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Mice deficient in Brain-Derived Neurotrophic Factor have Altered Development of Gastric Vagal Sensory Innervation

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ABSTRACT

Vagal sensory neurons are dependent on neurotrophins to survive programmed cell death during development. Here, the contribution of brain-derived neurotrophic factor (BDNF) to the survival of gastric vagal sensory afferents was investigated. Also, based on BDNF roles in other sensory systems, its effects on axon guidance and mechanoreceptor differentiation were examined. Postmortem anterograde tracing with 1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was used to selectively label vagal projections to the stomach on postnatal day (P)0, 3, 4, and 6 in wild types and heterozygous or homozygous BDNF mutants. Sampling sites distributed throughout the ventral stomach wall were scanned with a confocal microscope and vagal axon bundles, single axons, putative mechanoreceptor precursors (intraganglionic laminar endings, IGLEs; intramuscular arrays, IMAs) and efferent terminals were quantified. Also, myenteric neurons, which are innervated by IGLEs, were stained with cuprolinic blue and quantified. In homozygous mutants, at P0, IGLE density was reduced by 50% without any effect on myenteric neurons or vagal efferents. Also, putative IMA precursors exhibited truncated telodendria, and antrum innervation was disorganized. At P3-P6, the effects on IGLEs and antrum innervation still occurred, but to a lesser degree, some IMA telodendria were still truncated, and there was a large, but non-significant trend toward decreased IMA density (42%). These results suggest BDNF contributes to the survival of subpopulations of gastric IGLEs and possibly IMAs (or to IMA differentiation) and to patterning of antral innervation. Additionally, it is possible that other growth factors expressed in the stomach wall compensated for BDNF deficiency and reduced the losses of mechanoreceptors.

INTRODUCTION

The perinatal period is crucial to the proper development of complex systems of cells, tissues and organs. These systems, such as the digestive and autonomic systems, must act in concert to ensure homeostasis and the survival of an organism. In order to develop and establish these systems, an elegant and complex orchestration of both organ development and innervation must take place. A major autonomic channel for information flow between the brain and viscera is the vagus, or X^{th} cranial nerve. The vagus nerve contributes both sensory (afferent) and motor (efferent) innervation of organs and tissues of the neck, chest, and most of the abdomen. Among these, gastrointestinal (GI) organs are involved in digestive actions such as food absorption, gastric motility, gastric secretion, tissue defense, food intake and toxin alert. The majority (73%) of the axons of the abdominal vagus, which innervates most GI organs, are non-myelinated C-fiber afferents (Prechtl and Powley, 1990). These sensory axons are derived from pseudounipolar neurons of the nodose ganglion, which extend central processes to the nucleus of the solitary tract (NTS). These afferents send feedback to the central nervous system that contributes to the control of food intake, and they comprise the sensory arm of vago-vagal reflexes that produce gastric accommodation, antral peristalsis and enterogastric inhibition (Anand and Pillai, 1967; Andrews et al., 1980; Grundy et al., 1981; Iggo, 1957).

Two vagal mechanoreceptor classes that predominate in the GI muscle wall have been characterized. These include intraganglionic laminar endings (IGLEs) and

intramuscular arrays (IMAs). Intraganglionic laminar endings are more abundant and are distributed from the esophagus to the large intestine, but they are most dense in the stomach and duodenum (Berthoud et al., 1997a; Fox et al., 2000; Wang and Powley, 2000). These endings consist of terminal puncta arranged in flattened leaf-like structures that cover all or a portion of a myenteric ganglion. Moreover, IGLEs lie between the clusters of myenteric neurons and the ganglion capsule, and their terminal puncta extend into both the neuron and capsule layers. This anatomical arrangement suggested IGLEs transduce shearing forces due to tension and stretch of the muscle wall (Neuhuber and Clerc, 1990), a hypothesis supported by physiological studies (Yang and Liu, 2006; Zagorodnyuk et al., 2001). Intraganglionic laminar endings may also have a local effector role as some of their processes contain transmitter vesicles and appear to synapse on myenteric neurons (Neuhuber, 1987; Powley et al., 2008), and at least esophageal IGLEs exhibit vesicular glutamate transporter 2 (VGLUT2)-LIR, suggesting that they use glutamate as a transmitter (Raab and Neuhuber, 2003). Intramuscular arrays, on the other hand, are localized in the forestomach and the lower esophageal and pyloric sphincters, and to a lesser extent in the antrum of the mouse, and the duodenum and colon of the rat (Fox et al., 2000; Phillips et al., 1997; Wang and Powley, 2000). These receptors consist of arrays of parallel, rectilinear terminal processes (telodendria) interconnected by short collateral branches. They innervate the circular and longitudinal layers of the muscle wall, running in parallel with muscle fibers and intramuscular interstitial cells of Cajal (ICC-IMs). These ICCs form close appositions with IMAs through which they may influence each other's development or function (Berthoud and Powley, 1992; Fox et al., 2002; Fox et al., 2001a; Powley et al.,

2008). Based on this arrangement, IMAs are hypothesized to detect stretch of the muscle wall caused by organ or sphincter dilation (Wang and Powley, 2000; but see Zagorodnyuk et al., 2001).

In addition to these mechanoreceptors, another major pool of vagal sensory neurons supplies chemoreceptors, mechanoreceptors and polymodal receptors to the mucosal and submucosal layers of the GI tract, thermoreceptors to the intestine, and osmoreceptors to the liver (Adachi, 1984; Harding and Leek, 1972; Iggo, 1957; Mei, 1983). Little is known about the structure or density of these vagal afferents. Within the small intestine they travel through the muscle wall and submucosa to reach the mucosa where terminal arborizations either wrap around crypts, or ramify within villi (Berthoud et al., 1995).

Surprisingly little is known about the development of vagal sensory innervation of the GI tract, or about how development of its complex organization is orchestrated. In large part this has been due to the difficulty in selectively labeling developing vagal sensory fibers and terminals so they can be distinguished from the largely coextensive elements of the sympathetic, enteric and vagal efferent systems that innervate the same organs and tissues (Hayashi et al., 1982; Richards and Sugarbaker, 1995; Schafer et al., 1998). Some progress has been made with markers for esophageal IGLEs that take advantage of their neurochemistry, such as vesicular glutamate transport 2 (Raab and Neuhuber, 2003), and the purinergic receptor subunits $P2X_2$ and $P2X_3$ (Castelucci et al., 2003; Kestler et al., 2009; Wang and Neuhuber, 2003). However, they have not yet been established as reliable markers for IGLEs in other developing or mature GI organs.

Recently, building upon the DiI (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) anterograde tracing method of Sang and Young (1998), we were able to selectively label vagal innervation of the upper GI tract as early as embryonic day(E)12 and as late as postnatal day(P)8, ages involving key events in vagal development (Murphy and Fox, 2007). This enabled characterization of the temporal and spatial pattern of the normal perinatal development of vagal elements within the GI tract. In turn, this permits examination of perturbations in these patterns produced by pharmacological, surgical or genetic manipulations that can be used to investigate the mechanisms that regulate vagal development.

Here we have utilized this DiI method to investigate the role of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors in the development of vagal GI afferents. Neurotrophins play important roles in the development of several sensory systems, including the sensory component of the vagus nerve. BDNF is essential for a large proportion of vagal sensory neurons to survive programmed cell death during development (Conover et al., 1995; ElShamy and Ernfors, 1997; Erickson et al., 1996; Forgie et al., 2000). BDNF produces its effects by activating a high affinity receptor, trkB (Barbacid, 1994; Rodriguez-Tebar et al., 1992) and a low affinity receptor, p75 (Ernfors et al., 1990; Rodriguez-Tebar et al., 1990). There were two reasons we focused on BDNF. First, it met the several criteria developed for identifying genes that have a high probability of regulating development of vagal sensory neurons that innervate the GI tract; mechanoreceptors in particular (reviewed in Fox, 2006). Second, the available evidence suggests that at least some neurons supplying sensory innervation of the stomach depend on BDNF for survival.

The observation that survival of 97% of vagal sensory neurons is supported by BDNF, neurotrophin-3 (NT-3) or neurotrophin-4 (NT-4; Liu and Jaenisch, 2000) could imply that at a minimum one of these neurotrophins must support innervation of the stomach. Further, Brady et al. (1999) suggested that within the autonomic nervous system the survival effects of neurotrophins may be restricted to a single organ. Consistent with this organ-specific hypothesis, Fox et al. (2001b) found that NT-4 mutants sustained reductions in size and density of IGLEs in the small intestine, but not in the stomach, and Raab et al. (2003) established that esophageal IGLEs were dependent on NT-3 for survival. This leaves BDNF as the most likely factor to support survival of stomach innervation.

