Scuola Internazionale Superiore di Studi Avanzati

Afferent information modulates spinal network activity *in vitro* and in preclinical animal models

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DECLARATION

The data reported in the present PhD project have been published in the articles listed below.

Dingu N, Deumens R, Taccola G. (2016) Electrical stimulation able to trigger locomotor spinal circuits also induces dorsal horn activity. *Neuromodulation* 19:38–46.

Taccola G, Doyen PJ, Damblon J, Dingu N, Ballarin B, Steyaert A, Rieux AD, Forget P, Hermans E, Bosier B, Deumens R. (2016) A new model of nerve injury in the rat reveals a role of Regulator of G protein Signaling 4 in tactile hypersensitivity. *Experimental Neurology* 286:1–11.

The following study is currently under revision after first review.

Dingu N, Deumens R, Taccola G. Afferent input induced by rhythmic limb movement modulates spinal neuronal circuits in an innovative robotic *in vitro* preparation. *Journal of Neurophysiology*

The candidate carried out electrophysiological experiments and partecipated to behavioral and immunohistological studies. In addition, the candidate personally performed data analysis and contributed to paper writing.

ABSTRACT

Primary afferents are responsible for the transmission of peripheral sensory information to the spinal cord. Spinal circuits involved in sensory processing and in motor activity are directly modulated by incoming input conveyed by afferent fibres. Current neurorehabilitation exploits primary afferent information to induce plastic changes within lesioned spinal circuitries. Plasticity and neuromodulation promoted by activity-based interventions are suggested to support both the functional recovery of locomotion and pain relief in subjects with sensorimotor disorders. The present study was aimed at assessing spinal modifications mediated by afferent information.

At the beginning of my PhD project, I adopted a simplified *in vitro* model of isolated spinal cord from the newborn rat. In this preparation, dorsal root (DR) fibres were repetitively activated by delivering trains of electrical stimuli. Responses of dorsal sensory-related and ventral motor-related circuits were assessed by extracellular recordings. I demonstrated that electrostimulation protocols able to activate the spinal CPG for locomotion, induced primary afferent hyperexcitability, as well. Thus, evidence of incoming signals in modulating spinal circuits was provided. Furthermore, a robust sensorimotor interplay was reported to take place within the spinal cord.

I further investigated hyperexcitability conditions in a new *in vivo* model of peripheral neuropathic pain. Adult rats underwent a surgical procedure where the common peroneal nerve was crushed using a calibrated nerve clamp (modified spared nerve injury, mSNI). Thus, primary afferents of the common peroneal nerve were activated through the application of a noxious compression, which presumably elicited ectopic activity constitutively generated in the periphery. One week after surgery, animals were classified into two groups, with (mSNI+) and without (mSNI-) tactile hypersensitivity, based on behavioral tests assessing paw withdrawal threshold. Interestingly, the efficiency of the mSNI in inducing tactile hypersensitivity was halved with respect to the classical SNI model. Moreover, mSNI animals with tactile hypersensitivity (mSNI+) showed an extensive neuroinflammation within the dorsal horn, with activated microglia and astrocytes being significantly increased with respect to mSNI animals without tactile hypersensitivity (mSNI-) and to sham-operated animals. Lastly, RGS4 (regulator of G protein signaling 4) was reported to be enhanced in lumbar dorsal root ganglia (DRGs) and dorsal horn ipsilaterally to the lesion in mSNI+ animals. Thus, a new molecular marker was demonstrated to be involved in tactile hypersensitivity in our preclinical model of mSNI.

Lastly, we developed a novel *in vitro* model of newborn rat, where hindlimbs were functionally connected to a partially dissected spinal cord and passively-driven by a robotic device (Bipedal

Induced Kinetic Exercise, BIKE). I aimed at studying whether spinal activity was influenced by afferent signals evoked during passive cycling. I first demonstrated that BIKE could actually evoke an afferent feedback from the periphery. Then, I determined that spinal circuitries were differentially affected by training sessions of different duration. On one side, a short exercise session could not directly activate the locomotor CPG, but was able to transiently facilitate an electrically-induced locomotor-like activity. Moreover, no changes in reflex or spontaneous activity of dorsal and ventral networks were promoted by a short training. On the other side, a long BIKE session caused a loss in facilitation of spinal locomotor networks and a depression in the area of motor reflexes. Furthermore, activity in dorsal circuits was long-term enhanced, with a significant increase in both electrically-evoked and spontaneous antidromic discharges. Thus, the persistence of training-mediated effects was different, with spinal locomotor circuits being only transiently modulated, whereas dorsal activity being strongly and stably enhanced. Motoneurons were also affected by a prolonged training, showing a reduction in membrane resistance and an increase in the frequency of post-synaptic currents (PSCs), with both fast- and slow-decaying synaptic inputs being augmented. Changes in synaptic transmission onto the motoneuron were suggested to be responsible for network effects mediated by passive training.

In conclusion, I demonstrated that afferent information might induce changes within the spinal cord, involving both neuronal and glial cells. In particular, spinal networks are affected by incoming peripheral signals, which mediate synaptic, cellular and molecular modifications. Moreover, a strong interplay between dorsal and ventral spinal circuits was also reported. A full comprehension of basic mechanisms underlying sensory-mediated spinal plasticity and bidirectional interactions between functionally different spinal networks might lead to the development of neurorehabilitation strategies which simultaneously promote locomotor recovery and pain relief.

INTRODUCTION

1 Afferent feedback to the spinal cord

1.1 Somatosensation

Perception is mediated by the somatosensory system. The somatosensory system consists of peripheral receptors and neural pathways through which the central nervous system (CNS) is aware of events occurring along the skin surface (McGlone and Reilly, 2010), is provided of information about position and movement of parts of the body and receives feedback to motor control and coordination (Proske and Gandevia, 2012). His major functions are three: (1) exteroreceptive and (2) interoceptive, for our perception and reaction to stimuli originating outside and inside of the body, respectively, and (3) proprioceptive, for the perception and control of body position and balance (Abraira and Ginty, 2013).

Perception starts in the periphery where sensory receptors located in skin, muscles and joints detect mechanical, thermal or noxious stimuli. Sensory receptors are connected to the peripheral terminals of primary sensory neurons that convey information from the periphery to the spinal cord. Sensory neurons have cell bodies located in the dorsal root ganglia (DRGs), just outside the spinal cord for each spinal level, and two axonal branches, one extending to the periphery and the second that penetrates the spinal cord (pseudounipolar; Holinski et al., 2013). Axonal branches of primary sensory neurons converge and gather into primary afferent fibres.

1.1.1 Classification of primary afferent fibres with specific attention to the rat hindlimb innervation

Nerve fibres are classified based on anatomical and physiological properties and on peripheral end organs they innervate, as summarized by figure 1.

In 1924 Erlanger and Gasser obtained a first classification of peripheral nerve fibres (Erlanger et al., 1924; Erlanger and Gasser 1930, 1937; Gasser, 1941). They distinguished single fibres based on conduction velocities of the evoked compound action potentials (CAPs) in peripheral nerves. Based on this first classification, peripheral nerve fibres were divided into three main groups: (1) group A, further subdivided into α, β, γ and δ fibres, comprised myelinated somatic afferent and efferent fibres; (2) group B consisted of pre-ganglionic autonomic fibres; (3) group C included non-myelinated somatic afferent fibres and post-ganglionic autonomic fibres. It is important to highlight

that this first classification encompassed both afferent and efferent fibres, as Aα fibres enclosed also somatic motor axons from α-motoneurons and Aγ fibres corresponded to motor axons from γ-motoneurons.

In 1943 Lloyd introduced a new terminology referred only to afferent fibres (Lloyd, 1943). Considering fibre size, myelination and conduction velocity, he classified them into four groups: (1) group I, included Aα fibres of the previous classification by Erlanger and Gasser, and consisted in fibres coming from muscle spindles (Ia) and Golgi tendon organs (GTOs; Ib); (2) group II, comparable to the formerly described Aβ fibres, was associated to cutaneous low-threshold mechanoreceptors; (3) group III, equivalent to Aδ fibres, mediating fast mechanical pain; (4) group IV, representing the unmyelinated C fibres, responding to noxious mechanical stimuli and heat nociception. According to Lloyd's classification, group I and II refer to thick myelinated fibres having the highest conduction velocities, group III to thin myelinated fibres having intermediate conduction velocities, and group IV to unmyelinated fibres having the lowest conduction velocities.

In addition to conduction velocities, afferent fibres are further distinguished by adaptation properties to sustained mechanical stimuli. With this regard, they are classified as slowly, intermediately or rapidly adapting, according to changes in their firing rate when an external stimulus is applied and induces a mechanical deformation of the sensory nerve terminal (Hunt and Ottoson, 1975; Fitz-Ritson, 1982; Macefield, 2005; Abraira and Ginty, 2013). Moreover, in the rat DRG neurons innervating the glabrous skin of the hindpaw have been identified four types of currents based on constants of decay time: rapidly adapting currents (~ 3 – 6 ms), intermediately adapting currents (~15 – 30 ms), slowly adapting currents (~200 – 300 ms) and ultra-slowly adapting currents (~1000 ms; Hao and Delmas, 2010).

Lastly, primary afferents are discerned by the end organs with which they associate, consisting in highly specialized non-neuronal structures taking contact with the peripheral terminus of afferent fibres. Considering the rat hindlimb as an example, it is proven that it has a wide number of mechanoreceptors located within glabrous and hairy skin and of proprioceptors present in muscles, joints, ligaments and tendons. In the glabrous skin of the hindpaw, for instance, there are four types of mechanosensory end organs: Pacinian corpuscles, Ruffini endings, Meissner corpuscles and Merkel's discs (Abraira and Ginty, 2013). In the hairy skin of the hindlimb hair follicles play a major role in mechanosensation (Li et al., 2011). Hindlimb muscles contain highly specialized stretch receptors, the muscle spindles, which are very sensitive to length changes within the muscle

and that represent the sensory endings primarily responsible for proprioception (Banks, 2015). Unlike the muscle spindles that are arranged in parallel to the muscle fibres and activate during muscle stretch, other end organs located in tendons (Golgi tendon organs, GTOs), joint and ligaments (Pacinian corpuscles, Ruffini and Golgi-like receptors; Hildebrand et al., 1991; Delgado-Baeza et al., 1999) respond to forces generated by the contracting muscle and to joint movements in different directions and in more than one axis of rotation.

In peripheral tissues, there are also axon terminals which do not possess specialised end structures and for this reason are referred to as free nerve endings, usually associated to Aδ (group III) or C (group IV) fibres. These structures are called nociceptors because respond to mechanical, thermal and chemical stimuli that are tissue threatening or damaging. Nociceptors are widely present in the rat hindlimb, since they are located in both glabrous and hairy skin (McGlone and Reilly, 2010), in skeletal muscles where they represent more than 50% of the total muscle afferents (Laurin et al., 2015), and in ligaments and periligamentary tissue where they constitute 74% of the receptors (Delgado-Baeza et al., 1999).

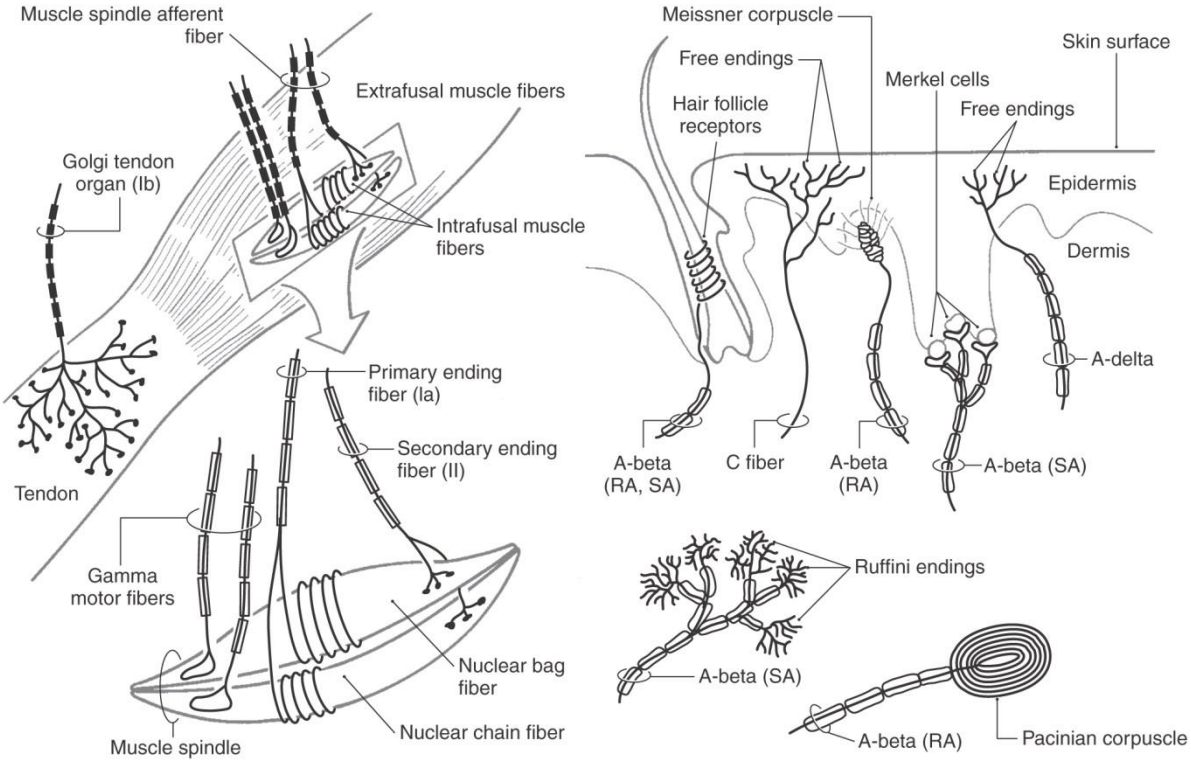

Figure 1: Overview of primary afferents and innervated end organs. Main proprioceptors (on the left) and skin mechanoreceptors (on the right) are shown. Primary afferent fibres are distinguished based on the two traditional classifications by Erlanger and Gasser and by Lloyd. Moreover, afferents are also classified by adaptation properties into rapidly adapting (RA) and slowly adapting (SA). (From Warren et al., 2015).

1.1.2 Basic insight into signal transduction of peripheral input

End organs and related innervation terminals from DRG neurons form complexes, referred to as peripheral sensors. They have a role in detecting different signals (e.g. mechanical, thermal, chemical) from peripheral tissues and in converting them into electrical signals. To carry out this task, peripheral sensors are enriched in membrane transducers inducing local receptor potentials which can reach threshold and generate action potentials that propagate through primary afferent fibres toward the central nervous system (CNS). Transduction pathways require a rapid and direct signaling provided by ion channels through interaction with both intracellular cytoskeletal and extracellular matrix proteins (Gillespie and Walker, 2001; Delmas and Coste, 2013). However, molecules underlying somatosensory transduction are not well defined. The first transduction channels have been identified in *Drosophila melanogaster* and *Caenorhabditis elegans* (Walker et al., 2000; O'Hagan et al., 2005). Recent studies have focused on homologs in mammals.

In mammals, the conversion of light touch and nociceptive skin sensation into electrical signal has emerged to be mediated by three main classes of ion channels: (1) transient receptor channels (TRP), (2) acid-sensing ion channels (ASICs) and (3) two-pore potassium (KCNK) channels (Tsunozaki and Bautista, 2009; Roudaut et al., 2012).

The TRP superfamily is known for sensing thermal information and chemical stimuli (e.g. capsaicin), and for mediating mechanical hyperalgesia during inflammation (Christensen and Corey, 2007; Damann et al., 2008). The ASICs not only respond to protons and are gated by pH variations, but also generate perception of sting and pain (Deval and Lingueglia, 2015). Both classes are associated to C- and Aδ-sensory neurons, demonstrating they play a major role in nociception (Deval and Lingueglia, 2015; Jardin et al., 2017).

KCNK members belong to the two-pore domain potassium channel family (K2P; Lesage, 2003) and contribute to the background potassium conductances that regulate the membrane resting potential of somatosensory neurons (Dobler et al., 2007). Somatosensory neurons express several KCNK subunits (Medhurst et al., 2001). For some of them it has been demonstrated a direct mechanical gating by membrane stretch (KCNK2 and KCNK4; Maingret et al., 1999 a; b), a role in mediating responses to light touch, as well as in inducing tingling sensation and painful mechanical stimuli (KCNK18; Bautista et al., 2008; Bhattacharya et al., 2008).

Lastly, it should be mentioned the role of end organs and other non-neuronal cells, such as keratinocytes that form the epidermis, in primary afferent fibres activation. It is possible that they trigger sensory afferents activity through secreted signalling molecules in response to mechanical

stimuli (Lumpkin and Caterina, 2007). For example, they may release ATP or other molecules, reinforcing main transmission pathways with protective roles (Koizumi et al., 2004; Hu and Lewin, 2006).

The sense of body and limb position is transduced by proprioceptors. They convey signals about the stretch and tension experienced by muscles, tendons, joints and skin and encode information for basic motor functions, such as standing and walking (Proske and Gandevia, 2012; Akay et al., 2014). In mammals, the molecular mechanisms underlying proprioception are very poorly understood.

Recently, Woo and co-workers have explored the role of the Piezo family in proprioception (Woo et al., 2016). Vertebrates have two Piezo members, Piezo1, barely detectable in DRGs, and Piezo 2, abundant in sensory neurons, whose structure remains to be determined (Coste et al., 2012). Woo's group has found that Piezo2, a mechanically-activated nonselective cation channel, is expressed in peripheral terminals of afferent fibres innervating muscle spindles and GTOs in mice. Moreover, conditional knockouts for Piezo2 show uncoordinated body movements and abnormal limb positions and *in vitro* recordings from muscle-nerve preparations of Piezo2-lacking mice display a decrease in stretch-sensitive nerve activity of muscle afferents. Based on these observations, they have demonstrated that Piezo2 is the principal transduction channel involved in mammalian proprioception.

Hong and collaborators have identified a new channel that appears to play a role in proprioception, Tentonin 3 (TTN3; Hong et al., 2016). The authors have demonstrated that TTN3 is expressed in muscle spindles and that TTN3-knockout mice show muscle weakness and loss of motor coordination. Even though little is known about TTN3 function, Hong's group has proved that this nonselective cation channel is activated by mechanical stretch and that it contributes to mammalian coordination.

Finally, the members of the DEG/ENaC superfamily have been considered to be good candidates for machanotransduction in muscle spindles (Bewick and Banks, 2015). However, their role in mammalian proprioception is controversial because the ENaC channel is not activated by mechanical stimuli and the genetic ablation of ENaC does not induce motor impairment (Rossier, 1998; Bewick and Banks, 2015).

Interestingly, muscle spindle primary afferent terminals investigated by electron microscopy have been shown to contain a population of vesicles with a clear lumen and a 50 nm-diameter, termed synaptic-like vesicles (SLVs; Bewick et al., 2005). SLVs undergo constitutive turnover at rest,

while their recycling is increased with mechanical activity. Moreover, vesicles contain glutamate (Bewick et al., 2005) and they express glutamate transporters (vGluT1; Wu et al., 2004). It has been demonstrated that peripheral glutamate released by SLVs is required to both maintain and increase spindle responsiveness to mechanical stimuli. The receptor involved in the spindle glutamate sensitivity is an atypical metabotropic glutamate receptor (mGluR) coupled to phospholipase D (PLD; Bewick and Banks, 2015), first identified in the hippocampus (Pellegrini-Giampietro et al., 1996). SLVs appear to be almost ubiquitous in primary mechanosensory nerve terminals and, therefore, of remarkable interest. Although the SLV-glutamate-PLD-mGluR system is not directly involved in mechanotransduction, it represents a complex and sophisticated example of peripheral control of afferent activity conveyed to the spinal cord.

1.2 Dorsal root ganglia are *en route* to the spinal cord

Signals generated in the periphery travel across DRGs before reaching the spinal cord. DRGs are located outside of the intervertebral neural foramen and contain the cell bodies of the primary sensory neurons separated from each other by an envelope of satellite glial cells (SGCs; Hanani, 2005). Within a typical DRG there are many thousands of sensory neurons, with lumbar DRGs ranging from 12.000 to 15.000 cell bodies (Schmalbruch, 1987). This raises the issue that DRG sensory neurons might be a greatly heterogenous population (Krames, 2015). On a histological section, they can be distinguished on the basis of cell diameter that allows defining three main subgroups, Aα/β, Aδ and C cells (Lawson and Waddell, 1991; Edwards et al., 1995). Moreover, DRG neurons can be functionally characterized by their response to peripheral stimuli into cell bodies of low-threshold mechanoreceptors and high-threshold mechanoreceptors (Koerber et al., 1988; Ritter and Mendell, 1992). They can also be classified on the basis of conduction velocity of the peripheral axon and on differences in the shape of the action potential (AP), such as variations in AP duration (Waddell and Lawson, 1990; Villière and McLachlan, 1996). Recently, a great effort has been made in trying to identify different DRG neuron subtypes based on their gene expression profile (Usoskin et al., 2015) and 14 subclasses have been determined using clustering gene analyses (Li et al., 2016). Thus, a complex picture of the DRG is taking shape, which was used to be considered relatively simple.

Primary sensory neurons are defined pseudounipolar neurons, as they have one axon that divides into two branches, the peripheral one traveling toward sense organs in skin, muscles etc., and the central one taking contact with the spinal cord (Devor, 1999). The peripheral and central axonal processes are connected to the cell body via a T-junction, as shown in figure 2. The T-junction of

sensory neurons influences the transmission of trains of APs that arise from peripheral terminals. It has been demonstrated that the T-junction represents an impediment to AP propagation towards the CNS, acting as a low-pass filter (Gemes et al., 2013). Propagation failure mechanisms are various and involve different ion channels, included Piezo 1 and Piezo 2 that have been found to act as high-pass, low-pass or band-pass filters depending on the waveform and duration of repetitive mechanical stimuli (Lewis et al., 2017). Thus, in the last few years it has been achieved strong evidence that DRG neurons do not represent mere metabolic factories or depots, where macromolecules involved in signal transduction and transmission at peripheral sensory endings are synthesized or stored. On the contrary, they take actively part to an early processing of the signal traveling toward the spinal cord, contributing to the sensory control of motor tasks (Weber et al., 2007; Holinski et al., 2013) and affecting peripheral nociceptive transmission (Gemes et al., 2013; Du et al., 2014).

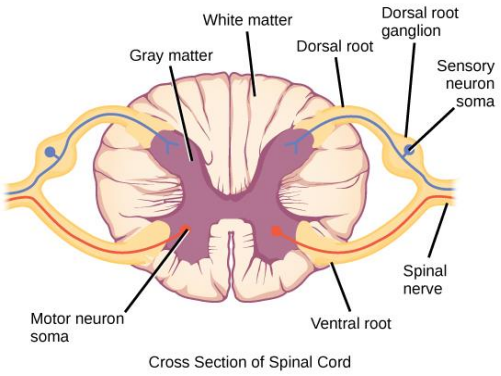

Figure 2: The dorsal root ganglion (DRG). DRGs contain cell bodies of primary sensory neurons. Sensory neurons are called pseudounipolar because they have an axon that bifurcates at the T-junction into a peripheral branch and a central one. Peripheral branches of sensory neurons (blue) and axons of spinal motoneurons (red) converge into spinal nerves, thus referred to as mixed since made up of afferent and efferent fibres. (From "OpenStax College, The Peripheral Nervous System. October 17, 2013").

1.3 Afferent projections to the spinal cord and their somatotopic arrangement

Sensory neurons in DRGs have central branches that terminate in the spinal cord. Once in the spinal cord, primary afferents take synaptic contacts either with spinal interneurons or directly with motoneurons. The spinal gray matter has a well-defined cytoarchitectonic laminar organization, as originally described in cat by Rexed (Rexed, 1952; 1954) and later on in rat by Molander and co-workers (Molander et al., 1984; 1989). As stated by these anatomical studies, a spinal transverse section can be divided into ten laminae based on cell morphology and density of cell packaging. Borderlines between different laminae are not so distinct in an individual section and the lamination pattern appears more evident in newborns than in adults (Rexed 1952; Molander et al., 1984). The laminar organization is hard to imagine when talking about lamina IX, that is actually split into

several subnuclei containing motoneurons which send their axons to muscles, as demonstrated in cat (Romanes, 1951) and rat (Nicolopoulos-Stournaras and Iles, 1983). The same as above for lamina X which surrounds the central canal.

Primary afferent fibres project to spinal laminae in a highly ordered and reproducible topographic arrangement. The spinal cord plays a key role in peripheral signal representation, integration and processing. Spinal processing of peripheral information takes into account (1) the fibre type, (2) the signal pattern, (3) the afferent terminal arborization, (4) the projecting lamina in the spinal cord, (5) the spinal microcircuit to whom the projecting neuron belongs. The spinal somatotopic arrangement of cutaneous mechanosensory fibres and proprioceptors is slightly different and it will be examined below.

The skin sensory innervation and projections to the spinal cord have provided an accessible model for first studying the organization of somatosensory input to the CNS. Over the last years, many studies investigated peripheral receptive fields of spinal neurons, defined as skin regions that, when appropriately stimulated, evoke a firing rate which significantly exceeds the resting discharge rate (Fitzgerald, 1985). Moreover, much more detailed information concerning the spinal projections of different subtypes of primary afferent fibres has been provided by neuronal tracers based on axoplasmic transport of marker substances (Wilson and Kitchener, 1996). Two main techniques, (1) transganglionic labeling applied to a peripheral nerve stump and (2) intraaxonal staining of single functionally-identified afferents, gave the first insight into somatotopic arrangement of primary afferent projections. However, many difficulties were encountered due to technical limitations and insufficient microanatomy knowledge of the primary afferent projections to the spinal cord (Wilson and Kitchener, 1996).

Recently, molecular biology and genetics approaches have allowed fast advancement in the field. A remarkable effort has been done in identifying unique molecular markers of physiologically distinct afferent classes and in designing genetic strategies for the visualization of axonal branches in the periphery and spinal cord. As reported by figure 3 for afferents from glabrous and hairy skin, cutaneous sensory fibres are endowed with peculiar branching properties before entering the dorsal horn and their collaterals terminate in different laminae. For example, in the processing of innocuous touch in glabrous skin, Aβ fibres bifurcate upon entering the dorsal horn and branches extend in opposite directions along the rostro-caudal axis, with a principal central branch travelling through the dorsal columns toward medulla and a secondary branch sprouting collaterals and taking synaptic contacts in deep dorsal laminae III-V (figure 3, panels C-D). On the contrary, Aδ and C

low-threshold skin mechanoceptors do not bifurcate, but extend few segments rostrally before entering the dorsal horn and terminating in distinct but partially overlapping laminae (II_{iv}-III; figure 3, panels A-B; Abraira and Ginty, 2013). In hairy skin, the three types of hair follicles, namely guard, zigzag, Awl/Auchene, have a unique and very complex projection pattern (inset in the bottom panel E of figure 3). In the spinal dorsal horn, the central afferent terminals that innervate the same or adjacent hair follicles are arranged in a characteristic manner consisting in narrow columns spreading from lamina II to lamina V (Li et al., 2011; figure 3, panel E). Lastly, noxious touch is detected by free nerve endings of high-threshold Aδ and C fibres in both glabrous and hairy skin, which project to the outermost laminae of the dorsal horn (I-II; Roudaut et al., 2012).

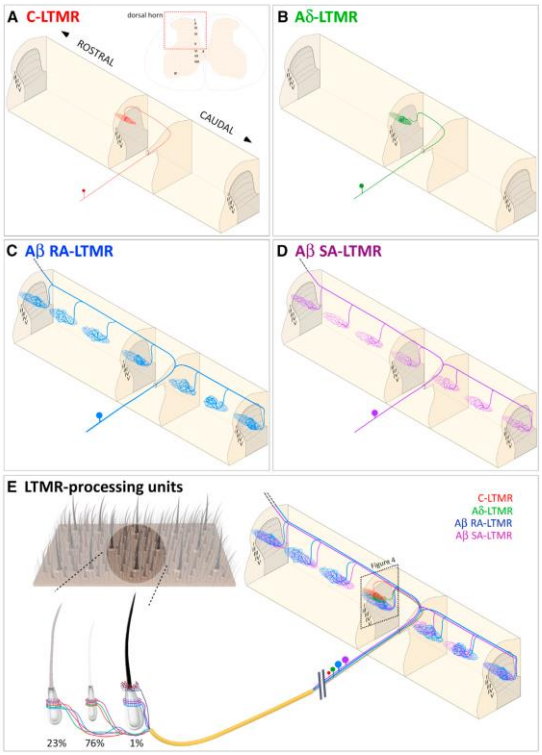

Figure 3: Cutaneous low-threshold afferent fibres projections to the spinal cord. Functionally and molecularly identified fibres from glabrous (A-D) and hairy (E) skin project to the spinal cord dorsal horn in a well-defined somatotopic arrangement. Reproducible branching properties and collaterals localization can be easily recognized. (From Abraira and Ginty, 2013).

Cutaneous primary afferents project to second order dorsal interneurons that play a key role in afferent signal processing and integration. Recently, Abraira and colleagues have identified a low-threshold mechanoceptor-recipient zone (LTMR-RZ) consisting in highly specialized dorsal interneurons involved in tactile perception. The authors have found that the LTMR-RZ is made up of seven excitatory and four inhibitory interneuron subtypes, which fine-tune and convey touch information to supraspinal centres (Abraira et al., 2017).

The spinal cord receives primary afferent fibre inputs from muscles, tendons, joint and ligaments as well as from skin.

Projections of muscle and tendon afferent fibres have been examined in detail in both cat (Jankowska and Lindstrom, 1972; Brown and Fyffe, 1978, 1979) and rat (Swett and Woolf, 1985; Molander and Grant, 1986, 1987). From these studies has emerged that group Ia axons from primary endings in muscle spindles distribute collaterals to laminae VI-VII, where interneuronal circuits involved in locomotion are located, and to lamina IX, representing motor nuclei of the spinal cord. Group II axons from muscle spindle secondary endings have specific branching patterns sprouting from lamina IV to lamina VIII, with collaterals reaching also lamina IX (Edgley and Jankowska, 1987). Group Ib axons from the GTOs project in a region including laminae V-IX. All three classes of primary afferents project to lamina IX, indicating the existence of monosynaptic contacts on motoneurons (Jankowska and Edgley, 2010). Interestingly, many spinal interneurons are co-excited by several types of primary afferents, consistent with a great overlap at spinal level (Harrison and Jankowska, 1985).

Joint and ligament afferents have been shown to contribute to joint stability, muscle coordination and proprioception through projections to spinal interneurons, α- and γ-motoneurons and several ascending pathways (Sjolander et al., 2002). In particular, reflexes involving α-motoneurons are characterized by excitatory effects on flexor motoneurons and both excitatory and inhibitory effects on extensor motoneurons (Holmqvist and Lundberg, 1961). It is supposed that these pathways are mediated by high-threshold fibres in the articular capsule and ligaments, which activate when the joint approaches the limits of its working range to avoid damaging hyperrotations (Sjolander and Johansson, 1997). Moreover, ligament afferents evoke strong excitatory and inhibitory reflex responses on γ-motoneurons (Sjolander and Johansson, 1995), generating optimal muscle spindle responses that have a significant impact on proprioception and motor control (Bergenheim et al., 1995).

Considering that a very small fraction of spinal neurons project to the brain, a major involvement of the spinal cord in peripheral information processing is arising. Indeed, spinal circuits represent an early decodification and integration site of incoming information. Moreover, local spinal networks appear to have a key role in shaping the output of projection neurons, thus substantially modifying the intrinsic nature of inputs reaching higher brain centers.

1.4 Postnatal development of sensory afferents

During the early neonatal life, peripheral sensory structures and primary afferent fibres are not fully developed. Moreover, during this period massive new peripheral somatosensory input is received by the spinal cord, where new sensory pathways and various control systems appear and mature (Fitzgerald, 1985).

One difference between afferents of very young and older animals is the degree of myelination. In rats, peripheral myelination has begun at birth but increases rapidly over the first two postnatal weeks. This leads to a progressive augment in conduction velocities with Aδ fibres reaching a complete myelination before Aβ fibres, the latter being thickly myelinated and requiring a higher number of lamellae to reach full maturation (Friede and Samorajski, 1968; Sima, 1974). Moreover, large diameter fibres seem to enter the cord first, while small diameter non-myelinated C fibres do not develop full contacts with spinal circuits until the second postnatal week (Fitzgerald, 1985), as demonstrated by the failure in evoking C-fibre specific noxious reflexes with the skin irritant mustard oil until postnatal day 10-11 (Fitzgerald and Gibson, 1984).

Different neurotrophins and several classes of transcription factors have been implicated in the survival and maturation of DRG neurons. Proprioceptive afferents terminating on muscle spindles (Ia afferents) or GTOs (Ib afferents) express the receptor tyrosine kinase TrkC (Chen and Frank, 1999; Patel et al., 2003). Its ligand, neurotrophin 3 (NT3), is released by satellite glial cells (SGCs) in the DRG, motoneurons and developing muscles (Oakley et al., 1995; Taylor et al., 2001), and is essential for the survival of proprioceptive sensory neurons (Ernfors et al., 1994; Klein et al., 1994). On the other hand, cutaneous afferents express TrkA and their survival depends on the release of nerve growth factor (NGF; Fundin et al., 1997).

In addition to neurotrophins, three main classes of transcription factors seem to play a major role in the development of sensory fibres: (1) the basic helix-loop-helix proteins neurogenin 1 (Ngn1) and neurogenin 2 (Ngn2), (2) the members of the heterodimeric core-binding factor/Runt family of transcription factors, (3) the winged helix-turn-helix Ets-domain transcription factor Er81. Ngn1 and Ngn2 are essential for DRG neurons differentiation, with most proprioceptive neurons derived from an early Ngn2 progenitor and the majority of cutaneous sensory neurons generated by an Ngn1-positive progenitor (Ma et al., 1999). Runx3, member of the Runt family of transcription factors, is associated to proprioceptive afferent development and survival and Runx3-knockout mice show severe limb ataxia and uncoordinated movements (Inoue et al., 2002; Levanon et al., 2002). Lastly, Er81 is responsible for the development of a proper connectivity of Ia afferents, since in

Er81-deficient mice Ia fibres terminate prematurely in intermediate laminae of the spinal cord, leading to an almost complete absence of monosynaptic connections between Ia afferents and α-motoneurons (Arber et al., 2000).

Maturation of muscle spindles depend on inductive signals provided by innervating fibres. It has been demonstrated that the removal of sensory (Kucera et al., 1993) but not motor innervation (Kucera and Walro, 1992) is detrimental for the differentiation of intrafusal muscle fibres from immature myotubes. The search for molecular markers has revealed a role for TrkC-NT3, since in both knockout strains proprioceptive neurons die before their axons reach the muscle and there is a complete absence of muscle spindles (Ernfors et al., 1994; Klein et al., 1994). Other markers important in muscle spindle development involve the Nrg1/ErbB system that promotes an early interaction between the Ia fibres and nascent muscle spindles (Andrechek et al., 2002; Hippenmeyer et al., 2002). Moreover, Nrg1/ErbB signaling regulates the expression of various transcription factors that take part to the initiation of muscle spindle differentiation (Burden and Yarden, 1997).
Similarly, end organs in glabrous and hairy skin require to be innervated for their development (Zelena, 1994). They usually express the TrkB receptor showing sensitivity towards brain derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4; Fleming and Luo, 2013). During early development, they also express another neurotrophic receptor tyrosine kinase, Ret (Luo et al., 2009). Ret-signaling is essential for the central projections of cutaneous afferents, since in Ret-deficient mice mechanoreceptors reach the dorsal horn but fail to extend collaterals to deeper laminae III-V (Honma et al., 2010). Their maturation is also influenced by the TrkA/NGF signaling (Fundin et al., 1997) and to a smaller extent by TrkC/NT3 signaling, in particular related to Merkel cells development (Cronk et al., 2002). The extrinsic neurotrophic signaling activates intrinsic transcriptional programs critical for innervation and maturation.
Therefore, neonatals represent a good experimental model due to easier manipulations and better *in vitro* viability, but it should be reminded they have still immature tissues and structures which could defer from the adult ones.

2 Spinal networks for rhythmic motor behaviors

Many forms of animal motor behavior are rhythmic. Rhythmic motor behaviors can be ongoing, such as breathing, chewing and walking, or episodic, like scratching, swallowing, coughing and sneezing (Marder and Calabrese, 1996). Interestingly, rhythmic motor programs also underlie the expression of emotions (LeDoux, 1996), self-survival-related behaviors, as the search of water and

food (Andersson, 1978), sexual behavior (Pfaff, 1999) and memory formation (Grillner et al., 2005). Rhythmic movements are governed by *central pattern generator* circuits (CPGs) that provide the timing of motoneuron discharge and determine appropriate sequences of muscle activation (Prinz, 2006). CPGs do not require sensory information and supraspinal input for their activity, even though they are needed for a dynamic adaptation to environmental changes and for initiation/termination of motor tasks, respectively (Grillner and El Manira, 2015). Invertebrates and vertebrates have a wide number of CPGs distributed along the neuraxis (Grillner, 2006). In mammals, the CPG controlling locomotion is utterly located in the spinal cord (Kiehn and Kjaerulff, 1998) and it is responsible for the rhythmic and coordinated movement of limbs during gait.

2.1 The mammalian *central pattern generator* (CPG) for locomotion

A considerable interest in unveiling the neuronal basis of locomotion has raised since the beginning of the 20th century and undoubtedly still represents a hot topic in current neurobiology (Clarac, 2008). Initially, it was generally accepted that locomotion could be assisted by different types of reflexes when elicited by appropriate stimulation, as demonstrated by Sherrington during his studies on decerebrated and chronic spinal cats. Sherrington coined the phrase "reflex stepping" to indicate that afferent input from muscle proprioceptors and cutaneous afferents could induce locomotion as a result of evoked reflexes (Sherrington, 1910; 1913a; b). In opposition to the reflex theory considering locomotion under a predominant peripheral control, Thomas Graham Brown suggested the existence of central neural oscillators called "half centers" (Graham Brown 1911; 1914). As reported in the cartoon on the top of figure 4, he recorded activity from tibialis anterior (ankle flexor) and gastrocnemius (ankle extensor) muscles in a decerebrated cat. An original record by Graham Brown (1911) shows that spontaneous rhythmic bursts of muscle contractions could be detected as soon as spinal dorsal roots were transected (traces underneath the cartoon in figure 4). He was the first who demonstrated that the isolated spinal cord can induce locomotor-related contractions in antagonist muscles independently from afferent sensory input. However, this idea did not receive support and the concept of locomotor CPG emerged only in the late 1960s (Jankowska et al., 1965; 1967; Grillner and Zangger, 1975; Grillner et al., 1976).

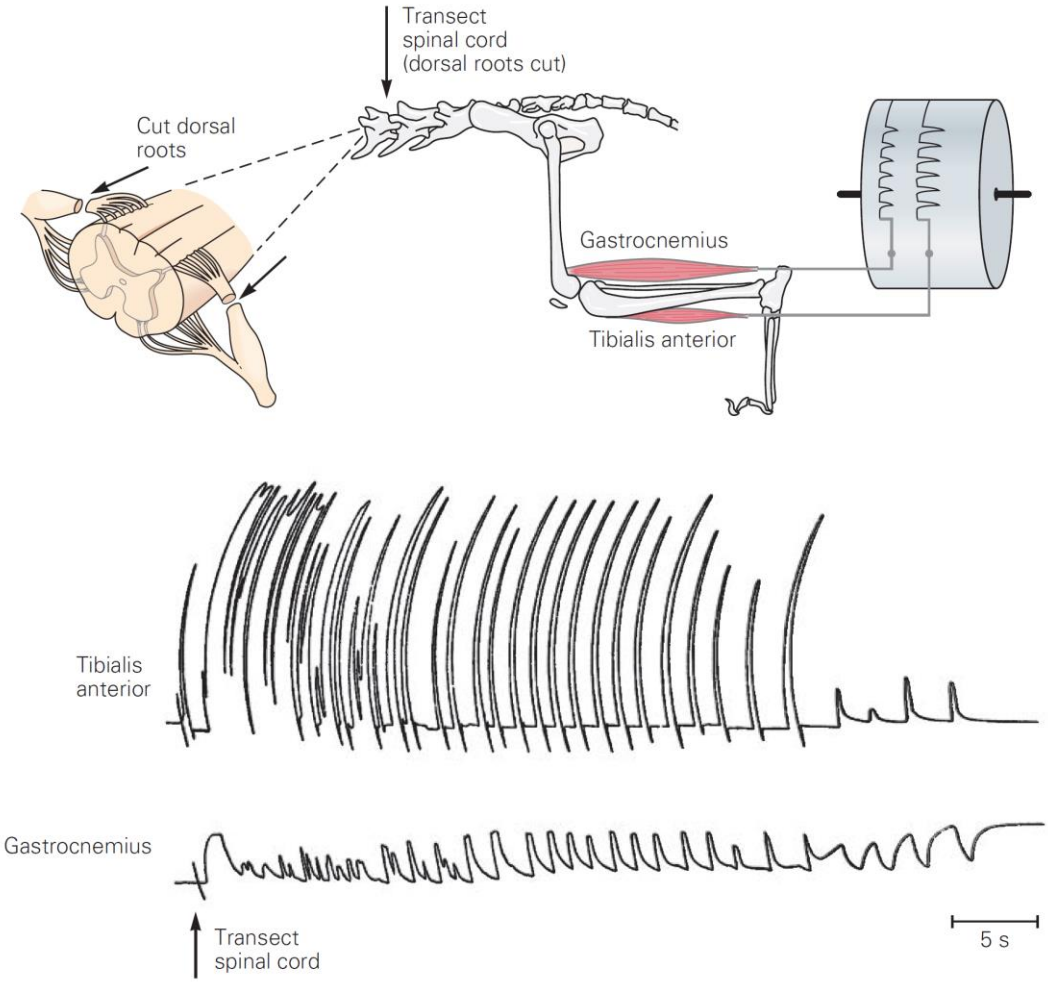

Figure 4: Graham Brown's pioneering experiment demonstrating the existence of neural circuits in the spinal cord able to generate locomotor patterns even in the absence of sensory input. In 1911 Graham Brown performed his experiment on a decerebrated and deafferented cat. He recorded the electromyographic activity from two antagonist muscles, tibialis anterior (ankle flexor; upper trace) and gastrocnemius (ankle extensor; lower trace). In traces the rise of the EMG signal denotes contraction, while the fall relaxation. A rhythmic and alternated activity could be recorded upon dorsal roots cutting. (From "Principles of neural science", fifth edition, chapter 36).

Research over the last years has provided detailed information about network structure and cellular composition of CPGs controlling locomotor behaviors in invertebrates (Friesen and Stent, 1978) and non-mammalian vertebrates, such as lamprey (Grillner et al., 1991) and *Xenopus* tadpole (Roberts et al., 1998; McLean et al., 2000). Compared with this extensive knowledge, still less is known about the mammalian CPG for locomotion. Although the vast complexity of the mammalian CNS, vertebrates share some basic and characteristic circuit components termed "building blocks" (Getting, 1989). As depicted by figure 5, in lower (lamprey) as in higher (primates) vertebrates, the mesopontine and diencephalic centres initiate locomotor activity through the activation of lower brainstem reticulospinal neurons. In turn, neurons in the brainstem activate the spinal CPG which generates a motor pattern dynamically adapted to external changes by the action of sensory feedback (Grillner et al., 1998). Thus, the main operation scheme for locomotion in mammals has

been defined, but the intrinsic organization and operation mode at a circuit and cellular level remain to be unveiled. A full comprehension would be of outstanding importance in improving neurorehabilitative interventions in subjects with spinal lesions or other neuromuscular disorders.

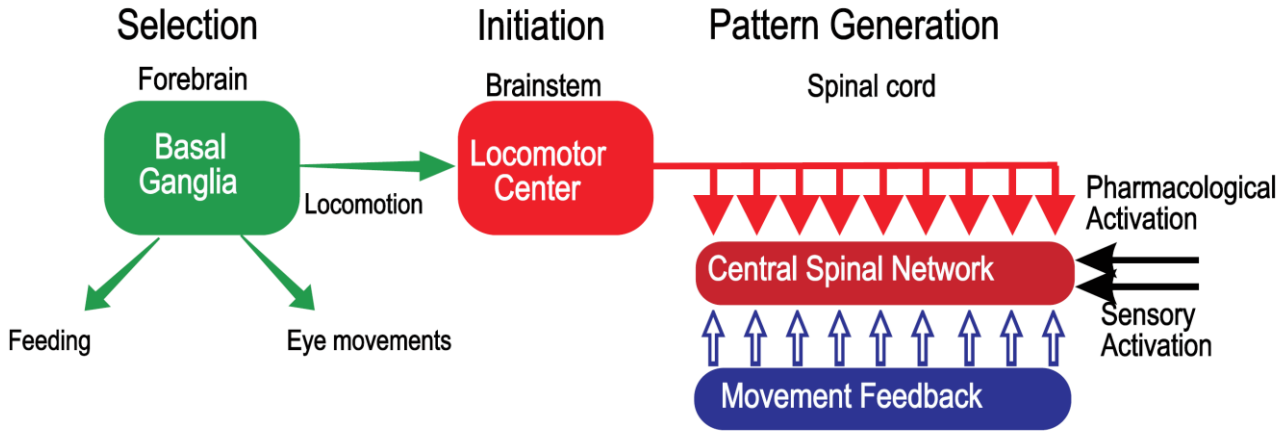

Figure 5: Schematic view of general control system of locomotion in vertebrates. Basal ganglia select a motor pattern through the tonic release of inhibitory neurotransmitter on other motor centres. Once a motor behaviour is selected (in the case shown in figure, locomotion), the corresponding locomotor centre in the lower brainstem becomes active. Neurons in the medial reticular formation of the brainstem initiate the locomotor pattern by projecting to the spinal CPG that executes locomotion. The output of the locomotor CPG is finely modulated by sensory afferent inputs from the periphery. The spinal CPG can also be pharmacologically activated. (Modified from Grillner et al., 1998).

2.1.1 Localization of the locomotor CPG

Over the last years, a great effort has been done in defining the exact location of locomotor circuits within the spinal cord. Research aimed at identifying the localization of the CPG network takes advantage of different techniques: (1) mechanical lesions, such as transections, gradual cuttings of the cord, sections along specific axis, quadrant or segment ablations (Bracci et al., 1996 b; Whelan et al., 2000; Taccola and Nistri 2006; 2007; Zaporozhets et al., 2011); (2) activity-dependent labeling using horseradish peroxidase (HRP) and its conjugates, sulforhodamine, c-fos etc., which label presumed rhythmically active neurons (Harrison et al., 1984; Kjaerulff et al., 1994; Jasmin et al., 1994); (3) calcium imaging (Bellardita et al., 2017); (4) extracellular and intracellular recordings (Kiehn and Kjaerulff, 1998).

These techniques have generally been used in combination and their results have sometimes given rise to scientific disagreements on the longitudinal distribution of motor CPG networks in the spinal cord (Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997). In the last few years, it has been reached a general consensus in stating that the rostrocaudal distribution of the rat CPG networks comprehends lumbar segments from L1-L3 to L4-L6 (Kiehn, 2006; figure 6). Moreover, the mammalian hindlimb CPG has a rostrocaudal excitability gradient, with the highest rhythmogenic capacity in the most rostral segments and the lowest excitability most caudally (Kjaerulff and

Kiehn, 1996; Kiehn and Butt, 2003; Kiehn, 2006). This was proved by partitioning studies using Vaseline barriers to obtain two hermetically sealed compartments in the recording chamber where the upper and lower lumbar cord could be selectively exposed to rhythmogenic drugs (Cazalets et al., 1995; Beato and Nistri, 1999; Bertrand and Cazalets, 2002; Taccola et al., 2010). Thus, when neurochemicals were applied to the upper lumbar segments (L1-L3), rhythmic activity could be recorded in the upper as well as in the lower lumbar ventral roots, whereas when the same solution was superfused in the lower compartment (L3-L6), only tonic activity could be detected. For this reason, it has been proposed that rhythmogenic circuits are localized to the upper lumbar segments, whereas lower lumbar segments do not have rhythmogenic properties.

While the longitudinal distribution of the locomotor network is controversial, there is little disagreement about its transverse distribution. A huge number of data has proved that the CPG circuit is located in the ventral spinal cord, in an area corresponding to laminae VII, VIII and X (figure 6). This has been confirmed by microlesion and electrophysiological studies (Bracci et al., 1996 b; Kjaerulff and Kiehn, 1996), and by activity-labeling studies (Cina and Hochman, 2000; Dai et al., 2005). Also with respect to the transverse plane, it has been recognized a rhythmogenic gradient with the lower rhythmogenic potential in the lateral direction and vice versa (Kjaerulff and Kiehn, 1996).

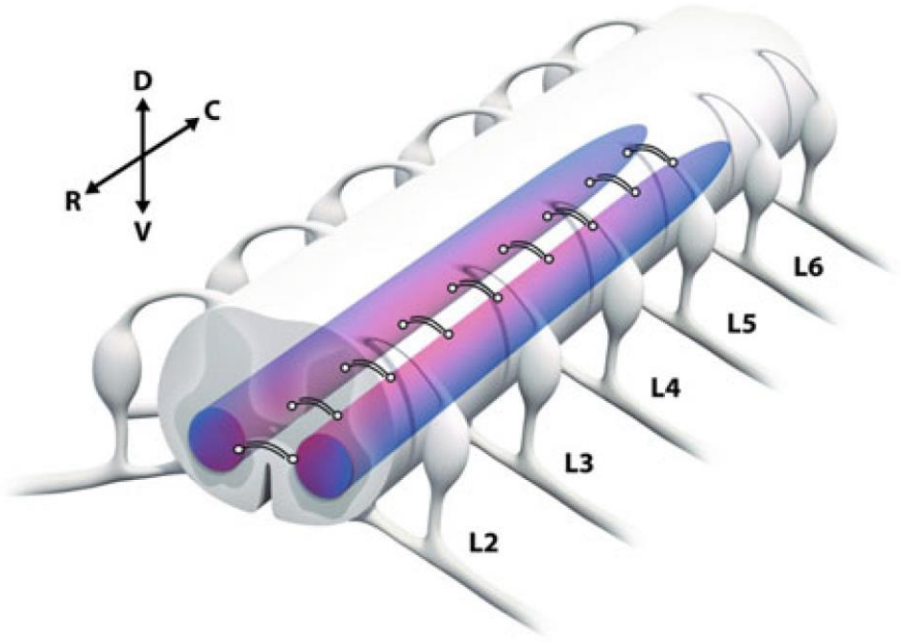

Figure 6: Localization of the locomotor hindlimb CPG in the rodent spinal cord. CPG networks are located in the ventral lumbar spinal cord with a L1-L6 rostrocaudal extent. The colour gradient indicates a higher rostral and medial ability to generate rhythmic activity (purple) and a lower caudal and lateral rhythmogenic capability (blu). (Adapted from Kjaerulff and Kiehn, 1996).

2.1.2 Organization of the locomotor CPG

The first general schematic for the description of the functional organization of the spinal CPG for locomotion was proposed by Graham Brown (1914). He suggested a half-center organization of the locomotor CPG which was later embraced by Lundberg and colleagues (Lundberg, 1981). The half-center model predicts that each limb has a separate CPG (the half-center) containing two populations of excitatory interneurons that project to flexor and extensor motoneurons, respectively. Between the half-centers there are mutual inhibitory interconnections to ensure that only one center is active at a time. When in the active half-center arise mechanisms of "fatigue" and its excitability falls below a critical level, the opposing half-center is released from inhibition and can become active. Thus, the half-center model consists in a single-level model in which the rhythm generation is produced by excitatory interneurons that have also the task to excite the corresponding motoneuron populations. In other words, the same interneuronal circuit operates as rhythm generator and unit pattern formation.

Graham Brown's idea has been further implemented in a considerable number of single-level models and it has represented a template for all the successive organization models of spinal locomotor networks. In 1981 another single-level model, the unit burst generator (UBG), was suggested (Grillner, 1981). The UBG model consists of multiple coupled oscillators (or units), each representing a half-center and directly controlling a subset of motor pools. In was proposed to explain that locomotion is not a strictly alternating pattern with all motoneurons classified as flexors and extensors, as indicated by the half-center model. On the contrary, locomotor activity is complex and may include motoneuron pools that display activity during both flexion an extension phases of the step cycle (Guertin, 2009). However, despite the proposed UBG architecture was more flexible than the classical half-center model, it could not explain other complex patterns of motoneuronal activity.

Single-level models have several limitations. Firstly, circuits generating the rhythm and those activating motor pools are the same, so any changes in excitability of the half-centers should simultaneously affect both cycle timing and motoneuron activity (McCrea and Rybak, 2007). Moreover, this simple architecture does not explain non-resetting deletions frequently observed in cat (Duysens, 1977; Lafreniere-Roula and McCrea, 2005; Duysens, 2006), rodent (Zhong et al., 2012), turtle (Lennard, 1985; Stein, 2008). Deletions are defined as the absence of one or few cycles of rhythmic bursts of motoneuron activity. They are classified as "non-resetting" when rhythmic motoneuron activity reappears without phase shift in the locomotor cycle (Lafreniere-Roula and McCrea, 2005). A reasonable explanation for non-resetting deletions is that the CPG contains

circuits that maintain the timing of the locomotor cycles during failures and that these circuits are different from the ones responsible for motoneuron excitation (Rybak et al., 2006 a). Therefore, it appears evident that single-level CPGs cannot explain non-resetting deletions and that a two-level model must be introduced (Lafreniere-Roula and McCrea, 2005; Rybak et al., 2006 a; b). It should be reminded that these findings have first been suggested by Lennard, who described resetting and non-resetting deletions during an electrically-elicited fictive swimming in an *in vitro* turtle preparation (Lennard, 1985). In the model shown in figure 7, the CPG contains a single half-center rhythm generator that controls the activity of multiple unit pattern formation modules connected to flexor and extensor motoneuron populations. Rhythm generator and unit pattern formation are similarly organized with excitatory interneurons in each half-center divided into extensor and flexor populations and reciprocal inhibition mediated by inhibitory interneurons between half-centers. Thus, in a two-level CPG organization rhythm generator and pattern formation networks are separate. In such a hierarchical model, non-resetting deletions occur at the level of pattern formation that projects to motoneurons, whereas resetting deletions are likely to represent a failure in the "clock" circuits generating the locomotor rhythm. The two-layer model, not only gives an explanation to non-resetting deletions, but also clarifies why afferent perturbation changes motor pattern activity without influencing the rhythm (Burke et al., 2001; Rybak et al., 2006 b) and why a change of amplitude in locomotor output can happen independently of a change in frequency (Kriellaars et al., 1994). In other words, such a layout in CPG organization elucidates the reason why the pattern can change independently of the rhythm. In conclusion, this model can explain many experimental phenomena and it realistically reproduces the main features of the real CPG operation.

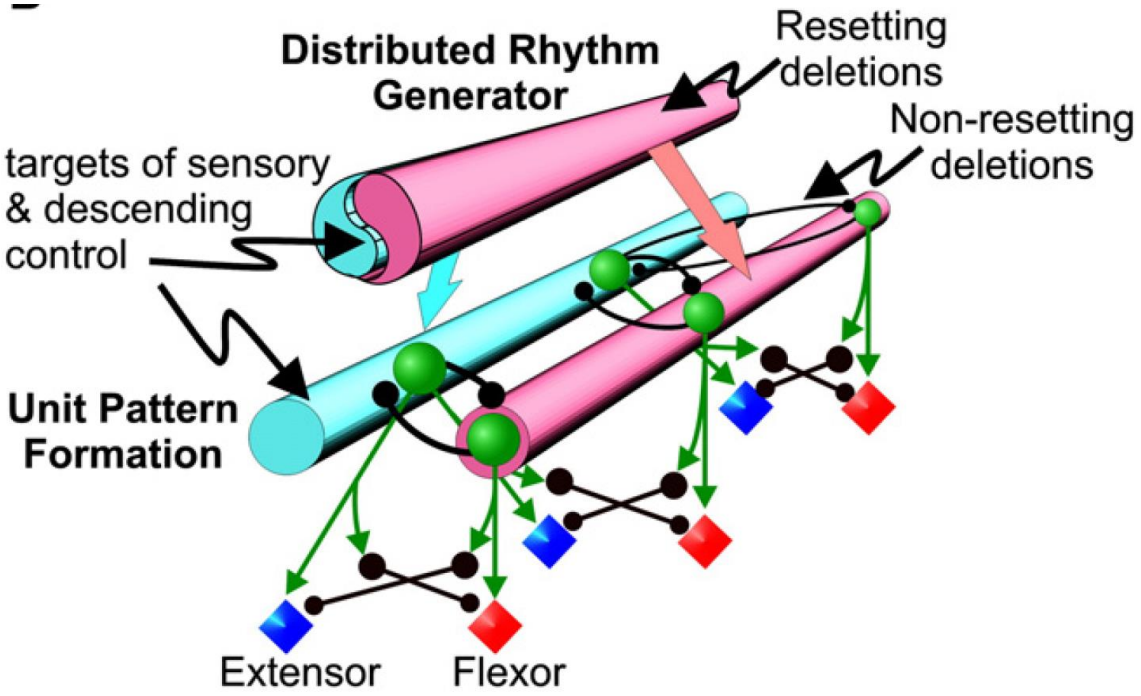

Figure 7: The two-level CPG organization. The model has a hierarchical structure with the rhythm generator and the unit pattern formation being separated. There is a single rhythm generator with a half-center-like organization and multiple concatenated unit pattern formation modules. Excitatory flexor (light red) and extensor (light blue) interneurons in the rhythm generator project (light blue and light red arrows pointing towards the lower level) to the corresponding subpopulations in the unit pattern formation modules, which in turn control activity of flexor (red) and extensor (blue) motoneurons. Inhibitory interneurons mediate reciprocal inhibition between half-centers. Excitatory and inhibitory connections are shown by green lines ending with arrowhead and by black lines ending with small circles, respectively. Afferent and descending inputs reach both the rhythm generator and the unit pattern formation modules, as depicted by black arrows on the top left. Resetting deletions are due to failure of the rhythm generator, whereas non-resetting deletions arise from errors in unit pattern formation modules. (Modified from McCrea and Rybak, 2008).

The model first suggested by Lennard and then validated by McCrea and Rybak has a symmetrical organization. However, experimental data demonstrate that the consequences of flexor and extensor deletions are quite different, indicating a basic asymmetry in the CPG network organization with a flexor-dominated architecture (Zhong et al., 2012). For this reason, Rybak and colleagues are revising their earlier model to incorporate the new data and to generate a thorough model of the rodent CPG, which can serve as a template for other and more complex neural networks in the CNS.

It should be mentioned that, besides the prevailing half-center model for rhythm generation, a new perspective has been proposed by Berg and colleagues (Berg et al., 2007). The traditional half-center model suggests a temporally segregated excitation and inhibition, where the excitation of one half-center drives the reciprocal inhibition of the antagonist half-center. On the contrary, Berg and co-workers have demonstrated that excitatory and inhibitory conductances are simultaneously increased during motoneuron membrane depolarizing waves of scratch-like episodes in an *in vitro* turtle preparation of isolated carapace-spinal cord. Thus, these authors suggest the coexistence of

balanced, rather than temporally segregated, inhibitory and excitatory synaptic activity driving spinal networks for locomotion.

