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Title: Rostral lumbar segments are the key controllers of hindlimb locomotor rhythmicity in the adult spinal rat

Abbreviated Title: Spinal segments that control rodent stepping

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Abstract (word limit 243/250)

The precise location and functional organization of the spinal neuronal locomotor-related networks in adult mammals remains unclear. Our recent neurophysiological findings provided empirical evidence that the rostral lumbar spinal cord segments play a critical role in the initiation and generation of the rhythmic activation patterns necessary for hindlimb locomotion in adult spinal rats. Since added epidural stimulation at the S1 segments significantly enhanced the motor output generated by L2 stimulation, these data also suggested that the sacral spinal cord provides a strong facilitory influence in rhythm initiation and generation. However, whether L2 will initiate hindlimb locomotion in the absence of S1 segments, and whether S1 segments can facilitate locomotion in the absence of L2 segments remains unknown. Herein, adult rats received complete spinal cord transections at T8 and then at either L2 or S1. Rats with spinal cord transections at T8 and S1 remained capable of generating coordinated hindlimb locomotion when receiving epidural stimulation at L2 and when ensembles of locomotor related loadbearing input were present. In contrast, minimal locomotion was observed when S1 stimulation was delivered after spinal cord transections at T8 and L2. Results were similar when the non-specific serotonergic agonists were administered. These results demonstrate in adult rats that rostral lumbar segments are essential for the regulation of hindlimb locomotor rhythmicity. In addition, the more caudal spinal networks alone cannot control locomotion in the absence of the rostral segments around L2 even when loadbearing rhythmic proprioceptive afferent input is imposed.

New and Noteworthy

The exact location of the spinal neuronal locomotor-related networks in adult mammals remains unknown. The present data demonstrate that when the rostral lumbar spinal segments (~L2) are completely eliminated in thoracic spinal adult rats, hindlimb stepping is not possible with neurochemical modulation of the lumbosacral cord. In contrast, eliminating the sacral cord retains stepping ability. These observations highlight the importance of rostral lumbar segments in generating effective mammalian locomotion.

Keywords

Spinal cord transection; epidural spinal cord stimulation; central pattern generation; lumbosacral spinal cord; serotonergic agonists

Introduction

Core components of the spinal neuronal networks that regulate hindlimb locomotion consist of what has become known as central pattern generators. Studies in the isolated neonatal rat spinal cord have shown that although the rhythm-generating networks are distributed within spinal cord segments L1-L6, the ability to generate fast and regular rhythmic activity decreases in the caudal direction. (Kjaerulff & Kiehn, 1996). Also it was demonstrated that either cervical, thoracic, lumbar, or sacrococcygeal networks can be activated in the neonate independently to generate a rhythmic fictive locomotor pattern (Cazalets *et al.*, 1995; Cowley & Schmidt, 1997; Kremer & Lev-Tov, 1997; Gabbay *et al.*, 2002; Cherniak *et al.*, 2014; Beliez *et al.*, 2015). In addition, a strong coupling between rostral and caudal networks with the rostral segments of the lumbar cord playing a leading role in the initiation of hindlimb locomotion has been reported in neonatal rats (Cazalets & Bertrand, 2000). These data suggest the existence of independent locomotor –related networks in cervical, thoracic, and lumbar spinal segments. In interpreting the above studies addressing issues of hindlimb locomotion in isolated preparations, it is important to recognize that the present *in vivo,* adult experimental model of load-bearing spinal locomotion is distinctly different from those models in which there is neither supraspinal nor peripherally-derived locomotion linked proprioceptive and cutaneous input to the lumbosacral segments of neonatal or adult animals.

In the adult rat after a thoracic spinal transection, epidural stimulation **(**ES) at either rostral lumbar (L2) or sacral (S1) spinal segments can enable step-like locomotor behaviors (Ichiyama *et al.*, 2005). Originally S1 stimulation was suggested by us empirically to control tonic activity of hindlimb muscles and provide optimal conditions for locomotion regulation during L2 stimulation. Indeed, we observed that simultaneous stimulation of the L2 and S1 segments evokes stronger stepping responses than stimulating at either segment alone (Courtine

et al., 2009). The main objective of the present study is to elucidate whether there are distinct contributions of the rostral vs. caudal segments of lumbosacral spinal cord in controlling and regulating hindlimb locomotion in adult rats. A key question is: Are there independent neuronal networks in the lumbar and sacral spinal segments controlling locomotor behavior?

We hypothesized that the sensorimotor networks intrinsic to the more rostral lumbar segments in the adult rat are more uniquely competent in generating rhythmic hindlimb locomotor patterns as compared to the sacral cord. To test this hypothesis, we used a double spinal transection model in adult rats, i.e., a complete spinal transection at T8 and then 22 days later a second transection at either the L2 or S1 spinal level; that is, we determined the locomotor ability in rats without either the rostral (L2) or caudal (S1) spinal segments. Our data indicate that rostral segments are essential and critical for regulation of hindlimb locomotor behavior and that caudal spinal networks alone cannot initiate or generate effective locomotion. However, they can modulate and control locomotor-related motor output when the rostrally localized networks are intact.

Materials and Methods

All research on animals reported in this manuscript was prospectively approved by the Institutional Animal Care and Use Committee of University of California Los Angeles and complied with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All data analysis procedures were performed at Stony Brook University, New York.

Experimental design

Twelve adult female Sprague-Dawley rats (200-250 g body weight) underwent electromyographic (EMG) recording and epidural stimulating electrode implantations and a complete first spinal cord transection (ST) at T8 as previously described (Shah *et al.*, 2012). Of these, eleven rats survived all surgical procedures and data herein are reported from eleven rats. Twenty-two days after the first ST at T8 (1ST), the rats underwent a second complete ST at either L2 (2ST-L2, n=5) or S1 (2ST-S1, n=5). One rat underwent a 2ST at L1. Surgical and housing conditions and post-operative care have been described in detail previously (Shah et al., 2016). The timeline for the experimental procedures is detailed in **Figure 1A**.

Spinal cord transection procedures

Spinal cord transections were performed as previously described (Shah *et al.*, 2012). For the first spinal transection within the T8 segment (1ST-T8), a dorsal mid-line skin incision was made from ~T6 to T10 vertebral level and the paravertebral muscles and fascia from ~T7 to T9 were reflected laterally to expose the vertebrae. A partial laminectomy was performed via removal of the spinous processes and a portion of the lateral bodies of the T8 and T9 vertebrae to expose the spinal cord. For the second transections within L2 (2ST-L2) and S1 segments (2ST-

S1), the mid-line skin incision was made from ~T10 to L6 vertebral level. Laminectomies involved removal of the spinous processes and a portion of the lateral bodies of the T12 and T13 vertebrae or L1 and L2 vertebrae to expose the L1/L2 and S1 spinal cord segments, respectively. For all spinal transections, the dura was picked up using fine forceps and micro-scissors were used to completely transect the spinal cord (including the entire extent of the dura) at each site. Two surgeons independently verified a complete spinal transection by gently passing a fine glass probe through the transection site and by lifting the cut ends of the spinal cord. Gel foam was inserted in the transection site to minimize bleeding and to separate (~2-3 mm) the cut ends of the spinal cord.

Electromyography (EMG) and epidural stimulation (ES) electrode implantation procedures

The EMG and ES implants were performed as previously described (Shah *et al.*, 2012). Chronic EMG electrodes were implanted into the tibialis anterior (TA) and medial gastrocnemius (MG) muscles bilaterally. For two rats, electrodes were also implanted into the vastus lateralis and semitendinosus muscle to obtain standing evoked response data (see results). All wires for the EMG and ES electrodes were connected to a common connector that was implanted on the skull.

Details of the location of the laminectomies and ES implant sites on the spinal cord are illustrated in **Figure 1B** (modified from Waibl, 1973). Briefly, a laminectomy at T12 vertebral body allowed accessing the L2 spinal segment and a partial laminectomy of the lower L1 and upper L2 vertebrae allowed access to the S1 segment (Waibl, 1973; Gelderd & Chopin, 1977; Harrison *et al.*, 2013). Wires were pulled from below the vertebral bodies, approximately 2 mm of the Teflon coating was removed over the wire to make an electrode, and the electrode then was secured over the spinal segment with 8-0 ethilon sutures. These surgeries are routinely

performed in our laboratory and in our experience the electrodes continue to be held in place for as long as three to four months. All EMG and ES wires were pulled subcutaneously to be secured on the skull as described previously (Roy *et al.*, 1991). Note that after the 1ST-T8, the spinal cord was epidurally stimulated at both segments. After the 2ST-L2 or 2ST-S1, the L2 or S1 wires at the transection site were removed to accommodate for the ST. As such, after the 2ST, only monopolar stimulation was used, where a single wire implanted epidurally over the spinal segment site served as the stimulating monopolar electrode. Accordingly, stimulation was delivered at L2 after 2ST-S1 and stimulation was delivered at S1 after 2ST-L2. In the rat with 2ST-L1 transection, the electrode was cut at the L2 spinal segment and spinal segment stimulated at S1. The reference electrodes for EMG and ES stimulation were placed under the skin at the level of inferior angle of the scapula.

Locomotor training procedures

After initial handling and training on the treadmill to step bipedally, rats underwent a two-week step-training period beginning 7 days after each transection surgery (**Figure 1A**). The rats were trained for 20 min/day, 3 days/week. The treadmill speed was gradually increased from 6 to 13.5 cm/s during each two-week period. An upper body harness system was used to position the rats over a treadmill belt and partially support their body weight during bipedal locomotion (Shah *et al.*, 2012). Bipolar ES at L2 and S1 (40 Hz, 500μs rectangular pulses) was delivered continuously during the training sessions after the first ST as previously described (Shah *et al.*, 2012), except that no pharmacological agents were administered in the present study during training. The training sessions after the second ST were divided into three 10-min sessions. The first session involved delivery of monopolar epidural stimulation ('ES' condition) at the same frequency and pulse duration as described above at the remaining site, e.g., L2 after transection

at S1. The second session ('Drugs' condition) involved administering two serotonergic agonists, i.e., quipazine (0.3 mg/kg of body weight, i.p.) and 8-hydroxy-dipropylamino-tetralin (8-OH-DPAT) (0.1 mg/kg of body weight, i.p.), as previously described (Courtine *et al.*, 2009). A 10-min period was allowed for the Drugs to take effect prior to the subsequent training session. The third session ('ES+Drugs' condition), after another 10-min break, involved testing the rats to step with the serotonergic agonists in the presence of ES.

Behavioral and EMG testing procedures

Kinematics and EMG data were collected from all rats (n=11) at day 3 prior to and then at 21 days after the 1ST-T8, and 21 days after the 2ST. Testing was performed with ES alone after the 1ST-T8, and with ES and the Drugs after the 2ST. Bipolar ES (40 Hz and 500μs duration rectangular pulses) between L2 and S1 was used during the training period after the 1ST-T8 due to its effectiveness in enabling locomotion in SCI rats (Lavrov *et al.*, 2008; Shah *et al.*, 2012). Locomotor ability, however, was tested using monopolar L2 or S1 stimulation so as to compare these data after a 2ST (because the electrode at L2 or S1 was surgically removed after the 2ST). After the 2ST, monopolar stimulation at the same frequency and pulse duration was used for both training and testing. The rats were tested with the above-mentioned ES, Drugs and ES+Drugs conditions 21 days after the 2ST. For all testing sessions, the optimal stimulation intensity used was that producing the best stepping ability as determined subjectively by the tester. This stimulation intensity, ranging from 1.0 to 3.5 V, was used during the kinematics and EMG data collection for all trials.

