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The soma and proximal dendrites of sympathetic preganglionic neurons innervating the major pelvic ganglion in female rats receive predominantly inhibitory inputs

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Abstract

Sympathetic preganglionic neurons (SPNs) in the intermediolateral (IML) and dorsal commissural nucleus (DCN) of the thoracolumbar segments of the spinal cord contribute to the autonomic control of the pelvic visceral organs. We examined the morphology of these neurons at the light and electron microscopic level and quantified the boutons apposing the soma and proximal dendrites of the SPNs innervating the major pelvic ganglion in female rats. The majority of these cells resided in the DCN ($61.6 \pm 6.2\%$) and IML ($33.2 \pm 4.4\%$) nuclei. Measurements of cell volume and shape revealed no differences between SPNs sampled from the DCN and IML populations. Ultrastructural studies of DCN and IML SPNs revealed that coverage of SPNs by synaptic inputs is sparse, with an average of $11.60 \pm 2.41\%$ of the soma membrane and $16.33 \pm 6.18\%$ of proximal dendrites apposed by boutons, though some somata exhibited no synaptic coverage. Three distinct types of boutons were found to appose the SPN somata and dendrites. The putatively inhibitory F-type bouton covered a significantly greater percentage of membrane on the soma ($8.48 \pm 2.12\%$) and dendrites ($12.65 \pm 4.34\%$), than the S-type bouton, a putatively excitatory bouton, which only covered $2.94 \pm 0.70\%$ of the somatic and $3.68 \pm 2.98\%$ of the dendritic membranes. Boutons with dense-core vesicles were rare. Our results demonstrate that SPNs of the DCN and IML of female rats are similar morphologically, and that synaptic input on these cells, though sparse, is predominantly inhibitory.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

Keywords

autonomic; electron microscopy; retrograde tracing; spinal cord; thoracolumbar; ultrastructure

INTRODUCTION

Sympathetic preganglionic neurons (SPNs) at the thoracolumbar levels of the spinal cord contribute to the autonomic control of the pelvic visceral organs, including the sexual organs (Dail et al., 1989, Houdeau et al., 1998) and the lower urinary tract (Kolbeck and Steers, 1993, Kihara and de Groat, 1997, Houdeau et al., 1998). In the rat, these neurons are principally found at the spinal segments L1 and L2. Most reside within two nuclei: the intermediolateral nucleus (IML) and the dorsal commissural nucleus (DCN), also known as the central autonomic area, with a few found in the intercalated nucleus (IC) in between (Neuhuber, 1982, Nadelhaft and McKenna, 1987). It is known that some of these neurons, contrary to the standard paradigm of sympathetic projections, actually extend their axons through the spinal chain ganglia and the inferior mesenteric ganglion without synapsing, as they are identifiable through application of retrograde tracers, such as horseradish peroxidase, to the transected stump of the hypogastric nerve (Nadelhaft and McKenna, 1987, Baron and Jänig, 1991). More recently, these neurons have also been traced through the injection of retrograde tracers such as cholera toxin subunit B (Hosoya et al., 1994, Llewellyn-Smith et al., 2005, Ranson et al., 2006) or Fluorogold (Harji et al., 1998, Chang and Havton, 2010) directly into the major pelvic ganglion (MPG) itself, where the parasympathetic preganglionic neurons (PPNs) in the lumbosacral spinal cord also project their axons via the pelvic nerve (Keast, 1995).

SPNs that reside along the thoracic and lumbar segments of the spinal cord are organized topographically, as cell bodies that regulate structures in the upper body are found at the rostral end of the spinal cord. Likewise, SPNs that innervate pelvic ganglia are found principally in segments L1-L2, the caudal end of the spinal distribution of SPNs (Strack et al., 1988, Harji et al., 1998, Llewellyn-Smith et al., 2005), resulting in some overlap of innervation that would necessitate retrograde tracing studies in order to distinguish, in the spinal nuclei, the subpopulations innervating the inferior mesenteric ganglion from those innervating the MPG.

Most studies involving retrograde tracers injected into the MPG of rats have been carried out in males, in part due to the anatomical prominence and the greater size of the male MPG, which contains up to two-and-a-half times more cells than the female MPG (Greenwood et al., 1985), thus making it a more accessible target for tracer injection. Past ultrastructural studies have shown that in males, over half of the somatic terminals contacting MPG-innervating SPNs in the IML are immunopositive for enkephalin (Llewellyn-Smith et al., 2005), though boutons positive for glutamate transporters (Llewellyn-Smith et al., 2007), as well as glutamate, glycine, GABA, and serotonin, though rare, have also been observed (Santer et al., 2002, Ranson et al., 2006). Through the timing of pseudorabies virus tracing studies (Cano et al., 2001) and studies of somatic inputs following spinal cord injury (Llewellyn-Smith et al., 2005), it is suspected that the majority of the contacts upon SPNs originate from spinal interneurons.

Despite previous studies investigating some of the neurochemical nature of synaptic contacts upon MPG-innervating SPN somata, a rigorous study of the ultrastructural features of the boutons contacting these somata and proximal dendrites has never been performed. Furthermore, we are aware of no studies that have performed analysis of synaptic inputs upon MPG-innervating SPNs in female rats.

The present study was performed to examine the ultrastructural features of synaptic contacts and appositions upon SPNs in the IML and the DCN innervating the MPG of adult female rats. SPNs were labeled with a bilateral injection of the retrograde tracer, FG, into the MPG. Light-microscopic studies were performed in order to measure and quantify positively labeled cells, while use of the freeze-substitution embedding technique allowed us to identify FG-positive SPNs using post-embedding immunogold techniques for analysis in the electron microscope.

