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Propriospinal neurons of L3-L4 segments involved in control of the rat external urethral sphincter

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Abstract

Coordination of activity of external urethral sphincter (EUS) striated muscle and bladder (BL) smooth muscle is essential for efficient voiding. In this study we examined the morphological and electrophysiological properties of neurons in the L3/L4 spinal cord (SC) that are likely to have an important role in EUS-BL coordination in rats. EUS-related SC neurons were identified by retrograde transsynaptic tracing following injection of pseudorabies virus (PRV) co-expressing fluorescent markers into the EUS of P18-P20 male rats. Tracing revealed not only EUS motoneurons in L6/S1 but also interneurons in lamina X of the L6/S1 and L3/L4 SC. Physiological properties of fluorescently labeled neurons were assessed during whole-cell recordings in SC slices followed by reconstruction of biocytin-filled neurons. Reconstructions of neuronal processes from transverse or longitudinal slices showed that some L3/L4 neurons have axons projecting toward and into the ventro-medial funiculus (VMf) where axons extended caudally. Other neurons had axons projecting within laminae X and VII. Dendrites of L3/L4 neurons were distributed within laminae X and VII. The majority of L3/L4 neurons exhibited tonic firing in response to depolarizing currents. In transverse slices focal electrical stimulation (FES) in the VMf or in laminae X and VII elicited antidromic axonal spikes and/or excitatory synaptic responses in L3/L4 neurons; while in longitudinal slices FES elicited excitatory synaptic inputs from sites up to 400 μ m along the central canal. Inhibitory inputs were rarely observed. These data suggest that L3/L4 EUS-related circuitry consists of at least two neuronal populations: segmental interneurons and propriospinal neurons projecting to L6/S1.

Keywords

spinal cord slice; transsynaptic tracing; pseudorabies virus

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Introduction

Coordination of the activity of the external urethral sphincter (EUS) striated muscle and the bladder (BL) smooth muscle which is essential for successful micturition is controlled by neural circuits located in the spinal cord and the brain stem (de Groat et al., 1981; de Groat et al., 2015; Hou et al., 2016). In the rat the main spinal circuits controlling EUS and BL activity are located in L6/S1 spinal segments (Chang et al., 2007). During urine storage bladder afferent activity induced by low intravesical pressures during bladder filling activates a spinal reflex pathway that induces tonic activity of the EUS to promote continence (the BL-to-EUS spinal reflex). On the other hand voluntary or reflex voiding in most species is mediated by supraspinal pathways involving the pontine micturition center in the brain stem that trigger a contraction of the bladder and simultaneous relaxation of the EUS (de Groat et al., 2015; Keller et al., 2018; Yao et al., 2019). However, EUS activity during voiding in rats and mice consists of rhythmic contractions separated by short periods of full relaxation of EUS muscle that functions as a pump to improve voiding efficiency (Yoshiyama et al., 2000; de Groat et al., 2001) and also to facilitate territorial scent marking (Cheng and de Groat, 2016; Kadekawa et al., 2016; Keller et al., 2018). In rats this activity termed EUS bursting is a prerequisite for efficient voiding because blocking EUS bursting with alpha bungarotoxin reduces voiding efficiency in rats (Yoshiyama et al., 2000). EUS electromyography (EUS EMG) during voiding in male and female rats exhibits bursting activity occurring at frequencies of ~ 4–5 Hz. The activity consists of an active period (duration, 70–80 ms) and a silent period (duration, 100–200 ms) (Cheng and de Groat, 2004; de Groat and Yoshimura, 2015). After transection of the spinal cord at T8-T10 segments tonic EUS activity mediated by the BL-EUS spinal reflex pathway persists but the micturition reflex and EUS bursting is initially lost. However 3–6 weeks after spinal cord injury (SCI) reflex bladder contractions, EUS bursting and voiding return although voiding efficiency is reduced due to increased EUS tonic activity and changes in the EUS bursting pattern (Cheng and de Groat, 2004). Return of lower urinary tract function is attributed in part to reorganization of spinal reflex circuits (de Groat and Yoshimura, 2006, 2012; Tai et al., 2006) and plasticity in bladder afferent neurons (de Groat and Yoshimura, 2010; Kadekawa et al., 2017).

If the spinal cord is transected at L4 or more caudally, reflex bladder contractions return but EUS bursting and voiding do not return (Chang et al., 2007). Furthermore, targeted electrical stimulation of L3/L4 promotes voiding in humans and animals (Chang et al., 2019; Herrity et al., 2018). This suggests the existence at the level of the L3/L4 spinal segments of a second component of spinal cord circuitry that contributes to the emergence of EUS bursting and BL-EUS coordination after SCI (Chang et al., 2007). The L3/L4 compartment of the EUS-related circuit was named Lumbar Spinal Coordinating Center (LSCC) to indicate its role in EUS-BL coordination (Karnup et al., 2017).

In this study we examined the morphology and electrophysiological properties of neurons in the LSCC of L3/L4 spinal cord that may control EUS function during micturition. The neurons were identified in spinal cord slices by retrograde transsynaptic tracing methods in which pseudorabies virus (PRV) co-expressing a fluorescent marker (PRV-GFP or PRV-

RFP) was injected into the EUS in juvenile (P18-P20) male rats 2 days prior to the experiments.

EXPERIMENTAL PROCEDURES

In this study all animal procedures were performed in accordance with University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) guidelines. A total of 28 juvenile male Sprague-Dawley rats (n=13 for PRV-injected rats and n=15 for control intact rats) between the ages of P18 and P20 were used because at this age the central circuitry underlying reflex voiding function is developed (Kruse et al., 1993; de Groat et al., 1981) and all major motor spinal circuits are fully wired (Etlin et al., 2010; Lev-Tov et al., 2010). In older rats (P21-P22) myelination of the spinal cord markedly reduces visualization of fluorescent cells in the spinal cord slice and reduces the ability to identify neurons in the grey matter around the central canal for patch clamping and electrophysiological recording. Therefore, P18-P20 was the optimal time window for our experiments. The animals were kept with their mother in the animal facility under a 12-h light-dark cycle with water and food *ad libitum*.

PRV injection

PRV is a retrograde axonal and transsynaptic tracer that is transported across synapses. Therefore it allows labeling of neurons upstream from the injection site with incremental time gaps. PRV injected in to the EUS spreads retrogradely to spinal motoneurons (EUS-MNs). In adult rats PRV is usually well expressed in EUS-MNs 2 days post-injection, and in 2.5 or 3 days a number of spinal interneurons become PRV-labeled as well (Nadelhaft and Vera, 2001). In most cases neurons presynaptic to EUS-MNs express a fluorescent marker carried by PRV about 12h after it is well expressed in the postsynaptic cell (Nadelhaft and Vera, 2001; Vizzard et al., 1995; Sugaya et al., 1997). This potentially allows the identification of different populations of neurons in a reflex pathway based on the timing of PRV infection starting from the efferent neurons in the chain: i.e., in our study the 1st order EUS-MNs followed by labelling of the 2nd order segmental INs and remote PSNs that are presynaptic to EUS-MNs.

For abdominal surgery and PRV injection, rats at P16-P18 were anesthetized with isoflurane. The EUS was exposed by a longitudinal midline abdominal incision and partial removal of the pubic bone. Using a microinjector Nanoliter 2010 (WPI, Sarasota, FL) we injected either PRV-512 (PRV-GFP) or PRV-614 (PRV-RFP) into striated muscles of the dorsal and lateral walls in the middle part of urethra. Total volume of injections was 3–4 µl. These two Bartha PRV strains (titer 1.3×10^9 pfu/ml) were kindly provided by L.W. Enquist (Princeton University, Princeton, NJ), supported by his Virus Center grant # P40RR018604. At each injection site the needle was left in place for 1 min after injection to avoid virus leakage. Control of possible leakage was made by adding a small amount of Fast Green FCF 0.5% (Electron Microscopy Sciences, Hatfield, PA) to the viral solution (0.2–0.3% of the total volume), so that any solution coming back would be immediately identified. In none of the cases did the virus solution leak back.