Kinematics data were collected using 3-D video recordings at 100 fps as described previously (Shah *et al.*, 2012; Shah *et al.*, 2013). The SIMI motion capture system (SIMI Reality

Motion Systems, Unterschleissheim, Germany) was used to obtain 3-D coordinates of limb markers attached bilaterally to bony landmarks (iliac crest, hip joint, knee joint, ankle joint, and fifth metatarsophalangeal joint) of the hindlimbs. EMG data were collected from eight to twelve consistent, consecutive step cycles bilaterally under conditions when bipedal stepping was possible, and for 60 continuous seconds when it was not (to ensure if stepping was indeed possible with the various conditions). The EMG signals were filtered (band passed, 30 Hz - 1 KHz) and amplified (x1000) using an analog amplifier (differential AC amplifier, AM-systems Inc., USA). The signal then was digitized at a 10 KHz sampling rate and stored on a computer using a data acquisition card (NI-DAQ; National Instruments Inc., US).

Kinematics analyses

The body of the rat was modeled as an interconnected chain of rigid segments. Joint trajectories were generated for both the swing and stance phases of the step cycles as previously described (Shah *et al.*, 2012). Six to twelve step cycles were taken when the rats were stepping consistently for each experimental condition. In the case when such continuous steps were not available, discrete steps were individually analyzed. A step was defined as complete if 1) the ankle joint was placed anterior to the hip joint at the beginning of the stance phase and as the foot went through mid-stance the ankle joint was placed posterior to the hip joint by the end of the stance phase, 2) the toe was lifted from the treadmill surface with concurrent hip and knee flexion to transition into the swing phase of the step cycle, and 3) the toe joint moved anterior to the knee joint during the swing phase. Partial steps were characterized by a reduction in the anterior-posterior excursions of the ankle and toe joints resulting in shorter stance durations and were not included in the calculation of step numbers. Rapid twitching or inconsistent non-rhythmic repetitive flexion-extension foot or leg movements were also not considered in the

quantification of complete steps. Instead, rats that exclusively exhibited these types of movements are described as non-stepping in the results. In addition to quantifying the number of partial and complete steps, qualitative observations of non-step related hip and knee movements were made.

Several kinematic gait parameters including the total number of complete steps, step cycle duration, step height (vertical ankle displacement) and stance length (horizontal toe displacement) were measured for each gait cycle. Complete steps were quantified as described above. Step cycle duration was measured from the onset of one swing phase in a step cycle to the onset of the next swing phase. Onset of swing was determined by the frame at which the foot initially ascended from the treadmill. Step height (vertical ankle displacement) was measured using the ankle joint marker: the ankle joint was chosen because it minimizes foot variability during swing without strongly impacting the magnitude of the vertical displacement during swing. Stance length (horizontal toe displacement) was measured using the toe joint since it best incorporates pre-swing drag of the foot in assessing total horizontal displacement during a stance phase.

Spectrum analysis of EMG Activity

Power spectra of EMG stepping was obtained to identify the unique spectral properties of each group and condition, to eventually decipher differences in EMG responses between stimulation and non-stimulation conditions. Fourier transforms were performed on ~10 steps of each limb for each group and condition. Activity synchronized with the application of ES was associated with spectral peaks at the 40 Hz stimulation frequency and at integer harmonics (i.e. 80 Hz, 120Hz, etc.). Power integrals across each of the ES harmonics were calculated between 0-

1000 Hz and normalized to the total power. Integral values were compared between 2ST-S1 and 2ST-L2 rats, when stimulation was delivered with and without administration of Drugs. Due to the similarities seen in the frequency spectrums between the TA and MG, spectral data from both muscles were combined to assess the integral power between conditions.

Evoked response analysis

Responses evoked in the TA and MG muscles by ES (40 Hz) were measured to report the functional state of the spinal cord. EMG bursts corresponding to best steps (each burst containing one evoked response window for every 25ms duration of the burst) were analyzed from each experimental condition as described previously (Lavrov *et al.*, 2006). Briefly, the middle response was considered as the result of a monosynaptic motor response from the ES that typically occurs 4-6ms after the ES pulse (Lavrov *et al.*, 2006). The full late response window, reflecting spinal polysynaptic activity that is predictive of the potential of the spinal circuitries to generate stepping (Lavrov *et al.*, 2006; Gad *et al.*, 2015), was set as 9-25ms, originating after the completion of the middle response and truncated by the onset of the subsequent ES pulse at 25ms. The 3ms gap between the middle and late response was included to insure no lingering components of the middle response were included in the late response due to the variabilities seen in the timing of the middle response. The number of unique peaks, was assumed to represent polysynaptic activity, during the late response waveform was determined using a custom MATLAB (Mathworks) script. Threshold for accepting a peak was set by an amplitude greater than 80% of the mean amplitude of the rectified burst during the LR. Note that an early response, reflecting direct stimulation of ventral roots, can be elicited only at higher stimulation intensities (Gerasimenko *et al.*, 2006; Lavrov *et al.*, 2006). The current stimulation protocol utilized stimulation intensities that were adequate to elicit the middle and late responses.

306 Since a more randomly generated late response waveform in an EMG burst is linked with
307 a more successful stepping pattern (Lavrov *et al.*, 2006; Gad *et al.*, 2015), we quantified this
308 variation using coefficient of variation (CV) values for the late response. This CV was calculated
309 at each point for each animal over the course of all late responses from each burst. Thus, greater
310 variations in the late response amplitude would suggest greater modulation of the spinal
311 interneuronal circuitry to generate stepping and would yield higher values of CV. The MG has a
312 longer activation period in a gait cycle and therefore the number of windows that were averaged
313 per muscle burst were greater in the MG EMG burst (about 5 windows of the late response
314 waveform per burst for TA and 30 for MG). At 10kHz sampling rate each late response contains
315 160 samples. The mean and standard deviations at each of the 160 points from the late responses
316 were calculated to determine the CV at each point. The CV's at all 160 points were then
317 averaged together for a single CV value per animal. The signals were rectified before calculation
318 such that the CV reflected only variations in the magnitude of muscle activation. reflected
319 variations in magnitude of muscle activation over time from onset of the ES pulse.
320 To verify if motoneuronal pools along the lumbosacral cord remained viable and were
321 responsive to stimulation, in two rats from each group, spinally evoked motor response data
322 were obtained from four hindlimb muscles including the vastus lateralis, semitendinosus, tibialis
323 anterior and gastrocnemius (0.3Hz, ~10 responses, 0.5ms pulse, 1-10V intensity). Data
324 from 4V stimulation voltages are presented here.
325
326 Statistical analyses
327 Datasets in this experiment underwent the Shapiro-Wilk test for normality. Statistical
328 comparisons were performed to determine the effects of ES, Drugs, and ES+Drugs only between
329 the two groups, i.e., 2ST-S1 and 2ST-L2 using a standard two-way multiple comparisons

ANOVA. Paired t-tests were run between the 2ST rats with the ES condition and their corresponding 1ST ES data. Control pre-injury data served to provide reference values for relevant outcomes i.e., number of steps, cycle duration, and step height and length. In all cases significance was set at $p < 0.05$ and a Bonferroni correction was performed to account for multiple comparisons. Of these comparisons, the resulting plots and graphs highlight the comparisons where the main variable is different at transection locations (2ST-L2 vs. 2ST-S1), which was our main outcome of interest. All statistical tests were performed using SPSS (IBM). Given the complexity of experiments involving two spinal transections, we analyzed each hindlimb separately in each rat. As such, each hindlimb per rat was treated as an independent unit. Statistical data are therefore from 6-10 limbs per group, depending upon whether the limb stepped or not and hence upon the availability of step data for the specific outcome.

Results

Bipedal stepping ability is retained after a spinal transection at S1 in rats previously spinalized at the T8 spinal segment.

Figure 2A shows kinematics of step movements from a representative non-injured rat (**Figure 2A: i-iv**). Three weeks after the 1ST-T8, L2 monopolar stimulation successfully enabled all rats (n=5, rats 1-5) to execute at least six continuous steps on the treadmill with at least one hindlimb (**Figure 2B, C;** representative rats 1 and 2 are shown). This level of stepping performance after 3 weeks of spinalization, with periods of co-activation between the extensor (MG) and flexor (TA) muscles, was expected with a monopolar stimulation configuration (Ichiyama *et al.*, 2005; Shah *et al.*, 2016).

After a 2ST-S1 in the same rats (n=5 rats), stepping was retained in all rats in the presence of stimulation (**Figure 2D,E;** two representative rats shown). Note that because the S1 segment was transected, stimulation in these rats was only possible and delivered at L2. All rats stepped continuously with complete steps. In most instances, the stepping was also bilaterally rhythmic. Interestingly, step heights, toe trajectories, and cycle durations were more consistent after the 2ST-S1 compared to after the 1ST-T8 (**Figure 2E: ii-iii**), The raw EMG signals showed distinctive reciprocal EMG bursts between the MG and TA muscles after than before the 2ST-S1 (**Figure 2D, E: iv**). This is most likely due to the longer time allowed for the lumbosacral networks to adapt to stepping behavior after the initial traumatic insult to the cord.

To assess the effects of activating the entire lumbosacral cord (versus predominantly the proximal lumbar segments alone as was done with the epidural stimulation alone) in facilitating stepping in the double spinalized rats, the serotonergic agonists quipazine and 8OH-DPAT were

administered (Barbeau & Rossignol, 1991; Ichiyama *et al.*, 2008). Four of the 5 rats retained bilateral stepping and consistency, taking 8+ consecutive steps with distinct stance and swing phases (**Figure 2F-H: i-iii,** representative rats, 1, 2 and 3 shown). Overall, the stepping patterns with Drugs more closely resembled stepping observed in non-injured rats. Raw EMG showed a clear antagonistic relationship between the TA and MG muscles in four out of the five animals tested in this group (**Figure 2F-H: iv**). The one rat that did not show this relationship generated only a few continuous complete steps and the majority of these steps exhibited strong ankle dorsiflexion associated with relatively brief bursts of MG activity and longer bouts of TA activity (**Figure 2I,** rat 4). Mean statistical data of multiple steps from the ten hindlimbs (all five rats), that quantifies systematically the number of steps, step cycle durations, step height and step length data are presented in Figure 4C and contrasted to similar outcomes after 2ST-L2.

Bipedal stepping ability is abolished after a spinal transection at L2 in rats previously spinalized at the T8 spinal segment.

Three weeks after 1ST-T8, stepping enabled by monopolar stimulation applied at S1 in five rats (rats 6-10) was similar to the stepping ability of rats 1-5 that were stimulated at L2 alone (compare **Figure 3A-B versus Figure 2B-C**, two representative rats shown). Such kinematics and EMG stepping patterns with S1 monopolar stimulation have been reported (Ichiyama *et al.*, 2005; Shah *et al.*, 2016).

