Short Thesis for the Degree of Doctor of Philosophy (PhD)

The lateralmost aspect of the superficial dorsal horn and the lateral spinal nucleus (LSN) of the lumbar spinal cord of the rat: anatomical description of neurons and electrophysiological study of their propriospinal connections

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INTRODUCTION

Relieving pain has been a mission of medicine from the beginning of times. It is important during the treatment of any kind of disease because it increases the patient's well-being, improves his cooperation and makes surgical interventions possible. Moreover, in terminal illnesses soothing pain can be the only thing a doctor can do for his patient. Nonetheless, after several scientific revolutions, in the age of modern technology, finding the ideal analgesic remains a challenge.

To move forward in the improvement of analgesics, it is indispensable to expand our knowledge about the nociceptive system itself. The better we understand the sensory processing of the painful stimulus from the peripheral nociceptor to the central nervous system, the more possibilities we have to intervene. In this information processing the spinal cord has long been thought to act as a relay station, which simply mediates the stimulus from the periphery to the central nervous system. However, today we already know that its role is much more complex than that. It performs the primary coordinative, regulatory and modulatory function in nociception. Since 1965, when Melzack and Wall proposed their gate-control theory, an increased attention has turned to the action of the eventual neural circuitry located in the spinal dorsal horn. Getting to know the specific neural populations in the spinal dorsal horn, describing their neurochemical and electrophysiological properties can lead to a better understanding of the complex operation of neural circuits in the spinal dorsal horn and their precise role in nociceptive information processing. Such knowledge would be fundamental to develop specific and effective analgesics, which lack supraspinal side effects.

THEORETICAL BACKGROUND

The grey matter of the spinal cord can be grossly divided to a ventral horn, location for neurons involved in motor functions and to a dorsal horn, which is responsible for sensory functions. Based on cytoarchitectonic features, the grey matter can be further subdivided into ten laminae, out of which lamina I and the outer two-third of lamina II (lamina IIo) is often mentioned as superficial dorsal horn, and bears special importance in the processing of painful stimuli, as it is densely innervated by nociceptive primary afferent fibers. The information conducted by

these small-diameter myelinated and unmyelinated fibers is processed by networks of local circuit neurons (LCNs) or interneurons and transmitted to projection neurons (PNs), which have a direct connection with supraspinal centers. The axons of these projection neurons form the ascending nociceptive pathways, the most significant of which is the anterolateral system (ALT) located in the anterior half of the lateral funiculus.

In the past decades several authors have described numerous hypothetical network models, which tried to interpret the operation of neuronal circuitry in the superficial dorsal horn and its role in the modulation of nociceptive information. These models focusing mostly on the neuronal circuits involving lamina II neurons, presume that the nociceptive information processing is performed by local, segmental neuronal circuits in the superficial dorsal horn, which lack the connection to other parts of the spinal cord. However, there are more and more evidence supporting the original results of axon degeneration experiments, carried out by Szentágothai, who emphasized the importance of propriospinal neuronal networks and reported the existance of short- and long-range propriospinal connections. Petkó and colleagues carried out axon tracing experiments to investigate these putative propriospinal pathways and their results not only reinforce the existence of widespread propriospinal connectivity, but also draw the attention to substantial differences between the propriospinal connections of the medial and the lateral aspects of the superficial dorsal horn. They found that the lateral aspect bears much more widespread propriospinal connections compared to the medial one.

The widespread propriospinal connections of the lateral aspect of the superficial dorsal horn can at least partly originate from lateral collaterals of projection neurons (LCT-PNs) in lamina I, as suggested by Szücs and colleagues. On the other hand, a significant part of these long-range connections may originate from interneurons, but mainly due to technical reasons, there is no excessive morphological description and analysis available in literature of the extension and branching pattern of the axon cloud of these neurons.

Besides the widely investigated superficial dorsal horn, it is tempting to assume that the lateral spinal nucleus (LSN) can be a potential target of these lateral long-range propriospinal connections. The LSN, also known as the nucleus of the dorsolateral funiculus, consists of neurons scattered among the fibres of the dorsolateral funiculus (DLF), and forms a continuous column ventrolateral to the

dorsal horn of the rat spinal cord from the spinomedullary junction to the sacral levels. Neurons of the LSN can be labelled by retrograde axoplasmic transport techniques from various supraspinal loci, including the parabrachial nucleus, the periaqueductal grey the nucleus of the solitary tract, the medullary reticular formation, the nucleus accumbens, the septum and the amygdala and orbital cortex, most of which are involved in sensory functions and some of them specifically in nociception. The diencephalic targets of LSN neurons include the hypothalamus and the thalamus. In contrast to the superficial dorsal horn, LSN projects bilaterally to the mediodorsal thalamic nucleus and some authors assume that this LSN-mediodorsal pathway may be involved in the emotional and cognitive aspects of pain. These results imply that the majority of LSN neurons are projection neurons, and this view is also supported by Réthelyi, whose morphological descriptions indicate that the axons of LSN neurons become myelinated soon after their origin, which is a common feature of projection axons. Besides supraspinal targets, LSN neurons also project to lamina I, II, V and VII of the spinal cord. Réthelyi also mentions that LSN neurons could be double labelled from two injection sites in the brainstem and diencephalon, as well as in the brainstem and spinal cord, which raises the possibility that these neurons probably take part in the establishment of propriospinal connections via collateral axon branches.

