Spinal cord associative plasticity improves forelimb sensorimotor function after cervical injury

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Abstract

Associative plasticity occurs when two stimuli converge on a common neural target. Previous efforts to promote associative plasticity have targeted cortex, with variable and moderate effects. In addition, the targeted circuits are inferred, rather than tested directly. In contrast, we sought to target the strong convergence between motor and sensory systems in the spinal cord.

We developed spinal cord associative plasticity (SCAP), precisely timed pairing of motor cortex and dorsal spinal cord stimulations, to target this interaction. We tested the hypothesis that properly timed paired stimulation would strengthen the sensorimotor connections in the spinal cord and improve recovery after spinal cord injury (SCI). We tested physiological effects of paired stimulation, the pathways that mediate it, and its function in a preclinical trial.

Subthreshold spinal cord stimulation strongly augmented motor cortex evoked muscle potentials at the time they were paired, but only when they arrived synchronously in the spinal cord. This paired stimulation effect depended on both cortical descending motor and spinal cord proprioceptive afferents; selective inactivation of either of these pathways fully abrogated the paired stimulation effect. SCAP, repetitive pairing of these pathways for 5 or 30 minutes in awake rats, increased spinal excitability for hours after pairing ended. To apply SCAP as therapy, we optimized the parameters to promote strong and long-lasting effects. This effect was just as strong in rats with cervical SCI as in uninjured rats, demonstrating that spared connections after moderate SCI were sufficient to support plasticity. In a blinded trial, rats received a moderate C4 contusive SCI. Ten days after injury, they were randomized to 30 minutes of SCAP each day for 10 days or sham stimulation. Rats with SCAP had significantly improved function on the primary outcome measure, a test of dexterity during manipulation of food, at 50 days after SCI. In addition, rats with SCAP had persistently stronger responses to cortical and spinal stimulation than sham stimulation rats, indicating a spinal locus of plasticity. SCAP rats had lasting improvements in H-reflex

modulation, signifying decreased hyperreflexia. The groups had no difference in the rat grimace scale, a measure of pain.

We conclude that SCAP strengthens sensorimotor connections within the spinal cord, resulting in decreased hyperreflexia and improved forelimb function after moderate SCI. Since both motor cortex and spinal cord stimulation are performed routinely in humans, this approach can be trialed in people with SCI or other disorders that damage sensorimotor connections and impair dexterity.

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Running title: Spinal cord associative plasticity

Keywords: plasticity; spinal cord; motor cortex; electrical stimulation; injury

Abbreviations: AAV = Adeno-Associated Virus; ADM = Abductor Digiti Minimi; CDP = cord dorsum potential; CNO = Clozapine-N-Oxide; CST = Corticospinal Tract; DREADD = Designer Receptor Exclusively Activated by Designer Drug; DRG = Dorsal Root Ganglion; IBB = Irvine, Beatties, and Bresnahan scale; MEP = Motor Evoked Potential; RGS = Rat Grimace Scale; SCAP = Spinal Cord Associative Plasticity; SCI = Spinal Cord Injury.

Introduction

Integration of sensory feedback with motor commands is required for skilled movement. Neuromodulation strategies have been developed to target sensorimotor integration in the cortex. Paired associative stimulation repetitively pairs median nerve stimulation with motor cortex stimulation timed to increase cortical excitability in healthy people[1] and improve arm and hand function after injury[2,3]. Using a similar logic but targeting the spinal cord, epidural stimulation has

been used to drive activation of spinal afferents that can be timed to converge with descending motor activation. This strategy helped to restore walking after SCI, but only when spinal stimulation was timed to coincide with motor cortex signals arriving in the spinal cord[4–6]. Our goal is to employ associative learning through pairs of electrical stimuli to strengthen residual connections after cervical SCI, the most common type[7]. For people with cervical SCI, recovery of arm and hand function is the highest priority[8].

In contrast to paired stimulation for sensorimotor integration, trials of paired stimulation targeting the spinal cord have targeted the motor system alone. Electrical stimulation of motor cortex has been paired with peripheral nerve stimulation to backfire spinal motoneurons. This motor system stimulation promotes lasting changes in motor evoked potentials and improved grip strength and finger control in people[9–11]. Intraspinal stimulation in monkeys has been timed with cortical activity so endogenous brain activity and exogenous spinal cord stimulation converge[12,13]. Repetitive pairing induced a small but significant increase in evoked potentials.

Since integration of descending motor control with spinal sensory feedback is critical for skilled movement, we targeted these interactions by pairing motor cortex and cervical spinal cord stimulation to mediate SCAP[14]. The convergence of subthreshold spinal cord electrical stimulation with a descending cortical volley robustly augmented the cortical motor evoked potentials (MEPs). When this pairing was performed repeatedly, there was an increase in excitability that lasts for at least an hour. This plasticity required paired stimulation delivered at the proper time, but the exact neural circuits that mediate the sensorimotor interactions are not known. Knowing the targets is critical to ensure strong and selective engagement and to understand the anatomical basis for SCAP. It is also not known whether the sparse connections that are spared after SCI are sufficient to support SCAP and recovery of function.

Spinal epidural stimulation offers several advantages as a target to activate sensory afferents. To test how SCAP works in awake and freely moving rats, we created spinal stimulating electrodes that can be inserted into the thin epidural space over the cervical spinal cord, are supple enough to conform to the spinal cord, and move with the neck[15,16]. These were used to measure and modulate the nervous system, which can be challenging in awake animals due to variability in MEPs[17]. We optimized the SCAP protocol to counter this variability and to improve efficacy. Together, these

experiments were designed to improve our understanding of SCAP mechanisms and to test its efficacy for recovery of dexterity after SCI.

Materials and methods

A detailed materials and methods are included in the supplementary text.

In awake and freely moving rats, we tested spinal cord associative plasticity (SCAP) and its efficacy on physiology and function in rats with SCI. First, we tested the motor cortex and spinal cord stimulation timing with the hypothesis that convergence in the spinal cord would produce the largest changes in physiology. Second, we tested the necessity of corticospinal and segmental afferent connections for paired stimulation by selectively inactivating each using chemogenetic tools. We then paired motor cortex and spinal cord stimulation repeatedly to induce plasticity (SCAP). We optimized the protocol by varying the frequency, number, and pattern of stimuli in uninjured rats. We tested SCAP in rats with SCI. Finally, we performed a randomized, blinded preclinical trial of 10 days of SCAP versus sham stimulation to determine the effects on physiology and behavior after a moderate C4 contusion SCI. The primary outcome measure was a behavioral test of food-manipulation; several physiological, behavioral, and anatomical measures were secondary.

Timing: In adult Sprague-Dawley female rats, we implanted three sets of electrodes: epidural screw electrodes over each motor cortex, EMG electrodes in each biceps muscle, and spinal epidural electrodes over the midline of dorsal C5-C6 spinal cord. To determine whether subthreshold spinal cord stimulation augments cortical motor evoked potentials (MEPs), we compared cortex stimulation only to motor cortex stimulation paired with spinal cord stimulation. We determined the threshold for provoking an MEP from motor cortex and from the spinal cord. We measured the time from motor cortex stimulation to the spinal cord dorsum potential (CDP) (Fig. 1A1). We then quantified the biceps MEP as the area under the curve in response to stimulation at 110% of threshold intensity (Fig. 1A2). In uninjured rats, this was compared against motor cortex stimulation paired with spinal cord stimulation at 90% of threshold intensity (Fig. 1B). This experiment quantified the size of the MEP with paired stimulation relative to cortex

stimulation alone using unpaired t-tests with Bonferroni correction. We also tested whether stimulating motor cortex at 90% of threshold with spinal cord stimulation also at 90% would provoke an MEP together when neither produced a response on their own. The magnitude of the MEP was compared to that of motor cortex stimulation at 110% of threshold (Fig.1C). Finally, we tested the effects of paired stimulations in rats, weeks after a moderate C4 contusion injury (Fig.1D, using the same methods as Fig.1B but in rats with SCI).

Inactivation: To target the corticospinal tract (CST) selectively, a Cre-dependent adeno-associated virus (AAV) encoding the Designer Receptor Exclusively Activated by Designer Drug (DREADD) was injected into the motor cortex and another encoding Cre-recombinase was injected into the cervical spinal cord (Fig. 2A1). Thus, only doubly infected neurons express the DREADD (Fig. 2A2), which can be inactivated by its ligand, clozapine-N-oxide (CNO)[18,19]. One week after AAV injections, animals were trained on the vermicelli handling task[20,21]. After reaching a stable baseline performance, the number of forepaw manipulations was measured before, during, and after inactivation (Fig. 2A3). One week after the behavioral test, we tested the effects of inactivation on the immediate effects of paired motor cortex and spinal cord stimulation (Fig. 2A4). Details in supplementary methods.

The necessity of afferents for the immediate effects of paired stimulation was tested using two inactivation strategies. For the first strategy, large diameter dorsal root ganglion (DRG) neurons were inactivated (Fig. 2B1). Large DRG neurons mediate touch, proprioception, and muscle length/tension[22]. The specificity of large-diameter DRG neurons was provided by the tropism of the AAV5[23]. An AAV5-hSyn-hM4D(Gi) was injected into each DRG at C5, C6, and C7 on one side of the spinal cord after partial laminectomy (Fig. 2B2). At the time of virus injection, stainless steel screw electrodes were placed over each motor cortex, spinal array on the epidural surfaces of C5-C6 cervical spinal cord, and EMG wires in the biceps muscle bilaterally. CNO was administered as described in the supplementary method, and responses to paired stimulation was measured (Fig. 2B3).

For the second strategy, we targeted the proprioceptive afferents, since these mediate the effects of spinal cord stimulation (Fig. 2C1)[24–26]. We used a genetic strategy to target DRG neurons that express parvalbumin, which are proprioceptors[27,28]. In transgenic rats that express Cre recombinase under the control of parvalbumin promoter PV-Cre rats (LE-Tg(Pvalb-iCre)2Ottc),

we injected a Cre-dependent DREADD (AAV5-hSyn-DIO-hM4D(Gi)-mCherry[29] into the C5, C6, and C7 DRGs as described above (Fig. 2C2). Inactivation with CNO and physiology measurement were also identical (Fig. 2C3).

Repetitive pairing (SCAP): We tested brain and spinal cord responses at baseline and then after the period of repetitive pairing (Fig. 3A1-A3), as we did in Mishra et al.,[14] but in awake rats with implanted electrodes. This differs from the immediate effects of stimulation (Fig. 1A3). Before modulation, we measured the stimulation threshold for both motor cortex and spinal cord, defined as the stimulation intensity needed to produce an MEP in 50% of trials. We then stimulated with 110% of threshold to determine cortical and spinal MEPs, which were quantified as the area under the curve. At baseline, cortical and spinal MEPs were measured singly and independently (Fig. 3A1). For repetitive pairing (Fig. 3A2), motor cortex stimulation was performed at 110 % of the threshold for evoking a cortical MEP followed 10ms later by spinal cord stimulation performed at 90% of the threshold for generating a spinal MEP. After repetitive pairing (Fig. 3A3), the same measures at baseline were taken immediately after pairing and every 20 min thereafter up to 120 min. We quantified excitability as 1/threshold and expressed it as % change from baseline.

Optimization: In order to maximize the efficacy of the SCAP protocol (5-minute SCAP, Fig 3B1), we varied four stimulation parameters: frequency, number of stimuli, number of trains, and time between trains. We kept the total stimulation period to 30 minutes, a period that could be used for stimulation in people. In two rats implanted with cortical and spinal electrodes, we first varied frequency by delivering 10 stimuli at 5Hz, 2 Hz, 1Hz, 0.5Hz and 0.2Hz and measured the size of the MEPs (Supplementary Fig. 3A). For the other parameters, we measured the change in cortical MEPs and cortical and spinal excitability. Spinal excitability was used since this was less variable than spinal MEPs, and out of concern that eliciting spinal MEPs might induce plasticity. For these trials, we used 5 Hz pairing and varied the number of stimuli by comparing 150, 75 & 38 pulses (Supplementary Fig. 3B, C & D1-D3). For subsequent trials, we used 150 stimuli, which we term a train, and varied the number of trains, either 10 or 20 (Supplementary Fig. 3B, C & F1-F3). For subsequent trials, we used 20 trains and varied the time between trains, either 1 or 5 minutes (Supplementary Fig. 3B, C & E1-E3).

Cervical spinal cord contusion (SCI): The C4 spinal cord contusion surgery was performed as previously described[30]. Briefly, the C3 to C5 vertebrae were exposed by skin incision and muscle separation, and laminectomy of C4 was performed. An Infinite Horizon impactor (IH-0400, Precision Systems & Instrumentation, LLC), was used to deliver 200 kilodynes force onto the spinal cord with a 3.5mm diameter tip. The force curve of the impactor was recorded (Fig. 7A). After the injury, the spinal electrode array was implanted as described above and rats received intense care, as described in supplementary method.

Preclinical trial: We designed a preclinical efficacy trial; the timeline is shown in Fig. 4A. Ten days after SCI, rats were randomized to receive real or sham SCAP for 10 days, a period that we have previously shown to be therapeutic[30,31]. The daily stimulation duration (30 minutes) and the number of sessions (10) were designed to be clinically viable. The primary outcome measure was the dexterity (Irvine, Beatties, and Bresnahan scale[32]) score, and secondary outcomes included performance of skilled walking, changes in MEPs (immediate effects), thresholds, lasting effects of pairing in stimulated rats, H-reflex modulation (a measure of hyperreflexia), and a pain measure (RGS). The study was powered based on the effect size we observe of a different neuromodulation strategy[30]. A priori power analysis suggested we need 8 rats in each group to achieve a power of 0.8. Rats were pseudorandomized into real (Stim) and sham (Control) groups with each cohort of up to 8 rats having rats assigned to each group. Rats were randomized immediately after SCI surgery, and experiments were carried out with blinded measures.

Behavior: Food Manipulation Task: To assess the rat's ability to manipulate, we used the Irvine, Beatties, and Bresnahan scale (IBB;[32,33], as we did previously[30]. **Horizontal ladder walking task**: The horizontal ladder walking task measured paw placement accuracy on irregularly spaced rungs, a task that requires the corticospinal tract[31,34–36]. **Rat Grimace Scale (RGS):** This scale is used to quantify pain based on rat facial characteristics in a way that is commonly used to assess pain in people[37].

Physiology: The immediate effects of pairing (Fig. 1A3) as well as thresholds were tested before the 30-mintue SCAP on each day of stimulation and every two weeks thereafter. The lasting effect of SCAP (Fig. 3A1) was measured on day 11 after SCI.

H-reflex testing: To track hyperreflexia, we measured the H-reflex at baseline, one week after SCI, just after 10 days of stimulation, and every two weeks post-stimulation (Fig. 4A). First, we tested responses to increasing stimulus intensities up to 2X threshold, which activates low threshold afferent fibers. Next, we tested the frequency-dependent depression of the H-wave[38].

