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GDNF availability determines enteric neuron number by controlling precursor proliferation

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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

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 hyperganglionosis (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, 1991; 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

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; Sanicola 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., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999), the phenotype of *Gdnf*$^{-/-}$ and *Nrtn*$^{-/-}$ 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). *Nrtn*$^{-/-}$ 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*$^{-/-}$/*Nrtn*$^{-/-}$). In addition, we have examined the ENS in mice heterozygous for GDNF (*Gdnf*$^{+/-}$), as well as *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$, *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 *Bid*$^{-/-}$ mice (Yin et al., 1999) were generous gifts of Dr Stan Korsmeyer. *Nrtn*$^{-/-}$ (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, undiluted) 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 (BAF560 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 mm^2 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 SigmaStat software (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 mm^2 grid. Cells in 20 randomly selected fields were counted from three animals of each genotype. 'Total neuron numbers' were determined by multiplying the neurons/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 neurons and neuronal 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*$^{+/-}$/*Nrtn*$^{-/-}$ mice. Both *Gdnf*$^{+/-}$ and *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$ 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 duodenum and distal colon. 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*$^{+/-}$/*Nrtn*$^{-/-}$ mice (Fig. 2A). Although there are slightly fewer neurons in all regions of the gut examined in *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$ 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 *Nrtn*$^{-/-}$ mice is normal. Interestingly, *Nrtn*$^{-/-}$ 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 *Nrtn*$^{-/-}$ 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 *Nrtn*$^{-/-}$ mice have smaller myenteric neurons than wild-type animals and a reduced neuronal fiber density in the

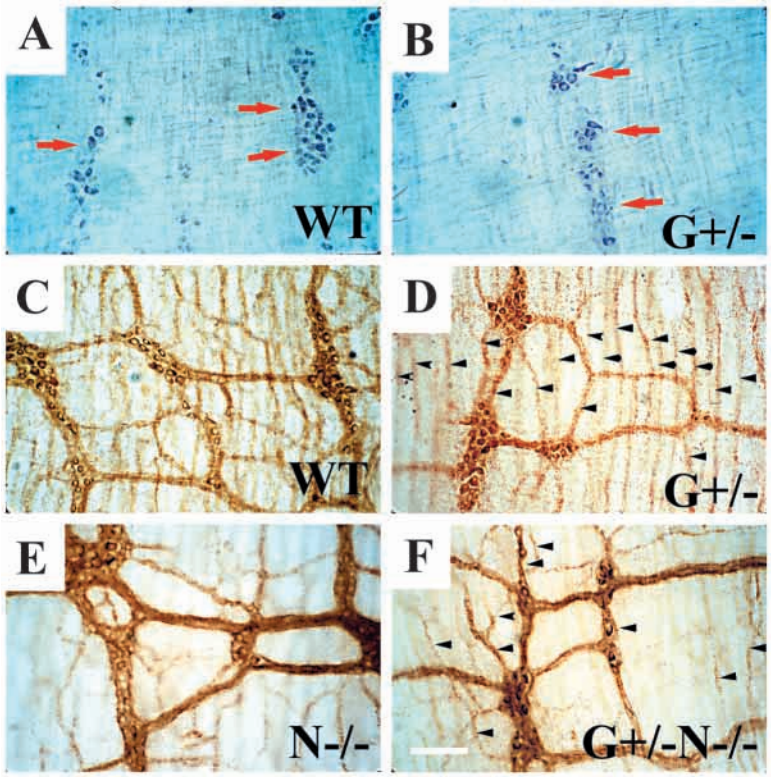


Fig. 1. Wholemount small bowel myenteric plexus staining for *Gdnf*$^{+/-}$, *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$, *Nrtn*$^{-/-}$ and wild-type mice. (A,B) Cuprolinic Blue staining highlights clustered neuronal cell bodies (red arrows) without showing neuronal processes. *Gdnf*$^{+/-}$ mice (B) have fewer neurons, 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). *Nrtn*$^{-/-}$ (E) and *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$ (F) mice have an obvious loss of small acetylcholinesterase-stained neuronal fibers. Black arrowheads identify small fibers and fiber bundles in *Gdnf*$^{+/-}$ and *Gdnf*$^{+/-}$*Nrtn*$^{-/-}$ mice (D,F). Scale bar: 100 μm.

