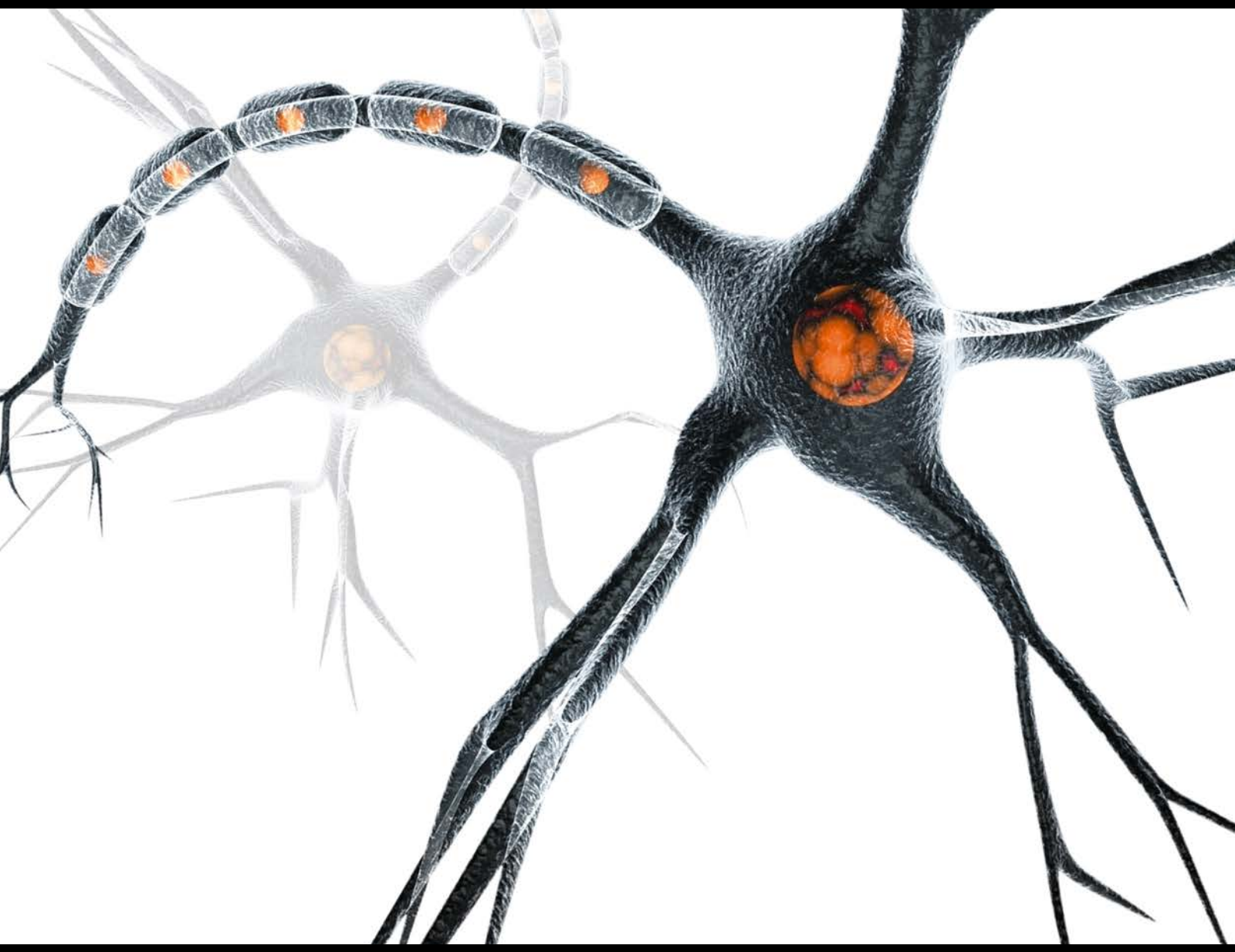
Neural Plasticity

Devising Novel Approaches in Neurorehabilitation: Lessons Learned from Motor Control and Motor Learning Studies

Guest Editors: Prithvi Shah, Alain Frigon, and Zaghloul Ahmed



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Editorial Board

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Research Article

Direct Current-Induced Calcium Trafficking in Different Neuronal Preparations

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The influence of direct current (DC) stimulation on radioactive calcium trafficking in sciatic nerve in vivo and in vitro, spinal cord, and synaptosomes was investigated. The exposure to DC enhanced calcium redistribution in all of these preparations. The effect was dependent on the strength of the stimulation and extended beyond the phase of exposure to DC. The DC-induced increase in calcium sequestration by synaptosomes was significantly reduced by cobalt and rupture of synaptosomes by osmotic shock. Although both anodal and cathodal currents were effective, the experiments with two electrodes of different areas revealed that cathodal stimulation exerted stronger effect. The exposure to DC induced not only relocation but also redistribution of calcium within segments of the sciatic nerve. Enzymatic removal of sialic acid by preincubation of synaptosomes with neuroaminidase, or carrying out the experiments in sodium-free environment, amplified DC-induced calcium accumulation.

1. Introduction

For the past twenty years, there has been a growing interest in noninvasive methods to stimulate the nervous system. One of them, rediscovered over fifteen years ago [1, 2], involves passing of the polarized, low-intensity current (1–3 mA) via electrodes located either on the scalp or in the proximity of the spinal cord. The effects occur relatively fast and often outlast the period of stimulation [3–6]. The consequences of DC stimulation are complex and seem to affect differently axonal [7] and synaptic components of the nervous system [4, 6, 8, 9]. The influence on the axon is presumably mediated by depolarization or hyperpolarization of the membrane [7, 8, 10]. Initial investigations of synaptic effects determined that hyperpolarization and depolarization increased and reduced the amount of neurotransmitter at neuromuscular junction, respectively [11, 12]. Recent research revealed that synaptic modulation exerted by DC is likely to occur via LTP- and LTD-like mechanisms implicated in synaptic plasticity [13–15]. While DC stimulation of the brain helps to ameliorate symptoms of psychological disorders [16–20], the exposure of the spinal cord to DC modulates spontaneous activity of the neurons [5, 8, 21] changing corticospinal interactions [5, 8, 21]. Those modulations are likely responsible for DC-induced improvement in the recovery after spinal cord injuries [5, 6, 9, 22–25]. It is well established that alteration of neuronal functions relies heavily on the spatially organized calcium signaling and changes in intracellular calcium concentration [26–28]. The release of neurotransmitters [29], neuronal migration [30], synaptic plasticity [31, 32], and organization of neuronal networks [33] are just a few specific examples of the processes which require very strict and precise control of calcium homeostasis and distribution within the neuron. Indeed, individual calcium channels are advantageously localized in the proximity of other signaling molecules (e.g., glutamate receptors, Ca^{2+} channels, and nitric oxide synthase), organized along the internodal axolemma under the myelin sheath in discrete "axonal nanocomplexes" [34]. Although overactivation of nanocomplexes during disease can lead to an excessive

increase in intracellular Ca^{2+} [35, 36], the influence of DC on these nanocomplexes and subsequent discreet elevation of intracellular calcium concentration could contribute to plasticity of neuronal networks, as observed in the CNS during induction of LTP and LTD [37]. Thus, one can assume that the influence of DC stimulation on CNS is at least partially mediated by modulation of the intracellular calcium concentration. Indeed, as reported by Ranieri and collaborators [14], the intensity of LTP was significantly changed by the exposure to DC. In subsequent, parallel experiments, Ahmed and Wieraszko [6] reported DC-induced modulation of the release of glutamate, a major neurotransmitter involved in induction and maintenance of LTP [38]. Conceding a strong influence of DC on neuronal activity in the brain [15, 39, 40] and spinal cord [5, 8, 9, 21], current investigation was focused on the influence of DC exposure on calcium trafficking in neuronal preparations in vivo and in vitro.

The changes in intracellular calcium concentration can be detected with either fluorescent probes [41, 42] genetically encoded calcium indicators [43] or radioactive tracer [44]. Most of the fluorescent probes enter the cell as hydrophobic esters and become charged in the cytoplasm. Therefore, their intracellular location and movement can be significantly altered by subsequent exposure to DC. Genetically encoded calcium indicators represent very promising but challenging method still under development [41, 42]. As confirmed by Islam and collaborators [44], the changes in calcium distribution in neuronal tissue can be estimated with radioactive calcium. However, their [44] radiographic quantitative data analysis is less reliable than determination of labelled calcium in the tissue prepared for qualitative scintillation counting. Additionally, the usage of autoradiography would be difficult for some of the preparations used and compared in our experiments. Therefore, as a method of choice in determination of the influence of DC exposure on the translocation of calcium in different preparations in vivo and in vitro, we used radioactive calcium. Its relocation from the incubation medium into the cellular compartment can be reliably and reproducibly followed both in vivo and in vitro.

2. Methods

2.1. In Vivo Experiments. We have used three different neuronal preparations to verify the hypothesis that exposure of neuronal tissue to DC stimulation modifies the concentration of intracellular calcium. As the first initial approach, we used the in vivo model system used by us previously [8]. Following exposure of the sciatic nerve in anesthetized mice, the petroleum jelly/silicone oil mixture was applied on the tissue to form a Ringer's solution-containing chamber with segment of the nerve inside of it (Figure 1(a), according to [8], modified). One rectangular electrode made of stainless steel (7 mm × 15 mm) was placed below the sciatic nerve which was insulated from the rest of the body by piece of rubber located underneath the electrode. The DC reference electrode was attached to the abdominal skin. The petroleum jelly/silicone oil mixture was used to create a second, smaller centrally located chamber on surface of stimulating plate (Figure 1(b)). The larger and smaller chambers were electrically insulated from each other with jelly/silicone oil mixture except for connection through the nerve. The segment of the sciatic nerve inside of smaller chamber and two segments of the sciatic nerve outside of the small chamber were termed as "inner" and "outer segments," respectively. For technical reasons only the pieces of the sciatic nerve from inner chamber and outer chamber distal to stimulating electrode were taken for subsequent analysis. The plate electrode was connected to either anodal or cathodal DC stimulator (ActivaDoseII, Iontophoresis delivery unit). $^{45}Ca^{2+}$ has been added to the central chamber to achieve the final concentration of 300 nM. Control experiments revealed that the seal was effective and there was practically no leak of radioactivity. Following stimulation period (0.8 mA, 20 min), the inner and outer segments of the sciatic nerve were dissected out, washed superficially in 100 ml of cold Ringer's solution, dried on the filter paper, weighted, measured in length, and homogenized in glass/glass homogenizer. The radioactivity of each homogenate was evaluated with the scintillation counter (Beckman Coulter LS 6500) and expressed in counts per minute per mm of the nerve (cpm/mm).

2.2. In Vitro Experiments

2.2.1. The In Vitro Experiments on Dissected Segments of Sciatic Nerve. As previously demonstrated by us, sciatic nerves maintained in vitro according to our procedure preserve evoked activity [5]. The nerve dissected out from anesthetized animal (2-3 cm) was preincubated at least one hour in Ringer's solution prior to experimental procedure to allow for sealing of cut, axonal ends [45]. The influence of DC stimulation on $^{45}Ca^{2+}$ accumulation and distribution within stimulated sciatic nerve was tested in a specially designed chamber (Figure 2), which was divided into two separate small pools with partition made of jelly/silicone oil mixture used for in vivo experiments as well. The only electrical connection between these two chambers was possible by the tissue of sciatic nerve which extended from one chamber to the other penetrating through insulating barrier (Figure 2(a)). Each pool had an electrode (10 × 6 mm) at the bottom and these electrodes were connected to either anodal or cathodal currents generated by ActivaDoseII, Iontophoresis delivery unit. $^{45}Ca^{2+}$ ($^{45}CaCl_2$, PerkinElmer, approximately 300.0 nM in the chamber) was always added to the chamber termed "inside." Following DC stimulation (3′, 0.1 mA), the nerve was cut at the partition, and the amount of $^{45}Ca^{2+}$ was determined in the nerve as described above for in vivo experiments. The distance between two electrodes used for in vitro stimulation was much shorter than the same distance in in vivo experiments. Therefore, to make the results more comparable, the intensity of the current was adjusted accordingly. The amount of $^{45}Ca^{2+}$ in Ringers of the outside chamber was also evaluated.

2.2.2. Segments of the Spinal Cord. The segments of the spinal cord (2-3 cm long) have been dissected from anesthetized animals, attached to the wooden stick as in our previous experiments [6], and placed in the plastic tube containing

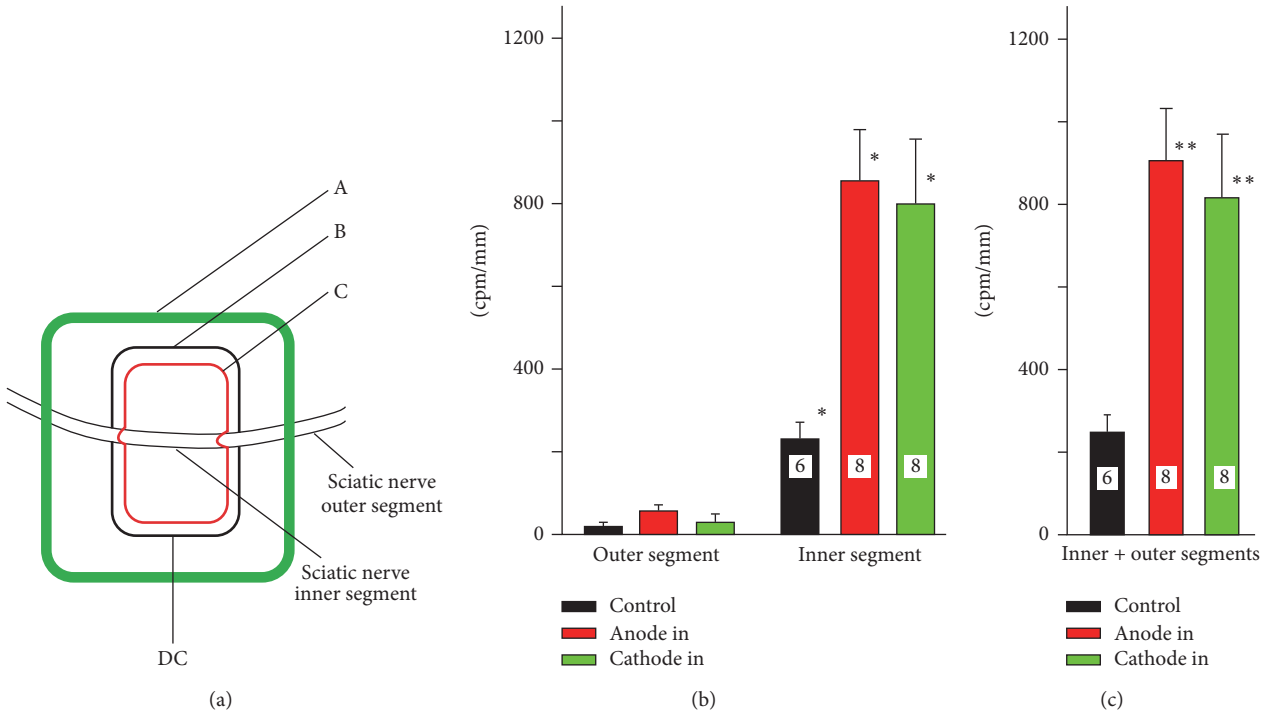

FIGURE 1: The accumulation of $^{45}Ca^{2+}$ by DC-stimulated sciatic nerve in vivo; (a) an experimental set up. The exposed segment of the sciatic nerve was surrounded by the barrier made of hydrophobic petroleum jelly/silicone oil mixture which formed the external pool (A). The second smaller (internal) pool (B) was made of the same petroleum jelly on the stimulation plate (C) inserted underneath the sciatic nerve. The stimulation plate (15 × 20 mm) was insulated from the tissue by the rectangular piece of the rubber located under the stimulation plate (omitted for the clarity from the picture). This experimental arrangement allowed keeping outer and inner segments of the sciatic nerve in different chambers which were electrically isolated from each other except for the connection made by the nerve itself. (b) $^{45}Ca^{2+}$ accumulated in the inner and outer segments (cpm/mm) in control experiments (no stimulation, black), and when either anode (red) or cathode (green) was connected to the stimulation plate ($^{*}p < 0.001$, one-way ANOVA followed by Dunn's test). (c) The total accumulation of $^{45}Ca^{2+}$ in inner and outer segments ($^{**}p < 0.001$, Mann–Whitney U Sum Rank test, control versus "anode in" and "cathode in" for combined segments). In this legend and all subsequent figure legends, the numbers inside of the bars indicate the number of separate experiments.

identical, stainless steel electrodes (30 × 7 mm; 210 mm^2 each, the distance between electrodes was 9 mm, Figure 3(a)).

2.2.3. Synaptosomes. Synaptosomes were obtained from cerebral cortex according to modified procedure described by Sawynok and collaborators [46]. The tissue has been homogenized in 0.23 M sucrose (1 : 10, tissue/sucrose ratio) in the teflon/glass homogenizer and centrifuged for 10 min at 5000 ×g. The pellet has been discarded and the supernatant was centrifuged for 20′ at 19000 ×g. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in Ringer's solution and used for the experiments. This procedure would yield suspension of synaptosomal vesicles of 0.5–0.6 μm in diameter. The synaptosomal fraction (1000 μl corresponding to 250 mg of wet tissue) was transferred to the plastic tube used for the experiments with the segments of the spinal cord (see above). The synaptosomes were stimulated after addition of $^{45}Ca^{2+}$ (final concentration was approximately 300 nM) for 3′ with DC varying in control experiments from 1 to 4 mA, while $^{45}Ca^{2+}$ was already present in the solution. In all subsequent experiments, synaptosomes were stimulated for 3′ with 3 mA current. In some experiments, brain synaptosomes were stimulated in the presence of 5 mM cobalt. In alternative sets of experiments, $^{45}Ca^{2+}$ was not present in the solution during the stimulation, but it was added 6 hrs after cessation of stimulation. The impact of modified composition of Ringer's solution by substituting NaCl with osmotically equivalent choline chloride was also tested. The influence of partial, enzymatic removal of sialic acid from the surface of brain synaptosomes on DC-induced $^{45}Ca^{2+}$ accumulation was assessed in separate set of experiments. The mixture of synaptosomes was incubated for 3 hrs at 33°C with neuroaminidase from Vibrio Cholerae (0.2 U/ml; Sigma). Then, the accumulation of $^{45}Ca^{2+}$ by control and DC-stimulated synaptosomes was evaluated. It has been of paramount importance to determine if the integrity of synaptosomes was compromised by DC stimulation. Therefore, the DC-induced $^{45}Ca^{2+}$ accumulation was compared between stimulated, control fraction of synaptosomes and fraction of synaptosomes exposed to osmotic shock. It is well known that synaptosomes burst in hypoosmotic environment transforming fraction of synaptosomes into suspension of membranes [47]. The synaptosomal suspension was transferred from isoosmotic Ringer's solution to water which would cause hypoosmotic destructions of synaptosomes. At the completion of all DC stimulation experiments, the synaptosomal

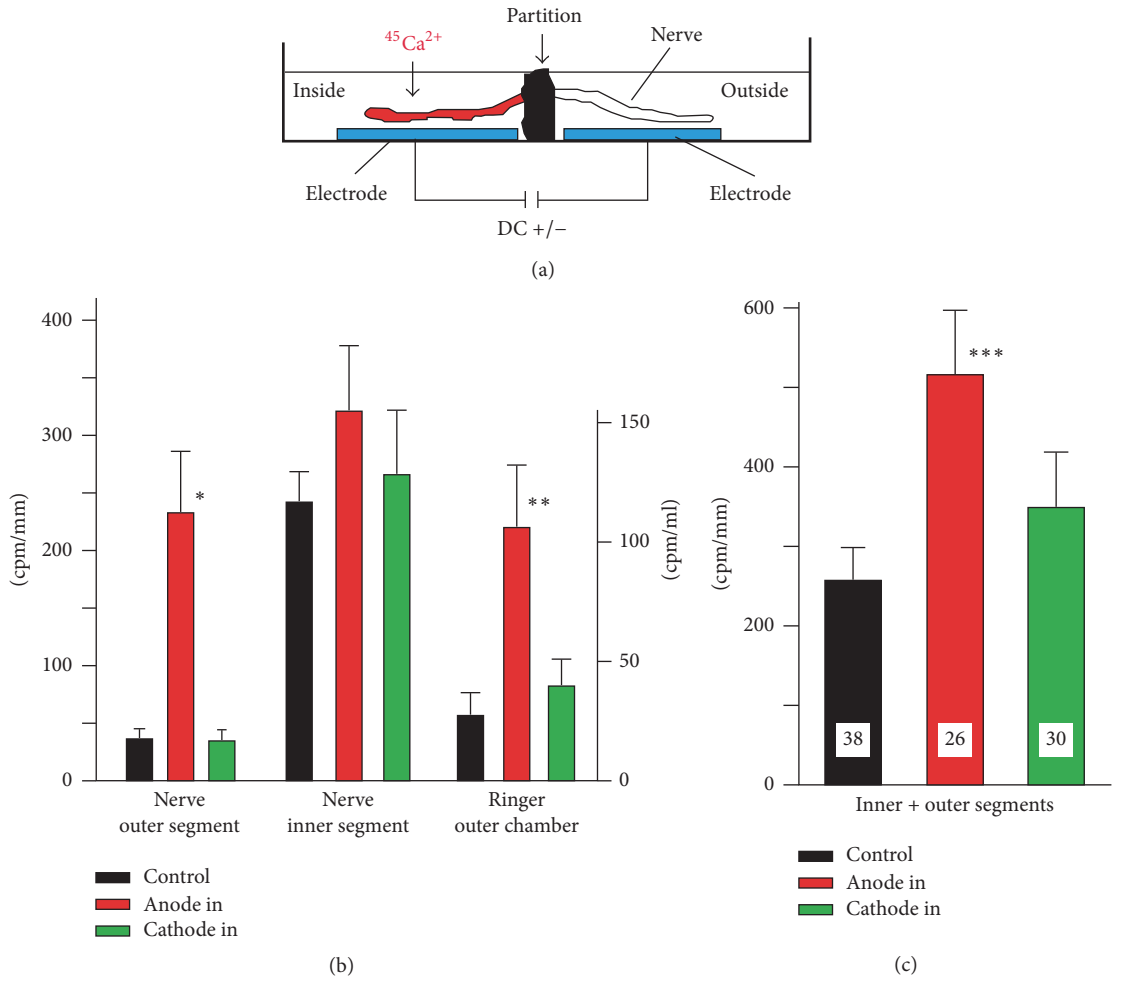

FIGURE 2: The accumulation of $^{45}Ca^{2+}$ by the DC-stimulated sciatic nerve in vitro. (a) An experimental setup. The Petri dish (3 cm in diameter) was divided into two parts with partition made of hydrophobic petroleum jelly/silicone oil mixture. There was a stainless steel electrode placed at the bottom of each part (6 × 10 mm). The segment of the sciatic nerve was extended through the partition. The only electrical connection between two parts of the Petri dish was through the sciatic nerve. The electrodes were connected to DC power supply delivering 0.1 mA for 3′. The reference electrode was much closer (approximately 8–10-fold) than in in vivo experiments. Therefore, to compensate for the distance and equalize experimental conditions, the intensity of stimulation for these experiments was adjusted accordingly. $^{45}Ca^{2+}$ was always added to the same part called "inner chamber." (b) $^{45}Ca^{2+}$ accumulation in the inner and outer segments exposed to either anodal or cathodal stimulation delivered to inner chamber. The anodal DC stimulation induced statistically significant increase in $^{45}Ca^{2+}$ accumulation in the outer segment ($^{*}p < 0.001$, Mann–Whitney U test) and in Ringer's solution collected from outer chamber ($^{**}p < 0.03$, Mann–Whitney U test). The anodal and cathodal stimulations induced a similar increase in the segment of the nerve located in the inner chamber, although those increases were not statistically significant. Note the increase in the concentration of $^{45}Ca^{2+}$ in the outer chamber (right side of Figure 1(b)). This diagram has two separate scales to express the amount of $^{45}Ca^{2+}$ in the nerve (cpm/mm, left scale) and the concentration of $^{45}Ca^{2+}$ in the Ringers of the outer chamber (cpm/ml, right scale). (c) The total accumulation of $^{45}Ca^{2+}$ in the inner and outer chambers; $^{***}p < 0.001$ as compared with control, and $p < 0.036$ as compared with "anode in"; Mann–Whitney U test.

suspension was filtered under vacuum (Whatman GF/B filters, soaked for 1 hr in 0.1% polyethylenimine solution before experiment), and the radioactivity remaining on the filters was counted in the scintillation vials after addition of scintillation fluid (Beckman Coulter LS 6500).

2.2.4. Stimulation with the Electrodes of Different Sizes. It is well documented that polarity of direct current is crucial for its effect [8, 48, 49]. Therefore, in separate experiments, the influence of anodal and cathodal stimulation on calcium accumulation by the spinal cord or synaptosomal suspension was tested in the tube with two electrodes of different sizes (Figure 6(a)). While one of the electrodes consisted of stainless steel plate (210.00 mm^2), the second electrode was made of stainless steel wire (11.75 mm^2). The distance between these two electrodes was 9 mm (Figure 6(a)). Following the addition of $^{45}Ca^{2+}$ (300 nM in the chamber) and stimulation (3 mA, 3′), the fraction of synaptosomal suspension or segments of the spinal cord were superficially washed and homogenized and the amount of radioactivity

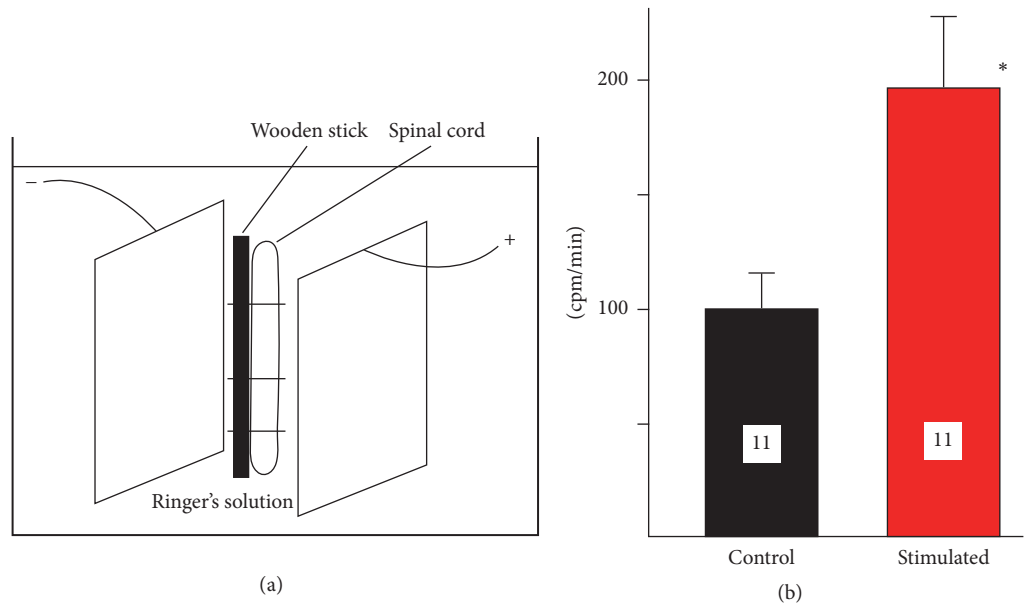

FIGURE 3: The $^{45}Ca^{2+}$ accumulation by the segments of the spinal cord stimulated in vitro by two electrodes of the same size. (a) An experimental setup. Two stainless steel plates (30 × 7 mm) were placed 9 mm apart inside of the plastic tube and connected to the source of DC. The segment of the spinal cord, attached to the wooden stick, was placed between two electrodes. (b) Accumulation of $^{45}Ca^{2+}$ in the segment of the spinal cord (in cpm/mm) following 3 mA, 3′ stimulation. The increase in stimulated spinal cord (190.9%) is statistically significant ($^{*}p < 0.029$, t-test).

was determined as described above. The type of test used for statistical analysis and calculated probability are illustrated in Table 1.

3. Results

3.1. Axonal, DC-Induced Accumulation of $^{45}Ca^{2+}$ In Vivo. In vivo experiments revealed that exposure to DC considerably enhanced accumulation of $^{45}Ca^{2+}$ by stimulated segment of the sciatic nerve located directly on the stimulation plate (inner segment, Figure 1). We used the term "anodal" or "cathodal stimulation" when the anode or cathode was connected to this plate, respectively. Anodal stimulation was more efficient in enhancing calcium accumulation. Over 90% of accumulated calcium could be recovered in the inner segment, although there was some accumulation in the outer segment as well, especially in the case of anodal stimulation. Since there was no leak of Ringer's solution between the inner and the outer chambers, the only source of $^{45}Ca^{2+}$ in the outer segment could be redistribution of $^{45}Ca^{2+}$ within the axon [28]. This relocation seemed to be more extensive in the case of anodal stimulation. The presence of $^{45}Ca^{2+}$ in the outer segments indicates that calcium, accumulated by the nerve under DC influence, was electrophoretically driven along the length of the axon further away from the site of the exposure towards anodal or cathodal end of the nerve. There was some negligible relocation of $^{45}Ca^{2+}$ from inner to outer chamber in control experiments (no stimulation) as well (Figure 1(b)). The difference between anodal and cathodal stimulation was statistically insignificant, although it became greater when the amounts of calcium accumulated by inner and outer segments were added together (Figure 1(c)). Anodal stimulation was significantly more efficient in stimulation of $^{45}Ca^{2+}$ accumulation than cathodal stimulation. Statistical analysis (one-way ANOVA followed by Dunn's test, $^{*}p < 0.001$) revealed that the difference between inner control segment versus "anode in" and "cathode in" was statistically significant at $p < 0.008$ and $p < 0.03$, respectively (Mann–Whitney U Rank Sum test). The difference between control and "anode in" and "cathode in" for combined segments (C) was also statistically significant ($^{**}p < 0.001$; Mann–Whitney U Sum Rank test). It should be emphasized that there were several factors which could influence recorded data. Some of those factors which were very difficult to control included fluctuation in the temperature of the exposed, dorsal part of the animal's body, the amount of the moisture in the vicinity of the plate, pulsation of the blood vessels, and movements due to breathing.

3.2. DC-Induced Accumulation of $^{45}Ca^{2+}$ by Axons In Vitro. The results of the subsequent in vitro experiments essentially reinforced the data obtained in vivo. Those experiments were designed differently (Figure 2(a)) and allowed for much more precise control of experimental conditions. Clearly, anodal stimulation was more effective in enhancing of $^{45}Ca^{2+}$ accumulation in the nerve segment ($^{*}p < 0.001$, Mann–Whitney U test, Figures 2(a) and 2(b)) and in the chamber ($p < 0.03$, Mann–Whitney U test, Figures 2(a) and 2(b)). The anodal current not only facilitated significant relocation of calcium ions within the axon but markedly increased the displacement of calcium ions from the nerve to the outer chamber (Figure 2(b)). Figure 2(c) depicts combined

Table 1: Neuronal preparations and statistical tests used to evaluate DC-induced calcium relocation.

Type of experiment	Statistical test	Probability
(i) Axon in vivo		
Anodal versus cathodal	One-way ANOVA followed by Dunn's test	$p < 0.001$
Anode in versus control in	Mann–Whitney *U* Rank Sum test	$p < 0.008$
Cathode in versus control in	Mann–Whitney *U* Rank Sum test	$p < 0.03$
Cathode versus anode (combined segments)	Mann–Whitney *U* Sum Rank test	$p < 0.001$
(ii) Axon in vitro		
Inner and outer chambers versus "anode in"	Mann–Whitney *U* Sum Rank test	$p < 0.036$
Inner and outer segments combined versus control	Mann–Whitney *U* Sum Rank test	$p < 0.001$
(iii) Spinal cord in vitro	*t*-test	$p < 0.029$
(iv) Synaptosomes		
^{45}Ca present during stim	ANOVA followed by Holm-Sidak	$p < 0.001$
^{45}Ca added 6 hrs after stim	*t*-test	$p < 0.003$
^{45}Ca in the presence of cobalt	*t*-test	$p < 0.001$
^{45}Ca/neuroaminidase/stim versus no neuroaminidase/stim	*t*-test	$p < 0.006$
Na^+ versus no Na^+, no stim	ANOVA followed by Dunn's test	$p < 0.001$
Na^+ versus no Na^+, stim.	*t*-test	$p < 0.029$
Osmotic shock	*t*-test	$p < 0.003$
Different electrodes	Mann–Whitney *U* Sum Rank test	$p < 0.001$

results obtained from inner and our chambers ($p < 0.036$, Mann–Whitney *U* test). The control, nonstimulated segments showed relatively high accumulation of $^{45}Ca^{2+}$ which might be the result of incomplete sealing of cut, exposed ends of the nerve [45].

3.3. DC-Induced Accumulation of $^{45}Ca^{2+}$ by Spinal Cord In Vitro. The tissue of isolated spinal cord is too delicate and fragile to be reliable and reproducibly placed in the chamber designed for sciatic nerve (Figure 2). Therefore, the spinal cord attached to the wooden stick for better stability (Figure 3(a)) was tested only for total accumulation of $^{45}Ca^{2+}$ during exposure to DC (3′, 3 mA). Like the sciatic nerve, the isolated segments of the spinal cord, located between two electrodes of the same size, accumulated more $^{45}Ca^{2+}$ when exposed to DC stimulation (190.9%, $^{*}p < 0.029$, *t*-test, Figure 3(b)). The effect was significant, although experimental design did not allow distinguishing between the influences of anodal and cathodal currents.

3.4. DC-Induced $^{45}Ca^{2+}$ Accumulation by Synaptosomes. In the pursuit of further characterization of the influence of DC stimulation on $^{45}Ca^{2+}$ trafficking, we used fraction of synaptosomes which can be prepared and tested in a very reproducible manner. Similarly as sciatic nerve and the spinal cord, synaptosomes also accumulated more $^{45}Ca^{2+}$ when exposed to DC. As depicted in Figure 4(a), increasing the intensity of the stimulating current induced proportional increase in $^{45}Ca^{2+}$ accumulation. Although 4 mA was the most effective within the tested range of intensities, 3 mA were employed in most of the other experiments. Synaptosomes damaged by osmotic shock and stimulated subsequently with DC accumulated over 500% less of $^{45}Ca^{2+}$ (533% ± 163%, $n = 3$, $p < 003$, *t*-test). Therefore, one can conclude that since 4 mA induced the highest calcium accumulation, there was no damage to synaptosomes by 3 mA current. The effect of DC stimulation was not limited to the duration of the stimulation but extended at least 6 hrs beyond the period of exposure. As depicted in Figure 4(b), DC-stimulated synaptosomes still accumulated 400% more calcium which was added 6 hrs after finishing the stimulation ($p < 0.003$, *t*-test, compared to nonstimulated controls tested after 6 hrs with experimental samples). Apparently, the accumulation of calcium occurred at least partially via calcium channels since it was significantly attenuated by 5 mM cobalt ($p < 0.001$, *t*-test, Figure 4(b)). Extracellular positively charged calcium ions are attracted by negatively charged molecules of sialic acid which is a major component of glycocalyx. Enzymatic partial removal of sialic acid with neuroaminidase significantly enhanced DC-induced calcium accumulation (Figure 5(a), $p < 0.006$, *t*-test).

3.5. The Influence of Na^+ and Size of the Electrodes on DC-Induced Calcium Trafficking. The change in the ionic environment of the preparation by omitting Na^+ ions also affected $^{45}Ca^{2+}$ accumulation. The osmolarity of the incubation solution remained the same since sodium ions were replaced with equivalent concentration of choline chloride. The synaptosomal suspension accumulated more $^{45}Ca^{2+}$ in the absence of sodium. The effect was relatively minor although statistically significant even without any electrical stimulation. However, application of DC to synaptosomes in sodium-free environment almost tripled the amount of accumulated $^{45}Ca^{2+}$ (Figure 5(b)). In the experiments described so far, each preparation was stimulated by two electrodes of identical size. In order to differentiate between the effects of anodal and cathodal stimulation, synaptosomes

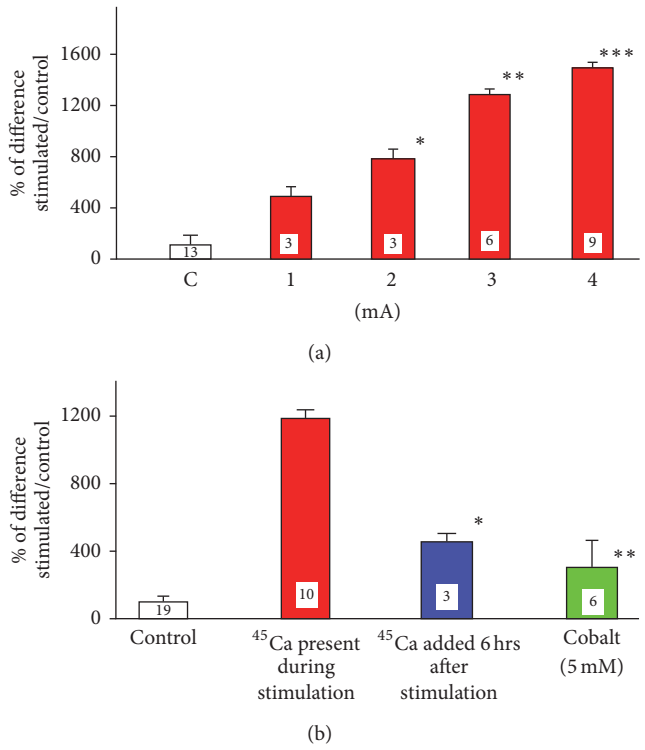

FIGURE 4: The influence of different experimental conditions on $^{45}Ca^{2+}$ accumulation by synaptosomes. (a) Accumulation of $^{45}Ca^{2+}$ by fractions of synaptosomes stimulated for 3 min by DC ranging from 1 to 4 mA. The results are presented as % of the difference between stimulated and nonstimulated preparations. ANOVA followed by Holm-Sidak, $p < 0.001$; $^{*}1$ versus 2 mA n.s.; $^{**}2$ versus 3 mA, $p < 0.001$; $^{***}3$ versus 4 mA, $p < 0.04$, t-test. (b) The accumulation of by stimulated synaptosomal suspension while $^{45}Ca^{2+}$ was either present in the tube during stimulation (red) or added to the tube 6 hrs after cessation of stimulation (blue); $^{*}p < 0.003$, t-test, as compared to nonstimulated control. The accumulation of $^{45}Ca^{2+}$ in the presence of 5 mM cobalt (green); $^{**}p < 0.001$, t-test, compared to synaptosomes stimulated in the presence of $^{45}Ca^{2+}$.

and segments of sciatic nerve were stimulated in vitro by exposure to DC generated by two electrodes of different sizes (Figure 6(a)). The connection of anode or cathode to the smaller electrode (wire) generated more intense anodal or cathodal current, respectively. The lower panel in Figure 6(a) illustrates the relative size of both electrodes and hypothetical lines and density of flowing current. Although both polarities enhanced accumulation of $^{45}Ca^{2+}$ by synaptosomes, cathodal stimulation was much more effective (Figure 6(b), $^{*}p < 0.001$, Mann–Whitney U test). The stimulation of the segment of sciatic nerve with two electrodes of different sizes yielded results very similar to stimulation of synaptosomal suspension (Figure 6(c)). The application of the cathodal current to the wire resulted in much greater concentration of radioactivity in the nerve ($^{*}p < 0.001$, Mann–Whitney U test) than anodal current. It has to be emphasized that, in the case of both of these preparations, namely, synaptosomes and sciatic nerve, the results represent sole accumulation of $^{45}Ca^{2+}$ as it was impossible to measure any translocation of accumulated calcium in this experimental arrangement.

4. Discussion

Considering the goal of our experiments, the employment of radioactive calcium seemed to be more appropriate than widely used calcium imaging [41]. Firstly, we wanted to compare three preparations: two in vitro and one in vivo. Considering technical challenges of calcium imaging [41], it has been concluded that using relatively simple but quantitative method for radioactive calcium determination will yield the data which can be compared with higher confidence. Secondly, calcium indicators have their own calcium-buffering abilities influencing free calcium concentration [42]. Undoubtedly, the employment of $^{45}Ca^{2+}$ seems to be much less invasive and damaging for cytoplasm than calcium imaging. Additionally, using radioactive calcium overcame the problem arising from limited dynamics of the radiometric imaging (saturation of different probes depending on their K_d values).

Finally, even a slight change in free calcium concentration due to calcium-induced calcium release may hypothetically obscure the data collected with calcium imaging procedures [50] more than that with radioactive tracer. Thus, it has been decided that in spite of its drawbacks the method of using $^{45}Ca^{2+}$ would be the most suitable one to achieve objectives of intended experiments.

Our method provided information about DC-induced, bulk relocation of calcium within an axon or between external environment (an incubation medium) and internal compartment of the stimulated neuronal preparation. Although one can be tempted to assume that an increase in radioactivity inside neuronal preparation is due to elevation in calcium accumulation, it may not be the only explanation. The DC-induced increase in internal radioactivity can only be the result of radioactive calcium accumulation. This can reflect an increase in radioactive and exogenous, nonradioactive calcium accumulation, an increase in the radioactive and endogenous calcium exchange, or combination of both of these processes occurring simultaneously during and/or after stimulation. Regardless of the interpretation, it is apparent that calcium metabolism and homeostasis were significantly modified by DC stimulation. The evaluation of $^{45}Ca^{2+}$ accumulation and relocation does not allow us to determine the destination of the calcium entering the cell and the cellular origin of the endogenous calcium which may exchange with radioactive ions. Nevertheless, we are convinced that the most of the observed increase in radioactivity results from increase in radioactive calcium accumulation. The rise in the exchange rate of endogenous/radioactive calcium would likely be significantly hampered by relatively slow diffusion of calcium [51, 52] within cytoplasm of the axon. In contrast, the DC-induced movement of $^{45}Ca^{2+}$ calcium observed in our preparations was massive and relatively fast. Additionally, we also observed an increase in radioactivity when $^{45}Ca^{2+}$ was added 6 hrs after the stimulation. If the increase in radioactive calcium would be the result of exchange, it

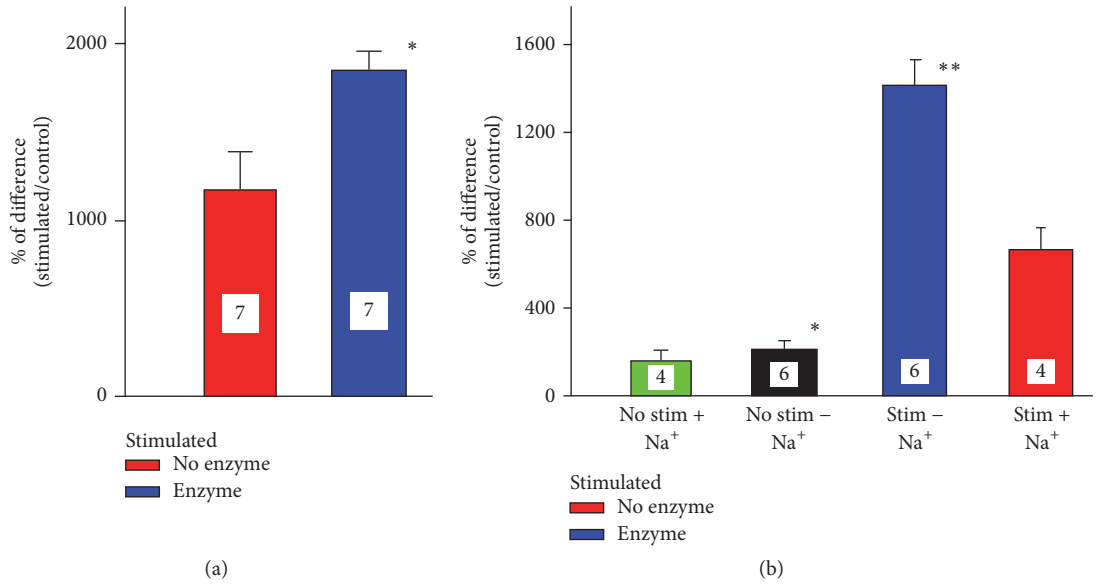

FIGURE 5: The modification of $^{45}Ca^{2+}$ accumulation by synaptosomes either treated for 3 hrs with neuroaminidase (enzyme) or incubated in sodium-free Ringer's solution. (a) Treatment of synaptosomes with neuroaminidase (0.2 U/ml) significantly increased DC-evoked $^{45}Ca^{2+}$ accumulation, as compared with nontreated ones and also by stimulated controls; $^{*}p < 0.006$, t-test. (b) The omission of sodium ions from Ringer's solution enhances $^{45}Ca^{2+}$ accumulation in DC-stimulated and nonstimulated synaptosomes. The control, nonstimulated synaptosomes accumulated more $^{45}Ca^{2+}$ in Na^{+}-free Ringer's solution (black bar), than in the presence of sodium (green bar); $^{*}p < 0.001$, ANOVA followed by Dunn's test; $^{**}p < 0.001$. Stimulated synaptosomes accumulated less calcium in the presence of sodium (red bar), than in Na^{+}-free solution (blue bar), $p < 0.029$, t-test.

would take place during stimulation and would most likely end before subsequent addition of ^{45}Ca. Therefore, since an increase was still observed 6 hrs later, it was probably due to persistent change in channels, carriers, or pumps responsible for the transport of calcium across the cellular membranes. The DC-induced increase in relocation of $^{45}Ca^{2+}$ in our preparations was dramatically reduced by cobalt, a general blocker of calcium channels. We believe that this reinforces our original assumption that the intracellular increase in radioactive calcium is a result of increased accumulation, not just an exchange, although our data do not allow us to draw any conclusions about possibility and intensity of this process. Nevertheless, this underlines the advantage of our technique which permits, in opposite to radiometric measurements, the determination of calcium source in this type of experiments. In conclusion, one can strongly advocate the idea that involvement of exchange process is likely to be minimal since it would involve relatively fast relocation of calcium from different intracellular compartments and move it out of the cell.

Our data in support of in vivo experiments on brain tissue [44], and human keratinocytes [53], convincingly show that the exposure to DC stimulation enhances accumulation/exchange of calcium by biological preparations. It was observed in vivo with the intact sciatic nerve and in vitro with the segments of sciatic nerve, spinal cord, and synaptosomes. The DC-induced amplification in $^{45}Ca^{2+}$ accumulation was much lower in the segments of the sciatic nerve and spinal cord than in synaptosomes. This difference is most likely related to considerably higher accessibility of the membranes of synaptosomes as compared to axons of the spinal cord surrounded by meninges and sciatic nerve embedded in epineurium. It also indicates that DC-induced increase in synaptosomal fraction is mostly due to calcium accumulation, not binding to external surface of synaptosomes, especially that it was attenuated by the presence of cobalt, which blocks most of the calcium channels [54], and was almost abolished by rupture of synaptosomes by osmotic shock [47]. Much greater accumulation of $^{45}Ca^{2+}$ by synaptosomes than by other preparations may be also related to geometry of these structures. Synaptosomes represent symmetrical, spherical vesicles which accumulate calcium into a single limited space without the ability to redistribute it along its length, while sciatic nerve and spinal cord are elongated preparations allowing entering calcium to be relocated along their long axis further away from the point of entry. This was clearly evident in sciatic nerve in vitro, where radioactive calcium was detected in the inside segments, as well as the outside segment and even in the solution of the chamber containing outside segment. This indicated that the forces responsible for redistribution of calcium were strong enough to draw it even outside of the tissue. Although the spinal cord was too fragile for this type of experiments, we assume that redistribution of calcium would be similar to what was observed in the sciatic nerve.

