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Smooth-muscle-specific expression of neurotrophin-3 in mouse embryonic and neonatal gastrointestinal tract

Edward A. Fox and Jennifer McAdams

Behavioral Neurogenetics Laboratory, Department of Psychological Sciences, Purdue University, 703 Third Street, West Lafayette, IN 47907, USA

Edward A. Fox: au_gc@psych.purdue.edu

Abstract

Vagal gastrointestinal (GI) afferents are essential for the regulation of eating, body weight, and digestion. However, their functional organization and the way that this develops are poorly understood. Neurotrophin-3 (NT-3) is crucial for the survival of vagal sensory neurons and is expressed in the developing GI tract, possibly contributing to their survival and to other aspects of vagal afferent development. The identification of the functions of this peripheral NT-3 thus requires a detailed understanding of the localization and timing of its expression in the developing GI tract. We have studied embryos and neonates expressing the *lacZ* reporter gene from the *NT-3* locus and found that NT-3 is expressed predominantly in the smooth muscle of the outer GI wall of the stomach, intestines, and associated blood vessels and in the stomach lamina propria and esophageal epithelium. NT-3 expression has been detected in the mesenchyme of the GI wall by embryonic day 12.5 (E12.5) and becomes restricted to smooth muscle and lamina propria by E15.5, whereas its expression in blood vessels and esophageal epithelium is first observed at E15.5. Expression in most tissues is maintained at least until postnatal day 4. The lack of colocalization of β -galactosidase and markers for myenteric ganglion cell types suggests that NT-3 is not expressed in these ganglia. Therefore, NT-3 expression in the GI tract is largely restricted to smooth muscle at ages when vagal axons grow into the GI tract, and when vagal mechanoreceptors form in smooth muscle, consistent with its role in these processes and in vagal sensory neuron survival.

Keywords

Autonomic nervous system; Development; Gastrointestinal mucosa; Vagus nerve; Visceral afferents; Mouse

Introduction

Despite the significance of vagal GI afferents for the regulation of food intake, body weight, and digestive functions, little is known about its functional-anatomical organization, or about the way that the development of this complex organization is controlled. Among the few genes known to regulate aspects of the development these afferents are the netrins and their receptor termed deleted in colorectal cancer (DCC), which may attract vagal axons to the stomach and intestine (Ratcliffe et al. 2006), laminin, which may terminate this attraction (Ratcliffe et al. 2008), and neurotrophins, which are essential for the survival of all vagal afferents (for a

Correspondence to: Edward A. Fox, au_gc@psych.purdue.edu.

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review, see Fox 2006). Neurotrophin-3 (NT-3), acting in part through the activation of the receptor tyrosine kinase *trkC*, the high affinity receptor for NT-3 (Lamballe et al. 1991), is essential for the survival of a large proportion of vagal sensory neurons: *NT-3* and *trkC* homozygous mutants have 34%–47% and 14% loss of neurons from the nodosepetrosal ganglion complex, respectively (Ernfors et al. 1994; Farinas et al. 1994; Liebl et al. 1997; Tessarollo et al. 1997).

The population of vagal sensory neurons that require NT-3 for survival is different from those supported by the other neurotrophins (ElShamy and Ernfors 1997). The survival effects of neurotrophins on vagal sensory neurons have been suggested to follow an “organ-specific” principle with each organ regulating the survival of all the sensory receptor types that innervate an organ system (Brady et al. 1999). This is in contrast to the modality-specific principle that appears to guide neurotrophin survival effects on somatosensory neurons (Farinas 1999; Snider 1994). Consistent with the “organ-specific” principle, vagal sensory innervation of the esophagus appears to be dependent on NT-3, as the low threshold slowly adapting intraganglionic laminar ending (IGLE)-type mechanoreceptors that predominate in the muscle wall are reduced by 65% and 40% in *NT-3* and *trkC* heterozygous mutants, respectively (Raab et al. 2003). In contrast, the vagal sensory innervation of the small intestine appears to be dependent on NT-4, because homozygous NT-4 mutants have an almost complete loss of IGLEs from the small intestine, but normal IGLE density and structure in the stomach (Fox et al. 2001).

Several actions of NT-3, in addition to its effects on neuron survival, have been characterized in the sensory and sympathetic nervous systems and could also affect vagal sensory development: roles in neuronal differentiation (Ernfors 2001), axon growth (Genc et al. 2004; Tucker et al. 2001), and nerve terminal formation, including size and structure (Lentz et al. 1999; Ulupinar et al. 2000), degree of contact with their accessory cells, and survival of their accessory cells (Albers et al. 1996), receptor maintenance (Airaksinen et al. 1996), and neurotransmission (Arvanov et al. 2000; Oestreicher et al. 2000).

The effects of NT-3 on the survival of vagal sensory neurons, as reviewed above, and other potential effects of NT-3 on vagal afferent development might be mediated by its expression in the region of the developing nodose ganglion (Ernfors et al. 1992) or by NT-3 being present in embryonic and postnatal GI tract tissues innervated by vagal sensory neurons. Indeed, NT-3 expression has been reported in the mesenchyme of the embryonic avian gut (Le Douarin and Kalcheim 1999), at the mesenchyme-epithelium boundary in the upper esophagus of the mouse embryo at embryonic day 16.5 (E16.5; Patapoutian et al. 1999), and in the postnatal mouse colon (Lommatzsch et al. 2005). However, only the upper esophagus and colon have been examined in the mouse, and details of the locations and timing of NT-3 expression are lacking for all of these species and organs.

Nevertheless, NT-3 expression within GI tract tissues is probably involved in mediating NT-3 effects on development of the vagal sensory innervation of the GI tract. Consistent with this hypothesis, *trkC* and *trkB*, which can also be activated by NT-3 (Farinas et al. 1998; Huang et al. 1999), are expressed by nodose ganglion neurons from E13-E18 (Ernfors et al. 1992; Huang et al. 1999; Huber et al. 2000), and NT-3 undergoes retrograde transport in vagal sensory axons, although this has only been demonstrated in adult rats (Helke et al. 1998). These findings are significant, because neurotrophin effects on neuronal survival are thought to be dependent on the retrograde transport of the internalized neurotrophin receptor complex to the cell body (e.g., Grimes et al. 1997, 1996; but see MacInnis and Campenot 2002). Similarly, NT-3 has been proposed to be expressed in the intestinal wall and to mediate the survival and differentiation of a subset of enteric neurons (Chalazonitis et al. 2001).

Investigation of the roles of target-derived NT-3 in the development of the vagal sensory innervation of the GI tract or in the development of intrinsic and other extrinsic innervation of the GI tract requires a much more detailed understanding of the spatiotemporal pattern of NT-3 expression in the developing GI tract than is currently available. Therefore, our aims have been to identify those tissues/cell types that express NT-3 within the developing GI tract and to characterize the time course of this expression.

