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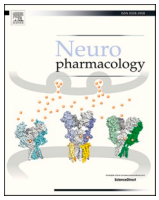

Novel aspects of signal processing in lamina I

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ABSTRACT

The most superficial layer of the spinal dorsal horn, lamina I, is a key element of the nociceptive processing system. It contains different types of projection neurons (PNs) and local-circuit neurons (LCNs) whose functional roles in the signal processing are poorly understood. This article reviews recent progress in elucidating novel anatomical features and physiological properties of lamina I PNs and LCNs revealed by whole-cell recordings in *ex vivo* spinal cord.

According to the classical point of view, the most superficial layer of the spinal dorsal horn, lamina I, relays information arising from nociceptive, pruritoceptive and thermoreceptive primary afferent fibers to the brainstem and thalamus. However, this simplistic view of lamina I as a signal integrator and transmitter overlooks two important factors. First, the major output elements, projection neurons (PNs), make up only about 5 % of the neuronal population in this layer, suggesting that the vast majority of lamina I neurons are local-circuit neurons (LCNs), which should play an important role in the signal processing. Second, the majority of lamina I PNs, like principal neurons in other CNS areas (e.g. cortical pyramidal neurons or spinal motoneurons), have local axon collaterals that target different intraspinal regions and may therefore be involved in information distribution within the spinal cord. Thus, a comprehensive understanding of the functional organisation of the neuronal network in lamina I requires detailed knowledge of the morphological characteristics of PNs and LCNs, their interconnections and the way they integrate peripheral input. It became clear that a major advance in this field could be achieved by using intracellular recordings from a spinal cord preparation, in which the entire neuronal structure, network connectivity and primary afferent input are preserved. For this purpose, more than a decade ago a novel *ex vivo* spinal cord preparation was developed for visually-guided patch-clamp recording from lamina I neurons. This article aims to review recent progress in elucidating the basic principles of the organisation of the nociceptive processing network in the superficial dorsal horn, as revealed by recordings from lamina I PNs and LCNs in the *ex vivo* spinal cord.

1. Oblique illumination imaging technique

The whole story began with the simple observation that oblique incident illumination from an optical fiber or a bare LED, which is widely used to view opaque specimens (Stephanides, 1947), allowed us to obtain high quality images of unstained cells on the sectioned surface of the tissue block from different areas of the CNS (Safronov et al., 2007). This finding was quite surprising, because it was generally believed that the single-cell imaging in a low-contrast living tissue, such as brain or spinal cord, requires application of sophisticated optical techniques, e.g., transmitted light differential interference contrast (DIC) microscopy. An another unexpected finding was that the superficial structures could be viewed at a resolution close to its theoretically

Abbreviations: DLF, dorsolateral funiculus; DRP, dorsal root potential; HO, high-output; HT, high-threshold; LCN, local-circuit neuron; LO, low-output; LSN, lateral spinal nucleus; LT, low-threshold; NK1, neurokinin 1; PAD, primary afferent depolarisation; PN, projection neuron; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter.

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predicted limit. This occurs because, unlike transmitted light DIC microscopy, the reflected incident light does not have to pass through the entire sample and is therefore much less affected by the unavoidable scattering in the tissue sample. Using a narrow-angle infrared LED it was possible, in some preparations, to visualise neuronal somata at a depth of ~200 μm (Szucs et al., 2009). However, the major advantage of the oblique LED illumination technique was that the high-resolution imaging was no longer limited to thin slice preparations, but could also be performed on unstained neurons, certain organelles (e.g. nucleus and rough endoplasmic reticulum), primary afferent fibers, as well as capillaries with erythrocytes in their lumen, on the surface of the intact spinal cord or brainstem (Szucs et al., 2009). This approach turned out to be especially advantageous for studying neurons located in the most superficial dorsal horn layer, lamina I, the lateral half of which in young rodents is virtually not covered by the white matter. These neurons could be accessed for the visually-guided patch-clamp recording in a non-sliced spinal cord preparation that preserved its entire segmental architecture and signal processing circuits (Fig. 1).

Oblique LED illumination was also used to visualise unmyelinated axons, motoneurons in the new-born rat, spinal neurons surrounding the central canal (lamina X), primary sensory neurons and satellite cells within the intact dorsal root ganglion, cerebellar neurons in the molecular layer and the Purkinje cell layer, and cells in the superficial nuclei of the brainstem (Safronov et al., 2007; Szucs et al., 2009; Krotov et al., 2017; Luz et al., 2019). The technique also proved useful for *in vivo* patch-clamp and optical recording in layer 1 of the cerebral cortex of mice (Xie et al., 2021). Later, the idea of oblique incident illumination was further used for diverse applications, e.g., phase-gradient microscopy in thick scattering samples (Ford et al., 2012) or non-invasive imaging of blood cells (Ledwig et al., 2018; McKay et al., 2020).

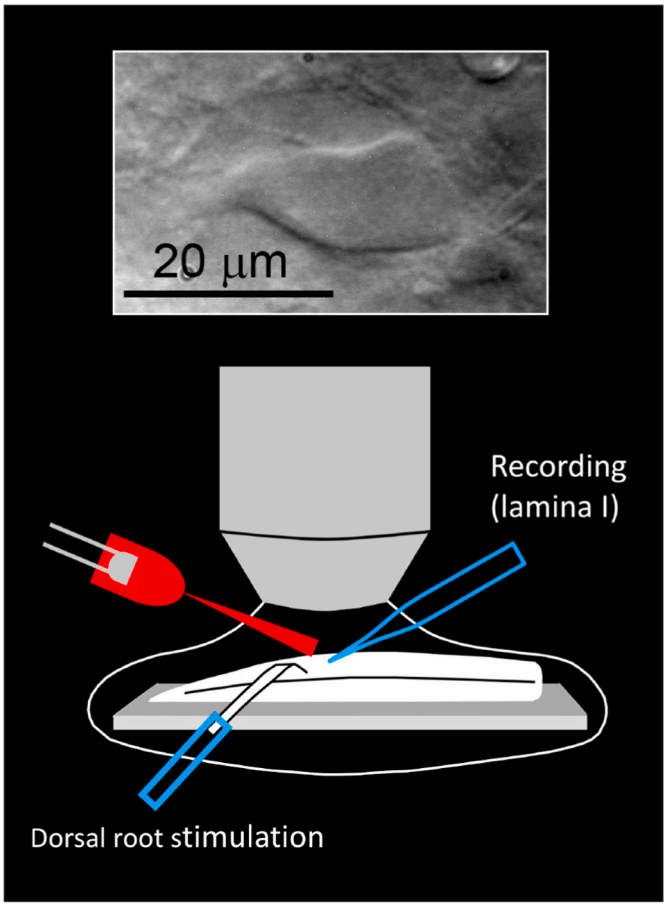

Fig. 1. Imaging of lamina I neurons. Lamina I neurons were viewed in the isolated spinal cord using oblique infrared LED surface illumination technique.

Oblique illumination produces a three-dimensional pseudo-relief image that depends on the direction of the rays, improving the resolution of some structures while rendering other invisible. The oblique light beam should be directed at right angles to the fine structures, axons or dendrites, that are to be observed. The basic principles of oblique illumination imaging have long been described for thin transparent specimens viewed by the transmitted light microscopes with a sub-stage condenser. According to classical theory, with oblique transmitted light, the objective captures positive diffraction orders from one side of the specimen and negative orders from the other side (Wayne, 2009). This causes one side of the detail to appear bright and the other side to appear dark. Cell visualisation using incident oblique LED illumination of the surface is based on the same principles of light diffraction and produces similar pseudo-relief images. However, the major advantage of oblique illumination in reflected light is that it allows high-resolution imaging of low-contrast structures in samples of unlimited thickness.

2. Development of *ex vivo* spinal cord preparation

Lamina I is an output unit of the spinal nociceptive processing network relaying information to the higher brain centres. It is the most superficial layer of the dorsal horn, which is also known as the marginal zone. With a thickness of about 20 μm, lamina I represents virtually a monolayer of scattered large neurons, most of which are nociceptive-specific and targeted by Aδ-afferents and C-afferents of somatic and visceral origin (Cervero and Iggo, 1980; Brown, 1982; Cervero and Tattersall, 1987; Willis and Coggeshall, 2004; Luz et al., 2015). A number of studies using sharp electrodes or patch pipettes for blind intracellular recording in the superficial dorsal horn provided important information about the properties of neurons in lamina I (Light et al., 1979; Woolf and Fitzgerald, 1983; Thomson et al., 1989; Lopez-Garcia and King, 1994; Han et al., 1998; Light and Willcockson, 1999; Graham et al., 2004). However, the blind approach could hardly be used for systematic recordings from loosely-packed cells in this thin layer. Alternatively, thin spinal cord slices are widely used for the patch-clamp recordings from the neurons visualised by means of the infrared DIC optics (Safronov et al., 1997; Prescott and De Koninck, 2002; Ruscheweyh and Sandkuhler, 2002; Dougherty et al., 2005; Li and Baccei, 2011). Unfortunately, lamina I neurons in the slice preparation not only lack a substantial part of their dendritic and axonal trees, but also most of their primary afferent input. Furthermore, many large neurons are damaged by slicing procedure and related thermal stress due to cooling down (from 37 °C to 0–4 °C, for slicing) and warming up the tissue (from 0 to 4 °C to 22–37 °C, slice incubation).

Thus, the *ex vivo* spinal cord preparation with preserved dorsal roots (ipsi- or contralateral) in a combination with the oblique LED illumination imaging technique offers a possibility for the visually-guided recording from intact lamina I neurons in their natural environment with well-preserved local network and multisegmental primary afferent supply. As a limitation, the isolated lumbar cord preparation lacks the tonic input from descending pathways. Nevertheless, effect of the descending tract activation on the spinal neurons could be studied in this preparation by directly stimulating preserved parts of the descending tracts (Krotov et al., 2022). The original *ex vivo* preparation for visually-guided recording (Szucs et al., 2009; Pinto et al., 2010) was then broadly modified to include the thoracic cord within the vertebral column with attached somatic and visceral nerves (Luz et al., 2015), the trigeminocervical complex with the trigeminal and cervical nerves (Kilinc et al., 2017; Luz et al., 2019; Fernandes et al., 2022b), the lumbar cord with dorsal root ganglia and associated muscle and cutaneous nerves (Li and Baccei, 2017) or saphenous nerve with hind limb skin (Hachisuka et al., 2016). Under these conditions, lamina I neurons could also be characterised on the basis of the modality of their peripheral input. As one of the next steps, the *ex vivo* spinal cord preparation can also be used for 2-photon Ca^{2+} imaging of neuronal activity at a

population level (Warwick et al., 2022).

3. Novel morphological features of lamina I neurons revealed by labelling in the *ex vivo* spinal cord

Visually-guided whole-cell recording was used to selectively fill lamina I neurons with biocytin for detailed *post hoc* 3-D reconstruction. This allowed us to reveal the almost complete structure of neurons from this previously overlooked population. The major advance of this approach was that, in addition to the well-characterised somatodendritic domain, axonal trees of lamina I PNs and LCNs could be recovered and reconstructed in detail. The resolution of the oblique LED imaging technique allowed a targeted selection of the neurons based on the shape of their somata and the structure of their proximal dendrites (Szucs et al., 2009; Luz et al., 2010). For large lamina I neurons, the multipolar somatodendritic type, more common for LCNs, could be clearly distinguished from the flattened, fusiform or pyramidal types characteristic of PNs (Lima and Coimbra, 1986; Luz et al., 2010; Szucs et al., 2010). In this way, putative LCNs and PNs could be pre-selected without retrograde labelling.

