Development 134, 2171-2181 (2007) doi:10.1242/dev.001388

Conditional ablation of GFRα1 in postmigratory enteric neurons triggers unconventional neuronal death in the colon and causes a Hirschsprung's disease phenotype

Toshihiro Uesaka[1], Sanjay Jain[2], Shigenobu Yonemura[3], Yasuo Uchiyama[4], Jeffrey Milbrandt[5,*] and Hideki Enomoto[1,*]

The regulation of neuronal survival and death by neurotrophic factors plays a central role in the sculpting of the nervous system, but the identity of survival signals for developing enteric neurons remains obscure. We demonstrate here that conditional ablation of *GFRα1*, the high affinity receptor for GDNF, in mice during late gestation induces rapid and widespread neuronal death in the colon, leading to colon aganglionosis reminiscent of Hirschsprung's disease. Enteric neuron death induced by *GFRα1* inactivation is not associated with the activation of common cell death executors, caspase-3 or -7, and lacks the morphological hallmarks of apoptosis, such as chromatin compaction and mitochondrial pathology. Consistent with these in vivo observations, neither caspase inhibition nor Bax deficiency blocks death of colon-derived enteric neurons induced by GDNF deprivation. This study reveals an essential role for GFRα1 in the survival of enteric neurons and suggests that caspase-independent death can be triggered by abolition of neurotrophic signals.

KEY WORDS: GFRα1, GDNF, Enteric neuron, Mouse

INTRODUCTION

During nervous system development, substantial numbers of immature neurons die by apoptosis. Such 'life or death' decisions of neuronal survival are controlled in part by the availability of neurotrophic factors (Yuan and Yankner, 2000). The actions of neurotrophic factors are regulated in a spatiotemporally restricted fashion, and the impairment of neurotrophic factor signaling can cause the abnormal loss of specific neuronal populations (Snider, 1994), leading to pathological conditions of the nervous system (Indo et al., 1996). Thus, identification of neurotrophic factors that support the survival of a given neuronal population is a vital step toward understanding the mechanisms underlying the formation of the nervous system in health and disease.

The enteric nervous system (ENS) constitutes one division of the peripheral nervous system and controls motility, secretion and blood flow in the gastrointestinal tract. The ENS is extraordinarily autonomic, being able to function by its own intrinsic neural circuits even when devoid of central afferent innervations. Structurally, the ENS is organized into myenteric (Auerbach) and submucosal (Meissner) plexi, each of which is comprised of interconnected ganglia containing diverse sets of neurons and glia. The majority of enteric neurons and glia originates from the vagal neural crest, which is formed at the level of somites 1-7 (Le Douarin, 1986). In mice, the vagal neural crest-derived ENS progenitors first enter the foregut between embryonic day 9.0-9.5 (E9.0-9.5) and successively undergo extensive rostrocaudal migration in the gut mesenchyme until they, along with their progeny, furnish the entire gastrointestinal tract at around E14.5 (Young et al., 1998). As they migrate, ENS progenitors proliferate to maintain cellular sources, and some of their progeny begin to differentiate, as indicated by the onset of pan-neuronal or neuronal population-specific marker (e.g. neurotransmitters) expression at developmentally determined times and locations (Young et al., 2003). Subsequently, neuronal and glial differentiation continues even after the gut is colonized by ENS progenitors (Young et al., 2003). Thus, the development of the ENS is a complex, asynchronous process that relies on the exquisite control of cell migration, proliferation and differentiation of neural crest-derived progenitor cells and their progeny in a spatiotemporally specific fashion.

Previous developmental studies have delineated a number of unique features of the ENS with respect to neuronal survival and death. First, unlike other areas of the nervous system, the developing ENS fails to exhibit evidence of neuronal apoptosis (Gianino et al., 2003; Kruger et al., 2003). Consistent with this observation, genetic ablation of the pro-apoptotic protein Bax, which increases final neuron counts in various systems by protecting neurons from apoptotic death (Deckwerth et al., 1996), fails to affect the number of neurons in the ENS (Gianino et al., 2003). These results suggest that physiological neuronal death may not take place during ENS development. Alternatively, enteric neurons may die in an unconventional (non-apoptotic) fashion during normal development. It remains undetermined whether the survival of enteric neurons depends on extracellular survival signals in vivo and, if so, whether the absence of such factors results in the activation of conserved molecular and morphological death pathways.

GDNF is a founding member of the GFL family and signals through a multicomponent receptor complex consisting of a glycosyl-phosphatidyl-inositol (GPI)-anchored cell surface protein, GFRα1, and the RET tyrosine kinase (Airaksinen and Saarma, 2002; Baloh et al., 2000). During ENS development, GDNF is expressed

[1]Laboratory for Neuronal Differentiation and Regeneration, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan. [2]Departments of Medicine, Renal Division and [5]Pathology and Immunology, Washington University School of Medicine, St Louis, MO 63110, USA. [3]Laboratory for Cellular Morphogenesis, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan. [4]Department of Cell Biology and Neurosciences, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan.

*Authors for correspondence (e-mail: jeff@pathbox.wustl.edu; enomoto@cdb.riken.jp)

Accepted 19 March 2007

in the gut mesenchyme, whereas both RET and GFRα1 are expressed in ENS progenitors and neurons. As suggested by these expression patterns, GDNF signaling via RET/GFRα1 is essential for development of the ENS in vertebrates. For instance, in humans, mutations in the *Ret* gene lead to increased susceptibility to Hirschsprung's disease, a congenital disorder characterized by the absence of enteric ganglia [intestinal aganglionosis (Newgreen and Young, 2002a; Newgreen and Young, 2002b)]. Mice deficient for *GDNF*, *GFRα1* or *Ret* display complete absence of enteric ganglia in the gut distal to the stomach (Airaksinen and Saarma, 2002; Baloh et al., 2000).

Detailed analysis of *Ret*-deficient embryos revealed that ENS progenitors are reduced in number and fail to enter the midgut, deficits that are discernible as early as E10.5 (Durbec et al., 1996). Although these studies demonstrate the requirement for GDNF signaling in the initial colonization of the gastrointestinal tract by ENS progenitors, this early developmental deficit leads to the complete depletion of the cellular source for the ENS in the small and large intestine, precluding analysis of the physiological role of GDNF signaling in later ENS development, especially in the distal portion of the gastrointestinal tract. It seems likely that GDNF plays a role in later ENS development, as its expression in the gastrointestinal tract persists after the deficits in migration are observed (Golden et al., 1999;Young et al., 2001). For instance, a mitogenic role for GDNF during ENS development was suggested by a study of embryos lacking one copy of the *GDNF* gene (Shen et al., 2002), in which the proliferating ENS precursor population in the midgut is significantly smaller at E12.5 than in wild-type littermates (Gianino et al., 2003). Nevertheless, many aspects of GDNF function remain unknown due to the remaining functional *GDNF* allele in heterozygous mice, and therefore the physiological functions of GDNF, GFRα1 and RET during the development of the ENS have yet to be fully explored.

We describe here the successful generation of a *GFRα1* conditional GFP reporter mouse line to elucidate the biological role of GFRα1. By crossing *GFRα1* conditional knockout mice with mice ubiquitously expressing inducible Cre protein, we inactivated *GFRα1* function in late ENS development, particularly after vagal crest-derived ENS progenitors have finished investing the entire gut. Disruption of *GFRα1* at these later stages of ENS development induced unexpected widespread death of enteric neurons specifically in the distal gastrointestinal tract, which led to colon aganglionosis reminiscent of Hirschsprung's disease at birth. This massive cell elimination in the *GFRα1* conditional knockout gut does not proceed by canonical apoptotic pathways. The present study uncovers unique features in the control of neuronal survival and death by *GFRα1* that may underlie development and pathology in the enteric nervous system.

MATERIALS AND METHODS

Generation of floxed *GFRα1* mice

A *Hin*dIII-*Eco*RV fragment of *GFRa1* genome encompassing exons 1-4 was used to construct a targeting vector. A gene cassette composed of the floxed mouse *GFRa1* cDNA-*SV40 intron-poly*(*A*) and followed by *GFP-poly*(*A*) and FRTed Tn5 *neo* was introduced into the second exon, which deleted 95 nucleotides containing the 5′-UTR, the initiator Met and the signal sequences. The deletion disrupts the expression of the endogenous *GFRα1* gene but allows the expression of the inserted gene (Enomoto et al., 2004). To facilitate identification of homologously recombined clones by Southern blot analysis, the vector was designed to introduce a mutation disrupting a *Bam*HI site located in the second intron. The floxed *GFRα1* allele was generated using embryonic stem cell-based homologous recombination, and successive removal of the neo cassette by crossing chimeric mice to *ACTB-Flpe* transgenic mice (Rodriguez et al., 2000) resulted in generation of heterozygous floxed *GFRα1* mice ($GFR\alpha1^{flox/+}$) (see Fig. S2 in the supplementary material). For time-specific inactivation of *GFRa1*, $GFRa1^{flox/flox}$ mice were crossed to *GFRa1* heterozygous mice ($GFRa1^{+/-}$) (Enomoto et al., 1998) ubiquitously expressing inducible Cre recombinase (*CAGGCre-ER*^{TM}; Jackson Laboratories) (Hayashi and McMahon, 2002), referred to as (*CAGGCre-ER*^{TM}; $GFRa1^{+/-}$). Cre activity was induced by single intraperitoneal injection of 4-hydroxytamoxifen (4-OHT; 0.5 mg per mouse, Sigma) into pregnant mothers. Conditional mutant mice used for this study were kept on a mixed 129/Sv × C57BL/6 background. Heterozygous mice carrying *GFRa1 GFP-knock-in* allele ($GFRa1^{GFP/+}$) were obtained by crossing $GFRa1^{flox/+}$ mice to β-actin-Cre mice. $GFRa1^{GFP/GFP}$ mice displayed a phenotype identical to that in $GFRa1^{-/-}$ mice (see Fig. S3 in the supplementary material) (Enomoto et al., 1998). Mice were cared for according to the RIKEN Center for Developmental Biology institutional guidelines.