LSN neurons may receive majority of their synaptic input from spinal interneurons. Presence of considerable amount of peptidergic varicosities is said to be characteristic of the nucleus and the number of peptidergic varicosities is not affected by dorsal rhizotomy or experimental lesions of the descending pathways, therefore several authors conclude that peptidergic input to LSN may arise from the same or nearby segmental levels of the spinal cord. On the other hand, occasional axon collaterals of lamina I projection neurons also enter the dorsolateral funiculus, which can provide substrate for a putative synaptic connection to the neurons of the LSN, although such synaptic connections have not yet been demonstrated by electrophysiological methods.

In contrast to the superficial dorsal horn, our knowledge of the LSN neuron morphology is far from complete. Despite the fact that most of the LSN neurons are supposed to be projection neurons, we don’t know anything about their axon trajectories in the spinal cord, although it is possible that they take part in the establishment of propriospinal connections via collateral branches.

To further investigate the intersegmental propriospinal connections, paired electrophysiological recordings would be indispensable. Unfortunately, due to technical limitations of the generally accepted thin-slice preparations, up till recently, such experiments could have been carried out only in vivo with very low yield. The recently described oblique infrared LED (IR-LED) illumination technique provides imaging by using reflected light, and can be successfully applied to visualize neurons through the overlying white matter in unsliced, intact lumbar spinal block preparation. Using such spinal block preparation during patch clamp recordings makes it possible to intracellularly label intact neurons without truncating the dendritic tree, the widespread axon of an interneuron or the ascending axon of a projection neuron. In addition it is also suitable for studying local microcircuits and could be potentially applicable to investigate short and long-range propriospinal connectivity. The efficiency of investigating neuronal circuits can be further enhanced by using the cell-attached stimulation technique, which allows testing an increased number of presynaptic neurons and putative neuronal connections compared to the conventional double patch clamp recording technique.

Combination of the oblique IR-LED illumination technique on intact spinal cord block preparation and cell-attached stimulation of putative presynaptic neurons provides a powerful tool for studying propriospinal neuronal networks.

OBJECTIVES

Our principal aim was to investigate the short and long range propriospinal connections in the lateral aspect of the superficial dorsal horn and analyse the neuronal participants by combined morphological and electrophysiological tools.

a) We aimed to provide an exhaustive morphological description of local circuit neurons and evaluate their putative contribution in propriospinal neuronal networks. In this aspect the axon bears special importance as it provides the output of these neurons and knowing its expansion, target regions and trajectory would help us to assess their putative synaptic contacts.

b) We also wanted to investigate the latero-lateral segmental and intersegmental propriospinal connections, a significant number of which may be given by connections between lamina I and LSN neurons, by *in vitro* electrophysiological tools.

c) Finally, we wanted to take advantage of the possibility to label intact LSN neurons during the electrophysiological recordings to study their detailed morphology and axon trajectory. We also aimed to compare their characteristics of adjacent neurons in lamina I and place them in the existing knowledge of the dorsal horn neuronal circuitry.

MATERIALS AND METHODS

Visualization and identification of neurons in lamina I and the LSN

The vertebral column of young laboratory Wistar rats of age P14-P21 was dissected and immersed in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. The lumbar segment of the spinal cord was cut out and glued to a golden plate by cyanoacrilate adhesive with the dorsolateral surface facing up.

Neurons were visualized by a Zeiss Axioskop FS microscope (Carl Zeiss Microscopy, USA) equipped with a X40 water immersion objective, custom made oblique infrared illumination and a CCD camera system (Hamamtsu, Japan). The lateral aspect of lamina I was identified as a territory of grey matter, formed by cell bodies of around 20 μm surrounded by a network of randomly oriented fibres. The LSN was found ventrolateral to lamina I and identified as large neuronal cell bodies scattered among the parallel, rostrocaudally oriented fibers of the dorsolateral funiculus.

Electrophysiological recording and intracellular labelling of neurons

Electrophysiological recordings from putative postsynaptic neurons in the lateralmost aspect of lamina I and the LSN were performed in whole-cell configuration. During the whole course of the experiment, oxygenated ACSF was perfused through the recording chamber, which was composed of: NaCl 115mM, KCl 3mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, NaH_2PO_4 1mM, $NaHCO_3$ 25 mM and glucose 11mM (pH was 7,4 when bubbled with 95%-5% mixture of O_2-CO_2).

Pipettes were pulled from a thick-walled borosilicate capillaries (BioMedical Instruments, Germany) by a vertical (Narishige PC-10, Japan) or a horizontal pipette puller (Sutter P-97, USA) and fire-polished afterwards (Narishige Microforge MF-830). The final resistance of the pipettes was ranging between 4-5 MΩ.

The pipettes were filled with an intracellular solution containing KCl 3mM, potassium-gluconate 150 mM, $MgCl_2$ 1 mM, BAPTA 1mM, Hepes 10 mM. The pH was adjusted by KOH to 7,3. The final $[K^+]$ was 160 mM. Before the experiment biocytin (0,5-1%) was added to the intracellular solution with which the recording pipette was filled. To enhance the passive diffusion of bioctyin into the fine neuronal

processes we applied repetitive depolarizing current pulses at the beginning and at the end of the recording session.

The experiments were carried out partly in the Instituto Biologia Molecular e Celular (IBMC, Rua do Campo Alegre 823, 4150-180 Porto, Portugal), where the amplifier used was EPC10-Double (Heka, Germany) and partly in the Department of Anatomy, Histology and Embryology (University of Debrecen, Nagyerdei krt. 98. 4032 Debrecen, Hungary), with an Axopatch 1D (Axon Instruments, USA) amplifier. The signal was low-pass filtered at 2.9 kHz and sampled at 10kHz. Offset potentials were compensated before seal formation. Liquid junction potentials were calculated (15mV) and corrected for using the compensation circuitry of the amplifier.