Anatomy: To determine the severity of SCI, we measured impact force during spinal cord contusion. We quantified the volume of spared gray and white matter at the injury site. We also quantified spared axons below the lesion in both groups. For CST axon quantification, BDA was injected into cortex and histology was performed on sections above (C3) and below (C7) the lesion site as in our previous studies[30]. For sensory axon quantifications, AAV1 was injected in C5-C7 DRG, and sensory afferent axons were quantified below the lesion segments.

Statistical Analyses: All the comparisons between stimulation conditions were done with paired/unpaired t-tests with Bonferroni correction or One-way ANOVA with Bonferroni corrections and Cohen's d was used effect size. All the statistical analysis was performed with SPSS (SPSS Statistics for Windows, version 27; IBM Corp., Armonk, NY, USA). We tested the normality of the data using the Shapiro-Wilk test. All data are expressed as Mean ± SEM.

Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Results

We hypothesized that repetitive pairing of motor cortex and spinal cord would augment spinal excitability and improve function in rats with SCI through spinal sensory and descending motor convergence in the spinal cord. This hypothesis was tested in steps. First, we tested whether convergence of paired stimulation was more effective in cortex or the spinal cord by altering the timing for paired stimulation. Second, we tested whether the connections preserved by cervical SCI were sufficient to enable paired stimulation. Third, to determine whether the CST and proprioceptors were necessary, we selectively inactivated each. Fourth, we optimized the repetitive application of paired stimulation (SCAP) protocol. Fifth, we tested this optimized protocol in rats

with SCI. Finally, we tested whether 10 days of repetitive SCAP improved physiology and behavior after SCI.

Spinal stimulation augments cortical responses when properly timed

We hypothesized that stimulating the spinal cord at the time that descending motor potentials arrive would most strongly augment cortical MEPs. The latencies shown in Fig. 1A1 are the averages of 20 responses each from three animals, while the waveforms are for a representative trial in one animal. As shown in Fig. 1A1, recorded C5–C6 spinal cord responses to cortical stimulation in awake rats. After the initiation of a train of 3 stimuli in the motor cortex, the CDP arrived at 9.17± 0.55ms (Mean ± SEM, n=3). Spinal cord stimulation augmented cortical MEPs at multiple time points (Fig. 1B) with a strong peak when the spinal cord was stimulated 10ms after the motor cortex (155% ± 27%, *df* =10, *Cohen's d* =14.32, $p < 0.0001$). There were also smaller increases in MEPs when the spinal cord was stimulated 20ms before cortex (17% ± 6%, *df* =16, *Cohen's d* =13.50, p = 0.019).

If the effects of motor cortex and spinal cord stimulation are synergistic, then subthreshold stimulation at each site may result in a suprathreshold response if they are properly timed. To test this hypothesis, stimulation intensities for both cortical and spinal stimulations were set to 90% of the threshold. Stimulation of the spinal cord only and motor cortex only produced no responses (Fig. 1C, insets left). Paired subthreshold stimulation generated an MEP with a peak effect at 10ms. The response was larger than cortical stimulation only at 110% of threshold (66 ± 38% larger; inset right) of 110% cortical threshold, p = 001; paired t-test). Thus, the synergistic effects of subthreshold stimulation were observed only with convergence in the spinal cord.

Neural circuits spared by SCI are sufficient to mediate the effects of paired stimulation

To determine if the neural circuits spared by SCI were sufficient to enable paired stimulation, we tested paired stimulation in rats with moderate C4 spinal contusion. Just as in Fig. 1A, 110% cortical stimulation was paired with 90% spinal stimulation. Augmentation of MEPs was observed with a peak at 10ms latency (Fig. 1D; 134 ± 20%, p = 0.0001, paired t-test). No augmentation was observed at any latency in which spinal cord was stimulated before cortex. This supports the

hypothesis that the spinal cord pathways spared by contusion can mediate the effects of sensory-motor convergence in the spinal cord.

Effects of paired stimulations are mediated by corticospinal and proprioceptive afferents.

To test the hypothesis that descending corticospinal circuits and large-diameter afferents are necessary for effects of pairing, we selectively inactivated each with a chemogenetic tool. In rats with injections into motor cortex and spinal cord (Fig. 2A1), layer 5 neurons in the motor cortex were selectively and robustly labeled (Fig. 2A2). These neurons were inactivated by administration of CNO, the ligand for the inhibitory DREADD. At the time of maximum inactivation (30 min; Fig. 2A3) the number of manipulations the rat made while eating uncooked pasta with the paw targeted for inactivation diminished to 39±7%. The number of manipulations partially recovered by 120 minutes, as the CNO washed out. Manipulations of the non-targeted paw were not highly affected. We then tested whether this inactivation abrogated the effects of paired stimulation (Fig. 2A4). At baseline, subthreshold spinal cord stimulation delivered 10ms after suprathreshold motor cortex stimulation strongly augmented biceps MEPs (>100%). Administration of CNO caused the MEP augmentation to be diminished compared to baseline, and at 30 minutes, spinal cord stimulation had no augmenting effect on motor cortex MEPs on the inactivated side. The effects of pairing recovered by the end of the 120-minute testing period. On the non-inactivated side, there was a less pronounced and more delayed effect of inactivation. Thus, corticospinal neurons are necessary for the effects of paired stimulation.

To determine if paired stimulation also depends on spinal afferents, we inactivated dorsal root ganglia (DRG) neurons using two methods. First, we targeted large-diameter neurons; an AAV5 encoding an inactivating DREADD was injected into DRGs of C5 and C6 on one side (Figure 2B1). Cells infected with the virus (Fig. 2B2) measured 68±18μm in diameter, like neurons that mediate touch, proprioception, and muscle length/tension[22]. Before inactivation, the pairing of the motor cortex and spinal cord stimulation caused a large augmentation of the biceps MEP (>100%; Fig. 2B3, red) compared to cortical stimulation only. Thirty minutes after CNO injection, the paired stimulation effect was abolished (-1.5% ±10.5%, $df = 4$, *Cohen's d* = 2.34, $p = 0.013$) and largely returned by 120 minutes. There was no significant effect on the side without inactivation.

For the second approach, we selectively targeted the proprioceptors. A Cre-dependent DREADD was injected into the DRGs (Fig. 2C1) of transgenic rats expressing Cre under control of parvalbumin, which is expressed selectively by proprioceptors (Fig. 2C2). Inactivation caused a large-scale, but incomplete, abrogation of paired stimulation (*28.6% ± 3.8%, df = 4, Cohen's d = 11.81, p = 0.017*; Fig. 2C3, green). Inactivation also decreases the motor cortex excitability (supplementary fig. 1A), spinal MEPs (supplementary fig. 1B), and spinal excitability (supplementary fig. 1C) in PV-Cre rats. The loss of paired stimulation effect with inactivation of proprioceptors was less than inactivation of many large-diameter DRG neurons (including mechanoreceptors and others). Thus, large-diameter sensory afferents, and proprioceptors in particular, are necessary for the effects of SCAP.

To ensure that the effects of inactivation were due to loss of function of specific circuits, we conducted two control experiments. We tested whether CNO injection itself changed physiological responses. In a blinded test of rats without viral injection, CNO had no effect on physiological responses (supplementary fig. 2A). In addition, administration of saline in rats with DREADD injection showed no change in paired stimulation effects on cortical MEPs, cortical excitability, spinal MEPs, or spinal excitability (supplementary fig. 2B1-4). Thus, neither CNO without DREADD administration nor DREADD injection without CNO affects physiological responses.

SCAP causes lasting changes in cortical and spinal cord evoked responses in awake rats with and without SCI.

In addition to the immediate effects of paired stimulation, repeatedly pairing motor cortex and cervical spinal cord stimulation at 10ms latency promotes spinal cord associative plasticity (SCAP). In these experiments, cortical and spinal responses were tested before and after a period of SCAP (Fig. 3A). SCAP delivered over 5 minutes (3B1) nearly doubled the size of both the cortical and spinal MEPs (3C1, dark and light brown lines), an effect that ebbed over 120 minutes. These changes were of a similar magnitude but much longer duration than we previously reported; anesthetized rats had no increase in MEPs after 60 minutes[14].

We also tested the effects of SCAP in rats with SCI. Following the 5-minute SCAP protocol, rats with SCI also showed lasting increases in the size of cortical and spinal MEPs (Fig. 3C1, dark and light blue lines). Similar effects were also observed with cortical and spinal thresholds in intact

and SCI rats (Supplementary Fig. 4A). We compared the average effects of paired stimulation delivered at 10ms latency versus several control conditions: motor cortex stimulation alone, spinal cord stimulation alone, pairing at 100ms latency, and 10ms pairing in intact and SCI rats (3D1). The control conditions did not induce plasticity (AVOVA with Bonferroni correction; $p=0.65$). However, both intact and SCI rats had significant increased cortical MEPs compared with these control conditions (AVOVA with Bonferroni correction $p< 0.001$); there was no significant difference between intact rats and rats with SCI (Intact=38±11; SCI=18±10; AVOVA with Bonferroni correction; $p=0.11$). The change in cortical MEP was averaged across the 120 minutes of testing. Thus, the circuits spared by cervical SCI were sufficient to enable SCAP.

In anticipation of using SCAP in a preclinical therapeutic trial, we optimized the protocol. We kept the duration to a maximum of 30 minutes, a period that is tolerable for clinical neuromodulation[39]. We tested 4 parameters: frequency of pairing, pulse number (length of train), time between trains and number of bursts/trains (supplementary Fig. 3). We tested five frequencies to determine if each pair of stimuli would cause inhibition or augmentation of subsequent stimuli. The 5Hz and 0.2Hz frequencies produced little change between stimuli, and we selected 5Hz to minimize the total duration of stimulation. We also observed that 1Hz was strongly inhibitory. Using the 5Hz frequency, we varied the number of stimuli; 150 stimuli were better than the others tested. We tested the time interval between bursts of 150 stimuli at 5 Hz; we selected 1 minute to balance effect size and the total duration of stimulation. Finally, we tested the total number of bursts. We observed that 20 trains of stimulation had a more durable effect than 10 trains with a similar overall magnitude. This resulted in the paradigm pictured in Figure 3B2 which delivers 3000 pairs of stimuli over 30 minutes.

We tested the effects of 30-minute SCAP in intact rats and rats with SCI. In intact rats, cortical and spinal MEPs were doubled immediately after 30-minute SCAP (Fig. 3C2, dark and light blue lines), and they were still ~50% elevated at 120 minutes. In SCI rats, following the 30-minute SCAP protocol (3B2) the size of both the cortical and spinal MEPs was increased 100-200% (3C2, dark and light brown lines), and the MEPs were still almost double at 120 minutes. Similar effects were also observed with cortical and spinal excitability in both intact rats and those with SCI (supplementary fig. 4B). The physiological effects of the 30-minute SCAP paradigm are more robust than the 5-minute SCAP paradigm in both intact and injured animals. Further, we compared the efficacy of 30-minute SCAP to control conditions (motor cortex alone, spinal cord alone, or

paired with spinal cord stimulation after a 100 ms delay) and convergence in motor cortex. Neither the control conditions nor convergence at cortex increased cortical or spinal MEPs (AVOVA with Bonferroni correction; p=0.20; Fig. 3D2) or changed cortical or spinal thresholds (supplementary Fig. 5). However, both intact and SCI rats had a significant increased cortical MEPs after 30-minute SCAP compared with these control conditions (AVOVA with Bonferroni correction p< 0.0001; Fig 3D2). There was no significant difference between intact rats and rats with SCI (Intact=82±9%; SCI=121±11%; AVOVA with Bonferroni correction; p=0.15). Finally, the similar magnitude and duration of effects in intact and injured rats suggest that circuits spared by SCI are sufficient to mediate pairing effects.

Repetitive SCAP after SCI improved forelimb dexterity without changing affective pain.

To determine whether SCAP might be used to restore sensorimotor function after SCI, we performed a preclinical efficacy trial (Fig. 4A). The pre specified primary outcome measure was the IBB test, a scale that measures paw function while eating pieces of cereal (Fig. 4B1). Before SCI, all rats performed at the top of the 9-point scale (Fig. 4B2). Twenty-eight days after SCI, the rats in each group showed severe impairment (stimulation=2.46±21; control=2.38±34), with difficulty holding and manipulating the cereal. By 50 days after injury, the last time tested, control rats scored 3.76±0.49, and rats with stimulation scored 5.18±0.25 (50days; *df*=12, *Cohen's d*=2.62, $p = 0.024$) demonstrating better grasp and support of the cereal (Fig. 4B2).

Our secondary behavioral outcomes were walking performance on the horizontal ladder (4C) and observed pain (4D). Rats performed equally before the injury. For walking, by 28 days, the first time that rats reliably perform the task, control rats made 73±7% errors, while rats with stimulation made 61±5% errors. At the end of the testing period, control rats made 66±8% errors, and rats with stimulation made 50±8% errors; this difference was not significant (50 days: *df* = 12, *Cohen's d* = 200.66, $p = 0.182$). To test the effects of stimulation on pain, we used the RGS, which uses facial features to rate severity on a three-point scale. All rats exhibited pain after SCI (Fig. 4D), despite being given analgesics, which diminished by 10 days and stayed low. There was no difference in pain scores between the groups at the end of the trial (50 days: *df* = 9, *Cohen's d* = 0.093, $p = 0.646$). Thus, ten days of SCAP improved dexterity and did not produce pain after SCI.

SCAP caused lasting increases in evoked responses and improved hyperreflexia over weeks.

We hypothesized that SCAP delivered daily over 10 days in rats with SCI would strengthen sensorimotor connections, as measured by evoked responses. We measured the immediate effect of paired stimulation; how strongly spinal stimulation augments the cortical MEP as the time they are paired. Since paired MEPs are always compared to cortical stimulation alone, this measure is stable and reflects the strength of convergent stimuli. In rats with SCI, the immediate effect of pairing was large at 11 days after SCI (Stim=162±22%, Control=153±11%) in both groups before the start of stimulation (Fig. 5A). In the stimulation group, cortical MEPs increased over the 10-day period to 259±12%, while the control group did not change (168±5.4%). The difference between groups was maintained at 50 days after SCI (Stim= 258%±21% vs Control = 169%±10%, $df = 12$, *Cohen's d* = 3.10, $p = 0.0001$). This indicates that repetitive pairing over a 10-day period creates a durable change in MEPs from paired stimulation.

We also measured cortical and spinal thresholds over the study. Cortical thresholds before SCI were above 1.2 mA in both stimulation (red line) and control (black line) rats (Fig. 5B). After SCI there was ~50% drop in the cortical thresholds in both groups of rats (ANOVA, $p<0.001$). After each 30-minute SCAP session, the cortical threshold (red dot) was lower than when it began (AVOVA, $p<0.001$; red line). There was a gradual increase in cortical threshold until the end of the study period. Similarly, the spinal thresholds (Fig. 5C) immediately after SCI were low in both stimulation (red line) as well as control (black line) rats (electrodes were implanted just after SCI). There was a lower threshold in the rats with stimulation compared to control rats (AVOVA, $p<0.001$, red line vs black line). There was a gradual increase in spinal threshold over the study period, as we have observed in implanted arrays[15].

