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Reaching training in rats with spinal cord injury promotes plasticity and task specific recovery

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In the current study we examined the effects of training in adult rats with a cervical spinal cord injury (SCI). One group of rats received 6 weeks of training in a single pellet reaching task immediately after injury, while a second group did not receive training. Following this period changes in cortical levels of BDNF and GAP-43 were analysed in trained and untrained animals and in a group with training but no injury. In another group of rats, functional recovery was analysed in the reaching task and when walking on a horizontal ladder. Thereupon, the cortical forelimb area was electrophysiologically examined using micro-stimulation followed by tracing of the lesioned corticospinal tract (CST). We found that trained rats improved substantially in the reaching task, when compared to their untrained counterparts. Trained rats however, performed significantly worse with their injured forelimb when walking on a horizontal ladder. In parallel to the improved recovery in the trained task, we found that the cortical area where wrist movements could be evoked by micro-stimulation expanded in trained rats in comparison to both untrained and uninjured rats. Furthermore, collateral sprouting of lesioned CST fibres rostral to the injury was increased in trained rats. Post-injury training was also found to increase cortical levels of GAP-43 but not BDNF. In conclusion we show that training of a reaching task promotes recovery of the trained task following partial SCI by enhancing plasticity at various levels of the central nervous system (CNS), but may come at the cost of an untrained task.

Keywords: spinal cord injury; plasticity; rehabilitation; training, cortical reorganization

Abbreviations: SCI = spinal cord injury; CST = corticospinal tract; CNS = central nervous system; RST = rubrospinal tract; CPG = central pattern generator

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Introduction

Spinal cord injury (SCI) is a devastating event, resulting in the loss of motor and sensory functions of the body innervated by the spinal cord below the injury site. Recovery following SCI is limited because severed axons of the central nervous system (CNS) are unable to regenerate spontaneously, and therapeutic strategies aimed at promoting significant regeneration are currently unavailable (Schwab *et al.*, 2006). Nevertheless, depending on the severity of the injury, some recovery of sensory and motor function will occur over the weeks following the injury in patients or animal models of SCI. The mechanisms of this recovery may include axonal sprouting, synaptic rearrangements and changes in cellular properties in spared neuronal circuits rostral and caudal to the lesion (Bennett *et al.*, 2001; Fouad *et al.*, 2001; Raineteau and Schwab, 2001; Weidner *et al.*, 2001; Bareyre *et al.*, 2004; Edgerton *et al.*, 2004; Ballermann and Fouad, 2006; Rossignol, 2006), often referred to as plasticity. A successful approach to further promote plasticity and recovery following SCI is intensive rehabilitative training. For example, treadmill training with partial weight support has been found to promote significant locomotor recovery in patients (Wernig and Muller, 1992; Dietz *et al.*, 1994) and animal models (Lovely *et al.*, 1986; Barbeau *et al.*, 1987). The underlying mechanisms involve the up-regulation of neurotrophic factors, changes in the spinal networks orchestrating the locomotor pattern (Gomez-Pinilla *et al.*, 2002; Griesbach *et al.*, 2004) and recently, increases of corticospinal tract (CST) function have also been reported to be involved (Thomas and Gorassini, 2005). Although the success of

treadmill training following SCI is striking, comparable intensive rehabilitative strategies for hand and arm function for patients with cervical SCI (accounting for ~50% of SCIs) have not been systematically explored. Support for the possible therapeutic benefit of training hand and arm function following CNS injuries, comes from the field of stroke and traumatic brain injury. Here, constraint-induced movement therapy [CIMT (reviewed in Taub *et al.*, 1999)] has established itself (in animal models and in the clinic) as a successful approach to promote functional rearrangements in corticospinal pathways and ultimately to promote functional recovery (Ostendorf and Wolf, 1981; Kleim *et al.*, 2004; Ramanathan *et al.*, 2006). Considering that following cervical SCIs even a small recovery in hand function could result in significant benefits in the quality of life, we examined the effects of training hand function in an animal model of SCI. To do so, we utilized the single pellet reaching task (Whishaw and Gorny, 1994) in rats with a cervical SCI, ablating the dorsal components of the CST and the majority of the rubrospinal tract (RST). Our analysis focussed on adaptations/plasticity in the motor cortex and its descending tract (i.e. the CST) and the functional benefits (on motor ability) following 6 weeks of intensive training. We found that this training up-regulated cortical levels of the growth-associated protein (GAP) 43, promoted sprouting of lesioned CST fibres above the injury, restored cortical maps and most importantly substantially promoted recovery in the trained task, but impaired the performance of a related but untrained task.

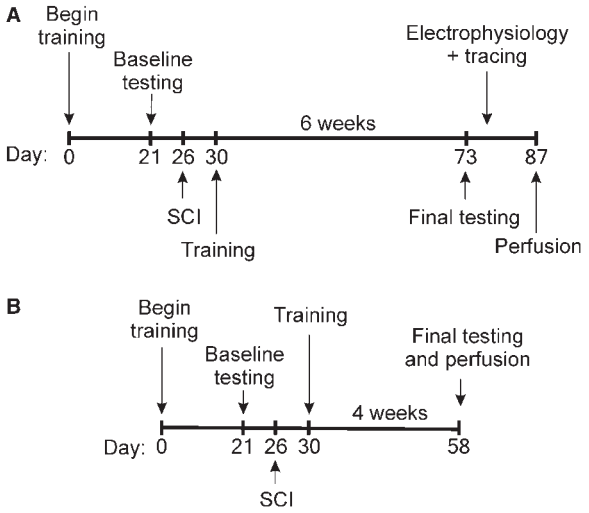

Fig. 1 Flow of the experiments. The first experiment (**A**) focussed on the effects of reaching training on recovery and neuroplasticity following cervical SCI in rats, the second (**B**) focussed on the effects of reaching training on the expression of BDNF and GAP-43 in the motor cortex.

Methods

Experiments studying the effects of rehabilitative training on recovery, CST sprouting and changes in cortical maps were performed in adult female Lewis rats (170–200 g). Experiments examining the effects of training on intra-cortical BDNF and GAP-43 levels were performed in adult male Long Evans rats (300–400 g). The use of different strains/genders is due the fact that these separate experiments were performed in different laboratories. The animals were group-housed and kept on a 12 h : 12 h light/dark cycle. To minimize grasping motions of the forelimbs in the cage, all rats were fed rodent chow mash (rodent chow and water). Water was provided ad libitum and the food was restricted so that the rats neither gained nor lost weight. This study was approved by local authorities complying with the guidelines of the Canadian Council for Animal Care. Figure 1 shows a timeline of the performed experiments.

