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3-D Reconstruction of Spinal Lamina I Neurons

Oleh Mytakhir

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Oleh Mytakhir

Master's Dissertation presented to Faculty of Sciences of the University of Porto, Institute of Biomedical Sciences Abel Salazar of the University of Porto

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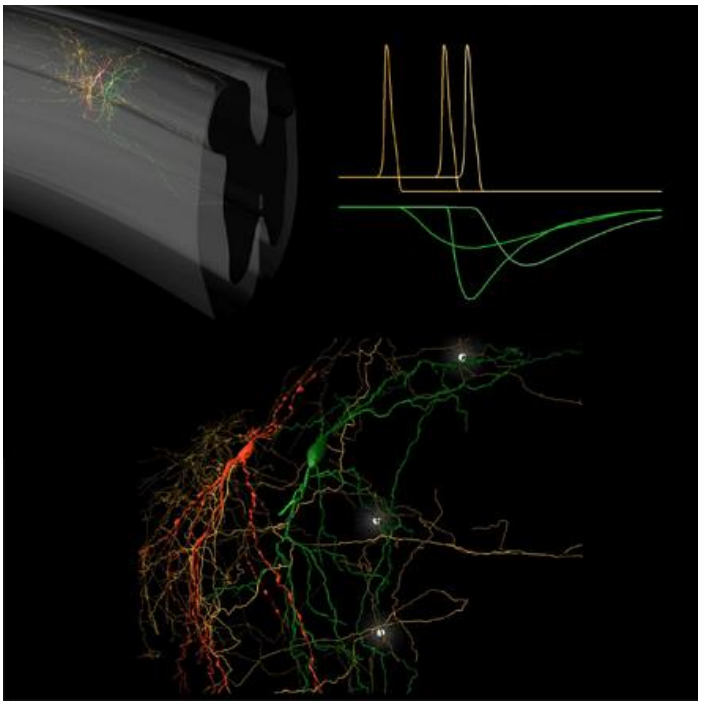

3-D Reconstruction of Spinal Lamina I Neurons

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Master's in Biochemistry

Department of Chemistry and Biochemistry
2015

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O Presidente do Júri,

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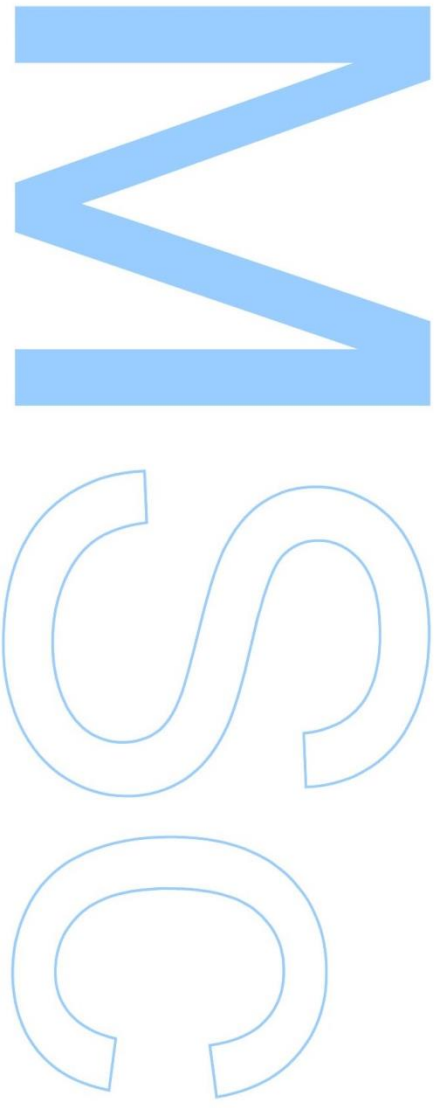

ACKNOWLEDGEMENTS

First of all, I would like to dedicate this dissertation to my parents. All that I have in this life was granted by them. The condition in which they came to the Portugal and still strive to achieve success through faith, hard work and love are characteristics I can only hope to achieve. The completion of this work would have been impossible without their love, support and patience.

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I could not have a dedication section in this work without special mention of my friends. They taught me lessons that cannot be learned from a book and it is a privilege to be their friend.

I would like to thank my dissertation committee, which has undoubtedly contributed to the improvement of this work.

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ABSTRACT

The superficial layer of the dorsal horn of the spinal cord, lamina I, is a key element of the pain processing system which integrates primary afferent input and relays it to the supraspinal centers. Although significant progress has been made concerning our knowledge of the morphological features of the lamina I neurons, our understanding of this layer is still incomplete. We still know little about the processing of inputs by its intrinsic network including local-circuit neurons and projection neurons. Although the somatodendritic architecture of lamina I neurons has been extensively studied, little is known about their axonal morphology.

We used the oblique infrared light-emitting diode (IR-LED) illumination technique to visualize and label lamina I neurons for reconstruction in 3-D and analysis of their dendritic structure and morphology of extensive axonal trees. The majority of lamina I neurons identified in this study were local-circuit neurons. These neurons had extensive local axonal trees that were centered on the cell body or shifted along the rostrocaudal axis. The extensive branching of these axons within and beyond the superficial dorsal horn demonstrates that lamina I is an interconnected layer involved in intralaminar, interlaminar and intersegmental spinal cord processing.

Some of the lamina I local-circuit neurons presented here had prominent ventrally protruding dendrites that reach laminae III-IV. Electrophysiological recordings have shown that these neurons receive monosynaptic inputs from myelinated low-threshold Aβ primary afferents. These findings provide morphophysiological evidence for the involvement of lamina I local-circuit neurons in the dorsal horn pathways that carry low threshold signals from deeper laminae to lamina I. More extensive morphological and neurochemical characterization of these lamina I LCNs will be necessary to better understand the functional role of these neurons in the dorsal horn synaptic circuits.

Keywords: spinal cord, dorsal horn, pain processing, neuronal networks, lamina I neurons, 3-D reconstruction, axon morphology.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

ACSF – artificial cerebrospinal fluid

CGRP – calcitonin gene-related peptide

CVLM – caudal ventrolateral medulla

DLF – dorsolateral funiculus

FRAP – fluoride-resistant acid phosphatase

GABA – γ-aminobutyric acid

GFP – green fluorescent protein

IR-LED – infrared light-emitting diode

LCN – local-circuit neuron

LTMR – myelinated low-threshold mechanoreceptors

NK1R – neurokinin 1 receptor

nNOS – nitric oxide synthase

NPY – neuropeptide Y

NTS – nucleus tractus solitarius

PAG – periaqueductal gray

Pb – parabrachial area

PN – projection neuron

SP – substance P

INTRODUCTION

The marginal layer of the dorsal horn, defined as lamina I (Rexed, 1952), is a key area for the processing of pain-related information and its transmission to the brain (Heinrich, 1992; Hunt & Mantyh, 2001; Todd, 2010). Neurons of the lamina I are integrated in the superficial dorsal horn network, establishing abundant intersegmental, propriospinal and interlaminar contacts.

The dorsal horn neurons receive sensory information from primary afferents that respond to specific types of noxious and non-noxious stimuli (Cervero & Tattersall, 1987; Christensen & Perl, 1970). Primary afferents terminate in the dorsal horn with a distribution pattern that is determined by their sensory modality and the region of the body that they innervate (Light & Perl, 1979). Sensory information is processed by complex circuits involving excitatory and inhibitory local-circuit neurons and is transmitted to projection neurons that relay it to the brain (Cervero et al, 1979; Dickenson et al, 1997). Activity of these circuits is modulated by descending axons from several supraspinal levels (Millan, 2002) and alterations in these circuits can lead to the development and maintenance of pathological condition.

Organization of the dorsal horn

The first subdivision of the dorsal horn into laminae was done in the cat dorsal horn (Rexed, 1952; Rexed, 1954). Rexed divided the grey matter of the dorsal horn into a series of laminae based on the morphological properties of the cells. It was later verified that this scheme could be applied to other species (Harmann et al, 1988; Ralston, 1979; Steiner & Turner, 1972). The lamination proposed by Rexed remains the reference in spite of the some criticism (Woodbury et al, 2000).

The marginal layer (lamina I) and substantia gelatinosa (lamina II) form the superficial dorsal horn. The superficial dorsal horn is important for processing of the nociceptive information and its transmitting to higher levels. Lamina III and lamina IV form the nucleus proprius, which was thought to be the non-nociceptive area of the dorsal horn. However, this area has neurons that respond to noxious stimuli and project to the supraspinal levels (De Koninck et al, 1992; Ma et al, 1996). Lamina I and lamina II are characterized by the presence of numerous small neurons. Lamina II is

divided into outer and inner parts, lamina IIo and lamina IIi, respectively. Lamina IIi has a lower density of neurons than a lamina IIo. The presence of some larger neurons distinguished Lamina III from lamina II. Lamina IV has lower cell density than lamina III and contains some large neurons (Rexed, 1952).

Primary afferent fibers

Primary afferents are classified according to their peripheral targets (e.g. cutaneous, articular, visceral afferents etc.), conduction velocity (depends on the fiber diameter and myelination), response properties (sensory modalities and the intensity of stimulus necessary for activation of afferents) and neurochemical phenotype (peptide expression) (Todd, 2010). The majority of primary afferents that relay pain-related (nociceptive) information are of small diameter and have unmyelinated or thinly myelinated axons, C and Aδ fibers, respectively (Braz et al, 2005; Cervero & Tattersall, 1987). Afferents that transmit pain-related information are called nociceptors. Large myelinated afferents (Aβ type) are low-threshold mechanoreceptors that respond to touch and hair movement. Although it is considered that Aβ fibers are non-nociceptive, it has been described that some of the myelinated nociceptors conduct in the Aβ range (Djouhri & Lawson, 2004).

