The locomotor central pattern generator of the rat spinal cord in vitro is optimally activated by noisy dorsal root waveforms

Giuliano Taccola1,2
1Neurobiology Sector, International School for Advanced Studies, Trieste; and 2Spinal Person Injury NeurorehabilitationApplied Laboratory, Istituto di Medicina Fisica e Riabilitazione, Udine, Italy

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Taccola G. The locomotor central pattern generator of the rat spinal cord in vitro is optimally activated by noisy dorsal root waveforms. J Neurophysiol 106: 872– 884, 2011. First published May 25, 2011; doi:10.1152/jn.00170.2011.—The spinal cord contains an intrinsic locomotor program driven by a central pattern generator that rhythmically activates flexor and extensor limb motor pools. Although long-lasting locomotor activity can be generated pharmacologically, trains of afferent stimuli trigger only few locomotor cycles. The present study investigated whether a new electrical stimulation protocol (termed FLstim) could elicit long-lasting fictive locomotion (FL) in the rat spinal cord in vitro. Thus, after inducing FL by bath application of N-methyl-D-aspartate and serotonin, the recorded waveform obtained from a lumbar ventral root was digitized and then applied to either a lumbar dorsal root or the cauda equina following washout of pharmacological agents. Two FLstim cycles were the threshold input to evoke an episode of FL from ventral roots. Longer cycles (up to 1 min) induced sustained FL (up to 1 min) with stereotyped periodicity (2.2 ± 0.5 s), despite changing frequency (2–4 s) or cycle amplitude of FLstim. Gradual filtering out of the noise from FLstim trace concomitantly decreased the efficiency of FL so that stimulation with equivalent pure sinusoids produced asynchronous, irregular discharges only that could not be converted to FL by adding spontaneous basal activity. This study is the first demonstration that epochs of rhythmic locomotor-like oscillations applied to a dorsal root represent an efficient stimulus to evoke FL as long as they contain the electrophysiological noise produced within FL cycles. These observations suggest novel strategies to improve the efficiency of electrical stimulation delivered by clinical devices for neurorehabilitation after spinal injury.

stochastic resonance; fictive locomotion; electrical stimulation

LOCOMOTION IS GENERATED by neuronal circuits, known as central pattern generators (CPG), localized in thoracolumbar segments of the spinal cord (Cazalets et al. 1995; Goulding 2009; Harris-Warrick 2010; Kiehn 2006). During gait, the CPG receives, through dorsal roots (DRs), a continuous sensory feedback (Windhorst 2007). The neuronal integration of this information within the spinal cord contributes to the refinement of motor output and represents an important source for postural control during locomotion (Grillner and Jessell 2009). In vitro preparations of isolated rodent spinal cord can mimic locomotion by producing alternating rhythmic activity from ventral roots (VRs) in the absence of any sensory input. This pattern is termed fictive locomotion (FL) (Cazalets et al. 1992; Kudo and Yamada 1987; Smith and Feldman 1987).

In addition, afferent stimuli are sufficient to activate the CPG. This has been demonstrated via experiments in which FL is recorded in response to trains of square electrical pulses applied in vitro either to a DR (Marchetti et al. 2001) or to the cauda equina (Etlin et al. 2010) of the isolated spinal cord. These studies have typically replicated the in vivo stimulation paradigm with intramuscular electrodes in cats (Guevermont et al. 2007) or with different parameters of peripheral (Selionov et al. 2009) or epidural electrical stimuli (Dimitrijevic et al. 1998) or transmagnetic stimulation in humans (Gerasimenko et al. 2010). In all these cases, however, stimulation was only effective in generating a few oscillatory cycles despite continuous stimulation. Efforts to optimize electrical stimulation have focused on varying frequency and intensity of rectangular train pulses. It is unknown whether variations in the pulse shape can improve the activation of locomotor patterns.

The in vivo intrinsic variability of each step, as indicated by kinematics analysis (Courtine et al. 2005), can be exploited to improve the effect of sensory stimulation, because the recruitment of network neurons within each locomotor cycle occurs according to a probabilistic criterion (Edgerton et al. 2006). Since neuronal network activity fluctuates during each cycle, this phenomenon may be described as stochastic resonance, a property whereby variable fluctuations (noise) are beneficial to the efficiency of the response in a nonlinear system (McDonnell and Abbott 2009). The concept of stochastic resonance, which has been reported in the human motor system (Martínez et al. 2007), has recently been applied to the contribution of intrinsic variability in increasing the performance of a neuronal network (Rabinovich and Abarbanel 1998). For instance, in sensory systems the presence of noise can significantly augment the ability to perceive weak stimuli (Simonton et al. 1997). In the present study, I investigated whether fictive locomotion-induced stimulation (FLstim; i.e., application of captured, digitized FL patterns previously recorded from a VR) applied to a single DR could activate locomotor patterns. Furthermore, I studied whether the characteristics of the “noise” contained in the FLstim were important for activating the CPG. The present results may suggest an innovative way to activate the CPG to be tested for gaining functional recovery with electrical stimulation during neurorehabilitation for spinal cord injury.

