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Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system

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Abstract

Neural crest cells (NCC) migrate, proliferate, and differentiate within the wall of the gastrointestinal tract to give rise to the neurons and glial cells of the enteric nervous system (ENS). The intestinal microenvironment is critical in this process and endothelin-3 (ET3) is known to have an essential role. Mutations of this gene cause distal intestinal aganglionosis in rodents, but its mechanism of action is poorly understood. We find that inhibition of ET3 signaling in cultured avian intestine also leads to hindgut aganglionosis. The aim of this study was to determine the role of ET3 during formation of the avian hindgut ENS. To answer this question, we created chick–quail intestinal chimeras by transplanting preganglionic quail hindguts into the coelomic cavity of chick embryos. The quail grafts develop two ganglionated plexuses of differentiated neurons and glial cells originating entirely from the host neural crest. The presence of excess ET3 in the grafts results in a significant increase in ganglion cell number, while inhibition of endothelin receptor-B (EDNRB) leads to severe hypoganglionosis. The ET3-induced hyperganglionosis is associated with an increase in enteric crest cell proliferation. Using hindgut explants cultured in collagen gel, we find that ET3 also inhibits neuronal differentiation in the ENS. Finally, ET3, which is strongly expressed in the ceca, inhibits the chemoattraction of NCC to glial-derived neurotrophic factor (GDNF). Our results demonstrate multiple roles for ET3 signaling during ENS development in the avian hindgut, where it influences NCC proliferation, differentiation, and migration.

Keywords: Enteric nervous system; Endothelin-3 (ET3); Endothelin receptor B (EDNRB); GDNF; Chick–quail chimeras; Coelomic transplantation; Hirschsprung's disease; Cecum

Introduction

Neural crest cells (NCC) migrate, proliferate, and differentiate within the gut wall to give rise to the neurons and glial cells of the enteric nervous system (ENS). Precursor cells of the ENS arise from two distinct regions of the neural tube. The majority of the ENS is contributed by the vagal neural crest (Le Douarin and Teillet, 1973). The hindgut ENS is derived mainly from vagal neural crest cells that enter the mesenchyme of the foregut and migrate in a rostrocaudal wave toward the cloaca. The sacral NCC migrates in a caudorostral direction within the intestinal mesenchyme, contributing a minority of the neural crest-derived cells of the distal hindgut (Jones, 1942; Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Serbedzija et al., 1991; Burns and Le Douarin, 1998). Avians have an additional sacral crest-derived structure, the ganglionated nerve of Remak, which extends within the mesentery from the cloaca to the umbilical level (Teillet, 1978; Catala et al., 1995; Doyle et al., 2004). Although the molecular mechanisms directing enteric neural crest formation are largely unknown, several factors that influence these processes have been identified.

Enteric NCC migration, proliferation, survival, and differentiation are primarily regulated by factors present in the gut mesenchyme. These proteins, together with their respective receptors expressed on the enteric NCC, are necessary for normal ENS formation. These essential factors include: glial cell line-derived neurotrophic factor (GDNF) and its receptors Ret and Gfrα1 (Schiltz et al., 1999; Homma et al., 2000; Natarajan et al., 2002); neurotrophin-3 and the TrkC receptor (Chalazonitis et

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al., 1994); bone morphogenetic proteins 2 and 4 (BMP2, BMP4) and the BMP receptors (Sukegawa et al., 2000; Kruger et al., 2003; Goldstein et al., 2005); netrin and deleted in colorectal cancer (Jiang et al., 2003); sonic hedgehog (Sukegawa et al., 2000; Fu et al., 2004); Indian hedgehog (Ramalho-Santos et al., 2000); endothelin-3 (ET3) and endothelin receptor-B (EDNRB) (Baynash et al., 1994; Hosoda et al., 1994; Kruger et al., 2003; Barlow et al., 2003). Mutations affecting many of these genes lead to a variety of developmental intestinal disorders in mammals, the most common of which is Hirschsprung's disease (HSCR), a severe intestinal motility disorder affecting 1 in 5000 human births and characterized by the absence of ENS along a variable length of colon. The majority of HSCR can be accounted for by mutations in Ret (Chakravarti, 2001), which is required for proliferation, migration, and differentiation of enteric NCC. Null mutations in GDNF and Ret in mice prevent enteric neural crest migration and result in aganglionosis of the entire midgut and hindgut (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

Mutations of the ET3-EDNRB signaling pathway have also been implicated in HSCR (Chakravarti, 2001). Absence of these genes in mice leads to aganglionosis limited to the distal colon (Baynash et al., 1994; Hosoda et al., 1994). While this suggests an essential role for ET3 in hindgut colonization, its mechanism of action remains controversial. The leading hypothesis is that ET3 signaling functions to maintain the pool of enteric NCC precursors by inhibiting neuronal differentiation and promoting proliferation of those cells. Loss of ET3 activity would thereby decrease cell proliferation and lead to premature neuronal differentiation, leaving an inadequate number of progenitor cells to populate the distal gut (Gershon, 1999). While several groups have confirmed the capacity of ET3 to inhibit neurogenesis in cultured NCC (Stone et al., 1997; Hearn et al., 1998; Wu et al., 1999), Kruger et al. (2003) recently showed in enteric neural crest stem cells that the absence of EDNRB does not increase neurogenesis. Similarly, the ability of ET3 to promote NCC proliferation has been supported by some (Stone et al., 1997; Lahav et al., 1996; Barlow et al., 2003) and refuted by others (Wu et al., 1999; Kruger et al., 2003; Woodward et al., 2003).

Much of what is currently known about the molecular regulation of ENS development has been learned from transgenic rodents and from cultured neural crest cells, each of which has some limitations. Mice, for example, are not easily amenable to experimental manipulation during embryogenesis. In vitro cell culture techniques lack the mesenchymal environment of the gut and are therefore limited in their applicability to the living animal. We have developed an assay for studying ENS development using the avian embryo that complements these other systems. In this assay, preganglionic quail hindgut is transplanted into the coelomic cavity of a chick embryo. We find that neural crest cells from the chick host populate the graft and give rise to two ganglionated plexuses containing differentiated neurons and glial cells. This method allows us to manipulate the molecular environment of the developing gut and to study the effect on ENS formation. We demonstrate the utility of this assay for studying both normal ENS formation and the role of ET3 during this process. Using coelomic transplants and intestinal organ culture, we find that ET3-EDNRB signaling has an essential role in promoting enteric crest cell proliferation, inhibiting neurogenesis, and modulating enteric NCC migration during ENS development in the avian hindgut.

Materials and methods

Animals

Fertilized White Leghorn chicken 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 the Hamburger and Hamilton (HH) tables (Hamburger and Hamilton, 1951) or the number of embryonic days (E).

Organ culture assays

To study the chemoattractive role of exogenous GDNF and ET3, the intestinal tract was removed from E8 chick embryos and placed in a three-dimensional collagen gel matrix. Collagen gels were prepared by adding 1 N NaOH and 1 mg/ml type I rat tail collagen (BD Biosciences) to DMEM medium (Gibco) supplemented with 1% penicillin–streptomycin. GDNF (10 ng/ml; R&D Systems), ET3 (250 ng/ml; Sigma), or BQ788 (5 μM; Sigma) was added to the collagen gel as previously described (Goldstein et al., 2005).

To study the role of ET3 on NCC migration and differentiation along the gut, E5 chicken embryonic midgut–hindgut was dissected from the umbilicus to the cloaca and embedded in collagen gel. The nerve of Remak was removed from the explants using tungsten needles. The collagen gel was supplemented with ET3 (250 ng/ml) or BQ788 (5 μM). After 3 days, the gut was removed and processed for immunocytochemistry.

Chick–quail intestinal chimeras

Hindgut was removed from E5 quail embryos. The ceca, cloaca, and nerve of Remak were removed and the hindgut was embedded in collagen gel. The collagen gel was supplemented with ET3 (250 ng/ml), GDNF (100 ng/ml), or BQ788 (20 μM). Controls consisted of media alone, without added proteins. After 24 h in culture, the hindgut was removed from the gel and processed for transplantation. For bead implantation, Affigel blue beads (BioRad) or heparin-acrylic beads (70–150 μm diameter; Sigma) were rinsed in phosphate-buffered saline (PBS) and then soaked in 30 μl of 10 μg/ml GDNF protein, 10 μg/ml ET3 protein, or 200 μM BQ788 at 37°C for 1 h. The mesenchyme of isolated E5 quail hindgut was punctured with electrolytically sharpened tungsten needles to create a space for the bead. The bead was inserted into the mesenchyme using a blunt-end glass needle. Hindguts were then transplanted into chick coelomic cavities. Both untreated guts and PBS-soaked beads served as controls.