The presence of GFP or RFP fluorescence in slices was examined on 1st, 2nd and 3rd days after injection of PRV-GFP/RFP into the EUS in order to choose the best time window for recordings from propriospinal neurons immediately presynaptic to EUS-MNs. No neurons in the spinal cord showed fluorescent labeling on day 1. Similar to adult animals, EUS-MNs showed bright fluorescence on day 2, but at the same time some spinal interneurons in L6/S1 and L3/L4 segments also exhibited a fluorescent tag. With high probability the brightly labeled interneurons should be considered as 2nd order neurons immediately presynaptic to EUS-MNs, whereas moderately or weakly fluorescent cells should be considered as interneurons of higher orders (3rd and 4th) infected with some delay through the 2nd order neurons. On day 3 the number of labeled interneurons increased; and the area in the vicinity of the CC exhibited fluorescent cellular debris. This indicated trans-synaptic spread of PRV to higher order intrasegmental interneurons and degeneration of some earlier infected 2nd order interneurons. Thus, the 2nd post-injection day was chosen as the optimal time slot to maximize chances of patching brightly labeled propriospinal cell, but not local interneurons. To identify propriospinal neurons presynaptic to EUS-MNs pseudorabies virus co-expressing either GFP or RFP was injected into the EUS two days before an experiment.

Preparation of spinal slices

Two days post-injection the rat was anesthetized with isoflurane and decapitated in accordance with Institutional Animal Care and Use Committee and National Institutes of Health guidelines. The spinal cord was removed and immersed in ice-cold sucrose-based solution equilibrated with carbogen (95% O₂+5% CO₂): (in mM) 26 NaHCO₃, 1 NaH₂PO₄, 3 KCl, 11 glucose, 234 sucrose, 10 MgSO₄, 0.5 CaCl₂. Using spinal roots and characteristic shape of the lumbar portion of the spinal cord for guidance, the L3-L4 segments were dissected. Then the L3-L4 fragment was cut to 300 µm thick slices on a vibratome Leica VT 1000S (Leica Biosystems, Buffalo Grove, IL) in ice-cold sucrose-based solution. After cutting, slices were warmed at 30°C for 0.5 hour and then incubated during an experiment at room temperature (22°C) in artificial cerebrospinal fluid (ACSF) saturated with carbogen: (in mM) 117 NaCl, 26 NaHCO₃, 3.6 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, and 11 glucose; 285–290 mOsm, pH 7.4. We used three types of slices: transverse, parasagittal and horizontal.

Electrophysiological methods

For whole cell recording, a single slice was placed in a chamber (~1 ml volume) and continuously perfused with ACSF at the rate 3–5 ml/min. All recordings were performed at room temperature. The recording chamber was installed on an upright microscope (Olympus BX51W1, Life Science Solutions, Global) equipped for epifluorescence and near-infrared differential interference contrast (DIC) optics. A CCD camera Hamamatsu C10600 ORCA-R² (Hamamatsu Photonics K.K., Japan) and MetaMorph software package (Molecular Devices, Sunnyvale, CA) were used for visualization of neurons. Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm outer diameter; World Precision Instruments Inc., Sarasota, FL) on a pipette puller (P-97; Sutter Instruments, Novato, CA) and were filled with a solution of the following composition (in mM): 114 K-gluconate, 5 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 HEPES, 5 EGTA, 0.3% biocytin hydrochloride. Osmolarity was adjusted to 270–275 mOsm, pH 7.3. The pipette resistance was 8–14 MΩ.

Whole-cell current- and voltage-clamp recordings were made with the MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and data acquisition software package Signal5 (Cambridge Electronic Design Limited, Cambridge, UK). There was no correction for the liquid junction potential (~10 mV) and no series resistance compensation. Only neurons that had access resistance of <30 M Ω were included in this study.

Neurons were classified into four types (tonic, phasic, delayed and single-spike cells) according to their firing patterns during injections of positive currents of 1–2 s duration. Within a few minutes after establishing whole cell configuration in current clamp mode and stabilization of the resting membrane potential (V_{mr}) all cells were tested by injections of negative and positive currents. Input resistance (R_{in}) and membrane time constant (τ) were measured with negative current steps resulting in 5 – 10 mV voltage deflections. Rheobase (R_h , the minimum positive current necessary to elicit an action potential) was measured from V_{mr} ; spike threshold was measured as a value of membrane potential (V_m) where rising part of the trace had a maximum of the second derivative; spike amplitude was measured from the spike threshold to its peak; spike duration was defined as the width of the first or second spike at half of its amplitude, i.e. as spike “half-width” (HW); amplitude of the fast after-hyperpolarization (fAHP) was measured from the spike threshold to the negative peak of post-spike hyperpolarization. These basic physiological characteristics were measured in current clamp for each cell.

For electrical stimulation in the slice we used a constant current stimulus isolator Iso-Flex (A.M.P.I., Jerusalem, Israel). Monopolar stimulation in white or grey matter was performed using a glass pipette 3–10 M Ω filled with ACSF. Duration of the stimulating current pulse was set to 1 ms in order to identify onset of an antidromic spike (<1 ms), otherwise spike latency would be masked by a rebound stimulus artefact. Stimulus intensities varied from 20 μ A to 50 μ A to identify response threshold and from 100 μ A to 300 μ A to test dependence of responses on stimulus strength. Usually stimuli were applied at 0.2 Hz, but in some cases 1–2 s trains of 10 to 100 Hz were used. Distances between the neuron and sites of stimulation varied from 150 μ m to 600–700 μ m. In voltage clamp mode EPSCs were measured at a holding potential $V_h = -70$ mV, and IPSCs at $V_h = 0$ mV. Monosynaptic responses were identified by a negligible latency jitter and lack of failures during standard 100 μ A, 0.2 Hz stimulation. Polysynaptic responses were less stable and had a variable latency.

Neuronal reconstruction

After a cell was recorded for 30–60 min and filled by diffusion with 0.5% biocytin hydrochloride a slice was fixed in 4% paraformaldehyde overnight. Fixed slices were used for morphological reconstruction of recorded neurons. They were processed using ABC-DAB reaction as described previously (Karnup and Stelzer, 1999). Briefly, sections were reacted with 1% H₂O₂, 0.5% Triton X-100, ABC complex, and Ni-DAB chromagen. After dehydration, slices were mounted in DPX. Reconstructions of biocytin-filled neurons were made with Neurolucida10 software (MicroBrightField, Colchester, VT) and figure preparation was done with the CorelDraw12 software.

Data analysis and Chemicals

Data analysis was performed using Origin8 and SigmaPlot12 software packages. Differences between means of electrophysiological data were estimated with Welch's t-test. Reconstruction of biocytin-filled neurons was done using Neurolucida10 software.

Animals were purchased from Envigo (Indianapolis, IN). Drugs and chemicals were purchased from 1) Sigma-Aldrich (St. Louis, MO): chemicals for preparation of ACSF, biocytin hydrochloride, DAB; 2) Vector Laboratories (Burlingame, CA): ABC kit.

RESULTS

Location of EUS-related spinal neurons

The presence of GFP or RFP fluorescence in slices was examined on 1st, 2nd and 3rd days after injection of PRV-GFP/RFP into the EUS. Motoneurons innervating the EUS (EUS-MNs) located in the Onuf's nucleus in the L6/S1 segments would be the first neurons infected by PRV; however, at 24h post-injection they did not express GFP/RFP. On the 2nd day GFP or RFP fluorescence was observed in EUS-MNs as well as in smaller neurons in the L6/S1 dorsal commissure (DCM) dorsal to the central canal (CC) (Fig. 1A) and in L3/L4 within lamina X dorsal and lateral to the CC (Fig. 1B). The small labeled neurons in L6/S1 DCM are considered to be segmental interneurons involved in EUS-related circuits (Blok et al., 1998), while at least one population of PRV-infected neurons in L3/L4 must be propriospinal neurons (PSNs) projecting axons to L6/S1. Because of the relatively short time for the appearance of PRV labelling in the PSNs it is presumed that they are second order neurons monosynaptically connected to EUS-MNs. Segmental interneurons synaptically connected to PSNs (i.e., third order neurons) might be another PRV-infected population in the L3-L4 spinal cord.