METHODS

Electrophysiological Recordings

In accordance with the guidelines of the National Institutes of Health and the Italian act Decreto Legislativo 27/1192 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on spinal cord preparations after isola-
tion from neonatal rats (0–5 days old), as previously reported (Taccola et al. 2008). All efforts were made to reduce the number of animals used and to minimize their suffering. Spinal cords (sectioned from the midthoracic region to the cauda equina) were mounted in a small recording chamber (at room temperature) and continuously superfused (5 ml/min) with oxygenated (95% O₂-5% CO₂) Krebs solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 MgCl₂, 7 H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃, and 11 glucose, pH 7.4. DC-coupled recordings from lumbar VRs were obtained using tight-fitting suction electrodes (Taccola et al. 2008). In a few experiments, AC-coupled records were acquired (range 0.1–10 000 Hz). As a routine, recordings were made from lumbar (L) 2 VRs, which primarily consist of axons from flexor motoneurons to hindlimb muscles, and from L5 VRs, which primarily consist of axons from extensor motoneurons to the same limbs (Kiehn and Kjaerulf 1996). The alternation of these discharges between flexor and extensor motor pools and between the left (l) and right (r) sides of the cord represents the trademark of FL (Juvin et al. 2007).

Parameters of Spinal Network Activities

DR electrical stimuli were used to evoke either single VR responses (recorded from the ipsilateral VR of the same segment once every 60 s) or cumulative depolarization, measured as detailed by Barbieri and Nistri (2001). Stimuli were considered as low or high threshold (Th) with respect to their ability to elicit fast synaptic responses from the corresponding VR (see Marchetti et al. 2001). Among 65 preparations, the average Th value was 22.62 μA (SD 11.30 μA). FL (Cazalets et al. 1992) was induced by the continuous bath application of N-methyl-D-aspartate (NMDA; 3, 4 or 5 μM; Tocris, Bristol, UK) plus serotonin (5-hydroxytryptamine, 5-HT; 10 μM; Sigma, Milan, Italy). FL cycles (at least 20) were analyzed for their periodicity (time between the onset of 2 cycles of oscillatory activity) and regularity expressed with the coefficient of period variation (CV between the onset of 2 cycles of oscillatory activity) and regularity.

Designing the FListim

First, FL induced by NMDA (5 μM) and 5-HT (10 μM) was recorded (sampling rate = 500 Hz, low-pass filter = 10 Hz) from VRs as described above, after which these agents were washed to allow the preparation to return to baseline conditions. Epochs (60 or 30 s) of FL traces were promptly processed for off-line analysis (Clampfit 10.1 software; Molecular Devices), from which records from one VR were selected for use. After the trace baseline was reset to the cycle trough, the sampled trace was imported into a spreadsheet of Origin 7.5 (OriginLab, North Hampton, MA), where the cycle trough, the sampled trace was imported into a spreadsheet of Origin Lab, North Hampton, MA, where the x-axis comprised each sampling time for the epoch duration and the y-axis was used for the corresponding current amplitude. The two columns of values were then exported (as an ASCII text file) to a multichannel stimulation device (STG 4004; Multi Channel Systems, Reutlingen, Germany).

The stimulating protocol resulting from this procedure was termed FListim (fictive locomotion-induced stimulation) and was applied to either one DR or the cauda equina through a bipolar suction electrode. No functional difference in VR responses was observed when FListim was applied to different DRs (L3–L7) as demonstrated with the example shown in Supplemental Fig. S1, in which FL cycles were recorded from the same preparation following stimuli applied to left and right DRs. (Supplemental material for this article is available online at the Journal of Neurophysiology website.)

Origin software enabled the amplitude of the FListim to be optimized for each preparation to evoke FL. To ascertain the role of oscillation frequency, FListims were acquired from preparations generating FL at different periodicities caused by varying NMDA concentration (5-HT concentration was kept stable). For further tests, the same fictive locomotion traces were sampled at a lower rate to minimize noise and provide a smoother signal.

