Restriction of developmental potential during divergence of the enteric and sympathetic neuronal lineages

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SUMMARY

In the peripheral nervous system, enteric and sympathetic neurons develop from multipotent neural crest cells. While local environmental signals in the gut and in the region of the sympathetic ganglia play a role in the choice of cell fate, little is known about the mechanisms that underlie restriction to specific neuronal phenotypes. We investigated the divergence and restriction of the enteric and sympathetic neuronal lineages using immuno-isolated neural crest-derived cells from the gut and sympathetic ganglia. Analysis of neuronal and lineage-specific mRNAs and proteins indicated that neural crest-derived cells from the gut and sympathetic ganglia had initiated neuronal differentiation and phenotypic divergence by E14.5 in the rat. We investigated the developmental potential of these cells using expression of tyrosine hydroxylase as a marker for a sympathetic phenotype. Tyrosine hydroxylase expression was examined in neurons that developed from sympathetic and enteric neuroblasts under the following culture conditions: culture alone; coculture with gut monolayers to promote enteric differentiation; or coculture with dorsal aorta monolayers to promote noradrenergic differentiation. Both enteric and sympathetic neuroblasts displayed developmental plasticity at E14.5. Sympathetic neuroblasts downregulated tyrosine hydroxylase in response to signals from the gut environment and enteric neuroblasts increased expression of tyrosine hydroxylase when grown on dorsal aorta or in the absence of other cell types. Tracking of individual sympathetic cells displaying a neuronal morphology at the time of plating indicated that neuroblasts retained phenotypic plasticity even after initial neuronal differentiation had occurred. By E19.5 both enteric and sympathetic neuroblasts had undergone a significant loss of their developmental potential, with most neuroblasts retaining their lineage-specific phenotype in all environments tested. Together our data indicate that the developmental potential of enteric and sympathetic neuroblasts becomes restricted over time and that this restriction takes place not as a consequence of initial neuronal differentiation but during the period of neuronal maturation. Further, we have characterized a default pathway of adrenergic differentiation in the enteric nervous system and have defined a transient requirement for gut-derived factors in the maintenance of the enteric neuronal phenotype.

Key words: Enteric, Sympathetic, Restriction, Neuroblast, Rat

INTRODUCTION

The generation of neuronal diversity is one of the fundamental issues in developmental neurobiology. During the formation of the peripheral nervous system, neural crest cells migrate to a number of embryonic locations where they give rise to distinct neural lineages (Le Douarin, 1980; Le Douarin and Dupin, 1992). Although postmigratory crest-derived cells develop along specific, stereotypic pathways, analysis of neural crest cell fate both in vivo and by clonal analysis in vitro has indicated that many crest cells are multipotent prior to their arrival at their final embryonic location (Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988; Ito et al., 1988; Duff et al., 1991; Fraser and Bronner-Fraser, 1991; Stemple and Anderson, 1992). Neural crest cells that aggregate proximal to the dorsal aorta develop into the cells of the sympathetic ganglia, while similar cells that migrate into the embryonic gut give rise to the enteric nervous system.

Neural crest-derived enteric and sympathetic progenitors express a number of common markers both while migrating from the neural crest and upon reaching the dorsal aorta and the gut, giving rise to the hypothesis that enteric and sympathetic neurons are derived from a common sublineage of the neural crest (Carnahan et al., 1991; Gershon et al., 1993). Tyrosine hydroxylase (TH) is a catecholaminergic marker of sympathetic neurons which is transiently expressed by enteric precursors in the gut (Teitelman et al., 1978; Baetge et al., 1990). TH expression is maintained in developing sympathetic neurons while expression is lost in the enteric lineage shortly after the arrival of neural crest cells in the gut (Jonakait et al., 1979; Baetge et al., 1990). In the rat gut, TH expression is no longer detectable by approximately E15 (Baetge et al., 1990;
Carnahan et al., 1991). Other markers, including SA-1, B2 and MASH-1, are transiently expressed (Carnahan et al., 1991; Groves et al., 1995; Blaugrund et al., 1996), while expression of the endothelin B receptor and the ret receptor tyrosine kinase are maintained in both the enteric and sympathetic lineages (Pachnis et al., 1993; Baynash et al., 1994; Hosoda et al., 1994; Lo and Anderson, 1995; Durbec et al., 1996; Nataf et al., 1996). This overlapping pattern of gene expression is suggestive of the divergence of a common pool of progenitor cells. Eventually sympathetic and enteric neurons develop into phenotypically and functionally discrete neuronal populations with the majority of sympathetic neurons expressing a noradrenergic phenotype, and enteric neurons forming a complex network of enteric plexuses expressing a diverse array of neurotransmitters and peptides (McConalogue and Furness, 1994).

The distinct differentiation pathways followed by enteric and sympathetic precursor cells suggest that the local microenvironments to which neural crest cells migrate control cell fate (Coulter et al., 1988; Ernberger and Rohrer, 1996; Groves and Anderson, 1996). Studies of overlapping catecholaminergic and neuronal markers have demonstrated that the effects of environmental cues in the gut are instructive, as opposed to selective (Baetge et al., 1990; Carnahan et al., 1991). While investigations into the specific molecular cues that promote adrenergic differentiation in the region of the sympathetic ganglia have begun, less is known about the factors that promote the acquisition of an enteric phenotype by developing neurons in the gut. Bone morphogenetic protein-2 (BMP-2) and BMP-4 promote the adrenergic differentiation of neural crest cells (Reissmann et al., 1996; Shah et al., 1996; Varley and Maxwell, 1996) and are expressed in dorsal aorta (Reissmann et al., 1996). BMPs are early players in a cascade of developmental events that include expression of the basic helix-loop-helix transcription factor MASH-1 and the transcription factor Phox2a, leading to neuronal specification and the expression of an adrenergic phenotype (Shah et al., 1996; Hirsch et al., 1998; Lo et al., 1998). The role of these factors in the development of the transiently catecholaminergic cells in the gut is not well understood, although BMP-2 and other members of the adrenergic cascade are also expressed in the gut (Bitgood and McMahon, 1995; Blaugrund et al., 1996; Lo et al., 1997).

A number of other peptide factors influence the development of neurons in the sympathetic ganglia and in the gut, but their contribution to the phenotypic maturation of enteric and sympathetic neurons is not clear. Neurotrophin-3 (NT-3), acting through the TrkC receptor tyrosine kinase, supports the survival of proliferating sympathetic neuroblasts (Birren et al., 1993; DiCicco-Bloom, 1993) and promotes neuronal and glial differentiation of enteric progenitors (Chalazonitis et al., 1994). Glial cell line-derived neurotrophic factor (GDNF) also enhances the number of enteric and sympathetic neuroblasts that develop (Buj-Bello et al., 1995; Hearn et al., 1998). The relationship between these factors, the final phenotype of the neurons that develop and the mechanism of restriction of enteric and sympathetic neuroblasts to their specific lineages is still largely unresolved.