2.1.3 Intrinsic rhythmogenic capacity of spinal locomotor networks

A key feature of spinal locomotor circuits is their intrinsic rhythmicity. The source of rhythm generation seems to be a core of glutamatergic excitatory interneurons, first identified in the swimming CPG of lamprey (Grillner, 2003). This network provides a rhythmic synaptic drive to spinal motoneurons and to circuits accountable for left-right and flexor-extensor coordination (Kiehn, 2016).

In experiments where there was a pharmacological block of inhibitory synaptic transmission, it was possible to generate spinal rhythmic activity. This result has been confirmed in lamprey hemicord (Cangiano and Grillner, 2003; 2005), in mouse (Bonnot et al., 1998; 2002), in rat (Bracci et al., 1996 a; Kremer and Lev-Tov, 1997), in cat (Kato, 1987; Noga et al., 1987). These data suggest that rhythm generation in the mammalian spinal cord is exclusively ascribable to excitatory networks that can generate the locomotor rhythm in the absence of inhibition (Kiehn, 2006).

Since excitatory CPG neurons are the only responsible for rhythm generation, it is supposed they should have some pacemaker-like properties (Ivanchenko et al., 2008; Brocard et al., 2013) or voltage-dependent membrane conductances (Tazerart et al., 2007; 2008) that support rhythmic firing. The pacemaker concept relies on neurons that have intrinsic oscillatory properties and generate the tempo of the rhythm, even when synaptic transmission is blocked. In this case, rhythmicity is due to a core of neurons with endogenous bursting properties that drive bursting in other cells. Such neurons are referred to as endogenous bursters, oscillators or pacemaker cells. They take synaptic contact with follower cells that burst as a consequence of synaptic input from pacemaker cells, but are tonically active or silent when synaptic transmission is pharmacologically blocked (Marder and Bucher, 2001). Importantly, it has been demonstrated that the persistent (slowly inactivating) sodium current (I_{NaP}) contributes to the generation of pacemaker activities in the locomotor CPG (Tazerart et al., 2008; Brocard et al., 2010) and that its blockade by riluzole abolishes locomotor-like activity in rodents (Tazerart et al., 2007; Zhong et al., 2007; Brocard et al., 2010). Recently, it has been shown that neuron pacemaker properties induced by I_{NaP} are switched on and tuned by activity-dependent changes in the extracellular concentrations of Ca^{2+} and K^{+} (Brocard et al., 2013).

Besides pacemaker systems, rhythmic patterns have also been proposed to arise from network mechanisms. The network hypothesis suggests that the rhythm arises from neurons that do not have

intrinsic rhythmogenic capacity, since they are silent or fire tonically when synapses are blocked (Ivanchenko et al., 2008). Thus, rhythmicity arises from network connectivity and rhythmic bursts are the result of cooperative, synapse-mediated instability (Nowotny and Rabinovich, 2007). Principles underlying network mechanisms of rhythmicity are still elusive and further investigation is required.

Defining sources of rhythmic activity is very complex if it is considered that in the spinal cord there are also neurons that possess conditional pacemaker properties. This is a minor percentage of unidentified neurons located in the ventromedial region, close to the central canal (Kiehn, 2006). The ability of these neurons to generate rhythmic activity in synaptic isolation depends on the presence of neuromodulators, such as NMDA (N-methyl-D-aspartate), 5-HT (5-hydroxytryptamine, serotonin) and muscarine (Hochman et al., 1994; Kiehn et al., 1996). Moreover, there is growing experimental evidence proving that some neural systems have a combined pacemaker-network mechanism for rhythm generation. Such a hybrid model of rhythmogenesis implies the existence of cooperation and competition mechanisms between pacemaker cells and network dynamics (Ivanchenko et al., 2008). This model has successfully been applied to the CPG for respiration in mammals (Rybak et al., 2004; 2007).

2.1.4 Circuits involved in locomotor coordination

Another feature of spinal locomotor networks is their capability to generate a well-coordinated pattern. During walking it is possible to recognize a double alternating coordination (1) between flexor and extensor muscles on the same limb and (2) between the left and right sides of the body. While the onset of rhythmicity requires only an excitatory drive, the appropriate sequence of motoneuron activation needs inhibitory networks for the execution of a coordinated motor pattern.

In flexor-extensor coordination, flexor and extensor motoneurons receive a rhythmic glutamatergic excitation which is alternated to a rhythmic glycinergic inhibition (Kiehn et al., 1997). When glycinergic inhibition is blocked, synchronous bursts emerge and this leads to a synchronous contraction of ipsilateral flexor and extensor muscles (Cowley and Schmidt, 1995; Beato and Nistri, 1999). Inhibitory circuits controlling flexor-extensor coordination are mostly unknown. It is thought that part of the inhibition could come from commissural interneurons (CINs), even though in sagittaly hemisected cords flexor-extensor alternation persists (Kjaerulff and Kiehn, 1996). Other inhibitory interneurons involved in flexor-extensor coordination might be Ia interneurons (Ia-INs) and Renshaw cells, both projecting to spinal motoneurons and rhythmically active during locomotion (Pratt and Jordan, 1987). However, in knockout experiments where Ia-INs and Renshaw

cells are markedly reduced or absent, it is still possible to detect a flexor-extensor rhythm (Kiehn, 2016). This suggests that there should be other inhibitory interneurons playing a role in the flexor-extensor coordinating circuitries and some effort has been done trying to identify a minimal inhibitory network that is needed to produce flexor-extensor alternation during locomotion (Talpalar et al., 2011). Moreover, it is currently under investigation the genetical identity of inhibitory interneurons mediating flexor-extensor interactions in the mammalian spinal cord (Shevtsova and Rybak, 2016).

Left-right coordination is mediated by CINs whose axons cross the midline via the ventral commissure. CINs have been extensively studied because they are of relatively simple identification. Based on their anatomical organization, CINs can be classified in different groups, mainly intrasegmental and intersegmental (Bannatyne et al., 2003). It is believed that these anatomically defined groups have distinct roles in locomotor circuits controlling left-right coordination through both excitatory and inhibitory synapses. Recently, two molecularly distinct classes of CINs have been identified and are thought to be involved in the relationship between left-right coordination and locomotor speed during different gaits (Talpalar et al., 2013). Lastly, several computational models are being implemented in the attempt to explain mechanisms of left-right coordination (Rybak et al., 2013; Molkov et al., 2015; Shevtsova et al., 2015).

2.1.5 Genetically-identified ventral interneurons contributing to the locomotor program

Functionally different interneuronal populations compose spinal networks for locomotion. Experiments combining genetics and molecular biology approaches have been useful in identifying key neuronal elements of the spinal CPG for locomotion. Based on the expression of transcription factors, four major classes of ventral horn interneurons have currently been suggested to take actively part to the locomotor program, consisting in V0, V1, V2 and V3 interneuronal populations (Jessell, 2000; Goulding and Pfaff, 2005).

The V0 interneurons are located in the ventromedial spinal cord and belong to genetically-identified CIN populations (Pierani et al., 2001). They can further be subdivided into three subtypes based on transmitter phenotype (Rybak et al., 2015): $V0_D$ are dorsally located inhibitory interneurons; $V0_V$ are ventrally located glutamatergic interneurons; $V0_C$ are cholinergic cells surrounding the central canal. Both the inhibitory $V0_D$ and the excitatory $V0_V$ subtypes are involved in left-right coordination (Lanuza et al., 2004; Talpalar et al., 2013). The inhibitory $V0_D$ CINs are thought to mediate a direct inhibition on the contralateral half-center when active (Talpalar et al., 2013). On the contrary, excitatory $V0_V$ CINs are supposed to mediate their task through two possible

pathways. For instance, when the ipsilateral extensor half-center is activated, they might inhibit the contralateral extensor half-center via excitation of interposed inhibitory neurons (Kiehn, 2011; Talpalar et al., 2013; Shevtsova et al., 2015). In alternative, they might also directly deliver excitatory input to the contralateral flexor half-center (Shevtsova et al., 2015). The genetical ablation of both $V0_D$ and $V0_V$ populations in knockout animals leads to left-right synchronization and hopping-like motor activity (Talpalar et al., 2013). A selective ablation of the inhibitory $V0_D$ CINs impairs left-right coordination at low frequencies, while maintaining alternation during a high frequency locomotor output. Vice versa, ablation of the excitatory $V0_V$ CINs preserves left-right coordination at low, but not at high motor frequencies (Talpalar et al., 2013). This suggests that $V0_D$ and $V0_V$ CIN populations control left-right coordination in a frequency-dependent manner.

The V1 interneurons include functionally different inhibitory neurons. Some V1 subpopulations take direct synaptic contacts with motoneurons, such as Ia inhibitory interneurons and Renshaw cells (Goulding, 2009). Other V1 interneurons have been shown to regulate the frequency of the locomotor CPG rhythm and to control the speed of locomotor outputs (Gosgnach et al., 2006; Zhang et al., 2014). Lastly, they are involved in flexor-extensor coordination (Goulding et al., 2014; Zhang et al., 2014). However, their selective removal of V1 cells results in a reduced frequency of locomotor activity, which is not completely abolished (Zhang et al., 2014). This suggests that other interneuron populations take place in flexor-extensor alternation.

The V2 interneurons can be further classified in two major subgroups: V2a excitatory interneurons and V2b inhibitory interneurons (Al-Mosawie et al., 2007; Lundfald et al., 2007). Similar to observations in $V0_V$ knockouts, V2a genetic ablation causes left-right synchrony at high locomotor frequency, while preserving left-right alternation at low locomotor frequency (Crone et al., 2008; 2009; Dougherty and Kiehn, 2010 a; b). In contrast, V2b selective ablation impaires flexor-extensor coordination, whereas a complete synchrony is achived only after simultaneous silencing of both V1 and V2b populations (Goulding et al., 2014; Zhang et al., 2014).

Lastly, the V3 interneurons are mainly excitatory CINs. Their silencing does not affect neither left-right nor flexor-extensor coordination (Zhang et al., 2008). However, when V3-mediated neurotransmission is selectively blocked, the regularity and stability of the locomotor rhythm are lost. Thus, V3 interneurons contribute to a balanced and symmetrical distribution of excitatory drive between half-centers during the locomotor program (Zhang et al., 2008).

Thus, genetic and molecular approaches have allowed identifying different ventral horn interneuron populations that belong to the spinal CPG for locomotion. Their exact functional role in the locomotor program must still be elucidated. To date, these four genetically-identified classes of

spinal interneurons do not give a full explaination to all electrophysiological data collected on locomotor CPG operation. This suggests that currently unknown interneuron populations must still be identified.

2.1.6 Supraspinal control of locomotion

Locomotion is finely controlled by many regions of the brain (see figure 8), each with a different regulation role of motor pattern. Neural structures involved in the supraspinal control of locomotion are: (1) the basal ganglia responsible for behavior selection; (2) the mesencephalic locomotor region (MLR) in the midbrain and the reticular formation (RF) in the lower brainstem that initiate the behavior; (3) the cerebellum that integrates sensory information with internal commands to organize a coordinated motor pattern maintaining posture and balance; (4) the cortex that performs visual adjustments of locomotor movements (Goulding, 2009). Hereafter, I am focusing on selection and initiation mechanisms of locomotion.

Selection mechanisms of an appropriate motor pattern take place in the basal ganglia. Neurons in the basal ganglia have axons projecting to the MLR. They are inhibitory GABAergic neurons with a high tonic activity at rest. When locomotion is selected, their inhibitory activity on the MLR decreases and the MLR neurons activate and initiate locomotion (Grillner and Robertson, 2015). Basal ganglia project also to the lateral and medial hypothalamus. When the lateral hypothalamus activates, it is selected a motor behavior consistent with food search. On the contrary, when he medial hypothalamus is turned on, a defensive or escape locomotor behavior is selected. Since these neuronal pathways are active in life-threatening situations, the hypothalamus may bypass the MLR and project directly to the RF in the brainstem for motor pattern initiation (Grillner et al., 2005).

The initiation commands for locomotion have origin in the MLR and in the brainstem RF. Neurons in the MLR are excitatory and contain glutamate and acetylcholine as their neurotransmitters. Instead of directly projecting to the spinal cord, MLR neurons take synaptic contacts with neurons in the RF in the lower brainstem that provide the final signal that initiates locomotion (Brocard and Dubuc, 2003; Kiehn, 2016). Neurons sending final initiation commands to the spinal cord are glutamatergic (found in all vertebrates) and serotoninergic (found only in mammals). The evidence for glutamatergic and serotoninergic descending pathways from the brainstem came from studies where the pharmacological blocking of glutamate and/or serotonin receptors interfered with the transmission of initiation commands to spinal locomotor circuits (Jordan et al., 2008; Kiehn et al., 2010).

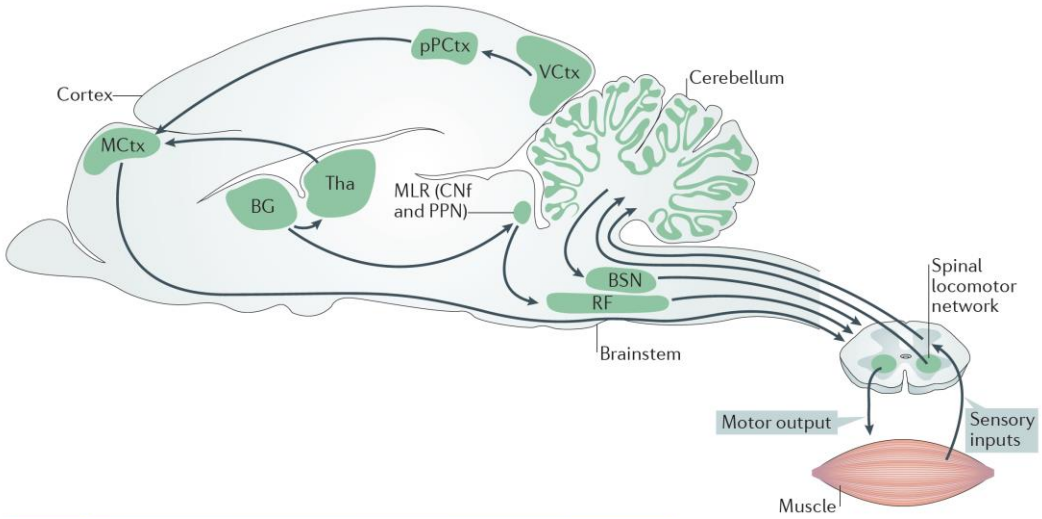

Figure 8: Representation of brain centers controlling locomotion. Basal ganglia (BG) select the locomotor behaviour. Initiation of locomotion is mediated by the mesencephalic locomotor region (MLR), including the cuneiform nucleus (CNf) and the pedunculopontine nucleus (PPN). MLR neurons project to neurons in the reticular formation (RF) in the lower brainstem that transmit final initiation commands to the spinal cord. The cerebellum coordinates the motor pattern and maintains posture and balance. The motor cortex (MCtx) uses visual information from the posterior parietal cortex (pPCtx) for visuomotor correction (VCtx) of locomotion and precise stepping movements. (From Kiehn, 2016).

2.1.7 Development of spinal locomotor circuits

Locomotor networks in the spinal cord have been extensively studied employing *in vitro* preparations from neonatal rats. In rat, the overall development lasts for at least 6 weeks, about 3 weeks in utero (E0-21) and 3 weeks in the nest with the mother (P0-21; Clarac et al., 2004). Thus, the rat is quite immature at birth, but undergoes a rapid maturation during the first two postnatal weeks (Vinay et al., 2000 a). However, CPG circuits for locomotion are functional at early stages, as demonstrated both *in vivo* and *in vitro* (Nishimaru and Kudo, 2000). In newborns, locomotion can be spontaneously evoked *in vivo* by reducing postural constrain due to musculo-skeletal immaturity, for example by lifting the animal in the air (air stepping; McEwen et al., 1997; Mendez-Gallardo et al., 2016) or by placing it in a pool (Cazalets et al., 1990; McEwen et al., 1997). In the rat fetus, coordinated movement resembling locomotion can be observed at embryonic day E20.5 (Bekoff and Lau, 1980; Robinson et al., 2008). *In vitro*, a locomotor-like pattern can be elicited through the bath-application of neurochemicals, such as NMDA and 5-HT (Cazalets et al., 1992), or electrically stimulating a dorsal root (DR; Marchetti et al., 2001 a; Taccola, 2011). All these observations demonstrate that neural circuits underlying locomotion are already operative at birth, but a still immature posture represents a limiting factor for spontaneous locomotion during the first postnatal days.

In the lumbar spinal cord in the rat fetus spontaneous and synchronized bursts of activity can be recorded from the embryonic (E) day 13.5 to E18.5, presumably mediated by GABA (γ-aminobutyric acid) and glycine that are excitatory at this age (Nishimaru et al., 1996). Kudo and Yamada first studied the maturation of spinal locomotor circuits (1987 a) and demonstrated that NMDA and/or 5-HT can induce a rhythmic spinal activity in different lumbar roots at E14 (1987 b). This indicates that the locomotor rhythm generator is active at this developmental stage (Kudo and Nishimaru, 1998; Nishimaru and Kudo, 2000). Moreover, transection studies have demonstrated that the rostral region in the embryo is endowed with a higher rhythm capability than the caudal one, confirming the existence of a rhythmogenic rostro-caudal gradient similar to that in the neonatal rat spinal cord (Nakayama et al., 1999).

Coordination mechanisms appear upon the rhythm-generating core becomes active. Left-right alternation is established between E16.5 and E18.5 and it seems to be linked to the maturation of the GABA/glycine-mediated synaptic transmission, that switches from excitatory to inhibitory exactly during this embryonic period (Wu et al., 1992). The switch is due to a shift of the reversal potential for chloride (E_{Cl}^-) towards hyperpolarized values (Ben-Ari, 2002), mediated by a down-regulation of NKCC1 (Na-K-Cl cotransporter 1) and up-regulation of KCC2 (K-Cl cotransporter 2; Rivera et al., 1999; Yamada et al., 2004). Moreover, the appearance of CINs at E14.5 and their maturation is essential for contralateral coordination (Nakajima et al., 2002). Flexor-extensor alternation has a later onset (E20.5), most likely because inhibitory circuits involved in flexor-extensor coordination are formed after E18.5 and start to function at E20.5 (Nishimaru and Kudo, 2000). Altogether, these data point out that CPG networks for locomotion are endowed with their key features, rhythmogenesis and coordination, during the embryonic period.

Spinal locomotor circuits receive sensory and descending projections. The development of afferent connections and supraspinal inputs has a different timing. Sensory collaterals reach the dorsal horn at E15.5, the intermediate region containing CPG circuits at E16.5 and motoneurons at E17.5 (Vinay et al., 2000 a). Indeed, the monosynaptic stretch reflex pathway is present at E18.5 (Kudo and Yamada, 1985; 1987 a). Initial connections are not all specific, with a peak of inappropriate contacts at postnatal (P) days 0-2. Within 1 week after birth, all redundant and inappropriate synapses become silent or are eliminated and the remaining appropriate connections are stabilized and refined (Seebach and Ziskin-Conhaim, 1994; Mears and Frank, 1997). Primary afferents are functional only at the end of the first week in controlling the posture of each limb (Brocard et al., 1999) and a full maturation is reached more than three weeks later (Clarac et al., 2004).

In addition to sensory afferent fibres, spinal networks receive descending projections. Brainstem projecting neurons are generated from E11 to E15 (Altman and Bayer, 1980 a; b; c; d) and the earliest projections are detected in the cervical cord at E13-14 and at lower thoracic levels at E14-15 (Lakke, 1997). Brainstem descending fibres reach the upper lumbar cord slightly before birth and the lower lumbar segments during the first postnatal days (Lakke, 1997), while the earliest corticospinal projections arrive at the end of the first postnatal week (Schreyer and Jones, 1982). A full development of most descending pathways is reached at P12-14 (Clarac et al., 2004). Thus, even though spinal locomotor networks are fully functional at birth, appropriate connections with the periphery and higher brain centers develop only during the first postnatal weeks.

Besides network maturation, motoneuron modifications during the early post-natal life have been extensively studied. Motoneurons are produced at E13-14 in the lumbo-sacral cord (Altman and Bayer, 1984) and most of them die during the embryonic period (Lance-Jones, 1982) and within the first 5-6 days after birth (Bennett et al., 1983).

During development, they undergo different morphological and physiological changes. Main morphological modifications affect the soma, which increases during embryonic stages and reaches adult size by birth, and the dendritic tree, which increases the total surface area and the average branch order becoming more complex (Cameron and Núñez-Abades, 2000; Carrascal et al., 2005).

Changes in somatodendritic morphology are accompanied by modifications in electrophysiological properties of lumbar motoneurons. It has been observed that during postnatal development there is a reduction in input resistance, consistent with the increase in motoneuron size and with variations of voltage–dependent and –independent conductancies (Fulton and Walton, 1986; Seebach and Mendell, 1996). Rheobase, the minimal injected current required to generate an action potential, is inversely correlated to input resistance and increases more than five times during postnatal development (Seebach and Mendell, 1996). These changes are consistent with a reduced excitability of lumbar motoneurons after birth. Furthermore, the duration of the action potential decreases between E16 and birth in the rat lumbar motoneurons (Gao and Ziskind-Conhaim, 1998), which, in turn, leads to a reduced afterhyperpolarization (AHP) duration (Viana et al., 1993). The shorter AHP duration parallels an increase in firing rate in mature cells with respect to newborn motoneurons (Viana et al., 1994). As a final result, the augmented firing rate would allow adult motoneurons to evoke faster muscle contractions (Kernell et al., 1999). Noteworthy, the maturation process is not simultaneous for all motor pools, but it is delayed in extensor motoneurons (E-MNs) with respect to flexor motoneurons (F-MNs), with a full development of F-MNs at P3-5 and only a

71 % maturation of E-MNs in the same postnatal period (Vinay et al., 2000 b). Part of this disparity can be attributed to differences in AHP duration, shorter in F-MNs than in E-MNs at birth, which, in turn, makes repetitive firing of E-MNs lag behind that of F-MNs (Vinay et al., 2000 b).
Lastly, changes in electrotonic coupling are observed during development. In the perinatal spinal cord, motoneurons innervating the same muscle are connected via gap junctions (Walton and Navarrete, 1991). Electrotonic coupling decreases with age and is no longer present after the first postnatal week. This is due to the disappearance of two out of five connexins present in the embyo and to the inactivation of the remaining three isoforms (Chang et al., 1999). The presence of not functional connexins in the adult spinal cord leads to the reestablishment of electrotonic coupling in certain circumstances, such as peripheral or central lesions (Chang et al., 1999).

In conclusion, during postnatal development, spinal locomotor circuits undergo a significant transformation concerning wiring changes, that drive the establishment of appropriate connections, and morphological and physiological cellular maturation. However, the neonatal spinal cord appears an appropriate model for the study of the locomotor CPG, since locomotor networks are fully functional at birth.

2.1.8 Evidence for a locomotor CPG in humans

While the existence of a CPG for locomotion has been directly demonstrated in lower mammals, there is indirect evidence that CPGs may also be a feature of the human spinal cord.
The first evidence proving the presence of spinal CPG circuits in humans is represented by the onset of rhythmic movements and contractions in subjects with a spinal cord injury (SCI). An early report claimed that a subject with a complete SCI could sometimes produce “self-propagating” stepping movements (Kuhn, 1950). Another report asserts that rhythmic myoclonic contractions of the trunk and lower limb extensor muscles could be evoked in a person with a spinal cord transection and that this rhythmic activity had only one step cycle duration (Bussel et al., 1988). Descriptions of rhythmic involuntary movements have been also reported in subjects with an incomplete SCI, where the presence of alternated lower limb activity is more common (Calancie et al., 1994; Dobkin et al., 1995). Moreover, in paraplegics it is possible to induce muscle leg activation resembling locomotion by means of external and manual control of stepping movements (Dietz et al., 1995; Dobkin et al., 1995).
Further evidence for a locomotor CPG in humans is achieved by experiments where the spinal cord is stimulated with electrical pulses at specific sites. The stimulus is generally represented by a

sustained, non-patterned train of electrical stimuli. The rationale of this approach is to replace the missing (because of the lesion) supraspinal tonic drive generated by brainstem neurons and mediated by descending pathways (Duysens and Van de Crommert, 1998). Dimitrijevic and coworkers first demonstrated that a tonic, non-patterned epidural stimulation of the lumbar spinal cord in subjects with complete, long-lasting SCI, can elicit step-like electromyographic (EMG) activity derived from antagonist limb muscles (figure 9; Dimitrijevic et al., 1998). Since then, several groups continue investigating properties of epidural stimulation (Minassian et al., 2007; Harkema et al., 2011; Sayenko et al., 2014). Moreover, a less-invasive spinal cord stimulation technique, the transcutaneous stimulation, has been shown effective in triggering step-like movements and, therefore, is collecting resounding interest (Gerasimenko et al., 2015; Minassian et al., 2016).

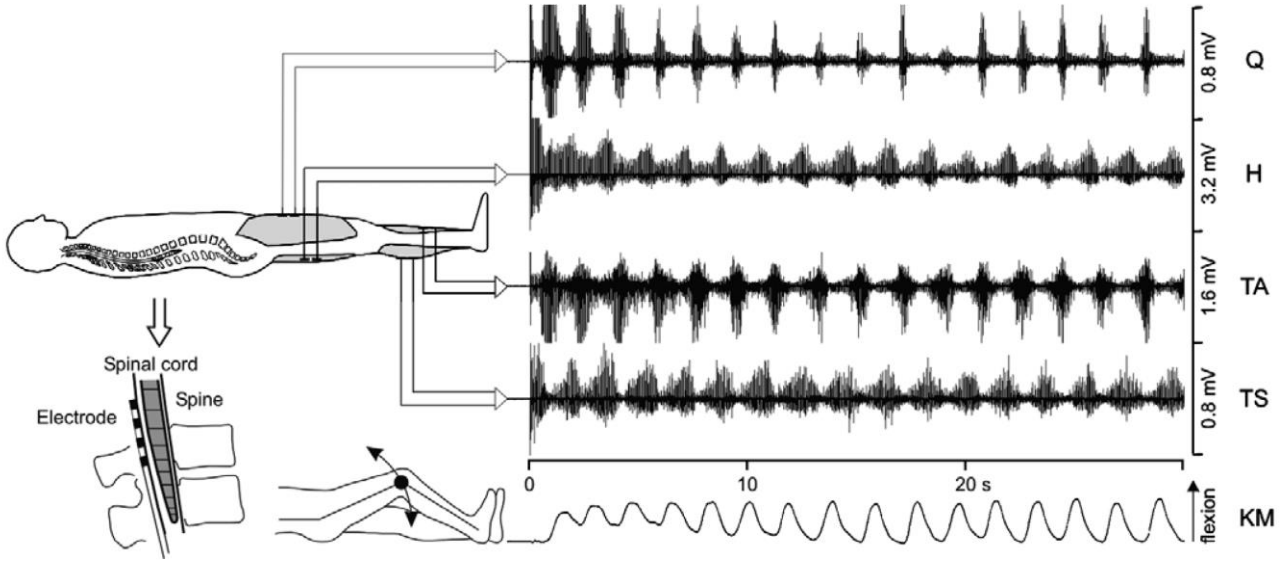

Figure 9: Reproduction of the experiment first performed by Dimitrijevic and colleagues, demonstrating the existence of a locomotor CPG in humans. The spinal cord injured subject has previously undergone surgical implantation of the stimulating epidural electrode at L2 spinal segment (T11-12 spinous processes). Noteworthy, all the enrolled subjects have a chronic, complete SCI at a high-thoracic or cervical spinal level, thus few spinal segments above the implantation site of the epidural electrode. During the experiment, the subject lies in supine position. Upon tonic epidural stimulation, EMG activity is recorded with surface electrodes from quadriceps (Q), hamstrings (H), tibialis anterior (TA) and triceps surae (TS). Note that activity is alternated between antagonist muscles (Q/H and TA/TS), clear sign of spinal locomotor circuits activation. Moreover, a position sensor is placed on the knee to trace rhythmic knee movements (KM) of the paralyzed lower limb. (From Minassian et al., 2007).

Another evidence has been found in newborn infants. When externally supported, they manifest an innate, stereotyped movement pattern (Forssberg, 1985). The gait pattern of human infants (7-12 month of age) has been investigated under body weight support on a split-belt treadmill, where treadmill belts run in different directions (Yang et al., 2005). The study is based on previous work on kittens, where the animal preserved coordination while stepping on a split-belt treadmill, indicating that in the postnatal period CPG circuits for each leg have some degree of autonomy

while interacting with each other for coordination (Robinson and Goldberger, 1986). Yang and colleagues have confirmed this observation in infant humans where legs can move independently on the split-belt, demonstrating the presence of an autonomous pattern generator for each limb.

Moreover, the fact that a locomotor CPG exists in humans is supported by the possibility to induce air-stepping, characterized by rhythmic EMG activity of antagonist muscles of the leg, through vibration of single muscles or of antagonistic muscles in the suspended leg (Kazennikov et al., 1997; Selionov et al., 1997). Air-stepping can also be induced by electromagnetic stimulation of the spinal cord at T11-12 spinous processes (Gorodnichev et al., 2010; Gerasimenko et al., 2010).

Lastly, sleep-related periodic leg movements (SRPLM or nocturnal myoclonus), are also consistent with the existence of a spinal CPG in humans (Yokota et al., 1991). They consist in stereotyped, periodic, repetitive movements involving one or both lower limbs. SRPLM have been suggested to show a spinal origin and to be related to spinal automatisms of locomotor circuits (Yokota et al., 1991).

In summary, these findings indicate that the lumbosacral spinal cord in humans hosts spinal locomotor circuits comparable to those observed in lower mammals. These circuits, called CPGs for locomotion, can generate a motor pattern when appropriately stimulated, even in the absence of supraspinal drive.

2.2 *In vitro* preparations for the study of the locomotor CPG

Neurophysiological mechanisms underlying locomotion can be uncovered only by using suitable experimental preparations. Pivotal studies that allowed defining the concept of CPG were first performed on decerebrate cat preparations walking on a treadmill after electrical stimulation of the MLR (Shik and Orlovsky, 1976). Since then, many invertebrate models (Friesen and Stent, 1978) and vertebrate systems with relatively few neurons (Roberts, 1983; Grillner and Wallen, 1985) have contributed to broaden our knowledge on spinal networks for locomotion. The need for models which are evolutionary closer to humans has pushed toward rodent preparations.

The neonatal rat preparation was first used in the form of an isolated hemisected spinal cord superfused with physiological solution (Otsuka and Konishi, 1974). Only few years later, a complete dissected preparation from the brainstem to the sacrococcygeal spinal cord was employed to investigate the respiratory rhythm (Suzue, 1984). First studies on locomotion were performed on this preparation applying in the perfusing medium different neuroactive substances to obtain a rhythmic motor pattern (Kudo and Yamada, 1987 b; Smith and Feldman, 1987).

The neonatal *in vitro* preparation has many remarkable advantages. First, surgical procedures can be done quickly and are simpler with respect to other mammals. Second, the preparation is relatively small and easily handled. Since the preparation is isolated from a neonate, the CNS is not myelinated yet, making it suitable to be kept in a recording chamber with an adequate perfusion system for hours without losing functionality. Moreover, the blood-brain barrier has been removed, therefore allowing for the administration of drugs that do not cross the hemato-meningeal barrier. In addition, all the chemical parameters of the perfusion solution mimicking the cerebrospinal fluid can be carefully controlled, such as pH, temperature and ion concentrations. Lastly, the dorsal input and the ventral output are preserved, unlike other *in vitro* models such as acute slices or organotypic cultures. All these features make the isolated spinal cord of the newborn rat a useful model to study properties of mammalian locomotion and the underlying circuitry.

Besides the isolated spinal cord, a more intact preparation has been proposed, consisting in a partially dissected spinal cord with the hindlimbs and their innervation intact (Hayes et al., 2009; 2012; Hochman et al., 2013). The main advantage of this preparation is that allows investigating the influence of sensory inputs on spinal locomotor circuits. Since primary afferents directly control and modulate CPG activity (Rossignol, 2006), their contribution to rhythmic motor patterns might be explored adopting this *in vitro* model. Furthermore, in this experimental condition, spinal circuits are provided with incoming information directly originating from hindlimb proprioceptors and mechanoreceptors during *in vitro* passively-driven stepping or cycling. The leg-attached preparation offers also a stable accessibility to spinal roots, allowing for long-lasting recordings of activity in spinal circuitries. This is enabled by the possibility to preserve viability for hours without significant changes in functionality of dorsal and ventral networks, similar to the isolated spinal cord.

2.3 *In vitro* activation of the locomotor CPG

A hallmark of CPG activation is the double alternation between flexor and extensor motor pools within the same side of the spinal cord and between left and right motor pools within the same spinal segment. *In vitro*, it is possible to record bursts of alternated activity bilaterally from lumbar L2 and L5 ventral roots (VRs), innervating flexor and extensor muscles of the rat hindlimb, respectively (Kiehn and Kjaerulff, 1996; Juvin et al., 2007). This rhythmic, oscillatory activity is called *fictive locomotion* (FL), since does not correspond to the real movement of hindlimbs during overground stepping (Forssberg et al., 1975). FL can be induced in the isolated spinal cord with or

without hindlimbs either by bath-applying neurochemicals in the perfusion system or by stimulating one dorsal root (DR) with electrical protocols.

2.3.1 Neurochemical activation

A broad spectrum of neuroactive substances is employed to evoke FL *in vitro*, as reported by many studies investigating the operation of the mammalian CPG for locomotion. Excitatory aminoacids (EAAs), such as glutamate and aspartate, and EAAs agonists, such as NMA (N-methyl-D,L-aspartate), NMDA and kainate, can trigger FL when added in the physiological solution (Kudo and Yamada, 1987 b; Cazalets et al., 1992). However, the role of NMDA receptor in rhythm generation in the mammalian spinal cord appears to be controversial. On one side, there is evidence that the NMDA receptor modulates membrane properties in spinal motoneurons and interneurons which significantly contribute to the operation of locomotor circuits (Schmidt et al., 1998). On the other side, FL can be evoked even in the presence of AP5 (D-2-amino-5-phosphono-valeric acid), a NMDA receptor blocker, when spinal networks are exposed to an appropriate excitatory drive mediated by non-NMDA receptors (Cowley et al., 2005). Moreover, it has been shown that CPG activity can be triggered after pharmacological block of either NMDA or non-NMDA receptors, although with a lower frequency, suggesting that both classes of glutamate receptors are not essential for rhythm generation and that the activation of one class at a time is sufficient to elicit FL (Beato et al., 1997).

Also the monoamine 5-HT can activate locomotor networks of the neonatal rat spinal cord *in vitro*. The serotonin-induced rhythm is very slow and its period is dose-dependent (Cazalets et al., 1992). When 5-HT is applied in combination with NMDA, it slows down and regularizes the FL typically elicited by NMDA alone, inducing a very stable pattern which lasts for several hours (Squalli-Houssaini et al., 1993; Kudo and Nishimaru, 1998; Taccola et al., 2012).

Another amine, noradrenaline (NA), has some particular rhythmogenic properties, since elicits an extremely slow alternating motor pattern (period of 80-90 s; Squalli-Houssaini and Cazalets, 2000). Moreover, when NA is applied during ongoing locomotor activity evoked by NMDA and 5-HT, it has a slowing effect on the rhythm without disrupting the alternated pattern (Kiehn et al., 1999). This suggests a very complex modulatory effect of this amine on spinal locomotor networks.

The role of dopamine (DA) on the locomotor CPG has also been characterized. Kiehn and Kjaerulff (1996) have found out that DA can induce rhythmic activity alone or in combination with 5-HT, and that DA-induced motor pattern is slow and irregular when compared with the FL evoked by 5-HT. Furthermore, DA preferentially facilitates extensor activity and supports a rhythm which is

closer to a stepping-like pattern in the *in vitro* preparation of neonatal rat spinal cord with one hindlimb attached.

Acetylcholine (Ach) has also been investigated and it has been proved that Ach cannot induce a locomotor-like pattern, since it evokes a left-right alternation but fails in eliciting a flexor-extensor alternation (Cowley and Schmidt, 1994). In addition, neuropeptides, like substance P, can induce only few VRs alternated discharges when applied alone, whereas heavily modulate a locomotor rhythm evoked by NMA and 5-HT (Barthe and Clarac, 1997).

Lastly, bath-application of high extracellular K^+ concentrations can generate motor patterns in the neonatal rat spinal cord (Bracci et al., 1998). The threshold concentration to observe the effect described by the authors is about 8 mM and there is a very narrow range to evoke a lasting FL. It is important to recall that the K^+ concentration in the cerebrospinal fluid of the developing rat at rest is arount 3 mM (Jones and Keep, 1987). On the contrary, the extracellular K^+ dose used by the authors falls within a range estimated in the interstitial fluid during episodes of FL triggered by electrical stimulation of afferent fibres (Marchetti et al., 2001 b). The rhythm evoked by high extracellular K^+ can still be elicited even in the presence of activity blockers of NMDA, non-NMDA and 5-HT receptors, only if extracellular K^+ concentration is further increased. Moreover, when subthreshold concentrations of K^+ are coapplied with subthreshold doses of NMDA or 5-HT, there is a mutual facilitation suggesting that an increase in network excitability is required for the operation of the spinal CPG for locomotion.

Thus, the huge amount of data collected, makes the *in vitro* preparation of neonatal rat particularly suitable for pharmacological studies that require a direct access to spinal locomotor networks bypassing the blood-brain barrier.

2.3.2 Electrical activation

Drug application is a useful tool in the investigation of spinal motor patterns, but it represents a condition far away from the physiological one, since the entire spinal tissue is in prolonged contact with substances that are usually released with finely time-regulated gradients *in vivo*. A more physiological way able to recruit spinal CPG networks is by repetitive stimulation of primary afferents either in lumbar roots (Marchetti et al., 2001 a) or in sacro-coccygeal roots (Lev-Tov et al., 2000; Gabbay et al., 2002; Taccola et al., 2011). This strategy determines the activation of spinal circuits for locomotion by inducing afferent fibres to release endogenous neurotransmitters. Electrical protocols consist in canonical trains of electrical stimuli delivered at a certain frequency (1 – 10 Hz; Marchetti et al., 2001 a) or in innovative noisy waves sampled from a chemically-

evoked FL *in vitro* (Taccola, 2011; Dose and Taccola, 2016) or from human motor patterns (Dose et al., 2013).

Canonical protocols are characterized by a repetitive stimulation, since single pulses are ineffective in evoking FL (Marchetti et al., 2001 a). Most probably, only a train of pulses can supply the network with a sufficient excitatory drive enrolling a neuronal population large enough to give rise to the locomotor pattern. An oscillatory rhythm arising from dorsal root stimulation shows the characteristic double alternation that marks out CPG activation. However, this pattern is short-lasting, showing an episodic nature unlike the ongoing rhythm elicited by NMDA and 5-HT. The decay in ventral oscillatory discharges during stimulation might be due to fatigue mechanisms taking place in the pathway upstream of the CPG, presumably ascribable to excitatory transmitter depletion in afferent terminals in the neonate (Lev-Tov and Pinco, 1992; Marchetti et al., 2001 a; Li and Burke, 2002). Moreover, a release of inhibitory neurotransmitters causing loss of rhythmicity cannot be excluded (Tabak et al., 2000; Marchetti et al., 2001 a). Noteworthy, changes in the frequency of stimulation, usually ranging between 1 – 10 Hz in canonical protocols, do not pair with variations in the frequency of the locomotor pattern, which preserves a typical periodicity of 1 – 2 seconds, as reported in pharmacological studies (Cazalets et al., 1992). This observation might be consistent with the existence of an intrinsic control system in spinal CPG circuits, a sort of filter with non-linear properties in the processing of incoming and outgoing inputs (Marchetti et al., 2001 a; Taccola et al., 2011). This hypothesis is supported by the finding that staggered delivery (delay of 0.5 – 2 seconds) of low-frequency pulse trains (0.33 and 0.67 Hz) to three different dorsal roots can reliably induce FL, while the synchronous delivery is ineffective (Dose et al., 2016). Thus, the spinal cord can somehow decode and process complex multisite afferent stimuli into a stereotyped pattern corresponding to the locomotor-like activity.

Innovative protocols of dorsal root stimulation imply the use of complex-shaped noisy waves containing a broad spectrum of frequencies. The first noisy protocol was obtained sampling a chemically-induced FL from a VR of an *in vitro* neonatal rat spinal cord (Taccola, 2011). This stimulating protocol was termed FL*i*stim (FL-induced stimulation) and it was characterized by a great intrinsic variability consistent with the fact that it derives from a biological signal. Since then, other noisy protocols have been constructed by sampling real human locomotion (ReaL*i*stim; Dose et al., 2013). Noisy waveforms are much more effective in inducing the locomotor rhythm with respect to canonical protocols, given that they are delivered at very low intensities (Taccola, 2011; Dose et al., 2013). Moreover, they show a synergistic effect with low doses of neurochemicals in triggering locomotor spinal networks (Dose and Taccola, 2012; Dose et al., 2014). Recently, the

complexity of noisy wave protocols has been dissected and a pair of frequencies (35 and 172 Hz) has been identified as the minimum stimulation paradigm able to optimally elicit FL when simultaneously delivered (Dose and Taccola, 2016).

Since electrical stimulation of afferent fibres activates the CPG for locomotion in the neonatal rat preparation, there is a clear demonstration that sensory input from the periphery takes direct contact with spinal locomotor networks at this developmental stage. The interplay between primary afferents and the CPG will be extensively discussed in the next section.

2.4 Interactions between afferent inputs and spinal locomotor activity

Dynamic sensorimotor interactions during locomotion between primary afferents and the spinal cord are a key feature underlying the locomotor program. On one hand, sensory feedback controls the activity of the spinal CPG for locomotion to adapt the motor output to external demands. On the other hand, the afferent inflow of information is continuously exposed to a central barrage in order to reduce or abolish exceeding and inappropriate sensory signals which could impair the locomotor pattern.

2.4.1 Sensory control of CPG activity

The locomotor CPG can generate a detailed rhythmic pattern which produces the precise timing and phasing of muscle activity needed to locomote in the absence of sensory information. Indeed, complete or partial removal of sensory afferents by dorsal rhizotomy does not prevent locomotion (Goldberger, 1983; Grillner and Zangger, 1984). Similarly, irreversible destruction of large-diameter sensory fibres after pyridoxine intoxication allows for a certain extent of locomotor recovery (Pearson et al., 2003). Moreover, in decerebrate and spinal cats where paralysis has been induced by a chemical neuromuscular blockade, FL can be triggered by administering L-DOPA (L-3,4-dihydroxyphenylalanine or levodopa; Grillner and Zangger, 1979; Pearson and Rossignol, 1991).

Although CPG circuits provide the basic locomotor rhythm, their activity is controlled and modulated by sensory signals adapting the operation of the CPG to the external environment and continuously adjusting its output (Frigon and Rossignol, 2006; Rossignol et al., 2006; Windhorst, 2007). For example, sensory control is responsible for allowing, preventing or selecting motor patterns, for the correct positioning of limbs and feet on the ground, for modifications in pattern frequency and amplitude and for regulation of phase transition (McCrea, 2001; Rossignol et al., 2006). Sensory afferents mediate a dual control on spinal locomotor networks: (1) a task-dependent

modulation where interactions between afferent inputs and the CPG vary according to the motor task (walking forward or backward, running, etc.), and (2) a phase-dependent modulation where changes happen with respect to the step cycle subphase (swing/stance; Frigon and Rossignol, 2006). Thus, mammals constantly use sensory information to adjust their stepping patterns to variations in external conditions and to unexpected perturbations. As depicted in figure 10, the limb provides sensory feedback principally via two classes of peripheral receptors: (1) proprioceptors in muscles and joints are involved in the regulation of stepping and furnish information about body movements encoded by stretch sensitivity from muscle spindles and tension/force sensitivity from GTOs (Windhorst, 2007; Jankowska, 2015); (2) cutaneous receptors induce stepping adjustments to external stimuli or obstacles and control foot positioning during locomotion (Panek et al., 2014; Bui et al., 2015).

Proprioceptive afferents can initiate or terminate locomotor movements. Powerful signals for locomotion initiation originate from the hip and ankle joints. Sherrington (1910) was the first who demonstrated that the stimulation of hip joint proprioceptors by extending the hindlimbs induces air stepping in chronic spinal cats. On the contrary, flexion of one hip joint abolishes the ability to step on that side, whereas the other side continues walking (Pearson and Rossignol, 1991). Concerning ankle joint, it has been reported that loading of ankle extensors during decerebrate cat walking inhibits flexor bursts of activity, whereas unloading of ankle extensors is essential to start the swing phase (Donelan and Pearson, 2004 a; b). Thus, proprioceptive information from hip and ankle joints strongly influences the locomotor CPG.
Moreover, muscle afferents regulate speed adaptation and interlimb coordination at different speeds by controlling step cycle duration and by facilitating the stance to swing phase transition. For example, in both normal and spinal cats walking on a treadmill, an increased belt speed couples with a shortening in stance phase, while the swing phase remains relatively constant (Forssberg et al., 1980; Halbertsma, 1983). These data suggest that afferent inputs regulate the duration of the stance phase by signaling its termination and the subsequent initiation of the swing phase, therefore adapting the speed of each hindlimb.

Cutaneous afferents provide information about the moving limb and the appearance of obstacles on the way. They allow avoiding hindrances through stumble-corrective reactions (Bouyer and Rossignol, 2003 a; b). Corrective responses have been proved to be phase-dependent, with the same stimulus exciting antagonist motor pools during different phases of the step cycle. Indeed, when a

mild mechanical stimulus is applied to the dorsum of the foot during swing phase, a considerable knee flexion is triggered which induces a consequent foot withdrawal followed by hip and ankle flexion to overstep the obstacle. On the contrary, when a similar contact occurs during the stance phase, it evokes the opposite response consistent with excitation of extensor muscles (if a flexion response were produced during the stance phase, the animal would collapse; Forssberg et al., 1977; Forssberg, 1979).
Similar to proprioceptive inputs, some cutaneous inputs can also trigger or stop locomotion. For example, perineal stimulation facilitates the expression of motor patterns (Pearson and Rossignol, 1991), whereas tonic stimulation of the back inhibits both real and fictive locomotion (Frigon et al., 2012).

Thus, sensory information is not necessary in the generation of a motor output. However, it is essential in shaping the locomotor circuits during development, since the removal of afferent feedback coming from the hindlimbs during the early neonatal period disrupts the ongoing locomotor activity due to a loss of excitatory drive from sensory afferents (Acevedo and Diaz-Rios, 2013). Furthermore, afferent fibres strongly modulate CPG activity due to a direct access to spinal circuits underlying locomotion (figure 10). The direct synaptic contact of primary afferents with CPG neurons is proved by the possibility to elicit FL through electrical stimulation of dorsal roots (Marchetti et al., 2001 a; Taccola, 2011), as well as by phase resetting an ongoing locomotor rhythm via electrical stimulation of afferent fibres (Conway et al., 1987; Kiehn et al., 1992; Iizuka et al., 1997). Lastly, the fine control that sensory inputs exert on spinal locomotor networks is associated to different mechanisms, such as presynaptic regulation of input transmission, selection of different and alternative neuronal pathways, changes in membrane properties of motoneurons and interneurons during locomotion (Rossignol et al., 2006).

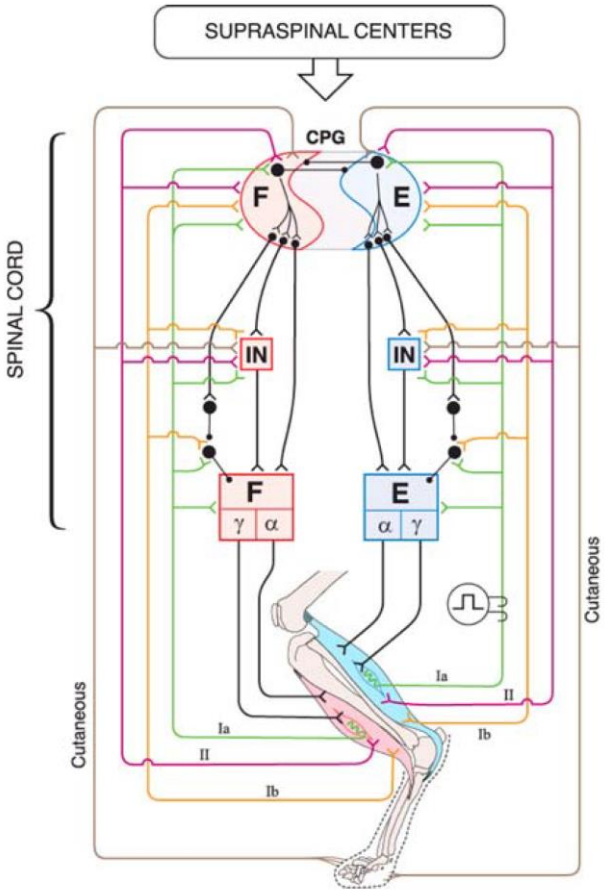

Figure 10: General scheme of sensory pathways controlling spinal circuits for locomotion. From the top to the bottom: higher brain centres involved in motor pattern selection and activation through descending pathways to spinal locomotor networks; the spinal CPG for locomotion represented with the two-level organization and the reciprocal inhibition between flexor (red) and extensor (blue) networks; motoneuron pools including both α- and γ- motoneurons projecting to extrafusal and intrafusal muscle fibres, respectively; hindlimb ankle flexor (red) and ankle extensor (blue) muscles depicted each with a muscle spindle. Afferents originating from primary and secondary spindle endings (group Ia and II, respectively), from GTOs (Ib fibres) and from skin (cutaneous afferents) take synaptic contacts with spinal locomotor circuits at different levels. The stimulation symbol on the right on the Ia afferent fibre from the ankle extensor illustrates the stimulus site for evoking the H-reflex. (From Frigon and Rossignol, 2006).

2.4.2 Primary afferent activity during *fictive locomotion*

Once spinal locomotor networks are in operation, the effectiveness of sensory transmission onto central circuits is regulated by presynaptic inhibition (PSI; Rudomin and Schmidt, 1999). PSI of the afferents represents a sophisticated mechanism of control that allows for the selection of sensory inputs entering the spinal cord, for instance to prevent undue responses in a given phase of the step cycle (i.e., contraction of ankle extensor muscles during swing; Hochman et al., 2013). For this reason, PSI occurs even before the first synapse between the primary afferent terminal and the second order neuron (Willis, 1999) and it can directly be modulated by the ongoing CPG activity during voluntary stepping in the intact cat (Prochazka et al., 1976; 1977), during FL in reduced (decorticate, decerebrate) cat preparations (Dubuc et al., 1988; Gossard et al., 1991; LaBella et al., 1992; Ménard et al., 1999; 2002; Beloozerova and Rossignol 1999; 2004) and during chemically-

evoked FL in *in vitro* preparations of neonatal rat (Kremer and Lev-Tov, 1998; Vinay and Clarac, 1999; Fellippa-Marques et al., 2000; Hayes et al., 2012).

PSI in afferents is typically thought to be mediated by the activation of the $GABA_A$ (γ-aminobutyric acid A) receptor. In the simplest configuration (represented in figure 11), the primary afferent terminal takes axo-dendritic contact with a lumbar motoneuron (postsynaptic cell) and axo-axonic contact with a GABAergic interneuron (Eccles et al., 1962; Rudomin and Schmidt, 1999). The latter, in turn, could be under the control of either sensory inputs, descending pathways or spinal locomotor circuits (Rudomin et al., 1993). Since primary afferents retain high intracellular chloride (Cl^-) due to a persistent expression of NKCC1 even in the postnatal life, $GABA_A$ receptor activation induces a Cl^- efflux and a subsequent primary afferent depolarization (PAD). PAD is thought to reduce glutamate release from the primary afferent terminal by inactivating sodium and calcium channels, thus preventing action potential propagation towards the spinal cord (orthodromic conduction) and reducing their amplitude (Miller, 1998; Rudomin and Schmidt, 1999). PAD is often so intense that it reaches the threshold for the generation of action potentials. These action potentials travel towards the opposite direction in dorsal fibres, that is from the spinal cord to the periphery (antidromic conduction), and they can be experimentally recorded as dorsal root potentials (DRPs; Barron and Metthews, 1938), that represent a measure of PSI (Wall, 1995; Lamotte d'Incamps et al., 1999). This complex mechanism is responsible for filtering or gating incoming sensory inputs from muscle (groups Ia, Ib, II) and cutaneous afferents both at rest and during a motor pattern.

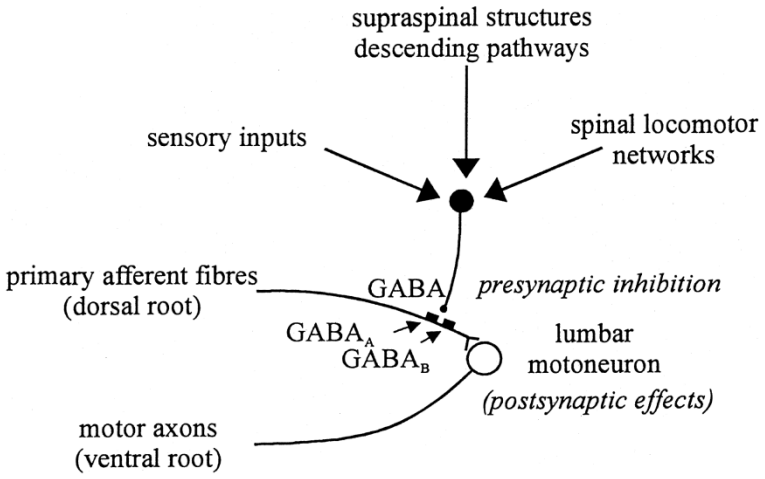

Figure 11: Simplified representation of presynaptic inhibition (PSI). The primary afferent fibre takes axo-dendritic contact with a lumbar motoneuron (postsynaptic cell) and axo-axonic contact with a GABAergic interneuron. This simplified system was first used by John Eccles to unravel inhibitory mechanisms of the spinal monosynaptic reflex pathway. The postsynaptic neuron could also be a dorsal or a ventral interneuron. Note that the GABAergic interneuron could be controlled either by sensory fibres, descending pathways or CPG neurons. The main mechanism for PSI involves the $GABA_A$ ionotropic receptor. A minor role is reported for the $GABA_B$ metabotropic receptor (Miller, 1998). For more detail refer to the text. (Modified from Vinay et al., 1999).

Focusing on antidromic activity generated during FL, it has been shown that DRPs have a rhythmic and phase-dependent pattern during the central locomotor program, being generally maximal during the flexion phase in most of muscle (Duenas et al., 1988) and cutaneous afferents (Gossard et al., 1989). Indeed, intra-axonal recordings have revealed that the membrane potential of muscle afferents exhibits rhythmic fluctuations during FL, with a maximal depolarization during the flexion phase (Gossard et al., 1991). A similar observation has been done relatively to membrane properties of cutaneous afferents where the flexor-related depolarization is ubiquitous, whereas the extensor-mediated depolarization is rare and more variable (Gossard et al., 1989). Studies here discussed have been conducted on curarized animals, where a phasic activity of peripheral origin must be excluded. Therefore, the observed phasic firing of muscle and cutaneous afferents during flexion phase should be due to central mechanisms originating within the spinal cord and mediating an antidromic back-propagation. Thus, it can be concluded that afferent pathways are phasically modulated during FL, with a relatively lower activity (higher PSI) during the flexor phase and vice versa a relatively higher activity (lower PSI) during the extensor phase. In other words, the efficacy of transmission of primary afferents can be altered more during the flexor phase rather than during the extensor phase.

Recently, the dependence of PSI on the phase of the step cycle has also been investigated (Hayes et al., 2012). Data have been collected using an *in vitro* neonatal rat preparation with hindlimbs attached, where an ongoing CPG activation is achieved by bath-applying neuroactive substances. The authors demonstrate that force-sensitive afferents (Ib) are recruited in the stance limb when it contacts the ground. Ib-sensory information evokes mechanisms of PSI and PAD on the swing limb (contralateral) via excitation of glutamatergic CINs. This crossed pathway is most probably under the control of the locomotor CPG, since it is suppressed when the limb is not in stance (Hayes et al., 2012; Hochman et al., 2013). Thus, sensory inflow of information is under a strict control from the spinal cord and a dynamic interaction takes place during locomotion.

3 Rehabilitative approaches in the management of neuromuscular disorders

Neurorehabilitation is a relatively new field. Standardized rehabilitation procedures have been established only in the past 20 years, but physicians still do not fully agree on the most effective approaches. As a result, neurorehabilitative strategies are deeply variable between rehabilitative centres and there is frequently lacking evidence for their effectiveness. The efficacy of a rehabilitative procedure is related to its ability in restoring a certain degree of sensory and motor

function following damage or disease of the CNS. This issue has become pressing over the last years, since it has been reported a progressive increase in the incidence of persons with disabilities due to traumatic events (e.g., spinal cord injury) or to neuropathological (e.g., stroke) and neurodegenerative (e.g., Parkinson's and Alzheimer's) diseases.

Several studies carried out in cat (Frigon and Rossignol, 2008; Barrière et al., 2010; Martinez and Rossignol., 2013; Gossard et al., 2015) and rat (de Leon et al., 2002; Cha et al., 2007; Hansen et al., 2012), as well as in humans (Maengele et al., 2002; Dietz and Müller, 2004; Grasso et al., 2004; Scivoletto et al., 2007; Curt et al., 2008; Molinari, 2009), show that neural networks underlying the generation of motor patterns are quite flexible after central or peripheral neuronal lesions. Indeed, even at the adult age, the nervous system exhibits a good adaptability mediated by mechanisms of neuroplasticity (Cai et al., 2006). Therefore, neurorehabilitative strategies are currently trying to exploit this intrinsic feature of the CNS, since it allows overcoming tissue loss due to the lesion and preventing subsequent sensorimotor deficits by promoting the regain of function in the spared tissue. Hereafter, effects of two therapeutic strategies, spinal cord stimulation and repetitive motor training, are being discussed with respect to potential improvements of the locomotor performance.

3.1 Spinal cord stimulation

Restorative interventions exploit the residual nervous system function to enhance functional recovery from CNS injury or disease. They consist in neuromodulation therapies that target different CNS areas, included the spinal cord. Spinal cord stimulation (SCS) belongs to the broad family of neuromodulation interventions used in restorative neurology (Minassian et al., 2012) and it is able to influence neuronal activity by the interaction of an electrical stimulating protocol with specific spinal networks (Holsheimer, 1998 a).

SCS involves the delivery of electrical current to spinal elements via electrodes placed with a well-defined geometry. To generate a potential difference, at least one cathode and one anode are required, which emit an electric field with lines orthogonally arranged to allow stimulation of spinal elements (Molnar and Barolat, 2013). Since stimulation should have the highest selectivity possible to avoid detrimental effects, stimulation parameters must be carefully selected, paying particular attention to amplitude and frequency (Tehovnik, 1996). Moreover, the spatial displacement of electrodes and the electric field lines they generate should be studied in depth (Molnar and Barolat, 2013).

After positioning electrodes for SCS, it should be considered that the higher the distance from the spinal cord the lower the efficiency of stimulation. The distance between electrodes and target

spinal elements depends on interposed anatomical structures and on different subject positions (e.g., lying supine vs prone; He et al., 1994; Holsheimer et al., 1994). For this reason, it is crucial that the subject does not move or change position during SCS.

The rationale behind SCS is that neurons are artificially depolarized by delivering current to a highly conductive extracellular milieu represented by the cerebrospinal fluid. A distribution of potentials between electrons in stimulating electrodes and ions in the extracellular fluid is generated and charge is transferred across the cell membrane. The electric field modifies the transmembrane potential of the neuron affecting passive membrane properties (capacitance and resistance) and different ion conductances (Molnar and Barolat, 2013). The net effect is an increase in membrane potential toward more depolarized values making the neuron prone to generate action potentials.

