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Differential Synaptic Inputs to the Cell Body and Proximal Dendrites of Preganglionic Parasympathetic Neurons in the Rat Conus Medullaris

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Abstract

Preganglionic parasympathetic neurons (PPNs) reside in the intermediolateral (IML) nucleus of the rat lumbosacral spinal cord and contribute to the autonomic control of visceral pelvic organs. PPNs provide the final common pathway for efferent parasympathetic information originating in the spinal cord.

We examined the detailed ultrastructure of the type and organization of synaptic inputs to the cell body and proximal dendrites of PPNs in the rat conus medullaris. The PPNs were retrogradely labeled by a systemic administration of the B subunit of cholera toxin conjugated to horseradish peroxidase.

We demonstrate four distinct types of synaptic boutons in apposition with PPN somata and proximal dendrites: S-type boutons show clear, spheroid vesicles; F-type boutons show flattened vesicles; DCV-type boutons show a mixture of clear and dense-cored vesicles; L-type boutons were rare, but large, exhibited clear spheroid vesicles, and were only encountered in apposition with the PPN dendrites in our sample. The membrane surface covered by apposed boutons was markedly higher for the proximal dendrites of PPNs, compared to their somata. The inhibitory synaptic influence was markedly higher over the PPN somata compared to their proximal dendrites, as suggested by the higher proportion of putative inhibitory F-type boutons in apposition with the soma and a higher frequency of S-type boutons per membrane length for the proximal dendrites. Our studies suggest that the synaptic input to PPNs originates from multiple distinct sources and is differentially distributed and integrated over the cell membrane surface.

Keywords

Electron microscopy; Ultrastructure; Autonomic; Spinal cord; Sacral; Retrograde tracing

The spinal parasympathetic nucleus contributes to the autonomic control of pelvic visceral organs, including the urinary bladder and distal colon (de Groat, 2006; Chung and Emmanuel, 2006). For this purpose, preganglionic parasympathetic neurons (PPNs), interneurons, and spino-bulbar neurons, which are located in the intermediolateral (IML) nucleus of the conus medullaris (Araki, 1994; Hamilton et al., 1995; Araki and de Groat, 1996; Birdier et al., 1999), contribute to the reflex control of the pelvic organs, and integrate synaptic inputs from primary visceral afferents, spinal interneurons, and supraspinal nuclei (de Groat, 2006).

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Early electron microscopic studies on the IML nucleus examined the fine structure of its synaptic inputs and the ultrastructural features of the neuronal somata. For instance, quantitative ultrastructural studies of synaptic profiles in the cat sacral IML nucleus showed the presence of boutons containing clear spherical vesicles, boutons exhibiting a mixture of clear and dense-cored vesicles, and boutons containing flattened vesicles (Nolan and Brown, 1978; Brown and Nolan, 1979). Electron microscopic studies of the IML nucleus in the thoracic spinal cord of the non-human primate *Macaca fascicularis* identified three main morphological types of neurons based on neuronal size, shape, relative prominence of the rough endoplasmic reticulum and Golgi apparatus, as well as patterns of synaptic inputs (Wong and Tan, 1980). However, these early ultrastructural studies did not include anatomical tracing techniques, or other methods to identify different functional subsets of neurons, and hence were unable to conclusively distinguish between e.g. PPNs and interneurons present in the IML.

As PPNs of the IML nucleus provide the final common pathway for parasympathetic information exiting the spinal cord, synaptic inputs to their somata and proximal dendrites are likely to exhibit a particularly prominent influence on the autonomic function of pelvic organs and have attracted detailed ultrastructural investigations. Electron microscopic studies of retrogradely labeled PPNs in the cat IML determined the relative contributions of different types of synaptic boutons to the PPN somata and proximal dendrites and demonstrated that the PPN proximal dendrites exhibit a higher degree of synaptic coverage than the somata (Mawe et al., 1986; Leedy et al., 1988). However, ultrastructural studies on the PPNs and the IML nucleus in rodents have been sparse.

The present study was performed to investigate the ultrastructural features of the synaptic inputs to PPNs of the IML nucleus in the adult female rat. A comparatively generous PPN sample was included in the studies to allow for detailed quantitative investigations of both prevalent and rare synaptic bouton types in a cell population with relatively sparse overall synaptic membrane coverage. Previous studies, using application of horseradish peroxidase (HRP) to the cut pelvic nerve, have demonstrated that PPNs are located in the IML of the L6 and S1 segments of the rat spinal cord (Hancock and Peveto, 1979; Nadelhaft and Booth, 1984). Here, we pre-labeled PPNs by a systemic administration of the B subunit of cholera toxin conjugated to horseradish peroxidase (B-HRP). Histochemical processing and plastic embedding of spinal cord sections allowed for the visualization of an HRP reaction product within retrogradely labeled PPNs in both the light and electron microscope (Havton and Broman, 2005).