The genotypes of mice carrying floxed *GFRa1* or *GFRa1 GFP-knock-in* alleles were determined by PCR using oligonucleotides (see Fig. S2C in the supplementary material): P1 (5′-CTTCCAGGTTGGGTCGGA-ACTGAACCC-3′); P2 (5′-AGAGAGCTCAGCGTGCAGAGATC-3′); P3 (5′-TTTACGTCGCCGTCCAGCTCGA-3′). Primers to genotype $GFRa1^{+/-}$ are described elsewhere (Enomoto et al., 1998).

Histological analysis

$GDNF^{lacZ/+}$ mice (Moore et al., 1996) (a kind gift from V. Pachnis, MRI-NIMR, UK) were used for the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. The gastrointestinal tract was fixed in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$, 0.1% NP-40, 0.1 M PBS (pH 7.2) for 30 minutes at 4°C and incubated in X-gal solution for 4 hours at room temperature.

Immunohistochemistry and acetylcholinesterase histochemistry were performed as described previously (Enomoto et al., 1998). The following antibodies were used for immunohistochemistry: chicken anti-GFP (1:1000, Aves Labs), goat anti-GFRα1 (1:500, Neuromics), rabbit anti-PGP9.5 (1:500, Ultra Clone), rabbit anti-cleaved caspase-3 and -7 (1:300, Cell Signaling), rabbit anti-phospho-ERK (1:500, Cell Signaling), rabbit anti-Phox2b [1:1000, a kind gift from J-F. Brunet, CNRS UMR, France (Pattyn et al., 1997)], rabbit anti-S100β (1:500, NeoMarkers), mouse anti-TuJ1 (1:500, Covance). Secondary Alexa 488-, 594- and 633-conjugated antibodies (used at 1:500) were from Invitrogen. Confocal images were acquired using a Zeiss LSM5 PASCAL system. Time-lapse imaging was performed using an Olympus ZDC-IMAGE system. Fluorescent and brightfield imaging was performed with a Zeiss Axioskop 2 FS plus system.

Cell culture

Small or large intestine was digested with collagenase/dispase (1 mg/ml, Roche) for 15 minutes at 37°C. After obtaining single cell suspensions, enteric neurons were immunopurified using the MACSelect LNGFR MicroBeads system (Miltenyi Biotec) according to the manufacturer's instructions. Following immunoselection, 1×10^4 cells were plated onto a single well of an eight-well slide coated with poly-D-lysine (0.1 mg/ml) and laminin (20 μg/ml). The culture medium contained DMEM-low (Invitrogen) with 1% N2 supplement, 2% B27 supplement (Invitrogen) and penicillin/streptomycin (Meiji Seika). Cells were cultured for 2 days in the presence of GDNF (100 ng/ml), then switched to medium containing either GDNF-neutralizing antibodies (no further addition of GDNF; GDNF-deprivation) or GDNF (100 ng/ml; control). Culture of sympathetic neurons [from superior cervical ganglia (SCG)] was performed as described previously (Martin et al., 1988). Other reagents and conditions are as follows: neutralizing antibodies for NGF (1:1000; SIGMA) or GDNF (0.5 μg/ml; R&D Systems), zVAD-fmk (100 μM; R&D Systems), Hoechst 33342 (1 μg/μl, Sigma), TMR red In Situ Cell Death Detection kit (Roche) for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). $Bax^{-/-}$ mice (Knudson et al., 1995) were obtained from Jackson Laboratories. For virus-mediated gene transfer, human *Bcl-X*$_L$ was cloned into FUW (Lois et al., 2002). High-titer viral particles were obtained as previously described (Miyoshi et al., 1998).

Transmission electron microscopy

Distal colon of E15.5 embryo, neurons cultured on the coverslip, and SCG of P0 mouse were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) followed by postfixation with 1% OsO_4 in the same buffer. After being stained en bloc with 0.5% aqueous uranyl acetate, samples were dehydrated with ethanol and embedded in Polybed 812. Semithin (0.5 μm) sections were stained with Toluidine Blue. Ultra-thin (80 nm) sections were stained doubly with uranyl acetate and lead citrate and examined under a JEOL JEM 1010 transmission electron microscope at 100 kV. Myenteric ganglia were clearly distinguishable from muscle cells, which had characteristic shapes and dense cytoplasm (Vannucchi and Faussone-Pellegrini, 2000). To examine death figures of ENS cells in conditional *GFRα1* mutant colon where massive ENS degeneration is undergoing, only regions where the presence of the ganglia (or degenerating ganglia) was confirmed were selected for the morphological analysis.

Statistical analysis

Statistical analysis was performed using Student's *t*-test and Mann-Whitney *U*-test. Results are expressed as means ± s.e.m.

RESULTS

Generation of conditional *GFRα1* mouse mutants

GDNF expression in gut mesenchyme persists throughout development (Golden et al., 1999; Young et al., 2001), suggesting it may be important for ENS formation throughout development. Expression of *GFRα1* displays spatiotemporally dynamic changes during the development of the ENS. During early ENS development (E9.5-11.5), *GFRα1* was expressed in virtually all ENS progenitors (data not shown). At E13.5, *GFRα1* expression was detectable in both ENS progenitors and the majority of differentiating neurons, although the levels of *GFRα1* expression in the former were higher than those in the latter (see Fig. S1A in the supplementary material). After E13.5, *GFRα1* expression levels in enteric neurons were reduced, especially in the small intestine, and neurons expressing high levels of *GFRα1* became confined to the colon at E15.5 (Fig. 1B). In contrast to the dynamic changes in *GFRα1* expression in neurons, enteric glial cells maintained high *GFRα1* expression from their emergence (~E13.5) to postnatal periods. After postnatal day 5 (P5), cells expressing high levels of *GFRα1* expression were confined to glia, although approximately 35% of enteric neurons expressed *GFRα1* at marginal levels (see Fig. S1B in the supplementary material).

We were especially intrigued by the high level of *GDNF* expression in the cecum and colon during late gestation (E15.5-19.5; Fig. 1A), when colonization of the gut by ENS progenitors has already completed and most ENS progenitors in the myenteric layer have exited the cell cycle and initiated neuronal or glial differentiation (Young et al., 2005). Although *Ret* was expressed in all enteric neurons (data not shown), *GFRα1* was differentially expressed in neurons of different gut regions. Nearly 100% of the enteric neurons in the colon expressed *GFRα1*, whereas it was expressed in only a small subpopulation of neurons in the small intestine (Fig. 1B,C). Because *GFRα1* is initially expressed in almost all ENS progenitors in early development (E11.5, data not shown), the shift in *GFRα1* expression in developing enteric neurons suggested that GDNF plays a spatiotemporally specific role dependent on the developmental context.

To investigate the physiological role of GDNF in late ENS development in vivo, we engineered mice in which the function of *GFRα1* can be conditionally inactivated using the Cre-*loxP* system. A gene cassette composed of floxed *GFRα1* cDNA followed by *GFP* cDNA was knocked into the first coding exon of the *GFRα1* gene by homologous recombination (Fig. 2A; see Fig. S2 in the supplementary material). Because Cre-mediated excision of floxed *GFRα1* cDNA converts the floxed *GFRα1* allele (functional) into a *GFP* reporter allele (null, see Materials and methods), this strategy allows facile detection of cells that have undergone Cre recombination through monitoring of GFP fluorescence (see Fig. S2D in the supplementary material). *GFRα1*$^{flox/flox}$ mice were viable, grew to adulthood and were fertile, validating the normal function of the floxed *GFRα1* allele. To temporarily control the inactivation of *GFRα1* function, we used *CAGGCre-ER*^{TM} mice (Hayashi and McMahon, 2002), which ubiquitously express chimeric Cre protein fused to the mutated ligand-binding domain of the estrogen receptor. We crossed *GFRα1*$^{flox/flox}$ mice to *GFRα1*$^{+/-}$ mice (Enomoto et al., 1998) harboring the *CAGGCre-ER*^{TM} transgene to obtain *GFRα1*$^{flox/+}$:*CAGGCre-ER*^{TM} or *GFRα1*$^{flox/-}$:*CAGGCre-ER*^{TM} embryos in the same litters (Fig. 2B). Treatment of pregnant mothers with 4-OHT induced Cre activity in utero, generating *GFRα1*$^{GFP/+}$:*CAGGCre-ER*^{TM} (control) and *GFRα1*$^{GFP/-}$:*CAGGCre-ER*^{TM} (conditional KO:cKO) embryos. In this setting, control and cKO cells differ only in whether they maintain *GFRα1* expression or not; other conditions, including 4-OHT exposure and transient Cre activation, were identical. Thus this strategy allowed us to reliably assess the physiological function of GFRα1 by comparing phenotypes between control and cKO cells.

