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Using EMG to deliver lumbar dynamic electrical stimulation to facilitate cortico-spinal excitability

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ABSTRACT

Background: Potentiation of synaptic activity in spinal networks is reflected in the magnitude of modulation of motor responses evoked by spinal and cortical input. After spinal cord injury, motor evoked responses can be facilitated by pairing cortical and peripheral nerve stimuli.
Objective: To facilitate synaptic potentiation of cortico-spinal input with epidural electrical stimulation, we designed a novel neuromodulation method called dynamic stimulation (DS), using patterns derived from hind limb EMG signal during stepping.
Methods: DS was applied dorsally to the lumbar enlargement through a high-density epidural array composed of independent platinum-based micro-electrodes.
Results: In fully anesthetized intact adult rats, at the interface array/spinal cord, the temporal and spatial features of DS neuromodulation affected the entire lumbosacral network, particularly the most rostral and caudal segments covered by the array. DS induced a transient (at least 1 min) increase in spinal cord excitability and, compared to tonic stimulation, generated a more robust potentiation of the motor output evoked by single pulses applied to the spinal cord. When sub-threshold pulses were selectively applied to a cortical motor area, EMG responses from the contralateral leg were facilitated by the delivery of DS to the lumbosacral cord. Finally, based on motor-evoked responses, DS was linked to a greater amplitude of motor output shortly after a calibrated spinal cord contusion.
Conclusion: Compared to traditional tonic waveforms, DS amplifies both spinal and cortico-spinal input aimed at spinal networks, thus significantly increasing the potential and accelerating the rate of functional recovery after a severe spinal lesion.

Introduction

The recovery of volitional motor control of paralyzed lower limbs observed in individuals with a chronic, functionally complete spinal cord injury following epidural electrical stimulation corresponds to the potentiation of synaptic activity in spinal networks [1–5]. Synaptic potentiation in the central nervous system can be induced based on the Hebbian principle of associative plasticity, by applying two converging inputs with a precise temporal order [6–9]. Indeed, two sequential electrical pulses applied to the cortex

Abbreviations: CV, coefficient of variation; DS, dynamic stimulation; EMG, electromyography; ER, early response; IP, intraperitoneal; ISI, inter stimulus interval; L, lumbar; l, left; LR, late response; MR, middle response; r, right; rDS, repetitive DS; S, sacral; Sol, soleus; TA, tibialis anterior; Th, thoracic.

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and to a peripheral nerve facilitated motor evoked EMG responses in healthy [10,11] and spinal cord injured persons [12–14]. In addition, electrical stimulation increases the basal excitability state of spinal networks, rendering them more responsive to proprioceptive input [15].

In the present work, we aimed at exploring whether basal excitability and synaptic activity of the spinal cord may be modulated by an epidural stimulation pattern that pairs pulses converging onto spinal circuits from different segments. To this purpose, we used an epidural multiple electrode interface that permitted the *in vivo* characterization of the effects of excitation coming from spatially different sources and the definition of their role in modulating the activity of spinal networks, also compared to traditional tonic input.

As highly varying waveforms sampled from multiple sources can increase the recruitment of neonatal spinal networks *in vitro* [16–18], we reasoned that a biologically-generated signal from a motor pool would more closely approximate the input that naturally projects from proprioceptive and cutaneous input, as well as from multiple brain sources, to spinal sensory-motor networks. Then, through the fully independent electrodes of the interface, we delivered a multifrequency stimulating paradigm named 'dynamic stimulation', consisting of highly varying patterns sampled from a leg EMG during real stepping. Furthermore, we investigated whether dynamic stimulation can facilitate sub-threshold cortico-spinal input to the motor pools controlling hind limbs, and also modulate synaptic transmission in the spinal cord after severe injury.

As previously demonstrated, strength of synaptic transmission in the spinal cord can be quantified by measuring the amplitude of EMG responses elicited by single electrical pulses delivered to the dorsum of the cord [19]. Based on the intensity of stimulation, a single epidural stimulus between L2 and S1 produces three types of evoked responses, i.e., early (ER; latency 1–4 ms), middle (MR; latency 5–10 ms), and late (LRs; latency 11–15 ms), in the bilateral vastus lateralis, semitendinosus, medial gastrocnemius, tibialis anterior and soleus muscles of intact [20] and spinalized [19,21] rats. While ERs likely correspond to the direct recruitment of motoneurons or ventral roots at higher stimulation intensities, MRs have some components consistent with monosynaptic reflexes and LRs with the recruitment of polysynaptic interneuronal spinal networks. We speculated that an increased excitability of spinal networks originating from the application of dynamic stimulation might modulate two inputs: EMG responses generated by a segmental and site-specific epidural stimulation of the spinal cord, on one hand, and cortico-spinal input elicited by sub-threshold cortical pulses, on the other.

Material and methods

The present work explored the functional modulation of spinal network excitability and cortical-spinal input in response to novel patterns of current derived from locomotion EMGs (named Dynamic Stimulation, DS) delivered to spinal networks via an epidural electrode array. Therefore, we initially collected the baseline trend of endogenous spinal modulation of the motor output during continuous threshold (as defined below) stimulation and compared it to the modulatory effects induced by both DS and traditional stereotyped protocols. Secondly, we wondered whether any increase in spinal network excitability induced by DS can facilitate sub-threshold cortico-spinal input. Finally, delivery of DS was tested after an experimental spinal cord injury in promoting residual spinal responses.

Experimental design

Data were collected from 35 adult female Sprague Dawley rats (250–300 g body weight). All procedures were approved by the Animal Research Committee at UCLA and are in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and with the European Union directive on animal experimentation (2010/63/EU). Epidural electrical stimulation of the spinal cord was used to generate motor evoked potentials recorded from selected hind limb muscles of animals under anesthesia. Animals were initially sedated with isoflurane gas at a constant flow of 1.5%–2.5% to immobilize them, followed by administration of urethane (1.2 mg/kg, IP). During surgery, toe pinch was performed periodically to assess the anesthetic level to be maintained with isoflurane gas, as needed. As soon as surgical procedures terminated, isoflurane gas was suspended and, during following recordings, animals remained under the influence of urethane only. At the end of experiments (9–10 h), animals were sacrificed with isoflurane and sodium pentobarbital (IP, 80–100 mg/kg). Five additional experiments were performed at the University of Leeds in order to test the effects of spinal neuromodulation on cortico-spinal input elicited by the selective activation of the lateral leg motor area. All procedures were approved by the UK Home Office and performed under the Animals (Scientific Procedures) Act 1986. Terminal recordings were performed from male Wistar rats (250–300 g body weight) anesthetized by intraperitoneal (IP) administration of a ketamine (100 mg/kg) and xylazine (5 mg/kg) mix. At the end of experiments, animals were euthanized by cervical dislocation.

Intramuscular EMG electrode implantation

Animals were kept under anesthesia over a heating pad (37 °C) during the bilateral implantation of intramuscular electromyography (EMG) recording electrodes [22,23] in the tibialis anterior (TA) and soleus (Sol) muscles. Skin and fascial incisions were performed to expose the belly of each muscle. Multistranded, teflon-coated stainless-steel wires (AS 632, Cooner Wire Co, Chatsworth, CA, USA) connected to a gold plated amphenol connector were passed subcutaneously. Pairs of wires were inserted into the muscle belly using a 23-gauge needle and anchored at their entry and exit from the muscle with knots made with 5.0 Nylon suture. A small notch (0.5–1.0 mm) in each wire was deprived from the insulation to expose the conductor and form the electrodes. Proper placement of electrodes was verified during surgery, by stimulating through the head connector. Bare wire tips were covered by gently pulling the Teflon coating over the tips. All EMG wires were coiled subcutaneously in the back region to relieve stress. A common ground (~1 cm of the Teflon removed distally) was inserted subcutaneously in the mid-back region for EMGs.

EMG recordings from bilateral TA and soleus muscles were band-pass filtered (gain 1000, range 10 Hz to 5 KHz and notched at 60 Hz), amplified using an 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). All surgical sites were closed in layers using 5.0 Vicryl® (Ethicon, New Brunswick, NJ, USA) for all muscle, connective tissue layers and skin incisions in the hind limbs, while 5.0 Ethilon® (Ethicon, New Brunswick, NJ, USA) was used to close skin incisions in the back.

Epidural multi electrode array implantation

To simultaneously deliver patterns of intrinsically varying signals to multiple segments of the spinal cord, it was necessary to develop a high-density platinum-based multi-electrode array consisting of three longitudinal columns and six horizontal rows of independent low-impedance electrodes (total of 18 independent electrodes) [24,25]. The high-definition and flexibility of the epidural interface was demonstrated by the selective activation of extensor or flexor motor pools while varying bipolar stimulation parameters (Supplementary Fig. 1).

To implant the multi-electrode array in the dorsal epidural space, a T12 to L2 vertebrae laminectomy was performed to expose the spinal cord. After epidural placement, the array was covered with small cotton balls rinsed in saline. Back muscles and skin were sutured using 5.0 Vicryl® (Ethicon, New Brunswick, NJ, USA), with leads to the array exiting through the skin. A common ground for all array electrodes, independent from EMG ground, was inserted subcutaneously in the left forearm. Throughout the text, segmental levels of the spinal cord are indicated in accordance to the topography of spinal roots entrance, as previously reported [26].

Electrical stimulation protocols

The experimental set up and the pattern of electrical stimulation used in this study are summarized in the cartoon in Fig. 1. The motor threshold intensity for each preparation was determined with a train of 40 rectangular pulses at 0.3 Hz. A set of five sweeps was delivered for each amplitude of stimulation and then increased by increments of 100 μA ranging from 100 to 800 μA. The motor threshold for each animal was defined as the minimum intensity required to elicit a detectable EMG response (considered as a deflection over five times the standard baseline deviation) acquired from at least one out of four recorded muscles. Threshold stimulation did not elicit any visible muscle twitches that would usually appear when increasing the stimulation intensity of 100 μA above the defined threshold. The input/output protocol was regularly repeated during the experiment to confirm threshold stability. Only in three out of 40 rats, threshold changed at the end of the experiment (at least 4 h) from 300 to 500 μA. After determining the threshold, a continuous train of 300–700 pulses (0.1 ms pulse duration, 0.3 Hz frequency, at threshold intensity) was delivered to a pair of array electrodes located at the same spinal segment to provide baseline responses.

We delivered a protocol of electrical stimulation named Dynamic Stimulation (DS) consisting of a 29.5 s segment of EMGs collected from the Sol muscle of a neurologically intact adult rat walking on a treadmill at the speed of 13.5 cm/s. The trace was acquired in AC mode (gain 1000, filter range 10 Hz to 5 KHz notched at 60 Hz) with an A-M Systems Model 1700 differential AC amplifier (A-M Systems, Sequim, WA, USA), then digitalized at 10 kHz (Digidata® 1440, Molecular Devices, LLC, CA, USA) and down sampled off-line to the final sampling rate of 2000 Hz, through Clampfit® 10.3 software (Molecular Devices, LLC, CA, USA).

