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"Fluoxetine treatment promotes functional recovery in a rat model of cervical spinal cord injury"

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Preface

This Thesis consists of two parts: in the first part, I studied the efficacy of fluoxetine on the recovery from spinal cord injury in rats; in the second part, I evaluated the effects of environmental enrichment on visual cortex plasticity in the aging rat. Both of the studies resulted in peer-reviewed publication (Scali et al., 2012; 2013).

When I started my PhD program, spinal cord injury was a completely new field in my lab. Under my Supervisor, Prof. Lamberto Maffei's, suggestion I moved to Cambridge (UK) to learn the necessary skills to bring the technique of the lesion model in Pisa laboratory, and after a necessary training I was able to start the experiments.

I chose to focus the main part of my dissertation on spinal cord injury because I devoted to this project most of my time. The study related to visual cortex plasticity in the aging rat is illustrated in the Appendix.

“When you examine a man with a dislocation of a vertebra of his neck, and you find him unable to move his arms, and his legs. His penis is erect; urine drips from his penis unknowingly. Then you have to say: A disease one cannot treat.”

Edwin Smith Surgical Papyrus, Seventeenth Century B.C

Introduction

Chapter 1: The spinal cord

Spinal cord anatomy

The spinal cord is the part of the central nervous system that controls limb and trunk movements and which receives sensory information from these areas; moreover, the spinal cord has an important role in viscera and blood vessel function of thorax, abdomen and pelvis. It looks like a dorsoventrally flattened cylinder coated by the meninges (dura mater, arachnoid and pia mater) and it lies inside the vertebral canal, where it extends from the base of the skull to the first lumbar vertebra. When observed in a transverse section, the cord appears in the shape of a butterfly constituted by central grey matter (which contains nerve cell bodies) surrounded by external white matter (formed by the axons of ascending and descending pathways and glial cells). The central grey locally organizes motor behavior and regulates sensory signals: the dorsal horns of the butterfly contain the cell bodies of ascending sensory fibers, while the ventral horns contain the motoneurons (Barson and Sands, 1977). The central region, which connects the dorsal and ventral horns, is called intermediate grey matter and surrounds the central canal (which is remnant of the embryological ventricular system and is continuous with the fourth brain ventricle; Figure 1).

The human spinal cord can be divided in 31 segments along its rostral-caudal axis, grouped by anatomists in five sections: cervical (C1-C8), thoracic (T1-T12), sacral (S1-S5), lumbar (L1-L5) and coccygeal (Co1). In other species the number of segments is different, as in the rat, which has 34 segments (C1-C8; T1-T13; S1-S6; L1-L4; Co1-Co3). Each segment is associated with a pair of sensory (arising from the dorsal horns) and motor (arising from the ventral horns) nerves that relay signals between periphery and the central nervous system; the nerve rootlets arising from the cord are bundled together so that one pair of spinal nerves

emerges from each segment. Because of the higher necessity for neural computation in limb regions, the grey matter is larger in cervical and lumbar areas, where the brachial and lumbosacral plexuses arise.

The central grey of was divided by Bror Rexed in ten layers, based on their cytoarchitercture (Rexed, 1952; 1954). The layers, called laminae, are organized in a dorsal-ventral fashion, except for lamina X, which is the neuroglial tissue that surrounds the central canal (Figure 1).

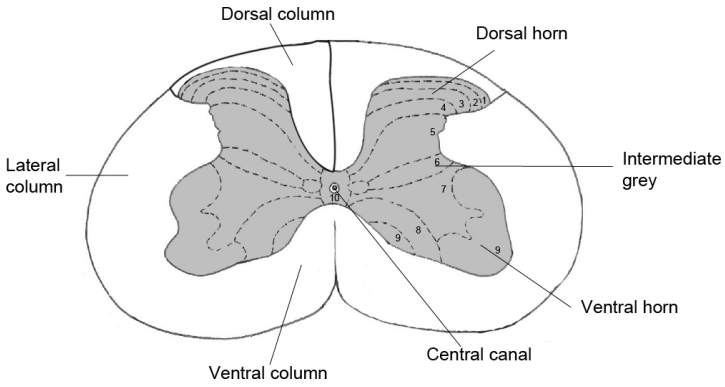

Figure 1. Transverse section of human spinal cord. Grey matter is indicated in grey color. Numbers indicate laminae. Adapted from Watson et al., 2009.

The dorsal horn is formed by laminae I to VI: laminae I-IV are the recipient of sensory input associated with cutaneous stimuli; lamina V is the thickest layer of the dorsal horn and receives afferents from viscera, skin and muscles, while lamina VI is primarily concerned with proprioceptive sensations that signal limb position and movement and is found only in the segments corresponding to cervical and lumbar enlargements. Lamina VII occupies the intermediate grey matter and the dorsal part of the ventral horn; it is constituted by interneurons that connect to motoneuronal pools and is involved in the control of posture and movement. In lamina VIII, propriospinal interneurons modulate motor activity, connecting motoneurons on the same and opposite side of the spinal cord. Lamina IX lies within the ventral horn and represents the main motor area. Axons from this region exit from the ventral root

and innervate muscle fibers. Lastly, lamina X, which surrounds the central canal, receives nociceptive information form skin and viscera (Grau et al., 2006; Watson et al., 2009).

The white matter is formed by longitudinally running fibers and glia. Groups of fibers can be classified based on the anatomical position and function: small bundles of axons located in a given area are defined fasciculi, while a group of fasciculi sharing common features is called funiculus; when a group of nerve fibers have the same origin, course, termination and function, the group is named tract. A group of tracts with a related function constitutes a pathway.

Grey matter horns divide the white matter in three distinct regions: dorsal, lateral and ventral columns (Figure 1). Dorsal columns are located between dorsal horns and are separated by the dorsal median sulcus; they are principally constituted by the central processes of dorsal root ganglion cells, which form the direct pathway conveying skin sensation and proprioception from the limbs and trunk to the brain. The direct pathway is constituted by the cuneate and gracile fasciculi, that ascend ipsilaterally and synapse respectively in the cuneate and gracile nuclei in the medulla oblongata; here, the fibers originating from these nuclei cross the midline and constitute the medial lemniscus pathway, which terminates in the ventroposterolateral nucleus of the thalamus. The gracile fasciculus contains the afferent fibers arising from lower trunk and extremities below the T6 spinal cord segment. In C1-T6 spinal cord segments, the gracile fasciculus is located medially with respect to the cuneate fasciculus, which constitutes the afferent pathway ascending from the upper trunk and extremities. These pathways are involved in transmitting sensations of discriminatory touch, deep pressure, proprioception, sense of position of joints, stereognosis and vibration. In rodents and many non-primate mammals, the ventral-most part of the dorsal column contains the dorsal corticospinal tract (CST), a bundle of fibers that originates primarily from the layer V of primary motor, premotor and somatosensory cortical areas; the axons of pyramidal neurons then

pass through the internal capsule, cerebral peduncle, longitudinal fibers of the pons, and medullary pyramid, to reach the caudal end of the brain stem, where most of them cross to the opposite side in the pyramidal decussation and then lie in the ventral part of the dorsal column of the spinal cord. In most mammals, the majority of CST fibers terminate on the neurons of the medial parts of the base of the dorsal horn and the intermediate grey matter (Rexed's laminae III-VI), which then send connections to lamina IX motoneurons. Differently from rodents, in primates and carnivores the major CST bundle is found in the dorsal part of the lateral column (see Figure 2); moreover, some of its fibers make direct synaptic connections with motoneurons in lamina IX (up to 20% in humans): this is correlated with the development of 'skilled' motor capacities, such as an increased dexterity of distal musculature (Heffner and Masterton, 1983; Watson et al., 2009); it is important to underline that in rodents, CST axons mediate fine movements of the limbs, whereas in humans the corticospinal tract is much more involved in almost all aspects of voluntary motor control (Blesch and Tuszynski, 2009).

The lateral and ventral columns are formed by a variety of ascending and descending fiber groups. The principal ascending tract is the spinothalamic tract, which transmits nociceptive, thermal, non-discriminative touch and pressure sensations to the somatosensory areas of the thalamus; another important ascending tract is the spinocerebellar one, which conveys informations from Golgi tendons and muscle spindles to the cerebellum, for the control of movement coordination. The descending tracts, besides CST tract (in primates), include: the rubrospinal tract, that arises form the red nucleus and is involved in the control of skilled movements (Whishaw et al., 1998); the reticulospinal tract, which originates in the reticular formation of the romboencephalon and is involved in postural control and modulation of some sensory and autonomic functions (Tracey, 2004); the vestibulospinal tract, that represents the main initiator of coordinated postural extensor activity in the limbs and trunk (Pom-

peiano, 1972); the raphespinal tract, that descends from raphe in medulla oblongata and has neuromodulatory influences on motor, autonomic, reproductive and excretory function and is also involved in modulating perception to pain (Tracey, 2004).

As well as these long ascending and descending projections, in the white columns there are many fibers, called propriospinal, that connect one spinal cord segment with another. These fibers lie very close to the grey matter and participate in a variety of physiological and behavioral processes, such as modulation of afferent and descending inputs to the central pattern generators (CPG) for locomotion and respiration, as well as autonomic functions like visceroreception and pain perception. The largest propriospinal pathways connect the brachial and lumbosacral enlargements to coordinate limb movements (Watson et al., 2009).

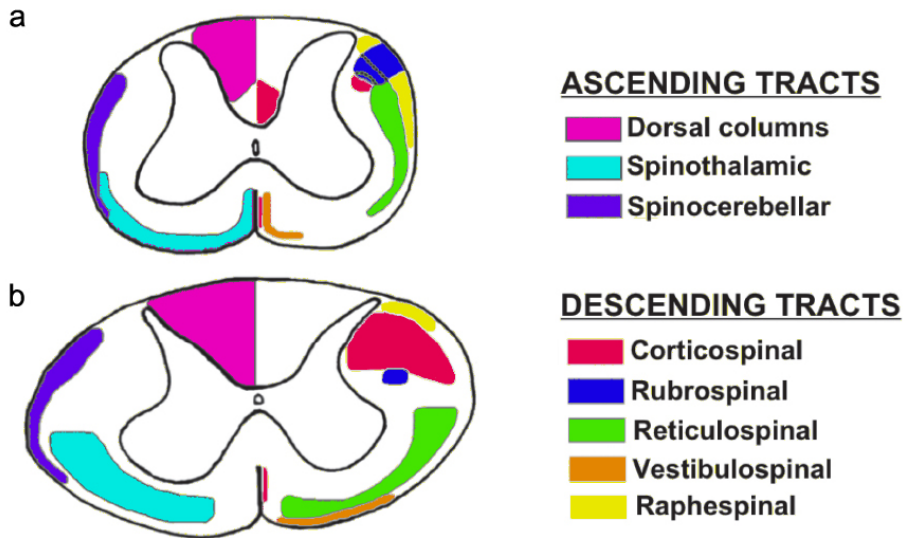

Figure 2. Approximate location of some ascending and descending tracts in rat and human spinal cord. **(a)** rat; **(b)** human. The diagrams are not drawn to scale. Dotted lines indicate areas where tracts appear to overlap. Left side: sensory; right side: motor. Adapted from Watson et al., 2009.

Spinal cord injury

General pathophysiology

Spinal cord injury (SCI) is the most common cause of invalidity in young adults. This condition affects 2.5 million people in the world, with an incidence of 130000 new cases every year (Thuret et al., 2006). The high majority of injuries have a traumatic origin (motor vehicle crashes, falls, violence and sport accidents) and affect principally young adults in the 15-29 years old age group. Non-traumatic injuries, as those caused by cancer, ischemia and multiple sclerosis, seem to be more related with old age. Moreover, the prevalence is 3.8 folds higher in males than females (Wyndaele and Wyndaele, 2006; van den Berg et al., 2010). Since quite half of injured patients is represented by young men that become unable to continue their job career and start to need assistance and special medical cares, social and economic costs of SCI are enormous.

Spinal cord injuries can be classified depending on the severity of the lesion (complete or incomplete), the segmental level (cervical, thoracic, lumbar or sacral) and the kind of insult that mechanically induced the injury (flexion, extension, rotation, compression or a combination of these forces; Schwab and Bartholdi, 1996). From the anatomical point of view, complete lesions are quite rare; anyway, when the damage to neural tissue is huge, a complete loss of function related to the interested area can be observed. The severity of the injury can be addressed analyzing the neurological outcome, and various scales can be adopted; for instance, the ASIA (American Spinal Injury Association) Impairment Scale, which is graded from A (complete sensorimotor injury) to E (normal), is widely accepted (Fawcett et al., 2006). Concerning the segmental level, cervical injuries are generally the most common. In very severe cases, cervical SCI causes tetraplegia, a condition that involves loss of function of upper and lower extremities, trunk and pelvis; however, only one third of patients experience

this state, while paraplegia, that afflicts principally the lower part of the body, is more frequent (Wyndaele and Wyndaele, 2006).

In spite of such a variety of causes and typologies, SCI pathophysiology seems to follow the same paradigm after the first insult (primary damage). In general, this process can be divided in an acute phase characterized by intertwined cellular death, hemorrhage and beginning of inflammatory response, and a chronic phase, in which the consequences of secondary damage started during the acute phase finally establish the formation of a glial scar at the injury site and, sometimes, of a cystic cavity full of liquid (Figure 3).

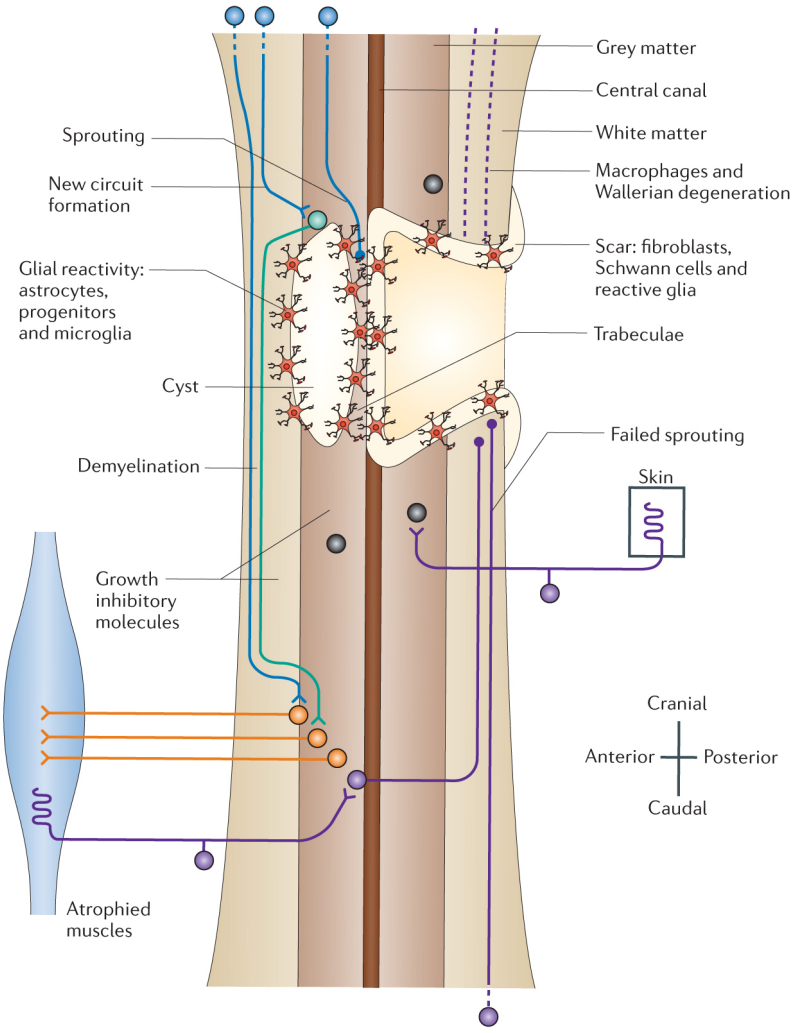

Figure 3. Diagram representing the spinal cord at the chronic stage of injury (adapted from Thuret et al., 2006).

The time at which a lesion reaches the "chronic stage" varies between species. Clinical data suggest that, for humans, an injury can be considered chronic 12 months after the first insult (Fawcett et al., 2006). The mechanisms of secondary injury have been deeply dissected in animal models of SCI (see below); nevertheless, recent studies indicate that similar mechanisms occur in humans as well (see Hagg and Oudega, 2006 for a review; Table 1).

Secondary response	*Rodent*	*Human*
Vascular response	Hemorrage, angiogenesis	Hemorrage, angiogenesis
Inflammation	Extensive	Much less pronounced, despite similar cytokine expression
Demyelination	Yes	Yes, but perhaps less pronounced
Axonal degeneration	Some die-back and Wallerian degeneration	Wallerian degeneration much more protracted
Glial scar	Extensive, with astroglial CSPG	Not extensive, CSPFs mostly in blood vessels
Cyst formation	Rat yes; mouse no	Yes
Schwann cell response	Some invasion	Extensive Schwannosis
CSPG: chondroitin sulphate proteoglycans		

Table 1. Similarities and differences between rodent and human response after SCI (adapted from Hagg and Oudega, 2006).

After initial trauma, cells near the site of injury immediately die. Disruption of blood flow due to death of endothelial cells brings to hemorrhage and intravascular thrombosis, which in combination with loss of autoregulation (Senter and Venes, 1979) and vasospasm of intact vessels (Attar et al., 2001), causes the anoxia and hypoperfusion of spinal tissue surrounding lesion and the appearance of edema (Tator and Fehlings, 1991). Vascular damage is one of the principal causes of secondary injury (Mautes et al., 2000): the high metabolic demand of grey matter cannot be satisfied because of incipient ischemia, moreover loss of autoregulation of microvascular tone leads to higher susceptibility of neurons to changes in systemic arterial pressure, a pretty frequent phenomenon in SCI patients (Levi et al., 1993). This process is

worsen by formation of free radicals (such as reactive oxygen species and peroxynitrite), compounds that induce peroxidation of lipid components of cellular membrane producing the disaggregation of membrane itself and the formation of new reactive species that continue the cascade (Cayli et al., 2004); besides, free radicals can damage mitochondrial respiratory chain enzymes, inducing the apoptotic death of the cell (Cuzzocrea et al., 2001; Tanaka et al., 2005).

The breakdown of blood-spinal cord barrier allows the entrance of inflammatory cells in the site of injury (Mautes et al., 2000; Whetstone et al., 2003; Habgood et al., 2007). The inflammatory response is a complex mechanism: it is initiated by the recruitment of neutrophils starting few hours after the SCI by chemoattractant molecules (chemokines, cytokines, adhesion molecules) that are increased by lysis of necrotic cells (Campbell et al., 2002; Kwon et al., 2004; Fleming et al., 2006; Donnelly and Popovich, 2008); neutrophils are then followed by macrophages derived from resident microglia (Popovich et al., 2002) and blood-born macrophages (Carlson et al., 1998). Infiltration of inflammatory cells allows the phagocytosis of tissue debris; moreover, these cells start to release pro-inflammatory cytokines (such as tumor necrosis factor alpha (TNFα) and interleukins 1 and 6), cytotoxic molecules (such as reactive oxygen species, nitric oxide) and proteolytic enzymes (such as metalloproteinases) that supply inflammatory responses and induce secondary damage (Schnell et al., 1999; Xu et al., 2001; Noble et al., 2002; Donnelly and Popovich, 2008).

An other important process related with the secondary damage of spinal cord is excitotoxicity due to change in ionic conductance and release of neurotransmitters. Cell lysis, demyelination due to apoptosis of oligodendrocytes and depolarization of neuronal membrane leads to increased intracellular concentration of sodium and calcium and extracellular concentration of potassium (Liu et al., 2011). This creates a loop in which increased intracellular calcium concentration allows the exocytosis of glutamate from synaptic vesicles, that in turn activates NMDA (N-methyl-D-aspartate) or AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxa-

zole- propionic acid)/kainate receptors. The hyperactivation of these ionotropic receptors results in increased intracellular sodium levels in neurons and astrocytes, that can cause the death of the cell due to water influx and lysis through depolarization (Matyja et al., 2005). Moreover, calcium influx trough NMDA and AMPA receptors provokes axonal injury and eventual apoptosis or necrosis via an increase in the activation of cellular enzymes, mitochondrial damage, acidosis, and production of free radicals (Schanne et al., 1979; Mody and MacDonald, 1995; Das et al., 2005).

As mentioned above, oligodendrocytes principally die by apoptosis after injury (Emery et al., 1998); oligodendrocyte death can be caused by microglial activation (Li et al., 2005), axonal degeneration (that causes the loss of trophic support to the oligodendrocytes; Barres et al., 1993) or interaction between NGF, which is increased by lesion, and pro-apoptotic receptor p75, which is expressed by oligodendrocytes (Casha et al., 2001). This process provokes demyelination of spared axons, with a consequent impairment of signal conductance and general physiology of the neuron, that eventually loses its function and degenerates (Waxman, 1989). Besides, a great loss of ascending and descending fibers is observed in the white matter one month after SCI (Bao and Liu, 2003; Wingrave et al., 2003; Araki et al., 2004; Iwata et al., 2004), with denervation and retraction of proximal axons.

The pathophysiology of the acute phase is completed by reactive gliosis. In response to cytokines and other molecules produced by inflammatory cells and degenerating axons, astrocytes start to overexpress the glial fibrillary acidic protein (GFAP) and to proliferate (Giulian and Lachman, 1985; Nishino et al., 1993; Chiang et al., 1994; Kahn et al., 1995; Hama et al., 1997; Rostworowski et al., 1997). These cells become hypertrophic and their processes start to overlap together with infiltrating fibroblasts, Schwann cells, macrophages and glial progenitors such as NG2 positive cells (Jones et al., 2002; Guest et al., 2005; Goritz et al., 2011), creating a glial scar between the spinal cord and the rest of the body in the at-

tempt to preserve the fragile neural tissue from further damage (Fitch et al., 1999; Myer et al., 2006). Unfortunately, the glial scar represents also a barrier for axonal regeneration, a condition that is reinforced by the production of transmembrane and secreted inhibitory molecules such as proteoglycans that form the extracellular matrix (ECM) (Jones et al., 2003), ephrin-B2 (Bundesen et al., 2003), semaphorin 3A (Pasterkamp et al., 2001), and tenascin (Apostolova et al., 2006) by the same astrocytes.

Experimental models of SCI

In order to study the mechanisms leading to secondary damage and to create tools to investigate the efficacy of possible therapies, researchers have developed several animal models mimicking different aspects of human SCI since Galen (2nd century A.D., Siegel, 1973). Injury models can be distinguished on the basis of the specimen, the level of injury and the kind of injury itself. Rodents and feline are the most used because of economic and practical advantages, although non-human primate models are emerging as the best choice for their similarities with humans (see Courtine et al., 2007 for a review).

Contusion and compression models

The models that best resemble the characteristics of human disease are those based on contusion or compression of spinal cord tissue, as the majority of patient lesions derive from blunt trauma and displacements of vertebral bone. These methods induce a mild to complete neurological lesion with the appearance of scar surrounding a fluid-filled cyst, then they are particularly suitable to study neuroprotective agents, tissue replacement and injury pathophysiology in general.

The first experimental contusion injury model was proposed by Allen in 1911: a weight-drop insult was applied on the exposed dorsal spinal cord of a dog, causing the contu-

sion and the displacement of neural tissue in the vertebral canal (Allen, 1911). This approach was then adapted to the rat (Gruner, 1992) and led to the development of the NYU/MASCIS (New York University Multicenter Animal Spinal Cord Injury Study) Impactor, a device that allows to induce a reproducible contusive damage to the rat spinal cord in controlled conditions (spinal level, drop weight, impact strength; Basso et al., 1996). Other tools created to induce contusion injuries in rodents are the OSU (Ohio State University) Impactor (Bresnahan et al., 1987; Stokes, 1992), which employs an electromechanical impact probe controlled by computer feedback, and the IH (Infinite Horizon) Impactor (Scheff et al., 2003), a device that applies a force-defined impact to the cord with a stainless steel-tipped impounder. All these instruments share similar advantages (reliability of performance and results, possibility to set and check all the parameters during and after the experiment, low maintenance costs), as well as similar limitations: while the force that induces lesion in humans is usually applied on the ventral surface of the cord or in a centripetal way (e.g. by torsion of the cord), the impactors described above act on the dorsal, surgically exposed surface of the neural tissue.

An alternative strategy to induce a lesion that might be more similar to human blunt SCI is represented by the clip compression model: after exposure by dorsal laminectomy, the spinal cord is compressed along the dorso-ventral axis between the arms of a modified aneurysmal clip (Rivlin and Tator, 1978; Joshi and Fehlings, 2002a; 2002b). The force and duration of compression are directly related with the functional outcome, so the severity of the lesion can be easily modulated; on the other hand, the principal disadvantage of this technique is represented by the difficulty of surgical manipulation of the cord. Compression injury can also be elicited by means of an inflated microscopic balloon inserted in the epidural space for a period that can vary from minutes (leading to a reversible functional lesion, Nesathurai et al., 2006) to hours (leading to irreversible damage because of secondary consequences de-

rived form initial compression, Lim et al., 2007). This method allows to operate on different species, from rodents to primate, and kindly resembles human injury.

Transection models

Transection models are based on the surgical cut of the spinal cord and can be divided in complete and partial transection models.

In complete transections, rostral and caudal segments of the spinal cord are fully dissociated; these models are used to mimic human complete injury, though in patients a complete anatomical lesion is very rare also in case of total functional loss. Nevertheless, a complete transection allows to evaluate the efficiency of treatments aimed at inducing axonal regeneration without the risk of misleading results due to the contribution of spared connections (Alilain et al., 2012; Lu et al., 2012). A complication related to complete transection models is the high post-operative care request to maintain critical physiological functions (as feeding and bowel/bladder function) and to control complications as infections, pain and dehydration of the animal.

Partial transections models are often criticized as they are less likely to represent human lesions than contusion models. On the other hand, incomplete transection is still a widely used model because it represent a valuable tool to study the contribution of particular tracts to motor and sensory function (e.g. CSTs, Bradbury et al., 2002; García-Alías et al., 2009) and to assess the effectiveness of pro-regenerative treatments; moreover, in case of dorsal or lateral hemisection, it allows to compare the injured circuit response with that of the healthy, uninjured counterpart (Muir and Whishaw, 2000; Anderson et al., 2007).

Ischemia models

Ischemic insults can be recapitulated by *in vitro* and *in vivo* models.

Organotypic spinal cord slices and specific cellular line cultures can be maintained under hypoxic conditions, enabling researchers to analyze the molecular cascades that lead to neuronal cell death (An et al., 2011; Lian Jin et al., 2011). Compared to *in vivo* models, the advantage in the use of slice and cell cultures is that they are far less expensive and do not require postoperative cares, while a limit is represented by the possibility to dissect just an aspect a time of a complex pathology as spinal cord injury.