By GFP and PGP9.5 double staining, the estimated recombination efficiency in enteric neurons was approximately 70% (Fig. 2C). No GFRα1 protein was detected in GFP-positive (GFP$^+$) cells in cKO embryos (Fig. 2D; gut from an E15.5 embryo treated with 4-OHT at E13.5 shown as an example). Furthermore, phosphorylation of

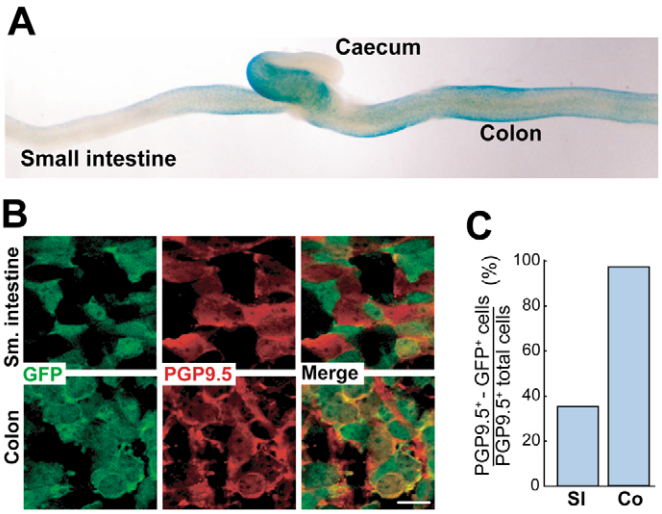

Fig. 1. *GDNF* expression is restricted to the distal gut after colonization of the entire gut by ENS progenitors. (**A**) *GDNF* expression in gut at E15.5. The gut isolated from *GDNF*$^{lacZ/+}$ mouse embryos was stained with X-gal histochemistry. Intense X-gal staining was observed in the cecum and colon. (**B**) Wholemount preparations of the small intestine (upper panels) and colon (lower panels) of E15.5 *GFRα1*$^{GFP/+}$ embryos (obtained by crossing *GFRα1*$^{flox/+}$ mice to β-actin Cre mice: see Materials and methods). *GFRα1*-expression (as revealed by GFP) was confirmed in almost all PGP9.5$^+$ neurons in the colon, whereas only a small neuronal population expressed *GFRα1* in the small intestine. (**C**) Quantitative analysis of *GFRα1*-expressing neuronal population. Percentage of GFP$^+$-PGP9.5$^+$ neurons / total PGP9.5$^+$ neurons is presented. Scale bar: 10 μm in B. Co, colon; SI, small intestine.

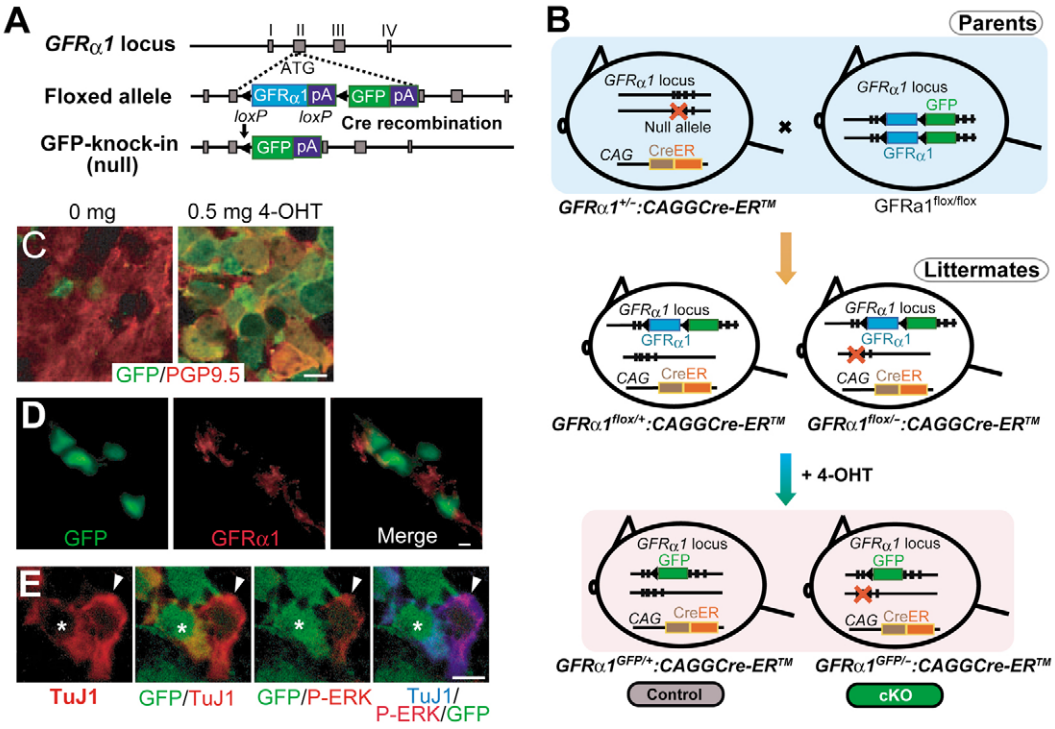

Fig. 2. Conditional inactivation of the *GFRα1* gene in mouse. (**A**) Schematic drawing of the *GFRα1* locus, the floxed *GFRα1* and *GFP* knock-in (null) alleles. Floxed *GFRα1* allele expresses *GFRα1* cDNA, thereby serving as a functional allele. Activation of Cre recombinase results in a removal of floxed *GFRα1*, simultaneously generating *GFP* knock-in (*GFRα1*-null) allele. (**B**) Schematic diagram of breeding strategy. *GFRα1*$^{+/–}$*:CAGGCre-ER*^{TM} mice were mated to *GFRα1*$^{flox/flox}$ mice to obtain *GFRα1*$^{flox/+}$*:CAGGCre-ER*^{TM} (control) and *GFRα1*$^{flox/–}$*:CAGGCre-ER*^{TM} (knockout, cKO) embryos in the same litter. Pregnant mice were given an intraperitoneal injection of 4-OHT at the desired time point to induce Cre activity. Following recombination, *GFP* reporter gene was expressed in the control and cKO embryos. (**C**) 4-OHT-induced Cre recombination in enteric neurons. Minimal 'leaky' recombination in the colon of E15.5 *GFRα1*$^{flox/+}$ embryo was observed in the absence of 4-OHT treatment (left panel). Administration of 4-OHT induced GFP expression in a large number of neurons in the colon, indicating that Cre recombination occurred in the majority of enteric neurons in the colon (right panel). (**D**) Histological analysis of cKO enteric plexus showing no GFRα1-immunoreactivity (red) in GFP^{+} cells (green). Note the expression of GFRα1 (red) in neighboring un-recombined cells. (**E**) Absence of pERK in GFRα1-deficient enteric neurons. Activation of ERK (pERK) was detected in GFP-negative-TuJ1-positive cells (arrowhead). By contrast, no pERK signal was observed in GFP-positive-TuJ1-positive cells (asterisk). Scale bars: 20 μm in C; 5 μm in D,E.

ERK, a signaling event downstream of RET activation, was not detectable in GFP^{+} cells, which was already evident 1 day after 4-OHT treatment (Fig. 2E, gut from an E13.5 embryo treated with 4-OHT at E12.5 shown as an example). These results validated the idea that the conditional ablation strategy was operational and suggested that GDNF signaling is impaired in enteric neurons rapidly after *GFRα1* inactivation.

GFRα1 inactivation during late gestation leads to drastic cell loss in the ENS

In order to investigate the function of GDNF signaling at late stages of ENS development, we chose E15.5 as a time point to inactivate *GFRα1*. Formation of the ENS proceeded normally in both *GFRα1*$^{flox/+}$*:CAGGCre-ER*^{TM} and *GFRα1*$^{flox/–}$*:CAGGCre-ER*^{TM} embryos without Cre activation (Fig. 3A). Pregnant mothers were administered 0.5 mg 4-OHT at E15.5, and the gut from their embryos was examined at E18.5 (3 days after *GFRα1* inactivation) by acetylcholinesterase (AChE) histochemistry, which labels the cell bodies and neurites of enteric neurons. A dense reticulate pattern of the enteric ganglia and their innervation was observed in the small intestine; the staining pattern was comparable in control and cKO embryos (Fig. 3B insets, SI). However, in the colon of the cKO embryos, ganglion structure was completely disrupted, and abnormally thick nerve bundles were observed (Fig. 3B, bottom). These nerve fibers were likely un-defasciculated extrinsic nerve fibers (Payette et al., 1987), similar to those observed in the colon of patients with Hirschsprung's disease. The results suggested that enteric neurons were lost by birth in cKO colon. Toluidine Blue staining of semi-thin longitudinal sections of the gut confirmed the nearly complete absence of the enteric ganglia in cKO colon (Fig. 3C). Thus enteric neurons and glia are eliminated rapidly after inactivation of *GFRα1*, revealing the essential role of GDNF signaling for the survival of cells in the ENS during late gestation.

