Passive Exercise of the Hind Limbs after Complete Thoracic Transection of the Spinal Cord Promotes Cortical Reorganization

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Abstract

Physical exercise promotes neural plasticity in the brain of healthy subjects and modulates pathophysiological neural plasticity after sensorimotor loss, but the mechanisms of this action are not fully understood. After spinal cord injury, cortical reorganization can be maximized by exercising the non-affected body or the residual functions of the affected body. However, exercise per se also produces systemic changes – such as increased cardiovascular fitness, improved circulation and neuroendocrine changes – that have a great impact on brain function and plasticity. It is therefore possible that passive exercise therapies typically applied below the level of the lesion in patients with spinal cord injury could put the brain in a more plastic state and promote cortical reorganization. To directly test this hypothesis, we applied passive hindlimb bike exercise after complete thoracic transection of the spinal cord in adult rats. Using western blot analysis, we found that the level of proteins associated with plasticity – specifically ADCY1 and BDNF – increased in the somatosensory cortex of transected animals that received passive bike exercise compared to transected animals that received sham exercise. Using electrophysiological techniques, we then verified that neurons in the deafferented hindlimb cortex increased their responsiveness to tactile stimuli delivered to the forelimb in transected animals that received passive bike exercise compared to transected animals that received sham exercise. Passive exercise below the level of the lesion, therefore, promotes cortical reorganization after spinal cord injury, uncovering a brain-body interaction that does not rely on intact sensorimotor pathways connecting the exercised body parts and the brain.

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Introduction

Limb amputation, damage to peripheral nerves and spinal cord injury can alter the somatotopic organization of the primary somatosensory cortex [1,2,3,4,5,6]. Cortical reorganization sub-serves functional recovery [7,8,9,10,11,12] but excessive reorganization can have pathological consequences, such as phantom limb sensations [13,14] and neuropathic pain [15,16,17,18]. It is therefore important to fully understand the mechanisms of cortical reorganization and to develop optimal strategies to modulate it after deafferentation [19].

Cortical reorganization after somatosensory deafferentation can lead to the enlargement of cortical representations of intact body areas into cortical representations of deafferented body areas. A conceptually similar cortical enlargement is induced by increasing the activity of a cortical area through enhanced sensory experience [20]. A natural strategy to maximize cortical reorganization after somatosensory deafferentation is therefore to increase the activity of the intact cortex by exercising the non-affected body or the residual functions of the affected body, as investigated in previous works in rat models [9,21,22] and in patients with spinal cord injury [23,24,25,26]. An alternative strategy would be to put the overall cortex in a more plastic state, e.g. using systemic drugs or other manipulations that generically promote cortical plasticity [27,28,29].

A possible intriguing way to generically promote cortical plasticity is indirectly suggested by exercise therapies employed after spinal cord injury, which are often applied to the affected body in order to improve functions below the level of the lesion [30]. For example, passive exercise of the lower limbs after spinal cord injury has been shown to reduce spasticity [31,32,33,34], reduce the rate of bone density loss [35,36] and reduce lower limb blood pooling [37]. Interestingly, passive exercise also produces the systemic effects that are typical of exercise per se, such as increases in cardiovascular fitness [38], improved circulation [39] and neuroendocrine changes [40]. These systemic effects of exercise are known to have a great impact on brain function [41], as they contribute to stimulate neurogenesis and to increase the levels of growth factors such as BDNF, thereby promoting brain plasticity.
Even though at first glance it might appear counter-intuitive, it is reasonable to suggest that exercise below the level of the lesion after complete spinal cord injury might promote cortical reorganization.

In order to directly test this hypothesis, we applied passive hindlimb bike exercise after complete thoracic transection of the spinal cord in adult rats. The rationale for employing the thoracic transection rat model of spinal cord injury is that it has been shown to produce cortical reorganization associated with alterations of BDNF regulation in the brain [46,47]. The rationale for employing passive hindlimb bike exercise is three-fold: (1) it is well established in rat models of spinal cord injury [48], (2) it has direct translational relevance for spinal cord injury patients, and (3) it specifically confines the exercise below the level of the lesion. We first investigated whether passive hindlimb bike training after thoracic transection of the spinal cord affected the expression in the somatosensory cortex of proteins associated with plasticity (Fig. 1A). We then verified that these changes in markers of cortical plasticity were associated with increased neurophysiological reorganization of the deafferented hindlimb cortex (Fig. 1B).