In addition, Clampfit software was used to create pure sinusoidal waves of predetermined amplitude and frequency. These stimuli were applied to the same preparations either separately or in conjunction.

Statistical Analysis

Data are means and SD, and n indicates the number of spinal cord preparations. After distinguishing between parametric or nonparametric data using a normality test, we analyzed all parametric values using Student’s t-test (paired or unpaired) to compare two groups of data, or ANOVA for more than two groups. For nonparametric values, Mann-Whitney test was used for two groups, whereas for multiple comparisons, ANOVA was first applied, followed by a post hoc test (Dunn’s method) for groups larger than two. Results were considered significant when P < 0.05.

RESULTS

FListim Induces FL on All Four Lumbar VRs

Figure 1A depicts a characteristic DC trace of FL in the presence of 5 μM NMDA and 10 μM 5-HT. In this example, the locomotor rhythm was stable [period = 1.92 s (SD 0.13 s); CV = 0.07], showing alternation among VRs on both sides as well as between pools of extensor and flexor motoneurons on the same side [CCF_homosegmental = −0.69 (SD 0.10); CCF_homolateral = −0.65 (SD 0.12)].

After washout and return to basal conditions, a 60-s segment of the record from VRrL5 (see shaded box in Fig. 1A) was taken for its transformation into the FListim protocol, to be applied to the DRrL5 of the same preparation (Fig. 1B). The stimulating wave amplitude (cycle peak to peak) was adjusted at a current intensity 0.3 times the one required by a single rectangular pulse to induce a detectable reflex response on the same preparation.

With a latency (from the stimulation onset) of 12 ms for the homologous extensor-related L5 VR and 27 ms for the flexor-related L2 VRs, VR depolarization started and reached a 0.68-mV level within 30 s (see asterisk in Fig. 1B), after which some fading appeared. In summary, among 20 preparations, the latency for depolarization onset was 43.34 ms (SD 29.38 ms) for the homologous VR and 60.88 ms (SD 31.39 ms) for the VRs farther away from the stimulating electrode. Superimposed on the depolarization plateau, locomotor-like oscillations appeared during the FListim pattern. In the example depicted in Fig. 1A, the episode of oscillations presented 19 cycles with a period of 2.21 s (SD 0.24 s) and a CV of 0.11. As for the example in Fig. 1B, the alternation between homolateral and homosegmental VRs, obtained from cross-correlation analysis, was CCF_homosegmental = −0.40 (Fig. 1C) and CCF_homolateral = −0.47 (Fig. 1D). After 42.31 s, these alternating oscillations spontaneously ceased and were replaced by a synchronous pattern, which followed the stimulating pattern with cessation after the end of FListim. The characteristics of the oscillatory activity obtained from 21 experiments are illustrated in Fig. 1E.
To summarize, the oscillations elicited by FLstim (fictive locomotion-induced stimulation) evoked locomotor-like oscillations from ventral roots (VRs). A: traces of fictive locomotion were simultaneously recorded from 4 lumbar (L) VRs in the presence of 5 μM N-methyl-D-aspartate (NMDA) and 10 μM serotonin (5-HT). 1, left; r, right. B: a 60-s trace, recorded from VRrL5 (see example in the shaded area) was then exported (ASCII format) and used to provide FLstim [0.3 threshold (Th)] to the dorsal root (DRrL5 on the same preparation, now perfused with physiological solution. In conjunction with FLstim, episodes of FL (alternating cycles between flexor and extensor motor pools and between right and left sides of the cord) emerged. The asterisk corresponds to the depolarization value indicated in the text. C and D: fictive locomotion (FL) characterized by the negative value of correlograms for lag period = 0. CCF, cross-correlation function. E: histograms summarize average properties of FL induced by FLstim in 21 preparations. OSCs, oscillations; CV, coefficient of variation.