SCS has first been used in the treatment of intractable, chronic pain (Shealy et al., 1967). Soon, its potential in ameliorating sensorimotor disorders was recognized and a remarkable spread of the technique has paralleled the technological advancement relatively to implantable stimulators (Mayr et al., 2016). SCS protocols can be distinguished with respect to the stimulation site into epidural and transcutaneous.

3.1.1 Epidural spinal cord stimulation

Epidural stimulation aimed at generating lower limb motor activity is obtained through an epidural lead positioned over the posterior aspect of the lumbar spinal cord, at the level of the second lumbar segment (L2), corresponding approximately to thoracic spinous processes 11–12 (T11–12; Dimitrijevic et al., 1998; Minassian et al., 2004). This precise location corresponds to the position of the spinal CPG for locomotion, as reported by Dimitrijevic and collegues in chronic, motor complete SCI subjects (previously discussed; Dimitrijevic et al., 1998). As schematized by the cartoon in figure 12, structures directly stimulated by epidural lumbar SCS correspond principally to afferent fibres of the dorsal roots and to the dorsal column (Murg et al., 2000; Rattay et al., 2000; Ladenbauer et al., 2010). Indeed, in rats with a complete spinal cord transection (at approximately T8 spinal segment) and a unilateral deafferentation (from dorsal roots T12 to S2), epidural stimulation could facilitate stepping-like movements on a rolling treadmill only on the non-deafferented limb, suggesting that triggering of motor patterns is primarily mediated by ipsilateral sensory information (Lavrov et al., 2008). Moreover, epidural stimulation supplies a tonic neural drive to lumbar spinal networks mimicking the excitatory activity of descending pathways which have been disrupted because of the lesion or the disease (Minassian et al., 2012). The concept that

epidural stimulation might exploit the volitional control mediated by supraspinal input is gaining an increasing clinical support (Angeli et al., 2014).

An appropriate control of lower limbs mediated by epidural stimulation requires a careful identification of effective stimulation parameters. Indeed, it has been reported that the stimulation can produce a variety of non-patterned and patterned motor outputs to the lower limbs, depending on the stimulation amplitude and frequency (Pinter et al., 2000). Focusing on frequencies, an epidural stimulation with a train of pulses within the range 5-15 Hz promotes a bilateral lower-limb extension in motor complete SCI subjects restoring weight-bearing (Jilge et al., 2004; Harkema et al., 2011), stimulation at 25-60 Hz generates or facilitates a rhythmic motor pattern confirmed by the EMG activity of flexor and extensor lower limb muscles (figure 12; Dimitrijevic et al., 1998; Gerasimenko et al., 2002; Minassian et al., 2004; Harkema et al., 2011), and frequencies between 50-100 Hz attenuate exaggerated lower limb activity affecting part of the SCI population (spasticity; Pinter et al., 2000).

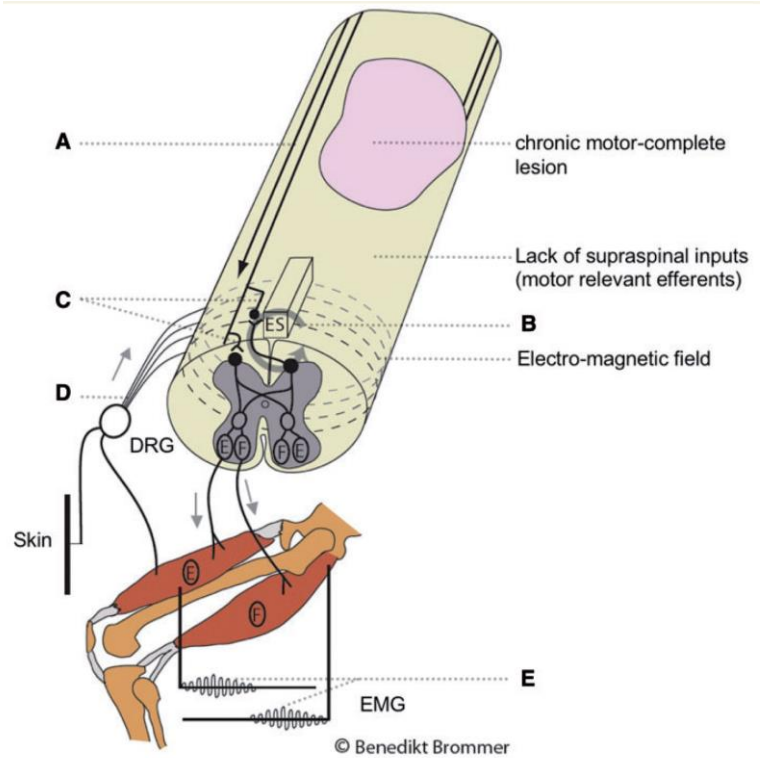

Figure 12: Epidural spinal cord stimulation for the facilitation of locomotor activity. (A) A motor-complete lesion is characterized by an almost complete isolation of spinal networks from higher brain centres. (B) Epidural spinal cord stimulation (ES) is administered through an electrode placed above the *dura mater* that generates an orthogonal electromagnetic field to the spinal cord. ES stimulates both residual ascending and descending fibres (C), as well as primary afferents (D). (E) ES evokes a rhythmic motor pattern derived from flexor (F) and extensor (E) muscles of the lower limb which burst in antiphase as recorded by the EMG. (From Schwab, 2014).

Many studies have evaluated the effect of a combined therapy of locomotor training and epidural stimulation. In chronic, motor complete SCI individuals, it has been observed that the rhythmic

EMG activity of lower limbs produced by manually-assisted treadmill-stepping with partial body weight support was increased when epidural SCS was delivered (Minassian et al., 2005; 2007). In these studies, as well as in others (Herman et al., 2002; Carhart et al., 2004; Ganley et al., 2005; Huang et al., 2006; Harkema et al., 2011), it is demonstrated that the spinal cord can integrate information from afferent fibre activity evoked by passive stepping and the tonic excitatory drive supplied by the stimulation. In all cases reported, an improved muscle activation pattern and an enhancement of EMG activity have been described when epidural SCS is applied during treadmill stepping.

Thus, epidural SCS facilitates standing and walking in subjects with SCI. However, neuromodulatory mechanisms underlying motor pattern formation during epidural stimulation are still unknown. Recently, Moraud and colleagues (2016) have shed light on this explaining that epidural stimulation triggers motor patterns through the modulation of muscle spindle feedback circuits. Therefore, the authors argue that sensory information plays a predominant role in the generation of complex and adaptive motor behaviors when supraspinal drive is disrupted. Moreover, they propose an innovative phase-specific protocol of epidural SCS, where stimulation frequency is varied throughout different phases of the gait cycle to correct gait asymmetries and to improve balance. Wanger et al. (2016), suggest a spatiotemporal epidural SCS, where stimulus parameters can be tailored to improve quality of the kinematic output and to enhance locomotor control. The study exploits a previous finding stating that hindlimb motoneuron pools show a rostrocaudal oscillation gradient of activity, with the most caudal segments of the lumbosacral enlargement displaying an intense motoneuron activity during the stance phase, whereas the most rostral segments exhibiting a peak of motoneuron activation during the swing phase (Yakovenko et al., 2002). Thus, epidural stimulation can be switched back and forth between the flexor and extensor hot spots via a control system that monitors real-time hindlimbs position with respect to the gait cycle. In another original study performed on Rhesus monkey (*Macaca mulatta*), the authors suggest a completely different approach with respect to the first two studies described above, that consists in bypassing the lesion through a brain-spine interface (Capogrosso et al., 2016). In this case, intracortical arrays placed on the leg motor cortex and decoding its activity are interfaced with a spatially selective epidural implant through a wireless control system. This system has been proved to be effective in restoring weight-bearing and locomotion of the paralyzed leg, thus being approved for investigations in humans. As proved by these recent studies, advancement in the field of SCS is strictly correlated to technological improvement of instrumentation, which could lead to an epochal breakthrough in the management of sensorimotor dysfunction. In parallel

to technological progress, a great effort should also be done in identifying basic mechanisms underlying benefits of SCS.

3.1.2 Transcutaneous spinal cord stimulation

The spinal cord can also be electrically stimulated with skin electrodes which represent a non-invasive method (Nardone et al., 2015; Gerasimenko et al., 2015; 2016; Minassian et al., 2016). The way electrodes are placed is fundamental in this stimulation technique, since current has to go through many impediments before reaching the target tissue. In figure 13 is shown the positioning of electrodes suggested by Minassian and co-workers, that consists in a pair of stimulating electrodes placed on the back over paravertebral skin between the T11-12 spinous processes, and a couple of large reference electrodes over the lower abdomen (Minassian et al., 2012). Other groups propose different electrode configurations, for instance using a 2.5-cm round cathode electrode placed midline at the C5, T11 or L1 spinous processes and two rectangular anode electrodes placed on the skin over the iliac crests (Gerasimenko et al., 2015 b). The electrode arrangement depends on stimulation parameters and it is the result of theoretical studies on the geometry of the electric field lines that better allows for a localized stimulation of dorsal root fibres or dorsal column (Struijk et al., 1992; 1993; Holsheimer and Wesselink, 1997; Holsheimer, 1998 b; Danner et al., 2011; Molnar and Barolat, 2013). Indeed, before reaching the well-conducting cerebrospinal fluid in the vertebral canal, the current generated by the transcutaneous electrode should cross two barriers. The first one is represented by the skin, connectives and muscles, while the second one by the spine. In the spine, the transversal electrical resistance is higher in correspondence to the bone making up vertebrae of the thoraco-lumbar segment, whereas it decreases allowing the passage of some current at the level of intervertebral discs between vertebral bodies and of ligaments between the spinous processes and laminae (Ladenbauer et al., 2010; Danner et al., 2011; Szava et al., 2011).

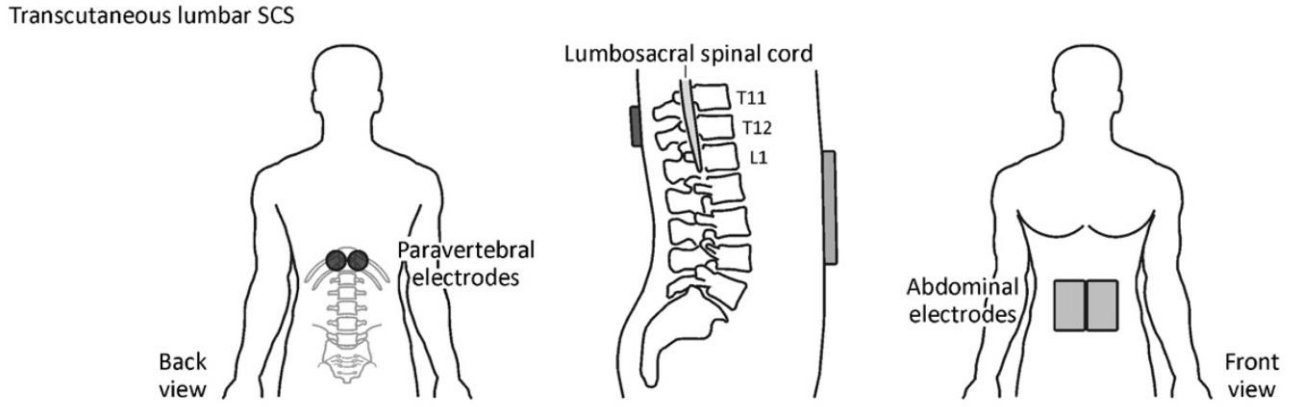

Figure 13: Electrode placement for transcutaneous spinal cord stimulation. The two stimulating (or paravertebral) electrodes are placed on the skin of the back between spinous processes T11-12 (approximately, L2 spinal segment; left). A pair of reference (or abdominal) electrodes is attached over the lower abdomen (right). In the middle, electrodes are shown with respect to the vertebral spine and spinal cord. (From Minassian et al., 2012).

Many studies are trying to clarify the efficacy of nonpatterned transcutaneous SCS in the enhancement of motor functions in people with sensorimotor impairment. Encouraging results have been achieved in decerebrated and spinal cats (Musienko et al., 2013) and in non-lesioned and lesioned subjects (Gerasimenko et al., 2015 c), where a rhythmic, oscillatory stepping-like activation of lower limbs was confirmed by kinematic analysis of the hip, knee and ankle joints and by EMG activity. Although the sole effect of transcutaneous stimulation must be further evaluated, its conjoint application with treadmill stepping appears more convincing. Indeed, tonic transcutaneous SCS delivered during manually-assisted and body-weight supported treadmill mobilization generates or modulates rhythmic lower limb EMG activities in motor complete and incomplete SCI subjects. Furthermore, this EMG pattern has a higher amplitude than the one produced during training alone (Dietz et al., 1995; Minassian et al., 2010; Hofstoetter et al., 2015).
Recently, a multi-site stimulation approach has been proved to evoke a more robust locomotor-like pattern than the canonical single-site stimulation (Gerasimenko et al., 2015 a; c). The protocol consists in delivering a simultaneous and independent electrical stimulation at three different vertebral levels (C5, T11 and L1). When the protocol is applied to a non-lesioned subject placed in a gravity-neutral position, coordinated oscillatory movements and alternated muscle contractions of the lower limbs are observed, with EMG bursts reaching the maximum amplitude within 2-3 step cycles. On the contrary, when stimulation is delivered at one site per time, it is able to elicit a rhythmic limb activation only during T11 transcutaneous SCS, even though with a clear reduction in EMG activity with respect to the multi-site protocol (Gerasimenko et al., 2015 a). Similar protocols in the motor-complete SCI subject increase the amplitude of knee displacement and EMG activity of leg muscles (Gerasimenko et al., 2015 c). Thus, multi-site transcutaneous stimulation activates motor pools at different spinal levels and maximizes their coordinated recruitment throughout the step cycle. Noteworthy, authors have considered only a simultaneous multi-site delivery of transcutaneous stimulation. However, a staggered multi-site stimulation of multiple dorsal roots has given promising results *in vitro* (Dose et al., 2016).

3.2 Training interventions

Over the last years has emerged a common opinion, that the injured or diseased human spinal cord is highly plastic and that plasticity can be driven by activity-dependent interventions, capable to

restore lost or impaired sensorimotor functions (Dietz, 2002; 2003; 2012; Grasso et al., 2004; Scivoletto et al., 2007; Molinari, 2009). An approach frequently applied for rehabilitation of gait is represented by an intense treadmill training capable to induce some functional regain of locomotor activity through the reactivation of spinal locomotor circuits (Barbeau and Fung, 2001; Ditz and Fouad, 2014). Training on a sliding treadmill belt is associated to a body weight support (BWS) system, first introduced by Finch and Barbeau (1985; figure 14). This system is used to unload lower limbs during walking through a fitted harness that allows for suspension in upright position (Barbeau et al., 1987; Norman et al., 1995). Indeed, subjects with sensorimotor dysfunctions (such as spinal lesions) may not have the balance or lower limb strength required to assume independently a standing position and bear their full weight on their legs. Thus, the introduction of a BWS system during training interventions is essential for the compensation of these deficits by providing weight-bearing and balance necessary for stepping. Moreover, leg movement during walking is assisted manually, generally during the first phase of the training in incomplete subjects (depending on the severity of paresis) and during the whole training period in complete paraplegic individuals (Dietz, 2008). It has been demonstrated that a partial weight bearing improves gait function in SCI subjects faster than a full body weight support (Visintin and Barbeau, 1989; Hesse et al., 1997). Thus, an adjustable system for weight bearing is more likely to promote a physiological gait pattern, since it evokes afferent inputs from load receptors which are crucial for the generation of a locomotor EMG pattern (Harkema et al., 1997; Dietz et al., 2002).

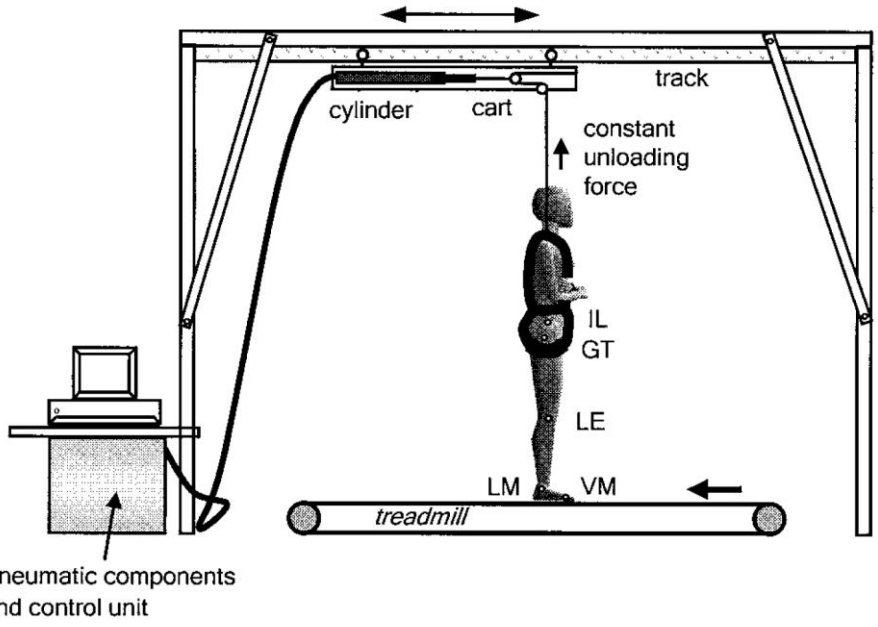

Figure 14: Body weight supported treadmill training for functional recovery of locomotion. The subject walks on a moving treadmill belt allowing for speed selection. A well-fitting harness is connected through a cable to a pneumatic device that exerts a defined unloading force. This system supports the subject in upright position, regulates weight-

bearing and balance and allow for stepping. Limb kinematics can also be recorded by monitoring the spatial coordinates of markers placed on the lower limbs. (From Ivanenko et al., 2002).

Incomplete SCI subjects gain major advantages from training with BWS, having a functionally better ambulation on the treadmill than before training and improving their locomotor performance on a static overground as well (Wernig and Müller, 1992; Barbeau et al., 1993; Visintin and Barbeau, 1994; Wernig et al., 1995; DeForge et al., 1996). A significant improvement in mobility mediated by training can be achieved also in chronic incomplete SCI individuals, where mechanisms of spontaneous recovery without training can be excluded (Wirz et al., 2005; Field-Fote and Roach, 2011). The improvement consists in the fact that the pattern of leg EMG activity and joint angular displacement induced by training are similar to those seen in non-injured subjects (Barbeau et al., 1993; Dietz et al., 1994; 1995). At the end of the rehabilitative training period, spinal incomplete subjects can also perform unsupported stepping movements relearning to walk independently (Dietz et al., 1994; 1995; Wernig et al., 1995).

In contrast, subjects with a complete paraplegia do not achieve unassisted stepping during treadmill training (Wernig et al., 1992; 1995; Dietz et al., 1994; 1995; Dobkin et al., 1995; Harkema, 2001; Hicks, 2005), but they experience positive effects concerning the cardiorespiratory and musculoskeletal systems (Liu et al., 2008; Hoekstra et al., 2013; Asselin et al., 2015). This suggests that humans have a greater predominance of supraspinal control over spinal neuronal mechanisms, unlike spinal complete cats that regain the ability to walk at the end of the locomotor program (Kuhn, 1950; Van de Crommert et al., 1998; Rossignol, 2000; 2006). Moreover, spinal complete subjects show an appropriate activation timing of leg muscles during training, but with a smaller amplitude of EMG bursts of activity (Dietz and Harkema, 2004). This may be related to the loss of inputs from descending noradrenergic pathways to spinal locomotor circuits (Barbeau and Rossignol., 1994).

Despite many studies attesting training effectiveness in the rehabilitation of sensorimotor systems involved in functional movements, the definition of the best training parameters remains controversial. For instance, there is no evidence about which should be the weekly frequency of training sessions, their duration and their total number within the neurorehabilitation period. Moreover, systematic studies are lacking that investigate the early timing of a training therapy after injury to define a therapeutic window within which the highest recovery is achieved and beyond which maladaptive mechanisms may be instated. As for the therapeutic window, some controversial results suggesting both a delayed (Krajacic et al., 2009) and an early (Norrie et al., 2005) onset of training have been described in animal studies. Similar results about the most proper timing of rehabilitation interventions have been achieved also in SCI subjects, with some evidence supporting

an early intervention (Dietz and Muller, 2004; Scivoletto et al., 2005), whereas other studies highlighting the importance of delayed rehabilitation (Scivoletto et al., 2006). Lastly, nothing is known about training intensity, even though this aspect has started to be investigated. With this regard, a randomized controlled trial is currently comparing regular and intensive exercise consisting in a 12-week program (Galea et al., 2013). Behrman and colleagues (2008; Fox et al., 2010) have presented a case report of a child with chronic, severe, incomplete cervical SCI who recovered walking with intense locomotor training and continued improving two years after training. An intensive treadmill training has been described to be effective also in subjects with a complete SCI (Murillo et al., 2012; Knikou and Mummidisetty, 2014). Zhu and co-workers (2008) have described an impressive effect on 30 subjects with a complete SCI for no longer than two and a half months. After orthopedic stabilization of the vertebral column, all recruited subjects underwent an early neurosurgical intervention consisting in epidural decompression to restore cerebrospinal fluid flow. Rehabilitation started 17 days after neurosurgery and it consisted in an intensive locomotor program of 6 hours walk per day, 6 days a week for three months. The authors assert that none of the subjects could stand or walk without assistance before neurosurgery. At day 17 after neurosurgery (before beginning neurorehabilitation), 10 subjects (33%) could walk using a rolling walker without assistance, thus showing a first beneficial effect mediated by the sole neurosurgical procedure. At the end of the training period, 18 subjects (60%) recovered walking with a rolling walker without assistance, indicating a further improvement induced by the intensive locomotor program.

Neurophysiological basis of such a recovery after training interventions in subjects with central movement disorders seem to be ascribable to flexibility of spinal and supraspinal networks underlying the generation and control of locomotion, respectively. Convincing evidence of use-dependent plasticity has been reported in animal studies (Lovely 1986; 1990; Edgerton et al., 1997; Pearson, 2000), where a reorganization of spared neural pathways has been proposed to take place (Curt and Dietz, 1997; Curt et al., 1998). It has been estimated that some functional recovery could be achieved whether at least 10-15% of descending fibres are spared (Basso, 2000; Metz et al., 2000), since a strengthening of spared descending pathways is still possible (Thomas and Gorassini, 2005). On the contrary, after a complete loss of supraspinal inputs to the spinal cord, the still intact spinal CPG for locomotion adapts to generate motor patterns in the absence of supraspinal drive (de Leon et al., 1998 a; b; Wirz et al., 2001). In the latter situation, only peripheral inputs continue to have access to the spinal circuitry representing the only source of control. Thus, the importance of

the peripheral sensory system is greatly enhanced in this clinical condition and an appropriate stimulation of afferents can induce the activation of spinal circuits for locomotion. Sensory information from load receptors and hip joints essentially contributes to the establishment of the locomotor pattern, to the control of phase transitions and to the reinforcement of the ongoing activity. Load information is suggested to be provided by proprioceptors located in extensor muscles and by mechanoreceptors in the foot sole (Dietz and Duysens, 2000), while signals of hip joint position are thought to originate mainly from proprioceptors in ligaments and muscles of the hip (Dietz, 2002). Furthermore, cutaneous afferents may be involved in the adaptation to actual ground conditions (Dietz and Fouad, 2014).

Recently, it has been reported that spinal circuits deprived of supraspinal drive develop maladaptive neurophysiological mechanisms leading to a loss of excitability below the level of the lesion (Lin et al., 2007). It has also been suggested that this condition might be reversed in SCI subjects by an intensive training (Hubli et al., 2012). These findings are in accordance with research on spinalized rats, where an imbalance between inhibitory and excitatory activity has been described after lesion, with a prevalence of inhibitory inputs onto the motoneuron (Ichiyama et al., 2011). In the same study, an intensive training program was able to reestablish a balanced synaptic transmission between excitation and inhibition (Ichiyama et al., 2011). Altogether, these studies demonstrate that activity-based interventions mediate their neurophysiological effect by providing spinal networks with a suitable sensory feedback and by preventing detrimental neuroplasticity mechanisms caused by sensorimotor dysfunctions.

3.2.1 Robot-assisted rehabilitation

Till the late 90s, functional training consisted in stepping on a treadmill with BWS in the presence of one or more physiotherapists giving manual assistance at each step. Thus, there was an impelling need to standardize locomotor training and to tailor it to the subject requirements. To the purpose, robotic training devises have been developed, capable to induce longer and more intensive stepping sessions while real-time monitoring kinematic parameters (Calabrò et al., 2016). Moreover, compared to traditional physical therapy, these assistive devices have the advantage of reducing the work of therapists, allowing for a prolonged motor exercise and for an improved reproducibility of the kinematics during the gait cycle (Chen et al., 2016).

Robotic systems for gait rehabilitation can be classified into stationary and overground walking systems. Stationary systems, such as Lokomat® (Jezemik et al., 2003), consist in a robotic gait orthosis, a BWS system, a moving treadmill and an optional monitor for virtual reality (figure 15).

The robotic exoskeletons are wearable devices secured to the lower limbs at multiple sites through padding and straps. Since paraplegic subjects are very susceptible to skin wounds and pressure sores, the pads must be carefully applied to prevent any skin complication during the use of the exoskeleton. BWS is mediated by a sophisticated counterpoise system which allows for a passive weight-bearing, only statically compensating for the gravitation load, or for an active and dynamic support, continuously modulating the level of assistance on the real need of the subject. The key core of the device, made up of the bilateral exoskeleton and the BWS system, ensures a precise match between the speed of the gait and the velocity of the sliding treadmill. This is achieved through an array of sensors detecting a wide range of biomechanical and kinematics parameters (Hussain et al., 2012). Virtual reality can be an integral part of the training program. It provides a direct feedback to the subject and represents a stimulating and engaging tool. Indeed, many interactive activities are proposed with the subject accomplishing different motor tasks, such as collecting or avoiding objects randomly distributed in the virtual environment. Moreover, there is the possibility to conform the task and the level of difficulty to specific needs of the subject, therefore personalizing the virtual feedback. The rehabilitative approach involving artificial reality is considered to have a great potential in enhancing improvements mediated by repetitive training. Recently, it has been assessed that the effects of training in a virtual environment are higher than those induced by robot training alone (Mirelman et al., 2010; Calabrò et al., 2017). Besides advantages, stationary systems have the main limitation that movements are constrained to the sagittal plane, not allowing for frontal and transverse plane rotations (Jezemik et al., 2003; Veneman et al., 2008).

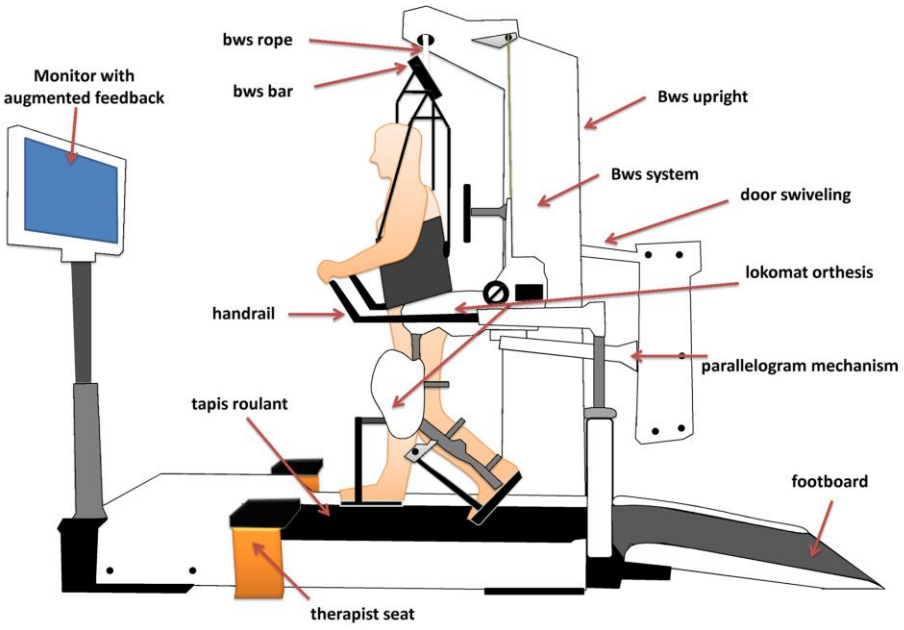

Figure 15: Schematic representation of Lokomat®, a stationary system for gait rehabilitation. The main components of the robotic device are a bilateral leg exoskeleton, a dynamic BWS system controlled by a sophisticated counterpoise, a sliding treadmill and a monitor for virtual reality. The subject is secured to the exoskeleton with his/her body weight supported by the system and the lower limbs mechanically stepping on the moving treadmill, while receiving a real-time feedback from the monitor. (From Calabrò et al., 2016).

Unlike stationary systems mainly used for rehabilitative purposes, overground walking systems have been developed to assist paralyzed subjects to accomplish motor tasks during the everyday life, such as standing up, sitting down and walking (Tsukahara et al., 2010; Esquenazi et al., 2012). Currently, ReWalk® is an overground walking exoskeleton that has received approval from the Food and Drug Administration (FDA) for home use for people with paraplegia (Contreras-Vidal et al., 2016). ReWalk® is an orthosis that mechanically moves the hip and knee joints and enables upright standing and walking. The device is powered by a battery located in a backpack carried on the wearer shoulders and it must be used with two Canadian canes to ensure stability during the execution of different motor tasks (Fineberg et al., 2013). Moreover, sensors measure joint angles, ground contact and upper-body tilt angle for initiation and regulation of the exoskeleton activity.

Robotic devices for gait rehabilitation can be controlled by two main mechanisms. The first one consists in trajectory tracking control, where predefined trajectories of the lower limb joints are recorded from healthy individuals and used as the control targets, therefore continuously comparing and adjusting the actual trajectory to the control (Jezernik et al., 2003). This kind of control system evokes a passively-driven training, since the subject follows a predefined reference trajectory and his/her active contribution to the task is completely abolished. There is clear evidence that after identical and repetitive patterns of activation the sensory system becomes less responsive (Dietz and Müller, 2004). To avoid the establishment of sensory adaptation to non-varying stimuli, a second control system for robotic devices has been developed, referred to as "assist-as-needed" (AAN; Aoyagi et al., 2005; 2007; Emken et al., 2008). In this case, assistance supplied to the subject is dynamically adjusted based on his/her physical condition and involvement in the rehabilitation session. As a consequence, the subject is strongly motivated to give the maximum voluntary contribution to gait rehabilitation (Riener et al., 2005; Duschau-Wicke et al., 2010).

Robot-assisted rehabilitation has shown a great technological advance in the last two decades. Assistive devices are thought to promote a plastic reorganization of the cortex, that can incorporate the assistive tool into the body neural schema (embodiment), thus enhancing potential beneficial effects of robot-assisted training (Lenggenhager et al., 2012; Pazzaglia et al., 2013; Pazzaglia and Molinari, 2016). However, it is still matter of debate whether robot-assisted rehabilitation is or not more effective than conventional physical therapy in the management of sensorimotor dysfunctions (Cao et al., 2014; Straudi et al., 2016; Taveggia et al., 2016; Wiart et al., 2016; Hwang et al., 2017;

Lefmann et al., 2017). Therefore, further investigations are needed to validate the role of robotic orthesis in clinical rehabilitation. To date, this kind of interventions should be considered as adjunctive tools rather than a substitute of standard treatment.

3.2.2 Preclinical models of passive training

A full understanding of central mechanisms evoked by training can be achieved in experimental animal models. This issue has first been addressed in different types of reduced preparations of kittens (Grillner, 1973; Forssberg et al., 1980 a; b; Edgerton et al., 1991) and adult cats (Barbeau and Rossignol, 1987; Edgerton et al., 1991; Bélanger et al., 1996; de Leon et al., 1998 a; b; Rossignol et al., 2000; 2002). For the first time in these studies, a common treadmill was adapted for *in vivo* studies. Thus, the cat was placed with its forelimbs standing on an immobile platform while the hindlimbs were put over the moving treadmill belt. The training setup did not include a BWS system and the hindquarters of the animal were manually hold by the experimenter.

Since then, robotic devices have been designed for assessing and training a certain locomotor task, for achieving very precisely controlled motor patterns and for defining critical elements for a successful training. De Leon and collegues (2002 a; b) have first developed a robotic device called "rat stepper". The components of the device include two robotic arms attached proximal to the ankles of the rat, a motorized BWS system and a treadmill. Thus, the rat steps bipedally while being supported by the system via the trunk with an automated adjustment of weight-bearing during the locomotor training. Moreover, it should be noted that the device controls only the position of the ankle joint for each limb, since authors have demonstrated that monitoring this single point is sufficient and has revealed being as effective as trying to control each joint of the limb (Nessler et al., 2004). The rat stepper supplies the necessary assistance to each limb during training and it provides all the tools for controlling experimental variables. By means of this systematic proceedings, it has been possible to obtain some basic insight into activity-mediated beneficial effects. Indeed, this device has been useful in investigating mechanisms of initiation of the swing phase in spinal rats, assessing that the probability of a successful swing phase significantly improves during robot-assisted hindlimb extension (Nessler et al., 2007). Moreover, the robot has been used to control the amount of loading that is sensed by the paw and that induces a response of the spinal circuitry after repetitive application during the stance phase, confirming that limb loading plays a major role in evoking stepping after spinal cord transection in rat (Timoszyk et al., 2002; 2005). It has also disclosed some mechanisms concerning coordination of motor pools, which is

likely to be improved by treadmill training through the refinement and elimination of non-functional synaptic contacts appearing after lesion (Ichiyama et al., 2008).

Other groups (Bose et al., 2012; Houlé and Côté, 2013) have proposed a rat bicycle to promote locomotion in injured rats. The bicycles have adjustable pedals and a harness that provides support of the trunk. Authors argue that stationary bicycles are spatially compact, economical to buy and can be safely accessed with minimal assistance.

It can be concluded that the comprehension of neuroplasticity mechanisms underlying motor regain of function after training is of outstanding interest. These mechanisms are observed and described in clinical studies, but their neurophysiological and molecular basis can be unveiled only in preclinical and in *in vitro* studies.

3.2.3 Neuroplasticity induced by training interventions

As asserted before, an intensive and repetitive execution of an impaired movement can improve motor function by exploiting neuroplasticity mechanisms of the CNS at different levels (Meeusen, 2005; Jakeman et al., 2011). Neuroplastic changes induced by training include axonal sprouting and re-wiring processes (Bareyre et al., 2004), modifications in synaptic transmission (Ichiyama et al., 2011), altered intracellular properties (Boulenguez et al., 2010; Murray et al., 2010). They are mediated by different molecular markers whose expression is modulated by training, becoming up- or downregulated.

One of the main effects mediated by training is the facilitation of neuronal excitability (Fouad et al., 2011). This is achieved by blocking the abnormally high levels of generalized inhibition resulting from the lesion. Indeed, spinal rats and cats express increased levels of GABA synthetic enzyme GAD_{67} (glutamic acid decarboxylase) and $GABA_A$ receptors in the lumbar spinal cord, whereas trained spinal animals show control levels of both markers (Edgerton et al., 2001; Tillakaratne et al., 2000; 2002). Inhibition is also sustained by astrocytes that have elevated immunostaining positivity for the $GABA_A$ receptor γ2 subunit, which is downregulated toward control levels after six weeks of step training (Bravo et al., 2002). Moreover, the γ2 subunit of the $GABA_A$ receptor is restored toward control values in motoneurons after training, indicating that motor training has an important role in the $GABA_A$ receptor trafficking and synaptic clustering (Khristy et al., 2009). Interestingly, transected rats subsequently trained by a robotic treadmill system with BWS, have shown an increased release of both glutamate and glycine in the lumbar spinal cord and an enhanced expression of VGLUT1 (vescicular glutamate transporter 1) and GLYT2 (glycine transporter 2).

The overexpression of these markers has been proved to positively correlate with an improved stepping performance (Cantoria et al., 2011).

Functional recovery is also associated to a regulation in chloride homeostasis. Data collected from spinalized rats, undergoing forced pedaling on customized bicycles, have shown a return to pre-injury expression levels of KCC2, a neuronal chloride exporter. Accordingly, the chloride importer NKCC1 has been demonstrated to be downregulated in trained animals with respect to non-trained transected rats (Côté et al., 2014; Chopek et al., 2015). This suggests that cation-chloride cotransporters (CCC) are targets for exercise-based neuroplasticity.

Moreover, cycling exercise provided to SCI animals has a modulatory effect on miRs (microRNAs, a class of small non-coding RNAs) involved in the regulation of apoptotic pathways. Indeed, cluster analysis has revealed that training promotes the expression of anti-apoptotic miRs, whereas downregulating miRs contributing to pathophysiological events secondary to SCI, such as neuroinflammation and apoptosis (Liu et al., 2010). In addition, miRs with a critical role in the control of cell proliferation, axonal outgrowth, protein synthesis, regeneration and synaptic plasticity in the CNS are also overexpressed (Liu et al., 2012).

Plasticity of spinal circuits may also be mediated by activity-dependent induction of neurotrophins (NTs; Grau et al., 2014; Skup et al., 2014). Besides their classical role in supporting neuronal survival, NTs modulate key steps of network construction and reconstruction promoting synaptic plasticity after injury or disease. Both NT transcript and protein levels are enhanced in the lumbar spinal cord by training (Keeler et al., 2012). Different neurotrophins can be expressed, such as BDNF (brain-derived neurotrophic factor; Gómez-Pinilla et al., 2001, 2002; Ying et al., 2005; 2008; Macias et al., 2009; Joseph et al., 2012; Keeler et al., 2012; Boyce et al., 2012; Weishaupt et al., 2013), GDNF (glial cell line-derived neurotrophic factor; Keeler et al., 2012; Han et al., 2014), NT-3 (Gómez-Pinilla et al., 2001; Boyce et al., 2012; Yang and Zhang, 2016) and NT-4 (Skup et al., 2002).

BDNF has been long studied and its role in neuroplasticity has been better characterized with respect to other NTs. There is strong evidence that long-term treadmill training of moderate intensity stimulates spinal circuitries to increase BDNF synthesis (Skup et al., 2002; Marcias et al., 2009). It is likely that BDNF reaches its maximum serum concentration after 20 minutes of exercise and returns to baseline after approximately 10 minutes of recovery from training (Schmidt-Kassow et al., 2012). Indeed, exercise-related increase in BDNF expression is dependent on maintenance of

sensory information being transmitted during training, since deafferentation abolishes exercise-dependent increase in BDNF levels (Ollivier-Lanvin et al., 2010).

BDNF release induced by training upregulates both excitatory and inhibitory synaptic transmission (Skup et al., 2014). On one hand, there is an overexpression of GAD_{67} in the terminals around motoneuron cell bodies, thus augmenting inhibitory inputs onto motoneurons (Ziemlinska et al., 2014). This is in agreement with previous results demonstrating that BDNF treatment mainly supports sprouting of F-type boutons with presumably inhibitory function (Novikov et al., 2000). Moreover, BDNF promotes the expression of KCC2 (Boulenguez et al., 2010). By modulating GABAergic neurotransmission and chloride homeostasis, BDNF may also enhance the functionality of isolated lumbar circuits, which in turn enable locomotion (Ziemlińska et al., 2014). Thus, spinal GABAergic transmission represents an important final target of exercise, further confirming the crucial role of GABA in organizing the locomotor pattern (Talpalar et al., 2011) and in integrating afferent stimuli (Fink et al., 2014). On the other hand, high levels of GABA are accompanied by an elevated expression of VGLUT2 in spinal rats treated with BDNF, supporting glutamatergic transmission (Ziemlinska et al., 2014). Furthermore, it is well established that TrkB (tropomyosin receptor kinase B) activation mediated by BDNF can induce an increase in NR2B subunit phosphorylation on NMDA receptors which, in turn, augments NMDA receptors open probability (Levine et al., 1998). More recently, it has been demonstrated a direct interaction between TrkB and NMDA receptor through the protein tyrosine kinase Fyn (Mizuno et al., 2003; Minichiello, 2009). BDNF has also been reported to modulate the expression and trafficking of NMDA receptors (Lau and Zukin, 2007; Carvalho et al., 2008) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (Malenka, 2003). Lastly, BDNF contributes to the control of the intrinsic excitability of motoneurons through the modulation of different ion channels (Skup et al., 2014).

Altogether these findings demonstrate that neuroplasticity promoted by activity-based interventions is very complex, involving different molecular markers and pathways. Our knowledge of these mechanisms is still insufficient and a better understanding will allow increasing the effectiveness of current neurorehabilitative approaches.

4 Spinal reflexes and training

Spinal reflexes are sensory-motor loops which establish early during development through highly selective synaptic connections mediated by specialized chemotactic and adhesion molecules (Chen et al., 2003). The stretch reflex (or myotatic reflex) is a monosynaptic excitatory reflex first

described by Liddell and Sherrington (1924), which represents the simplest proprioceptive feedback system. The tendon jerk (or tap) reflex is the visible manifestation of the stretch reflex. Briefly, when the knee extensor muscle is stretched, muscle spindle sensory feedback activates motoneurons innervating that muscle (Frigon and Rossignol, 2006). The tendon jerk reflex implies that when the knee extensor muscle (quadriceps) is contracted as a result of the stretch reflex, the knee flexor muscle (hamstrings) should be relaxed. Relaxation of the antagonist muscle is due to a disynaptic reflex, where sensory fibres activate an inhibitory interneuron that, in turn, inhibits flexor motor pools preventing flexor muscles contraction (Hultborn, 2006). Thus, the knee jerk induces simultaneously the contraction of the knee extensor muscle (net excitatory effect) and the relaxation of the knee flexor muscle (net inhibitory effect). So, it is made up of two spinal reflex pathways mediating knee extension, the stretch reflex and the disynaptic reflex.

The application of tendon jerks to elicit reflexes has obvious problems of reproducibility. Therefore, most studies take advantage of the possibility to stimulate the nerve electrically. The H-reflex is evoked by electrical pulses and it is analogous to the mechanically-induced stretch reflex. In humans, H-reflexes are obtained by stimulating the tibial nerve in the popliteal fossa and recording the EMG activity from the soleus (Stein and Thomson, 2006). Lag periods from one elicited H-reflex and the successive should be carefully assessed because of a characteristic postactivation depression, ascribable to muscle spindle afferents PSI (Luscher et al., 1983; Hultborn et al., 1996).

Finally, polysynaptic reflexes should be mentioned, which are involved in the fast activation of complex motor patterns aimed at safeguard and protection, such as the withdrawal and the flexion reflexes (Hultborn, 2006).

Spinal reflex pathways are dramatically impaired in case of sensorimotor disorders and the transmission to and from the spinal cord is markedly altered. Among other mechanisms for the regain of locomotion, it has been suggested that rehabilitative techniques reinstate function by normalizing reflex pathways (Frigon and Rossignol, 2006). Both step training (Trimble et al., 1998; Côté et al., 2003; Côté and Gossard, 2004) and cycle training (Skinner et al., 1996; Kiser et al., 2005; Reese et al., 2006) have been shown to modify reflex pathway transmission by shaping and reorganizing afferent input processing in the spinal cord.

It has been demonstrated that locomotor training modifies afferent transmission from load (Côté et al., 2003) and cutaneous (Côté and Gossard, 2004) receptors in chronic adult spinal cats. As for load pathways, it has been shown that step training reduces the amplitude of the monosynaptic stretch reflex and the disynaptic inhibitory reflex. Importantly, these changes are not due to modifications

in motoneuron intrinsic membrane properties, indicating a clear involvement of load afferent pathways. Both changes are positively correlated to an amelioration of side effects due to the chronic SCI, such as spasticity (a motor disorder characterized by an increase in tonic stretch reflexes caused by an abnormal intraspinal processing of primary afferent inputs). In turn, the normalization of these reflex pathways is considered to be critical for locomotor and weight-bearing recovery (Côté et al., 2003). Step training modifies also transmission of skin pathways signaling ground contact. Indeed, exaggerated cutaneous reflexes due to spinalization, are decreased and normalized to control values by training, thus facilitating the recovery of locomotor movements (Côté and Gossard, 2004). These results demonstrate that training influences afferent transmission in several reflex pathways by providing a functionally useful amount of sensory information and by counteracting hyperexcitability caused by the spinalization. In turn, the normalization of exaggerated reflex information can in part be responsible for the recovery of locomotion.

Accordingly, training modifies H-reflex excitability in chronic adult spinal rats. In these experiments, a high frequency stimulation resulted in a markedly reduced postactivation depression of the H-reflex in transected animals, whereas returning to control values in rats trained on a bicycle (Skinner et al., 1996). Since the postactivation depression of the H-reflex is related to PSI in muscle spindle afferents, modifications of this component indicate filtering impairments of incoming signals. The reinstatement of training-mediated postactivation depression of the H-reflex appears as early as 15 days after training, but a full restoration is achieved gradually over time (Reese et al., 2006). These data suggest that training restores and/or reorganizes presynaptic inhibitory mechanisms which may play a key role in locomotor recovery.

Similar studies have been carried out in humans with the aim to describe and quantify the influence that locomotor training has on the H-reflex pathway after a sensorimotor disorder (Trimble et al., 1998; Kiser et al., 2005). Subjects enrolled in the study of Kiser and collaborators (2005) underwent passive cycling five times a week for 13 weeks. Authors show that cycle training restores a normal postactivation depression of the soleus H-reflex, which parallels with improvements in spasticity. However, 4 weeks after training cessation there is a return to pre-training condition, indicating that cycle training must be continuously applied to retain ameliorations. In a case study of an incomplete SCI man, the postactivation depression of the soleus H-reflex elicited by a low frequency stimulation (1 Hz) has been assessed before and after 4 months of treadmill training (Trimble et al., 1998). The main conclusion is that low-frequency postactivation depression of H-reflexes is less before training and significantly increases at the end of the training program, which corresponds to improvements in locomotor performance. In accordance with animal studies, clinical research

confirms that training induces a general reorganization of reflex pathways. Therefore, rehabilitative approaches should also focus on the interplay mechanisms between reflexes and locomotion to strengthen the effectiveness of activity-based interventions.

5 Spinal motoneurons and training

Spinal motoneurons are located in lamina IX of the ventral horn and are found at all levels of the spinal cord. The large α-motoneurons innervate skeletal muscle fibres (extrafusal fibres) and drive muscle contraction, whereas smaller γ-motoneurons (or fusimotor neurons) innervate the intrafusal muscle fibres of muscle spindles and play a complex role in motor control (Hultborn and Fedirchuk, 2009). Moreover, a third less defined population has been identified innervating both intra- and extrafusal fibres, referred to as β-motoneurons (Kanning et al., 2010). Motoneurons innervating the same muscles are clustered in motor pools (Vanderhorst and Holstege, 1997). Motoneurons are directly affected by a prolonged neuromuscular training, which has been shown to promote locomotor recovery. However, plasticity mediated by physical activity and involving motoneuron modifications has been directly assessed only during the last several years.

Daily forced training is beneficial in restoring and maintaining locomotor patterns in SCI rats. Similar to observations in the uninjured animal (Beaumont and Gardiner, 2002; 2003; Cormery et al., 2005; MacDonell et al., 2012), the amelioration of locomotion after a stepping-based rehabilitation program might be related, among others, to the preservation of hindlimb motoneuron properties, mainly related to morphophysiological characteristics and efficacy in neuromuscular transmission (Petruska et al., 2007; Beaumont et al., 2008). There is strong evidence that passive exercise of the hindlimbs can prevent a small but functionally significant reduction in the number of motoneurons caudal to the lesion (Keefe et al., 2017). Moreover, morphological changes concerning the soma and axon diameter, as well as an increase in dendritic arbor, have been described after stepping training, even though these modifications appear to give minor contribution to the regain of function (Seburn et al., 1994; Gazula et al., 2004; Ishihara et al., 2004). Training-based modifications include also the dynamic interaction with the muscle spindle at the neuromuscular junction, where there is an increase in nerve terminal branching (Andonian and Fahim, 1988; Deschenes et al., 1993) and a higher synaptic efficacy due to enhanced neurotransmitter release (Dorlochter et al., 1991; Argaw et al., 2004). The augmented efficacy of the neuromuscular transmission may be related to an increased protein synthesis (Edstrom, 1957; Gerchman et al., 1975), also suggested by the finding that trained motoneurons seem to be able to transport larger amounts of protein in their axons in both anterograde and retrograde directions (Jasmin et al., 1987;

1988). In addition, the release of acetylcholine is promoted by a higher expression of SNAP25 (synaptosome-associated protein), involved in synaptic vesicle docking, which is selectively transported in higher quantities in axons of trained motoneurons (Kang et al., 1995). Subsequently to a higher protein synthesis, hindlimb motor training also facilitates axonal regeneration and sprouting in case of denervation signals coming from muscle fibres (Gardiner et al., 1984; Gardiner and Faltus, 1986; Soucy et al., 1996).

Recently, particular emphasis has been placed on the effect of activity-mediated neurotrophins on motoneurons. Among them, BDNF has been shown to promote plasticity within the spinal cord and to improve the ability of lumbar spinal networks to generate locomotion in SCI animals (Ying et al., 2008; Boyce et al., 2007; 2012). It is known that levels of endogenous BDNF are increased by hindlimb training (Hutchinson et al., 2004; Ying et al., 2005; Marcias et al., 2009; Cote et al., 2011). It is also well-established that motoneurons secrete BDNF in response to treadmill training (Marcias et al., 2009; de Leon et al., 2011). An intensive work has been done to clarify whether motoneurons synthesize BDNF by their own or whether BDNF derives from muscles. Firstly, it has been demonstrated that hindlimb muscles express a huge amount of BDNF during training, which is then anterogradely transported to motoneurons (Koliatsos et al., 1993; Gomez-Pinilla et al., 2002; Dupont-Versteegden et al., 2004). Recently, it has been reported that BDNF is also directly produced by motoneurons in SCI animals undergoing passive cycling (Keeler et al., 2012) or passive stepping (Joseph et al., 2012). Moreover, BDNF expression in spinal motoneurons is positively correlated to the intensity of training, with a higher amount of treadmill training resulting in a greater synthesis of BDNF by motoneurons (Joseph et al., 2012). This, in turn, has implications on the final outcome of training in terms of levels of synaptic plasticity and locomotor recovery.

Motoneurons undergo also differences in electrophysiological properties that are thought to mediate the effectiveness of training in injured animals. After spinal cord transection, the membrane resting potential and the voltage threshold for action potential generation become depolarized (Beaumont et al., 2004; 2008). In contrast, training induces a hyperpolarization of both parameters, which return to control non-injured condition (Beaumont et al., 2008). Moreover, the AHP depth is normalized, thus promoting the reestablishment of normal firing, which correlates with the recovery of stepping (Petruska et al., 2007). These results demonstrate that training influences motoneuronal excitability by acting on specific voltage-dependent ionic channels that modify the intrinsic response properties of motoneurons (Fulton and Walton, 1986, Kiehn et al., 2000). Moreover, it should be taken into account that electrical properties of spinal motoneurons are strongly influenced by descending monoaminergic pathways, which are partially or completely lost after injury determining some

compensatory mechanisms (Hultborn and Kiehn, 1992; Rekling et al., 2000; Harvey et al., 2006; Li et al., 2007; Perrier et al., 2013).

Lastly, synaptic inputs converging onto the motoneuron are also correlated with the success of treadmill step training. It has been demonstrated that a spinal cord transection induces a significant reorganization of incoming motoneuronal inputs, with an increase in inhibitory synaptic transmission that correlates with poor stepping abilities of the animal (Petruska et al., 2007; Ichiyama et al., 2011). However, locomotor training reverts the imbalance in inhibition and excitation to control ratios, resulting in improved stepping (Ichiyama et al., 2011). Altered inhibitory mechanisms onto the motoneuron are also supported by an overexpression of the γ2 subunit of the $GABA_A$ receptor in the transected rat, which is necessary for benzodiazepine binding (Gunther et al., 1995) and for $GABA_A$ trafficking and synaptic clustering (Kittler et al., 2000). Step training restores the $GABA_A$ γ2 levels towards control value, having a direct effect on those motoneurons that are involved with the motor task (Khristy et al., 2009). Moreover, daily training further normalizes inhibition through the upregulation of the potassium-chloride cotransporter KCC2, that shows a reduced expression in motoneurons after spinal transection (Chopek et al., 2015).

It is possible to conclude that neuroplasticity mechanisms involving the motoneuron must be better investigated in the next few years. Unraveling the way training operates on these effector neurons might allow to achieve useful tools for the improvement of rehabilitative interventions in the management of sensorimotor dysfunction.

6 Hyperexcitability of dorsal horn circuits

Beyond functional impairment of locomotion, nervous system disorders may result in a condition of chronic pain with central origin, referred to as neuropathic pain. Based on the IASP (International Association for the Study of Pain) definition, neuropathic pain is a type of chronic pain caused by a lesion (direct damage) or disease (indirect metabolic, autoimmune or inflammatory injury) of the somatosensory nervous system (Treede et al., 2008). In SCI subjects, neuropathic pain is clinically classified with respect to the lesion: at-level neuropathic pain is localized close to the major focus of damage to the spinal cord; below-level neuropathic pain is localized in the lower trunk and/or legs; above-level neuropathic pain is uncommon and it is localized in dermatomes rostral to the injury (Finnerup et al., 2003; Bryce et al., 2007; Richards et al., 2007; Widerstrom-Noga et al., 2008).

Typically, neuropathic pain includes both negative and positive sensory symptoms. Negative symptoms consist in a deficit or a complete loss of function that affects all or specific sensory

modalities (Nickel et al., 2011; von Hehn et al., 2012). Positive symptoms are further subdivided into spontaneous, like paresthesia (tingling, tickling, pricking, numbness or burning sensation), dysesthesia (abnormal, unpleasant, discomforting sensation) and ongoing superficial pain, and stimulus-evoked, such as allodynia (pain in response to a non-nociceptive stimulus) and hyperalgesia (increased pain sensitivity to a nociceptive stimulus; Baron et al., 2010).
Neuropathic pain is fostered by the onset of long-term plasticity mechanisms, actually consisting in maladaptive changes in the peripheral and central nervous system. Cellular and molecular mechanisms of central sensitization are going to be extensively discussed. It should be considered that also peripheral sensitization is established, but it is not going to be matter of dissertation. These mechanisms have been studies in animal models of neuropathic pain, which will be briefly described focusing on innovative models of chronification. Lastly, physical training will be presented as a promising non-pharmacological method in the management of neuropathic pain, when analgesics fail to relieve pain adequately and side effects largely exceed therapeutic ones.

6.1 Mechanisms of dorsal hyperexcitability

Neuropathic pain is characterized by the onset of central sensitization in the dorsal horn after nervous system lesion or disease. This consists in the establishment of hyperexcitability within spinal dorsal networks, leading to enhanced processing of incoming pain signals from the site of injury (hyperalgesia; Woolf, 1983) and to the condition in which innocuous stimulation of areas surrounding the injury is processed as painful (allodynia; Campbell et al., 1988).

Several mechanisms have been shown to support central sensitization of the dorsal spinal cord and they are summarized in figure 16. Glutamate is involved in one of these mechanisms ("1" in figure 16). Usually, painful stimuli from the periphery travel through thinly-myelinated Aδ fibres and unmyelinated C fibres. Once in the dorsal horn, primary afferent terminals of nociceptors release glutamate, which evokes an excitatory post-synaptic current (EPSC) in the second order dorsal horn interneuron. This current is primarily due to the activation of postsynaptic AMPA receptors, which in turn trigger NMDA receptors. In case of nervous system lesion or disease, there is an increased release of glutamate from the presynaptic terminal, which induces a persistent depolarization of the postsynaptic neuron. As a consequence of membrane depolarization, the magnesium block is removed from the inner pore of the NMDA receptor and sodium and calcium can enter the dorsal interneuron. In particular, a continuous calcium influx is responsible for the activation of intracellular pathways that modulate the activity of a wide number of targets, including

transmembrane receptors involved in cell excitability (Basbaum et al., 2009). As a result, AMPA and NMDA receptors are phosphorylated becoming constitutively active (Woolf and Salter, 2000; Ultenius et al., 2006; Latremoliere and Woolf, 2009), and voltage-gated sodium channels are overexpressed (Hains et al., 2004). Since this process implies a calcium inflow, it is comparable to plastic changes associated to long-term potentiation (LTP) in the hippocampus (Lynch, 2004; Castillo, 2012;). Thus, maladaptive mechanisms take place that make dorsal neurons become active not only by low level stimulation of Aδ and C fibres (hyperalgesia), but also by tactile-mediating Aβ fibres (allodynia; Nickel et al., 2011).

Besides an increased excitation, disinhibition of spinal dorsal networks plays an important role in central sensitization ("2" in figure 16). After a nervous system lesion or disease, there is an excitotoxic loss in inhibitory GABAergic interneurons in the dorsal horn (Moore et al., 2002; Scholz et al., 2005), although this claim is controversial due to an almost undetectable number of pyknotic nuclei in some pain models (Polgar et al., 2005; Taccola et al., 2010). Therefore, the reduction in GABA levels is most likely ascribable to a reduced expression in dorsal interneurons of the GABA-producing enzyme, GAD_{67} (Deumens et al., 2013). Moreover, inhibitory synaptic transmission mediated by GABA and glycine is decreased in superficial dorsal horn neurons (Zeilhofer, 2008). In particular, GlyRα3 glycine receptor subunit is phosphorylated rendering neurons unresponsive to the inhibitory effects of glycine (Harvey et al., 2004), while $GABA_A$ receptor subunits α2 and γ2 are downregulated (Obata et al., 2003; Lian et al., 2012). Other studies indicate that dorsal interneurons undergo also a shift in the Cl^- gradient, due to a downregulation of KCC2, such that activation of $GABA_A$ receptors results in depolarization rather than hyperpolarization of the dorsal interneuron, promoting a further excitability and pain transmission (Coull et al., 2003).

Despite postsynaptic changes, also the presynaptic terminal is affected by the lesion (Guo and Hu, 2014). Similar to observations made on postsynaptic cells, also here there is a reduction in GABA synthesis through the downregulation of GAD_{65}, which is selectively associated with presynaptic terminals of primary afferent fibres (Eaton et al., 1998). Furthermore, a phosphorylation-mediated increased activity of NKCC1 has also been reported in central sensory terminals (Pieraut et al., 2011; Wei et al., 2013; Modol et al., 2014). Enhanced PSI-PAD mechanisms lose their control function in gating incoming surplus and/or inappropriate signals and are suggested to play a major role in chronic pain onset (Willis, 1999). It is likely that the NKCC1 upregulation in DRG neurons

is transient and not maintained at later stages, probably being required for the initiation of neuropathic pain, but not for the maintenance of the chronic condition (Guo and Hu, 2014).
Spinal disinhibition in the dorsal horn may also be exacerbated by loss of descending inhibitory control from serotonergic, noradrenergic and dopaminergic pathways (De Felice and Ossipov, 2016). This could be due to an injury-induced apoptosis of neurons occurring at multiple supraspinal levels (Leong et al., 2011) or to the interruption of descending projections caused by a central lesion (Hains et al., 2002). Moreover, dysfunction in descending inhibitory pathways may be associated with the extent of hyperexcitability distribution with respect to the lesion, therefore resulting in below-level, at-level and above-level pain (Gruener et al., 2016). In particular, the above-level neuropathic pain is very rare. It is usually detected in animal models of incomplete SCI (Christensen et al., 1996; Christensen and Hulsebosch, 1997; Wang and Thompson, 2008; Carlton et al., 2009) and it never develops in animals with complete spinal transections (Hubscher et al., 2008; Densmore et al., 2010). This suggests that spared descending or ascending pathways crossing the site of SCI are likely to be involved in the development of above-level neuropathic pain.