Single pellet reaching task

Rats were trained and tested in a clear Plexiglas chamber (45 cm in height, 12.5 cm in width, 38.5 cm in length) based on reports by Whishaw *et al.* (1993). The floor of the chamber was raised 3 cm from the ground and consisted of Plexiglas in the back half and metal grating in the front (to ensure that a dropped pellet would remain irretrievable). A shelf where the pellets (45 mg chocolate flavoured sucrose pellets; Research Diets, New Brunswick, NJ, USA) were deposited was raised 3 cm from the base surface of the chamber. A narrow opening (1 cm wide, 10 cm high) allowed for one of the rat's paws to reach through for a pellet placed in a small indentation 2 cm from the inside wall of the chamber. This distance was far enough that the rat could not retrieve the pellet with its tongue. Pellets were aligned to either the left or right edge of the opening depending on the rat's innate handedness. When a pellet had been removed by a rat, the slot was refilled for a time period of 10 min. A successful trial was scored when a rat was able to retrieve and eat a pellet. Cases where the pellet was knocked off the shelf or pulled into the chamber and dropped through the metal grating were scored as failures.

Rats were trained 6 days per week for 3 weeks to grasp for pellets until baseline scores for all the animals reached a plateau (~65% success rate), before being subjected to a spinal cord lesion (see later). Several days prior to SCI, the rats' reaching style during the single pellet task was recorded for 10 min each using a digital video camera (JVC, 60 fields/s).

For the analysis of the very stereotypical single pellet reaching style from video recordings, a reach was broken down into seven components as described by Metz and Whishaw (2000). (1) Advance: the forelimb moves forward through the slot and moves toward the pellet. As the forelimb advances the elbow continues to move in. (2) Digits open: the digits open and partially pronates above the pellet. (3) Pronation: the elbow moves out, the palm is fully pronated over the pellet and the palm moves down in an arpeggio (one digit at a time) movement. (4) Grasp: the digits close around the pellet. (5) Supination I: the elbow moves in as the palm is withdrawn. As the palm is being withdrawn the palm turns 90°. (6) Supination II: the palm is withdrawn from the slot and the palm is again supinated so that the palm faces the rat's mouth. (7) Release: the rat sits back and places the food in its mouth. The rat also raises its other paw to assist the reaching paw with eating.

Each of the components was scored by an observer blind to the treatment of the animals on a 3-point scale and averaged from at least three separate reaches and expressed as a percentage of each rat's baseline score. A score of 1 was given if the movement

was present. A 0.5 was given if the movement was present but abnormal, and a score of 0 was given if the movement was absent. The absence of a movement component does not necessarily mean that the reaching was aborted, but that a compensatory (different) movement replaced the normal movement.

Horizontal ladder walking

The horizontal ladder is 1-m long and fitted with irregularly spaced metal rungs (inter-rung distances ranging from 1.5 to 3 cm). The entire apparatus is raised 30 cm from the ground. To familiarize the rats to the task, each rat crossed the ladder three times on two separate days prior to injury. For the final behavioural testing (see later), three horizontal ladder crosses were analysed per rat. Analysis focussed on counting slips (animal placed the forelimb on a rung, which then slipped off the rung before making a new step) and falls (paw is placed in between two rungs and did not make contact with any rungs before making a new step) made by the forelimb corresponding to the side of the SCI (i.e. the rat's preferred/dominant paw). Slips and falls were transferred into error rates by adding the errors (slips and falls) and dividing that number with the number of steps taken by that paw, and then multiplying by 10 steps (i.e. all errors were expressed as the number of slips and falls per 10 steps). Although horizontal ladder walking error rates were not expressed as a percentage of the baseline scores for each rat, the current and earlier studies demonstrate that uninjured rats make close to no forelimb placement errors on this task (Bolton *et al.*, 2006).

Spinal cord injury

Under gas anaesthesia (4% isoflurane for induction, 1.5% for maintenance) rats were mounted into a stereotaxic frame and their head tilted so that the spinal cord between C2 and C3 cervical vertebrae could be exposed without performing a laminectomy. Thereupon the dorsolateral quadrant ipsilateral to the preferred paw of each rat was lesioned using a micro-blade. The muscle and skin overlaying the injury site was sewn in layers. Directly following surgery, rats were placed on a heating pad until awake and received 0.03 mg/kg of the analgesic buprenorphine and 4 ml of saline. This was repeated following 8 h and repeated daily up to 72 h if the rats showed signs of pain or dehydration.

Post-injury training

Three days following SCI rats were visually inspected and ranked according to the level of paralysis. To do so, rats were placed individually into a cage and the use of their injured forelimb was observed and ranked according to the deficits/paralysis in their digits and paw. A score of 1 was assigned if the digits and paw were partially paralysed but rats were still able to stand on the limb, 2 if digits and paw were completely paralyzed and 3 if digits, paw and elbow were affected. Rats with bilateral deficits or deficits of the shoulder were removed from the study. Pairs of animals with similar scores were then separated into two groups (trained and untrained) ensuring that both groups consisted of animals with comparable injuries.

Starting on day 4 following injury, rats in the designated trained group were trained for 6 weeks (6 days/week, 10 min/day) or daily for 4 weeks in the groups designated for BDNF and GAP-43 analysis. In rats that tried to use the 'unlesioned' limb to reach for pellets, the paw was restrained using tape comparable to what was described by Whishaw *et al.* (1986). This was however only necessary in a single animal. Untrained rats were placed in the testing chamber without receiving food pellets to control for any possible differences in the familiarity to the testing environment and/or experimenter. To avoid influence of sucrose supplements these rats received diluted sucrose into their mash.

BDNF immunostaining and GAP-43 *in situ* hybridization

Following the 4 weeks of daily training in the reaching task (Fig. 1A) subgroups of rats (four trained lesioned, four untrained lesioned, four trained unlesioned) were euthanized with an overdose of sodium pentobarbital (1.6 ml/kg), transcardially perfused with warm saline and fixed with 4% formalin in 0.1 M phosphate buffer through the left ventricle prior to further histological analysis as described later. Regions of the brain corresponding to right and left forelimb motor cortex were dissected, post-fixed, cryoprotected in 20–30% sucrose, embedded in OCT compound (Tissue Tek, Miles Inc., Elkhart, IN) in a cryomold (Tissue Tek), frozen in isopentane cooled to –70°C and stored at –80°C until further processing.

