Development 126, 4351-4363 (1999)
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DEV3978

In vivo transplantation of mammalian neural crest cells into chick hosts reveals a new autonomic sublineage restriction

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Accepted 9 July; published on WWW 7 September 1999

SUMMARY

The study of mammalian neural crest development has been limited by the lack of an accessible system for in vivo transplantation of these cells. We have developed a novel transplantation system to study lineage restriction in the rodent neural crest. Migratory rat neural crest cells (NCCs), transplanted into chicken embryos, can differentiate into sensory, sympathetic, and parasympathetic neurons, as shown by the expression of neuronal subtype-specific and pan-neuronal markers, as well as into Schwann cells and satellite glia. In contrast, an immunopurified population of enteric neural precursors (ENPs) from the fetal gut can also generate neurons in all of these ganglia, but only expresses appropriate neuronal subtype markers in Remak's and associated pelvic parasympathetic ganglia. ENPs also appear restricted in the kinds of glia they can generate in comparison to NCCs. Thus ENPs have parasympathetic and presumably enteric capacities, but not sympathetic or sensory capacities. These results identify a new autonomic lineage restriction in the neural crest, and suggest that this restriction preceeds the choice between neuronal and glial fates.

Key words: Neural crest cells, Enteric neural precursors, Glia, Remak's ganglion, Cell fate, Cell transplantation, Rat

INTRODUCTION

Multipotent neural precursors utilize several strategies to generate different classes of neurons (Edlund and Jessell, 1999). In the developing cortex, stem cells undergo sequential symmetric and asymmetric divisions to produce a series of progenitors committed to different laminar fates (reviewed by Temple and Qian, 1996). In the retina (Cepko, 1999) and spinal cord (Tanabe and Jessell, 1996), postmitotic precursors can generate different types of neurons in response to signals that change over time and space, respectively. In the neural crest, pluripotent stem cells are thought to give rise to different classes of neurons via proliferating progenitor cells with restricted developmental capacities, in a manner analagous to hematopoeisis (Anderson, 1997; Sieber-Blum, 1997; Dupin et al., 1998). Defining these lineage restrictions is important for understanding the actions of genes that control neural crest development.

The issue of when the sensory, sympathetic, parasympathetic and enteric neurogenic sublineages segregate from the neural crest remains controversial. In avian systems, clonal lineage analyses in vivo (Bronner-Fraser and Fraser, 1988; Fraser and Bronner-Fraser, 1991) and in vitro (Baroffio et al., 1988; Sieber-Blum, 1989) have indicated that some neural crest cells can generate both sensory and sympathetic autonomic neurons; however, whether the same cell can generate sensory, sympathetic and enteric neurons has not been determined. Nevertheless, back-grafting of gut tissue fragments containing post-migratory crest cells into the neural crest of earlier host embryos led to colonization of sensory and sympathetic, as well as of enteric, ganglia by graft-derived cells (Rothman et al., 1990; Rothman et al., 1993b). These observations suggested that neural crest-derived cells in the gut, at least as a population, are multipotent and not restricted to an enteric fate.

Previously, we have approached the problem of lineage segregation in the rodent neural crest by analyzing the developmental capacities of antigenically homogeneous populations of crest cells in clonal culture (Stemple and Anderson, 1992; Lo and Anderson, 1995; Shah et al., 1996; Morrison et al., 1999). It would be desirable to challenge these different populations by transplantation in vivo, where the cells should encounter all the appropriate environmental signals necessary for the differentiation of various neuronal subtypes. This approach has been limited in rodents by the lack of an accessible host system. Trans-uterine grafting into rodent hosts is possible but technically difficult, and so far only melanocyte differentiation has been reported (Jaenisch, 1985; Huszar et al., 1991).

To overcome these limitations, we recently developed a system for transplanting rat neural crest cells into chick embryo hosts in ovo (Morrison et al., 1999). Several recent studies have demonstrated the feasibility of using mouse-chick chimeras to analyze the cell-autonomy of murine neural crest migration mutants (Rothman et al., 1993a; Serbedzija and McMahon, 1997). However, this approach has not yet been used to analyze lineage restrictions within wild-type neural crest populations. Indeed, although murine crest cells have been shown to

colonize the peripheral ganglia and nerves of avian hosts (Fontaine-Perus et al., 1997), differentiation markers have not yet been applied to determine whether murine cells differentiate appropriately in these locations.

We have now compared the differentiation of grafted post-migratory enteric neuronal precursors (ENPs) isolated from E14.5 fetal rat gut (Lo and Anderson, 1995), to that of migrating neural crest cells (NCCs) isolated from E10.5 rat neural tube explants (Stemple and Anderson, 1992), in chick hosts using an extensive panel of species-specific lineage markers. NCCs can differentiate into sensory, sympathetic, and parasympathetic neurons, as shown by the expression of neuronal subtype-specific and pan-neuronal markers, as well as into Schwann cells and satellite glia. In contrast, ENPs can also generate neurons in all of these ganglia, but only express appropriate neuronal subtype markers in Remak's and associated pelvic parasympathetic ganglia. ENPs also appear restricted in the kinds of glia they can generate in comparison to NCCs. Thus ENPs have parasympathetic and presumably enteric capacities, but not sympathetic or sensory capacities. These results identify a new autonomic lineage restriction in the neural crest, and suggest that this restriction preceeds the choice between neuronal and glial fates.

MATERIALS AND METHODS

Isolation of rat precursor cells

NCCs were obtained as previously described. (Shah et al., 1994) Briefly, female Sprague-Dawley rats (Simonson Labs) were killed at 10.5 days post coitum. Pieces of embryonic trunk just posterior to the heart and 10-12 somites in length were cut, digested in 1 mg/ml dispase (Gibco 17105-041) and 1 mg/ml collagenase (Wortington 4196) for 10 minutes at 4°C, and triturated to isolate neural tubes. Tubes were cultured on fibronectin-coated tissue culture dishes to allow neural crest to emigrate. See Stemple and Anderson (1992), for medium formulation; medium contained 15% chicken embryo extract and no retinoic acid. After 24 hours in culture, the neural tubes were scraped away, and neural crest cells were collected with a 10 minute EDTA incubation.

ENPs were obtained as previously described (Lo and Anderson, 1995). Female Sprague-Dawley rats were killed at 14.5 days post coitum. The stomach, small intestine, and hindgut were carefully removed from each embryo and dissociated with 1.5 mg/ml elastase (Sigma E-0127) and 1 mg/ml collagenase for 20 minutes at 37°C. Cells were stained with B2 antibody supernatant, then FITC-conjugated goat anti-mouse IgM (Jackson Labs), then the hamster anti-c-RET antibody cocktail, and finally with phycoerythrin-conjugated goat anti-mouse IgG (H+L) (Jackson Labs). All four incubations were done for 30 minutes at 4°C, and included 50 μg/ml DNAse I (Sigma DN-25). The c-RET$^+$ B2$^-$ population was positively sorted on a Beckman Vantage.

Analysis of marker expression in grafted cells at the time of injection into host tissue

To verify that grafted cells did not express differentiation markers prior to transplantation, we plated 200-400 donor cells and fixed them after 2 hours, in parallel with injections of cells into host embryos. Positive control cultures of superior cervical ganglia and dorsal root ganglia, both from newborn rats, were processed in parallel with experimental samples. mRNA for the following markers were not detected in NCCs or ENPs: P_0, GATA-2, tyrosine hydroxylase, and Brn3.0. In addition, NCCs did not express SCG10; however, between 2 and 4% of ENPs did express SCG10 mRNA in each experiment. Neither NCCs or ENPs expressed detectable levels of B2 epitope. Between 90 and 100% of both ENPs and NCCs expressed p75^{LNTR} surface protein in each experiment.

Grafting of cell suspensions

Grafting protocols were identical to those of Bronner-Fraser and Cohen (1980). Fertile white Leghorn eggs were incubated to Hamburger and Hamilton stage 18 (Hamburger and Hamilton, 1951). A small bolus of 10% india ink in Ca^{2+}- and Mg^{2+}-free Tyrode's salts was injected under the blastoderm to visualize the embryo. Cells, backfilled into drawn capillary tubes, were injected into the anterior, medial corner of one or two somites of each embryo, using an MM33 micromanipulator (Fine Science Tools) and very gentle air pressure. Manipulated embryos were incubated for an additional 3 days to stage 29, and fixed for analysis.

In preliminary experiments, some animals were fixed without incubation and processed for marker expression with incubated animals. From these controls we estimated that a typical injection consisted of 400 to 800 cells. 75-85% of injected animals had detectable rat cells 3 days post surgery (see Table 1).

Preparation of embryos for analysis

Embryos were fixed by immersion in fresh, ice cold, 4% paraformaldehyde in phosphate buffer for at least 16 hours, sunk in 15% sucrose, embedded in OCT, and stored frozen at –80°C. Embedded samples were sectioned at 15 μm. Normal rat and chick embryos used for controls were processed in parallel.