Glial cells also contribute to the central sensitization process leading to dorsal horn hyperexcitability ("3" in figure 16). Glia contribution in the initiation and maintenance of persistent pain states has been recently suggested (Ji et al., 2013). Glial cells are abundant in the spinal cord and they perform different tasks. Microglia cells are resident macrophages of the CNS and function as sentinels of injury or infection, whereas astrocytes are involved in fine-tuning synaptic transmission and in numerous homeostatic functions.
In case of injury, microglia switch to an activated state, consisting in swollen cell bodies and retracted processes. Microglia activation is principally mediated by ATP released by primary afferent terminals, which targets microglia P2-type purinergic receptors. Of particular interest in chronic pain are $P2X_4$ (Tsuda et al., 2003), $P2X_7$ (Chessell et al., 2005) and $P2Y_{12}$ (Haynes et al., 2006; Kobayashi et al., 2008) receptor subtypes. Activated microglia accumulate in the superficial dorsal horn within the projection zone of lesioned peripheral nerve fibres. Tissue infiltration is partly due to increased microglia proliferation and partly to permeabilization of the blood-brain barrier through the release of chemoattractant cytokines that cause the extravasation of circulating immune cells (Inoue et al., 2004; Tsuda et al., 2005). When in a pro-inflammatory state, microglia release a huge number of signaling molecules, including cytokines such as TNF-α (tumor necrosis factor) and various interleukins (DeLeo et al., 2007). The net result is that dorsal interneurons in the

outermost laminae are sensitized and their responses are enhanced, contributing to central hyperexcitability and persistent pain condition.

The contribution of astrocytes to central sensitization is less clear. Astrocytes are induced in the spinal cord after damage, becoming active and undergoing intense hypertrophy and proliferation. Their activation is generally delayed with respect to microglia and it is mediated by primary afferents release of ATP and microglia release of TNF-α and interleukins (Ji et al., 2013). Once activated, they start releasing pro-inflammatory molecules and ATP, which in turn enhance activity in the surrounding neurons and microglia (Sofroniew, 2014). Moreover, impairments in glutamate reuptake and in control processes of the extracellular potassium concentration should be considered (Ji et al., 2013). A possible explanation for astrocyte delayed activation is that they appear to be more critical to the maintenance and intensification of pain, rather than to the initiation of central sensitization (Bausbaum et al., 2009).

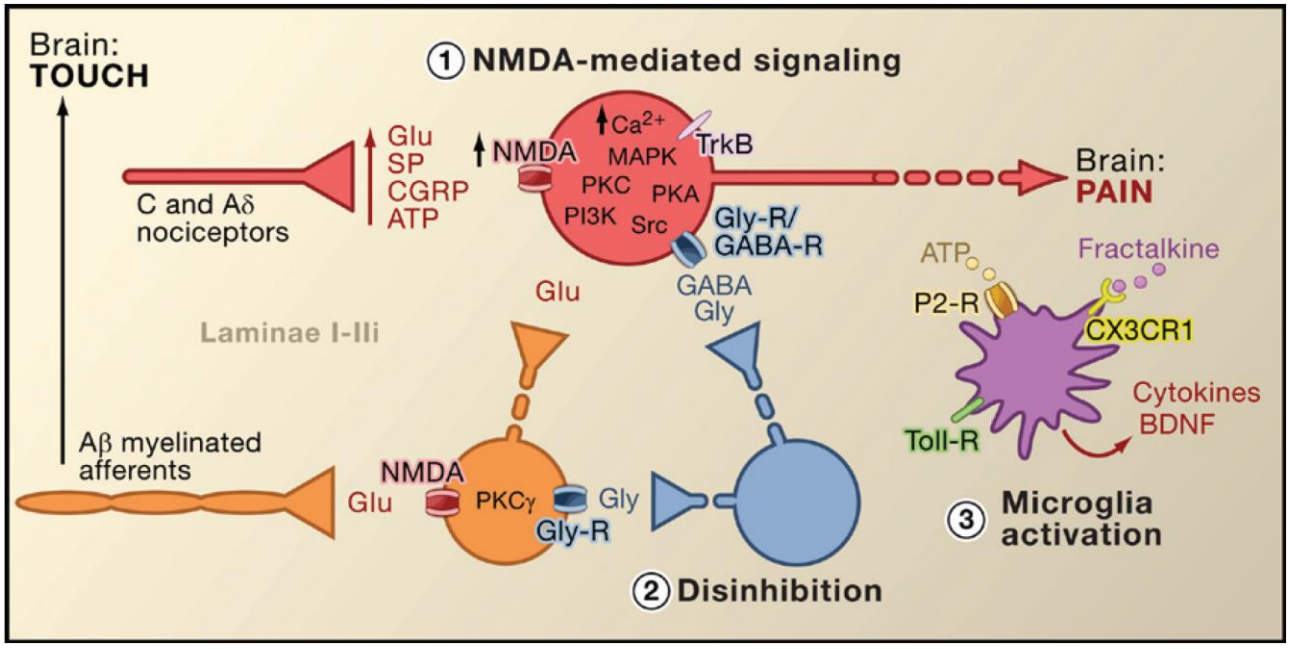

Figure 16: Mechanisms of central sensitization. Three main mechanisms of central sensitization are schematically depicted. (1) After injury or disease of the nervous system, Aδ and C fibres (red) are activated and release a variety of neurotransmitters in the dorsal horn. NMDA receptors in the postsynaptic neuron (red) loose the Mg^{2+} block, Ca^{2+} enters the cell and triggers different intracellular Ca^{2+}-activated pathways. As a result, excitability is increased and there is a facilitation in pain transmission signals to the brain. (2) In parallel, disinhibition mechanisms are established and consist in reduced neurotransmitters release from inhibitory interneurons (blue) and in impaired GABA and glycine transmission. As a consequence, Aβ fibres (orange), normally triggering innocuous touch stimuli, can now evoke painful sensations. (3) Moreover, microglia (violet) is activated subsequently to the release of ATP by Aδ and C fibre terminals (red) and contribute to the initiation and maintenance of central sensitization through the secretion of a wide number of pro-inflammatory cytokines and chemoattractant molecules. (From Basbaum et al., 2009).

Finally, it should be mentioned that ectopic activity originating from a damaged nerve fibre represents a major driver of central sensitization. Ectopic action potentials are generated at the site of injury, but also at more proximal axonal sites, including the soma of DGR sensory neurons

(Amir et al., 2005). After injury, spontaneous activity is evident in both damaged and neighboring undamaged nociceptive afferents (Wu et al., 2002). This is due to drastic changes in expression and distribution of many ion channels in primary sensory neurons, leading to alterations in intrinsic membrane properties. After injury, there is an overexpression of voltage-gated sodium channels (Na_v; Devor, 2006; Dib-Hajj et al., 2010). Between the nine isoforms identified so far, $Na_v1.7$ (Yang et al., 2004; Cox et al., 2006; Han et al., 2006), $Na_v1.8$ (Jarvis et al., 2007; Zimmermann et al., 2007) and $Na_v1.9$ (Wood et al., 2004) seem to play a major role in pain. Moreover, the two-pore-domain potassium channel TRESK is down-regulated by 30-40% (Tulleuda et al., 2011), as well as other voltage-gated potassium channels (Bahia et al., 2005), leading to a steady state depolarization of the membrane potential in DRG sensory neurons. Lastly, the expression and activity of many other receptors is altered, such as the TRPV1 (transient receptor potential vanilloid 1) involved in heat hyperalgesia (Caterina et al., 2001; Ma et al., 2005; Fischer et al., 2007; Biggs et al., 2008) and the TRPM8 (transient receptor potential melastatine family member 8) mediating cold allodynia (Wasner et al., 2004; Serra et al., 2009).

Thus, mechanisms leading to central sensitization are various and overlapping. Their net result is a generalized hyperexcitability of dorsal horn networks. Dorsal hyperexcitability can be experimentally assessed recording spontaneous antidromic activity (dorsal root potentials, DRPs; Barron and Metthews, 1938) and/or electrically-evoked antidromic activity elicited by single-pulse stimulation of a dorsal root, which may be many spinal segments far from the recording one (dorsal root reflexes, DRRs; Eccles et al. 1961).

6.2 *In vivo* models of neuropathic pain

Mechanisms of central sensitization have been studied in animal models of neuropathic pain. To date, a variety of rodent models exist, that reproduce different clinical conditions. In the majority of animal models, central sensitization is induced through a traumatic injury of the CNS or peripheral nervous system (PNS).

For the investigation of neuropathic pain after injury to the CNS, a wide number of animal models is available. In these experimental models, injuries to the CNS are induced via two different techniques. In “open injuries” the spinal cord is penetrated and this results in a partial or complete laceration of the tissue, while in “closed injuries” the spinal cord is compressed or contused (Hulsebosch, 2005). Among “open injury” models, spinal cord hemi-transection is the most extensively used lesion (Christensen et al., 1996). On the contrary, models of “closed injury” are

numerous and include spinal cord contusion (Gruner, 1992; Hulsebosch et al., 2000), spinal cord ischemia (Hao et al., 1991; Xu et al., 1992), excitotoxic spinal cord injury (Yezierski et al., 1998) and spinal cord compression (Bruce et al., 2002).

Most models of neuropathy arising from PNS lesions involve injuries to the sciatic nerve, its branches and contributory spinal roots or DRGs. The Chung model consists in L5 and/or L6 spinal nerve ligation (SNL) or transection (SNT; Kim and Chung, 1992; Chung et al., 2004). Injuries of the sciatic nerve, include the Seltzer and the Bennett models. The first consists in a partial sciatic nerve ligation (PSNL), where approximately half of the sciatic nerve is ligated (Seltzer et al., 1990). The second one is represented by a chronic constriction injury (CCI), in which ligatures are placed loosely around the main trunk of the sciatic nerve (Bennett et al., 2000). Lastly, the spared nerve injury (SNI) model consists in inducing a damage to peripheral branches of the sciatic nerve, where the tibial and the common peroneal nerves are ligated, transected or crushed, leaving the sural nerve branch intact (Decosterd and Woolf, 2000).

In animal models, central sensitization can be assessed through behavioral studies measuring paw withdrawal threshold (Berger et al., 2011). This method allows for the monitoring of allodynia (painful withdrawal responses to innocuous stimuli) and hyperalgesia (decreased withdrawal threshold to painful stimuli). Withdrawal thresholds to mechanical stimuli are determined with the von Frey test, applying hairs of increasing caliber to the hindpaw of the animal, whereas withdrawal thresholds to thermal stimuli are defined with the Hargreaves method, where the animal is placed on a heated platform (Bosier et al., 2015). Interestingly, in the SNI, von Frey hairs for the assessment of mechanical allodynia are applied to the territory innervated by the sural nerve, which represents the spared branch (Gallo et al., 2017). This suggests the establishment of central sensitization affecting not only projecting areas of the injured nerves, but the entire dorsal horn. Therefore, these models are suitable for the study of central sensitization.

6.3 Training-mediated attenuation of neuropathic pain

The clinical management of neuropathic pain is very complex. Pharmacological therapy is not always effective in mediating pain relief, since some kind of neuropathies may be insensitive to ordinary analgesics. To improve the current treatment of subjects with neuropathic pain, alternative strategies should be adopted. Activity-based interventions represent a potential approach which may lead to benefits for those who are suffering from this debilitating chronic condition.

Research on animal models has provided evidence for the positive impact of training on central sensitization and chronic pain. In rats with an incomplete SCI, intensive training both prevents and reverses the development of tactile hyperreactivity (Detloff et al., 2014; Ward et al., 2014; Dugan and Sagen, 2015). Moreover, an early exercise regimen (starting at day 1 post-contusion) appears to be more effective in reducing mechanical allodynia than a delayed training program (Brown et al., 2011). It has been reported that late interventions can even lead to the establishment of detrimental mechanisms resulting in the stabilization of aberrant nociceptive afferent plasticity and neuropathic pain condition (Detloff et al., 2016). Lastly, some training modalities seem to be more effective than others at decreasing tactile hyperreactivity, with treadmill training being superior to stand or swim training (Hutchinson et al., 2004). Indeed, swim training has been shown ineffective in attenuating neuroinflammation around the lesion site in animals with a thoracic injury (Smith et al., 2009).

In humans, the potential effect of motor training on the development or the reversal of central sensitization and neuropathy remains to be investigated. Although some benefits of locomotor training have been described in various populations of chronic pain (O'Connor et al., 2015), training effects on neuropathic pain have not yet been fully addressed. However, it has been established that in subjects with neuropathic pain, a repeated training exercise increases cutaneous (in particular, plantar) sensation (Li and Manor, 2010), the ability to perceive vibrations (Balducci et al., 2006) and improvements in trunk (Song et al., 2011) and ankle proprioception (Xu et al., 2004). Recently, it has been shown that robot-assisted gait training is associated with an immediate and long-lasting reduction in general pain intensity in subjects with chronic incomplete SCI (Labruyère and van Hedel, 2014). Moreover, a case report has demonstrated beneficial effects of neurologic controlled exoskeletal interventions on pharmacologically-resistant neuropathic pain (Cruciger et al., 2016). In this study, authors have used a hybrid assistive limb (HAL®) exoskeleton, consisting in a tailored approach where assistance in motor function is triggered by the patient voluntary drive. At the end of a 12-week training period with the HAL exoskeleton, both subjects enrolled in the study have displayed improved walking abilities and a significant reduction in pain severity (Cruciger et al., 2016). Assisted training in the presence of virtual reality has also been investigated, showing a stable reduction in neuropathic pain and suggesting virtual reality as a desensitization therapy (Wismeijer and Vingerhoets, 2005; Gold et al., 2007; Sharar et al., 2008; Villiger et al., 2013; Roosink and Mercier, 2014; Roosink et al., 2016). These promising results indicate that activity-based interventions may be a useful tool in the management of refractory neuropathic pain in humans.

It has been hypothesized that the reduction in pain perceiving observed after physical therapy might be explained by exercise-induced endogenous analgesia (Koltyn, 2000) or by a long-term anti-central sensitization that influences different molecular pathways (Nijs et al., 2015).

As for the first hypothesis, it has been shown that training promotes the release of endogenous opioids, such as met-enkephalin and β-endorphin, that lead to analgesia and feelings of well-being (Dobson et al., 2014). In rat models of neuropathic pain, exercise-induced increases in endogenous opiates are accompanied by higher thresholds in paw withdrawal (Bement and Sluka, 2005; Sluka et al., 2013). Moreover, pain relief mediated by regular exercise is dependent on the activation of endogenous opioids pathways located in the brainstem (Stagg et al., 2011).

Training mediates its beneficial effects also by directly modulating mechanisms fostering central sensitization in the dorsal horn. Between them, neuroinflammation promoted by glial cells greatly contributes to neuropathic pain. Exercise training reverses astrocytes and microglia hyperactivity in the dorsal horn (Cobianchi et al., 2010; Bernardi et al., 2013; Almeida et al., 2015), although this relationship needs better investigation. Moreover, a single training session can temporally inactivate GSK-3 (glycogen synthase kinase) in rodents (Aschenbach et al., 2006). This kinase is involved in a variety of intracellular pathways, mainly triggering glial cells activation, promoting the expression of pro-inflammatory mediators and, in turn, inhibiting the release of anti-inflammatory cytokines (Beurel et al., 2010; Kaidanovich-Beilin and Woodgett, 2011). Thus, training-induced inactivation of GSK-3 is consistent with a reduced neuroinflammation within the dorsal horn. Furthermore, training has a direct modulatory effect on the expression of the main cytokines mediating inflammation in the dorsal horn, namely TNF-α and IL-1β (Chen et al., 2012).

Besides central anti-inflammatory effects, training promotes pain relief by modulating the expression of neurotrophins. The role of BDNF in nociception is controversial. Indeed, excessive levels of BDNF in DRG sensory neurons and in the dorsal horn are associated with an abnormal nociceptive processing and the development of neuropathic pain (Zhu et al., 2014; Nijs et al., 2015). Exercise increases BDNF expression in motor but not sensory neurons in animal models of neuropathy (Keeler et al., 2012). Moreover, training reduces levels of BDNF in DRGs and decreases neuropathic pain in rat (Cobianchi et al., 2013; Almeida et al., 2015). Similarly, training normalizes spinal levels of GDNF, preventing sprouting of nociceptive fibres and reducing mechanical allodynia (Detloff et al., 2014). Lastly, exercise promotes the over-expression of NT-3 (Gomez-Pinilla et al., 2001), involved in neuron survival and differentiation and in synapse formation, that results in a reduction in neuropathic pain (Sharma et al., 2010).

Thus, locomotor training promotes a certain relief from neuropathic pain through the modulation of different pathways. Vice versa, it should be considered that motor recovery mediated by training might be potentially influenced by a persistent central sensitization (Nijs et al., 2012; Mercier et al., 2017). This issue is of outstanding importance when considering that neuropathic pain has a high incidence in subjects with sensorimotor dysfunctions, such as SCI (Werhagen et al., 2004; Margot-Duclot et al., 2009; van Gorp et al., 2015; Paolucci et al., 2016). Therefore, a large proportion of subjects receives motor rehabilitation in the presence of pain. In the current clinical practice, pain relief and motor recovery are examined as two independent problems, though it must be considered that they strongly interact with each other. Indeed, training-induced plasticity associated with both outcomes share similar mechanisms which communicate bidirectionally (Baumbauer et al., 2009; Ferguson et al., 2012). This robust interplay suggests that dorsal horn hyperexcitability, typical of neuropathic pain, might interfere with the effectiveness of motor rehabilitation (Mercier et al., 2017). Thus, a better understanding of these interactions can be used to develop rehabilitation strategies that may lead simultaneously to better motor recovery and reduced chronic neuropathic pain.

AIMS OF THE STUDY

Afferent sensory feedback has been shown to have a direct control on spinal networks, modulating both ventral and dorsal activity (Rossignol et al., 2006). These basic research findings have been exploited by current neurorehabilitation approaches to trigger locomotor recovery and to reduce neuropathic pain in subjects with sensorimotor dysfunctions (Mercier et al., 2017). Activity-based interventions represent a useful strategy to provide incoming inputs capable to induce plastic changes in the human spinal cord (Dietz, 2002). Insight into network, cellular and molecular mechanisms underlying training-mediated benefits can be obtained using *in vitro* (Hayes et al., 2009; 2012) and *in vivo* (de Leon et al., 2002 a; b; Bose et al., 2012; Houlé and Côté, 2013) models. A better understanding of the spinal modifications elicited by physical therapy will allow for the optimization of current neurorehabilitative techniques.

The present study aims at identifying effects that afferent input has on spinal networks. Afferent fibres were activated using different approaches, mimicking physiological or pathological conditions.

i. In the first part, primary afferent fibres were stimulated through trains of electrical pulses, previously demonstrated to trigger the spinal CPG for locomotion (Marchetti et al., 2001 a). Since a strong interplay between dorsal and ventral networks has been assessed, I wondered whether these protocols could modulate dorsal circuits activity, as well. Moreover, I wanted to determine whether the extent of dorsal modulation could be correlated to the degree of activation of the locomotor CPG.
ii. In the second part, a new *in vivo* model of neuropathic pain was developed, crushing a nerve of the hindlimb. The first goal was to confirm whether this pathological activation of peripheral afferent fibres led to dorsal horn circuits hyperexcitability, established in terms of paw withdrawal and glial activation in the injured animal. Once verified this condition, molecular markers were identified, responsible for mediating mechanical allodynia in this animal model.
iii. In the third part, I aimed at developing a new *in vitro* model where hindlimbs were passively driven by a robotic device integrated to the recording chamber. Firstly, I established whether an afferent feedback could be evoked in this model. Then, I determined effects of incoming information on spinal networks.

MATERIALS, METHODS AND RESULTS

(See enclosed papers).

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Electrical Stimulation Able to Trigger Locomotor Spinal Circuits Also Induces Dorsal Horn Activity

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Objectives: Investigate whether electrical stimulation of the spinal cord adapted to trigger locomotor patterns additionally influences dorsal horn networks.

Materials and Methods: An *in vitro* model of isolated neonatal rat spinal cord was used to repetitively deliver electrical stimuli to lumbar dorsal roots and record from homolateral lumbar dorsal roots and ventral roots.

Results: Repetitive electrical lumbar dorsal root stimulation can affect both locomotor rhythms derived from ventral neuronal circuits and activity from dorsal neuronal circuits.

Conclusion: These data suggest that neuro-electrostimulation protocols can simultaneously activate functionally distinct spinal neuronal circuits.

Keywords: Central pattern generator, fictive locomotion, isolated spinal cord, neurorehabilitation, rat

Conflict of Interest: The authors reported no conflict of interest.

INTRODUCTION

Rehabilitation medicine is witnessing major advances relating to the functional benefits that can be obtained with the use of electrostimulatory devices. These devices are a major asset in activity-based therapies that are considered to target the recovery of standing and walking based on activity-dependent neuroplasticity (1).

This neuroplasticity importantly depends on activation of spinal neuronal circuits. The ventral horns of the lumbar spinal cord contain neuronal locomotor circuits which generate locomotor activity (2). These locomotor circuits are comprised of a network of primarily interneurons, known as the locomotor central pattern generator (CPG) that alternatively sends locomotor-related signals, either to the same muscles on opposing sides of the lower extremities or to extensors and flexors in the same leg (3,4). The presence of a locomotor CPG has been evidenced in the lumbar spinal cord of humans (5) and in animal models, which have proven to be indispensable for functional investigations focused around CPG networks (6,7). Among such animal models is the isolated neonatal rat spinal cord that has rendered numerous important insights into the neuroanatomical and neurophysiological properties of the locomotor CPG (8,9).

During physiological locomotion, the CPG receives a continuous and, thus, repetitive proprioceptive input from peripheral sites like stretch receptors through dorsal root (DR) afferent fibers. Animal studies have shown that also electrical stimulation of a lumbo-sacral DR can potently trigger activity of the locomotor CPG (10), and repetitive stimulation protocols may therefore be regarded as a form of exercise mimetic therapy.

In addition to its use in treatment of motor problems, neuro-electrostimulation has a long history in treatment of neurological disorders that concern somatosensory functions. Pain conditions that show poor responsiveness to pharmacological intervention have benefited from invasive pain management therapies such as spinal cord stimulation (11). The mechanisms by which this type of electrostimulation has its beneficial effect remain largely unclear, even though modulation of dorsal horn circuits in the spinal cord through DR activation seems to play a key role (12). In contrast to typical analgesic stimulation protocols, electrostimulation for triggering activity in CPG neuronal circuits is done at supra-motor-threshold stimulation intensities (13). Nevertheless, it cannot be excluded that these stimulation parameters also affect neuronal circuits in the spinal cord dorsal horn. The objective of this investigation was to trigger an activity of locomotor neuronal circuits in the isolated neonatal rat spinal cord through repetitive electrical DR stimulation and determine whether this procedure influenced the activity of dorsal horn neuronal circuits. For this aim, we investigated both the spontaneous DR activity and DR potentials (DRPs) induced by activation of a lumbosacral DR.

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METHODS

All procedures were approved by the Scuola Internazionale Superiore di Studi Avanzati ethics committee and are in accordance with the European Union directive for animal experiments (2010/63/EU). Every effort was made to reduce the number of animals used and to minimize their suffering. All experiments were performed, in line with the guidelines provided by the Italian Animal Welfare Act, on spinal cords isolated from Wistar neonatal rats (0–2 days old). After decapitation and evisceration, a ventral laminectomy was performed. Spinal cords were surgically isolated from the midthoracic region to *cauda equina*, through the careful microdissection of all ventral roots (VRs) and DRs. Hereafter, preparations were then continuously superfused (5 mL/min) with oxygenated (95% O_2—5% CO_2) Krebs solution containing (in mM) 113 NaCl, 4.5 KCl, 1 $MgCl_2$ $7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose, pH 7.4. Nerve recordings were obtained from lumbar roots using monopolar suction electrodes (14,15). In detail, homolateral recordings were made from two lumbar (L) DRs and from L2 and L5 VRs, the latter of which carry signals driving flexor and extensor muscles of the hindlimb, respectively (16) (Fig. 1a). Single or repetitive stimulations were delivered using bipolar suction electrodes connected to a programmable stimulator (STG4004®, Multichannel System, Reutlingen, Germany). The stimulation threshold (Th) was defined as the minimum intensity of stimulation eliciting a noticeable reflex response from all recorded roots (44.231 ± 8.397 μA, Table 1). A single rectangular pulse (0.1 ms; 96.538 ± 14.596 μA, 2.537 ± 0.342 × Th, Table 1) was delivered to a single DR (typically from the DRL5 to sacrocaudal afferents) to elicit composite responses from DRs and VRs referred to as DRDRPs and DRVRPs, respectively. At least five events of DRDRPs and DRVRPs were averaged for each experiment followed by measurement of peak and area. Electrical stimulating protocols to elicit episodes of FL were delivered to a lumbo-sacral DR on the same side from which recordings were taken (Fig. 1a) and consisted of a stereotyped train of 120 square pulses (duration = 0.1 ms) at 2 Hz frequency (interstimulus interval = 500 ms, Fig. 1b) and a suprathreshold intensity (68.846 ± 12.316 μA, Table 1) (10). In response to stimulation, an episode of cumulative depolarization with superimposed alternated oscillations emerges between VRL2 and VRL5 and named fictive locomotion (FL) (17,18). A cycle of FL was

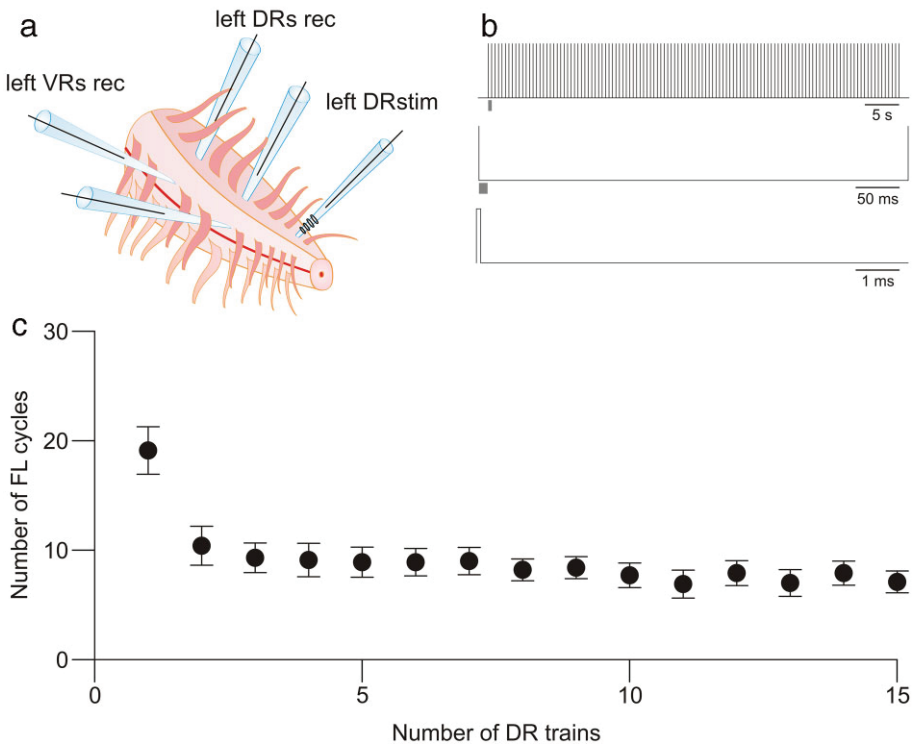

Figure 1. a. Cartoon to illustrate the isolated neonatal rat spinal cord (lateral view) with recording suction electrodes at homolateral dorsal and ventral lumbar roots and the stimulating suction electrode at a lumbosacral root on the same side of the recording electrodes. b. Top: A single train is composed of 120 stimuli for a total length of 60 s. b. Middle: A couple of pulses corresponding to the upper gray rectangle displays the interstimuli interval of 500 ms (2 Hz). b. Bottom: A segment corresponding to the gray square of the middle inset highlights the duration (0.1 ms) of each single rectangular pulse. c. The average number of oscillations recorded from VRs during subsequent DR trains ($N = 10$; note that three out of 13 samples were unsuccessful for the induction of such oscillations and therefore not included in this analysis).

Table 1. Stimulation Intensities for DR Trains and Single Pulse Delivery.

	Threshold (μA) (mean ± SEM)	Intensity of stimulation (μA) (mean ± SEM)	Intensity of stimulation (×Th) (mean ± SEM)	*N*
DR trains	44.231 ± 8.397	68.846 ± 12.316	1.610 ± 0.056	13
DRDRPs and DRVRPs	44.231 ± 8.397	96.538 ± 14.596	2.537 ± 0.342	13

defined as the interval spanning between the onset of the sustained depolarization to the end of repolarization to the initial baseline value. The number of FL cycles was quantified, and the phase relation among signal pairs was analyzed as cross-correlation function (CCF) with the software Clampfit® 10.3 (Molecular Devices Corporation, PA, USA). DR spontaneous activity was quantified as the root mean square (RMS) of the power spectrum. Data are means ± SEM, and *N* indicates the number of preparations. After testing for normality,

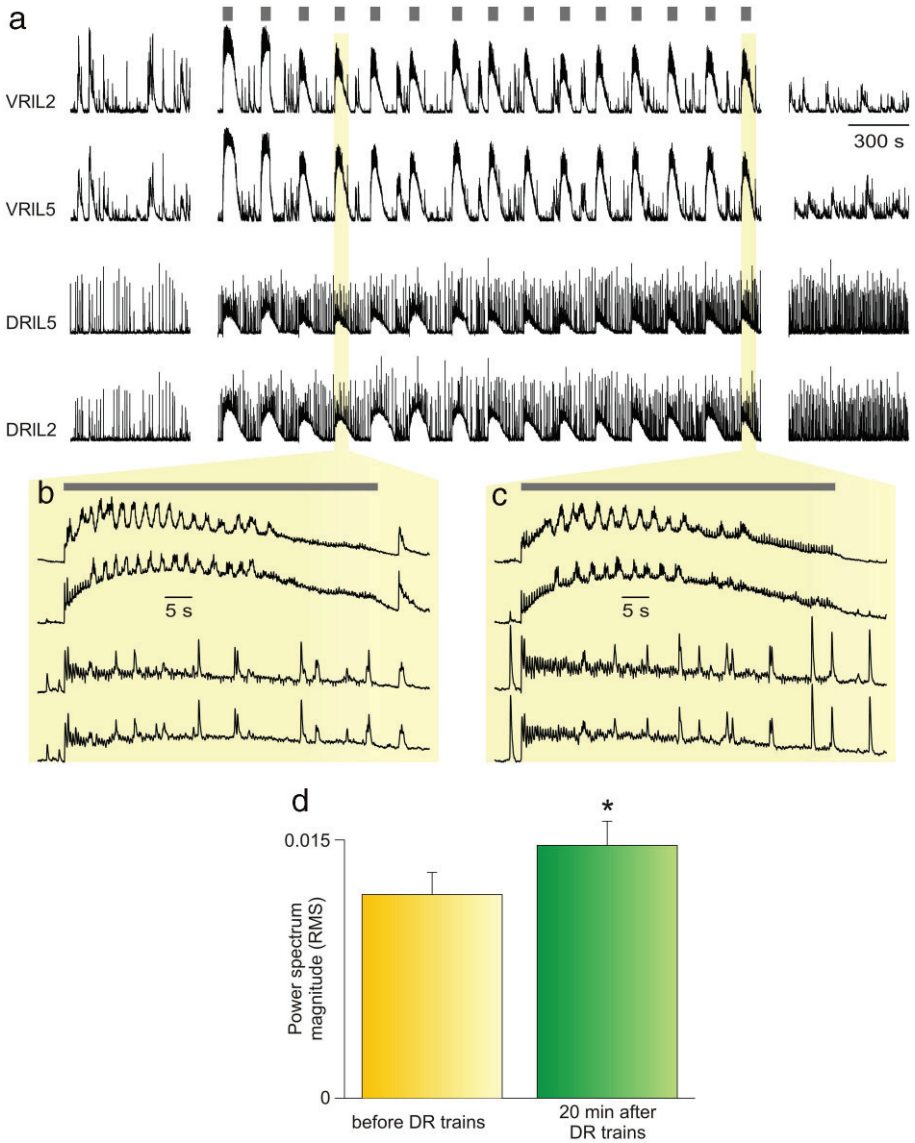

Figure 2. a. Traces obtained from the homolateral recordings of two ventral roots (left lumbar 2 and left lumbar 5; VRIL2 and VRIL5, respectively) and two dorsal roots (left lumbar 5 and left lumbar 2; DRIL5 and DRIL2, respectively) before (left panels), during (middle panels), and after (right panels) the application of 15 DR trains (upper gray bars, amplitude 23 μA, 1.5 × Th) to a DR on the same side where the recordings were performed (DRIL6). b. Larger magnification of root activities during the fourth DR train (upper gray rectangle) as an example. c. Larger magnification of root activities during the 15th DR train (upper gray rectangle) as an example. d. Spontaneous DRs activity, quantified as the magnitude of the power spectrum (root mean square [RMS]) during a 20-min interval either before or after the 15 DR trains (*N* = 13); the DR trains triggered a significant increase in DR activity (*p = 0.022, paired *t*-test on raw data, *N* = 13).

Table 2. Effect of DR Train Stimulation on Spontaneous DR Activity and Electrically Evoked DR and VR Responses.

	Before DR trains (mean ± SEM)	After DR trains (mean ± SEM)	*N*	*p* (statistical test)
Power spectrum magnitude (RMS)	0.012 ± 0.001	0.015 ± 0.001	13	0.022 (paired *t*-test)
DRDRPs peak (mV)	0.327 ± 0.095	0.278 ± 0.080	13	0.092 (paired *t*-test)
DRDRPs area (mV × s)	26.123 ± 7.004	25.213 ± 6.801	13	0.582 (paired *t*-test)
DRVRPs peak (mV)	0.483 ± 0.049	0.465 ± 0.064	13	0.635 (paired *t*-test)
DRVRPs area (mV × s)	382.799 ± 51.369	337.946 ± 50.411	13	0.219 (paired *t*-test)

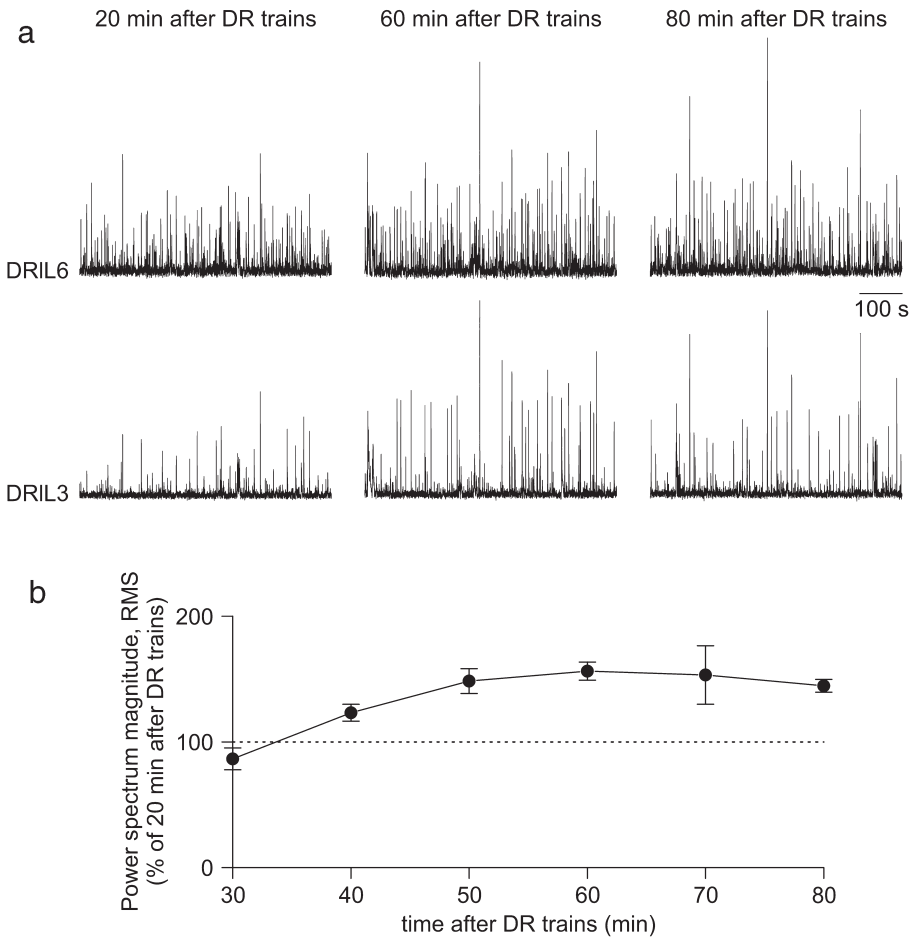

Figure 3. a. Recordings obtained from homolateral DRIL6 (upper traces) and DRIL3 (lower traces) showing DRs discharges after the delivery of DR trains (applied to left sacrocaudal rootlets) at three different time points in the following washout phase (20, 60, and 80, respectively). b. Time course assessing spontaneous DRs activity up to 80 min after last delivered train; antidromic discharges are quantified using the magnitude of the power spectrum (root mean square [RMS]) calculated for ten-minute bins. Data are expressed as percentage of the RMS value 20 min after electrostimulation.

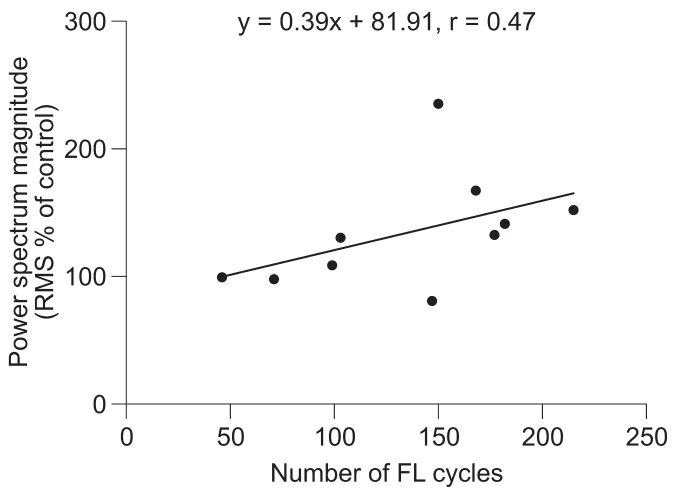

Figure 4. Linear regression analysis between the number of FL cycles (*x* axis) and spontaneous DR activity expressed as a percentage of control RMS (*y* axis). A weak correlation is achieved, confirmed by the low Pearson's *r* value (both regression equation and *r* value are reported on the top of the graph).

RMS, DRDRP and DRVRP area and peak were compared using the paired Student's *t*-test. A linear regression analysis was performed between the number of FL episodes and the RMS using ORIGIN® 9.0 software (OriginLab, Northhampton, MA, USA). Statistical analysis was performed using SIGMASTAT® 3.5 software (Systat Software Inc, CA, USA), and results were considered statistically significant when $p < 0.05$.

RESULTS

In order to achieve a continuous activation of the locomotor CPG *in vitro*, 2-Hz electrical pulses were repeatedly delivered to a lumbosacral DR, while homolateral DR and VR activities were simultaneously recorded (Fig. 1a). Within a time frame of 45 min, a total of 15 DR trains were delivered, each of 60 sec duration and interposed by two minutes of no stimulation. Each train was delivered at an intensity ($1.610 \pm 0.056 \times$ Th) that optimally elicited an episode of alternating oscillations. Although the first DR train induced the highest number of oscillations, also subsequent DR trains effectively triggered episodes of alternating cycles between homolateral L2 and L5 VRs (Fig. 1c).

We next studied the putative effect of the electrostimulation protocol on the spontaneous activity measured from DRs and on the

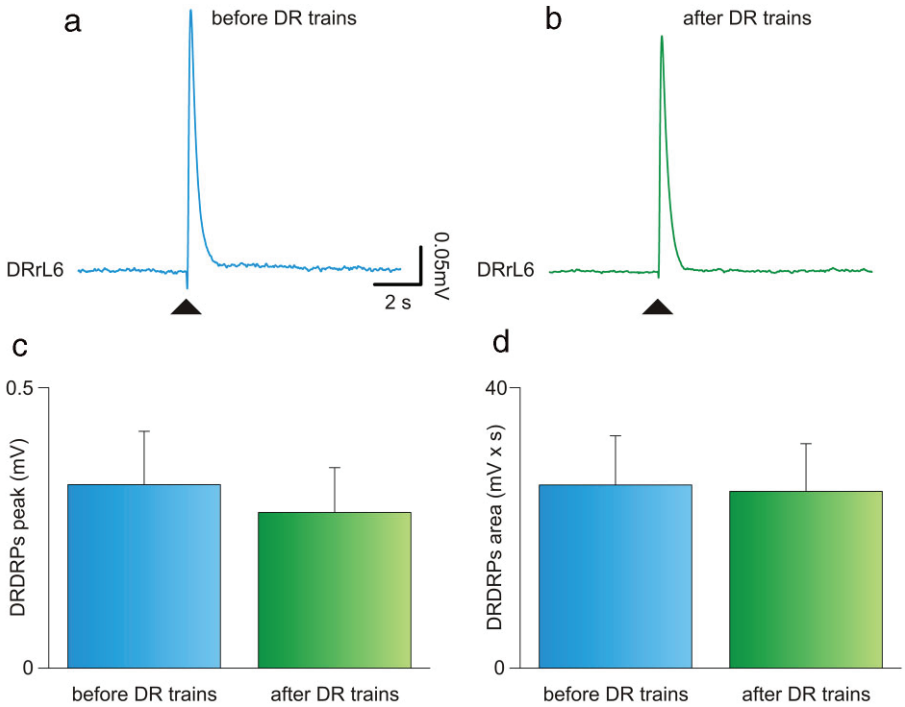

Figure 5. a, b. Sample mean traces (averaged from five independent episodes) recorded from DRrL6 in response to the delivery of single pulses (arrowhead) to the homolateral DRrS1 (duration = 0.1 ms, intensity = 37.5 μA, 1.5 × Th). Response before DR trains (left) is not different from the one evoked by the same stimulation right after 15 DR trains (right). c, d. Peak amplitude and peak area of DR potentials (DRPs) evoked by stimulation of a homolateral lumbosacral root, collectively termed DRDRPs before and after application of 15 DR trains to a homolateral lumbosacral DR ($N = 13$).

synchronous nonlocomotor-related activity (CCF = 0.91) recorded in control from VRs (Fig. 2a, lower and upper traces, respectively). Before the application of DR trains, the spontaneous activities are relatively modest (Fig. 2a, left). After the delivery of 15 DR trains, which effectively induced FL on VRs (Fig. 2b; number of oscillations = 16; CCF = –0.55; Fig. 2c; number of oscillations = 12; CCF = –0.53), an increase in spontaneous DR activity appeared (Fig. 2a, right). This was reflected by a significant increase in RMS within 30 min after DR trains compared with prestimulation values (Fig. 2d, $p = 0.022$, paired t-test on raw data, $N = 13$, Table 2). This increased spontaneous activity appeared to last for 40 min after cessation of the electrostimulation (data not shown). In order to assess whether the increased DR activity was maintained for a longer time, in one experiment, the post-DR trains period was extended up to 80 min (Fig. 3a). For the whole experiment, the magnitude of dorsal spontaneous activity remained augmented and not significantly different from the value reached after 20 min (Fig. 3b), indicating the persistence of DRs effect.

Finally, we tested whether FL was required for the observed increase in spontaneous DR activity. In only three samples (age P 0 and 2) repetitive trains (2 Hz, 120 pulses, intensity = 1.504 ± 0.016 × Th) were unable to elicit FL cycles. In these cases, DR spontaneous events were not significantly augmented ($RMS_{after\ DR\ train} = 0.013 \pm 0.003$ vs. $RMS_{before\ DR\ train} = 0.015 \pm 0.002$; $p = 0.548$, paired t-test on raw data; $N = 3$) suggesting that FL was required for the observed increase in spontaneous DR activity.

Nevertheless, linear regression analysis, considering only the experiments in which DR trains elicited the activation of locomotor CPG, showed that a weak relationship exists between the number of FL oscillations and RMS ($y = 0.39\times + 81.91$; Pearson's $r = 0.47$; $N = 10$, Fig. 4) indicating that the emergence of DR discharges due to electrical stimulation is a nonlinear feature.

Finally, we investigated whether the facilitatory effect of electrostimulation on DR activity was restricted to spontaneous activity or extended to evoked DR responses as well. DRDRPs (Fig. 5a,b) and DRVRPs (Fig. 6a,b) were found to be unaffected after DR trains (Figs. 5c,d and 6c,d; Table 2).

DISCUSSION

Delivery of electrical stimulation protocols to DRs has been successfully used to trigger activity in locomotor networks residing in the ventral horn of the lumbar spinal cord (10,13). The current investigation was performed on the basis of the hypothesis that such protocols also may influence neuronal networks other than the CPG, particularly neuronal circuits in the dorsal horn that are closely associated with or even *en route* with signals passing from the DRs to the CPG (19). We here show that such a modulatory influence exists and is specific for spontaneous dorsal activity. Electrically evoked responses represented by DRDRPs and DRVRPs are not modulated.

Our data are based on an *in vitro* model of isolated neonatal rat spinal cord with roots attached, allowing for functional investigations into spinal cord neuronal circuits within their native and integrated physiological organization. Within this context, we specifically studied not only spontaneous DR activity, but also DRDRPs and DRVRPs. DRDRPs are considered to relate to presynaptic inhibition of peripheral inputs (20), thereby representing a fundamental antinociceptive mechanism. DRVRPs represent the polysynaptic reflex responses evoked from motoneuronal pools by afferent inputs. In contrast to episodic low-frequency DR stimulation, application of repetitive trains causes an antidromic hyperactivity. This increase in activity might be ascribed to the known ability of high-frequency DR stimulation to produce long-term

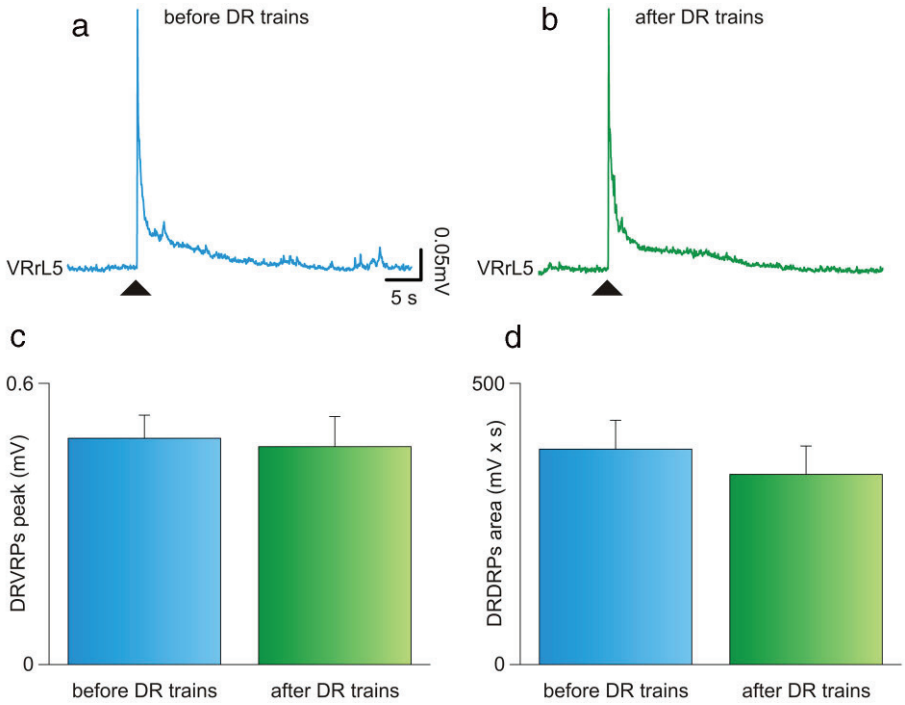

Figure 6. a, b. Sample mean traces (averaged from five independent episodes) recorded from VRrL5 in response to the delivery of single pulses (arrowhead) to the homolateral DRrL5 (duration = 0.1 ms, intensity = 135 μA, 3 × Th). Response before DR trains (left) is not different from the one evoked by the same stimulation right after 15 DR trains (right). c, d. Peak amplitude and peak area of VR potentials (VRPs) evoked by stimulation of a homolateral lumbosacral root, collectively termed DRVRPs before and after application of 15 DR trains to a homolateral lumbosacral DR (*N* = 13).

potentiation at the level of primary afferent synapses (21). In our experiments, DRDRPs and DRVRPs did not increase, as opposed to the spontaneous antidromic discharges, the latter of which reflects activity in dorsal horn networks. Therefore, the plastic events responsible for the observed effects in the current investigation ought to be linked to the synapses within the dorsal network responsible for the origin of discharges rather than to a synaptic potentiation at the level of primary afferent terminals in the spinal cord. This spinal cord plasticity may, then, have an influence on mechanisms of central sensitization in pathological contexts as such mechanisms directly involving dorsal horn nociceptive circuitries as well (22–24). The nociceptive networks residing in the dorsal horn of the lumbar spinal cord are, however, extremely complex and comprise a variety of synaptic contacts with inhibitory, excitatory, and other more modulatory effects (25). It thereby remains speculative whether an augmentation of activity in such complicated circuits impacts on nociception or pain. On the one hand, it is possible that it could relate to signs of discomfort that occur with neuroelectrostimulation at higher stimulation intensities in human individuals (26). On the other hand, activity originating from dorsal horn circuits and traveling antidromically through the DRs could form an impediment of peripheral signals on their way to the central nervous system. Through such mechanism, it could be expected that pain relief might occur (27).

The stimulation intensities required for engaging neuronal locomotor circuits in the ventral horn of the spinal cord are much higher than those known to modulate nociceptive circuits in the spinal cord dorsal horn (13). We showed that at these high stimulation intensities, spontaneous DR activity was augmented, but our data suggested only a minor relevance of ventral interneuronal networks in this effect. On the one hand, the three preparations which did not show FL induction were without increased spontaneous DR activity. On the other hand, a regression analysis performed for the preparations in which FL was induced showed only a weak correlation between spontaneous DR activity elicited by electrical stimuli and the number of FL cycles. A minor relevance of locomotor circuits in the spontaneous DR activities could be considered surprising. As part of the locomotor program, the spinal CPG phasically modulates sensory inputs through primary afferent depolarization (PAD) (28,29). Tonic and phasic depolarization of primary afferent terminals has been observed during both FL (30–32) and electrical nerve stimulation (33,34). These observations point to a specific timing of the flexor and extensor phases for the majority of afferents (32–34). PAD evoked by movement-related sensory feedback during real locomotion could work similarly (35). Also, in *in vitro* preparations can locomotor-related PAD and DR-evoked PAD be evoked. Here, dorsal cells phasically depolarize during each ipsilateral VR burst (36). Earlier reports have shown a role of (electrically induced) contralateral afferent activity in the generation of DRPs in the opposite limb during hindpaw swing (37). Similarly, literature has shown that, during FL, the sensory inflow to the swing limb can be increased by presynaptic inhibition based on forces generated by the (contralateral) stance limb (38). However, we previously reported on isolated neonatal spinal cord preparations that neurochemically induced FL did not evoke any DR discharges (39). We may therefore conclude that the increased DR activity reported after repetitive train delivery might be ascribed to nonlinear properties of CPG activation and/or directly to the barrage of incoming afferent signals reaching the spinal cord. In this regard, it is important to notice that comparable low-frequency trains of electrical pulses were found to recruit

mainly pain-related C fibers, while higher frequencies of stimulation triggered an activation of larger-sized A fibers (40).

Our repetitive DR stimulation protocol also was characterized by the induction of a strong locomotor response to the first DR train, while subsequent DR trains during the total length of the stimulation session elicited reliable episodes of slightly fewer FL cycles. The finding of more FL cycles initially following stimulation, but then declining, is reminiscent of the reduction in the number of FL cycles at the end of each single train. It is probable that the some of the elements that cause the decay in the locomotor response during the continuous delivery of a single train (membrane shunt due to the depolarization following increased potassium concentrations (41) and the release of inhibitory neurotransmitters, able to depress FL oscillation (42)) also might play a role in the progressive deterioration of episodes of FL when multiple stimulating trains are delivered in close sequence, as in our training mimetic protocol. The long-term expression of an optimal number of FL oscillations requires a recovery time of four minutes between two DR trains (43). In the present study, the resting phases of stimulation protocol were condensed to two minutes in order to mimic the prolonged activation of the CPG (1800 total pulses) for a total training session of 45 min, a duration typically used in training mimetic rehabitative therapies (44).

Although the present investigation used *in vitro* neonatal spinal cord preparations, the data may be considered of relevance to the field of neuro-electrostimulation in treatment of neurological disorders. First, spontaneous DR activity due to presynaptic inhibition of primary afferents is not specific for neonatal tissues as used in the present investigation. Indeed, it can also be evoked in the adult animal (45), suggesting that the present data may have biological relevance for the adult nervous system as well. While the organization of new-born rat spinal cord networks is similar to that of adult animals (e.g., cats) (46), this relatively young tissue presents clear advantages in terms of easiness of technical access to multiple DR and VR recordings and stimulations, as well as better *in vitro* viability compared with older tissues (47). Second, presynaptic inhibition is a process with direct relevance to neurological disorders. Both acute and chronic spinal cord injuries show systematic alterations in presynaptic inhibition within the lumbosacral cord and contribute to the clinical symptoms associated with reflex dysfunctions (48). While presynaptic inhibition is enhanced in acute lesion, leading to hyporeflexia associated with spinal shock, it does diminish in chronic SCIs, causing hyperreflexia associated with spasticity. Our results show an increase in spontaneous antidromic discharges in response to presynaptic depolarization of dorsal afferents following a repetitive electrical stimulation. We may speculate that this acquisition might reflect on the clinical use of epidural stimulation in case of a spinal cord injury. On the one hand, a similar increase in afferent excitation might be tied to an overall increase in presynaptic inhibition that would restore "spinal shock" in acute cases. On the other hand, in chronic lesions, where electrical stimulation is indicated for the neuromodulation of chronic pain or for the recovery of posture and gait, the increase in dorsal antidromic potentials might help to counteract spasticity and alleviate chronic pain, caused among others by the diminished presynaptic inhibition.

CONCLUSION

Basic research on *in vitro* preparations, computational studies and theoretical models, although far from clinical setting, has significantly advanced our understanding of the therapeutic effects of neurostimulation for rehabilitation and control of pain by unveiling some of the basic principles of extracellular electrical stimulation in spinal cord (49) and exploring the most suitable stimulating protocols to activate spinal networks (50).

In the current study, we conclude that the repetitive electrical stimulation of the DR that is able to evoke locomotor-like rhythmic motor activity in the lumbar VR also induces spontaneous activity observed in DRs. This remarkable observation will now set the basis for exploring the exact mechanism of this spontaneous DR activity as well as finding the neuronal population that constitutes the related "dorsal horn network" that is modulated by the stimulation protocol. These insights should be of great value for our understanding and optimization of clinical spinal cord stimulation protocols used for rehabilitation purposes and/or treatment of pain. Herein, *in vivo* experiments on adult rats will have an important role in addressing whether "locomotion-related" electrical protocols as used in the present investigation affect pain.

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Authorship Statements

Nejada Dingu conducted experiments, analyzed data, and prepared the manuscript. Dr. Ronald Deumens interpreted data and prepared the manuscript. Dr. Giuliano Taccola designed the study, conducted experiments, analyzed and interpreted data, and prepared the manuscript.

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COMMENTS

The study uses in vitro electrophysiology in isolated spinal cord models to test the ability of repetitive activation of sensory pathways in modulating specific (ventral or dorsal) spinal circuit operation. Particular attention is dedicated to the locomotor pattern activation, which is the objective of current stimulating protocols aimed at activating locomotor outputs in lesioned spinal cords. The study is timely in that the use of neuro-electrostimulation in treating neurological disorders is increasing, however our knowledge of the mechanisms responsible for any beneficial effects is still very limited.

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The experiments reported in this manuscript were performed by using electrophysiological techniques to record neural activity in isolated spinal cord models. The study was conducted to determine how repetitive stimulation of sensory pathways modulates specific ventral or dorsal spinal network function. The results lead to the suggestion that repetitive stimulation of lumbar dorsal roots has the capacity to influence both locomotor rhythms and dorsal horn neuronal circuitry. The translational importance of this study is that it may provide the background to use neurostimulation for gait rehabilitation and management of pain.

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Although electrical stimulation represents a promising rehabilitation method for inducing plasticity in activity-dependent mechanisms of the spinal cord, many basic issues regarding the mechanisms and consequences of this type of stimulation remain to be elucidated. In this paper, the authors report persistent changes in dorsal root activity following electrical-induced fictive locomotion in the isolated spinal cord of the neonatal rat. Specifically, dorsal root electrostimulation in vitro induced both fictive locomotor activity in ventral roots and increased spontaneous activity within the dorsal horn. It is an important consideration that dorsal horn networks are activated in tandem with locomotor

circuitry, highlighting that multiple distinct spinal networks are activated by electrical stimulation; however, the data presented here suggest that the relation between different networks may not be linear. It remains to be clarified how different levels of spontaneous activity in dorsal networks may impact sensory processing during execution of functional movement. In particular and as the authors point out, the influence of this activity on nociceptive circuits and signaling will be important to consider for protocols administering electrical stimulation to the injured spinal cord.

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Research Paper

A new model of nerve injury in the rat reveals a role of Regulator of G protein Signaling 4 in tactile hypersensitivity

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ABSTRACT

Tactile hypersensitivity is one of the most debilitating symptoms of neuropathic pain syndromes. Clinical studies have suggested that its presence at early postoperative stages may predict chronic (neuropathic) pain after surgery. Currently available animal models are typically associated with consistent tactile hypersensitivity and are therefore limited to distinguish between mechanisms that underlie tactile hypersensitivity as opposed to mechanisms that protect against it. In this study we have modified the rat model of spared nerve injury, restricting the surgical lesion to a single peripheral branch of the sciatic nerve. This modification reduced the prevalence of tactile hypersensitivity from nearly 100% to approximately 50%. With this model, we here also demonstrated that the Regulator of G protein Signaling 4 (RGS4) was specifically up-regulated in the lumbar dorsal root ganglia and dorsal horn of rats developing tactile hypersensitivity. Intrathecal delivery of the RGS4 inhibitor CCG63802 was found to reverse tactile hypersensitivity for a 1 h period. Moreover, tactile hypersensitivity after modified spared nerve injury was most frequently persistent for at least four weeks and associated with higher reactivity of glial cells in the lumbar dorsal horn. Based on these data we suggest that this new animal model of nerve injury represents an asset in understanding divergent neuropathic pain outcomes, so far unravelling a role of RGS4 in tactile hypersensitivity. Whether this model also holds promise in the study of the transition from acute to chronic pain will have to be seen in future investigations.

1. Introduction

The search for the cellular and/or molecular underpinnings of persistent pain states has intensified over the past decades. The advances in our mechanistic understanding of chronic pain, which affects 30–50% of the general population (Bouhassira et al., 2008; Torrance et al., 2006) are heavily based on animal models of peripheral nerve injury (Gregory et al., 2013). Chronic pain often has neuropathic characteristics (Lavand'homme, 2011) and, as chronic pain, neuropathic pain is highly intractable (Baron et al., 2010). Nevertheless, nerve lesions do not always lead to persistent pain as illustrated by half the surgical amputees who do not develop chronic postoperative pain (Kehlet et al., 2006). We still know only little about the factors that facilitate versus the factors that protect against a transition from acute to chronic pain (Deumens et al., 2013).