In situ hybridization

Brain sections were cut at 10 µm using a Microm HM500 cryostat (Zeiss, Canada), thaw mounted onto Probe-On+ slides (Fisher Scientific, Canada) and stored with desiccant at –80°C until hybridization. *In situ* hybridization was carried out on tissue using a 48 bp oligonucleotide probe (University Core DNA Services, Calgary, AB, Canada) complementary to GAP-43 [complementary to bases 70–117 (Karns *et al.*, 1987)]. The probe was checked against the GenBank database (NIH, Bethesda, MD) to ensure that no greater than 75% homology was found to sequences other than the cognate. The probe was labelled at the 3′-end with α-[^{35}S]dATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyl-transferase (Amersham Biosciences, Piscataway, NJ) in a buffer containing 10 mM $CoCl_2$, 1 mM dithiothreitol (DTT), 300 mM Tris base and 1.4 M potassium cacodylate, pH 7.2, and purified through Bio-Spin Disposable Chromatograph Columns (Bio-Rad Laboratories, Hercules, CA) containing 200 mg of NENSORB PREP Nucleic Acid Purification Resin (DuPont NEN, Boston MA). DTT was added to a final concentration of 10 nM. The specific activities ranged from 2.0 to 5.0 $\times$ 10^6 cpm/ng for each oligonucleotide.

In situ hybridization was carried out according to published procedures (Dagerlind *et al.*, 1992). Briefly, the sections were hybridized at 43°C for 14–18 h in a buffer containing 50% formamide (Sigma Aldrich, Oakville, ONT, Canada), 4 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 $\times$ Denhardt's solution (0.02% bovine serum albumin and 0.02% Ficoll), 1% sarcosyl (*N*-laurylsarcosine), 0.02 M phosphate buffer, pH 7.0, 10% dextran sulfate, 500 µg/ml heat-denatured sheared salmon sperm DNA, 200 mM DTT and 10^7 cpm/ml of probe. After hybridization, the slides were washed 4 $\times$ 15 min in 1$\times$ SSC at 55°C, dehydrated in ascending concentrations of alcohols, processed for radioautography as per Karchewski *et al.* (2002), and exposed for 12–80 days before developing in D-19 (Kodak, Rochester, NY).

The specificity of hybridization signal for each probe was ascertained by hybridization of labelled probe, labelled probe with a 1000-fold excess of cold probe (signal abolished), or labelled

probe with a 1000-fold excess of a dissimilar cold probe of the same length and similar G-C content (signal unchanged).

Immunohistochemistry

Sections were cut at 10 µm on a cryostat, thaw mounted onto Probe-ON^{+} slides (Fisher Scientific). To detect BDNF protein expression, immunohistochemistry was performed as previously described by Karchewski *et al.* (2002). The primary antibody, rabbit anti-BDNF [1 : 500; gift from Cindy Wetmore, Mayo Clinic, Rochester, MN, USA; Wetmore and Olson (1995)] was left on tissue overnight at 4°C. The next day, sections were incubated in secondary antibody Cy3-conjugated donkey anti-rabbit IgG (1 : 300; Jackson, Westgrove, PA) for 1 h in the dark at room temperature. Slides were coverslipped with glycerol/PBS.

Both, *in situ* hybridization and the immunostaining were analysed using greyscale densitometry with image analysis software (Scion Image, NIH). Since direct comparisons of the signal density in the corpus callosum of trained and untrained cortices mounted on one slide did not show differences, we used this area as a non-involved region to normalize cortical densities.

Evaluating functional recovery and plasticity

Following 6 weeks of training, groups of rats (six trained and eight untrained) were tested (and filmed) on the single pellet reaching task and the horizontal ladder walking task. Analysis of the rat's performance in the single pellet task was done as described for the baseline testing (see earlier). Rats were tested on the single pellet task over 3 days to ensure that the untrained rats (who had not been exposed to the reaching task for over 6 weeks) reached a plateau. On the final testing day, rats were video-recorded (digital video camera as above) crossing the horizontal ladder three times.

Electrophysiology

Cortical micro-stimulation was performed in all animals following their final behavioural testing. Animals were anesthetised (see above) and placed into a stereotaxic frame. The skin above the skull was cut and the anode was clamped onto the skin overlaying the skull. Two rectangular openings were drilled into the skull overlaying the forelimb motor area on both the left and right cortical hemispheres. The coordinates for the openings were: 1 mm lateral to 4 mm lateral to bregma and extended 3 mm rostral to bregma. A tungsten microelectrode (FHC Inc., Bowdoin, ME, USA, 8–10 MΩ, 250 µm shank diameter and a 'long thinned' profile) was used to deliver a train of cathodal pulses ($n = 30$, 0.25 ms, 330 Hz) to the cortex. During micro-stimulation, isoflurane levels were maintained low enough to allow for motor responses (tested on the unlesioned CST) but high enough to act as an anaesthetic (~1%). Care was taken so as to stimulate the same cortical areas in each rat. This was done by dividing each hemisphere into 25 spots or areas, with an electrode penetration spacing of ~400 µm. On average, 15 cortical spots were stimulated per hemisphere. The two limiting factors preventing micro-stimulation of every spot in the exposed hemispheres were the presence of superficial blood vessels overlaying the cortices, and the length of the procedure (since this was not a terminal experiment, care was needed so as not to lose an animal due to excessive surgical time).

Micro-stimulation studies were conducted as follows: for each cortical spot stimulated, the stimulating electrode was lowered into the sensory motor cortex to a depth ranging from 1.6 to 1.9 mm where usually the maximal movement response was noted (by visual inspection; stimulation intensity of 200 µA). Next, the threshold for the response was determined by decreasing the stimulus intensity 10 µA at a time until the response no longer occurred. The response type (e.g. wrist, shoulder, whisker movements) and the threshold to elicit the response were noted for each spot stimulated.

Cortical map

The cortical maps generated represent the occurrence rate of wrist and/or elbow movements at each stimulated spot in the forelimb cortical area and were calculated separately for each experimental group (trained and untrained rats as well as uninjured controls). For each point, the number of animals in which a forelimb (wrist and/or elbow) movement was elicited was taken over the number of times that spot was stimulated in each group. For example, if only one animal in a group responded to cortical stimulation at a cortical spot when five of the animals in the group were stimulated at that spot, the occurrence rate would be 0.2. To control for the fact that not every cortical spot was stimulated (due to the two limiting factors stated previously), the group cortical maps include only spots that were stimulated at least twice in a group (i.e. in two separate animals). If a spot was only stimulated once or not at all, the value for that spot was taken as the average of all adjacent spots. To visualize the organization of the cortical spots with higher occurrence rates of forelimb movements, a 4-colour colour code was given to the map. For statistical analysis we transferred the colour code into a score (0–3), and averaged the score of all 25 spots of all animals within the respective groups. This resulted in the average responsiveness score presented in Fig. 5.