In situ hybridization of sections and cultured cells

Antisense probes for rat mRNA were synthesized with digoxigenin-conjugated nucleotides, and antisense probes for chicken mRNA sequences were synthesized with fluorescein-conjugated nucleotides. Detailed protocols are available upon request. Briefly, samples were post-fixed, digested with proteinase K, and acetylated. Samples were then pre-hybridized for 1-3 hours at 65°C and hybridized with 1 μg/ml probe overnight at 65°C. Three high-stringency washes with 0.2× SSC were done at 65°C. Samples were incubated with 20% sheep serum, and then pre-absorbed alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody in 20% sheep serum, both for 1 hour at room temperature. Samples were developed with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoly phosphate (BCIP). In cases where rodent probes cross react with chicken sequences, a fluorescein-conjugated probe for the chicken gene was included in the hybridization step. Then, after NBT/BCIP development, the digoxigenin-conjugated AP was inactivated by heating to 85°C; the slides were incubated in AP-conjugated anti-fluorescein antibody, and developed with 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl tetrazolium chloride (INT) and BCIP, which yields a reddish product. This combination provided an accurate distinction between graft and host, because chick probes effectively competed for cross-hybridization between rodent and avian target sequences.

For non-radioactive in situ analysis of cultured cells, 2 hour cultures were fixed in 4% paraformaldehyde, acetylated, permeabilized with 0.2 N HCl, and prehybridized for 1 hour at room temperature. Hybridizations with digoxigenin-labeled rat probes were done at 65°C, and afterwards developed in an identical manner to the sections. Double labelings were not performed on cultured cells.

Table 1. Number of chimerae used in this analysis

Cell type	Number of cell preparations	Number of grafted embryos	Survivors 3 days post-surgery	Animals analyzed	Animals with grafted rat cells
NCC	6	71	58	45	38
ENP	6	85	57	47	36

Antibodies in analysis

The monoclonal mouse IgM B2 (Anderson and Axel, 1986) was revealed with peroxidase-conjugated secondary antibody (Jackson Labs) by developing with diaminobenzidine, intensified with nickelous sulfate.

RESULTS

NCCs can colonize chicken peripheral ganglia, and generate neurons and glia in appropriate locations

NCCs obtained from 24-hour explants of E10.5 rat neural tubes (Fig. 1A) were injected as dissociated cell suspensions into the somites of stage 18 chicken embryos, following procedures previously established for transplantation of avian neural crest cells (Bronner-Fraser and Cohen, 1980), and analyzed 3 days later at stage 29. We found transplanted cells located correctly in neural crest-derived structures, but the dorsal-ventral position of the grafted cells differed according to the stage of somite development at the time of grafting. Cells grafted into early somites at the epithelial ball stage (hindlimb level at stage 18), at the beginning of host crest migration, populated ventral derivatives such as the sympathetic ganglion, aortic plexus, and the peripheral nerves, similar to their host counterparts (Fig. 1C,E) (Serbedzija et al., 1989). In contrast, cells grafted into somites that had already dissociated into dermomyotomal and sclerotomal compartments (forelimb level at stage 18) primarily populated the dorsal root ganglion (DRG), as well as the peripheral nerve, and less frequently the sympathetic ganglion (Fig. 1C,D). Cells grafted into the sacral region contributed to Remak's ganglion in the hindgut and pelvic parasympathetic plexus, as do host crest cells from this region (Fig. 1E) (Pomeranz et al., 1991; Serbedzija et al., 1991; Burns and Le Douarin, 1998). These results are very similar to what has been published for avian donor cells (Bronner-Fraser and Cohen, 1980; Erickson et al., 1980; Rothman et al., 1993b; Sharma et al., 1995)

Initially, we asked whether NCCs could differentiate into both neurons and glia in dorsal root and sympathetic ganglia, and into glia in the peripheral nerve. Graft-derived rat neurons, defined by expression of the pan-neuronal marker SCG10, were found associated with both the DRG (Fig. 2A, purple, arrows) and the SG (Fig. 2B, purple, arrows) of animals engrafted with NCCs. The rat neurons were co-localized with host neurons, as visualized by double-labeling with a chick-specific SCG10 probe (Fig. 2A,B, red, arrowheads), although in the case of the DRG they tended to be at the perimeter of the ganglion. Graft-derived cells expressing the myelin gene P_0 were also detected in both DRG (Fig. 2D) and SG (Fig. 2E) of NCC-engrafted embryos. P_0^+ cells in the DRG were frequently seen associated with the capsule of the ganglion, in the dorsal root entry zone, and occasionally within the DRG itself. The location of these cells, taken together with their expression of P_0, is consistent with their differentiation to a satellite glial cell phenotype. In the peripheral nerve, elongated P_0^+ cells were observed in association with host axons (Fig. 2F), identified both by morphology (Fig. 2C,F) and by neurofilament immunohistochemistry on an adjacent section (data not shown). Ectopic rat-derived $SCG10^+$ somata in the peripheral nerve were rare (Fig. 2C). Quantification (Fig. 2G) indicated that comparable numbers of neurons and glia were detected in the DRG, but that glia were found much less frequently than neurons in the SG. In the peripheral nerve, primarily glia were detected.

NCCs can generate sensory neurons in the chick DRG

To determine whether NCC-derived neurons in chick DRG express a sensory phenotype, we used Brn3.0 RNA as a marker (Table 2). This gene is expressed early in rat DRG development, and persists to at least E17.5 (Fedtsova and Turner, 1995; and data not shown). Brn3.0 is not detectably

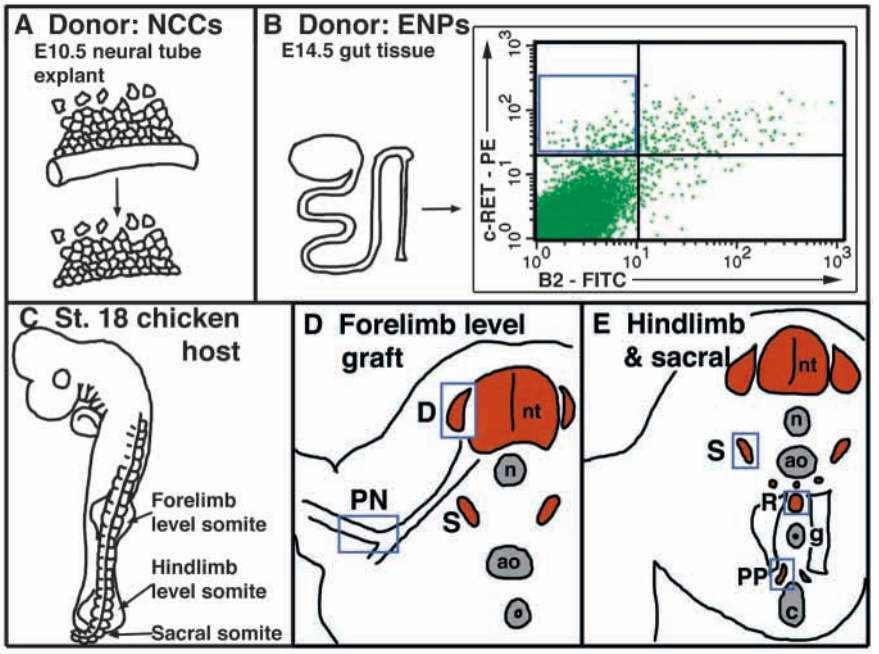

Fig. 1. Grafting and analysis diagram. (A) NCCs are obtained from explanted rat neural tubes after 24 hours of culture. See Methods for details. (B) Neural crest-derived ENPs are purified from both endodermal cells and from already differentiated neurons by sorting positively for c-RET and negatively for B2 (blue boxed region). (C) Donor cells are injected into somites at three distinct axial levels in stage 18 chicken hosts (72 hours incubation). (D) Analysis of forelimb level grafts. Forelimb level grafts contribute to more dorsal structures including the dorsal root ganglion, D, and peripheral nerve, PN, and less frequently to the sympathetic ganglion, S. (E) Analysis of hindlimb and sacral level grafts. Hindlimb level grafts contribute to more ventral structures like sympathetic ganglia, S, in addition to peripheral nerve (not shown). Sacral level grafts contribute to Remak's ganglion, R, of the hindgut; pelvic plexus, PP, by the cloaca; and also sometimes to sympathetic ganglia. In some sacral grafts, nearly all of the pelvic plexus neurons are rat-derived. Micrographs in this report are from the blue boxed regions in D and E. Landmarks: nt, neural tube; n, notochord; ao, aorta; g, gut; c, cloaca. Non-neuronal landmarks are gray; neuronal landmarks are brown.