Neurotrophins are also involved in other aspects of sensory neuron development, including axon growth and guidance (Fritzsch et al., 2005; Fritzsch et al., 2004; Krimm, 2007; Lykissas et al., 2007; McCaig et al., 2000; Paves and Saarma, 1997) and receptor differentiation (Fass et al., 2004; McFarlane, 2000; Miyamoto et al., 2006), as well as in neuronal maintenance (Airaksinen et al., 1996; Carroll et al., 1998) and function (Carroll et al., 1998; McIlwrath et al., 2005). Thus, in addition to hypothesizing that BDNF supports survival of vagal afferent innervation of the stomach, we suggest that should any gastric vagal sensory receptors survive in BDNF-deficient mice, a weaker form of the organ-specific principle would suggest that other aspects of their development may be regulated by BDNF, including axon guidance and receptor differentiation.

In order to examine these hypotheses, the effects of BDNF deficiency on vagal innervation were comparatively examined in the developing mouse stomach. Specifically, the number of vagal fibers, axon bundles and putative mechanoreceptors and efferent terminals in a large P0 sample and a smaller P3-P6 sample of wild-type mice (+/+) and mice lacking one (+/-) or both (-/-) copies of the *bdnf* gene were compared over the whole stomach wall. Additionally, since BDNF expression during development is restricted to specific stomach compartments (Fox and Murphy, 2008), vagal elements were grouped for analysis by location within either the antrum, corpus or forestomach. Further, although our previous work significantly extended available descriptions of vagal GI development, they were qualitative. Thus, the quantitative estimates of DiI-labeled vagal elements in the stomach of wild-type mice presented here at two development stages extend these findings and make it possible to compare distributions of these elements with those that have been estimated in adults.

METHODS

Animals. Mice heterozygous for a targeted mutation of *bdnf* were obtained from Jackson Laboratories (Bar Harbor, ME; stock no. 002266; Ernfors et al. 1994). Mice had ad lib access to food (Laboratory Rodent Diet 5001, PMI Nutrition International, Saint Louis, MI, USA) and water, temperature was maintained at 22-24°C, and a 14:10 light/dark cycle with lights on at 05:00 h was employed. Mice were genotyped by polymerase chain reaction (PCR) analysis of genomic DNA extracted from tail tips (PCR primer sequences: BDNF1: CAT GAA AGA AGT AAA CGT CCA C, BDNF2: CCA GCA

GAA AGA GTA GAG GAG; BDNF-PGK: GGG AAC TTC CTG ACT AGG GG). 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.

Mating. Heterozygous *bdnf* mutant mice were bred to obtain wild-type (*bdnf* $^{+/+}$), and heterozygous (*bdnf* $^{+/-}$) or homozygous (*bdnf* $^{-/-}$) mutant mice. Cages were checked daily at 1700 h for births and litters born that day were designated as P0. Additional litters were harvested on P3, P4, or P6.

Tissue Preparation. At P0, P3, P4, or P6, pups were deeply anesthetized with methohexital sodium (Brevital Sodium, Eli Lilly, Indianapolis, Indiana; 100 mg/kg) and perfused transcardially with warm saline for 5 min. For cuprolinic blue staining this was followed by ice-cold 4% paraformaldehyde (PF) in a phosphate buffered saline (PBS) for 30 min, and then tissues were stored 3 d at 4°C in 4% PF in PBS. For DiI nerve labeling the 4% PF in PBS contained 0.1% ethylenediamine tetraacetic acid (EDTA). EDTA was added to retard the diffusion of DiI out of membranes and therefore increase the specificity and longevity of the DiI label (Hofmann and Bleckmann, 1999; Murphy and Fox, 2007).

Experiment 1A: Effects of BDNF deficiency on gastric vagal innervation at P0

DiI Application. After 3 d fixation, DiI was used to label vagal fibers and terminals in the stomach of *bdnf* $^{+/+}$ (n=12), *bdnf* $^{+/-}$ (n=12), and *bdnf* $^{-/-}$ (n=11) mice as previously described (Murphy and Fox, 2007). Briefly, the stomach, esophagus and associated

vagal trunks and branches were exposed and one DiI crystal (D-3911, Invitrogen Corporation, Carlsbad, CA) was applied at the bifurcation of the hepatic and gastric vagal branches and then crushed into the nerve fibers (Fig. 1, top left panel) Precautions were taken to prevent redistribution of DiI crystal fragments as previously described (Murphy and Fox, 2007). Tissue was stored in 4% PF, 0.1% EDTA in PBS for 4 wk, light protected at 37°C.

Vagotomy. To verify the vagal origin of DiI labeled elements in the stomach wall, the anterior vagal gastric branch was sectioned between the DiI application site and the stomach immediately after DiI application, *bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$ (n=2/gp).

Tissue Preparation. The stomach along with the pylorus, esophageal sphincter, and associated vagal branches were dissected out, and any milk remnants removed by rinsing with PBS. Stomachs were prepared as wholemounts, separated into dorsal and ventral walls by making a longitudinal cut along the lesser and greater curvatures, and mounted directly in PBS with 0.5% sodium azide, mucosal side facing down on glass microscope slides, coverslipped, sealed with nail polish, and stored (light protected) at 4°C until imaged.

Microscopy. Specimens were first briefly examined using Leica DM5000 B microscope with filter cube Y3 to determine whether the DiI labeling of vagal fibers and terminals met several criteria required for inclusion in quantitative analyses: (1) A significant proportion of vagal fibers and terminals in all stomach compartments were labeled with DiI, and this labeling extended distally as far as the greater curvature and pylorus. (2) DiI labeling remained stable during transport and analysis, i.e. DiI did not leak out of vagal fibers. (3) DiI did not redistribute from the injection site to produce non-

specific labeling. Two specimens failed to meet all of these criteria and were not further examined (+/+, n=1; +/-, n=1). DiI fluorescence (460 nm excitation, 565 nm emission) was then visualized with standard rhodamine filters and optical sections obtained using an Olympus BX-DSU spinning disk confocal microscope equipped with a 60X water immersion objective (total magnification = 600X). Processing of three-dimensional image data was performed using Slidebook (v.4.1, Intelligent Imaging Innovations, USA). To control for fluorescence fading, all specimens were imaged within 5 h of initial inspection after removal from incubation and each was sampled in the same sequence.

Identification of Vagal Fibers, Bundles and Terminals. Criteria that have been published for identifying mature forms of vagal fibers, bundles, efferent terminals, IGLEs and IMAs were used to identify neonatal vagal fibers, bundles, efferent terminals and putative IGLE and IMA precursors as previously described (Murphy and Fox, 2007). Briefly, identification of IGLE and IMA precursors was possible because they exhibited some or all of the unique features of the mature mechanoreceptor forms, although the receptors were smaller in size and their nerve terminal components were less numerous or less dense. Also, the changes in their form over the course of development were characterized by selecting examples at P8 that could be matched to mature profiles and working backwards sequentially one day in age at a time to the ages utilized in the present study and as early as E16.

Criteria for identification of single fibers: (1) Consists of a single DiI-labeled axon coursing through the myenteric plexus, (2) not contained within a vagal fiber bundle.

Criterion for identification of an axon bundle: Consists of two or more DiI-labeled axons coursing through the myenteric plexus in parallel and in close apposition.

Criteria for identification of putative IGLE precursors: Consists of (1) a laminar (2) aggregate of fine terminal puncta (3) within the neuropil of a myenteric ganglion and (4) covering all or part of the ganglion (Berthoud et al., 1995; Neuhuber, 1987; Rodrigo et al., 1982; Rodrigo et al., 1975). For the present purposes, criterion (4) was determined by locating terminal processes in either the z-plane immediately above or below the planes containing myenteric neurons, the myenteric plexus, and vagal efferent terminals.

Criteria for identification of putative IMA precursors: Consists of (1) an array of parallel axonal telodendria (2) in close proximity, (3) interconnected by bridging axonal elements and (4) located in either the longitudinal or circular muscle layer (Berthoud and Powley, 1992; Fox et al., 2000; Wang and Powley, 2000). For the present purposes, criterion (4) was determined by locating terminal processes in the series of z-planes that included longitudinal or circular muscle layers either above or below the planes containing the myenteric plexus, respectively.

Criteria for identification of putative efferent terminal precursors: Consists of (1) a network of characteristic rings of fiber arborizations and terminal complexes (2) circling the myenteric neurons (3) within the myenteric ganglia (Holst et al., 1997; Kirchgessner and Gershon, 1989; Phillips et al., 2003).

