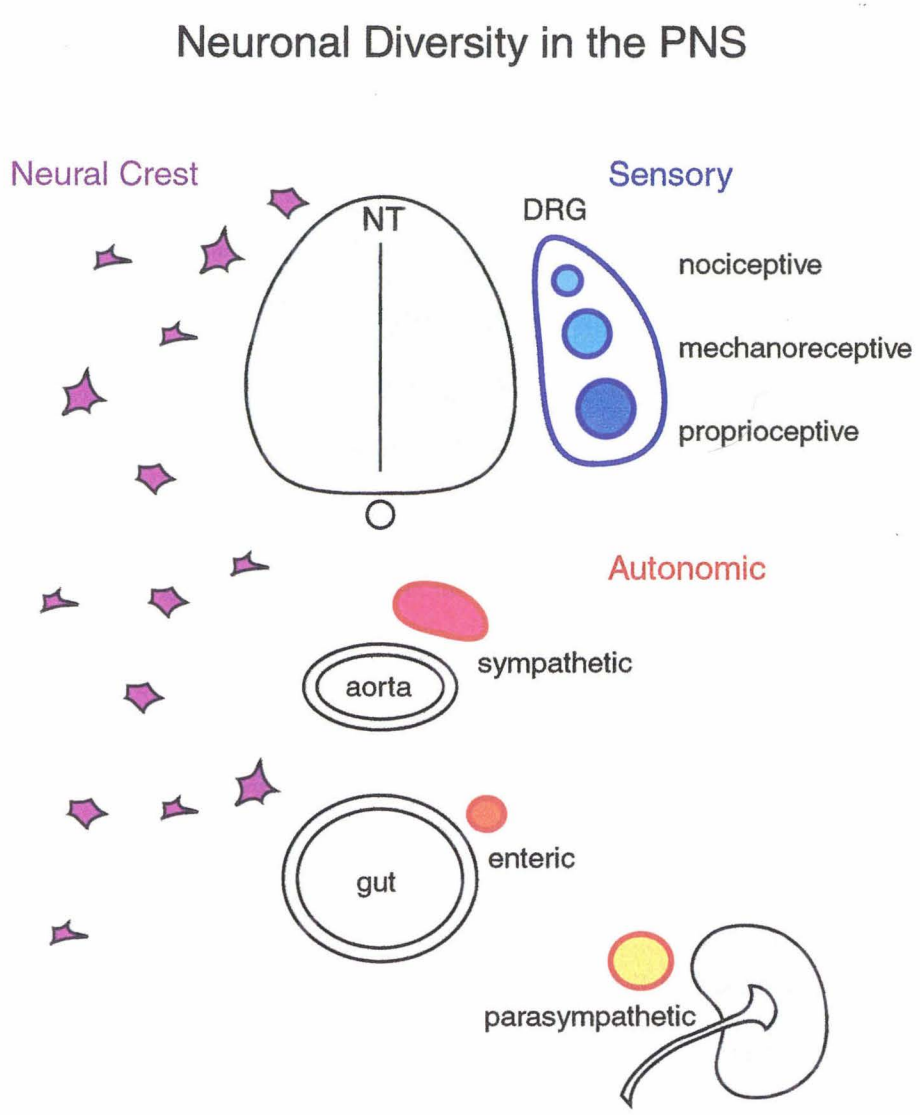


**Fig. 1** Neuronal diversity in the vertebrate peripheral nervous system (PNS). The schematic represents a cross-sectional view through an embryo (dorsal at top; ventral at bottom). There are two main classes of neurons: sensory and autonomic (right side). Different subtypes of sensory neurons, nociceptive, mechanoreceptive, and proprioceptive, are located together in dorsal root ganglia (DRG) adjacent to the neural tube (NT). In contrast, autonomic neuron subtypes, sympathetic, parasympathetic, and enteric, are generally located in separate ganglia at various sites in the ventral part of the body. All these neuronal types derive from neural crest cells that migrate from the dorsal neural tube earlier in development (left side).

sensory and autonomic neurons based on gene expression has been an important advance in the study of the development of these subtypes in vitro. In particular, differentially expressed transcription factors have been useful as specific markers of the classes of neurons in the PNS (reviewed in Anderson, 1997). Some important examples include members of the basic helix-loop-helix (bHLH) transcription factor family. For example, neurogenin-1 (ngn1; Ma et al., 1996), neurogenin-2 (ngn2; Gradwohl et al., 1996; Sommer et al., 1996), NeuroD (Lee et al., 1995), NSCL-1 (Begley et al., 1992) and NSCL -2 (Gobel et al., 1992), are expressed in sensory precursors and developing sensory neurons, but not in autonomic precursors or neurons. Conversely, the bHLH transcription factors MASH1 (Lo et al., 1991), eHAND (Cserjesi et al., 1995) and dHAND (Srivastava et al., 1995) are expressed in autonomic but not sensory precursors. Other transcription factors are maintained in differentiated neurons and thus provide good markers of mature neurons of each subtype. In the DRG, the POU-domain transcription factor Brn-3.0 (Fedtsova and Turner, 1995; Xiang et al., 1995) and the paired homeodomain transcription factor DRG-11 (Saito et al., 1995) are expressed in most sensory neurons. The mature neurons in autonomic ganglia express the paired-homeodomain transcription factors Phox2a (Valarché et al., 1993) and Phox2b (Pattyn et al., 1997), which are not expressed in sensory neurons of the trunk. Many of these transcription factors are required for the development of the subsets of neurons in which they are expressed (Guillemot et al., 1993; Blaugrund et al., 1996; Fode et al., 1998; Ma et al., 1998; Pattyn et al., 1999).

Both the sensory and autonomic classes can be further divided into many subtypes of neurons. The heterogeneity of sensory neurons in the DRG is apparent in terms of functional, structural, and molecular differences (reviewed in Scott, 1992), and it has been a major challenge to relate these characteristics (Fig. 2). Individual sensory neurons in the DRG mediate unique sensory modalities; they transduce information about pain

(nociception), thermal sensation (thermoreception), touch (mechanoreception), or spatial sense (proprioception). Moreover, they may innervate skin (cutaneous afferents), muscle (muscle afferents), or certain structures in the viscera, and display a wide range of electrophysiological properties. Although numerous methods have been employed to try to categorize sensory neurons, the heterogeneity among this population is large enough that it resists simple groupings. There are, however, general classes that can be described with the caveat that there is a fair amount of heterogeneity in each. The development of neuronal subtypes in the sensory ganglia is inherently related to the question of sensory neuron specification; it is not clear whether sensory neurons are specified generally as a class and then acquire different subtype fates, or whether sensory neuron and sensory subtype fate are specified simultaneously. The subject of autonomic subtype specification is beyond the scope of this thesis, however, but has recently been addressed in White and Anderson (1999).

Significant evidence has accumulated that sensory neurons with different functions have different neurotrophic requirements for survival (for reviews see Scott, 1992; Snider, 1994; Lewin, 1996). Thus, subtypes of sensory neurons can be characterized in part by their expression of high-affinity neurotrophin receptors. One class of sensory neurons expresses trkA and depends on nerve growth factor (NGF). These neurons have small to medium sized cell bodies, tend to express neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP), are excited by capsaicin via the vanillinoid receptor VR1, and typically innervate the skin to serve nociceptive or thermal functions. This class of neurons is lost in mice homozygous for the targeted deletion of trkA (Smeyne et al., 1994). Also lost in trkA-/- mice, however, is another population of nociceptive sensory neurons that do not express any trk receptors in

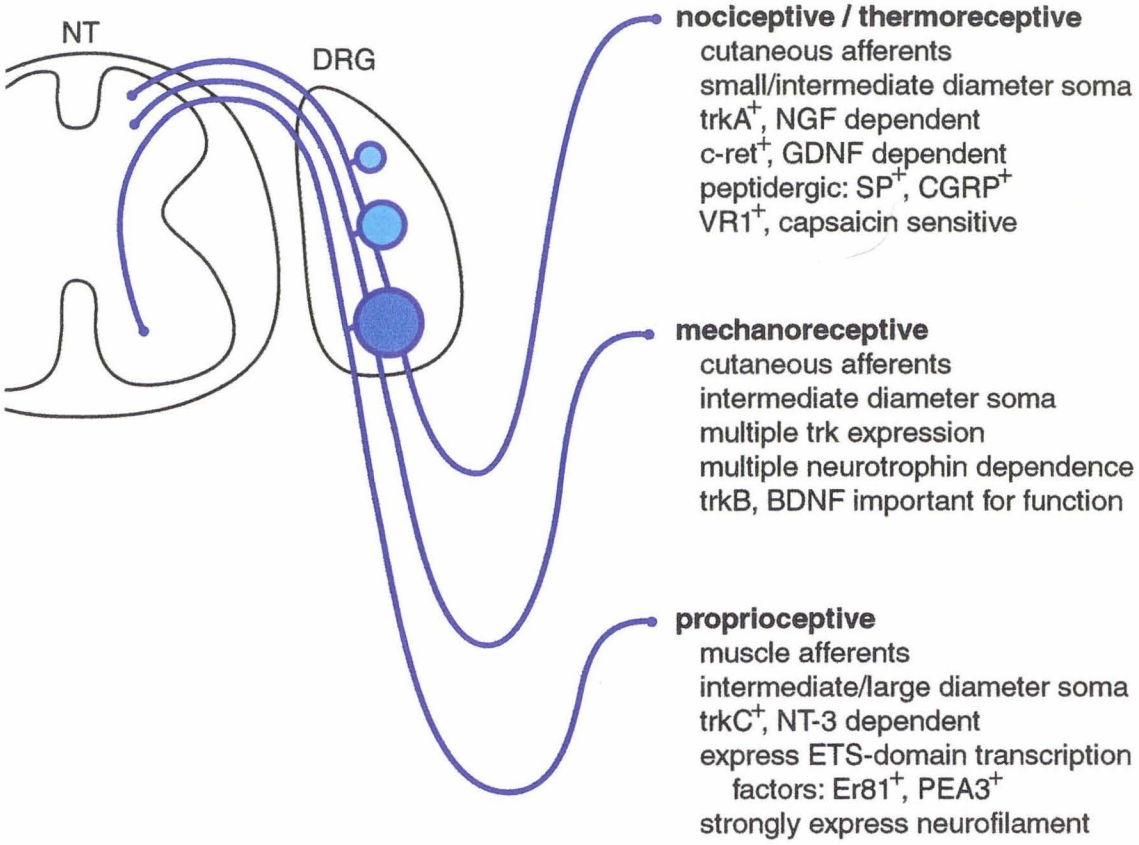


**Fig. 2** Sensory neuron subtypes. The schematic represents a cross-sectional view of the neural tube (NT) and dorsal root ganglia (DRG) containing three classes of sensory neurons (dorsal, top; ventral, bottom). Some properties of each class are listed on the right. Nociceptive sensory neurons mediate painful stimuli and make central projections in the dorsal horn of the spinal cord. Mechanoreceptive sensory neurons mediate different types of touch; these too innervate the dorsal horn. Proprioceptive sensory neurons mediate spatial sense and make central projections to the ventral horn of the spinal cord, some synapsing directly onto motor neurons.

the adult (Silos-Santiago et al., 1995). These have small cell bodies and can be identified by their binding to the lectin BSI-B4. It appears that these neurons express trkA and depend on NGF during development, but then undergo a trophic switch to become dependent instead on glial derived neurotrophic factor (GDNF) late in development (Molliver et al., 1997; Fundin et al., 1999). The $trkA^+$ and $trkA^-$ nociceptors both make central connections in the dorsal horn of the spinal cord, but terminate in different layers indicating they play different roles in transducing painful stimuli (Stucky and Lewin, 1999).

Many of the sensory neurons that express trkC and depend on neurotrophin-3 (NT-3) for survival are large-diameter muscle afferents that serve a proprioceptive function (Mu et al., 1993; Klein et al., 1994; McMahon et al., 1994). Large-diameter sensory neurons project to muscle spindles and Golgi tendon organs in the periphery and have central projections that terminate in the ventral horn of the spinal cord. These neurons express members of the ETS-domain family of transcription factors, such as ER81 and PEA3, that may play a role in the specificity of connections between sensory and motor neuron pools innervating the same muscles (Lin et al., 1998). Mice homozygous for the targeted deletion of trkC have obvious proprioceptive defects and are missing all innervation to the ventral horn of the spinal cord (Klein et al., 1994). In addition to proprioceptive defects, trkC -/- mice also have some defects in mechanosensation. Such a deficit is consistent with the observed loss of both large and intermediate sized sensory neurons in trkC-/- mice, and the fact that trkC is sometimes co-expressed with trkA and/or trkB (Wright and Snider, 1995).

The final class of sensory neurons is the most poorly defined. Sensory neurons that strongly express trkB have medium sized cell bodies and comprise less than 10% of the adult DRG (Mu et al., 1993; Wright and Snider, 1995). There are many cells, however, that co-express weaker levels of trkB with trkA and/or trkC, and it is likely that

this overlap is higher during development. A small number of sensory neurons are lost in mice with targeted deletions of trkB (Klein et al., 1993), but the function of these has not been determined. Although it was initially thought that mechanoreceptive cutaneous afferents would express trkB and depend on BDNF for survival, the story is much more complex. Different subtypes within the mechanoreceptive class appear to have multiple and overlapping neurotrophic dependencies (Fundin et al., 1997; Carroll et al., 1998). In addition, recent analyses of the combinatorial effects of different neurotrophin knockouts suggests that BDNF-dependent and NT-3-dependent neurons may derive from a common precursor that produces $trkB^+$ positive neurons in response to NT-4 signaling and $trkC^+$ neurons in the absence (Liebl et al., 2000).

The development of sensory neurons is an interesting, if complex, system in which to study how and when neural crest cells become specified to their different fates. Sensory neurons are just one general class of neural crest derivatives. The neural crest also produces the autonomic class of neurons as well as many non-neuronal cell types such as glia, smooth muscle, neuroendocrine cells, and melanocytes. It is equally important to identify cues that regulate particular aspects of sensory neuron identity as it is to characterize the developmental potentials of sensory precursors in terms of their abilities to contribute to sensory subtypes and other neural crest lineages.

## Levels of cell fate specification

The concept that progenitor cells acquire intrinsic developmental biases has been the subject of considerable discussion among biologists. The problem is that there seem to be different degrees to which a cell may be biased toward a particular fate. Operationally, there are three levels of bias that may affect a cell's potential. First, a cell is *unspecified* (unbiased) if it is multipotent and responds equally to different instructive cues or yields different cell types via stochastic events that are equally probable. Second,

a cell is *specified* (biased) if it produces the same cell type(s) when it is placed in a "neutral" environment as it would in vivo (Slack, 1991). Such a bias may not be irreversible. Finally, a cell is *committed* or *determined* (biased) to a particular fate if it does not respond to instructive cues or environments that promote alternate fates. Thus, to conclude that a progenitor is determined to a particular fate, experiments must be performed to challenge the cell with instructive signals or new embryonic environments. Note that it is possible for a cell to be both multipotent and biased if it produces more of one cell type than another by preferentially responding to particular cues or by stochastic events that are not equally probable. Although commonly used, I find that the term *restricted*, when unqualified, is not adequately descriptive and can lead to confusion. Cells can be restricted from or restricted to particular cell types both in terms of fate and potential. In this discussion I will use the other terminology defined above.

## Specification of the sensory class of peripheral neurons

### *Evidence for specified or determined sensory precursors*

Single-cell fate mapping in vivo has demonstrated that both premigratory and newly emigrated neural crest contains some cells that contribute exclusively to the DRG rather than to autonomic ganglia, the ventral root, or the dorsal migration pathway (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991; Raible and Eisen, 1994). Such data are consistent with the possibility that there are specified sensory precursors among the neural crest, but do not provide direct evidence toward such a conclusion. In vivo experiments have been performed that challenge the sensory potential of populations of neural crest. For example, it has been observed that the neural crest cells that migrate first along the ventromedial pathway contribute to autonomic derivatives while later migrating neural crest contribute to sensory ganglia (Weston, 1963; Serbedzija et al., 1989; Serbedzija et al., 1990). To determine whether

early and late migrating neural crest contain only committed autonomic or sensory precursors, respectively, grafting experiments were performed in which labeled donor neural tube fragments were heterochronically transplanted to host embryos (Weston and Butler, 1966). It was observed that both "young" (early migrating) and "old" (later migrating) neural crest have the potential to contribute to both sensory and autonomic ganglia. These results indicate that, as a population, early and late migrating progenitors are not strictly committed to colonizing particular ganglia. This study does not rule out the possibility that the neural crest at each stage is a mixture of specified sensory and autonomic precursors.

Neural crest cells that have recently delaminated from the neural tube are heterogeneous with respect to their expression of some sensory markers (Ciment and Weston, 1982; Gradwohl et al., 1996; Perez et al., 1999), and it has been hypothesized that the precursors that express sensory markers are specified to become sensory neurons. In the case of the neurogenins (*ngns*), it has been shown that ectopic expression of these genes in vivo biases the migration of neural crest cells to the DRG and induces expression of sensory markers in other cell types (Perez et al., 1999). Since the neurogenins are expressed in the neural tube and in delaminating neural crest, it is possible that endogenous *ngns* function to bias premigratory neural crest cells toward a sensory neuron fate. Ectopic expression of *ngn1* in the zebrafish CNS induces different types of neurons, the subtype of which is dependent on local cues (Blader et al., 1997). Preliminary studies of ectopic *ngn* expression in mammalian cells in vitro yield similar results (L. Lo and D.J.A., unpublished observation), underscoring the importance of identifying appropriately responsive precursors when studying the effects of a particular "determinant." Regardless, the *ngns* may play a role in the specification of the sensory fate from certain neural crest or neural tube precursors. We do not yet know the level of specification of *ngn* expressing cells in vivo. In particular, it will be important to

determine if all *ngn* expressing neural crest cells go on to make only sensory neurons and whether these *ngn*$^+$ cells are committed to the sensory fate.

Studies performed in vitro provide the strongest evidence for specified sensory precursors among recently emigrated neural crest. When neural crest was directly explanted from the midbrain region of quail embryos, neurons with substance P (SP) immunoreactivity were observed to differentiate from postmitotic precursors (Ziller et al., 1983; Ziller et al., 1987). The differentiation of such neurons was promoted by growth in a defined medium whereas these neurons did not appear in serum-containing medium. Although these data were interpreted as evidence for committed sensory precursors, the failure of sensory neurons to appear in medium that contained serum could be due to the differentiation of sensory precursors into another cell type. Other studies, however, have described similar neurons in cultures of both quail and mouse truncal neural crest (Sieber-Blum, 1989; Matsumoto, 1994a; Matsumoto, 1994b). These neurons were reported to differentiate from postmitotic precursors under conditions that were permissive for the division and differentiation of other neuronal types. The main reason for concluding that the precursors were specified to a sensory neuron fate was because they differentiated without dividing; by definition they did not produce any other cell types besides sensory neurons. Similarly, specification of neuronal identity is thought to occur concomitant with cell cycle arrest in other parts of the nervous system (McConnell and Kaznowski, 1991; Ezzeddine et al., 1997).

Recently, we have observed evidence of dividing, determined sensory precursors in cultures of rat neural crest (see Chapter 2, Greenwood et al., 1999). Sensory precursors were characterized in these cultures with a number of transcription factor markers that could unambiguously distinguish them from autonomic neurons. We observed that sensory neurons arose from dividing precursors even in the presence of the autonomic-inducing cue, bone morphogenetic protein-2 (BMP2). This study is the first

to challenge sensory precursors with conditions known to be instructive for a non-sensory fate. Operationally, these dividing sensory precursors appear to be committed to the sensory fate. The caveat, which exists for all experiments that challenge the potential of a cell, is that there may exist other instructive signals that can prevent these precursors from adopting a sensory fate. Additional experiments that challenge these precursors with other cues and environments will be required to more completely describe their state of commitment.

***Evidence for multipotent progenitors that contribute to sensory neurons as well as other major neural crest lineages***

Clonal analyses performed both in vivo and in vitro provide strong evidence that the neural crest does contain some precursors that produce sensory neurons in the DRG as well as contribute cells to other neural crest derivatives. When either individual neural tube cells or individual migrating neural crest cells in chicken embryos were labeled with a vital dye, at least some of the resulting clones were observed to contain cells in multiple neural crest derivatives including: neurons and non-neuronal cells in the DRG and sympathetic ganglion, glia in the ventral root, cells in the adrenal gland, and pigment cells beneath the ectoderm (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). Similarly, crest cells that generate sensory neurons as well as other types of progeny have been observed in zebrafish (Raible and Eisen, 1994). In vitro clonal analyses of quail neural crest cells have demonstrated that clones containing putative sensory cells, as identified by SSEA-1 immunoreactivity, can also contain pigmented cells and cells expressing autonomic markers such as tyrosine hydroxylase (TH) or dopamine-β-hydroxylase (DBH) (Sieber-Blum, 1989). These data suggest that individual neural crest cells can give rise to both sensory and autonomic neurons as well as other cell types. To date, it has not been possible to analyze individual

mammalian neural crest cells that have sensory potential. Sensory neurons have not been observed from mammalian cells grown at clonal density (A.L.G., unpublished observation). The identification of conditions under which mammalian sensory neurons can develop in clonal cultures would represent an important advance in neural crest biology.

One major question concerning such multipotent sensory precursors is when they become specified to the sensory lineage. Clonal analysis of the progenitors found in quail DRG and sympathetic ganglia suggests that multipotent precursors persist within the ganglia for quite a long time in development (Duff et al., 1991). Precursors from the DRG produced clones that contained SSEA-$1^+$ sensory-like cells, pigments cells, and autonomic-like cells even at two days after gangliogenesis (E6, st. 29). By four days after DRG formation (E8, st. 34) progenitors produced unpigmented colonies (without melanocytes) with both sensory- and autonomic-like immunoreactivities. The percentage of multipotent colonies appeared to decrease with time; this could either reflect the progressive restriction of a homogeneous pool of multipotent precursors or the loss of multipotent precursors from a heterogeneous pool. Quite similarly, an in vivo study in which DRGs were backgrafted to the crest migration pathway demonstrated that the grafted ganglia could contribute to neurons and glia in both the host DRG and sympathetic ganglia, but no longer could contribute to sensory ganglia if they were taken from the donor after E8 (Schweizer et al., 1983). Again, this study does not distinguish between the progressive restriction of multipotent precursors and the loss of a specified group of sensory progenitors. Taken together, however, these data suggest that some multipotent progenitors with sensory potential may persist in the DRG until E8 in quail.

It is interesting that cells with autonomic potential can be found in sensory ganglia well after the sensory neurons are born (Schweizer et al., 1983; Deville et al., 1992). The normal fate of these cells has not been established, but presumably they give

rise to glia or other support cells and could be similar or identical to precursors isolated from the sciatic nerve that have autonomic, glial, and myofibroblast potentials (Morrison et al., 1999). Whether autonomic ganglia contain cells with sensory potential is less certain. Autonomic ganglia from quail can produce colonies containing SSEA-1$^+$ cells for a limited time in development (Duff et al., 1991). By contrast, backgrafting studies in which autonomic ganglia were placed at the top of the crest migration pathway showed that these ganglia do not contribute cells to the DRG (Le Lievre et al., 1980). It is possible, however, that this assay does not provide an efficient enough challenge of precursors to reveal their sensory potential. Alternatively, culture conditions may have induced sensory capacity in Duff et. al. (1991). Identification of a strong, sensory neuron inducing cue would be of great use in resolving the question of which cells have sensory potential.

***Extrinsic signals implicated in the specification of the sensory neuron fate***

Although many growth factors appear to affect the development of sensory neurons and precursors, two in particular have been implicated in the specification of the sensory fate from neural crest cells: BDNF and leukemia inhibitory factor (LIF). The neurotrophin BDNF has been reported to upregulate the expression of the sensory marker SSEA-1 in clonal cultures of quail neural crest (Sieber-Blum, 1991). Importantly, this induction occurred without a concurrent increase of colony size. These data suggest that the effect of BDNF was to instruct neural crest precursors to adopt a sensory fate at the expense of other fates, rather than to promote the survival or proliferation of sensory precursors. It should be noted, however, that most of the colonies were three weeks old when they were analyzed for cell number, time enough to amplify slight differences in growth rate and survival. Such an instructive effect of BDNF has not yet been shown for mammalian precursors. In contrast, mammalian neural crest precursors respond to LIF by producing

sensory neurons. Addition of murine LIF to mouse neural crest cultures was observed to strongly promote the differentiation of $CGRP^+$ neurons from non-dividing precursors (Murphy et al., 1991). Although neuropeptides such as CGRP can be expressed in cultured autonomic neurons and thus are not specific sensory markers (Nawa and Patterson, 1990; Fann and Patterson, 1994b; Fann and Patterson, 1994a), a later study has shown that murine LIF promotes the differentiation of neurons that express the pan-sensory marker Brn-3.0 and have capsaicin sensitivity, a characteristic of a subtype of sensory neurons (Carey and Matsumoto, 1999). The effect of LIF to promote differentiation of non-dividing precursors into sensory neurons could be consistent with an instructive effect. Alternately, it may act permissively to allow the differentiation of precursors that were already specified for a sensory fate. A clonal analysis that determines the effect of LIF on other non-sensory fates, such as glia or autonomic neurons, could distinguish between these possibilities. It is also interesting to note that LIF appears to promote the differentiation of sensory neurons with a nociceptive phenotype.

Recent genetic evidence from zebrafish suggests a role for BMP family members in the specification of some sensory neurons during the neural plate stage, rather than after neural crest migration. In zebrafish, there are two types of sensory neurons: Rohon-Beard (RB) cells that serve mechanosensory functions in larvae and DRG neurons that derive from the classically recognized neural crest and survive into adulthood. The progenitors of RB cells and neural crest both arise from the same area of the lateral neural plate, the numbers of these cells generated is affected by levels of BMP activity (Barth et al., 1999; Nguyen et al., 2000). Recently it has been found that the gene narrowminded (*nrd*) is autonomously required for the development of both RB neurons and early migrating neural crest cells, suggesting, though not proving, the existence of a common progenitor for these cells (Artinger et al., 1999). Furthermore, parallels have been drawn

between the *ngn2* expressing sensory neurons in mammals and the RB cells because both develop from early emigrating precursors. Interestingly, ectopic expression of BMP4 or mutations which dorsalize embryos but have residual BMP activity greatly expand the areas of zebrafish *ngn* expression and the number of RB cells (Neave et al., 1997; Barth et al., 1999; Nguyen et al., 2000). This genetic analysis is consistent with a role of BMP family members in specifying a responsive zone in which sensory progenitors can form. It is likely that additional cues are necessary to actually specify the sensory neuron fate from these precursors because alterations of BMP levels do not change the density of neurons in the expanded neurogenic regions. It is tempting to speculate, however, that BMPs acting in the neural tube may affect sensory neuron production in mammals as well. Intriguingly, we have observed that treatment of neural tube explants with BMP2 also appears to expand the domain of *ngn* expression (see Fig. 8 in Chapter 2) and that exposure of neural tube cells to BMP2 can induce the expression of Brn-3.0 (see Fig. 9 in Appendix 2).

## Specification of sensory neuron subtypes

The question of whether particular sensory subtypes are specified at the time of neural crest migration is important in terms of understanding the initial source of heterogeneity among sensory neurons. At one extreme, all sensory neurons may be specified generally as a class and only later acquire subtype characteristics. In this case, sensory subtype fate could be specified by interactions between postmigratory sensory precursors, environmental influences at the time of differentiation, or interactions with peripheral or central targets after differentiation. This scenario predicts a fair amount of plasticity in the types of neurons that form under different conditions. At the other extreme, all precursors may acquire a subtype fate at the same time that they become specified as sensory neurons, perhaps some even before they migrate from the neural tube. This scenario predicts that there will be very early differences between classes of sensory

neurons and little plasticity. Clearly, there are many possibilities that lie between the two extremes. The way to discriminate between these possibilities would be to challenge sensory precursors with cues that are known to induce particular subtype fates and assess their response. At the present time, this sort of analysis is beyond our abilities. We are only beginning to identify the cues that regulate particular sensory characteristics and it is not known what factors are responsible for instructing particular subtype fates. Additionally, there are no known intrinsic determinants that distinguish between precursors fated to produce sensory neurons with different functions. Without these tools we cannot directly address the question, but an analysis of when sensory subtype heterogeneity first becomes apparent and how subtype characteristics are affected under certain conditions may provide important clues and allow us to rule out certain possibilities.

***Evidence for precursors that are specified to a particular sensory subtype fate***

Heterogeneity among sensory neurons in the DRG is apparent very early in gangliogenesis. Different types of neurons are born (become postmitotic) at different times: the first neurons born are fated to contribute to the large-diameter subclass and tend to occupy the ventrolateral (VL) part of the DRG. The small diameter sensory neurons are born a day later and occur in the dorsomedial (DM) part of the DRG (Lawson et al., 1974; Lawson and Biscoe, 1979; Kitao et al., 1996). Although this is generally true, sensory neurogenesis may go on longer than originally thought due to contribution of new progenitors from the neural tube (Sharma et al., 1995; Kitao et al., 1996). Clonal analysis in vivo of neural tube precursors that contribute to the DRG suggests that there are two types of precursors with respect to their subtype fates (Frank and Sanes, 1991). One class of precursors is observed when labeling is performed early. These cells divide only a few times and are fated to give rise to only large-diameter sensory neurons in the

VL part of the DRG ("VL only" clones). The second type of precursor is apparent when the labeling is done later. These typically undergo many divisions and produce sensory neurons with both large and small diameters located in both the VL and DM parts of the DRG ("VL+DM" clones). In this study, no precursors were observed to give rise to only small diameter sensory neurons. It is possible that no "DM only" clones were observed because the precursors analyzed did not include the latest wave of migrating cells. Regardless, the existence of clones that contain only one sensory subtype (VL only) is consistent with the hypothesis that some sensory precursors may be specified to the large-diameter (presumably proprioceptive, muscle afferent) fate when they emigrate from the neural tube. The ability to determine the potential of such precursors awaits a method to prospectively identify and then challenge them with cues that induce the small-diameter (nociceptive, cutaneous afferent) subtype fate.

Similar sensory precursors have been revealed in the analysis of *ngn1* and *ngn2* mutant mice. Sensory neurogenesis in mice depends on the *ngns* since *ngn1-/-;ngn2-/-* embryos lack DRGs but contain autonomic ganglia (Ma et al., 1999). In *ngn2-/-* mutants, neurogenesis is delayed, indicating a defect in the early wave of precursors. However, the DRGs of these fetuses contain the full complement of sensory subtypes, suggesting that *ngn1*$^+$ precursors have the potential to make both large and small diameter subtypes and may be similar to the multipotent precursors identified by clonal analysis in chick. In contrast, *ngn1-/-* mutants are missing the later wave of neurogenesis. The DRGs of these mice are depleted of trkA-expressing neurons (small-diameter, nociceptive). Thus, the *ngn2*$^+$ cells do not compensate for the loss of *ngn1*-dependent precursors. This phenotype is consistent with the notion that *ngn2*-dependent precursors do not have the potential to make trkA$^+$ sensory neurons; however, it is also possible that the early wave of neurogenesis is complete and *ngn2* expression is extinguished by the time the appropriate nociceptive-inducing signals appear. This question might be resolved by knocking the

*ngn2* gene into the *ngn1* locus to see if trkA$^+$ neurons can develop from *ngn2*-expressing cells.

Arguments for the early specification of sensory subtypes have also been put forth based on the projection patterns of sensory neurons identified by retrograde labeling of target tissues (Honig, 1982; Honig et al., 1998). The case that developing sensory neurons are prespecified to innervate particular muscle or cutaneous targets rests mainly on the observations that 1.) sensory axons projecting to a particular target appear to be bundled from the outset and to make distinct pathway choices within a very short distance from the DRG, and 2.) the cell bodies of sensory neurons projecting to a particular target are not all located near each other in the DRG (based on retrograde labeling of neurons with soma in the LV part of the DRG). An additional observation cited as support for this hypothesis is that individual neurons that project to a particular target often have cell bodies that appear in pairs or small clusters, suggesting that a prespecified precursor divided to produce a clone of neurons that all actively chose the same target. However, there are other interpretations of the data that do not require the early specification of precursors or neurons to a particular subtype fate. Also possible, I believe, is a scenario in which unspecified sensory precursors send out axons that follow the nearest available guidance cue. The first born sensory neurons in the VL part of the DRG would encounter a variety of different guidance cues, many of which are likely to be a motor axons on their way to the muscle. Sensory neurons born late in development would encounter a more "crowded" environment and would be more likely to follow other sensory axons, most of which had innervated a cutaneous target because the muscle axons were occupied. This model predicts that the cell bodies of the later born neurons should be more grouped with respect to their peripheral targets because the pathways of innervation are already well established by the time they are extending axons.

Retrograde labeling of targets that resulted in labeled cell bodies in the DM part of the DRG was not reported; it is possible these neurons are more clustered.

Although the available evidence does not directly demonstrate the existence of precursors that are specified to a particular sensory subtype, it does suggest that there are early differences in the types of precursors that contribute to the DRG. The ability to isolate different types of sensory precursors and the identification of cues involved in the specification of sensory subtypes would be an important step in resolving these questions. Chapter 3 of this thesis provides an initial characterization of some cues that affect the development of particular sensory subtype characteristics.

***Evidence for precursors among the neural crest that can generate multiple sensory subtypes***

Lineage and genetic analyses presented above suggest that there may be precursors among the neural crest that can make multiple types of sensory neurons. Individual progenitors labeled in the neural tube produce clones of cells in the DRG that include both large and small diameter sensory neurons (Frank and Sanes, 1991). Similarly, the population of *ngn1* expressing precursors can generate all subtypes of sensory neurons as assayed by expression of the trk receptors (Ma et al., 1999). It is likely that these studies reveal sensory precursors among the neural crest that are multipotent with respect to sensory subtype fate. Single cell analyses of migrating neural crest would bolster this conclusion. For example, it would be interesting to determine the range of sensory subtype fates of individual neural crest cells in the context of wild-type and *ngn2* mutant embryos. The recent advent of inducible, stable lineage markers, such as the Cre-recombinase system (Sauer and Henderson, 1988), may make such an analysis possible if these methods can be adapted to follow the progeny of individual cells.

Studies performed in vivo that alter the available targets for sensory innervation reveal a certain amount of subtype plasticity. In the normal course of development, each DRG innervates only targets within a particular, well-defined dermatome (segment) of the body. DRGs along the neuraxis differ in the composition of sensory neuron subtypes they contain based on the types of targets present in that particular segment. For example, in the bullfrog, DRG2 innervates the arm and contains a significant number of muscle afferents, whereas DRG3 innervates the ventral part of the thorax and contains mostly cutaneous afferents (Frank and Westerfield, 1982b). If sensory neurons were strictly specified to innervate particular targets, one might expect that they would not innervate structures in a different dermatome than their origin. It has been demonstrated, however, that upon removal of DRG2, the adjacent ganglia, DRG3, will innervate many of the muscles in the arm and form appropriate connections in the spinal cord (Frank and Westerfield, 1982a). Furthermore, if entire ganglia are transplanted from the mid-thoracic region to the location of DRG2, they also innervate peripheral and central targets appropriate for the new location (Smith and Frank, 1987). These results are particularly interesting since most of the neurons in the thoracic DRGs are cutaneous and not muscle afferents. The authors favored the interpretation that sensory subtype fate is plastic until late in development, and that the environment plays an important role in the specification of different subtypes. These analyses, however, did not follow the fate of individual neurons. It is therefore equally possible that the thoracic DRGs contain a mixed population of specified precursors or neurons and that the environment plays a selective, rather than instructive, role in matching the subtype of sensory neuron with the type of available target. Regardless, it is clear that sensory neurons are not restricted to innervating only targets in the dermatome from which they originate.

In vitro evidence has also been interpreted to suggest that early differentiating sensory neurons may not be initially specified with respect to their targets (Adams and

Scott, 1998). Cranial sensory ganglia, unlike DRGs, contain areas that can be prospectively identified to produce neurons of a particular subtype. When sensory neurons are isolated early in development from two areas that are fated to innervate either muscle or cutaneous targets, the growth cones of these axons respond similarly in vitro with respect to their avoidance of and attraction to particular target choices. However, if the sensory neurons from these structures are isolated at later times they respond differently to the targets. The authors suggested that one reason for such observations might be if the neurons isolated at early times did not have rigidly specified identities. Although this is possible, it is not clear how well such a target-choice assay challenges the identity of a neuron. The inability of immature neurons to discriminate between targets could simply indicate that their pathfinding machinery is not fully developed.

Given that the number of mature subtypes of neurons in the sensory ganglia is quite large, it is likely that some precursors are "multipotent" with regards to some aspects of identity. It is also possible that some subtype characteristics are specified early in neural crest development. The analysis of when particular sensory subtype characteristics become determined awaits the ability to efficiently follow the progeny of individual cells and the assess these clones for expression of subtype markers under conditions that challenge their fate.