Materials and methods

Subjects

The *NT-3^{LacZ}* mouse (Farinas et al. 1994) obtained on an ICR out-bred background was backcrossed for up to 11 generations with C57BL/6 mice obtained from Harlan Industries (Indianapolis, Ind., USA) and bred in our lab. Mice were maintained at 23°C on a 14-h:10-h light:dark schedule with *ad libitum* access to tap water and Laboratory Rodent Diet 5001 (PMI Nutrition International, St. Louis, Mo., USA). All procedures were conducted in accordance with Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

Rationale for using NT-3^{LacZ} mice—Mice that had the *LacZ* reporter gene “knocked into” the *NT-3* locus (*NT-3^{LacZ}* mice; Farinas et al. 1994) and that thus expressed β -galactosidase under the control of the *NT-3* promoter were utilized to characterize NT-3 expression in the developing GI tract. The rationale for using *NT-3^{LacZ}* mice in such studies has been discussed extensively (e.g., Farinas et al. 1996; Vigers et al. 2000, 2003). Of particular relevance, *NT-3^{LacZ}* mice have revealed detailed NT-3 expression patterns in many different organs and tissues in numerous studies (Bennett et al. 1999; Farinas et al. 2001, 1996; Hess et al. 2007; Tojo et al. 1995; Vigers et al. 2000, 2003; Wilkinson et al. 1996; Yee et al. 2003). Moreover, these mice have yielded higher resolution cellular expression patterns than achieved with *in situ* hybridization (e.g., somatosensory system; Farinas et al. 1994; Jones et al. 1994). Moreover, the patterns of NT-3 expression obtained in these mice accurately reproduced the patterns derived by using methods that revealed endogenous expression (Farinas et al. 1996).

Tissue preparation

Matings were set up between heterozygous *NT-3^{LacZ}* mice or between heterozygous *NT-3^{LacZ}* mice and wild-type mice. Noon of the day that a copulatory plug was observed was designated E0.5, with the day of birth as postnatal day (P)0. Embryos were harvested on E12.5, E13.5, E15.5, and E17.5, and GI tracts were dissected from P4 mice. Although NT-3 might be expressed earlier (Bernd 2008), our study was restricted to ages between mid-gestation (E12) and P4 when vagal axons grow into the GI tract and their nerve terminals begin to differentiate (Murphy and Fox 2007; Ratcliffe et al. 2006; Xiang and Burnstock 2004). Results were based on staining patterns in three or more independent litters at each age studied, and multiple embryos or neonates from each litter were examined. Homozygous and heterozygous mutants and wild-types were genotyped by polymerase chain reaction (PCR) with DNA extracted from a tail sample for mice or from the yolk sac for embryos (PCR primer sequences: wild-type allele - forward: ACT ACG GCA ACA GAG ACG CTA C, reverse: ACA GGC TCT CAC TGT CAC ACA C; mutant allele - forward: GTG CCA GCG GGG CTG CTA AAG CGC, reverse: CTG CAT TCT AGT TGT GGT TTG TCC AAA CTC ATC).

Embryos—Pregnant mice were killed by cervical dislocation, and then the uterus was removed and placed in chilled phosphate-buffered saline (PBS) on ice. Each embryo was dissected free of its decidua and yolk sac, and then the skin and muscle enclosing the abdomen were peeled back to expose the abdominal organs. Embryos were subsequently fixed for 30

min on ice with 1% paraformaldehyde, 0.02% glutaraldehyde, 0.5 mM EGTA, and 2 mM MgCl_2 in 0.1 M sodium phosphate buffer, pH 7.4.

Postnatal mice—Neonatal mice were deeply anesthetized with a lethal dose of methohexital sodium (Brevital Sodium, King Pharmaceuticals, Bristol, Tenn., USA; 100 mg/kg i.p.). When mice were unresponsive to nociceptive stimuli, the abdomen and thorax were exposed, and the animals were perfused transcardially at a flow rate of 3 ml/min with 0.9% saline for 5 min at room temperature followed by the same fixative used for embryos for 30 min at 4°C.

Histochemical staining of β -galactosidase

Immediately after fixation, the embryos or abdominal organs were stained with X-gal as previously described (Fox 2000). After staining was completed, tissue was postfixed for 48 h in 4% paraformaldehyde at 4°C, washed with PBS, and then transferred to 10% buffered formalin at 4°C for a minimum of 5 days. The tissues were then embedded in paraffin, sectioned at a thickness of 8 μm , and air-dried on gelatin-coated slides. Alternate ribbons of sections were counterstained with 0.1% neutral red, and all sections were dehydrated in a series of graded alcohols (70%, 95%, 2 $\times$ 100%; 2 min each), cleared in xylene (3 $\times$ 2 min), and coverslipped with Cytoseal (Richard Alan Scientific, Kalamazoo, Mich., USA). Additionally, immunohistochemical (IHC) detection of β -galactosidase in the E17.5 gut by using the methods described below was employed to validate the results obtained by X-gal staining.

Antibody characterization

Information on primary antibodies used in mouse embryonic tissues, including immunogens, is summarized in Table 1. Additional information on controls for specificity of each of these antibodies is provided below.

The β -galactosidase antibody employed was produced in guinea pigs, and its specificity was characterized by Yee et al. (2003). No immunoreactivity was detected after applying the antibody to mouse lingual tissue that did not contain β -galactosidase. Moreover, application of this antibody to tissues from several LacZ transgenic strains with tissues expressing β -galactosidase resulted in strong immunoreactivity. Additionally, when the β -galactosidase immune sera were preadsorbed with β -galactosidase, no immunoreactivity was detectable after its application to β -galactosidase-expressing mouse lingual tissue. In the present study, this antibody also showed complete correspondence with the X-gal staining of β -galactosidase expression in developing gastric and intestinal smooth muscle, esophageal epithelium, and gastric lamina propria.

Goat polyclonal anti-c-Kit (lot numbers H2907, C2108) was raised against a peptide from the carboxy terminus of mouse c-Kit. The molecular weight of precursor c-Kit is 120 kDa, and the mature form is 145 kDa. In Western blots of lysates (some immunoprecipitated) from several cell lines (manufacturer's technical information; Jahn et al. 2002) and cultured fetal cells (Nobuhisa et al. 2003), the antibody stained both the precursor and mature bands. Moreover, immunostaining by using this antibody on tissue sections was prevented by pre-incubation of the antibody with c-Kit blocking peptide (Schrans-Stassen et al. 1999). Additionally, this antibody has been used to label cells in the wall of the GI tract of the mouse, rat, and guinea pig with distributions and morphologies consistent with those of the several classes of interstitial cells of Cajal (ICC; Fox et al. 2000; Ho et al. 2003; Pham et al. 2002).

The mouse IgG2b neuronal protein HuC/HuD (HuC/D) monoclonal (clone 16A11) antibody (lot number 42804A) recognizes an epitope within the carboxy terminal domain of HuD, resulting in the labeling of neuronal cell bodies and nuclei (Marusich et al. 1994). This antibody recognizes neuronal proteins of the Elav family, including HuC and HuD, which are RNA-

binding proteins, and Hel-N1. The presence of Hu proteins acts as a marker for both newly committed postmitotic neurons and mature neurons (Barami et al. 1995; Marusich et al. 1994; Young et al. 2005). The staining pattern of cellular morphology and distribution observed in the enteric nervous system in the present study was the same as that previously described (Lin et al. 2002; Murphy et al. 2007; Young et al. 2005). Importantly, the staining pattern of biotin-conjugated HuC/D monoclonal antibody was identical to that of the unconjugated antibody (Hoff et al. 2008).

The polyclonal rabbit antibody directed against p75 (lot number 606031680), the low affinity neurotrophin receptor, was raised against the extracellular fragment specified by exon 3 sequences of the murine p75 gene (manufacturer's technical information). The specificity of this antibody was originally characterized by Huber and Chao (1995), and in Western blots, it labeled a single band of about 75 kDa in brain (Huber and Chao 1995), embryonic testis (Campagnolo et al. 2001), and importantly, GI myenteric plexus (Lin et al. 2005). In the present study, the morphology and distribution of immunoreactivity produced with anti-p75 was consistent with glial and neural precursors and with staining patterns produced by the same antibody in similar preparations (Lin et al. 2005; Vannucchi and Fausone-Pellegrini 2000; Yan and Keast 2008).