The increase in the strength of stimulation from 1 to 4 mA was followed by a gradual statistically significant enhancement in $^{45}Ca^{2+}$ accumulation (Figure 4(a)). The trend of enhanced $^{45}Ca^{2+}$ accumulation parallel to the increased strength of the stimulation was clear, although the difference

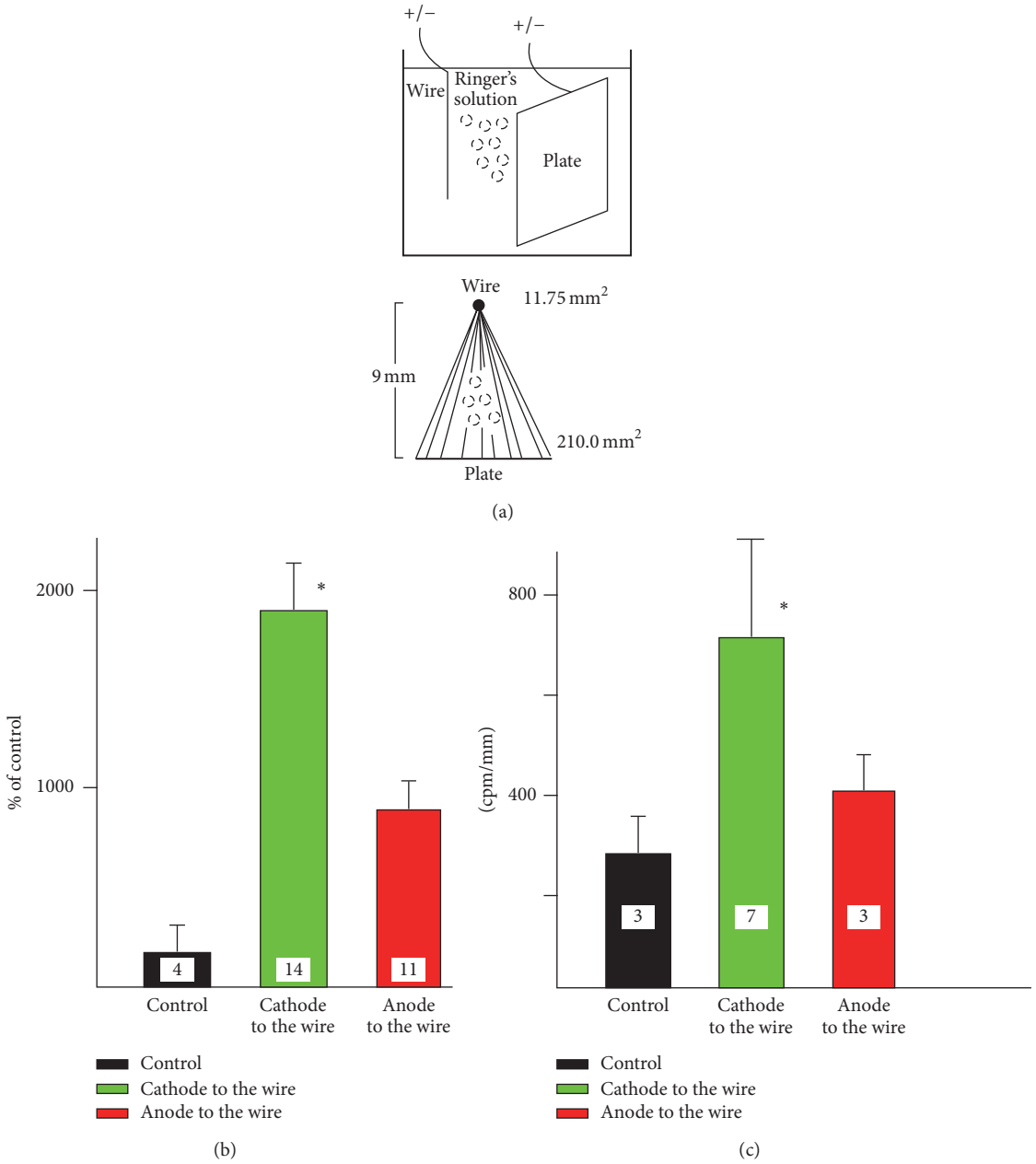

Figure 6: Accumulation of $^{45}Ca^{2+}$ by synaptosomes and segments of the sciatic nerve stimulated with two electrodes of different areas. (a) An experimental setup. The stainless steel plate (210 mm^2) was inserted into the plastic tube at the distance of 9 mm from the stainless steel wire (11.75 mm^2). The preparations (synaptosomes or segments of the sciatic nerve attached to the wooden stick) were placed in the tube and stimulated with DC (3′, 3 mA). The anode or cathode of DC was connected to either the wire or the plate. The broken lines in the lower panel of the figure illustrate hypothetical flow of the current. (b) The accumulation of $^{45}Ca^{2+}$ by synaptosomes, while anode (black) or cathode (red) was connected to the wire. (c) The accumulation of $^{45}Ca^{2+}$ by the segments of the sciatic nerve, while anode (black) or cathode (red) was connected to the wire; $^{*}p < 0.001$; Mann–Whitney U test.

between 1 and 2 mA was not statistically significant. The increase in calcium accumulation was observed when the calcium was present during stimulation and was also recorded when calcium was added several hours after termination of the stimulation. Although both anodal and cathodal stimulations were similarly effective with both electrodes of the same size, cathodal stimulation produced greater change when one of the electrodes was much larger than the other. While the concentration of radioactive calcium in our preparations was in the range of 3-4 nM, the concentration of endogenous calcium in the axon is in the range of 40–100 nM [55, 56]. Due to nearly 35-fold dilution of radioactive calcium, the accumulation/relocation of 1 nM of $^{45}Ca^{2+}$ observed in our experiments would reflect relocation of approximately 35

nanomoles of endogenous calcium. One has to also realize that the effect of DC-induced relocation of calcium to the cytoplasm, where most of the calcium targets are located, can be significantly influenced by endoplasmic reticulum and mitochondria [56]. While the concentration of endogenous cytoplasmic calcium is in the range of 100 nM, micromolar and even millimolar concentrations are observed in endoplasmic reticulum and mitochondria, respectively. Those two intracellular compartments have the ability to buffer intracellular calcium concentration reducing its toxicity [27, 56] and could sway DC-induced elevation in calcium trafficking. Our data supports and extends the results obtained in vivo by Islam and collaborators [44] who demonstrated that anodal stimulation applied in either single (30 μA, 30 min) or repetitive (5 times every 24 hrs) paradigms of stimulation induced an increase in $^{45}Ca^{2+}$ accumulation. The increase in vivo [44] was not the same in all brain structures and yielded damaged cells after repeated stimulations. Considering very high accumulation of $^{45}Ca^{2+}$ induced by 4 mA current (Figure 4(a)) and very low accumulation by osmotically damaged synaptosomes, it seems very unlikely that there was any DC-induced damage of synaptosomes, even by 4 mA. Since in Islam et al.'s [44] in vivo experiments and in some of our experimental design the increase in calcium accumulation and relocation was also observed when it was applied after cessation of DC stimulation, it is clear that not only DC itself but also the processes initiated by its application (e.g., trafficking of channels and receptors, relocation of internal organelles [57, 58]) persist beyond the period of stimulation and contribute to the observed effects. Our data obtained with sciatic nerve also unequivocally indicate that, as previously reported [59], not only the trafficking but also electrophoretically induced [60] redistribution of the calcium following its entrance to the preparation was modified by DC stimulation. This has been demonstrated by the experiments employing segments of sciatic nerve in vivo and in vitro. In both cases, the increase in calcium accumulation was noticed not only in the segments directly bathed in $^{45}Ca^{2+}$ but also in the adjacent areas. Calcium entering the axon is being relocated by DC to the adjacent segment of the sciatic nerve and even out of the axon to the extracellular Ringer's solution. This is particularly discernible in vitro in the case of anodal stimulation. One can speculate that positively charged, anodal electrode pushed calcium ions into the axon, while those ions were simultaneously pulled by negative, cathodal electrode on the other side of the partition resulting in the elevation of calcium concentration in the outer segment and even in extracellular Ringer's solution in the outer chamber. The inner segment under the anode would have $^{45}Ca^{2+}$ ions moving towards the cathode, away from inner segment. This repulsive action of anode towards positive calcium ions would be amplified by negativity of the entire outer segment. The negativity of the outer segment could even attract some radioactive calcium ions to Ringer's solution in the outer chamber (indeed, there is a slight increase). The cathode under inner segment would make the inner segment negative. This negativity combined with repulsive action of anode under the outer segment would push $^{45}Ca^{2+}$ ions into the nerve, but the movement of ions towards the outer segment would be attenuated by positivity of the anode under outer segment. Thus, the increase in $^{45}Ca^{2+}$ accumulation in the outer segment may be reduced by positivity of the entire outer segment. Therefore, in the case of cathodal stimulation, there was enhanced calcium accumulation in the inner segment but insignificant calcium redistribution. Hence, application of DC clearly enhanced calcium accumulation and polarity-dependent redistribution within the axon. The tangible content of $^{45}Ca^{2+}$ in the axon in vivo could be influenced by bidirectional exchange of axonal $^{45}Ca^{2+}$ with the interstitial fluid. Some calcium could diffuse back from the nerve to the interstitial fluid. It was technically impossible to measure this amount since it would be immediately diluted by the mixture of the interstitial fluid and Ringer's solution added to keep the preparation moist. As demonstrated by our previous electrophysiological experiments [8], this rapid calcium relocation could affect the amplitude of the compound action potential (CAP) recorded in vivo from DC-stimulated nerve [8]. The redistribution of calcium observed in current experiments and alterations in the amplitude of CAP [8] were both clearly related to the polarity and the strength of applied DC current. Moreover, the time frame of DC exposure required for both types of the changes to become apparent (1–3 min) was almost identical. Those correlations constitute a crucial link between DC-induced modulation of CAP and cellular background of this modulation observed as a change in the free calcium concentration/relocation inside of stimulated tissues.

Since movement of radioactive calcium is very slow following its injection into squid axon [61, 62], it is most likely driven by diffusion without participation of the axonal transport. In our experiments, anodal and cathodal stimulations significantly accelerated calcium relocation, most likely through electroosmosis in the axons and Schwann cells [63]. Since changes in DC-induced calcium redistribution were immediately apparent, it is unlikely that they resulted from the modification of the axonal transport. However, delayed, long-lasting effects of DC exposure on the axonal transport cannot be excluded and are worthy of further research. Although our results were obtained on sciatic nerve, the rate of movement would be most likely similar in other components of the nervous system.

Considering changes in calcium ionic activity following injury to the nervous system [64, 65], consequences of an increase in intracellular calcium concentration during and following DC exposure can be massive. As a second messenger, calcium ions are involved in several processes crucial for neuronal physiology including recovery of neuronal tissue from traumatic injury [5, 9, 25]. The involvement of calcium in neuronal excitability, synaptic activity and plasticity, extension of filopodia, formation of new synaptic contacts, guidance of synaptic sprouting [31], and receptor trafficking represents only a partial list of processes regulated by an increase in intracellular calcium concentration. The exposure to DC stimulation and subsequent free calcium elevation can alter not only biochemistry of the cell but also its morphology [59, 60]. The exposure of neurons in culture

to DC changes the location of intracellular organelles which favor cathode during their repositioning [60]. Those changes which also included polarization of the entire cell were evident within 1 hr of exposure and determined the migratory pattern of neurons during subsequent development [59]. As DC exposure dramatically enhances calcium trafficking through the membranes, it becomes an extremely potent tool which can significantly alter neuronal action [4, 6, 8, 9, 25].

The incubation of synaptosomes with neuroaminidase which removes sialic acid from extracellular glycoproteins and gangliosides modified DC-induced $^{45}Ca^{2+}$ accumulation. Sialic acid, which contributes to charged cloud on the cellular surface generated by glycocalyx, is especially important for nervous system function. It participates in neuroglial interactions [66], synaptic plasticity [67], and excitability [68] and is a structural component of sodium [69, 70] and calcium [71] channels. Remarkably, removal of sialic acid significantly alters exchange of ions in several biological preparations [72]. As evident from our data, depleting sialic acid and subsequent change in the charge of the membrane noticeably amplify ability of the calcium to penetrate through this modified membrane. As cobalt, known blocker of presynaptic calcium channels [46], reduced calcium accumulation, we propose that at least partial enhancement of the DC-induced calcium penetration through the membrane occurs via calcium channels. Alternative explanation could be offered assuming that charged calcium channels [72] could be persistently modified/activated after being electrophoretically relocated in the membrane by DC as reported for other functional proteins [70, 72]. Interestingly, an elevation in $^{45}Ca^{2+}$ accumulation was also observed in the experiments conducted in Na^+-free Ringer's solution. There was over 200% (233.3%) more $^{45}Ca^{2+}$ in synaptosomes stimulated in Na^+-free Ringer's solution than in control suspension stimulated in the presence of sodium. Moreover, in agreement with previously reported data [73], nonstimulated controls accumulated 25.5% more $^{45}Ca^{2+}$ in Na^+-free environment. The calcium concentration in neurons is regulated by plasma membrane Ca^{2+}ATPase and Na^+/Ca^{2+} exchanger [73–75]. While Ca^{2+}ATPase is more involved in calcium homeostasis, Na^+/Ca^{2+} exchanger counteracts significant changes in calcium concentration to prevent its toxicity [75]. The concentration of calcium inside of preparations evaluated in our experiments is apparently the results of dynamic equilibrium established by all processes which are forcing calcium in and out of intracellular space. It is clear that DC stimulation is shifting this equilibrium towards calcium accumulation/exchange which then exceeds exclusion of intracellular calcium. In support of this notion, we observed, as mentioned above, increased $^{45}Ca^{2+}$ accumulation in Na^+-free environment. Clearly, reduced Na^+ concentration affects the DC-modulated dynamics of calcium equilibrium. One can speculate that Na/Ca exchanger [74, 75] removes accumulated $^{45}Ca^{2+}$ less effectively when the counterion (Na^+) is missing. Alternatively, it is tempting to suggest that DC stimulation opens calcium channels and there is a massive influx of $^{45}Ca^{2+}$ into the synaptosomes. This massive influx and subsequent enhancement in intracellular calcium concentration stimulate compensatory activity of Na/Ca exchanger which increases pumping of calcium out in an attempt to readjust calcium concentration to its original equilibrium. However, in the absence of Na^+ the activity of this exchanger is attenuated and calcium entering synaptosomes during DC stimulation remains inside. Apparently, the Na/Ca exchanger works also without DC stimulation, since control synaptosomes in Na^+-free Ringer's solution accumulated more $^{45}Ca^{2+}$ than controls in Ringer's solution-containing sodium ions. Remarkably, treatment with neuroaminidase has similar effect. This would suggest that the presence of sialic acid is essential for full activity of the Na/Ca exchanger.

The geometry and size of the electrodes critically influenced the data. One can assume that the density of the current is inversely related to the surface area of the electrodes. Therefore, using electrodes of different sizes which would subsequently generate polarized currents of uneven strength would help to determine which of the two polarities is more effective. In stimulated sciatic nerve and synaptosomes (Figure 6), cathodal current was much more efficient than anodal in increasing calcium accumulation. While stimulation of synaptosomes with two electrodes of the same size (Figure 4(a), 3 mA) amplified accumulated radioactivity approximately 1,200-fold, cathode connected to the wire increased it by almost 2,000-fold (Figure 6(c)). This observation may be vitally important for future clinical applications of DC stimulation. The issue related to the geometry of electrodes is the relation between the electrodes and spatial position of the neurons in stimulated preparation. This factor may play very different role in synaptosomes, sciatic nerve, and the spinal cord. As mentioned before, the synaptosomes represent spherical structure. One can assume that the applied current would influence all of them in a very similar way. On the contrary, the nerve was stimulated by perpendicular current and the application of DC parallel to the axis of the axon could have different still unknown consequences. While long motor neurons run in the spinal cord parallel to its long axis, short interneurons may be spatially arranged in a variable way. Therefore, our experiments with the spinal cord represent the model reminiscent of the experimental arrangement used in vivo experiments to stimulate brain [44]. In both cases, stimulated tissue demonstrated increased concentration of $^{45}Ca^{2+}$ which persisted beyond the period of stimulation.

While application of DC is an emerging and very formidable procedure to persistently modify function of the nervous system [76], one has to realize that DC-induced redistribution of calcium ions is an indicative of electrophoretic processes which occur within the axon under the influence of direct current. Other ions, charged molecules, subcellular structures, and even some organelles can be redistributed under the influence of this current as well [62]. As such, if used in clinical setting, DC stimulation has to be applied with high attentiveness. It has been demonstrated that excessive calcium accumulation may be detrimental for cell physiology [56]. Also, the enhanced calcium accumulation can exert quite opposite effects on neuronal plasticity depending on the rate of calcium accumulation and its final

concentration induced by physiological process or experimental procedure [37].

Competing Interests

There is no conflict of interests related to this paper.

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Review Article

Paired Stimulation to Promote Lasting Augmentation of Corticospinal Circuits

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After injury, electrical stimulation of the nervous system can augment plasticity of spared or latent circuits through focal modulation. Pairing stimulation of two parts of a spared circuit can target modulation more specifically to the intended circuit. We discuss 3 kinds of paired stimulation in the context of the corticospinal system, because of its importance in clinical neurorehabilitation. The first uses principles of Hebbian plasticity: by altering the stimulation timing of presynaptic neurons and their postsynaptic targets, synapse function can be modulated up or down. The second form uses synchronized presynaptic inputs onto a common synaptic target. We dub this a "convergent" mechanism, because stimuli have to converge on a common target with coordinated timing. The third form induces focal modulation by tonic excitation of one region (e.g., the spinal cord) during phasic stimulation of another (e.g., motor cortex). Additionally, endogenous neural activity may be paired with exogenous electrical stimulation. This review addresses what is known about paired stimulation of the corticospinal system of both humans and animal models, emphasizes how it qualitatively differs from single-site stimulation, and discusses the gaps in knowledge that must be addressed to maximize its use and efficacy in neurorehabilitation.

1. Introduction

Many skills, including those most elemental to survival—eating, fleeing, and attracting mates—must be learned by associating a context with a function. The "rules" of synaptic learning have largely been gleaned from studies of the hippocampus and neocortex, both of which are highly adapted for learning. Three fundamental processes enable associative learning. First, learning depends on the relative firing of an input neuron and a receiving neuron that are connected by a synapse. This type of learning, originally proposed by Hebb, is known as Hebbian or spike-timing dependent plasticity [1, 2]. Second, associations are encoded by convergence of multiple stimuli onto a common target. For example, association cortex integrates multiple sensory modalities and enables learning about the relationship between them. Finally, learning can be modified by the state of excitability. Fear or arousal, likely through increased levels of monoaminergic neurotransmitters, can more strongly encode the association of a stimulus with the presence of a predator than when an individual senses safety [3].

Paired electrical stimulation for corticospinal system modulation has evolved to emulate these fundamental learning mechanisms with the goal of enhancing motor function. In this review, we group various paired stimulation paradigms that have been developed for modulation of the corticospinal tract (CST) into three fundamental strategies (Figure 1). First, we discuss Hebbian plasticity in experiments designed to modulate synapses directly between presynaptic corticospinal neurons and postsynaptic spinal motor neurons

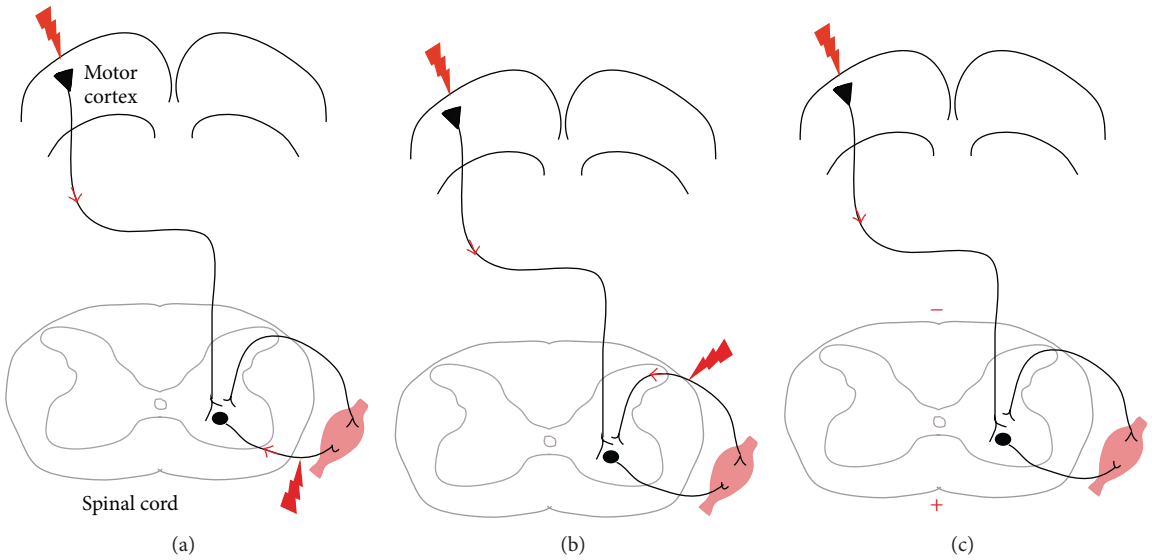

Figure 1: Three paired stimulation models. The corticospinal tract (CST) connects motor cortex directly to the spinal cord. The termination of the CST is largely (>80%) onto interneurons in humans and exclusively so in rodents, but for simplicity it is depicted only as synapsing onto motoneurons. (a) Pre-post synapse model. Repetitive paired stimulation of a presynaptic neuron and its postsynaptic target modifies the strength of the synapse connecting them. The timing of pre-post synaptic neuron firing determines whether the synapse is made stronger or weaker. This is also termed Hebbian or spike-timing dependent plasticity. For corticospinal modulation, this strategy usually pairs motor cortex stimulation with back-propagating peripheral motor nerve stimulation [1, 77–79]. (b) Convergent model. Two (or more) presynaptic neurons converge onto a common postsynaptic target. For corticospinal modulation, this strategy may pair motor cortex stimulation with afferent sensory nerve stimulation [87, 94]. (c) Phasic during tonic model. Adding tonic direct current stimulation concurrently with phasic stimulation at one or more sites can augment corticospinal circuit responses [95, 98, 99]. In this schematic, CST activation of motor cortex is modulated by direct current stimulation of the spinal cord.

(Figure 1(a)). The second strategy uses convergent inputs of paired stimuli onto a common postsynaptic target (Figure 1(b)). Sites for this modulation include cortex and spinal cord, usually achieved by pairing corticospinal and afferent stimulation, but at different interstimulus latencies. Finally, we discuss tonic stimulation at one site, for example, the spinal cord via direct current stimulation, to strengthen the effects of phasic stimulation at another site, for example, the motor cortex (Figure 1(c)). In all of these strategies, factors such as relative timing, intensity, and frequency of paired stimulation play crucial roles in determining the direction and duration of circuit modulation.

The review begins with a discussion of the organization of the CST and the different types of single-site modulation. We then review the application of paired stimulation through Hebbian, convergent, and tonic models. Like all models, these three models of paired stimulation are both useful and flawed. They are useful in that they illuminate general principles of paired stimulation interactions on a systems neuroscience level. This enables comparison of different stimulation protocols and emergence of common mechanisms. At this early stage, the models are flawed and incomplete because although a vast body of work has elucidated key synaptic learning mechanisms in vitro, it is difficult to pin down the basic synaptic mechanisms by which paired stimuli interact in the living organism. In addition, paired stimulation protocols may employ more than one overlapping mechanism to achieve modulation. Finally, even pairing aimed at specific circuit nodes likely act at several points in the network. But we believe that a conceptual framework organized into these three models is helpful to build mechanistic understanding and to identify patterns of effective neuromodulation.

2. The CST as a Target for Modulation with Paired Stimulation

The CST is a popular target for neuromodulation, in part, because it is so important for human health and function. The corticospinal tract is the direct connection between motor cortex and circuits within the spinal cord and is the principal pathway for skilled voluntary movement, particularly of the hands [4, 5]. Lesion of the CST strongly correlates with motor impairment [6]. In the setting of injury or disease, spared CST connections are largely responsible for motor recovery [7–10]. The CST's roles both in learning and executing motor skills and in providing the substrate for relearning skills after injury indicate that it is a malleable system [11]. This plasticity can occur within the CST itself [12] or as a result of spinal plasticity [13].

The CST is also an attractive target because it is accessible to external electrical stimulation. The motor cortex lies on the convexity of the cerebral hemispheres; the face and arm/hand representations are nearest to the scalp. This enables these regions to be stimulated noninvasively across the scalp, or without disturbing other neural tissues if electrodes are placed above (epidural) or below (subdural) the overlying

dura mater. Although spinal motor circuits lie deeper in the body, the spinal cord is still accessible through direct electrical stimulation or indirectly through stimulation of peripheral nerves. In humans, peripheral nerves and nerve roots are the most common targets for modulation of the spinal cord, through either orthodromic activation of afferent fibers or antidromic activation of efferent nerves.

A simplified schematic of the CST is shown in Figure 1. Importantly, the cortical motor system does not exclusively originate from primary motor cortex. Premotor, supplementary motor, and other cortical areas also make large contributions to descending CST pathways, with recurrent connections between motor cortex, sensory cortex, and thalamocortical pathways [14–17]. In the spinal cord, the CST largely terminates onto interneurons of the deep dorsal horn and the intermediate zone [15, 18, 19]. In primates, a small percentage of CST terminations contact motoneurons directly (15–20% in humans); with a few possible exceptions, direct cortex to motoneuron connections is present in rodents only early in development [20, 21]. CST terminations overlap extensively with terminations of large-diameter afferents, which encode joint position, muscle spindle tension, and other sensory modalities critical to skilled movement [22]. The extensive overlap of CST and afferent sensory terminations within the spinal cord provides a substrate for convergent modulation and plasticity of spinal motor circuits using paired stimulation.

While the spinal cord has been regarded in the past as a relatively simple conduit between the brain and periphery, it is now clear that the spinal cord harbors its own complex intrinsic circuitry [23]. Spinal circuits enable skilled movement through the coordination of agonist, antagonist, and stabilizing muscles across body regions [24–26]. A dramatic demonstration of this intricate circuitry is the ability of the lumbar spinal cord to support locomotion in spinalized animals. The intrinsic circuitry mediating these complex acts is termed the locomotor central pattern generator [27]. When given lumbar epidural stimulation, monoaminergic neurotransmitters, and afferent input below the injury, cats [28] and rats [29] with no brain-to-spinal cord connections can stand, walk, and even adapt to obstacles and changes in treadmill direction. Under these conditions, spinal circuits can respond to training by altering synaptic connections and strength, resulting in functional improvements in standing or walking [30, 31].

These results provide information about the use of paired stimulation in three ways. First, intrinsic spinal programs can be modified with experience: like the brain, the spinal cord can learn [32]. This makes the spinal cord an attractive target for modulation. Second, inputs from the brain, sensory afferents, or external electric stimulation do not need to encode the complexity of movement; rather, they can trigger intrinsic spinal cord motor programs to carry out those actions [25, 33]. Third, injury to the brain or spinal cord usually spares a portion of CST and other inputs to the spinal cord [34, 35]. The goal of external stimulation is to enhance connectivity made by these spared neural inputs onto intrinsic spinal circuits.

3. Stimulation Modality

To understand the effects of paired stimulation on the CST, we must first understand how single-site stimulation affects the system. Some basic technical concepts regarding individual stimulus modalities are introduced below, as the biophysics of each type of stimulation helps to determine which to use for paired stimulation. Stimulation is largely applied in one of two ways: phasic (short pulses lasting on the order of milliseconds) or tonic (applied at the same intensity over a period of minutes). The amplitude, position, orientation, and polarity of stimulation determine whether pulsed or tonic stimulation produces action potentials in the underlying tissue. In practice, phasic stimuli are more often used to depolarize underlying neurons synchronously with the stimulation; this may be performed repeatedly. On the other hand, tonic stimulation changes tissue excitability, which can alter the firing rate of neurons but without temporal specificity [36].

There is often a trade-off between stimulation focality and invasiveness: direct invasive stimulation on or within a neural target delivers more focal stimulation compared with noninvasive stimulation on the skin. However, even highly localized stimulation, such as that delivered with a sharp electrode into the central nervous system, has effects that spread throughout the interconnected neural circuits [37]. A potential advantage of paired stimulation over unpaired stimulation is that the effects may be constrained by the interaction between the two stimulation sites. This means that diffuse stimulation at one site might gain specificity through interactions at another site. The effects of stimulation intensity are also likely to be complex; like drug therapy, more is not necessarily better. This is true, in part, because more intense stimulation produces less targeted effects. Another potential advantage of paired stimulation is that the synergistic effects of stimulation at separate sites may allow lower stimulation intensity at each site compared to single-site stimulation.

Magnetic stimulation uses a transient focal magnetic field to induce a current in the neural tissue underlying the stimulating coil. When placed over the motor cortex, transcranial magnetic stimulation (TMS) can produce CST action potentials. The largest and fastest component of the motor-evoked potential (MEP) travels via the CST [38, 39]. However, polysynaptic pathways involving the reticulospinal, propriospinal, and other tracts contribute as well [40]. TMS offers the advantage of relatively high spatial and temporal specificity. Cortical TMS can be used diagnostically to measure CST function by testing the threshold to provoke motor responses as well as the amplitude, latency, and spatial distribution of those responses. As an intervention, repetitive TMS (rTMS) modulates brain function in rate-dependent fashion. Patterned "theta burst" rTMS (bursts of 3 pulses at 50 Hz, given at a rate of 5 bursts per second) may produce longer-lasting changes in cortical excitability [41, 42]. Excitatory rTMS (repetition rate of 1 Hz or greater) has been applied most extensively in stroke, where it has had a tendency to strengthen TMS-evoked responses and to improve some aspects of arm and leg function [43–45]. Inhibitory TMS (repetition rate of less than 1 Hz) has also been used effectively to dampen activity in the uninjured

hemisphere after stroke and thereby reduce interhemispheric inhibition [46].

TMS can also be applied to the spinal cord by holding the stimulating coil over the back of the subject. This approach likely recruits radicular inputs onto spinal cord circuits [47, 48]. For paired stimulation, this approach could be used to recruit afferents in the segment of the spinal cord underlying the stimulating coil, while a TMS coil over motor cortex could be used to stimulate motor cortex and the CST.

Direct current stimulation (DCS) uses surface electrodes to deliver continuous low intensity (e.g., 1-2 mA) electric current. A critical difference between DCS and TMS is that DCS delivers *tonic, sub*threshold stimulation rather than directly triggering action potentials. Although only a small fraction of the current crosses the skin, and despite the lack of direct evidence for which circuits DCS activates, data from numerous studies have suggested that DCS modulates underlying neuronal excitability [49–56]. Additionally, DCS has been used over the spinal cord, with possible effects on motor recruitment, pain, and spasticity [57–61]. DCS offers the advantages of lower cost and higher portability than other stimulation techniques. However, several major gaps in mechanistic understanding persist: there is no technique to directly map how the low-energy current is distributed within the body, which neural circuits are activated, or how individual variations in injury characteristics affect DCS circuit activation. Furthermore, the continuous nature of DCS means that it cannot be employed for timing dependent synaptic changes.

Intraspinal electrical stimulation through implanted electrodes is used to deliver *phasic* pulses at sub- or suprathreshold intensity directly to the spinal cord. This method is currently limited to animal models due to its highly invasive nature [62].

Epidural electrical stimulation delivers *tonic* pulses to the dorsal surface of the spinal cord that are usually subthreshold for activating motor neurons. This stimulation (usually in the range of 15–60 Hz) activates large-diameter sensory afferent fibers that enter the dorsal spinal cord and synapse onto interneuronal and motor circuits [63]. Although subthreshold epidural stimulation alone does not induce any movement, when combined with physical training or monoaminergic drug exposure, SCI animals and human subjects with implanted lumbar epidural stimulators have shown dramatic increases in volitional control of leg muscles below the injury level [29, 64–67]. Whether epidural stimulation directly facilitates increased responsiveness of spinal motor circuits, or whether individual epidural pulses stochastically interact with descending volitional signals to mediate spike-timing dependent synaptic plasticity, remains to be determined. These alternative hypotheses are not mutually exclusive.

Transcutaneous spinal electrical stimulation is applied noninvasively, usually at suprathreshold intensities (unlike DCS or epidural stimulation). At the lower range of stimulation intensity, this stimulus modality is thought to activate dorsal afferent fibers, whereas, at higher intensities, transcutaneous stimulation directly activates ventral efferent fibers [68–71]. For example, transcutaneous stimulation over the T11 level at 3 Hz induced coordinated walking movements in uninjured volunteers [47]. Adding simultaneous stimulation at the C5 and L1 levels (at 5 Hz) increased the coordination and range of motion achieved [72]. Delivered at a higher rate (50 Hz) and lower intensity (70% of motor threshold), lumbar transcutaneous stimulation reduced leg spasticity in three subjects with chronic incomplete SCI [73]. The most appealing aspect of this method is its noninvasiveness and portability; using simple adhesive electrodes, transcutaneous spinal stimulation could be given within the context of structured physical rehabilitation exercises.

4. Paired Stimulation Strategies

All paired stimulation paradigms share the same objective: to alter connections between specific target circuits. Relative to single-site stimulation, in which activation may spread to other areas connected to the target, paired electrical stimulation may narrow the effect to the site of interaction between multiple stimulation sites. Repetitive paired stimulation at two or more sites is designed to trigger lasting plasticity through synergistic mechanisms. Further considerations include site (brain, spinal cord, and peripheral nerve, each with varying levels of specificity) and whether stimulation is geared toward pre-post synaptic or convergent synaptic summation mechanisms. As demonstrated through decades of research in cellular and slice models, other major variables involved in paired stimulation include timing, intensity, and frequency [74].

Devising paired stimulation paradigms for neuromodulation of the CST involves integrating the systems neuroscience of sensorimotor interactions in the cortex and spinal cord with understanding of the biophysics of the stimulation modality. This is a necessarily iterative process because paired stimulation provides insight into interactions that cannot be achieved otherwise. Instead of a systematic review of the literature, we will highlight selected studies that demonstrate key concepts of using paired stimulation to target specific synaptic connections in animal models and humans. We will discuss progress and describe the main challenges that need to be addressed for paired stimulation to be successfully implemented in human neurological conditions.

Pre-Post Synaptic Stimulation. The classic Hebbian approach involves stimuli delivered in synchronous fashion directly to the two neurons connected by the target synapse; coordinated firing of a presynaptic neuron and its postsynaptic target adaptively alters the synapse that connects them [1]. This concept has been advanced experimentally in the hippocampus and other well-understood circuits, where it has been termed spike-timing dependent plasticity (STDP) [2, 74, 75]. We choose to call this approach "pre-post synaptic stimulation" because STDP and Hebbian plasticity have come to mean different things to different people.

The relative delay between pulse arrivals at pre-post synaptic sites dictates whether repetitive paired pulses potentiate or depress the targeted synapse. Work in cellular and slice models has shown that long-term potentiation (LTP) occurs after repetitive pulse arrival at an excitatory presynaptic terminal up to 20 ms prior to pulse arrival at

the postsynaptic terminal, whereas long-term depression (LTD) occurs after repetitive pulse arrival at the postsynaptic terminal between 20 and 100 ms prior to the presynaptic terminal [74, 76]. Note that, in vivo, consideration must be given to the latency between stimulation site and synapse arrival. These latencies vary depending on factors such as a subject's height, injury severity or disease status, and effort. Therefore, the interval between two stimulus sites/modalities may need to be individualized based on these factors and the desired site of synaptic interaction. Likewise, the relative intensity of the pre-post synaptic stimuli may affect the polarity and degree of synaptic modulation.

Nishimura and colleagues demonstrated pre-post synaptic stimulation in the CST of healthy primates. To test whether exogenous time-linked spinal stimulation would cause lasting modulation of corticospinal transmission, the investigators used intracortical electrodes to record activity in corticospinal motor neurons during free behavior [77]. Neurons that fired during specific arm movements were used to trigger delivery of intraspinal stimuli to cervical spinal motor neurons controlling the arm muscles that mediate the intended movements. When the latency between endogenous cortical spike and exogenous spinal stimulation was between 12 and 25 ms, corticospinal transmission (as determined by the correlation between cortical motor neuron spike activity and EMG facilitation) increased for at least 24–48 hours *after* stimulation. Conversely, when the investigators varied the timing such that spinal stimulation occurred several ms prior to arrival of the endogenous cortical signal, subsequent corticospinal transmission was depressed. Both of these time windows follow rules established in numerous classic Hebbian experiments [76].

In humans, the pre-post synaptic approach is best exemplified by pairing TMS with motor nerve stimuli such that the pulses arrive synchronously at synapses between corticospinal neurons and motoneurons within the spinal cord (Figure 1(a) and [78–80]). High-intensity electrical stimuli of peripheral nerves innervating arm or hand muscles travel antidromically to motoneurons in the cervical cord. In able-bodied volunteers and subjects with incomplete cervical SCI, a series of 50–90 TMS-peripheral nerve stimulation pairs timed such that TMS pulses arrived at cervical motor neurons 1-2 ms prior to retrograde nerve stimuli led to increased hand muscle motor-evoked potential amplitudes and fine hand dexterity for roughly 30 minutes after stimulation. Reversing the timing (peripheral stimulus arrival at cervical motor synapses 5–15 ms before TMS pulse arrival) resulted in either the opposite or no effect [78, 79]. Encouragingly, application of paired stimulation in the pre-post sequence resulted in transiently increased hand function, not just electrophysiological transmission. In able-bodied subjects, Janet Taylor's group observed increased strength of the targeted biceps muscle [78]. In both able-bodied subjects and those with incomplete cervical SCI, Monica Perez's group observed increased strength and EMG activity in the targeted first dorsal interosseous muscle, as well as increased agility on a skilled pegboard task [79].

Critically, as already described above in primate models, exogenous cortical stimulation could potentially be replaced by using endogenous cortical signals as the presynaptic pairing modality. The intent to move can be detected from intracortical (or less invasive scalp) electrodes and then used to trigger synchronized exogenously delivered spinal or peripheral stimuli. This volitionally driven approach could be used to *amplify* synaptic transmission within incompletely damaged native circuits. This is in distinction from the use of brain-computer interfaces as bypass routes to *replace* function of completely disconnected native circuits. As a large number of brain and spinal injuries spare at least some degree of volitional muscle activation, real-time electromyography (EMG) of the target muscle could serve as a simpler proxy for cortical intent, as demonstrated in rodent models [62]. In humans, an inverted approach has been tested, in which exogenous cortical stimulation is driven rather than replaced by peripheral signals. For example, TMS has been synchronized either with peripheral EMG activity or with timed physical arm movements, with mixed results [81–83].

The pre-post synaptic model represents the most straightforward approach to paired stimulation of the motor system, with timing and other parameters being well-delineated in slice and hippocampal models. However, the mechanistic challenge, especially in the case of volitionally driven human studies, is that it may be difficult if not impossible to precisely determine the circuit identities and synaptic mechanisms that contribute to observed changes in function. In the living organism it remains to be determined whether stimulation can be delivered precisely enough to modulate the targeted synapse without resulting in unintended collateral plasticity.

Convergent. In the convergent approach, rather than pairing stimulation between a single presynaptic neuron and postsynaptic neuron, stimuli are delivered to two or more presynaptic neurons that independently synapse onto a common postsynaptic target, resulting in summation of temporally paired inputs (Figure 1(b)). This mechanism was initially described in simplified in vitro and ex vivo preparations from *Aplysia* and neonatal rat spinal cord, where repeated paired activation of separate converging inputs facilitated responses of common target neurons to test stimuli [84–86]. In the living organism, all forms of external stimulation may in fact be at least partially "convergent," given the difficulty of limiting stimulation precisely to single pre-post synaptic neurons.

In the most highly cited demonstration of paired stimulation in humans, Stefan and colleagues paired median nerve electrical stimulation with TMS over the motor cortex area representing the abductor pollicis brevis muscle, a paradigm dubbed paired afferent stimulation (PAS) [87]. The median nerve was stimulated 25 ms before TMS to allow the median nerve signal to reach the motor cortex, presumably through ascending sensory projections to sensory cortex and then via cortico-cortico connections. A single pair of pulses delivered every 20 seconds for 30 minutes (90 pulses) resulted in increased cortical motor-evoked potential amplitudes at both the abductor pollicis brevis and abductor digiti minimi muscles; augmentation lasted for at least 30 minutes after pairing.

The site of PAS plasticity is likely in the cortex. There was no change in subcortical motor-evoked potential amplitude

or in F-wave responses, arguing against a subcortical or spinal locus of plasticity [87]. However, subsequent reports suggest some spinal cord changes in segmental reflexes (paired associative stimulation induces change in presynaptic inhibition of Ia terminals in wrist flexors in humans [88]). The timing dependent sensitivity of PAS was demonstrated by observing no effect when longer ISIs separated the median and TMS pulses and a *decrease* in median nerve-evoked sensory potentials when timing was reversed such that the TMS pulse arrived at somatosensory cortex 10–15 ms prior to the median nerve-evoked potential [89]. These time windows for synaptic potentiation and depression overlap with those seen in Hebbian pre-post synaptic plasticity, demonstrating the universal importance of timing in synaptic plasticity.

Although sometimes characterized as Hebbian, paired associative stimulation is more consistent with the convergent approach. Neither the afferent median nerve electrical impulse nor the cortical magnetic impulse takes direct routes to the target synapse: the afferent peripheral pulse synapses at the brainstem, thalamus, and sensory cortex before traversing intracortical fibers that are input onto pyramidal motor neurons. The TMS pulse also transits through intracortical fibers that converge onto the same pyramidal motor neurons [87, 90]. Thus, these stimuli lead to convergence of two or more presynaptic signals onto a common postsynaptic target—in this case, corticospinal motor neurons in layer V of motor cortex.

Convergence can be targeted to spinal rather than cortical circuits by altering stimulus latencies. For example, in the human, a motor cortical stimulus takes roughly 5–8 ms to reach synapses in the cervical spinal cord and 10–15 ms to reach synapses in the lumbar cord via the CST [79, 91–93]. Synchronized stimuli to afferent sensory inputs converge with descending corticospinal signals onto postsynaptic spinal motor neurons, modulating motor neuron responses depending on relative timing, intensity, and pattern. For example, a paradigm dubbed spinal associative stimulation (SAS) combines subthreshold cortical TMS pulses timed to arrive at soleus motor neurons roughly 5 ms prior to arrival of suprathreshold tibial nerve afferent pulses [94]. Pairing the pulses every 10 seconds for 15 minutes (90 pulse pairs) significantly increased tibial nerve H-reflex amplitude and sensitivity during and immediately after the stimulation period [94]. Whereas this paradigm increased H-reflex amplitude, F-waves were not measured, so the mechanism of increased spinal reflexes is unknown. Furthermore, postintervention TMS motor-evoked potentials were not reported, leaving the question open of whether corticospinal circuits were modulated. Another study targeting SAS toward cervical levels using suprathreshold TMS in combination with median nerve stimulation saw no change in the primary outcomes of TMS-evoked potentials and grip strength. The authors speculated that in this case the paired stimuli may have reached separate rather than common postsynaptic targets.

Convergent paired stimulation has several advantages as well as possible disadvantages compared with pre-post synaptic stimulation. The convergent approach has the advantage that spinal targets are more easily accessed via sensory afferent input than through antidromic motor stimulation, especially because the former can be delivered at lower (and more tolerable) stimulation intensities. In addition, sensory circuits are more easily accessible to surface (e.g., epidural) stimulation of the spinal cord. In addition, lower-intensity sensory stimulation may be easier to integrate with simultaneous physical rehabilitation exercises, providing an opportunity to supplement or supplant exogenous cortical stimulation with endogenous volitional motor signals. On the other hand, the convergent approach may have the disadvantages of more off-target effects and increased complexity by adding other synapses and circuits into the classic two-neuron pre-post synaptic picture.

Tonic during Phasic. Both pre-post synaptic and convergent plasticity rely on proper synchronization of paired stimulation on the order of milliseconds. In contrast, tonic stimulation is applied continuously over the course of minutes. Direct current stimulation (DCS) represents the most widely used form of tonic stimulation. For DCS, the positioning and polarity of stimulation, rather than timing, are critical to its effects. We will discuss tonic stimulation of the CST employing transspinal DCS (tsDCS; Figure 1(c)). The induced electric field of tsDCS alters the properties of the spinal cord, modulating responses to brain stimulation and spinal reflexes. Whether the cathode is placed dorsally and the anode ventrally (as shown in Figure 1(c)) or the polarity is opposite (cathode ventral and anode dorsal) has a major impact on the effects.

Both rodent and human experiments demonstrate effects of tsDCS on motor responses evoked by CST stimulation. In rodents, stimulating electrodes are placed subcutaneously to prevent the animal from removing the electrode. In humans, the electrodes are placed on the skin. The sites of stimulation include the neck, torso, and lower back. Mathematical modeling of current flow within the body suggests that the site of stimulation is critical to which peripheral nerves or spinal cord segments are affected by tsDCS [95]. Electrode size and stimulation amplitude, which together determine the current density, are other determinants of the effects of tonic stimulation [96].

A robust and reproducible finding across studies is that tsDCS causes greater augmentation of CST responses when the cathode is placed on the dorsal aspect and the anode ventrally (referred to as cathodal tsDCS). Tonic tsDCS has effects both during the stimulation period and for a period of minutes afterwards. The influence of polarity is particularly strong on the after effects, with cathodal tsDCS causing lasting augmentation of CST motor responses [97]. These effects are mediated by alterations in spinal cord synapses and axonal connections. Thus, cathodal tsDCS can augment CST motor responses when applied as single-site modulation.

The crucial question for paired stimulation is whether tonic tsDCS modulates the effects of concurrent phasic CST neuromodulation. Experiments in the John Martin Laboratory demonstrate that cathodal tsDCS strongly enhances the neuromodulation caused by repetitive motor cortex stimulation in rats. The brain stimulation paradigm used in these studies is intermittent theta burst stimulation, a paradigm involving "bursts" of three stimuli applied at 50 ms intervals

with electrodes implanted over motor cortex. As a single modality, theta burst stimulation causes lasting augmentation of CST responses both in rodents and in humans when applied via TMS [41, 98]. When paired with cathodal tsDCS in rats, the slope of theta burst augmentation increased. That is, tonic stimulation of the spinal cord caused larger increases in CST responses than theta burst motor cortex stimulation alone [98]. These effects lasted at least 30 minutes after paired stimulation was applied.

Importantly, pairing tonic with phasic stimulation improves CST *function* and motor skill in rodents with injury. Song et al. employed a cut lesion of the CST emanating from one hemisphere and paired intermittent theta burst stimulation of the spared CST with cathodal tsDCS over the cervical spinal cord beginning the day after injury, similar to a brain stimulation only protocol that was effective [19]. Paired motor cortex intermittent theta burst stimulation and cervical tsDCS were administered for 27 minutes a day for 10 days. This caused a decrease in the number of foot faults while walking across a horizontal ladder; improvement relative to sham tsDCS was sustained throughout the testing period of 31 days. In addition, the threshold of motor cortex stimulation to produce a motor response went *down* by more than 25% (indicating stronger CST responses) whereas the threshold for provoking responses in rats with sham tsDCS went *up* more than 50%. Finally, the protocol produced large-scale sprouting of spared CST axon endings in the gray matter of the cervical spinal cord; the cumulative axon length on the animals' impaired side was more than 5 times that of rats with sham tsDCS. Thus, this tonic during phasic protocol produced robust behavioral improvement that was accompanied by strengthening of CST physiology and function and abundant sprouting into largely denervated regions of the spinal cord.

Since tsDCS can enhance cortical neuromodulation, it may also increase the gain of other neuromodulation strategies. This includes corticospinal neuromodulation based on pre-post synaptic and convergent input. Experiments in Ahmed's laboratory have tested this hypothesis in the lumbar spinal cord of the mouse. One convergent input paradigm paired sciatic nerve stimulation with motor cortex stimulation (similar to PAS, but in the hind limb). When the sciatic nerve was repetitively stimulated up to 120 ms before brain stimulation, subsequent unpaired cortical test pulses were enhanced, demonstrating the lasting augmenting effect of pairing [99]. This convergence paradigm was then performed under tonic cathodal tsDCS, with markedly stronger augmentation of subsequent cortical test pulses. The effect of combining the convergence paradigm and tonic stimulation was larger than predicted by the individual effects, suggesting the synergistic potential of combining tonic stimulation with phasic paired stimulation strategies.

This protocol produced improvements in skilled locomotion in mice with spinal cord hemisection. Stimulation at cortex, sciatic nerve, and tsDCS was delivered beginning 13 days after hemisection at the caudal end of the thoracic spinal cord. Skilled locomotion was assessed using the horizontal ladder, similar to the Song et al. study. This protocol produced large-scale recovery of skilled locomotion; errors in hind limb stepping were reduced 77% in rats with stimulation compared to injury-only animals. Two groups of control mice (tsDCS only and paired motor cortex and sciatic nerve stimulation only) were reported to have improved less, although the data from these mice were not shown [99]. Together, these results suggest that adding tonic spinal cord stimulation increases the physiological and behavioral efficacy of motor cortex and peripheral nerve stimulation.

Clearly, this stimulation paradigm does not conform to the precisely time-locked Hebbian model of paired exogenous stimulation. Whether tonic stimulation itself prepares spinal motor circuits to become more responsive, or whether individual pulses stochastically interact with descending volitional signals at the correct synaptic latency, remains to be determined. Again, these scenarios are not mutually exclusive.

5. Gaps and Hurdles

Paired stimulation of the corticospinal system holds unique promise not only for gaining insight into systems-level organization of intact and injured motor control circuitry, but for potential application toward humans with neurological injury and disease. It also offers the possibility of modulating the CST in a circuit-specific manner, in which the effects of pairing are largely restricted to the site of interaction between two stimuli. The promise of paired stimulation is that its potential selectivity may boost efficacy and limit off-target effects, similarly to molecular medicines that specifically bind strongly to their target and limit side effects.

In order to clear the many hurdles impeding application of paired stimulation to humans for therapy, work is ongoing to address these critical questions.

Is Paired Stimulation Actually "Better" Than Unpaired Stimulation? This question has only been partially addressed by some of the paired stimulation studies highlighted in this review. These studies compared the effects of varying interstimulus intervals on acute outcomes, mostly related to electrophysiological rather than clinical function. As the paired stimulation field matures, more studies need to compare the effects of paired versus unpaired stimulation across multiple sessions, on meaningful clinical outcomes, in humans with relevant neurological conditions. It is critical to directly compare paired stimulation to unpaired (or sham) stimulation. In particular, for protocols that rely on precise timing of pairing, the most appropriate control will use paired stimulation, but at intervals that are ineffective at producing short-term physiological or behavioral changes.

How Does Stimulation Duration Influence Effect Duration? Most stimulation sessions last on the order of minutes. Effects have been measured over periods ranging from immediately after a single session to hours, days, or weeks after completion of multiple sessions. Some of these paradigms and schedules have been based on results of in vitro experiments of synaptic plasticity. Other schedules have been chosen to maximize convenience in human subjects. In many cases, stimulation schedules were chosen empirically and then

reproduced in subsequent studies while varying other factors. Is this an optimal approach? The entire field of neurostimulation desperately needs a more systematic approach to defining optimal stimulation schedules. How long should an individual session be? How many sessions should be applied? What are the best intersession intervals [100, 101]? To address these questions, an important assumption first needs to be validated: are short-term physiological effects predictive of long-term behavioral effects? If so, then baseline experiments can focus on short-term physiological effects and then subsequent experiments would aim to optimize longer-term physiological and behavioral effects. These studies would systematically alter the duration and frequency of stimulation in order to maximize the lasting effects. Optimization of these protocols should be a goal for the field.

How Do Relative Frequency and Intensity of Paired Stimuli Affect Outcome? Extensive literature documents the effects of interstimulus interval, frequency, and intensity when using single-site stimulation such as TMS. However, there is no clear consensus or formula that dictates which frequency or pattern to use for specific paired scenarios, or how to titrate relative intensity between two stimulation sites. To date, more attention has been directed toward the relative timing of paired stimuli arrival at target synapses. More effort needs to be devoted to optimizing paired pulse frequency and intensity in order to improve paired stimulation efficacy.

How Can Target and Off-Target Effects of Paired Stimulation be Monitored in Real Time? Stimulation of one node of a highly interconnected network makes it impossible to confine the effects exclusively to a target pathway. A more realistic goal is to maximize on-target relative to off-target circuit activation. To do this, we need to better understand the networks that are activated by paired stimuli. Ideally, this would involve visualization (or detection) of synaptic events in real time. For analysis of affected circuits, animal models offer advantages of invasive electrophysiology and imaging of neural activity within tracts and at synapses. This approach is likely to yield insights into the systems-level mechanisms of paired stimulation.

While animal studies can provide fundamental insight, the systems mechanisms of paired stimulation must also be studied in humans. In part, mechanistic studies are critical because of the myriad differences between humans and laboratory animals in the scale and organization of neural circuits. In addition, the stimulation protocols used in each species differ significantly. Modeling of current flow within tissues and mathematical predictions of circuit effects may prove helpful in translating animal studies to human studies [102–106]. But direct mechanistic studies of local and network effects of paired stimulation in humans are critical. This may involve use of established physiology techniques along with functional imaging of the human nervous system. In this way, mechanistic understanding and functional effects of paired stimulation may be translated from animal models into effective therapy for people with neurological impairments.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Spinal Plasticity and Behavior: BDNF-Induced Neuromodulation in Uninjured and Injured Spinal Cord

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family of signaling molecules. Since its discovery over three decades ago, BDNF has been identified as an important regulator of neuronal development, synaptic transmission, and cellular and synaptic plasticity and has been shown to function in the formation and maintenance of certain forms of memory. Neural plasticity that underlies learning and memory in the hippocampus shares distinct characteristics with spinal cord nociceptive plasticity. Research examining the role BDNF plays in spinal nociception and pain overwhelmingly suggests that BDNF promotes pronociceptive effects. BDNF induces synaptic facilitation and engages central sensitization-like mechanisms. Also, peripheral injury-induced neuropathic pain is often accompanied with increased spinal expression of BDNF. Research has extended to examine how spinal cord injury (SCI) influences BDNF plasticity and the effects BDNF has on sensory and motor functions after SCI. Functional recovery and adaptive plasticity after SCI are typically associated with upregulation of BDNF. Although neuropathic pain is a common consequence of SCI, the relation between BDNF and pain after SCI remains elusive. This article reviews recent literature and discusses the diverse actions of BDNF. We also highlight similarities and differences in BDNF-induced nociceptive plasticity in naïve and SCI conditions.

1. Introduction

After three decades of research, significant advances have been made in unraveling the cellular effects of brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family of growth factors that is encoded by the *bdnf* gene. BDNF was purified as the second member of the neurotrophin family in 1982 by Barde et al. [1]. Mature BDNF (14 kD) is cleaved from pro-BDNF by a series of serine proteases or convertase enzymes such as furin, PACE4, and PC5/6-B [2]. The serine protease tissue plasminogen activator has also been shown to play a role in the cleavage of pro-BDNF into mature BDNF. Mature BDNF exists as a dimer and mediates its cellular functions through two receptors: the high affinity, ligand-specific tropomyosin receptor kinase B (TrkB) and the p75 neurotrophin receptor ($p75^{NTR}$), which is a low affinity, nonselective neurotrophic receptor. Upon BDNF binding the TrkB receptor, a number of events follow. (i) The TrkB receptor dimerizes, which leads to (ii) autophosphorylation of the receptor and (iii) the subsequent activation of intracellular signaling cascades. These include the mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLC-γ), and phosphatidylinositol-3 kinase (PI3-K) cascades (see [3–7] and Figure 1). Activation of these pathways leads to a myriad of cellular actions including synaptic modulation and neuroplasticity, cell survival, axonal elongation, and neurite outgrowth. The activation of $p75^{NTR}$ similarly produces a variety of cellular events, ranging from neuronal differentiation to apoptotic cell death. Numerous studies have shown that $p75^{NTR}$ is the preferred receptor via which the precursor protein, pro-BDNF, mediates various cellular actions.

