Formation of a Novel Supraspinal-Spinal Connectome that Relearns the Same Motor Task after Complete Paralysis

Authors: L.S. Urban[1]*, M. Thornton[2], K. Ingraham Dixie[2], E. Dale[2], H. Zhong[2], P. E. Phelps[2], J. Burdick[1], V.R. Edgerton[2-6]

Affiliations:

[1] Computation and Neural Systems, California Institute of Technology, Pasadena, CA, USA

[2] Department of Integrative Biology and Physiology, University of California, Los Angeles, CA 90095 USA

[3] Department of Neurobiology, University of California, Los Angeles, CA 90095 USA [4] Department of Neurosurgery, University of California, Los Angeles, CA 90095 USA [5] Brain Research Institute, University of California, Los Angeles, CA 90095 USA [6] Institut Guttmann. Hospital de Neurorehabilitació, Institut Universitari adscrit a la Universitat Autònoma de Barcelona, Barcelona, 08916 Badalona, Spain

*Correspondence to: lurban@alumni.caltech.edu, Present address: EPFL/Campus Biotech, Chemin des Mines 9, 1202 Genève

ABSTRACT (201 words)

Having observed that electrical spinal cord stimulation and training enabled four paraplegic patients with motor complete paralysis to regain voluntary leg movement, the

underlying mechanisms involved in forming the newly established supraspinal-spinal functional connectivity have become of great interest. Van den Brand et al (2012) subsequently, demonstrated the recovery, in response to spinal electro-neuromodulation and locomotor training, of voluntary stepping of the lower limbs in rats that received a lesion that is assumed to eliminate all long descending cortical axons that project to lumbosacral segments. Here, we used a similar spinal lesion in rats to eliminate long descending axons to determine whether a novel, trained motor behavior triggered by a unique auditory cue learned before a spinal lesion, could recover after the lesion. Hindlimb stepping recovered one month after spinal injury, but only after two months the novel and unique audio-triggered behavior was recovered, meaning that not only was a novel connectivity formed but further evidence suggested that this highly unique behavioral response was independent of the recovery of the circuitry that generated stepping. The unique features of the newly formed supraspinal-spinal connections that mediated the recovery of the trained behavior is consistent with a guidance mechanism(s) that are highly use-dependent.

New & Noteworthy

Electrical spinal cord stimulation has enabled paraplegic patients to regain voluntary leg movement, and so the underlying mechanisms involved in this recovery are of great interest. Here we demonstrate in rodents the recovery of trained motor behavior after a spinal lesion. Rodents were trained to kick their right hindlimb in response to an auditory cue. This behavior recovered two months after paralyzing spinal cord injury, but only with the assistance of electrical spinal cord stimulation.

INTRODUCTION

A spinal cord injury (SCI) presents major challenges for functional recovery. First, loss of long-descending axons from multiple supraspinal nuclei precludes any direct brain signals from reaching spinal motor pools. Second, the direct damage caused by the injury disrupts spinal networks spanning multiple spinal segments, presenting a barrier for any reforming direct supraspinal-spinal connections. Despite these complications, there are now multiple individuals with clinically defined complete paralysis, lasting longer than a year, regaining voluntary control of their lower (11 ,9) and upper-limbs (7) while receiving electrical stimulation of selected spinal segments caudal to the lesion. Mechanisms enabling this recovery after paralysis are unknown, since the original purpose of electrical stimulation was only to re-engage local spinal networks distal to the lesion capable of coordinating muscle activity during locomotion, a task that does not require supraspinal control, at least in laboratory animal models of SCI (6).

A previous study demonstrated that spinal stimulation enables paralyzed rats to adjust bipedal locomotion in pursuit of a food reward (2). The experiment assumes that this change in locomotion is caused by a voluntary movement signal generated by the brain and transmitted across the spinal cord injury. This important observation raises several mechanistic questions regarding the specificity of the supraspinal signal that triggered this stepping response and the specificity of the resulting motor behavior. For example, the complexity of the supraspinal neural networks that recognize and generate the desire for feeding, and the numerous sources of stimuli, independent of supraspinal input, that can cause spinal networks to activate stepping behavior, leave open the

question of both the level of sensory specificity as well as to the specificity of the motor response that can be recovered after a complete separation from long descending supraspinal input. Is the response to the food attributable to the sight of the food, it's smell, or the rodent's hunger level? Do previous interactions between the rodent and experimenter influence the rodent's desire to chase a food reward held in the experimenter's hand? Almost certainly, all these factors play a role. Therefore, the voluntary movement signal generated in the brain is an unknown and complex mixture of multiple sensory modalities and internal motivations, making it difficult to identify the specificity of the neural pathway(s) responsible or necessary for the recovery of a learned, novel and specific supraspinally derived sensory- spinal motor response after a spinal cord injury.

It has also been argued that a supraspinal signal is not even necessary to modulate locomotion in this experimental setup, but that the modulation could be generated purely by neural networks caudal to the injury site, without any recovered neural connection crossing the spinal cord injury (18). This argument states that the spinal circuits that generate locomotion are very sensitive to hindlimb proprioception, and this proprioceptive signal is based on the rodent's body position. Since the spinal injury model used in the experiment only induced paraplegia, upper-body movement was preserved in these rodents. Therefore, the rodents could alter their body position by moving their upper-body in response to the food reward, and this in turn would change the proprioceptive signal generated from the hindlimbs (for example, shifting the upper body to the right will place more weight on the right hindlimb). This new hindlimb

86 proprioceptive signal will be fed back from the hindlimbs into the lumbosacral spinal
87 circuits, and these spinal circuits will incorporate this new proprioceptive signal into the
88 local spinal activity, which could result in a change in the gait pattern. Thus, the
89 modulation of locomotion can occur entirely caudal to the injury site, using only neural
90 connections between the lumbosacral enlargement and proprioceptive signals in the
91 hindlimbs, and without any direct neural connection recovering between the brain and the
92 lumbosacral spinal circuits (19).