We searched for putative presynaptic neurons in lamina I, one or two visual fields medially, and one to seven visual fields caudally from the recorded postsynaptic neuron. After establishing whole-cell configuration with the postsynaptic neuron, 2-3 landmark structures were noted at the caudal end of the view-field. Then, the objective of the microscope was moved caudally along the spinal cord until the landmarks disappeared at the rostral end of the view-field and putative presynaptic neurons were searched for in this region. The size of a single visual field under our conditions was approximately 180 μm. When measuring larger rostrocaudal distances, the above steps were performed repeatedly.

The stimulating pipette was filled with a solution containing 500 mM NaCl and 1,5% biocytin. The putative presynaptic cells were stimulated in cell-attached configuration by a 1ms long current pulse of 100 nA, at a frequency of 1 Hz using an EPC10-Double (Heka, Germany) or an Axoclamp 2B (Axon Instrments, USA) amplifier.

Identification of monosynaptically connected neurons

Pairs of monosynaptically connected neurons were identified by using a protocol that contained a pre-pulse period (200 ms) followed by a single current pulse (100 nA, 1 ms) and a post-pulse recording period (200 ms) in which the postsynaptic neuron was recorded for 200 ms preceding and 200 ms following the current pulse application to the presynaptic neuron, and by analysing the stimulus-evoked changes in the excitatory postsynaptic current (EPSC) numbers. In some cases the pre-pulse recording was shorter (20 ms). The protocol was repeated 90 times at 1 Hz. EPSC latencies in connections were calculated from the end of the 1

ms stimulation pulse to the time moment when the evoked EPSC reached 10% of its peak amplitude. EPSCs in the pre-and post-pulse period were detected automatically by using MiniAnalysis (Synaptosoft, USA). Postsynaptic responses were considered monosynaptic if evoked EPSCs showed a low failure rate and a latency variation below 4 ms. These criteria were based on a prior study in the intact spinal cord preparation showing that lamina I neurons are directly connected by several parallel synapses via axodendritic pathways of different length, and therefore, latencies of individual components of a monosynaptic EPSC recorded in one connection can differ substantially. The monosynaptic nature of the connection was also verified by confirming the presence of transitions between individual EPSC components. In case of those recordings, which were done with an Axopatch 1D amplifier (Molecular Devices, USA) the cell-attached stimulation of putative presynaptic neurons were carried out with an Axoclamp 2B amplifier (Molecular Devices, USA).

Histochemical processing

The preparations were fixed in 4% formaldehyde for at least 72 hours. After fixation the spinal cord was embedded in agar and 100 μm - thick sagittal sections were cut by a VT 1000S tissue slicer (Leica, Germany). To reveal biocytin, sections were permeabilized with 50% ethanol and treated according to the avidin-biotinyliated horseradish peroxidase (HRP) method (ExtrAvidin-Peroxidase, diluted 1:1,000; Sigma) followed by a diaminobenzidine (DAB) chromogen reaction. Sections were either counterstained on slides with 1% toloudine blue, dehydrated, cleared and coverslipped with DPX (Fluka, Switzerland) or treated with 1% OsO_4 and embedded in epoxy resin (Durcupan; Fluka, Switzerland). Phototmicrographs were taken using the 10x or 40x dry lens of a Primo Star (Carl Zeiss Microscopy, USA) microscope equipped with a Guppy (Allied Vision Technologies, Germany) digital camera. Contrast and brightness of the photographic images used in all the figures were adjusted using the Adobe Photoshop software.

3-D reconstruction of labelled neurons and analysis

Complete 3-D reconstructions were done from serial sections using Neurolucida (MBF Bioscience, USA).

Each section was completely traced, using a X40 (dry) objective, onto the corresponding section of a serial section data set. We aligned the sections and connected the matching pieces working always in the direction of the section containing the soma. As a result of shrinkage during the histochemical processing the thickness (Z dimension) of resin-embedded sections was 80-90% of the original 100µm. As this was comparable to the shrinkage along the X-Y axes, it was not corrected in these cases. In case of DPX-embedded sections, the shrinkage along the Z axis was corrected to reach the thickness of 80µm and to be comparable to the other reconstructions.

Morphometric analyses and basic quantitative measurements including Sholl analysis, dendritic segment length calculation, spine numbers, spine density and Fan-In projection were performed by Neurolucida Explorer (MBF Bioscience, USA).

Calculation and 3-D visualization of varicosity distributions and action potential propagation time maps

In order to perform spatially dependent morphometric analysis on the axonal trees, we created a set of specific functions (called Py3DN; https://sourceforge.net/projects/py3dn/) in the Python programming language. The Neurolucida data of the reconstructed axons were made available to the Python environment via a custom-made sparser.

For creating propagation time maps along the LCN axonal tree, we calculated the time needed for a hypothetical action potential to reach each point of the axon, starting from the initial segment. We assumed that the contribution of each segment to the propagation time depends on its length and diameter. The equation used for calculating the propagation time δ of myelinated regions was $\delta = l/(kd)$ where l is length, k is a constant factor, which was equal to 10m/second in case of

myelinated and 380 m/second in case of unmyelinated axonal segments, and *d* is the diameter. The diameter threshold we used to distinguish between myelinated an unmyelinated segments, was based on electron microscopic measurements. Finally we color-coded each point of the axon to represent the cumulative propagation time.