The polyclonal rabbit antibody directed against the established glial marker S100 (lot number 15317) stains the cytoplasm, labeling glial somata and processes. Specificity was demonstrated by two-dimensional immunoelectrophoresis, which identified one distinct double peak in human and cow brain extracts corresponding to S100a and -b and found no reaction with human plasma or cow serum (manufacturer's technical information). Further, an indirect enzyme-linked immunosorbent assay (ELISA) indicated that anti-S100 did not react with human plasma or cow serum.

IHC detection of sites of NT-3 expression (β -galactosidase protein)

IHC co-localization of an antibody that detects *LacZ* expression (β -galactosidase) with an antibody selective for neurons (anti-HuC/D), glia (anti-s100), undifferentiated neural crest precursors (anti-p75), or ICCs (anti-c-Kit) in heterozygous *NT-3^{lacZ}* embryos was utilized to determine whether NT-3 expression occurred in these cell types. Tissue sections from wild-types were stained in parallel to examine the specificity of the β -galactosidase antibody. Embryos harvested at E17.5 as described above were immersion-fixed in 4% paraformaldehyde at 4°C for 24 h, rinsed in PBS, incubated in 15% sucrose in PBS for 1 h at 4°C, followed by 30% sucrose overnight at 4°C and then a 1:1 mixture of 30% sucrose and OCT compound (Tissue-Tek, Miles) overnight at 4°C, and finally frozen in 100% OCT by using liquid nitrogen. Sections were cut at a thickness of 10 μ m and air-dried on gelatin-coated slides.

IHC procedures—All steps were performed at room temperature unless otherwise indicated. Tissue sections were washed in PBS, incubated for 1 h in blocking solution (10% normal donkey serum, 0.5% Triton X-100, 2% bovine serum albumin [BSA], 0.1% sodium azide), and then incubated overnight in the first primary antibody at 4°C. These and all other primary and secondary antibodies employed in the present experiments were diluted with 1% BSA, 2% normal donkey serum, 0.3% Triton X-100, and 0.1% sodium azide. After being washed with PBS, sections were incubated in the first secondary antibody for 2 h and then washed again with PBS. Next, the sections were incubated for 1 h in blocking solution and then in the second primary antibody, overnight, at 4°C. Sections were washed again in PBS and then incubated in the second secondary antibody for 2 h. After being washed in PBS, sections were mounted in glycerol, coverslipped, and sealed with nail polish. The secondary antibodies used included donkey rhodamine red X (RRX)-conjugated anti-goat or anti-guinea pig IgG (H+L) and donkey

fluorescein isothiocyanate (FITC)–conjugated anti-rabbit, anti-goat, or anti-guinea pig IgG (H +L; Jackson ImmunoResearch Laboratories). The mouse monoclonal HuC/D antibody employed was tagged with biotin, which avoided the use of an anti-mouse secondary antibody and the associated high background. To label this antibody, streptavidin tagged with tetramethyl rhodamine isothiocyanate (TRITC) or FITC was applied in the same manner as described for secondary antibodies. To control for antibody interactions, the majority of staining runs for each pair of primary antibodies included several sections that were taken through the entire double-staining protocol, but omitting the first primary antibody on some sections and the second primary on other sections (incubation in primary antibody diluent alone was substituted). Further, in order to control for non-specific staining by secondary antibodies, staining produced by each primary antibody alone was compared with staining following the omission of that primary antibody (incubation in primary antibody diluent alone was substituted), with all other protocol steps being kept constant. Finally, to control for non-specific staining by anti- β -galactosidase, it was tested on sections of wild-type specimens.

Microscopy and photomicrography

X-gal-stained tissue was examined with standard bright-field or differential interference contrast illumination, and rhodamine and fluorescein fluorescence was visualized by using standard filter sets (Leica DM5000 microscope; fluorescence filter cubes L5 and Y3, respectively). Photomicrographs were acquired directly with a video camera (Spot RT Slider; Diagnostic Instruments, Sterling Heights, Mich., USA). The fluorescently stained tissues were also scanned with an Olympus BX-DSU spinning disk confocal microscope operated with Slidebook software (v.4.1, Intelligent Imaging Innovations, USA). Image processing, three-dimensional reconstructions, and two-dimensional projections of z-series stacks of optical sections were also performed with the Slidebook software. Standard fluorescence microscopy was utilized to identify regions of tissue sections with potential for the co-localization of two different primary antibodies based on the apparent overlap of their respective rhodamine or fluorescein labels. Regions to be imaged were randomly selected, and then a series of confocal scanning optical sections through the z-axis of each region was collected. The series of sections collected at each site was examined to determine whether both labels were present in the same tissue elements. Three criteria were employed to determine co-localization: (1) in composite images of the FITC and TRITC or Texas Red channels, elements exhibited a yellow pseudocolor as a result of the combined green pseudocolored FITC channel and red pseudocolored TRITC or Texas Red channel, (2) the separate green- and red-labeled elements were in focus as determined by scanning through the z-series, and (3) the shapes of the green- and red-labeled elements exhibited partial or complete matching of in focus tissue elements. Photoshop software (version 6.0 Adobe Systems, Mountain View, Calif., USA) was used to apply scale bars and text, adjust brightness and contrast, apply color correction, and organize the final layouts for printing.

Results

β -Galactosidase expression was localized by using X-gal staining of the GI tract of embryonic and neonatal *NT-3^{lacZ}* mice, which expressed β -galactosidase under control of the *NT-3* promoter. Additionally, IHC detection of β -galactosidase in these mice at E17.5 was employed to validate the findings obtained with X-gal staining. This age was chosen because *LacZ* expression levels appeared to be high in most tissues, and all tissues had developed sufficiently to be clearly distinguished. β -Galactosidase expression was observed in embryonic tissues previously reported to express NT-3, including the ventral motor neurons within the spinal gray matter and hair follicles in the skin, suggesting that the accurate regulation of expression had been maintained in our line of *NT-3^{lacZ}* mice (not shown; Botchkarev et al. 2004; Buchman and Davies 1993; Ernfors et al. 1991). High levels of X-gal staining were apparent in whole-

mounts at one or more of the ages examined (E12.5, E13.5, E15.5, E17.5, and P4) in all the major GI organs studied, including the esophagus, stomach, pylorus, small intestine, cecum, and large intestine. No stain was observed in the GI tracts of wild-type embryos at ages E12.5–E15.5, but staining was present in the mucosa at many levels of the GI tract at E17.5 and P4, suggesting that the staining of this tissue in E17.5 and P4 transgenic specimens was artifactual. Artifactual X-gal staining attributable to endogenous enzymes with similar activity to the bacterial galactosidase has been reported in several tissues, including the adult mammalian intestinal mucosa (Coates et al. 2001; Lojda et al. 1974; Sanchez-Ramos et al. 2000; Weiss et al. 1999). Consistent with this interpretation, the pattern of NT-3 expression (X-gal staining) in *NT-3^{lacZ}* embryos as detailed below, was replicated in all tissues at E17.5 by β -galactosidase-like immunoreactivity (LIR), except for the mucosa of the stomach and intestines (Figs. 1e, 2a–c). This method also failed to label the mucosa of these organs in wild-type embryos. An additional implication of these findings obtained via IHC detection of β -galactosidase was that the masking of actual mucosal NT-3 expression by the artifactual X-gal staining of this tissue was unlikely.

The results in Table 2 summarize the localization of non-artifactual β -galactosidase expression at the five ages studied in detail: E12.5, E13.5, E15.5, E17.5, and P4.

