

ORIGINAL ARTICLE

Staggered multi-site low-frequency electrostimulation effectively induces locomotor patterns in the isolated rat spinal cord

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Study design: Experimental animal study.

Objectives: Epidural stimulation has been used to activate locomotor patterns after spinal injury and typically employs synchronous trains of high-frequency stimuli delivered directly to the dorsal cord, thereby recruiting multiple afferent nerve roots. Here we investigate how spinal locomotor networks integrate multi-site afferent input and address whether frequency coding is more important than amplitude to activate locomotor patterns.

Setting: Italy and Belgium.

Methods: To investigate the importance of input intensity and frequency in eliciting locomotor activity, we used isolated neonatal rat spinal cords to record episodes of fictive locomotion (FL) induced by electrical stimulation of single and multiple dorsal roots (DRs), employing different stimulating protocols.

Results: FL was efficiently induced through staggered delivery (delays 0.5 to 2 s) of low-frequency pulse trains (0.33 and 0.67 Hz) to three DRs at intensities sufficient to activate ventral root reflexes. Delivery of the same trains to a single DR or synchronously to multiple DRs remained ineffective. Multi-site staggered trains were more efficient than randomized pulse delivery. Weak trains simultaneously delivered to DRs failed to elicit FL. Locomotor rhythm resetting occurred with single pulses applied to various distant DRs.

Conclusion: Electrical stimulation recruited spinal networks that generate locomotor programs when pulses were delivered to multiple sites at low frequency. This finding might help devising new protocols to optimize the increasingly more common use of epidural implantable arrays to treat spinal dysfunctions.

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INTRODUCTION

A promising neurorehabilitative approach to provide functional benefits to persons with a chronic spinal injury is the direct electrostimulation of the dorsal cord via epidural electrodes.^{1,2} However, this procedure is invasive, because it requires surgical electrode implantation into the epidural space for the delivery of electrical impulses that can elicit a brief bout of alternating leg movements in paraplegic persons.³ The appearance of such locomotor-like episodes has been attributed to the reactivation of functionally impaired spinal neuronal circuits by inputs from dorsal root (DR) afferents.⁴ Because of recent upgrades in hardware technology, that is, grouping 16 electrodes into an epidural array, such a paradigm together with rehabilitative training is reported to facilitate voluntary motor control plus conferring other functional benefits in complete spinal cord-injured persons.² It should be noted that unlike single electrodes, these arrays excite a much wider area in the dorsal cord and may, thus, recruit a greater number of DR fibres establishing synapses with dorsal horn neurons.⁴ In previous studies, arrays have been used to activate spinal white matter tracts and to induce locomotor patterns in the isolated neonatal rat spinal cord,⁵ as

well as to facilitate locomotion through a new flexible implantable electrical epidural array in animal models of spinal lesion.⁶

With the perspective of further improvement in spinal neuro-rehabilitation, more advanced electrical stimulation paradigms are required to optimize the array construction, location and stimulation protocols. In particular, considering the latter issue, it is noteworthy that stimulation programs have remained unchanged in the past 15 years and still consists in stereotyped high-frequency trains of rectangular electrical pulses (25–40 Hz) simultaneously delivered through all electrodes in the array. Thus, future hardware developments should be associated with more performance-selective stimulating paradigms that are also expected to decrease the total amount of current supplied with decreased occurrence of side effects. The aim of the present work is to construct novel stimulation paradigms based on low-threshold amplitude and/or frequency that selectively activate the locomotor central pattern generator through a patterned stimulation of multiple afferent sites. Thus, we explored how spinal interneuronal circuits impinging on locomotor networks may integrate sensory afferent inputs arising from distinct segments in a concerted or staggered manner. Our data suggest that the delivery of staggered

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low-frequency stimuli to multiple sites of the isolated spinal cord could represent a suitable protocol to efficiently activate ventral root (VR) discharges, alternating among pairs of L2 and L5 on either side, defined as fictive locomotion (FL).⁷

MATERIALS AND METHODS

Isolation of spinal cord and rhythm induction

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. All procedures were approved by the Scuola Internazionale Superiore di Studi Avanzati ethics committee and are in accordance with the European Union directive for animal experiments (86/609/EEC). Every effort was made to reduce the number of rats used for the isolation of spinal cord and to minimize their suffering. All experiments were performed, in line with the guidelines provided by the Italian Animal Welfare Act, on spinal cord preparations isolated from neonatal rats (0–2 days old), as previously described.⁷ Spinal cords were dissected from thoracic level 5–6 to the cauda equina and care was taken to leave the DRs and VRs attached, which allows for the study of FL.⁸ FL is a locomotor-like rhythm originating in the spinal cord preparation and consisting of an episode of electrical discharges that can be measured from VRs. This FL is characterized by discharges alternating between pairs L2 and L5 VRs. Therefore, FL is considered to provide important information about the function of locomotor spinal circuits that can be observed in isolated neonatal rat spinal cord preparations.⁹ In our setting, we induced FL by applying a train of rectangular electrical pulses to a single DR or to sacrocaudal afferents (cauda equina), as described previously.^{8,10}

Electrostimulating paradigms

Thirty- or sixty-second trains of pulses (duration 0.1 ms) were delivered to several segmental levels (from L5 to the cauda equina), in a different range of frequencies (0.33–2 Hz) and with a variable strength (9–400 μ A). Although previous studies indicated that there was no significant change in FL features due to varying train frequency (1–50 Hz⁸), in this study we considered the 2 Hz protocol as a benchmark for comparing efficacy of the new multi-site stimulation protocols.

Nevertheless, in a subset of experiments, we reduced stimulating frequency to assess the lowest value able to induce an episode of FL, which was 1 Hz. In this case, FL was rarely evoked, but the features of FL episodes were similar to those evoked by the 2 Hz protocol.

Single pulses (duration=0.1 ms) were delivered at a strong intensity to induce phase resetting during FL.¹¹ In these experiments, absolute strength of stimulation spanned from 40 to 400 μ A at the lumbar level and from 90 to 400 μ A at the sacral level. We ascertained that the maximum strength of stimulation applied to a DR did not cause a non-selective activation of nearby DRs through leakage of current from the stimulating electrode. Therefore, we stimulated DR L6 at the maximum intensity used in the study (400 μ A), which evoked a reflex response from both the homosegmental VR and the adjacent VRS2. Similarly, stimulation of DR S2 at an intensity of 400 μ A induced a synchronous response from S2 and L6 VRs. After the complete horizontal transection of the spinal cord at the level of S1 segment, the stimulation of the same two DRs at the same intensity now only evoked a response from homolateral VRs, without affecting the disconnected portion of the cord (data not shown). This test proved that stimulation of a DR at maximal intensity did not evoke any aspecific activation of the adjacent DR, caused by leakage of current from miniature bipolar stimulating electrodes.