The sampled EMG trace was exported as an ASCII text file consisting of three columns of values. The first column of values corresponded to sampling time, the second to EMG amplitude. The third column duplicated the second column, but shifted in time by 0.5 s. A file header provided by the manufacturer's user guide was added to the ASCII file to make it readable from the closed-source proprietary software (MC_Stimulus II) controlling the programmable stimulation device (STG® 4008; Multi Channel Systems, Reutlingen, Germany). No additional algorithms were applied and the original ASCII file is provided to this manuscript as supplementary material. The software linearly converts ASCII text file's

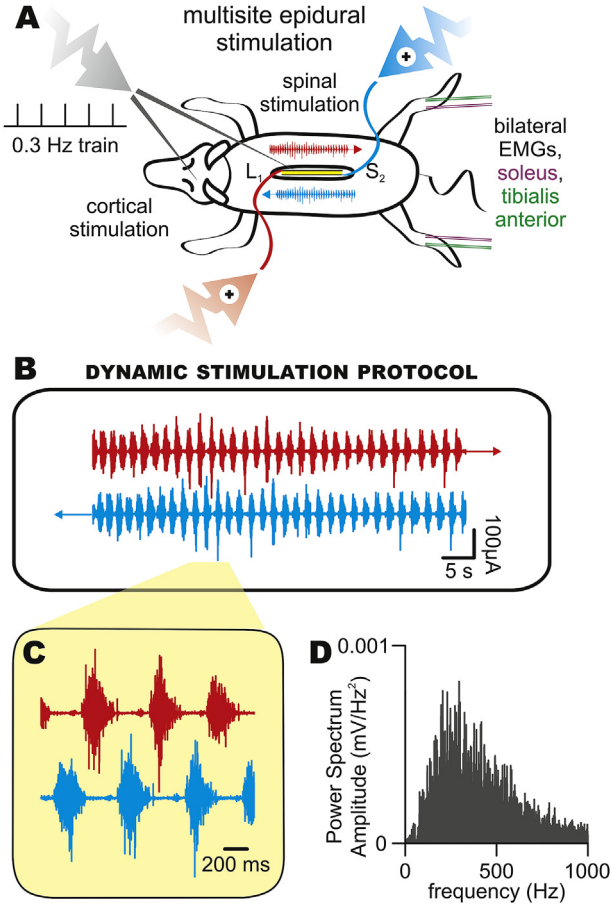

Fig. 1. The experimental setup allows for multiple sites of simultaneous stimulation and recording from an adult rat under urethane anesthesia. In A, three types of stimulation are delivered (zigzag arrows). First, a train of stimuli (0.3 Hz) at threshold intensity is continuously applied either to the dorsal spinal cord, or over the cortical motor area. During tonic stimulation at 0.3 Hz, a protocol of stimulation named Dynamic Stimulation (DS) is delivered over 4 spinal segments, from L1 to S2. Responses to stimulation were recorded from extensor (tibialis anterior, TA) and flexor (soleus, Sol) muscles through wire EMG electrodes bilaterally implanted in the muscle belly. DS is composed of a waveform sampled from the Sol EMG activity (29.5 s) during treadmill stepping (13.5 cm/s) in an intact rat. Through off-line analysis, the original EMG trace is duplicated and delivered staggered by 0.5 s (B). The two stimulating waveforms were supplied at the same time, through the electrodes located at the extremities of the array with opposite cathode location (blue waveform, cathode at S2, red waveform with cathode at L1, respectively). In the box in C, a magnification of two actual stimulating waveforms indicates that they are composed of rhythmic bursts characterized by higher frequency discharges (100–500 Hz), as confirmed by the Power Spectrum shown in D. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

voltage values to current values for the stimulator. The resulting time series of current amplitudes can be uniformly scaled by the user to the desired stimulation levels. Notably, the DS signals used here provide a safe charge-balanced stimulation. The prerecorded EMG signal carries a zero-average value, as the sum of positive amplitudes is periodically cancelled by the sum of negative ones. This is ensured by the fact that EMG signals were filtered from DC-10 Hz, thus cancelling out the slowly changing mathematical components of the signal, which would otherwise result in an average bias accumulation within the stimulation protocol (Supplementary Fig. 2). It is important to note that, if necessary, an additional circuit-based charge-balance mechanism could be applied to ensure long-term safety by periodically shorting the stimulating electrodes to ground [27]. The two time-staggered stimulating protocols were simultaneously delivered through two

independent outputs to the left and right external columns of array electrodes with opposite rostro-caudal cathode location. In more detail, during each experiment, the rostral cathode was indifferently placed on the right or on the left columns of the array and then a rostral anode was consequently placed on the other side. DS protocol was applied at different intensities (150, 245, 300, 375, 450, 600 μA), i.e. the difference between the maximum positive and the maximum negative amplitudes of waveforms (peak to peak). To better characterize the optimal intensity of DS to potentiate EMG responses elicited by single pulses applied to the spinal cord, in eight experiments, DS was serially applied at increasing amplitudes from 150 to 600 μA. At least 5 min rest was allowed between consecutive deliveries of DS. To test variations on the excitability of neuronal motor networks before and after DS, EMG responses were elicited through a train of rectangular monophasic pulses (100–400 pulses, pulse duration = 0.1 ms, frequency = 0.3 Hz) at threshold intensity, continuously delivered through a pair of electrodes (usually at L4). Repetitive DS (rDS) consisted of eight consecutive 30 s periods of DS separated by 1 min rest. After rDS, a long protocol of continuous stimulation (300–900 pulses, duration = 0.1 ms, frequency = 0.3 Hz, amplitude = threshold) was used to follow the baseline recovery for up to 45 min. No trend changes in the amplitude of responses occurred during continuous low-frequency threshold stimulation (Fig. 2). It is interesting to note that only electrodes connected to the stimulator for pulse delivery (DS or test pulses) were the active electrodes in use. The other electrodes in contact with the spinal cord were open circuited and thus did not directly impact on the spread of the electric field induced by active electrodes. This has been validated by the finite element model developed, which is consistent with previous modeling studies from our collaborators (see also independent stimulations in Supplementary Fig. 3 and in Supplementary Video).

Electrical stimulation of motor cortex

At the University of California, Los Angeles, in a subgroup of three adult female Sprague Dawley rats, trains of single monophasic electrical pulses (0.1–1 ms duration, 0.3 Hz frequency, Th) were delivered to the left sensorimotor cortex region representing the right hind limbs. Two small metal screws were implanted through the skull, one at 2 mm posterior to lambda and the other one at 8 mm anterior to bregma. Wires were connected at the top of each screw for bipolar electrical stimulation (STG 4002®; Multi Channel Systems, Reutlingen, Germany) with cathode rostral to anode. At the University of Leeds, a more selective stimulation of one side of the cortex, corresponding to either the left or right motor areas was performed by implanting an epidural cortical array (cathode rostral) under stereotaxic coordinates [28] in the additional five experiments performed on male Wistar rats. Single biphasic stimuli (duration 200 μs) or an epoch of three or five pulses (inter stimulus interval, ISI = 3 ms) were supplied at 0.3 Hz (808 ± 89 μA, ISO-STIM-01 M®, npi electronic GmbH, Tamm, Germany) and responses were recorded from contralateral TA muscles using wire electrodes (40 AWG; 79 μm diameter) inserted through the shank skin, connected to a differential amplifier (D160®, Digitimer Ltd, Hertfordshire, UK; Gain x2000, Lowpass filter 1 KHz) and then notched (Hum Bug®, A-M Systems, Sequim, WA, US). To exclude any spillover effects arising from cortical stimulation

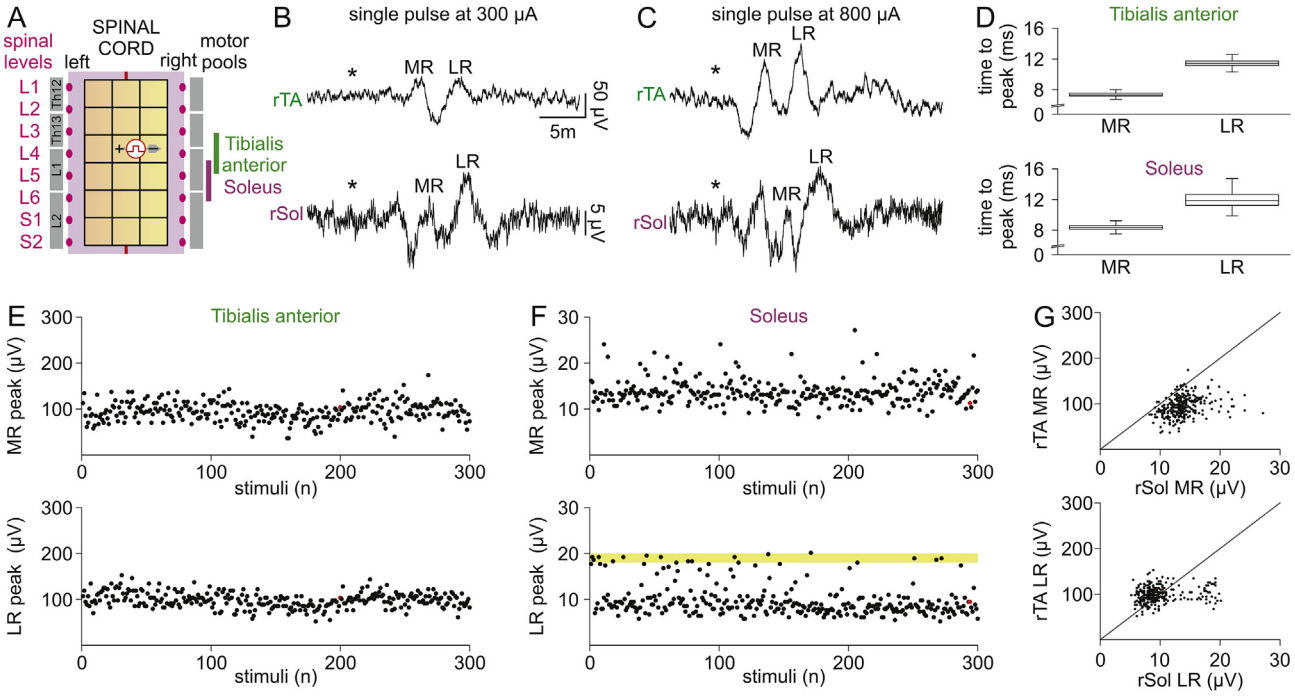

Fig. 2. Threshold electrical stimuli delivered through the epidural array generate small and variable responses from TA and Sol muscles. In A, a train of square monophasic threshold impulses (300 stimuli, 0.3 Hz, 300 μA, single pulse duration 0.1 ms) is continuously delivered (15 min) to the central sites of the array (L4, cathode on the left), as indicated in the cartoon with a calibrated representation of the array width and the distance between homosegmental dorsal roots. In B, recordings from right TA and Sol display small amplitude responses with a middle (MR) and a late response (LR), characteristically lacking an early response (ER) in the first five ms after the onset of stimulation (*). In C, by increasing the intensity of stimulation (800 μA), EMG responses increase in amplitude and are characterized by the appearance of an ER. In the box in D are reported the time to peak values of MRs and LRs for muscles TA (above) and Sol (below). In E, the time courses of peak amplitude of MRs (above) and LRs (below) over 300 consecutive pulses are recorded from a TA muscle. In F, the time courses of peak amplitude for MRs (above) and LRs (below) over 300 consecutive pulses are recorded from Sol muscle. Note also the greater probability of LRs to show a higher amplitude of about 18 μV (pale yellow field). The scatter plot in G reflects unique relationships between the TA and Sol for both MR and LR, with the ratio being higher in the Sol for the MR, but the opposite is true for the LR.

targeted to the left leg area, an expert experimenter assessed the absence of forelimb muscle contractions after implanting the cortical array, by sensing the lack of movement on shoulders and forelimbs during stimulation. Throughout the experiment, the absence of ipsilateral TA contractions was confirmed by continuous EMG recordings. Signals were acquired (Micro1401-3®, Cambridge Electronic Design Limited, Cambridge, UK) and stored in a PC for following off-line analysis (Signal® version 5.09, Cambridge Electronic Design Limited, Cambridge, UK).