NT-3 expression in the esophagus

No NT-3 expression was detected in the esophagus at E12.5 or E13.5. Moderate expression was first observed at E15.5 and was restricted to the epithelium (Fig. 1a). At E17.5, epithelial NT-3 expression was present throughout most of the length of the esophagus (Fig. 1b) but increased from moderate levels in the region of the lower esophageal sphincter to high levels in more anterior regions of the esophagus, whereas most of this expression at P4 was at high levels (Fig. 1c). Additionally, NT-3 expression in the circular muscle of the forestomach (see below) extended into the region of the developing lower esophageal sphincter (Fig. 1d). No other esophageal tissues exhibited NT-3 expression at any of the ages examined.

NT-3 expression in the stomach

The wall of the stomach in E12.5 embryos consisted of a thick layer of mesenchyme surrounding the single-cell-thick epithelial layer (Fig. 3a, b). NT-3 expression was present in the entire thickness of this mesenchyme but was stronger in the anterior portion of the caudal stomach region that develops into the corpus and antrum. In contrast, no expression was detected in the epithelium. A similar expression pattern was observed at E13.5, although the tissue layers of the stomach wall in some specimens had begun to differentiate, and NT-3 was expressed in those layers that develop into the lamina propria and circular and longitudinal smooth muscle (Fig. 3c, d). By E15.5, all of the layers of the stomach wall had become distinct in all specimens. Strong NT-3 expression at this age was restricted to the longitudinal and circular smooth muscle layers with the strongest expression in the antrum occurring on its anterior side (Fig. 3e, f, 4a, b). Additionally, expression in the forestomach was restricted to the circular layer of the muscle wall. Expression was also present in the lamina propria of the mucosa of the antrum and corpus but was consistently weaker than in the muscle layers. This overall expression pattern in the stomach wall was maintained at E17.5 and P4 (Figs. 4c, d, 5a–e) except that expression was slightly increased in the lamina propria on E17.5 and appeared to decline slightly in the muscle layers postnatally. No clear expression was detected in the submucosa, epithelium, or enteric plexuses at any of the ages examined.

NT-3 expression in the pylorus

The spatial and temporal patterns of NT-3 expression in the pylorus largely paralleled that of the adjacent caudal portion of the stomach. As early as E12.5 and E13.5, NT-3 expression was present in the mesenchyme of the pylorus, being stronger in the anterior portion of the wall.

Interestingly, this strong expression extended into the ventral mesogastrium (mesenchyme) immediately external to the pylorus and extended over short distances in the rostral direction (adjacent to the caudal stomach) and in the caudal direction (alongside the proximal duodenum). At later stages (E15.5 and E17.5), strong expression became concentrated in the external smooth muscle layers (Fig. 4a–d). In particular, the expression levels were high through the entire width of the pyloric muscle wall, which was predominantly a thick circular muscle layer. Postnatally (P4), this tissue began to show a decline in expression levels. Additionally, the weak expression that occurred in the lamina propria of the antrum extended into the pylorus and exhibited a similar time course. The only tissues that did not exhibit clear expression at any of the ages studied were the epithelium, submucosa, and enteric plexuses.

NT-3 expression in the small intestine

The NT-3 expression present throughout the thickness of the muscle wall in the caudal stomach and pylorus became restricted to the circular smooth muscle layer in the duodenum, an expression pattern that was dominant for the muscle wall in all the compartments of the small intestine. Expression in the small intestine muscle was first observed at E12.5 and, at this age and E13.5, was present in the mesenchyme or in the developing external muscle layers where it occurred predominantly in the circular muscle layer (Fig. 6). By E15.5, this expression was restricted to the circular muscle layer (Fig. 7a, b), and it remained so at E17.5 and P4, although the expression levels began to decline by P4 (Figs. 7c, d, 8). Tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, and enteric plexuses.

NT-3 expression in the cecum

The expression pattern that dominated in the cecum was similar to that in the small intestine. In most specimens, NT-3 expression was observed only in the circular layer of the muscle wall; this was stronger in the body of the cecum near its junctions with the large and small intestines and gradually became weaker and typically disappeared toward the apex of the cecum. The expression pattern near the junction region of the cecum and large intestine was, in some specimens, similar to that of the colon (see below); expression occurred in both smooth muscle layers but was typically weak in the longitudinal layer. This overall expression pattern was observed at E15.5 (Fig. 9a, b), E17.5 (Fig. 9c, d), and P4. At E12.5 and E13.5, the cecum was difficult to identify unequivocally in sections. The tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, enteric plexuses, and most of the longitudinal muscle layer.

NT-3 expression in the large intestine

In contrast to the pattern of NT-3 expression observed in the small intestinal muscle layers, the pattern of labeling in the large intestinal muscle layers at all ages examined was more similar to that observed in the stomach. At early ages, viz., E12.5 and some E13.5 specimens, the muscle layers were not differentiated, and NT-3 expression appeared diffuse throughout the mesenchyme (Fig. 6a–d, Supplementary Fig. 1a, b), whereas in other E13.5 embryos, it was mostly restricted to both layers of the forming muscle wall (Supplementary Fig. 1c–f). By E15.5 and at later stages, expression was present at high levels in the circular muscle and varied from undetectable to high levels in the longitudinal smooth muscle layer (Fig. 10, Supplementary Figs. 2, 3). Tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, and enteric plexuses.

NT-3 expression in tissues associated with abdominal GI organs

The smooth muscle of some mesenteric blood vessels exhibited NT-3 expression by E15.5 (Fig. 11a, b); by E17.5 (Figs. 9c, d, 11c, d) and P4, expression was also present in the smooth muscle of blood vessels that supplied the stomach (Figs. 2a, 5b, c) and liver, those that exited

the mesentery to supply the intestines (Fig. 8), and those within one of the liver lobes adjacent to the stomach. Interestingly, NT-3 expression was also observed in a thin layer at the edge of the liver lobe that was immediately anterior to the stomach/pylorus; this was observed at all ages examined (e.g., Fig. 3a, b). Moreover, at early stages, this portion of the liver was continuous with the ventral mesogastrium adjacent to the caudal stomach, which also exhibited NT-3 expression (see above).

NT-3 expression in enteric nerve plexuses

Based on an examination of the X-gal-stained tissues described above, no indication of significant NT-3 expression was found within the neurons and glial cells associated with the submucosal plexus in any of the organs studied. Moreover, no patterns of staining within the muscle walls of the organs examined were suggestive of myenteric ganglion structure. Nevertheless, small numbers of labeled cells in the myenteric plexus might have been obscured by the intense expression in the immediately adjacent smooth muscle layers. This possibility was examined by employing IHC double-labeling to determine whether markers for neurons (anti-HuC/D) or glial cells (anti-S100) or their precursors (anti-p75) were associated with NT-3 expression (anti- β -galactosidase). No evidence was found for NT-3 expression in any of these cell types in the myenteric plexus, as clear instances of double-labeled cells were not observed (Table 3; for example, see Fig. 12). All primary antibodies employed produced similar staining patterns when applied alone or sequentially with a second primary antibody. Additionally, none of the secondary antibodies produced non-specific labeling when the primary antibodies were omitted. The guinea pig anti- β -galactosidase applied to sections from wild-types produced weak non-specific β -galactosidase-LIR in the muscle wall of the esophagus, the circular muscle of the lower esophageal sphincter, and some regions of the stomach circular muscle. However, β -galactosidase-LIR in the last two mentioned structures in *NT-3^{lacZ}* mice was significantly brighter, and these tissues were clearly stained by X-gal in *NT-3^{lacZ}* specimens, but not in wild-types.