Electrophysiological recordings

Spinal cords were placed in a recording chamber, continuously superfused (5 ml min⁻¹) at room temperature (22 °C–24 °C) with carbogenated (95% O₂, 5% CO₂) Krebs solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 MgCl₂ × 7H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃ and 11 glucose, pH 7.4. Traces were obtained from lumbar (L) VRs, by using tight-fitting suction electrodes.⁷ DC-coupled recordings were acquired with a differential amplifier (DP-304, Warner Instruments, Hamden, CT, USA) at a sampling rate of 10 KHz, low-pass-filtered 10 Hz and high-pass 0.1 Hz, and digitalized

(Digidata 1440, Molecular Devices, Sunnyvale, CA, USA). The alternation of discharges between L2 and L5 VRs and between the left (l) and right (r) L2 VRs were considered distinct features of FL, as previously reported.¹²

Parameters of spinal network activities

Single or repetitive electrical pulses were delivered by a programmable stimulator (STG4004, Multichannel System, Reutlingen, Germany). DR electrical stimuli were adapted to evoke both single VR responses and cumulative depolarization⁸ originating from the addition of individual pulses delivered at each segments. Herein, the lowest stimuli able to elicit a detectable short-latency response (usually as long as 10 ms and as high as 3 × the width of baseline) from the corresponding VR were defined as threshold stimuli.⁸ Out of 31 preparations, the average threshold value of short-latency reflex response was 20.19 ± 22.05 μ A. Oscillations of FL were analysed based on their periodicity (time between the onset of two consecutive cycles of oscillatory activity) and regularity, expressed as coefficient of period variation (given by s.d. per mean). The strength of coupling among pairs of signals of VRs was measured using the cross-correlation function (CCF) analysis. When a CCF is higher than +0.5, two roots are synchronous, whereas a CCF smaller than -0.5 corresponds to full alternation.¹²

Data representation and statistics

Data are reported as mean ± s.d. values. Number of samples is indicated as *n* in the results. Normality testing showed a normal Gaussian distribution for all data sets. Accordingly, parametric data were analysed with the Student's *t*-test or one-way analysis of variance (ANOVA), whereas Kruskal–Wallis one-way ANOVA on ranks was used for non-parametric data. Differences were considered statistically significant when *P* < 0.05. Multiple comparisons were corrected for *post-hoc* analysis using pairwise multiple comparisons (Dunn's or Tukey's method).

RESULTS

Specificity of VR responses induced by electrical stimulation of multiple neighbouring DRs

As in this study we investigated the responses to electrical pulses applied to multiple DRs, it was important to exclude that, in our set up, electrical stimulation of a DR aspecifically activated closeby DRs simply by leakage of current through the stimulating electrode. Therefore, strong electrical pulses (intensity = 300 ± 100 μ A) were serially delivered to a pair of neighbouring DRs (L6 and S2). Evoked reflex responses were recorded from homosegmental VRs (peak_{VRIL6} = 0.32 ± 0.01 mV, peak_{VRS2} = 0.27 ± 0.03 mV, *n* = 3; Supplementary Figures 1a and b) and, with a delay of 11.6 ± 2.77 ms, from adjacent VRs (peak_{VRS2} = 0.16 ± 0.01 mV, peak_{VRIL6} = 0.14 ± 0.07 mV, *n* = 3; Supplementary Figures 1a and b). Next, we performed a complete transection at S1 level to obtain two separate portions of the spinal cord, which were left close to each other. Although distance between DRs was negligible, no spinal cord circuitry between the two tissue portions was preserved. Following electrical stimulation of a DR elicited a homosegmental reflex response (peak_{VRIL6} = 0.33 ± 0.03 mV, peak_{VRS2} = 0.19 ± 0.04 μ V, *n* = 3; Supplementary Figures 1c and d), but failed to induce responses in segments disconnected from the stimulating site (Supplementary Figures 1c and d). This observation demonstrates that intersegmental VR responses are driven by intact spinal circuitries rather than by a generalized electrical activation of closeby afferents.

Low-intensity simultaneous multi-site DR trains fail to elicit FL

Our first aim was to explore how spinal interneuronal circuits for locomotion integrate convergent sensory afferent inputs originating from different sites. The effect of stimulating a single DR, at low lumbar or sacral level, with pulse trains of high intensity was therefore