Experimental spinal cord injury

Spinal cords were injured using a calibrated customized device, consisting in a steel rod of 33.0 g weight dropped on the exposed cord from a height of 5 cm. The rod terminated with a cylindrical protrusion of 1 mm radius that impacted directly on the dorsal spinal midline at L4. After the impact, the impounder was left on the initial injury site for 10 s and then carefully raised from the surface of the cord. To stabilize the trunk during the impact, the belly of the animal was supported at a 2 cm height under the chest.

Data analyses

The EMG responses elicited by spinal cord stimulation were divided into early (ER, latency 1–4 ms), middle (MR, latency 5–10 ms) and late responses (LR, latency 11–15 ms) relative to the stimulation pulse [19]. The amplitude and time to peak of each EMG response was determined using Clampfit® 10.3 software (Molecular Devices, LLC, CA, USA) to plot time course graphs. Responses were statistically compared by individually calculating the peak of 20 (for single DS) or 100 (for repetitive DSs) consecutive sweeps immediately before and after the application of each protocol. Then, single peaks were averaged for statistical comparison. The ratio between the standard deviation and mean amplitude provided the amplitude coefficient of variation (CV), which is an index of consistency of EMG evoked responses (the lower the CV, the less variable the responses [29]). The power spectrum of the patterns composing DS was obtained through Clampfit® 10.3 software (Molecular Devices, LLC, CA, USA).

Statistical analysis

Data are indicated as mean ± SD values, with n referring to the number of experiments. After determining the normality of the distribution of data based on a Kolmogorov-Smirnov normality test, statistical analysis was performed using SigmaStat® 3.5 software (Systat Software, San Jose, CA, USA) to compare the mean ± SD of different experimental conditions. All parametric values were analyzed using Student's t-test (paired or unpaired) to compare two groups of data, or ANOVA for more than two groups. Among non-parametric values, Wilcoxon Signed-Rank test was adopted for two groups, and Kruskal-Wallis ANOVA on Ranks for more than two groups. For multiple comparisons, we applied either Tukey's methods or Dunn's methods, depending on data being parametric or non-parametric. Within each sample of non-parametric data, repeated measures were performed using Friedman test. Results reached significance when $P < 0.05$.

Results

Threshold pulses locally applied to the spinal cord evoked stochastically modulated EMG responses

As threshold modulation strengths render spinal networks more responsive to proprioceptive input [15], we explored the effects of continuous threshold stimulation on the excitability state of spinal networks, by analyzing the EMG responses induced by a train of epidural pulses (300 pulses, 0.1 ms, 0.3 Hz) delivered to L4 (cathode on the left, Fig. 2 A). A random sample of single EMG traces showed that when stimulation was delivered at threshold (300 μA; Fig. 2 B), motor responses were composed of two peaks that matched the latencies for MR and LR previously described [19,20] (time-to-peaks: $MR_{TA} = 7.6$ ms; $LR_{TA} = 12$ ms; $MR_{Sol} = 8.5$ ms; $LR_{Sol} = 12.8$ ms). However, at higher intensity of stimulation (800 μA; Fig. 2C) an additional peak appeared shortly after pulse delivery (ER; time-to-peaks: 1.5 ms for rTA, 1.7 ms for rSol; Fig. 2C), while the time to peak of both MRs and LRs was reduced (time-to-peaks: $MR_{TA} = 5.4$ ms; $LR_{TA} = 9.4$ ms; $MR_{Sol} = 4.9$ ms; $LR_{Sol} = 11.6$ ms).

Exemplary results are consistent with the mean time-to-peak for MRs and LRs collected from ten animals in response to both threshold stimulation (480 ± 148 μA; $MR_{TA} = 8.14 \pm 1.67$ ms; $LR_{TA} = 12.86 \pm 3.11$ ms; $MR_{Sol} = 8.19 \pm 1.45$ ms; $LR_{Sol} = 13.56 \pm 3.12$ ms) and to the highest intensity of stimulation (800 μA; $MR_{TA} = 7.44 \pm 1.51$ ms; $LR_{TA} = 12.08 \pm 3.56$ ms; $MR_{Sol} = 6.76 \pm 1.98$ ms; $LR_{Sol} = 10.72 \pm 1.20$ ms). For the same sample experiment as in B, the average time-to-peak (whiskers plots, Fig. 2 D) and the time courses of peak amplitude (total duration = 15 min) were plotted for both MRs and LRs recorded from TA (Fig. 2 E) and Sol (Fig. 2 F). Responses were characterized by a large variability in peak amplitude, as indicated by the high CVs ($MR_{TA} = 0.22$; $LR_{TA} = 0.17$; $MR_{Sol} = 0.20$; $LR_{Sol} = 0.35$). A random sample of six experiments reported CVs of peak amplitude consistent with the example ($MR_{TA} = 0.62 \pm 0.50$; $LR_{TA} = 0.45 \pm 0.31$; $MR_{Sol} = 0.54 \pm 0.32$; $LR_{Sol} = 0.48 \pm 0.31$). In order to define a reference value for the variability of responses, we serially delivered continuous pulses to six animals, at both threshold and maximal intensities (700–800 μA). Here, the peak amplitude of MRs from TAs at higher strengths was significantly more uniform than at threshold, as shown by the lower CV ($CV_{high} = 53 \pm 27\%$ of CV_{Th}; $P = 0.031$, Wilcoxon Signed Rank Test, $n = 6$).

Nevertheless, this great variability does not parallel any patterned modulation of the motor output between extensor and flexor muscles, as confirmed by the lack of a clear correlation among peaks of responses expressed in pairs of MRs and LRs reported for TA and Sol (Fig. 2 G).

In summary, tonic low frequency stimulation of the cord using threshold pulses elicited intrinsic fluctuations of small EMG responses, likely representing the spontaneous modulation of excitability in the specific spinal networks and motor pools being tested.

DS potentiated MRs induced by segmental single pulses, mostly on TA

To investigate whether DS modulates the motor output evoked by spinal cord stimulation, a train (0.3 Hz) of threshold electrical pulses (400 μA; 0.1 ms) was continuously delivered to L4 (cathode on the left) before, during and at the end of the protocol. In a sample preparation, small responses recorded from rTA (Fig. 3 B) and lSol (Fig. 3C) were potentiated immediately after 30 s of DS (see gray arrow) at 375 μA intensity. From the same experiment, 300 consecutive EMG responses (over 15 min) were singularly analyzed for MRs and LRs and used to draw time courses of peak amplitude for TA and Sol, respectively. Despite variability in pre-DS values, DS delivery strongly increased responses from rTA (1129% for MRs and 164% for LRs compared to pre-DS), starting from the beginning of DS and lasting for up to 4 min after the end of DS (Fig. 3 D). lSol muscle displayed a shorter (2 min) and milder effect for MRs (131% of pre-DS, black dots Fig. 3 E), while LRs remained unaffected (97% of pre-DS, red dots, Fig. 3 E).

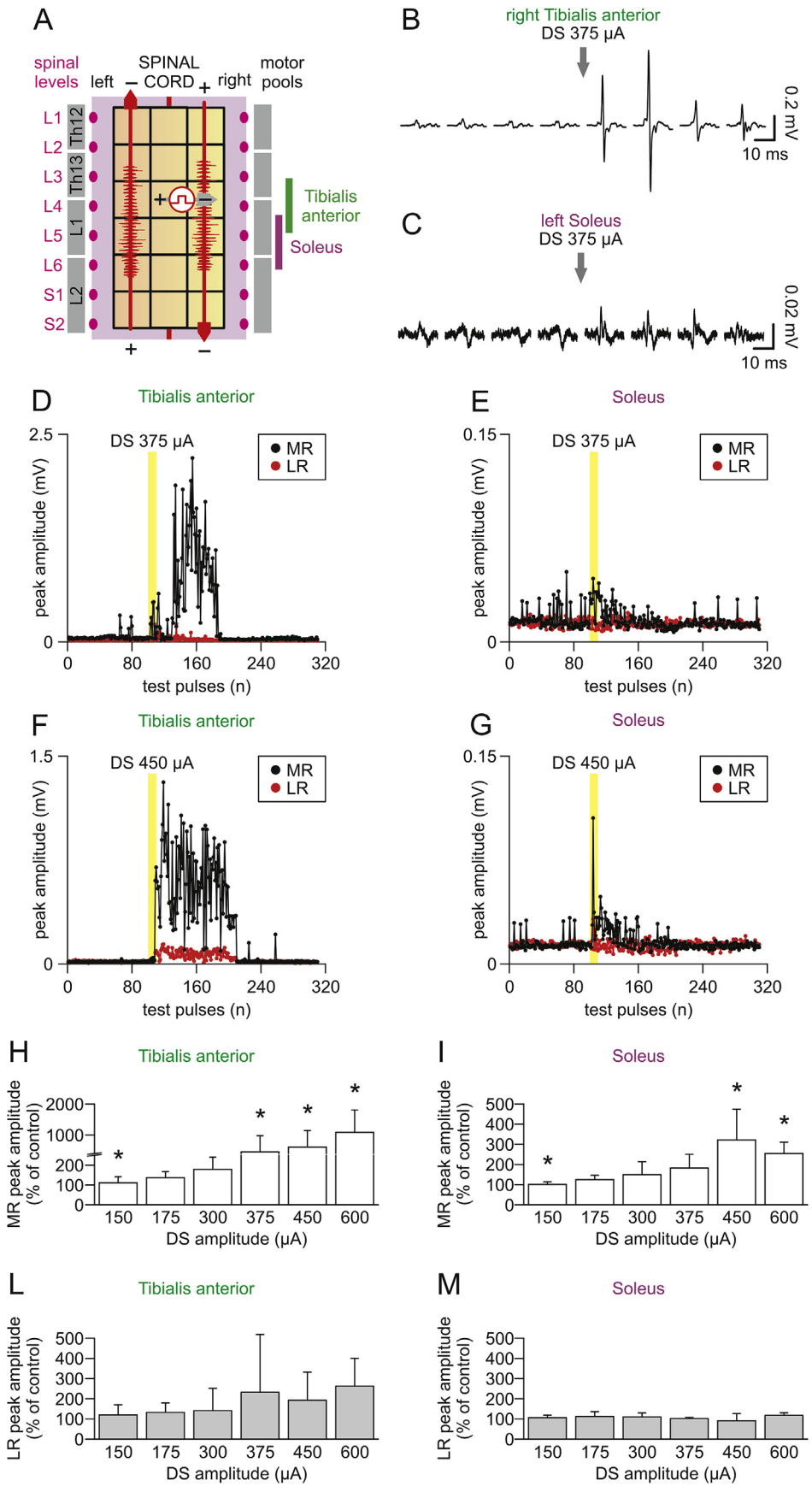

Fig. 3. DS increases the amplitude of MRs in EMG-evoked responses, even at the end of stimulation. In A, the cartoon summarizes the stimulation setting with a calibrated representation of the array width and the distance between homosegmental dorsal roots. Two highly varying waveforms are delivered longitudinally between the extremities of the

In the same preparation, increased DS intensity to 450 μA prolonged and strengthened MR potentiation on both lTA (6 min, 2989% of pre-DS left black dots, Fig. 3 F), and lSol (4 min, 148% of pre-DS; right black dots, Fig. 3 G). At the same time, LRs increased on lTA (370% of pre-DS; left red dots, Fig. 3 F), but not on lSol (90% of pre-DS; right red dots, Fig. 3G).