EXPERIMENTAL PROCEDURES

Animals

Adult female Sprague-Dawley rats (n = 8, 205 - 320 g, Charles River Laboratories, Raleigh, NC, USA) were used for these studies. All animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Chancellor's Animal Research Committee at UCLA. Care was taken to minimize the number of animals in the study as well as animal suffering during surgical and recovery procedures. Animals were housed in standard rat cages in a room with a 12:12 light:dark cycle at $24 \pm 1^\circ\text{C}$, with food and water available *ad libitum*. Animals were split into two groups, those used for light microscopic (LM) studies (n = 3) and those used for electron microscopic (EM) studies (n = 5).

Injection of retrograde tracer into the major pelvic ganglion

All animals received injections of the retrograde tracer, Fluorogold (Fluorochrome, Denver, CO, USA), bilaterally into the MPG in order to retrogradely label the pelvic-innervating sympathetic preganglionic neurons (SPNs) in the spinal cord. Tracer injections were performed according to previous protocols (Chang and Havton, 2010), with 1 μl of 5% FG solution in ddH₂O injected into each ganglion. Following injection, the area was rinsed with saline and both ganglia were visually inspected using a surgical microscope for verification that no tracer leakage took place. Buprenorphine (0.2-0.5mg/kg s.c., every 12 h for 48 h) and antibiotics (Trimethoprim/sulfamethoxazole oral suspension, USP, for the duration of survival) were administered post-operatively to control pain and as prophylaxis for infections, respectively. Animals were checked two or three times daily for well-being, and were allowed 4-7 days for proper retrograde tracer transport before sacrifice procedures.

Tissue collection and labeling verification

For the LM studies, animals were deeply anesthetized with a subcutaneous injection of urethane, and then perfused intracardially with 4% paraformaldehyde (pF) in phosphate-buffered saline (PBS). The L1-L2 spinal cord segments were dissected, post-fixed in 4% pF for 90 minutes, and then cryo-protected in 30% sucrose in PBS. The tissues were blocked in TissueTek O.C.T. compound (Sakura Finetek USA, Torrance, CA, USA) and then stored in a freezer at -80°C until sectioning. For the EM studies, animals were anesthetized with an injection of sodium pentobarbital, and perfused intracardially with 4% pF and 0.1% glutaraldehyde in PBS. L1-L2 segments of the spinal cord were dissected and post-fixed in 4% pF for 90 minutes. Whole transverse sections were cut on a vibrating tissue slicer (Electron Microscopy Sciences, Fort Washington, PA, USA) at a thickness of 250 μm in ice-cold PBS. These sections were briefly analyzed in a Nikon E600 light microscope equipped for epifluorescent detection of FG through a UV filter, where we confirmed that the pattern of FG fluorescence in SPNs was consistent with previously published studies (Harji et al., 1998) of MPG injection, with no evidence of systemic tracer leakage. Sections were cut to isolate the IML and DCN nuclei for embedding, and then cryo-protected through a graded series of glycerol in PBS, and stored overnight at 4°C in a solution of 30% glycerol.

LM immunohistochemistry, cell quantification and measurement

Transverse spinal cord sections were cut on a cryostat at a thickness of 30 μm and collected as free-floating sections for immunohistochemical analysis. Standard maps of rat cytoarchitecture were used to verify the segmental location of all tissues (Molander et al., 1984). Sections were first placed in 0.3% hydrogen peroxide to quench endogenous peroxidase activity before incubation with primary anti-rabbit FG (1:200, Fluorochrome) antibody. Sections were then processed with a biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA), and then incubated in an avidin-biotin complex (1:1:200; Vectastain ABC Elite kit, Vector Laboratories) and visualized as a light-stable product using diaminobenzidine (Sigma Aldrich, St. Louis, MO, USA) as the chromogen. FG-labeled sections were mounted on slides using Permount medium (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Unbiased cell counts of FG-positive SPNs in the IML, IC, and DCN nuclei were performed using a physical disector method (Coggeshall and Lekan, 1996). A total of 24 sections were sampled regularly in segments L1-L2, each section spaced 90 μm from the previous. Classification of nuclear location (IML, DCN, and IC) were determined by laminar location as established in previous studies (Nadelhaft and McKenna, 1987, Harji et al., 1998). Cells were traced at 100 $\times$ magnification using a camera lucida setup connected to a Nikon E600 scope, and were included if they displayed a definable nuclear envelope. For neuronal size measurements, we also included cells with a definable nuclear envelope that had been excluded from the physical disector counts. Cells were measured along the longest (A) diameter, and the longest diameter perpendicular to A (B). The formula for cell diameter was defined as $(A+B)/2$, and the formula for volume was used as that of an oblate spheroid rotated around the minor transverse axis, $(\pi/6)AB^2$ (Ulfhake, 1984).

Tissue embedding and sectioning for EM

Tissue sections were snap-frozen by immersion in liquid propane at -180°C using a Leica EM CPC cryo workstation (Leica Microsystems, Wetzlar, Germany). The frozen tissue sections were transferred to a Leica EM AFS2 automatic freeze substitution system (Leica Microsystems) and dehydrated in methanol containing 1% uranyl acetate at -90°C for 35 hours. The temperature was then raised by 5°C per hour for 9 hours to -45°C . The sections were then rinsed in methanol and infiltrated in graded solutions (2 hours each in 1:1, 2:1, 1:0, and then 1:0 overnight) of Lowicryl HM20 (Electron Microscopy Sciences). The sections were polymerized for 30 hours using ultraviolet light at -45°C before the temperature was gradually risen 5°C per hour to 0°C . Ultrathin sections (about 60-80 nm in thickness) were cut using a PowerTome X ultramicrotome (RMC Products, Boeckeler Instruments, Tuscon, AZ, USA) and collected on formvar-coated nickel grids.