We demonstrate the presence of four distinct synaptic types in apposition with PPNs in the IML of the rat conus medullaris. The most commonly encountered synaptic boutons exhibited clear, round vesicles (S-type), a mixture of clear and dense-cored vesicles (DCV-type), or flat vesicles (F-type). In addition, a rare synaptic bouton type was characterized by its very large size, presence of clear, round vesicles, and an affinity for making appositions with proximal dendrites (L-type). Overall, synaptic inputs to the PPN somata were relatively sparse, with their proximal dendrites showing a markedly higher degree of membrane coverage by synaptic boutons. However, the PPN somata demonstrated a relatively larger proportion of the putative inhibitory F-type boutons, suggesting that the somata may be under a more prominent inhibitory influence than the proximal dendrites. The present data will also serve as a reference base for future ultrastructural studies on nerve regeneration and plasticity of autonomic spinal reflexes, as the female rat has already demonstrated to be a valuable model for functional studies of both upper and lower motoneuron injury and repair (Pikov and Wrathall, 2001; Cheng and de Groat, 2004; Hoang et al., 2006).

EXPERIMENTAL PROCEDURES

Three adult female rats (150-225g, Charles River Laboratories, Raleigh, NC) were used for the studies. All animal procedures were performed in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the Chancellor's Animal Research Committee at UCLA. The animals were housed in a room with a 12 hour dark/light cycle with food and water access *ad libitum*.

Retrograde labeling and detection of efferent spinal cord neurons

Retrograde labeling and detection of efferent spinal cord neurons were performed according to our established protocol (Havton and Broman, 2005). In short, 0.2 mg of the B subunit of cholera toxin conjugated to horseradish peroxidase (B-HRP, List Biological Laboratories, Campbell, CA) was dissolved in 400 μ l of sterile water and administered by intraperitoneal (i.p.) injection.

Five days after the tracer injection, the rats were deeply anesthetized using sodium pentobarbital (100 mg/kg i.p.). A brief transcardial rinse with phosphate buffered saline (PBS, pH 7.4) was followed by tissue fixation by transcardial perfusion with a solution containing 1% paraformaldehyde and 2.5% glutaraldehyde in PBS at pH 7.4. A subsequent brief transcardial rinse with PBS removed any excess fixative from the vasculature. All solutions used for the tissue perfusion were kept at 4°C.

The spinal cord was removed, kept at 4°C, and the L6 and S1 segments were cut into serial 40 μ m transverse sections using an Oscillating Tissue Slicer (Electron Microscopy Sciences, Fort Washington, PA). The sections were reacted for HRP detection with 3, 3', 5,5'-tetramethyl benzidine (TMB) as a chromogen, using a modification of the protocol by Mesulam (1978) and Mesulam et al. (1980). Specifically, the stabilizing agent sodium ferrocyanide was replaced by ammonium molybdate (Olucha et al., 1985), to allow for a light insensitive reaction to take place near neutral pH. Here, 50 mg of ammonium molybdate tetrahydrate (Sigma A-7302) was dissolved in 20 ml of 0.1 M phosphate buffer (PB, pH 6.0-6.3) and kept at 4°C in an ice water bath. One TMB tablet (1 mg substrate, Sigma-5525) was dissolved in 0.5 ml absolute ethanol using a warm water bath and was added to the ammonium molybdate solution. Next, 10 μ l of 30% H₂O₂ was diluted in 1 ml of distilled water and added to the TMB/ammonium molybdate solution. The spinal cord sections were briefly rinsed in PB and incubated for 12-18 hours in the ammonium molybdate/TMB/H₂O₂ solution at 4°C. The sections were rinsed in PB and inspected in a dissection light microscope. Sections of the L6 and S1 segments demonstrating labeled cell bodies in the intermediolateral column (IML) were selected for EM processing.

Processing of sections for EM

Spinal cord sections, which exhibited cell bodies in the IML of the L6 and S1 segments were incubated in 1% osmium tetroxide in 0.1 M PB at pH 5.5 over 18 hours. This slow osmication at low pH allowed for the preservation of a TMB-molybdate reaction product that was more stable compared to the marked TMB reaction product loss when osmication occurs at neutral pH (Henry et al., 1985; Ralston, 1990). The sections were rinsed in PBS, dehydrated in graded ethanol, and embedded in Durcupan® ACM (Electron Microscopy Sciences). Ultrathin sectioning of tissue containing labeled neurons in the IML was performed using an RMC Products PowerTome ultramicrotome (Boeckeler Instruments, Tucson, AZ). The ultrathin sections (60-70 nm thickness) were serially collected on formvar-coated copper one-hole grids, and counterstained with uranyl acetate and lead citrate.