**Results**

**Hindlimb Bike Exercise after Thoracic Transection of the Spinal Cord Induces Changes in the Levels of Proteins Associated with Plasticity in the Somatosensory Cortex**

We performed western blot analysis to assess the levels of plasticity-related proteins in the somatosensory cortex of 32 rats, divided in 5 groups (Fig. 1A): one group of normal animals (n = 6); two groups of ‘bike-exercise’ transected animals, which received either 1 week (n = 8) or 8 weeks (n = 5) of hindlimb bike exercise after complete thoracic (T9/T10) transection of the spinal cord; two groups of ‘sham-exercise’ transected animals, which received either 1 week (n = 7) or 8 weeks (n = 6) of sham bike exercise after the spinal cord transection. Passive hindlimb bike exercise or sham bike exercise started the week after the spinal transection (two 30-min sessions per day on Monday, Wednesday and Friday) and animals were sacrificed within one hour after the last session. This exercise protocol is based on previous work showing that at least 1 week of hindlimb bike exercise after spinal cord injury is necessary to induce significant plastic changes in the spinal cord below the level of the lesion (Keeler et al., 2009). We focused on the following 6 proteins known to be involved in neuronal plasticity in the brain: ADCY1, BDNF and its receptor trkB, GAP43, LINGO-1 and p35 [49,50,51,52,53,54,55,56,57,58,59,60,61,62]. Data

![Figure 1. Experimental protocol.](https://www.plosone.org/doi/10.1371/journal.pone.0054350.g001)
increase tended to be stronger in animals that received 8 weeks plasticity-related proteins in the somatosensory cortex of our bike exercise after spinal transection increased the levels of exercise' and 'sham-exercise' animals (Fig. 2B). Passive hindlimb after spinal transection by jointly analyzing the data from 'bike-somatosensory cortex.

The levels of plasticity-related proteins in the rat primary protein-dependent (interaction exercise in the somatosensory cortex after complete spinal cord injury. p = 0.0141). These results show that exercise below the lesion way ANOVA, exercise: F = 10.7, p = 0.0034) and BDNF (F = 7.2, p = 0.0003). More specifically, the proteins that appeared more sensitive to hindlimb bike exercise were ADCY1 (follow-up two-way ANOVA, ADCY1: F = 27.4, p<0.0001; BDNF: F = 5.9, p = 0.0129; trkB: F = 0.93, p = 0.41; GAP43: F = 19.5, p<0.0001; LINGO-1: F = 3.6, p = 0.0524; p35: F = 11.2, p = 0.0009). Specifically, 1 week after spinal transection there was only a marginal increase in the level of ADCY1 (post-hoc: p = 0.0505) and BDNF (p = 0.0780), but 8 weeks after spinal transection all proteins but trkB were altered compared to 1 week: ADCY1, BDNF and p35 were decreased (p<0.0001, p = 0.0035, p = 0.0007), whereas GAP43 and LINGO-1 were increased (p<0.0001, p = 0.0279). ADCY1, GAP43, LINGO-1 and p35 were also significantly different 8 weeks after spinal transection compared to normal animals (p = 0.0001, p<0.0001, p = 0.0410, p = 0.0010). These data show that spinal cord injury per se affects the levels of plasticity-related proteins in the rat primary somatosensory cortex.

We then investigated the effect of passive hindlimb bike exercise after spinal transection by jointly analyzing the data from 'bike-exercise' and 'sham-exercise' animals (Fig. 2B). Passive hindlimb bike exercise after spinal transection increased the levels of plasticity-related proteins in the somatosensory cortex of our animals (three-way ANOVA, exercise: F = 8.7, p = 0.0076). This increase tended to be stronger in animals that received 8 weeks compared to 1 week of bike exercise after spinal cord transection (interaction exercise × weeks: F = 3.2, p = 0.0868), and was highly protein-dependent (interaction exercise × protein: F = 5.1, p = 0.0003). More specifically, the proteins that appeared more sensitive to hindlimb bike exercise were ADCY1 (follow-up two-way ANOVA, exercise: F = 10.7, p = 0.0034) and BDNF (F = 7.2, p = 0.0141). These results show that exercise below the lesion induces changes in the levels of proteins associated with plasticity in the somatosensory cortex after complete spinal cord injury.