3.1. Lamina I PNs

In addition to the numerous excitatory and inhibitory interneurons (Cervero et al., 1979; Hunt et al., 1981; Dickenson et al., 1997), spinal lamina I contains a small population of supraspinally projecting neurons, most of which have axons that cross the midline and ascend in the contralateral anterolateral tract (Ramon y Cajal, 1909; Kuru, 1949; Willis and Coggeshall, 2004). In contrast to humans, only a small percentage of rodent lamina I PNs project directly to the thalamus (Al-Khater et al., 2008), while the majority of them target the caudal ventrolateral medullary reticular formation (Lima et al., 1991), the nucleus tractus solitarius (Esteves et al., 1993), the parabrachial nuclei and the periaqueductal grey (Lima and Coimbra, 1989). Many of these supraspinal targets also receive projections from the ipsilateral lamina I PNs (Lima, 1998). A number of reports have analysed the somatodendritic structure of lamina I neurons (Ramon y Cajal, 1909; Light et al., 1979; Lima and Coimbra, 1986). In the rat, they have been classified as fusiform (IA and IB), multipolar (IIA and IIB), flattened (III) and pyramidal (IV) (Lima and Coimbra, 1986), with some confusion due to the fact that in many studies flattened and multipolar cells are not distinguished from each other (Zhang et al., 1996). The somatodendritic morphology of some PNs has been suggested to be related to their pattern of supraspinal connectivity (Lima, 1998) and the modality of peripheral input (Han et al., 1998), but later studies have contradicted this view (Todd et al., 2002; Spike et al., 2003).

Since lamina I PNs are output elements of the spinal nociceptive processing network, a special attention was paid to the analysis of their axonal arborisation (Szucs et al., 2010). The first striking observation was that in more than 70 % of PNs, the main axon originates from one of the stem dendrites. The dendritic origin of the axon has been reported in lamina I neurons (Hylden et al., 1986b; Cheunsuang and Morris, 2000), preganglionic sympathetic neurons (Morgan, 2001) and diverse types of CNS neurons (Hausser et al., 1995; Martina et al., 2000; Thome et al., 2014; Wahle et al., 2022), suggesting that it may represent a morphological substrate for excitability control by perisomatic inhibition (Hodapp et al., 2022). Thus, it is possible that a vast majority of lamina I PNs can be controlled by perisomatic inhibition mediated by dorsal horn neurons or descending pain modulatory pathways.

Detailed reconstructions of the axons of lamina I PNs revealed that they give rise to four major types of ipsilateral local collaterals (Szucs et al., 2010; Kokai et al., 2022). The thickness of these unmyelinated collaterals is about a quarter of that of the main axon, and they target specific regions of the spinal cord. They have numerous *en passant* varicosities and terminal boutons that are concentrated in a short fragment of the axon. A recent review suggested that placing PNs via their axon collaterals, as new elements in the spinal pain processing circuitry, may help to refine the connectivity principles and explain some of the ambiguous actions of nociceptive networks (Browne et al., 2020). Feedforward excitation as well as feedback inhibition, via inhibitory LCNs, were suggested as possible functions of PN collaterals (Browne et al., 2020; Kokai et al., 2022).

3.2. Dorsal collaterals

Dorsal collaterals either branch directly within the dorsal grey matter (laminae I-IV) or return to the superficial layers after branching from the main axon at the level of laminae III-IV (Fig. 2A). Dorsal collaterals may also enter the Lissauer tract in the overlying white matter and have numerous varicosities (Szucs et al., 2010; Kokai et al., 2022). These varicosities are thought to form synaptic contacts with neurons in this region or with those whose dendrites reach superficial laminae. In this way, lamina I PNs could recruit neighbouring neurons, some of which may also be PNs targeting the same (or other) supraspinal areas. In this case, the activity of a PN with dorsal collateral would amplify (or extend) the supraspinally directed signal. Recent quantitative analysis has shown that dorsal collaterals are less frequent than lateral or ventral collaterals, suggesting that lamina I PNs have most of their spinal targets in adjacent segments or deeper laminae (Kokai et al., 2022).

3.2.1. Lateral collaterals

Lateral collaterals of PNs occupy the dorsal part of the lateral funiculus (DLF, Fig. 2B). The majority of these run towards the rostral segments and only a few extend caudally (Szucs et al., 2010). Lateral longitudinal projections of axons of lamina I neurons have been reported in a number of studies (Szentagothai, 1964; Molenaar and Kuypers, 1978; Yezierski et al., 1980; Verburgh et al., 1989; Craig, 1991; Petko and Antal, 2000; Dutton et al., 2006). These results suggested that lamina I neurons, via thin unmyelinated slowly conducting axons (Cervero et al., 1979; Craig and Kniffki, 1985), are involved in the process of multisegmental integration, which must be slower than the acute nociceptive signal transmission. Postsynaptic targets of PN lateral collaterals may include dorsal horn neurons whose dendrites extend into the DLF, lamina I PNs (Luz et al., 2010) and LCNs, as well as neurons in the lateral aspect of laminae III and IV. Recruitment of lamina III-IV neurons via lateral PN collaterals would imply intersegmental integration of different ascending systems. Furthermore, these PN collaterals may also target neurons in the lateral spinal nucleus (LSN). Indeed, axons presumably originating from excitatory neurons contact LSN neurons (Olave and Maxwell, 2004). It is also possible that the PN axon collaterals in the DLF do not form synaptic contacts, but act on target neurons via a mechanism of volume transmission and increase their basal excitatory tone.

3.2.2. Ventral collaterals

Ventral collaterals are the most common collateral type observed in approximately one-third of lamina I PNs (Kokai et al., 2022). They usually remain within the segment where the soma of the PN is located, and make connections with neurons in laminae V-VII (Szucs et al., 2010) (Fig. 2C). These projections can explain an increase in the frequency of spontaneous action potential-dependent EPSPs in lamina V-VII neurons observed in the presence of substance P, as well as its abolition by a cut made at the level of lamina IV (Kokai et al., 2022). Through ventral collaterals, PNs expressing the neurokinin 1 (NK1) receptor may directly or indirectly contribute to this excitation. Ventral collaterals of lamina I PNs may also mediate some polysynaptic inputs from unmyelinated afferents to the neurons in laminae III-V (Braz and Basbaum, 2009). Thus, ventral collaterals can connect parallel pain pathways originating in laminae I and V (Braz et al., 2005) and contribute to the sensory information flow from the superficial to the deeper dorsal horn (Wall, 1960; Braz and Basbaum, 2009). They may also provide excitatory drive to the last-order premotor neurons of lamina VII and therefore be

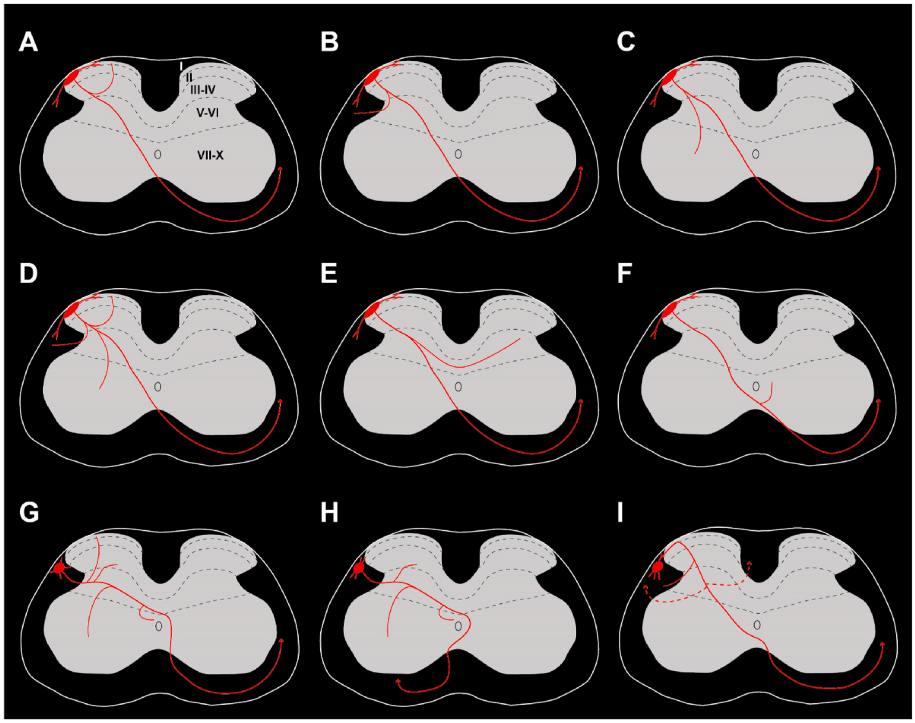

Fig. 2. Local axon collaterals of PNs. Lumbar lamina I PNs with axon collaterals of dorsal (branching in the superficial laminae) (A), lateral (running in the DLF) (B), ventral (reaching the deep dorsal horn and ventral laminae) (C), and mixed (D) types. Some PNs showed commissural collaterals that either crossed the midline in the dorsal commissure (E) or branched off the main axon after crossing the midline in the ventral commissure (F). PNs lateral to the borders of lamina I showed different axon trajectories (G–I). The main axon crossing in the dorsal commissure gave rise to the ipsilateral mixed type collaterals (G). PN with the main axon crossing in the dorsal commissure and re-crossing in the ventral commissure, ascending in the ipsilateral ALT; its ipsilateral collaterals branched in the deep dorsal horn and ventral laminae (H). PN whose main axon had two myelinated branches, one ascending in the contralateral ALT and the other in the ipsilateral DLF or dorsal funiculus; the axon collaterals remained in the DLF (I).

involved in the withdrawal reflex. In addition to the premotor apparatus, wide dynamic range neurons in deeper laminae may also be among the targets of ventral axon collaterals of lamina I PNs. Spinocerebellar and autonomic sympathetic preganglionic neurons in the thoracolumbar segments may also be targeted, suggesting a possible link between lamina I PNs and these ascending systems.

3.2.3. *Contralateral spinal collaterals*

Some collaterals of lamina I PNs reach the contralateral side of the spinal cord (Fig. 2E and F) by crossing the midline in the dorsal commissure, or originate from the main axon on the contralateral side (Kokai et al., 2022). Contralateral collaterals were observed in one-third of reconstructed PNs, but only in one of fifteen lamina I LCNs. Varicosities of these collaterals may form synapses on lamina X neurons, virtually all of which receive mono- and polysynaptic inputs from several types of thin afferents and play an important role in nociception (Krotov et al., 2019). Thus, lamina I PNs via their ventral and contralateral collaterals reaching laminae V and X, respectively, could recruit or modulate other supraspinally projecting cell populations and connect different ascending systems.

3.3. *Lamina I LCN*

The vast majority of lamina I neurons (>90 %) are excitatory or inhibitory LCNs (Cervero et al., 1979; Hunt et al., 1981; Bice and Beal, 1997; Dickenson et al., 1997; Spike et al., 2003). They were known to have axonal branches in laminae I-IV of the spinal and medullary dorsal horn (Light et al., 1979; Beal et al., 1981; Bennett et al., 1981; Hylden et al., 1986a; Cheunsuang and Morris, 2000; Li et al., 2000; Grudt and Perl, 2002). However, the first systematic study of the branching pattern and extent of LCN axons was carried out using biocytin-labelled neurons in an *ex vivo* spinal cord preparation (Szucs et al., 2013).

The main feature of the LCNs that distinguishes them from PNs is the absence of a single projection axon crossing the spinal cord midline. The cell body area of the LCNs ranges from small (mean, 212 μm^2 (Fernandes et al., 2016)) to large (mean, 351 μm^2 (Szucs et al., 2013); 434 μm^2 (Luz et al., 2015)). Small LCNs have mostly rostrocaudally oriented fusiform somatodendritic domains, whereas those of large LCNs are almost exclusively flattened or multipolar with a mediolateral orientation (Szucs et al., 2013).

Although lamina I neurons have the majority of their dendritic arbors confined within this lamina (Gobel, 1978), multipolar neurons send out prominent ventrally protruding dendrites (Lima and Coimbra, 1986) that can reach lamina III (Szucs et al., 2013; Kokai et al., 2022). Therefore, one could reasonably assume that, in addition to the source of primary afferent input common to lamina I, multipolar cells can integrate information from afferent fibers terminating in deeper laminae.

Similar to PNs, the axons of a high percentage of LCNs originate from stem dendrites, but at shorter distances from the soma. The axons of individual lamina I LCNs branch extensively over a significant rostrocaudal area of several segments and have a large number of varicosities, which can reach several thousand. The arbors of these varicosity-bearing axons overlap with the dendritic territories of lamina I neurons (Bennett et al., 1981). All LCNs have branches in the superficial dorsal laminae (I-II, occasionally extending into III and IV), whereas only about one-third of PNs have ipsilateral collaterals in the same region (Szucs et al., 2010, 2013; Kokai et al., 2022). The rostrocaudally oriented varicose axon branches of the LCNs run in the DLF and can relay information to adjacent segments (propriospinal connections) or to the LSN.

