INTRODUCTION

The enteric nervous system (ENS) is a complex network of neurons and glia within the wall of the intestine that controls intestinal motility, regulates mucosal secretion and blood flow, and also modulates sensation from the gut (Costa and Brookes, 1994; Grider and Foxx-Orenstein, 1998; Kunze and Furness, 1999). The cells that form the ENS migrate into the gut from the vagal, sacral and rostral trunk neural crest (Gershon et al., 1992; Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Young and Newgreen, 2001). During migration, neural crest cells actively proliferate to create enough neurons and glia to populate the gut. These cells then differentiate into a wide variety of neuronal subtypes that are required for normal intestinal motility and function. If there is inadequate proliferation, migration or survival of neural crest precursors, the distal intestine will be aganglionic. Distal intestinal aganglionosis causes tonic contraction of the intestinal muscle resulting in functional obstruction (i.e. Hirschsprung’s disease) (Martucciello et al., 2000; Skinner, 1996). Although Hirschsprung’s disease is the most dramatic example of disordered intestinal motility that results from abnormal ENS morphogenesis, more subtle changes in ENS structure also cause altered intestinal motility (De Giorgio et al., 2000; Kapur, 2001). This includes hypo- and hyper-ganglionosis (Shirasawa et al., 1997; Yamataka et al., 2001), as well as the loss of specific neuronal subpopulations (Blaugrund et al., 1996). These changes may cause functional constipation, intestinal pseudo-obstruction (Camilleri, 2001; Stanghellini et al., 1988) or irritable bowel syndrome (Aros and Camilleri, 2001; Quigley, 1999; Schmulson, 2001; Spiller, 1999).

In most parts of the central and peripheral nervous system up to twice as many neurons are produced as will be needed in the mature organism (Hutchins and Barger, 1998; Macaya, 1996; Oppenheim, 1996; Roth and D’Sa, 2001). Neurons that extend their projections to the correct target receive target-derived trophic factors that allow them to survive. Neurons that do not correctly reach their targets or that do not receive an adequate amount of neurotrophic factor are removed by programmed cell death (apoptosis). This allows the number of innervating neurons to be matched to their target size. In addition, the distance from the neuronal cell body to the innervation target of the neuron dictates the length of neuronal projections.

SUMMARY

To clarify the role of Ret signaling components in enteric nervous system (ENS) development, we evaluated ENS anatomy and intestinal contractility in mice heterozygous for Ret, GFRα1 and Ret ligands. These analyses demonstrate that glial cell line-derived neurotrophic factor (GDNF) and neurturin are important for different aspects of ENS development. Neurturin is essential for maintaining the size of mature enteric neurons and the extent of neuronal projections, but does not influence enteric neuron number. GDNF availability determines enteric neuron number by controlling ENS precursor proliferation. However, we were unable to find evidence of programmed cell death in the wild type ENS by immunohistochemistry for activated caspase 3. In addition, enteric neuron number is normal in Bax−/− and Bid−/− mice, suggesting that, in contrast to most of the rest of the nervous system, programmed cell death is not important for determining enteric neuron numbers. Only mild reductions in neuron size and neuronal fiber counts occur in Ret−/− and Gfra1−/− mice. All of these heterozygous mice, however, have striking problems with intestinal contractility and neurotransmitter release, demonstrating that Ret signaling is critical for both ENS structure and function.

Key words: GDNF, ENS, Apoptosis, Ret, Neurons, Mouse
fibers. For the ENS to function properly, enteric neuron number and the extent of neuronal fibers must also be closely controlled. Although neurotrophic factors essential for formation of the ENS have been identified, the biological processes regulated by these factors are not yet clear. Also, in contrast to the rest of the nervous system, the environment at the cell body and the tip of the axon are remarkably similar for many myenteric neurons. For this reason, it is more difficult to understand how ‘target-derived’ trophic factors could be used to determine which enteric neurons survive or the extent of axonal projections within the ENS.

To better understand the factors that regulate enteric neuron number and the extent of neuronal fiber projections within the ENS, we have conducted a detailed analysis of the enteric nervous system in mice with defects in the Ret signaling system. Ret is a transmembrane tyrosine kinase that is essential for formation of the ENS (Schuchardt et al., 1996) and acts as a receptor for the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs). There are four known GFLs [GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN)] (Baloh et al., 2000) that activate Ret. Ret activation also requires a glycosylphosphatidylinositol (GPI)-linked co-receptor (GFRα1,GFRα2,GFRα3 or GFRα4) that determines Ret ligand specificity. GFRα1 interacts preferentially with GDNF (Jing et al., 1996; Treanor et al., 1996), GFRα2 with NRTN (Baloh et al., 1997; Jing et al., 1997; Sanciona et al., 1997; Suvanto et al., 1997), GFRα3 with ARTN (Baloh et al., 1998a; Baloh et al., 1998b; Worby et al., 1998) and GFRα4 with PSPN (Enokido et al., 1998; Milbrandt et al., 1998). Both GDNF and NRTN are important for ENS morphogenesis and function. However, despite the comparable ability of GDNF and NRTN to promote ENS precursor proliferation and axonal extension in vitro (Chalazonitis et al., 1998a; Hearn et al., 2001; Heuckeroth et al., 1998), the phenotype of Gdnf+/− and Nrt+/− mice is very different. Gdnf+/− mice have hypoganglionosis in the stomach and aganglionosis of the small bowel and colon (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Nrt−/− mice have a normal number of myenteric neurons, but a reduced neuronal fiber density and abnormal intestinal contractility (Heuckeroth et al., 1999).