Hypoxia and ischemic damage can be induced also *in vivo* by means of vascular compression; the most characterized model is based on the insertion of a Fogarty balloon in the rat thoracic descending aorta; blood flow is blocked at the level of the subclavian artery by balloon inflation, then the spinal cord is reperfused. In rats, this kind of insult induces paraplegia accompanied by rigidity and spasticity (Lu et al., 2004a; Cizkova et al., 2007).

Chapter 2: Plasticity

Neural plasticity can be defined as the ability of the nervous system to functionally and structurally reorganize itself in response to experience: “The whole plasticity of the brain sums itself up in two words when we call it an organ in which currents pouring in from the sense-organs make with extreme facility paths which do not easily disappear” (James, 1890).

In 1960s and 1970s, Hubel and Wiesel examined the plasticity of cat and monkey visual systems and identified a ‘critical period’ during which deprivation of normal visual experience through the closure of an eyelid by surgical suture (monocular deprivation, MD) irreversibly altered neuronal connections and functions in the visual cortex (Wiesel and Hubel, 1963; Hubel and Wiesel, 1970; Hubel et al., 1977). Since then, the existence of critical periods in early postnatal life during which neural circuits display a heightened plasticity in response to external stimuli has been established for various brain regions subserving major behavioral functions, as for instance the organization of auditory maps for sound localization in barn owls (Knudsen and Knudsen, 1990), birdsong in zebrafinches (Brainard and Doupe, 2002) and language learning in humans (Newport et al., 2001. See Berardi et al., 2000 and Hensch, 2004 for a comprehensive review). Concerning the motor system, an example of critical period is represented by the sensitivity to tail suspension, a manipulation that allows hindlimb unloading: in young rats (postnatal day 8-13), tail suspension leads to permanent motor impairment in the spinal cord, while it is innocuous in older animals (Walton et al., 1992).

During critical periods, neural circuits reach maturity: starting from an initial pool of less precise connections, experience models neural circuits stabilizing some connections and removing others (Holtmaat and Svoboda, 2009; Fu and Zuo, 2011). Because of this “enhanced-plasticity status”, the recovery from injury is much more pronounced in the early

postnatal phase (Nicholls and Saunders, 1996; Payne and Lomber, 2001; Chen et al., 2002; Dumas et al., 2002; Mladinic et al., 2009; Choksi et al., 2010). When the development is completed, plasticity wanes in favor of the formation of stable, reliable networks with precise circuitries and connections. In the adult brain, even if decreased, synaptic plasticity continues through modifications of synaptic strength, as well as through formation and elimination of synapses (Holtmaat and Svoboda, 2009; Chen and Nedivi, 2010).

In the past years, a big effort was made to investigate the mechanism underlying the enhanced plasticity of immature circuits and the possibility to manipulate the capability of neural networks to respond to experience and insults after the closure of critical periods. Several factors have been identified using the visual system as a paradigmatic model (see Berardi et al. 2003; Tropea et al., 2009; Levelt and Hubener, 2012). Among them, the excitatory/inhibitory balance emerges as a critical regulator for neural plasticity. Inhibitory circuits sculpt the pattern and timing of neuronal electrical activity; for instance, the increase of GABAergic transmission in the visual cortex defines the opening and the closure of the critical period for ocular dominance plasticity (Hensch, 2005): when GABAergic input is reduced during development, as in mice knockout for GAD65 (the synaptic biosynthetic enzyme of GABA), the visual cortex does not respond to experience modification such as MD (Hensch et al., 1998). A similar reduction of MD sensitivity can be achieved increasing the efficacy of glutamatergic transmission during the critical period (Fagiolini et al., 2003). In physiological conditions, GABAergic transmission matures later than glutamatergic circuits (Jiang et al., 2005). If GABAergic tone is precociously increased during development through infusion of benzodiazepines, overexpression of BDNF, exposure to environmental enrichment (EE) or removal of PSA-NCAM (polysialic acid presented by the neural cell adhesion molecule), the onset and closure of the critical period are accelerated (Hanover et al., 1999; Huang et al., 1999; Iwai et

al., 2003; Cancedda et al., 2004; Di Cristo et al., 2007. See Sale et al., 2010 for a comprehensive review).

In the adult visual system, inhibitory circuitry stabilizes synaptic connections, thus reducing neural plasticity: pharmacological reduction of GABAergic transmission reactivates OD plasticity in response to MD in adult rats (Harauzov et al., 2010), and several lines of evidence demonstrate that inhibitory tone is reduced by manipulations that increase visual cortical plasticity, such as EE (Sale et al., 2007; Baroncelli et al., 2010), dark rearing (He et al., 2006) and fluoxetine treatment (Maya Vetencourt et al., 2008). Furthermore, GABA has been suggested to be crucial in masking existing horizontal connections and consequently in the rapid reorganization of cortical maps after, for instance, loss of afferents due to peripheral nerve transection or limb amputation (Jacobs and Donoghue, 1991; Chen et al., 1998; Wu and Kaas, 1999).

Plasticity in the spinal cord

As in other areas of the CNS, neuronal plasticity occurs in the spinal cord in response to experience through a variety of mechanisms. During development spinal cord plasticity is involved in standard behaviors like locomotion and rapid withdrawal from pain (Waldenstrom et al., 2003), while in the adulthood plasticity contributes to the acquisition and maintenance of new motor skills (see Wolpaw, 2007 for an extensive review). Moreover, spinal cord plasticity has a role in the mechanisms that lead to functional recovery (adaptive plasticity) or secondary adverse consequences (maladaptive plasticity) after an injury (Goldberger, 1977; Sanes and Donoghue, 2000; Blesch and Tuszynski, 2002; Raineteau et al., 2002; Edgerton et al., 2004). It has been estimated that if as little as 10–15% of the descending spinal tracts are spared, some locomotor function can recover (Basso, 2000; Metz et al., 2000). Based mostly on the results of studies using animal models, reorganization of the CNS, including synaptic

plasticity, axonal sprouting, and cellular proliferation, has long been known to spontaneously occur following spinal cord lesions. This reorganization occurs in the spinal cord circuitry and in supraspinal structures.

Early after SCI, in a time window varying between species, a certain degree of spontaneous recovery can occur (see Onifer et al., 2011 for a review). In this phase, the contribution of plasticity to functional return and recovery is difficult to discriminate from other possible processes including the recovery from spinal shock, a condition probably due to the breakdown of membrane potentials, excessive neurotransmitters levels, and the loss of neuromodulators regulating the excitability in the spinal cord (as the 5HT mediated system) that leads to the loss of muscle tone and segmental spinal reflexes (Smith and Jeffery, 2005). The onset for return of spontaneous function in both humans and animals after SCI could be due to the restoration of motoneuron excitability by constitutive expression of 5HT2C receptors (Fouad et al., 2010; Murray et al., 2010), adaptations in polysynaptic flexor reflexes involved in locomotor circuits (Lavrov et al., 2006; Dietz et al., 2009) and synaptic rearrangements (Rossignol, 2006).

As would also be expected, SCIs (both complete and incomplete) produce considerable reorganization in the spinal cord circuitry, with sprouting of collaterals from damaged fibers (regenerative sprouting) and sprouting of lesion-spared descending axons (compensatory sprouting, Tuszynski and Steward, 2012). Several experiments showed the existence of regenerative and compensatory sprouting by corticospinal tract axons (CSTs); for example, after cervical SCI in rats, spontaneous sprouting from the ventral CSTs occurred onto medial motoneuron pools in the cervical spinal cord, leading to spontaneous recovery (Weidner et al., 2001). Furthermore, Bareyre et al. reported that after incomplete spinal cord injury in rats, transected hindlimb CSTs sprouted into the cervical gray matter to contact short and long propriospinal neurons (PSNs). Over 12 weeks, contacts with long PSNs that bridged the lesion

were maintained, whereas contacts with short PSNs that did not bridge the lesion were lost. In turn, long PSNs arborize on lumbar motor neurons, creating a new intraspinal circuit relaying cortical input to its original spinal targets (Bareyre et al., 2004). Similarly, in rhesus monkeys a C7 spinal cord hemisection led to marked spontaneous plasticity of CSTs, with reconstitution of 60% of pre-lesion axon density arising from sprouting of spinal cord midline-crossing axons, and this extensive anatomical recovery was associated with improvement in coordinated muscle recruitment, hand function and locomotion (Rosenzweig et al., 2010). Other descending pathways, such as the neuromodulatory efferents, undergo anatomical plasticity after SCI. For example, compensatory plasticity of the rubrospinal tract was found to mediate the recovery of forepaw function following CST lesion in cats (Alstermark et al., 1987) and monkeys (Belhaj-Saif and Cheney, 2000). Similarly, rat cervical SCI led to increased sprouting of reticulospinal fibers rostral to the injury (Weishaupt et al., 2013), and several experiments showed that serotoninergic neurons sprout after spinal cord trauma (Sharma et al., 1990; Inman and Steward, 2003; Camand et al., 2004); intriguingly, in a cortical model of lesion, serotoninergic neurons displayed lack of axonal dieback and enhanced sprouting within the inhibitory environment of the glial scar, probably thanks to significantly higher amounts of growth-associated protein-43 and/or β1 integrin than cortical neurons (Hawthorne et al., 2011).

Some recovery of motor control after cord hemisection in cats has been attributed to collateral sprouting of primary afferent axons (Goldberger et al., 1993; Helgren and Goldberger, 1993), and early studies demonstrating enlarged excitatory postsynaptic potentials in chronic spinal cats attributed part of the increase to sprouting of primary afferent fibers (Nelson and Mendell, 1979).

In response to SCI, synaptic plasticity as well as anatomical reorganization can also occur at cortical and subcortical regions. Several studies have reported that cortical territories

controlling intact body parts tend to enlarge and invade cortical regions that have lost their peripheral target (McKinley et al., 1987; Jain et al., 1997; Fouad et al., 2001; Ghosh et al., 2010). The underlying mechanisms are hypothesized to be similar to those mediating reorganization after cortical injury, including disinhibition of latent cortical connections and axonal sprouting at multiple levels of the neuraxis (reviewed in Raineteau and Schwab, 2001; Fouad and Tse, 2008; Kaas et al., 2008). Another mechanism may be injury-induced structural plasticity in the dendritic spines of cortical motoneurons, as suggested by the observation that in rodents, after cervical SCI, dendritic spine density and morphology in neurons of the motor cortex is modified over 3 days to 2 weeks (Kim et al., 2006). TMS studies in human SCI patients revealed motor reorganization, as muscles immediately rostral to the lesion could be activated through bigger regions of the cortex (Levy et al., 1990; Topka et al., 1991). On the other hand, PET, EEG and fMRI studies showed that appropriate (e.g. foot, leg) motor areas can be activated by imagined movements, even in long-term paraplegic or tetraplegic patients (Cohen et al., 1991; Corbetta et al., 2002; Curt et al., 2002; see Endo et al., 2009 for a review).

Besides underlying spontaneous recovery, neuroplasticity can also lead to detrimental consequences (Brown and Weaver, 2012). Increase in neuronal excitability due to products of microglial activation and changes in sodium channel and glutamate receptor expression (Deumens et al., 2008), collateral sprouting of calcitonin gene-related peptide (CGRP)-containing primary afferent fibers (Christensen and Hulsebosch, 1997a; 1997b) and aberrant growth of descending serotoninergic axons in the spinal cord dorsal horn (Inman and Steward, 2003) have been indicated as the main causes of neuropathic pain, a condition that leads to abnormal sensations (dysesthesia) or to the insurgence of nociception after a non-painful stimulus (allodynia). In addition, SCI is often associated with autonomic dysreflexia, an abnormality of blood pressure control characterized by extreme hypertension accompanied by a

pounding headache and slow heart rate (see Weaver et al., 2006 and Mathias, 2006 for review): the entering of sensory input in the spinal cord below the level of the lesion leads to exaggerated sympathetic (autonomic) responses that can be associated with an increased CGRP-containing primary afferent arbor in the dorsal horn (Krenz and Weaver, 1998; Krenz et al., 1999), or in the presence of a normal arbor, with loss of descending inhibitory influences on spinal sympathetic reflexes (Gris et al., 2005). Moreover, modification in neuronal excitability after SCI can lead to the insurgence of spasticity, an involuntarily elevated muscular activity that can be triggered by a variety of sensory inputs, such as cutaneous stimulation (Norton et al., 2008). Notably, up-regulation of 5HT2 receptors and lowered expression of the potassium-chloride co-transporter KCC2 (which keeps Cl^- intraneuronal concentration low) in motoneuron membranes have been shown to be involved in the generation of this phenomenon (Lee et al., 2007; Boulenguez et al., 2010).

Chapter 3: Experimental therapies for spinal cord injury

In general, the spinal cord tries to repair itself after the initial insult: spontaneous recovery can be observed in animal models (Muir and Whishaw, 1999; Weidner et al., 2001; Fenrich and Rose, 2009; Rosenzweig et al., 2010) as well as in patients (Fawcett et al., 2006; Scivoletto et al., 2007). After injury, immediately early genes are overexpressed, recapitulating a situation typical of the developing nervous system (Maier and Schwab, 2006); signs of reorganization of neural circuits, for instance the appearance of regenerative or compensatory sprouting, have also been observed (Ramon y Cajal, 1928; Bradbury and McMahon, 2006). Some studies indicate that macrophages infiltrating the lesion site in the acute phase could contribute to neuroprotection (removal of cellular debris, release of protective cytokines) and to the reorganization of damaged tissue in particular temporal contexts and in species/strain specific manner, besides their well characterized pro-degenerative role (Schwartz, 2003; Donnelly and Popovich, 2008).

Acquired knowledge on processes that are occurring in acute, sub-acute and late phase of injury allowed researchers to develop and test various kinds of experimental therapies in the attempt to achieve axonal regeneration, remyelination of damaged axons and, in general, recovery of function. Some of these approaches have been demonstrated to be efficient in animals models and reached the clinical trial phase.

Cell transplantation therapies

The most evident anatomical consequence of spinal cord injury is the interruption of connections between the proximal and the distal segment of the spinal cord itself due to axon section and cell death; then, cell transplantation represent a promising strategy to replace damaged tissue, bridge the lesion cavity, counteract axonal demyelination and, moreover, can be used as a tool to create a neuroprotective and pro-regenerative environment for severed axons.

Peripheral nerve grafts

The first experiment of regeneration induced by cellular transplantation was carried out in 1911 by Francisco Tello, who transplanted portions of peripheral nerves in rabbit cerebral cortex and found out that cortical axons could enter in and elongate on the peripheral tissue (Tello, 1911). His pioneering work gave the basis for experiments with autologous peripheral nerve graft in injured spinal cord, which demonstrated that this treatment can induce axonal regrowth into the graft and functional improvement in rats with a complete spinal cord transection, alone (Richardson et al., 1980; Cheng et al., 1996) or in combination with other experimental strategies (Lee et al., 2002; Levi et al., 2002; Tom and Houlé, 2008), with one case of partial functional recovery in a patient with a chronic thoracic injury (Cheng et al., 2004).

Today, cellular transplants are preferred to nerve bridges: single cells can be purified and expanded *in vitro* to reach the high amount of material required to fill the cystic cavity and, most importantly, cells can be manipulated to produce and release factors at the lesion site (Ramer et al., 2005).

Schwann cells

Several studies demonstrated that the part of the peripheral nerve that actually works on axonal regeneration is represented by Schwann cells (SCs): this cells, indeed, create a more per-

missive environment after peripheral nerve injury by increasing the production of pro-proliferative factors (as neurotrophins and cell adhesion molecules) and decreasing the expression of inhibitory myelin proteins (see Oudega and Xu, 2006 for a review); in addition, SCs can recreate the myelin sheath around severed and intact central axons (Gilmore, 1971). Schwann cells have been successfully implanted in the rat spinal cord and regrowth of axons into the bridge graft was observed; a recovery of function has also been reported in some studies (Xu et al., 1997; Xu et al., 1999; Bunge, 2002; Takami et al., 2002). Unfortunately, regenerating axons were not able to cross the graft tissue and to reinnervate the host, and SCs seem to be ineffective on corticospinal axon regeneration (Fehlings and Vawda, 2011). For these reasons, combinatorial strategies with treatments aimed to reduce environment inhibition (as glial scar digestion, Fouad et al., 2005) and to increase pro-regenerative factors (as neurotrophins and cell adhesion molecules, Xu et al., 1995; Lavdas et al., 2010) have been evaluated. Schwann cells were tested in a study on patients with a chronic mid-thoracic lesion (Saberi et al., 2008); the study demonstrated that SCs could be safe for humans, as no negative consequences of the implant were recorded, but the beneficial outcome of this treatment was not clear (only one patient had a better performance after the therapy, but it was not possible to assess if it was caused by transplanted cells or not).

Olfactory ensheathing glia

Olfactory ensheathing glia (OEG) are cells derived from embryonic and adult olfactory bulb important for maturation and migration of olfactory neurons; these cells share some characteristics with SCs and astrocytes (Xu and Onifer, 2009), then they are considered a good tool to make a bridge between peripheral and central nervous systems. Olfactory glial cells have been tested in several injury models, giving encouraging results on regeneration of corticospinal axons and recovery of function in rats when used alone (Li et al., 1998, 2003; Ramon-Cueto

et al., 2000; Keyvan-Fouladi et al., 2003; Plant et al., 2003; López-Vales et al., 2007) or in combination with other treatments (Ruitenberg et al., 2003, 2005; Cao et al., 2004; Kubasak et al., 2008b); nevertheless, there have been cases in which a recovery was not observed (Takami et al., 2002; Ramer et al., 2004; Deumens et al., 2006; Lu et al., 2006; Bretzner et al., 2008), underlining the necessity to establish the optimal conditions for OEGs transplantation to work, as cell source, age of cells and graft strategy (Thuret et al., 2006). To date, OEGs have been used in some clinical trials, with beneficial effects observed in chronic patients (Lima et al., 2006; 2010).

Bone marrow stromal cells

Bone marrow stromal cells (BMSCs) have been used in transplantation therapy for many different diseases; they can be easily isolated from bone marrow, constituting a minimally invasive source for autologous cell transplantation; moreover, BMSCs have anti-inflammatory and immunomodulatory effects, mesodermal differentiation potential and secrete several neurotrophic factors (Fehlings and Vawda, 2011). Even though there is no conclusive evidence about their capability to differentiate in neural or glial cells (Lu et al., 2004b), BMSCs have been used in experimental SCI therapy in rats (Urdzíková et al., 2006; Lu et al., 2007; Novikova et al., 2011) and non-human primates (Deng et al., 2006) with beneficial effects on locomotion and axonal regrowth. Anyway, there is great variability in results depending on cell source (age, donor, culture conditions; Neuhuber et al., 2005; Ruff et al., 2012).

Despite the lack of consistency from preclinical studies, there are ongoing clinical studies with autologous BM-derived cells on small patient cohorts. The few studies carried out so far demonstrated that BMSCs are safe for human application, but no clear amelioration above spontaneous recovery was observed in these patients (Callera and do Nascimento, 2006; Chernykh et al., 2007; Yoon et al., 2007; Saito et al., 2008).

Neural progenitors

Neural progenitor cells (NPCs) represent a source for reconstituting damaged circuits, re-myelinating axons and increasing plasticity and/or axonal regeneration. They can be obtained from embryonic stem cells (eNPCs) or from adult stem cells (aNPCs).

Embryonic stem cells (ESCs) are obtained from the inner cell mass of the early blastocyst; they can potentially differentiate in every cellular type of an organism and, moreover, can indefinitely proliferate *in vitro* maintaining their pluripotency (Richards et al., 2002; Ko et al., 2007). Embryonic stem cells can be expanded *in vitro* as neurospheres containing precursors of neurons, astrocytes and oligodendrocytes, besides still pluripotent stem cells (Svendsen et al., 1999); studies on SCI models demonstrated that eNPCs transplantation resulted in migration of cells from injection site, differentiation in neurons, astrocytes and oligodendrocytes and improvement in function (McDonald et al., 1999; Ogawa et al., 2002; Meng et al., 2008). Unfortunately, the use of ESCs is subjected to some major caveats, such as teratogenicity *in vivo* (Fong et al., 2010), need to subject the host to immunosuppression before implantation (ESC are allogenic) and ethical questions around the use of human embryos for clinical research.

Adult stem cells (ASCs) can be found in mammals in the periventricular sub-ependymal layer and in the subgranular zone of the dentate gyrus of the hippocampus in the brain and in the ependymal regions lining the central canal of the spinal cord (Hawryluk and Fehlings, 2008; Barnabé-Heider et al., 2010). They represent an autologous source of stem cells, then reducing ethical concerns about cellular transplantation and risk of rejection. As eNPCs, aNPCs can be expanded *in vitro* as neurospheres (Weiss et al., 1996; Shihabuddin et al., 1997), but differently from eNPCs, adult progenitors tend to remain undifferentiated or to differentiate principally in glia when transplanted in the adult nervous system (Cao et al., 2001). Anyway, aNPCs can represent a good candidate for cellular therapy in humans, since

aNPCs transplantation induced functional recovery in experimental models of SCI (Karimi-Abdolrezaee et al., 2006; Bottai et al., 2008; Parr et al., 2008; Moreno-Manzano et al., 2009).

Oligodendrocyte restricted progenitors (OPCs) constitute a very promising cellular type for SCI therapy (Blakemore et al., 2000). They can be obtained from NPCs or from endogenous stem cells with the administration of particular transcription factors (as sonic hedgehog) and several growth factors expressed by the neural plate during development (Bambakidis et al., 2008). When transplanted in animal models of SCI, OPCs differentiated in mature oligodendrocytes, increased remyelination and promoted recovery of function (alone or in combination with viral vectors expressing neurotrophins, Cao et al., 2005; Keirstead et al., 2005). Human ESC derived OPCs were proposed for a phase I clinical study (by Geron Corporation, Menlo Park, CA) that is still underway.

In order to overcome the ethical questions regarding the use of ESCs and the limited availability of ASCs, scientists developed techniques to dedifferentiate somatic cells into induced pluripotent stem cells (iPSC) (Nakagawa et al., 2008; Okita et al., 2008; Yamanaka, 2010). The great advantage of these cells is that they are patient-specific, thus reducing reject risks; moreover, powerful reprogramming technologies have been developed to generate iPSC and differentiate them directly from skin or peripheral blood cells, avoiding the use of genetic and viral manipulations (Vierbuchen et al., 2010; Yamanaka, 2010).

Autologous macrophages

Since the observation that the inflammatory response differs in peripheral and central nervous systems after injury, a compromised recruitment of macrophages has been indicated as one of the causes of lack of regeneration in lesioned spinal cord (Hirschberg and Schwartz, 1995). To obviate to this defective response, an autologous transplant of activated macrophages was attempted in a rat model of SCI: the transplant induced a partial recovery of function (assessed

both behaviorally and electrophysiologically) and the regeneration of axons beyond the lesion site (Rapalino et al., 1998). These positive results, together with the manageability of cellular preparations for transplant, led to the first clinical trial for cellular transplantation therapy (PROCORD, Proneuron Biotechnologies Inc.). The study enrolled 16 patients with clinically complete SCI; after the treatment, five patients showed amelioration toward an incomplete injury status (three patients became ASIA C, two patients became ASIA B). Moreover, no adverse effect due to the transplantation was observed (Knoller et al., 2005). A phase II randomized multicenter trial was started but suddenly suspended for financial problems.

Conversely, the attempt to induce endogenous macrophages to be activated with microinjections of zymosan, a glucan prepared from yeast cell wall, induced detrimental effects on hindlimb function and tissue damage (Popovich et al., 2002).

Gene therapy

After injury, neurons display the expression of several growth associated genes, as the immediate early genes L1, c-Jun and c-Fos and GAP-43 (43 kDa growth-associated protein, Jenkins et al., 1993; Chaisuksunt et al., 2000), in a similar fashion to that appearing during development. Unfortunately, after the initial intrinsic growth response, axons fail to regenerate (Maier and Schwab, 2006). One possible cause for this phenomenon could be the lack of trophic factors, whose levels decrease dramatically in the adult (Maisonpierre et al., 1990). For this reason the administration of growth factors, such as neurotrophins (NGF, BDNF, NT-3, NT-4/5), cytokines (LIF, IL-6, CNTF), GDNF family ligands and insulin-like growth factor (IGF), represents a promising strategy to increase regeneration and plasticity after SCI.

The delivery of these molecules can be addressed directly by local injection at the site of injury (Schnell et al., 1994) or by intrathecal infusion with a catheter (Jakeman et al., 1998) or an osmotic minipump (Bradbury et al., 1999; Namiki et al., 2000). Otherwise, it is possible

to transduce cells - as fibroblasts (Jin et al., 2002), SCs (Weidner et al., 1999), BMSCs (Lu et al., 2005), OEGs (Cao et al., 2004), NPCs (Cao et al., 2005) - with viral vectors, such as adeno- and adeno-associated virus (AAV, Grieger and Samulski, 2012) and lentivirus (LV, Lundberg et al., 2008), containing genes encoding for growth factors and then graft these cells in the lesion site (Shumsky et al., 2003; Tobias et al., 2003; Tuszynski et al., 2003; Blesch et al., 2004; Mitsui, 2005; Sasaki et al., 2009). These vectors can also be used to directly infect the CNS *in vivo*: they can be transported retrogradely in the neurons and integrate in the host genome, producing a long lasting and stable over-expression of the desired gene in the infected cells (Liu et al., 1997; Romero and Smith, 1998; Blits and Bunge, 2006; Taylor et al., 2006a; Kwon et al., 2007). The regulation of delivered genes in the injured spinal cord is an important issue: the constitutive expression of trophic factors within the lesion site could, for instance, enhance the axonal growth into the graft while preventing their elongation beyond the graft itself. Moreover, the availability of growth promoting molecules for an indefinite time might cause the massive infiltration of Schwann cells in the lesion site, leading to increasing graft size (Blesch and Tuszynski, 2003; Blesch et al., 2004).

Concerning the molecules evaluated so far, neurotrophins appear to have a differential effect on different circuits in the spinal cord, consistent with the distribution of their respective receptors: NGF (nerve growth factor) promotes the sprouting and regeneration of cholinergic local motor, primary nociceptive sensory and coerulospinal axons (Tuszynski et al., 1994; Grill et al., 1997b; Romero and Smith, 1998). Adenoviral gene transfer of NGF to the dorsal spinal cord can also induce directed sensory axon regrowth across the dorsal root entry zone into the spinal cord (Romero et al., 2001). One of the major caveats linked with the use of NGF is the induction of severe hyperalgesia due to the effect on nociceptive fibers (Romero et al., 2000). In order to restrict sensory axon regeneration to their appropriate targets, NGF gene transfer to the superficial layers of the spinal cord was combined with a re-

pulsive signal for axon growth (semaphorin 3A) into deeper layers (Tang et al., 2004a, 2007): this strategy allowed to limit the expression of NGF in the ventral cord, thus limiting the penetration of nociceptive fibers in this region and reducing pain.