The phenotypes of sympathetic and enteric neurons diverge in their separate embryonic environments, but when and how developing neuroblasts become restricted in their developmental potential is unknown. There is evidence that post-migratory enteric progenitors retain some degree of phenotypic plasticity in the gut environment. Backtransplantation of quail gut segments into chick neural crest resulted in remigration of cells to multiple embryonic sites, including the identification of transplanted TH+ cells in the sympathetic ganglia (Rothman et al., 1990). Clonal analysis of avian and rat enteric neuroblasts revealed the potential of neural crest-derived cells in the gut to express an adrenergic phenotype (Sextier-Sainte-Claire Deville et al., 1994; Lo and Anderson, 1995). While these studies demonstrate that phenotypically plastic enteric neuroblasts are present in the embryonic gut, it is not clear whether these cells represent a small subpopulation of enteric neuroblasts, or whether maintenance of developmental potential is a general property of neural crest-derived enteric precursor cells. In addition, the timing of developmental restriction in the context of the gut environment is unknown.

Although sympathetic and enteric neurons arise from a similar progenitor population, the developmental potential of TH-expressing cells in the sympathetic ganglia is not known, and the restriction of enteric and sympathetic neuroblasts has not been addressed in a single study. Here we ask whether rat sympathetic and enteric neuroblasts are functionally equivalent in their capacity to alter their phenotype in response to environmental cues. We address the role of the local microenvironment in developmental restriction of the enteric and sympathetic lineages and examine the relationship between neuronal differentiation and developmental plasticity.

We have established in vitro environments for the development of enteric and sympathetic neurons and have determined that enteric and sympathetic neuroblasts from E14.5 rats are plastic with respect to their neuronal phenotype. By E19.5 the vast majority of enteric and sympathetic neurons no longer alter their phenotype in response to signals in an alternative environment; this restriction reflects intrinsic changes in the neuronal population. Restriction of developmental potential and neuronal differentiation are independently regulated in these cultures, with the timing of phenotype restriction corresponding to the period of neuronal maturation. During neuronal maturation in the gut, enteric neuroblasts also display a transient requirement for local gut-derived factors in order to maintain a TH− phenotype. These data define the timing of neuroblast/environment interactions in the peripheral nervous system, and reveal the consequences of these interactions for the restriction of the enteric and sympathetic neuronal lineages.

**MATERIALS AND METHODS**

**Primary cell cultures**

Cells were isolated from embryonic day 14.5 (E14.5) and 19.5 (E19.5) embryos from timed pregnant Simonsen Albino rats (Simonsen Labs, Sunnyvale, CA). The following tissues were removed and placed into air-buffered serum-free medium: thoracic sympathetic chains, dorsal aorta and the intestines (excluding stomach and colon). Tissues were enzymatically dissociated in 125-250 units/ml type I collagenase (Worthington) at 37°C for 30 minutes (E14.5) or 45 minutes (E19.5). Samples were triturated with fire-polished Pasteur pipettes and filtered through 60 μm sterile mesh. Single cell suspensions were either plated directly or used for the immuno-isolation of neuroblasts (see below).
Experiments. All cultures were grown in 2 dense monolayers. Neuroblasts were plated at 5,000 cells per well for end of the culture period in each environment the cells had formed coated with poly-d-ornithine (Gibco BRL) and laminin (Collaborative 7,000 cells per well in 24-well tissue culture dishes that had been before and after immuno-isolation. Isolated HNK-1 + cells were included in all antibody incubations. All cocultures were labeled with room temperature (Elberger and Honig, 1990). Goat serum (1%) was were fixed, washed and blocked (10% goat serum) in a sterile ways. Some cultures were equilibrated in 30% sucrose, frozen on dry (PKH26 or diI) during staining, we stained for intracellular antigens antibodies were used in these studies: rabbit anti-tyrosine hydroxylase antibodies diluted at 1:200 in preblock solution. The following incubations were for 2 hours at room temperature. Primary antibody incubations were overnight at 4°C. All were preblocked in 1% goat serum, 0.1% NP40 for 20 minutes at room temperature. Cells were After a 4 day culture period, cells were fixed in 3.7% formaldehyde, ·Staining was examined with an inverted Olympus IX70 Analysis of dye-labeled cells in complex environments ·Significant migration and cell death in the culture with fewer than 10% of the originally identified cells found at the end of the 4 day culture period. After fixing and staining, cells were found on the basis of their recorded position and scored for TH status. ·Cell death and proliferation assays DNA fragmentation indicative of apoptotic cell death was measured using terminal deoxynucleotidyl-transferase (TdT)-mediated DNA fragmentation indicative of apoptotic cell death was measured using terminal deoxynucleotidyl-transferase (TdT)-mediated biotinylated (dUTP) nick-end-labeling (TUNEL). Cocultures of dye-labeled HNK-1 + cells and environments were grown in 96-well tissue culture dishes and fixed with fresh 4% paraformaldehyde after 12 and 24 hours in culture. Cultures were processed using an In Situ Cell Death Detection Kit, Fluorescein (Boehringer-Mannheim) according to the manufacturer’s specifications with the exception that cells were permeabilized by freeze-thawing (see above) rather than NP40 treatment. Samples were treated with the TUNEL reagents for 2 hours at 37°C and the percentage of dye-labeled cells with TUNEL-labeled nuclei was determined. ·Cell division of dye-labeled cells in coculture was examined using bromo-deoxyuridine (BrdU) incorporation as previously described (Raff et al., 1988). Briefly, cells were cultured for 24 hours in the presence of 10 μM BrdU (Boehringer-Mannheim). After fixation in 70% ethanol for 20 minutes at −20°C samples were treated with 2N HCl and then 0.1 M NaB3O3-10H2O for 10 minutes each prior to blocking in 1% goat serum. Primary antibody against BrdU (DAKO) was used at a dilution of 1:400 for 2 hours at room temperature. Secondary staining was performed as described above and dye-labeled neuroblasts were scored for BrdU labeling. ·Comparative PCR RNA was prepared directly from HNK-1 + neuroblasts using a guanidinium thiocyanate and water-saturated phenol protocol (modified from Chomzynski and Sacchi, 1987). RNA was reverse-transcribed into cDNA using MMLV-reverse transcriptase (GibcoBRL) and random hexamer primers. Primer sets were obtained from the following sources; NF and trkC primers were designed using the Whitehead Institute Genome Center Primer3 program (Rosen and Skaltsky, 1996, 1997); trkA (Verdi et al., 1994); TH (Ohberg et al., 1995), actin (Dr Norman Davidson, Caltech). Cycle titrations were carried out with actin primers to determine the linear range for each cDNA sample. Based on actin data, cDNAs were diluted to permit us to use approximately the same amount of cDNA to amplify a specific mRNA from multiple samples. For each primer set, PCR reactions were run in triplicate and normalized to the average of parallel triplicate reactions run with actin primers. All reaction products were run on 1.5% agarose gels, stained with ethidium bromide and analyzed using the Molecular Analyst software (version 2.1.2). All data are shown as arbitrary units normalized to actin. A minimum of triplicate determinations were carried out on three independent RNA preparations for each primer set; errors shown are the standard error of the mean (s.e.m.). Each primer set yielded a single band whose identity was confirmed either by Southern hybridization or by direct sequencing of the PCR reaction product. Actin: 5'-CCACACCTT-TCTAAAAGAGAGGAGCAG-3' and 5'-CCGGAAGAGA-3'; trkC, 5'-CACGCCCTCTGAGACTCCTACC-3' and 5'-GAATGTGAC-GAGCGGAAGAGA-3'; trkA, 5'-GCTCCCACTTGAGAATG-3' and labeling status was not clear. Data are presented as the percentage of the total dye-positive neurons that expressed TH (% TH). In experiments in which individual cells were tracked and assayed for phenotype the following procedures were used in conjunction with the above techniques. First, a grid was etched into the bottom of the culture dish using a razor blade. Immuno-isolated, dye-labeled cells were plated onto dorsal aorta or gut environments on these dishes and allowed to adhere for 12 hours. At that time, wells were scanned for -process-bearing, dye-labeled cells and the grid location of the cells was recorded. Spot checks were done over the length of the culture period to follow the positional stability of the cells. We found significant migration and cell death in the culture with fewer than 10% of the originally identified cells found at the end of the 4 day culture period. After fixing and staining, cells were found on the basis of their recorded position and scored for TH status.