Discussion

Spatiotemporal pattern of NT-3 expression

Each GI organ studied exhibited NT-3 expression at one or more of the ages examined. The spatial pattern of NT-3 expression was characterized by two principles. First, NT-3 expression in the majority of the GI tract organs, including the stomach, small intestine, cecum, and large intestine, was smooth-muscle-specific: it was largely restricted to smooth muscle cells comprising the outer layers of the developing stomach, cecum, and intestines and the walls of blood vessels supplying the GI tract. The one exception to this principle was in the lamina propria of the stomach mucosa, although expression levels in this tissue appeared to be lower than those in the smooth muscle. Second, each organ investigated exhibited a unique expression pattern that differed from others in terms of which muscle layer exhibited NT-3 expression, and whether any additional tissues exhibited expression. For example, although NT-3 was expressed in the circular muscle layer of the stomach, intestines, and cecum, expression in the longitudinal muscle layer only occurred in some regions of the stomach and colon, thus distinguishing the expression patterns in these organs from those of the small intestine and cecum. Further, the lamina propria of the mucosa only exhibited expression in the stomach, thereby distinguishing the stomach pattern from that of the colon. NT-3 expression in the esophagus also exhibited a dramatically different pattern from all the other GI organs, being restricted to the mucosal epithelium.

Some differences in the temporal pattern of NT-3 expression appeared to occur across and within tissues. However, these observations must be interpreted cautiously because the persistence of β -galactosidase protein in the various stained tissues is not known (its half-life

in neurons is about 8 h; Pham et al. 1999). NT-3 expression in the mesenchyme of the GI wall was present at the earliest age examined, viz., E12.5, and started to become distinct in tissue layers developing into the smooth muscle and lamina propria at E13.5. In contrast, NT-3 expression in vascular smooth muscle and the esophageal epithelium was first observed at E15.5. Moreover, as early as E12.5, expression in outer gut wall smooth muscle appeared to be at high levels, whereas initially, at E15.5, expression in the vascular smooth muscle and esophageal epithelium was at low levels that increased gradually with age. Additionally, NT-3 expression was maintained in most of these tissues at least until P4, although levels in some tissues had begun to decline.

The present characterization of embryonic and neonatal NT-3 expression in the alimentary tract is consistent with the extremely limited information available from previous studies. *NT-3* mRNA has been demonstrated by in situ hybridization in the major thoracic and abdominal elastic vessels and their branches from the mid-fetal age to adulthood in the rat (Scarisbrick et al. 1993) and NT-3 protein by ELISA in the colon after birth (Lommatzsch et al. 2005). Moreover, one sentence describing unpublished work states that “*NT-3* mRNA can be found in the gut mesenchyme” (Le Douarin and Kalcheim 1999) and one image of mouse upper esophagus at E16.5 (Fig. 2f in Patapoutian et al. 1999) is accompanied by the statement: “Many of the tissues expressing *NT-3*, apart from being targets of axons, are also sites of epithelial-mesenchymal boundaries. These domains of expression include ... the digestive system, and arteries”. Finally, non-neural crest cells derived from rat intestine in vitro seem to secrete NT-3 (Chalazonitis 1996). Our results extend these findings by clearly demonstrating the outer smooth muscle as the major source of NT-3 in several organs of the developing GI tract. Additionally, we have identified, for the first time, low NT-3 expression levels in the embryonic and early postnatal mucosa of the stomach antrum and corpus and moderate to strong NT-3 expression levels in the walls of blood vessels supplying the GI tract. Previously, NT-3 expression had been observed in smooth muscle cells of blood vessels of the cardiovascular system, major abdominal vessels (Scarisbrick et al. 1993), and vessels associated with the stellate and superior cervical ganglia and their target organs (Francis et al. 1999). The sensitivity of the X-gal stain combined with its greater cellular resolution as compared with in situ hybridization may have permitted the visualization of sites such as the lamina propria in which expression levels are lower than in the mesenchyme or smooth muscle.

Since a subpopulation of myenteric neurons is dependent on NT-3 for differentiation and survival (Chalazonitis et al. 2001), and since small numbers of myenteric neurons have been observed to contain NT-3 in the mature GI tract of the rat (De Giorgio et al. 2000), we have investigated whether these neurons express NT-3 during development. However, the region of the GI tract wall that contains the myenteric plexus and is situated between the longitudinal and circular smooth muscle layers does not exhibit X-gal staining or β -galactosidase immunolabeling in *NT-3^{LacZ}* mice at any of the ages examined. These observations suggest that none of the myenteric plexus elements express NT-3, including the myenteric neurons, glial cells, undifferentiated neural crest cells, and ICC at the myenteric plexus (ICC-MYs). Consistent with this interpretation, immunolabeling for β -galactosidase at E17.5 does not co-localize with markers for any of the myenteric elements (HuC/D, S100, p75, or c-Kit).

Implications of spatiotemporal pattern of NT-3 expression

One interesting aspect of the various spatial patterns of NT-3 expression across the GI organs is that some of the organs or their compartments, including the small intestine and forestomach, exhibit expression in the circular muscle layer, but not the longitudinal layer, whereas the remaining organs/compartments show expression in both layers, a pattern that is highly consistent across specimens and ages. Developmental, structural, and functional differences between the longitudinal and circular muscle layers could be causally related to this expression

pattern (El-Yazbi et al. 2007; Shuba et al. 2006; Spencer et al. 2006; Takahashi et al. 1998). One particularly intriguing difference involves the localization of precursors of the classes of ICC, which depend on c-Kit and steel factor for proliferation during development. These include the intramuscular class (ICC-IM) of the forestomach and the ICC-MY of the small intestine (Burns et al. 1996; Fox et al. 2002; Huizinga et al. 1995; Ward et al. 1994). In the small intestine, these classes of ICC arise from mesoderm-derived precursor cells that are present in the longitudinal muscle layer: those that initially express c-Kit and smooth muscle myosin heavy chain (smmhc), and those that later (between E14.5 and E18.5) either downregulate smmhc and become ICCs or down-regulate c-Kit and become smooth muscle cells (Kluppel et al. 1998; Torihashi et al. 1997). Initially, c-Kit is also expressed in the circular muscle but is downregulated before precursors in the longitudinal layer begin to diverge toward ICC or smooth muscle fates. This raises the possibility that *NT-3* or a co-expressed gene suppresses c-Kit expression in circular smooth muscle precursor cells, resulting in the loss of their potential to differentiate into ICCs.

The most dramatic difference in the patterns of NT-3 expression is between the esophagus and the more distal GI organs, expression being restricted to the epithelium in the esophagus rather than to the external smooth muscle layers. This localization is consistent with previous observations of NT-3 expression at the mesenchyme-epithelium boundary in the upper esophagus at E16.5 (Patapoutian et al. 1999). The difference in NT-3 expression patterns suggests that the genetic program that regulates esophagus development is largely distinct from those for the more distal GI organs, consistent with additional differences in esophageal development as compared with the other GI organs. For example, a greater proportion of the neural crest cells that migrate to the esophagus originate from the trunk as opposed to the hindbrain (Durbec et al. 1996), and only the esophageal smooth muscle transdifferentiates into striated muscle (Kablar et al. 2000; Patapoutian et al. 1995; but see Rishniw et al. 2003). The role of NT-3 in the esophageal epithelium may be involved with the survival or development of its innervation or possibly with the survival or renewal of epithelial cells, which exhibit frequent turnover.

The lack of NT-3 expression in the muscle wall of the abdominal and thoracic esophagus between E13 and P4 appears inconsistent with earlier evidence suggesting the loss of vagal sensory neurons in *NT-3*-deficient mice, which would include the loss of esophageal IGLEs (Raab et al. 2003). However, taken together these results imply that the loss of these IGLEs is attributable to NT-3 deficiency in the region of the nodose ganglion, rather than to loss from the target organ (ElShamy and Ernfors 1997). The lack of NT-3 expression in the outer smooth muscle layers of the esophagus further implies that, once the axon terminals of IGLE vagal mechanoreceptors reach the esophagus during development, which begins around E15 (Sang and Young 1998), their survival and differentiation is supported by factors other than NT-3. However, the upper esophagus has not been examined at E17.5 and P4, and thus, NT-3 expression in striated muscle at this esophageal level cannot be ruled out.