After 2ST-L2 in the same rats, stimulation did not enable any complete steps in any of the 5 rats (**Figure 3C, D;** representative rats 6 and 7 are shown). Note that because the L2 segment was transected, stimulation in these rats was only possible and delivered at S1. Raw EMG signals from the TA and MG were saturated with unmodulated tonic activity induced by the ES, indicating strong, direct activation of both muscles without any distinct patterning of

activity that is typical of the activation of interneuronal/motoneuronal locomotor spinal circuitries (**Figure 3C: iv**). Similar observations were made from all rats in this group. In the best-case scenario, one of the 5 rats exhibited occasional repetitive dorsiflexion in one foot (**Figure 3D;** rat 7). Although these movements were correlated with distinct bursts of EMG in the TA, the ankle never elevated from the treadmill surface as would be required for normal plantar stepping.

After administration of Drugs, some minimal limb movement, particularly at the hip and knee, was observed in 3/5 rats in this group (**Figure 3E-G;** rats 6, 7, and 8). These joint movements, observed by watching videos, were arrhythmic, lacked any bipedal coordination, and involved sporadic and non-synchronous flexion-extension movements at the hip, knee, and/or ankle joints of one or both limbs. However, none of these rats were able to execute complete steps consistently. In the best-case scenario, rats occasionally elicited around 1-2 continuous or discrete complete steps per limb throughout the testing trial. Hindlimb movements and the accompanying muscle activity are shown in **Figure 3E-G**. One rat occasionally produced partial step movement of one hindlimb with toe-curling as the foot mainly dragged on the moving treadmill belt (**Figure 3E, rat 6**). These partial step movements were accompanied by moderate and rhythmic EMG bursts in the TA with some co-contraction of the MG, albeit at lower amplitudes. Two rats (**Figure 3F-G, rats 7 and 8**) produced bilateral non-alternating flexion-extension jump-like movements in the ankle and feet. This behavior is commonly observed in spinal rats after quipazine administration even in the absence of afferent feedback from the treadmill (Ichiyama et al. 2008). Importantly, the visible hindlimb joint movements did not display consistent or rhythmic swing and stance step cycle phases as normally occurs during treadmill walking. Although some patterned bursting was observed, there were co-contractions

of the MG and TA in all 3 rats (**Figure 3E-G: iv,** vertical red hashes). Lastly, rats 9 and 10 did not produce any movement either with ES or the Drugs (**Figure 3H;** rat 9 shown).

Stepping kinematics after the second spinal transection at S1 or L2:

Data from all rats **(Figure 4)** confirmed our observations on the stepping behavior demonstrated in Figures 2 and 3. In contrast to 2ST-S1, stepping in all rats after a 2ST-L2 was heavily compromised irrespective of the technique used in facilitating the lumbosacral circuitry to step. For the rats that showed some step-like movements (rats 6, 7, and 8), the number of complete steps in a trial averaged about 2 steps per hindlimb per trial. When these steps were used in the analysis of step kinematics, all mean measures of step kinematics confirmed that stepping quality was significantly compromised both in the timing (cycle duration) and the components of step trajectory length (step and stance lengths) compared to the corresponding stimulation condition after the 1ST. Additionally, all outcome measures were significantly lower in all stimulation and drug conditions when compared to 2ST-S1 rats **(Figure 4A-D** and **Table 1)**.

EMG Spectral analysis

The power spectra in **Figure 5A** show the unique spectral properties of each group (2ST-S1, 2ST-L2), condition (stimulation and/or Drug), and muscle. The power spectra analysis displays the distribution of the evoked response signal's energy capability in the frequency spectrum. Within each muscle-group plot it was observed that the ES power spectra had spectral peaks at each 40 Hz harmonic that decreased in amplitude as a function of frequency. Spectral peaks at ES harmonics are a result of the sum of spectral characteristics of action potentials of motor units (Okkesim *et al*., 2012) taking part during walking secondary to the stimulation and can be

associated with the direct application of ES. The Drugs spectra, in contrast, had no obvious spectral peaks, reflecting a frequency-independent effect of the Drugs. Within both groups, the interaction between Drugs and ES (ES+Drugs) resulted in reduced ES harmonic power relative to the ES condition, indicating a modulatory effect of the drug on ES facilitated muscle activity. Between groups, both muscles showed significantly larger spectral power in conditions with ES. ES harmonic power was significantly higher in 2ST-L2 than 2ST-S1, both before and after the addition of the Drugs (Figure 5B). This corresponded to the proportionally higher burst amplitudes seen in 2ST-L2. These data suggest that the energy resulting from the sum of spectral characteristics of action potentials of motor units was higher during L2 stimulation.

Spectral data for the Drugs condition were not different across groups and muscles. Hashed black line in Figure 5B refers to the Drugs-only power integrals using the same regions of integration that were used in the conditions where harmonics were present (ES, ES+Drugs). Note that the ES harmonic power for both ES and ES+Drugs conditions was larger than the power in the Drugs condition in the same region.

ES evoked waveforms as a window into the functional state of the spinal cord

After 1ST-T8, and with stimulation at either L2 or S1, all rats showed a step-phase dependent modulation that strongly inhibits the middle response (MR) during specific cycle periods, in the MG during the swing phase and the TA during the stance phase (**Figure 6A,B i, ii,;** one representative burst shown), as previously described for non-injured (Gerasimenko *et al.*, 2006) and spinal rats (Gad *et al.*, 2015). Such modulation of the MR wave has been linked to the ability of the spinal locomotor circuitry to effectively respond to feedback from the epidural stimulation. Physiologically, a balance is likely established in the excitatory and inhibitory networks between

472 the flexors and extensors to optimize stepping ability and coordination secondary to the
473 stimulation (Gad *et al*., 2015).
474 A classic late response waveform with multiple peaks was also observed in all rats during the
475 corresponding step phase from both the muscles (**Figure 6A, B; iii**). This modulation of the MR
476 along with the presence of the LR was present in all rats subsequently spinalized at S1 (**Figure 6**
477 **C**), but not in those spinalized at L2 (**Figure 6D**). This LR consisted of randomly generated
478 action potentials within the bursts, indicating engagement of polysynaptic spinal circuitry during
479 locomotion. These data are suggestive of an association between the ability to take steps (Figure
480 4) and the production of evoked waveforms from stimulation at L2.
481 The middle response in the 2ST-L2 group stayed elevated throughout the step cycle, and
482 the late response barely consisted of one or two peaks. In the presence of Drugs, the middle
483 response modulation and late response waveform with peaks continued to prevail in the 2ST-S1
484 rats (**Figure 6E**). After 2ST-L2 however, in trials where some steps were observed with the
485 drugs, there was some inhibition of the middle response in the TA and MG muscles, but the
486 signal was not completely suppressed during the step cycle, indicating co-contraction of muscles
487 during stepping (**Figure 6F**).
488 The number of peaks in the late response waveform were also significantly less after
489 2ST-L2 than before the injury or even when compared with 2ST-S1 **(Figure 7A).** The coefficient
490 of variation was used to capture the variation in the peak amplitudes during the late response that
491 was linked to better stepping. Larger coefficient of variation values reflect the temporal variation
492 at which the peaks occur in reference to the ES pulse, indicating their non-time-locked nature.
493 These values were lower in rats after 2ST-L2 in comparison to before the injury for the TA
494 muscle; and significantly lower than in rats with a 2ST-S1 (**Figure 7B**). These values were low

in the MG muscle too, but did not reach statistical significance, most likely due to the minimal number of EMG bursts that were actually available in the 2ST-L2 rats to detect the variation.

The motor evoked responses (waveforms, latency and amplitudes) from the vastus lateralis, semitendinosus, tibialis anterior and medial gastrocnemius muscles were identical after 2ST-S1 and 2ST-L2 (**Figure 8**, shown are raw data from one representative rat per group). Since elicitation of spinal evoked responses is mediated via stimulation of the low threshold dorsal root afferents (Gerasimenko *et al*., 2006; Lavrov *et al.,* 2008), these responses would not be obtainable if the nerve roots were accidentally cut during the transection surgery. As such, presence of evoked response data from multiple muscles by ES of the spinal cord verified the functional viability of the dorsal and ventral roots in the rostral lumbar segments after 2ST-L2.

Lastly, in one rat in whom the lumbar cord was transected at the L1 spinal segment (and not L2), the kinematics of stepping, EMG activity and characteristics of evoked responses during stepping were similar to before the spinalization (compare left and right panels in **Figure 9A-D**). The hindlimbs executed consecutive steps to result in full steps with adequate step height and lengths similar to what was seen prior to the 2ST-L1 **(Figure 9A and 9E).** The corresponding stepping EMG data were also similar before and after 2ST-L1, showing rhythmic high amplitude bursting from the agonist and antagonist muscles (Figure 9B). Evoked monosynaptic and polysynaptic responses specific to the step phase cycles waveforms in the TA and MG muscles also were comparable **(Figure 9C-D).** The number of late responses in the late response waveform were also similar **(Figure 9E).** These data indicate differences between outcomes obtained with a 2ST-L2 versus a 2ST-L1, and support our findings from this work that spinal neural networks at L2 play a predominant role in eliciting a stepping response.

Discussion

Our main finding here is that adult rats with a spinal transection at T8 were able to perform well-coordinated locomotion during ES at L2 even after a 2ST-S1. In contrast, S1 stimulation did not induce locomotion after a 2ST-L2 (**Figure 10**). We conclude that 1) rostral segments are essential and critical for regulation of locomotor behavior and 2) the more caudal spinal networks alone cannot initiate or generate effective locomotion. However, they alone can initiate and control locomotor-related networks when the rostrally localized networks are intact.

Rhythm-generating hindlimb locomotor networks in the lumbosacral spinal cord

In the original study performed by Iwahara and colleagues (Iwahara *et al.*, 1992), locomotion in decerebrated and spinal cats was induced effectively when epidural stimulation was applied to cervical or lumbosacral pre-enlargements. It has been suggested that the pre-enlargement spinal segments integrate descending information from motor pathways controlling stepping movements (Iwahara *et al.*, 1992). Further studies have shown that in the cat, the rostral segments of the lumbar cord are critical for the initiation of stepping movements (Marcoux & Rossignol, 2000; Langlet *et al.*, 2005) and for inducing rhythmic activity (Deliagina *et al.*, 1983). Experiments with spinal cord transection and with pharmacological blocking of

543 restricted spinal lumbar segments have demonstrated that the integrity of the L3–L4 segments in
544 cats is necessary to sustain the locomotor activity, and that the stepping movements in the cat
545 hindlimbs could not be generated after a spinal cord transection below the L4 spinal segment
546 (Marcoux & Rossignol, 2000).

547 At the same time there are experimental data showing that intraspinal microstimulation at
548 L7-S1 or ES at S1 can elicit stepping movements in the hindlimbs in spinal cats (Guevremont *et*
549 *al.*, 2006) and in spinal adult rats (review in (Shah & Lavrov, 2017), although it is unclear
550 whether these movements were the result of direct activation of caudal networks or mediated by
551 indirect activation of rostral networks. Recently, we demonstrated that initiating ES at L2 prior to
552 S1 almost always brought about consistent stepping in spinal rats in comparison to initiating the
553 stimulation at S1 spinal segment, suggesting that the rostral lumbar segments are the prime
554 controllers of rhythm generation (Shah *et al.*, 2016).