Primary afferents terminate in the dorsal horn with a distribution pattern that is determined by their functional class (Todd, 2010) (Fig. 1). Myelinated low-threshold afferents arborize in an area extending from lamina II (inner part) to lamina V. In turn, nociceptive and thermoreceptive Aδ and C fibers arborize in lamina I and much of lamina II. Nociceptive C fibers can be divided into two major neurochemical subpopulations: the peptidergic and the non-peptidergic. The peptidergic subpopulation expresses substance P (SP) and calcitonin gene-related peptide (CGRP) (Hunt & Rossi, 1985; Lawson et al, 1997). On the other hand, the non-peptidergic subpopulation expresses purinergic $P2X_3$ receptor, possesses fluoride-resistant acid phosphatase (FRAP) activity and binds the lectin GSA-IB4 (Hunt & Mantyh, 2001; Snider & McMahon, 1998). Non-peptidergic C fibers are associated with the skin, they innervate the epidermis (Taylor et al, 2009). Peptidergic fibers innervate deeper regions of the skin and various other tissues (Bennett et al, 1996; Perry & Lawson, 1998). To determine the relative proportions of afferents that belong to different classes is difficult. However, studies using the rat show that around 80% of cutaneous afferents are unmyelinated (Lynn, 1984) and about 50% of the lumbar dorsal root ganglion cells that form C fibers are peptidergic (Michael et al, 1997).

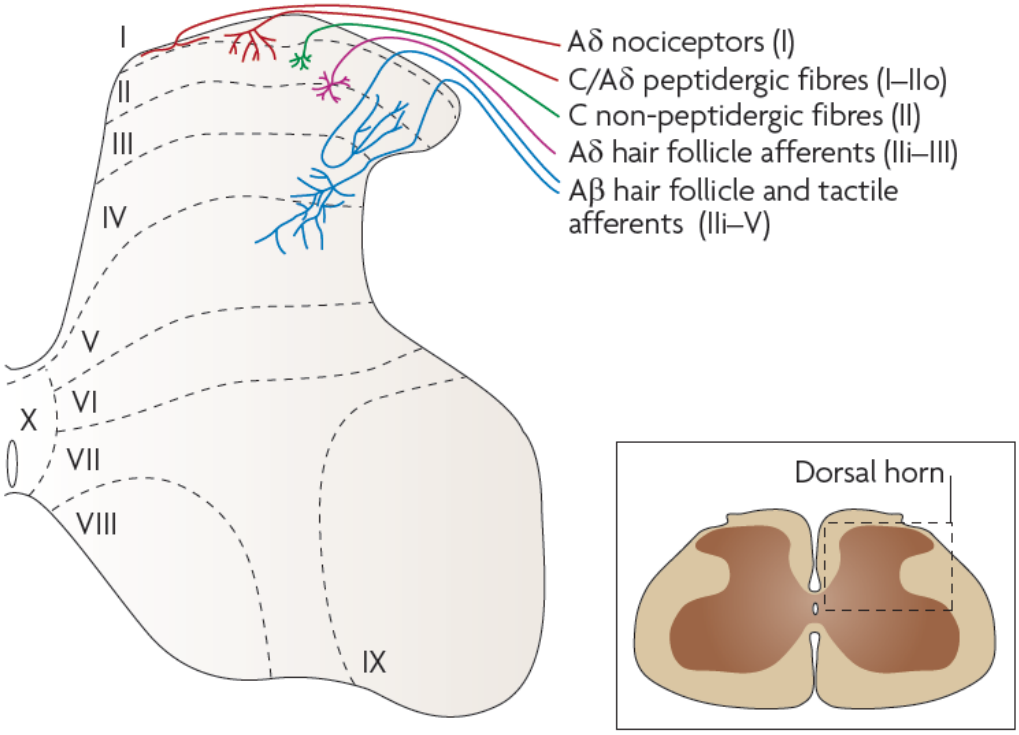

Fig. 1 – Primary afferent inputs in the dorsal horn. Primary afferents terminate in the dorsal horn in an orderly way. Myelinated Aβ tactile and Aβ hair afferents terminate mainly in lamina III–V, with some endings in lamina IIi (Hantman et al, 2004). Aδ hair afferents arborize in lamina II and lamina III, whereas Aδ nociceptors terminate mainly in lamina I (Light & Perl, 1979). C/Aδ peptidergic afferents arborize mainly in lamina I and lamina IIo, while non-peptidergic C afferents terminate in the lamina II (Lima & Coimbra, 1986). Adapted from (Todd, 2010).

The principal synaptic transmitter in all of the nociceptive fibers seems to be glutamate (De Biasi & Rustioni, 1988; Merighi et al, 1991). Like the nociceptive fibers, the non-nociceptive afferents are also glutamatergic (Battaglia & Rustioni, 1988; Merighi et al, 1991). Primary afferent axons form mostly axodendritic and some axosomatic synapses. However, primary afferents endings also form complex structure called synaptic glomerulus (Rethelyi et al, 1982; Ribeiro-da-Silva & Coimbra, 1982). Glomerulus is a complex synaptic arrangement in which a central axonal bouton of primary afferent forms synaptic contacts with several processes, including dendrites and peripheral axons.

Descending pathways

The dorsal horn receives a large number of fibers from the brainstem and other supraspinal levels. These fibers produce facilitatory or inhibitory effects on transmission

of pain-related information in the dorsal horn, designated as descending modulation (Millan, 2002). This descending modulation can operate through presynaptic and postsynaptic mechanisms, activating or inhibiting the targets. There are two main descending monoaminergic pathways: a serotonergic and noradrenergic. The serotonergic pathway originates in the nucleus raphe magnus, whereas the noradrenergic pathway originates in locus coeruleus and adjacent regions of the pons.

Dorsal horn neurons

The majority of neurons in each lamina are local-circuit neurons (interneurons), with axons that remain in the spinal cord and arborize locally. Local-circuit neurons can be classified as excitatory and inhibitory. The main neurotransmitter of the excitatory interneurons is glutamate and the inhibitory interneurons use γ-aminobutyric acid (GABA) and/or glycine. In laminae I, II and III, the proportions of GABA immunoreactive cells were 28%, 31% and 46%, respectively, whereas for glycine immunoreactive cells the proportions were 9%, 14% and 30% (Todd & Sullivan, 1990). Many inhibitory neurons co-release GABA and glycine (Yu et al, 2005) despite the identification of purely GABAergic and glycinergic interneurons (Keller et al, 2001).

Apart from local-circuit neurons, there are projection neurons that are concentrated in lamina I, virtually absent in lamina II and some of these neurons can be found in laminae III-VI. Electrophysiological studies have shown that most lamina I projection neurons are activated by noxious stimuli, although a few are activated by innocuous cooling (Bester et al, 2000; Han et al, 1998; Zhang & Giesler, 2005). Axons of the projection neurons cross the midline and ascend in the contralateral white matter, terminating in various brainstem and thalamic nuclei (Heinrich, 1992; Kuru, 1947). Anterograde and retrograde tracing studies have shown that lamina I projection neurons target the caudal ventrolateral medulla (CVLM) (Lima et al, 1991), the nucleus tractus solitarius (NTS) (Esteves et al, 1993), the parabrachial area (Pb), the periaqueductal gray (PAG) (Lima & Coimbra, 1989) and certain nuclei in the thalamus (Al-Khater et al, 2008). Most (if not all) of these neurons have axons that target more than one of these regions (Kuru, 1947). Studies of projection neurons in the lumbar segment of the rat spinal cord suggest that in lamina I there are approximately 5% of projection neurons (Polgar et al, 2004; Polgar et al, 2010b; Spike et al, 2003). Of these, 95% project to the parabrachial area, 30% to the periaqueductal gray, 25% to the nucleus tractus solitarius and 5% to the thalamus. A large number of supraspinal targets suggest that projection neurons are important for the sensory-discriminative

aspects of pain, as well as affective-motivational and autonomic aspects (Gauriau & Bernard, 2002; Gauriau & Bernard, 2004).

Despite the majority of the studies have been carried out in the rat, some information was obtained from other species. Many recent studies have been carried out in the mouse, due to the advances in molecular biological techniques. There seems to be a remarkable consistency in neuronal organization between the species despite some differences (Woodbury et al, 2000). It is important to pay attention when comparing data obtained from different species.

Lamina I

Lamina I neurons express the neurokinin 1 receptor (NK1R), which is the main target for peptide substance P. Substance P is released from the terminals of nociceptive afferents following noxious stimulation (Duggan et al, 1987). Ablation of NK1R-expressing neurons with a substance P-saporin conjugate prevents the development of hyperalgesia (Mantyh et al, 1997). Approximately 80% of lamina I projection neurons express NK1R (Al-Khater et al, 2008; Todd et al, 2000). Despite the receptor is also expressed by local-circuit neurons (Littlewood et al, 1995), its expression level is much lower than in projection neurons (Al Ghamdi et al, 2009). The effects of substance P-saporin should result from the loss of projection neurons in the lamina I.

Among the lamina I projection neurons that do not express NK1R, we can find a population of very large multipolar neurons that receive a dense inhibitory and excitatory inputs to the soma and dendrites (Polgar et al, 2008; Puskar et al, 2001). These "giant cells" are generally referred to as marginal cells of Waldeyer (Heinrich, 1992).

Several attempts have been made to classify neurons despite the fact that we still do not have a generally accepted classification. Morphological, electrophysiological, neurochemical and developmental criteria were used to classify the neurons into discrete populations (Kuru, 1947). Morphological classification is one of the most accepted. For this purpose, studies using the Golgi technique and single-cell labelling during electrophysiological recordings have been performed.

