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INTENSIVE MOTOR TRAINING ACCELERATES AXONAL REGENERATION FOLLOWING PERIPHERAL NERVE INJURY IN RATS

by

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AN ABSTRACT OF THE THESIS OF

Amir Wassim Madi for Master of science

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Title: Intensive Motor Training Accelerates Axonal Regeneration Following Peripheral Nerve Injury in Rats

Background: Peripheral nerve injury (PNI) results in a broad range of sensory and motor symptoms that depend on the severity of the injury and types of nerves involved. Many attempts to repair PNI have yielded limited outcome leading to sensory and motor deficits. Recent evidence has shown that physical training promotes the synthesis of nerve growth factors, needed to facilitate axonal regeneration.

Aim: To show that intensive motor training improves the sensory and motor functions in rats with sciatic nerve compression.

Methods: Adult male Sprague-Dawley rats had their right sciatic nerve crushed using fine forceps and were randomly divided into 4 groups and subjected for a month to different types of motor exercises (5 days/week). Groups 1 and 2 were trained for 1 hour (two 30 min interval separated by 10 min resting period). Rats in Group 1 were placed on a horizontal treadmill daily (8m/min), while those in group 2 were placed on the Rotarod (35 rpm; 8m/min). Group 3 were subjected to both the treadmill (30 min) and Rotarod (30 min) separated by 10 min resting period. Group 4 acted as control and were housed in standard cages for an equivalent period. To assess for nerve regeneration; behavioral, histological and electrophysiological tests were performed. All rats were evaluated for sensory recovery, and hypersensitivity to thermal and mechanical stimuli at 1, 5, 12, 19 and 26 days post injury. Locomotor performance was also assessed using the staircase test. Counting the number of grips and time taken to climb up and down the stairs were done before and at different time points post-surgery. Histologically, a whole mount immuno-florescence staining protocol was adopted to analyze the extent of regenerating axons using antibodies against neurofilament and myelin basic protein. Images of the stained nerves were then visualized using a laser scanning confocal microscope. The compound motor action potential (CMAP) were recorded twenty-six days after physical training to assess functional connections in the compressed sciatic nerve.

Results and Conclusions: Our behavioral data have shown that a combination of treadmill and rotarod exercises enhances sensory and motor recovery following sciatic nerve

compression. Electrophysiologically, the sciatic nerves of rats subjected simultaneously to both exercises showed shorter latency, higher amplitude and lower duration as compared to those of rats subjected to one type of exercise indicating a better functional neurological recovery. In summary, our results provide evidence that peripheral nerve regeneration could be enhanced substantially by combining exercises that activate additional mechanosensory and motor neurons and induce neuroplastic changes in different regions of the brain.

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ABBREVIATIONS

CNS	Central Nervous System
PNI	Peripheral Nerve Injury
BDNF	Brain Derived Neurotrophic factor
SFI	Sciatic Functional Index
IL 1β	Interleukin 1 beta
TNF-α	Tumor necrosis factor alpha
HSP 72	Heat Shock Protein 72
SCI	Spinal Cord Injury
NGF	Nerve Growth Factor
DRG	Dorsal Root Ganglion
NT-4	Neurotrophic factor 4
RNA	ribonucleic acid
mRNA	messenger RNA
rpm	rotations per minute
i.p.	intra-peritoneal
GAP-43	Growth Associated Protein 43
MRI	Magnetic resonance imaging
T	Treadmill trained group
R	Rotarod Trained Group
RT	Rotarod and Treadmill Combined Trained Group
C	Control Group
EMG	Electromyography
CMAP	Compound muscle action potential

CHAPTER 1

INTRODUCTION

Peripheral nerve injury (PNI) represents a significant medical problem worldwide. It is caused mostly by vehicle accidents, gunshot injuries, fractures or penetrating trauma after stabbing incidents, and stretching or crushing injuries after falls. Depending on the type of incidents, the nerves may be severely or moderately lacerated, displaced, stretched, or even transected. It is estimated that roughly twenty million Americans suffer from PNI caused by trauma and various medical disorders.

Although injured axons in the peripheral nervous system (PNS) have a better chance of recovery than those in the central nervous system (CNS), poor functional outcomes are usually observed following injury leading to long term disability especially at the level of daily activities (Brushart, 1998). Regeneration of injured peripheral axons is usually moderate and not all axons participate in this process, prompting functionally deficient muscle re-innervations (Fu and Gordon, 1995, 1997; Gordon, 2009). Moreover, under certain circumstances, regenerating motor axons are misled, thus re-innervating practically inappropriate targets (Evans et al., 1991; de Ruiter et al., 2008). More importantly, plastic changes occurring in the CNS have been shown to impact neuronal activities, leading to unsuccessful innervation of the denervated muscle (Alvarez et al., 2010). Based on the above reasons, it seems that different approaches have to be taken to successfully treat peripheral nerve injury. Recently, a number of studies have highlighted the importance of physical exercise in improving memory, strengthening synaptic connections and enhancing plasticity in different regions of the brain (Adlard and Cotman, 2004; Adlard et al., 2004). Exercise appears to be beneficial through a variety of cellular and biochemical mechanisms, and has

been shown to promote the synthesis of both brain derived neurotrophic factor (BDNF) and its receptor, trkB, in rats and to advance functional recovery after peripheral and CNS damage (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004; Molteni et al., 2004; Ploughman et al., 2005). Since physical training specifically engages primary afferent (sensory) and motor neurons, one may expect that increased activity in their axons could promote axonal regeneration and successfully lead regenerating axons to their appropriate targets. Furthermore, this activation may drive compensatory adaptation in central neurons that enhances behavioral recovery and improves patients' quality of life.

A. Peripheral Nerve Injury: Symptoms

Injury to a peripheral nerve induces structural alterations in both sensory and motor neurons. When neuronal impulses to and from the CNS are interrupted or perturbed, several symptoms, determined by many factors, may arise. The clinical presentation of peripheral nerve damage may vary from sensory deficit to motor loss or a combination of both. The symptoms can be different depending on the type and severity of injury. Some may include numbness, reduced or loss of cutaneous sensation and proprioception, pain and paresthesias in the distribution of the affected nerve.

Under certain conditions, such as trauma, immobilization, hypersensitivity or overuse, neuropathic pain may develop following nerve injury. It is usually associated with abnormal sensations of pain triggered by normally non-painful stimuli. In addition, within 10-14 days after injury, there is a loss of both superficial and deep reflexes. Severely damaged nerve fibers result in fibrillations and fasciculations during which muscles undergo atrophic changes. A flaccid type of paralysis would ensue if there were no regeneration due to a lack

of efferent impulses; the denervated muscles lose their tone, eventually shrink and are replaced by connective and adipose tissues.

In addition to sensory and motor deficits, autonomic changes are also present following injury. The skin of the area supplied by the injured nerve goes through a warm phase of vasodilatation, and passes into a cold phase after approximately twenty-one days. Changes in the skin's color and texture and the way sensations are felt are also very common in patients with peripheral nerve damage due to loss of cutaneous innervation (Menorca et al, 2013).

B. Effects of Exercise on Axonal Regeneration

Over the last decade, the question of whether exercise plays harmful or beneficial roles in peripheral nerve injury has been widely debated and reviewed. Following injury to the peripheral nervous system (PNS), functional recovery in patients remains poor, despite the well-demonstrated ability of axons to slowly regenerate and reinnervate denervated targets. Regenerating axons from the proximal segment of a cut nerve must be guided and directed to connect with the distal segment of the nerve, after which they should grow to reach their targets. For axons to regenerate and ensure functional recovery, they require the availability of growth-promoting molecules to support their survival, growth and elongation.

Recent studies have shown that neuronal activity in the affected nerve might contribute to the process of axon growth, survival and regeneration. Al Majed et al. (2000) demonstrated the beneficial effect of electrical stimulation of cut peripheral nerves on regenerating axons. They found that one hour of continuous supra-maximal stimulation of the proximal stump of a cut nerve could enhance regeneration of both sensory and motor axons (Al Majed et al, 2000). However, blocking the propagation of electrical activity from

reaching the cell bodies of these affected neurons resulted in a complete cessation of the enhancement induced by electrical stimulation, suggesting that the recovery effect is activity dependent (English et al, 2014). Furthermore, using transgenic mice that express the light-sensitive cation channel, channel rhodopsin (ChR2), in some but not all axons in peripheral nerves, scientists have found that increasing neuronal activity using light induces a selective enhancement of regeneration of motor axons expressing the transgene (Ward et al, 2016) and not the ones that lack it. Therefore, given the evident role of increased neural activity in promoting axonal survival, many researchers sought to examine the effect of exercise as a natural mean of activating neurons affected by injury. Data revealed that moderate daily exercise for 2 weeks has the ability to significantly enhance axonal regeneration, surpassing the effects of electrical stimulation.

Physical exercise, by means of voluntary or forced locomotion, is one of several possible strategies used to enhance peripheral nerve regeneration and improve target muscle reinnervation. Different exercise protocols such as swimming or rhythmic limb movement (Gordon and English, 2017) have been shown to achieve the same restorative effect in anesthetized animals. Although passive therapeutic exercise remains a common practice in the rehabilitation of PNS lesions (Pachter et al, 1989), only recently it was shown that active physical exercise and activity-dependent interventions have real impact on neurobiological mechanisms of peripheral nerve regeneration.

Studies reported that daily exercise could promote axonal regeneration after peripheral nerve injuries in both animal models and in human subjects. Although evidence has shown that exercise boosts regeneration of motor axons, its effect on the regeneration of sensory axons is still comparatively unknown. Furthermore, whether sensory and motor axons have the same requirements for increased activity to regenerate remains to be understood.

C. Effect of Treadmill Training on Enhancement of Axonal Regeneration

A number of studies have indicated that treadmill exercise training can improve peripheral nervous tissue regeneration following central and peripheral axonal injury. Treadmill training exerts its beneficial effect through natural activation of motoneurons via the spinal circuits that drive locomotion and leads to enhancement of axon regeneration without the increase in number of misdirected axons noted using electrical stimulation.

Following spinal cord injury, treadmill training performed in the recovery period has been shown to enhance neurological function, both in rodents and humans (Edgerton et al., 1997; Hutchinson et al., 2004). Edgerton and associates have upheld treadmill exercise as a treatment for patients with spinal cord injury. They demonstrated that both active exercise (Gomez-Pinillaet al., 2002; Engesser-Cesar et al., 2005) and treadmill exercise (Ying et al., 2005; Heng and de Leon, 2009) brought about significant increments in the expression of BDNF and neurotrophin-3 (NT-3) in the spinal cord, promoting the survival of local neuronal circuitry below the injury site (Funakoshi et al, 1995; Courtine et al., 2009). Even though limitations to this approach have been noted (Barbeau et al., 2006), various studies have been published highlighting the potential therapeutic effect of treadmill training in spinal cord injury. In light of these findings, various rehabilitation centers throughout the world have adopted this mode of exercise to enhance and speed up functional recovery in patients (Wessels et al., 2010).

On the other hand, the impact of physical training performed in the recovery period after peripheral nerve injury has been less extensively studied. Marqueste et al. (2004) demonstrated that treadmill exercise following transection of the common fibular nerve induced better sensory functional recovery while motor recovery remained undetermined Some had inferred that training has advantageous impacts (van Meeteren et al., 1997, 1998),

while others have contended that it doesn't yield the best outcome (Soucy et al., 1996). Feng et al (2000) studied the elongation of axonal regeneration in mice by using a subset of sensory and motor axons that are stamped by yellow fluorescent protein (YFP), to empower simple visualization of YFP+ axons. By utilizing a simple treadmill-training program, they demonstrated that 1 hour of daily treadmill training started on the third day following transection combined with careful repair of injured peripheral nerve in mice using grafts, brought about a striking increment in the length of recovering axons. Comparative improvement was observed in mice subjected to the same exercise regimen but nerves were repaired utilizing basic end-to-end anastomosis of the cut stumps (English et al., 2009). Taken together, these findings provide direct evidence for a positive effect of treadmill training on accelerating axonal regeneration following peripheral nerve injury.

