Neuroscience 157 (2008) 656–665

DIFFERENTIAL SYNAPTIC INPUTS TO THE CELL BODY AND PROXIMAL DENDRITES OF PREGANGLIONIC PARASYMPATHETIC NEURONS IN THE RAT CONUS MEDULLARIS

S. PERSSON AND L. A. HAVTON*

Department of Neurology, David Geffen School of Medicine at UCLA, Neuroscience Research Building, 635 Charles E. Young Drive South, Los Angeles, CA 90095, USA

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; dense-cored vesicle-type (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 with their somata. The inhibitory synaptic influence was markedly higher over the PPN somata compared with 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.

Key words: 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 spinobulbar neurons, which are located in the intermediolateral nucleus (IML) of the conus medullaris (Araki, 1994; Hamilton et al., 1995; Araki and de Groat, 1996; Birder 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).

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 (DCVs), 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

*Corresponding author. Tel: +1-310-206-5757; fax: +1-310-206-5533.
E-mail address: lhavton@mednet.ucla.edu (L. A. Havton).
Abbreviations: B-HRP, horseradish peroxidase-conjugated cholera-toxin subunit B; DCV, dense-cored vesicle; HRP, horseradish peroxidase; IML, intermediolateral nucleus; PB, phosphate buffer; PBS, phosphate-buffered saline; PPN, preganglionic parasympathetic neuron; TMB, 3,3′,5,5′-tetramethyl benzidine; VGLUT, vesicular glutamate transporter; VIP, vasoactive intestinal peptide.

0306-4522/08 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.09.017

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 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 (150–225 g, Charles River Laboratories, Raleigh, NC, USA) 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. Care was taken to minimize the number of animals in the study, as well as to minimize the suffering of the included animals. The animals were housed in a room with a 12-h 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 B-HRP (List Biological Laboratories, Campbell, CA, USA) was dissolved in 400 μl of sterile water and administered by 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 μm transverse sections using an oscillating tissue slicer (Electron Microscopy Sciences, Fort Washington, PA, USA). 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 waterbath and was added to the ammonium molybdate solution. Next, 10 μl of 30% H_2O_2 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 h in the ammonium molybdate/TMB/H_2O_2 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 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 h. This slow osmication at low pH allowed for the preservation of a TMB-molybdate reaction product that was more stable compared with 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, USA). 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 2900×. 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, USA). 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 7200× (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 were calculated. The percentage of synaptic coverage was calculated for both somata and dendrites as: (Sum of bouton apposition lengths×100)/(Total membrane length).