

**Functional stimulation system for rehabilitation
of gait and driving neural plasticity after spinal cord injury**

Sònia Trujillo Vázquez

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Presented by
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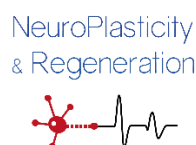
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Als meus pares

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I. Summary

Intensive sensory-motor rehabilitation is currently the most widely used and effective intervention to improve functional recovery after spinal cord injury (SCI), although the activity-dependent plasticity mechanisms induced within spinal networks remain incompletely understood. This thesis first compared the functional and morphological outcomes of graded intensity spinal cord contusions (100, 200, and 300 kdynes) to the thoracic segment in adult rats to characterize how lesion severity shapes the spinal cord “starting point”. More severe injuries produced progressively greater functional impairment and hyperexcitability spinal state, leading to hyperalgesia and hyperreflexia, together with maladaptive histopathological changes. Within this spectrum, the 200 kdynes contusion was identified as the most suitable model for rehabilitation studies, as it combined marked alterations with sufficient recovery potential to detect treatment effects.

Using this model, the second part of the thesis examined how different rehabilitation strategies modulate spinal excitability and plasticity after SCI. Bilateral alternating functional electrical stimulation (FES) of hindlimb extensor muscles in awake but resting animals was compared with daily treadmill locomotor training, a clinically consolidated paradigm. Both protocols improved motor and sensory function relative to non-rehabilitated animals and reached comparable BBB scores at the end of two-month follow-up, indicating a similar degree of locomotor recovery. However, FES was associated with better normalization of spinal reflexes and more pronounced shift toward an inhibitory state, accompanied by increased motoneuron preservation and enhanced KCC2 expression and serotonergic innervation within lumbar segments, suggesting a more effective restoration of segmental inhibitory control.

The thesis also explored the impact of advancing FES onset to very early post-injury stages, taking advantage of the possibility of applying stimulation even when the animal is not yet able to bear weight. In this early rehabilitation paradigm, FES did not substantially modify basal segmental excitability or the final level of locomotor recovery, but it accelerated the appearance of functional improvements and induced an “intermediate” state of spinal plasticity characterized by improved modulation of reflex responses during repetitive

Summary

stimulation and selective changes in KCC2 expression and serotonergic innervation in the lumbar cord.

Taken together, these findings show that contusion severity robustly determines the profile of post-injury deficits and support the selection of the 200kdynes model as an optimal platform for testing rehabilitation strategies. They also demonstrate that FES, applied both in subacute or very early phases, can specifically modulate spinal circuit excitability and organization, promoting a more favorable pattern of plasticity than treadmill training alone and highlighting the importance of its timing to fully exploit post-injury plasticity windows

II. Abbreviation

Ach: acetylcholine

AMPA: alfa-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: two-way analysis of variance

AUC: Area under the curve

BBB: Basso, Beattie and Bresnahan locomotor rating scale

BMI: Brain machine interfaces

ChAT: Choline acetyltransferase

CMAP: compound muscle action potentials

CnF: cuneiform nucleus

CNS: central nervous system

CPG: central pattern generator

CSF: cerebrospinal fluid

CSPGs: chondroitin sulfate proteoglycans

CST: corticospinal tract

DAPI: 4',6'-diamidino-2-phenylindole

DCML: dorsal column-medial lemniscus

DPX: Dibutyl phthalate xylene

DRG: dorsal root ganglia

EEG: electroencephalographic

EPSP: excitatory postsynaptic potentials

FBS: fetal bovine serum

FES: functional electrical stimulation

FF: fast fatigable

FFR: fast fatigue resistant

GFAP: Glial fibrillary acidic protein

Gi: gigantocellular nucleus

Iba1: Ionized calcium-binding adapter molecule 1

KCC2: Potassium-chloride cotransporter 2

LPgi: lateral paragigantocellular nucleus

MEPs: Motor evoked potentials

MLR: mesencephalic locomotor region

MN: motoneuron

NMDA: N-methyl-D-aspartate

OCT: optimal cutting temperature

PAG: periaqueductal grey

PAD: primary afferent depolarization

PBS: Phosphate-buffered saline

PBST: PBS with Triton 0,3%

PNS: peripheral nervous system

PPN: pedunculopontine nucleus

Abbreviations

RDD: rate-dependent depression

RNS: reactive nitrogen species

ROI: Region of interest

ROS: reactive oxygen species

RVM: rostral ventromedial medulla

SC: spinal cord

SCI: spinal cord injury

SEM: standard error of the mean

SFR: slow fatigue resistant

VGAT: Vesicular GABA transporter

VGLUT2: Vesicular glutamate transporter 2

III. Introduction

1. From Action Potential to Movement

Voluntary movement emerges from the coordinated transformation of electrical activity in neural circuits into mechanical work in muscles. Bioelectrical signals generated by ion fluxes across neuronal membranes propagate through distributed networks in the central and peripheral nervous system and ultimately reach muscle fibers, whose temporally precise activation underlies the execution of coordinated movements.

This transformation does not depend solely on descending motor commands from the brain to the muscles. In parallel, a continuous stream of sensory information from the body and the environment is integrated within spinal and supraspinal networks to dynamically adjust the pattern of muscle activation in real time. During locomotion, segmental spinal reflexes and sensorimotor interactions at the level of the spinal cord (SC) contribute critically to this process, enabling rapid and automatic adjustments that complement volitional control and support the generation of coordinated, adaptive and repetitive stepping.

Understanding how the central nervous system (CNS) integrates peripheral feedback and orchestrates complex motor patterns is essential for the development of strategies aimed at restoring locomotion after spinal cord injury (SCI). Locomotor behavior arises from the interaction between multiple reflex pathways and their modulation by supraspinal centers, which together shape the timing, amplitude and interlimb coordination of each step. Disruption of these pathways by SCI severely compromises the capacity to generate functional movement. In this context, functional electrical stimulation (FES) represents a valuable therapeutic tool: by artificially activating peripheral nerves and muscles, FES can reproduce key components of the locomotor pattern, drive activity-dependent plasticity within spinal circuits and partially re-establish the sensorimotor interactions required for gait recovery, thereby helping to bridge impaired neural pathways and facilitate the restoration of locomotion in individuals with SCI.

2. The course of a voluntary motor command along the Nervous system

The nervous system is a highly organized network that coordinates the responses of the organism to both internal and external stimuli. It is classically divided into two interconnected components: the central (CNS) and the peripheral nervous system (PNS). While the CNS functions as the main center for processing and integrating information, the PNS ensures efficient communication between the CNS and peripheral organs through cranial and spinal nerves distributed throughout the body. Within the PNS, sensory (afferent) pathways transmit information from receptors in the organism to the CNS, whereas motor (efferent) pathways convey commands from the CNS to muscles and glands, thereby enabling coordinated movement and functional regulation (Crossman, 2019; Kandel, 2013)

The CNS represents the most complex part of the nervous system, as it contains the majority of neuronal cell bodies and synaptic connections (*Kandel 2013 Principles of Neural Science, n.d.*). It is composed of the brain, the brainstem and the spinal cord, all of which are protected by bony structures: the skull encloses the brain, whereas the vertebral column shields the SC. In addition to this structural protection, the CNS is surrounded by three layers of connective tissue, the meninges. Between these layers circulates the cerebrospinal fluid (CSF), which bathes the CNS and flows through the ventricular system, contributing to mechanical protection, metabolic support and homeostasis (M. Murray, 2015). Together, these structures play a crucial role in maintaining the structural and functional integrity of the CNS.

Approximately 80% of neurons in the nervous system are directly or indirectly involved in the generation and control of movement, underscoring the fundamental importance of this function for survival and evolutionary success. As Charles S. Sherrington pointed out, “to move things is all that mankind can do, and for such the sole executant is muscle”, a statement that highlights the central role of the sensorimotor system, whose hierarchical organization spans from higher cortical areas to the SC and peripheral effectors (Grillner & El Manira, 2020).

Descending motor pathways originate in the cerebral cortex and are transmitted through multiple subcortical structures before reaching spinal circuits responsible for the final execution of movement. In parallel, highly specialized sensory feedback systems continuously inform the CNS about body position, movement dynamics and

environmental conditions. This information arises from proprioceptive, vestibular, visual and cutaneous afferents, and its integration with descending signals allows the generation of precise and adaptive motor responses.

Within this framework, the motor cortex plays an essential role in the planning and execution of voluntary movements, particularly those requiring precision and adaptation to complex environments. However, basic locomotor patterns can be generated in the absence of cortical control, highlighting the importance of subcortical and spinal circuits in movement production. The basal ganglia contribute to the selection of motor programs through disinhibitory mechanisms, whereas the cerebellum is involved in fine-tuning, temporal coordination and motor learning. The brainstem, in turn, acts as a key integrative center, channeling descending signals towards the SC (Grillner & El Manira, 2020; Bear, 2007).

2.1. Spinal cord

The SC represents the final common structure for motor execution and a fundamental center for sensorimotor integration. It is the site where descending commands from the brain converge with afferent information from the periphery, enabling the generation of motor responses that are both coordinated and context-dependent.

2.1.1. Spinal cord anatomy

From an anatomical perspective, the SC originates at the level of the medulla oblongata and extends caudally along the body axis to the conus medullaris. It has an ovoid morphology with a marked enlargement in the cervical region and regional variations in diameter along its longitudinal axis. Traditionally, it is divided into four main regions: cervical, thoracic, lumbar and sacral (Watson & Kayalioglu, 2009).

The SC is bilaterally organized and separated into two hemisections by the ventral median fissure. At each segmental level, pairs of spinal nerves emerge to innervate specific body territories. In humans, the SC terminates approximately at the level of the L1-L2 vertebrae, forming the conus medullaris, from which the lumbar and sacral nerve roots descend within the vertebral canal before exiting through their respective intervertebral foramina (Crossman, 2019).

This anatomical organization underlies the functional complexity of the SC as an integrative center for motor and sensory information. To understand the mechanisms by which these signals are processed, it is essential to examine its internal structure, particularly the arrangement and function of the gray and white matter.

In transverse section, the SC is composed of two main regions: a central core of gray matter surrounded by white matter. The gray matter, formed by the cell bodies of motoneurons, interneurons and glial cells, constitutes the principal site of segmental sensorimotor integration.

Macroscopically, gray matter is organized into dorsal and ventral horns, separated by an intermediate zone. The dorsal horn is primarily involved in the processing of sensory information, including nociceptive, thermal, mechanical and proprioceptive inputs, whereas the ventral horn contains the motoneurons responsible for muscle innervation and the generation of motor output.

Gray matter

The gray matter, in the shape of a butterfly, contains the soma of the SC neurons. From a cytoarchitectonic and functional perspective, the gray matter is organized into ten regions known as Rexed laminae, arranged in a dorsoventral sequence (laminae I–IX), with lamina X surrounding the central canal. This organization reflects a clear specialization in information processing. The dorsal laminae (I–VI) constitute the main entry point for peripheral sensory afferents and are involved in processing modalities such as pain, temperature and proprioception, with lamina VI playing a prominent role in the integration of signals originating from muscles and joints. Below this region, the intermediate zone (laminae VII and VIII) represents a major integrative center, containing networks of interneurons implicated in the generation and coordination of motor programs, including central pattern generator (CPG) circuits and interneuron populations that mediate bilateral coordination between both sides of the SC, and propriospinal connections between different cord segments. Finally, the ventral horn (lamina IX) contains somatic motoneurons organized somatotopically in longitudinal columns, such that neurons innervating proximal trunk musculature are located medially, whereas those projecting to distal

muscles in the limbs are positioned more laterally (Figure 1). This laminar arrangement enables the gray matter to function as an integrated unit in which sensory information is efficiently transformed into coordinated motor commands (M. Murray, 2015; Nógrádi et al., 2000; Watson & Kayalioglu, 2009).

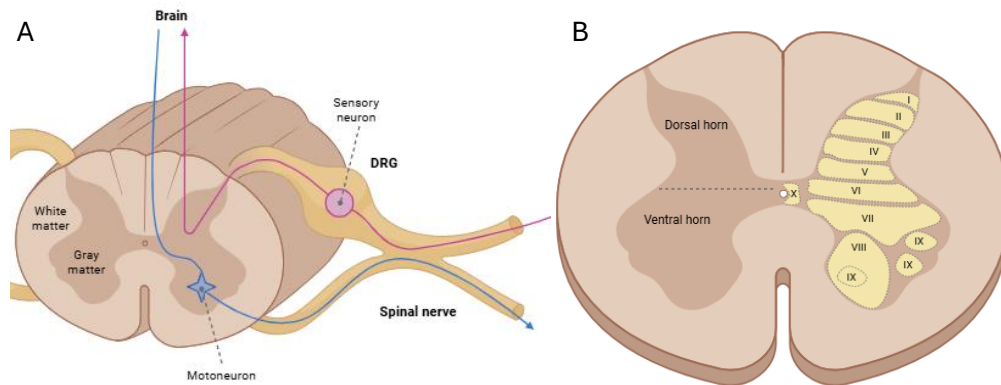


Figure 1. Schematic representation of SC anatomy. A. Cross-sectional view of the basic anatomy of the SC and their projections. B. Localization of Rexed laminae at the gray matter.

Spinal **interneurons** are widely distributed throughout the gray matter and constitute one of the main integrative elements of the CNS. These neurons link sensory afferents with descending pathways and can exert both excitatory and inhibitory actions, thereby contributing to the generation, coordination and modulation of complex motor programs. In this sense, the SC does not act merely as a relay structure, but as a processing center capable of integrating peripheral and supraspinal information to adjust motor output in a context-dependent manner (Nógrádi et al., 2000). According to their anatomical organization, spinal interneurons can be broadly classified into propriospinal neurons, whose ascending and descending projections interconnect different spinal segments, and local interneurons, whose axons remain within a limited segmental range and may project ipsilaterally or cross the midline, thus contributing to intersegmental and bilateral coordination of movement (Lane, 2011; Zholudeva et al., 2018). Within this latter group, Renshaw cells provide a well-characterized example of inhibitory local interneurons forming a recurrent circuit with alpha motoneurons, in which they are driven by motor axon collaterals and return powerful glycinergic (and partly GABAergic) inhibition onto motoneurons and premotoneuronal interneurons,

thereby stabilizing motoneuronal firing and shaping the temporal structure of the motor output (Côté et al., 2018; Windhorst, 1993).

Spinal **motoneurons** (MN) can be broadly classified as somatic or visceral. In the present work, the focus is on somatic motoneurons, which are cholinergic neurons located mainly in lamina IX of the ventral horn and whose axons project to skeletal muscle (Watson & Kayalioglu, 2009). These lower motoneurons are distinguished from upper motoneurons of cortical origin and constitute the final common pathway for muscle activation, directly linking central motor commands to peripheral effectors.

From a functional perspective, somatic motoneurons are subdivided into alpha (α) and gamma (γ) motoneurons according to the type of muscle fibers they innervate and their role in motor control (Burke, 2013). The α -motoneurons have larger soma and thicker axons, properties associated with higher conduction velocities and innervate extrafusal muscle fibers responsible for force generation and voluntary movement. Each α -motoneuron, together with the extrafusal fibers it innervates, forms a **motor unit**, and the population of motoneurons controlling a given muscle constitutes its motor nucleus. As the convergence point for sensory afferents, spinal interneurons and descending inputs from supraspinal centers, α -motoneurons represent the final common output stage of the motor system, where diverse signals are integrated before being transformed into muscle contraction (Burke, 2013; Guyton & Hall, 2021).

Activation of skeletal muscle fibers begins with the release of acetylcholine (ACh) at the neuromuscular junction, which triggers an action potential in the muscle membrane. This event initiates excitation–contraction coupling, involving calcium release from the sarcoplasmic reticulum and the interaction between actin and myosin, ultimately leading to contraction of each muscle fiber, followed by relaxation once calcium is sequestered again (Bear, 2007).

According to the physiological properties of the motor units they control, α -motoneurons are typically associated with slow fatigue-resistant (SFR), fast fatigue-resistant (FFR) and fast fatigable (FF) motor units. This organization reflects differences in neuronal size, recruitment threshold and firing pattern: in general,

smaller motoneurons have lower activation thresholds and are recruited first, whereas larger, less excitable motoneurons are recruited later and contribute to higher force levels (Bear, 2007; Burke, 2013). Moreover, the excitability of a motor pool can be markedly modulated by Ia afferents and by spinal inhibitory and facilitatory mechanisms, so that the motor response is not fixed but strongly dependent on the functional state of the system (Burke, 2013).

The γ -motoneurons, in turn, innervate intrafusal fibers of the muscle spindles and play a crucial role in regulating spindle sensitivity. During voluntary contraction, γ -motoneuron activation helps to maintain afferent discharge from the spindle, allowing the nervous system to preserve proprioceptive feedback even as muscle length and tension change (Burke, 2013). In this way, the gamma system is essential for adjusting spindle sensitivity and for supporting fine control of movement and posture.

White matter

The SC gray matter is surrounded by white matter, which consists mainly of myelinated nerve fibers and associated glial cells. These fibers are grouped into distinct bundles known as tracts or fasciculi, according to their common origin, destination or function. Some of these fibers interconnect adjacent or distant spinal segments and thereby support intersegmental coordination, whereas others extend over longer distances to link the SC with the brainstem and the brain, forming the ascending (sensory/afferent) and descending (motor/efferent) pathways

Ascending tracts convey two main types of information: exteroceptive signals, related to external stimuli such as nociception, temperature and touch, as well as proprioceptive signals that reflect the state of muscles and joints. These pathways are typically organized as three-neuron chains. First-order neurons, located in the dorsal root ganglia (DRG), conduct sensory information from peripheral receptors to the dorsal horn of the SC; second-order neurons act as intermediate relays and often decussate before ascending; third-order neurons, whose cell bodies reside in subcortical relay nuclei such as the thalamus, project to the cerebral cortex or cerebellum, where sensory information is integrated and reaches conscious perception (Burke, 2013).

The main ascending pathways of the SC can be grouped into three major systems. The **spinocerebellar tracts** transmit proprioceptive information from muscle spindles and Golgi tendon organs to the cerebellum, where it is used for the control of posture, muscle tone and ongoing movements; they include dorsal tracts, originating in Clarke's column (T1–L2), and ventral tracts that convey more integrated signals from spinal interneuronal networks. The **spinothalamic system** carries exteroceptive information related to gross touch, pressure, pain and temperature: second-order neurons located mainly in laminae I, II, V, VII and VIII of the dorsal horn decussate within the SC and ascend to the thalamus, from where projections reach cortical somatosensory areas. Finally, the **dorsal column–medial lemniscus (DCML)** pathway conveys vibration, fine touch and proprioceptive modalities; first-order axons ascend ipsilaterally in the gracile and cuneate fascicles to synapse in the corresponding nuclei of the medulla, from which second-order neurons decussate and ascend to the thalamus and on to the primary somatosensory cortex (Figure. 2) (Bear, 2007; Nógrádi et al., 2000; Tan et al., 2023).

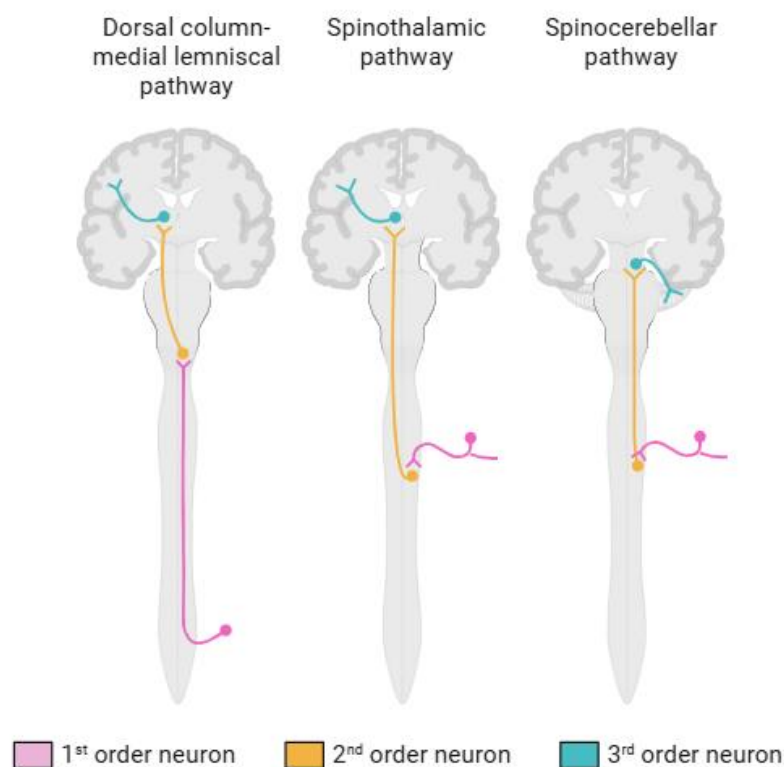


Figure 2. Schematic representation of the principal ascending tracts of the SC. Image adapted from Judith Sanchez Ventura's doctoral thesis (2023).

Descending tracts convey motor commands from the brain and brainstem to spinal α -motoneurons. In humans and non-human primates, they are classically described as a two-neuron system in which an upper motor neuron, located in the cortex or brainstem, synapses onto lower motor neurons in the ventral horn of the SC. However, anatomical and physiological studies have shown that upper motor neurons often transmit their influence via complex networks of spinal interneurons, adding additional levels of integration and modulation to these circuits (Bican et al., 2013; Massey et al., 2022; Nógrádi et al., 2000).

Descending motor pathways can be grouped into two major systems (Figure. 3):

The **lateral descending pathway** is the main system for the voluntary control of distal limb muscles and comprises the corticospinal and rubrospinal tracts (Bican et al., 2013; Nógrádi et al., 2000).

- The **corticospinal** or pyramidal (CST) tract is the largest descending tract in humans and reaches its maximal development in higher primates: approximately two-thirds of its fibers originate in the precentral motor cortex, while the remainder arise from somatosensory cortical areas. Around 90% of these fibers decussate in the caudal medulla to form the lateral corticospinal tract, whereas about 10% descend as the anterior corticospinal tract, which crosses later through the anterior white commissure; a small fraction remains uncrossed. Through direct and interneuron-mediated synapses onto spinal motoneurons, the corticospinal system exerts control over axial, proximal and especially distal musculature, supporting fine voluntary movements (Bican et al., 2013; Massey et al., 2022; Nógrádi et al., 2000). To note, that in lower level mammals, such as rodents, the CST occupies the inner part of the dorsal columns, anterior to the DCML tract.
- Complementary, the **corticobulbar** tract corresponds to the fibers that finish at the brainstem to control the motoneurons that innervate the cranial muscles.
- The **rubrospinal** tract represents a comparatively minor component in humans and originates in the magnocellular part of the red nucleus in the midbrain. Its fibers decussate soon after origin and descend contralaterally in the lateral funiculus to terminate mainly on interneurons in laminae V–VII,

which in turn facilitate the activation of proximal flexor muscles and inhibit extensors (Bear, 2007; Bican et al., 2013; Nógrádi et al., 2000). Although this pathway is more prominent in lower mammals, where it plays a major role in limb coordination, in primates it is thought to contribute to posture and gross limb movements, complementing corticospinal control.

In contrast, the **ventromedial descending pathway** primarily modulates posture, balance and orienting movements, and terminates mainly on interneurons controlling axial and proximal muscles (Bear, 2007; Massey et al., 2022; Nógrádi et al., 2000). It comprises:

- The **vestibulospinal** system comprises a lateral tract, which descends ipsilaterally and facilitates extensor motoneurons in the lumbar cord to support upright posture, and a medial tract, which projects bilaterally to laminae VII–VIII to regulate neck and back muscles and contribute to head–eye coordination and balance.
- The **tectospinal** tract arises in the superior colliculus and descends contralaterally to upper cervical segments, where it establishes polysynaptic connections with cervical motoneurons to orient the head and eyes towards salient visual, auditory and somatosensory stimuli.
- The **reticulospinal** system, originating in the pontomedullary reticular formation, comprises a medial (pontine) tract that facilitates antigravity and extensor posture and a lateral (medullary) tract that exerts an inhibitory influence on extensor motoneurons and an excitatory influence on flexor motoneurons. The functional balance between both reticulospinal pathways is strongly shaped by descending cortical inputs and is essential for postural control, locomotor initiation and the modulation of spinal reflex activity (Nógrádi et al., 2000).

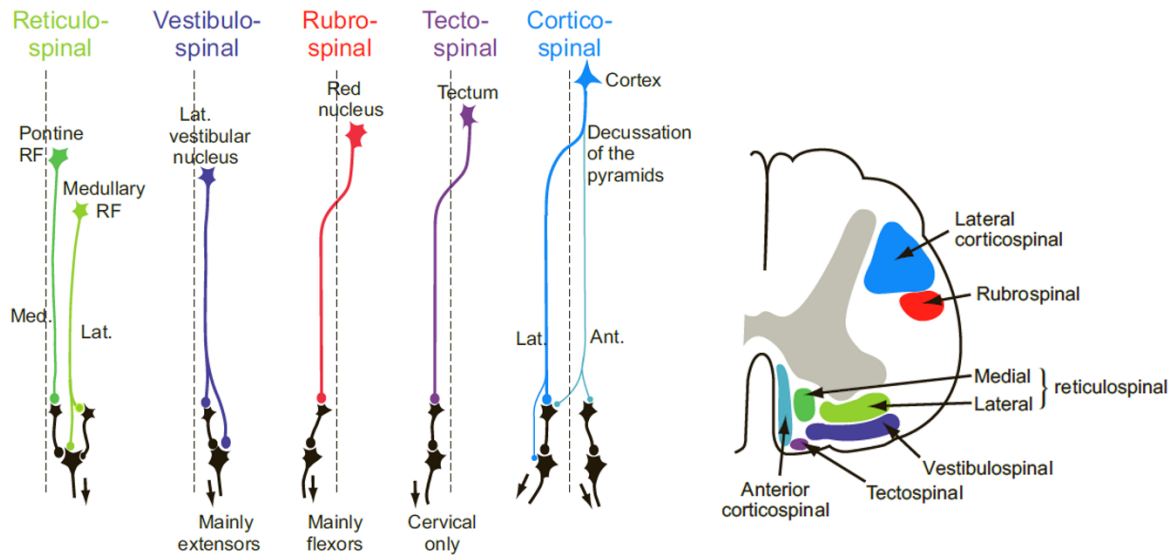


Figure 3. Schematic representation of the principal descending tracts of the SC. Image adapted from Carpenter's Neurophysiology.

Additionally, descending pain-modulatory pathways provide an extra level of control by inhibiting or facilitating nociceptive transmission within the SC dorsal horn. These pathways originate in supraspinal centers such as the periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM), and act primarily through monoaminergic systems, including serotonergic, noradrenergic and dopaminergic projections to interneurons in the dorsal horn (Kwon et al., 2014). By adjusting the gain of nociceptive signaling in a top-down manner, they play a critical role in both physiological pain modulation and pathological pain states.

2.1.2. Intrinsic spinal networks for sensorimotor control

Motor control does not rely solely on descending projections from supraspinal centers, but also on local spinal circuits that integrate and redistribute descending and afferent information. In this context, propriospinal pathways, spinal reflex networks, presynaptic inhibitory mechanisms and CPGs represent key elements for intersegmental coordination, modulation of motor excitability and the production of rhythmic movements such as locomotion. Together, these spinal circuits allow the SC to operate not merely as a relay, but as an active processing center that dynamically shapes sensorimotor output.

Propriospinal interneurons

Propriospinal interneurons form an intrinsic spinal network that coordinates motor activity across spinal segments, from cervical to lumbar regions, acting as functional relays between local circuits, CPGs and descending supraspinal pathways (Tan et al., 2023; Watson & Kayalioglu, 2009). They are commonly divided into short-projecting interneurons, which interconnect adjacent segments within the cervical and lumbosacral enlargements, and long-projecting interneurons, whose axons link these enlargements and are critically involved in synchronizing locomotor patterns and coordinating limb movements during gait (Côté et al., 2018).

Clinically, their relevance lies in their pronounced plasticity and differential resistance to SC injury, particularly in the case of long propriospinal interneurons, which tend to be better preserved than short thoracic interneurons after contusive lesions. This relative sparing allows them to form intraspinal “detour” pathways through collateral sprouting onto premotor and motor neurons caudal to the lesion, thereby bypassing damaged regions, partially restoring intersegmental communication and reactivating lumbar CPGs, which in turn contributes substantially to spontaneous and training-induced locomotor recovery after SCI (Conta & Stelzner, 2009).

Spinal reflexes

Spinal reflexes play a fundamental role in maintaining posture, joint stability and facilitating locomotion, regulation of muscle contraction in addition to their protective function. Spinal reflexes require at least four essential components: a somatic receptor, a sensory (afferent) neuron, a motor (efferent) neuron and the skeletal muscle; in more complex reflexes, spinal interneurons introduce flexibility and modulation by integrating sensory, supraspinal and local circuit inputs (Jankowska, 2013; Nógrádi et al., 2000).

Monosynaptic reflexes, such as the stretch reflex (Figure 4), are activated by group Ia afferent fibers from muscle spindles in response to changes in muscle length and establish a direct connection with α -motoneurons, leading to contraction of the agonist muscle. Their functional variability depends on temporal and spatial summation of excitatory postsynaptic potentials (EPSPs) and on key inhibitory

mechanisms: presynaptic and postsynaptic inhibition. **Presynaptic inhibition**, mediated by GABAergic interneurons forming axo-axonic synapses onto Ia/II afferent terminals, induces primary afferent depolarization (PAD) via GABA-A receptor activation, thereby reducing presynaptic Ca^{2+} entry and glutamate release and selectively attenuating the sensory signal before it reaches motoneurons (Bear, 2007; Willis, 2006). **Postsynaptic inhibition**, in contrast, directly hyperpolarizes the target neuron through inhibitory synaptic inputs (Nógrádi et al., 2000).

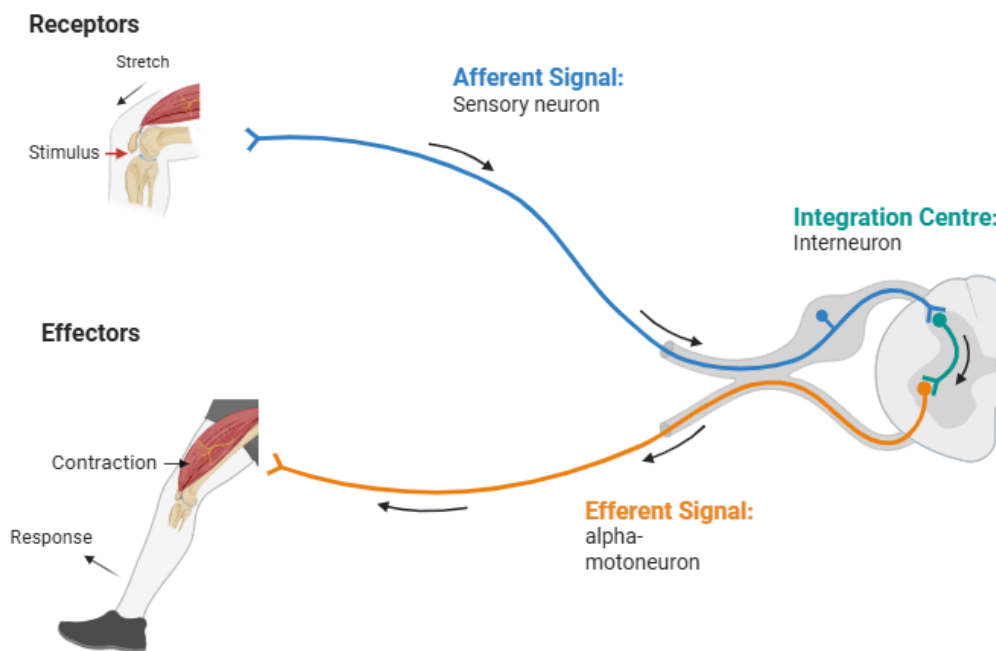


Figure 4. Schematic representation of the stretch reflex.

Electrophysiologically, these segmental reflexes can be quantified using the Hoffmann reflex (H-reflex) evoked by peripheral nerve stimulation. The H-wave reflects the excitability of the motoneuron pool and the efficacy of Ia afferent transmission, whereas the M-wave represents the direct activation of motor axons. Presynaptic inhibition selectively depresses the H-reflex without significantly affecting the M-wave, thereby scaling reflex gain according to postural and motor demands under the influence of local spinal circuits and descending pathways (Burke, 2013; Jankowska, 2013).

Polysynaptic spinal reflexes involve more complex interneuronal circuits that allow versatile and context-dependent responses, such as the flexor (withdrawal)

reflex, Ib reciprocal inhibition or recurrent Renshaw inhibition. A particularly relevant example in the context of locomotion is the stepping reflex, which under appropriate experimental conditions can generate rhythmic stepping movements in spinal preparations, indicating that rhythmic motor patterns do not rely exclusively on peripheral sensory input but can also emerge from intrinsic spinal central CPGs, i.e. autonomous networks capable of producing endogenous motor sequences that are modulated by peripheral afferents and supraspinal control (Jankowska, 2013; Nógrádi et al., 2000).

This organization shows that spinal reflexes go beyond simple stereotyped responses and are embedded within functional motor behaviors, sharing interneuronal networks with voluntary and locomotor movements, in which presynaptic inhibition and related mechanisms ensure the precision, timing and adaptability of motor output.

Central pattern generators

The SC is often described as a conduit relaying information between the brain and the periphery, but it also contains autonomous intrinsic circuits capable of generating complex motor behaviors. CPGs are spinal and brainstem neuronal networks that produce rhythmic and coordinated motor outputs, such as locomotion, breathing and chewing, through the alternating interaction of excitatory and inhibitory interneuronal populations (Grillner, 2006; Nógrádi et al., 2000).

These CPGs can operate independently of sensory input and supraspinal control, as first demonstrated by Brown (1911), who elicited rhythmic stepping movements in spinalized cats, indicating that the basic locomotor rhythm can be generated by spinal circuits alone. However, CPGs do not function as isolated hard-wired modules; rather, they are dynamically modulated by descending commands (corticospinal, reticulospinal, vestibulospinal) and by proprioceptive feedback from muscle spindles and Golgi tendon organs, allowing the locomotor pattern to adapt to changing environmental and physiological conditions (Grillner, 2006; Jankowska, 2013; Kiehn, 2006). The **CPG of locomotion** is a specialized neural network located within the last thoracic and first lumbar segments of the spinal cord, that generates rhythmic, coordinated motor outputs required for walking, running or swimming,

without needing continuous commands from the brain or sensory feedback (Grillner, 2006; Rossignol, 2009). More details are explained below in point 2.3.

This intrinsic spinal flexibility allows vertebrates to transition between different gaits and adapt their limb phase-relationships based on descending commands or sudden mechanical perturbations in the environment. While capable of operating autonomously, each CPG relies on descending signals from the brainstem to initiate movement and on sensory feedback from the limbs to dynamically adapt to uneven terrain.

2.2. Peripheral nervous system

Once the motoneurons are depolarized, bursts of action potentials will travel along the motor axons in the PNS. The peripheral nervous system serves as the functional link between the CNS and the peripheral structures of the body, allowing bidirectional transmission of sensory inputs from peripheral receptors and of motor commands to effector organs. The PNS is composed of nerves arising from the brainstem (cranial nerves) and SC (spinal nerves). Spinal nerves innervating the upper and lower limbs converge to form the brachial and lumbar plexuses, respectively, where fibers are redistributed among distinct peripheral nerves. The PNS also contains neuronal cell bodies located outside the CNS, grouped into the DRG, where the soma of sensory neurons resides (Crossman, 2019; Rigoard, 2017).

Axons in peripheral nerves are classified according to their degree of myelination, diameter and conduction velocity. The fastest fibers are the myelinated ones, categorized in descending order of diameter as $A\alpha$, $A\beta$, $A\gamma$, and $A\delta$. Additionally, autonomic B fibers are thinly myelinated, while C fibers are small-diameter and unmyelinated. Nerve fibers can be activated with different ranges of electrical pulse intensities, with a threshold inversely related to the axon diameter.

With respect to motor neurons, α -motoneurons conduct impulses through $A\alpha$ fibers that innervate extrafusal muscle fibers, whereas γ -motoneurons transmit signals via $A\gamma$ fibers targeting intrafusal fibers of the muscle spindle.

Sensory neurons located in the DRG exhibit substantial functional heterogeneity. $A\alpha$ and $A\beta$ fibers convey proprioceptive and fine touch information, while the smaller

A δ and C fibers are specialized in nociception, thermal, chemical stimuli, and gross touch (Usoskin et al., 2015).

2.3. Control of locomotion

Regarding motor action, the volley originating in the motor cortex for a voluntary action reaches the muscle fibers through a highly coordinated sequence that engages multiple levels of spinal processing. The cortical command is first relayed and distributed by propriospinal interneurons, then dynamically shaped by spinal reflex circuits and CPGs before converging onto α -motoneurons. The resulting action potentials travel along fast-conducting A α motor fibers to extrafusal muscle fibers, where they trigger coordinated contraction. In this hierarchical organization, the conduction velocity of the PNS ensures the rapid translation of centrally planned motor commands into effective muscle activation, thereby closing the sensorimotor loop that underlies both voluntary and automatic movements (Crossman, 2019; Jankowska, 2013).

Locomotion is a complex motor behavior that depends on precise coordination between flexor and extensor muscles bilaterally, mediated by spinal and supraspinal neural networks. This locomotor performance emerges from the interaction between motoneurons, spinal CPGs and multiple sensory feedback loops, which continuously adjust the motor pattern to mechanical demands, behavioral context and changes in speed (Côté et al., 2018; Danner et al., 2015, 2023).

Evidence from humans with SCI further supports the functional existence of spinal CPGs: even in the absence of voluntary control and step-specific feedback, the lumbar SC can produce rhythmic patterns of muscle activation closely resembling those generated by CPGs in isolated animal preparations, particularly when epidural or pharmacological stimulation is applied (Minassian et al., 2017). This suggests that, despite the loss of supraspinal input, key elements of the locomotor circuitry can still be recruited and modulated, providing a neurophysiological substrate for activity-dependent rehabilitation strategies after SCI.

The locomotor CPG is composed of networks of excitatory and inhibitory interneurons that generate flexion–extension alternation and coordinate limb

musculature. During each cycle, specific interneuronal populations facilitate activation of flexor muscles while inhibiting extensors and then reverse this pattern to produce the characteristic rhythmic alternation; commissural interneurons ensure left–right coordination, whereas other subsets specialize in flexor–extensor coupling (Goulding, 2009; Kiehn, 2006). Experimental work in the cat and in isolated rodent SCs has shown that rhythm-generating capacity is distributed along the lumbar enlargement, with a rostrocaudal gradient of excitability, and that critical CPG elements are concentrated in ventral regions of the grey matter (laminae VII, VIII and X) (Grillner & Zangger, 1979; Kjaerulff & Kiehn, 1996).