On post-infection day 3 the number of labeled cells in L6/S1 and L3/L4 increased, but the area in the vicinity of the CC exhibited fluorescent cellular debris indicating neuronal degeneration, and at the same time animals displayed signs of illness (rigidity, slow movements and ruffled fur). This suggests that on the 3rd day post-infection 2nd order cells were dying and the virus was spreading to higher order neurons in the spinal cord and the brainstem. Therefore, to minimize the probability of recording from damaged neurons and to minimize the probability of recording from 3rd order neurons we have chosen to study fluorescent cells in L3/L4 on the 2nd post-injection day assuming that they are 2nd order PSNs and possibly some of 3rd order interneurons. In addition, we focused our recordings on the larger rather than smaller cells, assuming that PSNs in comparison to local interneurons are likely to have a larger soma. However, the possibility of an occasional recording from a PRV-labeled 3rd order local interneuron cannot be totally excluded. Therefore, we will name neurons recorded in our experiments LSCC-Ns, i.e. neurons of the Lumbar Spinal Coordinating Center.

Morphology of EUS-related L3/L4 neurons

Reconstructions of PRV infected L3/L4 neurons filled with biocytin (n=21) during patch clamp recording revealed the location of the soma, 3D dendritic morphology within a slice

and in some cases the proximal part of an axon (Fig. 2). Cell bodies of labeled neurons were located either dorsal to the CC or up to 200 μm lateral to the CC (Fig. 1B and Fig. 2). In transverse slices ($n=12$ cells reconstructed) three dendritic patterns were identified: a predominant medio-lateral orientation, predominant dorso-ventral orientation or a stellate-like arborization (Figs. 2 and 3). Regardless of their preferred orientation the dendrites had few or no spines. These dendrites were predominantly confined to the central grey matter, i.e. to lamina X and medial lamina VII, although distal dendrites of some cells extended into the white matter of the dorso-medial (DMf) or the ventro-medial funiculi (VMf) (Figs. 2 and 3). In horizontal ($n=3$ cells reconstructed) and parasagittal slices ($n=6$ cells reconstructed) dendrites could be traced in the rostro-caudal direction for hundreds of microns typically close to the CC (Fig. 4). Axons were identified in 11 out of the 21 reconstructed neurons by even thickness throughout their length and in branches (when present), whereas dendrites tapered from proximal to distal parts and branches of a higher order were thinner than their parent branch. Axons of 3 cells in transverse and of 3 cells in parasagittal slices did not branch before projecting into the ipsilateral VMf (Fig. 3A). One neuron in a transverse slice exhibited an axon that crossed the midline and entered the contralateral VMf (Fig. 3B). All cells projecting axons into VMf were located lateral to the CC, but not dorsal to it. Two cells in horizontal slices projected axons caudally parallel to and in proximity to CC (Fig. 4A, B). One cell in a parasagittal slice had both local axonal ramifications as well as a branch descending into VMf (Fig. 4C). Four cells had only local axons in laminae X and VII consisting of 1–3 branches (Fig. 2C, D). In order to reveal the location of the main descending axonal pathway innervating EUS-MNs a small piece of lipophilic tracer Fast-DiI was inserted in Onuf's nucleus of L6 in the fixed spinal cord. After two months of incubation at 37°C staining was accumulated in the VMf at the level of L4 (Fig. 5A, B). This is consistent with our observation of biocytin labeled PSNs' axons directed towards the VMf and supports the notion that PSNs of the LSCC project their axons to EUS-MNs in Onuf's nucleus through the VMf. In summary, the anatomy of axonal projections is consistent with the view that the L3/L4 segments contain at least two populations of EUS related neurons: (1) propriospinal neurons that send axons caudally in the VMf and (2) interneurons with axons distributed locally in laminae VII and X.

Electrophysiological properties of EUS-related L3/L4 neurons

A total of 34 EUS-related LSCC-Ns were studied using current and voltage clamp recording. Depolarizing currents injected via a microelectrode elicited various firing patterns: tonic, delayed, phasic, or single spikes (Fig. 6A). The majority of cells exhibited tonic firing ($n=28$, 82.4%). Three cells had a phasic pattern (8.8%), one cell showed a delayed discharge (2.9%) and two cells fired a single spike (5.9%) in response to a broad range of depolarization intensities (Fig. 6B upper chart). Depolarizing currents of three times rheobase magnitude resulted in firing at moderate frequencies not exceeding 35 Hz (Fig. 6C). Passive membrane properties such as resting membrane potential (V_{mr}), input resistance (R_{in}) and membrane time constant (τ) were measured immediately after establishing the whole cell configuration (Table 1). The rheobase (R_h) was measured as an increase of injected current from V_{mr} to the threshold of the first spike (V_{thr}). Spike characteristics such as threshold, amplitude, half-width (HW, i.e. width at half-amplitude) and after-hyperpolarization (AHP) were measured at V_{thr} (Table 1). Since the recorded set of

LSCC-Ns could include physiologically distinctive populations we attempted to separate them based on membrane and spike characteristics. However, the measured physiological parameters in our limited sets of data did not allow a separation into distinct clusters nor reveal a correlation between their electrophysiology and morphology.

Synaptic responses of EUS-related L3/L4 neurons to focal electrical stimulation in the slice

Current clamp and voltage clamp recordings were performed on 27 LSCC-Ns while applying focal electrical stimulation at various sites in the slices in an attempt to identify the location of synaptic inputs to LSCC-Ns. To compare relative responsiveness of LSCC-Ns to stimulation from different sites we used standardized current pulses of 100 μ A (Fig. 7A lower trace). In most cases the threshold stimulating current for evoking EPSCs/EPSPs was 20–50 μ A (Fig. 8A fourth column), and an increase in stimulus strength to 100–150 μ A typically produced maximal amplitude EPSCs/EPSPs (Fig. 8A at $V_h = -70$ mV). Higher stimulus intensities of 300–500 μ A were used to test non-responsive sites to confirm the lack of inputs from these sites (Fig. 7A upper trace). Stimulations with 100 μ A elicited synaptic responses at 61 of 64 sites tested. The responses consisted of EPSC+IPSC complexes in 18 cases (Fig. 8A blue and grey traces), 43 responses consisted of only EPSCs (Fig. 8B, C blue traces) and none of responses consisted of IPSCs alone. IPSCs were unmasked by reducing the V_h from -70 mV to 0 mV (Fig. 8A). In 19 cells the evoked excitatory responses had steady amplitudes ranging from -200 pA to -400 pA at V_{mr} . Electrical stimulation in slices of different orientations revealed monosynaptic EPSPs/EPSCs predominantly from (a) within lamina X, (b) lamina VII at the level of the CC (Fig. 7), (c) VMf (Fig. 8B, C; Fig. 9B–D) and (d) DMf (Fig. 9C). Only polysynaptic EPSCs were elicited from the dorso-medial quadrant of lamina VII (Fig. 9C). The magnitude of the evoked EPSCs varied considerably between different locations (Figs. 9 and 10). Either no or weak polysynaptic responses were elicited when stimulation was applied in the lateral funiculus (Lf) at the level of the central canal in transverse slices (2 cells; Fig. 7A upper trace; Fig. 9A site 1) or horizontal slices (2 cells; Fig. 7B). However, stimulation in the grey matter from than half the distance between the Lf and the CC resulted in a monosynaptic EPSP with a short latency (3 ms, Fig. 7A lower trace) which was often accompanied by a slightly delayed polysynaptic component (Fig. 7B middle trace). Monosynaptic EPSCs had a jitter <1 ms and a latency of 2–5 ms. The most robust EPSCs which had steady amplitudes of -200 pA to -400 pA at V_{mr} were elicited predominantly from either: (a) the VMf in transverse and parasagittal slices (Fig. 8B, C blue traces; Fig. 9B, D; Fig. 10C, D), or (b) sites along the CC in horizontal or parasagittal slices (Fig. 10A, C). Pronounced monosynaptic EPSCs from VMf could be elicited by two types of inputs: (1) spinal tract axons descending from the brain or ascending from L6/S1 segments or (2) collaterals of PSN axons projecting into the VMf that generate antidromic spikes in response to VMf stimulation.

We have not found a correspondence between EPSC magnitude and the morphology of the cells or between EPSC latencies and distances to the site of stimulation. However, a decrease in amplitude was observed in the same cells when distances to a stimulation site increased. To illustrate this we tested from different distances and directions in 5 cells in transverse slices, 4 cells in parasagittal slices, and 4 cells in horizontal slices (Fig. 11).