Electron microscopy

Those spinal cords, which were intended to be sent for electron microscopic analysis, were fixed in a mixture of 4% formaldehyde and 0.1% glutaraldehyde. After fixation, 100-µm-thick serial sagittal sections were made by a VT1000S tissue slicer (Leica, Germany), and the sections were permeabilized by freeze-thaw cycles in liquid nitrogen. Biocytin was revealed according to the HRP-DAB protocol *(for details see chapter: Histochemical processing),* then sections were postfixed in 1% OsO_4, dehydrated, and embedded into epoxy resin (Durcupan; Fluka, Svájc) on glass slides.

Regions of interest were excised and re-embedded for ultrathin sectioning. Ultrathin sections were further contrasted by lead citrate and uranyl acetate, and then scanned by a JEM 1400 TEM type electron microscope (JEOL, Japan). We recorded images of the biocytn-filled axonal profiles with the help of an SC 1000 Orius CCD camera (Gatan, USA) at x 20,000 or x 50,000 magnifications.

Statistical analysis

All statistical analyses were performed with the SigmaStat 3.0 (Systat Software, USA) software. Comparison of morphometric parameters between lamina I neurons and cells in the lateral adjacent white matter and LSN was done by using Student´s t-test or Mann-Whitney Rank Sum Test. Between-group comparisons of EPSC kinetic parameters were done by Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn´s Pairwise Multiple Comparison. A $p<0.05$ was considered to be statistically significant.

Numbers are given as mean ± SEM unless otherwise mentioned.

RESULTS

Morphology of local circuit neurons in lamina I

Our research group performed a thorough morphological description of lamina I LCNs, involving eighty-two neurons, out of which thirteen have been completely reconstructed in 3-D, which allowed us to accomplish further analysis.

Extent of the axon

Although the axon of lamina I LCNs showed characteristic and distinguishing traits in some aspects, the lack of any axon in the contralateral white or grey matter and the presence of numerous ipsilateral, local axon collaterals with varicosities proved to be their common feature.

The axon of LCNs formed a local accumulation, which in most of the cases occupied the dorsal 100-120 μm thick band of the grey matter, corresponding the superficial dorsal horn and occasionally reached lamina III. While the mean mediolateral and dorsoventral extent of the labelled and recovered axon was 636 ± 27 μm and 295 ± 41 μm, respectively, the rostrocaudal spread was significantly higher, 2312 ± 116 μm, which corresponds to two to three lumbar spinal segments of rats at this age. The local network was either centred on the soma, or shifted along the rostrocaudal axis. In some cases, LCNs situated in the lateralmost part of lamina I had the majority of their axons located medially, several tens of micrometres away from the soma.

Apart from the above-mentioned local axon, the majority of lamina I LCNs had single or multiple, varicose or myelinated-appearing, solitary axon branches in the neighbouring white matter, including the Lissauer tract, dorsal funiculus (DF), dorsolateral funiculus (DLF), and lateral funiculus (LF). Some of these soliter branches gave rise to varicose unmyelinated collaterals of various density at certain distances form the main axon cloud. The presence of such remote targets suggested us to perform further analysis of the fine structure and organization of LCN axons.

Branching pattern and varicosity distribution along LCN axons

While some neurons of our sample possessed an axon, which only had three or four primary branches, and the proximal ones dominated the tree, in other cases the main axon gave rise to up to seven primary branches and the distal ones occupied more space. Regardless of the initial branching pattern, we found major or minor overlaps between target areas of the primary branches in all cases.

Mapping of varicosity density along the axon combined with path distance measurements and Sholl analysis revealed that LCN neurons presented the highest number of varicosities in the vicinity of, but not centred on, the soma. In some cases the number and density of varicosities gradually decreased farther from the soma. On the other hand there were cells in our sample, which presented fewer varicosities in the first 500 μm, but further local accumulations could be observed occasionally several hundreds of micrometers away from the soma in the rostrocaudal or in the mediolateral axis.

Fine structure of LCN axons and their effect on propagation time maps

The observation that sometimes remotely located varicosity bearing thin axon pieces were branching from myelinated appearing thicker axons raised the possibility of a presumable ultrastructural difference between the morphologically different parts. Therefore we performed electron microscopic analysis of a characteristic section, where both types of axon were present. We found that myelinated appearing axonal profiles presented several concentric layers of myelin without exception, and diameter measurements showed that these parts were 760 ± 28 nm (n=24) with, and 453 ± 18 nm thick without myelin sheath. On the other hand, axon profiles from a varicose axon region lacked myelin in case of both varicosities and intervaricosity segments, and the mean diameter of these unmyelinated parts was 311 ± 10 nm.

To determine how the different axon segments contribute to action potential conduction in the LCN axons we used a custom-made script, written in the Python programming language to create propagation time maps. Based on the means and histograms of the measured diameters, we determined a threshold (0,35 μm), above which the axon segment was considered myelinated. Conduction velocity in the unmyelinated part was set to 0,38 m/s for a uniform 1-μm thick axon, and to 10 m/s in case of myelinated axons. When propagation time maps were calculated

considering axon segments with a diameter above 0,35 μm as myelinated, the maximum propagation times dropped compared to those values, which were calculated by considering all axons unmyelinated. In case of LCNs with a remotely situated terminal branching area the maximum propagation time dropped to about 80% of the original value.