To determine whether GDNF and NRTN play distinct or partially redundant roles in controlling ENS morphogenesis, we have examined the ENS in mice deficient in both GDNF and NRTN (Gdnf−/−/Nrt−−). In addition, we have examined the ENS in mice heterozygous for GDNF (Gdnf+/−), as well as Gdnf+/−/Nrt−−, Ret+/− and Gfra1+− animals. These studies demonstrate that GDNF and NRTN perform largely non-redundant functions in the ENS. We have confirmed the recent observation (Shen et al., 2002) that Gdnf−/− mice have enteric hypoganglionosis. We have also demonstrated that this hypoganglionosis occurs because GDNF availability determines the rate of ENS precursor proliferation. Interestingly, we were unable to find any significant role for programmed cell death in controlling enteric neuron number. By contrast, NRTN availability determines acetylcholinesterase-stained neuronal fiber density in the mature ENS, but does not influence neuron number. In addition, we demonstrate that striking changes in intestinal contractility can occur in the absence of dramatic anatomic changes within the ENS.

**MATERIALS AND METHODS**

**Animals**

Ret+/− (Schuchardt et al., 1994) and Gdnf+/− mice (Moore et al., 1996) were generous gifts of Dr Frank Costantini and Genentech respectively. Bax−/− (Shirasawa et al., 1997) and Bcl−/− mice (Yin et al., 1999) were generous gifts of Dr Stan Korsmeyer. Nrm−/− (Heuckeroth et al., 1999) and Gfra1−/− (Enomoto et al., 1998) mice are described elsewhere. All mice were backcrossed two to four generations to C57BL/6. The adult ENS was analyzed at 14-20 weeks of age. For embryo studies, vaginal plug date was considered to be E0.

**Whole mount preparation**

Small bowel and colon were dissected from adult mice and mesentery was removed. Tissues were incubated one hour in oxygenated Krebs-Ringer solution (Sigma K4002) with nicardipine (2 mM) to relax muscles. Three (4 cm) pieces of proximal small bowel and two (2 cm) pieces of distal colon were analyzed. The most distal colon and two most proximal small bowel segments were acetylcholinesterase stained (Enomoto et al., 1998; Heuckeroth et al., 1999). Other segments were Cuprolinic Blue stained (Karaosmanoglu et al., 1996).

**Pgp9.5/BrdU double labeling**

E12 pregnant females were injected intraperitoneally with bromodeoxyuridine (BrdU) (50 μg/g body weight). Embryos were harvested 3 hours later, fixed (4% paraformaldehyde, 3 hours, room temperature) and paraffin wax embedded. Sections (6 μm) were cut and processed as described (Enomoto et al., 1998). Sections were incubated with PGP9.5 rabbit antibody (Biogenesis, 1:200) overnight, 4°C. A Cy3 tyramide signal amplification (TSA) kit (Perkin-Elmer Life Sciences) was used to identify the Pgp9.5-expressing cells. Sections were then rinsed in TBS, denatured in 1 N HCl (45 minutes), neutralized in 0.1 M sodium tetraborate (10 minutes) and blocked in 10% normal donkey serum in TBST (1 hour) before incubation with biotinylated mouse anti-BrdU antibody ( Oncogene, unlabeled) overnight. BrdU-positive cells were visualized with a Fluorescein TSA kit. A total of 150 Pgp9.5-expressing cells from three wild-type and three Gdnf+/− E12 embryos were evaluated for BrdU incorporation.

**Single label fluorescent immunohistochemistry**

Activated caspase 3 antibody (D175, Cell Signaling Technology, 1:100) staining was performed on 6 μm paraffin wax-embedded specimens. GFRα1 and GFRα2 (BASF560 and BAF429 respectively; R&D Systems, 1:40) immunohistochemistry was performed on 12 μm fresh frozen sections. Primary antibodies were placed on sections overnight at 4°C. Signals were detected using either a Cy3 or Fluorescein TSA kit.

