SUPPLEMENTARY MATERIALS: Jefferson SC et al. ***Cortical stimulation concurrent with skilled motor training improves behavioral function and enhances motor cortical reorganization following controlled cortical impact***

Supplementary Methods

Animals: *Male Long-Evans rats (Charles Rivers Laboratories) were housed* in pairs or triplets on a 12:12h light:dark cycle with water ad libitum, in standardized cages with supplements (a PVC and cardboard tubes, wooden objects and a complex food mixture). Rats were tamed by handling and placed on scheduled feeding (15g chow/day, gradually increased to permit normal age-related weight gain). Scheduled feeding insured animals were motivated to participate in the reaching task.

Single Pellet Reaching
The single pellet retrieval task is a sensitive measure of unilateral forelimb deficits and recovery and was used as the main behavioral outcome measure. As previously described in more detail [1] and trained to reach with their preferred forelimb through a 1cm window to retrieve a single (per trial) 45 mg banana-flavored pellet (Bioserve, Inc., Frenchtown, NJ) placed on a shelf (11.7 cm long x 5 cm wide x 3 cm high) in a shallow well aligned 1 cm from each edge of the window. Prior to surgery, rats were trained for 60 trials per day or 15 min to a minimum criteria of ~60% successful retrievals/reaches with the preferred limb. After reaching criterion, animals received a CCI over the caudal forelimb area (CFA) of motor cortex opposite the reaching limb. Following CCI, impaired forelimb function was probed with the single-pellet reaching task. Probe tests consisted of 30 reaching trials or 10 minutes, whichever came first. Percent reaching success was used as the primary behavioral outcome measure (%successful pellets placed in mouths/reaches).

Controlled Cortical Impact (CCI)
Traumatic brain injury was induced in the CFA using the controlled cortical impact method [2, 3]. This injury model produces a focal contusion injury and results in moderate to severe forelimb motor impairments. After anesthetization with Ketamine (90-110 mg/Kg; i.p.) and Xylazine (7-10 mg/Kg; i.p.) all animals received a unilateral 4 mm diameter craniotomy centered at 0.5 mm anterior and 4 mm lateral to bregma, delivered by a small-bore electromagnetically controlled device (Benchmark™ Stereotaxic Impactor, Cortech Holdings Co, St. Louis, MO). The electromagnetic controlled probe tip (3mm in diameter) penetrated the brain exposed at 3.0m/sec at a depth of 1.7mm below the cortical surface for 250 ms. After the impact, the wound was covered with gel foam and sutured. Topical antibiotics were applied to the incision and Buprenorphin (0.05mg/ml) was administered subcutaneously.

Electrode implantation
In all animals, electrodes were implanted directly following CCI. Following impact, the craniotomy was enlarged approximately 1 mm anterior and medial edges to expose peri-

injury motor cortex. Each electrode consisted of two 0.4mm wide by 2mm long parallel platinum wire strip contacts mounted on a 3mm by 3mm supporting plate extending from an electrode connector pedestal (Plastics One Inc., Roanoke, VA). Cathodal, 100Hz monopolar current flowed through the platinum contacts which were placed in a consistent manner relative to skull landmarks on dura and were orientated approximately parallel to midline. Anodal current flowed to a small metal disk implanted subcutaneously (contact facing skin) near Lambda and was used to ground the current. This electrode placement has been found to reliably enable post-operative stimulation-evoked contralesional forelimb, face and/or upper body movement [1, 4-6]. The electrode was secured to the skull using skull screws and cemented with a combination of standard dental cement and dental acrylic.

Assessment of Movement Thresholds
Weekly movement thresholds were determined only for the CS+RT group to set the stimulation current to be administered during post-CCI rehabilitation reach training and was defined as the minimal current necessary to produce visible movements of the forelimb, face or shoulder contralateral to the injury. Prior to the first day of training each week, rats were observed while a series of 3 sec trains of 1 msec pulses, were delivered with increasing amplitude at a frequency of 100 Hz. When a motor movement was observed, current amplitude was then lowered until the movement disappeared. Movement threshold was defined as the lowest current to evoke motor movement of the head, neck or forelimb.

Animals were placed into one of two groups matched for pre and post-CCI performance on the single-pellet reaching task. Following post-CCI probe testing, animals were randomly divided into cortical stimulation + rehabilitative training (CS+RT) and rehabilitative training only (RT) groups with the exception that they were matched as closely as possible for pre- and post-operative reaching performance on single pellet reaching test.

Rehabilitative training. Starting on day 10 post-CCI Animals received rehabilitative training on the tray-reaching task, in the same chamber described above. Four days per week animals were permitted to reach with the impaired forelimb for ~200 pellets placed on an inclined tray (25°) or for 20 min, whichever came first, while connected to electrode leads either with or without stimulation. Placing a wall ipsilateral to the reaching limb ensured use of the impaired limb.