Sabatier et al (2008) has demonstrated that short duration treadmill exercise, with varying intensity can exert a tremendous beneficial effect on facilitating axonal regeneration in the PNS. When Lerman et al (2002) subjected mice during the first two weeks post nerve transection, to as little as one hour of daily continuous treadmill locomotion at a slow speed, or two two-minute bouts of more intense treadmill locomotion at near maximal treadmill running speed, the cut axons showed significant regeneration. In contrast, reducing the quantity of training intervals to as few as two had no noteworthy impact on the measure of improvement of axon recovery. In addition, they showed that running for a single 2-min interval or broader interval training at moderate treadmill speeds was not sufficient to promote axon recovery suggesting a critical role for intensive treadmill exercises in mediating peripheral axonal regeneration.

Although treadmill training has been recommended for patients with spinal cord injuries for some time (Edgerton, et al., 2004), only recently, has the role of treadmill exercise in peripheral nerve injuries received considerable attention. Over the last decade, several animal studies highlighted the positive effect of treadmill training on facilitating nerve fiber regeneration. Interestingly, Molteni et al (2004) has provided evidence that exercise carried out before peripheral nerve injury has a protective effect and is thought to stimulate dorsal root ganglion neurons to provide the appropriate environment for axonal regeneration. In a sciatic nerve crush model, a positive effect of treadmill training on axon regeneration in rats has also been demonstrated (Seo, et al., 2006).

Many studies using distinctive training settings and various injury models, have reported advantageous impacts of treadmill training on axonal regeneration after peripheral nerve injury in rodents. Ilha et al. (2008) assessed the impact of modest speed treadmill training of just 9 m/min for 60 min and discovered changes in Sciatic Function Index (SFI) scores and morphology of recovering nerve in rats after sciatic nerve crush injury. Another group assessed the same injury model, using higher training intensity of 18 m/min for 60 min, and reported better axonal recovery (Seo et al., 2006). However, since the endoneurial tubes surrounding the nerves remain unblemished in crush injury, recovery was much better than those with nerve transection and repair injuries (Sunderland, 1990). Furthermore, Asensio-Pinilla and colleagues (2009) have reported that rats forced to walk on a treadmill at just 5 m/min for a total of 60 min/day (two 30-min intervals separated by 10min rest) after sciatic nerve transection, had an increased number of recovered myelinated axons.

On the other hand, passive exercises, electrostimulation of denervated muscle and locomotion training has been shown to be effective modalities to impede atrophy of muscles while improving its contractile responses after reinnervation. Udina el al (2010), have investigated the effect of passive and active exercise in axonal regeneration after peripheral nerve injury in

rats, and concluded that 1 hour of daily moderate exercise either active (treadmill) or passive (bicycle) improved the number of regenerating axons, improved muscle reinnervation while decreasing the excitability of spinal reflexes following nerve lesion.

Converging evidence, therefore, suggests that treadmill training following peripheral nerve injury leads to better axonal recovery independent of the type of injury or the different treadmill settings used. Nevertheless, further examinations are needed to discover the optimal treadmill parameters for faster recovery.

D. Mechanism of Action of Treadmill Training

To better understand the mechanism of action of treadmill training on axonal regeneration, axons of wild type host mice that were compelled to regenerate into grafts from mice in which the genes expressing BDNF were knocked out restrictively in all cells, or specifically in Schwann cells; there was no evidence of recovering axons. However, mice that were subjected to daily treadmill training for two weeks (continuous or interval training), had their regenerated axons increased in length as compared to the untrained wild type mice whose nerves were repaired with grafts from controls (Wilheml et al, 2009). Thus, the impact of treadmill training was shown to be independent of the medium through which axons recover and regenerate.

On the other hand, Young et al (2008) have used mice in which the gene expressing BDNF was knocked out restrictively in neurons. They demonstrated that neuronal BDNF Signaling was necessary for the effects of treadmill exercise on increasing synaptic input into axotomized motoneurons.

In view of the above findings, it seems that treadmill training exerts its advantageous effect on axon recovery through release of BDNF by the recovering axons and subsequent activation of BDNF receptors. Not surprisingly, treadmill training can bring out an expanded expression of neuronal BDNF, which can enable growth, and elongation of injured axons via autocrine or/and paracrine activity through trkB receptors exerted on the growth cones of those axons.

In support of this notion, it was shown that treadmill training doesn't exert its effect through mobilization of activity dependent molecules from the target muscles, as the enhanced recovery was initiated two weeks post nerve transection, long before any muscle re-innervation. It should be noted that any muscle-derived growth promoting molecules could not impact axon recovery systemically since axonal regeneration was absent in neuron specific BDNF knockout mice where muscle BDNF expression was normal. Therefore, the detected effect of treadmill training might be due to molecular changes in the neurons themselves.

It is likewise conceivable that treadmill training increases the expression of the trkB receptor in the growth cones of recovering axons. This increase would be required to bring about an enhanced sensitivity to BDNF in a few neurons and novel sensitivity to BDNF in others. The expression of TrK B receptor protein in the primary afferent neurons and motoneurons was shown to be higher after various types of treadmill training (Macias et al., 2009). Taken together, these findings suggest an important role for TrKB receptors in mediating the effect of treadmill exercise on regenerating axons.

By contrast, it has been shown that the intensity of treadmill modifies the effects of exercise, as large doses of BDNF have been found to play an inhibitory role in axonal regeneration (Boyd and Gordon, 2002). Consistent with these findings, Tam et al, (2001) confirmed the

inhibitory effect of BDNF *in vivo* following increased neuromuscular activity accompanying wheel running in rats.

While several studies emphasized the positive role of treadmill training in enhancing axonal regeneration and functional recovery following nerve injury, a growing body of evidence has demonstrated its considerable effect on decreasing acute and neuropathic pain, as well as inflammatory responses. Progressive exercise training has shown to decrease peripheral neuropathic pain as well as IL-1β and TNF-α overproduction while increasing HSP72 (heat shock protein 72) expression following chronic constriction injury of the sciatic nerve (Chen et al, 2012). Moreover, the impacts of various treadmill training protocols on the functional recovery of the sciatic nerve after chronic constriction injury in mice was studied and showed that short-lasting training (1 h/d for 5 days) diminished the neuropathy-induced mechanical allodynia and heat hyperalgesia. In contrast to other studies, it was found that long- lasting training (1h/d more than 5 days) was unfavorable for both functional recovery and allodynia. Interestingly, it has recently been demonstrated that the activity of serotonergic immunoreactivity in medullary raphe nuclei and spinal cord of rats suffering from sciatic nerve transection injury was upregulated following treadmill training (Korb et al, 2009) suggesting an important role for exercise in promoting serotonin release and modulating pain transmission.

More recently, the role of physical exercises in promoting the expression of neurotrophic factors has been validated in the brain and spinal cord (Boeltz et al, 2010). Jung et al, (2016) have shown that treadmill exercises facilitate recovery of locomotor function through axonal regeneration in spinal cord injuries rats via BDNF expression. As neurotrophins are recognized as the molecular framework for promoting spinal cord repair, the use of exogenous neurotrophins into the CNS has also been tested for motor regeneration following SCI (Ying et al., 2005). However, the intrinsic neural framework producing neurotrophins

appeared to be diminished (Ying et al., 2003). Hence, physical exercise was shown to be instrumental in enhancing endogenous neurotrophins expression in the injured spinal cords.

On the other hand, axonal growth supported by Schwann cells is accompanied by generating neurotophic factors, extracellular matrix molecules and cell adhesion molecules (Oudega and Xu, 2006). Schwann cell proliferation is shown to correlate functionally with axonal regeneration (Seo et al, 2006). Goulart et al (2014) observed that the combination of Schwann cells and Treadmill training treatment resulted in increased expression of BDNF, NGF and NT-4 in the sciatic nerve, DRG and spinal cord either by direct secretion from implanted cells or as a result of treadmill training. Collectively, these results suggest that the outflow of BDNF is due to the activity occurring in the neurons stimulated by treadmill training. (Vaynman and Gomez Pinilla, 2005).

It is worth noting that using an inclined treadmill, can expand the action in lower leg extensor muscles (soleus), yet causes a movement in flexor muscles (tibialis anterior), that is almost double the force found amid level walking (Sabatier et al., 2011), and improves motor axon recovery innervating flexor than extensor muscles. English et al (2009) have reported, using retrograde fluorescent markers, that mice trained on an inclined treadmill for 2 weeks, and had the sciatic nerve cut and repaired by end-to-end anastomosis, showed enhanced improvement in motor axon recovery than those trained on a level treadmill.

On the other hand, Cannoy and colleagues (2016) have investigated the effect of upslope treadmill exercise on axon regeneration and functional recovery following peripheral nerve injury. As moderate treadmill training had resulted in improved axonal regeneration and functional recovery, they assumed that the use of upslope treadmill training would result in greater motor axon regeneration and thus better functional recovery. Contrary to expectations, they concluded that slope training improves axon regeneration but not

functional recovery after sciatic nerve injury and repair. This poor improvement was attributed to several factors, including poor effect of upslope training on the proprioceptive sensory feedback from the reinnervated muscles, in addition to changes in muscle activity and limb movements in response to different biomechanical demands in upslope walking. Still, further studies are needed to examine the utility of different treadmill settings and their effect on promoting axonal reorganization and functional recovery after peripheral nerve injury.

E. Treadmill Training Stimulates Axonal Protein Synthesis

It has been widely accepted that axonal proteins are synthesized in the soma of neurons and then actively transported down the axon by microtubules at a relatively slow average daily rate due to frequent pauses of the cargoes (Weiss and Hiscoe, 1948; Brown, 2003). This form of anterograde transport is facilitated by kinesin, the same motor protein used in the much faster axonal transport of organelles (Brown et al, 1980). This same concept can be applied to the elongation of neurites from the proximal segments of cut nerves; considering this, the axon regeneration in cut peripheral nerves would be expected to proceed at the same slow rate (Lasek and Hoffman, 1976). A study by Hoffman et al. (2010) has shown that the cytoskeletal proteins, actin and tubulin, required for axon recovery are continuously transported in axons and are sufficient to account for the degree of axonal elongation past injury sites in cut peripheral nerves. However, if mice are subjected to treadmill training during the first week post-injury, twice as many axons are lengthened nearly twice as far as those of untrained mice. Since the traditional view of protein synthesis in the cell body and their slow axonal transport cannot account for the rapid elongation of injured axons following treadmill exercise, it would be reasonable to suggest that treadmill training enhances the

synthesis of cytoskeletal proteins in situ. Not surprisingly, accumulating evidence confirmed the synthesis of axonal proteins and the presence of their mRNAs in axons (Tobias and Koenig, 1975; Bassell et al., 1998; Krichevsky and Kosik, 2001). Therefore, fast recovery of axons in cut or crushed peripheral nerves after treadmill training may be due to the use of proteins that are synthesized and translated at or near the site of damage. Consistent with this notion, a study on mice found that hindering mRNA transport into axons significantly decrease the chance of axonal recovery after crush injury (Donnelly et al., 2011). The boosting effect of treadmill training on axonal growth has shown to be dependent on the response of growth cones to BDNF, which itself is dependent on local protein synthesis (Yao et al. 2006). Given this premise, it is likely that the rapid elongation of axons produced by treadmill training is caused by local axonal protein synthesis.

F. Treadmill Training Results in Less Misdirection of Regenerating Axons

Despite the ability of peripheral nerves to regenerate, the misdirection of the regenerating axons leads to inappropriate re-innervation of peripheral targets and such misdirection is said to produce the clinically poor functional outcomes following peripheral nerve injury (Fu and Gordon, 1997; de Ruiter et al., 2008).

In the mammalian CNS, there is a topographic localization of motor neurons innervating different groups of muscles with similar functions (Yakovenko et al., 2002) as their motor nuclei are located in spatially distinct regions in the CNS. However, single muscles or groups of muscles are likely to be innervated by different group of motor neurons following peripheral nerve damage. In an earlier study by Ito and Kudo (1994), it was shown that the motor neurons of injured facial nerve re-innervating the posterior digastric muscle have shifted their location in the motor nucleus, in a region that does not correspond with its

normal innervation. Moreover, immunohistochemical studies have looked into the topographic organization of the motor nucleus of the sciatic nerve and the impact of treadmill training on the degree of misdirection of regenerating axons after peripheral nerve transection and repair (English et al., 2005). Their results showed that the axons of motor neurons of the common fibular nerve are limited to the rostral part (60%) of the motor nucleus of the sciatic nerve in intact mice. However, after sciatic nerve transection, the cell bodies of motor neurons were found in the caudal part (40%) of this nucleus reflecting the misdirection of the tibial nerve regenerating axons (English, 2009). Interestingly, compared to untrained mice, those subjected to treadmill training, had only about 10% of motor neurons with regenerating axons in the common fibular nerve in topographically inappropriate region, indicating that treadmill training resulted in significantly less misdirection of newly regenerated axons. The above studies highlight the vital role of exercise in guiding peripherally regenerating axons toward their appropriate targets.