We still know little about the organization of lamina I neurons. Nevertheless, four morphological types of neurons (pyramidal, fusiform, flattened and multipolar) have been described in lamina I (Han et al, 1998; Lima & Coimbra, 1986; Zhang et al, 1996).

The classification is mainly based on soma shape and dendrite orientation. The dendrites of the most neurons remain in lamina I although some cell subtypes have dendrites that extend into deeper laminae (Fig. 2). Complete classification of the neurons requires 3-D reconstruction of their dendritic arborization (Yu et al, 2005).

Several evidences suggest a relation between the morphological characteristics of lamina I neurons and their function (Han et al, 1998; Prescott & De Koninck, 2002). A correlation between morphology and intrinsic electrophysiological properties in lamina I have been reported by Prescott and De Koninck. Some studies have found relationships between morphological classification and transmitter/receptor phenotype (Cheunsuang & Morris, 2000; Yu et al, 1999) and between receptor phenotype and afferent input (Yasaka et al, 2010).

Lamina II

There have been made many morphological analyses to classify lamina II interneurons. Perl and colleagues identified four main groups: islet, central, vertical and radial cells (Grudt & Perl, 2002; Lu & Perl, 2005). These different cells differ in their dendritic morphology. Identification of the neurotransmitter phenotype of lamina II interneurons allowed a comparison between the morphology and their functional properties (Hantman et al, 2004; Maxwell et al, 2007; Yasaka et al, 2010). Despite the relationship between morphology and the neurotransmitter phenotype, this relationship is not straightforward. All the islet cells were inhibitory, most vertical and radial cells were glutamatergic and central cells could be of either type. Limitation of these morphological studies is the presence of "unclassified" cells, about 30% (Grudt & Perl, 2002; Maxwell et al, 2007; Yasaka et al, 2010). So, we do not know whether the morphological classes identified (islet, central, vertical and radial) represent functional populations.

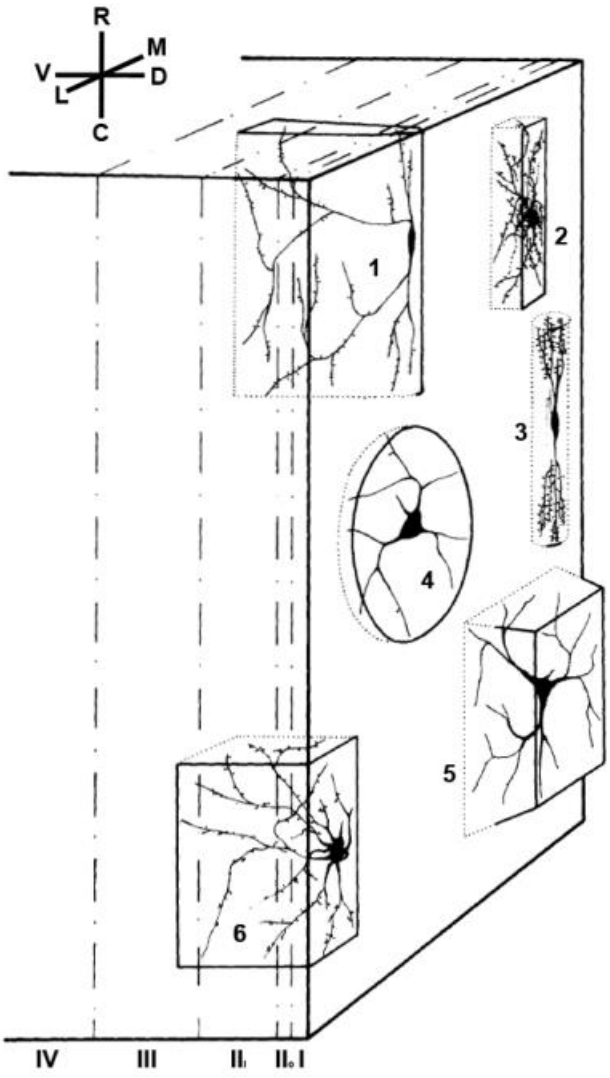

Fig. 2 – Representation of the somatodendritic features of the lamina I neurons. 1, fusiform neuron with longitudinal and ventral arbors; 2, multipolar neuron with many dendritic branches; 3, fusiform neuron with longitudinal arbors; 4, flattened neuron; 5, pyramidal neuron with dendrites in the white matter; 6, multipolar neuron with few dendritic branches, reaching lamina III. Rexed's laminae are indicated on the bottom. White matter lies to the right of the frame. R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral; M, medial. Adapted from (Lima & Coimbra, 1986).

Laminae III-IV

Lamina III and lamina IV possess both local-circuit neurons and projection neurons. Several studies have shown the involvement of these laminae in the nociception (De Koninck et al, 1992; Ma et al, 1997). Projection neurons of lamina III and lamina IV have similar supraspinal targets to the lamina I projection cells. While the dendrites of lamina I projection neurons remain within the lamina I, dendrites of the lamina III neurons have a more widespread distribution (Kuru, 1947). Large NK1R-

immunoreactive neurons whose somas are in lamina III and lamina IV with dorsally oriented dendrites provide an output to the superficial dorsal horn (Ma et al, 1997).

Synaptic circuits in the dorsal horn

The dorsal horn neurons are highly interconnected, establishing complex neuronal circuits. Although our knowledge concerning the dorsal horn circuits that process somatosensory information is still limited, some synaptic connections linking primary afferents, local-circuit neurons and projection neurons have been revealed.

It is likely that most dorsal horn neurons receive glutamatergic inputs from both afferent fibers and excitatory interneurons, as well as GABAergic and/or glycinergic inputs from inhibitory interneurons, differing only the specific subtypes and relative strength of these inputs (Todd, 2010; Todd, 2015). The axons of the inhibitory interneurons synapse with dendrites or cell bodies of other neurons (axodendritic or axosomatic synapses), underlying postsynaptic inhibition, which is the major form of inhibition in the spinal cord (Todd, 2015). However, most primary afferent axons receive axoaxonic synapses, which are the substrate for GABAergic presynaptic inhibition. Furthermore, there are dendrodendritic and dendroaxonic synapses, where the presynaptic element is the dendrite of the GABAergic interneuron (Todd, 2015).

Despite the local-circuit neurons are the main postsynaptic target for afferents, there are direct synaptic connections between primary afferents and projection neurons. Several studies have shown that projection neurons in lamina I and lamina III that express NK1R are densely innervated by peptidergic afferents, mainly substance-P containing primary afferents (Ma et al, 1997; Todd et al, 2002). These inputs constitute approximately half of the glutamatergic input to the lamina I neurons (Polgar et al, 2010a). Excitatory interneurons provide the remaining glutamatergic input to the projection neurons (Luz et al, 2010).

The dendrites of the NK1R-expressing projection neurons of the lamina III receive a moderate input from myelinated low-threshold afferents in laminae III-IV (Naim et al, 1998) and receive few contacts from unmyelinated afferents which do not contain substance P (Sakamoto et al, 1999). It is likely that these neurons receive mainly nociceptive inputs (Polgar et al, 2007). The giant lamina I projection cells apparently receive little or no primary afferent input (Polgar et al, 2008). Thus, the response to the noxious stimuli should be transmitted by polysynaptic pathways involving excitatory interneurons.

One of the functions of the excitatory interneurons that form synapses with lamina I projection neurons is to provide a polysynaptic input from low-threshold primary afferents. Recent studies have shown that lamina II vertical cells receive several inputs from myelinated low-threshold mechanoreceptors (LTMR), which suggests that these local-circuit neurons may establish connections between the afferents and lamina I projection neurons (Yasaka et al, 2014). Several studies report that the loss of inhibition should strengthen this polysynaptic low-threshold pathway, leading to allodynia in chronic pain states (Keller et al, 2007; Lu et al, 2013; Torsney & MacDermott, 2006)

Anatomical studies have permitted the identification of some patterns of connection between inhibitory interneurons and projection neurons. The NK1R-expressing projection neurons of the laminae III-IV receive numerous synapses from local inhibitory neurons that colocalize GABA and neuropeptide Y (NPY) (Polgar et al, 2011; Polgar et al, 1999) and receive few inputs from inhibitory interneurons that contain nitric oxide synthase (nNOS) and GABA (Todd, 2010). By contrast, the giant neurons of the lamina I receive a large input from nNOS-containing neurons, which provide one quarter of the GABAergic input (Puskar et al, 2001). In the dorsal horn, the balance between excitation and inhibition is essential for maintaining normal sensory function. Changes in the neuronal circuits have been implicated in the development and maintenance of the pain.

GOALS

Despite significant progress has been made concerning our understanding about the organization of the dorsal horn, we still know little about how the signal is modulated by dorsal horn networks that include local-circuit neurons (LCNs) and projection neurons (PNs). The main reason for this is the great heterogeneity of the neuronal population and the difficulty to properly identify subtypes of dorsal horn neurons in functional studies. Without a comprehensive classification it is not possible to establish the roles of different neurons within neuronal circuits.

The role of a neuron is determined by their input and output. From an anatomical point of view, it depends on the dendritic and axonal organization. The dendritic structure of lamina I neurons has been extensively studied (Lima & Coimbra, 1986; Zhang et al, 1996). However, little is known about the organization of the axonal trees. The lack of this information is in part due to the technical difficulty associated with blind filling of neurons and the unavoidable truncation of cell processes in slices, where neither the collateral nor the main axon could be followed. The use of the oblique infrared light-emitting-diode (IR-LED) illumination technique (Safronov et al, 2007; Szucs et al, 2009) in intact spinal cord solved this problem, permitting the recording, labeling and reconstruction of the complete dendritic and axonal trees.

