

# The Projections of Early Enteric Neurons Are Influenced by the Direction of Neural Crest Cell Migration

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The enteric nervous system arises from the neural crest. In embryonic mice, vagal neural crest cells enter the developing foregut at approximately embryonic day 9.5 (E9.5) and then migrate rostrocaudally to colonize the entire gastrointestinal tract by E14.5. This study showed that a subpopulation of vagal crest-derived cells, very close to the migratory wavefront, starts to differentiate into neurons early, as shown by the expression of neuron-specific proteins and the absence of Sox10. Many of the early differentiating neurons transiently exhibited tyrosine hydroxylase (TH) immunoreactivity. The TH cells were demonstrated to be the progenitors of nitric oxide synthase (NOS) neurons. Immunohistochemistry, lesions, and Dil tracing were used to examine the projections of developing enteric neurons. The axons of first neurons in the gut (the TH–NOS neurons) projected in the same direction (caudally), and traversed the same pathways through the mesenchyme, as the migrating,

undifferentiated, vagal crest-derived cells. To examine if the direction of migration and direction of axon projection are linked, coculture experiments were set up in which vagal crest-derived cells migrated either rostrocaudally (as they do *in vivo*), or caudorostrally (which they do not normally do), to colonize explants of embryonic aneural hindgut. The direction in which neurons projected was correlated with the direction of cell migration, but migration direction appears to be not the only mechanism influencing axon projection. Peristaltic reflexes involve both orally (rostrally) projecting neurons and anally (caudally) projecting neurons. Because few rostrally projecting neurons could be detected before birth, the full circuitry for peristaltic reflexes appears to develop after birth.

**Key words:** enteric nervous system; nitric oxide synthase; Sox10; axon projection; peristalsis; tyrosine hydroxylase

Enteric neurons and glial cells arise from neural crest cells that emigrate from two levels of the neural axis, vagal level (adjacent to somites 1–7) and sacral level (caudal to somite 24 in mice or somite 28 in chick) (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Burns and Le Douarin, 1998). Vagal level neural crest cells colonize the embryonic mouse gut in a unidirectional, rostral-to-caudal wave; crest cells enter the foregut at approximately embryonic day 9.5 (E9.5) and reach the caudal hindgut at ~E14.5 (Kapur et al., 1992; Young et al., 1998; Woodward et al., 2000). Although neural crest cells from the sacral level neural crest also contribute some neurons to the postumbilical gut, they do not enter the hindgut until after the gut has been completely colonized by vagal neural crest cells (Burns and Le Douarin, 1998; Kapur, 2000). While the vagal neural crest cells are migrating rostrocaudally through the foregut and midgut, ~15% of the cells transiently express catecholaminergic properties, including the synthetic enzyme, tyrosine hydroxylase (TH) (Cochard et al., 1978; Teitelman et al., 1978; Gershon et al., 1993; Young et al., 1999). Many of the TH+ cells have leading processes that project caudally, which is the same direction as the neural crest-derived cells are migrating (Young et al., 1999). At E10.5, the cell bodies of the TH+ cells are close to (sometimes only one cell behind), the migratory wavefront

in the midgut, but at later stages the TH+ cells become further from the migratory wavefront, and TH+ cells are never observed in the hindgut (Gershon et al., 1993; Young et al., 1999; Young and Newgreen, 2001).

It has recently been realized that axon guidance and cell migration are similar processes and can be influenced by the same molecules (Rakic, 1999; Song and Poo, 2001). In this study we examined the relationship between the direction of crest cell migration and the axon projections of early enteric neurons in the embryonic mouse gut. Because the TH+ cells have leading processes that project in the same direction as the crest-derived cells are migrating, it is possible that (1) their direction of projection is determined by the direction of cell migration, (2) there is a common guidance mechanism directing neural crest cell migration and the projection of leading processes, or (3) there are separate guidance mechanisms that happen to have the same polarization. The main aims of the study were to: (1) Determine whether the TH+ cells and their leading processes express molecules characteristic of neurons and axons. (2) Determine the relationship between the TH+ cells and NOS neurons, which are the first enteric neurons in the embryonic mouse gut to exhibit an adult-like neuronal phenotype (Branchek and Gershon, 1989). (3) Examine the development of neurons with different axon projections in the embryonic mouse gut, because different functional classes of enteric neurons have different projection patterns (Costa et al., 1996). (4) Determine the effect of caudal-to-rostral migration of vagal crest-derived cells (instead of rostral-to-caudal as normally happens) on axon projection direction.

## MATERIALS AND METHODS

Embryonic and adult BALB/c mice from an inbred colony were used. Timed, pregnant mice were killed by cervical dislocation, and the em-

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**Table 1. Primary antisera used**

Antiserum	Host	Dilution	Cells identified by antiserum	Source or reference
MAP2	Rabbit	1:2000	Neurons, predominantly in the dendrites	Chemicon, Temecula, CA
Neurofilament M, 145 kDa	Rabbit	1:100	Most neurons	Chemicon
Neuronal NOS	Rabbit	1:1000	Subpopulation of enteric neurons	Young et al., 1997
Neuronal NOS	Sheep	1:2000	Subpopulation of enteric neurons	Kind gift from Drs. P. Emson and I. Charles
p <sup>75NTR</sup>	Rabbit	1:250	Undifferentiated enteric neural crest-derived cells	Promega Corporation, Annandale, New South Wales, Australia
PGP9.5 (ubiquitin hydrolase)	Rabbit	1:1000	All neurons	Ultraclone, Isle of Wight, UK
PGP9.5 (ubiquitin hydrolase)	Sheep	1:50	All neurons	The Binding Site, Birmingham, UK
Phox2b	Rabbit	1:700	Undifferentiated enteric neural crest-derived cells	Pattyn et al., 1997
Ret	Goat	1:10	Undifferentiated enteric neural crest-derived cells	R & D, Minneapolis, MN
Sox10	Rabbit	1:50	Undifferentiated neural crest-derived cells and glial cells and their precursors	Chemicon
TH	Sheep	1:80	First enteric neurons to differentiate	Chemicon

**Table 2. Secondary antisera used**

Species in which primary antisera were raised	Secondary antisera
Rabbit	Goat anti-rabbit Alexa 488 (1:100, Molecular Probes, Eugene, OR)
Sheep or goat	Donkey anti-sheep Alexa 594 (1:100, Molecular Probes)
Rabbit + sheep or goat	Biotinylated donkey anti-rabbit (1:200, Jackson ImmunoResearch, West Grove, PA) followed by streptavidin Alexa 488 (1:100, Molecular Probes) and donkey anti-sheep Alexa 594 (1:100, Molecular Probes)

bryos were removed. Midday at the date at which a copulatory plug was observed was designated E0.5. Embryos at E13.5 and younger were also staged precisely using the staging system of Theiler (1989).

**Immunohistochemistry.** Whole-mount preparations of embryonic gut were fixed and processed as described previously (Young et al., 1999), using the primary and secondary antisera shown in Tables 1 and 2.

**Projections of neurons in E11.5 midgut and in cocultures.** The projections of the neurons was examined by performing circumferential (myotomy) lesions (Furness and Costa, 1987) of dissected E11.5 midgut or E11.5 hindgut explants that had been grown as cocultures for 3 d (see below). A semi-circumferential cut was made through the outer mesenchyme, severing the nerve fibers, using fine spring scissors or a fine scalpel blade. After 45 min to 2 hr in culture conditions, the lesioned gut was then fixed and processed for TH or protein gene product 9.5 (PGP9.5) immunohistochemistry. The directions in which nerve fibers were projecting were determined by the accumulation of immunoreactivity in the proximal stumps of the severed neurites.

**Catenary organ culture.** Explants of E10.5 and E11.5 gut were set up in catenary (suspended) organ culture as described previously (Hearn et al., 1999). Cocultures of E10.5 fore-midgut and E11.5 aneural hindgut were set up by suspending a segment of E11.5 postcaecal hindgut between the V shapes cut in the filter paper supports. The caudal end of the explant was indicated by cutting the corner off the filter paper support at that end. As neural crest cell donors, a small segment of E10-E10.5 gut, taken from the stomach swelling to the caudal end of the midgut, was then placed on the filter paper at either the rostral or caudal end and in direct contact with the E11.5 explant (see Fig. 11). Control cultures were set up in which the E11.5 hindgut explant was grown alone. The explants were grown for 4 d and then fixed and processed for immunohistochemistry. To compare the number of PGP9.5+ cells in the two sorts of cocultures (when the crest-derived cells migrated from rostral-to-caudal or

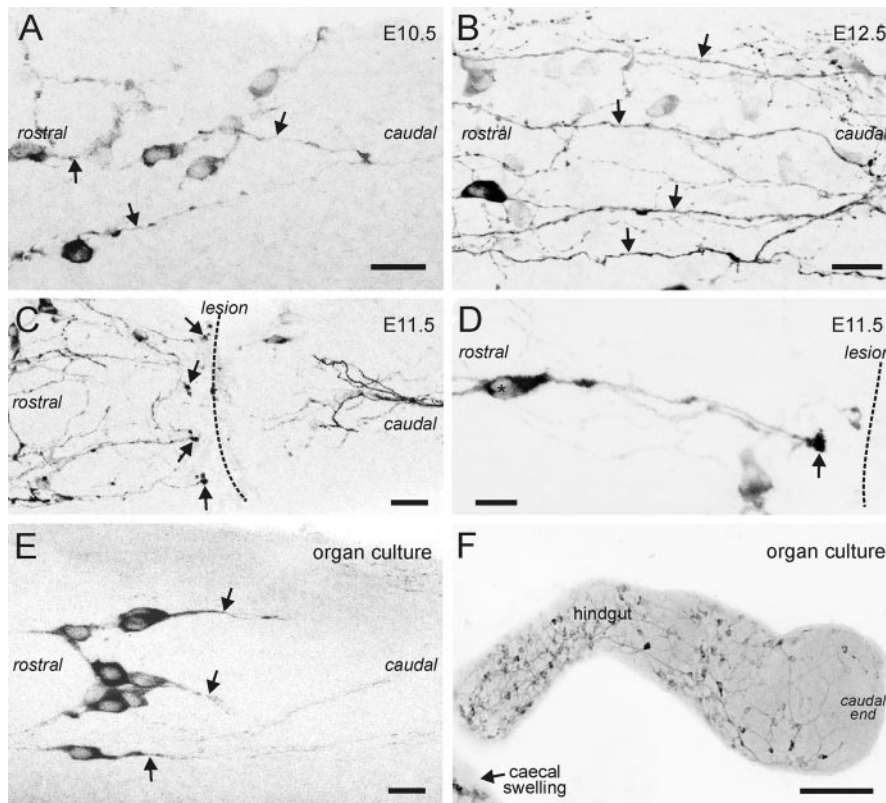
caudal-to-rostral), the number of PGP9.5+ cells on the top surface of the gut explants was counted using a 40 × objective and an eyepiece graticule. After processing for PGP9.5 immunohistochemistry, cells exhibiting a range of staining intensities were observed, probably reflecting varying degrees of neuronal differentiation. For the counts, cells were deemed to be PGP9.5+ if the nucleus was stained, and hence nucleoli discernible.