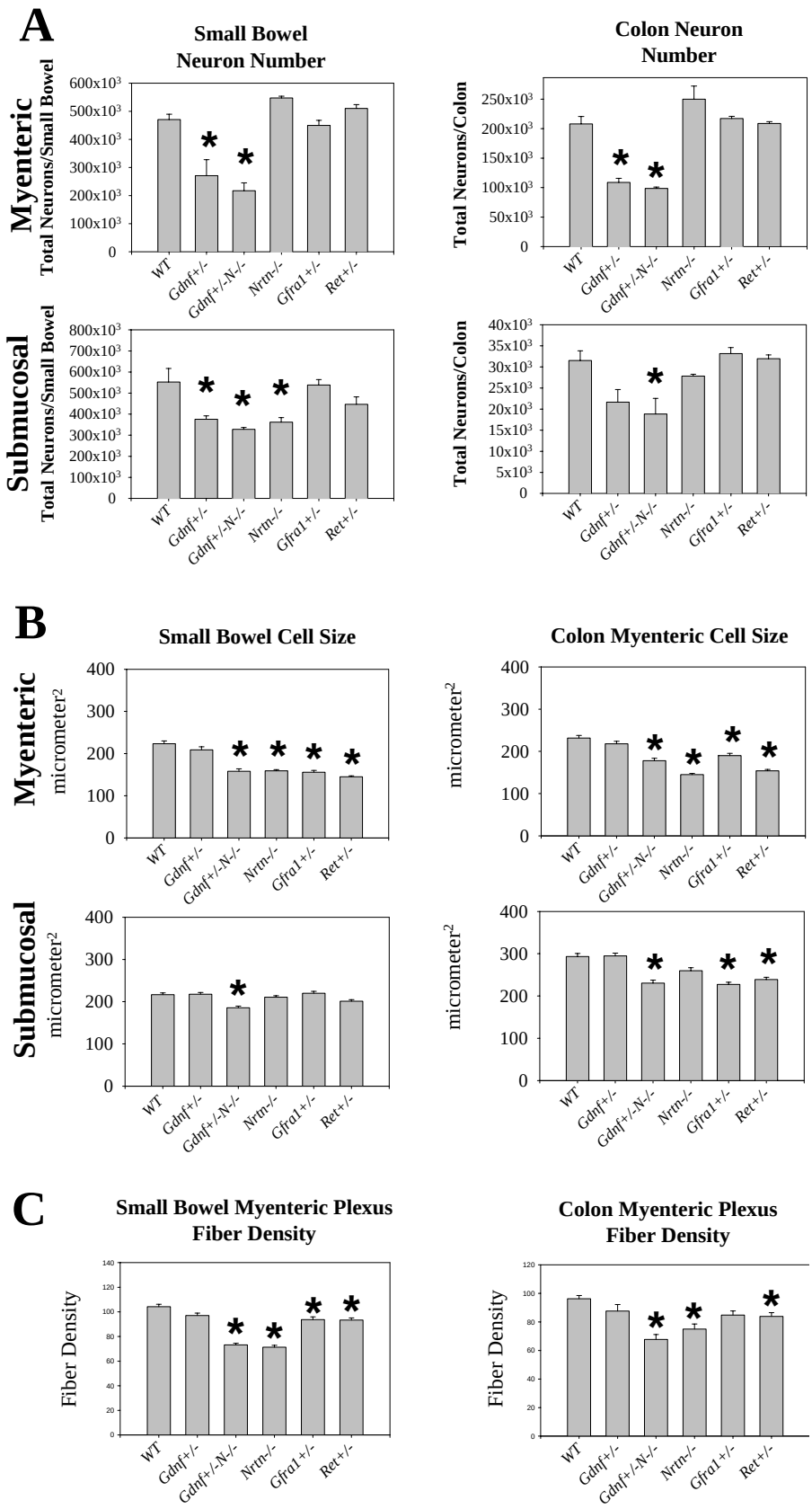


Fig. 2. Quantitative analysis of adult mouse small bowel and colon ENS structure. (A) Neuron number in wild-type and mutant mouse myenteric and submucosal plexus. (B) Cell size data. (C) Fiber density data for acetylcholinesterase-stained myenteric plexus. Error bars show s.e.m. *Differs significantly from wild type (P<0.05).

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.

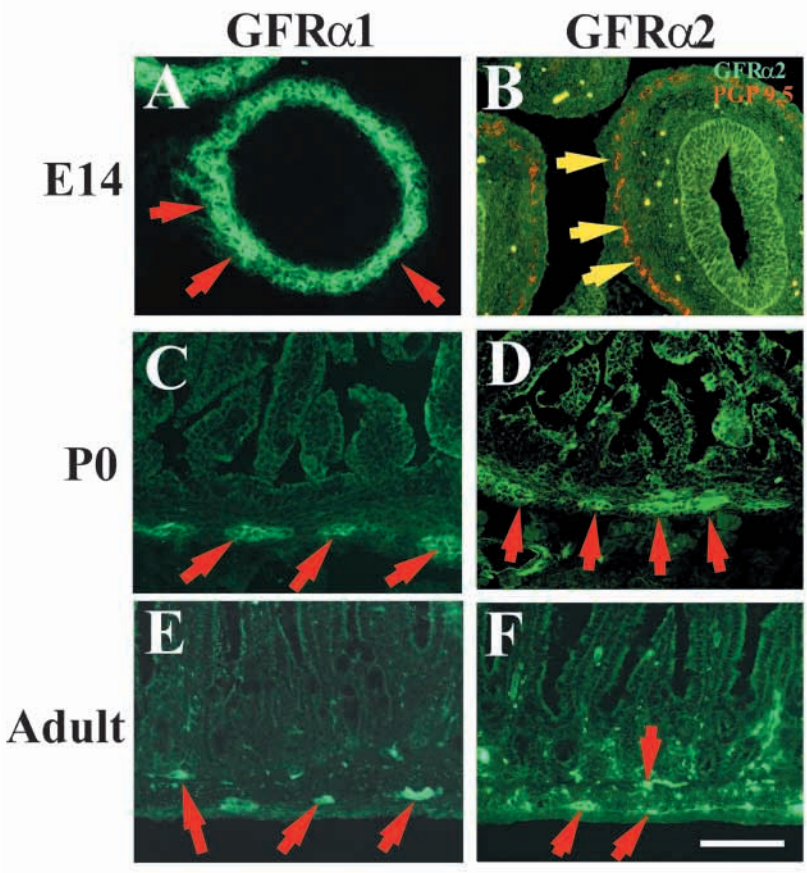


Fig. 3. GFRα1 and GFRα2 expression in the gut. (A,B) E14 gut expresses GFRα1 (red arrows, FITC) but not GFRα2 (FITC). Yellow arrows (B) show PGP9.5-expressing neural crest cells in the gut (Cy3 secondary), but there is no detectable GFRα2 staining. Newborn (C,D) and adult (E,F) small bowels express GFRα1 (FITC) and GFRα2 (FITC), but GFRα2 is much more easily detected in adult (F) than in newborn mouse gut. Scale bar: 100 μm.

