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Gdnf is mitogenic, neurotrophic, and chemoattractive to enteric neural crest cells in the embryonic colon

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Abstract

Glial-derived neurotrophic factor (Gdnf) is required for morphogenesis of the enteric nervous system (ENS) and it has been shown to regulate proliferation, differentiation, and survival of cultured enteric neural crest-derived cells (ENCCs). The goal of this study was to investigate its \textit{in vivo} role in the colon, the site most commonly affected by intestinal neuropathies such as Hirschsprung's disease. Gdnf activity was modulated \textit{in ovo} in the distal gut of avian embryos using targeted retrovirus-mediated gene overexpression and retroviral vector-based gene silencing. We find that Gdnf has a pleiotropic effect on colonic ENCCs, promoting proliferation, inducing neuronal differentiation, and acting as a chemoattractant. Downregulating Gdnf similarly induces premature neuronal differentiation, but also inhibits ENCC proliferation, leading to distal colorectal aganglionosis with severe proximal hypoganglionosis. These results indicate an important role for Gdnf signaling in colonic ENS formation and emphasize the critical balance between proliferation and differentiation in the developing ENS.

Keywords

enteric nervous system; glial-derived neurotrophic factor; Gdnf; Ret; Hirschsprung's disease; chick

Introduction

The enteric nervous system (ENS) comprises a network of neurons and glial cells responsible for multiple aspects of gut function, including regulation of intestinal peristalsis. The precursor cells that give rise to the ENS originate primarily from the vagal level of the neural crest (Le Douarin and Teillet, 1973). These cells migrate to the foregut mesenchyme and then continue in a rostrocaudal direction along the length of the intestine. Compared to the vagal contribution, a smaller number of colorectal ENS cells arises from the sacral neural crest (Burns and Le Douarin, 1998). Colonization of the gut by enteric neural crest-
derived cells (ENCCs) is influenced by interactions with the intestinal microenvironment that regulate ENCC survival, proliferation, migration, and differentiation. The importance of these is highlighted by the variety of disorders associated with abnormal ENS development. These include diseases characterized by lower than normal numbers (hypoganglionosis) or absence (Hirschsprung’s disease) of enteric neurons, as well as conditions with increased cell numbers (hyperganglionosis), as occurs in intestinal neuronal dysplasia. While formation of a proper ENS is essential for a normally functioning intestine, the mechanisms regulating ENCC development and the abnormalities that result in enteric neuropathies remain poorly understood.

One of the most important signaling pathways for the regulation of ENS development is mediated by glial-derived neurotrophic factor (Gdnf), which is expressed in the gut mesenchyme. Gdnf binds to a receptor complex formed by Ret and the co-receptor Gdnf family receptor α1 (GFRα1), expressed on the surface of migrating ENCCs. This Gdnf-Ret signaling between cell types has multiple roles during ENCC colonization, influencing survival (Heuckeroth et al., 1998; Taraviras et al., 1999), proliferation (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Worley et al., 2000; Barlow et al., 2003; Gianino et al., 2003; Flynn et al., 2007; Ngan et al., 2008), differentiation (Hearn et al., 1998; Taraviras et al., 1999; Ngan et al., 2008), and ENCC chemoattraction (Young et al., 2001; Natarajan et al., 2002). Null mutations of Ret, Gdnf, or GFRα1 in mice lead to total intestinal aganglionosis distal to the stomach (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto et al., 1998). In humans with Hirschsprung’s disease, coding sequence mutations in Ret are identified in 50% of patients (Amiel and Lyonnet, 2001), with nearly all other affected individuals having mutations in regulatory regions of the gene (Emison et al., 2005). Interestingly, while null Ret mutations cause total aganglionosis in mice, in 80% of humans with Hirschsprung’s disease the aganglionosis is limited to the distal colon and rectum (Amiel and Lyonnet, 2001).