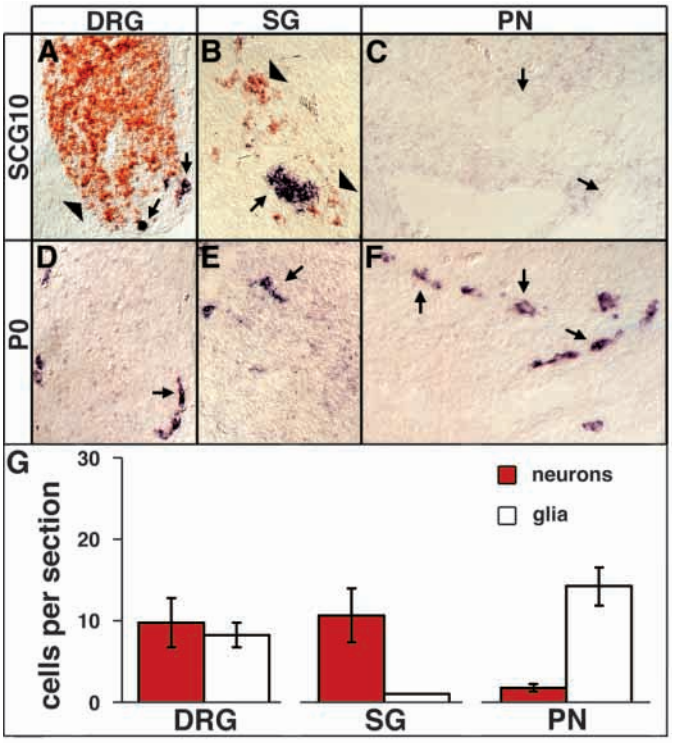

Fig. 2. Rat NCCs contribute neurons and glia to dorsal root and sympathetic ganglia, and glia to peripheral nerve. (A-C), SCG10 (neuron) two color in situ hybridization; (D-F), P_0 (glia) single color in situ hybridization. Rat-specific staining is purple (arrows); chick-specific staining is red (arrowheads). (A,D) Nearby sections through the dorsal root ganglion of a forelimb-level NCC chimera, showing that donor cells contribute both neurons (A) and glia (D) to this structure. (B,E) Nearby sections through the sympathetic ganglion of a hindlimb-level chimera, showing rat-derived neurons (B) and glia (E). (C,F) Adjacent sections through the peripheral nerve of a forelimb level chimera, showing that glia, not neuronal somata, are normally detected. (G) Quantification of donor neurons and glia per section (n=19 animals). For both DRG and SG, grafts typically extend 60-120 μm in axial length (neurons and glia found on 4-8 sections); for PN, grafted cells are found in a region extending 600 ± 120 μm in axial length, or an average of 40 sections.

expressed in NCCs prior to transplantation (data not shown). In NCC-derived grafts located in the DRG, however, 82% of animals showing rat neurons in the DRG also had rat Brn3.0$^+$ cells in adjacent sections, located in the same region (Fig. 3A, B, and Table 3). On average, the numbers of Brn3.0$^+$ cells and SCG10$^+$ cells in engrafted DRG on adjacent sections were similar, although Brn-3.0$^+$ cells were somewhat less frequent. These data suggest that many of the transplanted cells that differentiate as neurons in the DRG express Brn3.0. Our assay for neuronal subtype is indirect, because for technical reasons it was necessary to analyze individual markers on adjacent serial sections. However, the position of these markers in the same area of the sections makes it likely that many individual rat SCG10$^+$ cells coexpress Brn3.0.

As Brn3.0 is also expressed in a subset of dorsal interneurons of the neural tubes from which NCCs are isolated (Fedtsova and Turner, 1995; and data not shown), it was important to exclude the possibility that the rat Brn3.0$^+$ cells represented contaminating neural tube cells rather than sensory neurons. We therefore hybridized nearby sections with a BarH4.1 probe. BarH4.1 is expressed in the same region as Brn3.0 in the rat dorsal neural tube, but in contrast to Brn3.0, it is not expressed in DRG (Table 1; Saito et al., 1998). In all seven animals examined, BarH4.1 expression was not detected on sections adjacent to those containing Brn3.0$^+$ grafts in the DRG (Fig. 3C), although these sections still contained graft-derived cells as shown by expression of SCG10 on a subsequent section (Fig. 3C, inset). In control experiments, both Brn3.0 and BarH4.1 were easily detected on adjacent sections through fragments of E10.5 rat dorsal neural tubes grafted subectodermally into stage 18 embryos (Fig. 3D-F). Furthermore, in other control experiments, BarH4.1 was also readily detected in DRG of chick embryos engrafted with dissociated rat neural tube cells (data not shown). These data indicate that the presence of Brn3.0, combined with the absence of BarH4.1, permits identification of rat-derived sensory neurons in engrafted chick DRG. Moreover, they indicate that such neurons can readily differentiate in this location from transplanted NCCs (Table 3).

NCCs can generate sympathetic neurons in vivo

The presence of graft-derived neurons in the sympathetic ganglia and aortic plexii of NCC chimerae (Fig. 2B,E) prompted us to examine such chimerae for expression of markers consistent with a sympathetic fate. In both rat and chicken embryos, the transcription factor GATA-2 and the enzyme tyrosine hydroxylase (TH) are expressed in these structures soon after ventral crest migration is completed

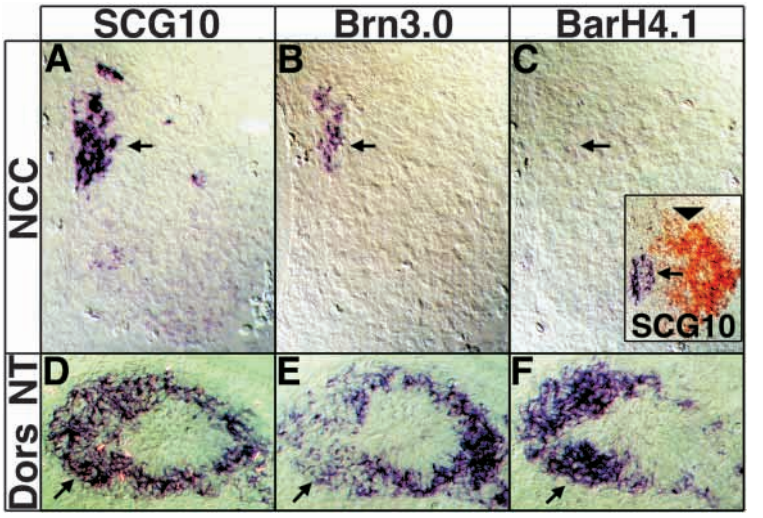

Fig. 3. NCCs express the sensory specific marker Brn3.0 in the DRG, and not the neural tube specific marker BarH4.1. (A-C and inset) Sequential sections through the DRG of an NCC chimera. (D-F), sequential sections through a fragment of rat dorsal neural tube implanted subectodermally in a chick host. Both DRG neurons (A) and dorsal neural tube neurons (D) express Brn3.0 (B and E, respectively), but dorsal neural tube neurons express BarH4.1 (F) and DRG neurons do not (C, arrow). Rat neurons can be detected on a subsequent section (inset, purple), indicating donor-derived neurons are likely to be present on the section shown in C. For quantification, see Table 3.

Table 2. Specificity of markers used in this analysis

Marker name	Nature of determinant	Expression in the midgestational rat nervous system					
		Sensory neurons[a]	Dorsal neural tube neurons[a]	Sympathetic neurons[b]	Parasym-pathetic neurons[c]	Enteric neurons[d]	Glia[e]
SCG10	Growth associated protein	+	+	+	+	+	–
P0	Myelin gene	–	–	–	–	–	+
Brn3.0	POU-class transcription factor	+	+	–	–	–	–
BarH4.1	bHLH transcription factor	–	+	–	–	–	–
B2	Surface carbohydrate epitope	–	–	+	+ (subset?)	+ (subset)	–
GATA-2	Zinc-finger transcription factor	–	–	+	+*	–	–
TH	Transmitter enzyme	–	–	+	–**	–	–
ChAT	Transmitter enzyme	–	–	–	+/–‡	+/–‡	–

Most of these data have already been published by others. We independently verified these expression patterns on E13.5, E14.5 and E17.5 rat embryos. Specific areas examined:

[a]Ganglia and neurons at the forelimb level.

[b]Ganglia at the hindlimb level.

[c]Pelvic ganglion (inferior hypogastric plexus).

[d]Stomach, small intestine, and large intestine.

[e]Both forelimb and hindlimb level nerves. It should be noted that in the caudad hindlimb, SCG10+ cells are occasionally detected in the peripheral nerves near the pelvic ganglion.

*GATA-2 is expressed in the ganglion as well as in the surrounding tissue. A similar expression pattern was seen in the E14.5 sphenopalatine ganglion

**By E17.5, a few TH+ neurons are detected.

‡Weak ChAT staining is observed at E14.5 and E17.5.

(Groves et al., 1995), and in the rat, both markers persist in sympathetic structures to at least E17.5 (data not shown).

