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Sympathetic axonopathies and hyperinnervation in the small intestine smooth muscle of aged Fischer 344 rats

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Abstract

It is well documented that the intrinsic enteric nervous system of the gastrointestinal (GI) tract sustains neuronal losses and reorganizes as it ages. In contrast, age-related remodeling of the extrinsic sympathetic projections to the wall of the gut is poorly characterized. The present experiment, therefore, surveyed the sympathetic projections to the aged small intestine for axonopathies. Furthermore, the experiment evaluated the specific prediction that catecholaminergic inputs undergo hyperplastic changes. Jejunal tissue was collected from 3-, 8-, 16-, and 24-month-old male Fischer 344 rats, prepared as whole mounts consisting of the muscularis, and processed immunohistochemically for tyrosine hydroxylase, the enzymatic marker for norepinephrine, and either the protein CD163 or the protein MHCII, both phenotypic markers for macrophages. Four distinctive sympathetic axonopathy profiles occurred in the small intestine of the aged rat: (1) swollen and dystrophic terminals, (2) tangled axons, (3) discrete hyperinnervated loci in the smooth muscle wall, including at the bases of Peyer's patches, and (4) ectopic hyperplastic or hyperinnervating axons in the serosa/subserosal layers. In many cases, the axonopathies occurred at localized and limited foci, involving only a few axon terminals, in a pattern consistent with incidences of focal ischemic, vascular, or traumatic insult. The present observations underscore the complexity of the processes of aging on the neural circuitry of the gut, with age-related GI functional impairments likely reflecting a constellation of adjustments that range from selective neuronal losses, through accumulation of cellular debris, to hyperplasias and hyperinnervation of sympathetic inputs.

Keywords

CD163; Gastrointestinal; MHCII; Muscularis; Myenteric; Resident Macrophages

1. Introduction

The intrinsic neural circuitry, or enteric nervous system, of the gastrointestinal (GI) tract sustains neuronal losses and reorganizes extensively as it ages (Phillips and Powley, 2007; Camilleri et al., 2008). These changes include progressive and selective loss of cholinergic neurons (Phillips et al., 2003; Abalo et al., 2005; Thrasivoulou et al., 2006; Bernard et al., 2009) and associated glia (Phillips et al., 2004), extensive accumulation of dystrophic neurites (Baker and Santer, 1988; Phillips et al., 2006; Phillips and Powley, 2007), and

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conspicuous aggregation of cellular debris, including deposits of the proteins alpha synuclein (Braak et al., 2006; Phillips et al., 2009, 2013) and hyperphosphorylated tau (Phillips et al., 2009).

In contrast to the detailed picture of the effects of aging on the intrinsic enteric circuits, age-related remodeling of the extrinsic inputs--specifically the sympathetic postganglionic projections--to the GI tract has not been thoroughly examined. Initial observations on the sympathetic inputs indicate that noradrenergic projections to the aged gut wall evidence reductions in the number of axonal varicosities and in the intensity of glyoxylic-acid-induced fluorescence (Baker and Santer, 1988) as well as parallel decreases in expression of tyrosine hydroxylase (TH; Phillips et al., 2006). Furthermore, past experiments have described age-related development of dystrophic and swollen noradrenergic axons both in the myenteric plexus and smooth muscle (Baker and Santer, 1988; Phillips et al., 2006).

The limited assessments of the effects of age on the efferent sympathetic projections to the GI tract have not, however, effectively evaluated the tenable and potentially instructive hypothesis that age-related reorganization of the sympathetic nervous system in the gut involves hyperinnervation of some GI tissues. Though this possibility has not been thoroughly evaluated in the aged gut, in other organs and tissues, noradrenergic fibers of the sympathetic nervous system are prone, in aging as well as in related diseases and traumas, to reorganize by hyperinnervation with different forms of dysplastic and ectopic projections (e.g., brain: Di Giulio et al., 1989b; heart: Hassankhani et al., 1995; lung: Hoyle et al., 1998; adipose tissue: Straub et al., 2011; blood vessels: Luff et al., 2005). Although not previously investigated systematically, similar plastic processes may also occur in the aged GI tract. Indeed, Belai et al. (1995) noted an increased density in noradrenergic fibers in the sphincters of the aged rat GI tract consistent with a hyperinnervation hypothesis. And, additionally, in diabetes, which often involves neuropathies similar to those associated with aging, Di Giulio and colleagues (1989a) observed that sympathetic axons hyperinnervate the duodenum (though apparently not jejunum).

The present experiment, therefore, evaluated the hypothesis that age-related changes in the sympathetic projections to the GI tract include hyperinnervation of the target tissues. To evaluate this idea, we employed a whole mount protocol that preserved key target tissues of the gut wall (i.e., the layers of smooth muscle and the myenteric plexus), permanent labeling immunohistochemistry that avoided complications commonly associated with fluorescent markers, and systematic sampling algorithms that would increase the likelihood of identifying hyperplastic terminal fields and ectopias. Additionally, the present survey employed double-labeling immunohistochemistry for TH-positive axons and immune cells to determine whether macrophage density might be interrelated with sympathetic axonopathies. The premise for this line of inquiry was based both on our earlier observations of macrophages in association with dystrophic fibers in the GI tract (Phillips and Powley, 2012; Phillips et al., 2013) and on the reports by others of sympathetic hyperinnervation in organs other than the gut (e.g., Hassankhani et al., 1995; Hoyle et al., 1998; Rush, 1997), in part, being orchestrated by activated macrophages (Wernli et al., 2009).

2. Materials and methods

2.1. Subjects

Virgin male Fischer 344 (F344; n = 42) rats were obtained at 3 (n = 14), 8 (n = 10), 16 (n = 6), and 24 (n = 12) months of age from Harlan Laboratory (Indianapolis, IN) or the National Institute on Aging colony maintained at Taconic Farms (Germantown, NY; sources determined by NIH supplier contracts). To minimize stress (Nadon, 2004), rats were group housed (n=2/cage) in polypropylene cages containing sterilized, dust-free Alpha-dri bedding

(Shepherd Specialty Papers purchased through Cincinnati Lab Supply, Cincinnati, OH), Nylabones (Bio Serve, Frenchtown, NJ), and polycarbonate tunnels (Bio Serv) in a room kept at 22-24°C on a 12:12 hour light:dark schedule. Solid chow (NIH-31M; Zeigler, Gardners, PA) and tap water were available ad libitum. Conditions in the AAALAC-approved colony approximated the housing, husbandry, and barrier conditions recommended by the National Institute on Aging, but did not provide a specific pathogen-free environment. All procedures were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (8th ed., The National Academic Press, Washington, D.C.), and were approved by the Purdue University Animal Care and Use Committee.

2.2. Celiac and superior mesenteric ganglionectomy

An immunohistochemical protocol for the localization of the noradrenaline synthesizing enzyme tyrosine hydroxylase (TH) was used to identify the sympathetic innervation of the jejunum. We used an affinity purified rabbit polyclonal antibody (P40101-0; Pel Freez Biologicals, Rogers, AK) specific for the 60 k TH protein. According to the technical information provided by the manufacturer, Western blots are performed on each lot of TH antibody to confirm its specificity.