Why the distal end of the bowel is particularly at risk in Hirschsprung’s disease remains unclear, and how Gdnf-Ret signaling is implicated in this distal aganglionosis is unknown. Since the overwhelming majority of ENCC colonization occurs in a rostrocaudal direction, perturbations of ENS development may manifest as a distal intestinal phenotype simply because of the distance ENCCs need to migrate to reach the distal gut. For a small population of vagal-derived neural crest cells to colonize the length of the intestine, extensive cellular proliferation is required, particularly considering the rapid intestinal lengthening occurring as ENS migration proceeds (Newgreen et al., 1996). Crest cell proliferation has been postulated to be one of the critical determinants of ENS development (Landman et al., 2007; Simpson et al., 2007), and the ability of specific axial levels of the neural crest to contribute a fully colonized ENS has been attributed to their proliferative advantage over other axial levels (Barlow et al., 2008; Zhang et al., 2010). The importance of ENCC numbers for full colonization is supported by the observation that a critical density of ENCCs is required at the wavefront to form the cellular strands that drive migration (Young et al., 2004; Druckenbrod and Epstein, 2007). Furthermore, a low density of ENCCs may delay the rate of their migration, leaving them unable to colonize a distal environment that has changed by the time the cells arrive (Druckenbrod and Epstein, 2009; Hotta et al., 2009). Similarly, premature neuronal differentiation, as caused by endothelin-3 defects, could arrest ENCC migration along its rostrocaudal path (Wu et al., 1999; Nagy and Goldstein, 2006), leaving the distal end aganglionic.

Since Ret activation promotes ENCC proliferation (Hearn et al., 1998; Barlow et al., 2003) and survival (Taraviras et al., 1999), a decrease in Ret activity could diminish the numbers of ENCCs and lead to distal aganglionosis. Uesaka et al showed that when Ret expression is
reduced to one-third of normal levels, colonic aganglionosis results (Uesaka et al., 2008). While this suggests that an inadequate number of ENCCs can lead to distal aganglionosis, a possible colon-specific role for Gdnf-Ret signaling may also exist. Conditional inactivation of Ret in late gestation, after ENS colonization is complete, results in enteric neuronal cell death specifically in the colon (Uesaka et al., 2008). Further, mice expressing only the Ret51 isoform are aganglionic in the terminal three-fourths of the colon (de Graaff et al., 2001). Since ENCC numbers are normal in the distal small intestine of these mice, the findings may suggest a specific defect in colonization of the colon.

We investigated the in vivo role of Gdnf on ENCC development in the colorectum using retroviral infection of avian embryos to modulate Gdnf expression in vivo and determine the effects on ENCC development in the distal intestine. Our results show that Gdnf has pleiotropic effects during colorectal ENS development, with mitogenic, neurotrophic, and chemoattractive effects on developing ENCCs. Furthermore, we show that the level of Gdnf available to migrating ENCCs is critically important, with increases and decreases both demonstrating phenotypic effects on ENS formation.

Results

Colonization of the post-cecal intestine requires Gdnf signaling

To test whether Gdnf signaling is required during colonization of the distal intestine, the intestinal tract from E5.5 chick embryo was removed and cultured for 3 days in a collagen matrix supplemented with anti-Gdnf function-blocking antibody. Explanted gut extended from the umbilical level to the junction of colon and cloaca. At this stage, the ENCC wavefront is located just above the ceca. In the absence of added factors, ENCC migration continues in organ culture and, after 3 days, ENCCs reach the distal colorectum, as shown by wholemount immunohistochemistry with Tuj1, an antibody recognizing neuron-specific class III β tubulin (Fig. 1A,B). When anti-Gdnf antibody is added to the matrix, migration is delayed (Fig. 1D) and severe hypoganglionosis develops, especially at the wavefront (Fig. 1E). The hypoganglionosis is characterized by few, isolated enteric neurons, without the rich network and cell clusters seen in controls (Fig. 1C,F).

In vivo modulation of Gdnf expression using retroviral vectors

Modulation of Gdnf expression was achieved by injecting the presumptive distal intestinal mesoderm of E2 chick embryos with RCAS virus. For Gdnf overexpression, the embryos were injected with a combination of RCAS(A)-Gdnf and RCAS(B)-Gdnf. Both RCAS subtypes were used together in order to maximize gene expression. Gdnf inhibition was achieved using a mixture of RCAS(A)-Gdnf-RNAi and RCAS(B)-Gdnf-RNAi. A combination of two different RNAi sequences was used as it achieved greater gene silencing than either alone. Control embryos were infected with RCAS-GFP. In situ hybridization with Gdnf riboprobe was used to confirm the effect on Gdnf expression. Fig. 2 shows representative in situ hybridization results on wholemount colorectum and on sections through the mid-colon. Normal Gdnf expression in the gut mesoderm is seen in Fig. 2A,D. Expression is stronger and more diffuse in RCAS-Gdnf-infected intestine (Fig. 2B,E), while significant downregulation of Gdnf is observed following RCAS-Gdnf-RNAi infection (Fig. 2C,F). Successful targeting and virus production are confirmed in infected intestines by staining with 3C2, an antibody to the retroviral coat. Infection is typically observed throughout the gut mesenchyme, but not in the epithelial layer (not shown).