Propriospinal connections in the lateral part of the lumbar spinal cord

During the experiments to investigate propriospinal connections, 86 cells were recorded in whole cell mode in the lateral aspect of the spinal dorsal horn and in the LSN. Although post-hoc visualization of biocytin in the recorded neuron was attempted in all cases, successful recovery, that allowed confirmation of the location and classification of the axon and somatodendritic domain, has only been achieved in 32 cases (recovery rate: 37%).

Synaptic connectivity

For 54 whole-cell-recorded cells we tested 241 putative presynaptic neurons by cell-attached stimulation (the numbers of trials for a single postsynaptic neuron ranged from 1 to 18). Most presynaptic cells tested were selected in the three neighbouring segments caudal to the location of the postsynaptic neuron. In 224 cases, stimulation of the presumably presynaptic cell did not evoke response in the postsynaptic neuron. The lack of possible mono- or polysynaptic connection was also apparent from the unchanged frequency of excitatory postsynaptic currents (EPSCs) in the 200-ms-long periods before and after the 1-ms-long presynaptic stimulation. These cells were evaluated as not connected.

In 12 cases, stimulation of the presynaptic neuron resulted in a reliably evoked EPSC with a mean latency of 5.7 ± 1.5 ms (n=12, range of the means: 1.7 – 19.8 ms). The mean amplitude of the monosynaptic EPSCs was 30.7 ± 5.2 pA (n=12, range of the means: 6.6 – 63.4 pA). The stable response caused marked increase in the frequency of EPSCs within the first 50-ms-period after the stimulation. The occurrence of monosynaptic connections was highest when looking for presynaptic neurons in the first visual field caudal to the postsynaptic recording site and decreased gradually when searching for presynaptic neurons more caudally. The most

distant monosynaptically connected neuron was found 4 visual fields (720 µm) away, from the postsynaptic neuron. This distance is comparable with the length of shorter lumbar segments in the juvenile rat spinal cord.

In case of 5 paired recordings stimulation of the presynaptic neuron initiated a huge increase of the basal EPSC frequency. Interestingly, this was sustained in the postsynaptic neuron for hundreds of milliseconds without further stimulation of the presynaptic cell, possibly due to activation of polysynaptic excitatory pathways. For this reason we called this type of connection polysynaptic. A well-defined monosynaptic component in these connections could not be identified due to the complex kinetics of the summed EPSCs. When normalized to the EPSC frequency of the 200-ms-long prestimulus period the EPSC increment was highest in the first 50-ms-long bin and showed a gradual decrease unlike the abrupt difference after the first 50-ms period of the monosynaptic connections.

Efficacy of the polysynaptic input, electrophysiological properties and synaptic connectivity

We repeated the same protocol (200 ms pre-stimulus and 200 ms post-stimulus periods) in current-clamp mode. The consecutive excitatory postsynaptic potentials (EPSPs) were capable of depolarizing the neurons above the firing threshold even from membrane potentials as low as -90 mV. Stimulation of the presynaptic neuron resulted in sustained tonic action potential discharge in the postsynaptic cell held at around -80mV. The frequency of the discharge evoked by this synaptic input stimulation was visibly lower than that evoked by a somatically injected current pulse causing depolarization of similar amplitude, about 10-15 mV.

When normalized to the pre-stimulus period, the mean number of action potentials (for all the neurons with polysynaptic input, n=5) showed a 5-fold increase in the poststimulus epoch.

Morphology of neurons in the LSN and in the lateralmost aspect of lamina I

General somatodendritic features of neurons outside the dorsal grey matter

From the 32 neurons, in which biocytin was successfully recovered, only 5 cells were located within the boundaries of lamina I. The rest of the cells were next to

the lateral edge of lamina I, among the superficial fibres of the DLF (n=18) or within the LSN (n=9). While neurons within lamina I could be classified according to the system introduced by Lima and Coimbra, neurons located more laterally, outside the dorsal grey matter and in the LSN, with the exception of three cells presented very different somatodendritic features.

We compared basic morphometric parameters of these neurons with a pooled sample of 3-D reconstructed projection neurons (PNs) from the work of Luz and colleagues and LCNs from our earlier study. Neurons lateral to lamina I and in the LSN had more stem dendrites and significantly longer total dendritic length. This difference was apparent in the generally more "bushy" appearance of neurons outside lamina I. Another striking feature of the neurons lateral to lamina I was the presence of numerous short spines along the dendritic surface. In some cases spines appeared to be present even on the neuronal soma itself. Indeed, the total number of spines and spine density both proved to be significantly higher in neurons outside lamina I.

Distinct axon trajectories of neurons outside lamina I and in the LSN

Regardless of the location of their somata, the recovered neurons could be grouped into distinct categories on the basis of the type and course of their axons. 16 out of 32 successfully recovered neurons could be classified according to earlier published criteria for projection- and local-circuit neurons.

In 7 cases the main axon crossed the midline in the posterior commissure and shortly after entered the medial side of the contralateral anterior column where it started to ascend.

Another set of neurons had axons with a similar initial course, but instead of ascending on the contralateral side these axons returned to the ipsilateral side by re-crossing in the anterior commissure and exited into the medial part of the ipsilateral anterior funiculus.

Finally, the main axon of the remaining 4 cells, before crossing the midline in the anterior commissure and ascending in the c-ALT, gave rise to two long axon branches one of which ascended in either the ipsilateral dorsolateral funiculus (i-DLF) or the ipsilateral dorsal funiculus (i-DF), while the other, showing characteristic features of unmyelinated collaterals, descended in the i-DLF.