We hypothesized that repetitive stimulation would help restore spinal reflex modulation. We tested H-reflex modulation before and after SCI (Fig. 6A). H-reflexes were elicited at different interstimulus intervals to modulate responses (Fig. 6B). Before injury in both rats with SCAP (Fig. 6C) and controls (Fig. 6D), shorter intervals between H-reflexes caused the second response to be diminished (blue lines). SCI diminished the rate-dependent decrease in the H-reflex (top lines), consistent with hyperreflexia. Ten days of SCAP largely restored rate-dependent depression of the H-reflex (6C; bright red lines). In contrast, control rats had little to no recovery of H-reflex

modulation (6D; gray lines). This effect was still persistent at 50 days after SCI (control 63%±1.5% vs Stim 45%±1.7%; *df* = 12, *Cohen's d* = 4.33, $p = 0.0001$). Thus, SCAP decreased hyperreflexia after SCI.

No difference in SCI and tissue sparing between stimulation and control groups.

To determine severity of SCI, we quantified the peak force rats received during C4 spinal contusion and the tissue preserved at the injury site at the end of the trial. The peak force that rats received is shown in Fig. 7A. There was no significant difference between the stimulation group (203.57±5.81 kilodynes) and the control group (209.1±7.13 kilodynes; t-test, $p = 0.28$). We also compared the amount of tissue spared at the site of injury. Representative spinal cord sections are shown in Fig. 7B. There was no significant difference between the control (3.13±0.46 mm^2) and the stimulation group (2.76±0.53 mm^2, independent t-test, $p = 0.61$). We concluded that the injuries rats received are similar between stimulation and control groups. We quantified the length of the spared descending motor and sensory afferent axons below the lesion in both groups. We did not observe a significant deference in total axon length of both descending motor or segmental sensory afferent axon lengths in between stimulation and control groups (data not shown). We conclude no difference in lesion and tissue sparing in between the groups after SCI.

Discussion

Our main hypothesis proved true: repetitive pairing of motor cortex and spinal cord augmented spinal excitability and improved function in rats with SCI through spinal sensory and descending motor convergence. Paired stimulation produces effects on 3-time scales: immediate, hours, and weeks. The immediate effects (Fig. 1) do not represent plasticity; they operate only at the time that subthreshold spinal stimulation augments cortical MEPs. For paired stimulation to promote plasticity, it must be repeated (SCAP). Our optimized repetitive stimulation protocol produces robust augmentation that was still 50% greater than baseline at 2 hours after 30 minutes of pairing. Thus, some effects are short-lived, on the order of hours. However, when SCAP was delivered for 10 days, both function and physiology were improved as long as they were measured, to 50 days. Dexterity improved with no increase in pain (Fig. 4). Physiology changes included stronger MEPs

with paired stimulation (Fig. 5) and decreased hyperreflexia (Fig. 6). These represent two critical functions of the sensorimotor system, to drive voluntary movement and to modulate segmental reflexes[40,41].

SCAP depends on sensorimotor convergence in the spinal cord.

Our model of SCAP as the convergence of descending sensory and segmental proprioceptors at the level of the spinal cord is supported by 3 lines of evidence. First, there is striking concordance between the time that motor potentials arrive in the spinal cord (9.17 ± 0.55ms; Fig.1A1) and the optimal latency for pairing for the immediate effects (10ms). Second, this immediate effect depends on the CST and proprioceptors, as demonstrated by abrogation of the effect with selective inactivation of each of these pathways (Fig. 2). Finally, pairing of motor cortex and spinal cord at a latency that has no immediate effect (100ms) also produces no plasticity when applied repetitively (Fig. 3D2 and supplementary Fig. 5).

We compared the effects of sensorimotor convergence at the level of the spinal cord with convergence in cortex, and the results challenge the most common clinical approach of targeting cortex. Paired afferent stimulation, which targets cortex, has been used to improve function in people, but the protocol does not work in all individuals, and the physiological effects are moderate in size[2]. In contrast, convergence in the spinal cord had a much larger immediate and lasting effect in the rats. Several groups have applied paired stimulation of the descending (upper) motor system and segmental (lower) motor system[11–13,42–44]. SCAP differs from these approaches by targeting descending motor and segmental afferents—sensorimotor integration rather than the motor connections in the spinal cord.

SCAP effective in rats with moderate cervical SCI.

SCAP was at least as effective in rats with C4 SCI as uninjured rats, indicating that sparse descending motor circuits mediate the plasticity effect, along with the intact sensory system below injury. The C4 contusion injury obliterates the gray matter and leaves just a thin rim of white matter, like human injury[45,46]. Interestingly, we observed sparing of <1% of the CST in rats with this injury[30]. This suggests that other descending motor pathways may be involved, as they are in recovery after human SCI[47]. Targeting the reticulospinal tract, which is slightly better preserved

by experimental SCI in rats, results in improvement of motor function, both in rodents[26,48] and in humans[10,49].

There is a striking concordance of physiological changes induced by SCAP at the cortical and spinal cord levels. This is likely explained by changes in responses in the spinal cord, possibly in the common target of descending motor and segmental afferent connections. These results contribute to our working model that convergent inputs to spinal neurons cause the synapses or the cellular targets themselves to undergo lasting change. Synchrony of input is critical, since paired stimulation with an inappropriate time difference between motor cortex and spinal cord stimulation was ineffective in producing plasticity.

Translation

We observed a striking concordance between these timescales; the latency that produces the largest immediate effects also produced the largest SCAP effects. This enabled us to optimize the protocol on a shorter timescale before application of the protocol in a successful preclinical trial. This process produced a large effect, but the assumption that optimal conditions for effects on one timescale are also optimal for another has not been tested. This may depend on the biological processes mediating each of these effects and whether they are conserved. In addition, like many neuromodulation approaches, we have not optimized the most durable effects, including duration, frequency and timing of repetitive SCAP. Still, this process may allow this protocol and others to be optimized in short-term studies to increase the chances that efficacy studies might have a higher likelihood of success.

This study provides important considerations for the application of paired stimulation to enhance sensorimotor function in people. The interactions between descending motor pathways and large-diameter afferents are largely conserved between rats and humans, but the direct connections between motor cortex and spinal motoneurons in humans may alter these interactions. In addition, the scale of the two species is very different, and it will be important to determine the proper timing of paired stimulation in people. Finally, it will be important to consider the differences in the location of tracts within the spinal cord and the pattern of injury to the spinal cord between human injury and experimental SCI in rats.

Acknowledgements

We thank James McIntosh for helpful discussions about the statistical methods. We thank Tong Chun Wen for help with image acquisition. We thank Aldo Sandoval-Garcia and Walter Voit for making the spinal electrodes.

Funding

Research reported in this publication was supported by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health under award number R01NS115470 and by the Travis Roy Foundation.

Competing interests

Jason Carmel is a co-inventor of a patent for the use of softening spinal electrodes. He also has equity in Backstop Neural, which seeks to commercialize the devices for humans. The authors declare no other competing financial interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Figures legends

Figure 1. The immediate effects of pairing depend on timing. **(A1)** Schematic of cervical spinal cord dorsum potential (CDP) recording. After the onset of 3 pulses of motor cortex stimulation at 110% of motor threshold, the latency of the response was 9.17 ± 0.55 ms (n = 3). (**A2).** Schematic of baseline testing after M1 stimulation at 110% of cortical threshold and EMG recorded from contralateral biceps. (**A3).** Immediate effect testing. Supra-threshold (110% of cortical threshold) stimulation of the motor cortex produces an MEP that is modulated by subthreshold (90% of spinal threshold) spinal cord stimulation. (**B).** Immediate effects of paired stimulation depend on latency. We tested different latencies between motor cortex and spinal cord stimulation from −30 ms to 30 ms. As indicated by the insets, negative times indicate that spinal cord was stimulated before motor cortex stimulation, and positive times indicate that spinal cord was stimulated after motor cortex stimulation. Maximum augmentation was found when spinal cord stimulation was delivered 10 ms after motor cortex stimulation. MEPs were significantly elevated at the time points indicated, as measured by t-test with Bonferroni correction compared to no spinal stimulation baseline, asterisks represent $p < 0.05$ (n = 9). (**C).** Immediate effects when both motor cortex and spinal cord stimulations were paired with their 90% of threshold intensities. The EMG recordings of stimulation of motor cortex only or spinal cord only at 90% are shown in the insets. The paired stimulations with latencies between ± 30ms were applied, and MEP responses were recorded. This figure shows % increase in the MEPs as a function of latencies. MEP responses started to appear with latencies around 10ms, and cortical convergence does not seem to cause any MEP response. MEP response becomes visible once the baseline EMG activity is doubled (n = 4). (**D**). We further tested this hypothesis in animals with C4 spinal contusion. A moderate C4 contusion injury was performed in two rats, and spinal electrode arrays were inserted over C5-C6. Two weeks later, paired stimulation was performed. In SCI rats when 110% cortical stimulation were paired with 90% spinal stimulation, we observed a similar augmentation in MEP as observed in uninjured rats with peak at 10ms latency (134 ± 20%, p = 0.0001)[50,51]. Error bars show SEM.

Figure 2. The corticospinal tract (CST) and spinal afferents are necessary for paired stimulation. (A) CST inactivation. (A1) AAV1-hSyn-DIO-hM4D(Gi)-mCherry was injected into the forelimb area of the left motor cortex and AAV2/retro-pmSyn1-EBFP-Cre was injected into the right side of the C5 & C6 spinal segments. (**A2)** Dual virus transduction drives robust

mCherry expression in corticospinal neurons located in motor cortex layer 5 (dotted lines). (**A3)** CST inactivation impairs skilled forepaw use. The number of adjustments of both forepaws was counted while the animal was eating uncooked pasta before and at 30, 60, and 120 minutes after clozapine N-oxide (CNO) injection. Inactivation significantly reduces the paw manipulations in the paw controlled by the inactivated CST (n = 3). (**A4)** CST inactivation abrogates the effect of paired stimulation. One week after the behavioral test, the CST was inactivated by CNO injection. The augmentation of paired stimulation was fully abrogated at 20-60 minutes in the inactivation side and gradually recovered (n = 3). (**B**) **Large-diameter afferent inactivation**. (**B1)** An AAV5 encoding the inactivating DREADD was injected into DRG on same site as the targeted side. (**B2)** DREADD expression in neurons (arrowhead), infected with the virus measured 67.9 ± 18.5µm in diameter, similar to neurons that mediate touch, proprioception, and muscle length/tension. (**B3)** Inactivation abrogates paired stimulation in rats infected with AAV5-DREADD, inactivation side (red line) compared to non-inactivated side (grey line, n = 2), 100% suppression at 40 minutes after CNO injection. **(C) Proprioceptor inactivation.** (**C1)** A Cre-dependent DREADD was injected into the DRG on the inactivation side of rats expressing Cre under control of parvalbumin, which is expressed selectively by proprioceptors. (**C2)** DREADD in proprioceptive neurons (arrowhead), infected with the virus. **C3.** Inactivation abrogates paired stimulation in PV-Cre rats, inactivation side (green line) compared to baseline (n = 3); more than 70% suppression of paired stimulation effect was observed at 40 minutes after CNO injection. More selective inactivation (green line) resulted in 25.8% ± 3.8% suppression of sensory afferents while non-selective (red line; -1.5% ± 10.5%) inactivation caused more robust suppression of paired stimulation responses on the targeted side of forelimb after CNO injection. Vehicle (normal saline) injection did not change MEPs compared to baseline on the inactivated side (data shown in supplemental Fig.1). Error bars show SEM.

Figure 3. Spinal cord associative plasticity (SCAP) protocol. (A1) Baseline measurements of cortical MEP, spinal MEPs and thresholds before repetitive pairing. Cortical and spinal stimulation at 110% of the cortical and spinal thresholds. MEPs recorded from the biceps. Threshold was determined by adjusting stimulation intensity to provoking a short-latency EMG in >50% of trials. (**A2)** Repetitive paired stimulation, repetitive pairing motor cortex and spinal cord stimulation at 10 ms latency for either 5 or 30 minutes. (**A3)** Testing the effects of both 5 and 30 minutes SCAP, cortical, spinal MEPs and thresholds were measured as it was done before SCAP. (**B1)** 5-minute

SCAP protocol (0.5 Hz, 150 pairs). (**B2)** A 30-minutes SCAP paradigm (5 Hz, 150 pairs of cortical and spinal stimuli 20 times with 1 minute gap between bursts, delivering a total of 3000 pairs). (**C1 – D2)** SCAP causes lasting changes in cortical and spinal cord MEPs in uninjured rats and rats with SCI**.** (**C1)** Cortical MEP augmentation after 5minute SCAP in uninjured awake rats (n = 6) as well as in rats with C4 SCI (n = 2). In both injured and uninjured groups of rats after 5-minutes of repetitive pairing, cortical evoked responses were augmented for more than 1 hours and this augmentation persisted even after SCI, then returned to the baseline. A similar effect was also observed with the spinal MEP and excitability increased after pairing (data shown in S. Fig. 4A). (**C2)** Cortical MEP augmentation after 30min SCAP in uninjured awake rats (n = 4) and in SCI rats (n = 7). In both SCI and intact (uninjured) groups of rats after 30-minutes of repetitive pairing, cortical evoked responses were augmented for more than 2 hours and this augmentation persisted even after SCI, similarly the spinal MEP and excitability increased for 2 hours after pairing (data shown in S. Fig. 4B). **(D1)** Average MEP augmentation over 120 minutes with 5-minutes SCAP conditions. Only motor cortex (4±7) or only spinal cord stimulation (8±6) or pairing at an inappropriate latency (100ms; 2±5)), do not result in elevated MEP responses. (**D2)** Average MEP augmentation over 120 minutes with different 30-min SCAP conditions. Only motor cortex (1.3±6) or only spinal cord stimulation (8±4) or pairing at an inappropriate latency (100ms; 0.2±3), do not result in elevated MEP responses. Pairing at -20ms aiming at cortical convergence, does not cause a strong MEP response (18±5). Error bars shows SEM. A One-way ANOVA was done for overall comparison and was highly significant followed by Bonferroni correction for multiple comparisons. * indicates $p < 0.001$, ns = non-significant.