Spatiotemporally specific regulation of enteric neuron survival by GFRα1

To understand the spatiotemporal specificity in GFRα1 regulation of ENS cell survival, we inactivated *GFRα1* function at different time periods. When embryos were treated with 4-OHT at E11.5 (in the midst of ENS progenitor migration) and their gut was examined at E13.5, we observed that the population of cells expressing GFP in cKO gut was significantly smaller than that in control (Fig. 4A). This was at least partly explained by impaired proliferation, as significantly fewer GFP^{+} cells incorporated BrdU in the cKO midgut than in control (cKO 12.2±1.4% versus control 24.4±3.4%; values obtained by BrdU^{+} cells/GFP^{+} cells, n=3 for each genotype,

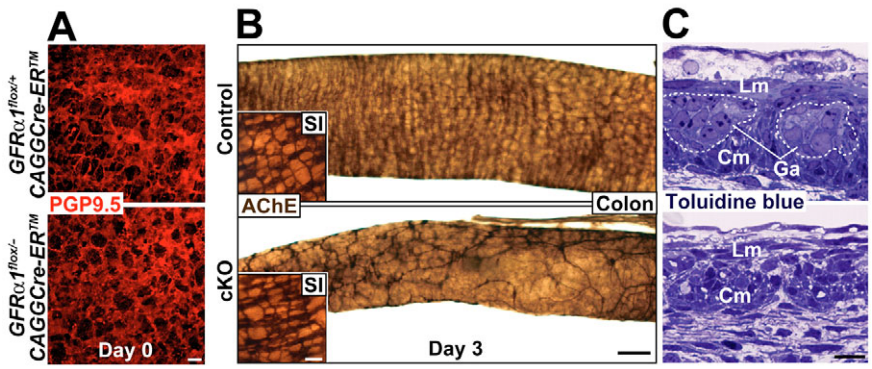

Fig. 3. Inactivation of *GFRα1* depletes enteric neurons in the colon. (**A**) PGP9.5 immunostaining of the distal colon in E15.5 control and cKO mouse embryos before injection of 4-OHT. Comparable ENS staining was observed in control and cKO colon, indicating normal development of the ENS before induction of GFRα1 inactivation. (**B**) Wholemount AChE histochemical analysis of E18.5 gut 3 days after inactivation of *GFRα1*. The highly organized meshwork-like pattern of ENS network observed in control (top) was dramatically disrupted in cKO (bottom). ENS structure in the small intestine was well maintained in both embryos (insets). (**C**) Toluidine Blue staining revealed the absence of enteric ganglia (Ga) in the myenteric layer (between Lm and Cm) of the colon in cKO embryos. Cm, circular muscle; Co, colon; Ga, enteric ganglia; Lm, longitudinal muscle; SI, small intestine. Scale bars: 20 μm in A; 200 μm in B; 10 μm in C.

$P<0.05$). We detected no sign of increased cell death of GFP-expressing cells by TUNEL in cKO gut (E12.5-13.5, data not shown). Because TUNEL was not an ideal marker to efficiently detect cell death in the ENS (see below), we also performed time-lapse microscopic analysis of cKO cells in gut explant culture. No abnormal cell death was detected even by prolonged time-lapse observation (up to 32 hours after 4-OHT treatment, data not shown). Those results demonstrate that, during E11.5-13.5, GDNF signaling plays a crucial role in cell proliferation (Gianino et al., 2003), but not cell survival.

We also treated embryos with 4-OHT at E13.5 [a period just after the entire small intestine and approximately two-thirds of the colon is colonized by ENS progenitors (Young et al., 1998)] and examined the gut at E18.5. Although intestinal aganglionosis was observed throughout the entire colon, just as seen in the colon subjected to *GFRα1* inactivation at E15.5, no structural abnormalities were detected in the enteric ganglia of the small intestine (Fig. 4B, $n=5$). This revealed that, unlike in the colon, widespread cell death does not take place in the small intestine, although the possibility that a minimal number of neurons may die in the small intestine after *GFRα1* inactivation cannot be excluded. The unequivocal phenotypic difference between the small intestine and the colon in conditional *GFRα1*-deficient embryos suggested that the requirement of GFRα1 for ENS cell survival is more related to the regional specificity of the colon rather than to the timing of colonization of the gut by ENS progenitors.

We also inactivated *GFRα1* in the postnatal ENS, and found no adverse effects on the structural integrity of the ENS when it was examined at P14 (Fig. 4C). The data collectively reveal that the requirement of GFRα1 for ENS cell survival is restricted to the colon during the late-gestational to early-postnatal period.

Lack of caspase activation during ENS degeneration of *GFRα1* cKO colon

Virtually all cells in the ENS were eliminated in cKO colon within 3 days after 4-OHT administration when GFRα1 inactivation was induced during late gestation. To determine the kinetics of cell death, we performed timecourse analyses. In both control and cKO colon, GFP expression became detectable 10-12 hours after 4-OHT administration (data not shown). By 16-21 hours after 4-OHT treatment, almost the entire myenteric layer was visualized by GFP in the colon of both control and cKO embryos (Fig. 5A, top). Because GFRα1 is known to be expressed in both enteric neural crest derivatives and smooth muscle cells at mid-gestation, we examined which cell types were marked by GFP in the colon. Most GFP-expressing cells were positive either for Phox2b, PGP9.5 and/or B-FABP (Fig. 5B, data not shown), indicating that the majority of cells marked by strong GFP fluorescence were immature neurons or glia at this developmental time period (Young et al., 2003). After 21 hours of 4-OHT administration, a progressive disappearance of GFP fluorescence was observed, and the signals were completely extinguished by 36 hours after 4-OHT treatment in most cKO colons, while GFP signals in the corresponding regions of control colon remained intact during the timecourse (Fig. 5A, cKO bottom and control). Complete absence of enteric ganglia in cKO colon (36 hours after 4-OHT) was confirmed by thionin staining of the colon sections (data not shown). These results indicated that enteric ganglion cells in cKO colon die within 36 hours after 4-OHT. Although the results also indicated that both enteric neurons and glia died in the absence of *GFRα1*, cells primarily affected by conditional *GFRα1* ablation were considered to be neurons because *GFRα1* is more highly expressed in colonic neurons at E15.5 (Fig. 1B) and because loss of *GFRα1* in non-neuronal cells does not affect ENS development (Enomoto et al., 2004) (see also Discussion).

As the peak of cell death was estimated to occur 24-30 hours after 4-OHT treatment, we attempted to visualize dying cells using TUNEL or antibodies that detect activated caspsase-3 or caspase-7. Surprisingly, despite the drastic cell loss, very few cells were detected by these methods. The total numbers of TUNEL-positive cells, generated by summing all consecutive sections of the entire colon, were 0.7±0.8, 7.0±1.4, 11.0±3.9 and 0 at 21, 24, 27 and 36 hours after 4-OHT treatment, respectively ($n=3$ for each time point). Activated-caspase staining detected even fewer cells (0.6±0.5 cells and 0.4±0.4 cells per entire colon for caspase-3 and caspase-7, respectively; only found at 27 hours after 4-OHT, $n=3$ for each time point). We failed to detect any TUNEL-positive cells that were also caspase-positive (Fig. 5C right). The staining pattern in the