Preganglionic hindguts from E5 quail embryos treated with ET3, GDNF, or BQ788 (either in organ culture or by bead implantation) were transplanted into the coelomic cavity of E3 chicks. Grafting experiments were performed as described for lymphoid organs (Nagy et al., 2004, 2005). Briefly, the HH stage 20 chick embryo was immobilized and the surface ectoderm removed with a tungsten needle. Quail hindguts were labeled with sterile charcoal and transferred to the right coelomic cavity of chicken embryos. Grafts were positioned longitudinally within the coelom, along the long axis of the embryo. Host chicken embryos were allowed to develop for 7 days following transplantation.

Chorioallantoic membrane transplants

The hindgut between the ceca and cloaca was dissected from E5 and E6 embryos and transplanted on the chorioallantoic membrane (CAM) of E8 chick embryos. Before transplantation, a small portion of the CAM was gently traumatized by laying a strip of sterile lens paper onto the surface of the epithelium and then removing it immediately. The dissected hindgut was placed over the junction of blood vessels on the CAM and incubated for 9 days. The graft, together with the surrounding CAM, was excised, fixed in 4% buffered formaldehyde, and embedded in gelatin.

Immunocytochemistry

For cryostat sections, guts were fixed in 4% formaldehyde in PBS for 1 h, rinsed with PBS, and infiltrated with 15% sucrose/PBS overnight at 4°C. The medium was changed for 7.5% gelatin containing 15% sucrose at 37°C for 1–2 h, and the tissues rapidly frozen at −60°C in isopentane (Sigma). Frozen sections were cut at 10 μm and collected on poly-L-lysine coated slides (Sigma). Cryostat sections were stained by immunocytochemistry according to standard techniques as described below. The following primary antibodies were used: HNK-1 (NeoMarkers, CA), which recognizes an epitope on the surface of migratory and postmigratory neural crest-derived cells; anti-HuC/D (Molecular Probes, OR), which recognizes a neuron-specific RNA-binding protein; Bfabp (brain fatty acid binding protein); 8F3, a chicken cell marker (Developmental Studies Hybridoma Bank, DSHB); QCPN, a quail cell nuclear marker (DSHB); 4H6, a neurofilament marker (DSHB); and QH1, a quail-specific endothelial cell marker (DSHB). After rehydration in PBS, sections were incubated with primary antibodies for 45 min, followed by biotinylated goat anti-mouse IgG, goat anti-mouse IgM, or goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) at room temperature. Endogenous peroxidase activity was quenched by incubation for 10 min with 3% hydrogen-peroxide (Sigma) in PBS. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphtol (Sigma). Sections were examined under a Nikon Microphot FXA microscope and digital images captured with a Spot camera and software version 3.3.1 (Diagnostic Instruments). Images were compiled using Adobe Photoshop.

NADPH-diaphorase

The histochemical method for the demonstration of nicotineamide adenine dinucleotide phosphate (NADPH) diaphorase activity was performed with modification as described previously by Burns and Le Douarin (1998). Briefly, frozen sections were incubated for 30–50 min at 37°C in PBS containing 1 mg/ml reduced β-NADPH (Sigma), 0.5 mg/ml nitroblue tetrazolium (Roche), and 3 μl/ml Triton X-100 (Fisher). Reaction product appeared as a blue granular staining and the reaction was stopped by rinsing sections with PBS.

BrdU labeling, apoptosis, and double immunofluorescence

For analysis of cell proliferation, ET3- or BQ788-treated guts were isolated from the collagen gel and incubated for 3 h in BrdU solution (DMEM medium containing 5 mg/ml BrdU; Roche). BrdU staining was performed on 10 μm cryostat sections. DNA was denaturated by incubating slides with 2 N HCl in H_2O at 37°C for 30 min. After neutralization with 0.1 M boric acid (pH 8.5) for 10 min, sections were stained with primary antibodies (QCPN, HNK-1, Hu, and fluorescein-conjugated anti-BrdU antibody; Roche). Fluorescent secondary antibodies included Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 goat anti-mouse IgM, and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). To detect apoptotic cells, sections were examined by double immunofluorescence using anti-Hu and anti-activated caspase-3 antibodies (Cell Signaling).

In situ hybridization

Whole embryos or dissected gastrointestinal tracts were fixed in 4% formaldehyde, dehydrated in methanol, and stored at −20°C until ready for processing. Riboprobe synthesis and whole-mount RNA in situ hybridization were performed as previously described (Jowett, 1999). Digoxigenin-labeled ET3 RNA probe was prepared from the full-length chicken ET3 cDNA. EDNRB riboprobe was generated from a partial clone from the quail, lacking the 5′ noncoding region and the translation initiation site. Both ET3 and EDNRB clones were kind gifts of Andrew Lassar. Antisense GDNF RNA probe was generated by amplifying a fragment of GDNF sequence from total RNA prepared from E9 chick gut using Trizol Reagent (Invitrogen). The following oligonucleotide primers were used: upstream primer, 5′-tgccagaggattacccagat-3′; downstream primer, 5′-aggtcatcgtcaaaggctgt-3′. The amplified product was cloned into pCRII-TOPO using TOPO TA Cloning (Invitrogen).

Results

ET3, EDNRB, and GDNF expression during avian gut development

Although ET3, EDNRB, and GDNF are known to be expressed in the avian gut (Nataf et al., 1996, 1998; Homma et al., 2000), a detailed analysis of their expression during ENS development has not been described. We examined the expression patterns of these genes by whole-mount in situ hybridization from HH 17 through 35 (E9). ET3 mRNA is expressed in the mesenchymal environment where crest-derived cells migrate. Between HH 18 and 21, ET3 is present in the subectodermal mesenchyme (Fig. 1A, arrows), where melanoblasts are migrating. At E4.5, ET3 is expressed specifically in the cecal primordia and cloaca (Fig. 1C), whereas it is expressed in the outer mesenchyme along the entire midgut and hindgut starting at E5 (Figs. 1B–F). EDNRB is expressed in the neural crest-derived cells of the developing gut. At E5, EDNRB expression is limited to the stomach and proximal duodenum (Fig. 1G). By E7 (Fig. 1H) and E9 (Figs. 1I–K), EDNRB-positive cells are present throughout the hindgut.

GDNF expression was present in the nephrogenic mesenchyme at HH 18 (Figs. 2A, B). Expression in the distal gut was evident at E5, where GDNF is present in the cecal primordia and cloaca (Fig. 2C). Longitudinal sections through the cecum show GDNF localized to the outer mesenchyme (Fig. 2D). Beginning at E7, GDNF expression was observed throughout the intestine, again restricted to the peripheral mesenchyme (Figs. 2E–H), similar to the region of ET3 expression (Figs. 1D–F).

Neural crest-derived cells reach the proximal hindgut at E6

To determine the relationship between ET3 and GDNF expression in the ceca and the timing of NCC arrival to that region of the gut, we established the time at which the migratory wavefront first reaches the proximal hindgut. A midsagittal section of an E6 embryo stained with Hu shows the presence of enteric crest cells in the distal midgut and ceca, with an occasional cell in the proximal hindgut (Fig. 3A, magnified view in panel B). The postcecal hindgut was removed at this stage and grown on the chorioallantoic membrane (CAM) of a host embryo for 7 days (number of experiments, $n = 16$). As shown in Fig. 3C, the CAM graft became completely populated by a normal-appearing ENS, suggesting that those rare cells in the proximal E6 hindgut are sufficient to populate the entire hindgut. CAM grafts were performed using E5 hindguts and these remain completely aganglionic (Fig. 3D), confirming that the host is not serving as a potential source of crest cells to the CAM graft.

EDNRB signaling is necessary for hindgut colonization

To establish that EDNRB activity is required for hindgut colonization, E5 chicken or quail guts (midgut + hindgut) were cultured in a three-dimensional collagen gel matrix for 72 h in the presence of ET3 or the EDNRB antagonist, BQ788. Hu