In NCC chimerae, 100% of animals with rat SCG10$^+$ cells in either the sympathetic ganglion or the aortic plexus also contained rat GATA-2$^+$ cells in these structures (Fig. 4A,B and Table 4). Double-labeling with chick (red, arrowheads) and rat (purple, arrows)-specific probes for these two markers revealed a close intermingling of the donor rodent and host avian cells. Rat-specific expression of TH was also detected (Fig. 4C, arrow), although only in 50% of animals with SCG10$^+$ cells in this region (Table 4). The frequencies of SCG10$^+$ and GATA-2$^+$ cells on adjacent sections were indistinguishable, whereas the frequency of TH$^+$ cells was somewhat lower (Table 4). These data are consistent with the idea that at least a subset of cells in the NCC population is competent to make sympathetic neurons in vivo.

NCCs generate parasympathetic neurons in the chick pelvic plexus

Unlike DRG and SG, the locations of parasympathetic neurons vary between avian and rodent species. We chose Remak's ganglion and the chick pelvic plexus to analyze the parasympathetic capacity of grafted cells. Remak's ganglion, a structure unique to avians, forms dorsal to its target, the caudal bowel (Suzuki et al., 1996). While most investigators consider Remak's ganglion a component of the enteric nervous system (see Burns and Le Douarin, 1998), this ganglion, together with the chick pelvic plexus, may be the avian counterpart of the rodent pelvic ganglion. In rodents, the pelvic ganglion innervates the reproductive organs, caudal bowel, and bladder (Keast et al., 1989). Neurons in the chick pelvic plexus and Remak's ganglion, and neurons in the rat pelvic ganglion, all express GATA-2 at early stages as well as the autonomic marker Phox2b (data not shown), and are cholinergic (Le Douarin et al., 1978). Thus, rat pelvic ganglion neurons have phenotypic similarities to avian pelvic neurons. These parasympathetic neurons can be distinguished from enteric neurons, which do not express GATA-2 (Groves et al., 1995), and from sympathetic neurons which do not express ChAT (although they do express GATA-2; Table 1). In addition, parasympathetic neurons can be distinguished from sympathetic neurons by a lack of TH expression.

Table 3. DRG neuronal phenotypes in NCC and ENP chimerae

Cell type	Number of cell preparations	Number of animals with rat cells	SCG10 in the DRG		Brn3.0 in the DRG		B2 in the DRG	
			No. of animals	Cells per section	No. of animals	Cells per section	No. of animals	Area[a] per section
NCC	2	15	11	19.1±4.1	9	13.0±3.6	0	0
ENP	4	16	10	17.8±6.4	2[b]	0.3	9	15.2±5.0

Quantification of neurons, and cells expressing neuronal subtype markers, on adjacent sections through the DRG region of NCC- and ENP-engrafted animals; cells per section is ± s.e.m. Both kinds of grafts generate equivalent numbers of neurons in the DRG. However, NCC-grafts express sensory (Brn3.0) and not autonomic (B2) markers, whereas ENP-grafts consist almost entirely of autonomic neurons.

[a]The area of B2 staining was measured on 40× micrographs of stained sections, and is normalized to the area of SCG10$^+$ cells from the same grafts. Area was used instead of cell number as the DAB reaction product obscured cell morphology in larger grafts.

[b]Both of these ENP-chimerae had only one Brn3.0$^+$ cell each in a large DRG graft. The probability that ENPs have the same capacity to produce Brn3.0$^+$ cells, and that only 2 Brn3.0$^+$ grafts were obtained by chance, is extremely low (P=0.00004).

Table 4. Sympathetic neuronal phenotypes in NCC and ENP chimerae

Cell type	Number of cell preparations	Number of animals with rat cells	SCG10 in the SG&AP		GATA-2 in the SG&AP		TH in the SG&AP	
			No. of animals	Cells per section	No. of animals	Cells per section	No. of animals	Area[a] per section
NCC	3	19	8	6.7±1.2	8	5.6±2.7	4	2.8±1.2
ENP	3	21	11	14.2±2.9	1[a]	0.2	1[b]	0.5

Quantification of neurons, and cells expressing sympathetic-specific markers, on adjacent sections through the sympathetic ganglia (SG) and aortic plexii (AP) of NCC- and ENP-engrafted animals; cells per section is ± s.e.m. SCG10, GATA-2, and TH are normally expressed by sympathetic neurons in both rats and chickens. Both NCCs and ENPs can generate neurons in these locations. Although ENP-derived neurons are twice as abundant as NCC-derived neurons, only NCC-derived cells also express sympathetic-specific (GATA-2, TH) markers.

[a]One GATA-2^+ cell was detected in a large graft.

[b]One TH^+ cell was detected in a large graft. The probability that ENPs have the same capacity to generate TH^+ cells as do NCCs, but that by chance only 1 TH^+ graft was obtained in a sample of 11, is very low (P=0.02).

Sacrally grafted NCCs migrated to both Remak's ganglion and the pelvic plexus, and differentiated as neurons (Fig. 5A,G) in 3 of 3 animals. Double labeling with rat- (purple, arrows) and chick- (red, arrowheads) specific probes revealed a close intermingling of graft and host $SCG10^+$ cells in Remak's ganglion. In adjacent sections, GATA-2 (Fig. 5B) and B2 (Fig. 5C) were detected. All three markers were also detected in sections through the pelvic plexus (Fig. 5G-I), as was Phox2b (data not shown). Rat-specific ChAT staining, weak but similar in intensity to that seen in control sections though normal rat pelvic ganglia, was also detected in the chick pelvic plexus (Fig. 5J, compare to Fig. 5R). Additional sections were hybridized for the sympathetic marker TH and the sensory marker Brn3.0, and were found to be negative (data not shown). As this marker profile ($SCG10^+$, GATA-2^+, $B2^+$, $ChAT^+$, TH^-) is identical to that of the normal E14.5 rat pelvic ganglion (Fig. 5O-R), we conclude that at least some of the rat neurons localized in the chick pelvic plexus are likely to be expressing a parasympathetic phenotype.

We did not observe rat neurons in either the myenteric or submucosal plexii of the post-umbilical bowel of embryos injected in the sacral region. However, new fate-mapping data indicate that, in contrast to previous reports (Pomeranz et al., 1991; Serbedzija et al., 1991), sacral crest-derived neurons are only detectable in these ganglia after stage 31 (E7.5) (Burns and Le Douarin, 1998). Incubation of chimerae to such late stages is complicated by decreased viability of the embryos. Experiments to determine whether sacrally grafted NCCs will contribute to the myenteric plexii are ongoing.

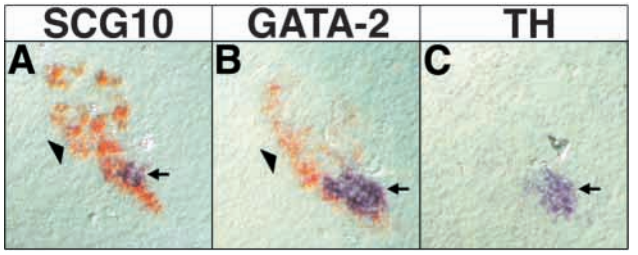

Fig. 4. NCCs express sympathetic neuron markers in the SG. Sequential sections from a hindlimb-level NCC chimera. Rat neurons (A, purple, arrow) intermingle with chick neurons (A, red, arrowhead) in the sympathetic ganglion. (B) In the next section, rat-specific GATA-2^+ neurons (purple, arrow) amid chick GATA-2^+ neurons (red, arrowhead), also in the sympathetic ganglion. (C) Rat-specific TH is detected on the next section. Double-labeling with a chick-specific probe was not performed in C. For quantification, see Table 4.

We attempted to transplant both NCCs and rat vagal neural crest cells into the chick vagal crest (4th somite) of stage 10-12 embryos. Of 23 chimerae generated, only one contained any rat $SCG10^+$ cells in the myenteric plexus, in the crop region. Although the graft contained rat c-RET^+ cells in this region as well, other expected autonomic markers, such as Mash-1, Phox2b, and B2, were not detected (data not shown). Thus, although these graft-derived neurons occupied the appropriate position for myenteric neurons, their failure to express the full panel of markers for an enteric phenotype precludes their definitive identification.

In summary, the foregoing data indicate that rat NCCs are able to migrate to the peripheral nerves, to the dorsal root, sympathetic, and Remak's ganglia, and to the pelvic plexus after engraftment in chicken hosts. In the peripheral nerves, P_0^+ glial cells, presumably Schwann cells, are primarily detected, not neurons. In the dorsal root ganglia, both neurons and glia differentiate. The expression of Brn3.0 on adjacent sections is consistent with a sensory phenotype, and the location of the glial cells in the DRG is consistent with satellite cell or boundary cap glial differentiation. In the sympathetic ganglia and aortic plexii, both neurons and glia are detected, as are sympathetic neuron-specific markers. Finally, in Remak's ganglion and in the pelvic plexus, we detected rat neurons and markers consistent with parasympathetic differentiation. None of the phenotypic markers was detectably expressed in NCCs prior to transplantation, indicating that their expression in vivo reflects de novo differentiation of the engrafted cells in the chick host. We conclude that NCCs as a population contain cells competent to generate sensory, sympathetic, and parasympathetic neurons and also Schwann cells and satellite (or boundary cap) glial cells. These data do not distinguish, however, whether individual NCCs possess the ability to make multiple classes of neurons or glia.