BDNF acts on the regeneration of raphespinal, coerulospinal, rubrospinal, reticulospinal, vestibulospinal, local motor and proprioceptive sensory axons (Kobayashi et al., 1997; Liu et al., 1999; Jin et al., 2002; Kwon et al., 2002; Ruitenberg et al., 2004; Lu et al., 2005). The regrowth has been associated with the increase of the expression of regeneration-associated genes such as GAP-43 and βIII-tubulin (Kobayashi et al., 1997; Ruitenberg et al., 2004; Kwon et al., 2007). Concerning CST fibers, instead, BDNF administration fails to induce sprouting or regeneration of lesioned axons (Lu et al., 2001; Brock et al., 2010), also if it enhances the survival of corticospinal neurons when delivered both in the cortex (near the cell soma) or in the spinal cord (Giehl and Tetzlaff, 1996; Lu et al., 2001; Brock et al., 2010).

Similar to BDNF, NT-3 expression enhances the regenerative response of raphespinal and proprioceptive sensory axons (Bradbury et al., 1999; Taylor et al., 2006b; Alto et al., 2009). Moreover, sprouting and regeneration of CST axons has been observed after NT-3 delivery (Schnell et al., 1994; Grill et al., 1997a; Blits et al., 2000; Tuszynski et al., 2003; Fortun et al., 2009), also if it is ineffective to protect CST neurons from lesion-induced atrophy (Brock et al., 2010). This differential effect of BDNF and NT-3 on CST somata and axons can be explained with the differential signaling and retrograde transport of their respective receptors (trkB and trkC) from the axonal compartment to the soma (Franz et al., 2012).

NT-4 acts on raphespinal, rubrospinal, reticulospinal, coerulospinal and propriospinal sensory axons (Bregman et al., 1997; Kobayashi et al., 1997; Blesch et al., 2004); this neurotrophin increases branching into cell grafts, also if growth beyond transplanted tissue has not been observed.

GDNF induces growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination; moreover, it ameliorates neuronal atrophy of CST motor neurons after injury (Bennett et al., 1998; Blesch and Tuszynski, 2003; Tang et al., 2004b).

IGF-I gene transfer supports CST neuron survival but, despite its essential role during CST development (Ozdinler and Macklis, 2006), fails to promote axonal regeneration in the adult injured spinal cord (Hollis et al., 2009b).

Until now, no clinical trial for gene therapy in human SCI has been attempted. A study carried out to treat Alzheimer's disease showed that the intracerebroventricular infusion of NGF induced pain and weight loss in patients during the course of treatment, leading to the stop of the trial (Eriksdotter Jönhagen et al., 1998). A more detailed knowledge about safety of transgene expression and regulation is still needed before translating the pre-clinical, encouraging results to humans.

Pharmacological therapies

Pharmacological therapies are meant to act on different aspects of SCI pathophysiology: avoid secondary damage (acute neuroprotective treatments), reestablish signal transduction, overcome the anti-regenerative barriers and activate or increase the intrinsic regenerative response.

Neuroprotective treatments

The most studied molecule in acute SCI therapy is methylprednisolone sodium succinate (MPSS), a drug belonging to the glucocorticoid class. Studies in animal models of acute SCI demonstrated that MPSS was neuroprotective when it was administered in high doses (Braughler and Hall, 1984; Braughler et al., 1987; Oudega et al., 1999). The neuroprotective

effect could be explained by MPSS antioxidant action through inhibition of lipid peroxidation and TNFα (that leads to modulation of the inflammatory/immune cells response), improved vascular perfusion and prevention of calcium influx and accumulation (Hall, 1992). The efficacy of MPSS was evaluated in three large scale acute clinical trials, named NASCIS (National Acute Spinal Cord Injury Studies) I, II and III (Bracken et al., 1985; 1990; 1992; 1997; 1998). The results emerged form these studies highlighted that MPSS exerted some neurological benefit when administered in high doses within the very acute phase of SCI (8 hours since the first insult), leading to the use of corticosteroids as a standard care for acute SCI patients. Further analysis of the clinical trial results showed that functional improvements were modest and present only in a small cohort of patients; moreover, increased rates of gastrointestinal hemorrhage, sepsis, pneumonia, delayed wound healing, and death due to respiratory complications were observed (Hurlbert, 2000; Bydon et al., 2013). Nevertheless, due to lack of alternative therapies, MPSS is still considered for use to treat acute SCI (Hawryluk et al., 2008).

Minocycline, a second-generation tetracycline antibiotic commonly used in dermatology, has been demonstrated to be beneficial in neurodegenerative conditions in which inflammation plays a major role, such as stroke, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis (Yong et al., 2004). In experimental models of SCI the systemic administration of minocycline inhibited microglial activation, reduced delayed oligodendrocyte death and attenuated axonal dieback (Stirling et al., 2004; Festoff et al., 2006; Yune et al., 2007), promoting functional recovery in mice and rats (Lee et al., 2003; Wells et al., 2003; Stirling et al., 2004; Teng et al., 2004). The promising data obtained from preclinical analysis led to a clinical trial from the University of Calgary. The study reached phase II and demonstrated that intravenous injections of minocycline were feasible and safe for patients with an acute, complete SCI; also if the results showed a tendency towards im-

provement in treated patients vs placebo, however, no clear functional recovery was observed (Casha et al., 2012).

In vivo and *in vitro* studies demonstrated that gangliosides, glycosphingolipids containing sialic acid that are abundantly expressed in the CNS, could mimic or at least increase the action of neurotrophic factors, thus enhancing neurite outgrowth and plasticity and reducing excitotoxicity and apoptotic cell death (Ferrari and Greene, 1998). A particular kind of ganglioside, the monosialotetrahexosylganglioside (GM-1 or Sygen, Fidia Pharmaceutical Corporation, Washington, DC) has been proven to induce neuroprotection and functional recovery when administered in an experimental model of SCI (Bose et al., 1986). The first clinical trial for GM-1 in acute SCI (the prospective randomized Maryland GM-1 study, enrolling 37 patients) showed that the GM-1 administration promoted a significant amelioration in the ASIA motor score (Geisler et al., 1991). The results led to the Sygen Multi-Center Acute Spinal Cord Injury Study, enrolling more than 750 patients. In this study GM-1 was administered after MPSS (along the NASCIS II protocol) because of ethical reasons (at that time clinicians believed that MPSS was safe and efficient, so all patients received it). Unfortunately, the functional outcome measured at the end of the trial evaluation (26 weeks after SCI) did not differ between GM-1 and placebo groups, also if a positive trend and a faster amelioration of sensitive and motor functionality was observed in GM-1 patients, in particular in those with incomplete SCI (Geisler et al., 2001).

Thyrotropin releasing hormone (TRH) is a tripeptide involved in the regulation of anterior pituitary gland function. The application of TRH induced neurological recovery in experimental SCI models in a dose-dependent fashion (Faden et al., 1984; Takami et al., 1991), indeed TRH antagonizes secondary injury effectors such as platelet activating factor, excitotoxic amino acids, endogenous opioids and peptidoleukotrienes (Dumont et al., 2001). The only clinical trial completed so far showed that the TRH induced a significant recovery of

function in case of incomplete SCI, whereas it was ineffective in complete lesions (Pitts et al., 1995).

Since the observation that endogenous opioids rise after SCI and participate to the acute phases of pathophysiology (Holaday and Faden, 1980; Krumins and Faden, 1986; Long et al., 1987; McIntosh et al., 1987), the use of opioid receptor antagonists has been investigated in experimental SCI models. These studies demonstrated that naloxone, a non specific opioid receptor antagonist, could increase sensorimotor recovery and prevent post-traumatic ischemia in several models of SCI (Young et al., 1981; Faden et al., 1984; Winkler et al., 1994). Regardless some discrepancies in preclinical data (Wallace and Tator, 1986; Haghighi and Chehrazi, 1987), naloxone was assessed for human therapy in a phase I clinical trial (Flamm et al., 1985) and as one of the treatment evaluated in NASCIS II (Bracken et al., 1990, 1992). The authors concluded that the use of naloxone could be beneficial for the treatment of incomplete SCI, but further analysis should be addressed to clarify drug dosage and administration timing (Bracken and Holford, 1993).

More results from neuroprotective treatment studies are summarized in Table 2.

Drug	*Molecular mechanism*	*Preclinical studies*	*Clinical trials*
21-Aminosteroids (Tirilizad mesylate)	Antioxidant without a glucocorticoid effect	Functional recovery in cats (Anderson et al., 1988, 1991; Hall et al., 1989) and rats (Holtz and Gerdin, 1992); reversal of ischemia (Hall, 1988).	NASCIS III: effectiveness similar to MPSS, no adverse secondary effect (Bracken et al., 1997; 1998), but the study did not include a placebo control.
Immunophillins (Tacrolimus)	Immunosuppressant (inibition of T-cell activation)	Dampened inflammatory response, improved locomotor score in rats (Madsen et al., 1998; López-Vales et al., 2005), increased oligodendrocytes survival (Nottingham et al., 2002), sprouting of ascending (Bavetta et al., 1999) and descending fibers (Voda et al., 2005).	A clinical trial for the effectiveness of a combination of tacrolimus and minocycline on acute SCI is underway at the Riyadh Armed Forces Hospital, Saudi Arabia.
Erythropoietin and erythropoietin analogues	Reduction of apoptosis, decreased oxidation, decreased inflammatory processes, and decreased autoregulatory vascular response	Improved motor function, decreased lesion severity in rats (Keswani et al., 2004; Vitellaro-Zuccarello et al., 2007; 2008). Other studies did not confirm neuroprotection (Mann et al., 2008; Pinzon et al., 2008).	Evaluation of Tolerability and Efficacy of Erythropoietin (EPO) Treatment in Spinal Shock: Comparative Study Versus Methylprednisolone (MP) (Italy). Suspended. Erythropoietin Spinal Cord Compression Randomized Trial (Canada), terminated for insufficient accrual
Anti-CD11d	Dampened neutrophil invasion	Improved motor score in rats, less allodynia and dysreflexia (Taoka et al., 1997; Gris et al., 2004; Oatway et al., 2005; Bao et al., 2008; Fleming et al., 2008); depletion of early neutrophils is detrimental in mice (Stirling et al., 2009).	None
TNFα antagonists (infliximab, etanercept, thalidomide)	Block of TNFα-mediated inflammatory response	Reduction of inflammation and apoptosis and improved motor performance in mice (Genovese et al., 2006; 2008); reduction of neuropathic pain and lipid peroxidation in rats (Kurt et al., 2009; Marchand et al., 2009).	None

Drug	*Molecular mechanism*	*Preclinical studies*	*Clinical trials*
Statins (Lipitor, simvastin)	Antinflammatory, antioxidant. Preservation of blood brain barrier integrity	Improved rat open field locomotor score (Pannu et al., 2005; 2007), reduction of apoptosis (Déry et al., 2009). Other studies showed a dampened astroglial response, but little motor recovery (Holmberg et al., 2008; Mann et al., 2010).	None

Table 2. Neuroprotective treatments.

Channel blockers

Ionic unbalance is one of the most important features of acute SCI pathophysiology (see above).

Riluzole (or Rilutek - Sanofi Aventis) is a benzothiazole Na^+ channel blocker; besides this principal effect, riluzole also inhibits presynaptic Ca^{2+}dependent glutamate release (Wang et al., 2004). Systemic administration of riluzole exerted neuroprotective properties by sparing gray and white matter in rostrocaudal regions surrounding the injury epicenter in rat models (Schwartz and Fehlings, 2001; Ates et al., 2007), alone or in combination with MPSS (Mu et al., 2000); moreover, regrowth of sensory axons has been observed in *in vitro* preparations (Shortland et al., 2006). Considering the promising experimental results and the knowledge deriving from the use of riluzole for ALS therapy (Miller et al., 2012), two clinical trials have been planned to assess safety and efficacy of riluzole treatment in acute SCI (ClinicalTrials.-gov Identifier: NCT00876889 and NCT01597518).

Glutamate level rises rapidly after CNS injury; the consequent overstimulation of NMDA receptors can lead to a massive influx of calcium that in turn can provoke axonal injury and apoptotic or necrotic cell death through the activation of enzymes, mitochondrial damage, acidosis and production of free radicals. Furthermore, Ca^{2+} homeostasis is important

for the maintenance of vascular smooth muscle physiology (Liu et al., 2011). Preclinical studies showed that administration of nimodipine, a member of the dihydropyridine class of Ca^{2+} channel blockers, could increase spinal cord blood flow (Guha et al., 1985) and reverse ischemic hypoperfusion (Fehlings et al., 1989), even though these results were not replicated by other groups (Ford and Malm, 1985; Haghighi et al., 1993). A clinical trial conducted in France failed to demonstrate the efficacy of nimodipine in SCI patients (Pointillart et al., 2000). Conversely, gacyclidine (GK-11, Beaufour-Ipsen Pharma), a non competitive NMDA receptor antagonist, improved function, histology, and electrophysiology in a rat model of SCI (Hirbec et al., 2001). In addition, GK-11 was substantially less neurotoxic than other NMDA antagonists (Gaviria et al., 2000), making it feasible for human application. A double-blind Phase II clinical trial was completed in France on 200 patients (Tadie et al., 1999) ; despite promising preliminary results, one year post injury analysis revealed a non significant trend toward improved function in treated patients, then GK-11 was no longer investigated as a therapeutic agent for SCI (Hawryluk et al., 2008).

Magnesium is an endogenous voltage-dependent blocker of NMDA receptors; moreover, Mg^{2+} blocks voltage-gated calcium channels, and improves vascular perfusion by inhibiting vasoconstriction (Vink and Cernak, 2000). Since Mg^{2+} levels decrease significantly within the injured spinal cord (Lemke and Faden, 1990) and brain (Vink et al., 1987), its exogenous application has been proposed for SCI cure. Different studies reported that Mg^{2+} improved biochemical, physiological, histological, and locomotor parameters after experimental injury (Süzer et al., 1999; Kaptanoglu et al., 2003a, b; Solaroglu et al., 2005; Wiseman et al., 2009), but the high dosage failed to reach the safety requirements for human therapy (Temkin et al., 2007). Recently, further analysis showed that a lower Mg^{2+} dose combined with polyethylene glycol (Medtronic, Inc) enhanced long-term clinical outcomes after SCI in rats (Di-

tor et al., 2007). A Phase II multicenter study on human SCI patients is currently being organized by Medtronic (Kwon et al., 2010a).

The demyelination phenomenon caused by SCI exposes fast voltage-gated K^+ channels at the internodal regions (Nashmi and Fehlings, 2001). The increased activity of K^+ channels results in the shunting of Na^+ current, short-circuiting of depolarization, and inhibition of the genesis of action potentials (Liu et al., 2011), thus provoking conduction failure. *In vitro* studies demonstrated that 4-aminopyridine (4-AP), a fast K^+ channel blocker, induces the increase of action potentials conduction in models of chronic SCI (Shi and Blight, 1997; Jensen and Shi, 2003). Since the preliminary study conducted in 1993 by Hansenbout and coworkers on 8 patients with an incomplete chronic SCI (Hansebout et al., 1993), various clinical evaluations showed that 4-AP can ameliorate motor and sensory function below injury site (Wolfe et al., 2001; Grijalva et al., 2003), while other studies failed to achieve functional benefits (van der Bruggen et al., 2001; DeForge et al., 2004). Additional research is aimed to identify K^+ blockers with greater affinity; recently, the novel K^+ channel blocker 4-AP-3-MeOH has been evaluated as an alternative choice for the reversal of conduction block in experimental SCI (Sun et al., 2010).

Modulators of myelin associated inhibitors

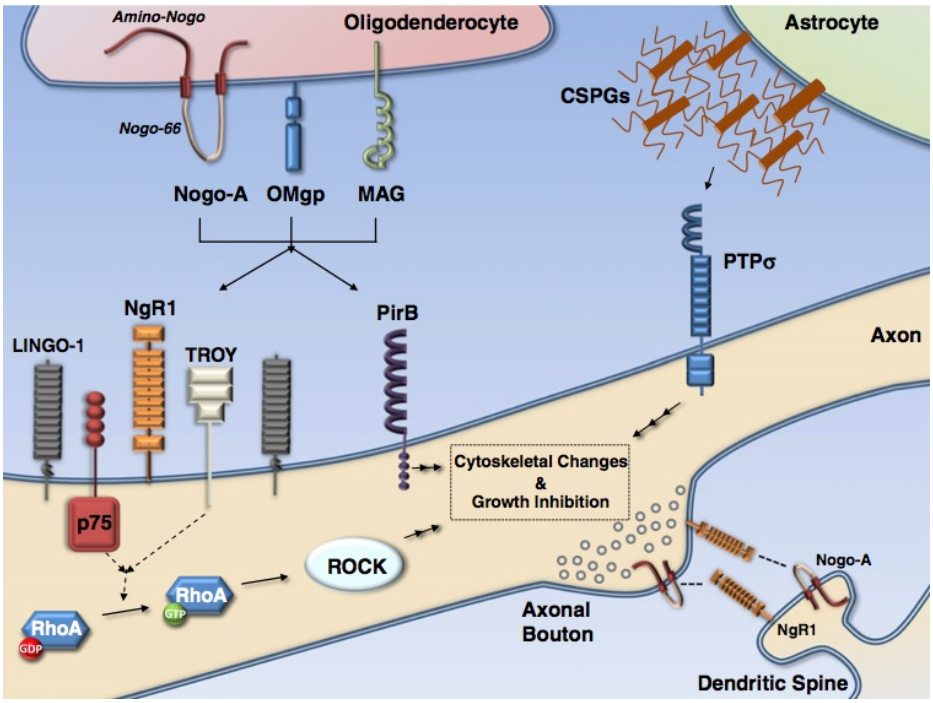

Figure 4. Growth inhibitors in the CNS. Abbreviations: CSPGs, chondroitin sulfate proteoglycans; ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; MAG, myelin associated glycoprotein; NgR1, Nogo-Receptor-1; OMgp, oligodendrocyte myelin glycoprotein; PirB, Paired- Immunoglobulin-like Receptor; PTPσ, Protein-Tyrosine-Phosphatase-σ; TROY, TNFα orphan receptor (from Akbik et al., 2012).

The inhibitory effect of white matter toward regeneration in the CNS was originally proposed by Ramon y Cajal in 1911. The direct experimental evidence was later provided by Schwab and colleagues who showed that CNS myelin, but not PNS myelin, possesses potent neurite growth inhibitory activity (Berry, 1982; Carbonetto et al., 1987; Caroni and Schwab, 1988; Crutcher, 1989; Savio and Schwab, 1989). Further research allowed to identify various myelin-derived inhibitory proteins (see Harel and Strittmatter, 2006 and Lee and Zheng, 2012 for a review); in particular, three molecules have been studied in deep in relation with CNS degeneration: myelin-associated glycoprotein (MAG, McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte myelin glycoprotein (OMgp, Mikol and Stefansson, 1988;

Wang et al., 2002b) and Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

Myelin associated glycoprotein is expressed by oligodendrocytes in the periaxonal layer of myelin during the first phases of axonal myelin ensheathment and persists in a strictly periaxonal distribution, suggesting a stabilizing glial-axonal interaction (Bartsch et al. 1989; Trapp 1990). Myelin associated glycoprotein is known to have a dual role in axon growth depending on the developmental stage of the neurons studied: growth promoting on younger neurons while growth inhibitory on older neurons, with this transition occurring at or soon after birth (Mukhopadhyay et al. 1994). Recombinant MAG potently inhibits neuronal cultures *in vitro*, and this inhibition is rescued by immunodepletion of MAG (McKerracher et al. 1994; Mukhopadhyay et al. 1994).

Originally isolated from CNS myelin, OMgp is detectable on membranes of several neuronal types, oligodendrocytes, and periaxonal myelin (Habib et al., 1998; Huang et al., 2005). Recombinant OMgp potently inhibits neurite outgrowth of cerebellar granule neuron cultures and induces growth cone collapse in adult dorsal root ganglion cell cultures (Kottis et al., 2002; Wang et al., 2002b).

Nogo (or Rtn4) is a membrane protein belonging to the Reticulon family. Although Nogo exists in three different isoforms (A, B, and C), Nogo-A is the principle one, and its expression in the CNS, but not in the PNS (Huber et al., 2002; Wang et al., 2002c), suggests that Nogo-A may account for limited CNS regeneration. Nogo-A is highly expressed by neurons during development, while in the adult mammalian CNS it is found mainly in oligodendrocytes (Josephson et al., 2001; Huber et al., 2002; Wang et al., 2002c). Nogo-A has two main inhibitory domains: amino-Nogo, which is unique to the Nogo-A specific N-terminus, and Nogo-66, a 66-amino-acid loop which is common to all three isoforms (GrandPre et al., 2000). Nogo-A has been repeatedly shown to initiate growth cone collapse and inhibit neurite

outgrowth *in vitro* by a wide number of laboratories (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Wang et al., 2002c), and to limit regeneration of the CST after spinal cord injury in mammalian animal models (Schnell and Schwab, 1990; Bregman et al., 1995; Fouad et al., 2004).

The identification of Nogo-A and the description of Nogo-66 inhibitory fragment led to the discovery of Nogo Receptor-1 (NgR1), a GPI-linked leucine rich repeat protein (Fournier et al., 2001). Interestingly, NgR1 constitutes a common receptor for Nogo-A, MAG and OMgp, despite the lack of homology in their sequences (Liu et al., 2002; Wang et al., 2002b). To initiate intracellular signal transduction, NgR1 requires the interaction with a membrane associate co-receptor (see Figure 4Figure 5), such as the low-affinity neurotrophin receptor p75 (Wang et al., 2002a), LINGO-1 (Mi et al., 2004) or TNFα orphan receptor TROY (Park et al., 2005). Moreover, in a screen for novel receptors for Nogo-66, Atwal and colleagues identified Paired Immunoglobulin-like Receptor B (PirB) as a high affinity receptor for Nogo-66, MAG, and OMgp (Atwal et al., 2008). Activation of NgR1 and signaling through its co-receptors leads to increased activation of the small GTPase RhoA and its effectors ROCK and LIMK1 (Fournier et al., 2003; Hsieh et al., 2006; Montani et al., 2009), while it reduces Rac1 activity (Niederost et al., 2002). These signaling cascades result in growth cone collapse and neurite outgrowth inhibition through modulation of actin polymerization, as seen in *in vitro* and *in vivo* models (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

The first strategy developed to counteract Nogo-A inhibitory effect consisted in the use of IN-1, an antibody raised against the myelin fraction containing Nogo-A (Caroni and Schwab, 1988). Afterwords, two new IgG anti-Nogo-A antibodies were developed (Buffo et al., 2000; Wiessner et al., 2003). The blockade of Nogo-A signaling with specific anti-Nogo-A antibodies enhanced regenerative CST sprouting and long-distance elongation in several

models of SCI; moreover, animals showed a remarkable functional recovery after treatment (Schnell and Schwab, 1990; Bregman et al., 1995; Thallmair et al., 1998; Brosamle et al., 2000; Raineteau et al., 2001; Fouad et al., 2004; Freund et al., 2006). Very similar results were obtained through the inactivation of Nogo-A by intrathecal infusion of a soluble decoy receptor, NgR(310)ecto-Fc (Fournier et al., 2002; Li et al., 2004; Robak et al., 2009) or by blocking NgR with NEP1-40, an antagonistically active Nogo fragment competitive for Nogo-66 binding site (GrandPre et al., 2002; Li and Strittmatter, 2003). In addition, blockade of NgR1 co-receptor LINGO-1 in rats promoted sprouting of both rubrospinal and corticospinal axons and improved functional recovery after SCI (Ji et al., 2006).

In 2006, Novartis sponsored the initiation of a multicenter, nonrandomized human clinical trial in Germany, Switzerland and Canada (ClinicalTrials.gov Identifier: NCT00406016) for the intrathecal infusion of the anti-Nogo-A antibody ATI355. In Phase I more than 50 acute ASIA A paraplegic or tetraplegic patients were enrolled to estimate safety, dosing, tolerance and pharmacokinetics; so far, no deleterious side effects were observed. A Phase II has been planned to evaluate the effects of acute treatment before the beginning of therapeutic rehabilitation (Starkey and Schwab, 2012).

A different strategy to promote axonal sprouting and recovery after CNS injury consists in the modulation of the Rac/Rho pathway, which has been found to constitute the common intracellular signaling cascade for the various myelin associated inhibitors (Figure 4). The application of Rho-A (the C3 transferase) or ROCK (the Y27632) inhibitors in the lesion site has been demonstrated to be neuroprotective (Rho activation contributes to apoptosis after neural damage) and to improve the behavioral outcome in partially transected mice (Dergham et al., 2002; Dubreuil et al., 2003; McKerracher and Higuchi, 2006; Dubreuil et al. 2003; McKerracher et al. 2006). Cethrin, a more cell-permeable version of C3, has been evaluated in two clinical trials in North America and Europe. The first study, sponsored by BioAxone

Therapeutic Inc., enrolled 37 ASIA A thoracic and cervical SCI patients that were treated with Cethrin within 7 days of their injury. The Phase II was concluded in 2007 and preliminary results suggest that part of patients experienced a partially recovery without major adverse effects (Kwon et al., 2010b). The second trial, promoted by Alseres Pharmaceuticals Inc., was a multicenter, randomized, double-blind, placebo-controlled, Phase II study and included adult patients with acute cervical SCI who received Cethrin within 72 hours from injury (ClinicalTrials.gov Identifier: NCT00610337). Recently, it has been demonstrated that some non-steroidal anti-inflammatory drugs like ibuprofen and indomethacin inhibit the Rho-A signaling pathway *in vitro* and *in vivo* (Zhou et al., 2003); moreover, these drugs promoted axonal regeneration and locomotor recovery via RhoA inhibition in rats subjected to SCI (Fu et al., 2007). Since these compounds are already widely used as pain relievers, they are likely to be transferred to human clinical trials for SCI therapy.

Targeting the Glial Scar

After SCI, activated hypertrophic glia forms the glial scar and produces high amounts of ECM molecules, which reinforce the scar structure. Chondroitin sulphate proteoglycans (CSPGs) are a class of inhibitory ECM molecules (aggrecan, brevican, neurocan, versican, phosphacan and NG2) characterized by a core protein covalently linked to linear chondroitin sulfate glycosaminoglycan chains (CS-GAGs) (Kjellen and Lindahl, 1991; Morgenstern et al., 2002). Chondroitin sulphate proteoglycan expression is important for the stabilization of synaptic connections in the CNS (Bartus et al., 2012): as the CNS matures and target innervation occurs, CSPG levels become down-regulated; conversely, their expression increases in areas where they are required for synaptic stability, forming lattice-like ECM structures called perineuronal nets (PNNs) around neuronal cell bodies and dendrites (Celio et al., 1998). Perineuronal nets maturation peaks in concomitance with the end of the critical period and the

reduction of spine dynamics; besides, PNNs expression appears to be restricted to GABA-expressing neurons (Hartig et al., 1992; Hensch, 2005).