Post-embedding immunogold detection of FG for EM

All grids containing ultrathin sections were placed in drops containing the reagents and kept in humid chambers for all immunogold incubation steps. Grids were first immersed into a 0.1% sodium borohydride + 50mM glycine in Tris-buffered saline (pH: 7.4) with 0.1% Triton X-100 (TBST) solution for 25 minutes, in order to block free aldehyde residues in the tissue. Grids were rinsed in TBST and then incubated with an anti-rabbit primary FG antibody (1:20, Fluorochrome) in Tris-buffer with 0.1% Triton X-100 for 48h at 4°C . Sections were rinsed again in TBST and then incubated with a secondary goat anti-rabbit antibody, conjugated to 15nm colloidal gold (1:10, GE Healthcare, Buckinghamshire, UK) at room temperature for 4h. Grids were contrasted with 8% uranyl acetate and lead citrate. Sections were examined in a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan). Cells were considered positively labeled if accumulation of gold particles in lysosomes was observed, consistent with patterns established in previous studies (Persson

and Havton, 2009). Images were captured at 4800× and 7200× on photographic film, scanned digitally at 1200 dpi, and then composited into a single image using Adobe Photoshop CS5 software (Adobe Systems, San Jose, CA, USA). All images were minimally corrected for brightness and contrast prior to analysis and publication. Due to the use of Triton X-100 for immunogold protocols, some ultrastructural characteristics were uninterpretable. When this occurred, we identified the same cell in contrast-only adjacent sections, and used these images for analysis instead.

Ultrastructural analysis of labeled neurons

Only cells exhibiting FG labeling and the nucleus in the plane of sectioning were included in the analysis. Diameters A and B, cell area, and perimeter were measured using the program SimplePCI (version 5.31, Hamamatsu, Sewickley, PA, USA). Cell volume was calculated using the formula, $(\pi/6)AB^2$ (Ulfhake, 1984). Cell nuclei with atypical invaginations were selected for separate analysis for cell membrane measurements. Total circumference of nuclear membrane exposure to the cytoplasm was compared against approximations of the same rounded, uninvaginated nucleus. Boutons in apposition with SPN somata and dendrites were characterized using ultrastructural features such as size and presence of synaptic vesicles, as well as synaptic vesicle size, shape, and electron density. In cases where synaptic specializations were also visible, they were included in the characterization. Measurements of individual boutons were performed at a primary magnification of 7200×. Soma apposition length of each classified bouton was determined using SimplePCI. The percentage of synaptic bouton coverage was calculated as $(\text{Sum of bouton apposition lengths} \times 100)/(\text{Total membrane circumference})$.

Statistical analysis

Comparisons of cell diameter, volume, and aspect ratio at the light microscopic level were performed using the Kruskal-Wallis one-way ANOVA on ranks, using Dunn's Method as a post-hoc test. For comparisons for values taken from electron micrographs, we employed a two-way ANOVA to compare cellular location (soma vs. dendrite) and bouton type (F vs. S), for values of apposition length, percentage membrane coverage, and bouton frequency per 100µm. The Holm-Sidak post-hoc test was used. We regarded $p < 0.05$ as a statistically significant difference between samples. All quantitative data are reported as mean $\pm$ standard error of the mean (S.E.). All statistical analyses were performed using Sigma Stat version 3.1 software (SyStat Software, Inc., Point Richmond, CA, USA).

RESULTS

We studied the pelvic-innervating SPNs in adult female rats at the light ($n = 3$) and EM ($n = 5$) levels after direct, bilateral injections of the retrograde tracer, FG, into the MPG. Thoracolumbar sections from all animals were examined in the light microscope. Retrogradely labeled SPNs were readily identified and no ectopically labeled motoneurons were encountered, confirming that the FG injections were successful and that the tracer did not leak from the ganglia into the vasculature.

Distribution and Size of MPG-innervating SPNs

In order to characterize the distribution and size of these MPG-innervating neurons, we first performed a bilateral FG injection into the MPG of adult female rats ($n = 3$). We found FG-positive neurons in the DCN, IC (Fig. 1A), and IML (Fig. 1B) nuclei of the spinal cord at levels L1-L2. Quantitative studies of a total of 87 SPNs distributed between the three rats demonstrated that cells present in the DCN, a midline nucleus situated dorsal to the central canal, were the most abundant, accounting for $61.6 \pm 6.2\%$ of observed SPNs. Cells in the bilateral IML nuclei combined accounted for $33.2 \pm 4.4\%$ of FG-labeled SPNs, and neurons

in the IC nucleus were relatively rare, only forming $5.2 \pm 2.4\%$ of the total MPG-projecting SPN population at the L1-L2 levels (Fig. 1C).

Measurements of cell shape and size characteristics were performed on cells exhibiting a clear nucleus in the plane of section, regardless of whether or not they had been counted with the physical disector method ($n = 118$). Calculations of soma diameter revealed a significant statistical difference, with IC ($21 \pm 1.8 \mu\text{m}$, $n = 8$) and IML ($18 \pm 0.67 \mu\text{m}$, $n = 33$) cells having larger diameters than DCN neurons ($16 \pm 0.35 \mu\text{m}$, $p < 0.05$, $n = 75$) (Fig. 1D). Calculations of cell volume, however, revealed no statistically significant differences between SPNs in the IML ($2200 \pm 250 \mu\text{m}^3$, $n = 33$), DCN ($1600 \pm 110 \mu\text{m}^3$, $n = 75$), and IC ($2500 \pm 440 \mu\text{m}^3$, $n = 8$) (Fig. 1E). Analysis of cell shape in the transverse plane using the ratio of the cell's short to long diameter revealed significant differences for the cells of the IC (0.43 ± 0.037 , $n = 8$, $p < 0.003$) compared to the DCN (0.64 ± 0.020 , $n = 75$) and IML (0.60 ± 0.021 , $n = 33$) (Fig. 1F), indicating that IC cells are significantly more elongated compared to the ellipsoid DCN and IML cells. Our results here demonstrate that despite the cells of these three nuclei all innervating the MPG, morphologically these cells exhibit different characteristics, with the soma of IC cells exhibiting more elongation than DCN and IML cells. DCN and IML cells share some morphological characteristics such as cell shape, but the IML and IC somata exhibit larger mean diameters than their midline counterparts, despite all cell bodies encompassing similar volumes.