In voltage-clamp mode at near threshold V_h LSCC-Ns could generate orthodromically evoked escape-spikes superimposed on EPSCs (Fig. 7B lower trace and Fig. 12). The latencies of orthodromic spikes were in the range of 2.5–11 ms with jitter >1ms which was slightly modulated by V_m , stimulus strength and position of a stimulating electrode (Fig. 12B). The spikes exhibited failures due to variability in EPSC amplitude or the holding potential (Fig. 12B).

IPSPs/IPSCs with short latencies <5ms were rarely elicited by focal electrical stimulation in the slice and always had latencies longer than corresponding EPSPs/EPSCs obtained at the same sites. Therefore, we conclude that recorded IPSCs were polysynaptic. Furthermore, when present IPSCs (in 12 of 27 tested cells) were extremely unstable and decayed to zero after 3–5 stimuli at 0.2 Hz (Fig. 8A upper red, blue, and green traces). Low amplitude rapidly fading but still distinguishable IPSCs were elicited in response to the few first stimuli at only 18 out of 64 stimulation sites, including large amplitude IPSCs (150–300 pA at $V_h = 0$ mV) at 4 sites.

To test whether other types of cells around the L3/L4 CC have a pattern of synaptic inputs similar to that exhibited by EUS related neurons we recorded from three non-fluorescent cells in this region while stimulating in the Lf. In contrast to EUS-related neurons that are unresponsive to Lf stimulation and have weak inhibitory inputs the non-fluorescent cells exhibited pronounced polysynaptic IPSPs and smaller amplitude polysynaptic EPSCs in response to Lf stimulation (not shown). Thus, EUS-related LSCC-Ns have synaptic connections that distinguish them from adjacent neurons that are presumably part of other circuits with different functions.

Non-synaptic responses of EUS-related L3/L4 neurons to focal electrical stimulation in the slice

Stimulation within or very near the VMf could elicit antidromic spikes (Fig. 8B, C black traces; Fig. 9B, D; Fig. 10A, D; Fig. 13; Fig. 14); this corroborates the notion that PSN axons project caudally within or near the VMf (as shown in Fig. 3 and Fig. 4). Seven of 17 cells stimulated with 100 μ A in VMf (41%) responded with an antidromic spike which had a latency of 1 ms, had no failures and exhibited no jitter at threshold V_m ($V_h = -40$ mV in Fig. 13B, C). At subthreshold V_m occasional failures and a noticeable jitter of less than 1ms occurred ($V_h = -50$ mV in Fig. 13B). At V_m close to a membrane potential which inactivates voltage-dependent Na^+ -channels ($V_h = -30$ mV in Fig. 13B) an antidromic spike at a latency of less than 1ms was strongly reduced in amplitude (underdeveloped spike) and had no jitter. Stimulation from the same sites with the same stimulus strength at hyperpolarized V_m always elicited either monosynaptic or polysynaptic EPSCs (Fig. 8B, C blue traces; Fig. 13A, B at $V_h = -70$ mV). Thus, in voltage-clamp mode antidromic escape-spikes exhibited all-or-none nature and no jitter only when V_h was near the spike threshold. At more negative V_h antidromic spikes had slightly longer latencies, a noticeable jitter and occasional failures (Fig. 13B, St = 100 μ A at $V_h = -50$ mV) probably because a stronger holding current slowed or partially suppressed activation of the voltage-dependent Na^+ -current underlying production of spikes. At a sufficiently negative V_h generation of

antidromic escape-spikes was completely neutralized by the holding current ($V_h = -70$ mV in Fig. 13B).

Application of trains of stimuli in voltage clamp mode revealed sensitivity of both postsynaptic currents and stimulus-elicited antidromic escape-spikes to stimulation frequency. Antidromic spikes followed high frequencies up to 50 Hz during train stimulations (4 cells tested), whereas EPSCs could not follow frequencies higher than 10–20 Hz (Fig. 14). At 100 Hz antidromic spike failures occurred in response to every second stimulus and groups of multiple failures occurred at 200 Hz (Fig. 14). When tested with the 50 Hz train, LSCC-Ns demonstrated a monosynaptic EPSC only in response to the first stimulus, thus limiting a possibility of temporal summation for successive inputs separated by <20ms intervals (Fig. 14). Hence, the high-frequency test indicates inability of L3/L4 LSCC-Ns of generating or reproducing bursts of spikes with interspike intervals of less than 10 ms and supports our observation that a preferable mode of activity for LSCC-Ns is tonic firing at relatively low frequencies.

Electrophysiological properties of L3/L4 neurons not infected with pseudorabies virus

To evaluate the effect of PRV infection on membrane properties of L3/L4 neurons we recorded 24 randomly selected unlabeled neurons in the same area where virus infected neurons were recorded. 17 cells were recorded in non-PRV injected rats ($n=6$ in transverse, $n=5$ in parasagittal, $n=6$ in horizontal slices) and 7 cells were recorded in transverse slices in PRV-injected animals ($n=7$). There were no differences in V_{mr} and R_{in} between the two groups, so they were merged. Among the control neurons (ConNs) 18 (75%) displayed tonic, 5 (20.8%) phasic and 1 (4.2%) delayed firing patterns (Fig. 6B, lower chart). None of ConNs responded with a single spike to incremental depolarizing current injections. Mean values of membrane characteristics such as V_{mr} , R_{in} , R_h , and V_{thr} for virus infected neurons and ConNs were significantly different ($p>0.05$, Table 1). Lower resting membrane potentials, decreased input resistance and rheobase of virus infected neurons are suggestive of increased membrane leakage caused by the PRV infection. Membrane time constants differed insignificantly but showed the same trend. However, at this stage of infection the neurons were capable of generating normal spikes and responded to synaptic or antidromic stimulation and the percentage of the neurons exhibiting different firing patterns was similar to that in non-infected ConNs. Increased number of spontaneously firing virus infected neurons (10 of 34) as compared to ConNs (1 of 24) may merely reflect a general depolarizing shift in V_{mr} . On the other hand, the differences of measured parameters might be partially due to random selection of control neurons which might have different properties. Therefore, despite an apparent slight deterioration of PRV-labeled cells we still could assess general physiological traits such as the dominant firing pattern and responsiveness to synaptic inputs.

DISCUSSION

This study examined the morphology and physiological properties of L3/L4 neurons presynaptic to EUS-MNs. We identified these neurons in slices using transneuronal tracing, recorded their activity with whole-cell patch clamp methods and then reconstructed their

processes and soma location. Among the neurons studied we presume that there are at least two populations: propriospinal neurons (PSNs) and interneurons that represent major components of the recently identified L3/L4 Lumbar Spinal Coordinating Center (LSCC) that is thought to be important for the control of the EUS bursting activity (Chang et al., 2007). To our knowledge this is the first electrophysiological evaluation of anatomically identified propriospinal circuitry involved in the control of a particular striated muscle.

Propriospinal neurons

Numerous types of PSNs are involved in coordination of striated muscles. They are distributed throughout dorsal, intermediate and ventral grey matter either as clusters or as scattered neuronal populations (Astelmark et al., 2011; Cowley et al., 2015; Dobberfuhr et al., 2014; Mitchell et al., 2016; Sun et al., 2009). PSNs give rise to ascending and/or descending axons that project ipsilaterally and contralaterally to adjacent and remote segments of the spinal cord (Reed-Magnuson, 2009; Flynn et al., 2017). PSN pools linked to different muscles may differ in size, location and chemical properties (Stepien et al., 2010; Ni et al., 2014; Mitchell et al., 2016; Brockett et al., 2013; Tripodi et al., 2011). For example, cell bodies of long descending propriospinal neurons (LDPNs with axons spanning >5 segments) retrogradely labeled with FluoroGold (FG) from lumbar segments in mice were identified ipsilaterally and contralaterally in the rostral thoracic and cervical spinal cord. The identical method of tracing in *GlyT2^{GFP}* and *GAD67^{GFP}* transgenic mice revealed that ~25% of cells among FG-labeled LDPNs were inhibitory neurons projecting ipsilaterally or near the midline, whereas other FG-labeled LDPNs widely distributed throughout the grey matter were presumed to be glutamatergic or cholinergic excitatory cells (Flynn et al., 2017). On the other hand, FG injections into the L2 spinal cord of rats retrogradely labeled short descending propriospinal neurons (SDPNs) in T7-T9 segments that have axons spanning 5 segments or less. These SDPNs were located primarily in the lamina VII and 90% were glutamatergic and only 10% were GABAergic, glycinergic or cholinergic (Deng et al., 2016).