Ultrastructural analysis of labeled neurons

Ultrathin sections containing retrogradely labeled PPNs were examined in a JEOL 100 CX electron microscope. Only labeled PPNs exhibiting the nucleus in the plane of sectioning were included in the analysis. Each PPN was analyzed in a single ultrathin section. The entire PPN, including all visible dendrites and its immediate environment, was captured on photographic film at a primary magnification of 2,900X. The negatives were scanned into digital files at a resolution of 137 pixels/ μm , and these were used for all subsequent measurements, using C-Imaging software (Compix, Sewickley, PA). Images were corrected for brightness and contrast prior to analysis and publication.

The mean soma diameter was calculated as the average between the longest soma diameter (A) and the longest diameter perpendicular to A (B). These measurements were also used to calculate the soma volume according to the formula for the volume of an oblate spheroid, $\pi/6AB^2$ (Ulfhake, 1984).

Boutons in apposition with PPN somata and proximal dendrites were characterized based upon their ultrastructural features, including size, presence of synaptic vesicles, mitochondria, as well as synaptic vesicle size, shape, and electron density. In the instances where a postsynaptic specialization, or active zone, was clearly discernible, its appearance was included in the characterization.

Measurements of individual boutons were performed at a primary magnification of 7,200X (digitized at a resolution of 340 pixels/ μm). The perimeter and soma apposition length of each bouton were determined. Next, the percentage of synaptic coverage and number of boutons per 100 μm of membrane was calculated. The percentage of synaptic coverage was calculated for both somata and dendrites as: (Sum of bouton apposition lengths X 100)/(Total membrane length).

Statistical analysis

Comparisons between samples were performed using the non-parametric Mann-Whitney U-Test (two-tailed) (SigmaStat 3.1, Systat Software, Inc., Point Richmond, CA). We regarded $p < 0.05$ as reflecting a statistically significant difference between samples. All quantitative data were expressed as mean $\pm$ standard error of the mean (SE).

RESULTS

The intermediolateral (IML) nucleus of the L6-S1 spinal cord segments was studied ultrastructurally in adult female rats after a systemic injection of B-HRP (Fig. 1A). PPNs were identified by the intracellular presence of the electron-dense HRP reaction product, which was detected in the nucleus and cytoplasm of somata, and within proximal dendrites (Fig. 1B). Every neuron included in the study was clearly labeled, typically with several instances of reaction product found in the cytoplasm and/or nucleus. Occasionally, the HRP reaction product was detected in synaptic boutons. This was presumably an effect of B-HRP uptake by primary afferents, and these boutons were typically in close apposition with non-labeled dendritic profiles in the neuropil. Only one instance was found of a labeled bouton apposing a labeled dendrite (Fig. 3F). It did not, however, display any clearly discernible synaptic specialization. These labeled boutons typically demonstrated spheroid synaptic vesicles with clear centers, although some labeled boutons exhibited a combination of clear and dense-core vesicles (Fig. 1C, D).

General ultrastructural features of PPNs

A total of 23 PPNs sampled from 3 adult female rats (6, 8 and 9 PPNs sampled from the respective animal) were included in the quantitative ultrastructural studies. The PPNs demonstrated a mono-phasic size distribution with a mean diameter of $19.5 \pm 1.0 \mu\text{m}$ ($n=23$) (Figure 2). The somata demonstrated an ellipsoid shape with an aspect ratio between the short and long axis of 0.67 ± 0.04 (Table 1). Using the formula for an oblate spheroid, the mean PPN soma volume was calculated as $3,538 \pm 864 \mu\text{m}^3$.

Three distinct synaptic bouton types are in apposition with PPN somata

We determined the ultrastructural features of a total of 165 boutons in close apposition with the somata of 23 retrogradely labeled PPNs. The boutons were classified into three distinct types based upon their ultrastructural features (Figure 3; Table 1).

The S-type boutons demonstrated clear and spheroid synaptic vesicles, mitochondria, and a mean PPN soma apposition length of $1.68 \pm 0.11 \mu\text{m}$ ($n=49$). Typically, S-type boutons exhibited an asymmetric synaptic complex with the soma membrane.

The F-type boutons displayed flat or pleomorphic synaptic vesicles, mitochondria, and a PPN soma apposition length of $1.61 \pm 0.07 \mu\text{m}$ ($n=82$). When visible, the synaptic specialization of the F-type boutons had a symmetric appearance.

The DCV-type boutons contained a mixture of dense-cored and clear, spheroid synaptic vesicles. The population of clear spheroid synaptic vesicles was typically intermingled with dense-cored spheroid vesicles, the latter ranging in numbers from two or three, up to dominating the appearance of the bouton. Their mean apposition length of $1.98 \pm 0.13 \mu\text{m}$ ($n=34$) was significantly longer than the apposition length of the S- and F-type boutons ($p<0.05$). DCV-type boutons typically exhibited mitochondria and an asymmetric synaptic complex with the soma membrane.