GI tract tissues and cell types that potentially utilize secreted NT-3

The main sources of NT-3 in the GI tract appear to be the outer smooth muscle layers of the GI wall and smooth muscle of blood vessel walls. Thus, these tissues or any cells with processes in close apposition to these tissues might utilize NT-3. A key requirement for such cells would be that they express the appropriate trk receptors, and possibly p75, during development to allow them to “transduce” the NT-3 signal.

Expression of NT-3 in vascular smooth muscle cells might support autocrine function. Consistent with this possibility, trk receptors, including trkC, are present in vascular smooth muscle cells and appear to mediate autocrine functions that are known to be regulated by neurotrophins such as cell migration and survival (Donovan et al. 1995). Additionally, NT-3

produced by vascular smooth muscle cells has been shown to have paracrine effects. For example, it provides an intermediate-target signal that promotes the growth of sympathetic axons in close proximity to blood vessels by activating trkA receptors on those axons and enabling them to reach their final target (Francis et al. 1999; Kuruvilla et al. 2004). These axons include those innervating the stomach and duodenum, axons that are reduced in density in *NT-3* knockout embryos. The potential for NT-3 produced in blood vessel walls to regulate vagal sensory axon development has not been directly examined, but since vagal axons also grow along blood vessels to reach and enter the gut wall, the expression pattern that we have observed is suggestive.

Since trk receptors have not been observed in smooth muscle cells of the developing GI wall (e.g., Lamballe et al. 1994; Sternini et al. 1996), NT-3 is probably secreted from these cells and has a paracrine role. Consequently, developing vagal and sympathetic extrinsic innervation and enteric intrinsic innervation of the outer muscle layers of the gut wall would have access to this source of NT-3.

Evidence is available that is consistent with enteric neuron uptake of NT-3 secreted from the muscle layers. In particular, a subpopulation of enteric neurons is dependent on NT-3 for survival and differentiation (Chalazonitis et al. 2001), and these neurons express trk receptors and p75 from mid-gestation to birth (Holmberg et al. 2001; Lamballe et al. 1994; Sternini et al. 1996; Sundqvist and Holmgren 2004). Vagal afferents that innervate the GI wall and are dependent on NT-3 for survival might also take up and utilize NT-3 secreted from the muscle layers, as they express trk receptors, including trkC, from mid to late gestation (Ernfors et al. 1992; Huang et al. 1999; Huber et al. 2000).

Implications of NT-3 expression pattern for organ-specific hypothesis

At face value, the pattern of NT-3 expression does not appear to support the organ-specific hypothesis of neurotrophin survival action on sensory neurons. In particular, NT-3 expression is distributed throughout similar tissues in several GI organs. If the function of NT-3 is similar in each of these organs, this would clearly be a violation of the principle. However, if NT-3 has different functions in each organ, then the survival function of NT-3 might be organ-specific. For example, NT-3 might be involved in the survival of vagal afferent innervation of one GI organ, have an effect on axon growth or guidance in another GI organ (Genc et al. 2004; Niwa et al. 2002; Paves and Saarma 1997), and act on receptor differentiation in yet another (Ming et al. 1997).

Many levels of the vagal sensory system are associated with potential sources of NT-3, including their central targets in the brainstem, the nodose ganglion in which their cell bodies are located, the blood vessel walls that their axons follow on their way to the gut, and within the gut itself. One way to distinguish the roles of each NT-3 source would be to manipulate one source at a time in vivo and to examine the effects on vagal development, for example, by using the cre-lox conditional knockout strategy (Sauer 1998). The present analysis represents a prerequisite for this strategy, which is to identify the ages at which this expression occurs and the cell types that express NT-3 in and near the target organs of vagal GI afferents.

Regulation of NT-3 expression pattern in developing GI tract

Little is known about the regulation of neurotrophin expression in the gut during development. A comparison of NT-3 expression patterns across organs has identified differences that must be accounted for by a regulatory scheme. For instance, as summarized earlier in this discussion, each GI organ exhibits a unique pattern of NT-3 expression.

Evidence is beginning to accrue suggesting that *Hox* genes play a key role in the gene cascade or genetic program that regulates the formation of the various GI organs from a relatively uniform tube. Therefore, these *Hox* genes are candidates for contributing to the regulation of organ-specific patterns of NT-3 expression. In compartments of the developing insect cuticle (segments) and the vertebrate hindbrain (rhombomeres), *Hox* genes exhibit overlapping nested expression patterns with unique anterior expression boundaries that coincide with the locations at which compartment boundaries later form (Lewis 1978; McGinnis and Krumlauf 1992). The result is a unique combination of *Hox* genes being expressed in each compartment; this acts as a code that confers the compartment's identity. The *Hox* proteins present in each compartment activate the expression of numerous genes that produce patterns of cell proliferation and migration that contribute to the formation of the unique structures present with each segment.

More recently, a similar relationship between *Hox* gene expression patterns and individual GI organs has been reported to occur by approximately E10, presaging the locations at which the boundaries between organs form (Kawazoe et al. 2002; Pitera et al. 1999; Sekimoto et al. 1998). Thus, should these organ-specific *Hox* gene expression patterns provide a code that confers identity to individual gut segments, they could directly or indirectly contribute to the organ-specific NT-3 localization patterns that occur in the GI tract. Moreover, some evidence exists that these expression patterns are maintained into maturity and determine the various identities of stem cell populations in each mature organ (Yahagi et al. 2004).

Consistent with a role for *Hox* genes in gut pattern formation, manipulations of *Hox* expression can alter the developmental trajectory of GI organ development and the location of organ boundaries. For example, ectopic expression of *Hoxc8* in the mouse stomach leads to an overgrowth of stomach epithelium and extension of the duodenum to the anterior pylorus (Pollock et al. 1992). Moreover, ectopic expression of the *IDX1* homeobox gene results in the alteration of the midgut-hindgut boundary and failure of the cecum to form (Heller et al. 1998).

Another potentially important facet of *Hox* expression in the developing gut is that the expression of many *Hox* genes is restricted to the mesoderm, suggesting the *Hox* code functions primarily in mesenchyme specialization and then leads to regional differentiation of gut subdomains through epithelial-mesenchyme interactions (Kawazoe et al. 2002). This regional differentiation might involve the activation of NT-3 expression in the mesenchyme and smooth muscle locations that we have observed. Further, some *Hox* genes are expressed in distinct mucosal tissues (Pitera et al. 1999) and therefore might control the differential NT-3 expression observed in them. For example, *Hoxc4* is the only gene among the *Hoxc* and *Hoxd* paralogs examined that is expressed in the endoderm of the esophagus at E12.5, the endoderm giving rise to the epithelium (Pitera et al. 1999). Thus, *Hoxc4* might activate the NT-3 expression observed in this tissue.