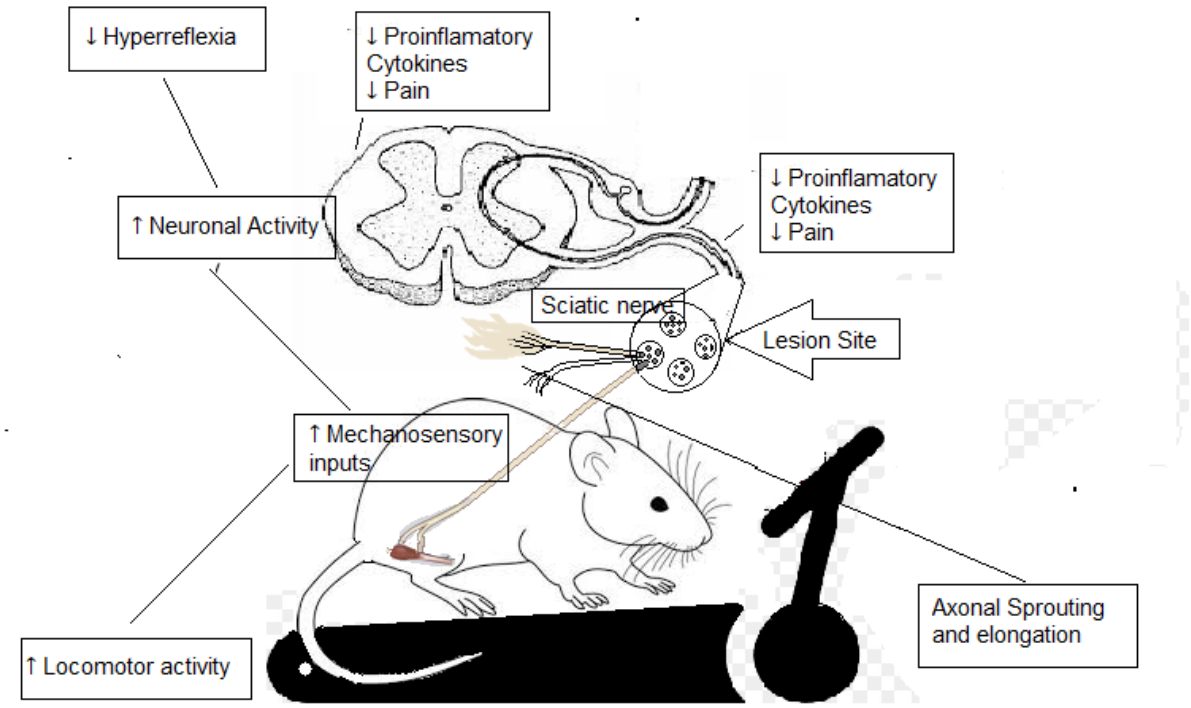


Fig.1 Schematic representation of treadmill training mechanism of action on nerve regeneration and functional recovery.

G. Treadmill Training Enhances CNS Plasticity

A series of cellular and molecular changes have been shown to take place in neuronal circuitries in certain areas of the brain and spinal cord following peripheral nerve injury. It has been reported that a large portion of the synaptic inputs into the proximal dendrites and cell bodies of motor neurons are removed following nerve transection possibly due to degeneration of primary afferent terminals or changes in motor neuronal properties (Lindå et al., 1992; Mendell et al., 2001; Oliveira et al., 2008). Even in the absence of sensory axons as in the facial nerve, synaptic input removal is found on brainstem motor neurons following its transection (Liebermann, 1971; Titmus and Faber, 1990). The withdrawal of afferent terminals from motor neurons appears to be permanent, regardless of whether the nerve was repaired, or axon recovery was effective. Synaptic terminals from primary afferent neurons that are found on motor neurons were less in number several months after nerve transection (Hughes et al., 2004; Alvarez et al., 2010; Chen et al., 2012) and were shown to correspond well with loss of stretch reflexes in self-reinnervated muscles (Alvarez et al., 2010).

Only recently has attention been devoted to studying the impact of treadmill training on the number and properties of synaptic input onto motor neurons in the spinal cord. A number of studies have demonstrated that retraction of synapses following nerve transection could be reversed by early application of recombinant BDNF and NT-3 to the proximal stumps of a transected abducens nerve (Davis-Lopez de Carrizosa et al., 2009a, b). This suggests that neurotrophins play a crucial role in strengthening synapses and restoring stripped synaptic inputs. Since treadmill training was shown to increase the expression of neurotrophins in spinal motor neurons (Gomez-Pinillar et al., 2001). Indeed, no loss of synaptic inputs from axotomized motor neurons was observed following 2 weeks of daily treadmill training (Krakowiak et al., 2010). More importantly, Krakowiak and colleagues have also found, using BDNF knocked out mice, that the effect of treadmill training was mostly dependent on

the accessibility of BDNF (Krakowiak et al., 2010). Collectively, these findings suggest that treadmill training may provide an essential therapeutic tool for patients with peripheral nerve injury, as it impacts both axonal regeneration and enhancing synaptic connections.

H. Role of Rotarod Training in Axonal Regeneration and Motor Control

Only few studies have examined the effect of rotarod training on enhancing axonal regeneration following peripheral nerve injury. Van Meeteren et al (1997), examined the effect of exercise training on improving nerve conduction velocity and functional recovery following sciatic nerve crush lesion in rats. The rats were trained to maximally erect on both hind paws to drink. They concluded that this exercise paradigm improved both sensory and motor functions evidenced by improved nerve conduction velocity. Voluntary wheel training for a time of only 3 and 7 days clearly improved the rate of neurite outgrowth in cultured exercise-conditioned dorsal root ganglion neurons and increased the number of recovering sensory neurons after sciatic nerve damage. This effect was shown to be associated with higher expression of BDNF and NT-3 in the sensory ganglia of crushed sciatic nerves, in addition to increased levels of synapsin I and GAP-43 (Molteni et al., 2004). On the other hand, when Badke and colleagues (1989) trained mice to run voluntarily on a running wheel following tibial nerve cut, impaired soleus muscle re-innervation was observed; however, synaptic transmission at the level of the neuromuscular junction was strengthened. Further analysis revealed that the synthesis of mature BDNF was enhanced following wheel running through activation of tissue-type plasminogen activator (Ding et al., 2011).

The rotarod test is generally used to assess motor coordination in rodents with neurological disorders. Among a few behavioral tests that measure motor activity, the rotarod has been considered an appropriate tool for assessment of motor deficits in rodents (Caston et al.,

1995; Lalonde et al., 1995) and shown to be a sensitive and effective test for assessing motor skill learning (Shiotsuki et al, 2010).

Buitrago and colleagues (2004) have characterized motor skill learning in the accelerated rotarod task, which included skilled forelimb reaching and acrobatic locomotor paradigms and determined that the rotarod can be considered a valid tool for motor skill learning over short and long-time periods. Gait analysis on the rotarod revealed a change in the gait patterns during training indicating the development of a new motor strategy that helped rats master the task. On the other hand, Wong et al (2013), aimed at studying the effect of combined treatment with progesterone and rehabilitation training using rotarod in improving functional recovery after cerebral ischemia. The training included entire body exercising while improving coordination and balance. Following training the brain function was enhanced in mice following stroke indicting the effectiveness of training in improving functional recovery. They inferred that combined treatment displayed improved adequacy in promoting functional recovery following brain injury. The effectiveness of rotarod in detecting motor deficits after mild and moderate brain injury was assessed and compared to beam balance and beam walking latencies tests. The results indicated that the rotarod task was more sensitive and efficient in testing motor impairment caused by brain injury (Hamm RJ. Et al, 1994). Moreover, it has been shown using multimodal MRI, that rotarod training task is associated with changes in brain regions that are involved in motor learning-related and task-associated brain regions (Scholz et al, 2015).

More importantly, early studies reported that complex motor training on rotarod involving balance and coordination played an essential role in improving motor function in ischemic rats, when compared with simple locomotor exercise on treadmill. Rotarod trained animals with or without ischemia exhibited enhanced motor performance, whereas animals trained for

up to 28 days on the treadmill did not show improved function, suggesting that complex motor training rather than simple exercise is more effective in generating positive functional outcome (Ding et al., 2011). Despite the beneficial effect of rotarod on motor functions, there has been limited research on the role of rotarod training in axonal regeneration and functional recovery following peripheral nerve injury.

I. Hypothesis and Aim of The Study:

Peripheral nerve injury (PNI) results in a broad range of sensory and motor symptoms that depend on the severity and types of nerves involved. Many attempts to repair PNI have yielded limited outcome leading to sensory and motor deficits. Converging evidence has shown that physical training promotes the synthesis of nerve growth factors and facilitates axonal regeneration. Based on these data, we aimed in the present study to examine and compare the effects of intensive motor training, including simple locomotor exercise on treadmill, complex motor training on rotarod or a combination of both, on axonal regeneration and functional recovery in rats following sciatic nerve compression. We hypothesize that a combination of both exercises yields the best functional outcome in rats.

CHAPTER 2

MATERIALS AND METHODS

A. Animal Model

Forty-three adult male Sprague-Dawley rats (4-5 weeks old), weighing 120-200g at the start of the experiment, were used in this study.

B. Experimental Design

Rats were used in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut and performed in accordance with relevant guidelines and regulations. All animals were housed in standard cages; each cage contained five animals, with free access to food utilizing standard diet regimen and water ad libitum. Animals were maintained in a controlled environment with temperature varying between 20 to 25° and kept in a 12/12 h light/dark cycle. Surgical procedures and behavioural tests were conducted during the light phase of the cycle by a researcher blind to the treatment conditions.

All rats were subjected to a compression injury of the right sciatic nerve and randomly divided into four groups in which each group included 10 rats:

1. Group T was subjected to a simple locomotion exercise on a horizontal treadmill for 1 hour (Two 30 min intervals separated by 10 min rest period) at a speed of 8m/min, (5 days per week) over a period of 1-month starting day 1 after surgery.

2. Group R was subjected to a more complex exercise on the rotarod at a speed of 35 rpm (8m/min) for 1 hour/day (Two 30 min intervals separated by 10 min rest period) for 5 days/week over a period of 1 month, starting day 1 after surgery.
3. Group RT was subjected to a combination of treadmill (30 min) and rotarod (30 min) (5 days per week) separated by 10 min rest period starting day 1 after surgery for 1 month.
4. Group C served as the control group. Rats were housed in standard cages for an equivalent period of time without any training.
5. Uninjured group was subjected to a skin incision without crushing the nerve and were housed in standard cages for an equivalent period of time without any training.