**DiI.** The gastrointestinal tract, from the stomach to the anus, from E10.5 to E18.5 mice, was fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After washing in PB, the tissue was pinned to dental wax using 100 μm entomology pins, or 50 μm tungsten wire for E10.5 embryos. The tips of the pins were dipped in DiI paste (Molecular Probes, Eugene, OR) before piercing the tissue. For E10.5-E12.5, two DiI-coated pins were applied to each preparation, one in the middle of the future small intestine, and another in the middle of the hindgut. For E13.5-E18.5 preparations, three DiI-coated pins were applied to each preparation, approximately one-third of the distance along the small intestine, approximately two-thirds along the small intestine, and in the middle of the hindgut. Segments of adult jejunum and ileum were opened down the mesenteric border, pinned out, and fixed as described above. After washing, the mucosa and submucosa removed, and DiI paste was applied to the external muscle using entomology pins. Both adult and embryonic preparations were then placed in a 37°C oven for 7–10 d (embryonic tissue) or 7 d to 2 months (adult tissue) in PB containing 0.5% sodium azide, and then mounted in PB and examined using a fluorescence microscope. Some of the preparations containing retrogradely labeled cells were subsequently processed for immunohistochemistry after being examined and photographed. These preparations were permeabilized in 70, 90 (both in 0.1 M PB), and 100% glycerol for 20 min each, and then washed in PB. E10.5 and E11.5 preparations were processed for TH immunohistochemistry, and older preparations were processed for NOS immunohistochemistry using the primary antisera shown in Table 1. The primary antisera were revealed using a donkey anti-sheep FITC (1:100; Jackson ImmunoResearch, Eugene, OR). Preparations were examined using a confocal microscope.

## RESULTS

### Leading processes of TH cells project caudally along the same pathway as undifferentiated neural crest-derived cells

Most of the TH+ cells in the E10.5 mouse foregut and midgut had one neurite that was considerably longer than the other neurites, and the longest neurite (leading process) projected caudally (Fig. 1A). However, some of the TH+ cells had either no stained neurites or were bipolar with two prominent neurites. At E11.5 and E12.5, the projections of individual TH cells were no



**Figure 1.** Inverted confocal microscope images of TH-immunoreactive cells in whole-mount preparations of embryonic gut. *A*, E10.5 foregut. Most of the TH+ axons have axons (arrows) that project caudally. Scale bar, 25  $\mu$ m. *B*, E12.5 midgut. The polarity of individual TH cells is not obvious. The TH+ processes (arrows) form bundles that run predominantly longitudinally down the gut. Scale bar, 25  $\mu$ m. *C*, Low-magnification image of TH cells in the E11.5 midgut after a circumferential lesion to the mesenchyme. Swollen processes (arrows) are present rostral to the lesion, indicating that the TH cells project caudally. Scale bar, 20  $\mu$ m. *D*, High-magnification image of a TH cell (asterisk) rostral to a lesion. There is a swelling of the process (arrow) emanating from the cell just rostral to the lesion site. Scale bar, 10  $\mu$ m. *E*, *F*, E10.5 gastrointestinal tract after growth in catenary organ culture for 3 d. *E*, Many of the TH+ cells have axons (arrows) that project caudally. Scale bar, 10  $\mu$ m. *F*, During the culture period, a caecal swelling develops in explants of embryonic gut, as they do *in vivo* (Hearn et al., 1999), enabling the hindgut to be identified. Low-magnification image of the post-caecal hindgut showing that TH+ cells are present along the entire length of the cultured gastrointestinal tract. Scale bar, 100  $\mu$ m.

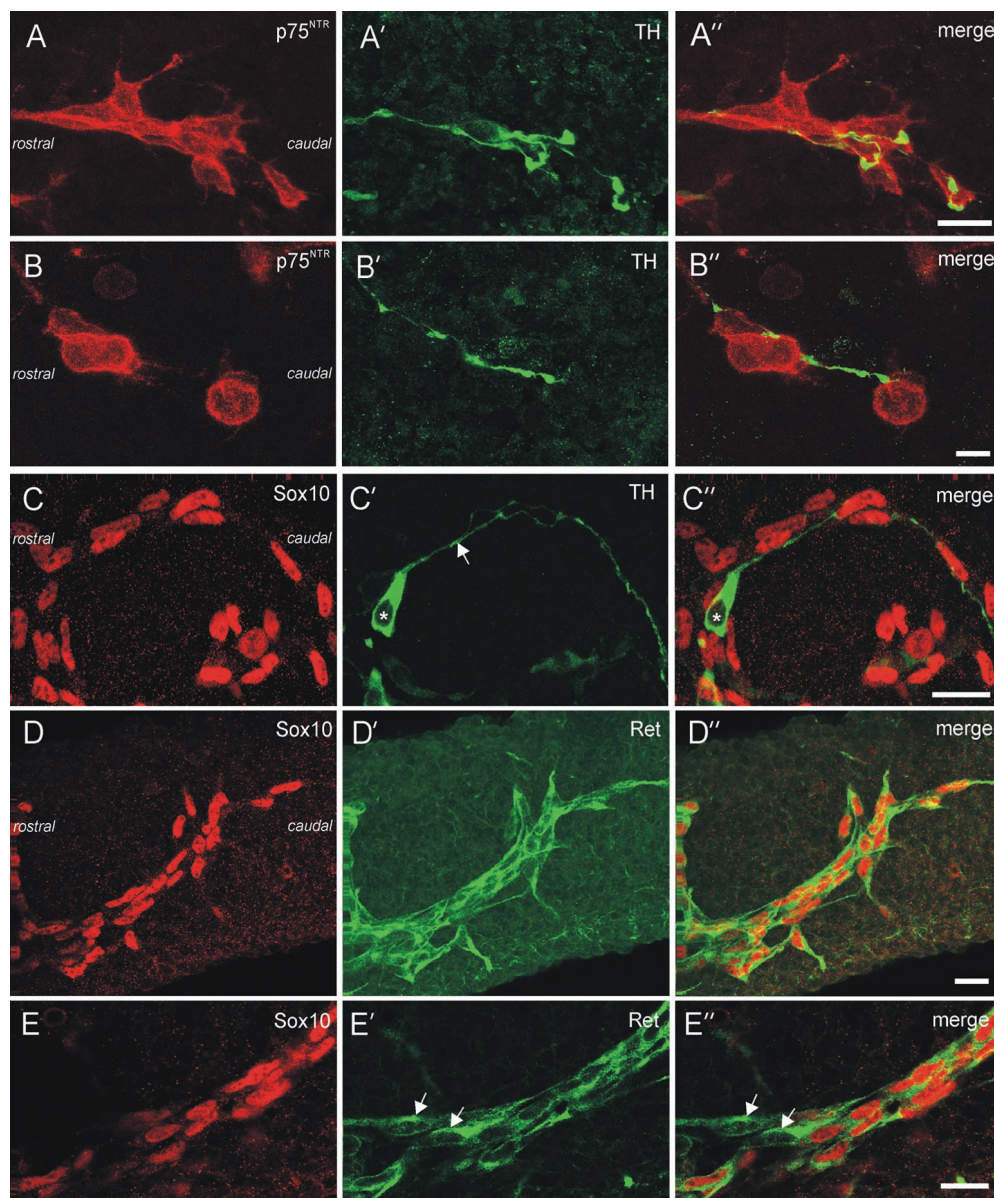
longer obvious because most of the TH nerve cell bodies were located on longitudinally oriented nerve bundles, and the neurites belonging to a single cell could not be discerned (Fig. 1*B*). To examine the direction of projection of the TH cells at E11.5, circumferential lesions were made through the mesenchyme of the middle part of the midgut, and the preparations were maintained in organ culture for 45 min to 2 hr before being processed for TH immunohistochemistry. In the majority of preparations, swollen TH+ processes were only found on the rostral side of the lesion (Fig. 1*C,D*), indicating that the TH cells projected caudally. However, in ~30% (3 of 11) of the preparations, one or two swollen processes were also observed on the caudal side of the lesion, indicating that (1) the TH cells do not always project directly caudally, (2) there is a small subpopulation of TH cells that project rostrally, and/or (3) there is a small subpopulation of bipolar TH cells (cells with processes that project both rostrally and caudally). It is unlikely that any of the swollen TH+ processes belonged to extrinsic neurons as vagal fibers only reach the stomach at E11 (Baetge and Gershon, 1989) and are therefore unlikely to have reached the midgut by E11.5, and sympathetic fibers have also not reached the gut at this stage. The TH+ fibers often formed bundles that ran predominantly rostrocaudally along the gut (Fig. 1*B*), suggesting the existence of rostrocaudal axon guidance cues. Using antisera to TH in combination with antisera to Phox2b, Ret, or p75<sup>NTR</sup> to label all neural crest-derived cells (Chalazonitis et al., 1998; Young et al., 1999), the leading processes of the TH+ cells were found to follow the same pathway through the gut mesenchyme as the cell bodies and processes of undifferentiated crest-derived cells (Fig. 2*A–B''*). In some E10.5 preparations, TH+ leading processes extended caudally beyond the most caudal crest-derived cell bodies, and in other preparations, the most caudal crest-derived cell bodies were more caudal than the most caudal TH+ process.

The gastrointestinal tract, from the stomach swelling to the anal end, was dissected from E10.5 mice, grown in catenary organ culture (Hearn et al., 1999) for 3–4 d, and then processed for TH immunohistochemistry. Some of the TH+ cells in the cultured explants had leading processes that projected caudally (Fig. 1*E*). *In vivo*, TH+ cell bodies are never observed in the hindgut (Gershon et al., 1993). At E10.5, when initially placed into organ culture, TH+ cells are present in the rostral one-third of the gastrointestinal tract only, approximately midway along the midgut (Young and Newgreen, 2001). However, after growth in organ culture for 3 d, TH+ cell bodies were present along the entire length of the explant, including the postcaecal hindgut (Fig. 1*F*), indicating that the conditions preventing the expression of TH by cells in the hindgut *in vivo* are not present in organ-cultured gut.