Thus, the main goal of this study was to provide a detailed morphological description of lamina I neurons in order to improve our understanding of the synaptic circuitry. We need to understand the functioning and organization of the synaptic circuits that process sensory information in the dorsal horn, because only then we can understand plastic changes that occur during chronic pain states.

MATERIALS AND METHODS

Ethical approval

Laboratory Wistar rats (2-3 weeks old) were killed in accordance with Portuguese national guidelines (Direcção Geral de Veterinária, Ministério da Agricultura) after anesthesia with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and subsequent check for lack of pedal withdrawal reflexes. The experiments were carried out according to the guidelines laid down by the study institution's animal welfare committee (Comissão de Ética do Instituto de Biologia Molecular e Celular).

Spinal cord preparation

The vertebral column was quickly cut out and immersed in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. The vertebral column was opened from its ventral side with scissors and the lumbar spinal cord with unilateral dorsal roots was dissected. The pia mater was locally removed in the region of interest with forceps and scissors to provide access for the recording pipette. The spinal cord was glued with cyanoacrylate adhesive to a golden plate with the dorsolateral spinal cord surface facing upward and transferred to the recording chamber (Fig. 3A). All measurements were performed at 22-24°C.

Identification of lamina I neurons

Lamina I neurons were visualized through the intact white matter in the lumbar spinal cord using the oblique infrared light-emitting-diode (IR-LED) illumination technique (Safronov et al, 2007; Szucs et al, 2009). The IR-LED (L850F-02U; Marubeni Japan) was positioned outside the solution meniscus. The LED had its emission peak at 850 nm, a narrow beam of ±5° and maximum radiant intensity of 270 mW/sr. The images were obtained with a digital CCD camera (C4742-95; Hamamatsu Japan). Lamina I was identified on the basis of orientation of myelinated fibers in the dorsolateral white matter (Pinto et al, 2010). Neurons were selected in the region between the dorsolateral funiculus (lateral border) and the dorsal root entry zone

(medial border) (Fig. 3B). The white matter covering this part of lamina I is thin in young rats allowing visually controlled tight-seal recordings from the superficial neurons. Lamina I neurons could be clearly distinguished from the more deeply located lamina II neurons, the soma of which were smaller and appeared as a densely packed cell layer (Szucs et al, 2009) (Fig. 3C).

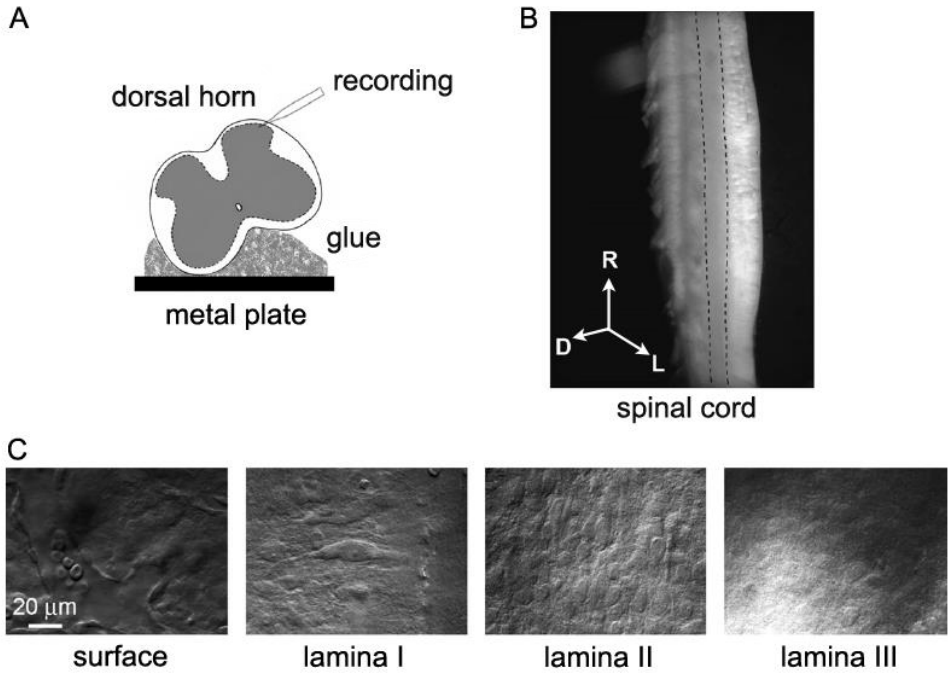

Fig. 3 – Visualization of lamina I neurons in the isolated lumbar enlargement. A. The spinal cord was glued to a golden plate with the dorsolateral surface facing upward. B. The spinal cord prepared for the visualization of neurons. Dashed lines indicate the dorsolateral region where neurons were visualized. R, rostral; D, dorsal; L, lateral. C. Visualization of spinal dorsal horn at different depths. The depth was measured from the surface of the preparation. Surface of the spinal cord at the level of the pia mater and white matter with blood vessels, erythrocytes and glial cells (depth, 0-5 µm); lamina I, large cell bodies (depth, 20-30 µm); lamina II, densely packed small cell bodies (depth, 40-80 µm); lamina III, small cell bodies (depth, 130 µm). Adapted from (Szucs et al, 2009).

Recording from lamina I neurons

Recordings from lumbar lamina I neurons were done in the whole-cell mode. The ACSF contained NaCl (115 mM), KCl (3 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), NaH_2PO_4 (1 mM), $NaHCO_3$ (25 mM) and glucose (11 mM; pH 7.4 when bubbled with 95%-5% mixture of O_2-CO_2). The pipettes were pulled from thick-walled glass (BioMedical Instruments GmbH, Zollnitz, Germany) and fire polished (resistance 4-5 MΩ). The pipette solution contained KCl (3 mM), K-gluconate (150 mM), $MgCl_2$ (1 mM), BAPTA (1 mM), HEPES (10 mM; pH 7.3 adjusted with KOH, final $[K^+]$ was 160 mM) and 0.5-1% biocytin.

Recordings were made with an EPC10-Double amplifier (HEKA Elektronik GmbH, Lambrecht/Pfalz, Germany). The signal was low-pass filtered at 2.9 kHz and sampled at 10 kHz. Offset potentials were compensated before seal formation. Liquid junction potentials were calculated and corrected for in all experiments using the compensation circuitry of the amplifier. In whole-cell mode, neurons were filled by passive diffusion of biocytin from the pipette. A current protocol consisting of depolarizing current pulses (500 msec) of increasing amplitude (10-170 pA, 10 pA increment) was repeatedly applied for 10 minutes to facilitate diffusion of biocytin from the recording pipette.

Dorsal roots were stimulated to record primary afferent inputs in lamina I neurons, as described previously (Pinto et al, 2008) (Fig. 4). Each root was inserted into a suction electrode fabricated from borosilicate glass tube. The electrodes were fire-polished to fit the size of the roots and mechanically fixed on a common holder controlled by a manipulator. An isolated pulse stimulator (2100, A-M Systems, Inc., Sequim, WA, USA) connected via a six-position switcher was used for a sequential stimulation of dorsal roots. Precautions were taken to avoid unspecific cross-stimulation of roots via neighbouring suction electrodes (Pinto et al, 2008). For this, each of six suction pipettes had its own reference electrode. Stimulation intensities used did not evoke a cross-stimulation of roots by neighboring suction electrodes.

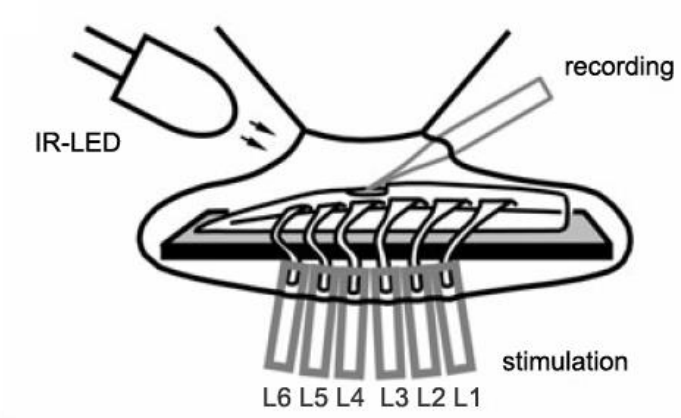

Fig. 4 – Recording from lamina I neurons in the entire spinal lumbar enlargement. Preparation of the lumbar enlargement with preserved unilateral six dorsal roots. The roots were stimulated through suction electrodes. The neurons in the lamina I were viewed using oblique illumination by IR-LED. Adapted from (Pinto et al, 2008).

To study the EPSC inputs to lamina I neurons, the roots were stimulated by 50 μs pulses of increasing amplitude to recruit Aβ and Aδ fibers. To activate Aδ and C fibers were applied 1 ms pulses. EPSCs were considered as monosynaptic if there were low failure rates and small latency variations (Pinto et al, 2010).

The conduction velocity was calculated dividing the conduction distance by the conduction time. The conduction distance included the length of the root from the

opening of the suction electrode to the dorsal root entry zone and the estimated pathway within the spinal cord. The spinal pathway was calculated as the sum of the rostrocaudal and mediolateral distances between the cell body and the corresponding dorsal root entry zone, measuring the distances on the digital micrographs. Conduction time for monosynaptic EPSC was calculated from its latency with a 1 ms allowance for synaptic transmission.