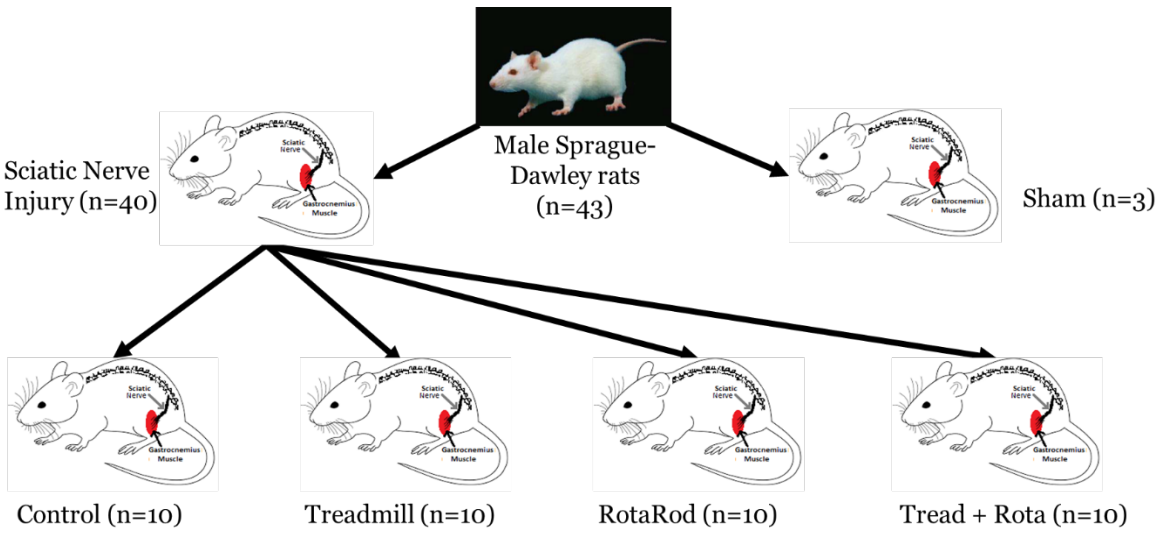


Fig.2 Schematic illustration of the experimental design.

C. Surgical Procedure

The animals were deeply anesthetised by administering a cocktail of ketamine (40-100 mg/kg) and xylazine (5-13 mg/kg) intraperitoneally (i.p.). The lower back was carefully shaved using a surgical clippers and hair depilation was done with Nair hair removal cream.

The rat was placed on a pre-heated homeothermic blanket system (Harvard Apparatus, 507222F) to maintain body temperature at 37°C. A small incision in the skin of the right hindpaw was made and the fascial plane between the gluteus maximus and the anterior head of the biceps femoris was opened using a retractor to expose the sciatic nerve. The nerve was then freed from the surrounding connective tissue and placed on the bottom jaw of a super-fine hemostatic forceps (van Meeteren *et al*, 1997) at 90 mm from the tip of the third toe. The hemostatic forceps have been engraved with a mark at 1.5 mm from their tip in order to place the sciatic nerve in line with this mark before compression. This procedure ensures a compression of uniform width. The nerve was crushed once for 30 seconds. Subsequently, the gluteal muscle and the opened skin were re-opposed and sutured using non-absorbable silk sutures.

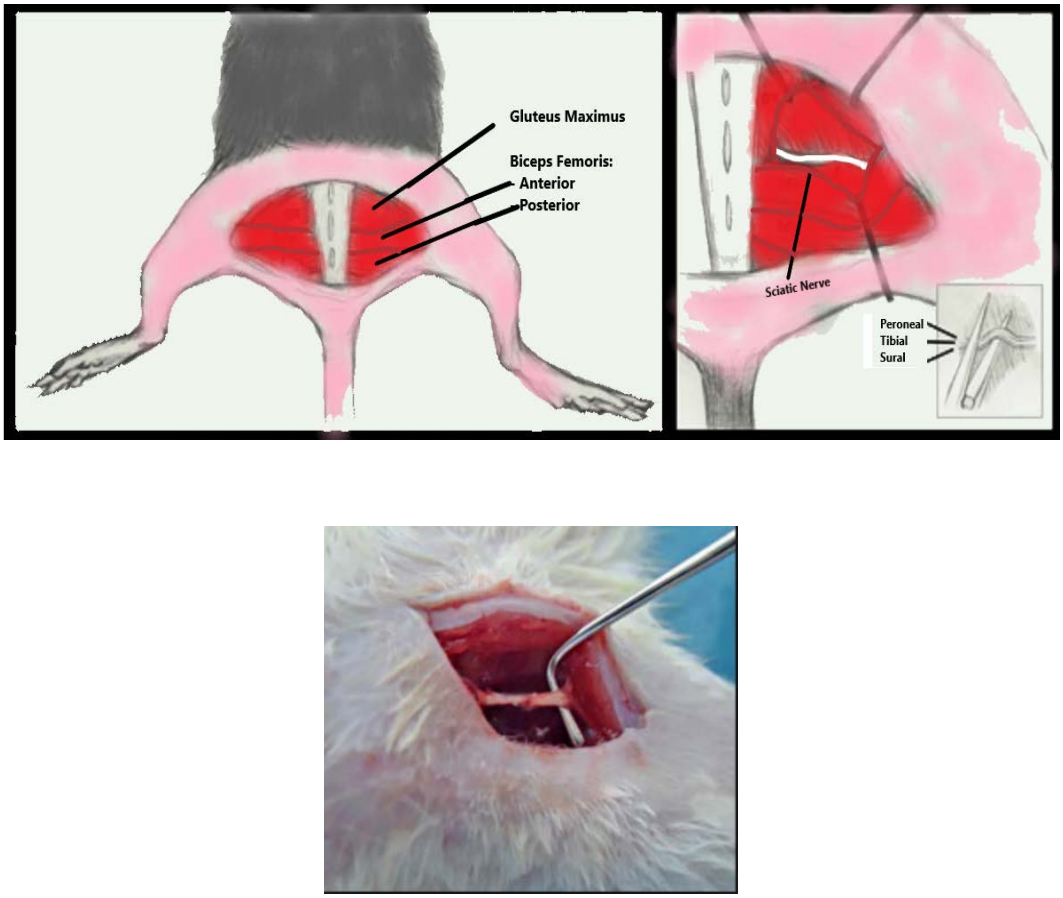


Fig 3. Surgical procedure of sciatic nerve injury model.

D. Training Protocol

- ***Treadmill Training:***

Treadmill training began in the first post-operative day, for five days per week over four weeks. Rats in group T (n=10) were subjected to one hour (two 30 min intervals separated by 10 min rest period) of continuous locomotion at a speed of 8m/min with no inclination (Seo *et al,* 2010). All rats were given 5 to 10 min treadmill pre-training once a day for two days prior to surgery. The treadmill incorporates a shock grid at the back to deliver a mild electric shock that forces the rats to run.

- ***Rotarod Training:***

Rotarod training began in the first post-operative day for five days per week for four weeks. Rats in group R (n=10) were subjected to one hour (two 30 min intervals separated by 10 min rest period) of continuous running on an accelerating rod at a speed of 35 rpm (8m/min) as described by Seo et al., (2010). All rats were pre-trained for 5 to 10 min once a day at accelerating speed (4–40 rpm) for two days prior to surgery. The rats were placed again on the rotarod if they fell off to complete their training period.

- ***Combination of Treadmill and Rotarod Training:***

Treadmill and Rotarod training began in the first post-operative day, for five days per week over four weeks. Rats in the RT group (n=10) were subjected to 30 minutes of treadmill raining at a speed of 8m/min, followed by 30 minutes of rotarod Training at a speed of 35 rpm (8m/min). All rats were pre-trained for 5 to 10 min once a day for two days prior to surgery.

E. Assessment of Sensory Recovery

A battery of behavioral tests was conducted to assess sensory recovery in rats subjected to different modality of exercises. Responses to thermal, mechanical and nociceptive stimulation were measured in the affected and intact hindlimb at 1, 5, 12, 19 and 26 days post-surgery.

- ***Thermal Stimulation***

Responses to thermal stimulation were assessed using the Hargreaves test (Hargreaves *et al*, 1988). Rats were placed in clear plastic chambers with a glass floor and allowed to acclimatize for 30 min prior to testing. The light emission of a projection lamp was focused from beneath the glass floor onto the plantar surface of the hind paw. Paw withdrawal latency was measured as the time it took the animal to withdraw his hindpaw from the heat stimulus. The latency was measured through a time-meter combined with infrared detectors. A baseline reading was obtained before starting the experiment. The test was repeated for at least 5 trials, separated by 5 min rest period, to obtain an average reaction time.

- ***Mechanical Stimulation***

To test for mechanical sensitivity, animals were placed into individual transparent plastic cubes with a wire-mesh floor. A mechanical stimulus using Von Frey hair was applied to the plantar surface of the hindpaw. Von Frey hair were inserted through the mesh to poke the animal's hindpaw. The exact force of the fiber is determined by its thickness, the 15g hair exerted a force of 147.10 mN. Normal reactions for rats include withdrawing or licking or shaking the paw. A baseline reading was obtained and the test was repeated for at least 5 trials, separated by 5 min rest period, to obtain an average reaction time. Paw withdrawal frequency recorded as the value for a test is the mean of three trials isolated by 5-minute rest

periods for both hind paws of every rat and these values were utilized for computing the percentage of the injured versus the intact hind paw.

- ***Pin-Prick Test***

For pin-prick test, the animals were also placed into individual transparent plastic cubes with a wire-mesh floor. A pricking stimulus using a needle was applied to the paw from the tip of the middle toe to the heel level until a withdrawal from the painful stimulus was elicited and recorded. Sensation level observed in this test was graded between 0 and 1, with 0 representing no sensation, 1 representing withdrawal response (Scharpf et al, 2003).

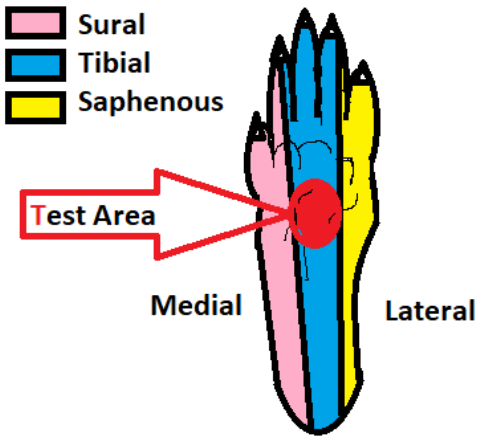


Fig. 4 Schematic representation of the thermal and mechanical stimulation sites in the rat's paw.

F. Assessment of Motor Response

- ***Ladder Climbing Test:***

Rats were prepared to ascend and descend a staircase runway. The assessment of locomotor execution of rats was done by counting the number of steps and time taken (in seconds) to climb up and down the runway. The performance of rats' hindlimbs and their ability to climb

up the stairs was noted and given a grade according to the following table (Anand et al, 2011):

0 points	No attempt to climb up the platform with its operated hindlimb
2 points	Mild attempt to climb up the platform with its operated hindlimb
4 points	Weak attempt to climb up the platform with slips and significant change in time
6 points	Good attempt to climb up the platform with slips and significant change in time
8 points	Good attempt to climb up the platform without any slips with significant change in time
10 points	Good attempt to climb up the platform with the performance near to normal animals

G. Electromyographic Recordings

Twenty-six days post-surgery, rats were placed under the same general anaesthesia (ketamine, 100 mg/kg and xylazine, 10 mg/kg) used for the surgical procedure with body temperature kept consistent at 37°C. The crushed site and the proximal and distal stumps of the sciatic nerve were located by separating the reattached biceps femoris muscle from the gluteus muscle. The nerve was given a supramaximal stimulus through a bipolar staineless steel electrode placed directly on the sciatic nerve trunk, 5-mm proximal to the crushed site. Evoked compound muscle action potential (CMAPs) were recorded from the gastrocnemius muscle utilizing microneedle electrode, while placing the reference electrode in the Achilles tendon. Latency, amplitude, and area of the evoked MAPs were recorded from the

gastrocnemius muscle. Before recording, and to ensure appropriate placement of the electrodes proximal to the sciatic nerve, current was passed through the recording electrode and muscle response was noted and observed. The signal from the recording electrode was fed into a differential amplifier, filtered, and monitored on an oscilloscope (Tektroniks Instruments). The analog signal was sampled in a 1401 data interface (CED, Cambridge, UK) and analyzed using Spike 2 software.

The latency was measured from stimulus to the takeoff of the first negative deflection. The amplitude and the area under the MAP curve from the baseline to the maximal negative peak were calculated. The MAP was then used to calculate the NCV, which was carried out by placing the recording electrodes in the gastrocnemius muscles and stimulating the sciatic nerve proximally and distally to the crush site. The NCV was then calculated by dividing the distance between the stimulating sites by the difference in latency time.

H. Statistical Analysis:

For the statistical analysis of behavioral and electrophysiological measurements of regenerated nerves, data were expressed as mean ± SEM. Comparisons between groups were made by the one-way analysis of variance using Sigma Plot v.14 software. The Tukey test was then used as a post hoc test for a multiple comparison. A p value of < 0.05 is considered statistically significant.