Although the major grouping criterion was the axon trajectory, neurons divided on this basis also showed other common characteristics described below.

Posterior commissural contralaterally projecting neurons

Neurons belonging to this group showed a spherical organization of their dendrites with the cell body located on the most superficial part rather than the center of this spherical structure. Dendrites protruded out from the soma in a multipolar fashion and occupied a large part of the DLF. Dendrites branched frequently even in the vicinity of the soma, thus dendritic segment were generally short.

This type of neuron possessed a high number of short spines. Fan-in projection revealed that dendrites showed no preferred orientation filling the space around the soma equally.

The axon of these neurons started off medially from the soma or proximal dendrites, entered the grey matter and aimed medially towards the central canal. The main axon gave collaterals on the ipsilateral side to various laminae, including lamina X around the central canal, and crossed the midline in the posterior commissure. The axon on the contralateral side took a short path ventrally before it exited into the medial side of the anterior column white matter.

Examination of the spontaneous synaptic input to these neurons revealed occasional, long-lasting and large amplitude EPSPs and EPSCs. Summation of these spontaneous events showing generally slow kinetics was not observed.

Double crossing ipsilaterally projecting neurons

In the sample of neurons with unique axon trajectories 5 neurons possessed a main axon that crossed the midline twice.

Dendritic arborisation of these cells were also dense with numerous branch points close to the soma and they also presented a high number of spines, although less, than neurons crossing in the posterior commissure. However, the orientation of the dendrites was more restricted: they were either going dorsally and followed the curvature of the dorsal grey matter, occupying mostly lamina I-II, or turned ventrally and branched in the DLF. For this reason Fan-in projection showed marked accumulation around 90 degrees.

The axon started off in the medial direction and aimed towards the posterior commissure giving ipsilateral collaterals in laminae of the deep dorsal horn, intermediate grey matter and around the central canal (lamina X). Interestingly none

of the 5 neurons gave collaterals to the superficial dorsal horn. The main axon then crossed the midline in the posterior commissure, took a short loop on the contralateral side and re-crossed in the ventral commissure to enter the medial aspect of the white matter in the ipsilateral anterior column.

Spontaneous synaptic input was more frequent than in the posterior commissure crossing cell group; both EPSPs and EPSCs were showing faster kinetics and occasional summation.

Bilaterally projecting neurons

Finally, 4 neurons, two in the LSN and two between the LSN and the edge of the dorsal grey, presented a main axon, that before crossing the line in the anterior commissure, gave rise to an equally strong myelinated axon branch that entered and ascended in the i-DF or i-DLF. Another characteristic morphological feature was the presence of single thin ipsilateral axon collaterals that had numerous varicosities and descended in the DLF or Lissauer´s tract.

The dendritic tree is quite asymmetric, having most branches filling a conical space in the DLF and having a single branch that spreads medially. Dendrites were more extensive and branched less frequently than in case of the previous two groups, resulting in longer and less dendritic segments. Spines were less numerous, not exceeding the values observed earlier in case of neurons in lamina I. Fan-in projection confirmed the conic arrangement of dendrites in DLF, showing a prominent accumulation of dendrites at 90 degrees.

Interestingly, despite the fact that these cells had few spines, the frequency of spontaneous excitatory activity was high and all cells in this group showed large number of various amplitude EPSPs and EPSCs that were often superimposed.

Differences in spontaneous EPSC and EPSP frequency and kinetics of distinct morphological neuron types

In order to quantitatively analyze the differences in the frequency of spontaneous postsynaptic events (sEPSCs and sEPSPs) we selected, from each neuron 10 traces (length: 200-800 ms) where the presynaptic neuron was not stimulated, calculated the sEPSC frequency for each trace and presented pooled data for the corresponding groups. Statistical analysis proved that bilaterally projecting neurons presented a significantly higher ($p<0.05$, Kruskal-Wallis One Way Analysis

of Variance on Ranks followed by Dunn´s Pairwise Multiple Comparison) mean frequency (23.4 ± 2.1 Hz) of spontaneous events than neurons with the other two types of axonal trajectories.

For measurements of sEPSP and sEPSC kinetics descriptions we selected 7-30 events of each type for each neuron in a given group. Spontaneous EPSCs were taken from traces where the presynaptic neuron was not stimulated, while sEPSPs were analyzed using current-clamp traces of the firing pattern protocol (at 0 current step) acquired for each cell. The mean amplitude of sEPSCs was significantly smaller ($p<0.05$, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn´s Pairwise Multiple Comparison) in neurons with double crossing ipsilaterally projecting axon. The mean sEPSP amplitudes, however, did not show significant differences. Mean rise time (measured as time-to-peak) was significantly shorter ($p<0.05$, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn´s Pairwise Multiple Comparison) in bilaterally projecting neurons for both sEPSCs and sEPSPs.

DISCUSSION

The experimental results described in the publications and presented in this thesis have shown that LCNs frequently possess a number of short and putative long propriospinal branches, and this feature provides morphological basis for their possible involvment in segmental interlaminar and propriospinal sensory information processing. We have also tested the hypothesis that rostrally oriented axons in the DLF, comprising, in part, the collaterals of PNs as well as local axon of LCNs, are providing synaptic input to dendrites of lateral lamina I neurons and to neurons located in the LSN. We proved that stimulation of superficial cells in the lateral aspect of lamina I could indeed evoke mono- and polysynaptic excitation of LSN and lateral lamina I neurons, although these connections were rare. On the other hand, by careful 3-D reconstruction of the neurons, we discovered, and described for the first time, previously undescribed axon trajectories of putative projection or propriospinal neurons in the lateral dorsal spinal cord.