***Extrinsic signals implicated in the specification of sensory neuron subtype fates***

Due to their ability to support the survival of different types of sensory neurons, it has been hypothesized that neurotrophins may play a role in the specification of subtype fate (Lewin, 1996). This hypothesis is intuitively appealing, but what is the evidence? Both the neurotrophins NT-3 and BDNF have the opportunity to exert early effects on subtype fate because they are present in developing DRGs (Ernfors et al., 1992; Schecterson and Bothwell, 1992). They have also been shown to affect the proliferation and maturation of

sensory neuroblasts before the period of neurotrophin dependence (Wright et al., 1992; Memberg and Hall, 1995), but there is no evidence that they directly specify the fates of NT-3- and BDNF-dependent neurons. Indeed, the observation that some sensory neurons appear to switch their neurotrophin dependency from NT-3 or BDNF to NGF (Buchman and Davies, 1993; Buj-Bello et al., 1994; Pinon et al., 1996) suggests that NT-3 and BDNF are not sufficient to specify subtype survival properties. Furthermore, when NT-3 is ectopically expressed in vivo using a muscle or skin specific promoter (Albers et al., 1996; Wright et al., 1997) , it selectively increases the number of neurons within appropriate subpopulations without depleting the others. Evidence presented in Chapter 3 indicates that the continued presence of NT-3 and BDNF does not prevent the differentiation of $trkA^+$ neurons in vitro. Although we cannot rule out the possibility that NT-3 or BDNF plays an instructive role in specifying the subtype fate of some precursors, it does not seem to be a general rule that sensory precursors or neurons become dependent on the first neurotrophins they are exposed to.

In the case of NGF, there is direct evidence to suggest that this signaling pathway is not required for the initial specification of the small-diameter nociceptive subtype. If it were, one would expect that the nociceptive fate would not be specified in the absence of NGF or trkA. The phenotype of trkA-/- and NGF-/- mutants, however, precludes the analysis of sensory subtype because the neurons are lost during the period of programmed cell death before they have any obvious identity (Smeyne et al., 1994). A recent and elegant study has addressed this problem: when the trkA-/- phenotype is analyzed in the context of the BAX-/- mutation, which prevents apoptotic death, many small-diameter neurons are found in DRGs that make appropriate connections to the dorsal horn of the spinal cord (Patel et al., 2000). These neurons do have defects in nociceptive function, presumably due to lack of peripheral innervation and expression of neuropeptides, but they appear to have retained a fundamentally nociceptive identity

based on their morphology, location, and central projections. Neurotrophins have pleiotropic and important effects on the development of sensory neuron characteristics, but it appears that they are not generally required for the initial specification of sensory subtypes. It will be very interesting to see how sensory subtype characteristics are affected in trkB-/- and trkC-/- mutants crossed into the BAX-/- background.

## Concluding remarks

The neurons in the PNS are an exceedingly heterogeneous group of cells and the sensory lineage especially so. Although many intrinsic and extrinsic cues have been discovered that regulate particular aspects of peripheral neuron identity, the challenge still remains to understand the main forces that initiate the diversity. Given that specification, as a general process, seems to work by the progressive subdivision of a progenitor population into groups that have different response properties, if we are to make sense of PNS subtype diversity it seems important to focus on the earliest specification events. Evidence presented here and elsewhere indicates that some, though certainly not all, of these decisions affecting the PNS may occur among premigratory or early migrating neural crest. This in itself emphasizes the need to characterize the developmental potentials of precursors within the neural tube and neural crest at different times. The isolation of precursors based on gene or protein expression should provide an important tool in this analysis. Additionally, the increasing ability to apply genetic analysis in mice and zebrafish to questions of PNS development will greatly contribute to the understanding of initial specification events.

It should also be pointed out that lineage relationships in the PNS may not correspond to progressive restrictions within the categories we have arbitrarily defined in this system. For example, one type of precursor may be limited to producing a subset of

sensory neuron types, while another may have the potential to produce a different subset of sensory subtypes plus autonomic, glial, and or myofibroblast fates. The characterization of the precursors that contribute to the PNS is not only important in terms of understanding what cells can and cannot respond to particular cues, but it may also reveal an underlying logic to the development of the system.

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# Chapter 2

## Identification of dividing, determined sensory neuron precursors in the mammalian neural crest

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# Identification of dividing, determined sensory neuron precursors in the mammalian neural crest

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## SUMMARY

**Sensory and autonomic neurons of the vertebrate peripheral nervous system are derived from the neural crest. Here we use the expression of lineage-specific transcription factors as a means to identify neuronal subtypes that develop in rat neural crest cultures grown in a defined medium. Sensory neurons, identified by expression of the POU-domain transcription factor Brn-3.0, develop from dividing precursors that differentiate within 2 days following emigration from the neural tube. Most of these precursors generate sensory neurons even when challenged with BMP2, a factor that induces autonomic neurogenesis in many other cells in the explants. Moreover, BMP2 fails to prevent expression of the sensory-specific basic helix-loop-helix (bHLH) transcription factors *neurogenin1*, *neurogenin2* and *neuroD*, although it induces expression of the autonomic-specific bHLH factor MASH1 and the paired homeodomain factor Phox2a in other cells. These data suggest that there are mitotically active precursors in the mammalian neural crest that can generate sensory neurons even in the presence of a strong autonomic-inducing cue. Further characterization of the neurons generated from such precursors indicates that, under these culture conditions, they exhibit a proprioceptive and/or mechanosensory, but not nociceptive, phenotype. Such precursors may therefore correspond to a lineally (Frank, E. and Sanes, J. (1991) *Development* 111, 895-908) and genetically (Ma, Q., Fode, C., Guillemot, F. and Anderson, D. J. (1999) Genes Dev. 13, in press) distinct subset of early-differentiating precursors of large-diameter sensory neurons identified in vivo.**

Key words: Neural crest, Sensory neurogenesis, Neurogenin, Brn-3.0, BMP2, NT-3, Rat

## INTRODUCTION

A fundamental question in development is how a diverse array of differentiated cell types can be produced from a multipotent population of progenitor cells. In vertebrates, this issue has been intensively investigated in the neural crest. Although a large body of evidence supports the idea that the neural crest contains multipotent progenitors whose choice of fate is determined by environmental signals, there is also evidence that the neural crest contains (or eventually generates) subsets of precursors whose fates appear restricted (for reviews see Le Douarin, 1986; Anderson, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991; Marusich and Weston, 1991; Weston, 1991; Anderson, 1993; Bronner-Fraser, 1993; Le Douarin et al., 1994; Anderson, 1997). It is therefore important to understand when particular neural crest cell fates become determined in relation to others, and how lineage segregation is regulated during neural crest development.

One setting in which such issues have been addressed is in the specification of the two major neuronal classes of the peripheral nervous system (PNS), the sensory and the autonomic (Le Douarin, 1986; Sieber-Blum et al., 1993; Le Douarin et al., 1994). Clonal analyses of neural crest in vivo (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991) and in vitro (Sieber-Blum, 1989; Duff et al., 1991) have identified individual precursors that can give rise to both sensory and autonomic neurons. Such observations, however, do not preclude the existence of separate sensory and autonomic precursors. Previous results from back-grafting studies in avian embryos have suggested the existence of dividing precursors that can contribute to the autonomic but not sensory derivatives of the neural crest. (Le Lievre et al., 1980; Schweizer et al., 1983; Le Douarin, 1986). Whether the neural crest contains dividing progenitors that can generate sensory neurons even in the presence of autonomic-inducing cues is not known.

To better understand the mechanisms that regulate the segregation of the sensory lineage, it is necessary to be able to identify and manipulate sensory precursors, challenge them with autonomic-inducing signals and determine whether they generate sensory or autonomic neurons. Recently, several transcription factors have been characterized that can be used to identify sensory neurons and/or their precursors and distinguish them from autonomic neurons and/or their precursors. Basic helix-loop-helix (bHLH) transcription factors such as the *neurogenins* (*ngns*; Gradwohl et al., 1996;

Ma et al., 1996; Sommer et al., 1996) and *neuroD* (Lee et al., 1995), as well as the POU-domain transcription factor *Brn-3.0* (Fedtsova and Turner, 1995; Xiang et al., 1995) are specifically expressed in the sensory lineage. Other transcription factors are expressed by autonomic but not sensory cells, including the bHLH factor MASH1 (Johnson et al., 1990; Shah et al., 1996) and the paired homeodomain factor Phox2a (Valarché et al., 1993; Lo et al., 1998).

Members of the bone morphogenetic protein (BMP) subfamily, such as BMP2 and BMP4, have been identified as autonomic neuron-inducing signals both in vivo and in vitro. Such factors can induce autonomic differentiation in mass cultures of avian neural crest (Reissman et al., 1996; Varley and Maxwell, 1996) and in clonal cultures of mammalian neural crest stem cells (Shah et al., 1996; Shah and Anderson, 1997; Morrison et al., 1999). In the latter system, BMP2 was shown to act instructively, promoting autonomic neurogenesis from multipotent neural crest cells at the expense of other fates. Overexpression of BMP4 in ovo also induces autonomic markers in neural crest cells (Reissman et al., 1996). BMP2/4 are likely to be inducers of the autonomic fate in vivo, as they are expressed by tissues neighboring autonomic ganglia (such as the dorsal aorta; Reissman et al., 1996; Shah et al., 1996) and these tissues can mimic the inductive effects of BMPs on neural crest cells (Groves and Anderson, 1996; Reissman et al., 1996) in a manner that is inhibitable by the BMP2/4-antagonist noggin (Zimmerman et al., 1996; A. Groves and D. J. A., unpublished data).

We have now used transcription factor markers to study the development of sensory neurons in cultures of rat neural crest. We have formulated a fully defined culture medium that permits the differentiation of sensory neurons from proliferating precursors that migrate from neural tube explants. Autonomic neurons do not develop under these conditions unless they are induced to differentiate by addition of BMP2. Strikingly, BMP2 does not appear to prevent the differentiation of most dividing sensory precursors or the expression of sensory precursor markers. These data suggest that the migrating neural crest includes a subpopulation of dividing precursors that are, operationally, committed to a sensory fate with respect to the autonomic-inducing signal BMP2. Interestingly, these precursors give rise to sensory neurons that exhibit a proprioceptive and/or mechanoreceptive, rather than a nociceptive phenotype. They may therefore correspond to a distinct subset of sensory precursors previously identified by retroviral lineage tracing in chick embryos (Frank and Sanes, 1991) and more recently by genetic analyses in mice (Ma et al., 1999).

## MATERIALS AND METHODS

### Rat neural tube explant cultures

Neural tubes were isolated from the trunk level of E10.5 Sprague-Dawley rats as described previously (Stemple and Anderson, 1992) but with the following modifications: trunk sections were enzymatically treated with 1.25 mg/ml collagenase (172 u/mg, Worthington) and dispase (0.77 u/ml, Gibco) in Hank's balanced salt solution (HBSS). The tissue was incubated for 5 minutes in ice-cold enzyme solution with trituration, then incubated for 10 minutes at room temperature, then gently triturated further until the neural tubes were free of other tissues. When neural tubes were grown in defined conditions, they were washed extensively in defined medium before plating. Explanted neural tubes were plated in 35 mm diameter Corning dishes on a fibronectin (BTI) substratum and grown at 37 C in 5% $CO_2$/95% air. Where stated, the neural tube was removed from the culture after 24 hours with a tungsten needle, but otherwise the explant was left intact.

### Media and additives

#### Defined medium

The recipe for defined medium (DM) was modified from that described previously (Stemple and Anderson, 1992) and is as follows: to L-15$CO_2$ (Hawrot and Patterson, 1979) add 100 μg/ml transferrin (Calbiochem), 5 μg/ml insulin (Sigma), 16 μg/ml putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM selenious acid (Sigma) 1 mg/ml bovine serum albumin, (crystallized, Gibco/BRL), 39 pg/ml dexamethasone (Sigma), 5 μg/ml α-d-1-tocopherol (Sigma), 63 μg/ml β-hydroxybutyrate (Sigma), 25 ng/ml cobalt chloride (Sigma), 1 μg/ml biotin (Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml glycerol, 100 ng/ml α-melanocyte-stimulating-hormone (Sigma), 10 ng/ml prostaglandin E1 (Aldrich). Basic fibroblast growth factor (bFGF; 10 ng/ml, UBI) was included in the medium for the first 2 days, but not thereafter. Where indicated, DM was supplemented with recombinant human BMP2 (50 ng/ml, gift from Genetics Institute).

#### Undefined medium

Undefined medium was also modified from that described previously (Stemple and Anderson, 1992). It contained the same basal components as the DM described above plus bFGF (4 ng/ml, R&D Systems Inc.), epidermal growth factor (EGF; 100 ng/ml, Sigma), retinoic acid (35 ng/ml, Sigma), nerve growth factor (NGF; 50 ng/ml, UBI), brain-derived growth factor (BDNF; 25 ng/ml), neurotrophin-3, (NT3; 25 ng/ml, BDNF and NT3 both provided by Dr G. Yancopoulos at Regeneron Pharmaceuticals), leukemia inhibitory factor (LIF; 25 ng/ml, R&D Systems Inc.) and 10% chick embryo extract (CEE) for the first 24 hours, 0.1% CEE thereafter.

### Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde and then processed for single or double antibody labeling or in situ hybridization (see below) followed by antibody labeling. Antibody-antibody double labeling involved detection of antigens using NiDAB and DAB substrates as described previously (Shah et al., 1994). Finally, cultures were incubated with 10 μg/ml DAPI for 15 minutes at room temperature to identify other nuclei and thus the extent of the outgrowth from the neural tube.

Primary antibody incubations were carried out at 4 C overnight. Affinity-purified rabbit anti-Brn-3.0 antibody (1:500; Fedtsova and Turner, 1995) and hamster anti-DRG11 hybridoma supernatant (Satto et al., 1995) were used to detect sensory neurons. Monoclonal antibodies to SCG10 (hybridoma supernatant; L. Lo and D. J. A., unpublished data), neurofilament 160 (1:250; NF160, clone NN18, Sigma), or NeuN (1:500, Chemicon) were used as general neuronal markers. Peripheral neurons were identified using the combination of Brn-3.0 expression and polyclonal anti-peripherin (1:2000; Chemicon) or monoclonal anti-c-RET (hybridoma supernatant; Lo and Anderson, 1995) antibodies. Rabbit polyclonal anti-Phox2a antibody (1:5000; gift from Drs. C. Goridis and J. F. Brunet; Tiveron et al., 1996) was used to identify autonomic cells. Autonomic precursors were stained using anti-MASH1 hybridoma supernatant (Lo et al., 1991). The monoclonal antibody RT97 (1:500; gift from Dr J. N. Wood; Lawson et al., 1984) and rabbit polyclonal anti-ER81 antibody (1:2,000; gift from Dr T Jessell; Lin et al., 1998) were used as markers of the large, proprioceptive subtype of sensory neurons. Rabbit polyclonal antibodies to Substance P (SP; 1:1000; Inestar), calcitonin gene related peptide (CGRP; 1:1000; Peninsula Laboratories, Inc.) and TrkA (1:500; gift from Dr L. Reichardt; Clary et al., 1994) were used as markers of the small nociceptive sensory subtype.

All secondary antibodies were used at a dilution of 1:200 and incubated for 1 hour at room temperature. Rabbit polyclonal antibodies were detected using an HRP-conjugated, goat anti-rabbit secondary antibody purchased from Vector Labs, Inc.. The monoclonal antibodies to SCG10, NF160 and MASH1 were detected with an HRP-conjugated, goat anti-mouse IgG secondary from Chemicon. Hamster monoclonal antibodies to c-RET and DRG11 were detected by a biotinylated goat anti-mouse IgG secondary antibody (Jackson Laboratories, Inc.) followed by the ABC amplification system (Vectastain ABC kit, Vector Labs, Inc.).

### In situ hybridization

In situ hybridization on explants was performed using a modification of procedures developed by Dr R. Li (Signal Pharmaceuticals, personal communication) and Dr D. Henrique (Henrique et al., 1995). Briefly, the explants were pretreated with acetic anhydride and 0.2 N HCl then prehybridized for 1 hour at room temperature. Hybridization in 1-2 μg/ml of digoxigenin (DIG)-labeled probe was carried out at 65°C overnight. After the hybridization, explants were washed at high stringency (0.2× SSC at 65°C for 1 hour), incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (Boehringer) and the signal developed using NBT/BCIP reagents. A more detailed protocol is available upon request. The probes used in this study include rat *ngn1* (Ma et al., 1996), mouse *ngn2* (Sommer et al., 1996) and mouse *neuroD* (Lee et al., 1995). For in situ antibody double labeling, explants were processed first for in situ detection of mRNA, then for immunocytochemistry as described above.

### BrdU analysis

To determine the mitotic status of sensory precursors in vitro, explants were grown in DM and supplemented with 175 nM BrdU at the time of plating (day 0) or after 1, 2 or 3 days of culture. After initial crest migration (24 hours), explants were modified by removing the neural tube from culture with a tungsten needle. Cultures were grown in the presence of BrdU for 24 hours, then fixed and processed for fluorescent Brn-3.0/BrdU double labeling. Rabbit anti-Brn-3.0 antibody (see above) was detected using a FITC-conjugated goat anti-rabbit secondary from Jackson Laboratories. After Brn-3.0 staining, BrdU incorporation was detected as described previously (Novitch et al., 1996) using a mouse anti-BrdU primary antibody (1:100, Caltag) and Cy5-conjugated goat anti-mouse secondary antibody (1:200, Jackson). Staining was observed using a 410 Ziess Axiovert LSM confocal microscope. Double-positive nuclei were scored by identifying individual Brn-3.0$^+$ cells in one channel (green) and checking for an identically shaped, BrdU$^+$ nucleus in the other channel (red). In cases where it was not possible to clearly determine if a Brn-3.0$^+$ cell had incorporated BrdU, these cells were omitted from the analysis; this situation occurred most often when the cells were tightly clustered in explants at day 1.

### Trophic support analysis

Neural crest explants were cultured in DM supplemented with 10 ng/ml bFGF for 2 days. On day 3, DM (without bFGF) was supplemented with growth factors singly or in combination at the following concentrations: NGF (100 ng/ml), BDNF (50 ng/ml), NT3 (50 ng/ml) and/or LIF (50 ng/ml). The medium and factors were replaced every 2-3 days until the explants were fixed at day 8 and processed for Brn-3.0 antibody staining. The number of Brn-3.0$^+$ cells in the dorsal outgrowth was scored for each explant, excluding cells tightly associated with the neural tube.

## RESULTS

### Both sensory and autonomic neurons are generated in rat neural tube explant cultures

To characterize the types of neurons that differentiated in rat neural tube explant cultures, we examined the expression of lineage-specific transcription factor markers in combination with general markers of peripheral neurons (Table 1). Both Brn-3.0, a POU-domain transcription factor, and DRG11, a paired homeodomain factor, are expressed by sensory but not autonomic neurons in the PNS in vivo (Fedtsova and Turner, 1995; Saito et al., 1995). Moreover, Brn-3.0 is genetically important for some aspects of sensory neuron development (McEvilly et al., 1996; Xiang et al., 1996). Although these transcription factors are also expressed by dorsal spinal cord neurons, Brn-3.0$^+$ or DRG11$^+$ peripheral sensory neurons can be distinguished from these CNS neurons by their expression


**Fig. 1.** Two populations of neurons develop in rat neural tube explant cultures. The view shown includes the dorsal neural tube (NT, top) and neural crest outgrowth (below dashed line) of an explant grown for 8 days in CEE-containing medium plus NGF, BDNF, NT3 and LIF. The explant is stained for both Brn-3.0 (dark blue, nuclear) and SCG10 (brown, cytoplasmic) via immunocytochemistry. Brn-3.0$^+$/SCG10$^+$ cells are located in the outgrowth proximal to the neural tube and have large cell bodies (region X). Brn-3.0$^-$/SCG10$^+$ cells are smaller and often found in clusters in the periphery of the outgrowth (region X′). The punctate staining in this latter region represents perinuclear SCG10 and not nuclear Brn-3.0 staining; see Fig. 2B′-D′ for higher magnification images showing that the neurons in this region do not express Brn-3.0.

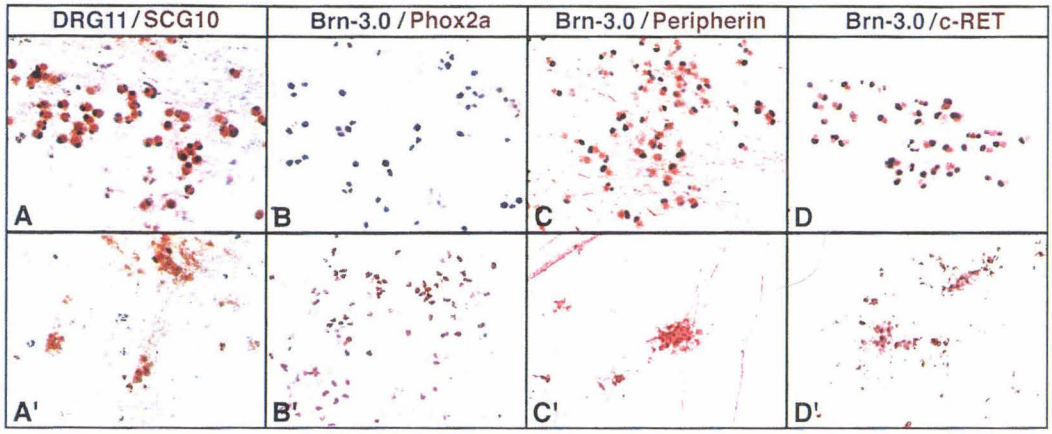


**Fig. 2.** Neural tube explant cultures contain peripheral sensory and autonomic neurons. Double-immunocytochemistry shows that large-diameter cells in the neural tube-proximal region of the outgrowth (Fig. 1, region X) express the sensory markers DRG11 (A) and Brn-3.0 (B-D; dark blue, nuclear). They also express SCG10 (A), peripherin (C) and c-RET (D; brown, cytoplasmic). Smaller-diameter cells in the same cultures (Fig. 1, region X′) express neither DRG11 (A′) or nor Brn-3.0 (B′,C′,D′), but do express SCG10 (A′), peripherin (C′), c-RET (D′) and the autonomic marker Phox2a (B′; brown, nuclear).

of peripherin (Parysek and Goldman, 1988) or c-RET (Pachnis et al., 1993). Peripheral autonomic neurons can be identified by their expression of the paired homeodomain factor Phox2a (Valarché et al., 1993; Tiveron et al., 1996) and either peripherin or c-RET, as well as by their lack of sensory markers (Table 1).

We first applied this array of markers to neural tube explants grown for 8 days in standard CEE-containing ('undefined') medium supplemented with the neurotrophic factors NGF, LIF, BDNF and NT3 (see Materials and Methods). Two populations of morphologically and antigenically distinct neurons were evident in the outgrowth of these cultures. Brn-3.0$^+$/SCG10$^+$ neurons had large somata and tended to be located proximal to the neural tube (Fig. 1X). Neurons in this location also expressed DRG11 (Fig. 2A), peripherin (Fig. 2C) and c-RET (Fig. 2D). We conclude that the large, neural tube-proximal neurons are sensory in nature. It was not possible to double-label such neurons for Brn-3.0 and Phox2a (Fig. 2B,B′) because the chromogen used to visualize Brn-3.0 labeling obscured that used for Phox2a. However, in cultures labeled only for Phox2a, the neurons with large cell bodies located proximal to the neural tube generally did not express the autonomic marker (data not shown).

**Table 1. Marker combinations identify peripheral sensory and autonomic neurons**

| | Brn-3.0[a] | DRG11[b] | Phox2a[c] | c-RET[d] | Peripherin[e] |
|---|---|---|---|---|---|
| PNS | | | | | |
| sensory neurons | + | + | − | + | + |
| autonomic neurons | − | − | + | + | + |
| CNS | | | | | |
| dorsal neural tube | + | + | − | − | − |
| preganglionic autonomic neurons | n.d. | n.d. | + | − | − |
| motor neurons | − | − | − | + | + |

The table summarizes previously published expression data in the rodent embryo at trunk level. (a) Fedtsova and Turner, 1995; Xiang et al., 1995. (b) Saito et al., 1995. (c) Valarché et al., 1993; Tiveron et al., 1996. (d) Pachnis et al., 1993; Lo and Anderson, 1995. (e) Parysek and Goldman, 1988. (+) expression in tissue indicated. (−) no expression in tissue indicated. (n.d.) not determined.

A separate population of smaller neurons was observed scattered throughout the outgrowth, often found in tight clusters in the periphery (Fig. 1X′). These smaller neurons did not express Brn-3.0 (Figs 1X′, 2B′,C′,D′) or DRG11 (Fig. 2A′), but did express Phox2a (Fig. 2B′). These small, Phox2a$^+$ neurons expressed peripherin (Fig. 2C,C′) and c-RET (Fig. 2D,D′), indicating a peripheral, autonomic phenotype. Thus, the use of specific transcription factor markers indicates that neurons of both sensory and autonomic lineages are generated in neural tube explant cultures grown in CEE-containing medium. Qualitatively, many more autonomic than sensory neurons appeared to develop under these conditions. It is striking that the sensory and autonomic neurons in these explants are located proximal and distal to the neural tube, respectively, as are sensory and autonomic derivatives in vivo.

## Defined medium permits sensory but not autonomic neurogenesis

A previous study suggested that the differentiation of sensory neurons may be promoted by growth in defined medium lacking serum or CEE (Ziller et al., 1987). We therefore formulated a defined medium (DM) with the intention of determining the growth requirements of sensory and autonomic neurons in the absence of CEE (see Materials and Methods). When neural tube explants were grown for 4 days in such DM, greater than 95% of the neurons in the outgrowth were Brn-3.0$^+$ as determined by double labeling for Brn-3.0 and NeuN, a pan-neuronal marker (data not shown). The Brn-3.0$^+$ cells had a neuronal morphology, expressed NF160 (Fig. 3A, inset)

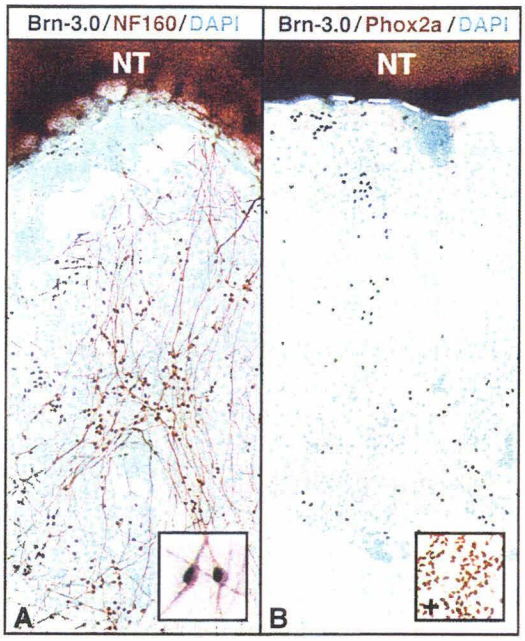


Fig. 3. Defined medium supports sensory but not autonomic neuronal differentiation. (A,B) Cells stained with an antibody to Brn-3.0 (dark blue, nuclear) are found in the crest outgrowth of explants grown for 4 days in defined medium. The cultures in (A) have been counter-stained with an antibody to NF160 (brown filamentous staining). The dashed line divides the neural tube (NT, top) and the outgrowth (below dashed line). (A, inset) Brn-3.0$^+$ cells co-express NF160 (brown, cytoplasmic; see also Fig. 4). (B) Cells in the outgrowth of these explants are not labeled with an antibody to Phox2a (brown, nuclear). Phox2a staining on BMP2-treated cultures (see also Fig. 6) serves as a positive control (inset in B). Unlabeled cells are visualized with DAPI fluorescence (aqua, nuclear).

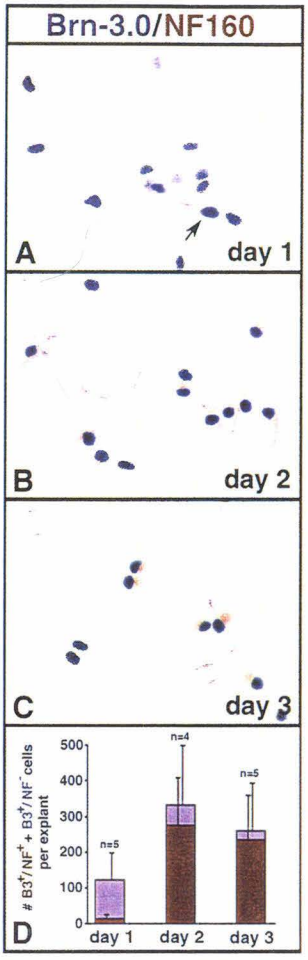


Fig. 4. Sensory precursors differentiate into neurons by day 2-3 in defined medium. (A-C) Double-labeling for Brn-3.0 (dark blue, nuclear) and NF160 (brown, cytoplasmic) antigens shows that after 1 day of culture, few Brn-3.0$^+$ cells in the neural crest outgrowth co-express NF160 (A, arrow). When this outgrowth (minus neural tube) is cultured until day 2 (B) or day 3 (C), a majority of Brn-3.0$^+$ cells co-express NF160. (D) The graph indicates the average number of Brn-3.0$^+$ cells per explant at each day, divided into Brn-3.0$^+$/NF160$^-$ (blue part of bar) and Brn-3.0$^+$/NF160$^+$ (brown part of bar) fractions. Note that as the total number of Brn-3.0$^+$ cells per explant increases from day 1 to day 2, the proportion of these cells that express NF160 increases as well. (n) is the number of explants scored from two experiments. Error bars represent standard deviations.

and peripherin (data not shown), and made up 6.6±2.8% of cells in the outgrowth. In contrast, autonomic (Brn-3.0$^-$/Phox2a$^+$) cells were almost never observed under these conditions (0.06±0.12% of cells in the outgrowth, Fig. 3B). Thus, this DM was permissive for sensory but not autonomic neuronal differentiation. The sensory neurons in DM cultures were not restricted to any particular region along the proximodistal axis of the outgrowth, in contrast to CEE-containing cultures where they were located proximal to the neural tube.

### Sensory neurons differentiate de novo from migratory precursors

It was formally possible that the sensory neurons that we observed in vitro did not differentiate de novo, but derived from already-differentiated neurons that simply migrated onto the culture substratum. To address this possibility, we compared the expression of Brn-3.0, which is found in both sensory precursors and sensory neurons (Fedtsova and Turner, 1995; Xiang et al., 1995), to that of NF160, an early marker of neuronal differentiation. In cultures grown for 24 hours in DM, only a minority of the Brn-3.0$^+$ cells that had migrated onto the substratum co-expressed NF160 (Fig. 4A,D). However, when such 24 hour outgrowth was cultured for an additional day following removal of the neural tube (to prevent the emigration of additional precursors), most of the Brn-3.0$^+$ cells co-expressed NF160 and had acquired a neuronal morphology (Fig. 4B,D). By day 3, no further increase in NF160 expression was apparent, although the neuronal somata appeared larger (Fig. 4C,D). The increase in the percentage of Brn-3.0$^+$ cells that co-expressed NF160 between day 1 and day 2 did not appear to be due to the selective death of undifferentiated Brn-3.0$^+$/NF160$^-$ cells, since the average number of Brn-3.0$^+$ cells per explant increased, rather than decreased, during that time period (Fig. 4D). These data suggest that the Brn-3.0$^+$/NF160$^+$ sensory neurons in these explants differentiate de novo from Brn-3.0$^+$/NF160$^-$ precursors between day 1 and day 2 of

culture. Such differentiation can occur in a fully defined medium lacking exogenously supplied neurotrophic factors.

### Sensory neurons develop from dividing precursors

We were interested to determine whether the sensory precursors in our cultures divided prior to differentiating. Previous studies have reported that the sensory neurons that differentiate rapidly in neural crest cultures are derived from postmitotic precursors (Ziller et al., 1983, 1987; Matsumoto, 1994b). To determine the mitotic activity of the sensory precursors found in our cultures, explants were exposed to 24-hour pulses of 175 nM BrdU beginning at the time of plating (day 0), or at day 1, day 2 or day 3. Immediately after the pulse, the cells were fixed and processed for Brn-3.0/BrdU double immunofluorescence. Again, the neural tube was removed from the cultures after 24 hours to avoid the influx of new precursors into the outgrowth.

Surprisingly, when BrdU was applied at the time of plating, greater than 95% of the Brn-3.0$^+$ cells in the outgrowth had incorporated the analog by the end of day 1 (Fig. 5A,E). During the next day (day 1-2), BrdU was incorporated into approximately 50% of Brn-3.0$^+$ cells (Fig. 5B,E). The percentage of BrdU$^+$/Brn-3.0$^+$ cells dropped dramatically on subsequent days of incubation: less than 5% of Brn-3.0$^+$ cells were labeled with BrdU when it was applied during day 2-3 (Fig. 5C,E), and there was essentially no labeling when it was applied during day 3-4 (Fig. 5D,E). These results indicate that most sensory precursors in our cultures are mitotically active as they leave the neural tube. Moreover, many of these cells divide during the subsequent day in culture. After two days, most of the sensory precursors have become postmitotic.

### BMP2 does not prevent either dividing or postmitotic precursors from executing a sensory fate

If the sensory and autonomic neurons that differentiate in explant cultures (Figs 1, 2) arise from a common precursor, conditions that promote differentiation of autonomic neurons might do so at the expense of sensory neurons. To explore this possibility, we challenged sensory precursors with 50 ng/ml of BMP2, a signal that instructs some neural crest precursors to become autonomic neurons at the expense of other cell fates (Shah et al., 1996; Shah and Anderson, 1997; Morrison et al., 1999). After removal of the neural tube at 24 hours, BMP2 was applied during the second day, a period during which similar cultures grown in DM contain many dividing sensory precursors (see Figs 4A, 5B,E, respectively). Following a 24 hour exposure of such cultures to BMP2, a vast number of cells expressed Phox2a (Fig. 6B, brown nuclei), approximately 5- to 10-fold more than the number of cells expressing Brn-3.0. Thus, although cultures grown in DM do not contain autonomic neurons, treatment of such cultures with BMP2 reveals a large population of neural crest cells with autonomic capacity. Despite this huge induction of autonomic neurogenesis by BMP2, the percentage of Brn-3.0$^+$ neurons per explant did not differ significantly when cultures were grown in the absence (Fig. 6A,C) or presence (Fig. 6B,C) of the factor. Furthermore, the exposure of explants to BMP2 did not seem to affect the total number of Brn-3.0$^+$ cells per explant ($z$=0.995, Mann-Whitney U-test, data not shown). These data suggest that the sensory precursors present in these cultures cannot be diverted to an autonomic fate by BMP2, although this factor clearly induces autonomic neurogenesis in many other cells in the explants.

As shown above, approximately 50% of the Brn-3.0$^+$ cells in cultures grown in DM come from precursors that divided between day 1 and day 2. (see Fig. 5B,E). Although BMP2 did not drastically reduce the percentage or number of Brn-3.0$^+$ cells per explant, the scatter in the data might conceal the inhibition by BMP2 of sensory differentiation from a subset of dividing precursors. When BMP2-treated cultures were labeled with BrdU during day 2, however, we found that the factor did not prevent the differentiation of Brn-3.0$^+$ neurons from

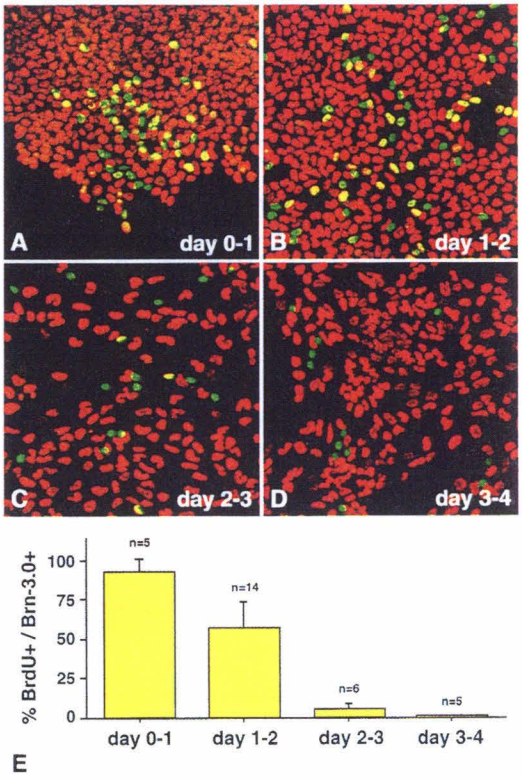


**Fig. 5.** Sensory precursors divide during the first two days of culture. (A-D) Confocal images show Brn-3.0 (green) and BrdU (red) double-immunofluorescence in cultures of neural crest outgrowth pulsed with BrdU for 24 hours at either the time of plating at day 0-1 (A), from day 1-2 (B), day 2-3 (C) or day 3-4 (D). Strongly double-labeled cells appear yellow. (E) The percentage of Brn-3.0$^+$ cells that incorporated BrdU during the previous 24 hours was quantitated per explant and is represented as an average. n is the number of explants from 2-3 experiments, error is given as standard deviation. Note that the high percentage of Brn-3.0$^+$ cells labeled by BrdU at day 0-1 and 1-2 is consistent with the more than doubling in the total number of Brn-3.0$^+$ cells per explant during this period (Fig. 4D).