CHAPTER 3

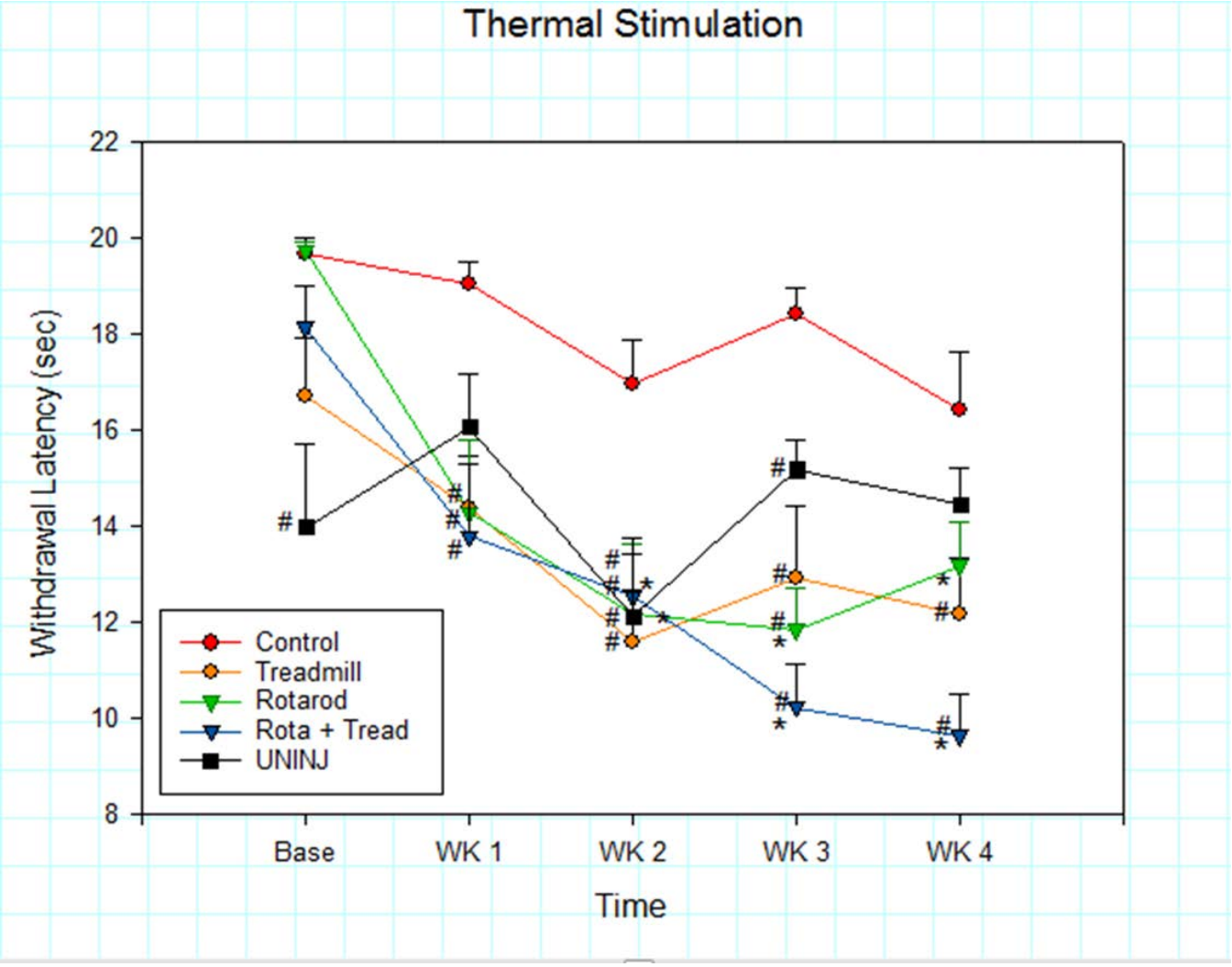
RESULTS

A. Intensive Motor Training Can Restore Thermal Sensory Loss Associated with Sciatic Nerve Compression in Rats:

Withdrawal responses to heat stimulation were assessed using the plantar test. They had a latency of 12s to 16s in the uninjured rats. In rats with peripheral nerve injury, there was no significant difference between the control (C group) and treadmill group (T group) throughout the treatment duration. However, in the combined group of rotarod and treadmill (RT group) and rotarod group (R group) alone there was a significant difference ($p<0.05$) starting at week 2 and throughout the treatment duration compared to the baseline where the values of the withdrawal latency for the RT was 9.623± 2.409 ms and for R 12.151±1.461 ms. On the other hand, the withdrawal latency was significantly reduced (<0.05) in the RT, R and T groups at week 1 when compared to the control group. Moreover, this difference remains the same in week 2 (12.531±3.428 ms, 12.152±4.132 ms, 11.573±3.201 ms respectively) and week 3 (10.220±2.508 ms, 11.838±2.481 ms, 12.899±4.281 ms, respectively). At week 4, although there was no significant difference between R and C groups it could still be observed that the R group had a shorter latency (13.170±2.631 ms). The T and RT groups showed better response at early time (week 1) and throughout the treatment duration as a significant difference was noted in week 4 compared to the control group (16.389±3.204 ms) with withdrawal latency of (12.152±3.383 ms) for T group and (9.632±2.409 ms) for RT group. The lack of withdrawal response is owing to the absence of sensation to the stimuli, since proximal muscles in the leg are typically innervated and permit withdrawal of the paw when the stimuli is applied to an innervated region such as the medial aspect of the hind paw innervated by the saphenous nerve which was intact (Casals-Díaz et

al., 2009). When comparing the RT group with the uninjured group, our results demonstrated a significant difference ($p<0.05$) indicating better performance than the uninjured group with a withdrawal latency of (9.632±2.409 ms) for RT group, and (14.445±2.210 ms) for the uninjured group. Thus, the combination of treadmill and rotarod showed better reinnervation at early times than single treatments and controls.

Fig.5 Time courses of thermal withdrawal latency in C, T, R, RT, and UNINJ rats, where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. The thermal withdrawal latency (sec) to heat stimulation were not significantly different between the C and uninjured group. Data are presented as mean ± SEM for 10 rats per group. The asterisk indicates P < 0.05 when the groups were compared within timeline; the pound symbol indicates P < 0.05 when the trained groups were compared with the control group (1-way analysis of variance of repeated measures using sigma plot).



B. Intensive Motor Training Can Improve Mechanical Sensory Loss Associated with Sciatic Nerve Compression in Rats:

Withdrawal reactions to mechanical stimulation in the rat's paws were found at 15g. When comparing the different groups throughout the treatment duration, there were no withdrawal reactions to mechanical stimulation in the injured paws until the second week of treatment in

the RT group as a significant difference ($p<0.05$) was observed with a value of (2 ±0.333). Other treatment groups including the control, treadmill group alone and rotarod group alone, showed a significant difference ($p<0.05$) at the third week of treatment with withdrawal response se of (1.208±0.198, 1.833±0.345, 2.238±0.376, respectively). On the other hand, even though there was no significant statistical difference between the trained groups including T group (1.833±0.345/ 1.625±0.369), R group (2.238±0.376/ 1.238±0.382), RT group (1.452±0.264/ 1.761±0.314) and the control group (1.208±0.198/ 0.958±0.247) on week 3 and 4, the values obtained were much more closer to the uninjured group (1.8333±0.319/1.833±0.319). The withdrawal reactions in the nociceptive tests were decreasing slightly throughout the treatment duration, demonstrating dynamic reinnervation of the skin, yet without noteworthy difference between the different groups at later times. Furthermore, there was no evidence of detectable hyperalgesia reactions (withdrawal limits lower than normal) were found in any of the treatment groups. Thus, changes in the withdrawal latencies with time in individual groups may reflect states of transient hyperalgesia in some animals, which has been reported during reinnervation after crush nerve injuries (Vogelaar et al., 2004; Casals-Díaz et al., 2009).

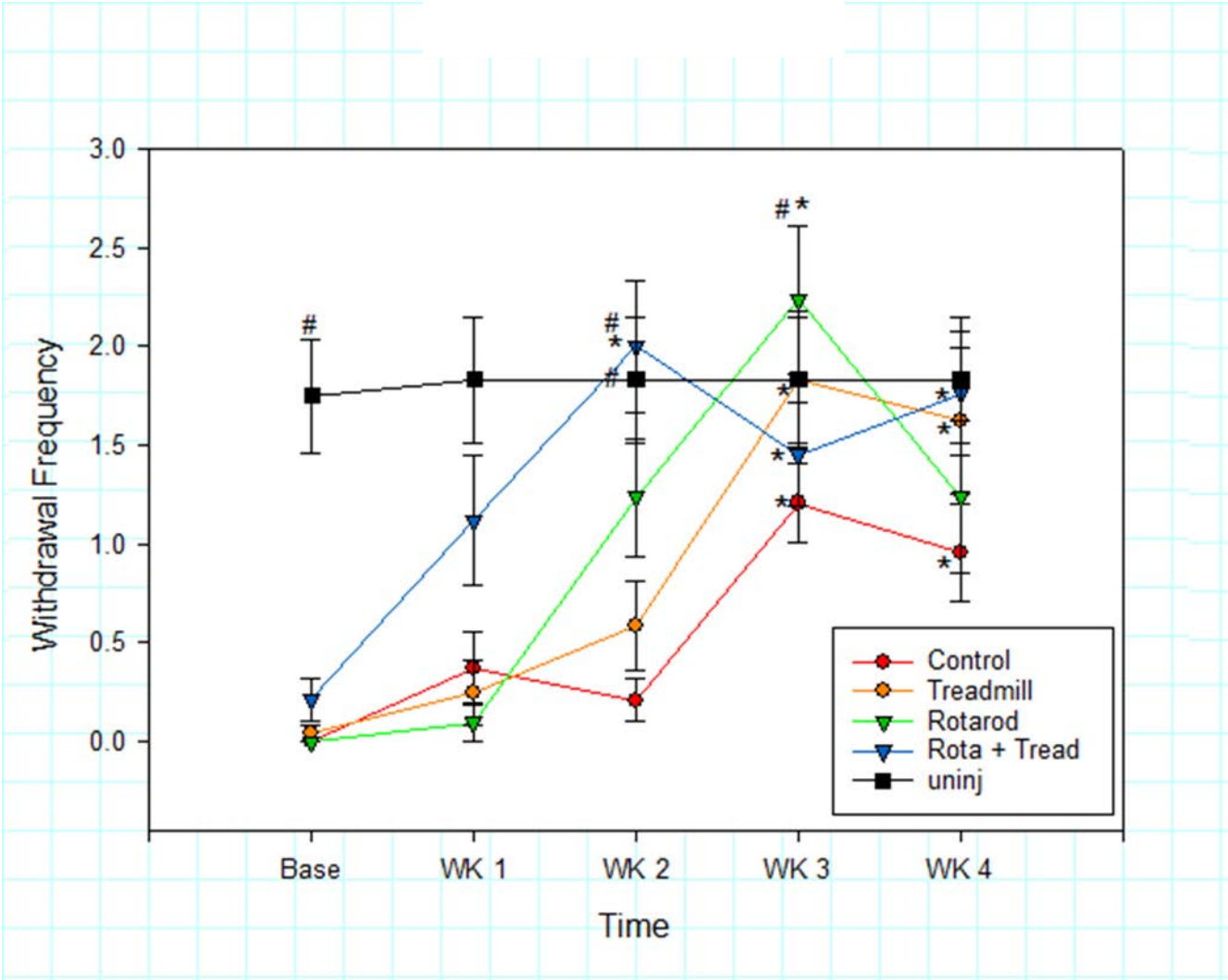
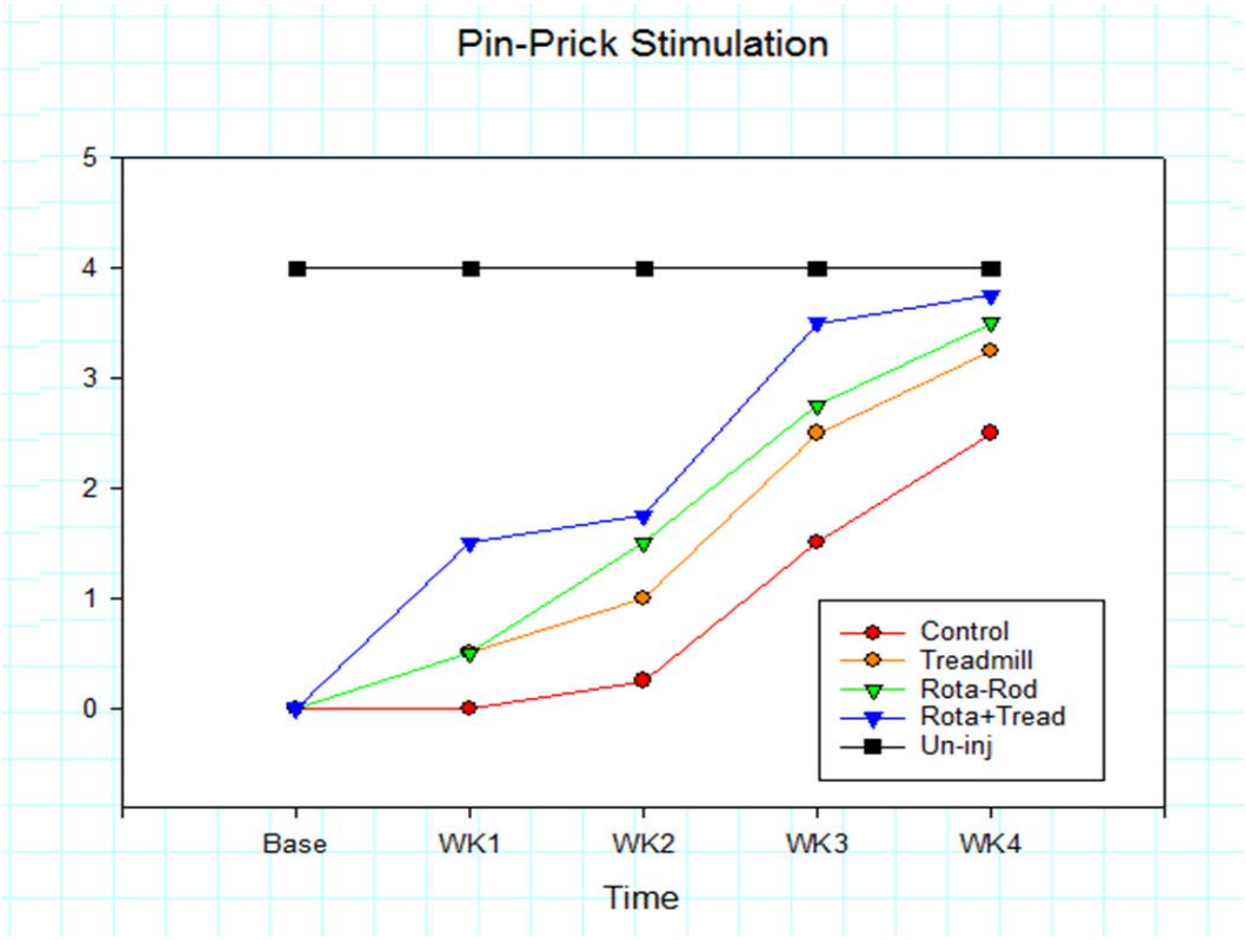