Across vertebrate species, this basic architecture is conserved from swimming systems (lamprey, tadpole) to terrestrial locomotion, with a dispersed spinal CPG that is strongly modulated by proprioceptive afferents and by descending reticulospinal, rubrospinal and vestibulospinal pathways (Goulding, 2009). In quadrupeds, segmental rhythm generators for each limb within the cervical and lumbar hemicords are coupled via commissural and long propriospinal interneurons, which determine interlimb coordination and the expression of speed-dependent gaits (Danner et al., 2023).

At the supraspinal level, locomotion is embedded in a distributed network encompassing the motor cortex, the mesencephalic locomotor region (MLR) and the pontomedullary reticular formation. The motor cortex contributes to the selection of locomotor strategies, to the voluntary initiation and termination of gait, and to the adaptation of the step pattern to task goals and environmental demands, with a particularly prominent role in bipeds, in whom continuous cortical engagement is required to maintain balance and coordinate complex tasks. Cortical commands reach the SC both directly, via the corticospinal tract, and indirectly, through projections to brainstem nuclei that recruit reticulospinal pathways. The MLR, consisting primarily of the cuneiform nucleus (CnF) and the pedunculopontine nucleus (PPN), acts as a key hub that transforms motor plans and motivational signals from the basal ganglia, thalamus and cortex into locomotor commands: graded activation of this region can initiate forward walking and scale speed, whereas its inhibition suppresses locomotor output (Goulding, 2009; Leiras et al., 2025). These commands are conveyed to the SC mainly by glutamatergic

reticulospinal neurons in the pontomedullary reticular formation, particularly in the lateral paragigantocellular nucleus (LPGi) and gigantocellular nuclei, which regulate the initiation and acceleration of locomotion, while reticulospinal populations in the gigantocellular nucleus (Gi) are specialized in active stopping and in the generation of asymmetries (turns) through unilateral adjustment of flexor rhythm (Capelli et al., 2017; Cregg et al., 2020). Within this hierarchical framework, the cortex specifies the “when” and “how” of locomotion, the MLR and reticular formation translate these decisions into descending commands for start, speed, stopping and direction, and spinal CPGs integrate these commands with peripheral sensory information to generate the final motor output.

Sensory feedback plays a critical role both at the level of motoneurons and directly on the CPG. Muscle spindles provide information about muscle length and stretch velocity, exerting monosynaptic excitation on motoneurons and modulating reflex gain in a phase-dependent manner, whereas Golgi tendon organs encode load and tension, prolonging the extensor phase when support is still required or facilitating flexion onset when load decreases (Grillner & Zangger, 1979; Pearson, 2004). Experiments in cats and other models have distinguished reflexes that act “downstream” on motoneurons from those that act “upstream” on the CPG, adjusting phase duration, cycle frequency and flexion–extension transitions according to signals related to load, hip position and movement or body curvature (Goulding, 2009; Grillner & Zangger, 1979). In addition, cutaneous afferents, including nociceptive inputs, trigger phase-dependent responses such as stumbling corrective reactions, which are smoothly integrated into the locomotor pattern to avoid obstacles without compromising overall stability. In humans, the functional presence of locomotor CPGs implies that these circuits can provide a feedforward component to generate basic flexor–extensor activation patterns and to regulate cycle frequency for a given target speed, while supraspinal centers select the locomotor mode, initiate and terminate movement, and adapt the pattern to task demands (Minassian et al., 2017) (Figure 5).

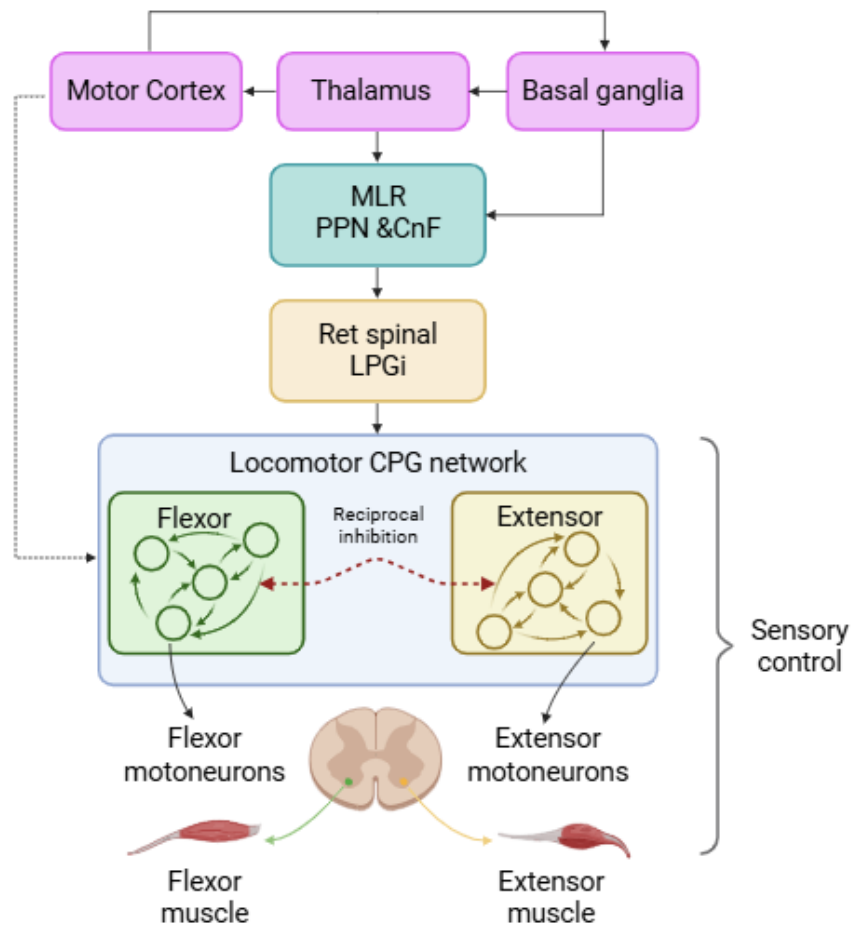


Figure 5. Schematic representation of the locomotor CPG. MLR: mesencephalic locomotor region; PPN: pedunculopontine nucleus; CnF: cuneiform nucleus; LPGi: lateral paragigantocellular nucleus.

Locomotor strategies differ across species. In quadrupeds, gait patterns are more automatic and rhythmic, strongly governed by spinal circuits and robust CPGs, with peripheral feedback acting as a control layer that optimizes an underlying synergy. In bipeds, by contrast, walking relies more heavily on continuous supraspinal input from the motor cortex, basal ganglia, and cerebellum to determine when to walk, at what speed and for what purpose, while brainstem circuits translate these decisions into precise commands to spinal locomotor networks (Goulding, 2009; Leiras et al., 2025; Minassian et al., 2017). This organization confers high flexibility and richness of locomotor patterns but also greater vulnerability to lesions affecting descending pathways or brainstem nuclei that form the obligatory interface between the brain and the SC.

3. Spinal cord injury

SCI profoundly disrupts all forms of sensorimotor function by compromising the generation, transmission and processing of impulses required for voluntary movements and for conscious sensations. It is defined as damage to the SC that results in motor, sensory and autonomic deficits, temporary or permanent, below the neurological level of injury, and may arise from traumatic causes (such as traffic accidents, falls or sports trauma) or non-traumatic (including degenerative, infectious, ischaemic or neoplastic processes). Globally, the mean incidence over recent decades has been estimated at approximately 20–30 new cases per million inhabitants per year, of which around 90% are of traumatic origin, with a clear predominance in males, increasing incidence with age, and with more severe clinical course in traumatic cases (Safdarian et al., 2023).

From an anatomical and clinical standpoint, cervical segments are typically associated with tetraplegia, whereas thoracic and lumbar injuries are linked to different patterns of paraplegia (Alizadeh et al., 2019). Beyond lesion level, SCI is classified according to its severity: in complete injuries there is a total loss of motor and sensory function below the neurological level, while in incomplete injuries some degree of function is preserved. The term “complete” does not necessarily imply a full histological transection, as residual connections across the lesion site are frequently present; this observation has led to the introduction of the concept of “discomplete” lesions, referring to cords that are clinically complete but anatomically partially preserved (Wahlgren et al., 2021).

3.1. Pathophysiology

Traumatic SCI most commonly occurs as a consequence of vertebral impact or dislocation that directly damages the SC tissue. The initial mechanical insult, known as the primary injury, produces immediate damage to neurons, glial cells and the associated microvasculature. Within minutes to hours, a secondary degenerative cascade is triggered, characterized by vascular disruption and altered permeability, ionic imbalance, glutamate-mediated excitotoxicity, metabolic dysfunction, an inflammatory response and the activation of processes leading to glial scar formation (Lima et al., 2022). In parallel, interruption of descending autonomic pathways contributes, from very early stages to systemic cardiovascular and

autonomic dysfunctions, which in the long term are associated with arrhythmias and increased cardiovascular risk in individuals with SCI (West et al., 2016).

From a temporal perspective, the evolution of SCI is usually divided into acute, subacute and chronic phases. The acute phase (first 24–48 hours) is characterized by hemorrhage, edema, vascular disruption and the onset of the immune response; together with cell death and inflammation, these processes promote ionic disequilibrium and excessive accumulation of excitatory neurotransmitters such as glutamate. The subacute phase (days to weeks) encompasses apoptotic cell death, axonal degeneration, demyelination, extracellular matrix remodeling, proliferation of reactive astrocytes and persistent inflammation; during this period, glial scar formation begins and, although some axonal plasticity and very limited regeneration may occur, repair remains highly restricted (Ahuja et al., 2017; Lima et al., 2022). The chronic phase (months to years) is characterized by cystic cavitation, axonal dieback and maturation of the glial scar, resulting in a strongly inhibitory microenvironment that limits plasticity and regenerative capacity and favors maladaptive changes such as spasticity, neuropathic pain and chronic autonomic dysfunction (Figure 6).

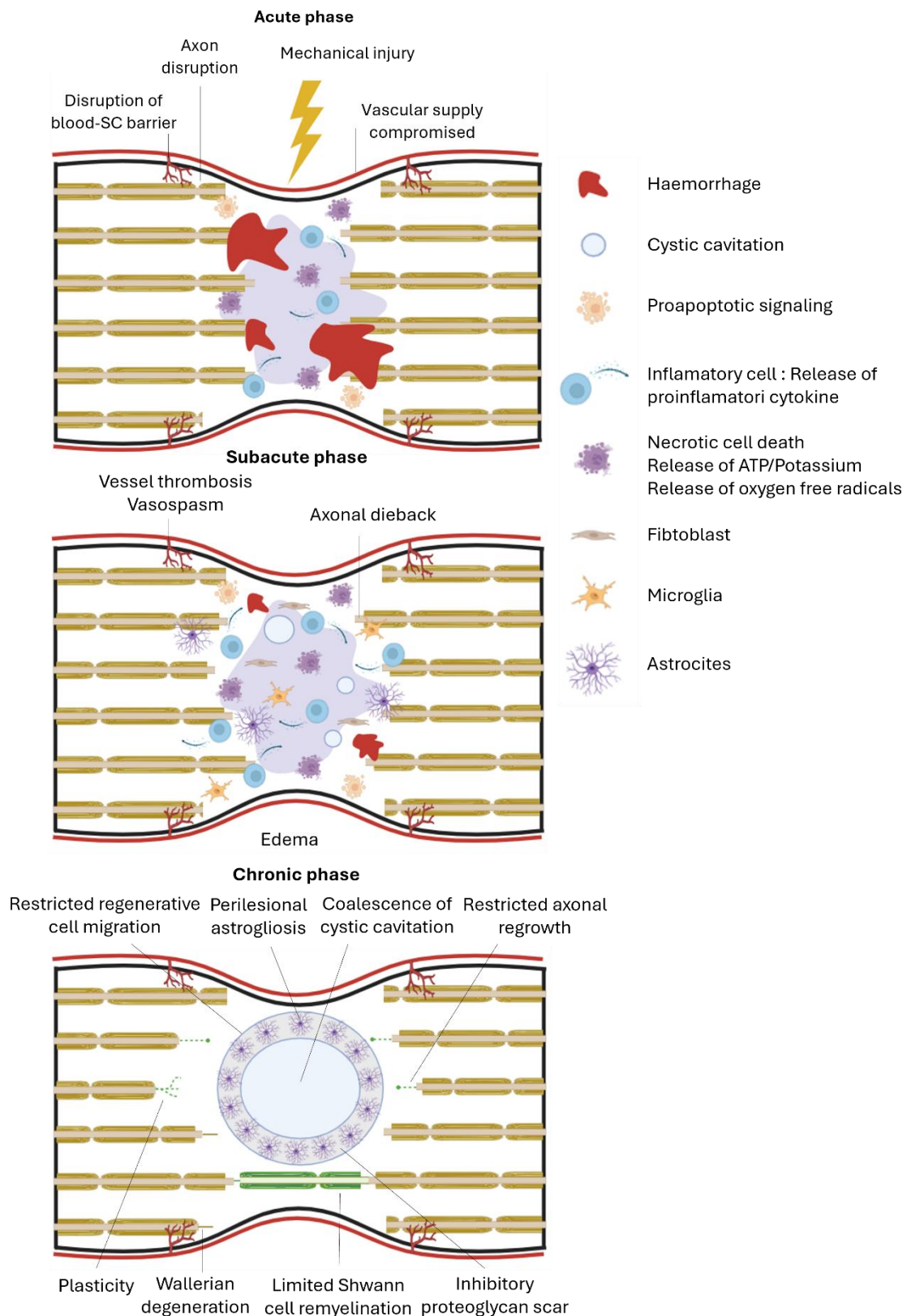


Figure 6. Pathophysiology of the Spinal cord injury. Main events in the acute, sub-acute and chronic phase of secondary injury after a SCI.

- **Permeability and vascular alterations**

Vascular damage is a major component of the pathophysiology of traumatic SCI, contributing to hypoperfusion, intraparenchymal hemorrhage and acute complications such as neurogenic shock and bradycardia (Ahuja et al., 2017; S. Li et al., 2022; Mautes et al., 2000). Disruption of blood flow and rupture of the spinal microvasculature promote the extravasation of blood and inflammatory cells, which increases local tissue pressure, further reduces perfusion and amplifies the inflammatory response and axonal damage during the first hours after injury (S. Li et al., 2022; Mautes et al., 2000). In addition, breakdown of the blood–SC barrier increases its permeability to proinflammatory cytokines and other neurotoxic factors, thereby indirectly contributing to secondary injury processes that unfold across the acute and subacute phases (Mautes et al., 2000; Yao et al., 2021).

- **Ionic Disruption and Glutamate excitotoxicity**

After a SCI, the sustained increase in extracellular glutamate concentrations induces persistent neuronal hyperactivation through the continuous stimulation of ionotropic AMPA and NMDA receptors, thereby triggering excitotoxic processes (Lima et al., 2022). This condition leads to widespread ionic dysregulation that affects both neurons and glial cells, rendering them highly vulnerable to degeneration and cell death.

In parallel, descending modulatory systems that normally regulate spinal motoneuron excitability, such as the serotonergic pathway, are disrupted, leading to a loss of inhibitory control over spinal circuits (Gerin et al., 2010). Within this context, a decrease in the potassium-chloride cotransporter KCC2 has been reported in motoneurons, which disrupts the intracellular chloride gradient and diminishes the efficacy of GABAergic and glycinergic inhibition (Boulenguez et al., 2010). The combination of glutamate-induced excitotoxicity, ionic imbalance, and KCC2 downregulation promotes a state of hyperexcitability and maladaptive reorganization of spinal networks, representing a key pathophysiological mechanism underlying spasticity and motor dysfunction following SC injury.

- **Oxidative stress**

Following the SCI, excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) leads to a state of oxidative stress that exacerbates secondary damage. This imbalance between free radical generation and antioxidant defenses causes oxidative damage to lipids, proteins, and nucleic acids, thereby compromising cellular integrity. Mitochondria are the primary source of ROS after SCI, due to respiratory chain dysfunction and calcium overload, while infiltrating inflammatory cells and iron released from hemorrhagic sources further enhance ROS formation. As a result, lipid peroxidation, mitochondrial dysfunction, and cell death occur, amplifying tissue degeneration and hindering functional recovery following SC injury (Lima et al., 2022; Yu et al., 2023).

- **Inflammatory response**

Following SCI, the innate immune response within the CNS is rapidly activated, giving rise to a state of neuroinflammation characterized by increased production of cytokines and chemokines by microglia, astrocytes, endothelial cells and peripheral immune cells that cross the blood–SC barrier. Although this early phase is necessary for debris clearance and containment of tissue damage, it frequently becomes chronic and contributes to secondary cell death and progressive tissue degeneration (Brockie et al., 2024; Kisucká et al., 2021; Lukacova et al., 2021).

Microglia act as the primary immune effector cells, responding rapidly to signals released by injured neurons and oligodendrocytes and accumulating around the lesion site, where they participate in glial scar formation. Their activation spans a continuum of states, ranging from proinflammatory phenotypes that produce cytokines such as TNF- α , IL-1 β and IL-6 and are associated with amplification of inflammation and chronic neurodegeneration, to more reparative phenotypes involved in debris phagocytosis and in the release of trophic and anti-inflammatory factors (BDNF, IGF-1, TGF- β , IL-10, arginase-1) that promote remyelination, neurogenesis and resolution of the inflammatory response (Brockie et al., 2024; Kisucká et al., 2021; Lukacova et al., 2021).

In a complementary manner, astrocytes undergo reactive astrogliosis, proliferate and migrate to form the glial scar, which is initially beneficial by isolating the lesion

core and limiting the spread of inflammation. However, the subsequent secretion of inhibitory molecules such as chondroitin sulfate proteoglycans can hinder axonal regeneration. In addition, astrocytes regulate blood–SC barrier permeability, immune cell infiltration and vascular repair, and, within the framework of the tripartite synapse, modulate glutamate uptake and energy metabolism, thereby helping to limit excitotoxicity (Brockie et al., 2024; Kisucká et al., 2021; Lukacova et al., 2021).

Taken together, microglia and astrocytes form an interdependent network that coordinates inflammatory responses, tissue repair and synaptic remodelling after SCI, such that the balance between proinflammatory and neuroprotective phenotypes is critical for functional recovery and represents a central target for future therapeutic strategies (Brockie et al., 2024; Kisucká et al., 2021; Lukacova et al., 2021).

- **Inhibitory environment**

In the CNS, SCI establishes a markedly inhibitory environment that severely limits the capacity for neuronal regeneration. Although an initial axonal growth response can be observed, it is soon halted when growth cones encounter the non-permissive microenvironment that develops within the lesion area. The abrupt rise in intracellular Ca^{2+} activates Ca^{2+} -dependent proteases that degrade the cytoskeleton, leading to growth cone collapse, retraction and the formation of dystrophic axonal endings, while the accumulation of myelin debris results in the release of myelin-associated inhibitors that block further axonal elongation. In parallel, the extracellular matrix is remodelled and enriched in molecules and proteoglycans that act as physical and chemical barriers: although they initially help stabilize the damaged tissue, over time they become a major obstacle to neural plasticity. Together, ionic imbalances, myelin debris and structural alterations of the extracellular environment generate a hostile microenvironment that prevents effective regeneration of nervous tissue (Lima et al., 2022).

- **Spinal cord scarring**

Following SCI, various cell types are recruited to the lesion site, where they contribute to the formation of both the fibrotic and glial scars. The fibrotic scar,

located at the core of the lesion, is primarily composed of fibroblasts and pericytes, which provide structural rigidity. These cells secrete extracellular matrix components such as collagen, fibronectin, and chondroitin sulfate proteoglycans (CSPGs), contributing to the establishment of an inhibitory microenvironment. Surrounding this structure lies the glial scar, which is mainly formed by reactive astrocytes, together with macrophages and microglia. The scarring process stabilizes within one to two weeks after injury, marking the maturation of the lesion site.

This scar complex creates a physical and biochemical barrier that, on one hand, plays a protective role by restoring the blood–brain barrier integrity, maintaining ionic homeostasis, and limiting immune cell infiltration. On the other hand, it restricts axonal regeneration and significantly impairs functional recovery (Clifford et al., 2023; Lima et al., 2022).

3.2. Regeneration and plasticity mechanisms after SCI

In the CNS, the capacity for regeneration after SCI is markedly limited compared with the PNS; nonetheless, a certain degree of structural and functional plasticity is preserved, allowing partial circuit reorganization and contributing to the spontaneous recovery of some functions (O'Shea et al., 2017; Wu et al., 2025). This recovery is supported primarily by two complementary processes: axonal regeneration and neural plasticity.

- Axonal regeneration

Adult neurons in the CNS exhibit a low intrinsic capacity for axonal growth, which is further constrained by the inhibitory microenvironment that develops at the lesion site (O'Shea et al., 2017). At the intrinsic level, injured neurons only partially reactivate the developmental growth programs; although transcription factors such as STAT3 and Sox11 can modestly enhance these programs, the extent of spontaneous regeneration remains very limited (O'Shea et al., 2017; X. Sun et al., 2025). In parallel, the extracellular environment contains multiple inhibitory signals previously described, which are not sufficiently counterbalanced by the endogenous production of trophic factors such as BDNF, NT-3 or NGF, whose magnitude and

spatiotemporal organization are inadequate to support effective regeneration (Zheng & Tuszynski, 2023). Consequently, current therapeutic strategies tend to focus more on enhancing the plasticity of preserved circuits than on achieving complete restitution of the original pathways.

- **Neural plasticity and functional reorganization**

Neural plasticity, rather than axonal regeneration, represents the principal mechanism underlying functional recovery after SCI. This process involves collateral sprouting of intact axons, synaptic remodelling and the formation of new circuits within and around the lesion site (Krucoff et al., 2016; O'Shea et al., 2017). In incomplete injuries, preserved descending fibers can establish collateral projections onto propriospinal interneurons, which act as relay pathways to partially restore communication between supraspinal centers and lumbar spinal networks (X. Sun et al., 2025).

Interventions such as locomotor rehabilitation and functional electrical stimulation promote synaptic plasticity, increase BDNF expression and contribute to the reengagement of spinal motor circuits (Lynskey, 2008; Wu et al., 2025). These therapies not only strengthen residual connections but also help bias plasticity in an adaptive direction, thereby reducing the likelihood of maladaptive reorganizations such as hyperexcitability and the development of neuropathic pain.

- **Maladaptive plasticity after SCI: neuropathic pain and spasticity**

As previously mentioned, the CNS is capable of remarkable plasticity following injury; however, this process is not always beneficial. After a SCI, maladaptive forms of plasticity may develop, the most prevalent being spasticity and neuropathic pain, which affect approximately 70% and 50–60% of patients, respectively (Finnerup, 2017). Both conditions typically emerge at delayed stages and often progress into chronic disorders, sometimes triggered by non-painful stimuli such as touch.

These syndromes share several pathophysiological mechanisms, including disinhibition of spinal circuits due to the loss of inhibitory interneurons and disruption of descending pathways, as well as neuronal hyperexcitability, synaptic reorganization, axonal sprouting, glial activation, and neuroinflammation.

The pain caused by a lesion or disease of the somatosensory nervous system is known as neuropathic pain. It manifests as spontaneous pain, allodynia, or hyperalgesia (Finnerup et al., 2020). This condition arises from hyperexcitability within the dorsal horn, caused by excessive glutamate release following SCI. The overactivation of glutamatergic receptors, combined with the loss of GABAergic inhibitory neurons, results in increased spontaneous activity of nociceptors and, consequently, heightened pain sensitivity ((Bhagwani, 2022; He et al., 2025). He et al. (2025) demonstrated in a rat model of neuropathic pain that SCI disrupts nociceptive pathways by increasing excitatory neural activity and impairing the excitatory–inhibitory balance, accompanied by enhanced activation of PKC γ -positive interneurons and noradrenergic neurons in the locus coeruleus.

Spasticity, on the other hand, is defined as the intermittent or sustained involuntary activation of muscles resulting from disordered sensorimotor control (Finnerup, 2017). It is primarily associated with hyperexcitability of spinal reflexes due to increased excitability of alpha motoneurons and a reduction in GABAergic tone, leading to an exaggerated stretch reflex (Adams & Hicks, 2005; Bhagwani, 2022). Although spasticity significantly impairs voluntary movement and functional control, in some cases it may provide postural support, albeit at the cost of fine motor function.

3.3. Evaluation of sensory-motor deficits and recovery following a SCI

The characterization of sensorimotor deficits and functional recovery after SCI generally relies on a combination of sensory assessments, motor measures and neurophysiological tests that probe the function of ascending and descending pathways. Sensory examinations allow investigators to characterize alterations across different modalities (touch, nociception, proprioception), to identify both loss of function and the emergence of maladaptive phenomena such as hyperalgesia and allodynia, and to delineate the level and extent of neurological compromise. Complementary motor outcome measures provide a global read-out of the functional capacity of the sensorimotor system and are widely used as primary endpoints in preclinical and clinical studies of SC repair (Dancause et al., 2015).

Within this framework, electrophysiology represents a fundamental tool for linking behavioral changes to the functional properties of spinal and supraspinal circuits. Neurophysiological recordings permit quantification of segmental excitability, transmission through afferent and efferent pathways and the effectiveness of descending motor activation, thereby providing information that is not accessible through clinical or purely behavioral observation. In experimental models of SCI, the integration of behavioral measures, sensory testing and electrophysiological parameters is systematically used to track the temporal evolution of deficits and to evaluate the functional impact of different repair and rehabilitation strategies within the broader context of regeneration and plasticity processes described in the preceding chapters.

3.4. Therapeutical strategies to promote plasticity and functional recovery

Numerous studies have demonstrated that therapies aimed at stimulating the preserved neural circuits can be highly beneficial for functional recovery following SCI. These interventions promote adaptive plasticity, which contributes to motor recovery and helps prevent maladaptive outcomes such as spasticity and neuropathic pain (Lynskey, 2008; Wu et al., 2025). Among the most extensively studied strategies are locomotor treadmill training and the use of neuroprosthetic technologies.

3.4.1. Locomotor rehabilitation

Treadmill locomotor training is one of the most extensively studied interventions to promote functional recovery after SCI. This therapy is based on the principle of activity-dependent plasticity, whereby the repeated activation of spinal and supraspinal circuits stimulates synaptic reorganization and strengthens residual connections (Lynskey, 2008; Wu et al., 2025).

Numerous experimental studies have demonstrated its efficacy in promoting both functional recovery and spinal plasticity. In rats with thoracic contusion, step training initiated one week after injury and maintained for 10 weeks produces a sustained improvement in BBB scores from the fourth week onward, together with more coordinated step cycles compared with untrained animals (T. S. Sun et al.,

2013). These functional gains are associated with increased expression of the growth-associated protein GAP-43 at the lesion site and a higher number of tyrosine hydroxylase-positive interneurons in the L2 segment, indicating enhanced axonal sprouting and interneuron plasticity in sublesional circuits (T. S. Sun et al., 2013). More recently, controlled treadmill training after mild SCI has been shown to improve locomotor scores, preserve spinal tissue and cavity size, and increase GAP-43 expression while modulating glial and inflammatory responses, further supporting its role in driving structural and molecular plasticity in the injured spinal cord (Ageeva et al., 2024). In addition, animal studies have demonstrated that locomotor training not only improves gait patterns but also reduces muscle atrophy and facilitates the activation of CPGs responsible for rhythmic locomotor control (Fakhoury, 2015; Lynskey, 2008).

At the systems level, cat and rat studies have shown that treadmill training can normalize initially asymmetric or unstable gait patterns and “imprint” spinal locomotor networks, such that trained animals retain more symmetrical and robust stepping even after additional lesions. These findings highlight the capacity of treadmill paradigms to reshape CPG activity and sublesional circuitry (Rossignol et al., 2015). However, the same work also illustrates that training confined to automated treadmill stepping with limited voluntary engagement primarily strengthens spinal circuits without fully restoring supraspinal control, whereas more demanding, task-oriented training paradigms are required to drive reorganization of descending pathways (Van Den Brand et al., 2012). In humans, body-weight-supported treadmill training, with or without robotic assistance, has been associated with improvements in gait speed, walking distance and functional independence, particularly in individuals with incomplete injuries, although the magnitude and durability of these benefits strongly depend on lesion severity, timing of intervention and training dose (Wu et al., 2025). In animal models, early initiation of training during the acute inflammatory phase did not produce lasting benefits, whereas delaying the onset until inflammation had subsided led to significant recovery of hindlimb function (Wu et al., 2025). Moreover, the effects of training-induced plasticity are partially reversible when therapy is discontinued, highlighting the importance of maintaining sustained rehabilitation (Lynskey, 2008).

A key factor that has gained increasing attention is training variability. Shah et al. (2012) demonstrated that introducing variations in stepping patterns, such as changes in speed, rhythm, or treadmill inclination, enhanced locomotor recovery in rats with SCI compared to repetitive, monotonous training. This variability appears to increase cortical engagement, reinforce sensorimotor pathways, and prevent the consolidation of maladaptive motor patterns. Thus, a dynamic and enriched training environment facilitates more effective and long-lasting plasticity (Shah et al., 2012).

Overall, treadmill training promotes the functional reorganization of locomotor circuits, stimulates activity-dependent neural plasticity, and contributes to motor recovery. Its effectiveness depends on the intensity, variability, and continuity of treatment, factors that must be optimized to maximize therapeutic outcomes (Lynskey, 2008; Shah et al., 2012; Wu et al., 2025).

3.4.2. Neuroprostheses

Neuroprostheses are devices designed to partially restore or replace lost motor, sensory, or autonomic functions following a SCI. These systems deliver electrical impulses to activate motor or sensory nerves that remain structurally intact, thereby generating muscle contractions that enable functional movements (Ajiboye et al., 2017; Gupta et al., 2023).

Neuroprosthetic systems can be classified according to their level of invasiveness, ranging from least to most invasive as follows: transcutaneous, percutaneous, and fully implantable devices (Figure 7).

Transcutaneous electrodes are placed superficially on the skin and represent the most commonly used option in clinical practice due to their ease of use and low cost. As they are non-invasive, they do not require surgery, although they demand higher stimulation intensities, particularly for deeper muscles, and provide limited selectivity and stability, which makes them less suitable for small muscle groups (Rupp, 2017).

Percutaneous electrodes consist of thin metallic wires that penetrate the skin to reach the vicinity of the target nerve or the motor end plate of the desired muscle. These electrodes offer greater stability and specificity than surface systems but

carry a risk of infection; therefore, they are typically used for short-term interventions (Marsolais & Kobetic, 1988).

Implantable neuroprostheses require surgical implantation around a nerve or within a muscle. This surgical approach enables highly selective and stable stimulation, making these systems suitable for long-term use. In addition, they allow the use of lower current intensities and reduce muscle fatigue (Memberg et al., 2014). However, their main limitations are the need for surgery and the associated risk of infection (Loeb & Davoodi, 2005).

Regarding their clinical application, neuroprostheses can be employed for either orthotic or therapeutic purposes. In orthotic use, the neuroprosthesis acts as a functional assistive device, replacing the lost function and requiring constant activation to perform it. In therapeutic use, neuroprostheses aim to harness activity-dependent plasticity to strengthen residual neural connections after injury. This therapeutic approach has been shown to promote cortical reorganization, reduce spasticity, and improve motor function in individuals with SCI or stroke (Rupp, 2017).

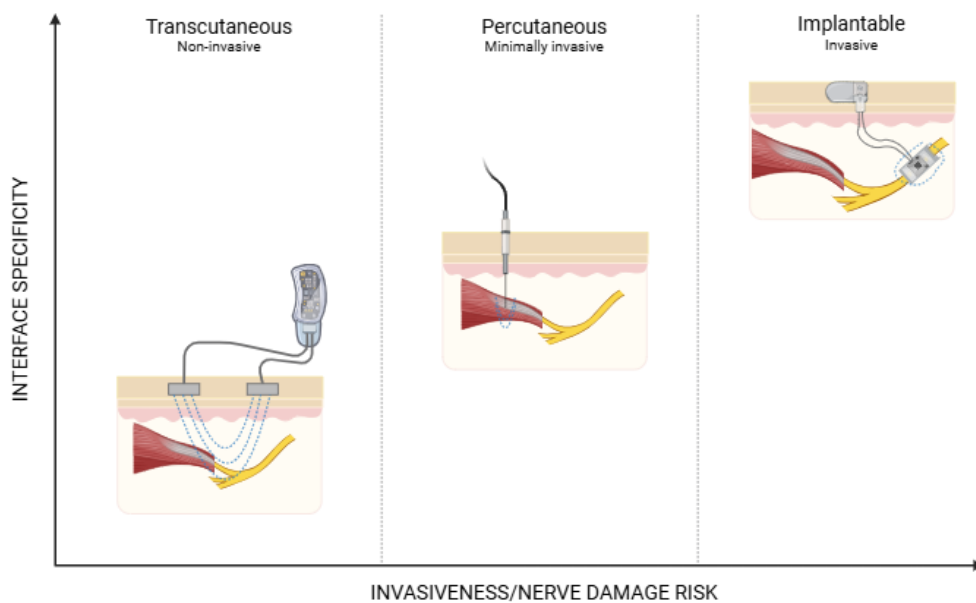


Figure 7. Invasiveness versus specificity of neuroprosthetic electrodes. Electrodes range from transcutaneous (non-invasive) to percutaneous and implantable systems. Increasing invasiveness enables greater selectivity and more precise targeting of neural structures, at the cost of higher surgical complexity and risk.

Beyond motor restoration, neuroprosthetic technology also encompasses neuromodulation, which involves the electrical activation of sensory or reflex pathways to modify neural network activity and enhance motor recovery. By stimulating afferent fibers or specific spinal circuits, neuromodulatory interventions can promote adaptive plasticity, modulate excitability, and contribute to functional improvements even in the absence of voluntary movement (Rupp, 2017).

The development of brain-machine interfaces represent a significant advancement within this field, as they enable the direct translation of neural activity into control signals for neuroprosthetic devices, enhancing both their functional and rehabilitative potential.

- **Brain machine interfaces (BMIs)**

Brain-machine interfaces are neuroprosthetic systems designed to record CNS activity and translate it into control commands that are sent to external devices or electrical stimulation systems, thereby generating an artificial output signal. Their main goal is to restore communication and motor control in cases where the descending pathways of the CNS are damaged but the generation of motor intention within the cerebral cortex remains intact (Müller-Putz & Rupp, 2021).

Most current systems use non-invasive electrodes to record cortical activity through electroencephalographic (EEG) signals. These recordings allow the detection of specific brain patterns associated with different mental states or motor tasks, such as event-related potentials or sensorimotor rhythm desynchronization (Pfurtscheller & Neuper, 2001).

In the field of rehabilitation, BMIs enable the synchronization of voluntary cortical activity with FES, thereby enhancing synaptic plasticity through Hebbian principles of associative activation. This coupling between cortical intention and peripheral response facilitates the functional reorganization of sensorimotor networks, contributing to the recovery of motor function after neurological injuries (Rupp, 2017).

3.4.3. Functional Electrical Stimulation

FES is a form of neuromuscular electrical stimulation specifically aimed at generating coordinated, functional movements in individuals with neurological impairments such as SCI or stroke (Gupta et al., 2023; R. Y. Kim et al., 2025; Marquez-Chin & Popovic, 2020). This technique focuses on reproducing organized motor patterns (such as reaching and grasping or locomotion) making it a highly valuable tool in neurorehabilitation. When used for this purpose, FES works as a neuroprosthesis, temporarily or permanently replacing motor functions that the CNS can no longer execute (Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021; Rupp, 2017). Beyond restoring partial mobility, these interventions have been shown to enhance independence, social participation, and quality of life in individuals with SCI (Figure 8).

FES uses intact peripheral nerves and muscles to elicit electrical excitation. Stimulation can be applied to motor nerves, triggering muscle contractions that enable functional movements, or directly to the muscle itself. Depending on the level of invasiveness and the placement of the electrodes, the effectiveness of stimulation can change. The main parameters that determine the physiological response are amplitude, pulse duration, and stimulation frequency, as these influence the number of fibers activated and the resulting type of contraction. The amplitude of stimulation directly affects which nerve fibers are recruited, with larger fibers, located closest to the stimulation electrode, being activated first. This recruitment order is opposite to that observed under physiological conditions. Medium frequencies (20–50 Hz) are most commonly used to generate sustained, tetanic contractions, while higher frequencies accelerate muscle fatigue, which remains one of the primary challenges of this technology (Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021).

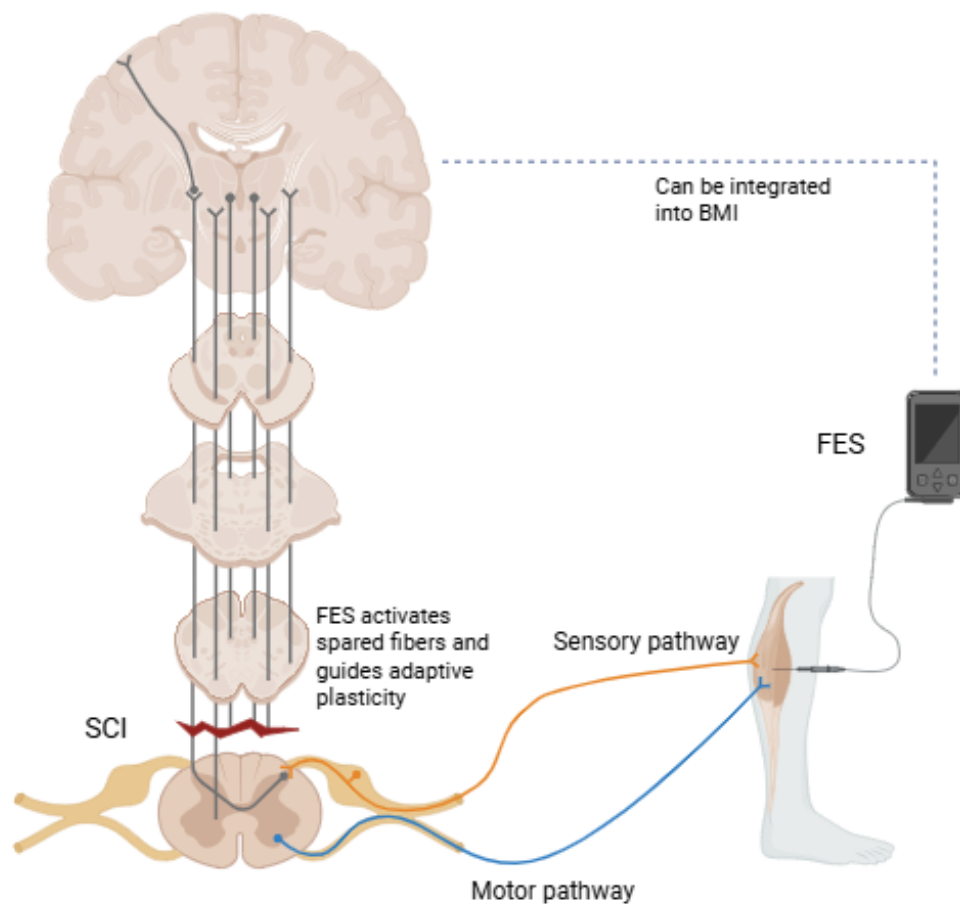


Figure 8. Schematic representation of FES after a SCI. FES activates spared motor pathways below the lesion to produce muscle contractions and functional movements, while sensory feedback contributes to adaptive plasticity.

