Neuron, Vol. 35, 643–656, August 15, 2002, Copyright ©2002 by Cell Press

Cell-Intrinsic Differences between Stem Cells from Different Regions of the Peripheral Nervous System Regulate the Generation of Neural Diversity

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Summary

Stem cells in different regions of the nervous system give rise to different types of mature cells. This diversity is assumed to arise in response to local environmental differences, but the contribution of cell-intrinsic differences between stem cells has been unclear. At embryonic day (E)14, neural crest stem cells (NCSCs) undergo primarily neurogenesis in the gut but gliogenesis in nerves. Yet gliogenic and neurogenic factors are expressed in both locations. NCSCs isolated by flow-cytometry from E14 sciatic nerve and gut exhibited heritable, cell-intrinsic differences in their responsiveness to lineage determination factors. Gut NCSCs were more responsive to neurogenic factors, while sciatic nerve NCSCs were more responsive to gliogenic factors. Upon transplantation of uncultured NCSCs into developing peripheral nerves in vivo, sciatic nerve NCSCs gave rise only to glia, while gut NCSCs gave rise primarily to neurons. Thus, cell fate in the nerve was stem cell determined.

Introduction

There is astonishing diversity in the neurons and glia that develop in different regions of the mammalian nervous system (McKay, 1989; McConnell, 1991). Neural diversity arises as a result of diverse environmental cues that regulate differentiation, as well as patterning differences that specify different cell fates in different regions of the nervous system. A number of extracellular signals, such as bone morphogenetic proteins (BMPs), instruct neural progenitors to acquire specific cell fates in the peripheral nervous system (PNS) (Shah et al., 1994, 1996; Morrison et al., 2000b) and central nervous system (CNS) (Johe et al., 1996; Williams et al., 1997; Gross et al., 1996). The responses of progenitors to these lineage determination factors not only determine neuronal versus glial differentiation, but may also determine neuronal subtype as well (White et al., 2001). The differential expression of these instructive cues throughout the nervous system is likely an important mechanism by which different types of cells are generated in different places (Lillien, 1997). In addition to these lineage determination factors, other types of extracellular signals, such as Sonic hedgehog, lead to regionalization and patterning within the nervous system (Wilson and Rubenstein, 2000; Jessell, 2000). Patterning is the process by which environmental signals in different regions of the nervous system cause progenitors to adopt region-specific cell fates (Shimamura et al., 1995; Puelles et al., 2000). Thus, neural diversity is encoded in the differential expression of lineage determination factors as well as in patterning that generates cell-intrinsic differences between progenitors in different locations.

Although the influence of lineage determination factors on stem cell differentiation has been extensively studied, the influence of patterning on stem cell differentiation has received very limited attention (reviewed by Anderson, 2001). One hypothesis is that stem cells from different regions of the nervous system are patterned to be intrinsically different in a way that heritably influences their differentiation (Anderson, 2001; Temple, 2001). Alternatively, stem cells may quickly repattern/reprogram themselves upon exposure to a new environment (Panchision et al., 1998; Hitoshi et al., 2002), or pattern formation may influence only restricted progenitors. In the latter case, stem cells from different regions of the nervous system would not be intrinsically different or would rapidly lose such differences upon transplantation into a similar environment.

Some studies suggest that stem cells cultured from different regions of the CNS do not exhibit cell-intrinsic differences in the way they differentiate. Neurospheres from different regions of the CNS expressed different markers of regional identity in culture (Zappone et al., 2000; Hitoshi et al., 2002); however, neurospheres from heterotopic locations were able to quickly express ventral forebrain markers upon coculture with ventral forebrain cells (Hitoshi et al., 2002). Moreover, when stem cells cultured from one region of the CNS were transplanted into other regions of the CNS, they often gave rise to cell types that seemed appropriate to the transplanted regions (Suhonen et al., 1996; Brustle et al., 1997; Takahashi et al., 1998; Fricker et al., 1999; Shihabuddin et al., 2000). These observations suggest that CNS stem cells often differentiate in a way that is appropriate to their new environment after transplantation.

The above experiments were performed on neural stem cells that were isolated retrospectively by expansion in culture. Recent experiments have demonstrated that some neural progenitors isolated by expansion in culture may lose patterning influences or broaden their developmental potential as a result of being cultured (Gage, 1998, 2000; Palmer et al., 1999; Kondo and Raff, 2000; Anderson, 2001). In order to test whether cell-intrinsic differences between stem cells influence their differentiation in vivo, it is preferable to prospectively identify and isolate uncultured stem cells from different regions of the nervous system at the same time in development. This makes it possible to compare the properties of different stem cell populations in vivo and acutely in culture. Unfortunately, only a few neural stem cell populations from different stages of development and different species have been prospectively identified (Morrison et al., 1999; Uchida et al., 2000; Rietze et al.,

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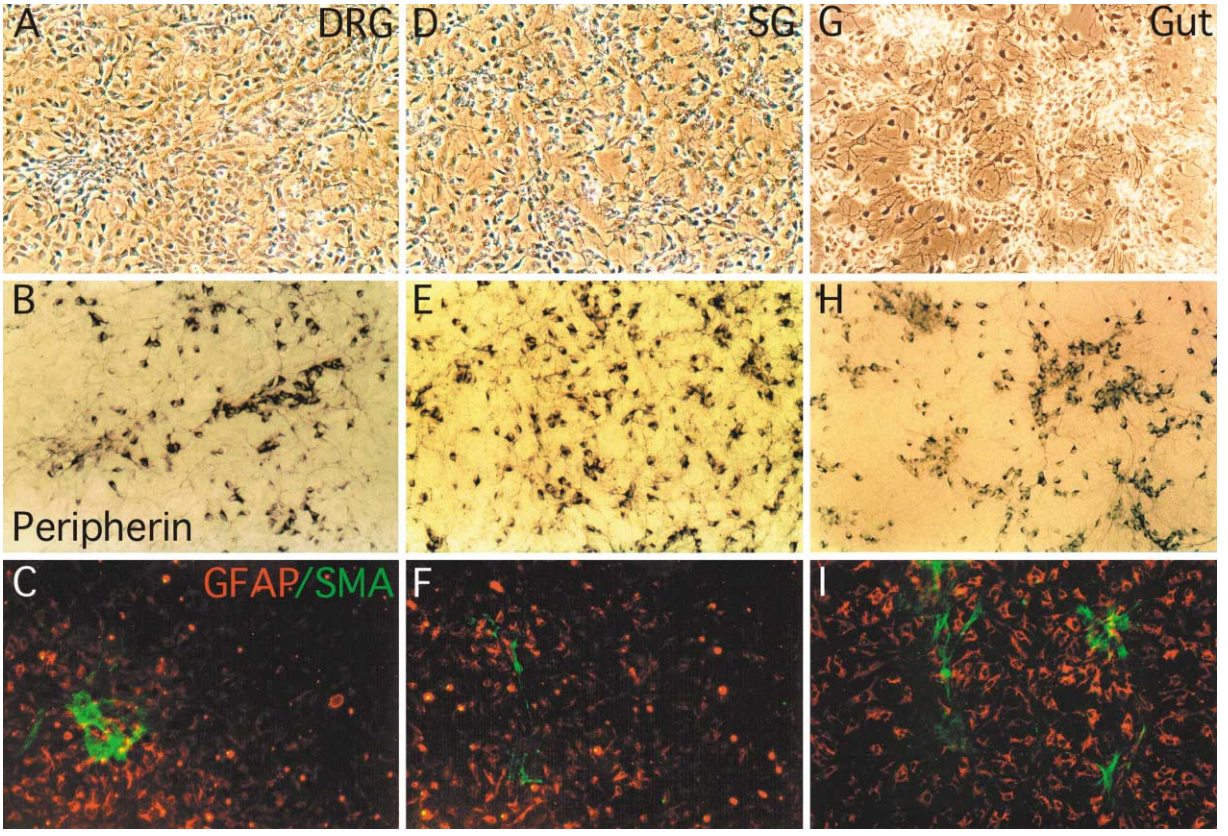

Figure 1. Multipotent Neural Crest Progenitors Persist Widely after Migrating

Dorsal root ganglia (DRG) (A–C), sympathetic ganglia (SG) (D–F), and guts (G–I) were dissected from E14.5 rat fetuses. Cells were dissociated and added to culture at clonal density (40 cells/35 mm dish) such that each cell was able to form a spatially distinct colony. After 2 weeks, the cultures were fixed and stained with antibodies against neurons (Peripherin; black in [B], [E], and [H]), glia (GFAP; red in [C], [F], [I]), and myofibroblasts (smooth muscle actin; green in [C], [F], [I]). Each column (A–C, D–F, or G–I) shows one field of view from a typical multilineage colony. All photos were taken using 20× objective magnification.

2001; Kawaguchi et al., 2001), so this has not yet been possible.