Downregulation of Gdnf expression in vivo results in distal colorectal aganglionosis

The presumptive hindgut mesoderm of E2 chick embryos was injected with one of 3 viral preparations: (1) RCAS-GFP, (2) RCAS-Gdnf (consisting of both RCAS(A)-Gdnf and
RCAS(B)-Gdnf), and (3) RCAS-Gdnf-RNAi (consisting of RCAS(A)-Gdnf-RNAi and RCAS(B)-Gdnf-RNAi. At E7 in infected controls, ENCCs have migrated approximately one-third of the length of the colorectum (Fig. 3A), whereas the wavefront in RNAi-infected guts remains at the level of the ceca (Fig. 3B). By E8, enteric neurons have colonized about 50% of the normal colon (Fig. 3C), while in RNAi-infected guts the wavefront is delayed by about 1 day (Fig. 3D, compare to 3A). At E9, control colorectum is nearly fully colonized (Fig. 3E), as is the Gdnf overexpressing gut (Fig. 3G). However, inhibition of Gdnf continues to cause a significant delay in migration, with roughly the distal half of the colorectum remaining aganglionic at this stage (Fig. 3F), constituting approximately a 24-hour delay.

To quantitatively compare ENCC migration following Gdnf overexpression and inhibition, we calculated the percentage of colorectum colonized in E8 and E9 intestines following injection with RCAS-GFP (n=5 E8, n=9 E9), RCAS-Gdnf (n=3 E8, n=10 E9), and RCAS-Gdnf-RNAi (n=4 E8, n=6 E9). As shown in Fig. 3H, inhibition of Gdnf led to a statistically significant delay in ENCC migration, with only 25% of the colon colonized at E8 and 45% colonized at E9. Gdnf overexpression did not lead to any statistically significant change in the rate of migration. We were unable to determine if the RNAi-infected colon eventually completes migration due to limitations of the system.

We calculated percent colonization of the colon as opposed to the absolute distance colonized to compensate for any possible differences in gut length resulting from the injections. In fact, we found that the total length of the E9 colorectum, measured from the base of the ceca to the junction of the colon and cloaca, differed between the 3 groups: RCAS-GFP (5.9±0.8 mm), RCAS-Gdnf (5.6±0.7 mm), and RCAS-Gdnf-RNAi (5.0±0.5 mm). RNAi-injected intestine was on average 15% and 11% shorter than RCAS-GFP and RCAS-Gdnf intestine, respectively, and these differences were statistically significant (p<0.05).

Gdnf inhibition was noted to have additional effects on ENCC number and morphology. Inhibition of Gdnf in vivo was associated with hypoganglionosis proximal to the migratory wavefront (Fig. 3, see insets), similar to the results observed in vitro with anti-Gdnf antibody (Fig. 1). The total number of ENCCs was counted for each of the three virus injections by staining cross-sections through the colon with HNK-1 antibody. RCAS-GFP control colon had an average of 221 ± 51 ENCCs per section (n=13), while RCAS-Gdnf had 205 ± 61 cells (n=10) and RCAS-Gdnf-RNAi had 166 ± 22 cells (n=5). RNAi-infected colon had significantly fewer ENCCs than controls (p<0.01). Whereas RCAS-GFP control guts display a network of enteric neurons with long, interconnected processes, RNAi-infected guts contain sparse, individual cells (compare insets in Fig. 3A and 3B).

Gdnf signaling affects development of the nerve of Remak

In order to determine the effect of Gdnf signaling on sacral-derived neural crest cells, we examined development of the nerve of Remak following retroviral infection. We measured the cross-sectional area of the nerve of Remak in colons injected with either RCAS-GFP (n=17), RCAS-Gdnf (n=18), or RCAS-Gdnf-RNAi (n=16). This area was used as a surrogate for the number of cells in the nerve of Remak, since the cells are tightly packed and very difficult to count individually. Since the nerve of Remak varies in size along the length of the gut, we avoided the cecal and cloacal regions and performed the measurements at the level of the mid-colon. ImageJ software was used to calculate the area (in arbitrary units). Results were as follows: RCAS-GFP=65242 +/- 22735, RCAS-Gdnf=54150 +/- 18906, RCAS-Gdnf-RNAi=40381 +/- 16247. The cross-sectional area of the nerve of Remak in RNAi-infected guts was, on average, 38% smaller than controls. This difference was statistically significant, with p<0.05. There was no statistical difference between
controls and RCAS-Gdnf infected guts. In addition, we observed that RNAi-infected intestine had markedly fewer neurofibres extending into the gut from the nerve of Remak when compared to controls and RCAS-Gdnf intestine (Fig. 3E–G).