### TH+ cells have characteristics of neurons

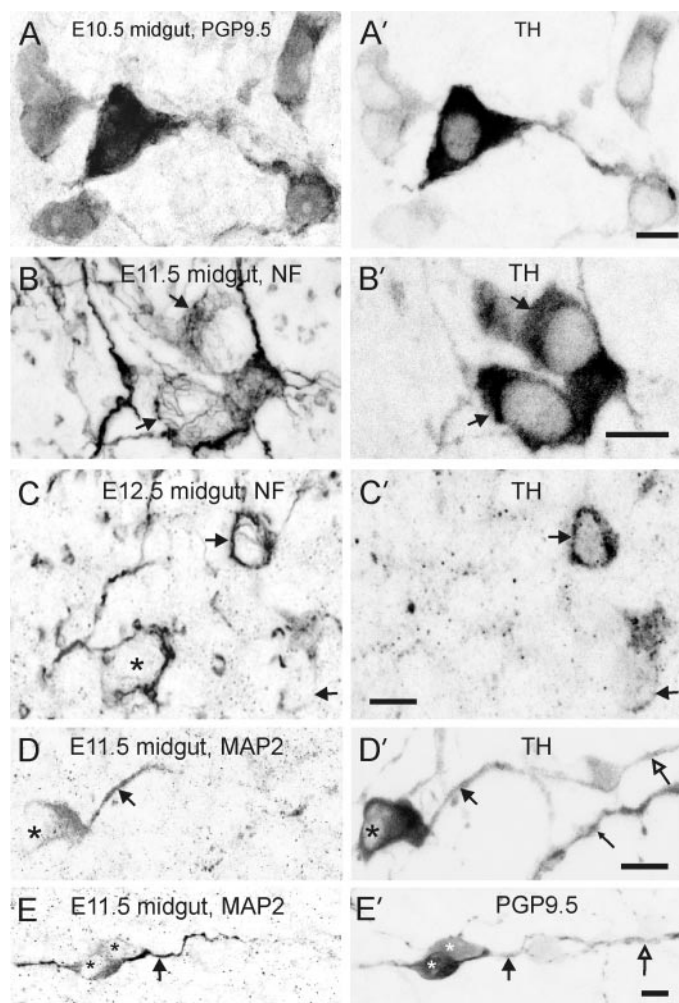
Some migrating neuron precursors, such as the migrating basilar pontine cells, extend long processes in the direction of cell migration, but the leading processes do not express neuron-specific proteins, and therefore cannot be considered to be axons (Yee et al., 1999). To determine whether the TH+ cells and their leading processes have characteristics of neurons and axons, we examined whether the TH+ cells express the neuron-specific proteins, neurofilament 145 and PGP9.5, and mitogen-activated protein (MAP) 2, which is localized mainly in the dendrites and cell bodies of mature neurons. Between E10.5 and E13.5, all of the TH+ cell bodies showed neurofilament and PGP9.5 immunoreactivity (Fig. 3*A,A',B,B'*), including the most caudal TH+ cells. At E10.5, the TH+ cells were the only PGP9.5+ or neurofilament+ cells in the gastrointestinal tract (Fig. 3*A,A',B,B'*). However, by E11.5 and E12.5, PGP9.5+ and neurofilament+ cells were observed that were not TH+ (Fig. 3*C,C'*). The leading

**Figure 2.** *A–B''*, Paired confocal microscope images of p75<sup>NTR</sup>+ neural crest-derived cells (red, *A,B*) and TH+ cells (green, *A',B'*) in two different preparations of E10.5 gut. p75<sup>NTR</sup> is expressed by all undifferentiated crest-derived cells. The p75<sup>NTR</sup>+ vagal crest-derived cells are migrating from rostral-to-caudal. The caudally projecting, TH+ axons (*A',B'*) are found in a similar location within the mesenchyme to the p75<sup>NTR</sup>+ cells, and merged images (*A'',B''*) show that the TH+ axons and p75<sup>NTR</sup>+ cells appear to be following the same pathway through the mesenchyme. Scale bars: *A*, 25  $\mu$ m; *B*, 10  $\mu$ m. *C–C''*, Paired, stacked confocal images of Sox10-immunoreactive nuclei of vagal neural crest cells in the foregut of an E10.5 mouse (*C*). *C'*, A TH+ cell in the same field of view (asterisk) does not show Sox10 immunoreactivity (*C''*). Note the caudally projecting process (arrow) of the TH+ cell. *D–E''*, Sox10 and Ret immunoreactivity in the midgut of an E11 mouse. *D–D''*, Paired, stacked confocal images showing that most of the Sox10+ cells (*D*) are also Ret+ (*D'D''*). *E–E''*, Single optical section showing two Ret+ cells (*E',E''*) that do not show Sox10 immunoreactivity (*E,E''*). Scale bars: *C–E*, 25  $\mu$ m.



processes of the TH+ cells showed both PGP9.5 and neurofilament 145 immunostaining. Most of the cell bodies of the TH+ and PGP9.5+ cells showed weak MAP2 immunoreactivity (Fig. 3*D,D',E,E'*). The proximal regions only of many of their processes also showed MAP2 immunostaining, whereas the more distal parts of the processes were MAP2-negative (Fig. 3*D,D',E,E'*). The prominent, longitudinally oriented TH+/PGP9.5+ nerve bundles lacked MAP2 immunostaining (Fig. 3*D,D'*). At E11.5, the most caudal TH+ cell bodies are in the caudal midgut, whereas TH+ nerve processes are found in the caecum, caudal to the most caudal TH+ cell body. None of the most caudal TH+ processes was MAP2+. The absence of MAP2, a dendrite marker, from the long, TH+/PGP9.5+ processes at E10.5–E11.5 suggests that these processes have some properties of axons. The enteric nerve processes within the circular muscle layer are exclusively axons. At E15.5, when the circular muscle layer has formed, the PGP9.5+ nerve fibers within this layer were MAP2-negative.

The transcription factor, Sox10, appears to play a role in early neural crest development and in the development of peripheral glia (Herbarth et al., 1998; Southard-Smith et al., 1998; Kapur, 1999; Britsch et al., 2001). Sox10 expression is initiated in neural crest cells as they emigrate from the neural tube (Southard-Smith et al., 1998), but expression is maintained only in the glial and melanocyte lineages (Herbarth et al., 1998; Kuhlbrodt et al., 1998). We compared the distribution of Sox10, TH, and Ret immunoreactivity in the E10.5 and E11.5 gut. Sox10+ cells were abundant, but none of the TH+ or PGP9.5+ cells showed Sox10 immunoreactivity (Fig. 2*C,C',C''*). All of the Sox10+ cells were also Ret+, but ~10–20% of the Ret+ cells did not show Sox10 immunoreactivity (Fig. 2*D,D',E''*). It has been previously shown that all of the TH+ cells in the embryonic mouse gut show strong Ret immunoreactivity (Young et al., 1999), and we therefore assume that most, or all, of the Ret+/Sox10-negative cells were the TH+ cells. Hence, the TH+ cells express neuron-specific proteins,



**Figure 3.** Paired, inverted confocal microscope images of whole-mount preparations of embryonic gut. All of the TH+ cells in the E10.5 midgut (A') also show PGP9.5 immunoreactivity (A), and vice versa. All of the TH+ cells in the E10.5 midgut (arrows, B') also show neurofilament immunoreactivity (arrows, B), and vice versa. By E12.5, although all of the TH+ cells are neurofilament+ (arrows, C, C'), there are neurofilament+ cells that do not show TH immunoreactivity (asterisk, C). D, D', A TH+ cell body (D') is also MAP2+ (D); MAP2 staining is present only in the proximal part of the process of the TH+ cell (arrow, D, D'), but not in the more distal parts of the TH+ process (open arrow, D'). A longitudinally running TH+ fiber (small arrow, D') is MAP2 negative. E, E', Two PGP9.5+ cell bodies (D') are also weakly MAP2+ (E); MAP2 staining is present only in the proximal part of the processes of the PGP9.5+ cells (arrow, E, E'). Processes forming a small, longitudinally running nerve trunk (open arrow, E') do not show MAP2 immunostaining. Scale bars, 10  $\mu$ m.

but lack Sox10, which has been implicated in gliogenesis (Britsch et al., 2001).

#### Location of NOS neurons in the hindgut in relation to the wavefront of migratory vagal neural crest-derived cells

The first enteric neurons to express a neurotransmitter synthetic enzyme expressed by mature enteric neurons are the NOS neurons (Branchek and Gershon, 1989). No cells showing detectable NOS immunostaining could be detected at E10.5 or E11.5, but NOS+ cells were present in the small intestine and rostral large intestine at E12.5. At E12.5, the most caudal vagal neural crest-derived cells are approximately one-third to one-half way along

the hindgut (Kapur et al., 1992; Young and Newgreen, 2001). We examined the location of the NOS neurons in relation to the wavefront of vagal crest-derived cells by combining NOS immunostaining with Phox2b immunostaining. Phox2b is a transcription factor that is expressed by all enteric neural crest-derived cells before and after their differentiation into enteric neurons (Pattyn et al., 1997; Young et al., 1999). In the hindgut of E12.5 mice, the most caudal NOS+ cell bodies and axons were rostral to the most caudal Phox2b+ cells by between 275 and 620  $\mu$ m (mean,  $415 \pm 77 \mu$ m;  $n = 4$  preparations) (Fig. 4). At E10.5, differentiating (TH+) neurons can be within 20  $\mu$ m of the migratory wavefront (Young and Newgreen, 2001). The increasing distance between the first differentiating neurons and the most caudal undifferentiated crest-derived cell with age may be attributable to the rapid growth of the gut as the crest cells migrate caudally (Newgreen et al., 1996).