Identification of SPNs in the electron microscope

A total of 18 retrogradely labeled SPNs were identified and analyzed in the electron microscope. The MPG-innervating SPNs were located in the DCN ($n = 15$) and IML ($n = 3$). No IC cells were included in the analyses.

SPNs were identified using post-embedding immunogold detection of FG. Labeled neurons exhibited a characteristic accumulation of gold particles inside small, enclosed, lysosome-like structures (Fig. 2). These structures were sometimes irregularly shaped and some exhibited electron-lucent vacuoles (data not shown), confirming previous studies (Persson and Havton, 2009). Only SPNs exhibiting unequivocally-positive FG labeling, as well as a nucleus in the plane of section, were included in the study.

General ultrastructural features of SPNs

Quantitative EM data obtained from MPG-innervating DCN and IML neurons were pooled, as our light microscopic studies of a larger sample of retrogradely labeled SPNs demonstrated that these neuronal subpopulations do not differ with regard to neuronal volume or shape. Pooling the ultrastructural data on soma size and shape from DCN and IML neurons yielded a skewed unimodal distribution of cell diameter (Fig. 3) with an average diameter of ($19.60 \pm 1.09 \mu\text{m}$, $n = 18$). The somata displayed an ellipsoid shape with a ratio between the short and long axes of 0.66 ± 0.03 (Table 1). Using the formula for an oblate spheroid, the mean soma volume was calculated to be $3487 \pm 677 \mu\text{m}^3$.

Most SPNs exhibited a round to ellipsoid nuclear envelope. However, some SPNs showed invaginations of the nuclear membrane. In our sample, a total of 6 of the 18 SPNs showed such atypical nuclear membrane structures (Fig. 4A-C). The nuclear invaginations were often associated with the accumulation of ribosomes. Examples of SPNs with invaginated nuclear envelopes were encountered in 4 of the 5 animals we studied. In addition, individual rats contained SPNs both with and without nuclear invaginations.

Boutons in apposition with SPN somata

We determined the ultrastructural features of a total of 106 boutons in close apposition with the somata of 18 MPG-innervating SPNs. Two cells, both in the DCN nucleus, exhibited no bouton appositions along the soma, membrane, although the surrounding neuropil did show synaptic contacts made with unlabeled dendritic profiles (Fig. 5). Most boutons could be classified into three distinct types based upon their ultrastructural features. The most common bouton was the F-type bouton, which displayed flat or pleomorphic synaptic vesicles and were found to make symmetrical synaptic specializations (Fig. 6A). Occasionally, these boutons would also exhibit multiple synaptic specializations along the same target (Fig. 6D). The average apposition length for F-type boutons was $1.40 \pm 0.07 \mu\text{m}$ ($n = 74$). S-type boutons exhibited clear, and spheroid synaptic vesicles, commonly with an asymmetric synaptic complex (Fig. 6B). S-type boutons on the SPN soma had an average apposition length of $1.24 \pm 0.11 \mu\text{m}$ ($n = 30$). Only two DCV boutons were observed, with a mixture of clear, spheroid vesicles and larger, dense-core vesicles (Fig. 6C). The mean apposition length of the DCV boutons were $1.41 \pm 0.07 \mu\text{m}$.

Quantitative analysis of appositions on the SPN soma (Table 1, Figure 7A) showed that these neurons exhibit relatively sparse synaptic inputs, but extensive variation between neurons was present. Some cells exhibited no synaptic appositions, and some had up to almost 26% of the soma membrane covered by ultrastructurally verified boutons. On average, $11.60 \pm 2.41\%$ of the cell soma received bouton appositions ($n = 18$). Specifically, F-type boutons covered an average of $8.48 \pm 2.12\%$ of the soma membrane surface and represented a significantly larger portion of the soma membrane compared to the $2.94 \pm 0.70\%$ coverage provided by the S-type boutons ($p < 0.05$) (Fig. 8A). The SPN somata demonstrated an average of 8.54 ± 1.62 boutons per $100 \mu\text{m}$ in close apposition with their membrane surface ($n = 18$). The cell body surface received 5.94 ± 1.40 F-type bouton appositions per $100 \mu\text{m}$, and 2.34 ± 0.48 S-type boutons per $100 \mu\text{m}$ (Fig. 8B). There was no statistically significant difference between these numbers for the F-type and S-type boutons. Portions of the SPN soma membrane not covered by boutons were apposed instead by glial processes, passing axons, or dendritic profiles. We did not observe any gap junctions or puncta adherentia in association with the SPN soma membrane and adjacent dendrites. However, we did observe two instances of boutons that formed synapses with adjacent dendrites in the neuropil that also abutted the cell soma membrane. No definite synaptic contacts were formed against the SPN soma membrane in these instances, and there was no clustering or docking of vesicles observed near the soma membrane. However, some ultrastructural features suggestive of a non-synaptic adherence formed between the apposing bouton and the SPN soma were present (Fig. 6E, F).

Boutons in apposition with SPN dendrites

A total of 7 of the 18 retrogradely labeled SPNs in our ultrathin sections exhibited dendritic branches extending from the cell body. Of these cells, 1 was located in the IML, and the rest were found in the DCN.