Sampling and Quantification. To assess innervation throughout the stomach wall, a sampling grid was used to locate equidistant sampling sites. The sampling grid established a proportional scale that normalized each wholemounted stomach so that the variability due to differences in stomach size or distention was minimized (Fig. 1, bottom panel). At each sampling location, a counting grid was used to quantify each

labeled vagal element as described below. Counts were made blind to the genotype of the specimen and independent counts of a small subset of the specimens were made by a second investigator to validate the identification criteria. There were no significant differences in estimations of any quantified vagal elements observed between counters (not shown).

At each sampling point a confocal microscope was used to scan a series of optical sections through the entire thickness of the stomach wall, which ranged from 20-150μm in thickness, at 1 μm intervals. Each optical section was 147μm X 114μm in size (16.76 mm^2 in area). Eighty sites were sampled for each stomach wall, covering about 6% of the stomach surface area, which appeared to sample a consistent portion of the vagal innervation in each sample, and thus provided an acceptable baseline for assessing changes in mutants.

The counting grid consisted of six equidistant rows and eight equidistant columns that spanned the entire width and height of an optical section and were drawn on transparency film paper that was attached to the computer screen where the optical sections were viewed. The location of each fiber on the counting grid was used to register positional information about individual fibers onto grid paper by assigning successive rows on the grid paper to successive optical sections (based on Cheng et al., 1997). In this way, each fiber was tracked along its 3-dimensional (3D) path so as to avoid counting a fiber more than once. Axons were then counted starting with the first section and then sequentially through each section. Using this technique, Cheng et al., (1997) were able to distinguish fibers separated by as little as 1 μm. The density of individual fibers and IMA telodendria were quantified by applying this method, and

counting the total number of gridline crossings that occurred. Numbers of IGLEs (aggregates of terminal puncta, or leaves) were estimated by counting each individual IGLE leaf identified within each sequential image (optical section) at each sampling site. Counts of vagal efferent terminals and axon bundles were made from 2D projections of the 3D series of optical sections collected at each sampling site. Each myenteric neuron encircled by DiI-labeled fibers was counted as one vagal efferent terminal in this 2D projection. Also, each vagal bundle present in a 2D projection was counted.

For statistical comparisons, data collected from sampling points throughout the stomach wall were also grouped by location within the forestomach, corpus, or antrum (Fig. 1, top right panel). These regions were established by dropping two vertical lines, one from each side of the outer surface of the lower esophageal sphincter resulting in the antrum consisting of columns 1-3, the corpus columns 4-6, and the forestomach columns 7-10.

Analysis and illustration. Results of quantification of fibers, endings and bundles are expressed as means and standard errors. These data were represented in graphs created using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Statistical analyses for all groups were carried out by one-way analysis of variance (ANOVA), followed by FSD-tests for normality and Tukey's post-hoc tests using Statistica 6.0 (StatSoft, Inc., Tulsa, OK). Photoshop software (version 6.0 Adobe Systems, Mountain View, CA) was used to a) apply scale bars and text, b) adjust brightness and contrast, and c) organize the final layouts.

<u>Experiment 1B: Effect of BDNF deficiency on gastric myenteric neurons at P0.</u>

The goal of this experiment was to determine whether changes in myenteric neuron number occurred in association with BDNF deletion since they are target cells for IGLEs. For this purpose a cuprolinic blue stain for enteric neurons in adult rodent GI wholemounts (Holst and Powley, 1995) was adapted to label these neurons in P0 mouse stomachs (*bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$; n=5/gp). Cuprolinic blue was chosen due to its greater completeness and specificity for staining myenteric neurons as compared with other methods (Heinicke et al., 1987; Karaosmanoglu et al., 1996). Cuprolinic blue (quinolinic phthalocyanine, Polysciences, Warrington, PA) stain was prepared by dissolving it in 0.05 M sodium acetate buffer (0.5% w/v, pH 4.9) and filtering it (0.22 μm; Millipore, Billerica, MA).

Tissue Preparation. Stomachs were prepared as wholemounts (as described above for preparation of tissue with DiI labeled elements) and the outer smooth muscle layers were separated from the mucosal and submucosal layers using #7 curved fine forceps and placed in a drop of cuprolinic blue on a glass microscope slide. The tissue was then covered with a piece of parafilm and stained for 1 h in a humidified slide-warming tray at 40 °C. After staining, wholemounts were drained and rinsed in distilled water, differentiated in 0.05M sodium acetate buffer (pH 5.6) and rinsed in 0.02M KPBS. Tissue was mounted on slides, air dried, dehydrated in a series of graded alcohols (70%, 95%, 2 x 100%; 2 min each), cleared in xylene (2 x 2 min), and then coverslipped with Cytoseal.

Imaging, Sampling, and Quantification. Myenteric neurons stained with cuprolinic blue were visualized with brightfield illumination using an Olympus BX-DSU microscope. For counting myenteric neurons, the same magnification and sampling grid

described above for counting DiI-labeled vagal fibers and terminals were used so these two sets of counts were obtained in as similar a manner as possible. At each sampling location all stained cells within the field of view were counted. Individual myenteric neurons are distinct – with blue stained Nissl substance clearly outlining the cytoplasm (which is unstained) and a stained nucleolus. Myenteric glial cells are not known to be stained by this method, so all of the stained cells were considered neuronal. Sampling points with excessive folds or tears in the tissue were excluded. For each stomach wall sampled, myenteric neuron numbers counted at each sampling site were totaled, and those from sampling sites within the antrum, corpus and forestomach compartments totaled separately. These data were represented and statistically analyzed as described for Experiment 1A. Images were acquired using a Leica DM5000 B microscope, a video camera (Spot RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI) and SpotSoftware (v4.5, Diagnostic Instruments) and adjusted using Photoshop software as in Experiment 1a.

Experiment 2: Effects of BDNF deficiency on gastric vagal innervation at P3-6

The goal of this experiment was to assess whether the effects of BDNF deletion on vagal innervation of the stomach were maintained as development continued beyond P0, became more severe, or less severe. To achieve this, several litters were permitted to survive beyond P0 to obtain the rare litters that contained a homozygous BDNF mutant that survived beyond P0. In such litters, vagal gastric innervation was labeled with DiI in *bdnf* $^{+/+}$ (P3, n=2; P4, n=2; P6, n=1; total, n=5), *bdnf* $^{+/-}$ (P3, n=2; P4, n=3; P6, n=1; total, n=6), and *bdnf* $^{-/-}$ (P3, n=1; P4, n=2; P6, n=0; total, n=3) postnatal mice.

Additionally, to verify the vagal origin of DiI-labeled elements in the stomach wall, one specimen of each of the three genotypes at each of the three ages examined was vagotomized after DiI application as described in Experiment 1A.

All methods were the same as described for Experiment 1A with the following exceptions. After DiI application to the abdominal vagus, tissue was incubated in 4% PF + 0.1% EDTA for 5 wk (P3, P4) or 6 wk (P6) at 37°C. The DiI labeling of vagal elements in one P4 specimen (*bdnf* $^{+/-}$) did not meet the criteria for sufficient labeling and therefore was not further analyzed. Since the stomach wall was larger at P3-6 as compared with P0, the percentage of the total surface area covered by 80 sampling points at P3-6 was less, approximately 4%. Nevertheless, this coverage still appeared to sample a consistent portion of the vagal innervation of the stomach, and thus provided a reasonable baseline for assessing changes in mutants.

RESULTS

Experiment 1A: Effects of BDNF deficiency on gastric vagal innervation at P0

Vagal Innervation in the stomach of wild-type mice at P0. DiI-labeled vagal fibers and terminals in the P0 stomach revealed a dense pattern of innervation reaching from the gastric branches traversing the lower esophagus to the greater curvature of the stomach. Dense innervation of the myenteric plexus included individual vagal fibers, bundles, putative IGLE precursors (Fig. 2A,B) and efferent terminals (Fig. 3). Putative IMA precursors innervated the circular and longitudinal smooth muscle layers with telodendria orientated in parallel to one another and to muscle fibers as previously

described for adult IMAs (Fig. 2C-E). Vagal innervation was also observed within the mucosa and submucosa, consisting mostly of fibers and free endings (Fig. 2F). The myenteric plexus in the antrum was more dense and tightly organized than in the forestomach (cf. Fig. 3A,B). Also, axon bundles were larger in the corpus (Fig. 3C) than in the antrum or forestomach probably because most bundles arising directly from the esophageal gastric branch initially traversed the corpus.