ENPs can generate parasympathetic neurons in the chick pelvic plexus

We next sought to determine the types of neurons that ENPs, immunoisolated from E14.5 fetal rat gut tissue by combined positive selection with anti-c-RET and negative selection with B2 (Fig. 1B), are able to generate in this assay. The results

obtained from NCC grafts served as a positive control, allowing us to compare the degree of developmental restriction of the two transplanted populations. To determine whether grafted ENPs can express a parasympathetic phenotype, we first assessed their differentiation in sacral grafts. Among 4 chimerae receiving sacral injections of ENPs, all had rat neurons intermingled with chick neurons in Remak's ganglion (Fig. 5D). Adjacent sections tested for the presence of GATA-2 and B2 revealed that these markers were also expressed (Fig. 5E,F). Expression of rat SCG10, GATA-2 and B2 was also seen in the pelvic plexus near the cloaca (Fig. 5K-M). Moreover, ChAT mRNA expression was seen in this region as well (Fig. 5N). Although the level of rat ChAT mRNA expression was weak, it coincided with the location of rat SCG10$^+$ and GATA-2$^+$ cells on nearby sections, and was similar in intensity to the labeling seen on control sections through rat pelvic ganglia. Additional sections in the grafts were tested for the expression of TH and Brn3.0 mRNAs, and were found to be negative (data not shown). These data reveal that ENPs can express a similar set of parasympathetic markers as NCCs when grafted to the sacral neural crest.

Although ENPs are isolated from the gut where they normally differentiate to enteric neurons, we were unable to demonstrate their differentiation to this phenotype in chick hosts. Two of the sacrally grafted ENP chimerae survived incubation to stage 35, when endogenous sacral crest derived neurons are differentiating (Burns and Le Douarin, 1998). Neither chimera showed evidence of rat-derived enteric neurons in the hindgut myenteric plexus, although both contained many Remak's ganglion and pelvic plexus neurons. Of 7 vagally grafted ENP chimerae, only one showed a few rat neurons in the myenteric plexus, and no autonomic markers (including c-RET) were detected. Thus, as was the case for NCCs, extensive engraftment of the host gut by ENPs was not achieved.

ENPs can engraft in other locations appropriate for neural crest and generate both neurons and glia

Next we injected ENPs into forelimb level and hindlimb level somites (Fig. 1C) of stage 18 chicken embryos to test if ENPs are able to make neurons and glia in trunk sensory and sympathetic ganglia. Graft-derived neurons are readily observed in dorsal root (Fig. 6A, purple, arrows) and sympathetic (Fig. 6B purple, arrows) ganglia. Glia, as indicated by P_0 staining, are frequently observed in the dorsal root entry zone (Fig. 6D), which is consistent with an interpretation of a boundary cap phenotype. Unlike NCC chimerae, in ENP chimerae P_0^+ cells were not observed in the capsule of the DRG, nor were they seen within the DRG proper. P_0^+ cells were also occasionally observed in the sympathetic ganglia (Fig. 6E). Surprisingly, in the peripheral nerves, P_0^+ cells were almost completely absent (Fig. 6F), whereas ectopic SCG10$^+$ neuronal somata were readily detected (Fig. 6C). In some animals, we also tested adjacent sections for expression of rat erbB3, a subunit of the GGF2/NRG1 receptor expressed by Schwann cell precursors in rat embryos throughout gestation, and found that it too was almost completely absent from peripheral nerve (data not shown). Quantification (Fig. 6G) showed that the frequencies of neurons and glia observed in the DRG of ENP chimerae were equivalent, and that the frequency of neurons was greater than that of glia in the sympathetic ganglia. These numbers are roughly similar to those seen with NCC chimerae (cf. Fig. 2G).

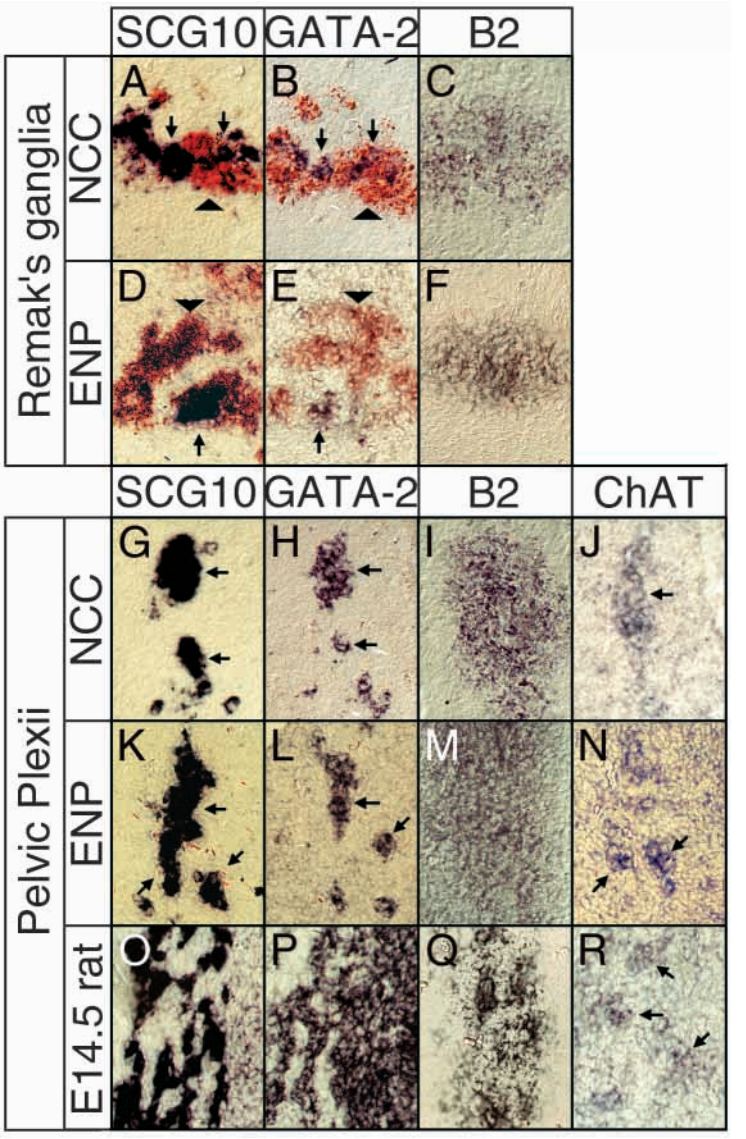

Fig. 5. Parasympathetic-like differentiation of NCCs and ENPs in Remak's ganglion and the chick pelvic plexis. (A-C,G-J) NCC chimera sections; (D-F, K-N) ENP chimera sections; (O-R) control sections from the pelvic ganglion of an E14.5 rat fetus. Both the NCC chimera and the ENP chimera were injected into sacral level somites. Rat neurons (A,D; purple, arrows) are detected amid host neurons (red, arrowheads) in Remak's ganglion within the post-umbilical bowel. The rat-specific markers GATA-2 (B,E; purple, arrows), and B2 (C,F) are also detected. No counterstaining for a chick marker was performed in C and F. More ventrally, both NCCs and ENPs generate neurons in the chick pelvic plexis (G,K) and the rat-specific markers GATA-2 (H,L; arrows), B2 (I, M), and ChAT (J,N; arrows) are detected. In these chimerae, most of the pelvic plexus neurons detected were rat-derived. ChAT staining in the chimerae is weak, but comparable in intensity to that in E14.5 rat pelvic plexus (R, arrows). TH and Brn3.0 were absent on additional sections (data not shown) although rat SCG10$^+$ cells were present both rostrad and caudad to those negative sections.