The glial scar represents a physical barrier to axonal regeneration; moreover, CSPGs inhibit the regenerative response through the Rho-ROCK signaling pathway (Borisoff et al., 2003; Sivasankaran et al., 2004; Koprivica et al., 2005) and the CSPGs receptor PTPσ (transmembrane protein tyrosine phosphatase sigma, Shen et al., 2009). Chondroitinase ABC (ChABC) is a bacterial enzyme which cleaves CS-GAG chains from the CSPG core protein (Suzuki et al., 1968; Yamagata et al., 1968). The application of ChABC in *in vitro* preparations demonstrated that the liberation of CS-GAG chains allows neurite outgrowth on tissues rich in CSPGs such as injury-induced gliotic cortical tissue or adult spinal cord slices (McKeon et al., 1995; Zuo et al., 1998). ChABC infusion in experimental animals digested scar tissue in the injured brain (Moon et al., 2001) and spinal cord (Lemons et al., 1999), and induced regeneration of lesioned axon (Moon et al., 2001; Bradbury et al., 2002). Since then, a lot of studies demonstrated that ChABC application is able to induce functional recovery in SCI models inducing the regeneration of injured fibers and the remodeling of injured and lesion-spared axons, alone (Bradbury et al., 2002; Caggiano et al., 2005; Barritt et al., 2006; Massey et al., 2006; Garcia-Alias et al., 2008; Tester and Howland, 2008; Starkey et al., 2012) or in combination with other treatments (Yick et al., 2004; Fouad et al., 2005; Houle et al., 2006; Massey et al., 2008; García-Alías et al., 2009, 2011; Tom et al., 2009; Karimi-Abdolrezaee et al., 2010, 2012).

Alternative strategies to counteract the inhibitory effects of the glial scar are based on the reduction of CSPG deposition (Logan et al., 1999; Davies et al., 2004; Minor et al., 2008; Fujiyoshi et al., 2010), the inhibition of CS chains glycosilation (Grimpe and Silver, 2004; Grimpe et al., 2005; Hurtado et al., 2008), the blockade of CS motifs responsible for CSPG-neuron interaction (Gama et al., 2006; Tully et al., 2006; Shen et al., 2009; Ohtake-Niimi et

al., 2010; Coles et al., 2011), or the modulation of CSPG signaling cascade, such as PTPσ knock-out (Shen et al., 2009; Fry et al., 2010) and Rho-ROCK pathway perturbation (Dergham et al., 2002; Sivasankaran et al., 2004; Koprivica et al., 2005; Lingor et al., 2007).

However, the glial scar constitutes also a protection from the spread of cellular damage, infections and infiltration of non-CNS constituents (Fitch et al., 1999; Myer et al., 2006). Matrix metalloproteinases (MMPs), which degrade extracellular matrix and proteins (Sternlicht and Werb, 2001) are up-regulated after SCI (Wells et al., 2003; Fleming et al., 2006; Buss et al., 2007), thus inducing an increase in blood brain barrier permeability. The genetic deletion of MMP-12 and MMP-9 allowed the recovery of function in a mouse model of SCI (Noble et al., 2002), indicating MMPs as a possible target for future therapies.

Modulation of intrinsic regenerative response

A powerful approach to enhance the effects of growth promoting molecules in the injured spinal cord consists in the induction of intrinsic regenerative response of damaged neurons. For instance, the association of subcortical BDNF-secreting grafts with the overexpression of trkB receptor in CST neurons has been showed to be effective in the induction of axonal regrowth into the transplanted tissue via the activation of the ERK pathway (Hollis et al., 2009a, Figure 4). Intrinsic response can be enhanced by gene transfer of transcription factors, like the cAMP response element binding protein (CREB, Gao et al., 2004), the activating transcription factor 3 (ATF3, Seijffers et al., 2007), or the signal transducer and activator of transcription 3 (STAT3, Qiu et al. 2005), or proteins important during CNS development such as retinoic acid receptor beta (RARβ, Wong et al., 2006; Yip et al., 2006) and neuronal calcium sensor-1 (NCS1, Yip et al., 2010). A particular case is represented by dorsal root ganglion neurons: the application of a “conditioning lesion” to the peripheral branch of sensory neurons leads to rapid and sustained changes in gene expression such as upregulation of cAMP signaling

pathways (Richardson and Issa, 1984; Neumann et al., 2002; Qiu et al., 2002; Lu et al., 2004c) and cytokines (Cafferty et al., 2004; Cao et al., 2006), and these changes are translated in increased axonal regeneration. Besides, the effectiveness of conditioning lesion has been validated in acute as well as chronic spinal cord injuries (Kadoya et al., 2009), confirming that sensory axons retain the ability to respond to regenerative stimuli.

The effect of intracellular cAMP elevation can be achieved through indirects methods. Forskolin, an adenyl cyclase activator, stimulated nerve regeneration in transected sciatic nerve *in vivo* (Kilmer and Carlsen, 1984). Injection of a cAMP homologue, dbcAMP, induced increased regeneration of spinal axons in *in vitro* and *in vivo* models (Neumann et al., 2002; Qiu et al., 2002; Lu et al., 2004c). Rolipram, an inhibitor of phosphodiesterase 4 (PDE4, a degrading enzyme of cAMP, Krause and Kuhne, 1988), increased 5HT axon regeneration, reduced astrogliosis and induced functional recovery in rats subjected to SCI and cell transplants (Nikulina et al., 2004; Pearse et al., 2004). Intriguingly, rolipram is able to cross the blood brain barrier, then it can be delivered orally or subcutaneously, making it a good candidate for use in patients

Modulation of serotoninergic tone

Serotonin is the most widely distributed transmitter in the brain (Dahlstrom and Fuxe, 1964; Steinbusch, 1981). In the mammalian brain, the cell bodies of serotoninergic neurons are found in and around the midline raphe nuclei of the brain stem (Dahlstrom and Fuxe, 1964; Azmitia and Whitaker-Azmitia, 2000); from these sites, 5HT cells send out the projections that innervate the diverse areas throughout the brain and the spinal cord (Figures 5 and 6, Jordan et al., 2008; Tuszynski and Steward, 2012).

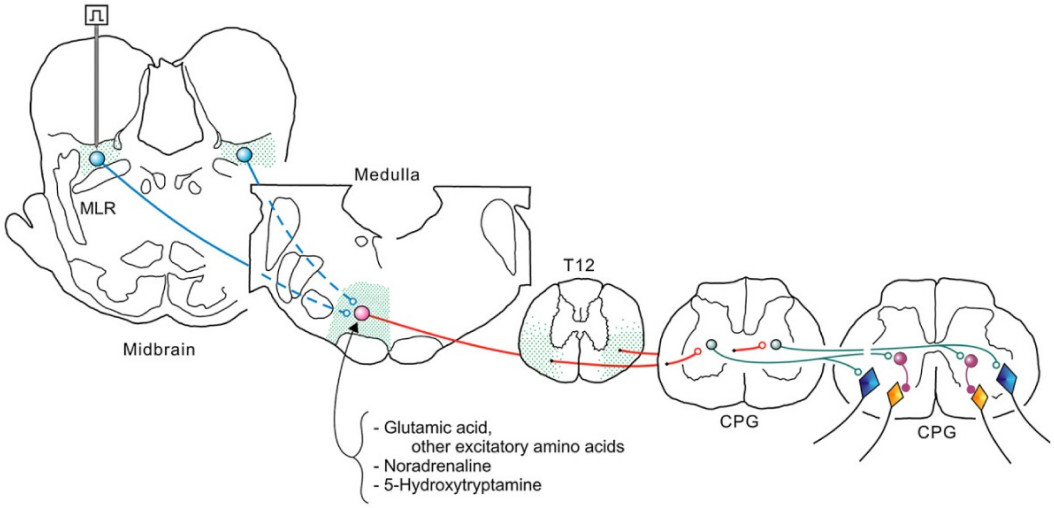

Figure 5. Serotoninergic projections. Schematic diagram showing the locomotor areas and putative pathways at brainstem and spinal levels. Blue circles represent neurons of the mediolateral raphe, and red circles represent medullary reticulospinal neurons of the locomotor pathway. Neurotransmitters thought to be present in reticulospinal locomotor neurons are indicated. At the spinal level, excitatory (green) and inhibitory (purple) cells represent neurons of the CPG. Motoneurons are shown in blue and orange (from Jordan et al., 2008).

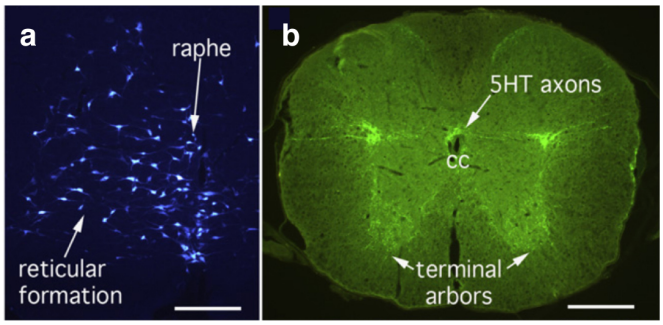

Figure 6. Serotoninergic projections. (**a**) Retrogradely labeled neurons in the midline raphe and reticular formation after injections of *true blue* into the spinal cord of a mouse. Neurons in the raphe nucleus give rise to serotoninergic (5HT) axons that project to the spinal cord. (**b**) Immunofluorescence for 5HT in the thoracic spinal cord. CC, central canal. Scale bars, 250 µm. Adapted from Tuszynski and Steward, 2012.

Accumulating data indicate that 5HT regulates neural plasticity during development and in the adult, modulating neural cell proliferation, cell migration and differentiation, neurite outgrowth, axonal guidance, synaptogenesis, and efficiency of trans-synaptic signaling (for review Gaspar et al., 2003; Daubert and Condron, 2010). Embryonic 5HT depletion af-

fects cortical development, reducing dendritic arborization of pyramidal neurons in the somatosensory cortex (Vitalis et al., 2007). *In vitro* research showed that 5HT directs interneuron migration (Vitalis et al., 2007; Riccio et al., 2009), thalamocortical axon pathfinding (Bonnin et al., 2007), and GABAergic circuitry maturation in the spinal cord (Allain et al., 2005). Moreover, besides its role in synapse formation and network construction during development, increasing evidence implicates 5HT in the regulation of cell adhesion molecules critically involved in the plasticity of the developing and adult brain (Yamagata et al., 2003; Dalva et al., 2007).

Serotoninergic inputs from the raphe nucleus and parapyramidal region of the ventral medulla (Ballion et al., 2002; Jordan et al., 2008) innervate the central pattern generators in the spinal cord, neuronal spinal networks capable to generate rhythmic motor activity in the absence of rhythmic input from peripheral receptors (CPGs, Grillner and Zangger, 1979; Grillner and Wallen, 1985). Experiments in cats demonstrated that pharmacological treatments with agonists of serotonin can evoke or modulate locomotor activity after chronic SCI (Barbeau et al., 1981; Barbeau and Rossignol, 1987, 1991; Gerasimenko et al., 2009). These results where confirmed in spinalized rodents, where the recovery of locomotor activity was achieved with the transplant of embryonic 5HT neurons below the lesion (Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000) and administration of 5HTR agonists (Feraboli-Lohnherr et al., 1999; Antri et al., 2002, 2003; Landry et al., 2006; Gerasimenko et al., 2007; Ung et al., 2008; Courtine et al., 2009; Dominici et al., 2012; Musienko et al., 2012).

Besides its immediate effect as a neurotransmitter, 5HT has other neurobiological functions, especially neurotrophic and neuroprotective effects (Azmitia, 2001). Recent studies carried out in animal models highlighted that selective serotonin reuptake inhibitors (SSRIs) , a class of drugs clinically used to cure mood disorders and depression, can induce functional recovery from neurological impairments due to brain injuries and degenerative diseases in

animal models and in patients (Narushima et al., 2007; Li et al., 2009; Yi et al., 2010; Chollet et al., 2011; Cirrito et al., 2011), encouraging the application of SSRIs in the regenerative therapy field.

Rehabilitative training

After injury, spontaneous recovery of function due to plastic response of injured and uninjured circuits has been observed in animal models as well as in patients with an incomplete lesion (Raineteau and Schwab, 2001). Since circuitry below the lesion site maintain the capability to respond to peripheral stimuli, rehabilitative training is a powerful tool to enhance the plastic response and increase locomotor recovery. Similarly to what happens during development, experience can model connections that are forming after SCI, strengthening active synapses and dismissing the less active (Hebb, 1949), than specific training can induce the recovery of the exercised function.

Rehabilitative training following SCI leads to use-dependent adaptive changes in the circuits below the lesion (Lovely et al., 1986; Barbeau and Rossignol, 1987), such as modulation of growth factors, adhesion and guidance molecules expression (Vaynman and Gomez-Pinilla, 2005; Maier et al., 2008), decreased inhibition (De Leon et al., 1999; Edgerton et al., 2001; Tillakaratne et al., 2002), increase of sprouting of lesioned and spared descending axons (Girgis et al., 2007; Krajacic et al., 2010), and modification of neuronal properties, such as motoneuron function normalization (Gardiner et al., 2006; Petruska et al., 2007; Beaumont et al., 2008). After rehabilitation, the spinal cord acts as a smart processing interface that continuously integrates multisensory input to control its motor output in partial or total absence of brain input (Musienko et al., 2012); moreover, repetitive exposure to specific sensory-motor patterns (i.e. the training) leads to the maintaining of a “memory” of these modification (Grau et al., 2006; Gomez-Pinilla et al., 2007).

Many experiments demonstrated the efficacy of rehabilitative training in the induction of recovery after SCI in animal models and in patients (Barbeau and Rossignol, 1987; Fung et al., 1990; Wernig and Muller, 1992; Donaldson et al., 2000; Beekhuizen, 2005; Smith et al., 2006; Girgis et al., 2007). Most of the studies focused on treadmill training, which has been demonstrated to induce recovery of weight supported stepping in case of thoracic incomplete injuries (Forssberg et al., 1980a, b; Lovely et al., 1986; Barbeau and Rossignol, 1987; van Hedel and Dietz, 2010). Assessing the effect of therapeutic approaches and rehabilitative training on forelimb/hand function is more difficult because their functions are much more complex (Maier and Schwab, 2006). Nevertheless, training the affected limb gave good results (Nudo and Milliken, 1996; García-Alías et al., 2009), in particular with the "constraint-induced therapy", which consists in the forced use of the affected limb while the unaffected limb is constrained (Maier et al., 2008).

The efficiency of locomotor rehabilitation is limited by the capability of the subject to perform the training, especially when the patient experience a complete lesion and/or is at a very old age. To overcome these problems, recently multi-system neurorehabilitation paradigms have been developed: in these protocols, locomotor training is coupled with epidural electrical stimulation and monoamine agonists supply to mimic the loss supraspinal input; this strategy leads to the emergence of a new functional state in the spinal cord that enables full weight-bearing treadmill locomotion in paralyzed rats that is almost indistinguishable from voluntary stepping (Courtine et al., 2009). More recently, the approach has been implemented with the use of robotic devices to help completely paralyzed subjects to execute the training. Locomotor recovery has been observed in rats as well as in humans (Bishop et al., 2012; Dominici et al., 2012; van den Brand et al., 2012).

A common strategy in rehabilitative training is to exercise a specific task to recover a specific function; unfortunately, it has been often observed that training in one task does not

necessarily translate into another (De Leon et al., 1998; Grasso et al., 2004; Smith et al., 2006; Bigbee et al., 2007) and, conversely, it can have a detrimental effect on the untrained function (De Leon et al., 1998; Bigbee et al., 2007; Girgis et al., 2007; García-Alías et al., 2009). Moreover, the combination between training and recovery-promoting treatments led to controversial results, since some studies demonstrated a synergistic effect of combinatorial strategies, with an increase of sensory-motor rescue (Kubasak et al., 2008a; Courtine et al., 2009; García-Alías et al., 2009), while others found that training and/or pro-recovery treatments were more efficient when administered alone, or even detrimental when applied together (Maier et al., 2009; Harel et al., 2010; Jakeman et al., 2011). It has been suggested that these effects could be induced by a bad timing in the application of different strategies, than more research about the temporal window to initiate the rehabilitation and to design meaningful treatment combinations needs to be addressed (Fouad and Tetzlaff, 2012).

Chapter 4: Aim of the thesis

In several studies it has been reported that, when SCI is incomplete, processes of partial spontaneous recovery may take place, both in animal models as well as in injured patients, and that the functional rescue is accompanied by anatomical reorganizations of neural circuits (Boulenguez and Vinay, 2009). Therefore, development of treatments aimed at increasing plasticity of spinal cord circuits emerges as one of the most promising strategies to restore motor function after injury.

Fluoxetine, a selective serotonin reuptake inhibitor (SSRIs), is a FDA-approved drug widely prescribed in the treatment of various neuropsychiatric disorders. Accumulating evidence suggests that SSRIs can boost neural plasticity in the adult brain, with beneficial effects on learning and memory, hippocampal neurogenesis, synaptogenesis and visual cortex plasticity (Malberg et al., 2000; Hajszan et al., 2005; Maya Vetencourt et al., 2008; Li et al., 2009). Strikingly, recent studies carried out in animal models and clinical trials highlighted that fluoxetine can induce functional recovery from neurological impairments due to brain injuries and degenerative diseases (Narushima et al., 2007; Li et al., 2009; Begenisic et al., 2013). Indeed, it has been demonstrated that an early prescription of fluoxetine coupled with physiotherapy enhances motor recovery in patients with ischemic stroke (Chollet et al., 2011).

In this study, I investigated whether increasing neural plasticity with fluoxetine could induce functional recovery in a rat model of spinal cord injury. I tested the impact of fluoxetine on a dorsal funiculi crush at the C4 spinal segment level, a widely studied model of cervical injury leading to partial loss of sensory inputs to the brain and descending corticospinal control of movements. In order to maximize the enhancement of neural plasticity operated by fluoxetine, I started the pharmacological treatment three weeks before performing injury, in agreement with a number of studies showing that at least 21 days of treatment are required to

induce beneficial effects in the central nervous system (Berton and Nestler, 2006; Baudry et al., 2010).

At first, I analyzed the motor performance of treated and untreated lesioned rats in several behavioral paradigms of forelimb function and fine sensorimotor skills. I chose the classical Montoya staircase (Montoya et al., 1991) and horizontal ladder (Metz and Whishaw, 2009) as sensitive tests to evaluate forelimb skilled reaching ability, and the footprint analysis of gait (de Medinaceli et al., 1982) as an estimation of general walking behavior of the rat.

At the end of behavioral assessment, in order to investigate possible mechanisms underlying the effects elicited by fluoxetine on motor behavior, I injected an anterograde tracer, BDA, in the forelimb representation of rat motor cortex, and I analyzed the sprouting of CST fibers caudal to the lesion site. Moreover, since serotoninergic axon plastic rearrangement has been suggested to be involved in the recovery of motor function after SCI (Sharma et al., 1990; Inman and Steward, 2003; Camand et al., 2004), I also measured 5HT fiber density around the lesion site with immunohistochemistry techniques.

Finally, based on the results on motor recovery and induction of anatomical plasticity, I examined the molecular changes underlying the enhancement of plasticity induced by three weeks of fluoxetine treatment. Given the well-known role of cortical inhibitory circuits in the regulation of plasticity time-course (Hensch, 2005; Sale et al., 2010; Baroncelli et al., 2011), I analyzed the levels of excitation and inhibition in the motor cortex and the cervical spinal cord of uninjured rats by semiquantitative Western Blot for the neurotransmitter transporters for glutamate (vGluT-1) and GABA (vGAT) and glutamate and GABA stimulus-evoked overflow from synaptosomes. In addiction, I measured BDNF and NT3 neurotrophin expression in the cervical spinal cord of treated and untreated rats using semiquantitative Western Blot (BDNF) and Enzyme Linked ImmunoSorbent Assay (ELISA, NT3).

A schematic diagram of experimental plan is provided in Figure 7.

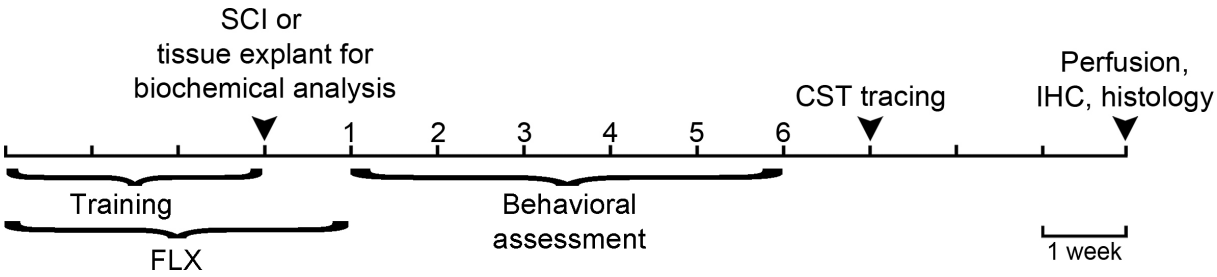

Figure 7. Time-line showing the experimental protocol. Abbreviations: SCI, spinal cord injury; FLX, fluoxetine; CST, corticospinal tract; IHC, immunohistochemistry.

Materials and Methods

Animal treatment

Adult (2-3 months) male Long Evans rats were used in this study, which was approved by the Italian Ministry of Health (Decreto N° 182/2011-B, 26/09/2011). Three weeks before spinal cord injury (SCI), rats were divided into two groups: control (CTR) and fluoxetine-treated (FLX). Fluoxetine (Fluoxetine-hydrochloride, Galeno, Prato-Italy) was administered in the drinking water (0.2 g/l) as previously described (Maya Vetencourt et al., 2008), corresponding to 16.45±0.36 mg/kg/day. Fluoxetine (or Prozac, Wong et al., 1974) is a highly active 5HT reuptake blocker *in vitro* and *in vivo*. After oral administration, fluoxetine is almost completely absorbed. Fluoxetine undergoes extensive metabolic conversion leading to the active metabolite norfluoxetine. Fluoxetine has a half-life of 1 – 4 days, whereas the half-life of norfluoxetine ranges between 7 and 15 days (Preskorn, 1997). At least 4 weeks of constant medication are necessary to reach steady state levels of fluoxetine (Vaswani et al., 2003). In rat, fluoxetine wash out requires about 3 days (Caccia et al., 1990). The treatment was not interrupted immediately after SCI, but continued until the end of the first postoperative week.

Spinal cord injury

Rats were deeply anesthetized with 1 ml/hg avertin (2.2.2 tribromoethanol solution; Sigma; 200 mg/kg, i.p.). After shaving the fur between the ears and the scapulae, the skin was sanitized with iodine solution (Betadine). A skin incision (2 cm) was performed in correspondence of the gap between the occipital bone and the dorsal edge of T2 vertebra. Scissors were inserted to blunt dissect the skin from muscles. A pillow was positioned under rat thorax in order to lift up the vertebral column in correspondence of cervical level. Hooks were inserted to pull aside the skin and expose muscles, and subsequently the muscle layers were cut along the

midline. Using blunt dissection, the superficial muscle layer was separated from the intermediate one, which was next blunt dissected from the deep muscle layer. Care was taken in order to avoid fat pat damage. The hooks were repositioned to reveal the deep muscle layer overlaying the vertebral column. Using scissors, the vertebral bone was exposed. C4 dorsal process was lift up with forceps, and the rostral and caudal ligaments of C4 vertebra were cut; after this, the dorsal lamina was removed with fine rongeurs. The dura mater was picked up with forceps and cut along the midline; then, 4-5 drops of the local anesthetic lidocaine were applied onto the spinal cord surface; after 3 minutes, the remaining fluid was removed and the spinal cord was rinsed in saline.

Spinal cord injury (SCI) was induced inserting the tips of fine forceps 2 mm in depth into the spinal cord parenchyma spanning the gap between the dorsal root entries (1.5 mm lateral to the midline) and down to the spinal canal and keeping them closed for 20 seconds. The injury included the descending dorsal corticospinal tracts (CSTs) and the ascending sensory dorsal columns. In a group of animals (CTR-sham control), only laminectomy was performed, without induction of spinal damage.

After SCI induction, the intermediate and superficial muscle layers were sutured with resorbable thread (Covidien Polysorb 5.0) and the wound was sanitized with Betadine. The skin was sutured with surgery staples. At the end of the surgery, rats were injected with desametasone (1 ml/kg, i.p.) and kept in a warm cage until recovery from anesthesia; afterward, rats were carried back in their home cage and were treated for 3 days with paracetamol (33 mg/kg/day, in drinking water).

Behavioral assessment

Montoya Staircase reaching task

In this task, animals had to grasp and eat sugar pellets (Bio-serv 45 mg Dustless Precision pellets) from a staircase (Campden Instruments Ltd.; Montoya et al., 1991) of seven small wells 8 mm deep at increasing distance from the rat on either side of a central divider. Before injury, rats were food deprived to 90% of free feeding weight to increase their motivation in test performance, then they were trained for three weeks to remove and eat as many sugar pellets as possible, until they reached at least 16 pellets eaten on a total of 28 (2 pellets for each well) in 15 minutes. All the animals that failed to achieve this number were discarded. After SCI, rats were tested once a week. Four measures were recorded in this test: eaten pellets, corresponding to the total number of retrieved and eaten; displaced pellets, which are the pellets that the rat can touch but not successfully retrieve; accuracy, i.e. the ratio between the number of eaten pellets and the sum of eaten and unsuccessfully displaced pellets; reached level, which is a measure of the maximum distance reached by the rat. A total of 6 CTR-sham, 13 CTR-inj (injured controls) and 10 FLX rats were included in this experiment.

Horizontal ladder

This task is designed to assess skilled walking in the rat. Briefly, animals were trained to walk along an apparatus that consisted of side walls made of clear Plexiglas and metal rungs (3 mm diameter), which could be inserted to create a floor with a minimum distance of 1 cm between rungs (Metz and Whishaw, 2009). The side walls were 1 m long and 19 cm high, measured from the height of the rungs. The ladder was elevated 20 cm above the ground, and a neutral start cage and a refuge (home cage) were positioned at each end. The animals were trained to cross the ladder from the neutral cage to reach their home cage, so the home cage provided the positive reinforcement for walking. Rats were trained for 3 days before injury, with 3 tri-

als for each session: in the first 2 sessions, the rungs were regularly spaced every 2 cm to allow the animals to become confident with the task; during the last session, an irregular pattern (with rungs differentially spaced from 1 to 5 cm) was used in order to avoid that the animals could learn the pattern.

Trials were video recorded over a 60 cm stretch and analyzed offline. The performance recorded in the last session was used as baseline measurement. After SCI, rats were tested once a week for 6 weeks on an irregular pattern, that was changed for each session. The average number of forepaw steps made over 3 trials per session and the number of footslips were recorded. Accuracy was evaluated as the percentage of correct steps on total. Behavioral testing in the horizontal ladder was performed in a subgroup of the same animals used for the Montoya staircase task (N = 6 CTR-sham, 7 CTR-inj and 7 FLX).