Cortical stimulation parameters during reach training
All rats were attached to stimulator cables and placed into the reaching chamber. For the CS+RT, continuous stimulation was delivered at 50% of that week's movement threshold. Stimulation was turned on when the rat was placed in the chamber and begun reaching. Cortical stimulation was delivered continuously as the animal retrieved the 200 pellets in the reaching tray or 20 min, whichever came first. The CS was a monopolar, 100 Hz pulse stimulation that was delivered epidurally as a train of continuous biphasic, charge balanced, and asymmetric pulses every 10^4 μs. Each biphasic, square pulse delivered voltage every 100 μs (first phase) and then the voltage was off for 9900 μs (second

phase). Stimulation amplitudes were adjusted as needed to accommodate changes in movement thresholds. No stimulation was delivered to the rats in the RT only group.

Intracortical Microstimulation Mapping

Intracortical microstimulation (ICMS) mapping of the motor cortex was used to reveal the functional integrity and organization of the injured motor cortex. ICMS evokes motor movements via direct and transynaptic activation of corticospinal neurons[7, 8]. The organization and size of movement representations is thought to reflect intracortical synaptic connectivity among neurons contributing to the movement. Thus, the loss of movement representation area likely reflects a loss or disruption of the connections that contribute to the movement. In intact brains, skilled reach training increases the distal forelimb (wrist & digit) representation in the motor cortex, as well as the number of synapses in this area [9]

Within 4 days of the last training session, animals were fully anesthetized with ketamine (i.p., 90-110 mg/Kg) and xylazine (i.p., 9-10 mg/kg) and received supplemental doses of ketamine (10-20mg/KG) or isofluorane gas (~.15%) to maintain appropriate levels of anesthesia. Electrodes were removed, skull was thinned over the SMC and was gently removed. The exposed cortex was covered in silicone oil (37°C) and cisterna magna was punctured to reduce brain swelling. A digital image of the exposed brain was taken and overlaid with a grid demarcating 500 um increments in order to demarcate the location of evoked movements. Movements were evoked via a platinum wire that was inserted into a glass microelectrode filled with 3M NaCl. A hydraulic microdrive was used to lower the electrode 1550 μm (layer V) to make systematic penetrations in the cortex 500μm apart. At each site, brief trains of stimulation (13, 200ms cathodal pulses delivered at 350Hz) were delivered from an electrically isolated stimulation unit. The current was increased until a discrete visible movement was observed or maximum amplitude of ≤60μA was reached. To further assess whether there were group differences in the threshold to elicit movement representations, we systematically tested the effects of stimulating up to 100 μA at any site in the injured cortex that was non-responsive at ≤ 60 μA. We used the total number of sites that could be elicited at ≤100 μA in assessing overall motor map plasticity. A surgical assistant was continuously monitoring the plane of anesthesia and was responsible for identifying the evoked motor movement. Both experimenters were blind to behavioral groups. The entire extent of the rostral and caudal forelimb areas and jaw and neck were mapped. At the conclusion of mapping procedures, animals were overdosed with sodium pentobarbital.

There were no significant differences between groups in total amount of ketamine [$F(1,16)=2.40$, $p = 0.631$] administered. Group means for ketamine administered during ICMS procedures are CS+RT = 162.7± 13.0 mg and RT = 175.0± 23.0 mg. There were also no differences in the total amount of xylazine administered during ICMS procedures between groups [$F(1,16)= 0.012$, $p = .915$]. The mean total amount of xylazine given per group is: CS+RT = 7.8± .7 mg and RT = 8.0± 1.1 mg. Thus, it is unlikely that differences in anesthetic level accounts for the results reported.

Histology

Following training, all animals underwent ICMS mapping and were then overdosed with sodium pentobarbital and transcardially perfused with .1 M phosphate-buffered saline (PBS) and 4% paraformaldahyde in the same buffer. Brains were removed and sectioned on a vibratome rostral to caudal throughout the cerebrum. Six rostral–caudal sets of 50 μm coronal sections through the cerebrum were collected and stored in cryoprotectant solution at -20°C before use. Each sixth section was placed in PBS for immediate slide mounting, and this set was stained with toluidine blue (0.25%; Sigma-Aldrich, St. Louis, MO), a Nissl stain, and used for the analysis of remaining cortical volume (an indirect measure of contusion size). Three animals were not included in analysis due to ICMS damage of tissue: RT+CS = 2 and RT = 1.

Measurement of remaining cortical volume

Injury size was inferred from volume measurements of remaining non-necrotic/nongliotic ipsiinjuryin the injured hemisphere cortex and the volume difference between ipsi- and contralesional cortices using Nissl stained coronal sections. Measurements included the first section caudal to the appearance of the forceps minor of the corpus callosum (~ +2.7mm anterior to Bregma) and eight additional caudal sections (to approximately 3.3 posterior to Bregma), each section was 600 μm apart. In each section, all remaining cortex and striatum was outlined using Neurolucida (MBF Bioscience) perimeter tracing software option, at a final magnification of 17X, to obtain cortical and striatal area measurements. This sampling scheme focuses the volume estimates in the SMC region, but does not limit the measurement to SMC because it is not possible to define the boundaries of this cortical region with great precision. Volume was then calculated using the Cavalieri method (Gundersen et al., 1988), as the product of the summed area and the distance between section planes. Injury size was calculated as the volume of the non-injured cortex-the injured cortex.