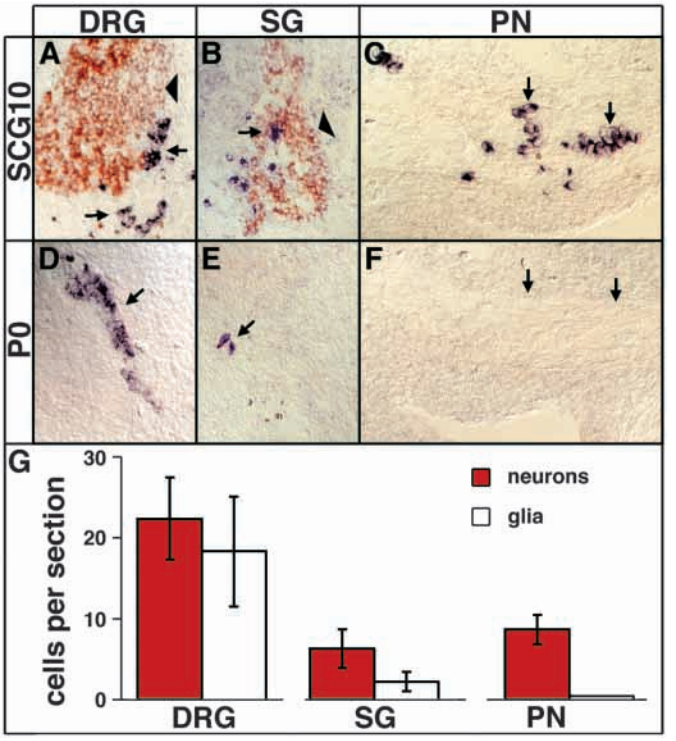

Fig. 6. ENPs contribute neurons and glia to the dorsal root and sympathetic ganglia, and ectopic neurons to the peripheral nerve. (A-C) SCG10 (neuron) two color in situ hybridization; (D-F) P_0 (glia) single color in situ hybridization. Rat-specific staining is purple (arrows); chick-specific staining is red (arrowheads). (A,D) Nearby sections through the dorsal root ganglion of a forelimb-level ENP chimera, showing that donor cells contribute both neurons (A, arrow) and glia (D, arrow) to this structure. D shows colonization of the dorsal root entry zone. (B,E) Nearby sections through the sympathetic ganglion of a hindlimb-level chimera, showing rat-derived neurons (B, arrow) and glia (E, arrow). (C,F) Adjacent sections through the peripheral nerve of a forelimb level chimera, showing that ENPs generate ectopic neuronal somata in the peripheral nerve (arrows); glia are absent (arrows). Compare with Fig. 2C,F. (G) Quantification of donor neurons and glia per section (n=12 animals). For both DRG and SG, grafts typically extend 60-180 μm in axial length (neurons and glia found on 4-12 sections); for PN, grafted cells are found in a region extending 480 ±120 μm in axial length, or an average of 32 sections.

In the peripheral nerve, however, the frequencies of neurons to glia are reversed in comparison to NCC chimerae.

ENP-derived neurons differentiating in DRG express an autonomic and not a sensory phenotype

As ENP-derived cells could be found in DRG and expressed pan-neuronal markers (Fig. 6A), we anticipated that these cells would, like NCC-derived neurons (Fig. 3), have a sensory phenotype. Surprisingly, however, in 80% of animals, ENP-derived grafts containing rat SCG10$^+$ cells in the DRG had no rat Brn3.0$^+$ cells on adjacent sections (Fig. 7D,E, and Table 3). 90% of these grafts, however, expressed B2, a rat autonomic marker not normally expressed by DRG neurons (Fig. 7F). The B2 contribution is indistinguishable from the SCG10 contribution in these ENP grafts (Table 3). In contrast, in NCC-derived DRG grafts B2 was never detected in sections adjacent to those containing SCG10$^+$ and Brn3.0$^+$ cells (Fig. 7A-C and Table 3).

In rare ENP-engrafted embryos, a single Brn3.0$^+$ cell was seen adjacent to rat SCG10$^+$ cells in the DRG. However, in these cases about 50 SCG10$^+$ cells were detected for each Brn3.0$^+$ cell. By contrast, in NCC-derived grafts the numbers of SCG10$^+$ and Brn3.0$^+$ cells on adjacent sections were similar (Table 3). The probability that the NCC and ENP populations have similar capacities to produce Brn3.0$^+$ cells, and that the observed differences are due to chance, is very low (Table 3; P<0.0004). Taken together, therefore, these data indicate that ENPs are significantly restricted in their capacity to generate sensory neurons in vivo, in comparison to NCCs. Moreover, despite this restriction, in host DRG, ENP-derived cells can differentiate to neurons expressing a position-inappropriate autonomic phenotype.

ENPs are not competent to generate sympathetic neurons in vivo

The observation that ENPs generate autonomic but not sensory neurons in DRG raised the question of whether these precursors can generate all classes of autonomic neurons, or only some. We therefore examined the sympathetic ganglia and aortic plexii of ENP chimerae for the expression of the two markers characteristic of the sympathetic autonomic sublineage, GATA-2 and TH.

Like NCCs, ENPs differentiated to rat neurons that were intermingled with chick neurons in the sympathetic ganglia (Fig. 8A, purple, arrows vs. red, arrowheads). In some grafts, B2 was detected (Fig. 8D), indicating that the neurons are autonomic. However, in contrast to NCC chimerae, in the vast majority of ENP chimerae neither GATA-2 nor TH were expressed, even though the average number of SCG10$^+$ cells

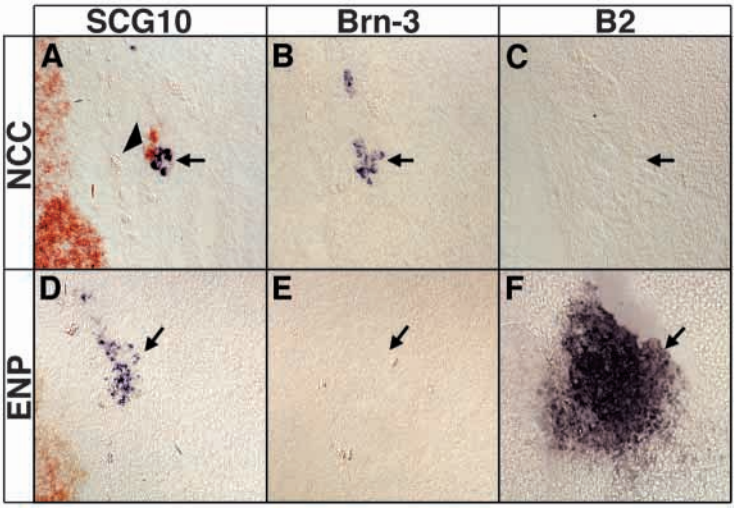

Fig. 7. ENP-derived neurons in the DRG are autonomic. (A-C) NCC chimera sections; (D-E) ENP chimera sections. DRG neurons in an NCC chimera (A, arrow) express the sensory marker Brn3.0 (B, arrow), but not the autonomic marker B2 (C, arrow). A subsequent section was positive for rat Brn3.0, indicating that the graft extended through this region (not shown). In contrast, DRG neurons of ENP chimera (D, arrow) express the autonomic marker B2 (F, arrow) and not the sensory marker Brn3.0 (E, arrow). See Table 3 for quantification.

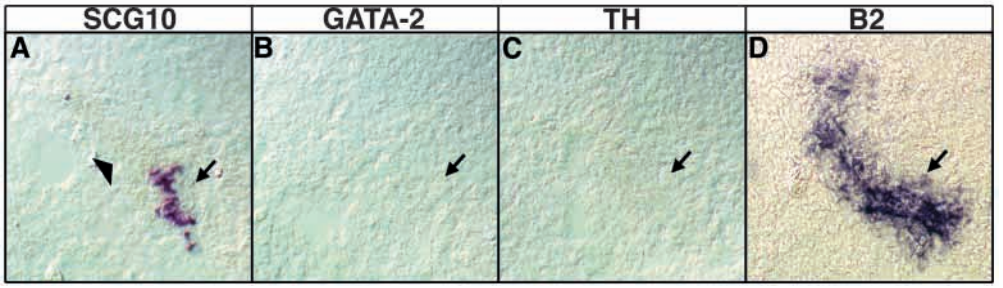

Fig. 8. ENPs do not express sympathetic neuron markers in the SG. Sequential sections through an ENP chimera, showing rat derived neurons (A, arrow) contributing to the sympathetic ganglion. GATA-2 (B, arrow) and TH (C, arrow) are absent, although the autonomic marker B2 (D, arrow) is detected on the subsequent section, indicating rat-derived neurons are likely present in the sections shown in B and C. The section shown in B was hybridized on a slide exhibiting rat GATA-2$^+$ cells in the pelvic plexus (data not shown), which provided an internal positive control for that marker. Only one rat TH$^+$ cell was ever observed in an ENP chimera, although ENP chimeric sections were often processed side-by-side with NCC chimeric sections that did exhibit rat TH staining. See Table 4 for quantification.

per section was twice that obtained with NCC grafts (Fig. 8B,C, and Table 4). A single cell expressing GATA-2, and a single cell expressing TH, were each detected in two different ENP chimerae (Table 4). However, given the frequency of detecting TH$^+$ cells in NCC chimerae (Table 4), the likelihood that ENPs and NCCs have similar capacities to generate TH$^+$ cells but that the observed difference is due to chance, is low (P<0.02). These data suggest that, in comparison to NCCs, ENPs are restricted in their capacity to generate not only sensory neurons but also sympathetic neurons as well.

