Universitat Autònoma de Barcelona

Activity-dependent treatments for neuropathic pain

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NeuroPlasticity & Regeneration

EPIONE

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ACADEMIC DISSERTATION

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INTRODUCTION

THE PHYSIOLOGY OF PAIN

Pain

Pain is defined by the IASP (International Association for the Study of Pain) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". It has a clear protective role in alerting us from potential damages to our body working through pain circuits to aware us from external (abnormal heating, pinch, …) or internal stimuli (cardiac ischemia) that would potentially hurt the tissues. It produces a continuous and unpleasant enough sensation so it cannot be ignored if the stimulus is present, to prepare an appropriate response driven by central processing. Different types of "normal" pain can be distinguished depending on their origin and characteristics: acute (or pricking), chronic (or burning) and continuous or visceral.

Nociceptors and nociceptive fibers

Nociceptors are free nerve endings with no specialized ending receptor structures. They grow from neuronal bodies in the dorsal root ganglion (DRG) (or trigeminal ganglion) sending axonal prolongations to a peripheral targeted tissue or enter the central nervous system (CNS) to synapse on nociceptive second order neurons. Stimuli can come from the skin, muscles, joint tissues or visceral organs traveling through the axons. Depending on diameter and myelination there are two main types of nerve fibers conveying pain information: Aδ fibers and C fibers (Fig. 1).

Aδ fibers are thinly myelinated, convey information from intense mechanical and thermal stimuli but generally nociceptive information. They can conduct a fast pain signal, at 5-30 m/s, reported as the first pain, the initial painful and sharp sensation just after the contact with the noxious stimuli.

C fibers mostly act as polymodal nociceptors, although a proportion seems to be sensitive only to mechanical or thermal stimuli. They are unmyelinated conducting slow pain signals at less than 2 m/s, reported as the second pain, that evokes a diffuse and long lasting painful sensation, more diffuse and prolonged than the pain evoked by the Aδ fibers.

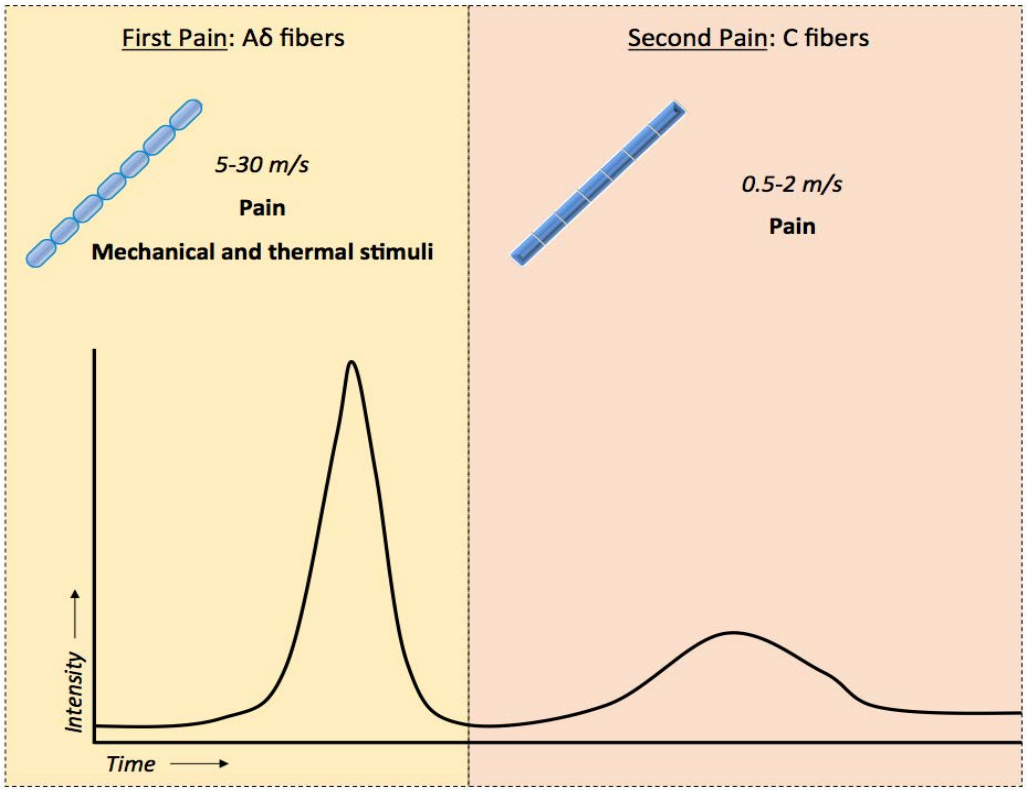

Fig. 1: First and second pain are transmitted by Aδ fibers and C fibers respectively.

All newly formed embryonic nociceptors express the tropomyosin receptor kinase A (TrkA) nerve growth factor receptor (Marmigere F. and Ernfors P., 2007). Following sensory neurogenesis, unmyelinated nociceptive afferents undergo two distinct differentiation pathways that lead to the formation of two major subpopulations of nociceptors, peptidergic and non-peptidergic nociceptors.

The peptidergic C nociceptors express substance P (SP) and calcitonin gene-related peptide (CGRP) and respond to nerve growth factor (NGF) produced by fibroblasts, keratinocytes and Schwann cells. Peptidergic nociceptors mediate the neurogenic inflammation induced by small vasoactive peptides, either directly or indirectly via mastocyte degranulation, which releases histamine.

The non-peptidergic C nociceptors contain a distinctive fluoride-resistant acid phosphatase (FRAP) activity, bind the isolectin B4 (IB4) and express a subset of purinergic P2X3 receptors whose naturally occurring ligand is adenosine triphosphate (ATP). Another feature of this group is a high density of tetrodotoxin-resistant sodium channels Nav1.8.

These two populations (Fig. 2) differ in neurotrophic support in the adult. In fact, during development, both populations require NGF for survival, but shortly after birth only the peptidergic continue to respond to NGF, whereas the non-peptidergic population starts to

respond instead to glial cell line-derived neurotrophic factor (GDNF) produced by Schwann cells via a common specific tyrosine-kinase receptor (RET). Accordingly, the peptidergic population expresses NGF high-affinity receptor TrkA, whereas the non-peptidergic expresses GDNF receptors.

Although the distinction between two populations of primary sensory fibers, peptidergic and non-peptidergic, seems attractive, it is not fully accurate as a small proportion of peptidergic sensory fibers (those that colocalize CGRP and somatostatin (SOM)) do not respond to NGF in the adult and bind the lectin IB4.

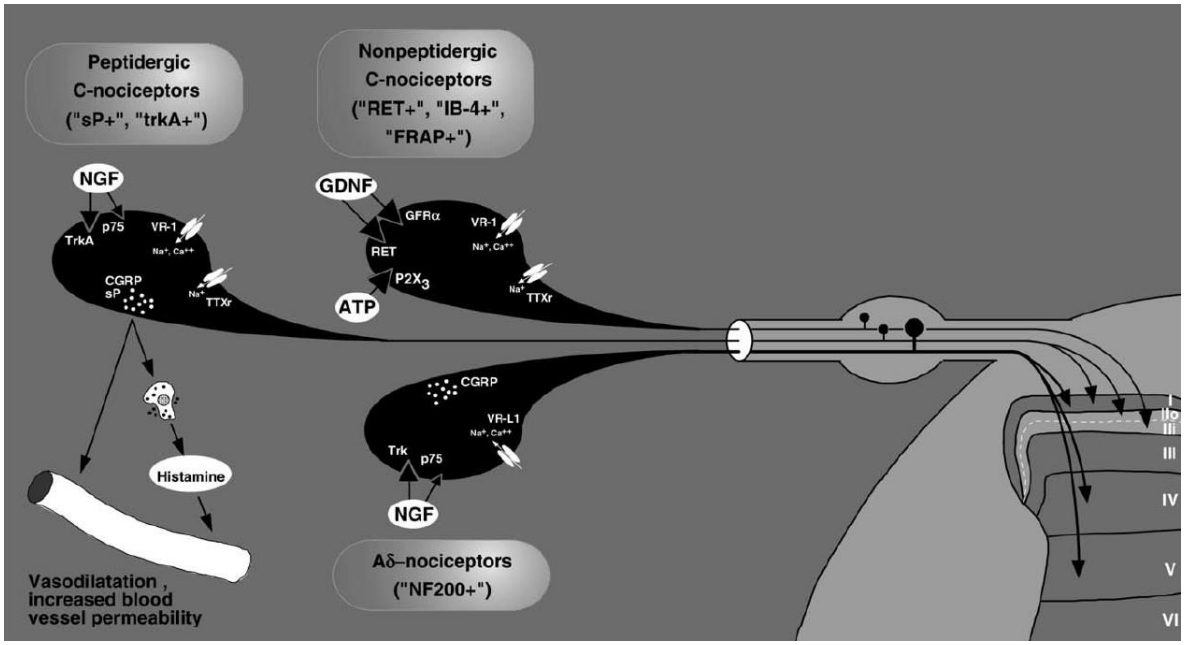

Fig. 2: Schematic representation of peptidergic and non-peptidergic C nociceptors and Aδ nociceptors. (Extracted from Coutaux et al., Joint Bone Spine, 2005).

In the spinal cord, sensory fibers that express SP and CGRP terminate mostly in laminae I and outer II, and some in lamina V; those that colocalize CGRP and SOM terminate in laminae I and II, and those that contain FRAP, bind the lectin IB4 and express the P2X3 receptor terminate mostly in the middle third of lamina II. Both groups of C fibers respond to similar types of noxious stimulation and express the capsaicin vanilloid receptor 1 (VR1), which transduces noxious chemical and thermal stimulation. It should be noted that other neuropeptides have been localized in the fibers that contain SP and CGRP: neurokinin A, galanin and the opioid peptide endomorphin-2.

The third group, Aδ nociceptors are myelinated and therefore can be characterized based on its neurofilaments (NF200). These nociceptors contain CGRP and other peptides. Their membrane expresses receptors for neurotrophins (TrkA, TrkB, and TrkC) and a high-temperature receptor different from VR1, the vanilloid receptor-like-1 (VRL1). These fibers project centrally to layers I, IV and V.

The transduction of the nociceptive information starts in the periphery, where a stimulus can activate the nociceptor endings, by stretching or bending the nociceptor surface or by promoting the activation of ion channels present in its membrane. At the site of injury substances such as proteases, bradikinin, ATP, and potassium ions are released. Due to the variety of stimuli that can elicit nociceptive signaling (thermal, mechanical and chemical stimuli), different specifics receptors have been described. Both Aδ and C fibers present transient receptor potential (TRPV) channels, which respond to heat and capsaicin, and even to mechanical stimuli. There are several TRPV channels, differing in the range of temperature that activates them. They resemble voltage gated ion channels, presenting six transmembrane domains and a central pore which allows an influx of sodium and calcium that initiates the generation of action potentials. These action potentials are transmitted to the spinal cord, where the signals will be integrated and transmitted to other areas.

Spinal cord circuits

There is evidence to indicate that the different subpopulations of primary afferent fibers convey nociceptive information through parallel spinal pathways. At every stage of the pain pathway (from sensory nerve to spinal cord, from spinal cord to brainstem and from brainstem to the forebrain) information signaling injury is subdivided or shared between these parallel systems. Molecular dissection has begun to reveal distinct functions for these separate pathways and their contribution to the final behavioral outcome.

Peptidergic primary afferents connect with the pathway that derives largely from lamina I neurons of the dorsal horn expressing the neurokinin 1 (NK1) receptor (Todd et al., 2002), and terminates within the thalamus, the parabrachial area and the periaqueductal grey. These areas in turn project on brain areas such as the hypothalamus and amygdala that modulate the affective dimensions of pain and control autonomic activity. The lamina I pathway is involved with signaling the intensity of pain, therefore these second-order neurons are capable of reliably detecting and transmitting precise quantitative information about noxious pressure and noxious heat to higher centers. Of interest, the selective destruction of lamina I results in a loss of the increased sensitivity to stimulation that follows inflammation or manipulation of the peripheral nerve.

On the other hand, recent evidence demonstrates that the IB4 binding subpopulation of non-peptidergic C fibers expressing the Nav1.8 sodium channel contacts neurons in inner lamina II of the spinal cord. These interneurons in turn contact projection neurons of lamina V, and many of these send axons to fourth-order neurons in the amygdala, hypothalamus, bed nucleus of the stria terminalis and globus pallidus (Braz et al., 2005).

Interneurons in this lamina also show increases in protein kinase C gamma (PKCγ) following inflammation which results in mechanical hypersensitivity (Malmberg et al., 1997). However, these interneurons predominantly receive myelinated, rather than unmyelinated, input from primary afferent terminals (Braz and Basbaum 2009; Neumann et al., 2008). This agrees with previous electrophysiological studies, which described that lamina II contained mostly innocuous mechanically responsive neurons and offers a possible mechanism by which nonnoxious stimuli activate PKCγ interneurons. In contrast, calbindin positive interneurons of lamina II are located postsynaptic to the IB4-positive subpopulation of non-peptidergic C fibers, but not to myelinated afferents. Taken together, these results illustrate the very complex connectivity of primary afferents with the interneurons of the substantia gelatinosa (lamina II).

Ascending pathways

The nociceptive information arriving from the periphery travels along the peripheral axonal branch of primary nociceptive neurons, whose soma are located in the dorsal root ganglia, and the central axon entering the spinal cord by the dorsal roots. After the dorsal root entry, they travel within the zone of Lissauer, in which axons move up or down a pair of segments before entering the gray matter of the dorsal horn, in a region called substantia gelatinosa. Central nociceptive terminals contact to second order neurons mainly placed in laminae I and II (pure nociceptive), and V (mixed nociceptive and mecanosensory). Sensory fibers that are peptidergic terminate mostly in laminae I and outer II, and a few in lamina V; those that are non-peptidergic (labeled by binding lectin IB4) terminate mostly in the middle third of lamina II. The main neurotransmitter involved in these first relays is glutamate, but also substance P, acting as cotransmitter in peptidergic nociceptors, is important to experience moderate to intense pain.

From the second order neuron, the thermal and nociceptive information crosses the midline and ascends to the brain in the spinothalamic tract. This decussation occurs at the spinal level and in two or three segments all the fibers are in the contralateral side. The ascending axons travel through the medulla, the pons and the midbrain without synapsing, until reaching the thalamus. From here, the information is conveyed to the primary somatosensory cortex. This route is followed in order to transmit the gross information of pain, the essential information for the brain

to note stimuli that threaten the integrity of the body. This route is called the spinothalamic pathway, part of the anterolateral system (Fig. 3).

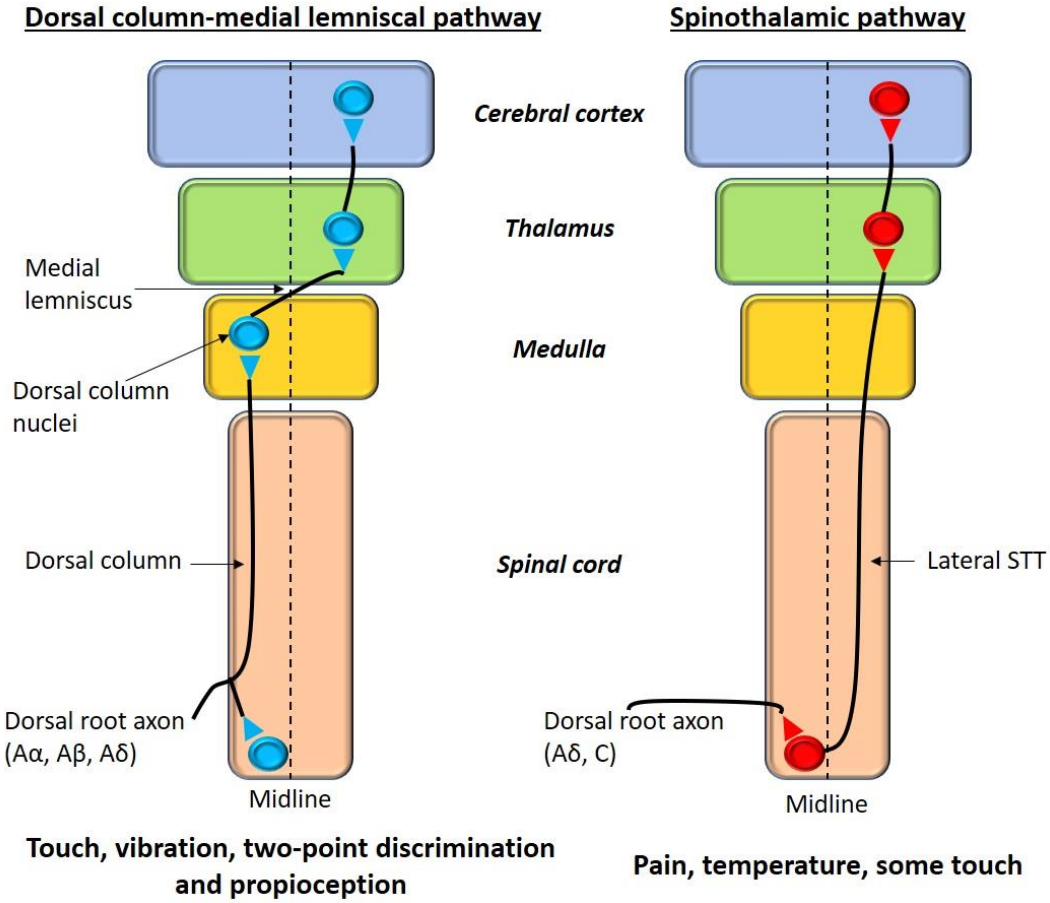

Fig. 3: Ascending pathways of somatic sensations.

The spinothalamic tract (STT), placed in the lateral and ventrolateral regions brings nociception, temperature, pressure and gross touch information to the somatosensory region of the thalamus. Axons from the second order spinal neurons make relays on different structures and nucleus, in order to mediate different aspects of the sensory and behavioral response to pain. One of these aspects is the sensory discrimination of pain (location, intensity and quality). The main responsible of this discrimination is the thalamus, in particular the ventral posterior lateral (VPL) nucleus. Another aspect is the affective or motivational, more related with the emotion that pain provokes in the individual who is suffering it (unpleasant feeling, fear, anxiety and secondary autonomic reactions). In this case the information travels by the spinoreticular and spinomesencephalic tracts, reaching several structures, such as the reticular formation, the superior colliculus, periaqueductal gray matter (PAG), hypothalamus and amygdala. In addition

to this, another group of neurons constituting the anterior spinothalamic tract, reach another structure in the thalamus, the midline thalamic nuclei, that will later connect with the anterior cingulate cortex and the insular cortex.

Apart from the anterolateral system, another fraction of information entering from the periphery travels along the dorsal columns, which will provide a more qualitative information of the stimulus that is reaching the nociceptive signaling system. This route is used essentially by the mechanoreceptive afferents, which provide information about touch, vibration and proprioception, and is known as the dorsal column-medial lemniscus system. This information travels directly by the central axons of primary sensory neurons in the dorsal columns ipsilateral to the site of entrance until the dorsal column nuclei in the medulla, where it decussates to reach the thalamus in the contralateral side and later the cortex. This system is also related to the discriminative aspects of pain.

Descending pathways

Once the nociceptive information arrives to the higher-level centers, it is integrated to elicit a complex physiological response in front of the noxious stimuli, and modulated in order to reduce the intensity of the painful sensation. The main mechanisms for pain modulation conform the descending pathway (Fig. 4). One of the most important regions is the PAG in the midbrain, but there are other regions in the brainstem also involved in this process: parabrachial nucleus, medullary reticular formation, locus coeruleus (LC) and raphe nuclei. These centers use noradrenaline, serotonin, dopamine, histamine and acetylcholine to exert both excitatory and inhibitory effects on different sets of neurons in the dorsal horn. Then, they can act on synaptic terminals of nociceptive afferents, interneurons (excitatory and inhibitory), synaptic terminals of other descending pathways, and projection neurons. These contacts do not only act inhibiting the transmission of nociceptive information but also modulating it, as well as controlling the balance between excitation and inhibition in the spinal cord.

The main action of the PAG is to modulate nociceptive signaling in the dorsal horn by secreting endogenous opioids (enkephalins, endorphins and dynorphins) on the dendrites of nociceptive neurons and wide dynamic range (WDR) neurons, causing the hyperpolarization of the second order neurons that implies its partial inactivation. They also release glycine on the terminals of primary afferents (A and C fibers), inducing a presynaptic inhibition that reduces the release of neurotransmitters on the second order neurons. Finally, the secretion of glutamate from the PAG excites the GABAergic interneurons in lamina II of the dorsal horn. This promotes the release of gamma-aminobutyric acid (GABA) on the second order neurons, hyperpolarizing them and therefore inhibiting them.

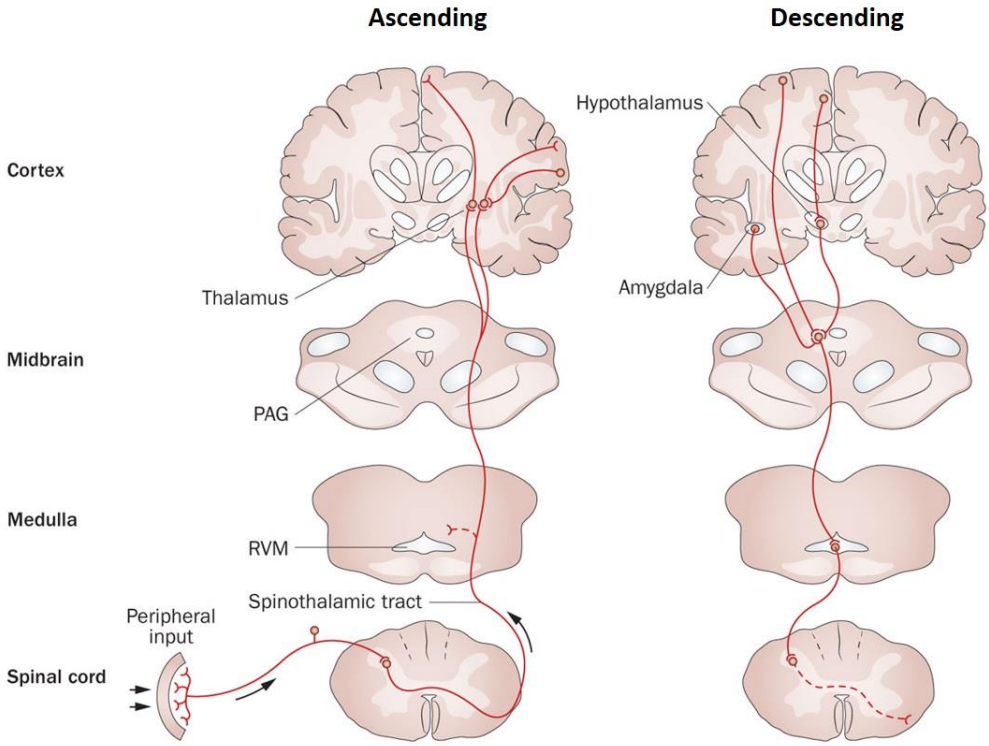

Fig. 4: Ascending and descending pain pathways. (Extracted and adapted from Zhou et al, Nat Rev Gastroenterol Hepatol, 2014)

The PAG also causes the depolarization of serotoninergic neurons in the nucleus raphe magnus (NRM), which project to second order neurons in the dorsal horn via the raphespinal tract. The binding of serotonin to receptors 5-HT1 and 5-HT2 induces an increase in the conductance of potassium and therefore the hyperpolarization of the second order nociceptive neurons. It also interacts with 5-HT3 receptors in the dendrites of GABAergic interneurons in lamina II, inducing the release of GABA and the inhibition of second order neurons. Something similar happens when PAG neurons secrete glutamate on the LC neurons. Once depolarized, these neurons release noradrenaline, which causes hyperpolarization of the nociceptive second order neurons by binding to α-adrenergic receptors.

Local circuits within the dorsal horn also play a role in modulating the nociceptive system. One of these systems was proposed by Melzack and Wall (Melzack and Wall, 1965), and called the "gate theory of pain", which is actually included under the afferent regulatory system of pain. This theory says that the activation of mechanoceptors (A fibers) can act on local interneurons to inhibit the transmission of information from C fibers to the dorsal horn projection neurons. This would explain how a mechanical stimulus such as scratching can temporarily give relief from pain in the same area.

Similarly, it has also been described a mechanism by which pain can inhibit pain. This phenomenon is called diffuse noxious inhibitory control (DNIC), or heterotopic noxious conditioning stimulation (HCNS) if strictly referred to human assays (Sprenger et al., 2011), and implies a spinal-medullary-spinal pathway. DNIC systems permits that a spinal neuron can be inhibited by a nociceptive stimulation applied in another part of the body (outside its receptive field), thus inhibiting the pain sensation after the application of a remote pain stimulation. WDR neurons and trigeminal nociceptive neurons play a key role in this phenomenon, which is subjected to regulation by serotoninergic pathways, and probably by opioids (Cervero, 2006). DNIC effects are usually contralateral and extrasegmental, and highly depend on the intensity of the stimulus. DNIC mechanisms are important since they may reflect alterations in the function of central descending inhibitory systems that could be potentially involved in chronic pain. In fact, research based on the use of DNIC has shown interesting results, since dysfunctions in DNIC were found in chronic pain conditions such as fibromyalgia or irritable bowel syndrome (van Wijk and Veldhuijzen, 2010).

Other elements are also involved in pain regulation, such as the endogenous opioids. Several brainstem regions, most of them conforming the descending system of pain control, are susceptible to the action of these molecules, provoking an important analgesic effect. Endogenous opioids are classified in three groups called enkephalins, endorphins and dynorphins, which present different distribution along the nociceptive system. Enkephalins, for example, can be released by local neurons on the dorsal horn, then impeding the release of neurotransmitters from the terminals onto the projection neurons, and therefore diminishing their level of activity. This local circuit can also be the target of other descending inhibitory projections, therefore constituting a powerful control mechanism of the amount of nociceptive information able to reach superior centers. Endorphins are released in pain states within some brain regions, but they can also provide tonic analgesic effect in the dorsal horn. Dynorphins have been described to increase after neural injuries, and are related to the development of thermal hyperalgesia by acting on the N-methyl-D-aspartate (NMDA) receptors and driving to spinal sensitization (Ossipov et al., 2000).

PERIPHERAL NERVE INJURIES

Peripheral nervous system

The peripheral nervous system (PNS) can also be divided into two separate systems: the autonomic nervous system (ANS) and the somatic nervous system (SNS).

The ANS regulates internal-organ function, by means of two complementary parts: the sympathetic and parasympathetic systems. The sympathetic nervous system activates the "fight or flight" response under sudden or stressful circumstances. It increases physical arousal levels, raising the heart and breathing rates and dilating the pupils, as it prepares the body to run or confront danger. The parasympathetic nervous system activates a "rest and digest" or "feed and breed" response after these stressful events, which conserves energy and replenishes the system. It reduces bodily arousal, slowing the heartbeat and breathing rate. Together, these two systems maintain homeostasis within the body: one priming the body for action, and the other repairing the body afterward.

The human SNS consists of 12 pairs of cranial nerves, 31 pairs of spinal nerves together with their ganglia. All of the spinal nerves are mixed containing both sensory and motor neurons. Primary sensory neurons transduce different stimuli into electrical signals that will be carried from the periphery to the CNS. Afferent information is received by different and specialized receptors responding to tactile, nociceptive, thermal and itching stimuli.

Peripheral myelinated axons are coated by Schwann cells producing myelin (Jessen and Mirsky, 2005). Myelin acts as an insulator of high resistance and low capacitance, so the ion current moves from node to node (saltatory conduction) increasing the conduction speed, where the nerve impulse jumps the spaces between the nodes of Ranvier. Conduction velocity in the unmyelinated axons is lower due to the absence of myelin and the impulse travels down the whole unmyelinated axon.

There are specialized tactile and proprioceptive neurons with large axons and thick myelin sheaths (Aα and Aβ fibers) and also protopathic tactile, thermoceptive and nociceptive neurons of small size (Aδ and C unmyelinated fibers). Within a nerve, each fiber is surrounded by a layer of connective tissue called the endoneurium. The axons are bundled together into groups called fascicles. Each fascicle is wrapped in a layer of connective tissue called the perineurium. Finally, the entire nerve is wrapped in a layer of connective tissue called the epineurium.

Definition, epidemiology, etiology and classifications

Peripheral nerve injuries (PNIs) cause partial or total loss of motor, sensory and autonomic functions of the denervated target segment due to interruption of axon continuity, distal degeneration of nerve fibers and death of axotomized neurons. These deficits can be compensated by the reinnervation of the target organs through three mechanisms: the regeneration of previously injured axons after Wallerian degeneration of distal part (Waller, 1850), the collateral branching of intact axons in the environment, and the structural and functional reorganization of the nervous system circuits involved in their actions. Although peripheral axons can regenerate

when the circumstances and the environment allow it to happen, functional recovery is not guaranteed (Allodi et al., 2012). The severity of the lesion would determine the final recovery grade. It should not be forgotten that not only regeneration of injured axons but also an adequate reinnervation of the target organs is necessary for a correct functional recovery.

PNI is a major clinical and public health challenge affecting more than one million people worldwide. In Europe, more than 300,000 PNIs are reported annually (Ciardelli and Chiono, 2006). Motor vehicle accidents, falls, domestic violence or sport activities (Ciaramitaro et al., 2010) are the main causes of injuries to peripheral nerves, plexuses and roots representing 5% of patients seen in civilian trauma centers (Robinson, 2000). Additionally, some nerve damages are due to carpal tunnel syndrome or secondary to diabetes (Aboonq, 2015; Vital et al., 1983). Complete paralysis, loss of muscle tone, atrophy, fibrillations, fasciculations, anesthesia or paresis (weakness) of the affected limb and development of neuropathic pain are typical symptoms of PNI resulting in sensory and motor function deficits (Siemionow and Brzezicki, 2009). The clinical significance, prognosis and treatment of PNIs depend on the site and extent of the injury.

PNI can be the result of a variety of causes that ultimately involve structural and functional lesions of the axons that compose it. Among the different causes, we can mention injuries due to ischemia, neurotoxic agents, radiation damage, metabolic disorders, immune attack, extreme temperature changes and mechanical injuries. Mechanical injuries are the most frequent affectations due to compression, friction or partial or total injury of the peripheral nerves due to traumatic accidents or because of surgical interventions. Mechanical injuries can cause different degrees of injury depending on its morphology, its therapeutic requirements and its prognosis. The most commonly employed clinical classifications are those of Seddon and Sunderland (Seddon, 1943; Sunderland, 1951) (Table 1).

PNI involving sensory fibers causes anesthesia or hypoesthesia of the different sensory modalities, forming the set of *negative symptoms* of the disorder. Parallelly, a series of *positive symptoms* are produced: isolated muscle fibers (fibrillations), complete motor units (fasciculations) or the whole muscle (spasms) are detected in the skeletal muscle. At the same level, hypersensitivity of the denervated organs to the neurotransmitters or agonists (Cannon, 1939) can occur, which may lead to an increase in the peripheral vascular tone, as well as compensatory sudomotor or vasoconstrictor activity increased in intact regions (Seddon, 1943). On the afferent sensory pathway, along with loss of sensation, spontaneous painful sensations appear in the absence of stimulation like major pain perception in presence of painful stimuli of low intensity (hyperalgesia), painful sensations in front of non-painful stimuli such as cold, heat or touch (allodynia) or phantom limb pain (PLP), characterized by the perception of having an amputated limb and pain referred to it by stimulating other areas (Omer et al., 1998).

Seddon, 1943		Sunderland, 1951	
Neuropraxia	Axons intact. Conduction blocked	***Grade 1***	Focal conduction block (Neuropraxia)
Axonotmesis	Axonal interruption. Endoneurium preserved	***Grade 2***	Axonal disruption (Axonotmesis)
		Grade 3	Axonal and endoneurium disruption (Axonotmesis)
Neurotmesis	Axonal interruption. Connective tissue disruption. No spontaneous regeneration	***Grade 4***	Axonal, endoneurium and perineurium disruption (Axonotmesis)
		Grade 5	Axonal, endoneurium, perineurium and epineurium disruption (Neurotmesis)

Table 1: Clinical PNI classifications by Seddon and Sunderland.

Regeneration process and amputations

After axons disconnection from the neuron soma, the distal segment degenerates in a so-called Wallerian degeneration, characterized by a progressive disintegration of axons and myelin sheaths, whereas a series of metabolic changes occur in the neuronal soma known generically as axonal reaction and chromatolysis (Selzer, 1980; Fawcett and Keynes, 1990). The functional meaning of Wallerian degeneration is to create a microenvironment in the distal segment (Zochodne, 2012) for the posterior regeneration of the surviving axons, whereas the neuronal retrograde reaction (Grinsell and Keating, 2014) and the chromatolysis contribute to the metabolic changes necessary for regeneration. The regeneration constitutes the set of cellular mechanisms that allows the elongation of axons (Selzer, 1980; Fawcett and Keynes, 1990; Fu and Gordon, 1997), with the functional objective of replacing the distal end of the axon that has been lost during degeneration and reinnervating the denervated target organs, allowing the restoration of their function and neural control. This process requires guidance signals different from those originated during developmental stages (Dudanova and Klein, 2013).

Neurons have an intrinsic growth capacity during the embryonic stage which is repressed during the adult transition to permit a suitable synaptic development (Allodi et al., 2012). However, after axotomy, the success of nerve regeneration depends primarily on the capacity of the injured neurons to survive, and to switch from a neurotransmitter to a regenerative phenotype. Neurons will express genes that encode for transcription factors which regulate other genes involved in cell survival and neurite growth (Navarro et al., 2007).

The neuronal retrograde reaction initiates a rapid influx of extracellular calcium and sodium ions to the injured axoplasm which causes an electrical response, inducing a high frequency burst of action potentials that propagate retrogradely (Lunn et al., 1990). Then, soma will suffer from chromatolysis with dissolution of Nissl bodies and disrupting retrograde transport of signals from target organs.

Wallerian degeneration initiates about 24-48 hours after nerve injury and can last for 1-2 weeks following a proximo-distal progression. The degenerative end products are eliminated by the cooperative action of Schwann cells and infiltrating macrophages and down regulate expression of myelin proteins and enhance neurotrophic factor and growth associated protein 43 (GAP-43) production. Denervated Schwann cells are able to phagocyte myelin debris to some extent; however, recruitment of macrophages is necessary to complete the phagocytosis of myelin and axonal debris. This clearance is important, since myelin debris can contain neurite outgrowth inhibitors.

Proximal to the lesion, the axons degenerate back to the adjacent node of Ranvier and growth cones emerge from the injured axons, induced by local factors. In the absence of a guiding structure, the re-growing axons form a neuroma composed of immature axonal sprouts and connective tissue (Siemionow and Brzezicki, 2009). In contrast, if the re-growing axons reach the distal nerve and find a suitable environment, they would elongate within the endoneurial tube. Neurotrophic factors secreted by Schwann cells (i.e. NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), GDNF), growth factors (i.e. basic-fibroblast growth factor (b-FGF), insulin-like growth factor (IGF)), cytokines (i.e. ciliary neurotrophic factor (CNTF), Il-1, Il-6, TNF-α) and neurotropic factors are needed during peripheral nerve regeneration and determine the response of the growth cone.

When limb amputation occurs, a neuroma is formed at the distal end of the sectioned nerve. The sectioned axons generate multiple regenerative buds, which cannot be elongated by the distal nerve, when it has disappeared. The neuroma represents a mass of axons that have been trapped in connective tissue, forming a ball of regenerative sprouts. The development, shape and size of the neuroma will depend on how the axons escape from their interrupted endoneurial tubes and go through the connective tissue. In amputated stumps, however, these characteristics assume particular importance because these stumps are unprotected in the extremity being subject to repeated blows, pressure and irritation. Chronic irritation of this nerve stump increases the size and sensitivity of the nerve. Under these unfavorable conditions this greater sensitivity becomes an acute hypersensitivity and becomes painful. Some of the neuromas end up being painful and others not.

Electrophysiological studies show that axons trapped in a neuroma tend to become more sensitive to mechanical, chemical, physical, and metabolic stimuli, with some spontaneous discharges of ectopic impulses (Devor, 1993). When there is a lesion of the sensory fibers, a pulse train of varying duration between seconds and a few minutes is produced immediately, which contributes to initiate the neuronal reaction but also to the central sensitization. Subsequently, there are notable changes in membrane excitability of motor and sensory neurons due to modifications in the expression of ion channels, mainly for sodium. Particularly, primary afferent neurons exhibit over-expression of type III sodium channels, which is sensitive to tetrodotoxin and is not normally found in spinal ganglion neurons, and a reduction in gene expression for sodium channels Nav1.8 and Nav1.9, which are resistant to tetrodotoxin (Waxman et al., 1999). These changes, which have also been demonstrated in inflammatory pain models, appear to be dependent on the deficit of neurotrophic factors such as NGF and GDNF. Reduction of N-type calcium channels in injured sensory neurons has also been described. These changes in sodium and calcium channels in response to nerve damage increase the excitability of neurons, leading to a predisposition to spontaneously stimulate and high frequency, resulting not only in spontaneous pain but also in central sensitization (Zimmermann, 2001).

The genesis of ectopic impulses originating in the neuroma may be due to the hypersensitivity of regenerative outbreaks to mechanical, thermal, chemical and inflammatory stimuli that occur in the local microenvironment. Ectopic impulses and the consequent sensation of pain may persist for long periods of time because of changes in neuronal excitability. A proportion of lesioned sensory fibers, both A and C, show oscillations of membrane potential leading to ectopic excitation. These discharges may be amplified by various mechanisms including: increase of post-discharges, cross-excitation to other fibers by local ionic changes or neuropeptide release, and ephaptic excitation due to membrane apposition of regenerative and demyelinated axons (Devor, 1993). It has been suggested that macrophages that are trapped within the neuroma could contribute to electrophysiological anomalies, either by releasing factors involved in regeneration or by creating amielynic areas susceptible to external stimuli (Frisen et al., 1993).

The main problem with neuroma is the neuropathic pain it causes. Several therapies have been used for the treatment of neuropathic pain with different degrees of success. Recent evidence suggests that neurotrophic factors could represent new treatments by reversing the process. Among the neurotrophic factors, we could highlight NGF, BDNF, NT-3 and GDNF (Sah et al., 2003). Another type of approach to avoid both formation of a neuroma such as the appearance of neuropathic pain, is surgery. These measures are aimed at promoting the dispersion of regenerated axons, suppressing axonal regeneration and limiting the growth of the final nerve stump, by implanting the distal stump into a muscle near the nerve in order to provide a better

environment in which may be extended or the stump encapsulation to avoid uncontrolled axonal growth (Sunderland, 1991).

NEUROPATHIC PAIN

Definition

The Assessment Committee of the Neuropathic Pain Special Interest Group (NeuPSIG) of the IASP defines neuropathic pain (NP) as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". Although sharing features with other kind of pain (inflammatory or cancer pain), NP presents some particular characteristics. Nociceptive and inflammatory pain can be both symptoms of peripheral tissue injury, and present a clear defensive, beneficial component, whereas NP is a symptom of neurological disease or injury, either affecting the peripheral or the central nervous system (Cervero, 2009), and instead of a defensive component it is considered as a maladaptive response. NP can be defined as central or peripheral depending on the site of the neural injury.

Neuropathic pain states are characterized by an almost complete lack of correlation between the intensity of peripheral noxious stimuli and of pain sensation, and are produced by neurological lesions that cause abnormal impulse activity generated in nerve sprouts, neuromas or dorsal root ganglion cells, ephaptic coupling between adjacent nerve fibers and abnormal responses of peripheral nociceptors and CNS neurons. Neuropathic pain syndromes produce pain sensations well outside the range of the sensations produced by the normal nociceptive system, even after serious peripheral injury or inflammation. Neuropathic pain may include spontaneous, or stimulus-independent, pain that has been described as shooting, burning, lancinating, prickling and electrical. Evoked, or stimulus-dependent, neuropathic pain includes allodynia, defined by the IASP as "pain due to a stimulus which does not normally provoke pain". These stimuli may be nonnoxious heat, light touch, or even a puff of cool air. Moreover, mechanical allodynia may be static, as evoked by light touch, or dynamic, as evoked by a light brush of the skin. Hyperalgesia is identified when a stimulus that normally produces nociceptive responses produces exaggerated responses (Porreca and Ossipov, 2009).

Epidemiological studies on the prevalence of neuropathic pain indicate a high incidence (~5%) (Bouhassira et al., 2008; Dieleman et al., 2008; Torrance et al., 2006). Associated risk factors include gender, age, and anatomical site of the injury. Emotional and cognitive factors influence how patients react to chronic pain (Haythornthwaite et al. 2003), but it is much less certain if these factors contribute to the risk of developing pain.

The different forms through which neuropathic pain manifests suggest that different mechanisms are likely to mediate the different features of the condition. Features that appear to be most often related to neuropathic pain include tingling or numbness, pain evoked by heat or cold, and especially, a sensation of heat or a burning-like quality is associated with the neuropathic pain state.

Phantom limb pain

The concept of phantom limb pain (PLP) as being the pain perceived by the region of the body no longer present was first described by Ambrose Pare, a 16_{th} century french military surgeon (Weinstein, 1998). Stump pain is described as the pain in the residual portion of the amputated limb whereas phantom sensations are the non-painful sensations experienced in the body part that no longer exists (Schley et al., 2008). Superadded phantom sensations are touch and pressure-like sensations felt on the phantom limb from objects such as clothing. Prevalence of PLP is more common among upper limb amputees than lower limb amputees and with higher incidence in females than in males.

Neuromas generate ectopic afferent impulses that may be perceived as pain by the brain. Early studies of animal models, such as rat sciatic nerve, demonstrated that the neuroma site is complicated and dynamic (Spring, 2010). Images of neuromas show multiple sprouts growing out from each cut axon and can travel in many directions, including back along the axon. There is an initial period during which each axon will produce a large number of sprouts, but many of these degenerate, leaving only a few that form the stable neuroma.

Ectopic activity has been demonstrated within all nerve fiber types. The neuroma shows sensitivity to a variety of compounds, especially norepinephrine (Black et al., 2009), which is possibly due to cytokine imbalance at the site of the neuroma and/or pathological receptor formation on the neuroma tissue. Support for this mechanism of PLP is provided by research demonstrating sodium channel expression upregulation and as well as damage to ion pumps leading to "leaky membranes" in these neuromas in the residual limb, both of which lead to hyperexcitability and spontaneous afferent impulses. PLP improves with sodium channel blockade in residual limb tissue. However, peripheral nerve blocks and lysis of neuromas do not reliably relieve phantom pain. Furthermore, patients with congenital absence of limbs experience phantom pain, but do not have classic neuromas from nerve resection (Fig. 5).

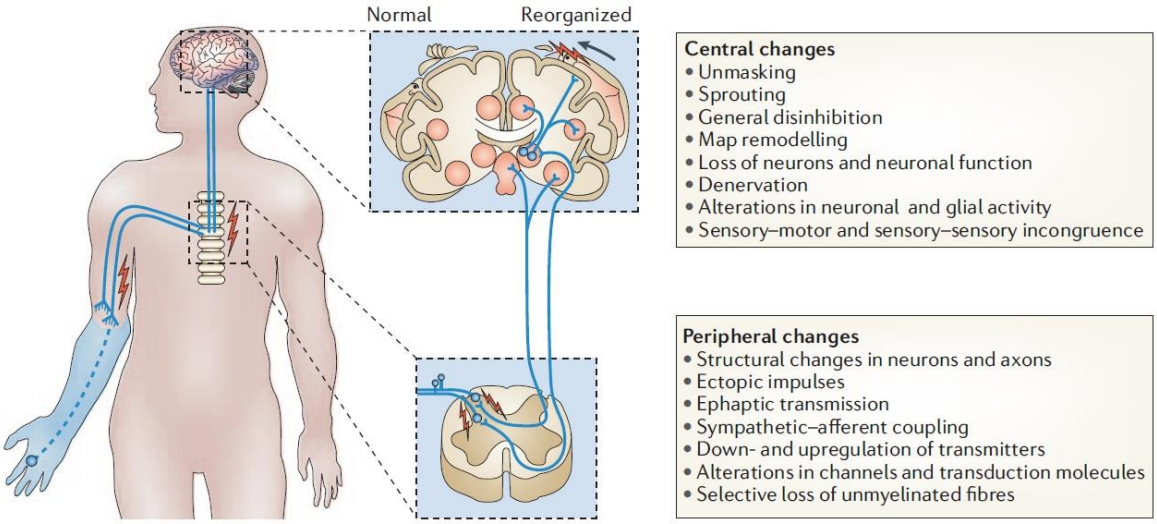

Fig. 5: Schematic representation of the areas involved in the generation of phantom limb pain and the main peripheral and central mechanisms. The peripheral areas include the residual limb and the dorsal root ganglion, and the central areas include the spinal cord and supraspinal centers such as the brainstem, thalamus, cortex and limbic system. The proposed mechanisms associated with phantom pain are listed for the PNS and CNS. (Extracted from Flor et al., Nature Reviews, 2006)

Mechanisms of neuropathic pain

Five key processes taking place in PNS and CNS are considered in the neurobiological approach to the mechanisms of pain and hyperalgesia: In the PNS (Fig. 6), the process of nociceptor activation and sensitization, responsible for the initial signaling of injury and the peripheral changes in the nociceptive system induced by a noxious stimulus; the process whereby activity in low-threshold sensory receptors from undamaged peripheral areas can access the nociceptive system and evoke pain sensations and hyperalgesic states (i.e. touch-evoked pain, tactile allodynia) (Cervero, 2009). In the CNS, the process of central amplification of nociceptive signals, known as central sensitization, generated by synaptic strengthening of connections between CNS neurons and responsible for the enhanced excitability that accompanies persistent pain states; the loss of endogenous inhibition produced at spinal level paired with changes in descending inhibitory pathways and finally, neuroplastic changes at subcortical and cortical levels.

Nerve injury-induced changes in transduction

Peripheral nerve injury leads to a redistribution of transducers in the wrong place within a neuron, and this event carries two consequences: the emergence of mechanical sensitivity at sites that are normally mechanically insensitive and mechanical allodynia. Even mechanical stimuli

associated with physiological functions, such as movement of tissue associated with blood flow, may also stimulate these transducers.

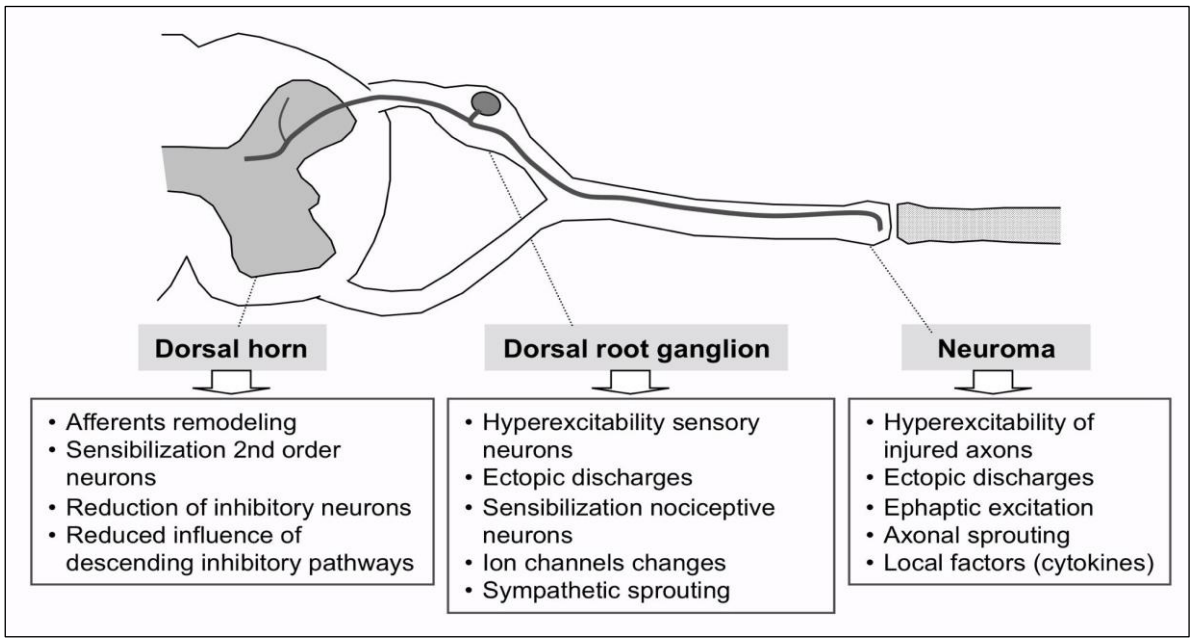

Fig. 6: Schematic representation of the main peripheral mechanisms involved in the generation of neuropathic pain after nerve injury.

Normal pain sensations are normally elicited by activity in Aδ and C primary afferent neurons. These nociceptors are usually silent in the absence of stimulation, and respond best to stimuli that are potentially noxious.

In neuropathic pain disease, after a peripheral nerve lesion, these neurons become abnormally sensitive and develop pathological spontaneous activity generated at any anatomical level proximal to those brain regions that mediate the sensory experience. These pathological changes are underpinned by dramatic molecular and cellular changes at the level of the primary afferent nociceptor that are triggered by the nerve lesion.

A long time ago, spontaneous ectopic activity was demonstrated in awakened human amputees with PLP, through microneurographic single-fiber recordings from afferent fibers projecting into the neuroma, as well as barrages of action potential firing (Nystrom and Hagbarth, 1981). After a nerve injury, there is an increase in membrane excitability. Spontaneous discharges in DRG neurons have been recorded from cells of both intact and injured nerves (Michaelis et al., 2000). There are phenotypic changes in injured neurons but more in uninjured ones (Decosterd et al. 2002). These changes are driven by cytokines and growth factors released by circundant dennervated Schwann cells (Wu et al. 2002).

An embryonic channel, Nav1.3, is upregulated in damaged peripheral nerves, and this is thought to promote ectopic spontaneous activity in primary afferent neurons. Also, genes for the voltage-gated sodium channels Nav1.8 and Nav1.9 are expressed selectively in nociceptive primary afferent neurons. These fibres acquire a unique sodium-channel expression profile after nerve injury (Wood et al., 2004). Clusters of these channels are responsible for the lowering of the action-potential threshold and consequent hyperexcitability (Lai et al. 2003). Their accumulation at the end bulb in a neuroma and also in dorsal root ganglion is a frequent explanation for abnormal electrical activity associated playing an important role in the genesis of neuropathic pain (Omana-Zapata et a1 1997).

Entrance of calcium ions into the nerve endings regulates growth-related proteins. In vitro, N and L-type calcium channels have been found to contribute to CGRP release from injured nerves. Blockade of N, T and P-type calcium channels reduces neuropathic pain (Kress et al., 2001).