Histological processing

The spinal cord was fixed (4% paraformaldhyde; 4°C for at least 12 hours) and embedded in agar. Sagittal or transverse serial sections were prepared with a tissue slicer (VT 1000S; Leica Microsystems GmbH, Wetzlar, Germany). Individual sections were collected serially into phosphate-buffered saline (0.1 M; pH 7.0-7.2) in the wells of a 24-multiwell flat-bottomed plate. To reveal the biocytin, the free-floating sections were permeabilized with 50% ethanol, treated according to the avidin-biotinylated horseradish peroxidase method (ExtrAvidin-Peroxidase, diluted 1:1000; Sigma-Aldrich Corp.) and the histochemical reaction was completed with a diaminobenzidine chromogen reaction. Sections were serially mounted on gelatin-coated glass slides and left to dry for at least 4 hours. After rehydration, sections were counterstained with 1% toluidine blue to help in determining borders of the gray matter and laminae during reconstruction. Finally, sections were dehydrated, cleared and coverslipped with DPX (Fluka; Sigma-Aldrich Corp.).

Visualization of neurons and measurements

Photomicrographs were taken using a Primo Star microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a Guppy digital camera (Allied Vision Technologies GmbH, Stradtoda, Germany). Contrast and brightness of the photographic images used in the figures were adjusted using Adobe Photoshop software. Distances were measured on the digital micrographs.

3-D reconstruction

Complete 3-D reconstructions were done from serial sections with Neurolucida (MBF Bioscience, Williston, VT). Each section was completely traced onto the

corresponding section of a serial section data set with a ×100 (oil immersion) objective. Caliber of the digitally traced processes was continuously adjusted during the tracing, covering the video image of the labeled process. Fiber caliber units for the selected lens were automatically set by the Neurolucida, based on prior calibration. The sections were aligned and the continuing processes were connected, working always toward the section containing the soma. As a result of shrinkage, the thickness (Z dimension) of sections was 70-80% of the original. Thus, the shrinkage was corrected to be comparable to the other reconstructions. Section contours, gray matter and central canal borders were traced at the bottom level of each section. Neuronal processes that could not be connected because of partial filling, distortion of the sections or any other technical problems were deleted from the data set. The estimated percentage of these deleted processes was below 5% in all cases.

RESULTS

The main goal of this study was to provide a morphological description of lamina I neurons. For this purpose, there have been successfully reconstructed more than a dozen lamina I neurons. Complete 3-D reconstruction was performed despite some sections were distorted and damaged during processing. Some of the neurons have been sectioned in the sagittal plane and other in transverse plane (Fig. 5 and Fig. 6). The greatest extent of the axonal arborization of the LCNs is in the rostrocaudal direction, so most neurons were sectioned in the sagittal plane. Furthermore, the main axons of the PNs had large projections in the sagittal plane. Therefore, fewer connections have been made between neighboring sections.

Lamina I neurons have been classified on the basis of the somatodendritic morphology (Lima & Coimbra, 1986). There have been identified fusiform, flattened and multipolar neurons, no pyramidal neurons were found. All multipolar neurons identified in this study were local-circuit neurons. Some of these neurons presented extensive ventrally oriented dendrites that reach lamina III and possibly lamina IV (Fig. 7), whereas dendrites of other LCNs were restricted to lamina I and lamina II. However, in the sagittal sections there is a difficulty of exact delineation of laminae. Electrophysiological recordings have shown that these LCNs received monosynaptic Aβ input providing morphological evidence for their involvement in the dorsal horn pathways that carry low threshold signals to the lamina I.

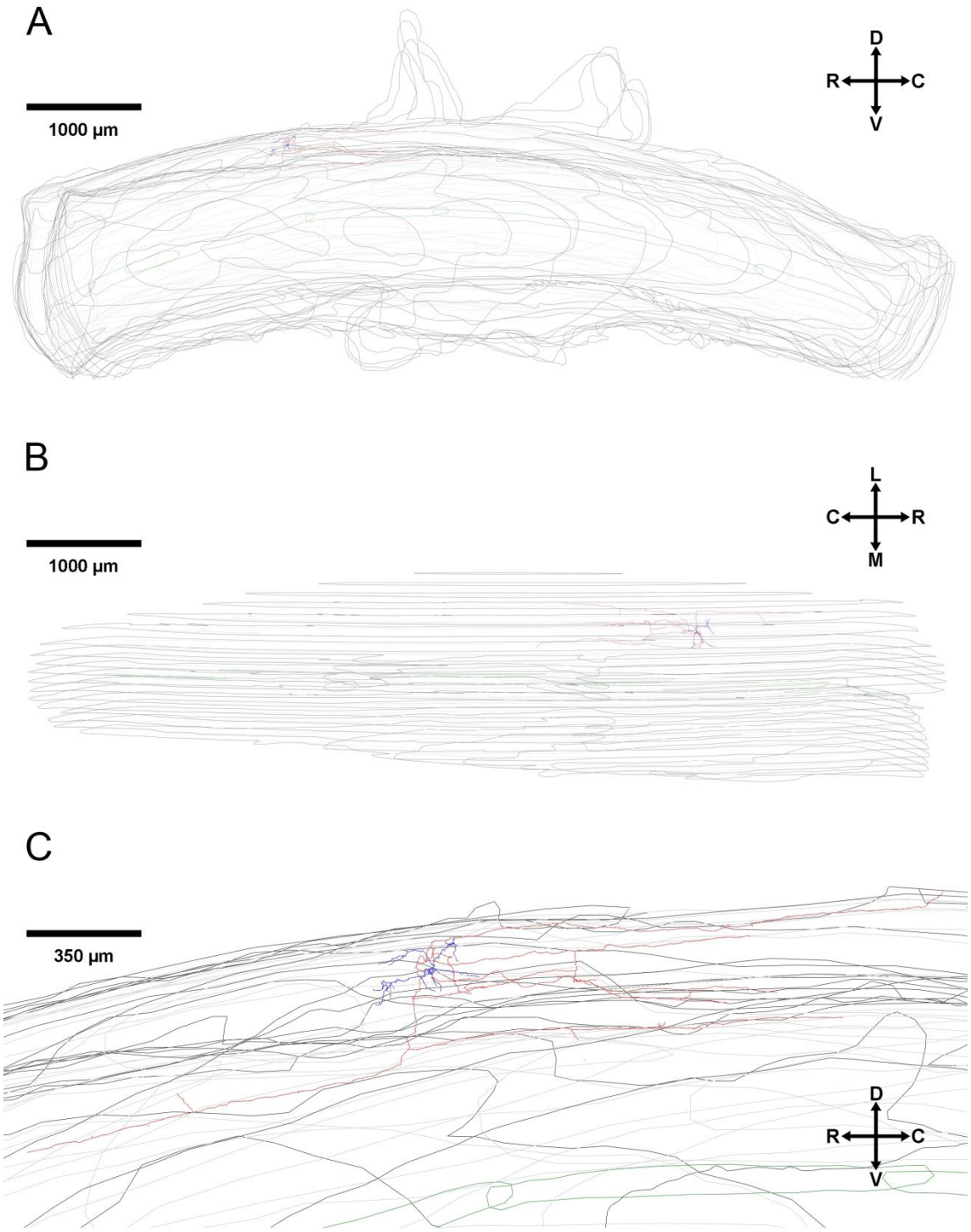

Fig. 5 – Reconstruction from sagittal serial sections. The spinal cord was sectioned in 23 sagittal sections (thickness, 100 µm). A. Sagittal, B. Horizontal view of a 3-D reconstructed neuron. First section at top is from that side of the spinal cord where the neuron was located. C. Multipolar LCN reconstructed from the overlaid and aligned serial sections. Dendrites and axons of this LCN occupy mainly laminae I-II with axons protruding into laminae III-IV. Cell body and dendrites are blue. Axons are red. Black lines indicate contour of the section. Gray lines depict the border of the gray matter. Green lines show the central canal. R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral; M, medial.