**Hindlimb Bike Exercise after Thoracic Transection of the Spinal Cord Enhances the Neurophysiological Reorganization of the Deafferented Hindlimb Cortex**

In order to verify the functional relevance of the above changes in proteins associated with cortical plasticity, we directly assessed whether passive hindlimb bike exercise after spinal cord transection affected the neurophysiological reorganization of the deafferented hindlimb cortex. To this end, we studied two additional groups of 'bike-exercise' transected animals (n = 5) and 'sham-exercise' transected animals (n = 4) (Fig. 1B). These new groups received passive hindlimb real or sham bike exercise for 8 weeks after transection. One week after the last bike session (i.e. 9 weeks after the spinal transection), animals were anesthetized to perform an acute, single-neuron mapping study in the deafferented hindlimb cortex. Similar to our previous study [9], high-impedance single tungsten microelectrodes were inserted into the hindlimb primary somatosensory cortex with multiple penetrations (4–6 per animal), and single neurons from the supragranular (bike: n = 142; sham: n = 64) granular (bike: n = 95; sham: n = 65) and infragrанныar (bike: n = 253; sham: n = 121) layers were isolated and identified as either responsive or not responsive to cutaneous stimulation of the forelimbs (Fig. 3A, Table 1).

In sham-exercise transected animals, the percentage of cells stereotaxically located in the deafferented hindlimb cortex that responded to stimulation of the intact forelimb was extremely low: 1.3% considering all cells (n = 233), 0% in the supragranular layers, 3.2% in the granular layers, and <1% in the infragranular layers. In bike-exercise transected animals, the percentage of cells stereotaxically located in the deafferented hindlimb cortex that responded to stimulation of the intact forelimb dramatically increased at all cortical layers: 11.6% considering all cells (n = 490, two-proportion test: p<0.0001), 14.8% in the supragranular layers (p = 0.0019), 20% in the granular layers (p = 0.0030) and 10.7% in the infragranular layers (p = 0.0014). Essentially the same results were obtained with a two-way ANOVA, considering each track as an independent sample (Fig. 3b). The data for each individual animal are given in Table 1. The completeness of the spinal cord transection was histologically verified in all animals at the end of the study (Fig. 3C). There were no differences (t-test, p = 0.53) in the rostrocaudal extent of the lesion between animals that received bike exercise (6.2±0.6 mm) and animals that received sham exercise (6.0±0.4 mm) after the spinal transection.

These results represent the electrophysiological counterpart to the exercise-induced protein regulation reported in the previous section and overall show that exercise below the level of the lesion promotes cortical reorganization after complete spinal cord injury.

**Discussion**

We previously showed that exercise (treadmill) above the level of the lesion promotes cortical reorganization in adult rats spinalized as neonates [9,22]. The main result of the present work is that passive exercise below the level of the lesion promotes cortical reorganization in adult rats spinalized as adults. Specifically, we show that passive hindlimb bike exercise after thoracic transection of the spinal cord induces changes in the levels of proteins associated with plasticity in the sensorimotor cortex and enhances the neurophysiological reorganization of the deafferented hindlimb cortex.

**Hindlimb Bike Exercise after Thoracic Transection of the Spinal Cord Induces Changes in the Levels of Proteins Associated with Plasticity in the Sensorimotor Cortex**

Spinal cord transection alone ('sham-exercise' transected animals compared to normal animals) had a profound impact on the levels of plasticity-related proteins in the somatosensory cortex. On the one hand, ADCY1 and BDNF tended to increase 1 week after spinal transection and significantly decreased 8 weeks after spinal transection – even below normal levels for ADCY1, together with p35. On the other hand, GAP43 and LINGO-1 increased 8 weeks after the spinal transection.