Figure 4. SCAP promotes motor recovery after SCI and does not affect pain. (A) Experimental timeline: Rats were trained on the food manipulation task and then subjected to C4 contusion injury. Rats were randomized to SCAP daily for 10 days (stimulation group) versus sham (no) stimulation (control group). Physiology and behavior testing was done before stimulation on and then weekly after stimulation. After 50 days, animals were sacrificed, and anatomical analysis was done. (**B1)** Schematics showing characteristic features of rat forelimbs during cereal manipulation task, the primary outcome measure. Images of a representative stimulation rat were generated from frames of live videos with the image tracing tool of Adobe Illustrator software. Score 9 at baseline before SCI: Volar (palm) support, paw adapts to the shape of the cereal. Score 5 after stimulation: Volar support, digit 2 contributes to cereal manipulation,

other digits extended. Score 3 after injury, before stimulation: Lack of volar support, extended digits, and paw doesn't adapt to the shape of the cereal. (**B2)** Average and individual rat's IBB scores. At each time point, the IBB score was averaged over four pieces of cereals for cereal manipulation trials. Thick red and black line are the average IBB scores for all stimulation group rats and controls respectively. Thin light red and black lines indicate individual stimulation and control group rats respectively. Error bar represents SEM. * unpaired t-test, $p = 0.024$, at 49 days after SCI ($n = 7$ in both stimulation and control groups). (**C)** Locomotor behavior on horizontal ladder shows % error that rats made while walking across the horizontal ladder over 20–24 trials at each time point, averaged over left and right forelimbs. Thick red and black lines are the average error rate for all stimulation group rats and controls respectively. Thin light red and black lines indicate individual stimulation and control group rats respectively. There was a decreased trend in % error rate in the rats in stimulation groups as compared to controls but was not significantly deferent between the groups at the final end point of testing (day 49, unpaired t-test, $p = 0.182$, $n = 7$ in each group). **(D)** Pain scores quantified pain using the rats facial characteristics. Thick red and black lines are the average pain scores for all stimulation group rats and controls respectively. Thin light red and black lines indicate individual stimulation and control group rats respectively. We observed no increase in pain score with 10 days of repetitive stimulation, rats in both the groups did not experience pain during the stimulation phase (unpaired t-test, $p = 0.646$, $n = 7$ in each group). Error bars show SEM.

Figure 5. Ten days of SCAP produces augmented MEPs for at least 50 days after SCI. (**A)** The immediate effects were not different before stimulation (day 11) in between the stimulation and control groups, in the stimulation group ($n = 7$), cortical MEP augmentation increased over the 10-day periods of stimulation to 259 ± 12%, while the control group ($n = 7$) did not change (168% +/- 5.4%). The difference between groups was maintained at 50 days after SCI (Stim = 258% +/- 21% vs Control = 169% +/- 10%, $df = 12$, *Cohen's d* = 3.10, $p = 0.0001$). This indicates that repetitive pairing over a 10-day period creates a durable change in physiology. (**B)** Cortical thresholds before SCI were above 1mA in both stimulation (red line) as well as control (black line) rats, after SCI there was a 50% drop in the cortical thresholds in both groups of rats, this may be due to hyperreflexia. There was gradual increase in cortical threshold after SCI but still below the before SCI value. The red line indicated the change in cortical thresholds in each session of repetitive paired stimulation (30-minute SCAP). After each session of 30-minutes of SCAP the

cortical threshold decreased (red dots) as compared to that of the pre-stimulation value, indicating increased cortical excitability after each session. (**C**) Spinal thresholds after SCI were low in both stimulation (red line) as well as control (black line) rats, the spinal threshold was measured only after SCI since spinal electrodes were implanted just after spinal contusion. There was a gradual increase in spinal threshold after SCI that we observed with our spinal electrodes due to an increase in impedance post implantation. The red line indicates the change in the spinal thresholds in each session of repetitive paired stimulation (30-minute SCAP). After each session of 30 minutes of SCAP the spinal threshold decreases (red dots) as compared to that of pre-stimulation value, indicating increase spinal excitability after each session. Error bars show SEM.

Figure 6. Reduced hyperreflexia with SCAP. (A) Method of H-reflex testing. (**B**) The second H-reflex is diminished (arrow) at short inter-stimulus intervals. (**C**) Rats with stimulation (n = 7). Less H-reflex depression in rats 5 days after SCI (dark red) compared to before (blue). After paired stimulation H-reflex depression was largely restored and maintained in the stimulation group. (**D**) Control rats (n = 7) do not show restoration of H-reflex modulation. M = M-wave, H = H-reflex. Error bars show SEM.

Figure. 7. Stimulation and control animals received similar injuries. (**A**) Force applied during cervical contusion (n = 7 for stimulation, n = 7 for control rats). (**B**) Representative spinal cord sections showing the extent of the lesion, scale bar: 1 mm. (**C**) Spared tissue area through the lesioned spinal cord. Error bars show SEM.

Figure 1.

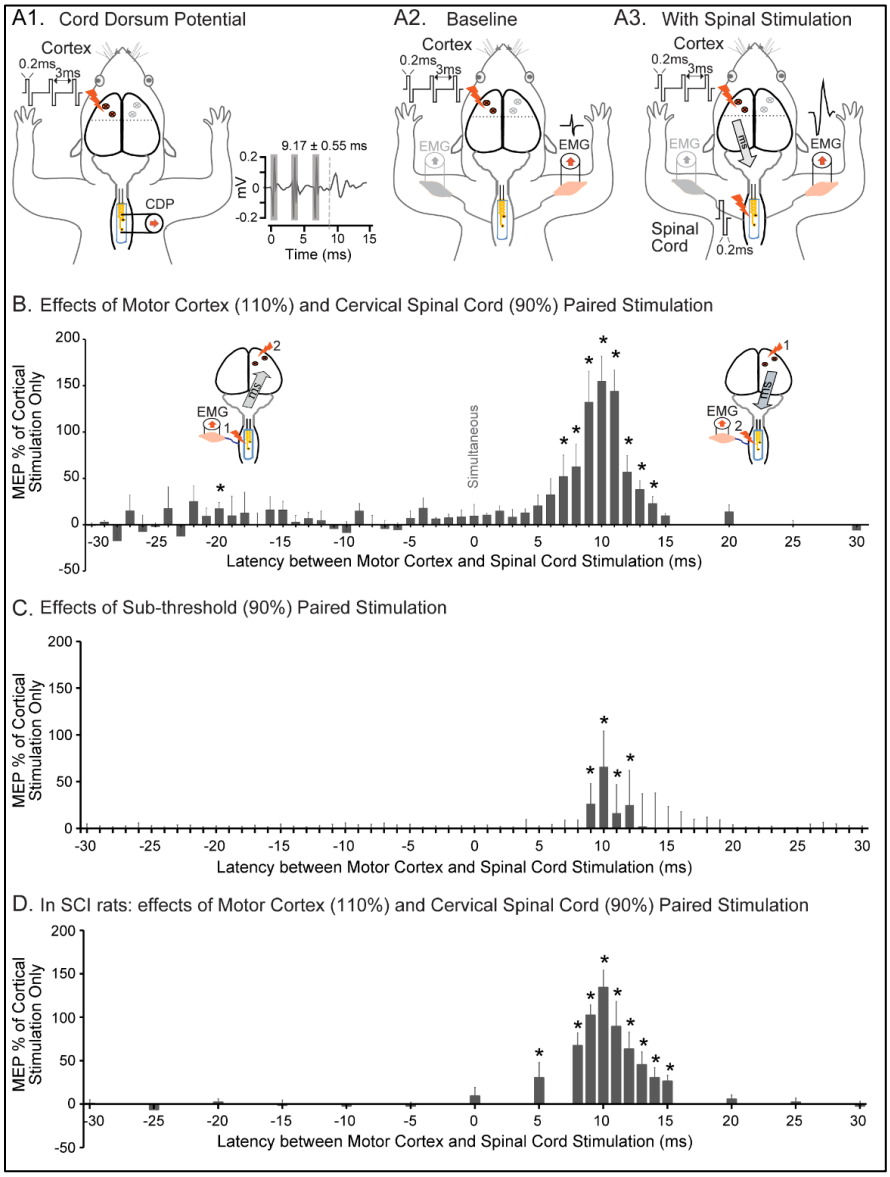

A1. Cord Dorsum Potential
Cortex
0.2ms
3ms
CDP
9.17 ± 0.55 ms
mV
Time (ms)
A2. Baseline
EMG
A3. With Spinal Stimulation
Spinal Cord
B. Effects of Motor Cortex (110%) and Cervical Spinal Cord (90%) Paired Stimulation
MEP % of Cortical Stimulation Only
Simultaneous
Latency between Motor Cortex and Spinal Cord Stimulation (ms)
C. Effects of Sub-threshold (90%) Paired Stimulation
D. In SCI rats: effects of Motor Cortex (110%) and Cervical Spinal Cord (90%) Paired Stimulation

Figure 2.

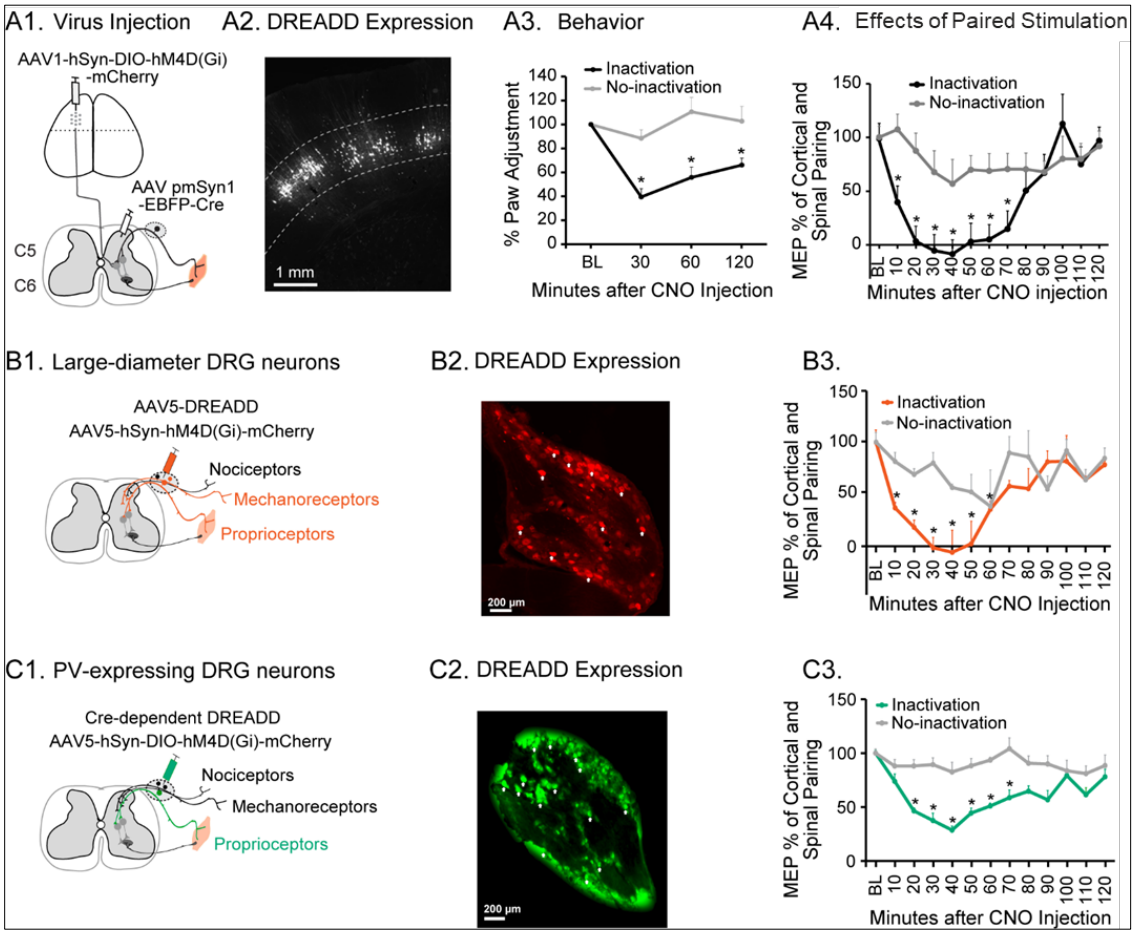

A1. Virus Injection
AAV1-hSyn-DIO-hM4D(Gi) -mCherry
AAV pmSyn1 -EBFP-Cre
C5
C6
A2. DREADD Expression
1 mm
A3. Behavior
Inactivation
No-inactivation
% Paw Adjustment
Minutes after CNO Injection
A4. Effects of Paired Stimulation
MEP % of Cortical and Spinal Pairing
Minutes after CNO injection
B1. Large-diameter DRG neurons
AAV5-DREADD
AAV5-hSyn-hM4D(Gi)-mCherry
Nociceptors
Mechanoreceptors
Proprioceptors
B2. DREADD Expression
200 µm
B3.
C1. PV-expressing DRG neurons
Cre-dependent DREADD
AAV5-hSyn-DIO-hM4D(Gi)-mCherry
C2. DREADD Expression
C3.

Figure 3.

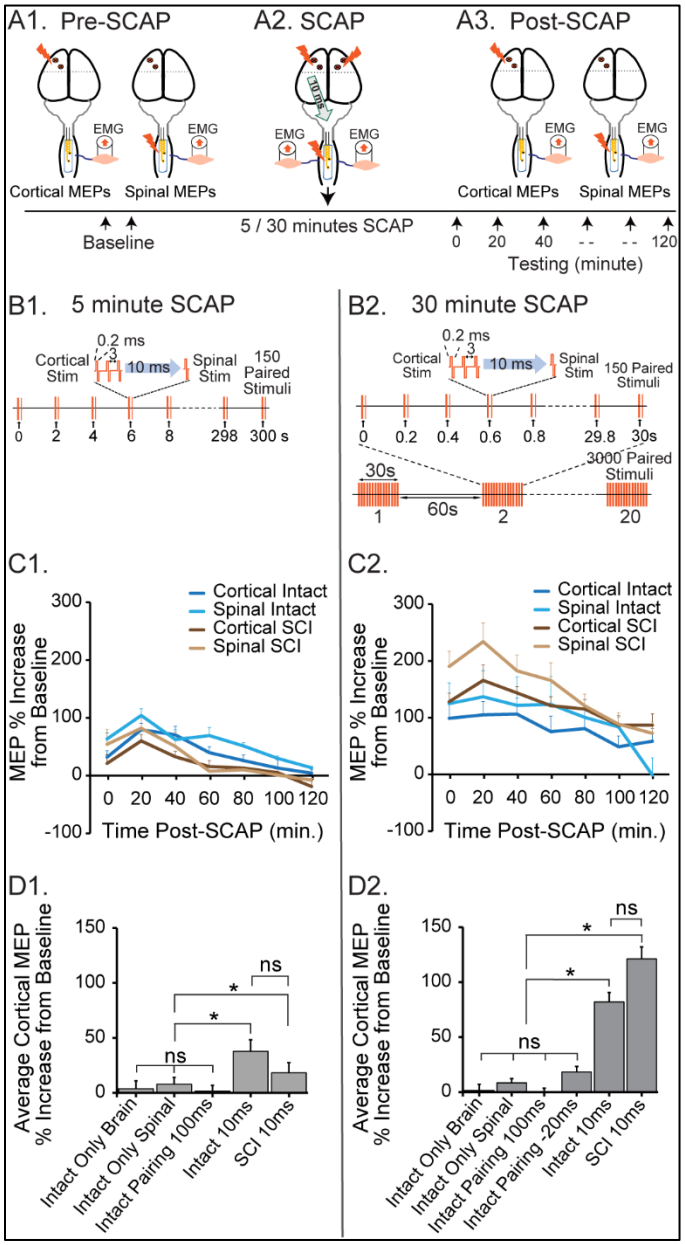

A1. Pre-SCAP
A2. SCAP
A3. Post-SCAP
EMG
Cortical MEPs
Spinal MEPs
Baseline
5 / 30 minutes SCAP
0 20 40 -- -- 120
Testing (minute)
B1. 5 minute SCAP
0.2 ms
Cortical Stim
10 ms
Spinal Stim
150 Paired Stimuli
0 2 4 6 8 298 300 s
B2. 30 minute SCAP
150 Paired Stimuli
0 0.2 0.4 0.6 0.8 29.8 30s
30s
60s
3000 Paired Stimuli
1 2 20
C1.
C2.
Cortical Intact
Spinal Intact
Cortical SCI
Spinal SCI
MEP % Increase from Baseline
Time Post-SCAP (min.)
D1.
D2.
Average Cortical MEP % Increase from Baseline
ns
*
Intact Only Brain
Intact Only Spinal
Intact Pairing 100ms
Intact Pairing -20ms
Intact 10ms
SCI 10ms

Figure 4.