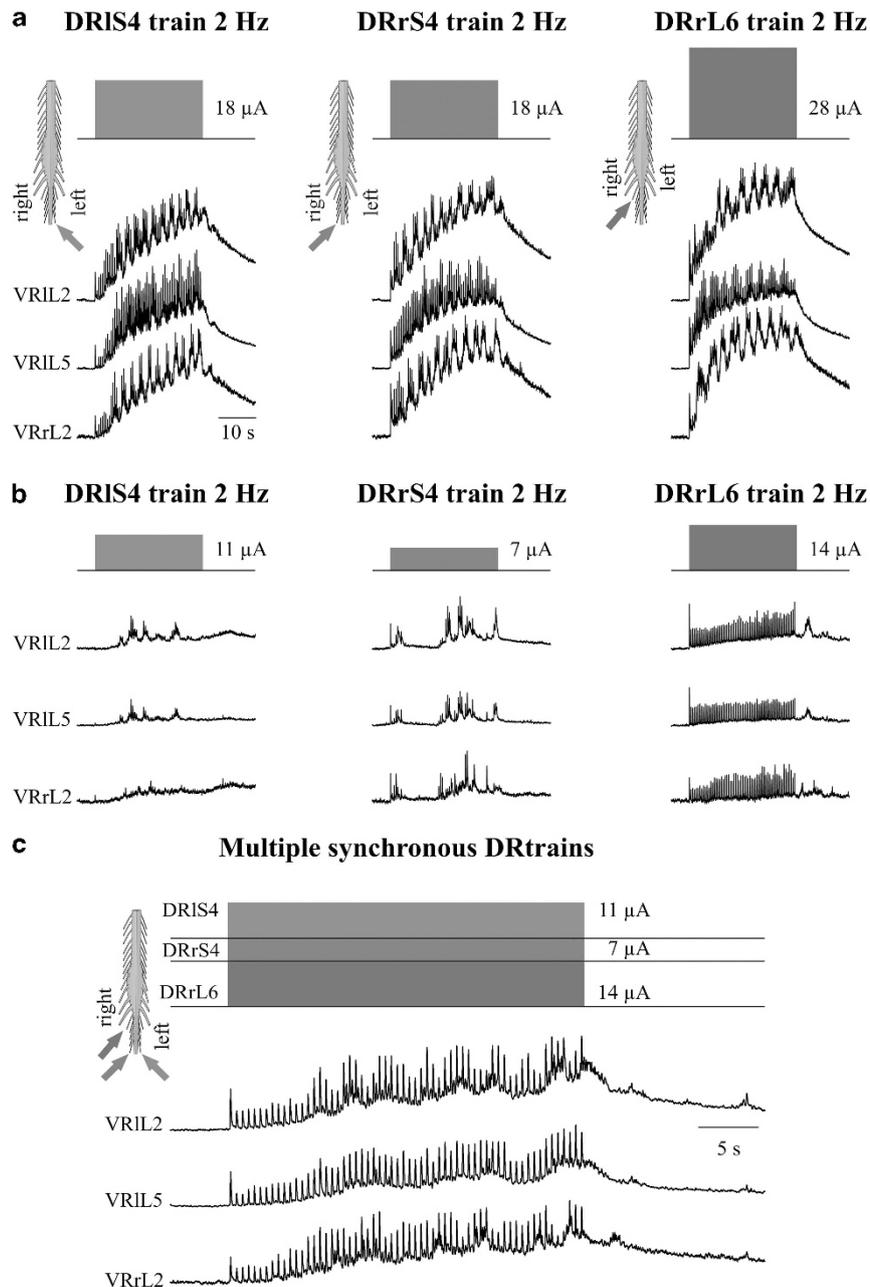


Figure 1 Multiple subthreshold amplitude DR trains do not reach threshold when delivered at once. (a, Left) A train of 60 stimuli at 2 Hz applied to DRIS4 (amplitude = 18 μA , 1.5 \times threshold) generates a cumulative depolarization and an episode of FL (CCF = -0.488). In the same preparation, an episode of FL is also evoked when the train at 2 Hz is applied to the contralateral DR (amplitude = 18 μA , 2 \times threshold; (a, middle) CCF = -0.570) or to DRrL6 (amplitude = 28 μA , 2 \times threshold; (a, right) CCF = -0.569). (b) When the amplitude of stimulation of each train is lowered, no cumulative depolarization is evoked and uncorrelated discharges replace locomotor oscillations (CCF = 0.109, 0.434 and 0.930, respectively). (c) Three trains of low intensity are now delivered synchronously, each to one of the three afferents of the same spinal cord. Although the sum of amplitudes of single protocols is above the value to trigger FL with individually applied trains, no locomotor response was observed (CCF = 0.647). CCF, cross-correlation function; DRIL, left lumbar dorsal root; DRrL, right lumbar dorsal root; DRrS, right sacral dorsal root; VRIL, left lumbar ventral root; VRrL, right lumbar ventral root.

compared with the stimulation of three DRs at lower intensity (that is, an intensity that was ineffective for inducing FL through a single DR). Initially, an episode of FL was observed as a series of oscillatory cycles recorded from L2 (flexor-related) and L5 (extensor-related) VRs, evoked by a train of 2 Hz stimuli delivered at optimal amplitude to just one DR among the three selected (Figure 1a; 18, 18 and 28 μA , respectively). Notably, these effects were not dependent on the DR selected. Whenever canonical 2 Hz DR trains⁸ were applied to various

DRs, the main properties of FL episodes (such as periodicity or number of oscillations superimposed on the cumulative depolarization elicited by the stimulus train) remained similar (Table 1). Table 1 also lists other parameters typical of FL such as the CCF, which according to its positive or negative value shows phase synchrony or alternation of cycles.¹² Likewise, when the same protocol was applied for an extended duration (60 s), the number of FL cycles did not increase (data not shown).

Table 1 Main features of FL episodes induced by a train of electrical pulses (2 Hz) delivered to a single DR at different spinal cord levels

Spinal level	Intensity of stimulation (\times threshold)	Duration (s)	Number of oscillations	Period of oscillations (s)	CV	Cumulative depolarization peak (mV)	CCF	n
L6–S1	1.60 \pm 0.62	25.95 \pm 2.58	11 \pm 2	2.58 \pm 0.41	0.16 \pm 0.04	0.94 \pm 0.46	-0.576 \pm 0.433	12
S2–S3	1.57 \pm 0.38	26.23 \pm 3.12	12 \pm 1	2.45 \pm 0.32	0.17 \pm 0.05	0.96 \pm 0.50	-0.747 \pm 0.100	6
S4–S5	1.65 \pm 0.44	25.54 \pm 2.33	11 \pm 1	2.45 \pm 0.19	0.15 \pm 0.03	0.58 \pm 0.29	-0.632 \pm 0.158	8
Cauda	1.73 \pm 0.25	25.71 \pm 3.66	12 \pm 3	2.38 \pm 0.34	0.13 \pm 0.02	0.75 \pm 0.14	-0.778 \pm 0.127	3
Statistics	$P=0.798$	$P=0.970$	$P=0.944$	$P=0.726$	$P=0.523$	$P=0.223$	$P=0.322$	

Abbreviations: CCF, cross-correlation function; CV, coefficient of period variation; DR, dorsal root; FL, fictive locomotion.

Next, we reduced the stimulus intensity down to a value that, per se, no longer generated locomotor-like responses (Figure 1b; 11, 7 and 14 μ A, respectively). The resulting low-intensity train was simultaneously applied to three distinct DRs (overall stimulus amplitude = 32 μ A). Although the total intensity of the three stimuli was greater than the value needed to activate FL with a single DR train at 2 Hz, it proved ineffective in inducing FL when distributed through three DRs, even though a slight cumulative depolarization with asynchronous slow oscillations appeared (Figure 1c). The same results were confirmed in further three experiments (CCF = 0.446 \pm 0.311, $n=4$). Even though the data were obtained on the basis of *in vitro* preparations, they indicate that the spinal locomotor networks process incoming stimuli in a complex manner, which is different from the mere summation of multiple, simultaneous single inputs originating from different afferent fibres.