BDNF is critical for neuronal growth and differentiation (Huang and Reichardt, 2001) and for the establishment of long-term synaptic plasticity [55,56], whereas LINGO-1 promotes neuronal survival and branching [54,58] but negatively regulates central myelination and axonal sprouting after spinal cord injury [52,53]. These two proteins have been previously implicated in brain reorganization after spinal cord injury, with complex temporal patterns of up-regulation and down-regulation [46,47,63,64]. In the only previous study specifically investigating the neocortex, Endo et al. [46] used in situ hybridization to quantify changes in the levels of BDNF and LINGO-1 mRNAs after spinal cord
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A

Effect of spinal cord transection (TX)

| Protein   | Normal | Sham-bike 1-week post TX | Sham-bike 8-weeks post TX |
|-----------|--------|---------------------------|---------------------------|
| ADCY1     | 100    | 150                       | 200                       |
| BDNF      | 150    | 200                       | 250                       |
| trkB      | 200    | 250                       | 300                       |
| GAP43     | 150    | 200                       | 250                       |
| LINGO-1   | 200    | 250                       | 300                       |
| p35       | 200    | 250                       | 300                       |

B

Effect of passive hindlimb bike exercise

1 week post TX

| Protein   | Normal | Sham-exercise | Bike-exercise |
|-----------|--------|---------------|---------------|
| ADCY1     | 100    | 150           | 200           |
| BDNF      | 150    | 200           | 250           |
| trkB      | 200    | 250           | 300           |
| GAP43     | 150    | 200           | 250           |
| LINGO-1   | 200    | 250           | 300           |
| p35       | 200    | 250           | 300           |

8 weeks post TX
After spinal cord transection in the absence of passive exercise, LINGO-1 mRNA was reduced in the deafferented hindlimb cortex and the adjacent forelimb cortex [46], while in our study protein levels are unchanged 1 week after transection and are even increased 8 weeks after transection. Endo et al. measured mRNA levels in discrete cortical sub-regions and the observed changes involved only a subset of these regions. Because we extracted total protein from the whole sensorimotor cortex, it is possible that changes affecting only part of it were diluted and hence not detectable. A more intriguing possibility, however, is that post-transcriptional and posttranslational modifications are responsible for the uncoupling of LINGO-1 mRNA and protein levels. One important mechanism is represented by micro-RNAs (miRNA), which affect protein translation by binding to their target mRNA and effectively controlling protein levels (see for example [65]). Moreover, LINGO-1 proteins form very stable membrane-bound homotetramers that act as a scaffold with multiple sites available for protein-protein interactions [66]. It is therefore likely that

### Figure 2. Changes in the levels of proteins associated with plasticity in the somatosensory cortex.

(A) Effect of spinal cord transection alone (‘sham-exercise’ transected animals compared to normal animals). (B) Effect of passive hindlimb exercise. Representative western blot bands are shown for each case. Error bars indicate standard deviations. (*) = p<0.1, * = p<0.05, ** = p<0.01.
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### Figure 3. Neurophysiological reorganization of the somatosensory cortex.

(A) Cartoon of the hindlimb (HL), forelimb (FL) and trunk (TR) areas in the primary somatosensory cortex in stereotaxic coordinates (left), and visual representation of responsive (filled squares) and unresponsive (empty squares) tracks in the electrophysiological study for sham-exercise transected animals (center) and bike-exercise transected animals (right). (B) Percentage of responding cells per track in sham exercise transected animals and bike-exercise transected animals, separated by cortical layer. supragranular (SP), granular (G) and infragranular (IG). In bike-exercise transected animals, the percentage of cells stereotaxically located in the deafferented hindlimb cortex that responded to stimulation of the intact forelimb dramatically increased (two-way mixed ANOVA, exercise: F = 7.54, p = 0.0091) at all cortical layers (interaction exercise × cortical layer: F = 0.88, p = 0.42). Error bars indicate standard deviations. (C) Nissl/myelin staining of the spinal cord of a representative animal that received bike exercise after spinal cord transection. No cell bodies or axons were observed in the transection site (marked by the black lines). The extent of this representative injury was comparable with the other injuries. Scale bar: 1.0 mm.
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numerous post-translational modifications may affect its subcellular targeting, processing and degradation rate, hence affecting the net protein level, at least in part independent of its mRNA levels. Since ours is the first study to address changes in cortical LINGO-1 protein levels after spinal cord transection, such possibilities cannot be ruled out and suggest interesting new lines of investigation on the mechanisms of cortical reorganization after spinal cord injury and new therapeutic options.