The specificity of the TH antibody used to label the sympathetic axons was further validated in the present study by surgically removing the celiac and superior mesenteric ganglion complex, which provides the majority of the efferent sympathetic innervation to the jejunum (Gillespie and Maxwell, 1971; Sclafani et al., 2003), followed by TH immunohistochemistry. The absence of TH staining in the jejunal muscularis following ganglionectomy validated the specificity of the antibody. Finally, the staining of the TH-positive innervation patterns of the myenteric plexus of surgical shams was identical to the pattern of staining previously reported by us for the same region of the intestine using an antibody to TH raised in mouse (Phillips et al., 2006).

Prior to surgery, each rat in the group to be ganglionectomized received an intraperitoneal (i.p.) injection of 5 mg of the fluorescent probe Fluoro-Gold (Fluorochrome, Inc., Denver, CO) suspended in physiological saline. Intraperitoneal injection of Fluoro-Gold labels all of the neurons in the sympathetic ganglia (Berthoud and Powley, 1993). Five days post-Fluoro-Gold injection, rats were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg). Six rats underwent sympathetic ganglionectomy consisting of the celiac and superior mesenteric ganglia being visualized under a surgical microscope using blunt dissection with forceps followed by removal of the ganglia with artery scissors (Holmes, 1953; Fu et al., 2011). To verify complete extirpation of the ganglia, the celiac and mesenteric arteries along with the descending aorta were inspected under UV light; the absence of Fluoro-Gold labeled neurons indicated a successful ganglionectomy (Berthoud and Powley, 1993). Two sham-operated controls underwent the same procedure, including i.p. injection of Fluoro-Gold, except the sympathetic ganglion complex was visualized through blunt dissection but otherwise left intact.

For pain management, rats received buprenorphine (0.02 mg/kg, i.p.) 15 min prior to surgery and then every 12 h for the next 72 h following surgery. Additionally, two 5 ml boluses of physiological saline, warmed to body temperature, were injected subcutaneously once a day for three days to prevent dehydration.

2.3. Fixation protocol and whole mount preparation

Rats were weighed, killed with a lethal dose of sodium pentobarbital (180 mg/kg, i.p.), and perfused through the left ventricle of the heart with 200 ml of 0.01 M phosphate-buffered

saline (PBS) followed by 400 ml of Zamboni's fixative. Given that the jejunal sympathetic innervation has previously been investigated in several species (e.g., rat: Baker and Santer, 1988; Phillips et al., 2006; mouse: Tan et al., 2010; guinea pig: Furness and Costa, 1974; human: Llewellyn-Smith, I.J. et al. 1984), providing useful comparisons, the present survey concentrated on the jejunum. The length of the entire small intestine was determined by measuring from the pyloric sphincter to the ileocaecal junction, and the jejunum was defined as the middle third of the small intestine (Hebel and Stromberg, 1976; Phillips and Powley, 2001). Whole mounts of the jejunum were fixed overnight in the same fixative, and then the mucosa and submucosa were removed.

2.4. Permanent immunohistochemistry

In addition to the eight rats described above (i.e., six ganglionectomized and two shams), which were single-labeled for TH, another 24 rats (n = 6/age) at 3, 8, 16, and 24 months of age were double-labeled for TH and anti-rat CD163 (MCA342R; AbD Serotec, Raleigh, NC). Finally, ten additional rats at 8 (n = 4) and 24 (n = 6) months of age were double labeled for TH and anti-rat major histocompatibility complex class II (MHCII; MCA46R; AbD Serotec).

According to the manufacturer's technical information, the AbD Serotec mouse monoclonal anti-rat CD163 antibody is purified by affinity chromatography and recognizes the rat CD163 cell surface glycoprotein, a 175 kDa molecule. The antibody is raised against rat spleen cell homogenate and is expressed by a majority of macrophages. While the antibody to MHCII is similarly an affinity purified mouse monoclonal anti-rat antibody, it is instead raised against rat thymocyte membrane glycoprotein and recognizes a monomorphic determinant of the rat RT1B MHC class II antigen present on B lymphocytes, dendritic cells, a subpopulation of macrophages, and certain epithelial cells.

Immunoperoxidase staining of free-floating whole mounts consisted of multiple rinses in 0.1 M PBS (pH 7.4), 30 min soak in methanol:H₂O₂ (4:1) to inhibit endogenous peroxidase activity, rinses in PBS, 4 d soak in normal serum block (0.5% Triton X-100, 5% normal goat serum, 2% bovine serum albumin, and 0.08% Na Azide in PBS), 24 h soak in rabbit TH (1:4000; Pel Freez Biologicals) in primary diluent (0.3% Triton X-100, 2% normal goat serum, 2% BSA, and 0.08% Na Azide in PBS), rinses in PBST (PBS + 0.3% Triton X-100), 2 h soak in biotinylated anti-rabbit IgG, raised in goat (1:500; 111-065-144; Jackson Immuno Research, West Grove, PA) in secondary diluent (0.3% Triton X-100, 2% normal horse serum, and 2% bovine serum albumin in PBS), rinses in PBS, 1 h incubation in ABC (PK-6100; Vector laboratories, Inc., Burlingame, CA) in PBS, rinses in PBS, 3 min reaction with DAB and H₂O₂ in tris buffered saline (TBS), rinses in cold dH₂O to stop the substrate reaction, rinses in PBS, overnight incubation in normal serum block, rinses in PBS, 15 min blocks in Avidin/Biotin (Avidin/Biotin Blocking Kit; SP-2001; Vector Laboratories, Inc.), 24 h soak in either mouse CD163 (1:1000; AbD Serotec) or MHCII (1:4000; AbD Serotec) in primary diluent, rinses in PBST, 2 h soak in biotinylated anti-mouse IgG, rat absorbed, raised in horse (1:500; Vector Laboratories, Inc.) in secondary diluent, rinses in PBS, 1 h incubation in ABC in PBS, rinses in PBS, 5 min reaction with Vector SG Peroxidase Substrate Kit (SK-4700; Vector Laboratories, Inc.) in PBS (working solution mixed according to the manufacturer's instructions immediately prior to use), and finally, cold dH₂O to stop the substrate reaction. The processing protocols, used consistently throughout the study, routinely produced staining of all TH-positive elements with a permanent brown chromogen and all macrophages stained for their phenotypic markers (i.e., either CD163 or MHCII) with a contrasting permanent black or dark blue/grey chromagen. Stained jejunal whole mounts were mounted on gelatin-coated slides, air-dried overnight, dehydrated in an ascending series of alcohols, cleared in two xylene steps, and coverslipped with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI).

2.5. Quantification of TH axonopathies

Distinct axonopathies consisting of markedly swollen TH-positive axons are routinely observed in the small intestine of aged rodents (Baker and Santer, 1988; Phillips et al., 2006). To generate a quantitative estimate of this phenomenon, each jejunal whole mount was scanned in its entirety by an experimenter blind to the age of the rat from which the tissue originated and the total number of dystrophic axons per whole mount was recorded.