Tracing

At the end of the electrophysiological experiments, the CST emanating from the forelimb area of the cortex of all rats was traced with 10% biotinylated dextran amine (BDA, 10 000 MW, Molecular Probes, Invitrogen Corporation, Burlington, ON). A 1 µl Hamilton syringe was inserted into the side of the cortex contralateral to the injury (i.e. to trace the injured CST) at 1.5 mm lateral and 1.5 mm rostral to bregma. Once lowered to 1.5 mm, 1 µl of BDA was injected over a period of 5 min.

Perfusions and histology

Two weeks following tracer injection, animals were euthanized and perfused as described earlier. Brains and spinal cords were extracted and post-fixed in 4% formalin (with 5% sucrose) overnight and then transferred to 30% sucrose for 3 days.

The cervical spinal tissue from each animal was divided into two blocks: a C1 segment (mounted for cross-sections and used to verify the number of fibres traced) and the remainder of the segments (mounted for horizontal sectioning and used to verify both lesion size and the number of collaterals rostral and caudal to the lesion). Both blocks were embedded in Tissue Tek (Sakura Finetek USA Inc., CA, USA) and frozen in 2-methyl-butane over dry ice (~–60°C). All tissue was sectioned at 25 µm in a cryostat at –20°C.

All sections were stained for BDA. All slides were reheated at 37°C for 1 h and then rehydrated with two 10-min washes in TBS

followed by two 45-min washes in TBS-TX. Slides were incubated overnight (at 4°C) in avidin–biotin complex (ABC) solution (Vector Laboratories, Burlingame, CA). The following day, after a rinsing step (two 10-min washes in TBS) slides were processed with DAB (Vector Laboratories) according to instructions. Briefly, DAB solution was placed over the slides for a maximum of 5 min and then the slides were placed in distilled water to halt the reaction. Slides were then washed (two 10-min washes in TBS) and dehydrated (2 min in each of the following: 50, 75 and 100% alcohol) and then cleared during two separate xylene washes. Sections were then coverslipped with Permount (Fisher).

To analyse the lesion size we reconstructed the lesion site from every fourth horizontal section with light microscopy with phase contrast (100× magnification). Using landmarks (i.e. grey/white matter interfaces, traced CST fibres, central canal) the maximal extent of the injury was determined on the horizontal slides and transferred onto a schematic cross section of the cervical spinal cord. The lesion size was calculated from the schematic cross section using Scion Image (NIH). This method was chosen as horizontal sections (as compared to cross sections) allowed a more convenient counting of collaterals rostral to the lesion.

To ensure comparable deficits we defined the following exclusion criteria. All animals that were included into the study had at least 95% injury to the dorsal CST components ipsilateral to the preferred paw and not more than 5% injury to the contralateral side (this ensures that grasping deficits are not due to deficits in the other forelimb, which is needed for standing and balancing during the grasping procedure). The dorsolateral quadrant had to be injured at least 75% and the ventro-lateral funiculus had to be at least 25% spared to be included. This ensured that a majority (60–100%) of the rubrospinal fibres were ablated, but reticulospinal fibres were spared.

Photographs of a processed and stained C1 cross section were taken using a camera mounted onto a Leica microscope (400× magnification) to count the total number of traced fibres in each animal. This value was later used to normalize the counted number of collaterals (see later).

Collaterals emanating from traced CST fibres were counted in a similar manner as previously described (Vavrek *et al.*, 2006). Each BDA positive collateral emanating from the CST and crossing into the grey matter for a distance of at least 30 μm was counted. The sum of the collaterals was normalized for interindividual tracing and analysis variability by dividing by the number of labelled CST fibres for each animal and by the total length of the spinal cord on which the counting was performed (to express the result as a percentage of CST fibre per millimetre).

Statistics

Statistical analysis was performed using a Mann–Whitney U Test (GraphPad Prism, San Diego, CA, USA) unless otherwise noted in the results. If variances between two groups were significantly ($P < 0.05$) different, a Welch's correction was performed. When comparing success rates in untrained animals during their final testing days, a one-way ANOVA was used. When quantifying cortical maps, a Wilcoxon matched pairs test was used. All data are presented as means ± SE.

Comparisons for the densitometry analysis were performed only between sections of different animals that were mounted on the same slide. Measurements were taken at comparable location and size ~1.5–2 mm lateral to the midline from the dorsal surface to 1.8 mm depth. Statistical comparisons between the different animal groups and the cortical hemispheres in one animal were performed using a two-sample student's *t*-test following normalization of the background brightness. In the process of normalization all yoked pairs were treated equally.

Results

Training of the single pellet reaching task increases the reaching success rate

The objective of this study was to examine whether task-specific training of a reaching task in rats with a cervical SCI could significantly promote functional recovery. Therefore, a group of rats with a lesion of the dorsolateral quadrant (ipsilateral to their preferred paw) was trained on the single pellet reaching task (Fig. 2A). Following a 6-week training period all rats (including untrained rats) were tested (over three separate days) in this task. For each rat, the best score achieved out of their three testing days was used and was normalized to the individual baseline score. Untrained rats were not exposed to the reaching task. When these rats were tested following 6 weeks of recovery they were able to reach an average success rate of $33.5 \pm 6.2\%$ ($n = 8$) of pre-lesion performance (Fig. 2B). Trained rats, were unable to perform the task in the first week of their training, however following 6 weeks they performed significantly better than untrained rats, reaching success rates of $60.9 \pm 4.5\%$ ($n = 6$), thus, nearly doubling the success rate achieved by the untrained rats ($P = 0.005$).

Considering that untrained rats were not exposed to food pellets over 6 weeks it could be argued that they simply 'forgot' how to perform the task. If this were the case, it would be expected that their success rate would improve significantly over the three testing days. This, however, was not found. The average success rate over the 3 days was 21.1 ± 6.1, 29 ± 7.6 and $24 \pm 7.4\%$. Furthermore, when rats initially are trained in the reaching task, their attempt rates are very low. During the first post-injury testing session however, a high number of unsuccessful attempts (data not shown) indicate that the rats did not 'forget' the task. Therefore, our results suggest that training promoted recovery in the reaching task.

Training does not alter the reaching style

Detailed analysis of the reaching style was performed in order to determine whether different movement strategies were adopted by the animals following injury and to determine whether these strategies were further altered by training. Seven different components in the reaching/grasping movement were analysed and quantified (see 'Methods' section). Figure 2C shows the results in the reaching style analysis in a score for both trained and untrained rats. Values of 1 represent a normal movement.