Phase resetting of electrically induced FL by delivering single pulses to a distant DR

In order to test whether different afferent inputs converge onto common spinal locomotor networks, we used the method of locomotor phase resetting, whereby the regularity of a rhythmic pattern induced by pulses delivered to a range of lumbosacral DRs is perturbed by a single pulse delivered to another DR. This phenomenon has been well-described for both *in vitro* chemically induced¹³ and *in vivo* electrically induced FL,¹⁴ to demonstrate how afferent signals of various origins affect the same network. As there are no data on phase resetting of the DR-evoked FL *in vitro*, we first studied its presence in our preparation. FL was induced by a single DR stimulation during which further stimuli were delivered through a distinct DR. In the example of Figure 2a, a series of electrical pulses (60 stimuli, 2 Hz, amplitude = 9 μ A) was applied to a sacral DR and induced an episode of locomotor-like oscillations recorded from L2 and L5 VRs. The regularity of FL was perturbed by two single stimuli (amplitude = 130 μ A; see vertical lines), delivered to a sacral DR of the opposite side and temporally separated by a 10-s interval. Figure 2b (note faster time base) clearly shows phase resetting: the single impulses delivered to IS2 (occurred in correspondence to the peak in the VRL2) lengthened the cycle ipsilateral to the stimulating site, while prolonging the trough in the contralateral VRL2 and VRL5. In five preparations, phase resetting of FL cycles was induced in 12 out of 24 impulses delivered as single sacral DR stimuli (mean stimulus strength equal to 222.50 \pm 116.39 μ A). Furthermore, electrical stimulation of lumbar DRs (mean amplitude 187.89 \pm 120.95 μ A) produced a comparable occurrence of phase resetting (19/34, $n=5$ cords).

Interestingly, the phenomenon was observed more often when single pulses were delivered in correspondence to peaks in FL cycles recorded from flexor-related VRs homolateral to the stimulating DR (16/24), rather than from contralateral flexor-related VRs (8/24). These data show that in our preparations, activity at lumbar or sacral

DRs possesses analogous resetting ability and, therefore, is most likely converging onto the spinal locomotor networks.

Multi-site delivery renders subthreshold frequency DR trains effective in triggering FL

So far, our experiments demonstrated that despite convergence onto the same spinal circuits, simultaneous weak stimulus delivery to various DRs was not able to evoke comparable amplitudes. For our next question, we then tested whether the spinal circuits could process stimulating frequencies in a different manner.

Thus, electrical trains were first delivered to one sacral DR as 60 rectangular pulses (2 Hz), to produce cumulative depolarization (0.64 \pm 0.26 mV peak; $n=18$) with superimposed oscillations alternating among homosegmental VRs (CCF = -0.667 \pm 0.153, $n=18$). FL lasted for the whole duration of the 2-Hz stimulating protocol (26.21 \pm 2.36 s, $n=18$; see examples in Figure 3a–c, left panels), with optimal strength amplitude of 1 to 2.5 \times threshold for inducing the highest number of alternating oscillations (11 \pm 2, $n=18$). This frequency of stimulation was then reduced by a third (0.67 Hz), while maintaining the same stimulation intensity. None of the resulting low-frequency trains was then able to activate the FL pattern when delivered alone, as low cumulative depolarization (0.36 \pm 0.17 mV, $n=9$) accompanied by reflex responses (CCF = 0.567 \pm 0.280, $n=9$) in correspondence to each impulse was detected (Figures 3a–c, right panels). Afterwards, the protocol of multi-site stimulation was applied as illustrated in Figure 3d. Three trains of low-frequency stimulation (0.67 Hz) were delivered together to three different sacral DRs of the same preparation, with a phase delay of 0.5 and 1 s for the second and third trains, respectively. By graphically superimposing the three single trains, we obtained the resulting multi-site stimulation protocol (Figure 3d, lower panel). This now showed an overall frequency of 2 Hz and was found to trigger FL with a number of cycles similar to single-site higher-frequency stimulation (Figure 3e) of similar amplitude.

We studied the effects of the multiple staggered low-frequency protocols in more detail in three preparations, where trains were applied to three different DRs (from L2 to the cauda equina) to generate a cumulative depolarization of 0.79 \pm 0.18 mV, accompanied by 10 \pm 1 locomotor-like cycles (CCF = -0.722 \pm 0.100). This effect was not significantly different from that evoked in the same preparations by single 2 Hz trains in terms of total duration of FL episodes (*t*-test, $P=0.297$; $n=3, 9$), mean cycle period (*t*-test, $P=0.907$; $n=3, 9$) and regularity, expressed as period coefficient of period variation (*t*-test, $P=0.078$; $n=3, 9$). Responses were not affected by the order in which various afferents were stimulated (data not shown). Delivery of multiple staggered DR trains of low frequency for 60 s did not prevent the spontaneous decay of locomotor response (mean duration of FL episodes = 38.63 \pm 7.23 s, $n=8$; Figure 4a), as noted when a single afferent was stimulated with a classical 2-Hz train.⁸