Footprint analysis of gait

This test allows a detailed analysis of motor coordination and synchrony of gait during normal walking (de Medinaceli et al., 1982; Brooks and Dunnett, 2009). Before SCI, the fore- and hindpaws were painted with dyes of different colors (red for forepaws and blue for hindpaws) and rats were encouraged to walk in a straight line along a 80 cm long runway over absorbent paper toward home cage. The footprint patterns were then digitalized and analyzed with Photoshop software for stride length, stride width and coordination.

Forepaw stride length was determined by measuring the distance between two consecutive prints, while stride width was determined by measuring the distance between two consecutive right and left forepaw prints. Coordination was measured as the distance between forelimb and hindlimb footprints. A series of at least five sequential steps was used to determine the mean values of each measurement. After injury, animals were tested once a week for 6 weeks. In this test, in order to avoid possible confounding effects in gait abilities due to

practice in other behavioral task, a separate set of animals were used (N = 6 CTR-sham, 9 CTR-inj and 14 FLX).

Corticospinal tracing and histological assessment

At the end of the functional evaluation, animals were deeply anesthetized with avertin and placed in a stereotaxic frame. With a dental drill, three holes were made bilaterally in the skull, over the underlying representation of the forelimbs in the sensorimotor cortex, and 0.25 µl of 10% biotinylated dextran amine (BDA-10.000, Invitrogen) solution in 0.01M PBS was slowly injected using a glass micropipette (Blaubrand) via each burr-hole. BDA was injected stereotaxically at a depth of 1.2 mm at six sites distributed over the sensorimotor cortex (stereotaxic coordinates: AP 0, ML ± 2; AP +1, ML ± 2; AP 2, ML ± 3). After the last injection, the skin was sutured and the animals were returned to standard housing conditions for three weeks and then were sacrificed by chloral hydrate overdose and transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains and the C3–C5 spinal cord segments were removed and postfixed in the same solution at 4°C overnight, followed by 30% sucrose. Tissue was frozen in Tissue-Tek OCT (Sakura, The Netherlands) and transverse 50 µm sections of the medulla oblongata and longitudinal sections of the C3–C5 spinal segments were cut using a CM 3050S cryostat (Leica Microsystems, Milan, Italy). BDA staining was performed with nickel-enhanced diaminobenzidine (DAB) protocol on slides. The accuracy of the lesion was assessed by the quantification of spared tissue above the central canal in sagittal sections of the C3-C5 spinal blocks. Animals which lesion was too superficial or too deep were excluded from analysis. Corticospinal sprouting axons were quantified in the gray matter from the longitudinal sections of the C3–C5 spinal blocks. Five vertical (0.5 mm, 1.5 mm, 2.5 mm, 3.5 mm, 4.5 mm) lines were superimposed on each of at least 10 spinal cord sections (Stereoinvestigator) as reference points for

crossing axons starting at the center of the lesion. To correct for variability in BDA uptake by CST neurons in the sensorimotor cortex, I normalized the quantitative data by counting BDA-labeled axons in the main pyramidal tract in three sections of medulla oblongata. A total of 7 CTR-inj and 7 FLX rats were included in this experiment.

Immunohistochemistry

Perfused cervical spinal cord blocks from CTR-sham, CTR-inj and FLX injured rats were frozen in Tissue-Tek OCT (Sakura, The Netherlands) and coronal 50 μm sections of C2 and C7 spinal segments were cut using a CM 3050S cryostat (Leica Microsystems, Milan, Italy). Sections were collected in serial order on SuperFrostPlus slides (Menzel-Glazer, Germany) and incubated for 1 h in a blocking solution (10% normalized goat serum in PBS, pH 7.4) and then immunostained overnight at 4°C with a rabbit polyclonal antibody to detect 5HT (1:2000, Immunostar). Antigen-antibody binding was revealed with a goat – anti-rabbit Alexa Flour 488 (1:400, Invitrogen) conjugated secondary anti-body. Sections were acquired at 63× magnification (1024×1024 pixels) using a confocal Leica microscope. Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant through the analysis.

The density of 5HT-IR raphespinal innervation of ventral horn was determined using NIH ImageJ version 1.45s. Labeled fibers were selected by thresholding, and average fiber length within gray matter was measured after using the skeletonize function in four sections per level (C2 or C7) from each rat. A total of 6 CTR-sham, 6 CTR-inj and 8 FLX rats were included in this experiment.

Western blot

Uninjured rats (FLX treated for three weeks and CTR) where decapitated after being deeply anesthetized with an overdose of chloral hydrate. Brain and spinal cord where rapidly re-

moved and the cortical area corresponding to the motor cortex and the cervical (C1-C8) portion of the spinal cord where dissected and frozen at -80°C until processing. Tissue explants were homogenized, and proteins were extracted with an isotonic lysis buffer (50 mM Tris HCl pH 7.6, 0.01% NP40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM PMSF, 0.1 Na3VO4, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Total concentration of samples was assessed with a Bradford assay kit (Bio-Rad, Hercules, CA). Fifteen or fifty (respectively, for vGlutT1/vGAT or BDNF quantification) micrograms of protein extracts were loaded on Tris-HCl 12% precast gels (Bio-Rad) and separated using SDS-PAGE (1 hr at 200 V), then blotted on nitrocellulose membrane (Bio-Rad). Blots were blocked using a solution of 4% milk and 0.2% Tween-20 in TBS for 2 hr at RT and then probed with anti-vGluT1 (1:2500, rabbit polyclonal antibody, Synaptic Systems, Tubingen, Germany), anti-vGAT (1:1000, rabbit polyclonal antibody, Synaptic Systems, Tubingen, Germany) or anti-BDNF (1:500, rabbit polyclonal antibody, Santa Cruz Biotechnology). Incubation lasted overnight at 4°C. Blots were then incubated with 1:20000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary anti-body (Jackson Immunoresearch, West Grove, PA) for 2 hr at RT. The signal was detected by enhanced chemiluminescence (ECL) using the luminol/enhancer system (Immun-Star Western C; Bio-Rad) and autoradiography films (HyperFilm; GE Healthcare). As an internal quantification standard, blots were also probed for α-tubulin. To do this, blots were blocked again for 30 min at RT with 5% milk, 0.2% Tween-20 in TBS, with 0.05% sodium azide added to extinguish the peroxidase activity of the anti-rabbit secondary antibody. Then, blots where incubated with anti-α-tubulin mouse monoclonal antibody (1:10000, Sigma, Germany) for 45 min at RT. Finally, blots were reacted with 1:10000 HRP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich Germany) for 45 min at RT and developed with the same ECL method. A total of 6 CTR and 6 FLX rats were included in the vGluT1/vGAT experiment, while 8 CTR and 8 FLX rats were processed for BDNF.

Enzyme Linked ImmunoSorbent Assay (ELISA)

The NT3 protein was measured with a sandwich ELISA using a NT3 immunoassay system (Uscn, Life Science Inc., China), according to the manufacturer's protocol.

Proteins from fresh C1-C8 spinal cord segments were extracted with an isotonic lysis buffer (50 mM Tris HCl pH 7.6, 0.01% NP40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM PMSF, 0.1 Na3VO4, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and the total concentration of the samples was assessed with a protein assay kit (Bio-Rad Laboratories Inc., CA, USA) using a bovine serum albumin (BSA)-based standard curve. The same amount of total proteins (in duplicate) and standards (in triplicate) were incubated in the same ELISA plate pre-coated with an anti-NT3 polyclonal antibody. Following the incubation and washing steps, an anti-NT3 monoclonal antibody was applied. Absorbance values were read at 450 nm in a plate reader (iMark Microplate Absorbance Reader, Biorad). NT3 levels were calculated from the standard curve prepared for the plate, using the Microplate manager software (Bio-Rad). The standard curve was linear in the range used (0-10 ng/mL) and the quantities of NT3 used in experimental samples were always within the linear range of the standard curve.

Neurotransmitter quantification

Synaptosome purification

Animals were sacrificed and then the brain and the spinal cord were rapidly removed; motor cortex area and cervical spinal cord (C1-C8) were dissected at 4°C. Synaptosomes were prepared essentially as previously described (Milanese et al., 2011). The tissue was homogenized at 4°C, utilizing a Teflon/glass homogenizer (clearance 0.25 mm), in 10 volumes of sucrose 0.32 M, buffered with Tris HCl at pH 7.4. The homogenized tissue was centrifuged (5 min, 1000 RCF at 4°C) in order to remove nuclei and cellular debris. Then, the supernatant was gently stratified on a four steps discontinuous Percoll® gradient (2, 6, 10, 20% v/v in Tris

HCl/sucrose) and again centrifuged (5 min, 33500 RCF at 4 °C). After centrifugation, the 10% and 20% Percoll interface, was collected, washed by centrifugation (15 min, 20200 RCF at 4°C) and then resuspended in a physiologic medium (pH 7.4), containing: 140 mM NaCl; 3 mM KCl; 1.2 mM $MgCl_2$; 1.2 mM $CaCl_2$; 1.2 mM NaH_2PO_4; 10 mM HEPES; 10 mM glucose. Total concentration of samples was assessed with a Bradford assay kit (Bio-Rad, Hercules, CA). A total of 6 CTR and 6 FLX rats were included in this experiment.

Release experiments

Synaptosomes were incubated at 37°C for 15 min; aliquots of synaptosomal suspension were layered on microporus filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Superfusion System, Ugo Basile, Comerio, Varese, Italy; Tardito et al., 2010). Superfusion was then started with standard medium at a rate of 0.5 ml/min and continued for 48 min. After 36 min of superfusion to equilibrate the system, samples were collected according to the following scheme: two 3-min samples (t = 36–39 min and t = 45–48 min; basal outflow) before and after one 6-min sample (t = 39–45 min; stimulus-evoked release). A 90 sec period of stimulation was applied at t = 39 min, after the collection of the first sample. Stimulation of synaptosomes was performed with 15 mM KCl, substituting for equimolar concentration of NaCl. Collected samples were analyzed for endogenous glutamate and GABA content. The stimulus-evoked overflow was estimated by subtracting the transmitter content of the two 3-min samples (basal outflow) from the release evoked in the 6-min sample collected during and after the depolarization pulse (stimulus-evoked release). Aminoacid release was calculated as pmol/mg of protein and expressed as Glutamate/GABA ratio.

Neurotransmitter release determination

Endogenous glutamate and GABA content was measured by high performance liquid chromatography analysis following pre-column derivatization with o-phthalaldehyde and gradient separation on a C18 reverse-phase chromatographic column (10 x 4.6 mm, 3 μm; at 30°C; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm;). Homoserine was used as an internal standard.

The following buffers were utilized: solvent A, 0.1 M sodium acetate (pH 5.8)/ methanol, 80 : 20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20 : 80; solvent C, sodium acetate (pH 6.0)/methanol, 80 : 20. The gradient program was as follows: 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 mL/min (Molinaro et al., 2013).

Statistical analysis

Statistical analysis was performed using Sigma Plot 11 (Systat Software, Chicago IL USA).

Multiple groups were compared by ANOVA followed by post-hoc comparisons applying Holm-Sidak test. When two groups were compared, t-test was applied. Normality and homoscedasticity of the data was checked. Data not normally distributed were compared using nonparametric Mann Whitney Rank Sum test (comparison between two groups) and Friedman Repeated Measures Analysis of Variance on Ranks (repeated measures). Significance level was equal to 0.05.

Results

Assessment of the lesion after SCI

Spinal cord lesion (SCI) was induced in Long Evans male rats inserting fine forceps down in the nervous parenchyma sparing the dorsal roots at the level of C4 spinal segments to reach the central canal (Figure 8a). This kind of lesion provokes the loss of fine motor control of the forelimbs: indeed, it disrupts the ascending sensory fibers of the dorsal columns and the descending dorsal corticospinal (dCST) axons and partially compromises the gray matter of the dorsal horns. Furthermore, it leads to the formation of a cystic cavity full of liquid (Figure 8b-d). Complete damage to the dCST was verified by observing complete transection of BDA-traced axons at the lesion site. The depth of the lesion was calculated and normalized on the total transverse width of the spinal cord at the injury site. All the animals in which lesion was too superficial or too deep were excluded from further analysis. The size and morphology of the cystic cavity formed was similar in all the animals and there were no significant differences between the experimental groups (control injured, CTR-inj, n = 7, median and interquartiles: 63.3, [53.7, 63.7], and fluoxetine treated, FLX, n = 7, 54.2, [53.3, 57.8]. Mann-Whitney Rank Sum Test, $p = 0.165$; Figure 8e).

Additionally, a group of control rats was subjected to laminectomy without induction of spinal damage, in order to discriminate behavioral outcome due to muscular and bone injury from SCI (CTR-sham).

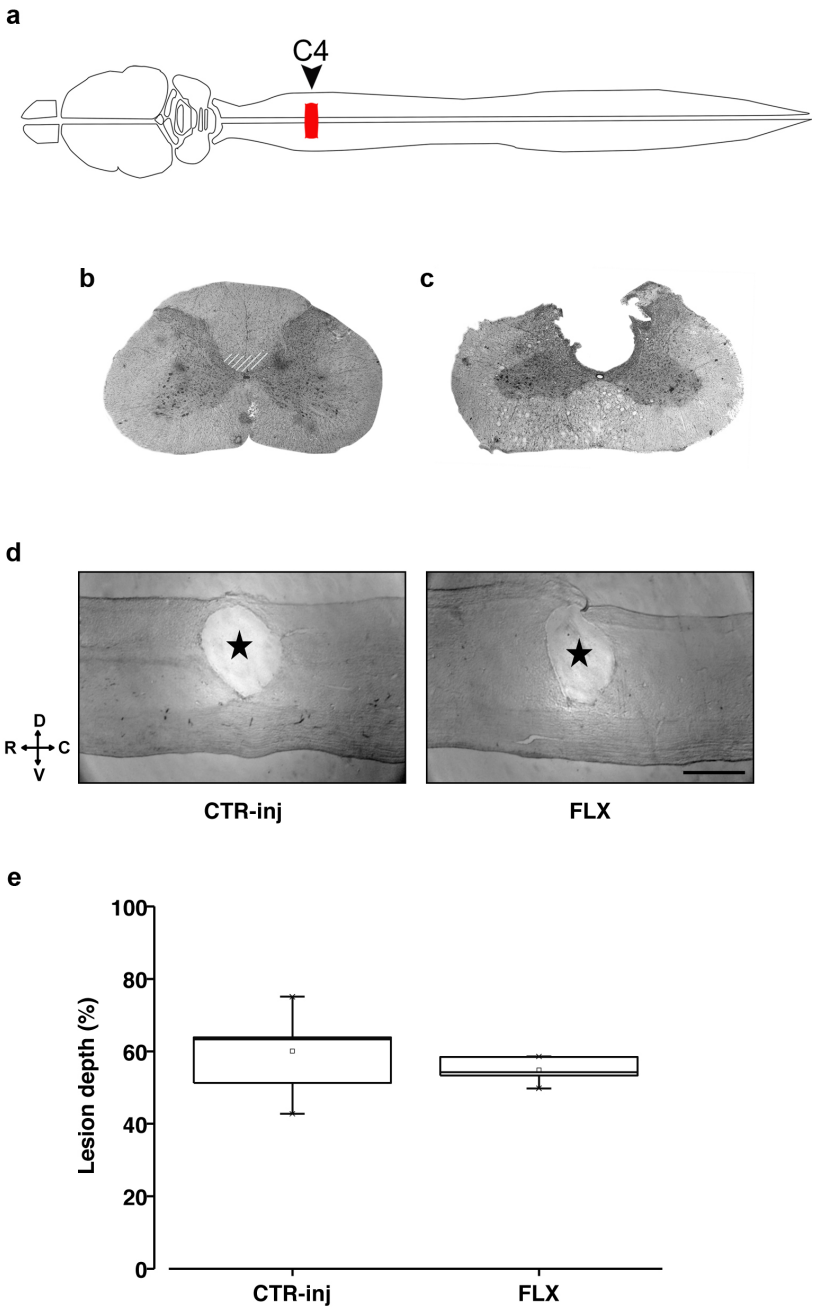

Figure 8: Spinal cord injury. (a) Representative diagram of rat spinal cord. The red box indicates the dCST lesion at C4 level. **(b, c)**. Histological trasverse sections of intact **(b)** and injured **(c)** C4 spinal cord. Dashed area indicates dCST. **(d)** Histological sagittal sections of CTR-inj (left) and FLX (right) cervical spinal cord. The star indicates the lesion cavity. **(e)** Quantification of lesion size in CTR-inj (n = 7) and FLX rats (n = 7) as the percentage of max transverse width of the spinal cord. There is no statistical difference between the two groups (Mann-Whitney Rank Sum Test, p = 0.165). Box-whisker plot: the horizontal lines in the box denote the 25th, 50th (median), and 75th percentile values; the small square inside the box represents the mean; error bars denote the 5th and 95th percentile values. X represents max and min values. Scale bar: 1 mm. Abbreviations: D, dorsal; V, ventral; R, rostral; C, caudal.

Fluoxetine promotes motor recovery in skilled-task after SCI

After injury, I analyzed the effects of fluoxetine treatment (FLX, 0.2 g/l in drinking water) on rat behavior in several motor tests and compared the performance of FLX group with that of CTR-inj and CTR-sham animals. In order to maximize the potential effect of fluoxetine on neural plasticity, the treatment started three weeks before SCI, in agreement with a number of studies showing that at least 21 days of treatment are required to induce beneficial effects in the central nervous system (Berton and Nestler, 2006; Baudry et al., 2010). The treatment was continued without interruption until the end of the first postoperative week.

Montoya staircase task

Rats were tested in the Montoya staircase task (Montoya et al., 1991), which allows to assess forelimb skilled reaching ability (Figures 9, 10). Before injury, baseline performance did not differ between FLX and CTR animals (Figure 10a-d, eaten pellets: FLX 22.5, [19.0, 23.0], CTR 22.0, [18.8, 25.3], Mann-Whitney Rank Sum Test, $p = 0.420$; displaced pellets: FLX 22.5, [19.0, 23.0], CTR 22.0, [18.8, 25.3], Mann-Whitney Rank Sum Test, $p = 0.763$; accuracy: FLX 91.0, [87.0, 93.0], CTR 90.0, [80.0, 95.4], Mann-Whitney Rank Sum Test, $p = 0.713$; reached level: FLX 6.25, [5.83, 6.67], CTR 6.50, [6.33, 6.96], Mann-Whitney Rank Sum Test, $p = 0.257$. FLX, $n = 10$ and CTR, $n = 19$ for all the measures). One week after SCI, all injured rats were unable to grasp and eat pellets, thus confirming the severity of the lesion received; moreover, they could not go further than the 4th well (Figure 10d). By the second week post-surgery, FLX animals ($n = 10$) began to significantly improve their performance compared to CTR-inj rats ($n = 13$; Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$ for both eaten pellets and accuracy). The improvement resulted in a performance of about 60% of pre-injury baseline in the sixth week after SCI, when the CTR-inj group displayed only a 30% of pre-injury performance (calculated on the accuracy parameter, Figure 10c). The performance

of CTR-sham rats (n = 6) did not change throughout the testing period (eaten pellets: One-Way RM ANOVA, p = 0.095; displaced pellets: Friedman Repeated Measures Analysis of Variance on Ranks, p = 0.996; accuracy: One-Way RM ANOVA, p = 0.881; reached level: Friedman Repeated Measures Analysis of Variance on Ranks, p = 0.225), demonstrating that laminectomy per se does not influence reaching ability in rats.

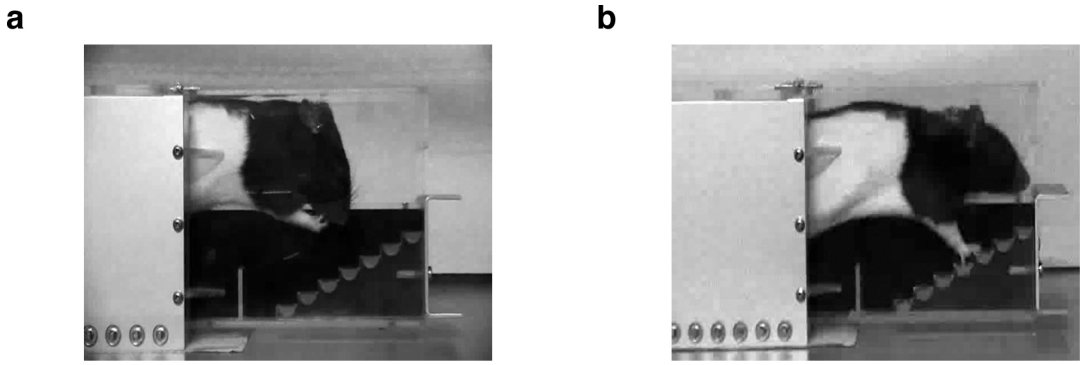

Figure 9: Fluoxetine induces recovery of grasping behavior after cervical spinal cord injury. (a, b) Pictures representing rats performing the staircase task. Before SCI rats learned to retrieve and eat pellets from the staircase **(a)**, but after injury they lost the capability to successfully grasp little objects **(b)**.

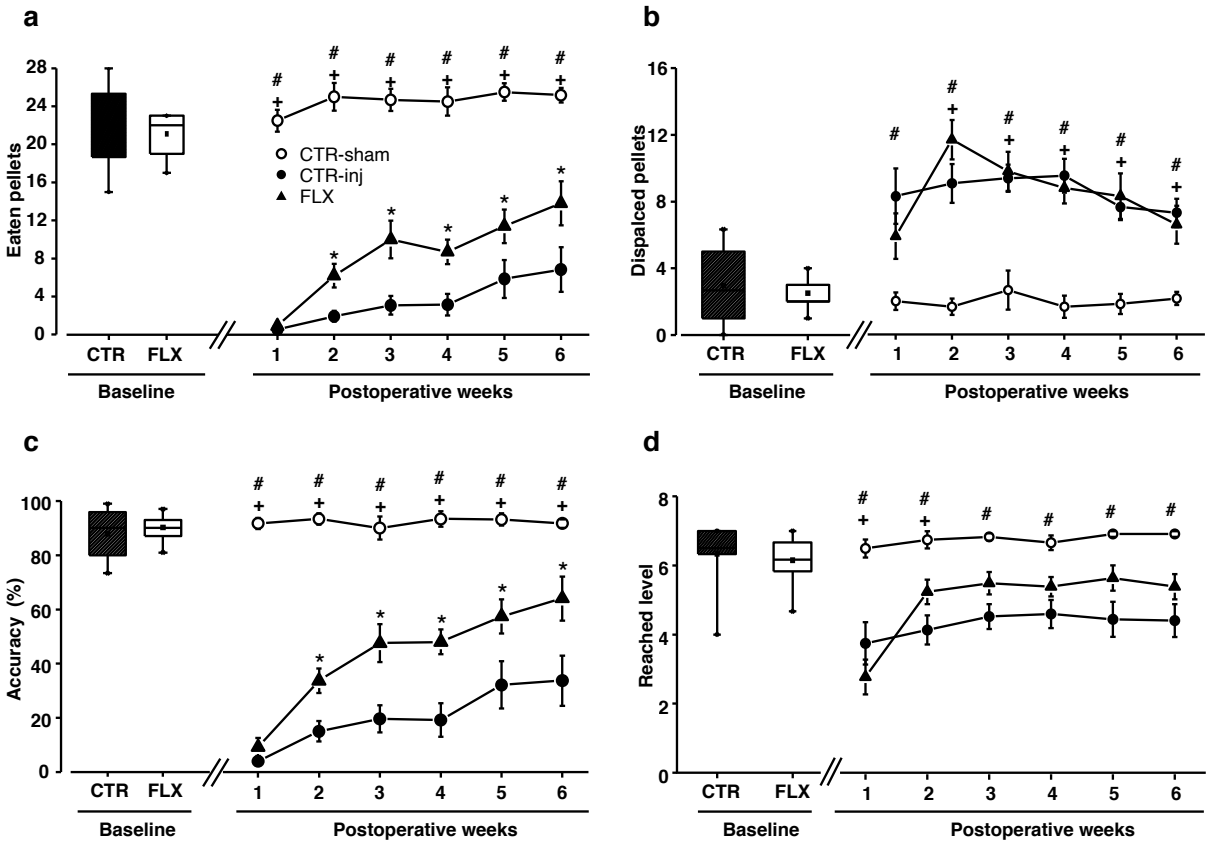

Figure 10: Fluoxetine induces recovery of grasping behavior after cervical spinal cord injury. (a) Quantification of the number of pellets eaten on the staircase. Starting from the 2nd week, FLX rats began to retrieve significantly more pellets than CTR-inj (FLX, n = 10; CTR-inj, n = 13; CTR-sham, n = 6. Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$). **(b)** Quantification of unsuccessfully displaced pellets. After injury all the lesioned rats displace an higher number of pellets compared to their baseline and to CTR-sham performance (Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$), indicating that injury affects precision but not motivation in task performance. **(c)** Accuracy. Accordingly with the measures obtained from the count of eaten pellets, FLX rats showed a greater accuracy (pellets eaten / (pellets displaced + eaten)*100) than CTR-inj (Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$) starting from the 2nd post-SCI week. **(d)** Reached level. Soon after SCI, lesioned rats could not go further than the 4th well. Starting from the 3rd week, FLX rats performance is indistinguishable from that of CTR-sham. The performance of CTR-sham rats did not change throughout the testing period for all the parameters (eaten pellets: One-Way RM ANOVA, $p = 0.095$; displaced pellets: Friedman Repeated Measures Analysis of Variance on Ranks, $p = 0.996$; accuracy: One-Way RM ANOVA, $p = 0.881$; reached level: Friedman Repeated Measures Analysis of Variance on Ranks, $p = 0.225$). Box-whisker plot: the horizontal lines in the box denote the 25th, 50th (median), and 75th percentile values; the small square inside the box represents the mean; error bars denote the 5th and 95th percentile values. X represents max and min values. Curves: error bars represent SEM. Symbols indicate statistical difference: CTR-sham vs CTR-inj, (#) $p < 0.05$; CTR-sham vs FLX, (+) $p < 0.05$; FLX vs CTR-inj, (*) $p < 0.05$.

Horizontal ladder

I further assessed skilled motor control in SCI rats running on a horizontal ladder (Metz and Whishaw, 2009, Figure 11). Baseline performance was equal between FLX and CTR groups (footslips mean ± SEM: FLX 2.143 ± 0.240, CTR 2.435 ± 0.225, t-test, $p = 0.420$; accuracy: FLX 84.3 ± 2.62, CTR 83.1 ± 1.24, t-test $p = 0.640$. FLX, $n = 7$ and CTR, $n = 13$ for both the measures). However, after injury both FLX and CTR-inj rats showed a dramatic increase in the number of errors made at crossing the ladder. I observed a robust acceleration in the time-course of the recovery process in FLX rats ($n = 7$) compared to CTR-inj animals ($n = 7$, Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$ for both footslips and accuracy). At the conclusion of the testing period, the performance of FLX animals was indistinguish-able from that of CTR-shams ($n = 6$, Two-Way RM ANOVA, post-hoc Holm-Sidak method; $p = 0.190$ for footslips and $p = 0.065$ for accuracy). The performance of CTR-sham rats did not change throughout the testing period (footslips: One-Way RM ANOVA, $p = 0.672$; accuracy: One-Way RM ANOVA, $p = 0.812$).