To further characterize dystrophic axons, axon diameter was determined for axons located at the base of Peyer's patches for the four ages sampled. We focused on the axons located at the base of Peyer's patches because these axons were typically swollen in the aged rats. Specifically, we identified the base of Peyer's patch by the distinct silhouetting of their follicular domes by macrophages, and then scanned the area under the lymphoid organ using high resolution light microscopy (100X oil objective, 1.30 NA; total magnification = 1000X; DIC). Once an axon was identified, it was visually scanned within the field of view for no indices that it was part of a bundle (i.e., consisting of 2 or more axons), such as, bifurcations or the presence of other converging axons that made the individual identity of the axon ambiguous. Individual axon(s) were then photographed using the Spot camera, and the diameter of the axon measured at three equidistant points along its length using the measurement function in the Spot Software program.

Finally, during routine scans, circumscribed regions that appeared to be hyperinnervated by TH-positive axons were identified. In these instances, neurite density was calculated by superimposing a 0.5 mm × 0.5 mm stereological grid (subdivided by 6 circular × 6 longitudinal lines) and then counting the number of intersections TH-IR axons made with the longitudinal lines orientated perpendicular to the circular muscle (Phillips et al., 2006).

2.6. Estimates of macrophage density and reconstructions of jejunal whole mounts

A design-based stereological sampling protocol was applied using Stereo Investigator (V.10; MicroBrightField, Inc., Williston, VT) to estimate the density of macrophages at the ages sampled (Mikkelsen et al., 2011; Phillips et al., 2013). Specifically, our protocol for generating unbiased areal densities (i.e., the number of cells per surface area of the muscle coat) consisted first of tracing the contour of each whole mount, followed by systematic random sampling of 30 to 60 sites using the software's fractionator probe (Glaser et al., 2007). A counting frame (0.5 mm × 0.5 mm) was then positioned at each of the sampling sites determined by the software, and the immune cells within the counting frame were counted through the full-thickness of the whole mount. The mean areal density was estimated as the sum of counted cells divided by the total number of sites sampled. Quantification of the macrophages was done by the same individual (author initials: CNH) who was blind to the age of the rat from which the whole mount originated. Contouring and 3D surface mapping of the unbiased estimates of macrophage areal density were generated using a grid-based graphics program (Surfer 10, V10.4.799; Golden Software, Inc., Golden, CO).

Moreover, as our initial observations (see Results) made it clear that sites of sympathetic axon hyperinnervation were co-located with focal concentrations of immune cells, which were distributed irregularly and randomly along the length and circumference of the jejunum, we considered that grouping immune cell counts across whole mounts would tend to “average out” local macrophage densities and result in a failure to identify a hyperinnervated site at a particular locus in a single animal. Thus, mosaics of individual whole mounts were scanned in their entirety, and any focal concentrations of macrophages, TH+ axon hyperplasia, or other detectable signs of remodeling were recorded and examined on a case-by-case basis. Finally, each of the data sets for the immune cell density estimates

from each age group sampled were analyzed using the “robust regression and outlier removal” (ROUT) method (Motulsky and Brown, 2006).

For the case-by-case scans, mosaics of entire jejunal whole mounts were generated using a Leica DM5500B (Leica Microsystems Inc., Buffalo Grove, IL) automated upright microscope running Surveyor with Turboscan (V. 6.0.1.4; Objective Imaging, Cambridge, UK). Areas of interest were identified for inspection at higher magnification by scanning the entire mosaic in a systematic fashion using Surveyor Viewer (OIVIEWER Application, V5.5.5.18; Objective Imaging).

A pattern observed with double labeling for TH+ processes and either of the macrophage markers was what appeared to be instances of neurite-macrophage contact. While definitive information on contacts requires EM analysis, of course, we did adopt criteria for identifying provisional contacts using light microscopy: When (1) a swelling or dilation in one stained element was observed in juxtaposition to a second stained process, and (2) no separation could be detected between the two elements with high resolution light microscopic optics (100X oil objective, 1.30 NA; total magnification = 1000X; DIC), we conditionally described the juxtaposition as a “contact” or “apposition” or instance of “tight registration.”

2.7. Statistical analysis

Statistical analyses and bar graphs were generated using GraphPad Prism (Version 6.02; GraphPad Software, Inc., San Diego, CA). Either t-tests or, as appropriate, one-way analysis of variance (ANOVAs) followed by Tukey's multiple comparison tests were used to determine statistical differences. Unless indicated otherwise the Mean $\pm$ SEM is reported. A p-value of less than 0.05 was required for statistical significance. Finally, the ROUT method of detecting outliers (Motulsky and Brown, 2006) was used to determine individual sites where the density of immune cells fell outside the normal distribution range of the total population of values sampled. The false discovery rate was set to a Q of 1%.

2.8. Brightfield image capture and image post-processing

Brightfield photomicrographs were acquired using a Leica DMRE (Leica Microsystems Inc.) microscope fitted with a Spot Flex camera controlled using Spot Software (V4.7 Advanced Plus; Diagnostic Instruments, Sterling Heights, MI). Focus stacking (Gulbins and Gulbins, 2009) was used to maximize the depth of field of images taken from thick GI whole mounts by merging a series of shots taken at different focal distances along the z-axis that contained the entire element(s) of interest. All-in-focus images were generated using Helicon Focus Pro X64 (Version 5.3; HeliconSoft Ltd., Kharkov, Ukraine). Photoshop CS6 (Adobe Systems, San Jose, CA) was used to: (1) organize the layout of the figures; (2) adjust color, hue, brightness, contrast, and sharpness of images; and (3) apply text and scale bars. The goal of these adjustments was to generate final images which accurately conveyed the innervation of the GI tract.

3. Results

3.1. Control assessments

In the immunohistochemical series for TH (single labeled) or TH and one of the macrophage markers (double labeled for either CD163 or MHCII), the longitudinal muscle, myenteric plexus, and circular muscle were innervated by axons immunoreactive for tyrosine hydroxylase (TH-IR); Fig. 1A-C. In contrast, omitting the TH primary or appropriate secondary from the staining protocol resulted in no observable labeling. Also, importantly, in the rats that received ganglionectomy surgeries, there was near-complete elimination of TH-IR fibers in the jejunum. Following the removal of the celiac and superior mesenteric

ganglia, only the occasional solitary TH-IR fiber was observed in the gut wall. Absence of almost all TH-IR fibers in ganglionectomized rats confirmed the specificity of the TH antibody for sympathetic nerves, and, equally critical, it established the extrinsic origin of the majority of the TH-IR innervation to the jejunum from the celiac and superior mesenteric ganglion complex; Fig. 1D.

A small number of isolated myenteric neurons, immunostained for TH, were observed in jejunal whole mounts from ganglionectomized rats (Fig. 1D), confirming the presence of a very small pool of intrinsic neurons that likely contributed the rare surviving TH-IR fiber. There was such a paucity of noradrenergic somata and neurites in the jejunum after ganglionectomy that we concluded that muscle wall patterns involving substantial numbers of TH-positive fibers must represent the sympathetic extrinsic projections to the small intestine.