555 In the present study we clearly demonstrate that ES at a caudal segment (S1) is not
556 effective for inducing locomotor activity in spinal rats without rostral (L2) spinal segments.
557 These observations suggest that the rostral segments of lumbar spinal cord are critical for
558 generating rhythmic locomotor patterns and that ES of the caudal segments can generate well-
559 coordinated, rhythmic stepping when L2 is intact. This issue is supported by the results of
560 examination of one rat in whom locomotion was possible to induce with S1 stimulation when the
561 spinal cord was transected at L1, instead of at L2 (**Figure 9**). Collectively, these data indicate
562 that the CPG driving hindlimb locomotion is centered at L2. This conclusion is consistent with
563 Cazalets et al. (1995) showing that locomotor network in newborn rats is restricted to a specific
564 part of the spinal cord (L1-L2), but is not distributed segmentally (Cazalets *et al.*, 1995).

565 Note that after the second transection at L2 or S1, the cord was stimulated at the intact
566 spinal segment and not at L3-L4. As such, the differences in stimulation site with different

conditions might have confounded our interpretation of the data (questioning for example, if the observed stepping possible with S1 stimulation is due to an intact S1 or the intactness of other spinal segments). Lastly, since the stepping quality was better after a S1 and T8 transection compared to a transection at T8 alone (**Figure 2B-E**), it is possible that the pattern generation circuits in the sacral spinal cord, such as those for swimming (Katz, 2016), conflicted with the hindlimb locomotion circuits. This could also help explain why an injury at L2 stopped locomotion, since the CPG networks for swimming located in the sacral region would have a conflicting influence on the CPG for hindlimb locomotion.

To examine the functional state of main locomotor hindlimb muscles after second spinal transection at L2 or S1 we have tested the motor evoked potentials in response to stimulation of S1 and of L2 spinal segments, correspondingly. We observed that after second spinal transection at L2, motor evoked potentials secondary to S1 stimulation were present and in fact similar to those seen with L2 stimulation both for the rostrally (vastus lateralis, tibialis anterior) and caudally situated motoneuronal pools (semitendinosus, and medial gastrocnemius) for the hindlimb muscles, but no stepping occurred with S1 stimulation at 30 Hz. In fact, lack of or moderate modulation of the evoked responses with the 40Hz S1 stimulation further suggested that crucial locomotion mediated neuronal pool activity was compromised after the L2 segment transection. The extent of polysynaptic activity was also heavily compromised with the L2 transection, reflecting lack of interneuronal coordination required to generate spinal stepping (Lavrov *et al.*, 2006). At the same time the motor evoked potentials, their modulation as well as locomotion was unchanged with L2 stimulation after second spinal transection at S1. These data lead us to conclude that the degradation of locomotion during S1 stimulation after a spinal transection at L2 is related to elimination of part of the neuronal locomotor network with trigger properties rather than directly destroying the locomotor motoneuronal pools themselves. Lastly,

591 tonic activity with S1 stimulation was observed not only in muscles that receive their innervation
592 from motoneurons located close to L2, but also in muscles whose motoneurons are not close to
593 the injury site, such as the medial gastrocnemius and semitendinosus muscles. These
594 observations indicate that the tonic activity does not necessarily reflect muscle spasms secondary
595 to the injury at L2, but instead a non-uniform excitation of motoneurons with S1 stimulation.
596 Lev-Tov and colleagues described ascending propriospinal circuits in caudal lumbar and
597 sacrococcygeal networks which project into and have an excitatory effect on rostral lumbar
598 locomotor networks (Lev-Tov *et al.*, 2000; Gabbay *et al.*, 2002; Cherniak *et al.*, 2017). These
599 combined results are consistent with our hypothesis that ES enables the sacral networks to
600 project locomotor-related sensory input to the more rostral networks (Etlin *et al.*, 2010; Lev-Tov
601 *et al.*, 2010; Cherniak *et al.*, 2014). How much of the caudally derived sensory input to the spinal
602 networks is processed by the more caudal networks vs. by the more rostral networks cannot be
603 determined by the present data. Based on these data and the results using unique spatiotemporal
604 epidural stimulation parameters of the lumbar (L2) and sacral spinal segments (S1) (Shah *et al.*,
605 2016), we suggest that rostral lumbar networks play a primary role in controlling locomotion
606 whereas the more caudal networks re-enforces the locomotor rhythmicity derived primarily from
607 the more rostral networks.
608
609 ***Pharmacological modulation of neuronal locomotor related networks***
610 The discrepancy in rhythmogenic capacity of rostral and caudal segments of the lumbar
611 spinal cord might be explained by different types and/or concentrations of transmitter agonists
612 and/or receptors located in those segments (Kiehn, 2006). Selective application of 5-HT to upper
613 lumbar cord segments induced rhythmic activity, whereas application of 5-HT to lower lumbar
614 cord segments failed to generate any hindlimb locomotor rhythm, and instead produced tonic

activity only (Cazalets *et al.*, 1995; Cowley & Schmidt, 1997). There is growing evidence that a functional segregation of 5-HT receptor subtypes exists in the spinal cord, and recently clusters of specific subtypes have been identified. Using synthesized receptor antibodies, dense populations of cells that are positive for the 5-HT_7 receptor subtype have been localized in the low thoracic-high lumbar spinal cord (Hochman, 2001), (Jordan & Schmidt, 2002). Thus, 5-HT_7 appears to have an active role in the region of the spinal cord suggested to be responsible for the generation of rhythmic stepping (Cazalets *et al.*, 1995). In a double-labeling experiment using the 5-HT_7 receptor antibody and the locomotor activity-dependent label sulforhodamine, the concentration of 5-HT_7-positive cells during fictive locomotion was significantly higher in the upper (rostral) lumbar spinal cord than in the lower (caudal) segments (Hochman, 2001).

In *in-vitro* experiments with neonatal rat preparations, Jordan and colleagues (Liu & Jordan, 2005) showed that blocking of the 5- HT_7 receptors in the rostral segments of the lumbar cord by clozapine decreased step cycle duration, whereas blocking of the 5-HT_2 receptors in the caudal segments of the lumbar cord by ketanserin did not influence step cycle duration. Also, it was concluded that the receptor subtypes 5-HT_{1A} and 5-$HT_{7,1}$ located in rostral lumbar segments are associated with an "induction" of locomotion, whereas 5-$HT_{2A/2C}$ receptors located in caudal lumbar segments are associated with "promotion" of locomotion (Jordan & Schmidt, 2002; Landry & Guertin, 2004; Landry *et al.*, 2006). The greater hindlimb locomotor rhythmicity intrinsic to the more rostral lumbar segments also seems consistent with data showing that these rostral segments contain interneurons controlling the hip muscles that largely define the kinematics of the hip joint during locomotion (Kiehn, 2006). Collectively, these data are consistent with the present results showing that elimination of the rostral lumbar segments by a complete spinal cord transection at L2 highly compromises the generation of a locomotor rhythm even when serotonergic agonists were administered after the L2 transection. In fact, the presence

of modest number of steps with the drugs was most likely due to the modest activation of neuronal circuits at the more caudal (L3 and downwards) lumbar segments.

Rhythmicity as a feed-forward mechanism regulation of hindlimb locomotion

Based on the examples noted above, the rhythmicity of locomotor patterns appears to follow a feed-forward mechanism, i.e., the rhythmicity is intrinsic to CPGs placed in the more rostral lumbar spinal cord segments, which drives hindlimb locomotion in a feedforward manner not needing sensory feedback (Gerasimenko *et al.*, 2017). The evidence that the fine details of the rhythmicity can be defined by the dynamics of the ensembles of its sensory input to all lumbosacral segments during locomotion (stepping forward, backward, sideways), however, is equally compelling (Courtine *et al.*, 2009; Shah *et al.*, 2012). On one hand, it seems obvious from numerous studies that the control of rhythmicity during locomotion is derived from a combination of the rhythmicity that is intrinsic particularly to the more rostral segments. On the other hand, it is equally obvious that the extrinsic sources of input generated by massively divergent proprioceptive and cutaneous sensors projecting to the spinal networks play a key role in defining a critical level of integrated bilateral hindlimb rhythmicity among multiple joints, which could not be effectively controlled by a single rhythmic network. We argue that this proprioceptive control of locomotion is derived from peripheral afferents projecting to the more sacral networks. The functional significance of these rostrally projecting, highly orchestrated and intricately timed locomotor generated signals that form the ongoing dynamic ensemble inputs, however, becomes greatly amplified within the more rostral networks. Perhaps, it is here where the analogy of the "final common pathway", i.e., those interneurons that project, to one or more motor pool at any given time fits with a comparable concept of "final common networks"

projecting to more rostral spinal and multiple supraspinal networks that could provide continuous updates of earlier locomotor patterns **(Figure 10)**.

Concluding remarks

A key question persists: what are the features of these rostral spinal networks that make them so critical? It cannot be their unique ability to generate a locomotor rhythm because it is clear that the eventual rhythm pattern is determined by the specific movements being generated. During forward, backward, or sideward spinal stepping or at any given speed, periodicity is controlled by the dynamics of sensory ensembles rather than the uniqueness of the rhythmicity intrinsic to the central pattern generators. We propose that the more rostral segments for locomotion is a reflection of the intrinsic properties of these networks that enables central pattern generation itself, i.e., its feed-forwardness. It is this feed-forwardness, not its rhythmicity per se, that provides the features necessary to process massive amounts of locomotor-linked data in real time to drive, with temporal precision, all of the necessary motor pools to generate load bearing stepping. The kinetics and kinematics of locomotion seems to be dictated by the dynamics of the sensory signatures generated by stepping in the chronic spinal rat. This sensory input provides a mechanism for synergizing the intrinsic mechanisms of rhythmicity within the more rostral segments with the peripherally derived sensory input. The present data derived from adult rats, without supraspinal control, under *in vivo* conditions with and without pharmacological modulation and free of any anesthetics, seems remarkably consistent with the above system-level concepts.