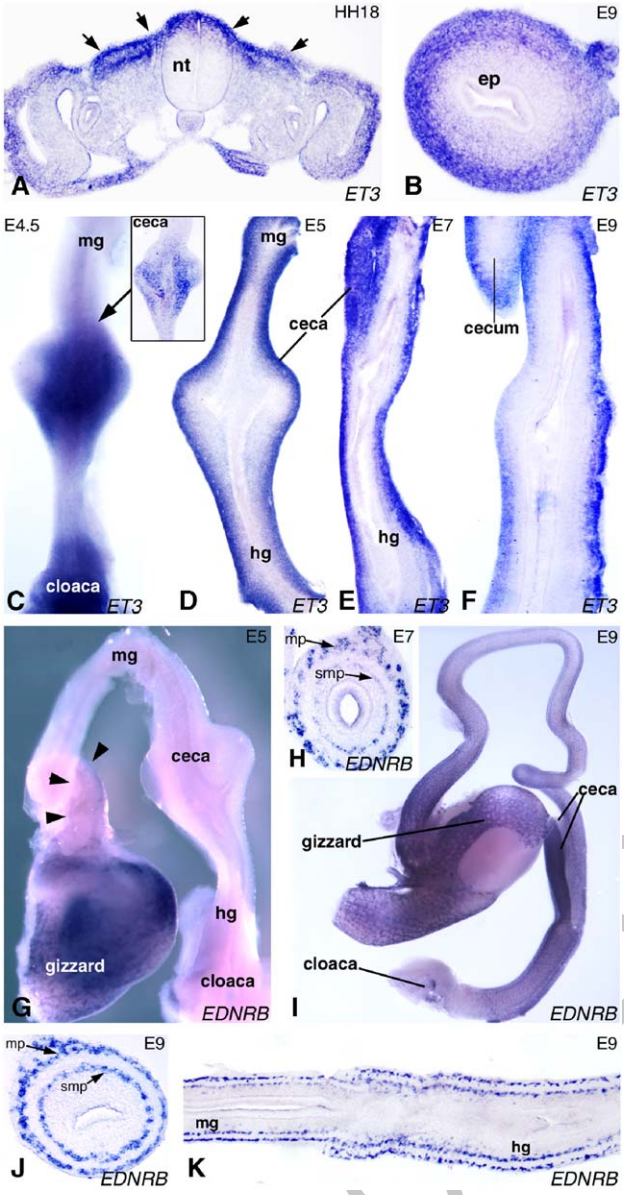

Fig. 1. Expression of ET3 and EDNRB during hindgut development. Cross-section of an E3 embryo demonstrates ET3 transcript in the dorsal subectodermal mesenchyme (A, arrows). Whole-mount in situ hybridization of an E4.5 gut shows ET3 expression concentrated in the ceca and cloaca (C). Inset shows a longitudinal section through the ceca, demonstrating mesodermal expression. From E5 through E9, ET3 is expressed in the outer mesenchyme along the entire midgut and hindgut, shown in longitudinal (D–F) and transverse (B) sections. Whole-mount of an E5 gut shows EDNRB-expressing neural crest cells in the gizzard, with the wavefront of EDNRB-expressing cells in the duodenum (G, arrowheads). Transverse section of an E7 proximal hindgut (H) shows EDNRB expression in the myenteric and submucosal plexuses. Crest cell expression is also evident at E9 in a whole-mount (I), midgut section (J), and longitudinal section of the distal gut (K). ep, epithelium; hg, hindgut; mg, midgut; mp, myenteric plexus; nt, neural tube; smp, submucosal plexus.

antibody was used as a marker of neuronal differentiation and HNK-1 as a marker of all enteric neural crest cells. In wild-type E5 embryonic gut, Hu-expressing crest-derived cells were present in the rostral midgut and absent from the caudal region of the midgut and the entire hindgut (Fig. 4A). By E8, Hu-positive crest-derived cells have reached the terminal hindgut (Fig. 4B). In control medium containing no added proteins (n = 24), complete colonization of the hindgut was observed after 72 h in culture (Fig. 4C). The same results were obtained in the presence of purified ET3 (Fig. 4D, n = 35). Explants grown in the presence of BQ788 (n = 35) demonstrated migratory arrest at the level of the ceca, leading to complete hindgut aganglionosis (Fig. 4E). HNK-1 immunostaining (Fig. 4F) confirmed the results obtained with Hu antibody, demonstrating that there is an arrest of enteric crest cell migration, not simply a delay in neuronal differentiation. These findings demonstrate that EDNRB signaling is necessary for NCC to colonize the hindgut.

Chick–quail intestinal chimeras

To investigate the role of ET-3 in development of the avian hindgut ENS, we developed a new application for avian coelomic transplantation. E5 quail hindguts, between ceca and cloaca, were microsurgically removed and transplanted into the coelomic cavity of E3 chick embryos (Figs. 5A, E) for 7 days (n = 32). At the time of transplantation, the E5 quail hindgut has not yet been colonized by crest-derived cells, as shown in a midsagittal section of an E5 embryo (Fig. 5B) and in a transverse section of an E5 hindgut (Fig. 5C). In all chick–quail transplant experiments, the nerve of Remak was removed to prevent it from serving as a source of enteric crest cells (Fig. 5D). The transplanted hindgut developed in the coelomic cavity of the host, as demonstrated in Fig. 5F. Fig. 5G shows the hindgut graft before (boxed area on right) and after 7 days of transplantation (left), demonstrating significant growth of the graft during the transplantation period. At the completion of the 7-day coelomic incubation, the graft is found attached to the liver and right lung. Although the graft is initially positioned adjacent to the foregut, at the time of harvesting the graft is found a distance from the esophagus and never attached to it.

Grafts were analyzed by immunohistochemistry using species-specific and cell-specific antibodies to look at the origin of the cells and their capacity to differentiate. As expected, the quail nuclear marker, QCPN, heavily stained the epithelium and mesenchyme of the graft, while it did not stain the presumptive neural plexuses (Fig. 5H, arrows). Those plexuses, both submucosal and myenteric, were strongly stained with the 8F3 antibody, which exclusively recognizes cells of chicken origin (Fig. 5I), suggesting that neural crest cells from the chick host colonized the quail hindgut. Immunostaining on serial sections shows that the 8F3-immunoreactive cells express the neural crest cell marker HNK-1 (Fig. 5J), neuron-specific Hu (Fig. 5K) and neurofilament (Fig. 5L), and glial-specific BFABP (Fig. 5M). We conclude that, in this chimeric system, NCC from the chick host colonize the quail hindgut graft and form two ganglionated plexuses containing neurons and glial cells. In addition to neural crest-derived cells, host endothelial cells are also found in the graft. Using QH1, a quail-specific endothelial cell maker, we find that the intestinal grafts have a chimeric vasculature, composed of both quail-derived (QH1+) and chick-derived (QH1−, 8F3+) endothelial cells (not shown).

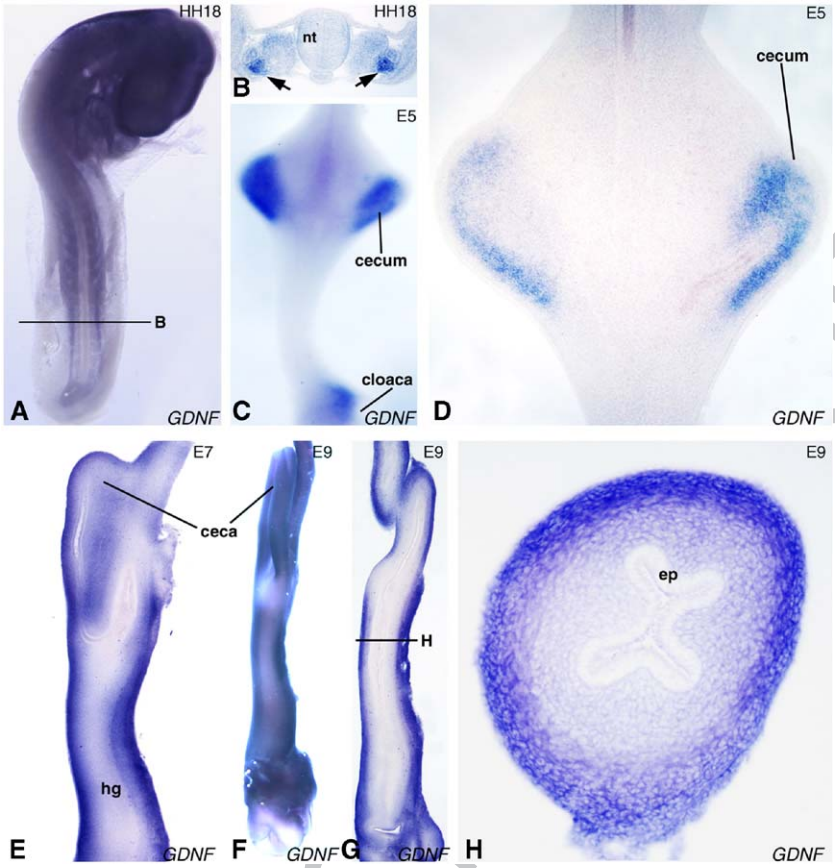

Fig. 2. GDNF expression during hindgut development. Dorsal view of a whole-mount in situ hybridization at HH 18 stained for GDNF (A). The line indicates the level of the transverse section shown in panel B, where GDNF expression is seen in the nephrogenic mesenchyme (arrows). E5 intestinal whole-mount shows expression in the cloaca and cecal primordial (C), where expression is localized to the mesenchymal cells (D). From E6 onward, staining is observed throughout the intestine, as shown at E7 (E) and E9 (F–H) in longitudinal (E–G) and transverse (H) sections. ep, epithelium; hg, hindgut; nt, neural tube.