In summary, we have shown ENPs are, like NCCs, able to generate parasympathetic-like neurons in Remak's ganglion and the pelvic plexus. ENPs are also competent to generate neurons in both the dorsal root and the sympathetic ganglia. Unlike NCCs, however, ENP-derived DRG neurons express the autonomic neuronal marker B2, and not the sensory marker Brn3.0. In the sympathetic ganglia, ENPs differentiate to neurons that express the autonomic marker B2, but do not express GATA-2 or TH, a combination of markers specific to the sympathetic lineage. Like NCCs, ENPs generate glia in the dorsal root and sympathetic ganglia; however, ENPs generate ectopic neurons, and not glia, in peripheral nerves. Thus in comparison to NCCs, the ENP population appears significantly restricted in its developmental capacities.

DISCUSSION

In vivo transplantation provides the most definitive test of a progenitor population's developmental capacities. Such tests have been difficult to perform on rodent neural crest cells because of the lack of an accessible host system. Here we have tested the capacities of two rat neural crest-derived progenitor populations, NCCs and ENPs, using chicken embryos as hosts and species-specific lineage markers to identify the fates of the grafted cells. NCCs isolated from neural tube explants are competent to differentiate to sensory, sympathetic and parasympathetic neurons, as well as satellite glia

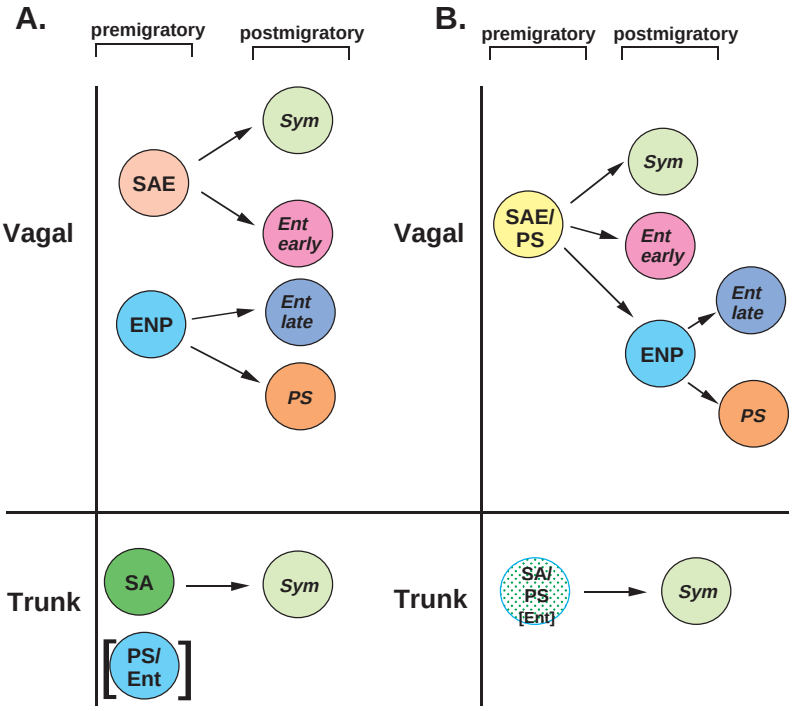

Fig. 9. Two models for the origin of para-enteric progenitors. c-RET$^+$/B2$^-$ ENPs isolated from E14.5 fetal rat gut (light blue circles) have the potential to generate parasympathetic (PS) and presumably late-differentiating enteric neurons (Ent late), but not sympathetic or sensory neurons. Relationship of ENPs to SAE precursors (Durbec et al., 1996) in the vagal neural crest: (A) ENPs may be distinct from SAE precursors in the premigratory vagal neural crest. (B) ENPs may share a common precursor with sympathetic and early-differentiating enteric neurons in the premigratory vagal crest (SAE/PS), but become restricted to late enteric and parasympathetic fates after migrating to the gut. Relationship of sympathetic to parasympathetic progenitors in the trunk crest: (A) The premigratory trunk crest contains a precursor that has sympatho-adrenal (SA) but not parasympathetic capacity. Separate precursors with parasympathetic and enteric capacities are postulated to account for the ability of the trunk crest to give rise to these derivatives in heterotopic transplantation experiments (Le Douarin, 1980); however in normal development the trunk crest does not generate parasympathetic derivatives (brackets). (B) Trunk crest contains precursors with sympathetic, parasympathetic and enteric potential. These potentials may not be equally available to such cells, however, since trunk crest does not fully populate the gut when grafted to the vagal region (Le Douarin, 1980; Fontaine-Perus et al., 1988). Note that the scenario for trunk crest illustrated in A could, alternatively, co-exist with that described for the vagal neural crest in B, or vice versa. Sensory lineages are excluded from this diagram for simplicity.

and Schwann cells. By contrast, c-RET$^+$/B2$^-$ ENPs immunopurified from E14.5 fetal gut are restricted from generating sensory and sympathetic neurons, but are competent to make parasympathetic and, presumably, enteric neurons. ENPs can also make glia in some but not all locations where NCCs differentiate to this fate. Taken together, these data suggest that the gut contains an autonomic neuro-glial precursor population displaying a novel pattern of developmental restriction.

NCCs are competent to generate neurons in three major peripheral lineages

Previous in vitro studies of rodent NCCs have shown that these cells can generate sympathetic (Matsumoto, 1994b; Maxwell et al., 1996) and other autonomic neurons (Stemple and Anderson, 1992; Shah et al., 1996), but whether they can also generate sensory (Murphy et al., 1991; Matsumoto, 1994a) and parasympathetic neurons has been less clear. We have applied an extensive battery of molecular markers for PNS neuronal subtypes to assess the differentiation capacities of rat NCCs after transplantation in vivo. This analysis has revealed that NCCs are able to generate neurons in three major peripheral neurogenic sublineages: sensory, sympathetic and parasympathetic. It is noteworthy that this is the first demonstration that cultured NCCs from any species can generate these three lineages after transplantation in vivo. Although this potential has so far been demonstrated only at the population level, it indicates that NCCs can now be used to investigate the segregation of these various neuronal sublineages in vitro. Our results also demonstrate that transplantation into chick hosts is a practical approach to assay lineage restrictions in vivo within different antigenically purified rodent crest-derived populations. In this respect, NCCs provide an important positive control for such experiments.

ENPs are partially restricted autonomic precursors

We have tested the capacities of ENPs to differentiate into sensory, sympathetic, and parasympathetic neurons as well as peripheral glia, following engraftment into chick embryos. Paradoxically, we have not been able to directly test their differentiation into enteric neurons. This may reflect difficulty in delivering the transplanted cells into a crest migration pathway providing access to the gut, or competition with endogenous crest cells for limited space. To our knowledge, there are no reports of successful engraftment of the gut in vivo by dissociated cell suspensions transplanted into the vagal or sacral crest. However, similarly isolated c-RET$^+$ enteric precursors from E12.5 mouse gut have recently been shown to generate enteric neurons and glia when injected into organ-cultured murine gut tissue (Natarajan et al., 1999). Therefore, it is a reasonable assumption that ENPs, positively selected from E14.5 rat gut using the same antibodies, also possess enteric potential.

With this assumption in mind, comparison of ENPs to NCCs in the transplant assay system suggests that the former population has a unique, previously undocumented pattern of lineage restriction. ENPs are not only restricted from a sensory fate, but also appear restricted from a sympathetic fate. The existence of autonomic precursors lacking sensory capacity is not surprising, and has previously been inferred from backgrafts of avian sensory ganglia (Le Lievre et al., 1980; Le Douarin, 1986; Fontaine-Perus et al., 1988). However, the idea that there are precursors restricted to generating subtypes of autonomic neurons has received less attention. Autonomic precursors in trunk DRG that have sympathetic capacity (Le Lievre et al., 1980) do not generate enteric neurons when transplanted to the vagal neural crest (Fontaine-Perus et al., 1988). However, a population that, conversely, can generate enteric and parasympathetic but not sympathetic neurons has not to our knowledge been demonstrated. Although sympathetic differentiation may be elicited from ENPs in vitro (see note added in proof), following transplantation in vivo these cells do not express sympathetic markers even though they differentiate to autonomic neurons in the para-aortic region; by contrast NCCs differentiate appropriately in this location. It has previously been suggested (Fontaine-Perus et al., 1988) that the capacity of post-migratory precursors in nodose ganglia to generate both sympathetic and enteric neurons (Ayer-Le Lievre and Le Douarin, 1982) might reflect distinct subpopulations restricted to sympathetic or enteric fates. Our results are consistent with this idea, and further suggest that the latter precursors may also have parasympathetic potential, although this has not been tested.

