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## Abstract

Epidural electrical spinal stimulation can facilitate recovery of volitional motor control in individuals that have been completely paralyzed for more than a year. We recently reported a novel neuromodulation method named Dynamic Stimulation (DS), which short-lastingly increased spinal excitability and generated a robust modulation of locomotor networks in fully-anesthetized intact adult rats. In the present study, we applied repetitive DS patterns to four lumbosacral segments acutely after a contusive injury at lumbar level. Repetitive DS delivery restored the spinally-evoked motor EMG responses that were previously suppressed by a calibrated spinal cord contusion. Sham experiments without DS delivery did not allow any spontaneous recovery. Thus, DS uniquely provides the potential for a greater long-term functional recovery after paralysis.

*Keywords*: neuromodulation, asynchronous noisy stimulation, spinal contusion, multi-electrode array, epidural interface, spinal reflexes.

## Introduction

A spinal cord injury significantly reduces the resting level of activity in caudal spinal neural networks (Frigon and Rossignol, 2008) and may reduce or even suppress evoked potentials that are spinally-induced from lesioned motor networks (Courtine et al., 2009). Likewise, excitability of networks caudal to the lesion are also largely altered, even in segments not visually affected by the initial trauma (Taccola et al., 2010).

However, their baseline excitability can be modified by neuromodulation via tonic electrical epidural or transcutaneous spinal cord stimulation and/or pharmacological activation. These protocols can modulate the excitability farther or closer to the motor threshold needed to generate action potentials within and among sensory-motor and autonomic networks in response to other sources of stimulation (Gerasimenko et al., 2015). Thus, tonic neuromodulation of spinal networks changes their physiological state, augmenting the probability to exceed the threshold of excitation. Changes in the basal excitability of spinal networks explain how cutaneous and proprioceptive input, as well as

input from descending motor pathways, allow to recover supra spinal-spinal connectivity after severe paralysis (Gad et al., 2013). These critical amounts of sensory excitation and/or supraspinal input, added to the elevation of baseline excitability levels, are the two main conditions to reach motor threshold and therefore generate movement (Taccola et al., 2018). Indeed, spinal cord networks have been converted from a non-responsive state to one that can generate sufficient depolarizing currents to induce action potentials among interneurons projecting to motoneurons of multiple motor pools.

To elicit locomotor-like patterns from spinal networks, a unique stimulating paradigm characterized by a noisy waveform was developed *in vitro* to optimally recruit neonatal spinal neuronal networks (Taccola, 2011; Dose et al., 2016). This stochastic pattern of modulation was then delivered dynamically to distinct sites of the spinal cord of fully anesthetized adult *in vivo* rats (Taccola et al., 2020). This method was named Dynamic Stimulation (DS), as opposed to the more static profile of trains of stereotyped pulses. DS generated patterns of muscle bursting followed by short-lasting rhythmic discharges (Taccola et al., 2020). Moreover, DS augmented distinct components of the EMG responses elicited by segmental epidural weak pulses, during and after the end of DS protocol delivery. Repetitive delivery of DS (rDS) further increased the amplitude of spinally-induced EMG responses (Taccola et al., 2020).

However, along with reduced background activity in spinal networks, we have demonstrated reduced amplitudes in spinal evoked motor responses within one week after a severe SCI (Lavrov et al., 2008). Further, the response intensities and latencies vary based on site of stimulation and duration of the injury (Gad et al., 2013). In addition, the time course of the reemergence of spinally-induced responses were similar to the recovery of stepping after a severe SCI, indicating that evoked responses from hindlimb muscles can represent a potential biomarker of the functional recovery after SCI (Gad et al., 2015). However, the mechanism linking the modulation of background activity in the spinal networks with the modulation of motor-evoked responses still remains poorly understood.

The objective of this study is to determine the efficacy of dynamic noisy patterns in restoring motor control after a calibrated spinal cord injury.

Experiments were performed on 11 adult female Sprague Dawley rats (250–300 g body weight). All procedures have been approved by the Animal Research Committee at UCLA and are in accordance with the guidelines provided by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and with the European Union directive for animal experimentation (2010/63/EU).