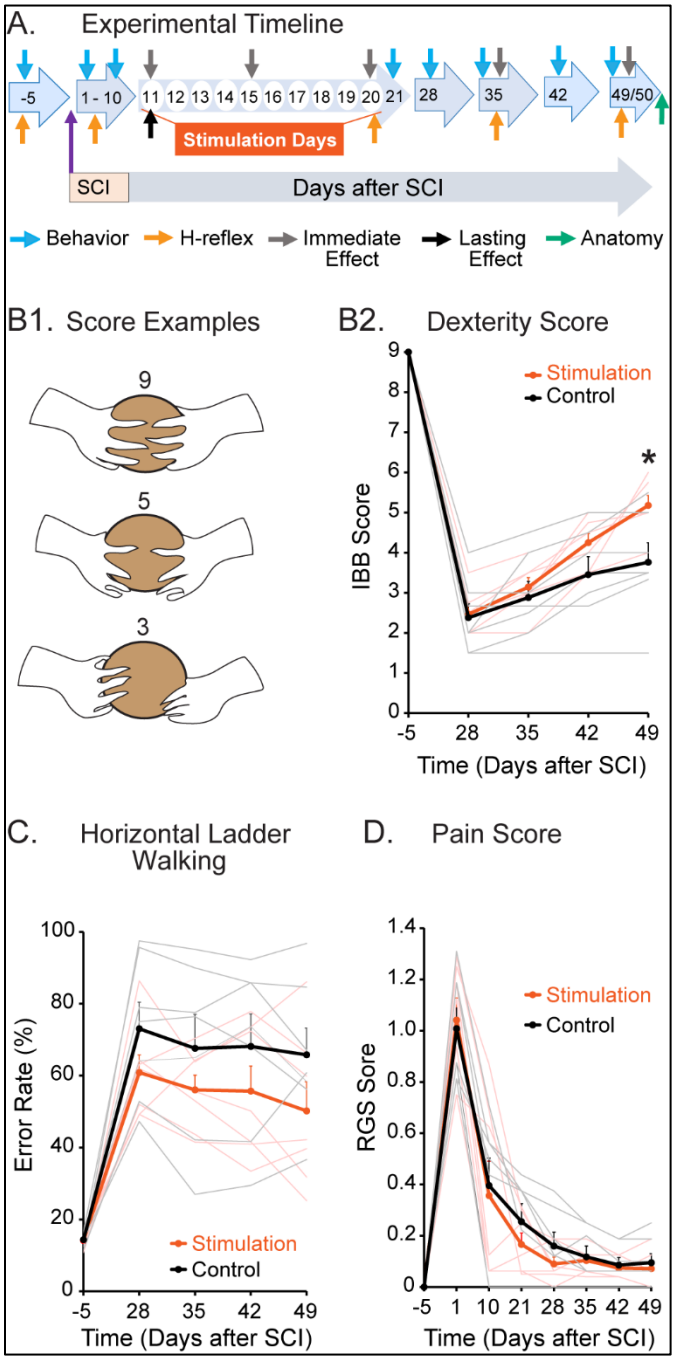

A. Experimental Timeline
-5
1 - 10
11 12 13 14 15 16 17 18 19 20 21
28
35
42
49/50
Stimulation Days
SCI
Days after SCI
Behavior
H-reflex
Immediate Effect
Lasting Effect
Anatomy
B1. Score Examples
9
5
3
B2. Dexterity Score
Stimulation
Control
IBB Score
Time (Days after SCI)
C. Horizontal Ladder Walking
Error Rate (%)
Stimulation
Control
Time (Days after SCI)
D. Pain Score
RGS Score
Stimulation
Control
Time (Days after SCI)

Figure 5.

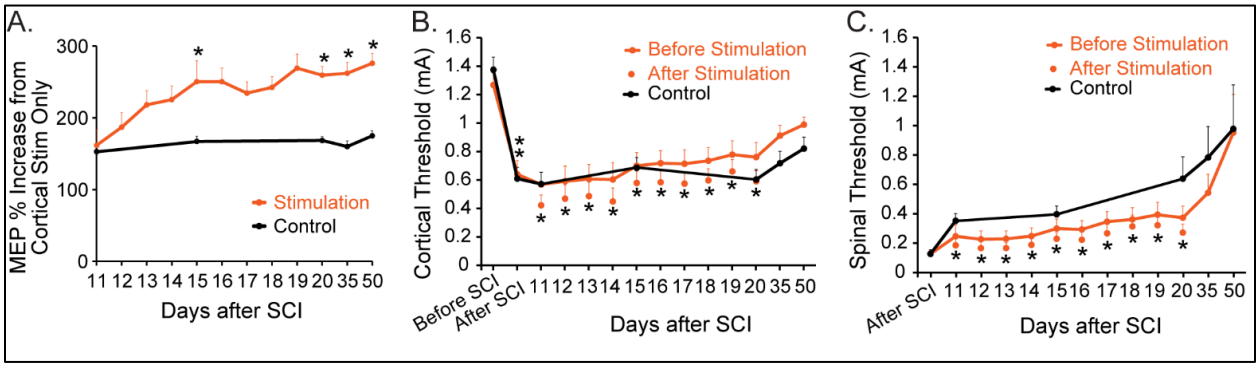


Figure 6.

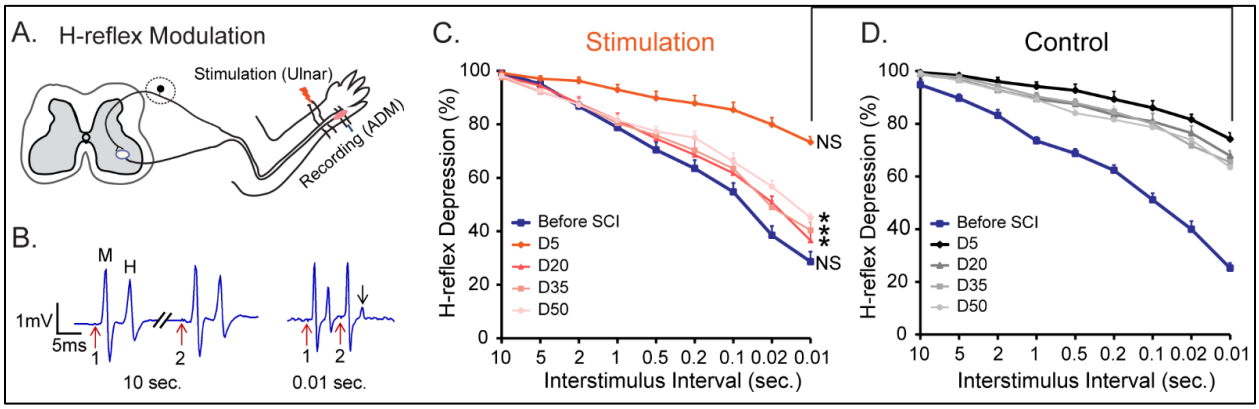
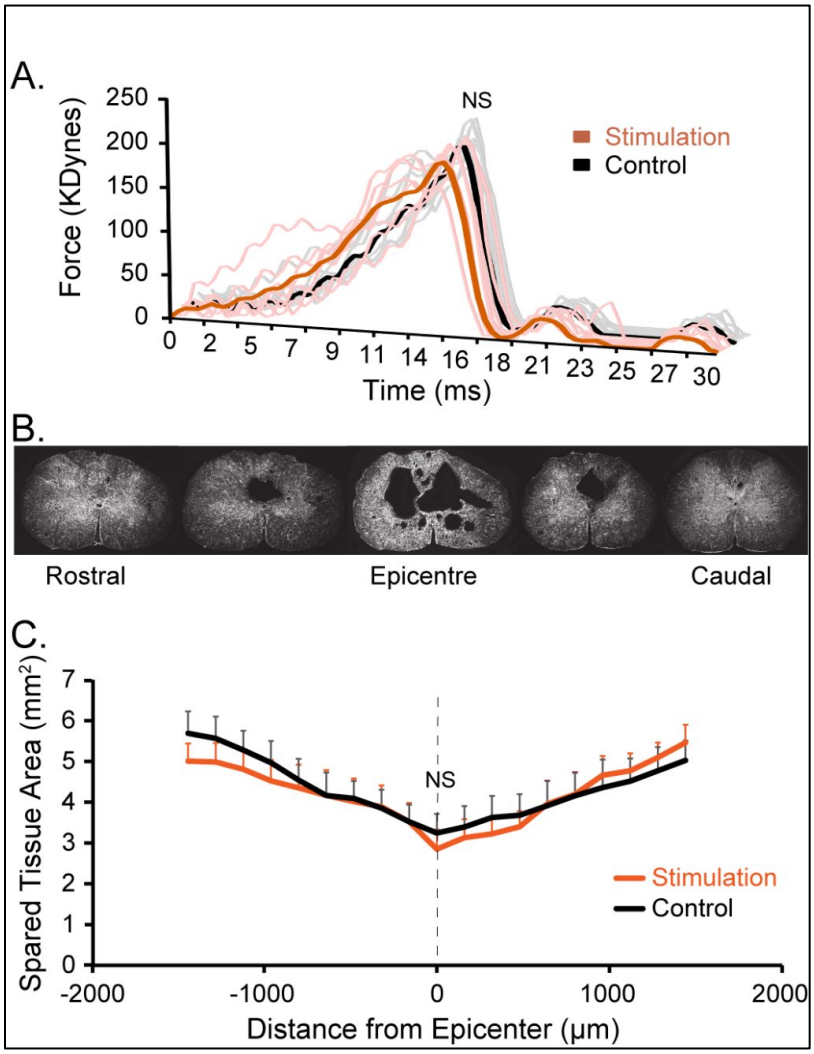


Figure 7.


A.
Force (KDynes)
250
200
150
100
50
0
NS
Stimulation
Control
0 2 5 7 9 11 14 16 18 21 23 25 27 30
Time (ms)
B.
Rostral
Epicentre
Caudal
C.
Spared Tissue Area (mm²)
7
6
5
4
3
2
1
0
NS
Stimulation
Control
-2000
-1000
0
1000
2000
Distance from Epicenter (µm)

Supplementary material

Materials and Methods

Descriptions of animals and surgical care

Animals:

Fifty-five adult female Sprague-Dawley rats (Charles River, bodyweight of 275 +/- 25 grams) were used in this study. Out of these 31 rats were uninjured and 24 rats received spinal contusion. Of the rats with spinal contusion, 8 died immediately after SCI. To test the proper timing of brain and spinal cord stimulation, a group of 9 healthy rats were tested on the immediate effect of adding subthreshold spinal cord stimulation to suprathreshold cortical stimulation (Fig. 1B), of these, 5 were used to test the effects of subthreshold stimulation both cortical and spinal (Fig. 1C). To understand which neural circuits mediate these effects, 8 rats had inactivation of neural circuits. Three rats were used for CST inactivation (Fig. 2A1-A4), two for sensory afferent inactivation (Fig. 2B1-B3), and Three female Long-Evans transgenic rats (250-275g), expressing Cre recombinase under the rat parvalbumin promoter {LE-TG(Pvalb-iCre)2Ottc, rat genome database (RGD) ID #10412329, RRRC line #773, NIDA, USA}, Pvalb-iCre rats were used for proprioceptor inactivation experiments (Fig. 2C1-C3). To test the effects of repetitive brain and spinal cord stimulation (SCAP), 14 healthy rats were used for the development and testing of SCAP (Figs. 3). To determine whether the neural circuits spared by SCI could support SCAP, 16 rats were subjected to a moderate cervical (C4) contusion. Two injured rats were initially used to test the short-term efficacy of SCAP. To test the long-term efficacy of SCAP to restore physiology and function, a group of 14 rats were randomized to real or sham (n=7, each) SCAP repeated over 10 days.

Surgery and Care:

For all surgical procedures, rats were anesthetized via intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg body weight). Local application of bupivacaine (0.25- 0.5%, 0.1-0.2mL) was done before making skin incision and buprenorphine (0.05 mg/kg

body weight, subcutaneous) was administered before and after the survival surgery and also after H-reflex testing to alleviate pain. Anesthesia levels were monitored by respiration and heart rate and responses to foot pinch. Animals were placed on heating pads (PhysioSuite, Kent Scientific Corporation, Northwest CT, USA) to maintain a body temperature of 37.5°C as measured continuously by a rectal probe (PhysioSuite). The animals were housed in individual cages with free access to food and water on a 12/12-hour light/dark cycle. All experimental procedures were in full compliance with the approved Institutional Animal Care and Use Committee Protocol of Columbia University.

Description of cortical electrodes, EMG wire, and spinal stimulator implantation: In a single procedure, motor cortex screw electrodes and EMG wires were placed and the head connectors brought to the common head cap. Rats were anesthetized and head-fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed and placement holes made using a Jobber Drill (number 60, P1 Technologies, Roanoke, VA, USA) without disturbing the dura mater. Stainless steel screw electrodes (1.19 mm diameter with a flat tip; Plastics One, Torrington, CT, USA) were implanted over the forelimb area of the motor cortex in one hemisphere at two locations as described in our previous work[1]. The screw electrodes were attached in advance to a head connector (Plastics One) that was secured with skull screws and dental acrylic. For electromyogram (EMG) recordings, we inserted supple stainless steel braided wire (diameter = 0.014mm; #AS 634, Cooner Wire Co., Chatsworth, CA, USA) electrodes into the biceps muscle (for MEPs) and adductor digiti minimi (#AS 631, for H-reflex). For the biceps, the other end was tunneled subcutaneously and attached in advance to a head connector, mounted on the skull, and also secured with skull screws and dental acrylic (Lang Dental Manufacturing Co., Inc., Wheeling, IL, USA).