The majority of lamina I LCNs have axons surrounding their dendritic arbors, but about a third of LCNs have axons that extend to the

medial edge of the dorsal horn, while most of their dendrites are restricted to the lateral aspect of the dorsal horn (Szucs et al., 2013; Kokai et al., 2022). These neurons bridge the lateral and medial primary afferent termination zones and may provide an anatomical substrate for the lateromedial spread of information, explaining radiating lumbar pain syndromes and certain types of headache (Defrin et al., 2020).

The NK1 receptor is expressed in ~45 % of lamina I neurons (Todd et al., 1998) and most of these are likely to be excitatory neurons (Littlewood et al., 1995). Approximately 80 % of PNs express the NK1 receptor (Marshall et al., 1996; Todd et al., 2000; Al-Khater et al., 2008). Although LCNs show only a weak NK1 receptor staining (Cheunsuang and Morris, 2000; Al Ghamdi et al., 2009), about one-third of them respond to substance P application (Luz et al., 2014). However, this response is substantially smaller than in PNs suggesting a significant difference in functional NK1 receptor expression in these cell types.

3.4. PNs in the lateral white matter and within the LSN

The DLF, the intrinsic longitudinal unmyelinated axon system, contains ipsilateral collaterals of lamina I PNs (Szucs et al., 2010) and numerous axons of lamina I LCNs (Szucs et al., 2013). These axons may contact neurons that are scattered between the fibres of the DLF outside the boundaries of lamina I or are located in a continuous column ventrolateral to the lateral edge of the dorsal horn, the LSN (Gwyn and Waldron, 1968, 1969). LSN neurons respond to noxious stimuli (Olave and Maxwell, 2004), receive input from C-afferents innervating viscera (Sugiura et al., 1989) and muscles (Olave and Maxwell, 2004), and project to the brainstem, hypothalamus and thalamus (Menetrey et al., 1982; Pechura and Liu, 1986; Menetrey and Basbaum, 1987; Leah et al., 1988; Burstein et al., 1990b). Lateral collaterals of PNs and axons of lamina I LCNs form functional short propriospinal connections with neurons in rostral lamina I and LSN (Antal et al., 2016).

3D reconstruction of individually labelled neurons revealed unique dendritic features and axon trajectories of PNs in the LSN and lateral white matter. The majority of these neurons could not be classified according to Lima and Coimbra (1986). They have more stem dendrites and a greater total dendritic length giving them a “bushy” appearance. Another striking feature is the presence of numerous short spines along the dendrites and, in some cases, on the soma. These features suggest that LSN neurons and PNs outside lamina I are contacted by numerous DLF axons and are likely to integrate a large number of inputs from different sources, including descending modulatory tracts (Millan, 2002).

While axons of all PNs in lamina I cross the spinal cord midline in the anterior commissure, those of PNs in the dorsal white matter and LSN show novel axon trajectories as described below.

3.4.1. PNs that cross the midline in the posterior commissure

Neurons in this group have a spherical dendritic organisation with the soma located at the most superficial part. Dendrites project from the soma in a multipolar fashion and occupy a large part of the DLF, often branching close to the soma. These neurons show a high number of short spines. The axon starts medially from the soma or the proximal dendrites, enters the grey matter and runs in the direction of the central canal. The main axon gives ipsilateral collaterals to several laminae, including lamina X (Fig. 2G), crosses the midline in the posterior commissure and enters the medial aspect of the ventral column (Antal et al., 2016). Midline crossing in the posterior commissure rather than the anterior commissure could be due to differences in responsiveness of pathfinding axon cones to attractants and repellents secreted by the floor and roof plates, such as Netrin-1 or Sonic-Hedgehog, (Augsburger et al., 1999; Escalante et al., 2013), or it could be due to temporal differences in neuronal development that result in neurons missing the time window for normal crossing in the anterior commissure (Antal et al., 2016).

3.4.2. PNs crossing the midline twice

Some PNs have a main axon that crosses the spinal cord midline twice. The dendritic arborisation of these cells is dense, with numerous branching points and a relatively high number of spines. Some dendrites follow the dorsal grey matter curvature, mostly occupying laminae I-II, while others turn ventrally, branching in the DLF. The axon starts medially and travels towards the posterior commissure, giving ipsilateral collaterals in deeper laminae (Fig. 2H), but avoiding the superficial dorsal horn. The main axon first crosses the midline in the posterior commissure, but then loops and re-crosses in the ventral commissure to enter the ipsilateral ventral column (Antal et al., 2016). While the finding that PNs can project ipsilaterally is not new (Lima, 1998), the observation that the projection axon crosses the midline twice is striking and difficult to explain with our current knowledge of axon guidance along the midline, which is dependent on floor plate-derived attractants (Kennedy et al., 1994; Charron et al., 2003) and repellents that prevent re-crossing (Zou et al., 2000; Long et al., 2004). Ipsilateral neurons whose axons grow close to the midline, but do not cross it, transiently activate the transcription factor Zic2 (Escalante et al., 2013). PNs in the LSN and lateral white matter differ in this respect, and may be controlled by other, as yet unknown, molecular mechanisms (Antal et al., 2016).

3.4.3. PNs with bilaterally ascending axon

In the third group of PNs in the lateral white matter or in the LSN, the main axon splits into two apparently myelinated branches of equal thickness. One ascends in the ipsilateral dorsal or dorsolateral funiculus, while the other travels in the contralateral anterolateral white matter. The main axon also gives rise to a single thin ipsilateral collateral (Fig. 2I), which has numerous varicosities and descends in the DLF or Lissauer tract. Although these PNs may have a double projection, it seems more likely that the ipsilateral myelinated branch makes long propriospinal connections, while the contralateral branch ascends to the supraspinal targets. Another interesting feature of this group of PNs is that although most of their dendrites are concentrated in a conical area of the DLF, a single dendrite extends medially and gives rise to the main axon (Antal et al., 2016). Bilaterally projecting PNs have been reported for the LSN and lamina I, but the axon of those cells branched and crossed the midline at the supraspinal level (Gauriau and Bernard, 2004; Al-Khater and Todd, 2009).

4. Intrinsic firing properties of lamina I neurons

The intrinsic firing properties are important determinants of the input-output characteristics of the neurons in the superficial dorsal horn (Lopez-Garcia and King, 1994; Graham et al., 2004). In lamina I, they have been intensively studied by whole-cell recordings both in spinal cord slices (Grudt and Perl, 2002; Prescott and De Koninck, 2002; Ruscheweyh et al., 2004; Dougherty et al., 2005; Li and Baccei, 2011) and *ex vivo* spinal cord (Luz et al., 2014, 2015, 2019; Fernandes et al., 2016; Agashkov et al., 2019). These experiments have shown that intrinsic properties are diverse and play a key role in determining how lamina I neurons convert their primary afferent input into the pattern of action potential discharges. Although the main types of intrinsic firing behaviour overlap, there are some discharge profiles that are unique to specific groups of lamina I neurons.

4.1. Lamina I PNs

Discharge properties were examined in lamina I PNs identified in two ways. The first approach was retrograde tracing from the supraspinal projection centres, the parabrachial area or the periaqueductal grey (Ruscheweyh et al., 2004; Li and Baccei, 2011; Agashkov et al., 2019). The other approach was based on the *post hoc* biocytin histochemistry and analysis of the course of the major PN axon which crossed the spinal cord midline and ran toward the contralateral anterolateral tract (Grudt and Perl, 2002; Luz et al., 2014). Although the whole-cell recordings

were made under different experimental conditions, e.g., internal solutions, holding potentials or stimulation protocols, several main patterns of intrinsic PN discharge were identified; tonic, bursting, gap and delayed firing. The tonic neurons produced sustained discharge of action potentials to depolarisation steps. The bursting PNs generated one or several short bursts of spikes during the tonic discharge. Gap firing PNs showed a long interval between the first two spikes in a train at strong stimulation; at weak stimulation, the first spike appeared with a typical delay. The delayed firing neurons responded to membrane depolarisation with a delayed generation of the first spike. Since neuronal discharge is regulated by the voltage-gated inactivating A-type K^+ currents and Ca^{2+} currents, the observed pattern of PN firing is dependent on the holding membrane potential. At less negative holding potentials, the gap, bust or delayed firing could be converted into tonic firing (Ruscheweyh and Sandkuhler, 2002; Ruscheweyh et al., 2004; Luz et al., 2014), the basic pattern of which is generated by the voltage-gated Na^+ and delayed-rectifier K^+ channels (Melnick et al., 2004b). These basic firing patterns were similar to those reported for neurons in spinal laminae V-VII (Szucs et al., 2003).

In the *ex vivo* spinal cord preparation, the firing patterns of lamina I neurons were studied by injecting depolarising current pulses at potentials close to the resting membrane potentials (Luz et al., 2014; Agashkov et al., 2019). Under these conditions, the predominant pattern of PN firing is tonic. Smaller fractions of PNs show burst, delayed and gap firing. It should be noted that PNs exhibiting gap firing when stimulated from their resting membrane potential were converted to tonic by a sustained membrane depolarisation to −70 mV (Luz et al., 2014). At early stages of development (P1-P5), some PNs show an intrinsic rhythmic-burst firing, i.e. slow plateau-like potentials with accompanying bursts of spikes at zero current injection, which can provide excitatory drive to supraspinal networks (Li et al., 2015b) or, via their collaterals, set a basal excitatory tone for neurons in central laminae (Kokai et al., 2022).

A remarkable correlation between the pattern of intrinsic firing of a PN and its response to the primary afferent input has been observed in experiments using *ex vivo* rat (Agashkov et al., 2019) and mouse (Li et al., 2023) spinal cords. Two major groups of lamina I spinoparabrachial neurons were classified as low-output (LO) and high-output (HO) PNs according to their response to single-pulse dorsal root stimulation (Agashkov et al., 2019). LO-PNs, the largest group of spinoparabrachial PNs, have a firing threshold in the C-fiber range and produce on average one spike to saturating dorsal root stimulation. In contrast, HO-PNs begin to discharge in the Aδ-fiber range and the number of evoked spikes progressively increases with the stimulation reaching ten-to-twenty in the C-fiber range. Interestingly, HO-PNs make up only 19 % of the spinoparabrachial lamina I PN population, but are able to generate 69 % of its output spiking activity (Agashkov et al., 2019). From a physiological point of view, an LO-PN simply relays C-fiber input to the higher processing centres, whereas an HO-PN can detect Aδ-input and encode the strength of the C-fiber input.

According to their intrinsic firing patterns, the majority of HO-PNs are bursting, while the remainder are tonic. The intrinsic bursts of few spikes are elicited in HO-PNs either by spontaneous synaptic activity or in response to the dorsal root stimulation. The intrinsic all-or-nothing bursts allow HO-PNs to amplify their response to the nociceptive Aδ-fiber input and represent one of the mechanisms responsible for the generation of multiple spikes upon the dorsal root stimulation at the C-fiber strength. In contrast, most LO-PNs are tonic or delayed firer, and only a few produce bursts with two spikes. These intrinsic firing properties, in a combination with a smaller number of direct Aδ- and C-fiber inputs, determine the one-spike response of the LO-PNs to saturating dorsal root stimulation (Agashkov et al., 2019). Thus, intrinsic firing properties play an important role in shaping the input-output characteristics of PNs.

4.2. Lamina I LCNs

Lamina I LCNs have been identified by biocytin labelling as neurons whose axons branch densely within the ipsilateral dorsal horn without crossing the spinal cord midline (Grudt and Perl, 2002; Luz et al., 2014, 2015, 2019; Fernandes et al., 2016), as neurons that remain unstained after the retrograde tracer injection into the parabrachial area or the periaqueductal grey (Ruscheweyh et al., 2004; Li and Baccei, 2011), or as GABAergic neurons expressing enhanced green fluorescent protein (Dougherty et al., 2005). Lamina I LCNs show a wide variety of discharge properties, depending on the size of the neuron, its function as an excitatory or inhibitory interneuron, and its developmental stage. In addition to the standard patterns, such as tonic, delayed and adapting, many lamina I LCNs generate intrinsic rhythmic discharges (Li and Baccei, 2011; Luz et al., 2014; Fernandes et al., 2016) and plateau potentials (Dougherty and Hochman, 2008; Fernandes et al., 2016).