**Myenteric plexus fiber counts**

Myenteric fiber counts were performed on acetylcholinesterase-stained wholemounts by counting fibers crossing a 0.12 mm2 grid (Heuckeroth et al., 1999) with five horizontal and six vertical lines. Twenty randomly selected fields per mouse from three animals of each genotype were evaluated. Data was analyzed using SigmaPlot and StatSoft for Windows (one way analysis of variants).

**Cell counts**

Cuprolinic Blue stained myenteric and acetylcholinesterase-stained submucosal neurons were counted to determine the number of neurons within a 0.25 mm2 grid. Cells in 20 randomly selected fields were counted from three animals of each genotype. ‘Total neuron numbers’ were determined by multiplying the neuron counts/unit area by the total area of the small bowel or colon. Because colon and small bowel sizes were similar in all animals, data in Figs 2 and 5 are similar to neuronal density data.
Cell size
Cell size was determined for acetylcholinesterase-stained submucosal neurons and Cuprolinic Blue stained myenteric neurons using the UTHSCSA Image Tool Program (http://ddsdx.uthscsa.edu/dig/download.html). Three animals of each genotype were evaluated (50 cells per animal).

Functional contractility studies
Contractility and transmitter release were measured in adult mouse small bowel and colon as previously described (Heuckeroth et al., 1999).

RESULTS
GDNF availability determines neuron number in the ENS
Although GDNF and NRTN are both important for ENS structure and function, it is not known whether these neurotrophic factors have redundant roles. To address this question, we compared the ENS in Nrtn+/–/Gdnf+/– with that in Gdnf+/– mice. Even though Gdnf+/– mice have severe intestinal aganglionosis, neurons are readily visible in the stomach and esophagus (Moore et al., 1996; Sanchez et al., 1996). If these few remaining neurons survive because of NRTN, then the aganglionosis in the Nrtn+/–/Gdnf+/– mice should be more extensive than in Gdnf+/– animals. Using acetylcholinesterase staining, a commonly employed technique to visualize enteric neuron cell bodies and fibers, we found identical defects in P0 Nrtn+/–/Gdnf+/– and Gdnf+/– mice (data not shown). This suggests that NRTN has minimal effect on enteric neuron survival or proliferation before birth.

As the severe loss of enteric neurons in Gdnf+/– mice might make it difficult to appreciate a subtle role for NRTN in determining neuron number, we examined the ENS in adult Gdnf+/– and Gdnf+/–/N rtn+/– mice. Both Gdnf+/– and Gdnf+/–/N rtn+/– mice appeared healthy. We did not observe animals with intestinal distension or evidence of obstruction; however, the ENS appeared hypoganglionic in Gdnf+/– mice (Fig. 1). To establish more definitively whether Gdnf+/– mice have an abnormal number of enteric neurons, we performed quantitative analysis of neuron number in the myenteric and submucosal plexus of the adult gut (Fig. 2A). To determine whether the distal bowel was more significantly affected than the proximal bowel, as it is in Hirschsprung’s disease, enteric neuron numbers were determined separately for proximal and distal bowel. This analysis demonstrated that Gdnf heterozygotes have 43% fewer small bowel and 48% fewer colonic myenteric neurons than wild-type littermates (Fig. 2A, P<0.001). Gdnf+/– mice also have fewer submucosal neurons, with a 33% reduction in the small bowel (P=0.028) and a 32% reduction in the colon (P=0.08). Because of variability between animals, the reduced number of colon submucosal neurons does not reach statistical significance. Nonetheless, Gdnf haploinsufficiency clearly causes significant reductions in enteric neuron number.

We next compared cell counts for Gdnf+/– mice with those in Gdnf+/–/N rtn+/– mice (Fig. 2A). Although there are slightly fewer neurons in all regions of the gut examined in Gdnf+/–/N rtn+/– mice compared with Gdnf+/– mice, these differences did not reach statistical significance (P>0.7). The relatively limited role for NRTN in determining enteric neuron number is also supported by the observation that the number of colonic submucosal neurons (P=0.85), and the number of colon (P=0.175) and small bowel (P=0.32) myenteric neurons in N rtn+/– mice is normal. Interestingly, N rtn+/– mice had 35% fewer small bowel submucosal neurons numbers than wild-type littermates (P=0.017), suggesting that NRTN plays a minor role in determining submucosal neuron number. Even in the small bowel submucosal plexus, however, GDNF is more important for determining neuron number, as N rtn+/– and Gdnf+/– mice have comparable neuron counts.