We find that postmigratory NCSCs persist widely throughout the late gestation PNS. The purification of uncultured NCSCs from embryonic day (E)14 gut and E14 sciatic nerve enabled us to ask whether these spatially, but not temporally, distinct stem cell populations have the same properties. In fact, these NCSCs exhibited heritable, cell-intrinsic differences in their responses to lineage determination factors. These differences biased gut NCSCs toward neuronal fates and sciatic nerve NCSCs toward glial fates, even though both cell types remained multipotent and able to generate neurons and glia in diverse locations in vivo. Upon engraftment into developing peripheral nerve in vivo, sciatic nerve NCSCs gave rise only to glia, while gut NCSCs gave rise primarily to neurons. This demonstrates that the nerve environment is permissive for neuronal differentiation even though only glia are generated in peripheral nerves during normal development. Instead of being environmentally determined, distinct cell types are generated in the nerve and gut as a result of cell-intrinsic differences in the stem cells from these locations. In other in vivo locations, sciatic nerve NCSCs gave rise to neurons, demonstrating that the local environment did influence their differentiation. These results suggest that neural diversity is generated by a combinatorial mechanism, involving both regional environmental differences as well as cell-intrinsic differences encoded in stem cells.

Results

Multipotent Neural Crest Progenitors Persist throughout the Late Gestation PNS

Uncultured, postmigratory NCSCs can be isolated by flow-cytometry from freshly dissociated E14.5 rat sciatic nerve as cells that express the neurotrophin receptor p75 but fail to express the peripheral myelin protein P_0 ($p75^+P_0^-$). More than eighty percent of $p75^+P_0^-$ cells are self-renewing, multipotent progenitors that give rise to neurons and glia in diverse regions of the embryonic chick PNS (Morrison et al., 1999; Morrison et al., 2000a; White et al., 2001). To test whether the sciatic nerve is unique in supporting the persistence of NCSCs into late gestation, we dissected gut, dorsal root ganglia, and sympathetic ganglia from E14.5 rat fetuses. Each tissue was dissociated and cells were cultured at clonal density. As shown in Figure 1, cells from each location gave rise to large multilineage colonies containing neurons,

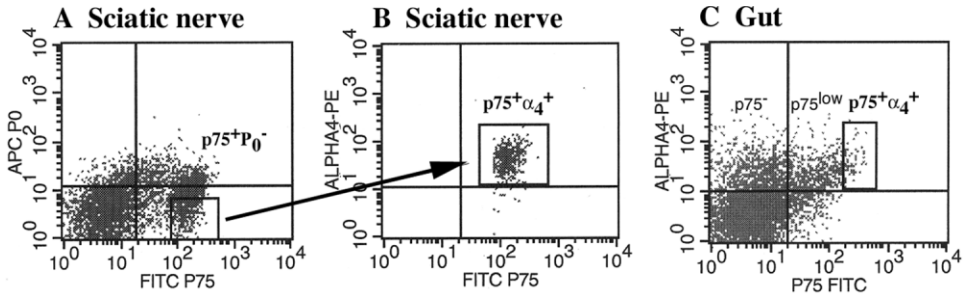

Figure 2. p75 and α_4-Integrin Mark NCSCs from Sciatic Nerve and Gut

E14.5 sciatic nerve (A and B) or gut (C) cells were dissociated and analyzed by flow-cytometry. (A) shows sciatic nerve cells stained with antibodies against p75 and P_0. The $p75^+P_0^-$ fraction of NCSCs (representing 10% of cells) is boxed. In (B), the $p75^+P_0^-$ cells from (A) (which had also been stained with an antibody against α_4 integrin) are shown with respect to p75 and α_4 integrin staining. 97% of $p75^+P_0^-$ cells were also α_4^+. (C) shows gut cells stained with antibodies against p75 and α_4 integrin. The boxed region contains 1% of gut cells that were $p75^+\alpha_4^+$ and highly enriched for multipotent progenitors (see Table 1). Although NCSCs in the gut and sciatic nerve were both $p75^+\alpha_4^+$, these sciatic nerve NCSCs were routinely isolated using the p75 and P_0 markers as has been reported previously (Morrison et al., 1999, 2000b).

glia, and myofibroblasts. The overall appearance of these colonies was similar to that of migrating NCSCs derived from E10.5 neural tube explants (Stemple and Anderson, 1992; Shah et al., 1994) or from postmigratory NCSCs obtained from E14.5 sciatic nerve (Morrison et al., 1999). These colonies also gave rise to many multipotent daughter cells when they were subcloned after 8 days in culture, demonstrating their self-renewal potential (data not shown). This indicated that NCSCs persist in diverse regions of the mammalian PNS after migrating. To study these cells in more detail, we decided to isolate the multipotent progenitors from the enteric nervous system (ENS; gut).

The Prospective Identification and Isolation of Gut NCSCs

The P_0 marker was not as effective in the gut as in the sciatic nerve at distinguishing stem cells from restricted progenitors. Therefore, we looked for additional markers that would allow us to isolate gut NCSCs. Using three-color flow-cytometric analysis, we found that $p75^+P_0^-$ sciatic nerve NCSCs also expressed α_4 integrin (Figure 2). In fact, a $p75^+\alpha_4^+$ population could be isolated from both the sciatic nerve and gut. Seventy-nine percent of sciatic nerve $p75^+\alpha_4^+$ cells gave rise to multilineage colonies in a clonal analysis, confirming that sciatic nerve NCSCs express α_4 integrin. Table 1 shows that the $p75^+\alpha_4^+$ population from gut was also highly enriched for multipotent neural crest progenitors. Ten percent of unfractionated E14.5 gut cells survived to form colonies in culture, and 11% of these cells formed multilineage colonies, indicating that around 1% of unfractionated gut cells are multipotent and survive in culture. All neural progenitor activity was contained within the $p75^+$ fraction of gut cells as would be expected, given that p75 consistently marks neural crest progenitors in the gut as well as in other locations (Stemple and Anderson, 1992; Lo and Anderson, 1995; Shah et al., 1994; Morrison et al., 1999). As p75 expression level increased, cells became increasingly enriched for the ability to form large multilineage colonies characteristic of NCSCs (Table 1). In order to maximize the enrichment of multipotent progenitors and include the majority of multipotent progenitors in the $p75^+\alpha_4^+$ population, we selected the 1%–2% of cells expressing the highest levels of p75 and α_4 integrin by flow-cytometry (Figure 2C). In a clonal analysis, around 70% of these $p75^+\alpha_4^+$ cells formed multilineage colonies in culture (Table 1).

We measured the self-renewal potential of the $p75^+\alpha_4^+$ multipotent progenitors by sorting individual cells into different wells of a 96-well plate, culturing for 8 days, and then subcloning individual colonies into secondary cultures to determine how many multipotent daughter cells were produced per multipotent founder

Table 1. Separation of E14.5 Gut Cells Based on the Expression of p75 and α_4 Integrin Allows the Prospective Identification and Isolation of Multipotent Neural Crest Progenitors

	Plating Efficiency (%)	Colonies that Contain the Indicated Cell Types (%)						
		N + G + M	N + M	N + G	G + M	G Only	M Only	N Only
Unfractionated	10.4 ± 4.5	11.2 ± 12.4	5.0 ± 6.0	0.4 ± 1.1	0.6 ± 1.6	0.1 ± 0.4	80.7 ± 19.9	0.1 ± 0.4
$p75^-$	6.7 ± 5.8	0.0 ± 0.0b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
$p75^{low}$	33.3 ± 17.5	30.8 ± 18.5	1.2 ± 2.0	3.2 ± 3.9	7.2 ± 14.1	2.0 ± 3.6	54.5 ± 24.0	0.5 ± 1.2
$p75^+$	28.1 ± 6.0	43.4 ± 17.8	6.3 ± 7.3	1.6 ± 1.8	6.1 ± 5.5	0.7 ± 1.4	40.4 ± 18.4	1.5 ± 1.7
$p75^+\alpha_4^-$	25.4 ± 22.0	43.4 ± 25.4	8.7 ± 15.1	1.7 ± 2.9	4.0 ± 3.6	0.6 ± 1.0	38.9 ± 34.7	2.8 ± 2.6
$p75^+\alpha_4^+$	49.3 ± 16.9	70.2 ± 13.1	2.0 ± 3.1	6.9 ± 7.4	4.1 ± 3.5	2.1 ± 2.5	13.0 ± 14.2	1.2 ± 2.1

Colonies were stained after 14 days in culture and contained different combinations of neurons (N; Peripherin$^+$), glia (G; GFAP$^+$), and myofibroblasts (M; SMA$^+$). For example, N + G + M colonies contained neurons, glia, and myofibroblasts, while M only colonies contained only myofibroblasts. The percentages in each row do not necessarily add up to 100 because up to 2% of colonies did not stain with any marker. The $p75^+\alpha_4^+$ population contained a significantly higher frequency of multipotent (N + G + M) colonies than any other population ($p < 0.05$).