Neural plasticity is essential to physiological functions. BDNF is a potent modulator of neural plasticity. It exerts diverse modulatory actions that range from neurogenesis to

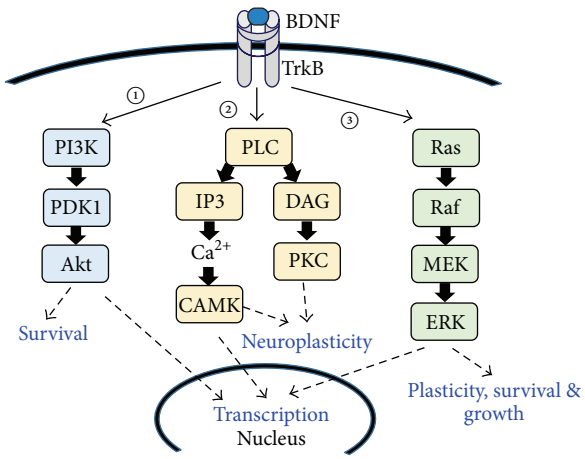

Figure 1: BDNF-TrkB dependent pathways: BDNF binds its high affinity receptor Tropomyosin-related kinase B (TrkB) and activates three main pathways (adapted from [3–5]): ① the PI3K/Akt pathway which has been shown to mediate cell survival function of BDNF, ② the phospholipase C (PLC) pathway which leads to the activation of protein kinase C (PKC), and ③ the Ras pathway which activates extracellular signal-regulated kinase (ERK). Activation of the kinases, PKC and ERK, leads to posttranslational modification such as phosphorylation and transcription. These processes are critical to neuroplasticity, including central sensitization and LTP. BDNF also binds the nonselective $p75^{NTR}$.

learning and memory formation. BDNF's actions appear synonymous with neuromodulation. While BDNF does not necessarily initiate an event such as a synaptic, sensory, or motor response, it can modify the response, exerting inhibition or facilitation. Such effects have been observed in the dorsal root ganglia, spinal cord, and various brain regions, where BDNF actions are typically associated with increased excitability, pronociception, learning, and memory. An important investigation into BDNF's effects focuses on its ability to modify injury-induced plasticity. BDNF has been shown to mediate inflammatory and peripheral injury-induced pain. However, as discussed below, after spinal cord injury (SCI), BDNF's actions appear to be more complex. Whereas treatments that increase spinal levels of BDNF are shown to promote functional recovery and adaptive plasticity, their effects on sensory functions, nociception, and pain remain elusive.

It should not be surprising that BDNF also exerts deleterious effects, which are typically associated with its overexpression. Although much can be said about the variety of outcomes that arise from pro-BDNF and mature BDNF interactions with both TrkB and $p75^{NTR}$, some of which we briefly discuss, in this article, our goal is to provide a comprehensive review on the neuromodulatory actions of BDNF, with greater emphasis on the effects of mature BDNF. We will (1) offer an overview of BDNF as a modulator of neural plasticity and (2) discuss the differential roles BDNF plays in spinal plasticity in intact and injured spinal cord, focusing on BDNF's effect on nociceptive plasticity. Despite the significant progress that has been made over the last 30 years, we will show that many questions remain unanswered.

2. BDNF Is an Important Modulator of Neural Plasticity

The heterogeneity of BDNF's actions is due to its ability to engage distinct signaling pathways (Figure 1). Early investigations into these signaling mechanisms showed that BDNF signaling is involved in the formation, maturation, and function of excitatory and inhibitory synapses [8–12] and in synaptic plasticity [12, 13].

2.1. Overview of Plasticity. Plasticity, "the ability to adapt," is an important feature of the central nervous system (CNS) [14–16]. Neural and functional changes that occur during development and maturation are crucial examples of plasticity. BDNF functions as a modulator of plasticity during development and into adulthood. A well-established role of BDNF is its ability to promote neuronal survival and development. This important function was initially demonstrated in the visual system, for example, [17, 18] and extended to other sensory systems such as the vestibular-auditory system [19]. Earlier studies revealed that, within the CNS, BDNF promotes neuronal development, although several of these studies indicated a preference towards sensory neurons, for example, [20–22]. The study by Jones et al. [20] showed that targeted disruption of the BDNF gene reduced the survival of offspring past 2 days after birth. Moreover, in offspring that survived, brain and sensory neuron developments were severely reduced. Along with the overwhelming number of reports demonstrating a preferential role of BDNF in developing sensory systems, several studies have shown that BDNF similarly functions as a survival factor for developing motoneurons [23, 24]. It is clear from these studies that, during early developmental periods, BDNF promotes adaptive plasticity, although the mechanisms that drive these actions have not been fully elucidated.

The fundamental ability of the CNS to function during postdevelopment and into mature stages relies largely on plasticity of synaptic connections. This includes changes in the number and efficacy of synaptic connections, which can ultimately direct neuronal activity. In addition to functioning as a critical survival factor during development, BDNF exerts numerous actions in developing and mature neural systems which draw on its ability to mediate or modify activity-dependent synaptic plasticity, learning, and memory. In many brain regions, BDNF plays a role in synaptic plasticity, although this has been most studied in the hippocampus, a brain region that encodes memory and learning. Long-term potentiation (LTP) is defined as an activity-dependent maintained increase in synaptic efficacy [25]. LTP, which is presumably the best described form of synaptic plasticity, has been proposed as a neural substrate of learning and memory. In general, its induction and maintenance require various cellular substrates such as the NMDA receptor, signaling kinases, posttranslational modification (e.g., phosphorylation), and transcription.

2.2. BDNF's Role in LTP, Learning, and Memory. As a modulator of neural plasticity, studies show that BDNF is implicated in the induction and maintenance of LTP.

Initial investigation showed that BDNF induces membrane depolarization and subsequent firing of action potentials in hippocampal neurons [26]. However, several additional studies reported a more direct role of BDNF in the induction of hippocampal LTP. For instance, exogenous administration of BDNF produces synaptic facilitation [27, 28]. Several studies using pharmacological or genetic approaches show that the synaptic facilitation is mediated primarily via the TrkB receptor. For example, administration of the fusion protein TrkB-IgG, which scavenges endogenous BDNF, attenuated the synaptic response induced by tetanic stimulation and the subsequent LTP [27]. BDNF similarly plays a role in the late phase of LTP [29], a process requiring the induction of gene transcription [30]. Late phase LTP is significantly attenuated when endogenous BDNF is sequestered with TrkB-IgG [31]. Genetic approaches have also been used to substantiate the importance of BDNF signaling via the TrkB receptor in the development of LTP. Minichiello et al. [32] showed that LTP in CA1 is severely impaired following conditional knockout of the TrkB receptor in the forebrain. In recent years, studies have continued to describe possible mechanisms by which BDNF contributes to hippocampal LTP. These include a recent study by Leal et al. [33] wherein it was reported that hippocampal plasticity, evidenced as an increase in synaptic activity, is accompanied by BDNF-mediated trafficking of ribonucleoproteins to dendrites. Similarly, Edelmann et al. [34] showed that, in the hippocampus, timing-dependent LTP induced by repeated pairing of one presynaptic action potential with four postsynaptic spikes requires BDNF/TrkB signaling and insertion of new AMPA receptors. Notably, these studies demonstrate how essential BDNF is to hippocampal LTP.

As previously stated, long-term maintenance of synaptic efficacy or LTP has long been identified as a possible neural mechanism of memory and learning. Since BDNF is necessary for hippocampal LTP and long-term maintenance of synaptic efficacy and/or LTP has been implicated as a mechanism of learning and memory, BDNF would be expected to affect behavioral manifestations of learning and memory *in vivo*. Several studies are consistent with this possibility. For example, in adult rats, exogenous administration of BDNF into the hippocampus protects from the development of stress-induced learning and memory impairment [35], and stress-induced impairment of learning and memory is marked by a decrease in hippocampal levels of BDNF mRNA [36]. Genetic, as well as pharmacological, approaches have also been instrumental in providing such evidence. Deficits in endogenous BDNF levels produce severe impairments in spatial learning and memory tasks in both mice [37] and rats [38]. The contribution BDNF makes to learning and memory extends to brain regions other than the hippocampus. For example, conditional knockout of TrkB receptors in the forebrain of mice resulted in impaired learning behaviors [32], and mice overexpressing TrkB receptors in the cortex and hippocampus showed facilitated learning [39]. A recent study by Ju et al. [40] reported that, in adult rats, BDNF signaling in the amygdala is necessary for conditioned place aversion (CPA) behavior induced by naloxone-precipitated morphine withdrawal. In this study, CPA was accompanied with elevated BDNF levels but was completely blocked by inhibition of BDNF in the amygdala. Many additional studies strongly implicate BDNF signaling through the TrkB receptor in learning and memory. Also, they typically illustrate a direct role of BDNF in both cellular/synaptic (e.g., LTP) and behavioral aspects of learning and memory.

2.3. Other "Deleterious" Effects of BDNF. The various actions of BDNF also include deleterious effects. Although not exclusively, these effects are more commonly linked to the overexpression of BDNF and $p75^{NTR}$ signaling. The expression of inflammatory and neuropathic pain could easily be seen as an important maladaptive effect of BDNF. As we discuss below, the development of pain is intricately associated with an increase in the expression of BDNF. However, there are several additional effects of BDNF that are deleterious in nature, which we briefly discuss in this section. First, epileptogenesis has been linked to the overexpression of BDNF in the hippocampus and/or cortex [41]. Supporting a role of BDNF in the development of epilepsy, a study by Kokaia et al. [42] showed that epileptogenesis is markedly suppressed in mutant mice expressing reduced levels of BDNF. BDNF's effect on epileptogenesis might be mediated through its high affinity receptor, TrkB. Several studies have shown that manipulations that perturb BDNF-TrkB signaling reduce the development of epilepsy. In contrast, overexpression of the full-length TrkB receptor, but not the truncated TrkB receptor, promotes epileptogenesis [43]; also see [44]. Given that the imbalance of inhibition and excitation is crucial to the development of epilepsy, these observations show that BDNF exerts modulatory control over both inhibitory and excitatory synaptic transmission in the brain.

Apoptotic cell death is a second deleterious effect of BDNF signaling system. However, this effect appears to be mediated by $p75^{NTR}$ which is not only a BDNF-specific receptor, but also activated by nerve growth factor (NGF), neurotrophin- (NT-) 3, and NT-4/NT-5. The role $p75^{NTR}$ plays in apoptotic cell death has been well studied. In a study by Roux et al. [45], it was reported that, after pilocarpine-induced seizure, the expression $p75^{NTR}$ is increased in neurons undergoing apoptosis. Studies from the Hempstead laboratory showed that $p75^{NTR}$ plays a role in producing apoptotic death of both neuronal [46, 47] and nonneuronal [48] cells. Additionally, they provided evidence that $p75^{NTR}$-mediated apoptosis can be initiated by pro-BDNF [46]. Mature BDNF activation of the $p75^{NTR}$ has also been shown to induce developmental apoptosis in sympathetic neurons [49]. Whereas studies consistently show that pro-BDNF-$p75^{NTR}$ signaling induces apoptosis (also see Koshimizu et al. [50]), it should be noted that this interaction results in neuronal remodeling [51] and shapes neurite outgrowth [52]. A study by Fan et al. [53] showed that pro-BDNF-$p75^{NTR}$ signaling can lead to selective death of axotomized sensory neurons following sciatic nerve transection. Clearly, pro- and mature BDNF actions through $p75^{NTR}$ signaling have important physiological and cellular functions.

BDNF is also implicated in the stress response. Glucocorticoid receptors can interact with the TrkB receptor to

modulate BDNF signaling [54, 55]. Studies typically show that glucocorticoids or their receptor ligands [55, 56] and stress [57–59] can suppress BDNF levels or its downstream signaling (also see reviews by Jeanneteau and Chao [60] and by Suri and Vaidya [61]). More recent studies implicating BDNF in stress have shown epigenetic regulation of the BDNF gene in response to various stress paradigms [62–64]. BDNF also plays a role in the pathophysiology of depression presumably by interacting with the monoaminergic systems (see reviews [65–67]). There are several other effects of BDNF signaling mechanisms that can produce devastating consequences. However, in the following sections of this review, we aim to discuss the role BDNF plays in nociceptive plasticity and pain in the intact and injured spinal cord.

2.4. BDNF's Role in Spinal Plasticity. The concept that chronic pain is maintained as a "memory trace" was first put forward by Coderre and Melzack [68]. This led to studies aimed at identifying commonalities between the neural mechanisms that underlie pain and those that encode learning and memory processes. Furthermore, as central sensitization, which is an increase in the excitability of the neuronal networks in the central nervous system, is considered the most likely neural substrate underlying spinal nociception and pain [69], many studies focused on mapping the neural processes and specific mediators involved. Given the similarity that exists between LTP and central sensitization at the cellular level, BDNF was initially proposed to play a critical role in central sensitization and inflammatory pain in the late 1990's with studies originating from the McMahon and Thompson laboratories. The results from these earlier studies suggested that, under inflammatory conditions, BDNF levels are increased in sensory neurons and this increase causes a subsequent facilitation of nociceptive spinal reflex excitability [70]. This study by Kerr et al. [70] first identified BDNF as critical to the development of inflammatory pain. Additionally, it identified small diameter "pain" fibers as a primary source of spinal BDNF, particularly after peripherally induced inflammation. To further demonstrate that BDNF plays a role in pain, it also showed that inflammatory pain induced by administration of formalin or carrageenan is significantly attenuated by TrkB-IgG. Thus, these observations demonstrated a novel function of BDNF; that is, elevated spinal levels of endogenous BDNF are necessary for inflammatory pain behaviors. In addition to these behavioral results, BDNF actions consistent with nociception were also observed at the cellular level. Specifically, Thompson et al. [71] showed that exogenous application of BDNF enhanced C fiber evoked ventral root reflexes and pretreatment with TrkB-IgG attenuated the amplitude of the ventral root potentials. Since these initial studies were undertaken, numerous studies have examined the role BDNF plays in sensory plasticity: central sensitization, nociception, and pain in intact CNS. The general consensus from many of these studies is that BDNF typically elicits pronociceptive actions, ranging from an increase in neuronal excitability to mediating pain behaviors, for example, [72–75].

Several additional studies have successfully associated BDNF with the development of pain. BDNF is upregulated in sensory neurons after peripheral inflammation induced by NGF or complete Freund's adjuvant [76–78]. In addition, inflammatory pain may cause a phenotypic switch of BDNF-expressing neurons, so that even large diameter sensory neurons express BDNF [79]. Lever et al. [80] showed that BDNF is released into the dorsal horn following electrical stimulation of afferent fibers at C fiber strength or after chemical stimulation by capsaicin. These apparent nociceptive actions of BDNF are not confined to DRG-to-dorsal horn neurons. Instead, even at supraspinal centers, BDNF-TrkB signaling appears to mediate descending pain facilitation [81].

BDNF is also implicated in the development of chronic neuropathic pain. For instance, several studies have shown elevated BDNF levels in sensory neurons and the dorsal horn in a variety of chronic neuropathic pain models [82–86]. Also, as observed in inflammatory pain, a phenotypic switch wherein larger diameter sensory neurons express BDNF is observed following sciatic nerve lesion [87]. The observation that BDNF is upregulated in sensory neurons of BDNF in these primary afferent fibers is consistent with its role in promoting nociceptive plasticity and pain (also see review by Pezet and McMahon [88]).

To investigate BDNF's actions as a mediator of nociceptive plasticity of spinal neuronal networks, in Mendell's laboratory, we performed electrophysiological experiments to assess BDNF's effects at the synaptic level [89]. We used a transverse spinal cord slice preparation in neonatal and young rats to study the effect bath-applied BDNF has on NMDA-induced currents and dorsal root-evoked synaptic responses in lumbar 2- (L2-) L5 lamina II neurons. After characterizing the synaptic responses as AMPA/kainate receptor mediated and elicited primarily by electrical stimulation of small diameter, high threshold afferents (C fibers), we found that BDNF produced a long-lasting (irreversible) potentiation of the synaptic responses. Bath-applied NMDA-induced inward currents were also facilitated by BDNF. We also showed that BDNF-induced facilitation of synaptic currents is blocked by NMDA receptor blockade with D-APV (Figure 2). A more thorough investigation into the underlying mechanisms of BDNF-induced synaptic facilitation revealed that postsynaptic NMDA receptors, postsynaptic PLC-δ-PKC signaling, and increases in intracellular calcium in the lamina II neuron are all required for BDNF facilitation. These observations, which are summarized in Figure 3, indicate the similarity in the underlying neural mechanism that produces central sensitization or nociception with that mediating BDNF-induced synaptic plasticity. Lamina II receives synaptic input primarily from C fibers and is the initial nociceptive processing site within the CNS. Therefore, our results suggested that BDNF's pronociceptive actions might arise from its ability to produce a maintained (LTP-like) synaptic modification of excitatory responses in lamina II neurons.

A prolonged facilitation of nociceptive synaptic responses in lamina II may represent one of several mechanisms that underlie BDNF's role in pain/nociception. Other investigators showed that an increase in BDNF from activated spinal microglia is critical to peripheral injury-induced pain [96–99]. Importantly, these observations were the first to show that activated microglia release BDNF. These studies also indicated that BDNF released from activated microglia could

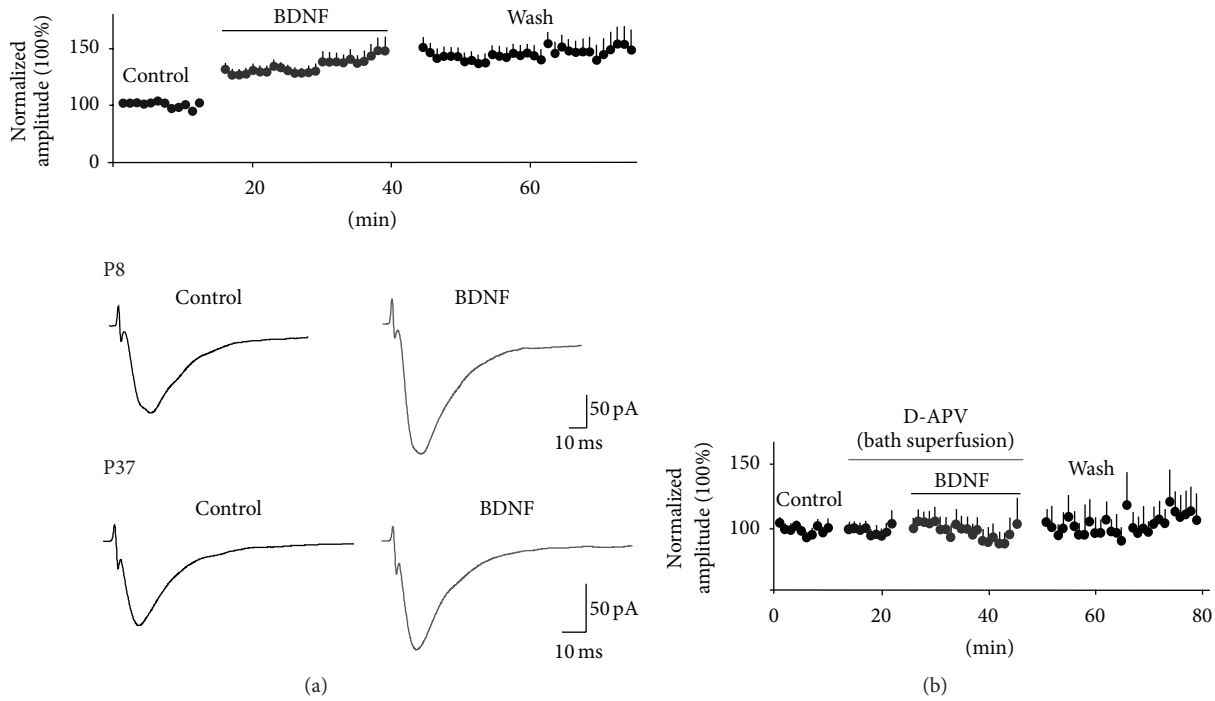

Figure 2: BDNF facilitates synaptic responses. (a) (Top) bath-applied BDNF potentiates dorsal root-evoked synaptic responses in lamina II neurons. In the presence of BDNF, synaptic amplitude is increased by ~30% and remains facilitated during wash. The data represent the average change in synaptic amplitude for all neurons, obtained from animals postnatal days 1–14. The average synaptic amplitude before BDNF application was computed for each neuron and was then normalized to the mean baseline amplitude denoted as 100%. The mean percent change in synaptic amplitude was calculated as the difference between the mean peak amplitude during drug treatment or wash and the mean value of the synaptic responses before drug (control). (a) (Bottom) the average synaptic responses are shown for recordings obtained in P8 and P37 rats before and during BDNF. Note: BDNF-induced facilitation is observed at both ages. (b) Bath application of the NMDA receptor antagonist, D-APV for 10 min prior to and during BDNF application blocks BDNF-induced synaptic facilitation ($n = 14$). Data previously reported in Garraway et al. [89, 90].

function as the critical signaling molecule that bridges glia and neuronal associations that underlie neuropathic pain. The study by Coull et al. [96] was groundbreaking on many fronts. It showed that neuropathic pain after nerve injury results from a BDNF-mediated shift in neuronal anion gradient, which causes the inhibitory neurotransmitter GABA to produce excitatory currents [96, 100]. These effects of BDNF result from the intricate interaction between BDNF and the chloride transporter, KCC2 (discussed below, also see Figure 7). In addition, for the first time there was evidence that resident spinal cells release BDNF. This assertion dispelled the previous dogma that small diameter primary afferents are the only source of spinal BDNF. Overall, the study reinforced the critical role BDNF and microglia play in injury-induced pain hypersensitivity [96].

2.5. BDNF-TrkB Signaling in Nociceptive Plasticity and Pain. As previously mentioned, BDNF binds the TrkB receptor with high affinity leading to the activation of the PI3K-Akt, PLC, and MAPK/ERK pathways. Many studies provided a direct evidence to support BDNF-TrkB signaling in the development of inflammatory and/or neuropathic pain. An earlier study by Mannion et al. [72] showed that peripheral inflammation and C fiber electrical activity that increased BDNF expression in the DRG also increased full-length TrkB receptor levels in the dorsal horn (also see [85, 101]). Even in the brainstem, TrkB levels are elevated in a model of chronic inflammatory pain [102].

The MAPK/ERK and PLC-γ-PKC pathways are shown to play a role in mediating the pronociceptive effects of BDNF. Activation of ERK and PKC pathways by BDNF can induce phosphorylation of the NMDA-R1 subunit [103]. Both activated ERK and phosphorylated NMDA-R1 are essential to the development of inflammatory pain [104, 105]. In our initial investigation into BDNF's actions on spinal pain systems, we showed that inhibition of PLC-γ-PKC signaling blocks BDNF-induced facilitation of C fiber evoked synaptic responses in lamina II [89]. Activation of the PLC-γ pathway leads to increases in intracellular calcium, either from internal calcium stores or via phosphorylated calcium-permeable glutamate receptors. Hence, to further support the importance of PLC-γ mediated pathways, our study also showed that BDNF-induced facilitation of synaptic and NMDA-induced currents were blocked when intracellular calcium in the spinal neurons was chelated with BAPTA [89] (Figure 3). These observations are consistent with the many reports showing that stimulation of the MAPK/ERK and PLC-γ-PKC, activation of critical downstream kinases, and elevated levels of intracellular calcium are crucial processes in

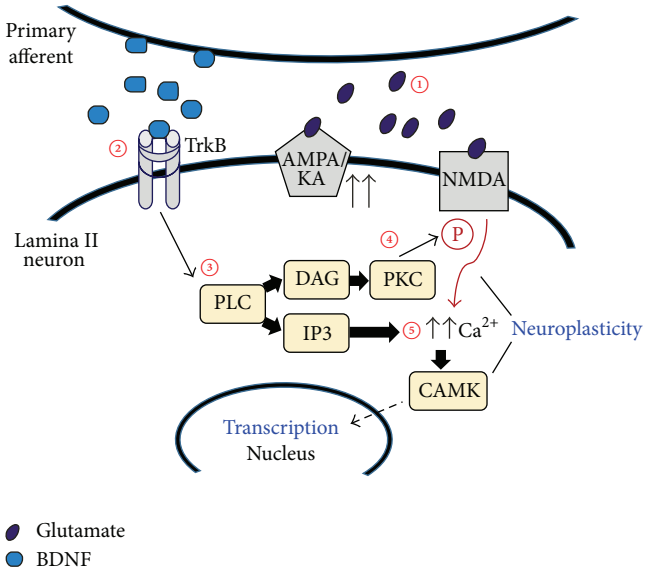

Figure 3: Proposed mechanism of BDNF-induced synaptic facilitation. Small diameter primary afferents express glutamate and BDNF. ① Under normal conditions, dorsal root stimulation evokes stable glutamatergic synaptic responses in lamina II neurons. ② Increased excitability of primary afferents causes the release of both glutamate and BDNF, which binds to TrkB receptors. Engagement of the TrkB receptors recruits the ③ PLC which can lead to activation of ④ PKC and ⑤ an increase in intracellular calcium $[Ca^{2+}]_i$. Both PKC and calcium dependent kinases such as CAMK can phosphorylate glutamatergic receptors, thereby increasing their calcium permeability. Consequently, these processes lead to an NMDA-R dependent facilitation of glutamatergic currents. Adapted from data reported in Garraway et al. [89].

the development of nociceptive plasticity, inflammatory, and neuropathic pain (Figure 8). They also suggest that BDNF can evoke pronociceptive actions by engaging these intracellular mechanisms, postsynaptically.

3. BDNF Impacts Plasticity after SCI

3.1. Overview of SCI. Spinal circuits are susceptible to long-term neuromodulation that can alter how they function. Research studies from several laboratories including ours have focused on the types of neuromodulation (plasticity) of spinal networks that arise after SCI. Having established that BDNF functions as a neuromodulator of spinal networks, many studies examined reciprocal interactions between BDNF function and SCI and the consequence these interactions have on spinal cord networks and functions. SCI results in a myriad of behavioral and cellular consequences. Behaviorally, the effects of SCI include paralysis, muscle spasticity, and impaired bladder, bowel, and sexual function. Pain, including chronic neuropathic pain, is also a common debilitating consequence of SCI. The cellular changes induced by SCI, which typically drive these pathologies, include an increase in the proliferation and activation of glial cells, the release of proinflammatory cytokines from activated glia, glutamate spill, and excitotoxicity, which can eventually lead to necrotic and apoptotic cell death, for example, [106–108]. Secondary to these cellular effects are morphological changes such as afferent sprouting, cavitation, and gliosis-induced scarring. The milieu created by the collusion of these cellular and morphological changes has impeded the prospect of full repair after injury.

3.2. BDNF's Effect on Axonal Regrowth and Recovery of Locomotion after SCI. In the developing nervous system, BDNF plays an important role of promoting neuronal growth and survival. Thus, it was posited that similar effects of BDNF could be observed following SCI. Many studies have assessed whether BDNF can repair the injured spinal cord and rescue locomotor function after SCI. Schnell et al. [109] first investigated the effect of the neurotrophins, BDNF, and neurotrophin-3 (NT-3) on sprouting of corticospinal tract fibers after SCI. They found that whereas NT-3 promoted regeneration of these fibers, BDNF did not. Meanwhile, reports coming out of other laboratories reported neuroprotective effects of BDNF, especially after injury [110, 111]. Following these initial studies, there was an overwhelming increase in the number of research studies that focused on unraveling the neuroprotective effects of BDNF. Numerous studies examined whether treatment with BDNF could promote axonal regrowth across the injury site or sprouting of supraspinal projections. In many of these studies, approaches were implemented that allowed for successful long-term administration of BDNF, applications such as the use of mini osmotic pumps [112–114], or cellular grafts genetically modified to secrete BDNF [115–119]. Commonly, the results showed that BDNF treatment resulted in neuroprotection, as well as promotion of regeneration and sprouting of axonal fibers. Studies from the Houle laboratory showed BDNF-induced regeneration of descending supraspinal neurons, including serotonergic and vestibulospinal tract neurons, for example, [120–122]. Quite often it was also reported that behavioral recovery complemented the cellular effects. For example, BDNF enhanced the recovery of locomotor functions after SCI [113, 118]. Critical to BDNF's role in promoting axonal growth, afferent sprouting, and functional recovery, it was also shown in later studies that BDNF suppressed apoptosis in neurons and oligodendrocytes following SCI [123, 124].

3.3. BDNF Promotes Adaptive Plasticity after SCI. For many years the Grau laboratory has undertaken studies on spinal plasticity using a simple instrumental learning paradigm in adult rats with a complete thoracic (T) level 2 spinal transection. They showed that the induction and maintenance of spinal learning require functional NMDA receptors [125], reviewed by [126]. NMDA receptor dependence is a feature common to most types of plasticity such as learning and memory [127–130], synaptic plasticity (LTP and LTD) [129, 131, 132], and nociceptive plasticity [104, 133, 134]. The spinally mediated form of instrumental learning studied here similarly requires many of the cellular processes that are involved in other forms of plasticity such as kinase activity and protein synthesis [126]. As discussed, BDNF plays a role in the induction and expression of LTP. It can also

induce synaptic facilitation and enhance excitability of spinal neurons, for example, [75, 89]. For these reasons, recent investigations focused on whether BDNF is equally involved in the processes that mediate isolated spinal learning. Using the standard Master-Yoke learning paradigm in T2 completely transected adult rats [135], cellular assays were undertaken to relate changes in the expression of BDNF and several "plasticity" genes with spinal learning [136]. For the Master-Yoke experimental design, rats are set up for instrumental learning in pairs. Each pair consists of one subject (Master), which is given response-contingent shock, wherein legshock is applied whenever the leg is extended and terminated when the leg is flexed (controllable shock). The second subject is experimentally yoked to the master rat and receives shock at the same time and for the same duration as the master rat but independent of leg position (uncontrollable shock, yoked). A third group, which serves as unshock controls, is set up in the same manner but does not receive any stimulation. Master-Yoke pairs and unshock controls undergo a 30-minute training phase. Following training, each subject undergoes instrumental testing for 30 minutes under the same conditions. During the testing phase, all three groups receive response-contingent shock. Immediately following instrumental testing, master rats that received controllable shock during the training phase and showed spinal learning during the testing phase had elevated spinal BDNF mRNA levels. In contrast, yoked subjects had decreased spinal levels of BDNF compared to unshock controls. Because yoked subjects fail to learn during the instrumental testing phase, these observations suggested that decreases in BDNF levels are detrimental to spinal learning [136].

In a more recent study, we used *in situ hybridization* and western blot to assess mRNA and protein levels, respectively, following Master-Yoke training [91]. Elevated BDNF mRNA was observed in master subjects in both dorsal and ventral spinal cords. There was no difference in mRNA levels in unshock controls versus yoked subjects. Similar effects were observed with protein, in that BDNF levels were increased in master subjects but unchanged in yoked subjects compared to unshock controls. Additional experimentation provided further support that endogenous BDNF is critical to the protective effect of spinal training processes and that administration of BDNF prevents the spinal learning deficit induced by uncontrollable shock (Figure 4). Together, our prior results are consistent with those observed in other CNS regions, such as the hippocampus, where BDNF is explicitly shown to be involved in the neural processes that underlie learning [35, 137].

The role BDNF plays in spinal learning and other forms of plasticity after SCI is unequivocally linked to changes in the expression and function of the BDNF receptor, TrkB. In regard to the effect SCI has on TrkB expression within the spinal cord, conflicting reports exist although the differences may be due to temporal and spatial experimental variables. Specifically, TrkB expression after SCI typically depends on the postsurgical time points and spinal segments used for the cellular assessment. Frisén et al. [138] reported elevated levels of TrkB mRNA and protein expression in the spinal cord after SCI. Interestingly, the increases were most pronounced between 3 and 6 weeks after injury. However, in contrast, several other researchers have reported that TrkB levels are decreased by SCI [139–141], generally in the acute stage of injury. Interestingly, after T2 spinal transection rats that received controllable stimulation had increased protein expression of TrkB compared to yoked and control subjects [91]. Moreover, the increased TrkB expression predominated in the dorsal spinal cord, but not ventrally. It should be noted that although we found adaptive plasticity, that is, spinal learning, to associate with elevated levels of both BDNF and TrkB, we also observed decreases in BDNF and TrkB levels immediately after SCI (discussed below).

3.4. BDNF's Effect on Pain and Nociception after SCI. It would appear from these aforementioned studies detailing the progress made at the basic science level that the clinical use of BDNF after SCI is inevitable. Surprisingly, according to the U.S. National Institutes of Health (ClinicalTrials.gov), there is currently no account of clinical studies where BDNF is used to promote functional recovery after SCI even though BDNF is the focus of several non-SCI related clinical studies. The reasons for this apparent discrepancy and the slow progression of BDNF from "bench to bedside" despite the overwhelming promise shown at the basic research level are unknown but need to be explored in more depth. The following section discusses nociceptive plasticity after SCI and the differential effects of BDNF.

Like peripheral injury, central injuries including SCI produce chronic neuropathic pain [142, 143]. It is generally assumed that the mechanisms that underlie pain resulting from inflammation or peripheral injury are identical to those that underlie SCI-induced pain, with the main candidate being central sensitization [69]. Over the years, many investigators have investigated the mechanisms that produce central sensitization and identified key mediators (reviewed by [144]). Most notable are the NMDA receptors, increases in intracellular calcium, signaling kinases, and BDNF. More recently, additional mediators have been identified. These include the small molecular weight signaling proteins known as the cytokines and chemokine [145–147]. These signaling factors are generally released from immune-inflammatory cells. These recent discoveries add a new dimension to studying the development of pain hypersensitivity after SCI. No longer should we limit our attention primarily to neuronal plasticity, but rather we can also focus on the direct contributions of astroglial cells and the immune systems, as well as their ability to influence neuronal functions.

A hallmark feature of injury to the CNS is the subsequent accumulation, proliferation, and activation of glial cells. The observation that there is formation of a glial scar after injury to the CNS dates back to early reports by Cajal [148]. Moreover, it was shown then that the glial scar serves as a major inhibitor of neural regeneration. This early discovery was followed up by studies that focused on characterizing the cellular structure of the scar and evaluating possible means of overcoming this barrier to regeneration (for recent reviews, see [149–152]). Studies now indicate that the function of glial cells extends beyond the formation of a physical barrier after injury to the CNS. Both infiltrating immune

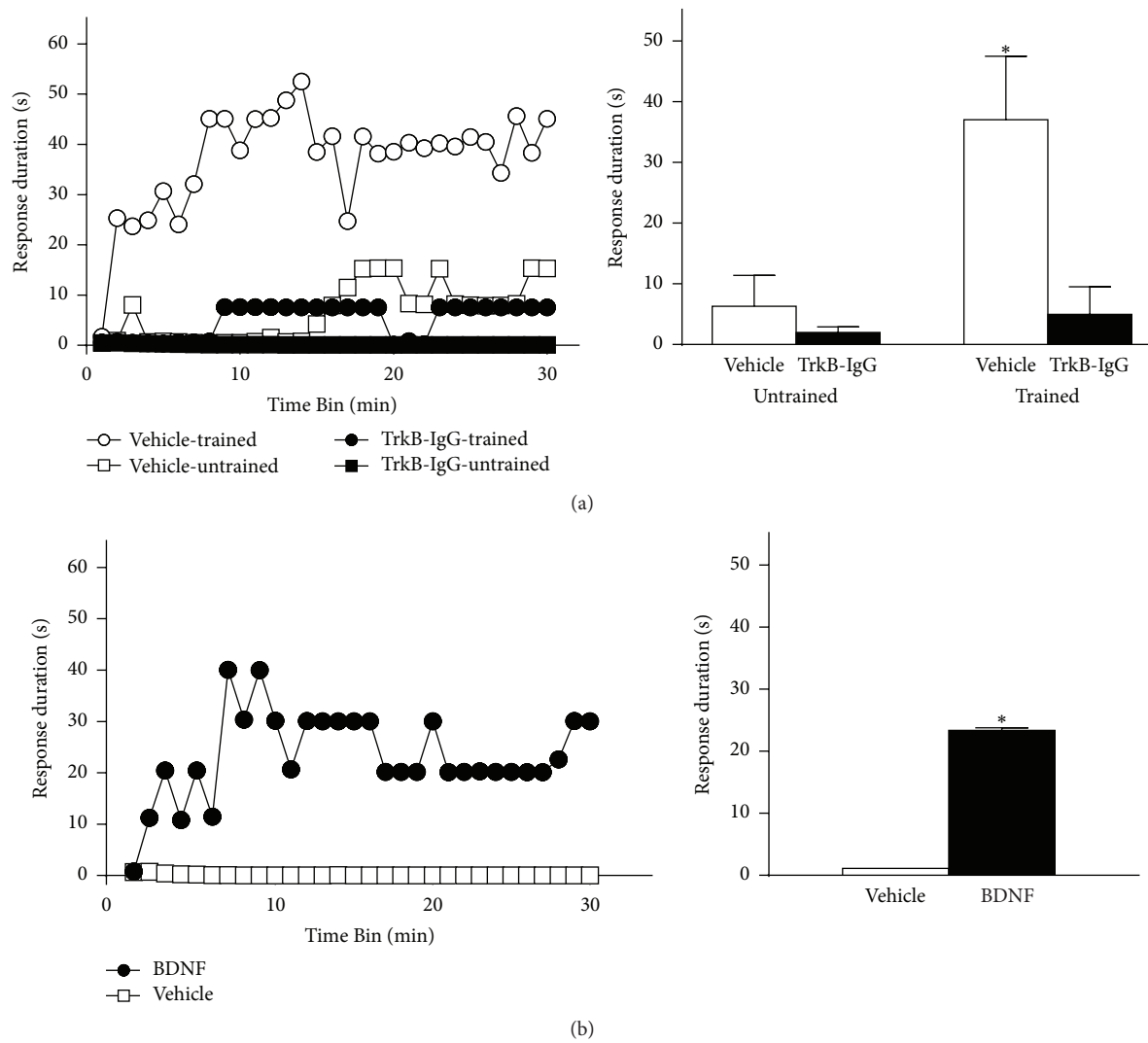

Figure 4: Role of BDNF in the protective effect of spinal instrumental training. (a) The necessity for endogenous BDNF in the spinal training effect. Rats received an intrathecal injection of either vehicle (saline) or the BDNF inhibitor TrkB-IgG and then either spinal instrumental training (trained) or none (untrained), followed by 6 min of uncontrollable shock. All subjects were then tested for instrumental learning over 30 minutes. Vehicle-treated subjects that were not trained prior to uncontrollable shock (vehicle-untrained) exhibited a learning deficit, whereas those prior training protected against this deficit (vehicle-trained). Subjects that received TrkB-IgG exhibited a learning deficit, regardless of training, indicating a necessary role for endogenous BDNF in the protective effect of spinal training. (b) To test the sufficiency for BDNF in the protective effect against uncontrollable shock, either vehicle or intrathecal BDNF was delivered prior to 6 minutes of uncontrollable shock. Rats that received vehicle prior to uncontrollable shock exhibited a deficit when tested for instrumental learning. Those treated with BDNF prior to uncontrollable shock were able to learn, indicating that BDNF is sufficient to protect against the maladaptive effects of uncontrollable shock ($^{*}p < 0.05$). Error bars indicate SEM. Adapted from Huie et al. [91].

cells and resident glia undergo structural and functional modifications after injury [153], ultimately enabling them to release several products. These include the inflammatory cytokines: interferon gamma, TNF alpha (TNFα) and the interleukins, and chemokines. Although they play various neural functions, many of these cytokines have been shown to promote deleterious effects after SCI, including the development of pain hypersensitivity and impaired locomotor functions. As reported by Coull et al. [96], activated microglia also release BDNF. Thus, one would expect that, together with the proinflammatory cytokines, the oversecretion of BDNF would only add to an environment that is favorable to maladaptive plasticity and pain hypersensitivity after SCI.

Despite the vast number of studies examining the behavioral effects of BDNF after SCI, few have looked at its cellular effects, especially in relation to nociceptive actions. This contrasts with the many studies aimed at understanding its role in neuropathic pain after peripheral nerve injury. Having shown that BDNF induces maintained facilitation of primary afferent-evoked synaptic responses in lamina II neurons in neonatal and young rats, an effect that is deemed pronociceptive [89], we turned our attention to investigate the actions

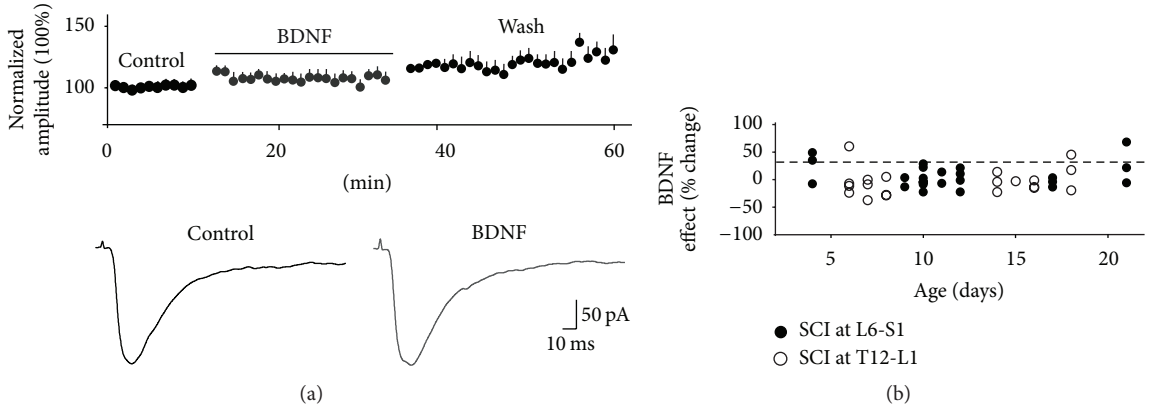

Figure 5: BDNF fails to induce synaptic facilitation after SCI. (a) Normalized data (mean ± SEM) averaged over all neurons show that synaptic facilitation was abolished after transection (at L6-S1 or T12-L1) or contusion (at T12-T13) SCI. (Some data points have smaller error bars than the points used to define the data.) Bottom: the average synaptic traces are shown for synaptic recordings obtained from a neonatal rat with contusion SCI before and during BDNF. (b) Mean percent change in synaptic amplitude in the presence of BDNF versus age is shown for each animal that received a transection injury. Although the effect of BDNF is somewhat variable, overall the effect is much reduced compared to the average facilitation of ~30% (represented by the horizontal dotted line) which was seen in uninjured animals of similar age. Also, BDNF's effects are not dependent on the location of injury. Recordings were done in L2–L5 lamina II neurons. From data previously reported in Garraway et al. [90, 92].

of BDNF after SCI. Following a transection SCI at T13/L1 or L6/sacral 1 (S1) or a contusion SCI at T12/T13, BDNF did not facilitate afferent-evoked synaptic responses in L2–L5 lamina II neurons [90, 92] (Figure 5), which was an unexpected finding. Equally surprising was the observation that BDNF's failure to induce synaptic facilitation was not dependent on the type or location of SCI. It seemed plausible that BDNF would induce an even greater facilitation of synaptic responses after SCI. The expectation was based on the notion that if peripheral injury increases both spinal levels of BDNF and the excitability of lamina II neurons, a similar effect could be anticipated after injury to the spinal cord. Additionally, it was already shown that SCI increases spinal neuronal excitability or output, although in nonpain processing spinal regions [154, 155], that SCI results in increased proliferation and activation of microglia, and that activated microglia release of BDNF plays a crucial role in neuropathic pain [96]. In contrast, we found that failure of BDNF to produce synaptic facilitation was linked to an injury-induced increase in $GABA_A$-mediated inhibition of spinal neurons coupled with suppression of NMDA receptor-mediated currents [92]. Although we did not observe any direct effect of BDNF on GABA currents, a previous study by Pezet et al. [156] showed that BDNF could directly stimulate the release of GABA from dorsal horn interneurons. Similarly, a study by Lu et al. [157] showed that, following long-term exposure of substantia gelatinosa neurons to BDNF, the amplitude of GABAergic inhibitory currents is enhanced. Together, these studies show a direct BDNF effect on GABAergic systems. However, it is unclear whether BDNF increases GABA levels after SCI.

The types of plasticity observed in developing nervous systems are not necessarily preserved into maturity. Therefore, before precise conclusions can be made on the kind of role BDNF plays in nociceptive plasticity after SCI, it is worthwhile to transition from a reduced preparation in juveniles to a mature, intact preparation which would allow for behavioral examination to be undertaken. We recently performed studies that investigated whether BDNF is involved in nociceptive plasticity and pain after SCI in an adult preparation, using the clinically relevant contusion SCI model at T12 [93]. The goal of the study was twofold: first, to assess the effect of SCI and noxious stimulation on the expression of key components of the BDNF signaling pathway and, second, to study interactions between the development of mechanical allodynia and changes in spinal expression levels of BDNF. Twenty-four hours after a moderate contusion injury, some adult rats received intermittent (~0.5 Hz) stimulation to the tail (SCI+SHK), at intensities that engage C fibers [158]. The remaining SCI subjects were placed in the shock apparatus but did not receive noxious tailshock (SCI+UNSHK). Our results first confirmed a prior study that showed that noxious tailshock negatively impacts the recovery of locomotion [159], as assessed by the Basso, Beattie, and Bresnahan (BBB) locomotor scale [160]. Our assessment of BDNF mRNA and protein levels showed that SCI alone significantly decreased both mRNA and protein levels at 25 hours and 48 hours after injury (equivalent to 1 hour and 24 hours after shock, resp.) (also see Strickland et al. [161]). However, noxious stimulation (SCI+SHK) produced an additional decrease in BDNF expression in the dorsal spinal cord. These changes in BDNF expression were nearly completely paralleled by TrkB mRNA and protein expression as well as that of the signaling kinases ERK1/ERK2 and CAMKII. In general, SCI decreased their expression levels during these early time points after injury, and noxious shock (SCI+SHK) produced additional decreases that were observed primarily in the dorsal spinal cord [93].

Our behavioral assessment showed that, during the acute postinjury period, when BDNF levels are reduced by SCI and noxious shock [93], the onset and magnitude of mechanical

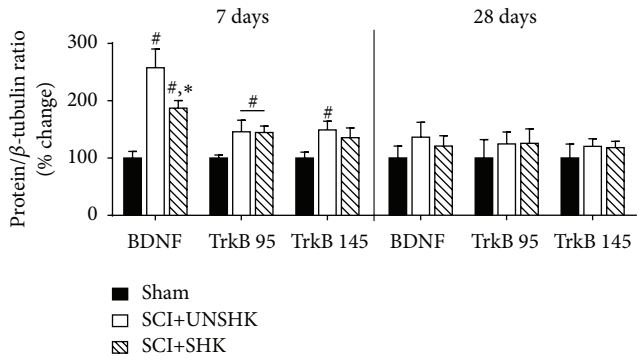

Figure 6: BDNF and TrkB expression in the dorsal spinal cord after SCI: Western blot was used to assess BDNF, $TrkB_{95}$ and $TrkB_{145}$ expression at 7 and 28 days after SCI in adult rats. Noxious tailshock was given 24 hours after SCI (SCI+SHK) to some animals. At 7 days after SCI, there was a significant increase in BDNF levels in the dorsal spinal cord of SCI animals that did not receive tailshock (SCI+UNSHK) and in SCI+SHK animals compared to sham controls. Although SCI+SHK animals had elevated levels of BDNF, shock treatment significantly decreased BDNF levels compared to SCI+UNSHK ($^{\#}p < 0.05$ indicates significance relative to sham and $^{*}p < 0.05$ indicates significance relative to SCI+UNSHK). Both SCI groups had elevated levels of $TrkB_{95}$ in the dorsal spinal cord compared to sham control, whereas $TrkB_{145}$ was only significantly increased in the SCI+UNSHK group of animals. At 28 days after injury, there were no differences in BDNF, $TrkB_{95}$, and $TrkB_{145}$ expression in the dorsal spinal cord among the three experimental groups.

allodynia are significantly increased [94]. These observations suggest that neither the induction of nociceptive plasticity nor the onset of neuropathic pain after SCI is dependent on elevated levels of spinal BDNF after SCI. A similar finding was reported by Ferguson et al. [162] which indicated that, after transection SCI, intermittent noxious shock that decreased BDNF levels also increased mechanical reactivity to von Frey stimulation. Overall the results from these studies undertaken in adult rats with either a moderate contusion or transection SCI indicate the likelihood that short-term maladaptive plasticity after SCI is associated with deficits in spinal BDNF levels not increases.

We extended the examination of the temporal changes in BDNF and TrkB expression in the dorsal spinal cord after SCI and noxious shock to 7 and 28 days after injury (Figure 6). The results revealed that SCI-induced reduction in BDNF and TrkB levels is transient. By 7 days following SCI, protein levels of BDNF, $TrkB_{95}$, and $TrkB_{145}$ were all elevated in SCI+UNSHK subjects compared to sham controls. Noxious stimulation produced a significant decrease in BDNF levels in the dorsal spinal cord, compared to SCI+UNSHK subject, although BDNF level remained elevated compared to sham controls. At 28 days after injury, BDNF levels and $TrkB_{95/145}$ in the dorsal spinal cord were similar in all three groups. In fact, other studies have reported that TrkB levels are increased in the later stages of SCI [138, 163]. Overall, these data sets fully support the concept previously stated that spatial and temporal variables are important in determining the expression level of BDNF and TrkB after SCI.

3.5. Maladaptive Plasticity and Impaired BDNF Signaling. From these aforementioned studies, we can posit that SCI-induced maladaptive plasticity following SCI is in part mediated by impaired BDNF-TrkB signaling within the spinal cord dorsal horn [91, 93]. Previous studies from the Grau laboratory proposed that shock-induced behavioral deficits after SCI are mediated by spinal mechanisms that produce central sensitization [162]. However, the current discussion pinpoints a fundamental difference between the mechanisms that produce central sensitization after peripheral and central injuries. While central sensitization driven by BDNF increase is implicated in peripheral injury- and inflammation-induced pain, after SCI, central sensitization-like mechanisms that do not involve elevated levels of BDNF are likely to promote the induction/onset of chronic neuropathic pain [93]. Yet, whether BDNF potentiates maladaptive plasticity including the maintenance of neuropathic pain or other behavioral deficits incurred in the chronic phase of injury cannot be discounted by these observations. Actually, Boyce et al. [164] showed that, in the chronic stage of injury (6 weeks), contused rats treated with a viral vector expressing BDNF had enhanced sensitivity to heat stimulus (Hargreaves' test) compared to subjects treated with NT-3 expressing or control vectors. The idea that BDNF could produce pronociception in the chronic phase of injury is supported by the fact that the early reductions in BDNF and TrkB levels after SCI are reversed at later stages after injury, when maintained mechanical allodynia is evident [94]. A probable scenario is that BDNF exerts bidirectional functions following SCI. In the acute stage of injury, reduced levels of BDNF produce maladaptive spinal plasticity and impair behavioral function. Under those conditions, treatments that increase BDNF levels are beneficial. For example, elevated BDNF levels, whether from endogenous or exogenous source, have been shown to promote adaptive plasticity [91, 136, 165, 166] and functional recovery [164, 167, 168] after injury. However, in the more chronic time point when BDNF levels have been restored, overexpression of BDNF can again induce maladaptive plasticity which could in turn lead to pain hypersensitivity [94, 164]. Together, these observations highlight a potential limitation to the use of BDNF after SCI, the apparent shift from exerting adaptive effects to actions that are maladaptive. Moreover, because TrkB expression is also increased during the chronic stage of injury, even minimal overexpression of BDNF can initiate maladaptive plasticity.