From a neurophysiological standpoint, FES is not only a means to produce movement but also a powerful activity-dependent driver of plasticity. By repeatedly pairing sensory inflow from the stimulated muscles with residual descending commands and spinal circuits, FES can strengthen preserved pathways, promote re-weighting of spinal reflex loops and facilitate the re-emergence of more physiological motor synergies (Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021). When delivered in functionally meaningful patterns (for example, timed to the gait cycle or to a reaching task), the intervention reinforces task-specific sensorimotor associations, which is thought to underlie many of the long-term therapeutic effects reported after intensive FES-based training (R. Y. Kim et al., 2025; Marquez-Chin & Popovic, 2020)). In this sense, FES bridges the gap between its use

as a neuroprosthetic tool and a purely restorative rehabilitation approach, providing both immediate functional assistance and potential carry-over effects when the device is switched off (R. Y. Kim et al., 2025).

A growing body of preclinical and clinical research supports the use of FES as an effective rehabilitation tool after SCI (Beaumont et al., 2014; Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021; Reinkensmeyer et al., 2023; Rupp, 2017; Tsai et al., 2025; Unger et al., 2025). In animal models, FES applied over lower limb muscles or peripheral nerves has been shown to enhance locomotor recovery, reduce muscle atrophy and promote task-specific reorganization of spinal circuits when combined with repetitive stepping or cycling paradigms (Beaumont et al., 2014; Tsai et al., 2025). In humans, upper-limb neuroprostheses based on FES, using either implanted or surface electrodes, have restored grasp and reach to a functionally meaningful degree in people with tetraplegia, enabling activities of daily living such as feeding or object manipulation with greater independence (Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021). Long-term follow up studies summarized in recent neurorehabilitation technology reviews indicate that these systems not only provide immediate compensatory benefits but can also induce therapeutic effects, such as improved voluntary activation and reduced reliance on external assistance, when used intensively within structured training programs (Marquez-Chin & Popovic, 2020; Reinkensmeyer et al., 2023).

Clinically, several modalities of FES have been developed, including surface stimulation for grasp and hand opening/closing, multi-channel systems for gait and cycling, and more invasive nerve or intramuscular implants for chronic users with high SCI level (Marquez-Chin & Popovic, 2020; Rupp, 2017). Across these implementations, common goals include reducing spasticity, preventing muscle atrophy, improving cardiovascular fitness and, crucially, enabling practice of functional tasks at intensities and repetitions that would not be possible through voluntary effort alone (R. Y. Kim et al., 2025; Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021; Unger et al., 2025). This combination of neuromodulatory and restorative effects explains why FES is increasingly considered a core component of modern rehabilitation programmes in SCI and other upper motor neuron

syndromes (Marquez-Chin & Popovic, 2020; Müller-Putz & Rupp, 2021; Reinkensmeyer et al., 2023).

The aim of this thesis is to determine whether functional repair after SCI can be promoted by enhancing spinal plasticity through targeted activation of muscle afferents. We hypothesize that repeated, task-directed activation of these segmental circuits can modulate spinal reflex function and facilitate the release of trophic factors, thereby creating a spinal environment that is more receptive to descending input and less rigid from a functional standpoint. In this framework, rehabilitation interventions designed to increase peripheral afferent activity are proposed to facilitate the adaptive reorganization of sensorimotor circuits and, consequently, to improve recovery after SCI. Taking together, these considerations define the central working hypothesis of the present thesis and provide the conceptual basis for the experimental approaches described in the following chapters.

IV. Hypothesis and objectives

The first hypothesis of this thesis is that thoracic spinal cord contusion injuries of increasing severity produce graded functional, electrophysiological, and neurobiological impairments that depend on the extent of spared tissue and spinal circuitry preservation. Furthermore, we hypothesize that activity-based rehabilitation strategies, including treadmill training and functional electrical stimulation, promote adaptive plasticity and improve functional recovery after SCI.

Thus, the overall objective of this thesis is to characterize the functional and neurobiological consequences of graded thoracic spinal cord contusion injuries and to evaluate the effects of activity-based rehabilitation strategies on spinal plasticity and functional recovery. The thesis has been divided into two chapters with the following specific objectives:

Chapter 1. Severity-dependent functional and neurobiological alterations following thoracic spinal cord contusion injury

Objectives

The main objective of this study is to investigate the functional and anatomical consequences of thoracic spinal cord contusion injuries of increasing severity by assessing behavioral and electrophysiological impairments, tissue disruption, plastic changes in spinal reflex circuitry, and the preservation or alteration of lumbar motoneuron pools.

Specific Objectives

1. To characterize the locomotor and sensory deficits induced by thoracic SCI of increasing severity.
2. To evaluate electrophysiological alterations associated with graded thoracic SCI.
3. To quantify tissue preservation and myelin integrity at the lesion epicenter following contusion injuries of different severities.
4. To evaluate the preservation or alteration of lumbar motoneuron pools following graded spinal cord contusion injuries.
5. To characterize microglial and astroglial responses in the lumbar spinal cord after thoracic contusion injury of increasing severity.

6. To evaluate changes in KCC2 expression in the lumbar spinal cord following graded thoracic SCI.
7. To correlate functional and electrophysiological impairments with morphological, and molecular alterations after SCI.

Chapter 2. Activity-based rehabilitation strategies promote functional recovery and spinal plasticity after thoracic spinal cord injury

Objectives

To evaluate the effects of activity-based rehabilitation strategies, including treadmill training, FES, and early FES followed by treadmill rehabilitation, on functional recovery and spinal cord plasticity after thoracic spinal cord contusion injury.

Specific Objectives

1. To evaluate the effects of treadmill rehabilitation on locomotor, sensory, and electrophysiological recovery after thoracic SCI.
2. To assess the effects of FES as an independent rehabilitation strategy on functional and electrophysiological outcomes following SCI.
3. To determine whether early FES intervention prior to treadmill rehabilitation accelerates or enhances recovery after SCI.
4. To characterize rehabilitation-induced changes in spinal sensorimotor plasticity using electrophysiological approaches.
5. To evaluate nociceptive sensory alterations associated with the different rehabilitation paradigms.
6. To analyze cellular and molecular changes in neuronal and glial populations associated with each rehabilitation strategy.
7. To correlate functional recovery with electrophysiological, cellular, and molecular markers of spinal cord plasticity.

V. Study design and methodologies

General issues

Ethical statement

All experimental procedures were approved by the Animal Experimentation Ethical Committee of Universitat Autònoma de Barcelona and complied with the European Communities Council Directive 2010/63/EU as well as Spanish National Law (RD 53/2013).

Animals

All the rats used in this work were acquired from accredited providers, and maintained at the Animal Service facilities of the UAB. They were housed in groups of 4 the first week and after implantations individually, under a 12h light-dark cycle, with room temperature at $22\pm 1^{\circ}\text{C}$, and unlimited access to food and water. Upon arrival, the rats were handled for one week before entering the studies.

Surgeries

The animals were anesthetized by a single intraperitoneal injection of ketamine (90mg/kg) and xylazine (10mg/kg). The surgical region was shaved and disinfected with 70% ethanol and povidone iodine. The rats were placed on a heating blanket at 32°C to maintain body temperature. At the end of each surgery, muscle layers and skin were sutured. With the animals awake, buprenorphine (0,05 mg/kg) was injected subcutaneously as analgesia. Amitriptyline (0,09 mg/l) and amoxicillin (0,5mg/ml, as antibiotic) were included in the drinking water.

Chapter 1: Severity-dependent functional and neurobiological alterations following thoracic spinal cord contusion injury

Animals

24 adult female Sprague Dawley rats, aged 12 weeks and weighing between 250-300g were used in this study. Of them, 5 were excluded from the analysis because the severity of the induced SCI did not match the criteria established for the experimental model. In these cases, the lesion was either more extensive or less severe than the target level intended for the study.

Experimental design

The animals were divided into three experimental groups according to the severity of the spinal cord injury induced at the T9 level of the thoracic region. The contusion force applied was either 100, 200, or 300 kilodynes, corresponding to mild, moderate, or severe injury, respectively. Functional assessments were conducted pre-surgery and at 7, 14, 30, and 60 days post-injury, including sensory and motor evaluations, as well as electrophysiological testing.

Surgeries

A midline skin incision was made from the T5 to T12 vertebral levels. The paraspinal muscles were carefully dissected and retracted to expose the underlying vertebrae. A dorsal central laminectomy was then performed at the T8 and T9 levels to expose the spinal cord and allow access to the subsequent injury procedure. The spinal cord was stabilized by fixing the T7 and T10 vertebrae with clamps applied to the spinous processes. A contusion injury was then induced at the center of the exposed spinal cord using the Infinite Horizon Impactor device (Precision Systems and Instrumentation). This instrument is computer-controlled, allowing precise definition of the impact force to produce injuries of varying severity. During the procedure, the device also records key parameters including the actual force delivered, the displacement of the piston, and the velocity of the impact, ensuring consistent and quantifiable injury profiles. Animals were assigned to experimental groups based on the displacement values recorded during injury induction. The

classification criteria were as follows: 100kdynes (0,8–1,0 mm), 200kdynes (1,1–1,3 mm), and 300kdynes (1,5–1,7 mm) (Figure 9) (Scheff et al., 2003).

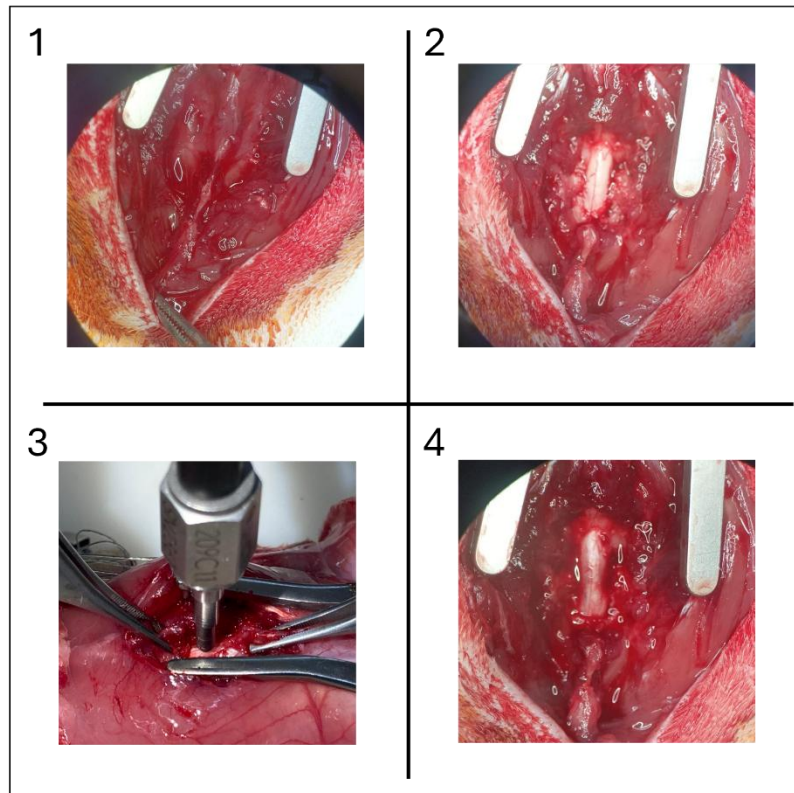


Figure 9. Spinal cord injury surgery. 1. Exposure of the vertebral column from T7 to T11. 2. T9 laminectomy. 3. Injury induced using an impactor. 4. Verification of the injury.

Functional outcome measures

- Open field test: BBB

Hindlimb locomotor function was evaluated in an open-field setting using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). This scale ranges from 0, indicating complete absence of hindlimb movement, to 21, which corresponds to normal locomotion characterized by coordinated gait, parallel paw placement, and stable trunk posture. Scores between 0 and 8 reflect isolated joint movements (hip, knee, and ankle), while scores from 9 to 13 indicate partial recovery, including occasional paw placement and some coordination with the forelimbs. Scores from 14 to 21 represent progressively more refined motor control, such as consistent stepping, toe clearance, trunk stability, and tail elevation. Each animal was assessed individually in an open enclosure (90 cm in diameter, 24 cm in

wall height) for a 5-minute observation period, during which free movement was encouraged. Evaluations were performed by two independent observers, and the final score was calculated as the average of their assessments. Locomotor performance was evaluated at 3 and, then, weekly for up to 56 days post injury (dpi).

- **Randall-Selitto Algesimetry**

Nociceptive withdrawal threshold was measured using a Randall–Selitto electronic algesimeter (IITC 2500 Digital Paw Pressure Meter, IITC Life Science, Woodland Hills, CA). Prior to testing, animals were gently handled for 5 minutes to facilitate habituation to the procedure. Each subject was manually restrained, ensuring stable positioning of the limb to be tested. A gradually increasing mechanical force was applied using the device probe, with the tip at the medial region of the plantar surface of the right hindpaw (Santos-Nogueira et al., 2012). The stimulus was terminated immediately upon eliciting a withdrawal response. To ensure consistency across sessions, the stimulus site was marked with ink. The maximum applied force was limited to 250 g to prevent tissue damage. Nociceptive response was evaluated presurgery and 7, 14, 30 and 60dpi.

- **Nerve conduction and spinal reflex tests**

To evaluate de neuromuscular function, electrophysiology recordings were performed. The compound muscle action potentials (CMAP) were recorded from the tibialis anterior and the plantar muscle (Valero-Cabre & Navarrò, 2001). Single electric pulses were delivered by monopolar needles inserted close to the sciatic notch. The signal was recorded by needle electrodes inserted on the belly of the muscle. The reference was placed at the second toe and the ground close to the knee. Measured parameters included M wave amplitude, H wave amplitude, H/M amplitude ratio, and H-reflex rate-dependent depression (RDD).

- **H/M ratio:** stimuli of increasing intensity were applied until a supramaximal M-wave response was obtained in the target muscle. The highest M-wave amplitude recorded was also used for analysis. The degree of hyperreflexia was calculated as the ratio between the maximal H-wave amplitude and the maximal M-wave amplitude. This calculation was performed for the two muscles tested.

- **H-reflex rate-dependent depression (RDD):** under the same conditions as above, 10 consecutive stimuli were delivered at the intensity required to elicit the maximal H-wave response. Series of 10 stimuli were applied at varying frequencies (1, 2, 5, 10, and 20 Hz). For each frequency, the amplitude of the H-wave evoked by the 10th pulse was compared to that of the first pulse (Meinck, 1976).

- **Motor evoked potentials (MEPs)**

Motor evoked potentials were recorded from the tibialis muscle of each animal. Transcranial electrical stimulation was delivered using two subcutaneous monopolar needle electrodes. The anode was placed over the sensorimotor cortex and the cathode at the base of the nose (García-Álías et al., 2006). Single rectangular pulses of 100 μ s and 70 mV were applied. Recording electrodes were positioned as described in the previous procedures. Muscle responses were displayed on an oscilloscope to measure amplitude and latency of the MEP.

Histological assessment

At the end of the experimental procedures, animals were euthanized with pentobarbital and transcardially perfused with 4% paraformaldehyde in 0,1M phosphate buffer. The thoracic (T7–T11) and lumbar (L1–L6) segments of the spinal cord were dissected and post-fixed in the same fixative for 24 h, followed by cryoprotection in 30% sucrose.

Spinal cords were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and rapidly frozen. Serial transverse sections, 25 μ m thick, were obtained using a cryostat, collected directly onto gelatin-coated slides, and stored at -20°C . Tissue sections were organized into series of five slides, each considered a replicate set.

- **Cresyl violet staining:** used to quantify motoneuron preservation across lumbar segments.
- **Fluoromyelin:** used to quantify myelin preservation along the injury.

- **Glial fibrillary acidic protein (GFAP):** used to assess astrocyte presence in the lumbar region and to evaluate glial scar formation at the lesion site.
- **Ionized calcium-binding adapter molecule 1 (Iba1):** used to assess microglial presence in the lumbar region.
- **Potassium-chloride cotransporter 2 (KCC2):** used to evaluate synaptic inhibition mediated by GABA/glycine.

Table 1. Antibodies used for tissue immunostaining/immunofluorescence.

	<i>Antigen</i>	<i>Dilution</i>	<i>Host</i>	<i>Reference number</i>
<i>Primary antibody</i>	GFAP	1:400	Mouse	Sigma G3893
	Iba1	1:400	Goat	Abcam ab5076
	KCC2	1:400	Rabbit	Millipore 07-432
	Fluoromyelin red	1:300		Thermo F34652
<i>Secondary antibody</i>	Alexa Fluor 488	1:200	Donkey x Mouse	Life Technologies A21202
	Alexa fluor 594	1:200	Donkey x Goat	Life Technologies A11058
	Alexa Fluor 647	1:200	Donkey x Rabbit	Abcam ab150075

For Cresyl violet staining, sections were incubated for 3 hours in a Cresyl violet solution, followed by dehydration through a graded ethanol series (70%–100%), clearing in xylene for 2 minutes, and mounting with DPX mounting medium.

For FluoroMyelin Red staining, sections were incubated with the dye for 20 minutes prior to blocking with fetal bovine serum (FBS).

For all immunostainings, tissue sections were first rehydrated in PBS and then permeabilized using PBS containing 0.3% Triton X-100. This was followed by a blocking step with 5% FBS for 1 hour to reduce non-specific binding. Sections were then incubated with the primary antibody (see Table 1) overnight at 4°C. The following day, sections were washed in PBS and incubated with the corresponding secondary antibody for 2 hours at room temperature. After additional washes, the tissue was mounted using Fluoromount-G with DAPI (ThermoFisher, 00-4959-52).

Image processing

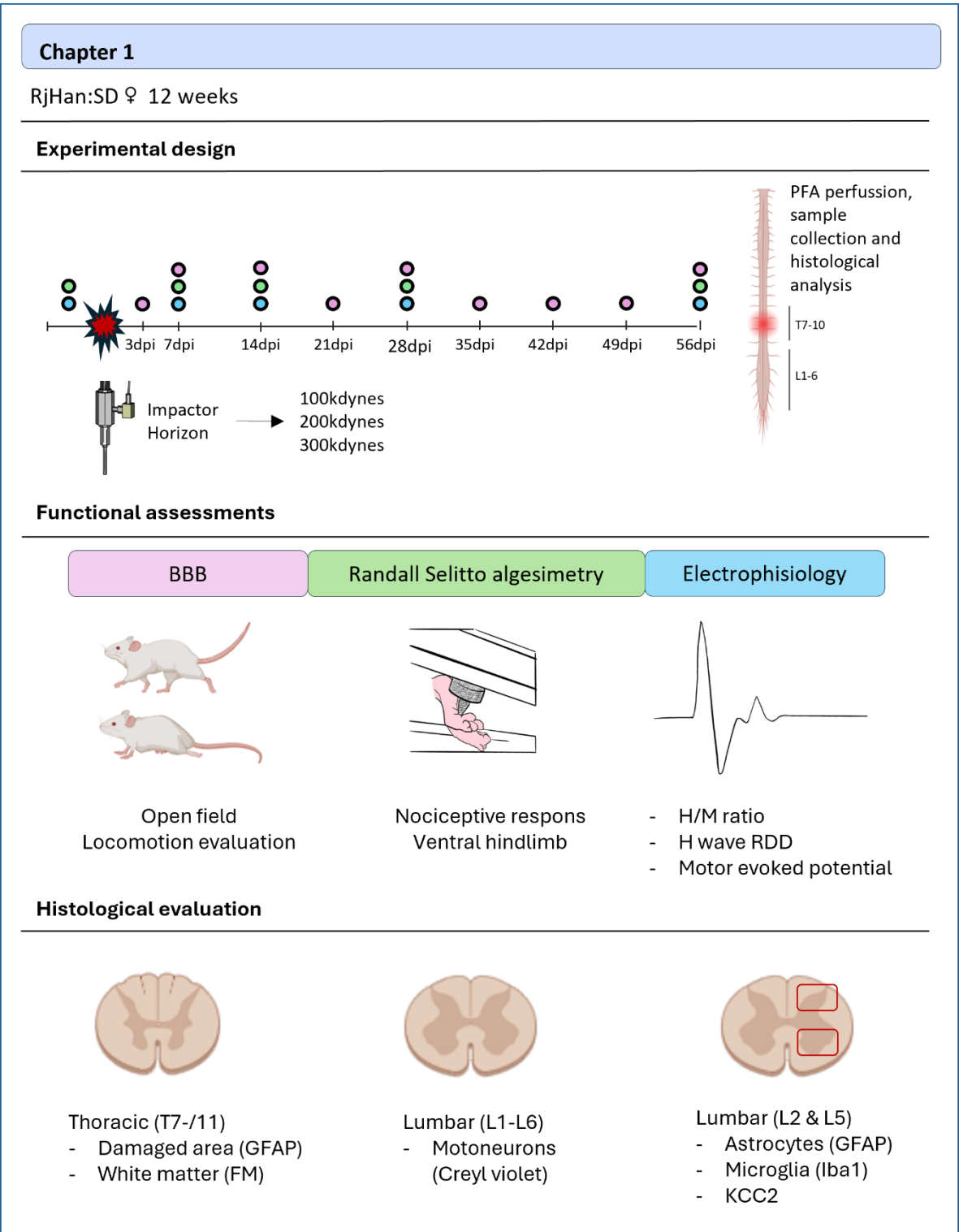
Histological sections were visualized in an Eclipse Ni epifluorescence microscope (Nikon), and Images acquired using a DS-Ri Nikon camera. High-resolution images of all stained and immunostained sections were obtained to assess tissue preservation, myelin integrity at the lesion site, motoneuron survival across lumbar segments, and the effects of different injury severities on microglia and astrocyte reactivity, and KCC2 expression.

Preserved tissue area was manually measured from GFAP-immunostained sections, while white matter preservation was assessed measuring the area of FluoroMyelin staining. Motoneuron survival was evaluated by manual counting motoneurons in the ventral horns of 4–6 sections per lumbar segment. Identification was based on morphological and size criteria, including polygonal shape, prominent nucleolus, and a diameter greater than 30 μm (Penas et al, 2009; Ferrucci et al., 2018).

Astrocytic and microglial responses were assessed by measuring the integrated density area of immunofluorescence. Due to variability between images, a manual threshold was applied in each case to accurately capture glial morphology. Similarly, KCC2 expression was evaluated by measuring fluorescence intensity with image-specific manual threshold adjustment. Fluorescence density measurements for GFAP, Iba1, and KCC2 were performed in both the dorsal and ventral horns of the L2 and L5 spinal cord segments. For the ventral horn, image stitching was performed to cover the entire region of interest. All analyses were performed using ImageJ software.

Statistical analyses

All data sets were analyzed using GraphPad Prism 10 (version 10.0.0). Data are expressed as mean $\pm$ standard error of the mean (SEM). Functional outcomes were analyzed using repeated-measures two-way analysis of variance (ANOVA), with time point as the within-subject factor and group as the between-subject factor, followed by Tukey's multiple comparisons test. For histological analyses, statistical significance was evaluated using one-way, two-way, or mixed-effects ANOVA, as appropriate, followed by Tukey's multiple comparisons test.



Chapter 2: Activity-based rehabilitation strategies promote functional recovery and spinal plasticity after thoracic spinal cord injury

Animals

52 adult female Sprague Dawley rats, aged 12 weeks and weighting between 250-300g were used in this study. Of them, 38 met the inclusion criteria and were retained for analyses. Animals were excluded from the study if: died postoperatively, SCI did not align with the defined parameters, or electrode failed prior to the completion of the experimental follow-up.

Experimental design

All animals received a standardized spinal cord contusion at the thoracic level T9 with an impact force of 200 kilodynes delivered by the Infinite Horizon impactor. In animals assigned to receive functional electrical stimulation, a prior surgical procedure was performed to implant intramuscular electrodes.

The animals were randomly assigned to one of four experimental groups:

- **Control group (n = 9):** Animals received the spinal cord lesion without any form of rehabilitation.
- **Treadmill rehabilitation group (n = 8):** Animals began physical rehabilitation using a motorized treadmill once they were able to stand unaided.
- **Functional electrical stimulation (FES) rehabilitation group (n = 9):** Animals underwent FES-based rehabilitation once they were able to stand, following the same functional criteria as the treadmill group.
- **Early FES + Treadmill group (n = 9):** FES rehabilitation was initiated three days post-injury, regardless of the animal's ability to stand. Once the animals could stand unaided, rehabilitation was continued using treadmill-based training.

All rehabilitation sessions were conducted for 1 hour per day, 5 days per week, over a period of 8 weeks following the injury. Rehabilitation protocols were standardized

across groups and administered according to fixed schedules. Animals were monitored regularly to assess functional recovery and to determine appropriate transitions in the rehabilitation protocol.

The animals were evaluated throughout the study with locomotor (BBB), sensory (Randall–Selitto), and electrophysiological tests, performed preoperatively and at 7, 14, 30, and 60 dpi. Additionally, a kinematic gait analysis on a treadmill was conducted before surgery and at 60 dpi to assess detailed locomotor patterns.

Electrode design

The electrodes were custom-fabricated by soldering six copper pins from a headplug (Plastic One Co) to multistrand tungsten/stainless steel wires (0.02" diameter) coated with PFA (total diameter 0,09"), each 25 cm in length. Prior to soldering, the PFA insulation was carefully removed from the contact region of the wires to ensure proper connection with the pins. The joints were then secured with epoxy and allowed to dry overnight. Electrode functionality was verified using a voltmeter, and the wires were labeled to facilitate identification during surgical procedures.

Surgeries

- Electrode implant

A midline incision was made along the scalp, together with a dorsal incision in the mid-back region and bilateral incisions in the hind limbs. The limb incisions allowed access to the quadriceps and gastrocnemius muscles. Subcutaneous tunnels were created between the cranial and limb incisions using blunt dissection to allow electrode wire routing.

Wires were passed subcutaneously from the cranial incision to each hind limb (three per limb), with the connector (pedestal/headplug) positioned on the skull. In each limb, electrodes were implanted in the gastrocnemius and quadriceps muscles. Briefly, a needle was used as a guide to position the electrode wire within the muscle, and a short segment of insulation was removed at the recording site to expose the conductive region. Electrodes were placed at the motor point and secured with a fine suture. A third wire, serving as a common anode, was de-insulated at its distal end and fixed subcutaneously near the knee. After electrode implantation, the incisions

were sutured, and the procedure was repeated on the contralateral limb. Electrode functionality was verified using a portable stimulator.

Finally, the electrode pedestal was secured to the skull with small screws, and fixed in place using dental cement, and the skin was sutured around the implant, leaving the connector exposed. The dorsal incision was then closed, ensuring proper positioning of the subcutaneous wires (Figure 10).

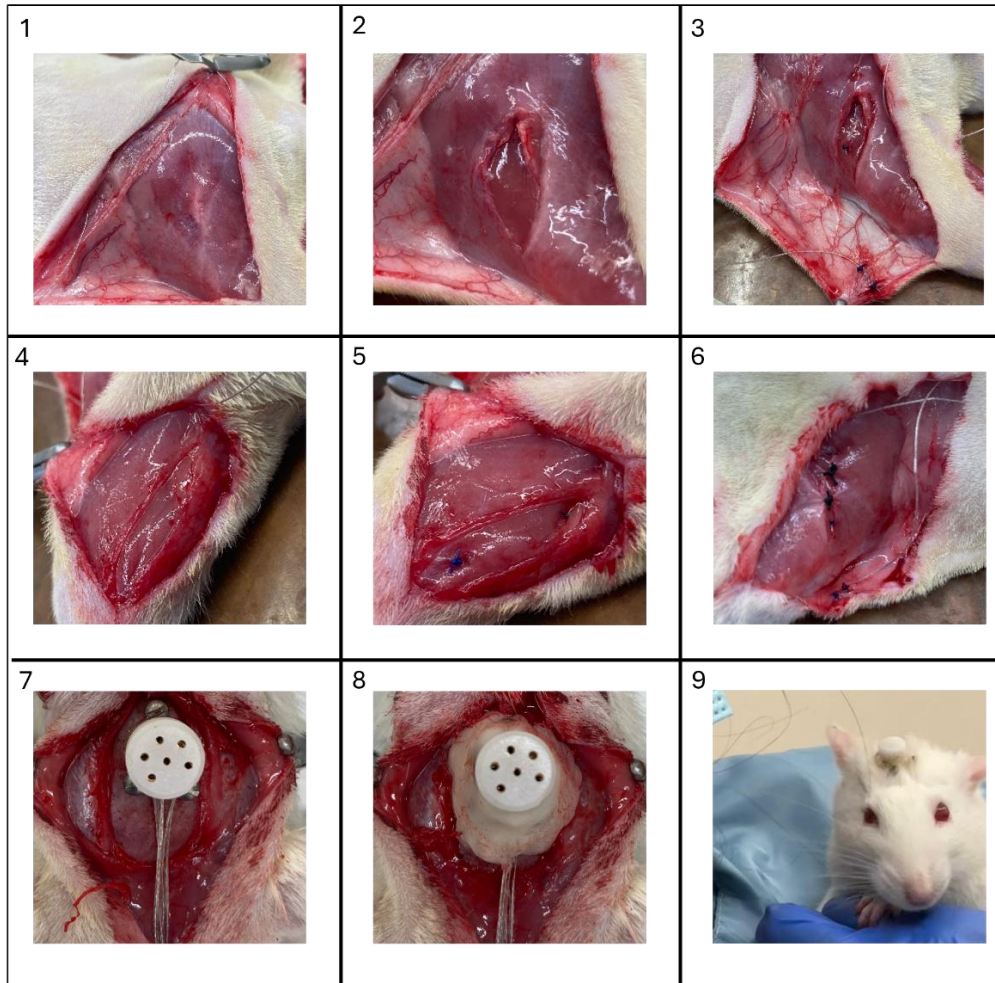


Figure 10. Electrode implantation surgery in the extensor muscles of the hind limbs. 1. Approach to the quadriceps muscle. 2. Exposure of the muscle. 3. Implantation of the intramuscular electrode and placement of the subcutaneous anode. 4. Exposure of the gastrocnemius muscle. 5. Intramuscular electrode implantation. 6. Closure of the musculature. 7. Placement of the headplate. 8. Fixation of the headplate using dental cement. 9. Skin suture and assessment of the animal condition.

- **SCI**

A 200kdynes contusive SCI was performed at T9. The spinal cord lesion procedure was conducted following the same protocol described in detail in Chapter 1, under the 'Surgeries' section.

Rehabilitation

- **Treadmill**

To ensure adaptation to the equipment, all animals underwent a preoperative habituation period to the treadmill apparatus (40cm x 10,3cm x 17,7cm, LE 8706, LSI Letica Scientific Instrument), during which they were trained to walk continuously for 30 minutes at the same speed (9 cm/s). This habituation aimed to reduce stress and facilitate smooth transition to the post-injury rehabilitation protocol. No aversive stimuli (e.g., electric shocks) were used at any point during habituation or rehabilitation. When necessary, gentle manual guidance was applied to support sustained locomotion.

Animals assigned to treadmill rehabilitation underwent training sessions consisting of 1 hour per day (two 30-minute walking periods separated by a 20-minute rest), 5 consecutive days per week, at a constant treadmill speed of 7-9 cm/s. Rehabilitation began once each animal in this group was able to stand unaided.

- **Functional electrical stimulation (FES)**

Animals assigned to the FES rehabilitation received stimulation for 1 hour per day, 5 days per week, while remaining inside their home cages. Electrical stimulation was delivered using a Multistim 8-channel programmable stimulator (Model 3800, A-M Systems). A biphasic stimulation train at 40Hz and at a voltage sufficient to elicit a visible muscle contraction, which ranged between 2–4 V across the time, was applied lasting 0.25 s duration. Each hindlimb received stimulation with an inter-train pause of 0.75 s, and a 0.25 s phase shift between the left and right hindlimbs was introduced to generate an alternating activation pattern of extensor muscles (quadriceps and gastrocnemius) in the hind limbs.

Functional outcome measures

Functional outcome measures were assessed using the same protocols described in Chapter 1. Briefly, locomotor function was evaluated using the open field test (BBB score), nociceptive response was assessed using the Randall–Selitto algesimetry test, and electrophysiological studies were performed, including H/M ratio, H-wave rate-dependent depression (RDD), and motor evoked potentials (MEPs). Detailed descriptions of these procedures are provided in the corresponding section of Chapter 1.

Histological assessment

Animals were perfused and the tissue was collected and processed following the same procedures described in Chapter 1. Briefly, after perfusion, spinal cord samples were carefully dissected, post-fixed, and prepared for subsequent histological analysis according to the previously established protocol. All steps, including tissue handling, preservation, and sectioning, were performed under the same conditions to ensure consistency across experiments. The staining and immunostaining procedures performed were as follows:

- **Cresyl violet staining:** used to quantify motoneuron preservation across lumbar segments.
- **Glial fibrillary acidic protein (GFAP):** used to assess astrocyte presence in both thoracic and lumbar regions, as well as to evaluate glial scar formation at the lesion site.
- **Ionized calcium-binding adapter molecule 1 (Iba1):** used to assess microglial presence in both thoracic and lumbar regions.
- **Potassium-chloride cotransporter 2 (KCC2):** used to evaluate synaptic inhibition mediated by GABAergic and glycinergic transmission.
- **Serotonin (5-HT):** used to assess serotonergic innervation in the lumbar spinal cord as a measure of neuroplasticity after injury.
- **Choline acetyltransferase (ChAT):** used to locate motoneuron in the ventral horn of lumbar spinal cord.

- **Vesicular glutamate transporter 2 (VGLUT2):** used to evaluate excitatory synaptic inputs.
- **Vesicular GABA transporter (VGAT):** used to evaluate inhibitory synaptic inputs and, together with VGLUT2, to assess the excitatory/inhibitory balance.

Table 2. Antibodies used for tissue immunostaining/immunofluorescence.

	<i>Antigen</i>	<i>Dilution</i>	<i>Host</i>	<i>Reference number</i>
<i>Primary antibody</i>	GFAP	1:400	Mouse	Sigma G3893
	Iba1	1:400	Chicken	Synaptic Systems 234 009
	KCC2	1:400	Rabbit	Millipore 07-432
	Serotonin 5-HT	1:2000	Rabbit	Inmunostar 20080
	VGlut2	1:1000	Guinea pig	Sigma AB2251-I
	VGAT	1:1000	Rabbit	Synaptic systems 131002
	ChAT	1:100	Goat	Millipore AB144P
<i>Secondary antibody</i>	Alexa Fluor 488	1:200	Donkey x Mouse	Life Technologies A21202
	Alexa Fluor 594	1:200	Goat x Chicken	Life Technologies A11042
	Alexa Fluor 647	1:200	Donkey x Rabbit	Abcam ab150075
	Biotinylated Rbt IgG	1:500	Rabbit	Vector BA-1000
	Alexa Fluor 488	1:200	Donkey x Rabbit	Life Technologies A 21206
	Cy3	1:200	Donkey x Guinea Pig	Jackson ImmunoResearch 706-165-148
	Alexa Fluor 488	1:200	Donkey x Goat	Life Technologies A 11055

Sections from the thoracic spinal cord region spanning the lesion were stained for GFAP and Iba1. In the lumbar region, the same markers were evaluated, together with Cresyl violet staining for motoneuron identification and immunostaining for KCC2, serotonin (5-HT), ChAT, VGLUT2, and VGAT.

For 5-HT immunostaining, sections from L2 and L5 were first rehydrated in PBS, permeabilized with 0,5% Triton X-100 in PBS, and subjected to antigen retrieval using boiling citrate buffer (10 mM citrate, pH 6,0) for 5 minutes at 90°C. After three washes, endogenous biotin was blocked using a commercial blocking kit

(ThermoFisher E-21390). Sections were then incubated in blocking solution containing 5% fetal bovine serum (FBS) and incubated with the primary antibody overnight at 4°C. The following day, sections were washed and incubated with a biotinylated anti-rabbit IgG for 2 hours at room temperature in blocking solution. After further washes, sections were incubated with the corresponding secondary reagent for 2 hours at room temperature and finally mounted using Fluoromount-G with DAPI (ThermoFisher, 00-4959-52).

For the remaining antibodies, immunostaining was performed following a standard protocol consisting of rehydration, permeabilization, blocking with 5% FBS, and overnight incubation with the primary antibody at 4°C in blocking solution. The following day, sections were washed, incubated with the appropriate secondary antibody for 2 hours at room temperature, washed again, and mounted using Fluoromount-G with DAPI (ThermoFisher, 00-4959-52).

Image processing

Images were acquired using a DS-Ri2 Nikon camera connected to an Eclipse Ni epifluorescence microscope (Nikon) at 4× magnification for Cresyl violet staining and at 10× magnification for GFAP, Iba1, and KCC2 immunostaining. Serotonin (5-HT), VGLUT2, VGAT, and ChAT images were captured using a Zeiss LSM 980 confocal microscope (PicoQuant), at 20× magnification for 5-HT and 25× immersion oil (37°C) for VGLUT2/VGAT/ChAT.

High-resolution images were obtained to assess lesion volume, motoneuron survival, astrocytic and microglial responses, synaptic inhibition through KCC2 expression, serotonergic plasticity, and excitatory/inhibitory balance based on VGLUT2 and VGAT expression around motoneurons. Lesion volume was evaluated by manually measuring the lesion area at 250 µm intervals along the rostrocaudal axis until the lesion was no longer detectable, using GFAP-immunostained sections. Motoneuron survival was assessed by manual counting following the same criteria described in Chapter 1.

Astrocytic and microglial responses, as well as KCC2 expression, were evaluated by measuring the integrated density of immunofluorescence with a manually adjusted threshold for each image. Serotonergic changes were assessed from 5-HT

immunostaining by generating maximum intensity projections (24µm depth) and measuring fluorescence density using the MaxEntropy threshold. For GFAP, Iba1, KCC2, and 5-HT analyses, measurements were performed in the dorsal and ventral horns of the L2 and L5 spinal cord segments using manually defined regions of interest (ROIs). For ventral horn analysis, image stitching was performed using ImageJ to cover the entire region of interest.

To assess the excitatory/inhibitory balance, confocal images of ChAT, VGLUT2, and VGAT immunostaining were processed by generating maximum intensity projections (16µm depth). Motoneurons were manually delineated based on ChAT immunostaining, and a 4 µm perisomatic band was generated around each cell. Fluorescence density for VGLUT2 and VGAT was then quantified within these regions using the Otsu threshold.