Table 2. E14.5 Gut p75$^+\alpha_4^+$ Multipotent Progenitors Self-Renew in Culture

	Subclones Per Multipotent Founder Cell					
Population	Total	N + G + M	N + G	G + M	G Only	M Only
Gut p75$^+\alpha_4^+$	987 ± 460*	876 ± 439	105 ± 168	2 ± 5	0 ± 0*	4 ± 11
SN p75$^+$P$_0^-$	613 ± 234	548 ± 279	58 ± 136	3 ± 5	5 ± 9	1 ± 2

Individual E14.5 gut or sciatic nerve cells were deposited into different wells of 96-well plates. After 8 days of culture, individual colonies (8 from sciatic nerve and 15 from gut) were subcloned into secondary cultures at clonal density. Secondary clones were cultured for 14 days under standard conditions and then were stained with antibodies against neurons (N), glia (G), and myofibroblasts (M) as described in Table 1. All primary colonies gave rise to multipotent secondary colonies (* significantly different, $p < 0.05$).

cell (Table 2). All 15 of the gut colonies that were subcloned gave rise to large numbers of multipotent daughter cells, as well as to various classes of restricted progenitors that were observed among freshly dissociated gut cells (compare Table 2 to Table 1). The ability of single p75$^+\alpha_4^+$ gut cells to form multilineage colonies and self-renew confirmed that they are NCSCs. Gut NCSCs gave rise to significantly more total subclones but significantly fewer glial-only subclones relative to sciatic nerve NCSCs (Table 2).

Uncultured Postmigratory Gut NCSCs Give Rise to Neurons and Glia In Vivo

To test the potential of gut p75$^+\alpha_4^+$ NCSCs in vivo, we injected freshly isolated, uncultured p75$^+\alpha_4^+$ cells into the hindlimb bud somites of stage 17–18 chick embryos. Three days after injection, the chick embryos were sectioned and hybridized with probes against rat *SCG10* (Anderson and Axel, 1985) to identify neurons and rat *P$_0$* (Lemke et al., 1988) to identify glia. As shown in Figure 3, the gut p75$^+\alpha_4^+$ NCSCs gave rise to neurons and glia in diverse locations of all five chicks that were analyzed. Engraftment included *SCG10*$^+$ neurons in the sympathetic chain (five of five chicks; 5.7 cells/positive section), aortic plexus (two of five chicks; 2.7 cells/positive section), Remak's ganglion (five of five chicks; 3.0 cells/positive section), gut (one of five chicks; 6.8 cells/positive section), and pelvic plexus (four of five chicks; 10.3 cells/positive section). All five of the chicks also had *P$_0$*$^+$ rat glia in the gut (two of five cases; 4.8 cells/positive section) and/or in other locations.

Neurogenic and Gliogenic Factors Are Expressed in Both the Nerve and Gut

Neural crest cells within the sciatic nerve generate glia, but not neurons (Jessen and Mirsky, 1992; Jessen et al., 1994), while in the gut, neurogenesis continues throughout fetal development (Pham et al., 1991). In the E14.5 gut, neural crest cells appear to be principally generating neurons, along with smaller numbers of glia (see Supplemental Figure S1 online at http://www.neuron.org/cgi/content/full/35/4/643/DC1). Thus, neural crest progenitors in the sciatic nerve are engaged primarily in gliogenesis, while neural crest progenitors in the gut are engaged primarily in neurogenesis.

If the local environment is responsible for this difference in cell fate determination, then gliogenic, but not neurogenic, factors should be expressed in the nerve, while neurogenic factors should be predominantly expressed within the gut. Neuregulin and the Notch ligand Delta have been shown to promote gliogenesis in the PNS. Neuregulin instructs glial differentiation by early migrating NCSCs and sciatic nerve NCSCs (Shah et al., 1994; Morrison et al., 1999) and is necessary for gliogenesis in vivo (Meyer and Birchmeier, 1995; Riethmacher et al., 1997). Neuregulin is known to be expressed in the fetal gut (Meyer and Birchmeier, 1994) as well as in the developing sciatic nerve (Dong et al., 1995). Delta instructs glial lineage determination by sciatic nerve NCSCs (Morrison et al., 1999) and is necessary for gliogenesis in vivo (M. Bronner-Fraser, personal communication). The Notch ligands *Jagged* and *Delta* were expressed within both peripheral nerve (Figures 4A and 4C) and gut (Figures 4B and 4D). *Notch 1* and *Notch 2* were also expressed within peripheral nerves (Figures 4G and 4I) and gut (Figures 4H and 4J). Previous studies have shown *Notch 1* and *Notch 2* expression within neural crest cells by in situ hybridization (Weinmaster et al., 1991; Williams et al., 1995). We confirmed this by quantitative PCR, observing similar levels of *Notch 1* and *Notch 2* expression in gut p75$^+\alpha_4^+$ NCSCs and sciatic nerve p75$^+$P$_0^-$ NCSCs (data not shown). Thus, *Notch 1* and *Notch 2* were expressed by both gut and sciatic nerve NCSCs, and Notch ligands were expressed in the gut and nerve environments.

BMPs are necessary (Schneider et al., 1999) and sufficient (Reissman et al., 1996) for autonomic neurogenesis in vivo and instruct early migrating NCSCs (Shah et al., 1996), sciatic nerve NCSCs (Morrison et al., 1999), and gut neural crest progenitors (Pisano et al., 2000) to undergo neuronal differentiation. *BMP4* was expressed in cells surrounding the axon bundles of peripheral nerves (Figure 4E) as well as in the gut mesenchyme (Figure 4F). *BMP2* was expressed in transverse streaks adjacent to peripheral nerves (data not shown), as well as in the gut epithelium (data not shown), as has been published previously (Bitgood and McMahon, 1995). Since we did not detect any obvious difference in the expression of known neurogenic and gliogenic factors between the nerve and gut, we decided to test whether there were differences in the sensitivity of neural stem cells from these locations to lineage determination factors.

A Cell-Intrinsic Difference in the Sensitivity of Sciatic Nerve and Gut NCSCs to BMP4

Addition of 50 ng/ml BMP2 or BMP4 to sciatic nerve NCSC cultures instructs neuronal differentiation, inducing the expression of the proneural bHLH gene Mash-1 within 24 hr and the mature neuronal marker Peripherin within 5 days in more than 80% of colonies (Morrison

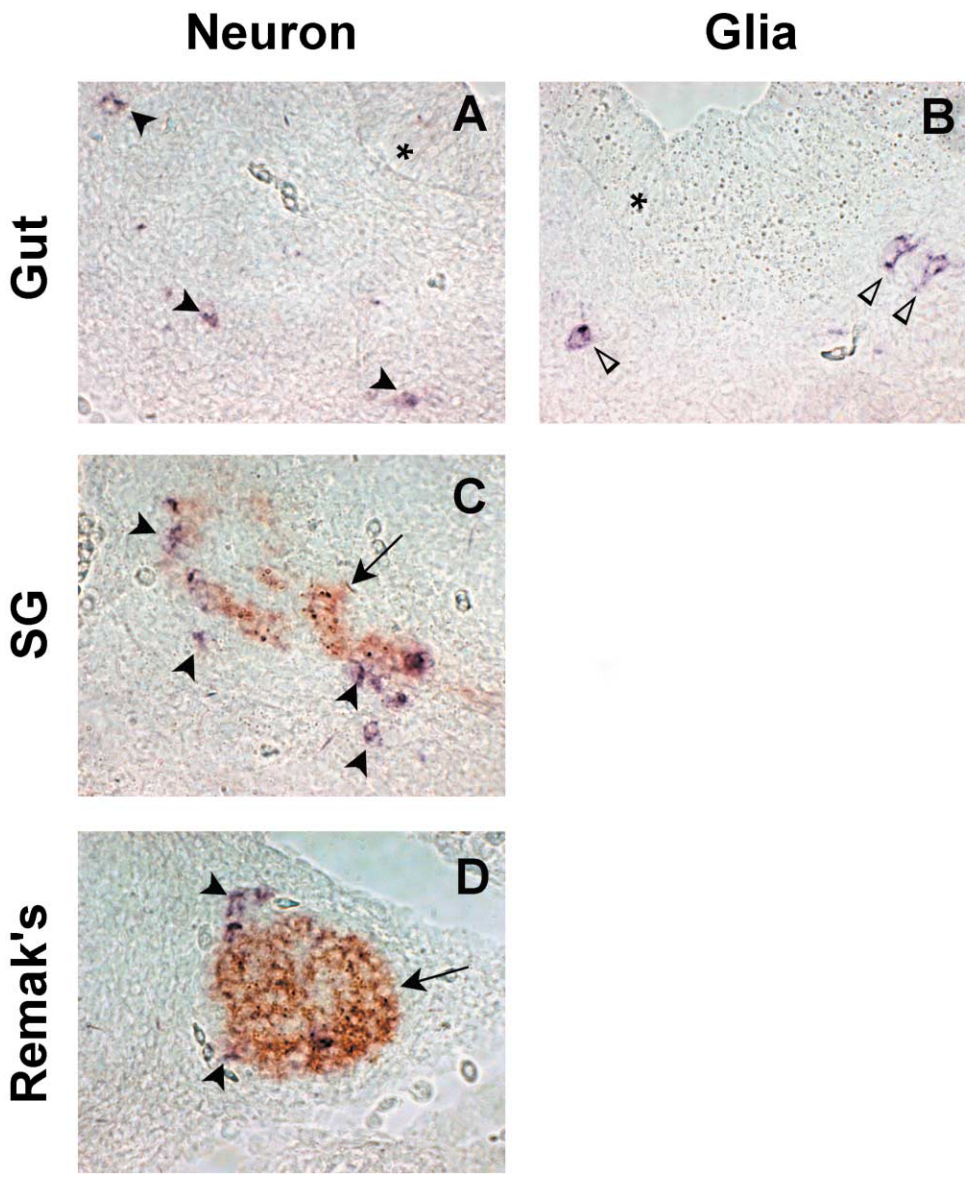

Figure 3. Gut NCSCs Give Rise to Neurons and Glia In Vivo

E14.5 rat gut p75$^+\alpha_4^+$ NCSCs were isolated by flow-cytometry and injected into the hindlimb bud somites of stage 18 chick embryos. Embryos were fixed and frozen after 72 hr of development (stage 29). Hindlimb level sections were hybridized in situ with rat- and chick-specific probes against the neuronal marker *SCG10* and the glial marker P_0. Hybridization with neuron-specific probes is shown in (A), (C), and (D), where the purple signal indicates rat neurons and the orange signal indicates chick neurons. Gut p75$^+\alpha_4^+$ cells gave rise to neurons in chick hindgut (A), sympathetic ganglion (C), and Remak's (parasympathetic) ganglion (D), in addition to aortic plexus and pelvic plexus (data not shown). Hybridization with the rat-specific glial probe (B) showed glial differentiation in the gut (B) as well as in some peripheral nerves (see Figure 6). Black arrowheads point to some of the rat neurons, black arrows point to some of the chick neurons, and open arrowheads point to rat glia. Asterisks mark the epithelial border of the gut lumen.