**Gdnf signaling regulates differentiation and proliferation in the colorectal ENS**

To determine whether the distal aganglionosis caused by Gdnf inhibition is associated with effects on ENCC proliferation or differentiation, E2 embryos were injected as described above, intestines collected at E9 and E12, and transverse sections of the colon prepared. To assess for neuronal differentiation, sections were stained with HNK-1 or p75NTR, which label crest-derived cells in the intestine, and Hu to mark neurons. The percentage of neuronal differentiation was calculated by dividing the number of Hu+ ENCCs by the total number of ENCCs. As shown in Fig. 4 (top panel), about 50% of ENCCs in control intestine have differentiated by E9, with this proportion increasing to 66% by E12, a statistically significant change. In contrast, RCAS-mediated overexpression of Gdnf led to a significant increase in the percentage of neuronal differentiation at E9 (60%), with no change during the ensuing 3 days of development (59% at E12). Similarly, inhibition of Gdnf expression using RCAS-Gdnf-RNAi led to 70% neuronal differentiation at E9 and 72% at E12. Thus, both overexpression and inhibition of Gdnf significantly increased the percentage of neurons at E9, but not at E12, consistent with inducing the premature neuronal differentiation of ENCCs.

ENCC proliferation was examined by counting the proportion of ENCCs incorporating BrdU following infection with the different viruses. The results are summarized in Fig. 4 (bottom panel). ENCCs in control E9 colon exhibit a 28% proliferation rate. Following treatment with RCAS-Gdnf, ENCC proliferation increased to 36%, comprising a 29% increase in the rate of cellular proliferation above baseline. This change was statistically significant. Consistent with this, Gdnf inhibition using RCAS-Gdnf-RNAi infection decreased the rate to 23%, an 18% drop in proliferation, although this change did not achieve statistical significance. At E12, with colorectal ENCC colonization complete, the rate of cell proliferation decreased dramatically. Proliferation rates at this stage for RCAS-GFP, -Gdnf, and –RNAi were 8.8%, 9.1%, and 8.3%, respectively. These values show similar trends as at E9, but were not statistically significant. No difference was identified in the rate of ENCC apoptosis, which was determined using antibodies to cleaved caspase-3 and HNK-1. Very few apoptotic crest-derived cells were identified in the injected colons, regardless of the retrovirus expressed (not shown).

**Gdnf is chemoattractive to ENCCs in the colon**

In addition to regulating cellular proliferation and differentiation, modulation of Gdnf expression could impact ENS colonization directly by affecting ENCC migration. To test this, beads coated with recombinant Gdnf protein or anti-Gdnf antibody were embedded into the hindgut mesenchyme prior to ENCC arrival. After 3 days in culture, during which time ENCCs normally colonize the colon, ENS development was visualized immunohistochemically on longitudinal sections. As shown by wholemount staining with Tuj1 antibody, PBS beads did not affect ENCC migration (Fig. 5A). ENCCs were able to migrate past the bead and into the distal colon. In contrast, both Gdnf- and anti-Gdnf-coated beads arrested migration (Fig. 5B,C). ENCCs stopped in the immediate vicinity of these beads, with only a few migrating a short distance beyond them. Longitudinal sections of guts stained with Hu antibody revealed more detail. The PBS bead had no impact on migration and ENCCs were able to migrate past the bead (Fig. 5D). We again observed that both Gdnf and anti-Gdnf beads arrested migration. Overexpression of Gdnf caused ENCCs to accumulate around the bead and also to differentiate, as shown by the strong Hu expression around the bead (Fig. 5E), preventing migration past the bead. In contrast, while...
anti-Gdnf beads also stopped migration, no ENCC accumulation occurred on the bead (Fig. 5F).