Possible roles of LCNs in local neuronal networks of the dorsal horn

The extensive branching of LCN axons suggest that, besides the anatomical divergence of primary afferent fibers, LCNs may provide further divergence of processed primary afferent information after integrating it with other sources of input. Furthermore, LCN axons occupy most dorsal laminae (I–II and occasionally III–IV), and this feature, theoretically, allows relaying of C-fiber information to deeper laminae. Consequently, neurons in laminae III–V may integrate direct primary afferent information with indirect processed form of the same information through lamina I LCNs.

Because of their extensive local axon, we can also presume, that relatively few LCNs could influence the superficial dorsal horn along several segments, in a sustained tonic manner. The large number of varicosities and the highly branched, extensive axon of LCNs also imply that these neurons may be involved in volume transmission.

Possible roles of LCNs in short and long propriospinal neuronal networks

A large percentage of LCNs had long, solitary axon branches, often with a myelinated appearance, in the DF, DLF and Lissauer's tract. These branches often ran for two to three segments in the rostrocaudal direction without any preferential direction, and then they faded below visibility. Similar axon branches originating from lamina II-III neurons have already been described in the dorsolateral white matter by earlier anatomical studies and intersegmental integration was also proposed recently for dorsal horn cholinergic neurons, with similar rostrocaudal axonal organization. However, morphological features of LCNs in our sample imply, that they can also act as short and in some cases as long propriospinal neurons.

It also has to be noted, that contribution to rostrocaudallly oriented varicose axon collaterals from ALT-PNs and LCNs is balanced in the DLF: 39% of LCNs and 40% of ALT-PNs (lateral and mixed collateral types) have collaterals in this region. Therefore, we can assume that besides the previously described lamina II and III neurons, both LCNs and ALT-PNs of lamina I may contribute to relaying local segmental information to neighboring segments and to neurons of the LSN.

Functional consequences of the organization of LCN axons: branching, varicosity distribution and spike propagation

Temporal dispersion of synaptic input is of crucial functional importance in case of complex axon arbors bearing thousands of varicosities, forming networks in the highly somatotopic spinal dorsal horn. The propagation time of an AP, initiated in the axon initial segment, is determined mostly by path length, axon diameter, myelination, and branching.

Path distance to a particular point of the axon is strongly dependent on branching. The alternating branching of primary collaterals from the main axon into the rostral and caudal directions, frequently observed in this study, seems to be an efficient solution for maximally filling the target space (laminae I–III) and, at the same time, equalizing path distance for the rostral and caudal portions of the axon tree. This setup, however, in a system with strong spatial boundaries, such as the spinal dorsal horn, results in an overlap between major branches. Target regions supplied by multiple overlapping branches may be activated repeatedly.

Short intersegmental connections of lamina I and the LSN are rare but some may be very potent

Our electrophysiological experiments have demonstrated that neurons outside the dorsal grey matter and in the LSN receive monosynaptic connections from lateral lamina I neurons. Connected neurons were rare and could not be detected at distances above 720 micrometres in the caudal direction. Such connections may be considered as intersegmental, since upper lumbar segments in animals of the experimental age we used have similar lengths.

In terms of functionality, these ultra-short propriospinal connections may increase the complexity of intraspinal divergence and convergence of primary afferent inputs. At the same time, a striking observation in this study was the presence of powerful polysynaptic excitation of some neurons in the DLF region. The stimulation of a single presynaptic lamina I neuron evoked long-lasting and suprathreshold excitation of postsynaptic cells. A putative circuitry behind such phenomenon would include a set of excitatory interneurons that spread and amplify the activity of a single "initiator" neuron via feedforward excitation. The sustained EPSC frequency increase may also be explained by disinhibition, the normally silent but now liberated excitatory neurons could be responsible for the frequency increase of EPSCs in target neurons. The "initiator" neurons may also be involved in pathological amplification of incoming signals during certain chronic pain conditions, thus identification of them would be crucial. A good candidate for the "conductor" neuron would be those excitatory and inhibitory LCNs in lamina I that have extensive axons that bridge several segments rostrocaudally and have thousands of varicosities, making them capable of providing high excitatory or inhibitory transmitter levels in a large volume of the spinal dorsal horn.

Neurons in the lateral aspect of the dorsal horn show morphological evidence for integration

With the exception of 5 cells the majority of neurons in the recovered sample fell outside the dorsal grey matter, some clearly within the boundaries of the longitudinal cell column of the LSN, while others between the edge of the dorsal grey and the LSN.

In general organization of the dendrites was very different from PNs and

LCNs of lamina I. Neurons close to the dorsal grey were asymmetric having most dendrites protruding into the DLF ventrolaterally, and only a few aimed to the medial direction, running close to the surface within lamina I.

Above the dense arborization of dendrites in the DLF, these lateral neurons also possessed a high number of dendritic spines, further increasing the putative contact surface. This efficient coverage of the DLF cross section is in line with the suggestion of that the LSN may function as an integrative nucleus, where neurons can receive inputs from primary afferents of visceral origin, interneurons and descending pathways from supraspinal loci.

Axonal architecture of neurons outside the superficial dorsal grey matter

There are several morphological evidence in literature, which imply that the majority of LSN neurons are projection neurons, and this view is also supported by Réthelyi, who observed that the axons of LSN neurons become myelinated soon after their origin; a common feature of projection axons. In good agreement with the above, in this study we found that most neurons outside the dorsal grey matter (24 out of 27) - including LSN neurons - had a myelinated-appearing projection axon and 20 had ipsilateral collaterals within almost all laminae of the spinal grey matter.