Quantitative analysis demonstrated relatively sparse synaptic input to PPN somata with less than one-fifth of the soma membrane being covered by ultrastructurally verified boutons (Table 1). Specifically, $5.9 \pm 1.1\%$, $9.2 \pm 0.9\%$, and $4.8 \pm 1.1\%$ of the cell body surface was covered by S-type, F-type, and DCV-type boutons, respectively, for a total soma coverage of $20.0 \pm 1.7\%$. The F-type boutons occupied a significantly higher proportion of the soma membrane than either S-type or DCV-type boutons ($p<0.05$; Fig. 5A). As the total soma membrane coverage is a function of both the number and size of boutons, we also calculated the number of boutons per PPN soma membrane length. The cell body surface exhibited 3.5 ± 0.6 , 5.8 ± 0.5 , and 2.4 ± 0.5 boutons per $100 \mu\text{m}$ length for a total of 11.8 ± 1.0 boutons per $100 \mu\text{m}$ soma membrane length. Here, the F-type boutons were more frequent than either S-type or DCV-type boutons ($p<0.05$; Fig. 5B). Portions of the PPN soma membrane not covered by boutons were apposed by glial processes, passing axons, or dendritic profiles. No gap junctions or puncta adherentia were encountered in association with the PPN soma membrane and associated proximal dendritic branches.

PPN dendrites and somata receive synaptic inputs with distinct features

A total of 16 of the 23 retrogradely labeled PPNs in the ultrathin sections studied here also exhibited dendritic processes extending from the cell body.

Electron microscopic analysis of the proximal dendritic arbors in direct continuity with the cell body of PPNs showed the presence of S-type, F-type, and DCV-type boutons, which appeared qualitatively similar to those encountered in apposition with the PPN somata. In contrast to what was observed on the PPN soma, there was no difference in apposition length for dendritic

contacts made by the S-type boutons ($1.74 \pm 0.12 \mu\text{m}$, $n=31$) F-type boutons ($1.76 \pm 0.11 \mu\text{m}$, $n=28$), and DCV-type boutons ($1.80 \pm 0.21 \mu\text{m}$, $n=21$). However, the PPN dendrites demonstrated an additional synaptic bouton type, which was not seen in apposition with any of the PPN somata in the present sample: the Large, or L-type, bouton, which is characterized by its large size, spheroid clear synaptic vesicles, and asymmetrical synaptic specialization. The mean apposition length of the L-type bouton was $2.98 \pm 0.67 \mu\text{m}$ ($n=4$) and it appeared markedly longer than the apposition length for the S-type, F-type, or DCV-type boutons. The L-type boutons were typically in apposition with the PPN dendrites at very proximal locations, including the dendritic side of the somato-dendritic junction. A total of four of the 16 PPNs exhibiting dendritic arbors in our sample, distributed over all three animals, demonstrated the presence of at least one L-type bouton in apposition with their dendritic arbors.

Next, we performed a quantitative comparison of synaptic inputs between PPN somata and their proximal dendrites. The analysis was restricted to the S-type, F-type, and DCV-type boutons and excluded the relatively rare L-type boutons (Table 3; Figure 5). The membrane coverage by apposing boutons was significantly higher for the proximal dendrites ($30.9 \pm 2.7\%$) than for the PPN somata ($19.1 \pm 2.2\%$; $n=16$; $p<0.01$). Similarly, the number of boutons per 100 μm membrane length was higher for the proximal dendrites (16.4 ± 1.2) than for the PPN somata (11.2 ± 1.4 ; $p<0.01$). Also in this sample of PPN somata, the F-type boutons exhibited a higher degree of membrane coverage than the S-type boutons ($p<0.01$) or the DCV-type boutons ($p<0.05$), whereas there was no difference in the relative degree of dendritic membrane coverage for these three bouton types (Table 2; Figure 5A). The PPNs also demonstrated a higher number of F-type boutons in apposition with the soma membrane per 100 μm membrane length compared to the S-type boutons ($p<0.05$) or the DCV-type boutons ($p<0.01$), whereas there was no difference in the frequency of these three bouton types in apposition with the membrane of the proximal dendrites (Figure 5B). However, the frequency of S-type boutons per 100 μm membrane length was higher for the dendrites than for the PPN somata ($p<0.05$; Figure 5B).