Related to thermal stimuli, normal body temperature can elicit spontaneous activity of primary afferents because of a change in the activation threshold of the noxious heat-sensitive TRPV1 channel (Biggs et al. 2008). Damage to peripheral nerves provokes upregulation of TRPV1 that are located predominantly on uninjured C fibers and A-fibers (Catarina et al., 2000) and also in medium and large injured DRG cells (Ma et al., 2005). These changes might contribute to the development of C-nociceptor sensitization and the associated symptom of heat hyperalgesia (Baron, 2000).

In transduction process for mechanical stimuli acid-sensing ion channels (ASICs) seems to be involved in static mechanical hyperalgesia (Price et al., 2001). Nerve lesion also triggers the expression of functional α1-adrenoceptors (α1a) and α2-adrenoceptors (α2a) on cutaneous afferent fibers developing adrenergic sensitivity. It is known that in amputees, the perineuronal administration of norepinephrine (NE) induced intense pain (Lin et al., 2006). Neuroma after injury has both afferent C fibers and efferent post-ganglionic sympathetic C fibers which release noradrenaline (NA) and adrenaline. With high sympathetic activity, there is a raised sensitivity of the regenerating sprouts towards the detection of nociceptive substances, such as bradykinin, serotonin, histamine, and capsaicin evoking depolarization and ectopic firing. Afferent excitability can also be increased by the combination of a downregulation of inhibitor transducers, as opioid receptors, and the upregulation of excitatory transducers as the ATP receptor P2X3.

After Wallerian degeneration, products such as NGF are released in the vicinity of spared fibers triggering the release of tumor-necrosis factor-α (TNF-α), channels and receptors expression (sodium channels, TRPV1 receptors, adrenoreceptors...) thereby altering the properties of uninjured afferents (Wu et al., 2001).

Low-Threshold Aβ Fiber-Mediated Pain

Neuropathic pain involves a profound switch in sensitivity such that low-threshold Aβ fibers, which normally signal innocuous sensations, may begin after neural lesions to produce pain (Khan et al., 2002; Witting et al., 2006). Differential block induced by compression of the radial nerve in patients with nerve injury or exposed to experimental pain clearly demonstrated that pain induced by light brushing was mediated through Aβ fibers, whereas thermal pain was mediated through unmyelinated C fibers (Koltzenburg et al., 1992, 1994). Intrathecal morphine reversed nerve injury-induced thermal, but not tactile, hypersensitivity (Bian et a1., 1995, Lee et a1., 1995). Mechanical allodynia evoked by probing with Von Frey filaments was blocked by systemic morphine or pregabalin, whereas allodynia in response to a light brush was blocked only by pregabalin in animals with spinal nerve ligation (SNL) (Field et al., 1999).

After peripheral nerve injury, the most spontaneously active fibers are Aβ and Aδ fibers (> 80%), and C fibers constitute a small (0-30%) portion of the active population (Kajander and Bennett, 1992). The hypersensitivity includes areas outside of injured nerve territories and occurs largely in the absence of peripheral sensitization. It is typically associated with a loss of C-fiber peripheral terminals (Devigili et al., 2008); and disappears when conduction in large myelinated fibers is blocked (Campbell et al. 1988). After nerve injury, polysynaptic and monosynaptic Aβ fiber inputs in the most superficial laminae of the dorsal horn are highly increased (Okamoto et al., 2001). This area normally only receives input from Aδ and C fibers (Lu and Perl, 2005). Thus, low-threshold input from large myelinated fibers is transferred from non-nociceptive to nociceptive circuits in the spinal cord.

Many changes either independently or combined can promote Aβ fiber–mediated pain: central sensitization, disinhibition, and central afferent terminal sprouting.

Central sensitization

Neuropathic pain could arise either because of peripheral sensitization of intact afferents (Fields et al. 1998) or due to central sensitization (Fig. 7). It consists of the amplification in the CNS of stimulus-evoked signals (Woolf & Salter 2000). Central sensitization has been defined as "a prolonged but reversible increase in the excitability and synaptic efficacy of neurons in central nociceptive pathways".

The function of nociceptive pathways is increased due to high membrane excitability, synaptic efficacy and reduced central inhibition. The net effect of central sensitization is to recruit previously subthreshold synaptic inputs to nociceptive neurons, generating an increased or augmented action potential output: a state of facilitation, potentiation, augmentation, or amplification (Latremoliere and Woolf, 2009). It is manifested as allodynia (touch-evoked pain),

enhanced temporal summation (escalating pain in response to a constant stimulus), hyperalgesia (exaggerated pain experience respect to a standardized noxious stimulus) and secondary hyperalgesia (pain and hypersensitivity beyond the dermatome of the nerve injury).

Peripheral nerve injury leads to an increase in the general excitability of WDR neurons with multiple synaptic inputs from nociceptive and non-nociceptive system. This central sensitization is initiated and maintained by activity in pathologically sensitized C fibers. After peripheral nerve injury, there are presynaptic functional changes that increase synaptic strength: the synthesis of transmitters and neuromodulators (Obata et al. 2003) and more calcium channel density (Hendrich et al. 2008). Aβ fibers express increased levels of neuropeptides such as CGRP and substance P and enhance activity of excitatory amino acid transmission via NMDA receptors.

Healthy nerve terminals uptake NGF and other growth factors from their target cells and transmit them by axonal transport to the DRG neurons. After nerve transection, sprouts can no longer take up these growth factors. The gene transcription and protein synthesis are altered. At the level of transcription control in the DRG neurons, the c-jun gene can be inducted 1 day after axotomy. It is well known that c-jun expression in the DRG neurons after nerve transection is associated with changes in neuropeptide levels: substance P and CGRP decrease; galanin and nitric oxide (NO) synthase (NOS) increase dramatically during the weeks and months following axotomy. The induction of c-jun of axotomized neurons has been closely related with inhibitory transynaptic neuron death or apoptosis by NGF starvation.

The increased release and production of NOS at the intraspinal presynaptic terminal may facilitate afferent synaptic transmission to the dorsal horn neurons contributing to spinal neuronal sensitization and hyperalgesia. Repetitive noxious stimulation leads to the increased activities of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which produce an influx of extracellular Ca2+ and activation of protein kinase C (PKC) in dorsal horn neurons. The increased intracellular Ca2+ induces the expression of c-fos. Fos protein is believed to be involved in the transcriptional control of genes that encode a variety of neuropeptides, including enkephalin and dynorphin. Enkephalin typically produces antinociceptive effects. Dynorphin has direct excitatory effects on spinal projection neurons and may also produce inhibition via a negative feedback mechanism on dynorphin-containing neurons. The net effect of these changes may have complex modulations in the development of central plasticity.

The enhanced synaptic transmission is manifested by long-term potentiation (LTP) following a short train of stimulation at C- fiber. The transition of LTP between spinal interneurons involves glutamate and NK1 receptors.

Postsynaptically, second-order dorsal horn neurons abnormally express Nav1.3 after peripheral nerve injury (Ji and Woolf, 2001). Physiologically, dorsal horn neurons receive a strong inhibitory

control by GABA releasing interneurons that are lost by apoptosis after partial peripheral nerve injury increasing central sensitization. Other postsynaptic changes involve phosphorylation of NMDA subunits (Ultenius et al., 2006) and increased receptor density due to trafficking and enhanced synthesis of ion channels and scaffold proteins (Cheng et al., 2008). Central sensitization occurs in the dorsal horn, amygdala, anterior cingulate gyrus, and prefrontal cortex (Pedersen et al., 2007).

Centralization is a different phenomenon. In centralization, there are intrinsic changes to the CNS: increased excitability (Balasubramanyan et al., 2006), structural alterations in synaptic circuitry (Woolf et al., 1992), degeneration of inhibitory interneurons (Scholz et al., 2005), and alterations in the brain stem regulation of nociceptive transmission (Vera-Portocarrero et al., 2006) independently of any ongoing peripheral input (Devor, 2006).

Continuous and sustained afferent inputs into the spinal cord cause a state of spinal sensitization. This is related to the demonstrated phenomenon of wind-up, in which noxious stimuli applied to the skin also enhance the excitability of dorsal horn units, producing exaggerated responses to subsequent stimuli (Mendell & Walsh 1965).

An alternative mechanism of intraspinal disinhibition following peripheral nerve injury involves a trans-synaptic reduction in the expression of the potassium–chloride cotransporter KCC2 in lamina I neurons, which disrupts anion homeostasis in these neurons. The effect is that GABA release from normally inhibitory interneurons and now exerts an excitatory action increasing central sensitization (Coull et al., 2003). Dorsal horn neurons receive a powerful facilitatory but mostly inhibitory descending modulating control from supraspinal brainstem centers. A loss of function in descending inhibitory serotonergic and noradrenergic pathways contributes to central sensitization and pain chronification. Peripheral nerve injury activates spinal cord glia and causes these cells to enhance pain by releasing neuroexcitatory glial proinflammatory cytokines and glutamate producing also central sensitization.

Changes in endogenous inhibitory pathways, disinhibition and plasticity

After nerve injury, there is a loss of pre- and postsynaptic GABAergic inhibition in the spinal cord. In nociceptive lamina I neurons, the transmembrane gradient for chloride ions change. GABA receptors no longer leads to hyperpolarization, instead depolarization is induced, provoking excitation and spontaneous activity (Keller et al., 2007). Independently, there is a loss of GABAergic interneurons by apoptosis compromising the dorsal inhibition (Scholz et al., 2005). It is known that after GABAergic or glycinergic control blockade or removal, a tactile allodynia is induced (Thompson et al., 1993) and increases synaptic currents from Aβ fibers to nociceptive lamina I neurons (Baba et al., 2003).

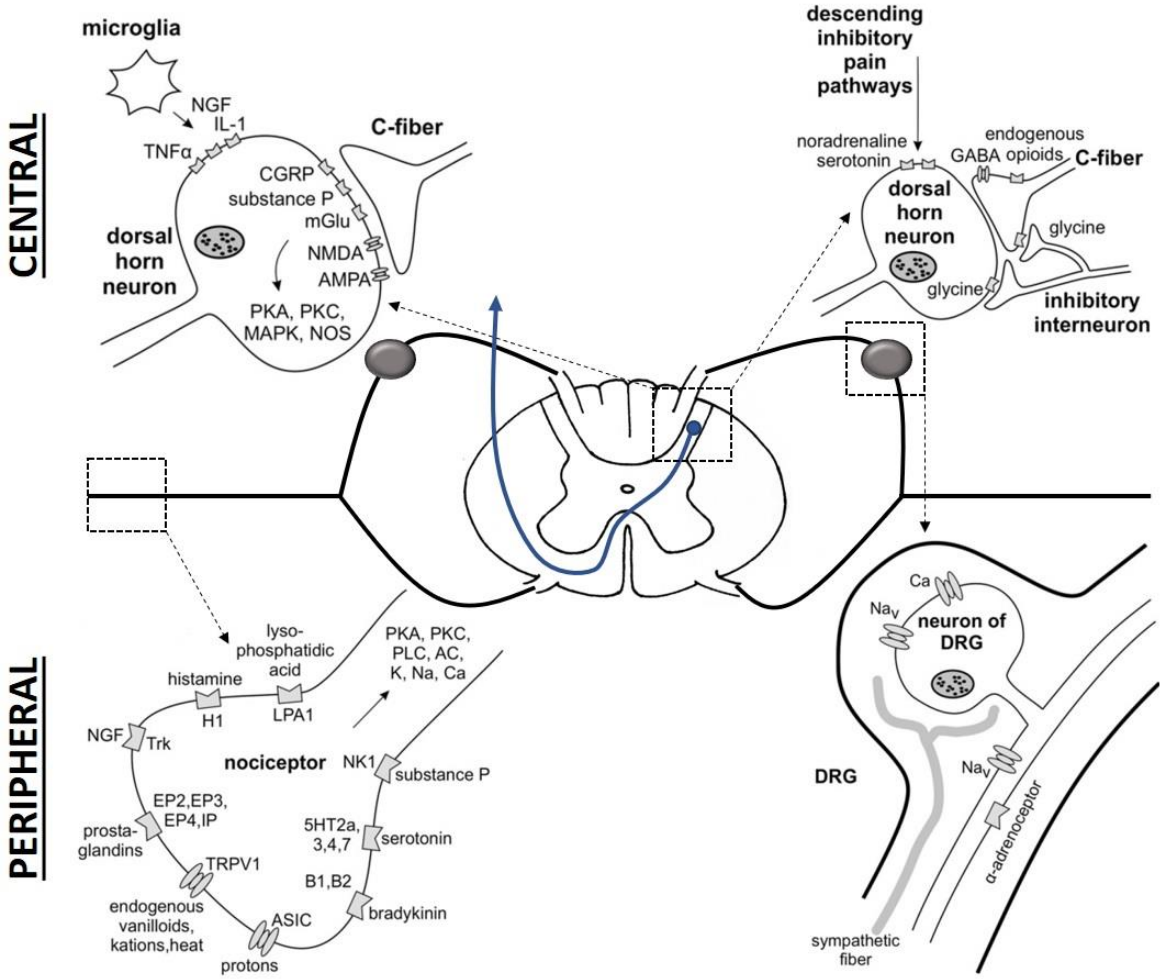

Fig. 7: Schematic representation of central and peripheral sensitization.

The tonic noradrenergic inhibition that acts on α2-adrenoceptors appears to be suspended after lesion (Rahman et al., 2008), thus, the net effect of descending serotoninergic input goes paradoxically, from inhibition to facilitation (Bee and Dickerson, 2008). Other descending pathway in the modulation of the nociceptive input are the μ opioid receptors which start reducing their expression together with less sensitivity of dorsal horn neurons to its agonists (Kohno et al., 2005).

These nociceptive neurons release glutamate, SP, CGRP and ATP acting as neurotransmitters and neuromodulators. After injury, hyperexcitability is established with a greater release of neurotransmitters in the spinal cord. They interact with NMDA, AMPA, mGluR, NK1R and P2X receptors causing depolarization of nociceptive projection neurons and scattering troughout spinothalamic pathway and with CGRP receptors of microglia and astrocytes. Glutamatergic

transmission in the ascending pain pathway related to neuropathic pain is mediated by isoforms Homer1b/c and Homer2a/b proteins (Obara et al., 2013).

Activated microglia synthesize and release prostaglandins, chemokines, NOS and cytokines (Interleukins 1 and 6 (IL1 and IL6), TNFα…) acting as chemical mediators to amplify the microglial reactivity favoring the elevation of these molecules in the dorsal horn (Zhuo et al., 2011). Reactive microglial cells are also responsible for the release of cathepsine-S protease that causes the proteolysis of a trans-membrane glycoprotein called fractalkine (CX3CL1) which interacts with its receptor (CX3XR1) located in the reactive microglial cell membrane, maintaining its reactivity and therefore contributing to the preservation of neuropathic pain. Microglia can also provoke neuronal death by producing ROS, pro-apoptotic cytokines and by a diminished glutamate uptake (Tawfik et al., 2008). Astrocytes also become activated after peripheral nerve injury with a slower onset and more prolonged time course than microglia, but also playing a role in the maintenance of neuropathic pain hypersensitivity.

The energy depletion in the injured neurons decreases the intracellular ATP concentration and consequent ATP-sensitive K^+ channel (KATP) activation. The activation causes potassium ions outflow leading to hyperpolarization, less excitability and reduction in the release of neurotransmitters. KATP expression is decreased after peripheral nerve axotomy of Aδ fibers enhancing hyperexcitability anyway (Zoga et al., 2010).

In thalamus, there is an upregulation of nicotinic and cannabinoid receptors after peripheral nerve injury, suggesting that supra spinal nicotinic and cannabinoid receptors in the thalamus may contribute to the modulation of neuropathic pain responses. On the other hand, μ-opioid receptor mediated G-protein activity is reduced producing desensitization in receptors from this region (Ueda et al., 2010). Also, NKCC1 and KCC2 disregulation in spinothalamic pathway is produced after sciatic nerve section and suture, suggesting that neuropathic pain is maintained by reducing inhibitory inputs at thalamus and cortex (Mòdol et al., 2014).

Activated microglial cells release BDNF after injury resulting in a reduction of KCC2 expression in a subset of neurons in the superficial lamina of the dorsal horn (Coull et al., 2005). Consequently, activation of GABAA receptors by GABA result in a diminution or absence of Cl- entry into the cell inducing disinhibition of these nociceptive neurons (Lu et al., 2009; Pitcher and Cervero, 2010).

Apart from the loss of descending inhibition, another feature contributing to the hyperexcitability in the spinal cord after injury is disinhibition. This can occur because of death of inhibitory interneurons caused by the excitotoxicity of the lesion, reduction in the release of inhibitory neurotransmitters from surviving interneurons, reduction in the expression of inhibitory transmitter receptors (Meisner, 2010; Costigan and Woolf, 2000). Another important

change to consider is the important plasticity and reorganization properties of the spinal circuits that imply functional changes of different elements. After an injury, the clear distribution of fibers in dorsal horn laminae can be lost, as some Aβ fibers arriving to lamina III-IV can produce aberrant sprouting and reach outer laminae. This may imply that some innocuous, tactile information will be processed abnormally in a nociceptive territory, constituting a potential mechanism for allodynia (Costigan and Woolf, 2000).

Silent circuits and synapses in normal conditions can also become activated after peripheral nerve injuries (Koerber et al., 2006). Neural plasticity occurring after neuronal lesions is also detectable in reflex circuits, which are usually used as an indirect measure of central hyperexcitability. Electrophysiological changes caused by the lesion and by following plastic reorganization can produce the appearance of hyperreflexia and an increase in wind-up responses that can eventually lead to spasticity and neuropathic pain.

Changes in subcortical and cortical regions

Peripheral nerve injuries results in loss of evoked activity by acute deafferentation in the corresponding cortical map. Then, plastic changes occur resulting in reduction of cortical field in favor of adjacent representations from intact sources with hardly discernible somatotopy. The brain plasticity is not very accurate *per se*, as it was demonstrated in several cases of nerve section and repair in humans where sensory mislocalizations persist for many years due to bad reinnervation.

The reorganization has different time courses depending on the severity of the lesion. After experimental sensory deafferentation of one finger in a human patient, the expansion of cortical representations of intact fingers is very fast and is recovered in minutes when the sensory blockade is stopped (Rossini et al., 1994). On the other hand, amputees suffer a slower brain plasticity process for a few weeks to become permanent with reduction of affected area and expansion of adjacent cortical regions (Pearson et al., 2001).

The basis of phantom sensations secondary to amputation seems to include reorganization phenomena at the cortical and subcortical levels. Furthermore, a sensory map corresponding to the different portions of the missing limb can be traced in the stump or in the face of such subjects (Ramachandran and Hirstein, 1998).

In chronic deafferentations, structural mechanisms like LTP or long-term depression (LTD) phenomena, sprouting for the formation of *de novo* connections and synaptogenesis can be reduced with NMDA receptor antagonist administration at the very beginning of the plastic process (Kass, 1991).

Plastic reorganization after nerve injuries is related with structural changes in dendritic arborization within the cortex (Hickmott and Steen, 2005). Adult reorganization seems to occur primarily through changes in the strength and efficacy of existing synapses, rather than implicate active remodeling of connections. At subcortical levels, transneuronal atrophy associated with retraction of axons and compensatory axonal sprouting seems to play a significant influence on the reorganization of somatotopic maps in the brain cortex.

Some groups of brainstem neurons are related with nociceptive modulation, forming the called 'brainstem pain modulating system'. It includes the midline PAG-Rostral ventromedial medulla (RVM) system, the more lateral and caudal dorsal reticular nucleus (DRt) and caudal ventrolateral medulla (cVLM). The descending projection of RVM includes the NRM and the adjacent reticular formation. Various neurotransmitters are involved in these descending pathways, but serotonin and noradrenaline are the main neurotransmitters. The predominant source of serotonergic input to the spinal cord arises from the NRM. Serotonin causes hyperpolarization of afferent nociceptive fiber terminals and dorsal horn projection and it produces excitation in spinal GABAergic interneurons. Noradrenaline causes hyperpolarization of projection neurons and over terminals of primary afferent fibers inducing excitation of dorsal horn inhibitory interneurons. There are wo types of neurons in the RVM: 'ON' cells facilitate, and 'OFF' cells inhibit, pain transmission (Fields et al., 2000).

Neuropathic pain induces hyperexcitation of specific nociceptive and WDR neurons in the spinal cord causing a potentiation of the 'ON' neurons response and a decrease of the 'OFF' neurons. The preferential activation of 'ON' cells located in RVM causes hyperalgesia, whereas hypoalgesia is achieved by the activation of RVM 'OFF' cells (Khasabov et al., 2012). A sensitization of 'ON'-RVM neurons is also induced by overexpression of NMDA/AMPA, TrkB and NK1 receptors, whereas μ opioid receptor expression decreases. Under these circumstances, 'ON'-RVM neurons do not respond to inhibitory signals from PAG, whereas they are highly stimulated by ascending inputs that release glutamate, SP and dynorphin over thalamic and brainstem neurons. PAG neurons are also sensitized due to overexpression of several receptors including NMDA/AMPA and SP/NK1 but also overexpression of glutamate and BDNF. These PAG-BDNF-positive neurons project their axons over the 'ON'-RVM neurons, enhancing their depolarization via TrkB and NMDA/AMPA receptors (Guo et al., 2006).

Furthermore, 'OFF'-RVM neurons mainly express NMDA/AMPA and TRPV1 receptors. After neuropathic and/or inflammatory pain, there are molecular changes in 'OFF'-RVM neurons such as an overexpression of GABA-A and κ opioid receptors (Heinricher and Ingram, 2009) provoking hyperpolarization and reduction of their anti-nociceptive effect on spinal cord neurons.

There is also an increase of microglial and astroglial reactivity in RVM, releasing several mediators that facilitate the excitation of 'ON'-RVM neurons and their excitatory effect on dorsal horn neurons (Wei et al., 2008).

In summary, neuropathic pain triggers plastic changes in the descending pain modulatory pathway: 'ON' cell activation and 'OFF' cell inactivation from the PAG-RVM system, and RVM glial reactivation to increase pain facilitation in the spinal cord.

Cutaneous nerve pharmacological blockade causes fast and reversible reorganization of dorsal column nuclei and thalamus (Nicolelis et al., 1993). In primates, immediately after section of ulnar and median nerves there is a change in hand representation mapping in their brainstem cuneate nucleus, maintained over several days (Xu and Wall, 1997). Loss of GABAergic inhibition is the main cause of the short-term plastic changes in the cortex (Chen et al., 2002). In the same way that occurs in the spinal cord, there is an upregulation of Nav1.3 in the third order nociceptive neurons in the thalamus resulting in hyperexcitability and expanded receptive fields (Zhao et al., 2006). Reorganization in cortex and thalamus delays few weeks to months depending on the growth of new connections and the expansion of thalamic receptive fields tend to be larger than somatosensory cortex. Cortical and subcortical reorganization and plasticity depend on projections from substructures (Krupa et al., 1999).

Related to forebrain in neuropathic pain states, there is a significant decrease in the levels of dopamine and serotonin in the orbital prefrontal cortex (OPC) and in the amygdala.

Changes in cortical caspase levels may represent an index of cell degenerative processes leading to cognitive deficits in neuropathic pain. In particular, caspase-1 plays a pivotal role in controlling the release of pro-inflammatory cytokines such as IL-1β, which appears to be upregulated in the prefrontal cortex of rats with neuropathic pain (Apkarian et al., 2006). Changes in microglia/astrocyte activation (through different expression of caspases and specific cytokines) serve as biomarkers of neural damage during chronic pain states. Drugs such as cannabinoid receptor subtype 2 (CB2) agonists can change the activation of caspases and the release of particular cytokines representing a tool to modulate chronic pain and avoid cortical impairment.

ANIMAL MODELS

The evaluation of neuropathic pain in humans is complex because most of the stimuli required to induce neuropathic pain produce irreversible damage. Consequently, the stimuli that do not produce irreversible harm can be used only in humans. Furthermore, it is also very difficult to recruit large number of humans for such type of testing. Therefore, there is a need of validated

and easily reproducible animal models of neuropathic pain to broaden the knowledge of the mechanisms involved in neuropathic pain and to evaluate the analgesic potential of novel pharmacotherapies for treating neuropathic pain. Several species have been used to study these events such as rat, mouse, cats, dogs, monkeys… although every species implies some pros and cons (Table 2). The ideal models should result in reproducible sensory deficits such as allodynia, hyperalgesia and spontaneous pain over a sustained period.

It is important to note that the terms "pain" and "nociception" are slightly different, and this is especially important to have into account when working with animals. Pain refers to the feeling or the sensation, the aversive and emotional reaction to a sensation coming from some part of the body. Nociception is the sensory process that provides the signals to trigger pain. Due to its emotional and conscious components, analysis of pain is usually restricted to humans, so the term nociception would be in fact more appropriate for animal models. Nevertheless, and for convenience, pain is also used when using animal models, but always with the semantic difference in mind.

Until the late 1970s, the basic science of pain concentrated almost only on the acute behavioral and electrophysiological reaction to transient noxious thermal or mechanical stimuli. The tail flick and the hot plate tests were the only tests employed for screening preclinical pharmaceutical analgesics. Therefore, the older models are relevant only for testing acute nociceptive pain and are of limited value to assess the hypersensitivities changes associated with chronic pain situations.

Algesimetry tests in animals such as electronic Von Frey for mechanical stimuli test or plantar test for thermal stimuli, become much more complex than in humans, since humans can express what are they feeling and where, and describe the sensations in understandable terms. For these reasons, it is important to understand that animal models do not completely mimic a human lesion and its consequences, but they are equally useful since they can reproduce most of the acute and long-term physiopathological events occurring after PNI, and can provide very important information for future treatments and therapies.

Wall and co-workers in the 1970s developed a chronic pain model by producing injury to a peripheral nerve and thus made a significant contribution in understanding the pathophysiological mechanisms in chronic pain, which is quite distinct from acute noxious pain. This led to attempts for developing different nerve injury models in animals as surrogates for neuropathic pain.

S. no.	Name of model	Principle of injury	Species
1	Axotomy (complete sciatic nerve transection)	Complete transection of sciatic nerve	Rats
2	Chronic constriction injury	Four loose ligatures around sciatic nerve	Rats, mice
3	Partial sciatic nerve ligation (Seltzer Model)	Tight ligation of one-third to half of sciatic nerve	Rats, mice
4	Spinal nerve ligation	Tight ligation of L5, L6 spinal nerves	Rats
		Tight ligation of L7 spinal nerve	*Macaca fascicularis*
5	Spared nerve injury	Axotomy of tibial and common peroneal nerves	Rats, mice
6	Tibial and sural nerve transection	Axotomy of tibial and sural nerves	Rats
7	Ligation of common peroneal nerve	Ligation of common peroneal nerve	Mice
8	Sciatic cryoneurolysis	Freezing of the sciatic nerve	Rats
9	Caudal trunk resection	Resection of caudal trunk	Rats, mice
10	Sciatic inflammatory neuritis	Injection of zymosan, HMG, TNF-alpha around sciatic nerve	Rats, mice
11	Cuffing-induced sciatic nerve injury	Implantation of polyethylene cuff around sciatic nerve	Rats, mice
12	Photochemical-induced sciatic nerve injury	Thrombosis in small vessels supplying sciatic nerve by photosensitizing dye and laser	Rats, mice
13	Laser-induced sciatic nerve injury	Radiation mediated reduction in blood supply to sciatic nerve	Rats
14	Weight drop or contusive spinal cord injury	Dropping a weight over the exposed spinal cord	Rats, mice
15	Excitotoxic spinal cord injury	Intraspinal injections of excitatory amino acids	Rats, mice
16	Photochemical spinal cord injury	Thrombosis in blood vessels supplying the spinal cord by photosensitizing dye and laser	Rats
17	Spinal hemisection	Laminectomy of T11-T12 segments	Rats
18	Drugs-induced		
(a)	Anti-cancer agents (vincristine, cisplatin, oxaliplatin, paditaxel)	Direct injury of drugs to the nerves of peripheral nervous system	Rats, mice, guinea pigs
(b)	Anti-HIV agents (2,3-dideoxycytidine, didanosine)		Rabbits, rats
19	Diabetes-induced neropathy	Persistent hyperglycemia-induced changes in the nerves	Rats, mice
(a)	Streptozotocin-induced		
(b)	Genetic models		
20	Bone cancer pain models		
(a)	Femur, calcaneus, tibial, humerus bone cancer pain	Inoculation of cancerous cells into respective bones	Rats, mice
(b)	Neuropathic cancer pain	Growing a tumor in vicinity of sciatic nerve	Mice
(c)	Skin cancer pain	Injection of melanoma cells in plantar region of hind paw	Mice
21	HIV- induced neuropathy	Delivery of HIV-1 protein gp120 to sciatic nerve	Rats
22	Post-herpetic neuralgia		
(a)	Varicella Zoster virus	Injection of viral infected cells in the footpad	Rats, mice
(b)	Herpes simplex virus	Depletion of capsaicin-sensitive	
(c)	Non-viral model	Afferents with resiniferotoxin	Rats
23	Chronic ethanol consumption/withdrawal	Administration of ethanol over extended period (around 70 days)	Rats
24	Pyridoxine-induced	Administration of high dose pyridoxine for long period	Dogs, rats
25	Trigeminal Neuralgia	Compression of trigeminal ganglion	Rats
		Chronic constriction injury to infra-orbital nerve	Rats
26	Orofacial pain	Injection of formalin, carragenan into temporomandibular joints and maxilla	Rats, mice
27	Acrylamide-induced	Administration of acrylamide for prolonged period	Rats

Table 2. List of different animal models of neuropathic pain. (Extracted from Jaggi et al., Fundam Clin Pharmacol, 2009)

Spared Nerve Injury (SNI)

This is a relatively novel animal model of neuropathic pain developed by Decosterd and Woolf (Decosterd and Wolf, 2000). In this model, rats are anesthetized, skin on the lateral surface of the right thigh is shaved and a division is made directly through the biceps femoris muscle. The sciatic nerve and its three terminal branches are exposed: the sural, the common peroneal, and the tibial nerves. Thereafter, the tibial and the common peroneal nerves are tightly ligated with 5–0 silk followed by axotomy of 2 mm of distal nerve. An enormous care is taken to avoid any contact with or stretching the sural nerve and thus, the sural nerve remain undamaged. The wound is closed in layers. In this model, one nerve (sural) is spared and other two nerves (tibial and common peroneal) are axotomized (Fig. 8).

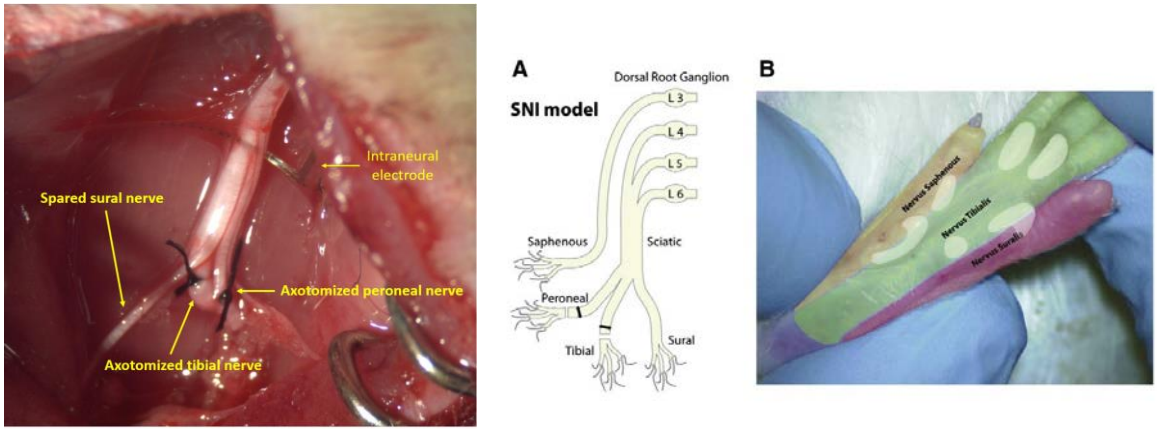

Fig. 8: Spared nerve injury surgery in rat with intraneural electrode implanted. (A) Schematic representation of SNI model. (B) Areas of the paw innervated by different nerves. The highlighted six areas on the foot sole represent the footpads. (Extracted from Duraku et al., Experimental Neurology, 2013)

The mechanical and thermal hyperalgesia and allodynia have been noted to occur within 3 days of injury, which persist for several weeks (up to 6 months) postinjury (Bourquin et al., 2006). The responsiveness to noxious and nonnoxious stimuli is increased in the ipsilateral sural and to a minor extent at saphenous region. The pain produced in SNI model is mechanically independent of the sympathetic system. It has been described that mice also show similar behavioral alterations, subjected to SNI as seen in rats. This model is different from other peripheral nerve injury models like chronic constriction injury (CCI), partial sciatic ligation (PSL) and SNL, because it allows the comparison of difference in mechanical and thermal sensitivities of non-injured skin territories adjoining to the denervated areas. This feature is important because it allows the simultaneous investigational changes in both injured primary sensory neurons and in neighboring unharmed

sensory neurons, so their relative contribution to the pathophysiology of pain could be investigated. The recent studies have highlighted the important contribution of non-injured neurons to neuropathic pain including ectopic activity in C-fibers, abnormal expression of sensory voltage-gated sodium channels, augmentation of TRPV1 and activation of Schwann cells. In this model, the changes in mechanical and thermal sensitivities are robust, substantial, prolonged time, and closely mimic many features of clinical neuropathic pain (Shields et al., 2003). The surgical procedure for creating this model is relatively easy compared with previous models, and there is relatively lesser variability in degree of damage.

Sciatic nerve transection and repair (SNTR)

SNTR is a model of injury clinically relevant for the study of peripheral nerve regeneration (Rodríguez et al., 2004), which allows reinnervation differently from models of partial sciatic lesion (Grelik et al., 2005; Ma and Bisby, 2000) in which sprouting and regenerative responses may be mixed (Fig. 9).

Neurotmesis of the sciatic nerve with distal stump excision causes that a large area of the hindpaw remains unresponsive, but the saphenous mediated hyperalgesia is sustained over weeks/months (Attal et al., 1994; Casals-Díaz et al., 2009; Kingery et al., 1994). In contrast, after SNTR, regeneration of sciatic axons allows for regaining skin innervation and initiates the resolution of hyperalgesia (Casals-Díaz et al., 2009; Kingery et al., 1994). However, areas of hyperalgesia and hypoalgesia may coexist in the affected hindpaw, due to variable extension of saphenous sensory fibers sprouting and incomplete sciatic nerve regeneration. For this reason, the assessment of somatosensory function mediated by the sciatic nerve after injury should be made in areas of the paw that are not affected by sprouting of the intact saphenous nerve, i.e. the lateral aspects of plantar skin and the lateral toes (Wood et al., 2011). After SNTR a primary source of early hyperalgesia is due to the saphenous nerve. The reinnervation of the skin by axons regenerating after SNTR start after 4 weeks, progressing from proximal to distal areas of the paw and gradually reaching sub-epidermal and epidermal layers up to the hyperreinnervation of the skin at 2 months. Sciatic reinnervation induced mechanical hyperalgesia in the lateral side of the paw, that reverted slowly over time (Lancelotta et al., 2003; Vogelaar et al., 2004).

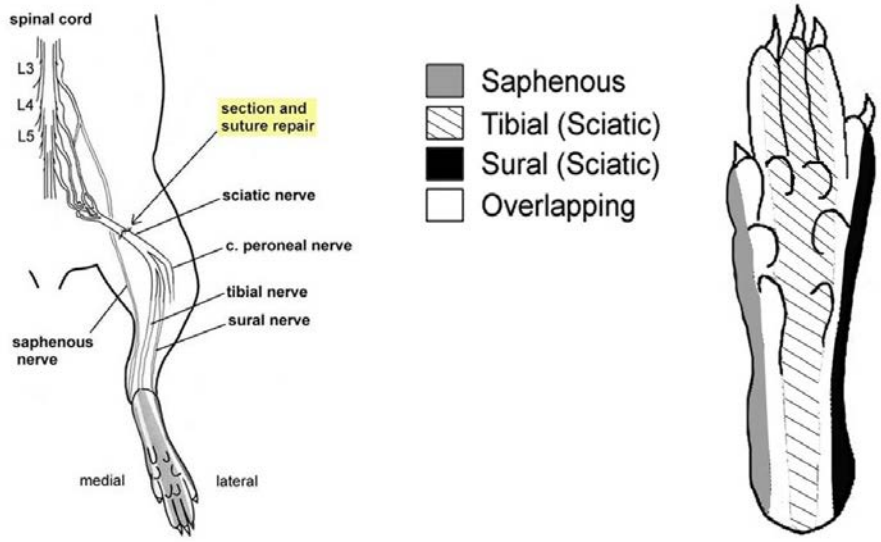

Fig. 9: Schematic representation of SNTR model.

PHARMACOTHERAPY

These therapies include the use of non-steroidal anti-inflammatory drugs (AIDEs), opioids, anticonvulsants, antiarrhythmics, tricyclic antidepressants and topical agents such as capsaicin, depending on the nature of the pain. It must be considered that all these treatments have many side effects besides acting more as palliatives than as curatives, with which the pathophysiological state of the nervous system persists and progresses. These drugs are aimed to treat NP by interfering pain processing and modulation for each different pain conditions.

The neuropathic pain may be caused by an increase in neuronal excitability. This increase in excitability may be due to both an increase in excitatory mechanisms and a loss or reduction in inhibition. Drugs such as opioids, antiepileptics and antidepressants act to increase inhibitory mechanisms, although some antiepileptics and tricyclic antidepressants have broad spectrum effects with several mechanisms of action that can both increase inhibition and lower excitation.

Since pain is usually debilitating and worsens the quality of life of those who suffer it, it is necessary to count on effective treatments with few side effects. Unfortunately, there are few really useful treatments, and NP is usually referred as refractory to common treatments. Here are shown the main groups of drugs used in the treatment of NP syndromes (Table 3).

Tryciclic antidepressants

The agents that are most commonly used in clinical practice are amitriptyline which is a noradrenaline reuptake inhibitor (Jain, 2008), and nortriptyline, although also imipramine, clomipramine, desipramine, maprotiline belong to this group of agents and have been studied in neuropathic pain syndromes. Their main functions are the inhibition of the presynaptic reuptake of noradrenaline and serotonin, the postsynaptic blockade of NMDA and α- adrenergic receptors, as well as the blockade of sodium and calcium channels. The main action of ryciclic antidepressants (TCAs) is enhancing endogenous pain modulation. Undesirable side effects, such as sedation, anticholinergic effects, and orthostatic hypotension, frequently occur with TCAs, particularly in the elderly.

Anticonvulsants

Drugs that suppress experimental and clinical seizures, defined as anticonvulsant or antiepileptic drugs, are classified as *first-generation* antiepileptics (i.e. benzodiazepines, carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and valproic acid), which were introduced between 1910 and 1970, and *second-generation* antiepileptics (i.e. felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, tiagabine, topiramate, vigabatrin, and zonisamide), which were introduced more recently (Loscher, 2002). Some drugs are designed to normalize membrane potentials and reduce hyperexcitability, such as carbamazepine, oxcarbazepine and phenytoin. Their action is aimed to block sodium channels, but some new blockers have been recently designed, and are supposed to act more specifically causing reduction in the release of glutamate and substance P, contributing to the decrease of hyperexcitability. Multiple pharmacological mechanisms have been elucidated for most antiepileptic drugs, including sodium channel blockade, calcium channel blockade, and suppression of glutamatergic transmission and/or GABAergic modulation (Dickenson et al., 2002). The potential for gabapentin and pregabalin to prevent and/or treat chronic pain after surgery has been studied in over a dozen trials that have yielded mixed results to date. In a recent systematic review of multiple, high-quality, chronic postsurgical pain prevention trials by our group, we reported that available evidence from 15 published RCTs does not support the efficacy of gabapentin or pregabalin in the prevention of chronic pain after surgery (Chaparro et al., 2013).

Opioids

Opioids are the most effective broad-spectrum analgesics available and are considered the cornerstone of therapy for moderate to severe acute pain or pain of similar intensity due to life-threatening illnesses, but their long-term use in non-cancer pain, is controversial due to their side

effects, and problems with tolerance (Finnerup et al., 2010a). Clinical trials assessing the efficacy of opioids for reducing neuropathic pain have been reported for more than 25 years, yet great variability in trial design in terms of the type of neuropathic pain syndrome treated, the type of opioid administered, and the duration of treatment has yielded contradictory results. Opioids that are useful for NP are oxycodone, with combined actions on μ- and K- opioid receptor, and tramadol that acts on μ-receptors and also has monoaminergic effects, inducing the release of serotonin and inhibiting the presynaptic reuptake of noradrenaline (Raffa et al., 1992).

NMDA antagonists

The NMDA receptors play a crucial role in the development of central sensitization, as well as in the appearance of tolerance to classical analgesics. NMDA antagonists are considered to be an important therapeutic option in the treatment of neuropathic pain. Currently, ketamine is mainly used intravenously as an antineuropathic treatment when more traditional treatments fail. Its topical use, alone or in combination with other agents especially amitriptyline, is increasing. Topical ketamine has also been reported to be effective in chemotherapy-induced neuropathic pain and radiation-induced neuropathic pain. Unfortunately, their use is limited because of their numerous side effects (Jain, 2008).

In the last years, as more and more mechanisms of NP are discovered, new drugs are created. Focused in neuromodulation of inflammatory processes in spinal cord (Iannotti et al., 2011; Esposito et al., 2012), as well as in the modulation of molecular targets (Ji and Suter, 2007; Gao et al., 2009). Some other experimental treatments, such as the use of chondroitinase or trophic factors (Pezet and McMahon, 2006), aimed to promote plasticity of the spinal nociceptive circuits (Xia et al., 2008; García-Alías et al., 2009).

The combination of different drugs acting on different mechanisms is a common strategy, especially in patients who do not respond to treatments of one single drug, or in cases of only partial efficacy. It is also important to consider the possible contraindications in each patient as well as the possible side effects of each treatment. In any case, it is also important to complement any pharmacological, biochemical or cellular therapy with rehabilitation therapies.

	Pain condition	Useful drugs
Peripheral pain	Painful diabetic neuropathy	TCAs and selective noradrenaline and serotonin reuptake inhibitors (SSNRIs)
	Nerve injury pain	Amitriptyline (TCA), topical lidocaine and topical capsaicine
	Painful neuropathy	TCAs, gabapentine, pregabaline, lamotrigine, tramadol, oxycodone
	Postherpetic neuralgia	TCAs, gabapentine, pregabaline, lamotrigine, tramadol, oxycodone, morphine, lidocaine
	Postamputation pain and PLP	Gabapentine, lamotrigine, pregabaline, carbamazepine
Central pain	SCI pain	Gabapentine, lamotrigine
	Multiple sclerosis	Cannabinoids

Table 3: Table of drugs with pain-relieving effects. (Modified from Sindrup et al., Handb Clin Neurol, 2006)

ACTIVITY-DEPENDENT THERAPIES

The primary goals of the management of neuropathic pain are to detect the underlying cause, to define the differential diagnosis and eliminate risk factors, and to reduce the pain. The physician should also know the functional and psychologic conditions of the patient. Therefore, a multimodal management plan in neuropathic pain is essential. Activity-dependent therapies are important options and must be considered when pharmacotherapy alone is not sufficient. In addition, psychosocial support and cognitive behavioral therapy could also be taken into consideration. It has been suggested that the importance of pain rehabilitation techniques will increase in time and these will take a larger part in the management of neuropathic pain.

Exercise

Routine exercise can be a beneficial addition to medical and pharmaceutical treatments for people with peripheral neuropathy. Some of several benefits of routine exercise include: enhanced macro- and micro-vascular health, reduced risk of hypertension, atherosclerosis and numerous cardiovascular diseases, decreased production of reactive oxygen species (ROS) and increased anti-oxidant defenses, reduced risks of certain types of cancer, increased muscle strength and cardiorespiratory endurance and reduction of neuropathic pain symptoms.

In rodents, aerobic exercise training has been shown to delay the onset of diabetic pain (Chen et al., 2013) and tactile hypersensitivity (Shankarappa et al., 2011), and to also reduce mechanical allodynia (Sharma et al., 2010; Bobinski et al., 2011; Stagg et al., 2011; O'Donnell et al., 2012; Cobianchi et al., 2013) and hyperalgesia (Hutchinson et al., 2004; Chen et al., 2013; Sluka et al., 2013) following injury. Reduction of allodynia after early short-lasting exercise correlated with reduced microglia and astroglia reactivity in the dorsal horn, but astrocytosis was not prevented by long-lasting exercise.

Similarly, routine exercise not only alleviates neuropathic pain in humans, but it has also been shown to increase cutaneous sensation (Li and Manor, 2010) and the ability to perceive vibrations (Balducci et al., 2006). Those sensory benefits, contribute to the positive effect that exercise training has on balance (Orr et al., 2006; Morrison et al., 2010; Song et al., 2011; Ahn and Song, 2012; Akbari et al., 2012; Li and Hondzinski, 2012) and functional mobility (Orr et al., 2006; Li and Manor, 2010; Song et al., 2011) in those with peripheral neuropathy.

In diabetic neuropathy in humans, routine exercise has been shown to both enhance peripheral nerve conduction velocity (Balducci et al., 2006; Hung et al., 2009) and increase intraepidermal nerve fibers (IENF) branching (Kluding et al., 2012) and density (Smith et al., 2006). On a related note, it is possible that exercise training could elicit favorable adaptions in the nervous system via plasticity mechanisms and by retraining neural pathways, but, to the best of the authors' our knowledge, this has not been investigated.

After PNI there is a disruption of injured axons paired with projections from intact neighboring axons. Since synaptic plasticity and sprouting of sensory fibers enhance pain perception during the denervation and reinnervation phases, activity treatments act on neuropathic pain processes and recovery of motor function. Combined rehabilitation of motor and sensory functions by passive or active exercise programs may eventually lead to a better coordination of sensorimotor tasks and restoration of adequate circuitry at the spinal level.

To promote functional recovery after neural injuries or in neurodegenerative diseases, electrostimulation or exercise have been shown to positively influence the neuromuscular functional outcome after experimental nerve injuries (Al-Majed et al., 2000; Asensio-Pinilla et al., 2009; Marqueste et al., 2004; Vivo et al., 2008) by enhanced sensory inputs and/or motor activity. Constant training is necessary to maintain the effect of improving tactile discrimination and threshold perception (Shieh et al., 1998).

Related to modifications in neurotrophins and cytokines expression after exercise, have been demonstrated that levels of pro-inflammatory cytokines in diabetic humans (Dekker et al., 2007; Balducci et al., 2010) and rats (Teixeira de Lemos et al., 2009) are decreased. As to BDNF, studies have demonstrated that exercise training tends to elevate the expression of BDNF in both the

CNS (Gomez-Pinilla et al., 2012; Rothman et al., 2012) and skeletal muscles (Gomez-Pinilla et al., 2012) and that those increases may be beneficial to cognition and the health of the brain (Rothman et al., 2012). However, excessive levels of BDNF in sensory locations like the dorsal horn and dorsal root ganglion are associated with abnormal nociceptive processing and the development of neuropathic pain. Similarly, Detloff et al. (2014) very recently showed that aerobic exercise can normalize spinal levels of GDNF, prevent excessive sprouting of pain afferents and reduce tactile allodynia in rats following spinal cord injury. NT-3, which is known to promote the survival and differentiation of existing neurons and to encourage the growth of new synapses and neurons is increased by aerobic exercise training in rodents in the spinal cord (Gómez-Pinilla et al., 2001) and skeletal muscles of both diabetics (Li et al., 2012) and those recovering from peripheral nerve injury (Hutchinson et al., 2004). Those observed exercise-induced elevations of NT-3 were associated with increases in peripheral nerve conduction velocity (Li et al., 2012) and reductions in neuropathic pain (Sharma et al., 2010).

Neurotrophins induce changes in the expression of Na^+ and K^+ channels and glutamate receptors, then down-regulation of NGF, BDNF and GDNF may reduce ectopic impulses generated in injured axons and sensory neurons, which contribute to the development of neuropathic pain (Navarro et al., 2007; Pezet and McMahon, 2006). The sustained reduction of hyperalgesia after exercise interruption may be explained by the reduction of early neurotrophins expression.

The transition from acute pain to chronic neuropathic pain is a highly complicated process. Exercise also reduces the activation of microglia in the dorsal horn of the spinal cord being a critical component in the initiation and maintenance of neuropathic pain (Scholz and Woolf, 2007; Leung and Cahill, 2010; Wen et al., 2011). Other studies also suggest that microglia activation can be related to the hyperalgesia observed after peripheral nerve injuries, and because their activation is more prolonged than that of microglia, astrocytes are required to perpetuate the neuronal hyperexcitability, neurotoxicity and inflammation that characterizes neuropathic pain (Ji and Suter, 2007; Gosselin et al., 2010; Zhuo et al., 2011)

In active microglia and astrocytes, glycogen synthase kinase 3 (GSK-3) promotes the release of IL1β, IL6 and TNFα and inhibits the release of anti-inflammatory cytokines like interleukin 10 (IL10) (Beurel et al., 2010; Kaidanovich-Beilin and Woodgett, 2011; Maixner and Weng, 2013). A single bout of exercise can temporarily phosphorylate (inactivate) GSK-3 in rodents, but other studies (Manabe et al., 2013) found that chronic exercise training resulted in prolonged increases. Therefore, whatever effects exercise has on spinal glia would have to be initiated in a different manner and, consequently, would likely involve different signaling pathways. While there is some evidence that exercise training alters the activity of astrocytes (Bernardi et al., 2013) and microglia

(Cobianchi et al., 2010), the relationship between exercise, glial activation and neuropathic pain requires much more investigation.

Endogenous opiates such as metenkephalin and β-endorphin is enhanced during exercise, and their action promotes analgesia and feelings of well-being (i.e. the "runner's high"). Furthermore, Stagg et al. (2011) demonstrated not only that aerobic exercise training reduced neuropathic pain, but that the analgesic effect depended on the stimulation of central opioid receptors.

Heat shock protein 72 (HSP72) is a member of a family of heat shock proteins which expression tends to decrease with diseases like diabetes (Atalay et al., 2004) but aerobic exercise enhances its expression throughout the body, including in the peripheral nerves, spinal cord (Chen et al., 2013) and in skeletal muscles (Ogata et al., 2009). HSP72 has been shown to increase tolerance to inflammatory cytokines, and there is some evidence that it may reduce their secretion by inactivating the nuclear factor kappa B (NF-κB) pathway (Yamada et al., 2008). Additionally, a very recent study by Chen et al. (2013) demonstrated that exercise-induced increases in HSP72 delayed the onset hyperalgesia and mechanical allodynia in diabetic rats (Table 4).