Overall these studies signify tremendous ambiguity as it relates to BDNF functions following SCI. First, it is clear that there is divergence in the mechanisms that underlie central sensitization and pain hypersensitivity after SCI, such that it no longer requires BDNF-induced facilitation of synaptic efficacy. Although there are many overlaps in the underlying cellular and neural mechanisms that underlie inflammatory, peripheral injury, and neuropathic pain [169], trauma to the spinal cord appears to fundamentally alter these basic processes. Second, they show a distinction between how BDNF influences spinal nociceptive processes in intact and injured subjects and also reveal an important difference in how BDNF influences motor versus sensory systems after SCI. Undoubtedly, BDNF enhances adaptive plasticity and

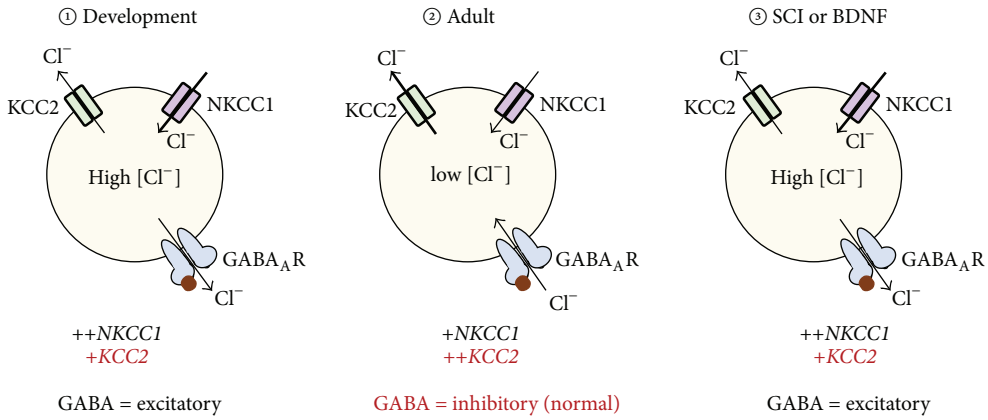

Figure 7: Plasticity in GABA-mediated chloride function; role of chloride transporters KCC2 and NKCC1. ① During development, CNS neurons express high levels of NKCC1 and low levels of KCC2. Upon GABA binding $GABA_A$ receptors, chloride ions $[Cl^-]$ exit the cell, thereby producing excitatory actions. ② In mature cells, the reverse occurs. The high concentration of KCC2 causes $[Cl^-]$ to enter the cell, which results in GABAergic inhibitory actions. ③ Both SCI and BDNF have been shown to decrease membrane bound KCC2 expression on neurons. This effect causes a shift in GABA function from inhibition to produce excitatory effects. This switch may contribute to pain after SCI.

promotes recovery of locomotor function but appears to be less influential in directing nociceptive plasticity, although there is a possibility that BDNF activates pain systems at later stages of injury. It appears that while underexpression of BDNF can mitigate adaptive plasticity, overexpression appears to be equally detrimental. This has been demonstrated in the forebrain where overexpression of BDNF was found to be detrimental to memory and learning [170]. We believe that spinal nociceptive networks are similarly vulnerable to maladaptive changes if BDNF levels are abnormally enhanced after SCI.

4. Mechanisms Mediating BDNF's Effects

4.1. GABA Signaling Mechanisms. As previously mentioned, a complex interaction between BDNF and GABA appears to play a pivotal role in neuropathic pain after peripheral injury [96]. Lu et al. [99] showed that, in young rats following sciatic nerve injury, BDNF alters the electrophysiological properties of dorsal horn neurons. Specifically, BDNF increases both the excitatory and inhibitory synaptic drives to putative excitatory interneurons while attenuating synaptic transmission to inhibitory GABAergic neurons.

The multifaceted BDNF-GABA relationship after peripheral and spinal cord injury is directly linked to the ability of both BDNF and SCI to independently regulate the expression and function of chloride transporters. K^+Cl^- cotransporter 2 (KCC2) plays an important role in regulating neuronal chloride homeostasis and is primarily responsible for the inhibitory actions of GABA [171, 172], unlike its depolarizing counterpart $Na^+K^+Cl^-$ cotransporter 1 (NKCC1) [173, 174] (Figure 7). In immature neurons where intracellular concentration of chloride ions $[Cl^-]_i$ is high, NKCC1 is highly expressed, thereby mediating the excitatory action of GABA seen during early development. However, in adult neurons, the inhibitory actions of GABA are due to a predominant KCC2 expression. GABA receptor functions and chloride homeostasis are influenced by manipulations that modify the expression of NKCC1 and KCC2. In hippocampal cells, BDNF decreased both the expression and phosphorylation of KCC2 [175, 176] and, in the nucleus raphe magnus, KCC2 levels were decreased by BDNF, producing an overall excitatory result that is mimicked by inflammatory pain [177]. This latter study suggests that, at supraspinal pain processing center, BDNF can regulate chloride homeostasis. It further implies that persistent pain resulting from decreased expression of KCC2 can be induced by BDNF's administration.

Nonetheless, different observations are noted after SCI. Boulenguez et al. [178] recently showed that SCI decreases the protein expression of KCC2 in the spinal cord (also see [179]), the same effect produced by BDNF. When BDNF was administered to spinal cord of intact subjects, there was a reduction in plasmalemmal KCC2 expression which is the converse of the increase caused by BDNF 15 days after SCI. Thus, it appears that BDNF, like SCI, can decrease KCC2, whereas BDNF plus SCI causes an increase in KCC2. Boulenguez et al. [178] also showed that the early decrease in KCC2 caused by SCI could be prevented by inhibiting endogenous BDNF with TrkB-IgG, which suggests that SCI-induced decrease in KCC2 is mediated by BDNF. Studies by Cramer et al. [179] and Hasbargen et al. [180] also reported that SCI dramatically reduces KCC2 but increases NKCC1 levels. When the results from these independent studies are collated, there is succinct evidence that BDNF-induced plasticity is highly susceptible to SCI-induced modification and *vice versa*. Therefore, it can be surmised from these studies that because a decrease in KCC2 expression is consistent with increase excitability, then BDNF exerts opposing actions in intact and injured spinal cord. Specifically, while it reduces KCC2 which promotes excitation (pronociceptive effects) in intact spinal cord, after SCI, BDNF could produce protective or antinociceptive effects by increasing KCC2 and GABAergic inhibition. Clearly, there are no solid explanations for these differential effects of BDNF or the mechanisms that trigger a tangible switch in BDNF functions after SCI. Both small diameter primary afferents and activated microglia

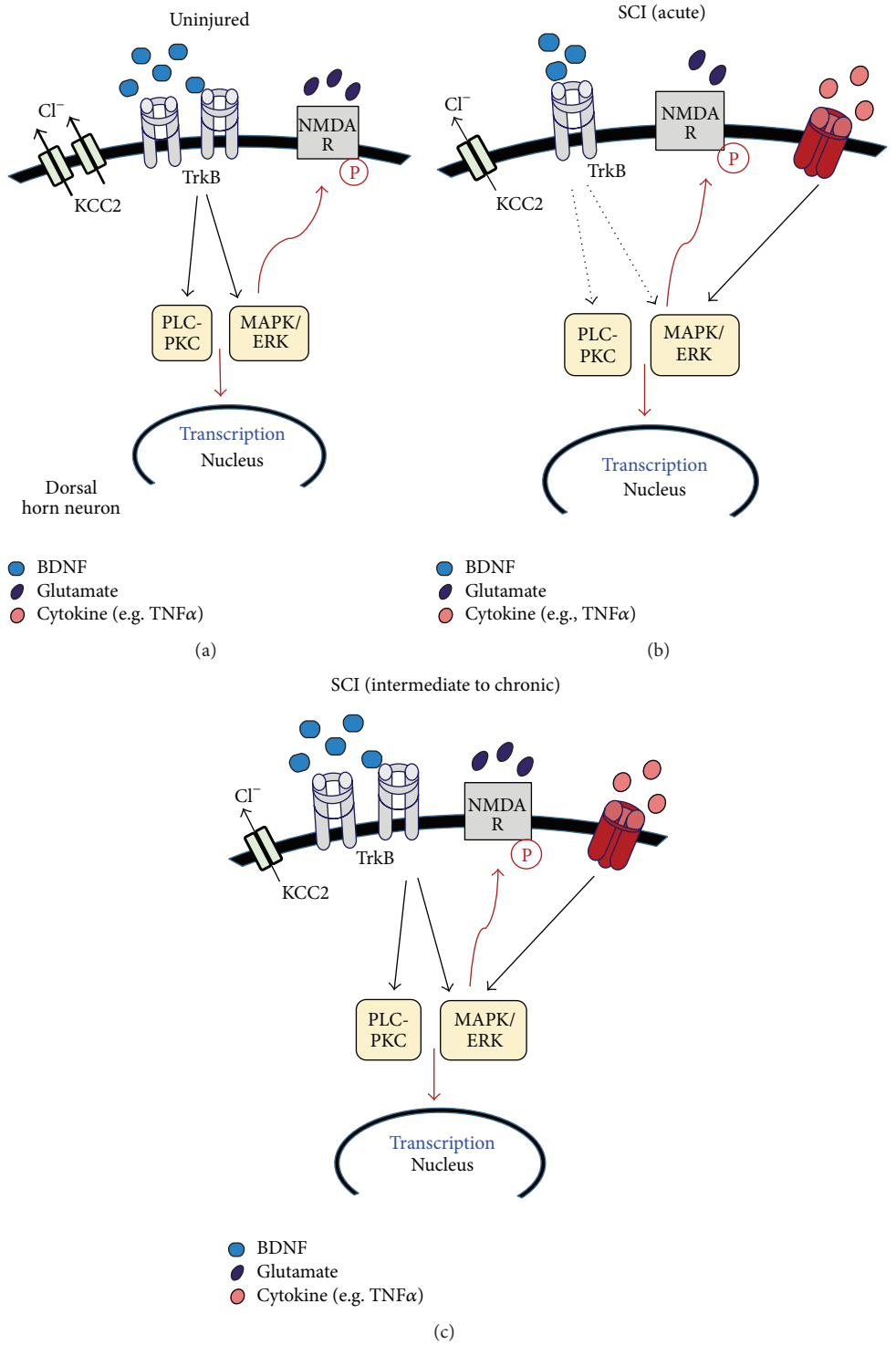

Figure 8: Potential BDNF-dependent and BDNF-independent mechanisms of pain in uninjured and injured spinal cord. (a) In the absence of SCI, BDNF-mediated effects contribute to pain. Constitutive increases in BDNF and TrkB expression activate MAPK/ERK and PLC-PKC kinase pathways, which in turn leads to transcription of pain genes and posttranslational modifications (e.g., phosphorylation of glutamate receptors). (b) In the acute stage of SCI, both BDNF and TrkB levels are decreased in the spinal cord dorsal horn [93]. However, during this stage, MAPK/ERK pathways could be activated by BDNF-independent mechanisms, presumably an alternate pathway such as the TNFα pathway (both TNFα and TNR1 expressions are upregulated by SCI [94]). A decrease in KCC2 by SCI which alters GABA-mediated chloride function is likely to also contribute to pain after SCI. (c) During the chronic stage of injury, pERK levels are increased [94, 95], which may result from increases in both BDNF-TrkB signaling and TNFα-TNFR signaling. Activation of these pathways could consequently increase kinase activity and the transition of pain genes. Overall, we propose that although BDNF may not necessarily initiate pain-producing pathway after SCI, it is likely to contribute to pain hypersensitivity during the chronic stages of injury.

are likely sources of increased levels of BDNF within the spinal cord. If high levels of BDNF are retained into the chronic stages of injury presumably after the primary injury has been healed, then probable mechanism of maintained pain hypersensitivity would be BDNF-induced reduction in KCC2 mediated GABAergic inhibition. However, although the effect BDNF has on KCC2 and GABA circuits in the chronic stage of injury is clearly speculative at this time, it seems that SCI-induced reduction in KCC2 expression is considered a possible mechanism that underlies acute or early onset pain after SCI.

4.2. ERK-pERK Signaling. As we discussed earlier, ERK-dependent signaling mechanisms are involved in the development of pain. Interestingly, in our studies, we observed a decrease in ERK1/ERK2 and activated ERK1 (pERK1) expression at the lesioned epicenter during the first 7 days after SCI [93] although a robust increase in pERK1/pERK2 was observed in the ventral spinal cord at 28 days after SCI, at a time when rats showed mechanical allodynia [94]. Crown et al. [95] reported an increase in pERK1/pERK2 in the spinal cord of SCI rats expressing neuropathic pain behaviors at 35 days after injury. Together, these studies suggest that, after SCI, ERK expression and phosphorylation correlate to the development of pain and this is not merely due to the SCI *per se*. It is not determined whether these changes in ERK expression are determined by BDNF-TrkB signaling or whether BDNF-induced activation of these neural substrates plays an important role in producing neuropathic pain after SCI particularly as ERK can be engaged by other signaling pathways such as TNFα. Moreover, as we and others have shown, both TNFα and its receptors (TNFR1 and TNFR2) are upregulated after SCI [94] and may contribute to chronic pain states after SCI. In Figure 8, we present a brief summary of these possible mechanisms of pain in uninjured and injured spinal cord.

Importantly, it should be noted that although SCI appears to mimic some of BDNF's detrimental actions, these effects may not be mediated by BDNF itself. The phenomenon termed "metaplasticity" by Abraham and Bear [181] is a change in the ability to induce subsequent synaptic plasticity. The fact that SCI impacts the manner and type of BDNF-induced plasticity particularly as it relates to the nociception and pain after SCI is an important example of spinally mediated metaplasticity. While additional experiments are needed to completely resolve this issue, the simplest conclusion to be made from these studies is that the use of BDNF following SCI does not present a challenge to spinal nociceptive and pain processing, at least acutely.

5. Conclusion

Studies of spinal plasticity have revealed that the processes involved are indeed complex. An even greater level of complexity arises after SCI, partly due to the enormous amount of cellular and morphological changes that ensue. BDNF has been classified as a neuromodulator and has been shown to be intricately involved in spinal plasticity. Clearly, there is no uniformity with BDNF actions as it has been shown that it can mediate both adaptive plasticity and maladaptive plasticity in spinal networks. For many years, our studies focused on unraveling these differential effects BDNF has on spinal networks in uninjured and injured spinal cord. Deductions made from several studies including some from our laboratories overwhelming show that as it relates to nociceptive processes, whereas BDNF exerts pronociceptive actions in the absence of injury, no such effect is observed after injury, at least acutely. Moreover, we found that functional deficits and mechanical sensitivity after SCI are associated with reduced BDNF levels, not increases. In contrast, increases in BDNF after SCI promote adaptive plasticity and functional recovery. The multifaceted effects of BDNF after SCI may be linked to SCI-induced changes in (i) expression of TrkB receptor and downstream kinases, (ii) GABA transmission due to altered expression of chloride transporters, and (iii) the activity of astroglial cells. The idea that BDNF plasticity is potentially susceptible to SCI-induced modifications indicates that metaplastic changes are essential to spinal function, particularly after injury to the CNS. In conclusion, we show that while significant progress has been made towards a clearer understanding of BDNF functions, after 30 years, additional research is warranted to elucidate the spinal effects of BDNF-TrkB signaling.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Stepping in Place While Voluntarily Turning Around Produces a Long-Lasting Posteffect Consisting in Inadvertent Turning While Stepping Eyes Closed

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Training subjects to step in place on a rotating platform while maintaining a fixed body orientation in space produces a posteffect consisting in inadvertent turning around while stepping in place eyes closed (podokinetic after-rotation, PKAR). We tested the hypothesis that voluntary turning around while stepping in place also produces a posteffect similar to PKAR. Sixteen subjects performed 12 min of voluntary turning while stepping around their vertical axis eyes closed and 12 min of stepping in place eyes open on the center of a platform rotating at 60°/s (pretests). Then, subjects continued stepping in place eyes closed for at least 10 min (posteffect). We recorded the positions of markers fixed to head, shoulder, and feet. The posteffect of voluntary turning shared all features of PKAR. Time decay of angular velocity, stepping cadence, head acceleration, and ratio of angular velocity after to angular velocity before were similar between both protocols. Both postrotations took place inadvertently. The posteffects are possibly dependent on the repeated voluntary contraction of leg and foot intrarotating pelvic muscles that rotate the trunk over the stance foot, a synergy common to both protocols. We propose that stepping in place and voluntary turning can be a scheme ancillary to the rotating platform for training body segment coordination in patients with impairment of turning synergies of various origin.

1. Introduction

When walking in everyday environments, subjects frequently change direction to negotiate corners and avoid obstacles. The ability to change direction and the ability to accurately control the curved trajectory while walking are essential components of successful navigation. Under the curved walking condition, the control of the muscle synergies takes into account not only the obligatory propulsion but also the equilibrium constraints connected to body rotation. Turning involves complex orientation of head, trunk, pelvis, and feet [1–5] and is accompanied by trunk inclination to the inner part of the trajectory to counteract the centrifugal acceleration acting on the walking body [1, 6, 7]. Also, motion of the lower limbs is asymmetric, whereby the leg inside the trajectory travels a shorter pathway than the outside leg [1–6]. Not unexpectedly, given the complex coordination and multisensory integration underlying curved walking [8], studies requiring subjects to travel both linear and circular pathways have detected abnormalities in patients with neurological disorders [9–13].

Rehabilitation of curved walking has been advocated by several investigators [7, 14, 15], and preliminary data on the potentially positive effect of circular treadmill training on curved walking in PD patients are available [16]. The improvement of the velocity of curved walking in these patients would possibly rest on the training of the neural circuits subserving the complex synergies for turning mentioned above.

The nervous system can learn to produce curved walking. Evidence thereof is represented by the so-called podokinetic after-rotation (PKAR). Previous studies showed that, after prolonged stepping in place on a rotating platform, subjects asked to walk normally on firm floor straight ahead without vision unknowingly generated a curvilinear path [14, 17]. In

addition, when subjects were asked to step in place without vision after having stepped on the rotating platform for a prolonged period, they continued to rotate in the horizontal plane around their vertical axis for a while after the halt of the platform [18–20]. This PKAR has been considered the effect of adaptation to the continuous perturbation of the foot position by the podokinetic stimulation (produced by the rotation of the platform upon which subjects step while keeping the orientation of trunk and head fixed relative to space) [20].

Thus, the podokinetic stimulation produces a rotation of the feet below the head and trunk, the orientation of which hardly changes with respect to the environment. The foot rotation is then counteracted by a corrective repositioning action, since the foot is moved to its original position again, in a direction opposite to the direction of the rotating platform, so that body orientation in space stays unchanged. Subjects are focused on maintaining stable head and trunk and rotate their feet back to the original position almost unconsciously, thereby neutralizing the effect of the platform rotation on the upper body. Then, when the platform is stopped but the subjects are asked to continue stepping in place, subjects continue turning in the same direction in which they rotated the feet during the counteraction that replaced them in the "right" position, necessary for keeping their body position fixed in space. Remarkably, such PKAR is not consciously perceived [14, 18]. Of note, a visual or haptic input given for few seconds during the PKAR period can reduce the PKAR velocity; when the new information is removed, the PKAR reappears [21].

Inadvertent rotation while stepping in place is not an odd or peculiar effect. Similar body rotation effects are obtained by the unilateral vibration of neck and trunk muscles while walking or stepping in place ([22, 23] and see [24]). Vibrating the sternocleidomastoid muscle, for example, compels the body to turn to the side opposite to vibration [23]. Moreover, rotation posteffects have been observed. A prolonged optokinetic stimulation causes a consistent posteffect. After that stimulation, blindfolded subjects turned around when attempting to step in place without turning [25]. Under the above conditions, as well as with PKAR, subjects were not aware of any body rotation while stepping in place with vibration or after optokinetic stimulation.

We put forward the hypothesis that a podokinetic aftereffect can take place after voluntary turning while stepping in place, that is, in the absence of the stimulation produced by the rotating platform. There is no quantitative information to date on the events occurring after a period of prolonged stepping in place while turning around the body's vertical axes. Contrary to what happens while stepping in place on the rotating platform, during voluntary turning subjects are certainly aware of the deliberate rotation of their feet in the direction they want to turn in. Moreover, head and trunk are not fixed in space but rotate continuously, and vision is removed in order to annul eye movements and optokinetic effects.

2. Methods

2.1. Subjects and Tasks. Sixteen healthy subjects (7 males and 9 females, mean age 27.5 yrs ± 6.4 SD, height 173.4 cm ± 7.9 SD, and weight 67.9 kg ± 10.15 SD) participated in the experiments. All subjects were naïve to the experimental procedure and all succeeded in performing the trials without difficulty. Experiments were performed in accordance with the Declaration of Helsinki. The ethics committee had approved the experiment (Central Ethics Committee, Fondazione Salvatore Maugeri, approval number 806 CEC). All procedures were carried out with the adequate understanding and written informed consent of each subject.

Subjects performed a trial in which they stepped in place with bare feet, eyes open, fixing a target at eye level at a distance of about three meters, at the centre of a disc of 2 m of diameter, rotating at a velocity of 60°/s in the counterclockwise direction for 12 min, thereby inducing a repetitive podokinetic stimulation. During this period, subjects maintained a roughly constant position of the body in space. They stepped at their own cadence, without any imposed cue. Following the 12 min period on the rotating platform, the platform was stopped. Subjects wore an eyemask on the forehead during the podokinetic stimulation and lowered it at eye level to block vision when the platform stopped. Then they were told to continue stepping in place for at least 10 min more.

In another trial, blindfolded subjects voluntarily turned around while stepping in place at their natural cadence and at their preferred angular velocity on a stationary surface (the same platform, motionless) for 12 min. After this period, an operator asked the subject to stop turning and continue stepping in place for at least 10 min more. During voluntary turning, subjects were asked to turn in place in clockwise direction. In this way, during the posteffect of the podokinetic stimulation and of the voluntary turning, blindfolded subjects rotated in the same direction while stepping. Subjects did not practice stepping prior to recording. The platform rotation and the voluntary rotation trials were performed in a different day and were randomized across subjects.

Under both conditions (podokinetic stimulation and voluntary turning) subjects stepped inside a plastic hula-hoop of 50 cm of diameter, loosely fixed at pelvic height by elastic straps secured to the platform outer railing. This hula-hoop prevented subjects' displacement from the platform rotation centre while stepping in place, in particular with eyes closed (during voluntary turning condition and the posteffect periods). Lightly touching the hoop with the pelvis occurred from time to time, but this gave no cue regarding the position in space, during either the rotation or the posteffect, as shown from the participants' report at the end of the experiments. Subjects' arms were folded under both conditions. Of note, no safety harness was employed nor did subjects hold onto a stable overhead [20, 26] or otherwise firm external structure.

2.2. Data Acquisition and Analysis. In order to capture both rotation in space and the feet stepping movements, eleven reflective markers were placed bilaterally on the following body positions: three markers were mounted on a light inner frame of a helmet in correspondence with vertex and lateral head position, and the others were placed on the acromion, lateral malleolus, posterior heel, and forefoot (dorsally, about

over the 1st metatarsophalangeal joint). Kinematic data were recorded by means of a device (Smart-D, BTS, Italy) composed of 12 optoelectronic cameras, at a sampling frequency of 100 Hz, and stored in a PC. The marker traces were filtered with a third-order low pass Butterworth filter with a cut-off frequency of 1.5 Hz (software developed in MATLAB, MathWorks Inc., USA). This frequency was chosen based on the frequency spectrum of the trace of shoulders marker displacement, which showed no frequency content >1.1 Hz in any subject. Off-line analysis was performed on the data acquired in a time-window that started 2 min before the platform stop, or 2 min before the signal to stop the voluntary turning, and lasted from 10 to 15 minutes.

For each trial of each subject, a software program developed in MATLAB calculated the angle described in the horizontal plane by the line-segment joining the markers placed on the shoulders within each 10 ms time interval (defined by the sample frequency). This was taken as the body rotation angle. The cumulative angle described by the body was calculated as the sum of the successive angles for the entire duration of the acquired epochs. The instantaneous angular velocity of the body rotation was the numeric derivative of the cumulative angle. A similar calculation was made for the angular rotation of the head, based on the recording of the two lateral markers placed on the helmet frame. The body angular velocity was then filtered with a low pass filter with a cut-off frequency of 2 Hz, just in order to clearly display the time course of the posteffects in Figure 1. From the head angular velocity (not filtered), we calculated the angular acceleration of the head rotation in the horizontal plane. The mean angular acceleration of the head was then obtained by averaging the rectified trace of the angular acceleration in the last minute of voluntary turning or stepping in place on the rotating platform and in a time period of one minute around the maximum peak of the rotation velocity during the two posteffects. The mean peak acceleration was also computed.

In order to estimate the time course of the posteffects induced by the stepping in place on the rotating platform or induced by the voluntary turning, the trace of shoulder rotation velocity in the postperiod was fitted with an exponential function $y = Ae^{-t/\tau_1} + Be^{-t/\tau_2} + C$. Based on visual checking of the data and on previously published analyses [21, 27–29], a function characterized by two time constants was chosen in order to describe the initial rise in the posteffect angular velocity, which is then followed by a slow decay over time. To this aim, the iterative conjugate gradient method of the Excel® Solver Utility was used, τ_1 and τ_2 being the time constants, C being the asymptotic value of the function, and $A + B + C$ being the intercept with the ordinate. The values of A, B, C, and τ_1 and τ_2 parameters were computed by using the minimum sum squared algorithm. The maximum value of the double-exponential function was assumed as the peak rotation velocity reached in the posteffect. The time at which the posteffects disappeared was estimated by $3 * \tau_2$, because at this time the rotation velocity has dropped to 5% of its peak value. The mean angular velocity of body rotation during voluntary turning was calculated in the last minute of this task before subjects were told to stop turning and continue to step in place. The rotation velocity while stepping on the rotating platform was simply the platform rotation velocity, since head and shoulders did not actually rotate in space while stepping on the rotating platform.

The time-relationship between head and shoulder rotation was computed while stepping on the rotating platform and thereafter and during voluntary turning and thereafter. The time lag was obtained by the cross-correlation analysis. To this aim, the filtered traces (high-pass filter with a cut-off frequency of 0.1 Hz) of the cumulative angles described by shoulder girdle and head axes on the horizontal plane were used. The time lag was the time interval at which the absolute value of the cross-correlation coefficient (R) was maximum. A negative time lag indicated that shoulders lagged behind the head movement.

Cadence, height reached by the feet (marker placed on lateral malleolus) during the swing phase, and duration of the stance phase (the time interval between the lowermost malleolus position and the subsequent malleolus off) were calculated by software developed in LabVIEW (National Instruments Corporation, Austin, TX). For each subject, the mean cadence, height of feet, and duration of stance phase were calculated within the last minute of the period of voluntary turning or stepping on the rotating platform and within one minute around the peak of velocity during the two posteffects. Further, by using the markers placed on the heel and forefoot, the step yaw angle of the foot of the side corresponding to the direction of rotation was calculated for each condition and subject by software developed in MATLAB.

2.3. Statistics. A 2-way repeated-measure ANOVA with experimental condition (podokinetic stimulation or voluntary turning) and pre- and posteffects (PKAR or vPKAR, i.e., the PKAR following voluntary turning) as factors was used to compare the following: rotation velocity of shoulders, head velocity and acceleration, time lags between head and shoulder axis rotations, and cadence and step angle. The time constants (τ_1 and τ_2) of the shoulder axis angular velocity during the two posteffect periods were compared by a 2-way repeated-measure ANOVA, with time constants and posteffect of voluntary turning (vPKAR) or podokinetic stimulation (PKAR) as factors. The duration of the stance phase and the height of foot lifting were compared by a 3-way repeated-measure ANOVA with conditioning procedure (voluntary turning or podokinetic stimulation), pre- and posteffects, and feet as factors. For all ANOVAs, the post-hoc test analyses were made with Fisher's LSD test. The software package used was Statistica (StatSoft, USA).

3. Results

3.1. Posteffect of Podokinetic Stimulation and of Voluntary Turning. After the 12 min period of stepping in place on the rotating platform, subjects showed a clear-cut PKAR. All the subjects, when the platform was stopped and they were asked to continue stepping in place, went on inadvertently turning around in the same direction as their feet had rotated

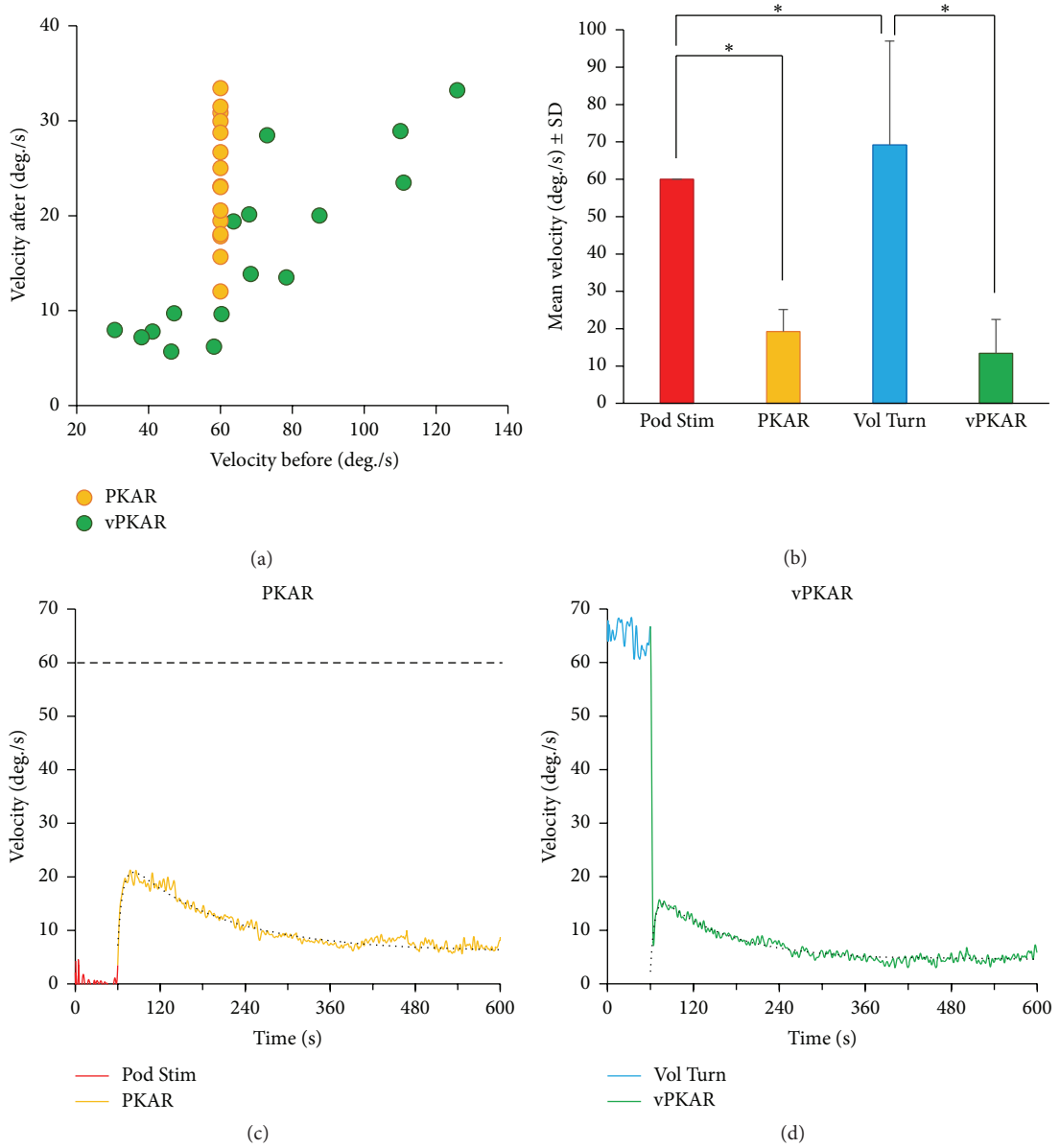

Figure 1: Posteffect of podokinetic stimulation and voluntary turning. In (a) the peak rotation velocity for each subject during the posteffect is plotted against the rotation velocity during the two conditioning procedures. (b) shows the mean angular velocity of the platform (Pod Stim) and of the body across subjects, during conditioning and posteffects. (c) shows the mean trace of the velocity of the body rotation (obtained by averaging the traces of all subjects) during the last minute of the podokinetic stimulation (Pod Stim, red colour, from 0 s to 60 s) and during the immediately following podokinetic after-rotation (PKAR, yellow color, 60 s to 600 s). The horizontal dashed line indicates the platform rotation velocity. (d) shows the angular velocity during the last part of voluntary turning (Vol Turn, blue, 0 s to 60 s) and the posteffect (vPKAR, green, 60 s to 600 s). The mean angular body velocity was almost null during Pod Stim but was more than 60°/s during Vol Turn (compare (c) and (d)). During the two posteffects, the mean velocities were just larger for PKAR compared to vPKAR but showed a similar initial rise and decay (the black dotted lines are the exponential fit). $*$ indicates significant difference ($p < 0.05$) between mean velocities.

to counteract the platform rotation (i.e., opposite to the direction of the platform movement). Likewise, after the 12 min period of voluntary stepping and turning, all subjects showed a posteffect (a PKAR following *voluntary* stepping and turning, vPKAR) and continued turning in the same direction as the direction of the voluntary rotation. This posteffect was broadly similar to that observed after the podokinetic stimulation. No subject, interviewed at the end of the experiment, reported any perception of turning during PKAR or during the vPKAR. For each subject and condition, the angular velocity recorded during the posteffect was fitted with a double-exponential function, where τ_1 described the initial rise and τ_2 described the decay of the rotational posteffect. The highest value of the function was the peak

rotation velocity reached during the posteffect. There was a remarkable analogy in the time constants and peak velocities between the two protocols, both within and across subjects.

Figure 1(a) shows the angular velocities of the rotating body (each symbol corresponds to one subject) observed in the postperiod (after the platform rotation or after the voluntary turning, inordinately), plotted against the corresponding angular velocities observed during platform rotation or during voluntary turning. The platform rotation velocities were identical for all subjects (60°/s, red bar in Figure 1(b)), while the velocities in the after-period (PKAR) peaked in a range from about 10°/s to 35°/s. The mean value across subjects was 19.2 ± 5.9°/s (Figure 1(b), yellow bar). Therefore, there was a mean reduction to about 30% with respect to the velocity of the platform. During voluntary turning (Figure 1(a)), the velocity of body rotation was largely different across subjects (they were free to select their velocity of turning while stepping), ranging from about 30°/s to about 120°/s. The mean value was 69.2°/s ± 27.7 (Figure 1(b), blue bar). After the period of voluntary turning, when subjects were asked to continue stepping in place without deliberately turning around, they continued to rotate in the same direction as that of the preceding voluntary rotation, with a mean velocity of 13.4°/s ± 9.7 (Figure 1(b), green bar). Clearly (Figure 1(a)), the velocity of rotation in the posteffect was proportional to the velocity of voluntary turning. On average, the vPKAR had an angular velocity of about 20% of the mean velocity during the voluntary turning.

ANOVA showed no significant difference in the mean angular velocity between voluntary turning and podokinetic stimulation (main effect, $F(1, 15) = 0.12$, $p = 0.72$). In the posteffects, the angular velocity significantly decreased with respect to that during both voluntary turning and podokinetic stimulation (pre- versus posteffect, $F(1, 15) = 245.86$, $p < 0.01$). However, there was a significant interaction between conditions and pre- and posteffects ($F(1, 15) = 11.1$, $p < 0.01$), since the mean angular velocity during voluntary turning was 15% greater than during platform rotation (post-hoc test, $p < 0.05$), while during vPKAR the mean angular velocity was smaller (even if not significantly so, post-hoc test, $p = 0.09$) than during PKAR.

Because the range of velocities during voluntary turning was large, the comparison was also done directly for the few participants that had voluntary turning velocities (6 subjects, mean velocity 65.26°/s ± 5.6) very close to that of the platform rotation. There were no difference in the angular velocities between voluntary turning and podokinetic stimulation ($F(1, 5) = 0.3$, $p = 0.61$), a significant difference between pre- and posteffect ($F(1, 5) = 692.24$, $p < 0.01$), and an interaction between condition and pre- and posteffect ($F(1, 5) = 98.83$, $p < 0.01$). The interaction was due to the significant difference between PKAR and vPKAR (post-hoc test, $p < 0.01$), since the angular velocity (13.3°/s ± 8.2) was smaller during vPKAR compared to PKAR (20.9°/s ± 5.1). Thus, there was a difference between the two conditions in terms of the posteffect relative to the preeffect.

Figure 1 also shows the time course of the posteffects. The mean trace of angular rotation velocity over time (all subjects' traces averaged) during the PKAR (c) and the vPKAR (d) is reported. In both cases, subjects briefly ceased turning for a moment (lasting less than 5 s, not obvious in the figure) when the platform stopped or at the end of voluntary turning, when they were told to continue stepping without turning. Then, they resumed stepping and turning around (involuntarily). In both cases, turning velocity rapidly increased to a maximum value, usually peaking in the first min or so. Next, the angular velocity slowly decreased until the end of the acquisition period.

Figure 2(a) shows that the time at which the maximum angular velocity was reached during the posteffects was similar for both conditions (29.5 s ± 18.9 for PKAR and 23.7 s ± 16.4 for vPKAR, t-test, $p = 0.37$). Figure 2(b) shows the mean values of the time constants: τ_1 was 13.8 s ± 10.1 for PKAR and 14.3 s ± 19.9 for vPKAR. The decay in the angular velocity had a mean τ_2 of 153.1 s ± 112.1 for PKAR. Therefore, on the average, after 459.4 s ± 336.2 ($3 * \tau_2$) the posteffect of podokinetic stimulation vanished. The angular velocity of vPKAR decreased with a mean time constant (τ_2) of 168.9 s ± 176.9. Therefore the posteffect of voluntary turning disappeared after 418.2 s ± 337.5. ANOVA showed no difference in the time course between the two conditions ($F(1, 15) = 0.2$, $p = 0.66$). Both conditions collapsed, and there was a significant difference between the time constants of the increase in angular velocity (τ_1) and of the vanishing of the posteffect (τ_2) ($F(1, 15) = 36.6$, $p < 0.01$). There was no interaction between time constants and conditions ($F(1, 15) = 0.16$, $p = 0.69$). Further, for each subject, the time constants describing the time course of the vPKAR were plotted against the velocity during the corresponding conditioning procedure (Figure 2(c)). There was no relationship between τ_1 and the angular velocity of voluntary turning ($R^2 = 0.05$, $p = 0.4$). However, the relationship between τ_2 and the angular velocity of voluntary turning reached significance ($R^2 = 0.25$, $p < 0.05$), in spite of the large variability across subjects.

3.2. Time Lag between Head and Shoulder Movement. The yaw angles described by head and shoulder axes of one subject during 10 s of podokinetic stimulation (a) and voluntary turning (c) and during the posteffects (PKAR (b) and vPKAR (d)) are reported in Figure 3. In (a), the traces are almost superimposable, indicating that head and shoulder girdle moved almost simultaneously. In order to estimate the time lag between head and shoulder angular rotation, cross-correlation analysis was performed on the traces of the yaw angle described by head and shoulder axes. Head and shoulder moved in phase under all conditions ($R = 0.69 \pm 0.16$ for podokinetic stimulation and $R = 0.90 \pm 0.05$ for PKAR; $R = 0.92 \pm 0.13$ for voluntary turning and $R = 0.94 \pm 0.04$ for vPKAR). Under all pre- and postconditions, shoulders and head moved almost simultaneously (Figure 3(e)) with time lags ranging across subjects and conditions from −40 ms to 60 ms. ANOVA showed no difference in time lag between voluntary turning and podokinetic stimulation ($F(1, 15) = 0.10$, $p = 0.75$), no difference between pre- and posteffects ($F(1, 15) = 0.69$, $p = 0.42$), and no interaction between conditions and pre- and posteffects ($F(1, 15) = 1.77$, $p = 0.2$).

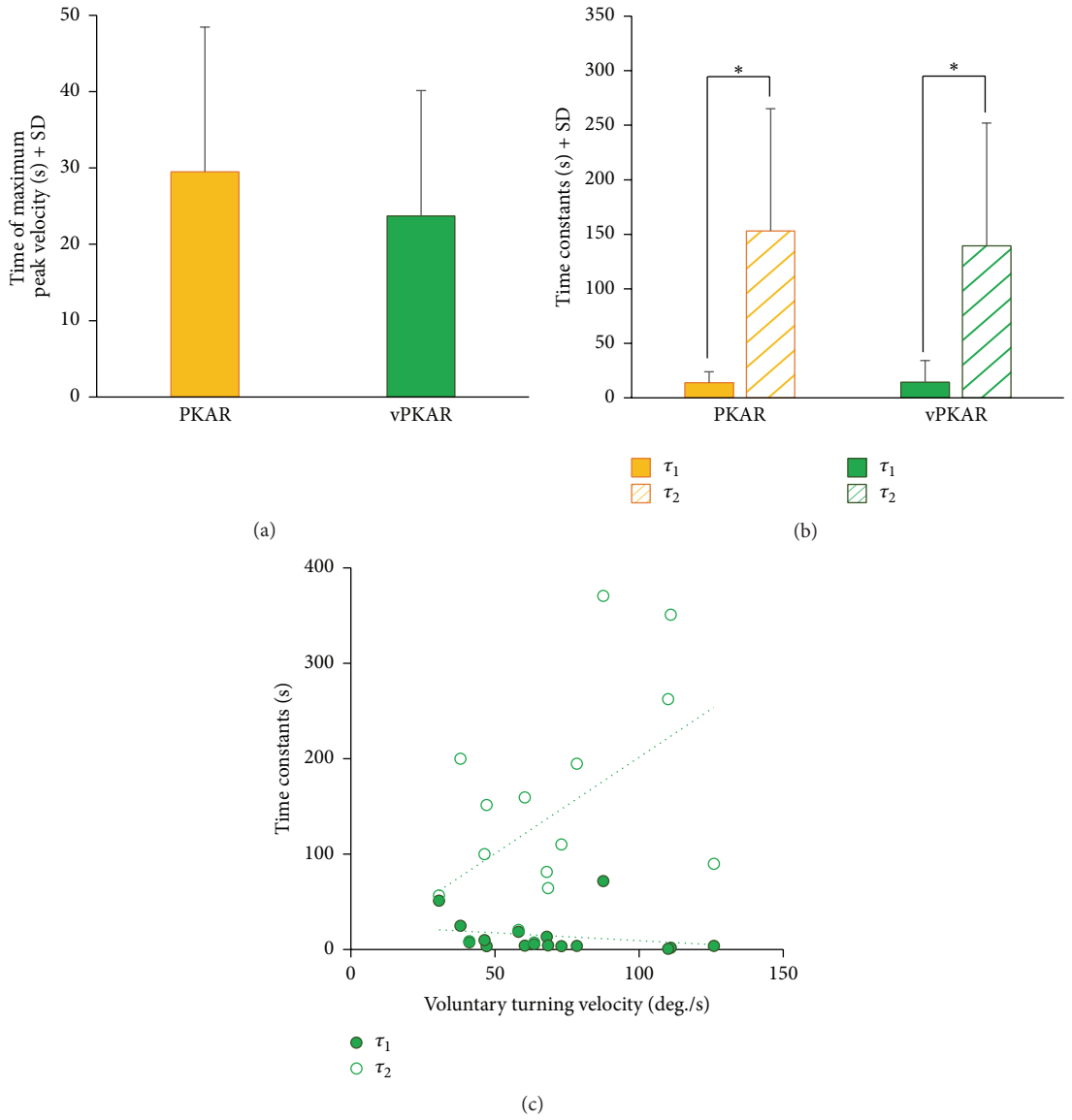

Figure 2: Peak velocity and time course of the posteffects. (a) Time at which rotation velocity peaked during PKAR (yellow bar) and during the posteffect of voluntary turning (vPKAR, green bar). (b) Time constants of the exponential fit to the time course of the two posteffects. τ_1 was the time constant of the initial rise and τ_2 was the time constant of the decay in angular velocity over time. (c) For each subject, the time constants (τ_1 green filled symbols, τ_2 open symbols) of vPKAR are plotted against the rotation velocity of voluntary turning. $*$ indicates significant difference ($p < 0.05$) between mean time constants (b).

3.3. Head Acceleration. The movement in the horizontal plane of the markers placed on right and left head side and on vertex and the head angular velocity and acceleration are reported in Figures 4(a)–4(h) for one subject. Data are reported for podokinetic stimulation (a and b), PKAR (c and d), voluntary turning (e and f), and vPKAR (g and h).

Across subjects, the mean head angular velocity (Figure 4(i)) was 69.1°/s ± 29.2 during voluntary turning and 0.01°/s ± 0.1 during podokinetic stimulation, while subjects tried to keep the head and trunk fixed in space. In the posteffects, head velocity was 18.9°/s ± 5.9 during PKAR and 13.9°/s ± 9.3 during vPKAR. ANOVA showed a significant difference in mean head angular velocity between conditions ($F(1, 15) = 40.64$, $p < 0.001$) and a significant difference between pre- and posteffects ($F(1, 15) = 31.35$, $p < 0.001$). There was a significant interaction between conditions (voluntary turning or podokinetic stimulation) and pre- and posteffects ($F(1, 15) = 227.38$, $p < 0.001$). The head angular velocity was different between voluntary turning and podokinetic stimulation (post-hoc test, $p < 0.001$) but not between the two posteffects (post-hoc test, $p = 0.17$).

The mean angular acceleration of the head (computed on the rectified acceleration trace; see Section 2) was 103.8°/s^2 ± 23.4 during podokinetic stimulation and 92.2°/s^2 ± 30.8 during voluntary turning. In the posteffects, head acceleration decreased to 78.3°/s^2 ± 24.5 for PKAR and to 79.1°/s^2 ± 24.3

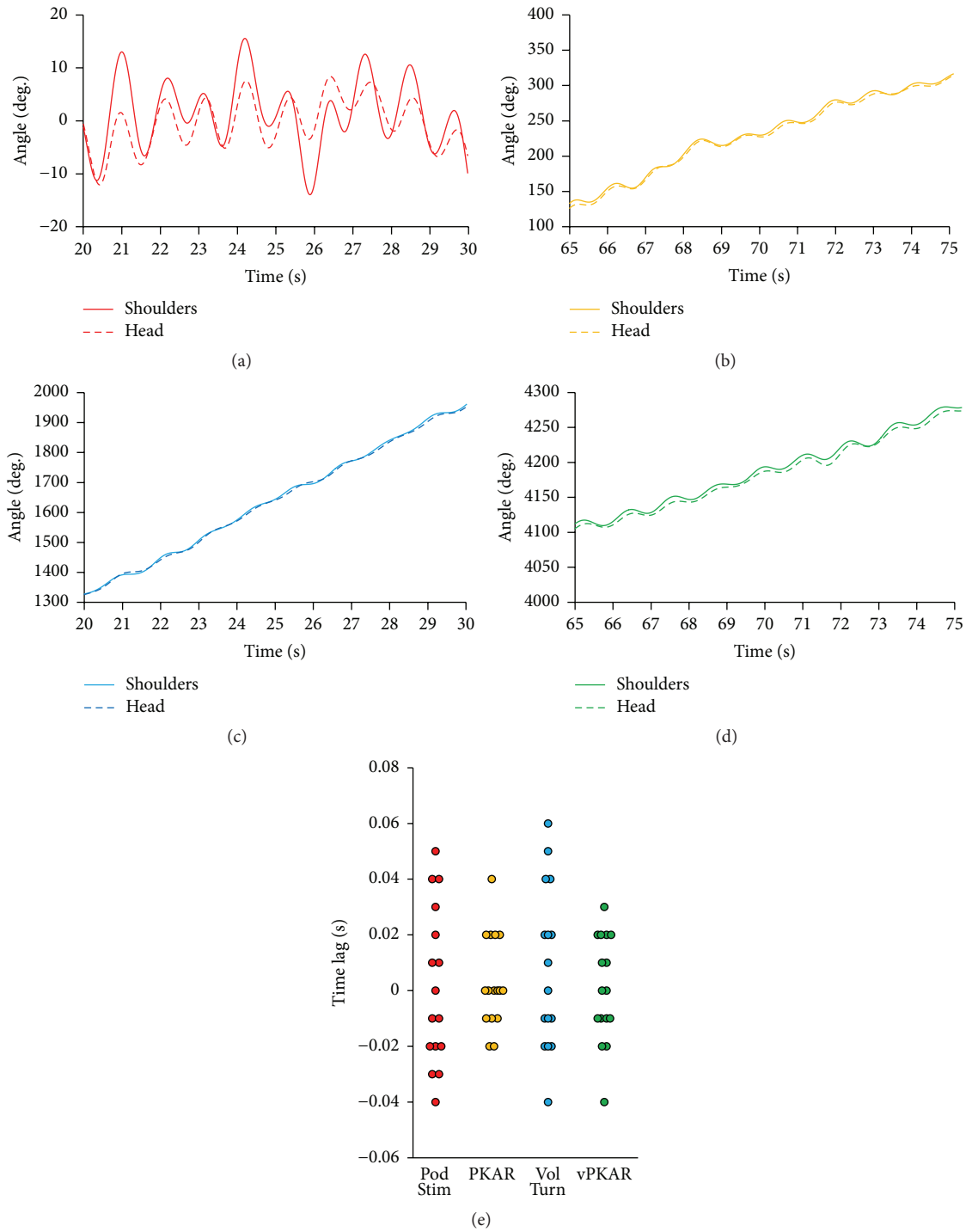

Figure 3: Coordinated head and shoulder movements. Angle described by the head (solid lines) and shoulders (dashed lines) mediolateral axis in a time period of 10 s during podokinetic stimulation (Pod Stim, (a)), PKAR (b), voluntary turning (Vol Turn, (c)), and its posteffect (vPKAR, (d)). During platform rotation, the body was kept almost fixed in space while head and shoulders showed minor left and right angular shifts. During voluntary turning (c) and during the two posteffects (b and d), head and shoulders continued to rotate in the horizontal plane, so that, in addition to their left and right yaw shift (a), the angle described by these segments continued to increase over time. The large differences in the y-scale amplitude between panel (a) and panels (b), (c), and (d) accommodate for the differences in the cumulative angle. The time lags between head and shoulders traces are reported in panel (e) for each subject. The 10 ms interval between the data points depends on the acquisition frequency; in many cases, several points coincide. There were no obvious differences across conditions.