Pooled data from eight experiments show that DS significantly modulated the peaks of MRs starting from 375 μA for TA (Fig. 3H; $P < 0.001$, Kruskal-Wallis One Way ANOVA on Ranks followed by multiple comparisons versus control group with Dunn's Method, $n = 6–8$) and 450 μA for Sol (Fig. 3 I; $P = 0.002$, Kruskal-Wallis One Way ANOVA on Ranks followed by multiple comparisons versus control group with Dunn's Method, $n = 4–6$), while LR components were not significantly modulated by DS even at the highest intensities (Fig. 3 L, M). Interestingly, short-lasting (at least 1 min) rhythmic discharges appeared right after DS supply (Supplementary Fig. 4) and ceased with a different timing for each muscle ($rTA = 295 \pm 227$ s; $lTA = 440 \pm 257$ s; $lSol = 196 \pm 125$ s; $n = 6$).

DS is more effective than standard stimulation in potentiating small EMG responses

To determine whether DS was more effective than a standard stimulation protocol at 40 Hz in modulating spinal networks, the two protocols were compared in the same animals and at the same intensity (375 μA), while threshold test pulses were segmentally delivered to the spinal cord at L6 (cathode on the left). Four traces from a sample preparation indicate that small responses evoked from rTA using threshold segmental stimulation greatly increased in amplitude during delivery of DS and immediately after stimulation (Fig. 4 A). After 10 min, the delivery of the standard protocol at 40 Hz also increased EMG responses over pre-40 Hz values. However, this effect was lower than with DS and responses quickly returned to pre-40 Hz values as soon as stimulation stopped (Fig. 4 B). The time course for 420 consecutive pulses (21 min) was plotted for the same exemplar experiment (Fig. 4C). DS largely potentiated the amplitude of MRs (1027% of pre-DS), which recovered their baseline after 2.5 min. After 10 min of rest, a train at 40 Hz facilitated MRs, but to a lesser extent (566% of pre-DS) than DS and only during the delivery of 40 Hz stimulation (30 s, Fig. 4C). Similar results were collected also when inverting the order of the two protocols shown in Fig. 4 A, namely 40 Hz train before DS (data not shown). Averaged data from six experiments, where mean peaks of MRs from 20 consecutive sweeps were compared before and right after the delivery of both protocols (Fig. 4 D), confirmed that DS significantly potentiates the amplitude of MRs compared to a train of stereotyped pulses at 40 Hz ($P = 0.031$, Wilcoxon Signed Rank Test, $n = 6$).

DS potentiated MRs induced by single pulses delivered lengthwise through the array

As reported above, DS increased the amplitude of MRs when EMG responses were induced by segmental stimulation of the spinal cord. We wondered whether spinal reflexes can display a similar modulating pattern when single pulses are rostro-caudally delivered along the cord for a more widespread stimulation rather than over specific circuits over a single spinal segment. To explore the effects of DS on the rostro-caudal connectivity along the lumbar cord (L1-S2), small responses from TA and Sol were induced by a train of test pulses (500 μA) applied along the entire length of the array at 0.3 Hz (Fig. 5 A). In a sample experiment, single pulses at an intensity of 500 μA generated small baseline responses with similar amplitude in both the rTA and rSol (Fig. 5 B, left). Addition of DS at 375 μA augmented the peak of both responses to 340% of pre-DS for rTA and 243% of pre-DS for rSol (Fig. 5 B, middle), an effect that persisted in both muscles even after protocol termination ($rTA = 356\%$ of pre-DS, $rSol = 223\%$ of pre-DS; Fig. 5 B, right). In the same exemplar preparation, the strength of DS was serially increased (150–600 μA), potentiating the peak of MRs on both TA (498% of pre-DS) and Sol (254% of pre-DS), already at 225 μA, but only during stimulation (Fig. 5C, D). However, starting from the intensity of 375 μA, facilitation of MRs persisted even after 1 min from the end of stimulation (374% of pre-DS for rTA; 214% of pre-DS for rSol), an effect further potentiated by higher strengths of DS (600 μA, 526% of pre-DS for rTA; 309% of pre-DS for rSol; Fig. 5C, D). In four experiments, in which 20 consecutive test pulses (375 μA,1 min) were considered before and after DS, MR peak amplitudes were significantly potentiated for both TA (Fig. 5 E, $P = 0.019$, paired *t*-test, $n = 4$) and Sol (Fig. 5 F, $P = 0.032$, paired *t*-test, $n = 4$), with TA responses higher than Sol. In summary, single pulses applied along multiple spinal segments induced greater EMG responses from TA and Sol following increasing strengths of DS.

Repetitive delivery of a longer DS affects EMG-evoked responses

The persistence of the facilitating effect elicited by DS shortly beyond protocol termination suggests that, apart from a mere summation of concurrent electrical pulses applied to different sources [30], DS could induce short term potentiation of synaptic activity. Indeed, we noted that following multiple and serial deliveries of DS, longer resting pauses were necessary to return to pre-stimulation baseline values, suggesting the additive effect of repetitive DS applications supplied at short intervals. Thus, a protocol named 'repetitive Dynamic Stimulation (rDS)' was designed by delivering eight slots of DS, regularly interposed by 1-min pauses, to reach the total length of 11 min.

In a sample experiment, small EMG responses were evoked by the continuous delivery of segmental pulses (500 μA) to the cord (L4, cathode on the left; Fig. 6 A). By the end of its delivery, the rDS protocol increased the peak of MRs to 779% of pre-DS on rTA (Fig. 6 B). In the same experiment, consecutive DRs progressively summate the amplitude of MRs, which remained higher than pre-DS (735%) for up to 8 min after the end of stimulation (time course in Fig. 6C). In seven animals, mean values obtained by averaging single peak amplitudes of 100 consecutive EMG responses (5 min) served to compare pre-rDS to the end of repetitive stimulation. The rDS protocol increased the peak of MRs for up to 5 min after ceasing

array on the two sides of the cord, while EMG responses are continuously elicited by single pulses delivered from two close electrodes placed at the center of the array (L4, cathode on the left). Before DS, small responses are induced by threshold single pulses (400 μA; 0.1 ms) from rTA (B) and lSol (C) muscles. Right after the delivery of DS for 30 s (gray arrows, 375 μA), the amplitude of EMG responses increased, mainly on TA. Time courses from rTA (D) and lSol (E) quantify the peak of MRs (black dots) and LRs (red dots) during the continuous delivery of 300 pulses (15 min). The supply of DS at the intensity of 375 μA for the duration of 30 s (yellow shaded rectangles) does not affect LRs (red dots), but augments amplitude of MRs (black dots), more dramatically on TA. Increasing the strength of stimulation (450 μA) potentiates MR peaks, mostly on lTA (F), with a feeble effect also on LRs (red dots). Also MRs from lSol become higher when DS is delivered at 450 μA (G). Histograms report the mean peaks of MRs after DS for 20 consecutive sweeps, expressed as the percentage of pre-DS values pooled from many experiments in which DS was serially increased from 150 μA to 600 μA. Pauses of 5 min are interposed between two consecutive DSs. MR peaks from TA statistically increase since DS delivery at 375 μA (H: *, $P < 0.001$), while, on Sol, DS increases the peak of MRs starting from 450 μA (I: *, $P = 0.002$). Similar analysis is performed for the same experiments as in H and I for LRs, showing no effect following DSs, even at higher amplitudes in both TA (L) and Sol (M) muscles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

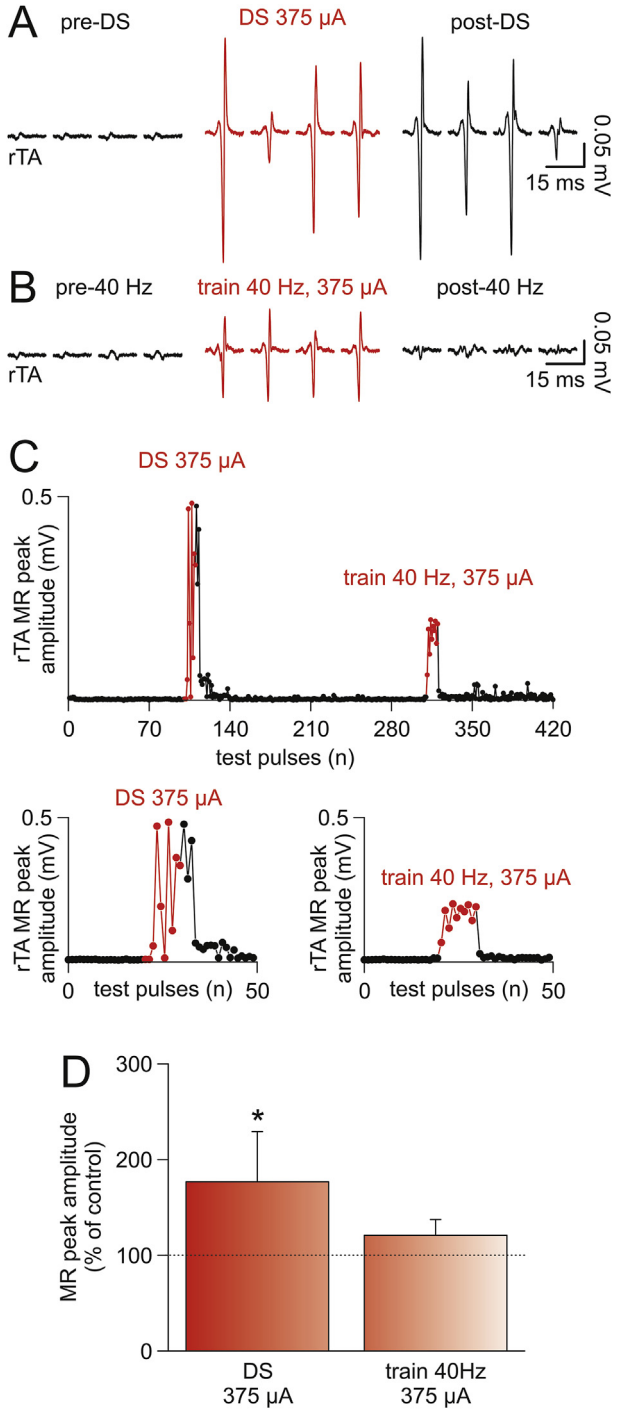

Fig. 4. Compared to a stereotyped train of pulses, DS largely increases motor evoked responses, even after protocol termination. In A, threshold single pulses (0.1 ms) applied to L6 (cathode on the left) induce small EMG responses from rTA in the absence of any continuous stimulation (pre-DS). During DS (375 μA), EMG responses largely increased (middle), persisting even after the end of stimulation (right). In the same preparation, a stereotyped train at 40 Hz augments motor responses only during protocol delivery, returning to baseline values as soon as stimulation ends (B). In C, complete time course for the experiment in A and B reports the peaks of middle responses (MR) for 420 consecutive single pulses (21 min). Single EMG responses after DS are higher than after the following delivery of a 40 Hz train. Note that prior to the delivery of a 40 Hz train, baseline completely recovered to pre-stimulus values. Below are reported the magnifications of the time courses during DS (left) and the 40 Hz train (right), stating that the potentiation of DS persists for 90 s after protocol delivery, while motor evoked responses return to pre-40 Hz values right after the end of the 40 Hz train. Histogram in D summarizes mean data from six tibialis anterior muscles. For each bar, mean peaks of MRs from 20 consecutive sweeps were compared before and right after the delivery of protocols. DS significantly potentiates the amplitude of MRs compared to a train of stereotyped pulses at 40 Hz (*, $P = 0.031$).

stimulation (Fig. 6 D, $P = 0.017$, paired *t*-test, $n = 7$), showing a more persistent effect than single applications.