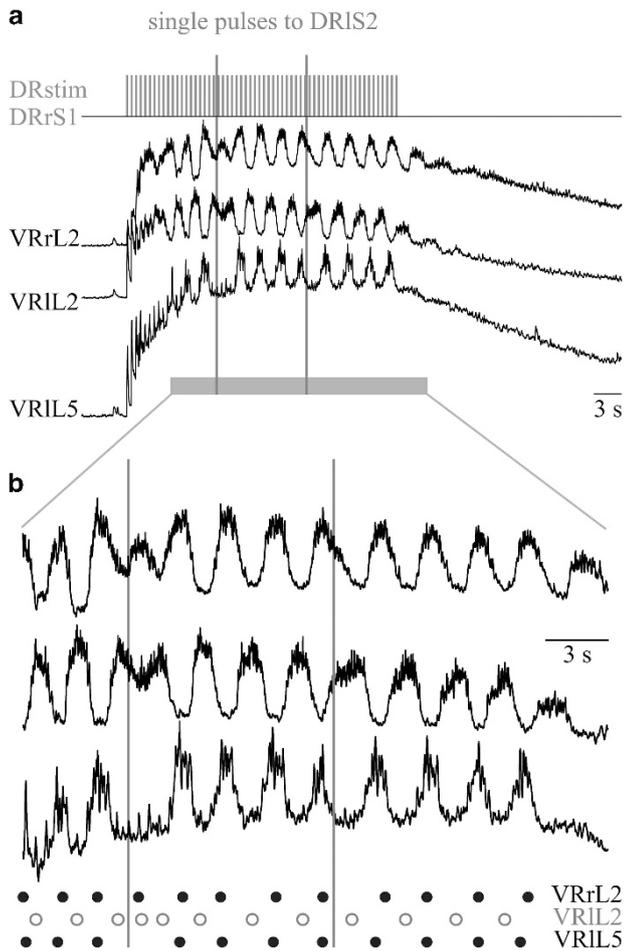


Figure 2 Single DR pulses evoke phase resetting of electrically induced FL cycles. (a) A train of 60 stimuli at 2 Hz (amplitude=9 μ A, 1 \times threshold) applied to DRIS1 induces an episode of alternating oscillations among right L2 and contralateral L2 and L5 VRs (CCF=-0.821). During train delivery, two other single impulses are added at a higher intensity (amplitude=130 μ A, 8.7 \times threshold) to DRIS2, which disturbed regularity of FL oscillations. (b) In correspondence to the first stimulus, delivered at the peak on the VRL2 homolateral to the site of stimulation, an early peak on VRrL2 appears, which is followed by an extra peak that prolongs the burst on VRIL2 and by a protracted pause on VRIL5. Note that traces are a higher magnification of VR recordings referring to the grey bars in a. On the other hand, during the first ascending phase of a cycle on the VRL2 homolateral to the stimulation site, the second impulse is delivered and delays the following peak on VRrL2, lengthens the burst of VRIL2 and slightly slows down rhythm on VRIL5. The scattered representation below highlights phase relation among peaks of oscillations in the three VRs. CCF, cross-correlation function; DRstim, dorsal root electrical stimulation; DRrL, right lumbar dorsal root; DRIS, left sacral dorsal root; VRIL, left lumbar ventral root; VRrL, right lumbar ventral root.

The minimum frequency of DR trains to effectively elicit FL was 1 Hz (Supplementary Figure 2a–c). Furthermore, the staggered multiple stimulation of three DRs at 0.33 Hz was still capable to evoke an episode of FL once applied with a phase delay of 1 s (Supplementary Figure 2d).

To verify whether the synchronous delivery of three trains at optimal intensity can evoke an FL notwithstanding low frequency of stimulation, we then simultaneously applied three 0.67 Hz trains to three DRs for 60 s (synchronous onset among three DRs; Figure 4b). Compared with staggered stimulation of three DRs in the same

preparation (Figure 4a), this protocol failed to generate FL (Figure 4b), as confirmed by cross-correlation analysis on pairs of VRL2 (mean CCF for staggered DR trains = -0.502 ± 0.138 vs mean CCF for synchronous DR trains = -0.162 ± 0.238 ; Mann–Whitney rank sum test, $P=0.005$; $n=8$). This suggests that efficacy of the staggered protocol does not originate from the sole simultaneous stimulation at optimal amplitude of multiple DRs, but also exploits the controlled time interval for the delivery of the trains.

Multiple delivery of random low-frequency trains still induced FL episodes, but with inferior coupling than canonical protocols

To clarify whether a staggered approach is absolutely necessary for the expression of FL episodes using low-frequency stimulation, multiple trains with the same number of pulses (mean frequency=0.67 Hz) were delivered randomly to three DRs.

As depicted in Figure 5a–c (left), single DR trains at classical 2 Hz evoked a cumulative depolarization with superimposed, fully alternated locomotor-like cycles. Conversely, FL was not elicited by a randomized delivery of stimuli with a mean frequency of 0.67 Hz to the same DRs, despite the similar amplitude of single pulses (Figure 5a–c, right).

On the other hand, the protocol resulting from the combined delivery of three random trains (Figure 5d) elicited FL cycles similar to the ones induced by staggered low-frequency trains, as exemplified in Figure 5e.

In four cords, 2 Hz DR trains (mean intensity = $1.56 \pm 0.19 \times$ threshold) induced a mean cumulative depolarization of 1.06 ± 0.53 mV, with an episode of 11 ± 1 locomotor-like oscillations for a total mean duration of 25.18 ± 1.59 s. Moreover, multiple random trains of identical amplitude evoked a comparable mean cumulative depolarization (1.00 ± 0.53 mV) with an FL epoch of similar duration (23.85 ± 0.93 s) and number of cycles (10 ± 1). Locomotor-like response evoked by multiple random trains was similar to the one elicited by the staggered protocol (cumulative depolarization = 0.93 ± 0.48 mV; duration = 23.93 ± 4.90 s; cycles = 11 ± 3).

In fact, the three different types of stimulation were not statistically different as for cumulative depolarization (one-way ANOVA, $P=0.898$; $n=12, 4, 4$), duration of FL episodes (one-way ANOVA, $P=0.447$; $n=12, 4, 4$) and number of oscillations (Kruskal–Wallis one-way ANOVA, $P=0.173$; $n=12, 4, 4$).

On the other hand, FL evoked by multiple random trains displayed a mean phase coupling among L2 VRs that was statistically lower than the one triggered by 2 Hz trains, which in turn was not statistically different from staggered protocols (Kruskal–Wallis one-way ANOVA followed by all pairwise multiple comparison with Dunn’s method, $P=0.011$; $n=12, 4, 4$; Figure 5f).

DISCUSSION

The principal finding of the present study is the experimental demonstration that optimal activation of the spinal locomotor program was achieved by delivering low-frequency trains in a staggered manner to multiple DRs of the rat spinal cord. Thus, locomotor spinal networks could decode and process multiple stimuli in a complex manner.

Indeed, locomotor spinal networks can sum frequency of stimuli reaching the central pattern generator from multiple afferents (frequency-related summation), while each afferent input is individually filtered out if below a threshold value (intensity-related occlusion).