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 expression was not detected at E14 (FITC staining, Fig. 3B). At P0, GFRα1 and GFRα2 are both visible within the ENS (Fig. 3C,D), but GFRα1 staining is less intense than at E14. GFRα1 is also more tightly associated with the developing ENS than at E14. Staining at P7 is similar to P0. In the adult mouse, GFRα2 continues to be strongly expressed (Fig. 3F) in the ENS. GFRα1 is also seen within myenteric and submucosal ganglia, but cells are less intensely stained than at E14 (Fig. 3E).

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

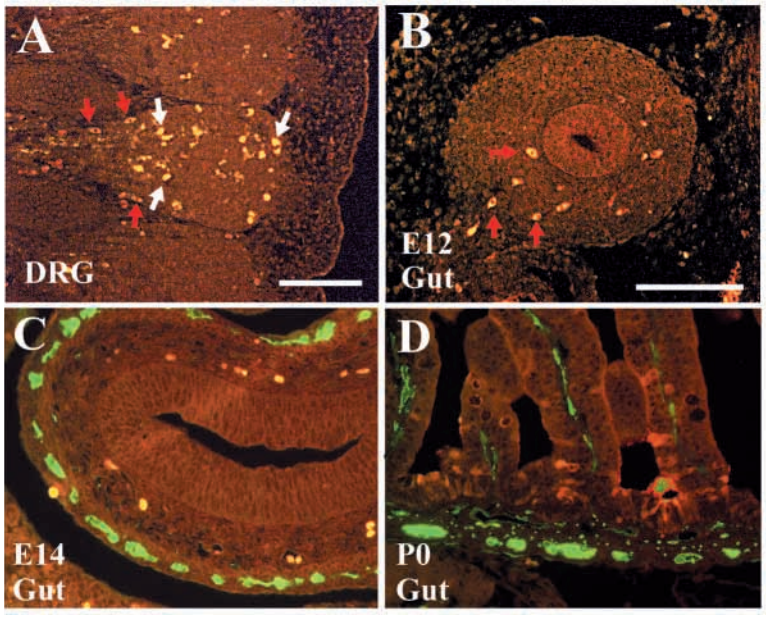


Fig. 4. Apoptotic cells are easily detected in the DRG, but not in the gut. (A,B) Activated caspase 3 immunohistochemistry easily identifies apoptotic cells in E12 DRG (A, white arrows), but not in the E12 ENS (B). Auto-fluorescent nucleated red blood cells are also seen (red arrows) in both the gut 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.

cells (Srinivasan et al., 1998). Using this antibody, apoptotic cells were easily identified in dorsal root ganglion (DRG) at E12 (Fig. 4A) and E14 (data not shown). However, we were unable to identify any activated caspase 3-positive cells in the ENS at E12, E14, E16, E18, P0, P7 and P14, or in adult mice. Fig. 4B-D shows the absence of activated caspase 3 staining in the gut at E12, E14 and P0.

The failure to identify apoptotic cells by immunohistochemistry for activated caspase 3, however, could be due to inadequate sampling. To look for further evidence that apoptosis was important for sculpting the ENS, we evaluated the ENS anatomy in *Bax*$^{-/-}$ and *Bid*$^{-/-}$ mice. Bax is a pro-apoptotic Bcl2 family member that is essential for 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