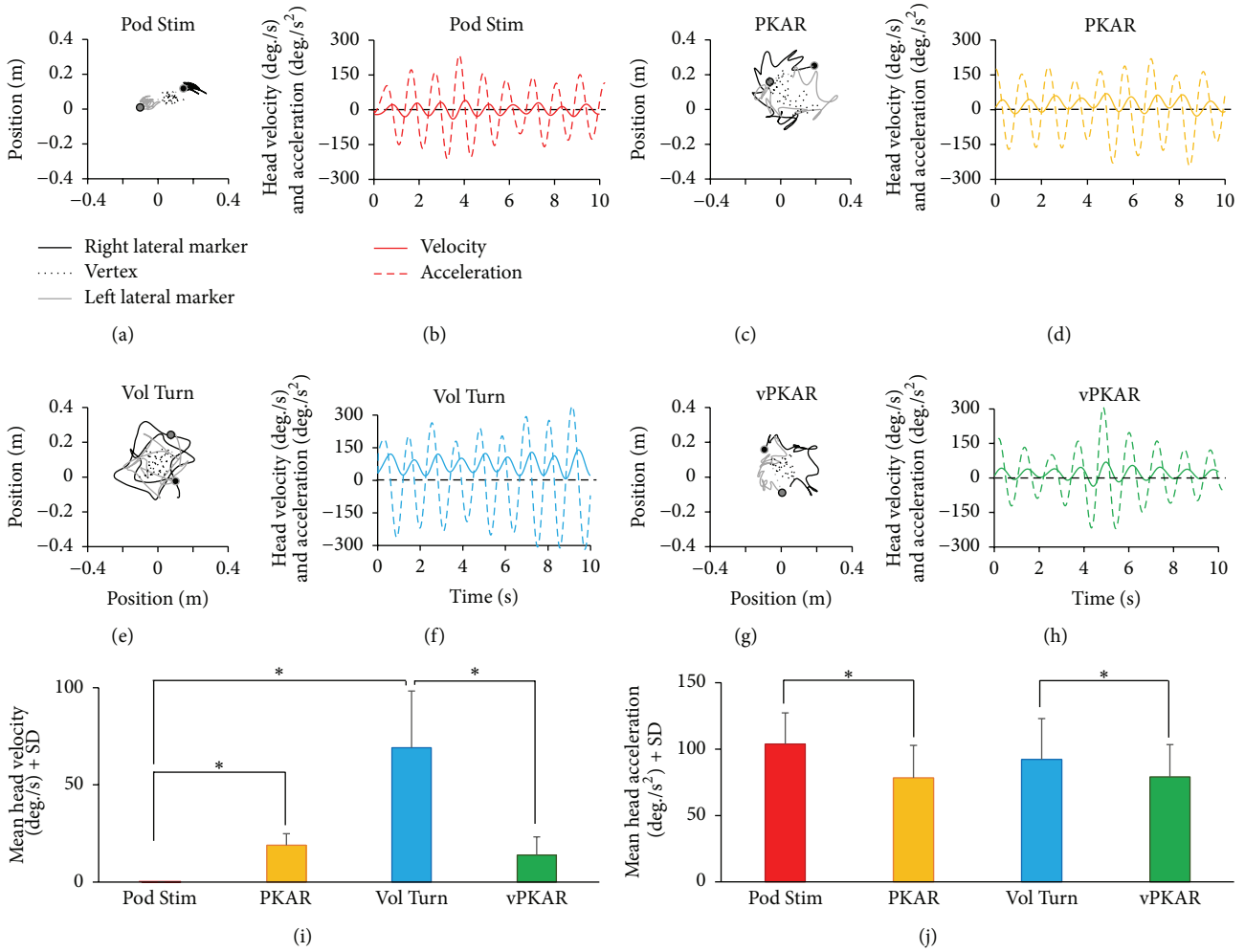

FIGURE 4: Head angular acceleration. (a) to (h) show the head movements in the horizontal plane during the podokinetic stimulation (Pod Stim, (a)), PKAR (c), voluntary turning (Vol Turn, (e)), and vPKAR (g). The two dots in (a), (c), (e), and (g) indicate the initial positions of the markers placed on the right (black dot) and left (grey dot) side of the head. Head velocity (solid line) and acceleration (dashed line) are reported in (b), (d), (f), and (h). The rotational effect of voluntary turning can be seen by the bias in the angular velocity profile (f). (i) and (j) show the mean values of head velocity (i) and acceleration (j) across subjects. Head showed angular acceleration (j) under both voluntary turning and podokinetic stimulation. There were no differences between the two posteffects for either head velocity or acceleration. $*$ indicates significant difference ($p < 0.05$) between mean head velocities (i) and accelerations (j).

for vPKAR. ANOVA showed no difference in head acceleration between podokinetic stimulation and voluntary turning ($F(1, 15) = 1.45$, $p = 0.25$). There was a difference between pre- and posteffects for the two conditions ($F(1, 15) = 24.8$, $p < 0.001$), since head acceleration was smaller in the posteffect than during the conditioning period (post-hoc test, $p < 0.05$, for the two comparisons). There was no difference in head acceleration between the two posteffects (PKAR versus vPKAR: post-hoc test, $p = 0.9$). A comparison was also done between mean peaks of head acceleration values. The mean amplitude of the peaks was 158.8 ± 39.4°/s^2 for podokinetic stimulation and 134.7 ± 44.3°/s^2 for voluntary turning. During the two posteffects, the mean amplitude of the peaks decreased to 121.4 ± 38.6°/s^2 for PKAR and to 118.8 ± 41.1°/s^2 for the vPKAR. ANOVA showed a minor difference between conditions ($F(1, 15) = 4.01$, $p = 0.06$) and a difference between pre- and posteffect ($F(1, 15) = 20.88$, $p < 0.001$). There was no difference between the posteffects (post-hoc test, $p = 0.8$).

3.4. Cadence, Stance Period, and Height of Feet Lifting during Voluntary Turning and Podokinetic Stimulation and during Their Posteffects. Figure 5 shows the mean cadence and mean duration of the stance period of stepping in place calculated in a time interval of 60 s during podokinetic stimulation and voluntary turning and around the time of the maximum velocity during the two posteffects. Mean cadence was 0.92 strides/s ± 0.14 during voluntary turning and 0.91 strides/s ± 0.11 in the posteffect. During the podokinetic stimulation, mean cadence was 0.97 strides/s ± 0.09 and 0.94 strides/s ± 0.1 during PKAR. There was no significant difference in cadence between voluntary turning and podokinetic stimulation ($F(1, 15) = 1.82$, $p = 0.19$). The small difference in cadence

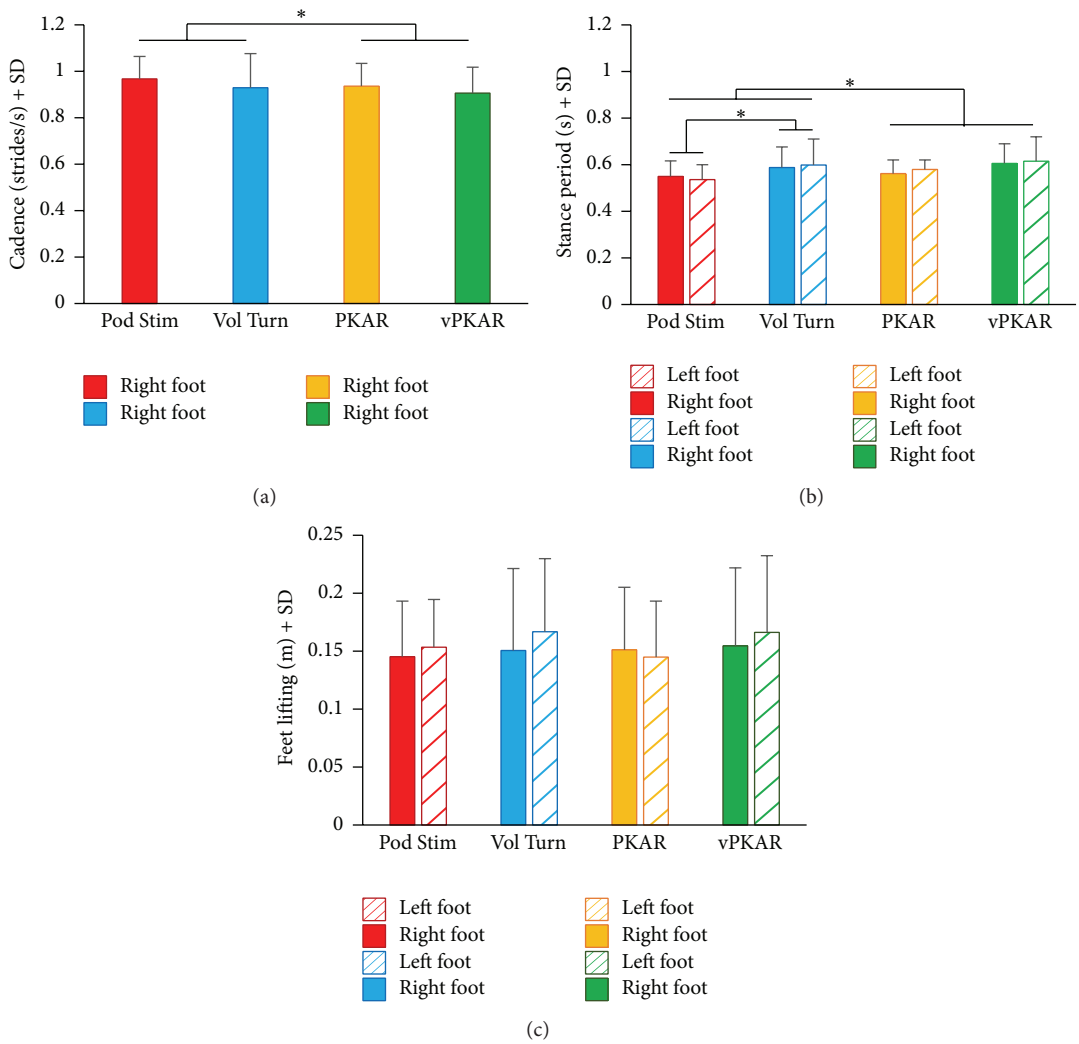

Figure 5: Cadence (a), duration of stance period (b), and height of feet lifting (c) during Pod Stim, Vol Turn, and the two posteffects. Cadence was not different between Vol Turn and Pod Stim but different between pre- and posteffects. The stance period (b) was somewhat longer during voluntary turning than during Pod Stim. Filled bars refer to the foot corresponding to the direction of rotation (right foot); striped bars refer to the foot opposite to the direction of rotation (left foot). There were no differences in height of feet lifting (c) between Vol Turn and Pod Stim or between the two posteffects. $*$ indicates significant difference ($p < 0.05$) between mean cadences (a) and mean stance period durations (b).

between pre- and posteffect was significant ($F(1, 15) = 5.9$, $p < 0.05$). However, the cadence during the posteffects in the 6 subjects turning at about the same velocities (mean velocity 65.26°/s ± 5.6) of the platform showed no significant difference between stimulation conditions ($F(1, 5) = 0.008$, $p = 0.93$) and no difference between vPKAR and PKAR (post-hoc test, $p = 0.2$).

The stance periods were calculated for each foot during the podokinetic stimulation, the voluntary turning, and their posteffects. The mean stance period was slightly longer during voluntary turning than during podokinetic stimulation ($F(1, 15) = 5.33$, $p < 0.05$). There was also a significant difference between voluntary turning or podokinetic stimulation and the two posteffects ($F(1, 15) = 16.52$, $p < 0.01$), since the stance period was just longer during the posteffects, consistently with the slightly lower cadence.

The mean height of foot lifting during voluntary turning and podokinetic stimulation and during the two posteffects is reported in Figure 5(c). There were no significant difference between voluntary and podokinetic condition ($F(1, 15) = 0.9$, $p = 0.3$) and no difference between pre- and posteffects ($F(1, 15) = 0.005$, $p = 0.95$).

3.5. Step Angle and Rotation Velocity. Figure 6(a) shows the mean foot angles calculated across subjects for each condition. The mean angle described by the foot on the horizontal plane during each step was 56.9° ± 6.5 during podokinetic stimulation and 21.4° ± 6.4 during the PKAR. The mean angle described by the foot during voluntary turning was 74.0° ± 27.5 and decreased to 15.6° ± 9.0 during the posteffect. ANOVA showed no significant difference between voluntary turning and podokinetic stimulation ($F(1, 15) = 1.35$,

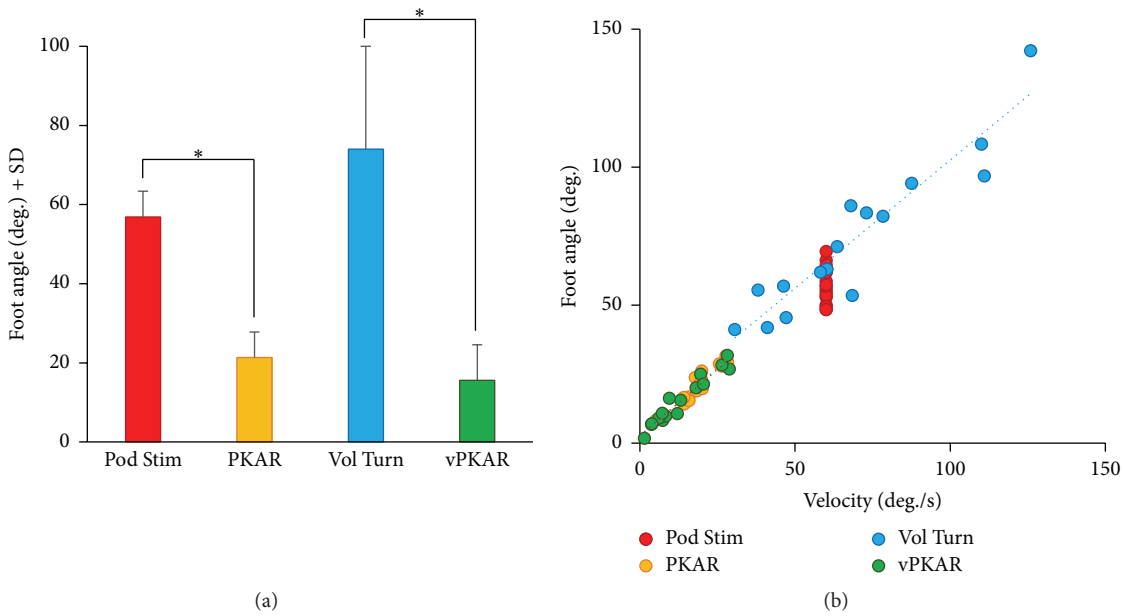

Figure 6: Foot angle (a) and its correlation with body rotation velocity (b). The mean foot yaw angle (a) between two consecutive stance phases was similar for podokinetic stimulation and voluntary turning. During the two posteffects, the mean angle diminished. In panel (b), the mean foot angle of each subject was plotted against the mean rotation velocity while stepping, for all conditions. There was a good relationship between foot angle and subject's angular velocity, both during voluntary turning and during the two posteffects. $*$ indicates significant difference ($p < 0.05$) between mean foot angles (a).

$p = 0.26$). There were a significant difference between pre- and posteffects ($F(1, 15) = 226.87$, $p < 0.01$) and an interaction between conditions and pre- and posteffect ($F(1, 15) = 29.14$, $p < 0.01$), since foot angle diminished slightly more during the posteffect of the voluntary turning than during the PKAR, in compliance with the overall slower turning velocity. Figure 6(b) shows the mean foot angle for each subject in each condition plotted against the mean angular velocity of the shoulders. There was a good relationship between the angle described by the foot and the subject velocities during both voluntary turning ($R^2 = 0.87$, $p < 0.01$) and the posteffects (vPKAR: $R^2 = 0.94$, $p < 0.01$, PKAR:$R^2 = 0.9$, $p < 0.01$).

4. Discussion

Turning around while stepping in place can be produced voluntarily or be the consequence of a stimulation applied during the stepping task, such as axial muscle unilateral vibration [22, 23, 30] or vestibular [31, 32] or optokinetic stimulation [25, 33]. One elegant way of producing inadvertent turning around while stepping in place eyes closed is to "induce" this behavior by having subjects stepping on a rotating platform while maintaining fixed heading by referencing body orientation to the seen environment [14, 20, 34]. In the literature, the platform training produces the podokinetic stimulation while the posteffect is called the podokinetic adaptation (podokinetic after-rotation, PKAR) [14, 18–20].

We tested the hypothesis that prolonged voluntary stepping in place while deliberately turning around on a motionless floor also produces a posteffect similar to PKAR. Hence, we sought an answer to the following questions: can a rotatory posteffect be produced by continuous, deliberate whole-body turning around while stepping (henceforth voluntary turning), as well as by the podokinetic stimulation consisting in a repeated displacement of feet orientation by a rotating platform (in turn counteracted by replacing the foot in a position compatible with heading maintenance)? If so, are there differences between the "true" PKAR and the posteffect of voluntary turning (vPKAR) in spite of the differences between the tasks? Can this comparison tell us something about the mechanisms underpinning PKAR?

At first sight, it would seem odd enough that a deliberate motor behavior contains in itself the potential for its inadvertent persistence after the termination of the specific voluntary command, not least, because of the presence of the efference copy during voluntary movement, which tends to cancel the feedback sensory information from the moved segments [35, 36]. However, examples are available for after-effects of deliberate motor actions, which normally tend to show features germane to the pristine task. For instance, a strong isometric effort of shoulder muscles for upper limb abduction, as by counteracting someone else's push against the hand for half a minute or so, produces an involuntary arm elevation (the Kohnstamm phenomenon) ([37] and see [38]) that inadvertently ensues at the end of the voluntary contraction. Further, walking on a linear treadmill, at variance with overground walking, produces an after-effect

on the orientation of the standing body in the form of a forward body inclination lasting for a few minutes [39]. In this case, body inclination would be the consequence of a change in the postural reference arising from treadmill locomotion itself, possibly connected to the peculiarities of this type of walking [40]. Moreover and more interestingly for the present account, when standing subjects oppose for a while a rotational torque applied to the pelvis, an involuntary postcontraction of the trunk muscles is observed. If these subjects start walking, they walk along a curved trajectory in the direction of the preceding torsion [41].

Hence, a posteffect of prolonged stepping in place while deliberately turning around may not be beyond belief. We show here that the posteffect of voluntary rotation (vPKAR) shares all the features of the PKAR induced by the podokinetic stimulation while stepping on the rotating platform. This applies to the evolution of the turning velocity over time, including rising time, peak velocity, and the decay after that peak. Indeed, no significant difference exists in the rise and decay time constants of the turning velocity profile between PKAR and vPKAR posteffects. Of course, larger interindividual differences were observed under the voluntary turning condition than under the podokinetic stimulation, since in the latter case the turning velocity was fixed, while in the former it was self-paced. Interindividual differences could also be observed during the posteffects. However, interestingly, the almost fixed ratio of the turning velocity post- (PKAR or vPKAR) versus prerotation (podokinetic stimulation or voluntary turning) was analogous, both within and across subjects, even if it is somewhat smaller for the vPKAR (about 20%) compared to PKAR (about 30%). This small difference observed in the grand means was reproduced when the posteffects were compared within a smaller subject subgroup for which the velocities were nondifferent during podokinetic stimulation and voluntary turning. Therefore, differences between voluntary turning (eyes closed) and platform stimulation protocols (eyes open) can affect, albeit to a limited extent, the process whereby the central nervous system integrates the podokinetic stimulation and sets the rotation velocity in the postperiod.

An apparently important difference between the two conditioning procedures is that vision was banned under the voluntary turning condition, which was performed being blindfolded. However, based on the similar quality of the posteffects, one would deduce that vision (a quasi-stable visual field is available *during* the podokinetic stimulation on the rotating platform) may not intrude at all in the acquisition of the PKAR features. As a limitation, we would note that our procedure did not allow assessing any effect of vision on the PKAR itself, since the podokinetic stimulation was always administered eyes open. This was necessary in order to have a constant orientation in space of the subjects, so that their feet and leg rotations counteracted closely the platform rotation. In other cases, the constant orientation in space was obtained by having subjects holding onto a hearth-fixed rail [17, 20, 26], but in no case were PKARs compared between podokinetic stimulations with and without vision. The role of vision may not be dissimilar to what happens while walking on the linear treadmill, where a mismatch also exists between the quasi-stable visual field and the "expected" visual flow connected to the virtual body progression. Yet, availability or suppression of vision has no major effects on dynamic stability in treadmill walking [42]. In passing and by necessity, vision has no effect on the PKAR itself or on the vPKAR, since eyes were closed in both tasks.

A vestibular input is certainly elicited by turning around the vertical axis [43] and it interacts with the generation of the PKAR [44]. The vestibular input could differ between voluntary turning and podokinetic stimulation and contribute to the differences observed in the peak velocity of vPKAR and PKAR. In the former case, during conditioning, the head undergoes a yaw rotation at about the same velocity and direction as the rotating body, while in the latter head and body do not quite rotate, unless for the minor yaw movements associated with the side-to-side displacement of head and trunk accompanying the stepping task. However, the mean angular head *acceleration* in the yaw plane proved to be not different during the voluntary rotation and the stepping on the rotating platform, since the value of the angular acceleration is mainly dependent on the small but fast horizontal to-and-fro yaw rotations of the head mentioned above. The ample but slow head rotations accompanying whole-body rotation while voluntarily stepping in place and turning around are relatively smooth and add little to the peak values of acceleration. Thus, the vestibular inflow may be comparable, under steady state, between the two conditioning procedures. However, Earhart et al. [27] have shown that the PKAR is somewhat enhanced in bilateral vestibular loss patients, suggesting an inhibitory action of the normal vestibular input on PKAR velocity. In this light, one might suppose a stronger vestibular input for voluntary turning compared to podokinetic stimulation, to account for the differences mentioned above. Indeed, the vestibular input must be stronger *just before* vPKAR compared to PKAR, since head velocity undergoes large changes at the end of the voluntary rotation (see Figure 1(d)). Instead, almost no changes are observed at the end of the podokinetic stimulation (see Figure 1(c)), because the head angular rotation was almost nil during the platform rotation (compare the angular velocity profiles in Figures 4(b) and 4(f)). Hence, the phasic vestibular stimulation occurring at the end of voluntary rotation could have prevented the peak rotation velocity of vPKAR to reach that of PKAR.

The global kinematic organization of the turning behavior in the posteffects was superimposable. The cadence of the stepping task, the duration of the stance period, and the height of feet lifting were not significantly different between the posteffect of podokinetic stimulation and that of voluntary turning. A strong relationship also appeared between amplitude of foot angular rotation and velocity of whole-body rotation, which somehow trivially shows that body rotation is dictated by the value of foot yaw rotation in the successive stance phases. This relationship held under both conditions, and its value was superimposable between the two posteffects. Also, the time lags between the periodic yaw rotations of shoulder and head were fully superimposed. Hence, both PKAR and vPKAR are characterized by *en* blochead rotation

and trunk rotation, in turn not different from that occurring during voluntary turning or stepping on the rotating platform. This conforms to the suggestion by Earhart and Hong [26], which advocated that PKAR is relayed through the same locomotor circuits active at the beginning of voluntary turning. Among the similarities of PKAR and vPKAR, it is important to mention that, under both voluntary turning and podokinetic stimulation conditions, the postrotation took place without the least perception of it by any subject, in spite of the yaw rotation velocity being certainly above the vestibular perception threshold at the yaw periodic rotations of about 1 Hz (or the stepping cadence) [45, 46]. Subjects themselves were startled when, well after their performance, they observed their final position and were told that that position was the final outcome of several spin movements.

During the podokinetic stimulation, feet (*and* legs) are passively rotated when they are placed on the platform during the stance phase, and subjects look at the surrounding environment that is their reference for keeping head and trunk almost fixed in space. Instead, during voluntary stepping and turning around, the supporting floor is stationary and the stance foot and leg are stable (they rotate during the swing phase), and trunk and head do rotate in space. A key event common to the two conditions is the rotation of the pelvis on the stance foot. During the voluntary rotation, one rotates the leg with respect to the pelvis (i.e., an external rotation of the leg, in the direction of the intended turning around) in the swing phase of stepping and rotates the pelvis pivoting on the stance foot in the same direction relative to space (i.e., an "active" internal rotation of the leg) during the stance phase. Conversely, during the platform rotation, the foot is being "passively" extrarotated in the direction of the platform rotation for the stance period, while the trunk rotates onto it for holding its orientation in space.

Hence, the truly common event to podokinetic stimulation and voluntary rotation is the active rotation of the trunk on the stance foot. This certainly requires the production of a nonnegligible force in the pelvic muscles that rotate internally the thigh, in order to have the heavy trunk keeping the pace with the foot extrarotation that has occurred during the immediately earlier swing phase (a task requiring minimal force). In this light, both PKARs would be produced by the voluntary effort of rotating the trunk on the feet, much as a voluntary deltoid effort produces the Kohnstamm arm-raising phenomenon or a voluntary trunk rotation effort produces a curved trajectory during a subsequent locomotion task [41]. Of note, a prolonged static twist of the trunk on the feet of 30° around the vertical axis during stance, maintained for 10 minutes, induced a subsequent unperceived postural reorientation but induced no PKAR when subjects were asked to step in place [34]. This indicates that PKAR is not an automatic consequence of postural reorganization but likely depends on the presence of a continuous motor output during the stepping condition [34]. The passive external rotation produced by the rotating platform would be the counterpart of the active (minimal-effort) external rotation during the swing phase in the voluntary rotation. In both cases, this extrarotation produces another event, that is, the passive stretch of the leg-intrarotating muscles. All in all, one might argue that the PKAR and the vPKAR rest both on the periodic active contraction of the lower limb intrarotating muscles, followed by their periodic passive stretch. The shortening-stretch cycle would appear the appropriate event, necessary for triggering an effective discharge in the muscle spindles, able to enhance the excitability of the locomotor circuits normally subserving turning (see [38], for a discussion of peripheral models of the Kohnstamm phenomenon generation).

We would only briefly speculate about the brain circuits that are potentially involved in the generation of PKAR and vPKAR in response to the periodic proprioceptive afferent volley from the muscles rotating the lower limb. Of note, during the Kohnstamm movement there is widespread activation of the cerebral cortex [47], and during the PKAR subjects show a direction-specific deviation of the subjective straight-ahead [48]. Therefore, the cerebral cortex, most likely the posterior parietal cortex, must be involved in the body orientation occurring during PKAR and vPKAR. In this light, subjects would trail their modified subjective straight-ahead while stepping in place, much as what occurs during neck muscle vibration [30], which is also known to affect the straight-ahead [49]. Courtine et al. [23] showed that axial but not appendicular muscle vibration produces a clear-cut deviation of the locomotor trajectory. In this vein, we would consider the (axial) pelvic muscles an important source of the proprioceptive input playing a role in the PKARs.

We would also note that the sum of the vestibular input and the proprioceptive input from the pelvis muscles might have affected the PKARs [50], much as what occurs for the sum of vestibular and neck input in the definition of the yaw motion perception [24]. In the cases mentioned in Pettorossi et al. [51, 52], the changes in the abnormal perception after a prolonged rotational vestibular stimulation and neck vibration are long-lasting. That duration is of the same order of magnitude as the duration of the PKARs, pointing to a central modulation of the deviated perception of the straight-ahead in both cases (see [24] for a review). We would also point out that while the repeated muscle contraction would create the background for a posteffect, the particular sequence of the active stepping movements would confer the peculiar rhythmic features to the posteffects. It is as if the build-up of the charge of the "integrator battery," wherever and whatever it is, contains in itself the memory of the pattern responsible for the process.

On the translation to the clinical side, this line of research goes in the direction of recent findings showing the relevance of practicing a specific task with added challenges to a training regimen ([53], in rats). Stepping in place while voluntarily turning can substitute and be effective almost as much as the rotating platform for training of the turning coordination [29, 54] in patients with impairment of turning synergies of various origin [9–11, 16, 55, 56].

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Training-Induced Functional Gains following SCI

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We previously demonstrated that daily, hour-long training sessions significantly improved both locomotor (limb kinematics, gait, and hindlimb flexor-extensor bursting patterns) and nonlocomotor (bladder function and at-level mechanical allodynia) functions following a moderate contusive spinal cord injury. The amount of training needed to achieve this recovery is unknown. Furthermore, whether this recovery is induced primarily by neuronal activity below the lesion or other aspects related to general exercise is unclear. Therefore, the current study objectives were to (1) test the efficacy of 30 minutes of step training for recovery following a clinically relevant contusion injury in male Wistar rats and (2) test the efficacy of training without hindlimb engagement. The results indicate that as little as 30 minutes of step training six days per week enhances overground locomotion in male rats with contusive spinal cord injury but does not alter allodynia or bladder function. Thirty minutes of forelimb-only exercise did not alter locomotion, allodynia, or bladder function, and neither training protocol altered the amount of in-cage activity. Taken together, locomotor improvements were facilitated by hindlimb step training for 30 minutes, but longer durations of training are required to affect nonlocomotor systems.

1. Introduction

From lower vertebrates to humans, it is known that locomotor activity is generated by spinal neuronal circuits referred to as the central pattern generator (CPG) [1]. Studies in lower vertebrates have been useful for modeling how the CPG performs in the absence of supraspinal and afferent feedback as well as determining the roles of neurotransmitters and afferent stimuli (load, speed, and perturbations). Within the context of spinal cord injury (SCI), central pattern generation has become a conceptual basis for locomotor training after injury [2, 3].

In rats, cats, and humans, a large amount of spontaneous recovery can occur following SCI, and this recovery is closely related to white matter sparing [4–6]. Specifically, this recovery has mainly been attributed to spared fibers in the ventral and ventral lateral funiculi where the rubrospinal, reticulospinal, and vestibulospinal tracts are located [7, 8]. Yet, the recovery achieved via training is generally specific to the task practiced, for example, stand, step, or swim training [9, 10]. The amount of activity imposed on the limbs is also a crucial component of locomotor rehabilitation. Importantly, step training on a treadmill using body weight support should provide a high number of repetitions to facilitate motor learning [11]. Recovery potential therefore is a function of (1) amount of sparing/injury severity, (2) task specificity, and (3) amount of activity (activity dependent plasticity). Other aspects of step training or general exercise may also contribute to SCI recovery, including environmental enrichment, intermittent hypoxia, and general improvements in body strength and psychological well-being. More studies are needed to determine the efficacy and mechanisms of training on functional outcomes (including locomotion, allodynia, and autonomic functions) after incomplete contusions in combination with appropriate control groups, kinematics, and overground locomotion (for review see [12]).

In humans, complete cord transection is rare. Therefore, a clinically relevant rat contusion model of SCI may provide

very useful information for the study and translation of locomotor training. The rat model exhibits similarities to human SCI progression [13]. Research with spinally complete and incomplete rodents has affirmed central pattern generation and identified important aspects of training and SCI locomotion [11, 14, 15]. While it is clear that training influences aspects of treadmill locomotion post-SCI, basic research studies have yielded conflicting outcomes regarding overground locomotion [16–19]. Furthermore, very little is known about the efficacy of step training on nonlocomotor functions.

We previously demonstrated that 60 minutes of training 7 days per week significantly improved both locomotor and nonlocomotor functions, such as open field locomotion, hindlimb kinematics, at-level mechanical allodynia, and bladder function in contused rats [20]. In the current study, we (1) tested the efficacy of a 30-minute body weight supported treadmill step training paradigm for the recovery of overground locomotion using qualitative scoring (BBB) and quantitative (kinematic) locomotor tests, (2) determined the contribution of in-cage activity to spontaneous locomotor recovery versus training-induced recovery, and (3) examined bladder function and allodynia (pain response to a nonnoxious stimulus). SCI control groups (nontrain, forelimb) were identically handled, harnessed, and tested. The SCI forelimb trained control group addressed the potential for exercise mediated improvements. Sham (laminectomy only) animals provided a standard comparison throughout the study for each parameter.

2. Materials and Methods

All animal procedures were performed according to the NIH guidelines, and the protocols were reviewed and approved by the Institutional Animal Use and Care Committee at the University of Louisville, School of Medicine. Thirty-eight male Wistar rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) approximately 60 to 70 days old weighing approximately 300 grams were individually housed in an animal room with a 12-hour light and dark cycle. Four animals served as shams (laminectomy controls). SCI animals were randomly divided into three groups before training began as previously described [20]. One group ($n = 14$) was quadrupedally trained; a second group ($n = 10$) served as nontrained controls; a third group was forelimb trained ($n = 10$). Training began at two weeks after SCI for thirty minutes per day, six days per week and continued for six weeks.

2.1. Spinal Cord Injuries. Animals were anesthetized with an intraperitoneal dose of ketamine (80 mg/kg, Ketoset®, Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10 mg/kg, AnaSed, Lloyd Laboratories, Shenandoah, IA) mixture. Chlorhexiderm scrub cleaned the shaved surgical site. Lubrication was applied to the eyes. The following were administered subcutaneously: 0.5 mL of dual penicillin (PenJect®; The Butler Company; Columbus, OH) single dose perioperatively as a general prophylactic, 5 mg/kg gentamicin (GentaFuse®, Butler Schein, Dublin, OH) once per day for 5 days to prevent bladder infections, 2.5 mg/kg ketoprofen (Ketofen®, Fort Dodge Laboratories, Fort Dodge, IA) twice per day for two days to alleviate postsurgical pain, and 10 mL saline, as previously described [20].

Heating pads maintained body temperature during surgery and throughout the recovery period. The T8 lamina was removed to expose the T9 spinal cord. Spinal clamps were applied to the T7 and T9 spinous processes to stabilize the spinal column. The Infinite Horizon impactor was used to deliver a 210 kdyn impact. Laminectomy shams had the T9 cord exposed but not contused. The muscle was closed with suture, the skin closed with Michel clips, and topical antibiotic applied. Animals were single housed on a 12 : 12 light : dark cycle.

2.2. Behavioral Procedures

Training Paradigm. Training interventions may be less effective when initiated in chronic SCI subjects. However, training interventions initiated too early after SCI may be detrimental to recovery efforts by exacerbating secondary injury cascades [21]. We initiated training at two weeks after SCI after the majority of spontaneous recovery had occurred according to past experience with this injury severity (based on locomotor scores and recovery of spontaneous bladder voiding) [22]. Step training was performed on the Exer3 Treadmill system (Columbus Instruments, Columbus, OH) customized with spring scales for body weight support. The animals were harnessed with lycra vests (Robomedica, Inc., Mission Viejo, CA) and hook-and-loop material and Velcro straps.

Trained animals began quadrupedal step training 2 weeks after SCI surgery, 6 days per week, 30 minutes per day, for 6 weeks, at 22 cm/s. Trainers adjusted the body weight support as needed using manual assistance at the hip flexor region to facilitate proper plantar placement, for example, complete toe extension, no ankle rotation, and incorporation of forelimb-hindlimb coordination with minimal assistance. Rats were encouraged to step independently as they began to gain consistent stepping and more stability without collapsing and dragging their hindlimbs. It is of note that animals were quadrupedally trained, as many studies utilize an upright bipedal training and testing position. The upright position alone can facilitate stepping [23]. Forelimb animals were harnessed and walked with forelimbs on the treadmill while a custom 4 × 6 inch metal platform supported the hindlimbs just above the moving treadmill belt. Nontrained animals were harnessed while a custom platform supported all limbs. For nontrained animals, the 4 × 12 inch metal platform was beside the treadmill belt. One trained, one nontrained, and one forelimb trained animal could be harnessed simultaneously. No body weight support was provided for forelimb and nontrained animals. Sham animals were not exposed to the treadmill system and were handled four times per week (minimum).

Rats were not required to complete the 30-minute session during the first week of training if they exhibited signs of stress, that is, porphyrin staining around the eyes or nose, irregular breathing, or excessive diarrhea. All animals were able to complete the 30 minutes by 6 sessions. Animals were

never stimulated to step by perineum or tail pinching. Noxious stimuli were avoided during training sessions; for example, if an animal had skin abrasion on a paw, animals ceased from training until the issue was resolved, as potentially noxious input may inhibit spinal learning [22, 24, 25].

Locomotor Assessment. An open field locomotor assessment, the Basso-Beattie-Bresnahan (BBB) scale, was used to evaluate hindlimb function in the rats [26]. Once per week, each animal was placed in an open field and tested for 4 minutes by the same two scorers, who were presented with the trained, forelimb, nontrained, and sham animals in random order (blinded to group). The BBB uses a 21-point scale for locomotion, which rates parameters, such as joint movements (0–8), weight support (9–13), and paw placement (14–21). Intact animals demonstrate a locomotor score of 21, whereas animals that exhibit complete paralysis of the hindlimbs are scored as 0. Baseline measurements were collected prior to SCI surgery followed by weekly testing thereafter.

Kinematic Data Acquisition. After the six-week training period the hindquarters were shaved and the bony landmarks on the lateral side of the left and right hindlimbs were marked with permanent marker: iliac crest, greater trochanter, lateral malleolus, and metatarsophalangeal joint (ilium, hip, ankle, and toe). The pad on the plantar surface of the paw was also marked. Each animal was then individually placed in a clear plexiglass runway (in random order). As the animal passed from one end of the tank to the other darkened side, cameras positioned on the side and underneath the tank captured the angles and footfall patterns [27]. 2D overground (unassisted) kinematics were analyzed using the MaxTraq motion analysis system (Innovision Systems, Columbiaville, MI). The iliac crest, hip, ankle (lateral malleolus), toe, and paw were digitized by a blinded observer, and the hip-ankle-toe (HAT) and iliac crest-hip-ankle (IHA) angles were marked to quantify the movement of the hindlimbs during overground stepping. Four animals ($n = 3$ forelimb; $n = 1$ nontrain) could not generate weight bearing steps (BBB = 8; 8 weeks after SCI); these animals did not participate in the RI or PSI tests.

Horizontal Ladder Walk. Animals were additionally tested for fine locomotor control by crossing a horizontal ladder of metal bars (Columbus Instruments, Columbus, OH) [28, 29]. Animals were tested for their ability to correctly place their hind paws while crossing the bars. Forelimb errors were not counted. Animals with more severe deficits (BBB score < 11) were not tested because their limbs drag across the rungs and have a falsely reduced error count. The animals readily crossed the runway with minimal encouragement. Two blinded raters manually counted the number of footfall errors (hind paw/limb slip or fall through the bars). After each crossing, the raters discussed and agreed on the error count. If, at the end of the crossing, the raters did not agree on error count within two errors, the trial was repeated. Footfall error is reported as the mean of three high quality passes (crossed with little or no hesitation and without changing direction; hindlimbs did not drag; raters agreed on error count).

At-Level Allodynia. Behavioral testing of SCI rats for sensitivity to normally innocuous stimuli (touch and gentle squeeze/pressure) was performed using our published grading scale for the scoring of pain-like behavior to trunk stimulation in the rat [30]. Detailed methods also are described in [20]. Once per week, testing sessions commenced at approximately the same time of day (9 am before the start of training). The dorsolateral trunk (T7–T9 dermatomal level) just above the T9 spinal injury level was tested bilaterally for at-level mechanical hypersensitivity to touch and gentle touch/squeeze. Two baseline measurements (at least 3 days apart) were performed before injury for all rats. Throughout the study, all allodynia testing was consistently performed by the same two experimenters, blinded to treatment groups.

At the start of testing, the top of the cage was removed and the animal was allowed to acclimate to the environment for 2 minutes. While in its cage, each animal was stroked at the dorsolateral trunk five times bilaterally with a number 5 paintbrush (1.5 × 0.5 cm bristles; average pressure, 15 g) in an alternating rostral/caudal plane [30]. An interstimulus interval of 1 min between sides was maintained. After each stroking stimulus, the presence/absence of any evoked responses that were indicative of pain was noted: (1) a freezing response (stopping of normal activity and staying still in response to the stimulus), (2) escape (any movement of the animal away from the stimulus probe), and (3) grabbing at or pushing away the stimulus probe with their forepaws. An animal must show an evoked pain-like behavioral response at least 60% of the time in a given session to be considered responsive to the testing stimulus (i.e., an animal responded to at least three of five stimuli/strokes per side) [31]. Responses to brush, if present, were further assessed for threshold values using a set of Semmes-Weinstein monofilaments (20-filament set, 15 of which are in the range of 0.008 g to 15 g; Stoelting Co., Wood Dale, IL). Animals designated as responders to brush (15 g stimulus) were then given a numeric score based on their associated responses to filament testing, receiving a minimum of 4 (4 = freeze, 5 = escape, and 6 = grab/push—as the aggressiveness of the behavioral response increases, so does the score) to a maximum of 10 (see [30] for scoring scale).

Depending on the behavioral response of the animal to brush, an initial filament stimulus was applied by pressing the tip of the filament into the dorsolateral trunk (T7–T9) until it bent. If the animal responded, a lower gram filament was applied to test the animal's sensitivity. In between filament probing, the animal was left alone for a 1 min interstimulus interval. The process was repeated until the lowest gram filament that the animal responded to 60% of the time was determined. If the animal was not responsive to the initial probing stimulus, the next greatest gram filament (and repeated if needed) was used to determine the threshold of the animal's sensitivity.

For those animals not responsive to brush stroke (i.e., evoked pain-like behavioral response to less than 60% of the stimuli—less than three of five strokes), a gentle squeeze/pressure test was conducted to determine if the animal had increased sensitivity to a stronger mechanical stimulus over a wider surface area (which also normally does not provoke

avoidance behaviors and is thus considered innocuous). In this instance, the animal's skin is gently squeezed with a pair of modified Adson tissue forceps (2.0 mm wide tips), which is equivalent to the 60 g Semmes-Weinstein monofilament. Gentle squeeze/pressure was applied to the dorsolateral trunk (T7–T9) five times bilaterally, with an interstimulus squeeze interval of 1 min. As with touch-evoked agitation, any evoked pain-like responses were observed and documented (0 = no response, 1 = freeze, 2 = escape, and 3 = grab/push). After the testing session, animals were scored for their degree of at-level allodynia based on a 10-point scale, with 10 being the maximum score an animal could receive [30]. Scores from each weekly testing were documented for each animal and averaged together to obtain a mean weekly allodynia score for each group.

Transvesical Catheter. After the last training session and all locomotor testing, a transvesical bladder catheter was implanted under 2% isoflurane (similar to [32]). Body temperature was maintained with a circulating water-heating pad. Briefly, the bladder was exposed via a midline abdominal incision through the skin and musculature. A purse-string suture (4-0 Ethilon) was placed in the urothelium of the bladder dome. PE-60 tubing (the tip previously heated to form a collar ~2 mm from the end) was inserted through the bladder dome within the suture limits and secured. The bladder was emptied, and the tubing was passed through the subcutaneous tissue and exteriorized behind the neck [33]. After a 1.5-hour recovery period, the animal was placed in a darkened box for cystometric recordings.

Urodynamic Analysis via Nonstop Transvesical Cystometry. The catheter was connected to an infusion pump and pressure transducer. Normal saline was infused into the bladder at a rate of 0.25 mL/min to evoke voiding contractions [34]. Urodynamic data (voiding and nonvoiding events, voided volumes, and animal movements or spasms) and experimenter notes were recorded on video for offline playback and analysis with Datawave software (http://www.dwavetech.com/). Voiding efficiency was calculated as the percent volume voided per volume saline infused. Cystometrogram (CMG) parameters are the mean of 5 consecutive cycles (which were sampled approximately 15 minutes after the start of saline infusion). CMGs were analyzed for resting pressure (mm Hg), maximal amplitude of contraction (mm Hg; peak pressure minus resting pressure), contraction time (sec), and intercontraction interval (sec).

Activity Meter. The in-cage activity of every animal was recorded using an Opto-M3 infrared activity monitor (Columbus Instruments, Columbus, OH). A cradle equipped with infrared beams, spaced one inch apart, was placed around the cage so that the infrared beams shined across the cage near the floor. The data were collected as ambulatory (number of two consecutive beam breaks; i.e., the animal was moving across the cage) and total movements (number of beam breaks) during the active phase (6 pm–6 am).

2.3. Histology of Lesion Epicenter. Each animal was deeply anesthetized with ketamine/xylazine and perfused transcardially with a solution of normal saline and heparin. The bladder was blunt dissected away from the prostate, blotted dry, and weighed. The spinal lesion area was removed and placed in 4% paraformaldehyde for at least 48 hours, followed by 30% sucrose/phosphate buffer solution with 1% sodium azide for at least 24 hours and until the tissue was cut on a cryostat (Leica CM 1850) at 18 μm thickness and stained with both Luxol fast blue and cresyl violet (Kluver-Barrera method). The lesion area was quantified as previously described [30] using Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and the Nikon E400 microscope. Briefly, white matter was divided into four regions (dorsal columns, dorsolateral funiculus, ventrolateral funiculus, and the ventral funiculus) and each area was subdivided into left and right sides. The gray matter was divided into dorsal and ventral regions. The central canal, medial edges of the dorsal horn, and the tips of the ventral horn were used as landmarks for the divisions. The percent of white matter sparing (WMS) was determined by dividing the white matter remaining at the epicenter, .5 mm rostrally, and 1.0 mm rostrally by the average area of white matter present in intact sections. The intact area of white matter for a given region was estimated by averaging together measurements from 2 sections 2 mm rostral to the epicenter.

2.4. Statistics. SCI animals were excluded from analysis if the recorded displacement of the impactor tip was less than 1.0 mm during the injury ($n = 3$); these animals typically have a very mild injury. An additional 2 animals were sacrificed during the first two weeks following injury due to autophagia. This resulted in the following groups: train, $n = 13$; nontrain, $n = 7$; forelimb, $n = 9$; sham, $n = 4$. Analyses were performed using SigmaStat and Microsoft Excel. Levene's test for inequality of variance was performed. One-way repeated measures analysis of variance (ANOVA) (fixed effects) was performed for tests of within subject and between subject effects followed by Bonferroni *post hoc* t-tests for the BBB, allodynia, and activity. One-way ANOVA (fixed effects) was used for cystometry parameters, bladder weight, gait, and kinematics followed by Bonferroni *post hoc* t-tests, significance level $p < .05$. For the regularity index (RI), *post hoc* tests approached significance and were followed by the Mann-Whitney U test. Percent animals with consistent weight support were analyzed with the binomial proportion test. Significance level was $p < .05$. All values reported in the paper are mean ± SD.

3. Results

3.1. Training Significantly Improved Overground Locomotion. All groups demonstrated significant spontaneous recovery from 1 to 2 weeks after SCI. At the initiation of training (2 weeks after SCI) all groups functionally displayed plantar paw placement with occasional weight support (BBB scores: trained: 9.61 ± 1.7; forelimb: 9.5 ± 2.2; nontrained 9.3 ± 2.0). After 3 weeks of training (5 weeks after SCI), trained

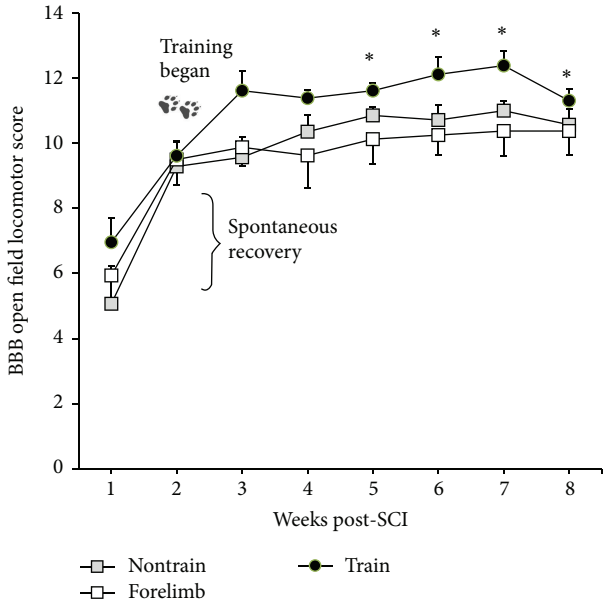

Figure 1: Weekly open field locomotor scoring of trained, nontrained, and forelimb SCI male rats. Statistically significant locomotor recovery occurred from week 1 to week 2 for all groups. At 2 weeks post-SCI training began. Only the trained group had significant increases from pretraining. Shams not shown, BBB = 21 (∗ versus pretraining; W2; repeated ANOVA with Bonferroni *post hoc t*-tests: nontrain $n = 7$; forelimb $n = 9$; train $n = 13$).

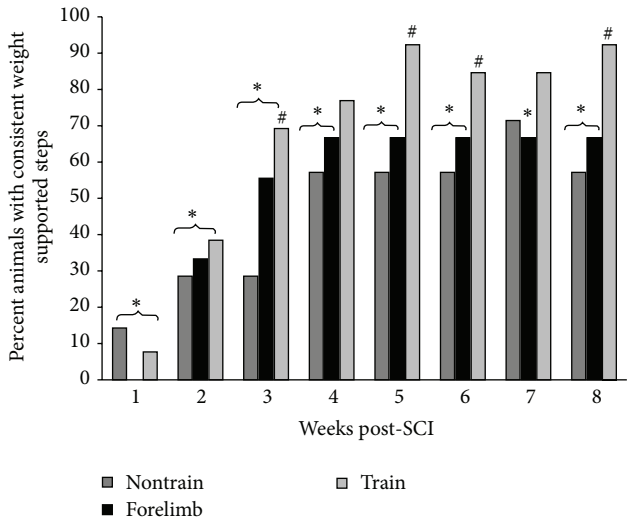

Figure 2: Percent animals with consistent weight support. Significant differences were found between sham and both the nontrained and forelimb groups (∗) at weeks 1–8, except week 7, as well as between the trained and nontrained groups (#) at weeks 3, 5, 6, and 8. No differences were found between sham and trained animals after week 3 (binomial proportion test: nontrain $n = 7$; forelimb $n = 9$; train $n = 13$).

animals' BBB scores were significantly higher compared to pretraining and remained significantly higher through the rest of the study (Figure 1). Forelimb and nontrain controls did not significantly improve from their pretraining BBB score. Consistent weight supported stepping (BBB ≥ 11) is a functional milestone on the BBB scale (which makes each animal eligible to receive a subscore). A significantly higher proportion of animals in the trained group achieved consistent weight support. No differences were found between sham and trained animals after week 3 (Figure 2). However, the trained group was also not significantly different from the forelimb or nontrain groups. Forelimb and nontrain groups had significant proportions of animals *unable* to consistently weight-support as compared to sham throughout the study.

Kinematic measurements of overground locomotion revealed that quadrupedal-trained animals more closely approximated the hindlimb angular movement of shams (no significant differences). However, the trained group was also not significantly different from the forelimb or nontrain groups. Both nontrained and forelimb trained control groups had significantly larger angular excursions of the hip and ankle, as well as significantly larger ankle extension (Figure 3).

The trained animals recovered to BBB scores necessitating the assessment of forelimb-hindlimb coordination. Importantly, although the BBB can be used to assess coordination, we also utilized a more objective assessment of coordination, the regularity index (RI) [35]. The RI is a score of plantar footfall pattern and limb coordination [36], and the trained group scored significantly better than the nontrained group (Figure 4). The plantar stepping index (PSI), which is a ratio of plantar hindlimb to forelimb steps [27], revealed no differences between trained and sham controls, while nontrained and forelimb controls scored significantly lower than shams (Figure 4). However, the trained group PSI was also not significantly different from the forelimb or nontrain groups. Analyses of gait parameters did not detect differences between groups for stride length, stride time, base of support, or toe velocity (data not shown).

The horizontal ladder demonstrated a large difference between injured and sham animals. Sham animals were able to cross the horizontal ladder with only one or two errors (1.1 ± 0.51). Although trained animals displayed improvements in overground locomotion and limb coordination, their ability to control fine placement of the hind paw remained impaired. There were no differences between any SCI group (train 7.67 ± 2.88; forelimb 8.43 ± 2.44; nontrain 7.42 ± 3.80).