Immuno-isolation of embryonic enteric and sympathetic neuroblasts

Neural crest-derived cells were isolated from gut and sympathetic ganglia by labeling cell suspensions with the HNK-1 antibody (Abo and Balch, 1981; Erickson et al., 1989; Pomeranz et al., 1993) followed by sequential incubations with paramagnetic bead (Milenyi Biotech) and FITC-conjugated secondary antibodies (Jackson Immuno Research). Labeled cell suspensions were passed over a column held in a magnetic field. Negative cells were washed off from the column, the column was removed from the magnet, and the positive cells eluted into a collection tube. This process was repeated twice to maximize cell number and purity. The purification was monitored by counting the percentage of FITC + cells in the sample before and after immuno-isolation. Isolated HNK-1 + cells were labeled with a lipophilic dye, either PKH26 (Sigma) or diI (Molecular BioProbes), according to the manufacturer’s specifications.

Immunocytochemistry

After a 4 day culture period, cells were fixed in 3.7% formaldehyde, 5% sucrose for 10 minutes at room temperature. Cells were preblocked in 1% goat serum, 0.1% NP40 for 20 minutes at room temperature. Primary antibody incubations were overnight at 4°C. All antibodies were diluted in preblock solution. Secondary antibody incubations were for 2 hours at room temperature with secondary antibodies diluted at 1:200 in preblock solution. The following antibodies were used in these studies: rabbit anti-tyrosine hydroxylase 1:500 (TH) (Eugene Tech), mouse IgG anti-neurofilament 160 1:100 (NF) (Sigma), rabbit anti-pheripherin 1:500 (Chemicon). In coculture experiments in which it was necessary to maintain dye-labeling (PKH26 or diI) during staining, we stained for intracellular antigens without the addition of detergents. Cells were processed in one of two ways. Some cultures were equilibrated in 30% sucrose, frozen on dry ice and thawed at 4°C prior to the addition of antibody. Other cultures were fixed, washed and blocked (10% goat serum) in a sterile environment and incubated with primary antibody for 72 hours at room temperature (Elberger and Honig, 1990). Goat serum (1%) was included in all antibody incubations. All cocultures were labeled with an anti-TH primary antibody and an FITC-conjugated secondary antibody. After processing, cells were stored at −20°C covered in n-propyl gallate.

Analysis of dye-labeled cells in complex environments

Staining was examined with an inverted Olympus IX70 Epifluorescence microscope. Wells were scanned at 20× to identify dye-labeled cells. Labeled neurons retain the dye in a distinctive punctate pattern and can be identified above background dispersion of the dye. Dye-labeled cells displaying a neuronal morphology were examined for TH staining. For a given condition, either every neuron in the well was scored, or a minimum of 100 cells per well, depending on the density of labeled cells. Cells were not counted if their dye-
Plated and photographed after 24 hours in culture. Bar, enteric (B) immuno-isolated cells were labeled with diI, and enteric neural crest-derived cells. Sympathetic (A) or E14.5 sympathetic 59.0±0.2 82.2±0.1 79.0±2.9 E14.5 enteric 37.8±2.3 76.6±0.3 0.0±0 E14.5 sympathetic 55.6±2.6 73.7±3.4 69.5±8.7 E14.5 enteric 17.4±6.5 30.3±1.2 15.9±2.4

Fig. 1. Neuronal morphology of E14.5 HNK-1+ sympathetic and enteric neural crest-derived cells. Sympathetic (A) or enteric (B) immuno-isolated cells were labeled with diI, plated and photographed after 24 hours in culture. Bar, 54 μm.

5'-ATGAGACCAGCTTCATC-3'; TH, 5'-GCGGAAGAGATTGCTACCTGGAAG-3' and 5'-GAAAATACAGGGCGGACAGTAGAC-3'; NF 160, 5'-CTTGGACCTTCTCGGTITC-3' and 5'-GACTTCAGCCGTCCTGTC-3'.

Statistics
All data from RT-PCR and culture experiments approached normal distributions. Data was analyzed using the JMP software package (version 3.1 for the Macintosh, SAS Institute, Inc.). Significance was assessed by ANOVA and subsequent Fisher’s Protected Least Significant Difference test using StatView software (version 4.5 for the Macintosh, Abacus Concepts). Single cell tracking data were analyzed using a paired Student’s t-test.