et al., 1999, 2000a). Gut p75$^+\alpha_4^+$ NCSCs exhibited a similar response as 50 ng/ml BMP4 caused 76% ± 14% (mean ± standard deviation) of colonies to contain Mash-1$^+$ cells after 24 hr and 82% ± 3% of colonies to contain Peripherin$^+$ neurons after 5 days in culture.

Although gut and sciatic nerve NCSCs exhibited similar responses to saturating concentrations of BMP4, we sought to determine whether these cells exhibit a difference in sensitivity to lower concentrations of BMP4 that probably more accurately reflect the limiting concentrations of BMPs encountered in vivo. E14.5 gut p75$^+\alpha_4^+$ NCSCs and sciatic nerve p75$^+$P$_0^-$ NCSCs were isolated by flow-cytometry, cultured for 24 hr in BMP4 concentrations that ranged from 0–100 ng/ml, and then stained with an antibody against Mash-1. Gut NCSCs exhibited a half-maximal response to BMP4 at around 0.5 ng/ml and a maximal response at 10 ng/ml, while sciatic nerve NCSCs exhibited a half-maximal response at around 5 ng/ml and a maximal response at 50 ng/ml (Figure 5). Thus, gut NCSCs were 5- to 10-fold more sensitive to the neurogenic effects of BMP4.

The observation that 45% of gut NCSCs expressed

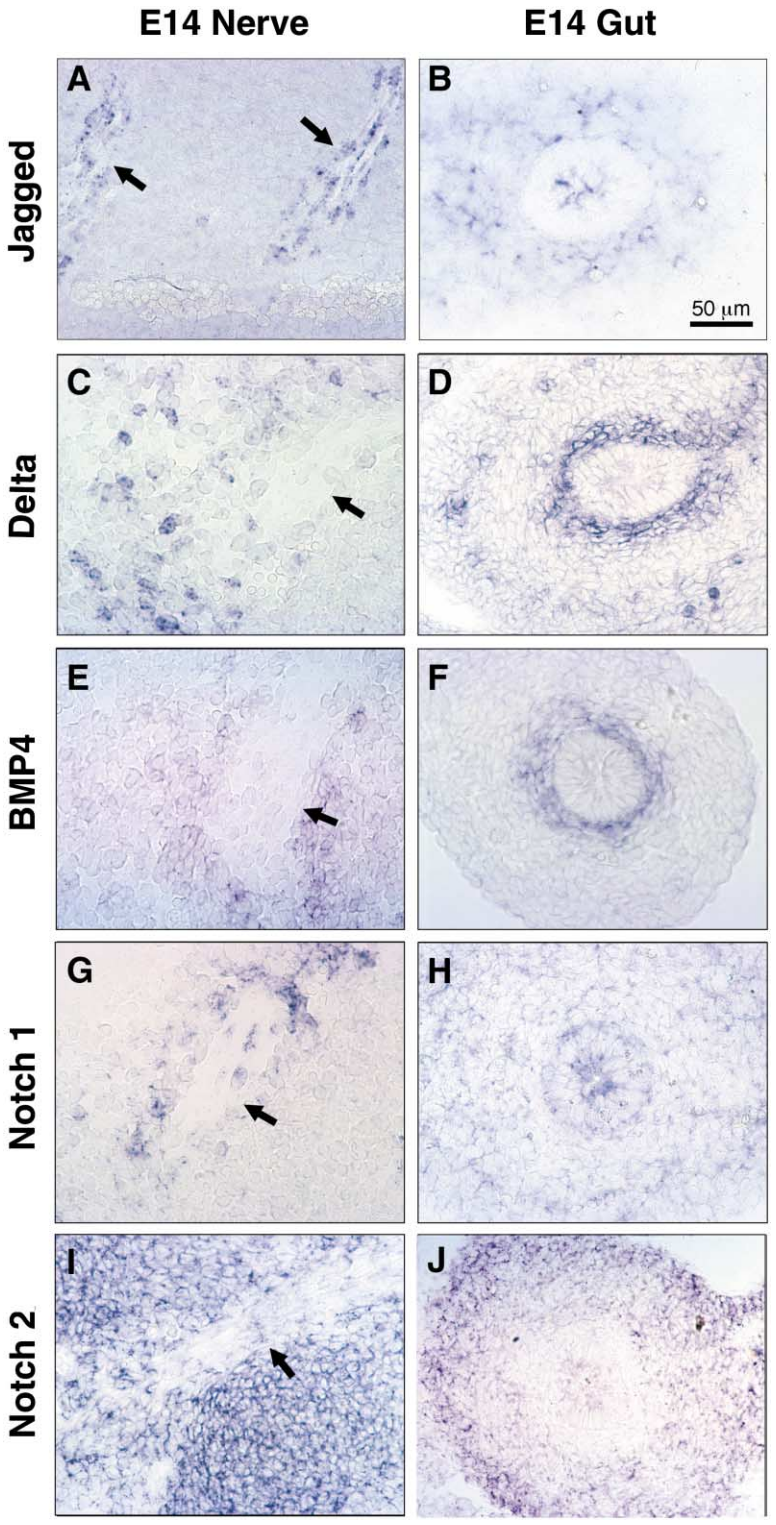

Figure 4. Neurogenic and Gliogenic Factors Are Expressed in the E14.5 Nerve and Gut

In situ hybridization (blue/purple signal) with anti-sense probes to *Jagged* (A and B), *Delta1* (C and D), *BMP4* (E and F), *Notch 1* (G and H), and *Notch 2* (I and J) mRNAs in oblique sections through E14.5 lumbosacral peripheral nerves (A, C, E, G, and I) and transverse sections of E14.5 gut (B, D, F, H, and J). Identification of nerves in sections was confirmed by staining adjacent sections with Peripherin (data not shown). Nerves were composed of a bundle of Peripherin$^+$ axons (arrows) surrounded and interspersed by S100β^+ neural crest progenitors. *Jagged* (A) and *Notch 1* (G) were expressed by cells within the axon bundles, while *Delta* and *Notch 2* were expressed in cells surrounding the axon bundles. The expression of *Notch 1* and *Notch 2* in sciatic nerve and gut NCSCs was confirmed by PCR (data not shown). *BMP4* was expressed in some cells surrounding the axon bundle (E) and in cells within the gut (F). *Jagged* (B), *Delta* (D), *Notch 1* (H), and *Notch 2* (J) were expressed in a pattern in the gut that was consistent with expression by neural crest progenitors as confirmed by staining sections with p75 (data not shown). In all cases, nearby sections were hybridized with sense control probes (data not shown). While we cannot be certain that gut and nerve NCSCs have direct contact with *BMP* or *Notch*-ligand expressing cells, the expression patterns are consistent with close contact.