Firstly, animals were sedated with isoflurane gas at a constant flow of 1.5%-2.5%, followed by urethane (1.2 mg/Kg, *i.p)*.

Subsequently, recording wire electrodes (AS 632, Cooner Wire Co, Chatsworth, CA, USA) for intramuscular electromyography (EMG) were implanted bilaterally in the tibialis anterior (TA) and soleus (Sol) muscles. EMG signals were band-pass filtered (gain 1000, range 10 Hz to 5 KHz and notched at 60 Hz), amplified (A-M Systems Model 1700 differential AC amplifier, A-M Systems, Sequim, WA, USA), and finally digitalized at 10 kHz (Digidata® 1440, Molecular Devices, LLC, CA, USA).

Delivery of signals was performed using a high-density platinum based multi-electrode array, structured in three longitudinal columns and six horizontal rows of paired electrodes (Chang et al., 2014; Taccola et al., 2020). Array implantation in the epidural dorsal space was performed after a T12 to L2 laminectomy, to dorsally expose the spinal cord.

To determine threshold intensity for each preparation, a train of 40 rectangular pulses at 0.3 Hz was adopted. Five sweeps were delivered for each stimulation amplitude, moving up by 100 µA increments, ranging from 100 to 800 µA. Threshold was defined as the minimum intensity for eliciting a detectable EMG response from any muscle. As recently reported (Taccola et al., 2020), DS consists of an EMG segment (29.5 s long) collected from the Sol muscle of a neurologically-intact adult rat during stepping. The trace, once acquired in AC mode (gain 1000, filter range 10 Hz to 5 KHz notched at 60 Hz) through an A-M Systems Model 1700 differential AC amplifier (A-M Systems, Sequim, WA, USA), was digitalized at 10 kHz (Digidata® 1440, Molecular Devices, LLC, CA, USA) and then reduced off-line at a sampling rate of 2000 Hz, using Clampfit® 10.3 software (Molecular Devices, LLC, CA, USA). Afterwards, the original EMG segment was duplicated, applying a staggered onset of 0.5 s and then exported (as an ASCII text file) to a programmable stimulator (STG 4002®; Multi Channel Systems, Reutlingen, Germany) to be applied to different electrode combinations within the array. The protocol was delivered to the two

lateral columns of electrodes in the array with opposite rostro-caudal cathode/anode polarity.

Spinal cord functionality was tested under urethane by applying trains of electrical pulses (test pulses) (0.1 ms duration, 0.3 Hz frequency). Pulse amplitude was increased after 5 sweeps in the range of 100 – 800 μA in order to define threshold intensity and trace a recruitment curve after injury. Severe spinal cord injuries abolishing spinally induced motor responses, were performed using a calibrated customized device, composed of a steel rod of 33.0 g weight dropping on the exposed cord from 5 cm of height. The end of the rod is a cylindrical protrusion of 1 mm radius to directly impact on the dorsal spinal midline at L4/L5. The impounder was left on the original injury site for 10 seconds before being carefully raised from the cord surface. During the impact, the trunk was stabilized by supporting the animal's belly with a rod, 2 cm high, under the chest. After 40-90 min from lesion, spinal cord functionality was tested under urethane by applying trains of electrical pulses (test pulses) (0.1 ms duration, 0.3 Hz frequency). Pulse amplitude was increased after 5 sweeps in the range of 100 – 800 μA in order to define threshold intensity and trace a recruitment curve after injury. The entire protocol for assessing spinal cord functionality spanned 40 min and was replicated twice before DS delivery. Repetitive DS consisted in the delivery of eight consecutive DS patterns of 30 s with 1 min intervals for a total duration of 11 min.