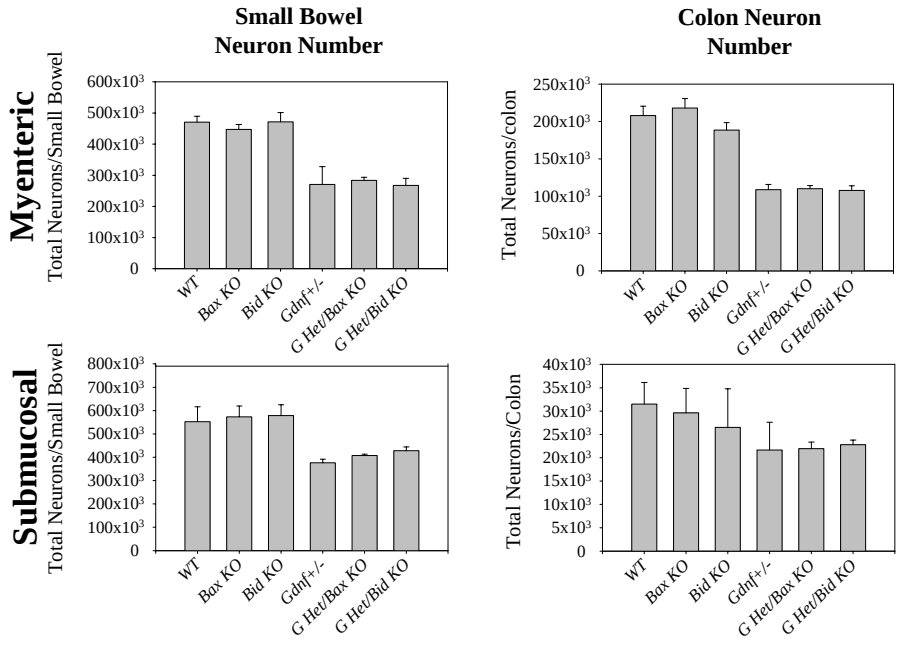


Fig. 5. Enteric neuron number is not influenced by Bax or Bid deficiency. Quantitative neuron counts in the myenteric and submucosal plexus of wild-type, *Bax*$^{-/-}$, *Bid*$^{-/-}$, *Gdnf*$^{+/-}$, *Gdnf*$^{+/-}$/*Bax*$^{-/-}$ and *Gdnf*$^{+/-}$/*Bid*$^{-/-}$ mice are presented. Although GDNF heterozygosity 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.

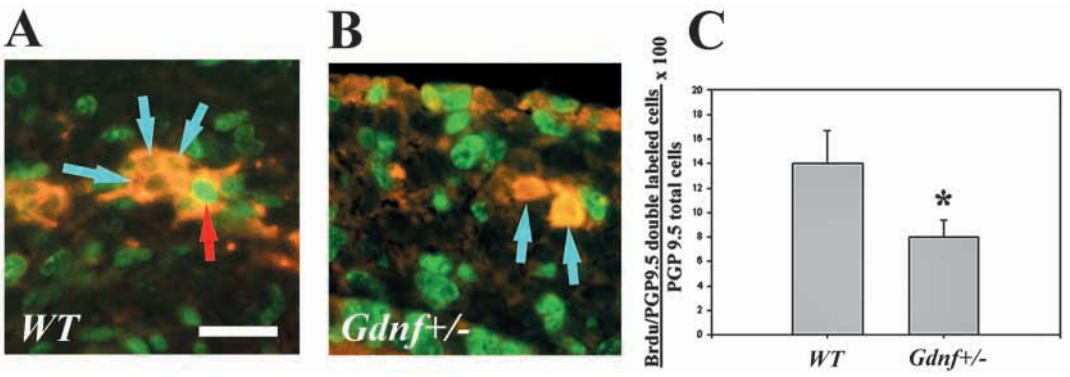


Fig. 6. GDNF heterozygous mice have a reduced rate of ENS neuronal precursor proliferation. (A,B) BrdU (FITC) and PGP9.5 (Cy3) double labeling in the E12 gut. The red arrow indicates a PGP9.5-positive cell that had incorporated BrdU. Blue arrows indicate PGP9.5-positive cells that have not incorporated BrdU. (C) Quantitative analysis of the rate of ENS neuronal precursor proliferation. **P*=0.01. Scale bar: 20 μm.

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.

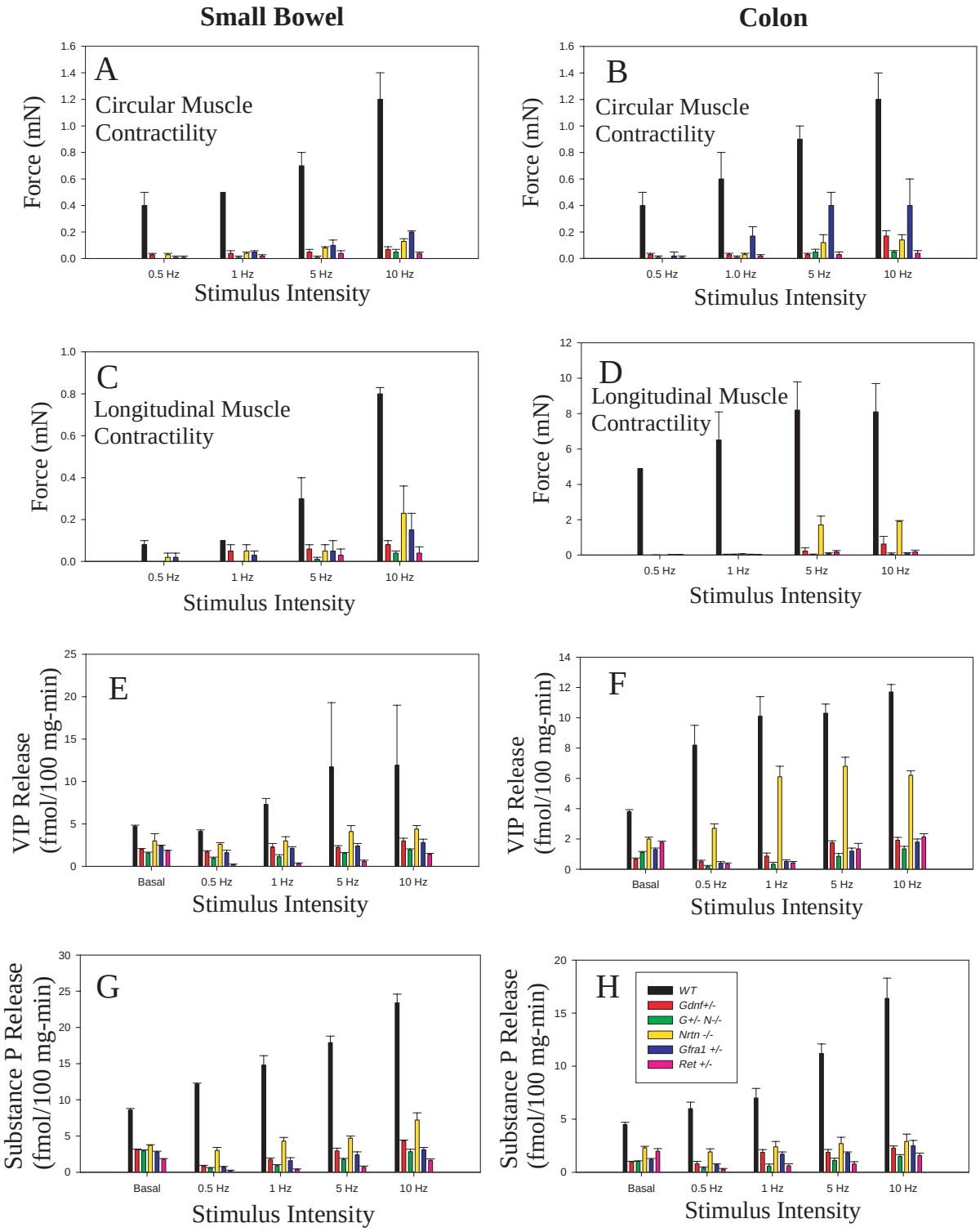
Nrtn$^{-/-}$, *Gdnf*$^{+/-}$, *Gfra1*$^{+/-}$ and *Ret*$^{+/-}$ mice all have abnormal intestinal contractility in vitro