DISCUSSION

The present study provides a detailed and quantitative assessment of the synaptic inputs to PPNs in the rat IML nucleus of the conus medullaris. We demonstrate that the putative excitatory S-type, putative inhibitory F-type, and neuropeptide-containing DCV-type boutons are in close apposition with both somata and proximal dendrites of PPNs. In addition, occasional L-type boutons were encountered and apposed to proximal dendrites. The density of synaptic inputs to the PPN cell bodies was relatively low, whereas the proximal dendrites showed a markedly higher density of boutons (Figure 4). Furthermore, the relative proportion of inputs by F-type boutons was higher for PPN somata, compared to their proximal dendrites. These results provide new insight into the processing of synaptic inputs to PPNs, the final common pathway of autonomic output from the conus medullaris of the spinal cord.

PPNs receive synaptic inputs with distinct ultrastructural features

Previous studies have demonstrated close relationships between the ultrastructural features of synaptic vesicles, transmitter contents, and ultimately function. For instance, synaptic boutons with clear spheroid vesicles are commonly associated with an excitatory function in the brain and spinal cord. Specifically, electron microscopy combined with immunohistochemistry has demonstrated that the excitatory transmitter glutamate exhibits a strong association with clear spheroid synaptic vesicles (Broman et al., 1993; Shupliakov et al., 1993; Örnung et al., 1998; Persson and Broman, 2004). Here, both S-type and L-type boutons exhibited clear spheroid vesicles. Glutamatergic synapses may now be further defined by the differential expression of vesicular glutamate transporters (VGLUTs) (Fremeau et al., 2004). In the spinal cord VGLUT1

is typically expressed in the central terminals of primary sensory afferents projecting to the deep dorsal horn and the motor nuclei as well as in the dorsal corticospinal tract, whereas VGLUT2 is prevalent in, for instance, the superficial dorsal horn and interneuronal projections (Oliveira et al., 2003; Todd et al., 2003; Alvarez et al., 2004; Persson et al., 2006). Sympathetic preganglionic neurons in the rat thoracic IML have been noted to receive inputs from both VGLUT2- and VGLUT3-immunoreactive terminals (Nakamura et al., 2004). Interestingly, recent studies have demonstrated a novel VGLUT1-immunoreactive primary afferent projection extending into the parasympathetic IML of the rat lumbosacral spinal cord (Llewellyn-Smith et al., 2007). However, the relationship between VGLUTs and synaptic inputs to the PPN somata remains to be elucidated.

An important inhibitory function has been proposed for both GABA and glycine in the control of bladder reflexes, as increased discharges were recorded from preganglionic parasympathetic neurons in the pelvic nerve of spinal cats following the co-administration of picrotoxin and strychnine, antagonists for GABA and glycine, respectively, but not when each drug was given individually (Thomson and Franz, 1981). The notion that both GABA and glycine mediate inhibition of PPNs has also been supported by recordings of inhibitory postsynaptic currents in slice preparations using the whole cell patch-clamp technique (Araki, 1994).

Ultrastructurally, an inhibitory action has been suggested for synaptic boutons containing pleomorphic or flat vesicles in the spinal cord, as the cat ventral horn exhibited GABA- and/or glycine-immunoreactivity in synaptic boutons containing flat vesicles (Örnung et al., 1994, 1996). However, an interesting exception to this association between synaptic vesicle shape and transmitter content has been suggested, as a subset of glycinergic synaptic boutons in the feline central nucleus of the inferior colliculus exhibited spherical synaptic vesicles and formed asymmetric synaptic specializations (Liu et al., 1999). On this note, previous ultrastructural studies have suggested that the appearance of elongated synaptic vesicles in the central nervous system is an effect of tissue fixation using aldehydes (Walberg, 1966). We speculate that the F-type boutons detected in the rat IML and in apposition with PPN somata and dendrites may also be associated with the storage and release of GABA and/or glycine. This may appear as a reasonable assumption, as electron microscopic studies have already demonstrated the presence of both of these inhibitory transmitters in the rat lumbosacral IML (Santer et al., 2002; Ranson et al., 2006). However, double-labeling ultrastructural studies are needed to determine whether individual terminals in the IML may express co-existence of GABA and glycine.

The identification of dense-cored vesicles in synaptic boutons is commonly associated with the presence of monoamines or neuropeptides (Peters et al., 1991). A combination of light and electron microscopic studies has demonstrated noradrenergic and serotonergic inputs to the IML in rats (Ranson et al., 2006). Immunohistochemical studies of the rat conus medullaris have also demonstrated in the IML the presence of several neuropeptides, including CGRP, substance P, vasoactive intestinal peptide (VIP), neuropeptide Y, galanin, and pituitary adenylate cyclase activating polypeptide (PACAP) (Gibson et al., 1986; Wang et al., 1998; Morgan et al., 1999; Vizzard, 2001; Zvarova et al., 2004, 2005). The association between neuropeptides and dense-cored vesicles in synaptic terminals of autonomic nuclei has also been supported by electron microscopy, as immunoreactivity for VIP has been detected ultrastructurally in synaptic terminals containing a mixture of clear and dense-cored vesicles in the sacral parasympathetic nucleus of cats (Morgan et al., 1999). Additional immunohistochemical studies combined with electron microscopy are needed, however, to determine which neuropeptidergic projections may exhibit a direct synaptic connection with the PPN somata and dendrites.