Analysis of the proximal dendritic arbors in direct continuity with the soma of SPNs (Table 2, Figure 7B) revealed the presence of only F-type and S-type boutons ($n = 45$). No DCV boutons were observed. The average apposition length of F-type boutons on dendrites was $1.65 \pm 0.16 \mu\text{m}$ ($n = 30$), while the average apposition of S-type boutons was $1.38 \pm 0.15 \mu\text{m}$ ($n = 15$). The distribution of both bouton apposition lengths revealed a unimodal distribution (Fig. 8C, D). Statistical analysis using a two-way ANOVA revealed no significant differences between apposition lengths of either bouton type or location (soma vs. dendrite). There were, however, differences between bouton type when comparing percentage coverage of the dendritic membrane, as F-type boutons ($12.65 \pm 4.34\%$, $p = 0.03$) covered a

significantly higher proportion of membrane than S-type boutons ($3.68 \pm 2.98\%$) (Fig. 8A). On average, $16.32 \pm 6.18\%$ of the dendritic membrane was apposed by boutons. The frequency of boutons per $100\ \mu\text{m}$ also showed a significant difference, with a greater frequency of F-type boutons (8.70 ± 3.17 per $100\ \mu\text{m}$, $p = 0.04$) than S-type boutons (2.70 ± 2.13 S-type boutons per $100\ \mu\text{m}$) (Fig. 8B), with an average of 11.40 ± 4.28 boutons per $100\ \mu\text{m}$ in apposition with the dendritic membrane.

DISCUSSION

This study provides a detailed and quantitative assessment of the location and size of MPG-innervating SPNs, as well as the synaptic inputs to these neurons in the female rat DCN and IML nuclei of the thoracolumbar spinal cord. We demonstrate that most cells are located inside the DCN and IML with only a few cells present in the IC. In addition, nearly all of the input onto the DCN and IML cells come from putative excitatory S-type and putative inhibitory F-type boutons apposing the soma and dendritic membrane, and that direct apposition by neuropeptide-containing DCV-type boutons is rare. The membrane density of these inputs is relatively low on average, even when these cells are compared to their lumbosacral parasympathetic counterparts (Persson and Havton, 2008). The amount and proportion of inputs onto the somata and dendrites are similar, however the SPN somata and proximal dendrites receive more input from F-type boutons than S-type boutons, suggesting that these cells may receive more inhibitory than excitatory influence. These results provide a new insight into the processing of synaptic inputs onto female rat SPNs and elucidate in part, a component of the nervous system of the pelvic organs.

Methodological considerations

The present study used an injection of a retrograde tracer directly into the MPG in order to label pelvic-innervating SPNs. This confirms numerous previous studies in both male and female rats that have shown that some thoracolumbar SPNs project their axons directly through the hypogastric nerve to the MPG without synapsing in the sympathetic chain and inferior mesenteric ganglia (Nadelhaft and McKenna, 1987, Baron and Jänig, 1991, Hosoya et al., 1994, Harji et al., 1998). Therefore, we believe that our injections of FG into the MPG have labeled preganglionic neurons, as FG is known to be a retrograde axonal transporter, not a transneuronal transporter (Schmued and Fallon, 1986). This is supported further by previous EM studies, which have shown that FG accumulates in the cytoplasm of neurons in lysosome-like structures (Persson and Havton, 2009).

Fewer IML cells were analyzed in our EM techniques, as there are far fewer retrogradely labeled IML cells than DCN cells according to our light microscopic data. This concurs with previous data of tracers injected into the hypogastric nerve, where it has been shown in females that as few as one-fifth of all SPNs reside in the IML (Nadelhaft and McKenna, 1987). This same study also showed that in comparison with previous studies in males, the total number of SPNs labeled through the hypogastric nerve in females is much lower than that of male rats (Nadelhaft and McKenna, 1987). Due to the nature of ultra-thin sectioning, immunogold labeling, and the scarcity of these neurons, these cells were difficult to detect in the electron microscope. As our light microscopic studies had shown that DCN and IML cells are of similar size, we chose to analyze the DCN and IML cells as one population of MPG-innervating SPNs.

Distribution and properties of labeled SPNs in the female rat traced from FG injection into the MPG

Unfortunately, it is not possible to determine the organ end-target through our method of retrograde tracing, as postganglionic cells in the MPG are known to innervate a number of

pelvic organs, including the reproductive organs, the colon and the lower urinary tract. Though there is a distinct topographic distribution of postganglionic neurons in the MPG of the male rat (Keast et al., 1989), we cannot distinguish which targets our observed sample of SPNs ultimately control. The evidence that the autonomic control of the pelvic viscera converge within a few peripheral ganglia may explain some of the observed heterogeneity in our measurements, as SPNs innervating, e.g., the uterus and the lower urinary tract, likely differ in afferent innervation and function.

Some SPNs exhibit invaginations of the nuclear envelope

Ultrastructural studies of other neuronal populations have demonstrated that neurons in other areas of the central nervous system also exhibit characteristics of nuclear invaginations, similar to those described in this study. Such nuclear features have been described in early studies in many developing neurons, and are often associated with rapid phases of neuronal and perikaryal growth, but the nuclear folds are lost with advancing maturity (LaVelle and LaVelle, 1970). However, pyramidal cells in the hamster motor cortex show invaginations of the nuclear envelope that persist through the stages of development and maturation, and into the stages of aging (Buschmann et al., 1981). It has been hypothesized that these nuclear folds are a result of an increase in protein synthesis (Hydén, 1943, Pico and Gall, 1989). Ultrastructural studies of the nucleus of the solitary tract have revealed that barosensitive neurons exhibit marked nuclear invaginations, which increase after being challenged by a hypertensive episode, however this was proposed to be associated with a mechanism inhibiting gene transcription, due to the simultaneous increase of heterochromatin in the nucleus (Chan et al., 2000). As such, it is unclear what the functional correlate of the invaginated nuclear envelope is.