PRV-tracing in the rat has also identified SDPNs in the L3/L4 spinal cord projecting caudally to lumbosacral (L5/S1) motor pathways controlling the bulbospongiosus muscle (BSM) that is responsible for seminal ejaculation (Xu et al., 2006) and to the levator ani muscle (Dobberfuhr et al., 2013). These SDPNs are located in lamina X around the central canal (CC) in the same location as the EUS-related neurons identified in the present experiments. PRV tracing from the EUS in rats by Nadelhaft and Vera (1996) has shown EUS-related neurons in the area near the CC in the L3/L4 segments. Because the BSM and EUS muscles exhibit bursting activity during ejaculation and voiding, respectively, and the bursting in both cases is dependent on integrity of connections between the L3/L4 and L6/S1 segments (Chang et al., 2007; Truitt and Coolen, 2002; Xu et al., 2006) it is possible that the L3/L4 propriospinal circuitry controlling these two muscles has a similar organization. The labeled cells relevant to BSM have been proposed to constitute the spinal ejaculation generator in rats (Staudt et al., 2012; Dobberfuhr et al., 2014; Xu et al., 2006; Truitt and Coolen, 2002; Allard et al., 2005) because a brief electrical microstimulation in laminae VII/X of L4 triggers rhythmic BSM contractions and a corresponding BSM-EMG bursting pattern lasting ~25s. Acute T8/T9 spinalization does not alter this activity; however,

injection of muscimol (GABA_A-R agonist) into L4 VII/X laminae interrupts ejaculation (Borgdorff et al., 2008). Electrical stimulation in L3/L4 also evokes EUS bursting (Abud et al., 2015) and transection of the spinal cord caudal to L3/L4 blocks EUS bursting induced by bladder distension or electrical stimulation of bladder afferent axons in the pelvic nerve (Chang et al., 2007). Possible overlap of the EUS and ejaculatory circuits has not been studied. Therefore, it will be important in future experiments to conduct PRV tracing from the EUS and the BSM in the same animals to determine if the L3/L4 circuitry controlling these muscles share a population of spinal interneurons or if the two circuits are totally independent.

Axons of LSCC-Ns

Because anatomical reconstructions of biocytin filled neurons in 300 μ m thick spinal slices are unlikely to provide a complete picture of axonal and dendritic structures due to transection of processes orthogonal to the slice plane, we attempted to minimize this deficiency by examining tissue in transverse, horizontal and sagittal slices. These data in combination with electrophysiological recordings have provided evidence consistent with the view that at least two populations of EUS-related neurons are present in the L3/L4 spinal segments. In transverse slices some cells (Type 1) had axons that were distributed entirely within the grey matter around the central canal (Fig. 2B–D); while others (Type 2) sent axons into the ventro-medial funiculus (VMf) ipsilaterally (Figs. 3A) or contralaterally (Fig. 3B). Type 2 neurons sending axons into the VMf were also identified in parasagittal slices (Fig. 4C). In horizontal slices some Type 1 neurons had axons that projected caudally within the grey matter adjacent to the central canal (Fig. 4A, B). In transverse slices this type of neuron with a caudally directed axon might not exhibit a detectable axon because it would project out of the plane of the section; and indeed this was observed in some transverse slices.

The demonstration of biocytin labeled EUS-related neurons sending axons into the VMf was confirmed by recording antidromically elicited somatic spikes in response to focal electrical stimulation in the VMf (Fig. 8B, C and Fig. 13). The antidromic spikes were of large amplitude (up to 80 mV in current clamp or 3000–3200 pA in voltage clamp), occurred at short latency in an all-or-none manner, and could be blocked by hyperpolarization of the neuron. They followed 50 Hz stimulation frequency, but failed to fully develop after every second stimulus at 100 Hz. This failure is probably due to slow or weak repolarization of the membrane insufficient to restore subthreshold V_m within 10 ms. Based on these electrophysiological and anatomical findings it is tempting to speculate that Type 2 neurons are PSNs that send axons caudally through the VMf to EUS-related neural circuitry in L6–S1. Our retrograde tracing of PSN axons with Fast DiI supports this assumption (Karnup et al., 2017). On the other hand, Type 1 neurons are likely to be local segmental interneurons (LINs) that make synaptic connections with other EUS-related neurons in laminae VII and X including PSNs.

Dendrites of LSCC-Ns

The dendritic patterns of biocytin-filled neurons were examined in transverse, horizontal and parasagittal slices to determine if preferred patterns occurred and if these patterns correlated

with the location of the cell and its axon or with the synaptic inputs evoked by focal electrical stimulation at different sites in the slice. In all cases dendrites were localized primarily in lamina X and/or lamina VII; but some cells exhibited thin branches of distal dendrites extending into the white matter of the DMf and VMf, suggesting possible synaptic contacts with ascending or descending axons. In transverse slices, the orientation of dendrites in different cells varied between dorso-ventral, or medio-lateral, oblique or no preferred orientation. Dendrites could also cross the midline above CC and project into lamina X on the contralateral side suggesting that LSCC-Ns receive a mixture of ipsi- and contra-lateral synaptic inputs. In horizontal and parasagittal slices the longest dendrites were oriented rostro-caudally and could project for up to 400 μ m. In horizontal slices some dendrites spread ipsilaterally in rostro-caudal direction, whereas other dendrites of the same neuron could cross the midline and project to the contralateral side. These observations indicate that LSCC-Ns integrate information: (a) from ascending and descending pathways, (b) from ipsi- and contralateral sides of the spinal cord but only within 200-300 μ m from the CC, as well as (c) from sites in laminae X/VII at distances up to 400 μ m along the CC. More data are needed to determine if LINs and PSNs have different dendritic patterns.

Synaptic Inputs to LSCC-Ns

In vivo physiological experiments indicate that EUS function is controlled by spinal and supraspinal mechanisms that are activated in part by afferent input from the bladder (de Groat et al., 2015). Tonic activity of the EUS during urine storage is controlled by spinal pathways (Chang et al., 2007), whereas bursting activity is dependent on input from the pontine micturition center (de Groat et al., 2015; Keller et al., 2018). However EUS bursting after chronic thoracic spinal cord transection is mediated by spinal pathways involving circuitry in the L3/L4 and L6/S1 segments of the spinal cord (Chang et al., 2007). Thus multiple afferent inputs to LSCC-Ns might arise from ascending and descending tracts in the adjacent white matter as well as from local spinal interneurons. To examine the origin of afferent inputs to LSCC-Ns we electrically stimulated various sites in a slice. Electrical stimulation in the lateral funiculus (Lf) in transverse and horizontal slices evoked no responses or only weak polysynaptic EPSCs, whereas robust mono- and polysynaptic responses were typically elicited from lamina VII at sites half the distance between the lateral funiculus and the CC. This suggests either that axons entering the grey matter from Lf do not synapse with LSCC-Ns and are not involved in EUS bursting function or that the LF axons pass tangentially through the grey matter and out of the plane of the section before making synaptic contact with the LSCC-Ns. The former seems to be more plausible as some of our control (PRV negative) neurons did respond to Lf stimulation.

Typically, mono- and polysynaptic excitatory responses to stimulation of incremental strength reached their maximum amplitude within a narrow interval above threshold indicating that a limited number of excited cells or fibers form synapses on a recorded neuron. Thus, we can speculate that LSCC-Ns receive inputs from a small number of local interneurons in the immediate vicinity, or that the presynaptic neurons are broadly scattered in the grey matter so that a strong stimulating current at a given site does not excite more presynaptic cells than does a moderate supra-threshold stimulus. Topography of synaptic

responses to stimulation from close and remote sites makes the first explanation preferable because the largest EPSCs were obtained from short distances not exceeding 300 μm .