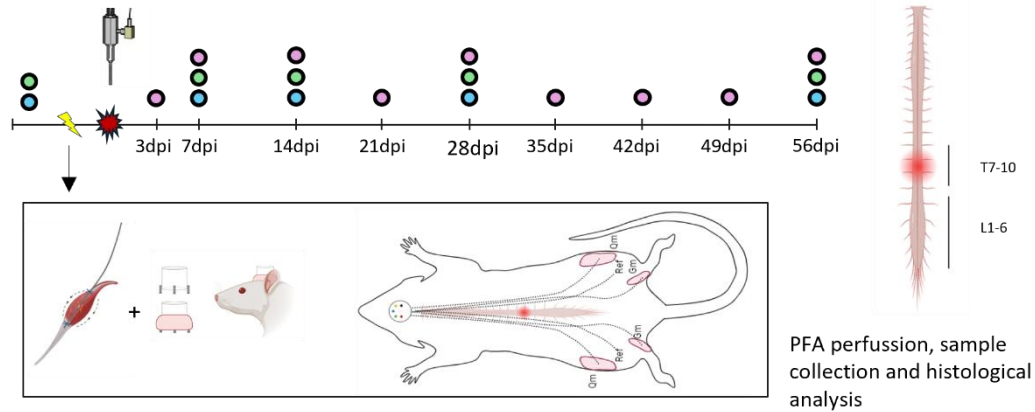
Statistical analyses

All data sets were analyzed using GraphPad Prism 10 (version 10.0.0). Data are expressed as mean $\pm$ standard error of the mean (SEM). Functional outcomes were analyzed using repeated-measures two-way analysis of variance (ANOVA), with time point as the within-subject factor and group as the between-subject factor, followed by Tukey's multiple comparisons test. For histological analyses, statistical significance was evaluated using one-way, two-way, or mixed-effects ANOVA, as appropriate, followed by Tukey's multiple comparisons test.

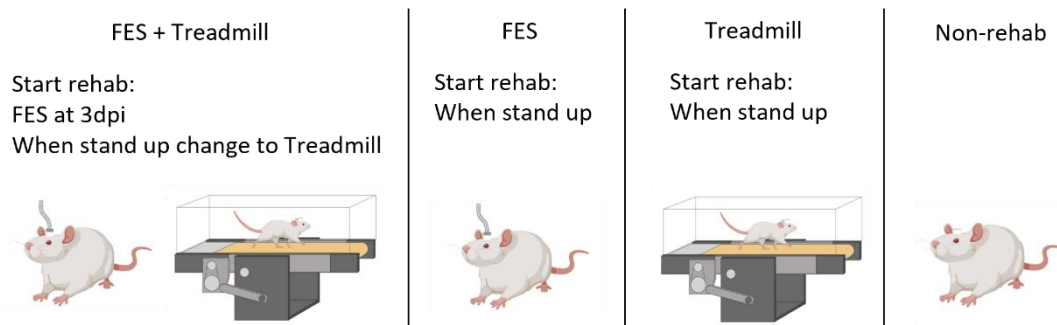
Chapter 2

RjHan:SD ♀ 12 weeks

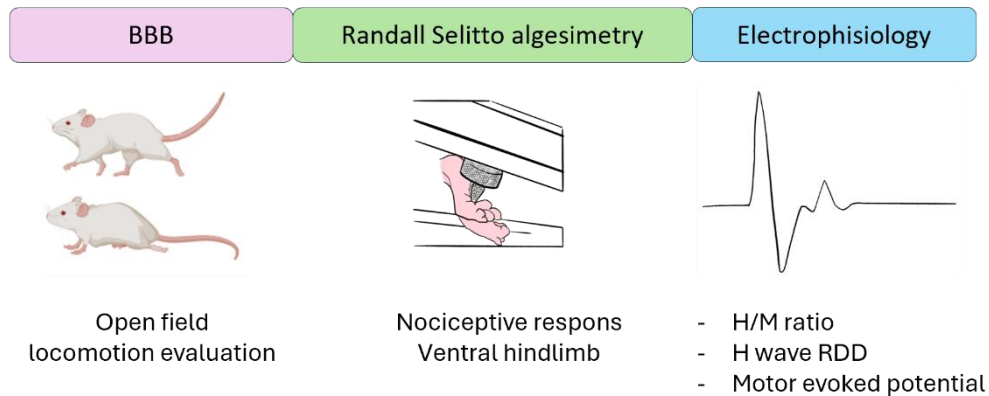
Experimental design



Experimental groups



Functional assessments



Histological evaluation



VI. Results

Chapter 1: *Severity-dependent functional and neurobiological alterations following thoracic spinal cord contusion injury*

The severity of the contusion determines the extent of spinal cord damage

To assess the extent of damage and tissue preservation across the different severities of SCI, we quantified the preserved tissue area and myelin content at the lesion epicenter and along the rostral and caudal affected spinal segments. This allowed comparing the magnitude of damage and tissue preservation between groups with different contusion force applied, and to relate the functionally observed deficits to the structural integrity of the spinal cord. The results are expressed as preserved tissue and myelin volumes, enabling evaluation of the progression of post-injury degeneration. In addition, the lesion cavity was characterized by delineating the GFAP labeled astrocytes surrounding the damaged area (Figure 11A).

At the lesion epicenter, the amount of preserved tissue was inversely related to the severity of injury: animals with the most severe lesion, 300 kdynes contusion, showed the largest volume of damaged tissue, followed by those with intermediate contusion of 200 kdynes, whereas animals with 100 kdynes contusion exhibited the largest preservation of spinal cord tissue. Consistently, analysis of total lesion volume revealed a progressive increase in damaged area with increasing contusion intensity (Figure 11B).

With respect to white matter, all groups showed a reduction of more than 50% of myelin labeling with FluoroMyelin at the lesion epicenter. White matter preservation was approximately 42% in animals with mild injuries, decreased to an average of about 21% in intermediate injuries, and was further reduced to around 8% in animals with the most severe lesions. Myelin loss was evident throughout the damaged segment, with a marked decrease in myelin signal in both central and perilesional regions (Figure 11C).

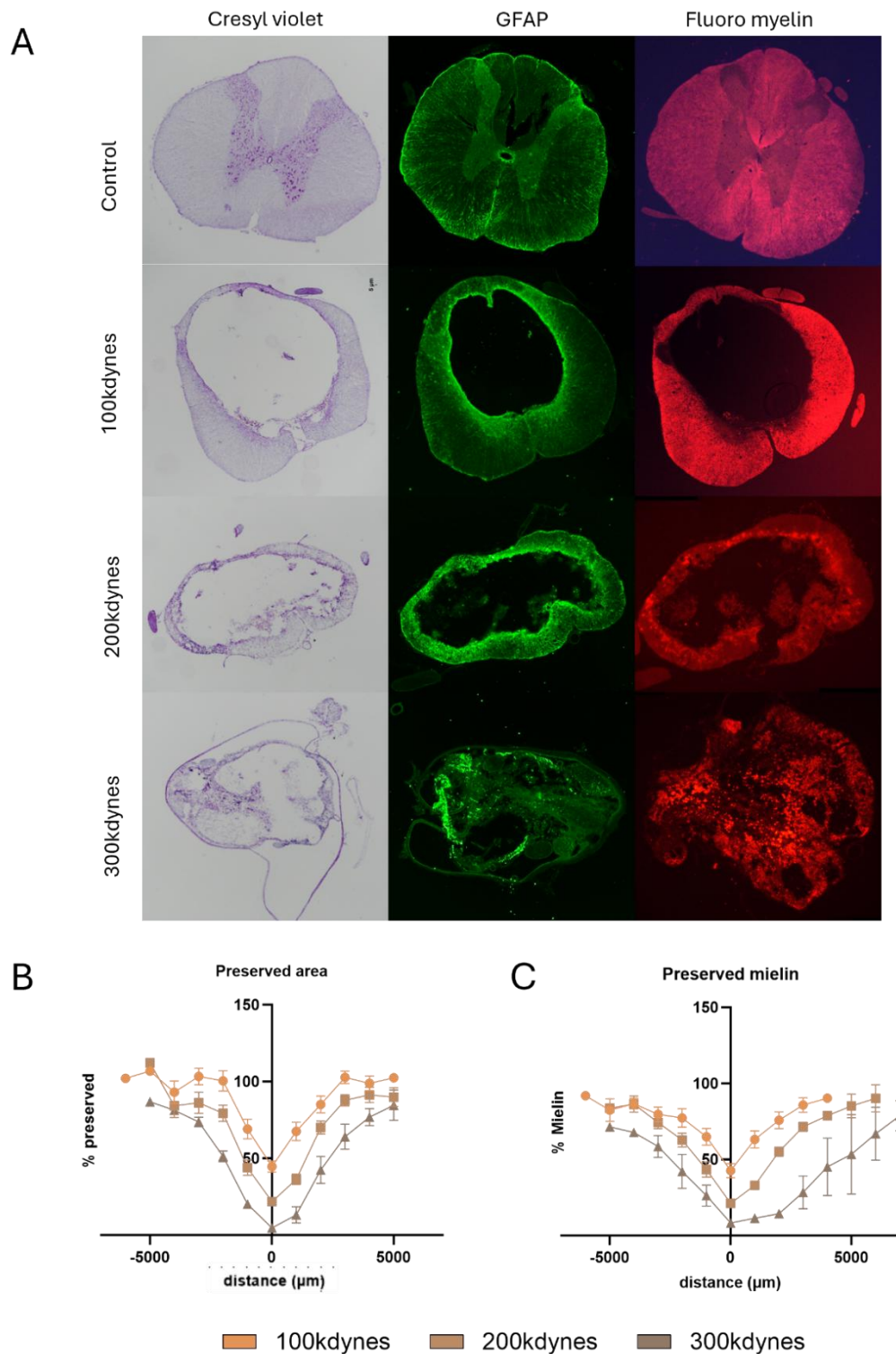


Figure 11. A. Representative images of the lesion epicenter from each experimental group. Cresyl violet staining is shown on the left, GFAP staining on the middle and FluoroMyelin staining on the right. B–C. Total preserved area (B) and preserved myelin (C) were quantified along the thoracic region from the lesion epicenter to the point where the lesion disappears, about 5 mm in both the caudal and rostral directions. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

More severe injuries induce higher locomotor deficits and lower functional recovery

The severity of the SCI was directly associated with the degree of hindlimb motor deficits observed throughout the study period. Animals exhibited progressively greater impairments as injury severity increased. The time required for animals to recover the ability to support their body weight after injury was evaluated. Animals with a 100 kdynes lesion either maintained weight-bearing ability from one day post-injury throughout the study. Animals with a 200 kdynes lesion required an average of 8 days to recover weight support, whereas those with the most severe injury (300 kdynes) required between 30 and 60 days, with a mean of 48 days (Figure 12A).

Locomotor recovery was evaluated using the Basso, Beattie, and Bresnahan (BBB) scale beginning at 3dpi and then weekly until the end of the study (Figure 12D). At 3dpi, animals with mild injury were able to support their weight, albeit with poor coordination. In contrast, animals with moderate and severe injuries exhibited only slight or inconsistent hindlimb movements, or no movement at all in the severe group (mean BBB scores: 100kdynes 10.71; 200kdynes 1.33; 300kdynes 0.29).

At 7dpi, all groups showed significant differences in locomotor performance. The moderate injury group displayed the most evident improvement, as animals were able to perform movements involving three joints, with minor movement in two of them (BBB scores: 100kdynes 11.71; 200kdynes 4.83; 300kdynes 0.64).

By the second week, locomotor improvement in the 100-kdyne group had stabilized for the remainder of the experiment. Although a slight improvement in coordination was observed, the BBB score increased only marginally from 11.86 at 14 dpi to 12.29 at 60dpi. In the 200kdynes group, animals began to recover partial weight support or showed markedly improved plantar placement of the hindpaws (BBB score: 8.91). Animals with the most severe injury exhibited limited hindlimb movements, such as extensive motion in a single joint (BBB score: 1.77).

At three weeks post-injury, further improvement was observed only in the moderate and severe groups. Animals with moderate injury displayed more consistent weight support, although coordination remained poor. Animals with severe injury showed

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extensive movements in all three hindlimb joints but were still unable to bear weight (BBB scores: 200kdynes 9.83; 300kdynes 7.35) (Figure 12D).

By one month post-injury, locomotor recovery in the 200kdynes group had plateaued, with BBB scores of 10.33 at 28 dpi and 10.75 at 56dpi, indicating weight support without coordination. In the 300kdynes group, at four weeks post-injury, animals exhibited extensive joint movements and occasional plantar stepping (mean BBB score: 7.71). During the second month, these animals slightly improved, with plantar placement becoming more consistent. By 56dpi, some animals were able to maintain static weight support (BBB score: 9.07).

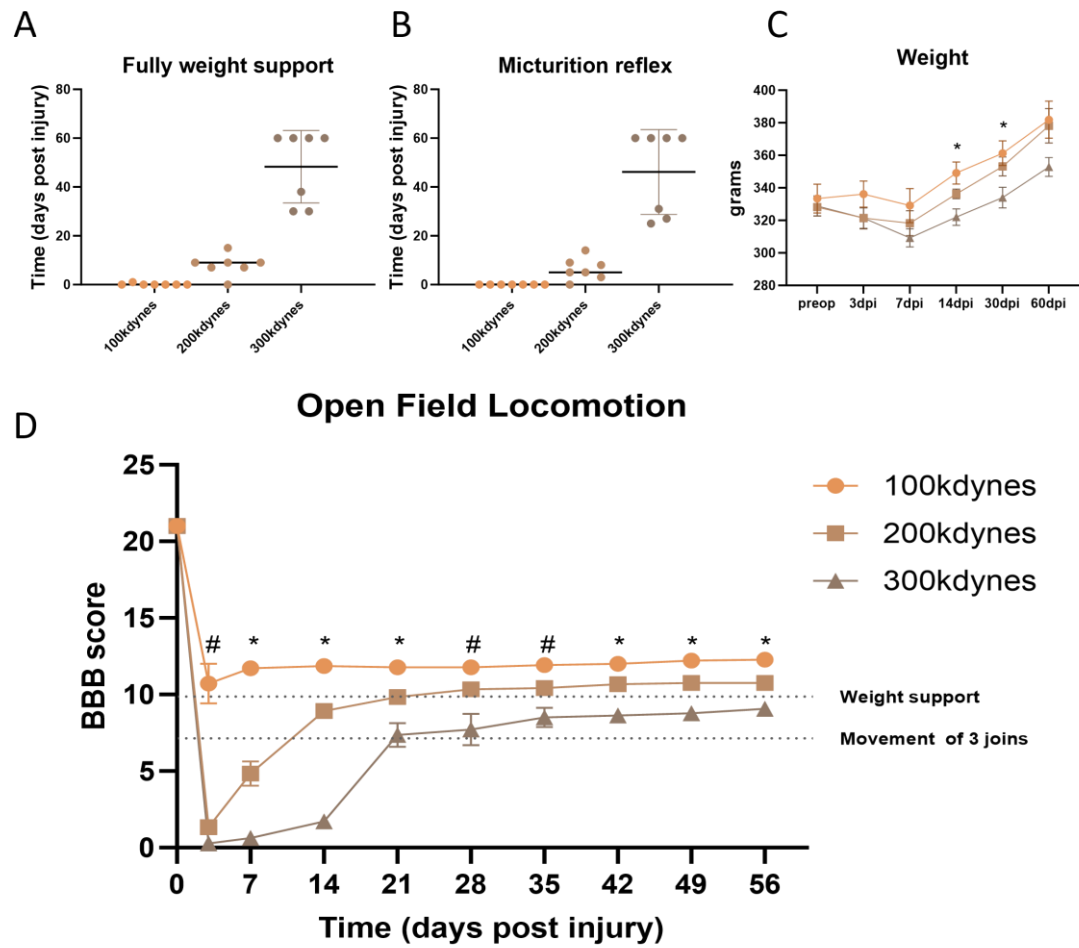


Figure 12: A. Day on which animals regained the ability to support their body weight. B. Day on which animals recovered autonomous micturition. C. Animals with more severe injuries show a greater reduction in weight. 14dpi 100kdynes vs. 300kdynes $P = 0.0203$; 30dpi 100kdynes vs. 300kdynes $P = 0.0422$. D. Locomotor performance evaluated using the BBB scale. Statistical comparisons: 3dpi 100kdynes vs. 200kdynes $P = 0.0006$; 100kdynes vs. 300kdynes $P = 0.0004$; 200kdynes vs. 300kdynes $P = 0.0591$. 7dpi 100kdynes vs. 200kdynes $P = 0.0003$; 100kdynes vs. 300kdynes $P < 0.0001$; 200kdynes vs. 300kdynes $P = 0.0064$. Statistical analysis was performed using two-way ANOVA for repeated measures, followed by Tukey's multiple comparisons test.

Body weight changes were also monitored throughout the study. In general, animals with more severe injuries showed greater weight loss than those with milder lesions during the first week. After SCI, rats in the 200- and 300 kdynes groups exhibited more pronounced decrease in body weight compared with the 100 kdynes group, although all animals progressively regained weight over the two-month follow-up. Significant differences were detected between the least and most severe injury groups at 14 dpi (100 kdynes: 349 g; 300 kdynes: 322 g) and 30 dpi (100 kdynes: 361 g; 300 kdynes: 334 g). This sustained lower body weight in the most severely injured animals is consistent with a greater degree of muscle mass loss (Figure 12B).

In addition, bladder function was also evaluated. Following SCI, animals with injuries of 200 and 300 kdynes lost micturition reflex, whereas those with 100 kdynes injury retained spontaneous micturition. The recovery of the micturition reflex occurred progressively and typically preceded the recovery of independent weight support by 1–5 days. Animals with severe injuries (300kdynes) that remained unable to support their body weight throughout the study did not recover the micturition reflex at any point and remained dependent on manual bladder expression for the two-month duration of the experiment (Figure 12C).

SCI induces persistent hyperalgesia dependent on severity of the injury

Randall–Selitto algosimetry was used to evaluate sensory function and hyperalgesia following SCI. All injured animals exhibited hyperreactivity to mechanical pressure stimuli applied to the ventral surface of the hindpaw, regardless of injury severity. This response was particularly pronounced in animals with more severe injuries,

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with pre-injury thresholds decreasing from $183 \pm 5\text{g}$ to $118,22\text{g}$ in the 100kdynes group, $109,29\text{g}$ in the 200kdynes group, and $63,77\text{g}$ in the 300kdynes group.

Throughout the two-month observation period, all groups showed partial normalization in their withdrawal response threshold; however, none reached pre-injury baseline levels. Comparison between groups revealed that animals with the most severe injury displayed greater hyperreactivity relative to those with mild or moderate injuries. At 14dpi, the pressure required to elicit a nociceptive response in the severely injured group was approximately half that observed in the mild and moderate injury groups (128g at 100kdynes, 120g at 200kdynes, and 68g at 300kdynes).

By one month post-injury, significant differences among all groups were observed, with the severely injured animals still being the most sensitive to the stimulus (88g), followed by the moderate (128g) and mild injury groups (155g). The data at 60dpi showed a similar trend, with mean thresholds of 144g in the 100kdynes group, 126g in the 200kdynes group, and 91g in the 300kdynes group (Figure 13).

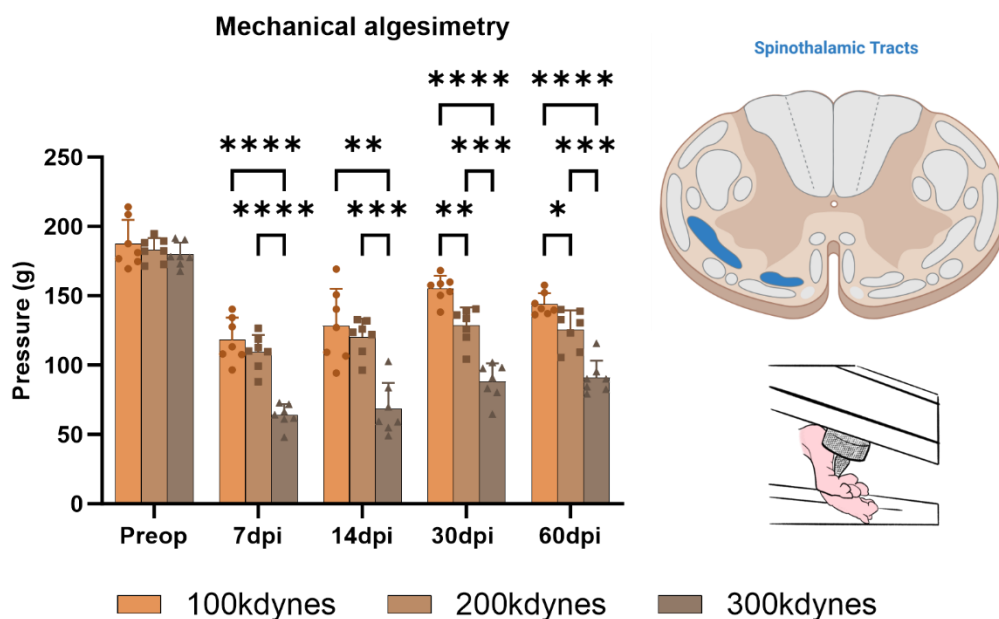
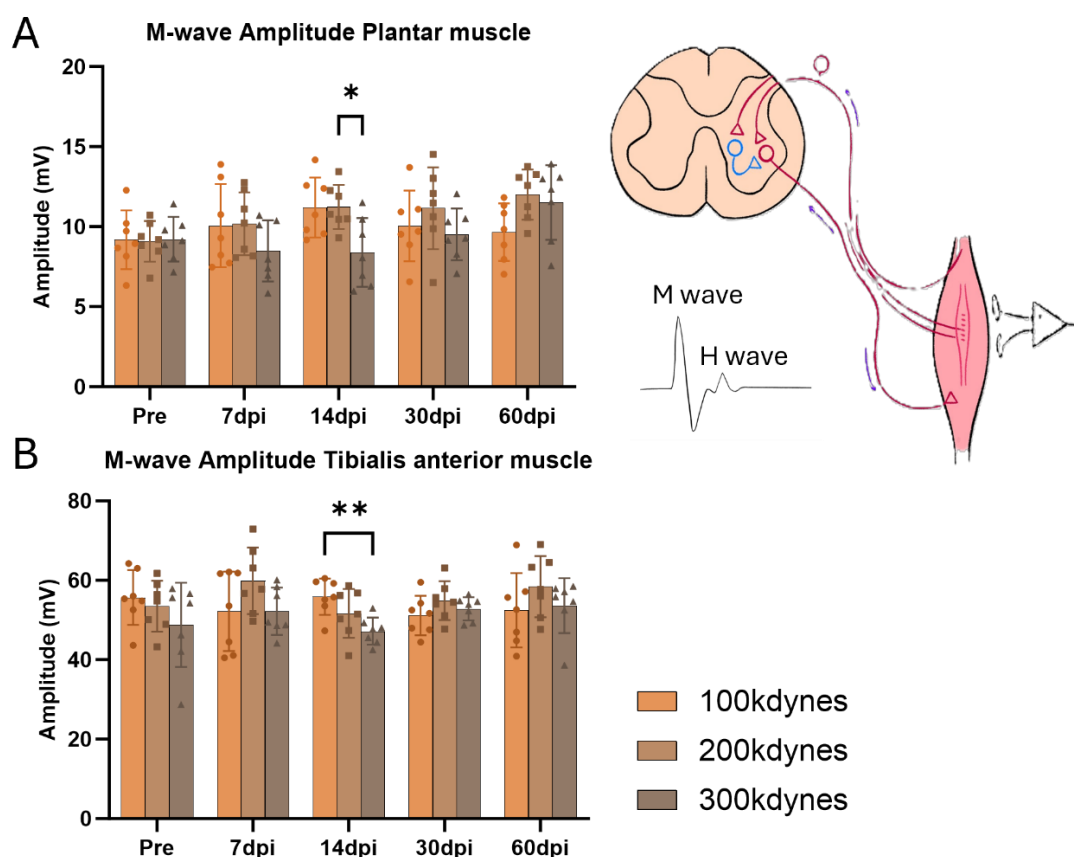


Figure 13. Assessment of mechanical hyperalgesia using the Randall–Selitto test. 7dpi 100kdynes vs 300kdynes $P < 0.0001$, 200kdynes vs 300kdynes $P < 0.0001$. 14dpi 100kdynes vs 300kdynes $P = 0.0015$; 200kdynes vs 300kdynes $P = 0.0003$. 30dpi 100kdynes vs 300kdynes $P < 0.0001$; 200kdynes vs 300kdynes $P = 0.0002$; 100kdynes vs 200kdynes $P = 0.0029$. 60dpi 100kdynes vs 300kdynes $P < 0.0001$; 200kdynes vs 300kdynes $P = 0.0009$; 100kdynes vs 200kdynes $P = 0.0323$. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

The amplitude of the M wave, but not the latency of the H wave, is affected by the severity of the injury

Electrophysiological recordings were performed mainly to evaluate spinal reflex activity following SCI. To assess the functional integrity of the neuromuscular unit, the amplitude of the M wave was examined in all groups after injury, in both the tibialis anterior and plantar interossei muscles. No significant differences in M-wave amplitude were observed between groups one week after injury. However, at 14dpi, slight differences became evident in both muscles. In the plantar muscle, a smaller M wave amplitude was observed in the group with the most severe injury compared with the other groups, although statistical significance was reached only when comparing the moderate and severe groups (Figure 14A). In the tibialis anterior, significant differences were found only between the severe and mild injury groups. At one and two months post-injury, no significant differences in M-wave amplitude were observed among groups in either muscle (Figure 14B).



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Figure 14. A–B. Graphical representation of M-wave amplitude recorded from the plantar and tibialis anterior muscles along the two-month study period. No significant differences between groups were observed, except at 14 dpi. In the plantar muscle, differences were detected between the 200 and 300 kdynes groups ($P = 0.0351$), with a tendency between the 100 and 300 kdynes groups ($P = 0.0563$). In the tibialis anterior muscle, significant differences were observed between the 100 and 300 kdynes groups at 14 dpi ($P = 0.0052$).

It is worth to note that the M wave amplitudes were not notably different than pre-operative control values, indicating preservation of the peripheral neuromuscular functionality.

Conduction velocity of the spinal circuit was further evaluated by analyzing the latency of the H wave. No significant differences in H wave latency were detected between groups throughout the follow-up. Nevertheless, in the plantar muscle a tendency was noted from 30dpi onward, with animals in the severe injury group showing longer H wave latency compared to those with mild injuries (100Kdynes vs. 300kdynes, 30dpi $P = 0.0747$; 60dpi $P = 0.0641$) (Figure 15).

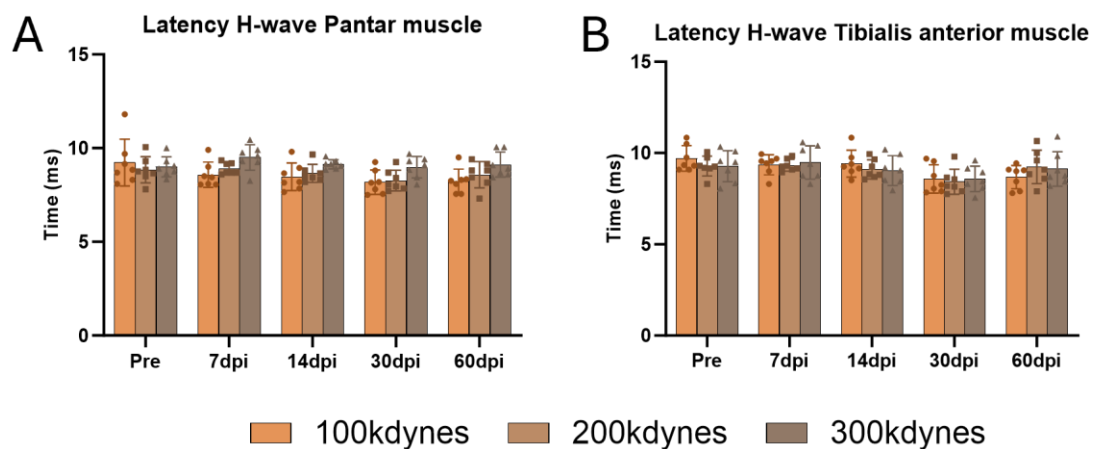


Figure 15: A–B. Graphical representation of H-wave latency following stimulation of the plantar and tibialis anterior muscles.

Increased H/M ratio indicates hyperreflexia after SCI

To evaluate the excitability of spinal reflex circuits, the H/M amplitude ratio was calculated for both plantar and tibialis anterior muscles. Already at 7dpi, a significant increase, about doubling, in the H/M ratio was observed in both muscles. Since no significant differences were detected in M-wave amplitude at this time point, this finding indicate that it is due to an increased H-wave amplitude.

Animals with more severe injuries presented significantly higher H/M ratio compared to those with mild injuries in the plantar muscle at one and two weeks postinjury (100kdynes: 31%; 300kdynes: 44%; $P < 0.05$). Similarly, in the tibialis anterior, values were about 7% in the mild injury group and 16% in the severe group ($P = 0.0092$).

No significant group differences were found at one or two months post-injury. In the plantar muscle, all SCI groups exhibited increased H/M ratios; at 30dpi (100kdynes = 41,38%; 200kdynes = 42,54%; 300kdynes = 46,30%) and at 60dpi (100kdynes = 41,63%; 200kdynes = 41,02%; 300kdynes = 47,96%) (Figure 16B).

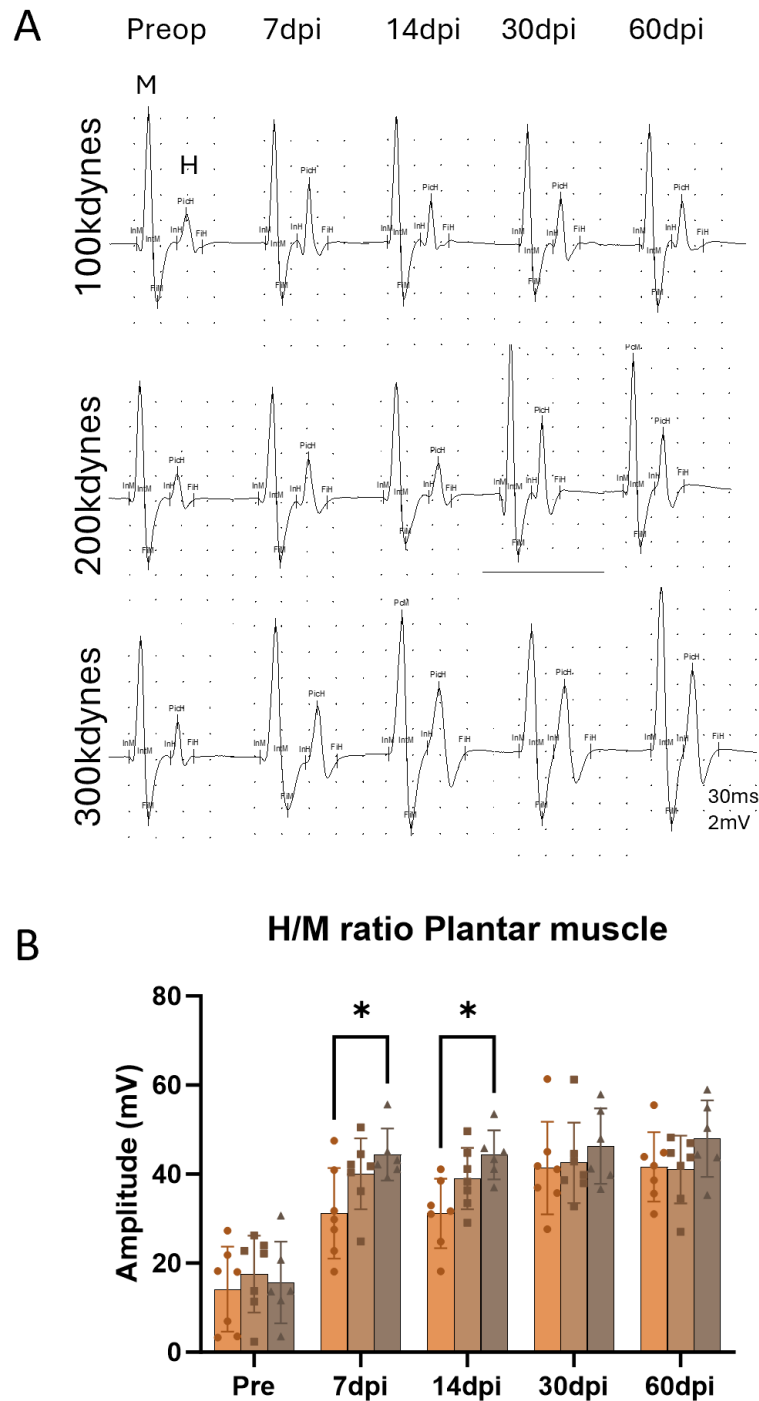
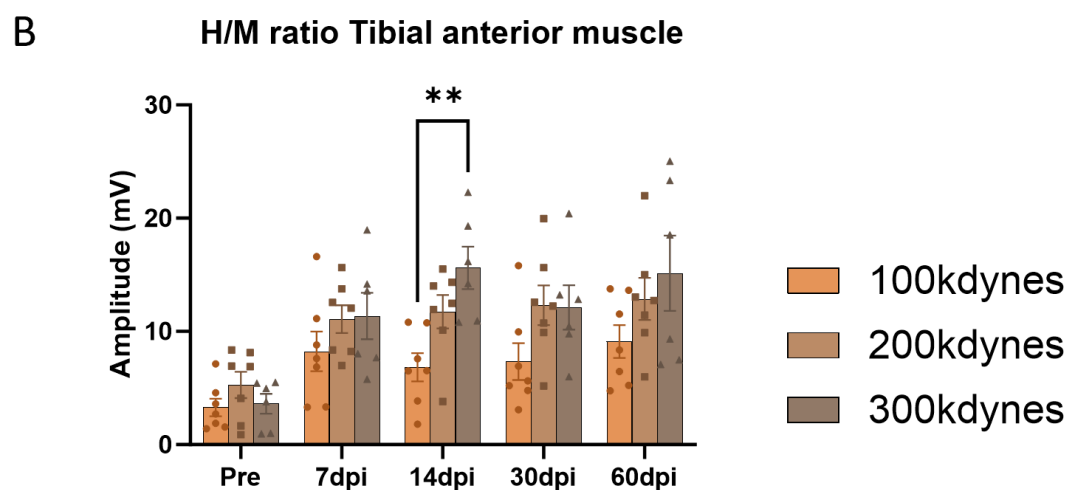
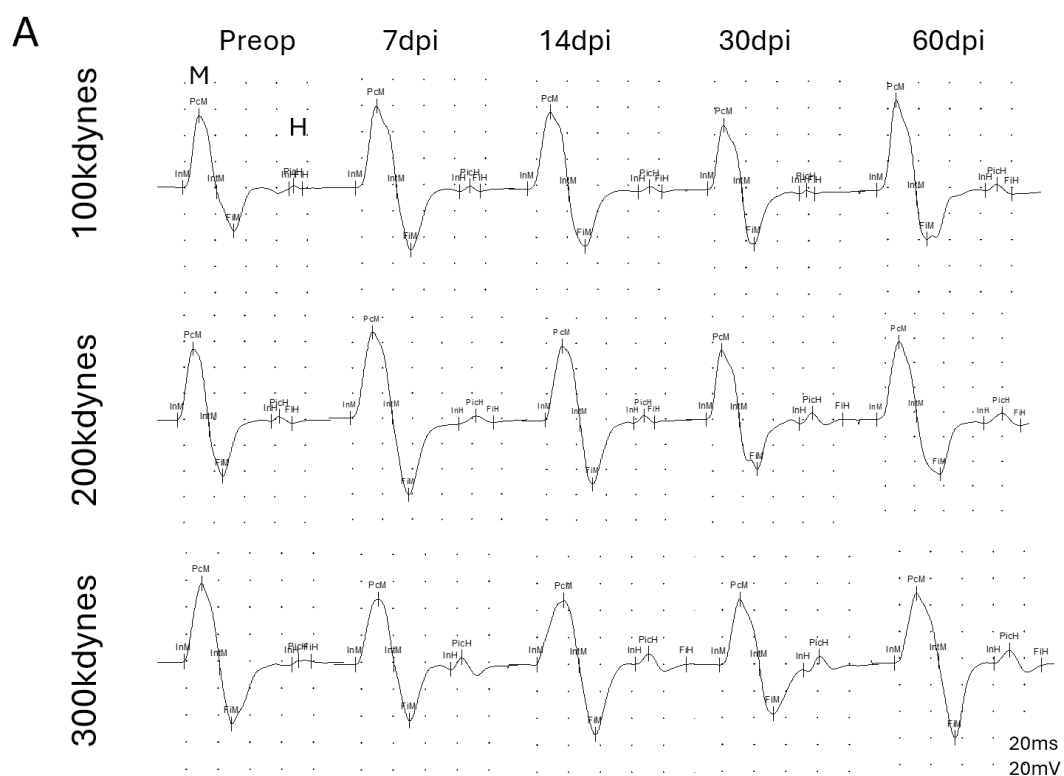


Figure 16: A. Representative electromyographic recordings of M- and H-waves in the plantar interosseus muscle. Following SCI, the H-wave amplitude increased whereas the M-wave remained relatively stable, resulting in an increased H/M ratio. This effect was more pronounced with increasing injury severity. B. H/M ratio in the plantar muscle. Significant differences were observed at 7 dpi (100 kdynes vs. 300 kdynes, $P = 0.0381$) and 14 dpi (100 kdynes vs. 300 kdynes, $P = 0.0122$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

In the tibialis anterior muscle (Figure 17A), a trend toward lower H/M ratios in the mild injury group compared to the other groups was observed, though the differences were not statistically significant at 30dpi (100kdynes = 7%; 200kdynes = 12%; 300kdynes = 12%) or at 60dpi (100kdynes = 9%; 200kdynes = 13%; 300kdynes = 15%) (Figure 17B),



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Figure 17: A. Representative electromyographic recordings of M- and H-waves in the tibialis anterior muscle. Following SCI, the H-wave amplitude increased whereas the M-wave remained relatively stable, resulting in an increased H/M ratio. This effect was more pronounced with increasing injury severity. B. H/M ratio in the tibialis anterior muscle. Significant differences were observed at 14 dpi (100 kdynes vs. 300 kdynes, $P = 0.0092$), with a tendency between the mild and moderate injury groups (100 kdynes vs. 200 kdynes, $P = 0.0651$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

The Rate-Dependent Depression of the H-Reflex Is Inversely Proportional to Injury Severity

To assess activity-dependent modulation of spinal reflex excitability, the rate-dependent depression (RDD) of the H-reflex was evaluated using repetitive nerve stimulation. In non-injured animals, repeated stimulation induced a marked reduction in H-reflex amplitude, consistent with intact inhibitory mechanisms. As shown by our results, stimulation at 1pps already induced an approximate 50% reduction in H-wave amplitude, while frequencies of 5pps and above produced reductions greater than 80%. In contrast, SCI animals exhibited a reduced H-reflex RDD, indicating impaired inhibitory control and increased excitability of the spinal reflex pathways (Figure 18A-B). In this study, RDD was analyzed at stimulation frequencies of 1pps, 2pps, 5pps, 10pps, and 20pps recording the H wave from both the plantar interosseus and tibialis anterior muscles.