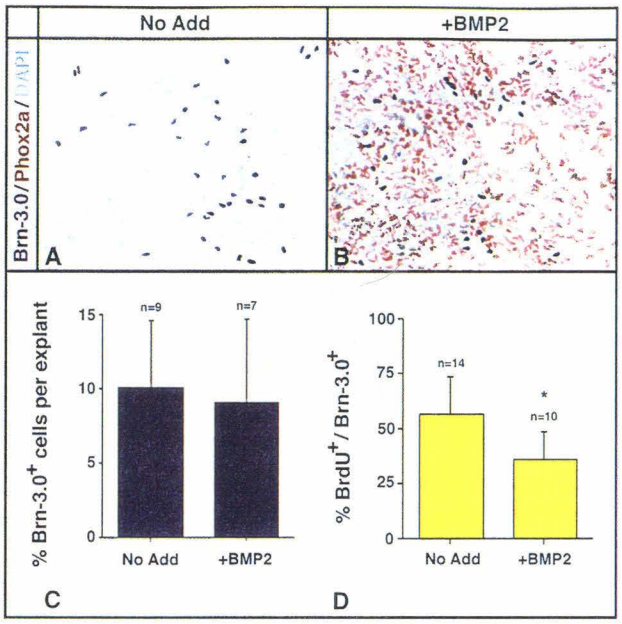


**Fig. 6.** BMP2 induces Phox2a but does not prevent expression of Brn-3.0 or division of sensory precursors. (A,B) Double labeling for Brn-3.0 and Phox2a at day 2 indicates that Brn-3.0$^+$ cells (dark blue, nuclear) are present in cultures of neural crest outgrowth which have been grown for 24 hours in either the absence (A) or presence (B) of BMP2. Phox2a$^+$ cells (brown, nuclear) are only found in BMP2-treated cultures (B). Unlabeled cells are visualized with DAPI fluorescence (aqua nuclei in A,B). Note that the massive induction of Phox2a by BMP2 in 24 hours is unlikely to be explained by the selective survival or proliferation of a subset of Phox2a$^+$ cells present in control cultures; therefore BMP2 is likely to act instructively rather than selectively. (C) Quantitation of the average percentage of Brn-3.0$^+$ expression per explant at day 2, after 24 hours of growth in 'No Add' or in 50 ng/ml BMP2. The data show that there is no statistically significant reduction in the percentage of Brn-3.0$^+$ cells by BMP2 ($P$=0.705, $t$-test). Similar results were obtained when the total number of Brn-3.0$^+$ cells per explant, rather than the percentage of Brn-3.0$^+$ cells per explant, was scored (not shown). (D) The average percentage of BrdU incorporation into Brn-3.0$^+$ cells per explant decreases slightly, but significantly (*$P$=0.004, $t$-test), in the presence of BMP2. In C and D, n is the number of explants counted over 3 experiments; error is represented as standard deviation.

dividing precursors, although it did reduce slightly (by about 35%) the proportion of Brn-3.0$^+$ cells labeled by BrdU (Fig. 6D). These data suggest that the majority of dividing sensory precursors in these cultures cannot be respecified by an autonomic neuron-inducing signal. The slight decrease in the percentage of Brn-3.0$^+$ cells that incorporated BrdU could indicate that BMP2 either promotes cell cycle arrest in a minority of sensory precursors, kills some of the cycling precursors, or else diverts a small subpopulation of them to an autonomic fate.

### Identification of neural crest cells expressing early sensory precursor-specific markers

The previous data indicated that neural tube explants grown in DM contain a population of mitotically active sensory neuron precursors that cannot be diverted to an autonomic fate by exposure to BMP2 during the second day of culture. However, these data did not exclude the possibility that more immature sensory progenitors, such as those that do not yet express Brn-3.0, may be respecified by the factor. We therefore sought to directly visualize such precursors in order to examine their response to BMP2. To do this, we used the bHLH transcription factors *ngn1*, *ngn2* and *neuroD* as early markers of sensory precursors. In vivo, *ngn2* is transiently expressed by some migrating crest cells, while *ngn1* is expressed slightly later following condensation of the sensory ganglia (Sommer et al., 1996; Perez et al., 1999; Ma et al., 1999). *neuroD* expression is downstream of and genetically dependent on the *ngns* (Ma et al., 1996, 1998; Fode et al., 1998). Expression of *neuroD* appears to overlap the period during which Brn-3.0 is expressed during DRG formation in vivo (Fedtsova and Turner, 1995).

In situ hybridization of 24 hour explant cultures revealed cells expressing *ngn2* mRNA both in the neural tube and in the immediately adjacent outgrowth (Fig. 7B,E, arrows). Combined in situ hybridization and antibody staining indicated that most of the *ngn2*$^+$ cells in the outgrowth did not co-express Brn-3.0 (Fig. 7E, arrowheads), although a few examples of *ngn2*$^+$/Brn-3.0$^+$ cells could be found (Fig. 7E, inset). This relative lack of overlap is consistent with the fact that expression of *ngn2* is initiated prior to that of Brn-3.0 in vivo (Fedtsova and Turner, 1995; Sommer et al., 1996; Ma et al., 1999), but the identification of double-labeled cells supports the idea that at least some Brn-3.0$^+$ sensory neurons derive from *ngn2*-expressing precursors (see below). Expression of *ngn1* was not detected among emigrating crest cells in such 24 hour explants (Fig. 7A), but was detected a day later (Fig. 8C), consistent with the fact that its expression follows that of *ngn2* in vivo (Sommer et al., 1996; Ma et al., 1999).

Numerous *neuroD*$^+$ cells were also found throughout the outgrowth (Fig. 7C,F arrows) as well as in the neural tube (data not shown). The *neuroD*$^+$ cells in the outgrowth tended to be located more distal to the neural tube than *ngn2*$^+$ cells (Fig. 7B,C), suggesting that the *neuroD*$^+$ cells may have emigrated early and have already downregulated expression of *ngn2* (Lee et al., 1995; Ma et al., 1996, 1998; Fode et al., 1998). In contrast to *ngn2*, most *neuroD*$^+$ cells co-expressed Brn-3.0$^+$ (Fig. 7F, arrows and arrowheads; inset). Given the genetic dependence of *neuroD* expression on *ngn2*, the *ngn2*$^+$/Brn-3.0$^-$ and the *neuroD*$^+$/Brn-3.0$^+$ precursors likely represent successive stages in sensory development. Consistent with this, forced expression of *ngns* can induce expression of Brn-3.0 both in vivo (S. Perez and D. J. A., unpublished data) and in vitro (L. Lo and D. J. A., unpublished data). Thus expression of *ngns* can be used to identify sensory precursors at an even

earlier stage than they can be identified by expression of Brn-3.0.

### BMP2 does not prevent expression of early, sensory precursor-specific markers

To determine if BMP2 might prevent the development of the early sensory precursors identified by expression of *ngn2* or *neuroD*, we exposed explants to this factor at the time of plating (before neural crest emigration) and assayed expression of these and other markers after 24 hours. Although BMP2 induced expression of both Phox2a and MASH1 in the outgrowth of neural tube explants (Fig. 8F,G, arrows, compare Fig. 8A,B), it did not abolish the expression of *ngn1*, *ngn2* and *neuroD* in cells adjacent to the neural tube (Fig. 8H-J). If anything, the expression of these sensory precursor-specific genes in the explants may have been slightly elevated by exposure to BMP2 (Fig. 8, compare C versus H, D versus I, E versus J). As expected from the results described earlier, addition of BMP2 at the time of plating also did not prevent the expression of Brn-3.0 (Fig. 8A,F, arrowheads). These data indicate, qualitatively, that the survival and/or proliferation of early sensory precursors identified by expression of *ngns* and *neuroD* was not prevented by addition of BMP2, and that the factor did not suppress expression of these sensory bHLH genes. Moreover, as *ngn2* is expressed earlier than Brn-3.0, these results suggest that sensory precursors may be refractory to the autonomic-inducing activity of BMP2 even before they express Brn-3.0.

Strikingly, the MASH1$^+$ autonomic precursors revealed by addition of BMP2 occupied a more distal region of the outgrowth relative to the neural tube than did the sensory precursors identified by expression of the *ngns* (cf. Fig. 8G, arrows versus 8H, I and J). This complementary distribution is similar to that of the differentiated sensory and autonomic neurons that develop from these precursors (Fig. 1, compare X versus X′, Fig. 8F, compare arrowheads versus arrows). The reasons for this patterned distribution are unclear but are currently under investigation.

### The phenotype of sensory neurons generated in defined medium includes the proprioceptive and/or mechanoreceptive, but not nociceptive, sensory subtypes

The relative rapidity with which the Brn-3.0$^+$ sensory neurons developed in our cultures suggested that they may correspond to the early-differentiating subset of sensory neurons in vivo. Early-differentiating neurons are large-diameter cells that comprise primarily the proprioceptive and mechanoreceptive sensory subtypes (Lawson et al., 1974; Lawson and Biscoe, 1979; Lawson, 1992). We were therefore interested in defining the subtype(s) of the sensory neurons that differentiate in our cultures. Since it is known that different subsets of sensory neurons require specific neurotrophins for their survival (reviewed in Snider, 1994), we sought to determine the trophic support profile of the sensory neurons generated in vitro as one criterion to identify their subtype. Proprioceptive or mechanoreceptive sensory neurons require NT-3 or BDNF, while nociceptive sensory neurons require NGF or LIF (for review see Snider, 1994).

Explants were grown for 8 days in DM or DM supplemented on day 3 with NGF, LIF, NT3 or BDNF alone or in combination. Explants grown in DM contain hundreds of sensory neurons at day 4 (Fig. 3A,B and data not shown), but very few Brn-3.0$^+$ cells survived after 8 days of culture (Fig. 9, 'No Add'), suggesting that these neurons die in the absence of exogenous trophic support. Addition of 100 ng/ml of NGF or 50 ng/ml of LIF at day 3 did not effect a significant increase in the number of surviving Brn-3.0$^+$ cells (Fig. 9, NGF and LIF). Medium supplemented with 50 ng/ml of NT3 or BDNF, however, supported the survival of significantly more sensory neurons than were obtained in NGF or LIF (Fig. 9, NT3 and BDNF). When explants were grown in both BDNF and NT3, more sensory neurons survived than with either factor alone (Fig. 9, +NT3 +BDNF). Similar numbers of Brn-3.0$^+$ cells were observed in cultures grown in NT3 plus BDNF compared to those cultured in the entire cocktail of factors (Fig. 9, +NGF +LIF +NT3 +BDNF). Therefore, our cultures contain sensory

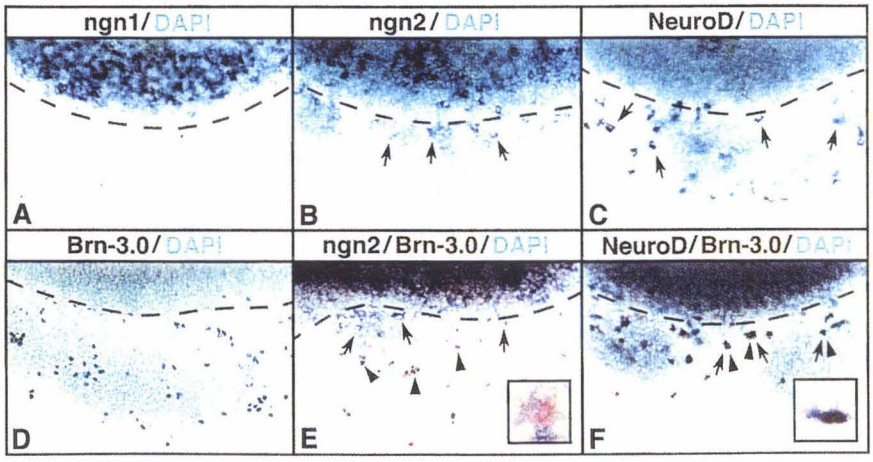


**Fig. 7.** Early sensory precursor-specific transcription factors are expressed in neural tube explant cultures at day 1. (A-F) Cells in the neural crest outgrowth (below dashed line) of explants grown in defined medium for 1 day express in situ signal (blue) for *ngn2* (B,E, arrows), and *neuroD* (C,F, arrows), and are immunopositive for Brn-3.0 (D, blue nuclei; E,F, brown nuclei, arrowheads). (A) At this stage *ngn1* expression is restricted to the neural tube (cells above dashed line). (E) Most *ngn2*$^+$ cells (arrows) do not co-express Brn-3.0 (arrowheads), although a few double-labeled cells can be found (inset). (F) In contrast, most *neuroD*$^+$ cells (arrows) do express Brn-3.0 (arrowheads; inset). The extent of the outgrowth is visualized with DAPI fluorescence (aqua).

neurons that can be supported by NT3 and BDNF, but not by NGF or LIF.

Different subpopulations of sensory neurons are also known to have distinct profiles of gene expression (e.g., see Lawson, 1992 for review). We therefore characterized the sensory neurons that appeared in explants grown for 8 days in DM supplemented with all four neurotrophic factors to determine if they expressed markers known to correlate with a particular class of sensory neurons. We found that sensory neurons grown under these conditions expressed a highly phosphorylated form of neurofilament recognized by the monoclonal antibody RT97 (Fig. 10A), the expression of which in vivo correlates with the large proprioceptive subpopulation of sensory neurons (Lawson et al., 1984). Additionally, the large neurons in neural

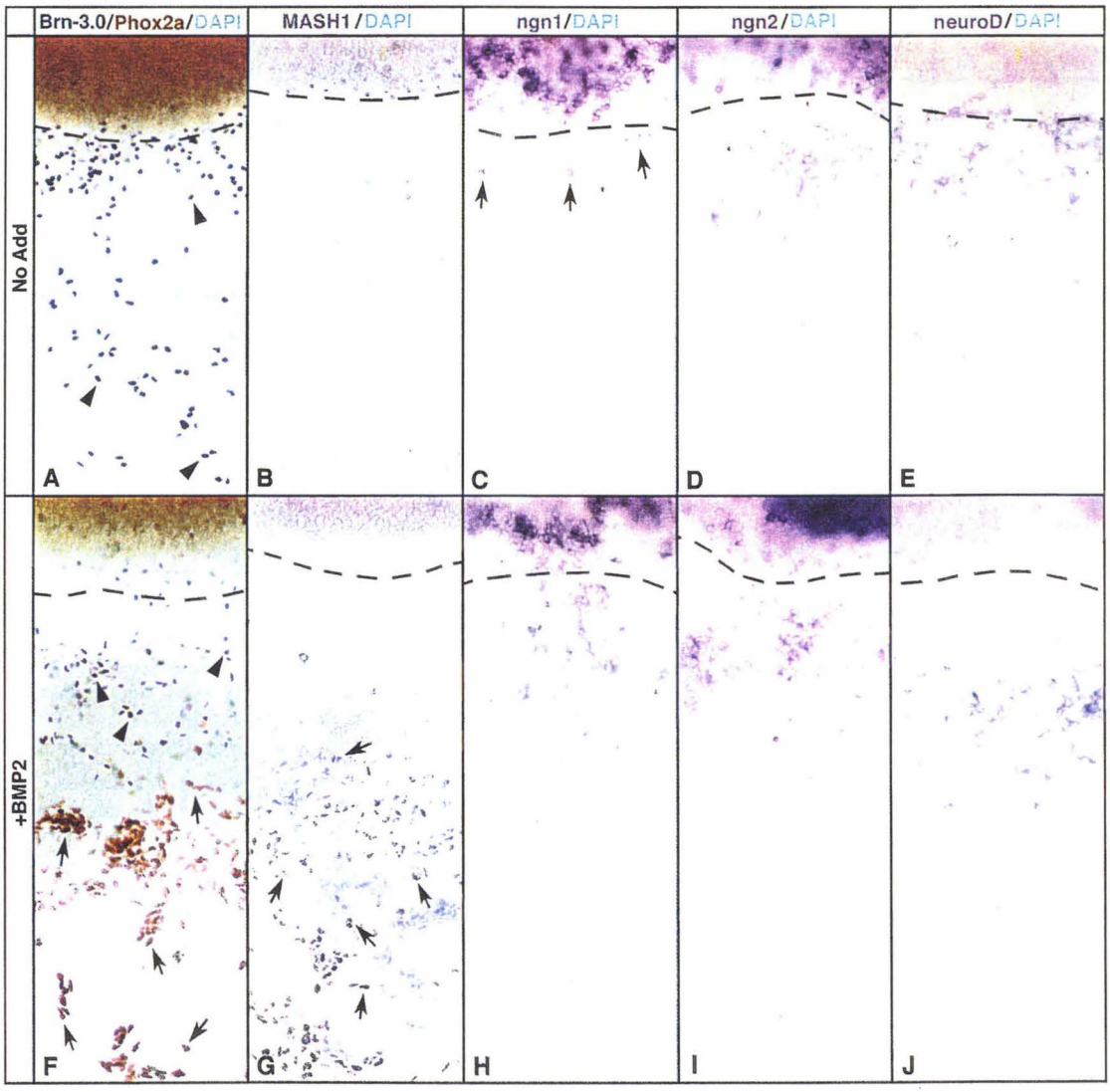


**Fig. 8.** Sensory precursor-specific markers persist in the presence of BMP2. Neural tube explants were grown in DM for 2 days in the absence (A-E) or presence (F-J) of BMP2. (A,F) Double labeling for Brn-3.0 (dark blue, arrowheads) and Phox2a (brown, arrows) shows that Brn-3.0$^+$ cells (arrowheads) are found in both conditions (A,F, arrowheads), but Phox2a$^+$ cells (arrows) are found only in the presence of BMP2 (F). (B,G) Similarly, cells immunopositive for MASH1 (arrows, purple nuclei) are found only in BMP2-treated explants (G) but not in 'No Add' conditions (B). (C-E versus H-J, purple in situ signal) At this stage, explants grown in the absence of BMP2 (C-E) contain a few cells in the outgrowth expressing *ngn1* (C, arrows), and many more expressing *ngn2* (D) and *neuroD* (E). Explants grown in the presence of BMP2 (H-J), contain cells expressing similar or greater levels of *ngn1* (H), *ngn2* (I), and *neuroD* (J).

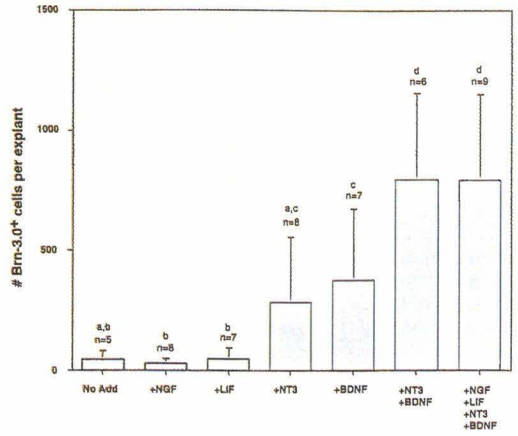


**Fig. 9.** NT3 and BDNF, not NGF or LIF, support significant populations of explant-derived sensory neurons. The graph represents the total number of Brn-3.0+ cells in the neural crest outgrowth per explant cultured for 8 days in defined medium in the absence or presence of different neurotrophins. There are more Brn-3.0+ cells in cultures grown in NT3 or BDNF than in cultures grown in DM ('No Add'), NGF or LIF. Cultures grown in NT3+BDNF and NGF+LIF+NT3+BDNF contain significantly more Brn-3.0+ cells than other conditions. n is the number of explants counted from 2-4 experiments. Error is represented as standard deviation. Statistics were compared by ANOVA followed by pair-wise *t*-tests. Statistically significant differences ($P<0.05$) are indicated by letters (e.g., a is different from b, but not from a or a, b).

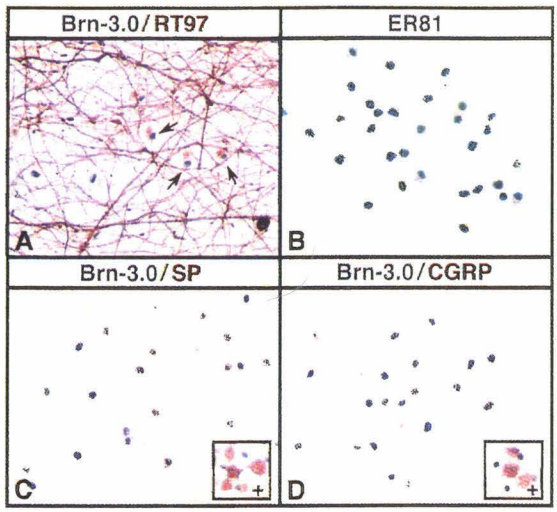


**Fig. 10.** Explant-derived sensory neurons express proprioceptive but not nociceptive, sensory subtype markers. (A-D) Neural tube explants were grown in defined medium in the presence of NGF, LIF, NT3 and BDNF for 8 days and stained for sensory subtype markers. (A) Double immunostaining for Brn-3.0 (dark blue nuclear) and RT97 (brown, cytoplasmic) reveals cells that co-express both markers (arrows) as well as many RT97+ processes. (B) The large diameter cells in these explants also express ER81 (dark blue, nuclear), a marker of proprioceptive sensory neurons (Lin et al., 1998). (C,D) In contrast, Brn-3.0+ cells in these cultures (dark blue, nuclear) do not express detectable levels of SP (C) or CGRP (D) staining (brown, cytoplasmic), compared to dissociated postnatal DRGs stained in parallel as a positive control (C,D insets).

crest explants expressed ER81 (Fig. 10B), an ETS domain transcription factor also restricted to the proprioceptive sensory subtype (Lin et al., 1998). Conversely, these neurons did not show detectable expression of markers that correlate with the nociceptive phenotype (for reviews see Hunt et al., 1992; Akopian et al., 1996): the neuropeptides, SP (Fig. 10C) and CGRP (Fig. 10D), or trkA, the high-affinity receptor for NGF (data not shown).

## DISCUSSION

The timing and mechanism of sensory and autonomic lineage segregation have been of interest for many years (Le Lievre et al., 1980; Schweizer et al., 1983; Le Douarin, 1986; Le Douarin et al., 1994). Here we have established a culture system in which mammalian sensory neurons differentiate from proliferating precursors in a defined medium. Although BMP2 induces massive autonomic neurogenesis in such explants, it does not prevent sensory neurogenesis. Furthermore, the survival and/or proliferation of early sensory precursors, identified by expression of several sensory-specific transcription factors, is not abolished by BMP2. These data provide evidence that a subset of neural crest cells are, operationally, committed to a sensory fate with respect to an autonomic inducing cue that is likely to be relevant in vivo (Reissman et al., 1996; Shah et al., 1996). Strikingly, the sensory neurons that develop from these precursors appear to assume a proprioceptive and/or mechanoreceptive, rather than nociceptive, phenotype. Such precursors may therefore correspond to a lineally (Frank and Sanes, 1991) and genetically (Ma et al., 1999) distinct subset of early-differentiating sensory precursors recently identified in vivo.

### Mammalian sensory neurogenesis in vitro

Considerable ambiguity has surrounded the identification of sensory neurons in mammalian neural crest cultures due to a lack of definitive markers. For example, large numbers of 'sensory-like' neurons, identified by expression of calcitonin gene-related peptide (CGRP) and SubstanceP (SP) were shown to differentiate in mouse neural tube explant cultures (Murphy et al., 1991, 1994). These authors acknowledged that CGRP and SP are not definitive sensory markers; for example, they can be expressed by sympathetic neurons under certain conditions (Nawa and Patterson, 1990; Fann and Patterson, 1994a,b). Indeed, other studies have shown that CGRP and SP can be co-expressed with autonomic markers in individual neurons that develop from mouse neural crest cells when they are grown in an undefined medium (Matsumoto, 1994a,b). In our hands, neural crest cultures grown for long periods (8 days) in an undefined medium contain, qualitatively, many more autonomic than sensory neurons. Therefore, it is possible that many, if not all, of the 'sensory-like' neurons observed in cultures of mouse neural crest grown in undefined conditions

(Murphy et al., 1991, 1994) may in fact have been autonomic in nature. Alternatively, these neurons may have been of the nociceptive sensory subtype, which express CGRP and SP in vivo and which do not develop under the culture conditions described here.

In both mammalian and avian systems, early-differentiating sensory-like neurons in neural crest cultures have been reported to arise from postmitotic precursors (Ziller et al., 1987; Sieber-Blum, 1989; Matsumoto, 1994b). Using BrdU labeling, we have demonstrated that virtually all the Brn-3.0$^+$ sensory precursors present in our cultures at day 1 are proliferating and that they continue to divide for the next 1-2 days. Consistent with this, cell division also persists after the initial expression of Brn-3.0 by sensory precursors in vivo (Fedtsova and Turner, 1995). A likely explanation for the difference between our results and those obtained in mouse neural crest cultures (Matsumoto, 1994b) is that the concentration of BrdU used in the earlier study (10 μM) was toxic to sensory precursors. In this study, we used 175 nM BrdU to label sensory precursors; we found that 10 μM BrdU disrupted explant morphology and abolished Brn-3.0 expression, although BrdU was incorporated into many cells (A. G. and D. J. A., unpublished data). The establishment of a system in which mammalian sensory neurons differentiate de novo from proliferating precursors in a defined medium now opens the way to more detailed studies of the cell-intrinsic and cell-extrinsic factors controlling their development.

### The neural crest contains dividing precursors that generate sensory neurons even in the presence of an autonomic-inducing signal

Our results point to the existence of a subpopulation of dividing neural crest cells that execute a sensory neuron fate whether or not they are challenged with BMP2, an autonomic-inducing cue. In this sense, these precursors appear 'committed' to a sensory neuron fate. Under these defined culture conditions, autonomic neurons do not differentiate unless BMP2 is added to the medium. The simplest explanation for these observations is that there are separate precursors for sensory and autonomic neurons in our cultures (Fig. 11A, $P_S$ and $P_{AGM}$, respectively). However, it is also formally possible that the cultures contain multipotent progenitors that generate both sensory and autonomic neurons in BMP2 (Fig. 11B, $P_{SAGM}$). In order to explain why BMP2 would not induce autonomic neurogenesis from such progenitors at the expense of a sensory fate, it is necessary to make two independent assumptions: first, these multipotent cells must be themselves unresponsive to BMP2 (Fig. 11B, $P_{SAGM}$), but divide to generate an autonomic

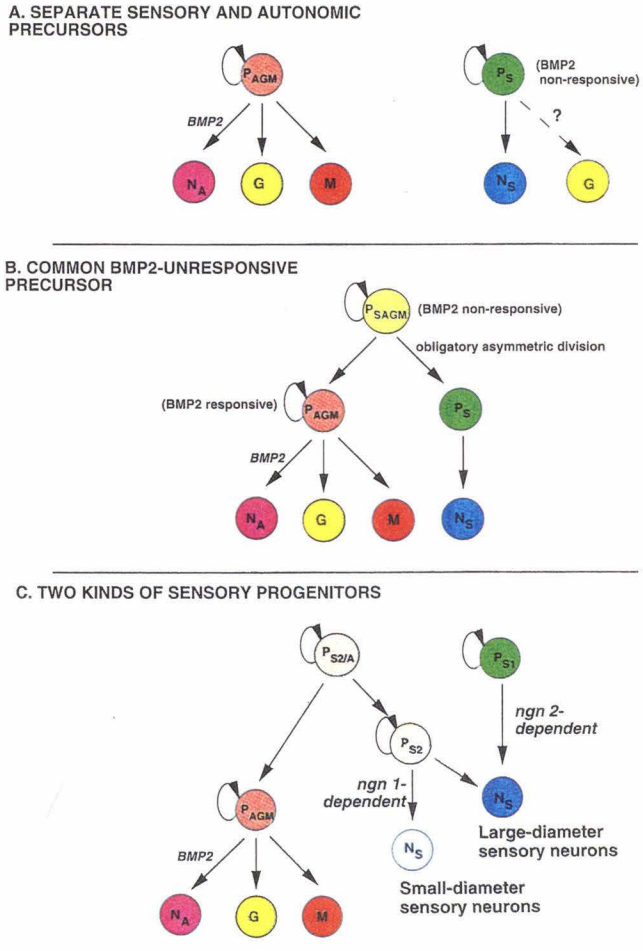


**Fig. 11.** Origins of sensory neurons in vitro and in vivo. (A,B) Alternative explanations for our in vitro observations; (C) represents a speculative model for sensory neurogenesis in vivo. In this schematic, straight arrows indicate potential and curved arrows indicate division, not self-renewal. (A) Sensory precursors ($P_S$) and their progeny do not have the potential to execute an autonomic neuronal fate ($N_A$) in response to BMP2. Instead, they generate only sensory neurons ($N_S$) or sensory neurons and glia (G). In this model, autonomic neurons are derived from a separate population of precursors ($P_{AGM}$), which are assumed to have glial and myofibroblast (M) potential like neural crest stem cells (Shah et al., 1996; Morrison et al., 1999). (B) A common precursor to sensory and autonomic neurons ($P_{SAGM}$) whose sensory differentiation is not inhibited by BMP2. Such a model assumes that this cell undergoes obligate asymmetric divisions that generate a sensory precursor ($P_S$) and a BMP2-responsive autonomic progenitor ($P_{AGM}$). The sensory daughter of the asymmetric division may divide symmetrically before it differentiates (not illustrated). (C) Two classes of sensory progenitors are postulated to exist in vivo: those committed to a sensory fate ($P_{S1}$) and those that can generate both autonomic and sensory neurons ($P_{S2/A}$). The former are *ngn*-2-dependent (Ma et al., 1999) and are fated to generate primarily large-diameter (proprioceptive/mechanoreceptive) sensory neurons (Frank and Sanes, 1991), but are not necessarily committed to this sensory subtype. The latter give rise to both autonomic progenitors ($P_{AGM}$) and *ngn1*-dependent sensory precursors ($P_{S2}$). These sensory precursors generate both large-diameter and small-diameter (nociceptive) sensory neurons (Frank and Sanes, 1991; Ma et al., 1999).

progenitor that *is* BMP2-responsive (Fig. 11B, $P_{AGM}$); second, such cell divisions must be asymmetric, so as to always generate a daughter that differentiates into a sensory neuron even in the presence of BMP2 (Fig. 11B, $P_S$). (Alternatively, equivalent cells could divide symmetrically to generate with equal probability either sensory or autonomic precursors.) In this way, such precursors would be 'committed' to generating sensory neurons in BMP2, but not be restricted from an autonomic fate. The inability to obtain sensory neuron differentiation in clonal cultures (A. G. and D. J. A., unpublished observations) and the low percentage of sensory neurons that develop in our explants makes it difficult to distinguish between these models by direct lineage analysis. However, we favor the former model (Fig. 11A) because it makes fewer assumptions.

Our use of BMP2 to challenge sensory precursors raises the question of whether this factor is a biologically relevant signal for testing the commitment state of these cells. BMP-family members have been shown to induce autonomic differentiation in a highly robust manner in multiple contexts. BMP2, BMP4 and BMP7 are able to induce expression of autonomic markers in cultures of neural crest cells from either avian (Varley et al., 1995; Reissman et al., 1996; Varley and Maxwell, 1996) or mammalian (Shah et al., 1996; Shah and Anderson, 1997; Morrison et al., 1999) embryos. Overexpression of BMPs in vivo has also been shown to induce autonomic markers (Reissman et al., 1996). In vivo, BMPs are expressed by tissues (e.g., dorsal aorta) near sites of autonomic differentiation (Reissman et al., 1996; Shah et al., 1996) and these tissues can mimic the inductive effects of BMPs (Reissman et al., 1996). Taken together, these data argue that BMP family members are likely acting as autonomic inducers in vivo. Therefore, although the sensory precursors that we have studied may not be committed with respect to other inductive cues, their insensitivity to BMP2 is likely to be of particular significance for sensory development in vivo.

BMP2 also constitutes a particularly stringent challenge of sensory precursors because it has been shown to act instructively on rat neural crest stem cells, causing them to adopt an autonomic neuronal fate at the expense of alternative fates (Shah et al., 1996). Because our assay for sensory neurogenesis could not be performed using clonal cultures, we were unable directly demonstrate that BMP2 acts instructively in this system. However, the large induction of autonomic markers that occurs during a 24 hour exposure to this factor (approximately, 0% Phox2a$^+$ cells in 'No Add' versus 25-50% in BMP2; see also Fig. 6) is consistent with an instructive effect of BMP2. Given that BMP2 has been shown to act instructively on rat neural crest cells isolated from two different sources and grown in two different culture media (Shah et al., 1996; Morrison et al., 1999), it is highly likely that the factor acts instructively in this system as well.

Earlier studies in avian embryos have been interpreted to suggest that the neural crest contains a subset of precursors restricted to a sensory fate (Ziller et al., 1987; Sieber-Blum, 1989; Duff et al., 1991). Although this general conclusion is similar to that which we have drawn here, the evidence supporting it differs in several important respects from our own results. In one series of experiments, sensory-like neurons were observed to differentiate in a defined medium; sensory differentiation was prevented by serum, which promoted autonomic neurogenesis (Ziller et al., 1983, 1987). These observations were interpreted to suggest that sensory neurons developed from separate precursors that were restricted from an autonomic fate. However, since sensory neurons failed to appear in serum-containing medium, it could not be determined whether the sensory precursors died or were converted to other fates. Other investigators have also concluded that some sensory precursors are restricted from an autonomic fate because they do not produce autonomic neurons under culture conditions that allow autonomic neurogenesis (Sieber-Blum, 1989; Duff et al., 1991). In neither of these studies, however, were the sensory precursors challenged by addition of defined, instructive autonomic-inducing signals. The fact that autonomic neurons differentiate under a given set of culture conditions may simply reflect the presence of factors permissive for such differentiation, in which case they are not an adequate challenge of the state of commitment of sensory precursors.

### Multiple sensory sublineages in the neural crest

The ability of some neural crest cells to give rise to both sensory and autonomic neurons in vivo is well-documented (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). Nevertheless, the notion that migrating neural crest also contains a subpopulation of precursors determined for a sensory fate is still consistent with the results of such lineage analyses. In one study, almost 50% (8/17) of migrating avian neural crest cells injected with a lineage tracer produced neurons exclusively in the DRG, rather than in both the DRG and sympathetic ganglia (Fraser and Bronner-Fraser, 1991). From such a lineage analysis alone, it is not possible to distinguish whether these clonal differences represent stochastic variations in fate among a developmentally equivalent precursor population, or rather distinct subpopulations that are intrinsically different. The present observations are consistent with the possibility that some lineage-marked crest cells that generated only sensory neurons in vivo were in fact determined for a sensory fate. Interestingly, a recent clonal analysis of migrating crest in vitro suggests that many precursors are fated to generate only neuronal progeny, although whether these neurons were sensory or autonomic was not investigated (Henion and Weston, 1997).

Our observations, taken together with previous studies, support the notion that there may be at least two classes of sensory precursors in the neural crest: those that are determined for a sensory fate early in neural crest ontogeny, and those that have a wider range of potentials including both sensory and autonomic fates (Bronner-Fraser and Fraser, 1988, 1989; Sieber-Blum, 1989; Fraser and Bronner-Fraser, 1991). Interestingly, sensory precursors in our cultures generate neurons that are predominantly of the proprioceptive or mechanoreceptive subtypes. Such cells may therefore correspond to a distinct sublineage of early-differentiating precursors that generate large-diameter sensory neurons (Fig. 11C, $P_{S1}$) that have been identified by a previous in vivo retroviral clonal analysis in chick (Frank and Sanes, 1991). Similar precursors are genetically dependent on *ngn2* but not *ngn1* in mice (Ma et al., 1999). This correspondence does not mean that *ngn2*-dependent precursors are necessarily committed to a proprioceptive/ mechanoreceptive subtype; for

example, they may simply differentiate at a time when signals that promote these subtypes are present.

If the progenitors that appear committed to a sensory fate in our cultures correspond to those fated to generate early-differentiating proprioceptive and mechanoreceptive neurons in vivo, then perhaps the common progenitors of sensory and autonomic neurons (Fig. 11C, $P_{S2/A}$) identified by in vivo lineage tracing (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991) are cells that give rise to the later-differentiating subpopulation of sensory precursors (Fig. 11C, $P_{S2}$; Lawson et al., 1974; Carr and Simpson, 1978; Lawson and Biscoe, 1979; Frank and Sanes, 1991). Such late-differentiating sensory precursors have been shown to be *ngn1*-dependent (Ma et al., 1999), and to give rise to both small-diameter (nociceptive) and large-diameter (proprioceptive/mechanoreceptive) neurons (Frank and Sanes, 1991; E. Frank, personal communication). At present it is not clear why only the early-differentiating sensory precursors develop in our cultures. One possibility is that the later-differentiating precursors may not migrate from the explanted neural tube. Alternately, they may migrate but not differentiate into, or survive as, nociceptive sensory neurons. In the absence of conditions to elicit differentiation of such nociceptive neurons, we cannot say whether the BMP2-responsive autonomic precursors do or do not have sensory potential. These cells may correspond to a common sensory-autonomic precursor from which we are currently able to elicit autonomic but not sensory neurogenesis (Fig. 11C, $P_{S2/A}$); alternately, they may already be restricted to an autonomic fate (Fig. 11C, $P_{AGM}$).

### Early determination of some sensory precursors may prevent them from responding to autonomic-inducing signals as they migrate

When do sensory precursors become determined? Our data reveal a correlation between the resistance of sensory precursors to an autonomic inducing signal and the persistence of *ngn2*-expressing cells despite the presence of this signal. Forced expression of *ngns* in chick neural crest cells in ovo can bias their distribution to the DRG and can induce sensory-specific genes including Brn-3.0 (Perez et al., 1999; S. Perez and D. J. A., unpublished data). These data are consistent with the idea that some progenitors may become determined for a sensory fate as early as the time they express *ngn2*. In vivo, *ngn2* is expressed by a subset of neural crest cells early in migration (Gradwohl et al., 1996; Sommer et al., 1996; Perez et al., 1999; Ma et al., 1999). Therefore, early expression of *ngn2* may identify a subset of neural crest cells that are specified for a sensory fate shortly after they emigrate from the neural tube, a conclusion consistent with some results from the in vivo lineage-tracing experiments mentioned earlier (Fraser and Bronner-Fraser, 1991). These cells may be similar or equivalent to those that we have studied here in vitro.

The early determination of some sensory precursors among the migrating neural crest may serve to make them resistant to the autonomic-inducing effects of locally expressed BMP-family members. Although these factors are likely to be important for autonomic neurogenesis in vivo, these same BMPs are also expressed in many tissues in the dorsal part of the embryo where autonomic neurons do not develop, including dorsal ectoderm (Liem et al., 1995), the dorsal neural tube (Liem et al., 1997) and the developing somites (Reshef et al., 1998). Because migrating neural crest cells are likely to encounter BMP family members in the dorsal part of the embryo, mechanisms must exist that prevent them from differentiating into autonomic neurons in this location. The early determination of some sensory progenitors with respect to BMPs may represent one such strategy. The fact that such determination occurs while the precursors are still mitotically active, moreover, would allow for the continued expansion of the sensory neuron population by postmigratory cells during the growth of the DRG (Duff et al., 1991). In this way, the early determination of a subset of dividing neural crest cells may play an important role in the growth and development of sensory ganglia.