#### Projections of NOS neurons

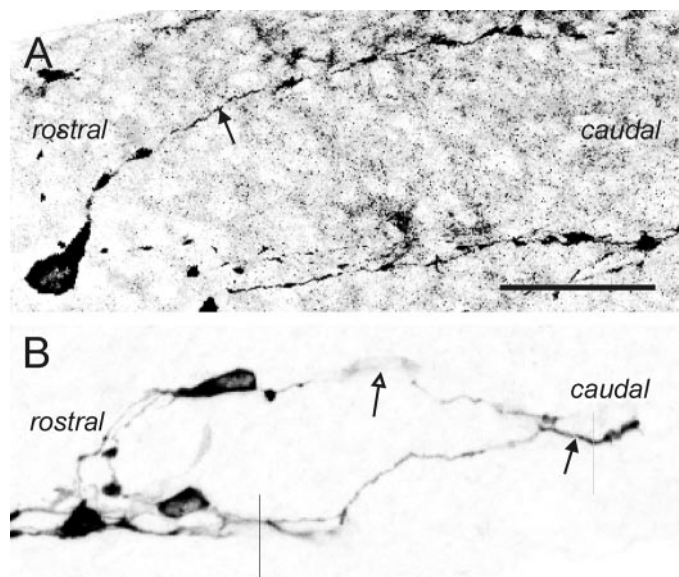
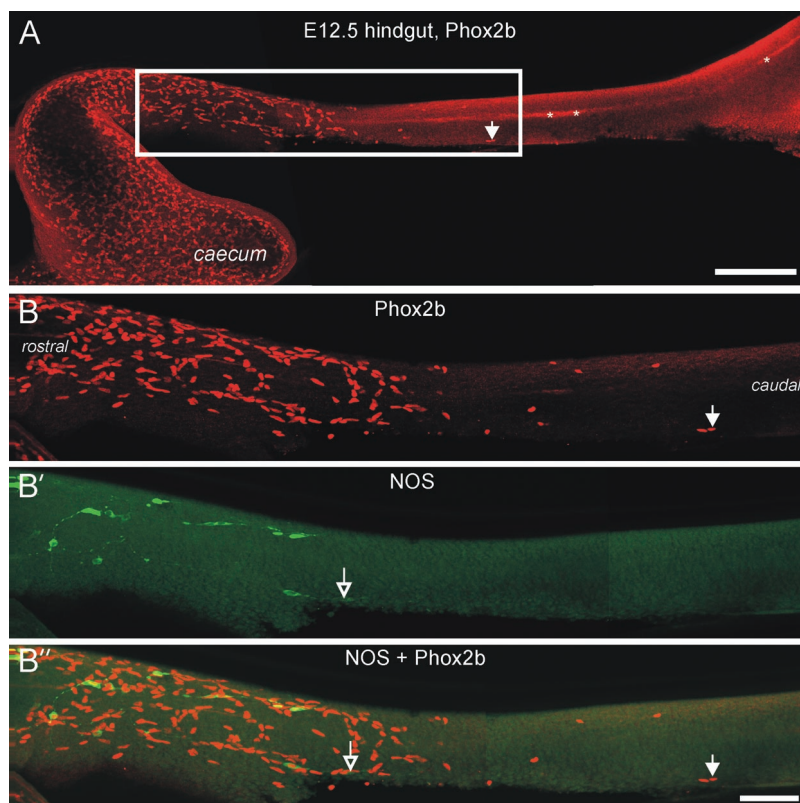
In the small and large intestine of adult mice, NOS neurons project anally (caudally) to innervate the circular muscle and other enteric neurons (Sang et al., 1997). Although the cell bodies of the NOS-immunoreactive neurons were well stained at E12.5, their processes were less well stained than the processes of the TH+ neurons. Nonetheless, an axon was discernible on a small percentage of the NOS cells, and in most such cases, the axons projected caudally (Fig. 5A). Moreover, NOS+ axons were present in the hindgut caudal to the most caudal NOS+ cell bodies, also indicating that at least some of the NOS neurons project caudally (Fig. 5B).

#### Overlap between NOS and PGP9.5 or TH immunostaining

At E12.5, all of the NOS+ cells showed PGP9.5 immunostaining, but not all of the PGP9.5+ cells were NOS+ (Fig. 6A, A').

The expression of TH by enteric neuron precursors is transient; at E12.5, the level of TH immunostaining is starting to decrease, and no TH immunoreactivity can be detected by E14.5. At low magnification, the TH and NOS cells appeared to form separate populations, because the cells showing the strongest NOS immunostaining appeared to be TH-negative, and the strongest TH+ cells appeared to be NOS-negative. However, at higher magnification, it became apparent many of the cells showing weak TH immunostaining also showed weak NOS immunoreactivity (Fig. 6B, B'). Because it was often difficult to decide whether particular NOS neurons showed TH immunostaining and vice versa (Fig. 6B, B'), precise counts of the degree of overlap between NOS and TH could not be made. Nonetheless, at E12.5, we estimate that approximately one-third of the TH+ cells showed definite NOS immunostaining, and vice versa. This raised the possibility that the TH+ cells are the precursors of NOS neurons and that the cells downregulate TH expression as they start to express NOS. We therefore exploited the findings of Baetge et al. (1990b) that, *in vitro*, the expression of TH by cells in the embryonic gut is not downregulated as it is *in vivo*, but is maintained for the life of the culture. We removed segments of E11.5 midgut and grew them in organ culture for 4–5 d and then examined the overlap between NOS and TH. Approximately 90% of the TH+ cells also showed definite NOS immunostaining and vice versa in the organ-cultured explants of gut (Fig. 6C, C') (in four explants,  $89 \pm 5\%$  of the TH cells was NOS+, and  $87 \pm 4\%$  of the NOS cells was TH+; 50 TH+ and 50 NOS+ cells were examined in each explant).

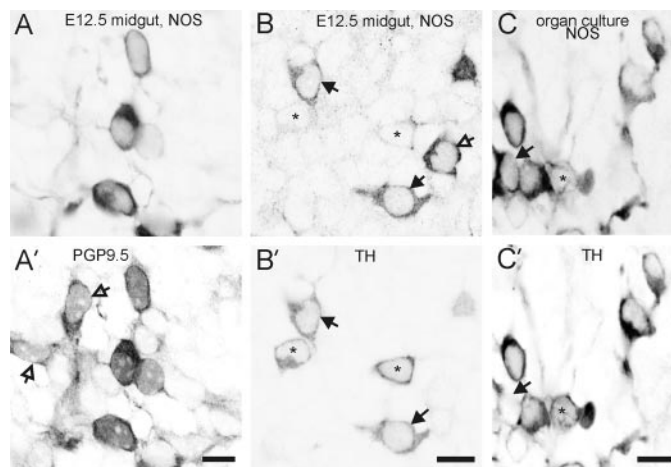
**Figure 4.** *A*, Whole-mount preparation of hindgut showing the location of Phox2b+ cells. Phox2b is expressed by all crest-derived cells, and the most caudal Phox2b+ cell (arrow) is approximately halfway along the hindgut. The Phox2b+ cells in the rostral hindgut are derived from vagal level neural crest, and although sacral level neural crest cells also contribute to the enteric nervous system in the hindgut of the mouse (Kapur, 2000), they do not enter the gut until after the vagal cells have colonized the entire hindgut. The region indicated by the rectangle is shown at higher magnification in *B*. The line of staining down the middle of the gut (asterisks) is nonspecific staining of the gut lumen. Scale bar, 250  $\mu$ m. *B*, *B'*, Paired, confocal microscope images of the region indicated in the square in *A* showing Phox2b immunostaining (*B*), NOS immunostaining (*B'*), and the merged images (*B''*). The most caudal NOS+ cell (open arrow, *B',B''*) in this preparation is rostral to the most caudal Phox2b+ cell (arrow, *B,B''*) by  $\sim 620$   $\mu$ m. Scale bar, 100  $\mu$ m.



**Figure 5.** Inverted, confocal microscope images of NOS-immunoreactive neurons in the E12.5 hindgut. *A*, An axon (arrows) can be identified on a small proportion of NOS+ neurons only, and the axon projects caudally. *B*, The most caudal NOS+ neurons in the hindgut. The most caudal NOS+ cell body (open arrow) shows only weak NOS immunostaining, and NOS+ axons (arrow) project caudally beyond the most caudal NOS+ cell body, also indicating that at least some NOS+ neurons project caudally. Scale bar, 50  $\mu$ m (applies to *A* and *B*).

#### Use of Dil to examine the development of neurons with different projection patterns *in vivo*

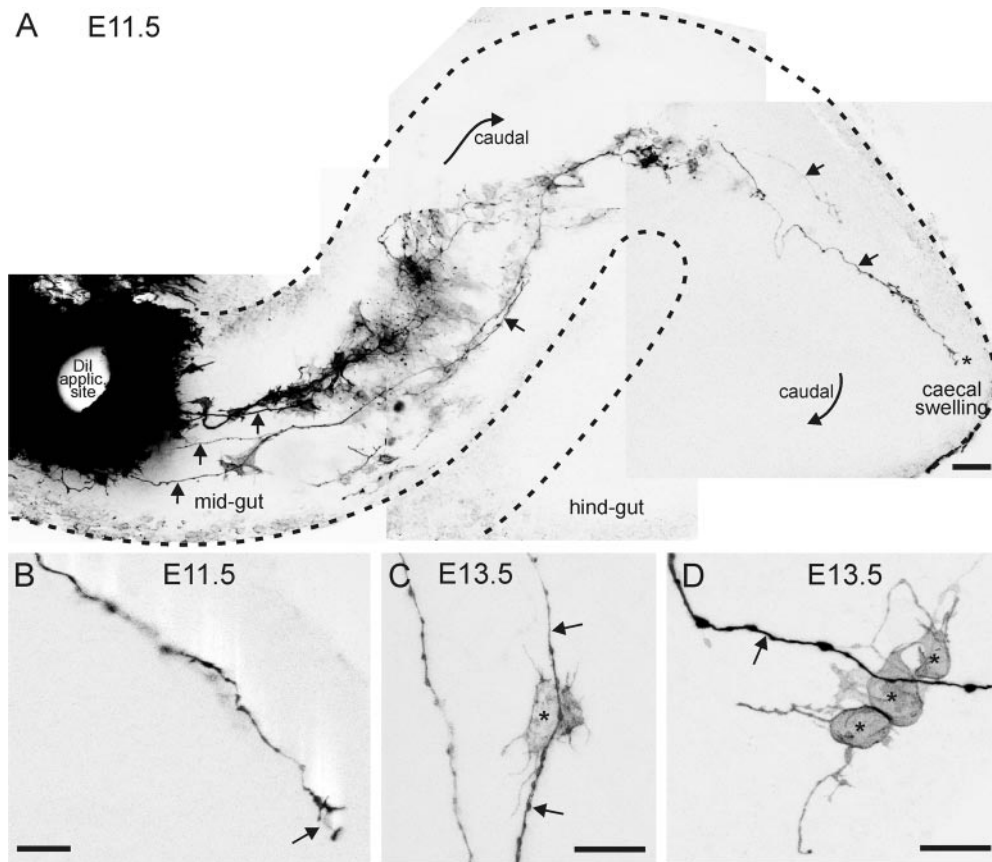
Dil was applied to fixed preparations of E10.5-E18.5 small and large intestine and adult ileum, and the preparations were left at 37°C for 7–10 d (embryonic gut) or 7 d to 2 months (adult preparations).