4.2.1. Large LCNs

Lamina I LCNs with a large somata (diameter, >20 μm; mean area, 351 μm^2) were studied in the *ex vivo* lumbar cord at P14-P24 (Szucs et al., 2013; Luz et al., 2014). Almost half of these LCNs show rhythmic discharge, a tonic firing of action potentials at zero current injection that persisted in the presence of blockers of fast synaptic transmission. These neurons fire rhythmically at a frequency of 2–7 Hz and the intervals between spikes can be shortened or lengthened by spontaneous EPSPs or IPSPs, respectively. Interestingly, the rhythmic firers represent the largest group of large lamina I LCNs, followed by tonic and gap firing neurons. The proportion of rhythmic LCNs is also high in the thoracic lamina I at P10-P14 (Luz et al., 2015). In the trigeminocervical complex, comprising the medullary and upper cervical C1-C2 lamina I, as well as in the C3-C4 cervical cord, two-thirds of neurons at P14-P20 generate intrinsic rhythmic discharges (Luz et al., 2019). Hence, rhythmic LCNs are widely distributed along the entire rostrocaudal axis of the spinal cord.

Such a high percentage of rhythmic LCNs could be explained by a good preservation of large neurons in the *ex vivo* spinal cord. The discharge properties of dorsal horn neurons depend on how efficiently their voltage-gated Na^+ and K^+ conductances, expressed in the axon initial segment (Safronov et al., 1997; Wolff et al., 1998; Safronov, 1999), drive the capacitive load of the dendritic tree. As shown by reconstructions, the axon and dendrites of LCNs remain intact in a non-sliced preparation (Luz et al., 2010; Szucs et al., 2013).

Large lamina I LCNs have a highly developed axonal network supplying the superficial dorsal horn of two to three lumbar segments, with a large number of varicosities, ramifications and terminals (Luz et al., 2010; Szucs et al., 2013). All LCNs that showed successful immunolabelling of varicosities expressed vesicular GABA transporter (VGAT), but not vesicular glutamate transporter 2 (VGLUT2), and were therefore inhibitory interneurons (Szucs et al., 2013). Importantly, eight of these inhibitory LCNs were rhythmic (unpublished data). Furthermore, rhythmic LCNs in the upper cervical lamina I express Pax2 (Fernandes et al., 2022b), a specific marker for inhibitory neurons (Larsson, 2017; Fernandes et al., 2022b). Thus, large rhythmic LCNs represent a group of lamina I inhibitory interneurons.

The functional role of large GABAergic rhythmic LCNs is not clear. Although their axon terminals intensely supply the superficial dorsal horn and in particular lamina I (Szucs et al., 2013; Luz et al., 2014; Kokai et al., 2022), we did not observe rhythmic IPSCs/IPSPs in thousands of lamina I neurons tested. It is therefore possible that their main function is not related to postsynaptic inhibition of lamina I neurons. Instead, rhythmic LCNs may release GABA from their nonsynaptic varicosities and induce tonic inhibition of the superficial dorsal horn neurons (Ataka and Gu, 2006; Takahashi et al., 2006; Takazawa and MacDermott, 2010) through the mechanism of volume transmission (Fig. 3). Another possibility is that rhythmic LCNs can form axo-axonic synapses and be involved in tonic presynaptic inhibition of primary afferent terminals. It

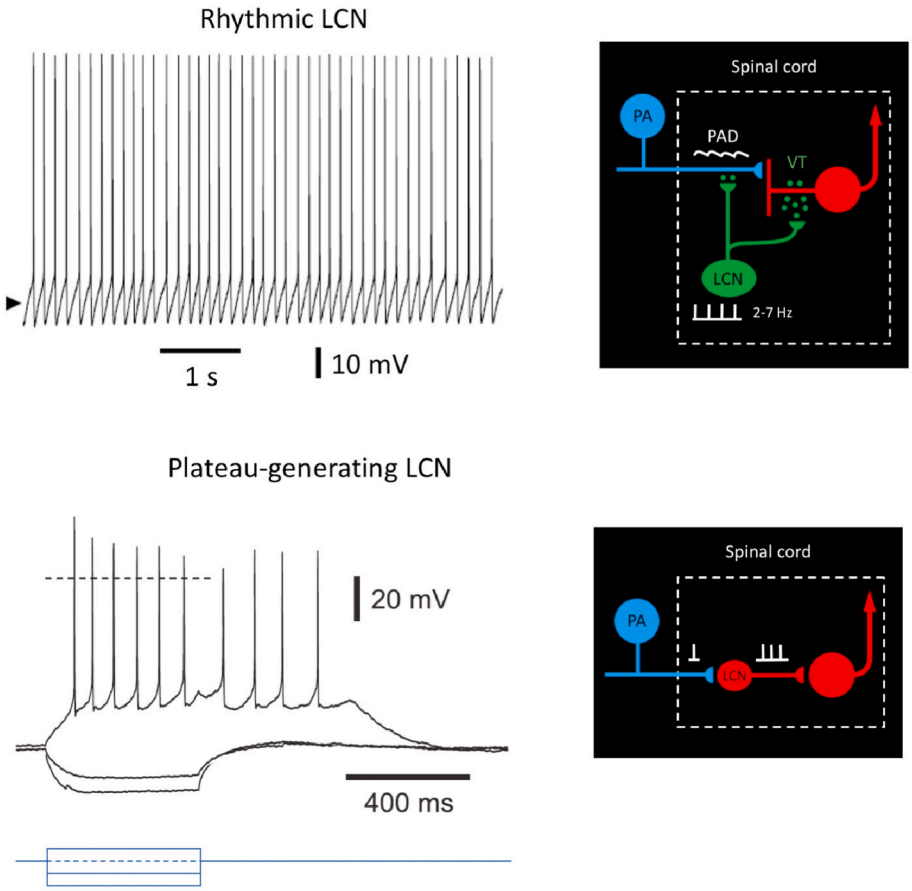

Fig. 3. Rhythmic and plateau-generating lamina I LCNs. A large LCN showing intrinsic firing at zero current injection in the presence of the mixture of CNQX, picrotoxin and strychnine (Luz et al., 2014). Right, GABAergic rhythmic LCNs may induce tonic inhibition of superficial dorsal horn neurons through the mechanism of volume transmission (VT) or provide continuous inhibition of primary afferent terminals. PA, primary afferent; PAD, primary afferent depolarisation; arrowhead, −70 mV. A small LCN generating plateau potential that supports ongoing firing (Fernandes et al., 2016). Right, plateau-generating lamina I LCNs may act as sensitive amplifiers of primary afferent input.

is known that presynaptic inhibition is caused by the primary afferent depolarisation (PAD), which is evoked by Cl^- efflux through $GABA_A$ receptors (Rudomin and Schmidt, 1999; Willis, 1999). The antidromic spread of PAD along the afferent fiber can be recorded as a dorsal root potential (DRP) (Barron and Matthews, 1938; Lloyd and McIntyre, 1949). The duration of the PAD, as estimated from our DRP recordings, is 300–400 ms (Fernandes et al., 2020). Therefore, a rhythmic GABA release from an LCN at a frequency of 2–7 Hz (Luz et al., 2014), i.e. every 140–500 ms, could be perfectly timed to provide a continuous inhibition of primary afferent terminals (Fig. 3).

A rhythmic discharge can be interrupted or enhanced by the primary afferent input (Luz et al., 2019; Fernandes et al., 2022b). Rhythmic LCNs can be silenced by injecting a small 5–20 pA current that hyperpolarises the membrane to approximately −70 mV (Luz et al., 2014). In this 'silenced' state, an LCN can respond to primary afferent input with a prolonged discharge (Luz et al., 2019). Thus, primary afferent input or hyperpolarisation can alter the functional state and processing mode of rhythmic LCNs. In addition, a third of these neurons respond to substance P (Luz et al., 2014). When applied to a 'silenced' neuron, substance P induces membrane depolarisation and ongoing action potential discharges (Luz et al., 2014). Hence, the firing behaviour of large LCNs and the efficacy of their afferent input can be regulated by different neuromodulatory systems; e.g., discharge can be 'silenced' by tonic GABAergic inhibition (Farrant and Nusser, 2005; Han and Youn, 2008), but restored by substance P release.

4.2.2. *Small LCNs*

There is a greater diversity of intrinsic firing patterns in small lamina I LCNs. These neurons are characterised by their relatively small soma diameter (<15 μm, (Fernandes et al., 2016) and area (mean, 154 μm^2 (Ruscheweyh et al., 2004); mean, 212 μm^2 (Fernandes et al., 2016)) or high input resistance (2–3 GΩ (Li and Baccei, 2011)).

During the neonatal period (P2-P3), a subpopulation of excitatory lamina I LCNs exhibits intrinsic rhythmic-burst firing, i.e. plateau potentials initiating bursts of spikes at zero current injection (Li and Baccei, 2011). These plateau potentials are triggered by persistent voltage-gated Na^+ currents previously described in superficial dorsal horn neurons (Safronov et al., 1997; Safronov, 1999; Prescott and De Koninck, 2005). Rhythmic-bursting is facilitated by Ca^{2+} influx through high-threshold (N- and L-type) voltage-gated Ca^{2+} channels. The axons of these neurons express VGLUT2 and target several areas of the spinal cord, including the superficial dorsal horn, deep dorsal horn and ventral horn. The percentage of the LCNs showing rhythmic-bursting is dramatically reduced at P9-P10, suggesting that these excitatory pacemaker neurons are likely to provide an endogenous drive necessary for the postnatal development of sensorimotor networks.

At more developed stages (P14-P26), the largest fraction (30–40 %) of smaller LCNs in both *ex vivo* spinal cord and spinal cord slices respond to a depolarising current injection with tonic firing (Ruscheweyh et al., 2004; Fernandes et al., 2016). Some of the tonic LCNs are inhibitory GABAergic interneurons (Dougherty et al., 2005). In the *ex vivo* spinal cord, the second major fraction (21 %) of small LCNs generate plateau potentials, a prolonged membrane depolarisation lasting 250 ms to 10 s

that persists after the end of the depolarising pulse (Fernandes et al., 2016). The plateau potentials are mediated by the voltage-gated L-type Ca^{2+}-channels and could evoke firing of action potentials in some neurons (Fig. 3). This pattern of activity is not seen in the samples of lamina I PNs and large LCNs and appears to be unique to the population of small neurons, some of which may be GABAergic (Dougherty and Hochman, 2008). Plateau potentials have also been observed in deep dorsal horn neurons and are thought to be an intrinsic component of the synaptically induced windup (Russo and Hounsgaard, 1996a; Morisset and Nagy, 1999, 2000; Derjean et al., 2003). In lamina I small LCNs (Fernandes et al., 2016), plateau potentials have a low threshold and are evoked by small (10–30 pA) depolarising currents. These neurons respond to primary afferent stimulation with prolonged after-discharges. Thus, plateau-generating lamina I LCNs may act as sensitive amplifiers of nociceptive afferent input and be involved in the spinal sensitisation to pain, particularly in neuropathies associated with upregulation of L-type Ca^{2+} channels (Fossat et al., 2010).

A significant fraction (13 %) of small LCNs in the *ex vivo* spinal cord generate intrinsic rhythmic discharge of action potentials at a frequency of 5–10 Hz (Fernandes et al., 2016). These rhythmic LCNs can be both excitatory and inhibitory (Li and Baccei, 2011) and can generate network activity in the dorsal horn (Sandkuhler and Eblen-Zajjur, 1994). In addition, rhythmic inhibitory LCNs may be able to release GABA for nonsynaptic activation of metabotropic $GABA_B$ receptors in a large population of neurons.

Some small LCNs also show delayed and adapting firing (Ruscheweyh et al., 2004; Fernandes et al., 2016). The latter could result from reduced expression of voltage-gated Na^+ channels compared to tonic neurons (Melnick et al., 2004a). Thus, regulation of Na^+ channel expression may be a factor that determines the firing properties of lamina I neurons.