The occurrence of nuclear invaginations has not been studied extensively in autonomic neurons of the spinal cord. However, a review of previously published literature on the ultrastructural studies of PPNs in female rats reveal examples of nuclei both with and without nuclear invaginations (Havton and Broman, 2005, Persson and Havton, 2008), though no quantitative assessments of the relative occurrence of these features were conducted. In addition, we have not found any literature addressing this characteristic in the rat dorsolateral nucleus, which contains motoneurons innervating the external urethral sphincter. Therefore, it is possible that the nuclear folds described here may be a feature of the pelvic-innervating autonomic neurons.

Under the assumption that the nuclear folding is indicative of protein synthesis, it is possible that the invaginations are indicative of the tonic activity the SPNs must maintain during continence. As the SPNs are a heterogeneous population that are responsible for targeting a variety of pelvic organs, it is also possible that the nuclear invaginations may represent unique functions in female rats, which may account for why the feature has not been previously described in ultrastructural studies, since male rats are preferentially used as subjects. Therefore, it is possible that the folds could represent different stages in the regulation of the estrus cycle. Since the exact function of the nuclear foldings remains to be determined, we cannot draw definitive conclusions.

SPN soma and proximal dendrites receive almost all input from two distinctive bouton types

Previous studies have demonstrated marked correlations between the ultrastructural features of synaptic vesicles, neurotransmitter contents, and therefore, function. F-type boutons, for example, have been shown to exhibit immunoreactivity for GABA and/or glycine in the cat ventral horn, thus suggesting that boutons with flat, pleomorphic vesicles are most likely inhibitory (Örnung et al., 1994, Örnung et al., 1996). Previous studies have detected the

presence of GABA and glycine in boutons in apposition with thoracolumbar SPNs in male rats (Santer et al., 2002). Past studies combining electron microscopy with immunohistochemistry have also demonstrated that the excitatory transmitter, glutamate, has a strong correlation with ultrastructural characteristics such as clear, spheroid synaptic vesicles (Broman et al., 1993, Shupliakov et al., 1993, Örnung et al., 1998, Persson and Broman, 2004). We therefore speculate that the S-type boutons here may be excitatory. Glutamate has also been demonstrated in boutons apposing SPNs traced from the MPG in male rats (Santer et al., 2002). Studies of the vesicular glutamate transporters, such as VGLUT2, are known to densely innervate the thoracolumbar sympathetic IML and the DCN, and boutons immunopositive for the VGLUT2 marker have been found to appose these SPNs associated with pelvic targets in male rats (Llewellyn-Smith et al., 2007). Compared to previous studies of pelvic-innervating PPNs in female rats (Persson and Havton, 2008), thoracolumbar SPNs exhibit far fewer appositions of DCV-type boutons, which are widely regarded as containing neuropeptide transmitters (Hökfelt et al., 1987).

SPN soma and proximal dendrites receive more putatively inhibitory input

Our studies revealed that F-type boutons cover a significantly larger percentage of the somatic and proximal dendritic membrane of SPNs than S-type boutons, though overall, coverage of these neurons was sparse. In comparison to previous studies, this coverage was even lower than the complementary pelvic-innervating PPNs in female rats (Persson and Havton, 2008). Our results are consistent with prior studies, which have noted that SPNs at the T1-T4 level receive few somatic inputs, despite many synaptic contacts being made on nearby dendrites in the vicinity the neuropil (Markham and Vaughn, 1990). These studies also noted, however, that the dendrites receive more bouton appositions than the soma (Markham and Vaughn, 1990), though this was not observed in our study, which compared inputs to soma and proximal dendrites. It has been suggested that due to the different peripheral targets and target organ functions, SPNs along the thoracic and thoracolumbar spinal cord exhibit rostrocaudal heterogeneity (Llewellyn-Smith, 2009), and therefore findings regarding SPNs at the upper thoracic level may not necessarily hold true for SPNs at the thoracolumbar level.

While somatic and proximal dendritic inputs on MPG-innervating SPNs in this study were relatively sparse, we do not claim that inputs upon these SPNs are scarce in general. It is known from previous morphological studies that SPNs in the IML and DCN exhibit extensive rostrocaudal, longitudinal dendritic bundles, as well as transverse dendritic bundles (Barber et al., 1984). As such, it is likely that SPNs receive most of their input from their distal dendrites, which we did not identify in the study, as detectable FG-labeling was almost exclusively isolated to the cytoplasm of the cell body. Previous studies have also shown that the distribution of glutamatergic and GABAergic inputs onto the soma and dendrites of SPNs innervating the superior cervical ganglion differ from those innervating the adrenal medulla (Llewellyn-Smith, 2002). Therefore, given the functional differences between the different visceral targets, it is difficult to precisely predict what the pattern of dendritic input will be on the MPG-innervating SPNs, but we speculate that innervation of the distal dendrites would be more plentiful than what we have found on the soma. It is likely that the tonic drive on these SPNs, in order to maintain the continence of the bladder, would likely rest on the distal dendrites, since there are very few putatively excitatory synaptic appositions to be found on the cell soma.

The predominance of putatively-inhibitory inputs onto the soma and proximal dendrites of MPG-innervating SPNs fits with some of the known evidence of sympathetic function in the pelvic viscera. In the lower urinary tract, the sympathetic system is tonically active in order to maintain continence. The activation of micturition, however, includes an inhibition of the sympathetic control, thus disinhibiting the detrusor and the bladder outlet (de Groat,

1993) and allowing for the expulsion of urine. A combination of tracing and spinal cord transection studies have shown that the majority of the inputs on SPNs come from intraspinal or supraspinal sources (Stornetta and Guyenet, 1999, Llewellyn-Smith et al., 2005, Llewellyn-Smith et al., 2006), which highlights the importance of interneurons mediating SPNs control, instead of direct contact by primary afferents.