Similar considerations hold for the difference between mRNA and protein levels of BDNF. Endo et al. [46] showed long-lasting up-regulation of BDNF mRNA levels in transected rats; on the contrary, 8 weeks after transection we observed increased BDNF levels only in transected animals under bike exercise regimen, while in non-exercising transected animals BDNF levels were actually decreased. Again, it is possible that post-transcriptional and post-translational mechanisms uncoupled the regulation of protein levels from mRNA levels, causing the discrepancy between the two studies. Our study further shows that other proteins known to be associated with different aspects of morphological and functional plasticity are regulated after spinal cord transection. These include ADCY1, which is involved in long-term synaptic plasticity, learning and memory [51]; GAP43, which is critical for the normal establishment of ordered topography in the developing barrel cortex [67,68] and mediates functional recovery after lesions in the adult central nervous system [21,57,60]; p35, which is regulated in neurodegeneration [61], has an important neuroprotective role in vivo [49] and contributes to different forms of synaptic plasticity [62]. A complex network of proteins is therefore involved in cortical reorganization after spinal cord injury.

Passive hindlimb bike exercise consistently modulated only the levels of two of the above proteins (increasing them): BDNF and ADCY1. On the one hand, exercise has been associated with increased brain levels of BDNF in several previous studies [45,69,70,71]. Our data suggest that exercise-dependent modulation of BDNF levels in the brain is not necessarily mediated by the sensorimotor system, but could also be mediated by cardiovascular and/or neuroendocrine mechanisms [41]. On the other hand, the involvement of ADCY1 is intriguing because of the critical role of this protein in multiple forms of plasticity, including the formation of cortical barrels [72,73,74], experience-dependent strengthening of whisker thalamic relay synapses [51] and the refinement of retinal projection maps [75,76] during development. It therefore seems possible that molecular mechanisms mediating cortical plasticity during development might be reactivated in adulthood after a massive injury such as spinal cord injury. The demonstration that cortical ADCY1 has a causal role in central sensitization and behavioral allodynia after peripheral nerve injury [77] and, more recently, that it affects behavioral sensitization by controlling PKMz levels independently of transcription [78] has important clinical implications, as its regulation may represent the mechanism underlying the emergence and maintenance of pain after spinal cord injury. Our data indicate that passive exercise, even in absence of sensorimotor spinal connections, can modulate these molecular mechanisms and suggest possible novel targets to selectively control central pain after spinal cord injury.

**Hindlimb Bike Exercise after Thoracic Transection of the Spinal Cord Enhances the Neurophysiological Reorganization of the Deafferented Hindlimb Cortex**

The altered levels of plasticity-related proteins were not sufficient – without passive bike exercise – to induce a detectable electrophysiological reorganization: in ‘sham-exercise’ transected animals, the probability of neurons in the deafferented hindpaw cortex to respond to forepaw/forelimb tactile stimulation 8 weeks after spinal transection was extremely low, as expected from normal animals under deep anesthesia [9]. This observation is consistent with the absence of cortical reorganization reported by Jain et al. (1995) based on the responses to tactile stimuli after dorsal columns section [79], and is not in contradiction with the reorganization observed with functional magnetic resonance imaging (fMRI), based on the responses to high-intensity electrical stimuli after complete transection of the spinal cord [46,80]. In fact, a similar dependence on stimulus-intensity is observed immediately after spinal cord injury [81], when the responses to low-intensity electrical stimuli are not sufficiently consistent to uncover cortical reorganization, but cortical reorganization is revealed by high-intensity stimuli. The dependence of long-term cortical reorganization after spinal cord injury on stimulus-intensity, possibly due to differences between dorsal columns inputs and spinthalamic inputs [82], is beyond the scope of the present study and will deserve further investigation.
The data presented here show that passive bike training after spinal cord transection is indeed sufficient to increase the neural responsiveness of the deafferented hindpaw cortex to tactile stimuli delivered to the forepaw/forelimb under deep anesthesia. This increase in responsiveness was observed at all cortical layers – differently from our previous experiments with forelimb training in neonatally spinalized rats, in which we did not observe changes in the granular layer [9]. Interestingly, a similar increase in responsiveness of the hindpaw cortex to tactile stimuli delivered to the forepaw/forelimb can be obtained by decreasing the depth of anesthesia [83]. This suggests that the exercise-dependent increase of neural responsiveness might be mediated not only by axonal sprouting [84,85,86], but also by the alteration of the dynamic equilibrium of dendritic spines and axonal boutons between interneurons and pyramidal neurons [10,87], possibly as a compensatory process to maintain the delicate cortical balance between excitation and inhibition [88], or even by the alteration of more subtle intrinsic properties of cortical neurons. Combining our electrophysiological and molecular observations, even though we did not show a causal link, it is tempting to suggest that BDNF and ADCY1 could be particularly critical for the reorganization of the cortical architecture dedicated to the processing of dorsal column inputs. Nonetheless, because the neurophysiological reorganization we observed in the deafferented hindlimb cortex was not layer-specific, we cannot exclude a subcortical contribution to the cortical reorganization [12,89,90,91,92,93]. We speculatively propose that the cardiovascular and neuroendocrine systemic effects produced by passive exercise can boost or simply accelerate the cascade of plasticity-related events that are already occurring in the massively deafferented somatosensory cortex after spinal cord injury.