One week after a cortical and EMG electrode implant, the rats went through another surgery to implant spinal stimulation electrodes, as described previously[2,3]. Briefly, the head was fixed and the cervical spine. The T1 spinous process was clamped to stabilize the spine. The C4 spinal cord was exposed by laminectomy. Array electrodes were placed in the dorsal epidural space in the midline of the spinal cord over C5-C6. Muscles and skin were sutured in layers. The array head connector was mounted on the skull and also secured with skull screws and dental acrylic.

Description of MEP measurements: All the MEP measurements were done in awake behaving rats in their home cages. Before the start of testing, electrodes were connected and the animal was left in the cage for 10-15 minutes to get habituated. We stimulated the motor cortex and measured MEPs from contralateral biceps muscle. Both motor cortices were stimulated independently and contralateral EMGs acquired from the biceps. A similar primary motor cortex and spinal cord stimulation paradigm were used as described by Mishra et al., 2017[1]. Briefly, a train of three biphasic square wave pulses (each pulse of 0.2 ms for each polarity; interstimulus interval of 3 ms; Fig. 1B) was used for motor cortex stimulation. Multichannel EMG signals were acquired before and after the repetitive stimulation at 5,000Hz sampling rate, with a CED (CED Micro 1401, Cambridge Electronic Design Ltd, Cambridge, UK) data acquisition system and a recording software (Signal 5.08, CED Ltd). The signals were amplified at a gain of x1000 and bandpass filtered 1–1000Hz with an integrated AC differential amplifier system (A-M Systems, Model 1700, Sequim, WA, USA). Motor cortex and spinal stimulation were performed using an isolated pulse stimulator (A-M Systems, Model 2100). MEPs were extracted, processed, and quantified using customized Motometrics analysis[4].

For the motor cortex stimulation we first measured the cortical threshold, defined as the stimulation intensity needed to evoke a short latency MEP in > 50% of trials, by stimulating cortex at increasing intensities. We then measured the MEP at 110% of the cortical threshold, which we call cortical MEPs. The same method was then used to determine the spinal threshold and to measure MEPs at 110% of spinal threshold, which we call spinal MEPs. For the paired stimulation, we stimulated the motor cortex at 110% of cortical threshold and the spinal cord was stimulated with 90% spinal threshold intensity. To quantify MEPs, the EMGs were rectified and then averaged across 20 trials. The area-under-the-curve (AUC) was calculated for MEPs and % change from baseline was quantified. To determine the most effective latency, we stimulated the spinal cord at various time intervals from 30 ms before motor cortex stimulation to 30 ms after motor cortex stimulation. Latencies were tested in a randomized sequence, with a minimum of 2 minutes between each test. For pairing at each latencies we first determined the cortical and spinal thresholds before the repetitive paired stimulation and 110% of cortical thresholds was used for baseline MEP recordings which only motor cortex stimulation. For pairing we used 110% of cortical threshold and 90% of spinal threshold intensities. At each latencies we measured spinal

threshold and pairing was done at 90% of the threshold using baseline cortical threshold. For Both Subthreshold stimulation, intensities for both cortical and spinal stimulations were set to 90% of their threshold values. First, as control recordings, only-spinal and only-cortical stimulations were applied to record the muscle activity during these subthreshold stimulations. Then, paired stimulations at 90% of thresholds with latencies between ± 30ms were applied, and MEP responses were recorded. The paired subthreshold stimulation generates an MEP response only when these two stimuli converge in the spinal cord, not in the cortex.

The corticospinal tract and spinal afferent inactivation

Corticospinal tract (CST) inactivation: The corticospinal tract was selectively inactivated using a Designer Receptor Exclusively Activated by Designer Drug (DREADD), during paired stimulation. To target the corticospinal tract selectively, a Cre-dependent adeno-associated virus (AAV) encoding the DREADD was injected into the motor cortex and another encoding Cre-recombinase was injected into the cervical spinal cord. Thus, only doubly infected neurons express the DREADD, which can be inactivated by the administration of its ligand, clozapine-N-oxide (CNO)[5]. Through a craniotomy, twelve injections of 200nl of AAV1-hSyn-DIO-hM4D(Gi)-mCherry {2 x 10^{12} vg/ml, pAAV-hSyn-DIO-hM4D(Gi)-mCherry was a gift from Bryan Roth (Addgene viral prep # 44362-AAV1 ; http://n2t.net/addgene:44362 ; RRID:Addgene_44362)[6] were made 1.5 mm from the pial surface into the forelimb area of the left motor cortex at the following forelimb motor cortex coordinates[7]: AP 0.5 ML 1.5; AP 0.5, ML 2.5; AP 0.5, ML 3.5, AP 1.5, ML 1.5; AP 1.5, ML 2.5; AP1.5, ML 3.5; AP 2.5, ML 1.5; AP 2.5, ML 2.5; AP 2.5, ML 3.5; AP 3.5, ML 2.0, AP 3.5, ML 3.0; AP 4.5, ML2.5. AAV retrograde; AAV pmSyn1-EBFP-Cre (5.5 x 10^{12} vg/ml, AAV pmSyn1-EBFP-Cre was a gift from Hongkui Zeng; Addgene plasmid # 51507; http://n2t.net/addgene:51507 ; RRID:Addgene_51507[8] was injected into the right side of the cervical spinal cord. 11 injections of 200 nl of AAV were made in C5 and C6, 0.8 mm lateral from the midline at the depth of 1.2 mm from the surface of the spinal cord, with 0.6 mm intervals between injections. One week after AAV injections, animals were trained on the vermicelli handling task[9,10]. After reaching a stable baseline performance, the number of forepaw manipulations was measured before, during, and after inactivation. The number of adjustments of each forepaw was counted while the animal was eating uncooked pasta before and at 30, 60, and 120 minutes after intraperitoneal injection of 2.4 mg/kg body weight of clozapine N-oxide (CNO).

One week after the behavioral test, we tested the effects of inactivation on the immediate effects of paired brain and spinal cord stimulation. Under deep anesthesia a cranial window was created to expose the forelimb motor cortex, and silver ball electrodes were placed over two locations: 1.0 mm anterior, and 2.0 mm lateral; and 3.0 mm anterior and 4.0 mm lateral to bregma. After that we exposed the C4-C6 vertebrae, and laminectomy was done at C5-C6 level to expose dorsal surface of spinal cord and a pair of silver ball electrodes were placed epidurally on the midline of C5-C6 cervical spinal cord. A pair of EMG wires were inserted in the biceps muscle bilaterally to record EMG. Systemic administration of CNO typically causes maximum inactivation of DREADD expressing neurons within 30 minutes, and CNO typically washes out within two hours[11]. This allows testing of physiology and behavior before, during, and after reversible inactivation of specific neural circuits. The MEP of each biceps was tested by independent stimulation of the contralateral motor cortex. Measurements were taken every 10 minutes for 2 hours. The cortical threshold changed during this time, so paired stimulation was always compared with cortex-only stimulation at the corresponding timepoint.

Afferent inactivation: The necessity of afferents for the immediate effects of paired stimulation was tested using two inactivation strategies. For the first strategy, large diameter dorsal root ganglion (DRG) neurons were inactivated. Large DRG neurons mediate touch, proprioception, and muscle length/tension[12]. The specificity of large-diameter DRG neurons was provided by the tropism of the AAV5[13]. One microliter of AAV5-hSyn-hM4D(Gi)-mCherry (2 x 10^{13} vg/ml, pAAV-hSyn-hM4D(Gi)-mCherry was a gift from Bryan Roth (Addgene viral prep # 50475-AAV5; http://n2t.net/addgene:50475 ; RRID:Addgene_50475) was injected into each DRG at C5, C6, and C7 on one side of the spinal cord after partial laminectomy. At the time of virus injection, stainless steel screw electrodes were placed over each motor cortex, spinal array on the epidural surfaces of C5-C6 cervical spinal cord, and EMG wires in the biceps muscle bilaterally. Similar to CST inactivation, four to five weeks after the virus injection, large-diameter afferents were inactivated by an intraperitoneal injection of 2.4 mg/kg body weight of CNO and MEPs measured over 2 hours. For the second strategy, we targeted the proprioceptive afferents for inactivation, since these mediate the effects of spinal cord stimulation[14,15]. We used a genetic strategy to target DRG neurons that express parvalbumin, which are proprioceptors[16,17]. In transgenic rats that express Cre recombinase under the control of parvalbumin promoter PV-Cre rats (LE-Tg(Pvalb-iCre)2Ottc, Optogenetics and Transgenic Technology Core, NIDA, MD, USA

obtained via Rat Research Resource Centre RRRC#773; the genotype was confirmed prior to virus injections by PCR using the primers, Pvalb forward: 5'-CCG TTC AGC TTG AAG GTG TC-3', iCre R12: 5'-CAC AGT CAG CAG GTT GGA GA-3') we injected a Cre-dependent DREADD (AAV5-hSyn-DIO-hM4D(Gi)-mCherry (2 x 10^{13} vg/ml, pAAV-hSyn-DIO-hM4D(Gi)-mCherry was a gift from Bryan Roth; Addgene viral prep # 44362-AAV5; http://n2t.net/addgene:44362 ; RRID:Addgene_44362[6] into the C5, C6, and C7 DRGs as described above. Inactivation with CNO and physiology measurement were also identical. In these rats cortical and EMG electrodes were implanted one week before DRG injections, and the spinal array was implanted just after the injection using the methods described above.

Inactivation controls: For CNO only control, immediate effects of paired stimulation was recorded from right and left forelimb biceps before CNO injection in a normal rat (no virus injection). After CNO injection immediate effects of paired stimulation was observed every 10 minute till 2 hours. For saline only control in two PV-Cre rats immediate effects of paired stimulation was recorded from both right and left forelimb biceps before normal saline (sterile injectable 0.9% NaCl, vehicle for CNO) injection. After saline injection immediate effects of paired stimulation effects was recorded every 10 minute till 2 hours. The cortical and spinal MEPs, as well as cortical and Spinal excitability were calculated as described below. The excitability was expressed as 1/ threshold, data was normalized to baseline values before injection.

The SCAP protocol and optimization

SCAP protocol: We tested brain and spinal cord responses at baseline and then after the period of repetitive pairing (Fig. 3A1-A3), as we did in Mishra et al., 2017[1] but in awake rats with implanted electrodes. This differs from the immediate effects of stimulation (Fig. 1A3). Before modulation, we measured the stimulation threshold for the motor cortex to produce a MEP in 50% of trials and also the spinal threshold. We then stimulated with 110% of threshold to determine cortical and spinal MEPs, using the area under the curve. At baseline, cortical and spinal MEPs, as well as cortical and spinal thresholds were measured singly and independently (Fig. 3A1). For repetitive pairing (Fig. 3A2), motor cortex stimulation was performed at 110 % of the threshold for evoking a cortical MEP followed 10 ms later by spinal cord stimulation performed at 90% of the threshold for generating a spinal MEP. After repetitive pairing (Fig. 3A3), the same measures

taken at baseline were taken immediately after pairing and every 20 min thereafter up to 120 min. We quantified excitability as 1/threshold and expressed as % change from baseline. Add description of each session threshold testing and quantification of excitability. Here we determined the cortical and spinal thresholds before the repetitive paired stimulation and 110% of cortical and spinal thresholds were used for baseline MEP recordings. For SCAP we used 110% of cortical threshold and 90% of spinal threshold intensities. After SCAP, each time points till 120 minutes we measured both spinal and cortical thresholds to calculate excitability (1/threshold) and MEP testing was done similar to baseline testing by stimulating cortex and spinal cord at 110% of threshold that was determined before SCAP.

SCAP optimization: In order to maximize the efficacy of the SCAP protocol (5-minute SCAP, Fig 3B1)[1], we varied four parameters in order to produce the largest physiological responses: frequency, number of stimuli, number of trains, and time between trains. In two rats implanted with cortical and spinal electrodes, we first varied frequency by testing 10 stimuli delivered at 5Hz, 2 Hz, 1Hz, 0.5Hz and 0.2Hz (Supplementary Fig. 3A). We have tested whether repeated stimuli suppressed the resulting MEP. For the other parameters, we examined the short term SCAP effects with the average percent increase in cortical MEPs, cortical and spinal excitability over the 120 minutes after the SCAP protocol (Fig. 3B2). Spinal excitability was used since this was less variable than spinal MEPs, and out of concern that eliciting spinal MEPs might induce plasticity. In addition, we intended to keep the total stimulation period to 30 minutes, a period that could be used for stimulation in people. For these trials, we used 5 Hz pairing and varied the number of stimuli by comparing 150, 75 & 38 pulses (Supplementary Fig. 3B, C & D1-D3). For subsequent trials we used 20 trains and varied the time between trains, either 1 or 5 minutes (Supplementary Fig. 3B, C & E1-E3). Further, for subsequent trials we used 150 stimuli, which we term a train, and varied the number of trains, either 10 or 20 (Supplementary Fig. 3B, C & F1-F3). The optimized 30-minute SCAP protocol is shown in Fig. 3B2. A train of 150 stimuli at 5Hz frequency (30 seconds) is followed by a 60-second gap. The train and gap is then repeated 20 times, delivering 3000 stimuli pairs in 30 minutes.

The rat C4 spinal contusion and SCAP after SCI

Cervical spinal cord contusion: The C4 spinal cord contusion surgery was performed as previously described[18]. Briefly, the C3 to C5 vertebrae were exposed by skin incision and muscle separation on the midline. Laminectomy of C4 was performed, taking care to ensure the dura remained intact. Following laminectomy, the rat was placed on the Infinite Horizon (IH) impactor stabilization platform (IH-0400, Precision Systems and Instrumentation, LLC), and the C3 and C5 spinous processes were clamped. A fine adjustment was made to the vertebral clamps to ensure that the exposed C4 spinal cord surface was flattened. The moderate contusion injury was made with a 3.5 mm impactor tip raised to three complete turns ~2 mm from the dorsal surface and was dropped with a preset 200 kdynes force on to the spinal cord with no dwell time. The force curve of the impactor was saved so that the rats in the Stim and Control groups could be compared. After injury, the rat was removed from the platform and the spinal electrode array was implanted as described above. Rats individually housed in separate cages provided with ALPHA-dri dust-free animal bedding (Shepherd Specialty Papers, Watertown, TN) and received intense care after SCI. This included placing their cage on a heating pad for 12 h, administering buprenorphine (4 doses in total, 0.05 mg/kg) every 8–12 h, and Baytril (5 mg/kg, Norbrook, Henry Schein, Melville, NY) daily for 5 days. For nutrition, Ringer's Lactate solution (10 ml, subcutaneous) was administered daily for 5–10 days and Diet boost Gel (ClearH2O, Portland, ME) was placed within reach until rats regained the ability to reach the food and water dispensers. Body weight, food and water intake was monitored daily. Bladders were palpated every 12 hours; none of the rats showed bladder dysfunction.