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# Chapter 3

## The regulation of subtype characteristics in sensory neurons generated from mammalian neural crest in vitro

## SUMMARY

Sensory neurons that differentiate from rat neural crest cells in a chemically defined medium exhibit characteristics of the muscle afferent sensory subtype, including trophic dependence on NT-3 and BDNF, expression of the ETS-domain transcription factor ER81, and lack of trkA expression (Greenwood, A. L., Turner, E. E., and Anderson, D. J. (1999) *Development* **126**, 3545-3559). NT-3 and BDNF can dramatically upregulate the expression of ER81 in such neurons after they have differentiated. In contrast, in medium containing either serum or BMP2 many of the sensory neurons that differentiate do not express muscle afferent markers, but rather express trkA and VR1, markers of the cutaneous afferent sensory subtype. Unlike the neurotrophins, serum acts on the neural tube to induce the production or migration of progenitors of $trkA^+$ (as well as $trkA^-$) sensory neurons. Importantly, NT-3 and BDNF do not prevent the development of $trkA^+$ sensory neurons in serum-containing cultures. Furthermore, these neurotrophins are not required for the development of $trkA^-$ sensory neurons. These data suggest that NT-3 and BDNF are neither necessary nor sufficient to specify sensory subtypes in vitro, but rather up-regulate expression of certain markers in neurons already specified for a muscle afferent fate. In contrast, serum and BMP2 act on the neural tube to influence, directly or indirectly, the fate of neural crest cells that subsequently migrate. These data provide evidence that the generation of different subclasses of sensory neurons from neural crest cells can be controlled by manipulation of the culture environment, and are consistent with the idea that these subclasses are generated from multiple subpopulations of sensory neuron precursors.


## INTRODUCTION

An important goal of developmental neurobiology is to identify extrinsic signals that regulate particular aspects of neuronal identity and to discover when and how they exert

their effects. Such signals may regulate the acquisition of either single or multiple neuronal characteristics and may act on undifferentiated precursors or on already-differentiated neurons. It is also important to identify which cells can and cannot respond to these signals. Environmental cues that affect the development of the two main classes of neurons derived from the neural crest, sensory and autonomic neurons, have received significant attention (Patterson, 1992; Le Douarin et al., 1994; Anderson, 1997; Groves and Bronner-Fraser, 1999). However, the factors that regulate neuronal subtype diversity within each class are still largely unknown.

Sensory neurons of the dorsal root ganglia (DRG) exhibit considerable phenotypic heterogeneity. In particular, two major sensory subtypes have been well described (Scott, 1992). One subtype consists of muscle afferents that receive information about spatial position (proprioception) and send their central projections to the ventral spinal cord. These neurons tend to have large-diameter cell bodies in vivo, depend on neurotrophin-3 (NT-3) for survival and express the corresponding receptor, trkC (Mu et al., 1993; Klein et al., 1994; McMahon et al., 1994). Many of these neurons also express ETS-domain transcription factors, such as ER81 and PEA3, the expression of which is regulated by unidentified target-derived factors in vivo (Lin et al., 1998). Another class of sensory neurons is comprised of the cutaneous afferents that transduce painful stimuli (nociception) and make central connections in the dorsal horn of the spinal cord. Many of these neurons have small-diameter cell bodies, respond to capsaicin, and express the vanillinoid receptor VR1 that mediates this sensitivity (Tominaga et al., 1998). The survival of most nociceptive, cutaneous afferent neurons depends on nerve growth factor (NGF) during at least some point in their development, and many of these neurons express the high affinity NGF receptor, trkA (Smeyne et al., 1994). Other types of sensory neurons, such as those that mediate the sense of touch (mechanoreception), appear to be more heterogeneous in their properties.

It is not known how sensory subtypes are initially specified during development. It has been suggested that neurotrophins may specify sensory subtype identity as well as supporting the survival of different subtypes (Lewin, 1996). Although this model is intuitively appealing, it has been recently demonstrated that the initial specification of nociceptive sensory neurons does not require NGF (Patel et al., 2000). The situation is not so clear for other neurotrophins. Brain derived neurotrophic factor (BDNF) has been implicated in the specification of generic sensory neurons from quail neural crest cells both in vitro and in vivo (Sieber-Blum, 1991). It has not been determined, however, whether early exposure of sensory precursors to NT-3 or BDNF affects the subsequent development of sensory subtype characteristics.

We have begun to investigate how extrinsic factors regulate sensory subtype characteristics in neurons that develop from rat neural crest cells in vitro. Previously, we characterized the sensory neurons that develop in a chemically defined medium. We found that these neurons resemble muscle afferent sensory neurons by numerous criteria including trophic dependence on NT-3 and/or BDNF, the expression of the ETS-domain transcription factor ER81, and the lack of expression of trkA (Greenwood et al., 1999). Cutaneous afferent-like sensory neurons were, however, conspicuously absent from these cultures. This observation prompted us to look for factors that would promote the development of the cutaneous afferent-like sensory subtype. We were interested to know whether signals that could positively affect the development of one sensory subtype would negatively affect the development of the other. Such an observation would imply that both muscle and cutaneous afferent sensory neurons derive from a common precursor pool; alternately, the independent regulation of muscle and cutaneous afferent development might imply that these neurons come from different precursors among the neural crest. We have now identified conditions that promote the development of the cutaneous afferent sensory neuron subtype. Our data are consistent with the idea that

there are different subclasses of sensory neuron precursors in the neural crest, though these precursors are not necessarily restricted to particular sensory subtypes.

## MATERIALS AND METHODS

### Rat neural tube explant cultures

Neural tubes were isolated from the trunk level of E10.5 Sprague-Dawley rats as described previously (Stemple and Anderson, 1992; Greenwood et al., 1999). Explanted neural tubes were plated in 35 mm diameter Corning dishes on a fibronectin (BTI) substrate, and grown at 37°C in 5% $CO_2$/ 95% air.

### Medium and Additives

#### *Defined medium*

The recipe for defined medium (DM) was modified from that described previously (Stemple and Anderson, 1992; Greenwood et al., 1999) and is as follows: to L-15$CO_2$ (Hawrot and Patterson, 1979) add 100 μg/ml transferrin (Calbiochem), 5 μg/ml insulin (Sigma), 16 μg/ml putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM selenious acid (Sigma) 1 mg/ml bovine serum albumin, crystallized (Gibco/BRL), 39 pg/ml dexamethasone (Sigma), 5 μg/ml ∝-d-1-tocopherol (Sigma), 63 μg/ml ß-hydroxybutyrate (Sigma), 25 ng/ml cobalt chloride (Sigma), 1 μg/ml biotin (Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml glycerol, 100 ng/ml ∝-melanocyte-stimulating-hormone (Sigma), 10 ng/ml prostaglandin E1 (Sigma), 67.5 ng/ml triiodothyronine (Aldrich), and 10 ng/ml basic fibroblast growth factor (bFGF, UBI).

#### *Additives*

Where indicated, DM was supplemented with one or more of the following: recombinant human bone morphogenetic protein-2 (BMP2 at 10 ng/ml; gift from Genetics Institute), brain derived growth factor (BDNF at 25 ng/ml), neurotrophin-3, (NT3 at 25 ng/ml; BDNF and NT3 both provided by Dr. G. Yancopoulos at Regeneron Pharmaceuticals),

nerve growth factor (NGF at 25 ng/ml; UBI), and/or 10% fetal bovine serum (FBS; Hyclone, lot AHC817).

**Immunocytochemistry**

Cultures were fixed in 4% paraformaldehyde and then processed for double antibody labeling. All primary antibodies were incubated overnight at 4°C. All rabbit polyclonal antibodies were detected using an HRP-conjugated, goat anti-rabbit secondary antibody purchased from Vector Labs, Inc., at 1:200 dilution for 1 hour at room temperature. Staining was detected using NiDAB and DAB substrates as described previously (Shah et al., 1994). In all cases the first antibody applied was developed with NiDAB (dark blue) and the second with DAB (brown).

Affinity-purified rabbit anti-Brn-3.0 (1:1000; (Fedtsova and Turner, 1995)) was used to detect general sensory neurons. Rabbit polyclonal antibodies to ER81 (1:12,000; gift from Dr. T. Jessell; (Lin et al., 1998)), TrkA (1:5000; gift from Dr. L. Reichardt, (Clary et al., 1994)), and VR1 (1:5000; gift from Dr. D. Julius) were used to detect these sensory subtype-specific antigens.

## RESULTS

### Defined medium promotes the development of sensory neurons that display characteristics of the muscle afferent subtype

Our previous study indicated that a chemically defined medium can support the differentiation of sensory neurons from rodent neural crest cells derived from neural tube explants (Greenwood et al., 1999). However, these sensory neurons do not represent the full array of sensory subtypes that appear in vivo. Explant-derived sensory neurons, as identified by expression of the POU-domain transcription factor Brn-3.0 (Fedtsova and Turner, 1995; Xiang et al., 1995), differentiate in the absence of neurotrophins, but depend on NT-3 and BDNF, but not NGF, for long-term survival in culture. This

observation indicates that these neurons may be of the muscle afferent or mechanosensory fate, but not of the nociceptive cutaneous afferent fate. In addition, we have observed that sensory neurons that develop in defined medium in the presence of NT-3 and BDNF express ER81, (Fig. 1C) a marker that largely correlates with the muscle afferent fate in vivo (Lin et al., 1998), and do not express trkA (Fig. 1A, B), a marker of the cutaneous afferent fate. Although it is not possible to precisely determine the functional specificity of the sensory neurons that develop in these cultures due to lack of appropriate targets, the ability to be supported by NT-3, the expression of ER81, and the lack of expression of trkA are consistent with these sensory neurons having a muscle afferent identity.

To determine whether ER81 expression is an inherent characteristic of sensory neurons that differentiate in defined medium or whether it is regulated by NT-3 and BDNF, we looked at the expression of this transcription factor in newly-differentiated sensory neurons before the period of trophic dependence. Neural tube explant cultures were grown in defined medium in the absence of neurotrophins. The neural tube was removed from culture on day 2 to isolate the neural crest, and the cultures analyzed on day 3 for the presence of ER81 and Brn-3.0 expression by antibody staining. As shown previously, the majority of Brn-3.0$^+$ neurons in similar cultures are post-mitotic and express neurofilament at this time (Greenwood et al., 1999). In the absence of neurotrophins, the cultures contained many neurons that expressed Brn-3.0 (Fig. 2A, arrows), but only a few that expressed low levels of ER81 (Fig 2A, arrowheads). However, as little as a 1 hour exposure to BDNF and/or NT3 on day 3 resulted in a striking upregulation of ER81 in the majority of sensory neurons (Fig. 2B-D). Such a brief exposure to neurotrophins is unlikely to significantly affect survival or proliferation. Therefore, these data indicate that exogenously added NT-3 and BDNF are sufficient to

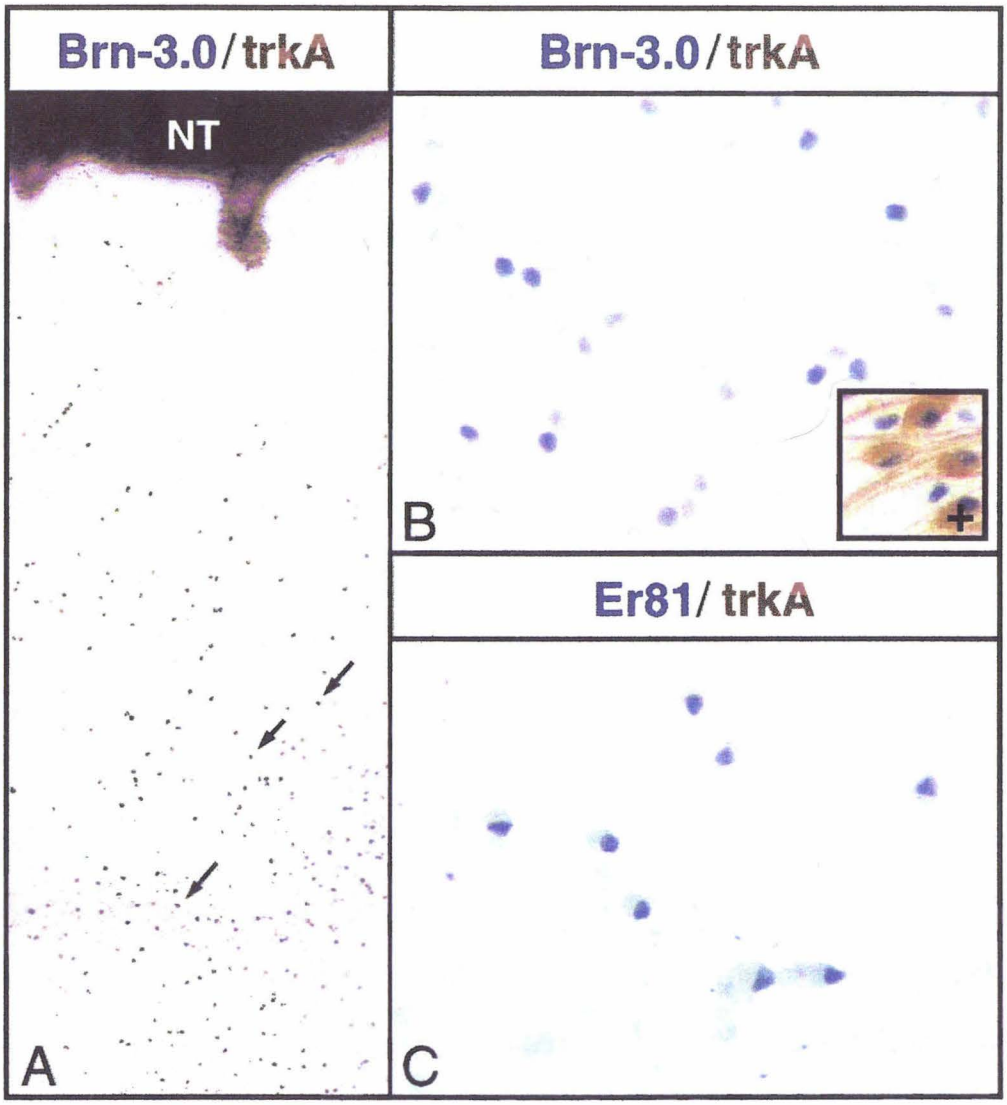


**Fig. 1** Defined medium supports the development of sensory neurons that have characteristics of the muscle afferent subtype. Rat neural tube explants were grown for 6 days in defined medium supplemented with NT-3 and BDNF (25 ng/ml ea.) and then double-labeled for Brn-3.0 (A, B; blue, nuclear) or Er81 (C; blue, nuclear) and trkA (A, B; brown, cytoplasmic) by immunocytochemistry. (A) The view shown includes the dorsal neural tube (NT; top) and neural crest outgrowth containing many Brn-3.0+/trkA- cells (arrows). (B) Higher magnification shows that the Brn-3.0+ cells clearly do not stain for trkA. (B, inset) TrkA staining on serum-treated cultures serves as a positive control. (C) The neurons grown under these conditions express Er81.

regulate ER81 expression in differentiated sensory neurons, but are not required for their initial development.

**Serum promotes the development of sensory neurons that display characteristics of the cutaneous afferent subtype**

Other studies of mammalian sensory neurons that differentiate in neural crest cultures have observed that these neurons express markers of the cutaneous afferent subtype (Murphy et al., 1991; Matsumoto, 1994a; Matsumoto, 1994b). In these studies, serum was included as a component of their medium. Therefore, we added serum to our medium to determine whether it would promote expression of cutaneous afferent markers and/or inhibit expression of muscle afferent markers. When neural tube explants were grown for 6 days in medium supplemented with 10% fetal bovine serum (FBS), we found that many Brn-3.0$^+$ neurons developed in the neural crest outgrowth. Most of these neurons were also immunopositive for trkA (Fig. 3A, B) and some expressed VR1 (Fig. 3C arrowheads), which is another marker of the cutaneous afferent phenotype. The Brn-3.0$^+$/trkA$^+$ sensory neurons in our explant cultures were found in large clusters adjacent to the neural tube (Fig. 3A). In the absence of exogenously added neurotrophins, sensory neurons in these cultures were only rarely immunopositive for ER81 (Fig. 3D). When these cultures were exposed for 1 hour to BDNF and NT-3 on day 6, ER81 was upregulated in minority of the sensory neurons (Fig. 3E). Most trkA$^+$ neurons did not express ER81 (Fig. 3E, -/+; 85.2 $\pm$ 3.7% of total trkA$^+$ cells), but of the cells that were induced to express ER81, 80.4 $\pm$ 5.2% were trkA$^+$ (Fig. 3E, +/+) and 19.6 $\pm$ 5.2% were trkA$^-$ (Fig. 3E, +/-). These data suggest that most of the sensory neurons that develop in serum-containing medium have a phenotype that is consistent with the cutaneous afferent fate; that is, they express trkA but not ER81 even in the presence of NT-3 and BDNF. The neurons that co-express trkA and ER81 in these cultures may represent a distinct

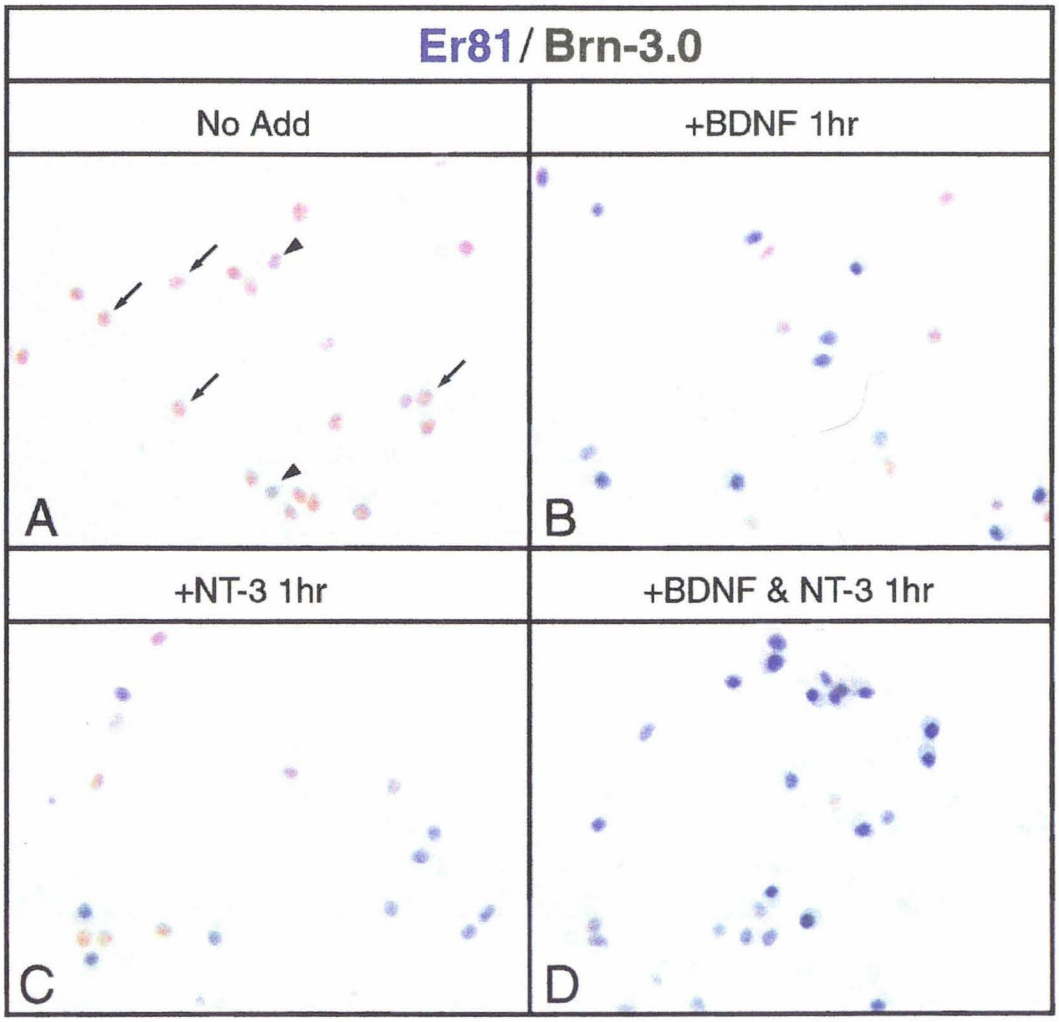


**Fig. 2** A one hour exposure to NT-3 or BDNF in the absence of the neural tube is sufficient to induce ER81 expression in sensory neurons grown in defined medium. The neural tubes were removed at day 2 from explants grown in defined medium; cultures were exposed to NT-3 and/or BDNF (25 ng/ml ea.) for 1 hour on day 3, fixed immediately afterward and processed for ER81 (blue, nuclear) and Brn-3.0 (brown, nuclear) antibody staining. (A) Only a small percentage of the sensory neurons (Brn-3.0 cells; arrows) are weakly positive for ER81 (arrowheads) in the absence of these factors. Cultures exposed for 1 hour to BDNF (B) or NT-3 (C) or both BDNF and NT-3 together (D) contain a much higher percentage of sensory neurons that strongly express ER81.

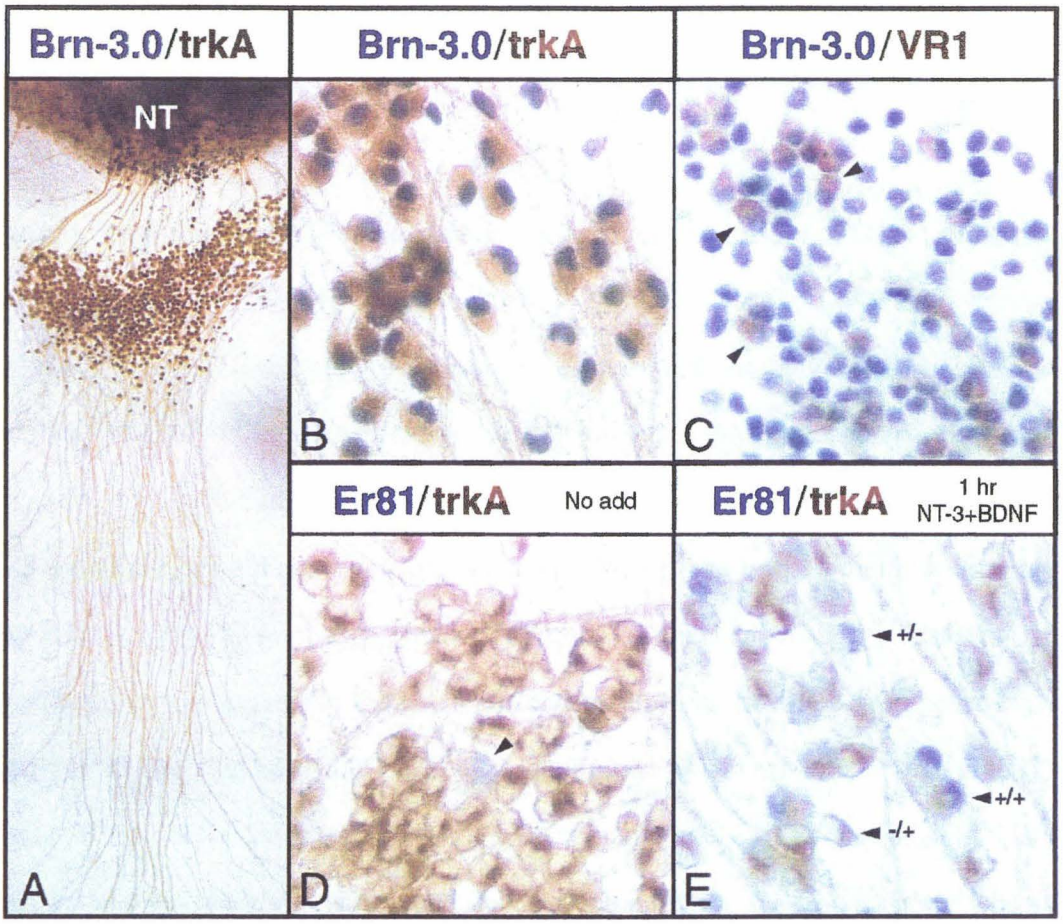


**Fig. 3** Serum-containing medium supports the development of sensory neurons that have characteristics of the cutaneous afferent subtype. Neural tube explants were grown for 6 days in medium supplemented with 10% FBS. (A) The view from the dorsal neural tube (NT, top) across the outgrowth shows many cells that co-express Brn-3.0 (blue, nuclear) and trkA (brown, cytoplasmic) in the area adjacent to the neural tube. (B) Higher magnification of similarly stained cultures shows that most Brn-3.0$^+$ cells express trkA. (C) Some of the Brn-3.0$^+$ cells in these cultures also express VR1 (brown, cytoplasmic; arrowheads). (D) Most of the trkA$^+$ cells in these cultures do not express Er81 (blue, nuclear), although an occasional weakly Er81$^+$ cell is observed (arrowhead). (E) When such cultures are exposed for 1 hour to NT-3 and BDNF (25 ng/ml ea.) on day 6, Er81 is upregulated in some trkA$^-$ (+/- arrowhead) and some trkA$^+$ (+/+ arrowhead) cells. Most trkA$^+$ cells do not express Er81 under these conditions (-/+ arrowhead).

subpopulation of trkA$^+$ neurons that co-express trkB or trkC, such as has been observed in vivo (McMahon et al., 1994; Wright and Snider, 1995).

**Serum is required for the development of significant numbers of trkA$^+$ sensory neurons in explant cultures**

To understand why trkA$^+$ sensory neurons develop in medium that contains serum, we examined whether serum is required and/or sufficient for the expression of trkA. As mentioned above, explants grown for 6 days in serum-free medium supplemented with NT-3 and BDNF do not contain trkA$^+$ cells, suggesting that defined medium does not support the development of trkA$^+$ sensory neurons. Consistent with this observation, we found that explants grown for 4 days in defined medium in the absence of neurotrophins contained mostly trkA$^-$/Brn-3.0$^+$ cells. These cultures, however, do contain a small fraction of Brn-3.0$^+$ cells that expressed trkA (approximately 10%), but most of these neurons had only weak expression and only occasionally did we find a strongly positive cell (Fig. 4A, arrowhead). Overall, the trkA$^+$ neurons found in explants grown in defined medium were far less numerous and had much weaker staining than those observed in explants that had been exposed to 10% FBS for the first two days of culture. In such serum-treated cultures, 28.0 $\pm$ 3% of the Brn-3.0$^+$ cells co-expressed trkA, and many of these had strongly stained cell bodies and fibers (Fig. 4B, arrowheads). Qualitatively, these cultures contained many more sensory neurons (Brn-3.0$^+$ cells) of both trkA$^+$ and trkA$^-$ (Fig. 4B arrows) subtypes, especially in the caudal part of the explants. Thus, exposure to serum for the first 2 days of culture is required for the development of large numbers of strongly trkA$^+$ sensory neurons.

There are two general mechanisms by which serum may promote the development of significant numbers of trkA$^+$ cells in explant cultures. First, serum may directly induce trkA expression in some sensory neurons or precursors that have already

migrated from the neural tube. Second, serum may induce the neural tube to produce sensory precursors, some of which have the potential to differentiate into the trkA$^+$ subtype. To address the first possibility, we allowed neural crest to migrate from the neural tube in defined medium for two days and then cultured these explants in medium containing 10% FBS until day 4. In contrast to explants that were exposed to serum for the first two days of culture, explants that were exposed to serum during the second two days of culture contained very few trkA$^+$/Brn-3.0$^+$ cells (Fig. 4C). Occasionally, however, a few trkA$^+$ cells were found adjacent to the neural tube under these conditions (Fig. 4C, inset). We hypothesized that these cells might have migrated from the neural tube after addition of the serum. Consistent with this idea, when the neural tube was removed from explant cultures at day 2 (before the exposure to serum) none of the Brn-3.0$^+$ cells in the outgrowth expressed trkA at the end of the culture period at day 4 (Fig. 4D). Serum, therefore, is required for development of significant numbers of trkA$^+$ sensory neurons in explant cultures, but is not sufficient to induce the expression of trkA in sensory neurons that have differentiated in defined medium.

**TrkA$^+$ sensory neurons develop in defined medium from explants that have been treated with BMP2**

One additional feature of serum-treated explants was that they contained autonomic neurons as assayed by Phox2a expression (data not shown). Such neurons are absent in explants grown in serum-free medium. As shown previously, autonomic neurons can be induced to develop in explant cultures grown in serum-free medium by exogenous addition of bone morphogenetic protein-2 (BMP-2; Greenwood et al., 1999). We were therefore interested to know if BMP2, like serum, could promote the development of trkA$^+$ sensory neurons in explants grown in defined medium in addition to promoting the development of autonomic neurons. As mentioned above, there is a low incidence of

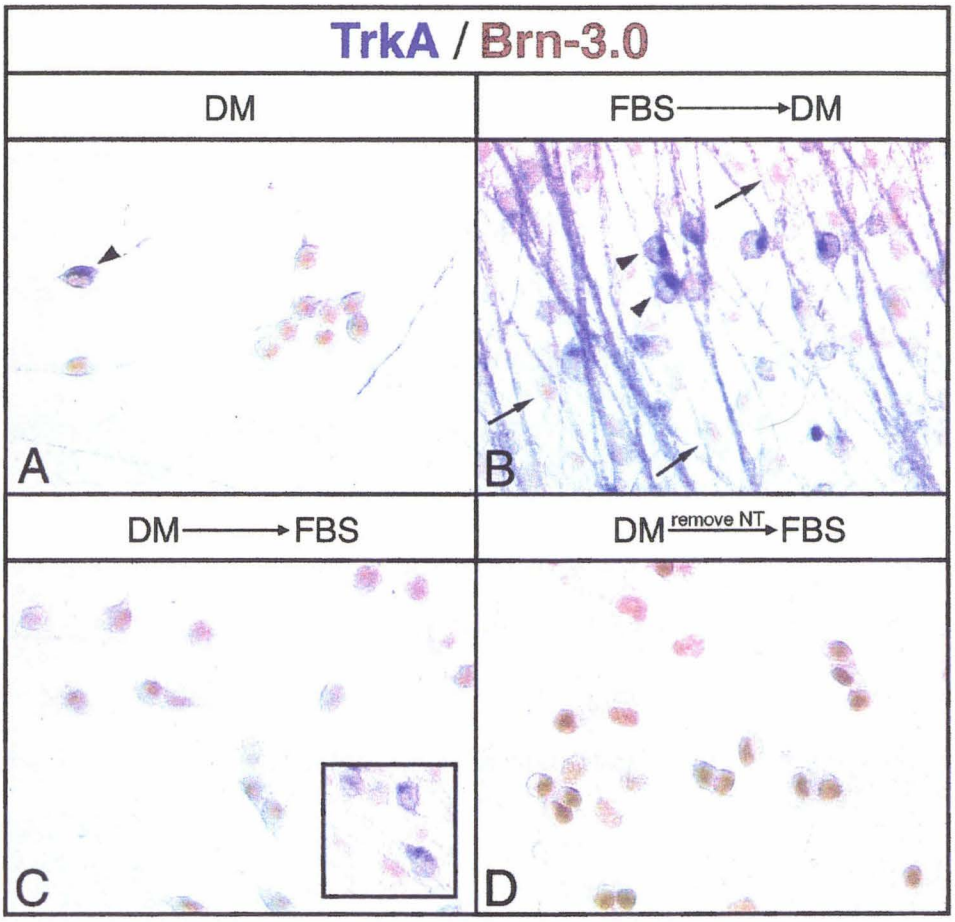


**Fig. 4** Serum induces the development of trkA$^+$ sensory neurons in explant cultures, but is not sufficient to induce trkA in sensory neurons that have previously differentiated in defined medium. Explants were cultured for 4 days then double-labeled for trkA (blue, cytoplasmic) and Brn-3.0 (brown, nuclear) by immunocytochemistry. (A) Most Brn-3.0$^+$ cells found in cultures grown continuously in defined medium (DM) for 4 days do not express trkA, although a few double-positive cells are observed (arrowhead). (B) Cultures grown for the first 2 days in the presence of serum (FBS) then in DM until day 4 contain many trkA$^+$/Brn-3.0$^+$cells (arrowheads) and many trkA$^-$/Brn-3.0$^+$cells (arrows). (C) However, when serum is added instead during the second two days of culture, most of the Brn-3.0$^+$cells do not express trkA. (C, inset) A few trkA$^+$ cells in these cultures are observed near the neural tube. (D) Cultures from which the neural tube was removed at day 2 before the addition of serum contained only Brn-3.0$^+$cells that did not express trkA.

trkA expression in the sensory neurons that differentiate in defined medium in the absence of exogenously added BMP2 (Fig. 5A, C). When BMP2 is included in the medium for the first two days of culture, the percentage of Brn-3.0$^+$ cells that express trkA at day 4 increases significantly from 10.6 $\pm$ 8.4% to 31.2 $\pm$ 11.8% (Fig. 5B, C). Due to the large and variable number of sensory neurons per explant (many hundreds to thousands), it has proven impractical to quantitate the effect of different conditions on total sensory neuron number. Qualitatively, however, the number of sensory neurons (total Brn-3.0$^+$ cells) appeared to increase in cultures treated with BMP2, though not as dramatically as in cultures treated with serum. In addition, BMP2, like serum, does not induce the expression of trkA in sensory neurons that have already differentiated in defined medium (c.f. Fig. 4; data not shown). These results indicate that BMP2 can affect the development of sensory neurons and their acquisition of subtype characteristics in a manner similar to serum.

**Serum acts on the neural tube to promote its ability to generate trkA$^+$ sensory neurons**

The foregoing experiments have demonstrated that when neural tube explants are exposed to serum during the first two days of culture, many trkA$^+$ sensory neurons can be found in the outgrowth at day 4 (Fig. 4B). As mentioned previously, it is possible that serum may increase the number of trkA$^+$ sensory neurons that develop in the outgrowth by affecting the type or number of precursors that migrate from the neural tube. In this case, the main effect of serum would be to act on the neural tube, the source of neural crest, rather than on already-emigrated neural crest cells. Examination of explants during the culture period suggested that the neural tube likely responds to serum by producing precursors that have the potential to differentiate into trkA$^+$ neurons. When explants were cultured for the first 2 days in medium supplemented with 10% FBS, the neural crest

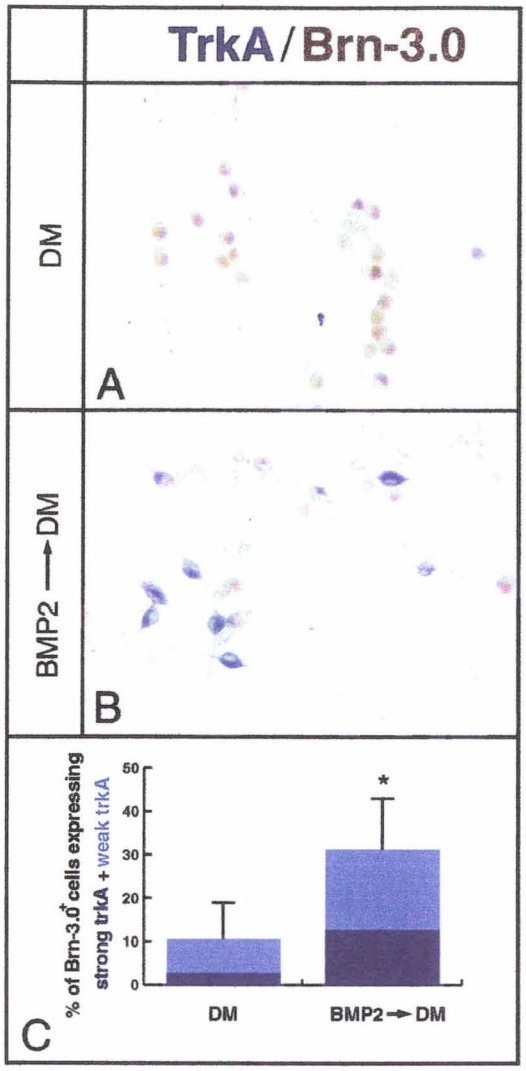


**Fig. 5** BMP2 induces the development of trkA$^+$ sensory neurons in explant cultures grown in defined medium. Explants were cultured for 4 days in either defined medium (DM) or DM supplemented with BMP2 (10 ng/ml) for the first two days. (A, B) Explants were double-labeled with antibodies for trkA (blue, cytoplasmic) and Brn-3.0 (brown, nuclear). (A) Explants grown in DM contain Brn-3.0$^+$ cells that are mostly trkA$^-$. (B) Explants exposed to BMP2 for the first two days contain a larger percentage of Brn-3.0$^+$ cells that express trkA. (C) The graph indicates the percentage of Brn-3.0$^+$ cells in each condition that express trkA strongly (dark blue part of bar) and weakly (light blue part of bar). n=6 explants for each condition; *P<0.01. Error is represented as standard deviation.

outgrowth was most prevalent along the rostral portion of the explant but only beginning to migrate from the caudal portion. However, following withdrawal of serum at day 2 and further culture until day 4, many cells migrate from the caudal portion of the explants. Most importantly, it is the caudal region that contains the most trkA and Brn-3.0 expression, although some sensory neurons were observed at all levels along the neural tube explant. These observations suggest that the precursors of $trkA^+$ sensory neurons are likely to migrate from the caudal neural tube after the serum withdrawal at day 2.