5. Synaptic connections between lamina I LCNs and PNs

Organisation of axon terminals and dendritic trees of lamina I neurons predicts intense interactions between cells in this layer (Lima and Coimbra, 1986; Luz et al., 2010; Szucs et al., 2013; Kokai et al., 2022). Accordingly, paired recordings in the *ex vivo* spinal cord revealed both direct and indirect connections, via intercalated neurons, between large excitatory LCNs and PNs (Luz et al., 2010). The direct connections have a common structural arrangement in which the axon of an LCN makes multiple synapses on the dendrites of a PN (Fig. 4). Furthermore, the pre- and postsynaptic neurons interact via both short and long axodendritic pathways, which can extend duration of the input from a neuron. The range of latencies for the components of monosynaptic input to a PN from a neighbouring LCN is unexpectedly wide, varying from 1 to 12 ms. These latencies are determined by both pre- and postsynaptic factors. The postsynaptic delay, resulting from the electrotonic EPSC/EPSP propagation in the dendrites of a PN was estimated to be at most 4 ms and therefore alone could not explain the longest latencies observed. The presynaptic delay due to the action potential propagation in a narrow (0.2–1 μm) highly branched axon of an LCN can be considerably longer, exceeding 10 ms. Thus, the conduction time in the long recurrent axons of LCNs is the main cause of their long-latency inputs to PNs.

From the functional point of view, such a structural arrangement of the axodendritic wiring between large LCNs and PNs may play a crucial role in increasing the efficacy of synaptic transmission between spinal neurons. In many cases, synaptic release from an LCN is sufficient to excite a PN. Furthermore, an action potential in an LCN that causes transmitter release at multiple synapses can evoke in a PN a complex monosynaptic EPSP whose overlapping, but temporally dispersed, components can cause prolonged postsynaptic depolarisation and multiple discharges. This mechanism of signal amplification by a multisynaptic transmitter release can convert a spike in an LCN into the discharge of multiple spikes in a PN. Besides, the functional strength of the LCN input to a PN could be further enhanced by indirect connections via intercalated excitatory neurons (Luz et al., 2010).

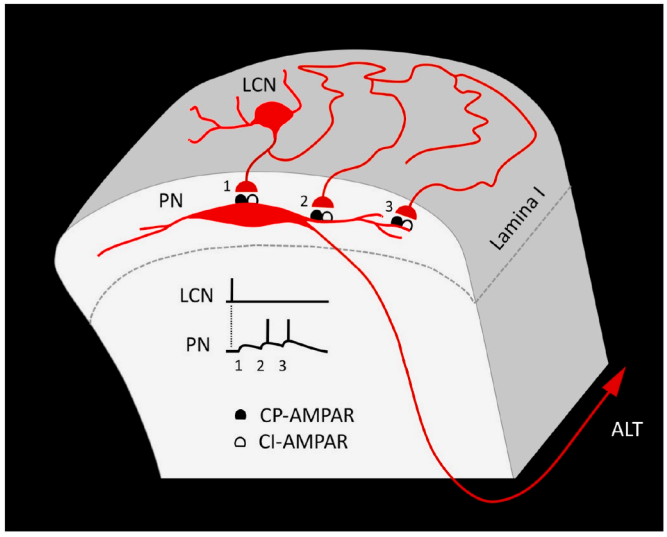

Fig. 4. Synaptic connections between lamina I large excitatory LCNs and PNs. An LCN and a PN can be connected via short and long axodendritic pathways. An action potential in an LCN can cause transmitter release at multiple synapses and evoke a complex monosynaptic EPSP and multiple discharges in a PN over a prolonged period of time. CP-AMPAR, Ca^{2+}-permeable AMPA receptors; CI-AMPAR, Ca^{2+}-impermeable AMPA receptors; ALT, anterolateral tract.

Synaptic transmission between lamina I LCNs and PNs is mediated by both Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA receptors (Luz et al., 2010). Expression of GluR2-lacking Ca^{2+}-permeable AMPA receptors and GluR2-containing Ca^{2+}-impermeable AMPA receptors in the superficial dorsal horn was demonstrated by immunocytochemistry and electron microscopy (Nagy et al., 2004; Antal et al., 2008; Larsson and Broman, 2008; Polgar et al., 2008). The former may provide Ca^{2+} influx that mediates synaptic plasticity in the superficial dorsal horn (Santos et al., 2009). The Ca^{2+}-permeable AMPA receptor-mediated plasticity in synapses of dorsal horn neurons (Santos et al., 2009) might act in a synergy with NMDA-receptor-dependent plasticity in synapses of primary afferents (Ikeda et al., 2003). The role of Ca^{2+}-permeable AMPA receptors in transmission in the superficial dorsal horn may be further enhanced in pathological conditions (Katano et al., 2008; Larsson and Broman, 2008; Vikman et al., 2008). Besides, multisynaptic wiring between individual neurons in lamina I may serve as a structural substrate for the functional plasticity associated with the activation of silent synapses (Santos et al., 2009).

Large lamina I LCNs, some of which are rhythmic, also receive monosynaptic inputs from other lamina I neurons (Luz et al., 2014). Similar to the LCN-to-PN connections, in most cases, a large LCN receives multiple active synaptic inputs from another lamina I neuron. The strength of such an input can be further increased by the activation of an intercalated excitatory neuron, which can trigger action potential firing.

Taken together, these results show that lamina I is an interconnected cell layer. In addition, lamina I neurons receive direct excitatory and inhibitory inputs from interneurons in lamina II (Lu and Perl, 2005; Graham et al., 2007; Santos et al., 2007, 2009; Kato et al., 2009; Luz et al., 2014).

6. Primary afferent supply

Lamina I neurons receive a complex pattern of primary afferent input. Afferents from several segmental dorsal roots or peripheral nerves can converge directly on individual lamina I neurons. This convergence determines the processing mode and integrative functions of the receiving neurons. The efficacy of peripheral input is subject to phasic

control by mechanisms of afferent-driven pre- and postsynaptic inhibition. Functional interactions between different classes of afferents supplying lamina I occur at segmental and heterosegmental levels.

6.1. Direct Aβ-, Aδ- and C-fiber inputs

In most cases, the dendritic tree of a lamina I neuron is restricted to the superficial dorsal horn, where fine-diameter myelinated Aδ- and unmyelinated C-afferents terminate (Lima and Coimbra, 1986; Todd, 2010). Accordingly, the vast majority of direct primary afferent inputs to lamina I PNs and LCNs are mediated by Aδ- and C-fibers (Grudt and Perl, 2002; Ruscheweyh et al., 2004; Pinto et al., 2010), which can be further divided into subclasses based on their conduction velocity and electrical activation threshold (Li et al., 2015a; Fernandes et al., 2016, 2022b).

In the *ex vivo* spinal cord, lamina I PNs are directly supplied by four types of thin afferents, termed Aδ, high-threshold Aδ (HT-Aδ), low-threshold C (LT-C) and C (Agashkov et al., 2019). The pattern of afferent supply has been shown to be an important determinant of the discharge characteristics of functional groups of lumbar PNs. Compared to LO-PNs, HO-PNs receive about twice as many direct inputs from thin afferents (5–6 monosynaptic EPSC components per cell). The most common type of input in all HO-PNs and LO-PNs is mediated by C-fibers. Aδ-fibers supply a significantly higher percentage of HO-PNs than LO-PNs. The majority of HO-PNs receive at least one LT-C-fiber input, whereas LO-PNs are almost deprived of it. Accordingly, lamina I PNs are directly contacted by boutons exhibiting immunoreactivity for VGLUT3 (Li et al., 2015a), a marker of LT-C-afferents (Seal et al., 2009) and Trpm8-expressing cold-sensitive afferents (Sharma et al., 2020). Furthermore, the overall mono- *plus* polysynaptic input in an HO-PN is about five times stronger than in an LO-PN. Thus, a higher number of mono- and polysynaptic inputs from different types of thin afferents determines the wide-range discharge characteristics of HO-PNs. In contrast, a typical one-spike firing in LO-PNs is triggered by a substantially weaker primary afferent input.

Large LCNs in the thoracic, upper cervical and medullary lamina I are also supplied by Aδ-, HT-Aδ-, LT-C- and C-afferents (Luz et al., 2015, 2019; Fernandes et al., 2022b). The input to these LCNs often consists of a monosynaptic excitatory and a polysynaptic inhibitory component. In a rhythmic LCN, a characteristic ongoing activity can be either enhanced or, in many cases, transiently interrupted by primary afferent stimulation. Since many of the large rhythmic LCNs in lamina I are GABAergic neurons supplying the superficial dorsal horn, it is possible that interruption of their firing causes a transient disinhibition of the nociceptive network and creates a window for increased excitatory drive to PNs.

Small LCNs in the lumbar lamina I receive different combinations of monosynaptic inputs from Aβ-, Aδ-, HT-Aδ- and C-afferents (Fernandes et al., 2016). The most common inputs are mediated by C- and Aδ-fibers. Of the neurons with direct inputs, about a third are supplied by only one specific type of fiber, either C or Aδ, while the remaining LCNs integrate broad inputs from several types of afferents. An interesting feature of some small non-rhythmic LCNs (tonic, adapting, delayed firing and plateau-generating) is a direct low-threshold Aβ-fiber input that appeared in a combination with C-, Aδ- and HT-Aδ-inputs. Biocytin-labelled LCNs with the Aβ-afferent supply had ventral dendrites that reached lamina III, the region where Aβ-fibers terminate (Fig. 5). However, it cannot be excluded that LCNs lacking deep ventral dendrites may also receive monosynaptic inputs from large calibre afferents whose "flame"-shaped arbors curve dorsally to reach lamina I (Szentagothai, 1964). These dorsally recurving arbors extending into lamina I appear before birth (Fitzgerald et al., 1994) and are maintained into adulthood (Boada and Woodbury, 2008).

Functionally, direct Aβ-fiber-evoked EPSPs in small LCNs are subthreshold, but induce a depolarisation that facilitates action potential initiation by higher-threshold afferents (Fernandes et al., 2016). However, Aβ-fiber input to lamina I can increase under pathological conditions, such as peripheral tissue damage (Li et al., 2015a). Thus, the wiring of Aβ-afferents to small excitatory LCNs may provide a substrate for functional plasticity, enhancing low-threshold afferent drive to the nociceptive network and contributing to the development of allodynia. On the other hand, some superficial dorsal horn LCNs that receive low-threshold afferent input are GABAergic (Daniele and MacDermott, 2009; Li and Baccei, 2019) and can supress PN activation.

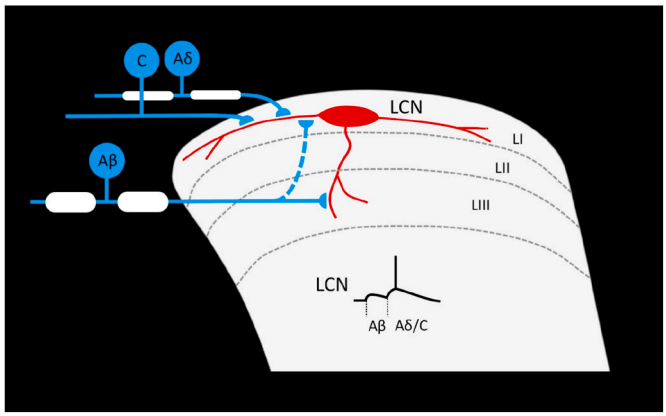

Fig. 5. Small lamina I LCNs with Aβ-afferent input. Small LCNs with long ventral dendrites can receive a combination of monosynaptic inputs from Aβ-, Aδ- and C-afferents. Direct Aβ-fiber-evoked EPSPs are typically subthreshold, but act as 'sensitizers' by inducing a depolarisation that facilitates spike initiation by higher-threshold afferents. In addition, some LCNs lacking deep ventral dendrites may also receive input from large calibre afferents whose "flame" shaped arbors curve dorsally to reach lamina I (dashed line).

In many small LCNs located in the L4 segment, Aδ-fiber inputs from the L4 and L5 roots are similar, regardless of their overall excitatory or inhibitory effect (Fernandes et al., 2016). This may be because such afferents can innervate the same peripheral point and transmit information to the same second-order neuron (Pinto et al., 2008). Both Aδ- and C-fibers can evoke a long-lasting depolarisation and after-discharges for hundreds of milliseconds in small LCNs, as previously described for deep dorsal horn neurons (King et al., 1988; Russo and Hounsgaard, 1996b; Morisset and Nagy, 2000). As most of these lamina I LCNs are tonic or plateau-generating, it can be assumed that a specific combination of synaptic and intrinsic properties is required for the generation of after-discharges. Functionally, the long-lasting discharge in lamina I LCNs may provide the excitatory drive that supports the long afferent-driven discharge in HO-PNs.