Independently of the exact mechanism, our data show that passive exercise of the lower limbs after complete transection of the spinal cord is indeed sufficient to promote cortical reorganization.

Conclusions

We showed that passive exercise below the level the lesion in rats both alters plasticity-related proteins in the sensorimotor cortex and promotes an electrophysiologically measurable reorganization in the deafferented hindlimb cortex after complete spinal cord injury. These results support the important role of physical exercise for learning and plasticity, suggest that exercise should be considered – among its other effects – as a rehabilitation strategy that generically promotes cortical plasticity, and uncover a brain-body interaction that does not rely on intact sensorimotor pathways connecting the exercised body parts and the brain.

Materials and Methods

A total of 41 adult Sprague-Dawley were used in this study. The different experimental groups are indicated in Figure 1. In 32 animals we used western blot analysis to assess whether passive hindlimb bike training after thoracic transection of the spinal cord affected the expression in the somatosensory cortex of proteins associated with plasticity (Fig. 1A). In 9 additional animals we performed single-neuron mapping in the deafferented somatosensory cortex at the end of the study to electrophysiological verify the presence of exercise-dependent cortical reorganization (Fig. 1B). All procedures were performed under the guidelines of the National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of Drexel University.

Spinal Cord Transection

Animals received a complete thoracic transection of the spinal cord with procedures that are similar to our previous studies [81,94]. Briefly, adult female Sprague-Dawley (Charles River) rats were anesthetized with isoflurane (2–3% with oxygen) and the spinal cord was exposed by laminectomy at the T8-/T9 level. The cord was transected with iridectomy scissors followed by aspiration of tissue within the cavity. A collagen matrix, Vitrogen, was injected into the site of the transection to fill the cavity. The muscle and skin were sutured in layers with 5-0 silk. Animals were then warmed, and when they became active, returned to their home cages. Bladders were manually expressed until the animals were able to void on their own. Animals were housed under a 12 h light/dark cycle (lights on at 07:00) with free access to food and water.

Passive Hindlimb Bike Exercise

As in previous studies, hindlimb bike exercise consisted of two 30-minute sessions with a 10-minute break, 3 days per week (M, W and F) [48]. This exercise regimen involved suspending the rats on a sling with the hindlimbs hanging down and the hind feet strapped onto the pedals of a bicycle-type device that was driven by a motorized belt. The exercise consisted of a pedaling motion that flexed one limb while extending the other without over-stretching the limbs. Cycling speed was 0.5 Hz. This was, therefore, a passive exercise of the hindlimbs only. Animals did not watch the exercised hindlimbs. Sham exercise consisted of placing the animals on the bike for 70 minutes, 3 days per week, but without moving the pedals.