3.2. At-Level Allodynia. Quantitative measurements of sensitivity to mechanical stimuli were obtained on two separate occasions prior to injury and then once per week for 8 weeks after SCI in the trained, nontrained, and forelimb groups. Preinjury measurements revealed that none of the groups demonstrated allodynic behavior to innocuous stimuli and were equivalent at baseline. One sham animal vocalized prior to injury and was excluded from this analysis only. The average sham score after surgery was 1.75 ± 2.4. Immediately after SCI, all rats exhibited a moderate degree of evoked at-level allodynia consisting of either freezing, escaping, or grabbing (with or without vocalization) towards a stimulus filament ranging from 15 g to 0.008 g. These pain-related aversion behaviors persisted throughout the course of training for all

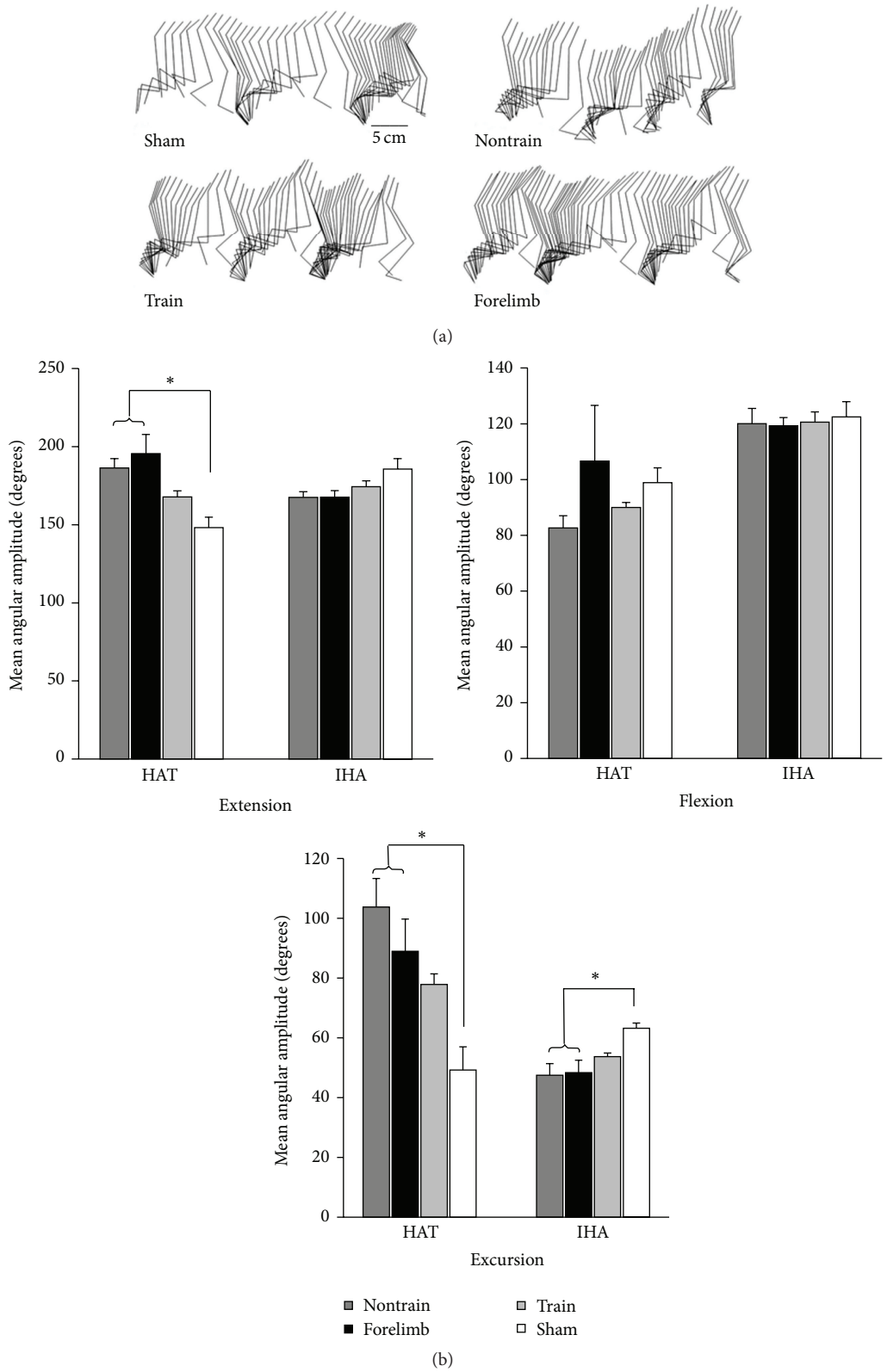

Figure 3: (a) Kinematic illustration of the hindlimb (iliac crest, greater trochanter, knee, lateral malleolus, and metatarsophalangeal joint) during stepping. (b) The maximum (extension) and minimum (flexion) angles as well as excursion (range of motion) of the hip-ankle-toe and iliac crest-hip-ankle angles were calculated and compared between trained (n = 13), nontrained (n = 7), forelimb (n = 9), and sham (n = 4) (ANOVA with Bonferroni *post hoc* t-tests). Significant differences were found for ankle extension and hip and ankle excursion (∗ sham versus nontrained and forelimb). Sham was not different from trained.

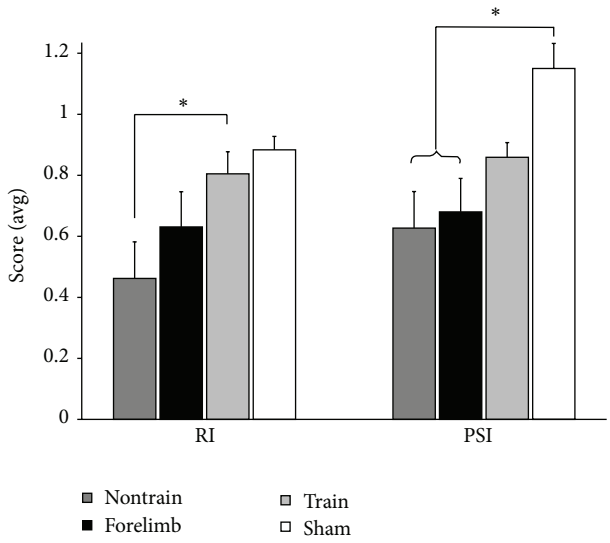

Figure 4: The regularity index revealed a significant difference between the trained and nontrained groups (∗) (Mann-Whitney U test). The plantar stepping index revealed a significant difference between the sham and both the nontrained and forelimb groups (∗) (ANOVA with Bonferroni *post hoc* t-tests). Parameters of gait were not significant: stride length, stride time, base of support, and toe velocity. Nontrain $n = 5$; train $n = 13$; forelimb $n = 6$; sham $n = 4$.

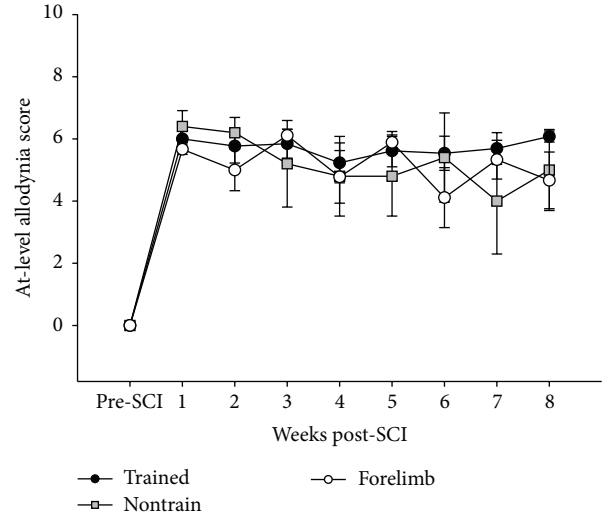

Figure 5: No animals were sensitive to touch or gentle squeeze prior to injury. Immediately after SCI, all rats exhibited a moderate degree of evoked at-level allodynia. There were no differences between groups regarding the course of at-level allodynia.

SCI groups (allodynia score at 8 weeks after SCI: train 5.72 ± 1.44; forelimb 5.19 ± 1.47; nontrain 5.23 ± 2.33) (Figure 5).

3.3. Cystometry and Bladder Weights. Autonomic dysfunction is of high priority for individuals with SCI [37]. We examined a total of 12 urodynamic parameters as well as bladder weights to identify possible treatment effects. Only a few are reported here. Shams were directly compared to SCI groups for bladder weight but not for urodynamics,

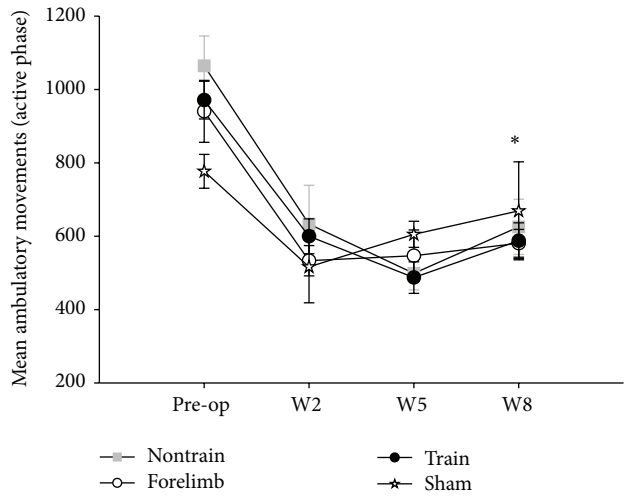

Figure 6: Home cage activity: in-cage activity assessments with an infrared activity monitor show no differences in the amount of ambulatory movements up to week 8 (W8), suggesting that the increased locomotor recovery of the trained group was a result of the step training paradigm and not due to "self-training." All animals significantly decreased activity with time from surgery (∗ preoperation versus W8 for each group; repeated ANOVA with Bonferroni *post hoc* t-tests; nontrain $n = 7$; train $n = 13$; forelimb $n = 9$; sham $n = 4$).

as the fill rate of .25 mL/min causes different physiological outcomes in a very small bladder compared to a bladder twice the normal size. No significant differences were observed between trained, nontrained, and forelimb groups (one-way ANOVA, $p > .05$). Bladder weights of all SCI animals were larger than shams (bladder weight in milligrams: sham 143.3 ± 25.2; train 226.5 ± 52.7; forelimb 287.2 ± 78.9; nontrain 240.0 ± 65.6; one-way ANOVA $p = .013$) [38, 39]. Intercontraction interval (ICI, seconds) was not different between groups (train 130.5 ± 88.8; nontrain 120.0 ± 51.1; forelimb 127.2 ± 77.4). Voiding efficiency was not different between groups (train 89.5 ± 9.8; nontrain 92.3 ± 8.3; forelimb 86.1 ± 15.2).

3.4. Home Cage Activity (6 pm–6 am). Another source of potential locomotor practice is in-cage activity. Home cage activity was monitored during the active phase and revealed that while all groups significantly decreased activity with time, there were no significant differences between any group when measuring ambulatory or total in-cage movements. All groups followed a similar pattern (Figure 6). This finding suggests that home cage activity was not a significant contributor to improved locomotion in the trained SCI group.

3.5. White and Gray Matter Spared. Histological assessment of the injury started with the epicenter and continued 1 mm rostrally. Figure 7 shows representative sections of the contused cord at the epicenter and .5 mm and 1.0 mm rostrally from the injury as well as an intact section. There were no significant differences between any group when analyzing weight gain (each group gained approximately 100 grams), injury parameters, white matter at the epicenter, .5 mm, or

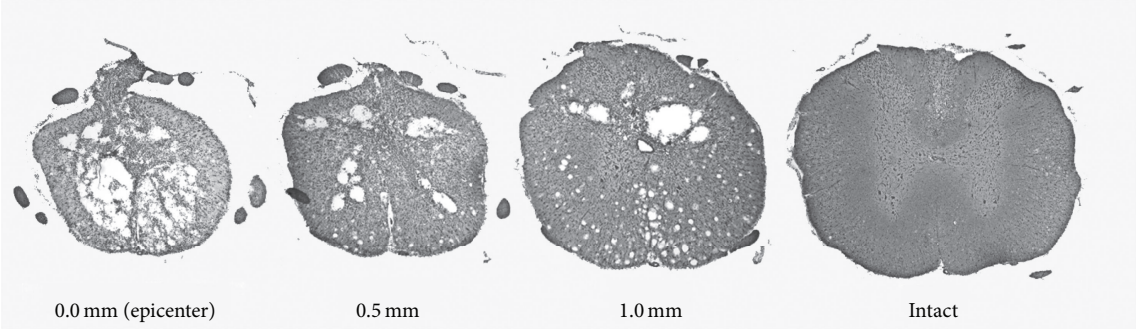

Figure 7: Representative spinal cord segments. Histological assessment did not reveal any differences between groups when analyzing total white or gray matter from the epicenter to 1.0 mm rostrally or when further subdividing the sections into areas of ventral and ventral lateral funiculi (ANOVA: nontrain $n = 7$; train $n = 13$; forelimb $n = 9$).

Table 1

Treatment	Force (kdyn)	Displacement (μm)	WMS% 0.0 mm	WMS% 0.5 mm	WMS% 1.0 mm	Body weight at week 8 (g)
Nontrain	215.57 ± 5.6	1269.4 ± 117.2	9.2 ± 5.1	28.3 ± 8.2	65.0 ± 5.7	417.57 ± 43.8
Forelimb	217.13 ± 4.6	1308.8 ± 140.0	11.4 ± 8.6	27.6 ± 9.0	59.4 ± 11.1	439.7 ± 43.1
Train	217.45 ± 6.6	1269.6 ± 158.9	12.8 ± 7.1	26.9 ± 8.8	59.6 ± 12.7	441.2 ± 35.0

1.0 mm (Table 1). The white matter was further subdivided into ventral and ventral lateral funiculi and gray matter of the ventral horn was also calculated at the epicenter, .5 mm, and 1.0 mm. There were no differences detected between any group when analyzing the ventral portions of the spinal cord. These data suggest that functional differences observed between groups cannot be attributed to differences in the amount of spared white or gray matter. Yet, plasticity within the spared pathways may facilitate functional recovery.

4. Discussion

The method of training used in this study facilitated additional recovery of the trained group (beyond the substantial amount of spontaneous recovery that occurred during the first two weeks following SCI [18, 26, 40]) and is the first to show overground improvements using quantitative kinematic and gait analyses in addition to qualitative open field scoring for contused male rats. The differences in locomotor parameters of trained versus nontrained and forelimb controls could not be attributed to differences in home cage activity, similar to a study comparing spontaneous exercise and enriched environment in which the amount of activity did not correlate to locomotor recovery [41]. Additionally, like other studies using activity based therapies [16, 18, 42], we found that training did not increase white matter sparing, even when analyzing subdivisions of the spinal cord, such as the ventrolateral funiculus. These results suggest that training reinforced or facilitated plasticity within the remaining pathways or lumbosacral circuits rather than promoting regeneration or sprouting of new pathways.

After 6 weeks of training, almost all trained SCI animals had achieved weight supported stepping and degrees of forelimb-hindlimb coordination. In contrast, while many animals in the SCI control groups recovered consistent weight supported stepping, recovery was slower and some animals never regained the ability to weight-support. These data are consistent with stand training in transected cats, which increased the duration of hindlimb standing [43, 44]. In rats with contusion or compression injury, treadmill training improved ankle extension [14] and weight bearing during open field locomotion [16]. Compared to our previous findings [20], as little as 30 minutes of daily locomotor training can improve weight bearing locomotor ability in SCI animals (60 minutes of daily training did not further improve BBB scores).

Many studies perform quantitative kinematic measurements on the treadmill (bipedal and quadrupedal) where the hindlimb is passively extended during stance, the trunk is supported by a harness, and partial body weight support is provided. Here, we used overground kinematic and gait analyses to quantify the training effects on full weight bearing overground locomotion compared to sham and SCI controls. Ankle extension and ankle and hip excursion were normalized by training (more similar to shams). Coordination and plantar paw placement were also normalized (RI and PSI). In cats, treadmill training increases the recruitment of flexor motor pools compared to nontrained cats [6]. Trained SCI cats and rats have also been shown to have greater paw lift and hip flexion, respectively, both allowing a reduction in paw dragging [6, 15, 45]. Our results extend these studies and indicate that training facilitates normal step cycle trajectories during *overground* locomotion by decreasing ankle extension and excursion, increasing hip excursion, and increasing the plantar stepping index.

Step training that provides alternate limb loading and rhythmic repeated steps can promote neural activity and improve EMG patterns and amplitudes [17, 46, 47], which

could explain how ankle extension (during stance), plantar paw placement, and weight support improved with training. Importantly, not all aspects of motor control improved with quadrupedal training. Although training promoted the recovery of overground locomotion, training did not improve the ability to cross a horizontal ladder. The ladder test correlates very well with injury severity [48]. However, this task requires precision paw placement and relies on proprioception [49, 50]. While our training paradigm improved gross locomotion (weight bearing, coordination, etc.), fine proprioceptive movements remained impaired, possibly due to the task specificity of training.

Locomotor training has been reported as having beneficial effects on bladder function in clinical settings [51–53]. Indeed, we previously demonstrated that 60 min of training significantly improved bladder function. However, our results according to cystometry and bladder weights in this study and SCI-induced polyuria in our previous study [54] indicate that 30 minutes of training did not affect bladder function after SCI. Thus, longer training durations should be further investigated for nonlocomotor functional recovery. Indeed, we found that, with 60 minutes of training, both quadrupedal and forelimb training improved the maximum bladder contraction amplitude and reduced SCI-induced polyuria [55].

With respect to sensory function, the emergence of neuropathic pain after SCI significantly impacts patients' quality of life and interferes with functional recovery [56–58]. In the clinical setting, exercise, including treadmill training, can positively influence neuropathic pain [59–61]. We previously demonstrated that 60 min of locomotor training resulted in a significant improvement in at-level allodynia scores compared to the nontrained group. This effect was observed after 3 weeks of training and remained consistent throughout the course of training [20]. Other rodent models of SCI, employing varying degrees of locomotor training intensity and duration, also report attenuations of allodynic responses following either below-level to the plantar aspects of the paws [62, 63] or at-level [64] mechanical threshold testing. In this study, 30 minutes of step training was not sufficient to ameliorate the onset of trunk at-level allodynia after SCI. Differences across studies may be attributed to the spinal location (cervical versus thoracic) and type of injury model (contusion versus compression), gender, magnitude, and intensity of training as well as the region being tested (trunk versus paw). It is not clear whether step training induces a level of resistance to the glabrous plantar aspect of the paw, perhaps influencing mechanical withdrawal thresholds. Overall, the degree of spared spinal pathways, spontaneous recovery, and the rhythmic weight bearing load during stepping, which may promote activation of cutaneous and proprioceptive afferents, may all be driving factors influencing improvements in tactile sensation [63]. A comparison between males and females may also be warranted as the development of pain and the requirements for differential effective exercise protocols may be sex-dependent [65–67].

Enriched environments and spontaneous exercise, such as wheel running, have been shown to improve locomotor recovery after SCI [41, 42, 68]. The possibility exists that exercise could promote post-SCI recovery through more general mechanisms, such as through alterations in inflammatory pathways, a "nursing effect" through neurotrophins, or increased well-being. In this study, we utilized a control group to induce exercise without specific activation of lumbar circuitry. This forelimb trained SCI control group did not show any benefits of exercise. In fact, although not statistically different, the forelimb trained SCI animals scored slightly worse on multiple locomotor parameters, including BBB score, ankle flexion and extension, and ladder errors. These findings suggest that step training improves locomotor recovery through direct activation of lumbar circuitry. In contrast, according to our finding that 60 minutes of forelimb training significantly altered some parameters of urinary tract function [55], other mechanisms must be responsible for these nonlocomotor improvements. In conclusion, a number of factors can enhance or prevent functional recovery after SCI. These factors are related not only to the injury but specific parameters of step training. These factors have been widely varied in experimental SCI and are likely contributing to different conclusions about step training's efficacy in an incomplete rat model of SCI. Our results suggest that 30 minutes of manually assisted step training initiated in the subacute stage of recovery can maximize potential locomotor gains. We found that 30 minutes of quadrupedal step training improved overground weight support, coordination, and ankle/hip range of motion. These improvements could not be directly attributed to lesion variability or home cage activity. We also found that 30 minutes of daily training did not improve precision paw placement, bladder function, or allodynia. While 60 minutes of daily training does not result in even higher BBB scores or an increase in the percentage of animals that can weight-support, longer daily training sessions result in additional benefits to nonlocomotor functions (allodynia and bladder function), which would substantially influence a patient's quality of life.

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

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Clinical Study

Exploiting Interlimb Arm and Leg Connections for Walking Rehabilitation: A Training Intervention in Stroke

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Rhythmic arm and leg (A&L) movements share common elements of neural control. The extent to which A&L cycling training can lead to training adaptations which transfer to improved walking function remains untested. The purpose of this study was to test the efficacy of A&L cycling training as a modality to improve locomotor function after stroke. Nineteen chronic stroke (>six months) participants were recruited and performed 30 minutes of A&L cycling training three times a week for five weeks. Changes in walking function were assessed with (1) clinical tests; (2) strength during isometric contractions; and (3) treadmill walking performance and cutaneous reflex modulation. A multiple baseline (3 pretests) within-subject control design was used. Data show that A&L cycling training improved clinical walking status increased strength by ~25%, improved modulation of muscle activity by ~25%, increased range of motion by ~20%, decreased stride duration, increased frequency, and improved modulation of cutaneous reflexes during treadmill walking. On most variables, the majority of participants showed a significant improvement in walking ability. These results suggest that exploiting arm and leg connections with A&L cycling training, an accessible and cost-effective training modality, could be used to improve walking ability after stroke.

1. Introduction

Body weight supported treadmill training therapy can be used for the recovery of walking after neurological damage. In this rehabilitation paradigm, participants walk on a motorized treadmill with a harness system allowing the weakened leg muscles to be freed from the necessity of body weight support and stepping is performed with the help of robotic interfaces or therapists. This protocol was initially utilized after spinal cord injury and may be equally beneficial for recovery of walking after stroke [1–5].

Results from this therapy are positive, but there are significant limitations that limit access for the broader stroke population. Body weight supported treadmill training therapy has significant labour requirements, requires specialized equipment, and is typically only available in restricted environments such as in rehabilitation centers [6, 7]. In addition, body weight supported treadmill training offers no additional benefit over conventional physical therapy, as demonstrated in a large randomized clinical trial [2]. A more cost-effective and generally accessible protocol based upon a device (e.g., arm and leg ergometer or a recumbent stepper) that could be more readily used in therapy would be of great benefit where less training is required for physical therapists to supervise training and participants may be more likely to comply with a community-based training regimen [2, 8].

In addition to finding a rehabilitation program that is widely accessible, exploiting the neural and mechanical linkages between the arms and legs that are inherent parts of human locomotion could enhance the recovery of walking [6, 9, 10]. Therefore, incorporating rhythmic arm movement paradigms for locomotor rehabilitation, such as with arm and leg (A&L) cycling, could be very beneficial to stroke locomotor recovery. Although there are differences in

kinematics, balance requirements, and loading of the arms between walking and A&L cycling, this type of training activates similar neural networks that are engaged during walking [11]. We have recently shown that, even following a stroke, neural commonalities between A&L cycling and walking persist, despite altered descending supraspinal input from the stroke lesion [12]. Given that A&L cycling and walking share common neural elements and that this persists following stroke, there is a reasonable basis for expectation of training transfer to improve walking.

The extent to which A&L cycling training can lead to training adaptations which transfer to improved walking function remains untested. Thus, the objective of this project was to test the efficacy of A&L cycling training to enhance walking after stroke. Given that A&L cycling and walking share a common core of subcortical regulation, we hypothesize that A&L cycling training will transfer to an improvement in walking. Improvements in walking function were gauged by changes in clinical walking status, strength, and walking performance. If indirect training with A&L cycling does improve walking function, this adjunct therapy could be used as an additional modality to improve walking ability after stroke.

2. Materials and Methods

2.1. Participants. Participant recruitment occurred through community stroke support groups, posters in medical offices/hospitals, and newspaper articles. As for inclusion criteria, participants were required to be a minimum of six months after infarct, after spontaneous poststroke changes are thought to have occurred [13], and able to stand free without assistive devices. Participants were screened with the Physical Activity Readiness Questionnaire to determine eligibility to participate in physical activity. If a response of "yes" was given for any of the questions in the questionnaire, indicating the presence of bone or joint problems or dizziness, written medical permission was obtained for that participant. A list of current medications was also obtained for each participant. Exclusion criteria included medications affecting muscle tone less than three months priorly and self-report of any cardiovascular, musculoskeletal, respiratory, or other chronic diseases. A sample size of twenty-five participants was recruited, in line with statistical reports that a sample size of 25 will specify a power of 0.80 at a large effect size and criterion value of $p = 0.05$ [14]. Sample size was based on previous studies of locomotor studies after stroke and other interventions yielding strength gains after stroke [15, 16].

To assist with determining participant's functional status, clinical assessments were performed by a licensed physical therapist. Muscle tone was measured using the Modified Ashworth Scale (5 points) at the ankle and knee for the lower limb [17, 18]. This is a graded rating of spasticity scored from 0 to 4, with 0 being flaccid and 4 being rigid. A measure of the basic motor skills necessary for functional ambulation was derived using the 6-point Functional Ambulation Categories Scale, where level 1 indicates that a patient is nonambulatory and level 6 indicates a patient is fully independent [19]. To measure general physical impairment, the Chedoke-McMaster Stroke Assessment [20] was used. Impairment in the arm (A), hand (H), leg (L), and foot (F) was determined using the 7-point activity scale, where a score of 1 represents complete independence and a score of 7 represents total assistance. Using the 5-piece Semmes-Weinstein kit of calibrated monofilaments (Sammons Preston Rolyan, Cedarburg, WI), ability to discern light touch and pressure was measured in the more affected hand and foot [21]. Reflexes obtained using a reflex hammer were graded on a 0 to 4+ scale, where 0 means a reflex is absent and 4+ represents a hyperactive reflex with clonus for hip flexion (L1) and ankle plantarflexion (S1) [22].

2.2. Ethics Statement. The authors confirm that all ongoing and related trials for this intervention are registered (ClinicalTrials.gov: NCT02316405). Informed written consent from each participant was obtained for a protocol approved by the University of Victoria Human Research Ethics Committee (Protocol number: 07-480-04d) and performed according to the Declaration of Helsinki. The study protocol that was registered was the same as the study protocol approved by the University of Victoria Human Research Ethics Committee prior to subject enrollment.

2.3. Training Protocol. Participants performed training three times a week, with 30 minutes of aggregate activity time per session, for a total duration of five weeks. Most participants completed training on Monday, Wednesday, and Friday. All experimental and training sessions took place in the Rehabilitation Neuroscience Laboratory at the University of Victoria.

For training, an arm and leg cycling ergometer with coupled upper and lower cranks was used (Sci-Fit Pro 2 ergometer). Dependent motion of the cranks for the arms and legs allows for passive assistance of weaker limbs during training. Mechanical modifications were made to the cycle ergometer to ensure a customized and comfortable fit for each training session. The cranks of the arm and leg ergometer were individually adjusted to the range of motion for each limb of each stroke participant and hand braces were worn when needed to ensure grip on the handle with the more affected (MA) hand. During each session, participants were allowed to take short 1-2-minute breaks during training if required, but the aggregate time for each session was always met. In fact, few participants took breaks and those who did only needed them in the early days of training. Participants were expected to tolerate the protocol very well as this was a modification of a previous protocol where chronic stroke participants performed four trials of six-minute bouts (totalling 24 minutes) of active A&L cycling [16].

To evaluate the physiological cost of training activity, heart rate (HR), rating of perceived exertion (RPE), and revolutions per minute (RPM) were collected every five minutes. Heart rate was monitored with a chest strap heart rate monitor (Polar Electro, Quebec, Canada) and recorded in beats per minute (bpm). The rating of perceived exertion was self-reported using the 10 pt scale [23]. A&L cycle ergometer

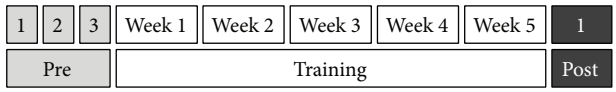

Figure 1: Illustration of the testing and training protocol. A multiple baseline within-subject control design was used for this study.

RPM were recorded visually from the digital display on the cycle ergometer as participants used this signal as a source of visual feedback for maintaining cadence. A single value for each variable was created each session by individually averaging the HR, RPE, RPM, and Watts over the 30 minutes of training and differences between the first and last training session were inspected.

Participants were encouraged to exercise at a moderate level sufficient to report a RPE value between three and five, corresponding to a target heart rate between 50 and 70% HR_{max} [24]. Target heart rate training zones were calculated with the Karvonen Formula taking into account heart rate reserve, and, if a participant reported being on beta blockers, adjustments to target heart rate goals were made [25]. The progressive element of this steady-state training included increasing the resistance of the ergometer over five weeks in order to maintain the same relative RPE. This is in line with many other poststroke treadmill training protocols where training volume was increased [26]. Increases in resistance were only required for 6 out of 19 participants and generally increases were made in 5 W increments to a maximum of 40 W. During the training and testing time, participants were also encouraged to maintain their normal activity levels but to not participate in additional research programs or interventions.

All exercise sessions were supervised by a Certified Exercise Physiologist with the Canadian Society for Exercise Physiology as well as several laboratory assistants to ensure appropriate monitoring. Exercise sessions were not initiated if participant's blood pressure exceeded 140/90 mmHg in accordance with Canada's Physical Activity Guidelines [27]. Exercise was terminated if HR exceeded 85% of the age-predicted maximum, if blood pressure exceeded 200/110 mmHg, and if the participant felt dizzy, nervous, or pain in the chest. Upon completion of the 30 minutes in each training session, participants were given three to five minutes to cool down and remained in the laboratory until blood pressure returned to preexercise values. All blood pressure values were obtained with a digital blood pressure cuff placed over the less affected arm.

2.4. Multiple Baseline and Posttest Measures. A multiple baseline within-subject control design was used for this study [28, 29]. Figure 1 illustrates the testing and training protocol. A multiple baseline design allowed for the creation of a reliable and consistent pretest measure, allowed for inspection of spontaneous recovery effects, and provided baseline data against which changes were evaluated. In this design, the control group is the experimental group. Multiple baseline measurements were obtained from participants in three baseline experimental sessions over a period of three to four weeks, with at least six days between baseline sessions. The posttest following training was performed within three days following training. As it was impossible to blind participants in this trial, several things were done to help control for potential sources of bias. At experimental sessions, the same tests were performed in the same order and environmental conditions (i.e., temperature, noise, lighting, and participant position) and session time of day were kept as consistent as possible [15, 30, 31]. These measures have been previously shown to have high reliability across multiple baseline points [29]. The project manager, who was in charge of participant recruitment and scheduling, did not take part in the assessment of outcomes, nor did the exercise supervisors. Analysis of data was mainly performed by laboratory assistants who were not involved in the design or interpretation of results.

2.5. Clinical Walking and Balance Measures. Clinical assessment of walking was performed by the same licensed physical therapist who was not involved in the study both before and after intervention. Tests included the Timed Up and Go test [32], timed 10 m walk test [33], and the 6-minute walking test [34]. These clinical walking tests assessed overground walking mobility, speed, and endurance. Balance was also assessed before and after intervention with the Berg Balance Scale [35].

2.6. Strength and Muscle Activation (EMG). Maximal voluntary isometric contractions were assessed for ankle dorsiflexion, plantarflexion, and handgrip, measured bilaterally. Similar to previous studies [15, 36], participants were assessed while seated in a custom-fit chair designed to minimize movement. Maximum forces produced during dorsiflexion and plantarflexion contractions were established via strain gauge (Omegadyne Ltd. Model 101-500) and converted to torque using a moment arm length of 0.15 m (measured from the heel block to the center of the strain gauge). Hand grip was performed with a commercial dynamometer (Takei Scientific Instruments Company Ltd., Niigata, Japan). In 10-second trials, following a silent period of 5 seconds, contractions were held for each limb separately. Following a brief warm-up, participants were given two attempts for achieving a maximum contraction.

Electromyographic (EMG) data from the soleus (SOL), tibialis anterior (TA), flexor carpi radialis (FCR), and posterior deltoid (PD), from the more affected (MA) and less affected (LA) limbs, were collected with surface electrodes placed in bipolar configuration over the muscle bellies of interest. Electrodes were placed on the skin and oriented longitudinally along the fibre direction, in accordance with SENIAM procedures [37]. Electrodes on the upper and lower limbs were placed in the same positions at each testing session. This was accomplished by recording cathode and anode electrode distances from anatomical landmarks and with pictures taken at the first session and the electrodes were placed by the same experimenter each time. As with other studies from this laboratory [11, 42], EMG signals were preamplified (×5000) and band-pass filtered (100–300 Hz) (Grass P511, Astro-Med). Data were converted to a digital signal, sampled at 1000 Hz using custom built continuous acquisition software (LabVIEW, National Instruments, TX,

USA) and stored to a PC for offline analysis. Using custom-written software programs (Matlab, The Mathworks, Inc., MA, USA), EMG data were full-wave rectified and low-pass filtered at 6 Hz using a 4th-order Butterworth filter to obtain the liner envelope. Maximum values were taken as the greatest reading generated over two trials by obtaining the mean value over 500 ms when force and EMG signals were highest.

2.7. Walking. Similar to previously reported methods [38], participants walked at a self-selected ("comfortable") speed on a motorized treadmill (Woodway USA, Waukesha, WI) while wearing an overhead safety harness (Pneu-Weight, Pneumex Inc., Sandpoint, ID, USA). All participants wore the safety harness without body weight support both before and after intervention and none wore an ankle foot orthosis. Participants were free to use hand-held railings in front or beside them during the trial and arm position did not change between pretests and the posttest. The self-selected treadmill speed (0.51 ± 0.32 mph) was held constant for that participant for baseline and posttraining tests to control for the effects of change in treadmill speed with changes in EMG [43].

EMG data for walking was collected in a similar manner as for strength but was normalized to maximal EMG recorded during walking. To quantify the rhythmic activation of muscles during walking, a modulation index ($\text{MI} = [(\text{EMG}_{\text{max}} - \text{EMG}_{\text{min}})/\text{EMG}_{\text{max}}] \times 100$) was calculated for each muscle across each movement cycle and averaged. This measure provides a means of comparing the extent to which muscles varied from phasic bursts of activity to alternatively tonic activity throughout the movement cycle [38, 39, 44, 45]. This measure provides an index of overall amplitude modulation across the movement cycle. Higher values, closer to 100%, indicate a larger range of modulation for a muscle with periods of contraction and periods of relaxation, while a lower value indicates that muscle's activity is more constant [45].

To detect joint kinematics, goniometers (Biometrics Inc., Ladysmith, VA) were used for both the LA and MA ankle (dorsiplantarflexion) and knee (flexion/extension). These devices were calibrated, output in degrees was determined, and data were sampled at 1000 Hz. Kinematic data were low-pass filtered at a cut-off frequency of 6 Hz with a fourth-order dual-pass Butterworth filter and were quantified by determining the range of motion by calculating the maximum and minimum angular excursions recorded through the stride cycle.

Similar to other studies [38, 46–49], custom-made force sensing resistors (FSR) (model 1027-1001-ND, Digi-Key, Thief River Falls, MN, USA) were inserted into both shoes under the heel and first metatarsal head of each foot. Heel-contact could not be precisely determined as there was some impairment in heel-strike for these participants; therefore, FSR signals from the foot sole were summed and used to define stride cycles as periods of stance (foot contact) and swing (no foot contact). The average duration between the starts of ipsilateral foot-contacts, duration of stance, and duration of swing were determined. Stride frequency was determined as the average number of strides taken in one second. EMG and kinematic data for the LA and MA sides were aligned to begin with foot contact for that respective side.

2.8. Cutaneous Reflexes. The pattern of cutaneous reflex modulation during walking was used to assess the strength of adaptation arising from A&L cycling training. Cutaneous reflexes were evoked via combined surface stimulation of the nerves innervating the dorsum of the hand (superficial radial; SR) and foot (superficial peroneal; SP) [11]. Electrodes for SR and SP nerve stimulation were placed just proximal to the radial head and on the crease of the ankle, respectively, on the LA limbs. A Grass S88 stimulator with SIU5 stimulus isolation and a CCU1 constant current unit (Astro-Med Grass Instrument, West Warwick, RI) were used to deliver stimulation in trains of 5×1.0 ms pulses at 300 Hz (P511 Astro-Med Grass Instrument). Perceptual and radiating thresholds (RT) were determined and nonnoxious intensities were found for each participant. Stimulation intensities were set to $2.2 \times$ RT for the SR nerve and $2.0 \times$ RT for the SP nerve. During treadmill walking, 120 stimulations were delivered pseudorandomly with an interstimulus interval of 1–5 seconds.

All data were sampled at 1 kHz with a 12-bit A/D converter connected to a computer running custom-written LabVIEW (National Instruments, Austin, TX) virtual instrument applications. Evoked reflexes in all muscles tested were aligned to delivery and averaged together. The stimulus artefact was removed from the reflex trace and data were then low-pass filtered at 30 Hz using a dual-pass, fourth-order Butterworth filter. To investigate phase-dependent modulation within each movement cycle, data were broken apart into 8 equally timed phases with phases 1–5 representing LA stance and phases 6–8 representing LA swing for walking [11]. For reflexes within each phase, the average trace from the nonstimulated data was subtracted from the stimulated average trace to produce a subtracted EMG reflex trace. Cutaneous reflexes were quantified as the average cumulative reflex over 150 ms following stimulation within each of the 8 phases [48, 50]. Background EMG levels between tests were also compared to inspect for a possible scaling effect on reflex activity. A modulation index (MI) for change in reflexes relative to maximum background activity (bEMG) across phases for each muscle was also calculated ($\text{MI} = [(\text{Reflex}_{\text{max}} - \text{Reflex}_{\text{min}})/\text{bEMG}_{\text{max}}] \times 100$).

2.9. Statistics. Using commercially available software (SPSS 18.0, Chicago, IL), pretest and posttest data were compared. Using commercially available software (SPSS 18.0, Chicago, IL), pretest and posttest data were compared. To evaluate the extent to which arm and leg cycling training altered walking ability, posttest data were compared to the 95% confidence interval (CI) created from three pretest sessions and compared to a pretest average for each participant. To establish the 95% CI for each measure, variability was computed from 3 pretest sessions and used to create a data range with which the posttest value was compared. If the posttest value fell outside the 95% CI range, it was considered significant for that participant. The total number of participants with a significant test is reported and dichotomous scores (1

Table 1: Participant data and clinical assessment parameters.

N	Sex/age/MA	Modified Ashworth (ankle/knee)	FAC (/6)	Chedoke-McMaster (A/H/L/F)	Monofilament (hand/foot)	Reflexes (S1/L1)	Years since stroke
1	M/74/R	3/1+	4	2/2/3/2	J 4.31/J 4.31	3+/1+	5
2	F/70/R	0/0	5	7/5/7/7	J 4.31/J 4.31	2+/2+	2
3	F/45/R	1/0	5	5/5/6/5	F 3.61/J 4.31	0/0	7
4	M/59/R	2/0	5	2/2/4/2	T 6.65/J 4.31	3+/3+	3
5	M/82/R	0/1	3	4/6/6/5	UTF/UTF	3+/0	3
6	M/86/L	1+/0	5	7/7/6/5	J 4.31/T 6.65	0/0	4
7	F/80/R	0/0	5	3/5/5/5	J 4.31/J 4.31	0/0	6
8	M/59/R	2/1	5	5/5/5/4	T 6.65/T 6.65	3+/4+	11
9	M/74/R	1/1	5	6/5/6/5	J 4.31/F 3.61	3+/2+	6
10	M/47/L	4/2	4	2/1/2/2	T 6.65/T 6.65	4+/3+	6
11	M/69/L	2/3	4	2/2/3/2	T 6.65/T 6.65	3+/3+	5
12	F/72/R	2/2	3	2/3/2/3	UTF/J 4.31	1+/3+	6
13	M/59/L	1/1	6	6/6/6/4	J 4.31/J 4.31	3+/2+	5
14	M/56/L	1/1	5	1/1/4/2	T 6.65/T 6.65	3+/3+	8
15	M/77/L	2/2	3	4/5/5/3	UTF/T 6.65	3+/3+	8
16	F/63/L	1/2	5	2/2/3/4	T 6.65/K 4.56	3+/1+	13
17	M/71/R	1/2	4	3/2/4/4	F 3.61/J 4.31	2+/3+	6
18	M/62/R	1+/2	4	4/3/4/5	D 2.83/D 2.83	3+/3+	8
19	M/78/L	3/1+	4	3/3/4/4	T 6.65/T 6.65	0/0	29

MA, more affected; M, male; F, female; L, left; R, right; FAC, Functional Ambulation Category; A, arm; H, hand; L, leg; F, foot; UTF, unable to feel; S1, 1st sacral vertebrae; and L1, 1st lumbar vertebrae.

representing a posttest score outside of the 95% CI range and 2 representing a score within the 95% CI range) for each participant for each measure were compared with the chi-squares test statistic to examine association.

For pretest data, a repeated-measures ANOVA was performed to examine difference across the three pretest sessions. If no difference was found, data were pooled together to create an average pretest value and compared to posttests values with paired-samples t-test (t). For each test, the degrees of freedom are reported in subscript. Assumptions for ANOVA and paired-samples t-tests were evaluated for parametric tests for a within-subject design. Pearson's correlation coefficients (r) were calculated between several variables and tested for significance. The observed effect for posttest differences for clinical measures, strength, and walking parameters is also reported as Cohen's effect size (d), where a small effect is $d = 0.2$, a medium effect is $d = 0.5$, and a large effect is $d = 0.8$ [51]. For priori hypotheses where direction of change was predicted, one-tailed paired-samples t-tests were performed. Statistical significance was set at $p \leq 0.05$.

3. Results

A total of 25 participants were recruited. Six participants were excluded because of self-withdrawal ($n = 1$), change in physical activity patterns ($n = 1$), and not meeting minimum inclusion criteria or were already participating in A&L cycling exercise ($n = 4$). Baseline and demographic data are reported for the remaining 19 participants (see Table 1). All participants contributed data to each measure.

3.1. Training Results. All participants completed the 15 sessions of A&L cycling training. Figure 2 shows the average HR, RPE, RPM, and Work in each of the 15 training sessions averaged across all participants. Within a session, HR increased between minute 5 and minute 30 from 76.0 ± 1.9 bpm to 98.9 ± 3.1 bpm and there was no significant difference between the first training session and the last training session. Across sessions, while there was no change in HR and RPE, there was a significant increase in RPM ($t_{(18)} = 2.399$ and $p = 0.014$, Figure 2(c)) and Work ($t_{(18)} = 6.475$ and $p = 0.000$, Figure 2(d)) between the first and last training session. Despite increases in RPM and Work, the same relative RPE was maintained.

3.2. Clinical Measures. A paired t-test revealed that there was a significant decrease (14.4% change ($t_{(18)} = 2.100$, $p = 0.025$, and $d = 0.350$)) in the time taken for the Timed Up and Go test where participants completed the test in 29.33 ± 25.83 seconds before training and 25.12 ± 22.14 seconds after training. Time taken for the 10 m walk test also significantly decreased where participants completed the test at 0.45 ± 0.50 m/sec before training and 0.51 ± 0.48 m/sec after training indicating a 13.3% improvement ($t_{(18)} = 2.342$, $p = 0.015$, and $d = 0.192$). The number of steps taken for the 10 m walk test also significantly decreased with 27.17 ± 12.44 steps before training and 25.69 ± 12.50 steps after training

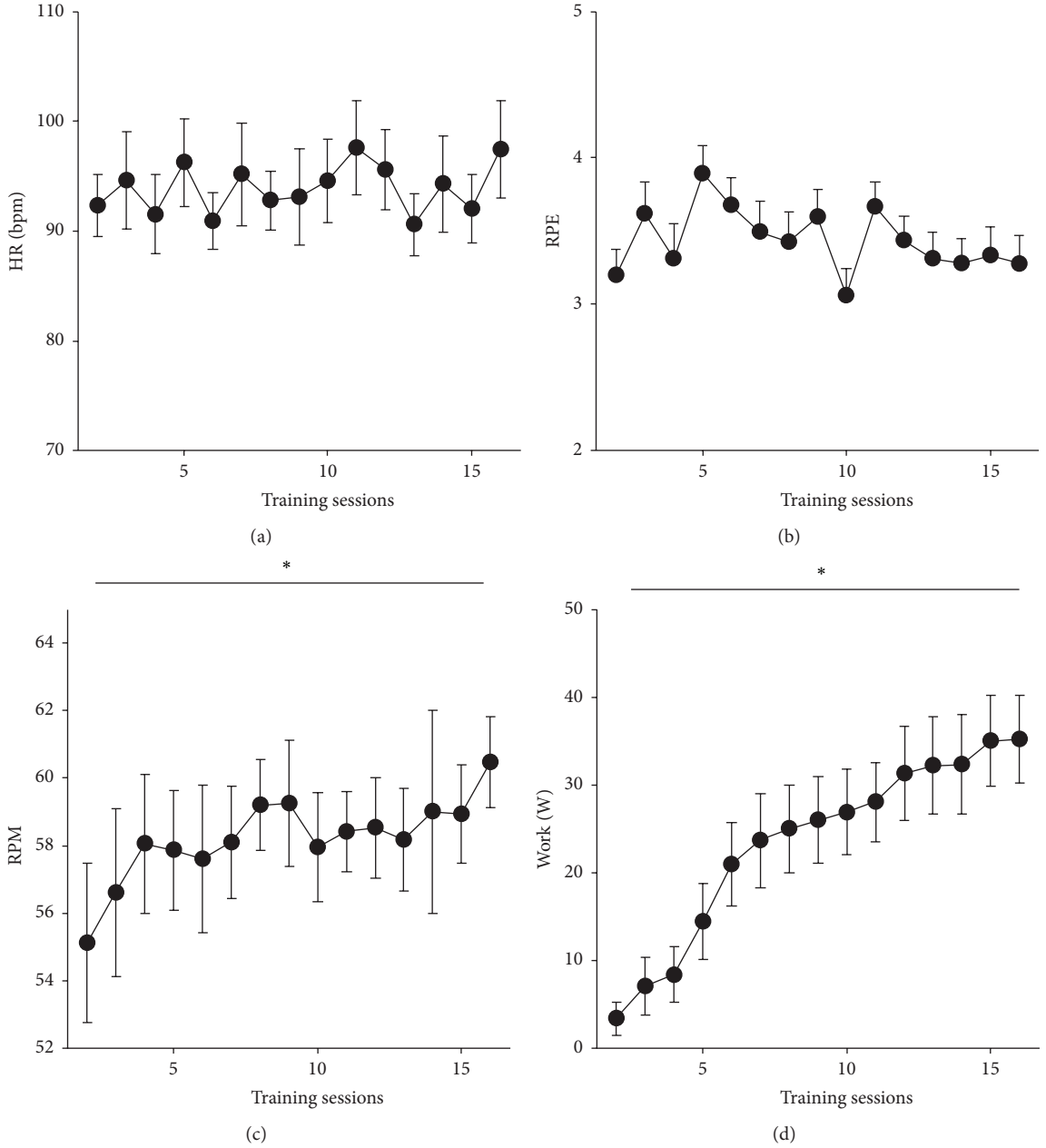

Figure 2: Training parameters for HR, RPE, RPM, and Work over 15 training sessions. Data are means (±sem) averaged across all participants for all training sessions. * indicates a significant difference between first and last training sessions.

indicating a 5.45% improvement ($t_{(18)} = 2.140$, $p = 0.023$, and $d = 0.239$). The total distance covered in the 6-minute walk test significantly increased between the pre- and posttest from 217.41 ± 107.67 feet to 252.43 ± 138.38 feet indicating a 16.10% improvement ($t_{(18)} = 3.586$, $p = 0.001$, and $d = 0.564$). The total score from the Berg Balance Scale significantly increased following A&L cycling training from a mean score of 42.04 ± 10.48 to a mean score of 45.06 ± 2.38 (median scores of 45 to 48 after training) indicating a 4.94% improvement ($t_{(18)} = 2.825$, $p = 0.005$, and $d = 0.528$).

Table 2 summarizes results from the single-participants statistical tests that are discussed below. The number of participants with a significant posttest value is reported for each variable in the table. For most variables, the majority of participants did show a significant posttest change.

3.3. Strength and EMG. Figure 3 shows peak EMG activity and force during plantarflexion, dorsiflexion, and handgrip averaged across all participants for three pretests and the posttest conditions. No significant differences were found for pretest baseline data. Following training, plantarflexion force was significantly increased on the LA side by 15.48% and on the MA side by 44.93% ($t_{(18)} = 2.061$, $p = 0.029$, and $d = 0.437$, Figure 3(b) and $t_{(18)} = 2.073$, $p = 0.029$, and $d = 0.439$, Figure 3(d) for the LA and MA sides, resp.). Maximal soleus EMG on the LA side also increased by 27.14%

Table 2: Single-subject analysis.

Measure	Number of participants (out of 19) with significant changes after training
Strength	
LA plantarflexion	10
LA SOL	10
MA plantarflexion	10
MA SOL	8
LA dorsiflexion	11
LA TA	7
MA dorsiflexion	12
MA TA	11
LA grip	14
LA FCR	7
MA grip	17
MA FCR	8
Walking bEMG modulation index	
MA SOL	13
MA TA	9
LA SOL	10
LA TA	12
MA FCR	12
MA PD	11
LA FCR	13
LA PD	12
Walking kinematics	
LA ankle	9
LA knee	9
MA ankle	10
MA knee	11
Walking parameters	
LA stride duration	8
MA stride duration	9
LA stance duration	11
MA stance duration	10
LA swing duration	14
MA swing duration	10
LA stride frequency	8
MA stride frequency	8
Walking cutaneous reflex modulation index	
MA SOL	13
MA TA	10
LA SOL	13
LA TA	12
MA FCR	13
MA PD	12
LA FCR	9
LA PD	12

MA, more affected; LA, less affected; SOL, soleus; TA, tibialis anterior; FCR, flexor carpi radialis; PD, posterior deltoid; bEMG, background electromyography.

($t_{(18)} = 2.154$, $p = 0.025$, and $d = 0.453$, Figure 3(a)). The increase in plantarflexion force and SOL EMG on the LA side was significantly correlated ($r = 0.499$ and $p = 0.045$). For dorsiflexion, LA force significantly increased by 16.61% and MA force significantly increased by 34.93% ($t_{(18)} = 1.821$, $p = 0.045$, and $d = 0.394$, Figure 3(f) and $t_{(18)} = 2.244$, $p = 0.021$, $d = 0.568$, and $d = 1.057$, Figure 3(h) for the LA and MA sides, resp.). Peak tibialis anterior EMG also significantly increased on the MA side by 27.91% ($t_{(18)} = 1.946$, $p = 0.036$, and $d = 0.417$, Figure 3(g)). The increase in MA dorsiflexion force and MA TA EMG activity was significantly related ($r = 0.742$ and $p = 0.001$). Handgrip strength significantly increased on the LA side by 16.74% and on the MA side by 44.78% ($t_{(18)} = 4.010$, $p = 0.001$, and $d = 0.687$, Figure 3(j) and $t_{(18)} = 5.026$, $p = 0.000$, and $d = 0.764$, Figure 3(l) for the LA and MA sides, resp.). There was an association between the likelihood of a significant increase in LA strength and MA strength ($\chi^2_{(1)} = 23.768$ and $p < 0.0001$).

3.4. Walking. Figure 4 shows EMG for the muscles of the LA and MA limbs averaged across all participants for three pretests and for posttest values during walking. Line graphs are data expressed as a percentage of the gait cycle where 0% indicates foot contact for that side. Bar graphs are background EMG modulation indices across muscles averaged for all participants. No significant pretest differences were found for any muscles. Following training, for the LA TA, there was a significant decrease ($t_{(18)} = 1.875$, $p = 0.041$, and $d = 0.398$, Figure 4(f)) in modulation by 6.4%. In the MA FCR, modulation significantly increased ($t_{(18)} = 2.134$, $p = 0.027$, and $d = 0.496$, Figure 4(k)) by 34.7% and modulation also significantly increased for both the LA and MA PD by 12.1% and 28.9% ($t_{(18)} = 2.975$, $p = 0.004$, and $d = 0.827$, Figure 4(n) and $t_{(18)} = 2.259$, $p = 0.021$, and $d = 0.649$, Figure 4(p) for the LA and MA PD, resp.). When comparing the ratio of modulation between the LA and MA sides for each muscle, there was a significant decrease of 49.2% in ratio for the PD ($t_{(18)} = 3.085$, $p = 0.009$, and $d = 0.423$).

Figure 5 shows kinematic data for the LA and MA ankle and knee averaged across all participants for three pretests and for posttest values during walking. Line graphs are from a representative participant aligned to begin at foot contact and bar graphs are ROM values averaged across all participants. No pretest differences were found for any kinematic variables. Following training, all variables showed statistically significant increases in ROM for posttest compared to pretest values (LA ankle: $t_{(18)} = 2.970$, $p = 0.004$, and $d = 0.558$, Figure 5(b); MA ankle: $t_{(18)} = 2.078$, $p = 0.027$, and $d = 0.426$, Figure 5(d); LA knee: $t_{(18)} = 2.561$, $p = 0.010$, and $d = 0.382$, Figure 5(f); and MA knee: $t_{(18)} = 3.404$, $p = 0.002$, and $d = 0.476$, Figure 5(h)). For the ankle, there was a 25.51% increase in ROM for the LA side and a 21.73% increase in ROM for the MA side. For the knee, there was a 19.37% increase in ROM for the LA side and a 22.21% increase in ROM for the MA side. There was a significant association between a change in LA and MA kinematics ($\chi^2_{(1)} = 3.979$ and $p = 0.046$).