To directly demonstrate that $trkA^+$ neurons are derived from cells that migrate from the neural tube after serum is withdrawn, we removed the neural crest outgrowth from explants at day 2 at the time of serum withdrawal and assayed whether the subsequent outgrowth contained $trkA^+$ neurons. As mentioned previously, by day 4 the caudal region of unoperated explants contained significant numbers of $trkA^+/Brn\text{-}3.0^+$ (Fig. 6A, arrowheads) and $trkA^-/Brn\text{-}3.0^+$ (Fig. 6A, arrows) cells. Similarly, the caudal outgrowth of explants from which neural crest had been removed at day 2 contained many $trkA^+$ (Fig. 6B, arrowheads) and $trkA^-$ (Fig. 6B, arrows) sensory neurons. These explants contained almost as many cells as seen in unoperated cultures. Therefore, sensory cells can migrate from the neural tube after serum has been withdrawn from culture and then develop into sensory neurons that express trkA. Note that the continued presence of serum is not required for the late migration of these precursors and their differentiation into $trkA^+$ cells. By contrast, when the neural tube, rather than the neural crest, was removed from explants at day 2 at the time of serum withdrawal, the isolated outgrowth contained a small population of $Brn3.0^+$ sensory neurons (Fig. 6C, arrows) that only rarely expressed trkA (Fig. 6C, arrowheads). This reciprocal experiment suggests that the neural tube is required for the development of $trkA^+$ sensory neurons because it produces most of the precursors to these neurons after the second day of culture.

**The neurotrophins BDNF and NT-3 induce expression of ER81 but do not prevent expression of trkA in sensory neurons**

We have shown that NT-3 and BDNF regulate the expression of ER81, one characteristic of the muscle afferent fate. Although a1 hr exposure to BDNF and NT-3 did not abolish the expression of $trkA^+$ in sensory neurons that had been cultured for 6 days in serum (Fig. 3), it was not clear whether these neurotrophins might prevent the development of $trkA^+$ sensory neurons if added at earlier times or for longer durations. Therefore, to determine whether these factors would prevent the development of $trkA^+$ sensory neurons, we included these factors in the medium for the duration of the culture period. Cultures grown in serum for 2 days and then in defined medium until day 4 contain many $trkA^-$ and $trkA^+$ sensory neurons in the absence of exogenously added neurotrophins (Fig. 7A). The expression of ER81 under such conditions is low (approximately 15%) and typically weak (Fig. 7B, note lack of blue nuclei). When explants were grown in similar conditions in the continued presence of 25 ng/ml of BDNF and NT-3 for four days, ER81 was strongly upregulated in many sensory neurons (Fig. 7D). However, such NT-3/BDNF treated cultures still contained many $trkA^+$/Brn-$3.0^+$ cells (Fig. 7C). However, such NT-3/BDNF treated cultures still contained many $trkA^+$/Brn-$3.0^+$ cells (Fig. 7C). BDNF and NT-3 have a similar affect on cultures grown in the continued presence of serum for 6 days; they do not abolish trkA expression or delay its onset although they do promote the presence (likely via survival) of a very large number of $trkA^-$ neurons that express ER81 (data not shown). These data indicate that NT-3 and BDNF can positively regulate the development of the muscle afferent subtype without preventing the development of the cutaneous afferent subtype.

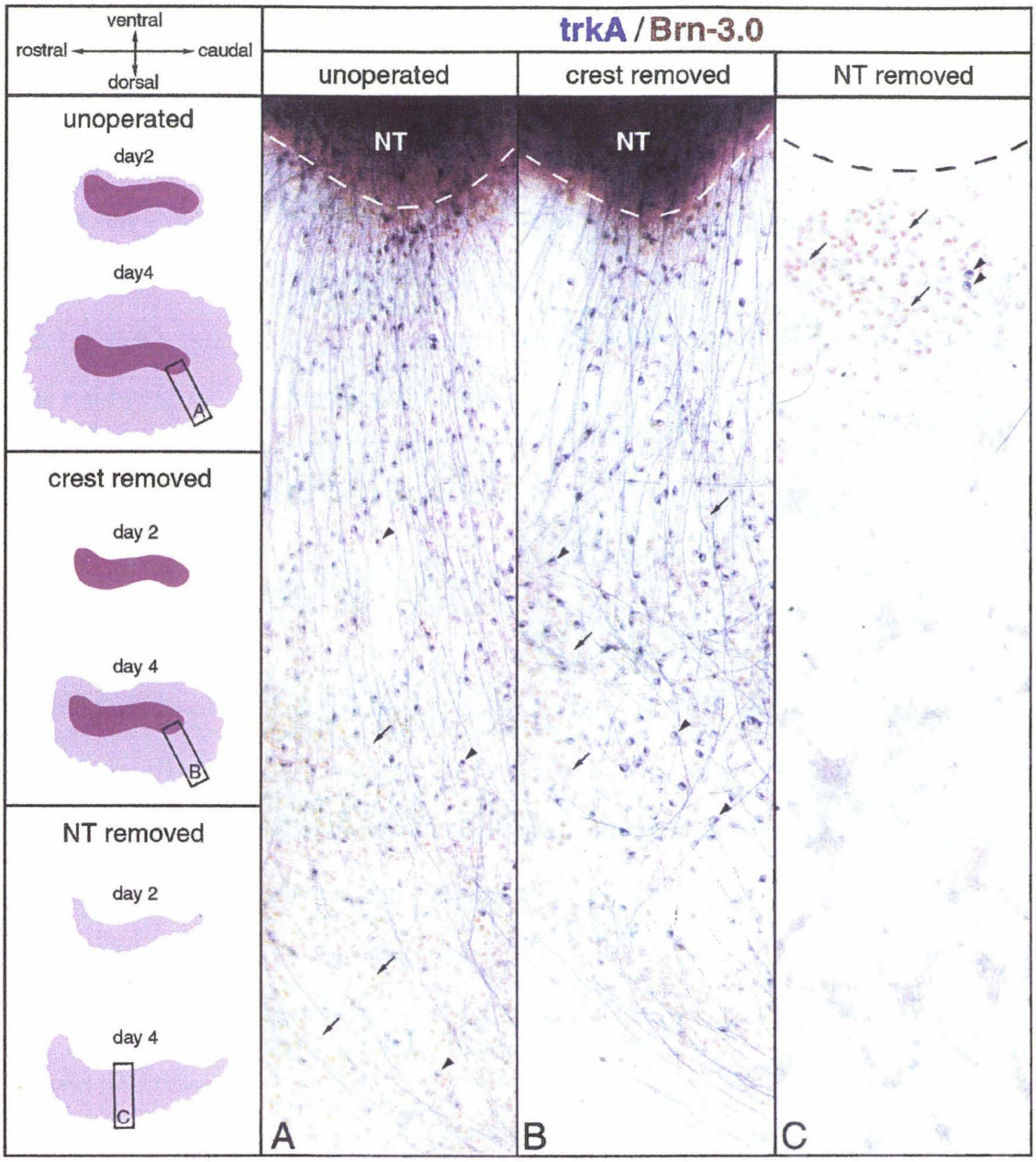


**Fig. 6** Serum acts on the neural tube rather than on already-migrated neural crest to induce the development of trkA$^+$ sensory neurons. Explants were grown in medium containing 10% FBS for two days, at which time the neural crest or the neural tube was removed from some cultures. Operated and unoperated explants were then cultured until day 4 in defined medium and stained for trkA (blue, cytoplasmic) and Brn-3.0 (brown, nuclear) expression by immunocytochemistry. Schematics to the left indicate the manipulations performed and the resulting morphology of the explants. (A) Unoperated

explants contain many Brn-3.0$^+$/trkA$^+$ (arrowheads) and Brn-3.0$^+$/trkA$^-$ (arrows) cells in the outgrowth from the caudal neural tube (NT, top). (B) Explants from which the neural crest was removed at day 2 also contain many trkA$^+$ (arrowheads) and trkA$^-$ (arrows) sensory neurons (Brn-3.0$^+$ cells) in the outgrowth from the caudal part of the neural tube. (C) Cultures from which the neural tube was removed at day 2 do not contain as many Brn-3.0$^+$ cells. Most of these do not stain for trkA (arrows) although a few Brn-3.0$^+$/trkA$^+$ cells can be found (arrowheads).

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## DISCUSSION

We have developed an in vitro assay system in which to study the factors that regulate the subtype characteristics of sensory neurons that differentiate de novo from the neural crest. Here we show that both serum and BMP2 promote the development of the cutaneous afferent sensory neuron subtype, as assayed by expression of trkA. Serum appears to exert its effects by inducing the neural tube to produce sensory precursors that then differentiate into both trkA$^+$ and trkA$^-$ sensory neurons. Neither serum nor BMP2 induces trkA expression directly in neurons that have previously differentiated in defined medium. In contrast, we have found that BDNF and NT-3 can directly induce expression of ER81, a marker of the muscle afferent sensory neuron phenotype, in already-differentiated neurons. NT-3 and BDNF do not prevent the development of sensory neurons that express trkA. Furthermore, serum-treated cultures contain many neurons that can respond to BDNF and NT-3 by upregulating ER81. These data indicate that serum/BMP2 modulate, either directly or indirectly, early aspects of sensory subtype identity while neurotrophins modulate later characteristics of sensory subtype identity.

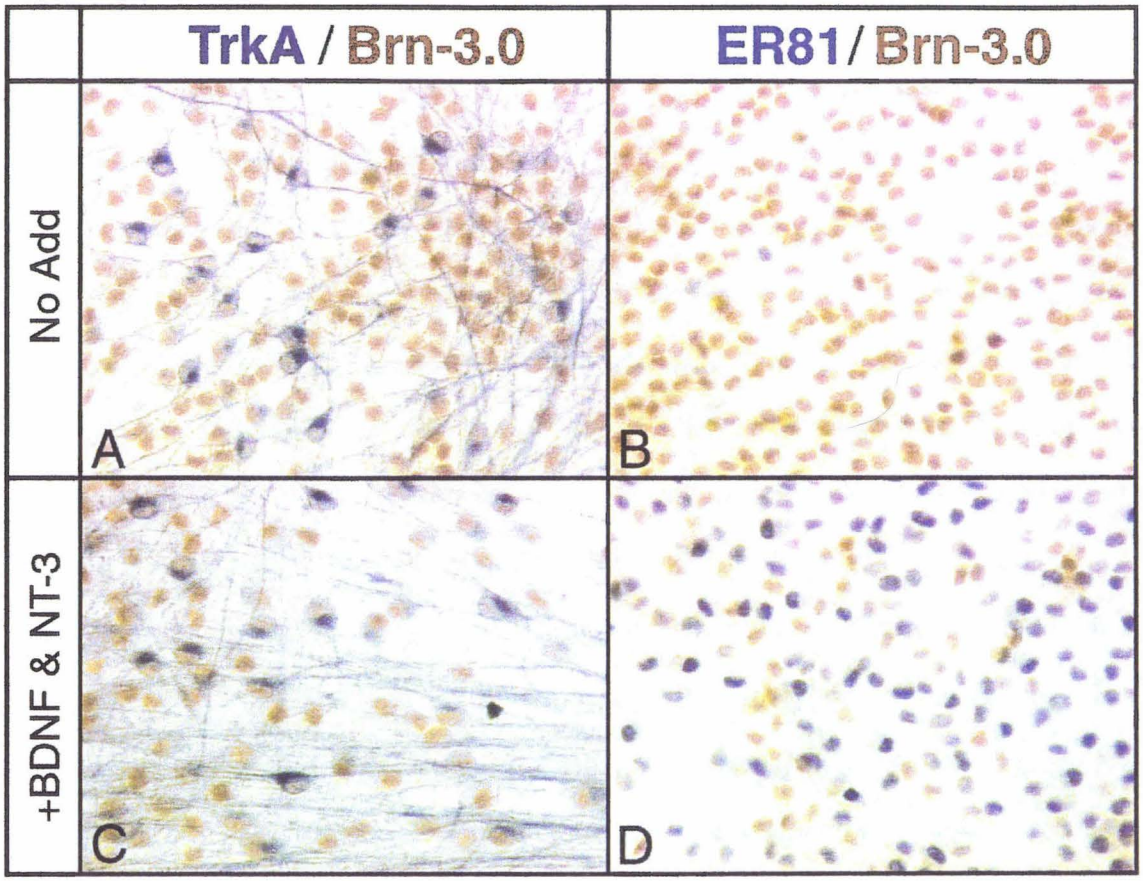



**Fig. 7** NT-3 and BDNF induce Er81 expression but do not prevent the development of trkA$^+$ sensory neurons. Explants were grown in the absence (No add) or continued presence of NT-3 and BDNF (25 ng/ml ea.) in serum washout conditions (2 days in medium containing 10% FBS followed by 2 days in defined medium), and then double-labeled with antibodies to trkA (blue, cytoplasmic) or Er81 (blue, nuclear) and Brn-3.0 (brown, nuclear). (A) Explants grown in the absence of NT-3 and BDNF contain many trkA$^+$/Brn-3.0$^+$and trkA$^-$/Brn-3.0$^+$cells. (B) Similar explants grown without factors contain sensory neurons (Brn-3.0+ cells) that express very little Er81. (C) In the continued presence of NT-3 and BDNF, many trkA$^+$/Brn-3.0$^+$cells still are observed. (D) A large percentage of the sensory neurons in cultures exposed to NT-3 and BDNF express Er81.

**Models for the mechanism by which serum and BMP2 affect the development of sensory neuron subtypes**

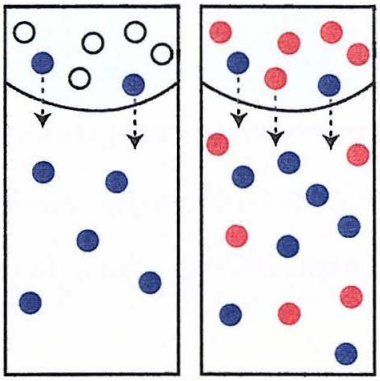
The ability of transforming growth factor-β (TGF-β) family members to affect neural tube patterning (Liem et al., 1997; Neave et al., 1997; Lee et al., 1998; McMahon et al., 1998; Nguyen et al., 2000), migration of the neural crest (Sela-Donenfeld and Kalcheim, 1999), and specification of peripheral neuron fates from the neural crest (Reissman et al., 1996; Shah et al., 1996; Schneider et al., 1999) has been well established. It is not surprising then that we have observed that BMP2, a member of the TGF-β family, and serum, which has been shown to have BMP-like activities (Ai et al., 1999), affect the development of sensory neurons in neural tube explant cultures. Serum and BMP2 have similar effects on the development of peripheral neuron subtypes in these cultures. Both induce the neural tube to produce more sensory precursors than would otherwise migrate in defined medium; these develop into both $trkA^+$ and $trkA^-$ neurons. Both induce the development of autonomic neurons from emigrated neural crest cells. Our evidence suggests that the ability of serum/BMP2 to affect the development of sensory subtypes in explant cultures is a result of an early action on the neural tube, and not due to an action on cells that have already migrated from the neural tube. There are, however, a number of cellular mechanisms by which this effect could be achieved.

There are two classes of possible mechanisms by which serum and BMP2 may act on the neural tube to cause it to produce sensory precursors, some of which differentiate into $trkA^+$ sensory neurons. These models must also account for the fact that many $trkA^-$ sensory neurons develop in the absence of serum/BMP2. The first class of models supposes that sensory neuron subtype fates are specified before neural crest precursors migrate from the neural tube. Serum/BMP2 could play a direct role in the specification of the cutaneous afferent fate from some neural tube cells only so long as there were already inherent differences that assured the development of the muscle afferent subtype

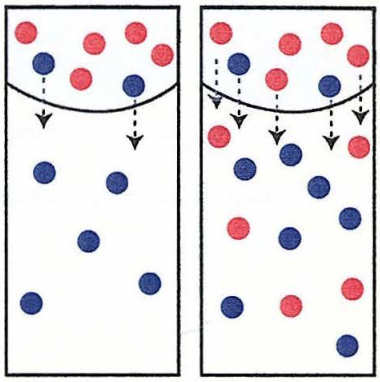
(Fig. 8A). Alternately, serum/BMP2 may not instruct the initial specification of sensory precursors, but may promote the migration of precursors that are already specified to produce trkA$^+$ neurons (Fig. 8B). Specification could also be achieved indirectly via another cell type (Fig. 8C). In addition, serum/BMP2 could somehow increase the number of premigratory neural crest, and lateral interactions within this population would specify some precursors to the cutaneous afferent fate (Fig. 8D). Given the previously demonstrated actions of BMP family members on the development of the neural tube and crest, this class of models seems plausible, though difficult to test. Possibly a clonal analysis of the sensory subtype fates of premigratory neural crest could distinguish between these models. At this point, we favor the model in which serum/BMP2 affects the migration of precursors from the neural tube (Fig. B) because the outgrowth of serum/BMP2 treated explants appears generally larger and contains more sensory neurons than in explants grown in the absence of these factors.

In the second class of models, serum may affect some aspect of neural tube or neural crest development that, in turn, specifies the subtype fate of sensory precursors after they migrate from the neural tube. For example, serum may induce the neural tube to produce some sort of signal that instructs sensory precursors or neurons in the outgrowth to assume a cutaneous afferent fate. A soluble signal (Fig. 9A) seems unlikely, however, since it should constrain the location of trkA$^+$ cells to the area immediately adjacent to the neural tube. Although we do observe such a phenomenon in cultures grown in the continued presence of serum, the trkA$^+$ cells in cultures exposed to serum or BMP2 for only 2 days seem well-distributed in the outgrowth, often far from the neural tube. It is also possible that the neural tube could produce a trkA-inducing cue in the form of a particular type of dorsal neural tube cell that, when contacted by sensory axons, specifies the cutaneous subtype fate (Fig. 9B). This too seems extremely unlikely, especially in light of the fact that sensory neurons in vivo express trkA immediately after

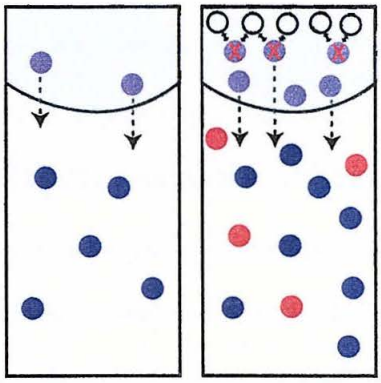
A. Serum specifies a subytype of sensory precursors within the NT.



B. Serum causes a specified subtype of sensory precursor to migrate from the NT.



C. Serum causes a non-sensory cell within the NT to specify the subtype of sensory precursors.



D. Serum produces more unspecified sensory precursors within the NT and lateral interactions specify subtype before they migrate.

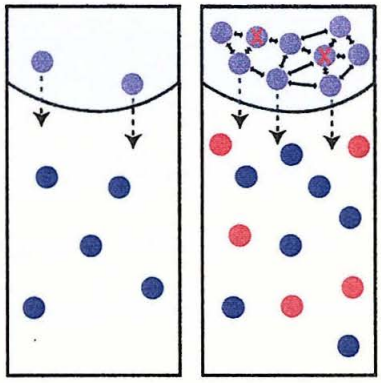


**Fig. 8** Models in which sensory subtypes are specified *before* precursors migrate from the neural tube. Grey regions indicate the neural tube. Circles indicate cells: purple represents unspecified neural crest, blue represents sensory precursors specified to the $trkA^-$ fate, red represents sensory precursors specified to the $trkA^+$ sensory fate. An "X" represents an act of specification. These models assume that the default sensory subtype fate is to become a $trkA^-$ sensory neuron. (A) Serum/BMP2 may directly specify the $trkA^+$ fate only if there is already subtype heterogeneity among the potentials of premigratory sensory precursors. (B) Subtype fates may be prespecified among sensory precursors within the neural tube by an unknown mechanism. Serum/BMP2 may cause sensory precursors to migrate from the neural tube including those with the potential to

make trkA$^+$ sensory neurons. (C) Serum/BMP2 may directly or indirectly cause a non-sensory cell within the neural tube to specify the trkA$^+$ subtype fate from some premigratory neural crest precursors. (D) Serum/BMP2 may promote the development of more premigratory neural crest cells that are not specified to any particular sensory subtype fate. Lateral interactions within this population may specify some to produce trkA$^+$ sensory neurons upon migration.

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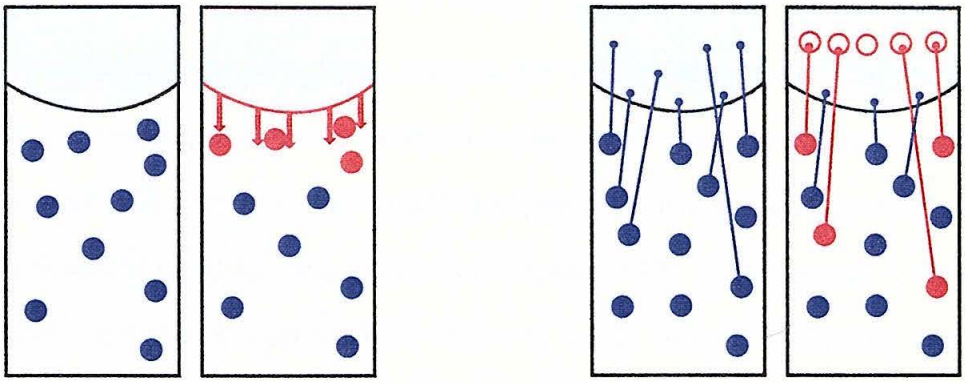
differentiation before their axons reach central targets (Fariñas et al., 1998). Other ways in which transient exposure of the neural tube to serum could affect the subtype fate of sensory precursors after they have migrated would be by increasing the density of these precursors to promote lateral interactions (Fig. 9C), or by promoting the specification or migration of a non-sensory cell that directly induces the cutaneous afferent fate (Fig. 9D). The ability to isolate sensory precursors from the migratory neural crest and grow them at different densities in the presence or absence of the neural tube would allow one to distinguish between these models.

**The role of NT-3 and BDNF in the development of sensory neuron subtypes**

Our results point to a role for the neurotrophins BDNF and NT-3 in the regulation of the muscle afferent phenotype. We have shown previously that the explant-derived sensory neurons that develop in defined medium depend on these factors for their long term survival in culture (Greenwood et al., 1999). In this study we find that a short (1 hour) exposure of these sensory neurons to NT-3 and BDNF is sufficient to upregulate expression of ER81. Given the rapidity of the response, it is likely that these factors are acting directly on the neurons themselves to upregulate expression of ER81 rather than promoting the survival or proliferation of ER81$^+$ cells. Interestingly, in chick, ER81

A. Serum causes the NT to produce a subtype-inducing diffusable signal.

B. Serum causes the NT to produce a subtype-inducing contact-mediated signal.



C. Serum causes the production or migration of more sensory precursors. Subtype is specified after migration via lateral interactions among these cells.

D. Serum causes the production or migration of a non-sensory cell that specifies subtype fate of sensory precursors.

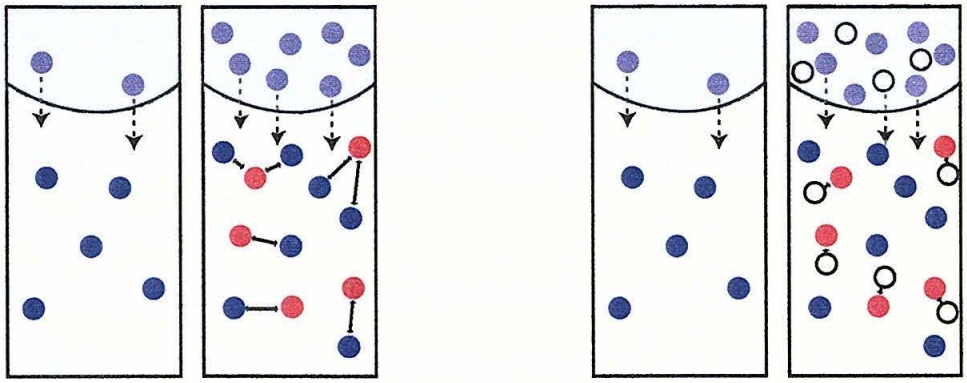


**Fig. 9** Models in which sensory subtype fates are specified *after* precursors migrate from the neural tube. (A) In the presence of serum/BMP2, the neural tube may produce a soluble factor that specifies the trkA$^+$ subtype fate from sensory precursors in the outgrowth. (B) In the presence of serum/BMP2, a cell type develops within the neural tube that specifies the trkA$^+$ subtype fate from sensory neurons upon contact with their central projections. (C) Serum/BMP2 may cause the neural tube to produce more sensory precursors. Lateral interactions within this population after they migrate specifies sensory subtype fate. (D) Serum/BMP2 may promote the development of a non-sensory, migratory progenitor. Signals from this cell type specify the trkA$^+$ subtype fate from sensory precursors after the cells have migrated from the neural tube.

expression is found in only a subset of the neurons that express trkC (Lin et al., 1998), and therefore there appear to be some neurons that are dependent upon NT-3 for survival but which do not upregulate ER81. Perhaps in this system there is a lower threshold of signaling required for survival than for ER81 expression. In contrast, recent data from mouse indicates that all $trkC^+$ sensory neurons express ER81 (S. Arber and T. Jessell, in preparation). We have observed that the ability to induce ER81 is not strictly specific to NT-3 and BDNF. A 1 hour exposure to NGF can elicit a modest but significant response (data not shown), and ER81 is also expressed in cultures treated with glial cell line derived neurotrophic factor (GDNF; data not shown). It is possible that multiple types of receptor tyrosine kinases can transduce signals to upregulate this gene. The mechanisms that restrict the expression of ER81 are not yet understood but could involve lineage restrictions that prevent subtypes of sensory neurons from expressing the gene in response to neurotrophins.

Our data suggest that BDNF and NT-3 are not required for the initial specification of the muscle afferent phenotype. Exogenous addition of these factors is not required for the differentiation of sensory neurons that ultimately will depend on them for survival and will respond to them by upregulating ER81. We cannot rule out the formal possibility that BDNF and NT-3 play an instructive role in specifying the fate of a subset of sensory precursors, but our results suggest they are not categorically required for development of the muscle afferent fate. Furthermore, these neurotrophins do not prevent the development of the cutaneous afferent ($trkA^+$) sensory neuron fate. This observation may imply that BDNF and NT-3 are not instructive factors for sensory subtype fates because they do not seem to affect the decision of precursors to become muscle or cutaneous afferents. Alternatively, BDNF and NT-3 might be instructive for the muscle afferent fate but they do not prevent the development of $trkA^+$ sensory

neurons because some sensory precursors are determined for the cutaneous afferent fate when they migrate from the neural tube. Clearly, neurotrophins play extremely important roles in the overall development of sensory neuron heterogeneity even if they are not responsible for the initial differences between sensory subtypes. It will be important to determine when sensory neuron subtypes first become different from each other and what signals impose the initial heterogeneity.

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# Chapter 4

# Summary and future directions

For any patterning or specification event in developmental biology, the objective is to find the source of information. Because the neural crest is a population of migratory cells, the goal is to learn what information about prospective fate is contained within individual premigratory or migratory progenitors, and what information is imparted by signals present in the embryonic environment. It appears that there is not an absolute rule governing the timing and nature of cell fate specification of neural crest precursors. Some migratory neural crest cells remain multipotent for significant periods of time, such as the stem cells that can be isolated from sciatic nerve that retain the potential to make glia, myofibroblasts, and autonomic neurons (Morrison et al., 1999). Others are determined to produce only one cell type before or shortly after they migrate from the neural tube, as is likely the case for the sensory progenitors described in Chapter 2 (Greenwood et al., 1999). Therefore, neural crest cells are heterogeneous in terms of their potentials even early in their development.

The central theme that can be drawn from the data presented in this thesis is that the diversity of peripheral neuron types is likely established early in neural crest development in a way that can be modulated by BMP signaling. This is not to say that individual progenitors are all determined for their peripheral neuron fates, but that interactions among neural tube and neural crest cells are sufficient to produce numerous peripheral neuron types in an orderly way that often resembles the in vivo patterning of these neurons. We have observed four ways in which BMP2 affects the development of peripheral neurons in neural tube explant cultures.

1.) BMP2 induces the autonomic neuron fate from migrating neural crest progenitors without preventing the differentiation of some sensory precursors (Chapter 2).

2.) We have noted, qualitatively, that BMP2 increases the number of sensory neurons present in explant cultures (Chapter 3).

3.) BMP2-treated cultures have an increased percentage of sensory neurons that exhibit characteristics of the cutaneous afferent phenotype (Chapter 3).

4.) BMP2 acts to pattern the proximodistal axis of the outgrowth such that sensory neurons form in the area adjacent to the neural tube while autonomic neurons form only in the periphery of the explant (Appendix 2).

Previous studies have shown that BMP2 can act instructively to specify the autonomic fate from some neural crest cells at the expense of glial and myofibroblast lineages (Shah et al., 1996). The induction of autonomic markers in the neural crest outgrowth is the only action of BMP2 we have observed that occurs independently of the neural tube. In contrast, the last three effects of BMP2 on the development of explant cultures are all dependent on the presence of the neural tube. These data indicate that the neural tube and the emigrated neural crest contain precursors that have inherently different responses to BMP signaling and/or that precursors rapidly change their responsiveness to BMPs.

Such data emphasizes an emerging focus in developmental biology: how can the same signaling molecules (or families of molecules) be used reiteratively during development to create diversity? Given any specification event, it is overwhelmingly likely that TGF-β, FGF, Wnt, hedgehog, and/or Notch/Delta signals or modifiers will be involved. How is it possible to use the same signals again and again in the context of a very small space to induce disparate developmental events without creating mass confusion? Within the development of the neural crest, there are numerous questions that can be posed. For instance, BMP signaling induces the neural tube to produce sensory

precursors and it also instructs neural crest to produce autonomic neurons. Since some sensory precursors are not shunted to an autonomic fate by BMP2, does this mean that they are all determined to the sensory fate? How can we reconcile this with the ability of some migrating neural crest cells to produce both sensory and autonomic neurons? If BMP signaling induces the migration of neural crest cells, some of which have autonomic potential, and it also induces them to become autonomic neurons, what prevents precursors from differentiating into autonomic neurons as soon as they migrate from the neural tube (i.e., in the area of the DRG)? Clearly, BMP responses must be well regulated.

There are two aspects of BMP signaling that may provide explanations for such questions. First, it is well known that BMPs can act as morphogens; depending on the dose, they can induce different responses from equivalent cells (for reviews see Smith, 1995; Dale and Jones, 1999). It is possible then that neural crest cells near the neural tube are exposed to a lower effective dose of BMPs than those in the periphery. Indeed, our preliminary data suggest that low doses of BMP2 induce the formation of sensory neurons from dissociated neural tube cells while higher doses induce the development of autonomic neurons (Appendix 2). This analysis should be extended, however, using better marker combinations to more carefully identify the different types of peripheral neurons that form under each condition. In contrast, when neural tube explants are cultured in the same high dose of BMP2, sensory neurons form near the neural tube and autonomic neurons form in the periphery. It seems important, though somewhat difficult, to determine the level of BMP signaling that is occurring in areas proximal and distal to the neural tube. Perhaps it may be possible to assay for the presence of activated SMAD proteins, which are known to transduce BMP signals upon phosphorylation (for review see (Massague and Chen, 2000), by staining with antibodies generated against phosphorylated SMADs or by monitoring the amount that translocate to the nucleus. A

lower level of activated SMAD proteins in cells near the neural tube would indicate that they were transducing less signal from BMP2 receptors. If such a difference in BMP activity were observed in the proximal and distal portions of the outgrowth, it may have arisen by an increased expression of BMP antagonists, such as noggin, in the neural tube and adjacent areas. In situ analysis of the expression patterns of BMP antagonists in neural tube explants grown in the presence and absence of noggin would be very interesting.

Another possibility for the difference in response to BMP signaling by different parts of the explant is that, instead of these areas being exposed to different effective levels of signaling, they may express different co-factors for activated SMADs that change their response to the incoming signal. It has been recently shown that transcription factors of the homeodomain and winged-helix families can act as co-factors for activated SMAD proteins to recruit them to distinct promoter elements (Germain et al., 2000). This implies an interesting strategy for characterizing the molecular differences between similar cell types that respond differently to BMPs (provided these cells can be isolated to obtain RNA with which to construct libraries). By using activated SMAD protein as a bait in an interaction screen, it may be possible to identify co-factors that are specific to each tissue type. Moreover, since assembly of transcriptional complexes may be dependent on the particular promoter, one could study the potential SMAD co-factors using a promoter element that contained SMAD binding sequences from a gene already known to be differentially expressed between the two cell types. For example, the *neurogenin* and *Mash* genes may be regulated directly by SMADs. If so, an analysis of the co-factors required for SMAD binding to their promoters may identify additional transcription factors involved in the specification of these particular cell fates.

The ideal experimental scenario would involve the ability to prospectively isolate and culture individual multipotent precursors from the rodent neural tube or neural crest

that can produce both sensory and autonomic neurons. At present, this requires that many conditions be tested empirically to define those that support the growth and direct the differentiation of these cells. Furthermore, the specific markers to identify and isolate such multipotent precursors have not yet been identified. It may therefore be valuable to explore whether any of the known genes which are differentially expressed in the neural tube can serve as markers for such multipotent precursors. This might be assayed by the use of transgenic reporter mice in which expression of lacZ, GFP, or some other sortable marker, is driven by regulatory elements of the candidate gene.

The ability to isolate multipotent progenitors and direct their differentiation is the true test of how well we understand the processes that control development. In addition, such technology holds significant promise for exciting clinical applications.

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# Appendix 1

## The extent of sensory neuron development in explants depends on the presence of the neural tube as a source of precursors

## INTRODUCTION

The neural tube is the ultimate source of sensory progenitors in the trunk region of the embryo. In addition, previous studies have indicated that the neural tube may provide signals important for the development of sensory ganglia in vivo (Kalcheim and Le Douarin, 1986; for review see Kalcheim, 1996). To further define the relationship between the neural tube and sensory neurogenesis, we took advantage of the ability to easily extirpate tissues from neural tube explants in vitro. We sought to address the following questions: first, is the neural tube required for the development of sensory neurons in explant cultures? Second, if the neural tube is required does it serve as a necessary source of signals, precursors, or both? Third, is the neural tube sufficient to support the generation or survival of sensory neurons in the absence of exogenously added neurotrophic factors?

## RESULTS AND DISCUSSION

### The neural tube is required for the extent of sensory neuron development in explant cultures

To determine if the neural tube was required for the development of sensory neurons in our cultures, we assayed the number of sensory neurons in the outgrowth of explants in which the neural tube had been removed after various durations of time. Explants were grown in a complex medium (containing chicken embryo extract and growth factors) that we had previously determined was permissive for sensory neurogenesis (see Materials and Methods in Chapter 2). The neural tube was removed from explants using a tungsten needle after 1, 2, 3, or 4 days of culture. The number of sensory neurons, identified as Brn-3.0$^+$/SCG10$^+$ cells by immunohistochemistry, was quantified per explant after 8 days of culture and compared to the number found in control explants in which the explant was left intact (see Materials and Methods in Chapter 2). Approximately a thousand

sensory neurons could be identified in the outgrowth of such undisturbed explants (Fig.1, Left in; Fig. 2, Left in). However, when the neural tube was removed after only one day of culture, there were strikingly fewer sensory neurons found in the outgrowth at day 8 ($27\pm$ 13 Brn-$3.0^+$/$SCG10^+$ cells; Fig. 1, Out day 1). The number of sensory neurons found in the outgrowth increased progressively with the length of time the neural tube remained in culture (Fig. 1, Out day 2, 3, and 4). This result indicates that the *extent* of sensory neurogenesis in explants depends heavily on the presence of the neural tube. However, the neural tube is not absolutely required for the presence of sensory neurons under these conditions, i.e., for survival.

The positive correlation between the number of sensory neurons and the presence of the neural tube in explants can be explained by two possible mechanisms. First, the neural tube may provide an extrinsic factor (or factors) that is necessary for the differentiation or proliferation of sensory precursors, but is not required for survival of sensory neurons under these conditions. Second, and not mutually exclusive with the first, the neural tube may contribute sensory precursors to the outgrowth in a progressive manner. The latter mechanism predicts that the thousand or so sensory neurons in an 8 day explant derive from precursors that migrate from the neural tube over an extended period of time. In attempt to provide evidence for the model that the neural tube supplies critical factors for sensory neurogenesis, we looked for an ability of neural tube conditioned medium or of neural tubes grown on transfilters to induce sensory neurogenesis from neural crest. We did not observe a significant increase in the number of sensory neurons in either case. Furthermore, data presented in Chapter 2 (discussed below) indicate that the neural tube is not absolutely required for the division or differentiation of sensory precursors in our cultures.