Neurturin availability determines cell size and neuronal fiber density for mature enteric neurons
Our previous analysis demonstrated that N rtn+/– mice have smaller myenteric neurons than wild-type animals and a reduced neuronal fiber density in the

Fig. 1. Wholemount small bowel myenteric plexus staining for Gdnf+/–, Gdnf+/–/N rtn+/–, Nrtn+/– and wild-type mice. (A,B) Cuprolinic Blue staining highlights clustered neuronal cell bodies (red arrows) without showing neuronal processes. Gdnf+/– mice (B) have fewer neurons, but normal neuronal cell size. (C-F) Acetylcholinesterase stains both neuronal cell bodies and fibers. Gdnf+/– mice (D) have a normal density of acetylcholinesterase-stained fibers compared with wild-type (C). N rtn+/– (E) and Gdnf+/–/N rtn+/– (F) mice have an obvious loss of small acetylcholinesterase-stained neuronal fibers. Black arrowheads identify small fibers and fiber bundles in Gdnf+/– and Gdnf+/–/N rtn+/– mice (D,F). Scale bar: 100 μm.
small bowel (Heuckeroth et al., 1999). To establish whether NRTN provides equivalent trophic support to small bowel and colonic myenteric neurons, ENS neuronal cell size was determined in both regions of the bowel (Fig. 2B). Myenteric neuron size was reduced by 29% in the small bowel and 33% in the colon of Nrtn−/− mice (P<0.001). In addition, the density of acetylcholinesterase-stained neuronal fibers in the myenteric plexus is reduced by 32% in the small bowel (P<0.001) and 22% in the colon (P<0.001) of Nrtn−/− mice (Fig. 2C). Thus, NRTN provides equivalent trophic support to small bowel and colonic myenteric neurons. The effect of NRTN on submucosal neuron size is more subtle (Fig. 2B). Although cell size is normal in both Nrtn−/− and Gdnf+/− mice, Gdnf+/−/Nrtn−/− animals have smaller than normal submucosal neurons in both the small bowel (14%, P<0.001) and in the colon (17%, P<0.001). This suggests that both GDNF and NRTN may provide trophic support for mature submucosal neurons.

To establish more definitively whether GDNF provides trophic support to mature myenteric neurons, we determined acetylcholinesterase-stained myenteric plexus fiber counts in the small bowel and colon of Gdnf+/− and Nrtn−/−/Gdnf+/− mice. If GDNF were important for determining neuronal fiber outgrowth from myenteric neurons, then Gdnf+/− mice would be expected to have fewer neuronal fibers than wild-type littermates. Surprisingly, acetylcholinesterase-stained myenteric plexus fiber counts in both the colon and small bowel of Gdnf+/− mice are normal, despite the 43-48% loss of myenteric neurons (Fig. 2C). The limited role for GDNF in determining the density of acetylcholinesterase-stained fibers within the myenteric plexus is also supported by the observation that neuronal fiber density is comparably reduced in Nrtn−/− and Gdnf+/−/Nrtn−/− mice (Fig. 2C). Although we cannot exclude the possibility that the normal density of acetylcholinesterase-stained fibers in the ENS of Gdnf+/− mice results in part from increased extrinsic innervation, these studies demonstrate that the acetylcholinesterase-stained fiber density in the ENS is critically dependent on the availability of NRTN and not GDNF.
Heterozygosity for GFRα1 or Ret causes only mild changes in ENS anatomy

Because Hirschsprung’s disease in humans is frequently caused by haploinsufficiency for Ret (Edery et al., 1994; Romeo et al., 1994), we examined the ENS in Ret+/– and Gfra1+/– mice. Remarkably, even with detailed quantitative analysis, myenteric (P>0.8) and submucosal (P>0.29) neuron numbers in the small bowel and colon of Gfra1+/– and Ret+/– mice (Fig. 2A) were identical to wild-type animals. Thus, in contrast to the results with GDNF, haploinsufficiency for Ret or GFRα1 does not appear to affect ENS neuron number in mice.

To establish whether Ret or GFRα1 heterozygosity influences mature enteric neurons, both cell size and fiber densities were determined for Ret+/– and Gfra1+/– mice (Fig. 2B,C). In the myenteric plexus there were significant reductions in cell size in the small bowel (35%; P<0.001), and colon (34%; P<0.001) of Ret+/– mice. Ret+/– mice also had 16% smaller colon submucosal neurons (P<0.001), but cell size was normal for small bowel submucosal neurons. These changes in neuronal cell size were similar to the reductions seen in Nrtn–/– mice. Myenteric neuron acetylcholinesterase-stained fiber counts were also reduced (11-13%, P<0.001) in Ret+/– mice, but not to the extent seen in Nrtn–/– animals (Fig. 2C).

Because GFRα1 is the preferred GDNF co-receptor and GDNF is not important for determining ENS neuronal cell size or acetylcholinesterase-stained fiber density, we expected these parameters to be normal in Gfra1+/– mice. However, neuronal cell size was reduced in colon submucosal neurons (19%; P<0.001), and in colonic (20% decrease; P<0.001) and small bowel (31% decrease; P<0.001) myenteric neurons (Fig. 2B) compared with wild-type littermates. Gfra1+/– mice also had 11% fewer small bowel (P=0.002) and a 12% fewer colonic (P<0.001) acetylcholinesterase-stained myenteric plexus fibers (Fig. 2C). These findings raise the possibility that GFRα1 and Ret heterozygotes may have abnormal intestinal motility, despite normal enteric neuron numbers, and imply that GFRα1/Ret signaling is important for the function of some mature enteric neurons.