Ninety minutes after the lesion, spinally-induced responses in TA and Sol muscles were suppressed (Fig. 1 A). Single pulses delivered at maximal intensity (800 μA) to the segment just below the injury site elicited no responses (Fig. 1 $A_1$). About three hours after injury, the rDS protocol was applied at the intensity of 600 μA (Fig. 1 B) followed by a long resting phase. Fifty minutes after the end of the protocol (Fig. 1 C), the same test pulses, delivered with the cathode on the right side, produced a consistent response from the muscles of the left leg, without any output from the right side (Fig. 1 $C_1$). Conversely, by inverting the cathode/anode polarity of the test stimuli (Fig. 1 D, cathode on the left side), TA and Sol on the right leg showed large muscle contractions without any responses from the left leg (Fig. 1 $D_1$). Similar observations were made in four animals,

when rDS was applied 190 ± 17 min after the impact. Likewise, spinally-evoked responses that were not present before rDS, reappeared when tested 228 ± 18 min after the impact. Restored responses elicited by 750 ± 100 μA, showed a mean amplitude of 0.23 ± 0.28 mV for Sol and 0.13 ± 0.15 mV for TA and a time to peak of 5.4 ± 1.0 ms for Sol and 5.6 ± 1.3 ms for TA (n=4). Responses evoked in intact cords with the same strengths of stimulation and electrode location showed amplitude and time to peak values similar to restored responses recorded from injured animals after rDS (for intact cords, amplitude = 0.32 ± 0.54 mV for Sol and 0.09 ± 0.08 mV for TA; time to peak = 6.6 ± 2.3 ms for Sol and 5.8 ± 1.9 ms for TA; n=3).

Further assessments were made to assure that recovery of spinally-induced responses was enabled by rDS and not by a spontaneous recovery over longer resting periods. Therefore, four sham experiments were performed, to replicate the same experimental procedures without any delivery of DS (Fig. 1 E-G). In a sample experiment, the lack of EMG responses from the injured cord segment was confirmed by continuous testing for up to 250 min after injury (Fig. 1 $E_1$, $G_1$, $H_1$). Moreover, suppression of spinally-induced responses extended also to more rostral and caudal segments, eventually demonstrating a worsening of the functional deficit within the first few hours after initial compression (data not shown).

In the present study, we exploited a recently designed protocol of multisite stimulation with noisy patterns, named Dynamic Stimulation, and its delivery through an epidural interface consisting in a multi-electrode array. Recently, we proved that these two resources modulate locomotor networks and facilitate the motor output induced by subthreshold cortical input. Here, in fully anesthetized animals, we demonstrated that the rDS paradigm of stimulation was linked to patterns leading to a greater recovery of motor output after a severe spinal cord injury.

Unlike many studies involving neurorehabilitation, our strategy did not target the lumbar central pattern generator for locomotion (Kiehn and Butt 2003), but was centered at the site of lesion to promote reconnection along adjacent segments. Another original point of this research was that the continuous electrostimulation of the lesioned cord was performed in an acute setting (in the first three hours after injury). This finding suggests

the possibility to employ novel dynamic stimulation paradigms, epidurally and/or transcutaneously, to the lesioned spinal cord as a first surgical intervention to limit the loss of functions following a spinal cord injury.

The manner in which acute multiple depolarizations, as the ones induced by rDS, counteract early functional impairments after SCI has not been explored so far and, likewise, the mechanisms of such recovery are far from being elucidated.

On the contrary, though, a spreading depolarization along the cord has been reported so far to contribute to secondary damage after an impact injury (Gorji et al., 2004), since it releases additional glutamate that reaches a toxic level for cells and leads to functional deficits (Hinzman et al., 2015). In the present study, the continuous delivery of DS, acutely applied across the lesion site, generated additional depolarizations that, paradoxically, not only did it not worsen the functional deficit, but in fact consistently facilitated the recovery of motor output.

This effect was robust, also, in spite of the possible spreading depolarization induced by damage had already concluded when we delivered rDS (3 hours after lesion). Alternatively, rDS might have confined the spreading depolarization triggered by the trauma, by generating multiple after-hyperpolarizations of cell membranes in response to the insurgence of action potentials evoked by rDS in the network neurons. Indeed, these asynchronous and diffused hyperpolarizing events throughout the network could act as unexcitable nodes along the path of spreading depression, limiting the massive propagation.