Longitudinal studies on postoperative patients show a 10–50% prevalence of chronic pain, strongly associated with nerve lesions (Kehlet et al., 2006). Clinical data suggest that tactile hypersensitivity early after surgery holds predictive value for chronic (neuropathic) postoperative pain (Lavand'homme et al., 2005; Martinez et al., 2012). While tactile hypersensitivity does not consistently develop after surgery or nerve injury in human, most animal models of peripheral nerve injury show consistent tactile hypersensitivity (Gregory et al., 2013). Interestingly, the prevalence of tactile hypersensitivity after injury to the rat spinal cord was found to depend on the extent of tissue trauma (Kloos et al., 2005). This made us wonder whether restricting the extent of tissue

Abbreviations: CB_1 receptor, cannabinoid type-1 receptor; DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; MPE, maximum-possible-effect; mSNI, modified spared nerve injury; PWT, paw withdrawal threshold; RGS4, Regulator of G protein Signaling 4; SNI, spared nerve injury; SSI, static sciatic index.

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trauma after peripheral nerve injury could reduce the prevalence of tactile hypersensitivity as well.

The prevalence of neuropathic pain-like behaviours such as tactile hypersensitivity may also depend on the choice of rat strain (Yoon et al., 1999). Nearly 50% of Holtzman rats were found to be protected against tactile hypersensitivity through an engagement of inhibitory projections descending from the brainstem to the spinal cord and modulating local nociceptive networks in the dorsal horn (De Felice et al., 2011). The failure to engage such inhibitory systems in the other 50% of rats remains largely unexplained, but disinhibition within the nociceptive system has been repeatedly documented after nerve injury, at multiple locations of the neuraxis (Blom et al., 2014; von Hehn et al., 2012).

A multitude of neuropathic mechanisms may cause disinhibitory states in the central nervous system (CNS), both of neuronal and non-neuronal (immune) origin. We recently demonstrated that the signaling efficacy of the analgesic cannabinoid type-1 (CB_1) receptors was reduced in the spinal cord of nerve injured rats due to an injury-induced up-regulation in Regulator of G protein Signaling 4 (RGS4) (Bosier et al., 2015). RGS is a family of multifunctional proteins that promote the termination of signaling through G protein-coupled receptors, and previous work already linked spinal RGS4 with a loss of opioid receptor signaling efficacy after peripheral nerve injury in the rat (Garnier et al., 2003).

In the present study, we modified the model of spared nerve injury in the rat in order to limit the extent of tissue trauma. Tactile hypersensitivity, which occurred in only 50% of rats with modified spared nerve injury, was found to be associated with an increased expression of RGS4.

2. Materials and methods

2.1. Animals: models of peripheral nerve injury

A total of 149 adult female Sprague Dawley rats, 10–12 weeks old, were used, 139 under ethical approval of the Belgium authority on animal experimentation (LA2230419) and 10 under ethical approval of the Italian authority on animal experimentation (the Scuola Internazionale Superiore di Studi Avanzati (SISSA) ethics committee). All experiments were conducted under strict regulations, respecting the European Community Council directive of 24 November 1986 (86–609/ECC) and the decree of 20 October 1987 (87–848/EEC). The animals were kept in groups of 2–3 animals per standard makrolon cage with ad libitum access to food at a regular 12:12 h light-dark cycle. An exception to this rule of social housing applied to animals receiving an indwelling intrathecal catheter, i.e. a total of n = 24 rats that were individually housed. Animals were either subjected to nerve injury (n = 124) or sham-surgery (n = 15) using methods reported previously (Decosterd and Woolf, 2000), but with slight modifications. In brief, the sciatic nerve was exposed *at random* on either the left or the right side under sevoflurane anaesthesia (6% in oxygen for induction; 2–3% in oxygen for maintenance) and aseptic conditions. Then, the three peripheral nerve branches of the sciatic nerve (i.e. tibial, common peroneal and sural nerve branches) were exposed through blunt dissection and freed from the surrounding connective tissue. Animals were *ad random* divided into three groups: (1) spared nerve injury (SNI; n = 21), (2) modified SNI (mSNI; n = 103), and (3) sham surgery (n = 15). For SNI, the tibial and common peroneal nerve branches were injured while for mSNI only the common peroneal nerve branch was injured. Injury was inflicted using a non-serrated nerve clamp, i.e. the De Beer clamp (Honer Medizin-Technik GmbH & Co., Spaichingen, Germany) exerting a force of 54 N over a period of 30 s (Luis et al., 2007). In both mSNI and SNI the sural nerve branch was left intact (spared). Sham surgery involved skin incision and the sciatic nerve branches were dissected free, but were not crushed. Then, wounds were closed using 4/0 prolene sutures and animals were returned to their home cage. Postoperative care did not include pain medication as this might interfere with the primary study outcome, i.e. the development of neuropathic pain-like behaviour.

2.2. Electrophysiology

Adult female rats were anaesthetized with CO_2 and then sacrificed through CO_2 asphyxiation followed by cervical dislocation in line with the guidelines provided by the Italian Animal Welfare act, following the European Directive for animal experiments 2010/63/EU. Sciatic nerves were carefully dissected out from dorsal vertebrae to the ankle. One sciatic nerve per animal was used and each time the side (right or left leg) was randomly selected (n = 10 animals; 5 right, 5 left). At the end of the experiment, a precision caliper was used to carefully measure the mean lengths of the three peripheral branches of the sciatic nerve (tibial branch = 40.85 ± 2.31 mm; common peroneal branch 34.60 ± 2.15 mm; sural branch = 28.62 ± 2.942 mm (n = 10)).

As for electrophysiological recordings, monopolar glass suction electrodes were used to draw in the peripheral extremity of each branch, while stimuli were delivered to the sciatic nerve using a concentric bipolar electrode (see cartoon in Fig. 1A). Signals were recorded in AC, amplified 1000 times (DP-304®, Warner Instruments, CT), digitalized (250 KHz, Digidata® 1440 A, Molecular Devices Corporation, CA) and stored in a personal computer for further analysis. Single pulses (total width = 0.2 ms) were delivered as cathodic-first charge-balanced biphasic rectangular current injections without a delay between cathodal and anodal phases. In order to obtain input/output curves, we delivered a train of stimuli (0.33 Hz) of increasing amplitude (80 stimuli, 5 pulses for each steps from 10 to 160 μA). The stimulating threshold was defined as the minimum pulse strength able to evoke an appreciable response (for tibial = 20.00 ± 1.67 μA; common peroneal = 23.33 ± 1.67 μA; sural = 23.33 ± 1.67 μA; n = 10). Nerve injury was inflicted onto both tibial and common peroneal branches (see cartoon in Fig. 1B) as described earlier. In order to prevent decay of signal amplitude with the slightest movements of nerve extremities during clamp manipulation, we released all suctions after control recordings. After lesion, the nerve clamp was removed and new suctions were performed with the same glass electrodes, to obtain an identical seal as in control conditions. The same procedures were performed on the unlesioned (spared) sural nerve, which served as an internal control. Time to peak was calculated as the time spanning from the first stimulation artifact to the peak of response. At least five traces were averaged for each stimulation intensity. Conduction velocity was expressed in m/s and resulted from a division of fiber length by the mean time to peak for the maximal stimulation strength applied (160 μA).

2.3. Algesimetry

After habituating the animals to the experimenter (R.D.), the animals were placed in transparent plastic chambers without floor, positioned on an elevated wire mesh. Acclimatization was allowed for a period of about 20 min after which the von Frey test was performed. Herein, a set of eight calibrated von Frey hair filaments (Stoelting, Wood Dale, IL, US) was used (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, 15.1 g). Filaments were applied to the plantar hind paw surface and held in a slightly buckled position for a period of around 8 s, starting with the 2 g-filament. The choice for the following filament was based on the response to the previous filament application, being the closest-lower filament in case of a positive withdrawal response ('x') or the closest-higher filament in case of a negative withdrawal response ('o'). A positive withdrawal response was defined by a paw withdrawal associated with aversive behaviour, such as keeping the stimulated paw elevated, licking the paw, or attacking or biting of the filament. This method of filament application was continued until a sequence of six filament applications was acquired starting either with 'o-x' or with 'x'. In case the upper-end filament (15.1 g) or the lower-end filament (0.4 g) was

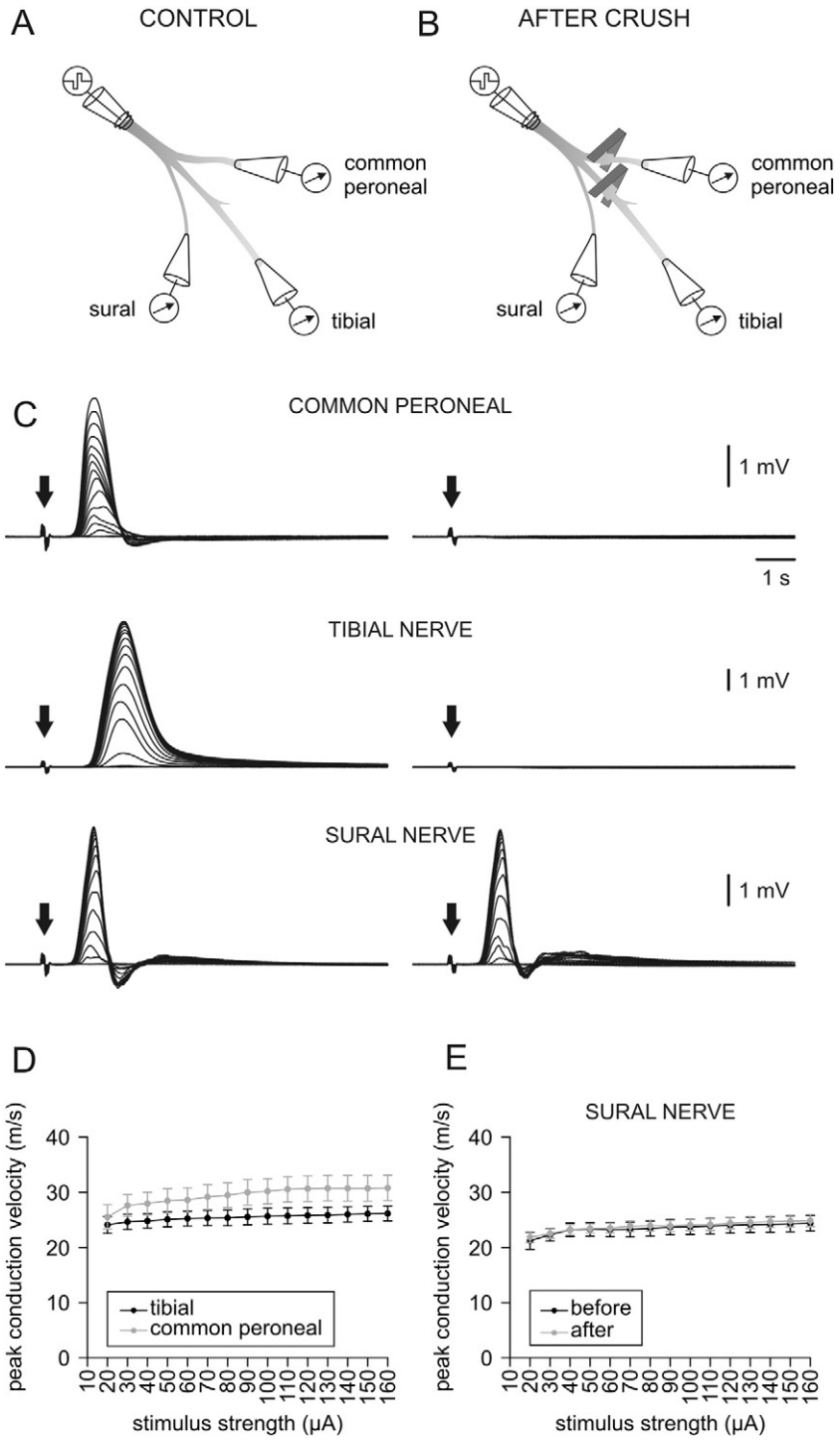

Fig. 1. Functional (electrophysiological) responses measured before and after crush injury. A; cartoon depicting the setting for electrophysiological experiments. A stimulating suction electrode was positioned at the proximal end of an isolated sciatic nerve while three suction electrodes recorded responses from the three peripheral ends of the sciatic nerve. B; sites of standardized crush are shown as sketched clamp positions. Traces in C result from input/output experiments performed in control (left) conditions and after lesion of the common peroneal and tibial branches (right). Note that injured branches were completely silenced while the unlesioned (spared) sural branch retains responses comparable to control. Arrows indicate stimulation artefacts; the different time to peak for the three responses corresponds to minimal differences in length of the three branches that, indeed, still maintained comparable conduction velocities. D; conduction velocities in control conditions for tibial (black) and common peroneal (grey) at increasing pulse strengths (n = 10). E; conduction velocities of the spared sural nerve before and after crush procedures (n = 10). Note superimposed dots before and after lesion indicating that sural responses were not affected by clamping procedures.

reached, no more filaments were applied any further. The 50% paw withdrawal threshold (PWT) was then calculated as described previously (Chaplan et al., 1994). In case of merely positive or merely negative responses to any filament, cut-off values were assigned (0.4 g and 15.1 g, respectively). Only the sural nerve territory at the glabrous plantar hind paw surface was stimulated throughout the experiment as this territory remained innervated in both injury models (SNI and mSNI), thus allowing for the assessment of stimulus-response behaviours. In order to determine on an individual level whether animals developed tactile hypersensitivity, we considered PWT-changes used in the literature to confirm efficacy of pain-treatments (Smits et al., 2006), i.e. a change of 50% in the PWT (in grams).

2.4. Static sciatic index

A subset of animals (n = 15) was used to monitor the static sciatic index (SSI) as a read-out of a sciatic nerve-dependent motor function. Briefly, rats were placed on a plastic surface in a plastic box and a webcam (Logitech HD webcam) positioned underneath the set-up was connected to a personal computer. Before and at multiple time points (until three weeks) after mSNI surgery a total of minimally 5 photographs were taken when animals were showing normal stance (non-rearing; all four paws in contact with the floor). SSI scores were calculated as described previously (Bervar, 2000). Briefly, the photomicrographs were loaded into NIH ImageJ software (version 1.45k) and four measures were taken: the outer toe spread (TS) of both the ipsilateral and contralateral hindpaws (distance between toes 1 and 5) and the inner toe spread (ITS) of both the ipsilateral and contralateral hindpaws (distance between toes 2 and 4). Then, the toe spread factor (TSF) as well as the intermediate toe spread factor (ITSF) were calculated: TSF = (ipsilateral TS – contralateral TS)/contralateral TS; ITSF: (ipsilateral ITS – contralateral ITS)/contralateral ITS. Finally, the SSI score was calculated per the following formula: SSI = (108.44 × TSF) + (31.85 × ITSF) – 5.49. Animals were finally divided into mSNI+ and mSNI− groups based on the 7-day PWT and respecting the 50% change in PWT as described earlier.

2.5. qRT-PCR

A subset of 17 rats was used for qRT-PCR analyses. The ipsilateral and contralateral lumbar (L4 and L5) DRGs and the ipsilateral and contralateral dorsal lumbar spinal cord were dissected one week after sham-surgery (n = 4), mSNI+ (n = 5) and mSNI− (n = 8). Total RNA was isolated using TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium), treated with the RQ1 RNase-free DNase kit (Promega, Leiden, Netherlands) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Nazareth, Belgium). Real-time PCR amplifications were carried out using the iCycler IQ™ multicolour real time PCR detection system (Bio-Rad Laboratories, Nazareth, Belgium), in a total volume of 25 μl containing 10 ng cDNA template, 0.3 μM of the primers (see hereafter) and the IQ™ SYBR Green Supermix using an annealing temperature of 60 °C. For quantitative analysis, a relative standard curve was generate using the same amplification conditions and with dilutions of a mix of cDNA templates (from 20 to 0.078 ng). Each sample was normalized to the relative amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeper gene. Relative quantification of mRNA in the samples was performed using the post-run data analysis software provided with the iCycler system. The following primer sequences were used; RGS4 forward primer: 5′ taacttgccagagggtgagc3′, reverse primer: 5′aaagctgccagtccacattc3′; RGS2 forward primer: 5′agcaaatatgggcttgctgcat3′, reverse primer: 5′ gcctcttggatattttgggcaatc3′; GAPDH forward primer: 5′gtctcctgtg acttcaacag3′, reverse primer: 5′agttgtcattgagagcaatgc3′.

2.6. RGS4 inhibitor experiment

A subset of animals (n = 24) received an indwelling intrathecal catheter, which was implanted immediately prior to mSNI. Under the same (sevoflurane) anaesthesia as described before, the atlanto-occipital membrane was freed and a slit was made through the underlying dura mater. The catheter was advanced into the intrathecal space up to 8.5 cm in order to have the catheter reach the lumbar enlargement. Prior to any further experimentation on day 7, 10 μl of 2% lidocaine was injected into the intrathecal catheter, followed by a 20 μl saline flush. All animals displayed a transient paresis of the hindpaws after this procedure, confirming the correct positioning of the catheter. Hereafter, algesimetric analysis was performed to distinguish between mSNI animals with and without tactile hypersensitivity. Out of the 24 animals, 11 showed tactile hypersensitivity and were used for further experimentation. Six of the latter animals received 0.5 μg CCG63802 (a specific RGS4 inhibitor; Tocris, cat. no. 4028, Bristol, UK; concentration of 0.05 mg/ml dissolved in 5% DMSO in saline) in a total volume of 10 μl, administered through the intrathecal catheter, as previously described (Bosier et al., 2015). The remaining five animals received 10 μl of vehicle solution (5% DMSO in saline). A 20 μl saline flush immediately followed the intrathecal injection of either CCG63802 or vehicle. Hereafter, algesimetric analysis was performed every 20 min for a total duration of 2 h. The results on PWT were then expressed as the maximum-possible-effect (MPE), calculated as follows: (posttreatment individual PWT – pretreatment average PWT/15.1 - pretreatment average PWT) × 100%.

2.7. Immunohistochemistry

At two weeks after surgery, rats (sham: n = 4; mSNI+: n = 6; mSNI−: n = 7) were euthanized using CO_2, immediately followed by transcardial perfusion with first approximately 100 ml of 0.1 M phosphate-buffered saline (PBS) and then 500 ml of 4% paraformaldehyde in 0.1 M PBS (PF). Spinal cords were post-fixed overnight in the same PF solution and then transferred to 10% sucrose in 0.1 M PBS for 24 h incubation. After a further 72 h incubation in 25% sucrose in 0.1 M PBS, the lumbar spinal cords were rapidly frozen using powdered dry ice and then stored at −80 °C until cryosectioning. The L4 and L5 spinal cord were embedded in Tissue-Tek (O.C.T., Sakura FineTek) and then transversally cut (30 μm-thickness), collecting every 12th section on Superfrost® Plus object glass slides (Thermo Scientific, Gerhard Menzel GmbH, Germany). Glass slides with transversal tissue sections were stored at −80 °C until immunohistological staining. Two immunostainings were performed in this investigation, targeting the microglial marker; ionized calcium binding adaptor molecule 1 (Iba1) and the astrocytic marker; glial fibrillary acidic protein (GFAP). Glass slides were thawed for about half an hour at room temperature and then washed three times with PBS. For Iba1 staining, sections were incubated for 1 h in PBS containing 1% Triton X-100 and 5% normal goat serum (NGS), followed by an overnight incubation in primary antibody solution at 4 °C (α-Iba1; WAKO, cat. no. 019/19741; 1:1000 in PBS-T containing 1% NGS). For GFAP staining, sections were immediately incubated overnight in primary antibody solution at 4 °C (α-GFAP; DAKO, cat. no. IS524; 1:1000 in PBS-T). Then next day, sections were incubated in secondary antibody solution for 1 h at room temperature; goat *anti*-rabbit Alexa-594 (Invitrogen, Belgium), diluted 1:100 in PBS and 1% Triton X-100 (for Iba1 staining this solution also contained 1% NGS). At the end of this incubation, glass slides were again washed three times with PBS and then coverslipped using 80% glycerol in PBS.

2.8. Image analysis

The immuno-stained sections were examined under a digital inverted EVOS microscope (Advanced Microscopy Group, Mill Creek, Washington) that uses a light-emitting diode (LED) illumination system and was equipped with a Texas Red light cube. At 4× magnification, photomicrographs were taken from the L4 spinal cord. A total of 5 photomicrographs per animal were used for image analysis. Photomicrographs were then loaded into *NIH* Image J analysis software (version 1.47) and background signals were subtracted. Then, the complete dorsal horn was selected as the region of interest (ROI) according to the rat atlas of Paxinos and Watson. The area-percentage within this ROI showing immunoreactivity (IR) for Iba1 or GFAP was determined. The percentages measured for each of the five photomicrographs were then averaged per animal and expressed relative to sham-operated rats. The analysis was performed by three independent investigators (J.D., N.D., B.Ba.), blinded for the experimental conditions.

2.9. Statistics

Data are reported as mean ± standard error (SE) values. Algesimetric data were analysed statistically using the log-values of the PWT. In order to test for differences between baseline PWT and 7-day PWT, paired student's t-tests were performed. Non-paired student's t-tests were performed to compare the day-1 SSI score of mSNI + and mSNI − rats as well as the MPE of CCG63802-treated rats and MPE of vehicle-treated rats at individual timepoints. qRT-PCR and immunohistochemical data of sham-operated, mSNI + and mSNI − animals were compared using a one-way analysis of variance (ANOVA) with a Dunnett post hoc correction (considering sham-operated rats as reference group). A two-way ANOVA with Bonferroni posthoc correction was used to compare SSI scores over time for mSNI + and mSNI − animals. Statistics and preparation of graphs was done using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA; www.graphpad.com). A p-value of 0.05 was regarded as the level of statistical significance.

3. Results

3.1. Clamp crush completely and specifically abolishes conduction along injured nerve branches

An ex vivo approach was used to determine whether the impact of the lesion procedure was restricted to the injured branches. Here, sciatic nerve samples were acutely isolated from adult rats and electrophysiological experiments were performed before and after crushing of the tibial and common peroneal, but not sural nerve branches (Fig. 1A, B). Before injury, responses from the three peripheral nerve branches increased gradually with augmenting pulse strengths (Fig. 1C, left). After injury, responses from the two crushed branches were completely abolished and remained absent throughout the observational period of up to 5.5 h (Fig. 1C, right), explaining a full lack of peak conduction velocities that had been recorded from these branches prior to crush (Fig. 1D). In contrast, conduction along the spared sural nerve remained unaffected after crush of the tibial and common peroneal branches (Fig. 1E). On the basis of this data we concluded that the crush procedure was suitable to induce lesions restricted to the injured branches of the sciatic nerve.

3.2. Restricting crush to the common peroneal nerve branch reduces prevalence of tactile hypersensitivity

Development of tactile hypersensitivity is a typical and consistent feature of rodent models of peripheral nerve injury. We here aimed to investigate whether the extent of tissue trauma in models of peripheral nerve injury influences the development of tactile hypersensitivity. For this purpose, we used the classical model of SNI in which two peripheral branches of the sciatic nerve are injured (Decosterd and Woolf, 2000) and compared tactile sensitivity at one week following surgery with that of a modified version of the SNI model (mSNI) in which the injury was restricted to only a single peripheral branch of the sciatic nerve, i.e. the common peroneal branch. The PWT at baseline was consistent for all animals, with values around the cut-off of 15.1 g. At one week after surgery, nearly all SNI animals had PWT values well below the baseline (Fig. 2A), while this was not the case for mSNI animals in which values were either around or below baseline (Fig. 2B). Sham-operated animals showed nearly identical PWT values before and one week after surgery (data not shown). mSNI animals were then subdivided based on a 7-day

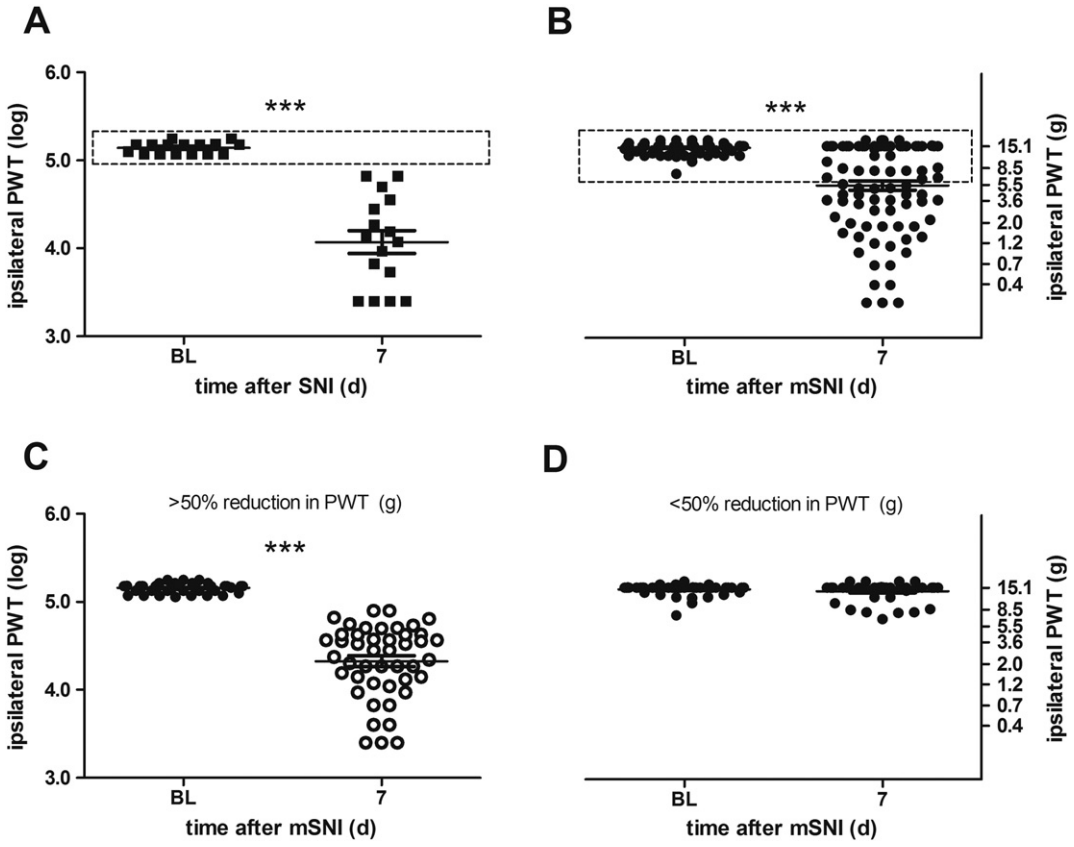

Fig. 2. Distinct outcomes for tactile sensitivity at one week after classical or modified spared nerve injury. A, B; PWT are shown before and after crush injury to either the common peroneal branch and tibial branch (SNI, n = 21, A) or the common peroneal branch only (mSNI, n = 103, B). The dashed boxes indicate the spread of individual PWT at BL. Note that all individual 7-days post-injury PWT are below those of BL for SNI, but not mSNI. Data of 103 mSNI rats were then clustered in two groups, i.e. one group in which the 7-days post-injury PWT (in grams) was 50% lower than at BL (C; n = 50) and one group where this difference did not exceed 50% (D; n = 53). *PWT*, paw withdrawal threshold; *g*, grams; *BL*, baseline; *(m)SNI*; (modified) spared nerve injury; *d*, days; ***, $p < 0.001$ (BL vs. 7-days post-injury).

PWT that was reduced by more or <50% of the individual baseline PWT value, respectively. While the former category was found to have a statistically significant reduction in PWT, the latter category did not show any statistically significant reduction (Fig. 2C–D). The mSNI animals with and without statistically significant reduction in PWT were designated mSNI+ and mSNI−, respectively. On the basis of a total number of 103 rats that underwent mSNI surgery, the ratio of mSNI+/mSNI− was 0.49/0.51.

3.3. Development of tactile hypersensitivity after mSNI shows no relation with the extent of motor deficits or recovery

The remarkable divergence in the presence or absence of tactile hypersensitivity after mSNI surgery made us wonder whether other lesion outcomes were affected by subgroup category. mSNI surgery was performed on 15 rats and these were followed daily for the static sciatic index (SSI), a sciatic nerve-dependent motor read-out (Bervar, 2000). Compared to baseline, animals in the mSNI+ and mSNI− group showed a similar drop in SSI score at one day after surgery (Fig. 3A). Likewise, the recovery of SSI scores that occurred spontaneously over the course of 2–3 weeks, was unaffected by subgroup category (Fig. 3B).

3.4. Role of RGS4 in tactile hypersensitivity at one week after mSNI

A divergence between development and absence of tactile hypersensitivity after nerve lesion has been previously linked to the functionality of inhibitory systems in the nociceptive neuraxis (De Felice et al., 2011) and we and others have implicated the spinal RGS4 in disinhibition processes after nerve lesion (Bosier et al., 2015; Garnier et al., 2003). Therefore, a putative role of RGS4 in tactile hypersensitivity following mSNI was explored here. Gene expression of RGS4 was found to be statistically significantly elevated in both the lumbar dorsal root ganglia and dorsal spinal cord, ipsilaterally, but not contralaterally to the nerve lesion in mSNI+ animals as compared to sham controls (Fig. 4A–D). mSNI− animals did not show such an elevation in gene expression. The possibility of an a-specific up-regulation of RGS members in mSNI+ animals was unlikely as RGS2 gene expression was also evaluated and not found to be regulated by either nerve lesion or subgroup category (Fig. 4E–H). As these data suggested an involvement of RGS4 in the onset of tactile hypersensitivity after mSNI, we designed an interference experiment using the specific RGS4 inhibitor CCG63802 as used previously (Bosier et al., 2015). This inhibitor was dissolved in vehicle solution (5% DMSO in saline) and administered intrathecally at 7 days after surgery to mSNI+ animals, after which tactile sensitivity was monitored every 20 min for a total duration of 2 h. Control mSNI+ animals underwent the same procedure with the exception that the intrathecal injection comprised 5% DMSO in saline without the RGS4 inhibitor CCG63802. mSNI+ animals treated with the inhibitor showed a rapid, but transient reduction in tactile hypersensitivity (Fig. 5). At 20 min, the effect was 59 ± 11% of what was maximally possible, and this was further increased to 70 ± 7% after 40 min. At 1 h after injection the effect was reduced to 29 ± 8% of the maximum-possible-effect (i.e. size of the effect in reference of control values) and hereafter PWT returned to pre-treatment hypersensitivity values. Saline treatment did not affect PWT at any time point within the 2 h-observation period (Fig. 5).

3.5. Persistence of tactile hypersensitivity at two weeks after mSNI and link with central gliosis

Clinical studies have proposed that early tactile hypersensitivity after surgery may be linked to persistent pain (Lavand'homme et al., 2005; Martinez et al., 2012). We therefore wanted to explore the persistence of tactile hypersensitivity in our model of mSNI. PWT were determined at two weeks after surgery for animals that were categorized as mSNI+ and mSNI− at one week after surgery. We found that 26 out of 33 animals (i.e. 79%) remained within the mSNI+ category, while this was the case for 28 out of 33 animals (i.e. 85%) within the mSNI− category (Fig. 6A). In a cohort of 15 animals that was followed for 4 weeks, the percentages of mSNI+ and mSNI− rats, which remained in the same category throughout the 4 week observation period were 84% and 89%, respectively (data not shown).

The persistence of pain hypersensitivity after nerve injury has been strongly linked to processes of gliosis within the CNS. Particularly, microglial reactivity and astrocytosis in the dorsal horn of the lumbar spinal cord have been implicated in chronic pain-like behaviours of models in which the sciatic nerve is injured (Ji et al., 2006; Tsuda et al., 2005). We therefore selected animals that showed consistence in mSNI+ or mSNI− at two weeks after surgery and performed an immunohistochemical analysis of microglial Iba1 and astrocytic GFAP expression in the ipsilateral lumbar dorsal horn and compared this with that of sham-operated control animals. We found a statistically significant up-regulation for both glial markers in mSNI+ animals, but not mSNI− animals, as compared to sham-operated controls (Fig. 6B, C). Representative images of the immunostained sections for both animal groups are shown in Fig. 7.

4. Discussion

In this manuscript we report on a new model of peripheral nerve injury in the rat in which the restriction of nerve trauma from two to a single injured branch of the sciatic nerve reduces the prevalence of tactile hypersensitivity. With this model we revealed a role of injury-induced RGS4 expression in the development of tactile hypersensitivity, while motor deficits seemed to be unrelated to RGS4, occurring to the same extent in animals with and without up-regulated RGS4. Finally, the presence or absence of tactile hypersensitivity was found to be mostly

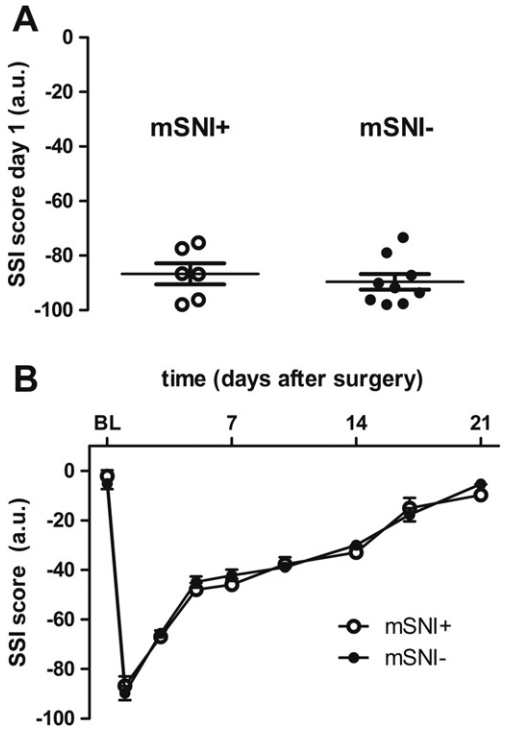

Fig. 3. Deficit and recovery of sciatic nerve-dependent motor function is similar between mSNI rats with and without tactile hypersensitivity. A; SSI scores at one day after mSNI+ (n = 6) and mSNI− (n = 9) animals. Note that the category 'mSNI+' and 'mSNI−' was determined at 7 days after nerve injury. B; evolution of SSI scores over the course of three weeks after mSNI surgery. *SSI*, static sciatic index; a.u.; arbitrary units; *mSNI+*; *d*, days; modified spared nerve injury with tactile hypersensitivity at 7 days after surgery; *mSNI−*; modified spared nerve injury without tactile hypersensitivity at 7 days after surgery.

persistent for at least another week, and could be associated with neuro-immune processes involving glial cells in the dorsal horn of the lumbar spinal cord.

Great advances have been made in our understanding of the mechanisms that underlie pain in the early phases after surgical insults such as nerve lesions (Berger et al., 2011; Ren and Dubner, 2010; Scholz and Woolf, 2007). The mechanisms of pain at later stages are also increasingly being explored (Ji et al., 2013; Milligan and Watkins, 2009), highlighting alterations in immune-related markers in chronic pain conditions (Marchand et al., 2005; Uceyler et al., 2007; Uceyler et al., 2010). In strong contrast, we only know little about what mediates the transition from acute to chronic pain or about what protects against

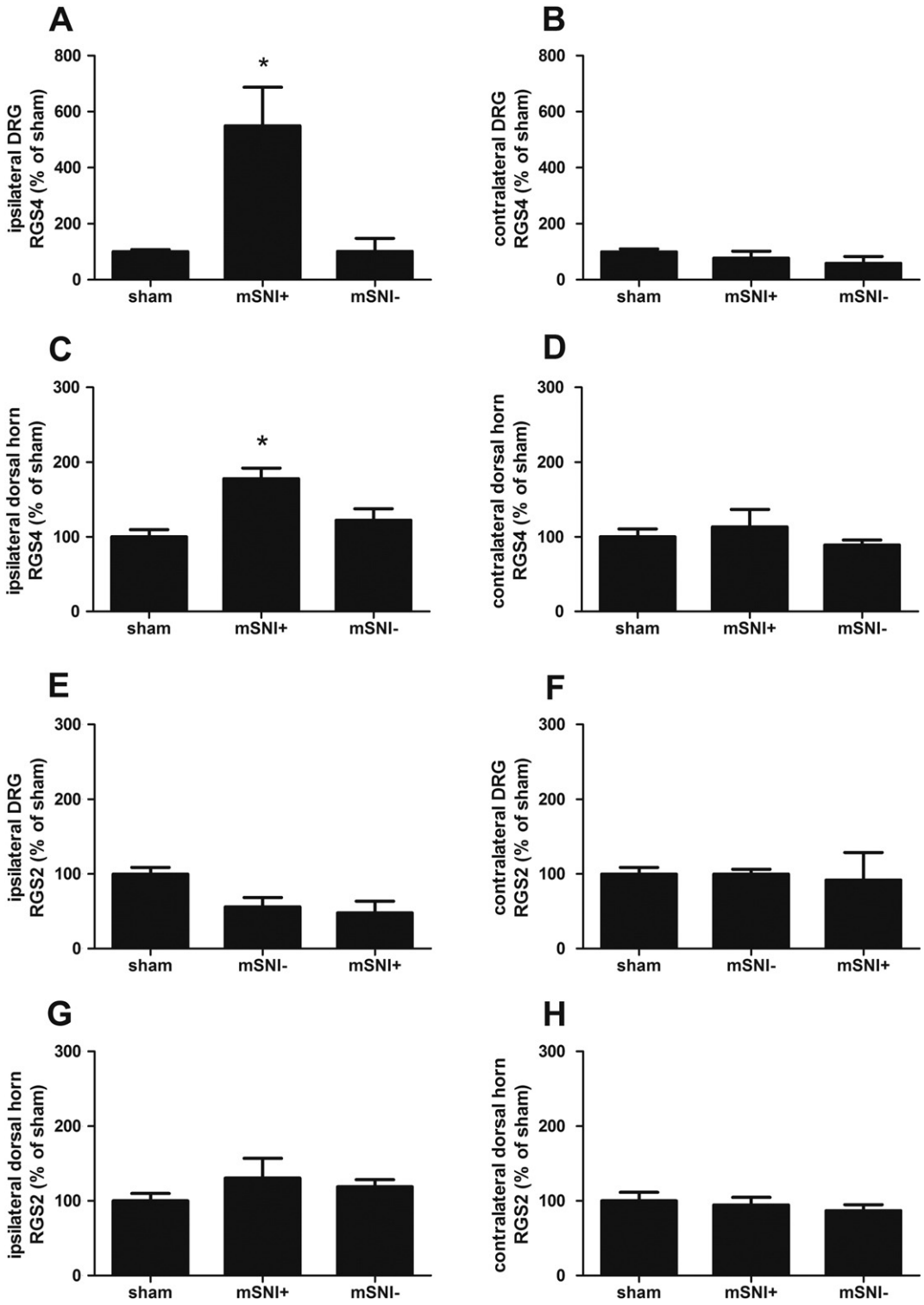

Fig. 4. RGS4 and RGS2 expression in lumbar DRGs and dorsal spinal cord at 7 days after mSNI. Animals were subdivided into mSNI + (n = 5) and mSNI − (n = 8) at 7 days after mSNI surgery and qRT-PCR for RGS4 and RGS2 was performed on lumbar (L4 and L5) DRGs ipsilateral (A, E) and contralateral (B, F) to the surgery with sham-operated (n = 4) animals serving as controls. The same was done for the ipsilateral and contralateral lumbar dorsal cords (C, D, G, H respectively). *DRG*, dorsal root ganglia; *RGS*, Regulator of G protein Signaling; *mSNI +*; modified spared nerve injury with tactile hypersensitivity at 7 days after surgery; *mSNI −*; modified spared nerve injury without tactile hypersensitivity at 7 days after surgery; *, $p < 0.05$ (vs. sham).

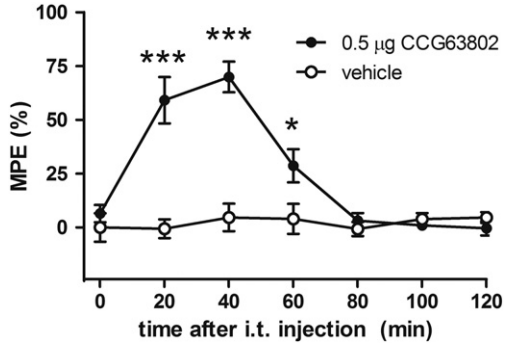

Fig. 5. Spinal RGS4 inhibition transiently reverses tactile hypersensitivity in mSNI + rats. A bolus injection of the RGS4 inhibitor CCG63802 or vehicle solution (5% DMSO) was given to mSNI + rats at 7 days after surgery. The PWT was determined every 20 min for a total duration of 2 h and results were expressed as MPE for CCG63802 (n = 6) and vehicle-treated rats (n = 5). *MPE*, maximum possible effect; *DMSO*; dimethyl sulfoxide; *i.t.*, intrathecal; *min*, minutes; *** and *, $p < 0.001$ and $p < 0.05$, respectively (CCG63802 vs. vehicle treatment).

the development of chronic pain states (Katz and Seltzer, 2009; Voscopoulos and Lema, 2010). Since tactile hypersensitivity in the early postoperative phase has been clinically implicated in the chronification of pain (Deumens et al., 2013) and tactile hypersensitivity is considered one of the most debilitating symptoms of neuropathic pain syndromes (Bennett, 1994), we here focused on this symptom. Previous work has shown that the genetic background of rodents (De Felice et al., 2011; Yoon et al., 1999), but also the amount of spared neural tissue (Kloos et al., 2005) can influence the prevalence of tactile hypersensitivity after neurotrauma. By reducing the extent of trauma to the sciatic nerve we could observe a reduction in the prevalence of tactile hypersensitivity in the present study. Nevertheless, the presence or absence of tactile hypersensitivity within the mSNI model seemed not to be caused by differential extents of tissue trauma for several reasons. First, the selected lesion procedure was robust and has been validated previously (Luis et al., 2007). Second, our electrophysiological data showed that the lesion procedure rendered a complete disappearance of signal conduction along injured fibers, but left it fully intact in spared nerve fibers. Third, the SSI, which is a reliable read-out for sciatic nerve function (Bervar, 2000) showed an identical drop and recovery for mSNI animals with and without tactile hypersensitivity.

In order to better understand this divergent pain-related outcome after mSNI, we decided to explore a possible role of RGS4. We recently reported RGS4 as a negative regulator of signaling through the analgesic CB_1 receptor, reducing its activity in the spinal cord ipsilateral to a partial sciatic nerve ligation (Bosier et al., 2015). This suggested that the up-regulation of RGS4 could be part of a nerve injury-evoked mechanism that causes disinhibition in the dorsal horn of the spinal cord. A divergence between disinhibition and engagement of inhibitory systems has indeed been previously linked to the presence and absence of tactile hypersensitivity after nerve injury (De Felice et al., 2011). Our current data showed that an up-regulation of RGS4 occurred in the lumbar DRGs and dorsal horns of only nerve lesioned animals that showed tactile hypersensitivity. Blocking of RGS4 through the intrathecal delivery of a specific inhibitor attenuated tactile hypersensitivity, an effect that was seen from 20 min after drug administration, but did not outlast the first hour. The transient nature of this effect may explain why, in previous work, we could not observe an effect of spinal RGS4 inhibition on tactile hypersensitivity when the interval between drug delivery and algesimetric testing reached several hours (Bosier et al., 2015).

Over the past years, members of the RGS family have been increasingly linked with nociception and analgesia (Han et al., 2010; Ibi et al., 2011; Psifogeorgou et al., 2011; Zachariou et al., 2003). More recent data also suggested a role of RGS in pathological pain states (Terzi et al., 2014; Yoon et al., 2015) (Mitsi et al., 2015; Salaga et al., 2016), which is further strengthened by the data on RGS4 in our current paper. Nevertheless, many unresolved issues remain such as 'what is the identity of the cells expressing RGS4?' The lack of suitable antibodies for immunohistochemical investigations limits advances in this direction, but the RGS-pain story may be further complicated by factors such as the lesion model. For example, sciatic nerve transection has been reported to reduce the number of primary sensory DRG neurons expressing RGS3 or RGS4 transcripts (Costigan et al., 2003) as opposed to an up-regulation of RGS4 transcripts in the DRG and spinal cord following partial sciatic nerve ligation and mSNI ((Bosier et al., 2015; Garnier et al., 2003) and data in the current study). Also the signaling events that are up-stream and down-stream of an RGS4 up-regulation after nerve injury remain unknown. While RGS proteins have been implicated in many different cellular functions (Hollinger and Hepler, 2002), RGS4 is just a small member of the family containing little

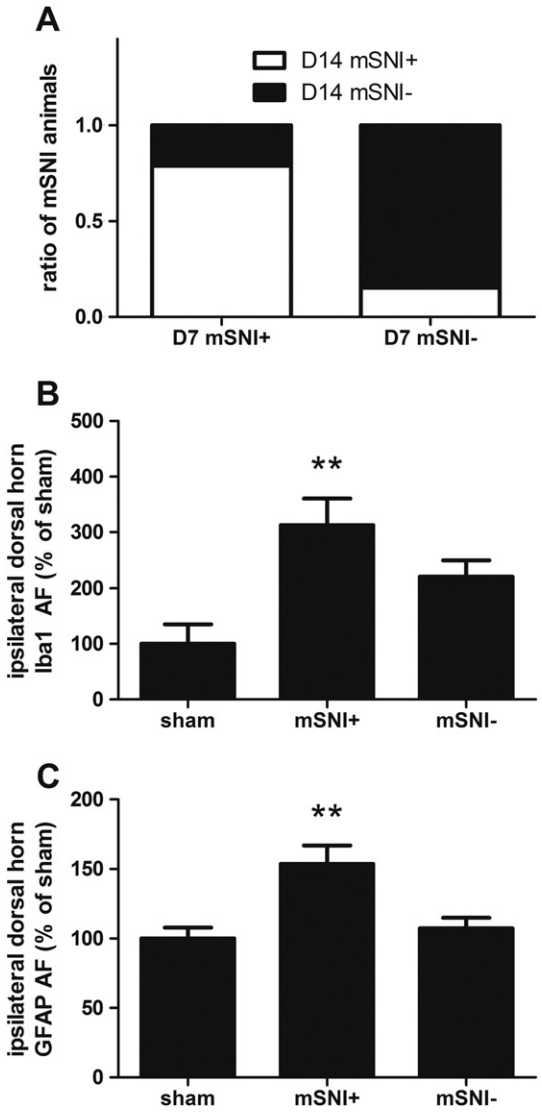

Fig. 6. Tactile sensitivity outcomes and glial reactivity in the lumbar dorsal horn at 14 days after mSNI. A, using the criterium of 50% decrease from BL-PWT values, rats were subdivided into mSNI + and mSNI − animals, both at 7 and 14 days after mSNI. Note in the stacked bars that the vast majority of mSNI + and mSNI − animals at 7 days remain in their respective category at 14 days. Microglial and astrocytic reactions of mSNI + (n = 6) and mSNI − (n = 7) rats with consistent outcomes were determined on the basis of immunohistochemical analysis, using sham-operated rats as controls (n = 4). The area fraction of the ipsilateral lumbar (L4-5) dorsal horn that was immunoreactive for either the microglial marker Iba1 or the astrocytic marker GFAP was determined. *Iba1*; ionized-calcium binding adapter molecule-1; *GFAP*, glial fibrillary acidic protein; *AF*, area fraction; *mSNI +*; modified spared nerve injury with tactile hypersensitivity at 7 days after surgery; *mSNI −*; modified spared nerve injury without tactile hypersensitivity at 7 days after surgery; **, $p < 0.01$ (vs. sham).

more than a single RGS domain that may relate to its full functionality. We and others have shown that RGS4 negatively influences the signaling through G protein-coupled receptors that are known for having analgesic effects, such as the CB_1 and μ-opioid receptors (Bosier et al., 2015; Garnier et al., 2003). Whether such action may explain the tactile hypersensitivity in 50% of mSNI animals remains to be investigated, but preliminary data already suggested no effect of mSNI surgery on the signaling efficacy through CB_1 receptors (data not shown). Even though the exact mechanisms by which RGS4 inhibition caused a reversal of tactile hypersensitivity remain unknown for now, data from the literature leads us to assume that specific targeting of the spinal cord was important for the observed effect. Indeed, systemic depletion of RGS4 fully preserved tactile hypersensitivity after SNI in mice (Stratinaki et al., 2013). This may be explained by an RGS4 action in supraspinal sites that opposes the therapeutic effect of spinal RGS4 inhibition. In support of this, depletion of RGS4 in the nucleus accumbens was found to reduce the analgesic effect of opiate analgesics (Han et al., 2010). While challenging in a clinical setting, a spinal targeting of RGS4 inhibition may be beneficial also for limiting unwanted side effects. In support of this, our data showed a specific association of RGS4 up-regulation with tactile hypersensitivity, but not sciatic nerve-dependent motor function, as assessed with the SSI. Also, previous work showed no motor impairments after intrathecal treatment with the same RGS4 inhibitor used in the current investigation (Bosier et al., 2015).

A great advantage of the mSNI model could lie in the possibility to distinguish between pathobiological alterations that specifically relate to the development of hypersensitivity as opposed to other injury-related symptoms and events. For now, an up-regulation of RGS4 appears to be part of a hypersensitivity-specific signature in the total of neurochemical alterations that follow peripheral nerve injury. Whether the model of mSNI will be a further asset in the search of pain chronification mechanisms remains to be determined in future investigations. While it is interesting to note that the vast majority of rats showing tactile hypersensitivity at one week after mSNI, retained this symptom for at least three more weeks, our study did not focus on persistence of pain states, which can be rather heterogeneous and reach well beyond tactile hypersensitivity (Dworkin, 2002). Notably, however, we did observe a higher glial reactivity in the dorsal horns of animals showing tactile hypersensitivity than in those without this pain-like behaviour. Microglia and astrocytes have been both heavily implicated in the inception and maintenance of neuropathic pain (Aldskogius and Kozlova, 2013; Ji et al., 2006; Tsuda et al., 2005) and chronic pain has even been considered as a possible result of 'diseased glia' (Ji et al., 2013). Then again, care should be taken with any conclusions in this direction, as injury-induced glial reactivity is not necessarily linked with painful outcomes (Gallo et al., 2015; Leinders et al., 2013). Many 'activation' states may exist, only some of which are 'pain-related' (McMahon and Malcangio, 2009). We speculate that the mSNI model with its divergent outcomes on tactile hypersensitivity, could help to distinguish between pain-related and non-pain-related activation states. The latter are even more interesting in light of recent work showing that microglial cells are not required for tactile hypersensitivity in female rats, as opposed to male rats (Sorge et al., 2015). In our investigation we exclusively used female rats as women seem to suffer from chronic

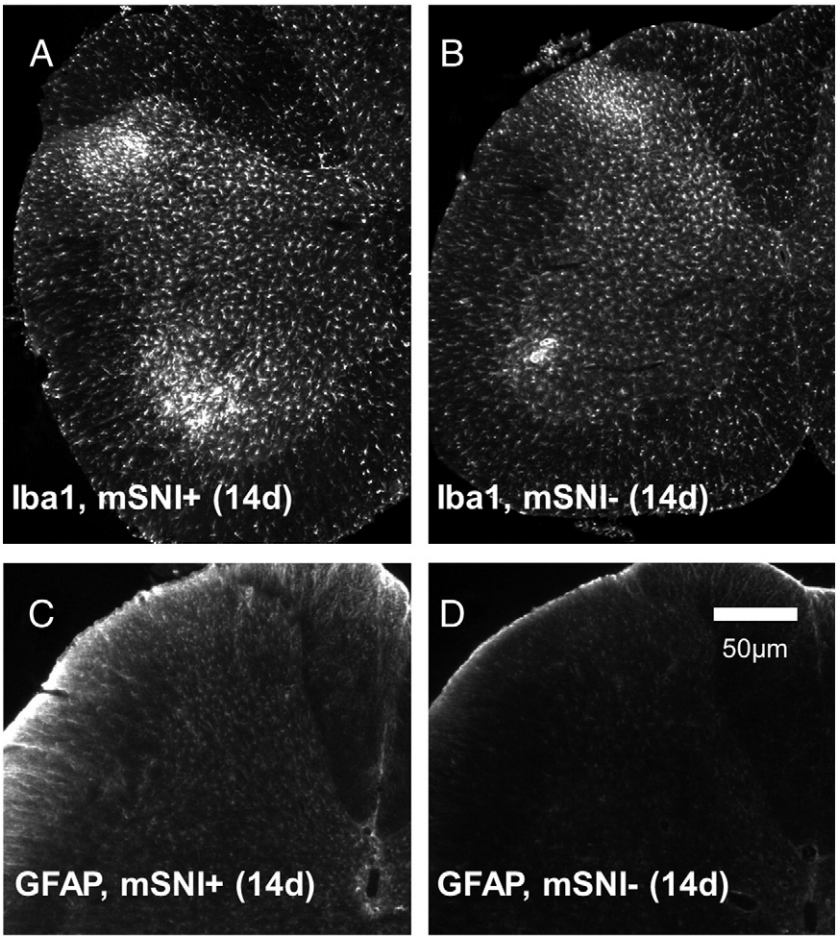

Fig. 7. Representative Iba1 and GFAP immunostainings of the L4 dorsal horn in mSNI + and mSNI − rats. Photomicrographs represent a section at the L4 spinal cord level, encompassing the full dorsal horn ipsilateral to mSNI, which was performed 14 days earlier. Iba1 immunoreactivity is shown for mSNI + (A) and mSNI − (B) rats; GFAP is shown for mSNI + (C) and mSNI − (D) rats as well. The scale bar in D applies to panels A-D. *Iba1*; ionized-calcium binding adapter molecule-1; *GFAP*, glial fibrillary acidic protein *mSNI +*; modified spared nerve injury with tactile hypersensitivity at 7 and 14 days after surgery; *mSNI −*; modified spared nerve injury without tactile hypersensitivity at 7 and 14 days after surgery.

pain disorders more than men (Berkley, 1997; Grosu and de Kock, 2011). As pathological pain mechanisms (including those following nerve lesion) may fundamentally differ between the two genders (Sorge et al., 2011), it remains to be seen whether the mSNI model with divergent tactile sensitivity outcomes applies to the male gender as well.

In summary, a multitude of animal models of peripheral nerve injury have been developed over the course of the last decades (Gregory et al., 2013). While the specific set of neuropathic pain-like behaviours, either spontaneous or evoked by thermal, tactile or chemical stimuli, may differ from model to model, a feature that is generally shared among models is the consistency in pain behaviours, such as tactile hypersensitivity. Since only a fraction of surgical patients develops tactile hypersensitivity (Lavand'homme et al., 2005; Martinez et al., 2012), current animal models with persistent tactile hypersensitivity may have strong limitations when trying to understand what happens in the early postoperative phase that may drive an acute postoperative pain to either spontaneous resolution or transition into a chronic pain problem. We here report on a new rat model of nerve injury in which only the common peroneal branch of the sciatic nerve is injured leading to an approximate 50% prevalence of tactile hypersensitivity. With this model we could, for now, demonstrate that RGS4 was specifically associated with the development of tactile hypersensitivity.

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Afferent input induced by rhythmic limb movement modulates spinal neuronal circuits in an innovative robotic *in vitro* preparation

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Afferent input induced by rhythmic limb movements modulates spinal neuronal circuits in an innovative robotic *in vitro* preparation

Nejada Dingu, Ronald Deumens, Giuliano Taccola

Abstract

We investigated the afferent inflow generated by passive hindlimb mobilization, modulating dorsal sensory-related and ventral motor-related spinal networks. Thus, we created a novel *in vitro* model, a robotic Bipedal Induced Kinetic Exercise (BIKE) device to simultaneously induce passive pedaling and record ventral and dorsal root (VR and DR) potentials in a preparation of isolated neonatal rat spinal cord with hindlimbs attached. BIKE evoked sporadic afferent volleys from DRs. Moreover, BIKE onset only rarely elicited brief episodes of fictive locomotion (FL) and ongoing pedaling did not affect the epoch of FL when trains of pulses were conjointly delivered to a DR. Nevertheless, switching off BIKE after a 30-minute session transitorily facilitated the electrically-induced FL, without affecting VR reflexes and DR potentials. However, after a 90-minute session, BIKE no longer facilitated FL and the area of VR reflexes was strongly depressed. Patch clamp recordings from single motoneurons after the 90-minute session indicated an increased frequency of both fast- and slow-decaying synaptic inputs to motoneurons. After 90-minute BIKE, antidromic discharges recorded from DRs were increased, an effect that substantially persisted into the post-BIKE resting phase. Moreover, 90-minutes of BIKE further increased 4AP-induced rhythmic antidromic discharges, mostly during BIKE training session. In conclusion, hindlimb rhythmic and alternated pedaling of different durations affects distinct dorsal and ventral spinal networks by modulating excitatory and inhibitory input to motoneurons. These results highlight the need to define an optimal therapeutic window for neurorehabilitation aimed at enabling spinal circuits.

Introduction

In the spinal cord dedicated neuronal networks, known as Central Pattern Generators (CPGs), drive limb locomotion (Kiehn, 2006), as demonstrated by the alternated hindlimb movements of the *in vitro* neonatal spinal cord with legs attached (Kiehn and Kjaerulff, 1996; Klein and Tresch, 2010).

The CPG-driven movement of limbs stretches muscles and joints and activates cutaneous receptors to generate an afferent feedback to the spinal cord (Loeb et al., 1977). The afferent feedback plays a crucial role in modulating sensory-motor processing (Mandadi and Whelan, 2009; Mandadi et al., 2013; Sirois et al., 2013) and locomotor patterns (Hayes et al., 2009; Brumley et al., 2017). Indeed, in neonatal rat spinal cord preparations with limbs attached, the locomotor pattern induced by neurochemicals is hindered by the removal of afferent inputs (Acevedo and Diaz-Rios, 2013). Moreover, the importance of afferent input in facilitating locomotor patterns is also proven by the appearance of an epoch of locomotor-like cycles in response to electrical stimulation applied to dorsal afferents (Marchetti et al., 2001; Taccola, 2011; Dose and Taccola, 2016; Dose et al., 2016) or to the repeated delivery of stimulating protocols, which eventually also increase spontaneous activity of dorsal horn networks (Dingu et al., 2016). Furthermore, the continuous flow of afferent inputs, evoked in clinics by sessions of repetitive and alternated limb movements, facilitates the re-expression of locomotor patterns after spinal lesion, probably due to plastic changes in spared spinal circuits (Dietz and Fouad, 2014).

Indeed, alternated and passive hindlimb mobilization increases the expression of genes involved in motoneuronal plasticity (Joseph et al., 2012; Keeler et al., 2012; Chopek et al., 2015), and restores the tuned balance between inhibitory and excitatory synaptic boutons to motoneurons (Ichiyama et al., 2011). The same positive influence on spinal circuitry has been somewhat obtained also on *in vivo* animal models through activity-based interventions such as passive cycling (Chopek et al., 2014; Côté et al., 2014).

While it is known that afferent inputs trigger locomotor-like cycles, it is unclear how the afferent feedback evoked by the continuous mobilization of limbs modulates the ongoing activity of dorsal sensory-related and ventral motor-related spinal networks. Likewise, it remains to be determined whether different length of passive exercise might selectively modulate distinct spinal networks and if functional effects on spinal circuits persist even after session ending.