SCAP in SCI rats: We designed a preclinical efficacy trial, as shown in Fig. 4A. Ten days of an optimized SCAP protocol was delivered in the subacute period, 10 days after injury[18,19]. The daily stimulation duration (30 minutes) and number of sessions (10) were designed to be clinically viable. The primary outcome measure was the dexterity (IBB) score, and secondary outcomes included changes in MEPs, H-reflex modulation (a measure of hyperreflexia), and a pain measure (RGS). The study was powered based on the effect size we observe of a different neuromodulation strategy[18]. A-priori power analysis suggested we need 8 rats in each group to achieve a power of 0.8. However, the physiological effects of SCAP were more robust than the previous paradigm,

suggesting a stronger behavioral effect. The post-hoc power with a total of 14 rats was 0.85 using the same parameters as the initial power calculation.

Behavior testing

Food manipulation task (IBB): To assess the rats ability to manipulate, we used the Irvine, Beatties, and Bresnahan scale[20,21], as we did before[18]. All rats were placed in a clear plastic cylinder on top of a rotating carousel so that they could be rotated in the direction of the video camera. The rats were habituated to the testing environment for 10 days, 15 minute per day. For testing, rats were video recorded while eating both donut-shaped (Froot Loops, Kellogg's NA Co.) and sphere-shaped (Reese's Puffs, General Mills Sales Inc.) cereal. Experimenters blinded to treatment recorded the rats eating the cereals before SCI and weekly from 4 to 7 weeks after SCI. Analysis of the recorded session was done offline by a blinded observer. The scores for the two different kinds of cereal and the two forepaws were averaged. A schematics showing characteristic features of rat forelimbs during cereal manipulation task is presented in Fig. 4B1. Images of a representative stimulation rat were generated from frames of live videos with the image tracing tool of Adobe Illustrator software.

Horizontal ladder walking task: The horizontal ladder walking task measured paw placement on the rungs, which were irregularly spaced[19,22,23]. After cortical electrode implantation, we trained rats 20 min daily for 10 days to ensure rats walked across the ladder without distractions. We motivated rats to cross the ladder with puffs from a can of compressed air and by offering a cotton swab dipped into 20% sucrose after every successful trial. The pattern of the irregularly spaced rungs was altered every 6 trials and the walking direction was changed after 12 trials. We video recorded the behavior at 50 frames-per-second. Experimenters blinded to the paired stimulation treatment analyzed the trials frame-by-frame to quantify the step quality. The start was defined when the rat placed all 4 limbs on the rungs and the end when it reached the last rung of the ladder. The criteria were used to establish when to begin and end scoring and also to measure the time taken to walk across the ladder. The steps were scored as a good step, overstep, under-step, or a missed step based on the placement of the forepaw on the rung; for the main analysis, this was divided only into good steps or errors. We excluded one rat that did not meet the baseline average of 15% error rate

Rat grimace scale (RGS): This scale is used to quantify pain using the rats facial characteristics[24], the way that is commonly used to assess pain in people. All rats were kept on a rotating carousel as in the case of the food manipulation task, and the facial features were recorded with a camera by a blinded experimenter at the times shown in Fig. 4. The facial features were analyzed offline by a blinded scorer.

H-reflex testing: To track hyperreflexia, we measured the H-reflex at baseline, one week after SCI, just after 10 days of stimulation and every two weeks post-stimulation (Fig.4A). The M-wave is evoked by the excitation of motor axons. The H-reflex is the electrophysiological equivalent of the spinal stretch reflex; Ia proprioceptive afferents synapse on and activate motor neurons in the spinal cord (Fig. 6A). Rats were lightly anesthetized with 60 mg/kg ketamine. A pair of braided wire electrodes were inserted across the medial plantar side of the wrist to stimulate the ulnar nerve (via a constant current isolated pulse stimulator, stimulus width 100µs, A-M Systems). Two recording electrodes were inserted into the abductor digiti minimi to record EMGs. The threshold was determined as the lowest stimulation intensity that elicited an H-wave response in at least 75% of the recordings[25]. First, we tested responses to increasing stimulus intensities up to 2X threshold, which activates low threshold afferent fibers. Next, we tested the frequency-dependent depression of the H-wave[25–27]. This is also commonly referred to as post-activation depression or as rate-dependent depression. We stimulated every 10 s with paired stimuli at inter-stimulus intervals from 10 s to 0.01 s. The H-wave area under the curve (AUC) of the test stimulus was normalized to the H-wave AUC of the conditioning stimulus. 25 paired stimuli per frequency were averaged and plotted as a frequency-depression curve (Fig. 6C & D).

Histological analysis: To determine the severity of SCI, we compared the parameter (at the time of SCI) and severity of injury between those with and without therapeutic stimulation. One week later, rats were deeply anesthetized and perfused, first with ~400 ml 0.1M phosphate-buffered saline (PBS) containing 5,000 UPS units/ml of heparin, and then with 600 ml 4% paraformaldehyde. Spinal cord (C1-T2) were dissected and post-fixed in 4% paraformaldehyde for 48 h. The tissue was then transferred to 30% sucrose at 4°C until it was cut in the coronal section at 40µm thickness on a cryostat. Tissue sections from C3 to C5 were processed for lesion reconstruction. Spinal cord sections were collected serially and directly onto slides to preserve tissue integrity. The tissue sections dried overnight and were coverslipped the next day. The outline

of the lesion, grey matter, and white matter were traced using darkfield microscopy and Stereo-investigator software (MBF Bioscience, Williston, Vermont, USA). A blinded experimenter used the Planimetry tool to reconstruct sections around the lesion epicenter to determine the lesion and total tissue section area around the lesion epicenter. The spared tissue area was computed by subtracting the lesion tissue area from the total tissue area. To compare the extent of injury in the two groups, spared tissue area data was quantified every 200 microns across the whole area[18].

Axon Quantification: For axon quantification, BDA histology was performed on sections above (C3) and below (C7) the lesion site. Briefly, sections from spinal cord levels C3 and C7 were rinsed with 0.1M PBS, then incubated for 2 hours in Alexa Fluor 594 streptavidin (1: 500 in PBS; Invitrogen #11227). After 2hrs of incubation sections were rinsed with PBS and sections were mounted on charged glass slides using Fluoroshield (Sigma-Aldrich # F6182) for preserving fluorescence of tissue, then sections were cover-slipped. Slides were stored at 4^0C till visualization. For each rat, data were collected from above and below the lesion site as described. Both the ipsilateral and the contralateral sides of the C7 sections were hand traced using Stereoinvestigator (MBF Bioscience, Williston, Vermont, USA). To correct for differences in the efficacy of the tracer, axon lengths were normalized by the efficiency of tract tracing[19,22,28]. The optical fractionator probe in Stereoinvestigator was used to determine an estimate of the number of labeled axons in the contralateral dorsal column at C3. A correction factor was calculated by dividing each rat's averaged dorsal column axon number by the average number of axons in the dorsal column. Some rats were excluded from the BDA quantification analysis because of too little BDA staining to be quantified.

For sensory axon quantifications, AAV1 was injected in C5-C7 DRG (7.74 $x10^{13}$; pAAV-hSyn-EGFP was a gift from Bryan Roth (Addgene viral prep # 50465-AAV1; http://n2t.net/addgene: 50465; RRID: Addgene_50465) and sensory afferent axons were quantified below the lesion segments with the Stereoinvestigator (MBF Bioscience, Williston, Vermont, USA). At spinal cord level C7, the space balls probe was used to estimate the axon length in the grey matter ipsilateral to the Virus injection. To correct for differences in tracer efficiency, axon length was normalized by computing the number axons in the ipsilateral dorsal column using the optical fractionator probe and a correction factor was calculated in the same way as for the motor axons.

Randomization and blinding procedures

Rats were pseudorandomized into real (Stim) and sham (Control) groups with each cohort of up to 8 rats having rats assigned to each group. Rats were randomized immediately after SCI surgery, and experiments were carried out with blinded measures. The experimenter who randomized rats into different groups and performed paired stimulation treatment, but was not involved in behavior tasks training, testing or scoring process or in histology. Experimenters who performed behavior training, testing, and scoring and histology did not participate in randomization, electrophysiology testing, or paired stimulation. Control rats were connected to the stimulator, but no stimulation was delivered. Since all rats had both cortical and spinal electrodes implanted, there was no way to distinguish by appearance whether rats received stimulation.

Statistical analyses

All of the comparisons between stimulation conditions were done with paired/unpaired t-tests with Bonferroni correction or One-way ANOVA with Bonferroni corrections and and Cohen's d was used effect size. To determine the latency for paired stimulation (Fig.1), we compared MEPs for pairing at different latencies to the baseline (only motor cortex stimulation at 110% of threshold). For the sub-threshold timing, the baseline MEPs were normalized and compared to the pairing at different latencies. For the inactivation experiments (Figs. 2), the immediate effects of the pairing on the targeted side at baseline was compared to the different times after CNO injection. For SCAP (Fig. 3), we used only descriptive statistics as percentage change in MEP. Independent cortex and spinal MEPs were compared to each time after SCAP. For the preclinical randomized trial, the Stim and Control groups were compared by an independent t-test with Bonferroni correction. The immediate effects of pairing was compared between the groups at several time points (Fig. 5). H-reflex modulation was compared only at the shortest (0.01s) inter-stimulus-interval (Fig. 6). The dexterity and pain scores were compared at the end of the trial (Fig. 4; day 49). To compare the injuries (Fig. 7), the peak force delivered to induce SCI (mean ± SEM force at the time of impact was presented) and percentage spared tissue area at the epicenter between the groups was compared independent t-test. All the statistical analysis was performed with SPSS (SPSS Statistics for Windows, version 27; IBM Corp., Armonk, NY, USA). We tested the normality of the data using the Shapiro-Wilk test. All data are expressed as Mean ± SEM.

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Supplementary Figure Legends

Supplementary Figure 1. (A) Cortical excitability decreased after inactivation. Inactivation decreases the motor cortex excitability in PV-Cre rats, inactivation side (black line) compared to baseline (BL, n = 2); more than 40% suppression of motor cortex excitability at 40 minutes after CNO injection. While on the non-inactivated side less than 10% change in cortical excitability was observed. (**B)** Spinal MEP decreased after inactivation. Inactivation abrogates paired stimulation effects on spinal evoked MEPs in PV-Cre rats, inactivation side (black line) compared to baseline (BL, n = 2); more than 70% suppression of paired stimulation effect at 20 minutes after CNO injection. While on the non-inactivated side no change in spinal MEPs observed. (**C)** Spinal excitability decreased after inactivation. Inactivation also decreases the spinal cord excitability in PV-Cre rats, inactivation side (black line) compared to baseline (BL, n = 2); more than 30% suppression of spinal excitability at 40-50 minutes after CNO injection. While in the non-inactivated side less than 20% change in spinal excitability was observed. Error bars show SEM, excitability = 1/ threshold.

Supplementary Figure 2. Inactivation Controls. (A) CNO only control. Immediate effects of paired stimulation were recorded from right (gray line) and left (black line) forelimb biceps before CNO injection in a normal rat (no virus injection). After CNO injection paired stimulation effects were recorded every 10 minutes for 2 hours. No change in paired stimulation effects on cortical MEPs was observed compared to before CNO injection. (**B1) Saline only control.** In two PV-Cre rats immediate effects of paired stimulation were recorded from right (gray line = no-inactivation side) and left (black line = inactivation side) forelimb biceps before normal saline (0.9% NaCl, vehicle for CNO) injection. After saline injection paired stimulation effects were recorded every 10 minutes for 2 hours. No change in paired stimulation effects on cortical MEPs was observation compared to before saline injection. (**B2)** Similarly cortical excitability was calculated before and after saline injection. No change in cortical excitability was observed compared to before saline injection. Similarly spinal MEPs (**B3**) and Spinal excitability (**B4**) also did not change after saline injection compared to before injection. Error bars show SEM, excitability = 1/ threshold.

Supplementary Figure 3. SCAP protocol optimization. (A) Effect of second stimulation over the first stimulation for different frequencies. (**B)** Protocol to test the effects of different numbers

of paired pulse trains, time intervals between the pulse trains and repeated trains. **(C)** Effects of number of stimuli in a train (150 stimuli are optimum), time interval between trains (1 min was as good as 5 min) and number of trains (10x and 20x are similar) on average MEPs over 120 minutes of testing. (**D1 – D3)** Using the 5Hz frequency, we tested for the length of the train, 150 stimuli were more effective than the others tested. (**E1 – E3) For** the time interval between two bursts, we observed that the 5-minute interval was slightly better than 1 minute, but we selected 1 minute to minimize the total duration of stimulation. (**F1 – F2)** Finally, we tested the total number of repetitions of trains. We observed that 20 trains of stimulation had a more durable effect than 10 trains with a similar overall magnitude. Error bars show SEM, excitability = 1/ threshold.

Supplementary Figure 4. SCAP causes lasting changes in cortical and spinal cord excitability in uninjured rats and rats with SCI. (**A)** Cortical and spinal excitability after 5-minute SCAP in intact rats (n = 6) as well as in rats with C4 SCI (n = 2). In both SCI and intact groups of rats after 5-minutes of repetitive pairing, both cortical and spinal excitability was augmented for more than 1 hour and this augmentation persisted even after SCI, then returned to the baseline. (**B)** Cortical and spinal excitability after 30-minute SCAP in intact rats (n = 4) and in SCI rats (n = 7). In both groups of rats after 30-minutes of repetitive pairing, both cortical and spinal excitability was augmented for more than 2 hours and this augmentation persisted even after SCI, similarly the spinal MEP and excitability was also increased for 2 hours after pairing. Error bars show SEM, excitability = 1/ threshold.

Supplementary Figure 5. SCAP controls. (A1) Effect of 30-minute SCAP in controlled conditions. Baseline cortical MEP, spinal MEP and thresholds were measured before 30-minue repetitive pairing. Cortical and spinal stimulation at 110% of the cortical and spinal thresholds. MEPs recorded from the biceps. Threshold was determined by adjusting stimulation intensity to provoking a short-latency EMG in >50% of trials. For only brain stimulation, motor cortex was stimulated for 30-minutes repetitively at 100% of cortical threshold, after 30 minutes of stimulation baseline measurements were done at each time-point for 2 hours. Similarly for spinal only, spinal cord was stimulated for 30-minutes at 90% spinal threshold and after that baseline measurements were done at each time-point for 2 hours. For pairing at 100ms, motor cortex and spinal cord stimulation were paired repetitively for 30-minutes. Here, first motor cortex was stimulated (at 110% of cortical threshold) then 100ms latter spinal cord was stimulated (at 90% of

spinal threshold). After 30 minutes of stimulation, baseline measurements were done at each time-point for 2 hours. To pair motor cortex and spinal cord stimulation at -20ms, first spinal cord was stimulated at 90% of spinal threshold, then 20ms latter motor cortex was stimulated at 110% of cortical threshold and repeated for 30-minutes. After 30 minutes of repetitive stimulation, baseline measurements were done at each time-point for 2 hours. (**A1)** There was a slight increase in cortical MEPs just after repetitive pairing in all the conditions and it came back to baseline quickly except for pairing at -20ms, here it was at an elevated level for 2hours. (**A2)** The cortical excitability was not changed as compared to baseline values in all the four different conditions. (**B1)** The spinal MEPs also follows similar trends to cortical MEPs but with more variability. (**B2)** Spinal excitability also follows the same trends to cortical excitability except for pairing at -20ms, here there was lots of variability between the rats. Error bars show SEM, excitability = 1/ threshold.