Moreover, the acute delivery of rDS might regress acute phenomena of network dysfunction (Taccola et al., 2010) by promoting activity-based plastic events (Ganguly and Poo, 2013). Indeed, DS provides a pattern of phasic stimulation derived from sampling traces from hind limb muscles during real locomotion. This pattern of input varies in amplitude and frequency and is comparable to that of afferent feedback during gait (Prochazka et al., 1976). According to this view, application of rDS a few hours after the trauma might promote activity-like signals mimicking a locomotor training session long before the subject is stabilized and prepared for neurorehabilitation protocols aimed at facilitating recovery of locomotion.

Extensive amounts of data are accumulating which points to activity-dependent mechanisms in play ranging from RNA expression and synaptic proteins to system-level learning phenomena within and among spinal networks (Kobayakawa et al., 2019) as well as the transformation of dormant to competent spinal connectivity in response to epidural and transcutaneous stimulation when combined with sensory-motor training (de Leon et al., 1998). Perhaps these phenomena can become even more robust in a time frame closer to the moment of injury.

Moreover, motor output potentiation winds up when DS delivery is repeated at short-time intervals (Taccola et al., 2020), as performed in this study. This event is likely due to the accumulation of a molecular factor released by rDS. For example, BDNF is upregulated following standard epidural electrical stimulation (Baba et al., 2009) and, in turn, spinal BDNF modulates the motor output (Côté et al., 2011). Interestingly, BDNF is involved in promoting recovery after lesion (Kim et al., 1996; Jakeman et al., 1998; Boyce et al., 2007). It is not known, however, whether the restorative effect of rDS can be replicated in the absence of BDNF.

The recovery of functions observed after an acute application of rDS is robust and adds a new element to the advantages of noisy stimulation (Taccola et al., 2020). Indeed, variable stimulation can work both toward increasing connectivity among spinal neurons spared by a lesion and toward rescuing them from the spinal shock.

These promising data, collected from terminal recordings in fully anesthetized animals, suggest the need for further studies to translate this neuromodulating strategy both in the acute stage of a spinal injury and in chronic scenarios in absence of anesthesia, to confirm the ability of rDS to restore functions. Collectively, these data provide compelling reasons why DS should be explored as a possible critical component in the acute phase of treatment that might limit the severity that emerges after a spinal cord injury.