Our hypothesis is that CPG-driven locomotor patterns are facilitated by afferent inputs generated during and after alternated leg movements.

The main aim of this study is thus to explore how afferent inputs evoked by repetitive and passive alternated movement of hindlimbs modulate patterns generated by spinal networks, as well as how exercise sessions of different duration affect distinct spinal circuits.

Specifically, we pursued this research adopting an innovative robotic model that generates passive pedaling in a neonatal preparation of isolated spinal cord with legs attached, while recording ventral and dorsal root (VR and DR) activity. Although immature, this preparation results useful to understand fundamentals of human neuro-motor system organization and basic mechanisms of clinical rehabilitation, since many "building blocks" common among the spinal circuitry of all mammalians are already present at birth (Getting, 1989; Stein, 1995; Nishimaru and Kudo, 2000). With this new model, the activity of motor-related and sensory-related networks can be induced by electrical or pharmacological direct stimulation. Rhythmic activity of ventral locomotor networks arises as epochs of electrical discharges alternating among homosegmental left and right VRs (FL, Juvin et al., 2007). In the same preparations, the activity of dorsal sensory circuits is probed with monitoring spontaneous or pharmacologically-elicited rhythmic antidromic discharges recorded synchronously among DRs (Vinay et al., 1999).

The question faced by the present paper relies on the key physiological mechanisms behind spinal processing of afferent input elicited by passive limb mobilization. This issue is at the base of numerous rehabilitative techniques, which are currently attempting to exploit passive and robotic walking for alleviating neuropathic pain and facilitating recovery of function in people with spinal cord injury (SCI, Harkema et al., 2012; Hubli and Dietz, 2013; Dugan and Sagen, 2015).

Methods

In vitro *preparations of neonatal rat spinal cord and nerves*

All procedures were approved by the International School for Advanced Studies (SISSA) ethics committee and are in accordance with the guidelines of the National Institutes of Health (NIH) and with the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European Union directive on animal experimentation (2010/63/EU). Experiments were performed on preparations of isolated thoraco-sacral (from T3-4 to *cauda equina*) spinal cord with hindlimbs attached, obtained from neonatal Wistar rats at postnatal (P) days 0-4. All efforts were made to minimize the number of animals used for the experiments and their suffering. Spinal roots were dissected from high thoracic spinal levels to the second lumbar segment (L2) included, leaving spinal segments below L2 (from L3 on) connected to the periphery. In a subset of experiments a mechanical compression of the hindpaw was performed to elicit the corresponding afferent feedback from lumbar DRs. Preparations were placed in a recording chamber continuously superfused (5 mL/min) with oxygenated (95% O_2 - 5% CO_2) Krebs solution containing (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose, pH 7.4.

A new device for the induction of passive training

We realized a novel device, named BIKE (Bipedal Induced Kinetic Exercise), to induce passive training in the isolated spinal cord with legs attached (Fig. 1 A). The preparation was placed in the recording chamber (maintained at room temperature, 23-25 °C), using acrylic glue to attach the hindpaws to the pedals, which were kept above the preparation. BIKE was connected to a stabilized power supply (K.E.R.T., Treviso, Italy), to allow an adaptable speed of rotation. The carefully shielded design of BIKE results in a low noise device to allow simultaneous long-term recordings without any electrical interference with electrophysiological equipment during passive movement of hindpaws. The absence of any electrical interference was tested with long recordings from an isolated spinal cord mounted in the BIKE chamber (Fig. 1 B). In this setting, the spontaneous electric activity recorded from both VRs and DRs was not affected by switching on of the device. A similar conclusion was obtained by the absence of any polarization of the ionic medium perfusing the chamber recorded during BIKE functioning (Fig. 1 C), and confirmed by the lack of main frequency of pedaling in the Fourier analysis (0.5 Hz; Fig 1 D). Sham samples were defined by maintaining the preparation in Krebs solution with hindpaws firmly fixed to BIKE pedals, while the device was switched off, for the same duration as a regular session of passive training with BIKE.

Nerve recordings

Using tight-fitting monopolar suction electrodes, simultaneous recordings were extracellularly obtained from whole L2 ventral roots (VRs) right (r) and left (l) and from the whole dorsal root (DR), either L1 or L2. Recordings of DR potentials were performed *en passant* by applying a negative pressure through a pipette close to the root surface. In order to isolate the sole contribution of the sensory input elicited in the periphery by passive limb mobilization, in a subgroup of preparations spinal nerves were transected and the distal stump was suctioned in glass pipette electrodes connected to an AC-coupled amplifier. Furthermore, a pair of hooked needle electrodes (Sei s.r.l., Padova, Italy) was used to record compound action potentials (CAPs) from one sciatic nerve (exposed proximally to its trifurcation) and dorsal afferent nerves following electrical stimulation of the territory of the hindpaw innervated by the sural nerve. AC- and DC-coupled recordings were acquired with a differential amplifier (DP-304®, Warner Instruments, CT, USA; low-pass filter = 10 Hz, high-pass filter = 0.1 Hz, gain = 1000) at a sampling rate of 10 or 50 kHz, digitized (Digidata 1440®, Molecular Devices Corporation, Downingtown, PA, USA), visualized real time with the software Clampex 10.3® (Molecular Devices Corporation, Downingtown, PA, USA) and stored on a PC for off-line analysis. A bipolar suction electrode connected to a programmable stimulator (STG4002®, Multichannel Systems, Reutlingen, Germany) was used to

deliver single or repeated electrical pulses to a DR (T13 - L2). Intensity of stimulation was determined in terms of threshold (Th), namely the lowest stimulus intensity capable of eliciting an appreciable response from all recorded spinal roots (Bracci et al., 1996a). Overall, the mean value of Th was 27.40 ± 18.35 μA. Responses were evoked by delivering single rectangular pulses (duration = 0.1 ms; intensity = 94.69 ± 42.56 μA, 3.15 ± 0.67 x Th) every 50 seconds. Episodes of fictive locomotion (FL) were induced by trains of electrical stimuli (120 rectangular pulses; frequency = 2 Hz; duration = 0.1 ms) delivered every 3 minutes to a DR at suprathreshold intensity (58.82 ± 35.80 μA, 2.07 ± 0.58 x Th). A couple of hooked needle electrodes connected to the programmable stimulator (STG4002®, Multichannel Systems, Reutlingen, Germany) was adopted to deliver single rectangular pulses (duration = 5 ms) every 50 seconds to the territory of the hindpaw innervated by the sural nerve. Since the stimulating electrode around the sural nerve was kept out of the electrolyte solution, the high resistance was overcome by increasing applied current (intensity = 16 mA; 4 x Th for eliciting an orthodromic compound action potential). The broad-spectrum potassium blocker 4-aminopyridine (4-AP; 10μM; Abcam, Cambridge, UK) was used to evoke rhythmic discharges from DRs and VRs (Taccola and Nistri, 2005).

Parameters of spinal network activity

Spontaneous activity was quantified in terms of power spectrum magnitude and expressed as root mean square (RMS; Deumens et al., 2013). Briefly, fixed-length time windows were decomposed using fast Fourier transform (FFT) analysis into a number of discrete frequencies and their power distribution was measured with Clampex 10.3® (Molecular Devices Corporation, Downingtown, PA, USA). Ventral reflexes and electrically-evoked antidromic activity were assessed through series of single electrical stimuli (0.1 ms duration, 0.02 Hz frequency and 3 x Th intensity) delivered to a DR in control, during training and after 30- and 90-minute BIKE sessions. At least 10 consecutive reflex responses were simultaneously recorded from one DR (dorsal root - dorsal root potentials, DRDRPs) and from one VR (dorsal root - ventral root potentials, DRVRPs; Kerkut and Bagust, 1995). For analysis, multiple sweeps were averaged and the mean peak amplitude and area were quantified. Episodes of locomotor-like activity were recorded from homosegmental L2 VRs, which predominantly consist in flexor motoneuronal axons (Kiehn and Kjaerulff, 1996). An alternated oscillatory pattern between left and right sides of the spinal cord represents a clear proof of FL driven by the CPG (Juvin et al., 2007). A FL oscillation was defined as a period of sustained membrane depolarizations remaining above a predetermined threshold (usually 5 times the standard deviation of baseline noise) for more than 400 ms (Bracci et al., 1996a).

Patch clamp recordings

Sacrolumbar cords were completely isolated from the leg-attached preparation and the dorsal surface of the cord was glued on an adjustable and articulated plastic support and bent at the level of upper sacral segments in a perpendicular upright position, with the rostral cord facing up, by means of a sylgard® 184 silicone elastomer cube (Dow Corning Corporation, Auburn, MI, USA). A horizontal section was made at lumbar (L) 4-5 spinal segments using a vibratome (Leica VT 1000 S, Leica Biosystems) to remove the caudal lumbar segments. This upside-down configuration allows patch clamp recordings from spinal motoneurons, still keeping intact the segments where the locomotor networks are mainly localized (Kjaerulff et al., 1994; Kremer and Lev-Tov, 1997; Cowley and Schmidt, 1997). The entire procedure from the end of BIKE to onset of patch clamp recordings requires at least 45 min. Patch clamp recordings in whole-cell configuration were made on L4-L5 motoneurons from isolated spinal cords, either after training (30, 90 min) or in sham preparations. In sham preparations, spinal cords were kept still in the BIKE recording chamber without BIKE functioning, for a time corresponding to the interval of trained cords. Note that experimental protocols adopted in sham preparations were identical to BIKE-trained preparations, with the exception that shams did not undergo any passive cycling. Recordings were performed in both voltage clamp (VC) and current clamp (CC) modes. Up-right bent spinal cords were continuously superfused with oxygenated Krebs solution (flow rate = 7 ml/min), illuminated by a far-red-emitting optical fiber (Scientifica Ltd, Uckfield, UK) and visualized through two switchable objectives (4x and 40x; Olympus, Tokyo, Japan) of an infrared video camera (Olympus U-TV1x-2, Tokyo, Japan) connected to the monitor through a C-mount adapter (Olympus U-CMAD3, Tokyo, Japan). Motoneurons were functionally identified by stimulating VRL5 with single rectangular pulses (duration = 1 ms; intensity = 800 μA) every 20 s. Once the correct area was identified, motoneurons were visually identified based on their morphology (21-25 μm diameter and one or two large processes; Fulton and Walton, 1986; Cifra et al., 2012) and location (close to the VR, in an area corresponding to the Rexed's lamina IX; Molander et al., 1984). Recordings were obtained using borosilicate pipettes with a mean resistance of 6.07 ± 2.08 MΩ and filled with a solution containing (in mM): 120 K gluconate, 20 KCl, 10 HEPES, 10 EGTA, 2 $MgCl_2$, 2 Na_2ATP, adjusted to pH 7.3 with KOH (Fabbro et al., 2012). Series resistance (lower than 18 MΩ) was monitored throughout the experiment at specific time points and was not compensated. Cells were discarded if series resistance was higher than 25 MΩ and if it varied more than 20% of the initial value (Bouhadfane et al., 2013; Tartas et al., 2010). Liquid junction potential in our experimental conditions was equal to 12.8 mV (Barry, 1994) and all membrane potential values were corrected. Electrophysiological responses were amplified using a differential amplifier (ELC-03XS Amplifier,

npi electronic GmbH, Tamm, Germany), digitized by Digidata 1440® (Molecular Devices Corporation, Downingtown, PA, USA) and visualized in real time with Clampex 10.3® (Molecular Devices Corporation, Downingtown, PA, USA). Data were acquired at a sampling rate of 10 kHz and subsequently analyzed off-line.

Parameters of motoneuronal activity

Spontaneous activity of lumbar motoneurons was recorded in VC mode, keeping cells clamped at -60 mV. Spontaneous post-synaptic currents (sPSCs) were selected using templates and, based on their decay time, classified as fast- ($\tau < 5$ ms) and slow-decaying currents ($\tau > 20$ ms). Main kinetic properties of fast and slow sPSCs were calculated with Clampex 10.3® (Molecular Devices Corporation, Downingtown, PA, USA).

Resting membrane potential (V_m = -82.90 ± 7.19 mV) of motoneurons was determined in CC mode without injecting any holding current (I = 0 nA). After injecting increasing steps of current, membrane resistance (R_m) was calculated as the slope of current-voltage (I-V) curve, which was linear in the interval considered. All membrane potentials were corrected for offset voltage (V_{off}), as obtained by raising the electrode from the cell at the end of each recording.

Statistical analysis

Data are expressed as mean ± SD. "n" indicates the number of preparations. A normality test was used to distinguish between parametric and non-parametric data. All parametric values were analyzed using Student's t-test (paired or unpaired) to compare two groups of data, or one way repeated measures ANOVA for more than two groups. Non-parametric values were analyzed with Mann-Whitney rank sum test (unpaired) and Wilcoxon signed rank test (paired) for two groups, and with Friedman repeated measures ANOVA on ranks for multiple comparisons. Multiple comparisons were followed by a post hoc test versus control (Bonferroni t-test). Statistical analysis was performed using SigmaStat® 3.5 software (Systat Software Inc, San Jose, CA, USA). Results were considered significant when $P < 0.05$.

Results

To assess whether afferent inputs from repetitive stepping facilitate spinal network activity, we devised a new tool that evokes a pattern of afferent inputs to the spinal cord, through a standardized and alternated mobilization of lower limbs. The increased number of alternating oscillations recorded from homosegmental VRs in response to a train of DR stimuli was used as an outcome to assess the effect of alternated limb exercise on the locomotor CPG. Whole-cell patch-clamp

recordings allowed us to trace modifications induced by exercise on the total number of synaptic inputs converging onto single motoneurons. Finally, increased antidromic discharges, either spontaneous or electrically-induced, pointed out to variations in the excitability of dorsal networks, but only for longer training sessions.

BIKE is a model to study locomotion and bipedal alternating movement in vitro

Up to date, studies about spinal changes determined by passive limb mobilization have only been performed on *in vivo* animal models (Gómez-Pinilla et al, 2002; Beaumont et al., 2004; Ichiyama et al., 2011; Joseph et al., 2012; Keeler et al., 2012; Chopek et al., 2015; Dugan and Sagen, 2015). Nevertheless, studying the *in vitro* spinal changes evoked by alternated limb exercise can provide crucial evidence on the physiological and basic mechanisms that drive activity-dependent plasticity. For this purpose, we created a robotic device named BIKE (Fig. 1 A), which allows stable recordings during passive hindlimb movement. Once a leg-attached isolated spinal cord preparation was placed in the recording chamber and hindpaws were fixed to the pedals, BIKE passively and alternatively propelled limbs. Movement was set at an operative speed of 30-35 cycles/min (pedaling frequency = 0.5 Hz) for all the experiments, to mimic standard periodicity of FL, as pharmacologically induced by NMDA (5 μM) and 5HT (10 μM; Dose and Taccola, 2012; Taccola et al., 2012). BIKE is tailored upon neonatal rats' dimensions, thus allowing the cord to remain still for a stable access to nerves. Moreover, the low-noise design of BIKE abolished electrical interference, as confirmed by the lack of electrical artefacts or an increase in baseline thickness during recordings in BIKE functioning (Fig. 1 B and C). Noteworthy, no electrical and mechanical interferences due to the pedaling engine or limb motion were detected by the probe electrode filled with Krebs solution and placed close to the recorded spinal roots (Fig. 1 C). Indeed, from the spectral analysis of the background noise detected during BIKE, the rhythmic component of the main frequency of pedaling (0.5 Hz) is absent (Fig. 1 D). These results confirm the usefulness of the proposed model in combining electrophysiological recordings and passive movement.

BIKE evokes afferent input

In DRGs, real locomotion in adult mammals generates a rhythmic and high frequency firing that reaches the spinal cord through dorsal sensory afferents (Loeb et al., 1977). This sensory feedback has been linked to the promotion of restorative spinal plasticity after lesion (Hubli and Dietz, 2013). Plastic variations in spinal network functionality can occur in response to incoming afferent patterns evoked by passive limb movement. In turn, we hypothesized that passive hindlimb movement *in vitro* can also evoke a sensory feedback to the spinal cord.

We tested this hypothesis using *en passant* AC-coupled nerve recordings from the whole DRlL5 (left L5 DR) in the isolated spinal cord with hindlimbs attached during a long (90-minute) control, as well as during training and resting phases (Fig. 2 A). The exemplar traces in A represent recordings in control of 10659 events, which increased to 12411 during BIKE and then returned to control values (9057). Pooled experiments showed an average increase of discharges during passive pedaling (138.11 ± 30.65 % of control) compared to control, which waned in the following resting phase (110.70 ± 36.39 % of control). To characterize the pattern of afferent discharges, fast Fourier transform (FFT) analysis was performed for the entire length (90 min) of the three experimental phases. During BIKE, a main rhythmic frequency component at 0.5 Hz appeared, which though was absent both during control (left) and after BIKE termination (right). The rhythmic component at 0.5 Hz was not an electrical interference produced by BIKE functioning, as it was not detected by a probe electrode measuring the polarization of the bath during pedaling (Fig. 1 D). These findings suggest that, during BIKE, a rhythmic component is elicited from afferents, which pairs the frequency of passive limb movement. However, this rhythm had small amplitude and became appreciable only at a higher time base scale, as it was covered by the baseline background noise (Fig. 2 C).

Since with *en passant* recordings both afferent input from the periphery and antidromic discharges from dorsal horns are detected, we wanted to isolate the sole contribution of sensory input. Thus, in a subgroup of preparations (data not shown), spinal nerves were transected and the distal stump was suctioned in glass pipette electrodes connected to an AC-coupled amplifier. In these experiments, the number of events recorded during BIKE was more than doubled when compared to control values (218.05 ± 65.81 % of control).

Therefore, even if it might be plausible that afferent inflow can be overestimated with *en passant* recordings, due to possible back-propagating discharges traveling across primary afferents, it was reported that limb-pedaling increases the number of afferent input reaching the spinal cord.

To exclude that these observations were biased by a possible damage of afferent pathways in our preparation, we recorded afferent discharges from dorsal nerves during peripheral stimulation of the hindpaw (Fig 3). AC-coupled traces and raster plots in Fig. 3 A show a considerable orthodromic activity recorded from DRlL4 during mechanical compression of the left hindpaw (an almost eight-fold frequency increase with respect to control), as highlighted by the magnification of selected events in Fig. 3 B. Moreover, in another subset of experiments (Fig. 3 C), electrical stimulation of the skin innervated by the sural nerve evoked responses from the sciatic nerve and, with a higher latency, from spinal afferent nerves L4 and L5 (Fig. 3 D). These results demonstrate that afferent

pathways conveying inputs from periphery to the dorsal spinal cord are fully intact and functioning in our *in vitro* preparation.

BIKE onset does not elicit FL per se, *nor does it vary an ongoing FL in the vast majority of cords.*
Motor exercise has been strongly suggested to enable spinal plasticity, reawakening the locomotor CPG in spinal cords isolated after lesion (Hubli and Dietz, 2013; Shah et al., 2013; Harkema et al., 2012). To confirm this hypothesis, we investigated the emergence of spontaneous locomotor activity from VRs during BIKE functioning. In 85% of experiments (52/61), no response was evoked during pedaling. In the remaining preparations (9/61), only a brief episode of spontaneous left-right alternating oscillations (0.55 ± 0.11 mV; 5 ± 3 cycles; data not shown) temporarily appeared as soon as BIKE was switched on. Likewise, during the continuous functioning of BIKE (90 min, Fig. 4 A), we observed no FL episodes and a consistent spontaneous synchronous activity was recorded in DC-mode from VRs. Spectral analysis of the spontaneous rhythmic activity at different time intervals during a long BIKE session (90 min, Fig. 4) confirmed that BIKE does not affect the spontaneous VR rhythmic pattern compared to control values (Fig. 4 B). Although this result indicates that BIKE onset only rarely elicited episodes of FL, we could not exclude that even a mild modulatory action of BIKE on locomotor networks might be sufficient to facilitate FL episodes evoked by electrical stimulation. To test this hypothesis, we analyzed the number of alternated oscillations recorded from VRs upon application of a train of rectangular pulses (2 Hz, 120 to 180 stimuli, 1.92 ± 0.38 Th) to different DRs (from DRT13 to DRL2). During pedaling, the conjoint delivery of a train of electrical pulses did not change the number of FL oscillations (108.93 ± 19.06 % of control, P = 1.000, Wilcoxon signed rank test, n = 5). These results indicate that addition of BIKE does not affect CPG functionality once already evoked by electrical stimulation.

Different durations of BIKE application affect locomotor spinal circuits in a nonlinear manner
Our experiments showed that the sudden start of BIKE does not activate the locomotor CPG in the vast majority of tested preparations. However, it would seem likely that BIKE cycling neuromodulates the locomotor pattern, in line with the concept that only an intense and repetitive training can elicit activity-dependent plasticity on spinal networks (Edgerton et al., 2004). Then, we wondered whether passive exercise of limbs could facilitate the locomotor pattern at the end of BIKE. To do so, we progressively lengthened BIKE sessions in order to verify whether spinal network excitability was ever affected by pedaling, once terminated. Therefore, a 2 Hz train of pulses was delivered before and right-after BIKE sessions of variable durations, to compare the ability to evoke an episode of locomotor-like oscillations.

Initially, we applied a 10-min session of BIKE to six samples and we did not observe any significant increase in number of oscillations (96.04 ± 33.87 % of control, P = 0.625, Wilcoxon signed rank test), although with a high variability. Thereafter, we considered prolonged BIKE sessions.

In Fig. 5 A, B are shown DC-coupled traces from an exemplar experiment where episodes of FL were electrically-elicited by DR stimulation before and after 30 minutes training. In control conditions, the electrical stimulation protocol induced a cumulative depolarization with 8 locomotor-like oscillations (Fig. 5 A). At the end of 30-min BIKE, the number of oscillations increased to 12 (Fig. 5 B). Pooled data from nine independent samples showed a statistically significant increase (142.36 ± 32.71 % of control) in the number of oscillations at the end of a 30-min BIKE session compared to control (Fig. 5 C, P = 0.008, Wilcoxon signed rank test). However, the number of oscillations was reduced to initial values (105.42 ± 37.98 % of control) after 20 min from ceasing BIKE training (Fig. 5 C, P = 0.039, Friedman repeated measures ANOVA on ranks, n = 5). These data suggest that FL was transitorily facilitated by 30 min of BIKE, even though BIKE does not *per se* induce CPG activity in the vast majority of cords.

Surprisingly, an extended training session of 90 minutes brought the number of FL cycles back to control levels and the transitory FL facilitation induced by 30 min of BIKE disappeared (Fig. 5 D; 61.20 ± 23.38 % of 30 min BIKE, P = 0.008, Mann-Whitney rank sum test, n = 9, 8). In order to confirm that our observations are determined only by passive hindlimb movement and not by any other experimental condition, sham experiments were performed. Results indicate that there was no difference in either the number of locomotor-like oscillations tested at the beginning or after 90 minutes (P = 1.000, Wilcoxon signed rank test, n = 3), nor in their cumulative depolarization (P = 0.750, Wilcoxon signed rank test, n = 3). Similarly, VR reflexes were unaffected by a long permanence in the recording chamber (peak: P = 0.250, Wilcoxon signed rank test, n = 3; area: P = 1.000, Wilcoxon signed rank test, n = 3). This outcome confirms that spinal circuits are equally functional even after long maintenance *in vitro*.

A long session of BIKE depresses VR reflexes

Notwithstanding the maintenance of stable conditions for more than 90 min, longer BIKE applications did not facilitate an electrically-induced FL, probably due to a depression in the sensory-motor connections caused by long pedaling. This point was explored by studying the sensory-motor reflex arc through the delivery of single pulses at low intensity (Th; 30.53 ± 9.66 μA) to a DR (T13 or L1), while recording potentials from the homologous VR. As reported in Fig. 6 A for a sample DC-coupled trace, a long BIKE session stably reduced the area of DRVRPs, an

effect that was still present even one hour after session end. On the other hand, the peak of DRVRPs was not affected by longer sessions of BIKE. In Fig. 6 B, the time course from many experiments demonstrates that the area of DRVRPs progressively decreased over the duration of BIKE, with a significant reduction reached at the end of the session (90 min; $P \leq 0.001$, one way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test versus control, n = 14). A subgroup of experiments, in which VR reflexes were not affected by a single 30-minute BIKE session (peak: P = 0.632, one-way repeated measures ANOVA, n = 5; area: P = 0.530, one way repeated measures ANOVA, n = 5) confirmed that sessions of BIKE shorter than 90 min did not affect the area of DRVRPs. The depressing effect of BIKE on DRVRPs appeared only for longer application of BIKE, which might alter the excitability of motoneurons.

A long session of BIKE affects spontaneous post-synaptic currents and membrane resistance of motoneurons

We then aimed at identifying the mechanism behind the effects of a 90 min session of BIKE, in terms of disappearance of FL facilitation and reduction in DRVRPs area. Thus, whole-cell patch clamp recordings were carried out on single antidromically-identified motoneurons as a read-out element for network activity. Afterwards, whole-cell patch clamp recordings were performed for up to five hours from the termination of 90-min of BIKE or sham experiments. After training, frequency of currents recorded from single motoneurons increased, as compared to sham experiments (Fig. 7 B, upper trace, and plot in C; P = 0.001, Mann-Whitney rank sum test, n = 32, 33), indicating an augmentation in inputs converging onto the motoneuron. However, this result was not sufficient to generate a spontaneous firing in current clamp mode, despite the presence of a more ragged baseline (Fig. 7 B, bottom trace), but was in line with the reduced membrane resistance of motoneurons after training (Fig. 7 D, E; P = 0.04, t-test, n = 5, 12).

Then, using kinetic analysis, we attempted to dissect out the following parameters for each contribution: current frequency (Hz), peak amplitude (pA), time of peak (ms), area (pA * ms), half-width (ms), rise time (ms), rise slope (pA/ms), decay time (ms) and decay slope (pA/ms).

After event selection, average traces for fast-decaying sPSCs (Fig. 8 A; $\tau < 5$ ms) and slow-decaying sPSCs (Fig. 8 B; $\tau > 20$ ms) were obtained and we found that BIKE increased frequency of both fast (Fig. 8 C; P = 0.03, Mann-Whitney rank sum test, n = 32, 33) and slow currents (Fig. 8 D; P = 0.03, Mann-Whitney rank sum test, n = 32, 33). Moreover, parameters of kinetic analysis were compared between sham and 90-minute trained motoneurons, but no differences were observed (data not shown).

According to literature, fast-decaying currents are mainly attributed to excitatory inputs mediated by AMPA receptors (Jonas, 2000), while slow-decaying currents are substantially ascribed to inhibitory input by GABA/glycine receptors (Galante et al., 2000). To confirm this issue, patch clamp recordings were performed at different holding potentials in order to selectively visualize fast glutamate-related currents and slow GABA/glycine-related currents. When comparing BIKE-trained and sham motoneurons, we observed that frequency of both fast ($P = 0.05$, Mann-Whitney rank sum test, $n = 5, 10$) and slow currents ($P = 0.05$, Mann-Whitney rank sum test, $n = 5, 10$) was increased.

Thus, we can conclude that training modifies the total number of synaptic currents and the excitatory and inhibitory input that converge onto the motoneuron.

Similarly, sPSCs were recorded from motoneurons after only 30-mins of BIKE, which correspond to the optimal duration to facilitate the FL induced by a train of DR pulses. Compared to sham experiments, the frequency of input to single motoneurons remained unchanged ($P = 0.681$, Mann-Whitney rank sum test, $n = 17, 20$), as also did fast-decaying and slow-decaying sPSCs (fast sPSCs: $P = 0.831$, Mann-Whitney rank sum test, $n = 17, 20$; slow sPSCs: $P = 0.286$, Mann-Whitney rank sum test, $n = 17, 20$).

However, the lack of any statistical results should be considered cautiously. Indeed, 30-min BIKE transiently facilitate FL within 20-min from turning off BIKE (Fig. 5 C), while spinal cord isolation for patch recordings largely exceeds this short time frame (see Methods). Therefore, it cannot be excluded that some modifications in the biophysical properties of motoneurons could transitorily take place in the first 20-min after BIKE.

Dorsal spinal networks are affected by training

Ninety-minute sessions of BIKE induced the disappearance of the facilitatory effect on FL, the reduction in DRVRP area, and the increase in slow-decaying sPSCs onto the motoneuron, mainly ascribed to a GABAergic nature. In turn, an increase in GABAergic transmission at the level of dorsal spinal networks augments antidromic reflex responses from DRs (DRDRPs; Barker and Nicoll, 1972; Bagust et al., 1985) and spontaneous antidromic discharges from DRs (Vinay et al., 1999; Fellippa-Marques et al., 2000; Bos et al., 2011). To clarify whether BIKE actually affected synaptic transmission in the dorsal cord, we first recorded DRDRPs, both in control conditions and after training. After 30 min of BIKE, peaks of DRDRPs did not change right after the training session (110.94 ± 26.18 % of control) and remained similar to baseline values for up to 1 hour of rest (111.75 ± 27.46 % of control; $P = 0.837$, Friedman repeated measures ANOVA on ranks, $n = 5$). On the other hand, when training was prolonged to 90 min, the peak of DRDRPs increased by

40% right after BIKE (Fig. 9 A). The amplitude of responses remained higher than in control even after a long rest (Fig. 9 B). In the time course shown in Fig. 9 B, overall DR responses augmented, with a significant increase in peak amplitude two hours after ending 90 min BIKE (Fig. 9 B, $P = 0.023$, Friedman repeated measures on ranks followed by Dunn's test versus control, $n = 5$). These results suggest that only prolonged exercise promotes a long-lasting potentiation of primary afferents, presumably of predominant GABAergic nature (Barker and Nicoll, 1972; Bagust et al., 1985). In Figure 8 C spontaneous antidromic activity is recorded in DC-mode during training from the left L2 DR, which displayed an increased frequency of antidromic discharges. The time course shown in Figure 9 D reports pooled data from many experiments, pointing out that the power spectrum magnitude of spontaneous activity on DRs significantly increased upon BIKE start. Significantly higher values than control persisted for the entire duration of training (Fig. 9 D, $P \leq 0.001$, one-way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test versus control, $n = 4$). Thus, initiation of BIKE can actually increase dorsal network excitability, as described by augmented antidromic discharges derived from DRs.

Ninety minutes of passive mobilization induce long-term increase in spontaneous dorsal discharges

Training exercise modulates spinal excitability in the lumbar spinal cord (Côté et al., 2014) and, in particular, it promotes dorsal network plasticity in the long-term (Detloff et al., 2014). In our study, we showed that spontaneous dorsal discharges increase with BIKE activation. Nevertheless, we wondered whether such augment in dorsal circuit excitability could persist even after the end of the pedaling session. Similarly, we cannot exclude that spontaneous activity recorded from VRs might also be affected by long sessions of BIKE, even once ended. Thus, VR and DR spontaneous activity was continuously recorded in DC-mode before, during and at the end of a 90-min session, even beyond two hours of rest (Fig. 10).

Figure 10 A shows that synchronous VR bursts emerged from fast single events during a 20-min control period, while a simultaneous intense tonic DR activity was measured (left panels). Recordings were continued during the subsequent 90-min BIKE period and showed an increase in DR activity, while VR discharges remained unaffected (middle panels). Twenty minutes after cessation of BIKE, only DR activity was strongly augmented both in frequency and amplitude (right panels). Power spectrum magnitude from many experiments confirmed that VR discharges were not significantly affected by 90 min training (data not shown, $P = 0.072$, paired t-test, $n = 8$), although the rhythm recorded from DRs significantly sped up with respect to control (Fig 10 B, $P = 0.005$, paired t-test, $n = 8$). To verify that these results were only ascribed to exercise, sham

experiments without BIKE demonstrated that maintenance of preparations *in vitro* for 90 minutes did not affect DR rhythm magnitude (data not shown, P = 0.929, paired t-test, n = 3).

We then aimed at assessing the minimal training duration able to elicit a significant increase in spontaneous dorsal activity after training. As depicted in Fig. 10 C, 30 min-long BIKE sessions were applied in sequence while DR rhythm magnitude was monitored at the end of each session. Exposure to cumulative BIKE shifts for less than 90 minutes did not change magnitude of dorsal discharges. Only a total duration of exercise of at least 90 minutes could evoke a statistical variation in DR rhythmicity that persisted after turning off BIKE (Fig. 10 D, P = 0.026, one-way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test versus control, n = 4).

In a subset of experiments, the magnitude of rhythms recorded from DR and VR, expressed as RMS of the power spectrum, was calculated in slots of 20 min for up to 140 minutes of rest. Time course in Figure 10 E shows that VR spontaneous activity was not significantly affected by 90 min BIKE even in the long run (Fig. 10 E, P = 0.077, one-way repeated measures ANOVA, n = 5). Conversely, DR discharges remained significantly higher than control for the entire observation period (Fig. 10 E, P < 0.001, one-way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test versus control, n = 5). These data suggest that 90 min of BIKE exercise is the shortest protocol to modulate spontaneous dorsal horn network activity, even in the following long resting period.

BIKE enhances 4-AP rhythm derived from DRs

A prolonged session of BIKE strongly and stably increased DR rhythmic discharges. We wondered whether exercise could still modulate dorsal networks when rhythmicity was already maximized by a conjoint pharmacological excitation. Thus, we applied micromolar concentrations of the broad spectrum K^+-channel blocker, 4-aminopyridine (4-AP), which significantly enhances reflex responses (Taccola and Nistri, 2004; 2005) and induces primary afferent depolarization, antidromically conducted along DRs (Dubuc and Rossignol, 1989; Taccola and Nistri, 2005). In particular, high concentrations (10 - 50 μM) of 4-AP were used to induce overexcitability of dorsal horn networks, which has been suggested to mimic activity related to neuropathic pain states (Ruscheweyh and Sandkühler, 2003).

In Figure 11, 4-AP (10 μM) was perfused for the whole experiment to increase typical DC-coupled DR spontaneous discharges to 314% of control in physiological solution (Fig. 11 A, left, top trace, P = 0.02, t-test, n = 5, 3) and to 562 % for VRs (Fig. 11 A, left, bottom traces, P = 0.032, Mann-Whitney rank sum test, n = 5, 4). Subsequently, in the continuous presence of 4-AP, BIKE was applied for 90 min. BIKE activation induced a sudden increase in DR activity, and DR discharges

remained higher throughout the training session (Fig. 11 A, middle, top trace). Conversely, VR rhythm was not further enhanced by BIKE (Fig. 11 A, middle, bottom traces). At the end of pedaling, DR and VR activities were monitored and we observed that, after a two-hour rest (Fig. 11 A, right), magnitude of DR and VR rhythmic discharges were comparable to the ones recorded right after BIKE cessation, which in turn were similar to control.

In Figure 11 B, averaged time courses from pooled experiments reported an increased activity during BIKE only for the rhythm recorded from DRs ($P = 0.003$, one way repeated measures ANOVA, $n = 3$) with VRs remaining unaffected ($P = 0.718$, Friedman repeated measures ANOVA on ranks, $n = 4$). Moreover, in the continuous presence of 4-AP, after session termination, rhythm on VRs matched the control ($P = 0.587$, Friedman repeated measures ANOVA on ranks, $n = 4$), and signals from DR faintly waned to control values ($P = 0.577$, one way repeated measures ANOVA, $n = 4$). Similarly, in 4-AP, after termination of a 90-min session, DRDRPs (peak: $P = 0.125$, Wilcoxon signed rank test, $n = 4$; area: $P = 0.171$, paired t-test, $n = 4$) were comparable to those of pre-BIKE conditions.

To confirm that BIKE specifically modulated the rhythm driven by dorsal networks, we performed sham experiments in which a long perfusion with 4-AP (at least five hours) did not modify magnitude of rhythmic activity, neither dorsally ($P = 0.964$, Friedman repeated measures ANOVA on ranks, $n = 4$) nor ventrally ($P = 0.802$, Friedman repeated measures ANOVA on ranks, $n = 4$).

Data collected suggest that, despite the strong network excitation induced by 4-AP, antidromic discharges could still be augmented in response to 90 min of conjoint exercise. This increase in DR rhythmic discharges did not persist after training nor for the entire period of observation. Interestingly, VR activity induced by 4-AP was not further modified by BIKE, indicating that such robust rhythm could be hardly modulated by training.

Discussion

In this study, we associated intra- and extra-cellular electrophysiological recordings with an innovative robot consistently able to generate afferent inputs to the DRs. We proved that 30 minutes of passive alternated mobilization of limbs increase the number of locomotor-like oscillations induced by DR electrical stimulation, without affecting VR reflexes and DR potentials. This facilitatory effect on the locomotor CPG, which likely arises from an early increase in putatively glutamatergic PSCs on motoneurons, was lost with longer training sessions, which in fact reduced the area of VR reflexes and increased antidromic discharges recorded from DRs. With longer training sessions, the increases in frequency of putatively GABAergic slow-decaying sPSCs on

single motoneurons, able to overwhelm the concurrent glutamatergic drive elicited by BIKE, may be involved in the disappearance of CPG facilitation.

A novel robotic instrument to induce a real alternate movement of hindlimbs in vitro

In our study we described a robotic device, BIKE, which was devised to allow the standardized mobilization of limbs in an isolated neonatal rat spinal cord preparation with legs attached. Previous results from *in vivo* animal models have already shown compelling evidence of activity-dependent plasticity in spinal cord tissue collected one hour after passive training (Gómez-Pinilla et al, 2002; Beaumont et al., 2004; Ichiyama et al., 2011; Joseph et al., 2012; Keeler et al., 2012; Chopek et al., 2015; Dugan and Sagen, 2015). Our innovative model has proven suitable to trace an earlier modulation of spinal networks, even during and immediately after the end of the passive exercise. In the past decades, several experimental models became available to study the modulatory effects of actual limb movement on *in vitro* CPG output (Wheatley and Stein, 1992; Hayes et al., 2009). In particular, Hayes and collaborators proposed a preparation in which the pharmacological induction of FL was paired with limb movement over a treadmill (Hayes et al., 2009). Although our model may look similar at first glance, there is a clear difference that gives both models their unique characteristics and advantages to investigate specific and fundamentally different research questions. Hayes and colleagues starts from a pharmacological activation of the CPG to induce real locomotion, and then studies how limb movements modulate the ongoing CPG rhythmic pattern that remains neurochemically-driven (Hayes et al., 2009). Our model, on the other hand, induces mere passive limb movement with the aim of studying its effects on the CPG. While Hayes and collaborators clarified how the pattern is refined both in different phases of gait and by environmental perturbations (Hayes et al., 2009; Hayes et al., 2012), our study aims at assessing how a session of passive mobilization can facilitate locomotor patterns. Our preparation has the great advantage that it can specifically allow to study the role of proprioceptive afferent feedback evoked by dorsal spinal nerves during the sole movement of limbs. Indeed, exclusive recordings of sensory feedback cannot occur if the whole preparation is in contact with neurochemicals, as these would inevitably contaminate afferent volleys by both inducing antidromic rhythmic activity along spinal dorsal networks (Kremer and Lev-Tov, 1998) and increasing excitability of spinal ganglia (Lovinger and Weight, 1988; Cardenas et al., 2001).

Afferent discharges emerge from pedaling

BIKE was able to increase the number of afferent discharges from DRs. This effect is consistent with that evoked on the same preparations by electrical stimulation of the skin and mechanic

compression of the paw. Presumably, also proprioceptive input should be included, because of the wide excursion of the hip and knee during each pedaling cycle. Nevertheless, we cannot exclude that even input from nociceptive and thermosensitive cutaneous afferents might be involved (Mandadi and Whelan, 2009). Unfortunately, though, no afferent feedback during passive limb mobilization has yet been reported on *in vitro* preparations, making it thus impossible for us to compare our afferent contributions with the results collected by others using different modes of peripheral stimulation.

The alternated rotation of paws induced by BIKE putatively produces a pattern of pressure and cutaneous rhythmic stimulation that activates peripheral receptors. This was confirmed by the main discharge frequency at 0.5 Hz during passive cycling, which corresponds to the pace of pedaling.

Finally, it remains to be noted that, although both our efforts in controlling experimental conditions and the more conservative surgery aimed at preserving the integrity of peripheral targets, as demonstrated by the stable output recorded for the whole period of observation, the afferent feedback from our experiments is of low amplitude and large frequency variability. On the one hand, this could be the consequence of the immaturity of neonatal preparations, which reach full development of afferent pathways over the first three postnatal weeks (Fitzgerald et al., 1994). On the other hand, it is interesting to note how, at birth, afferent feedback from periphery to central spinal circuits is only weakly inhibited presynaptically: hence, even a scarce peripheral feedback during early postnatal experiences (Sonner and Ladle, 2013) might contribute to optimally modulate the ontogeny of spinal circuits (Vinay et al., 2002).

Trains of electrical pulses to dorsal afferents induce FL during cycling

DR trains of stereotyped electrical stimuli were used to test a putative facilitation of spinal networks by BIKE. These trains have previously proven to reliably evoke locomotor patterns both in the deafferented isolated spinal cord (Marchetti et al., 2001) and in preparations in which sensory afferents remained intact (Klein et al., 2010), although without imposing any real outgoing movement. Our study exploited for the first time the integrity of afferents to generate a feedback through a real pedaling movement. The feedback generated by the real and passive limb movement might influence trains of stimulation applied to a DR, through phenomena of presynaptic inhibition or action potential collision (Willis, 1999). We observed, however, that during BIKE functioning, the response induced by a train of stimuli was equal to control conditions in the absence of any limb movement. We may speculate that the neuromodulatory effect induced by afferent input is mild compared to electrical DR activation through pulse trains. Moreover, the proprioceptive volleys evoked by limb movement did not perturb the train of impulses entering the stimulated root.

However, the use of DR stimulation represents a reliable and widely-used method to induce immediate and transient activation of locomotor networks, without the need to maintain a long perfusion with neurochemicals before establishing a FL (Marchetti et al., 2001; Dunbar et al., 2010; Juvin et al., 2012; Dose et al., 2016). In this manner, we could quantify the facilitatory role of afferent input on spinal network rhythmicity in terms of increased number of locomotor-like oscillations. Through a detailed time analysis, starting at the end of a training session and then with short intervals in the subsequent resting phase, we precisely quantified the duration of any putative BIKE effect.

BIKE facilitates excitatory and inhibitory input, with different timing

BIKE generated both excitatory and inhibitory effects. A short application of BIKE was able to transitorily increase the number of locomotor-like oscillations induced by trains of dorsal stimuli. On the other hand, a longer application abolishes this effect and reduces the area of DRVRPs. At the same time, a longer application also induces a potentiation of antidromic discharges, either spontaneous or induced by dorsal stimulation, that lasts even for several hours after the end of training.

Intracellular recordings showed that BIKE increased the number of total input directed to the single motoneuron, while the amplitude of these inputs remained unaltered, probably indicating a presynaptic effect on the motoneuron. The analysis of current kinetics intracellularly recorded from motoneurons after 90-min BIKE revealed an increase in the frequency of excitatory fast spontaneous currents, mainly attributed to AMPA receptors (Hestrin, 1993; Wyllie et al., 1994; Galante et al., 2000), paired with an increase in the slow spontaneous currents, putatively ascribed to a GABAergic nature (Lewis and Faber, 1996; Galante et al., 2000). On the other hand, shorter sessions of BIKE facilitated locomotor networks, but only with a transient effect that plausibly did not allow us to detect any changes on motoneurons.

The most parsimonious explanation as for the effects observed in this study is that excitatory and inhibitory contributions were recruited with a different timing during BIKE. This explanation, although not reported in this study, would predict an increase in DRVRPs for short BIKE periods. However, we could speculate that the facilitation of FL seen for shorter training sessions might reflect an early increase in spinal excitation, in line with the facilitation of weak electrical stimulation in the presence of low concentrations of glutamatergic agents (Dose and Taccola, 2012). In addition, in the present study, BIKE increased fast spontaneous PSCs attributed to AMPA glutamatergic receptors.

On the other hand, AMPA inputs on the motoneuron might be overwhelmed by longer sessions of BIKE because of the appearance, or the progressive increase, of GABAergic post-synaptic input (Fontana et al., 2001), which have been reported to depress FL (Cazalets et al., 1994; Cowley and Schmidt, 1995). Unfortunately, we could not test this hypothesis through selective pharmacological antagonism of AMPA or GABA receptors, as the administration of required drugs would have interfered with the expression of FL (Bracci et al., 1996b; Beato et al., 1997).

The alternative explanation, indicating that the potentiation of AMPA receptors (and the corresponding facilitation of FL) is lost with a prolonged application of BIKE as a result of the desensitization of AMPA channels (Ballerini et al., 1995; Tsvyetlynska et al., 2005), seems unlikely, since fast currents on the motoneuron are augmented after 90-min BIKE.

We can conclude that BIKE enhances spontaneous synaptic transmission within the spinal network. Likewise, training is known to affect the proportion of inhibitory vs. excitatory boutons in rat motoneurons (Ichiyama et al., 2011). Moreover, in correspondence to longer BIKE sessions, motoneuron membrane resistance was found to diminish, in line with previous reports on trained adult rats (Beaumont et al., 2004).

Antidromic discharges are affected by training

Our data indicate that BIKE sessions of increasing duration initially modulate rhythmicity of ventral networks generating FL (Kiehn, 2006), and, later, of dorsal networks that are considered responsible for antidromic discharges recorded from DRs (Dubuc and Rossignol, 1989). The biological meaning of these data remains to be investigated. From the perspective of the observed increase in dorsal network rhythmic activity, it is interesting to note that hyperexcitable dorsal networks are implicated in the development of neuropathic pain (Luo et al., 2008). It is, however, also important to mention that neuropathic pain has been previously attenuated by passive mobilization (Chen et al., 2012; Detloff et al., 2014; Dugan and Sagen, 2015). In the present study, dorsal network activity was assessed using two outcomes, i.e. the magnitude (root mean square, RMS) of the spectrum of spontaneous rhythmic discharges originating from dorsal circuits and the peak of dorsal reflex responses induced by electrical stimulation of an adjacent DR, in accordance with a previous report (Dingu et al., 2016). Antidromic responses recorded from DRs originate from primary afferent depolarization (Fellippa-Marques et al., 2000) and can reflect either presynaptic inhibition of incoming nociceptive input or the paroxysmal activity of dorsal horn neurons in the context of hyperalgesia (Willis, 1999). At the same time, antidromic discharges induced *in vitro* by 4-AP have been implicated in the processing of nociceptive input (Chapman et al., 2009; Visockis and King, 2013) and were found to be attenuated by classical analgesics that reduce neuropathic

pain as well (Ruscheweyh and Sandkühler, 2003). Our data show that a 90-min BIKE session increased the frequency of antidromic oscillations induced by 4-AP, an observation which may inspire further studies to explore the putative effect of prolonged training on nociception and/or pain. To better understand the translational relevance of the present study, future testing should assess the various durations of pedaling exercise in preclinical *in vivo* models of chronic pain. On the basis of our current *in vitro* data, we speculate that the duration of a BIKE session could differentially affect plasticity of both spinal locomotor circuits and nociceptive dorsal horn networks.

It is worth noting that, in the context of animal experimental models of SCI, various plasticity-promoting therapies have been associated with opposing effects on recovery of (locomotor) function and neuropathic pain (Deumens et al., 2008), two main scopes of rehabilitation therapy.

Conclusions and clinical perspectives

We are aware that neonatal rodents are not the best suited model to understand the mechanisms of passive pedaling in humans, adults in particular. Indeed, P0-P4 neonatal rats cannot bear their own weight and descending- and sensory input to motoneurons, as well as their membrane properties are still at an immature developing stage (Vinay et al., 2002; Clarac et al., 2004). Nevertheless, transient facilitation of locomotor patterns with a precise timing after passive cycling might inform us on the existence of an optimal therapeutic window for co-administering pharmacological agents and neurorehabilitation to activate spinal locomotor circuits after SCI (NCT01621113, NCT01484184, ClinicalTrials.gov).

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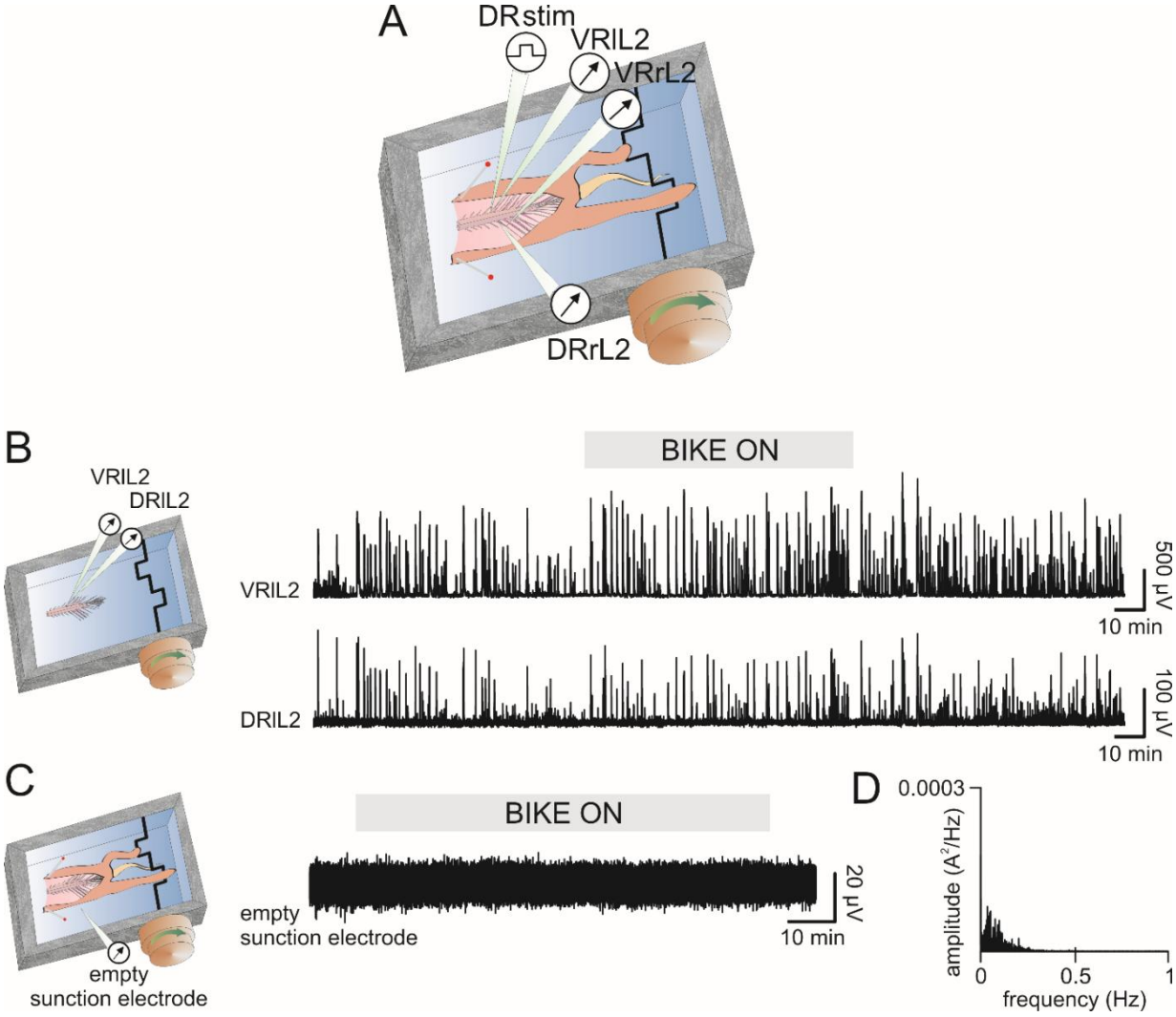


Figure 1. BIKE allows passive hindlimb movement while simultaneously recording from spinal roots.

A: The cartoon shows the experimental setup using a spinal cord with hindlimbs attached. Spinal roots were dissected from the midthoracic region down to the second lumbar segment (L2) included. Preparation was maintained in a continuously superfused chamber, with hindpaws firmly fixed to the pedals of BIKE above the chamber. Speed rotation was adjusted through a stabilized power supply at around 30 cycles/min (0.5 Hz). Only forward movement was applied, as indicated by the arrow. Simultaneous recordings were performed with suction glass electrodes from both right (r) and left (l) L2 ventral roots (VRs) and from a single dorsal root (DR), either in the presence or absence of DR stimulation. **B**: Sample traces recorded from VRlL2 and DRlL2 in an isolated spinal cord show that BIKE does not induce any electrical interference. **C**: Background noise recorded with a glass electrode placed close to L5 spinal segment demonstrates that passive hindlimb movement does not induce any baseline interference. **D**: The FFT analysis reports spectral analysis

for the trace in **C.** Note the absence of any rhythmic components around the frequency of pedaling (0.5 Hz).

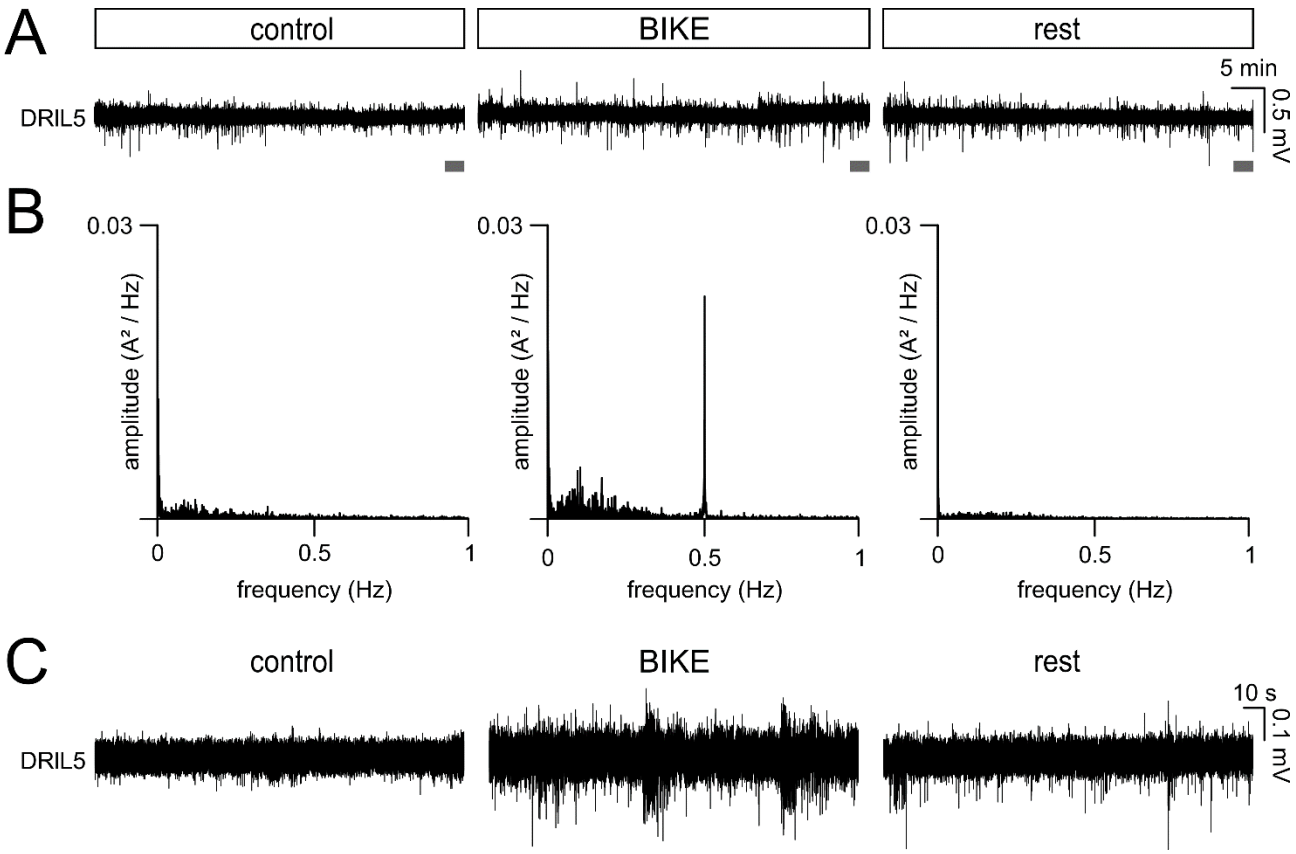


Figure 2. BIKE-induced passive training evokes afferent sensory feedback coupled with pedaling.

A: Sample traces recorded during 90 minutes control, BIKE and rest, as depicted by the protocol bars on top. Activity was derived from DRlL5 by applying a negative pressure on its surface through a suction glass electrode (*en passant* recordings). **B**: The FFT analysis for traces in **A** isolates a main frequency component at 0.5 Hz during BIKE (middle), which is coupled to the pedaling frequency. No components at 0.5 Hz could be detected in control (left) and rest (right). **C**: Higher magnification of traces in **A**, corresponding to grey rectangles, indicates the increase in afferent discharges during BIKE (middle) compared to control (left) and rest (right).

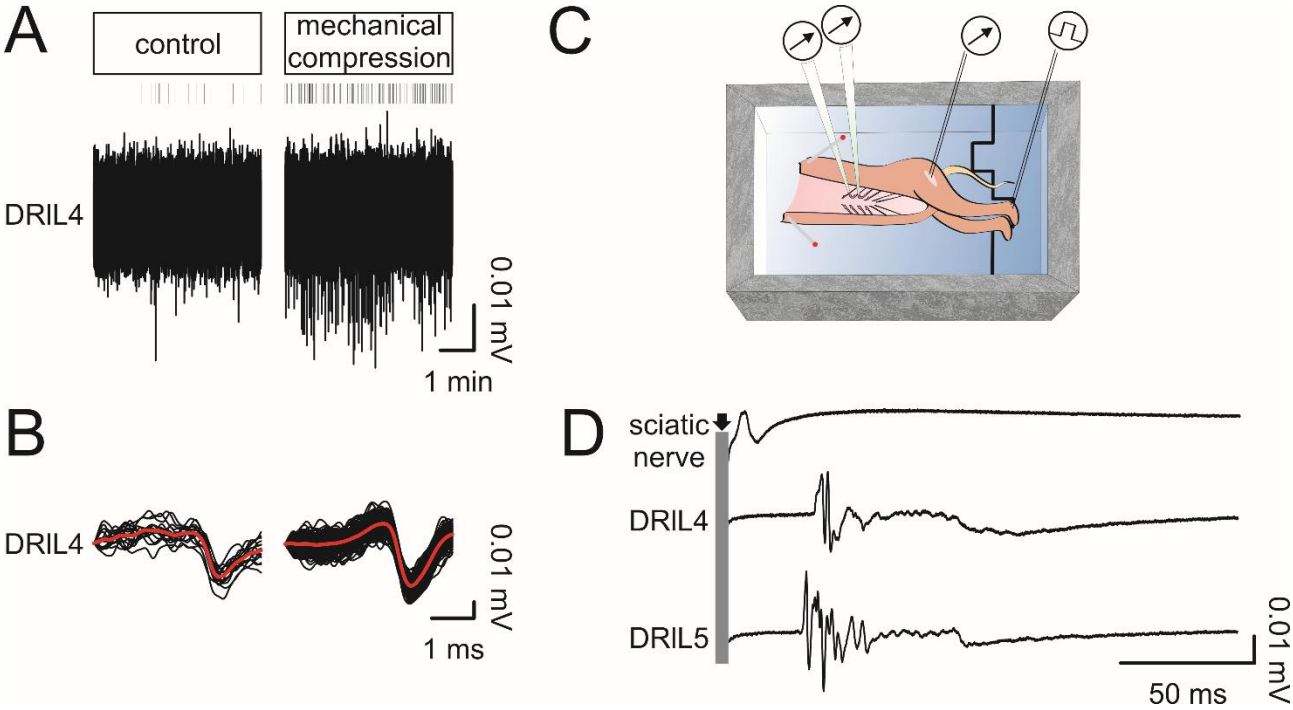


Figure 3. Afferent pathways to the spinal cord are fully intact and functioning in our *in vitro* model.