In the plantar muscle, animals subjected to mild SCI (100 kdynes) had a reduction of approximately 50% in H-wave amplitude at 2pps during the acute phase after injury (7 and 14 dpi). However, over time, higher stimulation frequencies were required to achieve the same degree of depression, being 5pps at 30dpi and 10pps at 60 dpi.

Comparison between injury groups revealed that increasing injury severity was associated with a progressively higher impairment in RDD, requiring higher stimulation frequencies to achieve similar reductions in H-wave amplitude. At 7dpi, animals in the 200kdynes group required stimulation frequencies of 5pps, whereas animals in the 300kdynes group required frequencies of 10pps to achieve a 50% reduction in H-wave amplitude (Figure 18C). At 14 and 30dpi, differences between the moderate and severe injury groups became less pronounced, with both groups

requiring stimulation frequencies of approximately 10pps to achieve a comparable reduction (Figure 18D-E).

This impairment in RDD became more evident with time after SCI. At 60dpi, animals in the severe injury group did not achieve reductions greater than 50% until stimulation at 20pps. In contrast, animals in the moderate injury group showed 80% reduction at 20pps, but above 50% reduction at 10pps. Animals in the mild injury group exhibited reductions greater than 50% at 10 pps, indicating a comparatively better recovery of inhibitory modulation (Figure 18F).

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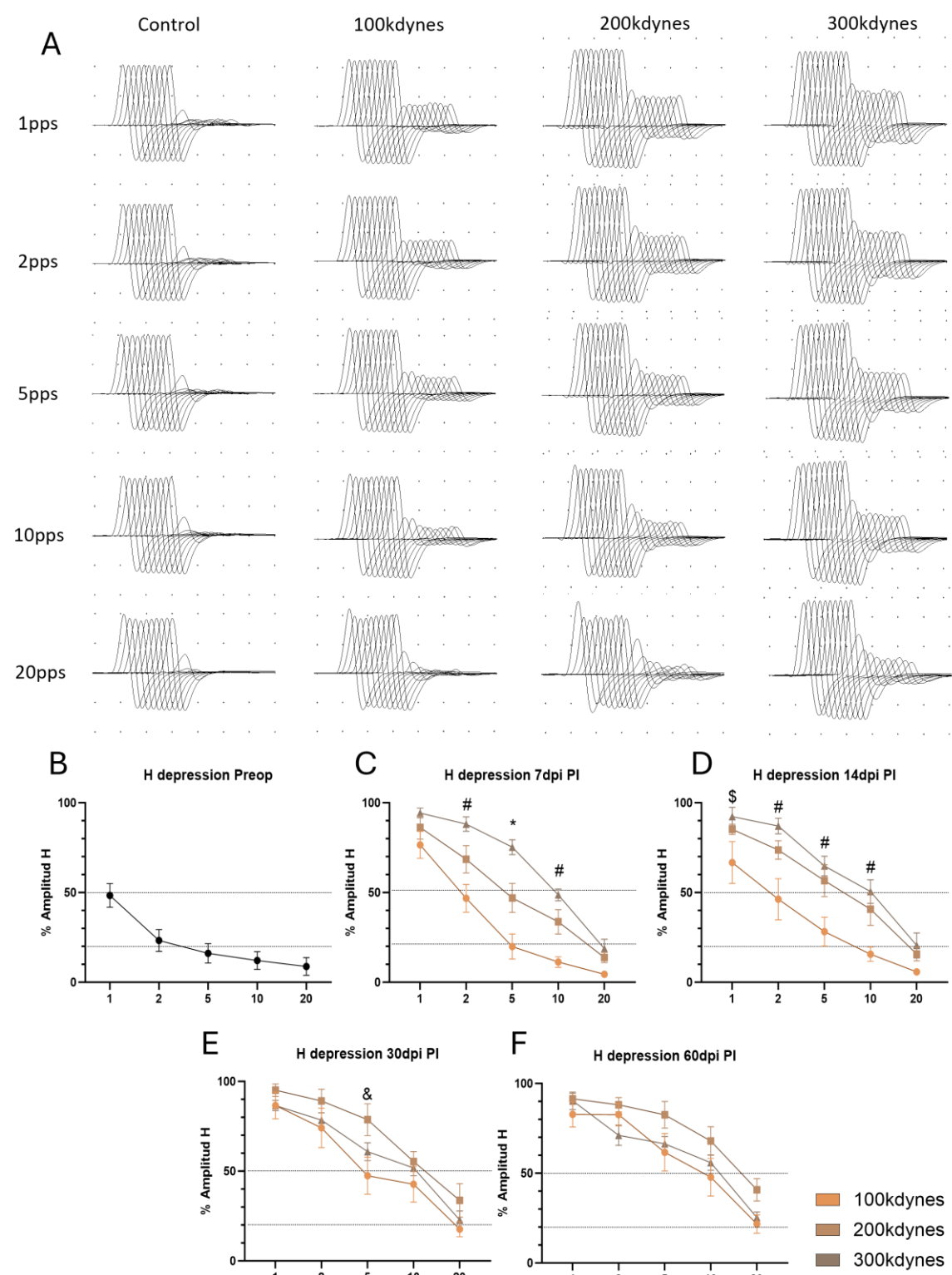


Figure 18: A. Representative recordings of M- and H-waves obtained from the plantar muscle at 60 dpi following delivery of 10 pulses at 1, 2, 5, 10, and 20 pps. in control group (uninjured) and SCI groups (100, 200, and 300 kdynes). B. Graph showing H-wave amplitude depression before SCI in all the animals, expressed as the ratio between amplitudes with the first and the last stimuli. C. H-wave amplitude depression at 7 dpi. Significant differences were observed at 2 pps (100 kdynes vs. 300 kdynes, $P < 0.0001$; 100 kdynes vs. 200 kdynes, $P = 0.0215$ (#)), 5 pps (100 kdynes vs. 300 kdynes, $P < 0.0001$; 100 kdynes vs. 200 kdynes, $P = 0.0030$; 200 kdynes vs. 300 kdynes, $P = 0.0029$ (*)), and 10 pps (100 kdynes vs. 300 kdynes, $P < 0.0001$; 100 kdynes vs. 200 kdynes, $P = 0.0171$ (#)). D. H-wave amplitude depression at 14 dpi. Significant differences were observed at 1 pps (100 kdynes vs. 300 kdynes, $P = 0.0360$ (\$)), 2 pps (100 kdynes vs. 300 kdynes, $P = 0.0004$; 100 kdynes vs. 200 kdynes, $P = 0.0168$ (#)), 5 pps (100 kdynes vs. 300 kdynes, $P = 0.0015$; 100 kdynes vs. 200 kdynes, $P = 0.0127$ (#)), and 10 pps (100 kdynes vs. 300 kdynes, $P = 0.0026$; 100 kdynes vs. 200 kdynes, $P = 0.0320$ (#)). E. H-wave amplitude depression at 30 dpi. Significant differences were observed at 5 pps (100 kdynes vs. 200 kdynes, $P = 0.0087$ (&)). F. H-wave amplitude depression at 60 dpi. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

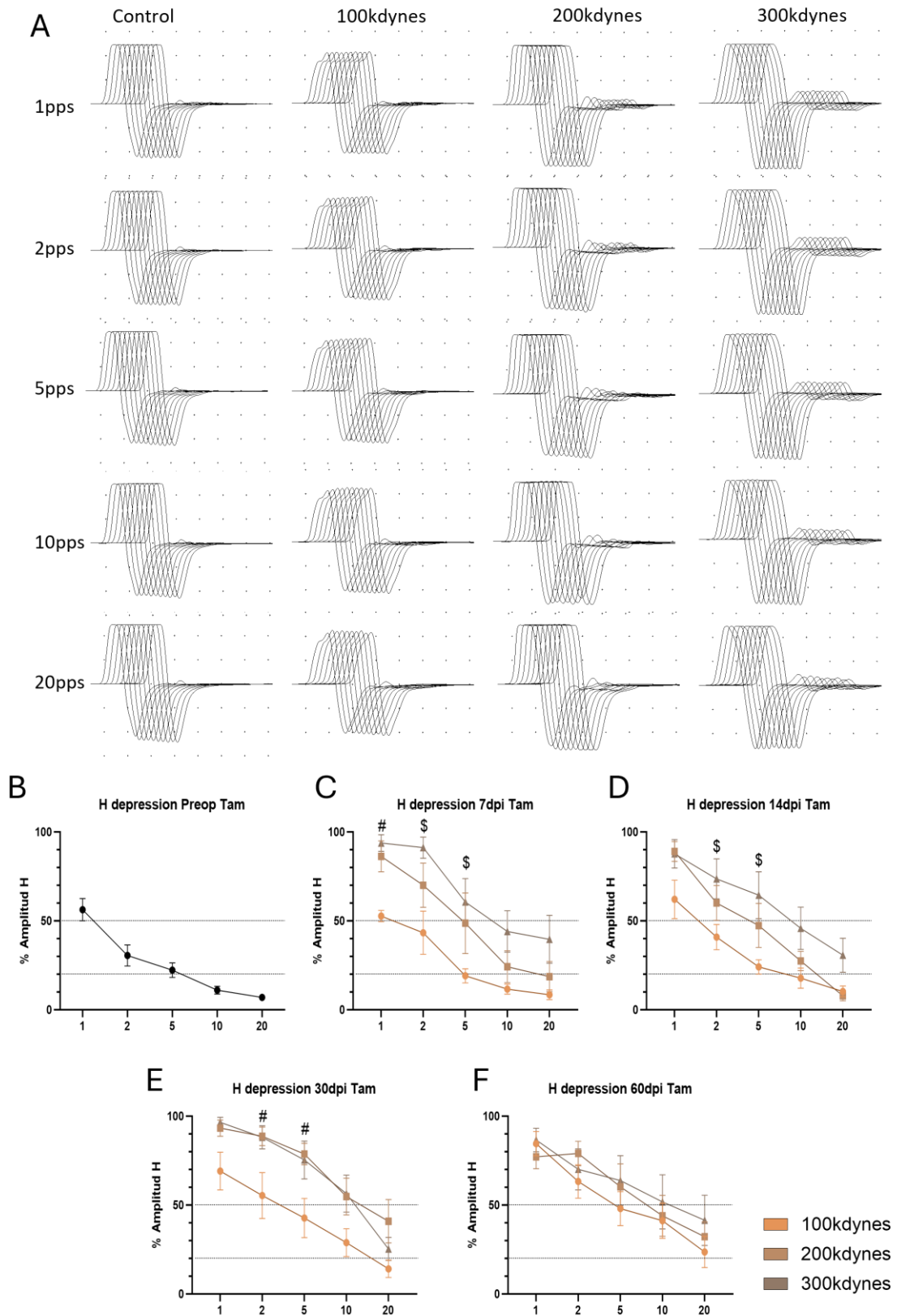
In the tibialis anterior muscle, intact rats showed also an approximately 50% reduction in H-wave amplitude at 1 pps and close to 80% at 5 pps, reflecting normal inhibitory modulation of the myotatic spinal reflex (Figure 19A-B). Animals with mild SCI (100 kdynes) displayed a similar pattern during the acute phase after injury (7 and 14 dpi) (Figure 19C-D). However, from 30dpi onwards, higher stimulation frequencies were required to achieve the same degree of depression, with frequencies of 5 pps needed to reduce H-wave amplitude by 50% (Figure 19E) and 20 pps required for reduction close to 80%. This pattern persisted at 60 dpi.

In animals with moderate and severe injuries (200 and 300 kdynes), the alterations were already evident during the acute phase after injury (Figure 19F). At 7 and 14 dpi, animals in the 200 kdynes group required stimulation frequencies of 5 pps, and animals in the 300 kdynes group required frequencies of 10 pps to achieve a 50% reduction in H-wave amplitude depression. Furthermore, while the 200 kdynes group achieved reductions greater than 80% at 20 pps, animals in the 300 kdynes group failed to reach this level of depression (Figure 19C-D). At 30 dpi, the differences between the moderate and severe injury groups became less pronounced. In both cases, frequencies of 10 pps were required to achieve reductions close to 50%. However, animals in the 200 kdynes group still exhibited a

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greater reduction at 20 pps (> 70%) compared to the 300 kdynes group (60%) (Figure 19E).

At 60 dpi, a similar trend persisted, with the severe injury group showing a response profile comparable to that of the moderate injury group. Although no statistically significant differences between groups were detected at this time point, animals in the mild injury group still achieved a 50% reduction in H-wave amplitude at 5 pps, whereas the moderate and severe injury groups required frequencies of at least 10 pps to reach the same degree of depression (Figure 19F).



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Figure 19: A. Representative recordings of M- and H-waves recorded from the tibialis anterior muscle at 60 days post-injury (dpi) following 10 pulses delivered at 1pps, 2pps, 5pps, 10pps and 20pps. *i*, control group (uninjured); *ii-iv*, injury groups (100, 200, and 300 kdyne). B. H-wave amplitude depression before SCI. Ratio between the first and last pulse amplitudes. C. Comparative graph at 7dpi. 1pps 100kdynes vs 300kdynes ($P= 0.0096$); 100kdynes vs 200kdynes ($P= 0.421$) (#). 2pps 100kdynes vs 300kdynes ($P= 0.0021$) (\$); 5pps 100kdynes vs 300kdynes ($P= 0.0088$) (\$); 10pps 100kdynes vs 300kdynes ($P=0.0517$); 20pps 100kdynes vs 300kdynes ($P= 0.0641$). D. Comparative graph at 14dpi. 2pps 100kdynes vs 300kdynes ($P= 0.0248$) (\$); 5pps 100kdynes vs 300kdynes ($P= 0.0042$) (\$). E. Comparative graph at 30dpi. 2pps 100kdynes vs 300kdynes ($P=0.0248$); 100kdynes vs 200kdynes ($P= 0.0219$) (#). 5pps 100kdynes vs 300kdynes ($P= 0.0254$); 100kdynes vs 200kdynes ($P= 0.0122$) (#). F. Comparative graph at 60dpi. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

Corticospinal excitability recovers only in mild SCI

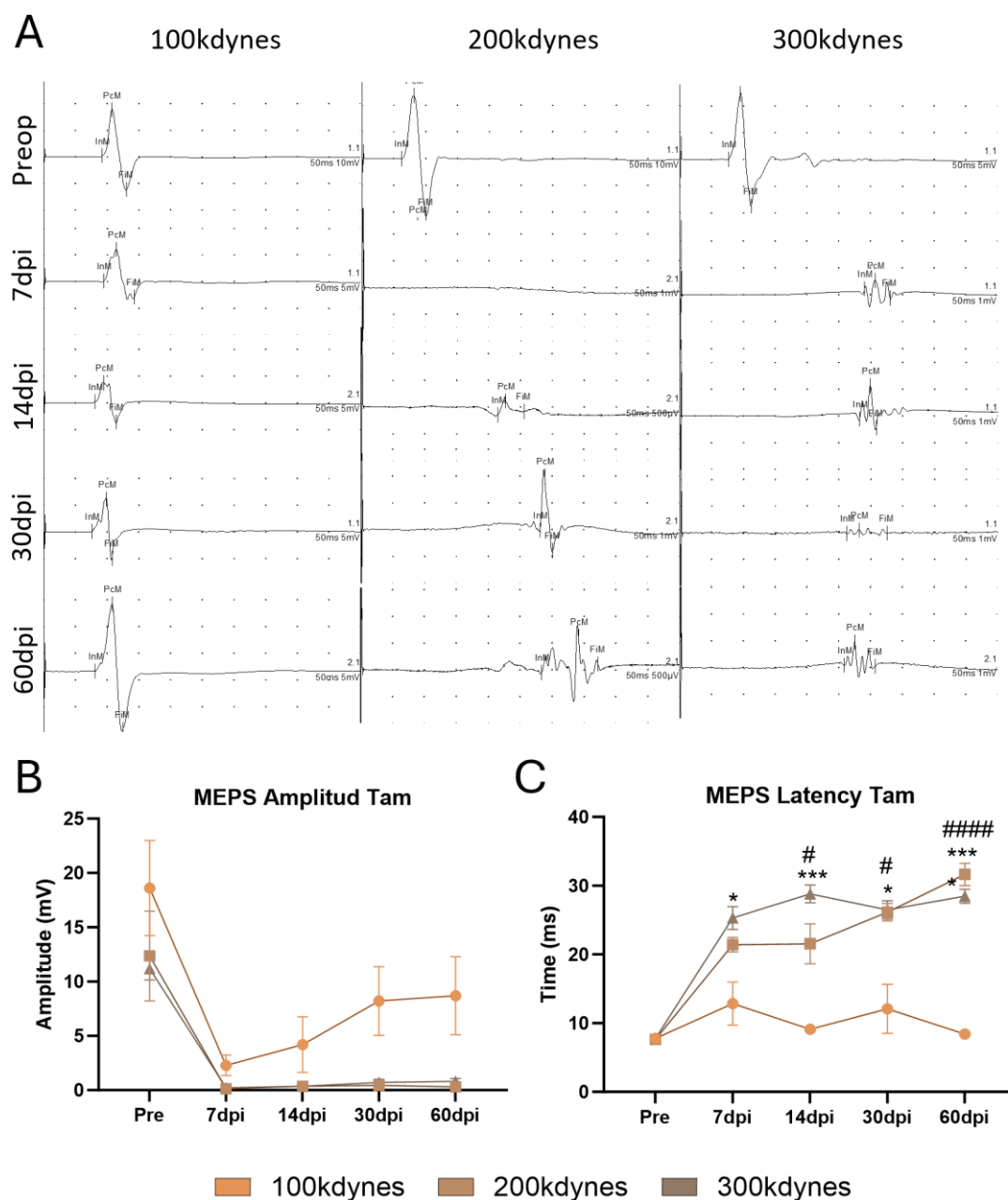
To evaluate corticospinal excitability and functional recovery following SCI, motor evoked potentials (MEPs) were recorded in animals before surgery and at 7, 14, 30, and 60dpi. Activity was recorded from the tibialis anterior muscle, allowing analysis of distal motor responses in relation to injury severity and applied rehabilitation strategies (Figure 20A).

MEPs were primarily quantified in terms of amplitude and latency, using pre-injury measurements as a baseline reference for each animal. This approach enabled the assessment of temporal changes in corticospinal excitability and the progression of motor function throughout the post-injury period.

Regarding MEP amplitude, a marked decrease was observed in all SCI groups at 7 dpi (mean <2.3 mV). Throughout the study, only the mild injury group showed a progressive recovery toward pre-surgical values (100kdynes: mean at 14dpi 4.2mV; 30dpi 8.2mV; 60dpi 8.7mV; 200kdynes and 300kdynes: mean at 14, 30, and 60 dpi <1 mV) (Figure 20B).

When examining the latency, differences between groups were even more pronounced. Following injury, latency increased in all groups, with greater increase observed in more severe injuries (7dpi: 100kdynes 12.9ms; 200kdynes 21.4ms; 300kdynes 25.3ms). These differences persisted up to 30 dpi, where the 200kdynes and 300kdynes groups showed similar values (30dpi mean: 100kdynes 12.1ms;

200kdynes 26.2ms; 300kdynes 26.5ms). This difference between the mild injury group and the other two groups was maintained until the end of the study, with the mild injury group reaching latency values close to pre-injury levels (60dpi mean: 100kdynes 8.4ms; 200kdynes 31.6ms; 300kdynes 28.5ms) (Figure 20C).



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Figure 20. A. Representative motor evoked potential recordings from the tibialis anterior muscle for each SCI severity group over the two-month study period. B. Graphical representation of MEP amplitude before injury and at 7, 14, 30, and 60 dpi. No statistically significant differences were observed between groups. C. Graphical representation of MEP latency. 7 dpi: 100 kdynes vs 300 kdynes ($P=0.0299$) (*); 14 dpi: 100 kdynes vs 300 kdynes ($P=0.0001$) (***), 100 kdynes vs 200 kdynes ($P=0.0270$) (#); 30 dpi: 100 kdynes vs 300 kdynes ($P=0.028$) (*), 100 kdynes vs 200 kdynes ($P=0.0308$) (#); 60 dpi: 100 kdynes vs 300 kdynes ($P<0.0001$) (****), 100 kdynes vs 200 kdynes ($P<0.0001$) (#####). Statistical analysis was performed using a mixed-effects analysis followed by Tukey's multiple comparisons test.

Histological evaluation

Motoneuron survival in the lumbar region decreases after injury and is dependent on injury severity

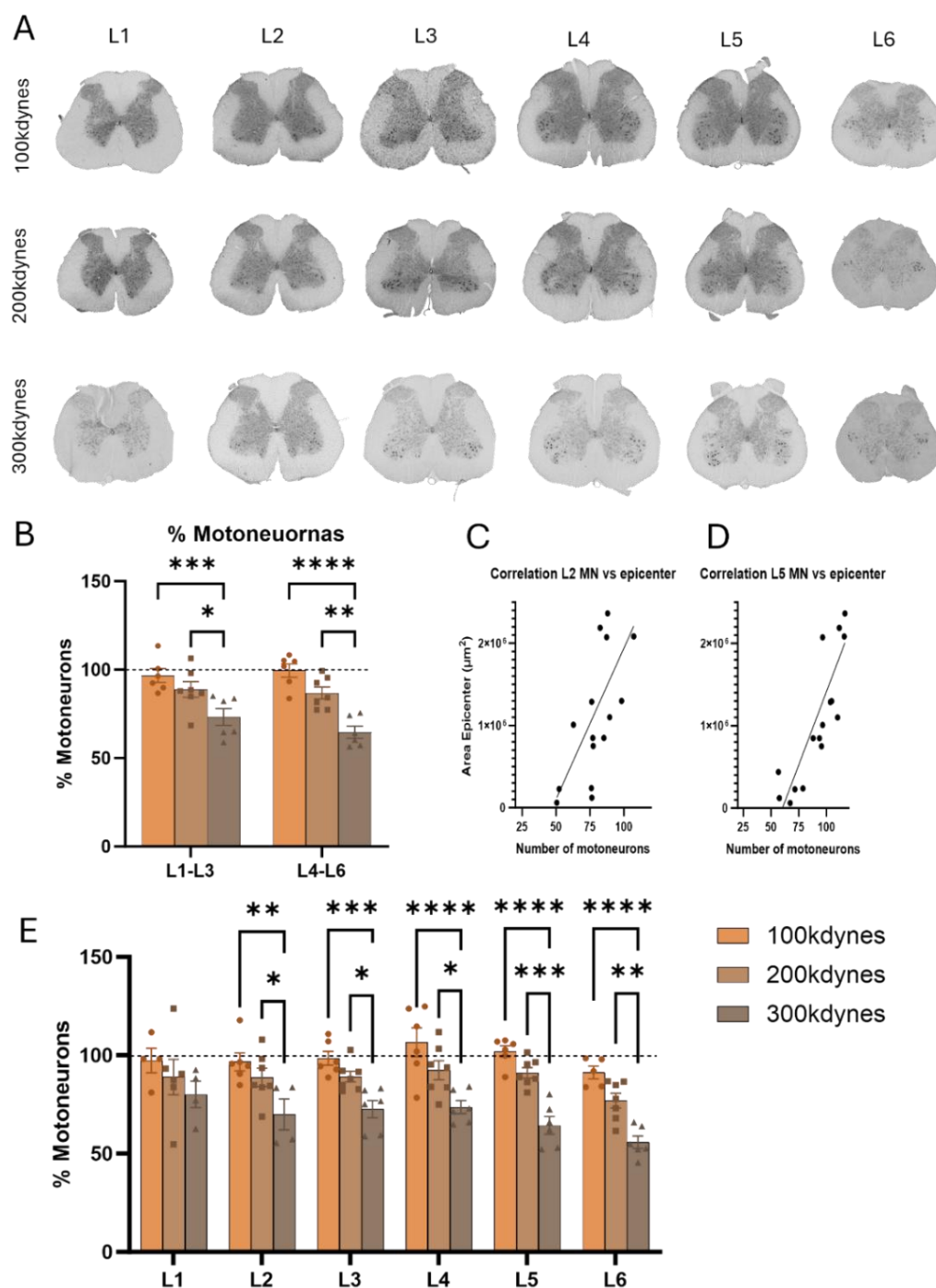
To evaluate neuronal survival following SCI, motoneurons were quantified along the lumbar spinal cord for characterizing the degree of neuronal loss associated with each level of injury severity. Quantification was performed along the entire lumbar axis, enabling analysis of motoneuron distribution in relation to the distance from the lesion epicenter (Figure 21A).

Motoneuron quantification revealed a progressive decrease in the percentage of motoneurons as injury severity increased. At all the analyzed cord segments, the mild injury group showed the highest motoneuron counts, followed by the intermediate group, while the severe injury group exhibited the lowest values (Figure 21B, E).

This pattern was consistently observed across the different lumbar levels, although differences between groups were more pronounced in segments L4–L6 (Figure 21B). In these regions, statistically significant differences were observed between groups, particularly between animals in the 100kdynes and 300kdynes groups, as well as between the 200kdynes and 300kdynes groups.

In more rostral segments (L1–L2), although a similar trend was observed, differences between groups were less marked, probably because there are lower number of motoneurons than at L4–L6 segments. Overall, these results indicate a

direct relationship between injury severity and motoneuron loss, with a greater impact on the more caudal lumbar segments (Figure 21 B-E).



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Figure 21. A. Representative Cresyl violet-stained images of the lumbar spinal cord from all experimental groups. B. Quantification of the number of ventral horn motoneurons in each lumbar segment. Statistically significant differences were observed between the most severe injury group and the other groups at L2 (100kdynes vs 300kdynes, $P=0.002$; 200kdynes vs 300kdynes, $P=0.0328$), L3 (100kdynes vs 300kdynes, $P=0.0007$; 200kdynes vs 300kdynes, $P=0.0349$), L4 (100kdynes vs 300kdynes, $P<0.0001$; 200kdynes vs 300kdynes, $P=0.014$), L5 (100kdynes vs 300kdynes, $P<0.0001$; 200kdynes vs 300kdynes, $P=0.0003$), and L6 (100kdynes vs 300kdynes, $P<0.0001$; 200kdynes vs 300kdynes, $P=0.0049$). C–D. Statistically significant correlations were found between lesion epicenter area and the number of motoneurons at L2 ($P=0.0059$) and L5 ($P<0.0001$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

The most severe lesions show a tendency towards more marked glial activation and lower KCC2 expression

To characterize the cellular and molecular changes associated with SCI, the immunoreactivity density of the markers GFAP, Iba1, and KCC2 were assessed in the lumbar segments L2 and L5. At each of these levels, the dorsal and ventral horn regions were evaluated separately. This approach enabled comparison of signal distribution across anatomical regions and experimental groups, providing insight into astrocytic activation (GFAP), microglial response (Iba1), and neuronal expression changes associated with synaptic inhibition (KCC2).

Regarding the astrocytic response, the results show similar integrated density values across the three groups in both lumbar segments and in both dorsal and ventral regions. In the latter case, some individual values suggest an increasing trend in the groups with greater injury severity; however, these differences did not reach statistical significance (Figure 22).

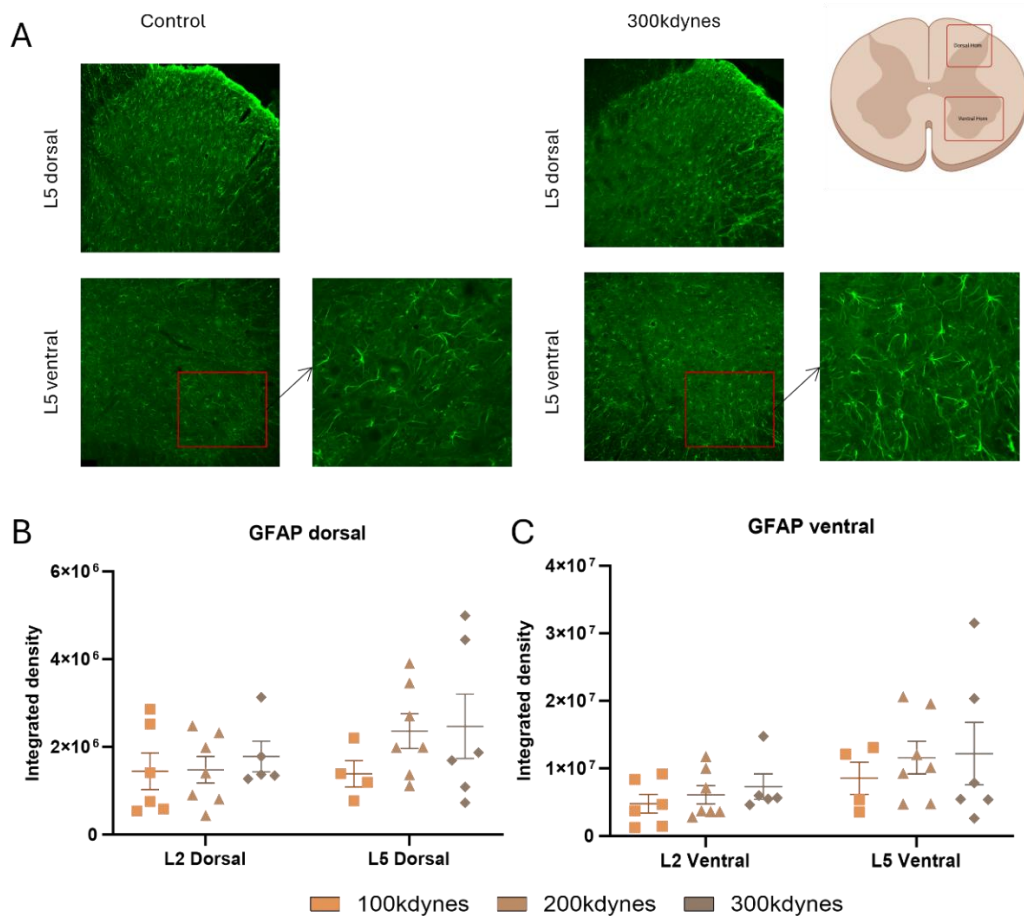


Figure 22. A. Representative images from sham and SCI 300 kdynes experimental rats of the dorsal and ventral horns at L5, showing astrocyte staining with the GFAP antibody. B–C. Quantification of integrated density of immunofluorescence in the dorsal (B) and ventral (C) horns of L2 and L5 spinal cord segments in the three SCI groups. No significant differences were found by two-way ANOVA.

With respect to the microglial response, the results suggest a greater activation with increasing injury severity across all regions and segments analyzed. However, due to intragroup variability, no statistically significant differences were observed (Figure 23).

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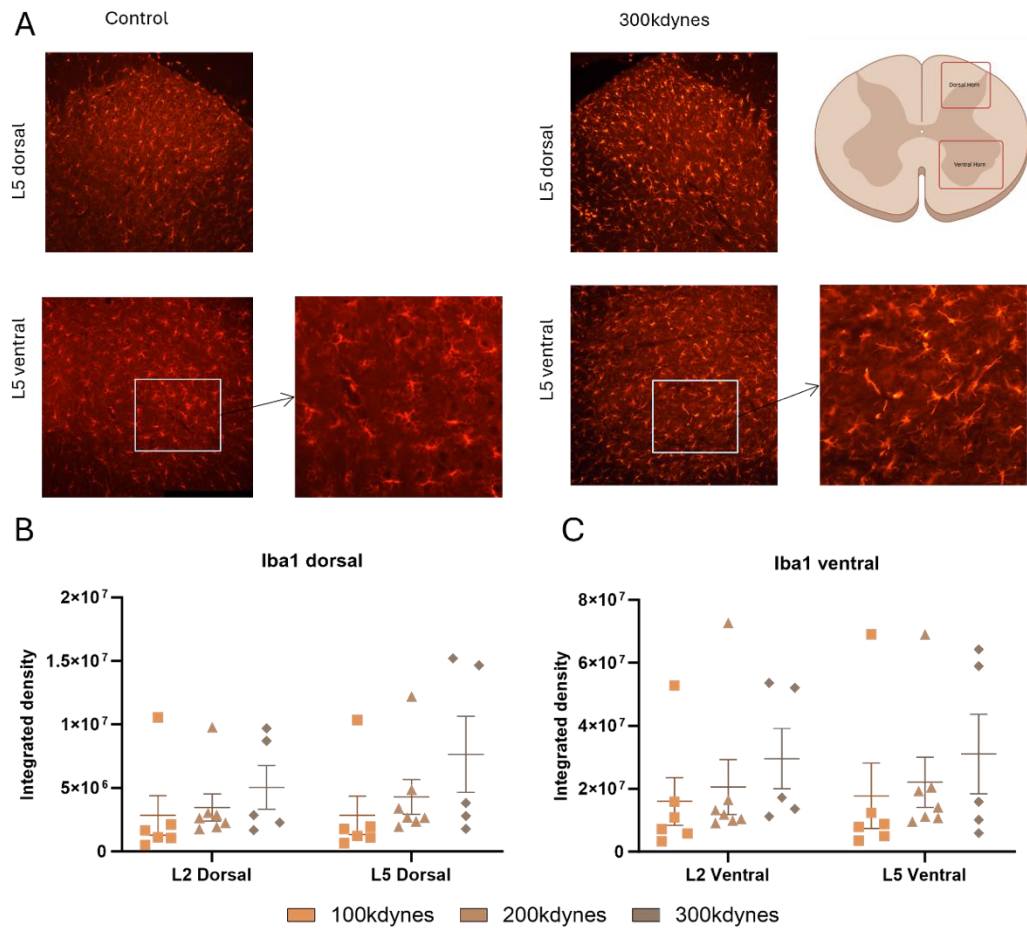


Figure 23. A. Representative images from sham and 300 kdynes SCI rats of the dorsal and ventral horns at L5, showing microglia stained with the iba1 antibody. B–C. Quantification of integrated density of immunofluorescence in the dorsal (B) and ventral (C) horns at L2 and L5 segments of the spinal cord in the three SCI groups. No significant differences were found by two-way ANOVA.

When examining changes associated with the KCC2 cotransporter, the results showed a lower signal presence for this marker in animals with more severe injuries. Although these differences were not statistically significant, they were more evident in the L2 region, which is closer to the lesion, and followed an inverse pattern, such that greater injury severity was associated with lower immunofluorescence density for KCC2 (mean L2 dorsal: 100kdynes 5.67651×10^6 ; 200kdynes 5.13225×10^6 ; 300kdynes 3.93982×10^6 ; ventral: 100kdynes 2.97897×10^7 ; 200kdynes 2.8621×10^7 ; 300kdynes 1.95031×10^7) (Figure 24).

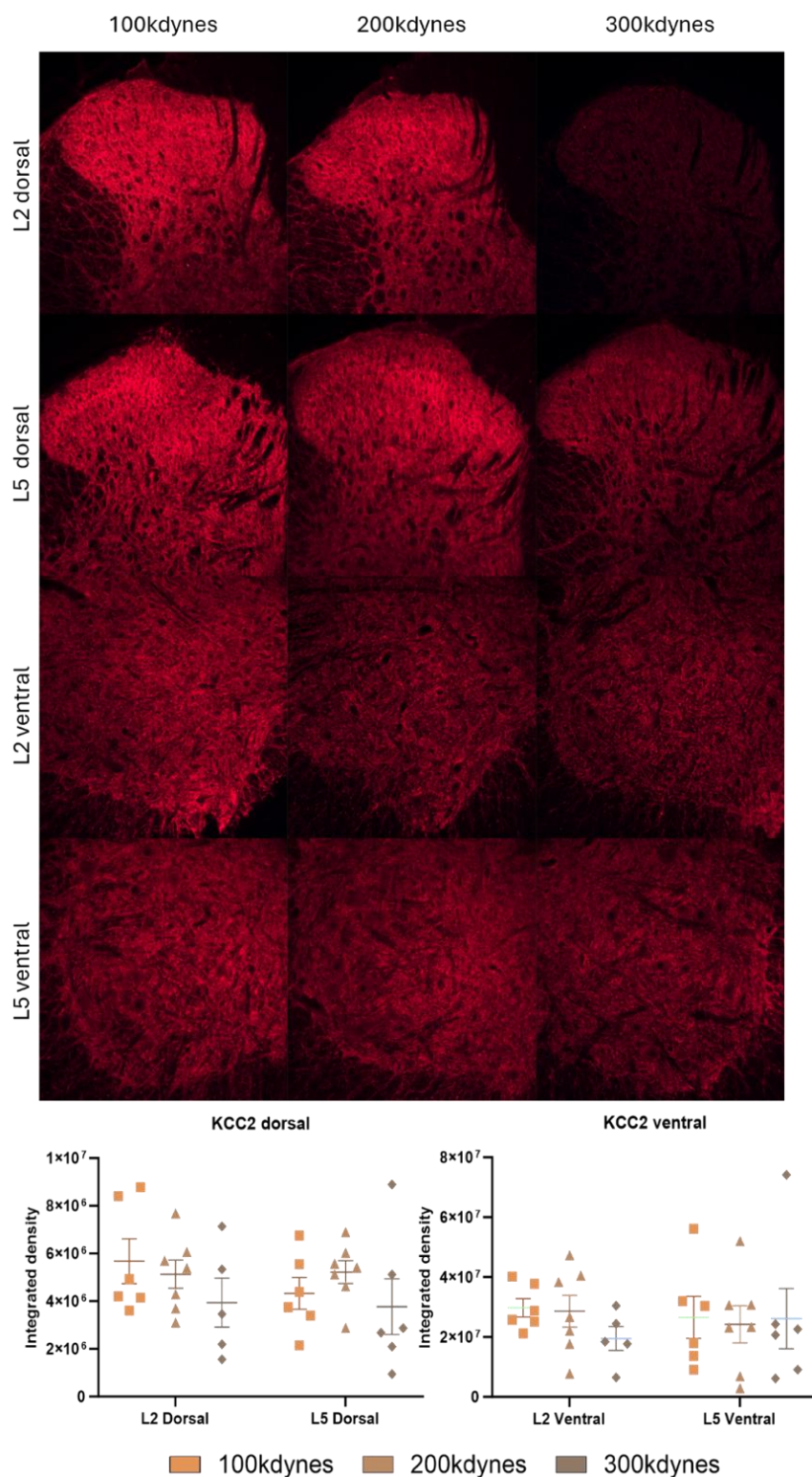


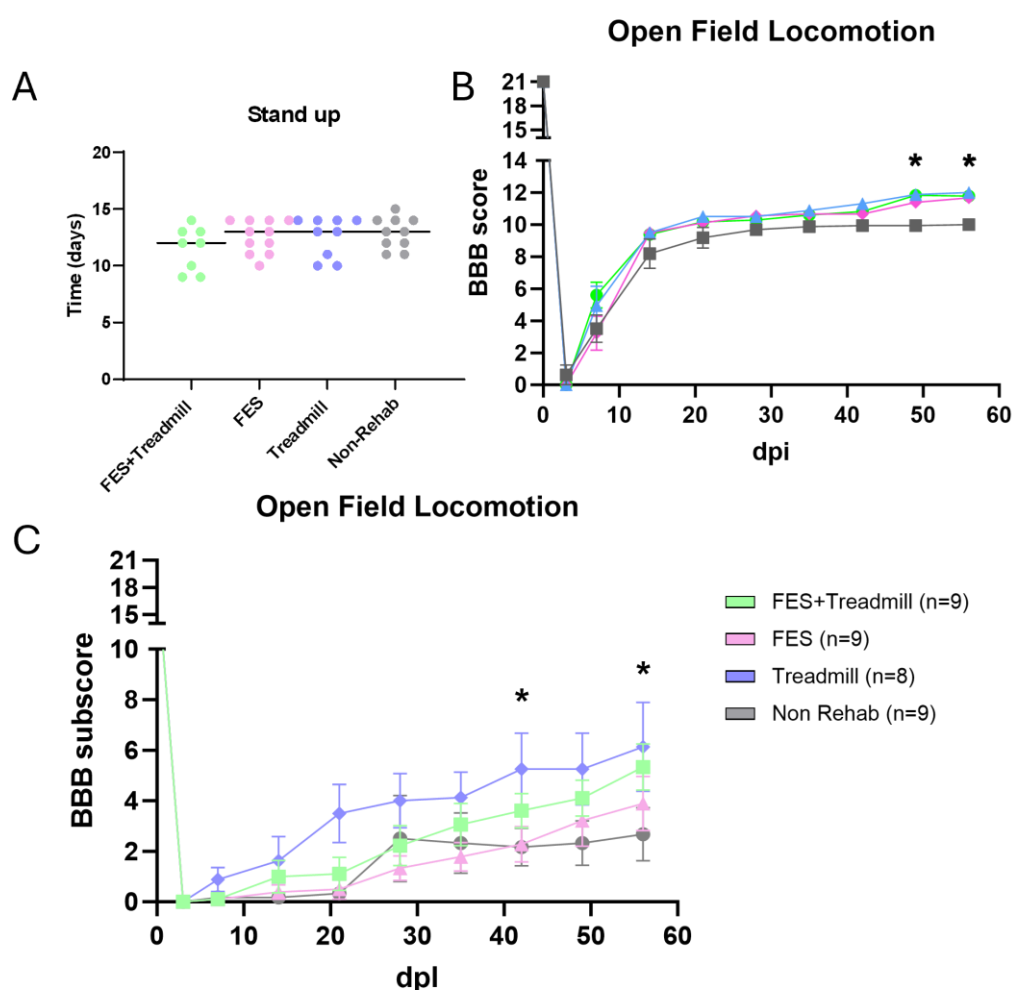
Figure 24. A. Representative images from each experimental group of dorsal and ventral horns at L2 and L5, showing co-transporter KCC2 labeling. B-C. Quantification of integrated density of immunofluorescence in dorsal (B) and ventral (C) horns of L2 and L5 spinal cord segments. No significant differences were found by two-way ANOVA.