Electrical stimulation

Electrical stimulation is not a new treatment for pain. The first report dates from the first century (Kelleway, 1946) when the electric torpedo fish was applied to the head of patients suffering chronic headaches. It was in the 19_{th} century that electrical stimulation reached its peak of popularity. The widespread application of this treatment to all forms of disease quickly lead to its disrepute and disuse. In 1963, Melzack and Wall propounded an explanation for the pain-relieving effect of electrical stimulation. Basically, they suggested that electrical stimulation of the sensitive, low threshold A fibers would interfere with or modulate the passage of impulses in the Aδ and C fibers, normally thought to subserve pain. It was considered that this modulation occurred at the dorsal horn level in the spinal cord. This proposition had an immediate effect in regenerating an interest in the clinical application of electrical stimulation to all parts of the nervous system.

Pharmacological relief of neuropathic pain may be sometimes insufficient, thus electrical neurostimulation may be applied. On average 30–40% of the patients do not achieve sufficient pain relief only with medication (Finnerup et al., 2005; Attal et al., 2006). Neurostimulation methods are used primarily for pharmacoresistant chronic pain, when long-term drug therapy is ineffective (Simpson 2003). The use of electricity to modulate pain has a surprising long history (Kozák et al. 2012). The neurostimulation techniques proposed for treating pain are: transcutaneous electrical nerve stimulation (TENS), peripheral nerve stimulation (PNS), nerve root stimulation (NRS), spinal cord stimulation (SCS), deep brain stimulation (DBS), epidural

motor cortex stimulation (MCS), and repetitive transcranial magnetic stimulation (rTMS). These techniques vary greatly in their degree of invasiveness, stimulated structures and effectiveness, but they are all reversible.

Activity	Injury	Duration	Nerve regeneration	Collateral sprouting	Neurophatic pain
Swimming	SNC	3 d	↑ Axonal growth		
		25 m/d, 7 wk			↓ Allodynia 5-7 wk
		2 h/d, 3-6 wk	↓ Reinnervation		
		1-2 h/d, 4-6 wk	↑ Reinnervation		
Wheel running	Intact	3, 7 or 28 d	↓ MAG, PKA ↑ BDNF, GAP-43, CREB		
	SNC	3, 7 d	↓ Regeneraion ↑ BDNF, NT3, GAP-43		
	SNF	17/34 wk	↑ Degeneration in tibialis a. (2 wk) ↑ Regeneration in soleus muscle (8-12 wk)		
	L4T, L5T	8 h/d, 4 wk		↓ Sprouting in tibialis a. muscle	
	LGST	30 or 90 d	↑ Contraction	↑ MU enlargement	
Treadmill running	Intact	1 h/d, 4 wk	↑ BDNF, NT4, TrkB		
		9 h/d, 1-7 wk	↑ Sprouting ↑ Fiber type change		
	PNTR	3 h/d, 10 wk	↑ CNAPs		
	CCI	1 h/d, 1 wk	↑ Regeneration		↓ Allodynia
		1 h/d, 8 wk			↑ Allodynia
	L4T	10 wk pre-injury		↑↓ Sprouting in plantaris in solueus	
	L4T, L5T	8 h/d, 3-28 d		↓ Sprouting and Schwann cells bridging	
	SNC	1-2 h/d, 2-6 wk	↑ Type II fibers in plantaris muscle		
		1 h/d, 14 d	↑ DRGs neurite growth, Schwann cells proliferation ↑ GAP-43		
	SNTR	1 h/d, 4 wk	↑ Regeneration and reinnervation		↑ Sensory recovery
		20 m/d, 2 wk	↑ Axonal elongation and sprouting		
		60 m/d,2 wk	↑ Axon elongation but not sprouting		
		1 h/d, 4 wk	↑ Regeneration and rinnervation		↓ H-reflex excitability
Bicycle training	PNC	2 h/d, 4 d	↑ Sprouts and reinnervation		
	SNTR	1 h/d, 4 wk	↑ Regenerated axons and reinnervation		↓ H-reflex excitability
Mechanical stimulation	Spared nerve injury	Daily, 8 wk	↑ Reinnervation		
	MTR		No effect		
	DCNT	2 wk		↑ Sprouting nociceptive fibers	↑ Sensitivity

Table 4. Summary of activity-induced effects in animal models of peripheral nerve injuries. (Extracted from Udina et al., Ann Anat, 2011)

Peripheral stimulation

The first device that provided 'electro-treatment' appeared in 1918 and was designed for electrical stimulation of peripheral nerves. In 1965, Wall and Sweet, electrically stimulated the infraorbital and ulnar nerve.

Peripheral stimulation comprises TENS, PNS and NRS. The method is used for pain and disability along the distribution of a peripheral nerve (Hassenbusch et al. 1996). TENS was introduced into practice between the years of 1967-1970 (Stanton-Hicks 2003). While more sophisticated in design, in function, the TENS unit was not dramatically different from earlier devices designed to stimulate the peripheral nervous system.

TENS is the best-known technique. In this procedure surface electrodes are located over the painful area or the nerve that innervates it. Pain must be confined to a relatively small area or a territory that is innervated by an easily accessible nerve. Two different approaches are used in TENS protocols. Stimulation delivered at high frequency and low intensity (below pain threshold), to produce an intense activation of Aβ afferent fibers and to evoke paresthesiae that cover the painful area. Patients with severe loss of such fibers are unsuitable for this protocol. Stimulation delivered at low-frequency and high-intensity stimuli that do elicit painful sensations (this technique is also called acupuncture-like). Because the pain relief is immediate but short lasting, many patients have the need to use a portable stimulator. The pain-relieving effect of TENS increases with dose (duration of the session, frequency of sessions and total duration).

PNS is used when a more stable effect is desirable. In this procedure, electrodes are percutaneously implanted to contact directly the nerve. NRS is applied to cover the painful areas that are not accessible from the surface.

For all the above techniques, the probable mechanism of action for the current of high frequency and low intensity, is inhibition exerted by large-size afferents on spinothalamic pathways. It is important to know that this inhibition is strictly homotopical and that pain relief rapidly declines after stimulation is stopped (Cruccu et al., 2007). The acupuncture-like stimulation is thought to activate the antinociceptive systems mediated by the opioid system (Fukazawa et al., 2005; Zhang et al., 2005).

TENS may be often used as an adjunctive therapy to drugs or other physical treatments. Contrary to this, PNS and NRS are used in pharmacoresistant patients (Cruccu et al., 2007).

SCS

In SCS the spinal cord is stimulated, most often the dorsal pathways, but sometimes the lateral pathways of the spinal cord are also partially stimulated.

Shealy, Mortimore and Reswick in 1967 were the first to refer to the current name of SCS. They were working on stimulating the dorsal columns of the spinal cord testing the 'gate theory' of pain. In the beginning, it was also called dorsal column stimulation or DCS. From 1970s SCS became the most widely accepted term for this technique.

The technique consists of implantation of electrodes into the posterior epidural space of the thoracic or cervical spine ipsilaterally to the pain (if unilateral) and at an appropriate level to evoke paresthesiae which are a pre-requisite condition for success. Catheter or wire electrodes can be inserted percutaneously under local or general anesthesia. Plate electrode systems require an open operation but may be more effective.

Pathophysiological mechanisms were explained with several theories. Based on 'gate theory' is thought that antidromic conduction stimulation of Aβ fibers in the dorsal columns reduced pain in the stimulated segment. The opioid theory says that SCS increases levels of endorphins which are produced mainly in the NRM and in PAG. Other theory is the activation of DNIC. It starts at the subnucleus reticularis dorsalis in the reticular formation of medulla oblongata neurons and ends on the WDR neurons in the spinal cord. Another SCS effect is the increased blood flow caused by sympathetic blockade or induced release of CGRP, which acts as a vasodilator in peripheral vascular tissues.

SCS can modulate different elements of neuropathic pain, including allodynia and additionally it has also an anti-ischemic action, both cardiac and in the periphery, and other autonomic effects including the normalization of the autonomic manifestations of complex regional pain syndromes.

DBS

The theory behind deep brain stimulation was to find places where pain pathways aggregate and then to interrupt the pain pathways through stimulation or destruction of structures associated with pain representation or manifestation. The technique of DBS has been used since the 1950s, nevertheless it should be treated a last-chance therapy in patients in whom all the less invasive procedures have failed, as it carries a small but serious risk of intracranial hemorrhage (1–5%).

Deep brain targets include the sensory part of thalamus (VPL) and periventricular gray matter (PVG) as well as PAG contralateral to the pain if unilateral, or bilaterally if indicated. Both locations have been targets in the treatment of pain with DBS for three decades (Hosobuchi et al., 1973). For target localization brain MRI, stereotactic computerized tomography and brain atlas are used. An electrode is stereotactically inserted into subcortical brain structures under local anesthesia. In general, combined stimulation of PVG and VPL has better effects single-lead stimulation (Rasche et al., 2006).

Animal studies have shown that thalamic stimulation suppresses deafferentation pain, probably via thalamo-corticofugal descending pathways. Presently it is believed that stimulation of ventral PVG engages nonopioid dependent analgesia pathways, whereas stimulation of dorsal PVG involves opioid-related analgesia with associated autonomic effects (Green et al., 2006). What is interesting, lower frequencies (5–50 Hz) have analgesic, whereas higher frequencies (>70 Hz) pain- provoking effect.

Candidates for DBS are patients with peripheral neuropathic pain, trigeminal neuropathic pain and/or dysesthesia dolorosa, phantom-limb pain and central pain syndromes. The results in patients with central pain syndromes are however not favorable.

MCS

In this method, epidural electrodes are implanted over the central brain area through the frontoparietal craniotomy. MCS uses 20-50 Hz stimulation and stimulates the motor cortex (gyrus precentralis) with one or two electrodes implanted over the motor representation of the painful area, either parallel or orthogonal to the central sulcus. The stimulation parameters are adjusted post-operatively, keeping the intensity below motor threshold.

MCS targets the brain's motor cortex and stimulates orthodromic conduction in the motor thalamic nuclei, which leads more releasing of GABA which inhibits the thalamocortical tracks projecting into the posterior gyrus. MCS-induced pain relief may relate to down activation of descending pain control systems going from motorcortex to thalamus, and perhaps to motor brainstem nuclei as well as to blunting of affective reactions to pain via activation of orbitofrontal-perigenual cingulate cortex. The fact that many of the regions activated by MCS contain high levels of opioid receptors suggests that long-lasting MCS effects may also involve secretion of endogenous opioids.

Seizures, wound infection, sepsis, extradural haematoma, and pain induced by MCS have been occasionally reported. Overall 20% of patients experience one or more complications, usually of benign nature.

rTMS

rTMS is a non-invasive, painless stimulation of the cerebral cortex using magnetic fields. The stimulation is performed by applying on the scalp, above a targeted cortical region, the coil of a magnetic stimulator. rTMS has become increasingly popular as a method for treating various neuropsychiatric diseases (Fregni and Pascual- Leone, 2007), and the number of clinical trials and clinical applications continues to increase dramatically. Transcranial stimulation can be used with the application of a pulse (single-pulse TMS) applied with a pair of pulses with variable intervals

(paired-pulse TMS) or repeating pulses (repetitive TMS). The methods are distinguished based on the stimulation frequency used: fast, high-frequency rTMS, which operates at frequencies over 1 Hz and a slow low-frequency rTMS at a frequency rate of 1 Hz or less.

Use of rTMS in healthy volunteers it decreased the sensory pain threshold. It was later revealed that this effect was also present in patients with various types of chronic pain (Lefaucheur, 2008). rTMS also reorganizes the cerebral cortex and other brain areas involved in the development of chronic pain. (Hirayama et al. 2006).

The rationale to use rTMS is the same as for MCS. The stimulation is thought to activate some fibres that run through the motor cortex and project to remote structures involved in some aspects of neuropathic pain processing. The greatest advantage is that the method is non-invasive and can be applied to any patient with drug-resistant, chronic neuropathic pain, who could be candidate for the implantation of a cortical stimulator.

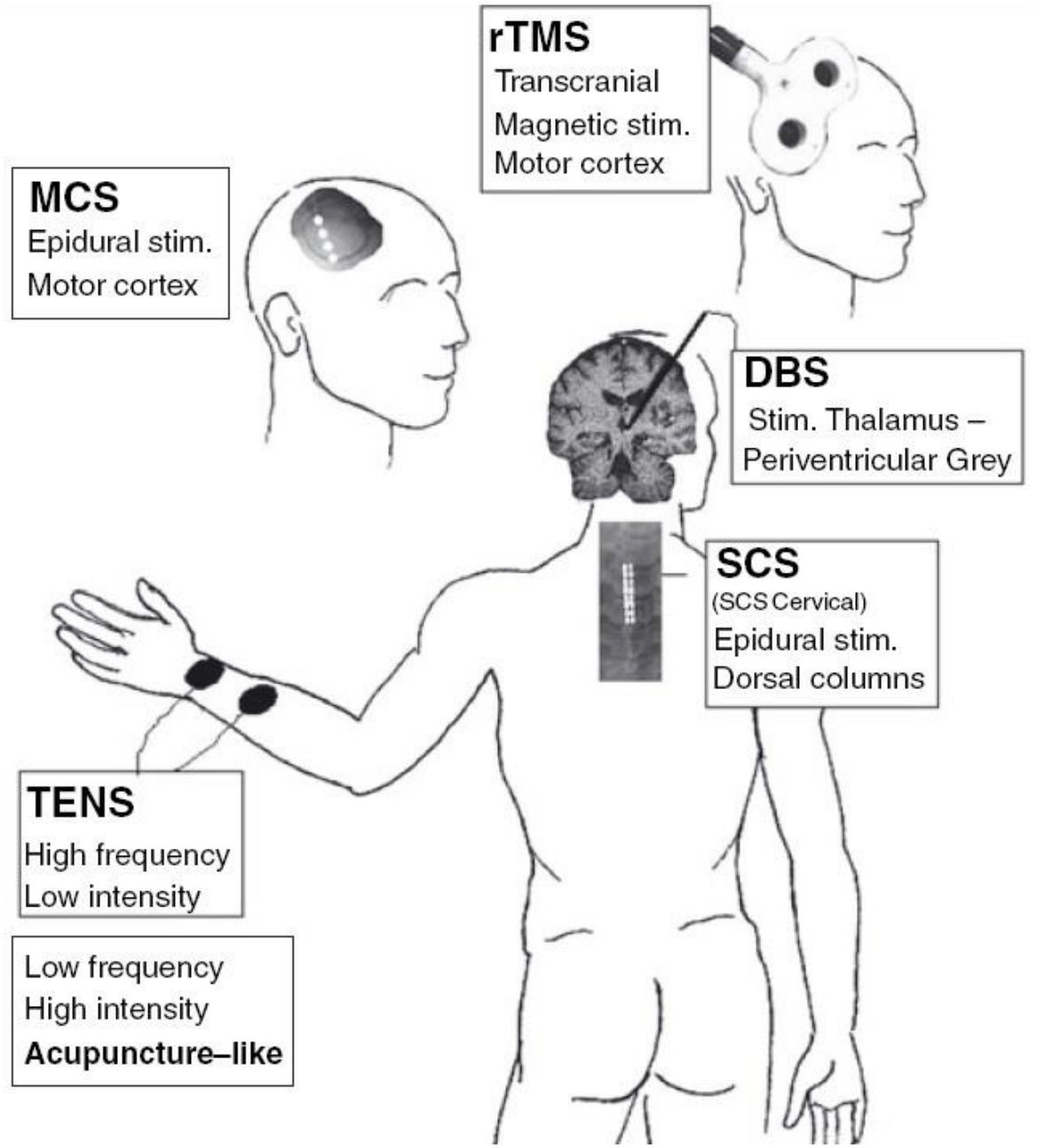

Fig. 10: Schematic representation of different neurostimulation procedures. (Extracted from Cruccu et al., Eur J Neurol, 2007)

OBJECTIVES

OBJECTIVES

The main goal of the present thesis was to validate the use of activity-dependent treatments like treadmill exercise and peripheral nerve stimulation for neuropathic pain. With this aim, we established the following specific objectives:

1. To characterize an effective protocol of treadmill exercise for the treatment of neuropathic pain.

2. To characterize an effective protocol of peripheral nerve stimulation for the treatment of neuropathic pain.

3. To reveal the mechanisms producing hypoalgesia by treadmill exercise in an experimental model of neuropathic pain.

4. To reveal the mechanisms producing hypoalgesia by peripheral nerve stimulation in an experimental model of neuropathic pain.

RESULTS

Chapter 1

Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury

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Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury

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Abstract

Activity treatments, such as treadmill exercise, are used to improve functional recovery after nerve injury, parallel to an increase in neurotrophin levels. However, despite their role in neuronal survival and regeneration, neurotrophins may cause neuronal hyperexcitability that triggers neuropathic pain. In this work, we demonstrate that an early increasing-intensity treadmill exercise (iTR), performed during the first week (iTR1) or during the first 2 weeks (iTR2) after section and suture repair of the rat sciatic nerve, significantly reduced the hyperalgesia developing rapidly in the saphenous nerve territory and later in the sciatic nerve territory after regeneration. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) expression in sensory neurons and spinal cord was reduced in parallel. iTR prevented the extension of collateral sprouts of saphenous nociceptive calcitonin gene-related peptide fibers within the adjacent denervated skin and reduced NGF expression in the same skin and in the L3 dorsal root ganglia (DRG). Injury also induced Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) upregulation in DRG, and K^+-Cl^- cotransporter 2 (KCC2) downregulation in lumbar spinal cord dorsal horn. iTR normalized NKCC1 and boosted KCC2 expression, together with a significant reduction of microgliosis in L3-L5 dorsal horn, and a reduction of BDNF expression in microglia at 1 to 2 weeks postinjury. These data demonstrate that specific activity protocols, such as iTR, can modulate neurotrophins expression after peripheral nerve injury and prevent neuropathic pain by blocking early mechanisms of sensitization such as collateral sprouting and NKCC1/KCC2 disregulation.

Introduction

Activity-based treatments such as treadmill exercise are used to improve functional recovery after both peripheral and central nervous system lesions.[30,66,73] Several studies have demonstrated that moderate-intensity treadmill exercise may enhance regeneration of injured axons and recovery of sensorimotor functions.[3,22,42,63] The most frequently reported underlying mechanism was an increase in neurotrophin levels, particularly BDNF, which is beneficial for survival and regeneration of injured axons.[48,77] However, intense and prolonged exercise reduces collateral sprouting of motor axons for compensatory reinnervation of denervated muscle fibers.[40,65] We previously found that prolonged electrical stimulation or intense locomotion training also have detrimental effects on peripheral nerve regeneration and neuropathic pain.[3,13] However, using an increasing intensity treadmill exercise (iTR) performed early after injury, significantly reduced mechanical allodynia after sciatic nerve chronic constriction injury and section and suture repair[12,13] without impairing nerve regeneration. Interestingly, hyperalgesia measured in the innervation territory of the uninjured saphenous nerve was significantly reduced, together with decreased levels of BDNF and NGF in dorsal root ganglia (DRG).[12] These findings suggest that treadmill training, when applied at increased intensity and only early after injury, may prevent neurotrophin-mediated hyperexcitability of injured and uninjured sensory neurons that underlies neuropathic pain.

Because the role of NGF and BDNF in activity-induced hypoalgesia is not well defined, a first hypothesis that we tested is that early applied iTR exercise may reduce the hyperalgesia occurring in the saphenous skin territory by preventing the sprouting of uninjured axons into the denervated adjacent skin. Collateral reinnervation of denervated targets is an adaptive mechanism for sensory recovery. Sensory fiber sprouting is induced by target-derived NGF,[18,43] mostly affecting peptidergic nociceptors that express TrkA receptor. Despite playing a role on neuronal survival and regeneration, NGF and BDNF may also trigger neuropathic pain.[53,58] In fact, anti-NGF and anti-TrK receptor treatments prevent the spread of sprouting into denervated skin and reduce hyperalgesia in nerve injury models.[17,67,74]

However, we assessed if iTR exercise was able to restore chloride homeostasis after peripheral nerve injury, which may be associated with a reduction in BDNF levels, as recently demonstrated after spinal cord injury.[14] BDNF released by activated microglia induces downregulation of the neuron-specific K^+-Cl^- cotransporter 2 (KCC2).[15,26] Along with Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1), present in primary afferent neurons, these cotransporters are responsible for setting the reversal potential for GABA and glycine receptors in the control of sensory inputs at the dorsal horn.[56,78] After nerve injury, disruption of chloride homeostasis by NKCC1 upregulation

and KCC2 downregulation has been linked to hyperalgesia.[12,46] We previously observed that iTR prevented microglia activation in the spinal cord dorsal horn and modulated the expression of neurotrophins in sensory neurons[12,13] after sciatic nerve lesions. Therefore, we investigated the effects of iTR on collateral reinnervation of denervated skin and changes in chloride cotransporters homeostasis to identify the mechanism that induces pain after nerve injury.

Materials and methods

Animals and surgery

Adult female Sprague-Dawley rats (240±30 g) were housed in standard cages with access to food and water ad libitum under a light–dark cycle of 12 hours. All the experimental procedures were approved by the Ethics Committee of the Universitat Autonoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU).

Rats were anesthetized by intraperitoneal injection of ketamine (10 mg/kg, Imalgene 500; Rhone-Merieux, Lyon, France) and xylazine (1 mg/kg, Rompun; Bayer, Leverkusen, Germany). The right sciatic nerve was exposed at the midthigh, transected at 92 mm from the tip of the third toe, and repaired by epineural sutures (10-0), maintaining the fascicular alignment of tibial, peroneal, and sural branches. The wound was closed in layers. Rats were kept in a warm environment until their recovery from anesthesia.

Experimental design

Seven days before surgery, all of the animals were habituated to the experimental device for treadmill locomotion (Treadmill LE 8706; LETICA, Barcelona, Spain) and pretrained to the task, by leaving them to explore the stopped treadmill for 5 minutes and then trained in a single iTR session. Each iTR session consisted of 1-hour running, starting at a locomotion speed of 10 cm/s that was increased 2 cm/s every 5 minutes, until a maximal speed of 32 cm/s.[12,13] At day 3 after surgery, animals were randomly selected to follow or not the iTR after injury. A group of rats was trained with daily sessions for 5 consecutive days, from 3 to 7 days postinjury (dpi) (iTR1 group, n=8), whereas another group continued training from 10 to 14 dpi (iTR2 group, n=7). A third group of injured rats was untrained and served as a control group (C group, n=8). Naive rats were used for immunohistochemical and biochemical assays, along with additional injured rats (n=4 per group at each time point). Sensory tests were performed during the morning, whereas treadmill running sessions were performed during the afternoon.

Nociceptive behavior tests for sensory threshold measurement

Seven days before surgery, all of the injured animals were habituated to the experimental devices, by allowing them to explore the apparatus for 20 minutes and then starting the test for baseline nociceptive thresholds recording. The nociceptive behavior tests for mechanical and thermal stimuli were performed on both hind paws before and at different days after injury. In postoperative sensory tests, the experimenter was blind to the assignment of rats to the different groups. Because in this injury model responses in the sciatic territory are completely lost until its regeneration, a medial and a lateral test sites were used[12] to differentiate changes in sensory thresholds produced by saphenous nerve sprouting from those because of sciatic denervation/regeneration.

Sensitivity to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 10 minutes before the experiment for habituation. A nonnoxious pointed probe was gently applied to each test site, and then the pressure was slowly increased. The threshold was expressed as the force (in grams) at which rats withdrew the paw in response to the stimulus. A cutoff force was set at 40 g, when the stimulus lifted the paw without response. The mechanical nociceptive threshold was calculated as the mean of 3 measurements per test site, with a 3-minute interval between each measurement.

Thermal sensitivity was assessed by means of a Plantar test algesimeter (Ugo Basile, Comerio, Italy). Rats were placed into a plastic box with an elevated plexiglass floor. The beam of a lamp was pointed at the same test sites as above in the hind paw plantar surface. Intensity was set to low power (40 mW/cm^2) with a heating rate of 1°C/s to elicit activation of unmyelinated fibers.[36] A cutoff time for the stimuli was set at 20 seconds to prevent tissue damage. Heat pain threshold was calculated as the mean of 3 trials per test site, with a 5-minute resting period between each trial, and expressed as the latency (in seconds) of paw withdrawal response.

Assessment of skin nociceptive reinnervation

The progression of nociceptive responses attributable to reinnervation of the hind paw plantar skin was assessed by the pinprick test.[12,49] Awake animals were gently restrained in a cloth with the injured paw plantar surface facing upwards, and the plantar skin was stimulated with a 24-G needle progressively from distal to proximal distinct areas (Fig. 1B). Responses were recorded as positive when an evident response (defined as fast paw withdrawal and vocalization) was triggered by the stimulus and taken as sign of skin functional reinnervation. An index of functional reinnervation was calculated as the number of responsive areas with respect to the total areas stimulated by pinpricking.

Immunohistochemistry of paw skin

To analyze if the early sensory changes measured at the medial test site were correlated with changes in nerve fiber sprouting, the plantar pad adjacent to the medial algesimetry test site was removed from both hind paws of rats at different days after sciatic nerve injury. Transcardiac perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2), the harvested samples were kept in fixative for 1 hour and then cryoprotected with 30% sucrose in PBS with azide. Cryotome sections 60 μm thick were incubated free floating overnight with primary antibody against protein gene product 9.5 (PGP, rabbit, 1:1000; ABD Serotec, Kidlington, United Kingdom) to label all the nerve fibers, or doubly with antibodies against calcitonin gene-related peptide (CGRP, goat, 1:500; Abcam, Cambridge, United Kingdom) and growth-associated protein 43 (GAP43, rabbit,1:1000; Millipore, Billerica, MA) for labeling peptidergic fibers and growing axons, respectively. Secondary antibodies were conjugated to Cy3 and Cy5 (1:200; Jackson ImmunoResearch, West-grove, PA, 1-hour incubation). Three footpad mid-plan sections from 4 samples per group were randomly used for quantification of skin innervation. The experimenter was blinded to the sample group. Intraepidermal nerve fibers (IENFs) were manually counted under an Olympus BX51 fluorescence microscope in a footpad epidermis field 500 μm long adjacent to the algesimetry test site. Footpad images for subepidermal nerve fibers (SENFs) measurement were captured with a Zeiss LSM 700 confocal microscope and analyzed using ImageJ software (NIH, Bethesda, MD). The length of SENFs was measured inside a 500 x 200 μm region of interest (ROI) drawn below the epidermal basal membrane adjacent to the site of algesimetry test. A diagram of the ROI used for IENFs and SENFs quantification is shown in Figure 2B.

Because inhibition of mature NGF degradation in the skin induces sensory fiber sprouting and significantly decreases sensory thresholds, as previously demonstrated,[54] we used an anti-NGF antibody (mouse, 1:200; Santa Cruz, Dallas, TX, overnight) that recognizes its mature form. Alexa 488 anti-mouse secondary antibody (1:200; Invitrogen, Carlsbad, CA, 1 hour) was used for this labeling. To assess NGF immunoreactivity in the intact saphenous innervated area, the medial plantar pads were harvested and processed as above. We quantified NGF immunolabeling in the same ROI drawn for SENFs measurement. A threshold for subtracting background was applied by defining a fixed grayscale cutoff point set from the same area of naive rats footpad subepidermal region. Then, the integrated density of NGF labeling was measured.

Immunohistochemistry of dorsal root ganglia and spinal cord

The L3-L5 spinal cord segment and the L3 DRG were removed from perfused animals and kept in fixative for 12 hours and 1 hour, respectively, and then cryoprotected with 30% sucrose

in PBS. Transverse sections, 25μm thick, of the lumbar tract of spinal cord (L3-L6) were cut on a cryostat and mounted on slides. Sections were first incubated overnight with anti-Iba1 (rabbit, 1:1000; Wako) or anti-BDNF (sheep, 1:100; Millipore) antibodies, in 0.3% Triton X-100 in PBS, and then washed and incubated for 1 hour with Alexa 594 anti-rabbit (1:200; Invitrogen) or Biotinylated anti-sheep (1:200; Vector) followed by Alexa 488 Streptavidin (1:200; Invitrogen). After washing in PBS, sections were coverslipped with a solution of DAPI (1 μL/mL) in Mowiol mounting medium. Transverse sections of DRG were cut at 20 μm thickness and processed as above. For double immunofluorescence staining, sections were first incubated overnight with anti-CGRP (goat, 1:500; Abcam) and anti-GAP43 (rabbit, 1:500; Millipore) antibodies, washed, and then incubated for 1 hour with Cy3 anti-goat (1:200; Jackson ImmunoResearch) and Alexa 488 anti-rabbit (1:200; Invitrogen). Three sections of 4 samples per group were randomly used for quantification of DRG and spinal cord immunolabeling. The experimenter was blinded to the sample group. Images of sections were acquired with a Zeiss LSM 700 confocal microscope and analyzed using ImageJ software (NIH). Counts were made by using thresholding as above across all the sample images of the same staining.

Quantification of microglia and BDNF immunoreactivity in spinal cord

To quantify Iba1-positive microglial cells in the spinal cord, 3 sections per animal were randomly selected from L3 (taken as saphenous projection) and from L5 (taken as sciatic projection) spinal cord. Confocal images of the ipsilateral side of the dorsal horn (laminae I-III) were captured at 340 using the same set of acquisition parameters. Areas of Iba1 immunoreactivity were outlined and expressed as percentage relative to the total image area. For double-labeling with BDNF, we calculated the integrated density (InDen) of BDNF immunoreactivity colocalizing in Iba1-positive areas using ImageJ program.

Protein extraction and Western blot

Subsets of 4 injured rats per group and 4 additional uninjured rats as controls were deeply anesthetized and killed at different times postinjury. Ipsilateral and contralateral medial plantar pads and L3-L6 DRG were harvested. The lumbar enlargement of the spinal cord was dissected and divided in quarters. We quantified the levels of NGF in footpad skin and DRG, of NKCC1 and pNKCC1 in DRG, and of KCC2 and pKCC2 in the dorsal horn. Samples were prepared for protein extraction and homogenized in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 1 mM EDTA) adding 10 μL/mL of protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) and PhosSTOP phosphatase inhibitor cocktail (Roche, Indianapolis, IA). After clearance, protein concentration was measured

by Lowry assay (Bio-Rad, Hercules, CA; DC protein assay). For Western blot, 20 mg of protein of each sample was loaded in SDS-polyacrylamide gels. The transfer buffer was 25 mM Trizma base, 192 mM glycine, 20% (vol/vol) methanol, pH 8.4. The membranes were blocked with 5% BSA in PBS plus 0.1% Tween-20 for 1 hour and then incubated with primary antibodies at 4˚C overnight. The primary antibodies used were mouse anti-GAPDH (1:20,000; Millipore), mouse anti-NGF (1:200; Santa Cruz), rabbit anti-phospho-^{Ser940}KCC2 (1:1000, PhosphoSolutions), rabbit anti-KCC2 (1:500; Millipore), mouse anti-NKCC1 (1:1000; Hybridoma bank), and sheep anti-phosphoNKCC1 (1:1000; Protein Phosphorylation and Ubiquitylation Unit, University of Dundee). Horseradish peroxidase–coupled secondary antibody (1:5000; Vector) incubation was performed for 1 hour at room temperature. The membranes were visualized using enhanced chemiluminiscence method, and the images were collected and analyzed with a Gene Genome apparatus, using Gene Snap and Gene Tools software (Syngene, Cambridge, United Kingdom). Western blot values are normalized to tubulin or GAPDH and presented as fold increase in injured samples (C and iTR groups) compared with naive animals.

Data analysis

Data are presented as mean ± SEM. Statistical analysis of nociceptive thresholds was made by 2-way analysis of variance with group and time after injury as factors, followed by Bonferroni's post hoc comparisons. Statistical significance for immunofluorescence and immunoblotting data was calculated by 1-way and 2-way analysis of variance (for multiple groups comparison) followed by Tukey's post hoc test when necessary. The level of statistical significance was 5% (P , 0.05) in all the analyses.

Results

iTR reduces hyperalgesia occurring in both saphenous and sciatic nerve territories

Sciatic nerve injury induced a fast decrease of withdrawal threshold to mechanical stimulation on the medial side that remained about 25% of contralateral values during the entire follow-up (P , 0.001 from 3 to 60 dpi) (Fig 1A, left panels). This reflected a state of chronic adjacent hyperalgesia in the saphenous nerve and adjacent skin territories. The decrease of thermal threshold in the medial side was slower but also significant (P , 0.001 at 15, 21, and 28 dpi; P , 0.05 at 40 dpi; P , 0.01 at 50 dpi), following a trend to recover.[72] The iTR1 significantly prevented the decrease of saphenous mechanical (P , 0.001 at 8 and 15 dpi; P , 0.05 at 21 dpi) and thermal (P , 0.01 at 8 dpi; P , 0.05 at 15 dpi) withdrawal thresholds. The reduction of mechanical hyperalgesia was

maintained for several weeks in the iTR1 rats. Prolonging the treadmill training until 14 dpi produced a similar increase in the mechanical threshold (iTR2 group, P , 0.001 at 15 dpi).

However, the sciatic lateral territory became insensitive because of denervation until 3 to 4 weeks after injury, when algesimetry responses returned parallel to reinnervation by regenerating axons (Fig. 1A, right panels), reflecting a course from hypoesthesia (P , 0.001 at 21 dpi) to mechanical hyperalgesia (P , 0.01 at 28 dpi; P , 0.001 at 40, 50, and 60 dpi), similar to that reported during reinnervation after a crush nerve injury.[8,59] Thermal withdrawal responses returned to normal levels from the fourth week after nerve injury. Even if treadmill training was concluded well before sciatic reinnervation, recovery of lateral mechanical responses was improved, as indicated by significantly higher thresholds compared with untrained rats.

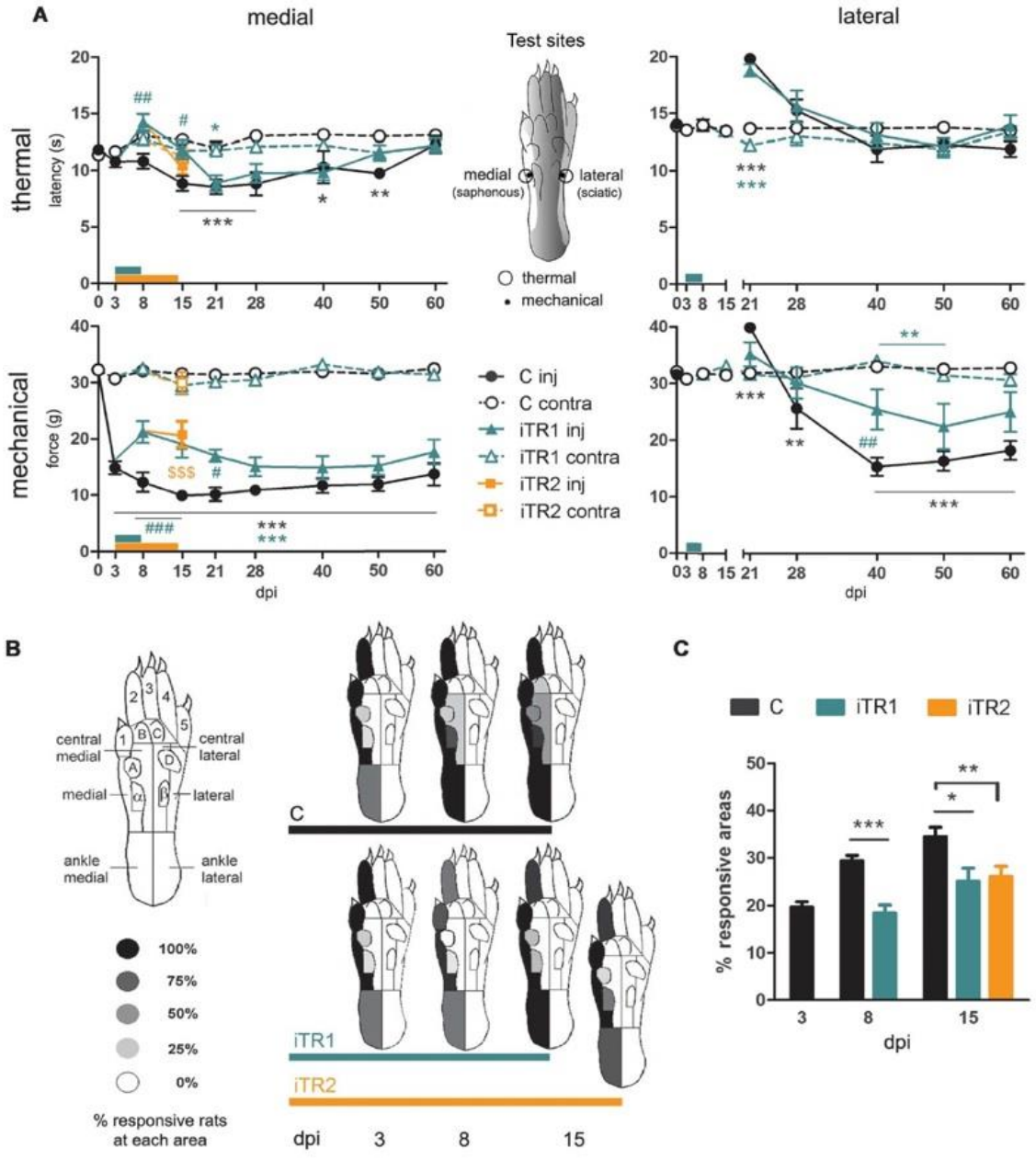

Figure 1: iTR reduces mechanical hyperalgesia and inhibits saphenous collateral sprouting after sciatic nerve injury. (A) Thermal (top plots) and mechanical thresholds (bottom plots) measured at the medial saphenous (left plots) are decreased in the injured paw of the untrained control group (C group, black line) in comparison with the contralateral intact paw. Animals trained from 3 to 7 dpi (iTR1 group, green line) showed significantly higher mechanical and thermal thresholds compared with the C group. Extension of exercise training until 14 dpi (group iTR2, orange line) did not increase further the thresholds compared with group iTR1, demonstrating that treadmill hypoalgesic effect was induced during the first week after injury. Threshold values measured at the lateral sciatic test site (right plots) showed an initial loss of sensitivity with gradual recovery of responses from 21 dpi in the injured paw of group C with the development of late mechanical hyperalgesia. Group iTR1 showed higher mechanical thresholds demonstrating that early exercise positively affected the recovery of sensitivity at long time. Drawing of the rat paw with indication of the test areas and the diameter of the stimuli (thermal and mechanical) is shown in the center. *P,0.05, **P,0.01, ***P, 0.001 for each color/group injured vs contralateral paws; #P , 0.05, ##P , 0.01, ###P , 0.001 for iTR1 group injured vs C group injured paw; $$$P , 0.001 for iTR2 group injured vs C group injured paw at 15 dpi. (B) Drawing of the rat paw with indication of the plantar areas stimulated by pinpricking to detect pain innervation. After sciatic nerve injury, rats of the C group showed positive pinprick responses only at the medial saphenous areas, but fast nociceptive reinnervation progressed toward sciatic areas. The percentage of responsive rats was increasing in central areas of the sole at 8 and 15 dpi. (C) Collateral reinnervation measured before sciatic regeneration measured as a plot of the group mean percentage of plantar areas responsive to pinprick. In iTR1 and iTR2 groups, the collateral reinnervation was inhibited because responses remained confined to the medial areas. The percentage of positively responsive areas of all the stimulated areas linearly increased in time in group C, whereas iTR rats did not show any increase at 8 dpi (***P,0.001) and lower expansion at 15 dpi (*P,0.05, iTR1 group; **P , 0.01, iTR2 group).

iTR reduces collateral sprouting and nociceptive reinnervation of plantar skin

Sciatic nerve injury induces intact saphenous nerve fibers to sprout and rapidly reinnervate the adjacent denervated skin from medial to central areas of the paw, invading areas normally innervated by the sciatic nerve.[12] The number of untrained rats responding to pinpricking (Fig. 1B) and the number of responsive areas (sprouting index; Fig. 1C) gradually increased from 3 to 15 dpi. In contrast, responses to noxious stimulation remained confined to medial saphenous areas in iTR1 rats (Fig. 1B), with a sprouting index significantly lower (P , 0.05) than in the C group (Fig. 1C). iTR2 rats showed a similar effect (P , 0.01 at 15 dpi).

We characterized the time course of reinnervation of the medial skin by immunohistochemical labeling for PGP and CGRP, markers of all nerve fibers and peptidergic nociceptive fibers, respectively (Fig. 2A). After injury, IENFs mostly disappeared, whereas many PGP-positive Langerhans cells were observed in the denervated epidermis. SENFs rapidly elongated through the dermal layers from the medial skin towards the previously denervated footpad and extended along the dermoepidermal junction without entering the epidermis, as we previously described.[12] Sprouting peptidergic SENFs expressed GAP43, which is detected at very low levels in the normal

skin. In the medial footpad of both iTR1 and iTR2 rats, there were almost no SENFs and shorter CGRP IR profiles at 8 and 15 dpi compared with untrained rats (Fig. 2A).

Intraepidermal nerve fibers and SENFs were distinctly quantified in areas of epidermal and subepidermal plexus layers, respectively (Fig. 2B, dotted boxes), representing the footpad skin fields just adjacent to the nonfootpad nociceptive behavior test site (Fig. 2B, asterisk). In these areas, iTR groups showed very few GAP43 IR profiles, further indicating poor nociceptive fiber sprouting into the denervated footpad. The counts of IENFs were markedly decreased after injury in untrained rats, but GAP43 IR IENFs increased (Fig. 2C). In parallel, the SENFs length increased across the subepidermal layer, with strong GAP43 expression (Fig. 2D). The mean number of PGP IR IENFs in iTR rats was similar to untrained C rats (Fig. 2C), but the length of SENFs and the percentage of nociceptive fibers expressing GAP43 were significantly reduced in the injured paw (Fig. 2D).

iTR reduces NGF expression in plantar skin

Sensory neurons expressing TrkA bind NGF synthesized in the skin and internalize the receptor–ligand complex, which is retrogradely transported to the cell body where it exerts biological actions on the neuron.[58,75,76] Mature NGF immunoreactivity in the normal rat footpad skin was observed in sparse keratinocytes and fibroblasts, mostly in the dermis and surrounding peptidergic fibers, marked with CGRP (Fig. 3A, naive group). In the medial footpad, an increase of NGF expression in dermal fibroblasts, keratinocytes, and vessels was observed at 8 dpi, being more evident at 15 dpi, when NGF IR profiles were seen in close proximity to sprouting CGRP fibers (Fig. 3A, C group). In contrast, iTR rats showed levels of NGF immunoreactivity in the medial footpad similar to naive rats (Fig. 3A, iTR1-2 groups). Treadmill training prevented the increase of NGF expression in the injured paw skin (Fig. 3B), both at 8 dpi (iTR1 group, P , 0.05) and at 15 dpi (iTR2 group, P , 0.05), observed in the C group. Western blot of NGF at 8 dpi (Fig. 3C) confirmed a significant reduction of mature NGF levels in the medial footpad of iTR1 rats samples (P , 0.05, C group vs iTR1 group), in contrast to the marked increase found in C group rats after sciatic nerve injury compared with naives.

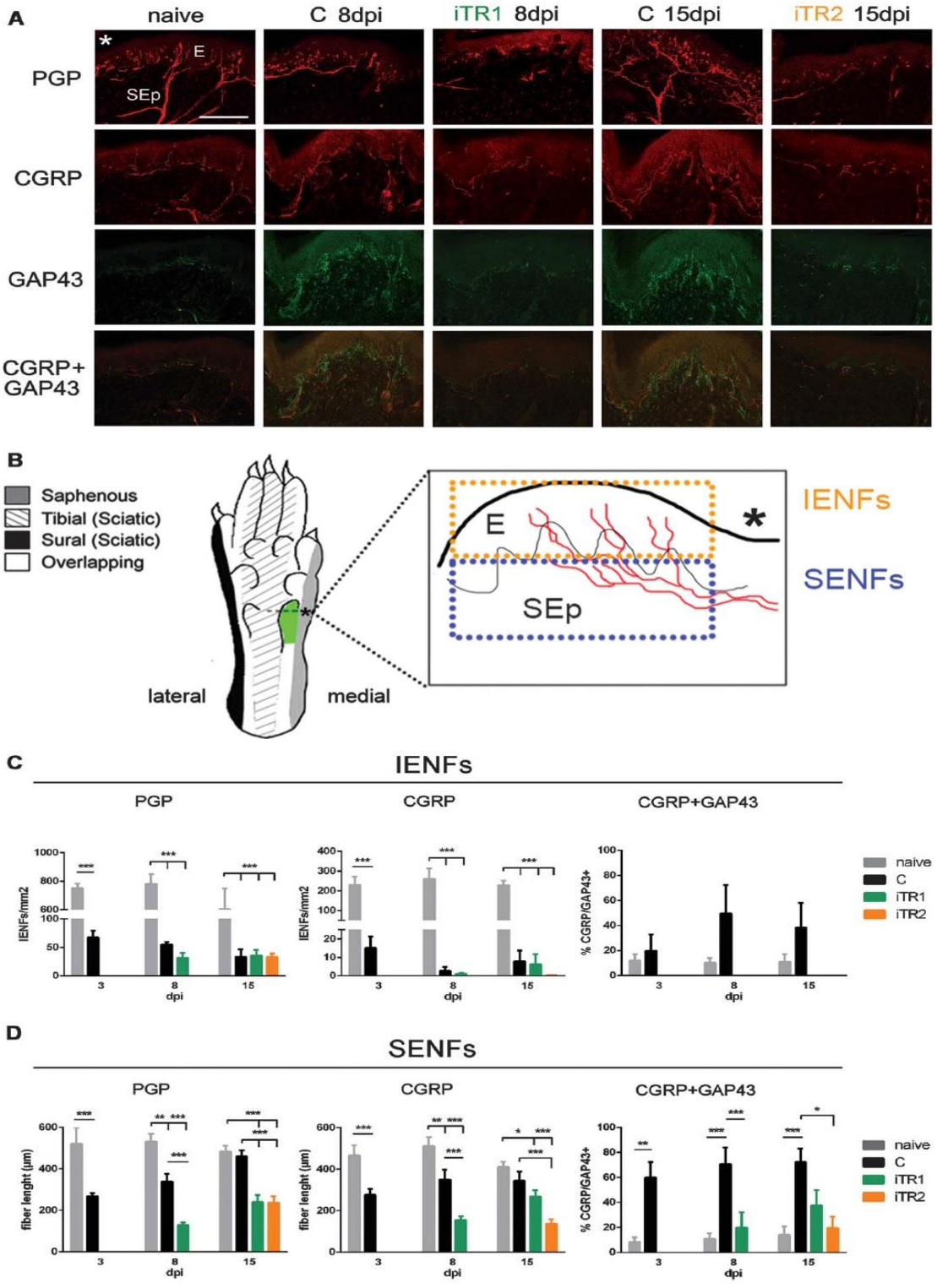

Figure 2: iTR blocked early collateral sprouting within the denervated adjacent skin. Pictures of medial footpad epidermal (E) and subepidermal (SEp) skin layers extending from the nociceptive behavior test site (asterisk) to the median footpad dermis. (A) After sciatic nerve injury, sensory PGP-IR fibers were markedly reduced (C group compared with naive). Some nerve profiles elongated from the test site through the subepidermal plexus from 8 to 15 dpi. Footpads of iTR1 and iTR2 rats showed almost no collateral reinnervating profiles at 8 and 15 dpi, respectively, after finishing the training period. Calcitonin gene–related peptide immunolabeling shows that peptidergic elongating profiles strongly expressed GAP43, which is scarce in the skin of naive rats. In contrast, iTR1

and iTR2 rats showed the absence of nociceptive fibers sprouting and no GAP43 expression. Scale bar: 200 μm. (B) Intra-epidermal nerve fibers (IENFs) and sub-epidermal nerve fibers (SENFs) profiles were distinctly quantified in areas of epidermal (E) and sub-epidermal (SEp) layers (yellow and blue dotted boxes, respectively), representing the medial footpad (green) skin fields just adjacent to the non-footpad nociceptive behavior test site (asterisk). Sciatic and saphenous territories of innervation are also represented in the plantar hind paw diagram). (C) After sciatic nerve injury, the density of peptidergic IENFs was strongly reduced at the medial footpad. (D) However, the length of SENFs increased with time in group C injured paws from 3 to 15 dpi. The proportion of CGRP-IR peptidergic fibers (60%-70%) expressing GAP43 largely increased in the injured paw, demonstrating a growing phenotype after lesion. iTR reduced the length of SENF of PGP and CGRP IR fibers and strongly decreased the rate of CGRP 1 GAP43 IR fibers in the injured paw compared with the C group. Data are shown as mean 6 SEM. *P , 0.05; **P , 0.01; ***P , 0.001.

iTR reduces NGF and GAP43 expression in the L3 dorsal root ganglia

Calcitonin gene-related peptide expression in sensory neurons colocalizes with the high-affinity NGF receptor TrkA, and its expression is mostly restricted to nociceptive neurons.[4,24] To assess the growing state of collaterally sprouting nociceptors, we first analyzed the expression of GAP43 in CGRP-positive neurons of the L3 DRG (Fig. 4A and B), which contains the soma of the saphenous neurons. Consistent with the growing phenotype of saphenous axons, GAP43 immunoreactivity was strongly in-creased in injured rats DRG neurons at 8 dpi, mostly in those expressing CGRP (Fig. 4A, arrows). In contrast, we found GAP43 expression strongly reduced in iTR1 rats, at least in the neuronal soma, with pattern of immunoreactivity appearing similar to normal. The number of CGRP neurons expressing GAP43 was significantly increased after injury (P , 0.001, C injured vs C contralateral; Fig 4B), but it was within the normal range in iTR1 rats (P , 0.01; iTR1 injured vs C injured; Fig. 4B), indicating that exercise counteracted the plastic change of nociceptive neurons.

The presence of NGF in L3 DRG neurons was analyzed by Western blot (Fig. 4C). NGF protein levels increased in untrained C group rats at 8 dpi, but returned to normal at 15 dpi, indicating that NGF production is upregulated during the first week. Applied within this time period, treadmill training significantly reduced NGF levels (P , 0.05, C injured vs iTR1 injured, 8 dpi) because iTR1 rats showed no significant differences with naive rats DRG.

These results demonstrate that inhibition of collateral sprouting induced by specific treadmill exercise is associated with modulation of peptidergic fiber plasticity and is parallel to reduction of NGF expression in sensory neurons and in target organs.