93 The presented data, however, demonstrate highly specific supraspinal-spinal
94 networks that can form novel functional connectivity capable of recovering a unique
95 trained motor behavior learned prior to a spinal lesion.

96 **MATERIAL AND METHODS**

97 Rodents (Sprague-Dawley rats; n=10) were trained to kick their right hindlimb in
98 response to a randomly occurring auditory cue, termed here as the flexion task. In
99 contrast to the modulation of locomotion, which was an untrained byproduct of the
100 animal's desire for food, our trained flexion behavior was directly triggered by an
101 auditory signal with a specific tone. This clearly identifies the auditory cortex as the
102 origin of the voluntary movement signal, and provides a precise time to align behavior to
103 study the recovered connection. To prevent upper-body movement from initiating this
104 flexion behavior, rodents in our study were secured in the prone position with their
105 hindlimbs hanging in free space.

All surgical and animal care procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California Los Angeles Chancellor's Animal Research Committee. This study involved adult female Sprague-Dawley rats, which were randomly assigned to the different cohorts.

Behavioral Training

Each rat was trained before the spinalization surgery by pairing the auditory cue (3kHz tone, 250 ms duration) with the vibration of a small motor temporarily taped to the rodent's right hindlimb. The initial vibration provoked a leg movement, which was positively reinforced with a chocolate food reward. The intensity of vibration was slowly lowered over multiple trials, until the rat performed the task without the vibration. Once the rat reliably performed the task, the motor was removed completely. This behavior took approximately one week to train, but one rat acquired the task in a single session.

In our experiment, rodents were trained on the behavioral task for one month prior to spinal cord injury. Baseline behavior was measured using video and EMG recordings, allowing each rodent's preinjury behavior to serve as their own control. The rodents then received a simultaneous double-hemisection spinal cord injury (left T7, right T10), after which the rodents were split into cohorts based on their treatment options. Pilot rats (n=2) were studied for two months after SCI, with spinal stimulation. Treated rats (n=4) were studied for four months after injury, with spinal stimulation. Untreated rats (n=2) received

the same training and injury, but never received spinal stimulation after injury. To ensure that any recovered behavior was not an artifact of the spinal stimulation, untrained rats (n=2) received the same injury and were treated with spinal stimulation as the pilot and treated cohorts, but were not trained on the flexion task. During recording sessions with the untrained rats, the auditory cue triggered a reward regardless of behavior. Finally, there were two incomplete-injury rats (n=2) that recovered weight bearing and stepping within ten days of spinalization.

Rodents underwent two surgical procedures; an implantation followed a month later with a spinalization. Trained surgical staff performed both surgical procedures using isoflurane anesthesia (1-2.5% via facemask) and aseptic techniques. The level of anesthesia was continuously monitored during both procedures. After each procedure, the rats were placed under observation and administered fluids, painkillers, and antibiotics.

Electrode Implant

The electrode implant consisted of insulated stainless steel wires (AS632, Cooner Wire) soldered into a percutaneous head-plug connector. Incisions were made on top of the skull and over the desired muscle groups. The wires were fed under the skin from the skull to the desired muscle group. A small notch was cut into the insulation of each wire to expose the underlying metal wire, which formed the electrode site. Rats were implanted with bipolar intramuscular EMG electrodes embedded into soleous (Sol), tibalis anterior (TA), vastus lateralis (VL), and sartorius (ST) muscles of the right (trained) hindlimb and in the tibalis anterior of the left hindlimb (TA-left). The untreated

147 and untrained rats had electrodes embedded only in the TA of the right (trained) hindlimb.
148 EMG wires were threaded through each muscle using a cannula. The wire was adjusted to
149 embed the electrode site in the belly of the muscle, and secured in place.

150 The stimulating electrodes were embedded by first performing a partial
151 laminectomy over the L2 and the S1 spinal levels. The stimulating electrodes were passed
152 under the spinous process, and sutured to the midline of the dura above and below the
153 electrode site. A set of common ground wires was inserted subcutaneously in the mid
154 back region. The percutaneous head-plug was fixed to the skull using bone-screws and
155 dental cement.

156 *Spinalization Surgery*

157 The double hemisection spinalization began by performing a laminectomy at
158 spinal levels T7 and T10 by incising the skin and separating the musculature over ~T6-
159 T11. A small incision was made in the dura just left of the midline of the spinal cord at
160 spinal level T7, and a left over-hemisection was performed via aspiration. The same
161 technique was used on the opposite side of the spinal cord to create the right hemisection
162 at spinal level T10. The muscle and skin were then sutured closed with 4-0 Vicryl and
163 Ethilon respectively. The bladder was manually expressed three times a day for two
164 weeks, until reflex voiding was established.

165 *Rehabilitation*
166 To encourage neural reorganization after injury, the treated and pilot rats received

in-cage multi-hour subthreshold stimulation sessions (8) and treadmill step training in the presence of tonic subthreshold stimulation, three times a week. During subthreshold stimulation the rat's headplug was connected to a long stimulating cable on a swivel, which allowed the animal to move freely around their home cage. EMG activity was not recorded during these sessions. Typical sessions lasted three hours. During step training, the rats, with spinal stimulation, were suspended in a harness above a treadmill to perform bipedal stepping. The treadmill speed was varied to evoke different stepping patterns. Kinematics were not recorded during these sessions. Typical sessions lasted fifteen minutes. Treadmill training was stopped one month after the injury, and multi hour stimulation was stopped after two months. The rats returned to the flexion task one month after the injury. Stimulation parameters during the flexion task were fixed to a 40 Hz pattern at 80-90% of motor threshold, and were not modulated to evoke behavioral response.

Experimental Setup

Recording sessions were conducted using custom MATLAB software (MathWorks, 2015). A computer would play a sound through a speaker at a random interval, and the experimenter could control the number of trials per minute. During the flexion task, rats were secured in place using a cloth harness. On the belly of the harness was a piece of Velcro that coupled to an experimental mount. The mount was a flat metal rod that was approximately the width of the rat. Notches were cut into the sides of the mount to allow the animal's legs to hang free. The mount was secured over the

experimenter's lap. Epidural stimulation parameters derived from prior studies (2) were: (40 Hz, L2 [anode], S1 [cathode], monophasic, rectangular waveforms, 200 microsecond duration, voltage controlled, 1-4 V).