Quantification of DiI-labeled elements in wild-type mice, including individual vagal fiber density, fiber bundle number, putative IGLE numbers, IMA density and efferent terminal number yielded no significant differences among the three stomach compartments, although there were trends toward increased innervation in the corpus and forestomach as compared with the antrum (Fig. 4A-E). For example, there was a 92% increasing trend in putative IMA density in the forestomach than in the antrum and 28% in the corpus. Interestingly, in each of the three major stomach compartments there were more than twice the numbers of longitudinal IMAs as there were circular ones (Fig. 5D).

Vagal Innervation in the stomach of BDNF-deficient mice as compared with wild types at P0. Visual inspection of confocal images from sampled sites revealed two striking differences in vagal innervation of the stomach in *bdnf* $^{-/-}$ mice compared to wild types that were not evident in statistical comparisons. These changes included altered organization of vagal innervation specific to the antrum and abnormal morphology of putative IMA precursors in the forestomach. First, vagal innervation in portions of the antrum appeared more dense and disorganized than in either *bdnf* $^{+/+}$ (cf. Fig. 6A,B; cf. also Fig. 6C,D in Fox and Murphy, 2008) or *bdnf* $^{+/-}$ mice. These changes appeared to

result from an increase in putative growth cones found in the myenteric plexus and a decrease in space between fiber bundles and ganglia. This decrease may have resulted from a change in the arrangement of axon bundles and an increase in their diameter. This altered organization was apparent in approximately 21% of sampled sites distributed throughout the antrum. Second, in the forestomach of *bdnf* $^{-/-}$ mice, the processes of putative IMAs appeared shorter in length and larger in diameter than those present in either *bdnf* $^{+/+}$ (cf. Fig. 6C,D) or *bdnf* $^{+/-}$ mice. This occurred in an average of 20% of sampled sites containing IMAs in the forestomach for all *bdnf* $^{-/-}$ mice, ranging in severity from 10-30%.

Quantification of numbers of vagal bundles, putative IGLE precursors and efferent terminals and densities of individual fibers and putative IMA precursors sampled across the entire stomach wall in *bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$ mice revealed a nearly 50% decrease in putative IGLE numbers in homozygous mutants as compared with wild types ($p<0.05$; Fig. 5C). Further, putative IGLE survival appeared to be sensitive to gene dosage as there was a 32% decrease in heterozygous mutants (Fig. 5C). In contrast, despite the morphological abnormalities exhibited by putative IMAs, their densities remained similar in mutant and wild-type mice, and this was also true for circular and longitudinal IMAs considered separately (Fig. 5D). Additionally, there were no effects of BDNF deficiency on numbers of vagal fiber bundles (Fig. 5A) or individual fiber densities (Fig. 5B). The lack of altered fiber densities could imply that vagal fibers innervating the mucosal and submucosal layers at this age, which were included in these counts, were not affected by BDNF deficiency. Finally, putative vagal efferent terminal numbers were similar in mutant and wild-type mice (Fig. 5E), suggesting that

the effects of BDNF deficiency were restricted to sensory innervation, and the decrease in IGLE numbers were not likely to have resulted from a general or non-specific effect on vagal innervation.

Comparisons of quantified elements of vagal innervation grouped by stomach compartment were not significantly different between *bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$ mice (Fig. 7A-E). Nevertheless, there were several interesting trends. For example, in parallel with the changes in antrum organization described above, there was a 42% increase in the number of axon bundles in *bdnf* $^{-/-}$ mice compared to both *bdnf* $^{+/+}$ and *bdnf* $^{+/-}$ mice in the antrum and also a 35% increase in the forestomach. Additionally, there were decreases in putative IGLE number in all three compartments, ranging from 40 - 59% in *bdnf* $^{-/-}$ mice compared to wild types (*bdnf* $^{+/+}$), and a similar gene dosage effect occurred in each, indicating that the loss of putative IGLEs was relatively evenly distributed across stomach compartments.

Vagotomies performed in *bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$ mice to verify vagal origin of DiI labeling prevented DiI diffusion in vagal axons from reaching the stomach in all cases, resulting in no DiI-labeled fibers observed (not shown).

<u>Experiment 1B: Effect of BDNF deficiency on gastric myenteric neurons at P0.</u>

Since BDNF deficiency could have produced the effects observed in Experiment 1A indirectly, for example by altering survival of target cells of IMAs and IGLEs, we attempted to stain these cells (ICC-IMs and myenteric neurons, respectively). We also attempted to stain growth cones in wholemounts and sections to verify the growth cone-like structures observed. A method for staining myenteric neurons in the neonatal

mouse stomach using cuprolinic blue was successful (Fig. 8A), but immunohistochemical detection of ICC-IMs and growth cones in the current paradigm was not.

Myenteric neuron numbers in the wild-type and BDNF-deficient stomach wall. Quantitative comparison of cuprolinic blue-stained myenteric neuron counts among the three stomach compartments in wild-type mice revealed a progressive decline in neuron numbers from the antrum to the corpus (34%) and then to the forestomach (58%; Fig. 8C). A similar decline in myenteric neuron numbers across these stomach compartments was present in *bdnf* $^{+/-}$ and *bdnf* $^{-/-}$ mice (Fig. 8C). The total number of neurons counted in the stomach muscle wall of *bdnf* $^{+/-}$ or *bdnf* $^{-/-}$ mice were not significantly different from the total in wild-type mice (Fig. 8B). However, non-significant trends in opposite directions in the *bdnf* $^{+/-}$ and *bdnf* $^{-/-}$ groups resulted in a significant difference between them (Fig. 8B). Analysis of neuron counts by compartment revealed that this difference was due mainly to differences in the antrum between these groups, the only compartment where this difference was significant (Fig. 8C).

Experiment 2: Effects of BDNF deficiency on gastric vagal innervation at P3-6

To assess whether the effects of BDNF deficiency observed at P0 persisted, recovered or worsened at later stages of development, DiI-labeled vagal innervation of the stomach of mice aged P3-6 was examined. Mice of several ages were combined because most homozygous BDNF mutants die shortly after birth, making it difficult to obtain them after P0. In fact, litters collected at P3-6 often included no homozygous BDNF pups.

Vagal Innervation in the stomach wall of wild-type mice at P3-6. DiI labeled vagal fibers and terminals in the P3-6 stomach revealed a dense pattern of innervation similar to that seen in Experiment 1A, although it was more mature in appearance, exhibiting more numerous vagal fibers, bundles and putative IMAs, IGLEs and efferent terminals that were slightly more similar to adult forms than was observed at P0 (Figs. 2,3 vs. 9,10). For example, myenteric vagal axon bundles appeared to be separated by greater distances, which may have been due in part to the growth of the stomach from P0 to P3-6, and IGLEs were larger in size, which made their laminar organization more evident (Fig. 9A,B). The putative IMAs that innervated the smooth muscle layers at P3-6 exhibited more numerous telodendria that were greater in length than at P0 (circular and longitudinal; Fig. 9C-E). Vagal innervation was also observed within the mucosal and submucosal layers (Fig. 9F). Among the three defined stomach compartments, qualitative differences observed at P0 were also present. The myenteric plexus in the antrum was much more dense and tightly organized than it was in the forestomach (cf. Fig. 10A,B). Also, larger bundles were observed in the corpus, as they descended from the esophagus, than were found in the antrum or forestomach (Fig. 10C).

Quantification of DiI-labeled elements of vagal stomach innervation yielded significant differences in IMA density and axon bundle number among the three stomach compartments in wild-type mice. There was a 97% increase in putative IMA density in the forestomach as compared with the antrum and 123% as compared with the corpus ($p<0.05$; Fig 11D). Also, there were 53% more fiber bundles in the forestomach than the antrum ($p<0.05$) and 43% more than in the corpus, although the latter trend was not significant (Fig. 11A). Additionally, there were non-significant trends

for greater numbers of putative IGLEs in the antrum as compared to the corpus (61%) and the forestomach (30%; Fig. 11C). In contrast, there were no differences observed for individual axon densities (Fig. 11B), or putative vagal efferent terminal numbers (Fig. 11E).