A weak excitatory input from the Lf to LSCC-Ns contrasts with the strong monosynaptic and polysynaptic EPSCs elicited in these neurons by low intensity stimulation within or close to the VMf. The VMf at the level of the lumbar spinal cord contains multiple axonal pathways some of which regulate the central pattern generator and locomotor function (Cherniak et al., 2014; Anglister et al., 2017; Lacroix et al., 2004). Therefore VMf inputs to PSNs could originate from neurons at many sites. In some cases the same VMf stimulation site elicited an antidromic spike preceding mono- or polysynaptic EPSCs (Fig. 8B, C). This combination of responses suggests that PSNs sending axons into the VMf may receive direct excitatory synaptic inputs from ascending or descending white matter tracts in the VMf as well as inputs mediated by recurrent collaterals of PSNs axons in the VMf. Another explanation for EPSCs from the VMf is that antidromic excitation of PSN axon collaterals activates a recurrent facilitatory mechanism mediated by local interneurons in the LSCC.

The possibility of ascending afferent inputs in the VMf from the sacral cord is of particular interest because EUS bursting in chronic spinal cord injured rats is generated by sensory input from the bladder mediated by a pathway from L6/S1 to the L3/L4 spinal cord (Chang et al., 2007). The existence of VMf-evoked monosynaptic EPSCs in LSCC-Ns that are antidromically activated in response to VMf stimulation also suggests that the VMf contains both the ascending and descending limbs of the bladder-to-EUS bursting reflex mechanism that exists in chronic SCI rats. It also raises the possibility that the input from L6/S1 may not require processing through interneuronal circuitry in L3/L4 but rather directly activates the PSNs projecting back to the EUS motor circuits in the L6/S1 cord.

The absence of inhibition in LSCC-Ns in response to electrical stimulation at most sites or only weak inhibition at other sites that rapidly declines during repetitive stimulation suggests that inhibition is of low functional importance in this population of neurons. This weak inhibition combined with the prominent excitatory inputs to LSCC-Ns potentially creates a network in which an initial external excitatory input from the bladder afferent nerves can be amplified and rapidly transformed into a massive output of the PSN population. Such amplification may play a role in the increase of L3/L4-to-L6/S1 modulation during recovery of voiding after spinal cord injury (SCI) (Chang et al., 2007) when pathways from supraspinal structures are disrupted and intrinsic spinal circuits have sole responsibility for BL-EUS coordination.

Intrinsic Electrophysiological Properties of EUS-related Neurons

Basic physiological parameters of PSNs are in the range of those for medium size neurons of the CNS (Williams et al., 1996; Davies and North, 2009; Gao et al., 2009; Kim and Chang, 2005). Distributions of these parameters among recorded neurons do not show definitive separation into groups. In our experiments firing patterns obtained by intracellular depolarization were predominantly tonic, although a few cells displayed delayed or phasic firing, or single spikes. Maximal firing rate to depolarizing current steps was 35 Hz. None of the cells demonstrated an intrinsic bursting discharge similar to that recorded from some cells in the DCM of L6/S1 (Lu et al., 2001). Classification of spinal neurons by their firing

patters (tonic, phasic, delayed and single-spiking) is common despite the lack of clear correlation with their roles in specific circuits (Lu et al., 2001; Breton et al., 2009; Bellardita et al., 2017; Krotov et al., 2017; Cui et al., 2016). Nevertheless, a type of firing pattern indicates whether a cell serves as: (a) a coincidence detector (characterized by a single spike or phasic response to depolarization) that is capable of fast transduction of an input signal or a set of synchronized inputs into an output signal or (b) an integrator (characterized by tonic or delayed firing) which collects multiple concomitant but non-correlated inputs and maintains its tonic output as long as a sum of inputs keeps the cell above the firing threshold (Ratté et al., 2013, 2015; Prescott and Koninck 2002; Prescott et al., 2008). Therefore, we suggest that most of EUS-PSNs integrate their excitatory inputs and send a tonic output to L6/S1 rather than generate temporally structured spike trains which could initiate the 4–8 Hz bursting activity of the EUS necessary for efficient voiding in spinal cord intact (Kruse and de Groat, 1993; Kruse et al., 1993; Dolber et al., 2007) and SCI rats (Yoshiyama et al., 2000). EUS EMG bursting consists of active periods with an average duration of approximately 70 ms separated by silent periods averaging 100 ms (Cheng and de Groat, 2004). Recordings of EUS motoneuron activity during voiding in the rat revealed burst firing in about 75% of EUS motoneurons consisting of 3–5 action potentials during each active period at dominant frequencies ranging from 45 Hz to 90 Hz (D'Amico and Collins, 2012). The majority of these neurons exhibit tonic activity during bladder filling which abruptly switches to bursting activity during a micturition reflex; while a minority of neurons are silent during bladder filling and only generate bursting during micturition. This switch-like behavior of EUS-MN bursting parallels the switch-like firing of parasympathetic neurons controlling the urinary bladder (de Groat et al., 2015) and is mediated by circuitry in the pontine micturition center (PMC) in spinal intact animals (de Groat and Wickens, 2013; Hou et al., 2016; Keller et al., 2018) but by lumbosacral spinal circuitry after chronic spinal cord injury (Cheng and de Groat, 2004; Chang et al., 2007).

The present experiments in spinal slices from spinal intact animals have not identified circuitry in the LSCC that can mediate the switch-like function or the high frequency firing of EUS-MNs during bursting. Excitatory synaptic inputs to EUS-related neurons in L3/L4 followed only low frequencies of stimulation and synaptically evoked EPSCs decayed rapidly within 3–5 repetitions at 20–50 Hz stimulation; thus it seems unlikely that the high frequency pattern of EUS MN firing during the active period of EUS bursting is generated directly by the output of PSNs in the LSCC. Furthermore, the 4–8 Hz pattern of bursting also seems unlikely to be generated directly by the LSCC network because we have not encountered rhythmic patterns of intrinsic activity among EUS-related cells in the L3/L4; nor did we find prominent inhibition that would be capable of producing silent periods of 100–250 ms duration necessary to generate the 4–8 Hz EUS bursting or the on-off switch-like property of bursting. Hence, the pattern of EUS bursting could be generated either by synaptic input to the LSCC or downstream of the output of the LSCC at the level of the EUS motor circuitry in the L6/S1 spinal cord. In spinal cord intact animals the switch-like activity of spinal efferent neurons during voiding is generated by descending excitatory input from switching circuitry in the PMC (de Groat et al, 1998; de Groat et al., 2015; Sasaki, 2005; Yao et al., 2019). This input is lost after spinal cord injury and EUS bursting is activated by afferent input from the bladder to the LSCC (Chang et al., 2007). However, because afferent

input from the bladder is tonic during bladder distension and/or contractions it seems unlikely to elicit EUS bursting unless the LSCC circuitry is remodeled after spinal cord injury. Alternatively bursting could be generated by the motor circuitry in the L6/S1 spinal cord in response to tonic excitatory input from the propriospinal neurons in L3/L4.

In conclusion, the present experiments have studied the neuronal circuitry in the L3/L4 spinal cord that is likely to modulate EUS activity during micturition. We have tentatively identified input and output pathways of this circuitry as well as two populations of neurons (interneurons and propriospinal neurons) that are major components of the circuit. While earlier reports (Chang et al., 2007) indicate that this group of neurons is essential for the generation of EUS bursting in chronic spinal injured rats, the present results obtained from LSCC neurons of spinal cord intact rats indicate that the output of these neurons is likely to be only a trigger for EUS bursting and does not generate the bursting pattern which must be produced by EUS motor circuitry in the L6/S1 spinal cord. A EUS inhibitory mechanism mediated by neurons located in the caudal lumbo-sacral spinal cord has been reported by Blok and Holstege (Blok and Holstege, 1998; Blok et al., 1998). Thus bursting activity of EUS-MNs might be dependent on combined inputs from bladder afferents, the PMC and from the LSCC. Impact of LSCC input should greatly increase in chronic spinalized animals when remodeled lumbar-sacral circuits are disconnected from the PMC.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
BL	bladder
CC	central canal
ConN	control neuron
DCM	dorsal commissure
DMf	dorso-medial funiculus
EUS	external urethral sphincter
EUS-MN	motoneuron of the external urethral sphincter
EMG	electromyogram
EPSC/P	excitatory postsynaptic current/potential
FG	Fluoro Gold

GFP	green fluorescent protein
HW	spike width at half amplitude
IPSC/P	inhibitory postsynaptic current/potential
IN	interneuron
Lf	lateral funiculus
LSCC	lumbar spinal coordinating center
LDPN	long descending propriospinal neuron
PMC	pontine micturition center
PRV	pseudorabies virus
PSN	propriospinal neuron
RFP	red fluorescent protein
R_{in}	input resistance
Rh	rheobase
SDPN	short descending propriospinal neurons
SC	spinal cord
SCI	spinal cord injury
V_{mr}	membrane potential at rest
VMf	ventro-medial funiculus
V_h	holding potential
τ	membrane time constant

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HIGHLIGHTS

- External urethral sphincter-related (EUS-R) spinal neurons were labeled by transneuronal virus tracing.
- One group of EUS-R neurons was identified in the L3-L4 spinal segments near the central canal.
- Properties of L3-L4 EUS-R neurons were studied in spinal slices with patch clamp and intracellular filling methods.
- EUS-R propriospinal neurons projecting into the ventral funiculus and interneurons were identified.
- L3-L4 EUS-R neurons are thought to have a role in coordinating bladder and EUS function during voiding.