3.2. Normative “adult” pattern of innervation of the jejunum by sympathetic fibers and distributions of macrophages at 3 and 8 months of age

Both the distribution of sympathetic axons immunoreactive for TH and the distribution and appearance of macrophages positive for CD163 or MHCII proved essentially identical at 3 and 8 months of age, making it practical and efficient to combine and describe the two ages together as “adults.”

In these adults, the longitudinal muscle, myenteric plexus, and circular muscle were innervated by sympathetic axons immunoreactive for tyrosine hydroxylase (TH-IR); Fig. 1A-C. The longitudinal muscle was innervated, albeit not densely, by TH-IR fibers; Fig. 1A. In these limited longitudinal muscle projections, TH-IR fibers ran parallel to the muscle cells close to the surface of the myenteric plexus without projecting deep within the longitudinal muscle. The myenteric plexus was innervated by a dense network of TH-IR axons; Fig. 1B. Axons within the connectives tended to be smooth in appearance, but the fibers became varicose upon entering a ganglion. Within a ganglion, individual TH-IR fibers were dotted with small varicosities and branched extensively as they encircled unstained neurons. Additionally, within the same layer of the muscularis as the myenteric plexus, varicose TH-IR fibers formed a tertiary plexus (Furness and Costa, 1974; Furness, 2006) with its perimeter delineated by the ganglia and connectives; Fig. 1B. Individual varicose TH-IR axons also coursed into and terminated within the circular muscle, parallel to muscle fibers and parallel to each other, and neurites were frequently connected by bridging elements; Fig. 1C.

Immunostaining for the antibodies to the macrophage proteins CD163 and MHCII labeled a dense population of immune cells within the jejunal whole mounts. An average of 39 sites per whole mount stained for CD163 were sampled for the two “adult” ages. Immune cells IR for CD163 were distributed in a fairly uniform fashion around the circumference and along the length of the whole mounts; Fig. 2A. The mean ($\pm$ SEM) density of CD163-IR cells was 71 ± 6 and 79 ± 6 cells/0.25 mm² at 3 and 8 months of age, respectively; Fig. 2B. No density outliers were detected following ROUT analysis of individual counts of CD163-IR cells. By comparison, the MHCII-IR immune cells were distributed in a highly variable or patchy pattern. At 8 months of age, an average of 53 sites were sampled per jejunal whole mount stained for MHCII, and cell density averaged 28 ± 4 cells/0.25 mm². Two outliers were identified following ROUT analysis of the data with a mean ($\pm$ SEM) density of 85 ± 15 immune cells/0.25 mm².

Macrophages were situated in close proximity to the TH-IR innervation of the jejunal smooth muscle. The immune cells routinely outlined the perimeter of the myenteric ganglia, but were also found, in some instances, to have infiltrated into the ganglia where they were

in close apposition to unstained neurons silhouetted by TH-IR axons. Small tendrils originating from the primary processes of macrophages were observed wrapped around the circumference of TH-IR axons; Fig. 3A,A'. Similarly, varicosities along the length of healthy appearing TH-IR axons were regularly observed in tight registration with macrophages, and even at high power magnification (x1000) separation between a varicosity and a macrophage was often not perceptible; Fig. 3B.

3.3. Remodeling of the sympathetic innervation of the jejunum occurred in the “aged” rats at 16 and 24 months of age

Four distinctive and conspicuous types of age-related remodeling of the TH-IR innervation of the jejunum occurred at both the 16-month-old and the 24-month-old time points. Though more frequent and severe in the 24-month-old rats compared to the 16-month group, qualitatively, the four patterns were similar at the two ages and are therefore described below as “aged.” These changes, described below, consisted of (1) dystrophic and swollen axons as noted previously (Baker and Santer, 1988; Phillips et al., 2006), (2) sites with tangled, apparently disorganized fibers, (3) localized sites of hyperinnervation, notably, around the bases of Peyer's patches, and (4) ectopic innervation of the serosa.

3.3.1. Dystrophic markedly swollen TH-IR axons—Only 6% of the jejunal whole mounts from 3-8-month-old adult rats (0.06 ± 0.1 dystrophic axons/whole mount) contained at least one dystrophic axon, whereas 88% of the whole mounts taken from 16-24-month-old aged rats (2.88 ± 0.6 dystrophic axons/whole mount) contained at least one dystrophic axon (Unpaired t-test; $p < 0.0001$); Fig. 4. Dystrophic TH-IR axons and terminals consisted of markedly swollen axons and terminals located within the muscularis whole mounts. Dilations occurred along the length of the swollen axons, and these swellings appeared to be packed with unstained or lightly stained organelles. Macrophages in close proximity to dystrophic axons were IR for either CD163 (Fig. 5A,C) or MHCII (Fig. 5B,D). Small thin tendrils were regularly observed along the primary processes of immune cells that were in close proximity to the dystrophic sympathetic axons; Fig. 5D.

3.3.2. Tangled, potentially reorganized TH-IR axons—A second subset of sympathetic neurites, observed in whole mounts from 16-24-month-old aged rats, exhibited tangled organizational patterns. In contrast to the dystrophic fibers described above, the tangled fibers did not have dilated and markedly swollen features as seen in dystrophic axons. Rather, except for their disordered, sometimes almost knotted, appearance, the neurites were otherwise normal and similar to the fibers seen in 3-8-month-old adult specimens. Specifically, in adult rats, TH-IR axons were both orderly in appearance and regular in distribution; Fig. 6A. In contrast, in the aged rats the normative adult pattern was interrupted at discrete loci by tortuous tangles of apparently reorganized TH-IR fibers; Fig. 6B,C. Further, while the calibers of presumably “healthy” TH-IR axons in adults were typically thin in connectives, with numerous small varicosities expressed along the lengths of the fibers as they entered a ganglion or innervated a sheet of smooth muscle (Fig. 1A-C and 6A), the patches of reorganized TH-IR axons in aged animals consisted of thick, smooth fibers; Fig. 6B,C.

3.3.3. Focal sites of hyperinnervation and macrophage aggregation in smooth muscle and at the base of Peyer's patches—Sites of hyperinnervation in the smooth muscle layers were observed in the wall of the 16-24-month-old aged small intestine. Locating and characterizing these features was facilitated by two features of the sampling protocol employed. First, the immunohistochemistry for immune cells proved critical insofar as dense clusters of macrophages were conspicuous and reliable indicators of areas of hyperinnervation. Second, the complete mosaic reconstructions of individual whole mounts

that we employed made it practical to survey and identify in individual cases “hotspots” consisting of both clusters of cells and hyperinnervation by neurites.

Importantly, because hyperinnervated hotspots were located unpredictably within a tissue sheet, counts averaged across animals within a group tended to “wash out” focal disturbances: when unbiased estimates of macrophage density were combined into averaged grouped data, there was no significant change with age in the overall mean density of CD163-IR macrophages (One-way ANOVA; $p = 0.51$); Fig. 2B. Similarly, there was no difference in the density of MHCII-IR cells between 8 and 24 months of age (Unpaired t -tests; p value = 0.80). Specifically, at 16 and 24 months of age, an average of 43 sites were sampled in the whole mounts stained for CD163 and the mean ($\pm$ SEM) density of CD163-IR macrophages was, respectively, 68 ± 6 and 78 ± 6 cells/0.25 mm². An average of 57 sites were sampled in the whole mounts from 24-month-old rats stained for MHCII and cell density consisted of 27 ± 4 cells/0.25 mm².