Fig. 1. Stimulation with FLstim (fictive locomotion-induced stimulation) evokes locomotor-like oscillations from ventral roots (VRs). A: traces of fictive locomotion were simultaneously recorded from 4 lumbar (L) VRs in the presence of 5 μM N-methyl-D-aspartate (NMDA) and 10 μM serotonin (5-HT). 1, left; r, right. B: a 60-s trace, recorded from VRrL5 (see example in the shaded area) was then exported (ASCII format) and used to provide FLstim [0.3 threshold (Th)] to the dorsal root (DRrL5 on the same preparation, now perfused with physiological solution. In conjunction with FLstim, episodes of FL (alternating cycles between flexor and extensor motor pools and between right and left sides of the cord) emerged. The asterisk corresponds to the depolarization value indicated in the text. C and D: fictive locomotion (FL) characterized by the negative value of correlograms for lag period = 0. CCF, cross-correlation function. E: histograms summarize average properties of FL induced by FLstim in 21 preparations. OSCs, oscillations; CV, coefficient of variation.
Fig. 2. FL stimuli recorded in DC or AC mode are equally effective in inducing locomotor-like oscillations. A: FL stimulus (Th; 60 s) constructed from traces recorded in DC mode induced episodes of locomotor-like oscillations with alternation among homosegmental left and right VRs and homolateral L2 and L5 VRs, as depicted in the enlarged inset (shaded bar). B: the same FL stimulus paradigm constructed from AC-coupled traces (Th; 60 s), which still evokes locomotor-like oscillations indistinguishable from those in A. C and D: correlograms show alternation among the signals obtained from the same pair of roots (VRrl2 and VRrl5) during FL stimuli obtained from traces recorded respectively in DC and AC mode. E: histograms indicate that stimulation with AC-mode FL stimulus elicited lower peak and area of cumulative depolarization (Cum Dep; for peak: $P = 0.029$, paired t-test on raw data; for area: $P = 0.017$, paired t-test on raw data; $n = 6$), despite the efficient production of FL.
Fig. 3. FLstim generates a longer lasting rhythm compared with that evoked by a train of square stimuli of the same duration. A: an episode of FL was induced by a DR train consisting of 120 square pulses (2 Hz; 1.5 Th). B: on the same preparation, an episode of FL was evoked by FLstim (0.3 Th, 60 s). Correlograms in C refer to the coupling among pairs of homosegmental (l) and homolateral (r) roots during the DR train. D: analogous analysis was performed on the oscillations induced by FLstim. Note that the values of the correlogram troughs for FLstim are larger, thus indicating more efficient alternation. E: histograms show the mean number of oscillations and their duration induced by FLstim as a percentage of those elicited by DR trains (*P = 0.008, Wilcoxon signed rank test; #P = 0.016, paired t-test; n = 5). F: homosegmental and homolateral CCF values for oscillations evoked respectively by FLstim (dark shading) and DR train protocols (light shading). In response to FLstim, CCFs are significantly closer to −1, indicating more efficient alternation among pairs of VRs (*P = 0.031, Wilcoxon signed rank test; #P = 0.004, Mann-Whitney rank sum test; n = 5).
DC, 27.57 s (SD 8.87 s) in AC; $P = 0.986$, paired $t$-test], with a similar number of cycles [13.33 (SD 5.12) in DC, 11.67 (SD 6.12) in AC; $P = 0.388$, paired $t$-test], period of oscillations [2.28 s (SD 0.79) in DC, 2.62 s (SD 0.83 s) in AC; $P = 0.052$, paired $t$-test], and CV [0.24 (SD 0.10) in DC, 0.24 (SD 0.09) in AC; $P = 0.844$, Wilcoxon signed rank test]. Amplitude and area of cumulative depolarization were, however, less efficient with the AC FLstim (Fig. 2E). On the same preparation, a single square pulse of amplitude equal to the FLstim did not evoke a detectable reflex response (Supplemental Fig. S2). Delivering the same stimulus after a short (40 ms) train of 20 stimuli at the same frequency of the FLstim sampling rate (500 Hz) generated a response from VRs (Supplemental Fig. S2C), indicating a rise in network excitability. The FLstim protocol was, thus, capable of activating a locomotor-like pattern at a much lower intensity than the one required to evoke standard reflex responses with square pulses.

**Comparison of FLstim With Square Pulse Trains to Evoke FL**

To compare the efficiency of FLstim vs. square pulse trains (DR train; 2 Hz) to induce FL, experiments were performed...
with these protocols on the same preparations ($n = 5$), as shown with the example in Fig. 3, A and B. Thus FLstim and a DR train of the same duration (60 s) were delivered to evoke FL, which, in the case of the DR train (Fig. 3A), decayed after about 35 s despite continuous stimulation, whereas FLstim (Fig. 3B) elicited robust FL throughout the whole length of the protocol.