Overall, our ultrastructural findings here are in good agreement with previous studies on PPNs in the sacral spinal cord of cats. In particular, S-, F-, and DCV-types of synaptic boutons were

also demonstrated to be in apposition with PPNs in the cat (Leedy et al., 1988). However, we demonstrated the presence of an additional bouton type, the L-type, in synaptic contact with PPNs in the rat conus medullaris. The L-type boutons contained synaptic vesicles that were similar to the clear vesicles encountered in S-type boutons but were here considered as a separate entity based on their large size, especially as we did not encounter boutons of an intermediary size.

Synaptic inputs to PPNs are differentially distributed over the soma and dendrites

The cell body surface and the dendritic membranes of efferent spinal cord neurons may show different degrees of synaptic coverage. We demonstrated that the proximal dendrites of PPNs in rat exhibit a markedly higher degree of membrane coverage by synaptic inputs than their somata. Previous studies of retrogradely labeled PPNs in the cat have also shown that proximal dendrites exhibit significantly more of their surface apposed by synaptic terminals than their somata (Leedy et al., 1988). The composition of synaptic types in apposition with the somata and dendritic trees may also differ. Here, PPN somata in the rat show significantly more F-type boutons than DCV- or S-type boutons, whereas the F-, S-, and DCV-types were apposed in similar frequencies with the proximal dendrites. Furthermore, the L-type bouton was only encountered in apposition with the PPN dendrites, not infrequently at the dendro-somatic junction. For comparison, previous ultrastructural studies on the synaptology of neurons in the sacral parasympathetic nucleus in cats demonstrated that F-type boutons were encountered most frequently in apposition with large dendrites, whereas the soma membrane exhibited primarily synaptic inputs from S-type boutons (Brown and Nolan, 1979). In all, the present and previous studies support the notion of a differential distribution of synaptic inputs over the PPN membrane surface.

Primary afferents project into the IML nucleus

In the present study, we used a systemic administration of B-HRP to retrogradely label PPNs in the L6 and S1 segments (Havton and Broman, 2005). This approach may also be used for transganglionic labeling of a subset of primary visceral afferents, which project to the IML of the sacral parasympathetic nucleus (Wang et al., 1998; Havton and Broman, 2005). Ultrastructurally, we encountered occasional synaptic boutons exhibiting the HRP reaction product in the IML neuropil. However, with a single notable exception, all encountered synaptic boutons were in apposition with unlabeled dendrites in the IML and not with retrogradely labeled PPNs, or their proximal dendrites. Thus, visceral afferents with an affinity for B-HRP appear more likely to make synaptic connections with other neuronal targets in the sacral parasympathetic nucleus. In contrast, following application of horseradish peroxidase (HRP) to the dorsal and ventral sacral roots in the cat, labeled primary afferents were demonstrated to make synaptic contacts with PPN somata in the sacral parasympathetic nucleus (Mawe et al., 1984, 1986). It is possible that the observed differences between the above studies may be attributed to circuit differences between species and/or the different uptake properties of the tracers used in the studies.

Non-synaptic neuronal interactions in the sacral spinal cord

Gap junctions may be encountered between neuronal elements in the adult mammalian spinal cord (Rash et al., 1998). Such specialized cell-to-cell contacts may contribute to the coordinated actions by specific neuronal populations with similar functions. For instance, electrotonic coupling may occur between sympathetic preganglionic neurons in the rat (Logan et al., 1996). In the rat and cat lumbosacral spinal cord, the motoneuron somata and dendritic bundles of the Onuf's nucleus homologue may demonstrate puncta adhaerentia or gap junctions (Kerns and Peters, 1974; Ramirez-León and Ulfhake, 1993; Matsumoto et al., 1988, 1989). However,

in the present sample, the somata and proximal dendrites of the labeled PPNs exhibited no gap junctions or other forms of non-synaptic contact specializations.

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ABBREVIATIONS

B-HRP, Horseradish Peroxidase-conjugated cholera toxin subunit B
 DCV, Dense-Cored Vesicle
 CGRP, Calcitonin Gene-Related Peptide
 GABA, Gamma-Aminobutyric Acid
 HRP, Horseradish Peroxidase
 IML, Intermediolateral Nucleus
 PACAP, Pituitary Adenylate Cyclase Activating Peptide
 PB, Phosphate Buffer
 PBS, Phosphate Buffered Saline
 PPN, Preganglionic Parasympathetic Neuron
 SE, Standard Error of the Mean
 TMB, 5,5'-Tetramethyl Benzidine
 VGLUT, Vesicular Glutamate Transporter
 VIP, Vasoactive Intestinal Peptide

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Figure 1.