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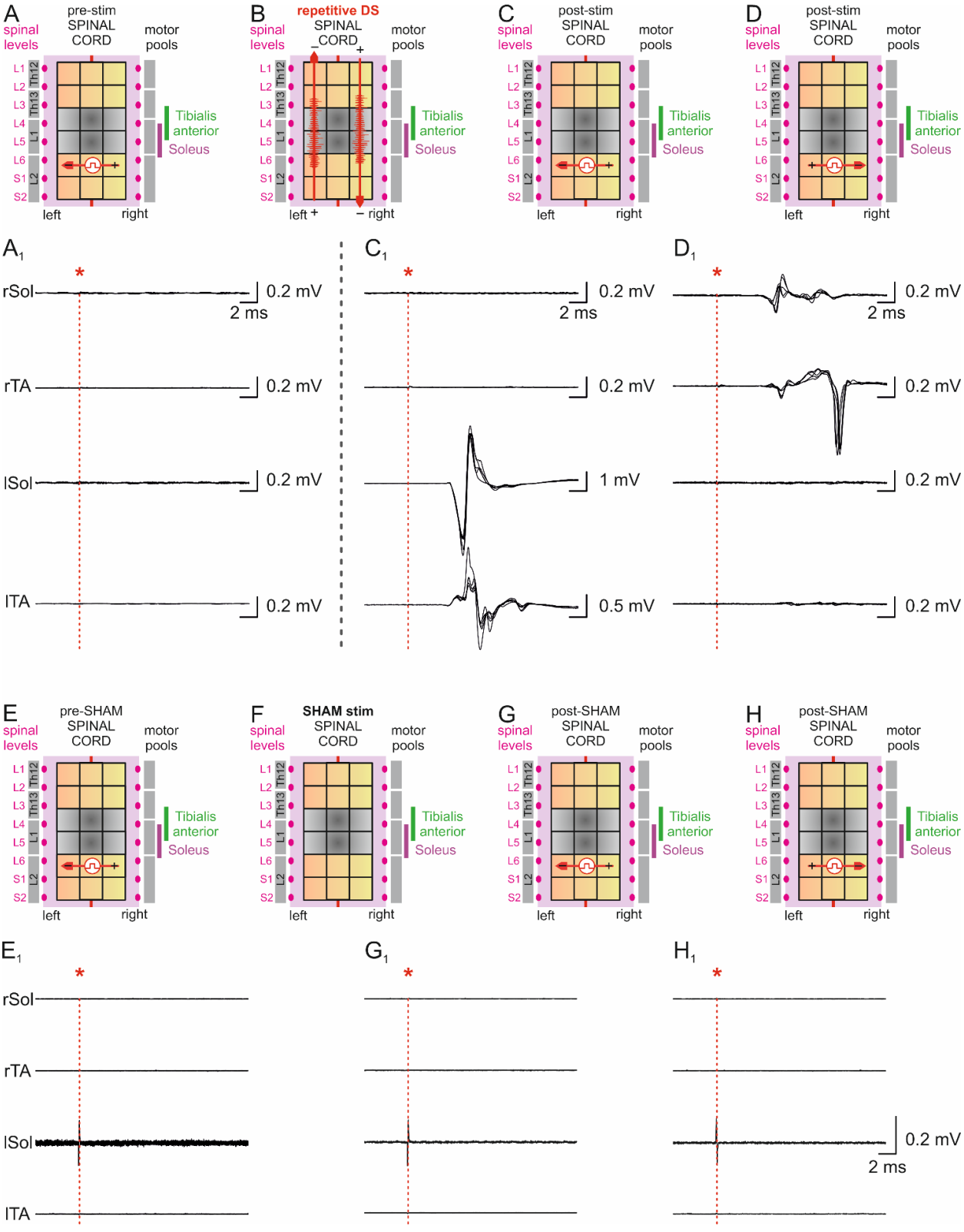
VRE, researcher on the study team hold shareholder interest in NeuroRecovery Technologies and hold certain inventorship rights on intellectual property licensed by The Regents of the University of California to NeuroRecovery Technologies and its subsidiaries. VRE, and PG, researchers on the study team hold shareholder interest in Spinex. Wentai Liu, researcher on the study team holds shareholder interest in Niche Biomedical Inc.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Soleus
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CORD
D
post-stim
SPINAL
CORD
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D1
rSol
rTA
lSol
lTA
0.2 mV
2 ms
1 mV
0.5 mV
E
pre-SHAM
SPINAL
CORD
F
SHAM stim
SPINAL
CORD
G
post-SHAM
SPINAL
CORD
H
post-SHAM
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CORD
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**Figure 1. rDS restores spinally-induced responses after acute spinal contusion.**
In A, the cartoon summarizes the stimulation setting, with a calibrated representation of the array width and the distance between homosegmental dorsal roots. 90 mins after a calibrate compression to the spinal cord at L4/L5, single pulses (red star and dotted line; intensity = 800 μA; duration = 0.1 ms) applied to L6 (cathode on the right) are unable to elicit any bilateral EMG responses from Sol and TA muscles ($A_1$). After additional 90 mins, rDS was supplied through the lesioned spinal cord (B) and 50 mins later (C, D), the same stimulation delivered in A now evokes spinally-induced responses from both the left leg ($C_1$, cathode on the right) and from the right one, as well, by swapping the position of the poles ($D_1$, cathode on the left). In E-H the same protocol in A-D was followed in an animal that did not receive any rDS. In the latter case, no spontaneous recovery was reported for either configuration of stimulation ($G_1$, $H_1$). In each panel $A_1$, $C_1$, $D_1$, $E_1$, $G_1$, $H_1$, five consecutive traces are superimposed.

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