Compared with a DR train, FLstim induced a higher number of alternated cycles (37 vs. 22) of similar period [1.61 s (SD 0.28 s) vs. 1.63 s (SD 0.35 s)] but of a greater regularity (CV = 0.28 vs. 0.35). The correlation analysis (Fig. 3C for DR trains and Fig. 3D for FLstim) indicates that the troughs of the correlograms for FLstim have values closer to −1 ($CCF_{homosegmental} = −0.76$ vs. −0.50; $CCF_{homolateral} = −0.87$ vs. −0.51), consistent with stronger cycle phase alternation. Figure 3E shows the average values from five spinal cords in which FLstim was clearly more effective than DR trains to significantly increase the number of oscillations [28 (SD 7) with FLstim and 21 (SD 4) with DR train, $P = 0.008$, Wilcoxon signed rank test] and the duration of FL episodes [58.26 s (SD 7.09 s) with FLstim and 47.05 s (SD 11.45 s) with DR train, $P = 0.016$, paired $t$-test].

Cycle period was, however, similar [2.16 s (SD 0.47 s) with FLstim and 2.32 s (SD 0.38 s) with DR train, $P = 0.26$, paired $t$-test], like the regularity of oscillations [0.30 (SD 0.08) with FLstim and 0.32 (SD 0.09) with DR train, $P = 0.652$, Wilcoxon signed rank test]. FLstim was significantly better for expressing segmental cycle coupling, as indicated by the mean values of the homosegmental CCF ($P = 0.031$, Wilcoxon signed rank test) and the homolateral CCF ($P = 0.004$, Mann-Whitney rank sum test; Fig. 3F).

**Characteristics of a FLstim Capable of Inducing FL**

**Stimulus intensity.** To investigate whether FL depended on the amplitude of the stimulating cycles, for each FL trace transformed into the FLstim protocol, the amplitude of the original cycles varied from 0.3 to 6 times $Th$ for the reflex. FLstim of different frequency evoke stereotyped episodes of locomotor oscillations. A–C: FLstim (0.3 $Th$; 30-s duration) of different frequency were captured from FL traces induced by decreasing concentrations of NMDA (from 5 to 3 $\mu M$). Note similar VR FLs despite changes in FLstim frequency. Shaded bars show records of FL cycles depicted on the faster time scale at bottom. D–G: histograms summarize unchanged period of oscillations ($P = 0.241$, one-way ANOVA; $n = 8$), period CV ($P = 0.119$, Kruskal-Wallis one-way ANOVA; $n = 8$), duration of FL episodes ($P = 0.261$, one-way ANOVA; $n = 8$), and number of oscillations ($P = 0.534$, one-way ANOVA; $n = 8$).

![Figure 5](http://jn.physiology.org/)

*Fig. 5.* FLstim of different frequency evoke stereotyped episodes of locomotor oscillations. A–C: FLstim (0.3 $Th$; 30-s duration) of different frequency were captured from FL traces induced by decreasing concentrations of NMDA (from 5 to 3 $\mu M$). Note similar VR FLs despite changes in FLstim frequency. Shaded bars show records of FL cycles depicted on the faster time scale at bottom. D–G: histograms summarize unchanged period of oscillations ($P = 0.241$, one-way ANOVA; $n = 8$), period CV ($P = 0.119$, Kruskal-Wallis one-way ANOVA; $n = 8$), duration of FL episodes ($P = 0.261$, one-way ANOVA; $n = 8$), and number of oscillations ($P = 0.534$, one-way ANOVA; $n = 8$).
response. Figure 4, A–D, shows episodes of FL elicited by FLstim (obtained from the same original trace) at increasing stimulation strengths. On average, in six experiments, for increases in FLstim cycle amplitude, the duration of each locomotor episode diminished (Fig. 4E) and the number of alternating cycles decreased (Fig. 4F), whereas period \((P = 0.435,\ one\-\way\ ANOVA)\), CV \((P = 0.260,\ Kruskal-Wallis\ one\-\way\ ANOVA\ on\ ranks)\), cumulative depolarization area \((P = 0.751,\ Kruskal-Wallis\ one\-\way\ ANOVA\ on\ ranks)\), and peak \((P = 0.873,\ one\-\way\ ANOVA)\) remained unchanged. In the example of Fig. 4, C and D, arrowheads indicate synchronicity between FLstim and VR oscillations for representative responses when the stimulus strength was 3 or 6 times Th.

As indicated in METHODS, the average threshold for inducing a VR reflex was 22.62 \(\mu\text{A} (SD 11.30 \mu\text{A})\). Since the electrophysiological setup did not allow testing current intensities below microampere, the lowest FLstim stimulus was 0.3 Th, which corresponded to about 1 \(\mu\text{A}\). The present recordings thus indicate that the optimal amplitude of the FLstim was in the range of 0.3–0.6 times Th for reflex. Larger intensities only induced a synchronous rhythm on all four VRs, time-locked with the stimulating pattern (see Fig. 4, B–D). The graph in Fig. 4G plots the CCF values for various VR patterns against the strength of FLstim delivered to DRIL4. As the increase in amplitude of stimulation grew, the oscillations recorded from VRs became synchronous with the FLstim pattern, up to a perfect match when intensity was 6–7 times Th. Furthermore, VRs closer to the stimulated DR were the first to be synchronized.