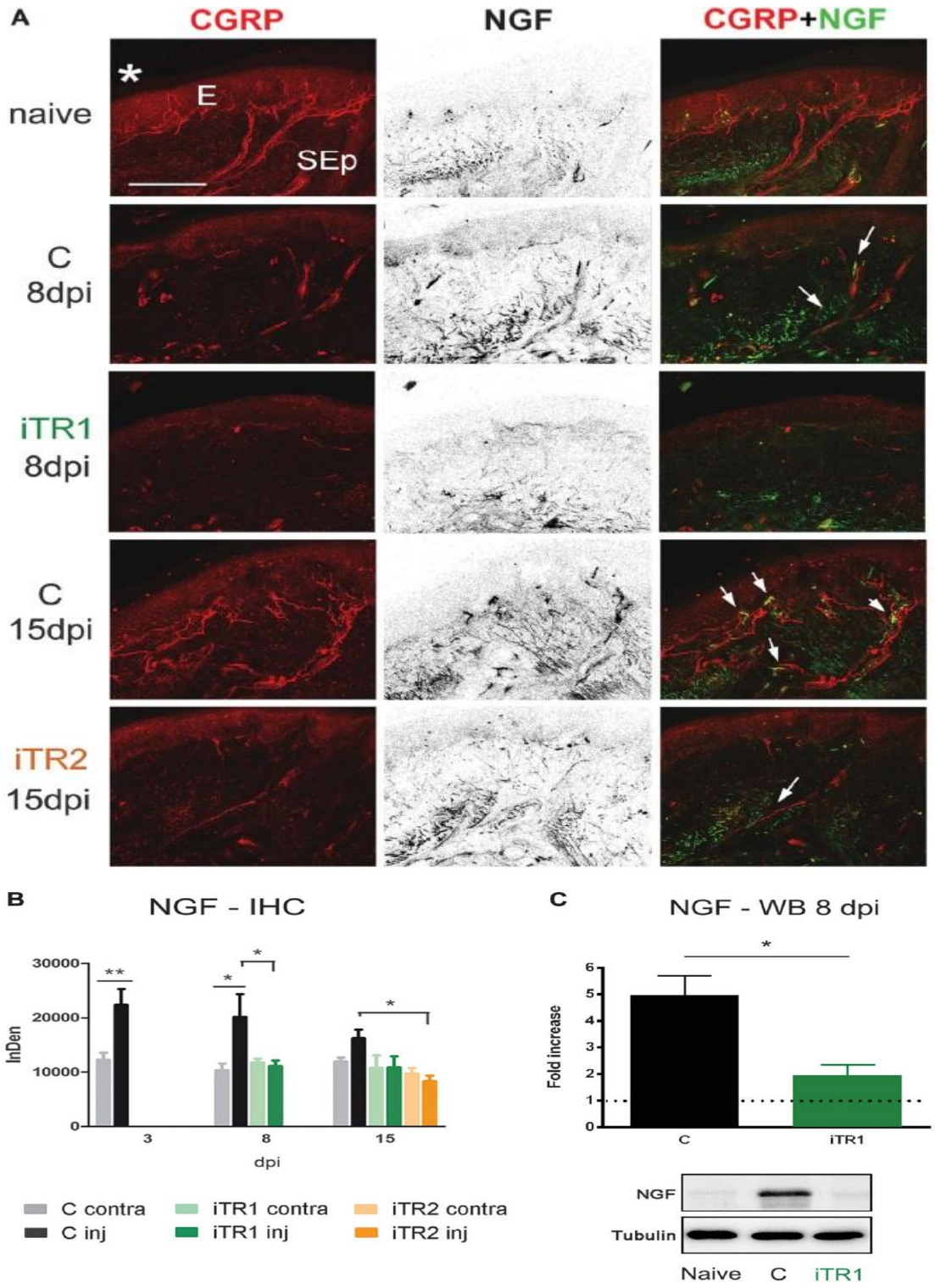

Figure 3: iTR reduces NGF expression in the partially denervated plantar skin. Pictures of medial footpad epidermal (E) and subepidermal (SEp) skin layers extending from the algesimetry test site (asterisk) to the medial footpad dermis. (A) NGF-positive dermal cells (central column panels, in black) increased after sciatic nerve injury (C group); NGF IR profiles (green in the right column panels) were observed in close proximity (arrows) to sprouting CGRP IR fibers (in red). iTR groups showed NGF immunoreactivity in the dermis similar to naive rats, and inhibition of CGRP fiber sprouting was accompanied by low NGF expression in the injured paw at both 8 and 15 dpi. Scale bar: 200 μm. (B)

NGF immunoreactivity (calculated as integrated density, InDen) increased levels at 3 dpi (**P ,0.01) and 8 dpi (*P , 0.05) in the injured paw medial footpad compared with the contralateral in C rats. iTR significantly decreased NGF immunoreactivity at 8 dpi (*P , 0.05 iTR1 vs C) and at 15 dpi (*P ,0.05 iTR2 vs C). (C) Western blot analysis confirmed the high increase in expression of NGF in the denervated medial footpad of group C rats compared with naive samples (dashed line), but iTR1 rats showed levels significantly decreased (*P , 0.05).

iTR prevents upregulation of NKCC1 in dorsal root ganglia and downregulation of KCC2 in dorsal horn

As we previously demonstrated,[46] sciatic nerve lesion induced a change in the expression of chloride cotransporters NKCC1 and KCC2 in the sensory pathway. We observed a progressive increase of NKCC1 phosphorylation in ipsilateral L4-L5 DRG neurons (Fig. 5B; pNKCC1, P , 0.05 at 8 dpi and P , 0.01 at 15 dpi, C injured vs C contralateral), with slight change of NKCC1 protein levels (Fig. 5A). Remarkably, iTR1 significantly abolished the increase of pNKCC1 levels (P , 0.01 at 8 dpi and P , 0.001 at 15 dpi, iTR1 injured vs C injured) and reduced the NKCC1 levels (P , 0.05 at 15 dpi, iTR1 injured vs C injured). The injury also induced an important downregulation of both ipsilateral and contralateral KCC2 protein (Fig. 5C) and phosphorylated active KCC2 (pKCC2, Fig 5D) in dorsal horn neurons, as shown in the C group at both 8 and 15 dpi. iTR1 significantly increased KCC2 levels at 15 dpi (P , 0.05 at 15 dpi, iTR1 injured vs C injured and iTR1 contralateral vs C contralateral), and pKCC2 levels at 8 dpi (P , 0.01, iTR1 injured vs C injured; P , 0.001, iTR1 contralateral vs C contralateral) and 15 dpi (P , 0.05, iTR1 injured vs C injured; P , 0.05, iTR1 contralateral vs C contralateral). These results demonstrate that the iTR training regime prevented the activation of NKCC1 and rescued the transsynaptic downregulation of KCC2.

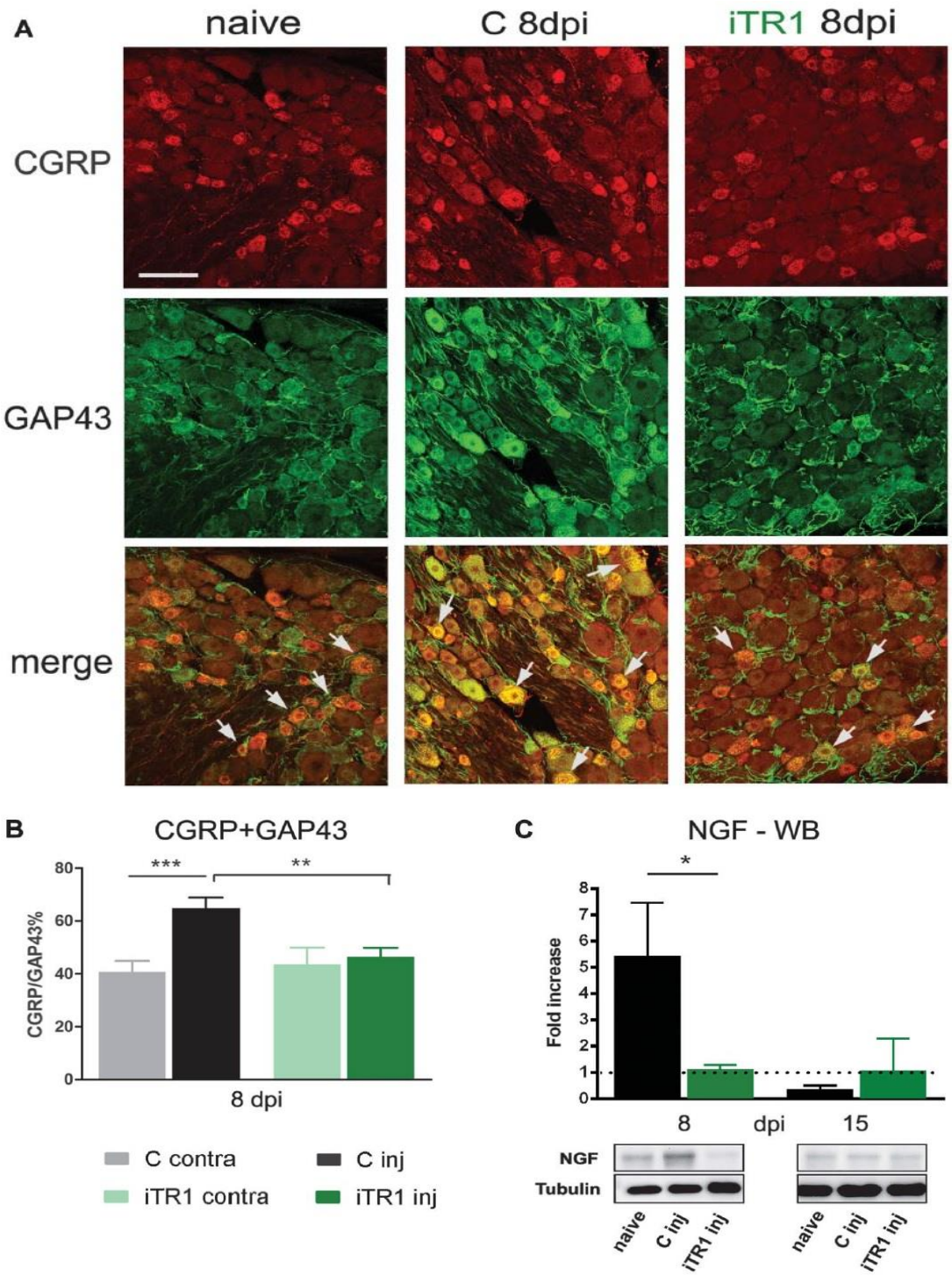

Figure 4: iTR reduces GAP43 expression of CGRP neurons and NGF expression in the L3 DRG. L3 DRG contains the soma of neurons projecting axons in the saphenous nerve. (A) After injury, GAP43 immunoreactivity was strongly increased in neurons of C group rats at 8 dpi and mostly in those expressing CGRP (arrows). In contrast, iTR1 group showed strongly reduced GAP43 expression. Scale bar: 100 μm. (B) The number of CGRP IR neurons double-expressing GAP43 increased in the L3 DRG after sciatic nerve injury (***P,0.001, C injured vs C contralateral), but treadmill reverted this effect (**P,0.01, iTR1 injured vs C injured). (C) Analysis of the expression of NGF in L3 DRG neurons by Western blot showed an increase of protein levels at 8 dpi in samples of injured rats compared with naive. In contrast, NGF levels were significantly decreased by treadmill (*P , 0.05, iTR1 injured vs C injured at 8 dpi) and iTR1 group had values similar to naive.

iTR reduces BDNF expression and microglial reaction in the dorsal horn

Microglia reactivity, labeled with Iba1, and BDNF expression was studied in the L3 spinal cord, the projection segment of the saphenous nerve, and in L5, a segment of the sciatic nerve projections, for comparing reactions in injured and uninjured levels. After sciatic nerve injury, strong activation of Iba1-positive microglia was observed in the lumbar ipsilateral dorsal horn not only at the L5 but also at the L3 spinal segment (Fig. 6A and B). Microglia in both L3 and L5 injured sides of C group rats showed a hypertrophic typical morphology of activated microglial cells, evident at 8 dpi and tending to increase at 15 dpi. In contrast, ameboid morphology of resting microglia was predominant in iTR1 rats at 8 dpi and more evident in iTR2 rats at 15 dpi. BDNF expression appeared mostly colocalized in Iba1-positive cells, and its expression increased after injury in activated microglia. In contrast, BDNF immunoreactivity appeared strongly reduced in iTR1 and iTR2 groups, particularly in microglial cells.

Iba1 immunoreactivity area (Fig. 6C, left panel) showed a significant increase in L3 dorsal horn in group C (P , 0.0001, C vs naive), which was prevented by iTR training (P , 0.05, iTR1 vs C, at 8 and 15 dpi). Similar results were obtained for Iba1 measurements in the L5 dorsal horn (Fig. 6C, right panel), with the Iba1 immunoreactivity area greatly increased at 8 and increased even more at 15 dpi in group C, whereas in groups iTR1 and iTR2 the increase was only about half as much (P , 0.01, iTR1 vs C, 8 dpi; P , 0.05, iTR1 vs C, 15 dpi; P , 0.01, iTR2 vs C, 15 dpi).

The expression of BDNF in microglia was greatly increased in untrained injured rats after sciatic nerve lesion similarly in the L3 and L5 dorsal horn (Fig. 6D; P , 0.0001, C group vs naive). Treadmill training significantly reduced BDNF expression in microglia, as observed in L3 (Fig. 6D, left panel, P , 0.05, iTR1 vs C, 8 dpi; P , 0.01, iTR1 vs C, 15 dpi) and in L5 (Fig. 6D, right panel, P , 0.05, iTR1 vs C, 8 dpi; P , 0.0001, iTR1 vs C, 15 dpi; P , 0.0001, iTR2 vs C, 15 dpi) segments. Some uninjured rats were trained as iTR2 to check whether the treadmill regime by itself could modulate microglia activation; however, these rats showed Iba1 and BDNF immunoreactivity similar to naive rats (Fig. 6C and D). These results demonstrate that iTR training effectively reduces injury-induced microglia reactivity in the dorsal horn of the spinal cord in injured and neighbor segments.

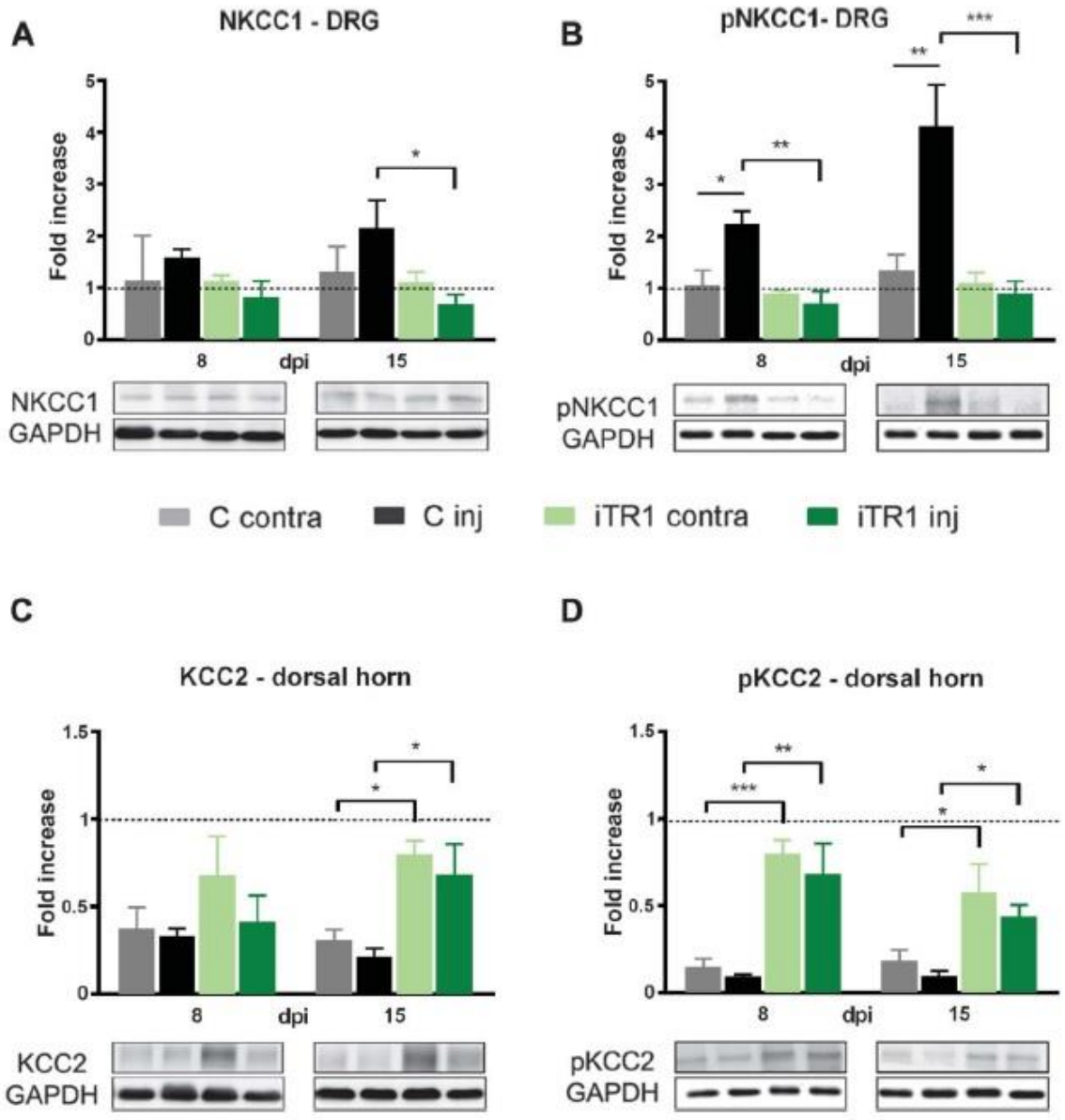

Figure 5: iTR prevents pNKCC1 increase in DRG and KCC2 dephosphorylation in the dorsal horn induced by peripheral nerve injury. (A and B) Changes in the expression of NKCC1 and pNKCC1 in DRG at 8 and 15 dpi analyzed by Western blot quantification. Increased NKCC1 and pNKCC1 levels were observed in the injured paw from 8 to 15 dpi in comparison with normal naive rats level (dashed line). iTR1 training significantly prevented NKCC1 and pNKCC1 increase (*P ,0.05, **P , 0.01, and ***P , 0.001). (C and D) Changes in KCC2 and pKCC2 in the dorsal horn of the spinal cord at 8 and 15 dpi analyzed by Western blot. Both KCC2 and pKCC2 were decreased at 8 and 15 dpi in both injured and contralateral sides in comparison with normal naive rats level (dashed line). iTR1 significantly enhanced KCC2 and pKCC2 levels (*P , 0.05, **P , 0.01, and ***P , 0.001) in injured and contralateral paws compared with C untrained rats paws. Data are shown as mean 6 SEM.

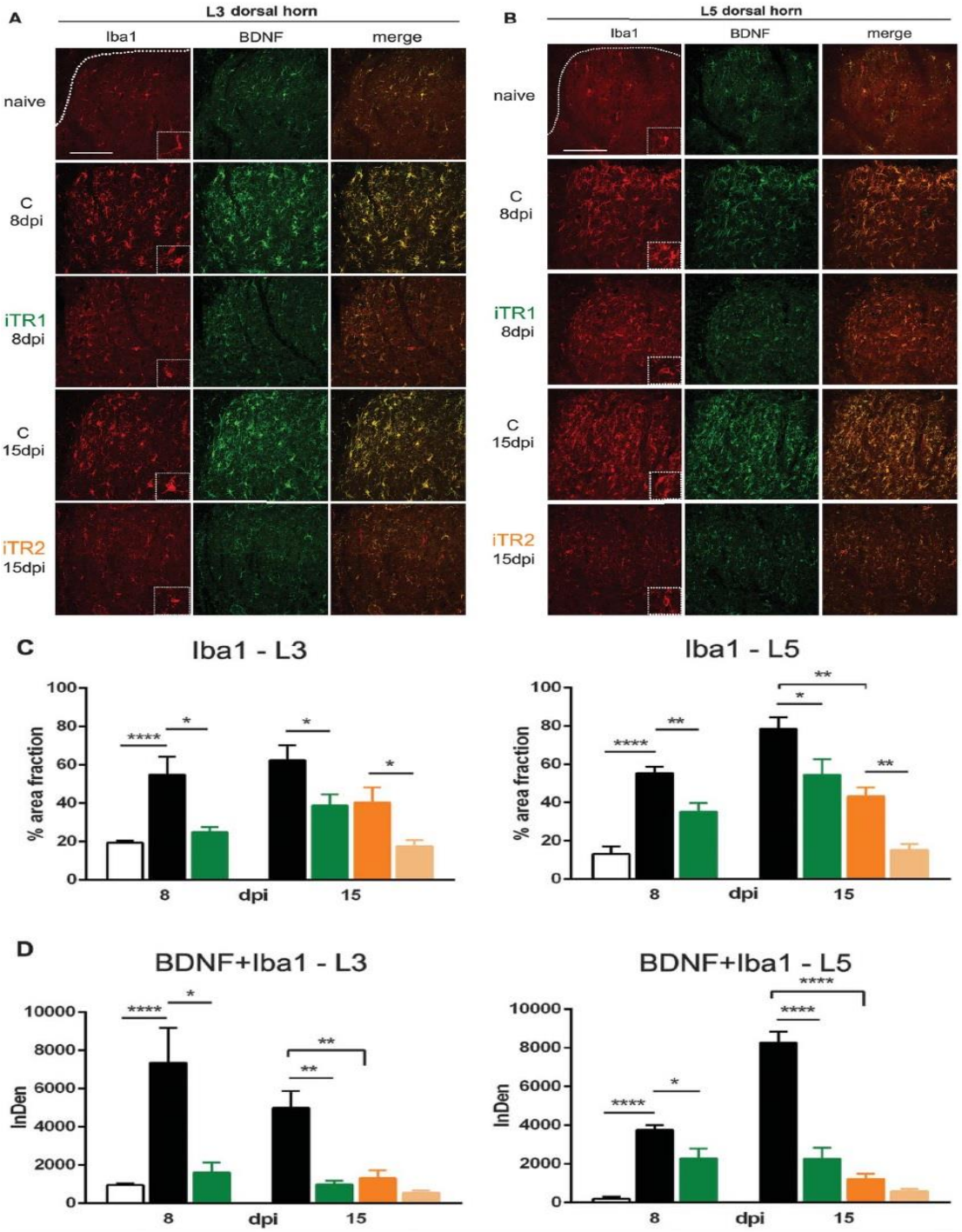

Figure 6: iTR reduces microglial activation and BDNF expression in the ipsilateral dorsal horn. (A and B) Immunoreactivity of Iba1 and BDNF in the dorsal horn of L3 spinal cord (A), which have projections of saphenous nerve, and of L5 spinal cord (B), which receives exclusively from sciatic nerve. BDNF immunoreactivity in superficial laminas (under the dotted line) of naive rats mostly colocalized with Iba1-expressing microglial cells, which showed resting phenotype (higher magnification in dotted boxes). Sciatic nerve injury induced strong activation and hypertrophia of microglia at 8 and 15 dpi in both L3 and L5 segments. However, resting-like and amoeboid morphology ofmicroglial cells was observed in iTR1 rats at 8 dpi and in iTR2 rats at 15 dpi. Along to Iba1, even BDNF immunoreactivity in microglial cells was strongly reduced by treadmill. Scale bars: 100 μm. (C) Quantification of Iba1 immunoreactivity performed in L3 (left panel) and L5 (right panel) dorsal horns of injured side spinal cord.

Iba1 immunoreactive area significantly increased in both L3 and L5 dorsal horns as shown by group C; however, it was strongly decreased in iTR1 group at 8 dpi and in iTR2 group at 15 dpi. (D) Quantification of BDNF immunoreactivity (calculated as integrated density, InDen) in Iba1-positive cells performed in L3 (left panel) and L5 (right panel) dorsal horns of injured side spinal cord. The expression of BDNF in microglia was strongly increased in C group similarly at L3 and L5 dorsal horns, however, strongly inhibited BDNF microglial expression as shown by iTR1 group at 8 dpi and by iTR2 group at 15 dpi. *P , 0.05, **P , 0.01, ***P , 0.001 and ****P , 0.0001 in all comparisons.

Discussion

In this study, we show that early increasing-intensity treadmill activity reduced mechanical and thermal hyperalgesia and modulated different peripheral and central mechanisms related to hyperexcitability. The iTR exercise regime applied reduced both the early hyperalgesia associated with collateral sprouting of intact nerve fibers and the late hyperalgesia subsequent to reinnervation by regenerating nerve fibers.[12]

iTR blocked sprouting of intact saphenous nociceptors into denervated sciatic areas in parallel to decreased production of NGF in the skin and in sensory neurons. This was associated with a reduction of the early adjacent hyperalgesia. We also demonstrated that the iTR exercise prevents NKCC1/KCC2 deregulation, which is a nerve injury–dependent mechanism of central disinhibition.[46] This was associated with the iTR reduction of mechanical hyperalgesia developing in the sciatic nerve territory after its regeneration.

In agreement with previous results,[13] we found that iTR reduced injury-induced microglia activation in the dorsal horn and also BDNF expression in microglia. The same treadmill protocol also induced a reduction of BDNF mRNA in DRG.[12] Thus, the present results indicate that iTR exercise modulates neurotrophin signaling–dependent mechanisms that regulate growth and excitability of sensory neurons after peripheral nerve injury. For the first time, a specific treadmill protocol is found to be beneficial in both preventing peripheral and central mechanisms of sensory neurons hyperexcitability, parallel to the tuning down of the early expression of NGF and BDNF, that during the first days after injury are generators of neuropathic pain.

Effects of iTR on sprouting and regeneration

A variety of treadmill exercise protocols have been applied in experimental nerve injury models, with conflicting results regarding beneficial and deleterious effects on axonal regeneration and pain.[69,66] The main difficulties in planning an exercise treatment are to establish the intensity, the duration, and the timing adequate to promote regenerative mechanisms and to prevent maladaptive responses. Different findings suggest that the outcome of activity intervention for

nerve insults is mostly related to an early time window, when different intensities of activity may stimulate or inhibit the axonal regenerative response, depending on the production of neurotrophic factors. The activation of neurotrophin signal transduction pathways during exercise is crucial in regulating the growth of sensory neurons.[48] A novel finding of this work is that iTR inhibits the denervation-induced early collateral sprouting without hampering longer duration nerve regeneration.[12] This is possible because the mechanisms influencing axonal regeneration and collateral sprouting after denervation display distinct characteristics. The local environment surrounding the regenerative growth cones influences axonal elongation through changes in reactive Schwann cells and production of neurotrophic and neurotropic factors.[1] However, collateral sprouting of undamaged axons occurs in response to denervation within the same target tissue and is dependent on local production of neurotrophins. Administration of NGF and other neurotrophins in vivo leads to potentiation of collateral sprouting within the denervated skin,[20] whereas application of NGF does not affect the rate of axonal elongation after axotomy but delays the onset of regeneration, probably by reducing the neuronal body response to injury.[47] This is supported by the findings that anti-NGF serum administration does not affect regeneration of injured nerves[19,35] but prevents collateral sprouting of nociceptive axons into denervated skin,[20,21,62] in parallel with a strong hypoalgesic effect.[55,67,74]

We found that iTR induced a reduction of NGF after sciatic nerve injury in the skin and in DRG. In a previous investigation,[12] we evaluated the time course of plantar skin reinnervation after sciatic cut and suture repair and demonstrated that nociception at medial areas is exclusively mediated by the saphenous nerve before sciatic regenerative reinnervation, because responses in this area completely disappeared after saphenous nerve cut at 15 or 28 dpi. Because the early hyperalgesic responses are strongly dependent on NGF that can drive the sensitization of saphenous afferents, in this model the early reduction of hyperalgesia is likely associated with the reduction of local NGF production.

If NGF inhibition is the mechanism for the prevention of collateral sprouting, the role of NGF in initiating neuropathic pain symptoms seems parallel to BDNF. By reducing NGF expression, iTR may influence dorsal horn neurons through reduction of BDNF release from primary afferents, as BDNF expression is induced within trkA/CGRP neurons by increased peripheral NGF.[2,44] Indeed, we found that iTR-induced reduction of nociceptive fiber growth was related to normalization of NGF and GAP43 expression in L3 DRG, suggesting that activation of saphenous neurons may be inhibited by iTR. We previously demonstrated[12] that nociceptive reinnervation of plantar lateral sciatic areas is not affected by iTR. Because iTR rats instead showed reduced hyperalgesia in this area, it is possible that the iTR hypoalgesic effect is not exclusively

limited to the blockade of collateral sprouting, but it may also be related to changes at central sensory circuits.

Effects of iTR on chloride cotransporters in nociceptive neurons

Cl^2 homeostasis in primary sensory neurons is mainly due to NKCC1, whose upregulation has been associated with the increased intracellular Cl^2 concentration in DRG neurons and with the spinal cross-excitation of primary afferent depolarization after nerve injury.[59,60] Sciatic nerve injury induces a transient increase of NKCC1 activity linked to reduced KCC2 activity at the dorsal horn of the spinal cord.[46] Postsynaptic disinhibition is regulated by BDNF through KCC2, unmasking the low-threshold input onto nociceptive-specific spinal projection neurons, and has been considered as a mechanism underlying mechanical hypersensi-tivity.[5,15,78] BDNF binding to presynaptic TrkB receptors also leads to a transient presynaptic disinhibition of $GABA_AR$ associated with development of thermal hypersensitivity.[10] Together with NKCC1 upregulation, BDNF-induced presynaptic disinhibition leads to increased calcium influx and neurotransmitter release from pre-synaptic terminals. We found that iTR prevents the injury-induced phosphorylation of NKCC1 and the increasing levels of BDNF, then avoiding the reduction of KCC2 after nerve injury. These results strongly suggest that an iTR exercise protocol may be an effective approach to restore loss of inhibition in primary and secondary nociceptive neurons.

Effects of iTR on microglia activation

BDNF released by reactive microglia causes an inversion of inhibitory GABAergic currents in a subpopulation of dorsal horn lamina I neurons, which is triggered by KCC2 downregulation.[16,26] GABAergic interneurons are particularly sensitive to altered BDNF signaling. Increased BDNF-mediated TrKB activation suppresses Cl-dependent fast GABAergic inhibition by downregulating KCC2,[61] inverting the anion flux on GABA receptors activation from inhibitory to excitatory.[6,59] BDNF expression in spinal microglia is significantly reduced after iTR, suggesting that increased locomotion can rescue KCC2 by reducing microglia reaction.[68]

Possible effects of exercise intensity on neurotrophins expression

Levels of neurotrophins, particularly BDNF, are significantly elevated in response to exercise,[23,50,51] with the increase being dependent on exercise intensity.[27,70] BDNF, unlike other neurotrophins, seems to be especially susceptible to regulation by activity, for both its expression and release,[9,34,38,41,48,64,71] whereas NGF secretion remains constitutive after activity.[25,41,64] In this scenario, the intensity of running may play a double role in sensory recovery. The hypoalgesia

induced by mild exercise protocols is associated with stimulation of neurotrophin-dependent adaptive mechanisms such as injured axons regeneration.[3,22,42,63,77]

However, high-intensity exercise regimes inhibit those mechanisms of neuropathic pain that may be maladaptive such as collateral sprouting and neuronal sensitization.[11,13,65] Similarly to microglial cells, different exercise intensities may stimulate or inhibit Schwann cells neurotrophins expression by means of an autocrine signaling pathway.[23,65]

Possible role of descending pathways

A mechanism that should be taken in consideration is the possible inhibition of proinflammatory molecules because of activation of pain descending pathways. The serotonergic system modulates both nociceptive and motor spinal cord circuits,[28,31,32,39,45,52,57] and motor activity increases the release of serotonin within spinal and supraspinal areas parallel to reduction of mechanical hyperalgesia.[29,33] Moreover, physical exercise decreases NKCC1 levels and restores KCC2 along with recovery of inhibitory reflex responses in spinal cord injured rats.[14] We suggest that the increased activity induced by our treadmill protocol may act similarly by upregulating serotonin activity and may stimulate PKC to restore KCC2-dependent disinhibition to pain after peripheral nerve injury.[7,37] Changes in neuronal excitability of supraspinal sensory projecting areas induced by KCC2 downregulation might significantly contribute to enhanced pain processing after peripheral nerve injury[46] and may serve as a further mechanism of spinal excitation through descending modulatory pathways.

In conclusion, our findings highlight that recodification of spontaneous neural activity after peripheral nerve injury by specific graded-intensity exercise may be a potent neurorehabilitation tool to prevent neuropathic pain generated from peripheral, spinal, and cortical plasticity and reorganization of sensory circuits. Modulation of neurotrophin expression and NKCC1/KCC2 unbalance play an important role in neuropathic pain[15,17]; however, they can be tuned by specific activity protocols resulting in reduction of pain after nerve injuries.

Conflict of interest statement

The authors have no conflicts of interest to declare. This work was supported by TERCEL and CIBERNED funds from the Fondo de Investigación Sanitaria of Spain, and Grant Nos. BIOHYBRID (FP7-278612) and EPIONE (FP7-602547) from the European Commission (EC).

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Chapter 2

Monoaminergic descending pathways contribute to modulation of neuropathic pain by increasing-intensity treadmill exercise after peripheral nerve injury

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Monoaminergic descending pathways contribute to modulation of neuropathic pain by increasing-intensity treadmill exercise after peripheral nerve injury

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Abstract

This study characterizes the impact of increasing-intensity treadmill exercise (iTR) on noradrenergic (NE) and serotonergic (5HT) modulation of neuropathic pain. Following sciatic nerve transection and repair (SNTR) rats developed significant mechanical and thermal hyperalgesia, that was partially prevented by iTR performed during the first 2 weeks after injury.

At 2 weeks after SNTR we found marked decrease in the expression of 5HT2A and α1A and β-, but not α2A adrenergic receptors in the spinal cord dorsal horn, which was significantly recovered by iTR, particularly in lamina II. In parallel, iTR significantly increased 5HT2A receptor expression in periaqueductal grey (PAG), raphe magnus (RM) and dorsal raphe nucleus (DRN), with a pattern suggesting reorganization of serotonergic excitatory interconnections between PAG and RN. iTR also increased the expression of α1A receptors in locus coeruleus (LC) and DRN, and β_2 receptors in LC, indicating that exercise enhanced activity of NE neurons, likely by activating the autologous projections from DRN and PAG.

iTR hypoalgesia was antagonized by blockade of β_2 and $5HT_{2A}$ receptors with administration of butoxamine and ketanserin. To study the contribution of LC NE neurons, the neurotoxin DSP4 was injected to induce depletion of NE projections from LC before starting iTR. DSP4 treatment worsened mechanical hyperalgesia, but iTR similarly produced hypoalgesia in DSP4-injected rats. All these results support a complex contribution of monoaminergic descending pathways in the hypoalgesic action of iTR, by activating NE and 5HT descending projections. The increased expression of $5HT_{2A}$ receptor in LC by iTR was unchanged after butoxamine treatment but further increased after DSP4 injection, suggesting an intrinsic regulation of 5HT and NE activity between PAG, DRN and LC neurons activated by iTR.

Finally, iTR significantly reduced microglial reactivity in LC and increased non-microglial BDNF expression, an effect that was reverted by butoxamine. Thus, changes in BDNF regulation may be also implicated in the central 5HT/NE actions on neuropathic pain.

Introduction

Increasing-intensity treadmill exercise (iTR), performed during the first days after injury, has been shown to significantly reduce hyperalgesia in neuropathic pain models, such as sciatic nerve chronic constriction injury (CCI) [1] and section and suture repair (SNTR) [2]. iTR decreased the expression of BDNF and other neurotrophins although without impairing nerve regeneration [2,3]. iTR-induced decrease of BDNF expression was associated to reduction of microgliosis and restoration of the expression of chloride transporters in the primary sensory neurons and along the central pain pathways [4]. Little is known, however, on the central mechanisms by which iTR induces hypoalgesia, and we hypothesize that it may act by activating descending pathways for inhibition of pain transmission at the spinal level.

Brain areas can modulate the ascending pain signals by serotonergic and noradrenergic projections to spinal cord neurons, which can facilitate or inhibit the afferent sensory neurons. Periaqueductal grey (PAG) areas receive pain and temperature fibers and activate defensive and stress responses by sending axons to both locus coeruleus (LC) and raphe magnus nucleus (RM), whose antinociceptive output trigger descending inhibition [5]. Serotonergic RM and noradrenergic LC projections normally activate spinal enkephalinergic and GABA/glycinergic interneurons. Enkephalin released from terminals of enkephalinergic dorsal horn interneurons acts on the opioid receptors located on the central processes of nociceptive primary afferents, reducing Ca^{2+} entry into their terminals and decreasing the release of nociceptive neurotransmitters such as glutamate and substance P [6]. Similarly, activation of dorsal horn interneurons containing GABA or glycine also inhibit the spinal transmission of noxious sensory signals, and previous studies indicate that spinal GABAergic inhibition is reduced after experimental nerve injury [7]. The loss of tonic inhibition by spinal interneurons is also associated with dysregulation of NKCC1/KCC2 chloride cotransporters expression, inducing an inversion of GABAergic depolarizing currents in neuropathic conditions [8], that is prevented by iTR [9].

Besides the demonstrated peripheral effects [2,9], we hypothesized that iTR may activate pain central inhibition normally gating the nociceptive input to supraspinal, medullary and cortical areas, which are decreased after peripheral nerve injury. By stimulating the descending noradrenergic and serotonergic projections to the dorsal horn, specific exercise training may result

in the activation of inhibitory circuits in the dorsal horn and in the consequent inhibition of second-order spinothalamic neurons by presynaptic and postsynaptic mechanisms.

We studied the expression of noradrenaline (NE) and serotonin (5HT) receptors in sensory neurons of the spinal cord dorsal horn, that participate in pain modulation. Among NE receptors, α_{1A} receptors are expressed in GABAergic and glycinergic neurons of dorsal horn lamina II, where they may participate in endogenous inhibition of afferent pain by exciting inhibitory interneurons [10]. α_{2A} receptors are expressed in the spinal cord predominantly on the terminals of primary C-fibers afferents, where they inhibit nociception [11]. β_2 receptor is an excitatory adrenoreceptor expressed in dorsal horn neurons [12], which activation induces antinociceptive effects [13,14]. We also assessed changes in serotonergic $5HT_{2A}$ receptor since it is involved in spinal chloride homeostasis, which dysregulation is associated with spinal disinhibition and neuropathic pain [15,16]. Under hyperalgesic states the $5HT_{2A}$ receptor was found to be expressed in laminae I-III NK1R-positive projection neurons [17] and in lamina II galanin-containing neurons expressing GABAergic boutons [18], receiving emerging interest as a potential target for treating nerve injury-induced pain and spasticity [19].

In this study, we investigated the changes induced by iTR on noradrenergic and serotonergic circuitry that may be related to antinociception. For this purpose, we analyzed the expression of adrenergic α_{1A}, α_{2A} and β_2 receptors, and serotonergic $5HT_{2A}$ receptor after injury to the sciatic nerve in rats, and their changes under increasing-intensity exercise when neuropathic pain is prevented.

Materials and methods

Animals and surgery

Adult female Sprague-Dawley rats (240 ± 30 g) were housed in standard cages with access to food and water *ad libitum* under a light–dark cycle of 12 hours. All the experimental procedures were approved by the Ethics Committee of the Universitat Autonoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). Rats were anesthetized by intraperitoneal (i.p.) injection of ketamine (10 mg/kg, Imalgene 500; Rhone-Merieux, Lyon, France) and xylazine (1 mg/kg, Rompun; Bayer, Leverkusen, Germany).

Rats were submitted to a sciatic nerve transection and repair (SNTR), a well characterized model that allows the evaluation of neuropathic pain and nerve regeneration [20]. The right sciatic nerve was exposed at the mid thigh, transected at 92 mm from the tip of the third toe, and repaired by epineurial sutures (10-0). The wound was closed in two layers and disinfected with povidone iodine. Rats were kept in a warm environment until their recovery from anesthesia.

Experimental design

Seven days before surgery, all the animals were habituated to the experimental device for treadmill locomotion (Treadmill LE 8706; LETICA, Barcelona, Spain) and pretrained to the task, by leaving them to explore the stopped treadmill for 5 minutes and then trained in a single iTR session. Each iTR session consisted of 1 hour running, starting at a locomotion speed of 10 cm/s that was increased 2 cm/s every 5 minutes, until a maximal speed of 32 cm/s [2,9]. All rats were evaluated during follow-up with sensory tests performed during the morning, whereas treadmill running sessions were performed during the afternoon.

At day 3 after surgery, animals were randomly selected to follow or not the iTR training. SNTR rats were divided in several groups: a group of rats performed iTR (SNTR-iTR group, n=10), and a second group remained sedentary (SNTR-sed group, n=8). Other groups performed or not iTR with pharmacological blockade of β_2-receptors with butoxamine (Sigma-Aldrich, 8 mg/Kg in saline, i.p.; SNTR-iTR+Bu group, n=6, and SNTR-sed+Bu group, n=6), or blockade of $5HT_{2A}$-receptors with ketanserin (Sigma-Aldrich, 8 mg/Kg in saline, i.p.; SNTR-sed+Ke group, n=6, and SNTR-iTR+Ke group, n=6). These drugs were administered each day of training, 30 minutes before starting the exercise. Control groups for both drugs were injected with saline vehicle only. A naive group of rats was added for comparison with injured rats (n=6).

Finally, other two groups of animals were injected with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4, Sigma-Aldrich, 50 mg/kg in saline, i.p.), a neurotoxin that selectively induces degeneration of NE neurons in the LC, thus depleting NE projections originating from the LC [21,22]. DSP-4 was injected within 10 min of preparation [23], and its administration was performed 4 days before the injury to ensure effect from the beginning of the training. One group of DSP4 injected rats followed iTR (SNTR-iTR+DSP4 group, n=8), and another remained untrained (SNTR+DSP4 group, n=10).

Nociceptive tests for pain threshold measurement

Three days before surgery, all the injured animals were habituated to the experimental devices, and then tested for baseline nociceptive thresholds recording. The nociceptive behavior tests for mechanical and thermal stimuli were performed on both hind paws before and at different days after injury (dpi), the experimenter being blind to assignment of rats to the different groups. Lateral and medial sites of the paw were tested to differentiate changes in sensory thresholds produced respectively by sciatic nerve injury from those due to saphenous nerve sprouting [20].

Sensitivity to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers. Then, a non-noxious pointed probe was gently applied to each test site, slowly increasing the pressure.

The threshold was expressed as the force (in grams) at which rats withdrew the paw in response to the stimulus. A cutoff force was set at 40 g, when the stimulus lifted the paw without response. The mechanical nociceptive threshold was calculated as the mean of 3 measurements per test site, with a 3 minutes interval between each measurement.

Thermal sensitivity was assessed by means of a Plantar test algesimeter (Ugo Basile, Comerio, Italy). Rats were placed into a plastic box with an elevated plexiglass floor. The beam of a lamp was pointed at the same test sites as above in the hind paw plantar surface. Intensity was set to low power (40 mW/cm2) with a slow heating rate. A cutoff time for the stimuli was set at 20 seconds to prevent tissue damage. Heat pain threshold was calculated as the mean of 3 trials per test site, with a 5 minutes interval between trials, and expressed as the latency (in seconds) of paw withdrawal response. For both mechanical and thermal thresholds, values are reported as the percentage ratio between the ipsilateral injured and the contralateral normal paw at each test day. This allows a representation of neuropathic pain induced by the injury since no significant variations of contralateral thresholds were found in previous studies [9].

Immunohistochemistry of spinal cord and brain

At the end of follow-up at 14 dpi, all rats were euthanized and spinal cord and brain samples collected for immunohistochemical assays. Lumbar (L4-L5) spinal cord segment and brain were removed from perfused animals and kept in fixative for 1 hour and 4 hours respectively, and then cryoprotected with 30% sucrose in PBS. Transverse sections, 25 μm thick, were cut on a cryostat and mounted on slides. Sections were blocked with specific serum and incubated overnight with primary antibodies in 0.3% Triton X-100 in PBS, used for staining 5HT and NE receptors in spinal cord and brain sections (anti-β_2 receptors, rabbit, 1:500, Santa Cruz; anti-α_{1A} receptors, goat, 1:500, Santa Cruz; anti-α_{2A} receptors, goat, 1:500, Santa Cruz; anti-$5HT_{2A}$ receptors, goat, 1:500, Santa Cruz), along with antibodies to identify 5HT and NE neurons (anti-tyrosin hydroxylase (TH), mouse, 1:500, Santa Cruz; anti-tryptophan hydroxylase (TPH), mouse, 1:500, Santa Cruz), microglial cells (anti-Iba1, goat, 1:200, Abcam) and BDNF (anti-BDNF, sheep, 1:200, Millipore).

The following day sections were incubated for 2 hours with Alexa Fluor 488 and/or Alexa Fluor 594 conjugated secondary antibodies (1:200, Life Technologies). After washing in PBS, sections were coverslipped with a solution of DAPI (1 mL/mL) in Mowiol mounting medium. For each marker, three sections from 4 samples per group were used for quantification of immunolabeling. The experimenter was blinded to the sample group. Anatomical structures of interest were previously identified under microscope in spinal cord and brain atlas coordinates using specific sections and Nissl or Hematoxylin staining. 10X and 20X images were captured with a Zeiss LSM 700 confocal microscope, and analyzed using ImageJ software (NIH, USA).

Thresholding of fluorescent signal was adjusted over the background level of a negative control. The integrated density of immunoreactivity was calculated within regions of interest drawn with ImageJ software tool. For spinal cord immunoreactivity, data are shown for both ipsilateral and contralateral sides. For brain immunoreactivity, data are shown only for projection areas of the injured side.

Data analysis

Data are presented as mean ± SEM. Statistical analysis of nociceptive thresholds was made by two-way analysis of variance (ANOVA) with group and time after injury as factors, followed by Bonferroni's post hoc comparisons. Statistical comparisons for immunofluorescence data were made by one-way and two-way ANOVAs followed by Tukey's post hoc test when necessary. The level of statistical significance was 5% ($p<0.05$) in all the analyses.

Results

iTR reduced hyperalgesia after SNTR

Changes in sensory thresholds were recorded at both medial and lateral sites of the hindpaw to monitor the contribution to hyperalgesia of collateral sprouting of saphenous nerve, and of denervation by the injured sciatic nerve [2,9].

After SNTR the lateral side of the injured hindpaw was unresponsive during the 14 days follow-up, since this territory remains denervated until at least 4 weeks after injury [20]. SNTR produced a marked decrease of the mean mechanical threshold at the medial side from 3 to 14 dpi (Fig. 1A; 48 to 32% of contralateral in SNTR-sed group). The decrease of thermal threshold was comparatively lower (Fig. 1B; 92 to 69% in SNTR-sed group). iTR significantly prevented the reduction of both mechanical and thermal thresholds at 7 and 14 dpi in the SNTR-iTR group (Fig. 1; $p<0.05$ SNRT-iTR vs. SNRT-sed). Since the development of thermal hyperalgesia was slower than the mechanical, we did not analyze changes of thermal thresholds in further experiments.

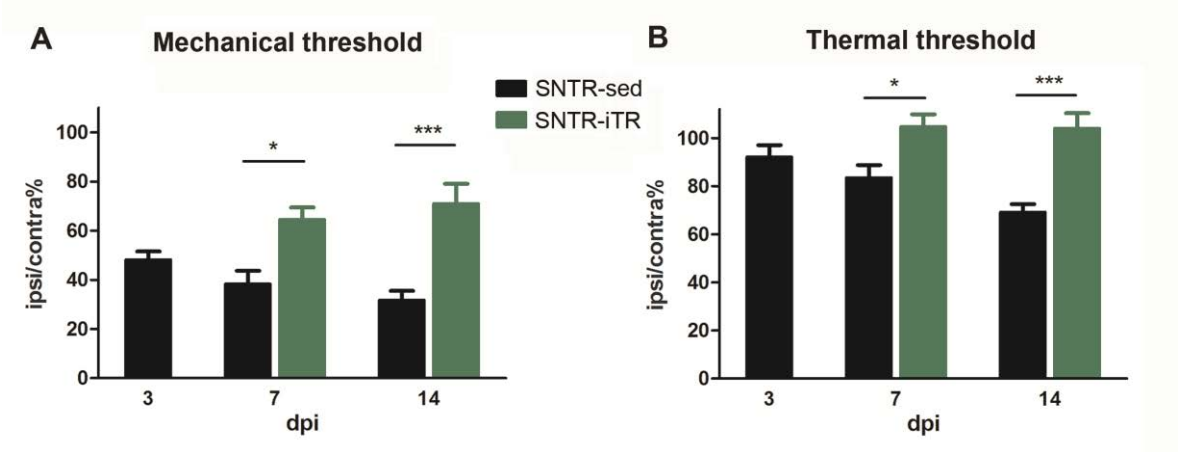

Figure 1. *Hyperalgesia is reduced by iTR after SNTR.* Changes in mechanical (A) and thermal (B) sensory thresholds recorded at the medial test site in rats after SNTR that were untrained (-sed) or followed daily iTR from 3 days (-iTR). Values are represented as the percent ratio between the mean ipsilateral and the contralateral paw threshold at 3, 7 and 14 days post-injury (dpi). * $p<0.05$, *** $p<0.001$.

iTR counteracted the decrease of α_{1A} and β_2 adrenergic and $5HT_{2A}$ serotonergic receptors expression in the dorsal horn after SNTR

We studied the expression of adrenergic and serotonergic receptors in laminae I, II and III of the spinal cord dorsal horn. We found α_{1A} receptor mainly expressed in lamina II and at lower intensity in laminae I and III (Fig. 2A). After SNTR, α_{1A} expression strongly decreased in all laminae of both dorsal horn ipsilateral and contralateral to injury (Fig. 2A, B).

The β_2 receptor was expressed in all laminae I-III of the dorsal horn in the naïve rats (Fig. 3A). SNTR reduced the expression of β_2 receptor in both the ipsilateral and contralateral sides of lamina I, II and III ($p<0.001$ naive vs. SNTR-sed; Fig. 3A, B). The expression of β_2 was significantly recovered by iTR in the lamina II bilaterally ($p<0.001$ SNTR-sed vs. SNTR-iTR) and in the contralateral lamina I ($p<0.01$).

In naïve animals, the $5HT_{2A}$ receptor was more densely expressed in lamina II, even if spot clusters of immunofluorescent labeling were present in all dorsal horn laminae (Fig. 4A). After SNTR, $5HT_{2A}$ immunoreactivity decreased significantly in lamina II bilaterally ($p<0.001$ naïve vs. SNTR-sed; Fig. 4B) and in the ipsilateral lamina I ($p<0.05$ naive vs. SNTR-sed). Interestingly, iTR significantly recovered $5HT_{2A}$ receptor expression in lamina II ($p<0.01$ SNTR-sed vs. SNTR-iTR), and in the ipsilateral lamina I ($p<0.05$, SNTR-sed vs. SNTR-iTR; Fig. 4B).

These results indicate that nerve injury determines a decrease of NE and 5HT tone in the dorsal horn neurons that may reflect the drive from normal to neuropathic conditions, and highlight the iTR potential to reverse these changes.