Abbreviations used:

ES : epidural stimulation

1ST-T8 : first spinal transection at the T8 spinal segment

2ST-S1 : second spinal transection at the S1 spinal segment

2ST-L2 : second spinal transection at the L2 spinal segment

2ST-L1 : second spinal transection at the L1 spinal segment

MG : Medial Gastrocnemius

TA : Tibialis Anterior

8-OH-DPAT : 8-hydroxy-dipropylamino-tetralin

CPG : central pattern generator

CV : coefficient of variation

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Table I : Summary of statistical results

Outcome	Group 1	Group 2	t	p
Figure 4				
Number of steps	1ST (ES at L2)	2ST-S1	1.416	0.1758
	1ST (ES at S1)	2ST-L2	8.218	0.0000
	2ST-S1, ES	2ST-L2, ES	3.573	0.0034
	2ST-S1, Drugs	2ST-L2, Drugs	3.001	0.0102
	2ST-S1, ES+Drugs	2ST-L2, ES+Drugs	8.603	0.00001
Cycle Duration	1ST (ES at L2)	2ST-S1	2.692	0.016
	1ST (ES at S1)	2ST-L2	13.75	0.0001
	2ST-S1 ES	2ST-L2 ES	5.837	0.0001
	2ST-S1 Drugs	2ST-L2 Drugs	4.853	0.007
	2ST-S1 ES+Drugs	2ST-L2 ES+Drugs	2.577	0.0242
Step height	1ST (ES at L2)	2ST-S1	0.524	0.6064
	1ST (ES at S1)	2ST-L2	6.936	0.0001
	2ST-S1 ES	2ST-L2 ES	5.717	0.0001
	2ST-S1 Drugs	2ST-L2 Drugs	2.249	0.0483
	2ST-S1 ES+Drugs	2ST-L2 ES+Drugs	4.387	0.0009
Stance length	1ST (ES at L2)	2ST-S1	1.017	0.3229
	1ST (ES at S1)	2ST-L2	8.047	0.0001
	2ST-S1 ES	2ST-L2 ES	3.112	0.009
	2ST-S1 Drugs	2ST-L2 Drugs	4.119	0.0021
	2ST-S1 ES+Drugs	2ST-L2 ES+Drugs	2.677	0.0202
Figure 5				
40 Hz Power	2ST-S1 ES	2ST-L2 ES	4.571	0.0006
	2ST-S1 ES+Drugs	2ST-L2 ES+Drugs	3.302	0.0068
Figure 7				
CV (TA)	1ST ES at S1	2ST-L2 ES	14.260	0.0001
	2ST-S1 ES	2ST-L2 ES	14.260	0.0014
CV (MG)	1ST ES at S1	2ST-L2 ES	4.128	0.069
	2ST-S1 ES	2ST-L2 ES	1.940	0.0763
Peaks	1ST ES at S1	2ST-L2 ES	2.662	0.0203
	2ST-S1 ES	2ST-L2 ES	3.159	0.0082
	1ST ES at S1	2ST-L2 ES	3.804	0.0025
	2ST-S1 ES	2ST-L2 ES	2.683	0.0199
Figure 9				
Cycle Duration	1ST (ES at L2)	2ST-L1	2.324	0.015
Step height	1ST (ES at L2)	2ST-S1	2.645	0.027

915

Figure and Table Legends:

Figure 1: Experimental design and timeline. (A) After initial handling and pre-training to bipedally step, adult rats (n=11) underwent chronic bilateral EMG recording electrode implantation in the medial gastrocnemius and tibialis anterior muscles. Pre-injury testing (EMG recordings from the implanted hindlimb muscles and 3-D video kinematics) for bipedal hindlimb stepping on the treadmill was followed by a 1ST-T8 and epidural electrode implantation over the rostral lumbar (L2) and rostral sacral (S1) spinal segments. After one week of recovery, the rats were step-trained bipedally on a treadmill for two weeks. Bipedal stepping ability was re-evaluated at 21d in the presence of ES at L2 and S1. Rats then underwent a 2ST-S1 or 2ST-L2 (n=5/group) or 2ST-L1 (n=1). After two weeks of additional bipedal step training, stepping ability was tested with ES (either at L2 or S1) with and without the administration of Drugs. **(B)** Schematic representation of the spinal cord and vertebral levels displaying the sites of the 1ST-T8 (thick black vertical line), the epidural electrode implants at L2 and S1 (black filled rectangles), and the sites of 2ST-L2 (red vertical line), 2ST-S1 (blue vertical line) or 2ST-L1 (green vertical line).

1ST-T8 : first spinal transection at T8; 2ST-S1: second spinal transection at S1; 2ST-L2 : second spinal transection at L2; 2ST-L1 : second spinal transection at L1; ES : epidural stimulation.

Figure 2: Stepping kinematics and EMG features before and after a second transection of the spinal cord at spinal segment S1. Data are during bipedal stepping from **(A)** a representative non-injured rat; **(B,C)** two rats after a 1ST-T8 enabled to step with ES at L2; (**D,E**) the same rats after a 2ST-S1 enabled to step with ES; **(F-I)** four rats after a 2ST-S1 enabled to step with the Drugs. Shown are representative: **(i)** stick figures during the swing and stance phases of a step cycle (red marks the foot distance from metatarsophalangeal joint to tip of

the toe), **(ii)** vertical ankle displacements (step heights) during consecutive steps, **(iii)** toe trajectories of consecutive steps, and **(iv)** 3s of band pass filtered EMG signals from the tibialis anterior (TA) and medial gastrocnemius (MG) muscles during bipedal treadmill stepping. Note in **B, C** that rats are able to step with ES after the 1ST-T8 (although uncoordinated). After the 2ST-S1, stimulation continues to facilitate stepping as shown in **D, E**. In the presence of Drugs (**F-I)**, stepping persists in all rats in this group (four rats shown). In all cases, the EMG patterns illustrate antagonist activity between the TA and MG muscles during stepping. In the schematic representation of the spinal cord on the far right, the red circle represents delivery of stimulation at L2 during testing and the horizontal blue line a 2ST-S1 in rats spinally transected at T8. 1ST-T8 : first spinal transection at T8; 2ST-S1: second spinal transection at S1; ES : epidural stimulation.

Figure 3: Stepping kinematics and EMG features before and after a second transection of the spinal cord at spinal segment L2. Data are during (attempted) bipedal stepping from **(A, B)** two rats with ES at S1 after 1ST-T8; **(C, D)** the same rats after a 2ST-L2 enabled to step with ES; and **(E-H)** four rats after a 2ST-L2 enabled to step with Drugs. Shown are representative: **(i)** stick figures during the swing and stance phases of a step cycle (red marks the foot distance from metatarsophalangeal joint to tip of the toe), **(ii)** vertical ankle displacements (step heights) during consecutive steps, **(iii)** toe trajectories of consecutive steps, and **(iv)** 3s of band pass filtered EMG signals from the tibialis anterior (TA.) and medial gastrocnemius (MG) muscles during bipedal treadmill stepping. Note in A and B that stepping is facilitated with stimulation at S1 after the 1ST-T8; but is almost completely abolished after the 2ST-L2 despite the stimulation (**C, D**). EMG activity is seen in the form of non-bursting tonic activity. In the presence of Drugs, few bilateral non-rhythmic flexion-extension movements of the hip, knee and/or ankle joint are

observed in three rats, with corresponding co-contraction observed between the TA and MG muscles (brown vertical lines in **E-G, iv**). In two rats, the Drugs did not elicit any joint movements and no EMG activity was detected (**H,** only one rat shown). The blue circles in the schematic representation of the spinal cord on the far right represents delivery of stimulation at S1 and the horizontal blue line a 2ST-L2 in rats spinally transected at T8. 1ST-T8 : first spinal transection at T8; 2ST-L2 : second spinal transection at L2; ES : epidural stimulation.

Figure 4: Average stepping kinematics after the second spinal cord transection. Mean (±SE) **(A)** number of steps**, (B)** step cycle duration, **(C)** step height, and **(D)** stance length from both limbs in rats (n=total of 6-20 limbs per group, depending on the rat group) after 1ST-T8 and 2ST-T8. 1ST-T8 data are in the presence of ES and 2ST data are under ES, Drugs, and ES+Drugs conditions. Note in A that the number of complete plantar steps significantly decreased after the 2ST-L2 in comparison to after the 2ST-S1. The individual or combined effects of ES and Drugs in the 2ST-L2 rats enabled a mean of ~2 complete steps per limb throughout a trial. These steps taken for kinematics analysis in **B-D** revealed significantly shorter cycle durations and joint movement trajectory measures (step height and length) in comparison to step kinematics obtained in rats with a 2ST-S1. The horizontal purple dotted line represents normative pre-injury data for each outcome. ***,** significant difference between groups at $p < 0.05$. 1ST-T8 : first spinal transection at T8; 2ST-S1: second spinal transection at S1; 2ST-L2 : second spinal transection at L2; ES : epidural stimulation.

Figure 5: Frequency spectral analysis after the second spinal transection. (**A)** Mean (±SE) power spectrum in the frequency domain of the tibialis anterior (TA) and medial gastrocnemius

(MG) muscles in response to ES), Drugs and ES+Drugs conditions after 2ST-L2 and 2ST-S1. For each muscle, in both injury types, 40Hz harmonics are observed with the ES condition, but not with the Drugs. The amplitude of harmonics is mid-way in the ES+Drugs condition. **(B)** Mean percentage (±SE) of the integral of power associated with harmonics in the presence of ES and Drugs. Black hashed horizontal line represents expected power in a given region of integration without ES modulated excitation (only Drugs condition). *, significant differences between groups at $p < 0.05$. 2ST-S1 : second spinal transection at S1; 2ST-L2 : second spinal transection at L2; ES : epidural stimulation.

Figure 6: Characteristics of spinal evoked responses during stepping before and after the second spinal transection. The evoked responses generated for each stimulation pulse in the tibialis anterior (TA) and medial gastrocnemius (MG) EMG bursts from two representative rats before **(A, B)** and after a 2ST-S1 **(C, E)** or 2ST-L2 **(D, F)** with and without the Drugs. Shown for each condition are: **(i)** raw EMG signals (1.5s) from the antagonistic muscles during treadmill stepping in the presence of 40 Hz ES; **(ii)** the 1.5s signal from **(i)** is depicted as a stack of sixty consecutive 25ms responses (that correspond to each stimulation pulse) from top to bottom chronologically, showing the middle response (MR) and late responses (LR); **(iii)** the average waveform during the late response window of the evoked responses obtained in **(ii)**. The red horizontal hash lines in **(iii)** represent the amplitude threshold for peak acceptance, the midpoint between the hashed lines indicates baseline amplitude of 0mV, and the blue circles represent peaks that were accepted and included as late responses (see *Methods* for threshold criteria for peak acceptance). Note that in the presence of ES and the Drugs conditions, there is the presence of several polysynaptic peaks during the LR along with marked retention in their variability in both muscles after a 2ST-S1, but not after 2ST-L2**.** The evoked responses are heavily time

locked to the stimulation between 5-8ms for the MG and 7-9ms for the TA after a 2ST-L2, especially in the ES condition (**D),** implying a lack of polysynaptic activity and hence lack of modulation of circuitry to generate a stepping pattern. 1ST-T8 : first spinal transection at T8; 2ST-S1 : second spinal transection at S1; 2ST-L2 : second spinal transection at L2; ES – epidural stimulation.

Figure 7: Analysis of the late response wave. (A) Mean (±SE) number of peaks during the late response (LR) before and after a 2ST-S1 and 2ST-L2 in the presence of ES. The number of peaks in the LR are significantly greater in the 2ST-S1 group as compared to the 2ST-L2 group and reflects greater polysynaptic reflex EMG activity secondary to the presence of stepping movements seen in 2ST-S1 rats (observed in Figure 4). **(B)** Coefficient of variation **(**CV) values during the late response that indicate the presence of muscle activity not time-locked to the ES pulse is significantly high in the 2ST-S1 group in comparison to the 2ST-L2 group for the TA muscle. ***,** significant differences between groups at $p < 0.05$. 2ST-S1 : second spinal transection at S1; 2ST-L2 : second spinal transection at L2; ES : epidural stimulation.