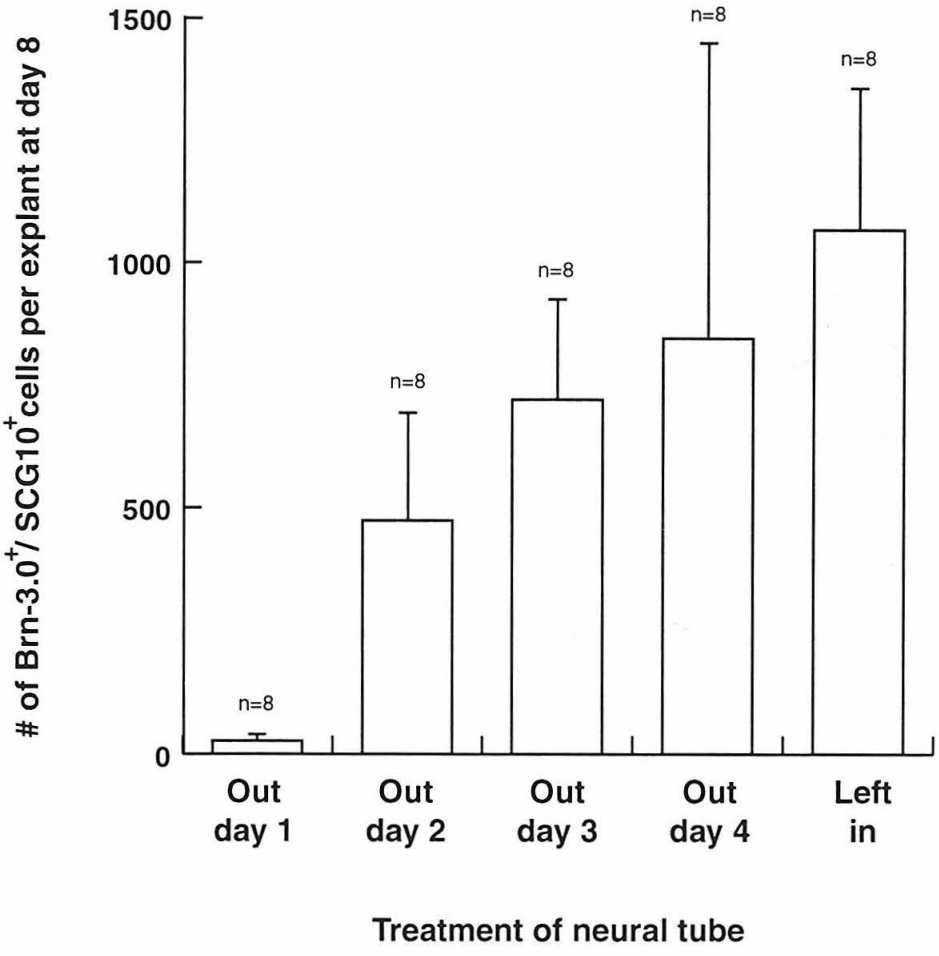


**Fig. 1** The neural tube is required for the extent of sensory neuron development in explant cultures. Bars indicate the total number of sensory neurons (Brn-3.0$^+$/ SCG10$^+$ cells) in the neural crest outgrowth per explant after 8 days of culture in complex medium supplemented with neurotrophic factors (NGF, BDNF, NT-3, and LIF). There are very few sensory neurons present in cultures in which the neural tube was removed at day 1 (Out day 1) compared to cultures left intact (Left in). The number of sensory neurons per explant increases progressively with the time the neural tube is left in culture. Error is represented as standard deviation. (n) is the number of explants scored from two experiments.

**The neural tube is a long-term source of sensory precursors in explant culture**

To address the role of the neural tube as a continual source of sensory precursors in vitro, we determined the number of sensory neurons produced by late migrating cells. The neural crest outgrowth was removed from explants using a tungsten needle at day 1, 2, 3, or 4. More cells were observed to migrate from the neural tube after this procedure, and the number of sensory neurons found in this new outgrowth was assayed at day 8 (Brn-3.0$^+$/SCG10$^+$ cells). When the neural crest was removed at day 1, the number of sensory neurons found in the outgrowth at day 8 was only slightly less than the number found in explants that were left intact (Fig. 2, Out day 1 vs. Left in). This suggests that the majority of sensory precursors that contribute to the sensory neuron population in vitro migrate from the neural tube after the first day in culture. When the neural crest was removed on subsequent days, progressively fewer sensory neurons were observed in explants at day 8 (Fig. 2, Out day 2, 3, and 4). There were very few sensory neurons found in 8 day explants in which the neural crest had been removed at day 4 (15 $\pm$12 Brn-3.0$^+$/SCG10$^+$ cells; Fig. 2, Out day 4), suggesting that the bulk of sensory precursors migrate during the first three days of culture.

This experiment suggests that one important reason the extent of sensory neurogenesis in explants depends on the neural tube is because it serves as a source of sensory precursors for numerous days in culture. A possible caveat is that the procedure of neural crest removal may somehow stimulate the migration of new precursors that normally would not have emigrated from the neural tube. Given that late migrating cells with sensory neuron potential have been observed in vivo (Sharma et al., 1995), it is unlikely that late migrating sensory precursors observed here in vitro are merely artifacts of the procedure. One way to directly address this issue, however, would be to label neural tube cells in an intact explant and observe when they migrate.

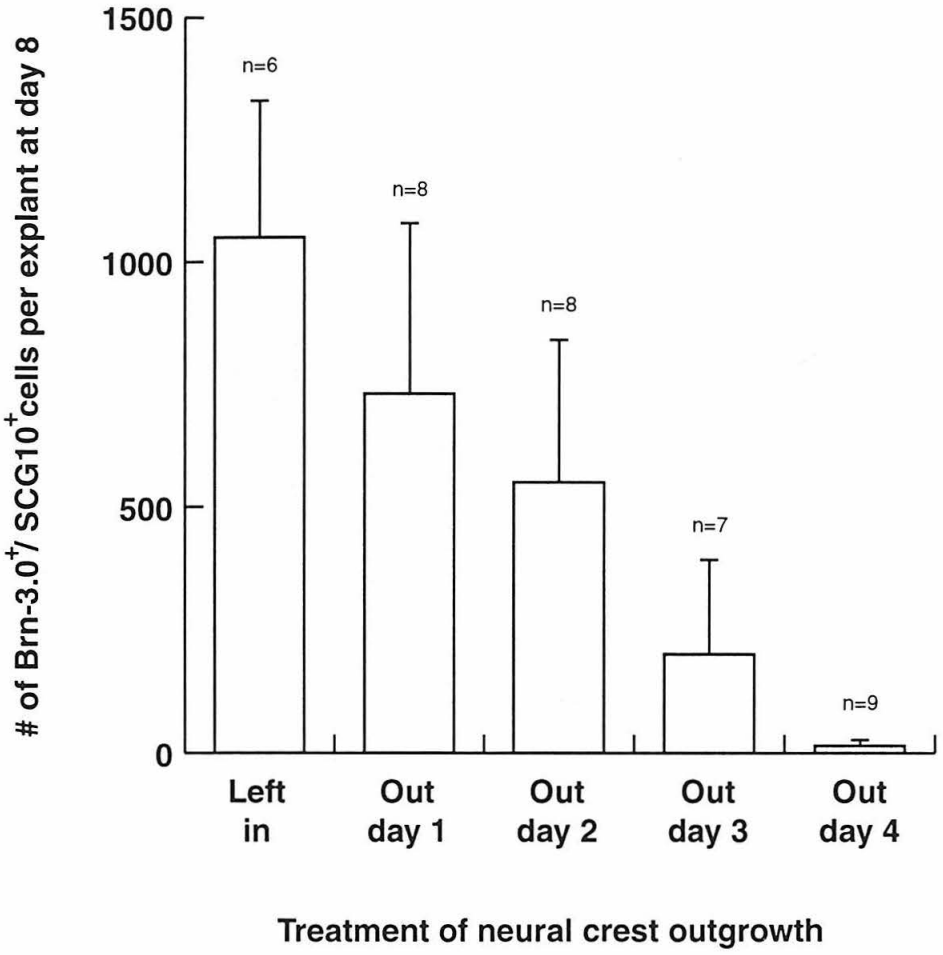


**Fig. 2** Sensory precursors migrate from the neural tube for numerous days in explant culture. Bars represent the total number of sensory neurons (Brn-3.0$^+$/ SCG10$^+$cells) in the neural crest outgrowth per explant after 8 days of culture in complex medium supplemented with neurotrophic factors (NGF, BDNF, NT-3, and LIF). When the neural crest outgrowth is removed at day 1 (Out day 1), more cells migrate from the neural tube and produce nearly as many sensory neurons as are found in intact explants (Left in). The number of sensory neurons per explant at day 8 decreases as the neural crest is removed on subsequent days of culture. Error is represented as standard deviation. (n) is the number of explants scored from two experiments.

**The neural tube is not required for sensory neurogenesis and is not sufficient for the long-term survival of sensory neurons**

The following data were presented in Chapter 2, but are discussed here in terms of the requirements of sensory neurons for the neural tube. In Chapter 2 we demonstrated that sensory precursors could differentiate in a fully defined medium in the absence of many exogenously added neurotrophic factors, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and leukemia inhibitory factor (LIF; see Chapter 2, especially Figs. 3 and 4). We found that the neural tube is not required for the process of sensory neurogenesis under these conditions since sensory precursors in the outgrowth can divide and differentiate even after the neural tube is removed from the culture (see Chapter 2, Figs. 4 and 5). Furthermore, the neural tube is not sufficient to support the long-term survival of sensory neurons under these conditions: although hundreds of sensory neurons could be found in explants after four days in culture, very few remained by day 8 unless the medium was supplemented with either neurotrophic factor-3 (NT-3) or brain derived neurotrophic factor (BDNF; see Chapter 2, Fig. 9).

The experiments presented above emphasize that the neural tube is important for the process of sensory neuron development in vitro because it is a source of precursors for an extended period of time. These data are consistent with the report that late migrating sensory precursors can exit the neural tube through the dorsal root entry zone (DREZ) to reach the sensory ganglia (Sharma et al., 1995). In addition, we have observed that sensory precursors can divide and differentiate in the absence of the neural tube in our defined medium. Superficially, this second observation appears to contradict a previous study that suggests neural tube derived signals are important for the development of sensory neurons in vivo (Kalcheim and Le Douarin, 1986). When silastic membranes were inserted between the neural tube and migrated neural crest,

markers of neural crest disappeared from the location of the presumptive DRG across from such membranes unless the membranes were coated with neural tube extract, BDNF, or basic fibroblast growth factor (bFGF) (Kalcheim and Le Douarin, 1986; Kalcheim et al., 1987; Kalcheim, 1989). It is important to note that the neural tube-independent sensory neurogenesis we observe in vitro occurs in a defined medium that *does* contain bFGF (though not BDNF or other neurotrophins). Explants grown in the absence of any bFGF did not survive well or produce much outgrowth. It would be interesting to know if withdrawal of bFGF from our cultures would have any effect on proliferation or survival or sensory precursors and whether neural tube derived factors could rescue such an effect.

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# Appendix 2

## Patterning of explants in vitro resembles the patterning of sensory and autonomic derivatives in vivo and is affected by BMP2 acting on the neural tube

## INTRODUCTION

The pattern of neural derivatives in the vertebrate PNS is manifested largely in the placement of different types of neuronal ganglia throughout the body. In contrast to patterning of the CNS, which is governed by interactions of cells within a neuroepithelial sheet, the arrangement of different neuronal types in the PNS is thought to depend on interactions between migrating neural crest cells and the embryonic environment. Our in vitro system afforded the opportunity to address whether interactions among the neural tube and neural crest were sufficient to impose a pattern on peripheral neuron fates, and whether such patterning could be influenced by manipulation of the culture medium. Any patterning observed in explants must be a result of cellular information contained only within the tissues present in culture: the neural tube and the neural crest.

Surprisingly, we observed a large degree of patterning of sensory and autonomic neurons along both proximodistal (P-D) and rostrocaudal (R-C) axes of neural tube explants that resembled patterning of sensory and autonomic derivatives in vivo. P-D patterning was induced by BMP2 or serum in a manner that requires the presence of the neural tube. In addition, we found that neural tube cells respond to different doses of BMP2 by expressing markers consistent with sensory or autonomic neurons. These data suggest that regulation of BMP-responsiveness among neural tube and crest cells is important to create the pattern of peripheral neuron derivatives observed in vivo.

## RESULTS AND DISCUSSION

### Patterning along the proximodistal axis of explants resembles the dorsoventral patterning of sensory and autonomic derivatives in vivo

Explants grown in defined medium lack overt P-D patterning. Sensory neurons, as identified by immunoreactivity for Brn-3.0, are found throughout the P-D axis of the outgrowth at both day 2 (Fig. 1A) and day 4 of culture (Fig. 2A). Under these conditions

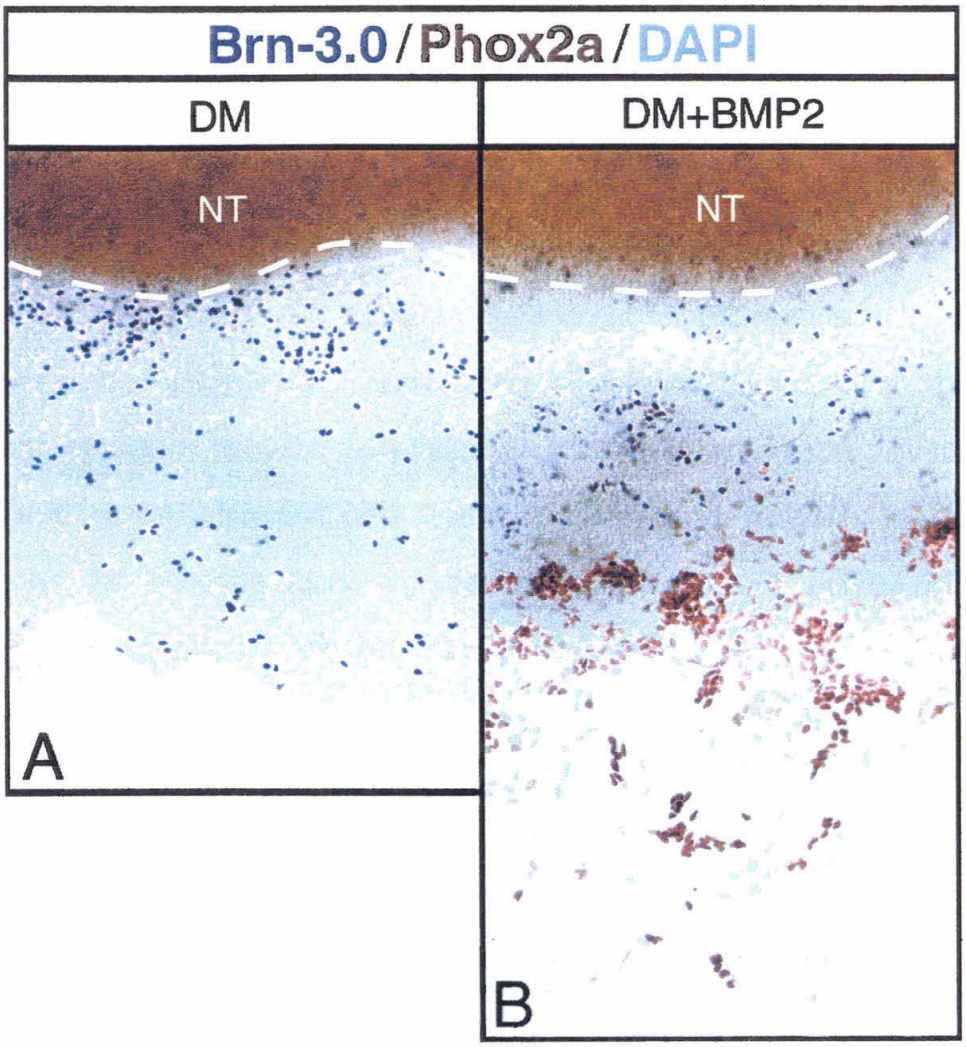


**Fig. 1** BMP2 induces P-D patterning of sensory and autonomic neurons in the outgrowth of neural tube explants. The views shown extend from the dorsal neural tube (NT; top) across the ougrowth (below dashed line) of explants grown in defined medium (DM) for 2 days. Double-labeling for Brn-3.0 (dark blue, nuclear) and Phox2a (brown,nuclear) shows the position of sensory and autonomic neurons, respectively. Other cells in the outgrowth are visualized by DAPI staining (aqua). (A) In explants grown without BMP2, Brn-3.0$^{+}$cells are scattered throughout the outgrowth and Phox2a is not expressed. (B) Explants grown in the presence of 50 ng/ml of BMP2 contain many Phox2a$^{+}$cells in only the distal part of the outgrowth; Brn-3.0$^{+}$cells are present in the area adjacent to the neural tube where Phox2a$^{+}$cells do not form.

no autonomic neurons (Phox2a$^+$ cells) are observed. Surprisingly, however, when the autonomic-inducing cue BMP2 is included in the medium, the outgrowth appears patterned: sensory neurons form only in the area adjacent to the neural tube and autonomic neurons form in the distal part of the outgrowth (Fig. 1B, 2B). Such patterning is also apparent when explants are grown in complex medium in the presence of chicken embryo extract (CEE; Chapter 2, Fig. 1) or serum (Chapter 3, Fig. 3). Note that the patterning has two potentially independent features: first, sensory neurons are located near the neural tube and, second, autonomic neurons are *not* located near the neural tube. The induction of autonomic neurons in the distal part of the outgrowth occurs both when BMP2 is added at the time of plating and explants are fixed at day 2 (Fig. 1B) or when BMP2 is added at day 2 after significant crest migration and then explants are analyzed at day 4 (Fig. 2B). The later the addition of the autonomic-inducing cue, however, the less likely are sensory neurons to be strictly localized near the neural tube (data not shown).

The arrangement of neural derivatives along the P-D axis in BMP-treated cultures bears striking resemblance to the arrangement of sensory and autonomic ganglia along the dorsoventral (D-V) axis in vivo. In explants, sensory neurons form near the neural tube; autonomic neurons form farther away from it (Fig. 3A). Similarly, in vivo, sensory neurons are found in dorsal root ganglia (DRGs) located immediately adjacent to the neural tube while autonomic neurons are located far from the neural tube in ganglia in the ventral part of the embryo (Fig. 3B). Although it has been demonstrated that DRGs contain cells with autonomic potential (Le Lievre et al., 1980; Schweizer et al., 1983; Duff et al., 1991), autonomic neurons do not differentiate in DRGs. Thus, autonomic neurons are excluded from the dorsal area near the neural tube in vivo even though there are cells that reside in or pass through the area that have autonomic potential. The similarity between the patterning of neuronal derivatives in vivo and in vitro raises the

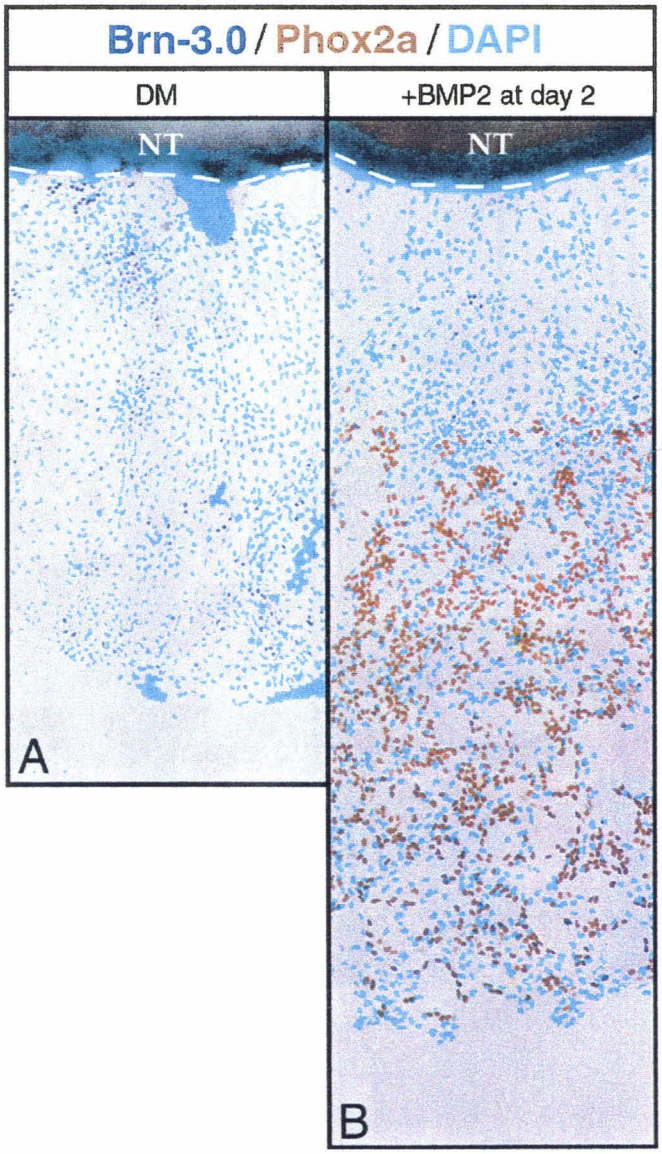


**Fig. 2** BMP2 induces P-D patterning of sensory and autonomic neurons in explants even after initial neural crest migration. Explants were grown in defined medium (DM) for 4 days and then double-labeled for Brn-3.0 (dark blue) and Phox2a (brown) to identify sensory and autonomic neurons, respectively. The dorsal neural tube appears at the top (NT); neural crest outgrowth is below the dashed line. DAPI staining (aqua) reveals the extent of the outgrowth. (A) Explants that were grown in the absence of BMP2 contain many Brn-3.0$^{+}$cells at all levels along the P-D axis. (B) Explants that were grown in the absence of BMP2 for 2 days and then exposed to 50 ng/ml of BMP2 contain Brn-3.0$^{+}$cells in the area near the neural tube and Phox2a$^{+}$cells in the distal region of the outgrowth.

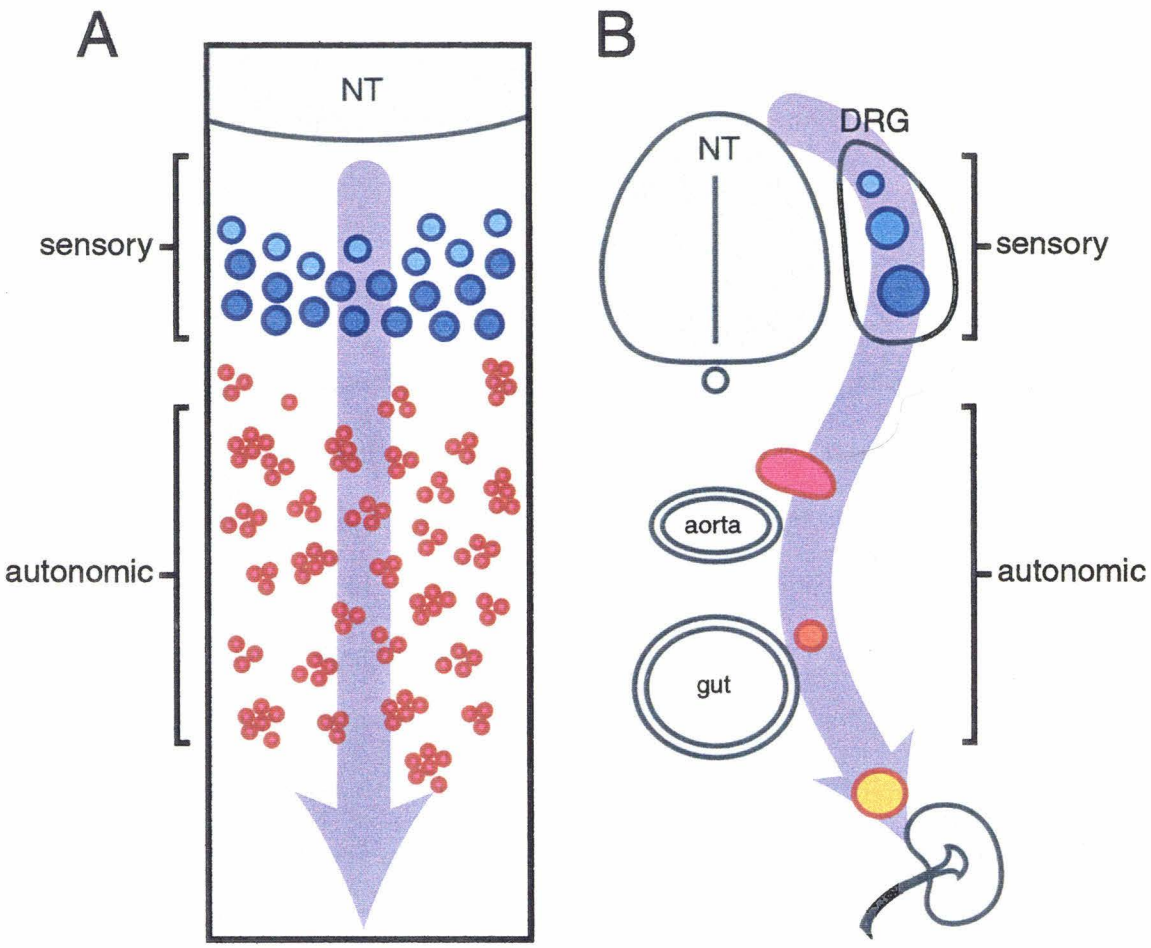


**Fig. 3** The patterning of explants in vitro resembles the patterning of sensory and autonomic ganglia in vivo. (A) In neural tube explants that have been treated with an autonomic-inducing cue, sensory neurons (blue circles) form in the area adjacent to the neural tube (NT) while autonomic neurons (red circles) form only the distal part of the outgrowth. (B) Schematic of the location of peripheral ganglia in vertebrate embryos. Sensory neurons (blue circles) are located in dorsal root ganglia (DRG) adjacent to the neural tube (NT) while autonomic ganglia (red circles) are located distal to the neural tube in the ventral part of the embryo. Purple arrows indicate the direction of neural crest migration.

possibility that similar forces are working in both systems. If so, interactions among cells of the neural tube and migrating neural crest may be more important in generating the pattern of peripheral ganglia than factors encountered locally by neural crest cells at the site of gangliogenesis.

**The neural tube is required for the proximodistal patterning of explants by BMP2**

Given that BMP family members are known to act on the neural tube to specify dorsal CNS cell fates (Liem et al., 1997; Lee et al., 1998) and thought to promote neural crest outgrowth (Sela-Donenfeld and Kalcheim, 1999), we were interested to determine whether BMP2 was patterning explants through an action on the neural tube. Alternatively, BMP2 could directly pattern the neural crest or simply reveal an inherent heterogeneity already present among neural crest cells. To address this question, explants were grown for 24 hours in defined medium in either the presence or absence of BMP2. During this time the neural tube is necessarily a part of the explant, and BMP2, if present, has an opportunity to act on this tissue. At the end of day 1, the neural tube was removed from the explant and the isolated outgrowth was cultured for another 24 hours in either the presence or absence of BMP2. (Experimental design is represented in Fig. 4E). Cultures that were never exposed to BMP2 had many sensory (Brn-3.0$^+$) cells scattered along the P-D axis of the outgrowth, but did not contain any autonomic (Phox2a$^+$) cells (Fig. 4A). When neural crest cells that had migrated in the absence of BMP2 were subsequently exposed to the factor after removal of the neural tube, many Phox2a$^+$ cells were observed, but these were freely interspersed with the Brn-3.0$^+$ cells along the P-D axis (Fig. 4B). Therefore, although autonomic cells were generated, the cultures were not patterned. Conversely, explants grown in conditions in which they were exposed to BMP2 during the first 24 hours showed a striking degree of pattering (Fig. 4C, D). Brn-3.0$^+$ cells were only located in the part of the explant that had been near the neural tube.

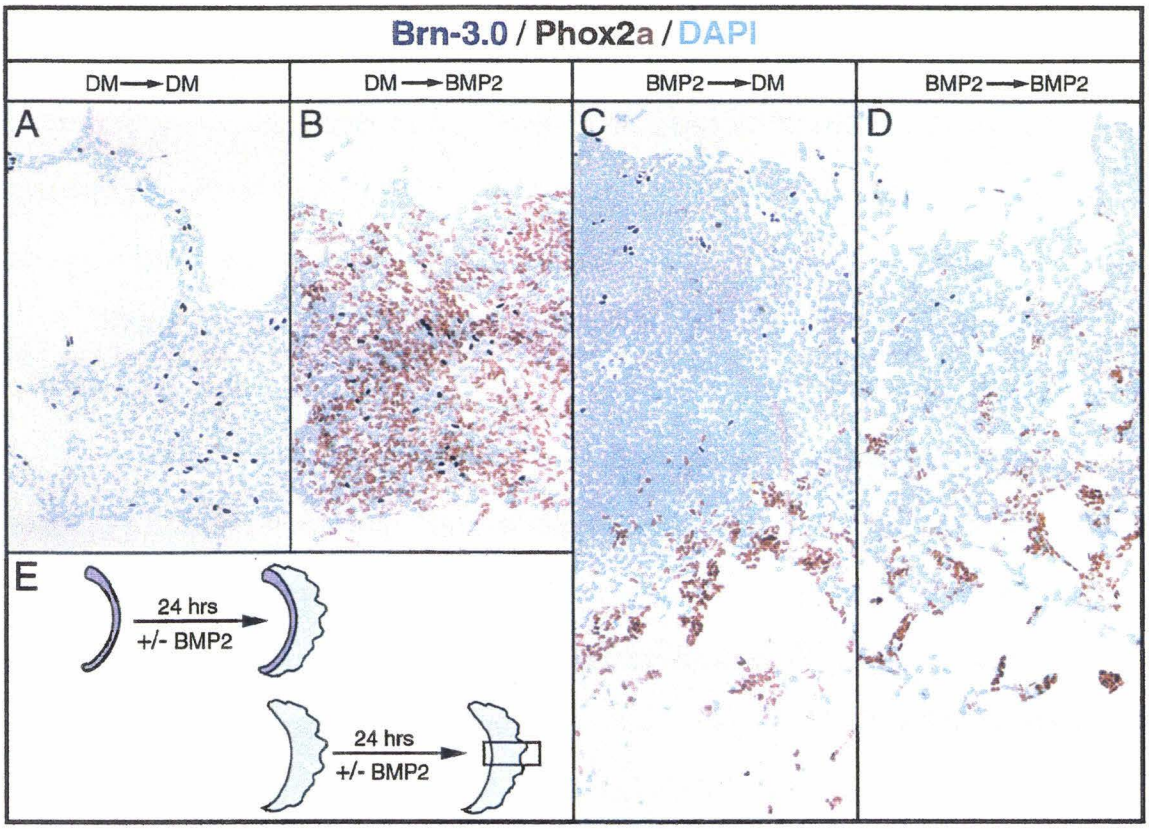


**Fig. 4** The neural tube is required for the P-D patterning of explants by BMP2. (A-D) Views show day 2 outgrowth stained for Brn-3.0 (dark blue) and Phox2a (brown) to identify sensory and autonomic neurons, respectively (outgrowth that was near the neural tube is at the top). DAPI staining (aqua) shows other nuclei. (A) Neural crest grown in defined medium (DM) without BMP2 was not patterned and contained only Brn-3.0$^+$ cells. (B) Neural crest that migrated in the absence of BMP2 and was then exposed to the factor after removal of the neural tube was not patterned and contained both Brn-3.0$^+$and Phox2a$^+$ cells. (C) Neural crest that migrated in the presence of BMP2 and was then switched into DM is patterned; Brn-3.0$^+$cells form in the area that was near the neural tube while Phox2a$^+$ cells are found in the distal outgrowth. (D) When the explants were continually exposed to BMP2, the outgrowth is patterned as in (C). (E) Schematic of the experimental design: neural tube explants were plated in the presence or absence of 50 ng/ml BMP2. The neural tube was removed from culture on day 1 when the explants were switched into new medium that did or did not contain BMP2 and grown until day 2.

This region also contained many cells that expressed neither neuronal marker. Phox2a$^+$ cells were only located in the distal portion of the explant. From these data we conclude that interactions between BMP2 and the neural tube are required to pattern the outgrowth into proximodistal regions that express either sensory or autonomic markers.

There are two general mechanisms by which the neural tube may be reacting to BMP2 to create an area that does not contain autonomic neurons. First, upon exposure to BMP2 the neural tube may inhibit the cells in the neighboring neural crest from becoming autonomic neurons. For example, in response to BMP2 the neural tube itself may produce an inhibitor, such as the BMP2/4 antagonist noggin, that prevents the induction of autonomic neurons in nearby regions. It could also, perhaps, begin a cascade of inhibitory cell-cell interactions by inducing expression of a BMP antagonist in the cells adjacent to the neural tube. In either case, it would be interesting to look at the expression of BMP antagonists in explant cultures grown in the presence or absence of BMPs. The second mechanism by which the neural tube could pattern the explant would be if BMP2 induced the migration of a different type of neural crest cell than normally migrates in the absence of BMP2. Given that the outgrowth appears larger in explants that have been treated with BMP2 (compare Fig. 4C, D to 4A, B), it seems likely that the neural tube is also producing more neural crest when it is exposed to BMP2. If so, the most interesting question is why those particular cells do not respond to BMP2 by becoming autonomic neurons.

Although the model presented above is directed at why autonomic neurons do not form near the neural tube, the question of why sensory neurons form only near the neural tube under autonomic-inducing conditions also needs to be addressed. We have observed that the placement of sensory neurons along the P-D axis of the outgrowth depends on how long they have been exposed to an autonomic-inducing cue such as BMP2 or serum. When explants are exposed to serum for only one day and then grown in defined medium

until day 4, both trkA$^+$ and trkA$^-$ sensory neurons at the caudal end of explants are observed to be scattered throughout the outgrowth (Fig. 5A). When explants are exposed to serum for two days before they are switched into defined medium, many more sensory neurons are found in the caudal outgrowth; these too are distributed evenly along the P-D axis (Fig. 5B). However, when explants are exposed to serum for three days before they switched into defined medium, many sensory neurons are observed only in the area adjacent to the neural tube (Fig. 5C). Because the presence of serum promotes the differentiation of autonomic neurons, one possibility is that autonomic neurons on the distal edge of the outgrowth inhibit the migration or differentiation of sensory precursors into that area. This hypothesis predicts, however, that clusters of sensory neurons near the neural tube should always be flanked by autonomic neurons, and this situation is not always observed. Another possibility is that autonomic inducing cues may directly affect the migration ability of sensory precursors, perhaps by promoting differentiation. This hypothesis is consistent with the observed small decrease in BrdU incorporation by sensory precursors in the presence of BMP2 (Chapter 2, Fig. 6D), but remains to be fully explored.

**Autonomic and sensory neurons form preferentially in the rostral and caudal portions of neural tube explants, respectively**

In addition to patterning along the P-D axis of neural tube explants, there is a significant degree of patterning along the R-C axis as well. In a defined medium that does not support autonomic differentiation, a greater percentage of sensory neurons (Brn-3.0$^+$ cells) are found in the caudal portion of the outgrowth than in the rostral portion in day 4 explants (Fig. 6). This discrepancy is increased in autonomic-inducing conditions, such as when explants are grown for two days in the presence of serum before they are switched into defined medium. Under these conditions, a significant amount of neural

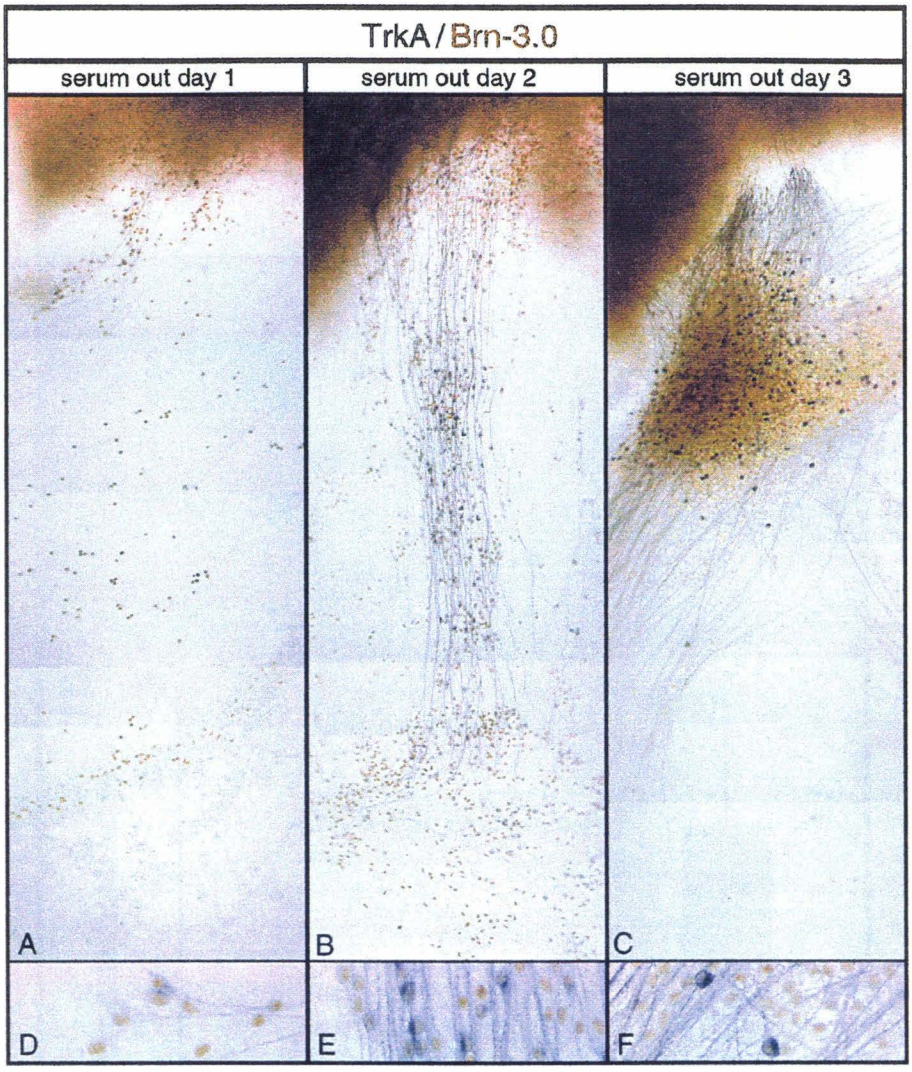


**Fig. 5** The P-D position of sensory neurons in the outgrowth depends on the length of exposure to serum. (A-C) View from the neural tube (top) across the outgrowth of 4 day explants double-labeled with Brn-3.0 (brown), which stains all sensory neurons, and trkA (blue, cytoplasmic), which stains a subset of sensory neurons. (A) Explants from which serum containing medium was withdrawn at day 1 contain Brn-3.0$^{+}$cells that are found throughout the outgrowth. (B) Explants from which serum was withdrawn at day 2 contain many Brn-3.0$^{+}$cells which are also dispersed along the P-D axis of the explant. (C) The Brn-3.0$^{+}$cells in cultures from which serum was withdrawn at day 3 are located in the area adjacent to the neural tube but not in the distal outgrowth. (D-F) High magnification views of Brn-3.0$^{+}$/ trkA$^{+}$ cells in (A-C), respectively.