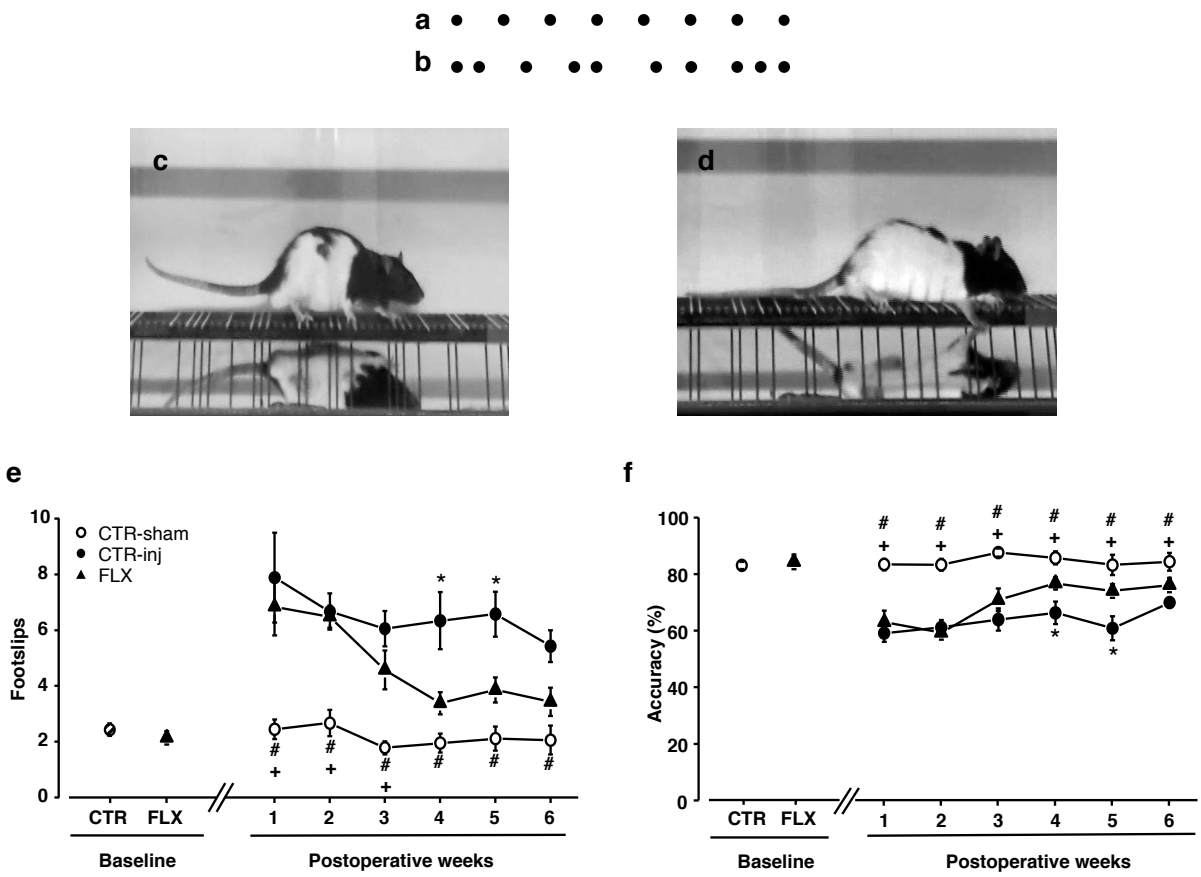

Figure 11: Fluoxetine accelerates the recovery of grasping behavior in horizontal ladder task. **(a, b)** Examples of rung arrangements: **(a)** regular; **(b)** irregular. **(c, d)** Representative pictures of rats before **(c)** and after **(d)** injury walking on the ladder. In **(c)** the animal was unable to correctly place its paws onto the ladder while walking across it. **(e)** Quantification of total forepaw slips. Baseline performance was similar in animals treated with FLX and controls (t-test: $p = 0.420$). After injury, the number of footslips was significantly reduced in FLX rats (n =7) compared to CTR-inj (n = 7) starting from the 4th week (Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$), while the CTR-sham group (n = 6) maintained the same performance recorded before surgery (One-Way RM ANOVA, $p = 0.672$). **(f)** Measure of accuracy (footslips/(total steps)*100) on ladder. Fluoxetine treated rats exhibited a more accurate performance than CTR-inj rats at the 4th and 5th week after SCI (Two-Way RM ANOVA, post-hoc Holm-Sidak method, $p < 0.05$). The CTR-sham group maintained the same performance throughout the testing period (One-Way RM ANOVA, $p = 0.812$). Box-whisker plot: the horizontal lines in the box denote the 25th, 50th (median), and 75th percentile values; the small square inside the box represents the mean; error bars denote the 5th and 95th percentile values. X represents max and min values. Curves: error bars represent SEM. Symbols indicate statistical difference: CTR-sham vs CTR-inj, (#) $p < 0.05$; CTR-sham vs FLX, (+) $p < 0.05$; FLX vs CTR-inj, (*) $p < 0.05$.

Fluoxetine induces a faster recovery of gait coordination in lesioned rats

Previous literature showed that a C4 dorsal funiculi crush can induce gait changes, with animals making shorter and wider frequent forelimb steps; moreover, injured rats displayed poor coordination (Bradbury et al., 2002; Garcia-Alias et al., 2008; Wang et al., 2011, Figure 12).

In order to study the effects of fluoxetine on walking pattern after SCI, I analyzed footprints left by rats as they walked. Baseline performances obtained in a single session before SCI did not differ between groups (coordination: FLX 1.97 ± 0.118, CTR 1.82 ± 0.106, t-test, $p = 0.367$; stride length: FLX 13.323, [11.919, 15.085], CTR 12.258, [12.167, 13.422], Mann-Whitney Rank Sum Test, $p = 0.169$; stride width: FLX 2.231 ± 0.147, CTR 2.341 ± 0.141, t-test, $p = 0.593$. FLX, $n = 14$, CTR, $n = 15$ for all the measures, Figure 12b-d). The assessment was then repeated once a week for six weeks after the induction of the lesion. While both injured groups exhibited a reduced coordination in forepaw-hindpaw stepping after SCI, FLX rats ($n = 14$) showed a marked recovery of gait coordination compared to CTR-inj animals ($n = 9$) three weeks after SCI (Two Way Repeated ANOVA, post hoc Holm-Sidak method, $p < 0.05$, Figure 12b). Otherwise, stride length and stride width did not seem to be influenced by the lesion and remained the same recorded before SCI for all the three groups (Figures 12c, d). The performance of CTR-sham rats did not change throughout the testing period (coordination: One-Way RM ANOVA, $p = 0.477$; stride length: One-Way RM ANOVA, $p = 0.145$; stride width: One-Way RM ANOVA, $p = 0.971$).

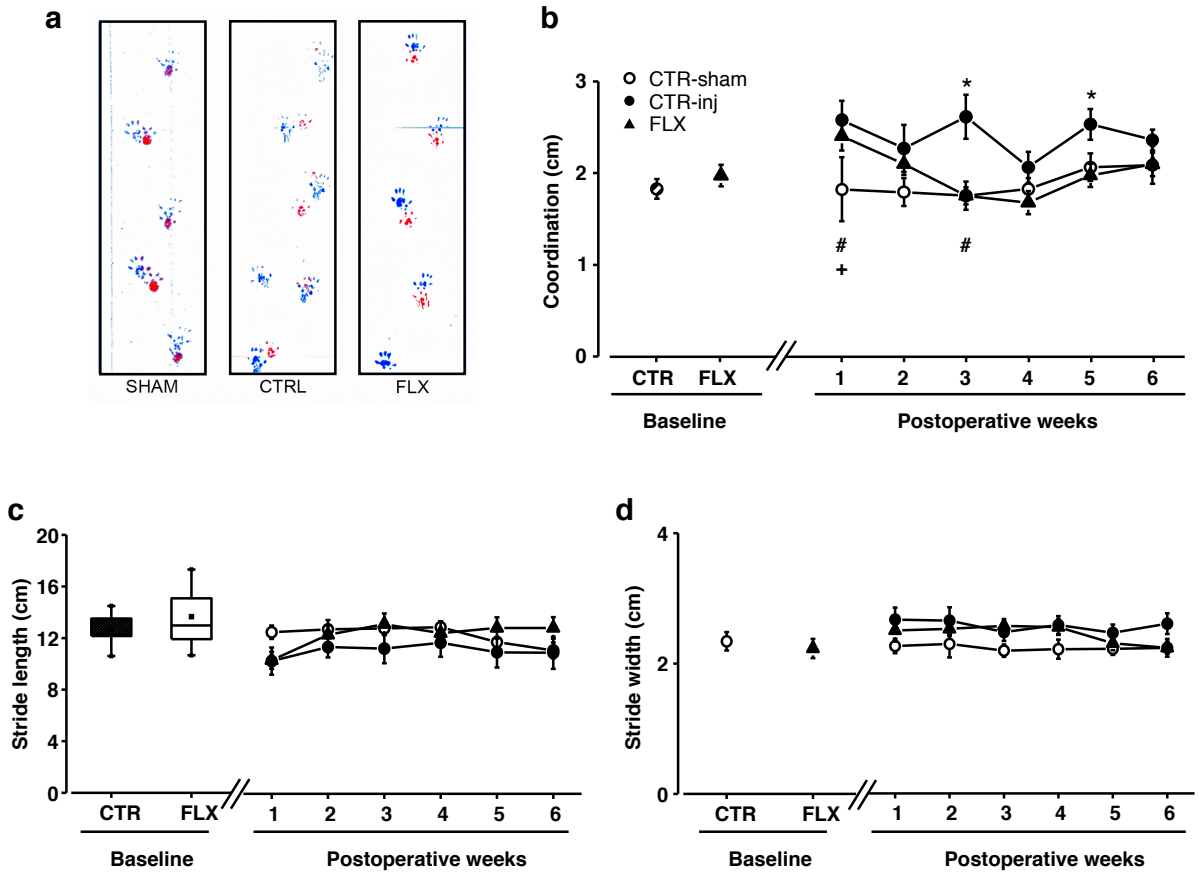

Figure 13: Faster recovery of gait coordination in FLX rats. (a) Typical footprints of animal walking 2 weeks after SCI. Red: forepaw footprints; Blue: hindpaw footprints **(b)** Forepaw-hindpaw footprint distance was used to assess the coordination of gait. After injury, FLX rats (n = 14) showed a marked recovery of coordination compared to CTR-inj (n = 9) at the 3rd (Two Way Repeated ANOVA, post hoc Holm-Sidak method, $p < 0.05$) and 5th (Two Way Repeated ANOVA, post hoc Holm-Sidak method, $p < 0.05$) testing week. The performance of CTR-sham rats (n = 6) did not change throughout the testing period (One-Way RM ANOVA, $p = 0.477$). **(c)** Stride length measures. Measures obtained before and after SCI did not differ between groups (baseline: Mann-Whitney Rank Sum Test, $p = 0.169$; postoperative weeks: Two Way Repeated ANOVA for treatment, $p = 0.475$). **(d)** Stride width measures. Measures obtained before and after SCI did not differ between groups (baseline: Mann-Whitney Rank Sum Test, $p = 0.593$; postoperative weeks: Two Way Repeated ANOVA for treatment, $p = 0.459$). Box-whisker plot: the horizontal lines in the box denote the 25th, 50th (median), and 75th percentile values; the small square inside the box represents the mean; error bars denote the 5th and 95th percentile values. X represents max and min values. Curves: error bars represent SEM. Symbols indicate statistical difference: CTR-sham vs CTR-inj, (#) $p < 0.05$; CTR-sham vs FLX, (+) $p < 0.05$; FLX vs CTR-inj, (*) $p < 0.05$.

Fluoxetine promotes sprouting in the injured spinal cord

Cervical dorsal funiculi crush results in an interruption of many descending motor and ascending sensory spinal tracts, and reestablishment of axonal connections across the injury site is fundamental for recovery of forelimb function. Regenerative and compensatory sprouting of descending fibers has been indicated as one of the mechanisms allowing spontaneous recovery after injury; for example, after cervical SCI in rats, spontaneous sprouting from the ventral CSTs occurred onto medial motoneuron pools in the cervical spinal cord, and this was correlated with recovery of forelimb function (Weidner et al., 2001). Furthermore, there is evidence that treatments that have been demonstrated to induce recovery of function, such as neurotrophin supply, myelin inhibitors and scar degrading agents, act through an increase of anatomical plasticity and subsequent formation of new connections in the injured spinal cord (Bregman et al., 1997; García-Alías et al., 2009; Lee et al., 2009).

Considering that fluoxetine promoted motor recovery in my experiment, I decided to study the effects of this treatment on the anatomical plasticity of the descending fibers involved in motor control in SCI rats.

Corticospinal tract (CST) plasticity

In order to understand if three weeks of fluoxetine could affect the spontaneous response of spinal circuits to the lesion, I started analyzing the sprouting of intact ventral corticospinal axons (vCST, Weidner et al., 2001).

At the end of the behavioral assessment, rats were placed in a stereotactic frame under deep anesthesia and the neuronal tracer biotinylated dextran amine (BDA) was bilaterally injected in the forepaw representation area of the sensorimotor cortex (Figure 13a). Animals were sacrificed after three weeks to ensure that the tracer reached the lesion site, and perfused spinal cords were dissected and processed for diaminobenzidine (DAB) staining. I counted

the labeled fibers in the central grey matter of sagittal C3-C5 sections at increasing distances from the center of the lesion (Figure 13c, e) in CTR-inj (n = 7) and FLX rats (n = 7). To correct for variability in BDA uptake by CST neurons in the sensorimotor cortex, quantitative data were normalized by counting BDA-labeled axons in the main pyramidal tract of medulla oblongata (Figure 13b, d). I found that FLX treatment was able to significantly increase CSTs sprouting at 1.5 and 4.5 mm caudal to the lesion compared to CTR-inj rats, in which few or none sprouting fibers were observed at any of the considered distances (Two Way ANOVA, $p < 0.05$, Figure 14).

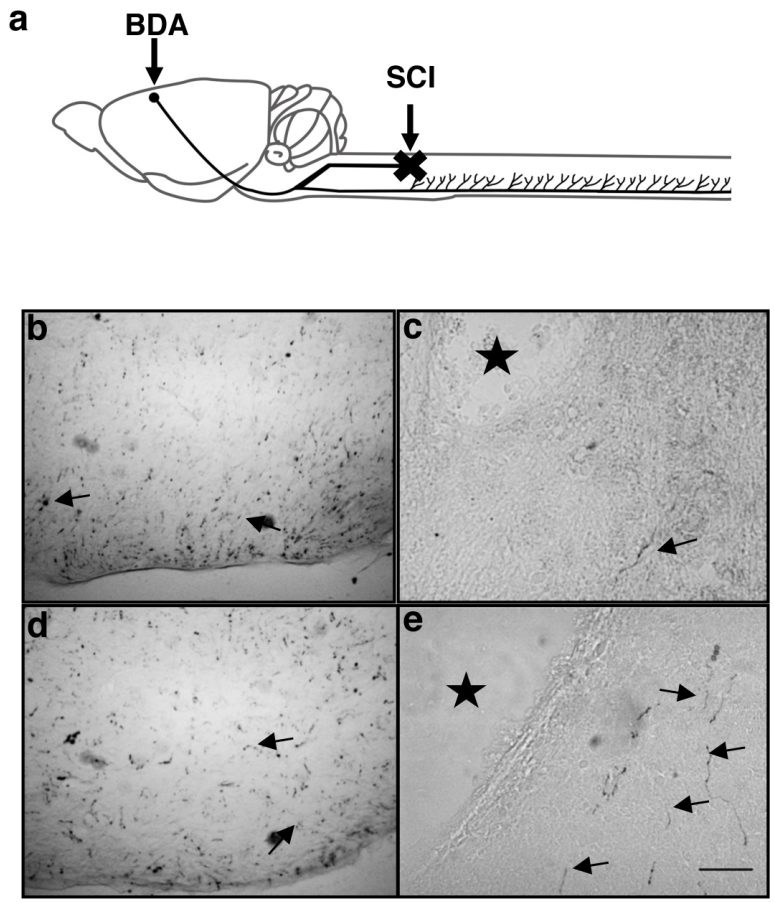

Figure 14: Fluoxetine increases vCSTs sprouting after SCI. (a) Representative diagram of neuronal tracer (BDA) injection in sensorimotor cortex corresponding to forelimb area. After three weeks, it is possible to detect the tracer in the spinal cord. **(b-e)** Representative pictures of fibers traced with BDA and DAB stained. After injury, spontaneous compensatory sprouting of the uninjured ventral corticospinal fibers occurs. **(b, d)** Coronal sections of brainstem pyramids of CTR-inj **(b)** and FLX **(d)** rats. **(c, e)** Sagittal sections of C3-C5 spinal sections at lesion site of CTR-inj **(c)** and FLX **(e)** rats. Scale bar: 50 µm.

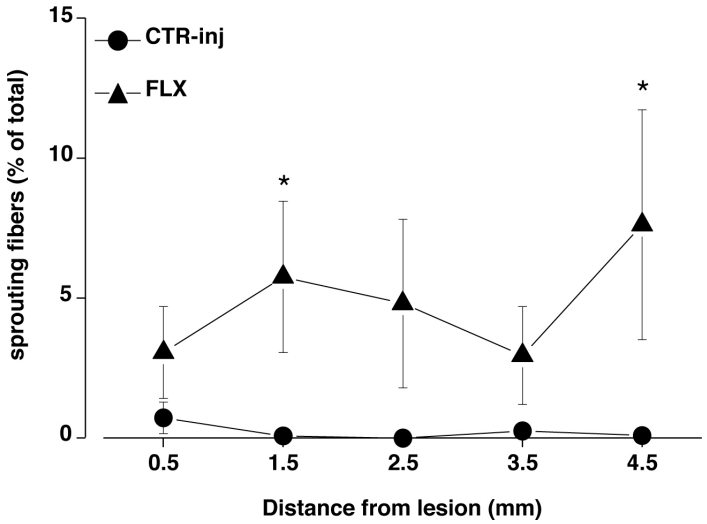

Figure 14: Fluoxetine increases vCSTs sprouting after SCI. Quantification of sprouting fibers in the ventral gray matter at 0.5, 1.5, 2.5, 3.5, 4.5 mm from lesion center normalized on total counts at medulla oblongata level (100%). FLX rats (n = 7) showed more sprouting fibers at 1.5 and 4.5 mm caudal to the lesion than CTR-inj (n = 7). Two Way ANOVA, post hoc Holm.Sidak method. Error bars represent SEM. Stars indicate lesion cavity. Arrows indicate traced sprouting CST fibers. Asterisks indicate statistical significance: (*) $p < 0.05$.

Serotoninergic fibers

Raphespinal axons originating in the brainstem descend bilaterally in the lateral columns and densely innervate both dorsal and ventral gray matter at all spinal levels (Figures 5, 6, Jordan et al., 2008; Tuszynski and Steward, 2012). Previous literature showed that raphespinal fibers contribute to locomotion (Lemon, 2008) and to forelimb functional recovery after SCI (Barritt et al., 2006; García-Alías et al., 2009; Lee et al., 2009).

To determine whether the growth of raphespinal axons is sensitive to fluoxetine administration, I assessed the growth pattern of 5HT positive axons in injured rats at C2 and C7 level, i.e. rostrally and caudally with respect to the lesion. The analysis was focused on the ventral horn of the spinal cord, where the serotoninergic fibers synapse on motor neurons (Cafferty et al., 2010, Figure 15a-i). I observed a slight increase in neurite density in C2 sections of injured rats, both CTR-inj (n = 6) and FLX (n = 7), compared to CTR-sham group (n = 6), indicating that injury itself induces sprouting of 5HT fibers in the ventral horn, but dif-

ferences between groups were not statistically significant (5HT fiber density: CTR-sham 91.3, [88.4, 119.9], CTR-inj 127.7, [96.7, 208.0], FLX 119.7, [93.8, 207.1], Kruskal-Wallis One Way Analysis of Variance on Ranks, p = 0.278, Figure 16a). In addition, no difference was observed in the C7 sections, indicating that neither the injury, nor the treatment, seems to affect 5HT axon distribution in the ventral horn caudally to a dorsal funiculi lesion (Figure 17b, 5HT fiber density: CTR-sham 100 ±17.1, CTR-inj 83.5 ± 10.6, FLX 107.2 ± 15.7, One Way ANOVA, p = 0.539. CTR-sham: n = 6; CTR-inj: n = 6; FLX: n = 8).

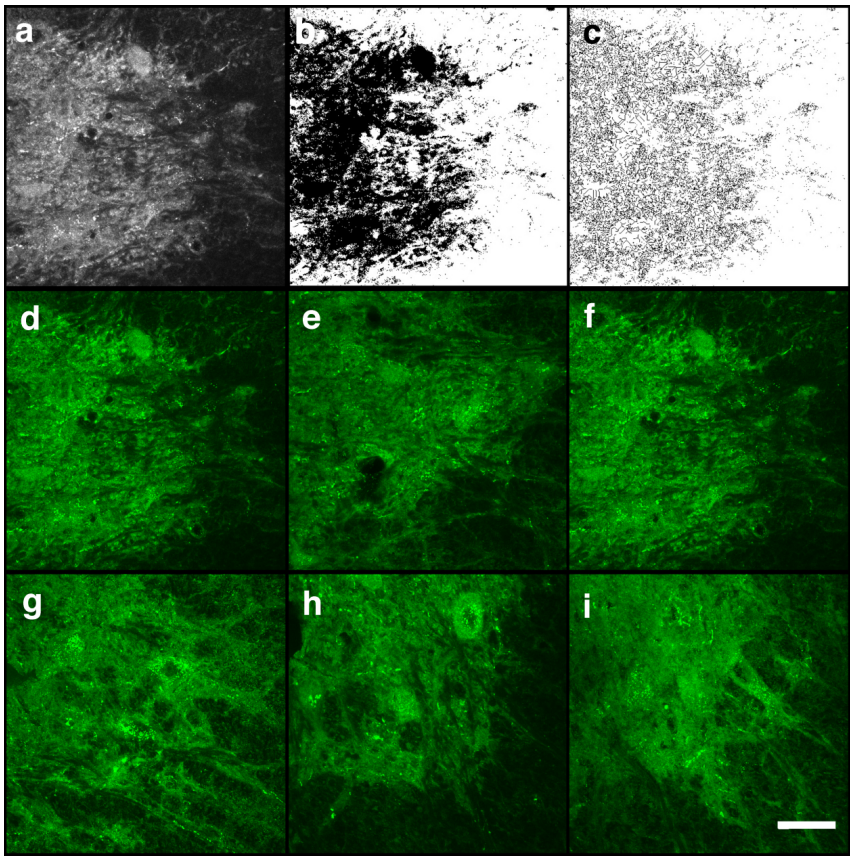

Figure 15: Distribution of serotoninergic fibers in the ventral horn. (a-c) Images of the analytical steps applied for automated quantification of labeled serotoninergic axons: **(a)** original photomicrograph of C2 ventral horn of a CTR-sham rat. Immunofluorescence (gray) shows 5HT signal; **(b)** threshold transformation of immunofluorescence represented in **(a)**; **(c)** same image processed in ImageJ for skeletonize function. **(d-f)** Original photomicrographs of C2 ventral horn of a CTR-sham **(d)**, CTR-inj **(e)** and FLX **(f)**. **(g-i)** Original photomicrographs of C7 ventral horn of a CTR-sham **(g)**, CTR-inj **(h)** and FLX **(i)**. Immunofluorescence (green) shows 5HT signal. Scale bar: 50µm.

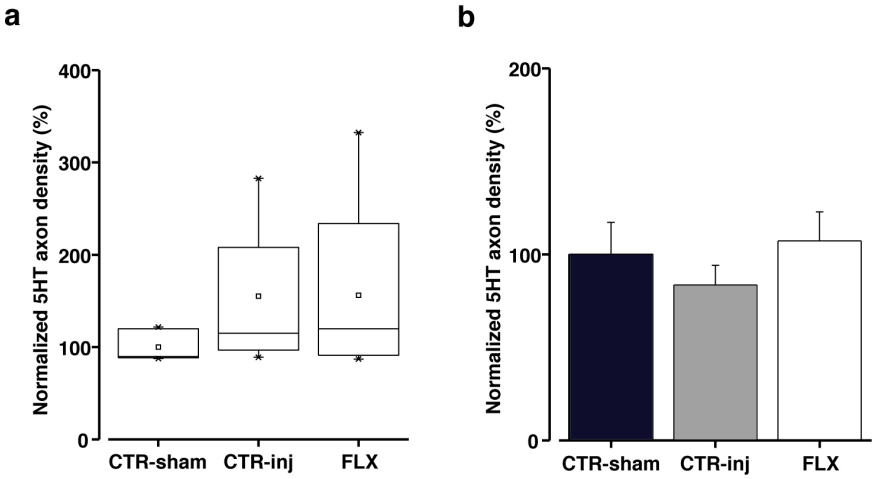

Figure 16: Distribution of serotoninergic fibers in the ventral horn. (a) Quantification of 5HT axon density in C2 ventral horn. No statistical difference was observed among groups (CTR-sham: n = 6; CTR-inj: n = 6, FLX: n = 7. Kruskal-Wallis One Way Analysis of Variance on Ranks, p = 0.278). **(b)** Quantification of 5HT axon density in C7 ventral horn. No statistical difference was observed among groups (CTR-sham: n = 6; CTR-inj: n = 6, FLX: n = 8. One Way ANOVA, p = 0.539)

Excitation/inhibition balance is modulated by fluoxetine

The relative strength of the excitatory and inhibitory connections is one major regulator of plasticity in the adult central nervous system (Hensch, 2005; Bavelier et al., 2010; Baroncelli et al., 2011). Accumulating evidence suggests that fluoxetine acts on excitatory/inhibitory balance, and this effect has been correlated with the enhancement of neural plasticity in cortical circuits induced by fluoxetine treatment (Maya Vetencourt et al., 2008; Chen et al., 2011).