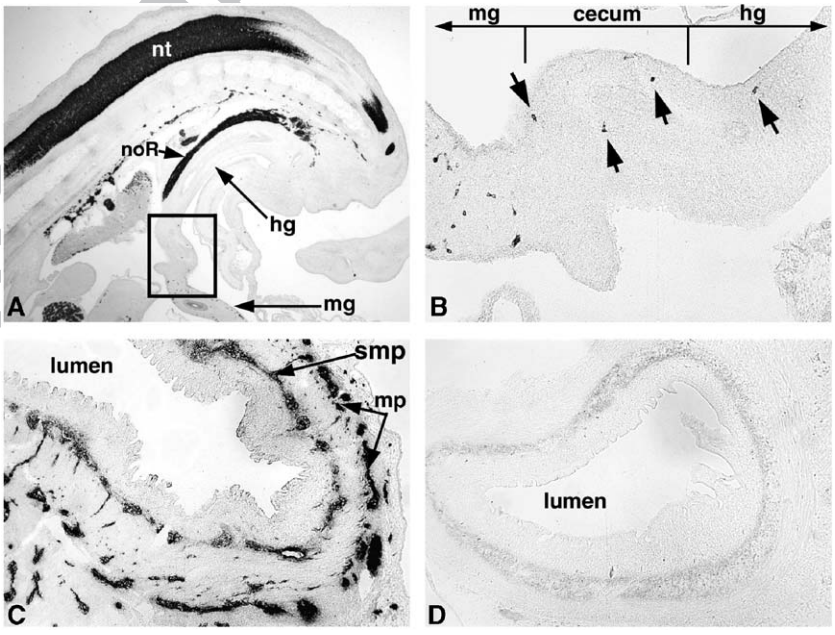

Fig. 3. The neural crest cell wavefront reaches the proximal hindgut at E6. Sagittal section of the caudal end of an E6 quail embryo stained with Hu (A, boxed area magnified in panel B), demonstrating a few NCC in the ceca and proximal hindgut (B, arrows). Removal of the postcecal hindgut at this stage and culture on the chorioallantoic membrane (CAM) for 9 days leads to complete colonization of the ENS from those few starting cells (C). CAM culture using an E5 hindgut, where NCC have not yet reached the hindgut, results in aganglionosis (D). hg, hindgut; mg, midgut; mp, myenteric plexus; noR, nerve of Remak; smp, submucosal plexus.

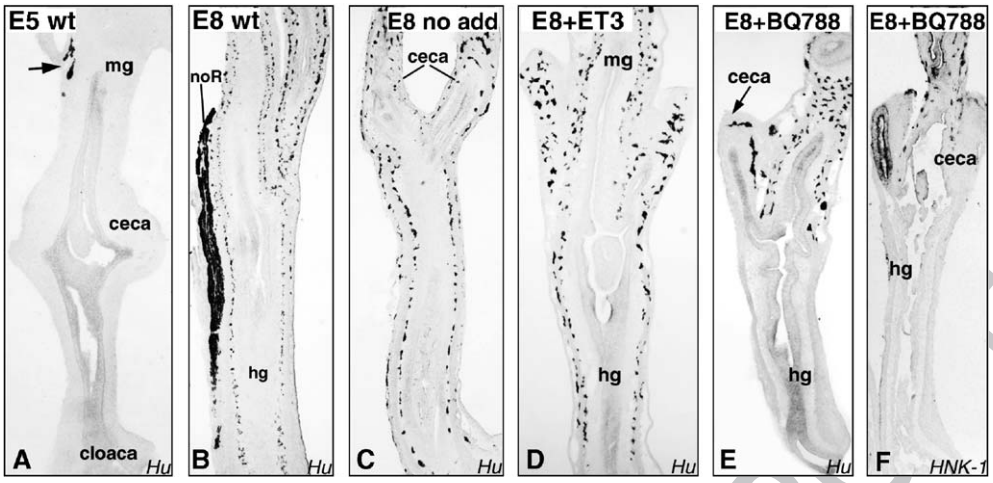

Fig. 4. ET3-EDNRB signaling is required for hindgut colonization. E5 gut was cultured in collagen gel for 72 h in the presence of ET3 protein or BQ788. Longitudinal sections stained with Hu antibody are shown. In wild-type E5 gut, NCC are present only in the proximal midgut (A, arrow) and absent distally. E8 wild-type intestine contains Hu-immunoreactive cells along its entire length (B). An E5 gut cultured for 72 h with no added proteins (C) looks indistinguishable from a wild-type E8 gut (B). When cultured in the presence of excess ET3 protein, no effect on migration is seen (D), while in the presence of BQ788, crest cell migration is arrested at the ceca and the hindgut is aganglionic, as shown by the absence of Hu+ (E) or HNK+ (F) cells. hg, hindgut; mg, midgut; noR, nerve of Remak.

Inhibition of EDNRB signaling leads to severe hypoganglionosis in the hindgut

The organ culture experiments shown in Fig. 4 demonstrate that NCC cannot migrate past the ceca in the absence of ET3 signaling. Therefore, in order to study the role of ET3 within the postcecal hindgut, we used chick–quail intestinal transplants. Preganglionic hindguts were isolated from E5 quail embryos. The ceca, cloaca, and nerve of Remak were removed to eliminate any intrinsic sources of NCC. Hindguts were then cultured overnight in collagen matrix containing media alone (controls, $n = 15$; Fig. 6A), 250 ng/ml ET3 ($n = 36$; Figs. 6B–E), or 20 μM BQ788 ($n = 42$; Figs. 6F, G). Following removal from the collagen gel, hindguts were transplanted into the coelomic cavity of E3 chicken embryos as described above. Analysis of the hindguts after 7 days of incubation revealed that chicken-derived neural crest cells, identified by their immunoreactivity to 8F3, HNK-1, and Hu, are present in the ET3-treated quail guts and form well developed submucosal and myenteric ganglia (Figs. 6B–E). These ganglia appear markedly increased in number and size as compared to untreated controls (Fig. 6A). Hindguts treated with BQ788, on the other hand, demonstrate marked hypoganglionosis, with the development of few and very small enteric ganglia (Figs. 6F, G). The total number of ganglion cells was counted in untreated, ET3-treated, and BQ788-treated hindguts and the data are summarized in Fig. 7. ET3 treatment produced significant hyperganglionosis, while inhibition of EDNRB activity yielded severe hypoganglionosis. The effect on ganglion cell number was seen equally in both submucosal and myenteric plexuses.

To locally inhibit EDNRB signaling and verify our findings, BQ788-coated beads were implanted into the wall of E5 hindguts prior to coelomic transplantation (Fig. 6H). After 7 days of transplantation, guts were sectioned and analyzed for the presence of NCC. Enteric ganglia adjacent to ET3 beads (Figs. 6I, J; $n = 25$) were larger than control ganglia (Fig. 6H, $n = 10$) and significantly larger than ganglia adjacent to BQ788-coated beads (Figs. 6K, L; $n = 25$). These results demonstrate that EDNRB signaling, independent of its role within the ceca, promotes the development of enteric NCC within the hindgut.

ET3 promotes proliferation of crest-derived cells in the hindgut

The effect of ET3 on ganglion size could result from an effect on NCC proliferation, survival, or differentiation. In order to study the effect on cell proliferation, E5 hindguts were cultured for 3 days in the presence of BQ788 or ET3. BrdU was added to the culture for the final 3 h. Longitudinal sections were then analyzed by double immunofluorescence using QCPN, Hu, HNK-1 and anti-BrdU antibodies. Numerous BrdU-positive epithelial cells, and a smaller number of proliferating mesenchymal cells, were detected in wild-type intestine (Fig. 8B). Occasional BrdU+ crest-derived cells were identified in the ENS (not shown). In BQ788-treated guts, only rare enteric crest-derived cells expressed BrdU (Figs. 8C, D). In contrast, guts cultured in the presence of ET3 contained numerous cells that coexpressed BrdU and HNK-1/Hu (Figs. 8E, F). Fig. 8A summarizes the results obtained from 15 hindguts, showing that the percentage of ganglia containing BrdU+ cells is 26% in wild-type hindguts, 10% in the presence of BQ788, and 70% in the presence of added ET3, suggesting that ET3 promotes proliferation of crest-derived cells in the avian hindgut.