Mash-1 after 24 hr in culture even without BMP4 addition (Figure 5) was consistent with the observation that these cells are engaged primarily in neurogenesis in vivo (Supplemental Figure S1). Although Mash-1 can be expressed by multipotent progenitors and Mash-1 expression does not indicate neuronal commitment (Lo and Anderson, 1995; Lo et al., 1997), we wanted to ensure that the gut p75$^+\alpha_4^+$ NCSC population was not significantly contaminated by immature neurons. E14.5 gut p75$^+\alpha_4^+$ cells were sorted into culture and stained 17 hr later for the early neuronal marker neuron-specific (class III) β-tubulin (TuJ1 antibody). Only 0.67% $\pm$ 0.58%

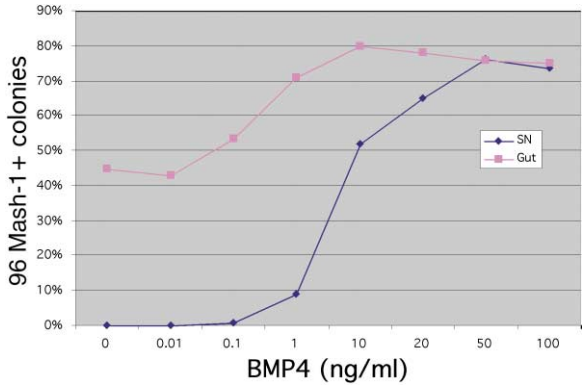

Figure 5. Gut NCSCs Were 5- to 10-Fold More Sensitive to the Neurogenic Effects of BMP4

$p75^+\alpha_4^+$ gut NCSCs and $p75^+P_0^-$ sciatic nerve NCSCs were cultured at clonal density and exposed to BMP4 concentrations from 0–100 ng/ml. After 24 hr, the cultures were stained with an antibody against the proneural bHLH protein Mash-1. Gut NCSCs exhibited half-maximal and maximal responses at around 0.5 and 10 ng/ml, respectively, while sciatic nerve NCSCs exhibited half-maximal and maximal responses at around 5 and 50 ng/ml, respectively. Each data point represents the average of ten independent experiments. At all BMP4 concentrations less than 50 ng/ml, the difference in Mash-1 expression between gut and sciatic nerve cells was statistically significant ($p < 0.05$).

of gut $p75^+\alpha_4^+$ cells expressed β-tubulin. No β-tubulin staining was detected among sciatic nerve $p75^+P_0^-$ cells.

The Increased Sensitivity of Gut NCSCs to BMP4 Persists Even after 8 Days in Culture and Subcloning

If cell-intrinsic differences in the sensitivity of NCSCs to lineage determination factors regulate differentiation in vivo, then such differences should be heritable and stable enough to persist even through changes in the cell's environment. To test whether the difference in BMP4 sensitivity was stable in culture, the two NCSC populations were cultured under standard conditions at clonal density for 8 days (12–14 population doublings). Then, individual colonies were subcloned into secondary cultures that contained no BMP4, 1 ng/ml BMP4, or 50 ng/ml BMP (Table 3). In these experiments, 58% of gut secondary colonies expressed Mash-1 after 24 hr in 50 ng/ml BMP4 (31% at 1 ng/ml). In contrast, only 27% of secondary sciatic nerve colonies expressed Mash-1 after 24 hr in 50 ng/ml BMP4 (7% at 1 ng/ml). Thus, as in the primary colonies, the gut subclones remained more likely to both express Mash-1 and respond to BMP4 than sciatic nerve subclones at all levels of BMP stimulation (Table 3; see Supplementary Figure S2 online at http://www.neuron.org/cgi/content/full/35/4/643/DC1). Although the gut subclones remained more responsive than sciatic nerve subclones to the neurogenic effects of BMP4, both types of subcloned progenitors exhibited reduced responsiveness to BMPs relative to what was observed upon initial isolation, consistent with a previous report (Kubu et al., 2002).

Cell-intrinsic Differences in the Sensitivity of Sciatic Nerve and Gut NCSCs to Delta and Neuregulin

Notch activation promotes rapid glial lineage determination among sciatic nerve NCSCs (Morrison et al., 2000b). One way in which this was demonstrated was by culturing sciatic nerve NCSCs in soluble Delta for 24 hr, followed by 4 or 5 days in 50 ng/ml BMP4. Without prior exposure to soluble Delta, BMP4 induces neurogenesis, but after exposure to soluble Delta, sciatic nerve NCSCs undergo gliogenesis even in the presence of BMP4 (Morrison et al., 2000b). To compare their responsiveness to Notch activation, gut and sciatic nerve NCSCs were cultured for 24 hr in soluble Delta, and then the Delta was washed out of the medium and the cells were cultured for 4 additional days in 50 ng/ml BMP4. Table 4 shows that whereas Delta caused extensive gliogenesis among sciatic nerve NCSCs, gut NCSCs exhibited virtually no gliogenic response. In the absence of soluble Delta, up to 66% of sciatic nerve colonies contained neurons (38% neuron only + 28% neuron + other), and fewer than 3% contained glia (Table 4). Exposure to soluble Delta significantly reduced the frequency of sciatic nerve colonies that contained neurons (to 4%) and significantly increased the frequency of colonies that contained only $GFAP^+$ glia (to 42%). In the absence of soluble Delta, gut NCSCs performed indistinguishably from sciatic nerve NCSCs, with up to 72% of colonies containing neurons (12%–13% neuron only + 44%–59% neuron + other) and no colonies containing glia. Exposure to soluble Delta only modestly reduced the percent-

Table 3. Gut NCSCs Exhibit Increased Sensitivity to BMPs Even after 8 Days in Culture

	Colonies Containing Mash-1+ Cells (%)		
E14.5 NCSC Subcloned	No BMP4	1 ng/ml BMP4	50 ng/ml BMP4
Gut $p75^+\alpha_4^+$	19 ± 17 c	31 ± 21 d	58 ± 18 e
SN $p75^+P_0$	3 ± 3 a	7 ± 5 b	27 ± 11 c, d

Gut and SN NCSCs were cultured at clonal density for 8 days (12–14 population doublings) and then subcloned into secondary cultures that contained 0, 1, or 50 ng/ml BMP4. The percentage of secondary colonies (mean ± standard deviation) that contained Mash-1+ cells after 24 hr is shown. 65% ± 13% of gut secondary colonies and 54% ± 15% of sciatic nerve secondary colonies made peripherin+ neurons after 5 days in 50 ng/ml BMP4, indicating that there was no overall difference between sciatic nerve and gut subclones in neuronal potential. All Mash-1 statistics were compared by paired t-tests, and significantly different statistics ($p < 0.05$) are followed by different letters. In each treatment, the percentage of colonies that contained Mash-1+ cells was higher among gut NCSC subclones than among sciatic nerve NCSC subclones. Data are based on 4692 subclones from 12 primary sciatic nerve NCSC colonies and 12135 subclones from 15 primary gut NCSC colonies.

Table 4. Notch Ligands Do Not Promote Gliogenesis by Gut NCSCs, in Contrast to Their Effect on Sciatic Nerve NCSCs

NCSC		Colonies Containing the Indicated Cell Types (% of All Colonies)					
		Neuron Only	Neuron + Other	Glia Only	Glia + Other	Myo Only	Myo + Other
SN	No add	38 ± 35	28 ± 31	0 ± 0	1 ± 2	14 ± 13	29 ± 29
	Fc	37 ± 34	24 ± 30	2 ± 4	1 ± 3	14 ± 12	25 ± 30
	Delta-Fc	0.4 ± 1*	4 ± 3	42 ± 27*	21 ± 14*	8 ± 10	21 ± 29
Gut	No add	12 ± 22	44 ± 21	0 ± 0	0 ± 0	16 ± 10	52 ± 32
	Fc	13 ± 20	59 ± 14	0 ± 0	0 ± 0	17 ± 14	64 ± 17
	Delta-Fc	2 ± 3	38 ± 22	1 ± 1	3 ± 3	29 ± 18	57 ± 21

NCSCs were cultured for 24 hr at clonal density (80 cells/35 mm dish) in either standard medium, medium supplemented with the Fc control, or with Delta-Fc; then these media were replaced with standard medium containing 50 ng/ml BMP4 for 4 additional days. Neuron + other colonies contained neurons as well as glia and/or myofibroblasts and/or undifferentiated cells, thus there is overlap between this category and the glia + other and myofibroblast + other categories. Among sciatic nerve NCSC treatments, there were no significant differences between no add and Fc only treatments, but Delta-Fc significantly (*; $p < 0.05$) reduced the percentage of neuron only colonies and significantly increased the proportion of glia only and glia-containing colonies based on paired t-tests. Note that variability between independent experiments was considerable, such that standard deviations sometimes exceeded means. Paired t-tests account for such variability by comparing treatments within multiple independent experiments. Delta-Fc had no statistically significant effect on the type of colonies formed by gut NCSCs. A total of 371 sciatic nerve colonies and 928 gut colonies were counted in five independent experiments.

age of colonies that contained neurons (to 40%) and slightly increased the percentage of colonies that contained glia (to 4%). Neither effect was statistically significant. Increasing soluble Delta concentrations 5-fold did not further decrease the proportion of neuron-containing gut clones or increase the proportion of glia-containing clones (data not shown). In contrast to the strong gliogenic response of sciatic nerve NCSCs to soluble Delta, gut NCSCs were insensitive to the gliogenic effects of Delta.

If gut NCSCs are generally insensitive to gliogenic factors, then they must also be insensitive to the gliogenic effects of Neuregulin. Table 5 shows that culture in Neuregulin-containing medium for 14 days caused glial differentiation by sciatic nerve NCSCs, but no statistically significant effect on gut NCSC differentiation. Under control conditions, 82% of sciatic nerve NCSCs formed colonies that contained neurons and nonneuronal cells (72% were N + G + M), while only 13% were glial only. In the presence of Neuregulin, only 2% of sciatic nerve colonies contained neurons, and 93% of colonies were glial only. Under control conditions, 88% of gut colonies contained neurons and glia and 6% were glial only, while in Neuregulin-containing medium, 84% of colonies contained neurons and glia, and 13% of colonies were glial only. Thus, Neuregulin promoted gliogenesis at the expense of neurogenesis among sciatic nerve NCSCs, but did not detectably affect the differentiation of gut NCSCs.