While immune cell densities derived by averaging the areal counts for each age were relatively homogeneous between the four ages sampled, contour and surface plots for each age (using the mean number of cells for congruent sampling points) were more informative. As mentioned above (Fig. 2A), contour and surface plots for 3-8-month-old adult rats were similar as CD163-IR cells were distributed uniformly across the length and width of the whole mounts; however, the plots became more variable in appearance when graphed for 16-24-month-old aged rats. In particular, noticeable peaks in CD163-IR cell density occurred randomly along the length and width of the whole mounts, and warranted investigation of individual rats, particularly at 24 months of age. Likewise, mosaics of the whole mounts from aged rats stained for CD163 had visible patches of dark macrophage staining that were not routinely observed in whole mounts from adult rats; Fig. 7A.

By plotting the distribution of CD163-IR macrophages within the jejunal whole mounts from individual 24-month-old rats, further analysis revealed that four of the six 24-month-old rats had dense clusters of CD163-IR macrophages localized to circumscribed areas. A ROUT analysis confirmed that there were 17 outliers in the counts consisting of a mean ($\pm$ SEM) of 129 ± 7 immune cells/0.25 mm². Similar sites of aggregated immune cells were detected in whole mounts from the 24-month-old rats stained for MHCII, and confirmed by ROUT analysis. Nineteen outliers were identified that had an average of 138 ± 7 cells/0.25 mm²; Fig. 8A.

Examination of sites identified using the ROUT analysis, which consisted of aggregated immune cells (i.e., hotspots), revealed hyperinnervation of the muscle sheets by TH-IR axons. Macrophages IR for either CD163 or MHCII were closely associated with the TH stained axons in the muscle; Figs. 7A and 8A. Sites of hyperinnervation of the muscle by TH-IR axons consisted of axons running parallel to each other and with the grain of the muscle (Fig. 7B, and Fig. 8C). Immune cells within these hotspots were located within the smooth muscle (Fig. 7B-E and Fig. 8C,D), but were surprisingly sparse within the layer of the myenteric plexus; Fig. 8B.

Similarly, although Peyer's patches were removed with the submucosa and mucosa during the dissection of the whole mounts, at the level of the deep circular muscle layer, the location of the lymphoid organ in the jejunum was still discernible based upon the silhouetting of the follicular domes by macrophages; Fig. 9A. The bases of six Peyer's patches were identified in the whole mounts from 3-8-month-old adults, and the pattern of innervation by TH-IR axon and macrophages beneath the follicular domes was similar to the innervation seen in adjacent myenteric regions without Peyer's patches; Fig 9B compared to Fig 1B. The bases of seven Peyer's patches were similarly identified in the whole mounts

from 16-24-month-old aged rats, however, the plexus beneath the follicular domes was distinctly different and consisted of large diameter, smooth TH-IR axons that were tangled in appearance and encircled the perimeter of the follicular domes; Fig. 9C. The mean ($\pm$ SEM) diameter ($1.4 \pm 0.06 \mu\text{m}$) of 21 axons located at the bases of seven Peyer's patches in whole mounts from 16-24-month-old aged rats was significantly greater compared to the mean ($\pm$ SEM) diameter ($0.61 \pm 0.02 \mu\text{m}$) of 18 axons located at the bases of six Peyer's patches in whole mounts from 3-8-month-old adult rats (Unpaired t-test; $p < 0.0001$). Finally, immune cells were densely packed around the perimeter of the follicular domes at the bases of aged Peyer's patches making it impossible to differentiate between individual cells for quantification of the macrophages; Fig 9C.

3.3.4. Characterization of “hotspots” within a whole mount from an aged rat—

An example of a jejunal whole mount from a single 24-month-old rat, which contained sites of aggregated CD163-IR macrophages, is shown in Fig. 10. Six randomly sampled sites from this whole mount were determined by the ROUT analysis to be outliers (i.e., “hotspots”), and ranged in immune cell density from 151 to 181 cells/ 0.25 mm^2 . The mean ($\pm$ SEM) density of cells ($164 \pm 5 \text{ cells}/0.25 \text{ mm}^2$) within the six hotspots was significantly (Paired t-test; $p < 0.0001$) greater compared to the mean ($\pm$ SEM) density of CD163-IR cells ($70 \pm 3 \text{ cells}/0.25 \text{ mm}^2$) at 21 random sites sampled within the same whole mount that did not meet the criteria of a hotspot and fell within the normal distribution of values for the specimen determined by the ROUT analysis. Furthermore, the mean density of cells within these 21 normal-appearing sites was similar to the mean density of cells in the intact muscularis of 3- and 8-month-old rats (i.e., 71 ± 6 and 79 ± 6 , respectively). Thus, the density of macrophages in the tissue surrounding a hotspot located in an aged rat was similar to the density of macrophages in whole mounts from adult rats without hotspots of CD163-IR cells.

Additional analysis of the TH-IR axons within these six hotspots determined that the density of sympathetic axons within the circular muscle layer was approximately twice that of randomly chosen sites in the same whole mount. Hyperinnervation by TH-IR axons running in the circular direction of the six hotspots averaged 193 ± 21 counting grid intersections compared to 86 ± 9 intersections for six randomly chosen sites within the same whole mount (Paired t-test; $p = 0.01$). The mean ($\pm$ SEM) intersections by TH-IR axons running in the circular direction of eight randomly sampled sites from an 8-month-old rat were similar (90 ± 6 intersections) to the number of intersections for the six randomly chosen apparently normal sites within the whole mount that contained the hyperinnervated hotspots (Unpaired t-test; $p = 0.66$).

Finally, the muscularis was significantly (Paired t-test; $p < 0.0001$) thicker at the centers of the six hotspots ($12.33 \pm 0.33 \mu\text{m}$) compared to six random sites within the same whole mount ($8.33 \pm 0.42 \mu\text{m}$). Estimates of muscle thickness were determined by using differential interference contrast microscopy to identify the upper and lower limits of the muscle layers and measuring the difference between the two in the coverslipped whole mount.

3.4. Hyperinnervation also occurred ectopically in the serosa

Surveys of sites of immune cell aggregation also revealed hyperinnervation and tangles of TH-IR axons ectopically located in the serosa/subserosa. Macrophages IR for CD163 and MHCII were closely associated with the TH stained axons in the serosa; Figs. 7C,E and 8D. Axonal tangles within the serosa coursed throughout the layer without any particular directionality. While primarily located within the serosa in close registration with serosal macrophages, these axonopathies likely penetrated into the longitudinal muscle as well. The

ectopic TH-IR axons within the serosa had thick diameters with a smooth appearance (i.e., there were no varicosities along their length); Fig. 7C,E and Fig. 8D.