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 anatomic 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



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.

Fig. 7. *Gdnf*$^{+/-}$, *Nrtn*$^{-/-}$, *Gdnf*$^{+/-}$/*Nrtn*$^{-/-}$, *Gfra1*$^{+/-}$ and *Ret*$^{+/-}$ mice all have abnormal intestinal contractility and reduced VIP and substance P release. Contractility of intestinal circular (A,B) and longitudinal (C,D) muscle in response to electric field stimulation. Different colored bars, as indicated in H, represent distinct mouse genotypes. (A-D) Intestinal segments from the small bowel (A,C) or colon (B,D) were stimulated with a 1 minute electric field stimulus at 80 V and 0.5, 1.0, 5.0 or 10.0 Hz. Contractile strength was measured with force transducers. Small bowel circular (A) and longitudinal (C) muscle, as well as colon circular muscle (B), contract during electric field stimulation. Colon longitudinal muscle relaxes during field stimulation, and then has a rebound contraction. The colon longitudinal muscle data (D) plots the rebound contraction phase. The relaxation phase that occurs during electric field stimulation was also reduced in all genotypes compared with wild-type mice, but the data are omitted to simplify the figure. Release of VIP (E,F) and substance P (G,H) from the small intestine (E,G) or colon (F,H) was measured either in the basal state or after electric field stimulation. The bars for transmitter release in response to electric field stimulation represent the increase in transmitter release over baseline. Error bars show s.e.m.. All of the mutant genotypes differ from wild type for all of the contraction and transmitter release parameters measured (P<0.05).

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; Korsching, 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 *Sox10*^{Dom}/*Sox10*^{Dom} 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 neuropoeitic 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 *Nt3*$^{-/-}$ and *Ntrk3*$^{-/-}$ (trkC) mice have a reduced number of enteric neurons and that only a subset of enteric neurons retrogradely transport NT3 (Chalazonitis et al., 2001). Similarly, CNTF receptor α and LIF receptor β are expressed on enteric neuron subsets, and activation of these receptors promotes the development of NADPH diaphorase-expressing neurons (Chalazonitis et al., 1998b). Thus, as in the DRG (Ernfors, 2001; Rifkin et al., 2000; Snider and Silos-Santiago, 1996), different functional classes of enteric neurons are likely to be supported by different trophic factors.

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 *Nrtn*$^{-/-}$ 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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REFERENCES

Aimi, Y., Kimura, H., Kinoshita, T., Minami, Y., Fujimura, M. and Vincent, S. R. (1993). Histochemical localization of nitric oxide synthase in rat enteric nervous system. *Neuroscience* **53**, 553-560.

Arenas, E., Akerud, P., Wong, V., Boylan, C., Persson, H., Lindsay, R. M. and Altar, C. A. (1996). Effects of BDNF and NT-4/5 on striatonigral neuropeptides or nigral GABA neurons in vivo. *Eur. J. Neurosci.* **8**, 1707-1717.

Armstrong, R. C. and Clarke, P. G. (1979). Neuronal death and the development of the pontine nuclei and inferior olive in the chick. *Neuroscience* **4**, 1635-1647.

Aros, S. D. and Camilleri, M. (2001). Small-bowel motility. *Curr. Opin. Gastroenterol.* **17**, 140-146.

Baloh, R. H., Tansey, M. G., Golden, J. P., Creedon, D. J., Heuckeroth, R. O., Keck, C. L., Zimonjic, D. B., Popescu, N. C., Johnson, E. M. J. and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* **18**, 793-802.

Baloh, R. H., Gorodinsky, A., Golden, J. P., Tansey, M. G., Keck, C. L., Popescu, N. C., Johnson, E. M. J. and Milbrandt, J. (1998a). GFRα3 is an orphan member of the GDNF/neurturin/persephin receptor family. *Proc. Natl. Acad. Sci. USA* **95**, 5801-5806.