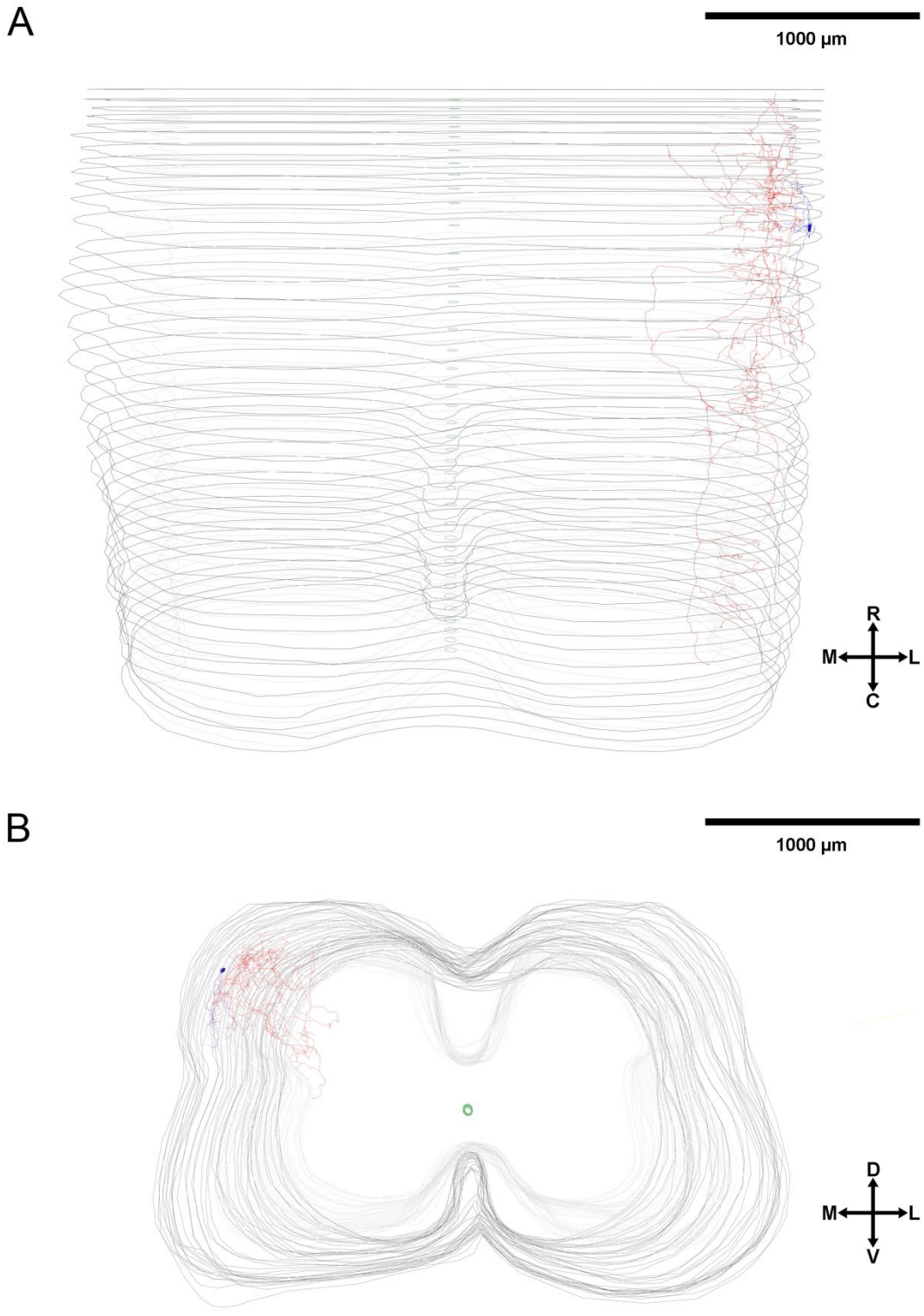

Fig. 6 – Reconstruction from transverse serial sections. A. The spinal cord was sectioned in 40 transverse sections (thickness, 70 µm). B. Transverse view of a 3-D reconstructed neuron. Mediolaterally oriented multipolar LCN with extensive axonal tree reaching laminae III-IV. Axon collaterals descend ventrally beyond the neck of the dorsal horn and some dendrites are located in the DLF. Cell body and dendrites are blue. Axons are red. Black lines indicate contour of the section. Gray lines depict the border of the gray matter. Green lines show the central canal. R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral; M, medial.

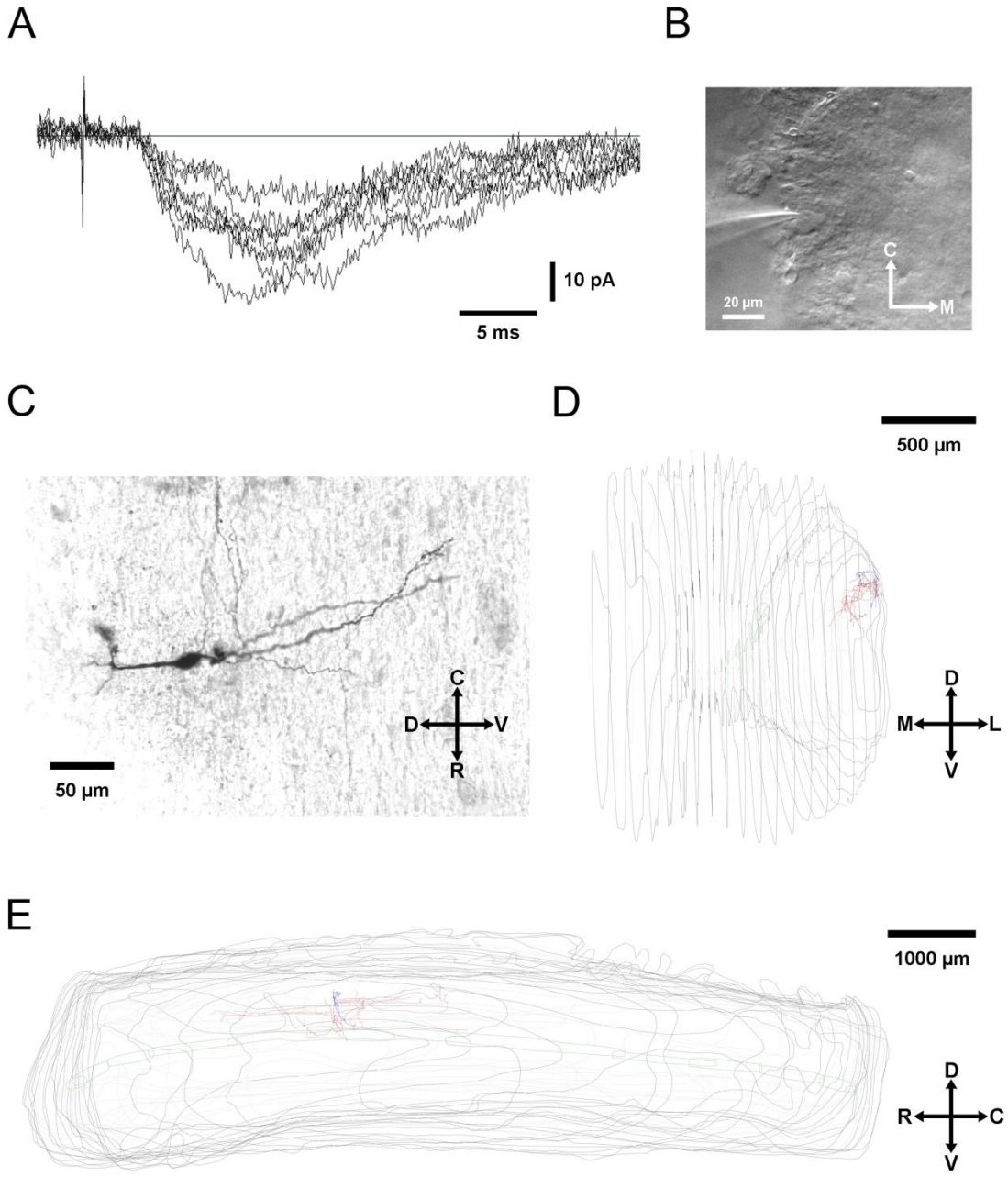

Fig. 7 – Fusiform LCN with dominating Aβ-fiber input. A. Monosynaptic Aβ-fiber-mediated EPSCs elicited in an LCN by stimulating dorsal roots with 50 µs pulses. The LCN was voltage-clamped at -70 mV. B. Image of LCN during the process of cell labeling. C. Photomicrograph of the soma, dendrites and axon branches of an LCN in a sagittal spinal cord section. D. Transverse, and E. Sagittal view of a 3-D reconstruction of the neuron. The neuron had mediolaterally oriented dendrites with ventral protrusions that reach laminae III-IV and had extensive local axon network centered on the soma. This LCN had several axon branches running caudally and rostrally in the DLF. Solid black lines indicate borders of the section; solid gray lines depict the border of the gray matter; solid green lines show the central canal; cell bodies and dendrites are blue; axons are red; R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral; M, medial.

Morphology of the axon of PN

It was possible to trace continuously the axon from the cell body to the ventral part of the dorsolateral funiculus (DLF) on the contralateral white matter. The main axon became gradually fainter when it crossed the midline in the anterior commissure (Fig. 8, inset 1). In some cases the main axon originated directly from the cell body but more frequently it originated from the stem dendrite. The branching point was frequently located after one or two dendritic branches. The axon exhibited ventromedial course although in some cases the axon formed the dorsomedial loop before take the ventromedial course toward the central canal. The main axon presented elongated swellings, resembling nodes of Ranvier (Morgan, 2001) (Fig. 8, inset 2). The main axon gave rise to one or more thin collaterals on the ipsilateral side (Fig. 8, inset 3). These collaterals presented numerous varicosities that are frequently accumulated along a short piece of the axon and appeared as slight thickenings of the axon.