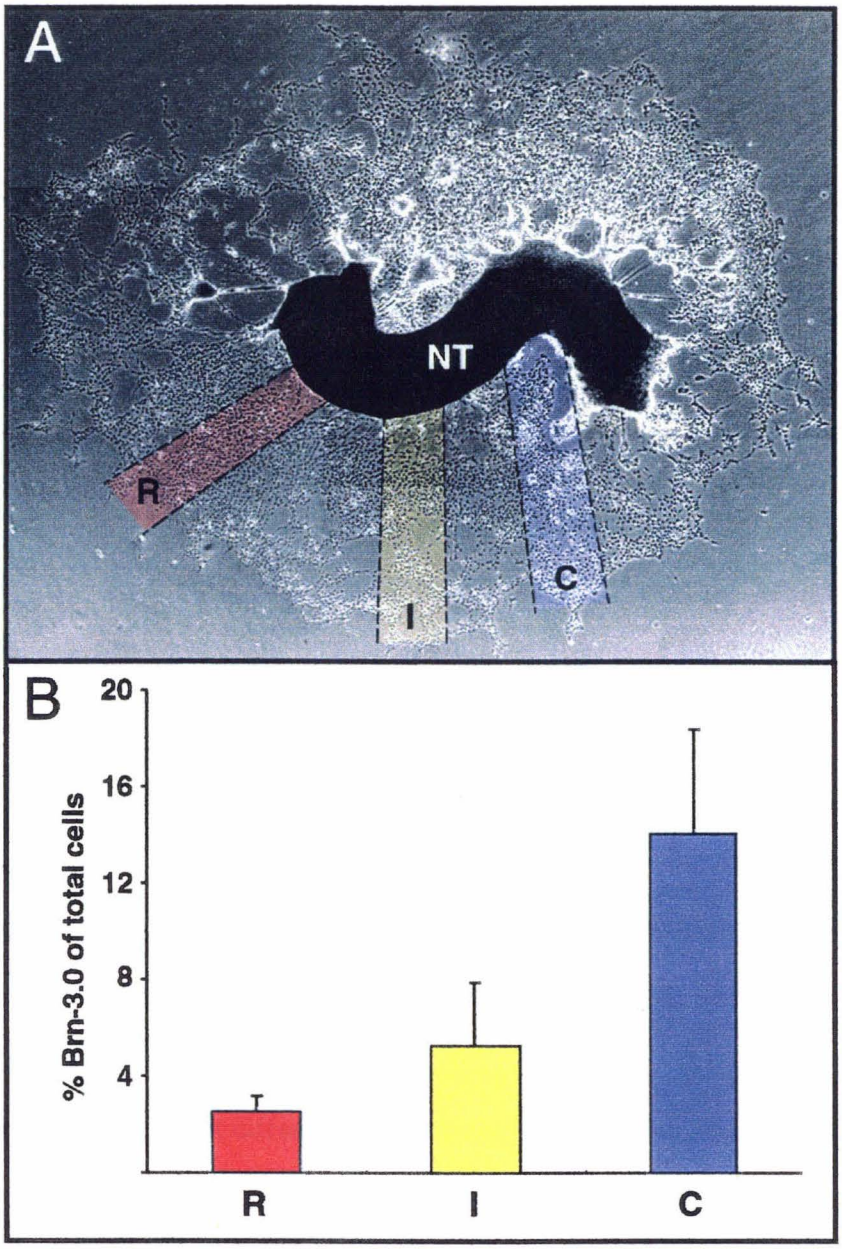


**Fig. 6** The percentage of sensory neurons along the R-C axis is higher in the caudal part of explants grown in defined medium (DM). (A) Phase microscopy of a day 4 explant grown in DM indicating the rostral (R), intermediate (I), and caudal (C) areas of the outgrowth from which the percentage of sensory marker expression was determined. (B) The graph represents the percentage of total cells (as identified by DAPI staining) that express Brn-3.0 in the R, I, and C parts of the outgrowth from 4 explants. Error is shown as standard deviation.

crest migrates first from the rostral part of the explant (Fig. 7A) and only later (after serum washout) from the caudal part (Fig. 7B). Most of the Phox2a$^+$ cells are found rostrally (data not shown) and most of the Brn-3.0$^+$ cells (both trkA$^+$ and trkA$^-$) are found in the caudal outgrowth that migrated after day 2 (Fig. 7C, D). The distribution of neuronal types can most easily be seen when the neural tube is divided evenly into rostral, intermediate, and caudal segments before culturing them as explants. When the segments are grown in serum-washout conditions, the rostral and intermediate explants produce overwhelmingly more Phox2a$^+$ cells than the caudal segments (Fig. 8A-C). Conversely, the caudal portion contains more Brn-3.0$^+$ cells than the rostral or intermediate portions, although some sensory neurons are found at all levels (Fig. 8D-E). Finally, trkA$^+$ cells, which have been shown to express the sensory marker Brn-3.0 in other experiments, are localized almost exclusively to the caudal explants (Fig. 8G-I). Simply put, the rostral, intermediate and caudal portions of the neural tube produce different kinds of neurons when cultured under the same conditions. These data suggest that different parts of the neural tube are predisposed to produce different amounts of autonomic and sensory neurons.

The observation that different proportions of sensory and autonomic neurons come from different parts of the neural tube in vitro could reflect two aspects of peripheral neurogenesis that have been observed in vivo. First, neural crest migration and subsequent gangliogenesis proceed in a rostral to caudal gradient such that the more rostral structures develop before caudal structures. At any particular rostrocaudal level of the trunk, the earliest migrating neural crest are fated to travel to the ventral part of the embryo and become autonomic neurons, while later migrating neural crest give rise to sensory neurons (Weston, 1963; Serbedzija et al., 1990). It is an interesting correlation that in vitro the early-migrating cells give rise preferentially to autonomic neurons and the later-migrating cells to sensory neurons. This suggests, perhaps, that early and late

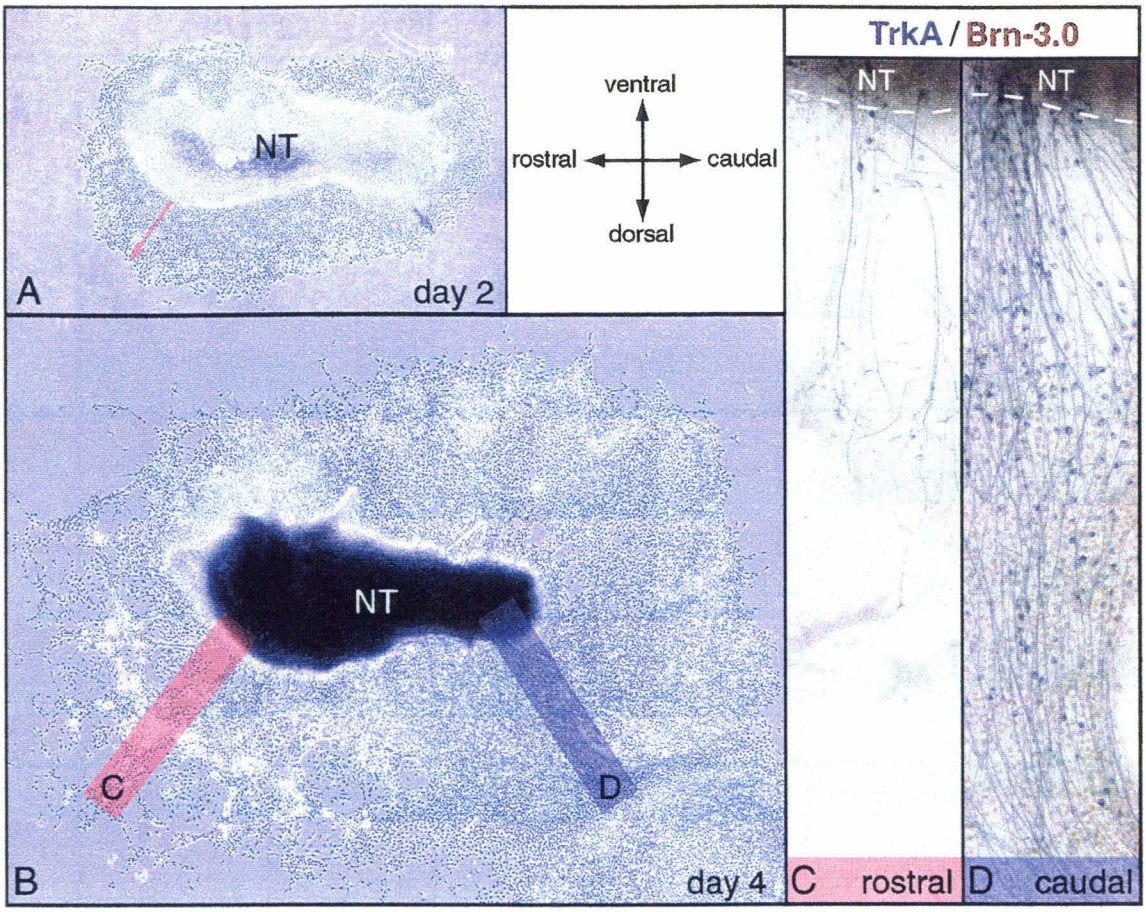


**Fig. 7** Both trkA$^+$ and trkA$^-$ sensory neurons develop preferentially in the caudal part of explants grown in serum-washout conditions. (A) Phase microscopy of an explant grown for 2 days in medium containing serum shows that more neural crest has migrated from the rostral part (red line) than the caudal part (blue line) of the neural tube (NT). (B) The same explant at day 4 after subsequent growth in defined medium has significant outgrowth in both rostral (red stripe) and caudal (blue stripe) regions. (C-D) Rostral and caudal views of the outgrowth stained for Brn-3.0 (brown) and trkA (blue) shows that there are more sensory neurons of both subtypes in the caudal (D) versus the rostral (C) parts.

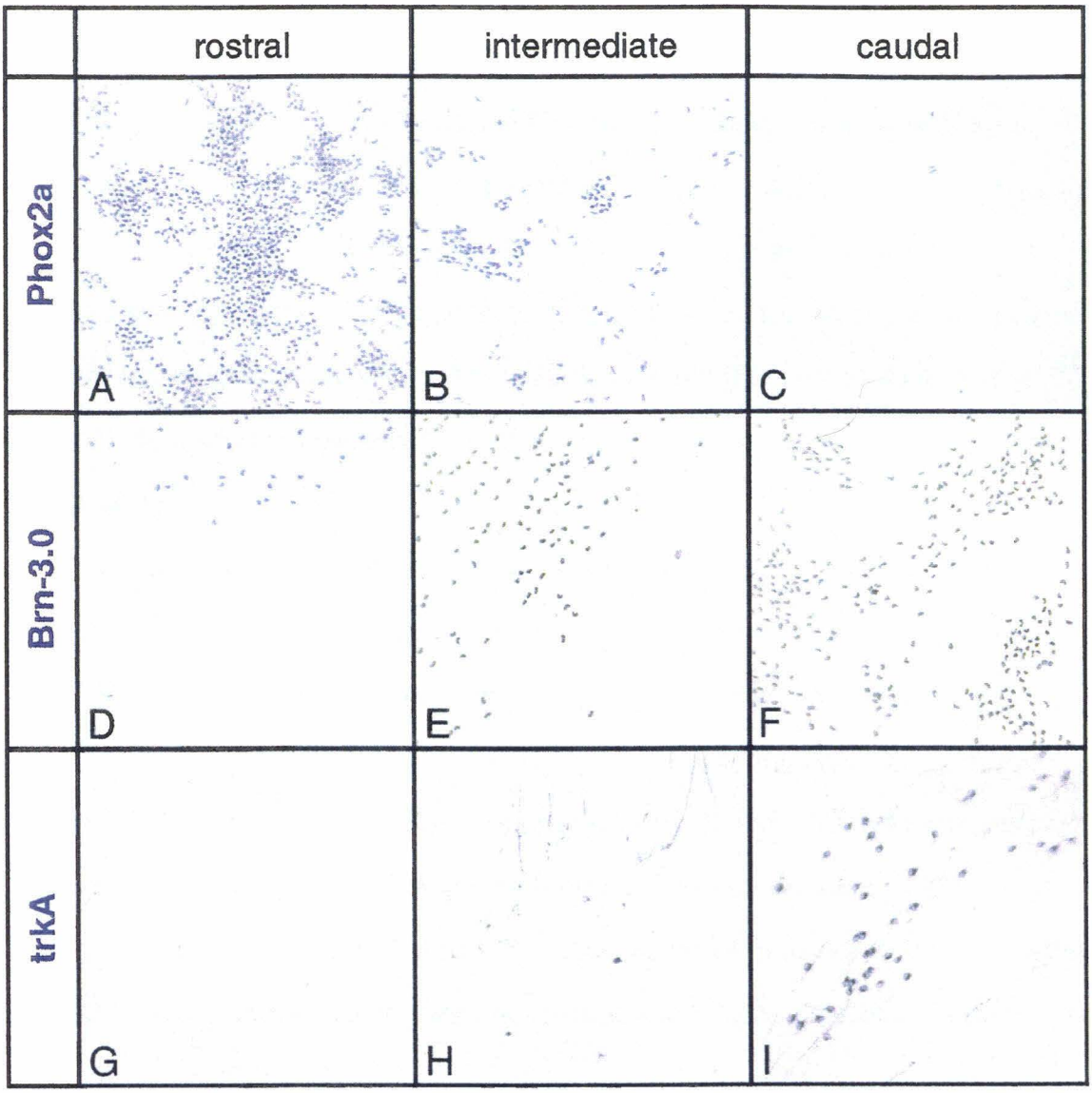


**Fig. 8** Autonomic and sensory neurons form in different parts of neural tube explants grown in serum-washout conditions. Explants were separated into rostral, intermediate, and caudal segments, then grown for 2 days in medium containing serum followed by 2 days in defined medium. (A-C) Labeling for the autonomic marker Phox2a reveals that there are many positive cells in the rostral (A) and intermediate (B) explants, but few in the caudal (C) segment. (D-F) The sensory marker Brn-3.0 is expressed in all segments, but more in the outgrowth of caudal (F) segment than in intermediate (E) or rostral (D) parts. (G-I) The sensory subtype marker trkA is expressed almost exclusively in caudal explants (I vs. G and H).

migrating crest have intrinsic biases to produce autonomic and sensory neurons, respectively. However, such a model predicts that the earliest migrating cells at *all* rostrocaudal levels will be biased to the autonomic fate. Instead, we observe that the first cells that migrate from the caudal part of the explant produce sensory instead of autonomic neurons. If the R-C patterning of explants is a reflection of the R-C gradient of development, then perhaps the reason it does not faithfully reproduce the in vivo pattern is because important environmental cues that control the timing of development are lacking.

Another possible explanation for the differences in the sensory and autonomic potential of rostral and caudal neural crest is that these differences may actually reflect the normal proportion in which these neurons produced in vivo. Although all segments in the trunk contain sensory ganglia, DRGs are much larger at limb levels. This size difference is apparent before the onset of cell death, which indicates that more sensory neurons are born (rather than simply survive) at limb levels (Goldstein, 1993). In our experiments, we isolate the neural tubes from the section of trunk which is caudal to the forelimb and thus represents only the mid-thoracic and lumbar segments. If different areas of the neural tube give rise to different proportions of sensory and autonomic neurons, it is possible that some of this information is contained within the neural tube itself and is not imposed only by somatic structures. It is intriguing that the cultured neural tube explants clearly produce more autonomic neurons from the rostral part and more sensory neurons from the caudal part. The relationship between this phenomenon and the in vivo processes is not yet understood.

**Different doses of BMP2 induce the expression of Brn-3.0 and Phox2a in cultures of dissociated neural tube cells**

The generation of both sensory and autonomic neurons from cells that migrate from the neural tube suggests that some precursors may be biased toward a particular neuronal fate at the time that they migrate. Furthermore, as shown above the patterning of these neurons appears to depend on interactions between the neural tube and BMP2. We were therefore interested to determine if BMP2 could have an affect on the types of peripheral neurons produced from neural tube cells. Members of the transforming growth factor-β (TGF-β) superfamily, which includes the BMPs, are known to pattern cell fates within the dorsal neural tube in a dose-dependent manner (Liem et al., 1997; Lee et al., 1998). Given this dose responsiveness, we looked at the ability of different doses of BMP2 to induce Brn-3.0 and Phox2a in dissociated cultures of neural tube cells grown in defined medium for 4 days. In the absence of BMP2, there was little expression of the markers by neural tube cells (Fig. 9A). In the presence of low doses of BMP2 (5-10 ng/ml), the cultures contained many Brn-3.0$^+$ cells and very few Phox2a$^+$ cells (Fig. 9B, C). When grown in 20 -30 ng/ml of BMP2, the cultures contained both Brn-3.0$^+$ and Phox2a$^+$ cells (Fig. 9D, E). In 50 ng/ml of BMP2, the cultures contained many Phox2a$^+$ cells and very few Brn-3.0$^+$ cells (Fig. 9F). Therefore, different doses of BMP2 can differentially affect the expression of these two markers in cultured neural tube cells: low doses promote Brn-3.0 expression, high doses promote Phox2a expression.

Although it is possible that different doses of BMP2 could specify different peripheral neuron fates from multipotent precursors within the neural tube (the most exciting interpretation), there are many caveats and alternate explanations that must be considered. First, the markers used in this experiment, Brn-3.0 and Phox2a, are expressed not only in sensory and autonomic neurons, respectively, but also in neurons of the dorsal neural tube. It is therefore possible that different doses of BMP2 are

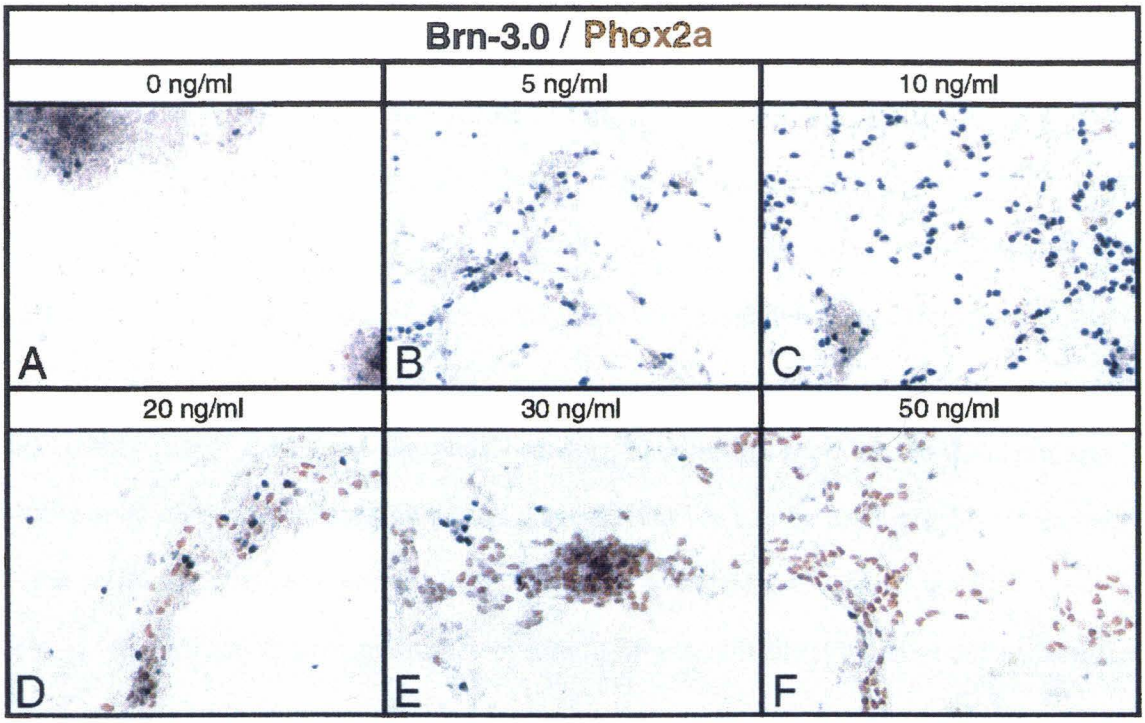


**Fig. 9** Different doses of BMP2 induce the expression of Brn-3.0 and Phox2a in cultures of dissociated neural tube cells. Double-labeling shows Brn-3.0 (dark blue) and Phox2a (brown) expression in dissociated neural tube cultures grown in defined medium for 4 days. (A) Little expression of either marker is seen in cultures that were not exposed to BMP2. (B, C) Many Brn-3.0$^+$ cells are found in cultures grown in low doses of BMP2 (5 and 10 ng/ml), but few Phox2a$^+$cells are observed. (D, E) At intermediate doses of BMP2 (20 and 30 ng/ml), cultures contain both Brn-3.0$^+$ and Phox2a$^+$cells. (F) At high doses of BMP2 (50 ng/ml), there are many Phox2a$^+$cells and few Brn-3.0$^+$cells.

modulating only the fate of dorsal neural tube cells, and not the production of peripheral neurons from premigratory neural crest precursors. To partially address this concern, dissociated neural tube cultures were grown in the presence of low doses of BMP2 (10 ng/ml) to promote differentiation of Brn-3.0$^+$ cells, then cultured in the presence of the neurotrophins NT-3 and BDNF (25 ng/ml) until day 8. When these cultures were stained for Brn-3.0 and peripherin, there were many neurons that expressed both markers indicating that at least some peripheral sensory neurons are produced in these cultures. A more detailed analysis in which neural crest derivatives can be distinguished from neural tube cell types will be important for the interpretation of these results.

In addition to the importance of identifying the different types of cells that arise in such cultures, it is also necessary to determine the mechanism by which BMP2 induces different markers. We are interested to know if it is acting instructively, selectively, or permissively on different sorts of neural tube progenitors. The standard method for analyzing such a question is via a clonal analysis; for technical reasons, however, this has not been possible with these cells. In the absence of such data, it is not possible to say how different doses of BMP2 are affecting neural tube cells. The preliminary observations, however, raise the interesting idea that heterogeneity among migrating neural crest cells, in terms of their abilities and biases to give rise to different kinds of peripheral neurons, may originate in the neural tube as a consequence of exposure to different factors and cell interactions. Such factors may include a differential response to BMP family members.

## MATERIALS AND METHODS

All immunocytochemical and tissue culture procedures described here are identical to those presented in Chapters 2 and 3.

### Cultures of dissociated neural tube cells

Neural tubes were isolated from the trunk region of E10.5 Sprague-Dawley rats as described previously (Greenwood et al., 1999). They were collected in defined medium in an Eppendorf tube and gently microcentrifuged at 5000 rpm for 1 minute. The medium was removed with a pipette and replaced with 1 ml of 0.5% Trypsin-EDTA (GibcoBRL), gently pipetted to resuspend the neural tubes. The Eppendorf tube was placed on its side in a tissue culture incubator and incubated at 37°C for approximately 10 minutes. (The timing of digestion is critical and should be determined for the amount of tissue and batch of enzyme being used). After incubation, medium containing 10% FBS was added to the tube, and then the tissue was again microcentrifuged to pellet the cells. The neural tube cells were washed again with medium containing serum and then twice with defined medium. Finally, the tissue was resuspended in 0.5 ml of defined medium and triturated with a fire-polished, pre-wet Pasteur pipette. Cells were plated on 35 mm plates coated with a fibronectin substrate (BTI). 1.5 ml of defined medium was added to plates, then greaseless, sterile cloning cylinders (4 mm internal diameter; Bellco) were placed on the dishes. 15,000 cells were delivered to each cloning cylinder (2-3 per dish). Cells were allowed to attach for 2-6 hours before removal of the cylinder. BMP2 was added after plating.

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# Appendix 3

## Identification by differential RT-PCR of a novel paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets

*Molecular and Cellular Neuroscience* **6**, 280–292 (1995)

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# Identification by Differential RT–PCR of a Novel Paired Homeodomain Protein Specifically Expressed in Sensory Neurons and a Subset of Their CNS Targets

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**Sensory neurons are a major derivative of the neural crest for which there have been no definitive molecular markers in mammals. We have developed a method that combines differential hybridization with degenerate RT–PCR to rapidly screen gene families for members exhibiting differential expression among tissues or cell types. We used this approach to search for transcription factor-encoding genes specifically expressed in mammalian sensory neurons. A novel paired homeodomain protein, called DRG11, was identified. DRG11 is expressed in most sensory neurons, including trkA-expressing neurons, but not in glia or sympathetic neurons. Unexpectedly, it is also expressed in the dorsal horn of the spinal cord, a region to which NGF-dependent sensory neurons project. These data suggest that DRG11 is not only a useful marker for sensory neurons, but may also function in the establishment or maintenance of connectivity between some of these neurons and their central nervous system targets.**

## INTRODUCTION

The study of cellular diversification during neurogenesis requires markers to identify different neural cell types. Recently it has become clear that transcription factors can serve as useful markers of neuronal identity. For example, the bHLH protein MASH1 identifies autonomic progenitors in the peripheral nervous system (Lo *et al.*, 1994). Similarly, Islet-1 (Ericson *et al.*, 1992) and additional recently characterized proteins in the *lim* homeodomain family mark subsets of functionally distinct motor neurons (Tsuchida *et al.*, 1994). These data suggest that the diversity of neuronal cell types can be defined in terms of an underlying diversity of expression of members of a transcription factor gene family.

Polymerase chain reaction (PCR)-based cloning using degenerate oligonucleotide primers has proven useful as a method to rapidly isolate members of a gene family (see, for example, Libert *et al.*, 1989; Wilkie and Simon, 1991). When cDNA rather than genomic DNA is used as the template, the method can selectively identify those members of a gene family expressed in a given tissue or cell type (Johnson *et al.*, 1990; Lai and Lemke, 1991). A limitation of this approach is that one must often sequence tens if not hundreds of PCR products to identify novel genes and then examine their expression patterns to identify those appropriate for further study. This makes it labor-intensive to apply this approach to several gene families simultaneously.

We have developed and applied a method, called differential reverse-transcriptase-based PCR (dRT–PCR), which combines RT–PCR with differential hybridization. This method identifies members of a gene family which are specifically expressed in a given tissue or cell type. We have applied this method to search for markers of mammalian sensory neurons, a major derivative of the neural crest for which there have been no definitive molecular markers described. Neuropeptides such as CGRP (Murphy *et al.*, 1991) and Substance P (Ito *et al.*, 1993) have been used as sensory markers, but these neuropeptides are not in fact sensory neuron-specific: for example, they

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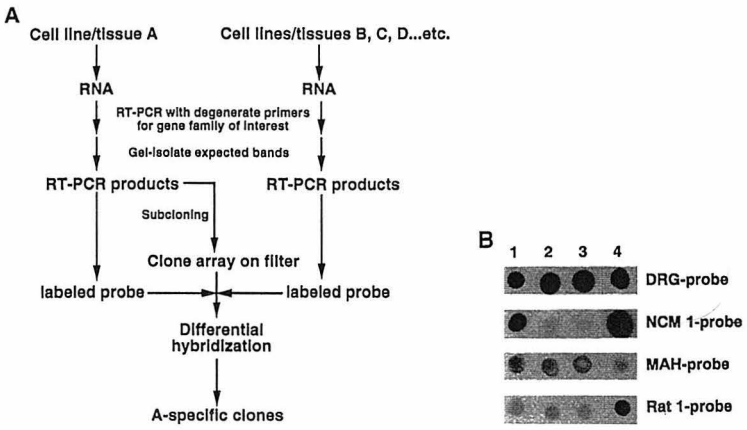


FIG. 1. (A) Schematic representation of the differential RT–PCR procedure and (B) an example of differential hybridization. See text for details.

can be induced in cultured sympathetic neurons by some cytokines (Fann and Patterson, 1994). In the absence of definitive sensory neuron markers, unambiguous identification of sensory neurons in mammalian neural crest cultures has been difficult (Matsumoto, 1994).

Using dRT–PCR, we screened four families of transcription factors for members specifically expressed in different neural crest derivatives and have thereby cloned a cDNA encoding a novel paired homeodomain protein called DRG11. DRG11 is specifically expressed in sensory neurons but not in autonomic neurons or glia. Unexpectedly, DRG11 is also expressed in a subset of the known functional targets of sensory neurons in the spinal cord. This transcription factor therefore provides a molecular marker to identify neurons in the peripheral sensory lineage. Moreover, its pattern of expression in the central nervous system suggests that it may function in regulating some aspect of the connectivity between these neurons and their central targets.

## RESULTS

### *Design and Application of a Differential RT–PCR Cloning Strategy*

A schematic outline of the dRT–PCR procedure is illustrated in Fig. 1A. cDNA from two or more cell types or tissues is amplified using degenerate oligonucleotide primers flanking a conserved domain from a given gene family. The PCR products from the "positive" tissue of interest (A cell in Fig. 1A) are subcloned, and the colonies are grown in a 12 × 8 array in a microtiter plate. Multiple replicas of this array are prepared by spotting small aliquots of each liquid bacterial culture onto nylon filters (Wilkie and Simon, 1991). These filters are then annealed with $^{32}P$ probes made from the same RT–PCR products, as well as from the RT–PCR products derived from one or more "negative" tissues (B, C, D . . . etc. cells in Fig. 1A). Clones displaying differential hybridization ($A^+B^-C^-D^-$) are picked for sequencing.

For simplicity, examples of only four clones (Fig. 1B, columns 1–4) derived from paired homeodomain (PHD) primer amplification of Embryonic Day 13.5 (E13.5) rat DRG cDNA, annealed in quadruplicate with four different probes, are illustrated. The probes used were PHD-amplified PCR products from: DRG, NCM-1 cells [a glial progenitor cell line (Lo *et al.*, 1990)], MAH cells [a sympathoadrenal progenitor cell line (Birren and Anderson, 1990)] and Rat-1 fibroblasts (a nonneural cell line). Two pieces of information are derived from this screen. First, the relative intensities of the hybridization signals exhibited by a single clone annealed with multiple probes gives an indication of its relative abundance within its gene family in different cells or tissues (Fig. 1B, read vertically). Second, the relative intensities of the hybridization signals exhibited by multiple clones annealed with a single probe provide an indication of their relative abundance within that cell or tissue type (Fig. 1B, read horizontally). Taken together, such information provides a unique "fingerprint" for each clone. Such fingerprint data allow a preliminary assessment of the number of distinct differentially expressed sequences within an array of clones.

In the example shown in Fig. 1B, clone 1 is strongly expressed in DRG, weakly in NCM-1 cells, and not above background in MAH and Rat-1 cells (background is de-

fined by the weakest hybridization signal observed on a clone array with a given probe); this clone was later identified as Pax3 (Goulding *et al.*, 1991). Clones 2 and 3 are expressed strongly in DRG, and not above background in NCM-1, MAH, and Rat-1 cells; this clone was subsequently identified as a novel PHD protein which we have called DRG11. Clone 4 is expressed in DRG and NCM-1 cells and weakly in Rat-1 cells. This clone encodes a novel PHD protein we have called NCM3 (data not shown). Similar analyses were carried out for three other transcription factor gene families [Ets-domain (Nye *et al.*, 1992), forkhead (Lai *et al.*, 1991), and scalloped/TEF (Campbell *et al.*, 1992) families] (data not shown). In all cases, differentially expressed sequences were obtained. Only the results of the PHD family screen will be presented here.

To further confirm the specificity of expression of the clones identified in the initial differential screen, the hybridization procedure was reversed: inserts from three of the clones (DRG11, Pax3, and NCM3) were used as hybridization probes on gel blots of total PHD RT–PCR products from various cell lines or tissues at different stages (Fig. 2). Consistent with the clone blot data (Fig. 1B), the DRG11 probe annealed to a single band in PHD RT–PCR products from DRG (Fig. 2C, lanes 7 and 8), but not in MAH, Rat1, or NCM-1 cells (Fig. 2C, lanes 1–6). By this analysis, DRG11 mRNA appears to be expressed in sensory ganglia but not in cell lines representing sympathetic neuron or glial precursors or in fibroblasts. As expected from the clone blot analysis, hybridization patterns distinct from that of DRG11 were obtained with the Pax3 and NCM3 probes (Figs. 2D and E).

To isolate full-length DRG11 cDNA clones a cDNA library was constructed using PCR-amplified cDNA from E13.5 DRG (see Experimental Methods) and screened using the DRG11 PCR product as a probe. The deduced amino acid sequence of the longest cDNA (2.4 kb) obtained is presented in Fig. 3A. The clone encodes a novel 28.6-kDa protein in the PHD family. As the 280-bp sequence upstream of the most N-terminal methionine contains an in-frame translational termination codon, we have tentatively assigned this methionine as the initiation codon. Attempts to determine the size of native DRG11 mRNA by Northern blotting of either total or poly($A^+$) RNA from rat embryos were unsuccessful, most likely due to the low abundance of the transcript. Moreover, the hamster anti-rat DRG11 monoclonal antibodies we generated (see below) did not work well in Western blotting experiments; therefore we are unable to establish the size of the native DRG11 protein. Thus, we cannot rule out the possibility that this protein is larger than that predicted by the deduced amino acid sequence from our cDNA clone.

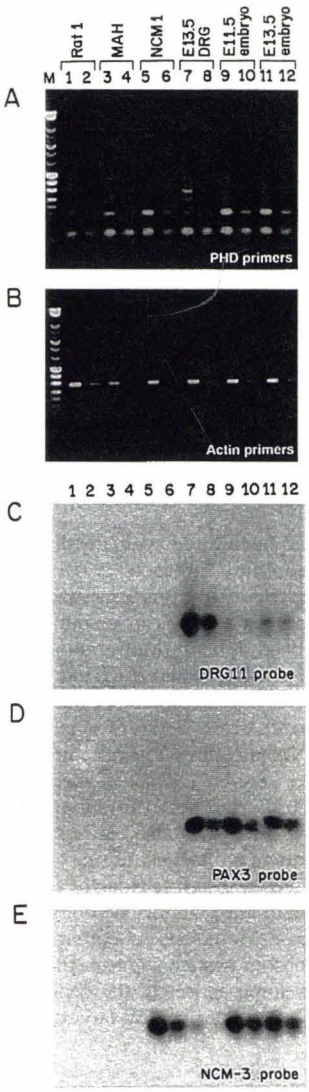


FIG. 2. (A) RT–PCR of paired homeodomain genes and (C–E) differential expression of the paired homeodomain genes. (A) Agarose electrophoresis of PCR products obtained from Rat 1 fibroblasts (lanes 1, 2), MAH (3, 4) and NCM1 cells (5, 6), E13.5 rat DRG (7, 8), E11.5 rat embryo (9, 10), and E13.5 embryo (11, 12) cDNA using the same degenerate primers as were used to clone the paired homeodomain genes. One microliter (odd-numbered lanes) or 0.1 $\mu$l (even-numbered lanes) of cDNA mixture equivalent to 40 or 4 ng, respectively, of total RNA (see Experimental Methods) were used as template for PCR. (B) Control RT–PCR from the same cDNA sources as in (A) performed using actin primers. (C–E) the PHD dRT–PCR products (A) were transferred to GeneScreen filters and hybridized using probes containing the homeodomains of (C) DRG11, (D) Pax3, and (E) NCM-3.

A

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MFYFHCPPQLEGTAPFGNHSTGDFDDGFLR   30
RKQRRNRTTFALQQLEALEAVFAQTHYPDV   60
FTREELAMKINLTEARVQVWFQNRRAKWRK   90
TERGASDQEPGAKEPMAEVTPPPVRNINSP  120
PPGDQARGKKEALEAQQSLGRTVGPAGPFF  150
PSCLPGTLLNTATYAQALSHVASLKGGPLC  180
SCCVPDPMGLSFLPTYGCQSNRTASVAALR  210
MKAREHSEAVLQSANLLPSTSSSPGPASKQ  240
VPPEGSQDKPSPTKEQSEGEKSV*  264
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B

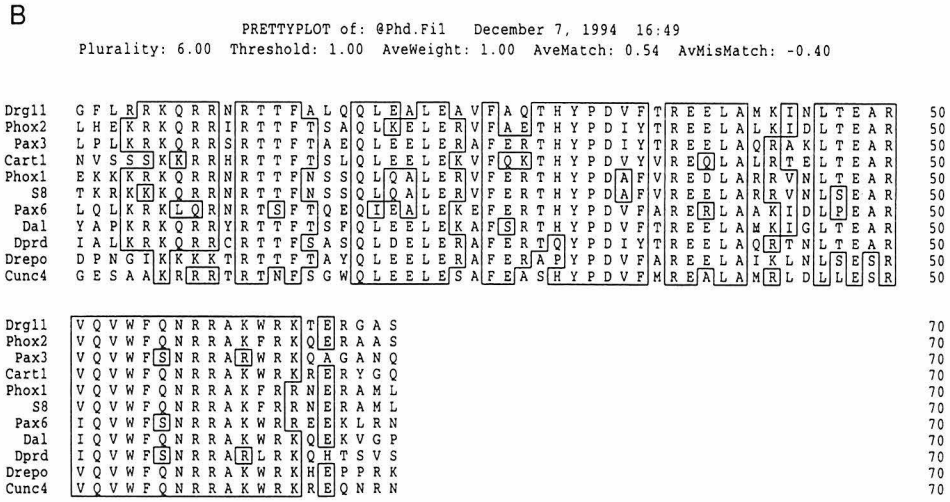



FIG. 3. (A) Deduced amino acid sequence of DRG11 protein; the homeodomain is underlined. (B) Comparison of homeodomain sequences among paired homeodomain proteins. The sources of the sequences illustrated are: Phox2 (Valarché *et al.*, 1993); Cart1 (Zhao *et al.*, 1993); Phox1 (Grueneberg *et al.*, 1992); S8 (Opstelten *et al.*, 1991); Pax6 (Walther and Gruss, 1991); Dal (Schneitz *et al.*, 1993); Dprd (Frigerio *et al.*, 1986); Drepo (Xiong *et al.*, 1994); Cunc4 (Miller *et al.*, 1992).