Western Blot Analysis

Immediately following their last exercise session, animals were anesthetized with isoflurane and then decapitated. The brain and spinal cord were immediately removed, sliced on ice into 2 mm thick coronal slices and flash-frozen on dry ice. We focused on the sensorimotor cortex. Each tissue sample was placed into a FastPrep 120 tube (MP Biomedicals, Irvine, CA) in cold homogenization buffer and homogenized with FastPrep 120 for 40 sec, centrifuged at 1000 g for 10 min, transferred into 1.5 ml microfuge tubes and centrifuged at 20,000 g for 15 min. Protein concentration was assessed by using BioRad DC protein assay method (BioRad, Hercules, CA). Homogenates were fast frozen on dry ice and stored at −80°C until further processing. Proteins were run on pre-cast 26 well SDS gels (BioRad) either 10% or 4–15% gradient, depending upon expected protein molecular weight. Protein standards (Biorad) were used to determine protein size. For each antibody an initial amount of 30 μg total protein/well was loaded at four different dilutions (1:1, 1:2, 1:5, 1:10). Once the optimal dilution was identified, it was held constant across all samples. Gelled proteins were transferred to 0.2 μ nitrocellulose membrane using 1 Blot system (Invitrogen). After transfer the membrane was incubated for 1 hr at room temperature in Li Cor western block (Li Cor, Lincoln NE) solution and then overnight at 4°C in primary antibody solution (Li Cor Block +0.1% Tween) with one of the following primary antibodies: adenylyl cyclase isoform 1 (ADCY1; Bioworld Bi2391), brain derived neurotrophic factor (BDNF; Santa Cruz SC-20981), tyrosine kinase B (trkB; Millipore 07–225), 43 kD growth associated protein (GAP43; Epitomics 2259-1), leucine-rich repeat and Ig domain-containing Nogo receptor-interacting protein (LINGO-1; Santa Cruz SC-134597), Cyclin-dependent kinase 5 activator 1 (p35; Bioworld Bi2065).
Antibody anti Beta-actin (Sigma A5441) was added to the incubation solution for endogenous control. After incubation in primary antibodies the blots were rinsed 3×10 min in PBS +0.1% Tween and incubated 1 hr and RT in block solution containing the following secondary antibodies developed in goat: anti-rabbit IgG IRDye800DX-conjugated, cat #611-130-122 and anti-mouse IgG IRDye700DX-conjugated, cat #610-132-121 (Rockland, Gilbertsville, PA) and HRP conjugated streptactin (BioRad 161-0382), then washed 3×10 min in PBS+TWEEN and rinsed in PBS. Processed membranes were exposed on Li-Cor Odyssey infrared imager at both 700 and 800 nm wavelengths for appropriate exposure times. All protein level values were obtained on the gel image by subtracting the background intensity from the signal for the protein of interest. The same procedure was performed for the control protein (Beta-actin) and each blot was normalized to its corresponding actin value. For statistical and illustration purposes, all western blot data for each animal and each protein were divided by the average value of the corresponding protein in the normal animals group and expressed as percentage. Note that in this way the average value for normal animals is 100% but the variability between animals (see error bars) is maintained.

Electrophysiology

Acute single-neuron mapping of the deafferented hindpaw cortex was performed at the end of the study with similar techniques as in our previous study [9]. Rats were anesthetized by intraperitoneal injection of urethane anesthesia (1.5 g/Kg) and placed in a stereotoxic frame. Craniotomies were performed over either the right or left cortex to expose the hindlimb representations in the primary somatosensory cortex. The stereotoxic coordinates for hindlimb craniotomy were from 0 to 3 mm posterior to bregma and from 2 to 3 mm lateral [95]. Electrode penetrations were defined using the stereotoxic coordinates for the hindlimb somatosensory cortex [96,97]. For all animals, the anesthesia level was maintained at Stage III-4 [98].

A high impedance (10 MΩ) tungsten microelectrode (FHC, Inc, Bowdoin, ME) was mounted on a stereotoxic electrode manipulator. A ground wire was inserted into the brain adjacent to the craniotomies. The microelectrode was then moved to the anterior-posterior and medial-lateral coordinates that defined a predetermined location above the hindlimb somatosensory cortex, and lowered, perpendicular to the surface of the brain, to penetrate the dura and pia. The microelectrode was then slowly inserted into the brain.