Fig.6 Time courses of mechanical withdrawal latency in control, T, R, RT, and uninjured rats, where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. The mechanical withdrawal latency (g) to mechanical stimulation were not significantly different between the C, trained and uninjured groups but the values of the trained groups were much closer to the uninjured group. Data are presented as mean ± SEM for 10 rats per group. The asterisk indicates P < 0.05 when the groups were compared within timeline; the pound symbol indicates P < 0.05 when the trained groups were compared with the control group (1-way analysis of variance of repeated measures using sigma plot).

C. Intensive Motor Training Can Improve Pinprick Sensory Loss Associated with Sciatic Nerve Compression in Rats:

The pinprick test was done because it is useful to assess progression of skin reinnervation with time, and to map the reinnervated territory. The score to pinprick was constructed by addition of the observed response in each of the four areas tested, from the ankle to the tip of the second digit. In the injured paws there were no withdrawal responses to the painful stimuli at baseline. Withdrawal responses increased progressively in all injured groups throughout the treatment duration. The final level of reinnervation was significantly higher in groups T (3.25), R (3.5) and RT (3.75) in comparison with groups C (2.5). Finally, the group of rats subjected to combination of treadmill and rotarod training had a significantly higher sensitivity to pinprick on all paw areas tested and at the different time intervals.



D. Intensive Motor Training Can Restore Motor Functions Following the Sciatic Nerve Compression in Rats:

Several behavioural tests to evaluate motor recovery following the injury were done. The ladder-climbing test was used to evaluate the sensorimotor capacities to correctly grip the rung while rats climbed up an inclined ladder. The number of grips and duration of stay on the ladder were calculated. All rats with sciatic nerve crush injury showed weak and delayed attempt in the climbing behaviour at baseline, week 1 and week 2. The performance of the injured animals had improved progressively in this test starting from week 3, in which significant difference ($p<0.05$) was noted between the control group (3.556±0.502) and all treatment groups including T (6±0.236), R (5.733±0.641), RT (5.933±0.279). The same behaviour was observed in week 4 in which the value of the control group was (4.889±0.502) while the values of the treated groups was (6.333±0.236) for group T, (5.933±0.435) for group R and (6.267±0.279) for the combined group RT indicating better motor recovery following peripheral nerve injury.

On the other hand, when comparing each treatment group throughout the treatment duration, the treadmill group alone and the rotarod group alone showed a significant difference ($P<0.05$) in the first three weeks of treatment T (6± 0.236) and R (5.733±0.641). this difference remained the same in the 4^{th} week with a value of (6.333±0.236) for the treadmill group and (5.933±0.435) for the rotarod group. However, the performance of combined treatment of treadmill and rotarod improved progressively starting from week 3 (5.933±0,124) and attained its maximum value at the ned of week 4 with a value of (6.266±0.124) were a significant difference ($p<0.05$) with respect to the baseline was observed.

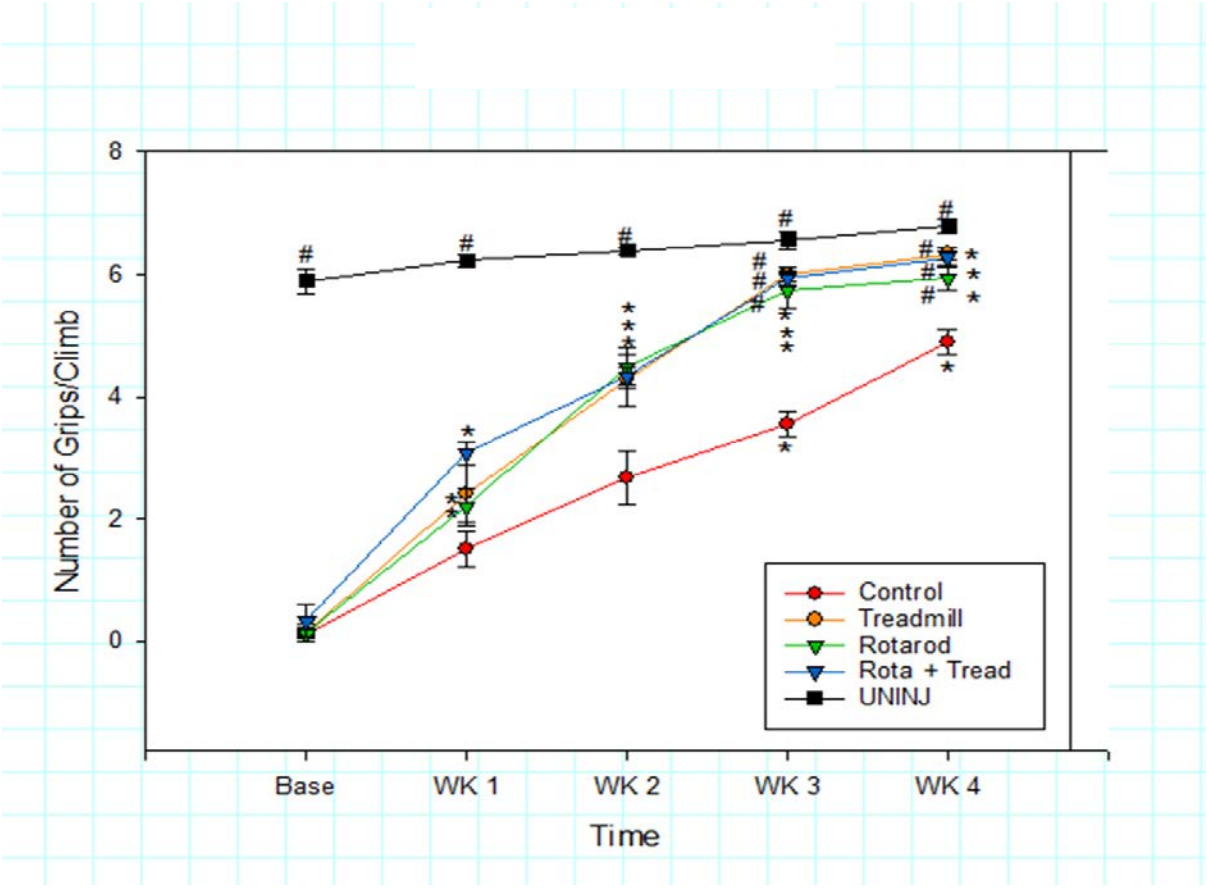


Fig.8 Time course of the recovery of locomotor function of sciatic nerve crushed rats following different exercise regimen where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. Functional recovery following sciatic crush injury is correlated to the improved number of grips using the ladder stair-climbing test. Data are presented as mean ± SEM for 6 rats per group. The asterisk indicates P < 0.05 when the groups were compared within timeline; the pound symbol indicates P < 0.05 when the trained groups were compared with the control group (1-way analysis of variance of repeated measures using sigma plot).

The second parameter that was used to assess locomotor behaviour is by recording the time taken (in sec) to climb up and down the runway. When comparing each group alone throughout the treatment duration, the control group and rotarod group alone showed a significant difference ($p<0.05$) starting from week 3 and 4 with a value of (11.611±1.756/12.056±1.482) for the control and (9.533 ±0.853/8.667±0.567) for the rotarod group respectively. However, the treadmill trained group alone and the combined treadmill and rotarod group showed a significant difference ($p<0.05$) starting from week 1 with a value

of (11.6±0.548) for the T group and (12.933±1.673) for RT group. This difference was sustained throw-out the treatment duration at weeks 2, 3 and 4 with values of (11.2±1.804/ 10.067±0.494/ 7.6±0.983 respectively) for treadmill group and (10.667±0.527/ 9±0.972/ 7.733±0.894 respectively) for RT group. Furthermore, when comparing the trained groups with the control and each other, there was a significant difference ($p<0.05$) between the control (at week 4:12.055±0.5060) and uninjured group (at week 4:7.222±0.329) throughout the treatment duration. However, when comparing the treated groups with the control, only the combined treatment of treadmill and rotarod showed a significant difference ($p<0.05$) starting from week 3 (9.00±0.972). The only significant difference ($p<0.05$) for the other groups (treadmill alone and rotarod alone) was observed on week 4 with a value of (7.600±0.983) for the treadmill group and (8.667±1.269) for the rotarod group indicating better and faster locomotor behavioural performance.