**Discussion**

Formation of the ENS requires the migration, proliferation, and differentiation of enteric neuronal precursors originating primarily from the vagal neural crest. Beginning as a small population of progenitor cells, the ENS relies on continued cellular proliferation to generate a sufficient pool of cells to colonize the entire length of the gut. While proliferation and migration are critical, some ENCCs must exit the cell cycle and differentiate into neurons to form the complex functional network that regulates intestinal motility. This balance between migration, proliferation, and differentiation is essential and abnormal migration, inadequate proliferation, or premature differentiation can all result in intestinal aganglionosis, as occurs in human Hirschsprung’s disease.

To determine the effects of Gdnf on ENS development, we modulated its activity in avian embryos using retrovirus-mediated gene overexpression and retroviral vector-based gene silencing. While the essential role of Gdnf signaling in ENS development has been established in humans (Amiel and Lyonnet, 2001), rodents (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), and zebrafish (Shepherd et al., 2001), most of these studies used cultured neural crest-derived cells devoid of the mesenchymal environment known to be important during ENS development (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Worley et al., 2000; Young et al., 2001; Natarajan et al., 2002; Ngan et al., 2008). Our approach allowed us to modulate Gdnf activity in vivo, specifically in the presumptive mesoderm of the distal gut, which has major clinical importance in neurointestinal diseases, such as Hirschsprung’s disease and intestinal neuronal dysplasia. We found that loss of Gdnf signaling causes a significant delay in ENCC colonization of the colorectum, leading to distal colorectal aganglionosis and severe hypoganglionosis proximally. This phenotype is consistent with that seen in rodent models, where the extent of ENS deficiency varies with Gdnf dosage. Null alleles of Gdnf lead to total intestinal aganglionosis (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), while heterozygous mice exhibit significant hypoganglionosis (Shen et al., 2002; Gianino et al., 2003; Flynn et al., 2007), especially affecting the distal gut (Shen et al., 2002). A similar dose-response is observed with Ret, where progressive reductions in Ret expression correspond with longer segments of intestinal aganglionosis (Uesaka et al., 2008). We find that the cause of the hypoganglionosis/aganglionosis is multifactorial, consistent with the known pleiotropic effects of Gdnf on ENCC proliferation, neuronal differentiation, and migration. Gdnf inhibition also resulted in a shorter length of colorectum, suggesting other Gdnf-mediated effects in the gut that need to be explored further. Additionally, inhibition of Gdnf resulted in a marked reduction in the size of the nerve of Remak and in the number of fibers extending into the colon. These findings suggest that, like the vagal crest-derived cells, sacral-derived ENCCs are also dependent on Gdnf signaling, which may explain why the terminal gut remains aganglionic in Hirschsprung’s disease and Ret-deficient mouse models.

**Gdnf promotes ENCC proliferation in the colorectum at E9 but not E12**

*In vivo* overexpression of Gdnf enhanced ENCC proliferation by 29%, while inhibition of the gene reduced proliferation by 18%. This ability of Gdnf to promote cellular proliferation has been demonstrated previously in cultured ENCCs (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Worley et al., 2000; Barlow et al., 2003; Ngan et al., 2008). The anti-mitogenic effect of downregulating Gdnf has been shown in Gdnf heterozygous mice, which demonstrate a significantly reduced rate of ENCC proliferation compared to wild-type mice (Gianino et al., 2003). Interestingly, the mitogenic
the effect of Gdnf was not observed in E12 embryos. A similar finding was reported by Chalazonitis et al (Chalazonitis et al., 1998), who showed that Gdnf stimulated the proliferation of neural crest-derived cells isolated from E12 intestine, but not from E14 or E16 gut. In both avians and rodents, the major proliferative effect of Gdnf appears to be present while ENCCs are still migrating along the gut. Thus the effect of Gdnf on ENCC development changes with time, highlighting the dynamic nature of its role during ENS development. The recent demonstration that inactivation of GFRα1 during late gestation in mice (at E15.5, well after colonization of the colon is complete) leads to enteric neuronal cell death specifically in the colon may be due to the fact that expression of both Gdnf and GFRα1 is markedly reduced in the small intestine and upregulated in the colon at this stage (Uesaka et al., 2007), illustrating the dynamic spatial and temporal role of Gdnf during ENS development.

Modulating Gdnf expression in vivo leads to premature neuronal differentiation

Our results demonstrate that both overexpression and inhibition of Gdnf induce premature neuronal differentiation. RCAS-Gdnf led to a 20% increase in the proportion of colorectal ENCCs that differentiated into neurons, while there was a 40% increase in RCAS-Gdnf-RNAi embryos. With both viruses, the proportion of differentiated ENCCs in the E9 colon (60% in RCAS-Gdnf and 70% in RCAS-Gdnf-RNAi) did not change significantly by E12 (59% in RCAS-Gdnf and 72% in RCAS-Gdnf-RNAi). In contrast, the proportion of neuronal differentiation in control guts increased from 50% to 66% between E9 and E12, respectively. These results demonstrate that modulating Gdnf expression either up or down during colonization of the colonic ENS induces premature neuronal differentiation.