EMG signals were differentially amplified (A-M system; Model 3500) and band pass filtered between 1 Hz and 5kHz. The signals were digitized (National Instruments; BNC-2111) and recorded to hard drive (LabView; custom). EMG data was processed offline using custom MATLAB software (MathWorks; 2015).

Additional Experiments

In addition to the standard auditory-flexion task, the rats were also tested in novel tone conditions, where the auditory cue was replaced with a sound the animals had not been exposed to previously (frequency chirp, 500Hz->3kHz, 250 ms duration). These novel tone sessions were recorded with the same equipment and in the same environment as the standard flexion task. During pharmacological testing strychnine or quipazine, was injected intraperitoneally (10) (quipazine - 0.3 mg/kg; strychnine 0.1 mg/kg). The drugs were never mixed. The auditory-flexion task was initiated 10 min after the drug was injected

Kinematics were recorded using video tracking software and visual markers placed on the rodent's hindlimbs. Baseline stepping kinematics were recorded immediately before the quipazine injection and without spinal stimulation. Rodents were

then injected intraperitoneally with quipazine (0.3 mg/kg), and the drug was given 10 min to take effect. The treadmill step testing was then repeated, again without spinal stimulation.

Histology

Rats were anesthetized with ketamine and xylazine, and perfused intracardially with paraformaldehyde [4%]. The spinal cord was dissected, cryoprotected with 30% sucrose, and embedded in OCT compound (Tissue-Tek). The lumbosacral enlargement was sectioned coronally (25 microns), whereas the thoracic region containing the injury site was sectioned horizontally (25 microns). Spinal cord sections were stored at 4°C in Millonigs buffer with azide.

Primary antibodies were used for immunolabeling neuronal cells (NeuN, mouse, Millipore, diluted 1:1500), neural activity (c-Fos, rabbit, Millipore, diluted 1:1500), choline acetyltransferase (ChAT, goat, Millipore, diluted 1:500), serotonin (5HT, goat, Immunostar, diluted 1:500), glial fibrillary acidic protein (GFAP, mouse, BD Biosciences, diluted 1:1000), or neurofilament (NF-H, rabbit, Millipore, diluted 1:1000). Sections were permeabilized with Triton-X (4%) and placed in species appropriate serum as a presoak (donkey, Jackson ImmunoResearch) before incubation with primary antibodies overnight. To visualize immunolabeling, species appropriate AlexaFluor 488, 555, or 647 secondary antibodies (diluted 1:100–500; Jackson ImmunoResearch) were used. Nuclei were stained with Hoechst dye (Sigma-Aldrich) for 5 min and sections were cover slipped with Fluorogel (Electron Microscopy Sciences). Spinal cord sections were

imaged with an Olympus AX70 microscope and Zen 2012 image capture software (Carl Zeiss) with the panorama module. The resulting images were processed using custom MATLAB software (MathWorks, 2015).

QUANTIFICATION AND STATISTICAL ANALYSIS

Muscle Activity

EMG activity of the tibialis anterior in the trained hindlimb was segmented around each trial (3 seconds before the auditory cue, 6 seconds after). EMG bursts were extracted manually. To test for significance, a histogram of the EMG burst onsets was computed, and a Pearson's Chi-Squared coefficient was calculated to test the likelihood that the timing distribution of TA burst was drawn from a uniform random distribution.

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EMG power in the tibialis anterior was also cross-correlated with EMG power from either the left tibialis anterior or the right vastus lateralis.

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The EMG power was defined as the instantaneous power in the raw EMG signal, band passed filtered between 0.1 Hz and 20 Hz. This cross-correlation was performed using a 4-second window of EMG activity occurring either immediately following or immediately preceding the auditory cue (Fig. S5).

Immunohistochemistry

Active neurons in the coronal sections from the lumbosacral enlargement were manually identified by colocalizing the immunohistochemical labeling of c-Fos and

NeuN. The total number of active neurons, and the number of active neurons located dorsal to the central canal, were counted for eight coronal sections per rat, spaced evenly between the L1 and L4 spinal levels. The mean and standard deviation in the total number of active neurons per coronal section, and the percentage of active neurons dorsal to the central canal, was calculated for each rat and displayed as a bar graph.

To study the spatial distribution of active neurons in the lumbosacral enlargement, the coronal sections were split into a 56x56 grid centered on the central canal. For each coronal section, the number of active neurons in each grid element was counted and the result was spatially smoothed by convolving with a two-dimensional Gaussian filter. These cell-count grids were aligned based on the central canal from each coronal section for every rat. Data from these cell-count grids was pooled into two groups based on the desired comparison (comparison 1 - group A: the treated and the incomplete injury rats, group B: the untreated and the untrained rats, comparison 2 - group A: only the treated rats, group B: the untreated and the untrained rats, comparison 3 - group A: the treated rats, group B: the incomplete-injury rats). Within each grid element, the cell counts from each of the eight coronal sections from all rats in group A were compared against all rats in group B using a one-way ANOVA. Grid elements with $p > 0.005$ were ignored. If the mean number of active cells in group A was larger than group B, the grid cell was colored on the red scale. If the mean number of active cells in group A was smaller than group B, the grid cell was colored on the blue scale. To provide anatomical landmarks, these results were then projected onto the average NeuN immunofluorescence image taken across all coronal sections from all rats in all cohorts (Fig. 4).