Vagal Innervation in the stomach of BDNF-deficient mice as compared with wild types at P3 - 6. Qualitative observations made by direct inspection of images from each of the sampled points revealed a persistence in some of the striking differences present in gastric vagal afferents at P0, whereas others appeared to be recovering toward wild-type patterns. In particular, the abnormal organization of vagal innervation observed in the antrum of *bdnf* $^{-/-}$ mice at P0 was still present at P3 – 6, but to a lesser degree (cf. Fig. 12A,B). In contrast, the process that resulted in aberrant morphology of putative IMA precursors in the forestomach of mice at P0 appeared to have continued to progress by P3-6. While some IMAs in P3-6 *bdnf* $^{-/-}$ specimens were in part similar to wild types, having longer, more mature processes than at P0, other IMAs had telodendria that appeared stunted compared to either *bdnf* $^{+/+}$ or *bdnf* $^{+/-}$ mice, and their overall density appeared reduced (cf. Figs. 9E and 12C).

Quantification of DiI-labeled vagal innervation in P3-6 *bdnf* $^{+/+}$, *bdnf* $^{+/-}$ and *bdnf* $^{-/-}$ mice over all the sampling sites of the stomach wall did not identify significant differences between these genotypes in putative IGLE precursor numbers or putative IMA precursor densities (Fig. 13C,D). Nor were there significant differences in vagal fiber bundle numbers (Fig. 13A), individual fiber densities (Fig. 13B), or putative efferent terminal numbers (Fig. 13E). However, there were trends consistent with the quantitative effects on IGLEs at P0 and the qualitative observations on IMAs at P0 and

P3-6. In particular, there was a 35% decrease in IGLE numbers in *bdnf* $^{-/-}$ compared to *bdnf* $^{+/+}$ mice, and a 42% decrease in IMA density.

Quantitative comparisons of vagal innervation among *bdnf* $^{+/+}$, *bdnf* $^{+/-}$ and *bdnf* $^{-/-}$ mice within each stomach compartment did not reveal significant differences for any of the DiI-labeled vagal elements examined (Fig. 14). However, there did appear to be strong trends across genotypes for putative IMAs (p=.06) and IGLEs (p=.15), especially in the antrum. For example, there was a 75% decrease in putative IMA density (Fig. 14D) and almost a 50% decrease in putative IGLE numbers (Fig. 14C) in the antrum of *bdnf* $^{-/-}$ mice compared to wild types. In contrast, efferent terminals were relatively unaffected (Fig. 14E). Additionally, IMA density in the forestomach showed trends toward reduction in both *bdnf* $^{+/-}$ (50%) and *bdnf* $^{-/-}$ (37%) mice relative to wild types (Fig. 14D).

Similar to vagotomies performed at P0 in *bdnf* $^{+/+}$, *bdnf* $^{+/-}$, and *bdnf* $^{-/-}$ mice to verify vagal origin of DiI labeling, those done for each genotype at P3-6 also prevented DiI diffusion in vagal axons from reaching the stomach in all instances (not shown).

DISCUSSION

Brain-derived neurotrophic factor, which is expressed in the stomach wall, in associated blood vessels and in the nodose ganglion during development (Ernfors et al., 1992; Fox and Murphy, 2008) supports survival of a large proportion of vagal sensory neurons (Conover et al., 1995; ElShamy and Ernfors, 1997; Erickson et al., 1996; Jones et al., 1994; Liu et al., 1995). The present study investigated the hypothesis that BDNF is essential for survival of neurons supplying vagal sensory

innervation of the stomach wall, and may have roles in axon guidance and mechanoreceptor differentiation. To examine this hypothesis, DiI was employed to label vagal projections to the early postnatal stomach wall in P0 BDNF KO mice and their heterozygous and wild-type littermates. First, vagal gastric innervation was examined in wild-type mice. At P0, vagal fibers, bundles, efferent terminals and putative IGLE and IMA precursors were present throughout the stomach wall, but the adult distributions of these mechanoreceptors across compartments were not evident yet and the morphology of these receptors was immature. By P3-6 the morphology of IMAs and IGLEs became slightly more mature and although IMAs had begun to concentrate in the forestomach as in adults, the IGLE distribution still had not made major strides toward maturation. Examination of this innervation in BDNF KO mice at P0 surprisingly revealed the presence of a significant amount of vagal stomach wall innervation. Nevertheless, consistent with our hypothesis, BDNF KO resulted in the loss of about 50% of IGLEs, an effect that was sensitive to gene dosage. Further, this appeared to be a direct effect of BDNF deficiency on IGLEs since myenteric neurons, which are the IGLE target cells in the gut wall, were normal in number and distribution. Additionally, BDNF deficiency was associated with aberrant morphology of some forestomach IMAs and abnormal organization of vagal innervation in portions of the antrum. To aid evaluation of these partial effects of BDNF KO, we examined whether they were maintained, strengthened or weakened at P3-P6. Effects on IGLEs and on organization of antrum innervation were still present, but they were reduced in magnitude. In contrast, there were still some IMAs with abnormal morphology and a large trend toward reduced IMA density had developed. These results suggest that BDNF contributes to

the survival of vagal sensory neurons that give rise to subpopulations of IGLEs and possibly IMAs that innervate the stomach, and may also play roles in axon guidance and IMA differentiation.

Organization of vagal stomach innervation in wild-type mice at early postnatal ages

Adult rats and mice have similar distributions of IMAs and IGLEs in the stomach (cf. Fox et al., 2000; Wang and Powley, 2000). In adult mice of a similar genetic background to those used in the present study, 50% of all IGLEs were present in the corpus, whereas progressively fewer occurred in the forestomach (36%) and antrum (14%). In contrast, circular and longitudinal IMAs were concentrated in the forestomach, with 86 and 100% occurring there, respectively, and the remaining 12 and 2% circular IMAs in the corpus and antrum, respectively. At P0 of the present study, there were no significant differences across stomach compartments for IMA density or IGLE number, or any other elements measured, which suggests that mature distributions have not yet begun to take shape. In contrast, the distribution of myenteric neurons was similar to that observed in adult male Fischer 344 rats. Their density was highest in the antrum and progressively lower in the corpus and then the antrum.

At the P3-6 ages examined in the present study the stomach had become larger, and the morphology and distributions of putative mechanoreceptors became more mature as compared with those observed at P0. For example, IGLEs became larger, making their laminar structure more apparent. Moreover, while there was not much increase in nerve fiber density, or numbers of axon bundles, IGLEs, or efferent terminals, IMA density had more than doubled, suggesting rapid growth of their

telodendria occurred as they matured from P0 to P3-6. At P3-6 in the present study, the distribution of IGLEs did not appear to be closer to the mature distribution than they were at P0 as there was no significant difference in their numbers across stomach compartments and the greatest concentration of IGLEs was in the antrum (41%) rather than the corpus (26%). However, the distribution of IMAs was closer to the adult pattern than at P0, with the majority (51%) in the forestomach and about 25% in each of the other compartments, and these differences were statistically significant. These results show that growth, maturation, and differentiation of vagal mechanoreceptors continue after birth, consistent with our previous qualitative observations made at several embryonic and postnatal ages (Murphy and Fox, 2007). Presumably, IGLEs and IMAs achieve their mature distributions and morphology by approximately P10 as has been observed in rats (Swithers et al., 2002).

<u>BDNF regulates survival of gastric IGLEs</u>

BDNF has been established as a survival factor for vagal sensory neurons, which reside in the nodose ganglion. Mice that lack BDNF exhibit up to 59% loss of these neurons (Conover et al., 1995; ElShamy and Ernfors, 1997; Erickson et al., 1996; Jones et al., 1994; Liu et al., 1995). While this loss in BDNF KO mice has been known for some time, and the target organs of large populations of NT-3- and NT-4-dependent vagal sensory neurons that innervate the GI tract have been identified, none of the GI targets of BDNF-dependent neurons had been determined. In fact, the only BDNF-dependent vagal sensory neurons that had been identified to date were those that supply arterial baroreceptor and chemoreceptor afferents (Brady et al., 1999; Erickson

et al., 1996), and they do not fully account for the large population of BDNF-dependent neurons. The results of the present study suggest that in addition to the subpopulation of BDNF-dependent vagal sensory neurons that innervate the arterial system, there is another subpopulation that constitutes a large proportion of the IGLEs that supply the stomach wall.

Approximately half of the IGLEs were absent from the stomach of BDNF KO mice at P0, an effect that was sensitive to gene dosage - the number of intact BDNF alleles present. A similar gene-dosage effect has been observed for the survival of the BDNF-dependent neuron population in the nodose and petrosal ganglia (Erickson et al., 1996), consistent with IGLEs being a subset of them. The loss of this subpopulation of gastric IGLEs in BDNF-deficient mice suggests that BDNF is required for their survival. Moreover, it is consistent with the apparent dependence of most small intestinal IGLEs on NT-4 (Fox et al., 2001b) and esophageal IGLEs on NT-3 (Raab et al., 2003) for survival. Similar to the lack of effect of neurotrophin deficiency on myenteric neuron survival in each of these studies, we found no effect of BDNF KO on myenteric neuron number, suggesting the reduction of putative IGLEs was not secondary to myenteric neuron loss. At P3-6, although the trend of IGLE loss persisted in BDNF KO mice, it was reduced in magnitude and was no longer statistically significant. Interestingly, at these ages the loss of IGLEs was greatest in the antrum, almost reaching significance in BDNF KO's as compared with wild types. This could imply that the BDNF expressed in the antrum smooth muscle normally contributes to the survival of these IGLEs.