**Cycle frequency.** To assess whether the locomotor response depends on the frequency of the stimulating waveform, records of FL at different periodicity, produced by applying various concentrations of NMDA (from 3 to 5 \(\mu\text{M}\)) were acquired and sampled to generate FLstim at different frequencies. Figure 4 depicts the alternating patterns recorded from VRs, with similar duration of oscillation episode (37.43 s from 3 \(\mu\text{M}\) NMDA, 38.48 s from 4 \(\mu\text{M}\) NMDA, 39.46 s from 5 \(\mu\text{M}\) NMDA) and number of locomotor oscillations (16 from 3 \(\mu\text{M}\) NMDA, 16 from 4 \(\mu\text{M}\) NMDA, 18 from 5 \(\mu\text{M}\) NMDA).

In the example in Fig. 5, oscillations obtained in response to different FLstim show similar period (2.18 s for 3 \(\mu\text{M}\) NMDA, 2.31 s for 4 \(\mu\text{M}\) NMDA, 2.17 s for 5 \(\mu\text{M}\) NMDA) and CV (0.25 for 3 \(\mu\text{M}\) NMDA, 0.24 for 4 \(\mu\text{M}\) NMDA, 0.17 for 5 \(\mu\text{M}\) NMDA), illustrating the stereotyped nature of these oscillations. Average results from eight experiments displayed no difference in locomotor-like oscillations, as for period (see Fig. 5D; \(P = 0.241,\ one\-\way\ ANOVA\)) and duration of episodes \((P = 0.261,\ one\-\way\ ANOVA)\), number of cycles \((P = 0.534\ one\-\way\ ANOVA)\), and period CV \((P = 0.119,\ Kruskal-Wallis\ one\-\way\ ANOVA\ on\ ranks)\).

**Minimal length.** The minimum number of stimulating cycles needed to induce a locomotor response was investigated by applying FLstim (constant cycle amplitude and period) for 2–60 s. Figure 6A demonstrates that a hemicycle could not induce alternating oscillations, although two cycles did so. A higher number of cycles increased the duration and the number of oscillations that did not outlast 30 s in this example (Fig. 6A). In general, the maximal length of FL was 1 min. Figure 6B shows that, on average, a longer FLstim was associated with a larger cumulative depolarization, yet an analogous number of oscillations. Hence, either 30-s or 60-s FLstim were used for further experiments.

FLstim applied to the cauda equina. The question then arises whether sensory afferents other than a single DR can be equally effective to activate the locomotor CPG. It was recently demonstrated that stimulation of the cauda equina induces locomotor responses in the in vitro spinal cord (Etlin et al. 2010). Thus a FLstim obtained after sampling the FL produced by NMDA (5 \(\mu\text{M}\)) and 5-HT (10 \(\mu\text{M}\)) was applied to the cauda equina rather than to a DR. Figure 7A shows an example of alternating oscillations recorded from four VRs after the application of this protocol. The cross-correlation analysis indicated that such oscillations alternated among homosegmental (Fig. 7B, CCF = −0.67) and homolateral VRs.
The number of oscillations (14 for square-pulse train vs. 24 for square pulse train vs. 53.54 s for FL discharges shorter than one induced by FL).

On the same preparation, a DR train of 30 square pulses (2 Hz; sinusoidal waveform failed to produce FL, as indicated in Fig. 8). VR before the application of NMDA and 5-HT) to the sinusoidal waveform of the same frequency as the FL alternating cycles (B) when DR stimulation was used with a pure sinusoid waveform of the same amplitude and frequency as the FL stim. Furthermore, addition of spontaneous baseline activity (CCF = −0.03), a negative correlation reappears when trains of square pulses were reapplied (CCF = −0.55).

In all tested cases (n = 10), stimulation with a pure sinusoid waveform of the same amplitude and frequency as the FLstim failed to generate FL, whereas VRs produced only irregular discharges, as confirmed by CCF analysis [−0.60 (SD 0.05) for FLstim, 0.05 (SD 0.27) for pure sinusoid; P = 0.002, Wilcoxon signed rank test].