RESULTS

Functional Recovery Two Months After Spinal Cord Injury

Before injury, all trained rats quickly and consistently responded to the auditory cue (Fig 1A, Fig 2A, movie S2). The median response time to the auditory cue before injury was approximately 600 milliseconds across all animals. The likelihood that this EMG activity was randomly occurring and unrelated the auditory cue was extremely low across all rodents ($p < 10^{-10}$). Immediately following injury, the rodents failed to generate useful muscle activity during spinal stimulation during the flexion task, standing, or stepping (Fig 1B). One month after injury, treated and pilot rats (n=6) recovered standing and stepping during spinal stimulation as has been observed in previous studies (5), but failed to generate any significant EMG activity during the flexion task (Fig 1C, Fig 2B). Two months after injury, however, spinal stimulation enabled all treated and pilot rats to recover the flexion response to the trained auditory cue (Fig 1D, F, G). The timing of the muscle activity in response to the auditory cue became more delayed and variable (Fig 2C, movie S3, movie S4). The median response time to the auditory cue after injury ranged from 1.3 seconds to 2.47 seconds, and again the likelihood that this EMG activity was randomly occurring and unrelated the auditory cue was extremely low ($p < 10^{-10}$) for all rodents. Without the sub-motor threshold spinal stimulation, these rats failed to generate leg movement (Fig 2D). Starting and stopping spinal stimulation enabled an immediate recovery and loss, respectively, of the trained behavior (Fig 2F, movie S5).

Recovered Performance Was Not An Auditory Reflex

To ensure that the recovered flexion movement was not a simple auditory reflex, but a trained response to a specific stimulus, we examined muscle activity after injury during the startle response and during novel-tone conditions. The classic startle response (a slight whole-body twitch, evoked using a loud burst of white noise) differed significantly from the recovered flexion movement (Fig S3A). The EMG activity triggered by the startle response occurred substantially faster (100 ms for startle activity, Fig S3A, vs 1-2 seconds for flexion response, Fig 2C) , and was significantly weaker and more stereotyped in all rodents. Treated rodents (n=4) also failed to generate leg movement after injury when the auditory cue was replaced with a novel tone that did not trigger a food reward (Fig S3B). Additionally, the untrained rodents, which received the same injury and treatment as the treated cohort, but were never trained to kick their hindlimb in response to the auditory cue, also failed to generate leg movement in response to the auditory cue both before and after spinal injury (Fig S7B).

Increased Correlation of Muscle Activities After Injury

Before injury, the flexion response to the auditory cue was a clear unilateral leg movement. After injury, the response became a bilateral and alternating movement, similar to stepping, and the patterns of flexor and extensor activation in the hindlimb muscles became more correlated. Performing a cross-correlation between EMG activity of the right and left tibialis anterior (TA) muscles showed a large spike in correlation occurring at approximately -100 ms offset, which was observed neither before injury nor

after injury in spontaneous muscle activity unrelated to the auditory cue (Fig 2 H). This spike in correlation after injury means a burst of activity in the right tibialis anterior (TA) was consistently preceded approximately 100 ms earlier by activity in the left (untrained) TA muscle. A similar spike in correlation between EMG activity was observed between the left (untrained) TA and the right vastus lateralis (VL). Performing the cross correlation analysis between EMG activity from these two muscle groups shows a spike of correlation at nearly 0 ms offset after injury, which was similarly neither observed before injury nor after injury in spontaneous muscle activity unrelated to the auditory cue (Fig 2 H). This spike in correlation after injury means that the left TA muscle and the right VL muscle co-contracted simultaneously in response to the auditory cue. As noted, this correlation between muscle activity of the different muscle groups did not occur in spontaneous muscle contractions occurring after injury and unrelated to the auditory cue. This suggests that the observed muscle co-contractions were a product of the supraspinal signal, and not a simply a generic motor action.

In a few early recording sessions, one treated rodent (rodent 4) failed to make visible hindlimb movements in response to the auditory cue, but generated tonic muscle activity only in the left (untrained) hindlimb (Fig S5D,E,F). Histology showed the T7 hemisection in this rat induced a large cyst, extending well across the midline, which was not seen in the other treated rats (Fig S4). While this particular rodent struggled to perform the flexion task in these early recording sessions after spinalization, in later recording sessions the rodent successfully performed clear visible kicks in the trained right leg in response to the auditory cue.

335 Four months after injury, there was no consistent further improvement in response
336 time in the treated rats compared to their initial recovered behavior at two months.
337 During the final recording session, one rat responded faster than its initial recovered
338 behavior, two responded slower, and the other showed no change (Fig S6).

339 *Quipazine Selectively Abolished Performance*

340 To explore the recovery mechanism, the treated rats received intraperitoneal
341 injections of either quipazine (a nonspecific 5HT agonist) or strychnine (a glycine
342 antagonist), both of which increase spontaneous leg movement and have been
343 successfully used to facilitate locomotion post SCI (10). Rats treated with strychnine
344 showed improved responses to the auditory cue (Fig 3B). Quipazine, on the other hand,
345 facilitated treadmill stepping (Fig 3D, Movie S6), but abolished performance during the
346 flexion task (Fig 3C). Quipazine did not interfere with the flexion response in uninjured
347 rats (Fig 3E).

348 *Recovered Performance Requires Behavioral Training and Electrical Spinal*
349 *Neuromodulation*

350 The recovered flexion response required both behavioral training and spinal
351 stimulation. Untreated rats (n = 2), which were trained on the task but received no spinal
352 stimulation, failed to generate leg movement after injury (Fig S7A). Untrained rats (n =
353 2), which were never trained on the task but received spinal stimulation after injury,
354 failed to generate muscle activity correlated with the auditory cue before or after injury

(Fig S7B). Rats with incomplete injuries successfully performed the flexion task without spinal stimulation after injury (n = 2) (Fig S7C).

Localized Patterns of Neural Activity in the Lumbosacral Enlargement Neural activity in the lumbosacral enlargement was studied using immunofluorescence labeling for the c-Fos protein, which is expressed in neurons that undergo a critical level of firing of action potentials (3). An hour after the final flexion test (lasting 45 minutes) rats were anesthetized with ketamine and xylazine, perfused, and prepared for immunohistochemistry. During this final test, treated rats (with stimulation) and incomplete-injury rats (without stimulation) successfully performed the flexion task, whereas untreated rats (without stimulation) and untrained rats (with stimulation) failed to generate leg movement.