**Figure 6.** Paired, inverted confocal microscope images showing the overlap between NOS and PGP9.5 (*A,A'*) and NOS and TH (*B,B',C,C'*). *A,A'*, In the E12.5 midgut, all of the NOS+ neurons also show PGP9.5 immunoreactivity, but not all of the PGP9.5+ cells (open arrows) show NOS immunoreactivity. *B,B'*, In the E12.5 midgut, some cells (arrows) are both NOS+ and TH+, some NOS+ cells (open arrow) are TH negative, and some TH+ cells (asterisks) show little, if any, NOS immunoreactivity. *C,C'*, Segment of E11.5 midgut grown in catenary organ culture for 3 d. At the beginning of the culture period, none of the cells in the explant was NOS+. After 3 d in culture, there are many NOS+ cells (*C*), and all of the NOS+ cells are also TH+ (*C'*), although some strongly NOS+ cells (arrow) show only weak TH immunostaining, and some strongly TH+ cells (asterisk) show only weak NOS immunostaining. Scale bars, 10  $\mu$ m.

#### Embryonic gut: nerve fibers

Approximately 60% of the Dil application sites in the small and large intestine had labeled nerve fibers associated with them (Figs. 7*A*, 8*A,A',B*), but mesenchymal cells (E10.5-E13.5) or



**Figure 7.** Inverted, confocal microscope images of fixed whole-mount preparations of embryonic gut 7–10 d after the application of DiI. *A*, Labeled fibers caudal to a DiI application site in the E11.5 midgut. This preparation had no labeled fibers on the rostral side of the application site. Most of the labeled fibers (arrows) project longitudinally down the gut, but often show some deviations in their pathways. The most caudal labeled fiber (asterisk) is at the level of the caecum. Although the most caudal labeled fiber is >1.1 mm from the DiI application site, this does not necessarily represent the projection length of an individual neuron, because DiI appears to be able to be transferred from cell-to-cell. Scale bar, 50  $\mu$ m. *B*, Higher magnification image of the most caudal labeled fiber from the preparation shown in *A*. At the distal tip (growth cone) of the axon (arrow), the axon is slightly swollen and gives rise to a number of small processes, which are probably filopodia. Scale bar, 10  $\mu$ m. *C*, *D*, “Indirectly” labeled cell bodies (asterisks) in preparations of E13.5 small intestine. The cell bodies are closely associated with a labeled, passing axon (arrows). The indirectly labeled cell bodies usually are more weakly labeled than the axon with which they are closely associated, and they have no detectable direct connection with the DiI application site. Scale bar, 25  $\mu$ m.

smooth muscle cells (from E14.5) were labeled at all application sites. Most, but not all, of the application sites in which nerve fibers were labeled possessed nerve fibers both rostral and caudal to the application site (Fig. 8*A,A'*). The labeled fibers presumably included anterogradely and retrogradely labeled fibers. No labeled fibers were observed associated with any of the DiI application sites in the hindgut of E10.5 and E11.5 preparations. At E10.5–E13.5, most of the DiI-labeled nerve fibers extended longitudinally (rostrocaudally) along the gut (Figs. 7*A*, 8*A,A'*, 9*A*). At E11.5, after application of DiI to the middle part of the midgut, labeled fibers were observed projecting as far as the caecum (Fig. 7*A*), which is around the location of the most caudal TH+ processes and only slightly rostral to the migratory wave-front of neural crest-derived cells at this stage (Young et al., 1999). After the formation of myenteric ganglia at  $\sim$ E14.5, the labeled fibers took more circuitous routes (Fig. 8*B*). The distal tips of the labeled fibers sometimes possessed small swellings and processes, which may represent the growth cone (Fig. 7*B*), but other labeled fibers showed no specializations at their distal tips. Before E16.5, the labeled fibers were in the same plane of focus as the myenteric ganglia, but from E16.5, labeled fibers were also observed within the circular muscle. Varicosities, which were up to 5  $\mu$ m in diameter, were prominent along many of the labeled fibers (Fig. 8*C*), particularly on the caudal side of the DiI application site.

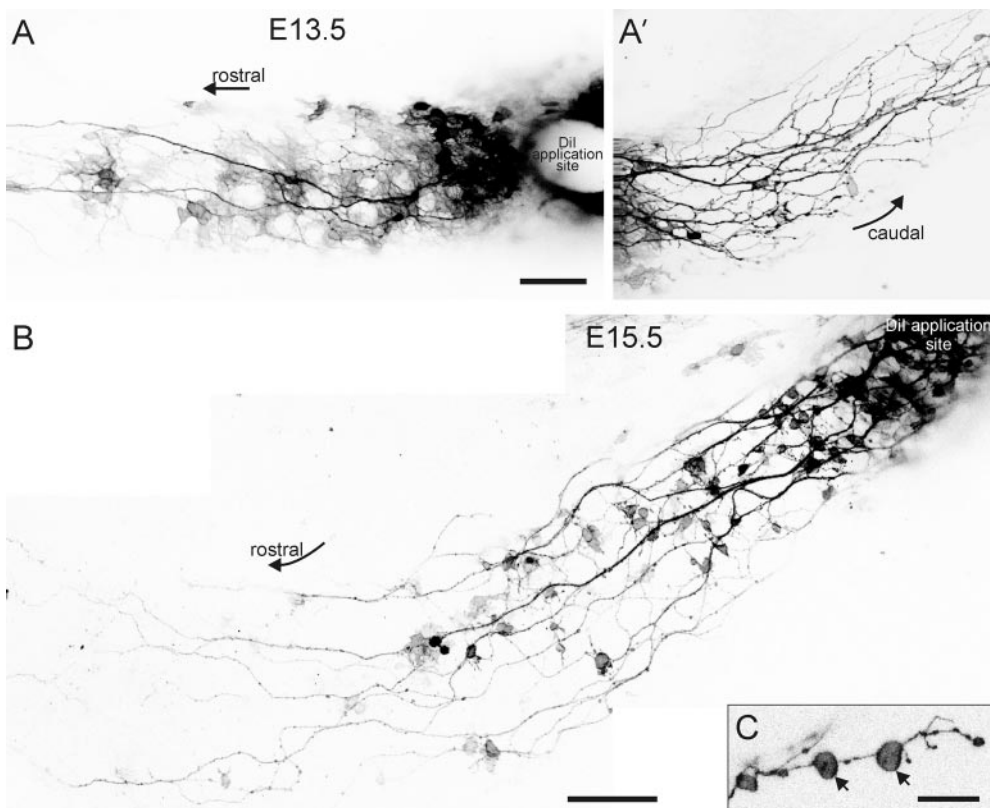
#### Embryonic gut: cell bodies

DiI-labeled cell bodies were observed on both the rostral (Fig. 8*B*) and caudal sides of many DiI application sites, but there were usually larger numbers of cell bodies on the rostral side of the

application sites than on the caudal side (Fig. 8*A,A'*). However, labeled cell bodies were very commonly observed that lacked processes projecting to the DiI application site but were in close apposition to DiI-labeled nerve fibers (Fig. 7*C,D*). These labeled cell bodies and fibers were usually more weakly stained than fibers that could be traced directly back to the DiI application site (Fig. 7*D*). Thus, it appears that many of the labeled cell bodies were not directly labeled from the DiI application site, but were labeled indirectly from leakage of DiI from adjacent, closely apposed, labeled nerve fibers. Because many of the DiI+ cell bodies may have been indirectly labeled, a DiI+ cell was only classified as being retrogradely labeled if the cell body was clear of any contact with neighboring cell bodies or fibers, and if it possessed an axon extending from the cell body to the DiI application site that was also clear of any contact with labeled cell bodies or axons (Fig. 9*A–F*).

The number of preparations set up, the number of retrogradely labeled cell bodies observed, and their polarity are shown in Table 3. Of the 60% of application sites that possessed labeled nerve fibers (see above), neurons that were definitively retrogradely labeled could only be identified in a small percentage of these preparations (Table 3). Of the 42 retrogradely labeled neurons observed, 40 (95%) had axons that projected caudally, and only two (5%) projected rostrally. Both of the rostrally projecting, retrogradely labeled neurons were in the small intestine, and all but one of the caudally projecting retrogradely labeled neurons was also found in the small intestine. The only retrogradely labeled neuron found in the hindgut projected caudally and was found in an E14.5 preparation. Because of the large size of the DiI application sites in relation to the diameter of the

**Figure 8.** Inverted, confocal microscope images of fixed whole-mount preparations of embryonic gut 7–10 d after the application of DiI. *A*, Labeled cell bodies and fibers both rostral (*A*) and caudal (*A'*) to a single DiI application site in a preparation of E13.5 small intestine. Compared with the rostral side, on the caudal side there are less labeled cell bodies and a higher proportion of varicose nerve fibers. Scale bar, 100  $\mu$ m. *B*, Labeled cell bodies and fibers rostral to a DiI application site in a preparation of E15.5 small intestine. Although labeled fibers are present >1.2 mm from the application site, they may not have been directly labeled from the DiI application site. Note that most of the labeled cell bodies lie on nerve bundles, and it is therefore difficult to trace an individual axon, unequivocally, back to the DiI application site. Scale bar, 100  $\mu$ m. *C*, High-magnification image of a single, DiI-labeled nerve fiber. Particularly on the caudal side of a DiI application site, many of the labeled fibers possessed large varicosities (arrows), up to 5  $\mu$ m in diameter. The nerve fiber also gives off some spines. Scale bar, 10  $\mu$ m.



embryonic gut, neurons with very short projections, and circumferentially projecting neurons would not have been detected in this study.