Previous studies have shown induction of neuronal differentiation by addition of Gdnf in vitro to cultured ENCCs (Chalazonitis et al., 1998; Hearn et al., 1998; Taraviras et al., 1999; Ngan et al., 2008), consistent with our in vivo findings that Gdnf overexpression induces differentiation. However, the finding that Gdnf inhibition also induces ENCC differentiation is interesting. Flynn et al (Flynn et al., 2007) analyzed neuronal differentiation in mice heterozygous for Gdnf and did not find a statistical difference compared to wild-type mice, although their data do show a consistent trend toward increased differentiation in the heterozygotes. One possible explanation for our findings is that since the loss of Gdnf expression delays migration, ENCCs are left with a prolonged exposure to as yet unidentified local factors in the colon that may drive neuronal differentiation. Further studies into the factors regulating ENCC differentiation in vivo are necessary. Nevertheless, our analysis demonstrates that Gdnf levels must be tightly regulated in the developing gut during the arrival of ENCCs, as any change in the baseline level of expression has a significant impact on the timing of neuronal differentiation, which can lead to downstream effects on ENCC migration. As expected, the increase in neuronal differentiation was associated with hypoganglionosis and distal aganglionosis in RCAS-Gdnf-RNAi guts. However, RCAS-Gdnf intestines were normally colonized by ENCCs despite their premature differentiation. We hypothesize that this is due to the marked increase in ENCC proliferation observed in these embryos, which may compensate for the premature differentiation and allow the generation of an adequate pool of precursor cells to complete ENS colonization of the distal gut.

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Altering local Gdnf levels in the colon arrests ENCC migration

Gdnf has been proposed to be chemoattractive to ENCCs based on in vitro studies using organ cultures (Young et al., 2001; Natarajan et al., 2002). The expression of Gdnf in embryonic mouse intestine is consistent with a potential role as a chemoattractant in the foregut and midgut, since Gdnf is initially expressed strongly in the stomach and later in the cecum at stages when ENCCs are still rostral to that level (Natarajan et al., 2002). However,
a similar pattern of Gdnf expression has not been observed in the hindgut (Natarajan et al., 2002). In avians, we find Gdnf mRNA strongly expressed in the ceca and cloaca at E5, with rapid expansion of expression throughout the length of the hindgut mesoderm by E7, when ENCCs are still in the proximal colon (Nagy and Goldstein, 2006). This pattern of expression makes it unclear whether Gdnf has a similar chemoattractive role in the distal gut. We found that the presence of Gdnf-coated beads in the colorectal mesenchyme was highly chemoattractive to ENCCs, which were drawn to the bead and failed to migrate far beyond it. These results confirm that colorectal ENCCs are chemoattracted to Gdnf. The additional effect of Gdnf to promote neuronal differentiation may have also contributed to the arrest of migration. Interestingly, beads coated with an antibody that blocks Gdnf function also resulted in aganglionosis distal to the bead, likely via a different, and multifactorial, mechanism. Local inhibition of Gdnf expression led to (1) an absence of the migratory cue, (2) promotion of neuronal differentiation, and (3) downregulation of ENCC proliferation. The finding that either too much or too little Gdnf expression interferes with ENCC migration again demonstrates the critical levels of the protein necessary to promote normal development of the colorectal ENS.

**Experimental Procedures**

**Animals**

Fertilized White Leghorn chicken (Gallus gallus) and quail (*Coturnix coturnix japonica*) eggs were obtained from commercial breeders and maintained at 37°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) tables (Hamburger and Hamilton, 1992) or the number of embryonic days (E).

**RCAS viruses**

Total RNA was obtained from E5-E10 chick guts using Trizol and cDNA prepared. The following primers were designed based on the chick Gdnf sequence (NCBI Accession #XM_425018): forward AGTAATGGGCAGAGCAGCTT, reverse TCAGACACATCCACACCTTT, with the addition of an upstream Kozak sequence and NotI/ClaI restriction sites. The amplified product was cloned into the replication-competent retroviral vectors, RCAS(A) and RCAS(B), and both vectors were injected simultaneously to maximize Gdnf expression.