Chapter 2: *Activity-based rehabilitation strategies promote functional recovery and spinal plasticity after thoracic spinal cord injury*

Rehabilitation promotes locomotor recovery after SCI

Following SCI, hindlimb mobility was assessed in all animals. At 3dpi, none of the animals exhibited movement in any of the hindlimb joints. By 7 dpi, animals in the non-rehabilitation groups began to show slight movements at the hip and knee, accompanied by minimal ankle movement (BBB score: 3–4), whereas animals in the early rehabilitation group (FES+Treadmill) demonstrated moderate ankle movement across multiple planes, occasionally allowing plantar surface contact with the ground (BBB score: 5–6).

From 14 dpi onwards, all animals were able to support their body weight during locomotion, although animals in the non-rehabilitation group displayed less consistent and more irregular weight-bearing. Focusing on this period, animals in the early rehabilitation group achieved weight-supported stance earlier than the other groups (11,12 vs 12,55–12,56) (Figure 25A).



Results

Figure 25. A. Plot showing the day on which animals were able to consistently support their body weight. B. Locomotor assessment using the BBB scale over the two-month study period following SCI. Statistically significant differences were observed between FES + treadmill vs non-rehab ($P = 0.0109$) and treadmill vs non-rehab ($P = 0.0112$) at 49 dpi, and between all rehabilitation groups and non-rehabilitated animals at 56 dpi (FES + treadmill vs non-rehab, $P = 0.0109$; FES vs non-rehab, $P = 0.032$; treadmill vs non-rehab, $P = 0.0082$). C. Evaluation of locomotion using the BBB subscore. Statistically significant differences were observed at 35 dpi between the FES and treadmill groups ($P = 0.0317$), and between the treadmill and non-rehabilitation groups ($P = 0.0458$). At 49 dpi, significant differences were also found between FES and treadmill ($P = 0.0342$), along with a trend toward significance between the treadmill and non-rehabilitation groups ($P = 0.055$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

Animals in the non-rehabilitation group also showed some degree of improvement, although to a lesser extent, developing longer steps but failing to achieve consistent forelimb–hindlimb coordination, reaching a maximum BBB score of approximately 10 (Figure 25B).

A more detailed analysis of locomotion using the BBB subscore for Fine Features revealed that animals in the non-rehabilitation group showed evident toe dragging during the swing phase, inconsistent paw placement, paw rotation, and low paw elevation (score: 3). The FES group showed a similar pattern until day 42, when improvements in paw placement and reduced toe dragging were observed (score: 4). In contrast, animals undergoing early rehabilitation demonstrated improved locomotion compared to the previous groups from day 35 onwards, presenting moderate paw elevation with a clearer swing phase, more appropriate paw placement, and reduced paw rotation (score: 5). Finally, the treadmill rehabilitation group showed greater improvement compared to the other groups, particularly from day 21 onwards, when all animals had undergone at least one week of rehabilitation. By day 58, no toe dragging was observed, paw placement was consistently correct, with good paw elevation during the swing phase and minimal paw rotation (score: 6) (Figure 25C).

All rehabilitation strategies improve mechanical hypersensitivity, with earlier recovery by the combined rehabilitation

All animals exhibited similar hyperreactivity to the applied pressure stimulus in the Randall-Slito test at 7dpi. From two weeks post-injury onwards, an improving trend in the response was observed in animals of the early rehabilitation group, which became significant compared to the non-rehabilitation group at 30dpi (Mean: FES+Treadmill 136.4; Non-rehab 110.6) and was maintained until the end of the study (Mean: FES+Treadmill 136.6; Non-rehab 11.,5).

In the case of the single-modality rehabilitation groups, slight improvements were observed over time, becoming significant compared to the non-rehabilitation group at 60dpi (Mean: FES 133.6; Treadmill 134.9).

On the other hand, no significant improvements over time were observed in the non-rehabilitation group, which continued to show increased sensitivity to a non-noxious stimulus, consistent with enhanced nociceptor activation. (Figure 26).

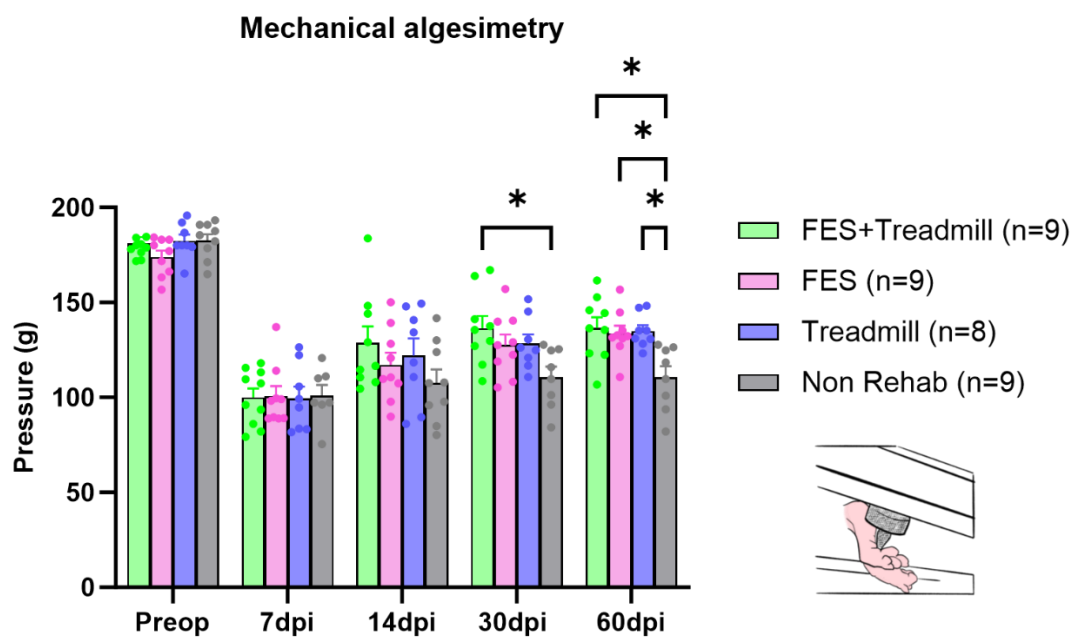


Figure 26. Evaluation of nociceptive response to a pressure stimulus applied to the hindlimb. Statistically significant differences were observed at 30 dpi between the early rehabilitation group and non-rehabilitated animals (FES+Treadmill vs non-rehab, $P=0.0381$). At 60dpi, all rehabilitation groups showed improvement compared to non-rehabilitated animals (FES+Treadmill vs Non-rehab, $P=0.0292$; FES vs Non-rehab, $P=0.0331$; Treadmill vs Non-rehab, $P=0.0189$). Statistical analysis was performed using a mixed-effects analysis followed by Tukey's multiple comparisons test.

Electrophysiological studies

For electromyographic analyses, the amplitude and latency of the M and H waves were assessed, together with the H/M ratio and the H-reflex RDD in the plantar interosseus and tibialis anterior muscles. In addition, MEPS were recorded for the tibialis anterior muscle.

Rehabilitation with FES induces a transient reduction in reflex hyperexcitability

The H/M ratio was used to assess changes in H-wave excitability. For each animal and time point, the maximal M-wave and H-wave responses were selected to calculate the Hmax/Mmax ratio. The results showed an increase in this ratio following SCI in all experimental groups. The hyperexcitability persisted over time in both muscles, although it was particularly evident in the plantar muscle (Figure 27).

Throughout the study, no significant differences between groups were observed in the tibialis anterior muscle (Figure 28). In contrast, in the plantar muscle, a reduction in the H/M ratio was observed in animals of the FES rehabilitation group compared to non-rehabilitated animals (Mean 30dpi: FES 46.2; Non-rehab 58.6), although this significant difference was no longer present at 60dpi (Figure 27).

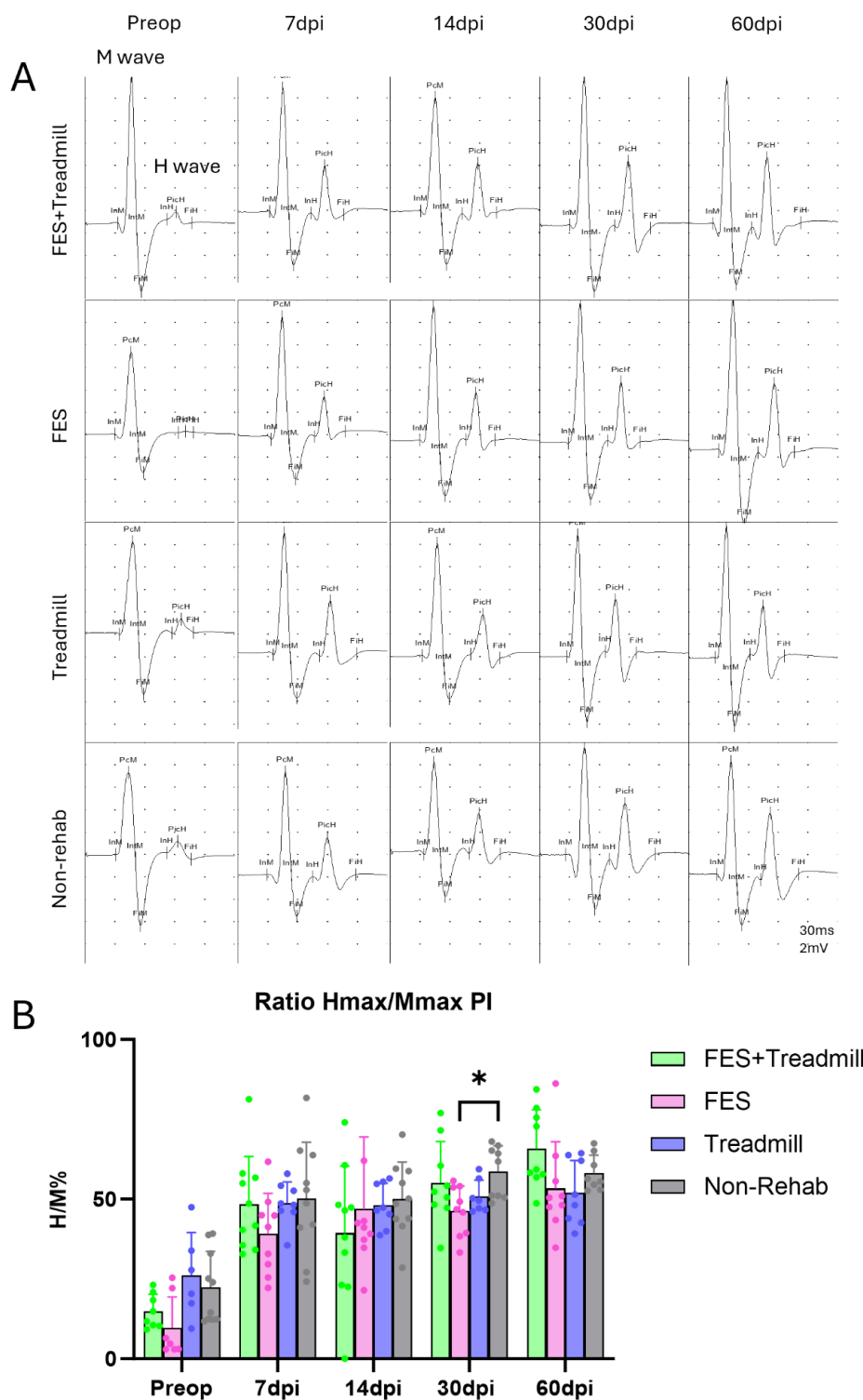


Figure 27. A. Representative recordings from each experimental group of the M and H waves of plantar muscle, before SCI and at 7, 14, 30, and 60dpi. B. Plot of the H/M ratio over the course of the study. An increase in the ratio was observed after injury in all groups, driven by an increase in H-wave amplitude. Statistically significant differences were found at 30dpi between the FES and Non-rehabilitation groups ($P=0.0217$), but this effect was not maintained at 60dpi. Statistical analysis was performed using a mixed-effects analysis followed by Tukey's multiple comparisons test.

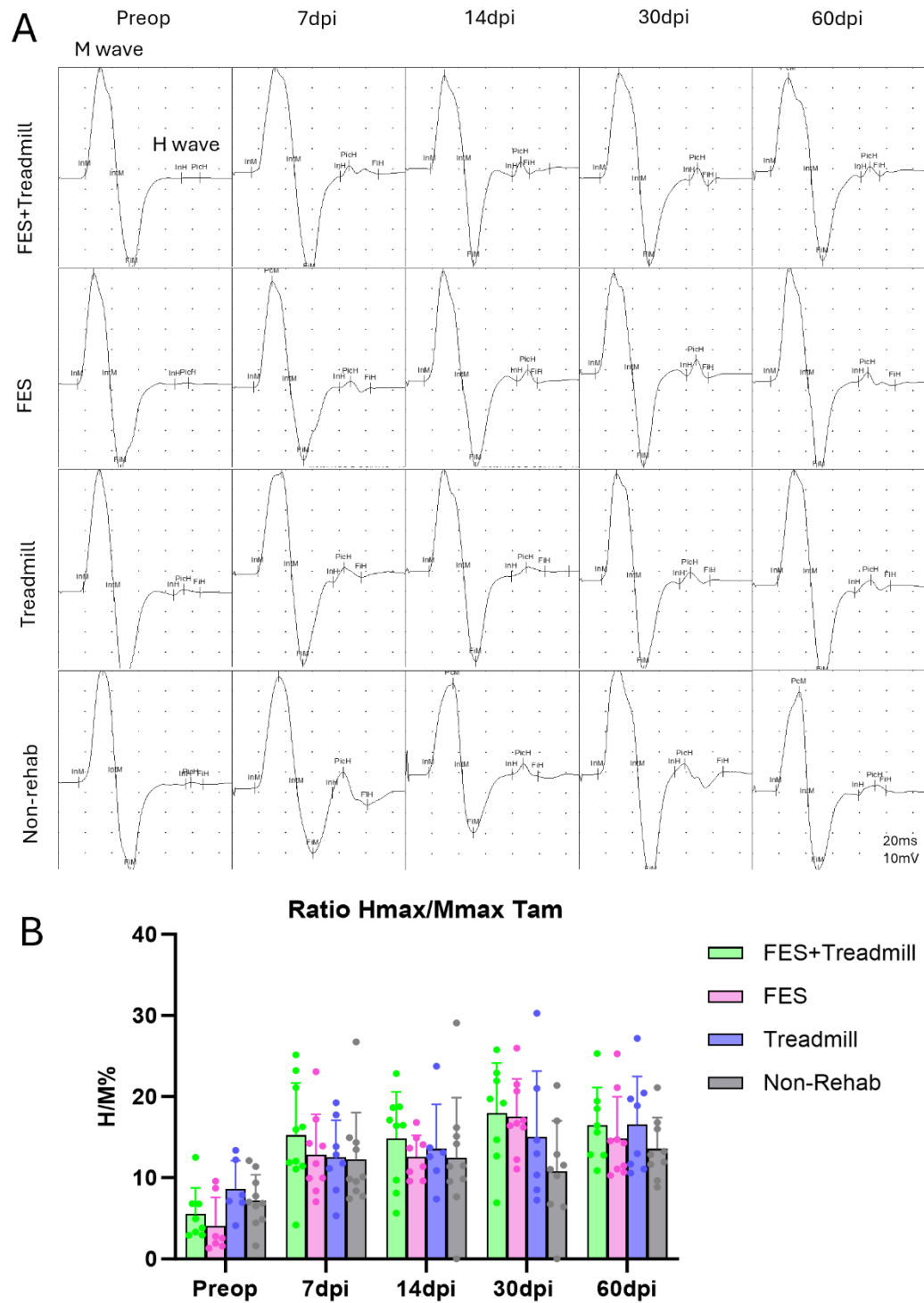


Figure 28. A. Representative recordings from each experimental group of the M and H waves of tibialis anterior muscle, before SCI and at 7, 14, 30, and 60dpi. B. Plot of the H/M ratio over the course of the study. An increase in the ratio was observed after injury in all groups, driven by an increase in H-wave amplitude, but without statistical differences between groups. Statistical analysis was performed using a mixed-effects analysis followed by Tukey's multiple comparisons test.

FES rehabilitation enhances H-reflex frequency-dependent depression

In control non-injured animals the H-wave amplitude showed a marked rate-dependent depression during repeated stimulation. Our reference values demonstrated an approximate 60% reduction in H-wave amplitude at 1pps, above 80% at 2pps and higher than 90% at 5pps. These results are consistent with the functional activity of inhibitory modulation of the spinal reflex pathway (Figure 29-30A).

In contrast, as soon as at 7 days following SCI, the H wave RDD was markedly reduced. All rats required stimulation frequencies above 5pps to achieve reductions greater than 50% in the H-wave amplitude of the plantar muscle, comparing the first and tenth pulses of each series. Reductions of the H wave greater than 80% were only achieved at 20pps stimulation, without significant differences between groups at this time point (Figure 30B).

Over time, this reduction in H-wave amplitude became progressively less pronounced, indicating sustained inhibition of the H wave RDD. At 14 dpi, the H wave amplitude at 5pps remained above 40% in all groups. However, animals of the FES rehabilitation group achieved a reduction greater than 50% from 2pps onwards, whereas the remaining groups required stimulation frequencies of 5pps to reach this level of depression. All groups achieved reductions close to 80% at 20pps, without statistically significant differences between them (Figure 30C).

At 30 dpi, improvements in the RDD became more evident in the FES group compared to non-rehabilitated animals. In the plantar muscle, animals undergoing FES rehabilitation had reductions in H-wave amplitude higher than 50% at 5pps, whereas the other rehabilitation groups showed reductions around 30%, and non-rehabilitated animals only approximately 20% at the same frequency. These differences were less marked as stimulation frequency increased (Figure 30D).

Finally, at 60 dpi, the H-wave amplitude remained above approximately 65% with stimulation at 5pps in all the groups. Animals of the FES and combined early rehabilitation groups achieved reductions greater than 50% in H-wave amplitude at 10pps, whereas the remaining groups did not. Furthermore, at 20pps, rehabilitation groups exhibited reductions close to 70%, while non-rehabilitated animals showed

Results

reductions of approximately 60%. However, no statistically significant differences between groups were observed at this time point (Figure 30E).

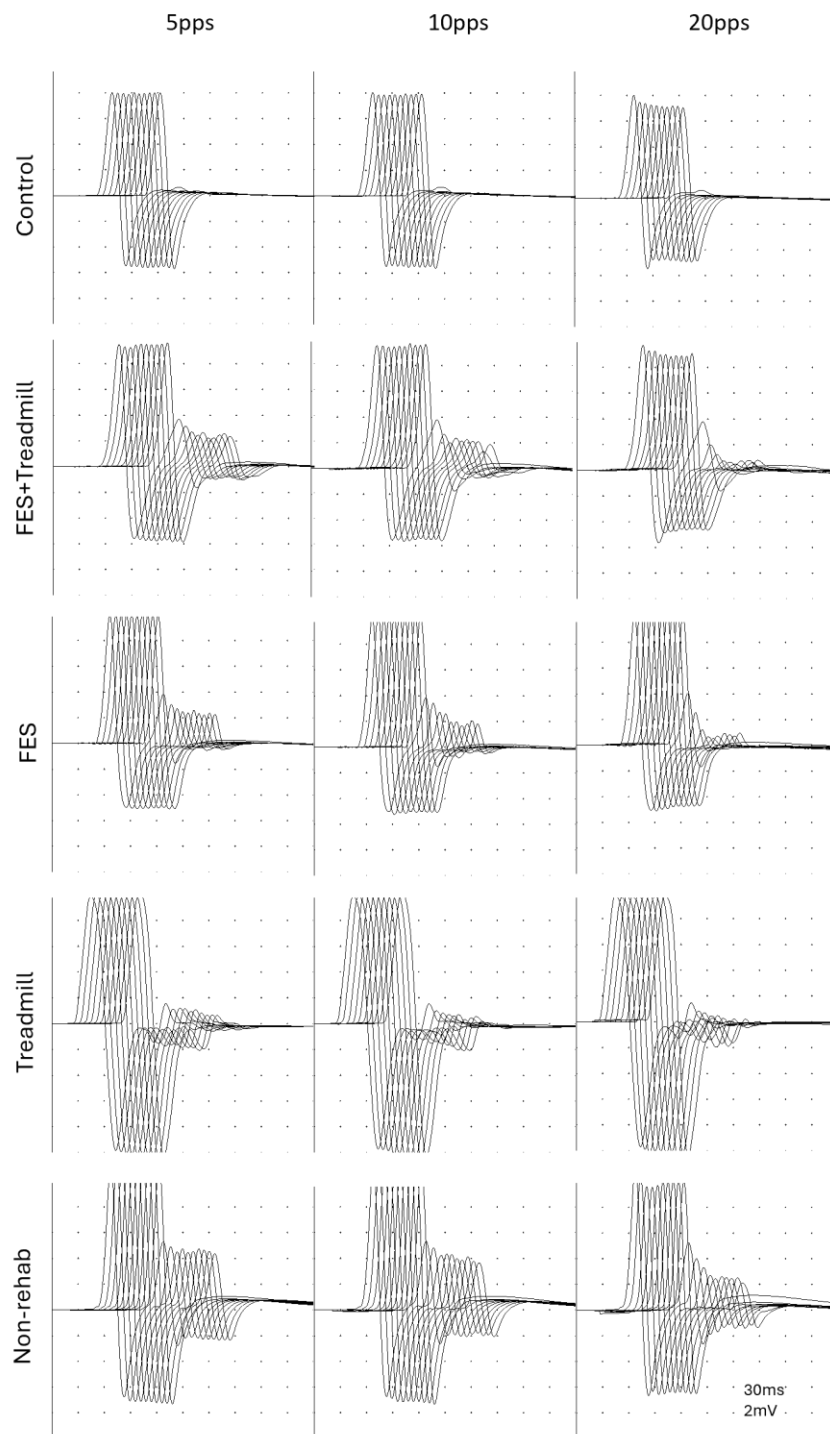


Figure 29. A. Representative electromyographic recordings from each experimental group for the H-reflex rate-dependent depression (RDD) study in the plantar muscle. Each trace represents 10 overlapping pulses, showing both M- and H-waves, in response to stimulation at frequencies of 5, 10, and 20pps, arranged from left to right.

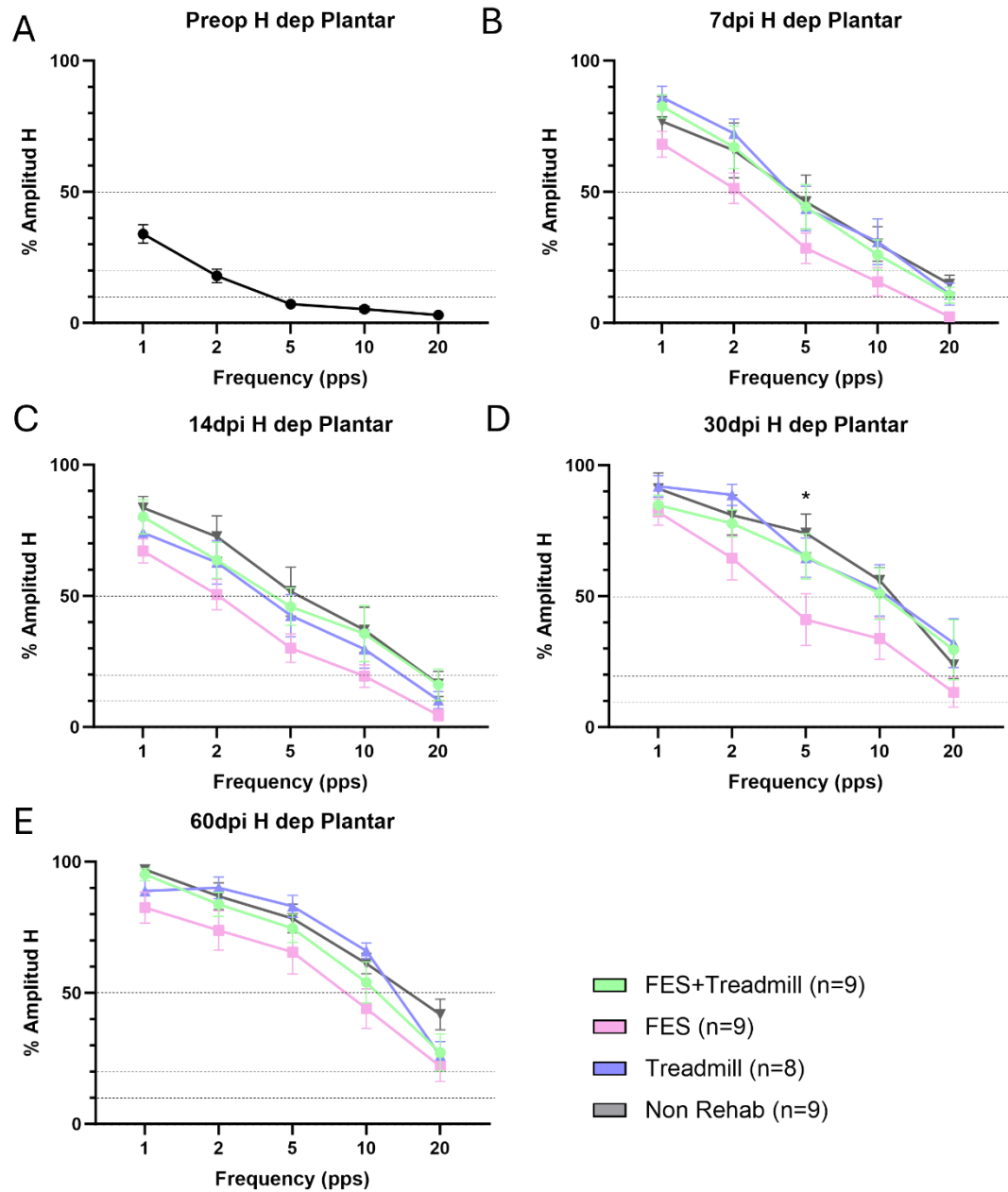


Figure 30. A-E. Evaluation of H-reflex RDD before SCI and at 7, 14, 30, and 60dpi, assessed by comparing H-wave amplitude between the first and the 10th pulse at stimulation frequencies of 1, 2, 5, 10, and 20pps. Statistically significant differences were observed only at 5pps, between the FES and Non-rehabilitation groups ($P=0.0121$) at 30dpi (E). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

The tibialis anterior muscle was also evaluated to analyze the RDD of the H wave under repetitive stimulation (Figure 31). In control animals, approximately 50% reduction in H-wave amplitude was observed with stimulation at 1 pps, around 80% at 5 pps and above 90% at 20 pps, reflecting preserved inhibitory modulation of the spinal reflex pathway (Figure 32A).

In the SCI groups, H-wave RDD was markedly reduced from the acute phase after injury, and slightly worsened along time. At 7 dpi, a reduction of approximately 50% in H-wave amplitude was only achieved at stimulation frequencies of 10 pps (Figure 32B). A similar pattern was observed at 14 dpi, indicating persistent impairment in inhibitory control of the spinal reflex circuit during the early stages after injury (Figure 32C).

From 30 dpi onwards, differences between groups emerged. At lower stimulation frequencies (up to 5 pps), all groups displayed a similar response profile. However, from 10 pps onwards, rehabilitation groups exhibited a greater reduction in H-wave amplitude compared to non-rehabilitated animals. These differences became more evident at 20 pps, particularly in the FES group, which showed an almost 90% reduction in H-wave amplitude, significantly greater than in non-rehabilitated animals, which exhibited reductions about 50% (Mean: FES 13,8; Non-rehab 45,52) (Figure 32D).

At 60 dpi, a similar pattern was observed. All rehabilitation groups showed improved RDD compared to non-rehabilitated animals at 10 pps, with statistically significant differences detected in the FES and combined early rehabilitation groups compared to non-rehabilitated animals (Mean: FES 45,78; FES+Treadmill 45,13; Non-rehab 77,31). However, despite these improvements, none of the rehabilitation groups achieved reductions greater than 80% in H-wave amplitude (Figure 32E).

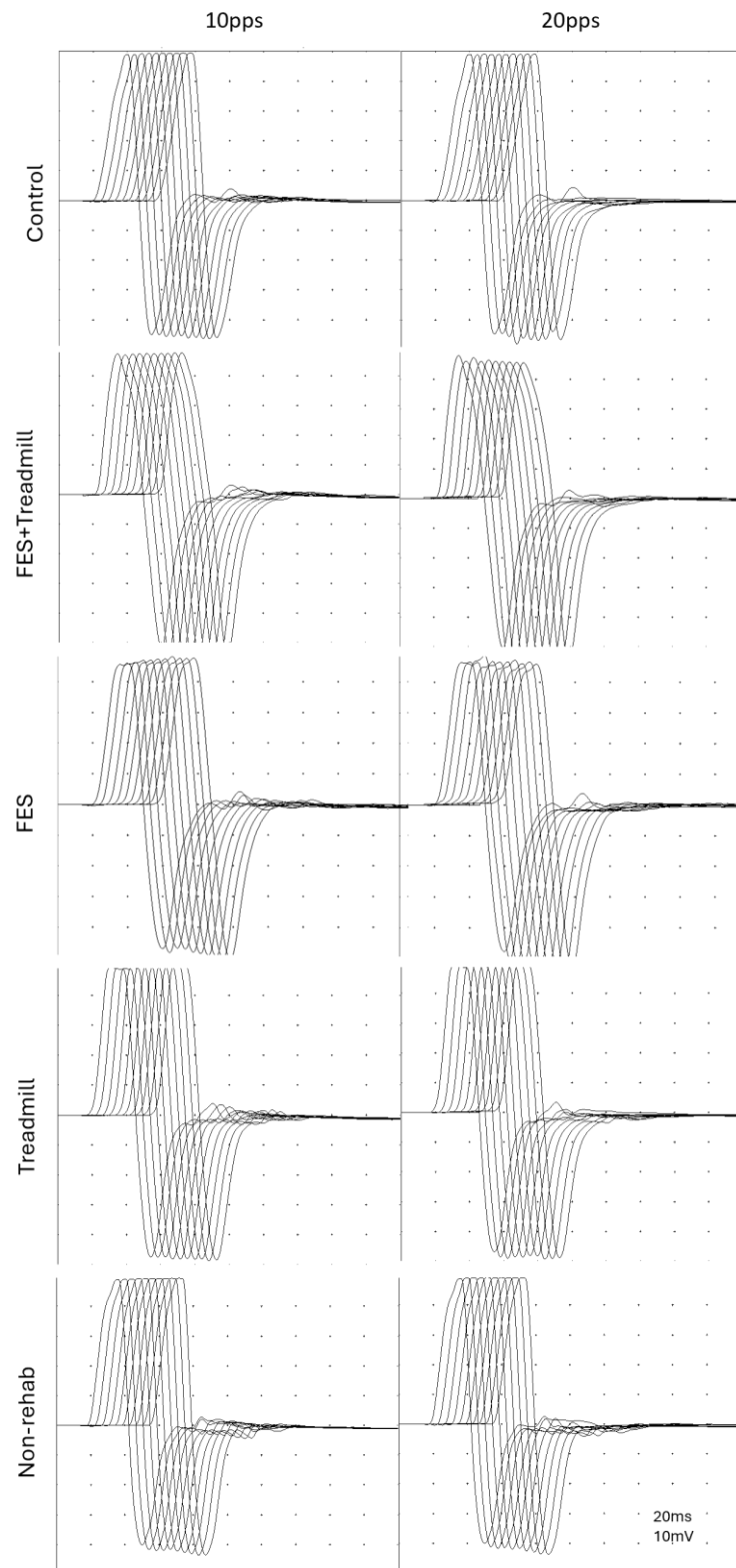


Figure 31. Representative electromyographic recordings from each experimental group for the H-reflex rate-dependent depression (RDD) study in the tibialis anterior muscle. Each trace represents 10 overlapping pulses, of both M- and H-waves. Recordings correspond to stimulation frequencies of 10 and 20pps.

Results

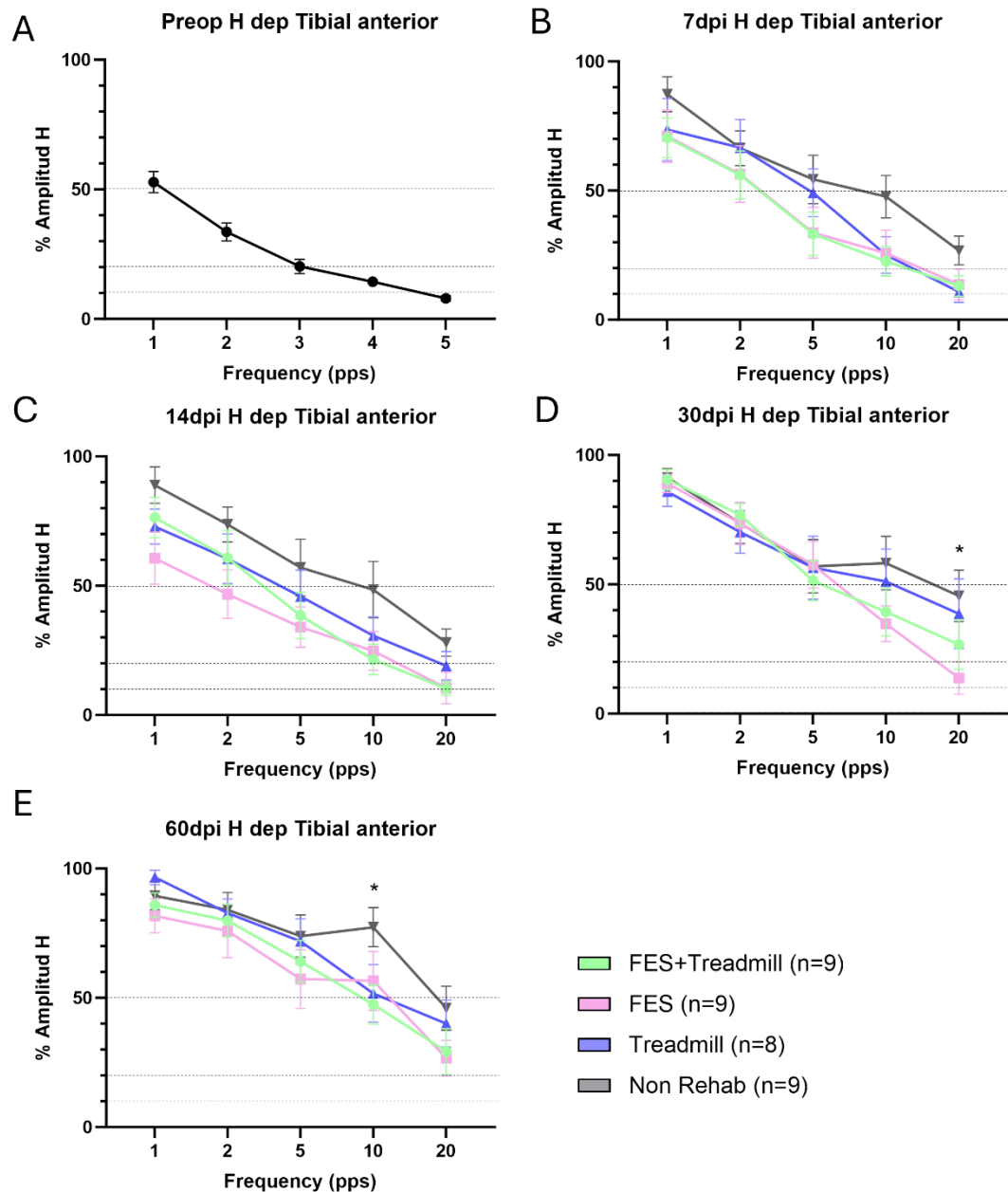


Figure 32. A-E. Evaluation of H-wave RDD before SCI and at 7, 14, 30, and 60dpi, assessed by comparing H-wave amplitude between the first and the 10th pulse at stimulation frequencies of 1, 2, 5, 10, and 20pps. Statistically significant differences were observed between the FES and Non-rehabilitation groups at 30 and 60dpi (30 dpi, 20pps: FES vs Non-rehab, $P=0.0428$; 60dpi, 10pps: FES vs Non-rehab, $P=0.0315$). Additionally, significant differences were observed between the early rehabilitation group and non-rehabilitated animals at 60 dpi at a frequency of 10pps (FES+Treadmill vs Non-rehab, $P=0.0218$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

Lack of improvements in MEPs by rehabilitation protocols

To evaluate corticospinal function, MEPs were recorded from the tibialis anterior muscle (Figure 33A). Following SCI, MEP amplitudes decreased markedly, dropping from pre-injury mean values of 7–14mV to 0.2–0.6mV at 7dpi. This reduction persisted throughout the two-month study period, without significant differences between groups (Figure 33B).