The loss of only about half of the putative IGLE precursors in BDNF KO mice at P0 could imply that BDNF provides support for only a subgroup of IGLEs. Consistent

with this hypothesis there is evidence for the existence of different IGLE populations. For example, NT-4 KO resulted in loss of most IGLEs from the small intestine, but a small proportion of them survived and virtually all of them innervating the stomach survived, suggesting they had different properties from those that were lost (Fox et al., 2001b). Also, phenotypic differences in IGLE morphology and function have been observed. In particular, gastric IGLEs exhibit a different morphology than small intestinal ones (Jarvinen et al., 1998). Additionally, some gastric and esophageal IGLEs have shown to be capsaicin-resistant, whereas the remaining IGLEs in these organs and most in the intestine are lost after capsaicin treatment (Berthoud et al., 1997b), consistent with each group being a different type of IGLE. Furthermore, mechanosensitive gastric afferents that expressed TRPV1, and thus were capsaicin-sensitive had a lower threshold for mechanical activation and responded to stomach stretch, whereas TRPV1-negative afferents had higher thresholds and did not respond to stretch (Bielefeldt et al., 2006). Thus, it is possible that BDNF-dependent IGLEs correspond to one of these phenotypically distinguishable IGLE populations.

Alternatively, it is possible that another factor expressed during development compensated for BDNF deficiency, preventing a more severe loss of putative IGLEs at P0. A good candidate for such a factor is another neurotrophin, NT-3. NT-3 KO results in a 41% loss of vagal sensory neurons and BDNF KO a 59% loss (ElShamy and Ernfors, 1997). If these NT-3- and BDNF-dependent populations are separate, then KO of both genes in one mouse should have additive effects and thus a loss of 100% of nodose neurons would be predicted. However, such *bdnf* $^{-/-}$ / *ntf-3* $^{-/-}$ double KOs exhibited a 66% reduction, which is only a 7% increase in addition to the loss produced

by BDNF KO, suggesting there is large overlap of the vagal sensory neuron populations supported by these neurotrophins (ElShamy and Ernfors, 1997). One possible explanation for this dual dependence is that some sensory neurons switch dependence from one neurotrophin to another as they progress from one stage of development to another, and in fact, the timing of cell loss from the nodose ganglion in BDNF and NT-3 KO mice is consistent with this possibility (ElShamy and Ernfors, 1997). The potential for NT-3 to compensate for BDNF deficiency is further supported by the overlapping expression patterns of NT-3 and BDNF in the stomach wall, associated blood vessels, and nodose ganglion (Ernfors et al., 1992; Fox, 2006; Fox and Murphy, 2008), and by the capacity for NT-3 to activate trkB, the high affinity receptor for BDNF (Barbacid, 1994). Moreover, the levels of neurotrophins, and in some instances NT-3 specifically, are upregulated in response to various factors or situations, including hormones (Krizsan-Agbas et al., 2003), inflammation (Woolf, 1996), neural injury (Friedman et al., 1996), and decreased signaling by other neurotrophins present in the same organ (Harrison et al., 2000). Presumably similar upregulation could result from decreased signaling due to reduced neurotrophin levels that occur with a knockout. Thus, if reduced BDNF levels in the stomach wall, associated blood vessels, or nodose ganglion resulted in increased NT-3 levels in any of these tissues, then this excess NT-3 would be available to support survival of nerve fibers in addition to those it normally supports. Consistent with these possibilities, Elshamy et al. (1996) found that even though a large population of sympathetic neurons failed to survive in NT-3 KO mice, some of the target organs exhibited normal innervation density, probably as a result of compensation by NGF or another factor supporting expansion of the innervation derived from the

surviving neurons. One approach for testing whether NT-3 or other factors can compensate for BDNF deficiency would be to investigate the effects of *bdnf* $^{-/-}$ / *ntf-3* $^{-/-}$ double KO (or double KO of BDNF and one of the other factors expressed in the stomach wall) on IGLE development. If NT-3 compensates for BDNF deficiency, its ability to do so should be compromised by the reduction of NT-3 levels in double mutants and therefore should result in increased loss of IGLEs as compared with the loss in BDNF KOs. This strategy successfully revealed the ability of NT-4 and BDNF to compensate for one another in supporting survival of neurons in the nodose and petrosal ganglia (Erickson et al., 1996).

If NT-3 or another factor did compensate for the lack of BDNF in supporting IGLE survival it might also account for the reduced gastric IGLE loss observed in BDNF KO mice at P3-6 (35%) versus P0 (50%). In fact, it is possible that BDNF deficiency in these older postnatal BDNF mutants did not result in as much IGLE loss or the same degree of abnormal antrum innervation as it did at P0 because these mice were even more effective at compensating for BDNF deficiency. For instance, most BDNF KO mice die shortly after birth. The occasional exceptions that do survive longer may do so because they do not experience as severe effects of BDNF deficiency as those that die at birth. One reason for these reduced effects could have been differences in their background genes that help protect against the effects of BDNF deficiency. Therefore, the mice examined at P3-6 may have belonged to a select group of BDNF KO mice, which had already experienced weaker-than-normal effects of BDNF deletion by P0. In fact, the variability observed in our P0 sample is consistent with this interpretation since a small percentage of P0 BDNF KO mice exhibited loss of IGLEs in the range observed at P3-6.

BDNF may regulate IMA differentiation or survival

A second deleterious effect of BDNF deficiency was on IMA development. At P0 the density of putative IMAs was similar to that of wild types, suggesting their survival, at least until this age, was not dependent on BDNF. However, a subset of the putative IMA precursors identified in the forestomach of BDNF homozygous mutants at this age displayed abnormal morphology. In particular, their telodendria appeared stunted, as if their growth was halted at an early stage of formation. By P3-6, some stunted telodendria were still present in BDNF KO mice, but additionally a strong trend toward decreased IMA density (42%) had developed, which might in part have reflected loss of the stunted putative IMAs present at P0. However, additional IMAs must have been lost as well since this trend was observed across all stomach compartments. It appeared that the main reason the substantial loss of IMAs did not reach statistical significance was due to large variation in IMA density among wild types, probably due to the small group size at P3-6.

These data suggest that BDNF may be required for specific aspects of IMA differentiation, including growth of terminal processes. Of all the elements measured in wild-type mice, the only large change that occurred between P0 and P3-6 was a significant increase in IMA density, indicating IMAs are in a dynamic phase of growth at this stage, which may include lengthening of telodendria and the formation of new ones. Therefore, in BDNF-deficient mice it is possible that the apparent failure of this IMA telodendria growth in a substantial subpopulation of IMAs ultimately resulted in loss of the affected IMAs, for example, by preventing them from obtaining sufficient levels of

growth factors. Alternatively, or in addition, BDNF may be required for survival or maintenance of this subgroup of IMAs. During early postnatal development BDNF is present in the antrum and portions of the corpus compartments of the stomach, but it is not expressed in the forestomach. This suggests that the effects on IMAs in the forestomach, which cannot have been due to loss of BDNF derived from the stomach wall, may have resulted from loss of BDNF normally expressed in the nodose ganglion, or in the blood vessel walls along which IMA axons grow to reach the stomach. In contrast, IMA loss from the antrum and portions of the corpus that express BDNF could have been due to BDNF deficiency in these tissues, as well as in the nodose ganglion or blood vessels. In fact, at P3-6, the loss of IMAs in BDNF KO mice as compared with wild types was greatest in the antrum, nearly reaching significance, consistent with a role for BDNF expressed in the wall of the antrum. Finally, the apparent trend of decreasing IMA density between P0 and P3-6 suggests that NT-3 or any other factors present in the stomach wall, blood vessel walls or nodose ganglion were not able to compensate for the effects of loss of BDNF from these tissues on IMAs.