DRG11 is closely related in sequence to Pax3 as well as to several other paired homeodomain proteins (Fig. 3B). Unlike the Pax genes, however, DRG11 lacks a paired domain (Fig. 3A). Interestingly, DRG11 has a Gln residue instead of a Ser at position 9 of the recognition helix (residue 56 in Fig. 3B); a substitution of Gln for Ser at this position also occurs in several other family members that lack a paired domain (e.g., Phox2 and Cart1 in Fig. 3B).

### *DRG11 Is Specifically Expressed in Sensory Neurons and in a Subset of Their CNS Targets*

We next performed *in situ* hybridization experiments using digoxygenin-labeled full-length DRG11 cRNA probes, on sections from rat embryos of different ages. The earliest stage at which DRG11 mRNA could be detected was at E12.5, 1–2 days following the initial condensation of neural crest cells to form dorsal root ganglia. Expression at this age was restricted to the nervous system and within the trunk region was restricted to the dorsal root ganglia; no expression was detected in the neural tube (Fig. 4A). By contrast, other transcription factors expressed in sensory neurons at this stage, such as Isl-1 and Pax3 (Fig. 4C, D) are also expressed in the neural tube.

By E15.5, DRG11 expression was strong in trunk DRG (Fig. 5A, arrowheads) and was also detected in the dorsal spinal cord. Positive cells were located both laterally near

the dorsal root entry zone and more medially near the ventricular zone (Fig. 5A, arrows). Between these two zones, there is a region containing only scattered DRG11-positive cells (Fig. 5A, open arrow), although this region does contain neurons as shown by SCG10 hybridization (Fig. 5B, solid arrow). This pattern suggests that DRG11 is expressed by cells which display the known migration pattern of newly born dorsal horn neurons (Langman and Haden, 1970): these cells are initially generated in the ventricular zone, then migrate laterally through an intermediate region to take up their final position at the dorsolateral margins of the spinal cord, where they create a second region of high DRG11 expression. The DRG11 expression pattern is distinct from that of a related PHD protein, Pax3 (Goulding *et al.*, 1991), which is also expressed in the dorsal spinal cord at this stage but only in the ventricular zone (Fig. 5C, arrowhead).

Although DRG11 was detected in sensory but not sympathetic ganglia (not shown) at E15.5, this apparent specificity could simply reflect the timing of differentiation in the two groups of neurons; sympathetic development is known to lag behind sensory development by several days. For example, trkA mRNA is initially expressed in sensory but not sympathetic neurons at E12.5 (Martin-Zanca *et al.*, 1990), but is later detected in sympathetic ganglia beginning on E16.5–E17.5 (Birren *et al.*, 1993). To determine whether this is also true for DRG11, we examined its expression in sections through the anterior trunk region of E17.5 embryos. These sections contain large sympathetic ganglia expressing SCG10 (Fig. 6B), trkA (Fig. 6D), and Isl-1 (Fig. 6C), but not DRG11 (Fig. 6A, arrows). This specificity of DRG11 expression in the PNS is also maintained at Postnatal Day 3 (not shown). At E17.5, DRG11 expression in the spinal cord appeared increased in the dorsal horns relative to E15.5 (Fig. 6A, open arrow). Interestingly, this region of the spinal cord receives synaptic input from the DRG (Kandel *et al.*, 1991). Examination at high magnification of sections hybridized with the DRG11 cRNA probe revealed that the DRG11$^+$ cells have a process-bearing morphology, suggesting that they are neurons (not shown). These data suggest that DRG11 is expressed both by sensory neurons in the DRG and by a subset of their synaptic target neurons in the dorsal spinal cord. At no time, however, did we detect DRG11 expression in the ventral spinal cord which also receives sensory innervation. No DRG11 expression was detected outside of the nervous system at any of the stages examined.

The expression of DRG11 in sensory ganglia as well as in a subset of their CNS targets raised the question of whether DRG11 is expressed only by those sensory neurons that project to the dorsal spinal cord. These neurons include the nociceptive, NGF-dependent subset (Ruit *et al.*, 1992) which expresses trkA. These neurons can be distinguished from other sensory neurons which express trkC, are NT-3-dependent, and project to the ventral spinal cord, many of which are proprioceptive (for review, see Snider, 1994). The expression of DRG11 in E13.5 trigeminal sensory ganglia (Fig. 7A, arrow) as well as in trunk DRG (Fig. 6A, arrowheads) appeared broader than that of trks A, B, or C in nearby sections, however (Figs. 6D, 7D–7F, and data not shown). Rather, the extent of DRG11 expression was similar to that of SCG10 (Stein *et al.*, 1988) or Isl-1, two markers which label all neurons in these sensory ganglia (Figs. 7B and 7C). In addition, DRG11 expression was detected in both small and large DRG neurons at E17.5; in contrast the larger neurons expressed trks B and C but not A (Figs. 7D–7F and data not shown). This suggests that, at least at this stage, DRG11 expression includes, but is not restricted to, the NGF-responsive subset of sensory neurons. That such neurons do express DRG11, however, is supported by double-label immunocytochemical labeling experiments (see below).

To determine whether DRG11 expression within ganglia is restricted to neurons or is common to neurons and nonneuronal (glial) cells, we examined its expression in dissociated postnatal DRG cultures using a monoclonal antibody raised against bacterially expressed DRG11 protein (see Experimental Methods). Staining of perinatal rat dissociated DRG cultures revealed nuclear immunoreactivity in many sensory neurons, but not in glia (Figs. 8A and 8B). Approximately 60% of the neurons were labeled by the antibody under these conditions (Fig. 8A, arrowheads). Whether the apparent lack of DRG11 expression in other sensory neurons reflects distinct sensory lineages or rather environmental modulation in culture is currently being investigated. As an additional control for the specificity of the antibody, similar dissociated cultures of superior cervical sympathetic ganglia (SCG) were stained. No labeling of any cells was detected (Figs. 8C and 8D). Thus, as predicted by its initial selection in the dRT–PCR screen based on expression in DRG but not in MAH or NCM-1 cell cDNA, DRG11 is expressed in many sensory neurons but not in sympathetic neurons or glial cells.

The availability of a monoclonal antibody to DRG11 allowed a preliminary assessment of whether this transcription factor is indeed expressed by the trkA-expressing subset of DRG sensory neurons, as suggested by the *in situ* hybridization data (see above). To address this question, dissociated cultures of E16.5 DRG were double-labeled with anti-DRG11 and a specific polyclonal antiserum to trkA (Clary *et al.*, 1994). Many trkA$^+$ neurons (Fig. 9A, arrowheads) coexpressed DRG11 in their nuclei (Fig. 9A, open arrows). Conversely, it appeared that the

majority of DRG11$^+$ cells also expressed trkA, although a few DRG11$^+$ trkA$^-$ cells could be observed. Finally, some neurons in the cultures expressed neither marker (Figs. 9A and 9B, solid arrow). These data confirm that DRG11 is expressed by NGF-responsive DRG sensory neurons, many of which are nociceptive neurons that project to the dorsal horn of the spinal cord (Snider, 1994) where DRG11 is also expressed. However, DRG11 expression is also detected in some sensory neurons that do not express trkA, consistent with the *in situ* hybridization data.

## DISCUSSION

Transcription factors have proven to be useful markers of cell type in the nervous system. Here we have developed and applied a method that combines degenerate RT–PCR with differential hybridization and used it to screen several families of transcription factors for genes that are differentially expressed among neural crest derivatives. We have identified a novel paired homeodomain gene, called DRG11, that is expressed in sensory but not sympathetic neurons and in dorsal horn neurons in the spinal cord. This transcription factor provides a molecular marker for the sensory lineage in the mammalian peripheral nervous system. Moreover, the expression pattern of DRG11 suggests that it may function in some aspect of the establishment or maintenance of appropriate connectivity between some sensory neurons and a subset of their central targets.

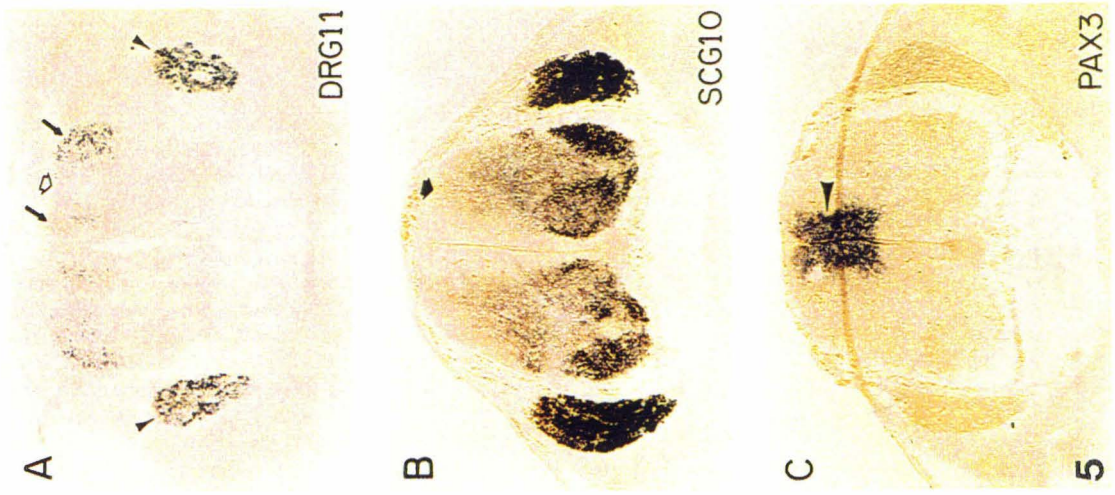
### *DRG11 Is a Sensory Neuron-Specific Paired Homeodomain Protein*

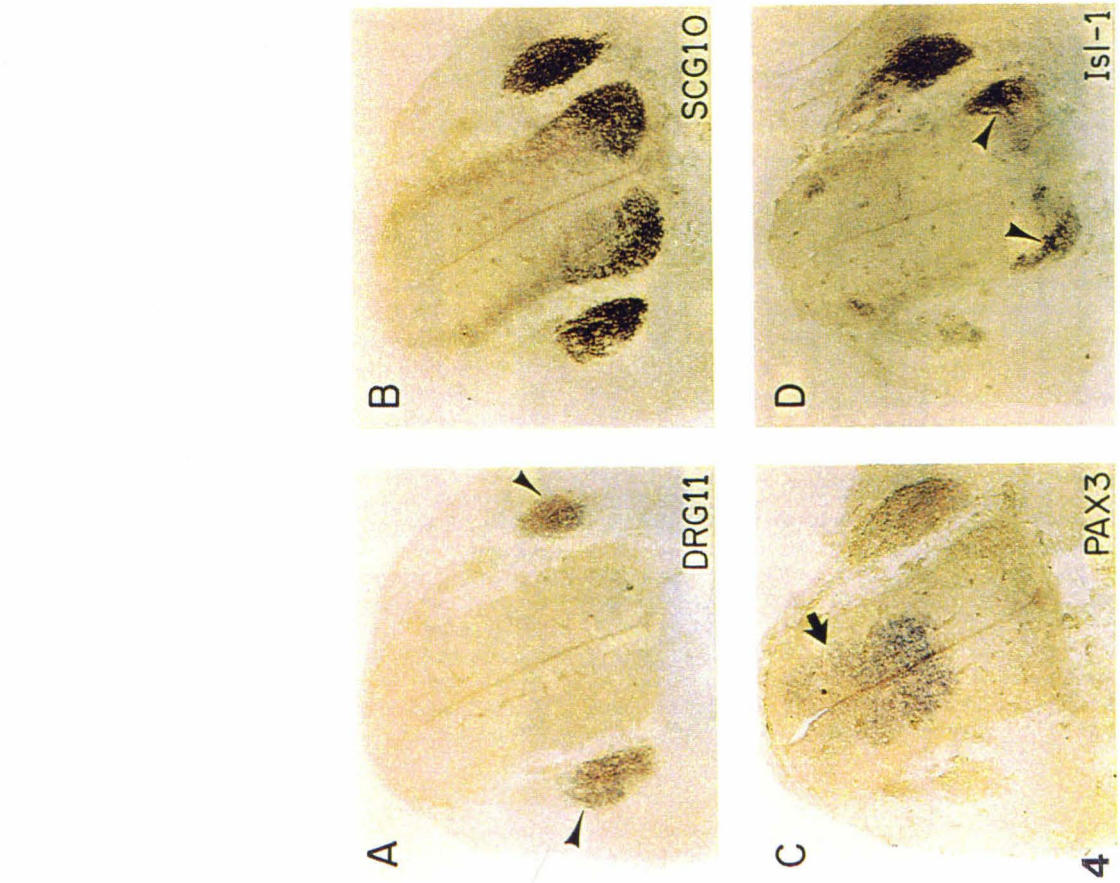
DRG11 is a transcription factor expressed in sensory but not in autonomic neurons. While other transcription factors have been reported to be specifically expressed in sensory neurons [such as Pax3 (Goulding *et al.*, 1991), the POU-domain protein Brn 3.0 (Gerrero *et al.*, 1993) and the bHLH proteins NSCL1, NSCL2 (Begley *et al.*, 1992; Göbel *et al.*, 1992), and NeuroD (Lee *et al.*, 1995)], in most of these cases a detailed temporal analysis of expression in autonomic ganglia was not reported. As mentioned earlier, the expression of several genes (such as trkA) in sympathetic ganglia (Birren *et al.*, 1993) lags 4–5 days behind their initial expression in sensory ganglia (Martin-Zanca *et al.*, 1990), perhaps in part because mitotic arrest of sympathetic neurons similarly lags behind that in sensory neurons (Rohrer and Thoenen, 1987; Verdi and Anderson, 1994). Most of the studies cited above examined expression at early gestational stages (E11.5–E13.5), and therefore expression of these genes in sympathetic ganglia at later stages might have been missed. Here we have shown that DRG11 is expressed by sensory neurons, but not by sympathetic and other autonomic neurons (such as parasympathetic and enteric neurons) even at late gestational and postnatal stages (Fig. 6 and data not shown).

The timing of DRG11 expression, 1–2 days after neurons are first detected in the DRG by expression of panneuronal markers such as SCG10, suggests that this putative transcriptional regulator is unlikely to be required for initial neuronal differentiation. Rather, this protein is likely to regulate later-developing aspects of sensory neuron phenotype or function. In this respect, the homology of DRG11 to another PHD protein, Phox2 (Fig. 3B), is of interest. Phox2 is expressed specifically in developing autonomic ganglia but not in trunk sensory ganglia (Valarché *et al.*, 1993), a pattern which is strikingly complementary to that of DRG11. Correlative data from *in vivo* and *in vitro* experiments, as well as DNA-binding data, suggest that Phox2 may be involved in the expression of neurotransmitter synthesizing enzymes such as DBH (Tissier-Seta *et al.*, 1993). By analogy, DRG11 could play a role in specifying some aspect of the complex neurotransmitter phenotype of sensory neurons. Alternatively, if as suggested below, DRG11 function is important in appropriate synapse formation, its downstream targets could include cell surface proteins important in establishing or maintaining proper connectivity. In this respect it is of interest that Phox2 has also been shown to regulate the promoter of *Ncam*, a cell surface adhesion molecule (Tissier-Seta *et al.*, 1993; Valarché *et al.*, 1993).

An intriguing feature of DRG11 expression is that it is detected both in sensory neurons and in a subset of their target neurons in the spinal cord, specifically those in the dorsal horn (Fig. 6A). A subset of these central neurons receive input from the NGF-dependent population of sensory neurons, which (as shown by double-labeling with antibodies to trkA) also expresses DRG11. This sug-

FIG. 4. Expression of DRG11 mRNA in the trunk spinal cord region of a rat E12.5 embryo. Serial sections were hybridized with anti-sense cRNA probes for (A) DRG11, (B) SCG10, (C) PAX3, and (D) Islet-1. Control hybridization using a DRG11 sense-strand probe gave no signal (not shown).
FIG. 5. Expression of DRG11 mRNA in the trunk spinal cord of an E15.5 rat embryo. Sections were hybridized using anti-sense cRNA probes for (A) DRG11, (B) SCG10, and (C) PAX3. Arrowheads indicate the DRG, arrows DRG11 expression domains in the dorsal spinal cord. No DRG11 expression was detected outside of the nervous system or in autonomic ganglia (not shown).




286

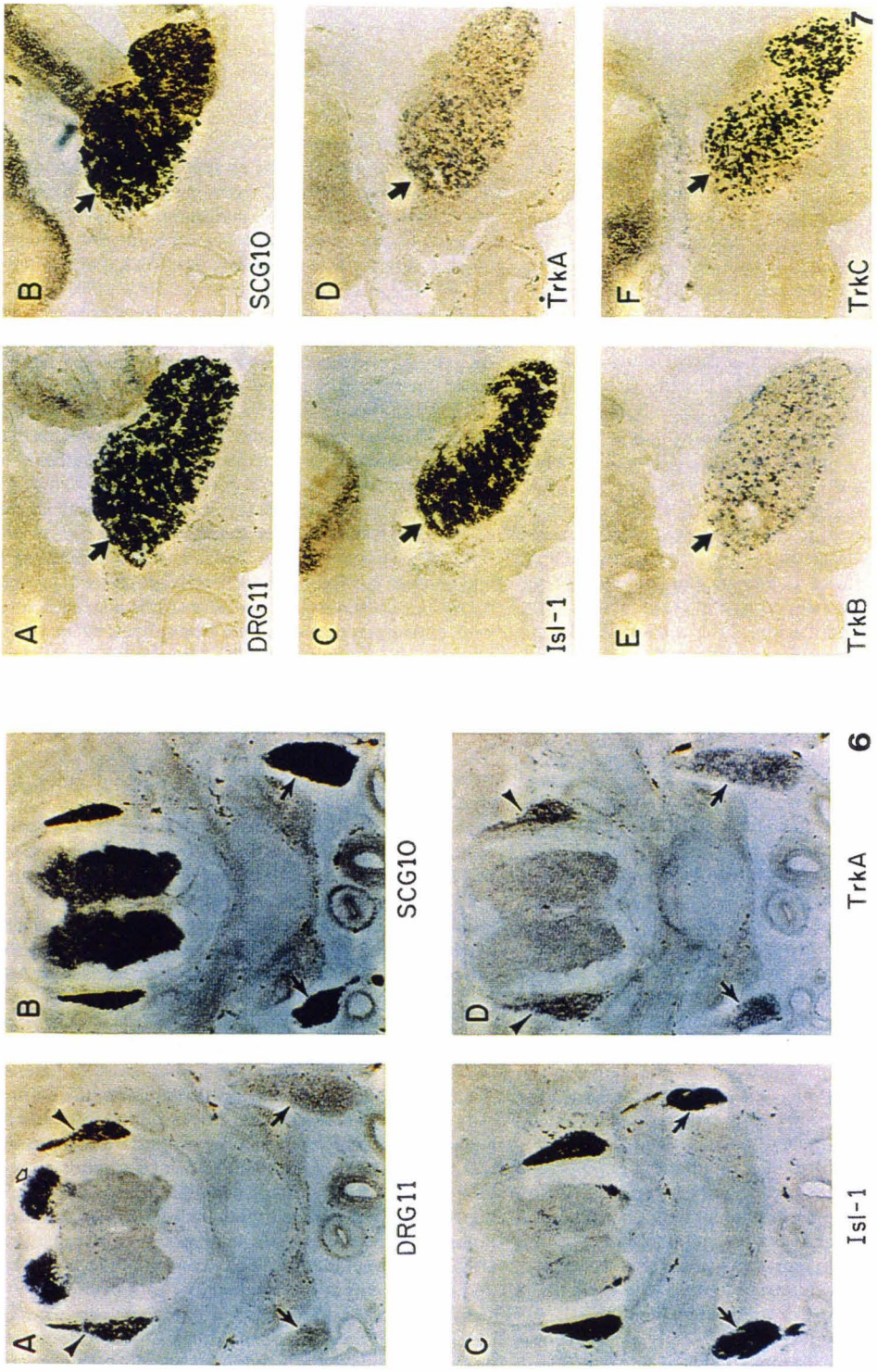



FIG. 6. Expression of DRG11 mRNA in the trunk region of a rat E17.5 embryo. Sections were hybridized with anti-sense cRNA probes for (A) DRG11, (B) SCG10, (C) Islet-1, and (D) TrkA. Arrowheads (A, D) indicate DRG, filled arrows (A–D) the sympathetic ganglia, and open arrow (A) the dorsal horn of the spinal cord.

FIG. 7. Expression of DRG11 mRNA in E13.5 trigeminal sensory ganglia. Serial sections through the same ganglion were hybridized with the probes indicated at the bottom. The number of cells expressing DRG11 appears greater than that obtained using trks A, B, or C probes (D–F) and comparable to that obtained using the panneuronal probes (B) SCG10 and (C) Isl-1.

gests that DRG11 might function in regulating some aspect of synapse formation between sensory neurons and their central targets. However, DRG11 expression within sensory ganglia was more extensive than that of trkA (Figs. 6 and 7); for example it was detected in large neurons which typically project to the ventral spinal cord (Snider, 1994). This suggests that DRG11 expression is not restricted to NGF-dependent sensory neurons. This indicates that DRG11 expression cannot be sufficient to specify the central connectivity of such neurons. However it could be necessary for some aspect of this process. This notion is strengthened by the fact that DRG11 first appears in the dorsal spinal cord at E15.5, about the time at which the first sensory afferents are growing into this region of the CNS. Studies in the chick indicate that some aspects of dorsal horn development depend upon sensory axon ingrowth (Sharma *et al.*, 1994). Experiments in progress are aimed at determining whether DRG11 expression in dorsal spinal cord neurons is dependent upon such ingrowth, or is, rather, autonomously controlled. In turn, functional perturbation experiments currently underway should address the role of DRG11 in the development of sensory neurons and their connections with the spinal cord.

### *Strengths and Limitations of dRT–PCR as a Method for Identifying Cell-Type-Specific Genes*

The dRT–PCR method we have developed has several advantages over conventional brute force RT–PCR using degenerate primers. First and foremost, only those RT–PCR products that display differential expression between cell types or tissues are selected for sequencing. This greatly reduces the number of PCR products that must be sequenced for any set of degenerate primers to identify interesting genes. Second, the intensity of the hybridization signals exhibited by a given clone annealed with multiple probes, as well as compared to other clones annealed with the same probes, provides a unique "fingerprint" for that clone. In our experience, clones displaying similar fingerprints usually contain the same insert sequence. Thus, by selecting for sequencing only those clones which exhibit different hybridization fingerprints, the characterization of redundant inserts is reduced. Third, although sequencing of inserts from a PCR experiment may no longer be rate-limiting due to automation, the analysis of the expression pattern of each of the PCR products still represents a major bottleneck. dRT–PCR reduces the number of genes whose expression patterns have to be characterized by identifying differentially expressed sequences at an early stage in the procedure. These three factors combined allow a relatively rapid assessment of whether a set of degenerate primers will identify gene family members differentially expressed among a set of tissues or cell types. This in turn permits rapid screening of multiple pairs of degenerate primers, either within a single gene family or among several different families. Similar approaches related to dRT–PCR have been described previously by others (Boehm, 1993; Lai and Lemke, 1991; Wilkie and Simon, 1991).

An unexpected problem we encountered is that the *in vivo* expression pattern of several differentially expressed dRT–PCR products was inconsistent with expectations based on their differential distribution in the original screen (T. Saito, L. Sommer, and D. Anderson, unpublished observations). In some cases, a gene was not detectably expressed in the expected tissue but rather was highly expressed elsewhere. For example, the NCM3 sequence, which behaved as if it were glial-specific in the dRT–PCR screen, was not detectably expressed by peripheral glial cells *in vivo*, but rather was abundantly expressed in cartilage, a tissue not examined in the original dRT–PCR screen (data not shown). Similar situations were encountered in a screen of receptor tyrosine phosphatases (L. Sommer and D. Anderson, unpublished data).

The reason(s) for this discrepancy is not clear. It may reflect aberrant expression of genes in some of the cell lines used as sources of cDNA. Alternatively, in cases where dissected tissues were used it could reflect the efficient amplification of sequences present in minor or contaminating cell types. These are biological problems rather than problems with the dRT–PCR method per se, but they illustrate the importance of judiciously selecting multiple tissues and/or cell lines for the initial dRT–PCR screen. Another potential explanation is that some genes may be preferentially amplified by a specific primer set, which would be a problem intrinsic to the PCR method rather than to dRT–PCR.

A final possibility, specific to dRT–PCR, derives from the fact that hybridization signal intensity in the clone blots actually reflects the relative abundance of that sequence *within the gene family of interest*, rather than in the cDNA population as a whole. In the limiting case where a given cell type expresses only a single family member, that sequence would represent 100% of the hybridization probe (and therefore would yield an intense hybridization signal from that cell type) even if it is actually expressed at very low levels in that cell or tissue. Such artifacts can be minimized by performing RNase protection experiments for individual clones on different tissues. In any case, the pattern of specificity exhibited by the dRT–PCR procedure should be considered tentative until confirmed by *in situ* hybridization data. This in turn raises another problem, in that the short (typically <250-

bp) PCR products provide insufficiently sensitive probes for the nonradioactive *in situ* hybridization procedure, necessitating additional time and effort to isolate longer cDNAs. The value of this method would be improved if sensitive and reliable nonradioactive *in situ* hybridization procedures could be performed directly with the PCR products. Nevertheless, the prescreening of clones in dRT–PCR still reduces the number of genes that have to be characterized by these more labor-intensive procedures.

In summary, our data support the idea that transcription factors provide useful and specific markers for subtypes of neurons in the vertebrate nervous system. We have developed and applied a method that combines degenerate PCR amplification with differential hybridization to allow rapid screening of gene families for members which are differentially expressed among populations of neural cell types. This screen has yielded a novel paired homeodomain protein, DRG11, that represents the first sensory neuron-specific transcription factor identified in mammals. The expression pattern of DRG11 suggests that its mechanism of regulation and function should be interesting subjects for future study. In addition, this gene may provide a useful marker for cell biological and genetic studies of the development of sensory and dorsal horn neurons.

## EXPERIMENTAL METHODS

### *Materials*

Rat embryos and pups were obtained from timed pregnant Sprague–Dawley rats (Simonson Laboratories). MAH, NCM1, and Rat1 cells were maintained as described previously (Birren *et al.*, 1993; Lo *et al.*, 1990).

### *Differential RT–PCR*

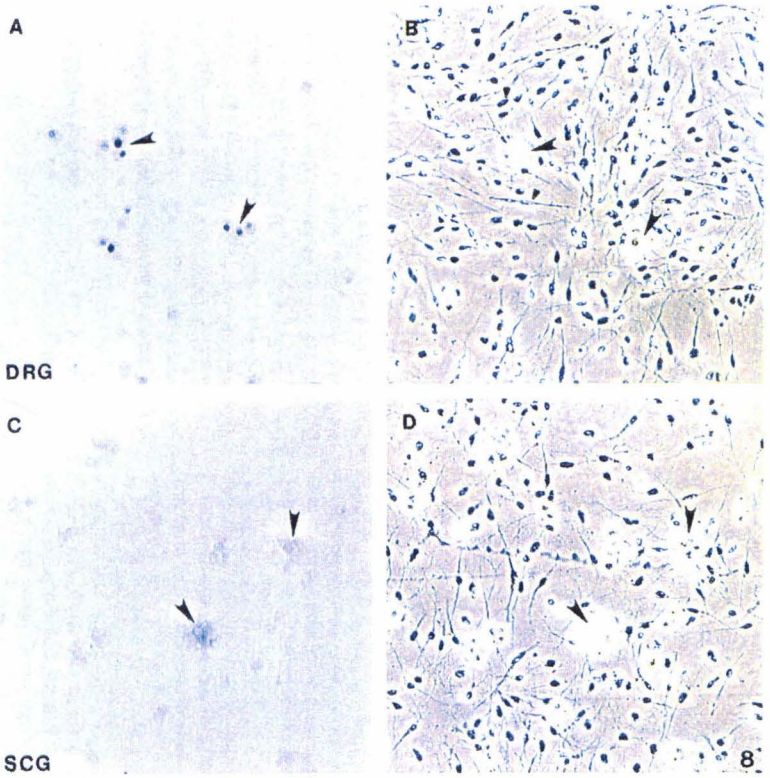
Molecular cloning was performed according to standard procedures (Sambrook *et al.*, 1989), with minor modifications. Total RNA was prepared by acid guanidinum thiocyanate method as described (Chomczynski and Sacchi, 1987) with slight modification. cDNA was synthesized from 2 $\mu$g of total RNA in 50 $\mu$l by using random hexamer primers and reverse transcriptase, and 1 $\mu$l of this reaction mixture was used for PCR. Degenerate oligonucleotide primers corresponding to the sequence coding for amino acids FTAYQLE and the complementary sequence coding for amino acids QVWFQNR [N-terminal and C-terminal portions of the paired type homeodomain from the *Drosophila* protein RK2/repo (Campbell *et al.*, 1994; Xiong *et al.*, 1994)] were used for PCR: CGGGATCCTT(TC)ACIGCITA(TC)CA(GA)-(TC)TIGA and CGGAATTC(GT)(GA)TT(TC)TG(GA)A-ACCAIAC(TC)TG. DNA was amplified by *Taq* DNA polymerase under the following conditions: 5 reduced stringency cycles using 94°C for 1 min, 42°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by 33 cycles using 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products were fractionated by agarose gels and DNA fragments of the expected size (~150 bp) were purified using the Mermaid kit (Bio101). Purified DNA fragments were reamplified using 28 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, purified by phenol extraction, and digested by *Eco*RI and *Bam*HI. Digested DNA fragments with a size of ~150 bp were purified from agarose gels (see above). An aliquot of the digested DNA fragments was ligated into pBluescript for subcloning and transformed into *Escherichia coli*. Another aliquot of these DNA fragments was saved for use as a probe in differential hybridization. Each transformant was transferred into 70 $\mu$l of LB-amp medium in a well of a 96-well plate and cultured at 37°C for 14 h with shaking. Five microliters of the liquid culture was spotted onto replica filters (Genescreen, Dupont) using a multichannel pipettor. The filter was treated according to the manufacturer's protocol and hybridized with a $^{32}$P-labeled probe prepared by random-primed labeling of the other aliquot of the restriction-digested PCR products. Nucleotide sequences of the clones which showed differential distribution were determined.

### *Southern Blotting of PCR Products*

PCR products of RT–PCR were fractionated by agarose gel electrophoresis, transferred onto replicate Genescreen filters, and hybridized with $^{32}$P-labeled probes derived from the DNA fragments encoding the homeodomains of DRG11, Pax3, or NCM3. As a control for the amount of amplified cDNA in the gel blot experiments, parallel aliquots were amplified for $\beta$-actin using gene-specific primers. The PCR product corresponded to a 310-bp fragment spanning nucleotides 767–1077 of the rat $\beta$-actin mRNA and was amplified with the following primers: TCATGAAGTGTGACGTTGACATCC and GT-AAAACGCAGCTCAGTAACAGTC. Conditions were 20 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min.

### *cDNA Library Construction and Screening*

Poly(A$^+$) RNA was purified from 1 $\mu$g of total RNA from E13.5 rat dorsal root ganglion by using oligo(dT) magnetic beads (Dynal) and converted to cDNA using the Superscript choice system (Gibco BRL). The cDNA was ligated to a pre-annealed mixture of oligonucleo-



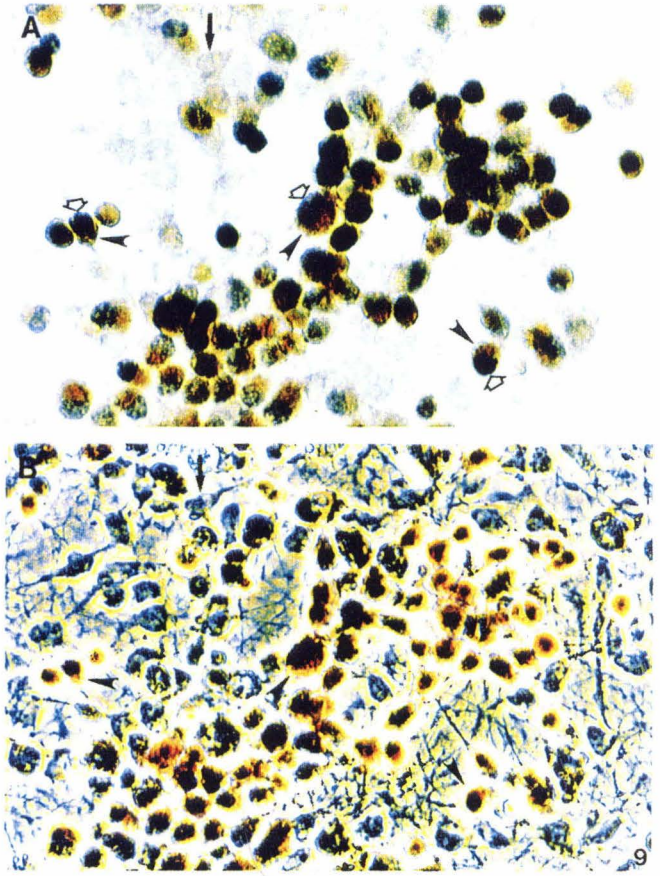




**FIG. 8.** Expression of DRG11 protein in dissociated sensory neurons. Postnatal Day 2 (A, B) dorsal root ganglia and (C, D) superior cervical ganglia were cultured overnight, fixed, and stained with monoclonal antibody to DRG11 protein. Note that the antibody stains the nuclei of several neurons in the DRG cultures (A, arrowheads), but not those in the SCG cultures (C, arrowheads), which exhibit only a low level of nonspecific background staining as determined by comparison to controls stained with an irrelevant primary antibody (not shown). (B, D) Phase-contrast micrographs of the bright-field images shown in (A, C) are provided to reveal glial cells (small arrowheads) which are not labeled in either culture.

**FIG. 9.** Coexpression of DRG11 and trkA in some sensory neurons. Embryonic Day 16.5 DRG were dissociated, cultured overnight, fixed, and double-labeled with monoclonal antibody to DRG11 (purple staining) and polyclonal antibody to trkA (brown staining). Open arrows (A) indicate neurons containing DRG11 in their nuclei which also express trkA (solid arrowheads). (A, B) Solid arrow indicates a neuron that expressed neither protein. The majority of $trkA^+$ neurons coexpressed DRG11 at this stage, but some $DRG11^+$ cells did not express trkA.

tides ACTGAAGCCAAGGTAGGATCCG and (phosphorylated) CGGATCCTACCTTGGCTTCAGTAG. The ligated cDNA was purified using the Spinbind PCR purification system (FMC) and amplified by 3 rounds of PCR (16 cycles, 12 cycles, and 9 cycles) using a phosphorylated oligonucleotide (CTACTGAAGCCAAGGTAGGATCCG) under the condition of 95°C for 1.5 min, 64°C for 2 min, and 72°C for 7 min. After each round of PCR, a small aliquot of the amplified DNA (one-tenth of the total reaction mixture) was used for the next round of PCR. [$^{32}$P]dCTP was added in the third round of PCR to calculate the amount of amplified DNA. Approximately 1.3 $\mu$g of DNA was obtained after the third round of PCR. cDNA fragments longer than 500 bp were separated on a size-fractionation column (Gibco BRL) and cloned into the λ ZapII vector (Stratagene).

The library was screened using a DNA probe containing the homeodomain of the DRG11 RT-PCR product. Two cDNA clones containing 2.4- and 1.0-kb inserts were obtained. Nucleotide sequences were determined on both strands using Sequenase (USB). The sequence of DRG11 has been submitted to GenBank under Accession No. U29174.

### *In Situ Hybridization*

Nonradioactive *in situ* hybridization was performed as described previously (Birren *et al.*, 1993). The following probes were used: SCG10 (Stein *et al.*, 1988); trkA, from pDM97 (a gift from L. Parada); trkB, from pFRK16; trkC, from pRtrkc8 (gifts from G. Yancopoulos). Islet-1 and PAX3 clones, which carry 0.9 and 1.2 kb of cDNA, respectively, were obtained by RT-PCR.

### *Antibody Production*

DRG11 protein expressed in bacteria was gel-purified and used to immunize Armenian hamsters. Hamster spleen cells were fused with P3X63Ag8u.1 mouse myeloma cells. Supernatants were first screened on dot blots of recombinant DRG11 protein, and positives from this screen were rescreened by immunofluorescence staining of CHO cells transfected with a mammalian DRG11 expression construct. Details of staining procedures, secondary antibodies, *etc.*, are available on request.

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*Note added in proof.* We have recently succeeded in identifying DRG11 transcripts by Northern blot. A single species of ~2.4 kb was detected in mRNA from DRG and neural tube. The size of this transcript is similar to the size of the DRG11 cDNA clone that was sequenced. This suggests that the clone is close to full-length and therefore that the deduced amino acid sequence of DRG11 represents the intact polypeptide.

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