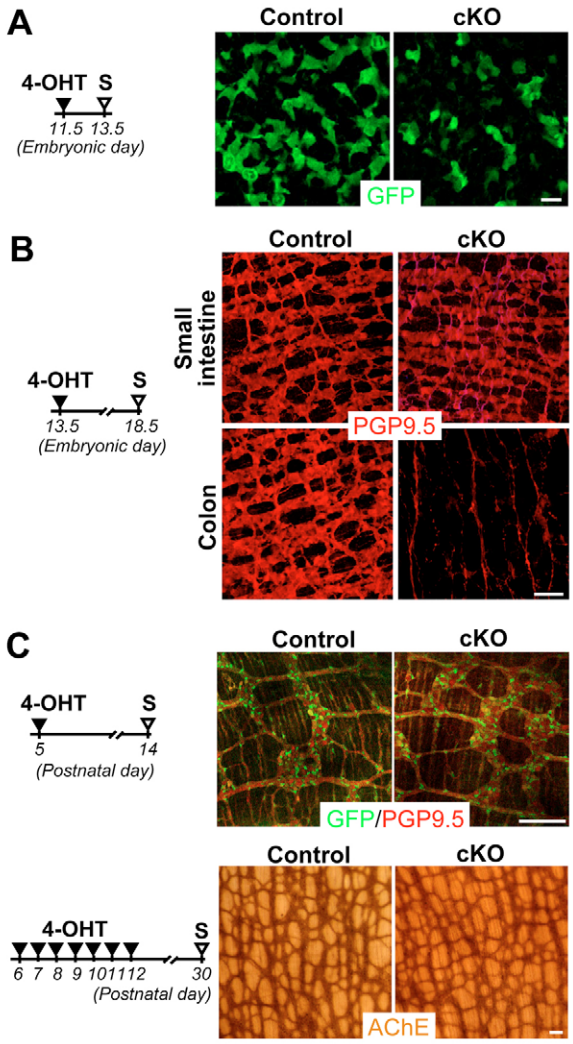

Fig. 4. Spatiotemporally specific effects of *GFRα1* inactivation on ENS development in mouse. (**A**) Confocal microscopic images of wholemount preparations of midgut from E13.5 control and cKO embryos in which *GFRα1* inactivation was induced at E11.5. GFP$^+$ cells were less dense in the cKO than control midgut. (**B**) Wholemount PGP9.5 staining of small intestine from E18.5 control and cKO embryos subjected to 4-OHT treatment at E13.5. Although total colon aganglionosis was observed in cKO embryos (bottom right), the ENS structure in the small intestine was well maintained (top right). (**C**) Wholemount preparation of myenteric plexus of the colon from P14 mice subjected to GFRα1 inactivation at P5. No obvious abnormalities were found in enteric neurons (PGP9.5$^+$) or GFP$^+$ cells of cKO myenteric plexus (upper panels). Even multiple 4-OHT injection did not affect ENS structure (lower panels). Recombination efficiency by single 4-OHT injection was estimated as 50% for A and B, and 40% for C. S, sacrificed. Scale bars: 10 μm in A; 50 μm in B; 100 μm in C.

degenerating ENS was quite unusual because, in other parts of the developing nervous system where neuronal apoptosis is taking place, activated-caspase-positive cells often outnumber and also include TUNEL-positive cells (Fig. 5C left, DRG shown as an example). Thus, neither TUNEL nor activated caspase efficiently detected cell death in the ENS after *GFRα1* inactivation, implying a unique regulation of cell death in *GFRα1*-deficient cells in the colon.

GDNF-deprived enteric neurons die in a Bax- and caspase-independent fashion

The absence of caspase activation in *GFRα1* cKO gut prompted us to examine the involvement of conserved death pathways in enteric neurons. We immunopurified enteric neurons from E15.5 embryonic gut, cultured them with GDNF for 2 days and then examined the effect of GDNF withdrawal. While most neurons from the small intestine survived for several days after GDNF withdrawal (Fig. 6A, SI), more than 70% of the neurons from the colon died within 24 hours after GDNF deprivation (Fig. 6A,B). Thus, the survival of the majority of neurons from the colon is solely dependent on GDNF under these culture conditions. Interestingly, neurons from the colon became more resistant to GDNF withdrawal when they were cultured initially for at least 3 days in the presence of GDNF (data not shown). Thus GDNF dependency by colonic neurons is limited to a relatively small time window, which appears to recapitulate the observation that GFRα1 is required for colonic neuron survival only during late gestation.

We analyzed the biological properties of the death process triggered by GDNF deprivation using enteric neurons isolated from the colon. NGF-deprived sympathetic neurons were separately prepared, as these neurons die by apoptosis in a caspase-dependent fashion (Deshmukh et al., 1996), thereby serving as control. In NGF-deprived sympathetic neurons (48 hours after NGF deprivation), chromatin compaction and nuclear fragmentation was easily discernible by Hoechst staining, and the staining overlapped with strong punctate signals by TUNEL (Fig. 6C, right panels). By contrast, dying enteric neurons displayed little chromatin compaction and were only weakly stained by TUNEL in a hazy pattern (Fig. 6C, left panels). The staining pattern by TUNEL in GDNF-deprived enteric neurons was reminiscent of that observed in neuronal death in caspase-3-deficient mice (Oppenheim et al., 2001). Thus we examined caspase activation and its involvement in enteric neuron death. Caspase activation was examined by counting the numbers of cells positive for active caspase-3 against numbers of remaining enteric neurons detected by TuJ1 immunoreactivity. Only a minor fraction of enteric neurons was stained by activated caspase-3 at 12 hours after GDNF deprivation (1.1±0.3% in GDNF-deprived versus 0.6±0.2% in control neurons). Similar results were obtained at 24 hours after GDNF deprivation. In addition, caspase-7 activation was also infrequently observed during the timecourse of cell death (data not shown). These observations contrasted sharply with the widespread caspase-3 activation that occurred in NGF-deprived sympathetic neurons (5.1±3.2% of control versus 26.3±2.9% in NGF-deprived neurons; 24 hours after NGF withdrawal). Furthermore, treatment of neurons with zVAD-fmk, a pan-caspase inhibitor, which completely protected sympathetic neurons from apoptosis, failed to rescue the survival of enteric neurons (Fig. 6D). Therefore, caspases are only minimally activated and are dispensable for enteric neuron death.

To investigate the potential involvement of the proapoptotic protein, Bax, we cultured enteric neurons from *Bax*$^{-/-}$ embryos and examined their response to GDNF deprivation. Although *Bax*$^{-/-}$ neurons tended to show a slight increase in viability, the difference in their survival was not statistically significant (three independent experiments, representative results shown in Fig. 6E). By contrast, *Bcl-X$_L$* overexpression in enteric neurons significantly rescued the survival of GDNF-deprived enteric neurons (Fig. 6F). Collectively, the results show that enteric neuron death requires neither Bax nor caspases, key components for apoptosis, suggesting the presence of non-canonical cell death machinery in GDNF-deprived enteric neurons that can be blocked by Bcl-X$_L$.

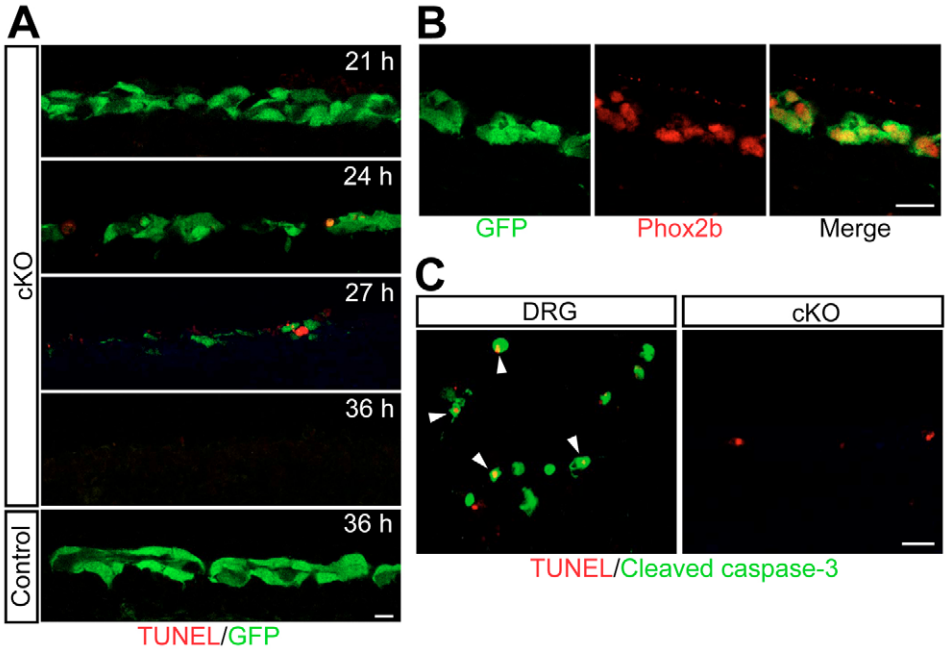

Fig. 5. Absence of caspase activation during ENS degeneration induced by inactivation of *GFRα1*. (**A**) Timecourse analysis of ENS degeneration after administration of 4-OHT (at 0 h). The gut samples collected at different time points were doubly stained by GFP immunohistochemistry and TUNEL. Note the progressive loss of GFP-positive cells in cKO colon 24-36 hours after administration of 4-OHT. TUNEL staining was observed in the colon 24-27 hours after 4-OHT treatment. GFP staining persisted in control colon (bottom). (**B**) Immunostaining of GFP (green) and Phox2b (red). Nearly complete overlap between GFP and Phox2b signals indicates that GFP predominantly labels enteric neurons and glia, but not smooth muscle cells, in the E16 distal colon. (**C**) Histochemical visualization of activated caspase-3- (green) and TUNEL-positive (red) cells. In embryonic DRGs, activated-caspase-3-positive cells outnumbered TUNEL-positive cells, and there was a significant overlap between those two cell populations. Caspase activity was not detected in any of the TUNEL-positive cells in cKO colon at any point during the timecourse of loss of GFP$^+$ cells. Scale bars: 20 μm.