Baloh, R. H., Tansey, M. G., Lampe, P. A., Fahrner, T. J., Enomoto, H., Simburger, K. S., Leitner, M. L., Araki, T., Johnson, E. M. J. and Milbrandt, J. (1998b). Artemin, a novel member of the GDNF ligand

family, supports peripheral and central neurons and signals through the GFRα3-Ret receptor complex. *Neuron* **21**, 1-20.

Baloh, R. H., Enomoto, H., Johnson, E. M. J. and Milbrandt, J. (2000). The GDNF family ligands and receptors – implications for neural development. *Curr. Opin. Neurobiol.* **10**, 103-110.

Bar-Peled, O., Knudson, M., Korsmeyer, S. J. and Rothstein, J. D. (1999). Motor neuron degeneration is attenuated in bax-deficient neurons in vitro. *J. Neurosci. Res.* **55**, 542-556.

Bennet, M. R., Gibson, W. G. and Lemon, G. (2002). Neuronal cell death, nerve growth factor and neurotrophic models: 50 years on. *Auton. Neurosci.* **95**, 1-23.

Blaugrund, E., Pham, T. D., Tennyson, V. M., Lo, L., Sommer, L., Anderson, D. J. and Gershon, M. D. (1996). Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers, and *Mash-1*-dependence. *Development* **122**, 309-320.

Camilleri, M. (2001). Enteric nervous system disorders: genetic and molecular insights for the neurogastroenterologist. *Neurogastroenterol. Motil.* **13**, 277-295.

Chalazonitis, A., Rothman, T. P., Chen, J. and Gershon, M. D. (1998a). Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRα-1 *in vitro* and *in vivo*. *Dev. Biol.* **204**, 385-406.

Chalazonitis, A., Rothman, T. P., Chen, J., Vinson, E. N., MacLennan, A. J. and Gershon, M. D. (1998b). Promotion of the development of enteric neurons and glia by neuropoietic cytokines: interactions with neurotrophin-3. *Dev. Biol.* **198**, 343-365.

Chalazonitis, A., Pham, T. D., Rothman, T. P., DiStefano, P. S., Bothwell, M., Blair-Flynn, J., Tessarollo, L. and Gershon, M. D. (2001). Neurotrophin-3 is required for the survival-differentiation of subsets of developing enteric neurons. *J. Neurosci.* **21**, 5620-5636.

Costa, M. and Brookes, S. J. H. (1994). The enteric nervous system. *Am. J. Gastroenterol.* **89**, S129-S137.

De Giorgio, R., Stanghellini, V., Barbara, G., Corinaldesi, R., de Ponti, F., Tonini, M., Bassotti, G. and Sternini, C. (2000). Primary enteric neuropathies underlying gastrointestinal motor dysfunction. *Scand. J. Gastroenterol.* **35**, 114-122.

Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, E. M., Jr, Snider, W. D. and Korsmeyer, S. J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* **17**, 401-411.

Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fekete, C., Ponder, B. A. J. and Munnich, A. (1994). Mutations of the *RET* proto-oncogene in Hirschsprung's disease. *Nature* **367**, 378-380.

Enokido, Y., de Sauvage, F., Hongo, J.-A., Ninkina, N., Rosenthal, A., Buchman, V. L. and Davies, A. M. (1998). GFRα4 and the tyrosine kinase Ret form a functional receptor complex for persephin. *Curr. Biol.* **8**, 1019-1022.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M. J. and Milbrandt, J. (1998). GFRα1 deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21**, 317-324.

Enomoto, H., Heuckeroth, R. O., Golden, J. P., Johnson, E. M., Jr and Milbrandt, J. (2000). Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. *Development* **127**, 4877-4889.

Ernfors, P. (2001). Local and target-derived actions of neurotrophins during peripheral nervous system development. *Cell Mol. Life Sci.* **58**, 1036-1044.

Furness, J. B. (2000). Types of neurons in the enteric nervous system. *J. Auton. Nerv. Syst.* **81**, 87-96.

Gabriel, S. B., Salomon, R., Pelet, A., Angrist, M., Amiel, J., Fornage, M., Attie-Bitach, T., Olson, J. M., Hofstra, R., Buys, C. et al. (2002). Segregation at three loci explains familial and population risk in Hirschsprung disease. *Nat. Genet.* **31**, 89-93.

Gershon, M. D., Chalazonitis, A. and Rothman, T. P. (1992). From neural crest to bowel: development of the enteric nervous system. *J. Neurobiol.* **24**, 199-214.

Giehl, K. M. (2001). Trophic dependencies of rodent corticospinal neurons. *Rev. Neurosci.* **12**, 79-94.

Grider, J. R. and Foxx-Orenstein, A. E. (1998). Neuroendocrine regulation of intestinal peristalsis. In *Gastrointestinal Endocrinology*. Chapter 11 (ed. G. H. Greeley), pp. 299-319. Totowa, NJ: Humana Press.

He, X., Yang, F., Xie, Z. and Lu, B. (2000). Intracellular Ca(2+) and Ca(2+)/calmodulin-dependent kinase II mediate acute potentiation of neurotransmitter release by neurotrophin-3. *J. Cell Biol.* **149**, 783-792.

Hearn, C. J., Murphy, M. and Newgreen, D. (1998). GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. *Dev. Biol.* **197**, 93-105.

Henshall, D. C., Bonislawski, D. P., Skradski, S. L., Lan, J. Q., Meller, R. and Simon, R. P. (2001). Cleavage of bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. *Neurobiol. Dis.* **8**, 568-580.