Our results show that no significant changes in the reaching score were detected between trained and

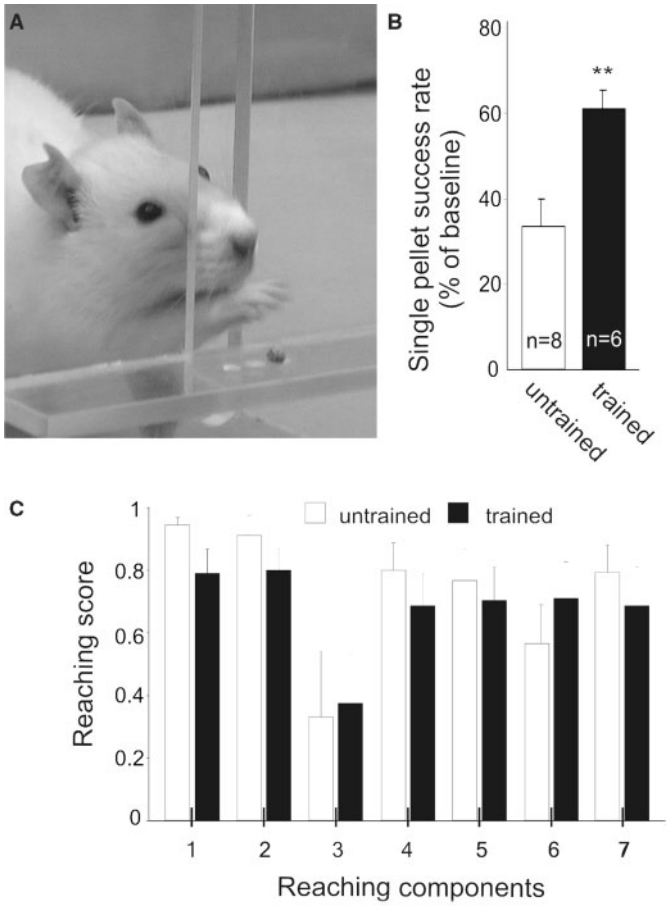

Fig. 2 The differences in the reaching task (A) between trained and untrained rats in their success rate (B) and the reaching style (C). The success rate which was normalized to the individual pre-lesion success rate shows that untrained rats recover to around 30% of their pre-lesion performance. This percentage is doubled in trained animals that reach success rates around 60% of their baseline (B). Reaching style was quantified by scoring seven phases of the reaching movement that are described in detail in the 'Methods' section: (1) Advance, (2) Digits open, (3) Pronation, (4) Grasp, (5) Supination I, (6) Supination II, (7) Release. Trained rats did not significantly differ from untrained rats. **indicates $P < 0.001$. Error bars show standard error of the mean.

untrained rats. In both groups, phase 3 of the grasp (pronation phase, where the palm is placed down upon the pellet and the digits close around it) is most affected, in keeping with previous reports (Whishaw *et al.*, 1998) showing the arpeggio (one digit closing around the pellet at a time) movement of the digits to be absent in animals with a CST and RST lesion. It is noteworthy, that although no statistical significance was found, the trained rats performed worse in most of the components (five out of seven), although their success rate was superior to untrained rats. Observations of their reaching movements indicate that trained rats adapt different strategies to grasp for the pellets, thus a lower score may represent a different adaptive strategy rather than only a less successful approach. As such strategies are variable between individuals rats quantification is difficult and was not performed.

Training increased cortical levels of GAP-43 but not BDNF

We suggest that the enhanced recovery we observed following intensive training of a reaching task is due to enhanced plasticity at the level of the brain, brainstem and spinal cord. A possible mechanism to translate physical activity or training into enhanced plasticity is the up regulation of growth promoting factors. To investigate this possibility we performed an experiment with a separate group of rats that received a SCI and subsequent rehabilitative training. In these animals we investigated the levels of BDNF and GAP-43 in the motor cortex representing the forelimb area using immunohistochemistry (BDNF) and *in situ* hybridization (GAP-43).

Following 4 weeks of training we did not find consistent changes of BDNF levels when directly comparing the cortex (contralateral to the trained side) from trained to untrained lesioned rats on the same slides ($P > 0.1$; data not shown) or when normalizing the density measures to that of the corpus callosum.

When analysing GAP-43 expression using densitometry (Fig. 3) we found no difference between the ipsi- and contralateral (controlling the preferred paw) side in trained rats without lesion or untrained rats with lesion (Fig. 3B). However in rats with lesion and training, the cortical hemisphere with ablated axons (contralateral to the spinal injury) showed a significant GAP-43 elevation. The normalized density was also higher when compared to lesioned untrained rats, but not when compared to trained rats without lesion (Fig. 3B). In order to examine whether GAP-43 was globally up-regulated over the entire cortex or whether changes were specific to the forelimb area we also analysed the S1 jaw region and oral surface. No differences were found between trained and untrained animals in this cortical region (Fig. 3C).

Lesion size is comparable between treatment groups

Equivalent deficits before a treatment are essential when comparing functional recovery between treatment groups. To ensure this, we employed two strategies: before the onset of training we paired the animals according to their deficits and distributed them into the trained and untrained group. Following the experiment we analysed the lesion size and removed animals from the study that did not follow a set of lesion criteria (see 'Methods' section). Figure 4A shows schematics of the largest (dark grey) and the smallest included lesion. When comparing the overall lesion sizes between untrained and trained rats there was no statistical difference (29.1 ± 2.5 versus $35.0 \pm 3.0\%$, respectively, Fig. 4B). Thus, we suggest that differences in recovery