Previous work in other systems is consistent with the possibilities of BDNF involvement in IMA differentiation, survival or maintenance. In culture, BDNF has been shown to stimulate increases in the length and number of growth cone filopodia (Fass et al., 2004). Moreover, Miyamoto et al. (2006) found that BDNF influences cellular morphology by activating endogenous Rho GTPases, which results in alteration of the actin cytoskeleton. More recently, another group identified geranylgeranyl-transferase I (GGT), which is activated by BDNF and mediates lipid modification, prenylation, as contributing to BDNF effects on the Rho GTPases (Zhou et al., 2008). Further, evidence

from the somatosensory system, including mechanoreceptors – a receptor type that may share properties with vagal mechanoreceptors (Fox, 2006) – has demonstrated that BDNF can have powerful control of the structure or survival of peripheral sensory nerve terminals. *In vitro* studies of embryonic DRG neurons (Lentz et al., 1999) found that BDNF significantly increased the terminal branching of subsets of these neurons. *In vivo* studies are consistent with these results since BDNF KO mice most often show decreases in size and density of the terminal fields of cutaneous receptors (including mechanoreceptors), whereas mice that overexpress BDNF in skin show increases (LeMaster et al., 1999; Rice et al., 1998). Additionally, similar to the present observations of IMA deterioration after initial formation, NT-3 or NT-4 deficiency resulted in loss of cutaneous mechanoreceptors that had developed normally until birth, or until a few weeks after birth, but were subsequently lost (Airaksinen et al., 1996; Stucky et al., 2002).

BDNF may regulate vagal axon guidance

Disorganized patterns of innervation in some regions of the antrum, including abnormal arrangement of myenteric axon bundles and increased density of growth cones or individual axons occurred in P0 BDNF KO mice and were still present although to a lesser degree at P3-6. This temporal pattern suggests that a factor may be compensating for BDNF deficiency to support development of antrum innervation as discussed above for IGLEs. Interestingly, the locus of this abnormal patterning largely coincided with the stomach region that expresses BDNF in the smooth muscle layers and the lamina propria of the mucosa. This raises the possibility that this BDNF

normally contributes to regulation of development of antrum innervation by acting as a guidance factor or chemoattractant. In particular, BDNF secreted from the antrum muscle wall adjacent to the axon bundles of the myenteric plexus could influence their formation, and thus loss of BDNF could disrupt this process. Also, BDNF secreted by the lamina propria of the antral mucosa may normally guide or attract axons to leave the myenteric plexus and enter the mucosa. Consequently, loss of BDNF from this tissue may have resulted in failure of some of these axons to exit the muscle layer, leaving them to wander in search of their missing guidance cue, or to sprout excess terminals. Similar disruptions of normal gastric sensory vagal organization in the stomach have been observed in c-Kit and steel mutant mice that lack ICC-IMs, the target cells of IMAs in the forestomach (Fox et al., 2002; Fox et al., 2001a). Consistent with this interpretation, numerous studies, mostly *in vitro*, but some *in vivo* have suggested BDNF has both axonal growth and guidance properties. *In vitr*o studies have demonstrated an ability of BDNF to produce neurite outgrowth from cultured neurons, including sensory ganglia (Li et al., 2005; Rochlin et al., 2000; Song et al., 2008; Ulmann et al., 2007), whereas *in vivo* manipulations of BDNF levels have shown that BDNF may be necessary for axons to find and enter a target tissue (Ernfors et al., 1994a; Fritzsch et al., 2005; Hellard et al., 2004; Krimm, 2007). Most recently, Krimm and colleagues (Ma et al., 2009) have shown that it is in fact the target-derived BDNF expressed in the gustatory epithelium that is necessary for gustatory axons to correctly localize to and innervate fungiform papillae.

Finally, the fact that changes in myenteric neuron or vagal efferent terminal number were not observed between wild-type and BDNF mutant mice suggests that the effects of BDNF deficiency on developing vagal gastric innervation were due to direct and specific effects of BDNF loss on the affected elements rather than to indirect or non-specific effects. However, these findings do not completely exclude the possibility that the primary effects of BDNF deficiency were on elements that were not directly examined such as the muscle wall itself, afferent or efferent sympathetic gastric innervation, or gastric myenteric axons or terminals. If such effects occurred, any or all of the changes we observed in vagal innervation could be secondary to them.

Significance and Future Directions

The present study represents the first examination of the role of BDNF in development of vagal sensory innervation of the stomach and lays the groundwork for future investigations of other factors affecting GI innervation. Importantly, it demonstrates that it is possible to use DiI labeling of vagal nerves in postmortem GI tissues during key phases of development to quantify elements of both normal and disrupted vagal innervation and to detect differences between them. In particular, among future studies of factors regulating vagal development it will be important to examine whether NT-3 or other factors compensate for BDNF deficiency. Additionally, targeting BDNF KO to the stomach wall or GI blood vessel walls by employing a conditional KO strategy could be used to test the hypothesis that these sites of BDNF expression play a role in vagal axon guidance or IGLE or IMA survival. Similarly, restricting BDNF KO to the nodose ganglion could be used to examine the contributions

this site of BDNF expression to these developmental roles. Moreover, targeted KO mice with altered vagal afferent development could be used to probe the roles of the altered elements in feeding behavior. Vagal afferents play key roles in meal patterning, especially determination of meal size through their activation of vago-vagal reflexes and signaling to feeding regulatory circuits in the brain. In fact, disruption of neurotrophic support (e.g., NT-4 KO) altered vagal sensory innervation of the GI tract and in parallel had significant effects on meal size and duration (Fox et al., 2001b). Since BDNF mutants die around the time of birth their feeding behavior cannot be studied, but restricting BDNF KO to the GI tract has resulted in viable mice that permit such examination (Fox, unpublished results). Further, the targeted KOs would minimize the contribution of altered BDNF levels in tissues outside the GI tract such as the brain to any changes in feeding behavior. Finally, the present evidence for BDNF roles in postnatal vagal afferent development at ages when sensory pathways become vulnerable to environmental influences, suggests a means by which changes in the perinatal environment could affect GI innervation. For example, any environmental factor that produced significant changes in the level, location or timing of BDNF expression (or expression of other factors essential to vagal sensory neuron development) could affect vagal mechanoreceptor formation.

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FIGURE LEGENDS

Fig. 1. The top left panel is a schematic diagram that illustrates the DiI (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) application site. A single DiI crystal was applied to the anterior vagal trunk at the bifurcation of the hepatic and gastric branches as they course along the abdominal esophagus. This location was determined to be optimal for comprehensive labeling of vagal innervation of the stomach wall (Murphy and Fox, 2007). The bottom panel is a schematic diagram that illustrates the sampling and counting method modified from Wang and Powley (2000) and Fox et al. (2000). Based on length and width of each ventral stomach wall sampled, eighty sampling points were established at the intersections of a sampling grid that consisted of 8 equidistant rows and 10 equidistant columns. The microscope objective was systematically moved from one intersection to the next, and the underlying tissue imaged. Then a counting grid was laid over each successive optical section of the z-series collected at each sampling site and used to quantify vagal fibers, bundles and terminals. Sampling points with less than half of the imaged area containing stomach tissue were not included in the quantification and are represented by gray circles in the sampling grid portion of the schematic. The top right panel is a schematic diagram adapted from Swithers et al. (2002) that illustrates where the estimated boundaries of three major stomach compartments were located in early postnatal mice and how the sampling sites were distributed among them. The antrum included columns 1-3 (light shading), the corpus columns 4-6 (dark shading), and the forestomach columns 7-10

(medium shading).

Fig. 2. Putative Intraganglionic laminar ending (IGLE; A,B) and intramuscular array (IMA; C,D,E) precursors and mucosal/submucosal processes and terminals (F) labeled with DiI in the stomach wall of wild-type P0 mice. (A) A confocal image illustrating putative IGLE precursors (in outlined area) in the forestomach. At this age IGLEs are small and often consist of only a few terminal processes that vary from small puncta to relatively large growth cone-like structures. (B) Higher magnification image of the area outlined in A that consists of optical sections restricted to a tissue plane immediately below a myenteric ganglion that contains the putative IGLE leaves (arrows). (C) A confocal image taken from the forestomach that includes optical sections through the longitudinal muscle, myenteric plexus and circular muscle layers that contain IMAs and myenteric vagal axons and fiber bundles. (D) A confocal image of the same region as in C, but optical sections were restricted to the circular muscle layer containing putative IMA precursors (arrows). (E) A confocal image of the same region as in C and D, but optical sections were restricted to the longitudinal muscle layer containing putative IMAs (arrows). (F) Vagal fibers (arrows) and free endings (arrowheads) with imaging restricted to the mucosal and submucosal layers of the forestomach. Scale bars = 25μm (A, C-F) or 10μm (B).