Rare boutons immunoreactive for the monoamine, serotonin, have been observed to appose MPG-innervating SPNs in male rats (Ranson et al., 2006), and opioid action through enkephalin has also been observed on MPG-innervating SPNs, with as much as half of the input on SPN soma ta exhibiting immunoreactivity for the marker (Llewellyn-Smith et al., 2005). The ultrastructural phenotypes associated with these transmitters has not been described in detail, however, and it is possible that some of these transmitters may coexist with others, as enkephalin may coexist in terminals with both glutamate or GABA in the medulla (Llewellyn-Smith et al., 2001).

Non-synaptic neuronal interactions in the thoracolumbar spinal cord

Only one example of a possible non-synaptic interaction between a bouton and an SPN was observed. The contact may represent a zona adherentia, a gap junction, or some other non-synaptic adherence between a bouton and a cell membrane. SPNs in the thoracic cord are known to have demonstrated electrotonic coupling (Logan et al., 1996), and motoneuron somata in homologues for Onuf's nucleus in the rat and cat spinal cord have demonstrated puncta adherentia and gap junctions (Kerns and Peters, 1974, Matsumoto et al., 1988, Matsumoto et al., 1989, Ramirez-Leon and Ulfhake, 1993). While the existence of these structures in the spinal cord has been demonstrated, non-synaptic neuronal interactions appear to be relatively rare among MPG-innervating SPNs in the rat spinal cord based on our findings.

Comparisons to PPNs

We are unaware of any previously published synaptology studies on the somatic motoneurons innervating the rat pelvic viscera. Thus, no direct comparisons can be made. However, previous studies have investigated the inputs on PPNs in female rats (Persson and Havton, 2008). Despite similarities in mean soma diameter, eccentricity, circumference, and volume, the overall input on SPNs appeared to be more sparse than the input on PPNs on both the soma and the proximal dendrites. PPNs exhibited greater frequency of DCV-type boutons, whereas they were rarely apposing the soma SPNs in our study, and none were discovered on the proximal dendrites. Dendritic input also appeared to be different from that of somatic input in PPNs, with an additional bouton type, the L-type, being observed at the somatodendritic junction in PPNs (Persson and Havton, 2008). We did not observe this bouton type in our sample. General results show that despite innervation of the same pelvic targets and some morphological similarities, the sympathetic and parasympathetic neurons exhibit different distributions of somatic and dendritic inputs. We speculate that the differences in synaptic innervations of the somata and proximal dendrites of PPNs and SPNs may reflect functional differences between the different neuronal types.

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LIST OF ABBREVIATIONS

DCN	dorsal commissural nucleus
FG	Fluorogold
IC	intercalated nucleus
IML	intermediolateral nucleus
MPG	major pelvic ganglion
PPNs	parasympathetic preganglionic neurons
SPNs	sympathetic preganglionic neurons

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HIGHLIGHTS

- Majority of SPNs innervating the MPG in female rats reside in DCN
- DCN and IML SPNs have similar cell volume and shape
- IC SPNs display significant shape elongation compared to DCN and IML SPNs
- Coverage by boutons of SPN soma and dendrites is sparse
- SPN soma and dendrites predominantly covered by putatively inhibitory boutons

Figure 1.

Representative transverse sections of positive FG-labeling by immunohistochemistry of MPG-innervating SPNs at the L1-L2 level. A) Depicts the cells of the DCN above the central canal (c.c.), as well as the IC neurons, which often have long, lateral projections. Scale bar = 250 μm. B) A higher magnification of positively labeled neurons of the IML. Scale bar = 50 μm. C) Distribution of labeled SPNs by nuclei. D) Comparison of SPN soma diameter. E) Comparison of SPN cell volume. F) Comparison of SPN elongation, as calculated by the ratio of the neuron's short (B) and long (A) diameter. *: $p < 0.05$

Figure 2.

Image depicting an SPN from the DCN exhibiting positive FG labeling in lysosome-like structures of the cytoplasm in proximity to the cell nucleus (nuc). Note that the FG-positive lysosomes (arrows) are dotted with round gold particles, which are much more regular in shape and more electron dense than other structural features of the cytoplasm, such as ribosomes. Thus, these structures are distinctly different from the mitochondria, which exhibit no gold. Scale bar: 1 μ m.

Figure 3.

The skewed unimodal size distribution of studied SPNs in the DCN and IML nuclei labeled through immunogold detection of FG. A neuron's size is expressed as the mean diameter, which is the average of the soma's longest diameter, and the longest diameter perpendicular to that.

Figure 4.

Representative samples of the atypical nuclear features found in a subset of SPNs. All cells depicted are from neurons from the DCN. Cell nuclei exhibited characteristics such as holes (arrow) in the nuclear envelope in the plane of section (A), invaginations (arrows) in the nuclear membrane (B), or two completely disconnected pieces of the nucleus (C), labeled “1” and “2.” All images are composites. A) and B) are contrast-only sections, while C) has been labeled with FG. Scale bar in all images is 5 μ m.

Figure 5.

A representative SPN from the DCN exhibiting an invaginated nucleus (as previously depicted in Fig. 4A) and no bouton appositions on the cell body. There are, however, a few synapses made in the surrounding neuropil, including S-type, F-type, and DCV-type boutons. S-type boutons are characterized by a thick and asymmetrical synaptic specialization (arrowheads), and clear, round vesicles (arrow). F-type boutons have a symmetrical synaptic specialization (arrowhead), and flat, small vesicles (arrow). DCV-type boutons, which are observed rarely, contain a combination of large round, electron-dense vesicles (black arrows), and round, clear vesicles (white arrow) with a symmetrical synaptic

specialization (arrowhead). Images are composites from a contrast-only section. Scale bar in whole-cell image is 5 μm , scale bar in bouton image represents 1 μm .