After 8 days in culture (12–14 population doublings), gut NCSC subclones remained less responsive to the gliogenic effects of soluble Delta and Neuregulin than sciatic nerve NCSC subclones (see Supplementary Table S1 online at http://www.neuron.org/cgi/content/full/35/4/643/DC1).

When Transplanted into Developing Nerve, Sciatic Nerve NCSCs Give Rise Only to Glia, While Gut NCSCs Give Rise Mainly to Neurons

Previous experiments have shown that sciatic nerve NCSCs give rise to glia, but not neurons, in developing peripheral nerves after transplantation into the neural crest migration pathway of stage 18 chick embryos (White et al., 2001). If the insensitivity of nerve NCSCs to neurogenic factors is responsible for the failure to generate neurons in peripheral nerve, then gut NCSCs should give rise to neurons upon transplantation into the nerve. We transplanted E14.5 gut $p75^{+}\alpha_4^{+}$ cells into the neural crest migration pathway of five chick embryos and then examined whether these cells gave rise to neurons and glia in peripheral nerves. As shown in Figure 6, the gut NCSCs gave rise to neurons in the peripheral nerves of all five chicks (8.3 neurons/positive section). Three of these chicks lacked any rat glia in peripheral

Table 5. Neuregulin Promotes Gliogenesis at the Expense of Neurogenesis in Sciatic Nerve NCSCs, But Not in Gut NCSCs

NCSC		Colonies Containing the Indicated Cell Types (% of All Colonies)					
		Neuron Only	Neuron + Other	Glia Only	Glia + Other	Myo Only	Myo + Other
SN	No add	0 ± 0	82 ± 10	13 ± 11	85 ± 11	2 ± 2	76 ± 15
	+Nrg	0 ± 0	2 ± 1*	93 ± 8*	7 ± 9*	0.5 ± 1	6 ± 9*
Gut	No add	0 ± 0	88 ± 14	6 ± 10	91 ± 10	2 ± 2	84 ± 13
	+Nrg	0 ± 0	84 ± 21	13 ± 23	84 ± 21	2 ± 2	45 ± 34

E14.5 NCSCs were cultured for 14 days either in standard medium (no add) or in standard medium supplemented with 65 ng/ml (1 nM) Neuregulin (+Nrg). As in Table 4, Neuron + other colonies contained neurons as well as glia and/or myofibroblasts and/or undifferentiated cells; thus there is overlap between this category and the glia + other and myofibroblast + other categories. Among sciatic nerve NCSC colonies, Neuregulin significantly reduced the frequency of neuron-containing and myofibroblast-containing colonies and significantly increased the frequency of glial only colonies (*; $p < 0.01$). Neuregulin did not have any statistically significant effect on gut NCSC differentiation. A total of 308 sciatic nerve colonies and 385 gut colonies were counted in four independent experiments.

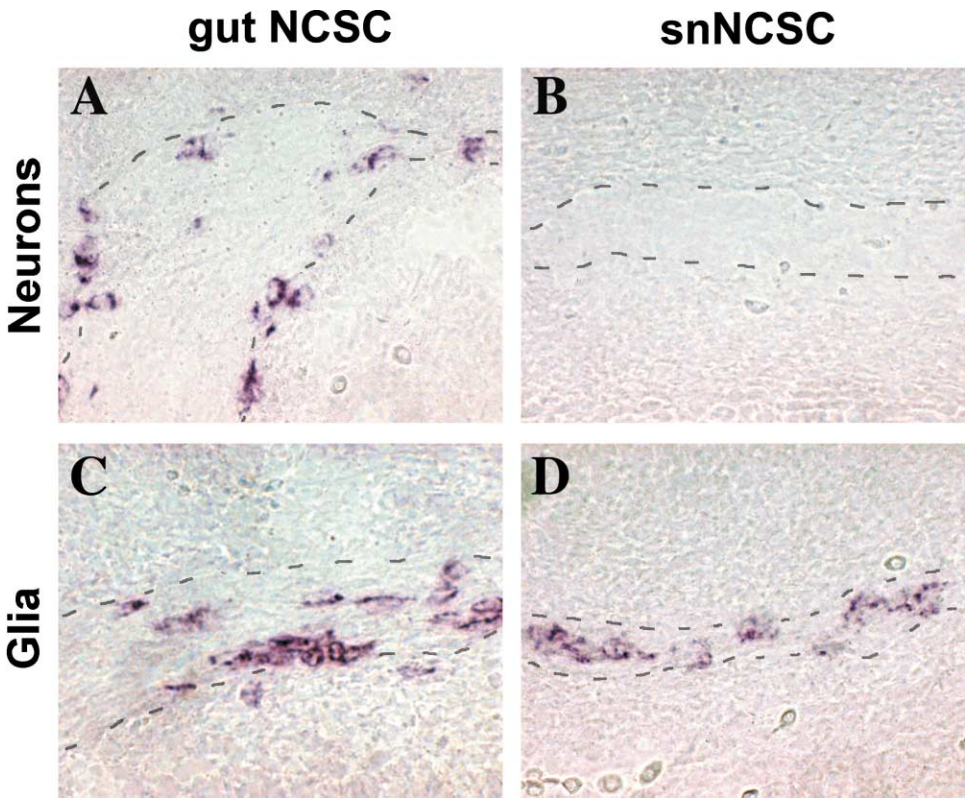

Figure 6. Gut NCSCs Give Rise to Neurons and Glia in Developing Peripheral Nerve, While Sciatic Nerve NCSCs Give Rise Only to Glia

Freshly isolated $p75^+\alpha_4^+$ gut NCSCs and $p75^+P_0^-$ sciatic nerve NCSCs were injected into hindlimb bud somites of chick embryos. Sections were hybridized in situ with rat-specific probes against the neuronal marker *SCG10* (A and B) or the glial marker P_0 (C and D). Gut NCSCs consistently gave rise to neurons (A) and glia (C) in the chick nerve, while sciatic nerve NCSCs gave rise to glia (D), but not neurons (B). Since the nerve at this stage of development consists of an axon bundle surrounded by neural crest cells, engraftment in the nerve was defined by the presence of rat cells within three cell bodies of the axon bundle. Axon bundles (demarcated by dashed lines in the photos) were identified based on position and morphology and confirmed in nearby sections by staining with neurofilament 200 (data not shown).

nerves, but the remaining two chicks did have rat glia in peripheral nerves (9.2 cells/positive section). When $p75^+P_0^-$ sciatic nerve NCSCs were injected into seven chick embryos, these cells gave rise to glia in the peripheral nerves of all seven chicks (10.7 cells/positive section). As observed previously (White et al., 2001), sciatic nerve NCSCs failed to generate neurons in the peripheral nerves of any chicks despite giving rise to neurons in other locations of the same chicks (Remak's ganglion, five of seven injected chicks, 1.3 cells/engrafted section). This demonstrates that the nerve environment is permissive for both neurogenesis and gliogenesis but that only glia are generated during normal development because nerve NCSCs are biased toward adopting glial fates.

If differences between NCSCs in sensitivity to lineage determination factors are generally important, then gut NCSCs should generate more neurons than sciatic nerve NCSCs in other locations as well. Both gut and sciatic nerve NCSCs consistently gave rise exclusively to neurons in Remak's ganglion, but gut NCSCs gave rise to more neurons per engrafted section (3.0 cells/section for gut versus 1.3 cells/section for sciatic nerve). The fact that sciatic nerve NCSCs gave rise exclusively to neurons within Remak's ganglion demonstrates that these NCSCs are not committed to glial fates but exhibit plasticity in response to different environments.

Gut NCSCs gave rise to neurons and glia in the gut of some chicks (Figure 3), but we did not detect any engraftment of sciatic nerve NCSCs in the chick gut. The failure of sciatic nerve NCSCs to engraft in gut may be due to cell-intrinsic differences in migratory properties. This is consistent with previous studies that found only cephalic or vagal neural crest-derived cells were able to migrate into the gut upon transplantation to trunk or sacral levels (LeLievre et al., 1980). The ability of E14.5 postmigratory gut NCSCs to migrate into the gut after transplantation into chick hindlimb somites thus distinguishes these cells from E10.5 migrating trunk NCSCs as well as E14.5 restricted enteric neuronal precursors, which failed to migrate into the developing chick gut in a similar assay (White and Anderson, 1999).

Discussion

We found NCSCs in the E14 gut, sympathetic chain, and dorsal root ganglia (Figure 1) in addition to peripheral nerves (Morrison et al., 1999). This is consistent with previous reports of multipotent neural crest progenitors in the E14 gut (Lo and Anderson, 1995) and dorsal root

ganglion (Hagedorn et al., 1999). These observations suggest that postmigratory NCSCs persist widely throughout the late gestation PNS. To characterize the gut NCSCs in more detail, we prospectively identified and isolated them by flow-cytometry as $p75^+\alpha_4^+$ cells (Figure 2). In clonal analyses, 70% of the colonies formed by this population contained neurons, glia, and myofibroblasts (Table 1). Gut $p75^+\alpha_4^+$ cells gave rise to neurons and glia in diverse locations throughout the developing chick PNS, just as sciatic nerve $p75^+P_0^-$ NCSCs (White et al., 2001) and early migrating NCSCs do (White and Anderson, 1999; White et al., 2001). Future studies will assess whether the neurons that engrafted in sympathetic, parasympathetic, and enteric ganglia (Figure 3) express markers that are appropriate for those locations.