Thus, lamina I LCNs are involved in a broad integration of Aβ-, Aδ- and C-afferent input and its relay to the PNs. This is important for processing different modalities of peripheral information and may contribute to spinal sensitisation to pain.

6.2. Receptors mediating primary afferent input

Fast synaptic transmission from primary afferents to lamina I PNs and LCNs is mediated by AMPA receptors, both Ca^{2+}-permeable and Ca^{2+}-impermeable, and NMDA receptors (Bardoni et al., 1998; Dahlhaus et al., 2005; Tong and MacDermott, 2006; Vikman et al., 2008; Pinto et al., 2010; Fernandes et al., 2016, 2022b; Li and Baccei, 2017; Luz et al., 2019). A combination of these receptors determines how a neuron responds to dorsal root stimulation. In HO-PNs, AMPA receptors provide the synaptic drive for the initial phase of afferent-evoked firing, while NMDA receptors support the long-lasting discharges (Agashkov et al., 2019). In one-fifth of lamina I PNs, NMDA receptors contribute to the induction of windup (Hachisuka et al., 2018). NMDA receptors and Ca^{2+}-permeable AMPA receptors are important for the induction of functional plasticity of primary afferent and interneuron synapses (Ikeda et al., 2003; Santos et al., 2009; Li and Baccei, 2017). However, in some cases, afferent-driven EPSCs are not completely blocked by a mixture of

the AMPA/kainate and NMDA receptor blockers (Luz et al., 2019), suggesting that other fast transmitter/receptor systems, such as ATP/P2X (Bardoni et al., 1997; Burnstock and Sawynok, 2010) may also be involved.

7. Feedforward inhibition of Aδ- and C-afferent input to lamina I

Functional interactions between primary afferents are critical for spinal nociceptive processing; they occur at both pre- and postsynaptic sites. Gate control theory (Melzack and Wall, 1965) suggested that nociceptive transmission in the spinal cord depends on the balance of activity in large- *versus* small-diameter afferents. Although presynaptic inhibition has been proposed as the main mechanism controlling nociceptive input, possible contributions of previously unrecognised postsynaptic mechanisms have not been ruled out (Melzack and Wall, 1965). *Ex vivo* spinal cord recordings have revealed several forms of pre- and postsynaptic feedforward inhibition of Aδ- and C-afferent input to lamina I neurons (Luz et al., 2014; Fernandes et al., 2016, 2020, 2022a).

7.1. Afferent-driven postsynaptic inhibition

In response to dorsal root stimulation, some PNs and large LCNs exhibit low-threshold short-latency disynaptic inhibition that often precedes monosynaptic Aδ-fiber-mediated excitation (Luz et al., 2014). Functionally, this disynaptic IPSP shunts monosynaptic Aδ-fibre EPSPs elicited by weak stimuli, so that stronger stimuli recruiting more fibers are required to evoke a spike (Fig. 6). In addition, discharge in lamina I neurons is controlled by the low-threshold long-latency inhibition, which could be mediated by polysynaptic pathways (Luz et al., 2014). The long-latency inhibition has no effect on the short-latency Aδ-fibre EPSPs, allowing reliable action potential initiation, but affects the longer-latency Aδ- and C-fiber inputs. These two types of low-threshold inhibition of PNs and large LCNs can be mediated by Aβ-afferents or low-threshold mechanical Aδ-afferents innervating hair follicles (Koltzenburg et al., 1997; Djouhri et al., 1998). In contrast, inhibited EPSPs are evoked by higher-threshold Aδ- and C-afferents. Thus, afferents that sense peripheral innocuous stimuli can induce inhibition, and therefore, stronger noxious stimuli are required to reach firing threshold in lamina I PNs. These postsynaptic mechanisms can contribute to the phenomenon of pain control by innocuous cutaneous stimulation (Willis and Coggeshall, 2004).

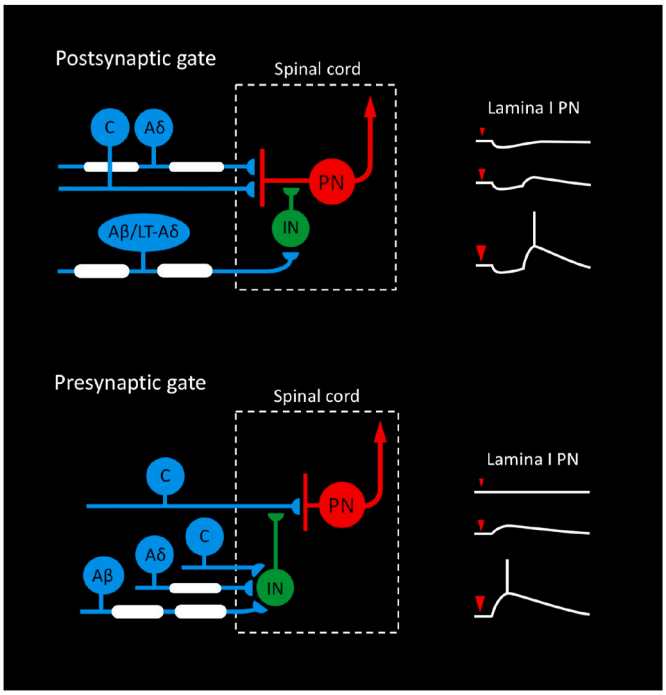

Fig. 6. Afferent-driven inhibition of lamina I neurons. Postsynaptic gate: low-threshold afferents mediate short- or long-latency disynaptic inhibition of a PN. Right, the short-latency inhibition precedes monosynaptic Aδ- and C-fiber-mediated excitation, so that stronger stimuli recruiting more afferents (red arrowheads) are required to evoke a delayed spike in a PN. The long-latency inhibition has no effect on the short-latency Aδ-fibre EPSPs, but affects the longer-latency Aδ- and C-fiber inputs (not shown). The laminar location of the inhibitory neuron (IN) is unknown. Presynaptic gate: presynaptic inhibition of C-fibers supplying lamina I neurons can be driven by Aβ-, Aδ- and C-afferents. Right, afferent-driven presynaptic inhibition controls direct C-fiber inputs. As a result, stronger stimuli recruiting more afferents (red arrowheads), or specific stimuli activating only C-fibers, are required to excite the lamina I neuron. For simplicity, one inhibitory neuron (IN) is shown for Aβ-, Aδ- and C-afferents.

Small LCNs in lamina I exhibit different forms of disynaptic inhibition mediated by Aβ-, Aδ- and C-fibers (Fernandes et al., 2016). The effect of the disynaptic IPSP depends on its latency and the firing properties of the receiving neuron. It can prevent spike initiation by weak EPSPs, regulate the discharge threshold and probability, prevent initiation of the second spike or after-discharge in small LCNs with strong inputs, and induce a transient interruption of rhythmic discharge.

The afferent-driven IPSCs/IPSPs in the superficial dorsal horn are mediated by both $GABA_A$ and glycine receptors (Yoshimura and Nishi, 1995; Luz et al., 2014). Detection of the low-threshold short-latency inhibitory inputs may critically depend on the preservation of long axon pathways in the *ex vivo* spinal cord, as many of them were recorded in the neurons after stimulation of the dorsal roots of adjacent spinal segments (Luz et al., 2014; Fernandes et al., 2016). Consistent with this, inhibition of the superficial dorsal horn neurons by cutaneous stimulation has been observed in the rat spinal cord–hind limb preparation (Lopez-Garcia and King, 1994) and in an *in vivo* mouse spinal cord (Graham et al., 2004).

The afferent-driven inhibition is one of the mechanisms that serve to uncouple lamina I PNs from the low-threshold afferent drive and thus prevent allodynia. Besides, the low-threshold afferents supply lamina I neurons via polysynaptic pathways that are normally suppressed by inhibitory circuitries (Torsney and MacDermott, 2006; Lu et al., 2013). These pathways can be opened in the presence of glycine and GABA receptor blockers, resulting in allodynia (Yaksh, 1989).

Afferent-driven postsynaptic inhibition also shapes the temporal pattern of discharge in lamina I neurons and is involved in the signal encoding in the superficial dorsal horn (Luz et al., 2014). More importantly, they can set the firing threshold in lamina I PNs, thus acting as a postsynaptic gate controlling the passage of peripheral input from high-threshold afferents to an important output unit of the spinal nociceptive network. Disinhibition of this pathway may result in hyperalgesia, since a decrease in the afferent-driven inhibition of lamina I PNs or LCNs increases the efficacy of the nociceptive input.

7.2. Afferent-driven presynaptic inhibition

Presynaptic inhibition of primary afferents is a powerful mechanism of sensory control in the spinal cord (Melzack and Wall, 1965; Rudomin and Schmidt, 1999; Willis, 1999). It is mediated by GABA released at axo-axonic synapses formed by spinal neurons at the central terminals of afferent fibers.

Presynaptic inhibition can block or reduce the amplitude of action potentials, thereby reducing transmitter release at the afferent terminals. Afferent-driven presynaptic inhibition was demonstrated for the muscle afferents supplying motoneurons and the low-threshold cutaneous afferents terminating in the deep dorsal horn (Koketsu, 1956; Eccles, 1964b; Rudomin and Schmidt, 1999; Hughes et al., 2012). However, until recently, there was no evidence for the afferent-driven inhibition of C-fibers supplying lamina I neurons, although this mechanism has long been proposed to control the passage of nociceptive information into the spinal cord (Melzack and Wall, 1965).

In recent years, significant progress has been made in this area by combining whole-cell recordings in the *ex vivo* spinal cord with the technique of selective activation of C-afferents by electrical stimulus of negative polarity (Fernandes et al., 2018), which induces anodal block of myelinated Aβ- and Aδ-afferents (Fernandes et al., 2020). These experiments have shown that C-fiber input to lamina I PNs and LCNs is subject to presynaptic inhibition mediated by Aβ-, Aδ- and C-fibers (Fig. 6). Thus, the low-threshold Aβδ-afferent-driven inhibition represents the presynaptic mechanism by which the gate control system processes nociceptive input. C-fiber-driven inhibition of C-fibers may function as another mechanism by which homotypic afferents control the flow of sensory information into the spinal cord and regulate the level of nociceptive afferent activation required to excite lamina I neurons (Fernandes et al., 2020; Krotov et al., 2023). Besides, C-fibers control Aδ-fibers that supply lamina I neurons, but have only a weak effect on Aβ-fiber input (Krotov et al., 2023).

Presynaptic inhibition of primary afferent input to lamina I neurons has a complex pattern, and results from interactions between homosegmental and heterosegmental afferents (Fernandes et al., 2020). Blocking of an EPSC component can be complete or partial, suggesting that the degree of reduction may be determined by the localisation of inhibitory sites. Important insights into a possible arrangement of the inhibitory circuitry have been obtained by analysing the ventral dendrites of the affected lamina I neurons. The structural basis for presynaptic inhibition is generally considered to be the axo-axonic synapse (Conradi, 1969; Conradi et al., 1983). Those targeting nonpeptidergic C-afferents are organised into structures called type I glomeruli, which are mostly found in the middle and ventral lamina II (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva et al., 1989; Todd, 1996). These glomeruli are involved in the inhibition of C-fibers supplying lamina I neurons whose ventral dendrites reach the middle or ventral lamina II. The glomerular mechanism can allow fine tuning of the inhibition of individual synapses and thus explain partial suppression of C-fiber input.

However, presynaptic inhibition also affected neurons with dendrites restricted to lamina I and outer lamina II (Fernandes et al., 2020), where glomeruli or simple axo-axonic synapses are rare (Ribeiro-da-Silva et al., 1989; Alvarez et al., 1993). In this case, the inhibition may be of a non-synaptic nature. The spinal branches and terminals of peptidergic and nonpeptidergic C-fibers express $GABA_A$ receptors (Knabl et al., 2008; Witschi et al., 2011; Paul et al., 2012; Lorenzo et al., 2014) that are not associated with synapses and can be activated by volume transmission. Volume transmission is less specific and may inhibit the parent branches of the C-afferent or its non-glomerular terminals. It can also result in a complete input block. In addition, non-synaptic transmission may play an important role in the afferent-driven inhibition of peptidergic C-afferents that terminate in lamina I and outer lamina II and have virtually no glomeruli or simple axo-axonic synapses (Todd, 2010). However, it cannot be excluded that rare axo-axonic synapses on peptidergic C-afferents may contribute to their presynaptic inhibition.