To validate the notion that signal noise was important for the effectiveness of stimulation, the same FLstim traces were acquired at a lower sampling rate (same amplitude and frequency), thus making them progressively smoother and noise free. Indeed, by varying the sampling frequency from 5,000 to 5 Hz, as depicted in Fig. 9A, the performance of the locomotor response clearly decayed in all its parameters. The histograms of Fig. 9B show the average fall in duration, number of oscillations, and area of cumulative depolarization for each reduction in FLstim sampling rate. Likewise, the mean peak of cumulative depolarization was reduced from 503 µV (SD 74 µV) at 5,000 Hz to 253 µV (SD 59 µV) at 5 Hz (P = 0.004; n = 3). Nonetheless, period (and CV) of locomotor oscillations remained unchanged (P = 0.597, one-way ANOVA for period; P = 0.838, Kruskal-Wallis one-way ANOVA on ranks for CV; n = 3).

**DISCUSSION**

The results of this study suggest that the locomotor CPG may be recruited in vitro by a novel protocol of electrical stimulation termed FLstim. This new experimental tool for eliciting CPG activity improves on the widely adopted techniques such as a cocktail of pharmacological drugs (Cazalets et al. 1992) or DR stimulation with trains of square impulses (Marchetti et al. 2001) or cauda equina stimulation (Eltin et al. 2010). By capturing and reapplying the motor output during canonical FL evoked by NMDA and 5-HT, the present study attempted to mimic the sensory feedback rhythmically relayed to the spinal cord during locomotion, to offer a more efficient protocol to activate the CPG. Hence, FLstim represents a more physiologically relevant method than bath-applied chemicals to activate the locomotor CPG and a more efficient protocol than just trains of afferent fiber square pulses. The present strategy is similar to that employed by Hayes et al. (2009), who used the isolated rat spinal cord with attached hindlimbs freely moving on a treadmill, with the additional benefit of analyzing in detail the electrophysiological properties of sensory inputs required to trigger the CPG.

**Properties of FL Induced by FLstim**

FL induced by FLstim applied to a single DR possessed periodicity and full alternation, as expected for FL, yet it was stereotypic despite wide changes in frequency and amplitude of the FLstim. This latter observation was in contrast with the fine modulation of chemically induced FL normally found by titrating the concentrations of neurochemicals such as NMDA and/or 5-HT (Cazalets et al. 1992), but it is reminiscent of the
effects of raising extracellular \( K^+ \), which stimulates the CPG over a very narrow range of frequencies (Bracci et al. 1998). It is, however, worth noting that bath-applied neurochemicals likely raise the excitability of a very widespread region of the spinal cord, possibly much larger than the few segments activated by stimulation of a single DR. Thus rhythm modulation might be due not only to stepwise changes in CPG network excitability (Bracci et al. 1998) but also to amplification of the functional coupling of distant unit burst generators (Grillner 2006), all contributing to a more refined motor output.

The present study did not identify the specific component neurons that are activated by FLstim and initiate the FL. These are presumed to be similar to those underlying chemically evoked FL in analogy with shared properties, as shown in Fig. 1. A detailed description of the neuronal wiring necessary for locomotion has recently started with the aid of mouse genetics, demonstrating distinct local neurons with individual contribution to the recorded pattern (Goulding 2009; Hägglund et al. 2010; Harris-Warrick 2010; Wu et al. 2011). Nonetheless, the wiring diagram remains to be fully understood and its applicability to the rat spinal cord to be firmly established.

FLstim wave amplitude was distinctly lower than the amplitude of the threshold square pulse necessary to elicit a monosynaptic reflex. This characteristic probably originated from differential thresholds of intraspinal neurons recruited into a patterned discharge mode only by stimulus cycles delivered in a sequence to gradually build up CPG excitability. This suggestion was supported by the observation that at least two full cycles of FLstim were needed to produce FL. In line with this notion, repeated subthreshold pulses applied to one DR generated a monosynaptic response, whereas a single pulse did not. DC- or AC-coupled FLstim elicited an epoch of FL shorter than the one in A. E–H: the cross-correlation analysis confirmed that cycle alternation was observed only when FLstim or square pulse DR trains were used (although less effectively in the latter case).