It is unlikely that the in vivo engraftment of the E14.5 gut NCSCs was significantly affected by the rare restricted neuronal progenitors that contaminated this population. Restricted neuronal progenitors and neuron-specific β-tubulin+ cells represented only 1.2% and 0.67% of $p75^+\alpha_4^+$ gut cells, respectively. Restricted enteric neuronal precursors (ENPs) from the E14.5 rat gut show an engraftment pattern in chick embryos that is distinct from $p75^+\alpha_4^+$ gut NCSCs (White and Anderson, 1999). For example, ENPs failed to migrate into the chick gut, while $p75^+\alpha_4^+$ gut NCSCs did give rise to neurons and glia in the gut (Figure 3). The conclusions of this study are also consistent with the conclusions of a companion study in which postnatal day (P)15 gut NCSCs, which are more responsive to gliogenic than neurogenic factors, gave rise almost exclusively to glia in developing chick peripheral nerves (Kruger et al., 2002). This was despite the fact that these cells had higher levels of contamination by immature neurons or restricted neuronal progenitors cells (around 4%) than E14.5 gut $p75^+\alpha_4^+$ NCSCs. The data argue strongly that the in vivo engraftment patterns that we observed were attributable to NCSCs rather than restricted neuronal progenitors.

Neural Crest Stem Cells from the Sciatic Nerve and Gut Are Intrinsically Different in a Way that Affects Their Differentiation

Our ability to isolate uncultured NCSCs from sciatic nerve and gut at E14 provided a unique opportunity to compare their differentiation in vitro and in vivo. During normal development, neural crest progenitors give rise to glia, but not neurons, in peripheral nerves (Jessen and Mirsky, 1992; Jessen et al., 1994). In contrast, neural crest progenitors in the gut are undergoing mainly neurogenesis at E14 (Supplemental Figure S1). There was no evidence for the differential expression of neurogenic and gliogenic factors between the nerve and gut (Figure 4). This suggested that cell-intrinsic differences between gut and nerve NCSCs, rather than environmental differences, likely explained the difference in cell fate determination between the nerve and gut. We found that sciatic nerve NCSCs were very sensitive to the gliogenic effects of Notch ligands (Table 4) and Neuregulin (Table 5), while gut NCSCs were insensitive to the gliogenic effects of these factors. Conversely, gut NCSCs were very sensitive to the neurogenic effect of BMP4, while sciatic nerve NCSCs were 5- to 10-fold less sensitive to the neurogenic effect of BMP4 (Figure 5). These differences in sensitivity to neurogenic and gliogenic factors persisted through 8 days (12–14 population doublings) in culture, followed by subcloning (Table 3 and Supplemental Table S1), demonstrating that these differences were stably encoded within the stem cells. Sciatic nerve NCSCs were biased toward glial fates, while gut NCSCs were biased toward neuronal fates even though both populations remained multipotent.

Gut and Sciatic Nerve NCSCs Adopt Different Cell Fates Even When Transplanted into the Same In Vivo Environment

If differentiation is primarily environmentally controlled or if the sensitivity of gut NCSCs to gliogenic factors can be repatterned by the migratory or nerve environments, then gut NCSCs should give rise to glia, but not neurons, in peripheral nerve since that is what occurs during normal development. Conversely, if differentiation in the nerve is stem cell-determined and the properties of gut NCSCs are not changed by the migratory or nerve environments, then the gut NCSCs should give rise primarily to neurons in the nerve, just as they normally do in the gut. To test this, E14.5 gut and sciatic nerve NCSCs were isolated by flow-cytometry and transplanted into the hindlimb bud somites of stage 18 chick embryos. These NCSCs were allowed to migrate through the neural crest migration pathway into peripheral nerve, and their differentiation was examined 3 days later. As we have reported previously (White et al., 2001), sciatic nerve NCSCs gave rise to glia, but not neurons, in peripheral nerves (Figure 6), despite giving rise to neurons in other locations of the same chicks. In contrast, gut NCSCs gave rise to neurons in the peripheral nerves of all chicks that were analyzed. This confirmed that the nerve environment is capable of supporting neurogenesis, as we had expected based on the local expression of *BMPs* (Figure 4). This experiment also showed that it is not the nerve environment at E14.5 that promotes gliogenesis and impairs neurogenesis but the intrinsic gliogenic bias of the stem cells within the nerve. Some of the information that goes into the process of generating different mature cell types in different locations is encoded intrinsically within stem cells.

But cell fates were not entirely stem cell determined as the environment clearly played a role in determining how NCSCs differentiated in other locations, just as local environmental differences have been shown to regulate CNS stem cell differentiation (Song et al., 2002). Although sciatic nerve NCSCs gave rise only to glia in peripheral nerves, they gave rise only to neurons in Remak's ganglion. This demonstrates that some environments can overcome the gliogenic bias of these cells and cause neurogenesis. Thus NCSCs remained plastic enough to acquire both neuronal and glial fates, and different fates were acquired in different locations. The differences in sensitivity to lineage determination factors influenced, but did not restrict, the differentiation of stem cells in vivo. Diversity is therefore generated by interactions between different types of stem cells and

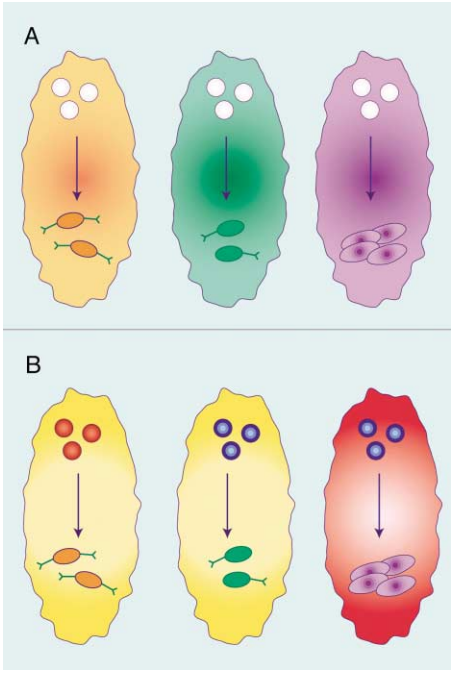

Figure 7. Neural Diversity Is Generated by Interactions between Intrinsically Different Types of Stem Cells and Different Environments

(A) Neural stem cells are sometimes thought of as a blank slate, able to give rise to appropriate types of differentiated cells in any environment they encounter. In this model, different populations of neural stem cells are equivalent, the local environment controls their differentiation, and diversity is encoded entirely by differences between local environments.

(B) Our data favor a model in which neural stem cells have very different sensitivities to the factors that regulate lineage determination. In this way, stem cells can remain multipotent but biased toward particular fates in certain environments. In this model, both cell-intrinsic differences within the stem cells as well as local environmental differences interact to generate diversity. Thus the nervous system would use a combinatorial mechanism to generate diversity. The environments that pattern the sensitivities of stem cells to lineage determination factors would impact how the stem cells differentiate in distant environments after migration. In this way, stem cells could transmit patterning information over long distances by their migration.

different local environments (Figure 7). Such a combinatorial mechanism would more efficiently encode diversity than if diversity was entirely determined by local environments. For example, instead of requiring 100 different environments to generate 100 types of cells, a combinatorial mechanism could, in principle, produce the same number of cell types through the interaction of 10 types of stem cells with 10 distinct environments. In this way, the nervous system might employ combinatorial interactions to encode diversity, just as the immune system generates diversity in T and B cell receptors through combinatorial rearrangements of genomic DNA.

Uncultured stem cells have not been purified from different regions of the CNS at the same time during development, but there are indications from studies of cultured stem cells and mixed populations of uncultured progenitors that there may also be regional differences in CNS stem cells that affect differentiation. E13 basal forebrain stem cells produced more GABAergic neurons in culture than E13 dorsal forebrain stem cells (He et al., 2001). Glial progenitors from different regions of the CNS exhibit divergent properties that correlate with regional differences in the timing of oligodendrocyte differentiation (Power et al., 2002). Mixed progenitors from the E12–E13 midbrain/hindbrain were not able to adopt local fates upon transplantation into the telencephalon (Campbell et al., 1995; Olsson et al., 1997). Mixed progenitors from E12–E14 ventral telencephalon failed to stop expressing a marker of telencephalic identity upon transplantation into the midbrain/hindbrain (Na et al., 1998). Since these latter studies employed mixed populations of progenitors, it remained possible that highly enriched stem cells would exhibit fewer regional restrictions upon transplantation in vivo. However, in this study we have isolated uncultured NCSCs from different regions of the PNS and found that they exhibit cell-intrinsic differences that dramatically affect the way they differentiate in vivo.