GFRα1 and GFRα2 expression patterns in the ENS reflect the function of these receptors

During development, ENS precursors actively divide within the intestine to produce enough neurons to populate the gut. Although GDNF and NRTN have similar effects on ENS precursors in culture (Heuckeroth et al., 1998), Gdnf+/– and Nrtn+/– mice have strikingly different ENS phenotypes. These differences would be best explained if GFRα1 was expressed at high levels early in development, while ENS precursors are proliferating and GFRα2 expressed at high levels in the mature gut. To test this hypothesis, immunohistochemical staining for GFRα1 and GFRα2 was performed on the gut of wild-type E14, P0, P7 and adult mice. At E14, GFRα1 is easily seen within the gut wall in a region broader than that occupied by the neural crest (Fig. 3A). This suggests that GFRα1 protein is present on both neural crest and mesenchymal components of the gut wall. The location and number of the neural crest cells within the gut is indicated by PGP9.5 staining (Fig. 3B). By contrast, GFRα2 staining is less intense than at E14 but there is no detectable GFRα2 staining. Newborn (C,D) and adult (E,F) small bowels express GFRα1 (FITC) and GFRα2 (FITC), but GFRα2 is much more easily detected in adult (F) than in newborn mouse gut. Scale bar: 100 μm.

GDNF controls enteric neuron number by regulating cell proliferation, not cell death

Because programmed cell death is the predominant mechanism for determining mature neuron numbers in most of the nervous system, we were interested in determining whether enteric neuron number is also controlled by apoptosis. We began looking for apoptosis using immunohistochemistry for activated caspase 3, a well-characterized marker for apoptotic...
programmed cell death in most of the central and peripheral nervous system (Bar-Peled et al., 1999; Deckwerth et al., 1996; Vekrellis et al., 1997; White et al., 1998). Bid is essential for tumor necrosis factor- and FAS ligand-mediated apoptosis (Yin et al., 1999), and is implicated in neuronal cell death in several nerve injury paradigms (Henshall et al., 2001; Plesnila et al., 2001). In agreement with the activated caspase 3 immunohistochemistry, the number of enteric neurons in the colon and small bowel of Bax- and Bid-deficient mice was the same as in wild-type animals (Fig. 5). To investigate the role of Bax and Bid further, we counted enteric neurons in Gdnf+/−/Bax−/− and Gdnf+/−/Bid−/− mice. Because the Gdnf−/− mice have 32-33% and 43-48% reductions in submucosal and myenteric neurons, respectively, relative to control animals, we reasoned that if Bax- or Bid-dependent apoptosis contributed to enteric neurons loss, deficiency in these pro-apoptotic molecules should prevent the neuron loss in the Gdnf−/− mice. The number of enteric neurons in Gdnf−/−/Bax−/− and Gdnf−/−/Bid−/− mice was, however, the same as in Gdnf−/− animals.

GDNF heterozygotes have reduced ENS precursor proliferation in vivo

Because we could not find evidence of apoptosis in the ENS, we performed BrdU/PGP9.5 double label immunohistochemistry to determine whether reduced ENS precursor proliferation could be responsible for hypoganglionosis in Gdnf−/− mice. PGP9.5 is expressed in the developing and mature enteric neural crest (Sidebotham et al., 2001), and BrdU is incorporated into proliferating cells. In wild-type embryos, 14% of PGP9.5-expressing cells were BrdU labeled (Fig. 6). Gdnf−/− embryos had 43% fewer BrdU+/PGP9.5+ cells (P=0.01). These

![Fig. 4.](image-url) Apoptotic cells are easily detected in the DRG, but not in the gut. (A,B) Activated caspase 3 immunohistochemistry easily identifies apoptotic cells in E12 DRG (A, white arrows), but not in the E12 ENS (B). Auto-fluorescent nucleated red blood cells are also seen (red arrows) in both the gut and DRG. (C,D) Sections of the gut at E14 (C) and P0 (D) were stained with antibodies to activated caspase 3 (Cy3 secondary antibody) and with antibodies to PGP9.5 (FITC secondary antibody) to show ENS precursors. Activated caspase 3 stained cells were not seen within the ENS at any age examined. Scale bars: in A, 100 μm for A; in B, 100 μm for B-D.