Increasing the excitability of spinal networks facilitates cortical-spinal input

We demonstrated that DS modulates EMG responses induced by pulses applied segmentally or rostro-caudally to the spinal cord. We questioned whether this effect is associated with a potentiation of cortico-spinal input, as well.

To better explore this possibility, motor responses from hind limbs were bilaterally evoked by broad cortical electrical stimulation (Fig. 7 A; 0.1–1 ms duration). In a sample experiment, at the strength of 800 μA, these motor potentials were characteristically composed of a first response at ~10 ms and a late, more varying, response at ~25–35 ms (Fig. 7 B, left). In the same experiment, sub-threshold pulses (500 μA) did not induce any motor-evoked potentials (Fig. 7 B, middle). Nevertheless, when single pulses at the same sub-threshold intensity were delivered in conjunction with DS, even at an intensity as low as 300 μA, TA motor potentials appeared (Fig. 7 B, right), albeit vanishing as soon as DS terminated (data not shown). In another animal, by doubling the strength of DS (600 μA), motor-evoked responses induced by sub-threshold single pulses persisted for even a couple of minutes after the end of spinal stimulation, eventually disappearing in the later post-DS resting phase (Fig. 7C). In a third animal, small motor responses were evoked by single pulses at 700 μA and were potentiated by DS (375 μA), persisting for 2 min after the end of DS (Fig. 7 D). As observed by serially and gradually increasing intensity of DS from 150 μA to 600 μA, 375 μA was the lowest intensity of DS for generating persistent cortically-evoked motor potentials higher than pre-DS (Fig. 7 E).

These observations were replicated in another set of five animals using a cortical epidural array recently designed for the selective stimulation of the hind limb motor area (Fig. 7 F, see Methods).

To confirm whether DS potentiates cortico-spinal input, experiments were repeated using fifty biphasic pulses epidurally delivered to the left cortical motor area through a selectively stimulating interface (see Methods) before DS, during DS (2.5 min, 600 μA) and right after DS (Fig. 7 G). Exemplar superimposed EMG responses (time-to-peak = 30–35 ms from the stimuli) were occasionally elicited from the contralateral TA only (Fig. 7 G and G_1, left) following single cortical pulses (840 μA, 0.2 ms, 0.3 Hz). Stimulation did not elicit any muscle activity from the ipsilateral leg or from forelimbs (data not shown). Delivery of DS (600 μA) induced higher and more consistent responses compared to pre-DS (469%; Fig. 7 G and G_1, middle), eventually fading away as soon as DS terminated (206%; Fig. 7 G and G_1, right). Averaged values from five animals show that delivery of DS in conjunction with cortical stimulation statistically increased the amplitude of cortically-evoked motor responses ($488 \pm 157\%$, Fig. 7H; $P = 0.024$, Friedman repeated measures ANOVA on ranks followed by all pairwise multiple comparison procedures with Tukey Test, $n = 5$), which returned to baseline values only after the end of DS ($132 \pm 50\%$ of pre-DS, Fig. 7H).

In summary, in eight animals, DS potentiated motor-evoked potentials induced by direct electrical stimulation of motor areas. Even more importantly, when paired with cortical pulses at sub-threshold intensity unable per se to generate any activity, DS facilitated the expression of motor responses. This makes the innovative protocol potentially noteworthy for exploiting spared electrically-incompetent motor fibers after spinal lesion to re-establish some longitudinal connectivity along the injured cord and enhance or even recover volitional motor control.

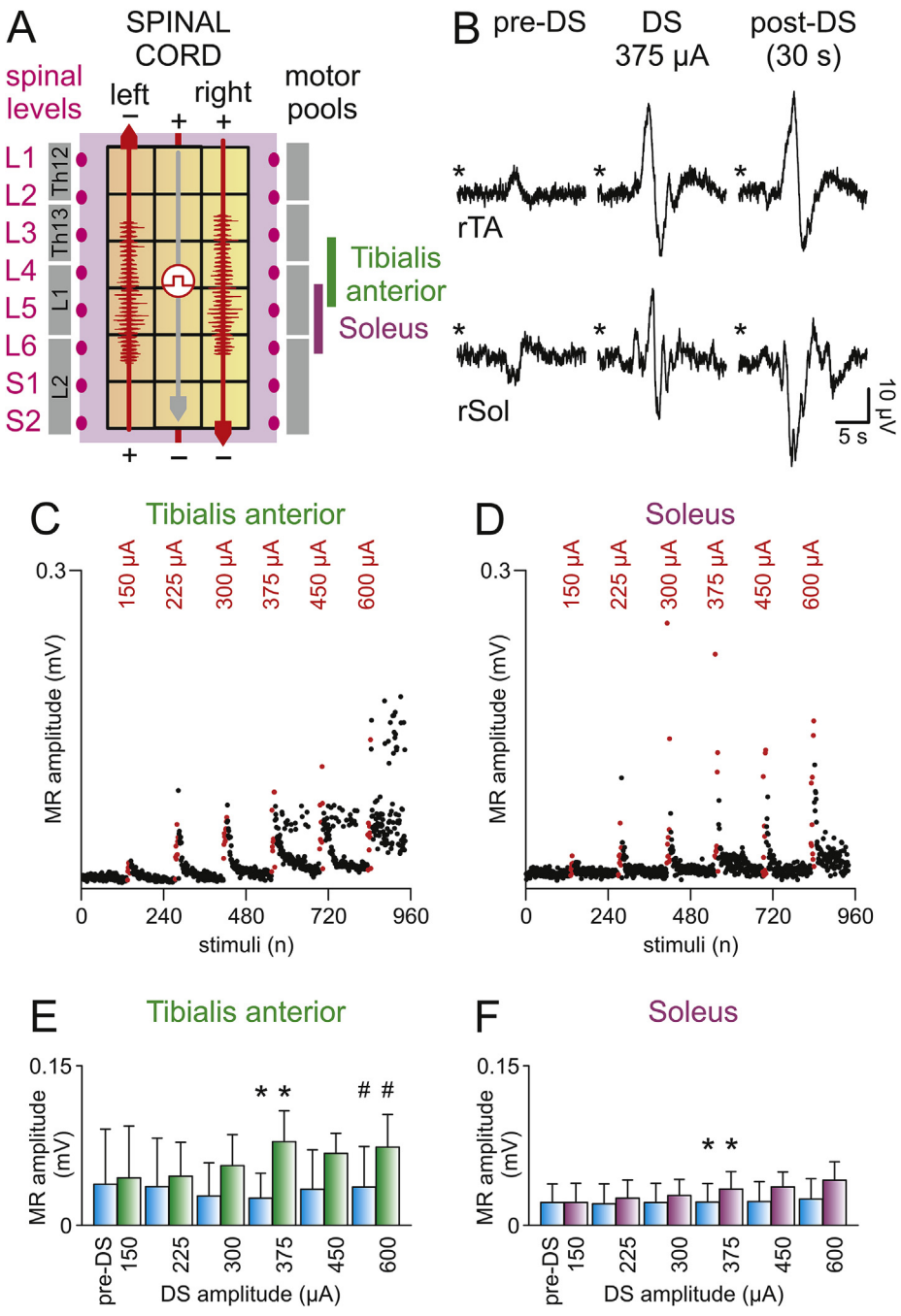

Fig. 5. DS facilitates MRs also in EMG responses elicited by longitudinal stimulation across the array. In A, the cartoon summarizes the stimulation setting with a calibrated representation of the array width and the distance between homosegmental dorsal roots. Two dynamically varying waveforms are longitudinally delivered between the extremities of the array on the two side of the cord, while motor responses are continuously elicited by single monophasic pulses rostro-caudally delivered along the entire length of the array (rostral cathode). In B (right), threshold single pulses (0.1 ms) induce small EMG responses from right tibialis anterior (TA, above) and right soleus (Sol, below) muscles. During delivery of DS (375 μA), the amplitude of EMG responses increases in both muscles (middle). The potentiation of MRs persists even after 30 s from the end of DS (right). In C and D, the entire time course (940 stimuli, 47 min total duration) is reported for the right TA (C) and right Sol (D) for a sample experiment in which DS intensity was serially increased from 150 μA to 600 μA. MRs of EMG evoked responses are continuously recorded during delivery of DS (30 s, red dots) and during each pause between two consecutive DSs (5 min, black dots). DS augments the amplitude of MRs, starting from the intensity of 225 μA. By increasing the strength of stimulation, the potentiation of MR peaks is higher and longer on both muscles. Histograms in E and F report from four experiments the mean peaks of MRs for right TA (E) and right Sol (F) as the average out of 20 consecutive sweeps before DS and the average of 20 consecutive sweeps right after DS termination at each different strength of stimulation. MR peaks statistically increase at 375 μA and 600 μA for TA (E: *, P = 0.019) and only at 375 μA for Sol (F: *, P = 0.032). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