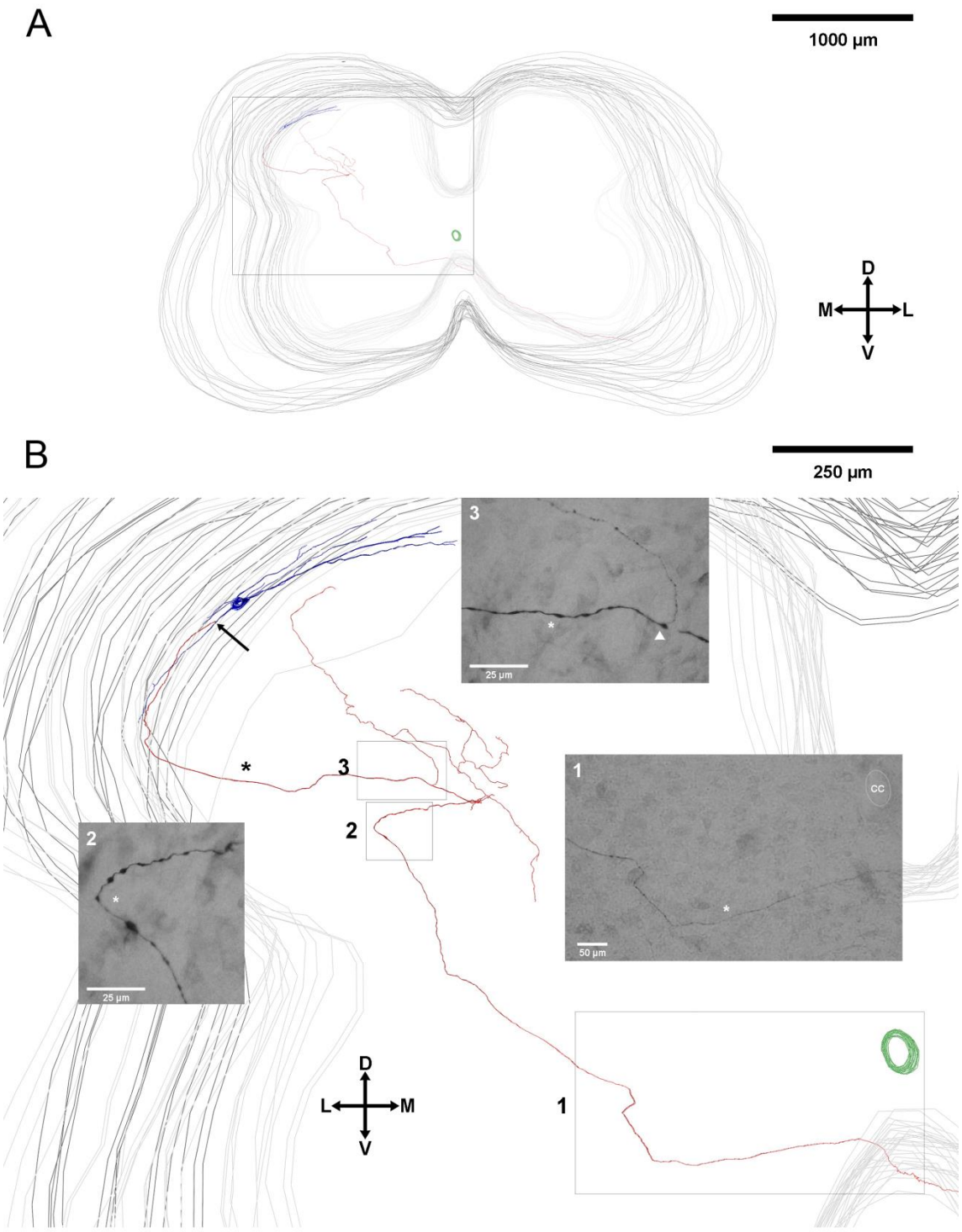

Fig. 8 – Morphological features of a PN. A. PN reconstructed from transverse serial sections. B. Flattened mediolaterally oriented lamina I PN with axon originating from primary dendrite. Arrow points on the axon origin. The main axon (asterisk) followed a contorted course and became gradually fainter when it crossed the midline (inset 1). The main axon presented numerous elongated swellings (inset 2). The axon gave rise to thin dorsal collateral on the ipsilateral side (inset 3, arrowhead). The collateral descended dorsally, giving side branches that presented a large number of varicosities. Cell body and dendrites are blue. Axons are red. Black lines indicate contour of the section. Gray lines depict the border of the gray matter. Green lines show the central canal. CC, central canal; D, dorsal; V, ventral; L, lateral; M, medial.

Morphology of the axon of LCN

The axonal tree of LCNs formed a dense local network that spanned one or even two segments rostrocaudally. In some cases, the axon reached one or both ends of the preparation and was therefore cut. Axon branches extended ventrally 100-120 µm, which correspond roughly to laminae I–II, and frequently reached the level of the notch at the neck of the dorsal horn, corresponding to laminae III–IV in lumbar segments. A few axon collaterals reached laminae below the level of the central canal (Fig. 9, red neuron). None of the LCNs had detectable axons crossing the midline and entering the contralateral white matter.

In some cases the main axon of LCNs originated from the cell body (Fig. 10, inset 1), however, in the majority of cases the main axon branched from one of the primary dendrites. The main axon had a myelinated appearance, similar to that of the main axon of projection neurons, giving several thinner branches (Fig. 10, inset 2). After two or three order branches the axon started to have varicosities. Most of the axons in the vicinity of the cell body presented a great number of varicosities (Fig. 10, inset 3). Apart from the local varicose axon network, LCNs had solitary axon branches with myelinated appearance in the neighboring white matter, including the Lissauer tract and DLF.

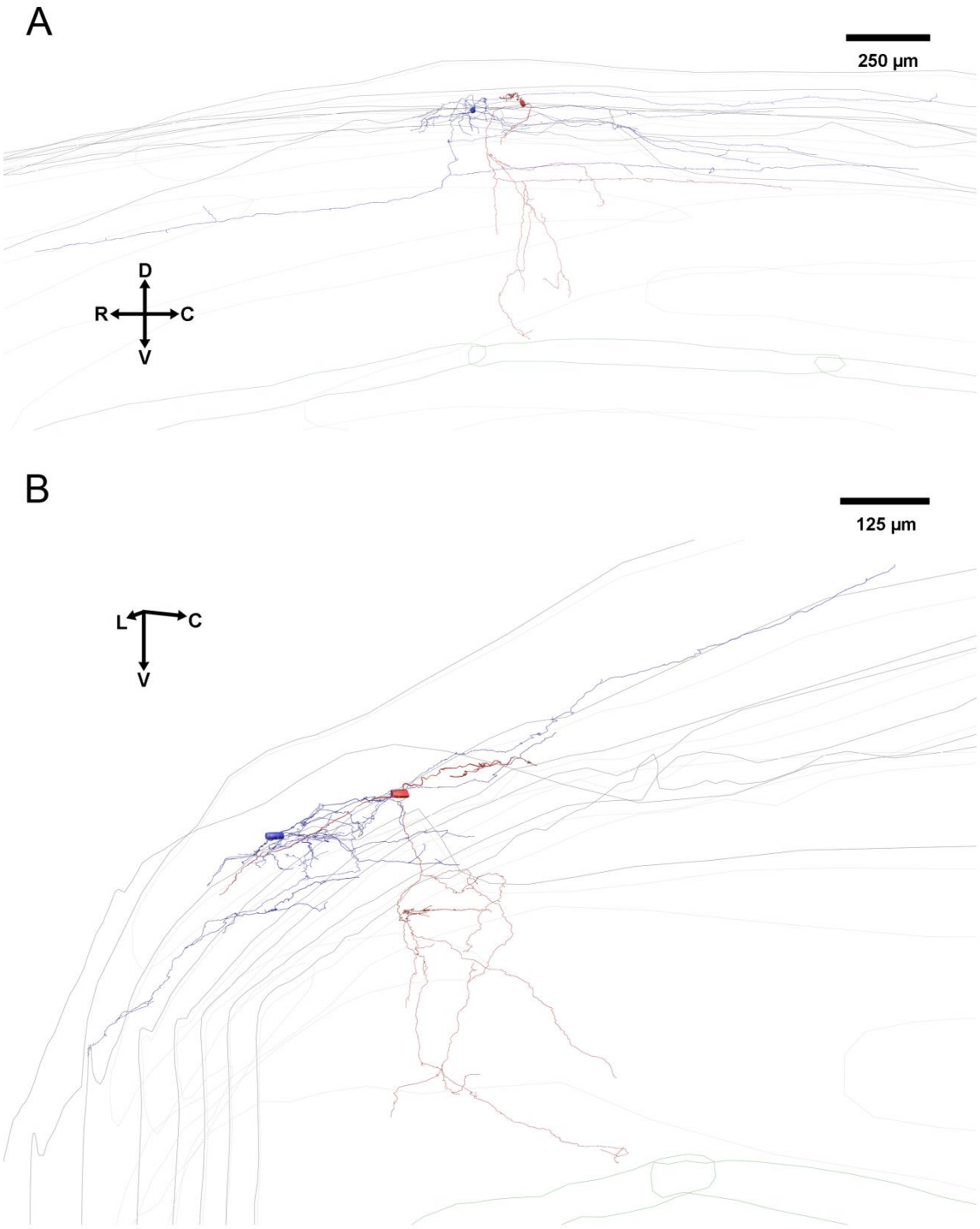

Fig. 9 – 3-D reconstruction of two LCNs filled in the same spinal cord sectioned in the sagittal plane. A. Sagittal, and B. Perspective view of a reconstructed LCNs. The blue neuron presented large axonal tree centered on the soma and long axons running along the rostrocaudal axis (rostrocaudal extent, 2838 µm). The red neuron presented long axon running ventrally and giving rise to several branches below the level of the central canal (dorsoventral extent, 857 µm). Black lines indicate contour of the section. Gray lines depict the border of the gray matter. Green lines show the central canal. R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral.