Thus, both synaptic and non-synaptic mechanisms are involved in the inhibition of C-afferents supplying lamina I neurons. The pattern of this presynaptic inhibition is quite heterogeneous, reflecting an involvement of lamina I neurons in processing different modalities of sensory information. Afferent-driven presynaptic inhibition can control the flow of sensory information into the spinal cord, lateral inhibition and signal transmission to selected targets (Eccles, 1964a; Lomeli et al., 1998).

8. Segmental organisation of primary afferent input

It has long been known that thin afferents entering the spinal cord terminate in the segment of root entry and one to two segments above and below (Szentagothai, 1964; Cruz et al., 1987; Traub and Mendell, 1988; Kato et al., 2004). This means that each spinal segment is supplied by thin afferents from several ipsilateral dorsal roots. Consistent with this, *ex vivo* spinal cord recordings have shown that approximately one-third of lumbar (L3 and L4) lamina II neurons receive converging monosynaptic Aδ- and C-fiber supply from two or three segmental roots (Pinto et al., 2008). Such convergence of afferents innervating one cutaneous region but entering the spinal cord via different segmental roots has been proposed to allow the formation of accurate and robust neural maps of the body surface at the spinal cord level. However, the converging input to individual lamina I neurons is broader and can originate from up to six segmental roots (Pinto et al., 2010). The strongest overall input arises from the dorsal root of the adjacent caudal segment, followed by the root of the segment in which the neuron is located. Thus, lamina I neurons act as integrators of broad heterosegmental afferent input (Fig. 7). Since afferents innervating one type of somatic structure (skin, muscles or joints) are unlikely to provide such extent of direct input, the multisegmental convergence of Aδ- and C-fibers onto lamina I neurons has been suggested to be involved in somatosensory and somatovisceral integration (Pinto et al., 2010). This was confirmed in the following studies, which analysed the supply of lamina I neurons by primary afferents from different nerves.

9. Somatovisceral convergence and referred pain

Referred pain is a phenomenon in which pain is felt at a site other than the site of the origin of the painful stimulus. It originates in the

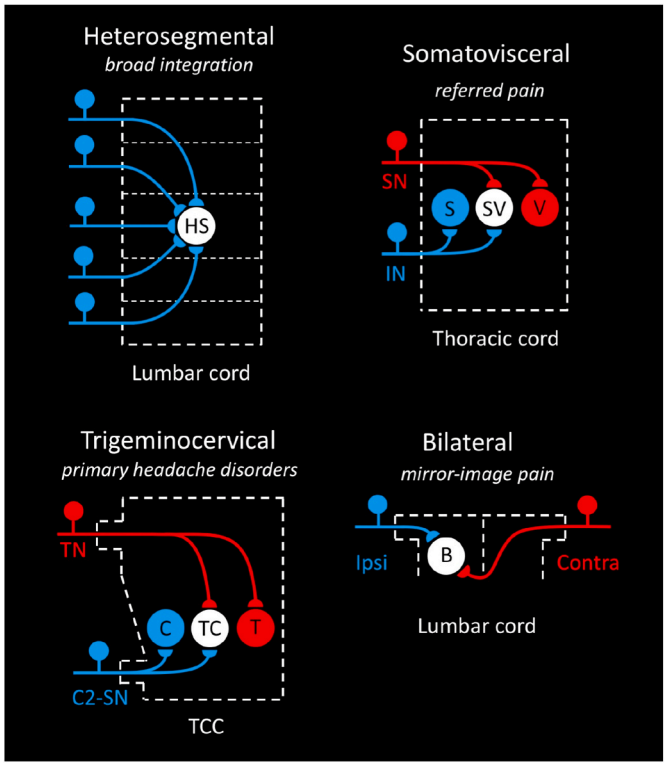

Fig. 7. Functional role of afferent convergence on lamina I neurons. Heterosegmental (HS) convergence of thin afferents onto lamina I neurons provides the basis for broad integration of primary afferent input. In the thoracic cord, direct monosynaptic convergence of thin afferents from sciatic nerve (SN, visceral) and intercostal nerve (IN, somatic) on lamina I neurons can provide a neurophysiological basis for referred pain. SV, somatovisceral convergent neuron, S and V, somatic- and visceral-specific neurons, respectively. In the trigeminocervical complex (TCC), convergence of afferents from the trigeminal nerve (TN) and the C2-spinal nerve (C2-SN) may contribute to various pain syndromes of trigeminocervical origin. TC, trigeminocervical convergent lamina I neuron, C and T, cervical- and trigeminal-specific neurons, respectively. Lumbar lamina I neurons receiving convergent bilateral input (B) may be involved in the induction of mirror-image pain.

viscera and is "referred" to the somatic tissues. Referred pain has been reported in a number of organs, and it is therefore reasonable to assume that its induction is caused by pathological alterations in the basic mechanisms that control the processing of somatic and visceral information. The most accepted theory of referred pain suggests that somatic and visceral processing pathways converge in the spinal cord (Ruch, 1961; Selzer and Spencer, 1969b). Consistently, single-unit responses of spinal second-order neurons to both visceral and somatic afferent stimulation have been recorded in *in vivo* studies (Gokin et al., 1977; Milne et al., 1981; Cervero and Tattersall, 1987; Akeyson and Schramm, 1994). Unit recordings revealed strong peripheral inputs that evoke postsynaptic action potentials, but provided little information about subthreshold events. However, a detailed analysis of somatovisceral processing in the spinal neuronal network also requires consideration of subthreshold excitatory and inhibitory inputs.

The *ex vivo* thoracic cord with preserved somatic (intercostal) and visceral (splanchnic) nerves was used for the whole-cell recording of synaptic inputs to lamina I neurons (Luz et al., 2015). This study has shown that thin somatic and visceral afferents converge directly on a group of lamina I PNs and large LCNs. Synaptic input from both afferents elicited reliable spike discharge in some PNs. Hence, lamina I is the first site in the central nervous system where somatic and visceral pathways merge together, and their convergence on the PNs provides the most direct central mechanism of somatovisceral integration (Fig. 7). However, the spinal neuronal network has a complex organisation and also includes visceral-specific, somatic-specific and inhibitory pathways.

Visceral-specific neurons, neurons receiving suprathreshold input only from the splanchnic nerve, could be further divided into three groups according to their somatic afferent supply; no input, subthreshold excitation or disynaptic inhibition driven by Aδ- and C-afferents (Luz et al., 2015). Such inhibitory somatic input to visceral-specific lamina I neurons can explain the previous observation that electrical stimulation of intercostal afferents suppresses the firing of thoracic spinal neurons evoked by noxious stimulation of visceral afferents (Qin et al., 2008). In this way, the neuronal network that processes somatovisceral information may not only evoke visceral referred pain, but also shut down visceral nociceptive pathways. The disynaptic inhibition of lamina I neurons by somatic Aδ- and C-afferents may also explain the classic observation that rather strong natural "counter-irritative" somatic stimuli are required to suppress visceral pain, whereas brushing of the hairs has little or no effect (Ruch, 1961; Selzer and Spencer, 1969a).

A number of lamina I neurons receive subthreshold inputs from both intercostal and splanchnic nerves (Luz et al., 2015) and may therefore play a critical role in pathological alterations of somatovisceral processing leading to the induction of referred pain. These inputs can undergo modality-specific sensitisation and change the processing mode of lamina I neurons after induction of functional plasticity at synapses of primary afferents (Ikeda et al., 2003) or excitatory interneurons mediating polysynaptic responses (Santos et al., 2009). This kind of plasticity can change the balance between somatic and visceral information flowing to the supraspinal centres.

Interestingly, some rhythmically firing large LCNs received inhibitory inputs from both nerves. As most of these are putative GABAergic neurons (see 4.2.1), convergent afferent-driven inhibitory input may transiently disinhibit their targets, thereby increasing the efficacy of somatovisceral excitatory drive to PNs.

Thus, functional coupling between thin afferents on lamina I neurons is the first step in the central integration of somatic and visceral inputs and can be considered as a neurophysiological substrate of referred pain. The emergence of referred pain may occur in the spinal lamina I network due to changes in the efficacy of somatic and visceral inputs.

Similarly, lamina I neurons are involved in the integration of different modalities of somatosensory input. Experiments using an *ex vivo* spinal cord preparation with preserved gastrocnemius and sural nerves showed convergence of muscle and cutaneous afferent input onto lamina I PNs (Li and Baccei, 2017).

10. Trigeminocervical processing in lamina I

The cervical C2 spinal nerve and the trigeminal nerve supply adjacent cranial areas involved in the pain associated with primary headache syndromes such as migraine, cluster headache or tension-type headache (Bartsch and Goadsby, 2003a; Goadsby et al., 2017). Nociceptive C2 afferents innervating the back of the head and neck (Bogduk, 2001; Kemp et al., 2011) terminate in the superficial dorsal horn of the C1-C3 spinal segments (Escolar, 1948; Kerr, 1961; Pfaller and Arvidsson, 1988; Garcia-Magro et al., 2018). Similarly, nociceptive trigeminal afferents supplying the front of the head, i.e., facial skin, cranial dura mater and blood vessels (Kemp et al., 2012; Edvinsson et al., 2020), enter the brainstem at the level of the pons and terminate in the trigeminal nucleus caudalis and upper cervical dorsal horn (Kerr, 1961; Pfaller and Arvidsson, 1988; Marfurt and Rajchert, 1991; Luz et al., 2019). A functional convergence of cervical and trigeminal afferents on neurons in the upper cervical dorsal horn (Kerr, 1961; Pfaller and Arvidsson, 1988; Bartsch and Goadsby, 2003b) may explain why pain in migraine and cluster headache is often perceived in the territories of both nerves. To investigate how the upper cervical lamina I neurons process trigeminal and cervical nociceptive input, the *ex vivo* brainstem-cervical cord preparation containing trigeminal and C2 spinal nerves was developed (Luz et al., 2019; Fernandes et al., 2022b).

10.1. Convergent and specific neurons

The trigeminal Aδ-, HT-Aδ-, LT-C- and C-afferents were found to project over long distances and directly supply lamina I neurons in the medullary, upper cervical (C1-C2) and, to a lesser extent, cervical C3-C4 cord (Luz et al., 2019). In the upper cervical cord, 50 % of neurons receive convergent monosynaptic input from both trigeminal and cervical afferents, with smaller fractions of neurons receiving direct input from either the C2 spinal nerve (35 %) or the trigeminal nerve (11 %) (Fernandes et al., 2022b). Considering the direct peripheral input as evidence of hard wiring to the corresponding processing lines, these neurons were classified as convergent, cervical-specific or trigeminal-specific (Fig. 7). The convergent and specific neurons were further characterised according to the strength of their overall excitatory drive and afferent-driven feedforward inhibition. In this way, ten different patterns of synaptic input from the C2 spinal and trigeminal nerves to lamina I neurons were identified. Such diversity of synaptic inputs to lamina I neurons is likely to reflect complex mechanisms of trigeminocervical processing in the superficial dorsal horn and may provide a substrate for functional plasticity.

For example, only about half of the convergent neurons receive suprathreshold drive from both nerves and are therefore excited by trigeminal and cervical afferents. A significant proportion of convergent neurons receive suprathreshold cervical but subthreshold trigeminal inputs and could function in control as transmitters of cervical information. However, the trigeminal input to these neurons may facilitate the initiation of action potentials by cervical afferents or may itself become suprathreshold if activity-dependent plasticity occurs in synapses of primary afferent fibers or excitatory interneurons. In this case, the trigeminal input enters the cervical and/or occipital processing circuitry, causing the trigeminal pain projection to the C2 spinal nerve territory.

In the cervical-specific group, some neurons receive inhibitory or no trigeminal input and thus function as elements of a pure cervical/occipital processing line. In addition, trigeminal afferent-driven inhibition of these neurons can suppress their cervical input, thereby raising the threshold for the perception of noxious stimuli. In contrast, some cervical-specific neurons receive a subthreshold polysynaptic trigeminal supply, which may facilitate discharge evoked by the cervical afferent volley.