The data also suggest that BQ788 treatment reduces the density of BrdU+ cells in enteric ganglia relative to controls. To ensure that this effect is specific to the ganglia and not a non-specific cellular toxicity, we counted the number of BrDU+ cells in the epithelium and non-ganglionic mesenchyme per high-power (40×) field. In ET3-treated, BQ788-treated, and control guts, respectively, there were 24 ± 14, 22 ± 5, and 25 ± 4 BrDU+ cells in the epithelium and 16 ± 6, 15 ± 1, and 15 ± 5 BrDU+ cells

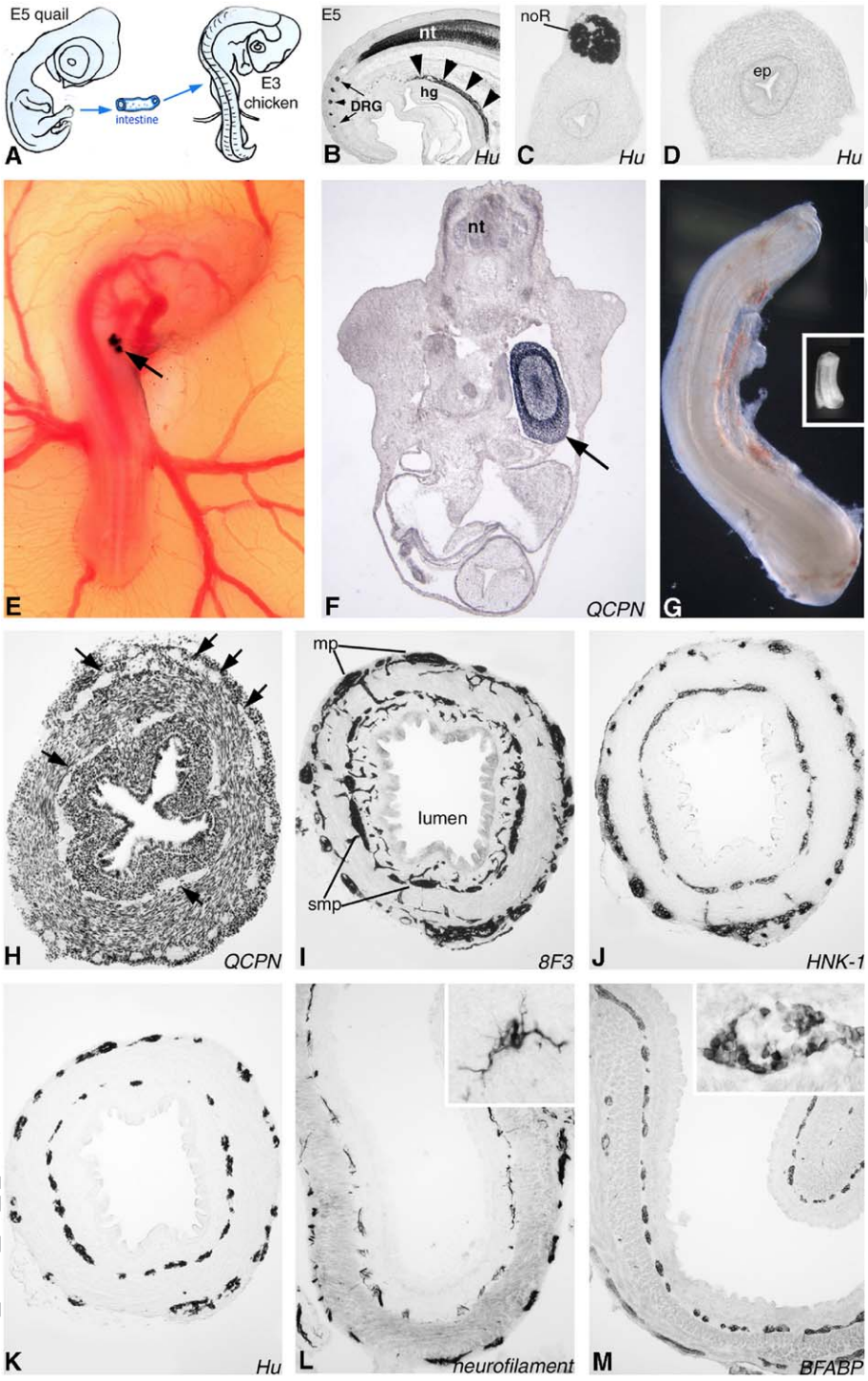

Fig. 5. Chick–quail intestinal transplants recapitulate normal ENS development. The E5 quail aneural hindgut was removed and transplanted into the coelomic cavity of an E3 chick embryo (A). Sagittal section of an E5 quail embryo stained with Hu (B) shows expression in the nerve of Remak (arrowheads), neural tube (nt), and dorsal root ganglia (DRG). Cross-sections from the isolated E5 quail hindguts before (C) and after (D) removal of the nerve of Remak (noR) are shown. Immediately following transplantation into the chick coelom, the carbon-labeled quail intestinal graft is visualized just ventral to the aorta (E, arrow). Two days after transplantation, QCPN-immunoreactive quail gut is seen adjacent to the host coelomic epithelium (F, arrow). The grafted intestine grows significantly after 7 days of incubation (G), as compared to the original size of the graft at the time of transplantation (G, inset). Seven days after transplantation, the quail hindguts were analyzed by immunocytochemistry. QCPN antibody demonstrates quail-derived cells throughout the gut, except for the neural plexuses, which remain unstained (H, arrows). These plexuses are comprised of 8F3-immunoreactive chicken cells (I), which also express the NCC marker HNK-1 (J), neuron-specific Hu (K) and neurofilament (L), and glial-specific BFABP (M). ep, epithelium; DRG, dorsal root ganglia; hg, hindgut; mp, myenteric plexus; noR, nerve of Remak; nt, neural tube; smp, submucosal plexus.

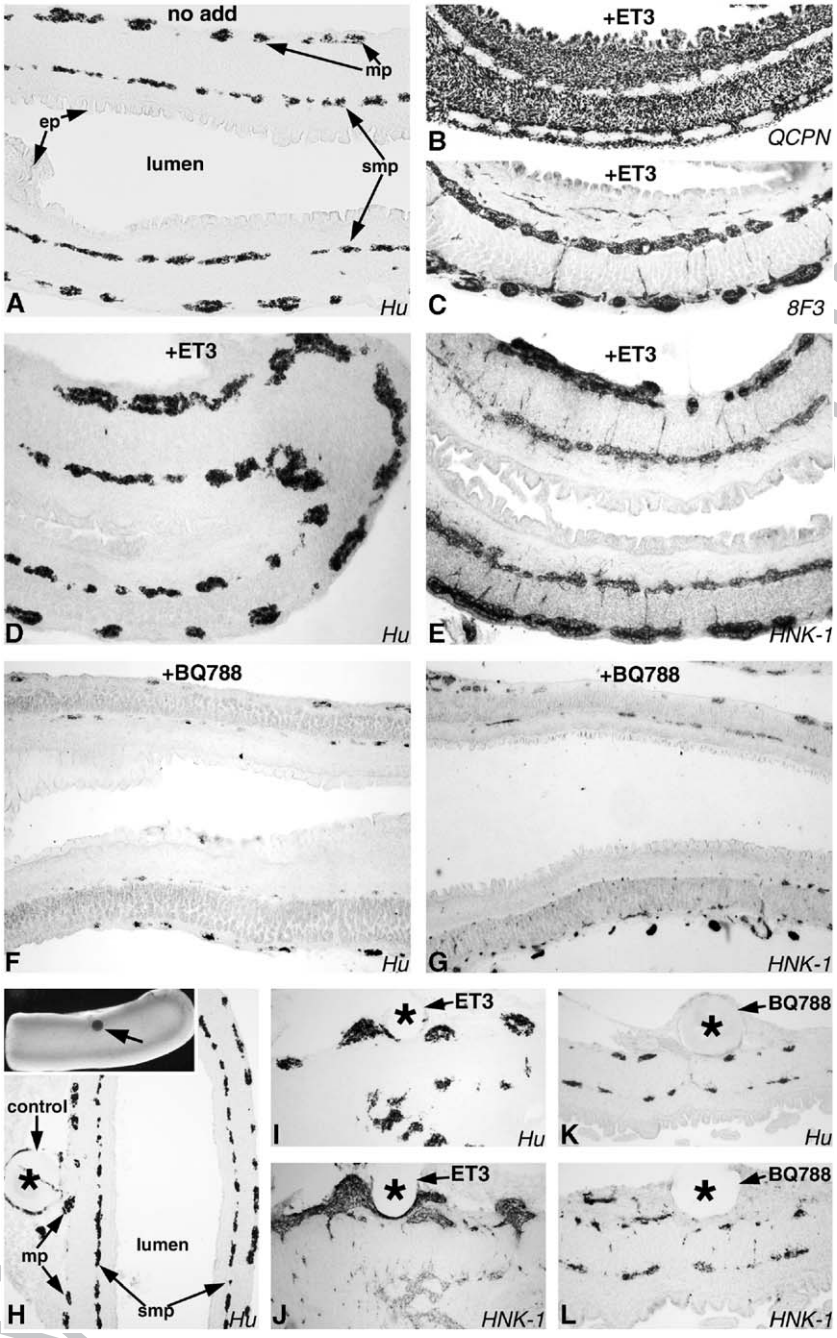

Fig. 6. The presence of excess ET3 leads to hyperganglionosis. E5 quail hindguts were treated with ET3 protein (B–E) or BQ788 (F, G) for 24 h, transplanted into the coelomic cavity of E3 chick embryos, and allowed to develop for 7 days. A longitudinal section from a control transplanted gut without additive demonstrates normal ganglia (A). In the presence of excess ET3, ganglia are significantly increased in size and number, as shown by QCPN (B), 8F3 (C), Hu (D), and HNK-1 (E) staining. Inhibition of ET3 signaling with BQ788 leads to a marked reduction in the size and density of ganglia, shown by immunoreactivity to Hu (F) and HNK-1 (G). Acrylamide beads coated with either ET3 (I, J) or BQ788 (K, L) were implanted into preganglionic E5 quail hindguts that were subsequently transplanted into E3 chick embryos. A hindgut treated with a control bead (H, asterisk) is shown. Inset demonstrates the intestinal graft with bead at time of isolation from the chick coelom. In the presence of an ET3-coated bead (I, J), enteric ganglia are significantly increased in size as compared to those exposed to BQ788-coated beads (K, L). ep, epithelium; mp, myenteric plexus; smp, submucosal plexus.

in the mesenchyme. None of these differences were statistically significant.