![Fig. 5.](image-url) Enteric neuron number is not influenced by Bax or Bid deficiency. Quantitative neuron counts in the myenteric and submucosal plexus of wild-type, Bax−/−, Bid−/−, Gdnf−/−, Gdnf−/−/Bax−/− and Gdnf−/−/Bid−/− mice are presented. Although GDNF heterozygosity caused reduced neuron numbers, Bax and Bid-deficient animals had normal numbers of enteric neurons. In addition, Bax and Bid deficiency did not rescue the reduction in neurons seen in the GDNF heterozygous mice.
findings suggest that the amount of GDNF available to proliferating ENS precursors critically determines the proliferative capacity of these cells and that the extent of ENS precursor proliferation ultimately determines the number of enteric neurons in the adult gut. Thus, in contrast to the rest of the nervous system, cell number in the adult ENS appears to be largely determined by cell proliferation rather than cell death.

**Nrtn**/–, **Gdnf**/–, **Gfra1**/– and **Ret**/– mice all have abnormal intestinal contractility in vivo

Although anatomical changes in the ENS of **Nrtn**/– and **Gdnf**/– mice are striking, changes in the ENS of **Gfra1**/– and **Ret**/– mice are very subtle. To ascertain whether any of these anatomical changes cause significant functional problems, intestinal contractility was measured for both colon and small bowel segments in an oxygenated organ bath. These studies demonstrated dramatic decreases in both longitudinal and circular muscle contraction in response to electric field stimulation in all of the mutant animals tested (Fig. 7A-D). Surprisingly, contractility was strikingly reduced even in the **Gfra1**/– and **Ret**/– mice that have only subtle changes in neuron size and neuronal fiber density compared to WT animals.

One possible explanation for these findings is that a small, but functionally important subset of enteric neurons is affected in the **Gfra1**/– and **Ret**/– animals. To determine whether these functional contractility problems are likely to reflect a defect in the major excitatory and inhibitory motoneuron populations rather than a defect in a small neuronal subpopulation, we measured release of the excitatory and inhibitory neurotransmitters substance P and VIP, respectively. These transmitters are expressed in largely non-overlapping subsets of enteric neurons that together account for 65% of myenteric neurons (Sang and Young, 1996). Both substance P and VIP release were reduced in all heterozygous mice tested (Fig. 6E-H). In most cases, the reduction in transmitter release was dramatic (70-95% reductions), although VIP release was relatively preserved in the colon of **Nrtn**/– mice (35-70% reduction). The finding that both VIP and substance P release is reduced in **Nrtn**/–, **Gdnf**/–, **Gdnf**/–/**Nrtn**/–, **Gfra1**/– and **Ret**/– mice demonstrates that a large percentage of the myenteric neurons are likely to be affected by these mutations and suggests that the functional defects do not simply result from loss of a small neuronal subpopulation.

**DISCUSSION**

The enteric nervous system is created by migration, proliferation and differentiation of vagal, sacral and rostral trunk neural crest cells precursors. Because the neural crest initially are a small population of cells, active proliferation is required to create enough cells to form the ENS. Once these cells differentiate into neurons, they extend processes to form an interconnected plexus that controls intestinal motility, mucosal secretion, and responds to sensory stimuli from the gut. For this system to work well, neuronal cell number and the extent of neuronal projections must be carefully regulated. By examining the ENS in mice with haploinsufficiency for Ret signaling system components, we have clarified the mechanisms that determine cell number and acetylcholinesterase-stained neuronal fiber density within the ENS. These studies lead to several key conclusions. First, Ret activation by GDNF is not only essential for formation of the ENS, but GDNF availability also determines the total number of enteric neurons in both the colon and small bowel. A recent study confirmed this observation in a much larger cohort of **Gdnf**/– mice (Shen et al., 2002). These authors also demonstrated functional motility problems in vivo in **Gdnf** heterozygotes that correlate with the reduced contractility observed in our in vitro system. Second, unlike most other parts of the nervous system, neuron number in the wild-type ENS appears to be largely determined by controlling ENS precursor proliferation rather than by programmed cell death. Third, although GFRα1 is still expressed in the adult mouse ENS, it is less important for trophic support of mature enteric neurons than NRTN and GFRα2. Fourth, NRTN and GFRα2 are not important for determining myenteric neuron number, but may play a minor role in determining submucosal neuron numbers. This is consistent with the observation that myenteric neurons are essentially post-mitotic by P0, whereas submucosal neurons continue to proliferate until P15 (Pham et al., 1991) and that GFRα2 is not expressed in the gut at the time of most active myenteric neuron precursor proliferation. Finally, these studies demonstrate that functional abnormalities in intestinal contractility may be more severe than anatomic defects within the ENS. This suggests that NRTN and GDNF influence aspects of enteric neuronal function (e.g. transmitter release, neuronal excitability) that are not identified with anatomic features.
techniques. Although this has not been previously demonstrated within the ENS, this hypothesis is supported by the observation that GDNF and NRTN increase spontaneous transmission frequency at the *Xenopus* neuromuscular junction (Wang et al., 2001; Wang et al., 2002). In addition, GDNF increases substance P levels in striatal neurons (Humpel et al., 1996). Other neurotrophic factors (e.g. neurotrophin 3, brain derived neurotrophic factor, neurotrophin 4/5) are also known to modulate neurotransmitter release and synaptic function (Arenas et al., 1996; He et al., 2000; Lessmann et al., 1994; Lessmann and Heumann, 1998; Li et al., 1998; Liou and Fu, 1997; Lohof et al., 1993). The role of GDNF and NRTN in synaptic transmission within the ENS will need further investigation.
Programmed cell death and the ENS