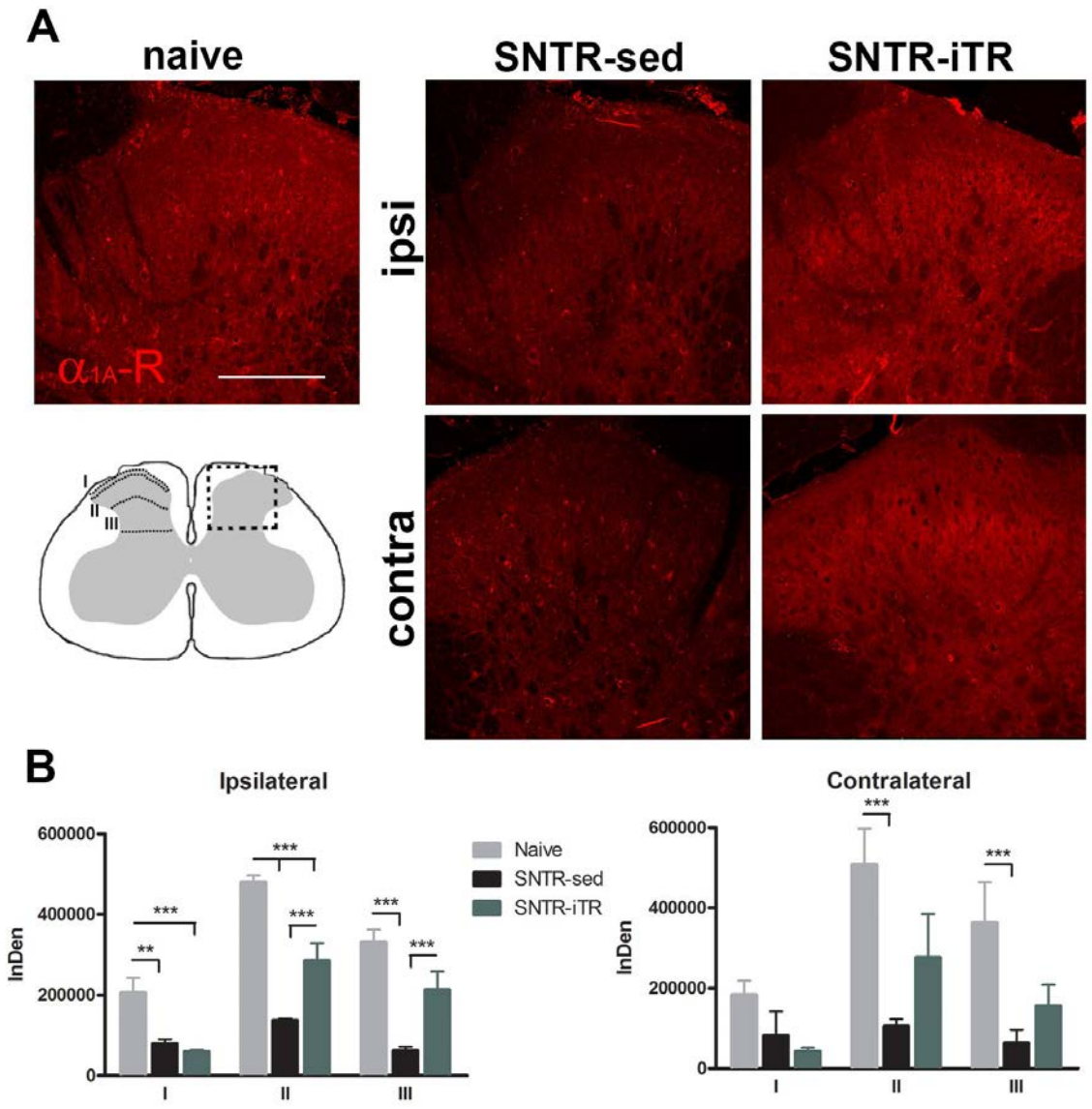

Figure 2. *iTR counteracted the decrease of a_{1A} receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of α_{1A} adrenergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100μm. (B) Quantification of α_{1A} immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared with to naïve rats. ** p<=0.01, *** p<0.001.

Changes in $5HT_{2A}$, α_{1A} and β_2 receptors expression in midbrain areas induced by SNTR and after iTR

Since iTR showed to significantly impact the expression of $5HT_{2A}$ serotonergic and α_{1A} and β_2 adrenergic receptors in the lumbar dorsal horn of spinal cord, we investigated the changes that peripheral nerve injury and iTR treatment determined on the expression of these receptors at higher integrating brain centers that participate in pain descending modulation, such the

periaqueductal grey matter (PAG), the locus coeruleus (LC), the dorsal raphe (DRN) and the raphe magnus nucleus (RM) (Fig. 5A).

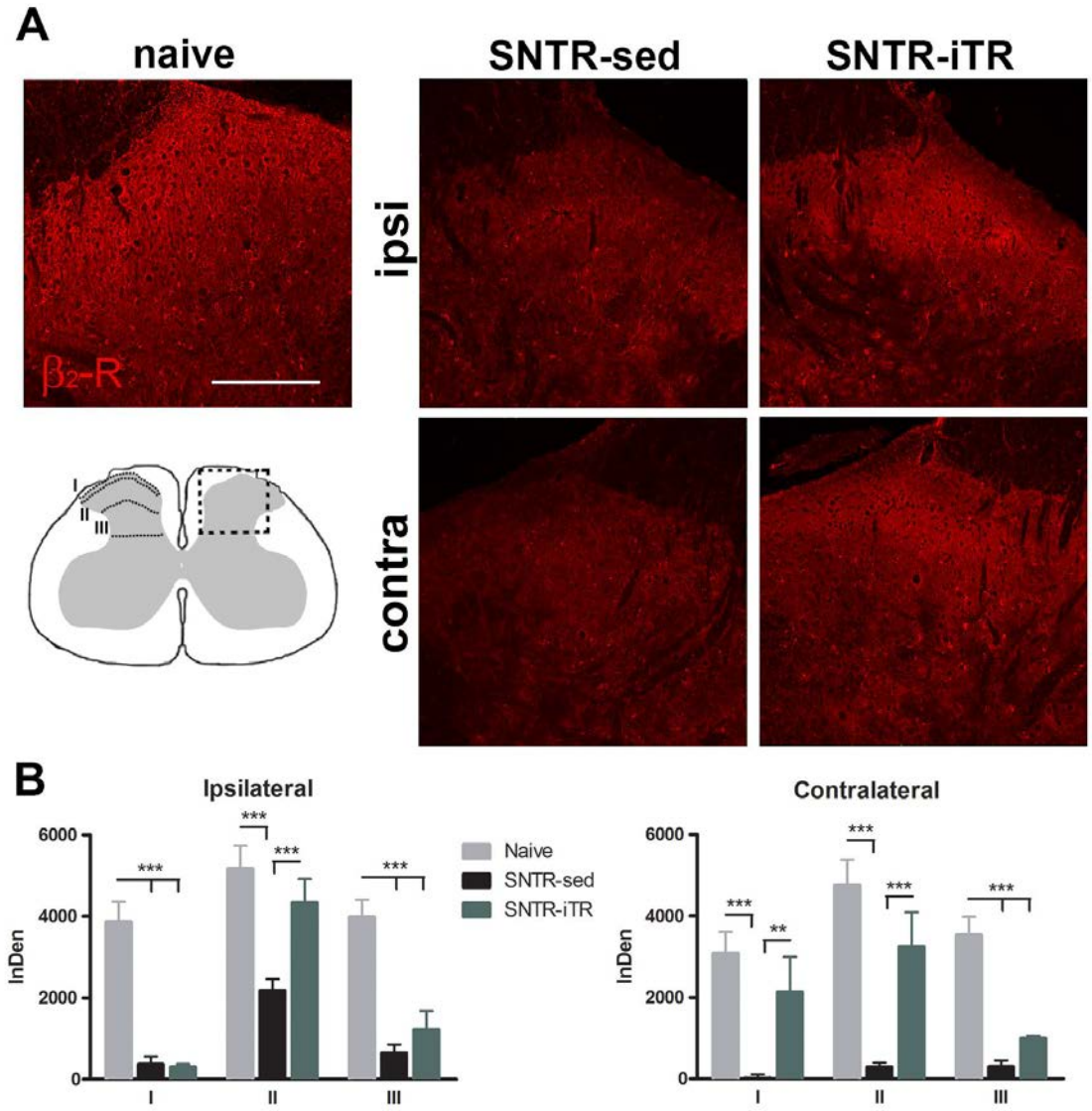

Figure 3. *iTR counteracted the decrease of β_2 receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of the β_2 adrenergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of β_2 immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared to naïve rats. ** p<=0.01, *** p<0.001.

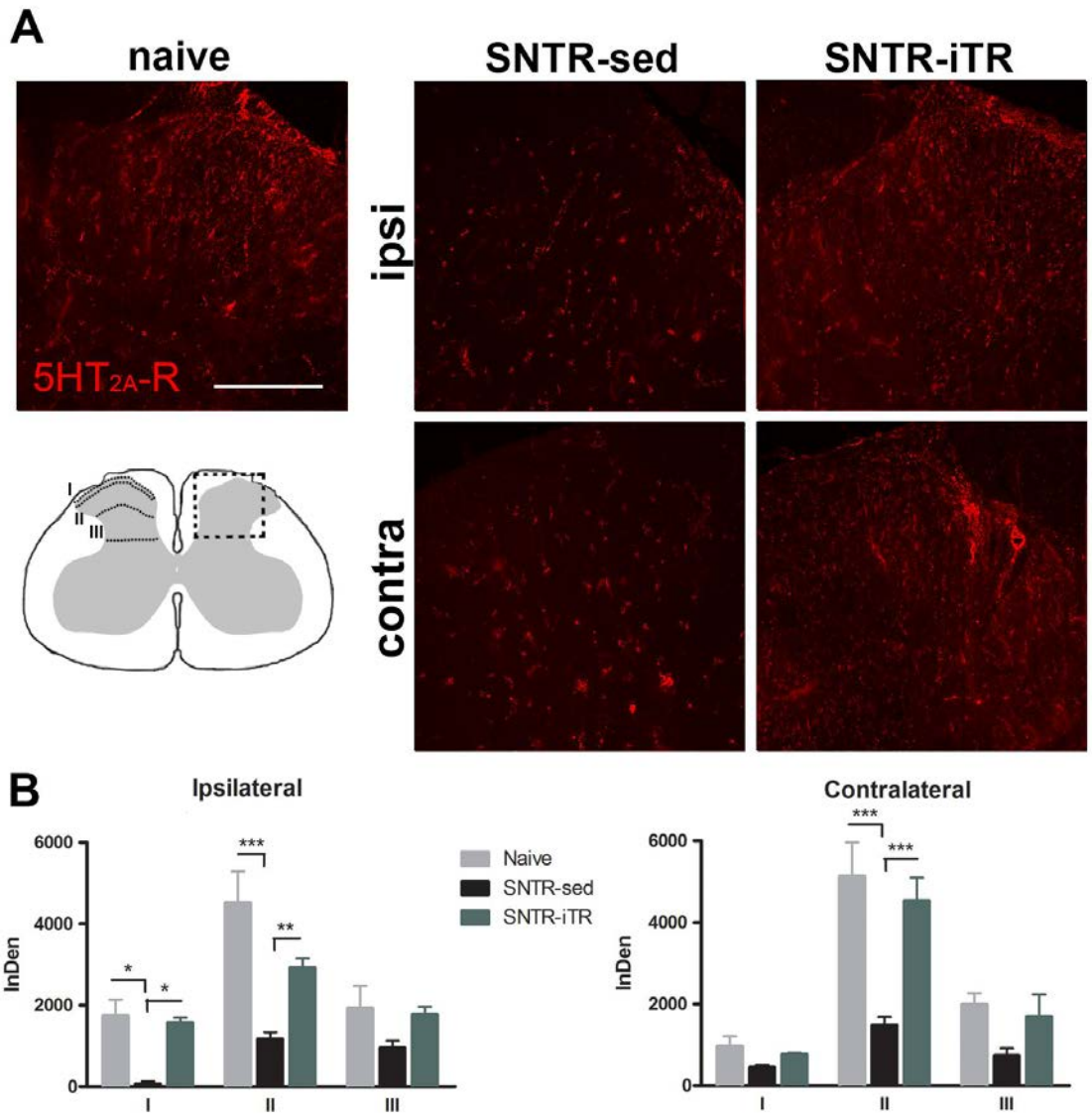

Figure 4. *iTR counteracted the decrease of 5HT$_{2A}$ receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of the 5HT$_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of 5HT$_{2A}$ immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.001$, ** $p<=0.01$, *** $p<0.001$.

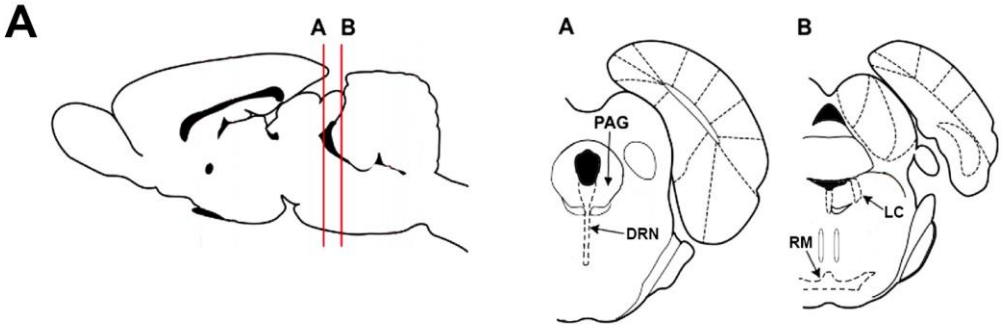

Figure 5. *iTR increased the expression of 5HT$_{2A}$ receptor in PAG, DRN and RM after SNTR.* (A) Graphic representation of PAG, RM, DRN and LC nuclei considered as regions of interest for quantification in coronal sections.

The immunolabeling of 5HT$_{2A}$ receptor in naïve rats showed positive clusters in PAG and DRN regions and rarely in the RM nucleus extending laterally into the adjacent reticular formation, where are large-size serotonergic neurons (Fig. 6A, naïve group). Peripheral nerve injury decreased the expression of 5HT$_{2A}$ in PAG and DRN (Fig. 6A-B, SNTR-sed group). The pattern of expression appeared different in injured animals, with higher presence of scattered 5HT$_{2A}$ clusters through dorsomedial areas of RM than in the PAG compared with naïve samples. iTR strongly increased 5HT$_{2A}$ receptor expression in PAG and DRN (Fig. 6A-B, SNTR-iTR vs. SNTR-sed $p<0.01$, and SNTR-iTR vs. naïve $p<0.05$). There was strong immunolabeling in the soma of many large-size neurons in these areas and even in dendrites of PAG and of DRN neurons projecting to other nuclei. Spot clusters indicating terminal 5HT$_{2A}$ contacts were significantly increased in the RM (Fig. 6A-B, SNTR-iTR vs. SNTR-sed $p<0.05$, and SNTR-iTR vs. naïve $p<0.01$). This result suggests a reorganization of serotonergic connections between PAG and raphe nuclei, with the possible function to increase their activity to descending output.

The normal expression of α_{1A} receptor was found in medium to large-size neurons of LC and DRN and on terminals of NE projections to RM (Fig. 7A, naïve group). Slightly stronger staining was observed in the DRN after injury (Fig. 7A, SNTR-sed group), in contrast with the reduced expression in the dorsal horn. iTR significantly increased α_{1A} immunoreactivity in LC and DRN, and in adjacent areas, such as the subcoeruleus nucleus, and in more neurons of dorsomedial DRN areas (Fig. 7A, SNTR-iTR group). Compared with LC and DRN, where the increase of α_{1A} immunoreactivity was significant (Fig. 7B; SNTR-iTR vs. naïve, $p<0.01$, and SNTR-iTR vs. SNTR-sed, $p<0.05$), only slightly increased amount of immunopositive clusters was observed surrounding the soma of RM neurons. The changes in the pattern of α_{1A} receptor expression

across LC and raphe nuclei suggest that autologous α_{1A} receptors in LC may have been activated in a positive loop to increase LC activity under exercise training.

Autologous β_2 receptors were also found in LC and sparse immunoreactivity in DRN and RM of naïve rats (Fig. 8A, naïve group). Nerve injury did not induce significant changes in β_2 receptors (Fig. 8A, SNTR-sed group). iTR rats showed immunoreactivity in a larger area of LC (Fig. 8A-B, SNTR-iTR vs. SNTR-sed $p<0.01$; SNTR-iTR vs. naïve $p<0.05$), and also a slight not significant higher density in DRN and RM, as terminals of noradrenergic projections from LC (Fig. 8B). These results confirmed that iTR enhanced the activity of NE LC neurons.

Blockade of β_2 or $5HT_{2A}$ receptors antagonized the iTR induced hypoalgesia after SNTR

We wanted to test if the activation of β_2 and $5HT_{2A}$ receptors was directly involved in the prevention of mechanical hyperalgesia observed in iTR rats after SNTR. Inhibition of β_2 receptors with butoxamine, a selective β_2 blocker, before starting iTR sessions antagonized its hypoalgesic effect at both 7 and 14 dpi (Fig. 9A, SNTR-iTR vs. SNTR-iTR+Bu $p<0.05$). However, butoxamine treatment alone had no effects on mechanical hyperalgesia, indicating a specific β_2 activity contribution on the iTR effects.

Similarly, treatment with ketanserin, an inhibitor of $5HT_2$ receptors (also with weak α_1 adrenergic blocking properties), significantly antagonized the iTR hypoalgesic effect, but only at 14 dpi (Fig. 9B). iTR hypoalgesia was only partially blunted at 7 dpi (Fig. 9B, SNTR-iTR vs. SNTR-iTR+Ke, $p<0.001$ at 14 dpi). Ketanserin treatment alone partially reduced SNTR induced mechanical hyperalgesia, indicating that $5HT_2$ receptors (and maybe α_1 receptors blocked by ketanserin) may mediate mechanisms of sensitization after nerve injury.

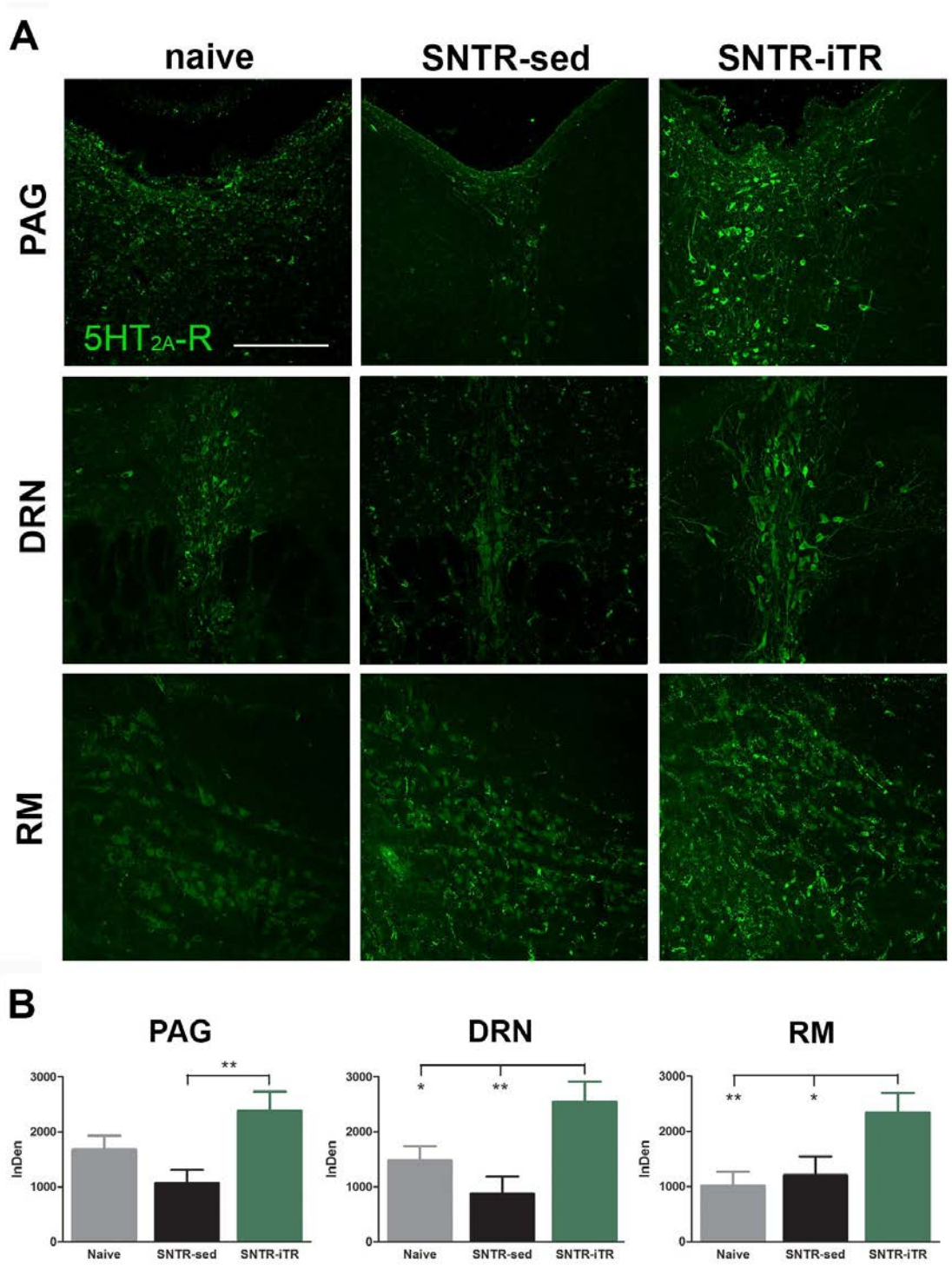

Figure 6: (A) Representative confocal images of the $5HT_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in PAG, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100µm. (B) Quantification of $5HT_{2A}$ immunoreactivity in PAG, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.05$; ** $p<0.01$.

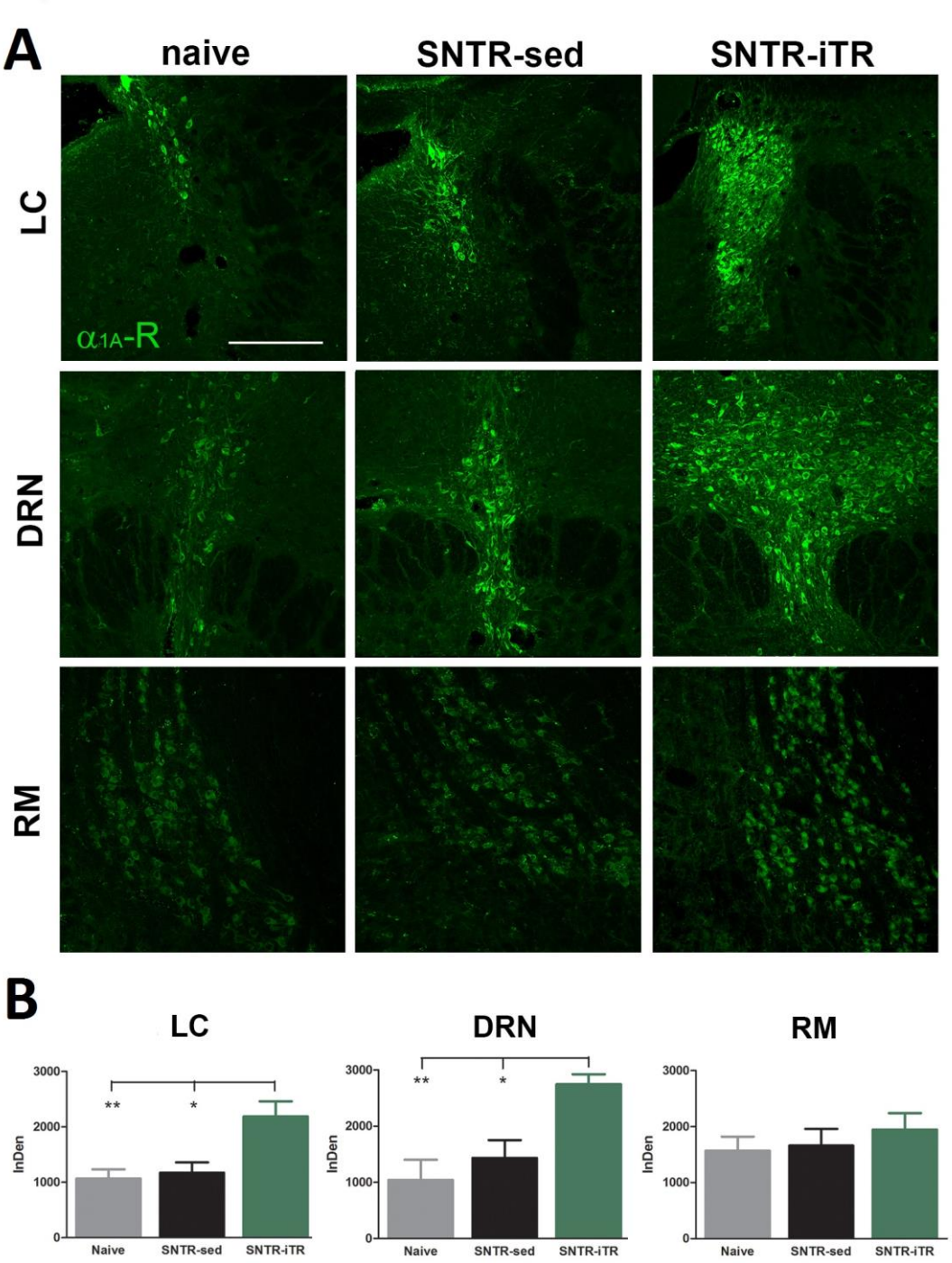

Figure 7. *iTR increased the expression of a_{1A} receptor in LC and DRN after SNTR.* (A) Representative confocal images of the α_{1A} receptor immunoreactivity at 14 dpi in LC, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100µm. (B) Quantification of α_{1A} immunoreactivity in LC, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.05$; ** $p<0.01$.

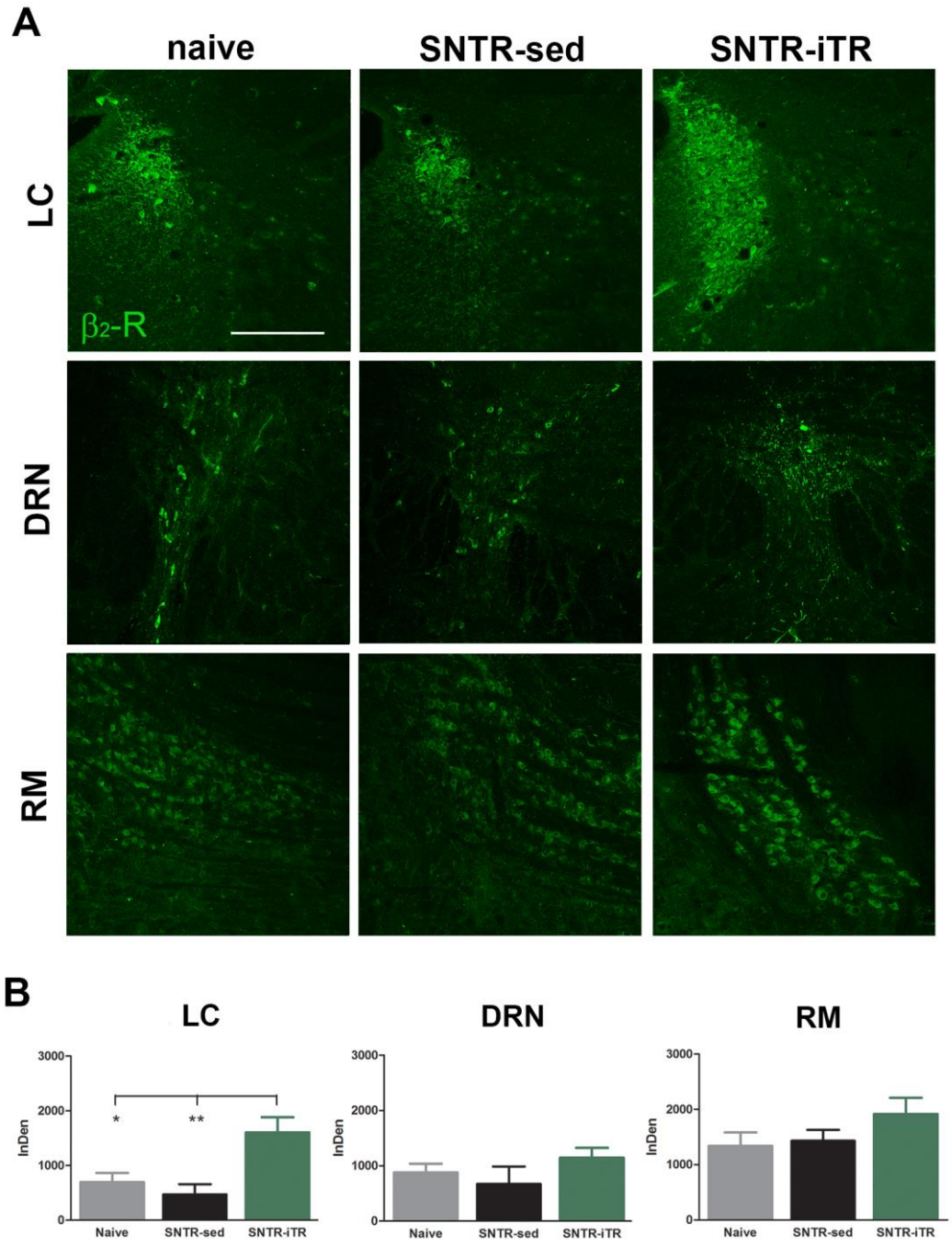

Figure 8. *iTR increased the expression of β_2 receptor in LC after SNTR.* (A) Representative confocal images of the β_2 adrenergic receptor immunoreactivity at 14 dpi in LC, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100µm. (B) Quantification of β_2 immunoreactivity in LC, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.05$; ** $p<0.01$.

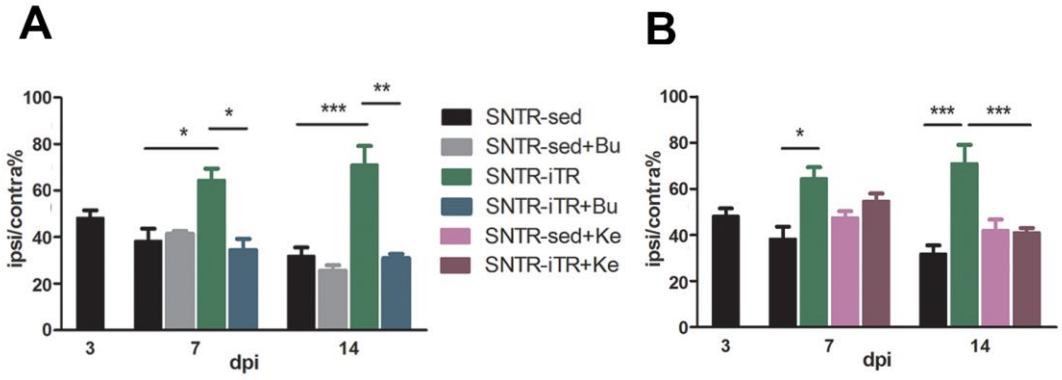

Figure 9. *Blockade of β_2 or $5HT_{2A}$ receptors antagonized the iTR induced hypoalgesia after SNTR.* (A-B) Changes in mechanical threshold of SNTR-sed and SNTR-iTR groups compared with rats treated with Butoxamine (A, SNTR-sed+Bu and SNTR-iTR+Bu groups) or Ketanserin (B, SNTR-sed+Ke and SNTR-iTR+Ke groups), recorded at medial test sites at 3, 7 and 14 days post-injury (dpi) and represented as the percent ratio between the ipsilateral and the contralateral paw. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Depletion of NE output from locus coeruleus with DSP4 did not modify the hypoalgesic effect of iTR

The NE specific neurotoxin DSP4 was injected to induce depletion of NE projections from the LC [23], thus preventing the activation of NE descending projections during iTR (Fig. 10). In rats receiving DSP4 injection the hyperalgesia was even slightly increased compared with untreated rats (Fig. 10A). However, iTR treatment similarly produced hypoalgesia in injured rats ($p<0.01$ SNTR-iTR+DSP4 vs. SNTR-sed+DSP4), despite the depletion of NE output significantly reduced the expression of β_2 and α_{1A} receptors in the LC, which was not compensated by iTR (Fig. 10B-C). This result suggests that the activation of NE descending projections may be not the only mechanism inducing pain relief by iTR, and points out the possibility that mesencephalic 5HT system play a supportive or modulatory role for NE.

To confirm this hypothesis, we looked at the $5HT_{2A}$ expression in LC. Sparse labeling of $5HT_{2A}$ receptors was found in the LC of naïve rats (Fig. 11A), probably corresponding to terminals of PAG excitatory and DRN inhibitory projections that regulate the LC activity [24,25]. SNTR decreased the expression of $5HT_{2A}$ receptors, which was prevented in the groups performing iTR (Fig. 11A-B). The inhibition of β_2 receptors by butoxamine did not have any effect on the expression of $5HT_{2A}$ in the LC of injured rats. In contrast, injection of DSP4 induced a significant increase of $5HT_{2A}$ immunoreactivity ($p<0.01$ and 0.05 SNTR-sed vs. SNTR-sed+DSP4 and SNTR-iTR+DSP4 respectively), even higher than by iTR alone. The strong effect induced by DSP4 on the expression of $5HT_{2A}$ receptors may suggest the activation of DRN 5HT to increase

the inhibition of depleting LC neurons, while iTR exercise seems to restore the normal tonic PAG and DRN 5HT control on NE activity. All together these results demonstrate that iTR hypoalgesia is conveyed by both β_2 and $5HT_{2A}$ receptors likely through parallel activation of $5HT_{2A}$ in PAG and DRN along to adrenergic LC neurons to balance the impairment of their activity due to injury.

iTR reduced microgliosis in locus coeruleus dependent on activation of β_2 receptor after SNTR

Since activation of microglia has been described to be regulated by β_2 adrenergic receptor activation [26,27], and plays an active role in the pathway that leads to BDNF regulation and KCC2 dephosphorylation in neuropathic pain states [4], we evaluated the microglial activation after SNTR (Fig. 12). We focused in LC in order to analyze the reaction of microglial cells adjacent to noradrenergic neurons projecting to the spinal cord. Iba1 immunoreactivity in the LC showed significant microglia activation after nerve injury compared to naïve rats (Fig. 12A-B, $p<0.01$, naïve vs. SNTR-sed).

Interestingly, iTR significantly reduced Iba1 immunoreactivity ($p<0.05$, SNTR-sed vs. SNTR-iTR). However, the effect of iTR was reverted by butoxamine injection ($p<0.001$, naïve vs. SNTR-sed+Bu and SNTR-iTR+Bu). Butoxamine treated groups showed highly ramified microglial cells suggesting that inhibition of β_2 receptors may favor proliferation of microglia (Fig. 12A). These results show that β_2 receptors are involved in the iTR reduction of microglial reaction after nerve injury in the LC, and this mechanism could represent a potential shift from hyperalgesic to hypoalgesic state.

Since the expression of injury-induced microglial BDNF reactivity in the spinal cord dorsal horn neurons is associated to neuropathic pain and is reduced by iTR [9], we also analyzed BDNF expression in the LC. BDNF immunoreactivity was scant in LC of naïve rats, and not colocalized with Iba1 (Fig. 12A, naïve group). The level of BDNF expression was not influenced by nerve injury, but it was present in reactive microglia (Fig. 12A, SNTR-sed group), and increased above normal levels by iTR (Fig. 11A, SNTR-iTR group). In contrast, BDNF immunoreactivity was lowered by butoxamine treatment (Fig. 12A, SNTR-sed+Bu and SNTR-iTR+Bu), although changes were not significant between the different groups (Fig. 12B). This result shows that iTR increases BDNF expression in midbrain areas but not dependent on β_2 receptors differently from the activation of microglia.

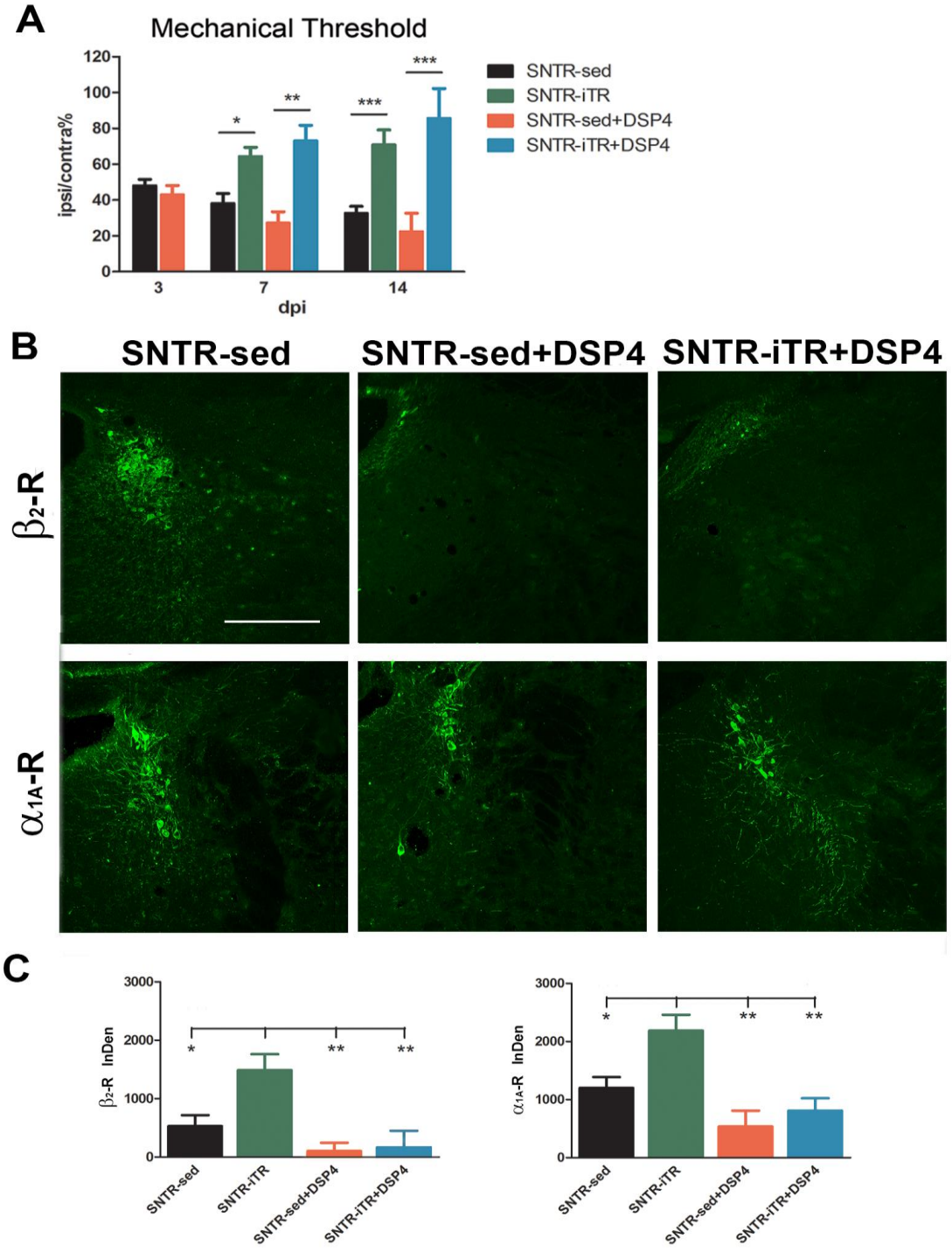

Figure 10. *Depletion of NE output from locus coeruleus with DSP4 did not affect the hypoalgesic effect of iTR*. (A) Changes in mechanical threshold of SNTR-sed and SNTR-iTR groups compared with rats treated also with DSP4, recorded at medial test sites at 3, 7 and 14 days post-injury (dpi) and represented as the percent ratio between the ipsilateral and the contralateral paw. (B) Representative confocal images of α_{1A} and β_2 adrenergic receptors immunoreactivity at 14 dpi in LC of SNTR-sed, SNTR-sed+DSP4 and SNTR-iTR+DSP4 rats. Scale bar 100µm. (C) Quantification of α_{1A} and β_2 immunoreactivity in LC of SNTR-sed and SNTR-iTR groups compared with rats treated with DSP4 (SNTR-sed+DSP4 and SNTR-iTR+DSP4 groups). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

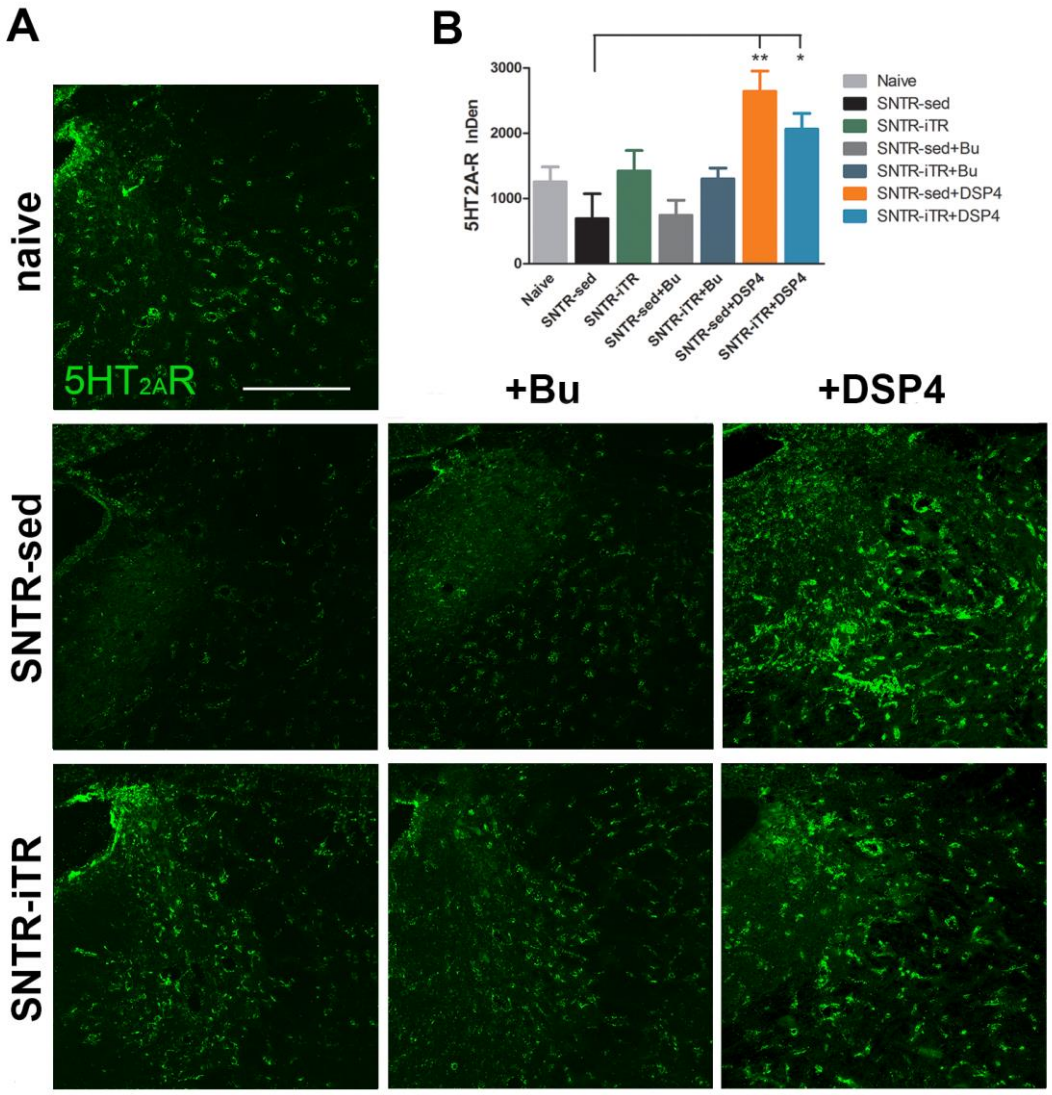

Figure 11. *The expression of 5HT$_{2A}$ receptor in locus coeruleus was normalized by iTR and further increased by DSP4 injection after SNTR.* (A) Representative confocal images of the 5HT$_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in LC of naïve, SNTR-sed and SNTR-iTR rats, and those treated with Butoxamine or with DSP4. Scale bar 100μm. (B) Quantification of 5HT$_{2A}$ immunoreactivity in LC of naïve, SNTR-sed and SNTR-iTR groups compared with the same groups treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups) or DSP4 (SNTR-sed+DSP4 and SNTR-iTR+DSP4 groups). * $p<0.05$; ** $p<0.01$.

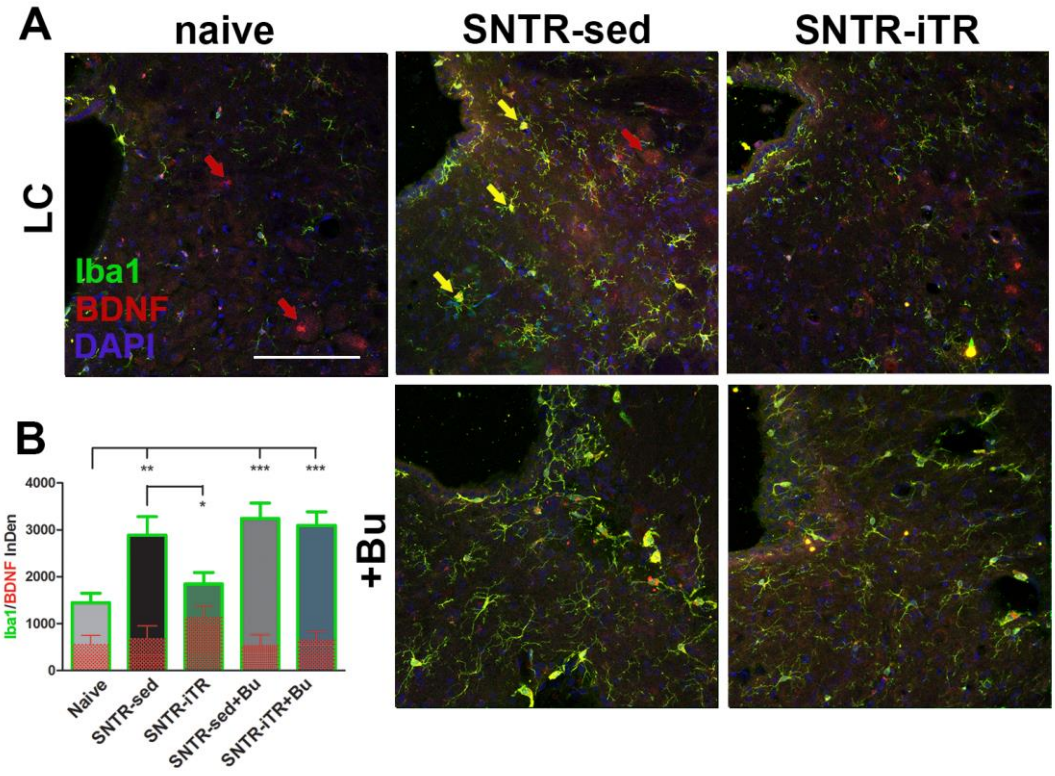

Figure 12. *iTR reduced microgliosis in locus coeruleus dependent on activation of β_2 receptor after SNTR.* (A) Representative confocal images of Iba1 and BDNF immunoreactivity at 14 dpi in LC of naïve, SNTR-sed and SNTR-iTR groups compared with groups treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups), colabeled with DAPI nuclear marker. Yellow arrows point to microglial cells expressing BDNF; red arrows point to BDNF labeling not colocalized with Iba1. Scale bar 100µm. (B) Quantification of Iba1 and BDNF immunoreactivity in double-labeled LC samples of naïve, SNTR-sed and SNTR-iTR rats compared with rats treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

In this study, we aimed to analyze the contribution of 5HT and NE descending pathways to the hypoalgesic effect of iTR on peripheral neuropathic pain. We showed that iTR reduced hyperalgesia was associated with parallel recovery in the expression of serotonergic $5HT_{2A}$ and noradrenergic α_{1A} and β_2 receptors in sensory neurons of lumbar spinal cord and in brainstem areas known to integrate sensory and motor inputs in order to modulate the descending responses. These results shed light on the potential use of specific intensity physical activity, such as iTR, to restore endogenous inhibition as a non-pharmacological adjunct therapy for pain management.

Effects of iTR on the expression of adrenoreceptors in dorsal horn after sciatic nerve injury

The immunohistochemical characterization of noradrenergic receptors expression revealed that they are mainly expressed in laminae II-III of dorsal horn, where they were reduced after SNTR. The recovery of α_{1A} and β_2 but not of α_{2A} receptors observed after iTR may be related to the inhibition of the afferent pain. Baba et al. [10] demonstrated the contribution of α_1 receptors to antinociception by activation of GABAergic and glycinergic inhibitory interneurons in the dorsal horn. Our results suggest that the downregulation of α_{1A} receptors in the dorsal horn interneurons contributes to hyperexcitability and spinal disinhibition after peripheral nerve injury. iTR training upregulated the α_{1A} receptor in the lamina II, a region rich of GABAergic and glycinergic interneurons, thus promoting their inhibitory action.

iTR also reverted the reduction of β_2 receptors, particularly in lamina II of the dorsal horn. The β_2 receptors are present in the central terminals of nociceptive afferents and on dendrites of superficial dorsal horn neurons in the spinal cord [12, 28, 29]. Interestingly capsaicin treatment in rats decreased the β adrenergic binding sites in the spinal cord [28], as we have found after sciatic nerve injury. The facts that iTR increased β_2 expression in the dorsal horn, and that inhibition of β_2 receptors with butoxamine antagonized the hypoalgesic effect of exercise, support the view that β_2 receptors are contributing to mediate the hypoalgesia induced by iTR. This suggestion is in line with the antiallodynic effect demonstrated by Yalcin et al. [13] by pharmacological stimulation of β_2 adrenoreceptors after sciatic nerve lesion.

Effects of iTR on the expression of $5HT_{2A}$ receptors in dorsal horn after sciatic nerve injury

Activation of $5HT_{2A}$ receptors is known to enhance glycine and/or GABA responses in spinal neurons [30-31], and to shift the chloride equilibrium potential in the hyperpolarizing direction [19], restoring endogenous inhibition at the dorsal horn. We have found that iTR increased $5HT_{2A}$ receptors expression in the dorsal horn, suggesting that activation of $5HT_{2A}$ mediated tonic inhibition may contribute to the iTR effect on reducing hyperalgesia after nerve injury. However, an increase in dorsal horn $5HT_{2A}$ receptor density was reported in a model of persistent pain [32], and upregulation and activation of $5HT_{2A}$ receptor was observed after spinal nerve ligation, associated to spinal hyperexcitability [33]. Furthermore, administration of a selective $5HT_{2A}$ agonist in the spinal nerve ligation model significantly increased C-fiber evoked potentials [33].