ET3 could also increase ganglion size by preventing apoptosis of crest-derived cells. Double immunofluorescence was performed using an antibody to activated caspase-3, a marker of apoptosis, and HNK-1. Apoptotic enteric crest cells were not seen in wild-type hindguts or in the presence of ET3 (not shown) or BQ788 (Fig. 8G). E6 dorsal root ganglion, which does contain apoptotic NCC, is shown as a positive control (Fig. 8H). The inhibition of enteric NCC proliferation observed with BQ788 treatment does not appear to be associated with increased cell death.

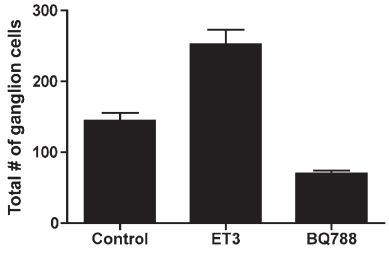

Fig. 7. Inhibition of EDNRB signaling produces severe hypoganglionosis. Guts treated with ET3 or BQ788 for 24 h, followed by coelomic transplantation, were stained with HNK-1 and analyzed quantitatively. The total number of ganglion cells per high-power (200×) field was counted in untreated, ET3-treated, and BQ788-treated guts ($n = 5$ from each group). Error bars represent standard error of the mean. Statistical analysis was performed by one-way ANOVA using Tukey's Multiple Comparison Test (PRISM software). A P value <0.05 was considered significant. The difference between each of the three groups was statistically significant at $P < 0.01$.

ET3 inhibits neuronal differentiation in the hindgut

The absence of hindgut colonization in the presence of BQ788 may result not only from inhibition of crest-derived cell proliferation but also from premature differentiation of those cells, preventing them from further migration. To assess for neuronal differentiation, we determined the presence of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity in hindgut explants. NADPH-diaphorase activity co-localizes with nitric oxide synthase (NOS) in the central and peripheral nervous system (Branchek and Gershon, 1989; Dawson et al., 1991; Balaskas et al., 1995; Burns and Delalande, 2005). In the ENS, neuronal NOS (nNOS) marks a subset of terminally differentiated enteric neurons (Young et al., 2005), unlike Hu and neurofilament, which are earlier markers of neuronal commitment (Payette et al., 1984; Fairman et al., 1995).

NADPH-diaphorase activity is present along the entire hindgut in E8 wild-type embryos (Fig. 9A). Following a 3-day incubation of an E5 hindgut with purified ET3 protein in organ culture, neurofilament is expressed along the length of the gut (Fig. 9B), similar to the expression of Hu (Fig. 4D). However, NADPH activity is only present in the midgut and ceca, and is absent from the entire hindgut (Fig. 9C), indicating a delay in neuronal differentiation. Glial differentiation, as detected by BFABP-immunoreactivity, is not inhibited (Fig. 9D). Guts exposed to BQ788 show expression of neurofilament, NADPH, and BFABP in the midgut and ceca (Figs. 9E–H), confirming the absence of ENS colonization of the hindgut and demonstrating that the most terminal cells in the ceca have undergone neuronal and glial differentiation. We conclude from these data that ET3 inhibits neuronal differentiation of enteric crest-derived cells.

ET3 inhibits the migratory response of neural crest-derived cells to GDNF

Recent studies in rodents have suggested that GDNF-mediated enteric neural crest cell migration is modulated by ET3 (Kruger et al., 2003; Barlow et al., 2003). To test this in the avian embryo, we cultured E8 guts in collagen gel ($n = 45$) in the absence (Fig. 10A) or presence of GDNF (Figs. 10B, C). Addition of GDNF to the culture medium resulted in significant emigration of crest-derived cells out of the midgut and ceca into the surrounding collagen matrix. Interestingly, GDNF did not promote any cell migration from the hindgut (Fig. 10C). The addition of ET3 alone did not promote enteric crest cell migration (Fig. 10D). However, when ET3 and GDNF were added together, migration was significantly decreased (Fig. 10E). ET3 inhibition of GDNF-induced migration was abolished after addition of BQ788, which resulted in enhanced emigration of HNK-1+ cells (Figs. 10F, G). These results indicate that ET3 inhibits the migration of midgut crest-derived cells in response to GDNF.

Discussion

The importance of ET3 signaling in ENS formation is well established (Baynash et al., 1994; Hosoda et al., 1994; Barlow et al., 2003; Kruger et al., 2003; Woodward et al., 2003), although its mechanism of action remains unclear. The most commonly held hypothesis is that ET3 activity maintains the pool of enteric NCC precursors by promoting their proliferation and inhibiting their differentiation into neurons (Gershon, 1999). In the absence of ET3, therefore, premature neuronal differentiation exhausts the pool before intestinal colonization is complete. The observation that the aganglionosis in ET3-deficient mice is limited to the hindgut suggests that in the absence of ET3 signaling there are only enough NCC precursors to populate the intestine to the level of the cecum. This suggests that ET3 may have a specific effect on migrating crest-derived cells as they reach the cecum, functioning to expand the population of precursors at that point so that they can populate the remaining intestine. Our findings in avians, together with previous experimental work in rodents, confirm that the cecum is central to ET3 function. For example, ET3 signaling is only required between E10.5 and E12.5 in mice, the time at which migrating crest cells are crossing the ileocecal junction (Shin et al., 1999; Woodward et al., 2000; Sidebotham et al., 2002; Lee et al., 2003). Moreover, NCC migration is normal in ET3 and EDNRB mutant mice until those cells reach the cecum, where there is a transient migratory arrest (Kapur et al., 1992, 1995; Lee et al., 2003). Similarly, we find that inhibition of EDNRB activity in the avian gut leads to arrested migration precisely at the level of the ceca (Fig. 4E).

We examined the expression of ET3 and GDNF during avian gut development and find both genes initially expressed in the ceca (Figs. 1 and 2), as has been demonstrated in mice (Leibl et al., 1999; Young et al., 2001). The cecal expression of ET3, however, is very transient, occurring just before E5 (Fig. 1C), and this may explain why it was not previously described in chicks (Nataf et al., 1998). GDNF is strongly expressed in the ceca at E5 (Figs. 2C, D). The timing of expression of both genes is notable in that it occurs just prior to the arrival of crest-derived cells at the cecal buds at E5.5 (Newgreen et al., 1996), suggesting that these factors may locally influence, directly or indirectly, enteric NCC at the migratory wavefront. Interestingly, using CAM grafts, we find that the first few crest-derived