In most parts of the central and peripheral nervous system, neurons are created by active proliferation of neuronal precursors. As in the ENS, these precursors often migrate during development to reach their final destination. The neurons then differentiate, stop migrating and extend neuronal processes toward their targets. The innervation targets secrete trophic factors that innervating neurons depend on for survival. Many neurons are particularly sensitive to trophic factor deprivation at the time of target innervation (Bennet et al., 2002; Ernfors, 2001; Giehl, 2001; Korschning, 1993). Neurons that fail to extend processes to their targets, or to compete for an adequate amount of trophic factor are eliminated by programmed cell death. This paradigm for developmentally regulated apoptosis is widespread in the peripheral and central nervous system. In fact, in most parts of the nervous system, 20-80% of all neurons produced during embryogenesis die before adulthood (Oppenheim, 1991). This process is thought to provide an efficient means for matching neuronal populations to the size of the innervation targets and for ensuring that neuronal processes are correctly targeted.

Although apoptosis in the nervous system is a common way of determining the final number of neurons, there are some neuronal populations that do not appear to undergo naturally occurring cell loss. This includes neurons in the pontine nuclei (Armstrong and Clarke, 1979), red nucleus, locus ceruleus (Oppenheim, 1981) and chick spinal cord interneurons (McKay and Oppenheim, 1991). Similarly, we have been unable to find evidence of naturally occurring cell death in the ENS using activated caspase 3 staining, a method specific for detecting cells undergoing apoptosis (Srinivasan et al., 1998).

To look for additional evidence that apoptosis is important for determining enteric neuron number, we analyzed the ENS in Bax- and Bid-deficient mice. For most parts of the nervous system, programmed cell death depends on the presence Bax (White et al., 1998). Bax deficiency in mice causes increased neuronal cell numbers and virtually eliminates apoptosis in spinal cord motor neurons, trigeminal ganglia, trigeminal brain stem nuclear complex, facial nucleus, DRG, sympathetic ganglia, cochleovestibular ganglia, cerebellum, post-natal retinal ganglion cells and in the hippocampus. The failure of Bax deficiency to alter enteric neuron number, even in the setting of GDNF haploinsufficiency, is therefore consistent with the idea that cell death is not a major determinant of neuronal cell number in the ENS. In some regions of the nervous system, however, cell death is Bax independent (e.g. retina from E11.5 to P1) (White et al., 1998), and it remains possible that other Bcl2 family members regulate cell death within the ENS. One potential candidate for regulating neuronal cell death in the ENS was Bid. Although Bid deficiency is not important for programmed cell death in most parts of the nervous system (Leonard et al., 2001), Bid has been implicated in neuronal apoptosis in the setting of nerve injury (Henshall et al., 2001; Plesnila et al., 2001). However, Bid deficiency also does not alter enteric neuron number.

Trophic factors and the ENS

These findings do not imply that enteric neurons or ENS precursors are trophic factor independent. In fact, the hypoganglionosis in Gdnf+/+ mice and the reduction in neuronal cell size and acetylcholinesterase-stained myenteric fiber density in Nrtn+/+ mice provides strong evidence that mature enteric neurons and their precursors are trophic factor dependent. The BrdU labeling studies, however, are consistent with the idea that enteric neuron number is determined by controlled cell proliferation rather than by apoptosis in wild-type mice. We do not propose that ENS precursors are resistant to apoptosis, but rather that apoptosis is not important for determining neuron number during normal ENS development. Indeed, cell death of ENS precursors is seen in the esophagus of Ret+/+ (Taraviras et al., 1999) and Phox2b+/+ mice (Pattyn et al., 1999), and in the mesenchyme surrounding the neural tube in Sox10Dom+/+ mice (Kapur, 1999; Southard-Smith et al., 1998). In addition, adult guinea pig enteric neurons undergo apoptosis in response to glutamate (Kirchgessner et al., 1997), and trkC-expressing rat ENS precursors undergo apoptosis in response to NT3 withdrawal (Chalazonitis et al., 2001). Thus, despite demonstrable trophic factor dependence, the absence of identifiable cell death in wild-type mice suggests that during normal development ENS precursors receive an adequate amount of trophic factor for survival.