In order to investigate whether three weeks of fluoxetine treatment were able to promote molecular changes which might favor plasticity in the motor cortex and in the spinal cord, I analyzed the excitation/inhibition balance in control and fluoxetine treated but not injured animals. Three weeks after the beginning of fluoxetine treatment, rats were sacrificed and the cervical portion of the spinal cord (C1-C8) and the cortical area corresponding to the motor cortex were rapidly dissected and stored for further processing. Tissues obtained from age matched rats reared in standard conditions were used as controls. I started with the quan-

tification of relative expression levels of the vesicular transporter proteins for glutamate (vGluT-1) and GABA (vGAT) which are, respectively, markers of the excitatory and inhibitory cortical tones (Mainardi et al., 2010, Figures 17a, b). Western blot quantification revealed that the vGluT-1/vGAT expression ratio was significantly increased in fluoxetine treated animals (n = 6) compared to controls (n = 6), both in the motor cortex and in the spinal cord (vGluT1/vGAT expression ratio in the motor cortex: FLX 1.889 ± 0.204, CTR 1.098 ± 0.178; vGluT1/vGAT expression ratio in the spinal cord: FLX 2.827 ± 0.565, CTR 1.281 ± 0.367; t-test, $p < 0.05$ in both cases, Figure 17b). To confirm this result, I further quantified neurotransmitter release in a synaptosomal preparation obtained from cervical spinal cord and motor cortex of treated (FLX, n = 6) and untreated (CTR, n = 6) animals (Figure 18a, b). In total agreement with the Western-blot results, I found a significant increase of the Glu/GABA stimulus-evoked overflow ratio in motor cortex and cervical spinal cord synaptosomes of FLX compared to CTR animals (Figure 18b, Glu/GABA stimulus-evoked overflow ratio in the motor cortex: FLX 3.7 ± 0.525, CTR 2.3 ± 0.337, t-test, $p < 0.05$; Glu/GABA stimulus-evoked overflow ratio in the spinal cord: FLX 2.59 ± 0.378, CTR 1.34 ± 0.195, t-test, $p < 0.01$).

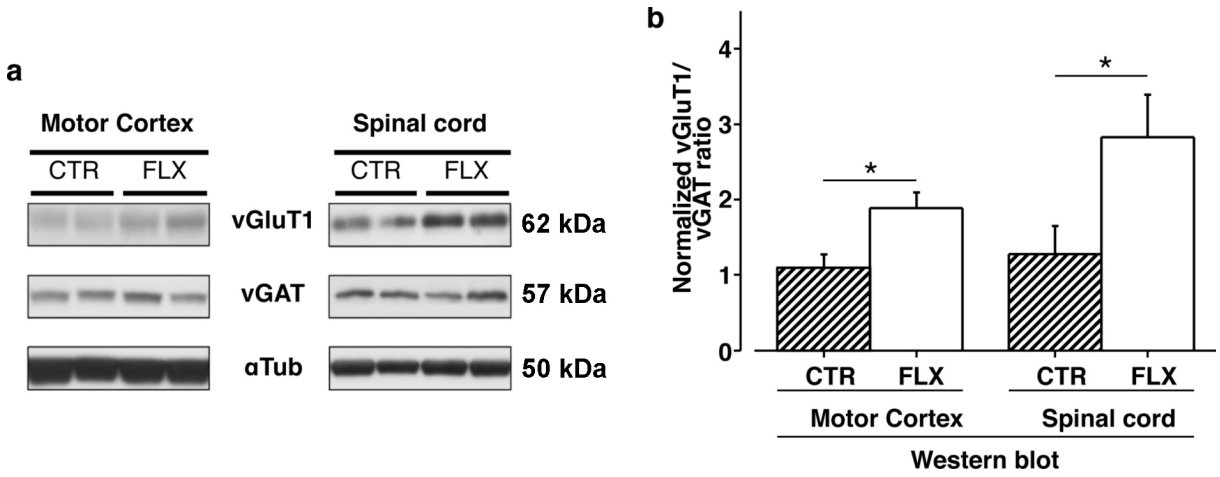

Figure 17: Fluoxetine increases the excitation/inhibition ratio in the motor cortex and the spinal cord. (a) Representative Western blots from control or fluoxetine-treated rat motor cortex (left) and spinal cord (right). α-tubulin was the loading control. **(b)** Quantification of the expression levels of the vesicular transporter proteins for glutamate and GABA (vGluT-1 and vGAT, respectively) by Western blot analysis. The vGluT-1/vGAT expression ratio was significantly increased after three weeks of fluoxetine treatment, both in the motor cortex and in the spinal cord (t-test, $p < 0.05$ in both cases). Error bars indicate SEM. Asterisks indicate statistical significance: (**) $p < 0.01$, (*) $p < 0.05$.

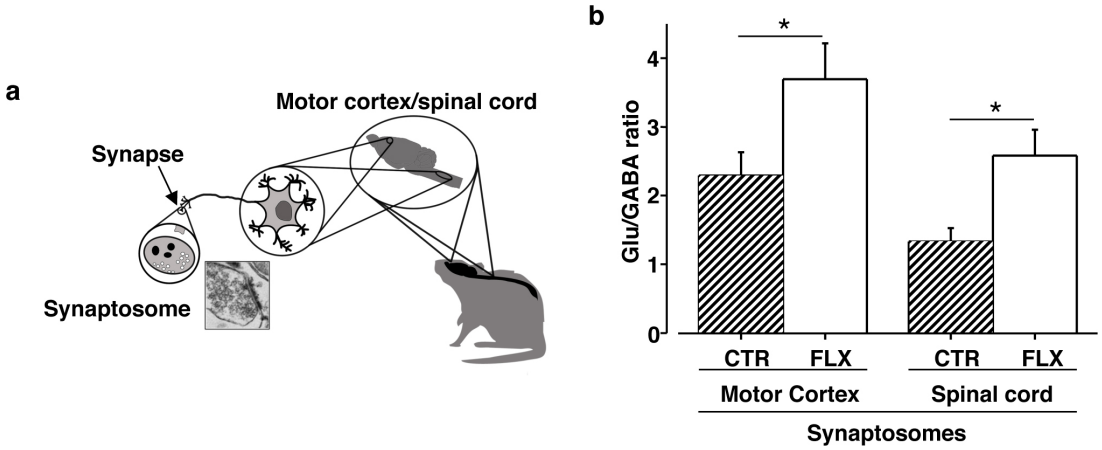

Figure 18: Fluoxetine increases the excitation/inhibition ratio in the motor cortex and the spinal cord. (a) Schematic diagram showing the synaptosome technique. **(b)** Quantification of glutamate and GABA released after a pulse of 15mM KCl by synaptosomes in superfusion obtained from the motor cortex and the spinal cord. The Glu/GABA stimulus-evoked overflow ratio was significantly increased in both the motor cortex and the cervical spinal cord tract of FLX compared to CTR animals (t-test, $p < 0.05$ and $p < 0.01$, respectively for the motor cortex and the spinal cord). Error bars indicate SEM. Asterisks indicate statistical significance: (**) $p < 0.01$, (*) $p < 0.05$.

Fluoxetine effect on neurotrophin expression in the intact spinal cord

Previous studies demonstrated that chronic antidepressant treatment up-regulates neurotrophin expression in the hippocampus and the cerebral cortex (D'Sa and Duman, 2002; Khawaja et al., 2004; Berton and Nestler, 2006). Neurotrophins are known to act as crucial regulators of synaptic plasticity and neuronal survival, with increasing amount of data suggesting that neurotrophins, and in particular BDNF and NT3, may play a pivotal role in neuroplasticity after stroke and SCI (see Hollis and Tuszynski, 2011 for a review).

To address if fluoxetine could act on neurotrophin expression in the spinal cord, and to investigate if neurotrophin levels could be involved in motor recovery after SCI in my experiment, I analyzed BDNF and NT3 expression in the cervical spinal cord of rats treated for three weeks with fluoxetine. Western blot quantification revealed that BDNF protein expression was slightly increased in FLX (n = 8) compared to CTR rats (n = 8), but the difference did not reach statistical significance (Figure 19a, b. BDNF expression: FLX 1.464 ± 0.452, CTR 0.941 ± 0.237, t-test, p = 0.323,). In addition, quantification of NT3 protein with an Enzyme Linked ImmunoSorbent Assay (ELISA) revealed that fluoxetine treatment did not influence the expression of this protein in the cervical spinal cord (Figure 19c. NT3 expression: FLX 2.042 ± 0.226, CTR 1.974 ± 0.201, t-test, p = 0.824. FLX, n = 11; CTR, n = 10,).

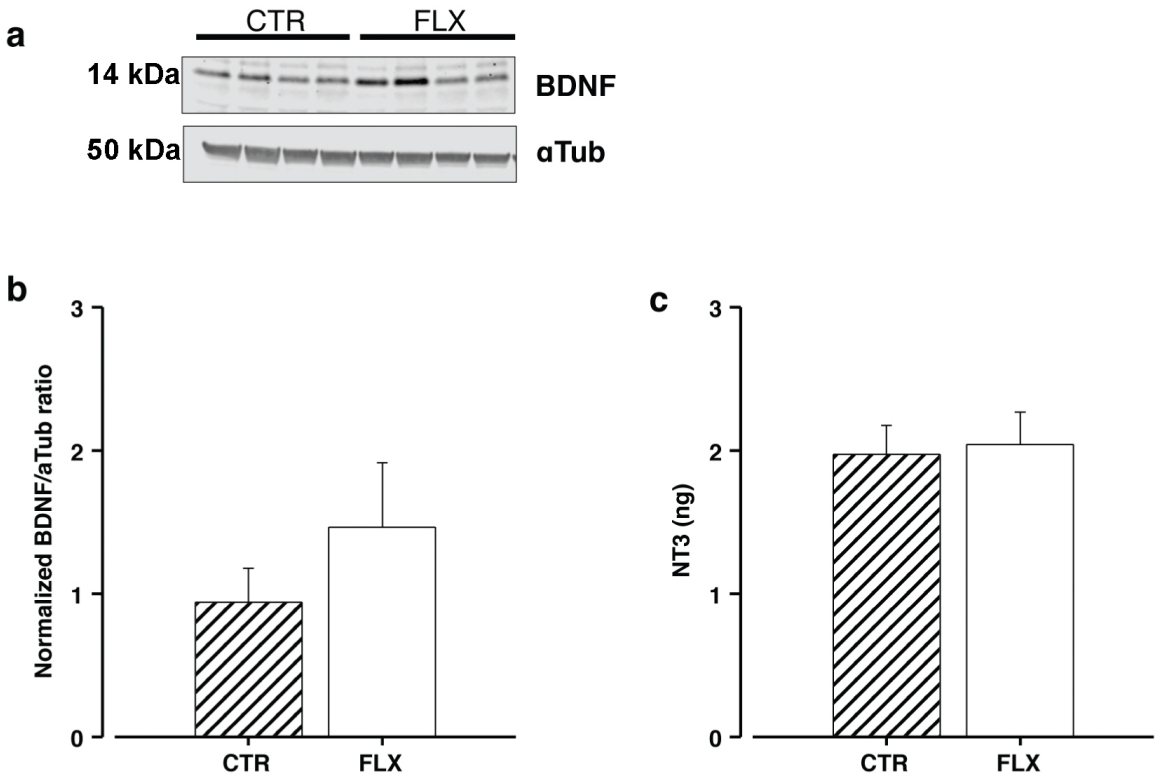

Figure 19: Neurotrophins expression. (a) Representative Western blot from control or fluoxetine-treated rat spinal cord. α-tubulin was the loading control. **(b)** Quantification of the expression levels of BDNF by Western blot analysis. The increase of BDNF in FLX treated animals is not significant (t-test, $p = 0.323$). **(c)** Quantification of the expression levels of NT3 by ELISA analysis. Fluoxetine did not change NT3 expression after three weeks of treatment (t-test, $p = 0.824$).

Discussion

Fluoxetine enhances motor recovery after spinal cord injury

Since the appearance of the "monoamine hypothesis" of depression, the development of drugs aimed at increasing the serotoninergic tone in the areas involved in the genesis of mood disorders, such as the frontal cortex and the hippocampus, has been strongly encouraged (Wenthur et al., 2013). These researches led to the discovery of fluoxetine (Wong et al., 1974), which was the first of a new class of antidepressants, the selective serotonin reuptake inhibitors (SSRIs). Despite the rapid kinetic of the action of fluoxetine on the 5HT reuptake transporter, the antidepressant effects are detectable only after a prolonged administration, arguing that other mechanisms should be involved in the response induced by fluoxetine treatment (Castren, 2005; Castren and Hen, 2013).

Accumulating evidence suggests that fluoxetine can promote neural plasticity in the adult brain: recent studies showed that chronic treatment of adult rats with fluoxetine induced a plastic state in the visual cortex that closely resembles that observed during the critical period (Maya Vetencourt et al., 2008). The reinstatement of juvenile like plasticity has also been found in a study that used the fear-conditioning paradigm to show that coupling of chronic fluoxetine treatment and extinction training increases neuronal plasticity in the amygdala and leads to long-term removal of the conditioned fear response (Karpova et al., 2011). Antidepressant treatment has been successfully used to counteract the effects of neurodegenerative disorders: recently, Cirrito and coworkers demonstrated that chronic treatment with a SSRI, citalopram, caused a 50% reduction in brain plaque load in a mouse model of Alzheimer disease, and retrospective analysis showed that Alzheimer patients treated with antidepressants during the past 5 years had significantly less amyloid load as quantified by positron emission

tomography (PET) (Cirrito et al., 2011). Fluoxetine administration restored neurogenesis and ameliorated hippocampal plasticity in mice models of Down Syndrome (Begenisic et al., 2013; Stagni et al., 2013). Furthermore, fluoxetine treatment exerted an acute neuroprotective effect and promoted hippocampal neurogenesis after experimentally induced ischemia in rodents (Windle and Corbett, 2005; Li et al., 2009; Lim et al., 2009), leading to the beginning of clinical trials for SSRIs application in post-stroke therapy (Acler et al., 2009; Jorge et al., 2010; Chollet et al., 2011). In particular, Chollet and coworkers found that a 3-month treatment with fluoxetine induced a positive effect on motor recovery in patients with acute ischaemic stroke (Chollet et al., 2011).

In this Thesis, I studied the effects of a long-term treatment with fluoxetine on forelimb function after a C4 dorsal funiculi crush in the rat. This kind of lesion leads to partial loss of sensory inputs to the brain and descending corticospinal control of movements (Bradbury et al., 2002; García-Alías et al., 2009). I analyzed the motor performance of treated and control rats using the classical Montoya staircase task (Montoya et al., 1991) and the horizontal ladder test (Metz and Whishaw, 2009) as sensitive assays to evaluate forelimb skilled reaching ability, and the footprint analysis of gait (de Medinaceli et al., 1982) as a measure of walking behavior. Lesion severity was confirmed by the poor performance recorded in all lesioned rats one week after spinal cord injury (SCI) compared to the sham operated group (Figures 9-12). After 2-3 weeks, however, fluoxetine treated rats displayed an increased recovery of motor performance in fine skills (Staircase, Figure 10; Horizontal ladder, Figure 11), as well as in general locomotor behavior (Gait analysis, Figure 12). The functional improvement that I observed was comparable with the kind of recovery induced by other well established treatments acting on neural plasticity, such as chondroitinase ABC (García-Alías et al., 2009) and anti-NogoA antibody IN-1 (Merkler et al., 2001). The enhancement of plasticity promoted by fluoxetine was assessed by quantifying the increase of the excitation/inhi-

bition ratio after three weeks of drug administration (Figures 17, 18). Moreover, the recovery process was accompanied by the sprouting of ventral corticospinal (CST) fibers in fluoxetine-treated animals, measured after the end of the behavioral assessment (Figures 13, 14).

Fluoxetine induces plastic rearrangements in the spinal cord

Anatomical plasticity

Several studies reported that partial spontaneous recovery may occur in case of incomplete SCI (Boulenguez and Vinay, 2009). Corticospinal tract plasticity has been shown to be the main mechanism at the basis of this functional recovery via the formation of new circuits through collateral sprouting (Raineteau and Schwab, 2001; Weidner et al., 2001). Weidner et al. showed that the functional recovery observed in rats with dCST lesion is abolished after a second injury of the ventral CST (vCST), indicating that vCST sprouting compensated the lack of innervation due to dCST transection (Weidner et al., 2001). Following this observation I analyzed the sprouting of vCST. I found that the number of vCST fibers observed within the spinal gray matter was increased in FLX treated rats compared to controls (Figures 13 and 14). This is in agreement with several works in which recovery of motor function was associated with an increase of CST sprouting caused by other treatments, such as neutralization of inhibitory CNS myelin components (Thallmair et al., 1998; Raineteau et al., 1999; Li and Strittmatter, 2003; Li et al., 2004; Li et al., 2005), the enzymatic degradation of glial scar (Bradbury et al., 2002; Barritt et al., 2006; Garcia-Alías et al., 2008, 2009; Wang et al., 2011), or administration of the neurotrophin NT3 (Schnell et al., 1994; Grill et al., 1997a; Blits et al., 2000; Tuszynski et al., 2003; Fortun et al., 2009).

Besides CST, also serotoninergic fiber sprouting has been associated with motor recovery in several SCI models (Sharma et al., 1990; Inman and Steward, 2003; Camand et al., 2004; Barritt et al., 2006; Lee et al., 2009; Garcìa-Alìas et al., 2009). A large amount of litera-

ture shows that serotonin is involved in the regulation of locomotion (Cazalets et al., 1992; Schmidt and Jordan, 2000; Lemon, 2008). In the mammalian brain, serotoninergic neurons are found in and around the midline raphe nuclei of the brain stem (Dahlstrom and Fuxe, 1964; Azmitia and Whitaker-Azmitia, 2000); from these sites, 5HT positive cells send out the projections that innervate the diverse areas throughout the brain and the spinal cord (Figures 6 and 7, Jordan et al., 2008; Tuszynsky and Steward, 2012). Normally, brainstem-derived 5HT facilitates a persistent calcium current in spinal motoneurons and interneurons through 5HT2 receptors, making spinal neurons ready to respond to fast glutamate synaptic inputs and allowing appropriate muscle contractions (Li et al., 2007; Murray et al., 2010). Moreover, pharmacological treatment with serotonin precursors, transplantation of embryonic 5HT neurons or administration of 5HT receptor agonists evoke or modulate locomotor activity after chronic SCI (Barbeau et al., 1981; Barbeau and Rossignol, 1991; Feraboli-Lohnherr et al., 1997; Feraboli-Lohnherr et al., 1999; Ribotta et al., 2000; Antri et al., 2002; Antri et al., 2003; Landry et al., 2006; Gerasimenko et al., 2007; Ung et al., 2008; Courtine et al., 2009; Gerasimenko et al., 2009; Filli et al., 2011; Musienko et al., 2012; Cristante et al., 2013).

To determine whether the growth of raphespinal axons is sensitive to fluoxetine administration, I assessed the growth pattern of 5HT-positive axons in the ventral horns of injured rats at C2 and C7 level, i.e. rostrally and caudally with respect to the lesion where serotoninergic fibers synapse on motor neurons (Cafferty et al., 2010, Figures 15 and 16). I did not observe any difference in the 5HT-positive fiber pattern in fluoxetine-treated rats compared to controls, suggesting that serotoninergic sprouting is not involved in the recovery of function after SCI mediated by fluoxetine treatment.

An increasing amount of literature shows that the sensory-motor cortex undergoes profound modifications after SCI, and several studies demonstrated that the reorganization of cortical circuitry plays a major role during the process of post-lesion recovery (Darian-Smith

and Ciferri, 2005; Giszter et al., 2008; Ghosh et al., 2009; Kao et al., 2009; Ghosh et al., 2010; Kao et al., 2011; Qi et al., 2011). In a recent research, Ganzer and coworkers demonstrated that the use of 5HT pharmacotherapy promoted a higher degree of circuitry reorganization in the motor cortex of rats after a complete spinal transection than what would be expected by transection alone; furthermore, the level of cortical rearrangement positively correlated with the behavioral outcome (Ganzer et al., 2013), confirming the idea that increasing cortical plasticity by means of serotoninergic manipulation could be a powerful tool to promote SCI rescue. In my study, I did not directly assessed the anatomical reorganization of the motor cortex; nevertheless, a biochemical analysis revealed that three weeks of fluoxetine treatment induced a strong increase of the excitatory/inhibitory balance in this cortical area (Figures 17, 18), suggesting that the establishment of a permissive environment for neural plasticity in the motor cortex could underlie the recovery of function observed after cervical injury.

Involvement of the excitatory/inhibitory balance

The excitatory/inhibitory balance is a major regulator of plasticity in the central nervous system (Berardi et al., 2003; Hensch, 2005; Bavelier et al., 2010; Baroncelli et al., 2011). In my experiments, I found that the excitation/inhibition ratio was increased after three weeks of fluoxetine treatment in non-lesioned rats, both in the motor cortex and in the spinal cord; thus, before the onset of SCI pathophysiology, fluoxetine led to molecular changes that could promote neural plasticity (Figures 17 and 18). This is in agreement with previous findings on the visual cortex (Maya Vetencourt et al., 2008; Chen et al., 2011), and suggests that a modulation of GABAergic inhibition accompanied by an increase of glutamatergic release might emerge as a critical regulator for plasticity enhancement not only in the adult cerebral cortex but also in the spinal cord.

There are various mechanisms through which fluoxetine could lead to the decrease of the GABAergic tone. Since the main function of fluoxetine, as a SSRI, is to increase the serotoninergic tone, I can hypothesize that the effect on inhibition is mediated by the action of 5HT on inhibitory interneurons: GABAergic neurons, indeed, appear to be the principal cortical target of 5HT fibers (DeFelipe et al., 1991; Hornung and Celio, 1992; Smiley and Goldman-Rakic, 1996), and several experiments demonstrate that 5HT inhibits GABA release from interneurons of various brain regions via a presynaptic mechanism mediated by 5HT1/2 receptor families (Schmitz et al., 1995; Schmitz et al., 1998; Koyama et al., 1999; Zhou and Hablitz, 1999; Xiang and Prince, 2003; Bramley et al., 2005; Lee et al., 2008), probably regulating the availability of transmitter vesicles (Wang and Zucker, 1998). Intriguingly, functional receptor binding analysis indicated that fluoxetine is an antagonist of 5HT2A and 5HT2C receptors (Koch et al., 2002). Furthermore, an increasing amount of data suggests that fluoxetine directly modulates inhibitory transmission: recent studies showed that the application of clinically relevant amounts of fluoxetine on hippocampal slices can induce a reduction of GABAergic signaling, both in the presence or in the absence of other SSRIs, thus demonstrating that the action of fluoxetine on the reduction of GABAergic transmission can be independent from serotonin (Mendez et al., 2012; Caiati and Cherubini, 2013). The results presented in these papers indicate that fluoxetine alters the release of GABA from the presynaptic terminal in the hippocampus; this is in accord with my findings, which show that three weeks of fluoxetine treatment induced a reduced expression of vGAT, the transporter that mediates the accumulation of GABA into synaptic vesicles, and the decrease of the evoked GABA release from synaptosomes in the motor cortex and in the spinal cord (Figures 17, 18).

The decrease of inhibitory signaling was accompanied by the increase of glutamatergic transmission, which was measured through vGluT1 expression and glutamate release quantification (Figures 17, 18). My results are in agreement with previous literature: indeed,

fluoxetine treatment enhanced vGluT1 mRNA expression in the hippocampus and in frontal, orbital, cingulate and parietal cortices in rats (Tordera et al., 2005). Furthermore, in chronically treated rats fluoxetine induced selective changes in glutamate receptor subunits which were associated with structural plasticity, and in particular with the upregulation of dendritic spine density and of large, mushroom-type spines in the cerebral cortex (Ampuero et al., 2010). Further experiments will be required to assess an analogous effect of fluoxetine in the motor cortex.

Neurotrophins

Previous studies demonstrated that chronic antidepressant treatment up-regulates neurotrophin expression, especially BDNF, in the hippocampus and the cerebral cortex (D'Sa and Duman, 2002; Khawaja et al., 2004; Berton and Nestler, 2006; Martinowich et al., 2007; Maya Vetencourt et al., 2008). Neurotrophins are known to be crucial regulators of synaptic plasticity and neuronal survival (Poo, 2001; Berardi et al., 2003), and an increasing amount of data suggest that neurotrophins, and in particular BDNF and NT3, may play a pivotal role in neuroplasticity after SCI (Schnell et al., 1994; Giehl and Tetzlaff, 1996; Grill et al., 1997a; Kobayashi et al., 1997; Bradbury et al., 1999; Liu et al., 1999; Blits et al., 2000; Lu et al., 2001; Jin et al., 2002; Tuszynski et al., 2003; Ruitenberg et al., 2004; Lu et al., 2005; Taylor et al., 2006a; Alto et al., 2009; Fortun et al., 2009; Brock et al., 2010).

To address if fluoxetine could act on neurotrophin expression in the spinal cord, and to investigate if neurotrophin levels could be involved in motor recovery after SCI in my experiment, I analyzed BDNF and NT3 expression in the cervical spinal cord of rats treated for three weeks with fluoxetine (Figure 19). My results show an increase, albeit not significant, of BDNF expression in fluoxetine treated rats, while NT3 levels were not affected. These results are not completely surprising, since previous literature showed that chronic fluoxetine in-

creased BDNF expression in the hippocampus but not in the spinal cord (Engesser-Cesar et al., 2007). However, since I did not evaluate neurotrophin concentration in the lesioned spinal cord of treated and control rats, I can not exclude the possibility that fluoxetine could act by counteracting the depletion of neurotrophic support which has been described after SCI (Nakamura and Bregman, 2001).

Furthermore, fluoxetine could increase BDNF expression in the motor cortex, where the CST fibers originate: several studies demonstrated that BDNF administration near the cell soma increases CST neurons survival after subcortical lesion (Giehl and Tetzlaff, 1996; Lu et al., 2001), then it is tempting to speculate that fluoxetine could exert a neuroprotective effect through BDNF in the motor cortex of fluoxetine treated rats.

Conclusions and future directions

The results presented in this Thesis show that the establishment of a more permissive environment through prolonged fluoxetine administration can enhance plasticity in the spinal cord and that this might favor recovery from SCI. A similar link between neural plasticity enhancement and functional improvement after SCI has been previously demonstrated for other treatments, such as chondroitinase ABC (García-Alías et al., 2009) and anti-NogoA antibody IN-1 (Merkler et al., 2001). In line with these studies, future work will focus on the potentially beneficial effects of post lesion treatment with fluoxetine. Given that fluoxetine requires a prolonged administration period to achieve its functional effects, it is likely that a protocol based on initiating fluoxetine administration immediately after SCI might represent a good strategy to induce recovery. Furthermore, it will be interesting to address the effects of fluoxetine in combination with sensory-motor rehabilitation. In agreement with this possibility, positive effects of fluoxetine coupled with physiotherapy have been recently shown in patients with ischemic stroke (Chollet et al., 2011).

The non invasive nature of the pharmacological paradigm reported in this study makes fluoxetine administration a treatment with a great potential for clinical application in the field of SCI and related neuropathologies.

Appendix: A rich environmental experience reactivates visual cortex plasticity in aged rats.

Introduction

In the past few decades, decreased birth rate and longer life expectancy due to improved environmental conditions and medical assistance caused a marked world population aging and the emergence of age-related diseases as major determinants of human mortality. Complementary to efforts aimed at the characterization of pathological aging, defining cellular and molecular mechanisms underlying normal aging and finding tools capable to prevent or reverse age-related decline in the organism function are primary goals of both basic and clinical research in Neuroscience (Bishop et al., 2010).