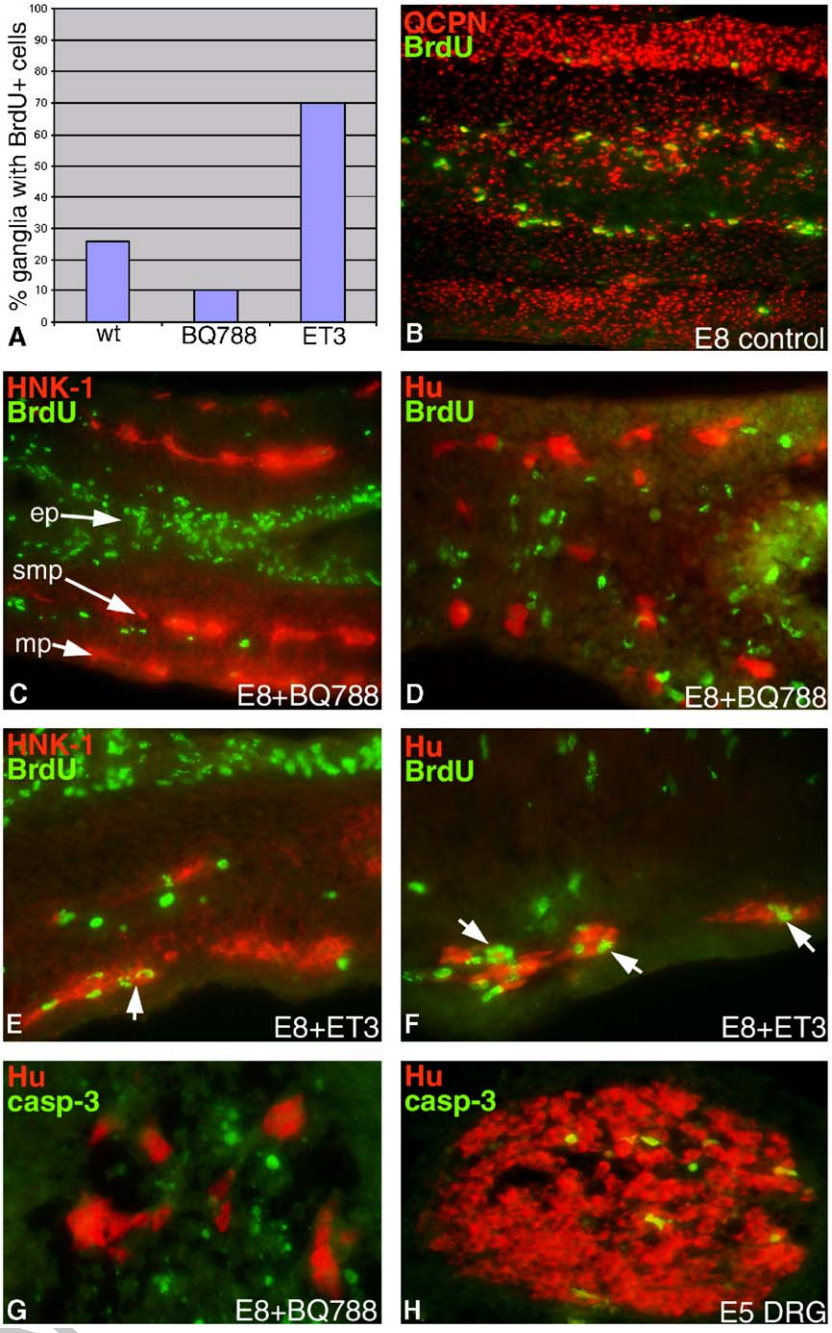

Fig. 8. ET3 promotes enteric neural crest cell proliferation in the hindgut. E5 hindguts were cultured for 3 days in the presence of BQ788 (C, D) or ET3 (E, F) and BrdU labeling performed. The presence of added ET3 led to a significantly higher percentage of BrdU-positive enteric ganglia as compared to controls and BQ788-treated guts (A). Double immunohistochemistry was performed on longitudinal sections through the midgut and hindgut. Numerous BrdU-positive epithelial cells, and scattered mesenchymal cells, were identified throughout the hindgut in an untreated control (B). Guts treated with BQ788 show very rare BrdU incorporation into HNK-1+ (C) or Hu+ (D) enteric neural crest cells in the midgut and ceca. In contrast, guts cultured in the presence of ET3 contain many ganglia in the hindgut that are positive for both BrdU and HNK-1 (E, arrows) or Hu (F, arrows). Sections from an E8 BQ788-treated gut (G) double-stained with an anti-activated caspase-3 antibody to detect apoptotic cells and anti-Hu to label neurons. Apoptotic epithelial and mesenchymal cells were present, but there was no evidence of colocalization of Hu with activated caspase-3 (G). The section shown in panel G is from the cecum of a treated gut. Apoptosis was detected in the dorsal root ganglion (H), used as a positive control. drg, dorsal root ganglion; ep, epithelium; mp, myenteric plexus; smp, submucosal plexus.

cells that reach the postcecal intestine, sometime between E5 and E6, are sufficient to populate the entire hindgut (Fig. 3). These results suggest two important aspects of the leading edge of migrating crest-derived cells when they arrive at the ceca: first, that they are exposed to a unique molecular environment rich in ET3 and GDNF and, second, that those few leading cells have a high proliferative capacity and can independently give rise to the entire hindgut ENS.

Since the cecal expression of both ET3 and GDNF occurs at least 0.5 days before NCC arrival, these factors may influence arriving crest-derived cells indirectly by altering the mesenchymal environment of the ceca in anticipation of NCC arrival.

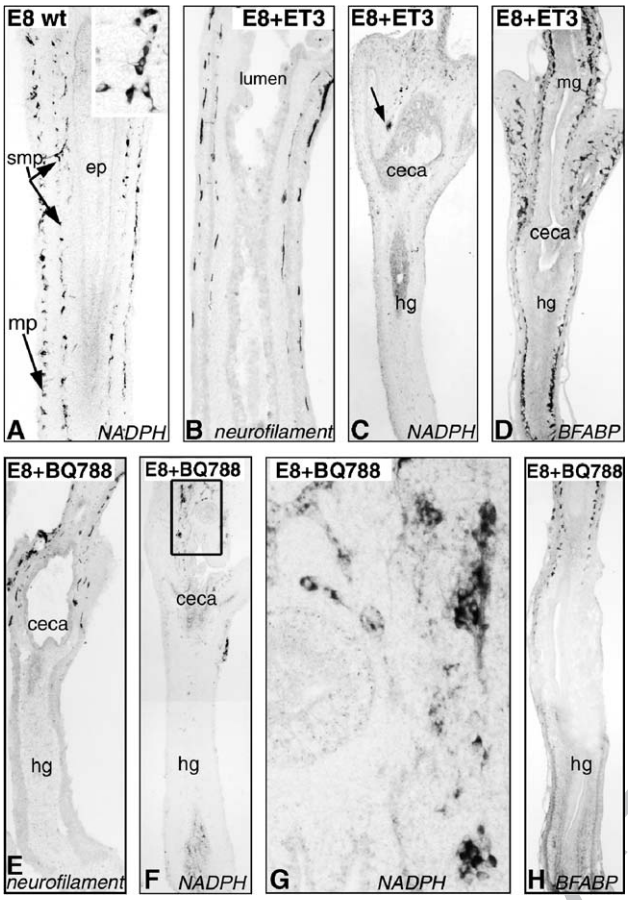

Fig. 9. ET3 inhibits neuronal differentiation in the ENS. NADPH-diaphorase activity is present along the entire hindgut in E8 wild-type embryos (A). E5 hindgut cultured for 3 days in the presence of excess ET3 shows neurofilament-positive cells along the length of the hindgut (B). However, NADPH activity is only present in the midgut and ceca, and is absent from the hindgut (C). Glial differentiation, as detected by BFABP-immunoreactivity, is present throughout the hindgut (D). Exposure to BQ788 led to expression of neurofilament (E), NADPH (F, boxed area magnified in panel G), and BFABP (H) in the midgut and ceca, consistent with normal differentiation of the most distal cells in these explants.

This idea has been proposed in mice, where EDNRB activation inhibits laminin α1 production (Wu et al., 1999). Since laminin α1 normally promotes neurogenesis (Chalazonitis et al., 1997), the loss of EDNRB activity would lead to an environment promoting premature neuronal differentiation. This model may not be relevant in avians, however, since EDNRB expression does not appear to be present in the non-NCC mesenchyme (Fig. 1; Nataf et al., 1996). ET3 and GDNF may have another effect, yet to be defined, on the molecular environment of the ceca and on the arriving crest-derived cells.

In order to investigate the role of ET3-EDNRB signaling in the avian hindgut ENS, we developed a method for studying ENS development using coelomic transplantation. Grafting of tissue into the avian coelom is a well-established technique that has been used to study development of the limb bud (Hamburger, 1939), lymphoid organs (Nagy et al., 2004), and hematopoietic tissue (Caprioli et al., 1997). Intestinal grafts have also been transplanted into the avian coelom to look at epithelial-mesenchymal interactions during gut development (Kedinger et al., 1981; Bolcato-Bellemin et al., 2003). However, ENS development has not been studied using this system. Preganglionic quail hindgut was transplanted into the chick coelom and allowed to develop for 7 days. We find that NCC from the chick host colonize the quail hindgut and form two ganglionated plexuses that are grossly indistinguishable from a normal ENS (Fig. 5). The fact that chick cells are able to colonize the quail gut is not surprising, given that others have previously created chimeras by coculturing the neural tube and intestine from each species onto the CAM (Teillet, 1978; Rothman et al., 1986; Hearn and Newgreen, 2000). By adapting the technique of coelomic grafting to create our chimeras, we have developed a useful model system in which we are able to manipulate the molecular environment of the aneural hindgut prior to grafting and to assess the subsequent effect on ENS formation. One of the advantages of this technique is that coelomic grafting can be performed on a 3-day-old chick embryo, unlike CAM grafting, which cannot be done prior to E8, allowing longer incubation times in the coelom. We are currently determining the origin of the donor cells to understand what molecular environment they arise in, and travel through, to get to the transplanted gut. Preliminary evidence using DiI labeling of the host neural tube suggests that the graft is colonized by vagal NCC from the host embryo (unpublished results).

These coelomic grafts are different from wild-type hindgut in several respects. Sacral crest cells from the host chick do not appear to contribute to the grafted hindgut, nor do quail sacral-derived cells, since the nerve of Remak is removed prior to grafting. While the nerve of Remak normally extends nerve fibers into the gut as early as E7.5, sacral NCC do not become incorporated into myenteric and submucosal ganglia until E10–E12 (Burns and Le Douarin, 1998, 2001), the stage at which our coelomic grafts are harvested (E5 graft +7-day coelomic transplantation). Even at E16, sacral crest-derived cells comprise <2% of ganglion cells in the submucosal plexus of the colorectum, while in the myenteric plexus they make up 0.3% of ganglion cells in the proximal colorectum and 17% distally (Burns and Le Douarin, 1998). The ENS effects we observed in our experiments were present equally throughout both plexuses, proximally and distally. We therefore believe that the absence of the nerve of Remak is unlikely to have a significant effect on our observations, although a better understanding of the role of this avian-specific structure is needed.