Fig. 1. EUS-related spinal neurons traced with PRV-GFP (PRV 512). **A** – GFP-labeled cells in the L6 spinal segment. Note large motoneurons in the Onuf's nucleus (On) in the ventral horn and small interneurons in the dorsal commissure above the central canal (CC). **B** – GFP-labeled cells in L4 spinal segment localized near the CC. DMf – dorso-medial funiculus; VMf – ventro-medial funiculus. Scale bar = 200 μ m. Distributions of labeled neurons are indicated in the insets on the right (zoomed with $k = 1.5$).

Fig. 2. Reconstructed lumbar spinal coordinating center neurons (LSCC-Ns) traced with PRV-GFP and subsequently filled with biocytin in transverse slices. Dendritic arbors in different neurons have a preferred orientation either in the medio-lateral (**A**, green cell; **D**) or dorso-ventral direction (**B**, **C**), or no preferred orientation (**A**, blue cell). A proximal part of an axon (ax, red) could be either non-branching (**B**) or poorly branching locally (**C**, **D**). A thick dendritic stub in **D** (left side of the soma) indicates that the entire left dendritic trunk was out of the slice and was lost. Axonal fragments are depicted in red. Scale bars indicate 100 μm.

Fig. 3. PRV-traced LSCC-Ns reconstructed after filling with biocytin in transverse slices project axons into the VMf. **A** – a non-branching axon of the blue neuron extends into the ipsilateral VMf. **B** – a non-branching axon crosses the midline and projects into the contralateral VMf. Scale bars indicate 100 μm .

Fig. 4.

A and **B** - Two PRV traced LSCC-Ns in the same horizontal slice reconstructed after filling with biocytin. Both neurons send dendrites ipsilaterally and contralaterally. Cell in **A** has also a long ipsilateral dendritic branch directed caudally along CC for 400 μm. Initial axonal segments (red) of both cells are directed caudally along the CC. **C** - a LSCC-N in a parasagittal slice exhibits rostral-caudal as well as dorso-ventral distribution of dendrites and an axon that has local branches and a short segment invading the white matter of the ventro-medial funiculus (VMf). Responses of this cell to electrical stimulation (site is depicted by a

red cross) are shown in Fig. 8B. At near-threshold holding voltage ($V_h = -40$ mV) 100 μ A stimulation resulted in an antidromic spike (Fig. 8B, black trace). Scale bars indicate 100 μ m.

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Fig. 5. Tracing of axonal pathways between the L4 segment and the L6 segment containing Onuf's nucleus (On). Insertion of Fast DiI in the On in the L6 segment of the fixed spinal cord (**B**) resulted in labeling of axons in the VMf in L4 segment (**A**). CC – central canal. Scale bar is 200 μ m.

Fig. 6.

A - Firing patterns of PRV-traced LSCC-Ns and control neurons (ConNs) not labeled with PRV. The majority of recorded neurons displayed tonic firing when injected with a depolarizing current (28 of 34 LSCC-Ns, upper trace and 18 of 24 ConNs). Three LSCC-Ns and five ConNs showed phasic discharges in response to depolarizations over a range of magnitudes of injected current (second trace from the top), two LSCC-Ns fired a single spike in response to a range of depolarizing currents (third trace from the top). One LSCC-N and one ConN fired tonically with a significant delay after being depolarized (lower trace, note different time scale). **B** - Charts showing the percentage of neurons exhibiting different firing types. The upper chart - LSCC-Ns and the lower chart - ConNs. **C** - Histogram of maximal firing rates of LSCC-Ns elicited by depolarizing currents at intensities three times the rheobase.

Fig. 7.

Diagrams of stimulation sites and evoked responses of neurons in transverse (A) and horizontal (B) slices. **A** – An LSCC-N (red dot) in the transverse slice exhibited a strong monosynaptic EPSP when stimulated with 100 μ A in the middle of lamina VII half way between the lateral funiculus (Lf) and the CC (St2, lower traces), but did not exhibit any response to a three-fold stronger stimulus applied in the white matter of the Lf (St1, upper traces). **B** – This cell in the horizontal slice responded with (a) long latency polysynaptic EPSCs to suprathreshold 50 μ A stimulation at the border between Lf and grey matter (St1, top trace), (b) combinations of mono- and polysynaptic EPSCs sometimes eliciting an orthodromic spike were elicited by stimulation more medially in lamina VII (St2, middle and St3, bottom traces). Responses of this cell to St3 = 100 μ A are also shown in Fig. 8C. Two dark grey traces in each record designate individual responses elicited immediately before and after the response shown in the red trace. Note, that synaptic efficacy varies in a set of three responses to the same stimulus.

Fig. 8.

Distant electrical stimulation at 0.2 Hz could elicit mono- and/or polysynaptic EPSCs that occurred in some recordings in combination with polysynaptic IPSCs (A) or somatic spikes (B and C). Records in A, B and C were obtained from three different cells. **A** – EPSCs were recorded at $V_h = -70$ mV (lower traces consisting of three superimposed recordings illustrated as one red trace followed by blue and green traces at a threshold intensity of 20 μ A and then incremental increases of stimulus intensity up to 100 μ A which increased EPSC amplitude with no further increase at 200 μ A. Site of stimulation is depicted in Fig. 10A, site #1). When responses were recorded in the same cell with the same stimulations at $V_h = 0$ mV (three upper traces) (red, blue and green numbered as 1, 2 and 3 on the right to indicate the sequence of recording) EPSCs alone were elicited at low intensities (20–50 μ A), but were followed by IPSCs at higher intensities of stimulation (100–200 μ A). Note that IPSCs rapidly dropped in amplitude having their maximum immediately after a switch of V_h from -70 mV to 0 mV, so that by the third response they almost disappeared. **B, C** – An antidromic escape-spike (with the latency of 1 ms in **B** and 1.5 ms in **C**) occurred at near threshold V_h (-40 mV, black traces). At hyperpolarized V_m (-60 mV and -65 mV) the spikes were blocked and these neurons exhibited mono- and polysynaptic EPSCs (blue traces). An antidromic spike in **B** resulted from direct excitation of an axon when stimulated in the VMf at site #1 (St1 = 100 μ A) in Fig. 9B. An antidromic spike in **C** was elicited by St3 = 100 μ A located in the medial lamina VII and depicted in Fig. 7B.

Fig. 9.