Trophic factor dependence changes during ENS development

Like many other neuronal populations, enteric neurons and their precursors appear to switch trophic factor dependence during development. In the early stages of cell migration and proliferation, GDNF activation of Ret via GFRα1 is absolutely required for both the survival and proliferation of all ENS precursors in the small bowel and colon. As development proceeds, many enteric neurons become dependent on neurturin and GFRα2 for trophic support. This is demonstrated by the reduced size of myenteric neurons within the ENS of adult Nrtn+/+ mice and by the loss of acetylcholinesterase-stained fibers in these animals. The switch in ENS trophic factor dependence from GDNF to NRTN is similar to the change in trophic factor dependence that occurs in the parasympathetic nervous system (Enomoto et al., 2000) where GDNF is required early for proliferation and migration of
neuronal precursors, but NRTN is essential for maintenance of neuronal projections in the mature animal. Because of the complexity of the ENS, however, it seems likely that subpopulations of enteric neurons will be supported by distinct neurotrophic factors and neuroepoietic cytokines. This hypothesis is supported by the observation that Gfra2−/− mice have normal appearing NADPH diaphorase-stained neuronal fibers (Rossi et al., 1998) and, at least in the rat, NADPH diaphorase and acetylcholinesterase stain largely non-overlapping populations of neurons (Aimi et al., 1993). The dependence of enteric neuron subsets on other trophic factors is also supported by the observation that both Nrtk3−/− and Ntrk3−/− (trkC) mice have a reduced number of enteric neurons and that only a subset of enteric neurons retrogradely transport its innervation target determines neuronal fiber length for DRG system. For example, the distance from the DRG cell body to peripheral nervous system. The reliance on controlled cell proliferation instead of cell death

Heterozygosity for Ret signaling components results in a range of abnormalities in the ENS structure and function

Gene dosage effects, such as the dramatic reduction in enteric neuron number seen in Gdnf−/+ mice, suggest that GDNF is produced in limiting quantities during murine ENS development. This is consistent with the idea that the quantity of neurotrophic factor available often limits neuronal survival or proliferation. The normal cell numbers in Ret−/+ and Gfra1−/+ mice implies that while ENS precursors are proliferating, wild-type mice have a significant excess of Ret and GFRα1 and thus ENS precursors proliferation is not affected by reduced Ret or GFRα1 gene dosages. The reductions in neuron size and acetylcholinesterase-stained fiber counts observed in adult Ret−/+ and Gfra1−/+ mice imply more limited production of these signaling components in the mature mouse ENS. This is consistent with the reduced intensity of GFRα1 immunohistochemical staining in the adult mouse ENS compared with the level seen at E14. Interestingly, Ret mutations that cause Hirschsprung’s disease (distal intestinal aganglionosis) in humans are typically inactivating mutations that are penetrant in the heterozygous state. This implies that human ENS precursors have more limiting Ret expression than the corresponding murine cells. Recent data also suggest that in human populations the penetrance of Ret mutations may depend on the presence of second site modifiers that could influence the level of Ret expression (Gabriel et al., 2002).

Enteric neuron number is controlled by regulating proliferation instead of cell death

Mechanisms that control many aspects of enteric neural crest patterning are similar to those used in other parts of the peripheral nervous system. The reliance on controlled cell proliferation in the ENS rather than programmed cell death to determine neuron number, however, may derive from anatomic differences between the ENS and other parts of the nervous system. For example, the distance from the DRG cell body to its innervation target determines neuronal fiber length for DRG neurons. Cells with inadequate dendritic extension to reach their targets are eliminated by apoptosis. Similarly, neurons whose processes extend to ‘incorrect’ targets do not receive the right type of neurotrophic factor and are also eliminated by apoptosis. For many neurons within the ENS, these mechanisms cannot apply. Several types of myenteric neuron, for example, extend their processes within the muscular layer of the gut (Costa and Brookes, 1994; Furness, 2000). For these neurons, the environment at the tip of the axon is similar to the environment near the cell body. If the only requirement for these axons is that the fibers reach a source of neurturin, this could be met within the tissues directly adjacent to the neuronal cell body eliminating the need for the axon to extend any significant distance. As an alternative strategy, it appears that the amount of neurturin available to each neuron determines the extent of acetylcholinesterase-stained fibers. This would explain why Ntrtn−/− mice have a reduction in acetylcholinesterase-stained fibers while Gdnf−/+ mice have an increased ratio of neuronal fibers to neuronal cell number, but a normal total number of fibers. Using apoptosis to eliminate neurons within the ENS with ‘incorrectly’ targeted axons would also be difficult for many myenteric neurons because within the myenteric plexus, the environment is similar in all directions. Instead, it appears that ENS cell number is largely determined by the ability of GDNF to control cell proliferation. Thus, although GDNF and neurturin availability, respectively, control neuronal cell number and neuronal fiber density within the ENS, mechanisms that control axon targeting within the ENS will need further investigation.

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