On the other hand, we showed that immunoreactivity to $5HT_{2A}$ after SNTR significantly decreased in laminae I-III, similarly to β_2 receptor, and the prevention of $5HT_{2A}$ receptor activation during exercise by pretreatment with ketanserin reduced the iTR hypoalgesic effect. These results suggest that if restoration of both spinal $5HT_{2A}$ and β_2 receptors is a direct

consequence of iTR, $5HT_{2A}$ receptor may be not directly involved as β_2 receptor in the mechanism of early pain suppression. This may be explained by the multiple roles that $5HT_{2A}$ receptors may play on neuropathic pain conditions, by differently acting on neuronal subpopulations of dorsal horn to inhibit or facilitate pain signals. $5HT_{2A}$ is an excitatory receptor expressed in the dorsal horn, where it mediates membrane depolarization through phosphoinositide hydrolysis [34]. After injury, a shift of $5HT_{2A}$ receptors to inhibitory interneurons was shown [33]. Since iTR counteracted the spinal loss of $5HT_{2A}$, we suggest that our treadmill protocol may potentiate the activity of inhibitory interneurons that intervene in nociception at the dorsal horn. Besides changes in the spinal cord, antinociception may be conveyed by iTR by activation of $5HT_{2A}$ receptors as well as adrenoreceptors at higher brain centers.

Effects of iTR on the expression of α_{1A}, β_2 and $5HT_{2A}$ receptors in midbrain areas after sciatic nerve injury

It is known that projections from PAG-RM and LC to the spinal cord play a pivotal role in pain control. Pain and temperature fibers project to PAG through the spinomesencephalic tract, and PAG can activate a mesencephalic control of afferent pain by means of parallel actions on RM and LC [35]. In these nuclei, antinociceptive mechanisms can be activated through μ_1 opioid, $5HT_{2A}$ and $5HT_{2C}$ serotonergic, and α_{1A} adrenergic receptors of the LC [36]. β_2 receptors are also expressed in areas directly participating in pain pathways [12], and human genetic studies confirmed the contribution of β_2 to chronic pain disorders [37]. Moreover, β_2 receptors are essential for the antiallodynic action of antidepressant drugs [38,39], since the absence or blockade of β_2 adrenoreceptors suppressed the hypoalgesic effect of antidepressant drugs on mechanical allodynia [13].

The actions played by iTR on mesencephalic nuclei to activate central pain control may be multiple, and conveyed through synaptic regulation of NE and 5HT interconnections. Indeed, PAG is known also to be excited or inhibited by $5HT_{2A}$ and $5HT_{1A}$ receptors expressing fibers from DRN, which form a regulatory circuitry negatively modulated by GABA and opioids [40,31]. The DRN is the largest serotonergic nucleus providing most supply of 5HT to forebrain, and its dorsal subnuclei are located adjacent to the PAG, where dense clusters of 5HT neurons project to the RM [41,42] and to the LC [25]. Activation of the brainstem 5HT system modulates both nociceptive and motor spinal cord circuits [43,44], and exercise increases the release of 5HT in supraspinal areas associated to reducing mechanical hyperalgesia [45,46]. Motor activity directly modulates also 5HT activity in midbrain and spinal cord [16,47]. In a recent work, Bobinski et al. [48] found increased brainstem levels of 5HT, its metabolites and $5HT_{1B/2A/2C}$ receptors after a 2-weeks low-intensity treadmill training following sciatic nerve injury. Interestingly, the inhibition

of 5HT but not of catecholamines reverses the hypoalgesic effect of such low-intensity exercise [48]. Our study confirms that the 5HT system is activated by exercise to induce hypoalgesia, but also suggests that, after nerve injury, NE antinociception can be further activated by increasing the intensity of exercise. Indeed, the iTR training induced an increase of α_{1A}, β_2 and $5HT_{2A}$ receptors expression in the PAG-DRN and PAG-LC pathways. Both 5HT and NE systems may interact in these areas of the midbrain during exercise. Earlier studies revealed that NE causes an increase in 5HT neuronal firing in DRN, mediated via activation of α_1 adrenoceptor located on 5HT neurons [49,50]. We hypothesize that the activation of α_{1A} receptor in the DRN may form part of a regulatory loop that is activated by iTR between DRN and LC to enhance LC and RM descending pain-suppressing neurons. NE axons could also activate α_1 receptors on GABAergic and glycinergic inhibitory interneurons leading to inhibition of pain-relay neurons [51].

On the other hand, pharmacological stimulation of β_2 receptor suppressed neuropathic pain after sciatic nerve insult [13]. We found that iTR strongly increased the expression of β_2 receptor in the dorsal horn lamina II interneurons and in the midbrain LC, and that activation of β_2 receptor was necessary to induce iTR hypoalgesia. To understand which part of this circuitry is involved in the hypoalgesic effect, we depleted the NE output from LC by using the neurotoxin DSP4 [21,22]. Administration of DSP-4 has been shown to revert antinociception in different pain models [52-54] and to regulate 5HT agonists induced analgesia [55]. In our model, pretreatment with DSP4 increased the hyperalgesia but did not block the hypoalgesic effect of iTR, even if it destroyed most β_2-expressing LC neurons and maybe part of those expressing also α_{1A} receptors. Since the activity of LC neurons is subordinated to the activation of 5HT projections from PAG and DRN, we suggest that iTR training can trigger NE-induced descending inhibition by reestablishing also $5HT_{2A}$ receptor activity in the midbrain. Our results highlight the 5HT and NE reciprocal actions under neuropathic pain and blockade of NE neurons.

Effects of the 5HT and NE systems activation on microglia and BDNF by iTR

Brain and spinal expression of neurotrophins, particularly BDNF, can be modulated by activation of NE and 5HT pathways, with relevant effects on motor and sensory recovery after nerve injuries. NE activation via β adrenergic receptors seems to be essential for exercise-induced BDNF regulation [55], and β adrenergic blockade significantly attenuates the increase of BDNF mRNA due to exercise in the cortex [56]. On the other hand, BDNF released by activated microglia triggers neuropathic mechanisms, such as downregulation of chloride cotransporter KCC2 [57,58], which is associated to disinhibition at spinal interneurons after peripheral nerve injury [8].

iTR increased the BDNF expression in LC parallel to decreased microgliosis and BDNF-expressing microglia, similarly to what we previously showed in the dorsal horn associated also to recovery of KCC2 levels [9]. The blockade of β_2 receptor increased microgliosis avoiding the effect of iTR, suggesting that activation of β_2 receptor during iTR plays a role in modulation of the neuroinflammatory response to nerve injury. $5HT_{2A}$ receptor activity has been described to downregulate the BDNF expression in the rat brain [59]. The reduction of neurotrophins NGF and BDNF observed in DRG sensory neurons after iTR [2,9] could be associated to the sustained increase of β_2 and $5HT_{2A}$ receptors expression in brain and spinal cord, which are reduced by sciatic nerve injury.

These results indicate that the increased neurotrophic factor production in sensory neurons after peripheral nerve injury can be reversely modulated by increased intensity exercise in order to prevent maladaptive plastic changes associated to neuropathic pain.

Conclusions

The results of this study bring new knowledge on the contribution of descending inhibition, by increasing activity of serotonergic and noradrenergic projections from brainstem centers, to the beneficial effects of specific exercise training programs in reducing neuropathic pain. Future studies aimed at understanding which neuronal populations and which molecular mechanisms underlying pain inhibition and central midbrain circuits are still needed to foster the therapeutic possibilities for treating sensorimotor disorders with specific exercise programs.

Conflict of interest statement

The authors have no conflicts of interest to declare. This work was supported by Grant EPIONE (FP7-602547) from the European Commission (EC), and TERCEL and CIBERNED funds from the Fondo de Investigacion Sanitaria of Spain.

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Chapter 3

Chronic electrical stimulation reduces hyperalgesia and associated spinal changes induced by peripheral nerve injury

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Manuscript.

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Abstract

This study characterizes the central changes produced and the impact of several electrical stimulation treatments in reducing neuropathic pain after two types of peripheral nerve injury. Following spared nerve injury (SNI) and sciatic nerve transection and repair (SNTR), rats developed significant mechanical hyperalgesia, that was partially prevented by peripheral nerve stimulation performed at the first 2 weeks after injury. Repeated electrical stimulations (rAES, 50 Hz, 1 h) at 3 days induced an immediate increase of mechanical pain threshold but it had no significant effect reducing hyperalgesia at long term. However, chronic electrical stimulation given daily with two different patterns varying frequency (CES and iCES, 1 h) reduced mechanical hyperalgesia from the very beginning until the end of follow-up in SNTR rats. The hypoalgesic effect of these stimulation treatments was lower in SNI rats.

We also studied several markers involved in the generation and maintenance of neuropathic pain. Chronic stimulation protocols were able to restore β2 adrenergic receptor expression that was highly reduced after SNTR. At 2 and 3 weeks after SNTR we found also a marked decrease in the expression of K^+-Cl^- cotransporter 2 (KCC2) in the spinal cord dorsal horn, which was significantly recovered by chronic stimulation protocols. In parallel, these treatments significantly decreased the activation of microglia and astrocytes after injury in the dorsal horn, thus partially preventing a proinflammatory and hyperalgesic state associated to neuropathic pain. Finally, we observed that GABA expression in the dorsal horn was partially lost after SNTR. rAES did not have any effect, but chronic stimulation protocols were able to increase GABA expression to naïve values in the dorsal horn.

In conclusion, chronic daily electrical stimulation of the injured nerve has a significant effect on signs and spinal changes of neuropathic pain.

Introduction

Neuropathic pain is often resistant to conventional pharmacological therapeutical interventions. Non-pharmacological approaches have been widely investigated for their potential in attenuating neuropathic pain. Relevant studies in animal models demonstrated that both active activity-dependent treatments (ADTs), like physical exercise such as swimming [1] or treadmill running [2, 3], and passive activities including several types of electrical stimulation (ES) [4, 5, 6] are able to reduce neuropathic pain. Although the underlying mechanisms are not yet perfectly understood, it has been postulated that maintenance of the activity in the neural circuits after lesions can modulate the plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs [7]. Peripheral nerve stimulation (PNS) has been shown to be an effective treatment for reducing hyperalgesia in neuropathic pain models [8, 9]. Experimental and clinical evidences suggest that electrical stimulation of injured peripheral nerves is an effective and minimally invasive procedure compared to other surgical interventions, for the treatment of neuropathic pain [10, 11]. The diverse peripheral neuromodulatory techniques applied vary on the type and location of electrode and intensity and frequency of stimulation. All these factors are closely related to the differences in the therapeutic outcome together with the pattern of peripheral nerve activation. The majority of previous studies used low intensity currents varying the frequency in a wide range (i.e. 2-100 Hz) to treat neuropathic pain [12, 13, 14]. We sought to assess the role of PNS but also treadmill running, as forms of passive and active ADTs respectively, in reducing mechanical hyperalgesia in two models of neuropathic pain induced by sciatic nerve injury, the spared nerve injury (SNI) and the sciatic nerve transection and repair (SNTR) in the rat. As a comparison, we also used a common pharmacological treatment with amitriptyline which has been demonstrated to reduce the development of autotomy due to neuropathic pain without affecting peripheral nerve function and regeneration [15], and is currently used in patients and animal experiments to treat neuropathic pain symptoms [16, 17].

It is known that noradrenaline and α1 and α2 adrenoreceptors are closely related with analgesic actions induced at the spinal cord level after peripheral nerve injury (PNI) [18]. Less studied, β_2 receptor has been demonstrated to reduce neuropathic pain with pharmacological stimulation after a sciatic nerve insult [19] and also participates in the reduction of hyperalgesia induced by treadmill running after nerve transection [20]. Following previous results, we think our PNS treatments might modify β_2 receptor expression regulating the activation of microglia produced after nerve injury [21, 22]. This glial activation triggers a big cascade of events leading to generation and maintenance of neuropathic pain. Numerous studies showing hypoalgesic effects derived from electrical stimulation procedures pointed to microglia and astrocytes activation and

proliferation as main actors implied [23, 24]. Therefore, we investigated if repeated single sessions or chronic electrical stimulation following SNTR modify the glial cells activity and neuropathic pain outcome. Reactive microglia also increase the production of some neurotrophic factors, such as BDNF. BDNF is able to downregulate the K^+-Cl^- cotransporter 2 (KCC2) in the dorsal horn neurons [25, 26] resulting in increased levels of spontaneous activity and hyperalgesia. The role of KCC2 in central modulation of pain is crucial due to its direct relation with the GABAergic inhibitory function [27]. All these possible changes produced at the dorsal horn network after a peripheral nerve injury contribute to the progression of neuropathic pain. Another mechanism that can be modified by PNS is the release of GABA by GABAergic interneurons in the dorsal horn [28]. We hypothesize that chronic electrical stimulation can increase the release of GABA in the spinal cord resulting in decreased activity of dorsal horn neurons and a hypoalgesic state.

Materials and methods

Animals and surgery

Adult female Sprague–Dawley rats (240±30 g) were housed in standard cages and kept on standard laboratory food and water ad libitum with a light–dark cycle of 12 h. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). Rats were anesthetized by intraperitoneal (i.p.) injection of ketamine (10 mg/kg, Imalgene 500; Rhone-Merieux, Lyon, France) and xylazine (1 mg/kg, Rompun; Bayer, Leverkusen, Germany) and lidocaine was applied at the skin prior to incision to minimize surgical pain.

The spared nerve injury (SNI), as described by Decosterd and Woolf [29], was performed in the right hindlimb of the rat. The branches of the sciatic nerve (common peroneal, tibial and sural nerves) were exposed. The common peroneal and tibial nerves were tightly ligated with a 6-0 suture, transected distal to the ligature, and a 2 mm portion of each nerve was removed. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The sciatic nerve transection and repair (SNTR) was performed also in the right sciatic nerve at the mid thigh. The sciatic nerve was transected at 92 mm from the tip of the third toe, and repaired by epineurial sutures (10-0), maintaining the fascicular alignment of tibial, peroneal and sural branches [30]. The wounds were closed in two layers and disinfected with povidone iodine. Rats were kept in a warm environment until their complete recovery from anesthesia.

Experimental design

Animals were randomly divided in 6 groups for SNI following different treatments: untreated rats (group SNI, n= 10), amitriptyline (group A, n=4), acute electrical stimulation (group AES, n=4), repeated acute electrical stimulation (group rAES, n=4), increasing-frequency chronic electrical stimulation (group iCES, n=4) and increasing-intensity treadmill running (group iTR, n=8).

There were also 7 groups for SNTR: untreated rats (group SNTR, n= 12), amitriptyline (group A, n=4), repeated acute electrical stimulation (group rAES, n=12), chronic electrical stimulation (group CES, n=12), increasing-frequency chronic electrical stimulation (group iCES, n=12), and increasing-intensity treadmill running (group iTR, n=9). A group of naïve rats (n=6) was added for comparisons with injured rats.

One week before surgery, all the animals were habituated to experimental devices for nociceptive baseline thresholds measurement, electrical stimulation and treadmill locomotion. Animals with electrode implanted were housed individually to prevent the implants from being damaged.

Electrical stimulation

Acute electrical stimulation (AES): immediately after injury, a needle was inserted at the sciatic notch as cathode and the anode was another thin needle inserted in a near muscle. The sciatic nerve was stimulated proximal to the lesion with pulses of 0.1 ms duration and suprathreshold intensity (3 V) delivered at 50 Hz (Grass S44, Quincy MA) for one hour.

Repeated acute electrical stimulation (rAES): an unipolar electrode of 1 channel made in stainless steel with ground (MS303/3-AIU/SPC, 5 cm length, PlasticsOne Inc., Roanoke, VA, USA) was coupled to a self-printed plastic pedestal (Fig. 1D). The fixation of the pedestal was performed suturing its four suture-holes to the fascia of multifidus and gluteus superficialis muscles at the back of the rat [31]. The electrode wires were placed in a stress-release loop to allow for leg movements without pulling and the tips were passed under the gluteus maximus muscle until an opening leading to the sciatic nerve, where the ground tip was trimmed to a shorter length and the cathode tip was twisted in hook shape and placed under the sciatic nerve proximal to the lesion. Stimulation was performed in individual regular cages with freedom of movement at 3rd, 7th, and 14th days post injury (dpi) for one hour each of these days, with pulses of 0.1 ms duration and suprathreshold intensity (3 V) at 50 Hz (Grass S44).

Chronic electrical stimulation (CES): the same electrode and implantation procedure than in the rAES was carried out. However, in this case, stimulation was given daily from the 3rd until the 14th dpi for one hour with the same pattern.

Increasing-frequency chronic electrical stimulation (iCES): electrode, implantation procedure and timing of stimulation were the same than for CES. The pattern of stimulation was designed to mimic locomotion steps performed by rats in the iTR protocol previously described [20, 30]. For this purpose, the frequency of steps was extrapolated into trains of pulses. Each train was composed of pulses of 0.1 ms duration at 50 Hz. The train duration was 400 ms and the train rate was increasing 0.13 trains per second (tps) every 5 minutes from 0.67 tps until 2.1 tps at the end of the hour of stimulation.

Treadmill running

Seven days before surgery, all the animals were habituated to the experimental device for treadmill locomotion (Treadmill LE 8706; LETICA, Barcelona, Spain), and pretrained to the task. Each iTR session consisted of 1 hour running, starting at a locomotion speed of 10 cm/s that was increased 2 cm/s every 5 minutes, until a maximal speed of 32 cm/s [2, 20, 30] . Animals were trained with daily sessions from 3 to 14 dpi.

Pharmacological treatment

Amitriptyline (Sigma-Aldrich) at 150 μg/mL in the drinking water was supplied from 5 days before surgery until the end of follow-up at 2 weeks [15].

Assessment of sensory function

Three days before surgery, all the injured animals were habituated to the experimental devices, and then tested for baseline nociceptive thresholds recording. The nociceptive threshold responses to mechanical stimuli were evaluated on both hindpaws by means of algesimetry tests at 0, 3, 7 and 14 dpi. Algesimetry tests were performed immediately after (-post), one hour (-post+1h) and five hours (-post+5h) after sessions (rAES, CES, iCES and iTR) to evaluate the progression of the hypoalgesic effects and how these were maintained or lost along a short period of time.

Two different areas of the plantar surface of the hindpaw were tested: the lateral (innervated by tibial and sural nerves) and the medial (innervated by tibial and saphenous nerves) regions [32]. Sensibility to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers 30 min before the experiment for habituation. Then, a non-noxious pointed probe was gently applied to each test site, slowly increasing the pressure. The threshold was expressed as the force (in grams) at which rats withdrew the paw in response to the stimulus. Mechanical nociceptive threshold was taken as mean of three measurements per paw region, with 3 min interval between each

measurement, and it was used for calculating the percentage of the injured vs the contralateral intact hindpaw. A cutoff force was set to 45 g at which stimulus lifted the paw with no response.

Values are reported as the percentage ratio between the ipsilateral injured and the contralateral paw at each test day. This allows a representation of neuropathic pain induced by the injury since no significant variations of contralateral thresholds were found in previous studies [33].

Immunohistochemistry of spinal cord

Following the algesimetry test results, we decided to study central mechanisms that could underly the hypoalgesic effect of electrical stimulation treatments in SNTR rats. At the end of follow-up at 14 dpi (for half of the rats per group) and at 21 dpi (for the rest, in order to see possible variations one week after treatment), animals were euthanized and spinal cord samples were collected for immunohistochemical assays. Lumbar (L4-L5) spinal cord segment was removed from perfused animals and kept in fixative for 1 hour and then cryoprotected with 30% sucrose in PBS. Transverse sections, 25 μm thick, were cut on a cryostat and mounted on slides. Sections were blocked with specific serum and incubated overnight with primary antibodies in 0.3% Triton X-100 in PBS, used for staining adrenergic receptors in spinal cord sections (anti-β_2 receptors, rabbit, 1:500, Santa Cruz), along with antibodies to identify microglial cells (anti-Iba1, goat, 1:200, Abcam), astrocytes (anti-GFAP, rabbit, 1:500, DakoCytomation), K^+-Cl^- cotransporter 2 (anti-KCC2, rabbit, 1:500, Millipore) and γ-Aminobutyric acid (anti-GABA, rabbit, 1:500, Sigma-Aldrich). The following day, sections were incubated for 2 hours with Alexa Fluor 488 and/or Alexa Fluor 594 conjugated secondary antibodies (1:200, Life Technologies). After washing in PBS, sections were coverslipped with a solution of DAPI (1 mL/mL) in Mowiol mounting medium.

For each marker, three sections from 4 samples per group were used for quantification of immunolabeling. 20X images were captured with a Zeiss LSM 700 confocal microscope, and analyzed using ImageJ software (NIH, USA). Thresholding of fluorescent signal was adjusted over the background level of a negative control. The integrated density of immunoreactivity was calculated within regions of interest drawn with ImageJ software tool. Data are shown for both ipsilateral and contralateral sides.

Data analysis

Data are presented as mean ± SEM. Statistical analysis of nociceptive thresholds was made by two-way analysis of variance (ANOVA) with group and time after injury as factors, followed by Bonferroni's post hoc comparisons. Statistical comparisons for immunofluorescence data were

made by two-way ANOVA followed by Tukey's post hoc test. The level of statistical significance was 5% ($p<0.05$).

Results

Hyperalgesia is reduced by chronic stimulation after SNTR

SNI produced a marked decrease of mechanical threshold at the lateral side from 3 dpi (Fig. 1; Fig. S1a, c). Electrical stimulation treatments, either acute or chronic, increased slightly, but not significantly, the mean mechanical pain threshold recorded at the lateral side (innervated by the spared sural nerve) from 3 to 14 dpi (Fig. 1).

After SNTR the lateral side of the injured hindpaw was unresponsive during the 21 days follow-up, since this territory remains denervated until at least 4 weeks after injury (data not shown) [30]. SNTR produced a marked decrease of the mean mechanical threshold at the medial side from 3 to 21 dpi (Figs. 2A-C). rAES, consisting in stimulations of 1 hour at 3, 7 and 14 dpi, induced a rapid but non-persistent hypoalgesic effect in SNTR+rAES group. Immediately after stimulation, the reduction of the mechanical threshold by SNTR group was prevented ($p<0.01$, $p<0.05$) but this effect was lost quickly at short-term (Fig. 2A). At the pre-stimulation tests performed at 7 and 14 dpi, the clear effect shown at short time after the stimulation session was lost. On the other hand, in chronic treatments with different patterns of stimulation like CES (Fig. 2B; $p<0.05$) and iCES (Fig. 2C; $p<0.001$, $p<0.01$, $p<0.05$), the prevention of reduction of mechanical threshold was better and sustained along the time. The maintenance of the immediate effect over time poststimulation increased in the iCES group compared with the CES and the rAES groups. Mechanical algesimetries showed in the plots were recorded at the medial side of the paws (Fig. 2D).

Detailed in a supplementary figure, we show how iTR or amitriptyline treatments were less effective in reducing hyperalgesia than electrical stimulation. The hypoalgesic effect was slightly better in SNTR compared to SNI rats from 3 to 14 dpi (Fig. S1). Following these insights, we decided to continue this work getting deeper into SNTR+ES groups to reduce the amount of data and be more exhaustive with the more successful treatment in reducing hyperalgesia.

Chronic stimulation reduced GFAP overexpression in dorsal horn after SNTR

Activation of microglia and also astrocytes is involved in neuropathic pain states. GFAP immunoreactivity was analyzed to identify activation of astrocytes in the lumbar dorsal horn (Fig. 3A). After SNTR, there was a GFAP overexpression in both sides showing hypertrophied astrocytes in the three dorsal laminae compared to naïve rats (Fig. 3B). Repeated-acute electrical

stimulation had no effect in reducing this overexpression at 14 or 21 dpi (Fig. 3B). However, both chronic electrical stimulation treatments induced a clear reduction in GFAP expression. iCES treatment was able to recover naïve expression levels bilaterally at 21 dpi (Fig. 3B; $p<0.05$ SNRT vs. SNRT+iCES) and in the contralateral side at 14 dpi (Fig. 3B; $p<0.05$ SNRT vs. SNRT+iCES), whereas the SNTR+CES group showed reduced GFAP expression at 21 dpi in the contralateral side (Fig. 3B; $p<0.05$ SNRT vs. SNRT+CES).

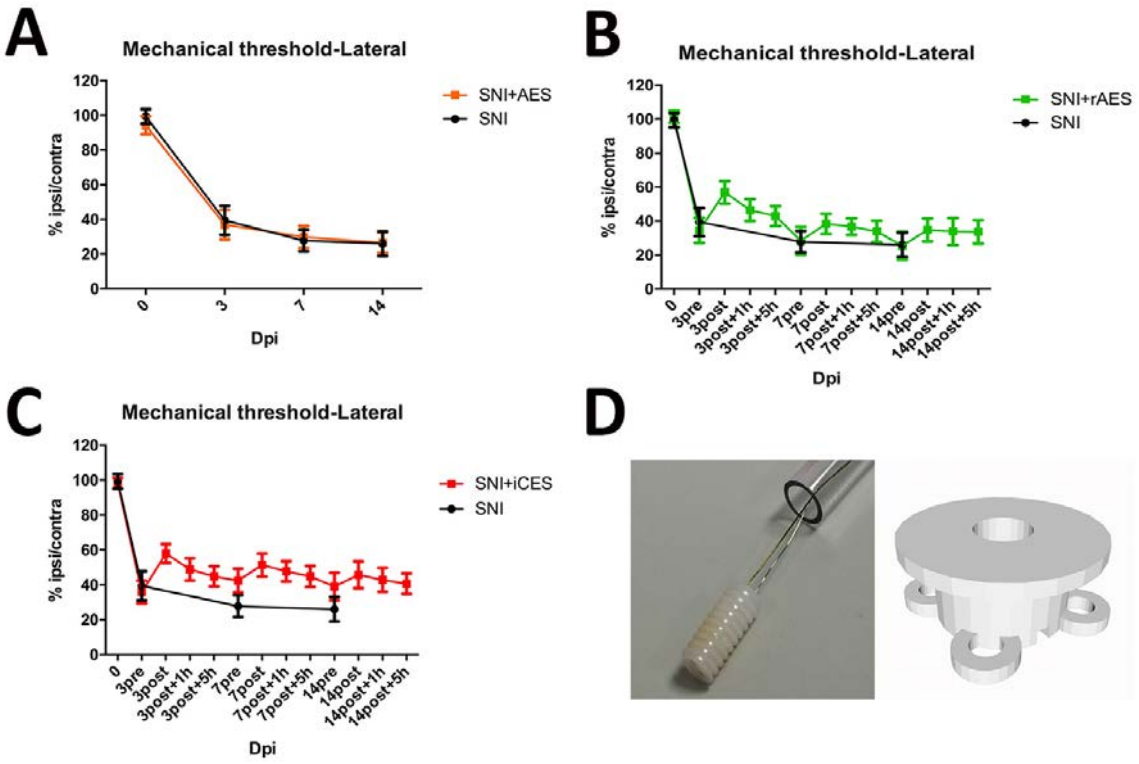
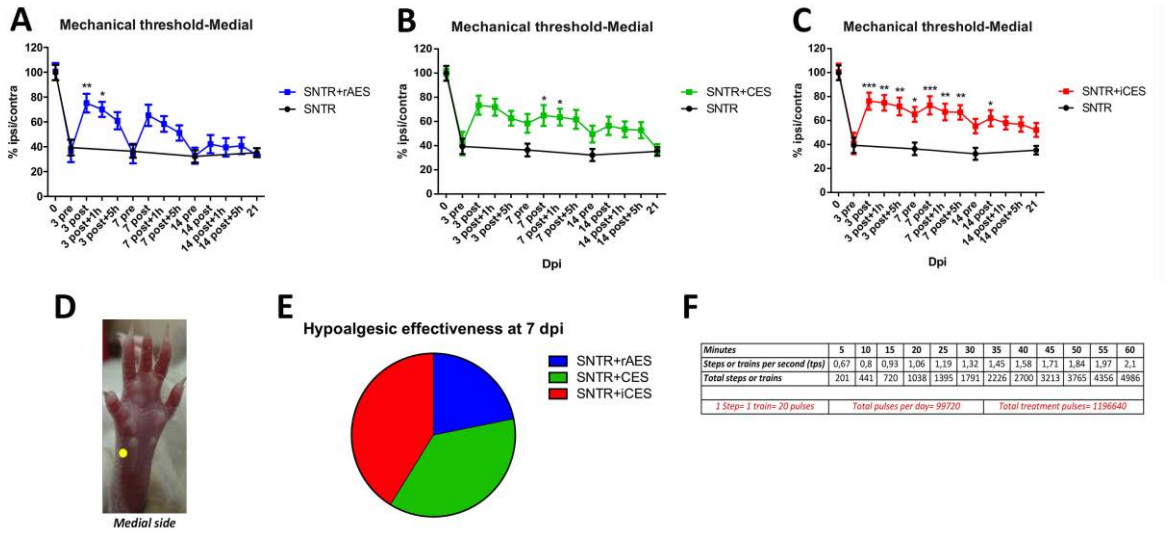


Figure 1. *ES treatments are not able to significantly reduce hyperalgesia after SNI*. Changes in mechanical sensory thresholds recorded at the lateral side in AES (A), rAES (B) and iCES groups (C) after SNI from 3 dpi pre-stimulation to 14 dpi. Recordings were performed also post-stimulation and at 1 and 5 hours post-stimulation. Values are represented as the percent ratio between the ipsilateral and the contralateral paw. Extraneural electrode used in rAES, CES and iCES groups for SNI and SNTR groups (D). 3D representation of plastic pedestal for electrode coupling (D).



Minutes	5	10	15	20	25	30	35	40	45	50	55	60
Steps or trains per second (tps)	0,67	0,8	0,93	1,06	1,19	1,32	1,45	1,58	1,71	1,84	1,97	2,1
Total steps or trains	201	441	720	1038	1395	1791	2226	2700	3213	3765	4356	4986
1 Step= 1 train= 20 pulses	Total pulses per day= 99720						Total treatment pulses= 1196640					

Figure 2. *Hyperalgesia is reduced by chronic stimulation after SNTR.* Changes in mechanical sensory thresholds recorded at the medial (D) test site in rAES (A), CES (B) and iCES groups (C) after SNTR from 3 dpi pre-stimulation to 21 dpi. Recordings were performed also post-stimulation and at 1 and 5 hours post-stimulation. Values are represented as the percent ratio between the ipsilateral and the contralateral paw. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Hypoalgesic effectiveness of different treatments at 7 dpi prestimulation (E). Extrapolation from treadmill training treatment (steps) to iCES (trains of pulses). Total pulses per day and per treatment (F).

Chronic stimulation reduced the increase of Iba1 expression in the ipsilateral dorsal horn after SNTR

After SNTR, there was a marked microglial reactivity, evidenced by an increase in Iba1 immunoreactivity in the dorsal horn compared to naïve rats (Fig. 4A). rAES treatment showed no significant differences with respect to the SNTR group at 14 or 21 dpi (Fig. 4B). iCES treatment counteracted the increase of Iba1 expression in the ipsilateral dorsal horn at 14 and 21 dpi (Fig. 4B; $p<0.05$ SNRT vs. SNRT+iCES), and the CES treatment also at 21 dpi compared to SNTR rats (Fig. 4B; $p<0.05$ SNRT vs. SNRT+CES). In this case, there was a tendency although not significant to reduce the Iba1 overexpression with chronic stimulation treatments in the contralateral dorsal horn.

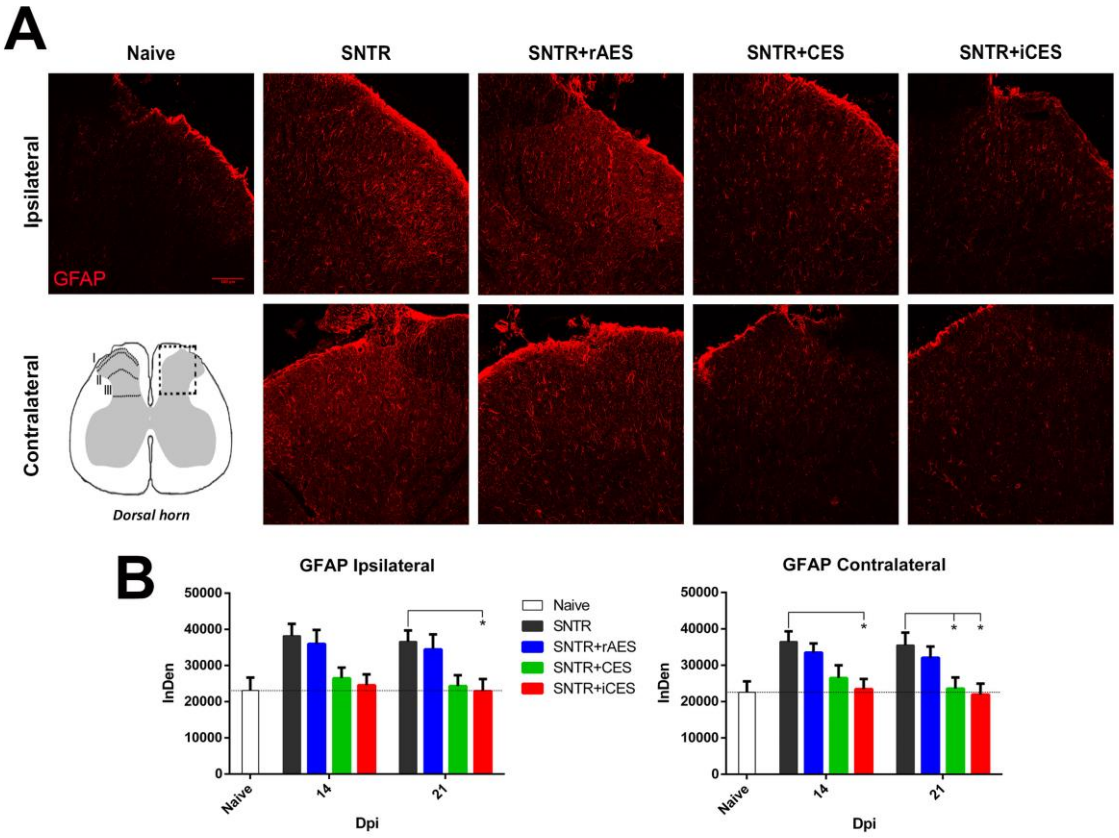


Figure 3. *Chronic stimulation reduced GFAP overexpression in dorsal horn after SNTR*. (A) Representative confocal images of GFAP immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral and contralateral dorsal horns of SNTR, SNTR+rAES, SNTR+CES and SNTR+iCES rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of GFAP immunoreactivity in the ipsilateral and contralateral dorsal horn of naïve, and SNTR rats compared with different ES treatments at 14 and 21 dpi. * $p<0.05$.

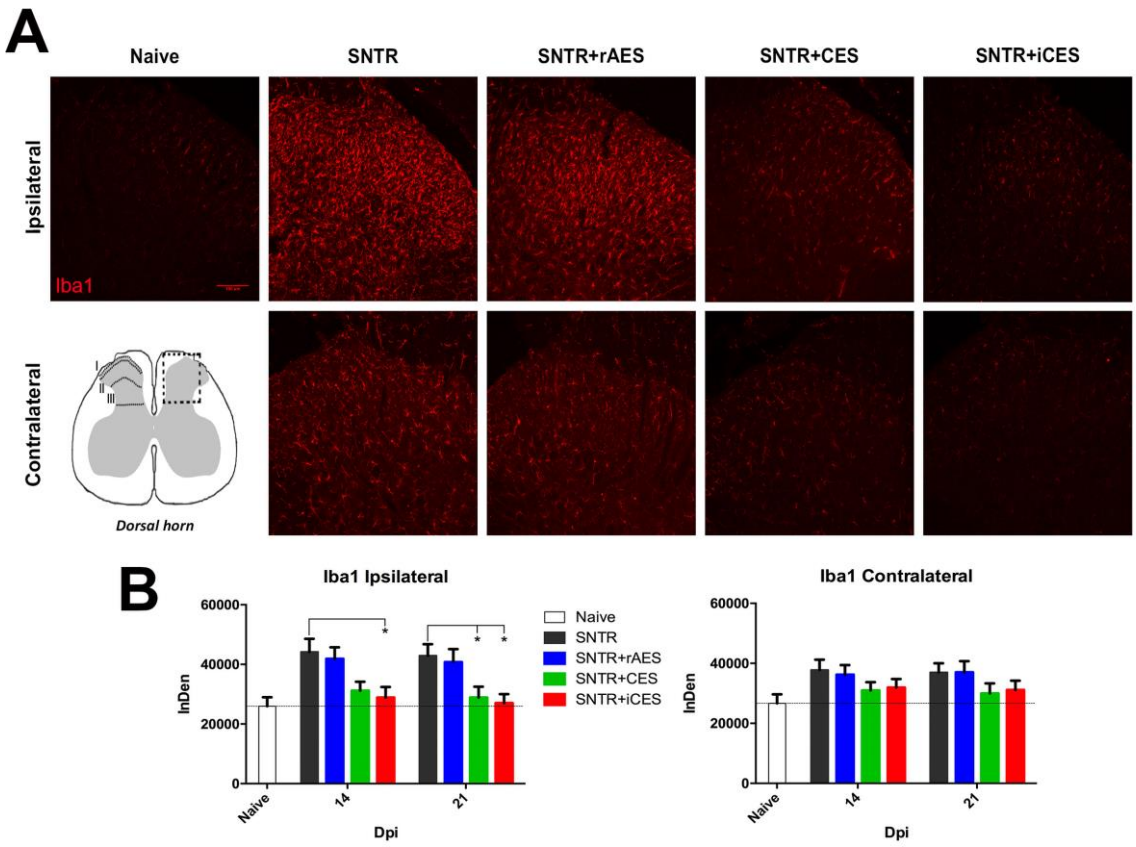


Figure 4. *Chronic stimulation reduced microglial reactivity in the dorsal horn after SNTR*. (A) Representative confocal images of Iba1 immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral and contralateral dorsal horns of SNTR, SNTR+rAES, SNTR+CES and SNTR+iCES rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of Iba1 immunoreactivity in the ipsilateral and contralateral dorsal horn of naïve, and SNTR rats compared with different ES treatments at 14 and 21 dpi. * $p<0.05$.

Chronic stimulation reverted the decrease of KCC2 expression in dorsal horn after SNTR

The decline in KCC2 expression following pathological changes in glial cells activity results in neuronal excitation since it results in a decline of the normally inhibitory interneurons [25]. After SNTR we found a marked and sustained reduction of KCC2 expression bilaterally for at least 3 weeks (Fig. 5A), in agreement with a previous report [34]. Chronic but not acute repeated electrical stimulation protocols were effective reverting the decrease of KCC2 expression. CES and iCES significantly counteracted the KCC2 reduction ipsilaterally at 14 (Fig. 5B; $p<0.05$ SNRT vs. SNRT+CES, $p<0.05$ SNRT vs. SNRT+iCES) and 21 dpi (Fig. 5B; $p<0.05$ SNRT vs. SNRT+CES, $p<0.05$ SNRT vs. SNRT+iCES), and in the contralateral side, group SNTR+iCES

also showed an increase in KCC2 expression at 21 dpi compared to SNTR rats (Fig. 5B; $p<0.05$ SNRT vs. SNRT+iCES).

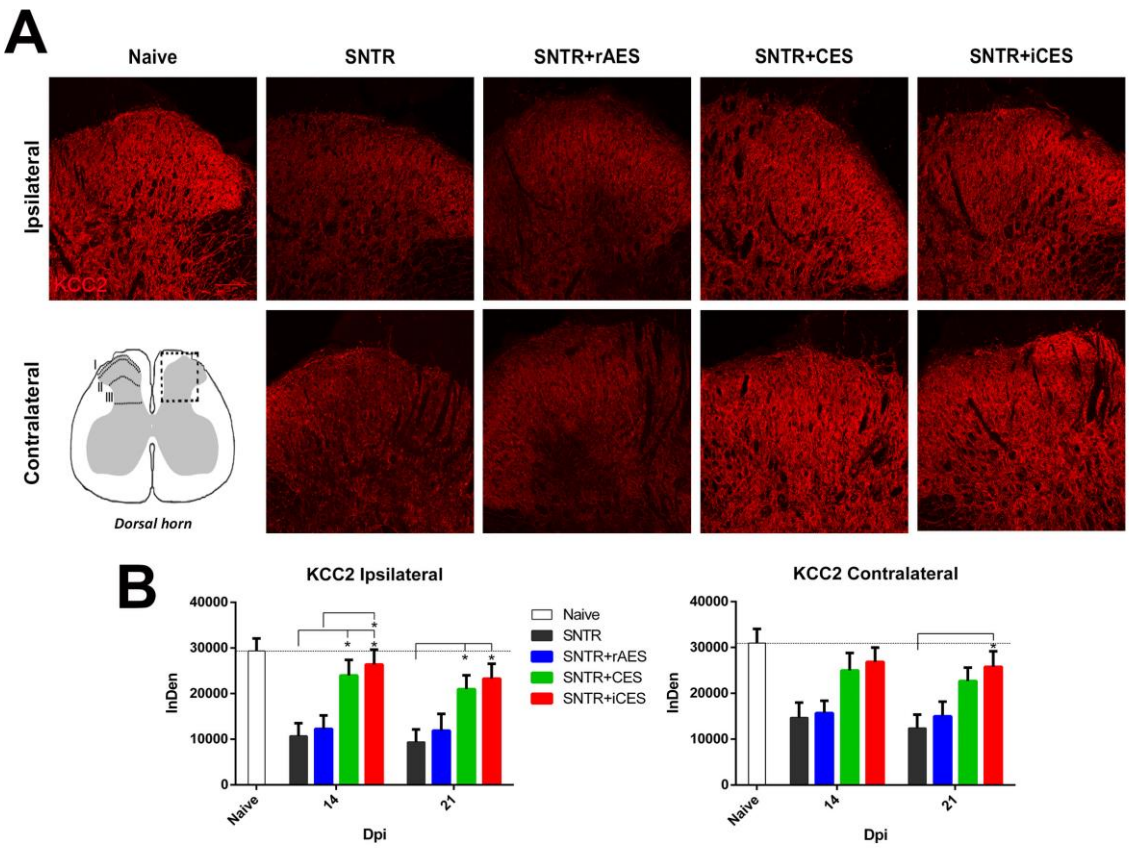


Figure 5. *Chronic stimulation reverted the decrease of KCC2 expression in dorsal horn after SNTR.* (A) Representative confocal images of KCC2 immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral and contralateral dorsal horns of SNTR, SNTR+rAES, SNTR+CES and SNTR+iCES rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100μm. (B) Quantification of KCC2 in the ipsilateral and contralateral dorsal horn of naïve, and SNTR rats compared with different ES treatments at 14 and 21 dpi. * $p<0.05$.

Chronic stimulation counteracted the decrease of β_2 receptor expression in dorsal horn after SNTR

We found β_2 receptor was expressed in laminae I-III of the dorsal horn in the naïve rats (Fig. 6A). SNTR reduced the expression of β_2 receptor in both the ipsilateral and contralateral sides of lamina I, II and III at 14 and 21 dpi. The expression of β_2 was not restored by rAES but chronic stimulation with CES and iCES protocols was effective in maintaining β_2 expression levels close to naïve values in the ipsilateral side at 14 and 21 dpi (Fig 6B). In the contralateral side, only iCES treatment counteracted the decrease of β_2 expression levels after SNTR (Fig. 6B).

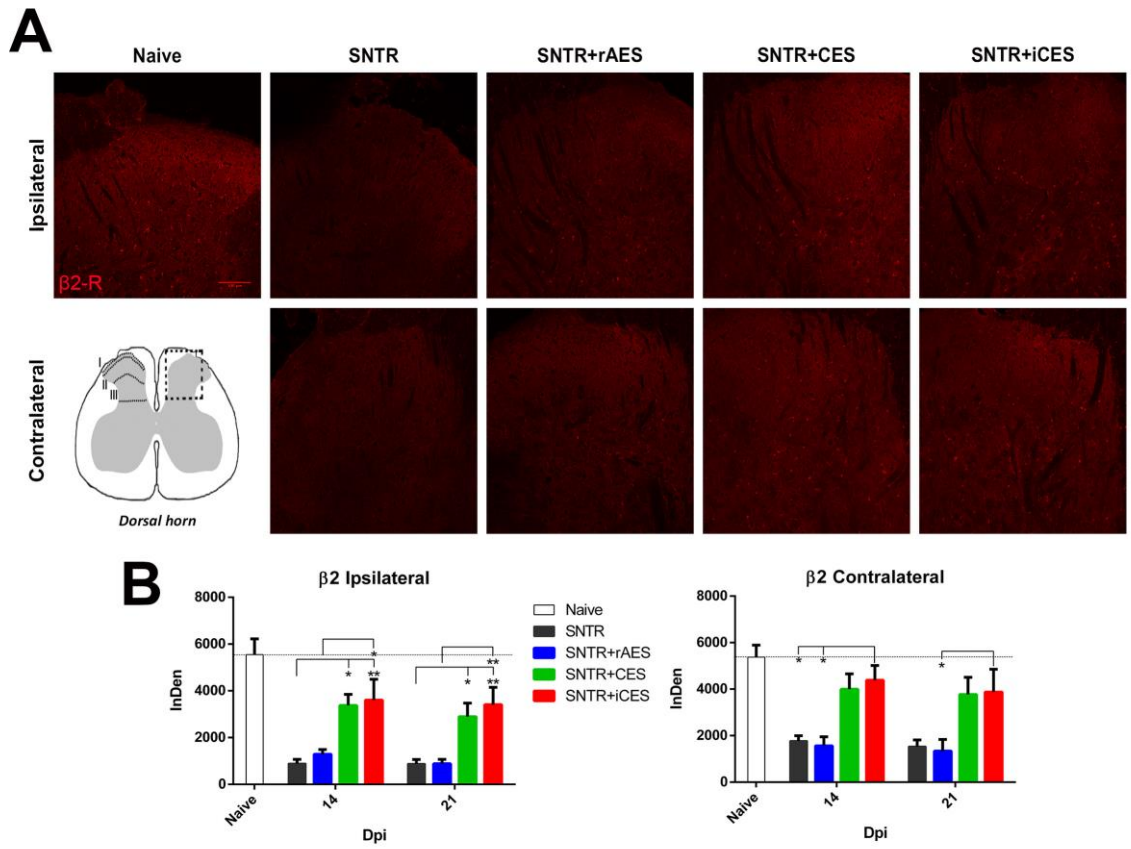


Figure 6. *Chronic stimulation counteracted the decrease of β_2 receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of β_2 adrenergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral and contralateral dorsal horns of SNTR, SNTR+rAES, SNTR+CES and SNTR+iCES rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of β_2 immunoreactivity in the ipsilateral and contralateral dorsal horn of naïve, and SNTR rats compared with different ES treatments at 14 and 21 dpi. * $p<0.05$, ** $p<0.01$.

Chronic stimulation prevented the decrease of GABA in dorsal horn after SNTR

Because of the relation between adrenergic receptors and GABA in spinal inhibition [35] and a previous study showing that GABA release in the spinal cord mediated the hypoalgesic effect of transcutaneous electrical nerve stimulation (TENS) [36], we studied the expression of GABA in the spinal cord dorsal horn. We found that the expression of GABA in lamina I, II and III was reduced in both sides of the spinal cord at 14 and 21 days after SNTR. CES and iCES, but not rAES, treatments significantly counteracted the decrease of GABA in the dorsal horn after SNTR (Fig 7B).

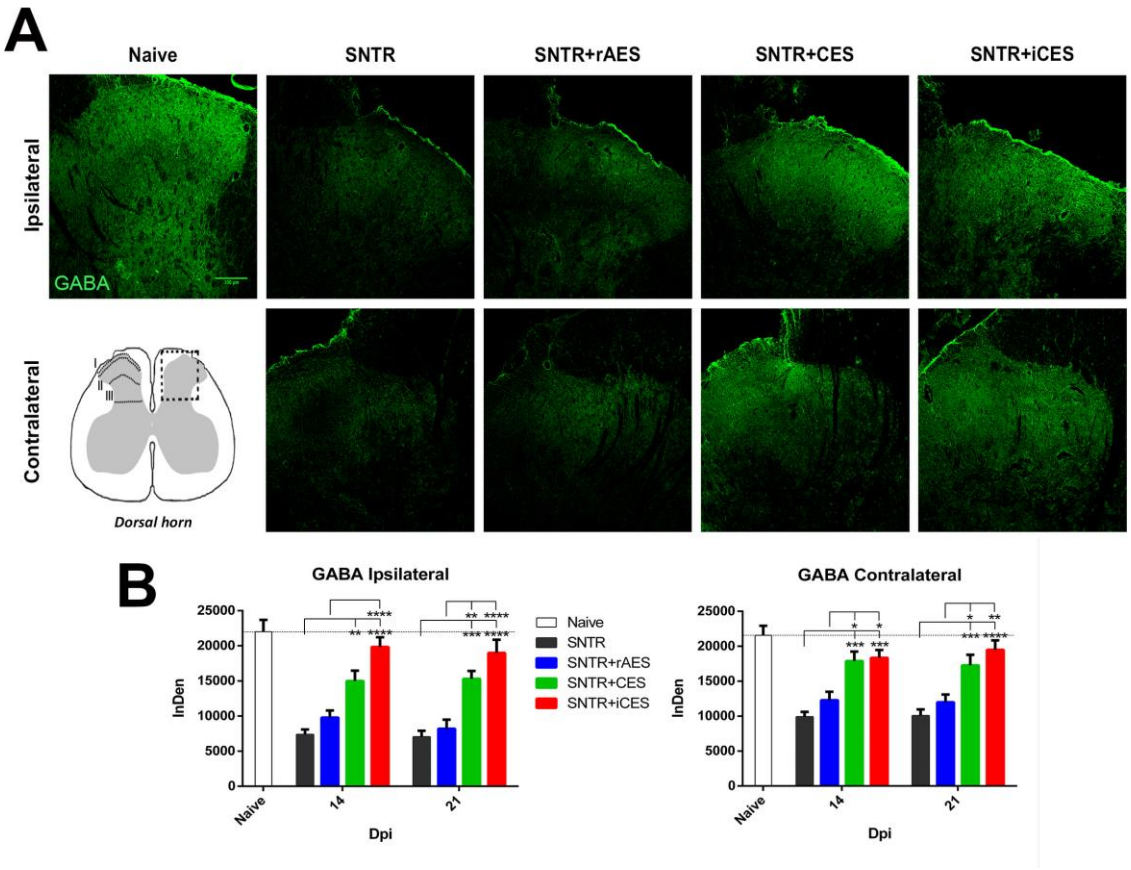


Figure 7. *Chronic stimulation counteracted the decrease of GABA in dorsal horn after SNTR.* (A) Representative confocal images of GABA immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral and contralateral dorsal horns of SNTR, SNTR+rAES, SNTR+CES and SNTR+iCES rats, with graphic representation of laminae I, II and III as regions of interest considered for quantification. Scale bar 100µm. (B) Quantification of GABA in the ipsilateral and contralateral dorsal horn of naïve, and SNTR rats compared with different ES treatments at 14 and 21 dpi. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Discussion

Peripheral nerve stimulation (PNS) is an easy to use and effective treatment for individuals with a variety of pain conditions being clinically effective in patients with neuropathic pain [9, 37, 38, 39]. Also, studies in animal models of neuropathic pain reported that PNS reduces hyperalgesia. Irrespective of the protocol used for PNS application (frequency, intensity and duration of application), PNS inhibits mechanical hyperalgesia [40, 41].