RESULTS

Divergence of the enteric and sympathetic lineages
The HNK-1 antibody recognizes migrating neural crest cells in avian embryos and has been used to identify neural crest-derived cells in peripheral embryonic structures of avian and rodent origin (Vincent and Thiery, 1984; Pomeranz et al., 1993; Chalazonitis et al., 1994). We used the HNK-1 antibody and a secondary antibody coupled to paramagnetic beads to immuno-isolate neural crest-derived cells from embryonic rat thoracic sympathetic ganglia and postumbilical gut (Chalazonitis et al., 1994). These cells were used to investigate the restriction of developing sympathetic and enteric progenitors to their specific neuronal cell fates. Following magnetic isolation we obtained cell populations containing 85–95% HNK-1+ cells. It has previously been reported that HNK-1+ cells isolated from E14 rat gut develop into both neurons and glia after 6-8 days in culture (Pomeranz et al., 1993). These cells were used to investigate the restriction of developing sympathetic and enteric progenitors to their specific neuronal cell fates. Following magnetic isolation we obtained cell populations containing 85–95% HNK-1+ cells. It has previously been reported that HNK-1+ cells isolated from E14 rat gut develop into both neurons and glia after 6-8 days in culture (Pomeranz et al., 1993). Similarly, we found that many HNK-1+ cells displayed a neuronal morphology at the time of isolation (Fig. 1). We used antibodies directed against peripherin and NF as independent neuronal markers to examine the expression of neuron-specific proteins in immuno-isolated HNK-1+ cells from the gut and sympathetic ganglia (Table 1). The expression of these markers indicated that distinct patterns of neuronal differentiation were present in the gut and sympathetic ganglia at E14.5. We found that 17% of E14.5 HNK-1+ cells isolated from the gut and 56% of HNK-1+ cells isolated from the sympathetic chain expressed NF at the time of isolation. For both enteric and sympathetic cells at this stage, a higher percentage of cells expressed peripherin than NF (30% peripherin+ for enteric precursors, 74% peripherin+ for sympathetic cells). At E19.5 the majority of sympathetic and gut HNK-1+ cells expressed peripherin, but only 38% of gut cells expressed NF compared to 59% of sympathetic cells. While there was little change in the expression of NF and peripherin in sympathetic neuroblasts between E14.5 and E19.5, enteric neuroblasts underwent a dramatic increase in the expression of these neuronal markers, suggesting that neurogenesis in the gut is delayed compared to the sympathetic ganglia.

Analysis of neuroblast-specific mRNA expression by RT-PCR
We used molecular markers to further characterize the phenotype of HNK-1+ cells isolated from the gut and sympathetic chains and to follow the divergence of the enteric and sympathetic lineages. Comparative RT-PCR was used to analyze the expression of mRNAs for trkA, trkC, NF and TH. By comparing PCR products for each primer set with the expression of actin in the same cells we were able to compare levels of RNA between different samples for each primer set (see Materials and Methods). Analysis of mRNA expression suggested that HNK-1+ cells from the gut initiated neuronal differentiation later than cells derived from the sympathetic ganglia and matured into neurons with distinct patterns of neuronal and phenotypic markers (Fig. 2). NF mRNA was higher in E14.5 sympathetic cells than enteric cells, consistent with immunostaining showing that neuronal differentiation was more advanced in the sympathetic ganglia at E14.5 (Table 1, Fig. 2A). RT-PCR also revealed that sympathetic ganglia-derived HNK-1+ cells contained higher levels of trkC and TH mRNA than similar cells in the gut (Fig. 2C,D), suggesting lineage-specific development patterns in the gut and sympathetic ganglia. Expression of trkA mRNA was low but detectable in cells derived from both gut and sympathetic ganglia at E14.5 (Fig. 2B), consistent with the reported lack of

Table 1. TH and neuronal markers in HNK-1+ populations

<table>
<thead>
<tr>
<th>Sample</th>
<th>% NF*</th>
<th>% peripherin*</th>
<th>% TH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5 enteric</td>
<td>17.4±6.5</td>
<td>30.3±1.2</td>
<td>15.9±2.4</td>
</tr>
<tr>
<td>E14.5 sympathetic</td>
<td>55.6±2.6</td>
<td>73.7±3.4</td>
<td>69.5±8.7</td>
</tr>
<tr>
<td>E19.5 enteric</td>
<td>37.8±2.3</td>
<td>76.6±0.3</td>
<td>0.0±0</td>
</tr>
<tr>
<td>E19.5 sympathetic</td>
<td>59.0±0.2</td>
<td>82.2±0.1</td>
<td>79.0±2.9</td>
</tr>
</tbody>
</table>

HNK-1+ cells were fixed and stained 3 hours after plating. A minimum of 100 cells per well was counted per experiment; each experiment was plated in 2-3 wells.

Data are shown as the mean of two independent experiments ±s.e.m.
NGF-responsiveness of embryonic sympathetic neuroblasts (Coughlin and Collins, 1985; Ernsberger et al., 1989; Birren et al., 1993).

We followed the developmental divergence of enteric and sympathetic neurons by examining the expression of mRNAs at E19.5. At this developmental stage we found little change in expression of NF mRNA from E14.5 in either sympathetic or enteric-derived HNK-1+ cells (Fig. 2A). We examined the expression of TH mRNA and found, like at E14.5, higher expression in the sympathetic HNK-1+ cells than the enteric HNK-1+ cells (Fig. 2D). Analysis of trkA mRNA expression at E19.5 revealed that although neuronal differentiation had initiated at earlier developmental times, sympathetic neurons underwent further neuronal maturation between E14.5 and E19.5 (Fig. 2B). We found a significant increase of trkA mRNA in immuno-isolated sympathetic cells at the later developmental time. This expression is appropriate for the acquisition of NGF dependence by sympathetic neurons during this developmental period (Birren et al., 1993; DiCicco-Bloom, 1993; Verdi and Anderson, 1994; Wyatt and Davies, 1995). Enteric neurons, which do not depend upon NGF for survival (Gershon et al., 1983), did not display an increase in trkA mRNA levels between E14.5 and E19.5.

**Changes in the noradrenergic phenotype of sympathetic and enteric precursor cells**

PCR data indicated that by E14.5 HNK-1+ cells in the gut express lower levels of TH mRNA than the equivalent sympathetic cells, consistent with previously published reports that gut cells transiently express TH and turn it off upon arrival in the gut environment (Teitelman et al., 1978; Baetge et al., 1990). We used immunocytochemistry to examine the expression of TH protein in HNK-1+ E14.5 enteric and sympathetic neuroblasts that had been plated for 3 hours. We found that 16% of the of the HNK-1+ gut cells were TH+ in comparison to 70% of the HNK-1+ sympathetic cells (Table 1). We investigated whether the local embryonic environment was required to maintain the phenotype of developing enteric and sympathetic neuroblasts at E14.5 by analyzing the expression of TH in immuno-isolated gut and sympathetic HNK-1+ cells cultured in the absence of their normal environmental milieu. HNK-1+ cells were isolated from E14.5 sympathetic ganglia and gut and cultured on a poly-d-ornithine/laminin substrate. After 4 days the cells were fixed, stained for TH, and the percentage of process-bearing neurons that expressed TH was quantified (Fig. 2). We found that approximately 10% of the neurons that developed in these cultures were TH+ (Fig. 3E). In contrast, neurons that developed from E19.5 sympathetic HNK-1+ cells cultured in parallel were 98% TH+. These results suggest that the majority of neuroblasts in the gut become restricted to a TH+ enteric phenotype during the embryonic period between E14.5 and E19.5.