Neurons co-expressing the c-Fos protein and the neural marker NeuN (16) were manually identified in eight 25 μm coronal sections of spinal tissue spaced evenly between the L1 and L4 spinal levels per rat. Similar numbers of c-Fos positive neurons were observed across all rodents (stimulated or unstimulated), suggesting the therapeutic effect of spinal stimulation is not to purely increase spinal activity (Fig 4A). The majority of c-Fos expressing neurons were located in the dorsal spinal cord in all rats (Fig 4B). The coronal sections were split into a 56x56 grid centered on the central canal, and active neurons in each grid element were counted. Results were spatially smoothed and all coronal sections were aligned to the central canal. To compare spatial distribution of neural activity between cohorts of rats, a one-way ANOVA was calculated in each grid

element that contained at least one active neuron in any coronal section from any rat (n =1235).

Rats that successfully performed the flexion task (treated and incomplete-injury cohorts) showed increased neural activity near the right (trained) side of the central canal and decreased neural activity in the right lateral spinal nucleus (LSN) compared to rats that failed (untreated and untrained cohorts) (Fig 4B). Treated rats showed broader neural activity along the medial side of the right dorsal horn, and an additional region of increased neural activity in the deep medial section of the left dorsal horn, in comparison with the untreated and untrained rats (Fig 4C). Increased activity in the left dorsal horn was unique to the treated rats (Fig 4D). Both regions of increased activity overlap with areas that normally receive dense corticospinal projections (20), and the region of decreased activity overlaps with the LSN which processes both sensory feedback and noxious stimuli from the hindlimb (14).

Cholinergic premotor interneurons, which modulate excitability of motor neurons (15), populate the area of active c-Fos near the central canal, and were identified using ChAT immunofluorescence in the same coronal sections described above. However, our analyses did not detect any ChAT-positive active neurons near the central canal in all rodent.

Surviving Direct Supraspinal Projections in Incomplete-Injury Rodents Anatomical analysis of the incomplete-injury rodents showed serotonergic axons, which originate in the raphe of the brain stem, were present in their lumbosacral enlargement (Fig S2A), showing that direct supraspinal projections survived in these two rodents. As noted, these

two incomplete injury rodents were able to perform the flexion task after injury without spinal stimulation, likely a result of these surviving long-range axons. Serotonergic axons were absent in the lumbar spinal cord of all other rodents (Fig S2B), and no other rodent demonstrated weight-bearing or walking after spinalization without the assistance of spinal electrical stimulation

DISCUSSION

Our results demonstrate that electrical neuromodulation of the lumbosacral enlargement can facilitate the formation of novel functional connections between highly specific supraspinal and spinal networks to restore a motor behavior learned prior to a spinal lesion. That is, novel connectivity was formed from the brain to specific motor pools necessary to respond to a specific auditory stimulus. While the novel supraspinal spinal functional connectivity was slightly less specific in the temporal pattern and complexity of the movement responses compared to the preinjury connection (i.e. delayed bilateral and alternating hindlimb movements vs fast unilateral leg kick), there was clear supraspinal influence on the acoustically unique initiated flexor response after spinal injury. This recovered connection reformed two months after injury, but remained dormant without the aid of spinal electrical stimulation. The flexion behavior was recovered within seconds of applying the spinal stimulation, demonstrating a fast-acting mechanism for recovery. Also, since the spinal stimulation was directly targeted to the lumbosacral enlargement, located a significant distance caudally from the site of the thoracic spinal injury, the mechanisms for this recovery is likely localized not only in the

lumbosacral segments but along the entire length of the spinal segments, often referred to as the propriospinal system, which is known to be involved in functional recovery after spinal cord injury (4). Most segments are synaptically connected by interneurons that project in one or more directions, caudally, rostrally and/ or bilaterally. These results demonstrate that spinal stimulation facilitated intersegmental communication among propriospinal system linking supraspinal and spinal networks. The role of spinal stimulation in recovery, therefore, likely facilitated the excitability of the spinal neurons to a physiological state that enabled the supraspinally derived signal sufficient to trigger a novel, learned spinal response.

This recovered supraspinal signal tracing through propriospinal relays can be characterized as traveling along a chain of multisynaptic connections down to the lumbosacral motor pools, resulting in transmission delays at each synaptic junction. This would account for the delayed motor response to the auditory cue observed in all six rodents that recovered behavior.

Additionally, due to the loss of direct monosynaptic connections between the motor cortex and individual motor neurons in the spinal cord, a recovered connection mediated through the propriospinal networks would have likely formed more diffuse connections with the lumbosacral spinal circuitry. These diffuse connections would result in less targeting specificity of individual motor pools, thereby causing less precise voluntary movement. For example, before injury the rodent's motor cortex could directly activate specific motor pools of the right hindlimb, resulting a clear unilateral leg movement. However, after injury, as the recovered supraspinal signal traces through

441 diffuse propriospinal relays, multiple motor pools were coactivated. Therefore, the
442 recovered supraspinal connections were less specific than preinjury with regard to the
443 number of motor pools activated and the number of EMG bursts per response to each
444 auditory cue.

445 The original purpose of epidural stimulation was to elevate the general level of
446 neural excitability in the spinal cord such that the automaticity of the spinal networks
447 could reengage and restore standing and walking in paralyzed patients (6). The
448 effectiveness of increased spinal excitability was discovered also using a mixture of drugs
449 like strychnine and quipazine (10). The selective interference of quipazine on the flexion
450 task in this study, while preserving standing and stepping, provides further evidence that
451 the supraspinal circuits that enabled the recovery of the learned motor task was distinct
452 from stepping and was governed by different excitatory mechanisms than the spinal
453 circuits traditionally studied during standing and stepping.