The brain aging process is typically associated with functional deterioration across multiple systems, including cognitive and sensory-motor domains, that can, in part, be explained by a progressive decay of neural plasticity, i.e. the capacity to reorganize cerebral circuits in response to instructive and adaptive signals from the surrounding environment (Burke and Barnes, 2006). Despite the increasing evidence that environmental enrichment (EE) attenuates age-related cognitive deficits (Frick and Benoit, 2010), little is known about the effects of enhanced environmental stimulation on experience-dependent plasticity processes in the cerebral cortex of aging animals. The visual system stands as the prime model for studying neuronal plasticity. Occluding one eye early in development (monocular deprivation, MD) leads to an ocular dominance (OD) shift of cortical neurons, that is, a reduction in the number of cortical cells responding to that eye and a robust increment in the number of neurons activated by the open eye. The same treatment is completely ineffective in the adult organism (Berardi et al., 2000).

Recently, studies conducted in this laboratory challenged this dogma, demonstrating that EE in adult animals reactivates juvenile-like OD plasticity in the visual cortex (Baroncelli et al., 2010). In the effort to start studying whether exposure to enhanced stimulating condi-

tions might favor experience-dependent plasticity in aged rats, I used *in vivo* electrophysiological recordings to measure OD plasticity in the visual cortex of monocularly-deprived aged rats exposed to either EE or normal standard rearing conditions. I provide evidence that aged rats are still sensitive to the reinstatement of plasticity induced by EE.

Materials and methods

Animal treatment

A total of 27 female Long-Evans hooded rats (350-400 g) were used in this study, approved by the Italian Ministry of Public Health. At 22-23 months of age the animals were transferred for three weeks to an environmental enrichment (EE) setting or maintained in standard conditions (SC). Environmental enrichment consisted of a large cage (100x50x82 cm) with three floors linked by stairs, containing several food hoppers, free-access running wheels (diameter 25 cm) and differently shaped objects, which were substituted with others at least once a week. Every cage housed 6-8 adult rats. Standard condition consisted of a smaller cage (40x30x20 cm) housing 2-3 adult rats. In both environmental conditions, animals were maintained at 21°C under a 12-h light/dark cycle and had ad libitum access to food and water. After 15 days in EE or SC, a group of rats was anesthetized with avertin (2.2.2 tribromoethanol solution; Sigma; 200 mg/kg, i.p.). Monocular deprivation (MD) was performed through eyelid suturing, after which the animals were returned to their respective housing conditions. Subjects with even minimal spontaneous eyelid re-opening were excluded from the study.

In vivo Electrophysiology

After 1 week of MD, animals were anesthetized with urethane (20% solution in saline; Sigma; i.p. injection; 0.5 ml/100g) and placed in a stereotaxic frame. Body temperature was maintained at 37°C. A hole was drilled in the skull, corresponding to the binocular portion of the primary visual cortex (binocular area, Oc1B) contralateral to the deprived eye. After the exposure of the brain surface, a micropipette filled with NaCl (3 M) was inserted into the cortex 5 mm lateral to the λ point. Both eyes were fixed and kept open by means of adjustable metal

rings surrounding the external portion of the eye bulb. The eyes were frequently inspected and rinsed with physiological solution to prevent the formation of cataracts.

VEP recordings

Ocular dominance (OD) was measured by calculating the contralateral to ipsilateral ratio of visual evoked potentials (VEPs), that is, the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording was performed. The electrode was advanced at a depth of 100 or 500 μm within the cortex, where VEPs had their maximal amplitude. Visual stimuli were horizontal sinusoidal gratings with a spatial frequency of 0.1 c/deg, generated by a VSG2/2 card running custom software, presented on a monitor positioned 20 cm from the rat's eyes, and centered on the previously determined receptive fields. Signals were band-pass-filtered (0.1–100 Hz), amplified, and fed to a computer for analysis. At least 128 events were averaged in synchrony with the stimulus contrast reversal. Transient VEPs in response to abrupt contrast reversal (0.5 Hz) were evaluated in the time domain by measuring the peak-to-baseline amplitude and peak latency. To prevent sampling bias, at least three penetrations were performed for each animal. Care was taken to equally sample VEPs across the two cortical depths so that all layers contributed to the analysis.

Single-unit recordings

Single-unit recordings were used to further assess plasticity in response to MD. Spontaneous activity, peak response and receptive field size were determined from peri-stimulus time histograms recorded in response to computer-generated bars (size: 3°; drifting speed: 38.15 °/sec; contrast: 100%), averaged over 15 stimulus presentations. Ocular dominance classes were evaluated according to the method of Hubel and Wiesel. For each animal, the bias of the

OD distribution toward the contralateral eye [contralateral bias index (CBI)] was calculated as follows: CBI = [(N(1) – N(7)) + 2/3 (N(2) – N(6)) + 1/3 (N(3) – N(5)) + NTOT]/2NTOT, where N(i) is the number of cells in class i, and NTOT is the total number of recorded cells in a specific animal. Additionally, for each cell an OD score was calculated as follows: {[peak(ipsi) – baseline(ipsi)] – [peak(contra) – baseline(contra)]}/{[peak(ipsi) – baseline(ipsi)] + [peak(contra) – baseline(contra]}, where peak is the maximal spike frequency evoked by visual stimulation, ipsi is the ipsilateral eye, baseline is the mean spiking frequency in the absence of stimulation, and contra is the contralateral eye. OD score cumulative distributions were computed for each group.

Immunohistochemistry

Visual cortex samples were collected from not deprived aged animals reared in SC or exposed to EE for 15 days in order to evaluate whether EE led to molecular changes, which could be permissive for plasticity recovery. Animals were anesthetized with an overdose of chloral hydrate (10% in saline; Sigma) and perfused transcardially with phosphate buffered saline (10 mM; PBS) followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4). Brains were removed and post-fixed for 6 hrs in the same fixative at 4°C, before being immersed in 30% sucrose. Free-floating sections were incubated for 1 h in a blocking solution (10% BSA in PBS, pH 7.4) and then overnight at 4°C in a solution of anti-GAD67 antibody (MAB 5406, 1:1000, Chemicon) or biotin-conjugated lectin Wisteria Floribunda Agglutinin (WFA, L1516, 10µg/ml, Sigma). Antigen-antibody binding was revealed, respectively, with Alexa Fluor 448 goat anti-mouse (1:400, Molecular Probes) and fluorescein-conjugated extravidin (1:400, Sigma). Sections of the two experimental groups were reacted together with the same immunohistochemical procedure. Sections were acquired at 32x magnification (1024x1024 pixels) using a confocal Leica microscope to analyze the number of GAD67-pos-

itive cells and WFA-positive perineuronal nets (PNNs). Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant through the analysis. Images were imported to the image analysis system MetaMorph and counts were done in blind on the entire thickness of Oc1B. For each animal, at least five Oc1B sections were analyzed.

Statistical analysis

Statistical analysis was performed using Sigma Stat 3.5. Normality and homoscedasticity of the data were checked. Multiple groups were compared by ANOVA followed by post-hoc Holm-Sidak test. Two-tailed t-test was applied in case of comparisons between only two groups. Differences between OD distributions were assessed using a χ^2 test. For OD score, statistical analysis was performed using Kolmogorov-Smirnov test. Significance level was $p < 0.05$.

Results

I evaluated OD plasticity in aged rats (22-23 months) housed either in environmental enrichment (EE) or in standard conditions (SC). Detrimental effects of aging, indeed, are self-evident in rats only after 20 months of age, with broadly expressed signs of deterioration in the limbic and sensory-motor systems not recognizable in younger adult animals (Frick and Benoit, 2010). After 7 days of MD, I recorded visual evoked potentials (VEPs) from Oc1B contralateral to the occluded eye, calculating the contralateral-to-ipsilateral (C/I) VEP ratio as a measure of OD properties of cortical neurons. While MD did not affect the C/I VEP ratio in SC animals (MD-SC, n = 5, C/I VEP ratio: 2.04 ± 0.13; One-Way ANOVA, post-hoc Holm Sidak method, p = 0.09), I observed a marked OD shift in monocularly-deprived EE rats (MD-EE, n = 5, C/I VEP ratio: 1.11 ± 0.14) with respect to that measured in SC animals not subjected to MD (n = 8, C/I VEP ratio: 2.39 ± 0.13; $p < 0.001$; Figure 20a).

I further assessed OD plasticity using extracellular single-unit recordings. Contralateral bias index (CBI) and OD class distribution of MD-SC (n = 4, CBI: 0.63 ± 0.05) animals did not differ from those measured in SC rats (n = 9, CBI: 0.65 ± 0.03; One-Way ANOVA, post-hoc Holm Sidak method for CBI comparison, p = 0.745; χ^2 test for OD class distribution comparison, p = 0.593), whereas a significant OD shift in favor of the open eye was detected in MD-EE animals compared to the SC group (n = 6, CBI: 0.54 ± 0.03; respectively, $p < 0.05$, $p < 0.001$; Figure 20b,c). Accordingly, the cumulative distributions of OD score in SC (216 cells) and MD-SC animals (93 cells) were completely superimposable (Kolmogorov-Smirnov test, p = 0.584); in contrast, MD-EE rats (130 cells) showed an OD distribution shifted towards the open eye and significantly different with respect to SC rats ($p < 0.05$; Figure 20d). I also checked that EE did not affect basic physiological response properties of visual cortical neurons, with both VEP and single-unit recordings. No differences among SC (contra: 115.23

± 7.48 ms; ipsi: 125.86 ± 6.36 ms), MD-SC (contra: 110.81 ± 4 ms; ipsi: 126.38 ± 3.55 ms) and MD-EE animals (contra: 116.13 ± 3.89 ms; ipsi: 125.15 ± 4.76 ms) were detected either in VEP latency values for both eyes (Two-Way Repeated Measures ANOVA, $p = 0.963$; Figure 21a), or in the spontaneous discharge of cortical neurons (SC: 1.11 ± 0.18 spike/s, MD-SC: 1.23 ± 0.37 spike/s, MD-EE groups; 0.78 ± 0.21 spike/s; One-Way ANOVA, $p = 0.449$; Figure 21b) or in the receptive field size distribution of the cell population (SC: 15.22 ± 1.46°, MD-SC: 16.25 ± 0.75°, MD-EE: 14.21 ± 1.47°; $p = 0.661$; Figure 21c).

Since a reduction of GABAergic transmission has been identified as a crucial hub for the restoration of plasticity processes in the adult brain (for recent reviews, see Bavelier et al., 2010 and Baroncelli et al., 2011), I quantified GAD67-positive cells ($GAD67^+$) in the visual cortex of SC and EE animals by means of semi-quantitative immunohistochemistry. The number of $GAD67^+$ interneurons normalized to the area of Oc1B was statistically lower in EE rats (n = 4, 80 ± 5.63 cells/mm^2) with respect to SC animals (n = 3, 137.69 ± 6.9 cells/mm^2; t-test, $p = 0.001$; Figure 22). Moreover, since the degradation of extracellular matrix perineuronal nets (PNNs) has been related to the reopening of cortical plasticity in the adult brain (Pizzorusso et al., 2002; Kwok et al., 2011), I examined PNN distribution in the visual cortex of SC and EE animals. Environmental enrichment led to a significant reduction in the density of PNNs in the visual cortex of aged rats (EE: n = 4, 78.67 ± 6.08 PNNs/mm^2) with respect to SC (n = 4, 108.4 ± 7.27 PNNs/mm^2; t-test, $p < 0.05$; Figure 23).

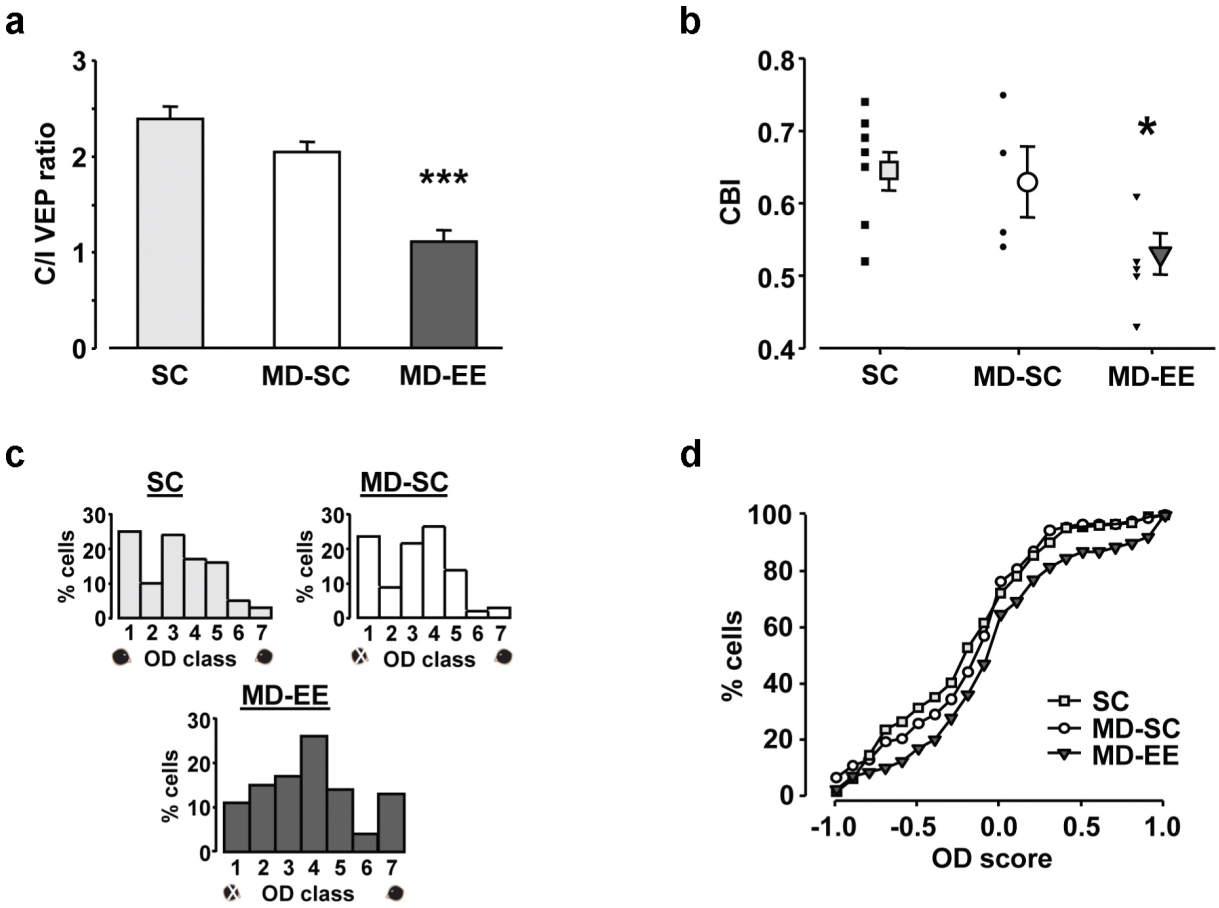

Figure 20. Environmental enrichment (EE) reactivates ocular dominance (OD) plasticity in the aged visual cortex. (a) VEP recordings revealed that, with respect to SC rats (n = 8), MD did not affect the C/I VEP ratio in MD-SC animals (n = 5; One-Way ANOVA, post-hoc Holm Sidak method, $p = 0.09$), whereas a significant decrease in the C/I VEP ratio of MD-EE animals was evident (n = 5; $p < 0.001$). **(b)** CBI values for SC, MD-SC and MD-EE groups obtained by single-cell activity recordings. Larger symbols represent the average CBI ± SEM for each experimental group; smaller symbols represent individual CBIs for each animal. The CBI of MD-SC rats (n = 4) did not significantly differ from that of SC animals (n = 9; One-Way ANOVA, post-hoc Holm Sidak method, $p = 0.745$), whereas the CBI of MD-EE rats was lower compared to that of the SC group (n = 6; $p < 0.05$). **(c)** A $\chi 2$ test showed that OD distributions for SC and MD-SC rats were superimposable ($p = 0.593$), whereas a significant OD shift towards the open eye was evident in MD-EE animals with respect to the SC group ($p < 0.001$). **(d)** OD score distributions for SC (216 cells) and MD-SC animals (93 cells) did not significantly differ (Kolmogorov-Smirnov test, $p = 0.584$) between each other, whereas OD score distribution for MD-EE group (130 cells) was statistically different from that of the SC group ($p < 0.05$). Asterisks indicate statistical significance: (*) $p < 0.05$; (***) $p < 0.001$. Error bars represent SEM.

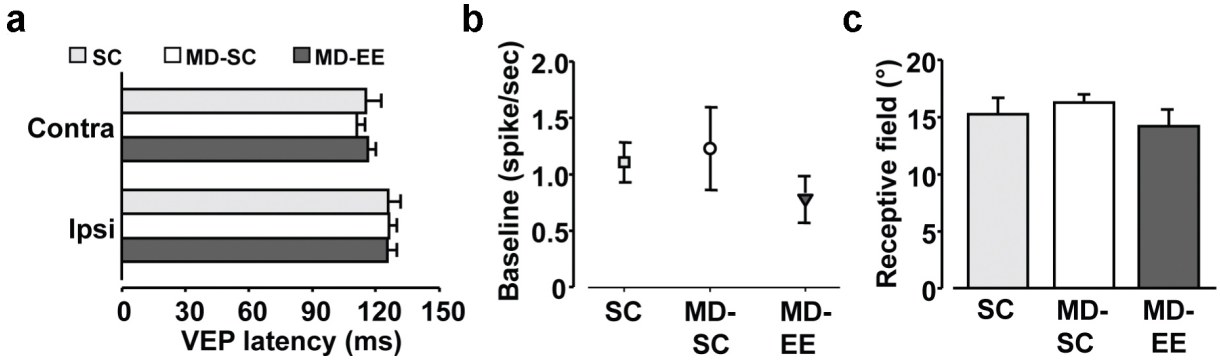

Figure 21. Environmental enrichment (EE) reactivates ocular dominance (OD) plasticity in the aged visual cortex. (a) VEP latencies obtained by alternatively stimulating the contralateral and the ipsilateral eye with respect to the recorded cortex at the spatial frequency of 0.1 c/deg were not different in SC, MD-SC and MD-EE animals (Two-Way Repeated Measures ANOVA, p = 0.963). **(b)** No statistical difference in spontaneous discharge of cortical neurons was present among SC, MD-SC and MD-EE groups (One-Way ANOVA, p = 0.449). **(c)** EE exposure did not alter the receptive field (RF) size distribution of the cell population (One-Way ANOVA, p = 0.661). Asterisks indicate statistical significance: (*) $p < 0.05$; (***) $p < 0.001$. Error bars represent SEM.

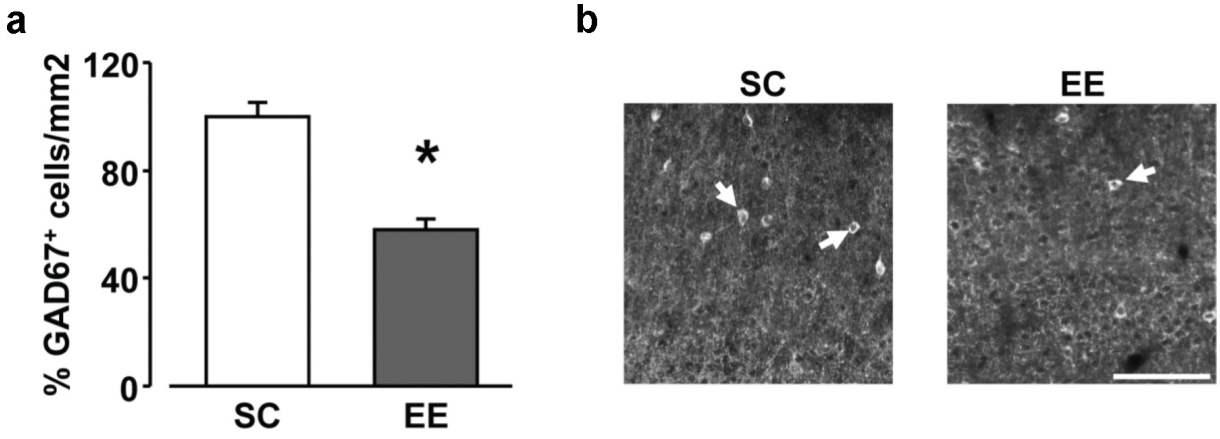

Figure 22. A role for intracortical inhibition in the induction of EE-dependent plasticity in the aged visual cortex. **(a)** Density of cells positive for GAD67 (GAD67+) in the entire thickness of the visual cortex was lower in EE animals (n = 4) with respect to SC rats (n = 3; t-test, p = 0.001). Cell density in EE visual cortex was normalized to the mean value in the SC cortex. **(b)** Illustrative images showing the reduction of GAD67+ cells in the visual cortex of EE animals. Arrows indicate representative examples of GAD67+ cells considered for quantification. Calibration bar: 100 μm. Asterisks indicate statistical significance: (*) $p < 0.05$. Error bars represent SEM.

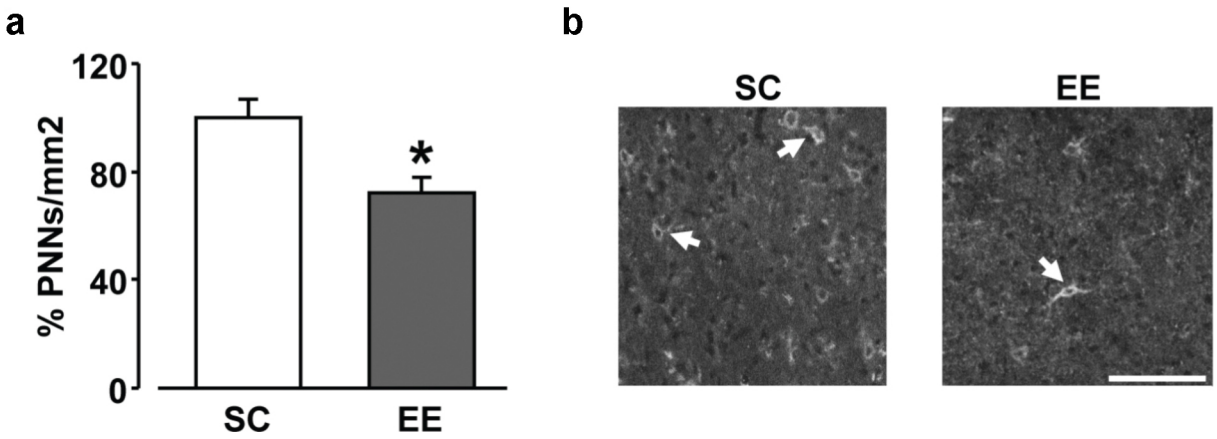

Figure 23. A role for extracellular matrix in the induction of EE-dependent plasticity in the aged visual cortex. **(a)** The number of WFA positive PNNs (WFA+) in the entire thickness of the visual cortex was significantly reduced in EE animals (n = 4) with respect to SC rats (n = 4; t-test $p < 0.05$). Density of WFA+ PNNs in EE visual cortex was normalized to the mean value in the SC cortex. **(b)** Illustrative images showing the reduction of WFA+ PNNs in the visual cortex of EE animals. Arrows indicate representative examples of WFA+ PNNs considered for quantification. Calibration bar: 100 μm. Asterisks indicate statistical significance: (*) $p < 0.05$. Error bars represent SEM.

Discussion

I have demonstrated that an enhanced environmental stimulation is able to restore OD plasticity in the aging visual cortex. To make sure that plasticity was not present only at the level of subthreshold modifications of postsynaptic potentials (detected by VEPs), I measured OD properties of cortical neurons also using single-cell recordings. I reported a significant OD shift in the visual cortex contralateral to the deprived eye in MD-EE animals, though slightly lower with respect to that measured in similarly treated adult rats (Baroncelli et al., 2010). Even if a connection between neural plasticity and EE in the aging brain has been suggested by previous results (e.g. Kempermann et al., 1998; Frick and Fernandez, 2003), this is the first time that the highly reliable and informative paradigm of OD plasticity has been studied in old rats.

It is generally assumed that EE provides the animals with a combination of multi-sensory and cognitive stimulation, high levels of physical activity, enhanced social interactions, and incentivizes explorative behavior, play, curiosity and attentional processes. I can speculate that this condition strongly increases the arousal state of animals setting in motion a molecular chain of factors that finally promote neural plasticity (see also Baroncelli et al., 2010). The excitatory-inhibitory balance is crucially involved in the regulation of plasticity during development and in adulthood (Bavelier et al., 2010; Baroncelli et al., 2011) and a decrease of intracortical inhibition levels is required for the reinstatement of neural plasticity elicited by EE in the adult visual cortex (Sale et al., 2007; Baroncelli et al., 2010). This correlation turned out to be true also in aged EE animals. Indeed, I found that the number of $GAD67^{+}$ cells in the visual cortex of EE rats was lower compared to that obtained for SC animals. Moreover, the decrease in GAD67-expressing neurons was accompanied by a remodeling of the extracellular matrix (ECM), with a significant reduction in the density of PNNs in the visual cortex of

EE rats with respect to SC. It is well-known that the extracellular milieu is one of the prime structural brake limiting brain plasticity in adulthood (Pizzorusso et al., 2002; Kwok et al., 2011) and the permissive action of ECM degradation on cortical plasticity could occur through a direct structural and functional remodeling of inhibitory synapses. My results suggest that molecular factors, which are known to be critically involved in the reinstatement of plasticity in the adult brain, may also underlie the EE effects in the aging visual cortex.

Given the complex nature of the EE setting and its capability to modify the cerebral biochemical milieu promoting visual cortex plasticity, I can assume that EE-dependent beneficial effects might be detected throughout the entire brain. Since deterioration in functional plasticity contributes to the decline of cognitive and sensory-motor abilities occurring during normal aging (Burke and Barnes, 2006), EE emerges as an elective strategy to counteract behavioral alterations observed in age-related disorders. It has been previously shown, indeed, that EE in aged rodents is able to promote an improvement of complex cognitive functions, accompanied by prominent changes at the anatomical and molecular level (for a recent overview, see Frick and Benoit, 2010).

Laboratory rodents are a powerful model system sharing many homologies with humans at the genetic and the physiological levels so that conclusions drawn in one species can be used to approach similar issues in the other. My results suggest that is never too late for the brain to get on track towards a rich environmental experience, encouraging the development of intervention protocols based on enriched experience aimed at maintaining a healthy and active lifestyle in aging people. Accordingly, strong correlative and epidemiological evidence shows that living habits, including occupation, leisure activities and physical exercise, has a direct effect on the risk of cognitive decline, with an increasing number of results indicating that a higher level and variety of mental and physical activity is associated with a lower cog-

nitive decline and a reduced risk for dementia (for a review, Nithianantharajah and Hannan, 2009).

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