A: Incoming discharges were recorded from DRlL4 in the leg-attached preparation. Recordings were performed in control condition and during a mechanical compression of the left hindpaw, as shown on the top. Raster plots above traces highlight a greater incoming activity during peripheral mechanical compression of the left hindpaw. **B**: Identified events were superimposed and shown in black, while averaged traces were depicted in red. **C**: In a subset of experiments leg-attached preparations deprived of the spinal cord were arranged in the BIKE recording chamber as depicted by the cartoon. The left hindpaw was firmly fixed to the right pedal to get access to the sural-innervated territory on the left hindpaw for single-pulse stimulation and to the left branch of the exposed sciatic nerve for recording. For this purpose, pairs of hooked needle electrodes were used for bipolar stimulation and recordings. Moreover, monopolar recordings with suction glass electrodes were performed from the spinal stump of DRlL4 and DRlL5. **D**: Average traces of 500 sweeps are reported. Compound action potentials could be elicited from both the sciatic nerve and, with a higher latency, from spinal afferent nerves L4 and L5. The grey rectangle on the left represents a 5-millisecond rectangular pulse applied to the sural territory on the left hindpaw.

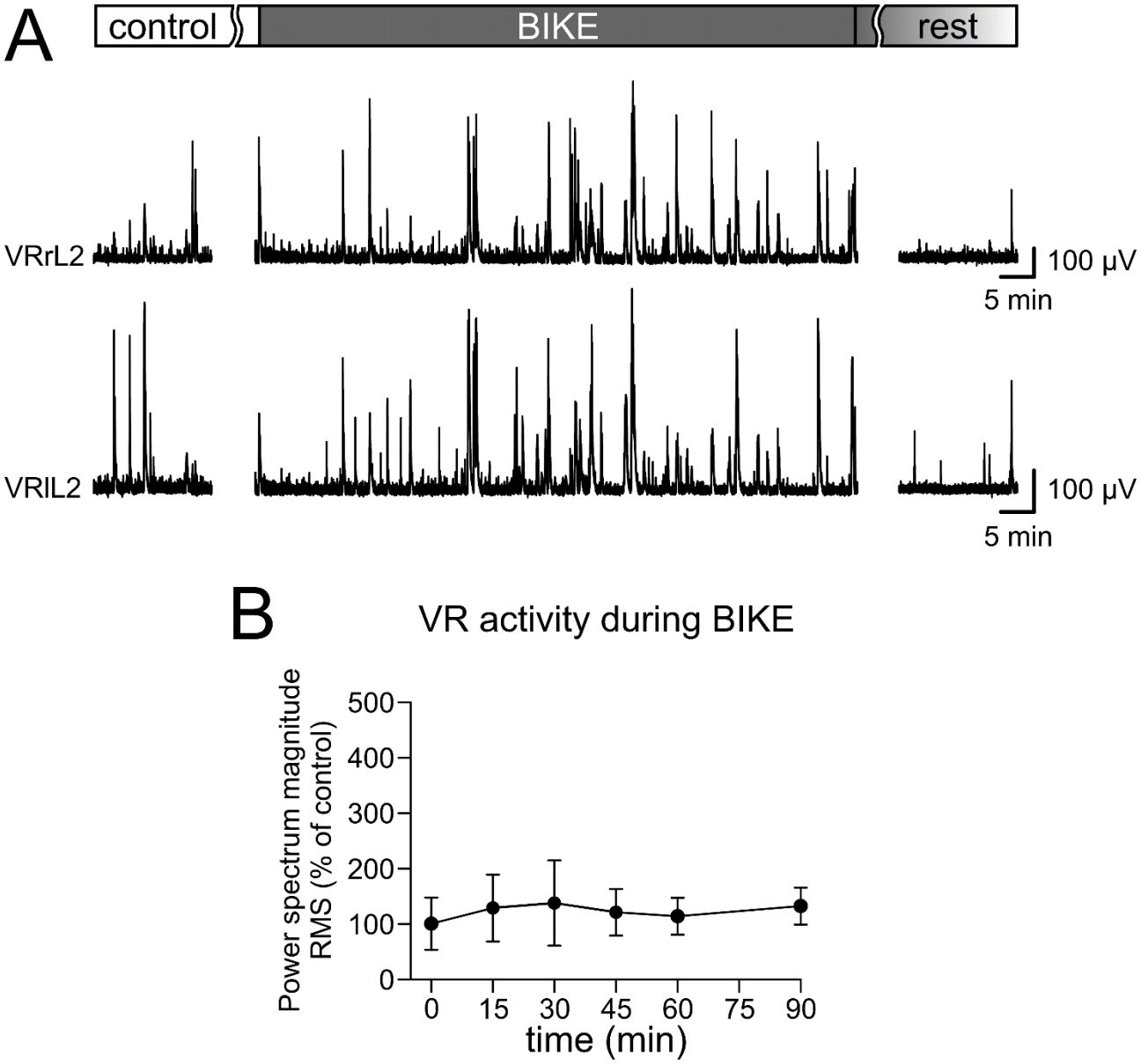


Figure 4. Long application of BIKE does not vary spontaneous VR activity.

A: Spontaneous activity was recorded from bilateral VRs L2 in control (left), during a BIKE session (90 min; middle) and at the end of training (right). Traces are interrupted in the correspondence of the artifacts from single or repetitive pulse stimulation (20 min). **B:** The time course points out that magnitude of the power spectrum for VR spontaneous activity during BIKE was not significantly different with respect to control conditions ($P = 0.267$, one way RM ANOVA, $n = 9$).

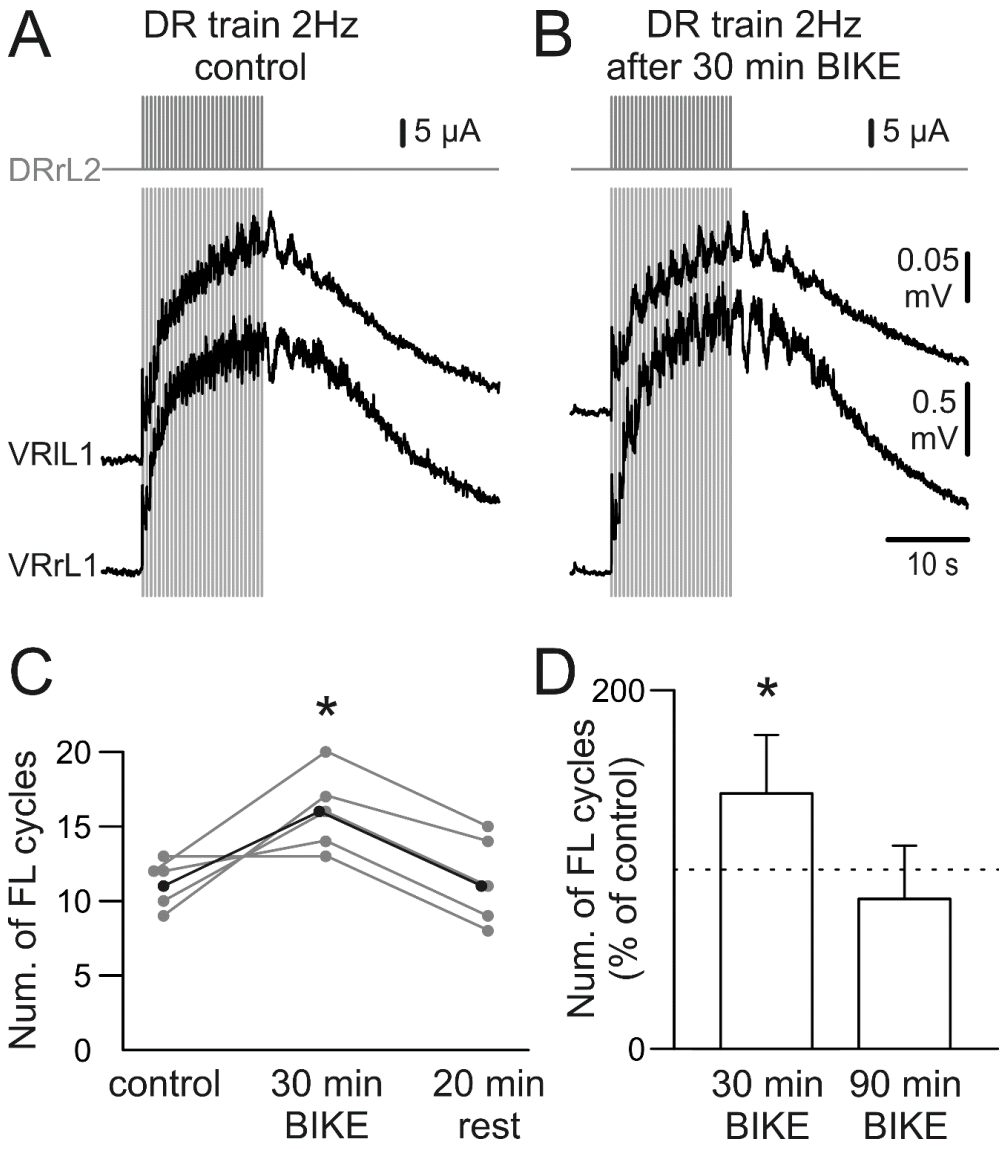


Figure 5. A 30-minute BIKE session transiently facilitates electrically-evoked FL.

A: Before BIKE, a train of stereotyped electrical stimuli (30 rectangular pulses; pulse duration = 0.1 ms; intensity = 45 µA, 3 x Th; frequency = 2 Hz) was delivered to DRrL2 (upper panel), inducing a characteristic episode of FL consisting in a cumulative depolarization with superimposed 8 alternating cycles recorded from homosegmental L1 VRs. **B:** At the end of a 30-minute BIKE session, the same train of pulses induced a higher number of locomotor-like oscillations (12 cycles; traces in lower panel). **C:** Plot summarizes for five experiments the time course related to the number of FL cycles induced by a train of stimuli, before, right after switching off BIKE (30 minutes) and after 20 minutes rest. BIKE transiently augmented the number of FL cycles, which returned to control values 20 minutes after end of training ($P = 0.039$, Friedman RM ANOVA on ranks, $n = 5$). Note that grey dots represent raw data and black dots indicate mean values. **D:**

Histogram compares the average increase in the number of cycles induced by DR trains in preparations subjected to 30 or 90 min BIKE. The facilitatory effect mediated by 30 minutes BIKE was lost if training was prolonged to 90 minutes ($P = 0.008$, Mann-Whitney rank sum test, $n = 9, 8$).

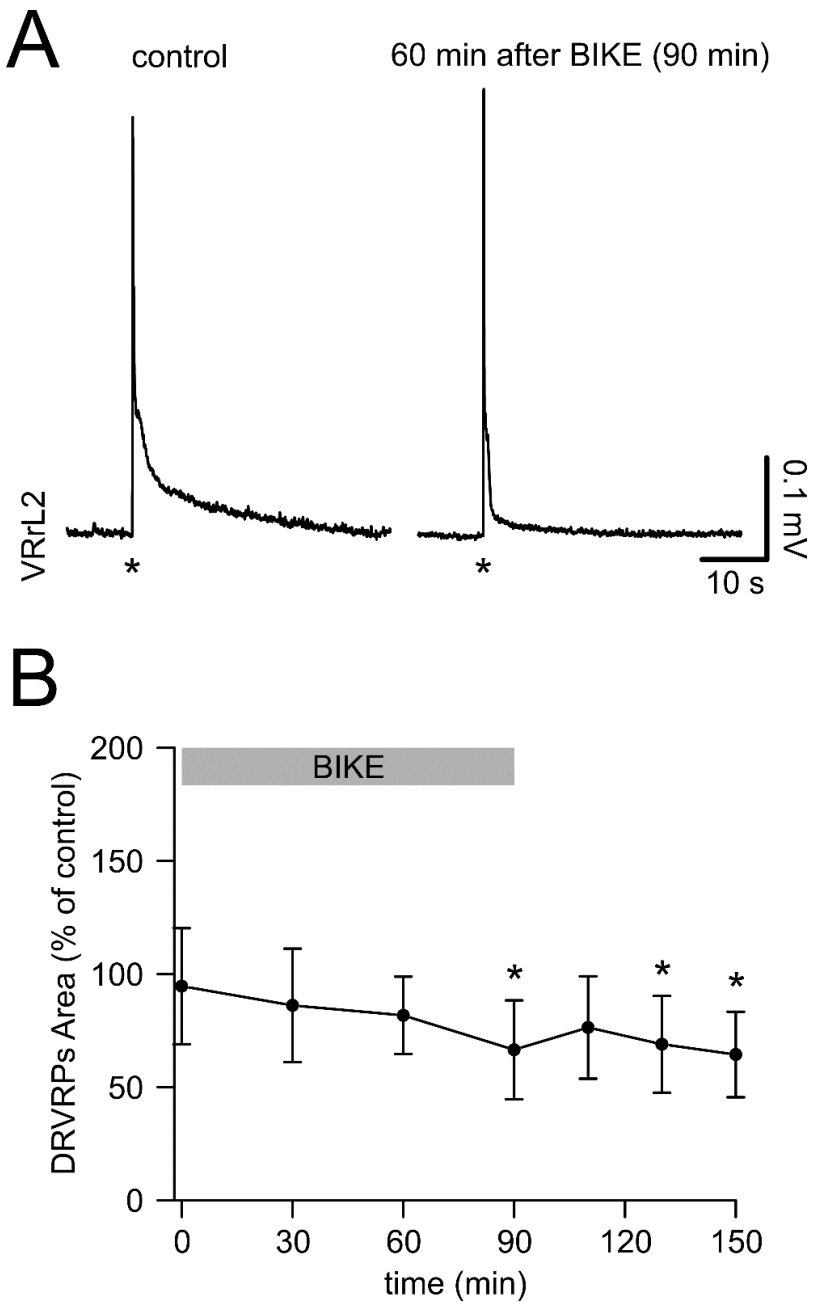


Figure 6. Only longer sessions of BIKE reduce the area of DRVRPs.

A: A spinal reflex (averaged traces from 10 sweeps) is induced by a single pulse (22 µA, Th, 100 µs) delivered at the time indicated by the stars on DRlT13. In control conditions (left), responses present an early peak followed by a slow decay repolarization. 1 h after a training session of 90 min BIKE (right), the same pulse elicited a response of smaller area while the peak remained unaffected. **B**: The time course plot summarizes mean values collected from 14 experiments, showing a progressive reduction in the DRVRP area by prolonging the duration of the BIKE session. After 90 min BIKE, a significant reduction of the reflex response is reached and maintained at least for the following hour ($P = <0.001$, one way RM ANOVA followed by post-hoc analysis with Bonferroni t-test versus control, $n = 14$).

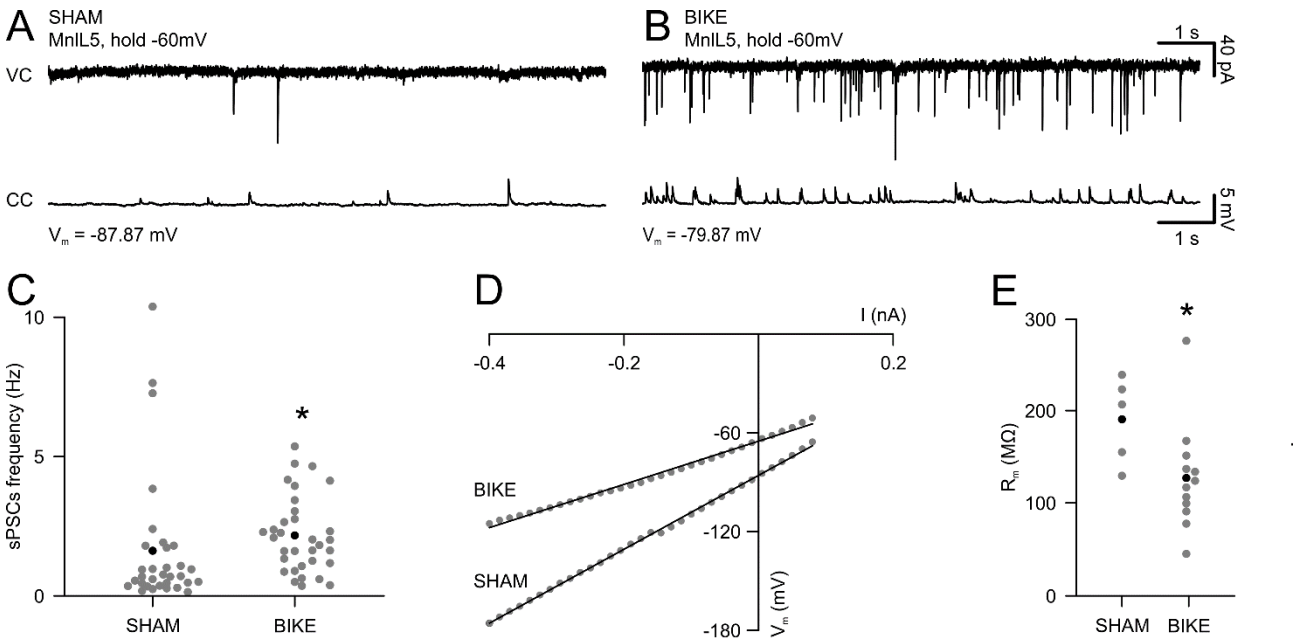


Figure 7. 90-minutes of BIKE increase number of spontaneous post-synaptic currents (sPSCs) and reduce membrane resistance in motoneurons.

A: Whole-cell patch clamp recordings were performed in voltage clamp (VC; top trace) and current clamp (CC; bottom trace) modes from the same lL5 motoneuron in a sham preparation. Holding potential in VC mode is -60 mV, while cell membrane potential (V_m) in CC mode, without injecting any holding current, is -87.87 mV. **B:** Recordings were carried out in VC mode (top) and CC mode (bottom) from the same lL5 motoneuron after 90 minutes of BIKE. Holding potential in VC mode was -60 mV, while V_m in CC mode was -79.87 mV. Recordings from motoneurons in **A** and **B** were obtained from an equivalent period of time after the isolation of the cord from the leg-attached preparation. Note the higher number of sPSCs (top trace) with respect to sham (top trace in **A**), as confirmed by the plot in **C** reporting a significant increase in sPSCs frequency after BIKE-training ($P = 0.001$, Mann-Whitney rank sum test, $n = 32, 33$). **D:** In the graph are shown the I-V curves for two sample cells obtained in CC mode, namely a BIKE-trained motoneuron and a sham motoneuron. **E:** The plot indicates a significantly lower membrane resistance (R_m) in BIKE motoneurons with respect to sham cells ($P = 0.04$, t-test, $n = 5, 12$); grey dots for raw data, black dots for mean values.

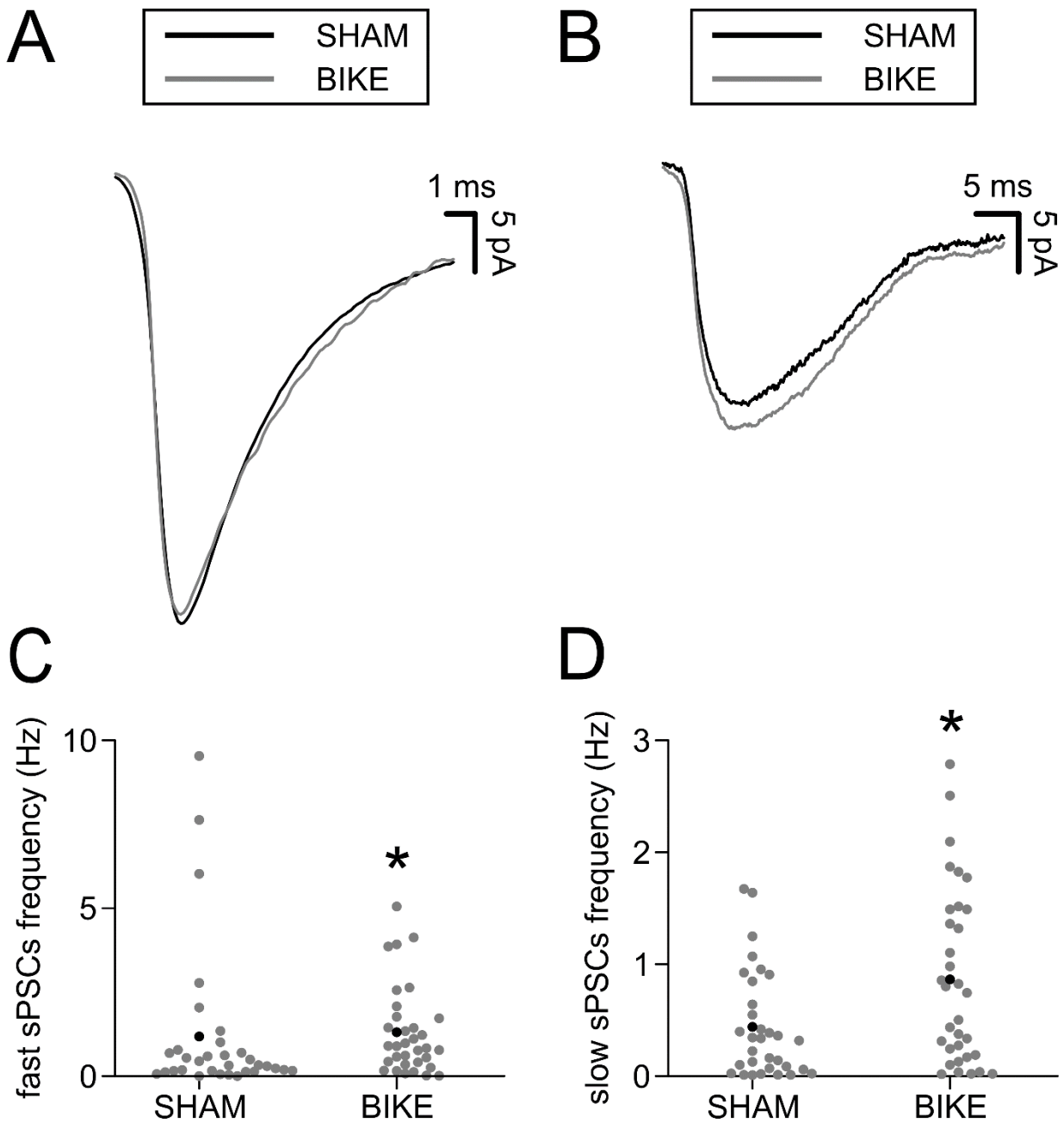


Figure 8. A long training with BIKE augments the number of both fast and slow currents reaching the motoneuron.

A and **B:** Superimposed averaged traces represent fast sPSCs (**A**) and slow sPSCs (**B**) from a pair of sample motoneurons in sham (black lines) and BIKE-trained preparations (grey lines). **C** and **D:** Plots indicate that 90 minutes of BIKE increased frequency of fast sPCSs (**C**; P = 0.03, Mann-Whitney rank sum test, n = 32, 33), and slow sPCSs (**D**; P = 0.03, Mann-Whitney rank sum test, n = 32, 33), compared to sham experiments. Grey dots represent raw data, black dots indicate mean values.

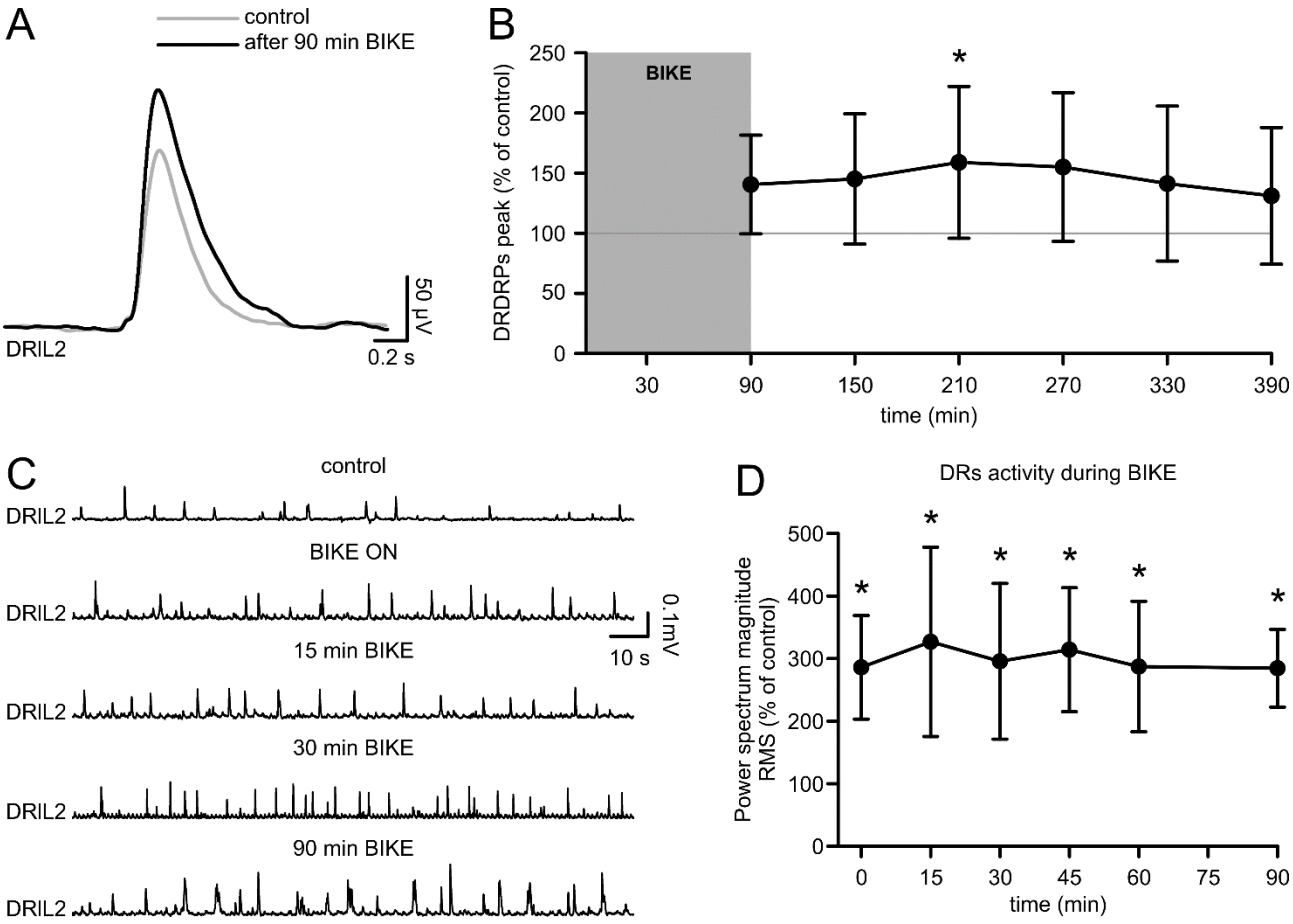


Figure 9. A 90-minute BIKE session stably increases DR reflexes and spontaneous discharges during training.

A: Averaged traces (mean of 5 sweeps) of DRDRPs were recorded from DRlL2 in response to electrical pulses applied to DRrL1 (rectangular pulses; duration = 0.1 ms; intensity = 80 µA, 4 x Th; frequency = 0.02 Hz) in control (grey trace) and after a 90-minute BIKE session (black trace). **B:** The time course of DRDRP peaks recorded for up to five hours after BIKE (grey rectangle) highlights a significant increase in DRDRPs amplitude at two hours after training ($P = 0.023$, Friedman RM ANOVA on ranks with Dunn's test for comparisons versus control, $n = 5$). **C:** Spontaneous activity was recorded from DRlL2 in control (top trace) and at different time points during BIKE training. **D:** The time course points out that magnitude of the power spectrum for spontaneous antidromic DR discharges was significantly increased as soon as BIKE was switched on and stably persisted throughout training duration ($P \leq 0.001$, one way RM ANOVA, $n = 4$).

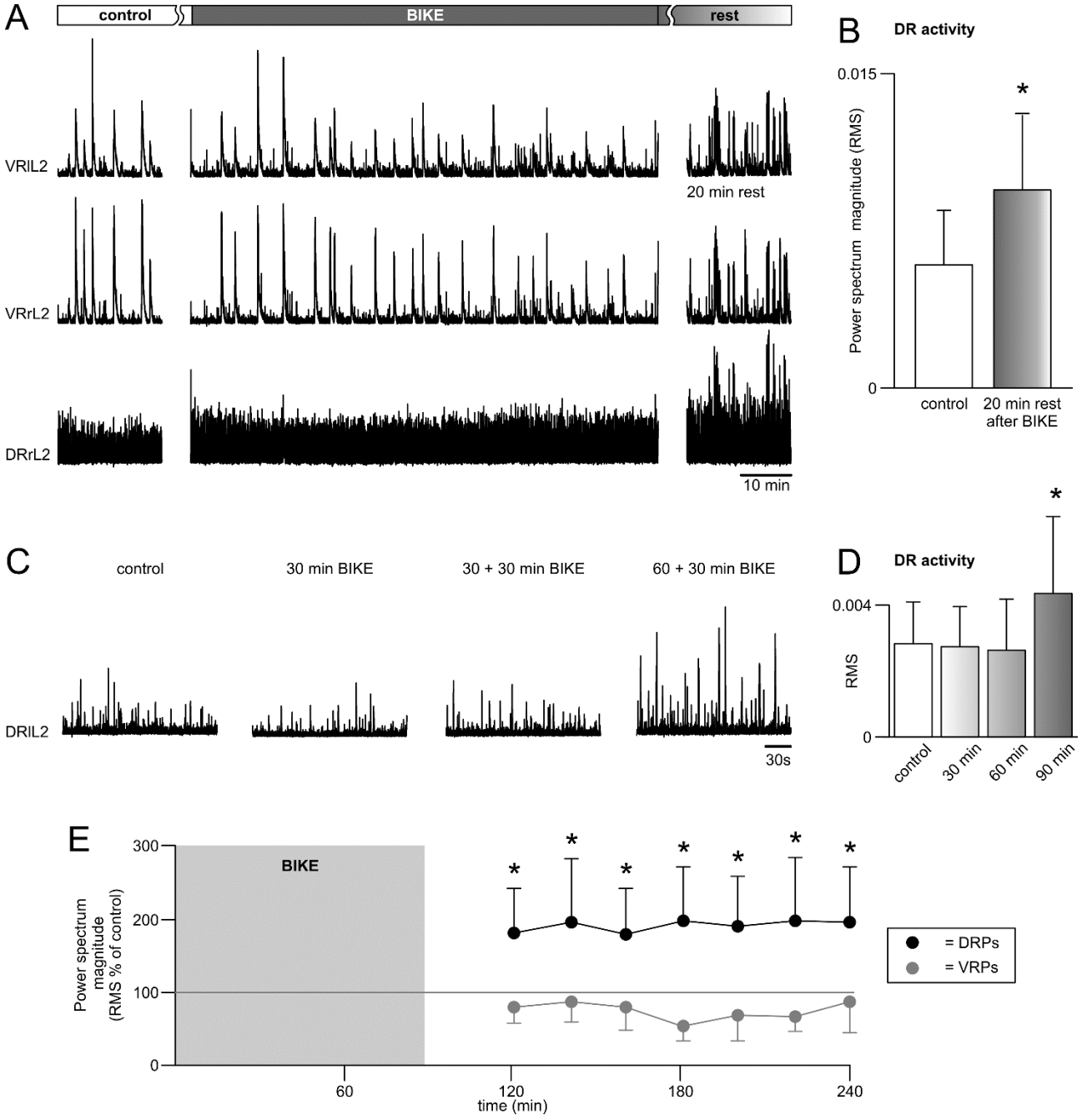


Figure 10. At least 90 minutes of BIKE are needed to increase spontaneous dorsal activity, which lasts throughout the following resting period.

A: As reported in the top bar, spontaneous activity was recorded from homosegmental L2 VRs and from DRrL2 in control (left traces), during a 90-minute BIKE session (middle traces) and for the first 20 minutes of rest (right traces). Note that, during BIKE, VR activity did not change (middle, top traces; see also Figure 4), while DR rhythm was increased (middle, bottom trace; see also Figure 9 C-D). Dorsal activity remained higher than control also at the end of training (right, bottom trace). **B:** This observation was replicated in different experiments. Histograms summarize average data of DR spontaneous discharges recorded after 20 minutes rest (P = 0.005, paired t-test, n = 8).

C: Three subsequent sessions of BIKE (30 minutes each) were cumulatively applied, meanwhile spontaneous dorsal activity was recorded from DRlL2 at the end of each session. **D:** Bars show that only three consecutive 30-minute sessions of BIKE (for a total training period of 90 minutes) significantly increased DR rhythm magnitude (expressed as root mean square; RMS; $P = 0.026$, one way RM ANOVA, $n = 4$), while a lower training duration was ineffective. **E:** The time course points out that after a 90-minute BIKE training (grey rectangle), DR spontaneous activity (black dots) remained higher than control for at least two hours of rest ($P \leq 0.001$, one way RM ANOVA, $n = 5$); on the other hand, VR spontaneous activity (grey dots) was unaffected by training ($P = 0.077$, one way RM ANOVA, $n = 5$). Spontaneous activity was assessed by 20-min bins, starting 10 minutes after the end of training.

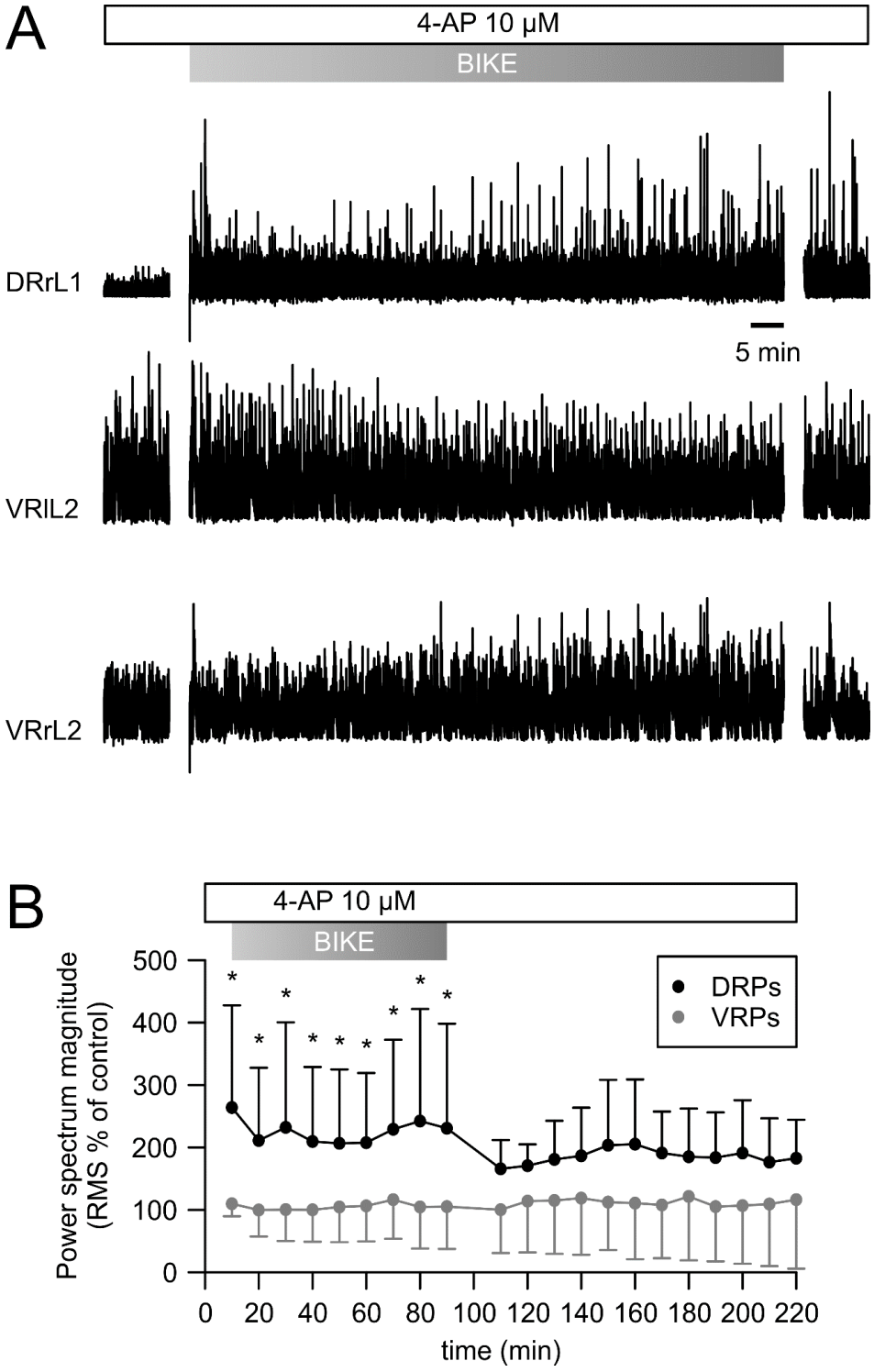
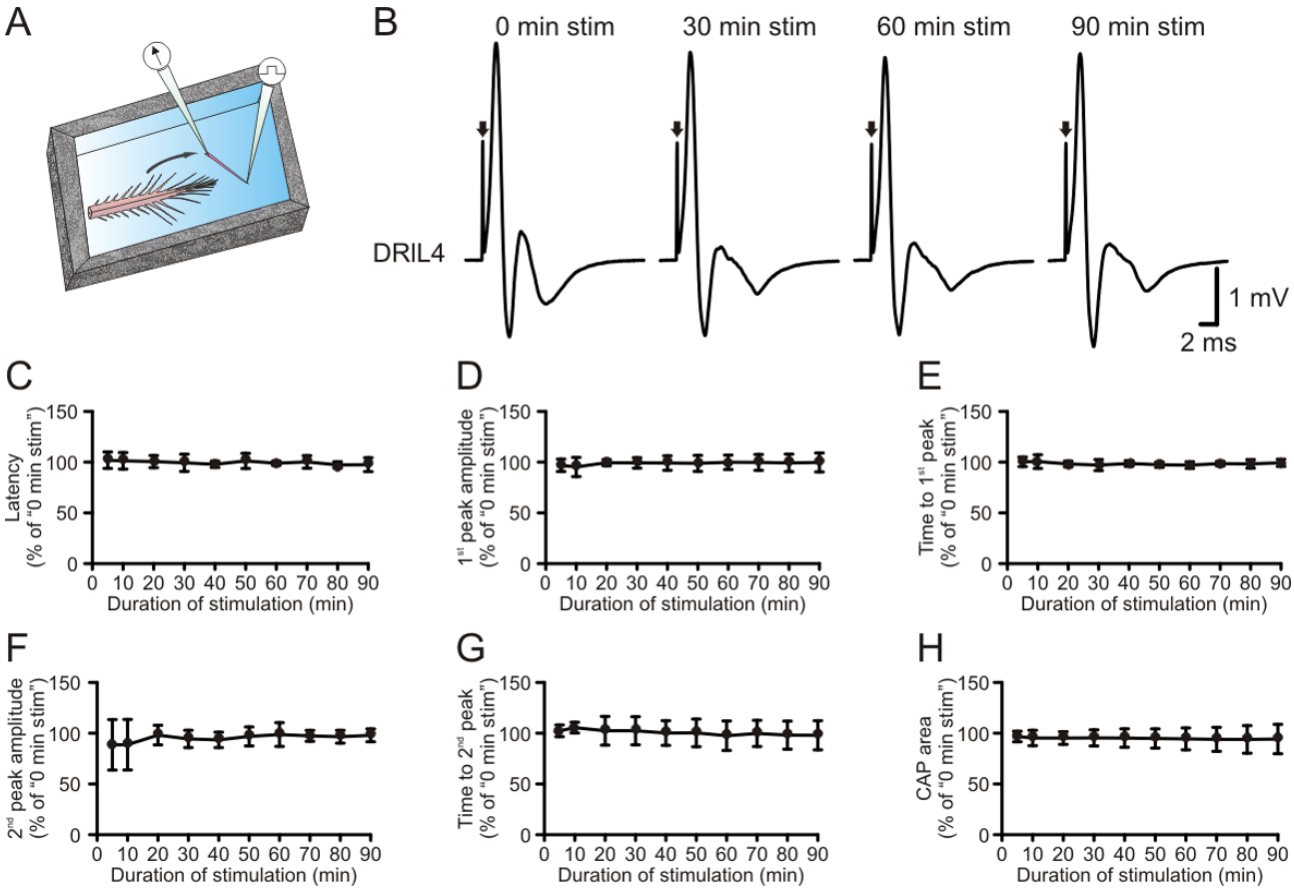


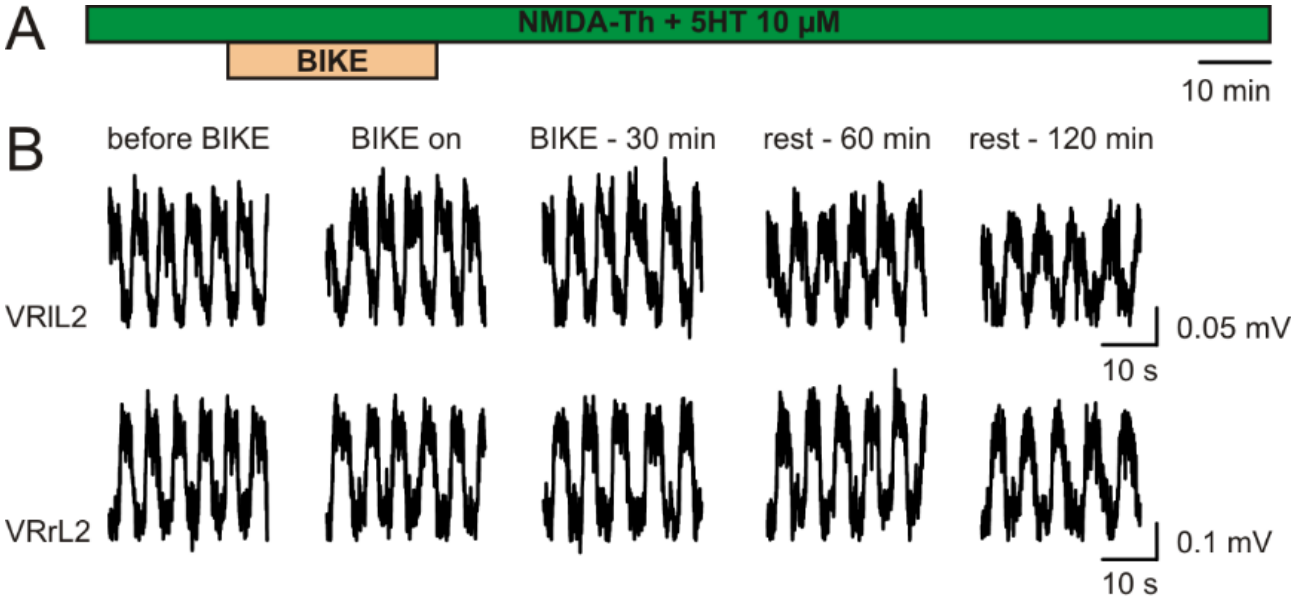
Figure 11. 4-AP-induced rhythmic discharges from DRs are further enhanced during BIKE. **A:** In the continuous presence of 4-AP (10 µM; white rectangle on the top), spinal activity was derived from DRrL1 and from homosegmental L2 VRs in control (left traces), during BIKE (90-minute session; grey bar on top, middle traces) and after a 2-hour rest (right traces). BIKE further increased DR activity (middle, top trace), while VRs activity remained similar to control (middle, bottom traces). **B:** Time course reports the mean value of rhythmic activity magnitude (expressed as

percent of control) in 4-AP (white bar), as recorded from DRs (black dots) and VRs (grey dots) during BIKE-training (grey bar) and during the following resting period. DR activity was significantly higher than control during BIKE functioning ($P = 0.003$, one way RM ANOVA, $n = 3$), but the effect was lost as soon as the device was switched off ($P = 0.577$, one way RM ANOVA, $n = 4$). On the contrary, VR activity remained unchanged during training ($P = 0.718$, Friedman RM ANOVA on ranks, $n = 4$) and after BIKE termination ($P = 0.587$, Friedman RM ANOVA on ranks, $n = 4$).

SUPPLEMENTARY RESULTS



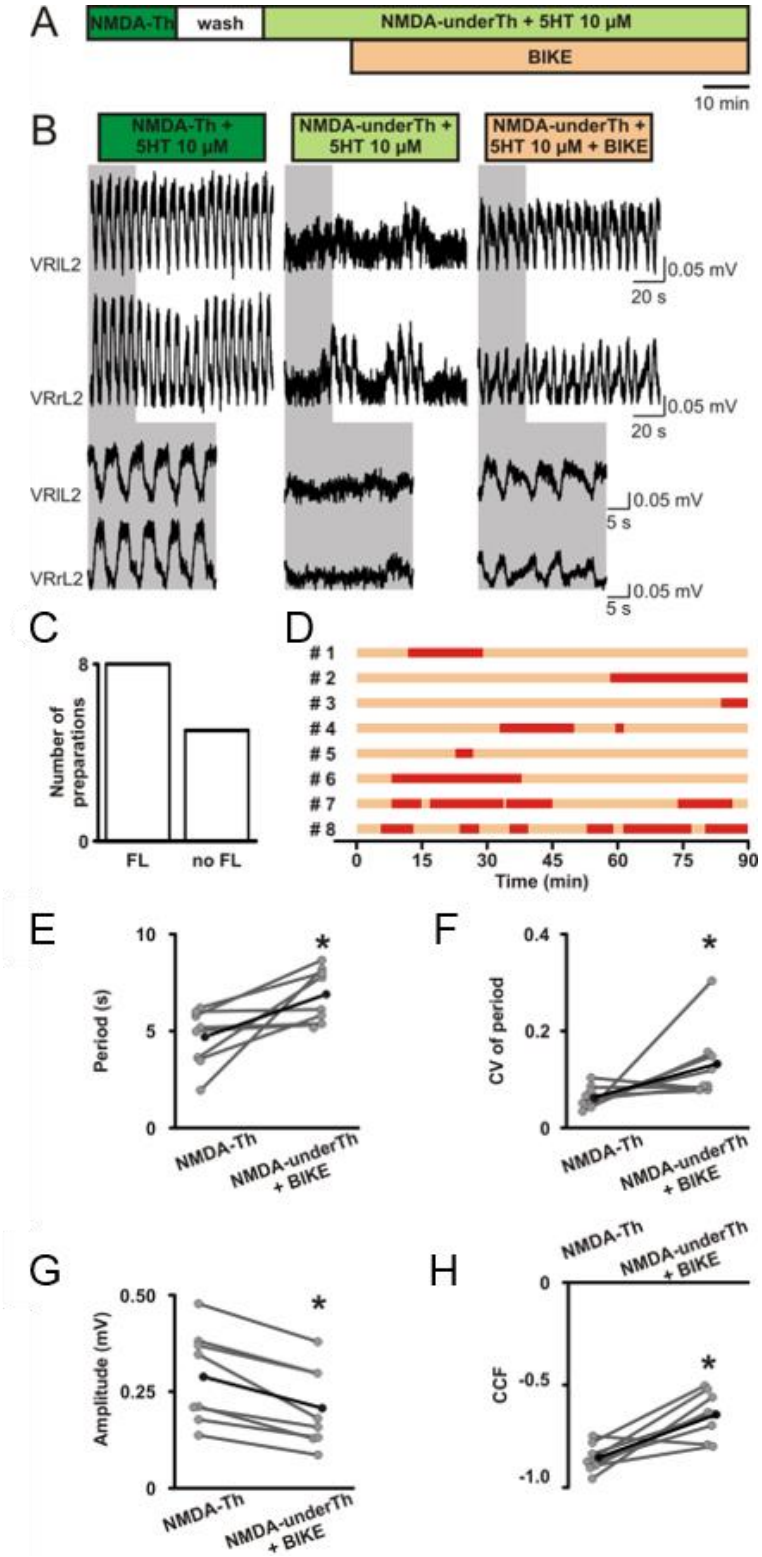
Supplementary figure 1: A prolonged stimulation of primary afferents at high intensity does not affect nerve conduction.
A: A lumbar DR, usually L4 or L5, is cut from the isolated spinal cord and used for electrostimulation and AC-coupled recordings. The distal DR stump is stimulated with a train of stereotyped pulses at high intensity (3xTh, threshold). Moreover, electrical stimuli are delivered at a frequency of 0.5 Hz (BIKE pedaling frequency) for a total duration of 90 minutes (corresponding to a long BIKE session). Electrically-evoked compound action potentials (CAPs) are recorded from the proximal DR stump. **B**: Exemplificative AC-coupled traces recorded from DRlL4 and showing electrically-elicited CAPs at four different time points during stimulation. Stimulus artefacts are indicated by black arrows. **C - H**: Latency, 1st and 2nd peak amplitudes, times to 1st and 2nd peaks and CAP area are calculated throughout the entire stimulation period and do not vary.



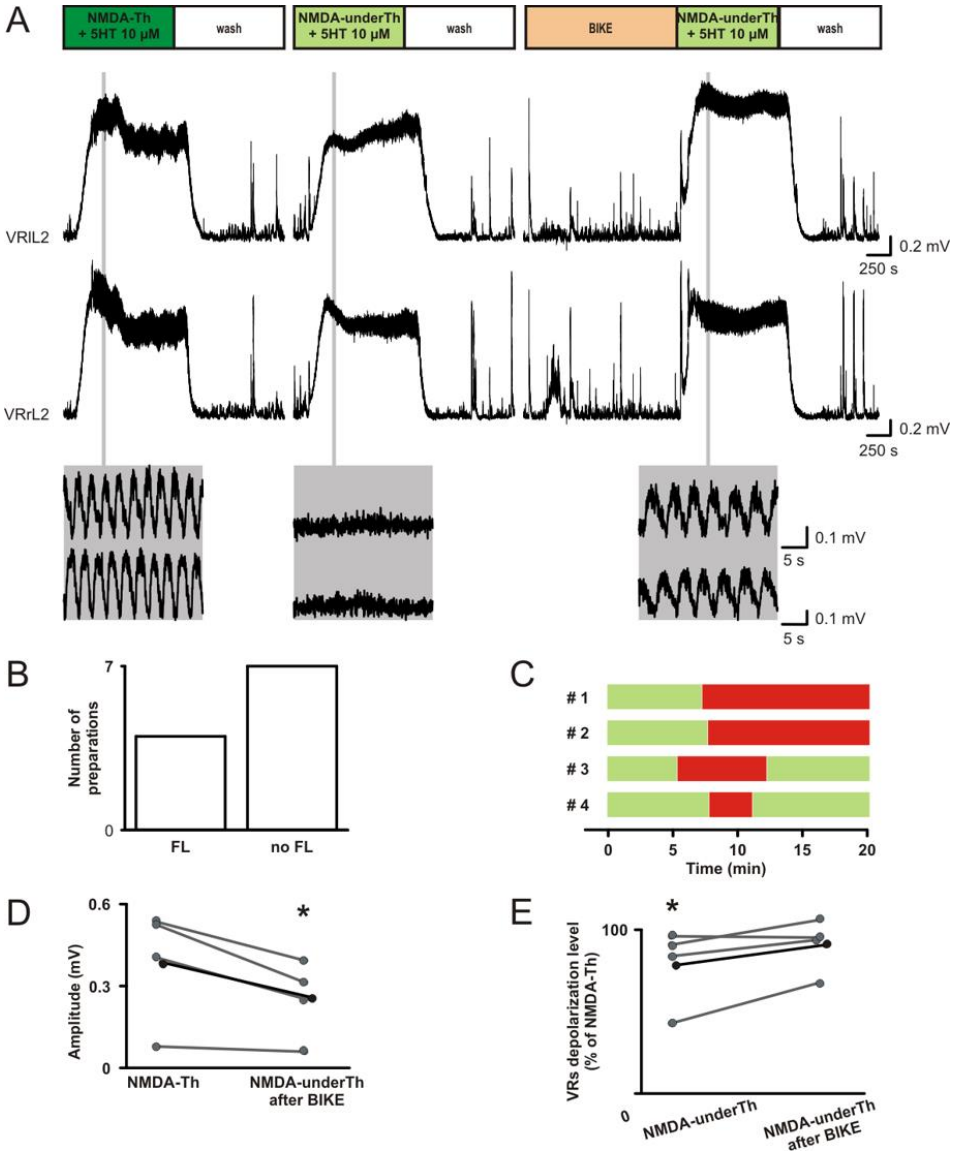
C

	before BIKE (mean ± SD)	BIKE on (mean ± SD)	BIKE - 30 min (mean ± SD)	rest - 60 min (mean ± SD)	rest - 120 min (mean ± SD)	*p* (statistical test)	N
Amplitude (mV)	0.22 ± 0.11	0.22 ± 0.11	0.23 ± 0.11	0.20 ± 0.12	0.18 ± 0.12	0.981 (One way ANOVA on ranks)	14, 5, 4
CCF	-0.82 ± 0.11	-0.79 ± 0.11	-0.82 ± 0.09	-0.84 ± 0.07	-0.77 ± 0.14	0.986 (One way ANOVA on ranks)	14, 5, 4
Period (s)	4.85 ± 0.91	5.05 ± 0.98	5.08 ± 0.82	4.66 ± 0.73	4.96 ± 1.02	0.992 (One way ANOVA on ranks)	14, 5, 4
CV period	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.476 (One way ANOVA on ranks)	14, 5, 4

Supplementary figure 2: The co-application of BIKE-training does not vary features of a chemically-induced FL. **A**: The two bars summarize the experimental protocol. The preparation is initially superfused with a solution of NMDA-Th (threshold) and 5HT at 10 µM (dark green bar). When a stable FL is obtained, BIKE is switched on for 30 minutes (orange bar) during the continuous application of neurochemicals. At the end of training, the preparation continues being superfused with the same solution of NMDA and 5HT up to two hours of rest. Note that determining NMDA concentration is a critical point in the experimental protocol. Indeed, minimal variations, equal to 0.5 µM, make the difference between inducing FL or simply uncorrelated electrotonic activity. So, for each preparation the threshold concentration of NMDA is identified by cumulative increases of 0.5 µM, starting from the initial value of 0.5 µM, until reaching the lowest concentration able to generate a stable FL. On the other hand, 5HT concentration is kept fixed at 10 µM. **B**: Sample DC-coupled traces recorded from VRlL2 and VRrL2 at different time points during the experimental protocol. **C**: The main features of the FL are not changed by training, as highlighted in the table.



Supplementary figure 3: BIKE facilitates the onset of FL when co-applied with neurochemicals at concentrations that do not induce locomotor-like activity *per se*.
A: Experimental protocol consisting in the induction of a stable FL with NMDA-Th and 5HT 10 µM (dark green bar), followed by an extensive wash out (white bar) and a subsequent bath-application of NMDA-underTh and 5HT 10 µM (light green bar), unable in itself to evoke FL. To the latter treatment is juxtaposed BIKE-training (orange bar). Note that NMDA-Th is determined as described in **Supplementary figure 2**. The under-threshold concentration of NMDA is obtained reducing NMDA-Th concentration of 0.5 µM and verifying that it does not evoke FL. So, NMDA-underTh concentration is the highest concentration of NMDA unable to induce FL. 5HT is maintained unvaried at 10 µM. **B**: DC-coupled traces derived from VRlL2 and VRrL2 during an ongoing locomotor rhythm (left panels) and during the superfusion with NMDA-underTh and 5HT 10 µM before BIKE (middle panels), when only uncorrelated activity is recorded, and during the co-application of BIKE (right panels), that is able to trigger FL. Grey rectangles in the bottom panel show traces at higher magnification. **C**: The histograms point out that the facilitatory effect of BIKE is observed in 8 preparations out of 13. **D**: For each of the 8 preparations in which FL is triggered, it is reported the training period (orange bars) and time-spans when FL appears during training (red bars), stressing that FL is not stably triggered. **E - H**: in plots are compared FL properties induced by NMDA-Th and 5HT 10 µM and FL features evoked by BIKE in combination with NMDA-underTh and 5HT 10 µM. Grey dots represent raw data, while black dots indicate mean values. Period (**E**) and coefficient of variation of period (**F**) are significantly increased, resulting in a higher variability of FL cycles. Amplitude (**G**) is decreased. Phase coupling among homosegmental VRs L2, quantified through the cross-correlation function (CCF; **H**), is altered indicating a lower degree of alternation between FL cycles.



Supplementary figure 4: Under-threshold neurochemicals evoke locomotor-like activity when applied right after the end of a short BIKE session.

A: The bars in the upper panel describe the experimental protocol: a stable FL is evoked (dark green bar); after wash out (white bar), a solution of NMDA-underTh and 5HT 10 µM is applied (light green bar); after a second wash out (second white bar), BIKE is switched on for 30 minutes (orange bar); at the end of training, the under-threshold solution is applied again (second light green bar). DC-coupled traces underneath the protocol bars report changes in the depolarization level of homosegmental VRs L2. Grey rectangles in the bottom panel show few locomotor cycles evoked by NMDA-Th and 5HT 10 µM (on the left), electrotonic activity due to NMDA-underTh and 5HT 10 µM (in the middle) and the re-establishment of FL induced by neurochemicals at under-threshold concentrations when applied after a 30-minute session of passive training (on the right). **B**: The facilitatory effect of BIKE is observed in 4 preparations out of 11. **C**: For each of the 4 preparations where FL is observed, coloured bars report the 20-minute application period of NMDA-underTh and 5HT 10 µM after training (light green bars) and time-spans where FL was induced (red bars), confirming variability in BIKE outcome. **D**: The dot plot indicates that the amplitude of FL cycles induced by NMDA-underTh and 5HT 10 µM after training is significantly reduced with respect to threshold concentrations. **E**: When applied before training, NMDA-underTh and 5HT 10 µM induce a clearly-distinguishable depolarization of VRs (as also depicted by traces in **A**), although significantly lower than depolarization observed at threshold. When NMDA-underTh and 5HT 10 µM are applied after training, VRs depolarization level becomes similar to the one at threshold.

DISCUSSION

In the present PhD project, a particular attention has been given to the complex interplay between afferent inputs and spinal networks. Sensory pathways have been activated by different mechanisms having various advantages. Recalling the "Aims" bulleted list, they could be summarized as follows:

i. The repetitive electrical stimulation of lumbar dorsal roots (DRs) in a completely isolated spinal cord allowed for a direct modulation of spinal circuitries, avoiding any filtering mechanisms mediated by peripheral sensors (Proske and Gandevia, 2012; Delmas and Coste,2013) and DRG neurons (Gemes et al., 2013; Holinski et al., 2013). Thus, a more reliable control of stimuli reaching the spinal cord was feasible.

ii. Although mechanisms of DR hyperexcitability can be studied *in vitro*, the establishment of a painful condition, such as tactile hypersensitivity (or mechanical allodynia), must necessarily be assessed *in vivo*. We recently developed a modified version of the SNI (mSNI). In our rat model, afferent and efferent fibres of the common peroneal nerve (mixed nerve) were crushed using a calibrated nerve clamp. This surgical procedure induced central sensitization, presumably driven by ectopic activity in injured primary afferents (Amir et al., 2005). Interestingly, the prevalence of tactile hypersensitivity in this new model was halved with respect to the classical one (SNI). Indeed, some animals developed mechanical allodynia, whereas others did not, although subjected to common peroneal nerve crush. This replicates clinical observations where only a fraction of patients suffering a certain nervous system lesion develops chronic pain, namely those who are prone to the onset of central sensitization. Thus, the model of mSNI is a useful tool in the search of mechanisms of pain chronification, distinguishing between pain-related and non-pain-related states.

iii. In the *in vitro* neonatal rat preparation of isolated spinal cord with hindlimbs attached, a passively-driven movement of hindlimbs allowed for the concurrent activation of peripheral sensors. Moreover, sensory pathways conveying inputs to the spinal cord were fully intact and functioning in this model. Thus, the spinal cord was exposed to a variety of incoming information, presumably showing a great variability when considering the peripheral source and the activation timing of different primary afferent classes during pedaling. This activation pattern allowed mimicking *in vitro* a more physiological condition with respect to the ones previously described. Indeed, in this new *in vitro* model, peripheral structures retain

their task of an early signal processing and the activation of primary afferents is not mediated by pathological mechanisms.