Regarding the MEPs latency, a marked increase was observed after injury, from a pre-surgical mean of approximately 8ms to values ranging between 23–26ms at 7dpi, remaining elevated over the two-month follow-up. No significant differences between groups were detected (Figure 33C).

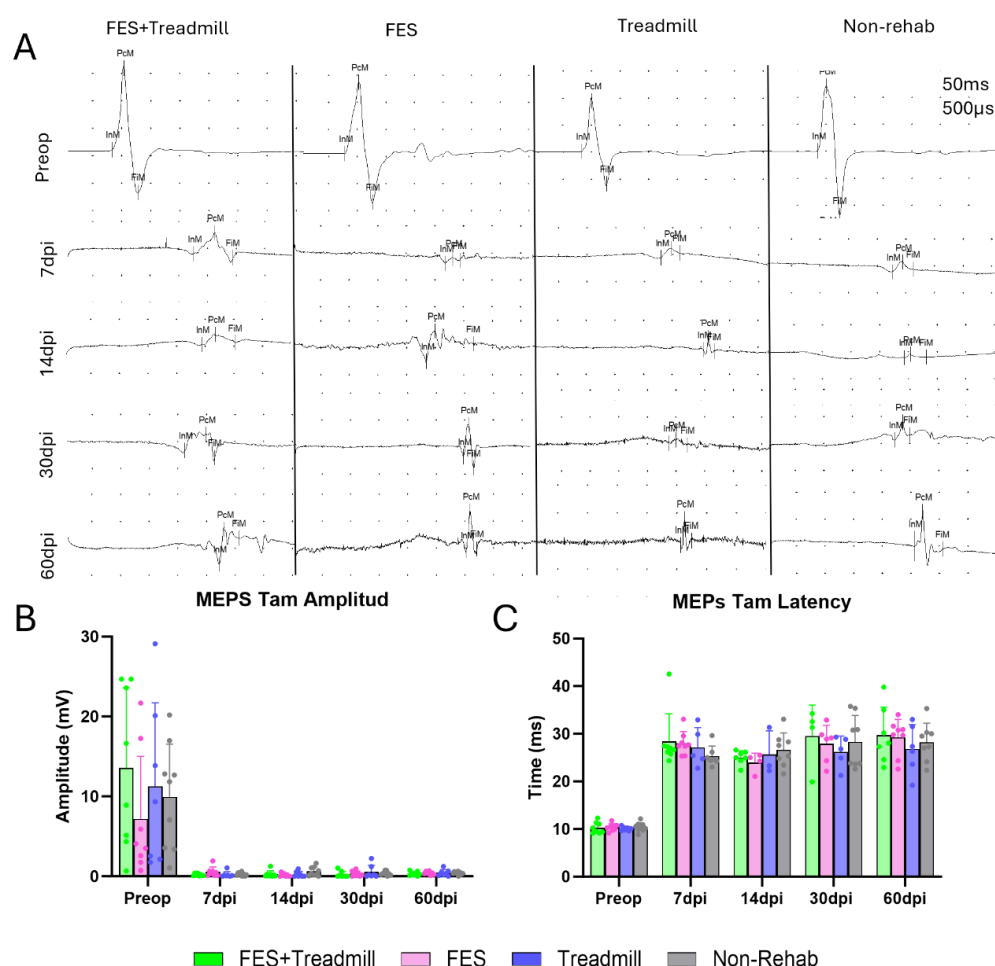


Figure 33. A. Recordings of MEPs from the tibialis anterior muscle before SCI and at 7, 14, 30, and 60 dpi. B. Graph of MEP amplitude over the course of the study in all experimental groups. C. Graph of MEP latency over the course of the study. An increase was observed in all groups after injury. No statistically significant differences between groups were found. Statistical analysis was performed using a mixed-effects analysis followed by Tukey's multiple comparisons test.

Histological evaluation

Spinal cord tissue preservation

To correlate functional deficits with the degree of injury, an evaluation of the damaged tissue area was performed (Figure 34). The results showed no differences between groups in the lesion epicenter area, which ranged from 817423 to 1128360 μm^2 (Figure 34B).

To further assess the extent of damage, the lesion volume was analyzed by calculating the area under the curve (AUC) of tissue preservation along 1 mm cord segment around the epicenter. Animals in the combined early rehabilitation group exhibited a lower AUC compared to the other groups, with a mean value of $1.49\text{e}+10$. Accordingly, this group showed a slightly greater lesion compared to the others, with average values of FES $1.77\text{e}+10$; Treadmill $1.69\text{e}+10$; and Non-rehab $1.67\text{e}+10$ (Figure 34C).

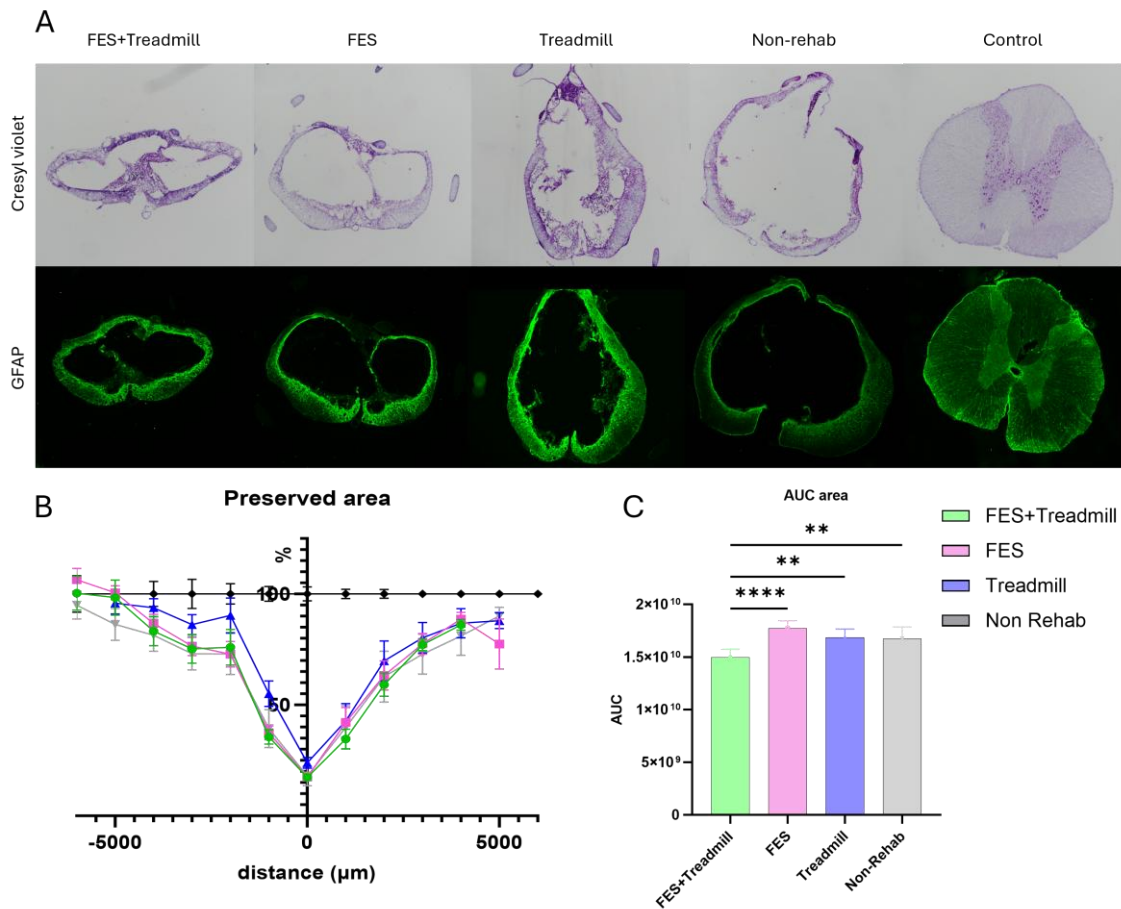


Figure 34. A. Representative images from each experimental group of the lesion epicenter, with Cresyl violet and GFAP staining. B. Graph of the percentage of preserved area from the epicenter to the point where the lesion disappears, in both rostral and caudal directions. C. Graph of the area under the curve of lesion volume for each group. Statistically significant differences were observed between the early rehabilitation group and the other groups (FES + treadmill vs FES, $P < 0.0001$; FES + treadmill vs treadmill, $P = 0.0017$; FES + treadmill vs Non-rehab, $P = 0.0032$), calculated using one-way ANOVA followed by Tukey's multiple comparisons test.

FES rehabilitation increases motoneuron survival after SCI

To evaluate motoneuron survival in the lumbar region following injury, motoneurons were quantified in each lumbar spinal segment (Figure 35). The results show that the lower lumbar segments (L4–L6) exhibit a higher number of motoneurons in animals of the FES rehabilitation group compared to non-rehabilitated animals, with mean values corresponding to 82% versus 68% relative to uninjured controls (FES vs non-rehab, p value = 0.0027). This effect was not observed in the more rostral lumbar segments (L1–L3), nor in the other rehabilitation groups (Figure 35B).

When analyzing each spinal segment individually, it was observed that the non-rehabilitated rats were the ones with lower number of surviving motoneurons, although the differences were not large. At the L4 level all rehabilitation groups had a higher number of motoneurons, approximately 90% of control values, compared to non-rehabilitated group with 75% (FES vs non-rehab, $P = 0.0014$; FES + treadmill vs non-rehab, $P = 0.0067$; treadmill vs non-rehab, $P = 0.0397$). However, in more caudal direction, significant differences were only maintained for the FES rehabilitation group, but not for the other rehabilitation groups. Thus, at L5, rats of the FES group showed 83% of motoneurons relative to controls compared to 69% in the non-rehabilitation group (FES vs non-rehab, $P = 0.0149$), and at L6, values were of 74% versus 59% (FES vs non-rehab, $P = 0.0065$) (Figure 35C).

Results

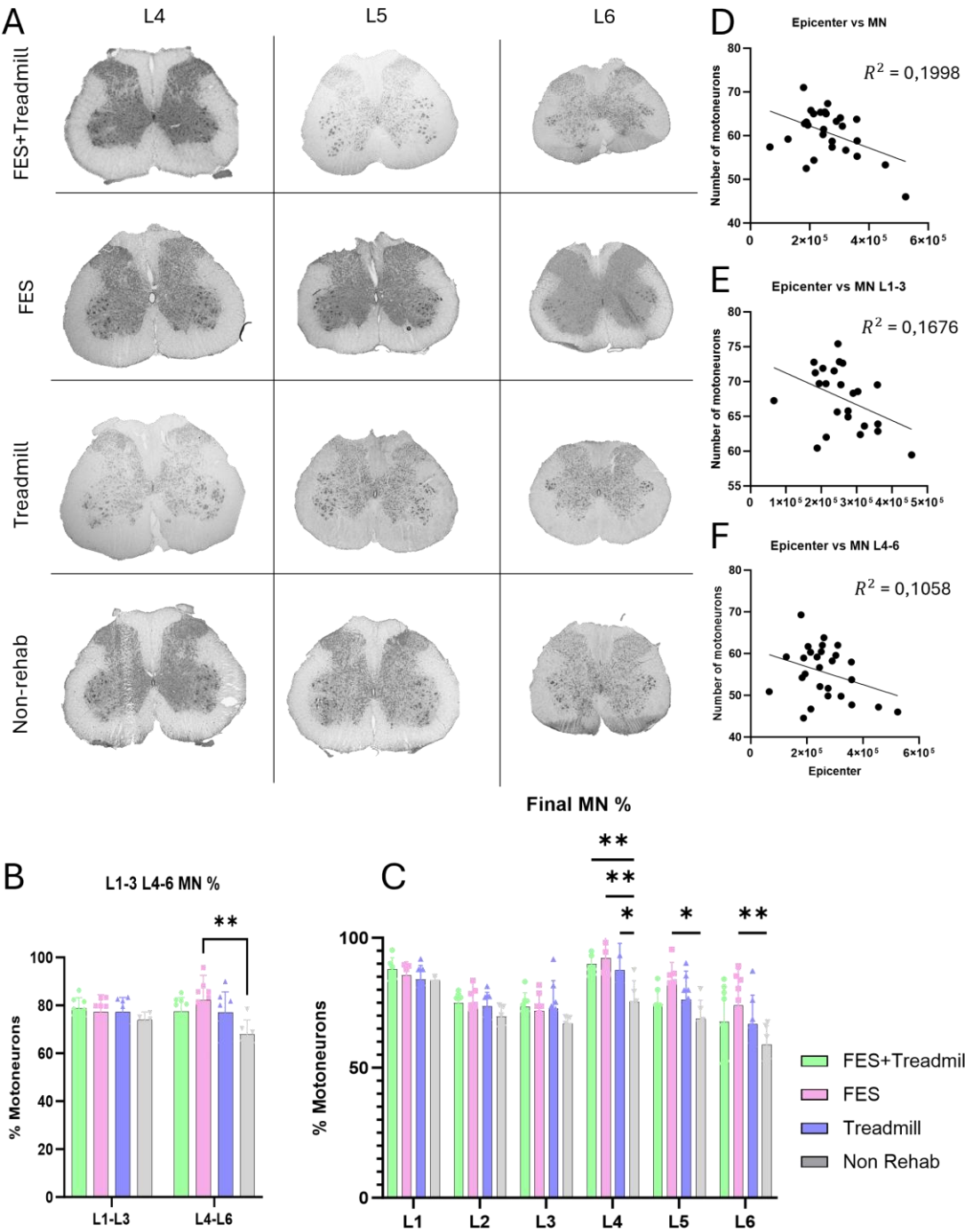


Figure 35. A. Representative images from each experimental group of Cresyl violet staining in the lumbar regions L4–L6 of the spinal cord. B–C. Quantification of motoneurons across all lumbar spinal segments. B. Statistically significant differences were observed between the FES rehabilitation group and the non-rehabilitation group in the more caudal lumbar regions (L4–L6), but not in the more rostral regions (L1–L3) (FES vs Non-rehab, $P = 0.0027$). C. When analyzing each segment separately, statistically significant differences were observed between all rehabilitation groups and the non-rehabilitation group at L4 (FES + treadmill vs Non-rehab, $P = 0.0067$; FES vs Non-rehab, $P = 0.0014$; treadmill vs Non-rehab, $P = 0.0397$). Differences were also observed at L5 and L6 between the FES group and the non-rehabilitation group (L5, FES vs Non-rehab, $P = 0.0149$; L6, FES vs Non-rehab, $P = 0.0065$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. D–F. Correlation between lesion epicenter area and total number of motoneurons (D), in segments L1–L3 (E), and in segments L4–L6 (F). A significant correlation was observed in the more rostral segments ($P = 0.047$), but not in the more caudal segments ($P = 0.0977$).

Rehabilitation does not induce changes in the lumbar glial response

The intensity of immunoreactivity for GFAP and Iba1 was quantified in the spinal cord, focusing on the lumbar segments L2 and L5. Within each segment, the dorsal and ventral horns were analyzed independently to characterize potential spatial differences in astrocytic and microglial responses.

The astrocytic response was assessed by measuring GFAP immunoreactivity. The results showed lower signal intensity in the dorsal horn compared to the ventral horn at both spinal levels. However, no statistically significant differences were observed between groups when the dorsal and ventral regions were analyzed separately in either L2 or L5 (Figure 36).

Results

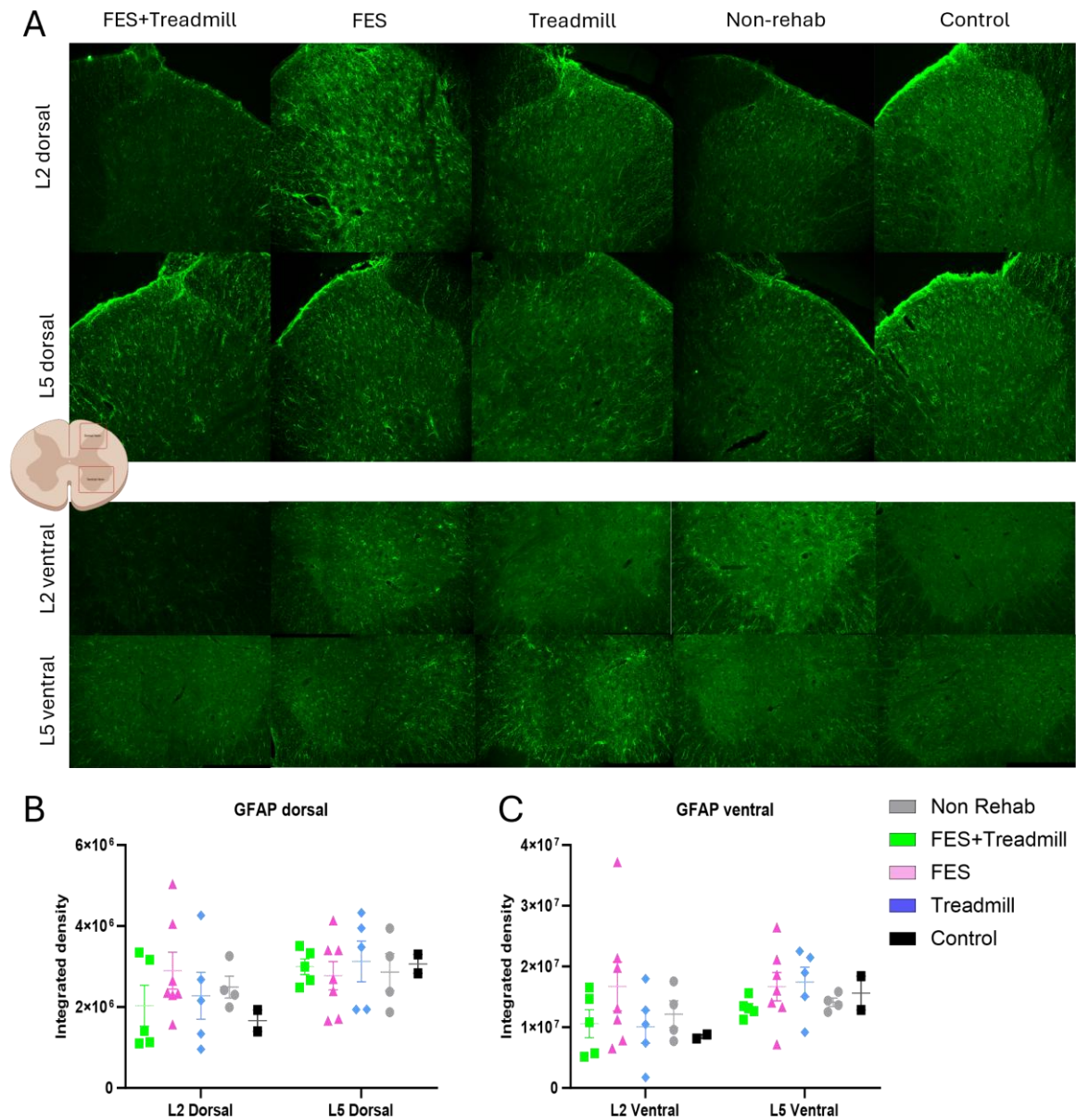


Figure 36. A. Representative images from each experimental group of the dorsal and ventral horns at L2 and L5, showing astrocyte staining with the GFAP antibody. B–C. Quantification of fluorescence integrated density in the dorsal (B) and ventral (C) regions of L2 and L5 of the spinal cord. No statistically significant differences were observed between groups.

Regarding microglial activation, Iba1 immunoreactivity was analyzed in the same anatomical regions. As observed for GFAP, a lower signal intensity was found in the dorsal horn compared to the ventral horn in both spinal segments analyzed. However, no statistical significance was found when comparing the experimental

groups independently for each region (dorsal and ventral horns) and spinal level (L2 and L5) (Figure 37).

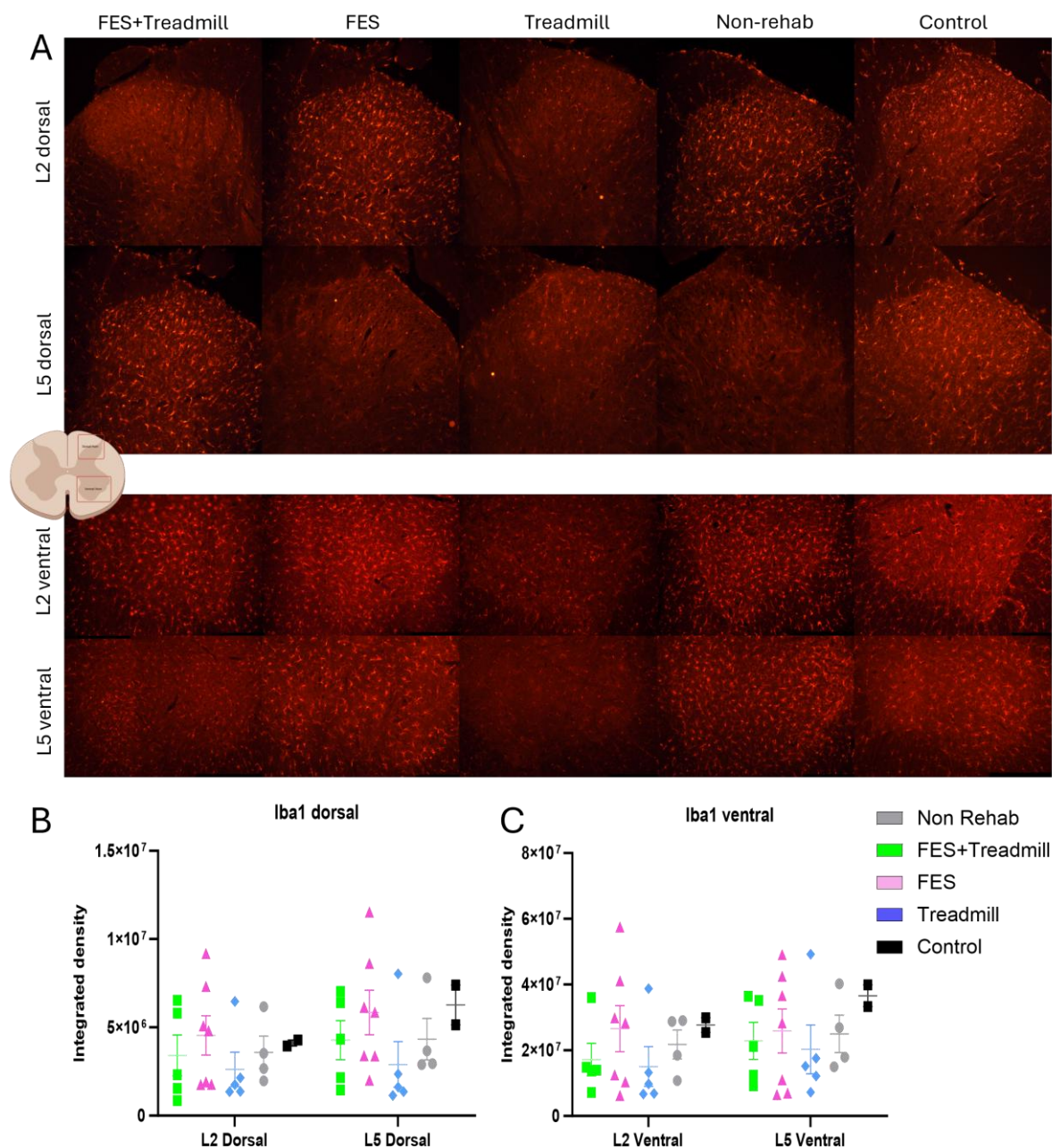


Figure 37. A. Representative images from each experimental group of the dorsal and ventral horns at L2 and L5, showing microglia staining with the Iba1 antibody. B-C. Quantification of fluorescence integrated density in the dorsal (B) and ventral (C) regions of L2 and L5 of the spinal cord. No statistically significant differences were observed between groups.

Electrical rehabilitation modulates the expression of KCC2

The intensity of the KCC2 co-transporter was evaluated as a marker associated with neuronal function and the regulation of synaptic inhibition. As observed for GFAP and Iba1, lower signal intensity was detected in the dorsal horn compared to the ventral horn (Figure 38).

When analyzing dorsal and ventral horns separately, the results revealed group differences following an anatomical region-dependent pattern. In the dorsal horn, no statistically significant differences were observed between groups at L2, although mean integrated density values in the rehabilitation groups showed an increasing trend compared to non-rehabilitated animals (mean values: Control 8.75×10^6 ; FES+Treadmill 7.04×10^6 ; FES 8.82×10^6 ; Treadmill 7.44×10^6 ; Non-rehab 4.65×10^6). At L5, a similar upward trend was observed in animals receiving FES stimulation (mean: 8.54×10^6) compared to the non-rehabilitation group (mean: 5.90×10^6), reaching statistical significance in the early rehabilitation group (mean: 9.77×10^6) when compared to non-rehabilitated animals (FES+Treadmill vs non-rehab, $P=0.0315$; FES vs non-rehab, $P=0.1878$) (Figure 38B).

In the ventral horn, all SCI groups exhibited a decrease in KCC2 fluorescence density at L2 (FES+Treadmill vs control, $P=0.0452$; FES vs control, $P=0.0286$; treadmill vs control, $P=0.0254$; non-rehab vs control, $P=0.0098$). In contrast, at L5, a statistically significant decrease was observed only in the non-rehabilitation group (non-rehab vs control, $P=0.0264$). Additionally, the results indicate that in the ventral horn of L5, KCC2 signal density was higher in animals of the FES rehabilitation group compared to non-rehabilitated animals over the two-month study period (FES vs non-rehab, $P=0.0406$) (Figure 38C).

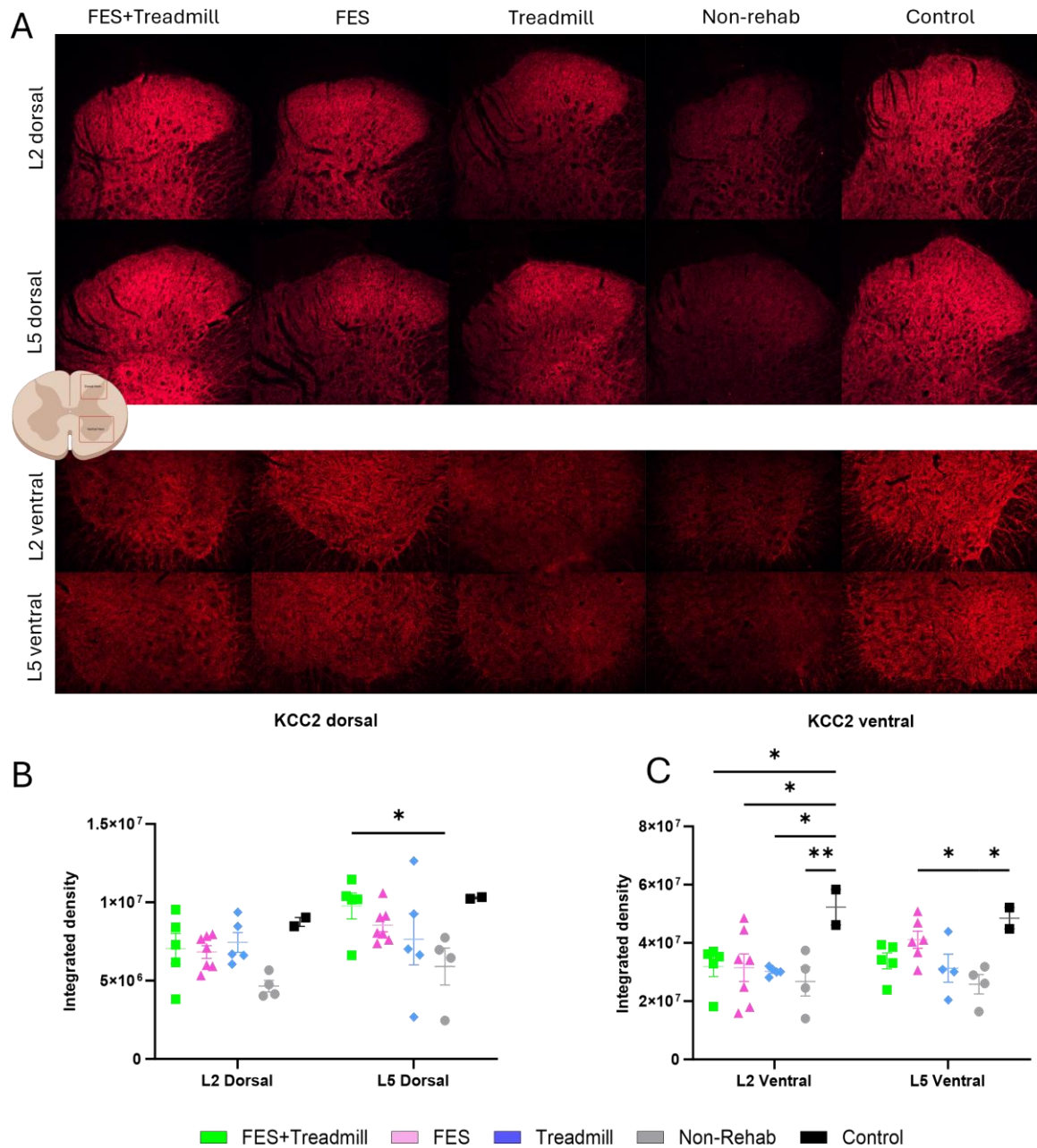


Figure 38. A. Representative images from each experimental group of the dorsal and ventral horns at L2 and L5, showing KCC2 transporter staining. B–C. Quantification of fluorescence integrated density in the dorsal (B) and ventral (C) regions of L2 and L5 of the spinal cord. B. Statistically significant differences were observed in the dorsal region of L5 between the early rehabilitation group and the non-rehabilitated animals (FES + treadmill vs non-rehab, $P = 0.0315$). C. At the ventral horn, statistically significant differences were observed between all experimental groups and the uninjured control animals at L2, but no differences between the experimental groups. However, at L5, significant differences were observed between the electrical rehabilitation group and the non-rehabilitation group (L5 ventral, FES vs non-rehab, $P = 0.0406$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

FES-based rehabilitation induces lumbar serotonergic plasticity

To evaluate potential changes in serotonergic spinal pathways plasticity following injury and after the application of different rehabilitation strategies, 5-HT immunoreactivity was analyzed in the lumbar spinal cord segments L2 and L5, distinguishing between the dorsal and ventral horns (Figure 39).

The results for the dorsal horn of both segments showed no differences between groups, except for the non-rehabilitation group, whose mean was significantly lower compared to the control group (mean non-rehab 9.42×10^9 ; mean control 4.04×10^{10} ; non-rehab vs control, $P = 0.0500$) (Figure 39B-C).

In contrast, in the ventral horn, notable changes were observed in some rehabilitation groups. At L2, the results showed an increase in 5-HT signal density in animals undergoing early rehabilitation compared to both the FES group and the non-rehabilitation group (FES + treadmill vs FES, $P = 0.0185$; FES + treadmill vs non-rehab, $P = 0.0094$) (Figure 39D). At L5, this pattern was maintained, with the early rehabilitation group showing significantly higher intensity compared to non-rehabilitated animals (FES + treadmill vs non-rehab, $P = 0.0136$) (Figure 39E).

In contrast to L2, the FES stimulation group at L5 showed a statistically significant increase in fluorescence density of serotonergic fibers compared to non-rehabilitated animals (FES vs non-rehab, $P = 0.0490$) (Figure 39E).

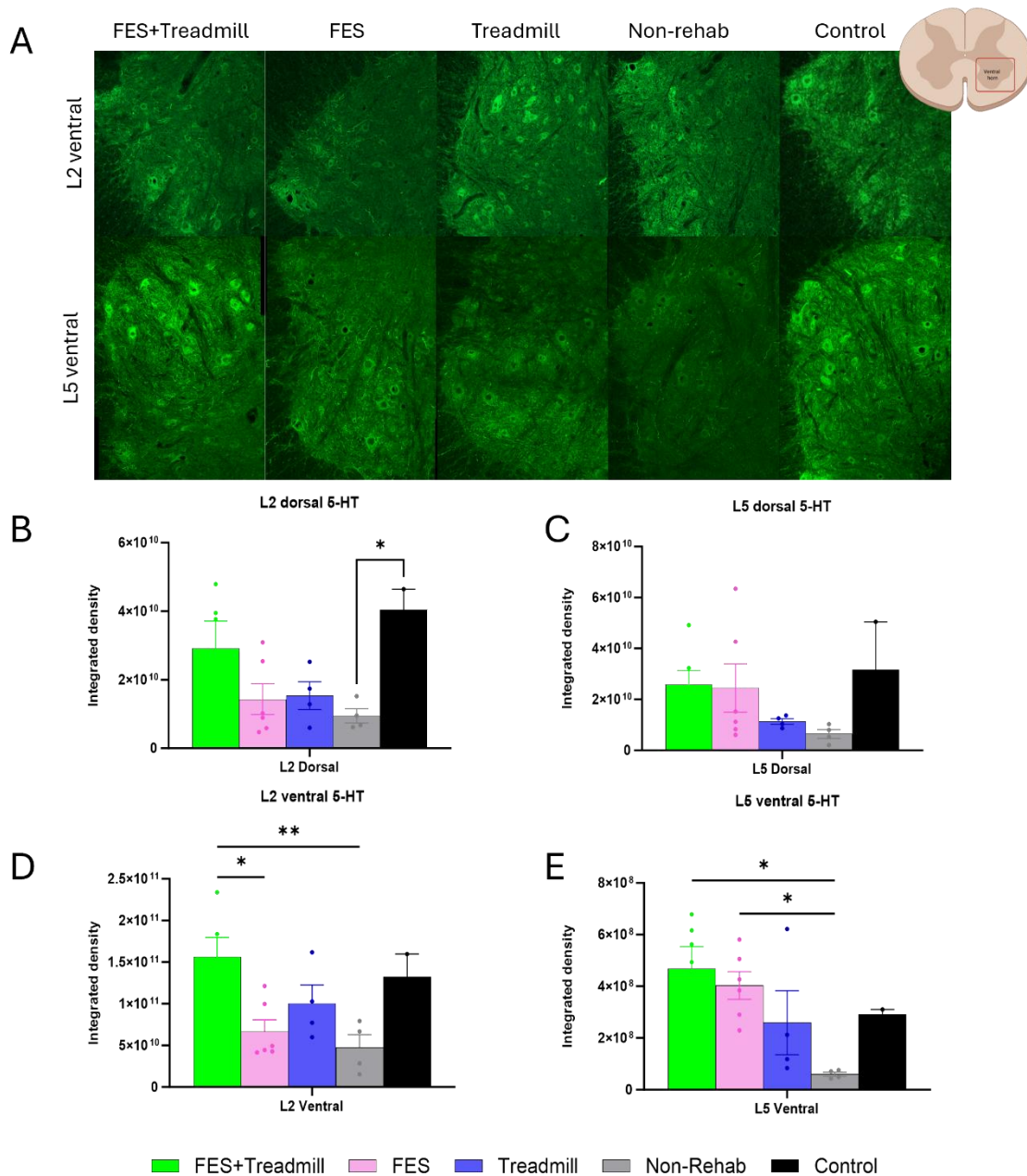


Figure 39. A. Representative images from each experimental group of the ventral horns at L2 and L5, showing serotonin staining with the 5-HT antibody. B–E. Quantification of fluorescence integrated density in the dorsal and ventral horns of L2 and L5. Statistically significant differences were observed between non-rehabilitated animals and uninjured control animals ($P = 0.05$) in the dorsal horn of L2 (B), but not in L5 (C). In the ventral horns, statistically significant differences were observed at L2 (D) between the early rehabilitation group and non-rehab groups (L2 ventral, FES + treadmill vs Non-rehab, $P = 0.0094$). Differences were also observed at L5, between the early rehabilitation group and the non-rehabilitated group, and between the FES group and the non-rehabilitated group (FES + treadmill vs Non-rehab, $P = 0.0136$; FES vs Non-rehab, $P = 0.049$). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

Limited impact of rehabilitation on excitatory–inhibitory inputs to lumbar motoneurons

To evaluate the effect of the different rehabilitation strategies on functional recovery, the expression of the excitatory marker VGLUT2 and the inhibitory marker VGAT were quantified around spinal motoneurons in the L5 segment, which were identified by ChAT immunostaining (Figure 40A). Although no statistically significant differences were observed for either VGLUT2 or VGAT due to the large variability, a downward trend in fluorescence density for the inhibitory marker was noted in non-rehabilitated animals (Figure 40B-C).

When assessing changes in the excitatory/inhibitory balance across rehabilitation groups, no statistically significant differences were detected, likely due to high intragroup variability (Figure 40D).