Fig. 3. The organization of vagal fibers and myenteric axon bundles is different in each of the three major stomach compartments at P0. (A-C) Confocal images of DiI labeled vagal bundles (arrows), fibers (smaller open arrows), and efferent terminals in myenteric ganglia (arrowheads) within the antrum (A), forestomach (B), and corpus (C). In the antrum fiber bundles were large in diameter and along with efferent terminals were

tightly packed, whereas in the forestomach axon bundles were smaller in diameter and along with fibers were more loosely organized. Organization of fibers, axon bundles and efferents and in the corpus was similar to the forestomach, but additionally large diameter bundles extended from the cardia. Scale bars = 25μm.

Fig. 4. Quantitative comparisons of DiI-labeled vagal elements among the antrum, corpus and forestomach of wild-type mice at P0. The quantified elements included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). Fewer of each of these five elements was present in the antrum as compared with the corpus and forestomach, but none of these trends reached significance.

Fig. 5. Quantitative comparisons of DiI-labeled vagal elements among wild types and heterozygous or homozygous BDNF mutants at P0. Elements were quantified over the entire ventral stomach wall and included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). IMA density in D plots circular and longitudinal IMAs separately and combined (total); the histogram bar shading represent the genotypes in a similar manner as in the other panels. The only significant difference was a 50% reduction of putative IGLEs in BDNF -/- mice compared with wild types. * Significantly different from wild type, $p<0.05$.

Fig. 6. At P0, BDNF KO was associated with altered vagal innervation of the antrum (A,B) and changes in the morphology of developing putative IMAs in the forestomach (C,D). In BDNF KO mice vagal innervation of the antrum appeared to become disorganized as compared with wild types and heterozygous mutants. In particular,

spacing between myenteric ganglia was reduced and there was an increase of growth cone-like structures. (A,B) Confocal images illustrating the normal organization of vagal fibers, bundles and ganglia in the antrum of a wild-type mouse (A), and the apparent disorganization of these elements in a BDNF KO mouse (B). (C) Confocal image that includes optical sections restricted to the circular muscle layer, demonstrating normal IMA structure (arrows) observed in the forestomach of a wild-type mouse. Normal IMA telodendria are long rectilinear processes. (D) Similarly restricted confocal image as in A, illustrating the shorter, larger diameter IMA telodendria (arrows) present in some regions of the forestomach of a BDNF KO mouse. Scale bars = 25μm.

Fig. 7. Quantitative comparisons of DiI-labeled vagal elements grouped by stomach compartment (antrum, corpus and forestomach) among wild types (open bars) and heterozygous (gray bars) or homozygous (black bars) BDNF mutants at P0. The quantified elements included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). There were no significant effects of BDNF deficiency on quantification of vagal elements within any of the stomach compartments. Nevertheless, it did appear that for putative IGLEs there were trends toward gene-dosage dependent reductions of their numbers in all stomach compartments.

Fig. 8. Analysis of gastric myenteric neurons. (A) Brightfield photomicrograph of a myenteric ganglion in the stomach muscle wall of a wild-type P0 mouse stained with cuprolinic blue (three neuron cell bodies are indicated by arrows). The differentiation of the stain from background was not as prominent as in the adult GI muscle wall, but was sufficient to identify neurons according to the criteria. Scale bar = 25μm. (B,C)

Quantification of myenteric neurons stained with cuprolinic blue at P0 among wild types and heterozygous or homozygous BDNF mutants, grouped by whole stomach (B) or by stomach compartment (C; antrum, corpus and forestomach). The histogram bar fills represent the genotypes in a similar manner as in B. When grouped by whole stomach neither heterozygous nor homozygous mutants were different from wild types (B). However, the decreased counts in heterozygotes compared with wild types were significantly different from the increased counts in homozygotes. This difference was mainly due to the significant difference between these groups in the antrum (C). Further, when grouped by stomach compartment a progressive reduction in the number of neurons present in the corpus and then forestomach as compared with the antrum was apparent for each of the three genotypes (C). # Significant difference between heterozygous and homozygous BDNF mutants at $p<0.05$ level.

Fig. 9. Putative IGLE (A,B) and IMA (C,D,E) precursors and mucosal/submucosal processes and terminals (F) labeled with DiI in the stomach wall of wild-type P3-6 mice. (A) A confocal image illustrating putative IGLE precursors (in outlined area) in the forestomach. At this age IGLEs were slightly larger than at P0 and often began to take on a more laminar appearance, but otherwise had not changed significantly in structure. (B) Higher magnification image of the area outlined in A that consists of optical sections restricted to the tissue plane that contained the putative IGLE leaves, which was immediately below a myenteric ganglion. (C) A confocal image taken from the forestomach that includes optical sections through the longitudinal muscle, myenteric plexus and circular muscle layers and thus contains putative IMAs and vagal axons and fiber bundles. At this age IMA telodendria appeared to have increased in number and

length as compared with P0. (D) A confocal image of the same region as in C, but optical sections were restricted to the circular muscle layer containing putative IMA precursors (arrows). (E) A confocal image of the same region as in C and D, but optical sections were restricted to the longitudinal muscle layer containing putative IMAs (arrows). (F) Vagal fibers (arrows) with imaging restricted to the mucosal and submucosal layers of the forestomach. Scale bars = 25μm (A, C-F) or 10μm (B).

Fig. 10. The organization of vagal fibers and myenteric axon bundles was different in each of the three major stomach compartments at P3-6. (A-C) Confocal images of DiI labeled vagal bundles (arrow), fibers (open arrow), and efferent terminals in myenteric ganglia (arrowheads) within the antrum (A), forestomach (B), and corpus (C). Although the vagal innervation pattern in each stomach compartment had matured slightly by P3-6 as compared with P0, the differences described between compartments at P0 were largely maintained at P3-6. Scale bars = 25μm.

Fig. 11. Quantitative comparisons of DiI-labeled vagal elements among the antrum, corpus and forestomach of wild-type mice at P3-6. The quantified elements included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). The forestomach contained more fiber bundles than the corpus and antrum, but only the trend for the antrum was significant (*, $p<0.05$). In contrast, the approximate doubling of IMA density in the forestomach as compared with the corpus and antrum was significant for both of these compartments (#, $p<0.05$).

Fig. 12. At P3-6, BDNF KO was associated with altered vagal innervation of the antrum (A,B) and changes in the morphology and density of developing putative IMAs in the

forestomach (C,D). In BDNF KO mice vagal innervation of the antrum still appeared to be disorganized and denser compared with wild types and heterozygous mutants, but to a lesser degree that observed at P0. (A,B) Confocal images illustrating these differences between the antrum of a wild-type mouse (A), and a BDNF KO mouse (B). The changes in IMAs observed at P0 appeared to have progressed in P3-6 mice. Some IMA telodendria were stunted, but normal diameter, and those that appeared normal in structure were decreased in density, suggesting some had failed to survive. (C) Confocal image that includes optical sections restricted to the longitudinal muscle layer, illustrating the shorter IMA telodendria (arrows) and reduced IMA density observed in the forestomach of a BDNF KO mouse. To compare these IMAs with those of a wild type see Fig. 9E, which is a similarly restricted confocal image from a wild-type at this age. Scale bars = 25μm.

Fig. 13. Quantitative comparisons of DiI-labeled vagal elements among wild types and heterozygous or homozygous BDNF mutants at P3-6. Elements quantified over the entire ventral stomach wall included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). A trend toward reduced putative IGLE numbers was still present in BDNF KO mice compared to wild types, but was smaller than at P0 and was not significant. Additionally, a new trend developed toward a substantial reduction in putative IMA density BDNF KO mice compared with wild types, but it was not significant either.

Fig. 14. Quantitative comparisons of DiI-labeled vagal elements grouped by stomach compartment (antrum, corpus and forestomach) among wild types (open bars) and

heterozygous (gray bars) or homozygous (black bars) BDNF mutants at P3-6. The quantified elements included axon bundle number (A), individual fiber density (B), putative IGLE precursor number (C), putative IMA precursor density (D), and efferent terminal number (E). There were no significant effects of BDNF deficiency on quantification of vagal elements within any of the stomach compartments. However, trends suggested that the reduced IGLE numbers in BDNF KO mice occurred mainly in the antrum and corpus, as compared with all compartments at P0. In contrast, IMA density exhibited trends toward reduction in all stomach compartments in BDNF KO mice, but only in the corpus and forestomach in heterozygous BDNF mutants, and these trends almost reached significance in the antrum $p = 0.15$, and forestomach, $p = .06$.