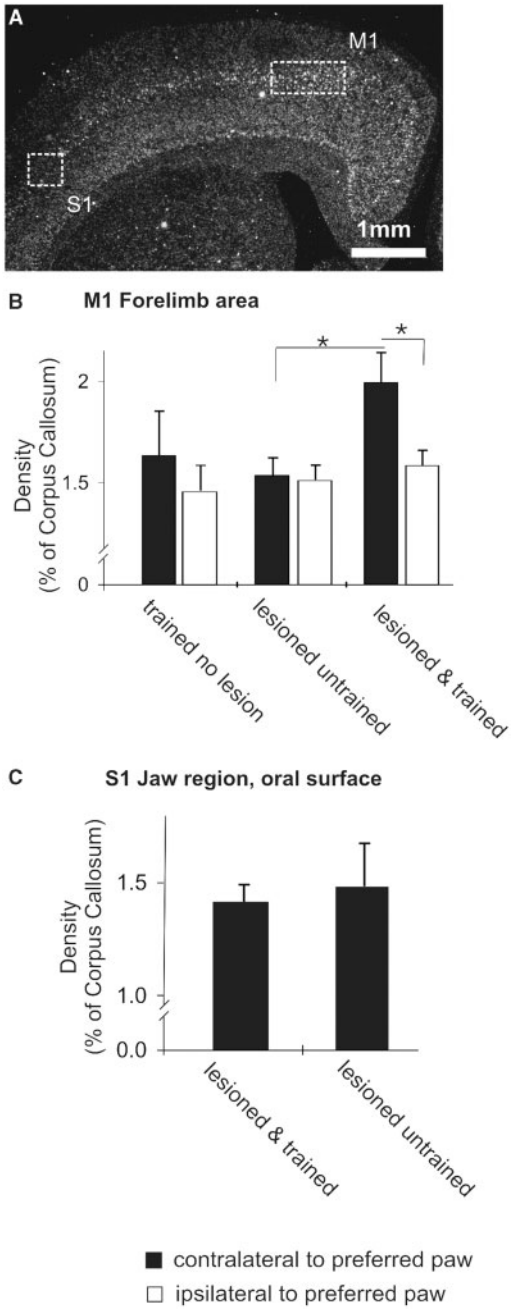

Fig. 3 The changes in GAP-43 expression following reaching training. Following *in situ* hybridization on cross sections through the cortical forelimb area, density measurements were performed at layer V, from 1 to 2 mm lateral from the midline (see rectangle in **A**). Another measurement was taken further laterally in the SI jaw and oral surface region (square in **A**). Comparisons of the density (expressed as percentage of the density measured in the corpus callosum) between the ipsi- and the contralateral side of the forelimb area did not show differences in trained unlesioned or lesioned untrained animals (Fig. 3B). In contrast in rats with lesion and training GAP-43 expression was enhanced in the MI region contralateral to the preferred paw. This density was also higher than that of lesioned but untrained rats (Fig. 3B). When comparing the normalized density of the SI jaw and oral surface region, no difference was found between lesioned trained and untrained animals. *indicates $P < 0.05$. Error bars show standard error of the mean.

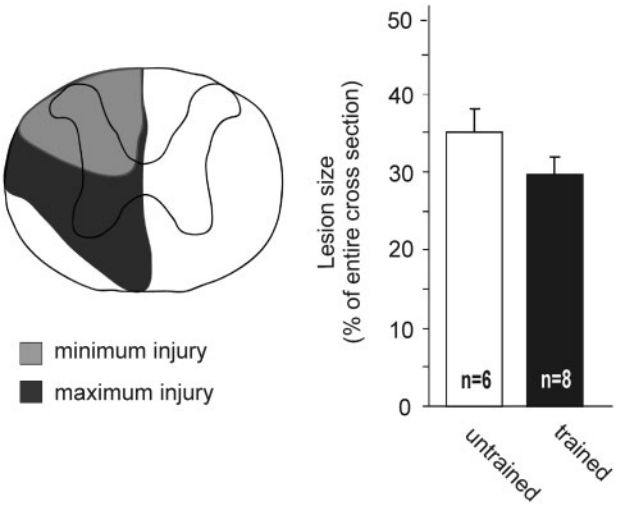

Fig. 4 The comparison of lesion sizes at spinal level C3, between trained and untrained rats. In (**A**) a schematic drawing of a spinal cord cross section illustrates the location and size of the biggest (dark grey) and smallest lesion (light grey) that was included into the study. A comparison of lesion size (in% of the complete cross section area) did not show a significant difference (**B**). Error bars show standard error of the mean.

between trained and untrained rats are not due to different lesion size or location.

Training increases cortical representation of the injured forelimb

Rearrangements of cortical maps are a frequently discussed mechanism in promoting recovery following stroke (e.g. Ramanathan *et al.*, 2006). Following SCI such map changes have also been reported (Bruehlmeier *et al.*, 1998; Fouad *et al.*, 2001). Here we investigated cortical map changes (location and excitability) focussing on forelimb (wrist and/or elbow) function using micro-stimulation in trained and untrained rats, as well as in three control (unlesioned and untrained) rats. Figure 5A illustrates changes in cortical maps between the different groups in the cortex ipsi- and contra lateral to the spinal lesion. Group data for every stimulated spot ($n = 25$) are expressed as the number of animals in which stimulation of this spot induced a contralateral forelimb movement, divided by the number of animals that were stimulated at that spot. This percentage was translated into a colour code (occurrence of wrist movements; Fig. 5A) and a number (0–3) for further statistical analysis. The results show that in control rats, wrist movements could most reliably be evoked at positions close to Bregma in the side contralateral to the spinal lesion (illustrated by three orange and one red square; Fig. 5A). Following a lesion this area became less reliable in triggering movements, however, locations formerly not responsive (light yellow) were able to trigger movements. Following training the connectivity of the cortical sites just anterior to Bregma was restored and this

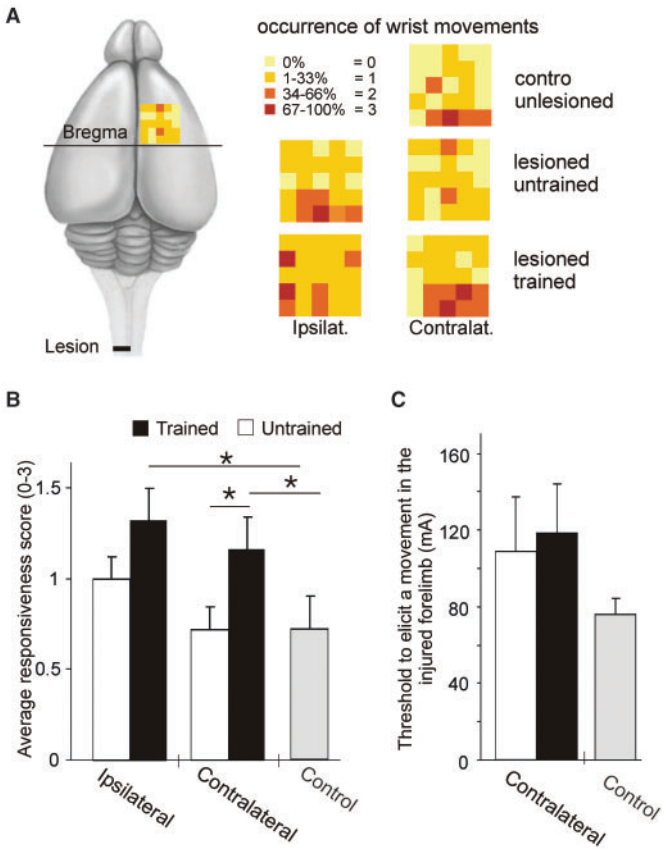

Fig. 5 The results from cortical micro-stimulation. Stimulation of up to 25 spots in the forelimb area of both cortical hemispheres were performed and the possibility to evoke a motor response in wrist extensor was scored and averaged between animals for each stimulated location (**A**). These values were used to create a map where darker colours indicate higher percentage of stimulation-induced wrist movements. The maps of both the ipsi- and contralateral cortex (to the spinal lesion) show adaptations following lesion only, and significant increases in expansions and excitability following training. Transferring the colour code into a score allowed creating an average responsiveness score, showing that training increased possibility to trigger wrist movements from both cortical hemispheres (always in the respective contralateral limb) when compared to control or untrained animals (**B**). The average thresholds to trigger wrist movements were increased following lesion however due to large SE this was not statistically significant (**C**). *indicates $P < 0.05$. Error bars show standard error of the mean.

area was even expanded, as illustrated by six squares in orange and two in red.