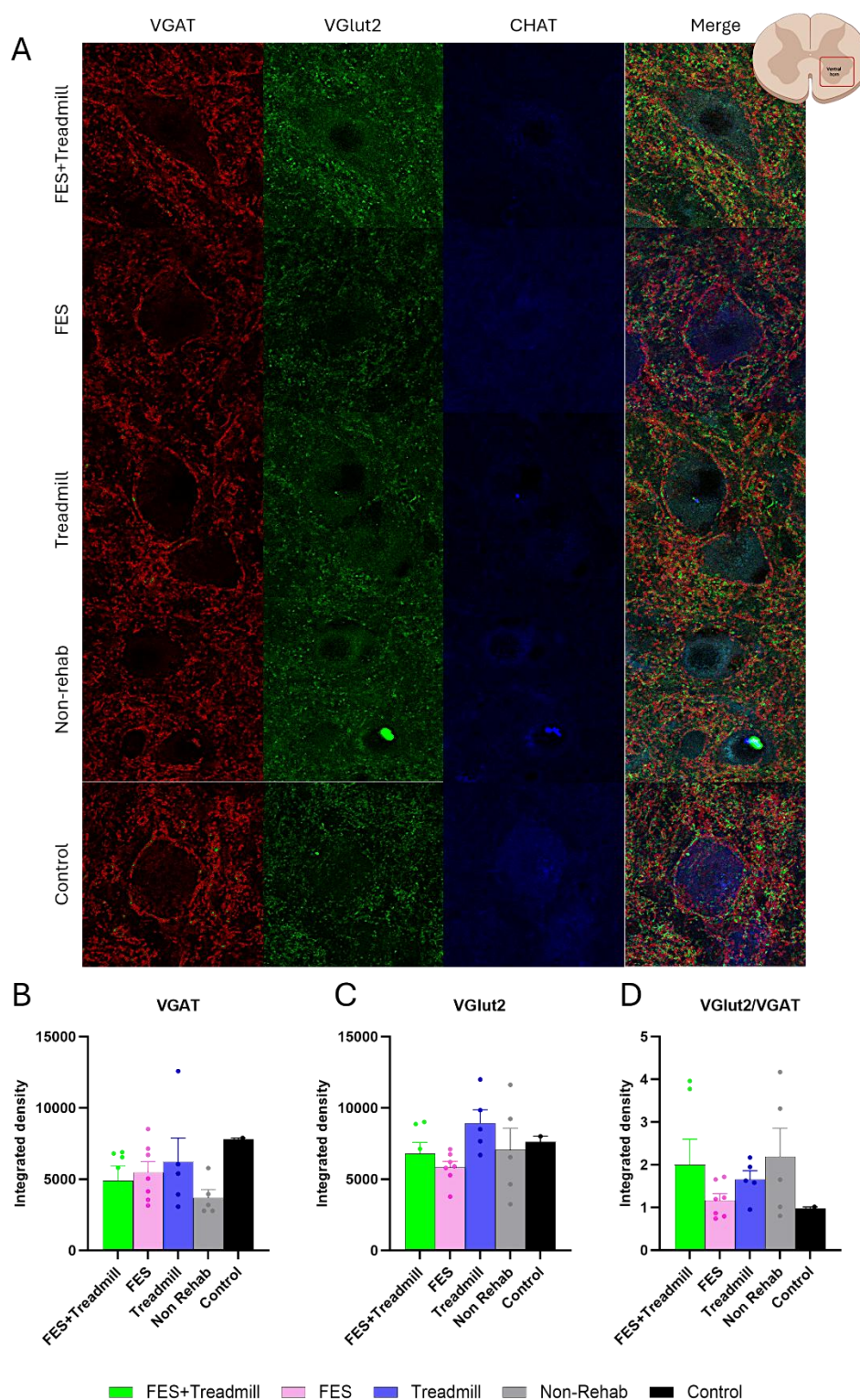


Figure 40. A. Representative immunofluorescence images showing VGAT, VGLUT2, and ChAT staining, and merged. B–C. Quantification of VGAT (B) and VGLUT2 (C) immunoreactivity within a 4 μ m perisomatic region surrounding ChAT+ motoneurons. D. VGAT/VGLUT2 ratio calculated per animal. No statistically significant differences were observed in any of the quantified parameters, as determined by one-way ANOVA followed by Tukey's correction for multiple comparisons.

VII. Discussion

Translational Research Using Animal Models

Investigating the pathophysiological mechanisms that follow SCI, as well as developing and evaluating therapeutic interventions, presents important limitations in humans. For this reason, animal models are widely used to study injury progression and assess rehabilitation strategies. Rats are the most commonly used species because they offer low cost, ease of handling, and a high degree of similarity to human SCI pathology, including the formation of a cystic cavity that closely resembles that observed in patients (Sharif-Alhoseini et al., 2017). In addition, their spinal circuits provide an appropriate platform for studying neuroplasticity, synaptic reorganization, and functional recovery under controlled experimental conditions that cannot be reproduced in humans.

The most commonly used experimental models of SCI include contusion, compression, and transection. Among these, the contusion model is considered the gold standard because of its high clinical relevance, as it reliably reproduces the mechanical forces involved in traumatic human injuries, such as those caused by traffic accidents or falls. Unlike human injury, rodents require a prior laminectomy, which allows the impact force to be applied in a controlled and reproducible manner while avoiding secondary bone damage that would complicate interpretation of the results. Modern electromechanical devices make it possible to precisely regulate impact velocity, force, and displacement, further improving experimental standardization (C. J. Lam et al., 2014). Compression models are particularly useful for studying ischemia–reperfusion processes and the effects of decompression timing, whereas partial or complete transection models are essential for investigating axonal regeneration and collateral sprouting (Hodgetts et al., 2009).

Beyond reproducing the injury itself, rat models are fundamental for studying rehabilitation approaches following SCI, including treadmill training, epidural stimulation, neuromodulation, and FES. Evidence obtained from these models demonstrates that early, activity-dependent rehabilitation can induce substantial reorganization within spinal and supraspinal circuits, facilitating partial recovery of locomotor patterns after severe injury (Loy & Bareyre, 2019; Tsai et al., 2025). In this way, the rat model constitutes an essential bridge between basic neuroscience and the development of translational therapies for SCI rehabilitation.

Nevertheless, the use of experimental animals inevitably raises ethical considerations. At present, there is no alternative capable of reproducing with the same fidelity the complexity of nervous system biology and physiology in the context of SCI and rehabilitation. However, advances in sophisticated *in vitro* models, organoids, and computational simulations raise the possibility that, in the medium and long term, dependence on animal models may be significantly reduced and some of these studies eventually replaced by alternative approaches. In this context, work with rats should be understood as a necessary and transitional step within a broader strategy aimed at achieving a deeper understanding of the mechanisms underlying injury and recovery, with the goal of minimizing the number of animals used and, whenever possible, replacing them with computational models and non-animal experimental systems.

Choice of the Model

In this thesis, we selected a thoracic contusion model at the T9 level because, among the wide range of experimental SCI models (contusion, compression, hemisection, complete transection, etc.), the contusion model most accurately reproduces both the biomechanics of trauma and the cascade of primary and secondary damage observed in the majority of traumatic SCIs in humans. Several reviews have highlighted that contusion models allow the generation of a controllable severity gradient with good reproducibility, more comprehensively recapitulate the acute and secondary phases of injury (hemorrhage, inflammation, central necrosis, cavitation, and gliosis), and are therefore considered the most translational model for evaluating therapeutic strategies aimed at preserving and modulating spared tissue (All & Al-Nashash, 2021; M. Q. Li et al., 2025). In contrast, transection models, although useful for studying regeneration in the context of complete pathway interruption, represent an extreme condition that is uncommon in current clinical practice and fail to capture the complexity of the contusion-compression pattern, which combines heterogeneous tissue damage with partially preserved pathways. Consequently, they are less suitable as a platform for analyzing rehabilitative interventions such as those proposed in this study (M. Q. Li et al., 2025).

In particular, low thoracic contusions in the T8–T11 segments, applied with intensities between 150 and 200 kdynes, are widely used because they produce

marked locomotor deficits in the hind limbs while still allowing partial spontaneous recovery that can be modulated by therapeutic interventions (Norrie et al., 2005; Tsai et al., 2025). In a study specifically focused on hind limb locomotion and the application of FES to extensor musculature, we selected a contusion at a low thoracic segment (T9), as it provides a motor deficit localized to the hind limbs with strong relevance for real incomplete SCIs in humans.

In the first chapter of this thesis, we analyzed which degree of SC contusion severity was most appropriate for studying the effects of treadmill-based rehabilitation and FES on locomotion. Although it may seem intuitive to associate a more severe injury with a greater functional deficit, it is important to consider not only the amount of damaged tissue, but also which tracts are affected, since different descending pathways contribute differently to motor function. In rats, locomotor deficits following SCI largely depend on which descending pathways are damaged, as each tract plays a specific role in gait control (Grillner, 1981; Pearson, 2004). Thus, reticulospinal and vestibulospinal tracts are particularly important for posture and weight support. Vestibulospinal neurons project to interneurons and extensor motoneurons and help stabilize the head and body in response to balance disturbances, whereas reticulospinal neurons integrate somatosensory afferents from all four limbs and generate coordinated motor patterns required for locomotion (Pearson, 2004). These systems are complemented by the rubrospinal and corticospinal pathways, which provide finer and more adaptive control of locomotor patterns by adjusting flexor–extensor activity and enabling precise step modifications (Grillner, 1981). Together, the combination and extent of damage to these tracts determine the severity of gait impairment after SCI and the capacity of the system to reorganize (Grillner, 1981; Peterson, 1984).

In the context of a progressive SCI, locomotor deficits also worsen in a stepwise manner as different descending tracts become successively compromised. In milder injuries, preferential damage to the dorsal corticospinal tract and, to a lesser extent, the rubrospinal tract mainly results in a loss of voluntary control and reduced ability to precisely adjust step amplitude and direction, while the basic locomotor pattern generated by the spinal CPGs may remain relatively preserved. As the injury expands and increasingly affects the reticulospinal pathways, the ability to initiate and

sustain rhythmic locomotion declines, leading to greater instability and fatigue during gait. Finally, when the damage reaches the vestibulospinal tracts and the more medial and ventral portions of the descending systems, weight support and postural control become critically impaired, causing the locomotor pattern to be severely disrupted or abolished, even though the spinal rhythm-generating circuits may still remain functional.

The locomotor deficits observed in our model follow this same logic of progression with increasing injury severity. Animals subjected to severe contusions (300 kdynes) were unable to support their own body weight throughout the two-month follow-up period and exhibited the greatest mechanical hypersensitivity, together with marked hyperexcitability of the H-wave in electrophysiological recordings. This profile indicates profound disruption of spinal circuits and a pronounced increase in pain sensitivity, but at the same time greatly limits the animals' ability to actively participate in locomotor training. Since one of the key requirements of the proposed intervention was that the animals be able to bear weight and walk independently on the treadmill, 300-kdyne contusions were excluded as the experimental model.

At the opposite end of the spectrum, mild injuries (100 kdynes) were associated with a high degree of spontaneous recovery across all functional tests, particularly in electrophysiological measures, where clear and consistent H-wave depression in response to repetitive stimulation and motor evoked potentials with greater amplitudes and preserved latency were observed. This pattern suggests substantial preservation of corticospinal transmission and segmental inhibitory mechanisms, thereby considerably reducing the margin for detecting additional improvements attributable to therapeutical interventions.

The 200 kdynes contusion produced an almost complete loss of hind limb mobility and absence of voluntary stepping during the acute phase, followed by progressive recovery toward limited but present weight support and locomotion, with intermediate BBB scores and clearly impaired coordination. The produced locomotor deficit appeared as sufficiently pronounced to detect therapeutic effects of rehabilitation without compromising participation in training or the residual plasticity upon which the intervention may act.

The H-wave hyperexcitability and the reduced depression in response to repetitive stimulation observed in the more severe groups are consistent with previous studies in murine models, in which SCI induces persistent reflex facilitation and incomplete H-wave depression during train stimulation (Lee et al., 2009). Similarly, Redondo-Castro et al. (2013) reported that rats subjected to 100 or 200 kdynes injuries show clear differences in evoked potentials: in milder injuries, the MEP is reduced but still present compared with healthy animals, whereas in 200 kdynes lesions the MEP is markedly attenuated or absent (Redondo-Castro, Torres-Espín, et al., 2013). Our results follow the same trend, as the MEP response in animals with injuries of 200 or 300 kdynes was notably reduced or abolished. At the histological and molecular levels, we also observed a severity-dependent gradient, characterized by progressive motoneuron loss, increased astrogliosis (GFAP) and microgliosis (Iba1), and a tendency toward reduced KCC2 expression as contusion severity increased, with intermediate values in the 200 kdynes group. These findings are consistent with the previously described relationship between injury severity, neuroinflammation, and spinal circuit remodeling reported by Redondo-Castro et al. (2013) (Redondo-Castro, García-Alías, et al., 2013).

Taken together, the convergence of locomotor (BBB), mechanical nociception, reflex and corticospinal excitability, and morphological observations indicates that the 200 kdynes contusion represents an intermediate degree of injury: more severe than 100 kdynes lesions, with clear alterations in excitability and neuroinflammation, but less extreme than 300 kdynes injuries, in which structural damage, hypersensitivity, and inability to support body weight compromise both participation in training and the potential for locomotor recovery. This profile makes the 200 kdynes model particularly suitable for the objectives of this thesis, as it provides a locomotor deficit sufficiently pronounced to detect FES-induced changes while preserving an anatomical and functional substrate upon which spinal and supraspinal plasticity mechanisms triggered by the intervention can operate.

Application of Locomotor Rehabilitation and FES in the Thoracic Contusion Model

In the second chapter of the thesis, the effects of different rehabilitation paradigms on locomotor recovery following a 200 kdynes thoracic cord contusion were analyzed.

Stimulation Pattern and Choice of Electrode Type

In this study, we employed muscle-targeted stimulation using intramuscular electrodes connected to a percutaneous connector, which provided a stable interface with the external stimulator and a relatively constant activation geometry throughout the training protocol. Although this approach requires somewhat higher current intensities than direct intra-nerve stimulation, the current intensity needed was still quite low, since it actually activates the intramuscular nerve. It offered an appropriate balance between selectivity, safety, and feasibility in a model of incomplete SCI. In addition, it avoided several limitations associated with surface electrodes, such as variability in placement, the need for high stimulation intensities, and the undesired activation of cutaneous mechanoreceptors and nociceptors. Previous studies, including Beaumont et al. (2014) and Jung et al. (2009), have also supported the use of this type of intramuscular stimulation (Beaumont et al., 2014; Jung et al., 2009).

Compared with fully implanted systems, the intramuscular configuration with a percutaneous output retained many of the functional advantages of implantable devices, including access to deep musculature and stable motor responses, while still sharing some of the limitations inherent to transcutaneous connections. This trade-off should be considered when extrapolating these results to more advanced clinical configurations.

Stimulation Parameters: Pulse Shape, Frequency, and Amplitude

In our protocol, symmetric biphasic stimulation at 40 Hz was used, with amplitudes ranging between 2–4 V adjusted to the motor threshold in each session. The choice of a symmetric biphasic pulse was based on safety considerations at the electrode–tissue interface. By reversing the polarity during each pulse phase, most of the potentially harmful electrochemical processes generated during the first phase are

effectively reversed, thereby reducing the risk of tissue damage and electrode corrosion associated with net charge accumulation. This type of pulse is the most recommended in FES systems intended for repeated or chronic use, as it helps preserve both tissue and implant integrity over the long term (Marquez-Chin & Popovic, 2020; Peckham & Knutson, 2005).

The selected stimulation frequency (40 Hz) lies within the range commonly used in FES to produce relatively smooth and functional tetanic contractions, avoiding discharges perceived as individual “jerks” and promoting a more continuous force profile. However, it is well established that FES tends to recruit motor units in a reverse physiological order, preferentially activating fast-twitch motor units, which generate greater force but are also more susceptible to fatigue. In addition, so-called low-frequency fatigue may occur within the 15–50 Hz range, as the coupling between neural excitation and muscle contractile response progressively deteriorates (Luo et al., 2020). Consequently, most neuroprosthetic systems regulate contraction strength by modulating pulse amplitude or duration while maintaining the stimulation frequency as low as possible within the range required to achieve a functional tetanic contraction, with the aim of minimizing premature fatigue. In our case, a fixed frequency of 40 Hz was selected, while the amplitude was adjusted between 2–4 V to achieve the minimum effective motor threshold in each session. This strategy ensured the desired muscle contraction while simultaneously reducing the total stimulation load applied to the muscle.

During follow-up, we observed that as the intervention progressed, progressively higher voltages were required to achieve an equivalent contraction. This could reflect several non-mutually exclusive phenomena, including local changes in tissue conductivity (for example, scar tissue formation around the electrode), some degree of cumulative muscle fatigue, or modifications in the impedance of the electrode itself. Although the study design does not allow us to precisely discriminate the relative contribution of each of these factors, this finding highlights the importance of dynamically adjusting and personalizing FES parameters over time and supports recent proposals advocating optimization of electrode placement, feedback control, and rehabilitation exercise dosing as key strategies to reduce FES-induced fatigue (Luo et al., 2020; Marquez-Chin & Popovic, 2020).

Importantly, the need to increase voltage emerged from the onset of stimulation rather than from the time of SCI itself. In other words, regardless of whether animals began FES rehabilitation at 3 dpi or 14 dpi, they initially showed similar sensitivity to stimulation, and it was approximately two weeks after the first stimulation sessions that voltage increases became necessary. This observation suggests that the stimulation itself may induce changes either in the electrode or in the surrounding tissue, resulting in a greater current requirement to achieve muscle tetanization and contraction. Although we verified that the implanted intramuscular electrode did not produce overt alterations in the muscle, it would be of interest to conduct a more detailed analysis of muscle and electrode interface changes following the daily stimulation protocol.

Temporal Pattern: Bursts and Alternating Extensor Activation

Regarding the temporal pattern, stimulation was delivered in 0.25 s bursts to the extensor muscles, alternating between both hind limbs. This duration was estimated taking into account that the average duration of a rat step during treadmill locomotion at low-to-moderate speed was approximately 0.5 s, consistent with the temporal structure described in kinematic studies of rat locomotion, in which cycle frequencies of approximately 1–2 Hz (cycle durations of 0.5–1 s) have been reported under comparable speed and gait conditions, both in intact animals and following SCI (Grillner, 1981). Since our paradigm employed an alternating bipedal pattern and stimulated only the extensors, which approximately correspond to half of a normal gait cycle, the burst duration was set at 0.25 s for each hind limb so that the electrical activation would respect the physiological timing of the rat locomotor cycle.

In this way, the stimulation pattern reproduces, in a simplified manner, the alternating flexor–extensor and left–right organization of locomotion, providing repeated but intermittent activation of the extensors in each limb, with periods of mechanical and metabolic recovery between bursts. This temporal alternation reduces the continuous load placed on the same muscle group, which likely contributed to limiting fatigue despite the use of a 40 Hz stimulation frequency. At the same time, it generates a rhythmic and bilateral afferent input pattern particularly suited to interact with the spinal locomotor circuits.

The decision to focus exclusively on extensor muscles was conditioned by the spontaneous progression of the model. Following the 200-kdyne thoracic contusion, animals transition from a phase of almost complete hind limb paralysis to a gradual recovery of mobility and, during the subacute weeks, regain the ability to stand. However, their resting posture clearly tends toward hind limb flexion (M. Q. Li et al., 2025). At this early stage, we considered it a priority to promote the less-used movement pattern (extension), allowing flexion to arise primarily from voluntary activation and the relaxation of extensors rather than being externally imposed by stimulation. Accordingly, the intervention was focused on antigravity extensor function and the early recovery of weight support.

Furthermore, studies in humans have shown that flexor and extensor muscles may differ substantially in their intrinsic excitability properties. Kim et al. reported that persistent inward currents estimated using the delta F method are greater in motoneurons of the tibialis anterior than of the soleus, suggesting an asymmetric neuromodulatory balance between these muscle groups at the ankle joint (E. H. Kim et al., 2020). In this context, choosing to stimulate only extensors allowed us to selectively reinforce weight-support function without indiscriminately increasing flexor excitability, while also accommodating the limitations of the available hardware (six channels in the headplug), which required prioritizing the quadriceps and gastrocnemius muscles of each hind limb as the main stimulation targets.

On the other hand, prioritizing extensor musculature without including flexors means that a portion of the muscles involved in gait did not receive stimulation or mechanical loading comparable to that provided during full locomotor training. Consequently, the muscle strengthening and intermuscular coordination induced were likely more modest and less global than those that would be achieved with an active walking protocol.

In addition, FES was applied in the home cage, with animals awake but mostly at rest or performing only brief movements, rather than during a sustained locomotor task that synchronized stimulation with the gait cycle. Ideally, stimulation should be coupled to locomotion through closed-loop systems or systematically combined with treadmill training. However, in our case, implantable solutions for detecting weight support proved technically unfeasible. Likewise, the simultaneous

application of FES during treadmill walking compromised the animals' balance, as previously described by Beaumont et al. (2014). Nevertheless, future developments enabling implanted FES coupled to active locomotion could provide a more comprehensive rehabilitative approach.

Comparison Between FES and Treadmill Rehabilitation

In this study, we aimed to evaluate how FES promotes plasticity following SCI in comparison with physical rehabilitation based on treadmill locomotion, which represents one of the standard therapeutic approaches used clinically. Overall, our results indicate that both interventions produce comparable functional improvements relative to non-rehabilitated animals, particularly when locomotor recovery is assessed using the BBB scale, in which both groups achieved similar scores during the final stages of the study. Numerous studies have demonstrated the beneficial effects of FES on locomotor recovery when applied intramuscularly, intraneurally, or superficially in both animal models and humans (Beaumont et al., 2014; Ho et al., 2014; Tsai et al., 2025; Unger et al., 2025). However, analysis of the BBB subscore revealed earlier and more pronounced improvements in the treadmill rehabilitation group, likely because this paradigm promotes a more complete and coordinated activation of the musculature involved in gait. In contrast, extensor FES applied in the home cage primarily recruits hind limb extensors and is delivered under conditions of limited spontaneous activity.

The interpretation of these differences should be framed within the concept of activity-dependent plasticity following SCI, according to which different exercise and stimulation paradigms generate specific patterns of supraspinal and spinal reorganization. Studies in both animal models and humans have shown that locomotor training and other forms of intensive rehabilitation induce changes in neurotrophic factors, cord electrophysiological properties, and circuit connectivity, and that these adaptations are largely specific to the type of activity applied (Lynskey, 2008). In this context, our results suggest that extensor FES and treadmill locomotion represent two distinct ways of “training” the lumbar spinal cord. Although both interventions improve overall locomotion, FES appears to promote a more focal form of plasticity within the L4–L6 segments, associated with greater

motoneuron preservation, more effective restoration of segmental inhibition, and neurochemical changes consistent with improved regulation of spinal excitability.

However, when segmental spinal excitability is considered, electrophysiological testing revealed important differences between both approaches. Specifically, the FES-treated group showed a more pronounced reduction in H-wave amplitude during the repetitive discharge depression (RDD) protocol, with statistically significant differences compared with the non-rehabilitated group. This finding indicates not only an attenuation of the basal hyperexcitability of the Ia–motoneuron reflex arc, but also a recovery of presynaptic inhibitory mechanisms and segmental inhibitory circuits capable of depressing Ia transmission during repetitive stimulation (Brangaccio et al., 2024; Jankowska, 2013; Knikou, 2008). The greater reduction of the H-wave at frequencies of 10–20 pps suggests a more substantial restoration of the ability to activate GABAergic interneurons that mediate presynaptic inhibition of Ia afferents, as well as improved efficacy of recurrent and reciprocal inhibitory pathways that together reduce reflex gain during repeated use (Côté et al., 2018; Jankowska, 2013; Tsai et al., 2025).

These modifications were less evident in the treadmill rehabilitation group, in which neither the H/M ratio nor H-wave depression differed clearly from the values observed in injured untreated animals. Thus, although treadmill training improves overall locomotion and weight-support patterns, FES appears to exert a more direct and specific effect on the excitability of spinal reflex circuits and on the capacity of the system to dynamically modulate Ia input.

On the other hand, nociceptive testing did not reveal clear differences between the two rehabilitation protocols, as both FES and treadmill training were associated with comparable improvements in nociceptive responses relative to the non-rehabilitated group. This suggests that, under the conditions of this study, changes in pain sensitivity may depend more on the presence of repeated activity and sensory stimulation than on the specific type of intervention applied.

At the cellular level, the functional differences between FES and treadmill rehabilitation were accompanied by clearly distinct patterns of spinal plasticity. The FES group showed greater motoneuron survival in the caudal lumbar spinal cord

segments (L4–L6) compared with both the physical rehabilitation group and the non-rehabilitated group, despite the fact that lesion area under the curve and lesion epicenter were similar across groups, ruling out the possibility that these differences resulted from reduced primary injury severity. This preservation of motoneurons is consistent with the fact that stimulation was applied to the quadriceps and gastrocnemius muscles, whose afferents and motoneuron pools are predominantly distributed within L4–L6. Repeated activation of these muscles and their corresponding nerves (femoral and sciatic) may maintain a continuous flow of afferent and efferent information, thereby preventing the chronic “functional disconnection” of these motoneuron pools and limiting their degeneration.

Although our findings were obtained in a spinal cord contusion model, they are consistent with observations from peripheral nerve injury models, in which pulsed electrical stimulation has demonstrated direct neuroprotective effects on spinal neurons, reducing cell loss and improving functional recovery (Pei et al., 2015). In contrast to studies using spinal cord stimulation (SCS/transspinal stimulation), where the main reported effects involve increased motoneuronal output and improved voluntary motor control without evident changes in motoneuron number (Balaguer et al., 2025; L. M. Murray & Knikou, 2019), our data suggest that chronic peripheral FES may not only functionally modulate the excitability of these circuits but also contribute to structural preservation of motoneuron pools within the stimulated lumbar segments.

In parallel, changes in the KCC2 cotransporter further support the idea of a more effective restoration of spinal inhibition in the FES group. Whereas all injured groups showed reduced KCC2 expression in ventral L2 compared with controls, indicating a persistent rostral inhibitory deficit, KCC2 signal in ventral L5 was greater in FES-treated animals than in non-rehabilitated animals, and no comparable increase was observed in the treadmill group. Given the role of KCC2 in maintaining low intracellular chloride concentrations and ensuring that activation of GABAA/glycine receptors produces hyperpolarizing IPSPs, this increase in ventral L5 is consistent with the reduction in the Hmax/Mmax ratio and the greater H-reflex depression observed following FES. More effective inhibition at the motoneuron level would facilitate both a reduction in the basal gain of the Ia–motoneuron reflex arc and an

enhanced ability to depress reflex responses during repetitive stimulation. In this regard, our findings are in agreement with studies in chronic SCI models showing that restoration of chloride homeostasis through increased KCC2 activity or expression is associated with improved rate-dependent depression of the H-reflex and reduced hyperreflexia and spasticity (Beverungen et al., 2020; Bilchak et al., 2021).

Finally, analysis of serotonergic innervation revealed an increase in 5-HT fiber density within the ventral horn of L5 in the FES group compared with the non-rehabilitated group, whereas this difference was not observed in the treadmill group. Since serotonin increases motoneuronal excitability and modulates persistent inward currents but may also promote KCC2 function and the recruitment of inhibitory circuits in the context of rehabilitation, the greater presence of serotonergic fibers in ventral L5 following FES suggests a stronger neuromodulatory drive over the segments controlling extensor musculature (Ghosh & Pearce, 2015; K. C. Murray et al., 2010). Taken together, the coexistence of greater motoneuron survival, increased KCC2 expression, and higher 5-HT density in L4–L6 in the FES group provides a structural and neurochemical substrate consistent with the reduction in reflex hyperexcitability and restoration of segmental inhibition, in contrast to the more limited impact of treadmill training on these parameters.

Overall, these findings reinforce the utility of FES as a rehabilitation tool following SCI, even when compared with widely used interventions such as treadmill training. The fact that relevant functional and spinal changes were observed using a relatively focused protocol targeting extensor musculature suggests that FES still has considerable room for optimization. It is reasonable to propose that more comprehensive stimulation protocols, integrating broader and more coordinated recruitment of the muscle groups involved in locomotion together with temporally optimized dosing strategies, could further enhance these beneficial effects and ultimately translate into more robust locomotor recovery.

Early Rehabilitation

It is well established that there is a critical window between the time of SCI and surgical decompression, during which secondary injury can be limited and

neurological prognosis improved (Ahuja et al., 2020; Pedro & Fehlings, 2022). This concept, commonly summarized as *time is spine*, highlights that earlier intervention is associated with fewer complications and better neurological outcomes. Extrapolating this idea to the field of rehabilitation, it is reasonable to propose that initiating rehabilitative therapies as early as possible could translate into faster and more effective recovery. In this context, one of the potential advantages of FES is that it can be applied even when the patient is unable to support body weight, allowing stimulation to begin at very early stages, including during bed rest in the hospital setting. For this reason, this thesis explored the application of FES starting at 3 dpi, with the aim of determining whether such an early intervention could trigger early plastic changes at the spinal level.

In previous studies, Beaumont et al. described improved locomotor recovery when FES was applied to the quadriceps and hamstrings muscles between 2 and 6 days post injury, advancing by several days the recovery of weight support and nociceptive function, although without increasing the final degree of recovery compared with later rehabilitation (Beaumont et al., 2014). These findings may indicate that early FES may not raise the ultimate functional “ceiling,” but it does accelerate the achievement of motor and sensory milestones, allowing rehabilitated animals to outperform untreated animals during the recovery process (Beaumont et al., 2014). Along the same lines, Tsai et al. demonstrated that daily electrical stimulation of both sciatic nerves initiated 3 days after contusion improves BBB scores more rapidly and to a greater extent than sham stimulation, while Norrie et al. showed that beginning training immediately after injury nearly doubles recovery compared with delaying rehabilitation by three months, despite similar lesion sizes (Norrie et al., 2005; Tsai et al., 2025).

These studies support the notion that advancing the onset of training or stimulation is more effective than applying the same protocol at later stages, and that delaying rehabilitation limits the ability of the nervous system to exploit therapy-induced plasticity. This concept of a “critical window” is further reinforced by the protocol proposed by Henry et al., which aims to directly compare early versus delayed locomotor training after incomplete SCI. Based on evidence from rodent models and retrospective human studies, this work identifies the first months after injury,

particularly the first ~3 months, as the period with the greatest capacity for functional recovery and prevention of neuropathic pain and neuroinflammation (Henry et al., 2024).

Our results are consistent with this concept of a “temporal benefit” rather than an increase in maximal recovery, and they allow a more precise characterization of the type of plasticity induced by early rehabilitation compared with other interventions. In the experimental model used, FES rehabilitation initiated at 3 days post injury did not produce clear differences in electrophysiological testing between the different rehabilitation groups, although consistent improvements were observed compared with non-rehabilitated animals. Unlike chronic FES, which was associated with a reduction in the Hmax/Mmax ratio and a more pronounced depression of the H-reflex, the early rehabilitation group displayed an H/M ratio similar to that of untreated injured animals, indicating that the basal hyperexcitability of the Ia-motoneuron reflex arc persisted. Nevertheless, these animals showed greater post-activation depression of the H-reflex than non-rehabilitated animals, particularly at frequencies of 10–20 pps, suggesting a partial recovery of the presynaptic and interneuronal inhibitory mechanisms that dampen reflex responses during repetitive stimulation. In contrast, treadmill-only training did not significantly modify either the Hmax/Mmax ratio or H-reflex depression relative to untreated animals, pointing to a more limited impact on segmental excitability when early rehabilitation does not include a component of targeted electrical stimulation.

At the histological level, the early FES group followed trends very similar to those observed in the treadmill rehabilitation group regarding motoneuron survival and microglial and astrocytic responses, despite presenting a larger lesion area under the curve than the other groups. More specifically, increased KCC2 expression was detected exclusively in the dorsal horn of L5 in animals receiving early rehabilitation compared with non-rehabilitated animals. This change was not observed in the other rehabilitation paradigms and suggests a localized modulation of sensory inhibition, without a global normalization of the excitatory/inhibitory balance or GABA levels. This pattern is consistent with the electrophysiological profile described above, in which the capacity to depress the reflex during repetitive stimulation recovers earlier than the basal excitability of the motoneuron pool.

Taken together, these findings indicate that early rehabilitation does not primarily act as a “neuroprotective” intervention that prevents the progression of secondary damage, but rather as a strategy that enhances the function and plastic potential of residual pathways. In other words, early FES appears to optimize the use of spared circuitry instead of structurally stabilizing the evolving lesion.

In parallel, the serotonergic changes observed in the ventral horns of L2 and L5, with a significant increase in 5-HT innervation in the early FES group compared with non-rehabilitated animals, and not reproduced by treadmill training, suggest that early electrical stimulation is capable of selectively activating key modulatory systems involved in motoneuronal excitability and locomotor circuit reorganization. Once again, this places early rehabilitation in an intermediate state between the untreated hyperexcitable spinal cord and the more complete normalization observed following chronic FES.

Taken together, the findings of Beaumont et al., the observations of Tsai et al. and Norrie et al., the temporal hypotheses proposed by Henry et al., and the results of this thesis converge on the idea that FES applied during the early post-injury window does not necessarily increase the final level of functional recovery achieved, but it does accelerate the appearance of motor and sensory improvements and triggers specific plastic changes at the spinal level. Furthermore, our findings suggest that this early rehabilitation induces an “intermediate” state of plasticity, characterized by partial recovery of dynamic reflex modulation mechanisms and activation of serotonergic neuromodulatory systems, even when the basal excitability of the Ia–motoneuron reflex arc and certain structural markers have not yet fully normalized. In this sense, early FES should be understood less as a means of halting secondary injury progression and more as an intervention that facilitates early engagement and reorganization of spared spinal and supraspinal circuits. These observations reinforce the hypothesis that there is a particularly sensitive window of plasticity during the subacute phase, in which interventions such as FES may modulate the trajectory of spinal and supraspinal reorganization, even when global locomotor scales do not reveal major differences at the final recovery endpoint.

For future studies, it would be particularly interesting to characterize in more detail the molecular state of the spinal cord between 14 and 30 days post injury, corresponding to the period in which the earliest functional improvements induced by FES are observed. A targeted analysis of markers related to excitability, neuroinflammation, and synaptic plasticity during this interval could help identify the mechanisms that distinguish early rehabilitation from other protocols and explain why certain functional gains emerge more rapidly without necessarily altering the final level of recovery achieved.

It would also be highly valuable to obtain a more refined characterization of locomotion. In this study, an attempt was made to implement kinematic analysis; however, limitations in the available equipment (an insufficient number of cameras to fully cover the recording environment) and the initial lack of experience in marker placement generated excessive variability, preventing reliable comparisons of the same animal before surgery and at day 60. Under these conditions, no clear differences between groups were detected beyond a tendency toward greater paw dragging during the swing phase and a lower hip position in injured animals. An optimized kinematic protocol, incorporating a large number of cameras, a better-defined capture volume, and rigorous standardization of marker placement, could reveal subtle patterns of locomotor recovery that are not captured by global scales such as the BBB.

Taken together, the current findings position early FES as an intervention capable of accelerating functional milestones and inducing an intermediate state of spinal plasticity, characterized by partial recovery of reflex modulation and activation of key neuromodulatory systems, even when basal excitability and certain structural parameters have not yet fully normalized. On the other hand, combination with later onset treadmill training showed an enhanced functional recovery of the animals after SCI.

FES, Regeneration, and Guidance of Plasticity

Beyond immediate functional improvement, a key challenge in rehabilitation after SCI is not only to promote regeneration and axonal sprouting, but also to guide this plasticity in order to avoid maladaptive configurations. It has been reported that the

orientation of electrical fields and, in particular, the relative position of the cathode influence the direction of axonal growth, which may affect both motor and sensory function (Hamid & Hayek, 2008). In this context, stimulation applied through the periphery is of particular interest because, by activating specific afferents that project to the dorsal horn and from there to defined motoneurons and segmental circuits, FES could help direct axonal sprouting and synaptic reorganization in a more selective manner for each muscle group, especially when delivered using discharge patterns that mimic locomotor activity.

This activity-dependent guidance could help reduce maladaptive plasticity, such as aberrant connections or excessively excitable circuits, while promoting more efficient circuits for functional recovery. Indeed, FES treatment reduced H hyperreflexia, a surrogate marker of spasticity. From this perspective, it would be highly interesting in future studies to analyze the orientation and organization of axons and synaptic terminals under different rehabilitation protocols, comparing FES, treadmill training, and the absence of rehabilitation. Likewise, it would be of great value to compare a nonspecific peripheral stimulation pattern with one that more faithfully reproduces the temporal and spatial characteristics of gait, in order to determine to what extent the “quality” of the activation pattern influences the direction and functional relevance of regeneration. Such studies could help define FES protocols not only as rehabilitation tools, but also as strategies to guide nervous system plasticity in a safe and effective manner following SCI.

Clinical Relevance

Although many of the changes described in this work may appear quantitatively modest within the experimental setting, their translation to the clinical context could have a highly significant impact on the daily lives of individuals with SCI. Improvements that in the laboratory are reflected as slight gains in strength, weight-support stability, or gait coordination may, in practice, represent the difference between needing assistance to climb a flight of stairs and being able to do so independently, or between requiring continuous support for transfers and performing them autonomously. From the patient’s perspective, these gains translate into a real increase in independence and participation in activities of daily

living, underscoring the importance of optimizing even those interventions that “only” produce gradual or partial improvements in functional outcomes.

In this regard, FES-based rehabilitation emerges as a particularly attractive therapeutic approach. In our experiments, FES demonstrated the capacity to modulate spinal excitability and promote patterns of plasticity that more closely resemble a physiological state. In addition, it offers the practical advantage of being applicable without requiring the patient to support body weight. This makes it especially suitable for individuals with more severe injuries and greater motor deficits, in whom other forms of locomotor training are difficult or impossible to implement during the early stages of recovery. Considering FES as a standard rehabilitation option, at least as an adjunctive therapy in more severe cases, could help expand the therapeutic window and provide opportunities for functional recovery to patients who currently have fewer therapeutic alternatives available.

Despite the substantial evidence supporting the benefits of FES, its accessibility and implementation in routine rehabilitation for individuals with SCI remain challenging. Logistical, economic, and organizational barriers continue to limit its widespread adoption, further compounded by the need for standardized clinical protocols, specialized staff training, and resources to individualize stimulation parameters (Brown et al., 2023). As discussed in previous sections, numerous studies have demonstrated consistent clinical benefits of FES, including improvements in muscle strength, body composition, and walking ability. These same studies also emphasize the requirement for specialized infrastructure and intensive therapeutic schedules, factors that restrict access in resource-limited settings (Giangregorio et al., 2012; T. Lam et al., 2007). Overall, the literature indicates that, despite its strong evidence base, the successful clinical implementation of FES depends on overcoming structural barriers such as cost, availability of trained personnel, therapeutic time allocation, and access to appropriate technology. Addressing these limitations will be essential to ensure that FES interventions can be reliably integrated into rehabilitation programs for individuals with SCI.

VIII. Conclusions

1. The severity of spinal cord contusion determines the degree of functional and electrophysiological deficits after injury, with more severe lesions being associated with poorer locomotor recovery, higher reflex responses, and a hyperexcitable spinal state that limits the extent of spontaneous recovery.
2. In parallel, increasing lesion severity leads to a progressive loss of lumbar motoneurons, a more intense glial response, and a reduction in KCC2 expression in the caudal spinal cord, creating a predominantly maladaptive plasticity environment characterized by decreased spinal inhibition.
3. Within the range of lesion severity studied, the 200kdynes contusion model offers the best balance between damage and recovery potential, as it produces clear motor and sensory deficits, while still allowing animals to regain weight-bearing and providing sufficient dynamic range to detect the impact of different rehabilitation interventions. Consequently, it emerges as an optimal preclinical model for therapeutic studies.
4. Bilateral FES-based rehabilitation of hindlimb extensor muscles yields locomotor and nociceptive improvements comparable to those obtained with treadmill training but it is associated with a higher normalization of segmental reflex excitability, larger motoneuron preservation in caudal lumbar regions, and a more effective restoration of KCC2-mediated inhibition, together with increased ventral serotonergic innervation in L5.
5. Early FES, initiated in acute post-injury phases, followed by subacute treadmill rehabilitation achieves functional recovery levels comparable to those of only treadmill rehabilitation started in the subacute phase, but accelerates improvement of nociceptive sensitivity and promotes specific reorganization of serotonergic projections in lumbar cord segments, suggesting that this intervention can exploit a critical plasticity window in the subacute period without necessarily altering the final functional level of recovery.
6. The lack of a clear correlation between functional improvements and lack of evidence for morphological cord preservation point out that the intensive rehabilitation protocols initiated after the SCI do not have a neuroprotective effect, although they help to recover partially normal function of spinal pathways.

IX. References

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