454 It appears that the disruption of the learned motor task was not simply the result
455 of a general increase in neural excitability introducing a significant level of noise in to the
456 recovered neural pathway. Thus, neurons expressing 5HT receptors appear to play a
457 critical role in the transmission of supraspinal signals after a spinal cord injury. The
458 specificity of this novel pathway also suggests it has the potential to be selectively
459 targeted pharmacologically.

460 This observation is not in conflict with the recovered performance observed in the
461 incomplete-injury rodents, which retained serotonergic projections from the brain to the
462 lumbosacral spinal circuitry after the spinal cord injury. The incomplete-injury rodents

had both a different behavioral response (successfully weight bearing and walking without therapeutic intervention) as well as a difference in anatomy (surviving long-range fibers reaching) in comparison to the other rodents in the study. Therefore, it is likely that the observed performance of these incomplete-injury rodents is due to a different mechanism than the recovery mechanism observed in the trained and pilot rodents. We expect that the recovered pathway in these incomplete-injury rodents is mediated at least in part via uninjured connections near and through the injury site. We also observed that, prior to injury, the administration of quipazine failed to abolish behavior during the trained flexion task in uninjured rodents (see Fig 3E). Likely, the residual connections after an incomplete injury are sufficiently normal to avoid a 5HT suppressive effect.

The possibility of a generating a unique and novel supraspinal-spinal neural network that can effect a highly specific trained response is also supported by the heterochronic emergence of a two-month delay in recovery of the trained flexion task compared to a one-month delay after injury for standing and stepping. If trained flexion response utilized the same neural networks required for standing and stepping, then one would expect both behaviors to recover simultaneously. These two behaviors did not recover simultaneously, therefore the mechanism enabling standing and stepping after spinal cord injury was insufficient for the recovery of voluntary movement. While the double hemisection SCI does not precisely match human SCI, it replicates the loss of long descending axons and induces damage across multiple spinal segments. Similar delayed responses and decreased muscle specificity is also observed in human subjects

(11,9), therefore, similar recovery mechanisms may occur in both cases. Modulation of locomotion has also been demonstrated after a controlled contusion SCI in rodents (1).

A unique feature of our flexion task is that the movement is discrete, highly repeatable, and locked to a qualitatively unique external auditory stimulus. By aligning neural activity to the auditory cue, our flexion task enables event-related techniques (13) for comparing functional properties of brain regions before and after injury in the same animal, and for functionally identifying spinal neurons involved in recovery. Our flexion task also provides quantifiable metrics (such as response latency and EMG amplitude) for optimizing electrode positions and stimulation parameters, which can then be quickly translated into clinical treatments.

Recovery of the flexion behavior after the elimination of long descending supraspinal connections demonstrates that: 1) supraspinal networks have access to a remarkable guidance phenomena extending all the way from the brain to the interneuronal networks projecting to specific lumbosacral motor pools, 2) this guidance specificity is not perfect (becoming bilateral), at least within the timeframe studied, but the relearned response was predominantly side specific 3) delayed responses were consistent with engagement of a greater number of synaptic links, probably via proprospinal intersegmental networks, 4) these processes were influenced and perhaps, necessarily driven by activity–dependent mechanisms and 5) functionally specific connectivity from supraspinal to the lumbosacral spinal segments, caudal to a mid thoracic spinal, complete lesion can be restored with a combination of spinal neuromodulation and task specific training.

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Data and materials availability: All data are available in the main text or the supplementary materials.

Supplementary Material Links:

Figures - https://doi.org/10.6084/m9.figshare.12613457

Videos - https://doi.org/10.6084/m9.figshare.12620066

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Fig 1. *Cartoon model of experimental timeline.* A) Before injury, a spinal network can successfully generate standing, stepping, and perform the learned flexion task requiring the supraspinal auditory cue. B) Immediately after the injury, spinal stimulation fails to recover any coordinated behavior from the spinal network (no standing, stepping, or learned behavior). C) One month after injury, standing and stepping recovers during spinal stimulation, but the animals fail to perform the trained flexion task. D) Two months after injury, a new connection between the brain and the spinal circuits has formed (colored red), and spinal stimulation enabled the spinal circuitry to stand, step, and incorporate supraspinal signals to perform the trained flexion task.

Fig 2. *Muscle activity during the flexion task before and after injury*. **A,B,C,D**) EMG traces from the right tibilalis anterior (TA) aligned to the auditory cue (blue line) during sequential trials of the flexion task, from the same treated rat (rodent 1) before and after injury, with and without stimulation. **E**) Right TA activity two months after spinalization from a treated rat (rodent 3) when switching spinal stimulation on (white) and off (red)

during sequential trials of the flexion task. **F**) Peristimulus time histograms of EMG bursts from the TA muscle in relation to the auditory cue before injury (red) and after (blue) in four treated rats. Triangles denote median response time. (* p < 10^-10, Pearson Chi-Squared). **G**) Median EMG power from the right sartorius muscle, aligned to the auditory cue before (red) and after (blue) spinalization in two additional pilot rodents. **H**) Cross-correlation of EMG activity between the left and right TA muscles (*left)* and between the right vastus lateralis (VL) and left TA muscle (*right*) during the flexion task in three conditions: 1) before injury (red), 2) after injury only using muscle activity occurring 4 seconds following the audio cue (blue), and 3) after injury only using muscle activity occurring 4 seconds before the audio cue.

Fig 3. *Pharmacology effects on auditory-flexion task.* **A,B,C**) Right TA muscle activity after SCI from the same treated rat during the flexion task with **A**) only spinal stimulation, **B**) stimulation plus strychnine or **C**) stimulation plus quipazine. **D**) Video frames of the right hindlimb at three time points during treadmill testing with and without quipazine (after SCI, without spinal stimulation). White dots are markers placed over the joints of the right hindlimb to visualize stepping patterns. **E**) Effect of quipazine on TA muscle activity before injury. **F**) Cartoon model showing the more systemic influence of quipazine on neuronal networks, and the selective loss of the learned flexion task after injury while receiving spinal stimulation, despite maintaining standing and stepping.