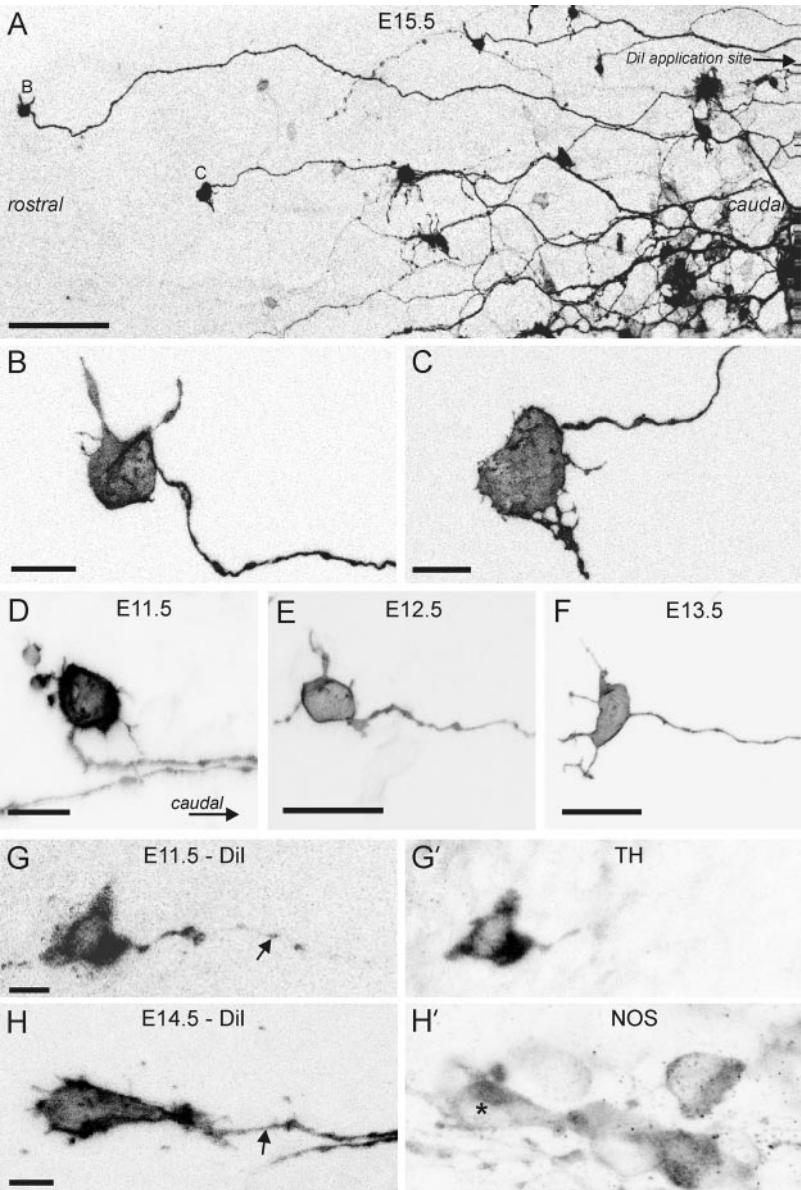
In fixed tissue, DiI inserts into the cell membrane and reveals all of the processes, including the finest extensions, of labeled cells (Godement et al., 1987). At all stages, including E10.5 and E11.5, the retrogradely labeled neurons had a single long process (an axon) and several short neurites (dendrites) (Fig. 9*A–F*). Thus, at E10.5 and E11.5, the morphology of the DiI-labeled cells was similar to that of most of the TH+ cells revealed immunohistochemically. A small number of preparations in which retrogradely labeled neurons had been identified were subsequently processed for either TH (E10.5 and E11.5) or NOS (E12.5 and older) immunohistochemistry. The data are shown in Table 4. In all of the preparations in which DiI-labeled neurons were successfully recovered, and in which the immunohistochemical staining was successful, the retrogradely labeled neurons projected caudally. Before E14.5, the caudally projecting neurons were all TH+ (at E10.5 and E11.5) (Fig. 9*G,G'*) or NOS+ (Fig. 9*H,H'*), but in the older embryos, two caudally projecting neurons that did not show NOS immunoreactivity were encountered (Table 4).

### Adult gut

DiI was applied to a small number of preparations of fixed adult small intestine. Labeled neurons were observed oral (rostral) (Fig. 10*A*), anal (caudal), and circumferential to the application sites. Unlike the embryonic preparations, there was little evidence of leakage of DiI, because it was common to observe ganglia containing many labeled nerve terminals, without labeled cell bodies (Fig. 10*B*).

### Effect of direction of migration on the projections of enteric neurons

To compare the effect of rostral-to-caudal colonization with caudal-to-rostral colonization of the hindgut by neural crest-derived cells on the development of neuronal polarity, segments of E11.5 caudal hindgut (which lack enteric neuron precursors) were grown in organ culture together with a segment of E10–10.5 fore-midgut, taken between the stomach swelling and the umbilicus, which contain vagal crest-derived cells (Young and Newgreen, 2001). The E10.5 gut segment was placed on the filter paper support at either the rostral or caudal end of the suspended E11.5 aneuronal explant (Fig. 11). Control cultures consisting of E11.5 caudal hindgut alone were also grown. After 3–4 d in culture, no PGP9.5+ neurons were observed in the E11.5 hindgut explants grown alone (Fig. 12*A*) ( $n = 6$ ), confirming that the E11.5 hindgut explants lacked enteric neuron precursors at the time of explantation. However, PGP9.5+ neurons were observed in the hindgut explants in which a source of vagal crest-derived cells (E10.5 midgut) was placed at the rostral end of the explant, and in the hindgut explants in which a source of vagal crest cells was placed at the caudal end of the explant (Fig. 12*C,D*). Thus, although vagal crest-derived cells normally migrate from rostral-to-caudal, at least some of them are capable of migrating from caudal-to-rostral. The number of PGP9.5+ neurons in each type of coculture was compared. Because of the tubular nature of the gut, it was not possible to count accurately the number of cells on the sides and bottom surface of the explants, so the number of PGP9.5+ cells on the top surface of the explants was counted. There were significantly more PGP9.5+ cells in the explants in which the crest cells migrated from rostral-to-caudal than in explants in which the cells migrated from caudal-to-rostral (Fig.



**Figure 9.** Inverted, confocal microscope images of neurons in the embryonic gut retrogradely labeled with DiI. *A*, Low-magnification image of the rostral side of a DiI application site in the small intestine of an E15.5 mouse. Two cell bodies (*B*, *C*) have axons that project toward the application site and are not closely apposed to other labeled neurons. These neurons were consequently identified as caudally projecting neurons. Scale bar, 100  $\mu$ m. *B*, *C*, High-magnification images of the retrogradely labeled neurons shown in *A*. Scale bars, 10  $\mu$ m. *D–F*, Examples of caudally projecting, retrogradely labeled neurons from preparations of E11.5 (*D*), E12.5 (*E*), and E13.5 (*F*) small intestine. Scale bars: *D*, 10  $\mu$ m; *E*, *F*, 25  $\mu$ m. *G*, *G'*, *H*, *H'*, Paired micrographs of retrogradely labeled neurons (*G*, *H*) that were subsequently processed for immunohistochemistry using antisera to TH (*G'*) or NOS (*H'*). Note that the DiI labeling becomes diffuse during the immunohistochemical processing. *G*, *G'*, A caudally projecting neuron from the midgut of an E11.5 mouse (*G*) is TH+ (*G'*). The axons of the retrogradely labeled neurons in *G* and *H* are indicated with arrows. Scale bar, 10  $\mu$ m. *H*, *H'*, A caudally projecting neuron from the small intestine of an E14.5 mouse (*H*) is NOS+ (asterisk, *H'*). Scale bar, 10  $\mu$ m. Note that it was not possible to determine whether the retrogradely labeled neurons were interneurons or motor neurons, because it was not possible, in embryonic gut, to apply DiI selectively to either the muscle or the ganglia (up until E14.5, the neurons have not even coalesced into ganglia).

**Table 3. Projections of neurons retrogradely labeled with DiI**

Age	E10.5	E11.5	E12.5	E13.5	E14.5	E15.5	E16.5	E18.5
No. of preparations examined	17	69	63	11	44	7	27	28
No. of rostrally-projecting neurons	0	0	1	0	1	0	0	0
No. of caudally-projecting neurons	1	4	7	4	13	5	2	4

12*B*) (unpaired *t* test, *p* = 0.03). Thus, although some vagal crest-derived cells are capable of migrating caudo-rostrally through explants of hindgut and differentiating into neurons, fewer do so than when they migrate rostrocaudally. The projections of the neurons in the hindgut explants was examined. Three cocultures in which the E10.5 midgut was placed

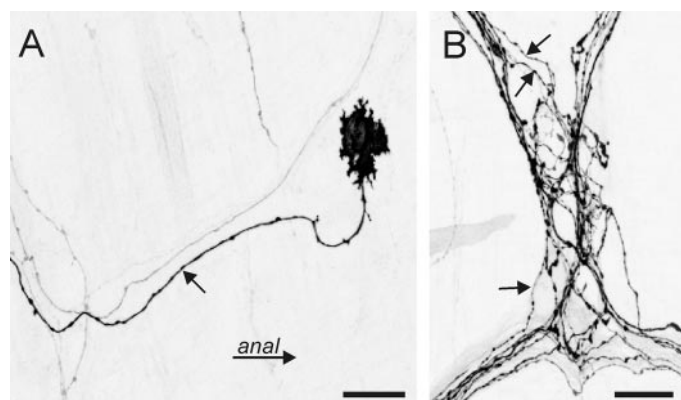
at the rostral end and three cocultures in which the E10.5 midgut was placed at the caudal end of the E11.5 hindgut explants were fixed, and then DiI was applied to the explants. Although some labeled nerve fibers and a small number of labeled cell bodies were observed on both sides of the application sites (Fig. 13*G,H*), no definitive, retrogradely labeled cell bodies were recovered. We

**Table 4. Immunohistochemical status of retrogradely-labeled neurons<sup>a</sup>**

Age	TH or NOS immunoreactivity <sup>b</sup>
E10.5	1/1 was TH+
E11.5	1/1 was TH+
E12.5	4/4 were NOS+
E13.5	1/1 was NOS+
E14.5	2/3 were NOS+
E16.5	0/1 was NOS+

<sup>a</sup>All of the neurons in which immunohistochemistry was successfully performed projected caudally.

<sup>b</sup>This table only includes preparations in which the immunohistochemistry was deemed to be successful, as judged by the presence of many TH+ or NOS+ neurons in the preparation. Many DiI-labeled preparations, particularly from the older embryonic stages, did not show any immunostaining after processing for immunohistochemistry.