A: camera lucida drawing of a tissue section from one of the studied cases. The gray trapezoid delineates the area subsequently analyzed in the electron microscope. B: EM micrograph of a labeled neuron and its proximal dendrite, in the intermediolateral nucleus. Reaction product indicating retrogradely transported B-HRP (arrowheads) is visible both in the cytoplasm and nucleus. Scale bar in B = 5 μm . C and D: two boutons containing TMB reaction product found in the neuropil. The bouton in C contains a mix of clear, round, and dense core (arrows) synaptic vesicles, while the visible vesicles in the bouton in D are only of the clear type. Scale bar in D (applicable to C and D) = 1 μm .

Figure 2.

The size distribution of the studied labeled PPNs. A neuron's size is expressed as the mean of the longest soma diameter, and the longest diameter perpendicular to that. All measurements are in μm .

Figure 3.

Photomicrographs of boutons apposing the somatic, or dendritic membranes of the studied neurons. A: S type bouton (S), with clear asymmetric postsynaptic specializations (arrowheads) apposing a dendrite. Insert: a magnified image of the area containing the postsynaptic density. Next to the S bouton is a bouton containing numerous dense core vesicles (DCV). B: F type bouton (F) with a symmetrical synaptic specialization (arrowhead) apposing a cell soma. Insert: a magnified image of the area containing synaptic cleft. C: Mixed clear, round and dense core vesicle containing bouton (DCV), with asymmetric synaptic specialization (arrowhead). Insert: a magnified image of the area containing the postsynaptic density, showing one of the bouton's dense core vesicles (arrow). D: Large type bouton (L) with several asymmetric postsynaptic specializations (arrowheads) at the somato-dendritic juncture. Insert: a magnified image of the area containing two of the postsynaptic densities. E: Large type bouton (L) whose apposition towards the dendrite (arrowheads) is small relative to the cross sectional area of the bouton. On the opposite side of the dendrite sits an F type bouton (arrow). F: the only example of a

TMB reaction product (arrow) containing bouton found apposing the proximal dendrite of a labeled cell (d1; arrowhead). It also apposes a distal dendrite (d2; arrowheads), without showing any clear synaptic specialization with either dendrite. Scale bar in F = 1 μm (applicable to all images).

Figure 4.

Two neurons representative of the studied sample, one with (A) and one without (B) a large-type bouton. In both panels, boutons are color coded according to: S, green; F, red; DCV, blue; L, yellow. Scale bar = 5 μ m.

Figure 5.

Distribution of boutons apposing the somatic and dendritic membranes, respectively. A: percentage of the membrane covered by each bouton type. B: total number of boutons of each type, per 100 μm of membrane surface. Note that the *Large* type boutons have been excluded, due to their small number. * $p < 0.05$, ** $p < 0.01$.

Table 1
Percentage coverage and number of boutons per 100 μm of soma membrane lengths for all neurons included in the study.