The ceca are also removed from the grafts prior to transplantation in order to eliminate quail-derived NCC. Migrating crest-derived cells thus enter the hindgut directly, without passing through the cecal environment. Whether this alters their development is unknown. Cocultures of ganglionated mouse midgut with aneural postcecal hindgut have been shown to result in grossly normal colonization of the hindgut, suggesting that migration through the cecum is not essential for hindgut ENS formation (Young et al., 2002). Our untreated coelomic grafts also appear to develop a normal ENS (Fig. 6A), although we cannot exclude the possibility that a molecular "modification" normally occurs during migration through the ceca. Thus, while coelomic grafts are a useful method for studying ENS development, the potential consequences of removing the nerve of

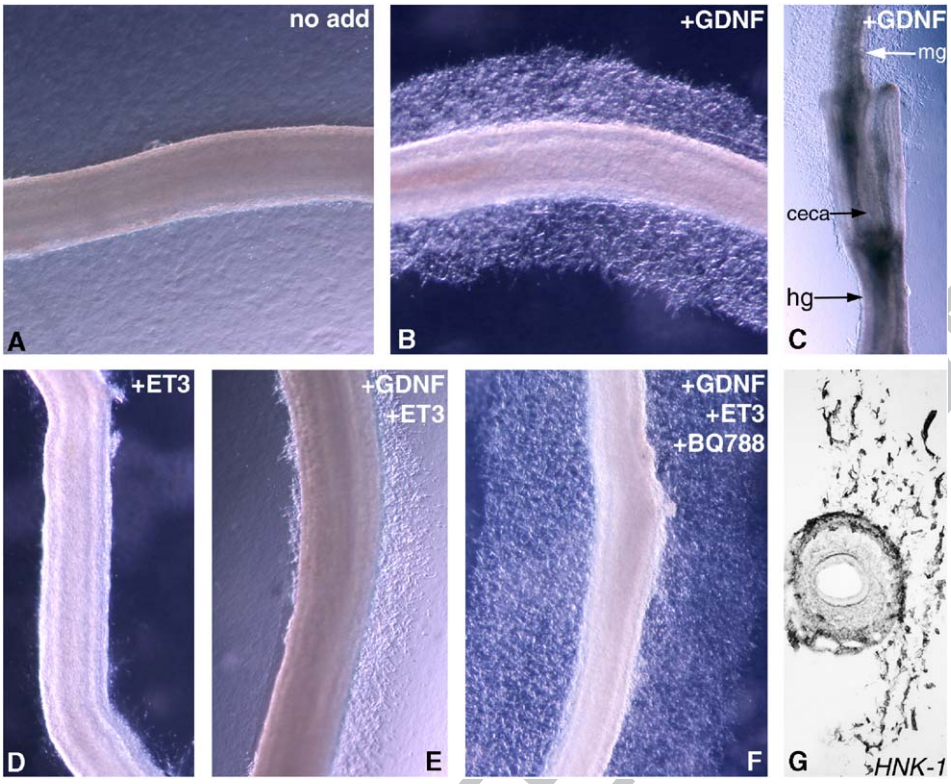

Fig. 10. ET3 inhibits the chemoattractive effect of GDNF. E8 gut was grown in collagen gel in the absence (A) or presence of 10 ng/ml GDNF (B, C), 250 ng/ml ET3 (D), GDNF+ET3 (E), or GDNF+ET3+ 5 μM BQ788 (F). While GDNF normally stimulates migration of neural crest cells from the midgut into the media (B, C), the presence of ET3 inhibits this GDNF-induced cell migration (E). The addition of BQ788 relieves the inhibitory effect of ET3 (F). The migrating cells are immunoreactive to HNK-1 antibody, confirming that they represent NCC (G). hg, hindgut; mg, midgut.

Remak and ceca should be considered. In general, when using avian embryos for studying ENS development, one should consider that the nerve of Remak and the cecal appendages in avians represent potentially important differences from mammals that could affect the results obtained.

Using coelomic grafts, we find that inhibition of EDNRB signaling in the hindgut leads to severe hypoganglionosis, while ET3 overexpression produces hyperganglionosis (Figs. 6 and 7). This impact on ganglion cell number can result from effects on NCC proliferation, survival, and/or differentiation. We find significantly more BrdU-positive ganglia in the presence of excess ET3 as compared to controls, and virtually no proliferating crest-derived cells in the presence of BQ788 (Fig. 8). The effect is specific to enteric ganglion cells, as we observe no difference in the density of BrdU-positive cells in the epithelium or in the non-NCC mesenchyme. This result is consistent with previous in vitro work showing that ET3 promotes proliferation of cultured NCC (Stone et al., 1997; Lahav et al., 1996) and of undifferentiated enteric crest-derived cells (Barlow et al., 2003; Hearn et al., 1998). We also find no evidence of increased apoptosis when EDNRB signaling is inhibited (Fig. 8G), in agreement with Lee et al. (2003), who did not observe increased apoptotic cell death in the ENS of EDNRB-deficient mice.

In addition to promoting proliferation of enteric crest-derived cells, ET3 also inhibits neuronal differentiation, as demonstrated by the absence of nNOS-expressing cells in ET3-treated hindguts (Fig. 9C). NOS is the first neurotransmitter synthetic enzyme expressed by mature enteric neurons (Branchek and Gershon, 1989). These terminally differentiated crest-derived cells are present in the ceca of BQ788-treated guts (Figs. 9F, G), reflecting their premature differentiation and accounting for their inability to migrate further along the gut, as initially suggested by Gershon (1999). Conversely, the absence of terminally differentiated neurons in ET3-treated hindguts (Fig. 9C) may account for the continued proliferation and migration of those undifferentiated crest-derived cells.

The importance of the ceca to ET3 function is further supported by the effect of ET3 on NCC migration. GDNF is known to be chemoattractive to enteric crest cells (Young et al., 2001). However, its strong expression in the ceca raises the question of how enteric NCC are able to migrate past that GDNF-rich segment of the gut. Recent work in rodents suggests that ET3 modulates the chemoattractive response of enteric NCC to GDNF (Barlow et al., 2003; Kruger et al., 2003). We find that this is also true in the avian gut, where ET3 inhibits the chemoattraction to GDNF (Fig. 10), and by so doing may allow crest-derived cells to migrate past the ceca and into the hindgut. BMP (Goldstein et al., 2005) and Shh (Fu et al., 2004) signaling has also been shown to modulate the migratory response to GDNF, suggesting potential interactions among these various pathways in the regulation of ENS migration. Once NCC are past the ceca and in the hindgut, GDNF no longer has a chemoattractive role (Fig. 10), suggesting the presence of other chemoattractive factors in the distal gut.

While our findings on the effect of ET3-EDNRB signaling on enteric crest-derived cell proliferation, differentiation, and migration corroborate previous observations in cultured NCC and enteric crest-derived cells from mice, we recognize that ET3 activity may exert slightly different effects in avians and mammals. The absence of EDNRB expression in the intestinal non-NCC mesenchyme of avians (Fig. 1; Nataf et al., 1996), for example, illustrates one such difference from mammals (Okabe et al., 1995; Pla and Larue, 2003) and may impact on the mechanism of action of this signaling pathway. Identifying other avian-specific differences will be important to interpreting results obtained from these and similar experiments.

Our results suggest a role for ET3-EDNRB signaling, not only in the ceca, but also in the hindgut, where ET3 becomes expressed as NCC are arriving (Figs. 1D, E). As shown in Figs. 4E, F, in the presence of ceca, EDNRB inhibition leads to hindgut aganglionosis. However, when ceca are excluded, as in the coelomic grafts, NCC enter the EDNRB-deficient avian hindgut, but only in very small numbers (Figs. 6F, G; Fig. 7). This hypoganglionosis suggests that EDNRB signaling may be necessary to ensure the development of a normal complement of enteric ganglion cells in the hindgut. The requirement for EDNRB signaling within the hindgut is in agreement with the findings of Wu et al. (1999), who demonstrated in explant cultures that the terminal colon of ET3-deficient mice remains aganglionic until exogenous ET3 is added to the culture medium, promoting the migration of crest-derived cells into the aganglionic region. The notion that ET3 activity not only maintains a pool of enteric crest cell precursors but also has a critical role within the hindgut itself may help to explain why sacral crest-derived cells do not contribute to the distal colorectum in ET3- and EDNRB-deficient mice (Baynash et al., 1994; Hosoda et al., 1994). If the defect was entirely due to premature neuronal differentiation and failure to complete migration, one would expect sacral crest-derived cells to contribute to the distal bowel in those mutants. Our results suggest that deficient ET3 signaling may create a hindgut environment that does not allow normal colonization by enteric crest-derived cells. The presence of a small number of ganglion cells in our grafts may be due to incomplete inhibition of EDNRB activity. In conclusion, ET3 signaling promotes enteric crest-derived cell proliferation, inhibits neuronal differentiation, and modulates migration in the avian hindgut ENS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.032.

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