Walking parameters including average stride, stance and swing durations, and stride frequencies from the LA and MA sides averaged across all participants for the three pretests and for posttest are shown in Figure 6. No baseline differences were detected for any walking parameter data. Following training, there was a significant decrease in stride duration on

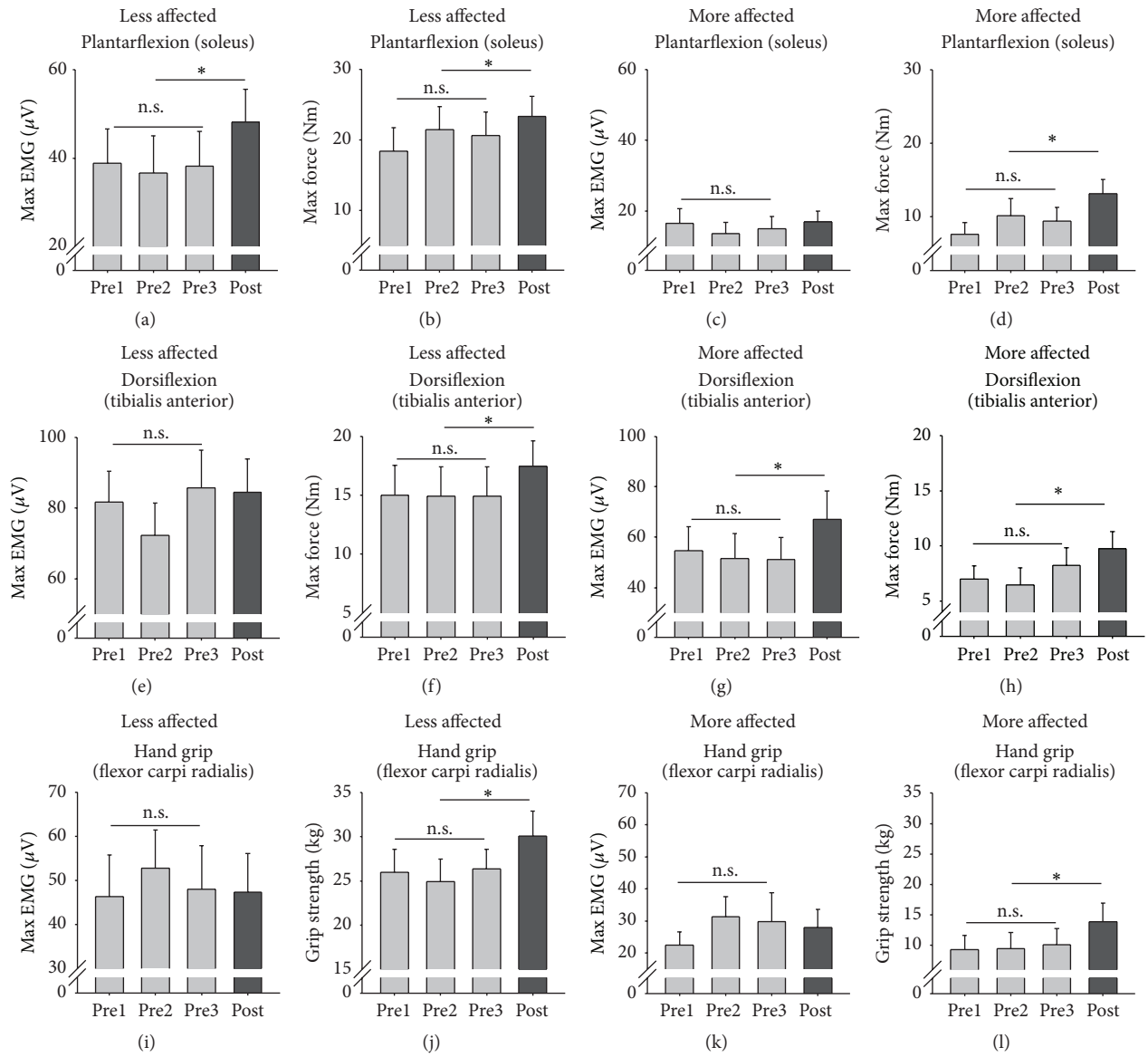

Figure 3: Plantarflexion, dorsiflexion, and hand grip strength and muscle activation. Bar graphs are means (±sem) for EMG and force during isometric strength tests averaged across all participants. * indicates significant differences between the pretest average and the posttest value and n.s. indicates a nonsignificant difference for the three baseline measures.

the LA and MA sides ($t_{(18)} = 2.448$, $p = 0.013$, and $d = 0.500$, Figure 6(a) and $t_{(18)} = 3.077$, $p = 0.003$, and $d = 0.587$, Figure 6(b) for the LA and MA sides, resp.) with a 5.25% and 8.74% decrease in LA and MA stride duration. Stance duration for the LA side significantly decreased ($t_{(18)} = 2.457$, $p = 0.013$, and $d = 0.501$, Figure 6(c)) by 12.53%, while swing duration increased ($t_{(18)} = 1.837$, $p = 0.042$, and $d = 0.397$, Figure 6(e)) by 11.29% following A&L cycling training. There were also significant increases in stride frequency compared to the pretest values for both the LA and MA sides ($t_{(18)} = -1.961$, $p = 0.033$, and $d = 0.419$, Figure 6(g) and $t_{(18)} = -2.114$, $p = 0.025$, and $d = 0.446$, Figure 6(h), for the LA and MA sides, resp.). Stride frequency increased by 3.82% for the LA side and 4.07% for the MA side. Percentage change in stride duration is significantly correlated with percentage change in stride frequency for both the LA ($r = -0.989$ and $p = 0.000$) and MA ($r = -0.702$ and $p = 0.001$) sides. There was a significant association between a change in MA and LA walking parameters ($\chi^2_{(1)} = 30.728$ and $p = 0.000$). These changes in walking parameters following training were independent of changes in speed as treadmill speed was held constant across all testing sessions.

3.5. Cutaneous Reflexes. Figure 7 shows data for all reflexes evoked during treadmill walking averaged across all participants. This process reveals the general trend in evoked responses but obscures phase-modulation. To quantify overall modulation of reflexes, a modulation index was quantified for the muscles on the LA and MA sides and shown as bar graphs on Figure 7. Following training, modulation was

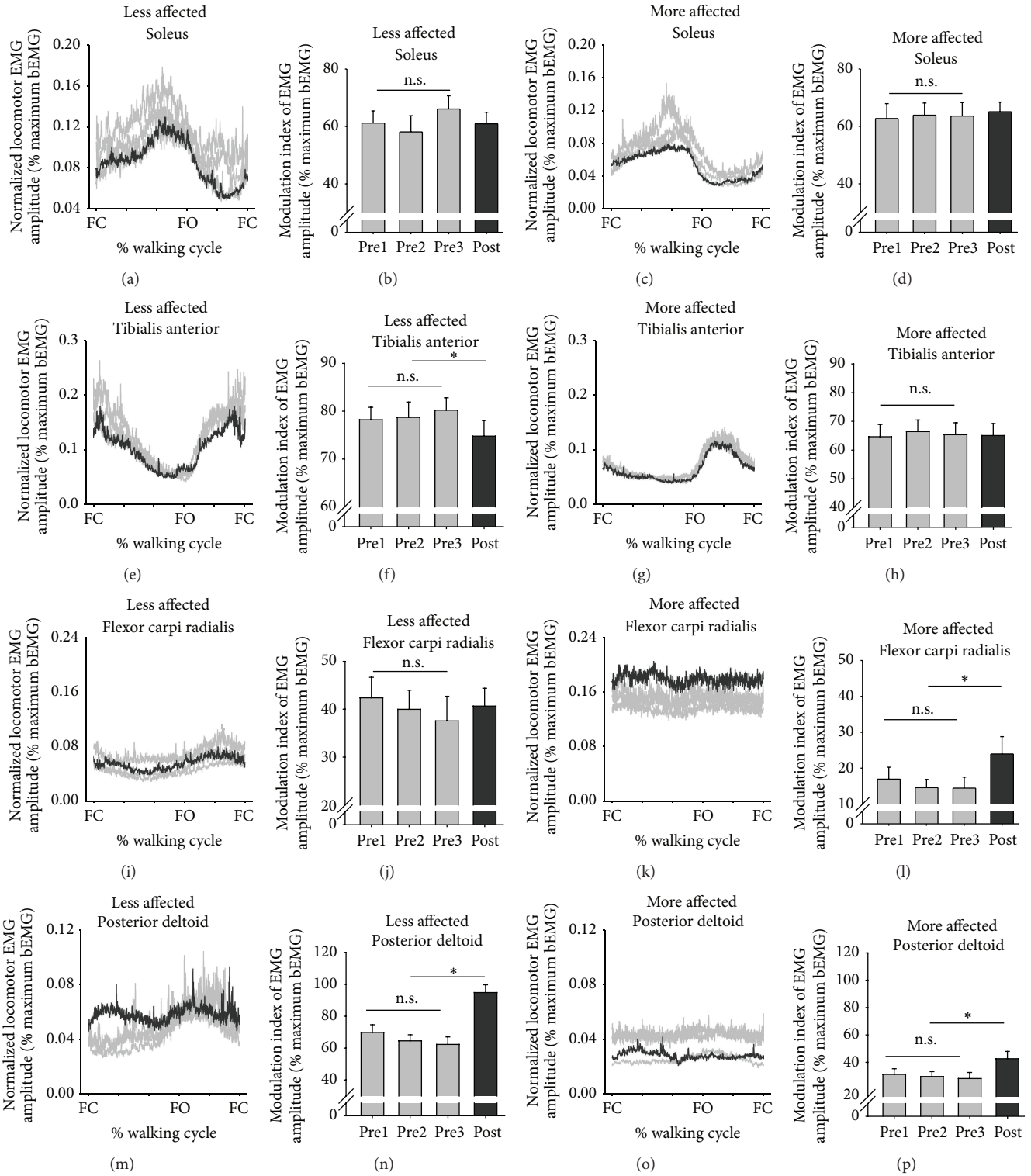

Figure 4: Background EMG during walking. Line graphs are normalized and averaged EMG for the walking cycle for three baseline tests (light gray lines) and for the posttest (dark gray lines). Foot contact (FC) and foot off (FO) times are indicated. Bar graphs are mean (±sem) modulation indices for all muscles averaged across all participants. *indicates significant differences between the pretest average and the posttest value and n.s. indicates a nonsignificant difference for the three baseline measures.

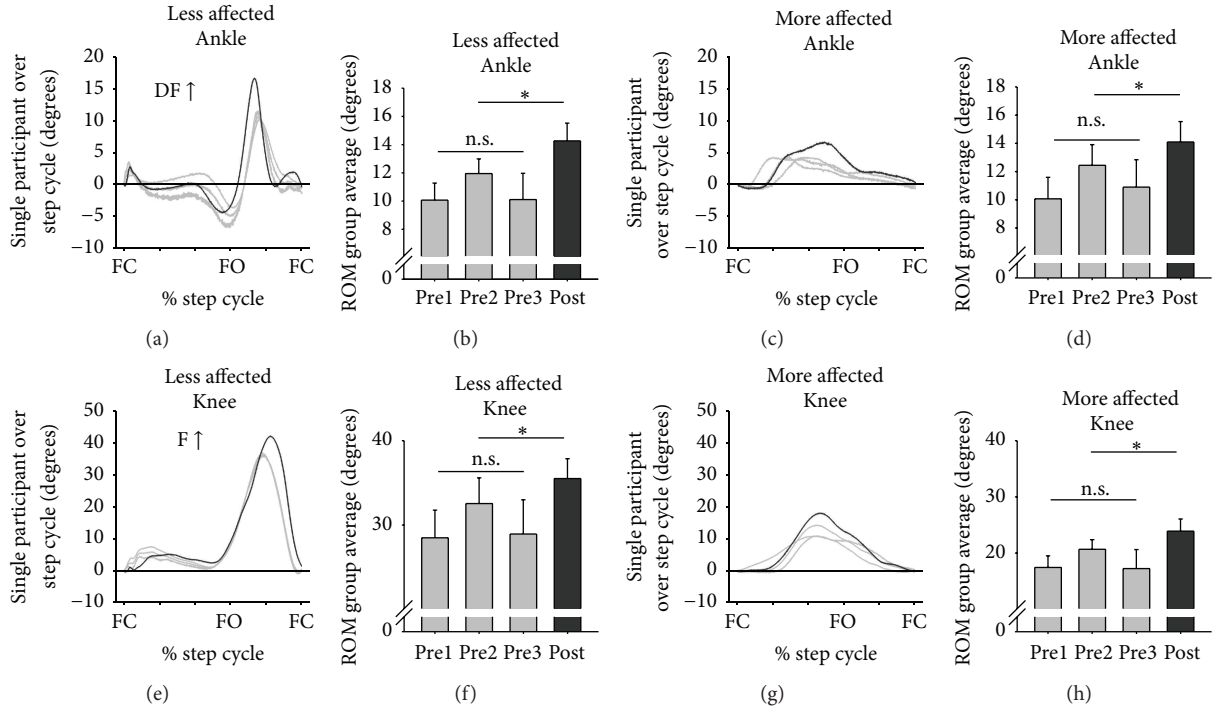

Figure 5: Kinematics during walking. Line graphs are single participant kinematics for the walking cycle for three baseline tests (light gray lines) and for the posttest (dark gray lines). Foot contact (FC) and foot off (FO) times are indicated. Dorsiflexion (DF) and flexion (F) increases are positive. Bar graphs are mean (±sem) range of motion values averaged across all participants. * indicates significant differences between the pretest average and the posttest value and n.s. indicates a nonsignificant difference for the three baseline measures.

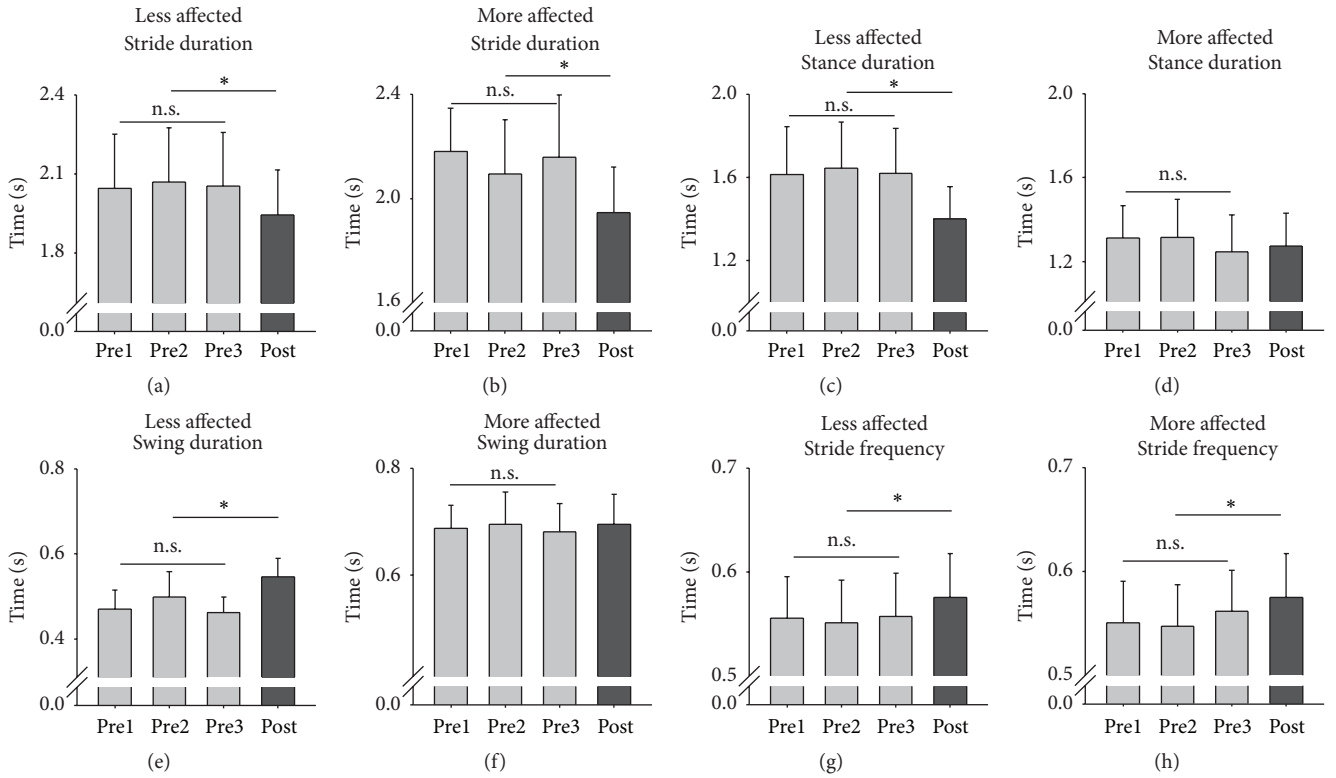

Figure 6: Temporal parameters of walking. Bar graphs are mean (±sem) values for stride duration, stance duration, swing duration, and stride frequency for three baseline tests and the posttest averaged across all participants. * indicates significant differences between the pretest average and the posttest value and n.s. indicates a nonsignificant difference for the three baseline measures.

significantly decreased for the LA SOL ($t_{(18)} = 2.217$, $p = 0.045$, and $d = 0.355$, Figure 7(b)) by 29.3%. For the LA TA, modulation significantly increased by 44.6% ($t_{(18)} = 3.493$, $p = 0.004$, and $d = 0.378$, Figure 7(f)), and, for the LA PD, modulation increased by 80.4% ($t_{(18)} = 2.197$, $p = 0.047$, and $d = 0.386$, Figure 7(n), resp.). There was a significant association between a change in MA and LA cutaneous reflex modulation ($\chi^2_{(1)} = 5.793$, $p = 0.016$).

Figure 8 shows cutaneous reflexes (bars) during walking at all phases. Since there were no significant differences between the pretest data, for simplification, the average value across the three tests is shown. For reflex amplitudes, there are significant differences between baseline and posttest values for several muscles, including the LA SOL, LA, and MA TA, and for the LA and MA FCR. For the LA SOL, there was a significant change in reflex amplitude for phase 2 ($t_{(18)} = 2.207$ and $p = 0.046$) and phase 7 ($t_{(18)} = 2.271$ and $p = 0.021$). For the LA TA, phase 1 and phase 8 showed significant differences in posttest values compared to the baseline average (for phase 1 $t_{(18)} = 2.271$ and $p = 0.041$ and for phase 8 $t_{(18)} = 1.871$ and $p = 0.042$). For the MA TA, a significant posttest difference was found for phase 1 ($t_{(18)} = 2.660$ and $p = 0.012$). For the LA FCR, phase 5 showed a significant posttest difference ($t_{(18)} = 2.718$ and $p = 0.018$), and, for the MA FCR, phase 1 showed a significant posttest difference ($t_{(18)} = 2.660$ and $p = 0.012$).

Investigating background EMG levels between tests allows for comparison of reflex amplitudes that cannot be explained by scaling with background EMG. Figure 8 shows bEMG (lines) during walking at all phases. For bEMG at specific phases of walking, there are significant differences between baseline and posttest values for the LA and MA FCR and LA PD muscles. For the MA FCR, significant differences were found for phases 2 ($t_{(18)} = 2.227$ and $p = 0.036$), 3 ($t_{(18)} = 2.142$ and $p = 0.044$), and 4 ($t_{(18)} = 2.406$ and $p = 0.033$). For the LA FCR, significant differences were found for phase 7 ($t_{(18)} = 3.578$ and $p = 0.004$). For the LA PD, significant differences were found for phases 1 and 2 ($t_{(18)} = 2.407$ and $p = 0.033$ and $t_{(18)} = 2.754$ and $p = 0.017$, resp.)

Figure 9 shows cutaneous reflexes during walking at specific phases of interest. Reflex modulation for the LA and MA TA and FCR is shown for specific phases of interest. At these phases, there are significant effects of training on posttest values and no significant differences in bEMG. Line graphs are of the subtracted reflex averaged across all participants for that phase.

4. Discussion

This project tested the efficacy of A&L cycling training for improving walking ability after stroke. Participants performed A&L cycling three times per week for five weeks for 30 minutes of exercise time each session. This aggregates 450 minutes of activity, performed at a moderate level, which improved walking after stroke. A&L cycling training improved clinical walking status, increased strength, range of motion, and temporal parameters of treadmill walking, and improved modulation of muscle activity and cutaneous reflexes. These results demonstrate that maximizing activity in inherent arm and leg connections spared after a stroke, with A&L cycling, could facilitate motor recovery. A&L cycling could be used as a novel rehabilitation modality to maximize functional motor recovery and improve walking ability after stroke.

A&L cycling training produced global changes in clinical status. There was a 4.19-second improvement in the Timed Up and Go test. This corresponds to a noticeable change in ability as the minimal detectable change for chronic stroke participants is 2.9 seconds [52]. Although there was an improvement in time taken for this test, values still fall below normative values for community-dwelling elderly people who finish the test in approximately 9–12 seconds [53]. For the 10 m walk test, speed increased by 0.06 m/s indicating a small but meaningful change [54]. Normative data for the stroke population ($n = 48$, age 68, with reduced muscle strength and walking capacity) is 0.84 ± 0.30 m/s. For the 6-minute walk test, participants improved by walking an additional 114.87 ft which is above a minimal detectable change of 112.76 ft for stroke participants [55]. Therefore, in summary, the walking tests showed minimal changes outside of error that reflect a true change between baseline tests and posttest values. For the balance test, scores on the Berg Balance Scale improved by 2.12 points which is just below the 2.5-point minimal detectable change criterion difference for a chronic stroke population [56]. We consider this change significant given that A&L cycling may not require the same trunk and pelvic control that walking does yet still improve balance after training.

Strength during isometric contractions increased for both the LA side and the MA side for plantarflexion and dorsiflexion following A&L cycling training. For the LA SOL and the MA TA, the increase in force was correlated with an increase in EMG. Handgrip strength also increased for both hands following training. A concomitant increase in EMG with force was not surprising as there is a linear correlation between the amplitude of EMG and the force produced during isometric contractions [57]. It is surprising however that no increase in EMG was recorded for the LA and MA FCR to match the increase in grip strength. Cocontraction of adjacent muscle groups, that were not being recorded, could account for this difference. Alternatively, perhaps no increase in EMG activity of these forearm muscles was observed because in some cases we used a hand brace to secure the weakened hand to the ergometer handle during training. Nevertheless, these results show an overall increase in strength resulting from A&L cycling training. A number of other studies also report improvements in strength following treadmill training interventions in those with spinal cord injury [4, 58–60]. Similar observations have been made in stroke and there is a positive correlation between strength gains and walking speed [61, 62]. Strength gains, an indirect result of A&L cycling training, likely contribute to the increase in walking ability seen here.

Changes in several variables measured during walking gauge training transfer effects following A&L cycling training. Variables include EMG modulation, kinematics, and stride parameters including duration, stance percentages, and

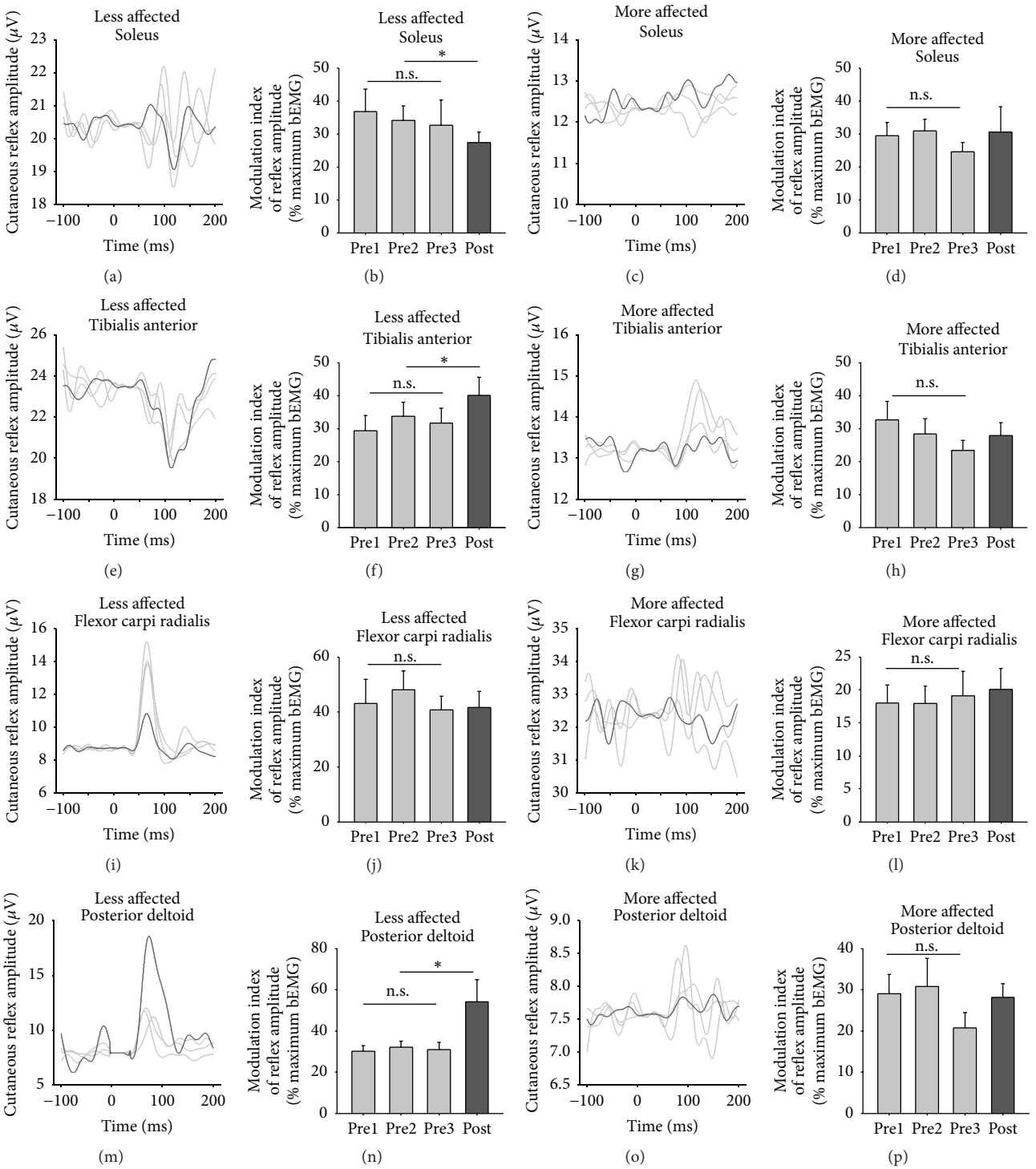

Figure 7: Cutaneous reflexes during walking. Line graphs are averages across all participants for three baseline tests (light gray lines) and for the posttest (dark gray line). The stimulus artefact beginning at time 0 has been blanked out and replaced with a flat line. Stimulation was applied to the superficial radial nerve of the hand and the superficial peroneal nerve of the foot on the LA side. Bar graphs are means (±standard error) averaged across all participants for baseline and posttest values. * indicates significant differences between the pretest average and the posttest value and n.s. indicates a nonsignificant difference for the three baseline measures.

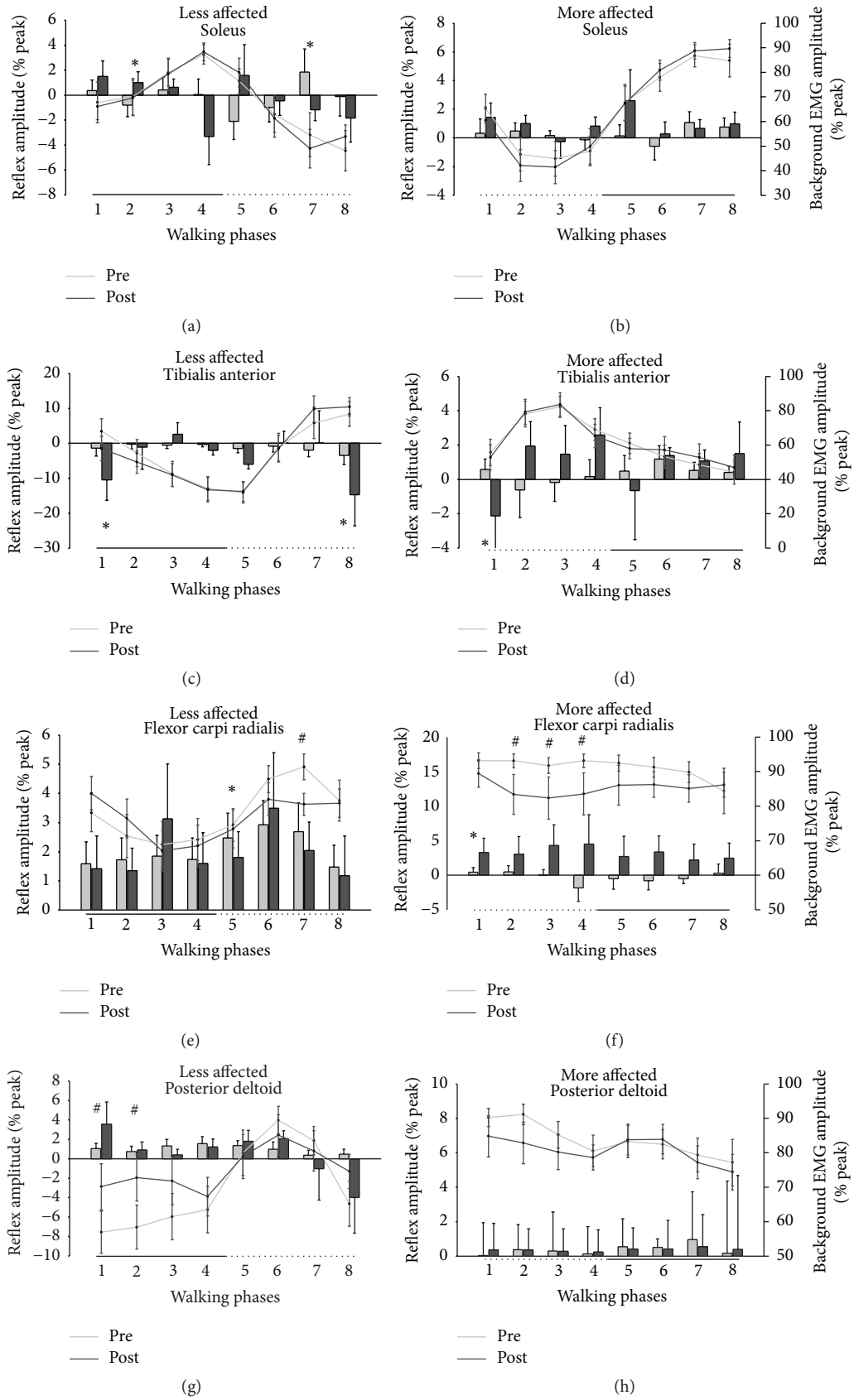

Figure 8: Normalized background EMG and reflex amplitudes during walking. Background EMG is shown in line plots and reflex amplitude is shown in bar plots. Values are means (±standard error) averaged across all participants and normalized to the peak undisturbed EMG during walking. The horizontal bars below the y-axes represent the stance (solid line) and swing (dotted line) phase of walking. Significant differences between the pretest average and the posttest value are indicated with # for background EMG and $*$ for reflex amplitude.

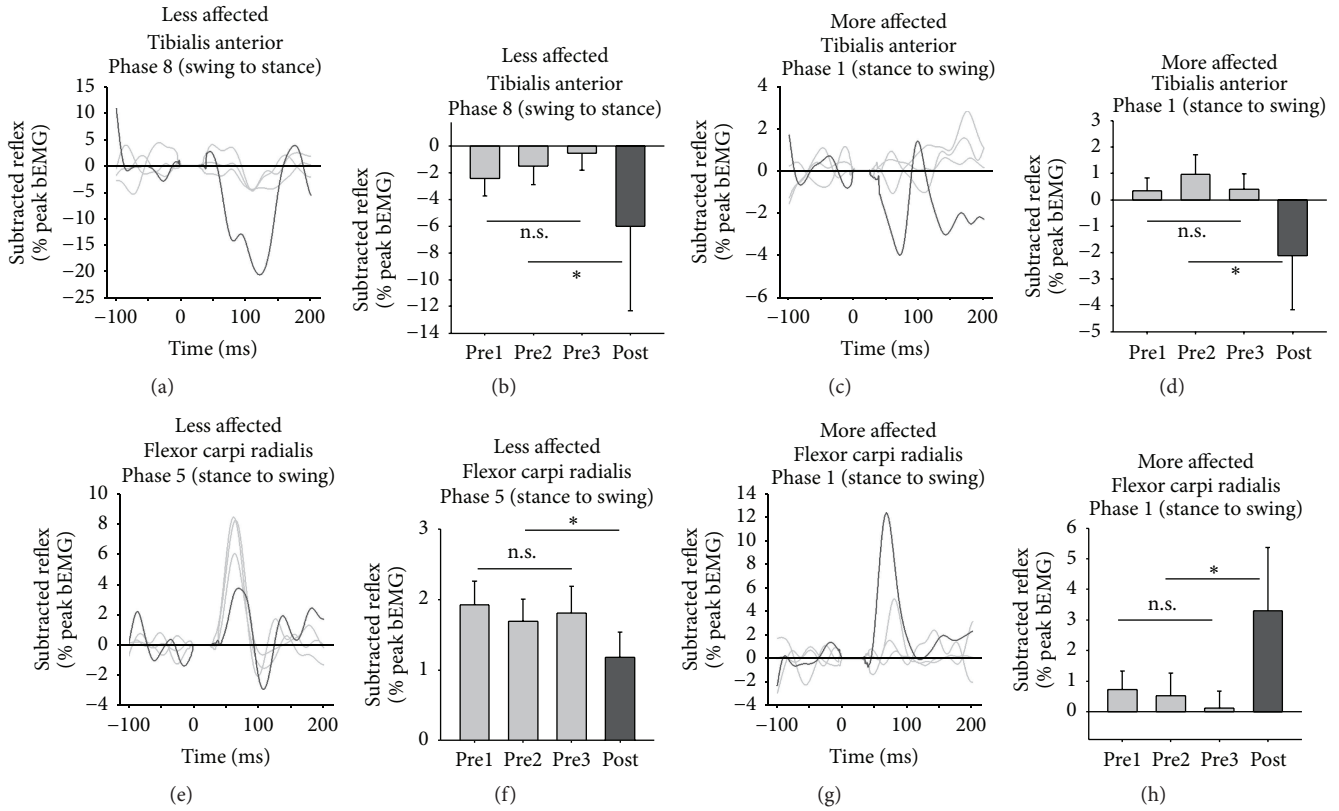

Figure 9: Normalized background EMG and reflex amplitudes at specific phase of interest during walking. Bar graphs are means (±standard error) for reflex amplitude averaged across all participants for baseline and posttest values. Line graphs are means (±standard error) for bEMG. *indicates significant differences between the pretest average and the posttest value. There were no significant differences for bEMG.

frequency. For depth of modulation following A&L cycling training, the LA TA showed decreased modulation representing a smoothing out of dorsiflexor activity. Increased control to eliminate unwanted dorsiflexor activity is required to increase walking endurance in people with hemiplegia after stroke [63]. For the arms, in general, the arm muscles showed increased depth of modulation after training. An increased depth of modulation, indicating an increase in the amount of phasic activity, which more closely represents what is found in neurologically intact participants [38], could have been due to changes in weight support borne through the arms after training. The significant changes in modulation indices and the pattern of EMG activity for FCR and PD muscles appear different between the MA and LA sides. This may be important for the A&L-induced walking improvements observed here where increased modulation of these muscles may decrease exaggerated interlimb neural coupling producing a "flexor synergy" that has been previously reported after stroke [64].

Walking kinematics for all joints tested increased range of motion following training with an average increase of 22%. The transfer from A&L cycling training to improve walking kinematics is particularly interesting given kinematics are constrained on the A&L cycle ergometer [11]. Several variables related to walking parameters were also changed by A&L cycling training. Stride duration was decreased following training related to an increase in stride frequency. Within a stride for the LA side, it was found that stance duration decreased, while swing duration increased. These changes in swing and stance duration represent a more normal gait pattern [65]. Treadmill belt speed between pretests and the posttest was held constant for that participant and cannot be implicated as a source of the change in walking parameters seen here.

Changes in cutaneous reflex modulation were taken as a proxy of spinal plasticity arising from the A&L cycling training. Overall reflexes showed some improved modulation patterns following training. Cutaneous stimulation produced reflex effects in all muscles tested and is modulated during walking in a similar way to that found in neurologically intact participants [66]. By using an index of modulation, it is possible to see how the depth of reflex modulation changed with A&L cycling training. In the LA TA and LA PD, reflex modulation increased, representing an overall increase in the depth of modulation, perhaps due to increased access to these interlimb networks following training.

When examining the grand average reflex traces from cutaneous stimulation, activity in the LA TA is mainly suppressive, while the MA TA shows mainly facilitations (see Figure 7). This is in line with previous observations of cutaneous responses in the TA following stroke [69], where, on the MA side, the decreased influence of the corticospinal

tract on reflex excitability, as a result of the stroke lesion, fails to produce the appropriate suppressions associated with normal reflex activity [67]. When examining specific functional phases for walking, adaptive plasticity was seen following training. Responses in the LA TA at phase 8, representing the swing to stance transition, showed increased inhibition. In the MA TA at phase 1, reflexes turned from facilitation to suppression following training. Normally at these phases, in neurologically intact participants, inhibitory responses are observed in the TA to aid with safe footfall allowing passive plantarflexion [48, 70–73] and the reemergence of end-swing suppressions following training reveals the normalization of reflexes as a result of A&L cycling training. In the arm muscles, stimulation following training produced decreased facilitation on the LA side and increased facilitation on the MA side, again representing a return to what one normally observes in modulation in these interlimb networks [38, 39].

Together these results demonstrate that adaptive plasticity in interlimb spinal networks is possible following rehabilitative training. It is unknown, however, how long these results persist and their functional implications. Further investigation of chronic plasticity in somatosensory pathways is warranted in order to fully understand motor adaptation to maximize functional recovery after neurological injury.

4.1. Task Transfer and Asymmetry of Changes between Sides. A bias between the observed training transfer effects between the LA and MA sides existed following A&L cycling training. A larger effect of strength gains following A&L cycling training was observed for the MA side for ankle dorsiflexion, plantarflexion, and handgrip. However, following training, an asymmetry was still observed between sides where MA posttest values were still below LA pretest values. An asymmetry in strength gains was also observed following treadmill aerobic exercise in patients with chronic hemiparesis following stroke where the greatest relative strength gains were seen in the MA limbs [74]. Asymmetry was also observed for EMG modulation following A&L cycling training where modulation was greater for the MA PD. The bias towards greater improvement on the MA side following A&L cycling training likely results from the increased potential for improvements on the MA side due to the higher degree of impairment [75]. Despite increased range of motion, alignment of walking kinematics between the LA and MA sides did not appear to improve after A&L cycling training. Therefore, although A&L cycling training does appear to result in a positive task transfer to improved walking, it does not produce a return to symmetry as kinematics on the MA side are still quite different from kinematics on the LA side.

A&L cycling training was an indirect training paradigm where walking was not the target of the training. The improvements in the trained task of A&L cycling transferred to improvements in the untrained task of walking. A few studies in stroke suggest partial transfer of a trained task on improving walking. For example, fitness training, high-intensity therapy, and repetitive task training all show beneficial improvements to walking after stroke [76–79].

The success of training transfer depended on how similar A&L cycling and walking are to each other and, indeed, all forms of rhythmic human movement share common neural elements [80]. The "common core" controlling cyclical limb movements is predicated upon multisegmental central pattern generating networks reinforced by somatosensory feedback regulated by supraspinal inputs [80–83]. Common neural elements are seen across different forms of walking (level, incline, and stair climbing; [46]), between different modes of rhythmic arm movement [9, 84], and between different modes of arm and leg coordination during recumbent stepping, cycling, and walking [85, 86]. The neuronal activity associated with generating rhythmic A&L cycling contains about 60% of what is found during treadmill walking [11] implying that rhythmic arm and leg movement performed in a task such as cycling could activate common locomotor networks.

Improvements in the temporal parameters of walking, kinematics, muscle modulation during walking, and clinical assessments of walking all demonstrate a positive transfer of A&L cycling training to enhanced walking function. The locus of task transfer is unknown but could originate from shared neural elements between the two tasks of A&L cycling and walking. More research on which physiological systems are affected by A&L cycling training is warranted.

4.2. Study Limitations. The observed improvement in walking could have been due to enhancements in cardiopulmonary fitness following A&L cycling training, a regular, prescribed fitness program. However, the level of training intensity for A&L cycling was quite low with little change in HR observed over a training session. The level of aerobic activity required to increase cardiopulmonary fitness in individuals with stroke is more intense than the level of exercise here [87, 88]. Future studies could, however, measure changes in cardiovascular function as a result of A&L cycling training. Another limitation of this study also has to do with the change in our intended sample. Although some participants did withdraw, significant effects were seen for many of the dependent variables indicating sufficient power. Additionally, intervention studies have often used reference untrained "control" groups to compare against the intervention or treatment groups. We have instead opted for the "multiple baseline" model where each participant serves as their own control and no committed volunteer participants are relegated to the role of an untrained control participant. In addition, in studying a patient population, there tends to be a large degree of between-subject variability as there is a wide range of abilities across participants. However, using a multiple baseline approach, we are able to mitigate this limitation as participants are instead compared against their own individual variabilities generated over multiple baseline sessions. We believe that multiple baseline measures should be considered a valid alternative or replacement to the concept of a control group.

4.3. Clinical Translation. Transfer of improvements following A&L cycling training to enhance walking could open the way to the development of a new approach for the rehabilitation of stroke patients. Current therapies for walking do not fully exploit the neuronal and mechanical linkages between the

arms and legs that are inherent parts of human locomotion [6, 9–11]. We have shown here that A&L cycling improves walking ability after stroke and suggest that A&L cycling be used as an additional training modality for locomotor recovery. A&L cycling is a safe and low-stress activity and the linked cranks allow for physical assistance to the weakened limbs to encourage rhythmic coordination. In addition, A&L cycle ergometers are widely available in most gyms and recreation centers and are relatively cheap to access. This type of community-based exercise allows for equalization of opportunity for training with increased equipment access outside of major rehabilitation centers. Increasing the ease of training based upon a device that could be more readily used in therapy would directly impact the health and quality of life for those who have suffered a stroke. Given that other types of training, such as strength training or treadmill training, also improve walking, we do not suggest that A&L cycling training be used to replace these therapies. Instead, we suggest that this therapy be used as an adjunct modality to improve walking ability after stroke and may be particularly valuable as a bridging approach for those who initially lack strength and balance control for independent walking. To fully understand the relative benefits of A&L cycling training to other therapies, a randomized controlled trial should be conducted. In addition, given the link between the arms and the legs, examining the benefits of just arm cycling training on enhancing walking ability after stroke should also be conducted.

5. Conclusion

A&L cycling training improves walking ability after stroke. Results showed improved clinical walking status, increased strength, improved physical performance on the untrained task of walking, and improved reflex modulation especially in the leg muscles. These results suggest that A&L cycling training, an accessible and cost-effective training modality, could be used to improve walking ability after stroke.

Competing Interests

None of the authors have potential competing interests to be disclosed.

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Review Article

A Review on Locomotor Training after Spinal Cord Injury: Reorganization of Spinal Neuronal Circuits and Recovery of Motor Function

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Locomotor training is a classic rehabilitation approach utilized with the aim of improving sensorimotor function and walking ability in people with spinal cord injury (SCI). Recent studies have provided strong evidence that locomotor training of persons with clinically complete, motor complete, or motor incomplete SCI induces functional reorganization of spinal neuronal networks at multisegmental levels at rest and during assisted stepping. This neuronal reorganization coincides with improvements in motor function and decreased muscle cocontractions. In this review, we will discuss the manner in which spinal neuronal circuits are impaired and the evidence surrounding plasticity of neuronal activity after locomotor training in people with SCI. We conclude that we need to better understand the physiological changes underlying locomotor training, use physiological signals to probe recovery over the course of training, and utilize established and contemporary interventions simultaneously in larger scale research studies. Furthermore, the focus of our research questions needs to change from feasibility and efficacy to the following: what are the physiological mechanisms that make it work and for whom? The aforementioned will enable the scientific and clinical community to develop more effective rehabilitation protocols maximizing sensorimotor function recovery in people with SCI.

1. Introduction

There are more than 250,000 persons living with spinal cord injury (SCI) in the United States and several million worldwide. Injuries of the spinal cord occur mostly in young adults who then require life-long healthcare. The impaired function of spinal circuitry, the impaired processing of afferent input by the spinal circuits, and the decline in transmission of uninjured fibers are clear markers of the central nervous system's (CNS) pathophysiological state after SCI [1–5].

The understanding of spinal control of locomotion has improved significantly since the times of Thomas Graham Brown and Sir Charles Sherrington [6–8]. Complex models are currently developed to address the function of the spinal networks that give genesis to single limb and bilateral right-left neuronal interactions [9, 10], as well as their reorganization abilities following locomotor training in animal preparations [11]. Based on the observations on the spinal neural control of locomotion and recovery of locomotion in spinalized animals, body weight support (BWS) on a treadmill with as-needed manual assistance by therapists [12, 13] and BWS on a treadmill with robot-driven leg assistance [14] are utilized to improve locomotor ability of these patients. In this review, we will provide an in-depth discussion about the manner in which spinal neuronal circuits are impaired after SCI, how they reorganize after locomotor training, the possible neurophysiological mechanisms underlying such reorganization, and the functional consequences of locomotor-training-mediated neuronal plasticity.

2. Neuromodulation as a Window of CNS Function

Representative examples of neuronal activity modulation, recorded through surface electromyography (EMG) upon peripheral skin/nerve or transcortical stimulation, while walking in humans are found in three examples: the Hoffmann (H) reflex, motor evoked potentials (MEPs), and the polysynaptic flexor reflex. First, the H-reflex, which presents the spinal part of the stretch reflex bypassing the muscle spindle and the fusimotor activity that may influence the sensitivity of the Ia afferents, is a powerful tool to probe the efficacy of Ia afferents to monosynaptically depolarize alpha motoneurons, the excitability state of spinal interneuronal circuits/pathways, and spinal integration of sensory afferent feedback [15]. Second, MEPs, which are the result of spinal motoneuron activation following single-pulse transcranial magnetic stimulation (TMS), can be used to assess corticospinal tract excitability while walking in humans [16]. Third, stimulation of the skin at varying multiples of perceptual threshold of the foot or a pure sensory nerve (sural) can evoke short-latency, middle latency, or even long-latency responses in flexors and extensors that have specific regulatory effects on locomotion [17].

In healthy humans, while stepping on a motorized treadmill, the soleus H-reflex amplitude increases progressively from midstance to late stance, decreases significantly at stance-to-swing transition, and remains depressed during the swing phase of gait (Figure 1(a)) regardless of the BWS level [18]. A gradually increasing H-reflex amplitude towards the end of the swing phase in healthy humans has also been reported (see [19, Figure 31]). A similar modulation pattern is also exhibited by the soleus MEP while walking. It increases progressively from early stance to midstance, reaching maximal amplitude at late stance, and is completely abolished during the swing phase with a gradually increasing MEP excitability at swing-to-stance transition (Figure 1(a)) regardless of the BWS level [16, 20]. Further, the short-latency tibialis anterior (TA) flexor reflex, evoked following innocuous stimulation of the skin over the medial arch of the foot, increases at heel contact, progressively decreases during the stance phase, and then increases throughout the swing phase in healthy humans while stepping on a motorized treadmill (Figures 1(c) and 1(d)), a pattern similar to that observed for the TA MEP (Figures 1(c) and 1(d)) [16, 21].

2.1. Spinal Reflexes and MEP Modulation. The amplitude modulation of soleus H-reflex, soleus MEP, and short-latency TA flexor reflex occurs largely in parallel with that of homonymous EMG activity [16, 21]. Because the soleus H-reflex remains depressed during the swing phase upon voluntary activation of the triceps surae, it is modulated in a similar manner to that observed while walking in absence of weight-bearing upon unilateral rhythmic leg movements, and the soleus MEP facilitation at swing-to-stance transition coincides with quiescent homonymous EMG [16, 22, 23], modulation of the soleus H-reflex and soleus MEP while walking cannot not be regarded simply as a sole reflection of background excitability changes of the motoneuron pool.

The soleus H-reflex amplitude modulation while walking can be partially ascribed to (1) presynaptic regulation of synaptic transmission from group Ia afferents to motoneurons and interneurons, (2) presynaptic regulation of GABAergic inhibition acting on dorsal root afferents, (3) phasic depolarization of group I afferents, and (4) tonic decrease in the excitability of the afferent fibers (animal data: [24–27]; human data: [28, 29]). Further, Ib facilitation [30, 31] and reciprocal Ia inhibition from flexor nerve afferents onto extensor motoneurons [32] also constitute spinal segmental mechanisms that contribute to the soleus H-reflex amplitude modulation at the stance and swing phases, respectively. Presynaptic inhibition of Ia afferent terminals and Renshaw cells acting on Ia inhibitory interneurons have also been documented [33]. The phase-dependent modulation of soleus and TA MEPs may be attributed to excitability changes of corticomotoneuronal cells [34], corticospinal volleys activating mutual reciprocal inhibitory interneurons [16, 35, 36], and cortically mediated ongoing changes in presynaptic inhibition of Ia afferents [37]. Excitatory and inhibitory interneurons in the motor cortex may contribute to MEP excitability changes while walking [38, 39]. The phase-dependent modulation pattern of the short-latency TA flexor reflexes can be partly attributed to amplitude modulation of presynaptic inhibition of cutaneous afferent volleys [25, 40].

For a phase-dependent modulation of soleus H-reflex, soleus/TA MEPs, and short-latency TA flexor reflexes to occur, locomotor neuronal networks need to be appropriately engaged at each phase of a step cycle, and thus these networks can depict both the physiological function and the underlying neuronal reorganization of spinal locomotor circuits in spinal-injured humans after repetitive step training.

3. Plasticity of Neuronal Activity after Locomotor Training

Following induction of SCI, animal studies have shown that locomotor training improves locomotor capacity beyond spontaneous recovery, full weight-bearing ability is prolonged, and improved locomotion persists up to 6 weeks after training stops [41, 42]. Similarly, in humans with SCI, locomotor training improves limb coordination, limb kinematics, step symmetry, walking speed, endurance, and balance [43–49], reduces systolic blood pressure and heart rate [50], improves respiratory function [51], and reduces inflammatory status [52]. Improvements in standing, walking, and respiratory capacity are likely due to plasticity of spinal interneuronal circuits. Below, we discuss evidence surrounding plasticity of neuronal activity after locomotor training in people with SCI.