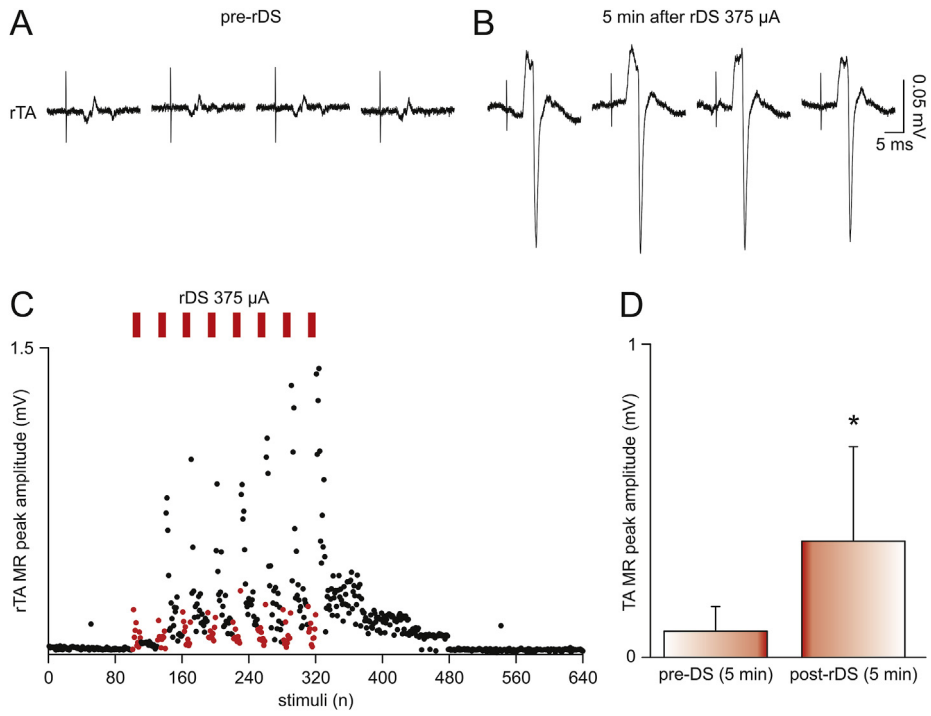

Fig. 6. Repetitive delivery of DS potentiates EMG evoked responses during and after stimuli stopped. In A, small EMG responses on right (up) and left (below) TAs were induced by a train of square pulses (frequency 0.3 Hz; intensity = 500 μA; duration = 0.1 ms), segmentally applied at L4 (cathode on the left). Eight slots of DS (30 s) were serially delivered with 1 min pause from one another in a protocol named repetitive DS (rDS, total duration = 11 min). At the end of the last DS delivered, the same single pulses used before rDS now increase responses from rTA (B). In C is reported the time course of the entire experiment shown in A and B, where 640 single pulses are delivered at 0.3 Hz for a total duration of 32 min rDS is reported as a serial alternation of 8 DS repetitions (red) spaced by resting pauses of 1 min during which no DS was supplied. MRs from rTA are quite consistent before rDS. Amplitude then increases after each slot of DS, reaching the maximum value at the end of rDS and remaining higher than pre-rDS for at least the following 8 min. In D, bars report mean data about MRs, calculated in seven experiments by averaging 100 consecutive responses from TA (total duration = 5 min) before and after rDS, which show the significant increase in motor responses induced by rDS (*, P = 0.017).

Repetitive DS restores evoked motor responses suppressed by spinal cord injury

All the data presented so far is collected from intact rats. To ascertain whether the ability of the DS protocol in potentiating motor output persists even in severely contused spinal cords, we considered a subgroup of animals that underwent a calibrated drop-weight impact to the dorsal midline of the cord, at L4. After injury, the functional interruption of white matter tracts along the lesioned cord was repetitively confirmed as the inability of single pulses (duration = 0.1 ms) delivered to the rostral pairs of array electrodes (L1) to elicit any muscle responses even at the maximal strength of stimulation (800 μA). Nonetheless, 120 min after the injury, MRs were still induced by a series of pulses (0.3 Hz, intensity = 800 μA; duration = 0.1 ms) supplied below the site of lesion through the caudal electrodes of the array (L5; 400 μA; Fig. 8 A). In the sample experiment in Fig. 8 B, although baseline responses were smaller (12 ± 2 μA) than in an uninjured animal (94 ± 21 μA; Fig. 2 E), DS (375 μA) still augmented the peak amplitude of MRs to 483.73%. On average, in three animals, DS was supplied after 217 ± 95 min from the experimental injury and increased MRs of TA to 310.24 ± 152.23%.

Discussion

Dynamic vs. tonic stimulation patterns

In the current study, we adopted a stochastic pattern of modulation dynamically delivered to multiple sites along the spinal cord. These dynamically varying patterns consisted of continuously changing frequencies, amplitudes and polarities, as opposed to the traditional rectangular waves of single frequency and amplitude. We call this method of modulation: dynamic stimulation (DS), in contrast to the more static profile of trains of stereotyped pulses.

In fully anesthetized adult rats, after the end of DS, spontaneous stereotyped rhythmic activity was observed in bilateral TA and Sol muscles, likely reflecting a short-lasting increase in spinal network excitability. Furthermore, DS increased the middle EMG response (MR) elicited by segmental epidural pulses at threshold intensity in TA and Sol muscles, an effect that persisted beyond the end of protocol in animals, either intact or after a severe spinal contusion. Efficacy of DS depends upon its strength, with results already visible at low intensities (about 225 μA) of stimulation. Moreover, compared to a standard tonic 40 Hz stimulation, DS was more effective at similar intensities in augmenting the amplitude of EMG responses elicited by single pulses applied to the spinal cord, even after protocol termination. Responses were also facilitated when elicited by threshold pulses applied rostro-caudally along the cord or over the cortical motor area. Repetitive delivery of DS greatly lengthened the duration of EMG responses. Thus, stimulation with asynchronous DS patterns increased spinal responses and facilitated cortico-spinal input to spinal networks in the adult rat *in vivo*. Although an increase in synaptic activity of spinal networks, as well as in cortico-spinal axonal conduction, can be responsible for the facilitatory effect reported here, a plausible explanation should consider that the same descending conduction has also a greater impact on more responsive spinal interneurons and motoneurons.

Moreover, a few critical features of spinal networks must be considered to better understand the importance of variability offered by DS. Firstly, spinal stimulation taps into the intrinsic feedforward feature of spinal networks. Indeed, maintaining a same pattern of stimulation may fail to trigger the next phase of "feed-forwardness" [31], a requirement that may be addressed by the DS protocol. While step training spinal rats, the intensity of stimulation needs to be modulated from every few tens of seconds to minutes to maintain a stable pattern of stepping (unpublished observation). Further, as the spinal networks are trained to stand or step, the threshold of activation changes (generally reduces), suggesting the constantly modulating network at work. Using DS, we may be enabling some of these feedforward features of the spinal networks at different time frames (seconds to minutes).

Variability of motor responses elicited during threshold stimulation

Spinal reflexes induced by spinal cord electrical stimulation were reported to characteristically span along multiple segments of the spinal cord [3,32]. In the present study, once the dorsal surface is stimulated with single pulses at motor threshold intensity, simultaneous EMG responses were obtained from distinct muscles, each characterized by a different latency. Moreover, by increasing the strength of stimulation, responses appeared with a first intermediate latency, followed by a later one. Short latencies were only observed with higher intensities of stimulation, corresponding to the direct activation of ventral motoneurons [19,20]. It has been previously reported that the amplitude of each evoked response is modulated by increasing stimulation intensities up to a maximal muscle recruitment [19,20]. Low intensity stimuli predominantly used in this study elicited responses with intermediate and late EMG latencies, reflecting the recruitment of multiple combinations of synapses. In this study, DS selectively modulated MRs in EMG-evoked responses. Previous reports on awake spinalized animals highlighted the impact of neuromodulation on LRs [19] and the prognostic value of the reappearance of LRs for the recovery after injury [33]. However, LRs were not affected by DS, even at higher intensities of stimulation. We suppose that this might be due to anesthesia depressing the connectivity across a wider network, in turn confirming the local impact of DS on the directly stimulated sacro-lumbar circuits [34].

On the other hand, when threshold impulses were delivered at low frequency over a long period, motor-evoked responses were spontaneously modulated. Indeed, amplitude and latency of baseline motor responses varied randomly, without any correlation with the activation of motor pools from flexors and/or extensors. This phenomenon reveals that random, spontaneous synaptic events might be an intrinsic feature of the neuromotor system and can be exploited using an asynchronous pattern of stimulation to induce resonance [35]. This effect was observed in adult rats under anesthesia and presumably would be even more dramatic in an awake rat, where the spinal network's state of excitability is higher and changes continuously as it processes vast multi-modal input [20]. Spontaneous modulation intrinsic to spinal networks presumably plays a role in defining the effect of direct electrical stimulation of the spinal cord, especially at low intensities. Further, if frequency of stimulation is a crucial factor for reaching a certain level of selectivity to tune distinct motor outputs [36], the real impact of stimulation relies on the ensemble of multimodal inputs to the spinal interneurons which defines the physiological states of the spinal networks that project to a given combination of motor pools. Indeed, the intrinsic frequency of modulation of network's excitability is crucial, especially at low intensities of stimulation, for summating or filtering external pulses and then defining the effective frequency of stimulation received by the network. Although DS overall increased the amplitude of EMG responses, they remained variable, suggesting that DS exploits the same endogenous mechanisms of modulation of the motor output, even in the anesthetized state. DS showed slight differences in the modulation of the motor output from TA and Sol, as TA was generally modulated by lower intensities of DS. Although a systematic approach in determining the impact of DS on different muscles was beyond the scope of this first study introducing the new protocol, we cannot exclude that different muscles may have a different sensitivity and/or responsiveness to neuromodulation [19,20]. However, the lower intensity of DS necessary to modulate TA compared to Sol, often reported through the study, can be ascribed to the configuration of single pulses delivered to test synaptic activity. Indeed, the fully independent electrodes of the interface demonstrated a selective recruitment of either TA or Sol, alternatively, depending on the site of delivery of single pulses and their cathode and anode polarity compared to the localization of specific motor pools.

Stimulation with dynamically varying waveforms

The mechanisms of traditional epidural stimulation using trains of stereotyped impulses and the adoption of selected frequencies (15–40 Hz) that can selectively activate stepping or standing after lesion are still poorly understood. Likewise, the mechanisms by which DS waveforms increase motor evoked responses remains unclear. In the populated power spectrum of DS, also the frequencies from 15 to 40 Hz are present and likely responsible for the modulation of spinal excitability reported after standard neuromodulation in humans [1–5] and rats [19–21]. However, the intrinsic baseline variability of DS, or some distinct frequencies within its spectrum, may be responsible for the longer and greater facilitation of the motor output when compared to the use of standard paradigms.

Moreover, in neonatal *in vitro* preparations, "noisy" waves were delivered to the lumbar spinal networks to trigger an epoch of locomotor-like discharges [16]. Contrariwise, here, asynchronous stimulation was primarily delivered to the lumbo-sacral propriospinal networks to affect the excitability of circuits modulating the motor output. However, by applying DS to anesthetized adult animals *in vivo,* more questions arise regarding the level of complexity required to elicit an optimal response. In addition, the more intact preparation makes it difficult to distinguish slight effects on the motor output, i.e., the role played by each single feature of DS, when using two waveforms instead of one, with staggered onset and opposite cathode location of the two patterns. Nonetheless, the configuration of DS was chosen to provide high levels of variability in amplitude, frequency and direction of the electric field.

At the same time, the rhythmic spontaneous activity appearing among multiple motor pools a few minutes after DS delivery demonstrates a transient modulation of interneuronal networks that select the different motor pools to activate within the lumbosacral segments. A similar increase in the spontaneous activity of motor pools did not occur in reduced preparations, even when undergoing longer periods of stimulation [37]. On the other hand, *in vivo* experiments involving weight bearing stepping will likely reveal a much higher level of variable, but also patterned, multi-modal input also in relation to different physiological states.