Heuckeroth, R. O., Enomoto, H., Grider, J. R., Golden, J. P., Hanke, J. A., Jackman, A., Molliver, D. C., Bardgett, M. E., Snider, W. D., Johnson, E. M., Jr et al. (1999). Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* **22**, 253-263.

Heuckeroth, R. O., Lampe, P. A., Johnson, E. M. J. and Milbrandt, J. (1998). Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. *Dev. Biol.* **200**, 116-129.

Humpel, C., Marksteiner, J. and Saria, A. (1996). Glial-cell-line-derived neurotrophic factor enhances biosynthesis of substance P in striatal neurons in vitro. *Cell Tissue Res.* **286**, 249-255.

Hutchins, J. B. and Barger, S. W. (1998). Why neurons die: cell death in the nervous system. *Anat. Rec.* **253**, 79-90.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R. et al. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. *Cell* **85**, 1113-1124.

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P. L., Boone, T., Delaney, J., Schultz, H., Zhou, R. and Fox, G. M. (1997). GFRα-2 and GFRα-3 are two new receptors for ligands of the GDNF family. *J. Biol. Chem.* **272**, 33111-33117.

Kapur, R. P. (1999). Early death of neural crest cell is responsible for total enteric aganglionosis in *Sox10^{Dom}/Sox10^{Dom}* mouse embryos. *Pedatric and Developmental Pathology* **2**, 559-569.

Kapur, R. P. (2001). Neuropathology of paediatric chronic intestinal pseudo-obstruction and related animal models. *J. Pathol.* **194**, 277-288.

Karaosmanoglu, T., Aygun, B., Wade, P. and Gershon, M. (1996). Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. *Anat. Rec.* **244**, 470-480.

Kirchgessner, A. L., Liu, M.-T. and Alcantara, F. (1997). Excitotoxicity in the enteric nervous system. *J. Neurosci.* **17**, 8804-8816.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. *J. Neurosci.* **13**, 2739-2748.

Kunze, W. A. A. and Furness, J. B. (1999). The enteric nervous system and regulation of intestinal motility. *Annu. Rev. Physiol.* **61**, 117-142.

Le Douarin, N. M. and Teillet, M. A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. *Embryol. Exp. Morphol.* **30**, 31-48.

Leonard, J. R., D'Sa, C., Cahn, B. R., Korsmeyer, S. J. and Roth, K. A. (2001). Bid regulation of neuronal apoptosis. *Dev. Brain Res.* **128**, 187-190.

Lessmann, V. and Heumann, R. (1998). Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. *Neuroscience* **86**, 399-413.

Lessmann, V., Gottmann, K. and Heumann, R. (1994). BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. *NeuroReport* **6**, 21-25.

Li, Y. X., Zhang, Y., Lester, H. A., Schuman, E. M. and Davidson, N. (1998). Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. *J. Neurosci.* **18**, 10231-10240.

Liou, J. C. and Fu, W. M. (1997). Regulation of quantal secretion from developing motoneurons by postsynaptic activity-dependent release of NT-3. *J. Neurosci.* **17**, 2459-2468.

Lohof, A. M., Ip, N. Y. and Poo, M. M. (1993). Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* **363**, 350-353.

Macaya, A. (1996). Apoptosis in the nervous system. *Rev. Neurol.* **24**, 1356-1360.

Martucciello, G., Ceccherini, I., Lerone, M. and Jasonni, V. (2000). Pathogenesis of Hirschsprung's Disease. *J. Pediatric Surg.* **35**, 1017-1025.

McKay, S. E. and Oppenheim, R. W. (1991). Lack of evidence for cell death

among avian spinal cord interneurons during normal development and following removal of targets and afferents. *J. Neurobiol.* **22**, 721-733.

Milbrandt, J., Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S. et al. (1998). Persephin, a novel neurotrophic factor related to GDNF and Neurturin. *Neuron* **20**, 245-253.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichart, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.

Oppenheim, R. W. (1981). *Neuronal Cell Death and Some Related Regressive Phenomena during Neurogenesis: A Selective Historical Review and Progress Report*. New York: Oxford University Press.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**, 453-501.

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J.-F. (1999). The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. *Nature* **399**, 366-370.

Pham, T. D., Gershon, M. D. and Rothman, T. P. (1991). Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. *J. Comp. Neurol.* **314**, 789-798.

Pichel, J. G., Shen, L., Hui, S. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76.

Plesnila, N., Zinkel, S., Le, D. A., Amin-Hanjani, S., Wu, Y., Qiu, J., Chiarugi, A., Thomas, S. S., Kohane, D. S., Korsmeyer, S. J. et al. (2001). BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia. *Proc. Natl. Acad. Sci. USA* **98**, 15318-15323.

Pomeranz, H. D., Rothman, T. P. and Gershon, M. D. (1991). Colonization of the post-umbilical bowel by cells derived from the sacral neural crest: direct tracing of cell migration using an intercalating probe and replication-deficient retrovirus. *Development* **111**, 647-655.

Quigley, E. M. (1999). Disturbances in small bowel motility. *Baillieres Best Pract. Res. Clin. Gastroenterol.* **13**, 385-395.

Rifkin, J. T., Todd, V. J., Anderson, L. W. and Lefcort, F. (2000). Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. *Dev. Biol.* **227**, 465-480.

Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Serl, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. et al. (1994). Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* **367**, 377-378.

Rossi, J., Luukko, K., Poteryaev, D., Laurikainen, A., Sun, Y. F., Laakso, T., Eerikainen, S., Tuominen, R., Lakso, M., Rauvala, H. et al. (1998). Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. *Neuron* **22**, 243-252.