Fig 4. *Spatial distribution of active neurons in coronal sections of the lumbosacral enlargement four months after injury*. **A**) The mean and standard deviation in the number of active neurons from eight coronal sections spaced evenly between L1-L4 spinal levels per rat, and **B**) percentage of those active neurons located in the dorsal spinal cord. **C,D,E**) Spatial distribution comparisons of the number of active neurons between cohorts of rats. The treated rats (Group A, n=4) were treated with spinal stimulation and successfully performed the flexion task. The untrained rats (Group B, n=2) received spinal stimulation, but failed to perform the flexion task since they were never trained on it. The untreated rats (Group C, n=2) did not receive spinal stimulation and failed to perform the flexion task. Finally, the incomplete-injury rats (Group D, n=2) were not treated with spinal stimulation but successfully performed the flexion task. These two incomplete-injury rats had recovered standing and walking within one week of spinalization. For each of the three comparisons, the coronal sections were discretized, smoothed, and a one-way ANOVA was computed in each grid element ($p > 0.005$ were ignored). In the heat maps, orange denotes increased activity, blue denotes decreased activity.

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FIG 1

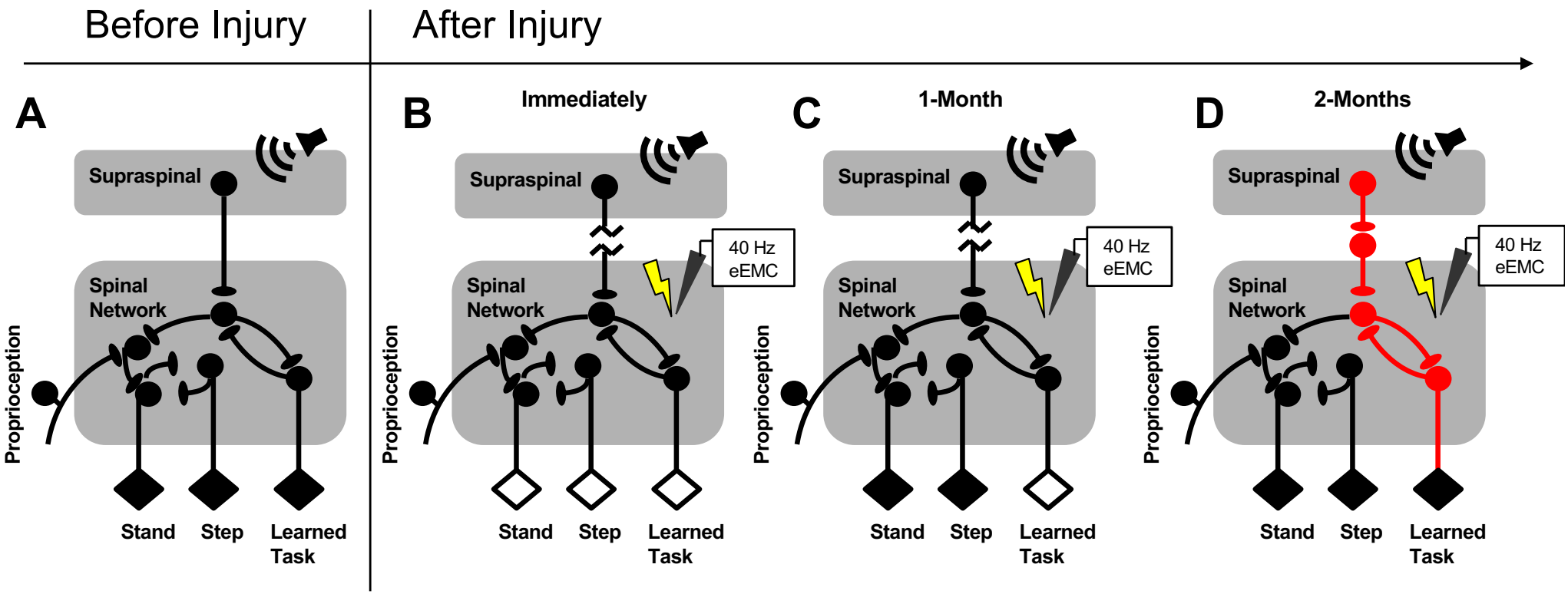

FIG 2

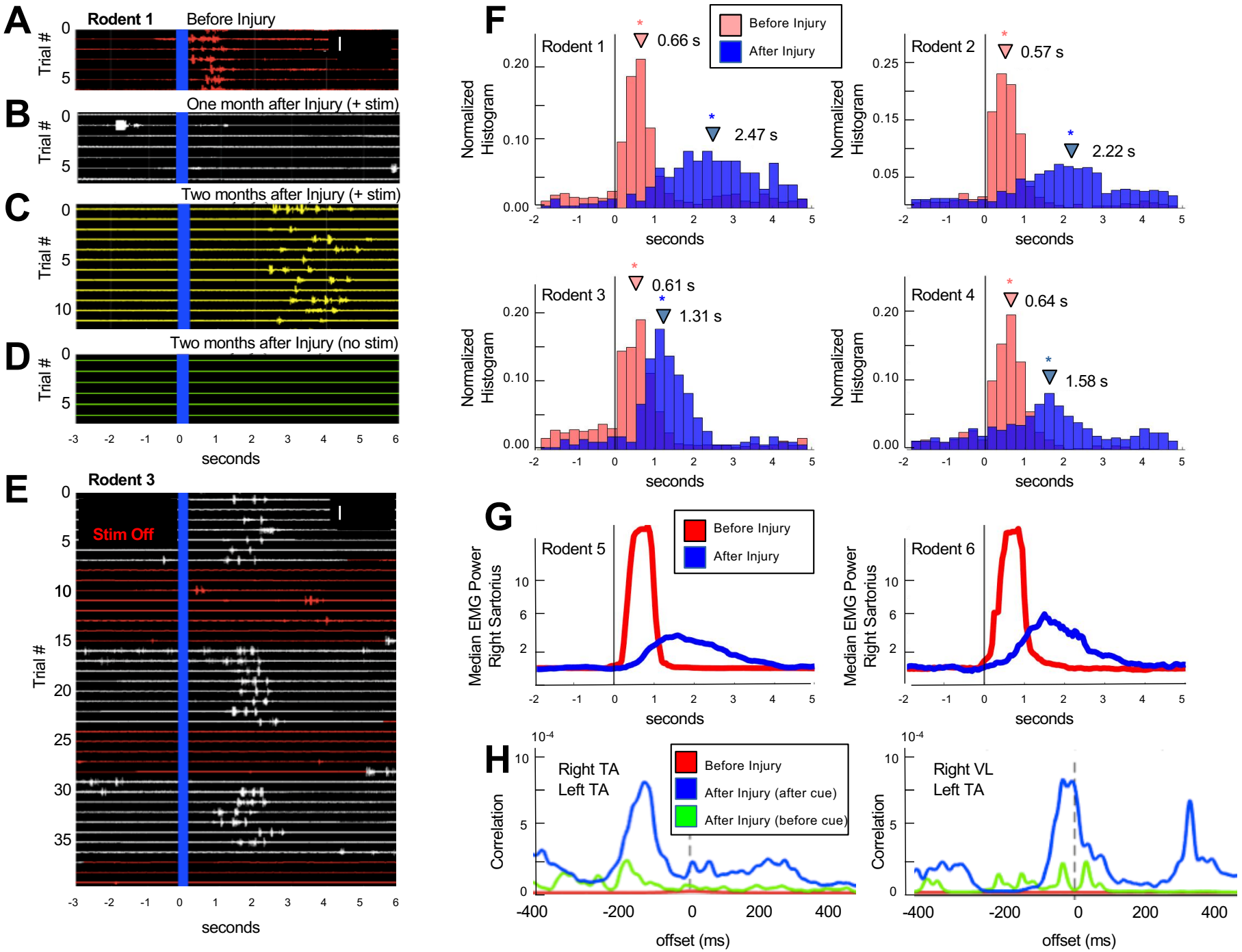

FIG 3

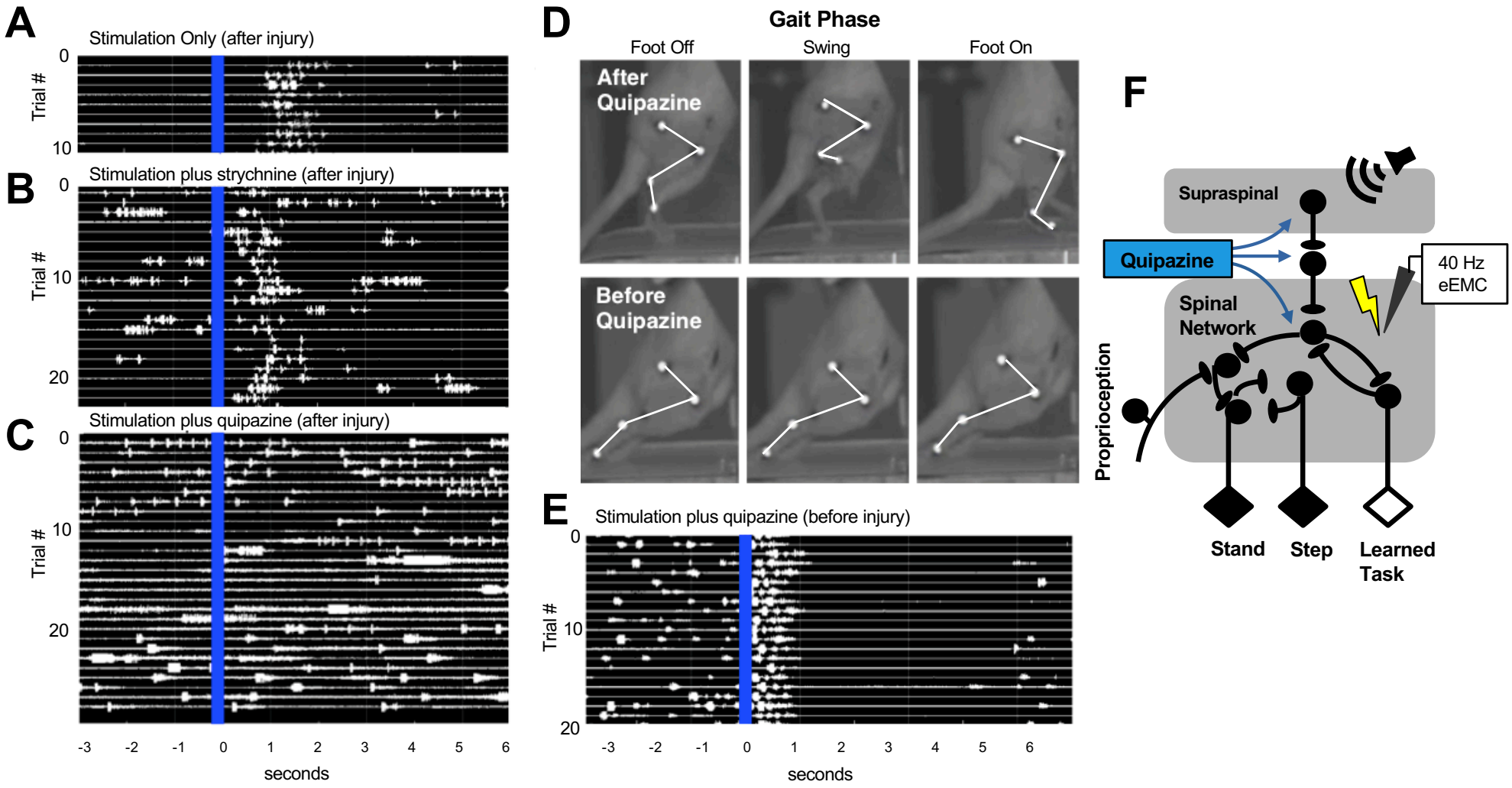

FIG 4