4. Discussion

Previous observations on the sympathetic projections to the aged gut, most of them made in broad surveys, have noted dystrophic catecholaminergic axons and terminals in the myenteric plexus, smooth muscle layers, and submucosal plexus (Baker and Santer, 1988; Phillips et al., 2006, 2007). Our present findings replicate those observations but also establish a relationship between macrophages and the swollen dystrophic features of TH-IR axons. Furthermore, by focusing on the sympathetic projections, concentrating on the jejunum, using a different whole mount preparation, and systematically surveying sympathetic endings with an unbiased stereological sampling technique, with the ROUT outlier analysis, and with case-by-case inspections of all specimens for macrophage aggregations, the present experiment also identified three additional types of regularly-occurring neuropathic profiles, including axon tangles, loci of hyperinnervation, and ectopic hyperplasias in the serosa.

It should be stressed that the axonopathies and hyperinnervation we report may be characteristic of multiple catecholaminergic projections to the gut, not merely the sympathetic projections. Given the locations, innervation patterns, and dense distributions of the TH+ fibers we examined, the bulk of the neurites were presumably sympathetic efferents, and we, for the simplicity of discussion, have used the terms *TH+*, *catecholaminergic*, and *sympathetic* interchangeably. Nonetheless, it is important to note that the neuropathies of aging we have observed could occur in a variety of catecholaminergic processes, including the more limited populations of TH+ processes that project to the GI tract from the dorsal motor nucleus of the vagus (Hayakawa et al., 2004; Tsukamoto et al., 2005), dorsal root ganglia (Brumovsky et al., 2012; Tan et al., 2010), and intrinsic enteric plexuses (Li et al., 2004; present observations).

As discussed below, after a consideration of the normal adult profile of jejunal catecholaminergic fibers, the patterns of age-related remodeling and the macrophage distributions associated with them suggest some mechanisms that could be involved in the axonopathies of gut aging.

4.1. Normative pattern of sympathetic innervation in adult rats

The basic patterns of sympathetic innervation observed in the healthy 3-8-month-old adult rat jejunums (and, notably, even in the 16-24-month-old aged rats, in unaffected jejunal sites where no axonopathies were observed) were comparable to those described in a report on the extrinsic innervation of the adult mouse jejunum (Tan et al., 2009). Specifically, TH-IR axons extensively innervate the myenteric plexus and the smooth muscle layers of the muscularis. Most of the TH-IR axons are smooth in appearance along the majority of their length, until they approached the target(s) that they innervate, where the axons then became varicose. Such targets include, for example, myenteric ganglia where TH-IR axons formed loose networks of varicose neurites around enteric neurons. Similarly, sympathetic axon terminals within the smooth muscle, particularly within the circular muscle sheets, became varicose as they formed long, parallel arrays within the muscle layer. Consistent with previous reports for rats (Phillips et al., 2006), mice (Tan et al., 2009), and guinea-pigs (Furness and Costa, 1974; Browning et al., 1999), TH-IR axons and terminals were not observed, in the present study, within the serosa or subserosal layer(s) of the adult rat small intestine.

In the adult jejunal whole mounts, macrophages, particularly CD163+ macrophages, were distributed throughout the length and circumference of the intestinal wall. Immune cells were often observed in close apposition to TH-IR axons, and in particular, to varicosities along the length of the fibers. Because immune cells migrate freely in the gut wall (Bain and Mowat, 2011), these associations could reflect random encounters between the cells and the sympathetic axons, however, the sympathetic axon-immune cell appositions occurred frequently in the jejunum of both adult and aged rats, and the macrophages involved were typically well-formed with a mature morphology (i.e., either bipolar or stellate in appearance rather than simple monocytes).

4.2. Sympathetic axonopathies in the aged intestine include focal hotspots

Sympathetic axonopathies of aging were distributed irregularly. Particularly striking was the presence of markedly swollen dystrophic axons restricted to narrow, circular strips of the tertiary plexus of the myenteric layer, suggestive of a discrete topographical projection of a terminal field by an individual sympathetic neuron. Such punctate and isolated fields of swollen and dystrophic axons in the aged intestine (also noted in a previous experiment—Phillips et al., 2006) are counter to what would be predicted for a generalized condition, such as, an infection, inflammation, or a catastrophic insult (i.e., large areas of tissue or entire whole mounts with the majority of the nervous innervation remodeling). It therefore seems likely that the sympathetic axonopathies, restricted to small localized areas, reflect some focal insult(s) such as hypoxia or ischemia (or pathogen invasion) associated with either a small artery or capillary supplying the immediate area. Or, alternatively, it could be argued that the axon-by-axon pattern would similarly occur if a process leading to dysplasia originated in individual sympathetic neuron cell bodies and then spread anterogradely to the axon terminals.

Consistent with the inference of a localized-insult, Downman (1952) determined that the region of intestine supplied by sympathetic axons is coextensive with the area supplied by an artery and its branches after entering the gut wall from the mesentery, and that only a small area of overlap exists between areas supplied by paravascular sympathetic axons following adjacent arteries. Furthermore, when Downman (1952) interrupted the nerve fascicles following a particular artery, the terminals in the area supplied by its branches degenerated with little change in the adjacent fascicles. Conversely, if he left intact the axons following one artery while interrupting those on either side of the artery then the terminals in the intact field remained while those on either side degenerated. Thus, Downman's (1952) axotomy experiments and our observations of patchy axonopathy patterns are consistent with the hypothesis that the dystrophic terminals in the tertiary plexus of the aged rat jejunum represent the terminal field of an individual fiber (or fibers) innervating a small area that had suffered either an ischemic or hypoxic event (or a breach of immune barrier function). This inference is further bolstered by the fact that macrophages were in close contact with the dystrophic terminals at these sites, since immune cells are well known for accumulating in diseased hypoxic/ischemic tissue (Murdoch et al., 2005).

Like the locations of dystrophic axons, sites of hyperinnervation were also not evenly distributed. In examining this present series of whole mounts, we evaluated the hypothesis that the extrinsic sympathetic inputs to the aged gut might evidence profiles of hyperinnervation similar to those described for other organs (e.g., brain: Di Giulio et al., 1989b; heart: Hassankhani et al., 1995; diabetic intestines: Di Giulio et al., 1989a; lung: Hoyle et al., 1998; adipose tissue: Straub et al., 2011; blood vessels: Luff et al., 2005). In keeping with such observations on non-GI tissues, we observed that sites of hyperinnervation in the gut also occurred in punctate, spatially random patterns. The tissue layers affected by hyperinnervation were predictable, but the locations of the hotspots around the circumference and along the length of the organ were not. The loci of hotspots of

macrophage aggregation and axonal hyperplasia occurred, seemingly randomly, at sites typically surrounded by an apparently normal field of undistorted adult-like innervation.

4.3. Age-related sympathetic axonopathies are associated with macrophages whose distribution and phenotypes are characteristic of different activation states

Recent analyses of the innate immune system have emphasized that macrophage mobilization is multifaceted (e.g., Bain and Mowatt, 2011). Whereas innate immune cells were classically considered as resident and either inactive or active, macrophages are now thought to express a spectrum (Mosser, 2003; Mosser and Edwards, 2008) or continuum of phenotypes involving different combinations of functions (Town et al., 2005). Specifically, immune cells are now routinely categorized as either pro-inflammatory (i.e., M1 or classically activated) or anti-inflammatory (M2 or alternatively activated) depending on the presence of different markers within the cell.