**Figure 10.** Inverted, confocal microscope images of DiI labeling in the myenteric plexus of the small intestine of adult mice. *A*, An orally (rostrally) projecting neuron labeled by application of DiI to the circular muscle. The axon (arrow) projects rostrally toward the DiI application site. *B*, DiI-labeled nerve fibers (some of which are arrowed) in a myenteric ganglion. Despite the presence of many labeled nerve terminals, there are no labeled cell bodies, indicating no leakage of DiI from nerve fibers into cell bodies as occurs in embryonic tissue. Scale bars, 25  $\mu$ m.

therefore examined the projections of the neurons in the E11.5 hindgut explants by performing circumferential (myotomy) lesions. The cocultures were grown for 4 d, and then a circumferential cut was made through the outer mesenchyme of the E11.5 hindgut explant, severing the nerve fibers running within the explant. After a further 45–60 min in culture, the explants were fixed and processed for PGP9.5 immunohistochemistry. The directions in which nerve fibers were projecting were then determined by the accumulation of immunoreactivity in the proximal stumps of the severed neurites. When the E10.5 midgut explants were cultured abutting the rostral end of the E11.5 aneural

hindguts, 58% (7 of 12) of the hindguts possessed swollen varicosities only on the rostral side of the lesion, indicating the presence of caudally-projecting neurons only (Fig. 12*A–C*), 42% (5 of 12) had swollen varicosities on both the rostral and caudal sides of the lesion, indicating the presence of both rostrally and caudally projecting neurons (Fig. 12*D*), and none of the cultures had swollen varicosities only on the caudal side of the lesion (Table 5). Some of the preparations in which there were swollen processes on both sides of the lesion, possessed approximately equal numbers of varicosities on the rostral and caudal sides of the lesion (Fig. 12*D*), whereas others had more swollen processes on the rostral side than the caudal side. When the E10.5 midgut explants were cultured abutting the caudal end of the E11.5 aneural hindgut, none of the hindgut explants had swollen varicosities on the rostral side of the lesion only, 44% (4 of 9) had swollen varicosities on both the rostral and caudal sides of the lesions, and 56% (5 of 9) of the hindguts possessed swollen varicosities only on the caudal side of the lesion (Fig. 12*E*), indicating the presence of rostrally projecting neurons only. In some cocultures in which cells had migrated from caudal-to-rostral, the density of neurons was low at the rostral end of the recipient gut explant and individual PGP9.5+ neurons could be observed. All of the individual neurons in which an axon could be discerned projected rostrally (Fig. 12*F*).

## DISCUSSION

### The first enteric neurons are TH+

Transiently catecholaminergic (also called TC or TH) cells are present in the mouse gut during development (Cochard et al., 1978; Teitelman et al., 1978). At E10.5, TH cells comprise ~15% of vagal crest-derived cells within the gut, and they are close to the migratory wavefront as the cells migrate rostrocaudally (Young and Newgreen, 2001). This study and previous studies (Baetge and Gershon, 1989; Baetge et al., 1990a) have shown that TH cells express a range of neuron-specific proteins, including neurofilament 145 kDa, PGP9.5, peripherin, GAP-43, MAP2, and MAP5, but lack Sox10, a molecule involved in peripheral gliogenesis. Thus, a subpopulation of vagal crest-derived cells very close to the migratory wavefront starts to differentiate into neurons. At E10.5 the early neurons all express TH, but by E11.5, cells expressing neuron-specific proteins are present that are not TH+.

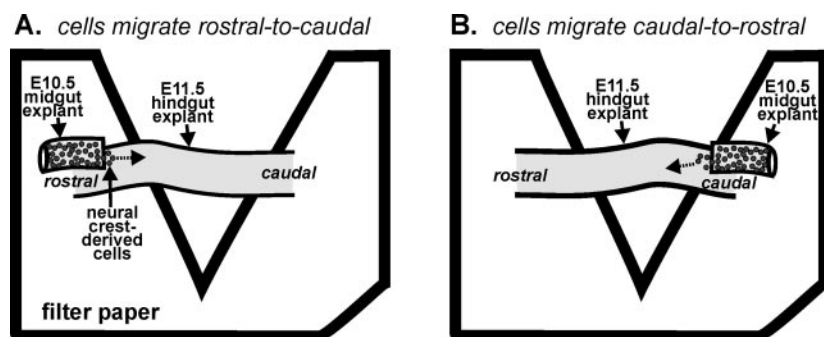
### TH cell derivatives

TH is expressed transiently. 5-HT neurons appear to be a derivative of TH cells because *Mash1*  $-/-$  mice lack both TH+ cells and 5-HT neurons (Blaugrund et al., 1996). The current study suggests that NOS neurons are also derivatives of TH cells. NOS neurons comprise 25–30% of myenteric neurons in the mouse small intestine, whereas the 5-HT neurons comprise only 1% of

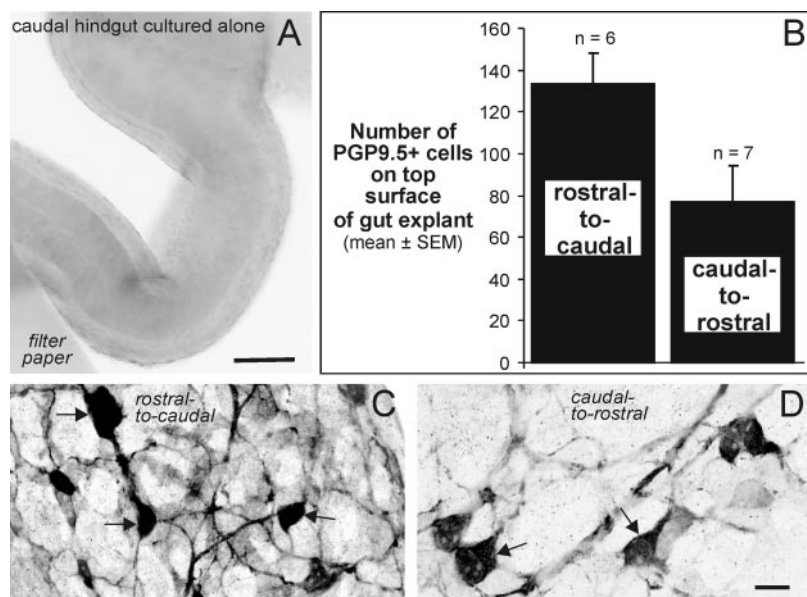
**Table 5. Presence of swollen nerve fibers at lesion sites in explants of aneural hindgut grown as cocultures with a source of vagal neural crest cells (E10.5 midgut)**

Direction of migration	Swollen processes only on rostral side of lesion	Swollen processes on both rostral and caudal sides of lesion	Swollen processes only on caudal side of lesion
Rostral-to-caudal	7/12	5/12	0/12
Caudal-to-rostral	0/9	4/9	5/9

In some cultures, the source of neural crest cells was placed at the rostral end of the explant so that the crest-derived cells migrated from rostral-to-caudal (as they do *in vivo*), and in the other cultures, the vagal crest derived cells were placed at the caudal end of the hindgut explant so that the crest-derived cells migrated from caudal-to-rostral, which they do not normally do. The presence of swollen processes on the rostral side of the lesion indicates the presence of caudally projecting neurons, and the presence of swollen processes on the caudal side of the lesion indicates the presence of rostrally projecting neurons.



hindgut explant and migrate from rostral-to-caudal. In *B*, the crest-derived cells originating from the E10.5 gut segment will migrate through the E11.5 hindgut from caudal-to-rostral.



**Figure 11.** Diagrams showing the arrangement of E10.5 midgut and E11.5 hindgut segments in cocultures. The postcaecal hindgut from E11.5 mice is suspended across the V shapes cut in a piece of filter paper, and the caudal end is indicated by cutting the corner off the filter paper. *A*, In some preparations, segments of E10.5 gut, taken from between the stomach swelling and the umbilicus, are placed on the filter paper at the rostral end of the E11.5 hindgut explant. *B*, In other cocultures, the E10.5 explant is placed on the filter paper at the caudal end of the E11.5 hindgut explant. No crest-derived cells are present in the E11.5 postcaecal hindgut at the time of explantation. In *A*, the vagal crest-derived cells originating from the E10.5 explant enter the aneural E11.5

**Figure 12.** *A*, Inverted fluorescence micrograph of a whole-mount preparation of postcaecal, caudal hindgut grown alone in catenary organ culture on filter paper supports for 4 d and then fixed and processed for PGP9.5 immunohistochemistry. No PGP9.5+ cells are present. Scale bar, 100  $\mu$ m. *B*, Number of PGP9.5+ cells on the top surface of hindgut explants in which vagal crest-derived cells migrated from rostral-to-caudal ( $n = 6$  cocultures) or caudal-to-rostral ( $n = 7$ ). There were significantly more PGP9.5+ cells in the gut explants in which they migrated from rostral-to-caudal than in explants in which they migrated from caudal-to-rostral (unpaired  $t$  test,  $p = 0.03$ ). *C*, *D*, Inverted fluorescence micrographs of explants of aneural hindgut grown in coculture for 4 d with a source of neural crest cells at either the rostral (*C*) or caudal (*D*) end and then processed for PGP9.5 immunohistochemistry. PGP9.5+ cells (arrows) are present in both types of coculture. Scale bar, 10  $\mu$ m.

neurons (Sang and Young, 1996); the 5-HT neurons may therefore arise from the 10% of TH cells that were NOS-negative. Although NOS neurons appear to express TH transiently during development, TH expression is not an obligatory step in enteric NOS neuron development, because although TH cells are absent from the gut of *Mash1*<sup>−/−</sup> mice and from avians, enteric NOS neurons are present in *Mash1*<sup>−/−</sup> mice (Q. Sang and H. M. Young, unpublished observations) and avians (Li et al., 1994).

### TH cells in the hindgut

Although TH cells can be within one cell of the migratory wavefront at E10.5, over the next 24–48 hr of development they become progressively further from the wavefront, and are never observed in the hindgut. When the gut of E10.5 mice was grown in organ culture, TH+ cells were found in the hindgut. The presence of TH cells in cultured hindgut may be attributable to the persistence of expression of TH *in vitro* (Baetge et al., 1990b). Alternatively, although differentiation and migration occur in organ culture similar to *in vivo*, the growth of cultured gut is considerably less than *in vivo* (Hearn et al., 1999). Thus, TH cells may be able to colonize the hindgut *in vitro* because they have less distance to migrate than *in vivo*; as the TH cells have neuronal characteristics, they may have poor migratory abilities.