Experiments performed in intact animals hint at the possibility that DS recruits long loop effects through the spinal cord and the brain stem, as well as through cortical or other higher systems [38]. Nevertheless, facilitation of spinal reflexes obtained in severely

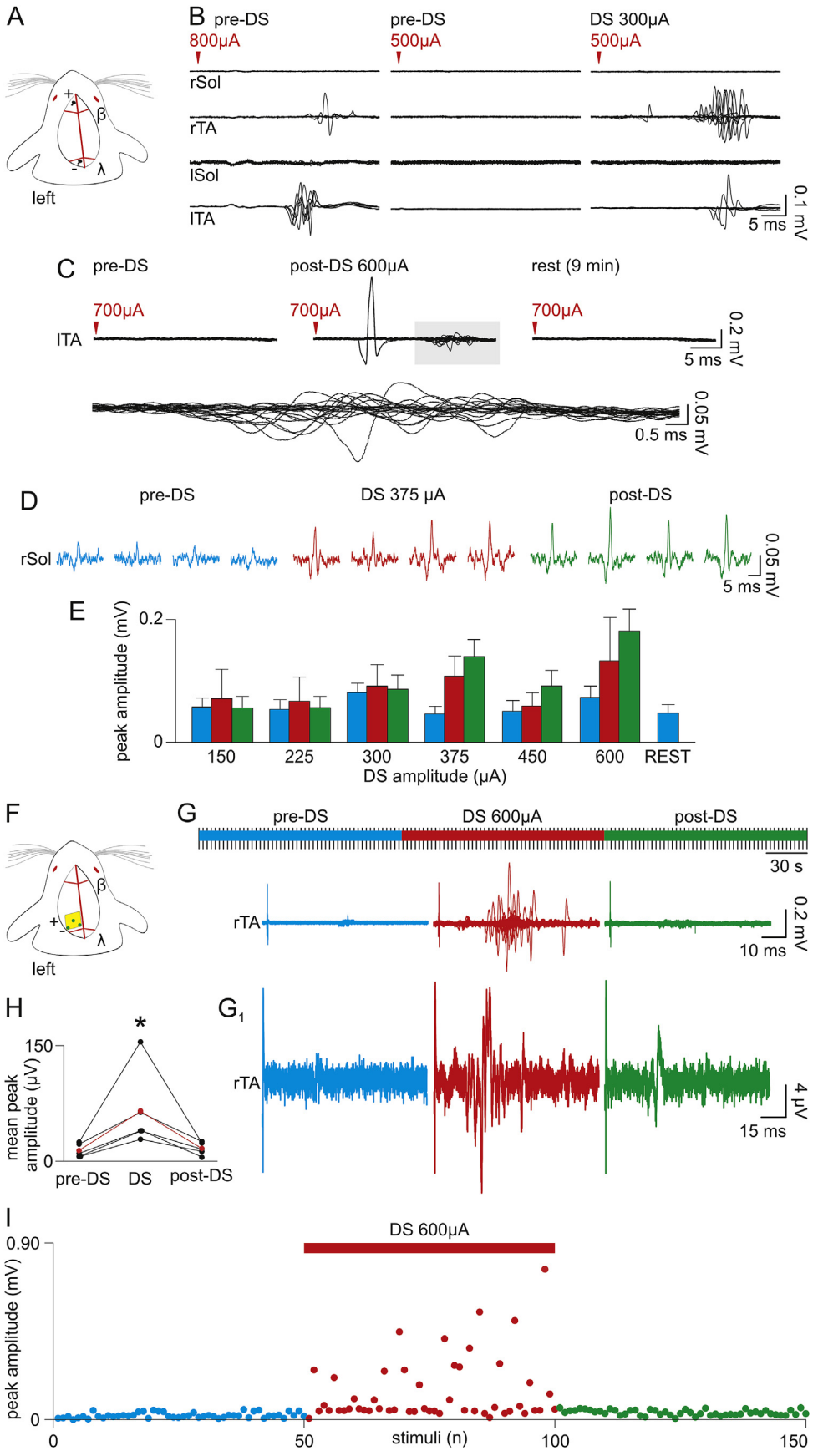

Fig. 7. DS facilitates the reappearance of cortically-evoked EMG responses. In A, a cartoon of the rat's head depicts the setting for the broad cortical stimulation using two metal screws placed on the left side of the skull, rostral to bregma (β) and caudal to lambda (λ, see methods), respectively. In B, consecutive traces are superimposed during the delivery of

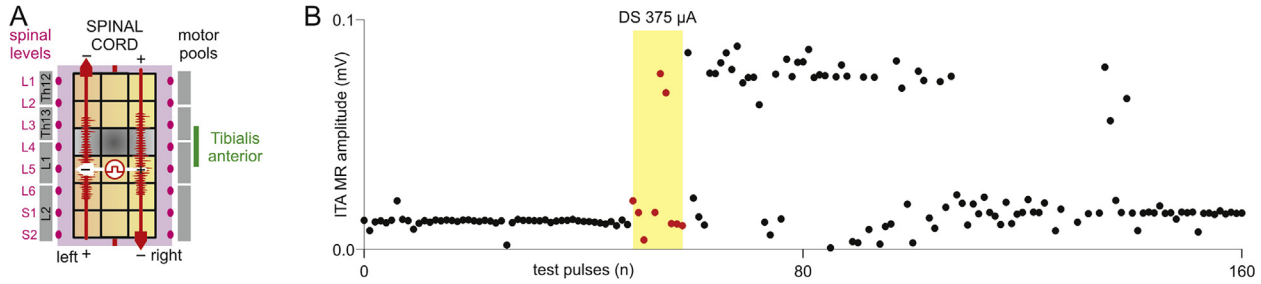

Fig. 8. DS enhances spinal network excitability after an acute spinal contusion. In A, single pulses (0.3 Hz, intensity = 800 μA; duration = 0.1 ms) are continuously supplied to the L5 of a spinal cord, severely contused at L4 level. In B, fifty MRs are acquired before delivering DS (375 μA, 30 s, pale yellow field), which largely increases the peak of responses (red dots) even after DS termination. Only after 2.5 min from the end of DS, amplitude of MRs declines towards pre-DS values. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

contused cords demonstrates that DS still potentiates the motor output even when the lumbar cord is functionally disconnected from higher centers. Therefore, DS seems to locally affect the stimulated spinal cord region, likely through the recruitment of more excitable, or possibly larger, interneuronal networks. Perhaps, after DS, a single electrical stimulus at threshold applied to the dorsum of the cord might activate a greater number of motoneurons and motor pools to generate a more robust motor response. Moreover, the less dramatic increase of MR amplitude by DS in contused versus intact rats suggests that a broader multisegmental lumbar network is required to exploit neuromodulation.

A novel paired associative stimulation confined to the cord

Ascending and descending stereotyped trains of pulses delivered to the extremities of the motor pathway (the peripheral nerve and the cortex) and converging onto spinal networks effectively facilitate the motor output even after injury [10–14]. Recent confirmation was obtained by shortening the distance between the two sites of paired associative stimulation, i.e. pulses applied to the cortex and to the spinal cord directly [39,40]. However, a more local associative stimulation of spinal networks has been hindered so far by the lack of epidural arrays with fully independent electrodes to allow simultaneous stimulation of two segments of the cord with different patterns. Our study used a novel interface that allows pairing of two pulse-patterns converging onto spinal networks and delivered rostrally and caudally along four spinal segments. In our protocol, two dynamically varying waveforms were continuously delivered with a staggered onset. The large number of spikes, the range of frequencies and the variability within the two waves of DS may converge with optimal latency onto the spinal network to exploit phenomena of spike timing–dependent plasticity [7–9]. In particular, in the lumbar network, DS might optimally pair the antidromic depolarization of post-synaptic terminals with the orthodromic depolarization of pre-synaptic terminals, as previously reported using paired-pulse stimulation protocols that increased amplitude of EMG responses [12].

In addition, in individuals with a clinically-defined complete spinal cord injury, it might be difficult to find one single optimal latency out of the two associated stimuli to facilitate the motor output, because of the wide variability in real-world spinal injuries. Indeed, each lesion can trigger a unique pattern of polysynaptic tract rerouting, which can potentially request different pairing latencies. Our epidural stimulation protocol consisting of two patterns of different wide spectrum harmonics should provide multiple combinations for a tailored coupling of ascending and cortico-spinal input, increasing the probability to more optimally tune the latency of pairing to benefit a higher number of individuals. Indeed, terminal recordings reported here from acutely injured spinal cords might support a more dramatic impact of DS on chronic spinal cord injuries, which already overcame the acute depression of neuronal activity and might allow behavioral improvements in response to DS.

Conclusions

In the present study, we explored a novel pattern of highly varying stimulating patterns delivered through an innovative spinal epidural interface. These two resources provided the means for testing the potential of variable and multisite spinal stimulation, by generating an increase in spinal network excitability and a more robust modulation of lumbosacral networks compared to tonic

a train of single cortical pulses (arrowheads; duration = 1 ms, frequency = 0.3 Hz). Single pulses at 800 μA (left, pre-DS, 10 consecutive traces are superimposed) elicit consistent EMG responses from ITA and some variable responses from rTA. Responses from both muscles disappear when the strength of stimulation is lower (500 μA, middle, 10 consecutive traces are superimposed). During DS delivery at low intensity (300 μA), even sub-threshold single pulses (500 μA) are now able to reinstate repetitive responses from rTA and ITA (right, 10 consecutive traces are superimposed). In C, from a different animal, 20 consecutive traces are superimposed during the delivery of a train of single cortical pulses (arrowheads; duration = 1 ms; intensity = 700 μA; frequency = 0.3 Hz). In the absence of DS (pre-DS), no responses were recorded from ITA. Right after DS delivered at the intensity of 600 μA (middle), a single maximal peak and a consistent bundle of lower and late potentials appear on ITA. These effects persist for about 1 min after the end of DS, as emphasized by the magnification below that corresponds to the shaded gray rectangle above. EMG responses disappear after 9 min from the termination of DS (right). In D, in a third animal, 4 consecutive small EMG responses evoked by cortical stimulation (duration = 0.1 ms; intensity = 700 μA; frequency = 0.3 Hz) are recorded from rSol before DS (pre-DS, left, blue traces). During DS at 375 μA (middle, red traces), EMG responses are potentiated for up to 3 min after the termination of DS (right, green traces). In E, from the same experiment as in D, peak amplitudes of 20 consecutive sweeps are averaged before DS (blue), during DS supplied at increasing intensities (150–600 μA; red) and after DS delivery (green). Note that the potentiation post-DS (green bar) is higher at the maximal strength (600 μA).

The cartoon in F schematized the selective stimulation of the hind limb cortical motor area using a customized array (see Methods). In G, top, the protocol is summarized by three colored rectangles (2.5 min long each). Fifty single biphasic pulses (840 μA; 0.2 ms; vertical black bar) were consecutively delivered to the left motor area before DS (pre-DS, blue), during DS supply (600 μA, red) and post-DS (green). Below, EMG responses (50 consecutive superimposed traces) elicited from the contralateral TA (ITA, blu traces) increase in number and amplitude during DS (red traces) and then reduced after DS termination (green traces). The averaged traces for each slot are reported in G_1, visualizing how DS increases cortically-induced responses with a latency around 30–35 ms from the pulse artifact. In H, values of peak amplitude were reported for five experiments (black dots and lines) before DS (pre-DS), during DS and post-DS. Mean values are reported in the plot as red dots and lines (*, P = 0.024). In I, the entire time course of MR amplitude is plotted from TA during the whole experiment reported in G. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

patterns of stimulation. Further, the DS paradigm of stimulation was linked to patterns associated with the facilitation of the motor output induced by subthreshold cortical input.