In the present study, we used an antibody to CD163 because it is a scavenger receptor involved in the uptake of extracellular hemoglobin complexes released from dying cells (Fabrick et al., 2009; Colton and Wilcock, 2010), and is considered a marker for M2 macrophages involved in the disposal of necrotic fibers and also tissue repair (Zhang et al., 2011); whereas, the MHCII molecule is mainly involved in the presentation of extracellular pathogens to helper T cells and the triggering of an appropriate immune response, and is predominantly expressed by M1 cells but can occasionally be found in M2 cells. Additionally, while MHCII can be a useful marker for classically activated macrophages (Mikkelsen et al., 2011) care must be taken because it is also expressed by multiple cell types and cannot be used to unequivocally distinguish between macrophages and dendritic cells.

In view of such complexities of immune cells in the gut, an examination of the macrophages expression of the phenotypic markers CD163 and MHCII yields too incomplete a picture to provide a full functional analysis of the different patterns we observed. Short of full phenotypical profiles, though, the presence of the two markers in macrophages does suggest some basic inferences. In the case of two of the axonopathy patterns observed, namely the swollen dystrophic axons and the axon tangles, the presence of evenly distributed macrophage stained robustly for CD163 were consistent with phagocytosis and general housekeeping (Mosser, 2003). Presumably, such responses would limit and minimize the cellular debris generated by dystrophic fibers and might, theoretically, be able to reverse some features of the disturbance (Rotshenker, 2011). Conceivably, the fact that the axon tangles varied in the extent to which they were associated with the “alternatively activated” macrophages may reflect the tangled fibers reaching a stable condition in which innate immune cells are no longer elicited by the affected neurite (or of course, conversely, the profile could reflect a stage in the development of the tangles, preceding macrophage mobilization towards a housekeeping role in maintaining a stable environment).

The fact that the other two axonopathy patterns, namely the hyperinnervation profiles, including the Peyer's patch-related hyperinnervation and the serosal sites of hyperplasia, involved a different profile of macrophage activity underscores the distinctive nature of the types of axonopathies and suggests something of the potential processes associated with hyperinnervation. In this case, more particularly, the fact that the characteristic hyperinnervation hotspots involved aggregation of CD163+ and MHCII+ cells at the sites, suggest a different set of signals and responses. In general, the staining phenotype of macrophages appeared to have somewhat more of an M1 or pro-inflammatory profile (Zhang et al., 2011).

The contrasting profiles of macrophage response seen with the different axonopathies in this initial set of observations underscore a set of questions that will need to be addressed if the cellular mechanisms controlling the axonopathies are to be understood. The sympathetics are well known to depend on trophic factors to support their differentiation, outgrowth, and survival (e.g., Hassankhani et al., 1995; Hoyle et al., 1998; Rush et al., 1997). Hence, underlying changes in the trophic factor signaling in the target area--whether occasioned by ischemia, pathogen invasion, tissue wear and tear or other processes correlated with age--are one primary candidate mechanism to account for the neuropathic remodeling that occurred. The signaling sequences are less clear, however, conceivably, the local tissues may elaborate trophic factors or cytokines (See et al., 1988; Peters et al., 1998), which in turn induce axonal reactions that then elicit macrophage responses, or, conversely, the tissue insults might elicit macrophage responses which in turn affect the trophic environment (Batchelor et al., 1999; Starke-Buzettie and Oaks, 2008; Laskin et al., 2011) that shapes the axonal responses and structure. Or, of course, the interactions could well be reciprocal (Desai et al., 2010).

4.4. Sympathetic neurons with age-related axonopathies apparently remain viable

Though dystrophic catecholaminergic axons in the wall of the aging gut have been previously reported, it has been unclear whether (a) such dystrophic TH-IR neurites in the wall of the GI tract presage a later loss of the axons and, potentially, even a loss of the postganglionic somata or (b) the dystrophic processes might be viable or even, perhaps, repairable. On the one hand, the reduction in glyoxylic-acid-induced fluorescence varicosities (Baker and Santer, 1988), the generalized reduction in TH expression (Phillips et al., 2006), the knotted, varicose, and swollen dystrophic neurites (present results, also Phillips et al. 2006; Phillips and Powley, 2007), and the appearance of alpha-synuclein aggregates in TH-IR fibers (Phillips et al., 2009) would all be features that might be found in neurons beginning a process of degeneration and eventual cell death.

On the other hand, in the aged F344 rat, nitrergic axons located in the smooth muscle wall of the gut develop dystrophic features similar to those observed in sympathetic fibers, apparently without any associated loss of nitrergic myenteric neurons (Phillips et al., 2003; Phillips and Powley, 2007). Such survival of nitrergic neurons with dystrophic axons suggests the possibility that the sympathetic axonopathies might not lead to degenerative changes and losses of postganglionic motor neuron pool. Consistent with such potential viability, Schmidt and colleagues (Schmidt, 2002; Schmidt et al., 1983, 1990) have reported that, with age, the celiac and superior mesenteric ganglia do not sustain reductions in postganglionic somata numbers, though they do accumulate neuroaxonal dystrophies. This observation suggests a parallel with the pattern that we previously observed for nitrergic myenteric neurons and underscores the possibility that the sympathetic neurons with age-related dystrophic swellings in their neurites could remain chronically viable, even though they might, perhaps, lose some or all functionality or eventually even come to disrupt normal GI physiology.

In addition to the argument that neurons with dystrophic axons can be viable, the presence of hyperinnervated sympathetic terminal fields and substantial ectopias is consistent with the observation that the sympathetic neuronal cell bodies in the celiac and superior mesenteric ganglia are not only spared (Schmidt, 2002; Schmidt et al., 1983; 1990), but that they actually hypertrophy (Baker and Santer, 1988a) with aging. Though any conclusions about long-term viability based on structural observations at a particular time point must be provisional, the hyperplasias seen in the hyperinnervated fields and ectopias are also not consistent with the postulate that the parent postganglionic neurons are entering a pathway of degeneration and death.

4.5. Functional consequences of the sympathetic axonopathies of aging

This initial survey based on widely separated time points over the lifespan and a limited number of histochemical markers cannot provide unequivocal inferences about the functional impact of the neuropathies. Nonetheless, the patterns of remodeling are suggestive and can be combined with other observations in the literature to delineate some of the possibilities that should be examined.

Assuming that the axonopathies are chronic and viable (see section 4.4 above), it would seem likely that the dystrophic endings and axon tangles diminish the overall efficiency of the parent fibers. Conceivably, and perhaps most likely, these losses in efficiency would translate into functional reductions and some of the impairments that compromise the aged intestines. That said, a conceivable, though seemingly less likely, alternative is that some loss-of-function in the sympathetic projections is a compensatory mechanism to minimize other impacts of aging. For example, given that there is a relatively dramatic reduction in enteric cholinergic neurons with age (Phillips et al., 2003; Abalo et al., 2005; Thrassivoulou et al., 2006; Bernard et al., 2009) and that the sympathetic projections often operate to establish a balance with cholinergic tone, it might be the case that the axonopathies reflect trophic-factor mediated adjustments elicited to balance catecholaminergic versus limited cholinergic tone in the aging intestinal wall.