**Fig. 2.** Expression of neurofilament (NF), trkA, trkC and tyrosine hydroxylase (TH) mRNA in HNK-1+ enteric and sympathetic cells at different developmental times demonstrates lineage divergence and neuronal differentiation. Comparative RT-PCR was used to measure the level of the different RNAs in comparison to actin levels in the same cells. Data are comparable within each primer set. (A) neurofilament, (B) trkA, (C) trkC, (D) TH. Light gray bar, immuno-isolated HNK-1+ cells from E14.5 and E19.5 gut (enteric); dark gray bar, immuno-isolated HNK-1+ cells from E14.5 and E19.5 sympathetic ganglia. Values are mean ± s.e.m. of a minimum of three independent mRNA preparations. *Significantly different from age-matched sample, P<0.02. ‡Significantly different from tissue-matched sample, P<0.01.
E14.5 and E19.5. While these experiments demonstrate progressive loss of developmental plasticity in HNK-1+ enteric neuroblasts, they leave open questions about developmental restriction of HNK-1+ sympathetic neuroblasts and regarding the role of the embryonic environment in the restriction of developmental potential.

Phenotypic plasticity of HNK-1+ cells from the gut and sympathetic ganglia

We investigated the restriction of developmental potential in sympathetic as well as enteric neuroblasts by challenging the cells with in vitro environments derived from embryonic structures known to promote noradrenergic or enteric differentiation. A number of studies have demonstrated that explants of gut are capable of suppressing the expression of TH in cultured neural crest cells (Coulter et al., 1988; Mackey et al., 1988; Groves and Anderson, 1996). Sympathetic neurons develop in close proximity to the embryonic dorsal aorta (DA) and factors produced by the dorsal aorta have been shown to promote adrenergic differentiation of neural crest cells (Ernsberger and Rohrer, 1996; Groves and Anderson, 1996; Reissmann et al., 1996). Immuno-isolated enteric and sympathetic cells were labeled with a lipophilic dye to permit their identification on complex cellular environments. Dye-labeled cells were then plated onto gut or DA environments to determine the capacity of the cells to alter their phenotype in response to environmental cues. Using TH-immunoreactivity as a marker of a sympathetic phenotype we analyzed the percentage of TH+ dye-labeled neurons that developed from HNK-1+ neuroblasts after 4 days of coculture (Fig. 4). In control experiments we determined that the dye-labeled neurons analyzed for TH expression arose from the HNK-1+ population.

We tested whether dissociated monolayers of E14.5 gut could support the development of dye-labeled gut-derived neuroblasts to TH+ enteric neurons. We showed that 15% of the neurons that developed from these cells expressed TH at

Fig. 3. E14.5, but not E19.5 enteric HNK-1+ cells, express an adrenergic phenotype when cultured in the absence of their normal environment. Immuno-isolated HNK-1+ cells from the gut and sympathetic ganglia were stained for TH following 4 days in culture. Neurons were identified by morphology and the percentage of TH+ neurons was determined. Sympathetic neurons (A,B) and enteric neurons (C,D) were derived from E14.5 (A,C) and E19.5 (B,D) embryos. The arrow in D indicates an E19.5 TH+ enteric neuron. All exposures are matched. Bar, 54 μm. (E) Combined data from three independent experiments, each with triplicate determinations, showing the mean ± s.e.m. *E19.5 enteric neurons significantly different from E14.5 enteric neurons, P<0.001.

Fig. 4. Experimental design for coculture of neuroblasts with sympathetic and enteric environments. Culture environments that supported the differentiation of sympathetic or enteric neurons were established by plating dissociated dorsal aorta or gut cells and culturing them overnight. Immuno-isolated HNK-1+ sympathetic and enteric cells were labeled with a lipophilic dye and plated onto these environments. After 4 days in culture, the cells were fixed, stained for TH and the percentage of dye-labeled neurons expressing TH was determined.
Peripheral neuron lineage restriction

the end of the culture period (Fig. 5G-I). Since over 55% of enteric neuroblasts developed into TH+ neurons in the absence of the gut environment (Fig. 3E), these results indicate that interactions with the gut environment lead to the suppression of TH expression by developing enteric neurons.

We next asked whether E14.5 sympathetic neuroblasts were developmentally restricted to a sympathetic neuronal cell fate, or whether, like the enteric neuroblasts, they could respond to factors in the gut environment to downregulate the noradrenergic phenotype. When cultured in the presence of gut monolayers, 42% of neurons that developed from dye-labeled E14.5 sympathetic neuroblasts expressed TH (Fig. 5C,D,I). This is in contrast to 85% TH+ neurons when cultured in the absence of any environment cells (Fig. 3E), indicating that at E14.5, at least a subpopulation of TH+ sympathetic neuroblasts were plastic with respect to their final neuronal phenotype. This number underestimates the number of dye-labeled cells that responded to the gut environment, as many of the cells that did not completely lose TH immunoreactivity showed a decreased level of staining (Fig. 5D).

We also investigated the expression of TH in enteric neuroblasts cultured with an environment that supports the differentiation of TH+ sympathetic neurons. E14.5 sympathetic neuroblasts were labeled with a lipophilic dye, plated on a dissociated monolayer of E14.5 dorsal aorta cells for 4 days, fixed and stained for TH. Greater than 80% of the dye-labeled neurons were TH+ (Fig. 5A,B,I). Since the E14.5 HNK-1+ sympathetic population is predominantly TH+ at the time of isolation (Table 1), this experiment demonstrated that dorsal aorta monolayers provided an environment that was sufficient for the maintenance of TH expression and the development of a neuronal morphology. When dye-labeled E14.5 enteric neuroblasts were cultured on the dorsal aorta environment, 51% of the dye-labeled neurons were TH+ (Fig. 5E,F,I). In contrast, 15% of enteric sympathetic neurons grown on gut environments expressed TH. This increase in TH-immunoreactivity was similar to that observed for enteric neuroblasts cultured alone (Fig. 3E). These results suggest that the bias toward a sympathetic phenotype displayed by enteric neuroblasts is not further enhanced by factors in the dorsal aorta. Cells from both the gut and sympathetic ganglia remain competent to respond to signals from the gut environment that suppress TH expression, indicating that at E14.5 sympathetic and enteric neuroblasts are developmentally plastic.

Table 2. Single cell tracking of HNK-1− cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of cells scored</th>
<th>Number of neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5 enteric</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>E14.5 sympathetic</td>
<td>64</td>
<td>3</td>
</tr>
<tr>
<td>E19.5 enteric</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>E19.5 sympathetic</td>
<td>52</td>
<td>0</td>
</tr>
</tbody>
</table>

HNK-1− cells were identified 3 hours after plating. After 4 days in culture, colonies were scored as either neuronal or non-neuronal. Table shows combined data from 5 independent experiments.