Figure 8: Representative spinal evoked motor response data during standing after the first and second spinal transection. (A-B) Raw EMG traces in a representative rat during treadmill stepping after the 1ST-T8 and after the 2ST-S1 from the vastus lateralis (VL), semitendinosus (ST), tibialis anterior (TA) and medial gastrocnemius (MG) muscles in response to L2 stimulation at 40Hz. **(C)** In the same rat, shown are an average of ~10 motor evoked responses during weight-bearing bipedal standing after 1ST-T8 and after 2ST-S1 with L2 stimulation at 0.3Hz. **(D-E)** Raw EMG traces in a representative rat during treadmill stepping after the 1ST-T8

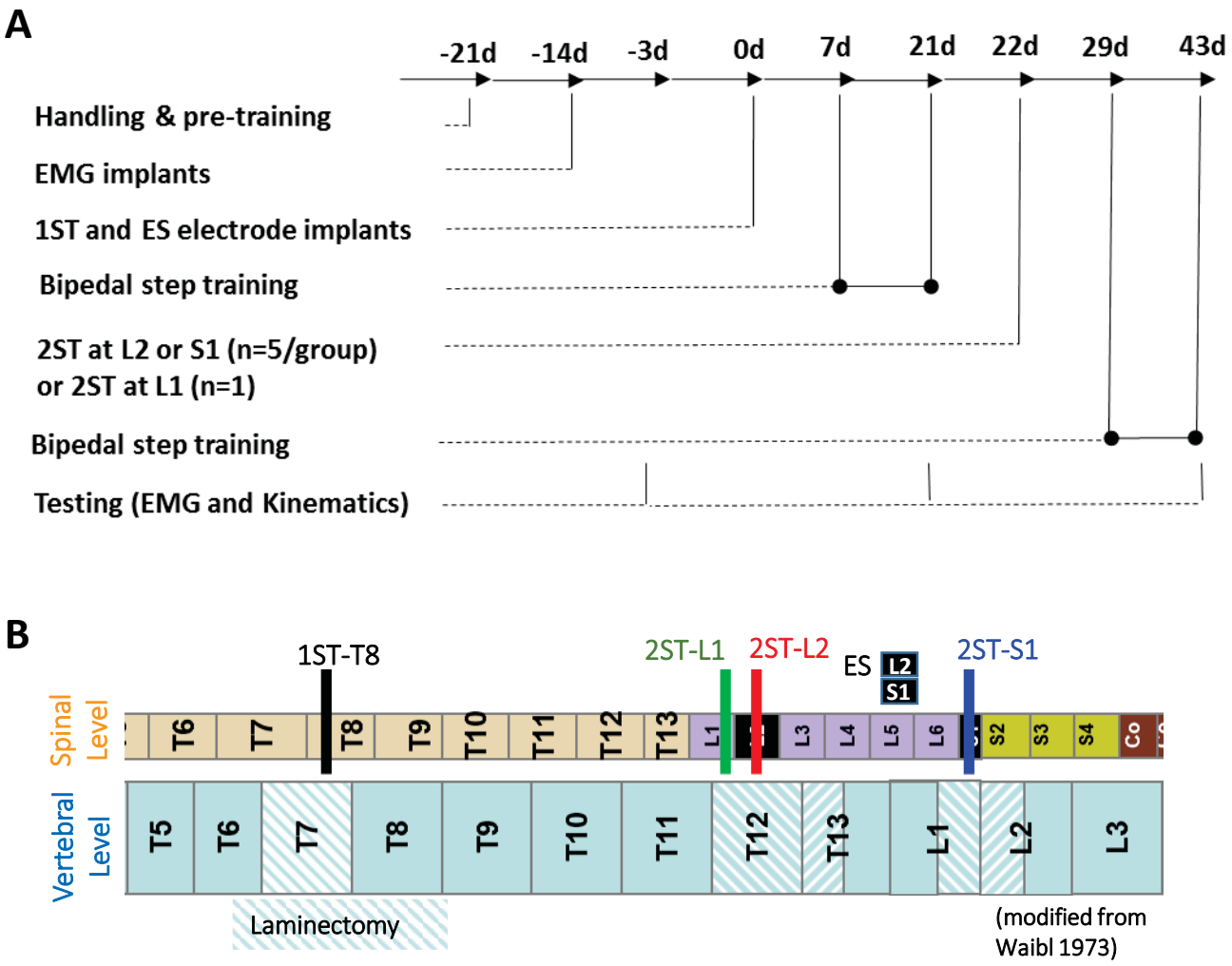
and after the 2ST-L2 from the same muscles in response to S1 stimulation at 40Hz. **(F)** In the same rat, shown are an average of ~10 motor evoked responses during weight-bearing bipedal standing after 1ST-T8 and after 2ST-L2 with S1 stimulation at 0.3Hz. Note that the evoked response profiles are comparable whether rats were transected at the S1 and L2, reflecting the responsiveness and therefore viability of dorsal and ventral roots after the second transection. 1ST-T8 : first spinal cord transection at the T8 spinal segment; 2ST-S1 : second spinal transection at S1; 2ST-L2 : second spinal transection at L2.

Figure 9: Stepping kinematics and EMG features before and after a second transection of the spinal cord at spinal segment L1 in one rat. The left panel shows data after the 1ST-T8 and the right panel shows data after a 2ST-L1 in the same rat. Note that the overall stepping kinematics and electrophysiology data are similar before and after the 2ST-L1. (A) Representative stick figures during the swing and stance phases of a step cycle (red marks the foot distance from metatarsophalangeal joint to tip of the toe) along with vertical ankle displacements (step heights) and toe trajectories of consecutive steps; **(B)** 3s of band pass filtered EMG signals from the tibialis anterior (TA.) and medial gastrocnemius (MG) muscles during bipedal treadmill stepping; (C) the EMG signal from B is depicted as a stack of consecutive 25ms responses (that correspond to each stimulation pulse) from top to bottom chronologically show middle response (MR) and late response (LR); **(D)** the average waveform during the late response window of the evoked responses obtained in **C**. The red horizontal hash lines in represent the amplitude threshold for peak acceptance, the midpoint between the hashed lines indicates baseline amplitude of 0mV, and the blue circles represent peaks that were accepted and included as late responses; **(E)** Mean (±SE) cycle duration, step height, stance length and total number of peaks in the late response waveform before and after 2ST-L1. ***,** significant

1058 differences between groups at $p < 0.05$. 1ST-T8 : first spinal cord transection at the T8 spinal
1059 segment; 2ST-L1 : second spinal transection at L1.

1060 **Figure 10: Schematic of the conceptual hypotheses for the observed results. (A)** Cartoon of
1061 the lumbosacral cord after the 1ST-T8 shows that when the L2 and S1 spinal segments are intact,
1062 ES at L2 or S1 (yellow thunderbolt arrows) modulates the locomotor circuitry at L2 in the
1063 presence of proprioceptive feedback from the hindlimbs. The resultant outcome is a robust
1064 stepping response from one or both hindlimbs, as evidenced by the foot trajectory plots. **(B)**
1065 After a 2ST-S1, ES at L2 and proprioceptive feedback from the hindlimbs continue to activate
1066 the rostral lumbar locomotor circuitry to evoke less robust stepping. **(C)** After 2ST-L2, however,
1067 the rostral locomotor circuitry is compromised after the injury. Despite the presence of
1068 proprioceptive feedback and ES at S1, stepping ability is almost lost. 1ST-T8 : first spinal cord
1069 transection at the T8 spinal segment; 2ST-S1 : second spinal transection at S1; 2ST-L2 : second
1070 spinal transection at L2.
1071
1072 **Table I:** Statistics on kinematics and EMG data. Red letters represent significance after
1073 Bonferroni correction for multiple comparisons.
1074

Figure 1



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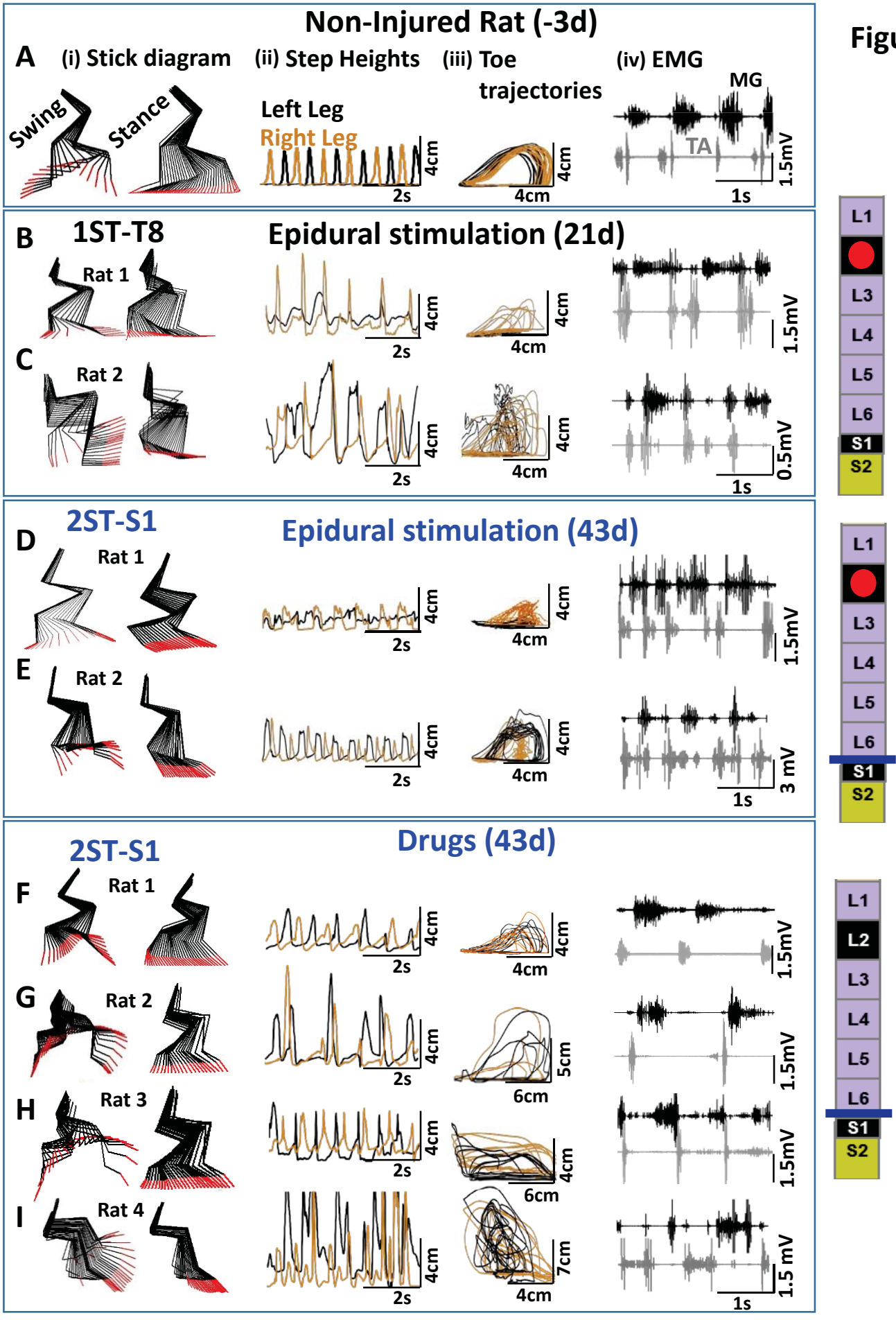