Ultrastructural analysis reveals atypical death of enteric neurons in vivo

Analysis of cultured enteric neurons revealed that enteric neurons die in a non-apoptotic and caspase-independent fashion in vitro. To elucidate the morphological attributes of ENS cell death, we examined the ultrastructure of enteric ganglia by transmission electron microscopy in control and cKO colon 20-48 hours after 4-OHT treatment of pregnant mothers at E15.5.

Because of the immaturity of enteric ganglion cells in the colon at this developmental stage, we were not able to distinguish neurons from glia clearly. Nevertheless, consistent morphological changes in cellular architecture were detectable in myenteric ganglion cells during the course of ENS degeneration in cKO colon. In control colon, most myenteric ganglion cells displayed ovoid cytoplasm containing round or football-shaped nuclei with finely dispersed chromatin (Fig. 7A,C). In cKO ganglia with their integrity relatively well preserved (e.g. Fig. 5A, 24 h), the only discernible change was found in the nucleus: many cKO ganglion cells displayed abnormal indentations in the nuclear membrane (Fig. 7A, right). Occasionally, more than one nuclear profile was found in a single cell plane (Fig. 7A, marked in green or pink in cKO). The results suggest that these alterations in nuclear morphology are the first event in the cell death process. In enteric ganglia where cell loss or reduced ganglion size was recognizable (intermediate stage), most cells displayed an abnormal constriction of the nuclear membrane, which resulted in multilobulation of the nuclei (Fig. 7B). Cells were shrunken, while marginal heterochromatin became highly condensed in irregularly shaped nuclei, and small chromatin masses were dispersed in the karyoplasm (Fig. 7C, right). Despite these changes, whenever identified, mitochondria were evenly scattered in the cytoplasm, their matrices were pale with electron density equivalent to that of the surrounding cytoplasm, and the overall structural integrity as short barrels with cristae remained intact (Fig. 7C, arrowheads). Other membranous structures including polysomes, profiles of ER and Golgi apparatus also appeared normal in cKO ganglion cells (data not shown). In the myenteric layer region, where ganglion structure was almost completely disrupted (terminal stage), the diminution of the cytoplasm and heterochromatin condensation in the nuclei was markedly enhanced (Fig. 7E, left). A number of cells in the degenerating ganglia possessed heterophagosome-like vacuoles at this stage (Fig. 7D, upper panel). Notably, despite our extensive search, we never observed typical nuclear hallmarks of apoptosis, such as globular or crescent-shaped chromatin compaction, in any individual cells at any stages of cell death. On very rare occasions nuclei with highly condensed chromatin were detected, but this was found exclusively in heterophagosome-like vacuoles (Fig. 7D, lower panel). These results suggest that chromatin compaction does not take place by cell-autonomously initiated mechanisms in dying enteric neurons. We occasionally observed cells with single-membraned vacuoles containing partly digested cytoplasmic organelles, which are characteristic of autolysosomes (Fig. 7E, middle), although such cells were few in number. In addition, a few degenerating cells with numerous vacuoles of various sizes were also found in the terminal stage cKO colon (Fig. 7E, right). Although this result suggested the

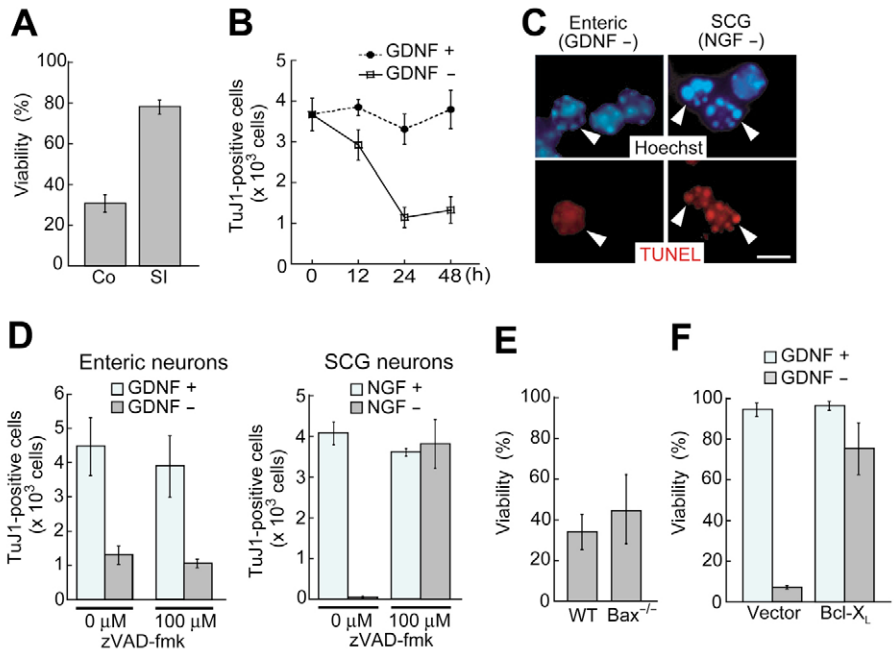

Fig. 6. Caspases are not required for the death of GDNF-deprived enteric neurons. (**A**) Enteric neurons from distinct gut regions respond differentially to GDNF deprivation. Enteric neurons isolated from mouse E15.5 colon and small intestine were cultured for 2 days and then switched to GDNF-deprived conditions. The ratio of surviving neuron numbers (48 hours after GDNF deprivation) to initially plated cell numbers is shown. (**B**) Timecourse analysis of colonic neuron survival. TuJ1-positive neurons were counted at 0, 12, 24 and 48 hours after GDNF deprivation (–) or in the presence of GDNF (+). (**C**) Nuclear staining by Hoechst 33342 (upper panels) and TUNEL (lower panels) of enteric neurons 12 hours after GDNF deprivation (left panels) or of SCG neurons 24 hours after NGF deprivation (right panels). Condensed chromatin and nuclear fragmentation associated with strong TUNEL-reactivity, salient features in dying SCG neurons (arrowheads in right panels), were never observed in GDNF-deprived enteric neurons (arrowheads in left panels). (**D**) Treatment with the broad-range caspase inhibitor zVAD-fmk (100 μM) rescues NGF-deprived SCG neurons (right) but not GDNF-deprived enteric neurons (left). Survival of enteric neurons and sympathetic neurons was examined 2 days after GDNF deprivation or 5 days after NGF deprivation, respectively. (**E**) Responses of *Bax*-deficient enteric neurons to GDNF withdrawal. No significant differences were observed in the survival of colonic neurons between *Bax*-deficient and wild-type embryos (E15.5). Values represent the percentages of living cells normalized to the GDNF-maintained neurons. (**F**) Bcl-X_L overexpression rescues the survival of GDNF-deprived enteric neurons. Enteric neurons were infected with lentivirus expressing Bcl-X_L or vector alone several hours after plating. Surviving neurons (TuJ1$^+$) were counted 48 hours after GDNF deprivation. Scale bar: 10 μm in C. Co, colon; SI, small intestine; WT, wild type.

potential involvement of autophagy, we rarely detected the emergence of typical autophagosomes at any stage of cell death. Thus autophagy does not seem to be a prevalent process in *GFRα1*-deficient degenerating ENS cells. Finally, necrotic cells were never found in the myenteric layer of cKO colon. Collectively, these studies revealed that, unlike cell death that occurs in many other parts of the nervous system, dying enteric ganglion cells exhibit molecular and morphological features distinct from those of apoptosis.

DISCUSSION

It is widely accepted that GDNF signaling via GFRα1/RET is central to the control of ENS development, because homozygous null mutations in either of these genes affect ENS progenitor cells early during ENS development and cause the nearly complete absence of the enteric ganglia throughout the entire gastrointestinal tract. In contrast to the importance of GDNF signaling in early ENS development, however, the physiological role of GDNF signaling in later ENS development has not been explored extensively. By engineering mice in which GFRα1, the high affinity GDNF receptor, can be conditionally inactivated, we were able to assess directly, for the first time, the physiological function of GFRα1 in ENS development during late gestation. Our analysis has revealed a previously unforeseen function of GFRα1 in cell survival control of the ENS, providing novel insights into the regulation of ENS development by GFRα1.