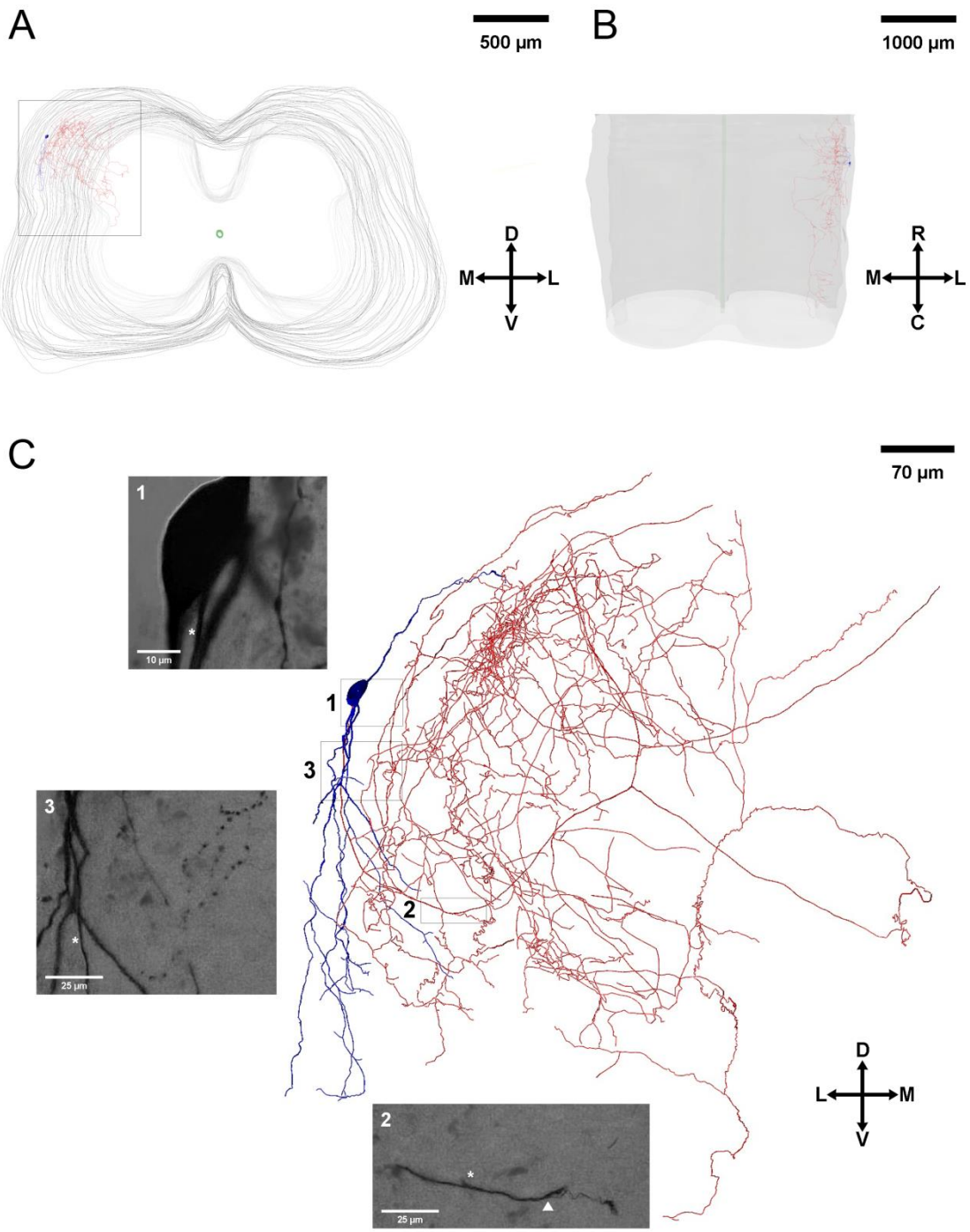

Fig. 10 – Morphological features of an LCN. A. Multipolar LCN reconstructed from transverse serial sections. B. Horizontal view of a 3-D reconstruction of the neuron. C. Mediolaterally oriented lamina I local-circuit neuron with its dense axonal tree. The main axon originated from the cell body (inset 1, asterisk) and gave rise to several primary branches (inset 2, arrowhead). After two or three order branches the axon started to have varicosities. There were found several fine terminal branches enriched with varicosities in the vicinity of the cell body (inset 3). Cell body and dendrites are blue. Axons are red. Black lines indicate contour of the section. Gray lines depict the border of the gray matter. Green lines show the central canal. R, rostral; C, caudal; D, dorsal; V, ventral; L, lateral; M, medial.

DISCUSSION

The oblique IR-LED illumination technique allowed the identification and labeling of lamina I neurons in a non-sliced tissue. The use of intact in vitro spinal cord preparation allowed complete reconstruction of these neurons. Despite the great heterogeneity of lamina I neurons it was possible to identify some common anatomical features.

In this study we used young animals (2-3 weeks old) in which visually controlled recording combined with labeling of surface neurons had a high success rate (Szucs et al, 2009). Therefore, one cannot exclude that morphological features of more mature lamina I neurons may be different. Results presented on this study are based on neurons from the lateral two-thirds of lamina I. The medial part of the dorsal horn surface (dorsal root entry zone) is rich in myelinated fibers and was excluded because of the decreased visibility in this region. Thus, we cannot exclude that medially located neurons may have different anatomical characteristics.

Lamina I neurons reconstructed in this study have been classified on the basis of their somatodendritic architecture (Lima & Coimbra, 1986). There have been identified fusiform, flattened and multipolar neurons, no pyramidal neurons were found. All multipolar neurons identified in this study were LCNs. This is in good agreement with previous studies on lamina I neurons (Szucs et al, 2010; Szucs et al, 2013).

The LCNs had extensive local axonal trees that were centered on the cell body or shifted along the rostrocaudal axis. The axonal arborizations of these LCNs occupy mainly laminae I-II, occasionally protruding into laminae III-IV. The distribution of the axonal trees suggests that the main postsynaptic target areas of these neurons are neurons in laminae I-II. However, the extensive branching of the axons that reach deeper laminae (Fig. 9, red neuron) suggests that these lamina I LCNs relay information to these areas, supporting the hypothesis of sensory information ‘‘flow’’ from lamina I to deeper laminae (Braz & Basbaum, 2009). This demonstrates the involvement of these LCNs in intralaminar and interlaminar connectivity. Detailed description of synaptic connections of these neurons is necessary for a better understanding of their roles in neuronal circuits.

A large percentage of LCNs presented in this study had long axonal branches, often with myelinated appearance, running in the DLF. These branches never crossed the

midline and ran in the rostral or caudal direction until they faded or reached the end of the spinal cord preparation. These long branches may be long propriospinal branches, indicating that these LCNs may be important on distal spinal cord regions. To confirm this hypothesis, detailed morphometric analysis of LCN axons combined with retrograde labeling from distal spinal cord regions will be necessary.

The majority of lamina I neurons described in this study had the main axons originated from primary dendrites and rarely from the cell body. The high proportion of axons with dendritic origin has been previously reported in other studies of lamina I neurons (Cheunsuang & Morris, 2000; Szucs et al, 2010; Szucs et al, 2013). Dendritic origin of the main axon may have a functional significance. Targeted synaptic input to the dendritic region between the cell body and the axon origin can effectively modulate spike generation. Determining the ultrastructure of the axon initial segment and the dendrite giving rise to it will be necessary to explain the physiological role of this anatomical variation (Duflocq et al, 2011).

Some of the interneurons presented here have prominent ventrally protruding dendrites that reach lamina III and possibly lamina IV (Fig. 7), although lamina I neurons have the bulk of their dendritic trees confined within laminae I-II (Lima & Coimbra, 1986). Electrophysiological recordings have shown that these lamina I local-circuit neurons are activated by monosynaptic Aβ primary afferent inputs. These findings show that these neurons integrate information from deeper laminae and should be treated as a separate group of lamina I LCNs, demonstrating one more time the great diversity of lamina I neurons and their functions. It would be important to know whether the interneurons identified were excitatory or inhibitory. To make it possible it would be necessary to test the immunoreactivity of these neurons.

The neurotransmitters, GABA and glycine exert strong inhibitory control over the dorsal horn neurons, including lamina I neurons (Bardoni et al, 2013; Takazawa & MacDermott, 2010). The inhibition maintains separation between touch sensitive afferent input and transmission of information about noxious stimuli to supraspinal levels. Loss of this inhibition contributes to the generation and maintenance of chronic pain (Schoffnegger et al, 2008; Torsney & MacDermott, 2006). Inhibitory interneurons are mainly GABAergic in laminae I–IIo and glycinergic neurons are prevalent in the laminae II–III (Inquimbert et al, 2007; Todd et al, 1996). Removal of glycine inhibition activates the polysynaptic excitatory pathway triggered by low-threshold mechanical input, leading to the excitation of nociceptive lamina I projection neurons (Miraucourt et al, 2009). One proposed circuit involves lamina III excitatory LCNs with monosynaptic

Aβ input and axons that reach lamina I (Schoffnegger et al, 2008). However, most axons of the lamina III neurons do not arborize extensively in the superficial dorsal horn (Schneider, 1992). Therefore, it seems that lamina II vertical cells work as interconnecting neurons that establish connections between the primary afferents and lamina I projection neurons (Grudt & Perl, 2002; Yasaka et al, 2007).

The results obtained in this study provide morphological evidence for the involvement of lamina I LCNs in the dorsal horn pathways that carry low threshold signals from deeper laminae to the lamina I. The lamina I interneurons identified can be inhibitory interneurons that suppress activation of nociceptive specific neurons in lamina I. On the other side, these interneurons can also function as excitatory interneurons providing a synaptic input from low-threshold primary afferents to the lamina I projection neurons. More extensive morphological and neurochemical characterization of these lamina I LCNs will be necessary to clarify and understand the role of these neurons in the dorsal horn synaptic circuits.

Despite significant recent progress in our knowledge about the organization of the dorsal horn circuits, our understanding of the signal processing in spinal cord is still incomplete and little is known about the changes that occur in pathological pain conditions. This is in part due to the heterogeneity of neuronal populations in each lamina and the difficulty to identify these different subtypes of neurons in functional studies. For instance, our knowledge about the organization of inhibitory circuits comes from purely morphological and immunocytochemical studies. There are few functional studies and the main reason is the difficulty to identify GABAergic and glycinergic LCNs for targeted electrophysilogical recording. The growing availability of transgenic mice that express green fluorescent protein (GFP) in neurochemically defined neuronal subpopulations should allow targeted recording from these neurons and thus reveal their physiological roles and synaptic connections (Dougherty et al, 2009; Ganley et al, 2015; Heinke et al, 2004).

The designing of classification schemes, based on combinations of morphological, immunocytochemical and electrophysiological properties are critical for understanding the organization of neuronal circuits in the dorsal horn. Although combined studies using electrophysiological recording and intracellular labeling with immunocytochemical analysis are technically demanding, they achieve a more complete picture of the neuronal organization and its function. Future investigations of the neuronal network organization are necessary to understand the nociceptive processing and the changes

that occur in the dorsal horn in chronic pain states. These findings will allow us to identify potential targets for the development of novel analgesics.

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