The most thoroughly described incidence of sympathetic hyperinnervation, a pattern apparently analogous to what we observed in the aged gut, occurs in the ventricles of the heart following myocardial infarction (Hasan et al., 2006; Oh et al., 2006), with the presence of macrophages considered a prerequisite for sprouting of sympathetic nerves. In particular, hyperinnervation is highly localized to the infarct border zone (Wernli et al., 2009) which contains large numbers of macrophages that are thought to create a growth-permissive environment for sympathetic plasticity to take place, with excessive amounts of neurotrophic substances resulting in uncontrolled neurite growth. Congruent with the findings in the present study is the fact that sympathetic hyperinnervation of the heart occurs selectively in regions containing abundant macrophages. The inflammatory cells appear prior to axonal ingrowth, and nerve hyperinnervation and degeneration occurs in the same region (Wernli et al., 2009). From a functional perspective, unfortunately, the hyperinnervated regions of the heart are thought to contribute to arrhythmias following myocardial infarction, so it is anything but clear that the sympathetic plasticity in the aged gut works to conserve or restore normal function.

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Figure 1.

Pattern of innervation of the adult jejunum by noradrenergic sympathetic axons immunoreactive for tyrosine hydroxylase (TH-IR). (A) Sympathetic innervation of the longitudinal muscle was sparse, consisting primarily of fibers (arrowheads) running parallel to the longitudinal muscle. (B) The sympathetic innervation of the myenteric plexus, however, was extensive with TH-IR axons throughout the ganglia (G), connectives (CN), and tertiary (T) plexus. (C) Sympathetic axons within the circular muscle were regularly spaced, parallel to each other (arrowhead), with occasional bridging element (arrow) connecting adjacent axons. (D) The origin of the TH-IR axons in the jejunum from the celiac and superior mesenteric ganglia was confirmed by removal of both (CGX and SMGX, respectively) resulting in almost complete elimination of TH-IR innervations of the muscularis other than a few TH-IR neurons in the myenteric plexus. Scale bar = 20 μm in B (applies to A-D).

A: Distribution of CD163+ cells in the adult jejunum **B: Mean ($\pm$ SEM) CD163+ cells in the jejunum** **Figure 2.**

A design-based stereological sampling protocol was used to quantitatively describe the distribution of macrophages in the smooth muscle of the rat jejunum. (A) Macrophages IR for CD163 were distributed in a relatively uniform manner throughout the longitudinal and circular axis of the adult jejunum. (B) The mean density of CD163 cells was similar in the jejunums across the four ages sampled.

Figure 3. Macrophages were in close proximity to the TH-IR axons. (A,A') Filepodia-like tendrils (arrowheads), emanating from the process of a macrophage, encircle a TH-IR axon. (B) A varicose TH-IR axons in tight registration with the soma of a macrophage (arrow). Scale bars = 20 μ m in A,B; 10 μ m in tracing.

Figure 4. Dystrophic markedly swollen TH-IR axons were found throughout the jejunal whole mounts from aged rats. (A,B) Numerous dilations (arrowheads) occurred along the length of swollen TH-IR axons. Scale bars = 40 μ m in A,B.

Figure 5.

The immune response appeared to be localized to the immediate vicinity of the markedly swollen TH-IR axons. Macrophages expressed either CD163 (A) or MHCII (B), and had morphologies consistent with a phagocytotic response. Specifically, the presumptive phagocytes (shown in panels C,D are high power enlargements of the boxed regions in, respectively, panels A,B) were tightly apposed to degenerating axons and were swollen and dilated by comparison to macrophages located in adjacent “healthy” regions of the same whole mounts. Phagocytes had numerous small tendrils and filopodia in contact with the dystrophic axons; arrowheads in panel D. Scale bars = 40 μ m in A,B; 10 μ m in D (applies to C,D).

Figure 6.

The presence of smooth axons, well labeled for TH-IR, with complex looping tortuous paths within the whole mounts from aged rats, were considered evidence for potentially reorganized sympathetic axons. The innervation of the tertiary (T) plexus in an adult rat was orderly in appearance (Panel A) compared to the looping (arrowheads), overlapping, sinusoidal course of potentially reorganized TH-IR axons in aged rats (Panels B,C). Scale bar = 50 μ m in C (applies to A-C).

Figure 7.

The presence of clusters of CD163-IR immune cells revealed focal sites of sympathetic hyperinnervation. (A) Two sites of aggregated macrophages, consisting of dense clusters of CD163-IR cells, are shown surrounded by relatively normal distributions of CD163-IR cells and TH-IR axons. (B,D) The circular muscle at these sites was hyperinnervated by TH-IR axons. (C,E) The serosa was innervated by ectopic TH-IR axons. Scale bar = 250 μ m in A; 25 μ m in E (applies to B-E).

Figure 8.

Aggregates of MHCII-IR immune cells, similar to CD163-IR immune cells, indicated sites of sympathetic hyperinnervation. (A) A dense cluster of MHCII-IR cells, localized to a small discrete region, surrounded by normally distributed macrophages and sympathetic axons. Sympathetic innervation of the myenteric plexus (B) appeared normal, whereas the circular muscle (C) was hyperinnervated by TH-IR axons and the serosa (D) was innervated by ectopically located tangles of TH-IR axons. Macrophages within these foci were clustered in dense clumps within the circular muscle and serosa, but not the myenteric layer. Scale bars = 150 μ m in A; 10 μ m in B,C,D.

Figure 9.

The base of Peyer's patches in the aged jejunum were hyperinnervated by sympathetic axons. (A) Although the lymphoid organ was removed with the mucosa and submucosa, the base of the Peyer's patches were easily identified in adult rats based on the characteristic accumulation of macrophages that silhouetted the follicular domes. (B) Sympathetic innervation at the base of the domes in adult rats was characteristic of the TH-IR innervation of the myenteric plexus; macrophages were similarly uniform in appearance. Panel B is a high power enlargement of the boxed region in panel A. (C) The base of Peyer's patches in aged rats, however, were hyperinnervated by TH-IR axons that densely encircling the perimeter of the follicular domes, and complete loss of integrity of the myenteric plexus within the dome. There was a similarly dense accumulation of immune cells at the base of the follicular domes of aged rats. Scale bars = 500 μ m in A; 50 μ m in B,C.

Figure 10.

A contour and surface plot of the densities of CD163-IR cells sampled at systematically random sites from the jejunal whole mount of a single 24-month-old rat, illustrates “hotspots” consisting of dense clusters of immune cells. Red peaks in the surface plot represent sampled sites with the highest density of cells. The contour of the whole mount shows the 55 randomly sampled sites (designated by red boxes) where counting frames were superimposed and macrophages counted to determine the areal density of the cells. Darkly stained regions in the mosaic of the jejunal whole mount demarcate foci of aggregated macrophage and contrast with adjacent lighter regions that have relatively normal numbers of immune cells. Because macrophage hotspots are localized to circumscribed regions of the tissue, our random sampling protocol resulted in underestimates of the number of hotspots in whole mounts and, in some cases, likely missed hotspots altogether when the whole mount contained only a few such sites. Scale bar = 3 mm.