It should be mentioned that our *in vitro* model does not allow us to fully analyse the fine-tuning of motor control observed with kinematic

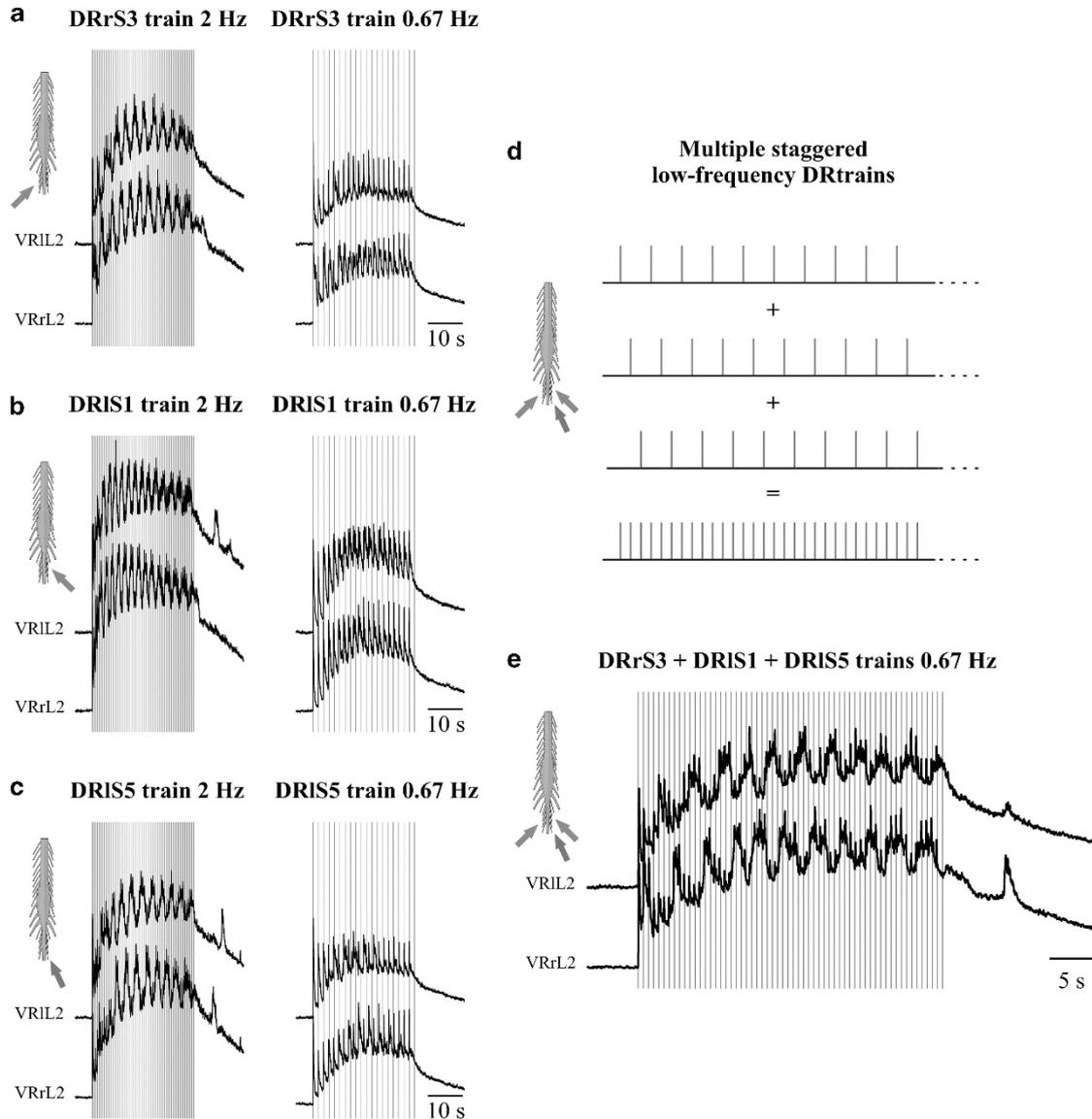


Figure 3 Low-frequency trains of stimuli applied to multiple DRs with staggered onsets elicit an episode of FL oscillations. (a) A train of 60 stimuli to DRrS3 induces a locomotor response when applied at 2 Hz (left; $CCF = -0.654$), but not when delivered with a lower frequency (0.67 Hz; right; $CCF = 0.555$), although with equal intensity ($18 \mu\text{A}$, $2 \times \text{threshold}$). In the same preparation, a similar trend is observed when trains at 2 and 0.67 Hz are serially delivered to DRIS1 (amplitude = $25 \mu\text{A}$, $2 \times \text{threshold}$; (b) $CCF = -0.500$ and $CCF = 0.870$, respectively) or DRIS5 (amplitude = $25 \mu\text{A}$, $2.5 \times \text{threshold}$; (c) $CCF = -0.629$ and $CCF = 0.600$, respectively). (d) Sketches how the stimulating protocol is prepared, by superimposing three trains of low frequency (0.67 Hz), each one applied to a distinct afferent of the same cord and with an onset staggered by 0.5 s from the previous train. In e, this protocol proves (same preparation as in a–c) to be able to activate an episode of FL ($CCF = -0.608$) comparable, as for number and regularity of cycles, to the ones evoked from single DR trains at 2 Hz when delivered separately. CCF, cross-correlation function; DRIL, left lumbar dorsal root; DRrS, right sacral dorsal root; DRIS, left sacral dorsal root; VRIL, left lumbar ventral root; VRrL, right lumbar ventral root.

analysis in walking animals. Nevertheless, our method has the important advantage of recording motor output of pure neuronal origin without any influence of either compensatory muscle activation or modulators of the peripheral circulatory stream.