Similarly to earlier observations, dendritic origin of the axon was also frequent among neurons outside lamina I and in the LSN. The most remote axon initiation (~90 μm) occurred in one of the bilaterally projecting neurons, where the axon originated from the single medially oriented loopy dendrite. This observation enforces earlier results of computer simulations and suggestions that both the distal and proximal part of the dendrite, which give rise to an axon may have very important roles in the regulation of action potential initiation. Indeed, with the help of modern techniques, in a recent study authors provided functional evidence for the influence of dendritic axon origin on action potential initiation.

Projecting axon of neurons located outside the dorsal grey matter may also cross the midline in the posterior commissure

The mechanisms that govern axon midline crossing during development have been well studied, particularly at the level of the spinal cord. Attracting and

repellent factors such as Netrin1 and Sonic-Hedgehog, secreted by the floor-plate and roof-plate were shown to act in a complementary and sequential manner to guide sets of axons within the developing CNS.

While lamina I projection neurons in earlier works, without exception, crossed the midline in the anterior commissure, neurons outside the dorsal grey matter and in the LSN showed other axon trajectories. In 7 cases the main axon crossed the midline in the posterior commissure and continued to ascend in the medial part of the anterior column white matter. Midline crossing in the posterior instead of the anterior commissure may be the result of different responsiveness of the path-finding axon cone or a temporal difference in neuronal development that results in missing the time window for regular crossing in the anterior commissure. Anterograde tracing of posterior commissure crossing axons will be important to define targets and function of these neurons.

Axon trajectory of double crossing ipsilaterally projecting neurons oppose our present knowledge on axon midline guidance

The fact that the projection axon in these 5 cases crosses the midline twice is not compatible with our present knowledge on axon midline guidance. Prior to midline crossing, comissural axons are attracted by floor plate-derived Netrin-1 and Sonic Hedgehog. After crossing, repellents of the Slit and Semaphorin families expel axons from the floor plate and prevent re-crossing.

Our results provide evidence, however, for the first time, that there are ipsilaterally projecting neurons that cross the midline twice: first in the posterior commissure and then, after taking a short path in the contralateral grey, for a second time in the anterior commissure. Our observation suggests that there are other, yet unresolved molecular mechanisms that govern axon crossing through the midline. Further tracing and lineage analysis studies will be necessary to elucidate the function and origin of these neurons.

Bilaterally projecting axons

Apart from the neurons with posterior commissure crossing and double-crossing projecting axons, 4 cells had bifurcating myelinated-appearing axons. The main axon in these cells bifurcated within the dorsal grey matter and while one branch followed the regular path of PNs, the other one entered the dorsal funiculus

and ascended there in the ipsilateral side. Lamina I PNs with collaterals crossing the midline at the supraspinal level and thus achieving bilateral projections have been reported before. In case of the four bilateral projection neurons in our sample the splitting of the main axon occurs earlier and the two equally strong branches seemingly ascend in different tracts from the beginning.

Indeed, LSN neurons could be double labelled from two injection sites in the brain stem and diencephalon, as well as in the brainstem and spinal cord, which raised the possibility that these neurons probably take part in the establishment of propriospinal connections via collateral axon branches.

Furthermore, when the retrograde tracer cholera toxin beta-subunit (CTB) was used to trace long ascending propriospinal projections from neurons in the lumbosacral spinal cord to the upper cervical gray matter some lumbosacral superficial dorsal horn neurons were identified. These propriospinal projections were suggested to be involved in coordinating head and neck movements during locomotion or stimulus-evoked motor responses. In addition, LSN neurons in the upper cervical segments can be labeled from the upper thoracic segments. Conversely, anterogradely labeled axons of the LSN neurons located in cervical segments have been detected around the neurons in the intermediolateral nucleus in the middle thoracic segments. This caudal propriospinal connection is supported by our finding that bilateral neurons in the LSN and outside the dorsal grey also issued long but thin caudal collaterals in the ipsilateral DLF or Lissauer-tract.

KEY WORDS

spinal cord, dorsal horn, lateral spinal nucleus, dorsal horn circuitry, 3-D-reconstruction, projection neuron, local circuit neuron, axon trajectory

SUMMARY

Our findings indicate that the widespread axonal arbor of lamina I LCNs and frequent collaterals in the dorsolateral white matter designate these neurons, besides some lamina I PNs, as candidates for establishing short- and long-range, intrasegmental and intersegmental propriospinal connections. We have demonstrated the existence of these theoretical propriospinal connections, from lamina I PNs and LCNs onto other lamina I and LSN neurons, which are not frequent but some are extremely potent. The latter may be important in sustained nociceptive signaling during pathological conditions.

We revealed unique axon trajectories of putative projection or propriospinal neurons in the lateral aspect of the dorsal horn and in the LSN that suggests a development different from the regular lamina I projection cells. While LSN is not present in all species the novel axon trajectories revealed in our study may help to identify cell groups with analogous function and developmental origin in other species.

Our findings emphasize the importance of understanding the connectivity matrix of the superficial dorsal horn in order to decipher spinal sensory information processing.

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List of publications related to the dissertation

1. **Antal, Z.**, Luz, L.L., Safronov, B.V., Antal, M., Szűcs, P.: Neurons in the lateral part of the lumbar spinal cord show distinct novel axon trajectories and are excited by short propriospinal ascending inputs.
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