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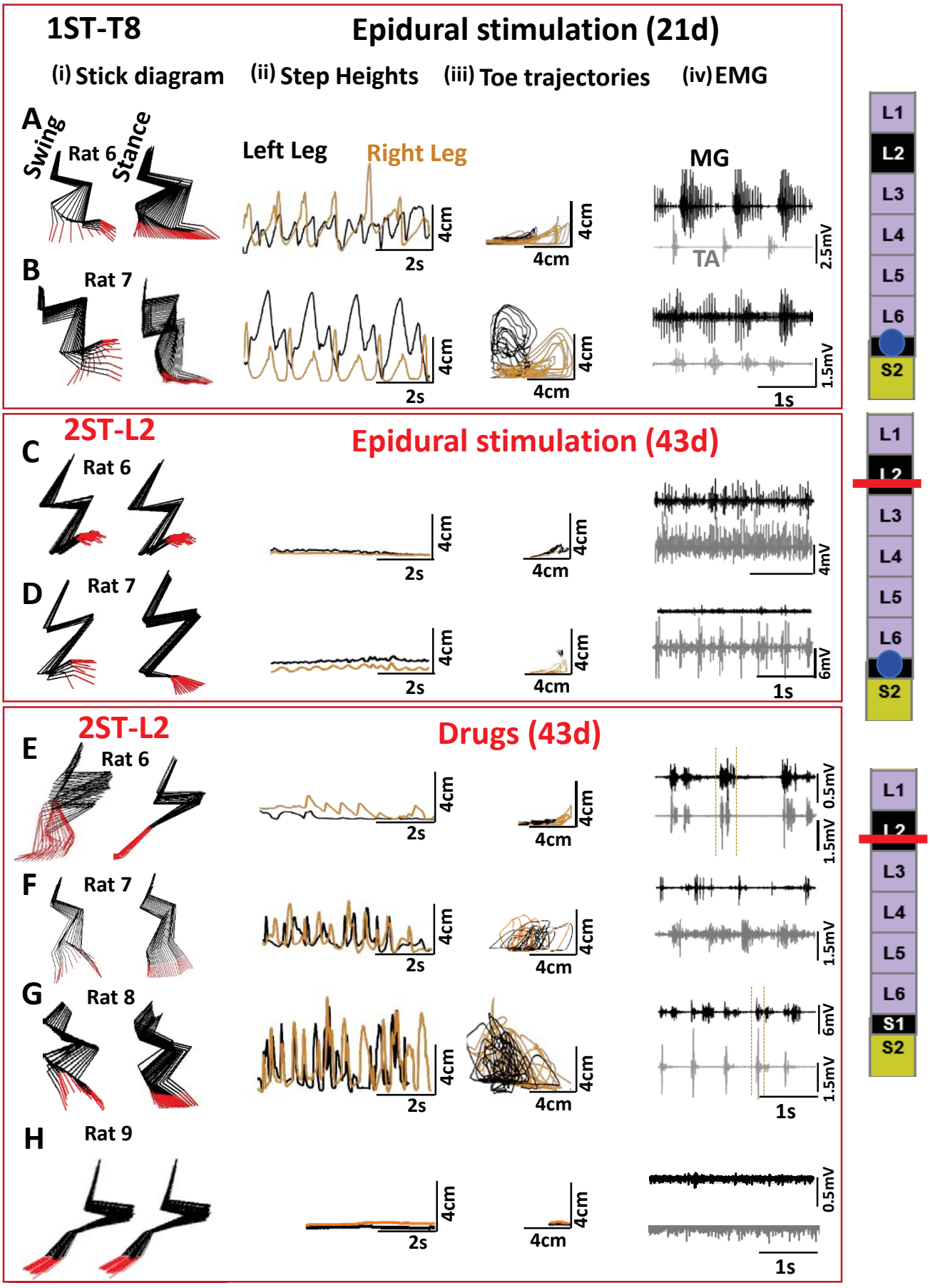

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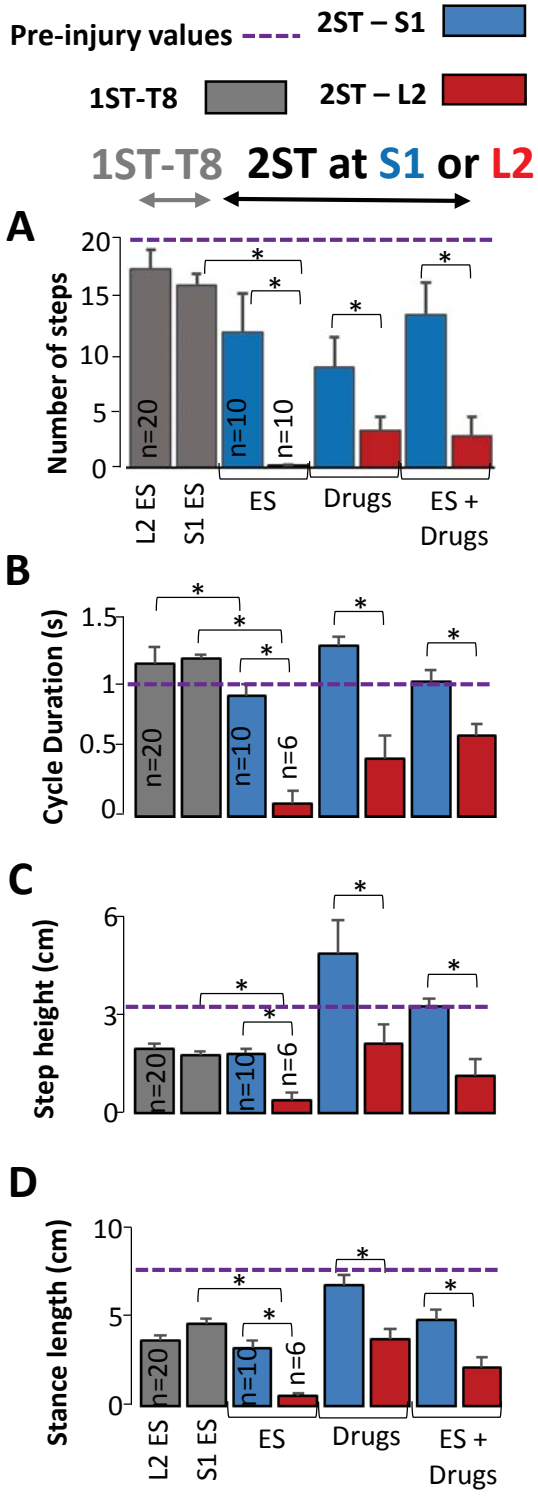

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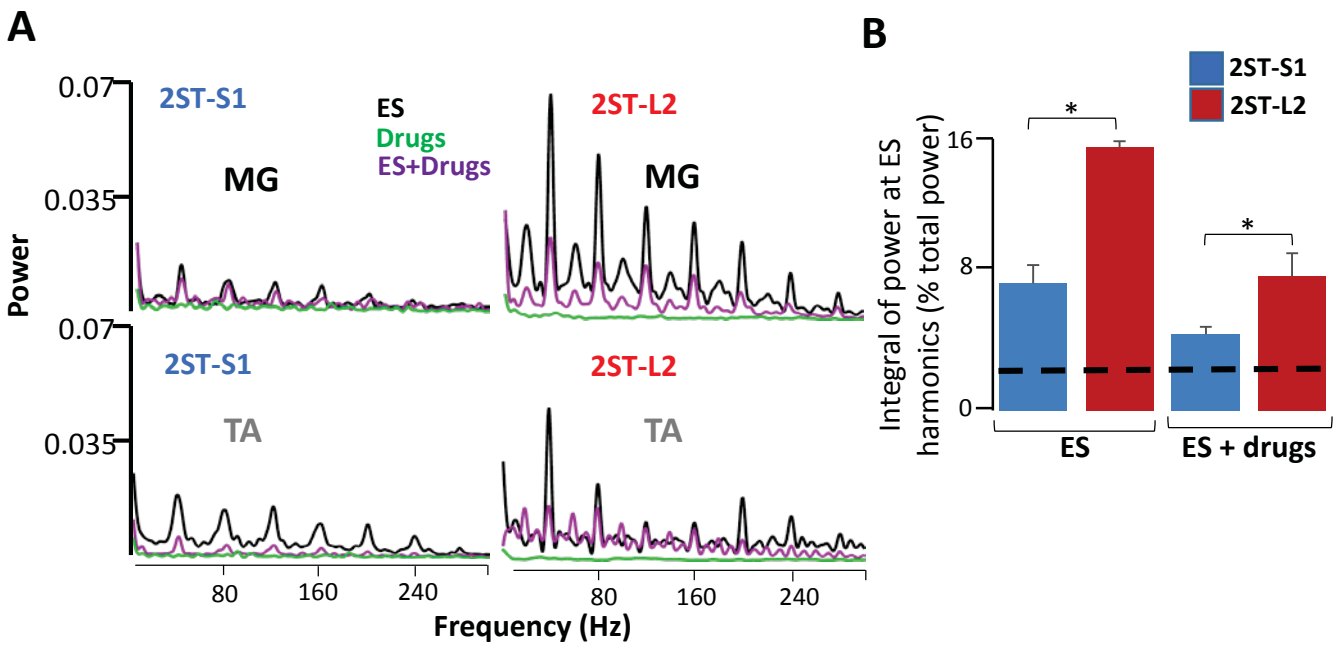