Fig. 5. Plasticity of E14.5 sympathetic and enteric neuroblasts. (A,C,E,G) Dye-labeled cells. (B,D,F,H) TH staining. (A-D) Dye-labeled sympathetic neuroblasts were cultured for 4 days on environments derived from gut (GT) or dorsal aorta (DA). Culture of sympathetic neuroblasts on the gut environment led to the downregulation of TH expression (C,D), compared to that of sympathetic cells cultured on dorsal aorta (A,B). The arrow in D shows an E14.5, dye-labeled, TH− neuron derived from a dye-labeled sympathetic precursor cell. (E-F) Dye-labeled enteric neuroblasts expressed TH when exposed to the dorsal aorta environment. When grown on the gut environment TH expression was much lower (G,H). The figure shows matched exposures for all TH staining. Bar, 54 μm. (I) Light gray bars, dorsal aorta environments; dark gray bars, gut environments. Values are mean ± s.e.m. for three independent experiments, each with triplicate determinations. *Sympathetic and enteric neurons on gut environments were significantly different from the same cells on dorsal aorta, P<0.001.
In all of these experiments, we observed intrinsic, unlabeled neurons in the sympathetic and enteric differentiation environments. These neurons developed both in cultures grown in the presence of dye-labeled exogenous cells and in control cultures grown in the absence of added cells. In the gut environments these neurons derive from the intrinsic neural crest-derived neuroblasts and were negative for TH immunoreactivity. In cultures derived from embryonic dorsal aorta the neurons were TH+ and presumably were derived from the thoracic sympathetic ganglia. The sympathetic ganglia develop in close proximity to the dorsal aorta and are co-isolated with this structure during embryo dissections. These neurons were not labeled with diI or PKH26 and therefore were not counted in the above experiments. In addition to providing internal controls for TH staining, these cells form an intrinsic part of the sympathetic and gut environment. One interesting question for future studies is the contribution of these intrinsic neurons to the TH regulation of the dye-labeled, cocultured neurons.

One question arising from these experiments is whether selective cell death of the sympathetic neuroblasts on the gut environment could account for the growth of a TH− population. We used the TUNEL technique to label apoptotic nuclei containing fragmented DNA and analyzed the number of dye-labeled, HNK-1+ sympathetic-derived cells with TUNEL+ nuclei in different culture conditions. Likewise we measured the extent of DNA fragmentation of gut-derived HNK-1+ cells cultured with either dorsal aorta or gut environments. 12 hours after plating 3±1% of sympathetic cells cultured on gut environments contained apoptotic nuclei compared to 4±1% of sympathetic cells cultured on dorsal aorta (Fig. 6A). Gut-derived HNK-1+ cells were also 3±1% TUNEL+ when cultured on gut and 4±1% when cultured on dorsal aorta. After 24 hours of culture we found no significant changes any of the conditions tested compared to the 12 hour point (data not shown). Together these experiments show similar patterns of apoptotic DNA fragmentation on dorsal aorta and gut environments, suggesting that selective survival does not account for the changes in TH expression observed in the different culture conditions.

We also examined the incorporation of BrdU into E14.5 neuroblasts as a measure of cell proliferation in different environments. Dye-labeled sympathetic and enteric neuroblasts were cultured on gut or dorsal environments in the presence of BrdU for 24 hours. The cultures were fixed and stained for BrdU, and the percentage of BrdU+ dye-labeled neuroblasts was determined. We found that neither the gut nor dorsal aorta environment significantly or preferentially promoted proliferation of cocultured neuroblasts, although there was a slight trend toward lower proliferation on the gut environment (Fig. 6B).

The extent of BrdU incorporation by enteric and sympathetic neuroblasts suggested that the neuroblasts continued to divide even after initial neuronal differentiation. To directly test this possibility we identified individual, process-bearing enteric and sympathetic neuroblasts and showed that after a 4 day culture period both types of neuroblasts gave rise to multi-cell colonies (Table 3). Thus, as previously described for sympathetic neurons (Rohrer and Thoenen, 1987; Birren et al., 1993), enteric neurons continue to divide following the initiation of neuronal differentiation.

**Phenotypic restriction is not linked to neuronal differentiation**

While our experiments demonstrate developmental plasticity of sympathetic and enteric neuroblasts at E14.5, a subpopulation of neuroblasts do not alter their phenotype when challenged with an alternative environment. Since a significant proportion of enteric and sympathetic neuroblasts have initiated neuronal differentiation at E14.5 (Table 1, Fig. 1), we
investigated the possibility of a link between initiation of neuronal differentiation and the restriction of developmental plasticity. We identified and tracked individual dye-labeled sympathetic neuroblasts which had extended neuronal processes 12 hours after they were plated onto gridded dishes containing gut or dorsal aorta monolayers. Cultures were grown for 4 days, fixed and stained for TH. Identified, dye-labeled sympathetic neurons were 96% TH+ when cultured on the dorsal aorta environment. When grown in the gut environment, however, only 34% of the identified sympathetic neurons were TH+ (Table 4). These data indicate that the initiation of neuronal differentiation is not concomitant with the restriction of sympathetic neurons to a TH+ phenotype. In control experiments we fixed cells at the 12 hour point and determined that the sympathetic neuroblasts were TH+ (data not shown), indicating that a switch to a TH− phenotype did not take place between the time of plating and the time of scoring neuronal morphology.

**Enteric and sympathetic neuroblasts undergo a loss of developmental potential by E19.5**

Within the sympathetic nervous system neurons are capable of undergoing switches in neurotransmitter phenotype at late developmental stages (Landis, 1990). Taken together with our demonstration of phenotypic plasticity of E14.5 sympathetic and enteric neuroblasts, we investigated the possibility that, in the peripheral nervous system, plasticity is an intrinsic property of neurons, independent of developmental stage. We immunolabeled HNK-1+ cells from the sympathetic chains and guts of E19.5 embryos and analyzed TH-immunoreactivity following culture on environments established from the dorsal aorta and gut of the same stage embryos (Fig. 7). In the presence of a gut monolayer greater than 87% of the sympathetic neurons maintained TH expression. Dye-labeled E19.5 enteric neuroblasts did not express TH when cultured on either a gut or dorsal aorta environment. Together, these data show that both enteric and sympathetic neuroblasts undergo a significant loss in the capacity to express the TH-phenotype associated with an alternative peripheral lineage by E19.5.