Frequency of incoming inputs is more important than intensity

Although previous reports point out that also capsaicin-sensitive pain-related fibres are involved in the modulation of ongoing FL,¹⁵ modelling studies have shown that DR electrical stimulation activates the locomotor pattern mainly due to a selective recruitment of multiple fibres carrying tactile and proprioceptive inputs. These fibres are characterized by a wide diameter and low excitation threshold,

thus being unable to discriminate small variations in stimulus amplitude.⁴ This characteristic can account for the inability of simultaneous stimuli of low amplitude to summate and generate a locomotor pattern. We, therefore, propose the existence of a gating system that filters the amplitude of afferent stimuli. Such a system is reminiscent of the relay neurons in the sacrococcygeal spinal cord that were previously suggested to drive lumbar rhythms.¹⁰ On the other hand, previous studies have shown that stimulation frequency might sculpt circuit synapses through different plasticity mechanisms: modifying either the amount¹⁶ or type¹⁷ of released neurotransmitters, reversing the balance between excitation and inhibition,¹⁸ or limiting negative-feedback pre-synaptic processes.¹⁹ In addition, circuit

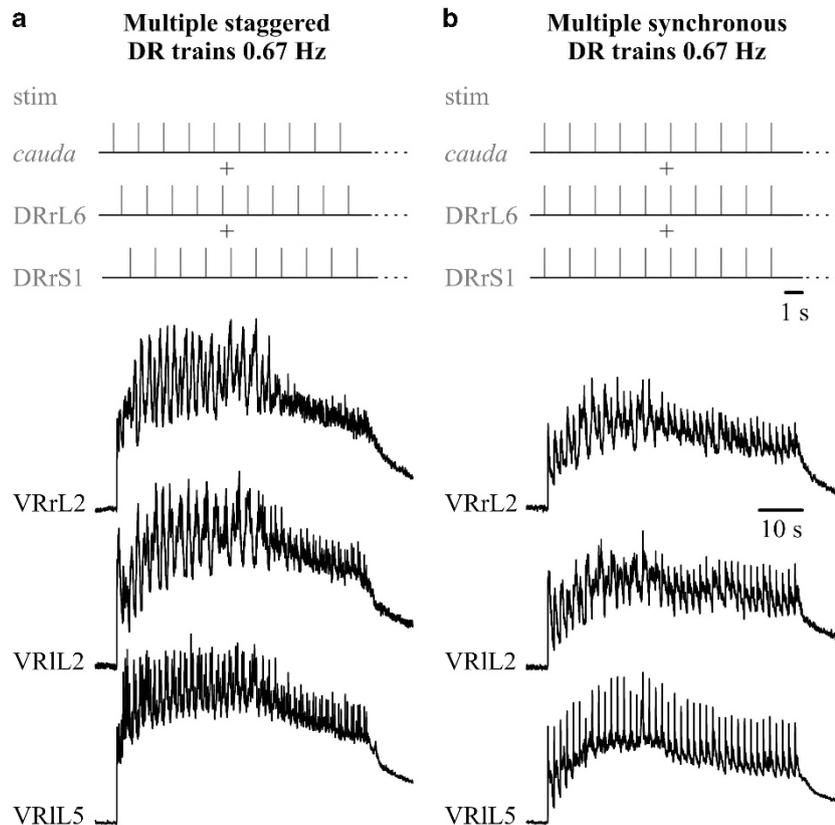


Figure 4 Low-frequency multi-site synchronous stimulation fails to induce FL. (a) A sixty-second protocol of multiple staggered trains of pulses applied at low frequency (0.67 Hz) to the cauda equina (amplitude = 30 μ A, 1.5 \times threshold), DRrS1 (amplitude = 15 μ A, 1.5 \times threshold) and DRrL6 induced an episode of FL (CCF = -0.539) that does not last beyond 38 s, whereas a depolarizing plateau with superimposed tonic activity replaces the locomotor-like cycles during the rest of the stimulation. When trains at 0.67 Hz are delivered synchronously to the three afferents, alternating cycles of FL are replaced by tonic responses time-locked with each of the train's stimuli (b, CCF = +0.136). It is noteworthy that the amplitude of the pulses is kept unvaried for each afferent during delivery of protocols in a and b. CCF, cross-correlation function; DRrL, right lumbar dorsal root; DRrS, right sacral dorsal root; VRIL, left lumbar ventral root; VRrL, right lumbar ventral root.

architecture can be flexibly reconfigured by distinct stimulation frequencies. Indeed, they may either recruit distinct types of interneurons²⁰ or add previously 'silent' frequency-dependent spinal pathways to the ongoing locomotor pattern.²¹ Our data here reported may spur further studies on these findings also for the spinal locomotor networks.

Afferent stimuli modulate locomotor circuits

Interneuronal circuits embedded in locomotor networks of the mammalian spinal cord are known to span several lumbar segments.⁹ Within these segments, many features of the rhythmic pattern are affected by inputs from the periphery.¹⁴ One of our main findings is that within the *in vitro* neonatal rat spinal cord, inputs coming from multiple DRs converge onto the same locomotor circuits. This consideration is based on the observed phase-shift in the oscillatory rhythm of FL induced by stimulation of a distant DR, with the implication that the locomotor network is accessible also via inputs from anatomically different locations. To the best of our knowledge, no phase resetting has ever been reported during electrically induced FL *in vitro*.

Multiple-source inputs facilitate neuronal networks

Multiple-source stimulation is a concept with wide support in the field of neuromodulation, as it has been used to activate different types of

neurons and pathways,²² and to promote behaviours, *viz.* learning and associative plasticity.²³ Simultaneous electrical stimuli delivered to numerous spinal segments facilitate involuntary stepping movements and limb oscillation amplitude in non-injured human subjects, thus proving synergy among inputs converging onto locomotor circuits.²⁴

In our experiments, electrical stimulation with staggered trains of low frequency was simultaneously applied to multiple DRs and sacrocaudal afferents. Although each train on its own was ineffective, their combination activated the locomotor program and the delay in latency at which each DR was stimulated proved to be essential to this effect. The responsiveness of locomotor networks to electrical stimuli from multiple DRs supports the concept that spatial and temporal addition of numerous afferent inputs is fundamental for activating spinal locomotor networks. This phenomenon is closely related to the role had by stochastic variability in the recruitment of neuronal networks, as previously described.²⁵

There is further evidence in support of the concept that multiple types of afferent stimuli are required to activate the locomotor circuits in the ventral spinal cord. FL can be induced by stimulating a single DR or sacrocaudal afferent root, which contains several types of afferent fibres, each with their own conduction velocities.²⁶ In contrast, electrical stimulation of only a fraction of the fibres that reside in a nerve afferent failed to activate the locomotor pattern.²⁷

