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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 phenotypical 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.

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 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, noradrenergic fibers of the sympathetic nervous system are prone, in other organs and tissues, 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

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Giulio et al. (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; Rush et al., 1997; Hoyle et al., 1998), 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 that 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 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:H_2O_2 (4:1) to inhibit endogenous peroxidase activity, rinses in PBS, 4 day 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%