The signals from the microelectrode were continuously monitored on the oscilloscope and audio speakers as the electrode was lowered. When a neuron was encountered, the dorsal/ventral coordinates of the cell were noted. Two experimenters then determined whether the identified cell responded to sensory stimulation. The first experimenter, with knowledge of the electrode placement, used wooden probes to touch the hair/skin on the forelimb and shoulder. The second experimenter, blind to the position of the electrode and treatment group of the animal, determined if the cell responded to the stimulus, predominately by listening for a change in firing rate. If the cell did not modulate its firing rate in response to the stimulation, the cell was noted as negative. If the cell did modulate its firing rate, the cell was noted as positive. If the cell was noted as positive, then the receptive field of the cell was identified by tapping locations on the body rostral to the level of the injury. Stimulation of any body surface that modulated the cell’s firing rate was considered part of the cell’s receptive field. To ensure that tapping forces between animals and across sites were uniform, the responses elicited by the wooden probe were periodically compared to responses elicited by von Frey filaments to calibrate the stimulus applied by the wooden probe. The stimulation consisted of pressing a filament gently against the skin, perpendicular to its surface until the filament bent 90 degrees. This procedure was done 5 times for each filament and skin site, to ensure reproducibility of the results. The filament necessary to elicit a response similar to the wooden probe was noted and compared across animals and locations. The filaments required to produce an equivalent response ranged from 4.31 (bending force of 2 g) to 4.93 (bending force of 8 g) across animals and animal groups. There were no identifiable differences in the distribution of filaments used between the groups.

After a cell was characterized, the microelectrode was moved at least 50 microns deeper (with respect to the cortical surface) before another cell could be identified in the same penetration to ensure a new cell was encountered. For every cell identified, the stereotoxic coordinates of the microelectrode position were identified allowing us to evaluate neuronal responsiveness for each layer of the cortex. To minimize tissue damage and its possible effects on cell responsiveness during later penetrations, no more than 6 penetrations were performed per animal.

Perfusion and Histological Processing of the Brain and Spinal Cord

At the end of the mapping sessions, the rats were perfused transcardially with buffered saline, followed by buffered 2% paraformaldehyde, and then by buffered 2% paraformaldehyde containing 10% sucrose. The cortex was removed and flattened between two glass slides. The tissue was cryoprotected in 30% sucrose and sectioned (70 microns parallel to the pial surface) frozen on a sliding microtome. Series of these sections from the cortex were stained for cytochrome oxidase (CO) activity [99]. Spinal cords were removed and placed in phosphate buffer containing 30% sucrose for 72 h. Specimens were frozen in OCT and sectioned on a freezing microtome at 20 μm. The transection segments of the spinal cords were sectioned parasagittally, and alternate sections were Nissl-myelin stained. The resulting sections were examined under a microscope to confirm completeness of the transection.

Statistical Analyses

To assess the effects of spinal cord transection alone on plasticity-related proteins in the sensorimotor cortex, we compared ‘sham-exercise’ transected animals to normal animals by entering the data into a two-way independent-measures analysis of variance (ANOVA). The factors were ‘protein’ (ADCY1, BDNF, trkB, GAP43, LINGO-1 and p35), and ‘time from lesion’ (normal, 1 week after transection, 8 weeks after transection). In case of significant interaction, we performed follow-up one-way ANOVAs on individual proteins, followed by Fisher’s post-hoc test (as in Endo et al., 2007).

To assess the effects of passive hindlimb bike exercise after spinal transection on plasticity-related proteins in the sensorimotor cortex, the data from ‘bike-exercise’ and ‘sham-exercise’ animals were entered into a three-way independent-measures analysis of variance (ANOVA). The factors were ‘exercice’ (bike or sham), ‘protein’ (ADCY1, BDNF, trkB, GAP43, LINGO-1 and p35) and ‘weeks’ (1 or 8 weeks). In case of significant interactions, we performed follow-up two-way ANOVAs on individual proteins.

To assess the effects of passive hindlimb bike exercise after spinal transection on the electrophysiological reorganization of the deafferented cortex, we performed two types of analyses. First, the percentage of cells stereotaxically located in the deafferented hindlimb cortex that responded to stimulation of the intact forelimb were compared between bike animals and sham animals.
using two-proportion tests, separately for all cells and for each cortical layer. Second, the percentage of cells per track stereotaxically located in the deafferented hindlimb cortex that responded to stimulation of the intact forelimb were entered into a two-way mixed ANOVA, considering each track as an independent sample. The facilitatory factors were exercise (bik e or sham, independent measures) and cortical layer (supragranular, granular, infragranular, repeated measures).

Results were considered significant at p<0.05.

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Author Contributions

Conceived and designed the experiments: KAM JS AG. Performed the experiments: KAM JS AG EBK. Analyzed the data: KAM AG GF. Contributed reagents/materials/analysis tools: KAM JS AG. Wrote the paper: KAM AG GF.

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