In agreement with the above studies, we demonstrated here the positive effects of three different patterns of electrical stimulation with an extraneural electrode placed proximal to the injury site near the sciatic nerve. Interestingly, these effects only occurred when PNS was applied daily during at least 2 weeks after injury (CES, and iCES), but not when stimulation sessions were

repeated sporadically along the time (rAES). With a daily stimulation at constant frequency of 50 Hz from 3 to 14 dpi we demonstrated that chronic stimulation is more effective than acute in reducing hyperalgesia. The pulse frequency is a key factor and several studies of PNS for neuropathic pain have shown good results in reducing hyperalgesia by applying electrical stimulation at different frequencies [12, 13, 14, 42]. Alternating the frequencies of stimulation induces a variation in the pain thresholds [43] and high frequencies are known to be able to block C-fibers in a concrete range [44]. Active ADTs, like treadmill running, have been also demonstrated to be effective in reducing neuropathic pain and one of the most efficient treadmill protocols for injured rats was described recently, consisting in an increasing-intensity (velocity) running (iTR) [20]. Taking all these works into account, we mimicked the inputs of the running locomotion of the rats and we extrapolated the iTR to a protocol of stimulation called iCES in which the rats were chronically stimulated with trains of stimulation increasing in frequency over an hour of session. The results indicate that this chronic stimulation protocol was the most effective in reducing mechanical hyperalgesia during the first 3 weeks after injury compared to rAES and CES treatments.

Effects of chronic stimulation on glial reactivity in the dorsal horn after SNTR

Peripheral nerve injuries provoke changes in spinal microglia including proliferation, hypertrophy, and overexpression of neuropeptides, cytokines, chemokines, nucleotides and various receptors, all together leading to sustained changes in the dorsal horn environment and contributing to neuropathic pain [45, 46]. Microglia play a role in the initiation of neuropathic pain in the early induction phase [47, 48]. Activation of microglia has been described to be regulated by β_2 adrenergic receptor activation [21,22] and, on its side, playing an active role in the pathway that leads to BDNF upregulation and KCC2 dephosphorylation in neuropathic pain states [34]. We found a significant capacity of chronic stimulation treatments to reduce microglial activation in the dorsal horn of the spinal cord after SNTR. Our data also showed that suppression of aberrant astrocytic activation in spinal cord after SNTR also occurred after chronic but not acute electrical stimulation.

Effects of chronic stimulation on mediators of hyperexcitability in the dorsal horn after SNTR

Activated microglia is known to release BDNF after a peripheral nerve injury that controbutes to trigger neuropathic mechanisms, such as downregulation of chloride cotransporter KCC2 [25, 26]. This reduction of KCC2 in the second order neurons of the pain pathway results in neuronal hyperexcitability when normally inhibitory GABAergic synapses are activated. Activation of

GABAergic inputs to neurons in which the KCC2 transporter is disrupted results in increased levels of spontaneous activity [49]. Indeed, dorsal horn projecting neurons sensitized following a neurogenic injury may be rescued through the KCC2 activator CLP257 [50]. Our work indicates that intense activity-dependent treatments, like iTR [20] and chronic electrical stimulation protocols (present study) restored almost to normal values the decreased expression of KCC2 after SNTR, normalizing the functioning of inhibitory GABAergic interneurons.

The relation between adrenoceptors and pain is complex and it likely depends on the subtype of receptor and on the subtype of pain that is considered. It has been demonstrated that an increase in spinal cord noradrenaline contributes to the antihyperalgesic effect of antidepressants after peripheral nerve injury [51]. In fact, adrenergic as well as cholinergic neurotransmission are two important mechanisms of analgesia in electrical stimulation treatments like spinal cord stimulation (SCS) [52]. Several studies suggested that electrical stimulation induces release of noradrenaline in the spinal cord and among adrenergic receptors, α1 and α2 are shown to be largely involved in pain modulation [53]. Noradrenaline enhances the spinal GABAergic and cholinergic transmission by activating α1 and α2 adrenoreceptors. The tricyclic antidepressants (TCA) analgesic effect concerns the recruitment of noradrenergic descending pathways that inhibit nociceptive responses and its action is considered to be mainly exerted through the α2 receptor.

However, β_2 receptors are also known to be expressed within the dorsal horn of the spinal cord [54] which is a critical relay for nociceptive information. Moreover, pharmacological stimulation of β_2 receptor suppressed neuropathic pain after sciatic nerve insult [19]. In previous studies in our laboratory, we found that iTR treatment strongly increased the expression of β_2 receptor after peripheral nerve injury inducing a reduction of hyperalgesia (unpublished observations). The results shown in this work follow the same line in that chronic stimulation protocols counteract the reduction of β_2 receptor expression after SNTR. The blockade of β_2 receptor increases microgliosis avoiding the effect of iTR, suggesting that activation of β_2 receptor during iTR plays a role in modulation of the neuroinflammatory response to nerve injury.

Alterations in dorsal horn network connectivity provoked by a progressive loss of inhibitory mechanisms contributes to the progression of neuropathic pain. Following a nerve injury, there is a loss of A-fiber mediated inhibition in sensory dorsal horn neurons [55], and it can be explained in part by the reduction in GABA-mediated inhibitory postsynaptic currents in the dorsal horn due to the death of GABAergic interneurons or reductions in the amount of GABA released into the dorsal horn [28]. Some studies showed that low frequency TENS induces release of serotonin in the spinal cord resulting in release of GABA that subsequently decreases activity of dorsal horn neurons. Also, high frequency TENS activates supraspinal δ opioid receptors that release GABA

and enkephalins in the spinal cord resulting in decreased release of glutamate and decreased activity of dorsal horn neurons [36].

Conclusions

The results of this study bring new knowledge on the contribution of chronic peripheral nerve electrical stimulation in reducing neuropathic pain. Our data suggest that chronic stimulation triggers a series of changes in the spinal cord, such as the restoration of expression of KCC2 and β_2 receptor that act directly on the increase of GABA release in spinal cord facilitating the reduction of mechanical hyperalgesia in the paw of rats after sciatic nerve injury. Future studies are needed to understand more about these interactions and which neuronal populations and molecular mechanisms are implied. Also, more work is needed to elucidate differences between SNI and SNTR models in terms of response to ADTs and to optimize the protocols for ameliorating hyperalgesia.

Acknowledgements

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Supplementary data

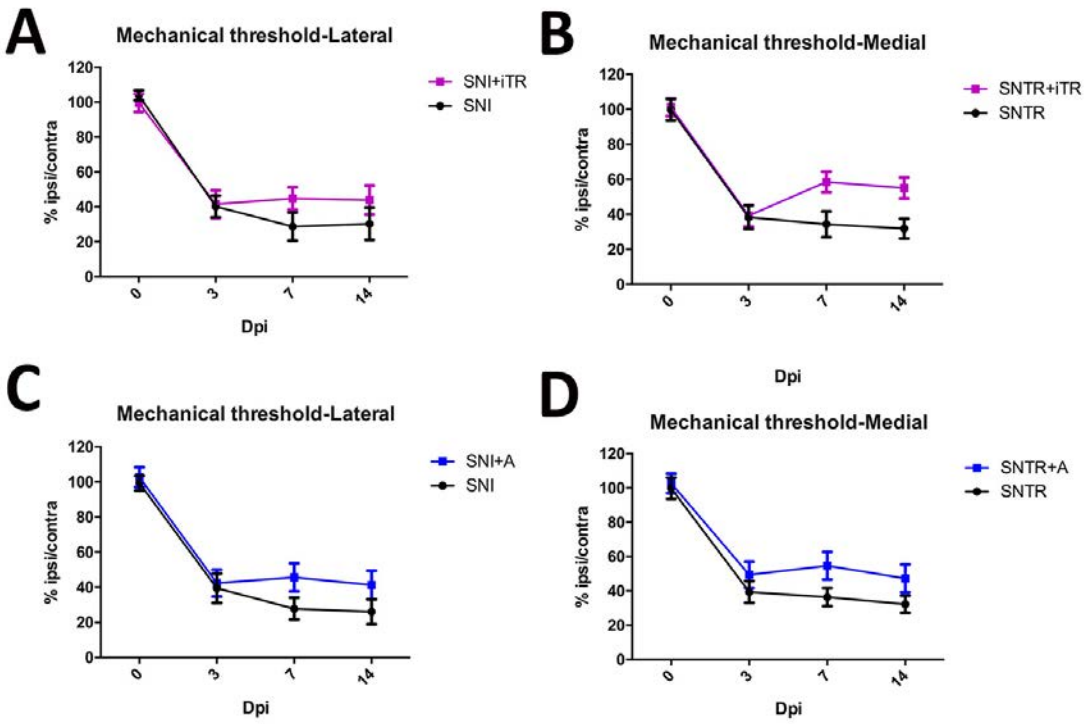

Figure S1. *iTR and amitriptyline were less effective than ES in reducing hyperalgesia after SNI or SNTR.* Changes in mechanical sensory thresholds recorded at the lateral side in iTR (A), and A groups (C) after SNI. Changes in mechanical sensory thresholds recorded at the medial side in iTR (B), and A groups (D) after SNTR. Recordings were performed at 3, 7 and 14 dpi. Values are represented as the percent ratio between the mean ipsilateral and the contralateral paw.

DISCUSSION

DISCUSSION

Neuropathic pain produced after peripheral nerve injuries induces severe and abnormal pain sensations including spontaneous pain, allodynia and hyperalgesia (Cervero, 2009). Changes produced after PNI affect the whole nervous system, both peripheral and central nervous systems, involving disinhibition, sensitization, changes in transduction and plasticity at spinal cord and brain areas. Neuropathic pain associated to peripheral nerve injuries have a high incidence and poor prognosis, despite pharmacological and surgical approaches used to ameliorate its symptoms. Using two different experimental models of neuropathic pain induced after PNI, we focused into activity-dependent treatments, such as exercise and electrical stimulation, to ameliorate the pain symptoms and to investigate more deeply the several mechanisms related with the general hypoalgesic effect induced.

Relevant studies with animal models demonstrated that treadmill running as an active activity-dependent treatment (ADT) but also passive activities like several types of electrical stimulation (Matsuo et al., 2014; Maeda et al., 2008; Cruccu et al., 2007) are able to reduce neuropathic pain through different underlying mechanisms. Maintenance of the activity in the neural circuits after lesions can modulate the plastic changes that neurons suffer due to the loss of synaptic and neurotrophic inputs. Active and conscious locomotion with treadmill exercise ameliorates functional recovery after peripheral nervous system injury but, depending on intensity and duration, different effects have been described. Moderate-intensity exercise recovers sensorimotor functions and enhances regeneration after peripheral nerve injury (English et al., 2011; Marqueste et al., 2004; Sabatier et al., 2008) while intense and prolonged exercise reduces collateral sprouting of motor axons for compensatory reinnervation of denervated muscle fibers (Tam and Gordon 2003; Love et al., 2003) but have also some detrimental effects on peripheral nerve regeneration and neuropathic pain (Asensio-Pinilla et al., 2009; Cobianchi et al., 2010). After some years of investigation, we introduced a new protocol of treadmill exercise, by increasing the velocity progressively during one hour (iTR). Using the SNTR model, we found that iTR protocol performed early after injury reduces hyperalgesia by recodification of spontaneous neural activity, modifying neurotrophins levels, preventing the disruption of chloride cotransporters and counteracting microglial reactivity at central areas. iTR also increased the activity of serotonergic and noradrenergic projections from brainstem centers partially restoring the central disinhibition induced after the nerve injury.

Electrical stimulation as a passive ADT is an effective and minimally invasive procedure compared to other surgical interventions, for the treatment of neuropathic pain (Long et al., 1981; Ma and Sluka, 2001). The therapeutic outcome together with the pattern of peripheral nerve

activation, are closely related to the differences in the type and location of electrode, intensity and frequency of stimulation. We used peripheral nerve stimulation instead of the most common TENS applied to human patients due to its effects are more stable and persistent for animal models. In this thesis, we have analyzed some variations in the type of electrode, the intensity, frequency, duration and timing of the treatment after the injury.

We began by discerning the frequency of stimulation producing the better reduction of hyperalgesia with acute stimulation after injury. Once we discarded the lowest and highest frequencies (1 and 100 Hz), we decided to improve the type of electrode and location by designing a system to make the treatment more durable and stable for at least two weeks in the rat. With the help of a plastic pedestal designed and printed in our laboratory, we were able to attach different types of electrodes implanted in the lower back of our animals to treat them with chronic stimulation applied daily in the freely moving animal. In the first experiments with this setting, we applied repeated acute stimulations sporadically over two weeks and finally chronic stimulations of one hour daily from 3 dpi to 14 dpi. These chronic stimulations were performed with cuffs, intraneural and also extraneural electrodes with the tip placed under the sciatic nerve proximal to the lesion.

After numerous comparisons between our different groups, we found that in rats with SNTR an iCES protocol applied with extraneural or intraneural electrodes, produced the greatest reduction of mechanical hyperalgesia. Our iCES protocol consisting in an increasing-frequency pattern of stimulation, similar to the iTR protocol, triggers a series of changes at central levels, such as the restoration of expression of KCC2 and β2 receptor that act directly on the increase of GABA release in spinal cord facilitating together with the decrease of microglial and astrocytic reactivity, the reduction of mechanical hyperalgesia produced after SNTR.

The experimental models

In this thesis, we used two models well described in the literature to mimic neuropathic pain condition. Before the translation to clinical trials in human patients of new therapies for any injury or disease, an extensive basic research including adequate preclinical in vivo models is required. Nonetheless, there are frequent failures due to the experimental model itself is not reproducing exactly the pathogenetic mechanisms of the human disease, or to methodological problems in the techniques used for assessment. The research on pain has considerable shortcomings, since animals lack the capability of expressing the subjective sensation regarding quantity and quality of pain.

The SNI model is a type of injury recently described by Decosterd (Decosterd and Woolf, 2000) consisting in the complete section of two branches of the sciatic nerve without further

repair. This lesion is very severe and stable and has been described a high incidence of mechanical hypersensitivity with respect to other models such as peroneal axotomy, tibial axotomy, tibial tight ligation and partial tibial tight ligation (Li et al., 2006). The positive response of the SNI model to electrical stimulation treatments such as SCS when compared to these models is also very low (below 10% of the response rate). However, since it was a good established model in the literature, it was our first choice when we started our first experiments with peripheral electrical stimulation (acting in a more proximal way to the injury than SCS does). Probably due to the neuromas formed in these axotomized nerves and the inability to regenerate, PNS had very discrete effects in the reduction of mechanical hyperalgesia.

The SNTR model is also well established in the literature (Cobianchi et al., 2014) and consistent with more frequent clinical situations in which a nerve transection is surgically repaired. In contrast with the SNI model, the SNTR model allows the regeneration of the injured axons which is an essential point for the reduction of the ectopic discharges from transected axons and the hyperalgesia produced by a neuroma as it happens with the SNI model.

The dilemma: promote regeneration versus maladaptive responses

One of the great challenges when designing a treatment for a nerve lesion is to promote a proregenerative environment on the injured nerve (such as in the section and repair model) to recover as much as possible the functionality without negatively affecting other maladaptive responses, like neurosensitization or inaccurate collateral sprouting. After the injury, the peripheral neuron is able to initiate a regenerative response for at least 12 months after injury. Proximal to the lesion, growth cones emerge from the injured axons induced by local neurotrophic and neurotropic factors determining its response. The surrounding Schwann cells support and guide the regenerating axons to their target organs but sometimes, in absence of a guiding structure, the re-growing axons form a neuroma composed of immature axonal sprouts and connective tissue (Siemionow and Brzezicki, 2009).

In an early time window, the presence of local factors is crucial to stimulate or inhibit the axonal regenerative response and a great variety of treadmill exercise protocols have been studied for this purpose regarding beneficial but also deleterious effects depending on the intensity, duration and the timing applied (van Meeteren et al., 1997; Udina et al., 2011). The intensity factor seems to be important producing beneficial effects at mild and high intensities. A mild exercise is associated with stimulation of neurotrophin-dependent adaptive mechanisms such as injured axons regeneration (Asensio-Pinilla et al., 2009; English et al., 2011; Marqueste et al., 1985; Sabatier et al., 2008; Ying et al., 2005). However, high-intensity exercise regimes inhibit those mechanisms of neuropathic pain that may be maladaptive such as collateral sprouting and

neuronal sensitization (Tam and Gordon, 2003; Cobianchi et al., 2010; Cobianchi et al., 2013). For these reasons, we designed in our laboratory a treadmill protocol trying to combine both beneficial effects with the increasing-intensity treadmill training protocol (iTR).

The reactive Schwann cells producing neurotrophic and neurotropic factors surrounding the regenerative growth cones influence axonal elongation (Allodi et al., 2012). However, collateral sprouting of undamaged axons is dependent on local production of neurotrophins. Different exercise intensities may stimulate or inhibit Schwann cells neurotrophins expression by means of an autocrine signaling pathway (English et al., 2014). NGF have a special role leading collateral sprouting of denervated areas (Diamond et al., 1992) but curiously the local administration of NGF after axotomy delays the onset of regeneration (Mohiuddin et al., 1999). Other studies support the findings with anti-NGF serum administration (Diamond et al., 1992; Lankford et al., 2013; Doubleday and Robinson, 1992; Ro et al., 1999; Owolabi et al., 1999; Ugolini et al., 2007).

Previously in our laboratory, we demonstrated than after SNTR the nociception corresponding to the medial area was exclusively mediated by saphenous intact nerve (Cobianchi et al., 2014). In this thesis, we found that iTR reduced significantly the NGF expression in the skin and the L3 DRG corresponding to the saphenous nerve. GAP-43 is a protein associated to the basal lamina of the growth cones which, when phosphorylated, allow the extension and ramification of the growth cone (Van Lookeren Campagne et al., 1989). iTR is also capable to normalize its expression in L3 DRG. Thus, the neuronal sensitization and collateral sprouting by intact saphenous nociceptors to medial areas of the paw were strongly reduced by iTR and consequently the early adjacent hyperalgesic responses associated to it. However, reinnervation by regenerating nerve fibers was not negatively affected by iTR and the late hyperalgesia subsequent to it was also reduced.

BDNF is another important neurotrophin in the central hyperexcitability control. Its levels are normally elevated after exercise depending on the intensity (Ferris et al., 2007; Neeper et al., 1995; Neeper et al., 1996; Vaynman and Gómez-Pinilla, 2005). However, BDNF expression unlike other neurotrophins, can be regulated by activity (Chen et al., 1999; Kuczewski et al., 2008; Lesmann and Brigadski, 2009; Lu and Chow, 1999; Schinder and Poo, 2000; Vaynman et al., 2003). With the iTR treatment NGF expression was normalized and reduced respect to injured animals, influencing dorsal horn neurons through reduction of BDNF release from primary afferents, as BDNF expression is induced within trkA/CGRP neurons by increased peripheral NGF (Michael et al., 1997; Apfel et al., 1996).

iTR exercise modulates neurotrophin signaling-dependent mechanisms that regulate axonal growth preventing peripheral and central mechanisms of sensory neurons hyperexcitability,

parallel to the tuning down of the early expression of NGF and BDNF, that during the first days after injury are generators of neuropathic pain.

iTR also positively reduced hyperalgesia in the lateral sciatic area demonstrating that the iTR hypoalgesic effect is not exclusively limited to the blockade of collateral sprouting. Changes at central sensory circuits are also important.

Neuroplasticity: counteracting central disinhibition

Besides the demonstrated peripheral effects, we hypothesized that iTR may activate pain central inhibition normally gating the nociceptive input to supraspinal, medullary and cortical areas, which are decreased after peripheral nerve injury. One of the principal mechanism related with central inhibition we wanted to study was the descending noradrenergic and serotonergic projections to the dorsal horn from brainstem centers and its modulation at the spinal cord inhibitory circuits acting over second-order spinothalamic neurons by presynaptic and postsynaptic mechanisms.

The brain areas we studied (periaqueductal grey area, locus coeruleus and raphe magnus nucleus) trigger this descending inhibition (Millan, 2002). Pain and temperature fibers project to PAG through the spinomesencephalic tract, and PAG activate a mesencephalic control of afferent pain by means of parallel actions on RM and LC (Basbaum and Fields, 1978). Motor activity have been described to reduce mechanical hyperalgesia by releasing serotonin within spinal and supraspinal areas (Gerin et al., 2008; Grackiere and Vinay, 2014; Jacobs and Fornal, 1997; Pearlstein et al., 2005). Recently has been demonstrated the increased brainstem levels of $5HT_{2A}$ receptor after two weeks of low intensity treadmill after sciatic nerve injury (Bobinski et al., 2015) as we showed in this thesis with iTR protocol. The interconnections between NE and 5HT regulates the central pain control by multiple actions. Moreover, the hypoalgesic effect of antidepressant drugs on mechanical allodynia (Yalcin et al., 2010) are directly related with β_2 receptors participating in pain pathways contributing also to several pain disorders (Nicholson et al., 2005; Diatchenko et al., 2006). Same as $5HT_{2A}$ mostly in PAG, β_2 receptor was strongly increased in LC. The NE output from LC was depleted producing increased hyperalgesia but it did not block the hypoalgesic effect of iTR probably by the subordination of LC neurons to 5HT projections activation from PAG and DRN. α_{1A} receptor in the DRN was also increased probably forming part of a regulatory loop that is activated by iTR between DRN and LC to enhance LC and RM descending pain-suppressing neurons. The iTR training induced an increase of α_{1A}, β_2 and $5HT_{2A}$ receptors expression in the PAG-DRN and PAG-LC pathways indicating that both 5HT and NE systems clearly interact in these areas of the midbrain during exercise.

In the dorsal horn, $5HT_{2A}$ receptors play multiple roles on neuropathic pain conditions, being an excitatory receptor where it mediates membrane depolarization through phosphoinositide hydrolysis (Hoyer et al., 2002) shifting to inhibitory interneurons after injury (Aira et al., 2010). Its expression in laminae I-III interneurons, similarly to β_2 receptor, was decreased after SNTR and iTR counteracted that, inducing an hypoalgesic effect which was lost by preventing its activation with ketanserin pretreatment. In the NE system, α_{1A} receptors have an important role in endogenous inhibition and they are expressed in GABAergic and glycinergic neurons of dorsal horn lamina II exciting inhibitory interneurons (Babe et al., 2000). α_{2A} receptors on the other hand, are expressed predominantly on the terminals of primary C-fibers afferents (Stone et al., 1998). After SNTR we found a reduced expression of both and iTR was able to counteract the α_{1A}, but not the α_{2A} decrease indicating that may be related to the inhibition of the afferent pain. The downregulation of α_{1A} receptor in the dorsal horn interneurons after SNTR contributes to hyperexcitability and spinal disinhibition, but its recovery by iTR enhances the inhibitory action of GABAergic and glycinergic interneurons. Adrenergic and cholinergic neurotransmission are two important mechanisms of analgesia in electrical stimulation treatments like SCS (Schechtmann et al., 2008) and β_2 receptor is an excitatory adrenoreceptor expressed in the central terminals of nociceptive afferents and on dendrites of superficial dorsal horn neurons in the spinal cord (Nicholson et al., 2005), which activation induces antinociceptive effects (Zhang et al., 2016). Our chronic stimulation protocols (specially iCES) counteracted the reduction of β2 receptor expression after SNTR in the same way as the iTR did particullary in lamina II. Its blockade by butoxamine, antagonized the hypoalgesic effect of exercise, supporting other studies where an antiallodynic effect was shown by pharmacological stimulation of β_2 adrenoreceptors after sciatic nerve lesion (Yalcin et al., 2010). The recovered expression of serotonergic $5HT_{2A}$ and noradrenergic α_{1A} and β_2 receptors in sensory neurons of lumbar spinal cord and in brainstem areas by ADTs, indicate the integration of sensory and motor inputs in order to modulate the descending responses.

The spinal enkephalinergic and GABA/glycinergic interneurons are activated by serotonergic RM and noradrenergic LC projections. Enkephalin acts on the opioid receptors located on the central processes of nociceptive primary afferents and dorsal horn interneurons containing GABA or glycine also inhibit the spinal transmission of noxious sensory signals. Following SNTR, there is a loss of A-fiber mediated inhibition in sensory dorsal horn neuron (Woolf and Wall, 1982) normally explained by the reduction in GABA-mediated inhibitory postsynaptic currents in the dorsal horn due to the death of GABAergic interneurons or reductions in the amount of GABA released into the dorsal horn (von Hehn et al., 2012). The release of GABA is increased by low and high frequency TENS by releasing serotonin or δ opioid receptors resulting in decreased

release of glutamate and decreased activity of dorsal horn neurons (Maeda et al., 2007). In our case, the chronic stimulation treatments (CES and iCES) increased also GABA release after its reduction by SNTR. Under hyperalgesic states $5HT_{2A}$ receptor was found to be expressed lamina II galanin-containing neurons expressing GABAergic boutons (Tiong et al., 2011). Its expression is compromised after SNTR and iTR as an ADT, is capable again to restore it recovering the decreased GABA release and reducing hyperalgesia.

Cl^- homeostasis in primary sensory neurons is mainly due to NKCC1. Its upregulation after PNI has been associated with the increased intracellular Cl^- concentration in DRG neurons and with the spinal cross-excitation of primary afferent depolarization (Price et al., 2005; Price et al., 2009). A reduced KCC2 activity at the dorsal horn of the spinal cord was also induced by SNTR (Modol et al., 2014). BDNF released from activated microglia causes the shift in neuronal anion gradient underlying neuropathic pain. The reduction of KCC2 in the second order neurons results in neuronal hyperexcitability when normally inhibitory GABAergic synapses are activated and iTR prevents the injury-induced phosphorylation of NKCC1 at the DRG and the increasing levels of BDNF in the spinal cord, avoiding the reduction of KCC2 after nerve injury. Activation of GABAergic inputs to neurons in which the KCC2 transporter is disrupted results in increased levels of spontaneous activity (Keller et al., 2007). iTR and CES protocols probably also act by upregulating serotonin activity and stimulating PKC to restore KCC2-dependent disinhibition. These novel findings suggest that ADTs are effective approaches to restore loss of inhibition in primary and secondary nociceptive neurons.

ADTs as modulators of glial reactivity

After a PNI there is an increased glial reactivity and overexpression of neuropeptides, cytokines, chemokines, nucleotides and various receptors, all together contributing to neuropathic pain. Activation of astrocytes is also involved in neuropathic pain, especially during the extension of the chronic phase of pain (Svensson and Brodin, 2010). Microglia, on the other hand, play a role in the initiation of neuropathic pain in the early induction phase (Echeverry et al., 2008). BDNF spinal expression can be modulated by activation of NE and 5HT pathways. β2 adrenergic receptor has been described to regulate the activation of microglia (Fujita et al., 1998) playing an active role in the pathway that leads to BDNF upregulation and KCC2 dephosphorylation in neuropathic pain states. The downregulation of chloride cotransporter KCC2 which is associated to disinhibition at spinal interneurons after SNTR is triggered by the BDNF released by activated microglia and in agreement with other studies (Cobianchi et al., 2010), we found that iTR reduced injury-induced microglia activation in the dorsal horn and also the associated BDNF expression. The butoxamine blockade of β_2 receptor increased microgliosis avoiding the effect of iTR,

suggesting that activation of β_2 receptor during iTR plays a role in modulation of the neuroinflammatory response to SNTR. Our CES treatments also showed a significant capacity to reduce microglial and astrocytic activation in the dorsal horn of the spinal cord after SNTR.

CONCLUSIONS

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The main conclusions of the present thesis are:

Chapter I: *Early increasing-intensity treadmill exercise reduces neuropathic pain by preventing nociceptor collateral sprouting and disruption of chloride cotransporters homeostasis after peripheral nerve injury*

1. The increasing intensity treadmill exercise protocol (iTR) reduces mechanical and thermal hyperalgesia recorded at medial and lateral sides of the paw after sciatic nerve section and repair.
2. The early hyperalgesia appearing in the hindpaw after sciatic nerve injury is due in part to hyperexcitability of collateral sprouts from the intact saphenous nerve.
3. iTR inhibits collateral sprouting and reinnervation of plantar skin by nociceptive C fibers, in association with reduced neurotrophin expression in the denervated skin and in the dorsal root ganglion.
4. iTR counteracts central disinhibition after sciatic nerve injury produced by disruption of chloride cotransporters expression and increased BDNF and microgliosis in the dorsal horn of the spinal cord.

Chapter II: *Monoaminergic descending pathways contribute to modulation of neuropathic pain by increasing-intensity treadmill exercise after peripheral nerve injury*

5. Nerve injury induced a marked decrease in the expression of $5HT_{2A}$ and α_{1A} and β adrenergic receptors in the spinal cord dorsal horn, indicating disruption in the descending pathways for pain control.
6. iTR counteracted the decrease of α1A and β2 adrenergic and $5HT_{2A}$ serotonergic receptors expression in the dorsal horn after sciatic nerve injury.
7. iTR was able to increase also the expression of β2 receptor in locus coeruleus. α1A receptor in locus coeruleus and dorsal raphe nucleus, and $5HT_{2A}$ receptor in midbrain areas.
8. The activity of β_2 and $5HT_{2A}$ receptors is needed for the hypoalgesic effect induced by iTR after sciatic nerve section, since this effect was blocked by administration of selective blockers.
9. iTR significantly reduced microglial reactivity in locus coeruleus and increased BDNF expression, an effect that was reverted by butoxamine, a β_2 blocker.

Chapter III: *Chronic electrical stimulation reduces hyperalgesia and associated spinal changes induced by peripheral nerve injury*

10. The effectiveness of peripheral nerve stimulation therapy is lower in the SNI model compared to the SNTR experimental model.
11. Chronic stimulation treatments (CES and iCES) reduce significantly the mechanical hyperalgesia produced by SNTR. The hypoalgesic effect is prolonged during one day after cessation of the stimulation session.
12. CES and iCES reduce the increased microglial and astrocytic reactivity in the dorsal horn of the spinal cord after sciatic nerve injury.
13. Chronic stimulation treatments (CES and iCES) act directly at central level, restoring KCC2 and β2 receptor expression and inducing an increase of GABA release to revert central disinhibition, that coupled to a reduced glial reactivity lead to a mechanical hypoalgesic effect.

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ABBREVIATIONS

α1a: α1-adrenoceptors

α2a: α2-adrenoceptors

ADT: activity dependent treatment

AIDEs: non-steroidal anti-inflammatory drugs

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANS: autonomic nervous system

ASICs: acid-sensing ion channels

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

b-FGF: basic-fibroblast growth factor

CB2: cannabinoid receptor subtype 2

CCI: chronic constriction injury

CGRP: calcitonin gene-related peptide

CNS: central nervous system

CNTF: ciliary neurotrophic factor

CX3CL1: fractalkine

DBS: deep brain stimulation

DCS: dorsal column stimulation

DNIC: diffuse noxious inhibitory control

DRG: dorsal root ganglion

FRAP: fluoride-resistant acid phosphatase

GABA: gamma-aminobutyric acid

GAP-43: growth associated protein 43

GDNF: glial cell line-derived neurotrophic factor

HCNS: heterotopic noxious conditioning stimulation

HSP72: heat shock protein 72

IASP: International Association for the Study of Pain

IB4: isolectin B4

IENF: intraepidermal nerve fibers

IGF: insulin-like growth factor

IL1: interleukin 1

IL6: interleukin 6

IL10: interleukin 10

KATP: ATP-sensitive K+ channel

KCC2: K^+-Cl^- cotransporter 2

LC: locus coeruleus

LTP: long-term potentiation

LTD: long-term depression

MCS: motor cortex stimulation

NA: noradrenaline

NE: norepinephrine

NeuPSIG: Neuropathic pain special interest group of IASP

NF200: 200-kDa neurofilament, characteristic of myelinated peripheral fibers

NF-κB: nuclear factor kappa B

NGF: nerve growth factor

NK1: neurokinin 1 receptor

NKCC1: Na^+-K^+-$2Cl^-$ cotransporter 1

NMDA: N-methyl-D-aspartate receptors

NO: nitric oxide

NOS: nitric oxide synthase

NP: neuropathic pain

NRM: nucleus raphe magnus

NRS: nerve root stimulation

NT-3: neurotrophin-3

NT-4: neurotrophin-4

NT-5: neurotrophin-5

OPC: orbital prefrontal cortex

PAG: periaqueductual gray matter
PKC: protein kinase C
PKCγ: protein kinase C γ
PLP: phantom limb pain
PNI: peripheral nerve injury
PNS: peripheral nervous system
PNS: peripheral nerve stimulation
PSL: partial sciatic ligation
PVG: periventricular gray matter

RET: tyrosine-kinase receptor
ROS: reactive oxygen species
rTMS: repetitive transcranial magnetic stimulation
RVM: rostral ventromedial medulla

SCS: spinal cord stimulation
SNI: spared Nerve Injury
SNL: spinal nerve ligation
SNS: somatic nervous system
SNTR: sciatic nerve transection and suture
SOM: somatostatin
SP: substance P
STT: spinothalamic tract

TCA: tryciclic antidepressants
TENS: transcutaneous electrical nerve stimulation
TNF-α: tumor-necrosis factor-α
TrkA: tropomyosin receptor kinase A
TrkB: tropomyosin receptor kinase B
TrkC: tropomyosin receptor kinase C
TRPV: transient receptor potential channel

VPL: ventral posterior lateral
VR1: vanilloid receptor 1

VRL1: vanilloid receptor-like-1

WDR: wide dynamic range neurons

ANNEXES

Neuroprotective effects of exercise treatments after injury: the dual role of neurotrophic factors

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Abstract

While the relationship between increased physical activity and neuroprotection has been studied for decades, only recently the mechanisms underlying this relationship began to emerge. Several evidences suggest that physical activity offers an affordable and effective method to improve functional recovery in both peripheral and central nerve injuries and to delay functional decay in neurodegenerative disorders. In addition to improving cardiac and immune functions, physical activity modulates trophic factors signaling and, in turn, neuronal function and structure at times that may be critical for neurodegeneration and regeneration. Sustained exercise, particularly if applied at moderate intensity and early after injury, exerts anti-inflammatory and pro-regenerative effects, and may play a role in preserving cognitive and motor function in aging and neuropathological conditions. However, recent evidence suggests that exercise modalities can differently affect the expression of brain-derived neurotrophic factor and other neurotrophins involved in the generation of neuropathic conditions. These findings may lead to the identification of new exercise strategies with therapeutical benefits for nerve injuries. Given the growing number of individuals with peripheral nerve and spinal cord injuries worldwide, better understanding of how modulation of neurotrophic factors contribute to exercise-induced neuroprotection and regeneration is a relevant topic for research, and constitutes a first step toward developing non-pharmacological therapeutic strategies to improve the outcome of rehabilitation of neural disorders.

Introduction

Exercise is able to generate endogenous neuroprotection through the activation of multiple mechanisms, such as promoting neurogenesis, improving the neurovascular unit integrity,

decreasing apoptosis, and modulating inflammation. Through these mechanisms, exercise can be used as a treatment to protect from nerve damage and stroke, and to promote regeneration of injured axons and reduce neuropathic pain. In general, exercise is conceived to provide substantial neuroprotection through a variety of mechanisms all which increase neuronal survival. These protective effects mainly revolve around the neurovascular unit, composed of neuronal, glial and vascular cells. This unit is significantly enhanced by exercise training and is injured in nerve lesions. Excessive excitability, chronic inflammation and microgliosis, added to maladaptive plasticity of injured nerve pathways, are major contributors of neural disorders and neuropathic pain conditions. In this scenario, neuroprotection is conveyed by exercise partly through the upregulation of neurotrophins expression, including brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF). These important regulatory proteins increase neurogenesis, providing a richer neuronal network prior to injury and potentiating neuronal regenerative response. Exercise modalities specific to injury can convey more potent neuroprotection, and animal studies have recently started to tune up training paradigms of different intensities and at critical time windows for treating injuries to the peripheral and central nervous systems. In this view, it seems also to open a strategy for setting up an endopharmachology of pain and regeneration, based on the modulation of endogenous levels of neurotrophins through different exercise protocols. This may depend on the dual role that neurotrophic factors (particularly BDNF and NGF) play after nerve injury, and the opposite effects they can exert on stimulation of neurogenesis and regeneration but also on inhibition of chronic pain and maladaptive plasticity.

Mechanisms of exercise-induced neuroprotection

Effects on the neurovascular unit

Exercise based therapies assume that repeated neuronal activity has to be supported by the neurovascular unit, then gradual training can induce an effective adaptation and reinforcement of its integrity as a whole. Composed of capillary endothelial cells, the basal lamina and astrocytic-glial cells, the blood brain barrier (BBB) provides a robust filtration mechanism which serves as the functional barrier for neurovascular unit integrity in the setting of a neuronal damage. Exercise induces reduction of physical damage to the neurovascular unit, as the most basic structure for neuroprotection and stability, and this effect is conveyed by strengthening the BBB [1,2]. The integrity of the blood brain barrier (BBB) is paramount to maintaining proper filtration of

nutrients and toxic molecules from the vascular system and in providing the necessary structure to the neurovascular unit.

The basal lamina provides the central structural support and selective permeability necessary to maintain a healthy neuronal environment. Various proteins of the extracellular matrix compose the basal lamina, including collagen type IV, laminin, heparan sulphate, proteoglycan and fibronectin, which are produced by surrounding endothelial cells and astrocytes. When this barrier is damaged, its ability to selectively permeate products in the neurovascular system is compromised, which is clinically evident as a vasogenic edema. Under ischemic conditions, the lack of selective permeability and sustained edema lead to cellular swelling. In the brain, swelling of astrocytes and endothelial cells separate them from the basal lamina, further promoting leakage of vascular contents into the cerebral interstitial spaces [2]. Exercise training increases basal lamina thickness, adding both strength and stability to the BBB [3]. Following CNS injury, these effects are associated with decreased cerebral edema and stroke volume, along to improved neuronal recovery. Collage type IV, a major component of the basal lamina, is found to be upregulated in exercised rats, and exercise reduced the net loss of collagen type IV after stroke [3]. The increase of collagen type IV in exercised animals was correlated also to a reduction of behavioral deficits after stroke [3].

Similar to collagen expression, integrins also provide stability to the basal lamina and the BBB, providing additional support to the neurovascular unit. Composed of α and β heterodimers, these proteins serve as cell adhesion molecules within the basal lamina and extracellular matrix, anchoring astrocytes and endothelial cells together, ultimately maintaining the integrity of these structures [4,5]. Integrins act as receptors for numerous ligands and proteins within the basal lamina matrix, primarily collagen and laminin [2, 5, 6]. These structural proteins also serve as signaling receptors for astrocytes and endothelial cells, allowing for dynamic alterations of the BBB in response to exercise, injury, and noxious stimuli. Exercised rats showed significantly higher integrins expression in astroglia and endothelium following muscle [8] and CNS damage [6], correlating with a decrease in behavioral deficit [9].

Astrocytes play a key role in maintaining the neuronal framework. Astrocytosis is a major effect often observed both in brain [10] and in spinal cord [11] after chronic exercise. Some neuroprotective functions are played by astrocytes in order to strengthen the BBB and the neurovascular unit. First, they provide a rigid framework to withstand insults against the neurovascular unit. Astrocyte expansions cover 90% of the cerebrovascular surface with a primary function in restriction of permeability across the damaged BBB [2, 12, 13, 14]. Exercise-induced astocytosis is associated to better outcomes in recovery from injuries [10].

Exercise training may transform the neurovascular system and develop a vital metabolic response to CNS injury. Under normal conditions, angiogenesis and endothelial cell proliferation is scant in the adult brain. Previous studies have shown that locomotion on a treadmill increases blood vessel density in the brain [15, 16, 17, 18] and cortical and striatal angiogenesis [19, 20]. In addition, exercise also increases arteriogenesis, which promotes cerebral blood flow, increases collateral circulation, and ameliorates neuronal injury and death [21, 22]. Through increasing the metabolic demand, exercise may provide an increased blood supply allowing the increased delivery of nutrients to active neurons and leading to further angiogenesis [16, 23]. These changes may reduce brain damage as well [19]. In addition to CNS lesions, exercise can improve blood flow overall in the whole animal, and resulted in higher levels of neurotrophic factors and improved activation of Schwann cells in peripheral nerves [24, 25]. Physical activity has been shown to result in neovascularization and increased peripheral blood flow, and this effect is due to increase in expression of angiogenesis related genes in skeletal muscles [26]. The structural alterations seen in angiogenesis are driven by regulatory growth factors and proteins, namely vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and angiopoietins (Ang) 1 and 2. VEGF and IGF-1 expression is increased in the periphery by exercise and cross the BBB to enter the brain [20, 27, 28, 29]. Blocking either IGF-1 [27] or VEGF [28] peripheral entry to the brain prevented the exercise-induced proliferation of neural precursors in the hippocampus, and reduced the survival-promoting effect of exercise on newly generated neural precursors [27]. VEGF mRNA expression is exponentially higher with increasing duration of exercise training, and exercise-induced angiogenesis is associated with an increase in brain VEGF mRNA and protein [9, 29]. This increase has potent mitotic activity specific to vascular endothelial cells, affecting proliferation, survival, adhesion, migration and capillary tube formation [30]. A role for BDNF in neurogenesis or angiogenesis seems to be also played during exercise, since the induction of BDNF participates in increasing proliferation and survival of new neurons and BDNF regulates baseline neurogenesis in vivo [31].

Effects on the inflammatory response

The neuroprotective action of physical activity has been linked to the ability of preventing and modulating inflammatory conditions. Several studies have shown that individuals who regularly participate in physical activity appear to have fewer viral and bacterial infections [33, 34], a lower incidence of systemic low-grade inflammation [34, 35], and a lower incidence of neurodegeneration and cognitive decline [36]. Experimental studies converge on demonstrating that sustained physical activity include overall enhancement of immune function and anti-inflammatory processes strongly enhancing neuroprotection. These effects seem to depend upon

two critical parameters: intensity and duration of exercise. While chronic exercise leads to a reduction in chronic inflammation, acute exercise conversely may promote or reduce inflammation by increasing the release of pro-inflammatory or anti-inflammatory cytokines respectively, depending upon the intensity of acute exercise bout [37]. Kohut et al. [38] studied older adults participating in aerobic or flexibility exercise for 10 months and found a significant reduction in plasma levels of interleukin 6 (IL-6), interleukin 8 (IL-8), C-reactive protein (CRP), and tumor necrosis factor α (TNFα), linking chronic exercise to anti-inflammatory processes. However, a meta-analysis [39] reported that individuals diagnosed with type I diabetes mellitus, cystic fibrosis, and chronic obstructive pulmonary disease demonstrated an elevated inflammatory response after participation in a single bout of exercise, whereas patients diagnosed with chronic heart failure and type II diabetes mellitus demonstrated an attenuated systemic inflammatory response after participation in chronic endurance training programs. Thus, the analysis of the effects of exercise on particular inflammatory disorders should be conducted in relation to the mechanism by which inflammatory mediators and modulators are produced in the pathology and by the type of exercise.

Effects on inflammatory modulators

The long-term effect of chronic exercise could be ascribed to the anti-inflammatory response elicited by an acute bout of exercise, which is partly mediated by IL-6. IL-6 is a cytokine which may have both pro- and anti-inflammatory effects, and is released in the periphery by T-cells, macrophages, fibroblasts, endothelial cells and osteoblasts [40]. IL-6 plays a critical role in the metabolic regulation of muscle cells and is released in response to eccentric muscle contraction [41]. Moderate levels of aerobic exercise lead to release of IL-6 from muscle, with circulating levels increasing up to 100-fold for up to 1h following activity [42]. Other reports indicate increases of 100-fold in IL-6 after strenuous exercise, such as that encountered when running a marathon. Physiological concentrations of IL-6 stimulate the appearance in the circulation of the anti-inflammatory cytokines IL-1α and IL-10, and may inhibit the production of the pro-inflammatory cytokine TNFα [42]. Moreover, IL-6 stimulates lipolysis as well as fat oxidation. The neuroprotective effects of exercise-induced pro-inflammatory IL-6 was first related to TNFα- insulin resistance. IL-6 -mediated inhibition of TNFα production was suggested by in vitro [43] animal [44, 45] and human [46] studies. Since IL-6 easily crosses the BBB [47], it can impose significant functional alterations on neurons and glial cells, with controversial effects on neuroprotection. Overexpression of IL-6 in mouse astrocytes has been linked to neurodegenerative alterations in age-dependent learning [48]. Strenuous exercise-induced increase in IL-6 could result in inflammation in the brain; however a recent study demonstrated that a

combination of gradual resistance exercise and protein enriched diet can reduce the levels of IL-6 [49], suggesting that the role of IL-6 in exercise-induced neuroprotection requires further investigation.

Differently from IL-6, IL-8 or chemokine (CXC Motif) ligand 8 (CXCL8) is a chemokine with neuromodulatory effects. It is expressed in neurons, glia and endothelial cells of the BBB. Neuroprotective effects of exercise-induced IL-8 may relay in promoting local angiogenesis in muscle [50]. IL-8 is produced in response to high [51] but not moderate intensity exercise [52, 53].

The link between exercise and the immune system implicates the modulation of various inflammatory processes that can be activated by factors implicated in cognitive dysfunction, aging and neurogenerative diseases, extending well beyond classical chemotactic functioning. For example, exercise is likely to suppress TNFα also via IL-6-independent pathways, as demonstrated by the finding of a TNFα decrease in exercised IL-6 knockout mice [54]. Moreover, brief exercise has been shown to significantly increase a pleiotropic chemokine called stromal cell-derived factor-1 (SDF-1), also known as C-X-C motif chemokine ligand 12 (CXCL12). CXCL12 participates in adaptive immune responses and angiogenesis by recruiting endothelial progenitor cells from the bone marrow [55, 56, 57]. It has been shown that 3 weeks of free wheel running lead to a significant increase in CXCL12 gene expression, protein levels, and learning and memory performance in Tg2576 mouse model of AD [58]. CXCL12 is extensively expressed in the CNS, with CXCL12 mRNA and protein being detected in cholinergic, dopaminergic and AVP-ergic neurons in the brain [59].

A crucial role in the human immune system is played by the C-reactive Protein (CRP), and exercise showed to modulate its expression. CRP is an acute-phase protein found in the blood; it is involved in activation of the complement system and is synthesized primarily in the liver, adipose tissue and vascular smooth muscle cells [60]. Pyramidal neurons of the hippocampus express CRP [60] and these neurons show increased CRP expression in people with neurodegenerative diseases [61]. In the opposite, a number of cross-sectional studies demonstrated an inverse correlation between physical activity and CRP levels [62]. However, the clinical data on the effects of exercise on CRP is inconsistent, since CRP levels were significantly reduced in only half of the studies using aerobic exercise regimens in children, adult and elder subjects [63]. All these evidences confirm a direct action of physical activity on critical inflammatory players that may promote neuroprotection from nerve insults as well as from neuronal aging and degeneration.

Effects on microglia and other factors

One of the key effects of physical activity in inducing neuroprotection after neural damage, seems to be the inhibition of microglial activity, as demonstrated in some recent studies [11, 65, 66, 67]. Microglial cells typically exist in a relatively quiescent form, however when detecting damage, they proliferate and form thick clusters, turn in an amoeboid morphology and start to phagocyte cellular debris and foreign material. Microglial activity is triggered by complex signaling cascades, and after nerve injury is regulated by activity-dependent mechanisms [68, 69].The potential immunomodulatory effects of exercise within the brain are of particular interest in the context of neuroprotection against chronic inflammation and aging, as normal aging primes microglia towards the classic inflammatory phenotype. Voluntary exercise can modulate basal changes in microglia activation in aged mice, however the effects vary with age, sex, and brain region [65]. Microglial activation in the central nervous system depends on the presence of purines, cytokines and chemokines that can induce their activation and stimulate protective or toxic activity after peripheral or central injuries. Particularly, chemokines such as CCL2 and CCL21, proteases such as MMP-9 and cathepsin-S (which release further cytokines and chemokines, such as interleukin-1β and fraktalkine), growth factors such as neuregulin-1 (NR1), and purines such as adenosine triphosphate (ATP) have been described [70]. All these signals stimulate the proliferation and chemotaxis of microglia in regions of the CNS in proximity to damage or to which injured afferents project, can be enhanced by increased neuronal activity, and may be modulated by physical activity. Activity-dependent increase of ATP interacting with P2 purinergic receptors has been identified as a particularly critical event for activating microglial cells and generating pathologic inflammatory and painful states [70, 71]. In turn, activated microglia and astrocytes release cytokines such as IL-1β, TNFα, IL-6 and chemokines. Cytokines enhance neurotransmitter release by primary afferents and promote numerous actions associated with neuronal damage, including amplification of microglial activation [72], infiltration of phagocytic neutrophils[73], damage to Schwann cells and to axons [74]. At this level, a critical role seems to be played by BDNF released from microglia, which instead of having a neurotrophic effect as under normal conditions, after injury increases the excitability of nociceptive neurons [68] and reduces the expression of the potassium-chloride cotransporter 2 (KCC2), altering the anion gradient in GABAergic interneurons to have an excitatory rather than an inhibitory action [75, 76, 77]. The overall effect is enhanced excitation, neurotoxicity and induction of hypersensitivity.

In this pathologic environment, specific exercise training could be effective in reducing or preventing microglia activation and associated inflammatory and neuropathic mechanisms, promoting neuroprotection and resolution of the disease. Thus, increasing-intensity treadmill

training reduced sciatic injury-induced microglial activation in the dorsal horn [11] and BDNF expression in microglia [64]. The same treadmill protocol also induced a reduction of BDNF mRNA in dorsal root ganglia (DRG) [78], indicating that intermediate intensity exercise can down-regulate neurotrophin signaling–dependent mechanisms that regulate maladaptive excitability of sensory neurons after peripheral nerve injury, without significantly altering the regeneration of injured axons [64, 78]. GABAergic interneurons are particularly sensitive to altered central BDNF signaling, and increased BDNF-mediated TrKB activation suppresses Cl-dependent fast GABAergic inhibition by downregulating KCC2 [79], inverting the anion flux on GABA receptors activation from inhibitory to excitatory [80, 81]. BDNF expression in spinal microglia was significantly reduced after increasing-intensity treadmill, suggesting that increased locomotion can rescue KCC2 by reducing microglia reaction. Chronic exercise may reduce microglial reactivity and inflammation through regulation of multiple metabolic and transcriptional processes that start in the skeletal muscle. The rate of both glucose uptake and glycogen synthesis are acutely raised in skeletal muscle during exercise. Glycogen synthase kinase 3 (GSK-3) is a major regulator of the balance between the pro-inflammatory and anti-inflammatory mediators in immune cells, including microglia [82]. Chronic adaptations in skeletal muscle with long-term exercise training showedan inverse [83, 84, 85]. In active microglia, GSK-3 promotes the release of IL-1β, IL-6 and TNFα and inhibits the release of anti-inflammatory cytokines like IL-10 [86, 87, 88]. GSK3 controls many neuronal functions, including neurite outgrowth, synapse formation, neurotransmission and neurogenesis, working as a sensor for determining cell fate in brain. The extracellular signals that are known to inhibit GSK-3 and may be activated by exercise include epidermal and platelet-derived growth factors [89, 90], α1A adrenergic receptor stimulation [91] and insulin [92]. It is noteworthy that significant/prolonged inactivation of GSK-3 in microglia and astrocytes shifts balance of secreted cytokines from pro-inflammatory to anti-inflammatory [86, 93, 94].