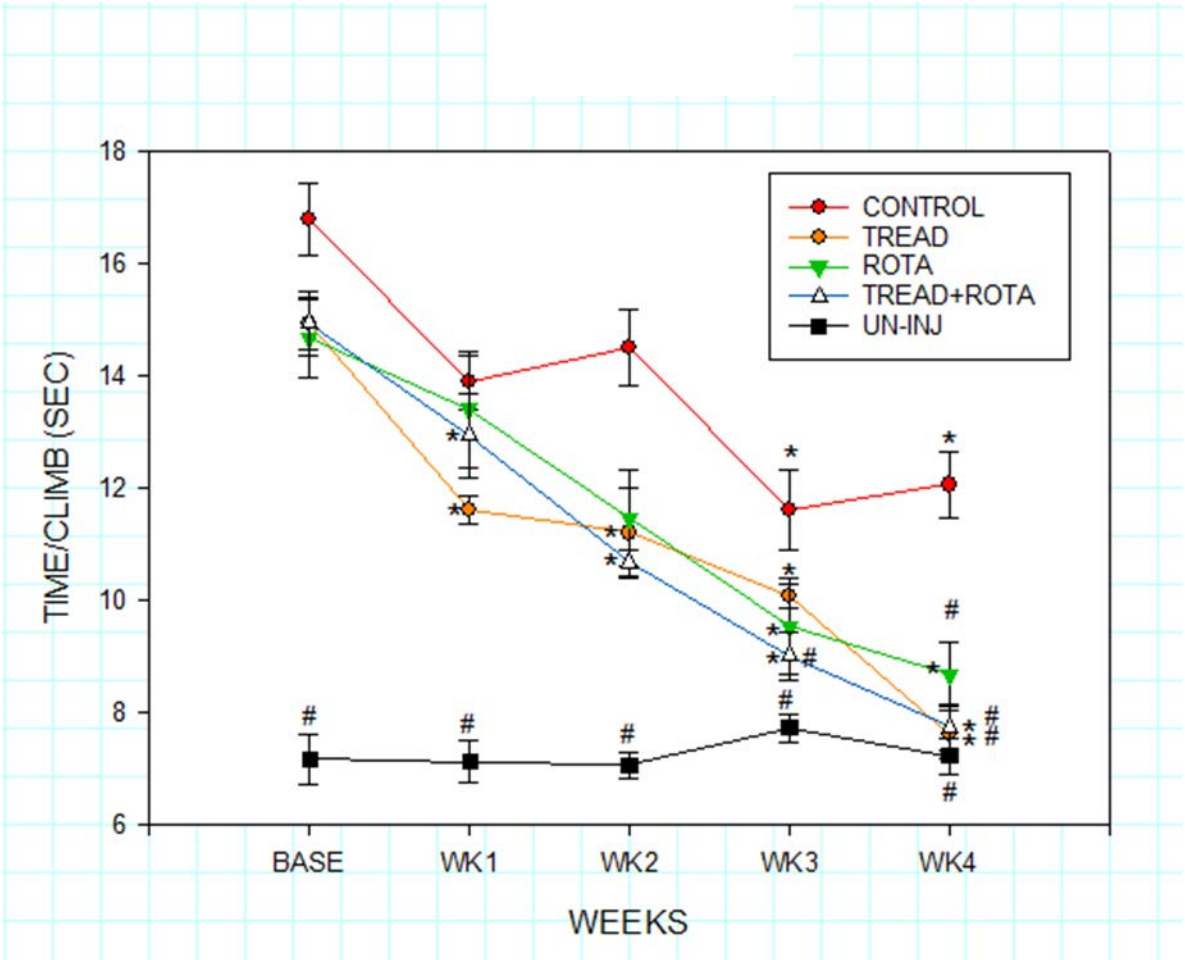


Fig.9 Illustrated the locomotor behaviour of rats by recording the time taken (in sec) to climb up and down the runway of all groups, where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. Functional recovery following sciatic crush injury is correlated with a decreased duration for climbing up and down the runway. Data are presented as mean ± SEM for 6 rats per group. The asterisk indicates P < 0.05 when the groups were compared within timeline; the pound symbol indicates P < 0.05 when the trained groups were compared with the control group (1-way analysis of variance of repeated measures using sigma plot).

The locomotor behaviour of the rats was assessed with a motor scale based on their behaviour during climbing the ladder. In this test the locomotor functions of the rats with sciatic nerve injury were difficult to quantify accurately. All injured rats showed a mild movement in the injured limb by the end of first postoperative week (2 points). The improved performance of treadmill group alone started from week 2 were a significant difference ($p<0.05$) was noted (3.600±0.894) and this difference was sustained throughout the treatment duration (week 3:5.200±1.095/week 4:7.20±1.095). Moreover, the control group, rotarod group and combined group of treadmill and rotarod showed a significant difference ($p<0.05$) starting at week 3 and week 4 (3.667±0.333/5.333±0.421; 5.200±0.489/7.200±0.489; 6.800±0.489/7.600±0.40 respectively). On the other hand, when comparing the trained groups with the control group only (week 3: 3.667±0.333; week 4: 5.333±0.421), the combined group of treadmill and rotarod showed a significant difference ($p<0.05$) starting from week 3 (6.800±0.489) and thus this difference was further improved at week 4 (7.600±0.400). There was no significant difference in the other trained groups, including R group (5.200±0.489/7.200±0.489), T group (5.200±0.489/7.200±0.489), although the values obtained are much higher than the control group (3.667±0.333/5.333±0.421). Thus, by the fourth postoperative week, walking and climbing pattern of operated animals subjected to combined training of treadmill and rotarod quickly improved and recovered to near normal levels.

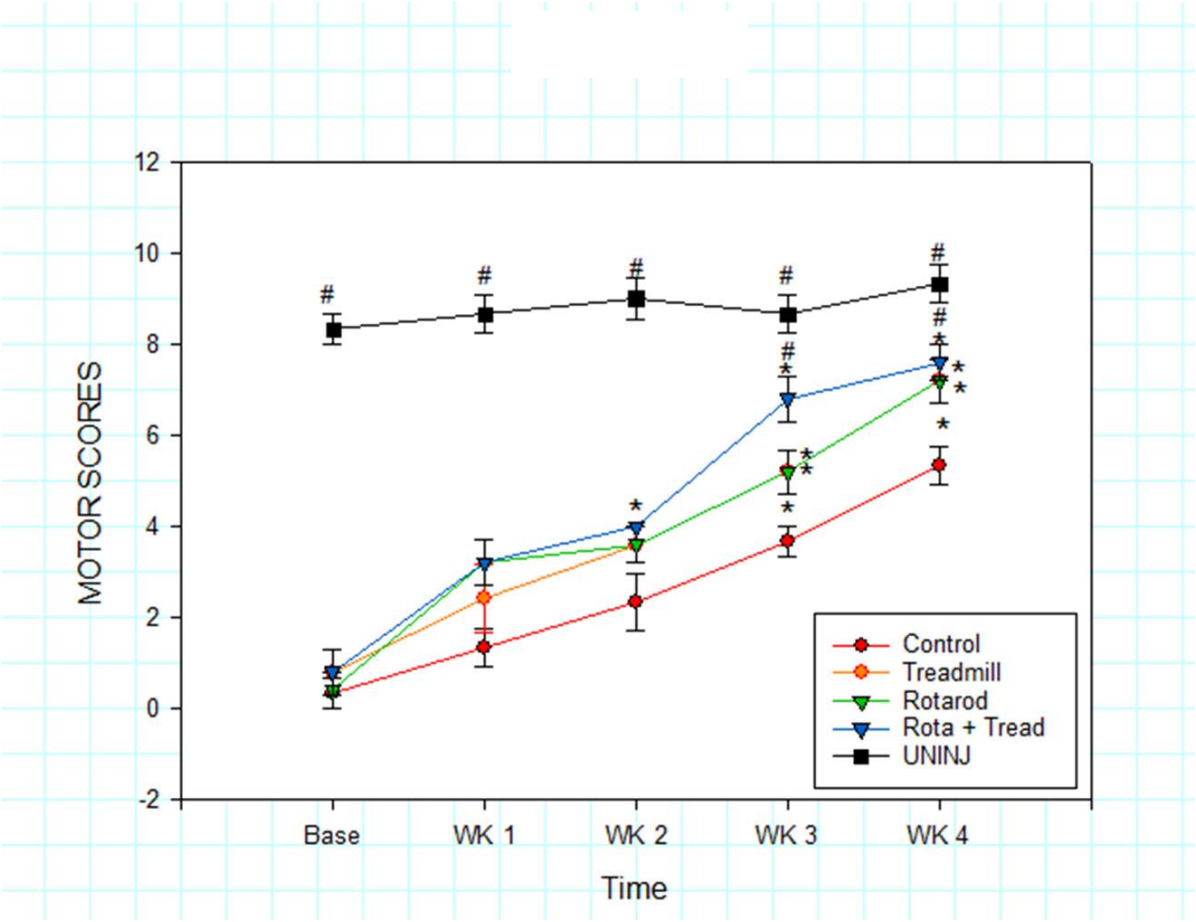


Fig.10 The value of motor score scale in control, T, R, RT, and uninjured rats, where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. Functional recovery following sciatic crush injury is correlated with the improved Motor Scale. Data are presented as mean ± SEM for 6 rats per group. The asterisk indicates P < 0.05 when the groups were compared within timeline; the pound symbol indicates P < 0.05 when the trained groups were compared with the control group (1-way analysis of variance of repeated measures using sigma plot).

Table 1. This table summarizes the significant behavioural changes observed in the three trained groups as compared to the untrained control in weeks 3 and 4 after injury.

	Treadmill	Rotarod	Treadmill + Rotarod	Treadmill	Rotarod	Treadmill + Rotarod
	Week 3			Week 4		
Thermal Stimulation	*	*	*	*		*
Mechanical Stimulation	–	–	–	–	–	–
Pinprick	*	*	*	*	*	*
Number of Grips	*	*	*	*	*	*
Duration of Climbing			*	*	*	*
Motor Scale			*			*

(*): Significantly different ($P<0.05$).

(-): No significant difference ($P>0.05$).

E. Intensive Motor Training Reinstate the Compound Muscle Action Potential of Injured Sciatic Nerves in Rats:

Four weeks after sciatic nerve compression, the electrophysiological studies were performed to investigate the motor functional recovery. The CMAP in the injured side of each group was measured and compared to control value. The CMAP peak amplitude, CMAP latency, and nerve conduction velocity values were calculated. The three bar graphs show the latency in milliseconds, the amplitude in millivolts and duration in millisecond of the Compound motor action potential recorded from the sciatic nerves of rats subjected to different training regimen. There was no significant difference in latency of CMAP between trained groups (T group alone and R group alone) and control groups ($P > 0.05$), whereas the amplitude of

CMAP of the right hindlimb was significantly greater in the trained groups (T group alone and R group alone) than in the control group ($P < 0.01$). Moreover, when comparing the duration of CMAP recorded, there was no significant difference in latency of CMAP between trained groups (T group alone and R group alone) and control groups ($P > 0.05$). However, sciatic nerves of rats trained with a combination of treadmill and rotarod presented a significant statistical difference ($p<0.05$) with a minor latency, higher amplitude and lower duration as compared to those of rats subjected to one type of exercise indicating a better functional neurological recovery.