Nonlinear Properties of CPG Operation vs. Inputs Provided by FLstim

The stereotypical CPG output raised the issue of intrinsic self-limiting properties. In favor of this hypothesis is the finding that when the frequency of FLstim was increased to overcome the stereotypic output, the pattern was converted from VR alternation to VR synchronization and thus lost its fundamental FL feature. Although the present experimental model tested the effect of just one DR stimulation and thus differed from the in vivo condition whereby multiple DRs are coactivated during walking, it is clear that the one important aspect of FLstim was the use of weak DR stimuli, often subthreshold as single pulses for evoking VR responses. Future studies are necessary to explore whether topographically different sites of DR stimulation or coapplication of FLstim to multiple DRs can modulate CPG output. Notwithstanding these current constraints, it is clear that FLstim is a far more
efficient method to generate FL than trains of square or sinusoidal DR pulses. In accordance with this notion was the demonstration that longer episodes of FL with more efficient cycle phase alternation were recorded following FL i stim compared with similar lengths of DR trains on the same preparations.

The Transient Nature of Locomotor-Like Oscillations Evoked by FLstim

With fine-tuning of the number of stimulatory cycles required for FL induction, it became apparent that FL started with a delay from the beginning of the FLstim application and could outlast it. Although this result distinguished CPG output from simple reflex activity and implied functional reconfiguration of the spinal networks into alternating VR output, an episode of locomotor oscillations induced by FLstim inevitably decayed even when FLstim persisted. Previous studies have demonstrated a strong synaptic depression of afferent inputs in the neonatal rat spinal cord (Lev-Tov and Pinco 1992), probably because of development of synaptic fatigue (Lee and O’Donovan 1991). It is, however, interesting that the amplitude of cumulative depolarization underlying these stimulatory cycles did not significantly decline, probably indicating that the network activity had remained above threshold.

Why Is FLstim Efficacy Different From an Artificial Waveform?

FLstim can be viewed as a certain application of the dynamic clamp technique developed to retrace organization and functioning of a network by perturbing the system in different modes and studying the consequences (Robinson and Kawai 1993; Sharp et al. 1993a, 1993b). The use of digital parameters (square or sinusoidal waves), devoid of any intrinsic noise, likely underestimates the role of intrinsically variable components in the operation of neuronal networks (Le Masson et al. 2002; Prinz et al. 2004). Previous studies have shown how the introduction of physiological noise into a network enhances neuronal firing properties (Stacey and Durand 2002; van Rossum et al. 2003).

In particular, on hippocampal pyramidal neurons, association of noisy stimuli with synaptic activity is a powerful tool to preserve Na+ current efficiency, despite a background of increased membrane conductance, and to reduce spike frequency adaptation. Hence, high-frequency periodic discharges ensure enhanced spike rates, indicating that the neuronal integrative properties depend on the frequency and magnitude of voltage fluctuations (Fernandez et al. 2011).

In line with this notion, the present experiments indicated that the “noisy” nature of this stimulus was a very important property to induce FL. It was not possible, with VR recordings, to identify exactly what constituted the noise in the traces captured and converted into FLstim. Nevertheless, one may surmise that it represented a stochastic resonance phenomenon at CPG level so that detection of a low-level signal is enhanced in a nonlinear system by the introduction of noise (Stacey and Durand 2001). Recent investigations (Geertsen et al. 2011) have shown that, during the “inactive” phase of fictive locomotion, the network noise is predominantly made up by inhibitory synaptic potentials that pace the motoneuron firing. It is likely that the noise present in the VR records used for FLstim contained a similar type of signal, in addition to excitatory ones (Raastad et al. 1996). It has recently been shown that global network transitions between bistable states can be triggered by introducing physiologically sparse activity (Fröhlich et al. 2010). The present study, however, indicates that adding baseline spontaneous activity to a sinusoidal input cannot evoke a...
stable pattern of fictive locomotion, demonstrating the special characteristics of the noise intrinsic to the locomotor pattern. In addition to the noise arising from intraspinal network activity during FL, further sources of noise may be subpopulations of propriospinal interneurons (Berg et al. 2009), ephaptic interactions between the fibers of the intraspinal white matter (Jefferys 1995), and stochastic variations of cord potentials (Cuellar et al. 2009).

Clinical Perspectives

The results of this study may have interesting implications in the field of neurorehabilitation, which uses peripheral functional electrical stimulation (FES) to contrast the functional deficit of spinal cord-injured persons (Ragnarsson 2008). Although the performance of these devices has been improved, the nature of square pulse trains has not been changed (Mushahwar et al. 2007). Unfortunately, FES cannot be used in all scenarios of spinal cord injury due to the high stimuli intensities required, which generate, in many cases, side effects including spasticity, neuropathic pain (Ashley et al. 1993), and muscle fatigue (Tepavac and Schwirtlich 1997). The introduction of innovative stimulation protocols, based on the principles of subthreshold noisy stimulation, may provide better functional access to the CPG operation and may improve the techniques of FES.

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