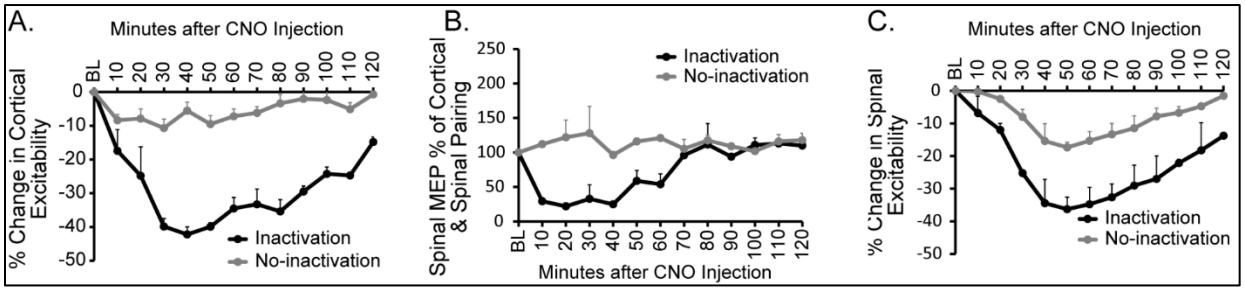
Supplementary table 1: Number of animals (n) used in the immediate effect of motor cortex and spinal cord stimulation latency testing.

Latency (ms)	Supra-threshold motor cortex and subthreshold spinal cord stimulation (Fig. 1B)	Subthreshold motor cortex and subthreshold spinal cord stimulation (Fig. 1C)	Supra-threshold motor cortex and subthreshold spinal cord stimulation after SCI (Fig. 1D)
-30	9	4	2
-29	5	3	NA
-28	5	3	NA
-27	5	3	NA
-26	5	3	NA
-25	9	4	2
-24	5	4	NA
-23	5	4	NA
-22	5	4	NA
-21	5	4	NA
-20	9	4	2
-19	5	4	NA
-18	5	4	NA
-17	5	4	NA
-16	5	4	NA
-15	9	4	2
-14	6	4	NA
-13	6	4	NA
-12	6	4	NA
-11	6	4	NA
-10	6	4	2
-9	6	4	NA
-8	6	4	NA
-7	6	4	NA
-6	6	4	NA
-5	6	4	2
-4	5	4	NA
-3	5	4	NA
-2	5	4	NA
-1	5	4	NA
-0	6	3	2

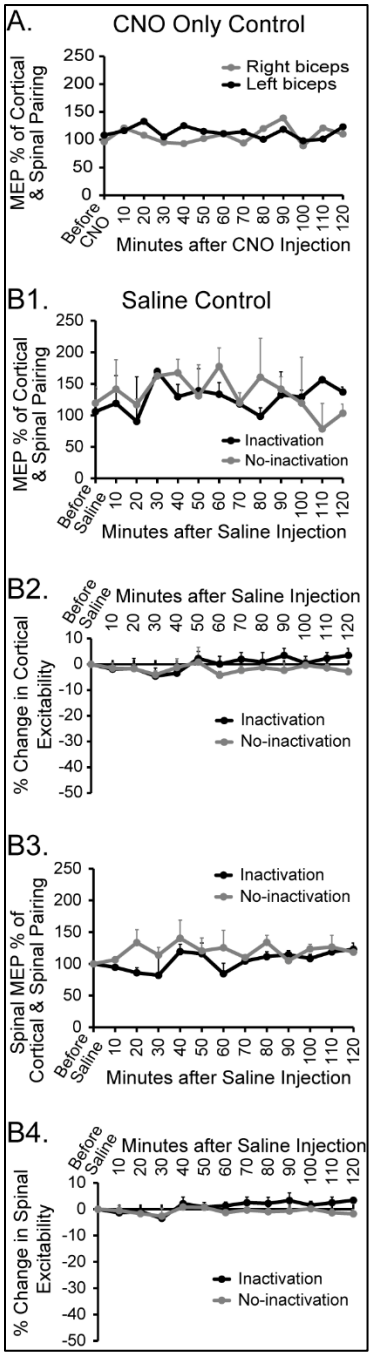
1	5	3	NA
2	5	3	NA
3	5	3	NA
4	5	3	NA
5	6	4	2
6	6	4	NA
7	6	4	NA
8	6	4	2
9	6	4	2
10	6	4	16
11	6	4	2
12	6	4	2
13	6	4	2
14	6	4	2
15	6	4	2
16	NA	4	NA
17	NA	4	NA
18	NA	4	NA
19	NA	4	NA
20	6	4	2
21	NA	3	NA
22	NA	3	NA
23	NA	3	NA
24	NA	3	NA
25	6	4	2
26	NA	2	NA
27	NA	2	NA
28	NA	2	NA
29	NA	2	NA
30	6	3	2

NA = not applicable

Supplementary Figure 1.



Supplementary Figure 2.



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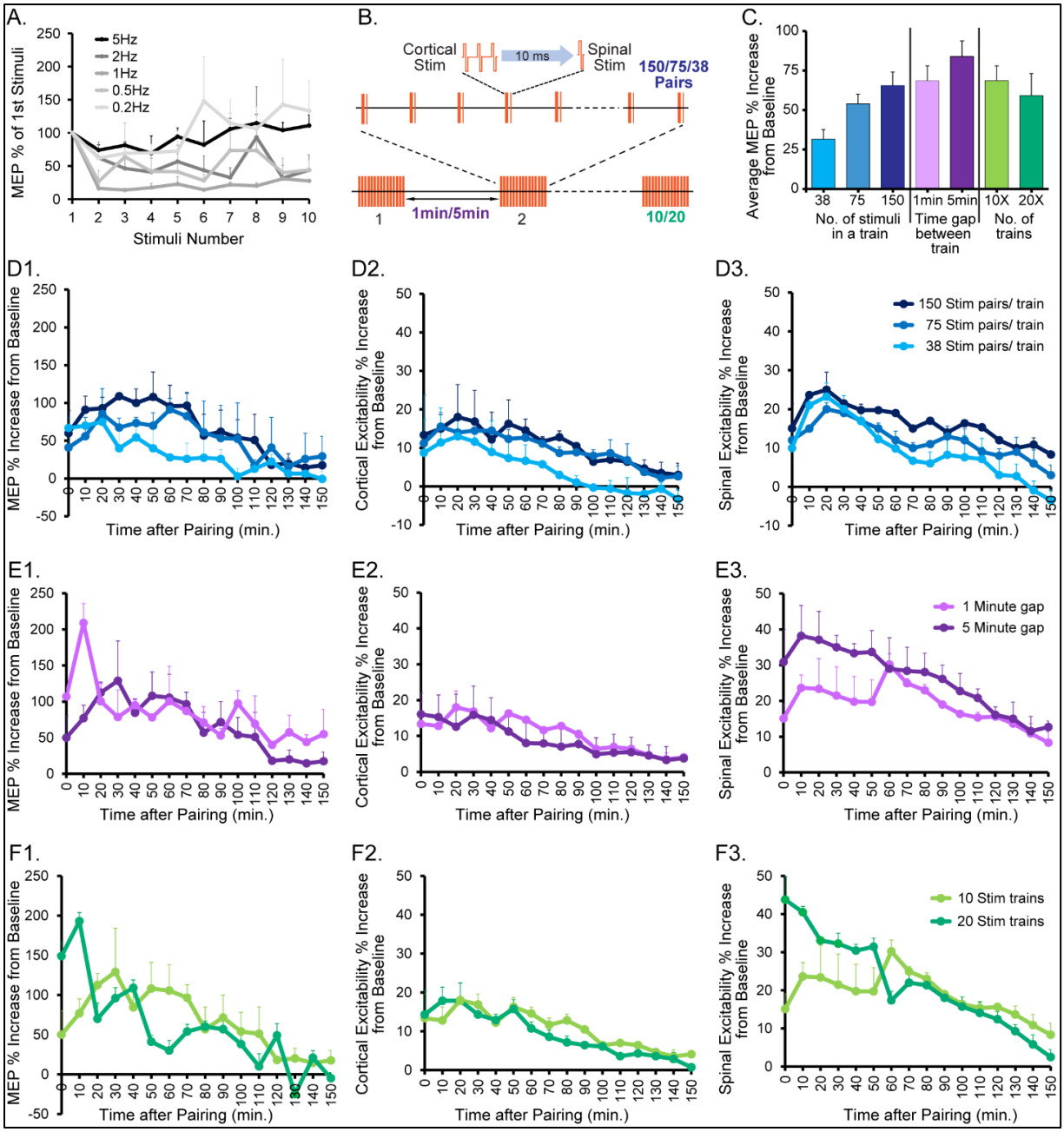

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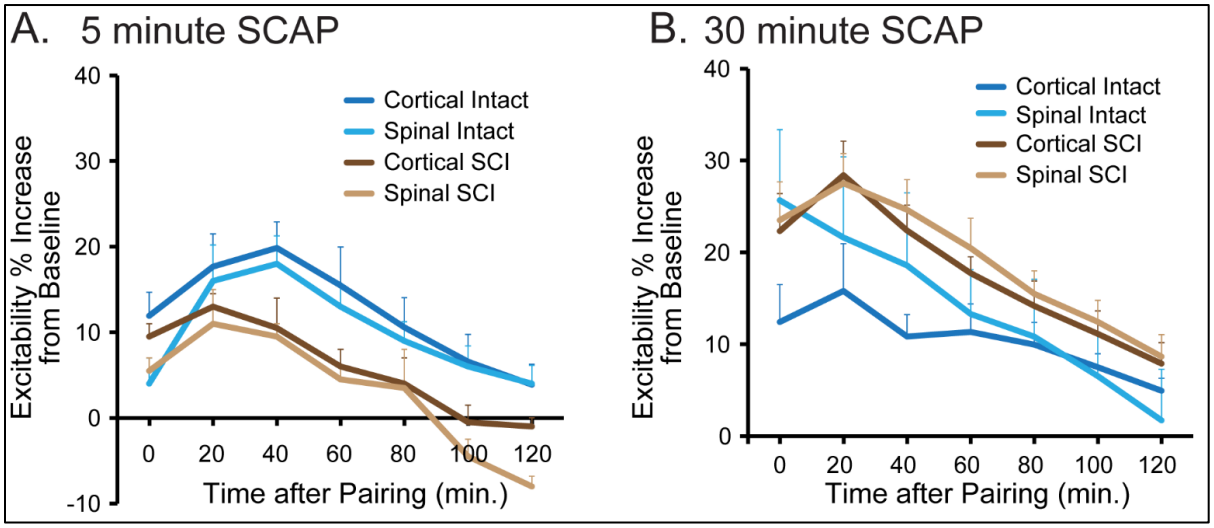
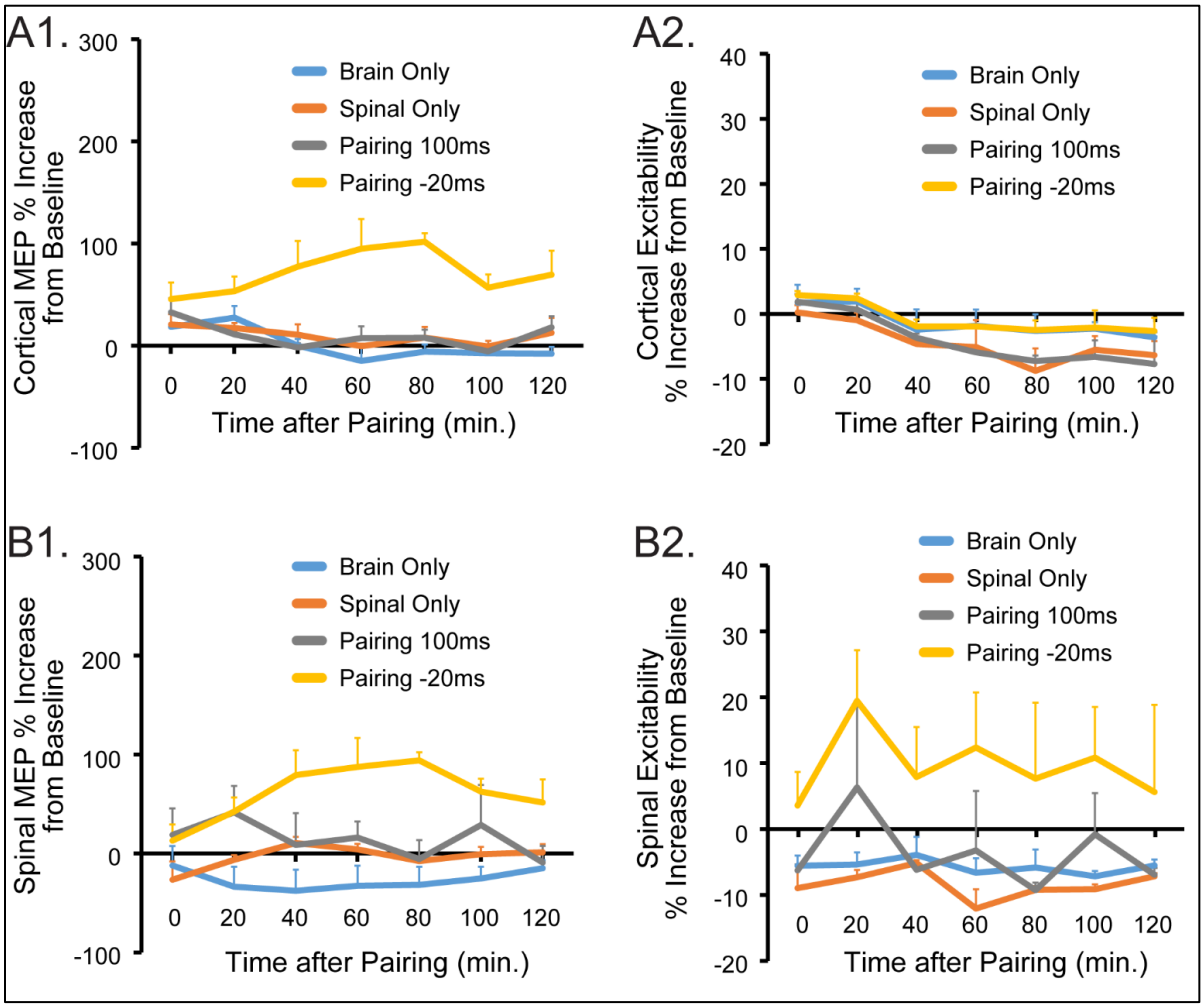
Supplementary Figure 5.