GDNF is a physiological survival factor for enteric neurons

Previous mouse genetic studies have identified several genes that play crucial roles in ENS development, some of which are shown to be potentially involved in the regulation of cell survival. For instance, increased cell death of ENS progenitors is observed in the esophagus of *Ret*$^{-/-}$, *Phox2b*$^{-/-}$ and *Sox10*^{Dom}/*Sox10*^{Dom} (Dom, Dominant megacolon mutation) embryos (Kapur, 1999; Pattyn et al., 1999; Taraviras et al., 1999). Moreover, decreases in enteric neuron numbers are observed in mice lacking *NT-3* or *TrkC* (also known as *Ntf3* and *Ntrk3*, respectively, – Mouse Genome Informatics) (Chalazonitis et al., 2001). However, due to the complex nature of ENS development, in which cell migration, proliferation, differentiation and survival proceed in a significantly overlapping manner, it has been difficult to deduce the precise function of those molecules by simply examining ENS phenotypes of mutant mice. By generating conditional *GFRα1* mouse mutants and disrupting *GFRα1* gene function during late gestation, we were able to examine the biological role of GFRα1 specifically in

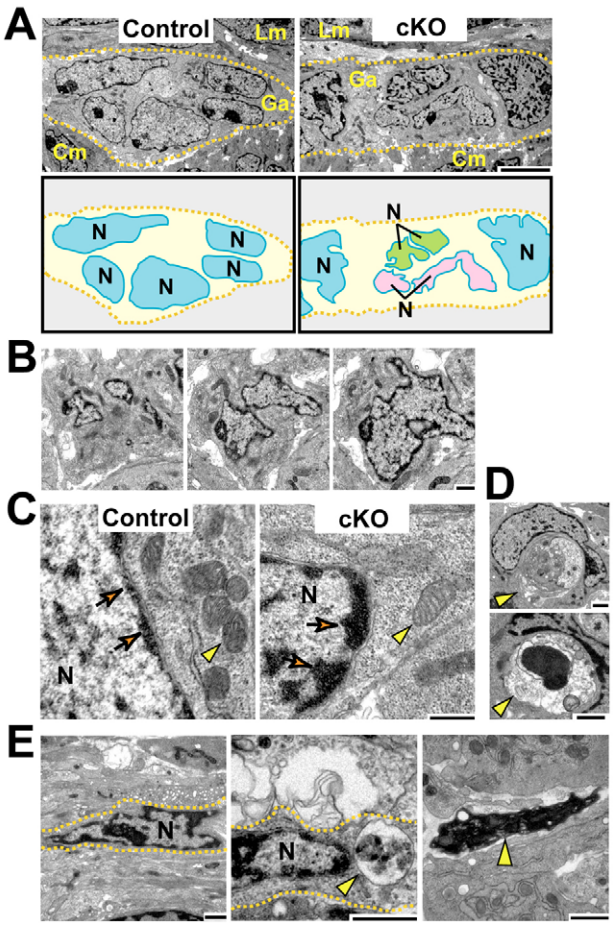

Fig. 7. Morphological characteristics of dying enteric ganglion cells. (**A**) Longitudinal section of enteric ganglia in a segment of the colon from E16.5 control and cKO mouse embryos 1 day after inactivation of *GFRα1*. Many nuclei exhibited constricted and irregularly shaped morphologies in cKO embryos (top right). (Bottom) Schematic figures depicting the location of the myenteric ganglia (circled by dotted lines) and the positions of the nuclei in myenteric ganglion cells (indicated by N). Multiple nuclear profiles observed in a single cell plane are marked by pink and green. (**B**) A parallel section series of a single cell in cKO colon, revealing abnormal lobulation of the nucleus, but not fragmentation. (**C**) High-magnification view of perinuclear regions of control and cKO cells. Note that electron-dense structures in the nucleus (N) are more prominent in cKO than in control cells (right, arrows). No significant changes were observed in mitochondrial morphology (arrowheads). (**D**) Clearance of dying cells by large vacuoles (arrowheads) in cKO enteric ganglia. Conditional KO colon 27 hours after inactivation of *GFRα1* shown as an example (upper panel). A phagocytotic vacuole containing cell debris of high electron density, possibly representing a condensed nucleus of the engulfed cell (lower panel). (**E**) Left: a typical degenerating cell with severely depleted cytoplasm in the cKO distal colon 27 hours after inactivation of *GFRα1*. Middle: representative image of a single-membraned vacuole containing cellular components (arrowhead) in a degenerating ganglion cell. Right: a degenerating cell with high electron-dense cytoplasm throughout the entire cell body. Note that the cell also contains multivesicles (arrowhead). Broken lines depict cell margins. Scale bar: 5 μm in A; 1 μm in B,D,E; 0.5 μm in C. Cm, circular muscle; Ga, enteric ganglia; Lm, longitudinal muscle; N, nucleus.

postmigratory differentiating cells in the ENS. *GFRα1* inactivation rapidly triggered cell death and nearly completely disrupted the ENS structure in the normally formed colon, providing compelling evidence that *GFRα1* inactivation affects cell survival during late gestation. Because GFRα1/RET receptor complex mediates GDNF signaling, and because *NRTN*- or *ARTN*-deficient mice do not display aganglionosis of the colon (Heuckeroth et al., 1999; Honma et al., 2002), the ligand that associates with GFRα1 in vivo to support colonic neuronal survival is likely to be GDNF.

In *GFRα1* cKO gut, not only neurons but also glial cells die, as virtually all cells are eliminated in the myenteric layers of *GFRα1* cKO colon by birth. Because GFRα1 is expressed in enteric neurons, glia and smooth muscle cells in the colon during late gestation, one might speculate that *GFRα1* inactivation affects all of these cell types and that neurons die secondarily due to death of those non-neuronal cells. However, mice in which the elimination of *GFRα1* expression in the gut is restricted to smooth muscle cells and glia (*cis*-only mice) (Enomoto et al., 2004) display normal glial cell numbers in the ENS (see Fig. S4 in the supplementary material), indicating that the loss of *GFRα1* in non-neuronal cell populations in the gut has no adverse effects on the survival of glia in the ENS. Thus, the cells that are primarily affected in *GFRα1* cKO colon are neurons, and neuronal cell death occurs in a cell-autonomous fashion. We speculate that enteric glia die secondarily to enteric neuron death in *GFRα1* cKO colon owing to the sudden abolition of trophic support from neurons, similar to the massive glial death that is observed in response to neuronal insults in other regions of the developing nervous system (Grinspan et al., 1996; Riethmacher et al., 1997; Trachtenberg and Thompson, 1996).

Our electron microscopic analysis of the colon of *GDNF* heterozygous embryos failed to detect the presence of death figures in the developing ENS (T.U., unpublished). This is perhaps because the early reduction of proliferating ENS progenitors diminishes the number of enteric neurons at later stages and thereby decreases the vulnerability to reduced GDNF levels in heterozygous animals. Thus, the survival-promoting actions of GDNF signaling could have not been uncovered without conditional gene ablation. Collectively, our study, along with previous evidence, establishes GDNF as a pleiotropic factor controlling multiple facets of enteric neuron development, including cell migration, proliferation, differentiation and survival.

Unique features in the control of survival and death of enteric neurons by GDNF signaling

Our histological and biochemical characterization of enteric neuron death delineates crucial features of the death of these neurons. A detailed temporal analysis of *GFRα1*-deficient colon revealed that the death process occurred fairly rapidly, resulting in the almost complete elimination of neurons within 36 hours after induction of *GFRα1* inactivation, as judged by GFP fluorescence. Considering the time required for Cre-mediated *GFRα1* cDNA excision, GFRα1 degradation and GFP maturation in those neurons (more than 8-10 hours in total), the estimated time for enteric neuron death is less than 26-28 hours after actual loss of *GFRα1* function.

Although we observed the emergence of TUNEL-positive cells in the ENS after *GFRα1* inactivation, the total numbers of TUNEL-positive cells in the entire colon even at multiple time points were less than 20, which is remarkably few, considering that as many as 20,000 neurons (Gianino et al., 2003) succumb to death in *GFRα1*-deficient colon within 36 hours, and in comparison to the significantly higher levels of TUNEL-positive cells observed in other parts of the nervous system during the naturally occurring cell death period (White et al., 1998). We did not observe activation of caspase-3 or caspase-7 in *GFRα1*-deficient colon even at the peak of the cell death period. Thus, neither TUNEL nor activated caspase

immunoreactivity efficiently detected ENS cell death after *GFRα1* inactivation. Consistent with these findings, pan-caspase inhibition does not block death of GDNF-deprived colonic neurons in vitro. Furthermore, hallmarks of apoptosis (chromatin compaction and mitochondrial pathology) were rarely detected in dying enteric neurons in ultrastructural analyses. The results demonstrate that the death machinery employed by enteric neurons is distinct from that utilized by other types of neurons undergoing apoptosis. Although our morphological examinations delineated crucial features of dying enteric neurons, they failed to definitively classify enteric neuron death into one of the currently proposed death forms such as apoptosis, autophagic death or necrosis (Clarke, 1990; Nelson and White, 2004). Enteric neuron death is also distinct from previously reported atypical death of GDNF-deprived sympathetic neurons (Yu et al., 2003) in that enteric neuron death proceeds in a caspase-independent fashion. To our knowledge, this is the first evidence that caspase-independent cell death is triggered by disruption of neurotrophic factor signaling. The unconventional nature of enteric neuron death may explain why previous studies failed to detect apoptosis during the development of the ENS. Physiological cell death in the ENS, if it in fact occurs at all, may not take the form of apoptosis, as we observed in *GFRα1* cKO colon.