Cortical areas ipsilateral to the lesion also undergo changes. The cortical area to reliably evoke movements in the 'unlesioned' forelimb expands following lesion and increases further following training of the other 'unlesioned' forelimb. An increase in ipsilateral evoked responses however was not observed. Only one out of the eight trained rats and none of the untrained rats showed an ipsilateral response.

A quantification of the cortical mapping is shown in Fig. 5B. Following rehabilitative training the occurrence of wrist/elbow movements increases compared to uninjured (control) ($P = 0.03$) and untrained ($P = 0.04$) rats. This includes increased responsiveness to micro-stimulation in spots that are excitable in some but not all unlesioned animals, and responses from areas that were unresponsive in all unlesioned animals.

A different pattern is seen in the ipsilateral (to injury) cortex. Trained rats show a significant increase in the occurrence of stimulation induced wrist/paw movements when compared to control uninjured rats ($P = 0.01$). There was no significant difference between trained and untrained rats on the ipsilateral side of the cortex.

These results show that the cortex is adapting to a spinal injury, both in the hemisphere projecting to the injury site, and in the hemisphere projecting through the unlesioned side of the spinal cord.

Changes in stimulation threshold can provide information on the mechanisms of the observed cortical adaptations. We found however no significant differences. The average minimum threshold to evoke a movement of the injured forelimb following stimulation of the contralateral cortex was enhanced following lesions but statistically not significantly different in any group following training (Fig. 5C).

Training following SCI increases the number of CST collaterals rostral to the injury

Plasticity following injuries of the CNS has not only been observed at cortical levels. In this analysis we investigated whether rehabilitative training could enhance sprouting of the injured CST fibres rostral to the injury comparable to earlier reports following thoracic injury only (Fouad *et al.*, 2001; Bareyre *et al.*, 2004) or BDNF administration to the cortex (Vavrek *et al.*, 2006). The number of CST collaterals emerging from the forelimb cortical area rostral to the injury was calculated and normalized as described previously (Vavrek *et al.*, 2006). Figure 6A shows a segment of a horizontal section of the spinal cord from an injured rat, with CST collaterals growing into the grey matter (arrows). We found that injury alone did not significantly enhance the number of collaterals. Although there was no statistical difference between trained and untrained lesioned rats, the trained group had significantly more collaterals than the uninjured and untrained controls ($P < 0.05$; Fig. 6B). This shows that training can enhance injury-induced collateral sprouting within the spinal cord.

Reaching training interferes with the ability to walk on a horizontal ladder

The horizontal ladder walking task is a task requiring fine motor control (Fig. 7A) and, similar to the single pellet reaching task, requires the integrity of the CST and RST. To determine whether improvements on the single pellet reaching task could translate into improvements in this

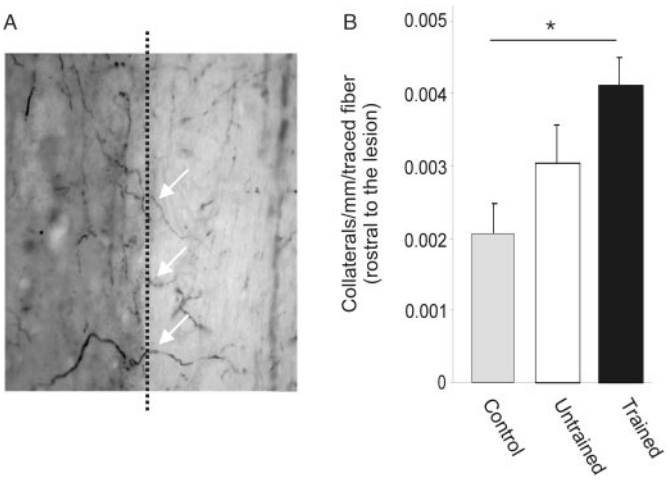

Fig. 6 Collaterals emanating from the lesioned CST (white arrows) rostral to the lesion and growing into the grey matter (**A**). The dashed line indicates the grey–white matter interface. When collaterals were normalized to the number of traced axons and the distance they were counted on, we found an insignificant increase when comparing lesioned to unlesioned animals (**B**). This increase was greater following training and was significant when compared to controls. *indicates $P < 0.05$. Error bars show standard error of the mean.

related task, videotape analysis was performed on all rats crossing the horizontal ladder. The error rate was expressed as the number of slips and falls made by a paw (ipsilateral to the injury) per 10 steps. In contrast to the hindlimbs, rats rarely produce errors with their forelimbs. The lesion of the dorsolateral quadrant caused the rats to make errors with their 'injured' forelimb, but not with the 'uninjured' forelimb (data not shown). Figure 7B shows the error rates of the injured forelimb in trained and untrained rats, when walking on the horizontal ladder. In the trained group, there was a significant increase in the errors (trained: 1.91 ± 0.4, untrained: 0.70 ± 0.3; $P = 0.03$ (unpaired t-test), showing that training on the single pellet reaching task did not only transfer into improvements on the horizontal ladder walking task, but also caused a decrease in performance.