Diagrams showing the variations in the magnitude of synaptic responses of four LSCC-Ns in transverse slices elicited by focal electrical stimulation (100 μ A) at different sites in the ipsilateral spinal cord. Blue areas designate the LSCC. Recorded neurons are depicted as yellow dots. Black diamonds indicate stimulation sites. Red, orange or magenta stripes designate excitatory inputs; and green stripes designate inhibitory inputs. Thickness of stripes corresponds to amplitudes of averaged EPSCs at $V_h = 70$ mV or the first IPSCs at $V_h = 0$ mV (see black scale bars on the right). **A** – Stimulation in the lateral funiculus at site **1** with 100 μ A did not elicit a response and stimulation with 300 μ A induced only a weak polysynaptic EPSC, whereas 100 μ A stimulation at half the distance between the lateral funiculus and CC (site **2**) elicited pronounced mono- and polysynaptic EPSCs. **B** – LSCC-N displayed pronounced monosynaptic EPSCs from three sites ventral to the cell (**1**, **2**, **3**). Site **1** was located in the upper part of the VMf, site **2** was ~ 100 μ m lateral to site **1** in the grey matter and ~ 50 μ m from the VMf. Site **3** was at a distance of 300 μ m from the white matter in the ventral horn. Polysynaptic EPSCs from sites **4** and **5** were much weaker. IPSCs were not elicited by stimulation at any site. Stimulation at sites **1** and **2** also produced antidromic spikes (symbolically depicted in red) in addition to synaptic responses. **C** – This cell had a strong excitatory input from site **2** in the DMf and a weaker but well expressed excitatory synaptic input from VMf (site **1**) and from sites **3** and **4** dorsal to lamina X. Very weak polysynaptic EPSCs were evoked from site **5**. Measurable IPSCs (in a few initial responses at $V_h = 0$ mV) were obtained from points (**1**) and (**3**). **D** – LSCC-N received substantial excitatory synaptic inputs in response to stimulation in the VMf at the ipsilateral sites including strong EPSCs from sites (**1**) and (**3**) and a weaker input from site (**4**). Stimulation in the contralateral VMf at site (**2**) did not induce any responses. An antidromic spike (shown as a red spike between panels **B** and **D**) was evoked from sites (**1**) and (**3**).

Fig. 10.

Diagrams of stimulation sites and evoked responses of four LSCC-Ns in horizontal (*A, B*) or parasagittal (*C, D*) slices. *A* – LSCC-N (reconstructed in Fig. 4A) received strong synaptic excitatory inputs from rostral and caudal ipsilateral remote sites (400 μ m from the cell) located near CC in the horizontal slice. *B* – Another LSCC-N in which the same configuration of stimulation points **2** and **3** along the CC did not elicit pronounced synaptic responses; and stimulation at site **1** close to the lateral funiculus at the same distance from the soma gave a similarly weak response. *C* – In a parasagittal slice well expressed EPSCs were obtained from sites **3** and **4** along the CC and from the site **2** in the VMf just ventral to the recorded cell. Stimulation at more distant point **1** in VMf gave only a weak polysynaptic response. A polysynaptic IPSC was obtained only from site **3**. *D* – Stimulation in the VMf at a site ventral to the neuron elicited a strong monosynaptic EPSC and an antidromic spike.

Fig. 11.

Magnitude of an excitatory input in slices depends on a distance and location of an electrically stimulated site. Averaged amplitude of EPSC declines with the distance to the stimulation site regardless of its position in all types of slices. Stimulus intensity 100 μ A.

Fig. 12.

A – Superimposed recordings in voltage clamp configuration of monosynaptic EPSCs and orthodromic spikes with occasional failures at $V_h = -50$ mV. Responses were evoked by 30 μ A stimulation at a site near the CC and 250 μ m rostral to the neuron in a horizontal slice preparation (recording corresponds to “c3d” in **B**). Despite the constant latency (3.3 ms) of the EPSCs the spikes had variable latencies with ~ 4 ms jitter. **B** – Chart showing the substantial variation in the latency of orthodromic escape-spikes recorded from six neurons (C1-C6) in voltage-clamp configuration at $V_h = -50$ mV and elicited by electrical stimulation at different sites in the spinal cord and at different intensities. Each symbol represents a single evoked response. Some symbols overlap. Average spike latencies were changed by the following factors: 1. V_h (c3a vs. c3b); 2. stimulus strength (c4a vs. c4b); 3. position of the stimulating electrode in the VMf in transverse (c2) or parasagittal (c5, c6) slices; 4. position at remote sites near the CC caudally (c3a, c3b, c3c) or rostrally (c3d, c4a, c4b) in horizontal slices; 5. position in medial lamina VII in a horizontal slice (c1). The right column shows the type of slice: horizontal (H), transverse (T) and parasagittal (P), the number and percentage of recordings exhibiting spikes.

Fig. 13.

A - Slightly suprathreshold stimulation with 50 μ A in VMf (transverse slice) evoked only monosynaptic EPSCs at all V_h values which are designated on the left of each recording while the numbers of superimposed traces are indicated above each set of responses in panels **A** and **B**). The initial truncated upward deflection in the recording is the stimulus artifact. **B** - Doubling the stimulus strength to 100 μ A elicited an antidromic spike at near threshold $V_h = -40$ mV and at more hyperpolarized $V_h = -50$ mV (The large amplitude spikes are truncated at this amplification.). **C** - The insets indicated by arrows represent responses depicted in panel **B** at $V_h = -40$ mV and -50 mV at lower amplification and at a faster time base on the extreme right side of the figure. Note the increase of spike and EPSC amplitudes as well as of the spike jitter with hyperpolarization. At $V_h = -30$ mV antidromic spikes were substantially reduced in amplitude (underdeveloped) due to partial inactivation of voltage-dependent Na^+ channels.

Fig. 14.

Voltage clamp recordings at $V_m = -50$ mV showing responses of a propriospinal neuron in the LSCC evoked by $100 \mu\text{A}$ stimulation in the VMf at different frequencies (10–200 Hz). Both EPSCs (*) and antidromic spikes (←) shown in the top right record) reliably occurred in response to each stimulus at 10 Hz. At 50 Hz the EPSC amplitude decayed rapidly but spikes were reproduced in each response. At 100 Hz only the first EPSP occurred and full somatic spikes initiated by every second stimulus were separated by underdeveloped spikes (+). At 200 Hz rare full spikes occurred sporadically within a train of underdeveloped spikes. In all traces full spikes are truncated. Insets on the right are the expanded fragments delimited by a dotted box on the left.

Table 1.

Electrophysiological characteristics of recorded neurons.

		PRV(GFP/RFP) labeled LSCC-Ns		Control unlabeled neurons near CC (ConNs)	
Electrophysiological parameter	Significant difference of means (*) (p<0.05)	M±SE, n=35	Range	M±SE, n=25	Range
V _{mr} [mV]	* t(df)=5.18 df=55	-50.2 ± 1.0	-66 ÷ -40	-57.7 ± 1.0	-66 ÷ -45
R _{in} [MΩ]	* t(df)=10.8 df=30	565 ± 51	150 ÷ 1480	975 ± 108	282 ÷ 2550
τ [ms]	t(df)=1.3 df=57	80 ± 9	15 ÷ 208	62 ± 7	21 ÷ 143
Rh [mV]	* t(df)=2.08 df=40	0 (n=10) 37 ± 6 (n=24)	-2 ÷ 160	0 (n=1) 12 ± 2 (n=23)	-2 ÷ 35
Spike threshold [mV]	* t(df)=5.38 df=55	-31.1 ± 0.8	-40 ÷ -22	-38.7 ± 1.1	-48 ÷ -28
Spike amplitude [mV]	t(df)=0.42 df=44	65 ± 2.1	38 ÷ 90	63.5 ± 2.9	41 ÷ 94
Spike HW [ms]	* t(df)=2.17 df=42	1.31 ± 0.06	0.86 ÷ 2.27	1.55 ± 0.09	0.74 ÷ 2.31
AHP amplitude [mV]	* t(df)=4.56 df=42	24.9 ± 0.8	-39 ÷ -24.5	18.1 ± 1.2	-34 ÷ -6.8
I _h presence		No I _h - 5 cells Weak I _h - 24 Moderate I _h - 5 Strong I _h - 0		No I _h - 14 cells Weak I _h - 9 Moderate I _h - 1 Strong I _h - 0	

Membrane properties of PRV-(GFP/RFP) labeled neurons of LSCC significantly differ (*) from those in randomly recorded non-labeled control neurons near the central canal in L3/L4. In all cases they show deterioration of membrane properties due to virus infection, but at this stage neurons are still capable of responding to synaptic or antidromic stimulation as well as of spike generation with characteristic firing patterns. Note that Rh=0 for ten LSCC-Ns and one ConN because those cells fired spontaneously at V_{mr} and did not require an additional current injection; Rh = 0 for all other neurons which were silent at V_{mr}.

Abbreviations: LSCC-Ns - neurons of Lumbar Spinal Coordinating Center, ConNs – control neurons, V_{mr} – membrane potential at rest, R_{in} – input resistance, τ – time constant, Rh – rheobase, spike HW – width of a spike at half-amplitude; AHP – post-spike afterhyperpolarization, I_h – hyperpolarization-activated inward current, t(df) – t value in Welch's t-test, df – calculated degree of freedom.