Author contributions

Designed research: GT, PG; RE; Performed research: GT; Contributed unpublished reagents/analytic tools: WL, SC; Analyzed data GT, SC; Wrote the paper GT, PG, SC, RI, VRE.

Declaration of competing interest

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2019.09.013.

References

[1] Harkema S, Gerasimenko Y, Hodes J, Burdick J, Angeli C, Chen Y, Ferreira C, Willhite A, Rejc E, Grossman RG, Edgerton VR. Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: a case study. Lancet 2011;377:1938–47. https://doi.org/10.1016/S0140-6736(11)60547-3.

[2] Angeli CA, Edgerton VR, Gerasimenko YP, Harkema SJ. Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. Brain 2014;137:1394–409. https://doi.org/10.1093/brain/awu038.

[3] Gerasimenko YP, Lu DC, Modaber M, Zdunowski S, Gad P, Sayenko DG, Morikawa E, Haakana P, Ferguson AR, Roy RR, Edgerton VR. Noninvasive reactivation of motor descending control after paralysis. J Neurotrauma 2015;32:1968–80. https://doi.org/10.1089/neu.2015.4008.

[4] Grahn PJ, Lavrov IA, Sayenko DG, Van Straaten MG, Gill ML, Strommen JA, Calvert JS, Drubach DI, Beck LA, Linde MB, Thoreson AR, Lopez C, Mendez AA, Gad PN, Gerasimenko YP, Edgerton VR, Zhao KD, Lee KH. Enabling task-specific volitional motor functions via spinal cord neuromodulation in a human with paraplegia. Mayo Clin Proc 2017;92:544–54. https://doi.org/10.1016/j.mayocp.2017.02.014.

[5] Taccola G, Sayenko D, Gad P, Gerasimenko Y, Edgerton VR. And yet it moves: recovery of volitional control after spinal cord injury. Prog Neurobiol 2018;160:64–81. https://doi.org/10.1016/j.pneurobio.2017.10.004.

[6] Zhang LI, Tao HW, Holt CE, Harris WA, Poo M. A critical window for cooperation and competition among developing retinotectal synapses. Nature 1998;395:37–44.

[7] Song S, Miller KD, Abbott LF. Competitive Hebbian learning through spike-timing-dependent synaptic plasticity. Nat Neurosci 2000;3:919–26.

[8] Dan Y, Poo MM. Spike timing-dependent plasticity: from synapse to perception. Physiol Rev 2006;86:1033–48.

[9] Caporale N, Dan Y. Spike timing-dependent plasticity: a Hebbian learning rule. Annu Rev Neurosci 2008;31:25–46. https://doi.org/10.1146/annurev.neuro.31.060407.125639.

[10] Poon DE, Roy FD, Gorassini MA, Stein RB. Interaction of paired cortical and peripheral nerve stimulation on human motor neurons. Exp Brain Res 2008;188:13–21. https://doi.org/10.1007/s00221-008-1334-8.

[11] Cortes M, Thickbroom GW, Valls-Sole J, Pascual-Leone A, Edwards DJ. Spinal associative stimulation: a non-invasive stimulation paradigm to modulate spinal excitability. Clin Neurophysiol 2011;122:2254–9. https://doi.org/10.1016/j.clinph.2011.02.038.

[12] Bunday KL, Perez MA. Motor recovery after spinal cord injury enhanced by strengthening corticospinal synaptic transmission. Curr Biol 2012;22:2355–61. https://doi.org/10.1016/j.cub.2012.10.046.

[13] Tolmacheva A, Savolainen S, Kirveskari E, Lioumis P, Kuusela L, Brandstack N, Ylinen A, Mäkelä JP, Shulga A. Long-term paired associative stimulation enhances motor output of the tetraplegic hand. J Neurotrauma 2017;34:2668–74. https://doi.org/10.1089/neu.2017.4996.

[14] Urbin MA, Ozdemir RA, Tazoe T, Perez MA. Spike-timing-dependent plasticity in lower-limb motoneurons after human spinal cord injury. J Neurophysiol 2017;118:2171–80. https://doi.org/10.1152/jn.00111.2017. Epub 2017 May 3.

[15] Gad P, Choe J, Shah P, Garcia-Alias G, Rath M, Gerasimenko Y, Zhong H, Roy RR, Edgerton VR. Sub-threshold spinal cord stimulation facilitates spontaneous motor activity in spinal rats. J NeuroEng Rehabil 2013;10:108. https://doi.org/10.1186/1743-0003-10-108.

[16] Taccola G. The locomotor central pattern generator of the rat spinal cord in vitro is optimally activated by noisy dorsal root waveforms. J Neurophysiol 2011;106:872–84. https://doi.org/10.1152/jn.00170.2011.

[17] Dose F, Menosso R, Taccola G. Rat locomotor spinal circuits in vitro are activated by electrical stimulation with noisy waveforms sampled from human gait. Physiol Rep 2013;1(2). https://doi.org/10.1002/phy2.25. e00025.

[18] Dose F, Taccola G. Two Distinct stimulus frequencies delivered simultaneously at low intensity generate robust locomotor patterns. Neuromodulation 2016;19:563–75. https://doi.org/10.1111/ner.12402.

[19] Lavrov I, Gerasimenko YP, Ichiyama RM, Courtine G, Zhong H, Roy RR, Edgerton VR. Plasticity of spinal cord reflexes after a complete transection in adult rats: relationship to stepping ability. J Neurophysiol 2006;96:1699–710.

[20] Gerasimenko YP, Lavrov IA, Courtine G, Ichiyama RM, Dy CJ, Zhong H, Roy RR, Edgerton VR. Spinal cord reflexes induced by epidural spinal cord stimulation in normal awake rats. J Neurosci Methods 2006;157:253–63.

[21] Gad PN, Roy RR, Zhong H, Gerasimenko YP, Taccola G, Edgerton VR. Neuromodulation of the neural circuits controlling the lower urinary tract. Exp Neurol 2016;285:182–9. https://doi.org/10.1016/j.expneurol.2016.06.034.

[22] Fournier M, Roy RR, Perham H, Simard CP, Edgerton VR. Is limb immobilization a model of muscle disuse? Exp Neurol 1983;80:147–56.

[23] Roy RR, Hirota WK, Kuehl M, Edgerton VR. Recruitment patterns in the rat hindlimb muscle during swimming. Brain Res 1985;337:175–8.

[24] Gad P, Choe J, Nandra MS, Zhong H, Roy RR, Tai YC, Edgerton VR. Development of a multi-electrode array for spinal cord epidural stimulation to facilitate stepping and standing after a complete spinal cord injury in adult rats. J NeuroEng Rehabil 2013;10:2. https://doi.org/10.1186/1743-0003-10-2.

[25] Chang CW, Lo YK, Gad P, Edgerton R, Liu W. Design and fabrication of a multi-electrode array for spinal cord epidural stimulation. Conf Proc IEEE Eng Med Biol Soc 2014;2014:6834–7. https://doi.org/10.1109/EMBC.2014.6945198.

[26] Lavrov I, Courtine G, Dy CJ, van den Brand R, Fong AJ, Gerasimenko Y, Zhong H, Roy RR, Edgerton VR. Facilitation of stepping with epidural stimulation in spinal rats: role of sensory input. J Neurosci 2008;28:7774–80.

[27] Lo YK, Kuan YC, Culaclii S, Kim B, Wang PM, Chang CW, Massachi JA, Zhu M, Chen K, Gad P, Edgerton VR, Liu W. A fully integrated wireless SoC for motor function recovery after spinal cord injury. IEEE Trans Biomed Circuits Syst 2017;11:497–509.

[28] Russell C, Kissane RWP, Steenson DP, Chakrabarty S. A novel simplistic fabrication technique for cranial epidural electrodes for chronic recording and stimulation in rats. J Neurosci Methods 2018;311:239–42.

[29] Taccola G, Nistri A. Characteristics of the electrical oscillations evoked by 4-aminopyridine on dorsal root fibers and their relation to fictive locomotor patterns in the rat spinal cord in vitro. Neuroscience 2005;132:1187–97.

[30] Dose F, Deumens R, Forget P, Taccola G. Staggered multi-site low-frequency electrostimulation effectively induces locomotor patterns in the isolated rat spinal cord. Spinal Cord 2016;54:93–101. https://doi.org/10.1038/sc.2015.106.

[31] Gerasimenko Y, Sayenko D, Gad P, Liu CT, Tillakaratne NJK, Roy RR, Kozlovskaya I, Edgerton VR. Feed-forwardness of spinal networks in posture and locomotion. The Neuroscientist 2017;23:441–53.

[32] Minassian K, Persy I, Rattay F, Dimitrijevic MR, Hofer C, Kern H. Posterior root-muscle reflexes elicited by transcutaneous stimulation of the human lumbosacral cord. Muscle Nerve 2007;35:327–36.

[33] Gad P, Lavrov I, Shah P, Zhong H, Roy RR, Edgerton VR, Gerasimenko Y. Neuromodulation of motor-evoked potentials during stepping in spinal rats. J Neurophysiol 2013;110:1311–22. https://doi.org/10.1152/jn.00169.2013.

[34] Paasonen J, Stenroos P, Salo RA, Kiviniemi V, Gröhn O. Functional connectivity under six anesthesia protocols and the awake condition in rat brain. Neuroimage 2018;172:9–20. https://doi.org/10.1016/j.neuroimage.2018.01.014.

[35] Martínez L, Pérez T, Mirasso CR, Manjarrez E. Stochastic resonance in the motor system: effects of noise on the monosynaptic reflex pathway of the cat spinal cord. J Neurophysiol 2007;97:4007–16.

[36] Minassian K, Persy I, Rattay F, Pinter MM, Kern H, Dimitrijevic MR. Human lumbar cord circuitries can be activated by extrinsic tonic input to generate locomotor-like activity. Hum Mov Sci 2007;26:275–95.
[37] Dingu N, Deumens R, Taccola G. Electrical stimulation able to trigger locomotor spinal circuits also induces dorsal horn Activity. Neuromodulation 2016;19:38–46. https://doi.org/10.1111/ner.12354.
[38] Chofflon M, Lachat JM, Rüegg DG. A transcortical loop demonstrated by stimulation of low-threshold muscle afferents in the awake monkey. J Physiol 1982;323:393–402.
[39] Dixon L, Ibrahim MM, Santora D, Knikou M. Paired associative transspinal and transcortical stimulation produces plasticity in human cortical and spinal neuronal circuits. J Neurophysiol 2016;116:904–16. https://doi.org/10.1152/jn.00259.2016.
[40] Mishra AM, Pal A, Gupta D, Carmel JB. Paired motor cortex and cervical epidural electrical stimulation timed to converge in the spinal cord promotes lasting increases in motor responses. J Physiol 2017;595:6953–68. https://doi.org/10.1113/JP274663.