Statistical Analyses

The behavioral and histological data were collected from the same animals. Behavioral data were analyzed using SPSS (SPSS, Inc., Chicago, IL) repeated-measures analyses of variance (ANOVAs). Volume was analyzed using one-way ANOVAs. Significance levels were set at α level 0.05.

References

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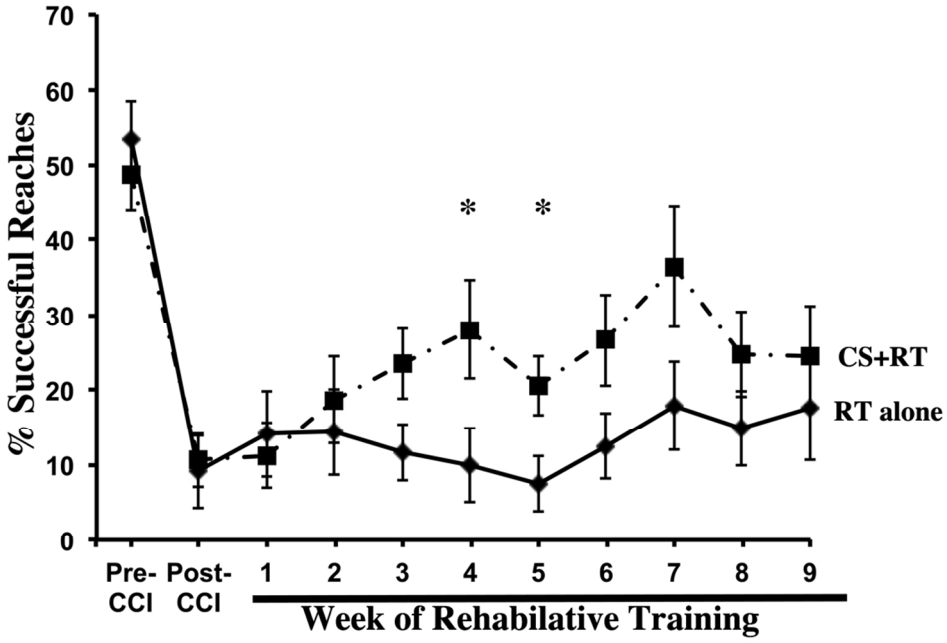

Following a unilateral CCI centered over the CFA, reaching performance with the impaired forelimb drastically declined. All animals improved after 9 weeks of RT, although far from pre-injury levels. CS+RT significantly increased reaching performance compared to RT alone. Data are means ±SEM. *p ≤ 0.05.
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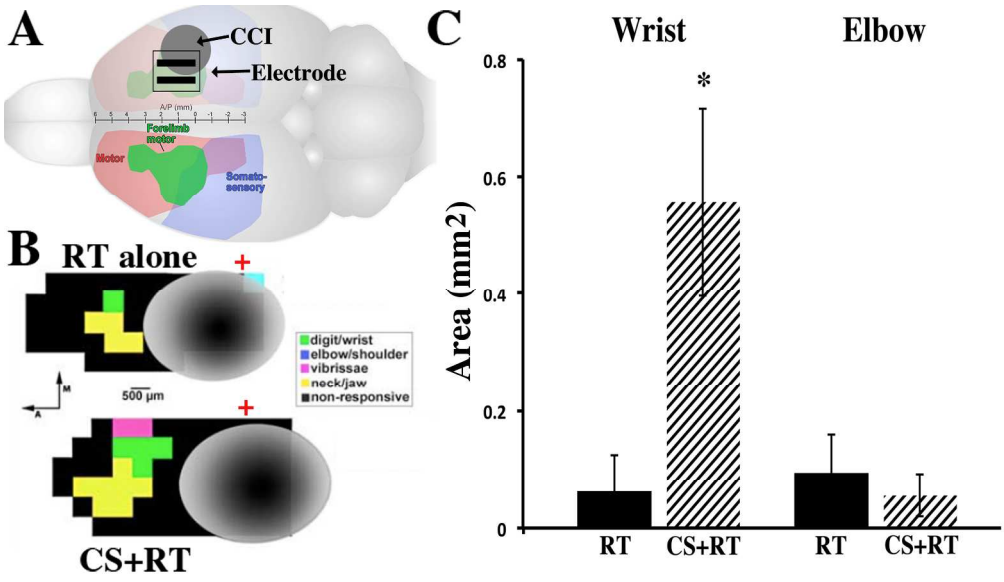

A, As seen in the rat brain schematic, CCI (grey circle) was induced over the forelimb overlap area of the sensory motor cortex (arrow). Epidural electrode contacts (parallel black bars) were placed rostral and medial to the injury over the remaining forelimb area (CFA and RFA, in green). B, Representative ICMS derived motor maps surrounding injured tissue (grey) following 9 weeks of RT alone (top) and CS+RT (bottom). Green squares are wrist, yellow are jaw, pink are whisker and black are non-responsive sites. Red crosses denote Bregma. C, CS significantly increased the total area of wrist movement representation compared to RT alone, but not elbow, movement representation area. Data are means ±SEM. *p<0.05
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