Hox genes continue to be expressed at ages when NT-3 is expressed and therefore could activate and maintain gut NT-3 expression directly. Indeed, evidence exists for direct *Hox/NT-3* interactions in other systems. For example, *Barhl1*, a homeobox gene, and NT-3 exhibit similar expression patterns in association with cerebellar granule cell development. Further, knockout of either gene or of NT-3 receptors has similar deleterious effects on granule cell migration and survival during development (Li et al. 2004). Moreover, the 5' flanking sequences of the NT-3 gene have been found to contain at least two potential *Barhl1* homeodomain-binding sites. Similarly, evidence has been presented that a neurotrophin closely related to NT-3 (brain-derived neurotrophic factor), which is also expressed in gut-associated smooth muscle (Fox 2006), is regulated by the homeobox genes *Pitx3* and *Hoxa1* (Martinez-Ceballos et al. 2005; Peng et al. 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Photomicrographs demonstrating the restriction of NT-3 expression in the wall of the esophagus to the epithelium shown here at three different stages of development: E15.5 (**a**), E17.5 (**b**, **e**) and P4 (**c**, **d**). These cross sections through the thoracic (**a**, **b**) and abdominal (**c**–**e**) esophagus were reacted with X-gal, which gave a blue stain in cells expressing β -galactosidase (*bv* blood vessel, *ep* epithelium, *mw* muscle wall). In a longitudinal section through the P4 esophagus and lower esophageal sphincter and stained with X-gal and neutral red (**d**), NT-3 expression in the circular muscle of the forestomach extended into the lower esophageal sphincter (*les*). A confocal microscope image of an oblique section through the abdominal esophagus at E17.5 (**e**) was immunostained for β -galactosidase. β -galactosidase-like immunoreactivity (LIR) was restricted to the epithelium, thus confirming the X-gal staining of this tissue layer. The weaker staining in the outer muscle layer was tissue autofluorescence, because it also occurred when the primary antibody was omitted, and it was not stained by X-gal (**a**–**c**). Bar 100 μ m (**c**, applies to **a**–**d**), 100 μ m (**e**)

Fig. 2.

NT-3 expression, detected as β -galactosidase-LIR, confirmed the detection of β -galactosidase protein with X-gal staining as observed in the smooth muscle layers of the GI organs and some blood vessels (see Fig. 1 and subsequent figures for examples of X-gal-staining patterns). In contrast, X-gal staining observed within the mucosa/submucosa at E17.5 and P4 was not confirmed by β -galactosidase-LIR in any of the organs studied, with the exceptions of the esophageal epithelium (Fig. 1e) and weak staining in the lamina propria of the stomach antrum and corpus (a). Confocal images of examples of the β -galactosidase-LIR in smooth muscle shown here include (a) the stomach and a GI blood vessel (*bv* blood vessel, *cm* circular muscle, *lm* longitudinal muscle, *lp* lamina propria, *mp* myenteric plexus), (b) the small intestine (*vi* villi), and (c) the large intestine (*muc* mucosa). Bar 100 μ m (a, also applies to b, c)

Fig. 3. NT-3 expression patterns in the stomach wall at E12.5 (**a, b**), E13.5 (**c, d**), and E15.5 (**e, f**). Photomicrographs of X-gal-stained stomach sections (**a, c, e**) side-by-side with matching sections counterstained with neutral red (**b, d, e**). Initially, at E12.5, NT-3 was expressed in the mesenchyme, and by E13.5, as this tissue began to differentiate, strong expression became largely restricted to the smooth muscle layers, with weaker expression limited to the lamina propria (*li* liver, *mes* mesentery, *sub* submucosa; other abbreviations as in Figs. 1, 2). In contrast, no expression was seen in the epithelium (especially clearly shown by comparison of *insets* in **a, b**, which are regions of **a, b** shown at higher magnification, viz., $\times 1.6$). By E15.5,

most tissue layers could be clearly and consistently identified, and NT-3 expression was completely restricted to the smooth muscle layers and lamina propria. *Bars* 100 μm

Fig. 4.

NT-3 expression patterns in the smooth muscle layers of the antrum, pylorus, and duodenum. Photomontages of X-gal-stained sections of antrum, pylorus and anterior duodenum (**a, c**) are shown immediately above matching sections counterstained with neutral red (**b, d**). At E15.5 (**a, b**) and E17.5 (**c, d**), NT-3 expression in the antrum occurred in both the circular and longitudinal smooth muscle layers and rapidly transitioned to expression throughout the smooth muscle of the pylorus, which was dominated by a thick circular muscle, and then rapidly transitioned to expression that was restricted to the circular muscle layer of the duodenum (abbreviations as for Figs. 2). Bars 100 μ m

Fig. 5.

NT-3 expression patterns in the stomach at P4. Photomontage of the corpus-forestomach transition region stained with X-gal and neutral red (a), and photomicrographs of X-gal-stained sections of antrum (b) and forestomach (d) above matching sections counter-stained with neutral red (c, e). At P4, similar to earlier ages, NT-3 was expressed in the circular and longitudinal muscle layers of the antrum and some regions of the corpus and only in the circular layer of the remaining regions of the corpus (*ep* epithelium; other abbreviations as for Figs. 2). At the border between the corpus and forestomach, a rapid transition occurred from expression in both smooth muscle layers to expression only in the circular muscle of the forestomach (a). NT-3 expression patterns are shown at higher magnification for the antrum (b, c) and forestomach (d, e). Blood vessel walls that expressed NT-3 (b, c) in the submucosa are indicated by arrowheads in c. Bars 100 μ m

Fig. 6.

NT-3 expression in the intestines within the abdomen (**a, b**) or the intestinal sac (**c, d**) at E12.5 and in the small intestine within the abdomen at E13.5 (**e, f**). Photomicrographs of X-gal-stained intestine sections (**a, c, e**) are shown side-by-side with semi-adjacent sections counterstained with neutral red (**b, d, e**). Section in **f** was taken at the level of the bile duct (*bd*). Within the abdomen at these ages, some regions of the intestines consisted of mesenchyme (*mes*) and epithelium (*ep*; **a, b**; intestine *left*; **c, d**), whereas in other regions, the muscle wall (*mw*) had begun to differentiate (**a, b**; intestine *right*). NT-3 expression was present in the mesenchyme, where it was the predominant tissue layer. As the muscle wall formed, it also became the site

of the bulk of NT-3 expression. Weak expression occurred in the remaining mesenchyme (*cm* circular muscle, *ys* intestinal sac). *Bars* 50 μ m

Fig. 7.

NT-3 expression in the small intestine at E15.5 (**a, b**) and E17.5 (**c, d**) was restricted to the circular smooth muscle layer (abbreviations as for Figs. 2, 3). Photomicrographs of X-gal-stained intestine sections (**a, c**) are shown above (**a**) or side-by-side (**c**) with semi-adjacent sections counterstained with neutral red (**b, d**, respectively). By E15.5, villi had formed in the small intestine. The weak X-gal stain present in the villi at E17.5 (**c, d**) was most likely an artifact, as this tissue was not stained at E15.5 (**a, b**) and, at E17.5, did not exhibit β -galactosidase-LIR (Fig. 2b). Bar 100 μ m

Fig. 8.

NT-3 expression in the small intestine at P4 was restricted to the circular smooth muscle layer (abbreviations as for Fig. 2). Photomicrographs of an X-gal-stained section of small intestine (**a**) is shown above an adjacent section counterstained with neutral red (**b**); an enlarged image (**c**) is shown of the *lower left-hand corner* of **b**. The weak X-gal stain present in the villi is most likely an artifact (see Fig. 7). A blood vessel that expressed NT-3 (*arrowhead*) and part of the bile duct (*bd*) are present in these sections. Bars 50 μ m

Fig. 9.

NT-3 expression in the cecum at E15.5 (**a, b**), E17.5 (**c, d**), and P4 (not shown) was similar to that observed in the small intestine, i.e., largely restricted to the circular smooth muscle layer (abbreviations as for Fig. 2). Photomicrographs of X-gal-stained cecum/intestine sections (**a, c**) are shown above semi-adjacent sections counterstained with neutral red (**b, d**). The section at E15.5 includes the junction of the large intestine (*lg intest*) with the cecum, whereas at E17.5, the junction of the small intestine (ileum) with the cecum is shown. All but one of the cross-sectioned mesenteric blood vessels in **d** (*arrowheads*) exhibit NT-3 expression (**c**). *Bar* 200 μm

Fig. 10.