Retroviral vector-based Gdnf gene silencing was achieved using the method described by Das et al (Das et al., 2006). Two RNAi target sequences were selected (GTGATGCGTGGACAGCCACGTA and ACTCTAATATGCCAGGATTA) and each was cloned into a microRNA operon in the pRFPRNAiC shuttle vector (ARK-Genomics). The operon was excised with NotI and ClaI and directionally cloned into RCAS. One RNAi target was cloned into RCAS(A) and the other into RCAS(B) and both injected in ovo simultaneously to achieve maximal gene silencing.

DF1 cells transfected with the viral construct were grown to confluence and the supernatant harvested. Viral harvesting, concentration, and titering were performed as described (Cepko, 1991). Control infections were performed using an RCAS-green fluorescent protein (GFP) vector.

**In ovo viral infection**

Embryos were incubated until E2 (~HH12), windowed, and viewed under a Nikon SMZ800 dissection microscope. Approximately 1 µl of virus (~1×10⁵ cfu) was injected into each side of the embryo, directed at the presumptive gut mesoderm, based on established chick fate.
maps (Matsushita, 1995). The eggs were then sealed, returned to the incubator, and harvested at various time-points, 5–10 days after infection.

**Organ culture**

To study migration of ENCCs along the intestine, E5 (HH27) quail or E5.5 (HH27) chick gut was dissected from the umbilicus to the cloaca and embedded in a three-dimensional serum-free collagen gel matrix, prepared as described (Nagy and Goldstein, 2006). The collagen gel was supplemented with Gdnf (10 ng/ml; R&D Systems) or anti-Gdnf function-blocking antibody (10 µg/ml; R&D Systems). After 3 days, the explants were removed and processed for immunohistochemistry.

For bead experiments, heparin-acrylic beads (70–150 µm diameter; Sigma) were rinsed in PBS, soaked in protein (100 ng/ml Gdnf or 100 µg/ml anti-Gdnf) at 37°C for 1 hour and 4°C overnight. The intestine was dissected from E5.5 (HH27) chick, from umbilicus to cloaca, and the hindgut incised with tungsten needles to create a space in the mesenchyme for the bead. The bead was inserted with a blunt-end glass needle and the guts embedded in a three-dimensional collagen gel matrix for 3 days (Nagy and Goldstein, 2006).

**Immunohistochemistry**

Guts were fixed in 4% formaldehyde, gelatin-embedded, frozen, and sectioned at 12 µm. Primary antibodies included Tuj1 (1:1000; Covance), p75NTR (kind gift of Louis Reichardt) (Weskamp and Reichardt, 1991), HuC/D (1:100; Molecular Probes), HNK-1 (1:100; Fisher), cleaved caspase-3 (1:100; Cell Signaling), and 3C2 (1:5; Developmental Studies Hybridoma Bank). Detection was by visible light or fluorescence. For visible light, sections were incubated with antibody for 45 min., followed by biotinylated goat anti-mouse IgM or IgG (Vector Labs, Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Labs). Endogenous peroxidase activity was quenched by incubation for 10 min. with 3% hydrogen peroxide (Sigma) and primary antibody binding sites visualized with 4-chloro-1-naphthol (Sigma) or diaminobenzidine (DAB). For fluorescence, secondary antibodies included Alexa Fluor 594 and 488 goat anti-mouse IgG, Alexa Fluor 594 and 488 goat anti-mouse IgM, and Alexa Fluor 594 and 488 goat anti-rabbit IgG (Molecular Probes). Cell nuclei were stained by DAPI (Vector Labs).

For wholemount immunofluorescence, intestines were fixed with 4% formaldehyde for 3 hours. After several washes in PBS, specimens were incubated overnight with primary antibodies (Tuj1 or p75NTR) followed by secondary antibodies (Alexa Fluor 594 anti-mouse IgG or Alexa Fluor 488 anti-rabbit IgG) for 5 hours.

Cell proliferation was determined by incubating the gut for 4 hours in 5 mg/ml BrdU (Roche) solution. DNA was denatured by incubating slides with 2N HCl in H2O at 37°C for 30 minutes. After neutralization with 0.1 M boric acid (pH 8.5) for 30 minutes, sections were stained with fluorescein-conjugated anti-BrdU antibody (Roche).