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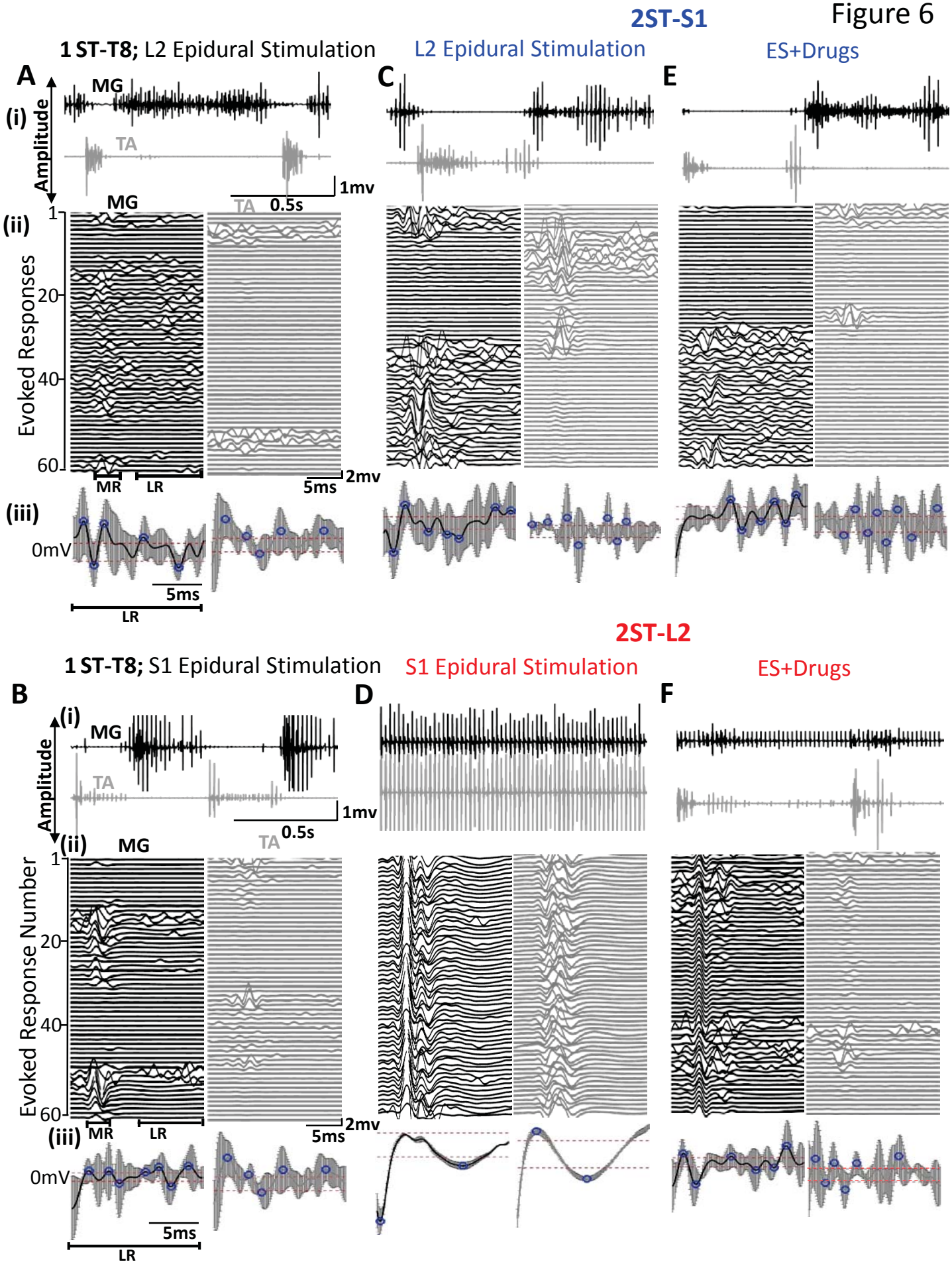

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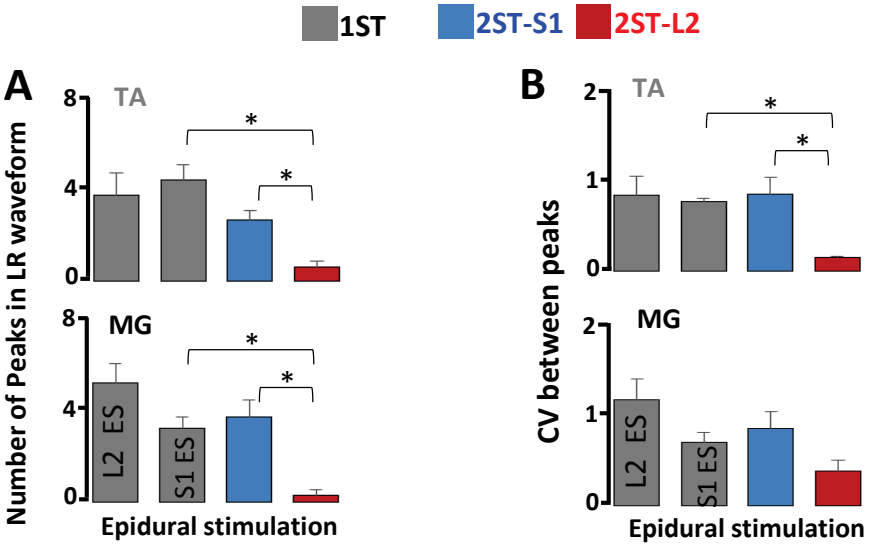

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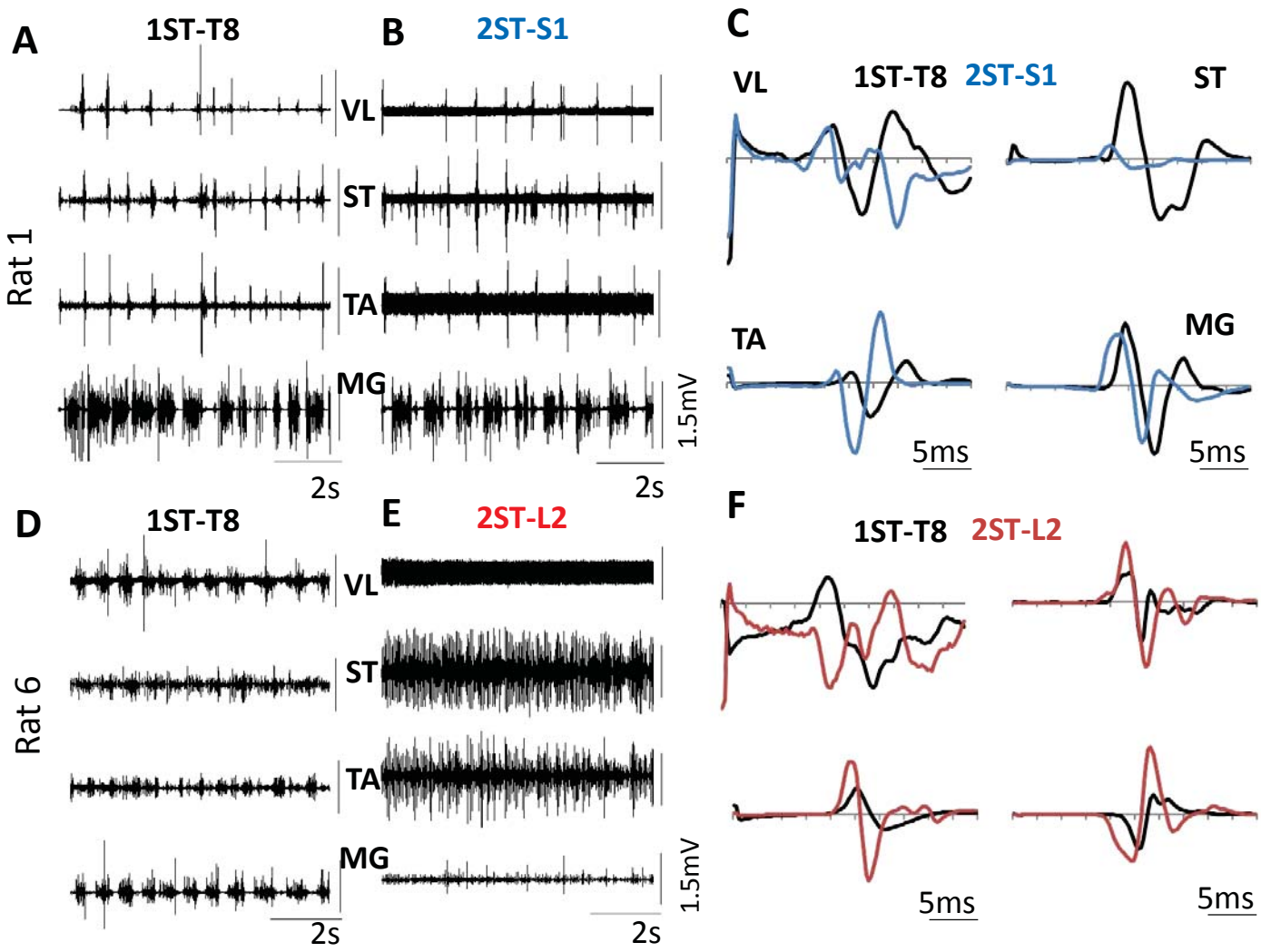

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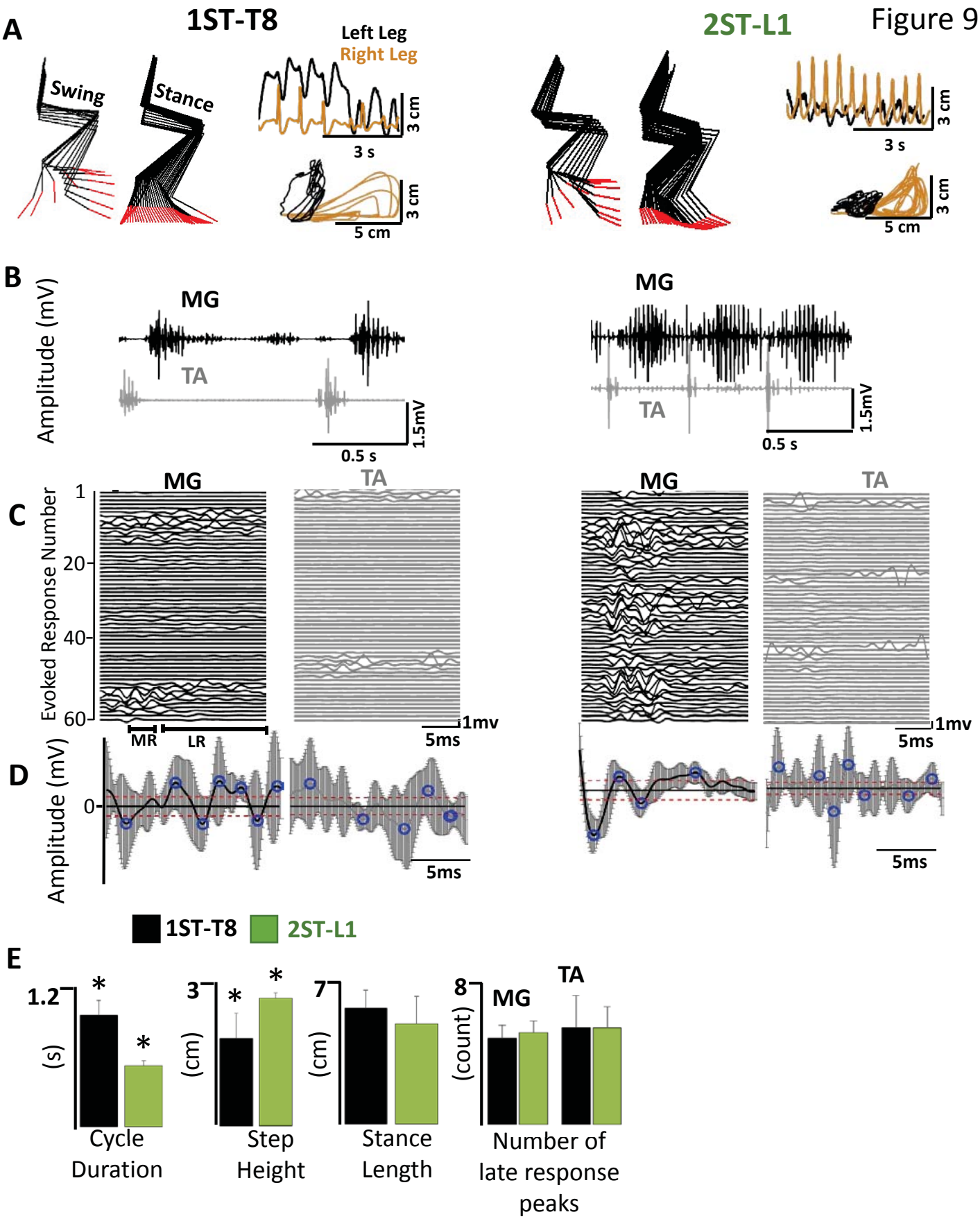

Figure 10