Our results may appear, on the surface, inconsistent with a body of evidence suggesting that sympathetic and at least some enteric neurons share a common progenitor (reviewed by Gershon, 1997). The posterior vagal neural crest has been shown by diI labeling to contain a precursor population generating both enteric and sympathetic neurons (Durbec et al., 1996). These so-called sympatho-enteric (SAE) precursors (Durbec et al., 1996) transiently express sympatho-adrenal markers such as TH at early stages of gut development (E11.5-E14.5), and differentiate into early-born enteric neurons such as the serotonergic subset (Baetge and Gershon, 1989; Carnahan et al., 1991; Blaugrund et al., 1996). This population is selectively eliminated in *Mash1*$^{-/-}$ embryos, while later-differentiating enteric neurons are unaffected (Blaugrund et al., 1996). Our isolation procedure selects against neuroblasts or neurons expressing the differentiation antigen B2 (Anderson et al., 1991) at E14.5, and therefore enriches for later-differentiating enteric precursors. Since the derivatives of SAE precursors are likely to express B2 by E14.5 (Blaugrund et al., 1996), they should be excluded from the c-RET$^+$/B2$^-$ ENP population. Similarly, the adrenergic potential of avian enteric neural crest-derived cells in vitro was shown to decline with developmental time (Deville et al., 1994)

The idea that ENPs represent a distinct late-differentiating subpopulation of enteric precursors that lacks sympathetic capacity could also explain an apparent inconsistency between our results and those of (Pisano and Birren, 1999), who observed TH$^+$ neurons in cultures of rat E14.5 enteric cells immuno-selected using HNK-1. The population selected with this antibody includes neuroblasts and TH$^+$ cells (Pisano and Birren, 1999). In contrast, ENPs plated and fixed 2 hours after isolation did not express TH, implying that the HNK-1$^+$ subset includes an additional population not present in the c-RET$^+$/B2$^-$ subset. Furthermore, about half of cultured HNK-1$^+$ enteric neurons did not express TH under any in vitro conditions tested (Pisano and Birren, 1999), consistent with the idea that HNK-1 may additionally select precursors that lack sympathetic capacity i.e., ENPs. The notion that SAE and ENPs represent different subpopulations of precursors in the

gut could also explain why backgrafts of gut tissue fragments into the avian neural crest yielded sympathoadrenal derivatives (Rothman et al., 1990), whereas immunopurified ENP cells did not. It remains to be determined whether the segregation of these two putative enteric precursor subtypes occurs prior to (Fig. 9A, ENP), or only after (Fig. 9B, ENP), emigration of crest cells to the gut.

The observation that ENPs have parasympathetic but not sympathetic capacity is surprising in light of the widely held assumption that these two autonomic neuronal subtypes share a common precursor. Despite this assumption, fate-mapping studies have indicated that sympathetic and parasympathetic neurons derive for the most part from distinct regions along the rostrocaudal neuraxis: the former from the trunk and the latter from the vagal region (Le Douarin, 1986). Nevertheless, vagal neural crest can generate sympathetic neurons when grafted to the trunk region (Le Douarin and Teillet, 1974). However, the possibility that this plasticity reflects distinct precursors with parasympathetic or sympathetic capacity intermingled in the premigratory vagal crest has not been formally excluded (Fig. 9A, Vagal). Such distinct sympathetic and parasympathetic precursors could also be present in the premigratory trunk neural crest (Fig. 9A, Trunk). If the latter also had enteric capacity (like ENPs) and are less numerous than in the vagal region, it could explain why trunk crest does not efficiently populate the gut when grafted to the vagal crest (Le Douarin, 1980; Fontaine-Perus et al., 1988). Indeed, no common precursor of sympathetic and parasympathetic neurons has been identified by single cell lineage analyses in vivo (Fraser and Bronner-Fraser, 1991). A compromise view is that parasympathetic potential may be present in different types of precursors, some of which also possess sympathetic capacity (Fig. 9B, Trunk, SA/PS) and some of which do not (Fig. 9A,B; Vagal, ENP).

It is interesting to find a precursor population with both parasympathetic and enteric capacity, in view of the commonalities in the differentiation of these neuronal subtypes. Parasympathetic and enteric neurons tend to arise from similar regions of the neuraxis, which are distinct from those that produce sympathetic ganglia (Le Douarin, 1982). Furthermore, unlike sensory and sympathetic neurons, which reside in metameric chains of ganglia near the spinal cord and distant from their targets, both parasympathetic and enteric neuronal cell bodies are located within their target tissues. Thus, the precursors to parasympathetic and enteric neurons have to migrate longer distances, and presumably proliferate more extensively before they differentiate, than do sensory and sympathetic precursors. This may require a mechanism to delay differentiation of parasympathetic and enteric neurons, one not utilized by sensory or sympathetic precursors. Interestingly, primitive vertebrates such as the lamprey have parasympathetic- and enteric-like ganglia, but not sympathetic chains (Hirata et al., 1997), suggesting that the capacity of neural crest cells to produce sympathetic ganglia may be a more recent evolutionary invention. In this way, the developmental restriction exhibited by ENPs may reflect an evolutionarily more primitive capacity of the neural crest.

Neuron subtype restriction may precede commitment to a neuronal fate

Do ENPs become restricted to the para-enteric sublineage before or only after they commit to a neuronal fate? Because the ability of individual ENPs to make neurons and/or glia has previously been analyzed in clonogenic cultures (Lo and Anderson, 1995), the present study provides an opportunity to assess the relationship of neuron subtype restriction to the segregation of neuronal and glial lineages. This issue has not been addressed in previous grafting studies, as the clonogenic differentiation capacities of the transplanted cells were not assessed.

Clonal analysis of ENPs in vitro has indicated that all cells able to make glia can also make neurons (Lo and Anderson, 1995; Lo et al., 1997). Since grafted ENPs generate both neurons and glia in DRG and sympathetic ganglia, yet do not express sensory or sympathetic markers in these locations, the implication is that restriction to a para-enteric sublineage can occur prior to the choice between neuronal and glial fates. This conclusion must be tempered by the caveat that although individual ENPs can produce both neurons and glia in culture, it is technically difficult to show that individual grafted cells are similarly bipotent in vivo. Therefore, we cannot exclude that all of the grafted cells that exhibit restricted neuronal subtype capacities in vivo derive from the subset of ENPs committed to a neuronal fate in vitro (so-called NPs; Lo and Anderson, 1995). Nevertheless, it seems more parsimonious to assume that if many ENPs produce both neurons and glia in vitro, then they will do so in vivo as well. Indeed, transplantation of single c-RET$^+$ ENPs into organ-cultured murine gut yielded clones containing both neurons and glia (Natarajan et al., 1999). The idea that neuron subtype restriction can precede the neuron-glial decision has precedent in the *Drosophila* retina, where multipotent precursors become restricted to making a particular photoreceptor subtype (R7) before choosing between photoreceptor and glial (cone cell) fates (Zipursky and Rubin, 1994).

If bipotent neuro-glial ENPs are restricted in the subtypes of neurons they can generate, are they similarly restricted to producing subtypes of glia? Interestingly, whereas grafted NCCs contributed to glia in both the capsule and the dorsal root entry zone of the DRG, ENP-derived glia were found only in the latter location. Furthermore, although grafted NCCs differentiated into Schwann cells in peripheral nerve, ENPs did not, even though some grafted ENPs migrated to this location and differentiated into neurons (which likely developed from committed NPs; Lo and Anderson, 1995). This latter observation implies that gliogenic ENPs differ from NCCs in their ability to colonize, differentiate or survive in peripheral nerve.

Taken together, these data suggest that multipotent ENPs are restricted in the subtypes of glia, as well as of neurons, that they can generate. It is likely that in vivo ENPs normally generate enteric glia, which differ from Schwann cells in their antigenic phenotype and proliferative properties (Eccleston et al., 1987; Dulac and Le Douarin, 1991). Thus, ENPs may be restricted to producing subtypes of glial cells that are uniquely suited to the functions performed by the kinds of neurons that these precursors can also generate. In this way, the neural crest-derived cells that migrate over long distances to form the parasympathetic and enteric nervous system would be endowed with the capacity to generate the appropriate types of both neurons and glia for these distal autonomic ganglia.

We would like to thank the following people for their guidance with

various techniques: Li Ching Lo, for the isolation of ENPs; Marianne Bronner-Fraser, for making chimerae; Nirao Shah, for the isolation of NCCs, Rochelle Diamond and Patrick Coen, for FACS assistance; Andrew Groves, for the in situ protocol; Tetsuchiro Saito, for double label protocols; and Amy Greenwood, for Photoshop tips. Eric Turner and Tetsuchiro Saito kindly provided us cDNA for Brn3.0 and BarH4.1, respectively; we would also like to thank Jean-Francois Brunet and Christo Goridis for helpful discussions. Sherry Perez, Alice Pacquette, Kai Zinn, Marianne Bronner-Fraser and Andrew Groves made comments on this manuscript. Finally, we would like to thank an anonymous reviewer for the suggestion to assay for parasympathetic phenotypes. This research was supported by a grant from the NIH. D. J. A. is an Investigator of the Howard Hughes Medical Institute.

Note added in proof

We have recently determined that under certain culture conditions, a small proportion of ENP-derived cells can express TH in vitro. The vast majority of ENP-derived neurons, however, do not express TH.

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