Thereafter, spinal integration and processing of incoming information was studied and dorsal and ventral network modifications were described. The overall findings reported here may provide an early nurture to exploit useful sensory information for gait rehabilitation and pain management. These results will be discussed in the following paragraphs, with particular emphasis on the translational potential into clinical practice.

1 *In vitro* dorsal root fibre hyperexcitability induced by repetitive electrical stimulation

In the first part of the present PhD project, electrostimulation protocols were repetitively delivered to a DR. The repetitive stimulation of a DR with electrical pulses has been previously demonstrated to be effective in activating the spinal CPG for locomotion (Marchetti et al., 2001 a). Here, I adopted canonical 2 Hz-trains of stimuli, while monitoring DR and VR activity. The main finding was that electrical protocols capable to trigger CPG activity in the ventral horn, influenced DR activity, as well. Changes in primary afferents activation might be ascribable to an increase in the extracellular concentratin of potassium mediated by the repetitive stimulation (Marchetti et al., 2001 b). They might also be related to a network contribution within the dorsal horn through mechanisms of PSI-PAD, a possibility that should be better investigated. Moreover, the enhancement in DRs spontaneous activity appeared to be persistent in our *in vitro* condition at the end of electrostimulation. Since the reuptake of extracellular potassium is relatively fast in the spinal cord after stimulation of primary afferents (Cordingley and Somjen, 1978), it is likely that DRs persistent activation might be supported by dorsal network mechanisms.

We also hypothesized a possible interplay between sensory and motor circuits within the spinal cord. However, it is hard to establish whether the sensorimotor interplay might be driven by DRs hyperactivation or by CPG operation, since both eventualities are equally plausible. Indeed, electrostimulation might enhance DRs activity through an increase in extracellular potassium concentration, which in turn triggers CPG activation. On the other side, trains of electrical pulses activate spinal locomotor circuits (Marchetti et al., 2001 a), whose operation has been demonstrated to increse the extracellular potassium concentration as well (Marchetti et al., 2001 b), thus contributing to DR hyperexcitability.

It is well-established that antidromic DRPs are phasically modulated both in cat (Dubuc et al., 1988; Gossard et al., 1991; LaBella et al., 1992; Beloozerova and Rossignol 1999; 2004; Ménard et al.,

1999; 2002) and rat (Kremer and Lev-Tov, 1998; Vinay and Clarac, 1999; Fellippa-Marques et al., 2000; Hayes et al., 2012) preparations during FL. However, there is currently missing evidence about persistent modifications in DR activity at the end of CPG operation in the above-listed experimental models. To the best of my knowledge, our report first addressed this issue, considering a possible interplay between CPG and spontaneous dorsal activity and assessing changes in antidromic discharges at the end of a sustained CPG activation.

Unlike other groups, in our *in vitro* preparation, we elicited a locomotor pattern via electrical stimulation rather than bath-applying neurochemicals. Although an electrically-evoked FL has an episodic nature, this choice was adopted to avoid non-specific alterations in spinal activity due to the prolonged contact with neuroactive substances in the perfusion solution. Moreover, this approach appears to have a reasonable translational prospective, since SCS is currently used in everyday clinical practice, whereas most molecules experimentally adopted to induce a stable FL are toxic and life-threatening for humans.

In the present study, DR hyperexcitability was assessed in terms of spontaneous antidromic activity. We can speculate on the meaning of such an activation of DRs at the end of the electrostimulation protocol. DRPs are thought to contribute to sensory processing by filtering and gating incoming information (Rudomin and Schmidt, 1999). Thus, it could be hypothesized that antidromic activity might represent a physical impediment for peripheral stimuli travelling toward the spinal cord (Oakley and Prager, 2002; Buonocore et al., 2008). The physical barrage mediated by electrostimulation might avoid the establishment of central sensitization during the very first months after a peripheral lesion or disease of the somatosensory nervous system. Different considerations should be envisioned when thinking about central lesions, where spinal disinhibition might be prevented by electrostimulation. Indeed, the prevalence of pain is particularly high at the subacute stage after SCI, with almost 80% of individuals reporting at least moderate neuropathic pain at 1 – 6 months postinjury (Finnerup et al., 2014). Therefore, an early electrostimulation program might contribute to an adaptive reorganization of spinal circuitries avoiding chronification. This has been confirmed in *in vivo* models of neuropathic pain where SCS has been proven to reduce the release of EAAs and increase GABA levels within the dorsal horn, showing an overall suppression of tactile allodynia (Cui et al., 1997). On the contrary, non-responder animals have been reported to retain unchanged levels of EAAs and GABA with respect to the pre-SCS condition (Stiller et al., 1996; Cui et al., 1997). The plastic adaptations mediated by electrostimulation appear to be supported mainly by GABAergic mechanisms, since the intrathecal administration of gabapentin and pregabalin ($GABA_A$ agonists) at low doses potentiates the analgesic effect of SCS

in responder animals and evokes analgesia in the non-responder ones (Cui et al., 1997; Wallin et al., 2002). However, it remains speculative whether electrostimulation protocols used in the present *in vitro* investigation may positively impact on pain management and further experimentation on preclinical animal models is required. Indeed, an excessive DR hyperexcitability might have the opposite effect, fostering mechanisms of central sensitization and neuropathic pain (Cervero and Laird, 1996; Willis, 1999). Unfortunately, little is known about the transition from adaptive to maladaptive plastic changes within the dorsal horn and more investigation is needed.

Futhermore, few considerations on the stimulation parameters adopted in the current study are necessary. First, the stimulation intensity we selected to trigger CPG activation is high, in line with previous *in vitro* studies (Lev-Tov et al., 2000; Marchetti et al., 2001 a; Gabbay et al., 2002;) and with epidural SCS intensities aimed at evoking a motor output (Dimitrijevic et al., 1998; Minassian et al., 2004; 2007). On the contrary, SCS intensities for pain relief are usually low, reaching maximum 90% of the motor threshold (Meyerson and Linderoth, 2006; Sato et al., 2013; Gong et al., 2016). As for stimulation frequency, we adopted a 2 Hz-train of pulses, whereas frequencies commonly used in the management of neuropathic pain are much higher (Deer et al., 2013; Kim et al., 2015; Gong et al., 2016; McRoberts, 2016). Thus, it would be interesting to test *in vivo* the potential role that a CPG-activating electrical protocol might have on chronic pain syndromes. Moreover, innovative protocols of DR stimulation could be good candidates to be studied in animal models of neuropathy, such as FL*i*stim (Taccola, 2011) and a two-frequency protocol (Dose and Taccola, 2016). Noteworthy, both paradigms are characterized by low intensities and high frequencies of stimulation, making them similar to SCS protocols currently used for pain relief. An advantage of these novel protocols with respect to canonical clinical electrostimulation is their capacity to optimally trigger the CPG for locomotion, thus boosting a favorable sensorimotor interplay which might counteract detrimental perception outcomes.

2 Training mimetic protocols may reproduce beneficial effects mediated by physical therapy when exercise cannot be administered

As described in the above paragraph, electrostimulation protocols consisting in 2 Hz-trains of stimuli were repetitively applied to a DR aiming at a sustained activation of the locomotor CPG. In detail, a total number of 15 trains of electrical pulses were delivered, each train lasting one minute and applied every three minutes to the same DR. Thus, the overall duration of the electrostimulation

protocol was 45 minutes, in line with the typical duration of a neurorehabilitative training session employed in the vast majority of studies (Schück et al., 2012; Geigle et al., 2013; Mirbagheri et al., 2013; Duffell et al., 2015; Lam et al., 2015). During the electrostimulation program, DR trains were intentionally delivered with a 2-minute pause from the end of the previous train. This time-window represents a good compromise between the need of a continuous and prolonged activation of spinal locomotor networks and the prevention from deterioration of the locomotor output caused by a repetitive stimulation (Marchetti et al., 2001 a; b; Taccola et al., 2012; Diaz-Ríos et al., 2017). As a result, the locomotor program is persistently triggered by electrostimulation, similar to the activation mediated by training interventions. For this reason, we suggest that our electrostimulation program might be defined as a training mimetic protocol, since it mimics the effect of a passively-driven training. Moreover, we hypothesize that a repetitive electrical stimulation might replace physical therapy in mediating functional recovery and pain relief in bedridden subjects. Indeed, during the acute phase of sensorimotor disorders or related pathophysiological complications, hospitalized subjects cannot undergo intense physical training. In these conditions, a rehabilitative approach which does not foresee mobilization, although retaining most of the advantages of training interventions, might be a more advisable choice. Furthermore, the possibility to bring the rehabilitation treatment to the bedside allows to anticipate interventions aimed at locomotor recovery and hypersensitivity prevention. As a result, protective neuroplasticity mechanisms might be promoted during these very early stages, competing and overcoming maladaptive changes within spinal networks.

3 The flip side of dorsal hyperexcitability: a novel *in vivo* model of neuropathic pain

DR hyperexcitability is often related to the establishment of a painful condition. However, as argued in previous paragraphs, this might not always be the case. Dorsal circuits associated to perception and nociception are very complex. Indeed, many distinct dorsal horn interneuronal populations are thought to have a role in signal processing, but their molecular and physiological characterization is still elusive. Given this great complexity, it is difficult, if not impossible, to associate an *in vitro* DR hyperexcitability state to pain. Moreover, since pain has been defined as an unpleasant sensory or emotional experience associated with actual or potential tissue damage (World Health Organisation, WHO), processing mediated by higher brain centers is implied, as well. For all these reasons, in the second part of this project, I switched from a simplified *in vitro*

model for the study of DR hyperexcitability to a more complex *in vivo* model of neuropathy, where the animal provides an immediate pain feedback when appropriately stimulated.

Our animal model consisted in a mSNI, where only the common peroneal nerve was crushed. Interestingly, around 50% of animals undergone this surgical procedure developed tactile hypersensitivity. Such a partially effective model in the induction of neuropathic pain might be a powerful tool for the simultaneous research of mechanisms of pain nourishment and prevention. On one side, it appears to be particularly suitable for the study of pain onset, maintenance and chronification. On the other side, it might allow for the investigation of protective mechanisms actuated in the group of animals that does not show tactile hypersensitivity.

Tactile hypersensitivity in our model was assessed by quantifying a reduction in paw withdrawal threshold after stimulation of the territory innervated by the sural nerve. Since the sural nerve was spared in our mSNI model, tactile hypersensitivity in the sural territory represented an indirect demonstration of central sensitization. This was further supported by the finding that, at the end of behavioral studies, when animals were sacrificed, a higher glial reactivity was reported in the group of animals showing tactile hypersensitivity with respect to both treated animals without any hypersensitivity outcome and sham-operated animals. Thus, this model allowed for a direct correlation between a behavioral observation, such as tactile hypersensitivity, and a pathophysiological condition, like central sensitization.

In these experimental conditions, it would have been interesting to assess a possible DR hyperexcitability by the mean of *in vivo* electrophysiological recordings. Indeed, antidromic activity is not only a peculiarity of neonatal preparations (Vinay et al., 1999), but it can be recorded also in adult animals (Rudomin, 1990; Rudomin et al., 1993; Gossard, 1996; Nusbaum et al., 1997; Rossignol et al., 1998; Gossard et al., 1999), as well as in humans (Stein, 1995; Katz, 1999; Aymard et al., 2000). In our mSNI model it might be presumably possible to quantify at which extent DR hyperexcitability is useful or even protective against the development of neuropathic pain and when it exceeds the therapeutic effect becoming harmful. It can be hypothesized that the mSNI animals with and without tactile hypersensitivity can be employed to define a clear borderline in the amount of antidromic activity with a filtering or gating role against ectopic activation arising from the injured nerve and having a major role in driving central sensitization and chronification (von Hehn et al., 2012). Such an indication obtained in *in vivo* studies might be useful in *in vitro* investigations, currently limited to the assessment of dorsal hyperexcitability without any possibility to link it to pain or non-pain states.

Our mSNI model revealed a role of the regulator of G protein signaling 4 (RGS4) in the development of tactile hypersensitivity, since it was overexpressed in the dorsal horn and DRGs ipsilaterally to the injury. RGS4 is a small member of the RGS family, made up of more than 30 highly different and multifunctional signaling proteins which directly bind to the Gα subunit (Hollinger and Hepler 2002). The RGS/Gα interaction leads to the Gα subunit deactivation and to the termination of downstream pathways (Hollinger and Hepler 2002). In particular, RGS4 upregulation is associated to an attenuation of opioid receptors (Garnier et al., 2003) and cannabinoid type-1 receptors (CB1; Bosier et al., 2015) signaling, thus mediating insensitivity to analgesics in animal models of neuropathic pain. Besides its role in pain, RGS4 has also been linked to motor behavior modulation. Indeed, in zebrafish it has been shown that a knockdown model for RGS4 results in an aberrant motor pattern (Cheng et al., 2013). Moreover, RGS4 has been demonstrated to regulate motor control through the modulation of dopamine-mediated endocannabinoid pathways in mouse (Lerner and Kreitzer, 2012). A post-transcriptional inhibition of RGS4 has also been found to positively correlate with a decrease in abnormal involuntary movements in rat models of Parkinson's disease (Ko et al., 2014). However, the role of RGS4 in spinal locomotor networks remains poorly understood. It can be hypothesized that its persistent overexpression in the spinal cord might induce motor network depression through modulatory mechanisms involving CB1 receptors (Veeraraghavan and Nistri, 2015). This suggests that RGS4 upregulation might potentially interfere with the effectiveness of motor rehabilitation. Therefore, the search for mechanisms which downregulate its expression or selectively inhibit its biological function might presumably be beneficial in simultaneously inducing pain relief and promoting an enhanced motor recovery.

Besides RGS4, it would be interesting to investigate a possible differential expression of other molecular markers in our mSNI model, such as those responsible for PSI-PAD. For instance, the assessment of NKCC1, $GABA_A$, GAD_{65} (Eaton et al., 1998) on presynaptic terminals and DRG neurons would be an important indication allowing for a link between DR hyperexcitability and tactile hypersensitivity.

4 A new *in vitro* model of passive cycling

Physical training represents the principal intervention of current neurorehabilitation. It has been shown to promote a certain regain of locomotor function and it has also been suggested as a non-pharmacological method for the management of neuropathic pain. It is proposed that activity-based interventions mediate their therapeutic effects by providing an afferent sensory feedback that

induces spinal circuit plasticity. However, intrinsic mechanisms underlying clinical improvements are currently obscure. In the third part of this PhD project, I investigated on network, synaptic and cellular modifications driven by passive training within the spinal cord.

To the purpose, I adopted an *in vitro* neonatal rat preparation of hindlimb-spinal cord. In this preparation, spinal roots from the third lumbar segment (L3) to *cauda equina* were intact and functionally connected to their physiological targets in the periphery. In contrast, L2 spinal roots were cut in this model, as well as roots above the L2 spinal segment. In rat, the hindlimb is innervated by several mixed (sensorymotor) nerves, conveying both afferent and efferent information. Primary afferent fibres from hindlimb nerves project to the lumbar spinal cord, mainly from L2 to L6 spinal levels (Swett and Woolf, 1985; Molander and Grant, 1986). Major nerves innervating rat hindlimbs are the tibial, common peroneal, sural and saphenous nerves. Their somatotopic projections to the spinal cord are shown in figure 17 (Molander and Grant, 1986). In our preparation, afferent information from common peroneal (Pe) and sural (Su) nerves was fully conveyed to the spinal cord, since related DRs were intact. On the contrary, information carried from L2 DRs was lost, thus a small part of the sensory feedback traveling through tibial (Ti) and saphenous (Sa) afferent fibres did not reach spinal circuits. In our *in vitro* model, L2 spinal roots were used to deliver single or repetitive electrical pulses, to monitor spinal networks activity and to assess changes in locomotor CPG operation, which has been demonstrated to be located at L2-6 spinal segments in the neonatal rat (Kjaerulff and Kiehn, 1996). Thus, in such a preparation L2 DRs did not contribute in transmitting sensory information from the innervated peripheral tissues of the hindlimb. However, the vast majority of hindlimb afferent input entered the spinal cord and could directly mediate modulatory effects on both ventral and dorsal circuitries.

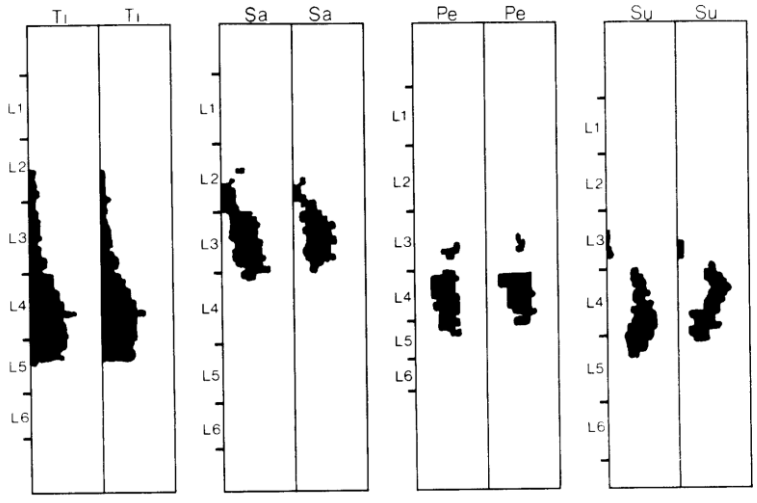


Figure 17: Spinal projections of four major hindlimb nerves in the rat. Horseradish peroxidase (HRP) has been applied to distal cut ends of peripheral nerves on both hindlimbs. The tracer has been retrogradelly transported to the spinal cord and maps of the resulting projections have been obtained. Four hindlimb nerves have been investigated:

tibial nerve (Ti), saphenous nerve (Sa), common peroneal nerve (Pe) and sural nerve (Su). Each pair of columns represents the two sides of the spinal cord from four animals. On the sagittal plane, medial corresponds to the left border of each column, while lateral to the right border. (From Molander and Grant, 1986).

In the leg-attached preparation, primary afferents were activated by passively moving the hindlimbs. A continuous and repetitive cycling was driven by a low-noise robotic devise integrated to the recording chamber, called BIKE (Bipedal Induced Kinetic Exercise). Before starting the experiment, the preparation was placed inside the recording chamber and well-stabilized for long-term recordings, with the hindpaws fixed to BIKE pedals. Presumably, every time BIKE is switched on, a variety of somatosensory stimuli start being perceived, such as muscle stretch and tension, joint displacement, skin indentation and other light touch sensations. Unless other similar preparations (Hayes et al., 2009; 2012; Hochman et al., 2013), in our model the movement of hindlimbs was mediated by BIKE, instead of pharmacologically evoked. This more physiological condition allows to reproduce *in vitro* the clinical circumstance where a subject with a SCI has his legs automatically moved by an assistive device, with the advantage that we can monitor real-time spinal network activity. However, our preparation lies on the back, with the hindlimbs placed in a gravity-neutral position above the recording chamber. This arrangement might cause a partial loss of load information, conversely investigated in the *in vitro* model of Hayes and colleagues, where each hindpaw supports the preparation weight and is placed on two 2D force platforms for the measurement of limb endpoint forces (Hayes et al., 2009; 2012; Hochman et al., 2013). Although in our model the paw sole does not fully bear the body weight, it might be assumed that a continuous flexion/extension of the ankle joint during pedaling mediates a rhythmic pressure on the paw soles. Load receptors have been suggested to play a major role in the generation of a locomotor EMG pattern in humans (Harkema et al., 1997; Dietz et al., 2002). Thus, it would be interesting to directly assess load contribution to the motor output by comparing muscle activation in Hayes' *in vitro* model and in our BIKE model.

5 BIKE passive training evokes afferent discharges from the periphery

I first wondered whether primary afferents were actually activated by BIKE during hindlimb passive cycling in our *in vitro* preparation. Presumably, as soon as hindlimbs start being moved, the spinal cord is supplied with information from cutaneous mechanoreceptors, from muscle, tendon, joint and ligament proprioceptors and from high-threshold nociceptors of the hindlimb (Lewis and

McMahon, 1991 a; b). To verify this hypothesis, I performed *en passant* recordings of incoming inputs from a DR immediately before its insertion site into the spinal cord.

First, I observed that in our *in vitro* model sensory feedback was provided at some extent to the spinal cord even before turning on BIKE, while the preparation was lying in the recording chamber. This finding should not be surprising, since hindlimbs are fixed to BIKE pedals with acrylic glue. Thus, skin mechanoreceptors are activated, as well as mechanical or chemical high-threshold nociceptors cannot be excluded to directly contribute to the generation of afferent signals at rest. However, it should be considered that large diameter Aβ fibres enter the spinal cord first, whereas small diameter non-myelinated C fibres develop mature synaptic contacts with spinal networks only at the second postnatal week (Fitzgerald and Gibson, 1984; Fitzgerald, 1985). Therefore, it is likely that in our P0-4 preparations most of the incoming signal originates from low-threshold fibres.

Furthermore, it should be considered that *en passant* recordings might be contaminated by antidromic discharges which back-propagate through primary afferents. Indeed, the neonatal rat spinal cord is characterized by a sustained spontaneous activation of dorsal circuits (Vinay et al., 1999), which is presumably detected when recording *en passant*. This phenomenon was assessed by cutting the recording DR at its insertion site into the dorsal horn and recording incoming signals from the dorsal stump. Although a certain degree of back-propagating antidromic activity cannot be excluded in the *en passant* configuration, incoming sensory activity was always enhanced when BIKE was switched on. Moreover, the frequency of primary afferent discharges elicited by passive cycling was shown to be paired with BIKE pedaling frequency (0.5 Hz). This might suggest a direct correlation between the mechanical stimulus (BIKE-induced pedaling) and the evoked firing activity in sensory fibres, most likely due to intrinsic properties of different peripheral elements involved in signal transduction.

However, the frequency of incoming inputs detected in our model was much lower than firing frequencies reported in previous studies. In adult cat, recordings from single muscle spindle afferents (Prochazka et al., 1976; 1977; 1979; Loeb and Duysens, 1979; Prochazka and Gorassini, 1998 a; b; Ellaway et al., 2015) and GTOs afferents (Prochazka et al., 1976; Appenteng and Prochazka, 1984; Prochazka and Gorassini, 1998 a; b) during locomotion report very high mean firing rates, in the range of 50 – 110 Hz, with peak rates of 150 – 200 Hz. Similar results have been obtained also in adult rat (Haftel et al., 2004; Vincent et al., 2015; Smilde et al., 2016; Carrasco et al., 2017) and mouse (Nakanishi and Whelan, 2012; Wilkinson et al., 2012; Carrasco et al., 2017). In humans, firing rates are usually reported to be lower than 20 Hz (al-Falahe et al., 1990;

Macefield, 2013; Fuglevand et al., 2015; Day et al., 2017), suggesting that species differences are involved in primary afferent discharge frequency (Prochazka and Gorassini, 1998 b; Carrasco et al., 2017).

Inter-species variations might be related to ion channels underlying somatosensory transduction or to voltage-gated channels responsible for the generation of ionic conductances that sustain firing in primary afferents. Recently, it has been demonstrated that three voltage-gated sodium channel isoforms, $NaV_{1.6}$, $NaV_{1.1}$ and $NaV_{1.7}$, show a similar distribution pattern in the spindle primary sensory ending of cat, rat and mouse (Carrasco et al., 2017). However, authors argue that differences in Ia afferent firing between the three species might be related to possible variations in the expression levels of NaV isoforms. Similarly, it might be hypothesized that in the neonate these ion channels might be differentially expressed with respect to the adult or might present a still immature subunit composition providing the channel with different kinetic properties, such as rising and decaying times contributing to the open probability.

Since primary afferent discharge frequencies observed in our *in vitro* model during BIKE did not fit with findings obtained by others in adult animals during walking, different possible reasons were investigated. A first hypothesis in the attempt to explain my data was the possibility of having collected biased results due to unintentional damage of afferent pathways during dissection. This eventuality was confuted by experiments where a high intensity mechanical stimulation of the hindpaw (compression) was effective in evoking an afferent feedback to the spinal cord in all tested preparations. Furthermore, the electrical stimulation of the territory innervated by the sural nerve was proven to evoke compound action potentials (CAPs) both from the sciatic nerve and DRs.

Once excluded a possible functional impairment of peripheral sensory structures and projecting pathways, a second hypothesis was a potential fatigue of primary afferent fibres consequent to a prolonged stimulation (BIKE training session), that might induce putative changes in fibre intrinsic wiring properties. Also this possibility appears to be unlikely, since I have demonstrated that DR stumps can be stimulated at high intensity for more than 90 minutes with a 0.5 Hz-train of electrical pulses without showing any failure or modification in CAP shape and transmission (supplementary figure 1).

Besides, the developmental stage of our neonatal rat preparation should be considered. Indeed, in adult animals, it has been reported that muscle spindle activity is controlled by skeletal muscles, with spindle firing rates being directly modulated by the frequency of muscle contraction during voluntary locomotion (Prochazka and Gorassini, 1998 a; b). It might be hypothesized that in a

neonatal model this modulatory control is missing because of a still immature musculoskeletal system, resulting in a much lower discharge frequency of primary afferents with respect to the adult. It can also be speculated that mature muscle fibres might release activity-mediated molecules, such as BDNF (Koliatsos et al., 1993; Gomez-Pinilla et al., 2002; Dupont-Versteegden et al., 2004), during rhythmic contractions. In turn, these molecules might promote the expression and maturation of ion channels on primary afferents, reaching firing rates detected in the adult.

5.1 Metabolic by-products of hypoxia and muscle fatigue might contribute to afferent discharges evoked by BIKE

In our *in vitro* preparation, nerve and muscle structures of the hindlimb are preserved, but blood circulation is absent. Hypoxia in hindlimb tissues is most probably established immediately after dissection and lasts for the entire experimental protocol. Thus, hindlimb muscles are deprived in oxygen for almost one hour before BIKE is turned on and afferent discharges start being recorded. Moreover, a possible muscle fatigue might be promoted during BIKE training. In humans, muscle fatigue is characterized by a deprivation in oxygen of muscle fibres, that induces the release of a wide range of metabolic by-products, including lactate and protons (Sahlin et al., 1976). For instance, during moderate anaerobic exercise, intramuscular pH decreases to approximately 6.6 (Spriet et al., 1986). Although in our *in vitro* study muscle oxygenation was not directly assessed, it cannot be excluded that a slight but persistent acidosis arose in hindlimb muscles as a result of blood stream lack and BIKE training.

It has been demonstrated that reductions in extracellular pH occurring within the physiological range are sensed by ASICs (Waldmann et al., 1997 b; Light et al., 2008). ASICs are cationic channels mainly permeable to Na^+ (Waldmann et al., 1997 a), although a small conductance for Ca^{2+} has also been reported (Waldmann et al., 1997 b). DRG neurons express both ASIC1 (Benson et al., 2002; Alvarez de la Rosa et al., 2003; Hughes et al., 2007; Calavia et al., 2010; Walder et al., 2010) and ASIC3 (Benson et al., 2002; Molliver et al., 2005; Hughes et al., 2007; Ikeuchi et al., 2008; 2009; Walder et al., 2010).

Besides their physiological role in mechanosensory function and touch perception (Omerbašić et al., 2015), ASICs are also involved in chemosensation (Sluka and Gregory, 2015). Indeed, decreases in pH produce inward ASIC-like currents in muscle spindle primary afferents (Gautam et al., 2010; Gautam and Benson, 2013). ASIC1 and ASIC3 are the most sensitive members of this ion channel family, sensing pH variations as small as 0.4 and 0.2 units from the physiological pH 7.4, respectively (Yagi et al., 2006; Lingueglia, 2007; Deval et al., 2008). Thus, it is likely that afferent

discharges recorded in our experimental conditions are strongly sustained by ASIC channels, although this hypothesis needs to be tested in pharmacological studies.

Moreover, it is known that, once active, ASICs generate sustained currents that last as long as the extracellular pH remains acid (Deval et al., 2010; Baron and Lingueglia, 2015; Grunder and Pusch, 2015). Indeed, it has been reported that some ASICs, in particular ASIC1 and ASIC3, do not desensitize during prolonged exposure to protons (Waldmann et al., 1997 a; Yagi et al., 2006; Springauf and Grunder, 2010). Translating these findings to my experiments, it might be speculated that in our *in vitro* preparation primary afferents are constitutively depolarized as a result of muscle acidosis mediated by hypoxia and training and that this depolarization is supported by ASICs. This condition might explain the reason why the coupling between afferent discharge frequency and pedaling is not so manifest in our experimental model and an analysis of frequency components is required. Indeed, if ASICs contribution is excluded, it is expected that activity recorded from afferent fibres is enhanced at a certain phase of the gait cycle (for example, stance) and depressed in the next phase (swing). This rhythmic pattern depends on an appropriate stimulation of the peripheral innervated target subsequent to hindlimb repetitive movement. Since I could not record an afferent feedback which appeared clearly phase-related to pedaling, I suggest that this rhythmic activity might somehow be masked by a constitutive firing of primary afferents supported by peripheral membrane transducers, such as ASICs. However, these speculations require further investigations.

Besides protons and lactate, potassium is another major by-product of hypoxia and muscle fatigue. It is released by skeletal muscles during a prolonged anaerobiosis (McKenna, 1995; McKenna et al., 1996; Hostrup and Bangsbo, 2017) and it can reach a more than doubled extracellular concentration (Sejersted and Sjøgaard, 2000). An increased extracellular potassium induces membrane depolarization (Walton and Chesler, 1988; Bracci et al., 1998; Sejersted and Sjøgaard, 2000; Marchetti et al., 2001 a) and is involved in the early onset of acidosis consequent to hypoxia and muscle fatigue (Nielsen et al., 2004; Gunnarsson et al., 2013). So, it is likely that also an accumulation of potassium in the interstitium of skeletal muscles might be correlated to the pattern of incoming signals recorded during BIKE.

Lastly, it is important to mention that accumulation of metabolites in skeletal muscles after intense exercise is associated with a decreased sensitivity of spindle primary endings (Gandevia, 2001; Proske and Gandevia, 2012), and even damage of muscle proprioceptors (Mense, 1996). As a result, proprioceptive signals conveyed to the spinal cord are strongly disturbed (Skinner et al., 1986; Saxton et al., 1995; Brockett et al., 1997; Ribeiro et al., 2007). Thus, it seems possible that a similar

condition might take place in our *in vitro* model, contributing to the low firing rate recorded from primary afferents.

5.2 DRGs might modify BIKE-induced afferent feedback

In our *in vitro* model, afferent activity was recorded upstream of the DRG. Afferent impulses running from the peripheral sensory ending are thought to pass through the T-junction and continue straight into the DR and spinal cord, with the cell soma being avoided by pulse propagation. Based on this, all information generated in the periphery should theoretically reach the spinal cord without undergoing any modifications.

However, this traditional theory is currently considered to be obsolete. Indeed, in most DRG neurons, afferent discharges propagate along the T-junction and invade the soma (Amir and Davor, 2003). Moreover, it has been found out that DRG neurons are endowed with intrinsic membrane excitability properties (Devor and Wall, 1990; Utzschneider et al., 1992; Liu et al., 1999; Amir and Devor, 1996; 2000), with certain cell bodies exhibiting repetitive firing capability (Matzner and Devor, 1992; Amir et al., 1999; Devor, 1999; Amir et al., 2002). Thus, it is probable that afferent discharges propagating to the soma might opportunely trigger the generation of action potentials in primary sensory neurons (Devor, 1999). In turn, these DRG-originating spikes might directly contribute to the firing rate in the branch entering the spinal cord.

Moreover, repetitive mechanical stimuli are processed in DRG sensory neurons by Piezo 1 and Piezo 2 channels (Coste et al., 2010; 2012; Pathanak et al., 2014; Woo et al., 2014; 2016; Ranade et al., 2015; Hung et al., 2016; Chesler et al., 2016; Lewis et al., 2017). Piezo channels act as pronounced frequency filters of both the onset and continuation of repetitive mechanical stimuli. It has been shown that Piezo channels faithfully transduce slow repetitive stimuli (< 2 Hz), whereas fast repetitive stimuli (> 20 Hz) are transduced well at their onset, but inefficiently during continuous exposure (Lewis et al., 2017). Thus, their transduction efficiency is mainly related to stimulus frequency, although other variables are also involved (Lewis et al., 2017). Overall, these studies suggest that the mean discharge frequency that I record from primary afferents in my experiments has extensively been modified by the DRG, that can directly contribute to the firing rate by either enhancing or filtering inputs originating in the periphery.

Lastly, it is well-established that DRGs act as chemosensors (Devor, 1999). Indeed, they are located outside of the blood-nerve barrier (Jacobs et al., 1976; Wadhwani and Rapoport, 1987; Allen and Kieman, 1994), they are endowed with abundant microvilli that expand the membrane surface available for metabolite exchanges (Shinder and Davor, 1994; Pannese, 2002), and they express a

huge number of receptors on their soma, such as ASIC1 and ASIC3 (Alvarez de la Rosa et al., 2003; Molliver et al., 2005; Hughes et al., 2007). So, cell bodies of DRG neurons can directly sense changes in the extracellular milieu, such as hypoxia-induced acidosis (Burchiel, 1984; Henrich and Buckler, 2008). This might lead to modifications in gene expression and a net effect on the afferent firing rate cannot be excluded, as well. In our *in vitro* model, experimental conditions in the recording chamber are under a strict control. However, hindlimbs are located outside the continuously superfused and oxygenated recording chamber. Thus, changes to peripheral tissues appear to be plausible and to affect primary afferent discharges evoked by BIKE.

6 BIKE effect on spinal locomotor networks

After having verified that an afferent feedback to the spinal cord could be evoked in our experimental model, I aimed at assessing its effect on spinal networks. In particular, I extensively investigated the spinal CPG for locomotion. As previously discussed, the locomotor CPG can generate a rhythmic and coordinated movement of lower limbs in the absence of sensory information, although required for a dynamic adaptation to the environment (Rossignol et al., 2006). Moreover, afferent signal promotes spinal plasticity of locomotor circuits, which is thought to have a key role in the regain of function induced by activity-based interventions adopted in current neurorehabilitation (Harkema et al., 1997; Dietz and Duysens, 2000; Barbeau and Fung, 2001; Dietz, 2002; 2003; 2012; Grasso et al., 2004; Scivoletto et al., 2007; Molinari, 2009; Ditz and Fouad, 2014).

First, I determined whether spinal locomotor networks were triggered by BIKE activation. As soon as hindlimbs started being moved by the device, a brief episode of locomotor activity was boosted, although spontaneously decaying within few seconds during continuous BIKE operation. The transient activation of the locomotor CPG mediated by BIKE was observed only in a very restricted group of preparations (15 %). Since CPG circuits have been demonstrated to be very sensitive to frequencies (Dose and Taccola, 2016; Dose et al., 2016), it might be hypothesized that BIKE pedaling frequency (0.5 Hz) does not represent an optimal stimulus for the activation of spinal locomotor circuits. This hypothesis is supported by *in vivo* studies where spinal cats initiate stepping on a treadmill only after intense perineal stimulation (Pearson and Rossignol, 1991; Alluin et al., 2015), confirming that a low-frequency afferent stimulation barely activates spinal locomotor networks. However, it has also been reported that trains of electrical pulses delivered to a DR at low frequency (1 – 4 Hz) are effective in eliciting FL in the isolated spinal cord *in vitro* (Marchetti et al., 2001 a), which preserves the typical periodicity of rhythmic activity induced by the combined

application of NMDA and 5-HT (Cazalets et al., 1992; Beato et al., 1997). Nevertheless, it should be considered that Marchetti and colleagues have adopted low-frequency trains of supra-threshold (2 – 5 x Th) stimuli, defined as the minimum intensity to elicit a detectable response in all recorded VRs (Bracci et al., 1996 b). In my BIKE experiments, I could easily control the pedaling frequency by setting the speed of rotation on the stabilized power supply, but I did not have any information in terms of motor threshold on signal intensity generated by passive cycling. Thus, it can be speculated that the low efficiency of BIKE in inducing spontaneous locomotor activity is ascribable to a low intensity stimulation. Nevertheless, it should be considered that a low-frequency passive stimulation might modulate the spinal CPG for locomotion, rather than directly activate it.

So, it was hypothesized that passive training could facilitate an electrically-induced FL by promoting its onset and maintenance. To the purpose, canonical 2 Hz-trains of impulses were delivered to a DR during BIKE pedaling. Then, I quantified the number of locomotor-like oscillations, alternated between homosegmental VRs, as it represents a clear hallmark of left-right coordination during CPG operation (Butt et al., 2002; Rybak et al., 2013; Talpalar et al., 2013; Molkov et al., 2015; Shevtsova et al., 2015). However, no appreciable changes in the number of FL oscillations could be detected during the combined application of electrostimulation and training. It can be supposed that the two approaches antagonize each other, since they both exploit primary afferents to convey inputs to the spinal cord. It is also probable that the strongest inputs, those mediated by electrical DR stimulation, overcome weak sensory signals evoked by passive cycling. This was supported by the fact that electrically-induced locomotor-like responses during BIKE were comparable to those recorded when hindlimbs were still and no afferent feedback was generated.

I also investigated the effect of BIKE training on a chemically-induced FL through bath-application of NMDA and 5-HT (supplementary figure 2). This approach is interesting since both drugs act centrally on spinal locomotor networks, thus theoretically setting afferent fibres free to convey peripheral information to the spinal cord. However, also in this experimental condition no modulatory BIKE effect could be detected. Noteworthy, when the preparation was superfused with neurochemicals at under-threshold concentrations, that do not induce FL *per se*, a combined application of BIKE triggered CPG activation (supplementary figure 3). Overall, these results suggest that BIKE training has a mild neuromodulatory effect. Indeed, it rarely elicits a direct activation of the locomotor program and it does not modulate an already established locomotor activity, triggered either electrically or chemically. However, when spinal networks already possess a certain degree of excitation, although insufficient to trigger locomotor-like activity, BIKE is able to fill the gap and provide the missing excitatory drive necessary for CPG activation. This effect is

presumably mediated by inducing primary afferents to release glutamate. BIKE-mediated glutamate should necessarily be released in exiguous quantities, since it is overwhelmed by excitation levels taking place within spinal circuitries when the locomotor program is active.

I further evaluated whether passive hindlimb training could induce plastic changes on spinal locomotor networks, persistent over time and detectable also when BIKE was switched off. Again, I used 2 Hz-trains of electrical stimuli to elicit FL before and after exercise. In this situation inputs arising from the periphery or from DR stimulation travel through primary afferents at different moments, avoiding possible wiring obstructions resulting in loss of incoming information. Sessions of passive cycling were progressively prolonged in time duration to identify the optimal training window capable to positively modulate CPG activity. I found out that a 30-minute BIKE session represents the shortest training window capable to facilitate spinal locomotor networks, although transiently. Similar observations were replicated also when neuroactive substances (NMDA and 5-HT) were bath-applied at under-threshold concentrations right after the end of 30 minutes BIKE, showing the onset of FL and confirming that BIKE mediates its effect through the release of glutamate (supplementary figure 4). I also demonstrated that longer BIKE sessions were ineffective in promoting facilitatory mechanisms on an electrically-evoked locomotor pattern. Apparently, these results might be explained in light of further data collected through patch clamp recordings on motoneurons, implying training-mediated modifications in synaptic transmission. This evidence will extensively be discussed later on (paragraph 8). Here I would like to stress a bit more the concept of frequency as a main tool in CPG activation. Recently, it has been demonstrated that a two-frequency protocol can optimally activate the spinal CPG for locomotion (Dose and Taccola, 2016). Hence, it could be envisioned a training protocol which exploits these basic research findings. Thus, each limb might be driven at two different frequencies that should be finely tailored to promote a motor pattern, as reported *in vitro* (Dose and Taccola, 2016), *in vivo* (Fujiki et al., 2015; Frigon et al., 2015; 2017; Kuczynski et al., 2017) and in humans (Forssberg, 1985; Prokop et al., 1995; Yang et al., 2005). Since the spinal cord is able to decode and process complex multisite afferent stimuli into a stereotyped pattern corresponding to the locomotor-like activity, it might be hypothesized that such a rehabilitation approach could enhance locomotor recovery, making the training protocol more efficient and allowing for a reduction in the training session duration. Moreover, it would allow to reduce habituation to the sensory input mediated by a repetitive and stereotyped mechanical stimulation and it would supply spinal networks with the required level of step-to-step variability (Ziegler et al., 2010; Shah et al., 2012). Indeed, besides preferential

frequencies, intrinsic signal variability has been shown to be another important feature promoting CPG activation (Taccola, 2011; Dose et al., 2013).

7 BIKE effect on spinal reflexes

Training has also been shown to induce a general reorganization of reflex pathways and to influence afferent transmission by providing a functionally useful amount of sensory information. Rehabilitation approaches aimed at normalizing exaggerated spinal reflexes are correlated to improvements in locomotor performance (Skinner et al., 1996; Trimble et al., 1998; Côté et al., 2003; Côté and Gossard, 2004; Kiser et al., 2005; Reese et al., 2006; Frigon and Rossignol, 2006). Therefore, reflex activity and locomotion show strong interplay mechanisms. For this reason, in the present study, the investigation of passive cycling effects on spinal locomotor networks ran parallel to the assessment of changes in spinal reflexes.

I demonstrated that in our *in vitro* model a 30-minute BIKE session, reported to be the shortest training duration capable to facilitate the locomotor CPG, did not affect spinal reflexes. On the contrary, a long BIKE session, which mediated the disappearance of facilitation on spinal locomotor networks, induced a progressive and persistent reduction in the area of ventral reflexes, as well. Reasons underlying these latter effects might be various, including modifications at peripheral and central levels mediated by prolonged training. In the periphery, a possible functional impairment of sensor organs, such as the muscle spindle, cannot be excluded (Mense, 1996; Gandevia, 2001; Proske and Gandevia, 2012), due to exercise-mediated accumulation of different metabolites, like protons, lactate (Sahlin et al., 1976; Spriet et al., 1986) or potassium (McKenna, 1995; McKenna et al., 1996; Hostrup and Bangsbo, 2017). On the other side, a potential fatigue of primary afferents subsequent to the prolonged BIKE training appears to be unlikely, since the conduction ability of DR stumps is unaltered for more than 90 minutes of high intensity stimulation with low-frequency (0.5 Hz) trains of pulses (supplementary figure 1). Moreover, fatigue interesting the pre-synaptic terminal of primary afferents might be a reasonable mechanism, putatively ascribable to a depletion in glutamate vesicles caused by the continuous training (Tabak et al., 2000; Miller et al., 2011). Besides glutamate, primary afferents release also inhibitory neurotransmitters within the spinal cord (Bowery and Smart, 2006), as well as other inhibitory molecules, such as ATP and adenosine; which might promote a sustained inhibition of spinal networks (Marchetti et al., 2001 a; Taccola et al., 2012). Furthermore, changes in VR reflexes mediated by a long BIKE session suggest a clear involvement of motoneurons, where output variations directly reflect modifications in intrinsic membrane properties and synaptic transmission.

8 BIKE effect on spinal motoneurons

Motoneurons have been shown to be directly affected by a prolonged and intense exercise, both in uninjured (Beaumont and Gardiner, 2002; 2003; Cormery et al., 2005; MacDonell et al., 2012) and injured (Petruska et al., 2007; Beaumont et al., 2008) animals. Thus, I aimed at evaluating training-mediated neuroplasticity mechanisms involving the motoneuron in our *in vitro* model.

Whole-cell patch clamp recordings were performed on spinal motoneurons from trained preparations to assess passive membrane properties and synaptic transmission. Two session durations were considered, a short/30-minute session and a prolonged/90-minute one. The purpose was to understand why a short BIKE training is effective in promoting CPG facilitation, although transiently, whereas a long exercise results to be ineffective.

Surprisingly, I could not describe any changes, neither in biophysical membrane properties nor in synaptic inputs, in motoneurons recorded from preparations previously undergone 30 minutes of passive cycling. However, it should be considered that these data might be biased because of technical issues. Indeed, I demonstrated that the facilitatory effect of a 30-minute BIKE session is transient and lasts for approximately 20 minutes after switching off BIKE. On the contrary, the time necessary to isolate the spinal cord from the leg-attached preparation and to arrange it for patch clamp recordings is longer, largely exceeding the 20-minute time window where BIKE facilitation is still detectable. Thus, it cannot be excluded that some modifications might occur in spinal motoneurons, including an increase in excitatory post-synaptic currents (EPSCs) compatible with the higher number of locomotor-like oscillations reported at the end of 30 minutes training. The transient nature of FL facilitation might be explained by non-persistent changes in spinal motoneurons due to the fact that BIKE signal appears to be weak. Probably, such a signal is not enough vigorous to promote long-term modifications onto the motoneuron, for instance, by inducing the expression of membrane ion channels or other molecular mediators involved in neuronal excitability.

However, when the same type of recordings was performed on motoneurons after 90 minutes training, an increased frequency of PSCs was observed. When PSCs were further dissected on the basis of kinetic properties or through recordings at different holding potentials, it was evident that both AMPA-related EPSCs and GABA/glycine-related inhibitory PSCs (IPSCs) were enhanced by a prolonged exercise. In parallel, a consequent reduction in membrane resistance was detected, in line with previous observations (Beaumont and Gardiner, 2002; 2003; Gardiner et al., 2006; MacDonell et al., 2012). These results suggest that a long BIKE session influences synaptic transmission onto the motoneuron and modifies its intrinsic membrane properties.

Given these data, some speculations can be done in light of previously described observations. On one side, it might be hypothesized that a short training can selectively and transiently increase EPSCs, since a facilitation of the locomotor CPG is observed. However, this increase should be limited and should contribute to the locomotor pattern with a very mild synergistic network effect, since spinal reflexes and spontaneous ventral activity are not affected by a short BIKE session. On the other side, a prolonged training seems to stabilize EPSCs and, in parallel, promotes IPSCs. Inhibitory inputs onto the motoneuron might overcome excitation and be responsible for the loss of FL facilitation and for the progressive and persistent depression of spinal reflexes mediated by a long BIKE training. Although locomotor exercise has been shown to promote a balanced inhibition and excitation, it should also be considered that in these experimental studies the training session does not exceed 30 minutes (de Leon et al., 2002; Timoszyk et al., 2002; 2005; Ichiyama et al., 2011; Singh et al., 2011; Bose et al., 2012), unlike our long BIKE session. This points out a crucial issue in neurorehabilitation, which deals with defining an optimal therapeutic window for activity-based interventions. Data presented here and by others indicate that special attention should be paid in the assessment of an appropriate training duration. Indeed, the therapeutic window should not be exceeded, since beyond this beneficial time span muscle fatigue and other detrimental peripheral and central mechanisms could establish. Furthermore, the therapeutic window has been shown to promote the facilitation of neuronal excitability (Bravo et al., 2002; Fouad et al., 2011), the regulation of chloride homeostasis (Côté et al., 2014; Chopek et al., 2015) and the expression of different neurotrophins (Grau et al., 2014; Skup et al., 2014). As for BDNF, it is important to remind that it reaches the maximum serum concentration after 20 minutes of exercise (Schmidt-Kassow et al., 2012), thus training sessions which largely exceed this time span should be reconsidered. Moreover, BDNF concentration returns to baseline approximately 10 minutes after the end of training (Schmidt-Kassow et al., 2012), in accordance with the disappearance of FL facilitation promoted by a short BIKE session. Since BDNF expression is strictly dependent on maintenance of sensory information transmitted during training (Ollivier-Lanvin et al., 2010), it is likely that it contributes only partially to long-term plastic changes observed at the end of exercise and that other molecular pathways should be involved.

In conclusion, the effect of BIKE cycling on the ventral spinal cord might be explained by admitting a weak neuromodulatory effect of passive training in our *in vitro* model. Moreover, it is likely that BIKE-evoked exercise has the capability to recruit excitatory and inhibitory contributions with a different timing, although further investigation is required to better assess this point. This

finding might be exploited to determine an appropriate training window able to maximize the outcome of current rehabilitation protocols.

9 BIKE effect on dorsal root activity

Physical therapy has also been proposed as a non-pharmacological method in the management of drug-resistant neuropathic pain. Training interventions have given promising results in terms of pain relief both in animal models (Hutchinson et al., 2004; Brown et al., 2011; Ward et al., 2014; Dugan and Sagen, 2015; Detloff et al., 2014; 2016) and in humans (Wismeijer and Vingerhoets, 2005; Gold et al., 2007; Sharar et al., 2008; Villiger et al., 2013; Labruyère and van Hedel, 2014; Roosink and Mercier, 2014; Cruciger et al., 2016; Roosink et al., 2016). In our *in vitro* model, I investigated on the outcome that training sessions of different duration have on DR activity.

I report here that a 30-minute cycling session capable to facilitate the locomotor CPG did not modify neither spontaneous antidromic activity nor electrically-evoked dorsal activity, when assessed immediately after switching off BIKE. Since dorsal activity might be sustained by GABAergic mechanisms of PSI and PAD (Rudomin, 1993; Wall, 1995; Rudomin and Schmidt, 1999; Willis, 1999; Vinay et al., 1999; Lamotte d'Incamps et al., 1999), this finding is in line with what has been suggested before, that a short BIKE session does not enhance GABA-sustained activity.

Then, I assessed the shortest training session able to elicit a significant DR hyperexcitability. The protocol consisted in a progressive cumulative application of 30-minute BIKE sessions. I found out that a training duration of at least 90 minutes was necessary to trigger DR activation. So, while 30 minutes corresponded to the shortest BIKE session capable to facilitate the locomotor CPG, 90 minutes represented the minimum exercise duration able to induce a significant DR hyperexcitability. Therefore, the two training durations used in the present study were identified based on functional modifications of different spinal circuitries.

Long BIKE sessions of 90 minutes could always induce an increase in DR activity, which persisted for hours after the end of training. This indicates that a long cycling exercise might promote an intense GABAergic activation, as confirmed by the increased frequency in IPSCs recorded on motoneurons, by the loss in FL facilitation and by the depression in spinal reflexes. If this hypothesis is true, it might be validated by demonstrating an overexpression in $GABA_A$, GAD_{65} and NKCC1 on primary afferent terminals, three major players in PSI-PAD (Rudomin and Schmidt, 1999; Willis, 1999). Moreover, the pharmacological block of NKCC1 (Alvarez et al., 1998; Willis et al., 1999), selectively expressed in the pre-synaptic terminus, should disrupt the rhythm recorded

from DRs, proving that it is sustained by GABA-mediated PSI-PAD. Interestingly, spontaneous dorsal activity was shown to increase upon BIKE start. This might suggest that training does not only mediate its effect by inducing long-term modifications through changes in gene expression, but also promotes fast adaptation mechanisms within the dorsal horn, such as receptor trafficking and rapid exposure onto the pre-synaptic terminal. Moreover, an increase in extracellular potassium concentration cannot be excluded, as well.

The functional meaning of DR hyperexcitability induced by a long BIKE training is unkown. As already discussed in previous paragraphs, antidromic activity might promote analgesia, for instance by filtering exaggerated incoming discharges through mechanisms of spike collision and PSI-PAD when a peripheral lesion or disease of the somatosensory system is established (Willis et al., 1999; Oakley and Prager, 2002; Buonocore et al., 2008). At the same time, an excessive DR hyperexcitability might support central sensitization and neuropathic pain (Baron et al., 2010; Nickel et al., 2011; von Hehn et al., 2012). An *in vitro* model allows to ascertain the establishment of DR hyperexcitability, but it does not supply any information about the functional meaning of an enhanced activation of spinal DRs. To answer the question, our *in vitro* model should necessarily be translated onto *in vivo* models. A 90-minute BIKE session should be tested in both no-pain and pain animals to assess potential changes in paw withdrawal threshold. Preclinical studies appear to be essential also in light of controversial results obtained with 4-AP (4-aminopyridine). 4-AP induces a peculiar dorsal rhythmicity *in vitro*, associated to nociceptive inputs (Chapman et al., 2009; Visockis and King, 2013) and attenuated by classical analgesics adopted for the relief of neuropathic pain (Ruscheweyh and Sandkühler, 2003). I observed that the conjoint application of BIKE during a 4-AP rhythm further enhanced dorsal networks activity. Since training has been demonstrated to promote pain relief both in animals and humans, a BIKE-induced decrease in 4-AP rhythm would have been expected. Nevertheless, 4-AP has a complex modulatory effect on spinal networks (Chapman et al., 2009; Visockis and King, 2013), making BIKE task not so easily achievable.

Overall, these data demonstrate a strong and persistent effect of a long training session on the dorsal spinal cord. In contrast, any comparable long-lasting outcome could be reported on spinal locomotor circuits. In future studies, it should be tried to develop new training protocols capable to promote a long-term facilitation or even a direct activation of the locomotor CPG, by looking for optimal frequencies and by adding variability to the rehabilitation program. Indeed, spinal networks retain a great potential, but currently adopted protocols are not capable to fully exploit it.

CONCLUSION

The present project has investigated the capability of afferent information in modulating spinal circuit activity. Network, synaptic, cellular and molecular modifications have been demonstrated to take place within the spinal cord after a repetitive and prolonged activation of primary afferents. Pathways conveying incoming signals have been triggered by delivering electrical stimulation protocols (i), by lesioning a peripheral nerve (ii), or by passively moving lower limbs (iii). From data presented here, it is possible to draw some conclusions, which might be translated to current clinical practice and rehabilitation approaches.

i. Electrostimulation protocols aimed at activating the locomotor program simultaneously affected primary afferent fibres. The meaning of DR hyperexcitability is poorly understood. Beyond speculations on putative analgesic effects, a clear evidence of sensorimotor interplay was presented, which might directly influence neurorehabilitation outcomes. Indeed, modulation of primary afferent activity might impact on sensory processing when applying SCS protocols for the execution of functional gait in subjects with sensorimotor disorders.

ii. A new molecular marker, RGS4, was shown to contribute to tactile hypersensitivity in a rat model of peripheral neuropathic pain. Interestingly, RGS4 might affect spinal locomotor networks, as well. To date, a direct demonstration of the modulatory effect of RGS4 in spinal CPG operation is missing. Thus, RGS4 represents a good molecular candidate for further investigations aimed at developing innovative pharmacological approaches to simultaneously promote pain relief and motor recovery.

iii. Passive training was reported to mediate a weak effect on spinal locomotor networks. Indeed, a short session of rhythmic hindlimb movement transiently facilitated the spinal CPG for locomotion. Mild neuromodulatory mechanisms elicited by passive cycling in our *in vitro* model might underlie poor outcomes mediated by activity-based interventions for gait rehabilitation in clinics. To date, rising evidence seems to suggest the potential role of combined strategies to improve locomotor performance in subjects with neurological disorders, by simultaneously providing pharmacological therapies, weight-bearing training and electrostimulation (Edgerton et al., 2004). Interestingly, in our study, a prolonged

training session failed in facilitating the locomotor pattern, presumably through modifications of motoneuron synaptic transmission. This suggests that the length of single training sessions should be carefully assessed within a rehabilitation program aimed at motor recovery.

On the contrary, passive cycling promoted a long-lasting hyperexcitability of primary afferents. As stated before, the meaning of DR hyperactivation is unknown and it should be investigated in preclinical models. However, this finding further supports the hypothesis of strong interactions between dorsal sensory-related and ventral motor-related spinal circuitries.

Lastly, a clear indication for training length was first provided, by reporting exercise-mediated differential effects on spinal circuits. Although basic mechanisms should be better and deeply investigated, these results might represent an early observation for spurring future studies aimed at identifying optimal training durations for motor regain of function and/or pain relief.

LIST OF ABBREVIATIONS

5-HT – 5-hydroxytryptamine (serotonin)
AAN – assist-as-needed
Ach – acetylcholine
AHP – afterhyperpolarization
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP – action potential
AP5 – D-2-amino-5-phosphono-valeric acid
ASIC – acid-sensing ion channel
ATP – adenosine triphosphate
BDNF – brain derived neurotrophic factor
BG – basal ganglia
BWS – body weight support
CAP – compound action potential
CB1 – cannabinoid type-1 receptor
CCC – cation-chloride cotransporter
CCI – chronic constriction injury
CINs – commissural interneurons
CNf – cuneiform nucleus
CNS – central nervous system
CPG – central pattern generator
DA – dopamine
DRG – dorsal root ganglion
DRPs – dorsal root potentials
DRRs – dorsal root reflexes
DRs – dorsal roots
EAAs – excitatory aminoacids
ECl- - chloride reversal potential
EMG – electromyography
E-MNs – extensor motoneurons
EPSC – excitatory post-synaptic current
ES – epidural stimulation
FDA – Food and Drug Administration
FL – fictive locomotion
F-MNs – flexor motoneurons
GABA – γ-aminobutyric acid
GABAA receptor – γ-aminobutyric acid A receptor
GAD – glutamic acid decarboxylase
GDNF – glial cell line-derived neurotrophic factor
GLYT2 – glycine transporter 2
GSK-3 – glycogen synthase kinase 3
GTO – Golgi tendon organ
HAL – hybrid assistive limb
IASP – International Association for the Study of Pain
KCC2 – K-Cl cotransporter 2
KCNK – two-pore potassium channel
L-DOPA – L-3,4-dihydroxyphenylalanine (levodopa)
LTMR-RZ – low-threshold mechanoceptor-recipient zone

LTP – long-term potentiation
MCtx – motor cortex
mGluR – metabotropic glutamate receptor
miRs – microRNAs
MLR – mesencephalic locomotor region
mSNI – modified spared nerve injury
NA – noradrenaline
Nav – voltage-gated sodium channels
NGF – nerve growth factor
Ngn1/2 – neurogenin1/2
NKCC1 – Na-K-Cl cotransporter 1
NMA – N-methyl-D,L-aspartate
NMDA – N-methyl-D-aspartate
NT3 – neurotrophin3
NT4 – neurotrophin4
NTs – neurotrophins
PAD – primary afferent depolarization
PLD – phospholipase D
PNS – peripheral nervous system
pPCtx – posterior parietal cortex
PPN – pedunculopontine nucleus
PSI – presynaptic inhibition
PSNL – partial sciatic nerve ligationRF – reticular formation
RGS4 – regulator of G protein signaling 4
SCI – spinal cord injury
SCS – spinal cord stimulation
SGC – satellite glial cell
SLV – synaptic-like vesicle
SNAP25 – synaptosome-associated protein 25
SNI – spared nerve injury
SNL – spinal nerve ligation
SNT – spinal nerve transection
TNF-α – tumor necrosis factor α
Trk – receptor tyrosine kinase
TrkB – tropomyosin receptor kinase B
TRP – transient receptor channels
TRPM8 – transient receptor potential melastatine family member 8
TRPV1 – transient receptor potential vanilloid 1
TTN3 – tentonin 3
UBG – unit burst generator
VCtx – visuomotor correction
vGluT1 – vesicle glutamate transporter 1
VGLUT1/2 – vescicular glutamate transporter 1/2
VRs – ventral roots

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