Effects on neurotrophins expression

Neurotrophins are polypeptides belonging to a closely related family that regulate a variety of neuronal functions including proliferation, survival, migration, and differentiation [95, 96], thus fundamental for neuroprotection [97, 98]. They include NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT4/5). They are synthesized by target neurons and glial cells, and their biological action is mediated by two different classes of receptor systems, the low-affinity P75 neurotrophin receptor (P75NTR) and the tropomyosin related kinase (Trk) family of high affinity tyrosine kinase receptors [99, 100]. Following binding to their receptors, neurotrophins are internalized along with their receptor and transported via retrograde axonal transport to the cell

soma, where they initiate multiple survival promoting effects within the nucleus [99]. A third of sensory neurons do not express receptors for neurotrophins, but receptors for GDNF signaling (RET, GFRa-1, and GFRa-2; Bennet et al., 1998). GDNF is a member of the transforming growth factor-b (TGF-b) superfamily, and has been demonstrated to have effects similar to neurotrophins as potent survival promoter on brain dopaminergic neurons, motor and sensory neurons.

Several works demonstrated that a few days of exercise induce increased production of BDNF and NT-3 in muscles [101], in dorsal root ganglia (DRG) [102] and in spinal cord [103, 104, 105], enhancing survival and regeneration of injured axons. Other factors have also been involved in the exercise-dependent signaling for growth and innervation, such as NGF [106, 107] and NT-4/5 [108]. In uninjured rats, chronic treadmill exercise locomotion of moderate intensity induces the most important changes in muscle and brain BDNF expression, the latter mostly in the grey matter, while NT-4 expression increases in the white matter of the spinal cord, where astrocytes may be additional source of neurotrophins. On the other side, GDNF protein content in skeletal muscle seems to be directly controlled by stretch, while membrane depolarization by acetylcholine (ACh) decreases its content [109]. Expression of GDNF increases in muscles and spinal cord after treadmill exercise [110, 111], and induces morphological changes at the neuromuscular junctions (NMJ) that are activity dependent [110, 112].

The most disabling problem after peripheral nerve injury is the slow growing of injured axons impairing functional recovery with significant misdirection of regenerating axons reinnervating inappropriate targets and inducing also changes of the central neural circuitry [113]. Peripheral nerves are able to regenerate and there is sufficient endogenous neurotrophic factors supply in axotomized motor and sensory neurons and denervated Schwann cells to support nerve regeneration. However a reduced supply in chronic lesions must be supplemented when target reinnervation is delayed, particularly after severe injuries requiring long time for functional recovery [114]. After peripheral nerve injury, increased BDNF and NGF induced by exercise significantly enhanced axonal regeneration [115, 116, 117]. However, findings that exogenous BDNF and GDNF did not increase the number of neurons that regenerate their axons in freshly cut and repaired rat nerves, but did increase the numbers significantly after chronic axotomy [114], are consistent with evidences that increased neurotrophic production by physical activity may be critical in long-term neurorehabilitation. However, the same neurotrophic factors may promote excitability and maladaptive plasticity, such excessive collateral sprouting, that in turn may exacerbate symptoms of pain in peripheral neuropathies. In this view, setting exercise protocols to decrease instead of increasing neurotrophins signaling may be a more efficacious

strategy for treating pathologies characterized by hyperexcitability of peripheral and central nervous system.

Despite their involvement in axonal regeneration, neurotrophins, particularly NGF and BDNF, are well-known mediators and modulators of pain [118], and have several roles in the development of neuropathic pain [119, 120]. Anti-NGF and anti-TrK receptor treatments significantly reduced neuropathic pain in sciatic nerve injury models and prevented the spread of nociceptive collateral sprouting into the denervated skin [120, 121, 122, 123]. Recently, swimming training was shown to significantly reduce mechanical allodynia associated to normalization of increased BDNF, NGF and GDNF expression in DRG after partial sciatic nerve ligation [124]. These results are in concordance with studies in our group using an increasing-intensity treadmill training (iTR) [11, 64], that produced reduction of neuropathic pain along with decreased BDNF, NGF and GDNF up-regulation in DRG [78], BDNF expression in the dorsal horn and NGF expression in the denervated skin [64]. These changes were accompanied by strong reduction of microgliosis in the dorsal horn of the spinal cord [64, 124], normalization of cation chloride cotransporters expression and inhibition of nociceptors collateral sprouting [64]. Elevated BDNF and NGF levels in sensory pathway are associated with neuropathic pain and inflammatory conditions. Activated microglia release BDNF, which increases excitability of nociceptive neurons [125] leading to allodynia [126]. Mechanical hyperalgesia as induced by delayed onset muscle soreness (DOMS) after unaccustomed strenuous exercise has been associated to elevated NGF and GDNF upregulation in muscles [127]. On the other side, after spinal injuries oppositely a loss of neurotrophic factors has been associated to neuropathic pain conditions. Hutchinson et al. [128] found that resolution of allodynia after spinal cord injury was concomitant to normal mRNA levels of BDNF in both lumbar spinal cord and soleus muscle of treadmill trained rats, but not in swimming nor in standing upright trained rats. Upregulation of BDNF in the CNS is considered one of the most important effects of exercise for modulating spinal and brain circuitries, and promoting neuronal repair, memory and learning. Therefore, the effects of increased physical activity on functional recovery mediated by neurotrophins may be opposed in central and peripheral neural lesions.

Neeper and colleagues initiated the study of exercise-induced BDNF and NGF expression in the brain in 1995. They first showed a positive correlation between mean distance run on a running wheel and mRNA for BDNF in the hippocampus and caudal neocortex in rats. The elevation of neurotrophins levels in brain circuits has been related also to neuroprotection and cognitive improvement in neurodegenerative disorders. Several studies have established links between low levels of BDNF and conditions such as depression, schizophrenia and dementia [129, 130]. Specifically, exercise has been used to delay neurodegeneration in aged AD transgenic

mice [131, 132, 133], and humans [134, 135, 136]. In these pathologies, exercise reduces the levels of detrimental factors, for example oxidative stress and inflammation [58, 131, 132, 133], contrary to increase the levels of neurotrophic factors, such as BDNF and IGF-1 [36, 137]. One of the mechanisms proposed for changes produced by exercise in the brain is the promotion of demethylation of BDNF promoter IV in rat hippocampus enhancing brain function and plasticity [138]. BDNF produced by exercise also induced long-term potentiation (LTP) by tetanic stimulation in the hippocampus, which showed only short-term potentiation (STP) in absence of BDNF [139]. The BDNF upregulation in the hippocampus induced by exercise was parallel to an improvement of cognitive function, including memory in rodents [140], whereas downregulation of BDNF or TrkB impaired the formation of memory [141].

Conversion of pro-neurotrophins into mature forms

Neurotrophins actions can change in dependence of their secretory pathway, and they can be altered not only at transcriptional mRNA level but also at the protein level [142]. Neurotrophins are initially synthesized as a precursor protein in the endoplasmic reticulum. Following cleavage of the signal peptide, pro-neurotrophin is transported to the Golgi apparatus for sorting into either constitutive or regulated secretory vesicles, and may be intracellularly converted into the mature form [143]. Different biological actions of neurotrophins, either neuroprotection or neurodegeneration, can be induced by interaction with different receptors, as previously discussed, and regulated by proteolytic cleavage. Pro-neurotrophin forms preferentially activate p75NTR to mediate apoptosis, while mature forms selectively activate Trk receptors to promote survival [144, 145]. For instance, proBDNF and mBDNF elicit opposing synaptic effects through activation of the two distinct receptors, p75NTR and TrkB [146]. Moreover, high-frequency neuronal activity may regulate opposing functions of BDNF isoforms, that may be achieved through different type of neurotrophin receptors. Both low- (inducing long-term depression, LTD) and high- (inducing long-term potentiation, LTP) frequency neuronal stimulation increased proBDNF in the extracellular milieu, but only high-frequency neuronal activity resulted in extracellular conversion of proBDNF to mBDNF [147]. Berchtold et al. [148] reported that 28 days of daily voluntary wheel running increased BDNF concentration in the hippocampus as a result of increasing the conversion of proBDNF into the mature form. These results suggest that different mechanisms of plasticity may be activated in place of different types of neuronal stimulation, and can be induced as a result of different intensities of exercise in the intact as in the damaged neurons.

Effects on neuronal death and survival signaling pathways regulated by neurotrophin receptors

Despite the neuroprotective and pro-regenerative roles of neurotrophic factors have been extensively associated to physical activity, they also participate in apoptotic processes and may generate neurotoxic effects. The biological actions played by neurotrophins determining the fate of neurons are regulated by two different receptors: Tyrosine kinase receptors (Trk) and the p75NTR [149, 150]. NGF binds most specifically to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC. On the other hand, p75NTR can bind all the neurotrophins, but has the additional capability of regulating the affinity of Trk receptor for its cognate ligand. There are some evidences that Trk and p75NTR receptors could form complexes, and p75NTR can increase the ligand selectivity of Trk receptors [151]. p75NTR also appears to function as constituent of other receptor complexes, most notably the Nogo receptor complex [152], that participates in regulation of myelination and axonal regeneration. Despite this, these two types of receptors are not allowed to bind each other directly. Thus by binding to p75NTR, neurotrophins can stimulate apoptosis in different cell types [153]. Proneurotrophins, as proNGF, have been demonstrated to exert this apoptotic function [154].

The biological action of neurotrophins can be regulated by proteolytic cleavage, with pro-forms preferentially activating p75NTR to mediate apoptosis and mature forms selectively activating Trk receptors to promote survival [144, 145]. Apoptosis by p75NTR is facilitated by binding to pro-neurotrophins and sortilin, wich is a trafficking receptor. Cell death through p75 signaling has been observed during stress, injury or inflammation conditions. Thus, p75NTR could be a pro-apoptotic receptor during developmental cell death and after nervous system injury [155]. The p75NTR regulates three main signalling pathways. NF-ϰB activation results in transcription of multiple genes, including several involved in neuronal survival. Activation of the Jun kinase pathway similarly controls activation of several genes; in this case, some of which promote neuronal apoptosis. Ligand engagement of p75NTR also regulates the activity of Rho, which controls growth cone motility. Moreover, it is know that pro-neurotrophins are more effective than mature NGF in inducing p75NTR-dependent apoptosis [151].

On the other side, neurotrophins promote cell survival and differentiation through activation of Trk receptors [150]. Each Trk controls three basic signaling pathways. After neurotrophin binding, Trks activate and recruit adaptor Src homology 2-containing proteins (Shc) and fibroblast growth factor receptor substrate 2 (FRS2), and effectors such as phosphoinositide 3-kinase (PI3K) and phospolipase C-γ (PLC-γ). Synaptic plasticity is promoted by the activation of PLC-γ1 which binds to Tyr790, and this interaction has been proposed to facilitate interactions with ion channels, such as the vanilloid receptor-1 (VR-1) channel, finally resulting in activation

of Ca2+ and protein kinase C (PKC) regulated pathways. Although Tyr490, Shc or FRS2 become tyrosine phosphorylated and provide a scaffold for other signaling proteins leading to activation of the Ras/MAPK (mitogen-activated protein kinase) or PI3K/Akt pathways, this phosphorylation event has many consequences [144, 145]. Activation of Ras triggers the MAP kinase-signalling cascade, which promotes neuronal differentiation and neurite outgrowth. Moreover, activation of PI3K through Ras or Gab1 promotes survival and growth of neurons and other cells.

A number of neurotrophic factors are implicated in the survival, differentiation and function of central nervous system neurons. Among these factors, BDNF, NGF, NT-3 and IGF-1 have been involved in mediating neuroprotective effects of exercise against apoptosis [36, 156, 157]. Aerobic exercise has shown to modulate the β-CaMKII and the PI3K/Akt pathways via neurotrophic factors activation [157, 158]. Activation of Akt is more sensitive to IGF-1R activation [159], whereas β-CaMKII is more robustly activated by TrkB [160]. Different authors have shown that exercise-induced BDNF enhancement is associated with increased survival signaling through PI3K and MAPK pathways [157, 161, 162]. Moreover, blockade of TrkB in the hippocampus abolishes the effects of exercise on hippocampal plasticity and molecular changes, such as increases in Ca2+/calmodulin-dependent kinase II (CaMKII) and MAPK [163, 164]. BDNF also protects against hypoxia–ischemia induced brain damage and inhibits apoptosis by blocking caspase 3 activation. Anti-apoptotic effects of treadmill exercise have been associated to modulation of Bcl-2 family molecules that inhibit the expression of caspases after central injuries [157, 165, 166]. GDNF is also able to inhibit caspase-3 activation and then antagonize neuronal apoptosis following hypoxic–ischemic injury [167, 168]. Neurotrophins and GDNF treatment was proposed as neuroprotective and regenerative therapy for brain neurons in neurodegenerative diseases [169, 170, 171].

Effects on neurodegenerative diseases

The protective impact of exercise on neurodegenerative processes is still under evaluation, and the mechanisms underlying the potential benefits have not been yet determined particularly for pathologies affecting mid to aged humans such as Parkinson's disease (PD) and Alzheimer's disease (AD), or to devastating younger onset diseases like Amyotrophic Lateral Sclerosis (ALS). For PD and AD, current pharmacological therapies are mainly symptomatic and offer only short-term benefits before disease symptoms and drug adverse reactions worsen. There is growing evidence suggesting that physical activity and exercise can generally slow down aging, prevent chronic diseases, and promote health [172, 173].

PD is a neurodegenerative disease characterized by dopaminergic neurons loss in the nigrostriatal system with clinical symptoms such as resting tremor, rigidity, akinesia, and disturbances of postural reflex [174]. A meta-analysis demonstrated that exercise might improve physical functions, health-related quality of life, strength, balance, and gait speed of PD patients [175]. Moderate exercise training exerted neuroprotective effects in PD model of rats, enhancing subventricular zone neural progenitor cell proliferation and migration parallel to increase in BDNF and GDNF levels, with subsequent improvement in deteriorated motor function [176]. Usually both BDNF and GDNF are significantly reduced in the substantia nigra of patients with PD [177]. In another animal model by systemic administration of lipopolysaccharide (LPS) to induce nigral cell loss and parkinsonism, exercise blocked these negative outcomes in proportion to the exercise duration, apparently mediated by elevated BDNF levels [178]. How such animal models relate to PD is open to debate. However, the experimental studies suggest that exercise-induced neuroplasticity is operative in cortical circuits to delay the onset of degeneration. This may be achieved by modulation of neurochemical status in the striatum of rats, particularly increasing neurotrophic factors and possibly by improving oxidative stress. Treadmill exercise in PD rats increased the levels of BNDF and oxidative stress markers such as sarcoplasmic reticulum Ca2+-ATPase (SERCA II), superoxide dismutase (SOD) and catalase (CAT), thus decreasing oxidative damage in lipids and protein [179]. BDNF and GDNF levels were increased in substantia nigra and striatum, respectively, after 18 weeks of treadmill, with associated restoration of mitochondrial respiration, ATP and antioxidant SOD levels, and improvement of motor behavior [180].

The role of oxidative stress in switching from normal brain aging to pathological aging and the benefits of physical activity on AD are corroborated by studies using animal models of AD, particularly the 3xTg and the Tg2576 [158, 181, 182, 183]. AD is a heterogeneous neurodegenerative disorder characterized by amyloid-beta plaques (Aβ), neurofibrillary tangles, inflammation, and neuronal loss [184]. The effects of exercise included improvement in mitochondrial condition and reduction in oxidative damage, but also neurogenesis and angiogenesis through secretion of neurotrophic factors, strengthening of synaptic neurotransmission, increase of brain volume, epigenetic changes, partial reduction of amyloid pathology, and systemic effects of cardiovascular and neuroimmunoendocrine rejuvenation [158, 181, 182, 183, 185, 186]. These beneficial mechanisms are also believed to occur in humans [36, 187].

AD is associated with decreased levels of NGF and significantly increased levels of immature NGF, pro-NGF [188]. It appears that Aβ-peptide accumulation curbs the maturation of NGF, which then results in increased concentrations of pro-NGF. On the other hand, exercise training

has been shown to significantly increase the levels of NGF [107, 189, 190, 191]. The latter study showed that exercise training attenuated the age-associated decrease in BDNF and NGF, which strongly affects long term potentiation (LTP).

Low circulating levels of BDNF have been suggested to predispose patients to AD. A recent Finnish study showed that indeed, circulating BDNF can serve as a marker of impaired memory in aging women [192]. As a result of exercise, the hippocampus and cortex release BDNF into circulation, due to increased BDNF mRNA and protein synthesis in these brain regions. It has been suggested that the brain contributes to about 70-80% of the circulating BDNF [193]. Exercise has the capability of significantly inducing BDNF and NGF and thereby neurogenesis in the hippocampus, resulting in enhanced cognitive function. These processes are just opposite to the deleterious effects of AD. Besides BDNF, GDNF also plays an important role in cell survival and brain plasticity-enhancing properties of neurons, especially in the hippocampal region [194]. Although the available information on the effects of exercise on GDNF is limited, some observations suggest that exercise induces GDNF content, which is involved in the neuroprotective effects of exercise [176, 195].

Additional neuroprotection in AD is conveyed by exercise through modulation of inflammatory factors such as TNFα. Increased levels of TNFα are a prominent risk factor for AD in old adults [196]. A number of studies have linked TNFα to AD pathology and a positive correlation has been found between the levels of TNFα and the severity of AD dementia [197, 198, 199]. Moderate chronic exercise reduces the levels of TNFα [200], resulting in a significant reduction in TNFα gene expression in muscles in normal elderly [201].

Therefore, modifying lifestyle with increased exercise activity would be a non-pharmacological neuroprotective approach for averting neurodegenerative processes as well as to avoid the excessive use of drugs. On the other hand, the benefits of exercise appear to be inverted in the case of ALS, or at least when physical activity is intense, as in high-performance athletes. A recent retrospective study of thousands football players from the Italian professional league established that morbidity ratios were increased for the development of ALS, particularly of young onset, and footballers who played for more than 5 years were at the highest risk [202]. Other studies have suggested a raised risk of ALS in marathon runners and rugby players populations. The exact pathophysiological mechanisms underlying neurodegeneration in both familial and sporadic ALS have yet to be defined. Inheritance of familial ALS is usually autosomal dominant, often linked to mutation of the copper/zinc superoxide-dismutase-1 gene (SOD-1) resulting in the typical adult-onset ALS phenotype [203]. The key function of the SOD-1 enzyme involves free radical scavenging, with the enzyme catalyzing the conversion of the superoxide anion to molecular oxygen and hydrogen peroxide. The pathophysiology of ALS is multifactorial, involving complex

interaction between genetic factors and molecular pathways, with resultant damage of critical target proteins and organelles within the motoneuron. Possible pathophysiological links between exercise and the development of ALS comprise oxidative stress, excessive free radical production and increased glutamate stimulation, all mechanisms that contribute to motoneuron death. These mechanisms may become neurotoxic as a result of exercise and excessive neuronal activation in susceptible individuals. On the other hand, recent studies are in favor of specific low-intensity exercise programs, particularly swimming [204] or moderate treadmill locomotion [205], that increase the lifespan and delay the motor decline in the transgenic SOD1G93A mouse model of ALS. Recent pilot studies involving inspiratory muscle training in patients suggest potential benefit of exercise [206]. Moreover, flexibility, balance, strength and aerobic exercises are indicated in the management of ALS patients for postponing muscular loss, maintain aerobic responses and reduce spasticity [207, 208, 209].

Dosing neuroprotection by tuning up exercise parameters

To date, no specific guidelines have been established in human studies to convey adequate or optimal neuroprotection by exercise training after injury. A variety of treadmill exercise protocols have been applied in experimental nerve injury models, with conflicting evidences regarding beneficial and deleterious effects on neuronal survival, regeneration and pain [210, 211]. Although exercise has been shown to produce endogenous neuroprotection and cardioprotection [212, 213], the intensity, time and duration of different types of exercise have not been conclusively studied. Despite this, some studies revealed that moderate or intermediate intensity and duration of exercise, as opposed to mild or strenuous exercise, correlates with better outcomes and life improvements [214]. Intermediate levels of exercise intensity seem to promote long-term adaptation and induce beneficial plasticity and neuroprotection. In order to optimize the exercise for rehabilitation after nerve damage, the neurotrophic contribution can be modulated by tuning the parameters of the training protocol. For example, a day session of exercise is known to increase plasma and serum BDNF levels probably proportionally to intensity [215] in the same way as does a shorter but intense bout of exercise. Resting BDNF levels also increase after training although ambiguity exists regarding the duration, type and intensity of training required to maintain this effect steady in time [216]. A better understanding of the possible neuroprotective versus neurotoxic effects of exercise after nerve injury can be traced back to the results of modulation of its main variables.

Forced versus voluntary exercise

Voluntary exercise is able to increase the expression of several molecules associated with the action of BDNF on synaptic function and neurite outgrowth in the lumbar region of the spinal cord and the hindlimb muscles [103, 217]. However, forced exercise may elicit responses of both transient and chronic stress, that after nerve injury can boost the loss of neuronal factors and produce most robust neuroprotective effects, along to analgesic effects [218, 219, 220, 221, 222]. Although most forced exercise studies have evaluated nociceptive thresholds after exercise-induced acute stress responses have resolved, prolonged forced exercise gives rise to chronic stress in some cases [218, 220].Voluntary exercise may also elicit a stress response [219]; on the contrary, various studies showed anxiolytic effects of prolonged voluntary wheel running in cases of mild to moderate stress [223, 224, 225, 226, 227].

Rats forced to exercise on a treadmill tend to have better neurologic outcomes after stroke than those under voluntary running on a running wheel [228]. Sheanan et al. [229] recently showed that, contrary to what was demonstrated with forced exercise paradigms, voluntary short-duration wheel running does not improve pain symptoms in mouse models of acute inflammation and peripheral nerve injury. The discrepancy in the effects on neuroprotection of voluntary versus forced exercises seems to be due to the different behaviors. Previous studies have shown that forced exercise on a treadmill is slower but more constant than voluntary exercise, the latter occurring in shorter spurts with faster speed, although the total distance may be equal in the two groups [220]. Neuroprotection by forced exercise led to decreased stroke volume, improved neurologic deficit, upregulation of heat shock proteins, increased neurogenesis and cerebral metabolism [218, 228, 230]. These findings indicate that intermediate exercise intensity over a longer time period, as provided by programming treadmill training, conveys more efficient and greater neuroprotection than more vigorous exercise over shorter time periods as in voluntary running. It is conceivable that effective achievement of a beneficial plastic adaptation to exercise has the cost of the initial effort to increased neuromuscular activity, with self-perceived fatigue representing a first limiting issue for the patients.

Intensity of exercise

A major problem in planning an exercise treatment is to establish the adequate intensity of exercise to activate neuroprotective and regenerative mechanisms but also to prevent or inhibit maladaptive responses such as pain and excessive excitability of motor and sensory neurons. Evidences that neurotrophic factors expression may be related to the amount of activity performed during exercise training suggest a key role of the intensity parameter in positive or

negative modulation of neurotrophins. While some studies reported an increase [104, 105, 217, 231], others showed a decrease of neurotrophic factors following exercise [232, 233]. This discrepancy may rely on variations in the intensity of exercise applied. BDNF and NT-3 in the lumbar spinal cord are known to increase following low-to-moderate intensity exercise [101, 102, 107]. However, moderate-intensity running, that elevated blood lactate and corticosterone levels, caused a depression of BDNF mRNA, but not its protein [234]. An increasing-intensity treadmill exercise protocol decreased BDNF, NGF and GDNF mRNA levels in the rat DRG [78], and BDNF expression in the spinal cord was also reduced [64]. High-intensity treadmill exercise decreased BDNF levels in the frontal cortex of mice [235] independently if continuous or intermittent [236]. Thus, it seems that moderate-to-high intensities of exercise may trigger responses opposite to low intensity exercise on neurotrophins production, as a regulatory negative feedback similar to that observed under sustained neural activity and stress. These results are consistent with previous studies reporting a decrease in BDNF mRNA expression in the hippocampus induced by stress and glucocorticoids, as well as immobilization and exhaustive exercise [237]. However, more studies are needed to accurately understand the interplay between exercise intensity and neurotrophic factors production.

In the muscles, neurotrophic factors expression may be different depending on the type of muscle that is more stimulated by specific activities. There may be an activity-dependent relationship that determines the amount of neurotrophin protein levels in different types of skeletal muscle, since their production seems to depend on the intensity of exercise training [238, 239]. Low- to intermediate intensity locomotion likely activates slow-twitch muscle fibers to a greater extent than fast twitch muscle fibers. Treadmill exercise increased the expression of BDNF in the soleus muscle (containing slow-twitch fibers) but no differences in the gastrocnemius muscle (containing fast-twitch fibers) [240]. GDNF protein content also increased in the soleus muscle and decreased in the extensor digitorum longus muscle (with high proportion of fast-twitch fibers) following low-intensity exercise [109]. A greater expression of NT-4 was observed in the slow fibers of the soleus compared to the fast fibers of the gastrocnemius muscle [238].

Exercise intensity may also affect the neurotrophic response to injuries depending on their severity. Acute and prolonged passive cycling treatment in spinalyzed rats increased the mRNA of BDNF, GDNF and NT-4 in motoneurons compared with little mRNA increase following the injury [241], differently to what was observed after peripheral nerve injuries [242]. In contrast, in large DRG neurons, mRNA of neurotrophic factors and their receptors were largely unaffected by either spinal cord injury with or without exercise [241]. These results show that intensity of

training should be taken in consideration and adapted to different types and grades of injury in planning rehabilitative exercise protocols.

The debate is open regarding how and to what extent the exercise intensity may be added to the type and degree of nervous injury, in order to generate an optimal neurotrophic contribution. The possible mechanisms for an increase in neurotrophins following moderate intensity exercise are considered the increased conversion of pro-neurotrophins to the mature form [243], and the induction of IGF-1 [244, 245] and estrogens [246, 247] as well as the reduction of leptin [245] and corticosterone concentrations [246]. It is still unclear, however, how high intensity exercise may induce the opposite effect and, therefore, trigger a downregulation of neurotrophic factors. Both high [248] or low [246] intensity exercise trainings enhanced BDNF concentration in the brain due to the reduction of corticosterone concentration, suggesting that mechanisms for inducing neurotrophin downregulation may be more than just activation of a cortical stress response.

Observation of rodents preferences for training to continuous forced running over a treadmill at sustained speed led us to formulate an increasing-intensity treadmill protocol (iTR) [11, 64, 78]. iTR running in rats starts at normal locomotion speed (10 cm/sec) and gradually increases (2 cm/sec every 5 minutes) until 1 hour of training session. In this way injured animals [11, 78] do not refuse the final high speed running (32 cm/sec) if slowly adapted to it without stressors or electric shocks. Resembling this protocol, intermediate levels of exercise intensity may be applied to human patients by similarly starting with normal kinesis and gradually increasing velocity until elevated but sustainable levels of activity. iTR performed early and short-lasting after injury significantly reduced mechanical allodynia after sciatic nerve chronic constriction injury and section and suture repair [11, 249], without impairing nerve regeneration. Interestingly, hyperalgesia was significantly reduced, together with decreased levels of BDNF and NGF in DRG and spinal cord, inhibition of microgliosis and normalization of chloride cotransporters homeostasis [64, 249]. Compared to a low-intensity protocol, the iTR protocol demonstrated neuroprotective effects counteracting the destruction of perineuronal nets (PNN) and reducing the motoneurons synaptic stripping, partially protecting Vglut1 synaptic buttons reduction on motoneuron pools, after sciatic nerve lesion [250] (Figure 1). Taken together, these findings suggest that increasing intensity exercise, such as the iTR protocol described, may be a strategy to optimize neurotrophins actions on both sensory and motor neurons in order to reduce early neurotrophin-mediated hyper-excitability in sensory neurons but in parallel stimulate neuroprotection of spinal motor circuitry.

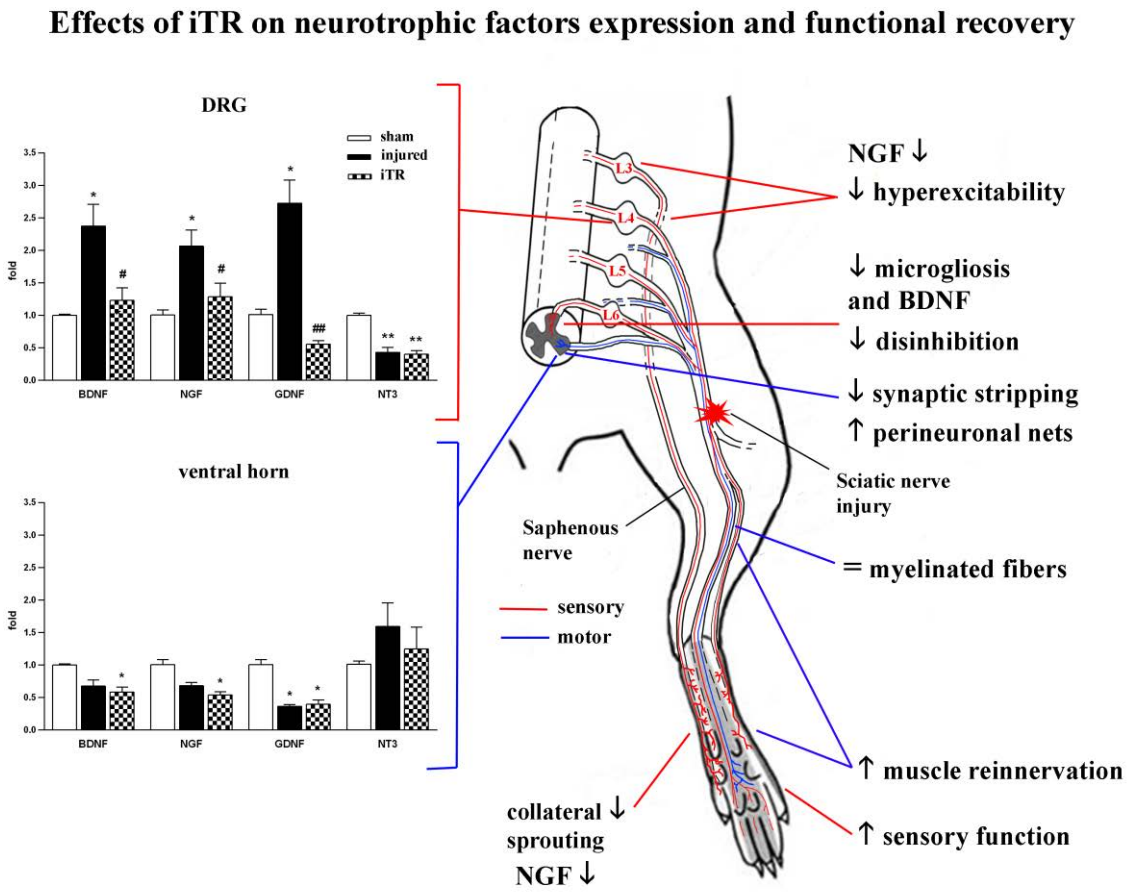

Fig. 1: Effects of iTR on neurotrophic factors expression and functional recovery.

Duration of exercise

Duration of exercise bouts following an injury is also an important variable for setting activity treatments, since a greater duration of exercise seems to lead to greater stimulation of neurotrophic system. Recently serum BDNF levels were measured in adult human males after a vigorous or a moderate intensity aerobic cycle exercise bout. All the exercise types caused a substantial BDNF increase, but long duration (40 min) exercise offered the greatest probability of a significant BDNF elevation [251]. Both acute and chronic aerobic exercise lead to an increase of BDNF levels, but this is not induced in case of strength training [252, 253, 254]. These results suggest that aerobic exercise effects on neuroprotection should take into account the volume of neurotrophin release over time.

Animal studies have consistently shown that chronic aerobic exercise elevate baseline BDNF levels in the hippocampus, striatum and various cortical regions [19, 107, 163, 193, 255, 256]. Resistance exercise can also elevate BDNF levels in the hippocampus [257]. Unlike other neurotrophic factors which showed tolerance to chronic exercise, BDNF levels remained upregulated in the rat hippocampus after 28 consecutive days of exercise [148, 258]. In animal

models of disease, chronic exercise has provided BDNF-related benefits, such as cell survival [259], decreased depressive symptoms [260], and functional recovery after traumatic brain injury [261]. Furthermore, chronic aerobic exercise seems to have a robust effect on cognition, as various intensities and durations of voluntary and forced exercise have consistently improved learning and memory in healthy laboratory animals, whether assessed by Morris water maze [140, 237, 262], radial arm maze [263], Y-maze [264], object recognition tasks [265], or pain avoidance training [191, 266].

Despite these results, there is controversy about the dependence of BDNF and GDNF expression on exercise duration. Some studies showed that short-term treadmill running (3, 7, 15 days training periods) resulted in an equal BDNF in the hippocampus and basal forebrain in both control and exercise trained groups [267, 268]. On the other hand, a recent study showed that both high intensity interval training and continuous training increased rat brain BDNF and GDNF levels [269]. Different studies have shown that long-term exercise training at moderate intensity increased GDNF concentration in striatum, spinal cord and sciatic nerve [180, 270], while short-term exercise training at low to moderate intensity did not influence the GDNF concentration in the striatum and substantia nigra [178]. Therefore, production and activation of neurotrophins during exercise seem to be dependent on the duration of exercise protocols, and the time spent in performing the activity can be tuned up to gradually increase the intensity of a specific exercise. Duration of training may also affect the maintenance of neurotrophic supply. In basal serum, after increasing long-term training, BDNF expression values returned to baseline after 8 weeks of detraining [253].

If chronic exercise provides the injured nervous system with a higher neurotrophic supply, excessive neuromuscular activity may produce opposite deleterious effects on muscle reinnervation [271], reducing axonal regeneration [115, 272] and Schwann cell proliferation in the regenerating nerve [273], and also inhibit collateral sprouting in partially denervated muscles [274, 275, 276]. Prolonged electrical stimulation or treadmill running also resulted detrimental for peripheral nerve regeneration and neuropathic pain [11, 277].

Another critical variable for setting the rehabilitative exercise treatment is the time of administration with respect to a nerve injury. If application of exercise prior to injury or pre-training is thought to have a neuroprotective effect, for example priming neurons for increased axon regeneration following injury [102], emerging evidence suggests that better recovery may be achieved when exercise is applied at early times after injury [11]. Exercise, either acute or prolonged, may affect the early expression of genes involved in plasticity and apoptosis as well as neurotrophic factor production in a cell specific manner (differently in motor than in sensory neurons) after spinal cord injury [241]. The first week after injury seems to be a crucial time

window, during which starting activity at different intensities may stimulate or inhibit the neuronal regenerative response. The activation of neurotrophin signal transduction pathways peaks within 7 days for NGF, and around 7-14 days for BDNF and GDNF in injured peripheral nerves; on the contrary, other neurotrophins such NT-3 and CNTF are downregulated [5, 242, 278]. The application of exercise during the first days after nerve injury seems to be critical in regulating the neurotrophic response to stimulate survival and growth of sensory [102, 279] and motor [279, 280, 281] neurons, or to inhibit excessive hyperexcitability, maladaptive collateral sprouting and mechanisms of neuropathic pain [11, 64, 78, 241].

On the other side, central injuries, such as spinal cord injury, induce a loss of some neurotrophic factors support, so that early applied exercise may boost and normalize their levels. Hemisection of the spinal cord induced a decrease in BDNF and synapsin-I levels, that were restored by voluntary running exercise [282]. Of several neurotrophic factors analyzed, spinal cord injury increased the early expression of mRNA for GDNF [283] and NT-3 together its receptor TrkB [241]. However cycling exercise increased the mRNA for both BDNF and GDNF, and attenuated the expression of those increased by spinal cord injury. Taken together, all these studies support the idea that starting exercise during the first days after injury may promote functional restoration by modulating the changes in neurotrophic factors induced by neural injuries (Figure 2).

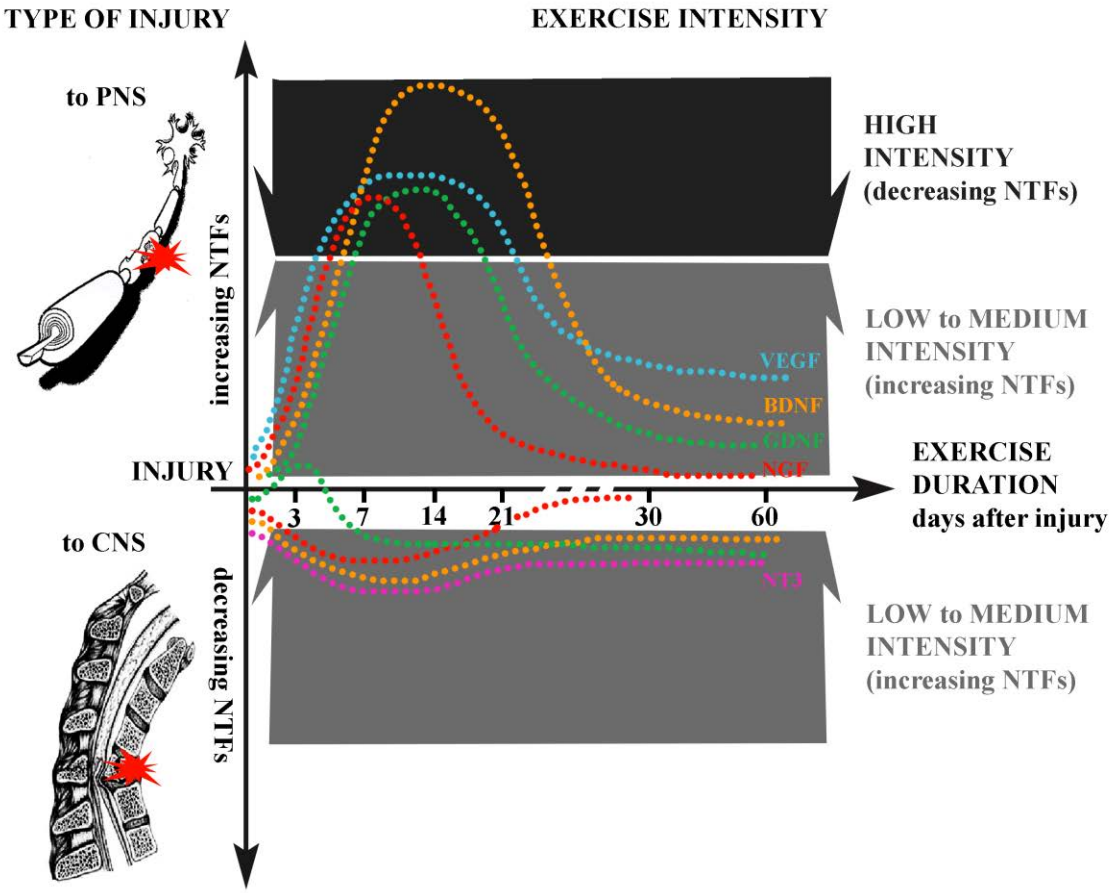

Fig. 2: Schematic representation of neurotrophic factors progression.

The double role of neurotrophic factors in exercise

The dichotomy of exercise effects on endogenous neurotrophins-mediated neuroprotective versus excitotoxic effects may be explained by the dual role that neurotrophic factors play under activity-dependent increasing stimulation following nerve injury and denervation. In this context, intensity and duration of stimulation may differently convey neuroprotection by producing an enhancement or a decrease of neurotrophic factors. The expression or inhibition of neurotrophic factors, such as BDNF, can promote or inhibit pain and regeneration, depending on whether exercise activity activates the neurotrophin signaling and excites neurons at peripheral or central levels.

Peripheral versus central neurotrophin expression

A systematic review [284] on the exercise-induced response of peripheral BDNF suggests that acute aerobic, but not acute strength exercise increases basal peripheral BDNF concentrations, although the effect is transient. Circulating BDNF seems to originate both from central and peripheral sources. It seems that exercise temporarily elevates basal BDNF and upregulates cellular processing of BDNF (i.e. synthesis, release, absorption and degradation). From that point of view, exercise would result in a higher BDNF synthesis following an acute exercise bout compared with untrained subjects. Subsequently, more BDNF could be released into the blood circulation which may, in turn, be absorbed more efficiently by both central and peripheral tissues where it may induce a cascade of neurotrophic and neuroprotective effects if exercise is repeated in subsequent training.

Aerobic but not strength training leads to an increase of peripheral BDNF levels [252, 253, 254]. This is due to the fact that muscle BDNF is not released into systemic circulation, and also that muscle is probably not a source of peripheral BDNF during chronic exercise. In fact, brain appears to be the main source of circulating exercise-induced BDNF, while peripheral blood mononuclear cells and endothelial cells may contribute approximately to 20–30% of peripheral levels [193, 285]. Moreover, aerobic exercise may be a potent stimulator of neurotrophins both in the peripheral and the central nervous system, but central expression may be different from peripheral. For example, voluntary wheel running increased mRNA levels of NT-3 and its receptor TrkC in the spinal cord and the soleus muscle, but changes in NT-3 protein were found only in the spinal cord, suggesting that the central nervous system may produce its own neurotrophins in response to exercise [217].

Brain and spinal expression of neurotrophic factors can be modulated by direct activation of noradrenergic and serotonergic pathways, with relevant effects on neuroprotection and functional recovery after injuries. Noradrenergic activation via β-adrenergic receptors seems to be essential for exercise-induced BDNF regulation [286], and β-adrenergic blockade significantly attenuates the BDNF mRNA elevation due to exercise in the cortex [287]. In addition, some cognitive benefits of exercise, such as the augmentation of contextual fear conditioning, are also attenuated by blockade of β-adrenoreceptors [288]. Moreover, it is known that exercise mediates increased BDNF expression by stimulating the PKA pathway following β-adrenergic G-protein-coupled receptor activation via the transcription factor CREB [289]. CREB is a nuclear transcription factor ubiquitously expressed and has been implicated in cell proliferation, differentiation, adaptation and survival [290]. Through β-adrenergic receptors physical activity may not only maintain muscle metabolism after injury but also activate important pathways, such as mitogen-

activated protein kinases (MAPKs) and phosphoinositol 3-kinase (PI3K)-Akt signaling pathways [291, 292, 293, 294].

Motor activity directly modulates also serotonergic activity in brain and spinal cord [295], and prominent effects are observed when exercise is applied after peripheral and central nervous injuries [296, 297]. For this reason, exercise-induced BDNF alterations via direct serotonergic activation may be more complicated. 5-HT2A/C receptors blockade may alter exercise-induced BDNF mRNA levels [287], particularly activation of 5HT2A receptors are relevant in strengthening the locomotor alternating pattern after injury and in rescuing the lost post-synaptic inhibition by restoring spinal chloride homeostasis, potentially counteracting central disinhibition, spasticity and neuropathic pain [297]. 5-HT1A receptor blockade did not affect exercise-induced BDNF, but significantly enhanced levels above those achieved with exercise alone in the anterior cingulate cortex [287].

These findings show that exercise may activate several mechanisms to differently modulate neurotrophin expression in peripheral and central neurons.

Positive and negative feedback for neurotrophic factors expression

A delicate interplay between glutamate and neurotrophic factor signaling systems is at the heart of activity-dependent neuroplasticity, during development as well as in the adult [298]. Neurotrophic factors can modify glutamate signaling directly, by changing the expression of glutamate receptor subunits and Ca2+-regulating proteins, and also indirectly by inducing the production of antioxidant enzymes, energy-regulating proteins and anti-apoptotic Bcl2 family members [299]. On the other side, glutamate stimulates the production of neurotrophic factors such as BDNF, which, in turn can promote neurogenesis, neurite outgrowth and synaptogenesis. An activity-dependent survival mechanism is believed to underlie the ability of exercise and intermittent fasting to prevent the death of neurons in experimental models of stroke [300].

It is usually accepted that low or intermediate intensities of exercise produce neuroprotection for the brain, while high intensities can cause damage until cell death. Excessive activation of glutamate receptors, such under conditions of intense and prolonged activity, may contribute to neuronal dysfunction and degeneration [301]. Indeed, harmful effects could appear in undue conditions of physical or psychological stress. These conditions may reverse neurotrophic factor production by providing a negative feedback regulation of the hypothalamo-pituitary-adrenocortical (HPA) axis, and trigger oxidative and stress responses [302]. Imbalance of glutamatergic neurotransmission could contribute to maladaptive serotonergic neurotransmission, and negative regulation of neurotrophic factors may be parallel also to extensive activation of noradrenergic and serotonergic pathways. However, after nerve injury,

chronic exercise may affect the noradrenergic and serotonergic systems in similar ways to the effects of pharmacological interventions, increasing excitatory noradrenaline and serotonin activity and storage in the brain [303, 304, 305]. Increased serotonergic activity, such after treadmill exercise [296] has shown to induce downregulation of BDNF expression in the brain [306]. We found reduced expression of NGF, BDNF and GDNF in DRG and spinal cord after increasing-intensity treadmill locomotion in sciatic nerve injured rats [64, 78]. Interestingly, the reduction of neurotrophin expression was associated to an increased expression of β2-adrenergic and 5HT2A serotonergic receptors in lumbar spinal cord, which were reduced after sciatic nerve injury (unpublished data). In this context, the increased neurotrophic factor production observed after peripheral nerve injury can be modulated by exercise in order to preserve and maintain the central circuitry from maladaptive plastic changes that are typically associated to neuropathic conditions [64].

On the other side, sustained and excessive activation of glutamate receptors is known to be excitotoxic, particularly under conditions of reduced energy availability and increased oxidative stress. Exercise can induce a long-term enhancement of glutamatergic activity through upregulation of NMDA receptors [307]. Additionally, enhanced transmission through AMPA receptors can promote Ca2+-mediated BDNF release and signaling through TrkB [308, 309]. High intensity exercise in BDNF levels that may lead to states of neuronal hyperexcitability [310]. The resulting BDNF-mediated effects on synaptic plasticity may further induce enhancement of excitatory glutamatergic transmission. This positive feedback loop between BDNF and glutamatergic activities accounts for the increased seizure vulnerability and excitotoxicity seen following experimental manipulations that enhance BDNF function, particularly in the hippocampus [311, 312, 313]. This potential state of hyperexcitability following BDNF upregulation may also account for the increased vulnerability to kainic acid-induced excitotoxicity following direct injection into the hippocampus of rats that had undergone several weeks of exercise [314].

Conclusions

Physical activity clearly demonstrated neuroprotective effects that are conveyed through multiple mechanisms to peripheral and central neurons. The neuroprotection resulting from exercise training is an endogenous effect that may be best afforded by specific exercise protocols. Neuroprotective and pro-regenerative effects of exercise have been related to modulation of neurotrophic factors. Enhancement of neurotrophic factors expression is connected to a number of reactions, such strengthening the neurovascular unit, induction of astrocytosis, angiogenesis and arteriogenesis, decrease of inflammatory response and microgliosis, and decrease of apoptotis, that all together

protect neurons from the consequences of nerve injuries and neurodegenerative diseases. However, the effects of increasing neurotrophic factors are dichotomous, ranging from neuroprotection and nerve repair to cell death, hyperexcitability and induction of pain. Significant reduction of neuropathic pain was recently achieved through moderate-to-high intensity exercise that was associated to a decrease of neurotrophic factors, particularly NGF, BDNF and GDNF levels in sensory neurons, after peripheral nerve injuries [64, 78, 124]. Furthermore, high intensity exercise reduces brain neurotrophins similarly to activation of metabolic and glucocorticoid stress responses [235, 236, 237].

From a practical point of view, exercise protocols applied to induce neuroprotection in the setting of rehabilitation after nerve injuries should take into account several parameters that have been demonstrated to be critical. First of all, the intensity of activity can be tuned up to reach more efficaciously the aimed outcome of training. It may be increased in order to enhance neurotrophic factor production (for example to stimulate motor and sensory nerve regeneration), and further increased till high levels to trigger the opposite effect and decrease neurotrophic factors (for example, to modulate plasticity, pain and hyperexcitability). The type of injury (peripheral versus central) is crucial to decide the outcome of different intensity exercises, since neurotrophic factor response to insult may be opposite: peripheral nerve injuries are known to induce an increase of neurotrophic factors expression as spontaneous response to regenerate, but after spinal cord injuries generally a loss of neurotrophic factors has been observed and even associated to pain conditions. Moreover, exercise intensity is relevant in promoting neuroprotection at different sensory and motor modalities: for example, low-to-intermediate intensities of exercise activate slow-twitch muscle fibers to a greater extent than fast twitch muscle fibers, and stimulate sprouting and regeneration of sensory neurons, but contrarily adaptation of fast twitch muscles and reduction of sensory fiber sprouting were observed after high intensity exercise.

The time window of exercise treatment application after injury is another critical parameter. The dual effects of chronic and acute physical activity on neuroprotection, particularly in those individuals with an underlying inflammatory condition, should be better understood such that the greater effects of exercise take place within the time of greater vulnerability and plasticity of the pathways affected by the damage. This seems to be the earliest possible time after the insult, when modulation of neurotrophic factors transcription and activation may play a greater effect in protecting neurons from primary and secondary damage.

Physical activity offers an affordable and effective method to increase neuroprotection and improve functional recovery by acting on neurotrophic factors, representing a multiple endogenous treatment. Although different exercise treatments have uncovered diverse cognitive and behavioral phenotypes in animal models of nerve injuries, more recent studies, including those described above, underscore the multiple levels by which BDNF and other factors function can be manipulated with

activity-dependent therapies. The use of distinct intensities of activity, the preferential application at critical phases of neurotrophins expression, the regulation of pro-neurotrophins to mature neurotrophin cleavage, and the design of paradigms to differentially stimulate peripheral versus central and sensory versus motor neurons, provide a diverse repertoire of regulatory mechanisms that may be optimized for each individual pathology.

Conflict of interest

The authors have no conflicts of interest to declare. This work was supported by TERCEL and CIBERNED funds from the Instituto de Salud Carlos III of Spain, and Grants BIOHYBRID (FP7-278612) and EPIONE (FP7-602547) from the European Commission (EC).

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