Roth, K. A. and D'Sa, C. (2001). Apoptosis and brain development. *Ment. Retard. Dev. Disabil. Res. Rev.* **7**, 261-266.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-73.

Sang, Q. and Young, H. M. (1996). Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tiss. Res.* **284**, 39-53.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R. et al. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. *Proc. Natl. Acad. Sci. USA* **94**, 6238-6243.

Schmulson, M. J. (2001). Brain-gut interaction in irritable bowel syndrome: new findings of a multicomponent disease model. *Isr. Med. Assoc. J.* **3**, 104-110.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Constantini, F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-383.

Schuchardt, A., D'Agati, V., Pachnis, V. and Costantini, F. (1996). Renal agenesis and hypodysplasia in *ret-k-* mutant mice result from defects in ureteric bud development. *Development* **122**, 1919-1929.

Shen, L., Pichel, J. G., Mayeli, T., Sariola, H., Lu, B. and Westphal, H. (2002). Gdnf haploinsufficiency causes hirschsprung-like intestinal obstruction and early-onset lethality in mice. *Am. J. Hum. Genet.* **70**, 435-447.

Shirasawa, S., Yunker, A. M., Roth, K. A., Brown, G. A., Horning, S. and Korsmeyer, S. J. (1997). Enx (Hox11L1)-deficient mice develop myenteric neuronal hyperplasia and megacolon. *Nat. Med.* **3**, 646-650.

Sidebotham, E. L., Woodward, M. N., Kenny, S. E., Lloyd, D. A., Vaillant, C. R. and Edgar, D. H. (2001). Assessment of protein gene product 9.5 as a marker of neural crest-derived precursor cells in the developing enteric nervous system. *Pediatr. Surg. Int.* **17**, 304-307.

Skinner, M. (1996). Hirschsprung's disease. *Curr. Probl. Surg.* **33**, 391-461.

Snider, W. D. and Silos-Santiago, I. (1996). Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**, 395-403.

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox 10 mutation disrupts neural crest development in Dom Hirshsprung mouse model. *Nat. Genet.* **18**, 60-64.

Spiller, R. C. (1999). Disturbances in large bowel motility. *Baillieres Best Pract. Res. Clin. Gastroenterol.* **13**, 397-413.

Srinivasan, A., Roth, K. A., Sayers, R. O., Shindler, K. S., Wong, A. M., Fritz, L. C. and Tomaselli, K. J. (1998). In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ.* **5**, 1004-1016.

Stanghellini, V., Corinaldesi, R. and Barbara, L. (1988). Pseudo-obstruction syndromes. *Bailliere's Clinical Gastroenterol.* **2**, 225-254.

Suvanto, P., Wartiovaara, K., Lindahl, M., Arumae, U., Moshnyakov, M., Horelli-Kuitunen, N., Airaksinen, M. S., Palotie, A., Sariola, H. and Saarma, M. (1997). Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptor beta, a homologue to GDNFR-alpha. *Hum. Mol. Genet.* **6**, 1267-1273.

Taraviras, S., Marcos-Gutierrez, C. V., Durbec, P., Jani, H., Grigoriou, M., Sukumaran, M., Wang, L.-C., Hynes, M., Raisman, G. and Pachnis, V. (1999). Signalling by RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development* **126**, 2785-2797.

Treanor, J. J. S., Goodman, L., Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F. et al. (1996). Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80-83.

Vekrellis, K., McCarthy, M. J., Watson, A., Whitfield, J., Rubin, L. L. and Ham, J. (1997). Bax promotes neuronal cell death and is downregulated during the development of the nervous system. *Development* **124**, 1239-1249.

Wang, C. Y., Yang, F., He, X., Chow, A., Du, J., Russell, J. T. and Lu, B. (2001). Ca(2+) binding protein frequenin mediates GDNF-induced potentiation of Ca(2+) channels and transmitter release. *Neuron* **32**, 99-112.

Wang, C. Y., Yang, F., He, X. P., Je, H. S., Zhou, J. Z., Eckermann, K., Kawamura, D., Feng, L., Shen, L. and Lu, B. (2002). Regulation of neuromuscular synapse development by glial cell line-derived neurotrophic factor and neurturin. *J. Biol. Chem.* **277**, 10614-10625.

White, F. A., Keller-Peck, C. R., Knudson, C. M., Korsmeyer, S. J. and Snider, W. D. (1998). Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. *J. Neurosci.* **18**, 1428-1439.

Worby, C. A., Vega, Q. C., Chao, H. H.-J., Seasholtz, A. F., Thompson, R. C. and Dixon, J. E. (1998). Identification and characterization of GFRα-3, a novel co-receptor belonging to the glial cell line-derived neurotrophic receptor family. *J. Biol. Chem.* **273**, 3502-3508.

Yamataka, A., Hatano, M., Kobayashi, H., Wang, K., Miyahara, K., Sueyoshi, N. and Miyano, T. (2001). Intestinal neuronal dysplasia-like pathology in Ncx/Hox11L.1 gene-deficient mice. *J. Pediatr. Surg.* **36**, 1293-1296.

Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A. and Korsmeyer, S. J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* **400**, 886-891.

Young, H. M. and Newgreen, D. (2001). Enteric neural crest-derived cells: Origin, identification, migration and differentiation. *Anat. Rec.* **262**, 1-15.