### Vagal crest cells can migrate caudorostrally

Vagal crest-derived cells migrate rostrocaudally through the gut. In cocultures performed in this study, we showed that vagal cells

are also capable of migrating caudorostrally and differentiating into neurons. However, significantly less neurons were present within gut explants after caudal-to-rostral than rostral-to-caudal migration. This may be because (1) for vagal cells, the most distal hindgut is less attractive than the rostral hindgut to migrate into, (2) survival and/or proliferation of vagal cells is higher when they migrate rostrocaudally, or (3) a greater proportion of vagal cells differentiate into neurons, or they differentiate faster, when they migrate rostrocaudally.

### Correlation between direction of vagal crest migration and axon projection

Immunohistochemistry, lesion experiments, and DiI labeling showed that the first neurons in the gut (TH–NOS neurons) project predominantly caudally, which is the same direction as the vagal crest cells are migrating, and the axons of developing neurons were closely associated with undifferentiated crest-derived cells. Associations between migrating neural crest cells and outgrowing axons are also observed outside of the gut. Before entering the gut, vagal-level crest cells migrate along the same pathway as the vagal fibers that enter the stomach, although the migration of crest cells precedes that of the outgrowing vagus nerve (Baetge and Gershon, 1989). *In vitro*, sympathetic cell bodies migrate along neurites (Kawasaki et al., 2002). During peripheral nerve development, crest-derived Schwann cell precursors migrate along the same pathway as emerging motor and

sensory nerve fibers. However, it is unclear whether the Schwann cell precursors follow nerve fibers (Carpenter and Hollyday, 1992) or vice versa (Noakes and Bennett, 1987; Noakes et al., 1988) or whether they comigrate (Noakes et al., 1993), and whether the mechanisms guiding Schwann cell migration and peripheral nerve fiber navigation are common. Although both motor axons and crest cells traverse the same pathway through the rostral halves of the somites, the molecular guidance mechanisms within the somites appear to be different (Koblar et al., 2000).

It has recently been realized that axon guidance and cell migration are similar, and some molecules (e.g., Slit) can influence both processes (Li et al., 1999; Rakic, 1999; Wu et al., 1999; Song and Poo, 2001; Wingate, 2001). The main difference between migration and axon navigation is that the cell body remains stationary in axonal navigation (Rakic, 1999). It is feasible that similar mechanisms contribute to both rostrocaudal crest cell migration and caudally directed, initial axon projection within the embryonic gut, but that the response to the guidance cue or cues depends on the state of differentiation: undifferentiated cells migrate, and differentiating neurons extend an axon. Multiple mechanisms are likely to be responsible for the migration of vagal crest cells through the gut (Taraviras and Pachnis, 1999), including the presence of chemoattractive molecules, such as GDNF, in the gut mesenchyme (Young et al., 2001). "Population pressure" also appears to influence migration because when the number of premigratory vagal crest cells is reduced surgically, the caudal regions of the gut are not colonized (Yntema and Hammond, 1954; Peters-van der Sanden et al., 1993; Burns et al., 2000).

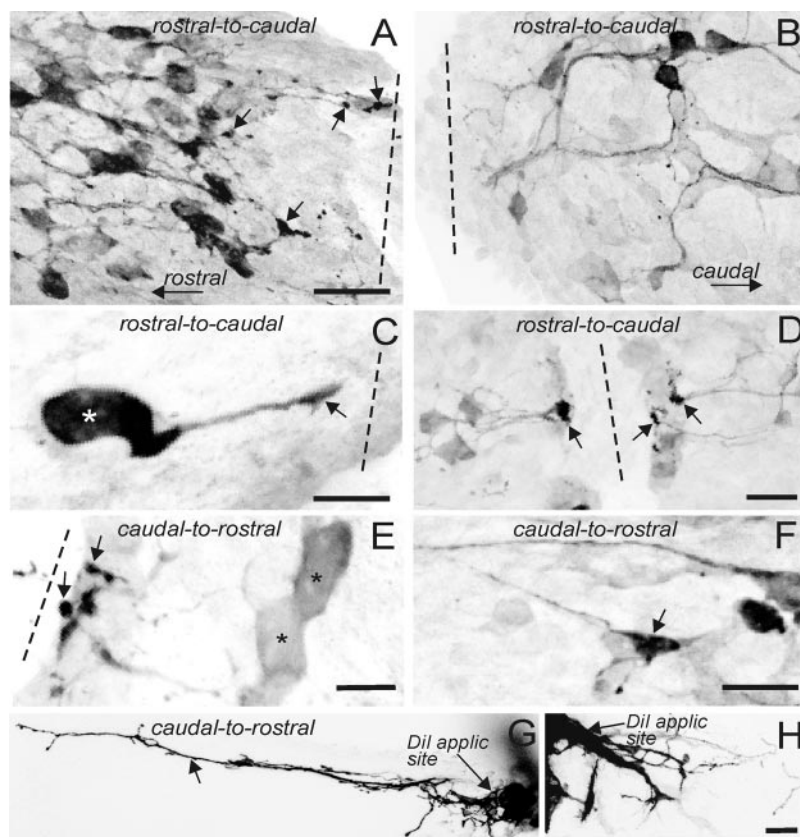
We examined the projections of neurons in explants in which vagal crest-derived cells migrated rostrocaudally or caudorostrally. Migration direction appears to influence axon projection

because in the majority of explants in which the crest cells migrated from rostral-to-caudal, only caudally projecting neurons could be detected and in over one-half of the explants in which cells migrated from caudal-to-rostral, only rostrally projecting neurons were observed. Surprisingly, however, 42% of the control cocultures in which vagal crest cells migrated from rostrocaudally (as they do *in vivo*) had swollen processes on both the rostral and caudal sides of the lesions, indicating that both rostrally and caudally projecting neurons were present. Although a small proportion of lesioned E11.5 midgut preparations also had some swollen processes on the caudal sides of the lesion, they were vastly outnumbered by swollen processes on the rostral sides of the lesions. In contrast, there were sometimes equal numbers of swollen processes on the rostral and caudal sides in the cocultures. It therefore appears that some of the mechanisms determining axon projection are not reproduced in organ culture. The projections of enteric neurons are probably determined by multiple mechanisms; the direction of cell migration appears to be one of the mechanisms affecting the first enteric neurons, but other factors such as the longitudinal growth of the gut (which is not reproduced in culture), and the presence of attractive or repulsive substances within the gut mesenchyme, may also be important.

### Functional significance

The neuronal circuitry underlying peristalsis has both orally (rostrally) projecting, cholinergic neurons responsible for ascending excitation and anally (caudally) projecting, neurons, most of which contain NOS, that are responsible for descending inhibition (Costa et al., 1996; Furness, 2000; Brookes, 2001). This study has shown that the anally projecting, NOS neurons develop early, and throughout embryonic development, the vast majority of neurons

**Figure 13.** Inverted fluorescent micrographs of whole-mount preparations of aneural E11.5 hindgut cocultured with E10.5 fore/midgut explants at either the rostral (*A, C, D*) or caudal (*B, E–H*) end (Fig. 11) for 4 d, then lesioned and processed for immunohistochemistry using antibodies to the neuron-specific protein, PGP9.5. In all images, rostral is to the left and caudal is to the right, and the sites of lesions are marked with dotted lines. *A–D*, Effect of lesions on explants in which the E10.5 segment was placed at the rostral end of the E11.5 hindgut explant. In one preparation, swollen processes (arrows) are present on the rostral side of the lesion (*A*), but no swollen processes are present on the caudal side (*B*) of the lesion, indicating that only caudally projecting neurons were present. Scale bar, 25  $\mu$ m (applies also to *B*). *C*, High-magnification image of a cell body close to the rostral side of a lesion. The cell body (asterisk) gives rise to a caudally projecting axon that terminates in a swollen process (arrow) at the lesion site. Scale bar, 10  $\mu$ m. *D*, In a different preparation in which vagal crest cells had migrated from rostral-to-caudal before lesioning, swollen processes (arrows) are present in approximately equal numbers on both the rostral and caudal sides of the lesion, indicating the presence of both rostrally and caudally projecting neurons. Scale bar, 25  $\mu$ m. *E, F*, Preparations in which vagal crest cells had migrated from caudal-to-rostral before lesioning. *E*, Swollen processes (arrows) on the caudal side of a lesion, indicating the presence of rostrally projecting neurons. The asterisks indicate cell bodies close to the lesion. Scale bar, 10  $\mu$ m. *F*, Rostral end of a gut explant (away from the lesion site) where the density of neurons is low. Rostrally projecting PGP9.5+ neurons (arrow) can be clearly seen. Scale bar, 25  $\mu$ m. *G, H*, DiI was applied to a preparation in which vagal crest-derived cells migrated from caudal-to-rostral. DiI-labeled fibers (which may have been labeled retrogradely or anterogradely) are present both rostral (*G*) and caudal (*H*) to the application site. Scale bar, 50  $\mu$ m.



projected caudally. Functional studies of peristalsis have yet to be performed in embryonic mice. Nonetheless, meconium is present in the hindgut of late fetal mice, indicating that intestinal contents do move in an anal (caudal) direction before birth. As few rostrally projecting neurons were detected in the embryonic gut, the movement of intestinal contents in an anal direction during fetal stages may be mediated by (1) descending relaxation only or (2) both excitatory and inhibitory pathways, but the excitatory pathways are short, local pathways and were not detected in the current study. The latter possibility seems unlikely because cholinergic properties (choline acetyltransferase and vesicular acetylcholine transporter immunoreactivity) cannot be detected until ~E18.5 to postnatal day 0 (H. M. Young and B. R. Jones, unpublished observations). Future studies are required to determine if there is a pool of undifferentiated cells that persists until after birth that gives rise to cholinergic, rostrally projecting neurons.

### Leakage of DiI between neurons during embryonic development

Many of the neurons labeled with DiI in the embryonic gut appear to have been “indirectly” labeled by leakage of DiI from neighboring labeled neurons. In contrast, there was little evidence of DiI spread between neurons in adult gut. In the developing CNS, gap junctions appear to be important in producing functional neuronal assemblies (Kandler and Katz, 1995). Neural crest cells (Lo et al., 1997; Huang et al., 1998; Bannerman et al., 2000), including those in the gut (Lang et al., 2000), also appear to possess gap junctions, which may be responsible for the transfer of DiI between developing enteric neurons. Alternatively, it is possible that DiI can cross the “immature synapses” that are present between developing enteric neurons (Vannucchi and Faussone-Pellegrini, 2000).

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