Discussion

In this study, we showed that reaching training in rats with a cervical SCI can substantially promote the recovery of that particular task. An important difference to the well-established paradigm of treadmill training (Dietz and Colombo, 2004; Behrman *et al.*, 2005; Dobkin *et al.*, 2006) is that reaching involves fine motor control that is controlled directly by brain and brainstem centres, whereas repetitive locomotor patterns (as during locomotion) are orchestrated by spinal networks, termed central pattern generators (CPGs). Training-induced adaptations in CPGs were attributed to be involved in the recovery of walking in animal studies (de Leon *et al.*, 1999; Tillakaratne *et al.*,

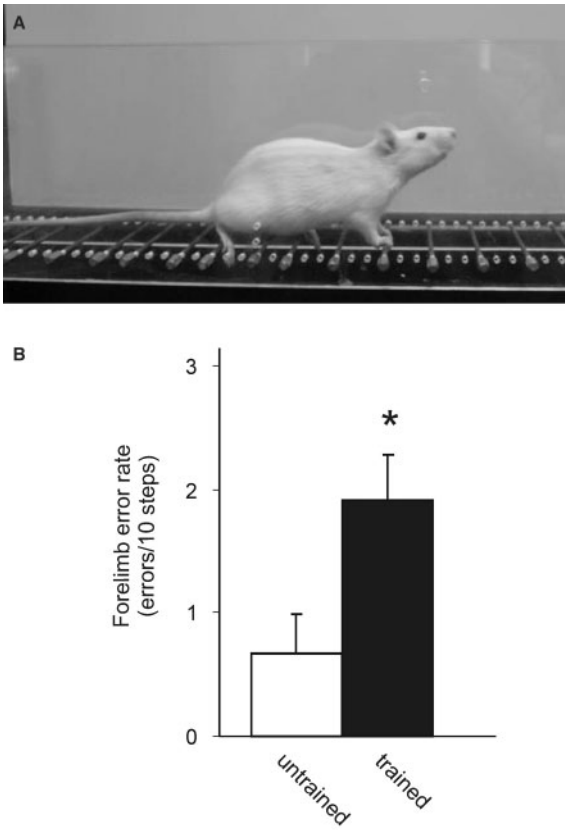

Fig. 7 A cost for improvements following reaching training when testing the animals in an untrained task that also involves fine motor control, i.e. walking on a horizontal ladder (**A**). Rats that underwent reaching training made significantly more errors than untrained rats (**B**).

2000, 2002), however recently also changes in CST function following training were reported in patients (Thomas and Gorassini, 2005).

In the present study we report that training in uninjured rats results in the up-regulation of GAP-43 in the cortical hemisphere contralateral to the trained paw. Following spinal cord lesion and training, the difference between GAP-43 levels between both hemispheres is not found anymore, indicating that either the lesion reduces the signal in the lesioned CST cell bodies, or the cortical hemisphere ipsilateral to the lesion is influenced by the training. The first argument is supported by reports that following cervical lesions CST neurons might degenerate or die (Hains *et al.*, 2003). However, when comparing GAP-43 levels in rats that had been lesioned but did not receive training, no differences between the cortical hemispheres were found. It is thus likely that training promotes plasticity in both cortical hemispheres, which is supported by our finding that training significantly promoted cortical map rearrangements in the 'non-trained' hemisphere ipsilateral to the lesion. Overall, the changes in GAP-43 levels are in parallel with results found with cortical micro-stimulation (using comparable stimulation paradigms or longer stimulation trains) following stroke where training

had significant effects on cortical maps (Kleim *et al.*, 1998; Ramanathan *et al.*, 2006).

A possible mechanism contributing to the cortical changes following SCI is the sprouting of lesioned and unlesioned CST fibres. As the corticospinal tract in rats does not exclusively project in the dorsal funiculus but also has a ventral and lateral portion, it is likely that spared fibres respond to injury of dorsal CST components and training by increased sprouting of spared fibres as described following lesion only by Weidner *et al.* (2001). In this study we focussed on plasticity rostral to the injury, comparable to what has been described earlier (Fouad *et al.*, 2001; Bareyre *et al.*, 2004; Vavrek *et al.*, 2006) and found that it is enhanced in animals that underwent rehabilitative training.

It is unlikely that sprouting of lesioned and spared axons within the spinal cord is the only mechanisms for the restoration of cortical areas able to trigger forelimb movements. Recovery and the reconnection of lesioned CST fibres could be attributed to the strengthening of cortio-rubro or cortico-reticular connections, as both spared rubro and reticular fibres indirectly connect to forelimb motoneuron pools and have been reported to be involved in the recovery of grasping movements in cats and primates (Pettersson *et al.*, 2007). A comparison to rats could be drawn as the CST in rats serves the same function as in primates, i.e. fine motor control, especially of distal muscles. Evolutionary changes in the anatomy of the CST/motor cortex are paralleled by increased dexterity. For example, a difference between rats and primates is the lack of direct connections from CST fibres onto motoneurons innervating the forelimb in rats, which emerged during primate evolution. Another major difference is the location of the main portion of the CST that travels in rats within the ventral parts of the dorsal column, and in primates in the lateral column.

In conclusion, it cannot be clearly delineated to what extent the cortical rearrangements and sprouting of lesioned CST fibres are involved in the observed functional recovery, however our study clearly demonstrates that rehabilitative training promoted recovery by enhancing injury-induced repair mechanisms.

An important issue for the clinical application of rehabilitative training is the onset of training. An early start is considered most effective as injury induced up-regulation of various growth-promoting factors (Hayashi *et al.*, 2000; Song *et al.*, 2001; Di Giovanni *et al.*, 2005) might support greater benefits. On the other hand, findings in the stroke model have taught that starting training too early possibly exacerbates the lesion (Humm *et al.*, 1998). Importantly, we started training 4 days following lesion, which did not alter the lesion size between the trained and the untrained group. When comparing the lesion sizes the trained group appears to have a slightly smaller lesion (not statistically significant), however, one may argue that the improved recovery in the reaching task is due to this small difference. We would like to argue against this as, 3 days following lesion, we matched the groups with rats having comparable deficits, and also found that trained rats performed significantly worse on an untrained but related task (i.e. the horizontal ladder test).

Importantly, the benefits found on a trained task (reaching/grasping) did not transfer onto another related task (walking on a horizontal ladder), but rather caused further impairment. This is an interesting similarity to studies in patients with incomplete SCI, where patients trained to walk forwards on a treadmill had more problems walking backwards, than untrained patients (Grasso *et al.*, 2004). Similarly, in cats with complete spinal cord ablation training to walk on a treadmill interfered with their ability to stand and the other way around (de Leon *et al.*, 1994). As no descending connections were spared in these animals it was discussed that this effect was caused by disinhibition within the pattern generating network (de Leon *et al.*, 1999). A potential mechanism for the detrimental effect of reaching training on the abilities to walk on the horizontal ladder could be a competition of neural substrates, as a large percentage of fibres controlling fine motor movements were ablated. Reaching training might strengthen connectivity between brain areas controlling reaching and the spared descending fibres, thus competing for the limited post-synaptic space with unused pathways.

In conclusion, rehabilitative training of a reaching task following cervical SCI in rats promotes plasticity at various levels of the CNS and substantially promotes functional recovery in the trained task. Thus, our study supports the idea of increased post-injury patient care, to provide intensive physical therapy, as large benefits may be achieved in patients. Task-specific training, however, might come at the price of reduced function in untrained tasks indicating that training of a range of tasks might be more beneficial.

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