By Stably Encoding Differentiation Biases within Stem Cells, Patterning Environments Can Have Long-Distance Effects on Differentiation

Although gut NCSCs were transplanted into somites near the beginning of the neural crest migration pathway, they still gave rise mainly to neurons after migrating into peripheral nerves. This indicates that neither the migration pathway nor the peripheral nerve environment was able to repattern the gut NCSCs toward a gliogenic fate in the nerves. One possibility is that patterning of NCSCs only occurs in the premigratory neural tube environment where NCSCs are born. However, recent results suggest that cranial neural crest cells are patterned by their migratory environment (Trainor et al., 2002; Couly et al., 2002). In view of this, another possibility is that the cell-intrinsic differences between sciatic nerve and gut NCSCs were established during or after migration, but once established were slow to change in response to new environments. Perhaps there is a critical period during which migrating NCSCs can be patterned, after which NCSC properties change only very slowly.

Although the differences between sciatic nerve and gut NCSCs were not reversed by transplantation into similar in vitro and in vivo environments, our observations do not imply they are immutable. Postnatal gut NCSCs are much more responsive to gliogenic factors than neurogenic factors and give rise predominantly to glia in chick peripheral nerve (Kruger et al., 2002). This suggests that the sensitivities of neural stem cells to lineage determination factors may change over longer periods of developmental time or there may be specific environmental signals that are restricted in their expression and that can quickly repattern neural stem cells.

Whether the sensitivities of NCSCs to lineage determination factors are patterned before or after the onset of migration, the stability of these differences in vitro and in vivo suggest that they can be transmitted over long distances as the stem cells migrate. In principle, this could allow patterning influences to be transmitted by the stem cells from the environment in which they are born or begin migration to the environments in which

they eventually differentiate. Thus patterning of gut and sciatic nerve NCSCs in or near the neural tube could lead to neurons being generated throughout the gut and glia throughout peripheral nerves, even though NCSCs must migrate long distances in order to complete gut and nerve development.

We do not yet know the molecular basis for either the patterning difference between gut and sciatic nerve NCSCs or the specific differences in sensitivity to neurogenic and gliogenic factors. Gut NCSCs arise primarily from the vagal neural crest, while sciatic nerve NCSCs arise at more caudal levels of the neural tube. This suggests that factors that lead to rostrocaudal patterning of the neural tube could lead to patterning differences in NCSCs as well. Irrespective of the molecular mechanism, our data demonstrate that cell-intrinsic regional differences in the responsiveness of neural stem cells to lineage determination factors play an important role in the generation of neural diversity.

Experimental Procedures

Isolation of Sciatic Nerve and Gut NCSCs

Pregnant Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). E14.5 sciatic nerves were dissected into ice cold Ca, Mg-free HBSS and dissociated by incubating for 4 min at 37°C in 0.005% trypsin + 0.002% EDTA (BioWhittaker, Walkersville MD; product 17-161E diluted 1:10 in Ca, Mg-free HBSS) plus 0.25 mg/mL type 4 collagenase (Worthington, Lakewood NJ). The digestion was quenched with 2 volumes of staining medium: L15 medium containing 1 mg/mL BSA (Sigma product A-3912, St. Louis, MO), 10 mM HEPES (pH 7.4), penicillin/streptomycin (BioWhittaker), and 25 μg/mL deoxyribonuclease type 1 (Sigma, product D-4527). After centrifuging, nerve cells were triturated, filtered through nylon screen to remove aggregates of cells, and resuspended in staining medium. E14.5 gut, including stomach, small intestine, and hindgut were dissected from the same embryos and dissociated by incubating for 2 min at 37°C in 0.5mg/ml deoxyribonuclease type 1 (Sigma) in Ca, Mg-free HBSS.

All sorts and analyses were performed on a FACSVantage dual-laser flow-cytometer (Becton-Dickinson, San Jose, CA). Sciatic nerve cells were stained with antibodies against p75 (192Ig, directly conjugated to FITC) and P_0 (P07, a gift of J.J. Archelos). Gut cells were stained with antibodies against p75 and α4 integrin (Becton Dickinson, MRα4-1 clone, directly conjugated to phycoerythrin). After washing off unbound antibodies, cells were resuspended in staining medium containing 2 μg/ml 7-AAD (Molecular Probes, Eugene, OR). Dead cells were eliminated from sorts and analyses as 7-AAD$^+$. Sciatic nerve NCSCs were isolated as p75$^+$$P_0^-$, and gut NCSCs were isolated as p75$^+$$\alpha_4^+$. When sorting into culture, great care was taken to maintain a physiological pH in the culture medium by keeping the plates sealed in plastic bags containing 6% CO_2 before and after sorting.

Cell Culture

Cells were typically cultured in 6-well plates (Corning, Corning, NY) at clonal density so that individual colonies were spatially distinct (fewer than 30 clones per well for 14 day cultures) as previously described (Morrison et al., 1999, 2000a). Plates were sequentially coated with 150 μg/ml poly-d-lysine (Biomedical Technologies, Stoughton, MA) and 0.15 mg/mL human fibronectin (Biomedical Technologies) as described (Stemple and Anderson, 1992). The culture medium contained DMEM-low (Gibco, product 11885-084) with 15% chick embryo extract (prepared as described in Stemple and Anderson, 1992), 20 ng/mL recombinant human bFGF (R&D Systems, Minneapolis, MN), 1% N2 supplement (Gibco), 2% B27 supplement (Gibco), 50 μM 2-mercaptoethanol, 35 mg/mL (110 nM) retinoic acid (Sigma), penicillin/streptomycin (Biowhittaker), and 20 ng/ml IGF1 (R&D Systems). IGF1 promotes the survival of neural crest progenitors without influencing their differentiation (data not shown). This medium composition is described as standard medium. Under standard conditions, cells were cultured for 6 days in this medium, then switched to a similar medium (with 1% CEE and 10 ng/mL bFGF) that favors differentiation for another 8 days before immunohistochemical analysis of colony composition. All cultures were maintained in gas-tight chambers (Billups-Rothenberg, Del Mar, CA) containing decreased oxygen levels, as previously described, to enhance the survival of NCSCs (Morrison et al., 2000a). Delta-Fc or Fc was added to some cultures as previously described (Morrison et al., 2000b).

Immunohistochemistry

Cultures were fixed in acid ethanol (5% glacial acetic acid in 100% ethanol) for 20 min at −20°C, washed, blocked, and triply labeled for Peripherin (Chemicon AB1530; Temecula CA), GFAP (Sigma G-3893), and αSMA (Sigma A-2547) as described (Shah et al., 1996). MASH-1 staining was performed as described (Shah et al., 1994). TuJ1 antibody was obtained from Covance and used at a dilution of 1:500.

In Vivo Transplantation of Neural Crest Progenitors

Fertile White Leghorn eggs (Bilbie Aviaries, Ann Arbor, MI) were incubated to Hamburger and Hamilton stage 17–18 (Hamburger and Hamilton, 1951) and injected with sciatic nerve or gut NCSCs. Approximately 30,000 gut NCSCs were isolated by flow-cytometry, backloaded into a drawn glass capillary tube (World Precision Instruments, Sarasota, FL), centrifuged for 2 min at 52 × g, and injected into the anterior, medial corner of one somite in the hindlimb bud region of each embryo. To avoid any underestimation of neuronal engraftment by sciatic nerve NCSCs, 85,000 sciatic nerve cells were loaded into glass capillary tubes and injected into two hindlimb bud somites per chick. Injections were performed using a Narishige H-7 pipette holder mounted on a MM-33 micromanipulator (Fine Science Tools, Forester City, CA) and very gentle air pressure. By labeling injected cells with DiI and then sectioning through somites immediately after injection, we counted an average of 1410 ± 670 cells injected per somite. Remaining details are as previously described (White and Anderson, 1999; White et al., 2001). Injected embryos were incubated for an additional 3 days to stage 29, fixed, embedded in OCT, and cryostat sectioned. In situ hybridization methods were adapted from prior protocols (White and Anderson, 1999; Barthel and Raymond, 1993). To control for nonspecific hybridization, sections from E16.5 rat and uninjected stage 29 chicks were processed in parallel as positive and negative controls, and sections adjacent to those showing engraftment were probed with the sense strands of *SCG10* and *P_0*. Since embryos were unilaterally injected, the contralateral side to the injection also served as a negative control.

Acknowledgments

This work was supported by the National Institutes of Health (R01 NS40750-01), the Searle Scholars Program, and the Howard Hughes Medical Institute. J.T.M. was supported by a fellowship from the University of Michigan Cancer Biology Training Program. Thanks to Mark Kukuruga, Anne Marie Deslaurier, Mark Kiel, and the University of Michigan Flow-Cytometry Core Facility for flow-cytometry. Flow-cytometry was supported in part by the UM-Comprehensive Cancer NIH CA46592 and the UM-Multipurpose Arthritis Center NIH AR20557. Thanks to Elizabeth Smith in the Hybridoma Core Facility for antibody production, which was supported in part through the Michigan Diabetes Research and Training Center (NIH5P60-DK20572) and the Rheumatic Core Disease Center (1 P30 AR48310). Thanks to Chris Edwards, Elizabeth Horn, Mark Deming, and Thom Saunders for microscopy and technical assistance. Thanks to David Anderson, Gerry Weinmaster, Greg Lemke, Bruce Rutherford, and Kate Barald for providing primers and plasmids. Thanks to Jack Parent, Rolf Bodmer, Theo Ross, and David Anderson for helpful comments.

Received: March 20, 2002
Revised: June 13, 2002

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