NT-3 expression patterns in the large intestine at E17.5. Photomicrographs of X-gal-stained sections of large intestine (**a, c**) are shown side-by-side with semi-adjacent sections counterstained with neutral red (**b, d**). Similar to the expression pattern at E15.5, NT-3 expression in the large intestine at E17.5 occurred in the circular smooth muscle layer (**a–d**) and was equally strong in **c, d** and weaker in or absent from various anterior-posterior levels of the longitudinal smooth muscle layer (abbreviations as for Fig. 2). By E17.5, the formation of the mucosa was largely complete. Bars 200 μm (**b**), 50 μm (**d**)

Fig. 11.

NT-3 expression in mesenteric blood vessel walls at E15.5 (**a, b**) and E17.5 (**c, d**).

Photomicrographs of X-gal-stained sections containing GI organs and mesentery (**a, c**) are shown side-by-side with adjacent sections counterstained with neutral red (**b, d**). The blood vessels (*arrowheads* in **b, d**) exhibit NT-3 expression in their walls (**a, c**, respectively).

Insets in **c, d** Higher magnifications ($\times 2.2$) of the cross-sectioned blood vessel in **d** (*bv* blood vessel, *mst* mesentery, *mst att* mesenteric attachment, *sm intest* small intestine, *lg intest* large intestine, *stom* stomach). Bar 100 μm (**b**), 50 μm (**d**)

Fig. 12.

Confocal microscope images illustrating the apparent lack of NT-3 expression (β -galactosidase-LIR) in myenteric ganglia at E17.5. Each image in each of **a–d** is oriented with the circular muscle *left*, the myenteric plexus/ganglia *middle*, and longitudinal muscle *right*. NT-3 expression (**a**; FITC-labeled secondary antibody) and myenteric neurons that exhibited HuC/D-LIR (**a'**; RRX-labeled secondary antibody; *arrows*) did not co-localize (**a''**; merge of NT-3 expression and HuC/D-LIR); stomach wall. NT-3 expression (**b**; RRX-labeled secondary antibody) and undifferentiated neural crest cells (neuronal and glial precursors) that exhibited p75-LIR (**b'**; FITC-labeled secondary antibody; *arrows*) did not co-localize (**b''**; merge of NT-3 expression and p75-LIR); small intestine wall. NT-3 expression (**c**; RRX-labeled secondary antibody) and glial cells that exhibited S100-LIR (**c'**; FITC-labeled secondary antibody; *arrows*) did not co-localize (**c''**; merge of NT-3 expression and S100-LIR); large intestine wall. NT-3 expression (**d**; RRX-labeled secondary antibody) and interstitial cells of Cajal at the myenteric plexus (ICC-MYs) that exhibited c-Kit-LIR (**d'**; FITC-labeled secondary antibody; *arrows*) did not co-localize (**d''**; merge of NT-3 expression and c-Kit-LIR). In addition to ICC-MYs, some cells in the muscle layers expressed c-Kit but also did not co-localize with NT-3 expression; stomach wall. *Bars* 10 μ m

Table 1

Primary antibodies used on sections from mouse embryos

Antibody	Immunogen	Host	Dilution	Source
β -Galactosidase, polyclonal	β -Galactosidase, <i>E. coli</i> origin	Guinea pig	1:500–1:2000	Thomas Finger, University of Colorado, Aurora
c-Kit, polyclonal	c-Kit, c-terminus peptide mouse origin	Goat	1:10	SC-1494 (M-14); Santa Cruz Biotechnology, Santa Cruz, Calif., USA
HuC/D-biotin, monoclonal (clone 16A11)	HuC, HuD, Hel-N1 proteins, human origin	Mouse	1:2–1:5	A21272; Molecular Probes Eugene, Ore., USA
p75, polyclonal	p75, amino acids 43-161, mouse origin	Rabbit	1:1000	AB1554; Chemicon, Temecula, Calif., USA
S100, polyclonal	S100, from cow brain, bovine origin	Rabbit	1:500–1:1000	Z 0311; Dako, Glostrup, Denmark

Table 2

NT-3 expression (β -galactosidase) in the GI tract of NT-3^{LacZ} embryos and neonates (– no label, +/- faint X-gal stain in some specimens, + faint X-gal stain, ++ medium X-gal stain, +++ dense X-gal stain)

Organ/tissue	E12.5	E13.5	E15.5	E17.5	P4
Esophagus					
Muscularis externa	–	–	–	–	–
Submucosa	–	–	–	–	–
Mucosa	–	–	+	++	+++
Epithelium	–	–	–	–	–
Lamina propria	–	–	–	–	–
Stomach, forestomach/lower esophageal sphincter					
Mesenchyme	++	–	–	–	–
Muscularis externa	–	–	–	–	–
Longitudinal m.	–	–	–	–	–
Circular m.	–	+	++	++	+/++
Submucosa	–	–	–	–	–
Mucosa	–	–	–	–	–
Epithelium	–	–	–	–	–
Lamina propria	–	–	–	–	–
Stomach, antrum/corpus					
Mesenchyme	+++	–	–	–	–
Muscularis externa	–	+++ ^a	+++ ^a	+++ ^a	+++ ^a
Longitudinal m.	–	–	–	–	–
Circular m.	–	++	++	++	++
Submucosa	–	–	–	–	–
Mucosa	–	+/-	+	++	++
Epithelium	–	–	–	–	–
Pylorus					
Mesenchyme	+++	–	–	–	–
Muscularis externa	–	+++	+++	+++	++
Longitudinal m.	–	+++	+++	+++	++
Circular m.	–	–	–	–	–
Submucosa	–	–	–	–	–
Mucosa	–	–	–	–	–
Epithelium	–	–	–	–	–
Lamina propria	–	+/–	+	+	+
Small intestine					
Mesenchyme	+++/+++	+/–	–	–	–

Organ/tissue	E12.5	E13.5	E15.5	E17.5	P4
Muscularis externa	++	-	-	-	-
Longitudinal m.	-	-	-	-	-
Circular m.	++	+++	+++	++	-
Submucosa	-	-	-	-	-
Mucosa	-	-	-	-	-
Lamina propria	-	-	-	-	-
Cecum					
Muscularis externa			+/-	+/-	-
Longitudinal m.					
Circular m.			++	++	+/-
Submucosa			-	-	-
Mucosa			-	-	-
Lamina propria			-	-	-
Large intestine					
Mesenchyme	++/+++	++/+++			
Muscularis externa	++	++			
Longitudinal m.	-	-	++/+++ ^a	++/+++ ^a	-
Circular m.	-	-	++	+++	++
Submucosa	-	-	-	-	-
Mucosa	-	-	-	-	-
Lamina propria	-	-	-	-	-

^a Stain in the longitudinal muscle layer of the stomach corpus and large intestine was intermittent

Table 3

Percentage of confocal sampling sites that exhibited co-localization of a cell type marker (antibody 2) with NT-3 expression (β -galactosidase) in the myenteric plexus (number of sampled sites in parentheses)

Antibody 1	Antibody 2	Stomach	Small intestine	Cecum	Large intestine
β -Galactosidase	HuC/D	0 (44)	0 (38)	0 (17)	0 (33)
	S100	0 (38)	0 (25)	0 (11)	0 (22)
	p75	0 (27)	0 (45)	0 (13)	0 (35)
	c-Kit	0 (38)	0 (33)	0 (13)	0 (20)