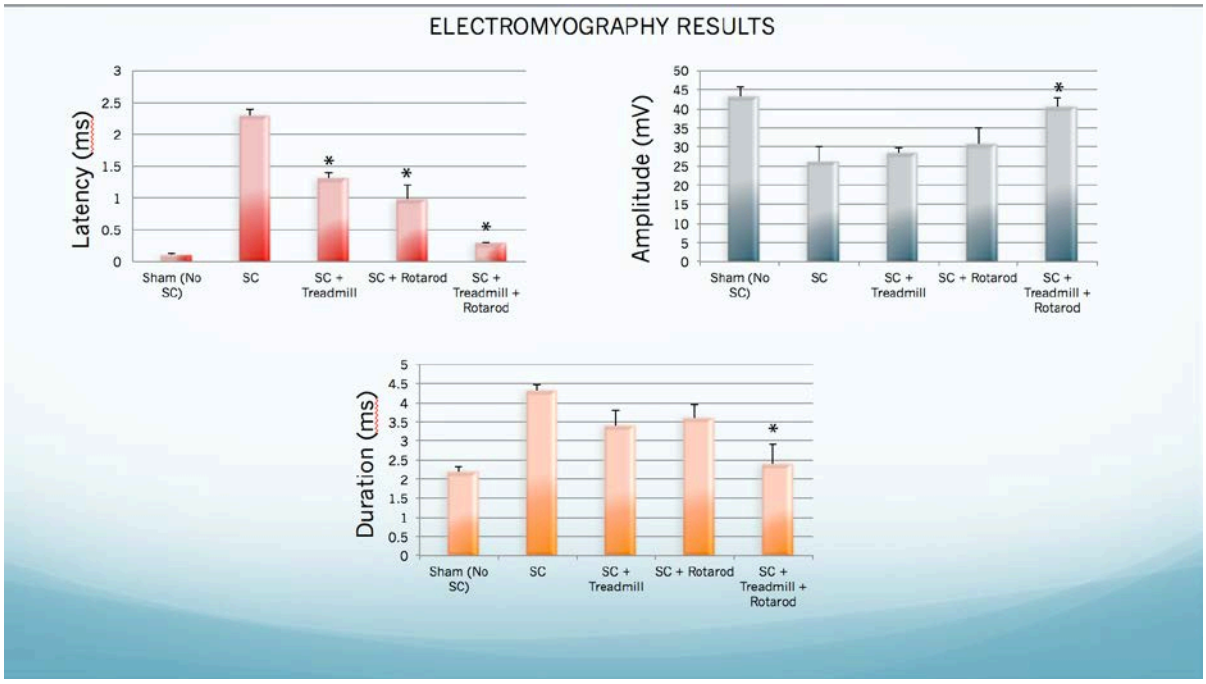


Fig.11 The three bar graphs show the latency in milliseconds, the amplitude in millivolts and duration in millisecond of the Compound motor action potential recorded from the sciatic nerves of rats subjected to different training regimen, where C: Control group; T: Treadmill Trained group; R; Rotarod trained group; RT Rotarod and Treadmill trained group. Sciatic nerves of rats trained with a combination of treadmill and rotarod had decreased latency, higher amplitude and lower duration as compared to those of rats subjected to the untrained control indicating a better functional neurological recovery. Data are presented as mean ± SEM for 3 rats per group.
 # indicates $P < 0.05$ when the trained groups were compared with the untrained control group (1-way analysis of variance of repeated measures using sigma plot).

CHAPTER 4

DISCUSSION

Injured axons in damaged peripheral nerves can considerably regenerate, yet the functional outcomes remain very disappointing and result in a long-term disability (Brushart, 1998; Frostick et al., 1998). Different factors have been identified and shown to contribute to failed recovery. These include misdirection of regenerated motor axons (Evans et al., 1991; de Ruiter et al., 2008), functional inadequate re-innervation of muscles (Fu and Gordon, 1995, 1997; Gordon, 2009) and plastic changes in the central nervous system (CNS) (Alvarez et al., 2010). Despite advances in technologies and our knowledge of the molecular and cellular basis of axonal injuries and repair, there is no therapy proven to treat peripheral nerve injury. Physical exercise, by means of voluntary or forced locomotion, is one of several possible strategies used to enhance peripheral nerve regeneration and improve target muscle re-innervation. Although therapeutic exercise remains a common practice in the rehabilitation of PNS lesions, only recently was it shown that physical exercise and activity-dependent interventions have real impact on neurobiological mechanisms of peripheral nerve regeneration. Exercise, tends to naturally activate sensory mechanoreceptors and motor fibres innervating skeletal muscles, induce the synthesis of both brain derived neurotrophic factor (BDNF) and its trkB receptor (Gomez-Pinilla et al., 2001; Hutchinson et al., 2004; Molteni et al., 2004; Ploughman et al., 2005), increase the level of NGF in sciatic nerve (Goular et al, 2014), and stimulate neurogenesis in the hippocampus while prompting recovery after CNS injury (Liu and Nusslock, 2018). However, the effects of intensive training using a combination of simple and complex exercises on enhancing axonal regeneration and

improving the sensory and motor functional outcomes in rats with sciatic nerve compression are less clear and poorly understood.

In the present study, we investigated the effects of simple and complex exercises on axonal regeneration in a rat sciatic nerve compression model. We observed enhanced regeneration using a variety of outcome measures when rats were exercised for about an hour 5 days/week for four weeks. Our results confirm previous reports and extend the beneficial effect of intensive exercise to another peripheral nerve injury model. We demonstrated the effects of three different training exercise regimen on functional behavioral improvement in four different tests that evaluated sensory and motor recovery, and on electrophysiological measurements that evaluated motor re-innervation of the gastrocnemius muscles. Of the three behavioral outcome measures, accelerated sensory recovery in rats subjected to combined training exercises was evident as they showed increased responses to thermal, mechanical and nociceptive stimulation as early as 1 week post injury. In addition, combined treadmill and rotarod training induced better myelination of the regenerated axons as indicated by reduced distal latency, decreased duration and increased amplitude of the evoked motor response. Nerve functions in the untrained group with sciatic compression were significantly different than those of the sham and the three trained groups confirming the loss of axons in the sciatic nerve. Moreover, the withdrawal latencies to thermal stimulation for the untrained group were significantly higher than the sham and trained groups, suggesting that subsets of sensory axons were damaged or interrupted by sciatic nerve compression. The pinprick test also revealed a significant decrease in sensation over the plantar surface of the paw supplied by the sciatic nerve in the untrained group suggesting loss of sensory activity in the sciatic nerve. In contrast, an improvement in sensory functions was clearly observed in the trained groups, but was more pronounced in rats subjected to the combination of treadmill and rotarod training. Taken together, our findings confirm the positive effect of exercise on

enhancing sensory functions post nerve injury. A number of studies have shown that several factors might influence the outcome measures following exercise such as the intensity and duration of training, the pattern and frequency of training, and the starting time of exercise (English et al., 2014). This has been demonstrated in a study by Van Meeteren et al (1998), in which treadmill running was shown to significantly delay the gradual return of motor function without any impact on sensory recovery. The negative effect in their study was attributed to stress and to the severity of the training. More studies have demonstrated harmful effects on axon regeneration and muscle damage after high intensity swimming or treadmill training (Herbison et al., 1974a, 1980b; Guttmann and Jakovlek, 1963; Van Meeteren et al., 1998), indicating that the intensity, mode and duration of exercise appear to play an important role in modifying neuronal responses to optimally enhance regeneration and achieve successful recovery.

In the present study, the impact of training on motor recovery was also evaluated using the climbing ladder test. Untrained rats with sciatic nerve compression exhibited impairment in motor functions as evident in the reduced number of grips/ climb and the longer time needed to climb the ladder. However, training with treadmill, rotarod or a combination of both has significantly improved the rats' motor functions, as they showed enhanced gripping of the rungs and faster climbing. The significant enhanced motor recovery was seen as early as 1 week post injury for rats trained with simple and complex exercises, while it took the untrained control group 3 weeks to show significant improvement; This is possibly due to the enhanced regeneration of axons that innervate the hindlimb muscles. These results are in agreement with other studies, which demonstrate that mild exercise stimulates axonal regeneration. The use of continuous low-intensity treadmill training for one hour per day or interval high-intensity training have showed improved recovered axons after nerve transection and repair in mice (Sabatier et al., 2008). Likewise, increased physical activity

has a significant effect on the sensory and motor recovery after crushed and transacted sciatic nerves in rodents (Van Meeteren et al., 1997; Marqueste et al., 2004). Furthermore, Seo et al. (2006) also demonstrated that motor training on a treadmill showed a positive effect on axon regeneration following sciatic nerve crush injury (Seo et al, 2006).

One-hour of daily treadmill, or rotarod training performed during the first four weeks after nerve crush injury, can to a certain extent, enhance growth of regenerating axons and improve sensory and motor recovery. The rate of recovery was faster in the first 2 weeks of training than the last two weeks possibly due to enhanced release of neurotrophins in the acute post-injury response. Although treadmill training alone or rotarod alone ameliorated sensory and motor activities following injury, their influence was more effective when combined to produce the desired outcome. This was consistent with the results of others (Al-Majed et al., 2000a, b; English et al., 2006, 2007, and 2009) showing the beneficial effect of training exercise on facilitating axonal regeneration following surgically transected or repaired nerves. The observed improvement in our study could be due to upregulation in BDNF and trkB in the recovering axons (Al-Majed et al., 2000a, b) and other neurotrophins in the CNS (English et al., 2006, 2007). Motor training tends to increase BDNF and its receptor in the brain (Berchtold et al., 2005) and spinal cord (Gomez-Pinilla et al., 2002, Zaheer et al., 2006; Macias et al., 2007). One study by Boyd and Gordon (2002) has demonstrated that the effect of BDNF on axon regeneration is dose dependent. They showed that large amounts of BDNF could exert an inhibitory effect on regenerating axons.

The enhancement in sensorimotor functions seen in our study could be due to the increase in the number of motor neurons recruited. Even though subtle differences were observed between treadmill training alone and rotarod training alone, the overall effect of motor training was improvement of motor responses. It is likely that forced exercise has led to

increased activation of affected motor neurons or recruitment of new ones causing stronger muscle contraction and resulting in enhanced gripping of ladder rung and improved climbing. Moreover, motor training could have induced changes in the metabolic properties of muscle fibres by increasing the oxidative potential of trained muscles in response to different exercise regimen (Saltin et al., 1977) or by increasing the blood flow to the injured areas as motor training had shown to increase neovascularization and the expression of angiogenesis related genes (Gustafsson et al, 1999).

Based on previous studies, the neuronal adaptations to training were observed to be at the alpha motor neurons level. These neurons demonstrate evidence of improved proteins synthesis, dendrite rebuilding, improved axon proteins transport and changes in electrophysiological properties (Gardiner et al, 2006) with increased voluntary or forced training. Adjustments in alpha-motoneurons with exercise training may include modifications in ion conductances, which incorporate changes in the expression of certain genes of the ion channels subunits. This probably explains the fast recovery of crushed axons in trained rats, particularly those submitted to combination of treadmill and rotarod training as evident in our behavioural and EMG data. With combined exercises (treadmill and rotarod), the EMG data obtained in our study indicate enhanced re-innervation of muscles as this was evident in the shorter latency, higher amplitude and lower duration of the CMAP recorded in these rats. This was consistent with other studies that reported improved CMAP as already reported in other studies (Van Meeteren et al., 1997; Marqueste et al., 2004; Sabatier et al., 2008). However, more research is required to build up the connections between exercise training, resting and dynamic motoneuron sensitivity, modulation of ions channels, and the impacts of neuromodulators.

Exercise can also enhance motor recovery through stimulation of sensory afferents (Molteni et al., 2004). Walking on a treadmill or a rotarod sends a barrage of sensory information from mechanoreceptors and proprioceptors to the spinal cord to modulate responses of motor neurons. This enhanced sensory activity might generate an increased released of glutamate, neuropeptides and neurotrophins from primary afferents driving and facilitating cellular proliferation in the spinal cord and promoting axonal regeneration. Manual sensory stimulation of the crushed facial nerves has been shown to enhance functional recovery in rodents, suggesting that stimulation of intact sensory afferents could improve motor recovery (Angelov et al., 2007). Additionally, recent studies have found that sensory stimulation by TENS exerts beneficial effect on motor recovery following a stroke, particularly when used in combination with active training. Taken together, our results support the argument that increased sensory stimulation induced by both treadmill and rotarod exercises may underlie the enhanced sensory and motor recovery observed post sciatic compression.

In conclusion, our findings provide significant new evidence substantiating a role for intensive training exercises in promoting successful axon regeneration in the peripheral nervous system following sciatic nerve compression in rats. The results have left us with so many assumptions and questions that we will attempt to answer in future studies. For example, the optimal parameters of each type of training have yet to be identified and tested to better understand the mechanisms underlying axonal regeneration. In addition, we would like to examine whether intensive training affects cellular proliferation and neurogenesis in the spinal cord, a mechanism that might account for the enhanced axonal regeneration. Through this project we hope to develop an innovative approach to exercise to maximize its impact on sensory and motor recovery in patients with traumatic nerve injury or neuropathies.

Our study has certain limitations, which have to be highlighted. A clear limitation is the nerve injury model used. The degree of individual variation among rats subjected to sciatic nerve compression affects the outcome of the experiments. The force of crush produced by the forceps can vary; however, this can be partially overcome by using surgical forceps integrated with force sensors, so a constant force could be applied for all nerves. Studying the mechanisms underlying motor recovery of the hindlimb of rats is somewhat problematic. Since rodents are quadrupedal and typically uses their forelimbs in locomotion, it is likely that motor skill acquisition with the forelimb influences sensorimotor function of the affected hindlimb. Finally, in the ladder-climbing test, there was a lack of high precision force sensor for determination of gripping strength.

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