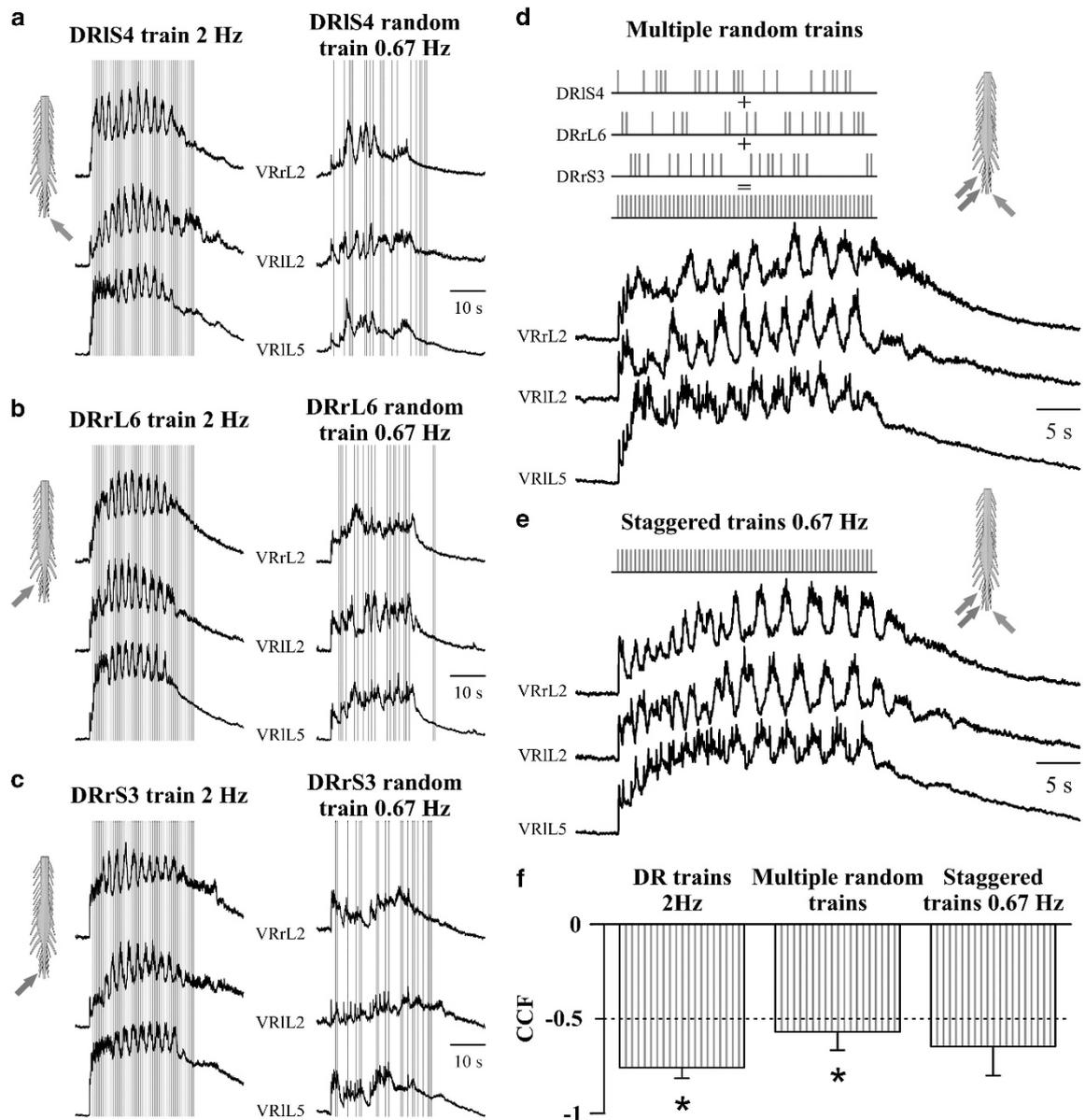


Figure 5 Low-frequency trains of pulses randomly delivered to three DRs induced FL. (a, Left) A 2-Hz train (intensity=13 μ A) applied to DRIS4 elicited a stable FL (CCF=-0.870). A new protocol (mean frequency of 0.67 Hz), designed by randomly selecting only one-third of pulses from the 2 Hz train, was delivered to the same DR without evoking any locomotor patterns (a, right) CCF=-0.296). (b, Left) The 2-Hz train delivered to DRrL6 (intensity=7 μ A) also induced an FL episode (CCF=-0.852), where the multiple random protocol failed to do so ((b, right) CCF=-0.347). (c, Left) FL (CCF=-0.761) was elicited by the 2-Hz train (intensity=20 μ A) to DRrS3 contrary to the random protocol applied to the same root ((c, right) CCF=0.088). The three multiple random trains (mean frequency=0.67 Hz) were now simultaneously applied to the three DRs, as graphically schematized in d (top), showing how the superimposition of single trains recomposed the overall 2 Hz frequency. This stimulating pattern evoked an episode of alternating cycles (CCF=-0.507), similar to multiple staggered trains at low frequency ((e) 0.67 Hz; CCF=-0.748). FL episodes evoked by the three protocols were compared in f as for phase coupling between the pair of homosegmental L2 VRs. All three protocols induced locomotor-like cycles (CCF < -0.5, see dotted line), although the 2-Hz train evoked a statistically stronger alternated coupling among oscillations than did multiple random trains (*Kruskal-Wallis one-way ANOVA followed by all pairwise multiple comparison with Dunn's method, $P=0.011$, $n=12$, 4, 4). CCF, cross-correlation function; DRrL, right lumbar dorsal root; DRrS, right sacral dorsal root; VRIL, left lumbar ventral root; VRrL, right lumbar ventral root.

CONCLUSIONS

A multi-site stimulating protocol with low-frequency trains, delivered with a controlled staggered onset, effectively activated the locomotor central pattern generator in the neonatal rat spinal cord *in vitro*. As the lumbosacral circuitry controlling locomotion in rats and humans has been considered strikingly similar,²⁸ our data cast light on the intrinsic logic adopted by spinal networks to integrate

peripheral stimuli during locomotion and thereby propose new protocols of low-energy multi-site stimulation to improve rehabilitation and/or chronic pain neuromodulation. Moreover, advantages of the proposed stimulation protocol will extend to a reduced energetic demand that increases the life span of batteries in implantable stimulators, thereby increasing the interval between battery replacements. Finally, this protocol should reduce adverse effects, typically

associated with high-energy stimulation, for example, muscle fatigue and spasticity.^{29,30}

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Spinal Cord website (<http://www.nature.com/sc>)