3.1. Monosynaptic-Polysynaptic Motoneuron Responses While Walking. In people with SCI, the soleus H-reflex modulation pattern while walking varies considerably between patients, from being relatively normal in some to being completely absent in others [2, 53, 54]. The most common abnormal patterns we have observed in people with SCI, regardless of the American Spinal Injury Association Impairment Scale (AIS), are lack of reflex depression during the swing phase

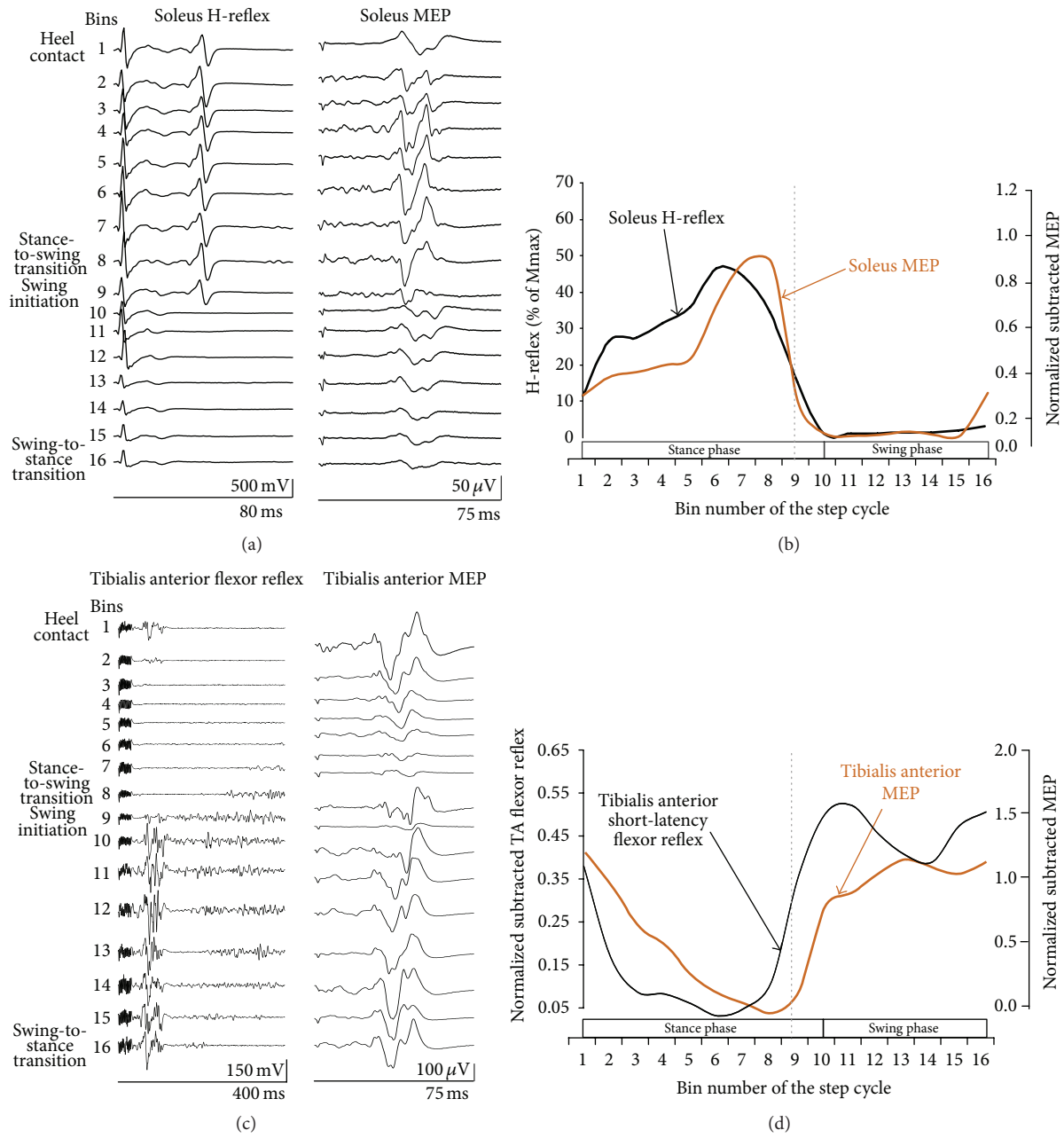

Figure 1: Modulation of neuronal activity while walking in uninjured humans. (a, b) Soleus H-reflexes and soleus motor evoked potentials (MEPs) amplitude at each bin of the step cycle while stepping on a motorized treadmill for single subjects (a) and for a group of healthy subjects (b). (c, d) Short-latency tibialis anterior (TA) flexor reflexes and TA MEPs amplitude at each bin of the step cycle while stepping on a motorized treadmill for single subjects (c) and for a group of healthy subjects (d). For the grouped data, for each bin of the step cycle, the soleus H-reflex was normalized to the maximal M-wave evoked 60–80 ms after the test H-reflex, and the short-latency TA flexor reflexes and soleus/TA MEPs were normalized to the maximum homonymous locomotor EMG having subtracted the control EMG (EMG without stimulation) at identical time windows and bins. Each step was divided into 16 equal bins based on the signal from the right foot switch. Bin 1 corresponds to heel contact. Bins 8, 9, and 16 correspond approximately to stance-to-swing transition, swing initiation, and swing-to-stance transition, respectively. Vertical dotted lines designate the stance-to-swing transition phase. Data adopted and modified from [2, 16, 21, 22].

and a disruption of sustained reflex excitability during the stance phase [54]. Similarly, the most common change we have observed after locomotor training regardless of the AIS is reestablishment of reflex depression during the swing phase that promotes reciprocal activation of ankle flexors and extensors [54]. Also importantly is our observation that reflex depression at mid-late swing was restored in two cases of motor complete SCI (AIS A-B) (see [54, Figure 2A]). We also observed that this neuronal reorganization was not distributed equally in the more impaired leg compared with

the less impaired leg, as the soleus H-reflex during the stance phase was moderately decreased across all patients after locomotor training in the more impaired (right) leg compared to the less impaired (left) leg (compare Figures 2 and 3 in [54]).

An additional neuronal characteristic of SCI is the dominance of late long-lasting flexor reflexes over the short-latency flexor reflexes [1, 55–57]. While the late long-lasting flexor reflexes in human SCI have a similar interneuronal reorganization to that reported in acute spinal cats treated with L-DOPA (reviewed in [58]) and are due largely to the absent mutual inhibitory actions from early onto late flexor reflex interneuronal networks, their relative behavior signifies the altered interneuronal reorganization after injury. We recently reported that locomotor training in people with chronic SCI results in reappearance of short-latency TA flexor reflexes (see [28, Figure 3]), reduces the amplitude of long-latency TA flexor reflexes in the more impaired right leg (see [21, Figure 2]), increases the amplitude of long-latency TA flexor reflexes in the less impaired left leg (see [21, Figure 2]), and promotes a phase-dependent modulation of both short-latency and long-latency TA flexor reflexes during assisted stepping [21].

3.2. Spinal Inhibition. Impaired function of many different spinal inhibitory pathways has been implicated as one of the main causes of pathological movement and muscle tone after SCI, related to reduced GABAergic and glycinergic inhibitory neurotransmission/reception [59]. Physiological measures of neuronal activity, discussed below, strongly support that the main underlying neurophysiological mechanism of locomotor training is the return of the lost spinal inhibition in people with chronic SCI.

3.2.1. Homosynaptic Depression. Homosynaptic (or low-frequency) depression is a form of presynaptic inhibition (Figure 2(a)) attributed mostly to a decrease in the amount of released neurotransmitters by the previously activated Ia afferents [60–62], depletion of releasable vesicles, failure of action potential conduction at axonal branches [63], decrease of presynaptic quantal size [64], and adaptation of exocytosis machinery [65]. Impaired function or completely absent homosynaptic depression in people with chronic SCI has been linked to stretch reflex hyperexcitability, clonus, and cocontractions due to altered or abnormal synaptic efficacy of afferent impulses [66–68].

Limited evidence exists on the reorganization of homosynaptic depression in animals and humans. Homosynaptic depression was potentiated after passive exercise of complete spinal transected rats [69], after 10 locomotor training sessions in one SCI person capable of ambulation [70] and after cycling in one person with spastic tetraplegia [71]. We recently reported that repetitive locomotor training restores soleus H-reflex homosynaptic depression, but we found significant differences among patient groups [72]. In summary, we found that soleus H-reflex homosynaptic depression was restored in two people with motor complete SCI in both right and left legs, and it became stronger after training in the more impaired right leg compared to the less impaired left leg regardless of the AIS (see [72, Figure 4]). Last, we found that, in cases where some homosynaptic depression was present before training, locomotor training further potentiated the soleus H-reflex homosynaptic depression (see [72, Figure 4]). In Figure 2(b), representative examples of this neuronal organization are indicated from one person with AIS B who received 53 locomotor training sessions. These recordings clearly indicate that the soleus H-reflex amplitude exhibited a strong stimulation frequency-dependent depression after locomotor training even in cases when descending control is greatly impaired or absent [72].

3.2.2. Presynaptic Inhibition. The synaptic efficacy of afferent volleys before they reach their target neurons can be adjusted by presynaptic inhibition (Figure 3(a)). Methods have been developed to probe presynaptic inhibition exerted only at Ia afferent terminals [71]. This is because only Ia afferents have monosynaptic projections to motoneurons and separation from motor fibers based on stimulation intensities and respective thresholds is possible. Presynaptic inhibition was originally described in the cat by Frank and Fuortes [73], is associated with primary afferent depolarization (PAD), is mediated by axoaxonic synapses [74], and involves modulation of transmitter release at the Ia-motoneuron synapse by means of $GABA_A$ receptors, which consequently increase the efflux of Cl^- ions and produce depolarization of the afferent terminals [75].

Presynaptic inhibition is modulated in a phase-dependent manner during fictive and real locomotion in animals, including humans [25, 28, 76, 77], and accounts to a great extent for the differential soleus and quadriceps H-reflex amplitude modulation while walking in uninjured humans [29, 78]. Functionally, increased presynaptic inhibition of the soleus Ia excitatory feedback may be needed to prevent excessive activation of ankle extensor motoneurons at mid-to-late stance phases (causing a stiff gait), while decreased presynaptic inhibition of the quadriceps Ia excitatory feedback at early stance prepares the knee joint to accept loading. The soleus H-reflex facilitation following femoral nerve stimulation at group I threshold is exerted from quadriceps afferents onto soleus motoneurons via monosynaptic connections, and increases or decreases of this facilitation have been ascribed to changes in the ongoing presynaptic inhibition [79]. The excitatory influence of Ia afferents onto synergistic muscles, as is the case with quadriceps afferents acting onto soleus motoneurons, is also known as heteronymous Ia facilitation. In people with traumatic SCI, the increased heteronymous Ia facilitation supports decreased presynaptic inhibition [80]. The complete disappearance of presynaptic inhibition of Ia afferent terminals of the flexor carpi radialis H-reflex, elicited by electrical stimuli applied to the nerve supplying antagonistic muscles at long conditioning-test intervals, in two patients with tetraplegia due to a spinal cord lesion at C5-C6 [81], supports further abnormal premotoneuronal control after SCI. It has also been shown that the level of presynaptic inhibition declines over time after SCI [66]. The decrease of presynaptic inhibition after SCI is likely related to impaired function of the descending pathways that ensure suppression of inhibitory interneurons transmitting cutaneous inhibition of first-order PAD interneurons [82].

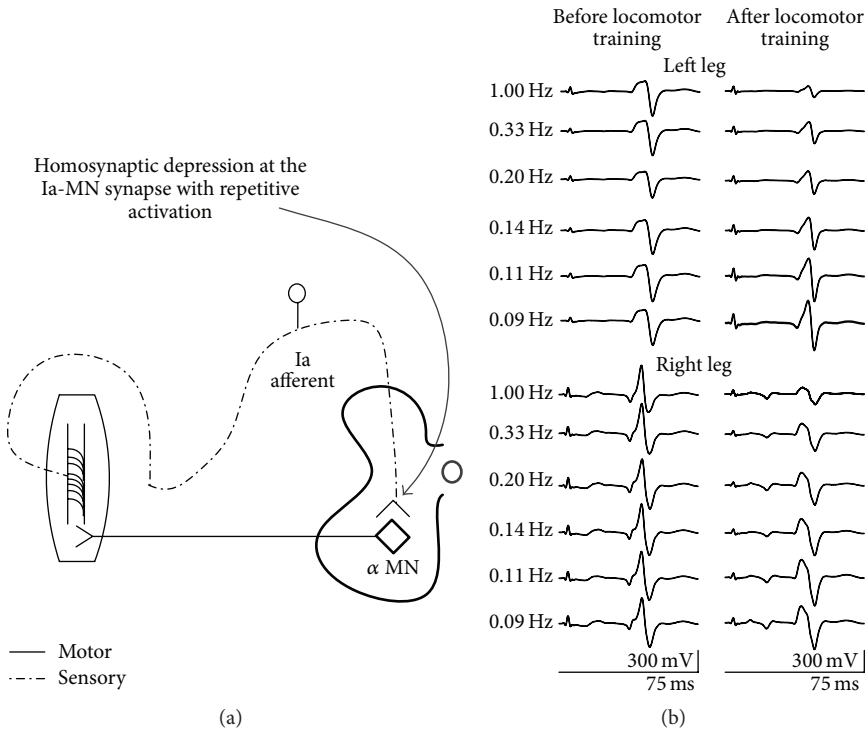

Figure 2: Functional reorganization of homosynaptic depression after locomotor training in SCI. (a) Schematic diagram of the soleus H-reflex homosynaptic depression exerted at Ia-motoneuron synapse with repetitive activation of Ia afferents. (b). Nonrectified waveform averages of soleus H-reflexes recorded at different stimulation frequencies from one AIS B patient before and after locomotor training for both legs. The soleus H-reflex amplitude exhibited a strong stimulation frequency-dependent depression after locomotor training. Data adopted and modified from [72].

We recently reported that presynaptic inhibition of soleus Ia afferents, assessed as the amplitude of the conditioned soleus H-reflex by excitation of antagonistic group I afferents at long conditioning-test intervals in the seated position [15], was reorganized in motor incomplete SCI (AIS C-D) but not in motor complete SCI (AIS A-B) after locomotor training (Figure 3(b)) [72]. We also found that, during assisted stepping, the modulation of presynaptic inhibition occurred at different phases of the step cycle before training when compared to that observed after training [72], and this change was comparable to the modulation pattern we have reported for uninjured human subjects during assisted stepping [77]. Reorganization of presynaptic inhibition can partly account for the return of the physiological soleus H-reflex amplitude modulation while walking after locomotor training found for the same patients [54].

3.2.3. Reciprocal Ia Inhibition. The neuronal pathway from the large muscle spindle (Ia) afferents to antagonistic alpha motoneurons is the most known and well-studied spinal inhibitory pathway in the mammalian CNS (Figure 4(a)), described originally by Lloyd [83–85], with vestibulo-, cortico-, and rubropropriospinal tracts and cutaneous and flexor reflex afferents to affect transmission in the Ia interneurons and their subsequent synaptic inputs onto motoneurons [86]. Ia afferent-mediated reciprocal inhibition is effective in blocking antagonist motoneuron activation at birth in hemisected spinal cord preparations and in humans when rhythmic motor programs have not been developed, used, or stored [87, 88]. A high specificity of neuronal connections from quadriceps Ia afferents to posterior biceps-semitendinosus motor neurons is reported at birth in mice [89].

The functional significance of reciprocal Ia inhibition is apparent when one considers that this neuronal pathway operates only between flexor and extensors and not between abductors and adductors [91]. Thus, the role of reciprocal Ia inhibition in the alternating activation of flexors and extensors during locomotion might be to eliminate excitatory effects during the passive (swing) phase of the step cycle and remove the enduring Ia excitation during the shifts between flexion and extension phases [92]. Recordings from Ia inhibitory interneurons during fictive locomotion in complete spinally transected cats showed that hyperpolarization of extensor alpha motoneurons during the swing phase is directly related to their activity [93–95], largely determined by intraspinal rhythmic processes [96].

SCI in humans is associated with pathologic changes of reciprocal Ia inhibition, with alterations reported in strength, timing, and modulation at rest, during contraction, and while walking [97–101]. Reciprocal facilitation is related to poor motor recovery of legs, while stronger reciprocal inhibition is linked to less spasticity (1 and 2 on the Ashworth score) [102].

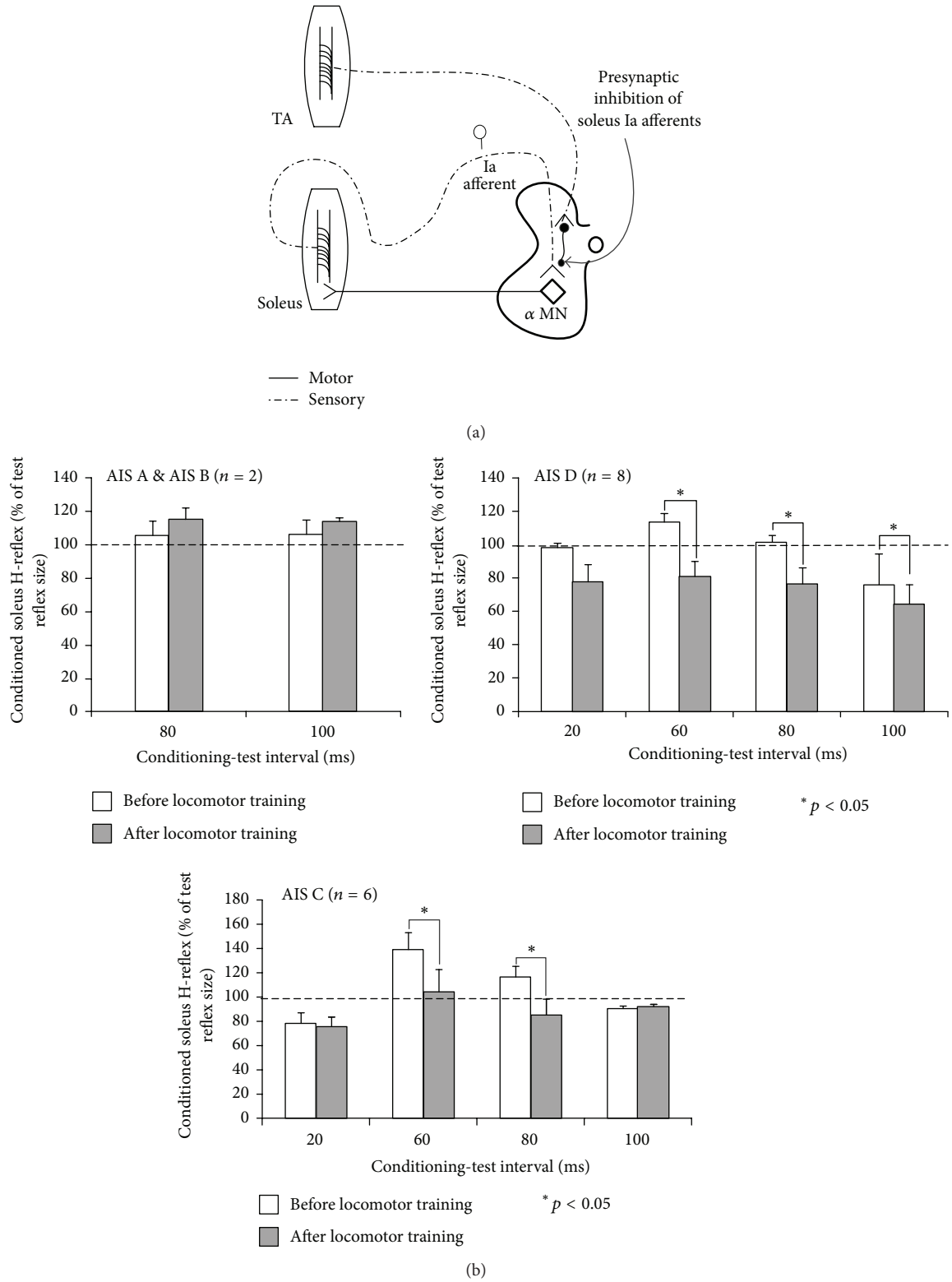

Figure 3: Functional reorganization of presynaptic inhibition of soleus Ia afferents after locomotor training in SCI. (a) Schematic diagram of the neuronal pathway of presynaptic inhibition of soleus Ia afferents. In this paradigm, presynaptic inhibition of soleus Ia afferents is induced by a conditioning afferent volley following common peroneal nerve stimulation at long conditioning-test (C-T) intervals. (b) Mean amplitude of the conditioned soleus H-reflex as a percentage of the unconditioned H-reflex recorded at each C-T interval tested before and after locomotor training from the right leg, grouped per AIS, in the seated position. $^{*}p < 0.05$ indicate statistically significant differences of the conditioned H-reflexes recorded before and after locomotor training. Data adopted and modified from [72].

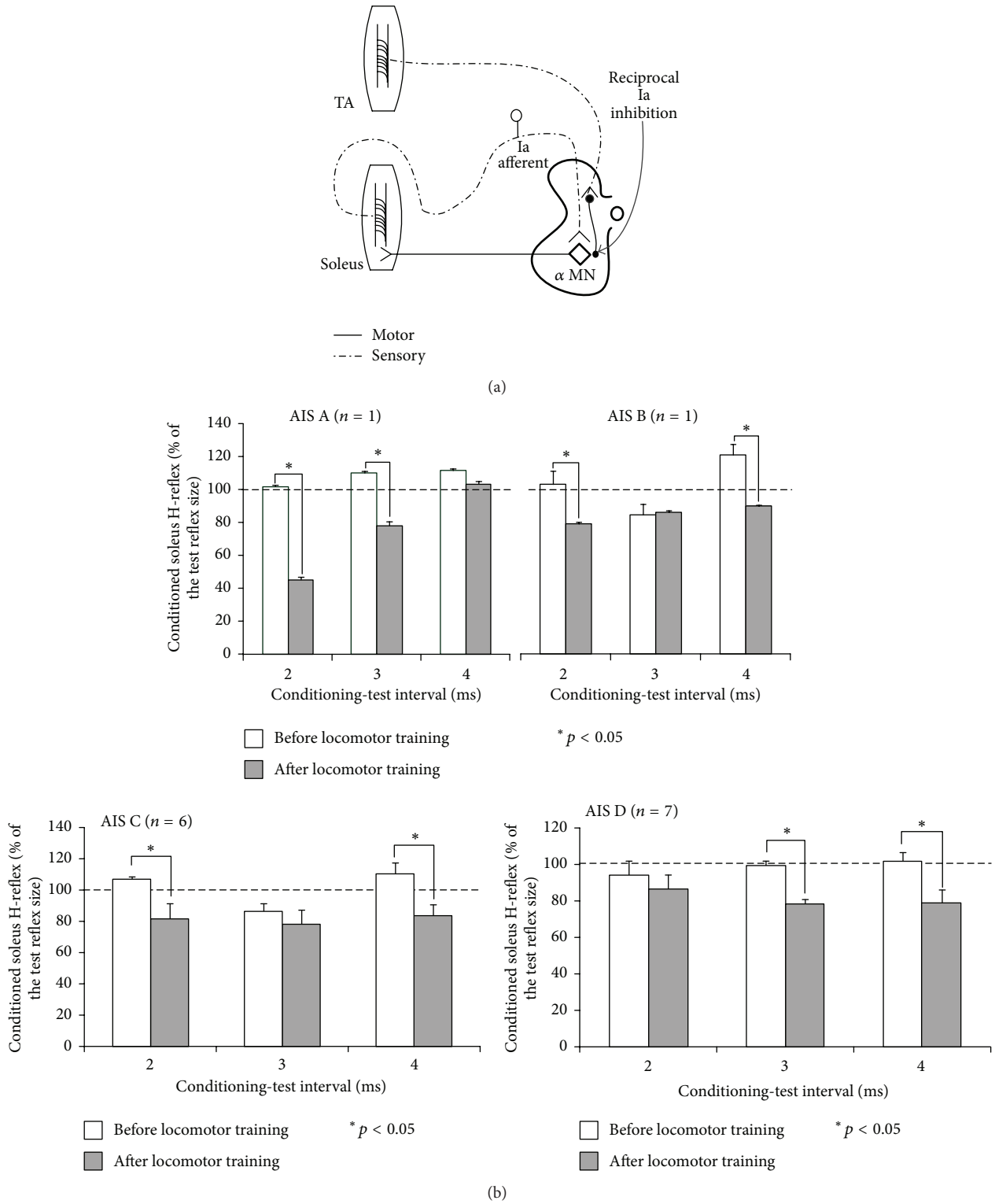

Figure 4: Functional reorganization of reciprocal Ia inhibition after locomotor training in SCI. (a) Schematic diagram of the neuronal pathway of reciprocal Ia inhibition mediated by a conditioning afferent volley induced by stimulation of the ipsilateral common peroneal nerve at short conditioning-test (C-T) intervals. (b) Mean amplitude of the conditioned soleus H-reflex as a percentage of the unconditioned H-reflex recorded at each C-T interval tested before and after locomotor training from the right leg, grouped per AIS, in the seated position. $^{*}p < 0.05$ indicate statistically significant differences of the conditioned H-reflexes recorded before and after locomotor training. Data adopted and modified from [90].

In a group of people with SCI, we studied to what extent reciprocal Ia inhibition of soleus motoneurons, assessed as the soleus H-reflex amplitude conditioned by excitation of TA group I afferents at short conditioning-test intervals while seated [15], is restored after locomotor training. We found that reciprocal facilitation was replaced by reciprocal inhibition regardless of the AIS level in the seated position (Figure 4(b)) [90]. However, during assisted stepping the changes were not uniform across AIS patients, because we found that reciprocal Ia inhibition recovered at a greater level in AIS C than in AIS D after locomotor training (see [90, Figure 2]). Reciprocal inhibition was profoundly decreased during the stance phase and increased during the swing phase in AIS C after locomotor training, while, in AIS D, reciprocal inhibition was mostly decreased (see [90, Figure 2]). The reduced amounts of reciprocal inhibition in AIS D can explain the lack of full soleus H-reflex depression during the swing phase we observed in these patients. It is possible that more training sessions or more intense training (i.e., more steps/session) [103] may be required to increase the amount of reciprocal inhibition in some patients with SCI.

3.2.4. Nonreciprocal Ib Inhibition. The views pertaining to the functional role of Ib afferents (Figure 5(a)) have changed substantially from a simple autogenic protective reflex response to the more complicated view that these afferents continuously provide information about the amplitude of muscle contraction. Ib interneurons that mediate such information are widely distributed, reaching almost all motoneuron pools of the ipsilateral limb [104]. Ib interneurons can participate in alternative pathways allowing for excitation or inhibition depending on the selected subpopulation of interneurons [105] and receive extensive convergence from other afferents and descending tracts [106]. It is well established that Ib afferents participate in a reflex reversal during fictive locomotion in decerebrate cats, known as locomotor-related group I excitation, which utilizes a different circuit organization compared to that observed at rest and is transmitted through the extensor half centre [107, 108]. These Ib locomotor excitatory interneurons are located in the intermediate zone in mid to caudal parts of the lower lumbar spinal cord [30]. In summary, group I (mainly Ib) afferents of ankle extensors shape the amplitude, duration, and timing of ipsilateral extensor activity and depending on the timing that excitation occurs they can increase the activity of extensor motoneurons at the stance phase, initiate extension, and terminate or delay flexor bursts in the ipsilateral hind limb [107, 109–112]. A similar facilitatory locomotor group I pathway also exists in humans [113], with Ib inhibition decreasing while loading and reversing to excitation while walking [31].

In people with chronic spinal cord lesions, conflicting evidence exists on this pathway, as nonreciprocal Ib inhibition is reported to be either physiologic or pathologic at rest and during assisted stepping [114–116]. Indeed, we recently reported the presence of short-latency soleus H-reflex depression following medialis gastrocnemius (MG) nerve stimulation at short conditioning-test intervals (attributed mostly to Ib inhibition) in two persons with AIS A and AIS B while seated (Figure 5(b)) [90]. In addition, locomotor training potentiated the preexisting Ib inhibition at rest in AIS A, AIS B, and AIS C (Figure 5(b)), but during assisted stepping we found that the reorganization was different for AIS C and AIS D [90]. In general, changes in Ib inhibition were noted mostly during the swing phase in AIS C patients, while in AIS D patients Ib inhibition was increased at midstance [90]. While these findings are consistent with the reduced short-latency group I inhibition of synergists at the stance phase of walking in healthy humans and during fictive locomotion in spinal animals [113, 117], locomotor training did not induce, as expected, an extra facilitation of soleus motoneuron responses by group Ib afferents during the stance phase. Strengthening Ib polysynaptic excitation with locomotor training during the stance phase of walking may require more training sessions, more steps per session, more body loading, greater allowance for manageable errors, and/or training at different levels of environmental constraints [103, 118].

3.3. Alpha Motoneurons. Altered excitability of spinal neurons is considered a key pathophysiological event after an injury to the spinal cord. The monoamines serotonin and norepinephrine, which are released from pathways originating in the brainstem, substantially modulate spinal motoneuron excitability. Activation of monoamine receptors enhances intrinsic low-voltage-activated persistent inward currents (PICs) that produce plateau potentials and self-sustained firing in both the somata and dendrites, also regulating the gain of the motoneuron pool [119–123]. PICs amplify both synaptic excitation and inhibition, are critical for the dynamic transformation of synaptic inputs, and provide a sustained excitatory drive that allows motoneurons to fire repetitively following a brief synaptic excitation [124–126]. Inhibitory synaptic inputs can exert considerable control over alpha motoneuron discharge by regulating intrinsic PICs activation/deactivation [127].

Despite the lost or reduced brainstem-derived serotonin with chronic SCI, PICs are enhanced due to compensatory upregulation of constitutively active 5-HT_2 receptors [128]. PICs that drive self-sustained firing in motoneurons have been related to the development of muscle spasms and hyperreflexia to nonnoxious stimuli and clonus [129]. Additionally, the voltage threshold of slow motoneurons changes, axonal conduction velocity, and rheobase current increases, afterhyperpolarization duration decreases [130, 131], short pulse current threshold increases [131], and resting threshold and resting membrane potential decrease [131, 132]. Further, spinal cord transection leads to changes in the rhythmic firing patterns of motoneurons in response to injected currents [132]. In people with SCI, alpha motoneuron PICs and associated self-sustained firing facilitated the firing of motor units during the prolonged muscle spasms that can continue for many seconds, even minutes, at very low discharge rates [133].

Evidence from animal studies suggests that intrinsic properties of motoneurons recover after locomotor training. Motoneurons of trained rats have lower hyperpolarized resting membrane potentials, decreased spike trigger threshold levels (membrane potential at which an action potential is triggered), increased amplitudes of after hyperpolarization (reflecting a decrease in membrane excitability) [134–136],

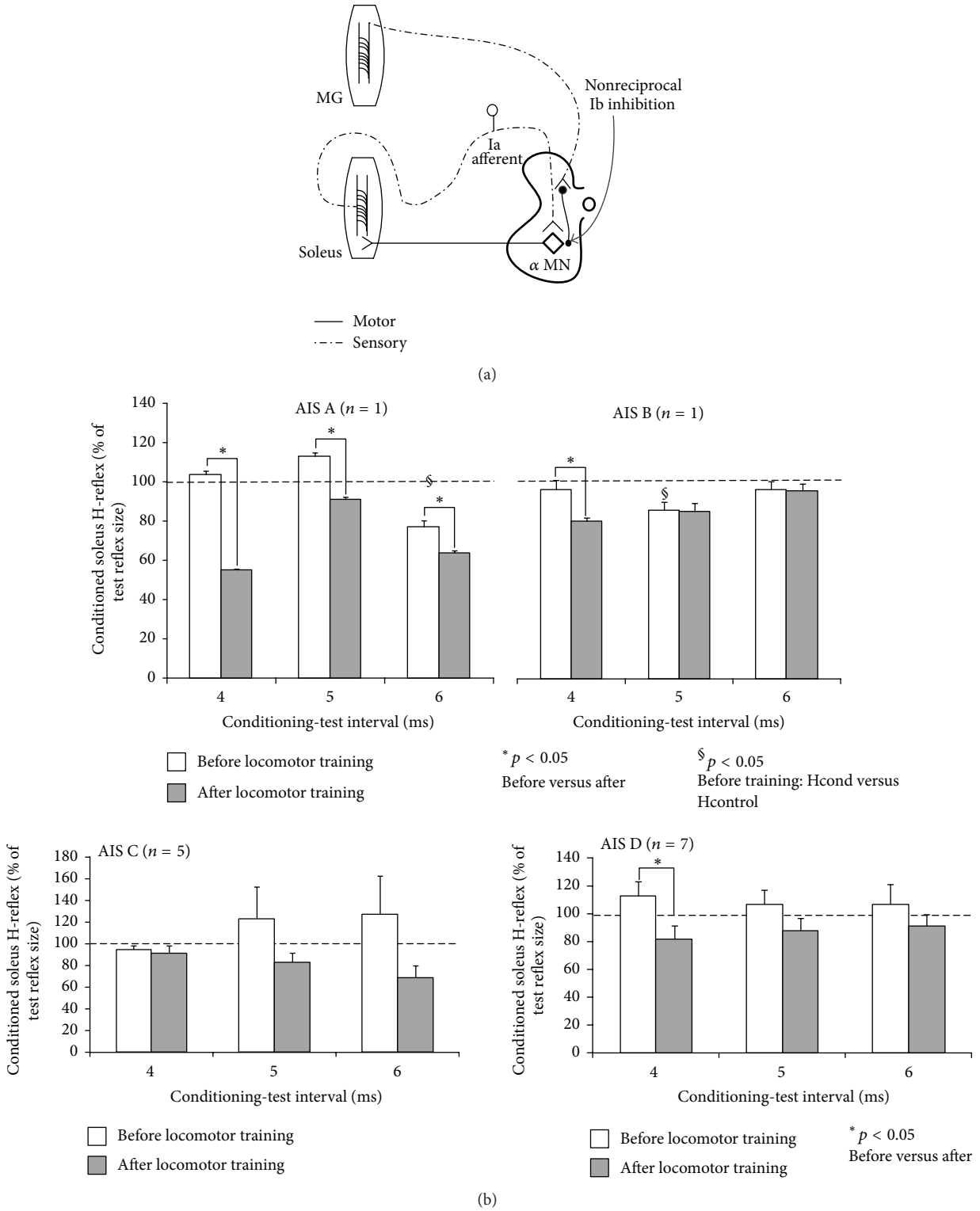

Figure 5: Functional reorganization of nonreciprocal Ib inhibition after locomotor training in SCI. (a) Schematic diagram of the neuronal pathway of nonreciprocal Ib inhibition mediated by a conditioning afferent volley induced by stimulation of the ipsilateral medialis gastrocnemius nerve at short conditioning-test (C-T) intervals. The facilitatory locomotor Ib pathway is not indicated. (b) Mean amplitude of the conditioned soleus H-reflex as a percentage of the unconditioned H-reflex recorded at each C-T interval tested before and after locomotor training from the right leg, grouped per AIS, in the seated position. $^{*}p < 0.05$ indicate statistically significant differences of the conditioned H-reflexes recorded before and after locomotor training. Data adopted and modified from [90].

stabilized dendritic tree structure of motoneurons [137], altered synaptic inputs from the spinal white matter [138], and a soma size and Na^+, K^+, and ATPase activity similar to uninjured animals [139]. The duration of training is critical in changing the intrinsic properties of motor neurons, as 3 weeks of training does not restore their electrical properties [140].

Direct changes in the electrical and biophysical properties of motor neurons in SCI patients following locomotor training are difficult to document given the methodological limitations in human studies. However, the amplitude of monosynaptic motoneuron responses at different stimulation intensities along with excitation thresholds can help us draw conclusions on these characteristics. The amplitude and stimulation threshold intensities of the soleus monosynaptic motoneuron responses are not adjusted in untrained SCI patients in the supine and standing positions compared to those observed in uninjured subjects [18]. We found that these parameters were remarkably modified in a body position-dependent manner that depended largely on the leg motor impairment after locomotor training [141]. The maximal H-reflex (Hmax) size was decreased after training while seated and while standing in AIS A and AIS B subjects [141]. The soleus H-reflex size, from the onset of the recruitment curve until its maximum amplitude, was decreased in the right leg in AIS D and in the left leg in AIS C while seated and was increased while standing in both legs in AIS C but not in AIS A, AIS B, and AIS D [141]. Further, the stimulus corresponding to H-threshold, 50% Hmax, and Hmax was increased in AIS D, in whom the reflex excitability was decreased in the right leg while seated after training [141, Table 1]. This means that, after locomotor training, more stimulation intensity is required to activate the most excitable Ia afferent fibers that subsequently depolarize the lower threshold (most excitable) soleus motoneurons. The stimulus corresponding to H-reflex threshold expresses the number of active motoneurons or the spinal excitability level, which reflects the balance of excitatory and inhibitory inputs acting on the motoneuron pool [142]. The decreased spinal reflex excitability with the concomitant increased soleus H-reflex threshold indicates that motoneuron excitability was altered along with the excitability level of Ia afferents.

The increased soleus H-reflex excitability we observed in AIS C subjects while standing after training, compared to that observed before training [141], may enhance ankle stability and thus contribute to an improved leg function while standing. It is known that in uninjured humans, while standing, Ib inhibition exerted from MG to soleus motoneurons is decreased, presynaptic inhibition of soleus Ia afferents is increased, and reciprocal inhibition is decreased when compared to that observed while seated [31, 143, 144]. Thus, both presynaptic inhibition and Ib facilitation after locomotor training may reinforce H-reflex excitability while standing, promoting weight-bearing in people with motor incomplete SCI. The neuronal activity changes we have recently reported after repetitive locomotor training in people with chronic motor complete and incomplete SCI [21, 54, 72, 90, 141] are summarized in Table 1 based on body position, motor task, and AIS. These changes can be summarized as follows: (1) monosynaptic motoneuron responses are adjusted in a body position manner, (2) soleus H-reflex phase-dependent modulation is restored, (3) soleus H-reflex homosynaptic depression is restored regardless of AIS, (4) presynaptic inhibition of the soleus Ia afferents evoked by a conditioning stimulus recovers only in AIS C and AIS D, (5) reciprocal Ia inhibition from flexor group I afferents on soleus motoneurons is absent before training and returns regardless of AIS after training, (6) Ib inhibition from MG group I afferents on soleus motoneurons is present before training and is increased after training in AIS A, AIS B, and AIS D, and (7) short-latency flexor reflexes reappear and both short- and long-latency flexor reflexes are modulated in a phase-dependent manner [21, 54, 72, 90, 141].

4. Recovery of Motor Activity after Locomotor Training in SCI

Motor output can be viewed, without excluding the descending pathways, as the net result of function of motor neurons and interneurons at multisegmental spinal levels. Based on our latest completed locomotor trial in people with SCI and other studies, the changes in motor function can be summarized as (1) increase in peak EMG amplitudes of ankle muscles and decrease in peak EMG amplitudes of medial hamstrings and hip adductor gracilis muscles in the more impaired right leg (medial hamstrings and hip adductor gracilis muscles are known to contribute primarily to the spastic gait pattern and to the pathological leg spasticity pattern at rest) [72, Figure 5B], (2) restoration of biphasic EMG activity (when a muscle contracts in more than one phase within a single step cycle) (see [72, Figure 5A]), (3) onset changes of EMG activity while stepping, (4) reduced cocontraction levels between ankle and knee antagonistic muscles (see [72, Figure 5C]), (5) improvements in the alternating activity of the same muscle between the left and right legs, and (6) reduced EMG clonic activity of ankle extensors at rest and on the treadmill [54, 72, 145, 146]. However, locomotor training does not restore motor activity similarly in complete and incomplete SCIs. An example of an episode of muscle activity during assisted stepping after locomotor training is shown in Figure 6. The EMG bursts clearly indicate that the ankle antagonistic muscles were activated in a reciprocal pattern after locomotor training in the incomplete SCI subject (AIS D, R014), while a complete absent phase-dependent activity is evident in the complete SCI subject (AIS B, R06) after 53 locomotor training sessions (Figure 6). The lack of distinguished antagonistic EMG bursts with clear onset and offsets in the person with motor complete SCI after locomotor training clearly supports pronounced differences between recovery in animals and humans [147], and thus we need to be cautious when animal data are translated to humans.

The profound changes in motor activity after locomotor training in motor incomplete SCI coincided with changes in gait parameters. The BWS required while stepping was decreased by an average of 55%, the gait speed was increased by 58%, and the leg guidance force by the robotic exoskeleton was decreased by 43% [72]. Furthermore, in the motor incomplete subjects, locomotor training improved their lower extremity motor scores, assessed manually by a physical

Table 1: Summary of neuronal activity changes in SCI after locomotor training[#].

(a)

Neuronal activity	AIS A/B		AIS C		AIS D	
	Seated	Stepping	Seated	Stepping	Seated	Stepping
Soleus H-reflex phase-dependent modulation	NA	↓ stance phase* ↓ swing phase*	NA	↓ swing phase*	NA	↓ swing phase*
Homosynaptic depression	Restored*	NA	Restored*	NA	Restored*	NA
Presynaptic inhibition of Ia afferents	No change	Not tested	Restored*	↓ stance phase* ↑ swing phase*	Restored*	↓ late stance* ↑ swing-to-stance transition *
Reciprocal Ia inhibition	Restored*	Not tested	Restored*	↓ stance phase* ↑ swing phase*	Restored*	↓ stance phase* ↓ swing phase*
Nonreciprocal Ib inhibition	Restored*	Not tested	No change	No change in stance* ↓ early swing* ↑ late swing*	Restored*	↓ stance phase*
Long-latency flexor reflexes	↓ R-Leg* ↑ L-Leg*	Improved*	↓ R-Leg* ↑ L-Leg*	Improved*	↓ R-Leg* ↑ L-Leg*	Improved*
Short-latency flexor reflexes	Reappeared in R-Leg*	Phase-dependent modulation emerged in R-Leg*	Reappeared in R-Leg*	Phase-dependent modulation emerged in R-Leg*	Reappeared in both legs*	Phase-dependent modulation emerged*

(b)

Neuronal activity	AIS A/B		AIS C		AIS D	
	Seated	Standing	Seated	Standing	Seated	Standing
Soleus motoneuron excitability (Hmax)	No change in R/L-Legs	No change in R/L-Legs	No change in R-Leg* ↓ L-Leg*	↑ R/L-Legs*	↓ R-Leg* No change in L-Leg*	No change in R/L-Legs
H-threshold	No change in R/L-Legs	No change in R/L-Legs	No change in R/L-Legs	No change in R/L-Legs	↑ R-Leg* No change in L-Leg*	↓ L-Leg* No change in R-Leg*

[#]Neuronal activity changes after locomotor training in people with SCI are from authors' recent published work [21, 54, 72, 90, 141]. R: right; L: left; and NA: not applicable; symbols ↑/↓ indicate increased or decreased neuronal activity. ∗ refers to neuronal activity changes.

therapist, with the more impaired right leg improving by 10% and the left leg improving by 6.4% [21], a motor improvement also reported for cervical and thoracic AIS D patients [148].

5. Pathways and Circuits Underlying Neuronal and Motor Plasticity after Locomotor Training

The CNS adapts and reorganizes continuously based on motor experience and use. This *natural* reorganization is the result of physiological, anatomical, and functional neuronal changes along the neuroaxis (cortex, cerebellum, spinal cord, and nerve axons) [149]. After an injury to the spinal cord, neuronal reorganization occurs that eventually results in neural circuits/pathways with altered properties and functions. The findings on the neuronal activity changes in animals and in humans detailed in the above paragraphs support that locomotor trainingcan promote *functional* neuronalreorganization [150]. A major drive to the neuronal reorganization after locomotor training is reinforcement of activity-dependent sensory feedback from receptors (including but not limited to plantar mechanoreceptors and hip proprioceptors) that can adjust the operation of the CPG [11, 151, 152].

Sources for neuronal activity changes in people with SCI after locomotor training could include modifications in the intrinsic properties and function of the somata and dendrites of neurons, excitability profile of motoneuron pools, excitability thresholds of muscle afferents, modulation of EPSPs from afferents, and modifications on the descending control of spinal reflex networks involving synaptic and nonsynaptic mechanisms. These changes most likely occur simultaneously at differing strengths during the course of locomotor training, while adjustments made to the BWS, treadmill speed, and

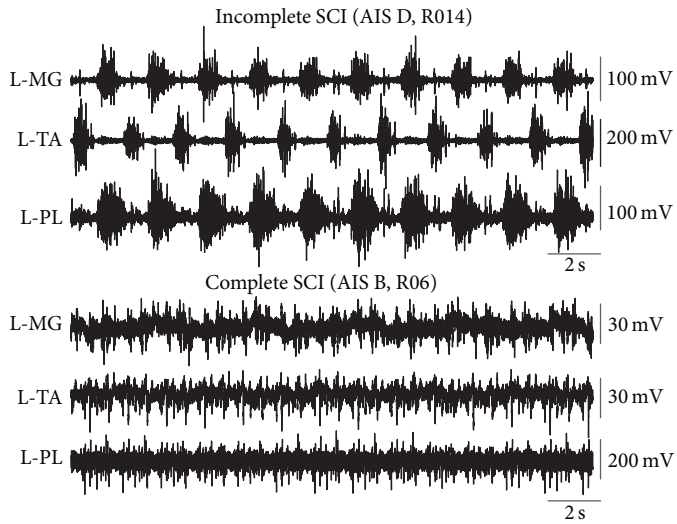

Figure 6: Motor activity after locomotor training in incomplete and complete SCI. Nonrectified electromyographic (EMG) activity from 10 consecutive steps of medialis gastrocnemius (MG), tibialis anterior (TA), and peroneus longus (PL) muscles from the left legs in one motor incomplete SCI subject (AIS D) and in one motor complete SCI subject (AIS B) during assisted stepping after locomotor training. Note in subject R014 that MG and PL occur in a reciprocal pattern with the TA, but distinctive EMG bursts are absent in subject R06.

leg guidance force during the course of training [54] affected the reorganization of spinal neuronal pathways integrating information about body loading and muscle stretch.

The restored soleus H-reflex depression during the swing phase in motor complete SCI we observed after locomotor training points towards three directions: (1) the soleus H-reflex depression during the swing phase cannot be attributed solely to reciprocal Ia inhibition between ankle antagonistic muscles because the physiological supraspinal control of Ia inhibitory interneurons (animal: [98, 153, 154]; human: [98, 155]) is greatly impaired in AIS A and AIS B, (2) reciprocal Ia inhibition can become functional after locomotor training even when descending control is impaired, and (3) functional behavior of reciprocal Ia inhibition is not depicted well in the EMG bursts during assisted stepping (see EMG bursts of AIS B subject in Figure 6). While all of these directions entail limitations with respect to the relevant contribution of reciprocal inhibition to the reflex depression during the swing phase of gait, it is possible that this change represents the capacity of intrinsic properties of the spinal cord to alter rhythmic motor activity after training [156].

Differences between right-left leg soleus H-reflex modulation changes suggest that the connections made by commissural spinal cord interneurons to motoneurons [156, 157] might have been affected differently by locomotor training in some patients compared to others. Commissural interneurons interact with 5-HT and GABA systems, form excitatory and inhibitory connections onto contralateral motoneurons at latencies consistent with monosynaptic and polysynaptic pathways, are under descending influence, and support locomotor rhythm generation in response to brainstem stimulation [157–163]. Further, midline lesions and photoablation affecting the axons of these neurons eliminate rhythmic ventral root bursting, alter the symmetry of ventral root bursts, and can eliminate rhythmic bursting [163], supporting the contribution of commissural interneurons to rhythmogenesis.

In healthy humans, ipsilateral posterior tibial nerve stimulation or knee extension joint rotation produces inhibition in both the contralateral soleus motoneurons and the reflex responses in the contralateral biceps femoris muscle, both being modulated according to the phase of walking [164–167]. Crossed postsynaptic inhibition in contralateral soleus motoneurons from ipsilateral groups I and II afferents at short latencies (3–7 ms), similar to those reported for the feline spinal cord, has recently been described for humans [168]. Further, activation of contralateral hip proprioceptors results in ipsilateral soleus H-reflex depression [169]. Taken altogether, differences between right-left leg H-reflex changes during the stance phase may thus represent plastic changes of commissural interneurons, but it is evident that there is a need for in-depth exploration of the physiological changes of commissural interneurons in people with SCI after locomotor training.

Changes in presynaptic and postsynaptic inhibition after locomotor training may be related to changes in the strength of the depolarization of muscle afferents [24, 170, 171] or may be the result of transformations in the intrinsic properties of spinal neurons and afferents after locomotor training. For example, in anesthetized chronic spinal cats there is an overall increase in Ia excitatory postsynaptic potentials (EPSPs) in ankle extensor motoneurons [172]. Plantar mechanoreceptors interact with presynaptic inhibitory interneurons, in humans at rest and in spinalized cats during fictive locomotion [54, 173]. Additionally, plantar mechanoreceptors evoke a phase-dependent modulation of primary afferent depolarization [24, 25], alter their effects on spinal motoneurons in spinalized cats after step training [173], and normalize the function of monosynaptic spinal reflexes while stepping in untrained spinal cord-injured patients [174]. Changes in the intrinsic

properties of spinal neurons after locomotor training are supported by the increased density of the glycinergic axonal terminals and decreased size of both glycinergic and GABAergic axon terminals in complete spinal trained transected rats compared to nontrained transected rats [175]. Because plasticity of the glycinergic system, which mediates inhibitory neurotransmission, occurs independently of supraspinal influence [176] and reciprocal Ia inhibition was potentiated after training in complete SCI at rest but recovery while stepping varied between patients, direct descending inputs on Ia inhibitory interneurons may not be a key source for neuroplasticity. However, this may be required for long-term support of inhibitory synaptic transmission and regulation of the depth of reciprocal Ia inhibition during locomotion.

The aforementioned are possible physiological changes in complete SCI, but the neuronal reorganization is more complex in incomplete SCI because neuronal structures above the lesion site might adapt the function and behavior of spinal neuronal circuitries known to control locomotor activity through remnant descending pathways. In incomplete SCIs, the plasticity of uninjured fibers plays an important role in functional recovery. In cats and monkeys, as little as 25% of remaining white matter tracts can allow for recovery of voluntary locomotor ability [177, 178], and a similar observation was found in humans following partial spinal cord transection to provide cancer pain relief [179]. Animal studies provide substantial direct evidence that, following a hemisection injury to the corticospinal tract, transected fibers sprout into cervical gray matter to communicate with propriospinal interneurons, whose propriospinal neurons then relay the motor command to distal lumbosacral motoneurons [180–185]. In rats, this corticopropriospinal connection can be enhanced pharmacologically [186] and with locomotor treadmill training [187]. Considerable indirect evidence suggests that this pathway is preserved in humans [188–192] and may be probed utilizing TMS and peripheral nerve stimulation [192, 193]. Future research on this pathway in humans with SCI and the effects of locomotor training is warranted.

6. Functional Consequences of Neuronal and Motor Plasticity

A question that arises is as follows: to what extent is plasticity of neuronal activity related to improvement of motor function in SCI patients? Although improvements in gait parameters were noted over the course of training, overground walking ability assessed by the 6-min walk test, and the number of sit-to-stand repetitions completed within 30 s, and the time needed to rise from a chair, walk for 3 m, and return to the chair were not improved after locomotor training in AIS C and AIS D [54]. Lack of changes in these walking ability variables could be related to (1) number of training sessions per participant, (2) small number of participants, (3) existence of nonresponders within the group, and (4) the fact that the 6-min walk test may not be sensitive enough to detect improvements in quality of walking of patients with SCI [194]. It may be also the case that the benefits seen with robotic-assisted treadmill training did not carry over into the task-specific overground testing of the 6 min walk test. However, previous literature involving locomotor training with as-needed manual assistance or with robotic-driven leg assistance in motor incomplete SCI demonstrated improvements in the walking index for SCI version II (WISCI-II) scale, overground walking speed, Berg balance scores, and 6 min walk test [146, 195–198]. Further, locomotor training improves lower extremity motor scores in both motor complete and motor incomplete SCIs [199, 200]. Taken altogether and including gaps in the literature, it is apparent that the time course of neuronal plasticity with corresponding motor recovery needs to be established.

7. Concluding Remarks

Locomotor training of persons with clinically complete, motor complete, or motor incomplete SCI induces reorganization of spinal neuronal networks that coincides with improvements in motor activity and decreased pathophysiological phenomena of the spasticity syndrome. However, to maximize recovery of motor function in patients with SCI, we need to utilize both established (i.e., locomotor training, spinal cord stimulation) and contemporary (i.e., brain controlled intraspinal microstimulation) technologies/interventions simultaneously and change the focus of our research questions from "feasibility" and "efficacy" to "what are the physiological mechanisms that make it work?" and "for which category of patients?" Additionally, while we need to better understand the physiological changes underlying locomotor training, especially of the uninjured fibers, research efforts should concentrate on providing strong scientific evidence when more than one intervention is utilized concomitantly. Over the course of treatment, physiological signals can be used to (1) probe recovery, (2) develop algorithms that may be used to define the approach of locomotor training for each patient, and (3) predict functional recovery. These approaches will enable the scientific and clinical community to develop more effective rehabilitation protocols.

Abbreviations

AIS:	American Spinal Injury Association Impairment Scale
BWS:	Body weight support
CNS:	Central nervous system
CPGs:	Central pattern generators
C-T interval:	Conditioning-test interval
EMG:	Electromyograms/electromyography
EPSPs:	Excitatory postsynaptic potentials
Hmax:	Maximal H-reflex
MG:	Medialis gastrocnemius
MEP:	Motor evoked potential
PAD:	Primary afferent depolarization
SCI:	Spinal cord injury
TA:	Tibialis anterior
TMS:	Transcranial magnetic stimulation.

Disclosure

This review does not represent the views of the NIH, Craig Neilsen Foundation, or NYSDOH.

Competing Interests

No conflict of interests, financial or otherwise, is declared by the authors.

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