10.2. Presynaptic interactions between trigeminal and cervical afferents

Trigeminal and cervical afferents supplying the upper cervical lamina I PNs and LCNs demonstrate a wide range of presynaptic interactions (Fernandes et al., 2022a). Recording of DRPs has shown at a population level that stimulation of the trigeminal and cervical nerves causes reciprocal induction of PAD, and that these afferent systems may be under presynaptic control. The $GABA_A$ receptor blocker picrotoxin suppressed only the initial phase of DRPs in a manner similar to that described for the rat spinal cord (Kremer and Lev-Tov, 1998; Fernandes et al., 2020). Hence, inputs to dorsal horn neurons may be subject to presynaptic inhibition mediated, at least in part, by $GABA_A$ receptors (Fernandes et al., 2020). The remaining PAD in both afferent systems can be mediated by ionotropic glutamate receptors (Russo et al., 2000; Zimmerman et al., 2019) or metabotropic glutamate or $GABA_B$ receptors.

The *ex vivo* brainstem-cervical cord preparation was used to study in detail the pattern of afferent-driven presynaptic inhibition of upper cervical lamina I neurons (Fernandes et al., 2022a). This form of inhibition was found to control monosynaptic and polysynaptic Aδ- and C-fiber-mediated inputs to a substantial population of PNs and LCNs. Suppression of the monosynaptic components of the trigeminal and cervical inputs could be complete or partial, being caused by the inhibitory action on the afferent parent or terminal branch, respectively (Fernandes et al., 2020).

In convergent neurons, monosynaptic trigeminal Aδ- and C-afferent inputs are controlled by cervical afferents and *vice versa*. The direct C2 spinal nerve inputs to the cervical-specific neurons are controlled by trigeminal afferents, whereas the direct trigeminal nerve inputs to the trigeminal-specific neurons are inhibited by cervical afferents. In this way, even if one nerve does not directly supply the neuron, it can regulate its monosynaptic input from the other nerve. Such an organisation makes it possible to control the monosynaptic input to cervical- and trigeminal-specific neurons.

The presynaptic interactions between the trigeminal afferents and the cervical afferents can be unidirectional and reciprocal. The unidirectional effect, most commonly seen in convergent neurons, can occur in either direction. This mechanism represents a trigeminocervical form of presynaptic lateral inhibition, in which one afferent inhibits another that innervates adjacent territory. The reciprocal control of trigeminal and cervical afferents occurs in convergent and cervical-specific lamina I neurons. It acts as a reciprocal form of lateral presynaptic inhibition for afferents supplying neighbouring cranial territories. Under normal conditions, these mechanisms can prevent PNs from receiving nonspecific input. Therefore, different forms of unidirectional and reciprocal presynaptic control may shape the pattern of trigeminal and cervical afferent supply of neurons in upper cervical lamina I.

Afferent-driven presynaptic inhibition provides feedforward control of peripheral drive to lamina I neurons, regulating their convergent and cervical- or trigeminal-specific processing modes. Consequently, its disruption may contribute to the pain referral to cervical and trigeminal nerve territories observed in patients with primary headache syndromes.

11. Lumbar lamina I and mirror-image pain

Mirror-image pain, a mysterious phenomenon in which pain is perceived to originate from the body region contralateral to the actual site of injury, is caused by changes in the nociceptive network that controls the functional lateralisation of primary afferent input. It is associated with many clinical syndromes and is characterised by allodynia and hyperalgesia (Coderre and Melzack, 1991; Coderre et al., 1993; Huang and Yu, 2010; Konopka et al., 2012). This type of pain is thought to be caused by changes in signalling pathways, such as glial or neurochemical pathways, that connect the two sides of the body (Coderre et al., 1993; Koltzenburg et al., 1999; Milligan et al., 2003; Huang and Yu, 2010; Cheng et al., 2014). Unilateral sciatic nerve lesions have been shown to affect bilateral inhibitory mechanisms within the spinal dorsal horn (Ibuki et al., 1997; Simpson and Huang, 1998) and to alter the contralateral expression of receptors, ion channels and neuropeptides (Koltzenburg et al., 1999). Mirror-image thermal hyperalgesia is mediated by substance P acting on the NK1 receptor (Coderre and Melzack, 1991), and lamina I is likely to play an important role in this process, as both PNs and LCNs in this layer express the NK1 receptor (Al Ghamdi et al., 2009) and are depolarised by substance P (Luz et al., 2014).

At the level of the spinal cord, the morphophysiological basis of mirror-image pain is provided by the bilateral organisation of the nociceptive network, which includes decussating afferents (Culberson et al., 1979; Light and Perl, 1979a; Marfurt and Rajchert, 1991), commissural interneurons (Petko and Antal, 2000; Petko et al., 2004), bilaterally projecting spinothalamic and spinoparabrachial neurons (Burstein et al., 1990a; Spike et al., 2003), and lamina I neurons projecting via the ipsilateral anterolateral tract (Antal et al., 2016). Decussating nociceptive afferents are numerous in the medullary, cervical, thoracic and sacral cord (Culberson et al., 1979; Light and Perl, 1979a; Marfurt and Rajchert, 1991), that can explain a high incidence of the mirror-image pain associated with the structures innervated by these afferents (Khan et al., 2007; Mathew et al., 2008). However, the decussating thin afferents are much less common in the lumbar spinal cord (Culberson et al., 1979; Light and Perl, 1979a; Shehab and Hughes, 2011), and it was not clear whether they could provide an excitatory drive to the contralateral lamina I PNs that might be sufficient to explain the induction of mirror-image pain associated with pathology of the lumbar afferent system (Coderre and Melzack, 1991; Milligan et al., 2003; Konopka et al., 2012; Cheng et al., 2014; Su et al., 2018).

The recent study using *ex vivo* lumbar cord with preserved bilateral dorsal roots has provided new insights into the spinal mechanisms of mirror-image pain (Luz et al., 2023). It has been shown that some decussating thin primary afferents project to the contralateral superficial dorsal horn. These fibers with bouton-like enlargements extend along the dorsal surface of the contralateral grey matter in the termination field of the ipsilateral peptidergic, CGRP-positive, and non-peptidergic, IB4-positive, nociceptors. In addition, the varicosities of contralateral afferents contact somata and dendrites of lamina I and II neurons. Accordingly, about a quarter of the neurons, including PNs, in lateral lamina I receive mono- and/or polysynaptic excitatory input from the contralateral Aδ- and C-afferents. All these neurons also receive ipsilateral input and are therefore involved in bilateral processing (Fig. 7). The contralateral input in these bilateral neurons is significantly smaller than the ipsilateral input, which may explain why the mirror-image pain on the uninjured side is usually less severe than the pain on the injured side (Konopka et al., 2012).

Contralateral Aδ- and C-fiber input to lamina I is under diverse forms of phasic and tonic inhibitory control (Luz et al., 2023). Attenuation of afferent-driven presynaptic inhibition and disinhibition of the dorsal horn network increased contralateral synaptic drive to second-order neurons and its ability to evoke spikes. In addition, the contralateral myelinated Aβδ-afferents presynaptically control the ipsilateral C-fiber input to lamina I neurons. This means that some lamina I neurons are wired to the contralateral afferent system, whose input is normally under inhibitory control. Pathological disinhibition of these decussating pathways may open a gate that controls the flow of contralateral information to the nociceptive PNs and thus contribute to the induction of mirror-image pain.

12. Similarities between lamina I and lamina X neurons

Lamina X, the grey matter surrounding the central canal, has a complex organisation and includes the dorsal and ventral grey commissures and the substantia gelatinosa centralis (Rexed, 1952). Lamina X neurons are involved in a number of functions, including motoneuron

control (Stepien et al., 2010; Bertrand and Cazalets, 2011), autonomic regulation (Deuchars and Lall, 2015), somatosensory integration and visceral nociception (Ness and Gebhart, 1987; Lanteri-Minet et al., 1993; Cervero and Laird, 2004; Eijkelkamp et al., 2007). Several types of primary afferents terminate or decussate in this area (Light and Perl, 1979b; Morgan et al., 1981; Sugiura et al., 1989; Luz et al., 2023) and its neurons respond to noxious stimulation of somatic and visceral structures (Nahin et al., 1983; Honda, 1985; Honda and Perl, 1985). However, little was known about the afferent supply of lamina X, which still remains one of the least studied regions of the CNS.

The hemisected *ex vivo* spinal cord preparation was used for recording from lumbar lamina X neurons (Krotov et al., 2019), which were found to be similar to lamina I neurons in many respects. The most common pattern of intrinsic firing in lamina X is tonic, followed by adapting, delayed and bursting discharges. In contrast, this layer contains no rhythmic neurons, and all spontaneous action potential discharges are driven by the ongoing synaptic activity in the local neuronal network. A striking feature of lamina X neurons is the lack of input from low-threshold Aβ/δ-afferents. However, virtually all neurons in lamina X receive input from HT-Aδ- and C-fibers. Two-thirds of these neurons receive monosynaptic input and may therefore be directly targeted by primary nociceptors. In addition to excitatory inputs, about half of the neurons also receive polysynaptic inhibitory supply, which is mainly mediated by C-fibers. Interactions between excitatory and inhibitory synaptic components determine the output properties of the neurons, a third of which fire action potentials in response to the saturating dorsal root stimulation. In this respect, the grey matter surrounding the central canal is similar to the superficial dorsal horn, the main spinal nociceptive processing area. Thus, neurons in lamina X integrate direct and indirect inputs from several classes of thin afferents and are involved in nociception.

Besides, afferent input to lamina X neurons is subject to segmental and descending control (Krotov et al., 2022, 2023). Aδ- and C-afferents supplying lamina X are presynaptically controlled by homo- and heterosegmental afferents as well as descending fibers from the corticospinal tract, dorsolateral funiculus and anterior funiculus. Activation of descending pathways suppresses primary afferent-driven spikes, but can also evoke excitatory or inhibitory postsynaptic responses. Thus, the primary afferent input to lamina X is subject to both spinal and supraspinal control and is regulated by at least five different pathways.

13. Conclusions

Lamina I is more than just an output element of the nociceptive network that transmits primary afferent input to higher brain centres. It is a complex processing unit involved in a number of different functions. Lamina I represents an integrative network of interconnected neurons that receive highly convergent primary afferent input regulated by various forms of inhibitory control. Alterations in signal processing in lamina I neurons can lead to a number of pathological conditions. Dysfunction of the somatovisceral integration can cause referred pain. Pathology of the trigeminocervical afferent system may contribute to pain and its referral in primary headache syndromes. Pathological changes in bilateral input processing can cause mirror-image pain. Further studies are needed to elucidate the specific roles of lamina I PNs and LCNs in the peripheral input processing under physiological and various pathological conditions.

14. Perspectives

The neurophysiological and neuroanatomical findings described here are the results of a long series of experiments carried out in rats, a species used in studies that paved the way for creating prototypes of functional and anatomical maps of the spinal dorsal horn as we know it today. Recent advances in the field of genetic and molecular biology techniques have generated an enormous amount of data and suggested new classification schemes. Single-cell RNA sequencing has been used to create a neuronal atlas of the mouse dorsal horn, helping to link transcriptional cell types to sensory input (Haring et al., 2018). Viral vectors and optogenetics have been used extensively in mice to study the spinal dorsal horn circuits involved in processing information about pain, itch and innocuous temperature changes (Foster et al., 2015; Acton et al., 2019; Pan et al., 2019; Petitjean et al., 2019; Choi et al., 2020; Chisholm et al., 2021), revealing new gating (Duan et al., 2014; Sun et al., 2017; Boyle et al., 2019) and descending control mechanisms (Francois et al., 2017; Gao et al., 2019). It would be an important future step to reconcile these new mouse data with the electrophysiological and anatomical findings of the early rat and cat literature. The combination of the oblique IR-LED imaging with the novel approaches in transgenic mice could be a prospective step towards identifying specific functional roles of different types of lamina I PNs and LCNs.

CRediT authorship contribution statement

Boris V. Safronov: Writing – original draft, Conceptualization. **Peter Szucs:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

No data was used for the research described in the article.

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