The phenotypic restriction of E19.5 enteric and sympathetic neuroblasts could reflect developmental alterations in the properties of the neuroblasts that limit their ability to respond to signals in an alternative environment. It is also possible that E19.5 neuroblasts from the gut and sympathetic ganglia remain plastic while changes in the local environment result in the absence of specific signals required for phenotypic switching. We cultured E19.5 sympathetic and enteric neuroblasts on environments established from E14.5 dorsal aorta and gut to test the possibility that these neuroblasts could respond to lineage cues found in earlier embryos (Fig. 8A). Neurons that developed from E19.5 sympathetic neuroblasts were over 95% TH+ when cultured on E14.5 dorsal aorta or gut monolayers. Neurons derived from E19.5 enteric neuroblasts were over 75% TH+ under these culture conditions. These data indicate that between E14.5 and E19.5 alterations take place within the population of differentiating enteric and sympathetic neuroblasts, resulting the loss of developmental potential and the expression of a specific neuronal phenotype. Further, we tested whether changes in the local environments between E14.5 and E19.5 resulted in the loss of extrinsic cues capable of regulating TH expression in responsive E14.5 neuroblasts. E14.5 enteric and sympathetic neuroblasts altered their TH expression when cultured with gut or dorsal aorta environments derived from E19.5 embryos (Fig. 8B), demonstrating that these environments continue to express regulatory factors even after the bulk of enteric and sympathetic neuroblasts had lost the potential to respond to alternative developmental cues.

**DISCUSSION**

The loss of TH expression by transiently catecholaminergic enteric neuroblasts in the gut is a key step in the divergence of the enteric and sympathetic neuronal lineages (Teitelman et al., 1978; Jonakait et al., 1979; Baetge et al., 1990; Carnahan et al., 1991; Gershon et al., 1993). Here we present evidence that enteric neuroblasts retain their developmental potential even after downregulation of TH expression and initial lineage divergence in the gut. Moreover, TH− enteric neuroblasts display a transient requirement for gut-derived factors to maintain their enteric phenotype. In the absence of these signals, enteric neuroblasts undergo differentiation to noradrenergic neurons. In the sympathetic ganglia, sympathetic neuroblasts express TH from early developmental stages (Cochard et al., 1978; Shirley et al., 1996), yet even following neuronal differentiation these cells maintain the capacity to respond to signals in the gut environment to turn off TH expression. Intrinsic cellular changes during the period of neuronal maturation lead to the restriction of developing neurons to an enteric or sympathetic phenotype, with the majority of neuroblasts losing the capacity to respond to alternative environmental cues by E19.5 in the rat. This study demonstrates that both sympathetic and enteric neuroblasts maintain the capacity to respond to alternative environmental cues even after initial phenotype divergence and that neuronal differentiation and the restriction of developmental potential are independently regulated.
Phenotypic plasticity and expression of sympathetic and enteric traits

We have shown that E14.5 rat enteric neuroblasts, which are predominantly TH- at the time of dissection (Table 1), express TH when cultured in the presence of dorsal aorta (DA) cells also derived from E19.5 embryos. Cultures were stained for TH and the percentage of TH+ neurons was determined. Neurons that developed from sympathetic neuroblasts were TH+ when cultured either on dorsal aorta or gut (B,D). Enteric neuroblasts developed into TH- neurons in either environment (F,H). Arrows in F and H show TH+ dye-labeled neurons. TH staining is shown as matched exposures. Bar, 54 μm. (I) Combined data from three independent experiments, each with triplicate determinations shown as mean ± s.e.m. Light gray bars, dorsal aorta environments; dark gray bars, gut environments.

Experimental systems. Back-transplantation of quail bowel segments to the neural tube of younger chick embryos resulted in re-migration of neural crest-derived cells and the identification of TH+ quail cells in the sympathetic ganglia (Rothman et al., 1990). These experiments may suggest that plasticity is a consequence of interactions with adrenergic-inducing cues from the sympathetic environment. Alternatively, it is possible that the expression of a noradrenergic phenotype by enteric neuroblasts is due to the absence of signals from the gut, rather than the presence of exogenous signals. We have demonstrated that immuno-isolated enteric neuroblasts are largely TH- when cocultured with gut monolayers (Fig. 5), but that TH is upregulated when these cells are cultured in the absence of other cell types (Fig. 3). Likewise, TH expression has been observed in clonal cultures of chick enteric neuroblasts (Sextier-Sainte-Claire Deville et al., 1992), immuno-selected chick HNK-1+ enteric cells (Pomeranz et al., 1993) and rat ret+ enteric cells (Lo and Anderson, 1995).

The expression of TH and other markers in both the developing enteric and sympathetic lineages has been cited as evidence that neurons in the sympathetic ganglia and gut are derived from a common precursor population (Carnahan et al., 1991; Gershon et al., 1993; Blaugrund et al., 1996). The capacity of enteric cells to express a noradrenergic phenotype has also been used as evidence of the unrestricted developmental potential of enteric neuroblasts. In addition, the expression of TH in cultured enteric neuroblasts suggests that some of these cells express a noradrenergic phenotype in the absence of local gut signals. This phenotypic alteration could reflect the effects of adrenergic differentiation signals from embryonic structures such as the neural tube, notochord and dorsal aorta (Teillet et al., 1978; Howard and Bronner-Fraser, 1985; Groves et al., 1995; Ernsberger and Rohrer, 1996) acting on migrating neural crest cells prior to their colonization of the gut. If such a noradrenergic differentiation pathway exists, environmental cues in the gut can override the developmental program, as indicated by the loss of TH expression in enteric neuroblasts maintained in a gut environment (Teitelman et al., 1978; Baetge et al., 1990) (Fig. 5). Eventually the developmental plasticity of these cells is lost; enteric neuroblasts that reside in the gut environment for extended periods lose the capacity to express a noradrenergic phenotype either when cultured in the absence of embryonic gut cells (Sextier-Sainte-Claire Deville et al., 1992) (Fig. 3), or in the presence of cells from the dorsal aorta (Fig. 7). The fact that this restriction of neuroblasts in the gut takes place...
Peripheral neuron lineage restriction subsequent to the downregulation of TH indicates that gut-derived factors, in addition to initially suppressing TH expression, are transiently required for the maintenance of the TH^- phenotype. By E19.5, when most enteric neuroblasts have undergone a loss in developmental potential, the requirement for signals to maintain a TH^- phenotype is lost.

Unlike enteric neuroblasts in the gut, sympathetic neuroblasts do not turn off TH expression in vivo or when cultured in the absence of their local microenvironment. Despite this continuous expression of a noradrenergic marker, E14.5 sympathetic neuroblasts are competent to respond to signals in the gut environment to turn down expression of TH. We found that 58% of the neurons that developed in a gut environment from immuno-selected sympathetic neuroblasts were TH^- in contrast to less than 20% TH^- neurons that developed on dorsal aorta or in the absence of any exogenous cells (Figs 3, 5). While the embryonic gut is known to negatively influence TH expression in neural crest cells (Mackey et al., 1988), this is the first demonstration that the neurotransmitter phenotype of postmigratory sympathetic neuroblasts can be altered in response to factors in the gut environment.

Studies of the loss of TH expression in transiently catecholaminergic enteric neuroblasts have suggested that the process is instructive and not selective (Baetge et al., 1990; Carnahan et al., 1991). We investigated whether changes in proliferative capacity or survival could account for alterations in TH expression in enteric and sympathetic neuroblasts in different environments. Analysis of BrdU incorporation and DNA fragmentation indicative of apoptotic cell death did not reveal significant differences in either cell division or programmed cell death of enteric and sympathetic cells cultured on gut or dorsal aorta environments (Fig. 6), suggesting subpopulations of these neuroblasts are not differentially selected in the different environments.