Although our data have revealed an unconventional form of enteric neuron death, the underlying mechanisms remain elusive. Efficient blockade of enteric neuron death in vitro by Bcl-X_L overexpression suggests that enteric neuron death triggered by GDNF does not reflect a global collapse of cellular homeostasis, but rather a molecularly regulated process. The finding also suggests the potential involvement of mitochondria in the death process, although only minimal morphological abnormalities were detected in the mitochondria of dying enteric neurons in vivo. Alternatively, autophagy may in fact play a role in GDNF deprivation-induced enteric neuron death, because Bcl-2, one of the closest relatives of Bcl-X_L, inhibits autophagy and autophagy-induced cell death by interacting with Beclin 1 (Pattingre et al., 2005). Consistent with this possibility, we observed a few degenerating cells with multivesicular lysosomal structures in cKO colon, suggesting that at least a fraction of enteric neuron death is associated with the abnormal activation of autophagic processes. However, treatment of enteric neurons with 3-methyladenine, a pharmacological inhibitor of autophagy, did not block the death or prolong the survival of GDNF-deprived neurons (our unpublished data), arguing against the notion that conventional autophagy plays a pro-death role, at least in our culture paradigm. As Bcl-X_L interacts with a number of molecules and mediates a variety of functions dependent on its subcellular localization, determining the site of action of Bcl-X_L (e.g. mitochondria or ER membrane) in cell death inhibition may help to clarify the mechanisms underlying enteric neuron death.

Finally, the unconventional features of the death of GFRα1-deficient colonic neurons raise the possibility that, in some pathological conditions, neuronal death in the enteric ganglia is indiscernible by conventional methods such as TUNEL or immunodetection of activated caspases. Especially, it is important to consider the potential involvement of cell death in the etiology of Hirschsprung's disease, because mutations in the *Ret* gene are the primary cause of the disease and because aganglionosis is restricted to the distal colon in most patients. In this respect, it is noteworthy that RET can function as a dependence receptor and induces cell death in certain in vitro paradigms (Bordeaux et al., 2000). Thus, it would be of interest to investigate whether RET dysfunction caused by Hirschsprung's disease-associated *Ret* mutations induces cell death in the colon and to examine whether the death, if any, is triggered by dependence-receptor function of RET in future studies.

We thank Eugene M. Johnson Jr, Masatoshi Takeichi, Yoshiki Sasai, Masahiko Hibi, Masayuki Miura, Douglas Sipp, Shigeo Hayashi, Raj K. Ladher and Thomas W. Gould for critical reading of the manuscript. We are indebted to Vassilis Pachnis, Jean-Francois Brunet, Carlos Lois and Hiroyuki Miyoshi for providing us with valuable research materials. We also thank Kazuyo Misaki and members of the Enomoto laboratory and of the Laboratory for Animal Resources and Genetic Engineering (LARGE), especially to Kazuki Nakao and Hitoshi Miyachi, for their excellent technical assistances. This work was supported by RIKEN, MEXT 'The Project for Realization of Regenerative Medicine' (H.E.) and by Grant-in-Aid for Young Scientists (B, 18790154) from the ministry of Education, Science, Sports and Culture, Japan (T.U.), HOPE Center for Neurological Disorders, NIH grants HD047396 (S.J.), AG13730 and NS39358 (J.M.).

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/11/2171/DC1

References

Airaksinen, M. S. and Saarma, M. (2002). The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* **3**, 383-394.

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

Bordeaux, M. C., Forcet, C., Granger, L., Corset, V., Bidaud, C., Billaud, M., Bredesen, D. E., Edery, P. and Mehlen, P. (2000). The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *EMBO J.* **19**, 4056-4063.

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.

Clarke, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol.* **181**, 195-213.

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.

Deshmukh, M., Vasilakos, J., Deckwerth, T. L., Lampe, P. A., Shivers, B. D. and Johnson, E. M., Jr (1996). Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J. Cell Biol.* **135**, 1341-1354.

Durbec, P. L., Larsson-Blomberg, L. B., Schuchardt, A., Costantini, F. and Pachnis, V. (1996). Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development* **122**, 349-358.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M., Jr and Milbrandt, J. (1998). GFR α1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21**, 317-324.

Enomoto, H., Hughes, I., Golden, J., Baloh, R. H., Yonemura, S., Heuckeroth, R. O., Johnson, E. M., Jr and Milbrandt, J. (2004). GFRalpha1 expression in cells lacking RET is dispensable for organogenesis and nerve regeneration. *Neuron* **44**, 623-636.

Gianino, S., Grider, J. R., Cresswell, J., Enomoto, H. and Heuckeroth, R. O. (2003). GDNF availability determines enteric neuron number by controlling precursor proliferation. *Development* **130**, 2187-2198.

Golden, J. P., DeMaro, J. A., Osborne, P. A., Milbrandt, J. and Johnson, E. M., Jr (1999). Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. *Exp. Neurol.* **158**, 504-528.

Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulaloglou, M. and Scherer, S. S. (1996). Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J. Neurosci.* **16**, 6107-6118.

Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* **244**, 305-318.

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 [see comments]. *Neuron* **22**, 253-263.

Honma, Y., Araki, T., Gianino, S., Bruce, A., Heuckeroth, R., Johnson, E. and Milbrandt, J. (2002). Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. *Neuron* **35**, 267-282.

Indo, Y., Tsuruta, M., Hayashida, Y., Karim, M. A., Ohta, K., Kawano, T., Mitsubuchi, H., Tonoki, H., Awaya, Y. and Matsuda, I. (1996). Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat. Genet.* **13**, 485-488.
Kapur, R. P. (1999). Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. *Pediatr. Dev. Pathol.* **2**, 559-569.
Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A. and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270**, 96-99.
Kruger, G. M., Mosher, J. T., Tsai, Y. H., Yeager, K. J., Iwashita, T., Gariepy, C. E. and Morrison, S. J. (2003). Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. *Neuron* **40**, 917-929.
Le Douarin, N. M. (1986). Cell line segregation during peripheral nervous system ontogeny. *Science* **231**, 1515-1522.
Lois, C., Hong, E. J., Pease, S., Brown, E. J. and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-872.
Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G. and Johnson, E. M., Jr (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* **106**, 829-844.
Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. and Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *J. Virol.* **72**, 8150-8157.
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.
Nelson, D. A. and White, E. (2004). Exploiting different ways to die. *Genes Dev.* **18**, 1223-1226.
Newgreen, D. and Young, H. M. (2002a). Enteric nervous system: development and developmental disturbances – part 1. *Pediatr. Dev. Pathol.* **5**, 224-247.
Newgreen, D. and Young, H. M. (2002b). Enteric nervous system: development and developmental disturbances – part 2. *Pediatr. Dev. Pathol.* **5**, 329-349.
Oppenheim, R. W., Flavell, R. A., Vinsant, S., Prevette, D., Kuan, C. Y. and Rakic, P. (2001). Programmed cell death of developing mammalian neurons after genetic deletion of caspases. *J. Neurosci.* **21**, 4752-4760.
Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D. and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927-939.
Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. *Development* **124**, 4065-4075.
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.
Payette, R. F., Tennyson, V. M., Pham, T. D., Mawe, G. M., Pomeranz, H. D., Rothman, T. P. and Gershon, M. D. (1987). Origin and morphology of nerve fibers in the aganglionic colon of the Lethal Spotted (ls/sl) mutant mouse. *J. Comp. Neurol.* **257**, 237-252.
Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.
Rodriguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F. and Dymecki, S. M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat. Genet.* **25**, 139-140.
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.
Snider, W. D. (1994). Functions of the neurotrophins during nervous system development: What the knockouts are teaching us. *Cell* **77**, 627-638.
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 the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development* **126**, 2785-2797.
Trachtenberg, J. T. and Thompson, W. J. (1996). Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379**, 174-177.
Vannucchi, M. G. and Faussone-Pellegrini, M. S. (2000). Synapse formation during neuron differentiation: an in situ study of the myenteric plexus during murine embryonic life. *J. Comp. Neurol.* **425**, 369-381.
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.
Young, H. M., Hearn, C. J., Ciampoli, D., Southwell, B. R., Brunet, J. F. and Newgreen, D. F. (1998). A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture. *Dev. Biol.* **202**, 67-84.
Young, H. M., Hearn, C. J., Farlie, P. G., Canty, A. J., Thomas, P. Q. and Newgreen, D. F. (2001). GDNF is a chemoattractant for enteric neural cells. *Dev. Biol.* **229**, 503-516.
Young, H. M., Bergner, A. J. and Muller, T. (2003). Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. *J. Comp. Neurol.* **456**, 1-11.
Young, H. M., Turner, K. N. and Bergner, A. J. (2005). The location and phenotype of proliferating neural-crest-derived cells in the developing mouse gut. *Cell Tissue Res.* **320**, 1-9.
Yu, L. Y., Jokitalo, E., Sun, Y. F., Mehlen, P., Lindholm, D., Saarma, M. and Arumae, U. (2003). GDNF-deprived sympathetic neurons die via a novel nonmitochondrial pathway. *J. Cell Biol.* **163**, 987-997.
Yuan, J. and Yankner, B. A. (2000). Apoptosis in the nervous system. *Nature* **407**, 802-809.