**In situ hybridization**

Tissues were fixed in 4% formaldehyde, dehydrated in methanol, and stored at −20°C until ready for processing. Riboprobe synthesis and wholemount RNA *in situ* hybridization was performed as previously described (Jowett, 1999). Antisense Gdnf RNA probe was generated by amplifying a fragment of Gdnf sequence from total RNA prepared from E9 chick gut (Nagy and Goldstein, 2006).

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Figure 1. Gdnf is required for colonization of the colorectal ENS

E5.5 intestine was cultured in serum-free collagen matrix in the presence (D–F) or absence (A–C) of anti-Gdnf antibody. After 3 days in culture, wholemount immunohistochemistry was performed with Tuj1 (A,B,D,E). The proximal end of the colon is marked with an asterisk and the migratory wavefront with an arrow (A,D). The wavefront region is magnified in B,E. Severe hypoganglionosis is seen throughout the treated colon (D,E), with very sparse and isolated Hu-immunoreactive ganglion cells in cross sections near the wavefront (F). The nerve of Remak (NoR) is smaller in the absence of Gdnf expression (F).
Figure 2. Gdnf expression can be modulated in ovo with RCAS
RCAS virus encoding GFP, Gdnf, or Gdnf-RNAi were injected into the presumptive distal intestinal mesoderm of E2 embryos, and the intestines harvested at E9. Gdnf expression was detected by in situ hybridization on wholemount colorectum (A–C) and on sections through the mid-colon (D–F).
Figure 3. Inhibition of Gdnf produces distal colorectal aganglionosis

Intestines infected with RCAS-GFP (A,C,E,), RCAS-Gdnf-RNAi (B,D,F), or RCAS-Gdnf (G) were collected at E7 (A,B), E8 (C,D), and E9 (E–G). ENS was visualized by wholemount Tuj1 (A–D) or p75NTR (E–G) immunohistochemistry. Distal is to the right in all panels. The proximal end of the colon is marked with an asterisk and the migratory wavefront with an arrow. Insets in A,B are magnified views of the wavefront. Insets in C,D correspond to the proximal colon (1) and the wavefront (2). Magnified views in E-F are from the mid-colon. ENCC migration is markedly delayed in RNAi-infected guts at all stages examined, with the wavefront only reaching the mid-colorectum by E9 (F), when migration is nearly complete in control (E) and RCAS-Gdnf (G) guts. The percent
colonization of the colorectum at E8 and E9 is summarized in H, showing a significant delay in ENCC migration in RNAi-infected intestine (*p<0.05 compared to RCAS-GFP and to RCAS-Gdnf).
Figure 4. Gdnf induces premature neuronal differentiation and promotes ENCC proliferation
(Top panel): The percentage of neuronal differentiation was calculated in E9- and E12-
injected embryos. Intestine was harvested at those stages and immunohistochemistry
performed on cross-sections through the mid-colorectum using antibodies to ENCCs
(p75<sub>NTR</sub> or HNK-1) and neurons (Hu). Panel on left shows a myenteric ganglion from an
E12 RCAS-GFP hindgut. The number of neurons and ENCCs was counted to determine the
percentage of Hu-immunoreactive ENCCs (arrows). (Bottom panel): ENCC proliferation
was determined using BrdU incorporation into HNK-1+ ENCCs. Panel on left shows a
myenteric ganglion from an RCAS-GFP infected hindgut with arrows marking BrdU+ cells.
The percentage of proliferating ENCCs was calculated by dividing the total number of
HNK-1+ ENCCs by the number of BrdU+/HNK-1+ double-immunoreactive cells. The graphs show the average and standard deviation for each group. Lines with asterisk denote statistical significance (p<0.02).
Figure 5. Gdnf is chemoattractive to migrating ENCCs in the colorectum

E5.5 intestine was isolated and the colon implanted with a PBS bead (A,D), Gdnf bead (B,E), or anti-Gdnf bead (C,F), then cultured in collagen gel for 3 days. Wholemount Tuj1 staining shows ENCC migration continuing past the control bead (A), while Gdnf- and anti-Gdnf-coated beads arrested migration (B,C). The proximal end of the colon is marked with an asterisk and the migratory wavefront with an arrow in A–C. Insets show magnified views of the area near the bead. Longitudinal sections (proximal end to the left) were labeled with Hu antibody. ENCCs migrate around the PBS bead (D). In contrast, the Gdnf-coated bead attracts enteric neurons, causing them to cluster around the bead (E). Beads coated with anti-Gdnf antibody stop migration, but do not lead to neuronal clustering (F).