Figure 6.

photomicrographs of boutons apposing the somatic or dendritic membranes of the studied SPNs. A) Two F-type boutons, with flat, pleomorphic vesicles (white arrow) and symmetric synaptic specializations (black and white arrowheads). B) Two S-type boutons with round, spherical, clear vesicles (black arrow) and asymmetric synaptic specializations (black and white arrowheads). C) Bouton demonstrating the large, dark, dense-core vesicles characteristic of DCV-type boutons. D) F bouton apposing a dendritic membrane exhibiting multiple synaptic specializations (black arrowheads). E) Bouton adjacent to soma membrane, forming synaptic contact (black arrowheads) with recruitment of vesicles (black arrows) to a dendrite in the neuropil. Note that vesicles are not docking near the membrane surface abutting the soma (white arrowheads). F) Bouton adjacent to soma membrane, also forming synaptic contact (black arrowheads) against a dendrite in the neuropil. Note that the vesicles (black arrows) are not docking to the membrane abutting the soma. Adherence to the soma membrane does appear, however (white arrowheads), and is in stark contrast to area of the membranes not forming adherence (grey arrowheads). All images are from contrast-only sections, with the exception of C), which has been immuno-labeled for FG. Scale bar in all images is 1 μ m.

Figure 7.

Two neurons representative of the diversity of the studied sample, one from the IML (A) with a high amount of bouton coverage and no dendrite, and a soma from the DCN (B) with an associated dendritic process (C), both with sparse bouton appositions. All images are composited from 7200 $\times$ magnification plates. Contrast differences in images represent artifacts from film processing. In both panels, boutons are color-coded. S: red, F: blue. Scale bar in all images: 5 μ m. All images are taken from contrast-only sections.

Figure 8.

Distribution of boutons apposing the somatic and dendritic membranes. A) Percentage of the membrane covered by each bouton type. Both in the soma and the dendrites, the F-type boutons cover a significantly higher percentage than the S-type boutons. B) Total number of boutons of each type per 100 μm of membrane surface. Analysis showed significantly higher number of F-type boutons than the S-type on the dendrites. * $p < 0.05$. C) Frequency distribution of apposition lengths of F-type boutons on SPN soma and dendrites. D) Frequency distribution of apposition lengths of S-type boutons on SPN soma and dendrites.

TABLE 1

Measurements of bouton appositions on the soma membrane

CellID	Nuclear Location	Mean diameter (μm)	B:A aspect ratio ^a	Circumference (μm) ^b	Volume (μm ³)	Percentage Coverage				Number of boutons/100μm			
						S	F	DCV	Total	S	F	DCV	Total
1	DCN	14.62	0.70	48.49	1309	0	0	0	0	0	0	0	0
2	DCN	17.52	0.78	58.23	2432	1.90	34.1	0	36.0	1.72	18.9	0	20.6
3	DCN	16.05	0.62	54.84	1572	0	4.75	0	4.75	0	3.65	0	3.65
4	DCN	16.09	0.54	54.97	1386	2.03	2.45	0	4.48	1.82	1.82	0	3.64
5	DCN	24.82	0.84	82.30	7269	7.49	10.6	0	18.1	3.65	7.29	0	10.9
6	DCN	17.75	0.69	60.31	2320	6.97	6.70	0	13.7	4.97	3.31	0	8.28
7	DCN	18.58	0.67	61.95	2592	0.916	0	0	0.916	1.61	0	0	1.61
8	DCN	17.8	0.77	60.85	2572	9.93	13.4	0	23.3	4.93	8.2	0	13.1
9	DCN	12.87	0.67	42.23	865	1.79	0	0	1.79	2.37	0	0	4.16
10	DCN	17.11	0.68	57.05	2035	0	0	0	0	0	0	0	0
11	DCN	18.87	0.42	66.39	1736	0	12.5	0	12.5	0	6.02	0	6.02
12	DCN	30.58	0.68	99.92	11619	2.98	9.99	0	16.5	2.00	7.01	0	10.0
13	DCN	17.15	0.43	60.08	1335	2.73	0	0	2.73	1.66	0	0	1.66
14	DCN	18.37	0.66	61.35	2486	2.06	13.2	0	15.3	1.63	11.4	0	13.0
15	DCN	27.07	0.49	92.90	5982	3.61	2.36	3.04	9.01	5.38	3.23	2.15	10.8
16	IML	20.47	0.92	66.45	4283	5.57	20.3	0	25.9	4.51	16.6	0	21.1
17	IML	25.56	0.81	86.08	7764	4.97	16.0	0	21.0	5.81	13.9	0	19.7
18	IML	21.1	0.53	72.43	3208	0	6.32	0	6.32	0	5.52	0	5.52
Mean		19.60	0.66	65.93	3487	2.94	8.48	—	11.60	2.34	5.94	—	8.54
± S.E.		1.09	0.03	3.60	677	0.70	2.12	—	2.41	0.48	1.40	—	1.62

^a aspect ratio between the neurons short (B) and long (A) diameters.^b Circumference of soma membrane visible in the section.

TABLE 2

Measurements of bouton appositions on the dendritic membrane

Cell ID	Nuclear Location	Membrane length (μm) ^a	Percentage Coverage			Number of boutons/100 μm		
			S	F	Total	S	F	Total
4	DCN	79.80	1.96	2.80	4.76	1.25	1.25	2.50
5	DCN	84.97	21.4	21.4	42.8	15.3	11.8	27.1
7	DCN	23.71	0	3.39	3.39	0	4.22	4.22
9	DCN	10.13	0	8.74	8.74	0	9.87	9.87
11	DCN	82.82	0	12.0	12.0	0	6.04	6.04
13	DCN	47.49	0	5.90	5.90	0	2.11	2.11
17	IML	42.96	2.41	34.3	36.7	2.33	25.6	27.9
Mean		53.13	3.68	12.65	16.33	2.70	8.70	11.40
± S.E.		11.40	2.98	4.34	6.18	2.13	3.17	4.28

^a total length of the dendritic membrane visible in the section.