Cell ID	Mean Diameter (μm)	B:A Aspect Ratio ¹	Circumference (μm) ²	Volume (μm ³)	Percentage coverage			Number of boutons/100 μm				
					S	F	DCV	Total	S	F	DCV	Total
1	13.8	0.44	53.8	709	1.9	6.9	0	8.8	1.9	5.6	0	7.4
2	13.6	0.66	34.7	998	8.3	9.2	10.6	28.1	7.7	5.8	3.8	17.3
3	13.4	0.77	45.6	1083	12.3	12.2	4.3	28.8	4.4	8.8	2.2	15.3
4	14.1	0.64	54.0	1099	4.5	6.7	2.5	13.8	1.8	5.6	1.8	9.2
5	17.9	0.43	63.1	1511	3.9	4.9	4.9	13.6	3.2	6.4	3.2	12.7
6	15.1	0.77	49.9	1547	2.0	9.2	13.1	24.2	2.0	8.0	6.0	16.0
7	16.8	0.53	52.4	1550	4.1	9.1	14.8	28.0	3.8	7.6	5.7	17.2
8	15.4	0.87	53.1	1775	0	1.2	3.6	4.8	0	1.9	1.9	3.8
9	19.1	0.42	48.8	1788	19.1	13.7	1.4	34.1	10.2	8.2	2.0	20.5
10	16.1	0.80	64.9	1912	1.9	13.9	0	15.8	1.5	7.7	0	9.2
11	17.0	0.85	63.7	2327	16.7	12.2	0	29.0	4.7	6.3	0	11.0
12	22.0	0.37	66.2	2353	7.8	3.7	0	11.5	4.5	1.5	0	6.0
13	19.3	0.75	59.9	3169	2.6	20.6	0	23.3	1.7	8.4	0	10.0
14	18.5	0.95	43.5	3205	3.8	5.3	6.3	15.4	3.3	3.3	4.9	11.5
15	25.0	0.37	85.5	3471	5.7	9.1	2.2	17.0	3.5	3.5	1.2	8.2
16	21.7	0.61	52.9	3830	0	8.9	0	8.9	0	5.7	0	5.7
17	21.0	0.72	49.3	3971	7.6	7.6	15.1	30.3	5.4	3.6	7.2	16.2
18	25.3	0.40	78.9	4000	0	5.0	10.9	15.9	0	2.5	5.1	7.6
19	20.7	0.92	70.3	4457	11.4	12.0	5.0	28.4	8.5	10.0	2.8	21.3
20	21.7	0.79	78.0	4653	2.8	8.7	1.5	13.1	2.6	6.4	1.3	10.2
21	20.9	0.95	93.6	4677	6.6	8.3	12.6	27.5	4.6	5.8	4.6	15.0
22	24.7	0.64	66.0	5915	4.0	8.9	2.5	15.4	1.3	5.0	1.3	7.6
23	35.3	0.88	70.6	21374	8.7	15.6	0	24.3	4.2	7.1	0	11.3
Mean	19.5	0.67	60.8	3538	5.9	9.2	4.8	20.0	3.5	5.8	2.4	11.8
±SE	±1.0	±0.04	±3.0	±864	±1.1	±0.9	±1.1	±1.1	±0.6	±0.5	±0.5	±1.0

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¹ Aspect ratio between the neuron's short (B), and long diameter (A).

² Circumference of soma membrane visible in the section. Where applicable, the parts of the membrane belonging to the PPN dendrite(s) has been excluded.

Table 2
Percentage coverage and number of boutons per 100 μm of dendrite membrane lengths for all dendrites.

Cell ID	Membrane Length (μm) ¹	Percentage coverage					Number of boutons/100 μm				
		S	F	DCV	L	Total	S	F	DCV	L	Total
1	9.4	11.1	0	5.3	0	16.4	10.7	0	10.7	0	21.4
6	19.6	0	0	25.1	24.2	49.3	0	0	15.3	5.1	20.4
7	7.1	0	0	29.6	0	29.6	0	0	14.0	0	14.0
8	71.2	15.0	6.6	0	4.2	25.8	7.0	4.2	0	1.4	12.6
9	20.0	4.2	17.7	7.3	0	29.2	5.0	10.0	5.0	0	20.0
10	47.9	7.3	18.8	12.6	3.2	41.9	4.2	6.3	6.3	2.1	18.9
12	31.7	24.3	4.9	21.5	0	50.7	12.6	3.2	6.3	0	22.1
13	24.8	10.6	5.7	5.4	0	21.7	5.4	5.4	5.4	0	16.2
14	21.5	9.4	9.5	10.3	0	29.2	9.3	4.6	9.3	0	23.2
15	20.7	0	16.2	0	0	16.2	0	9.7	0	0	9.7
16	83.7	8.1	10.2	5.5	0	23.8	4.8	4.8	2.4	0	12.0
17	40.5	2.4	24.3	4.5	6.6	37.8	2.5	12.3	2.5	2.5	19.8
18	79.5	12.4	5.5	2.8	0	20.7	7.1	4.2	1.4	0	12.7
19	13.4	0	12.0	32.6	0	44.6	0	7.5	15.0	0	22.5
22	24.8	19.0	10.2	0	0	29.2	8.0	4.0	0	0	12.0
23	32.0	15.5	5.7	0	0	21.2	9.4	3.1	0	0	12.5
Mean	34.2	8.7	9.2	10.2	2.4	30.9	5.4	5.0	5.8	0.7	16.4
±SE	±6.1	±1.8	±1.8	±2.8	±1.5	±2.7	±1.0	±0.9	±1.4	±0.4	±1.2

¹Total length of the dendritic membrane visible in the section.

Table 3

Total membrane covered by boutons on soma and dendrites of the studied PPNs^{*l*}, expressed as mean $\pm$ SE.

	Soma	Dendrite	<i>n</i>
Percentage Membrane Coverage	19.1 $\pm$ 2.2	30.9 $\pm$ 2.7**	16
Number of Boutons per 100 μ m	11.2 $\pm$ 1.4	16.4 $\pm$ 1.2**	16

^{*l*}Measurements refer to PPNs displaying dendrites and have been calculated as percentage of the membrane length apposed by all bouton types, and frequency of bouton coverage expressed as number of boutons per 100 μ m of membrane length, respectively.

** indicates a value significantly higher than in soma ($p < 0.01$).