Sympathetic neurons are known to switch from a noradrenergic to a cholinergic neurotransmitter phenotype during the innervation of the rat sweat gland in vivo (Landis and Keefe, 1983), and in response to cholinergic differentiation factors such as leukemia inhibitory factor (LIF) (Yamamori et al., 1989) and ciliary neurotrophic factor (CNTF) in vitro (Saadat et al., 1989). Thus, sympathetic neurons retain phenotypic plasticity even after birth. By E19.5 however, most sympathetic neuroblasts have lost the capacity to respond to signals in the gut environment to downregulate TH expression. These results argue that developmental plasticity is independently regulated for different environmental interactions. It has been proposed that neural crest-derived cells undergo progressive developmental restrictions resulting in the emergence of distinct neural lineages (Anderson, 1989; Lo and Anderson, 1995). Our data argue that one such progressive restriction event is the loss of competence of sympathetic neurons to switch their neurotransmitter phenotype in response to factors in the gut environment.

Neuronal differentiation is not coupled to restriction of developmental potential

In addition to demonstrating the developmental potential of postmigratory enteric and sympathetic neuroblasts we have defined the timing of the loss of this potential within the context of ongoing developmental changes in the peripheral nervous system. We analyzed expression patterns of neuron-specific mRNA and protein as well as markers that define the divergence of the enteric and sympathetic lineages (Table 1, Fig. 2). RT-PCR and immunocytochemistry revealed the initiation of neuronal differentiation by E14.5, a developmental age at which many enteric and sympathetic neuroblasts have the potential to express alternative phenotypes. Lineage divergence had also commenced at E14.5, as indicated by the differential expression...
of TH mRNA and protein and trkC mRNA in enteric and sympathetic neuroblasts. These experiments also document ongoing neuronal differentiation and maturation between E14.5 and E19.5 with evidence for the development of a mature, trkA-positive sympathetic phenotype by E19.5. Given that a large proportion of enteric and sympathetic neuroblasts demonstrate a loss of developmental potential during this period, these results raise the question of the relationship between neuronal differentiation, maturation and developmental restriction.

Not all enteric and sympathetic neuroblasts switch their TH expression when cultured with environment cells from the other lineage. 42% of sympathetic neuroblasts remained TH+ when cultured with a gut environment, and as many as 50% of enteric neuroblasts were TH- when cultured alone or on an environment of dorsal aorta cells. While we have not ruled out the possibility that our culture conditions are not fully optimized for phenotype switching, these results suggest that by E14.5, subpopulations of HNK-1+ enteric and sympathetic neuroblasts have become restricted in their developmental potential. Since subsets of the E14.5 neuroblasts express a neuronal phenotype at the time of plating (Table 1, Fig. 1), we examined a possible link between neuronal differentiation and restriction of developmental potential. Neuron-specific markers are expressed early in the development of the sympathetic and enteric lineages, with some enteric precursor cells expressing neurofilament and a neuronal morphology even before their final arrival in the gut (Ciment and Weston, 1983; Payette et al., 1984). In the sympathetic ganglia the early neuronal marker neuron-specific tubulin is expressed even before adrenergic markers (Shirley et al., 1996), and axonal outgrowth of sympathetic fibers has been observed as early as E12 in the rat (Rubin, 1985). We addressed the question of whether cells that had initiated neuronal differentiation shortly after plating were restricted in their capacity to respond to environmental signals to switch their neurotransmitter phenotype. We carried out single cell tracking experiments on process-bearing, dye-labeled sympathetic neuroblasts plated at clonal density on gut or dorsal aorta environments (Table 4). After 4 days of culture on the different environments, the identified cells were tracked and analyzed for TH expression. We found that while over 95% of the process-bearing sympathetic cells developed into TH+ neurons when cultured on the dorsal aorta environment, fewer than 35% of the tracked cells on the gut environment expressed TH. It is interesting to note that the decrease in TH expression was greater in this experiment compared to mass cultures of sympathetic neuroblasts on gut environments. While the basis for this difference is not clear, one possibility is that sympathetic-derived cells plated at higher density produce factors that limit the extent of phenotype switching. These experiments demonstrate that neuronal differentiation can precede the loss of competence of neuroblasts to switch neurotransmitter phenotype in response to alternative environmental cues. Since significant restriction takes place in the neuroblast population between E14.5 and E19.5 (Figs 5, 7), we conclude that the loss of developmental potential is associated with neuronal maturation, as opposed to initial neuronal differentiation.

**Intrinsic changes in neuroblast populations underlie developmental restriction**

We have demonstrated that most E19.5 sympathetic and enteric neuroblasts do not change their TH expression in response to cues from alternative environments. Two mechanisms could result in this loss of developmental potential: first, intrinsic changes in the neuroblasts that result in a loss of responsiveness to local environmental cues, and second, developmental changes in the environments leading to altered expression of signaling molecules. We distinguished between these possibilities by demonstrating that environment cells from E14.5 embryos, which permit phenotype switching in E14.5 neuroblasts, were not capable of triggering changes in TH expression in E19.5 neuroblasts, while cues derived from E19.5 environments were capable of altering TH expression in E14.5 neuroblasts (Fig. 8). These results indicate that during development, enteric and sympathetic neuroblasts lose responsiveness to inappropriate environmental cues, defining the cellular basis of developmental restriction in these peripheral lineages.

Although neuronal differentiation is initiated in the gut and sympathetic ganglia at a developmental time at which neuroblasts are not yet restricted (Figs 1, 5; Table 1) (Ciment and Weston, 1983; Payette et al., 1984; Lo and Anderson, 1995), developing neurons undergo a continuing maturation process that is marked by changes in gene expression between E14.5 and birth. In the enteric nervous system the expression of mature neurotransmitters and neuropeptides increases over developmental time (Pham et al., 1991; Sextier-Sainte-Claire Deville et al., 1992). Many of these enteric markers are also expressed in the maturing sympathetic nervous system (Rao and Landis, 1992). In the sympathetic ganglia, developmental changes in the expression of neurotrophin receptors take place with increases in TrkA (Fig. 2) and p75 expression and downregulation of TrkC (Birren et al., 1993; DiCicco-Bloom, 1993; Verdi and Anderson, 1994; Wyatt and Davies, 1995). Our demonstration of restriction of developmental potential during this period of neuronal maturation raises new questions about the molecular basis of developmental restriction and provides a system in which to investigate these molecular cues. By determining the timing of developmental restriction and defining the interactions between neuroblasts and their environments this study sheds light on the mechanisms of developmental restriction in the peripheral nervous system.

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